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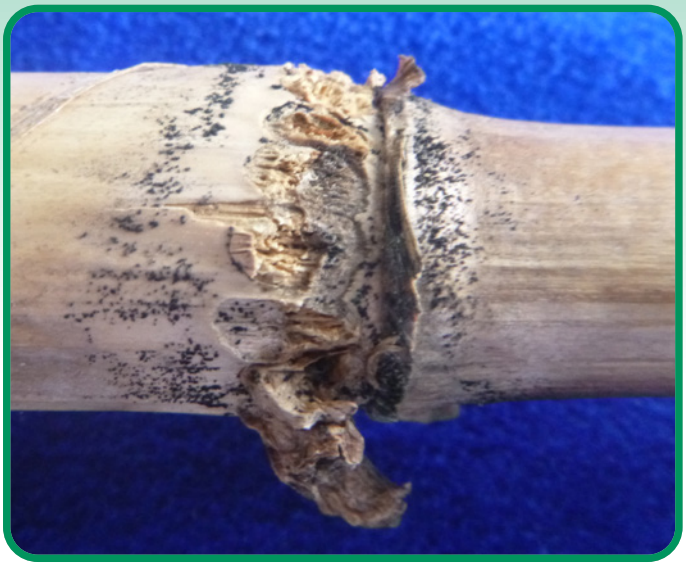

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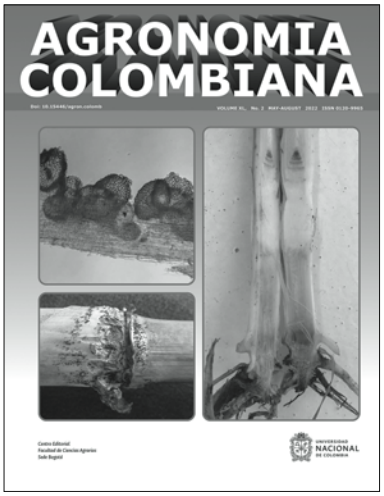

Our cover:

Fusarium species that cause corn stalk rot in the Ubaté valley of Cundinamarca, Colombia

Article on pages 237-248

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Hernán Darío Suárez Rodríguez[1], Diego Andrés Benítez Duarte[2], Alejandro Chaparro-Giraldo[1†], and Orlando Acosta[3]*

ABSTRACT

Off-patent corn (event TC1507) contains genes coding for CRY1F and PAT proteins, which confer resistance to lepidopteran insects and tolerance to the herbicide glufosinate-ammonium. We employed the substantial equivalence approach to investigate the compositional safety of the corn *off-patent* event (TC1507). The levels of the nutritional contents of proximate analytes in grain and forage tissues of *off-patent* genotypes of transgenic maize plants and conventional corn genotypes were compared. The levels of the analytes evaluated in the transgenic plants were found to be within the ranges published in the literature for non-transgenic corn and were statistically indistinguishable from the conventional corn from which they are derived (elite corn lines), indicating substantial equivalence between the *off-patent* (event TC1507) and its conventional counterpart. These results constitute key evidence of the safety evaluation of the world's first transgenic corn developed from technologies that are in the public domain.

Key words: transgenic corn, genetically modified *off-patent* crops, substantial equivalence, food safety, compositional analysis.

RESUMEN

El maíz *off-patent* (evento TC1507) contiene los genes que codifican para las proteínas CRY1F y PAT que le confieren resistencia a insectos lepidópteros y tolerancia al herbicida glufosinato de amonio. Empleamos el enfoque de equivalencia sustancial para investigar la seguridad composicional del maíz *off-patent* (evento TC1507). Se realizaron comparaciones de los contenidos nutricionales de los analitos proximales en los tejidos de grano y forraje de los genotipos *off-patent* de plantas de maíz transgénicas y de los genotipos de maíz convencional. Los niveles de los analitos evaluados en las plantas transgénicas se encontraron dentro de los rangos publicados en la literatura para el maíz no transgénico y fueron estadísticamente indistinguibles del maíz convencional del cual derivan (líneas elite de maíz), lo que indica la equivalencia sustancial entre el *off-patent* (evento TC1507) y su homólogo convencional. Estos resultados constituyen una evidencia clave de la evaluación de seguridad del primer maíz transgénico en el mundo desarrollado con base en tecnologías que están en dominio público.

Palabras clave: maíz transgénico, cultivos transgénicos *off-patent*, equivalencia sustancial, inocuidad de los alimentos, análisis composicional.

Introduction

The genetic modification of plants originated in the early 1980s when new genes of interest were introduced into a plant (*i.e.*, *Nicotiana tabacum*) using *Agrobacterium tumefaciens* as a vector (Bevan *et al.,* 1983; Herrera-Estrella *et al.,* 1983; Basso *et al.,* 2020). Since the mid-1990s, the first genetically modified (GM) crops from numerous public and private laboratories have been commercially introduced. In 2019, the area cultivated with GM crops was estimated to be 190.4 million ha in the world, successfully adopted in 29 countries (ISAAA, 2019), with more than 50% of the world's population. The majority of the cultivated area is represented by the most important domesticated species including corn, soybeans, cotton, and canola (Duke & Cerdeira, 2010; Brookes & Barfoot, 2020). According to Brookes (2020), a total of 1.07 million ha have been planted with cotton and corn containing transgenic

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traits in Colombia since 2003, and farmers benefited from an increase in income of US $301.7 million.

The first patents granted to GM crops have started to expire as these patents are valid for 20 years. These crops are considered *off-patent* events. These *off-patent* events compare to generics in the pharmaceutical and agrochemical industry. However, in contrast to the development of the generic industry in the pharmaceutical and agrochemical fields, the lack of harmonization of the current regulatory frameworks of the countries on the sowing, commercial release and use as human and animal food of *off-patent* crops presents challenges (Rüdelsheim *et al.,* 2018).

A GM event has been defined as the insertion of DNA into the plant genome resulting from a single transformation process (Pilacinski *et al.,* 2011; Basso *et al.,* 2020). However, individual GM events may contain one or more transgenes. In other words, a GM event refers to the precise location of an expression cassette in the host genome that encodes a trait of interest (Bell *et al.,* 2018). GM events that confer tolerance to herbicides and/or resistance to insects are the most widely applied engineered traits. These genetic modifications have benefited adopting farmers through higher yields, reduced input costs and less environmental pollution (Carpenter, 2010; Cerdeira *et al.,* 2011; Brookes & Barfoot, 2018).

The timeline for the commercialization of an event is relatively long (the first commercial launch took around 14 years) (Fraley, 2015) and a large investment is needed to comply with all regulatory requirements (McDougall, 2011). In addition, Proprietary Regulatory Property (PRP) holders must obtain regulatory approvals in the countries where they intend to commercially release or export events (Rüdelsheim *et al.,* 2018).

Substantial equivalence of a GM crop has been defined as a new product that must be the same as the non-GM crop except for the traits that were enhanced, added, or removed through genetic engineering (OECD, 2002; Parrott *et al.,* 2010; ISAAA, 2018). The concept of substantial equivalence has been used to investigate the compositional safety of transgenic crops (Codex Alimentarius Commission, 2009; Privalle *et al.,* 2013). This approach recognizes that, although no crop or food can be shown to be 100% safe, GM crops can be compared to crops that have a history of safe use (Cheng *et al.,* 2008; Harrigan, Glenn *et al.,* 2010). The most common approach has been to compare the transgenic line with its isogenic version without the transgene, using compositional analysis to identify potential differences in the levels of each of the nutritional components. If no significant differences are found, or are within the expected natural variation, or within ranges previously reported in the literature for each component, the results of this evaluation are presented as a fundamental part of the requirements of the regulatory authorities (OECD, 2003; Harrigan *et al.,* 2010).

Fall armyworm (*Spodoptera frugiperda*) is one of the most common pests of corn and it has been responsible for large economic losses in Colombia (Gómez *et al.,* 2013; Jaramillo-Barrios *et al.,* 2019). The introduction of insect resistance genes in Colombian maize genotypes is a potential alternative for the control of this harmful pest. For this purpose, the Corn Genetic Improvement Program of the National Cereal and Legume Research Center (CENICEL) of the National Federation of Cereal and Legume Growers (FENALCE), with the support of the research group of Genetic Engineering of Plants of the Universidad Nacional de Colombia (IGP-UN), obtained an *off-patent* maize hybrid from the TC1507 event. This hybrid was obtained through the backcrossing method using elite Colombian corn lines crossed with Herculex® I corn (GM corn event TC1507), followed by complementary field tests and immunostrip assays (Jiménez *et al.,* 2016). In this way, elite maize lines of the Colombian genotypes with the TC1507 event (*cry1f and pat*) were obtained. Subsequently, these elite lines with the introgressed event were hybridized to obtain an *off-patent* maize hybrid with the TC1507 event resulting from the best hybrid combinations of the breeding program (Jiménez *et al.,* 2016). The purpose of the present study was to evaluate the substantial equivalence of this hybrid. The levels of the nutritional components of the proximal analytes in the grain and forage tissues involving the *off-patent* genotypes (event TC1507) of GM corn plants were compared with the conventional corn genotypes from which they were derived; this hybrid has the same genetic background as *off-patent* hybrid TC1507 but does not contain the genetic modification.

Materials and methods

Field trial samples

A field trial to obtain grain and forage samples for compositional analysis was carried out. The corn was grown in the CENICEL Paraguaicito experiment station, in the municipality of Buenavista (Quindío), located in the natural subregion of the Colombian coffee zone. This crop had normal pest control and maintenance practices (irrigation, fertilization, herbicide and pesticide applications, etc.), consistent with maize production and applied uniformly

to each entire trial area. The planting design consisted of a random block design, with three replicates per block, where six genotypes were distributed: two elite *off-patent* lines, two conventional elite isolines, one *off-patent* hybrid (conventional elite line X elite line *off-patent*), and a conventional hybrid grown locally. From each of the genotypes, three samples of two types of tissues were collected: 1) the samples of the corn grain in its state of maturity (phase R6) and 2) the leaf samples at the same stage. The lines worked were Hybrid Transgenic line (HT), Transgenic line I (T1) and Transgenic line II (T2) of *off-patent* maize TC1507 event; the non-transgenic control samples lines were Hybrid Conventional (HC), Conventional line I (C1) and Conventional line II (C2).

Compositional analysis

The samples were kept on dry ice until they were transported to the Nutrianalisis laboratory in Bogotá (Colombia) where each of the analytes was quantified. Humidity was determined gravimetrically by placing the samples in a hot-air drying oven at approximately 135°C for 2 h (AOAC method 925.09). Total protein content was determined as total nitrogen using digestion with H_2SO_4 followed by distillation and titration (Kjeldahl method - AOAC method 979.09). Fat from the corn kernel samples was determined gravimetrically using a Soxhlet extraction with pentane as solvent (AOAC method 922.06). The crude fat of the forage samples was determined gravimetrically by means of an acid hydrolysis procedure (AOAC method 945.02). Crude fiber was estimated using a neutral detergent solution and a thermostable amylase to dissolve easily digestible proteins, lipids, sugars, starches, and pectins. A fibrous residue was obtained which consisted mainly of cell wall components including both nitrogen and indigestible nitrogenous matter (NTC method 5122). Ashes were determined by placing the samples in an electric oven at 550°C until they were incinerated. The residual ash was quantified gravimetrically (AOAC method 923.03). Carbohydrate content was estimated using the calculation previously described by Herman *et al.* (2004) and Cong *et al.* (2015).

Statistical analysis

Statistical analysis of nutrient composition data was performed using R software (R Core Team, 2021). To perform the statistical analysis, four functions were written. The first function, which was the basis for the other functions, allowed the generation of a database for graphic analysis and statistical tests of differences between groups. The second function performed an exploratory data analysis and obtained a box-and-whisker plot resulting from each analyte per genotype. The third function established the confidence intervals of each analyte according to the Composition Database of the International Institute of Life Sciences (Ridley *et al.,* 2004; AFSI-CCDB, 2020). Finally, the fourth function performed the difference tests between the genotypes according to each analyte analyzed. The database includes data from the evaluation of conventional crop samples using validated analytical methods, providing a robust collection of high-quality non-gene compositional data for various crops (Suit *et al*., 2016). The statistical tests performed included the Shapiro–Wilk test for normality, the Levene test for homoscedasticity and the Kruskal-Wallis test for differences between groups and their respective Wilcoxson *post hoc* test (Bonferroni test). Two separate statistical analyzes of the compositional data were performed. The quantification of the analytes made it possible to evaluate the data of the replicas of each of the corn grain and forage analytes using the Kruskal-Wallis test to identify statistically significant differences (α=0.05) between the genotypes and their respective Wilcoxon *post hoc* test. This evaluation allowed corrections on the level of significance to avoid increasing the type 1 error and to identify the significant differences between each of the genotypes (α=0.05).

Results and discussion

Grain analytes

The results of the proximate analysis from the grain samples collected during the field trial did not show statistically significant differences between the transgenic genotypes [Hybrid Transgenic (HT), Transgenic line I (T1) and Transgenic line II (T2)] of *off-patent* maize TC1507 event and the non-transgenic control samples [Hybrid Conventional (HC), Conventional line I (C1) and Conventional line II (C2)] (Fig. 1). For instance, the Kruskal-Wallis test showed differences for grain components such as moisture (*P*-value=0.0323), protein (*P*-value=0.0093), fat (*P*-value=0.0237), and carbohydrates (*P*-value=0.0085) in at least two of the genotypes in comparison. However, after performing a *post-hoc* test, the Mann-Whitney test (Wilcoxon rank sum test) did not show significant differences between the genotypes for all analytes that initially seemed to differ. The Wilcoxon rank sum test corrects the level of significance to avoid increasing the type I error and shows in which groups there are differences.

Figure 1 shows that, for each of the analytes corresponding to each genotype, there are no differences with the maximum and minimum ranges reported by the literature (Ridley *et al.,* 2004; AFSI-CCDB, 2020).

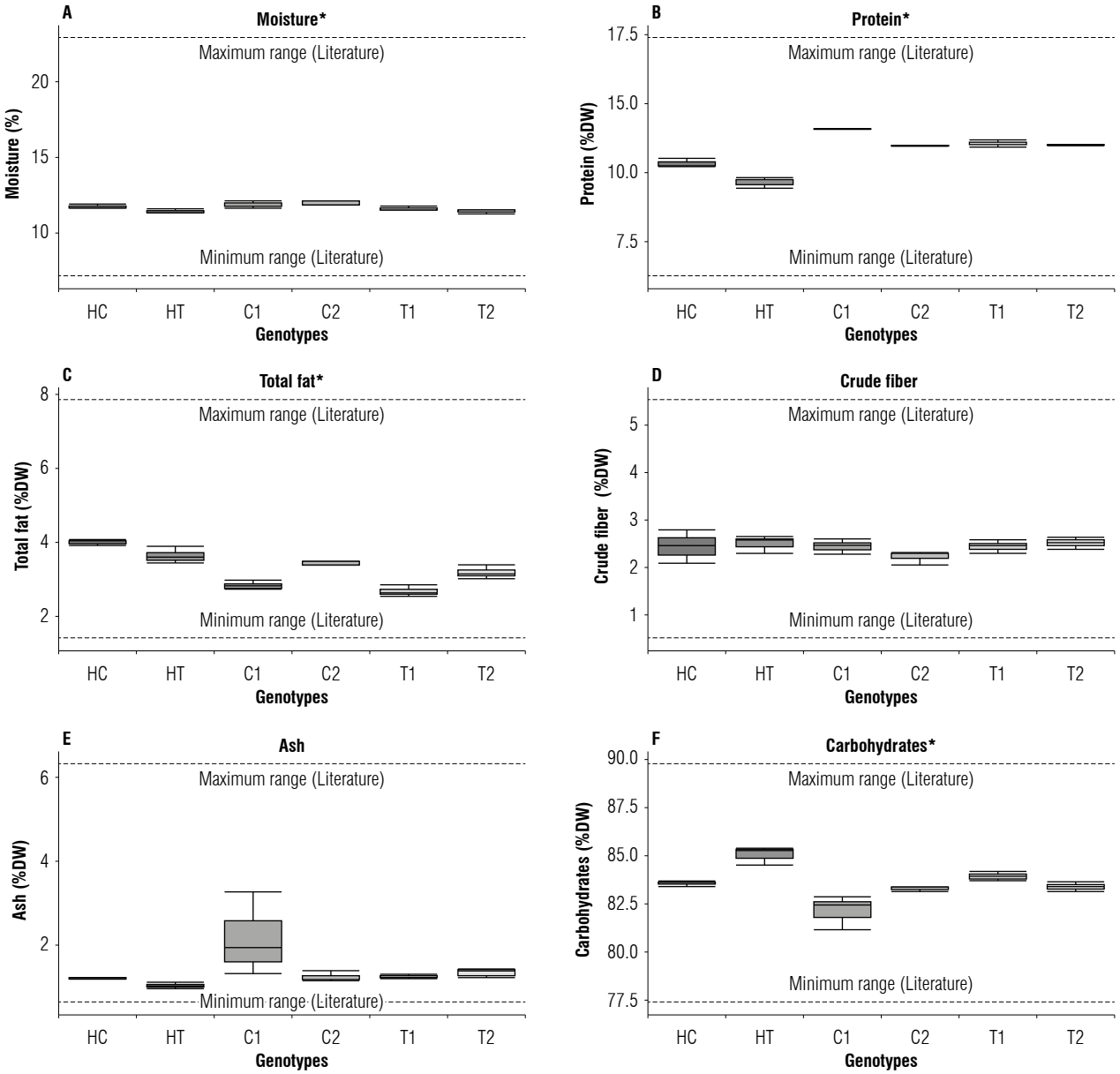

FIGURE 1. Variability of percentage values for proximate analytes from corn grains. A) Moisture; B) Protein; C) Total fat; D) Crude fiber; E) Ash; and F) Carbohydrates. [Hybrid Transgenic (HT), line Transgenic line I (T1) and line Transgenic line II (T2)] of *off-patent* maize (TC1507 event) and the non-transgenic control samples [Hybrid Conventional (HC), Conventional line I (C1) and Conventional line II (C2)]. Values are expressed as medians (±IQR, n=3) from three independent experiments. ***** indicated the *P*-value < 0.05 for Kruskal-Wallis test.

For each of the different analytes studied (Fig. 1A, D), the variation for moisture and fiber is very similar for each of the compared genotypes, although there are slight differences for other analytes (Fig. 1B, C, E, F). For total fat, the conventional hybrid tends to have higher levels than the transgenic hybrid. The comparison of the lines shows unexpected data: the transgenic line presents a variation similar to that of the comparable non-transgenic line. For protein, the transgenic hybrid presented the lowest values together with the conventional hybrid, while the C1 had the highest values in contrast to its transgenic isoline and the T2 and C2. For ash, only the C1 presented a great variation unlike the other genotypes analyzed that maintained a similar variation. For carbohydrates, the transgenic hybrid had higher levels, while the C1 showed lower values. The remaining genotypes maintained similar variations. All these apparent differences are within natural variations. All the values for analytes examined were found within the values reported in the literature (Watson, 1982; Lundry *et al.,* 2013; Cong *et al.,* 2015; AFSI, 2020) (Tab. 1).

TABLE 1. Medians, minimum and maximum values reported from the proximate grain analysis of *off-patent* maize hybrid TC1507 (transgenic), *off-patent* maize lines TC1507 I and II (transgenic), their respective conventional isolines I and II (non-transgenic) and a commercial maize hybrid (non-transgenic) from the samples collected from the field trials in Colombia.

Variable (% dry weight)	Hybrid Transgenic (TC1507)	Transgenic line I	Transgenic line II	Conventional line I	Conventional line II	Hybrid Conventional	Literature range*
	(HT)	(T1)	(T2)	(C1)	(C2)	(HC)	
Total fat	3.54 ± 0.355 (3.41 - 3.84)	2.58 ± 0.15 (2.52- 2.82)	3.09 ± 0.18 (2.99 - 3.35)	2.76 ± 0.12 (2.70 - 2.94)	3.40 ± 0.045 (3.35 - 3.44)	3.97 ± 0.15 (3.88 - 4.04)	1.363 – 7.83
Protein	10.34 ± 0.24 (10.05 - 10.53)	12.17 ± 0.17 (11.99 - 12.33)	12.10 ± 0.03 (12.07 - 12.13)	12.84 ± 0.015 (12.83- 12.86)	12.06 ± 0.05 (12.06 - 12.07)	11.15 ± 0.21 (11.05 - 11.47)	5.72 – 17.26
Fiber	2.56 ± 0.185 (2.25 - 2.62)	2.42 ± 0.13 (2.28 - 2.54)	2.48 ± 0.125 (2.35 - 2.60)	2.42 ± 0.165 (2.24 - 2.57)	2.25 ± 0.13 (2.02 - 2.28)	2.41 ± 0.355 (2.05 - 2.76)	0.49 – 5.5
Ash	1.05 ± 0.07 (0.99 - 1.13)	1.24 ± 0.05 (1.23 - 1.33)	1.38 ± 0.095 (1.24 - 1.43)	1.92 ± 0.96 (1.34 - 3.26)	1.19 ± 0.12 (1.17 - 1.41)	1.22 ± 0.01 (1.21 - 1.23)	0.616 – 6.282
Moisture	11.33 ± 0.105 (11.29 - 11.50)	11.43 ± 0.03 (11.40 - 11.74)	11.44 ± 0.13 (11.25 - 11.51)	11.87 ± 0.255 (11.58 - 12.09)	11.92 ± 0.14 (11.79 - 12.07)	11.68 ± 0.12 (11.62 - 11.86)	7 – 23
Carbohydrates	85.26 ± 0.43 (84.50 - 85.36)	83.91 ± 0.26 (83.68 - 84.20)	83.40 ± 0.26 (83.15 - 83.67)	82.46 ± 0.835 (81.21 - 82.88)	83.33 ± 0.09 (83.17 - 83.35)	83.65 ± 0.125 (83.44 - 83.69)	77.4 – 89.7

* Watson (1982), Lundry *et al.* (2013), Cong *et al.* (2015), Anderson *et al.* (2019), AFSI (2020).

Forage

The results of the proximate analysis for the forage samples collected during the field trial did not show statistically significant differences between the transgenic genotypes (HT, line T1, and line T2) of *off-patent* maize event TC1507 and the non-transgenic control samples (HC, line C1, and line C2). The Kruskal-Wallis test showed differences only for the fat component (*P*-value=0.03), finding that at least two of the genotypes in comparison exhibited differences. However, after performing a *post-hoc* test, the Mann-Whitney test (Wilcoxon rank sum test) showed no significant differences between the genotypes (Tab. 2).

There were no cases in which the analyzed analytes showed differences in their maximum and minimum ranges with those reported in the literature (Fig. 2). For moisture, protein, fiber, fat, ash, and carbohydrates, the observed variations were very similar between each of the compared genotypes, although at first glance there are slight differences. For moisture, only the HT presented lower values than the other genotypes, which presented values slightly above the maximum limit reported in the literature (Fig. 2A). For fat, the HT presented lower values and T1 higher values compared to the other genotypes (Fig. 2C). For crude fiber, only the hybrids showed slight differences, while the

TABLE 2. Medians, minimum and maximum values reported from forage proximate analysis of *off-patent* maize hybrid 1507 (transgenic), *off-patent* maize lines 1507 I and II (transgenic), their respective conventional isolines I and II (non-transgenic) and a commercial maize hybrid (non-transgenic) from samples collected in field trials in Colombia.

Variable (% dry weight)	Hybrid transgenic (TC1507)	Conventional transgenic line I	Conventional transgenic line II	Conventional line I	Conventional line II	Hybrid Conventional	Literature range
	(HT)	(T1)	(T2)	(C1)	(C2)	(HC	
Total fat	1.28 ± 0.135 (1.11 - 1.38)	2.46 ± 0.43 (1.69 - 2.55)	2.07 ± 0.095 (1.90 - 2.09)	1.65 ± 1.41 (1.49 - 1.97)	1.57 ± 0.19 (1.29 - 1.67)	1.81 ± 0.225 (1.52 - 1.97)	1.04 – 6.755
Protein	14.21 ± 0.57 (13.88 - 15.02)	16.04 ± 0.285 (15.72 - 16.29)	16.03 ± 0.33 (15.42 - 16.08)	16.09 ± 0.645 (14.93 - 16.22)	16.06 ± 0.435 (15.23 - 16.10)	16.07 ± 0.36 (15.62 - 16.34)	3.14 – 16.32
Fiber	24.74 ± 0.97 (24.49 - 26.43)	22.29 ± 2.11 (19.79 - 24.01)	21.31 ± 1.005 (21.13 - 23.14)	20.12 ± 1.92 (19.52 - 23.36)	22.37 ± 0.9 (22.30 - 24.28)	19.54 ± 1.985 (18.15 - 22.12)	12.5 – 42
Ash	12.06 ± 0.41 (11.60 - 12.42)	10.85 ± 0 .305 (10.78 - 11.39)	10.87 ± 1.295 (9.66 - 12.25)	10.50 ± 2.045 (9.99 - 14.08)	11.28 ± 1.375 (8.62 - 11.37)	12.32 ± 0.47 (12.04 - 13.10)	0.66 – 13.2
Moisture	73.81 ± 4.93 (68.93 - 78.79)	74.14 ± 5.725 (67.91 - 79.36)	71.79 ± 0.295 (71.29 - 71.88)	72.66 ± 1.405 (71.62 - 74.43)	72.33 ± 0.47 (72.31 - 73.25)	74.69 ± 3.125 (70.26 - 76.51)	55.3 – 87.1
Carbohydrates*	71.45 ± 0.945 (71.35 - 73.24)	69.97 ± 1.25 (68.38 - 70.88)	69.98 ± 0.895 (69.43 - 71.22)	69.31 ± 3.545 (66.34 - 73.43)	71.75 ± 1.67 (68.86 - 72.20)	67.54 ± 0.685 (66.65 - 68.02)	66.9 – 92.9

* Watson (1982), Lundry *et al.* (2013), Cong *et al.* (2015), Anderson *et al.* (2019), AFSI (2020).

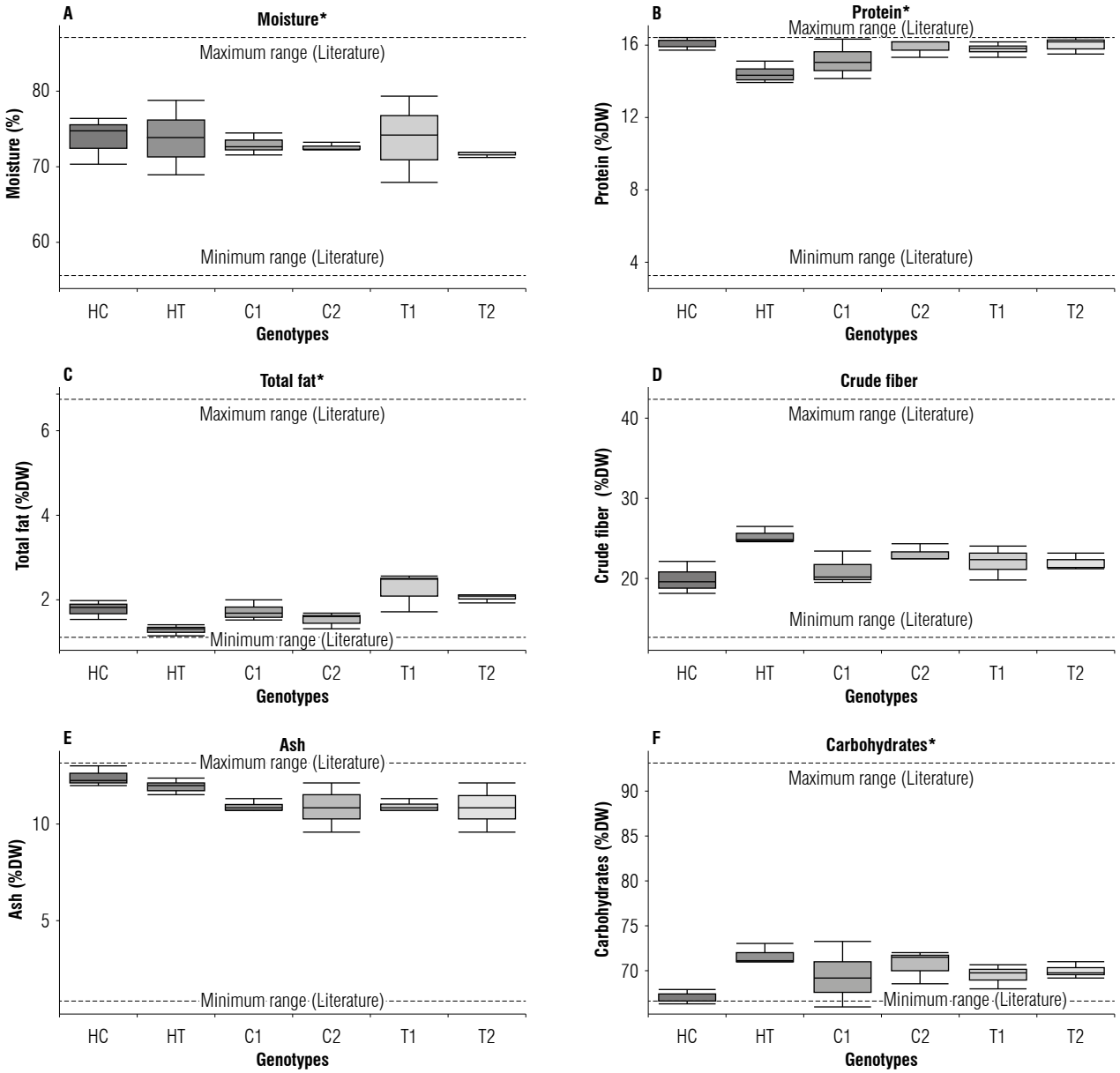

FIGURE 2. Variability of percentage values for proximate analytes from corn forage. The methods used for estimating the percentage values for each genotype are described in the Material and methods section. A) Moisture; B) Protein; C) Total fat; D) Crude fiber; E) Ash; and F) Carbohydrates. [Hybrid Transgenic (HT), line Transgenic line I (T1) and line Transgenic line II (T2)] of *off-patent* maize (TC1507) event and the non-transgenic control samples [Hybrid Conventional (HC), Conventional line I (C1) and Conventional line II (C2)]. Values are expressed as medians (± SD, n=IQR, n=3) from three independent experiments. * indicated the *P*-value < 0.05 for Kruskal-Wallis -test.

conventional and the transgenic lines showed lower and higher values, respectively (Fig. 2D). For moisture, differences were not observed, although greater variations were observed for both the HT and the T1. For ash, the HC showed slightly higher values compared to the other genotypes (Fig. 2A and E, respectively). For carbohydrates, the conventional hybrid showed slightly lower values than the other genotypes (Fig 2F). The conventional hybrid had a carbohydrate value lower than that reported in the literature, while its other values (highlighting the median) were found within the ranges reported. All these apparent differences were found within natural variation. All the values for the analytes examined were found within the values reported in the literature (Watson, 1982; OECD, 2002; Lundry *et al.,* 2013; Cong *et al.,* 2015; Anderson *et al.,* 2019; AFSI, 2020) (Tab. 2).

Analysis strategy of the substantial equivalence of *off-patent* corn

The developers of different transgenic events, both simple and stacked, have obtained authorizations in Colombia for human and animal consumption using data portability, since the trials in most cases were not carried out in Colombia but in northern agroecosystems (ICA, 2011, 2013; MPSP, 2012a, 2012b, 2012c, 2014; INVIMA, 2018). These developers have performed substantial equivalency assessments based on the compositional analysis of proximate analytes.

Based on the principle of equality, the strategy for *off-patent* crops with transgenic events that have been previously authorized in Colombia could be incorporated into conventional breeding since it is in the public domain (its use does not violate the rights of third parties). In addition, the analysis of freedom of operation (Jiménez *et al.,* 2016; Rojas *et al.,* 2017) makes it possible to carry out field trials with a minimal design, where a limited number of proximate analytes equal to the number examined by developers of the specific event that allowed to support the substantial equivalence of stacked events that contain the same event.

The case of stacked events (stacked traits or crop stacking) originating from conventional plant breeding, where two or more parents with a single transgenic event are crossed to produce a progeny containing two or more transgenic events that provide a useful grouping of traits would be the closest issue to *off-patent* events. The discussion that has arisen today could help resolve the regulatory gap and allow the authorization for human and animal consumption of these derived crops, where substantial equivalence and other safety assessments play a fundamental role (Kok *et al.,* 2014). From a regulatory point of view, stacked trait and *off-patent* crops differ from single trait crops, since they contain events that are not new to regulatory authorities considering that they have been fully evaluated in the single events that make them up. This distinction is crucial since the concern for the safety of transgenic crops is centered around the safety of the introduced trait and the possibility of unintended adverse effects from its introduction (Acosta & Chaparro, 2008; Codex Alimentarius, 2009; Herman & Price, 2013).

Safety assessment approaches for stacked trait crops vary globally. There are regulatory bodies that address a crop of stacked traits as if it were a crop with a new event that requires a de novo assessment, despite the existence of evidence and safety evaluations demonstrating the safety and substantial equivalence for each one of the individual transgenic events that make up the crop with stacked events. This approach is based on the concern about the possible interactions between events that could give rise to characteristics of the plant different from the expected sum of the stacked events. Steiner *et al.* (2013) extensively analyzed the overall potential for event interactions in stacked event crops. The authors highlighted that since the functional characteristics of the introduced expression cassettes are known, it is possible to develop hypotheses as to whether a specific combination of traits would interact to affect plant metabolism in a novel way. It was also pointed out that if a hypothetical interaction posed any risk, for example as in the case of stacking of tolerance traits to glyphosate and dicamba through conventional plant breeding, the molecular mechanisms of the enzymes that mediate these stacked tolerances would exclude any plausible interactions. In fact, the substantial equivalence of a crop with stacked traits that confers tolerance to glyphosate and dicamba has been empirically demonstrated (Taylor *et al.,* 2017). Specifically, for corn containing traits of resistance to insects and tolerance to glufosinate-ammonium, there is no plausible hypothesis that their combined presence affects the metabolism of the plant and leads to compositional differences. Likewise, it is even less plausible that undesirable interactions occur in an *off-patent* obtained from the crossing between a transgenic hybrid and a conventional maize line.

In the global landscape of regulations on genetically engineered crops, there are currently two general approaches to assess food and feed safety for stacked event crops that could be applied for *off-patent* crops. In the first approach, no additional data or evaluations are required for a stacked trait crop generated by conventional plant breeding whose constituent events have previously been evaluated and approved. Regulatory agencies may require written notification for the stacked event product to be marketed. Examples of agencies that follow this approach include the FDA and APHIS-USDA of United States, the Canadian Food Inspection Agency, the Canadian Ministry of Health, the Australian and New Zealand Food Standards Agency, and the Food Safety Commission and the Ministry of Agriculture, Forestry and Fisheries of Japan (Pilacinski *et al.,* 2011; Steiner *et al.,* 2013; FSCJ, 2016; Goodwin *et al.,* 2021). In the second approach, a safety assessment is required for any stacked trait crop, regardless of whether its constituent events were previously assessed and approved. Some examples of regulatory agencies that follow this approach are the European EFSA, the Ministry of Food and Pharmaceutical Safety of the Republic of Korea, the Food and Drug Administration of Taiwan, and the Federal

Commission for the Protection against Sanitary Risks of Mexico (Pilacinski *et al.,* 2011; Steiner *et al.,* 2013; FSCJ, 2016; Goodwin *et al.,* 2021).

In rational and scientifically sustainable risk assessment (Pilacinski *et al.,* 2011; Steiner *et al.,* 2013; FSCJ, 2016; Goodwin *et al.,* 2021), it is important to follow the case of stacked trait crops that could contribute to solving the regulatory challenge of *off-patent* crops. In this context, the formulation and evaluation of a risk hypothesis for a crop in question is central in determining whether further evaluation of the safety of a product with stacked traits is needed.

Today there is a growing body of empirical data in reports and/or in scientific articles evidencing the safety of genetically modified crops of relevant single events as well as the safe historical use of conventional plant breeding. Therefore, there is valid scientific justification to eliminate or make more flexible the mandatory requirements aimed at presenting and evaluating characterization, safety, and substantial equivalence data for each new crop of stacked events (Goodwin *et al.,* 2021) and, therefore, for *off-patent* crops. Pragmatically, as proposed by Bell *et al.* (2018) and Goodwin *et al.* (2021), a gradual approach could start with flexibility and end with the elimination of the requirements for safety assessments for the crops of stacked events (and therefore *off-patent* crops). Along these lines, the first step could be to refine the approach towards a single specific analysis of stacked events. This approach would provide an empirical bridge to the previous evaluations performed for the approved transgenic events that make up the stacked events. In this way, a simplified evaluation of the stacked event or of the *off-patent* crop would focus on the composition, evaluating the substantial equivalence from the proximate analytes of the grain (and from the forage, in the case of resistance to insects) in the context of known natural variability. Such an assessment could be used to complement previously reached safety conclusions for constitutive individual trait products (Bell *et al.,* 2018; Goodwin *et al.,* 2021).

The comparative analysis of corn during field trials carried out in Colombia showed no significant differences between *off-patent* hybrids of lines 1507 I and II obtained by conventional crossing with their non-genetically modified counterparts. Small exceptional variations were not indicative of a general pattern of changes derived from crossing with a genetically modified hybrid; these are explained by the well-known natural variation (genotype-environment interaction). These variations in composition arise due to a wide range of factors specific to the biology of the species and, particularly, to the growing conditions (location, irrigation, type of soil, etc.) that influence the growth and biochemistry of plants. In the present study, no significant biological differences were found between *off-patent* TC1507 event corn and non-transgenic conventional corn. Therefore, the *off-patent* hybrid is substantially equivalent from a compositional point of view to its conventional counterpart, except for the genetically engineered characteristics. The composition of the *off-patent* TC1507 corn hybrid was found within the normal ranges of variation observed in non-genetically modified varieties with recent biotechnological tools.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

HS and ACG formulated the research objectives. ACG supervised and directed the planning and execution of the research activities. HS, DB, and ACG performed the experimental design. HS carried out the research and investigation process, particularly conducting field trials and data collection. HS and DB applied statistical techniques and wrote the code to synthesize and analyze compositional data. OA discussed the results, performed a critical review of the methodological and regulatory aspects, revised the initial version of the manuscript, and wrote the English version. All authors, except ACP (R.I.P.), reviewed the manuscript.

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Application of the BLUPe predictor in the selection of potential soybean varieties for Orinoquia

Aplicación del predictor BLUPe en la selección de variedades potenciales de soya para la Orinoquía

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ABSTRACT

The Colombian Orinoquía and the Altillanura subregion show comparative and competitive advantages for soybean production (edaphoclimatic conditions, cost-benefit ratio, potential area, and location) essential food with a high protein content (~37%) and used mostly for poultry and pig nutrition. However, this immense region has scarce varietal alternatives of high grain yield and quality that are adapted to its climatic and edaphic conditions. The current research is based on the selection of superior lines or potential varieties with high genetic merit using the restricted maximum likelihood/standardized best linear unbiased predictor (REML/BLUPe) procedure. Sixty advanced lines and four commercial varieties were tested in an 8x8 alpha lattice design. Grain yield (GY) oscillated between 1,117 and 4,431 kg ha^{-1}, the population average yield was 2,682 kg ha^{-1}, and BLUPe predictors ranged between 5.37 and -3.71. With a t-test at a significance of 5% (1.67) and a predictor comparator of (t1-t2)≥1.67 (√2), six outstanding lines were identified with superior BLUPe values compared to the mean (P<0.05) and GY>3,500 kg ha^{-1}. In descending order (kg ha^{-1}), the GY was: L-041 (4,431), L-019 (4,326), L-104 (3,923), L-149 (3,832), L-202 (3,536), and L-201 (3,519 kg ha^{-1}). The BLUPe standardized predictor allowed an effective selection (92%) of lines.

Key words: Altillanura, genetic breeding, mixed models, REML.

RESUMEN

La Orinoquía colombiana y en particular, la subregión de la Altillanura presenta ventajas comparativas y competitivas para la producción de soya (condiciones edafoclimáticas, relación costo-beneficio, área potencial y ubicación), alimento esencial por su contenido proteico (~37%), especialmente para aves y cerdos. Sin embargo, las alternativas varietales adaptadas y de alto rendimiento de grano son escasas para esta vasta región. Por ello, la presente investigación fue orientada hacia la selección de líneas mejoradas o variedades potenciales con alto mérito genético mediante la aplicación del mejor predictor lineal insesgado estandarizado (REML/BLUPe). Se evaluaron 60 líneas avanzadas y cuatro variedades comerciales en un diseño Alfa látice 8x8. El rendimiento de grano (RG) osciló entre 1117 y 4431 kg ha^{-1}, el promedio poblacional fue de 2682 kg ha^{-1} y los predictores BLUPe oscilaron entre 5.37 y -3.71. Con una significancia t del 5% (1.67) y un comparador de predictores (t1-t2)≥1.67 (√2), se identificaron 6 líneas sobresalientes con BLUPe superiores a la media (P<0.05) y RG>3500 kg ha^{-1}. En orden descendente el RG fue: L-041 (4431), L-019 (4326), L-104 (3923), L-149 (3832), L-202 (3536) y L-201 (3519 kg ha^{-1}). El predictor estandarizado BLUPe permitió una selección efectiva (92%) de líneas.

Palabras clave: Altillanura, mejoramiento genético, modelos mixtos, REML.

Introduction

The Orinoco basin is shared by Colombia and Venezuela and has an area of 981,446 km^2. Thirty-five percent of this vast region is located in the equatorial zone of Colombia between 2°N and 10°N and 60°W and 75°W (Vásquez Cerón *et al*., 2019). In the savannah complex of the Orinoco basin, the Colombian Altillanura includes 2.8 million ha that, according to the CONPES document No. 3797 (DNP, 2014), has high agricultural potential with typical oxidic soils, where soybean is projected to have comparative and competitive advantages as a first-class raw material as balanced feed, especially for poultry, pigs, and the production of vegetable oil.

In the Altillanura only 39,793 ha are planted per year, including two sowing cycles, with a limited varietal offering and a production of close to 119,412 t of grain (FENALCE, 2021). This supply, representing less than 7% of the national demand, has motivated agribusinesses to approach national soybean production as an inclusive, economically viable, and ecologically sustainable regional agricultural development. Currently, the Corporación Colombiana de Investigación Agropecuaria - Agrosavia is carrying out a

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soybean breeding program for Oxisols from the Orinoquía region. This development aims to generate varieties adapted to the low tropics with high yield potential, vegetative cycles compatible to integrated crop rotation systems, tolerant to adverse biotic and abiotic factors. The importance of expanding the existing varietal genetic base for this vast region and the need to close the grain yield gap with varieties that exceed 3,400 kg ha^{-1} is highlighted.

The process of genetic breeding is arduous and complex. Its initial phase implies the introduction, characterization, and evaluation of diverse germplasm, a crossing plan in search of the best genetic combinations, and later a selection of superior individuals that will become advanced lines or potential varieties. These lines, their components, and sanitary behavior are evaluated in a first cycle through preliminary yield trial (PYT). Then, the best lines are subjected to multi-environment trials (MET) to determine their level of phenotypic stability. The most outstanding ones are subjected to regional (RT) or agronomic evaluation trials (AET), regulated by the Colombian Agricultural Institute (ICA, 2020), where the superior line or lines with identity attributes, homogeneity, and stability are registered as new cultivars.

Grain yield and other agronomic traits in genetic breeding programs have traditionally been valued using the classical generalized linear model (GLM). However, these models do not satisfy the statistical assumptions, particularly with unbalanced databases (Resende, 2007) nor through the predominant application of fixed factors usually analyzed through mean comparison tests such as DHS Tukey, Scheffé, and LSD, etc. There are statistical procedures, such as restricted maximum likelihood/best linear unbiased prediction (REML/BLUP), generically called a mixed model methodology (Sturion & Resende, 2010; Bandera-Fernández & Pérez-Pelea, 2018) that improves the estimation and prediction of genetic parameters and the effectiveness of selection to reduce these biases. Mixed models have provided valuable information for selecting lines by genetic merit. According to Piepho *et al.* (2008), BLUP is better in genetic breeding and variety evaluation precision than the best linear unbiased estimator (BLUE). The BLUP predictor has advantages over BLUE by reducing the response due to environmental effects and efficiently discriminating genotypes with high varietal potential (Pacheco *et al*., 2020). The mean associated with fixed-effects in BLUE is an average performed over all the effect levels in the population, while BLUP is a regression towards the general mean based on the variance and covariance components associated with the random-effects model (Casanoves & Balzarini, 2002). BLUP has a shrinkage property, so the prediction values tend to be less separated from the mean than the original values, increasing the precision of the analysis (Baselga & Blasco, 2008). In this sense, BLUP is a standard method for random effects and requires the estimation of the genetic variance ($\sigma^2 g$) and residual (σ^2) components, preferably through REML (Mora & Arnhold, 2006). The latter compensates for the loss of degrees of freedom that results from the estimation of the fixed effects and produces less biased estimates (Patterson & Thompson, 1971). Overall, for authors such as Searle *et al*. (1992), Robinson (1991), Piepho and Möhring (2006), Resende (2016), and Volpato *et al*. (2019), REML/BLUP has become an effective procedure for estimating parameters and predicting genetic values to optimize selection.

Resende and Duarte (2007) suggest subjecting experiments with more than 10 cultivars or genotypes to a BLUP analysis as a random source of the mixed model. The genotype ranking could be similar for phenotypic selection and the one performed by BLUP when data are balanced. Although it is essential to link pedigree information in the genetic merit analysis, for Piepho *et al*. (2008), it is easy to exploit the information via BLUP through a simple mixed model without explicit reference to the pedigree. BLUP maximizes the correlation between the real and predicted genotypic values, *i.e*., the main objective of the breeder. In this regard, Panter and Allen (1995), evaluating highly related individuals, found that the kinship matrix link in the BLUP analysis is not justified. However, if the data of the historical parents are available in crosses that are not closely related, pedigree data should be included because it can improve the prediction accuracy of progeny performance.

Accordingly, the current research was carried out to select advanced soybean lines or potential varieties with high genetic merit and grain yield >3,500 kg ha^{-1} in a preliminary yield trial for Oxisols of the Colombian Orinoquean region, using the REML/BLUP methodology and its standardized BLUPe value.

Materials and methods

Study area

A preliminary yield trial (PYT) of 60 advanced soybean lines of the Agrosavia genetic breeding program and four commercial varieties (C. Superior 6, C. Achagua 8, C. Primavera 11, and Soyica P-34) was carried out during the second period of 2020 at the research center La Libertad,

Villavicencio, Meta (Colombia). The study site is located at 4°22' N and 72°13' W, in Oxisols with a pH of 4.9, 5.9 mg L^{-1} of phosphorus, 2.4% of organic matter, and a base saturation of 57.3%.

Plant material, trials, and variables

The soybean lines and varieties were planted in plots with four rows of 5 m long, 0.45 m apart, with a distance between plants of 0.07 m. Mineral and biological fertilization with *Bradyrhizobium japonicum* was uniform according to soil analysis and crop requirements. The experimental units were distributed in an alpha lattice design proposed by Patterson and Williams (1976) of 8x8 with two replicates. Grain yield was evaluated as the response variable of interest for the BLUP analysis, and the following phenotypic variables that characterize the genotypes under study were registered.

Grain yield (GY) included grain weight in kg ha^{-1} per experimental unit and grain moisture contents of 14%; days to flowering (DF) was the number of days between emergence and flowering in the upper nodes of the plant; days to physiological maturity (DM) was the number of days from emergence to reaching 95% of mature pods in each plot, at which point the average height (H) and the number of nodes (NN) per plant were also recorded.

Additionally, the qualitative description of the lines was made using flower color (FC), growth habit (GH), and pubescence color (PC).

To determine water excess and deficits during the crop cycle, the hydric balance model (Allen *et al*., 2006) and the database of the meteorological station La Libertad ascribed to IDEAM were used. Real evapotranspiration was obtained using the tank coefficient (Kp) (Cruz Valderrama, 2015) and the FAO cultivation coefficients (Kc) (FAO, 2000).

Statistical analysis

In the analysis of variance, the statistical model described by Singh and Bhatia (2017) for incomplete blocks was applied (Equation 1):

$$Y_{ijk} = \mu + G_i + \gamma_j + \rho_{k(j)} + \varepsilon_{ijk} \quad (1)$$

Where Y_{ijk} is the phenotype of i^{th} genotype in j^{th} replication and k block; G_i is the genotype effect i=1,2……..k; γ_j is the replicate effect j= 1,2,……r; $\rho_{k(j)}$ is the block within replicate effect k= 1,2….S; ε_{ijk} is the random error. For the statistical analysis, the matrix structure of the mixed linear model (Piepho *et al*., 2008) was applied using the SAS 9.4 software (SAS Institute, 2014). The variance components were estimated with the PROC MIXED/REML procedure of the SAS System (Bueno Filho & Vencovsky, 2000). This analysis considers lines and the block effects within the repetition as random effects. The comparison of means was carried out using the BLUP predictors (best linear unbiased prediction) that represent the predicted value for each genotype with respect to the general mean (Biasutti, 2012). The comparison of the BLUPs between lines/varieties was performed with the t statistic (Yan & Rajcan, 2002). This statistical comparator called BLUPe (standardized BLUP) was obtained from the relationship between the predicted empirical BLUP value and the associated prediction error. The BLUPe (standardized BLUP) was obtained from the BLUP predictor or predicted value for each genotype concerning the general mean (Yan & Rajcan, 2002). For the comparison analysis of the standardized predicted values, the genotypes are considered different if the BLUPe values met the following condition, with a t-test significance of 5%: (t1-t2)≥1.67 (√2) (Yan *et al*., 2002). Additionally, the probabilistic values generated by SAS/Mixed for each BLUPe were used to determine the superiority ($P \leq 0.05$) or inferiority ($P > 0.05$) of the lines/varieties concerning the general mean (Casanoves & Balzarini, 2002). The BLUPe pair GY were ordered in descending order to identify the genotypes or superior lines. This methodology allowed comparing free genetic values of environmental effects and not the phenotypic means to improve genetic gain in the subsequent selection cycle.

The elevated kinship of the parents that gave rise to the group of lines makes using the coefficient of coancestry in the BLUP analysis unnecessary since it does not represent a change in the results. Furthermore, when the purpose is to estimate the total genotypic value, it is reasonable not to use the coefficient of coancestry (Piepho *et al*., 2008).

The level of experimental precision in selecting superior lines was measured based on the square root of broad-sense heritability, calculated on a mean plot basis using the following equation (Hacker & Cuany, 1997):

$$H^2 = \frac{Vg}{\left(Vg + \frac{Ve}{r}\right)} \quad (2)$$

Where *Vg* represents genetic variance, *Ve* is the environmental variance, and *r* is the repetitions.

Results and discussion

The descriptive analysis of the variables DF, DM, H, NN, and GY (Tab. 1) allowed inferring a high variability as a genetic source for varietal improvement. The wide ranges of DF and DM (22 and 29 d, respectively) underlined the differential behavior of the genotypes in precociousness, together with the high variation of H, NN, and GY. In general, the lines and varieties with indeterminate GH showed higher H than those with semi-determined GH, and these, in turn, were higher than those with determined GH. Plants with indeterminate GH continue to grow for a long time after flowering, while those with determinate GH finish stem growth when or shortly after flowering begins, often resulting in fewer nodes than the indeterminate GH (Fehr & Caviness, 1977). These differences in H and GH do not necessarily represent lower yields in those with determined GH since agronomic-importance traits such as GY are quantitative (Volpato *et al*., 2019), where the environmental effect represents a large part of the variation. In the analysis of variance, highly significant differences ($P<0.01$) between lines and varieties were ratified for the case of GY by BLUPe predictor values ranging between 5.37 and -3.71. The GY range was between 1,117 and 4,431 kg ha^{-1}. It is noteworthy that, in the edaphoclimatic conditions of the current study, there were no phytosanitary problems that affected the GY response variable.

A highly significant positive Pearson correlation ($P<0.01$) was found between GY and DM (r=0.32) that means that the higher the DM, the higher the GY under the agroclimatic conditions of the preliminary yield trial. In contrast, a shortening of the filling period is frequently associated with decreasing grain weight (Kantolic *et al*., 2004).

BLUPe vs. grain yield

The BLUP and BLUPe predictors and the significance of the random effects for GY are presented in Table 2. The BLUPe (t1-t2)≥1.67 ($\sqrt{2}$) comparator allowed an effective differentiation of the genotypes; by ordering them in descending order, a range with a maximum of 5.37 and a minimum of -3.71 for lines L-019 and L-078 was reached. Genotypes with positive BLUPe for GY were classified as higher, and those with a negative value were considered lower with respect to the general mean of 2,682 kg ha^{-1}. The higher the BLUPe, the greater the probability of success in selecting superior lines. The standardized BLUP (BLUPe) is more discriminant than the empirical BLUP when selecting outstanding lines because the prediction error adjusts it (Casanoves & Balzarini, 2002). Therefore, a higher mean does not necessarily represent a higher BLUPe, as occurs between lines L-041 and L-104. The lines with GY≥3,519 kg ha^{-1} showed statistical differences ($P>|t|$) concerning the general mean and positive BLUPe ≥ 2.52 (Fig. 1), and they constituted promising lines or potential varieties. In descending order, the GY (kg ha^{-1}) were: L-041 (4,431), L-019 (4,326), L-104 (3,923), L-201 (3,832), L-149 (3,536), and L-202 (3,519), with higher GY for the Colombian Orinoco region.

The genetic base of these lines includes the varieties that were developed for the Colombian Orinoco, such as Orinoquía 3, C. Libertad 4, and C. Taluma 5, sources of adaptation to the low tropics, with prominent differences in precocity and high yield potential that were crossed with elite materials from the EMBRAPA variety bank (Brazil).

The only control variety that exceeded the general average was C. Primavera 11, although with a BLUPe significantly lower than L-019. The other controls did not differ statistically from the mean, where the C Achagua 8 variety had a positive BLUPe (0.55), while in the Soyica P-34 and C. Superior 6 varieties, the predictor was negative (-0.03 and -1.71). The upper lines, representing a selection pressure of 10% with an estimated genetic gain of 37.2% concerning the average, were subjected to multi-environment trials to assess their behavior in different agroclimatic conditions and establish their phenotypic stability.

TABLE 1. Descriptive analysis of agronomic and yield characteristics of lines evaluated.

Statistics	DF	DM	H (cm)	NN	GY (kg ha^{-1})
N	127	124	123	124	116
Minimum	31	80	43	11	1,117
Maximum	53	109	112	25	4,431
Mean	40	93	77	17	2,682
SD	5	8	18	3	690
CV (%)	13	8	14	16	26

DF: days to flowering; DM: days to physiological maturity; H: average plant height at maturity; NN: average number of nodes; GY: grain yield; N: data number; SD: standard deviation; CV: coefficient of variation.

TABLE 2. Grain yield (GY, kg ha^{-1}), BLUP and BLUPe predictors, and significance level of soybean lines and varieties in the preliminary yield trial (PYT), Research Center La Libertad, Orinoquía, 2020.

Line/Variety	GY	BLUP	BLUPe	Pr>|t|	Line/Variety	GY	BLUP	BLUPe	P>|t|
L-019	4,326	1,377.76	5.37	<.0001	L-052	2,632	-30.02	-0.12	0.9076
L-104	3,923	1,042.97	4.06	0.0002	L-003	2,620	-40.06	-0.16	0.8769
L-041	4,431	1,253.49	3.81	0.0005	L-051	2,615	-43.94	-0.17	0.8651
C.Primavera11	3,556	737.77	2.87	0.0067	L-139	2,588	-66.87	-0.26	0.7960
L-149	3,536	721.54	2.81	0.0079	L-171	2,515	-127.22	-0.5	0.6232
L-202	3,519	706.98	2.75	0.0091	L-123	2,502	-137.68	-0.54	0.5951
L-201	3,832	827.67	2.52	0.0163	L-029	2,459	-173.67	-0.68	0.5031
L-058	3,371	584.31	2.28	0.0288	L-006	2,416	-209.55	-0.82	0.4197
L-085	3,292	518.88	2.02	0.0506	L-154	2,406	-217.57	-0.85	0.4023
L-102	3,288	514.99	2.01	0.0523	L-071	2,277	-278.12	-0.85	0.4031
L-061	3,281	509.10	1.98	0.0549	L-143	2,396	-226.27	-0.88	0.3839
L-145	3,276	505.16	1.97	0.0567	L-174	2,231	-311.09	-0.95	0.3502
L-153	3,267	498.20	1.94	0.0600	L-022	2,193	-337.79	-1.03	0.3109
L-049	3,208	448.79	1.75	0.0888	L-124	2,034	-450.82	-1.37	0.1786
L-103	3,205	446.59	1.74	0.0903	L-193	2,236	-359.24	-1.4	0.1702
L-136	3,178	424.16	1.65	0.1071	L-055	2,228	-365.60	-1.42	0.1629
L-090	3,150	400.47	1.56	0.1274	L-050	2,203	-386.95	-1.51	0.1404
L-140	3,090	350.37	1.36	0.1807	L-007	2,187	-399.55	-1.56	0.1282
L-068	3,076	339.00	1.32	0.1949	L-166	2,173	-411.66	-1.6	0.1174
L-016	2,971	251.41	0.98	0.3339	C.Superior 6	2,141	-438.31	-1.71	0.0962
L-115	2,946	230.62	0.9	0.3750	L-183	2,076	-492.40	-1.92	0.0629
L-167	2,924	212.71	0.83	0.4128	L-189	2,028	-531.79	-2.07	0.0454
L-204	3,032	259.02	0.79	0.4359	L-175	2,026	-533.44	-2.08	0.0448
L-048	2,910	201.18	0.78	0.4384	L-047	2,011	-546.06	-2.13	0.0402
C. Achagua 8	2,837	140.08	0.55	0.5887	L-106	1,985	-567.57	-2.21	0.0334
L-217	2,875	147.07	0.45	0.6573	L-180	1,377	-918.42	-2.79	0.0082
L-150	2,789	100.82	0.39	0.6969	L-229	1,380	-916.17	-2.79	0.0084
L-215	2,811	101.94	0.31	0.7583	L-107	1,803	-719.40	-2.8	0.0080
L-082	2,741	60.20	0.23	0.8160	L-216	1,762	-753.15	-2.93	0.0057
L-056	2,695	22.11	0.09	0.9319	L-182	1,637	-856.78	-3.34	0.0019
Soyica P-34	2,658	-8.48	-0.03	0.9738	L-002	1,117	-1102.91	-3.35	0.0018
L-147	2,638	-24.94	-0.1	0.9232	L-078	1,523	-951.85	-3.71	0.0007

BLUP: best linear unbiased predictor; Standardized BLUP: BLUPe=BLUP/prediction error; $P>|t|$: t-value probability; GY: grain yield (kg ha^{-1}).

The correlation between grain yield (GY) and BLUP predictors was 0.997, and the BLUPe value was 0.985, meaning that phenotypic selection and predicted values are equally effective for selection in the target population. However, there is no doubt that BLUPe is very useful when deciding about the upper lines, mainly when the data are unbalanced, while Tukey's (5%) mean comparison test did not allow the precise discrimination of the lines. Several researchers have shown significant and positive correlations between predicted genetic and observed yield values (Casanoves & Balzarini, 2002; Milla-Lewis & Isleib, 2005), a highly dependent situation on trait heritability.

Grain yield showed a genetic variance of 381,356 (P<0.01), an error variance of 137,426 (P<0.01), a heritability value of 84.7%, and 92% efficiency in selecting the best lines, consistent with Falconer (1981), where the selection of lines for their phenotypic values is effective when the heritability is high

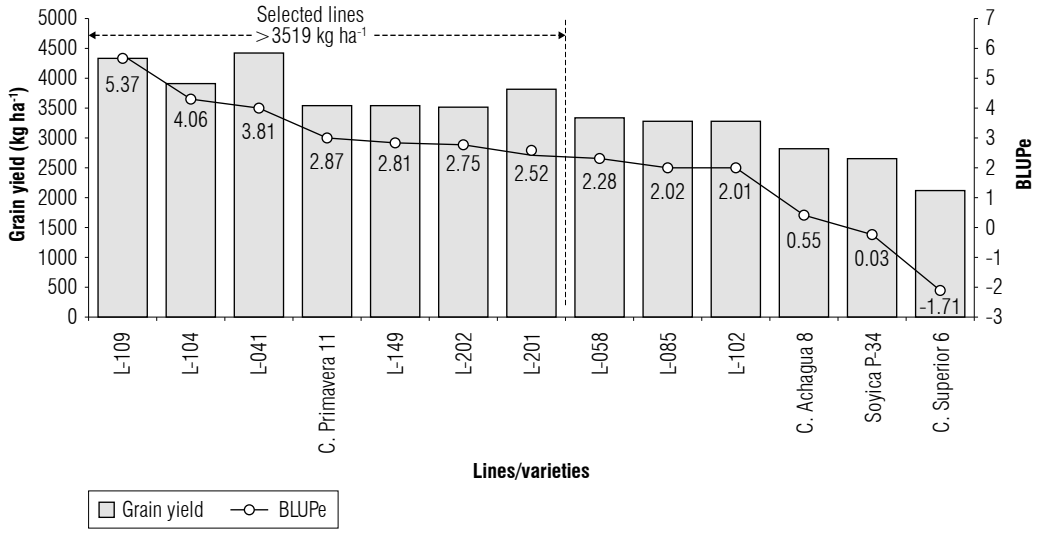

FIGURE 1. BLUPe vs. grain yield of various soybean lines/varieties, Orinoco region, 2020.

In this sense, Souza *et al*. (2000) finds that when heritability is low or very low, the correlation between genetic values predicted by BLUP and phenotypic values is also low. If heritability is moderate, the genetic values obtained by BLUP will allow a better classification of genotypes than the phenotypic values for a more efficient selection.

Generally, the genotypic variance in its optimal environment in soybean is higher than in stress environments. However, Ceccarelli (1989) states that even when the heritability of a specific trait in a stressful environment is always lower than in the optimal environment, this is not clear evidence that selection should be conducted only in optimal environments. Therefore, following the criteria stated by Allen *et al*. (1978) concerning heritability and how to conduct selection, promising lines should be subjected to multi-environment trials to identify the more stable and profitable ones for farmers in the Colombian Orinoco region.

Hydric balance vs. grain yield

During the experimental crop cycle, precipitation was variable in frequency and intensity, reaching a total volume of 618 mm from sowing to harvest, close to the water requirement of the crop in the Orinoco of 350 mm and 550 mm (Almansa, 2006). The hydric balance (Fig. 2) diagram elaborated for soybean in the study site showed a marked variability when excesses and deficits occurred. Although the excesses in the Colombian Orinoco are more noticeable in the first semester of the year and the deficits in the second semester, it is frequent to observe marked variations in the same semester. These variations in water resource availability affect plants according to the moment of occurrence of the stress and the genotype. In general, the lines similar to the early control C. Superior 6 with DM between 81 and 84 and with a determined or semi-determined GH were highly affected by a water deficit of 13.87 mm between September 15 and 21, and very marked on September 17 and 18 (3.9 and 4.7 mm, respectively), with temperatures above 31°C, coinciding with the reproductive phase of the beginning of pod formation (R3). Almansa (2006) determines that soybean cultivation consumes 4.5 mm of water per day, and an absence of rain for four days makes irrigation necessary. According to Giménez (2014), severe water deficiencies can produce very substantial yield losses (40% or more), mainly when they occur in the critical period of pod formation and filling (Fehr & Caviness, 1977; Sawchik *et al*., 2013). If the water deficit occurs between R3-R5, it significantly affects the number of grains, and if it does so later, it affects the weight of the grains. Additionally, high temperatures with water deficit during flowering and grain filling cause physiological changes such as stomatal closure that in turn causes premature leaf and flower drop, embryonic abortions, pod drop, and reduced grain yield.

Water excesses in the filling phase and physiological maturity were also decisive when selecting promising lines. In this sense, the control variety C Primavera 11 with a semi-determined GH and an intermediate cycle (102 DM), although it reached a GY of 3,556 kg ha^{-1}, also showed high foliar retention and a non-uniform population that negatively affected harvest and grain quality. Carvajal *et al*. (2017) find that alternate periods of wetting and drying the grains inside the pods result in a marked grain or future seed deterioration. In contrast, the selected lines used in this research had uniform maturity and drying.

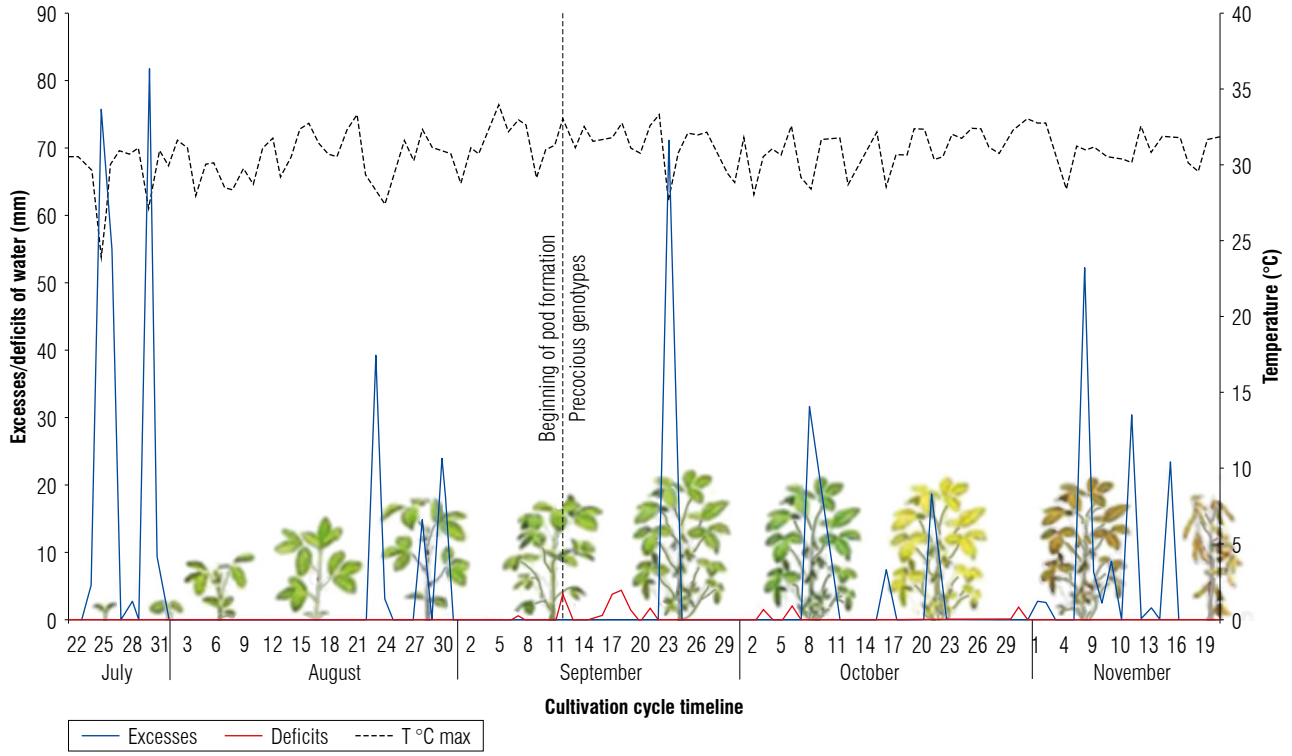

FIGURE 2. Water excesses and deficits, and average temperature during the soybean cultivation cycle. Research Center La Libertad, Orinoquía, 2020.

It is indisputable that the significant differences in GY between lines and varieties are due to the genetics and physiology of the plants in response to local environmental conditions, particularly associated with water and thermal availability. These occur during plant development and differentially influence cultivars according to their early, intermediate, or late cycle, also dependent on the moment of occurrence and intensity of the stress, the genotype, and its photoperiodic sensitivity (Sawchik *et al*., 2013). Therefore, a timely sowing date, adequate plant population, and water and nutrient availability in the critical phases (R3-R6) will maximize grain production.

It is essential to highlight that in the edaphoclimatic conditions of Orinoquía the selection of very precocious genotypes can reduce plant growth and final grain yield. In contrast, in the very late genotypes, the maturity of the pods is ostensibly delayed, favoring the incidence of diseases, pests, weed competition, and poor seed quality due to the variable occurrence of rainfall after maturity. The selected lines have as a comparative advantage an intermediate ripening period (88-105 d after emergence), uniform drying, and good grain quality. Some morphoagronomic traits of the promising lines are presented in Table 3.

The subsequent evaluation of these lines in MET should guarantee the genotype-by-environment interaction (GxE) assessment to select the future phenotypically stable variety or varieties with a higher genetic potential to be cultivated in Oxisols of the Orinoquía region. However, although

TABLE 3. Morphoagronomic traits of selected soybean lines from the Research Center La Libertad, Orinoquía, 2020.

Line/Variety	FC	PC	GH	DF	DM	H (cm)	NN
L-019	W	B	SD	53	105	64	16
L-041	P	B	I	36	88	103	18
L-104	W	G	D	39	89	60	13
L-149	P	B	I	38	88	92	18
L-201	P	B	I	37	97	109	22
L-202	W	G	I	37	97	100	20

FC: flower color [(white (W) and purple (P)]; PC: pubescence color [(brown (B) and gray (G)]; GH: growth habit: [determinate (D), semi-determinate (SD), and indeterminate (I)]; DF: days to flowering; DM: days to physiological maturity; H: average plant height at maturity; NN: average number of nodes.

there was broad variability in the population of soybean lines/varieties under study, the genetic base of the genetic breeding program remains narrow. Access to foreign germplasm and the implementation of moderate genotyping and high-performance phenotyping tools are necessary to accelerate the genetic gain of future cultivars for current and potential areas of Colombia through genomic selection.

Conclusions

The application of mixed models using the REML/BLUP procedure allowed the generation of a standardized predictor (BLUPe) useful for the effective classification and selection of potential lines or varieties due to the high genetic merit obtained. With a heritability of 84.7% for GY and a selection pressure of 10%, six lines with positive BLUPe values ≥ 2.52 and grain yields higher than 3,519 kg ha^{-1} were identified: L-019, L-041, L-104, L-149, L-201, and L-202. These lines have an intermediate period to physiological maturation of 88-105 d after emergence, uniform drying, and good grain quality. These lines will be subjected to multi-environment yield trials to determine their phenotypic stability and select the line or lines with the highest genetic potential for ICA registration as a variety or varieties for the Colombian Orinoco.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

RAVR, leader of the Soybean Orinoquía Project, designed the experiments, performed statistical analysis and interpretation of the results, and contributed to the writing of the manuscript; YSTA carried out planting activities, data collection in the field, and contributed to the writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Combining ability and selection of wheat populations for a tropical environment

Aptitud combinatoria y selección de poblaciones de trigo para un medio ambiente tropical

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ABSTRACT

The selection of segregating populations with the potential for derived lines is essential for breeding programs. The present work analyzes the potential of tropical F_2 wheat (*Triticum aestivum* L.) populations originated from complete diallel cross combinations. For this purpose, eight tropical wheat cultivars were combined in a complete diallel design in 2019 after F_1 seeds were multiplied in a greenhouse and the seeds of 56 F_2 populations, plus the eight parents, were evaluated in the field in Viçosa, MG, Brazil in the winter harvest of 2020 using a simple lattice design (8×8). The trait scores of (1) severity of tan spot (*Pyrenophora tritici-repentis*), (2) severity of wheat head blast (WHB) (*Magnaporthe oryzae* pathotype *Triticum*), (3) days to heading, (4) spike height, (5) and total grain weight of the plot were evaluated. We performed a diallel analysis using mixed models to obtain the effects of general combining ability (GCA), specific combining ability (SCA), and estimation of population genotypic values. The additive effect predominated for the control of all traits, except for spike height. There were greater GCA effects for the set of parental maternal plants. Heritability, in the narrow sense, ranged from 0.20 (blast) to 0.66 (heading). There was an effect of maternal GCA for all variables, while for paternal GCA the effect was only for days passed for head and total grain weight. Populations derived from the cultivars TBIO Aton, TBIO Ponteiro, and TBIO Sossego had lower disease severity, while the combinations from BRS 254, BRS 264, and BRS 394 had earlier maturation time. The most promising combinations to derive lines for the set of traits were BRS 254 × CD 1303, BRS 394 × TBIO Aton, TBIO Aton × BRS 254, CD 1303 × BRS 254, and CD 1303 × BRS 264.

Key words: additive effect, diallel analysis, mixed models, segregating population, *Triticum aestivum* L.

RESUMEN

La selección de poblaciones segregantes con potencial para derivar líneas es esencial para los programas de mejoramiento. El presente trabajo presenta el potencial de las poblaciones tropicales de trigo F_2 (*Triticum aestivum* L.) generadas a partir de combinaciones de cruces dialélicos completos. Para ello, se combinaron ocho cultivares de trigo tropical en un diseño dialélico completo en 2019 después de multiplicar semillas F_1 en invernadero y se evaluaron en campo las semillas de 56 poblaciones F_2, más los ocho progenitores, en la cosecha de invierno de 2020 en un diseño reticular simple (8×8) en Viçosa, MG, Brasil. Se evaluaron las variables: severidad de la mancha amarilla (*Pyrenophora tritici-repentis*), severidad del tizón (WHB) (*Magnaporthe oryzae* pathotype *Triticum*), número de días hasta embuchamiento, altura de la espiga y peso total de grano en la parcela. El análisis dialélico se realizó utilizando modelos mixtos para obtener los efectos de capacidad combinatoria general (GCA), capacidad combinatoria específica (SCA) y estimación de valores genotípicos de la población. El efecto aditivo predominó para el control de todas las variables, excepto para la altura de la espiga. Hubo mayores efectos de GCA para el conjunto de plantas madres progenitoras. La heredabilidad, en sentido estricto, osciló entre 0.20 (tizón) y 0.66 (embuchamiento). Hubo efecto de la CGA materna para todas las variables, mientras que para la CGA paterna solo para número de días hasta embuchamiento y peso total de grano en la parcela. Las poblaciones derivadas de los cultivares TBIO Aton, TBIO Ponteiro y TBIO Sossego presentaron menor severidad de la enfermedad, mientras que las combinaciones de BRS 254, BRS 264 y BRS 394 presentaron un tiempo de maduración más temprano. Las combinaciones más promisorias para derivar líneas para el conjunto de variables evaluadas fueron BRS 254 × CD 1303, BRS 394 × TBIO Aton, TBIO Aton × BRS 254, CD 1303 × BRS 254 y CD 1303 × BRS 264.

Palabras clave: efecto aditivo, análisis dialélico, modelos mixtos, segregación de población, *Triticum aestivum* L.

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Introduction

Because of its amount of protein and the possibility of derived products, wheat (*Triticum aestivum* L.) is an important part of the human diet. It is currently the main source of protein and carbohydrates for humans (Shewry *et al*., 2016). In this context, wheat corresponds to 30% of world grain production (United States Department of Agriculture – USDA, 2018). In Brazil, wheat production occurs mainly in the southern region that generated about 90% of the 6.2 million t produced in 2020 (Companhia Nacional de Abastecimento - CONAB, 2021). Even so, Brazil needs to import about 5.8 million t of wheat (Conab, 2021). Pasinato *et al.* (2018) point out that despite the low participation in the total wheat production, the Cerrado region of Brazil is a potential for production of this crop, increasing national production. However, the expansion of agricultural frontiers depends on the generation of cultivars adapted to new locations, like the Brazilian Cerrado. National expansion is fostered especially by the participation of wheat in the country's food security, since it is an affordable source of nutrition for a large part of the population, especially those with low-middle income. In addition, wheat is essential in sustainable agricultural systems (Hickey *et al*., 2019).

The plant breeding pipeline depends on the formation of segregating populations with a potential for selection. To this end, it is necessary that parental lines concentrate favorable alleles for the traits of interest, allowing the production of superior individuals after hybridization (Fasahat *et al.,* 2016). In breeding programs, several segregating populations are produced and evaluated. However, only a small portion of them has the potential to obtain superior genotypes. This slows the progress, because of unsatisfactory segregating populations requiring work and resources. Methodologies capable of making the process more efficient are used, such as genomic selection (Merrick *et al*., 2022) or diallel analysis. The latter is more suitable to the reality of Brazilian public research, since the implementation of genomic selection is the enormous cost of genotyping, as well as the need to develop infrastructure for genotyping thousands of progenies (Juliana *et al*., 2020).

Diallelic analysis allows studying and selecting the best combinations of parents through the evaluation of the generated progenies. This evaluation seeks to identify parents with a high allelic complementarity. In addition, it allows the determination of the predominant gene action in the expression of a trait (Cruz *et al*., 2014). Some previous studies describe diallel analysis on wheat (Pimentel *et al.,* 2013; Pagliosa *et al.,* 2017; Akel *et al.,* 2018; Pelegrin *et al*., 2020). The difficulty in obtaining a satisfactory number of seeds in the F_1 generation limits the use of robust statistical designs or a greater number of repetitions. Thus, generations with a greater seed availability, such as F_2, can be used efficiently in the diallel analysis of wheat, resulting in accurate predictions (Masood & Kronstad, 2000). There is also the mixed model methodology, where the use of the best linear unbiased predictor (BLUP) that considers components of variance estimated by restricted maximum likelihood (REML), allows breeders to select and predict genetic values efficiently and accurately (Resende, 2016).

It is common in wheat breeding programs to evaluate several genotypes and select promising ones according to the main traits of interest. When talking into account a large number of traits, truncated and indirect selection is limited, since it becomes efficient only for traits with high genetic association. With the use of selection indexes, we seek to obtain simultaneous gains for several traits, facilitating decision-making for the selection of populations (Céron-Rojas & Crossa, 2020). In addition, the gains are better distributed among the set of traits considered important for selection in the breeding program.

Studies on improvement of tropical wheat in Brazil are still scarce and contrast with the need to achieve self-sufficiency in wheat. According to Pereira *et al.* (2019), the great potential of wheat in tropical regions requires the development of genotypes that are adapted to local climatic conditions and show a desirable agronomic level. Thus, the objective of this work was to select promising tropical F_2 populations of wheat based on estimations of combining ability of elite parent plants using REML/BLUP.

Materials and methods

Cultivar combinations

For crosses, eight wheat cultivars recommended for cultivation in the Brazilian tropical region (Registro Nacional de Cultivares – RNC, 2022) were selected based on agronomic parameters and technological quality of the flour (Tab. 1). The cultivars were combined in a complete diallel design, resulting in 56 hybrid combinations. The crosses were carried out between August and October 2019 in a greenhouse belonging to the Departamento de Agronomia da Universidade Federal de Viçosa, in the state of Minas Gerais, Brazil.

Generation advance

The seeds from crossings were harvested at physiological maturity, manually treshed, and stored in a cold chamber.

TABLE 1. List of wheat parents combined in a complete diallel arrangement to obtain segregating populations.

Code[1]		Description of parents				
♀	♂	Parents	Provenance	Release year	Class	Cycle
A	1	CD 1303	Coodetec	2016	Bread/ Improver	Early
B	2	BRS 254	Embrapa	2005	Improver	Early
C	3	BRS 264	Embrapa	2005	Bread	Early
D	4	BRS 394	Embrapa	2014	Improver	Early
E	5	TBIO Aton	Biotrigo	2018	Bread	Medium
F	6	TBIO Duque	Biotrigo	2018	Bread/Bleacher	Early
G	7	TBIO Ponteiro	Biotrigo	2017	Bread	Medium/Late
H	8	TBIO Sossego	Biotrigo	2015	Bread	Medium

♀: code for maternal parent; ♂: code for paternal parent.

The F_1 seeds of each combination were sown in February 2020 in a greenhouse to advance the generation and production of F_2 seeds. Harvesting was carried out in mid-May 2020. The spikes were trailed and the F_2 was sowed in a field at the beginning of June 2020.

F_2 population evaluation

The field experiment was carried out during the 2020 winter at the Universidade Federal de Viçosa, Viçosa, MG, Brazil experimental area (20°45'14"S; 42°52'55"W; 648 m a.s.l.). The seeds harvested from F_1 plants (56 F_2 combinations) and the eight parents comprised the 64 treatments. These were sown in a simple lattice design (8×8), with 2 replicates. The experimental plots were composed of three 3 m long lines spaced 0.2 m apart, totaling plots of 0.6 m^{-2}. The sowing density was ten seeds per linear meter, that is, with spaced plants and following the pedigree conduction method, as used by McVetty and Evans (1980).

Fertilization consisted of 300 kg ha^{-1} of N-P-K (08-28-16) plus a cover fertilization of 90 kg ha^{-1} of N divided in two phenological stages, 50% at the beginning of tillering and 50% in the heading phases 21 and 45, according to the scale of Zadoks *et al.* (1974). The nitrogen source used urea (46% N), totaling 200 kg ha^{-1}. Chemical weed control was performed using the active ingredient metsulfuron methyl at a dose of 5 g ha^{-1} of the commercial product approximately 20 d after plant emergence. For chemical control of aphids (*Metopolophium dirhodum* and *Sitobion avenae*), the active ingredient acetamiprid was used at a dose of 375 g ha^{-1} of commercial product at the post-anthesis phase. For diseases, chemical control was not performed in order to verify the natural reaction of the genotypes. The experiment was carried out with sprinkler irrigation according to the water needs of the genotypes. The climatic conditions of the field assessment cycle are displayed in the Table 2.

Evaluated traits

The following traits were evaluated: severity of tan spot (*Pyrenophora tritici-repentis*) following the scale proposed by Lamari and Bernier (1989) (1 = resistant, 5 = susceptible), wheat head blast (WHB) severity (*Magnaporthe oryzae* pathotype *Triticum*) following an adapted scale, initially proposed by Maciel *et al.* (2013) (1 = 0%, 12 = 100%). Days to heading were evaluated in days began from the phase 10 (first leaf appearance after the coleoptile) to the phase 54 (half of inflorescences emerging from the sheath of the flag leaf) following Zadoks *et al.* (1974). Spike height was measured by a ruler graduated in cm from the base of the plant to the spike insertion, and total weight of dry grains of the plot was measured in grams using a precision scale.

Statistical analysis

Data were evaluated according to the methodology of maximum restricted likelihood/best linear unbiased prediction (REML/BLUP). To estimate the variance components (REML), predict the genotypic values (BLUP), and estimate the values of general combining ability (GCA)

TABLE 2. Weather conditions during the growth season.

Month	Precipitation, mm	Mean temperature, °C	Maximum temperature, °C	Minimum temperature, °C	Relative air humidity, %
June	2.60	17.28	18.00	16.61	84.06
July	2.60	17.45	18.18	16.78	82.62
August	21.40	17.17	17.97	16.40	78.53
September	30.20	20.64	21.50	19.84	71.67
October	184.40	21.54	22.16	20.97	78.45

and specific combining ability (SCA), the model 192 of the software Selegen-REML/BLUP was used (Resende, 2017). It simultaneously fits one model for crosses and another one for parents.

The following model was used to adjust intersections:

$$y = X_c + Z_p + W_m + S_d + T_b + e,$$

where y is the phenotypic vector, c is the fixed effect of the general mean of crosses, p is the vector of effects of the general combining ability of paternal (random) parents ($p \sim N(0, \sigma^2_p)$), m is the vector of effects of general combining ability of maternal (random) parents ($m \sim N(0,\sigma^2_m)$), d is the vector of effects of specific combining ability of (random) crosses ($d \sim N(0, \sigma^2_{SCA})$), b is the vector of (random) block effects ($b \sim N(0, \sigma^2_b)$), and e is the vector of (random) error effects and ($e \sim N(0, \sigma^2_e)$). X, Z, W, S, and T are incidence matrices for their respective effects.

The adjustment of effects of parents was performed using the following model:

$$y = X_u + H_p + T_b + e,$$

where y is the phenotypic vector, u is the fixed effect of the general mean of parents, p is the vector of additive genetic effects of parents (random) ($p \sim N(0, \sigma^2_g)$), b is the vector of (random) block effects ($b \sim N(0, \sigma^2_b)$), and e is the vector of (random) error effects ($e \sim N(0, \sigma^2_e)$). X, H, and T are incidence matrices.

The additive genetic variance ($\hat{\sigma}^2_a$) was estimated as follows:

$$\hat{\sigma}^2_a = \{[4/(1 + F)]\ \hat{\sigma}^2_p + [4\ (1 + F)]\ \hat{\sigma}^2_m/2,$$

where $\hat{\sigma}^2_p$ is the genetic variance of paternal parents, $\hat{\sigma}^2_m$ is the genetic variance of maternal parents, and F is the inbreeding coefficient (F=1).

Variance due to dominance deviations ($\hat{\sigma}^2_d$) was estimated according to the formula:

$$\hat{\sigma}^2_d = [4/(1 + F)]\sigma^2_{SCA},$$

where σ^2_{SCA} is the variance of specific combining ability.

Heritability in the narrow sense ($\hat{h}^2_n$) and dominance ($\hat{h}^2_d$) were estimated as follow:

$$\hat{h}^2_n = \frac{\hat{\sigma}^2_a}{\hat{\sigma}^2_{pF2}} \text{ and } \hat{h}^2_d = \frac{\hat{\sigma}^2_d}{\hat{\sigma}^2_{pF2}}$$

respectively, where $\hat{\sigma}^2_{pF2}$ is the phenotypic variance of the populations evaluated.

The genotypic values (GV) of the F_2 populations were expressed as:

$$VG = u + GCA_i + GCA_j,$$

where u is the general mean of the trait, GCA_i is the value of the general combining ability of parent i, GCA_j is the value of the general combining ability of parent j.

Simultaneous selection on genotype values (GV) of all the evaluated traits was applied considering the selection of 20% of the superior populations. We calculated the multiplicative index (Subandi *et al.,* 1973) according to the following expression:

$$I_{S(i)} = \prod_{j=1}^{n} w_i,$$

where $I_{S(i)}$ is the multiplicative index for the populations i; $w_i = x_{ij} - k_j$, where x_{ij} the observed population value for population i in trait j (VG), k_j is the selection criterion for the trait j assuming $w_i = 0$, if $x_{ij} - k_j > 0$ for the traits tan spot, blast, heading and spike height and if $x_{ij} - k_j < 0$ for the trait total weight. The k_j is defined as the maximum or minimum value established for each trait. In this study, the criterion we adopted was the mean genotypic value of the trait ± genotypic standard deviation. For the traits that satisfy the condition (k_j), the value of the difference $x_{ij} - k_j$ is used in productive ($\prod$). Populations that do not meet the criteria receive a value of zero, making $I_{S(i)}$ null, so it cannot be selected. Statistical analyses were performed using the software Selegen REML/BLUP (Resende, 2016) and the graphs were plotted using the software R (R Development Core Team, 2020).

Results and discussion

Estimates of genetic parameters by REML

Table 3 shows a significance of 5% for the variance between maternal parents ($\hat{\sigma}^2_{GCAm}$) or for the general combining ability for maternal parents for all analyzed traits. In turn, considering the paternal parents, the general ability to combine paternal parents ($\hat{\sigma}^2_{GCAp}$), the traits of days to heading, and total grain weight were significant. The specific combining ability ($\hat{\sigma}^2_{SCA}$) only for the trait spike height was significant. In general, there is a greater participation of additive effects in relation to the effects of dominance. This is because most traits have a higher significance for the effects of GCA, either by the paternal or maternal parent, than for the effects of SCA.

TABLE 3. Estimation of genetic parameters for the traits tan spot (scale), blast severity (scale), days to heading (d), spike height (cm), and total weight of dry grains (g) in 64 tropical wheat genotypes and average genotypic values of maternal parents (♀) and paternal parents (♂).

Parameter	Tan spot	WHB	Days to heading	Spike height	Total grain weight
$\hat{\sigma}^2_{GCAm}$	0.28	0.17	15.03	4.27	1745.85
$\hat{\sigma}^2_{GCAp}$	0.03	0.00	4.69	0.56	975.31
$\hat{\sigma}^2_{SCA}$	0.01	0.01	2.05	10.51	1001.50
$\hat{\sigma}^2_{parents}$	0.00	0.04	0.08	0.05	2746.58
$\hat{\sigma}^2_g$	0.32	0.18	21.78	15.32	3722.66
$\hat{\sigma}^2_a$	0.31	0.17	19.73	4.82	2721.16
$\hat{\sigma}^2_d$	0.01	0.01	2.05	10.51	1001.50
$\hat{\sigma}^2_b$	0.00	0.01	0.18	0.01	16.93
$\hat{\sigma}^2_e$	0.92	0.68	7.97	17.74	7125.84
$\hat{\sigma}^2_{pF2}$	1.24	0.86	29.93	33.08	10865.42
$\hat{\sigma}^2_{parents}$	0.92	0.72	8.24	17.80	9889.34
C^2_{GCAm}	0.23*	0.20*	0.50*	0.13*	0.16*
C^2_{GCAp}	0.03^{ns}	0.00^{ns}	0.16*	0.02^{ns}	0.09*
C^2_{SCA}	0.01^{ns}	0.01^{ns}	0.07^{ns}	0.32*	0.09^{ns}
$C^2_{parents}$	0.00^{ns}	0.06^{ns}	0.01^{ns}	0.00^{ns}	0.28^{ns}
C^2_b	0.00^{ns}	0.01^{ns}	0.01^{ns}	0.00^{ns}	0.00^{ns}
$\hat{h}^2_n$	0.25	0.20	0.66	0.15	0.25
$\hat{h}^2_d$	0.01	0.01	0.07	0.32	0.09
$\hat{h}^2_b$	0.26	0.20	0.73	0.46	0.34
$\hat{r}_{\hat{a}a}$	0.51	0.45	0.85	0.68	0.59
Mean	1.86	1.57	65.91	75.33	487.65

Parents	Genetic value									
	Tan spot		WHB		Days to heading		Spike height		Total grain weight	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
A	2.10	1.93	1.77	1.57	68.34	66.74	75.33	75.33	547.38	541.80
B	2.47	1.86	2.22	1.58	66.51	67.11	77.37	75.40	516.07	508.82
C	2.31	1.99	2.10	1.58	65.91	65.91	76.64	75.75	520.63	491.46
D	2.88	2.03	1.98	1.58	67.31	66.35	75.52	75.62	487.65	487.65
E	2.01	1.90	1.63	1.58	69.60	68.43	75.74	75.46	528.08	521.73
F	2.18	1.95	1.85	1.58	68.96	67.66	75.94	75.54	502.97	501.63
G	1.86	1.89	1.69	1.58	71.81	67.85	76.24	75.73	508.31	493.98
H	1.94	1.91	1.57	1.57	70.52	68.04	79.23	75.66	494.37	497.28
Mean	2.22	1.93	1.85	1.58	68.62	67.26	76.50	75.56	513.18	505.54

* significant and ^{ns} non-significant by the χ^2 test at 5% probability; $\hat{\sigma}^2_{GCAm}$= variance of the GCA of the maternal parents; $\hat{\sigma}^2_{GCAp}$ = variance of the GCA of the paternal parents; $\hat{\sigma}^2_{SCA}$= variance of the SCA of the F_2 populations; $\hat{\sigma}^2_{parents}$= genotypic variance between parents; $\hat{\sigma}^2_g$= genetic variance; $\hat{\sigma}^2_a$= additive genetic variance; $\hat{\sigma}^2_d$= dominance variance; $\hat{\sigma}^2_b$= block variance; $\hat{\sigma}^2_e$ = error variance; $\hat{\sigma}^2_{pF2}$= phenotypic variance between F_2 populations; $\hat{\sigma}^2_{parents}$= phenotypic variance between parents; C^2_{GCAp} = coefficient for determining the GCA effects of paternal parents; C^2_{GCAf} = coefficient for determining the GCA effects maternal parents; C^2_{SCA}= coefficient for determining the SCA effects; $C^2_{parents}$= coefficient for determining the parents effects; C^2_b = coefficient for determining the block effects; $\hat{h}^2_n$= narrow-sense heritability; $\hat{h}^2_d$= heritability of values due to deviations in dominance; $\hat{h}^2_b$= broad-sense heritability; $\hat{r}_{\hat{a}a}$= accuracy; A= CD 1303; B= BRS 254; C= BRS 264; D= BRS 394; E= TBIO Aton; F= TBIO Duque; G= TBIO Ponteiro; H= TBIO Sossego; WHB= wheat head blast.

Both heritability in the narrow sense ($\hat{h}^2_n$) and in the broad sense ($\hat{h}^2_b$) obtained the highest values for the trait days to heading. Such heritability, according to Resende (2002), is considered moderate ($0.15 < h^2 < 0.50$) and high ($h^2 > 0.50$). According to the classification proposed by Resende and Duarte (2007), the selective accuracy is classified as moderate ($0.40 < \hat{r}_{aa} < 0.65$) for tan spot, blast, and total weight, and high ($0.65 < \hat{r}_{aa} < 0.85$) for days to heading and spike height.

The decomposition of the phenotypic variance component of the F_2 populations ($\hat{\sigma}^2_{pF2}$) (Tab. 3) into genetic variance (σ^2_g), additive genetic variance (σ^2_a) and genetic variance due to dominance deviations (σ^2_d) allows verification of a greater participation of additive effects in all traits with the exception of spike height, for which the variance of dominance effects had the highest proportion. Ljubicic *et al.* (2017) demonstrated predominant dominance effects when studying productive variables in diallel analysis. When identifying and selecting superior genotypes, σ^2_a is a fundamental component to quantify the potential of populations (Cruz *et al.,* 2014). Its greater participation in the composition of genetic variance allows using direct strategies to increase the frequency of favorable alleles to a given trait. This also happens with the presence of the significance of GCA effects in comparison with non-significant effects of SCA. In this sense, it is noteworthy that 98.1% of variation effects for the trait tan spot severity are due to GCA effects ($\hat{\sigma}^2_{GCAm} + \hat{\sigma}^2_{GCAp}$), which is similar to blast severity (97.70%), as well as days to heading (90.40%), and total grain weight (73.10%). Spike height, in turn, differs from the others. There is a greater share of effects of specific combining ability σ^2_{SCA} in relation to GCA effects with σ^2_{SCA} corresponding to 68.5% of the variation. There are similar results when analyzing wheat productivity (Pimentel *et al.,* 2013; Pagliosa *et al.,* 2017), plant height, and days to heading (Akel *et al.,* 2018).

Estimates of GCA and SCA

Figure 1 shows the estimates of the effects of general combining ability (GCA) of each parent for each trait, whereas Table 3 shows the means of each parent. Although with different magnitudes, there was agreement between the estimates of GCA effects for parents considering maternal and considering paternal parents.

There were negative effects of GCA for the two disease severity traits, tan spot and blast, for the parents CD 1303 (A), TBIO Aton (E), TBIO Duque (F), TBIO Ponteiro (G), and TBIO Sossego (H). They ranged from -0.03 (TBIO Aton) to -0.58 (TBIO Ponteiro) (Fig. 1A) and from -0.09 (TBIO Duque) to -0.39 (TBIO Sossego) (Fig. 1B) for tan spot and blast. Still, among the traits in which negative GCA effects are desired days to heading (Fig. 1C) the group of parents belonging to the breeder company, Embrapa and Coodetec showed negative GCA effects. These effects ranged from -0.01 (CD 1303) to -4.23 (BRS 264). For spike height (Fig. 1D), only the parent TBIO Sossego had no negative GCA effect considering both paternal and maternal parents. The CD 1303, TBIO Aton, and TBIO Duque parents showed negative GCA effects simultaneously for both maternal and paternal parents.

In general, the effects of specific combining ability (SCA) were of low magnitude (from -0.01 to 0.01) for both tan spot severity (Fig. 2A) and WHB severity (Fig. 2B). Populations with negative SCA effects and the presence of at least one parent with a negative GCA for these traits stood out among ten F_2 populations for severity of tan spot and five F_2 populations for severity of WHB (B1, C1, C8, D5, and D7). For days to heading, 25 F_2 populations showed a negative SCA effect (Fig. 2C) that varied from -0.03 (A3) to -1.72 (G3). The total variation of the negative SCA effects for spike height was -7.99 (G2) to 4.39 (B5) (Fig. 2D). However, when targeting populations with negative SCA effects and at least one parent with a negative GCA, 24 populations stood out. The trait total grain weight (Fig. 2E), combining positive SCA and GCA effects, comprises a group of 20 promising F_2 populations with a SCA variation from 1.00 (F5) to 24.47 (A7).

The estimate of SCA of parents (A1, B2, C3, D4, E5, F6, G7, and H8) (Fig 2A-E) showed an alternation of signals: for tan spot and WHB, the SCA was zero. However, for days to heading, the parents A1, D4, E5, and G7 had a positive SCA, and the others had a negative SCA. For the trait spike height, the parents who had positive SCA were D4, F6, G7, and H8, while for total grain weight, the parents with positive SCA were A1, C3, E5, F6, and H8.

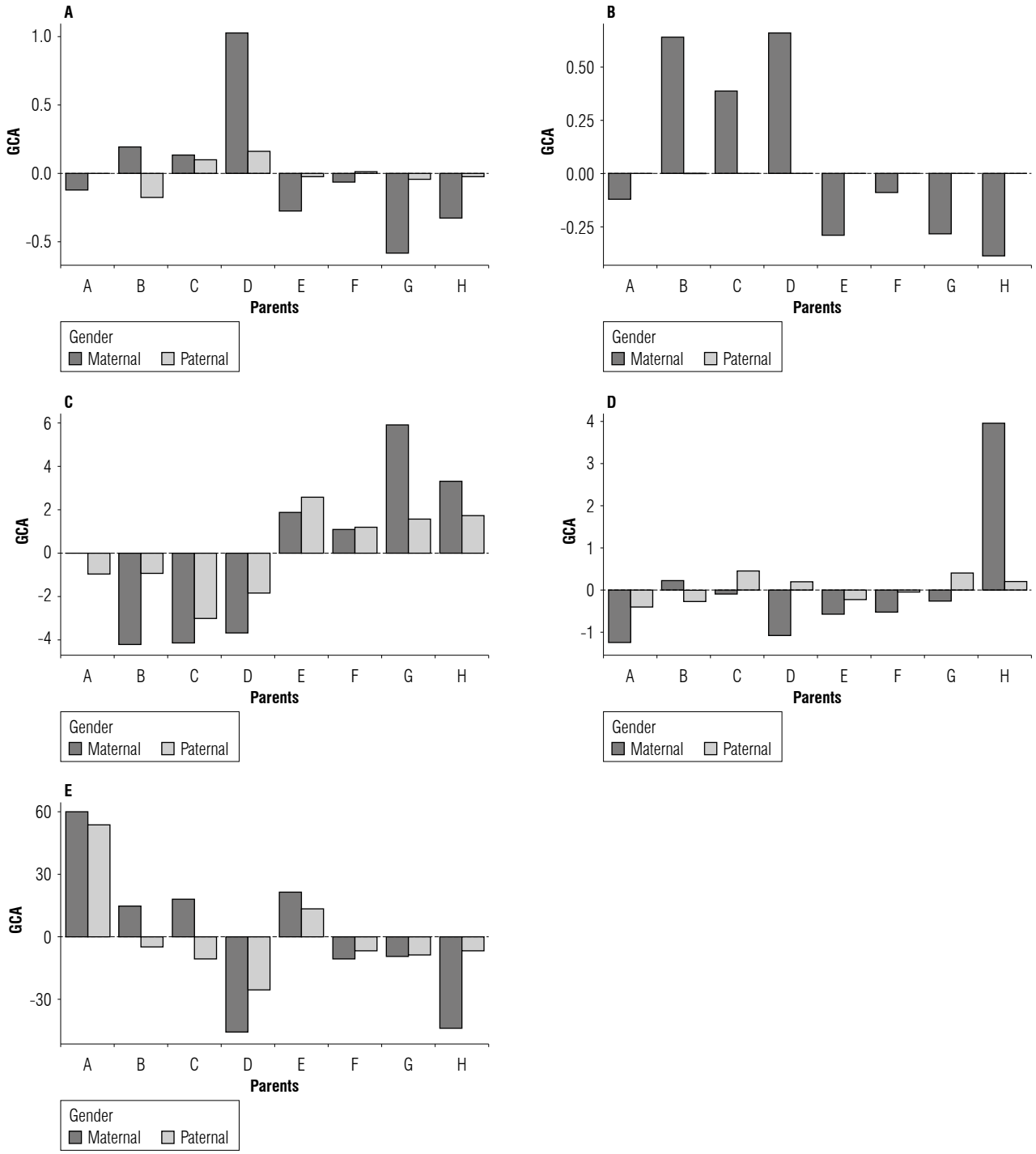

FIGURE 1. General combining ability (GCA) for tan spot severity (A), wheat head blast-WHB severity (B), days to heading (C), spike height (D), and total grain weight (E) of eight tropical wheat parents by diallel analysis via REML/BLUP.

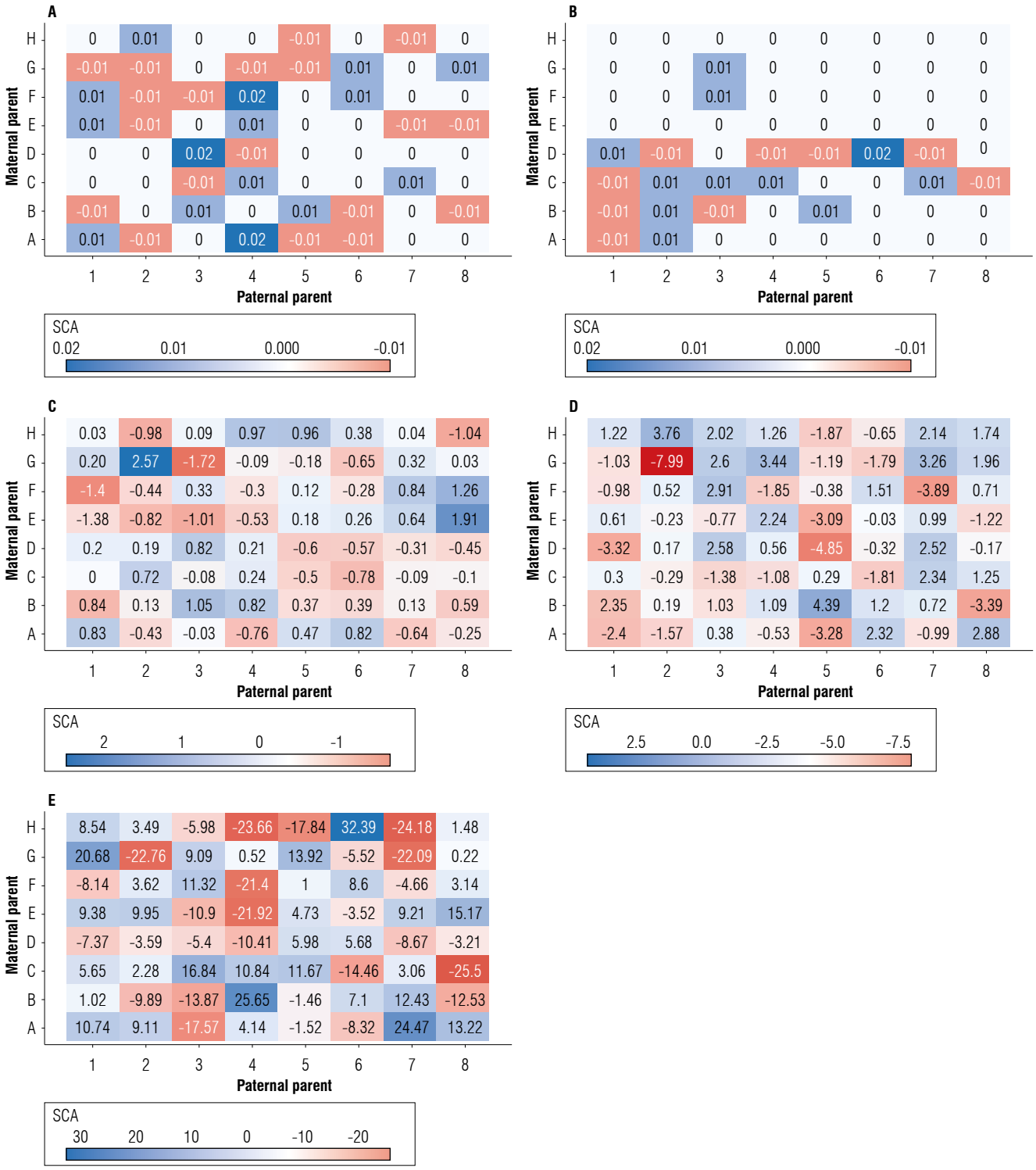

FIGURE 2. Specific combining ability (SCA) for tan spot severity (A), whea head blast-WHB severity (B), days to heading (C), spike height (D), and total grain weight (E) of eight tropical wheat parents by diallel analysis via REML/BLUP.

Considering paternal parents, the lack of significance of GCA effects for some traits (Tab. 3) showed that the mean number of favorable alleles is similar between genotypes. This may be because the parents used here were commercial cultivars with already desirable agronomic traits; therefore, they already shared a set of favorable alleles. The SCA was expressed as a function of deviations in dominance and genetic diversity between parents. Although there was diversity between parents, the non-significance of SCA effects for tan spot, WHB, days to heading, and total grain weight could have been the result of a small contribution of dominance effects on the expression of traits in the evaluated generation (F_2) in which heterosis was reduced to half its initial value. Low contributions are stressed by the low values of $\hat{h}^2_d$ by Pimentel *et al.* (2013), who also did not observe a heterotic effect for productivity, although other authors observe this in studies with durum wheat (Akel *et al.,* 2018).

In the improvement of self-pollinated species such as wheat, the final selected progenie plants are homozygous lines, which suggest the additive effects explained by the value of heritability in the narrow sense $\hat{h}^2_n$. Non-additive effects, such as dominance deviation effects, make improvement more complicated since their expression occurs in heterozygous individuals. Along with the advance of generations, heterozygosity reduces by half. Thus, the effects of additive predominance reported in this work are satisfactory for obtaining superior lines, confirmed by observing the values of $\hat{h}^2_n$. Elias *et al.* (1989) evaluate F_4 and F_5 populations of wheat and observe a $\hat{h}^2_n$ mean of 0.73 for tan spot, a value higher than that reported in the present study. However, the generations considered in that study have a higher degree of homozygosity, in which effects due to deviations in dominance become insignificant, remaining only additive effects. Juliana *et al.* (2020) find resistance to WHB in 1.11 accessions of wheat from the germplasm bank of the International Maize and Wheat Improvement Center (CIMMYT) in different environments, and they report a $\hat{h}^2_n$ ranging from 0.49 to 0.87. Previous research analyze the $\hat{h}^2_n$ days to heading and plant height. The latter is correlated to spike height in F_2 populations of wheat (Afridi *et al.,* 2017), observing values of $\hat{h}^2_n$ of 0.35 for days to heading and 0.45 for plant height. Pimentel *et al.* (2014), in turn, studying the $\hat{h}^2_n$ of grain productivity in F_2 populations of tropical wheat report a $\hat{h}^2_n$ of 0.01, lower than that of the present study.

Selection based on GCA and SCA

Considering that scales of tan spot and wheat head blast (WHB) scores used in the study considered resistant plants those with the lowest scores, we recommend the selection of parents with a negative GCA. Thus, the maternal parents TBIO Aton, TBIO Ponteiro, and TBIO Sossego (Fig. 1A-B) contributed more to reduce the mean of severity scores of tan spot and WHB. When those parents participated in crosses as pollen-donor parents, there was a greater probability to give rise to superior-health populations. In contrast, the genotypes from Embrapa (BRS 254, BRS 264, and BRS 394) showed GCA effects with greater negative magnitudes (Fig. 1C), for an earlier maturation cycle. Casagrande *et al.* (2020) report similar results when studying the genetic diversity of 32 tropical wheat genotypes. The cultivars from Embrapa show earlier maturation when comparing to cultivars from Biotrigo seed company, in contrast, those with a long cycle showed greater health.

With respect to spike height and total grain weight (Fig. 1D), only the parents CD 1303 and TBIO Aton had the effects of paternal GCA and maternal GCA. It was negative for spike height; however, a height reduction is desired for these parents in order to avoid lodging, especially in irrigated areas of the Brazilian Cerrado (Richards *et al.,* 2019). The result was also a positive GCA for total grain weight (Fig. 1E). GCA refers to the mean performance of the parent present in hybrid combinations. Thus, it is associated with genes with additive effects; in turn, SCA designates cases in which hybrid combinations are superior or inferior than expected based on the mean parents performance and their gene complementarity, since SCA depends on genes with dominant effects and on their interactions (Fasahat *et al.,* 2016).

The estimation of parent's SCA effects is relevant because it becomes an indicator of the existence or not of unidirectional dominance. SCA values are negative when dominance deviations are predominantly positive; otherwise, they are positive (Cruz *et al.,* 2014). The discrepancy in the SCA values of parents observed in the present study for the traits days to heading, spike height, and total grain weight, (that is, positive and negative values of SCA) points to the absence of unidirectional dominance for these traits. Therefore, dominant and recessive genes determine the traits.

The aim of breeding programs is based not only on the improvement of one trait but also the selection of progenies that present the combination of desirable traits. However, depending on the number of genes that control a trait, it becomes difficult to find a homozygous genotype that includes all genes for all selected traits. Thus, it is necessary to select segregating populations that have such desirable

traits, such as populations that have desirable SCA effects for each trait and that bring together at least one of the parents with desirable GCA effects (Chagas *et al.,* 2019).

In this sense, three combining populations have desirable SCA effects (Fig. 2) for most selected traits, namely, B1 (BRS 254 × CD 1303), D5 (BRS 394 × TBIO Aton), and E2 (TBIO Aton × BRS 254). B1 and D5 combine the traits WHB lower severity, days to heading, spike height, and total weight, while the F_2 E2 population differs in relation to the first trait: it is resistant to tan spot. Another six F_2 populations stand out for combining SCA effects for three other traits, as follow: A2 (CD 1303 × BRS 254), A4 (CD 1303 × BRS 394), A7 (CD 1303 × TBIO Ponteiro), F2 (TBIO Duque × BRS 254), F3 (TBIO Duque × BRS 264), and G1 (TBIO Ponteiro × CD 1303). Different previous studies report efficiency in the use of diallel analysis to select promising wheat populations (Sharifi *et al*., 2019), so it is worth recommending these populations.

Simultaneous selection

As a second criterion for selecting the most promising populations, the genotypic value (GV) shown in Table 4 in which the simultaneous selection methodology used is based on the method of Subandi *et al.* (1973). Adopting a selection pressure of 20%, 13 best populations were selected, the gain with selection was suitable for all traits: 6.84% for tan spot severity, -5.98% for WHB severity, 0.00% for days to heading, -1.37% for spike height, and 6.28% for total grain weight with a total gain of 20.57%.

The average of the selected populations ($\bar{X}_S$) was 2.13 tan spot severity, 1.77 for blast severity, 76.988 cm for spike height, and 561.86 g for total weight. Considering the severity scales proposed by Lamari and Bernier (1989) (tan spot) and by Maciel *et al.* (2013) (WHB), these selected populations are considered resistant or moderately resistant. The average of the populations selected for heading, of 69.573 d, is inferior to commercial cultivars CD 1303, BRS 254, BRS 264, BRS 394 and TBIO Duque that are considered early cycle (Tab. 1). The superior performance of these selected F_2 populations, even in a state of segregation, demonstrates the potential for derivation of superior lines since there are of transgressive individuals.

When considering the simultaneous selection by Subandi *et al.* (1973) and the selection based on the values of GCA and SCA, the populations F_2 A2 (CD 1303 × BRS 254), A4 (CD 1303 × BRS 394), A7 (CD 1303 × TBIO Ponteiro) and E2 (TBIO Aton × BRS 254) were selected on both ways. These populations have desirable genetic values for all traits, *i.e*., values of tan spot and WHB severity, days to heading, and plant height below the general mean, and total grain weight above the mean of the populations. Therefore, the selection for the derivation of lines is promising.

TABLE 4. Estimates of original average ($\bar{X}_0$) and average of selected populations ($\bar{X}_S$), selection differential (SD), selection gain (SG), and selection gain in % (SG%) in the simultaneous selection for 10 superior tropical wheat populations F_2 for the traits: tan spot severity (%), wheat head blast-WHB severity (%), days to heading (d), spike height (cm), and total grain weight (g).

Trait	$\bar{X}_0$	$\bar{X}_S$	SD	SG	SG%
Tan spot	2.30	2.13	-0.16	-0.07	-6.89
WHB	1.88	1.78	-0.11	-0.06	-5.61
Days to heading	69.57	69.21	-0.36	-0.01	-0.52
Spike height	78.06	76.59	-1.47	-0.02	-1.88
Total weight	528.64	564.41	35.77	0.07	6.77
Total					21.67

Population	Tan spot	WHB	Days to heading	Spike height	Total grain weight
CD 1303	2.15	1.77	69.57	75.74	612.28
CD 1303 × BRS 254	2.02	1.84	68.93	75.98	565.11
CD 1303 × BRS 264	2.27	1.83	67.48	77.44	549.47
CD 1303 × BRS 394	2.32	1.82	67.88	76.60	553.94
CD 1303 × TBIO Aton	2.13	1.79	71.14	75.66	574.34
CD 1303 × TBIO Ponteiro	2.10	1.80	70.09	76.42	577.97
TBIO Aton × CD 1303	2.05	1.72	69.96	77.52	592.31
TBIO Aton × BRS 254	1.95	1.65	69.70	76.93	543.73
TBIO Aton × BRS 264	2.09	1.67	68.32	77.21	523.26
TBIO Duque × BRS 254	2.23	1.91	69.06	76.36	551.68

Conclusions

The additive effect predominates in the genetic control of tan spot severity, blast severity, days to heading, and total grain weight. The effect resulting from dominance deviations predominates in the genetic control of the trait spike height. There are greater GCA effects for the set of maternal parents compared to GCA effects for the set of paternal parents. Populations derived from the parents TBIO Aton, TBIO Ponteiro and TBIO Sossego have a greater potential for obtaining superior lines with lower disease infection, while populations from the parents BRS 254, BRS 264, and BRS 394 have a greater potential for showing early maturation cycle lines. The most promising populations to derive lines for the set of traits evaluated are BRS 254 × CD 1303, BRS 394 × TBIO Aton, TBIO Aton × BRS 254, CD 1303 × BRS 254 and CD 1303 × BRS 264.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

HCM, CRC, AB and MN designed and conducted the research; HCM, CRC, CMS and GWL performed the field experiment; HCM and MN performed the statistical analyses; AB and MN supervise, validate, and acquire funding for the research; HCM wrote the manuscript; and HCM, CRC, CMS, GWL, AB and MN reviewed the manuscript.

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Genetic improvement of faba bean (*Vicia faba* L.) genotypes selected for resistance to chocolate spot disease

Mejoramiento genético de genotipos de haba (*Vicia faba* L.) seleccionados con resistencia a la enfermedad de la mancha chocolate

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ABSTRACT

Inter-varietal hybridization is a powerful tool for genetic improvement and production of new genotypes for a trait of interest. Four parents of faba beans (*Vicia faba* L.) were hybridized using agromorphological and molecular characterization to obtain genotypes resistant to the chocolate spot disease. The study was done at the Nubaria Research Station, Giza, Egypt. Eight traits including resistance to chocolate spot, days to flowering, plant height (cm), number of branches/plant, number of pods/plant, number of seeds/plant, 100-seed weight (g), and seed yield/plant were estimated during the three growth seasons of 2016/2017, 2017/2018, and 2018/2019. Genetic parameters revealed by RAPD and ISSR markers assessed the genetic variation of genotypes with their generations. Crosses 1 (P_1 "Nubaria-1" x P_2 "Sakha-1"), 2 (P_1 "Nubaria-1" x P_3 "TW"), and 3 (P_1 "Nubaria-1" x P_4 "Camolina") revealed high resistance to disease with high yield. Markers patterned specific loci of resistant parents at a length of 360, 470, 450, 660, and 140 bp in RAPD and 1100, 810, 650, 700, 480 bp in ISSR. Inter-varietal hybridization between the resistant and susceptible genotypes is considered one of the most promising methods to obtain germplasm with resistance and high yield.

Key words: hybridization, ISSR, genetic resistance, plant breeding, RAPD, yield components.

RESUMEN

La hibridación intervarietal es una herramienta poderosa para el mejoramiento genético y la producción de nuevos genotipos prometedores para un rasgo de interés. Cuatro progenitores de haba (*Vicia faba* L.) fueron cruzados para obtener genotipos resistentes a la enfermedad de la mancha chocolate mediante caracterización agromorfológica y molecular. El estudio de campo se llevó a cabo en la Granja Experimental de la Estación de Investigación de Nubaria, Giza, Egipto. Se estimaron ocho características, incluidas el grado de resistencia a la mancha chocolate, los días a floración, la altura de planta (cm), el número de ramas/planta, el número de vainas/planta, el número de semillas/planta, el peso de 100 semillas (g) y el rendimiento de semillas/planta, durante las tres temporadas de crecimiento de 2016/2017, 2017/2018 y 2018/2019. Los parámetros genéticos se estimaron mediante marcadores RAPD e ISSR para evaluar la variación genética de los genotipos con sus generaciones. Los cruces 1 (P_1 "Nubaria-1" x P_2 "Sakha-1"), 2 (P_1 "Nubaria-1" x P_3 "TW") y 3 (P_1 "Nubaria-1" x P_4 "Camolina") revelaron alta resistencia a la enfermedad de la mancha chocolate con alto rendimiento. Los marcadores modelaron loci específicos de padres resistentes a una longitud de 360, 470, 450, 660 y 140 pb en RAPD y 1100, 810, 650, 700, 480 pb en ISSR. La hibridación intervarietal entre los genotipos resistentes y susceptibles es considerada uno de los métodos más promisorios para obtener germoplasma con resistencia y alto rendimiento.

Palabras clave: hibridación, ISSR, resistencia genética, fitomejoramiento, RAPD, componentes de rendimiento.

Introduction

Faba beans (*Vicia faba* L.; 2n=12) are one of the most common field pulses in Egypt (Bakry *et al*., 2011; Mohamed *et al*., 2012) and one of the oldest crops cultivated worldwide (Link *et al*., 1995; Zong *et al*., 2009). They belong to the Fabaceae family, the subfamily of Papilionoideae, and the tribe of Viceae (Duc, 1997). Faba beans are consumed for their green pods and dried seeds (Duc *et al.,* 2010). They are considered a main source of cheap protein and energy in Africa and some parts of Asia and Latin America, where many people cannot afford a meat source of protein (Duc, 1997; Alghamdi, 2009).

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A fungal disease called chocolate spot, caused mainly by *Botrytis fabae* (Sardina) and by *B. cinerea* (Pers.), is one of the most damaging diseases to this plant (Harrison, 1988; El-Komy *et al.,* 2015; Haile *et al*., 2016; Aguilar-Luna *et al*., 2021). Symptoms oscillate from some spots on leaves to complete covering of the plant. Under severe conditions, the disease spreads from affected leaves into stems, flowers, and pods, causing damages (Bernier *et al*., 1993; Rahman *et al.,* 2002; Villegas-Fernández *et al*., 2012; Haile *et al*., 2016). Various approaches are employed to control the disease, including genetic improvement (Wilson, 1937; Sahile *et al*., 2008; Abou-Zeid & Hassanein, 2000; Aguilar-Luna *et al*., 2021).

Quantitative trait loci (QTL) utilizes our knowledge of the effect of genetic control tools for selection in crop breeding programs. Various approaches are used for selecting promising traits, especially those for adapting to the local environment (Mahdy & El-Sharabasy, 2021). Phenotypic-revealed markers assess genetic diversity and performance of germplasm versus the attribute (Mahdy, 2012). These markers are based on visual observations (El-Sharabasy *et al*., 2021; Mahdy & El-Sharabasy, 2021) and screen the quantitative traits to increase field crop production.

PCR (polymerase chain reaction) - based markers are applied for genetic improvement and breeding, genetic diversity, and genetic relationships (Chen *et al.,* 2008; Tomás *et al.,* 2016). PCR is fast, reproducible, simple, and low-cost procedure. PCR-based markers are very practical in multi-disciplines, including genetic diversity and genetic improvement programs (González *et al.,* 2005). Various PCR-based markers were applied on crops, *i.e*., Random Amplified Polymorphic DNA (RAPD) (Link *et al*., 1995), Inter-simple Sequence Repeats (ISSR) (Terzopoulos & Bebeli, 2008; Aguilera *et al.,* 2011; Abdel-Razzak *et al.,* 2012; Mahdy, 2012; Asfaw *et al*., 2018) on jew's mallow, Restriction Fragment Length Polymorphism (RFLP) (Torres *et al*., 1993), Start Codon Target (SCoT) (Mahdy *et al*., 2021) on cowpea, and Conserved DNA-Derived Polymorphism (CDDP) (Ghazzawy *et al*., 2021) on date palm.

We chose four faba bean parents for (1) improving genetic resistance against the chocolate spot, (2) determining the genetic variations, (3) evaluating the performance under the infection of chocolate spot, (4) measuring genetic distance, and (5) generating a molecular profile using agromorphological traits and RAPD markers.

Materials and methods

Faba bean materials, planting, and field experiment

Four parents, as shown in Table 1, were sown on the experimental farm at the Nubaria Research Station, Agricultural Research Center (ARC), Egypt, during the seasons of 2016/2017, 2017/2018, 2018/2019, to evaluate their performance via the measurement of agromorphological traits. There is a high incidence of chocolate spot disease in Nubaria; moreover the disease is spread widely in the northern region of the Nile Delta of Egypt, with low temperatures and high relative humidity (Khalil *et al.,* 1993). Four parents were hybridized in 2016/2017 to secure F_1 hybrid seeds in the 2016/2017 season. In the 2017/2018 season, parents were re-hybridized; their F1 hybrids were grown in a randomized complete block design with three replicates under insect-free cages. In the 2018/2019 season, parents with F_1 and F_2 generations were artificially inoculated with *Botrytis fabae* fungus, under insect-free cages, then purified and identified according to Morgan (1971). Each plot comprised six rows 3 m long, with 0.60 m distance between rows, and 0.2 m between mounds with two seeds in each.

Measurement of agromorphological traits

Eight traits were measured: plant height (cm), number of branches/plant, days to flowering, seed number per plant, pod number per plant, seed yield per plant (g), 100-seed weight (g), and reaction to chocolate spot disease. An assessment scale of response to chocolate spot disease was estimated using a quantitative scale of 0-5, where 0 (very highly resistance) indicates no visible chocolate spot, 1 (high resistance) indicates a few chocolate spots, 2 (resistance) indicates increased and scattered spots, 3 (moderately resistance) indicates larger spots , 4 (susceptible) indicates necrotic spots reaching half of the leaf, 5 (highly susceptible) indicates majority of necrotic spots and leaf abscission (ICARDA, 2005). Homogeneity of the

TABLE 1. Pedigree and origin of four parents.

ID	Parents	Origin	Pedigree	Botanical group	Foliar disease reaction
P1	Nubaria1	Egypt	Single plant selection form Giza Blanka	Large	Resistant
P2	Sakha1	Egypt	620/283/85x716/724/88	Medium	Resistant
P3	TW	Sudan	Sudan	Medium	Susceptible
P4	Camolina	Spain	Imported from Spain	Small	Susceptible

variance across environments was tested according to the Bartlett test (Steel & Torrie, 1980). Parents and their F_1 and F_2 hybrids were evaluated for the experiment traits and measurements without reciprocal.

Molecular analysis

DNA extraction

DNA was extracted from twenty samples of the faba beans (4 parents + 6 hybrids F_1+ 10 hybrids F_2) using the DNeasy Plant Kit (Qiagen, Germany). Nanodrop was used to determine the DNA concentration and quality.

PCR analysis

Six RAPD primers and five ISSR primers were used to detect the polymorphism among the twenty samples, which were synthesized by Metabion Corp., Germany (Tab. 2).

The amplification reaction was done in 25 µl reaction volume containing 12.5 µl Master Mix (sigma), 2.5 µl primer (10 pcmol), 3 µl template DNA (10 ng), and 7 µl dH_2O, according to Ibrahim *et al.* (2019). The PCR was processed with a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, USA) adjusted to fulfill 40 cycles. The initial denaturation cycle was for 5 min/94°C. Each was at 94°C in 45 s for the denaturation step and 72°C in 60 s for the elongation step. The annealing temperature was adjusted as in Table 2 for 50 s. The extension was adjusted at 72°C in 7 min in the final cycle. The amplified products were run in a 1.5% agarose gel containing ethidium bromide (0.5 µg ml^{-1}) in 1X TBE buffer at 95 V. A 100 bp DNA ladder (Promega, USA) was standardized to determine the PCR product sizes. Gel images were visualized using a UV transilluminator and photographed using a Gel Documentation System (BIO-RAD 2000, USA). A binary matrix as present (1) or absent (0) was scored for the PCR products. The final data sets included both polymorphic and monomorphic bands.

TABLE 2. List of primers and their nucleotide sequence.

No	Name	Sequence	Temperature, °C
1	OPA-07	5'-GAAACGGGTG-3'	36
2	OPA-10	5'-GTGATCGCAG-3'	36
3	OPA-17	5'-GACCGCTTGT-3'	36
4	OPB-05	5'-TGCGCCCTTC-3'	36
5	OPG-19	5'-GTCAGGGCAA-3'	36
6	OPG-20	5'-TCTCCCTCAG-3'	45
7	ISSR-1	5'-AGAGAGAGAGAGAGAGTC-3'	45
8	ISSR-2	5'-AGAGAGAGAGAGAGAGTG-3'	45
9	ISSR-3	5'-ACACACACACACACACAT-3'	45
10	ISSR-4	5'-ACACACACACACACACTG-3'	45
11	ISSR-5	5'-GTGTGTGTGTGTGTGTAG-3'	45

Statistical analysis

A randomized complete block design (RCBD) with three replicates was used according to Gómez and Gómez (1984) and analyzed using MSTATC computer software. Average values and analysis of variance were conducted for all studied traits of 20 faba bean genotypes (four parents: (1) P_1, (2) P_2, (3) P_3, (4) P_4, six F_1 generation: (5) P_1xP_2, (6) P_1xP_3, (7) P_1xP_4, (8) P_2xP_3, (9) P_2xP_4, (10) P_3xP_4, and ten F_2 generation: (11) P_1xP_2, (12) P_1xP_3(R), (13) P_1xP_3(S) (14) P_1xP_4(R), (15) P_1xP_4(S) (16) P_2xP_3(R), (17) P_2xP_3(S), (18) P_2xP_4(R), (19) P_2xP_4(S) and (20) P_3xP_4 (S×S)) in each generation according to Steel *et al.* (1997). The least significance difference (LSD) test ($P \leq 0.05$) was calculated (Steel & Torrie, 1980).

We calculated the number of total bands, unique bands, polymorphic bands, and the percent of polymorphism. We also estimated some genetic parameters. Shannon information index was calculated $\{I = -1 \times (p \times \text{Ln}(p) + q \times \text{Ln}(q))\}$ according to Shannon (1948). The observed number of alleles (N_a), effective number of alleles $\{N_e = 1 / (p^2 + q^2)\}$, Nei genetic diversity $\{h = 1 - \Sigma (p^2 + q^2)\}$, and Unbiased Diversity $\{{}_u h = (\text{N} / (\text{N} - 1)) \times h)\}$; where p = band frequency and $q = 1 - p$ estimated according to Hartl and Clark (1997) and Liu and Muse (2005). Nei's genetic identity and distance used Nei (1972) and Nei (1978). Nei's genetic identity was calculated with the following formula: $NeiI = \frac{Jxy}{\sqrt{JxJy}}$, where $Jxy = \sum_{i=1}^{k} PixPiy$, $Jx = \sum_{i=1}^{k} Pix^2$, $Jy = \sum_{i=1}^{k} Piy^2$. Nei genetic distance was estimated with the formula $NeiD = -lnI$, where I is the genetic identity. F-test was determined trait-marker associations. Power marker software V3.0 was fed. The association analysis selected markers with a high P-value ($P > 0.01$).

Results and discussion

Phenotype-based traits

The average values of the agromorphological traits were calculated (Tab. 3). The results showed broad significance, as evidenced by the characteristic ranges for chocolate spot disease (1.30 to 5.63, resistant to susceptible, respectively), days to flowering (40.80 to 63.66), plant height (93.30 to 131.66 cm), number of branches per plant (3.10 to 10.80), number of pods per plant (25.46 to 111.36), number of seeds per plant (83.13 to 295.83), 100 seed weight (47.30 to 137.20 g), and seed yield per plant (56.37 to 199.96).

TABLE 3. Performance of growth and yield characteristics.

Genotypes	Chocolate spot	Days to flowering	Plant height (cm)	No. of branches/plant	No. of pods/plant	No. of seeds/plant	100-seed weight (g)	Seed yield/plant
Parents								
P_1	1.30	63.66	130.80	10.80	44.50	152.33	129.30	187.16
P_2	1.87	42.93	112.20	4.50	42.33	131.73	100.36	132.70
P_3	5.63	42.56	102.63	3.10	38.73	104.03	55.20	56.73
P_4	5.40	40.80	102.17	5.10	46.56	137.80	47.30	64.93
F_1 generation								
$P_1 \times P_2$	1.86	41.90	125.80	6.53	40.73	119.00	137.20	155.66
$P_1 \times P_3$	2.73	51.83	127.47	5.63	44.33	132.13	93.26	129.00
$P_1 \times P_4$	2.20	44.96	126.10	7.56	82.46	250.86	78.10	199.96
$P_2 \times P_3$	2.30	46.63	119.13	5.90	67.46	205.20	71.83	147.96
$P_2 \times P_4$	2.86	44.63	111.90	6.96	98.50	295.83	62.53	181.20
$P_3 \times P_4$	3.86	42.06	103.83	5.53	111.36	265.96	54.53	134.06
$LSD_{0.05}$	1.18	3.33	5.35	1.03	9.27	35.34	7.61	30.35
F_2 generation								
$P_1 \times P_2$ (R×R)	1.73	50.20	131.66	6.90	47.40	147.30	132.13	172.36
$P_1 \times P_3$ (R)	2.44	49.63	125.38	5.42	44.33	132.13	93.26	129.00
$P_1 \times P_3$ (S)	3.53	47.00	122.20	4.20	25.46	83.13	76.40	62.60
$P_1 \times P_4$ (R)	2.22	49.43	136.18	7.56	73.58	242.08	78.16	169.88
$P_1 \times P_4$ (S)	3.53	48.63	132.43	6.56	53.86	175.73	76.40	123.06
$P_2 \times P_3$ (R)	2.34	45.83	117.73	5.92	65.77	203.82	71.93	149.76
$P_2 \times P_3$ (S)	3.40	42.93	116.63	4.46	55.86	173.13	70.46	119.80
$P_2 \times P_4$ (R)	2.48	45.13	113.82	5.87	91.73	276.91	61.84	171.68
$P_2 \times P_4$ (S)	4.50	42.86	111.06	4.60	73.06	158.90	60.03	95.76
$P_3 \times P_4$ (S×S)	5.20	43.33	93.30	5.36	61.40	181.70	51.90	93.50
$LSD_{0.05}$	1.01	4.10	6.13	1.44	8.07	32.92	6.52	29.82

As shown in Table 3, Nubaria 1 recorded the highest mean values of 100-seed weight, seed yield/plant, and plant height (cm). Meanwhile, Camolina recorded a higher number of pods/plant, seed/plant, and branches/plant than the other parents. On the other hand, TW recorded the lowest mean values of the number of pods and seeds per plant. The two parents, Nubaria 1 and Sakha 1, gave the highest mean values of plant height. The parents, Nubaria 1 and Sakha 1, were considered highly resistant; their estimated mean values were (1.30 and 1.80) for chocolate spot infection. The parents TW and Camolina had the highest susceptible values (5.63 and 5.40) for the chocolate spot.

F_1 generation

The mean values of the sex-tested hybrids were calculated (Tab. 3). Results indicated that the cross (P_1xP_2) had the highest mean value of 100-seed weight (137.20 g). The two crosses, P_1xP_3 and P_1xP_4, produced the last cross. For plant height, the crosses, P_1xP_3 and P_2xP_4, had the highest mean values (127.47 cm and 126.10 cm, respectively) of plant height and the lowest value (103.83 cm) obtained from the cross (P_3xP_4). The genotypes Camolina, Sakha1, and TW were the parents which flowered quickest. Also, cross P_1xP_2 (41.90 d) was the cross which flowered quickest, followed by the cross P_3xP_4 with a mean value of 42.06 d. Concerning the number of branches/plant, the results showed that three crosses (P_1xP_4), (P_2xP_4), and (P_1xP_2) had the highest values.

Concerning the number of pods per plant, the parental variety Camolina (P_4) showed the highest mean value (46.56), whereas cross (P_1xP_2) gave the lowest number of pods/plant. For the number of seeds per plant, the cross P_2xP_4 gave the highest mean value (295.83), followed by the two crosses P_3xP_4 (265.96) and P_1xP_4 (250.86).

The two crosses (P_1xP_2) with 137.20 g and (P_1xP_3) with 93.26 g had the highest mean of 100-seed weight and highest seed yield per plant.

The highest seed yield per plant could be attributed to the high number of seeds and seed weight/plant. The two mentioned crosses were the most promising for yielding ability and tended to combine high seed yield and its components.

F_2 generation

The cross (P_1xP_2) gave the highest mean values of the number of seeds per plant, seed yield per plant, and 100-seed weight and gave the lowest infection value for chocolate spot (1.73) (Tab. 3). The cross (P_1xP_3) had the lowest mean values for number of pods per plant and number of seeds per plant and the highest infection value of the cross P_1xP_2 (3.53). The cross (P_3xP_4) had a low mean value for 100-seed weight, and the reaction for chocolate spot gave the highest susceptible value (5.20).

Three crosses, P_2xP_3, P_2xP_4, and P_3xP_4, were the earliest crosses for the flowering date. The cross (P_2xP_4) gave the highest number of pods per plant, number of seeds per plant, and the highest susceptible value for reaction chocolate spot (4.50). The lowest values were scored by the two crosses, P_1xP_4 and P_2xP_3, which also gave the lowest value for chocolate spot infection (3.53 and 3.40, respectively). Those genotypes considered promising for chocolate spot resistance and the best performing F1 crosses had mean values slightly better than those of F_2 crosses in most of the studied traits.

Significant differences among faba bean genotypes in all studied traits were considerable evidence for the existence of a suitable amount of genetic diversity valid for further assessments (Ahmed *et al*., 2016; Abdalla *et al*., 2017; Hamza & Khalifa, 2017; Abou-Zaid & El-Gendy, 2019; El-Abssi *et al*., 2019).

The results suggest that these genotypes carry genes for resistance to chocolate spot disease, coming from their parents according to their pedigree (Tab. 1). Similar results have been reported for faba bean yield traits and components, as well as for disease resistance traits (Abid *et al.,* 2015; Zakaria *et al*., 2015; Beyene *et al*., 2016; Eldemery *et al.,* 2016; Belal *et al.,* 2018; El-Rodeny *et al.,* 2020).

Polymorphism revealed via RAPD markers

Performance among twenty faba bean genotypes was analyzed by PCR-based markers, RAPD and ISSR (Figs. 1-2). Table 4 summarizes the data resulting from all analyzed loci that resulted from 148 amplified fragments ranging from 60 for ISSR and 88 for RAPD. The RAPD markers ranged from 130-2000 bp while the ISSR markers oscillated from 160-1500 bp. The polymorphism averaged a score of 74%, oscillating from 57% for ISSR to 74% for RAPD. RAPD markers revealed a high percentage of polymorphism compared with ISSR. The RAPD markers scored four positive unique bands by the primer OPA-07. Unique bands were at 1350 and 140 bp for P_1xP_2 (F_1), 920 and 130 bp for P_1xP_4 S and P_1xP_3 S (F_2), respectively. On the contrary, the ISSR revealed negative unique bands across the F_2 generation. The primer ISSR-1 scored a negative unique band at a distance of 160 bp (P_1xP_4 R); ISSR-3 scored at 650 bp (P_2xP_4 R), 360 (P_1xP_3 S), and 250 bp (P_2xP_3 R); and ISSR-5 scored 529 bp (P_2xP_4 R).

TABLE 4. Polymorphism revealed by ISSR and RAPD markers.

Primers	MW (bp)	Total (AF)	M_b	U_{b+}	U_{b-}	P_b	P (%)
			RAPD				
OPA-07	130 -1350	19	5	4	0	14	74
OPA-10	160 - 1500	14	3	0	0	11	79
OPA-17	140 - 860	14	5	0	0	9	64
OPB-05	140 - 820	10	3	0	0	7	7
OPG-19	160 - 2000	15	2	0	0	13	87
OPG-20	180-1800	16	5	0	0	11	69
Total $_{RAPD}$	130-2000	88	23	4	0	65	74
			ISSR				
ISSR-1	1530 -1100	14	4	0	1	10	71
ISSR-2	170 - 800	12	6	0	0	6	5
ISSR-3	200 - 1500	14	4	0	3	10	71
ISSR-4	160 - 530	11	8	0	0	3	27
ISSR-5	239 - 950	9	4	0	1	5	56
Total $_{ISSR}$	160 - 1500	60	26	0	5	34	57
Total		148	49	4	5	99	67

MW=molecular weight, AF=amplified fragment, M_b=monomorphic bands, U_{b+}=positive unique bands, U_{b-}=negative unique bands, P_b=polymorphic bands, P=polymorphism percentage.

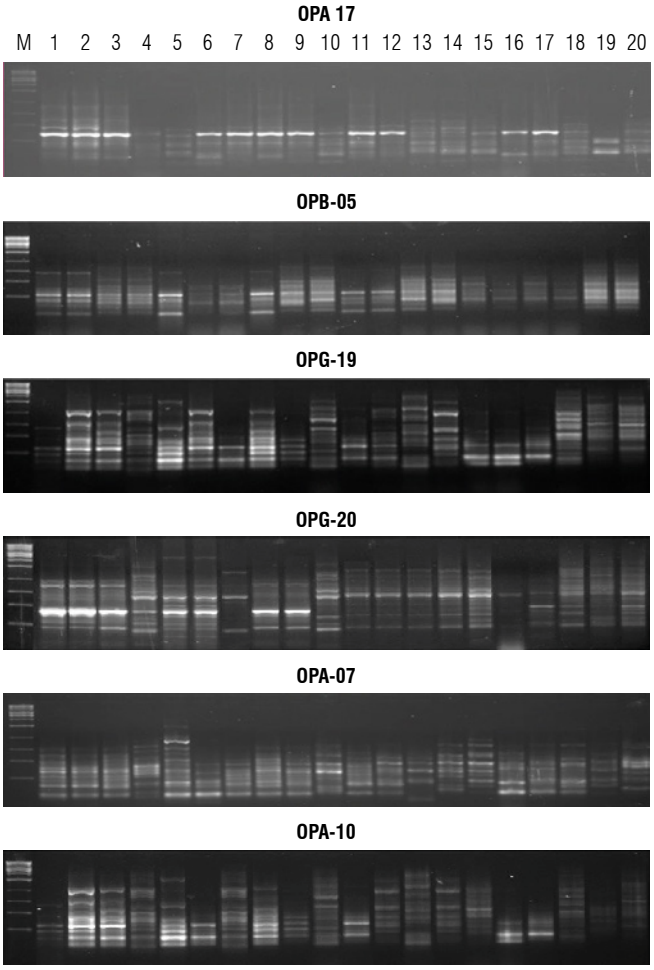

FIGURE 1. Profiles of twenty faba bean genotypes revealed via RAPD.

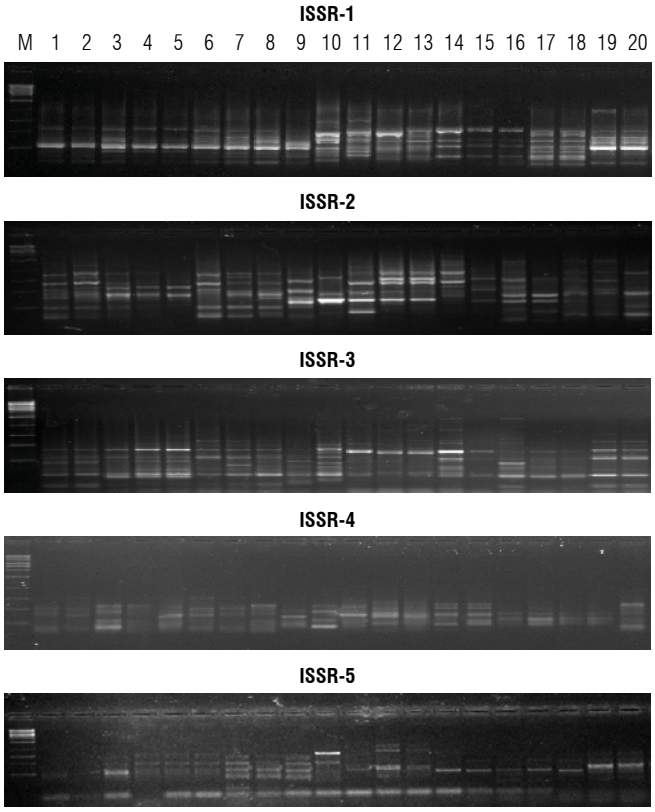

FIGURE 2. Profiles of twenty faba bean genotypes revealed via ISSR.

Table 5 summarizes specific loci in parents segregated during subsequent generations. These loci can be used directly for the breeding program for resistance to chocolate spot disease. RAPD markers patterned specific loci of resistant parents at a length of 360 bp for OPA-07, 470 bp (P_2), and 450 bp (P_1 and P_2) for OPA-17, 660 and 140 bp (P_1 and P_2) for OPB-05. Primers, OPA-10, OPG-19, and OPG-20, patterned specific loci of susceptible P_4 except the primer OPA-17 owing to a specific locus for susceptible parent (P_3) at a distance of 400 bp. ISSR patterned resistant specific loci at a length of 1100 & 810 bp by ISSR-1, 650 bp by ISSR-2, and 700 & 480 bp by ISSR-3 for parents 1 and 2. The primers ISSR-2 and ISSR-5 patterned susceptible specific loci at 800 & 170 bp for P_3 and P_4 and 390, 281 and 239 bp, respectively. The primer ISSR-1 characterized P_3 only at a length of 200 bp.

We found the RAPD markers showed higher polymorphism than the ISSR, in contrast to other results detected in wheat (Nagaoka & Ogihara, 1997) and vigna beans (Ajibade *et al.*, 2000). Terzopoulos and Bebeli (2008) found 68% polymorphism between the Greek faba bean populations using eleven ISSR primers. Khalaf *et al.* (2015) found 73% polymorphism between seven faba bean genotypes using fifteen ISSR primers.

Genetic parameters, identity, and distance

Genetic parameters are vital for the efficiency of a marker technique used in discriminatory objects based on polymorphism. The number of genetic parameters was estimated to evaluate the informative and discriminatory power during subsequent faba beans generation, as shown in Table 6. Heterozygosity (*h*) averaged 0.18, oscillating from 0.16 to 0.20 for RAPD, from 0.15 to 0.23 for ISSR, and from 0.16 to 0.21 for both markers. *h* reveals important polymorphism information content (*PIC*), with high informative marker value.

The Shannon index (*I*) revealed an average of 0.271 for both markers used. The RAPD markers ranged from 0.229 for Parents to 0.304 for F1, with an average of 0.275. The ISSR markers oscillated from 0.23 (F_1) to 0.33 (F_2), with an average of 0.266. The effective number of alleles (N_e) of RAPD markers ranged from 1.275 (Parents) to 1.311 (F_1), while ISSR revealed the range from 1.26 (F_1) to 1.1 (F_2), with a grand average of 1.306.

TABLE 5. Specific loci related to performance of resistance and susceptible genotypes.

Primer	MW	P_R	P_S	F_1	F_2
OPA-07	360	P_1, P_2		P_1xP_3	P_1xP_2, P_2xP_3(R), P_2xP_3 S, P_3xP_4
OPA-10	1200	---	P_4	P_3xP_4	P_1xP_3S
	810	---	P_4	P_1xP_4 & P_3xP_4	P_1xP_3S & P_1xP_4S & P_2xP_4R & P_3xP_4
OPA-17	470	P_2	---	-----	P_2xP_4S & P_3xP_4
	450	P_1 & P_2	---	P_1xP_4 & P_2xP_3 & P_3xP_4	P_1xP_2 & P_1xP_3R & P_1xP_3S & P_1xP_4R & P_2xP_4R
	400		P_3	P_1xP_2	P_1xP_3S & P_1xP_4R & P_1xP_4S
OPB-05	660	P_1 & P_2	---	P_1xP_2 & P_2xP_3	P_2xP_4S & P_3xP_4
	140	P_1 & P_2	---	P_1xP_2 & P_2xP_3	P_1xP_2 & P_1xP_3R & P_2xP_4S & P_3xP_4
OPG-19	2000	---	P_4	-----	P_2xP_4R
	840	---	P_4	P_2xP_4 & P_3xP_4	P_1xP_3R & P_1xP_3S & P_2xP_4R & P_2xP_4S
	600	---	P_4	P_2xP_4	P_1xP_3S
OPG-20	1500	---	P_4	P_3xP_4	P_1xP_2 & P_2xP_4R & P_2xP_4S
	1050	---	P_4	P_3xP_4	P_2xP_4R & P_2xP_4S & P_3xP_4
	210	---	P_4	P_1xP_4 & P_3xP_4	-----
ISSR-1	1100	P_1 & P_2	---	P_1xP_2	P_1xP_4R
	810	P_1 & P_2	---	P_1xP_2 & P_2xP_4	P_1xP_2 & P_2xP_4R & P_2xP_4S
	200	---	P_3	P_1xP_3 & P_1xP_4 & P_2xP_3 & P_2xP_4 & P_3xP_4	P_1xP_3R & P_1xP_4S & P_2xP_3R & P_2xP_4R & P_3xP_4
ISSR-2	800	---	P_3 & P_4	P_3xP_4	P_1xP_3R & P_1xP_3S & P_1xP_4R & P_1xP_4S & P_2xP_3R & P_2xP_3S & P_2xP_4R & P_2xP_4S
	650	P_1 & P_2	---	P_1xP_2 & P_2xP_4	P_1xP_2 & P_2xP_4R & P_2xP_4S
	170	---	P_3 & P_4	P_3xP_4	P_3xP_4
ISSR-3	700	P_1 & P_2	---	P_1xP_3 & P_1xP_4 & P_2xP_3 & P_2xP_4 & P_3xP_4	P_1xP_2 & P_1xP_3R & P_1xP_3S & P_1xP_4R & P_1xP_4S & P_2xP_3R & P_2xP_3S & P_2xP_4R & P_2xP_4S
	480	P_2	---	P_2xP_3 & P_2xP_4	P_1xP_2 & P_1xP_3R & P_1xP_4R & P_2xP_3R & P_2xP_3S & P_2xP_4S
ISSR-5	390	---	P_3 & P_4	P_2xP_3 & P_3xP_4	P_1xP_3R & P_1xP_3S & P_1xP_4R & P_1xP_4S & P_2xP_3R & P_2xP_3S & P_2xP_4R & P_2xP_4S & P_3xP_4
	281	---	P_3 & P_4	P_2xP_4 & P_3xP_4	P_1xP_3S & P_1xP_4S & P_2xP_3S & P_2xP_4R & P_2xP_4S & P_3xP_4
	239	---	P_3 & P_4	P_3xP_4	P_1xP_3S & P_1xP_4S & P_2xP_3S & P_2xP_4S & P_3xP_4

TABLE 6. Genetic parameters calculated among parents and their generations.

Generation	Na	Ne	I	He	uHe	%P
			RAPD			
P	1.148	1.275	0.229	0.16	0.178	38
F_1	1.568	1.311	0.304	0.20	0.213	64
F_2	1.568	1.297	0.292	0.19	0.197	64
Total	1.428	1.294	0.275	0.18	0.196	56
			ISSR			
P	1.33	1.30	0.24	0.17	0.19	0.38
F_1	1.38	1.26	0.23	0.15	0.17	0.42
F_2	1.57	1.41	0.33	0.23	0.24	0.57
Total	1.428	1.324	0.266	0.18	0.199	45.56
			Grand markers			
P	1.223	1.286	0.232	0.16	0.183	39.19
F_1	1.493	1.291	0.272	0.18	0.194	54.73
F_2	1.568	1.342	0.309	0.21	0.216	60.81
Total	1.428	1.306	0.271	0.18	0.197	51.58

Na=number of alleles, Ne=effective number of alleles, I=Shannon index, He=expected heterozygosity, uHe=unbiased expected heterozygosity, and %P=percentage of polymorphism.

Duc *et al.* (2010) reported an enormous genetic variability for faba beans, useful for breeding purposes. The results of *h* agree with those of Suresh *et al.* (2013), who recorded PIC values of 0.45 in faba beans genotypes revealed by developing 55 novel polymorphic cDNA–SSR markers. Also, Oliveira *et al.* (2016) recorded PIC values from 0.07 to 0.66, with an average of 0.33. Hemeida (2008) used PIC to evaluate the primer efficiency of ISSR and established the relationships, and successfully discriminated among the genotypes tested.

The difference between the mean diversity (*h*) of both markers and between markers was undoubtedly due to mirror inbreeding or selection method against heterozygotes. The nature of used markers might be due to the level of observed heterozygosity resulting in the non-detection of homozygotes from heterozygotes because of the presence of null alleles. The heterozygosity represents the direct count of heterozygosity in the population and is estimated based on the allele frequency of individuals in that population according to the Hardy-Weinberg equilibrium. The *PIC* evaluates the informative potential of markers in different germplasm (Grativol *et al.*, 2011; Mahdy, 2018). The *h* value of a marker with many amplicons desirable for variation splits into three main classes based on Botstein *et al.* (1980). The PIC values are more than 0.5 for highly informative markers, between 0.25-0.5 for reasonably informative markers, and less than 0.25 for slightly informative markers. The Shannon information index (*I*) is one of the most important genetic diversity measurements (Sherwin *et al.*, 2006). The effective number of alleles (N_e) is a reciprocal of gene homozygosity (Hartl & Clark, 1997). The N_e is used as a corollary to *h*; when *h* is high, N_e will be the high.

Table 7 summarizes the estimates of genetic distance and genetic identity between generations and their parents according to the Nei coefficient (Nei, 1972; Nei, 1978). It reflects the genetic relationships and the direction of the genetic improvement process. Results show that the RAPD markers revealed high genetic identity and distance values. The highest genetic distance value scored by RAPD was 0.089 on parents versus F_1, and the lowest value scored 0.028 on F_1 versus F_2. Genetic identity oscillated from 0.914 (parent versus F_1) to 0.972 (F_1 versus F_2), as revealed by RAPD.

Nei genetic identity ranges from 0 to 1. Consequently, Nei genetic distance ranges from 0 to infinity (Nei 1972; Nei, 1978). ISSRs are more efficient markers for polymorphism and potent for intra- and inter-genomic diversity than other arbitrary markers like RAPDs (Zietkiewicz *et al.*, 1994). Both markers target different portions of the genome.

TABLE 7. Genetic and identity distance among generations and parents.

Parameter	P versus F_1	P versus F_2	F_1 versus F_2
RAPD			
Nei genetic identity	0.924	0.914	0.972
Nei unbiased genetic identity	0.947	0.932	0.989
Nei genetic distance	0.079	0.089	0.028
Nei unbiased genetic distance	0.055	0.070	0.011
ISSR			
Nei genetic identity	0.951	0.942	0.954
Nei unbiased genetic identity	0.973	0.964	0.969
Nei genetic distance	0.050	0.059	0.047
Nei unbiased genetic distance	0.028	0.037	0.031
All			
Nei genetic identity	0.935	0.925	0.964
Nei unbiased genetic identity	0.957	0.945	0.981
Nei genetic distance	0.067	0.077	0.036
Nei unbiased genetic distance	0.044	0.057	0.020

Genetic variations in genotypes of interest may be more directly due to polymorphism detected by technique rather than which techniques are employed.

Cluster analysis and similarity between twenty genotypes

Cluster analysis derived from both markers based on UPGMA in accordance with Jaccard (1908; Fig. 3). The twenty genotypes divided into two main groups at a distance of 0.65. Cluster I (right cluster) divided into two subgroups at a distance of 0.668. The first subgroup (left subgroup) consists of P_1 and P_2 in the sub-sub group and P_1xP_2 (F_1) only in a sub-sub group. The second subgroup (right subgroup) split further into sub-sub groups, which include the genotypes of $P_1xP_2(F_2)$ and $P_2xP_4(F_1)$ grouped in a cluster, $P_1xP_3(F_1)$ only in a group, $P_2xP_3(F_1)$ only in a group, and P_2xP_3 $S(F_2)$ and $P_2xP_3R(F_2)$ together in a group.

Cluster II (left cluster) separated into two subgroups at a distance of 0.672. Each split further into sub-sub groups. The first sub-sub group (right one) includes $P_1xP_2R(F_2)$ and P_2xP_4 $R(F_2)$ together, P_4 and $PxP_4(F_1)$ in a cluster, and P_1xP_4 $R(F_2)$ and P_1xP_4 $S(F_2)$ in a group. The second one consists of P_2xP_4 $S(F_2)$, P_1xP_3 $S(F_2)$, and $P_3xP_4(F_2)$ in a separated cluster for each and P_3 and $P_3xP_4(F_1)$ in a cluster.

El-Ghadban *et al.* (2017) and Mahdy *et al.* (2021) reported similar results. The differences in the clustering pattern of genotypes may be due to marker sampling error, the level of polymorphism, or the number of loci and their coverage across the genome (Loarce *et al.*, 1996).

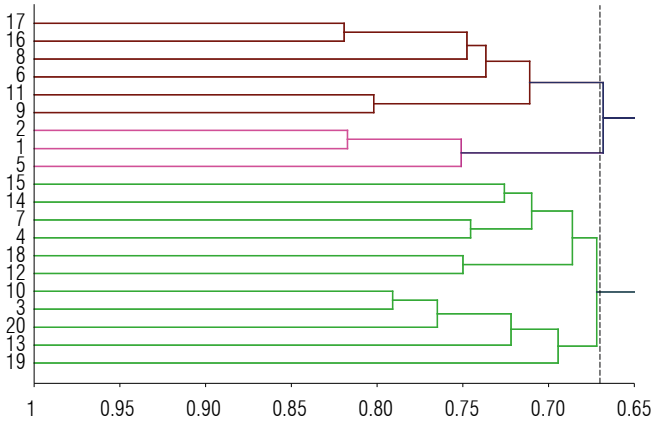

FIGURE 3. Dendrogram tree constructed using UPGMA according to Jaccard similarity. Cluster analysis based on RAPD and ISSR markers; (1)-P_1,(2)-P_2, (3)-P_3, (4)-P_4, "six F_1" (5)-P_1xP_2, (6)-P_1xP_3, (7)-P_1xP_4, (8)-P_2xP_3, (9)-P_2xP_4, (10)-P_3xP_4, "ten F_2" (11)-P_1xP_2, (12)-P_1xP_3 (R), (13)-P_1xP_3 (S) (14)-P_1xP_4 (R), (15)-P_1xP_4 (S) (16)-P_2xP_3 (R), (17)-P_2xP_3 (S), (18)-P_2xP_4 (R), (19)-P_2xP_4 (S) and (20)-P_3xP_4 (S×S)".

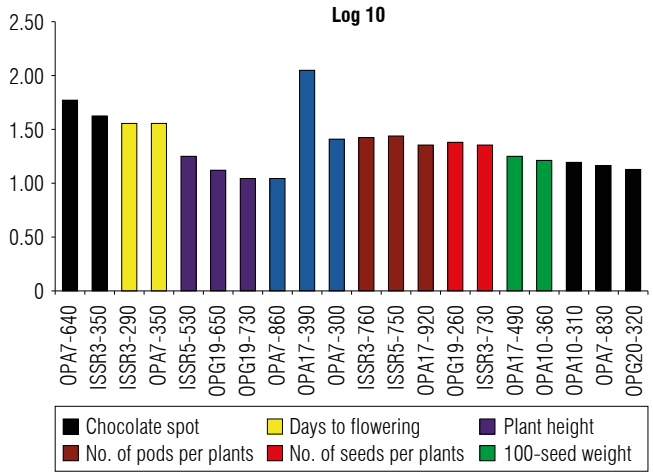

FIGURE 4. Association analysis. Trait-marker association was achieved through F-test analysis. Power marker software V3.0 was used and markers with high *P*-value ($P>0.01$) were selected in the association analysis. X and Y axes refer to -Log P values and the name of primers associated with certain traits.

Association analysis

Trait-marker association was analyzed using a single locus F-test module in Power maker software (Fig. 4). The results show the property of one trait for one locus characterized. The characterized locus generated by OPG-20 was associated uniquely with seed yield per plant. Moreover, several traits were associated with one or more than one locus. Amplified loci of OPA-07 were associated with the different characterized chocolate spots, days to flowering, the number of branches per plant, and seed yield per plant.

Previous results have suggested the high potential use of molecular markers in search for genes affecting crop productivity and resistance to chocolate spots. Also, defense mechanisms against biotic and abiotic stress factors identified statistical associations between the genetic markers and the traits of interest.

Marker-trait associations were generally diverse in the amount of genetic variation valid for assessment. The results exhibited that a greater number of primers was possibly involved in controlling traits at resistance to chocolate spot disease. This variation described by identified associations for each trait may be attributed to the role of many minor genes controlling the trait, performance, and reaction of faba bean genotypes to chocolate spot disease, markers exhibiting minor quantitative effect, rare alleles, and complex allelic interactions (Yang *et al*., 2010; Debibakas *et al*., 2014). These results correspond with the findings of Lou *et al*. (2015) and Sun *et al*. (2015). Association data can be used for faba beans breeding, especially in terms of its resistance to biotic and abiotic stresses.

Conclusion

The parents Nubaria1, Sakha1, TW, and Camolina could be considered good for crossing for resistance to foliar chocolate spot disease. Crosses 1, 2, and 3 (P_1xP_2 Nubaria-1 x Sakha-1, P_1xP_3 Nubaria-1 x T.W, P_1xP_4 Nubaria-1 x Camolina, respectively) showed resistance to disease during both generations, F_1 and F_2 with high values for yield and its components, especially date of flowering and 100-seed weight per plant. Both markers exhibited interest specific loci relating to performance of chocolate spot that are 360 bp (OPA-07), 450 and 470 bp (OPA-17), 140 and 660 bp (OPB-05), 810 and 1100 bp (ISSR-1), 650 bp (ISSR-2), and 480 and 700 bp (ISSR-3). The genetic distance valued by RAPD was 0.089 on parents versus F_1, and the lowest value scored 0.028 on F_1 versus F_2. Genetic identity oscillated from 0.914 (parent versus F_1) to 0.972 (F_1 versus F_2), as revealed by RAPD. Estimating genetic relationships and differences generated by both markers could be moderately clarified by the product number of PCR, the number of bands, and coverage along the genome with the association of agromorphological traits. The current research could provide information on the morphological and molecular characteristics of faba beans chocolate spot disease. Intervarietal hybridization (resistant and susceptible) is still considered one of the most promising methods for obtaining germplasm with resistance and high yield.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

EM, MI, AM, and HEH designed the experiments. HEH, EM, and MI carried out the formal analysis. MI and HEH provided all resources. EM, MI, and AM oversaw and led the research activity and execution. All authors wrote the original draft. EM, HEH, and EMBM authorized, edited, and reviewed the manuscript.

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Critical dilution curves for calcium, magnesium, and sulfur in potato (*Solanum tuberosum* L. Group Andigenum) cultivars Diacol Capiro and Pastusa Suprema

Curvas críticas de dilución de calcio, magnesio y azufre en cultivares de papa (*Solanum tuberosum* L. Grupo Andigenum) Diacol Capiro y Pastusa Suprema

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ABSTRACT

Diagnostic tools must be developed to optimize the management of calcium (Ca), magnesium (Mg), and sulfur (S) in potato crops. This research aimed to develop the critical dilution curves for Ca, Mg, and S in potato (*Solanum tuberosum* L. Group Andigenum), establishing harvest indices and characterizing the nutrient relationships. Four field experiments were established in two growth cycles in the localities of Facatativá (high fertility soils) and Chocontá (low fertility soils) in Colombia. Two cultivars (Diacol Capiro and Pastusa Suprema) and two levels of fertilization (0 and 100% of macro and micronutrients) were evaluated. The dry biomass and Ca, Mg, and S concentration in tubers and aerial parts were measured from the formation of main stems until tuber maturation; this information was used to calculate the critical concentrations (Cac, Mgc, Sc), harvest indices, and nutrient correlations. The critical curves established were for Capiro: $Cac = 1.7326W^{-0.2956}$, $Mgc = 0.7191W^{-0.2803}$, $Sc = 0.6461W^{-0.3904}$ and for Suprema: $Cac = 1.523W^{-0.2559}$, $Mgc = 0.6507W^{-0.236}$, $Sc = 0.7669W^{-0.3932}$. Critical levels were established for five phenological stages. Capiro had a higher accumulation of Ca, Mg, and S in the tubers independently of locality, while Suprema had better performance in Chocontá. The accumulation of mineral nutrients in the tubers followed the order Ca<Mg<S. Capiro was a genotype with greater Ca-Mg-S uptake and better adaptation to locations. The Cac, Mgc and Sc curves provided a tool to carry out the nutritional diagnoses at critical stages of development and they are the first ones reported for potato of Group Andigenum.

Key words: nutrient concentration, nutrient diagnostics, nutrient harvest index, secondary macronutrients.

RESUMEN

Herramientas de diagnóstico deben ser desarrolladas para optimizar el manejo de calcio (Ca), magnesio (Mg) y azufre (S) en cultivos de papa. La investigación tuvo como objetivos desarrollar las curvas críticas de dilución para Ca, Mg y S en papa (*Solanum tuberosum* L. Grupo Andigenum), establecer sus índices de cosecha y caracterizar la relación entre nutrientes. Se establecieron cuatro experimentos en campo en dos ciclos, en las localidades de Facatativá (suelos de alta fertilidad) y Chocontá (suelos de baja fertilidad) en Colombia. Se evaluaron dos cultivares (Diacol Capiro y Pastusa Suprema) y dos niveles de fertilización (0 y 100% de macro y micronutrientes). Se midió la biomasa seca y concentración de Ca, Mg y S en tubérculos y parte aérea, desde la formación de tallos principales hasta maduración del tubérculo y se calcularon las concentraciones críticas (Cac, Mgc, Sc), índices de cosecha y correlaciones entre nutrientes. Las curvas criticas establecidas para Capiro fueron: $Cac = 1.7326W^{-0.2956}$, $Mgc = 0.7191W^{-0.2803}$, $Sc = 0.6461W^{-0.3904}$ y para Suprema: $Cac = 1.523W^{-0.2559}$, $Mgc = 0.6507W^{-0.236}$, $Sc = 0.7669W^{-0.3932}$. Se establecieron niveles críticos para cinco etapas fenológicas del cultivo. Capiro presentó mayor acumulación de Ca, Mg y S en el tubérculo independiente de la localidad, mientras Suprema tuvo mejor desempeño en Chocontá. La acumulación de nutrientes minerales en los tubérculos siguió el orden Ca<Mg<S. Capiro mostró ser un genotipo de mayor consumo de Ca-Mg-S y de mejor adaptación a localidades con condiciones edafoclimáticas contrastantes. Las curvas de Cac, Mgc y Sc proporcionan una herramienta para realizar el diagnóstico nutricional en etapas críticas del desarrollo y son las primeras reportadas en cultivos de papa del Grupo Andigenum.

Palabras clave: concentración de nutrientes, diagnóstico nutricional, índice de cosecha de nutrientes, macronutrientes secundarios.

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Introduction

The potato (*Solanum tuberosum* L.), a species native to South America, has wide adaptability to different edaphoclimatic conditions and is cultivated in countries in temperate, tropical, and subtropical regions (Campos & Ortiz, 2020). Potato tubers are rich in carbohydrates, low in fat and have an adequate balance of vitamins and mineral nutrients (Handayani *et al*., 2019; Gaj *et al*., 2020). Due to its high nutritional quality and potential yield, the potato is a species of great importance for food security in the world (Raymundo *et al*., 2018). In recent years, there has been a progressive world increase in potato cultivation; currently 19 million ha are cultivated with an approximate production of 378 million t (Campos & Ortiz, 2020). In Colombia, the departments with the highest production are Cundinamarca with 37%, Boyacá with 27%, and Nariño with 20% (Minagricultura, 2019).

The cultivars Diacol Capiro and Pastusa Suprema from the Andigenum Group are among the most widely consumed in Colombia and are appreciated for their qualities for industrial processing (chips or cane) and fresh consumption (Barrientos & Ñústez, 2014; Gómez *et al*., 2019a). Both cultivars are tetraploid, produce tubers under short-day conditions, have good frying behavior, and potentially yield more than 40 t ha^{-1} (Guerrero-Guio *et al*., 2019; Campos & Ortiz, 2020).

The potato crop has a high demand for mineral nutrients compared to other short-cycle crops (Helal & AbdElhady, 2015); fertilization can represent about 24% of the total costs (Fedepapa, 2018). Nutritional research is extensive for nitrogen (N), phosphorus (P), and potassium (K) (Koch *et al*., 2020). However, the information on calcium (Ca), magnesium (Mg), and sulfur (S) necessary to obtain optimal tuber yield and quality is scarce (Hauer-Jákli & Tränkner, 2019; Koch *et al*., 2020; Naumann *et al*., 2020). These elements play fundamental roles, for example in cell walls and membranes, signaling, activation of enzymes, energy metabolism, formation of amino acids, sulfolipids, etc. (Koch *et al*., 2020).

The traditional management of crops with contributions focused on NPK in continuous production cycles, added to the natural processes of nutrient loss (leaching, erosion, fixation, among others), decreases the natural contents of secondary macronutrients in soil and generates imbalances that limit the availability of Ca, Mg, and S (Aula *et al*., 2019; Wang *et al*., 2020). The application of Ca-Mg-S is frequently carried out following general recommendations, with over- or under-dosage of nutrients (Koch *et al*., 2019). This problem is not necessarily detected during the production cycle; its negative effects on the yield and quality of the tuber are not evident until harvest. In this context, a diagnostic tool must be developed that allows the nutritional evaluation of Ca-Mg-S during the production cycle, to detect and correct excesses or deficits opportunely.

An adequate supply of Ca-Mg-S has positive effects on yield (Muthanna *et al*., 2017; Seifu & Deneke, 2017; Wang *et al*., 2020) and tuber quality (Singh *et al*., 2018; Koch, Naumann, *et al*., 2019; Assunção *et al*., 2020). A deficit of these nutrients leads to less translocation of photoassimilates to the tubers, low mechanical resistance, low specific gravity, and multiple physiological disorders, such as hollow heart (Koch *et al*., 2019; Schabow & Palta, 2019). In contrast, doses higher than the optimum can lead to negative effects due to excess of other nutrients, loss of fertilizer, and cost overruns (Wang *et al*., 2018; Barroso *et al*., 2021).

The critical dilution curve (CDC) of a mineral nutrient is an allometric relationship between its concentration in the plant and the biomass or leaf area (Lemaire *et al*., 2019). This tool is based on the principle of the existence of a minimum concentration required to achieve the maximum growth of the crop, which varies with the age of the plant and its biomass (Wang *et al*., 2018; Carciochi *et al*., 2019). The CDC is a useful tool for carrying out a quick nutrient diagnosis *in situ* at different stages of the crop, allowing timely corrections in nutrition to reach maximum development (Greenwood *et al*., 1990; Giletto & Echeverría, 2015; Lemaire *et al*., 2019). The CDC approach has been successfully used for the management of N in various crops in cereal research (Lemaire *et al*., 2019; Chen *et al*., 2021). Likewise, the CDC of P, K, and S has been calculated for crops such as corn, canola, and wheat (Carciochi *et al*., 2019; Lemaire *et al*., 2019). In the potato, the CDC has been established for N (Giletto & Echeverría, 2015), P (Zamuner *et al*., 2016; Gómez *et al*., 2019b) and K (Cogo *et al*., 2006; Gómez *et al*., 2019b), however, we found no reports for Ca-Mg-S.

In order to improve our knowledge of the nutrition of potato with Ca-Mg-S, it is of interest to establish what type of relationship exists between these nutrients as the crop develops and to understand the accumulation dynamics in the harvest organs (Duarte *et al*., 2019; Naumann *et al*., 2020). The objectives of this research were to develop the critical dilution curves and harvest indices of Ca, Mg, and S in the potato (*Solanum tuberosum* L. Group Andigenum) and to analyze the relationships among these nutrients. The

results will provide a new tool, useful to potato producers, for the nutrient diagnosis and management of Ca-Mg-S during the productive cycle of two potato cultivars of importance in Colombia.

Materials and methods

Study site

The research was carried out at two localities in the Cundinamarca-Boyacá highlands (Colombia): Facatativá with Andic Eutrudepts (saturated bases, high fertility) and Chocontá with Humic Dystrudepts, (desaturated bases, acidic, low fertility) (Tab. 1). The localities were selected as representative of potato production in Colombia, showing high productive potential (> 50 t ha^{-1}) with contrasting edaphoclimatic conditions. Two production cycles were evaluated at each locality (2013-2016), each one with a total duration of 150-160 d after sowing (DAS). Soil analyses were carried out for each cycle and each locality for the arable layer (0-30 cm), prior to the establishment of the crop.

Experiment design and crop management

For each location and production cycle, an experiment was established in divided plots with four replicates distributed in completely random blocks. The main plot corresponded to the cultivars Capiro and Suprema and the subplots to the fertilization levels (0 and 100% of macro and micronutrients). Each experimental unit was 5 m x 10 m, with a distance between rows of 1 m and between plants of 0.37 m. As plant material, tubers of 70 g on average were used and sown manually. 135 plants were planted per plot with a density of 27,000 plants ha^{-1}. The fertilization doses (100% level) for each locality-cycle were established using the soil-plant balance method (Castro & Gómez, 2013) (Tab. 2). The 0% level corresponded to treatment without fertilization and represented the natural fertility conditions of the soil.

TABLE 1. Climatic variables and soil characteristics of the experiment locations.

Climatic variables * \ Location	Facatativá		Chocontá	
	Cycle 1 (2013-I)	Cycle 2 (2015-I)	Cycle 1 (2013-II)	Cycle 2 (2016-I)
Altitude (m a.s.l.)	2597	2597	2780	2710
Latitude	4°49'26.9" N	4°49'39.9" N	5°5'30.37" N	5°6'23.94" N
Longitude	74°22'29.7" W	74°22'49.3" W	73°43'2.04" W	73°40'48.53" W
Annual precipitation (mm)	951	850	1295	1058
Annual precipitation/cycle (mm)	397	415	712	803
Evapotranspiration per cycle (mm)	454	382	640	603
Max air temperature (°C)	18.1	18.5	16.2	16.5
Min air temperature (°C)	7	7.2	4.4	10.1
Mean temperature (°C)	12.7	12.5	10.6	12.9
Soil properties**	Andic Eutrudepts		Humic Dystrudepts	
Texture	Loam	Loam	Clay loamy	Clay loamy
Soil fertility	High	High	Low	Low
pH	6.4	5.8	5.5	5.3
Al (cmolc kg^{-1})	0	<0.1	0.1	0.5
Organic matter (g kg^{-1})	166.7	127.1	67.7	85.9
CEC (cmolc kg^{-1})	31.95	19.14	9.52	7.90
N (g kg^{-1})	8.3	6.4	3.3	4.3
P (mg kg^{-1})	39.64	70.16	18.18	41.50
K (cmolc kg^{-1})	3.14	0.87	0.68	0.84
Ca (cmolc kg^{-1})	24.26	15.98	7.20	5.90
Mg (cmolc kg^{-1})	4.36	2.14	1.57	1.40
S (mg kg^{-1})	29.53	29.53	11.52	11.52

* Data on climatic variables obtained from IDEAM. ** The physical chemical analysis of the soil was carried out according to IGAC (2006). The methods used were as follows: Al: Yuan's method; Organic matter: Walkley-Black; P: Bray II-colorimetry; K, Ca, Mg: ammonium acetate-atomic absorption; S; monobasic phosphate-colorimetry. Soil classification was done according to the USDA (Soil Survey Staff, 2014).

TABLE 2. Nutrient contributions by location and growth cycle for 100% fertilization treatment.

Locality	Cycle	Fertilization dose* (kg ha^{-1})	Mineral nutrient dose (kg ha^{-1})										
			N	P	K	Ca	Mg	S	B	Zn	Mn	Fe	Cu
Facatativá	1	1582	171	113	149	23.2	70	74	3.4	5.6	7	2.8	0.56
	2	1900	164	126	195	34.1	80	150	2.3	4.6	5.6	2.2	0.45
Chocontá	1	2175	192	148	289	46.3	56	120	1.2	2.4	3	1.2	0.24
	2	2000	191	165	262	46.3	40	38	4.4	4.8	5.4	2.2	0.43

*The fertilizer sources were diammonium phosphate ($(NH_4)_2HPO_4$), potassium chloride (KCl), potassium sulfate (K_2SO_4), calcium nitrate ($Ca(NO_3)_2$), magnesium sulfate monohydrate ($MgSO_4H_2O$), and Nutricomplet® (Ingeplant, Colombia. Source of B, Zn, Mn, Fe, and Cu). Adapted by permission from Springer Nature: Nutrient Cycling in Agroecosystems. Nitrogen, phosphorus and potassium accumulation and partitioning by the potato group Andigenum in Colombia, Gómez MI *et al.* Copyright 2019.

Fractionation was carried out according to the historical management of the study sites (yield history per harvest > 50 t ha^{-1}) as follows: N 55% at sowing and 45% at 45 DAS; P 80% at sowing and 20% at 45 DAS; K 12% at sowing and 88% at 45 DAS; Ca, Mg, S and lower, 63% at sowing and 37% at 45 DAS. Phytosanitary management of the crop was carried out according to local practices.

Sampling and measurements

Five destructive samplings were carried for the five phenological stages (Roveda *et al.*, 2010): stage I, 50 to 55 DAS (formation of primary stems); stage II, 70 to 75 DAS (formation of secondary stems and beginning of tuberization); stage III, 90 to 100 DAS (flowering, maximum tuberization, and beginning of tuber filling); stage IV, 120 to 125 DAS (end of flowering, tuber filling); stage V, 150 to 160 DAS (leaf senescence, maximum filling, and tuber maturation). In each sampling, three (cycle 1 at both localities and cycle 2 in Facatativá) or four (cycle 2 in Chocontá) plants were harvested per experimental unit and their organs were sectioned (leaves, aerial + underground stems, and tubers). For quantification of mineral nutrients, the plant material was washed with deionized water, the same organs from the four plants were mixed and a 200 g subsample was oven-dried at 70°C until constant weight to determine the dry weight (Gómez *et al.*, 2019a). The concentration of Ca, Mg, and S per organ was determined by chemical analysis according to IGAC (2006). The total contents of Ca, Mg, and S were estimated by multiplying the concentrations of nutrients in the organ (g 100 g^{-1} dry weight) by the amount of dry biomass accumulated in each stage (Abdallah *et al.*, 2016).

Critical dilution curves

The critical dilution curve from dry matter (DW) for Ca (Cac), Mg (Mgc), and S (Sc) was calculated according to Equation 1, proposed by Greenwood *et al.* (1990),

$$Nut_c = aW^{-b} \quad (1)$$

where Nut_c corresponds to the critical concentration of the nutrient in the biomass (g 100 g^{-1}), W is the total dry weight of the biomass (t ha^{-1}), the coefficient *a* is the concentration of the nutrient when the biomass is ≤1 t ha^{-1}, and the coefficient *b* (dimensionless) is a dilution coefficient that describes the curvature or decrease of the nutrient as the total biomass increases (Giletto & Echeverría, 2015).

For the calibration of the critical curves, the identification and selection of data for which fertilization did not significantly limit the growth of the crop (total dry biomass) was carried out. The following steps were followed: i) the principles of normality and homoscedasticity were evaluated; ii) the analysis of variance (ANOVA) of the total biomass under the different fertilization levels was carried out for each combination of factors (cycle x locality x cultivar x phenological stage); iii) means were compared using the minimum test significant difference (LSD) (P<0.05); iv) the data of the fertilization level with the highest biomass production were selected. When there were no significant differences, the lowest dose was chosen (Abdallah *et al.*, 2016; Wang *et al.*, 2017). From the selected data, the dilution curve for each nutrient was constructed by calculating the coefficients *a* and *b*, their standard errors, and 95% confidence intervals using the PROC NLIN procedure (SAS Institute, 2017). The model's coefficients were compared based on the method of intervals of confidence described by Cumming *et al.* (2007). Based on total biomass measured and the critical dilution curves developed, critical dilution values for each phenological stage were calculated.

Harvest indexes

Harvest (150 DAS) indexes for Ca (CaHI), Mg (MgHI), and S (SHI) were calculated, dividing the amount of nutrients accumulated in the tuber (Nut_{tub}, kg ha^{-1}) by the total accumulation (leaves, aerial stems, stolons, tubers) in the plant (Nut_{tot}, kg ha^{-1}) as shown in Equation 2,

$$NHI = (Nut_{tub} / Nut_{tot}) \quad (2)$$

where NHI is the nutrient harvest index) (Giletto & Echeverría, 2015). Roots were not considered in the analysis. For the analysis, the PROC MIXED procedure (SAS Institute, 2017) was used, taking the repetitions as a random effect and the cultivar, location, cycle, and fertilization level as fixed effects. For each level of interaction between factors, the least square means were calculated; based on these means, comparisons were made with the adjusted Tukey statistic (P<0.05) for mixed models.

Correlation and linear regression

The correlations between variables were calculated from the Spearman correlation coefficient using the PROC CORR procedure (SAS Institute, 2017). A total of n=130 observations (measured plants: locality x cycle x cultivar x phenological stage x plants measured) were used for each cultivar and significance was established with a 95% confidence limit. Linear models of the form "y=bx+a " were established by linear regression, where a is the intercept on the y-axis and b is the slope of the line. For each model, the coefficient of determination (R^2) was calculated. The models were compared by their coefficients based on the method of intervals of confidence described by (Cumming *et al*., 2007). The proportions of mineral nutrients in the plant were determined from the equations established for the linear model between the total contents of the nutrients, dividing 1 by the coefficient b (slope of the curve).

For all variables, the principles of normality and homoscedasticity were evaluated using PROC UNIVARIATE; an analysis of variance (ANOVA) was performed using PROC GLM in SAS 9.4 software (SAS Institute, 2017). The graphs were developed with ggplot2 (Wickham, 2016).

Results

Critical dilution curves

The dilution curves of Cac, Mgc, and Sc followed a potential negative model in relation to the increase in total dry biomass in the two cultivars (Fig. 1). The average dry biomass during the evaluation cycle was in the range of 1.7 to 22.6 t ha^{-1}. The coefficients a and b of the critical curves of the three nutrients did not show significant differences between cultivars (Tab. 3); however, a differential tendency was seen between the two Sc models. Capiro on average had 0.05 percentage unit lower concentration compared to Suprema throughout the growth cycle (Fig. 1C). For the biomass of 6 t ha^{-1}, the means averages of measured concentrations of Ca, Mg, and S in Capiro were 0.3, 0.2 and 0.1, higher than those established by the model (Fig. 1).

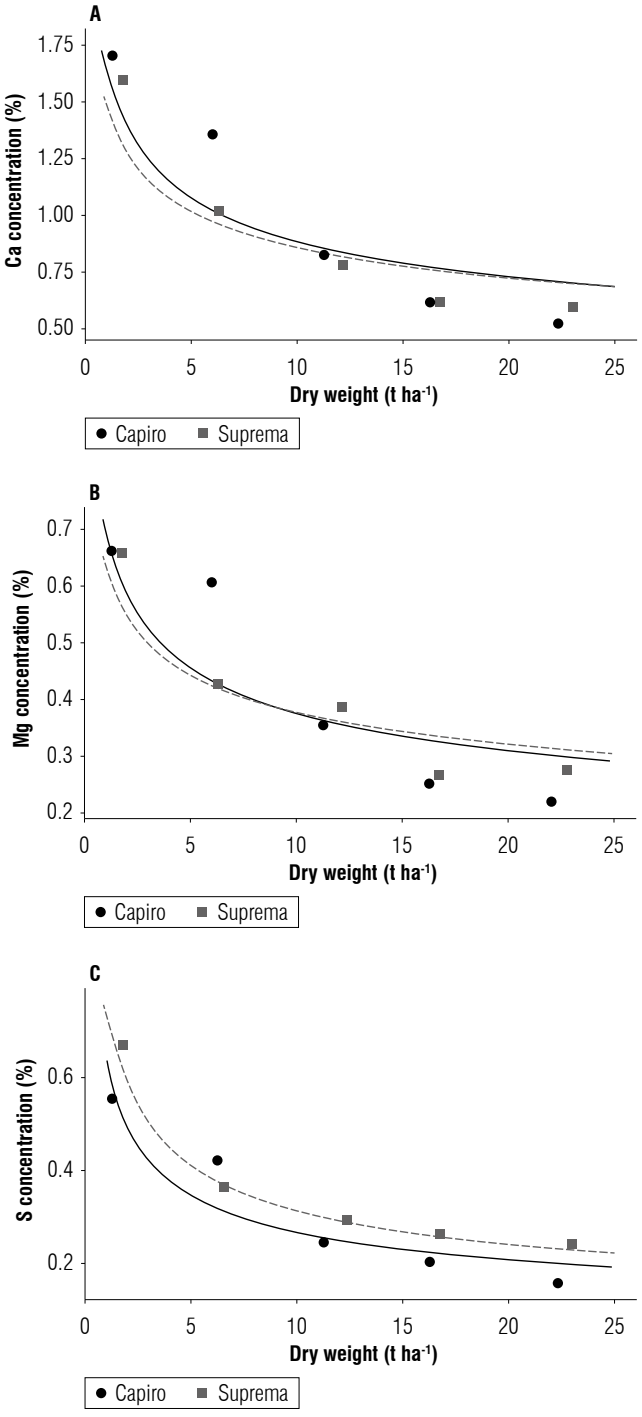

FIGURE 1. Critical dilution curves of A) Ca, B) Mg, and C) S for Diacol Capiro and Pastusa Suprema of *Solanum tuberosum* L. Andigenum Group under non-limiting nutrient conditions. The points constitute the data from which the critical curves were developed. Each point represents the average of 13 values taken per cultivar and phenological stage. The dotted and solid lines represent the CDC for each cultivar for a biomass greater than 1 t ha^{-1}.

TABLE 3. Coefficients of critical Ca, Mg and S dilution curves developed for Diacol Capiro and Pastusa Suprema from the total dry biomass (W) under non-limiting mineral nutrition conditions.

Nutrient	Cultivar	CDC ($N_c = aW^{-b}$)	RMSE	CI (*a*) 95%	CI (*b*) 95%	SE *a*	SE *b*
Calcium	Capiro	$1.7326W^{-0.2956}$	0.2133	1.3862- 2.0790 ns	0.1797- 0.4115 ns	0.1733	0.058
	Suprema	$1.523W^{-0.2559}$	0.1622	1.2172- 1.8288 ns	0.1508- 0.3610 ns	0.153	0.0526
Magnesium	Capiro	$0.7191W^{-0.2803}$	0.5493	0.5812 - 0.8570 ns	0.1731- 0.3875 ns	0.069	0.0536
	Suprema	$0.6507W^{-0.236}$	0.4199	0.5341- 0.7673 ns	0.1440- 0.3280 ns	0.0584	0.0460
Sulfur	Capiro	$0.6461W^{-0.3904}$	0.1480	0.5465- 0.7458 ns	0.2915- 0.4893 ns	0.0499	0.0495
	Suprema	$0.7669W^{-0.3932}$	0.2152	0.6090- 0.9249 ns	0.2660- 0.5204 ns	0.0791	0.0636

CDC: critical dilution curve; CI: confidence interval; SE: standard error; RMSE: root mean square error; ns: no significant differences found between the cultivars.

TABLE 4. Critical concentrations of Ca, Mg and S (%) by phenological stage under non-limiting conditions of mineral nutrition for Diacol Capiro and Pastusa Suprema.

Phenological stage	Capiro			Suprema		
	Ca_c	Mg_c	S_c	Ca_c	Mg_c	S_c
Vegetative growth	1.56	0.65	0.56	1.29	0.56	0.61
Initial tuberization	1.02	0.43	0.31	0.95	0.42	0.36
Flowering - maximum tuberization	0.85	0.36	0.25	0.80	0.36	0.28
End of flowering - tuber filling	0.76	0.33	0.22	0.74	0.33	0.25
Maximum filling - tuber maturation	0.69	0.30	0.19	0.68	0.31	0.22

The highest "dilution" of mineral nutrients was observed when the total dry biomass was between 1 and 6 t ha^{-1} (Fig. 1) in the tuberization initiation stage (Tab. 4). The average concentration of Ca in the plants decreased from a maximum of 1.6% (Capiro) and 1.3% (Suprema) in the vegetative growth stage to a minimum of 0.7% in the tuber maturation stage (Fig. 1A, Tab. 4). For Mg, the values were from 0.7% (Capiro) and 0.6% (Suprema) to 0.3% (Fig. 1B, Tab. 4) and for S the values varied from 0.6% to 0.2% (Fig. 1C, Tab. 4). The Cac and Sc curves had a greater fit with root mean square error (RMSE) values in the range of 0.1 to 0.2, while Mgc had an RMSE of 0.4 to 0.5 (Tab. 3). The Ca and Mg concentration of Capiro in the initial stage (stage I: vegetative growth) was 17% and 14% higher compared to Suprema, while that of S was 8% higher in Suprema (Tab. 4).

Harvest indexes

The CaHI values were in the range of 0.01-0.11 with a mean of 0.06. MgHI and SHI were in the range of 0.13-0.50 and 0.16-0.65 with means of 0.34 and 0.45, respectively (Fig. 2). The harvest indices showed differences among the localities, cycles, and cultivars (P<0.001). Capiro showed, on average, higher CaHI, MgHI and SHI values of 37%, 44%, and 57% compared to Suprema. The Mg and S indices of Suprema showed high variation between localities with a standard deviation (SD) of 0.14 and 0.20. The highest values were observed in Chocontá (CaHI: 0.05, MgHI: 0.39 and SHI: 0.50). Capiro had similar MgHI and SHI values in both locations with a SD of 0.08 and 0.06, respectively. Capiro had the highest CaHI value in Chocontá-1 and Suprema had the lowest value in Facatativá-1.

Relationship between Ca, Mg, and S in plant organs

The MgHI-SHI relationship showed the highest correlation (r=0.97) followed by CaHI-MgHI (r=0.94) and CaHI-SHI (r=0.91). The models established that MgHI and SHI increased by 4.38 and 4.92 units for each CaHI unit in Capiro and by 3.84 and 5.42 in Suprema. For each unit of MgHI, the SHI increased by 1.33 and 1.22 units in Capiro and Suprema, respectively. The determination coefficients (R^2) were greater than 0.7 except for the CaHI-SHI relationship. The values obtained for Capiro had lower dispersion and greater fit to the linear model with respect to Suprema. Between harvest indices these showed a positive linear behavior (Fig. 3, Tab. 6).

The correlations between the nutrient content in the aerial part of the plant and the tuber were 0.46, 0.41 and 0.29 for Ca, Mg, and S, respectively. Among the total nutrient contents, the highest correlation was between Ca-Mg (r=0.95), followed by Mg-S (r=0.88) and Ca-S (r=0.85).

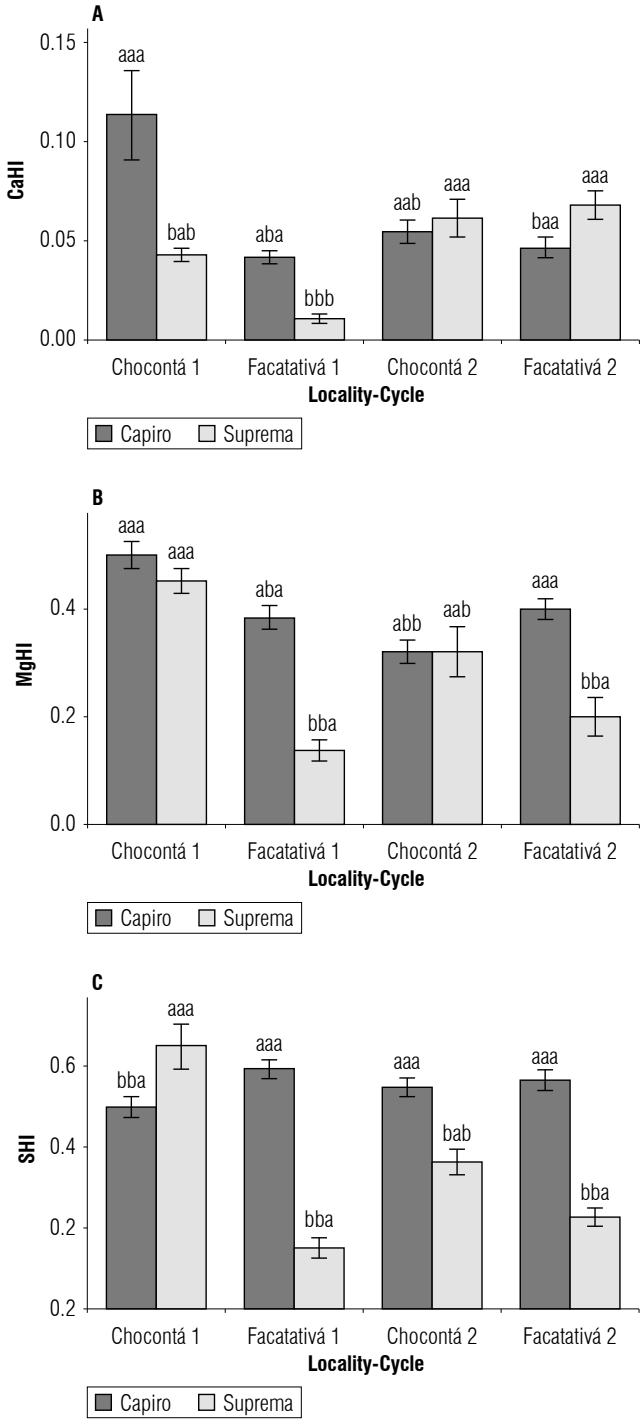

FIGURE 2. Harvest indexes (HI) for A) Ca, B) Mg, and C) S for Diacol Capiro and Pastusa Suprema in soils with low (Humic Dystrudepts, Chocontá) and high (Andic Eutrudepts, Facatativá) fertility, in two productive cycles (2013-2016). The first letter indicates significant differences between cultivars within the same cycle and locality; the second letter indicates significant differences between localities within the same cultivar and cycle; the third letter indicates significant differences between cycles within the same cultivar and locality. These were significant differences according to the Tukey's test ($P<0.05$). The error bars indicate the standard error.

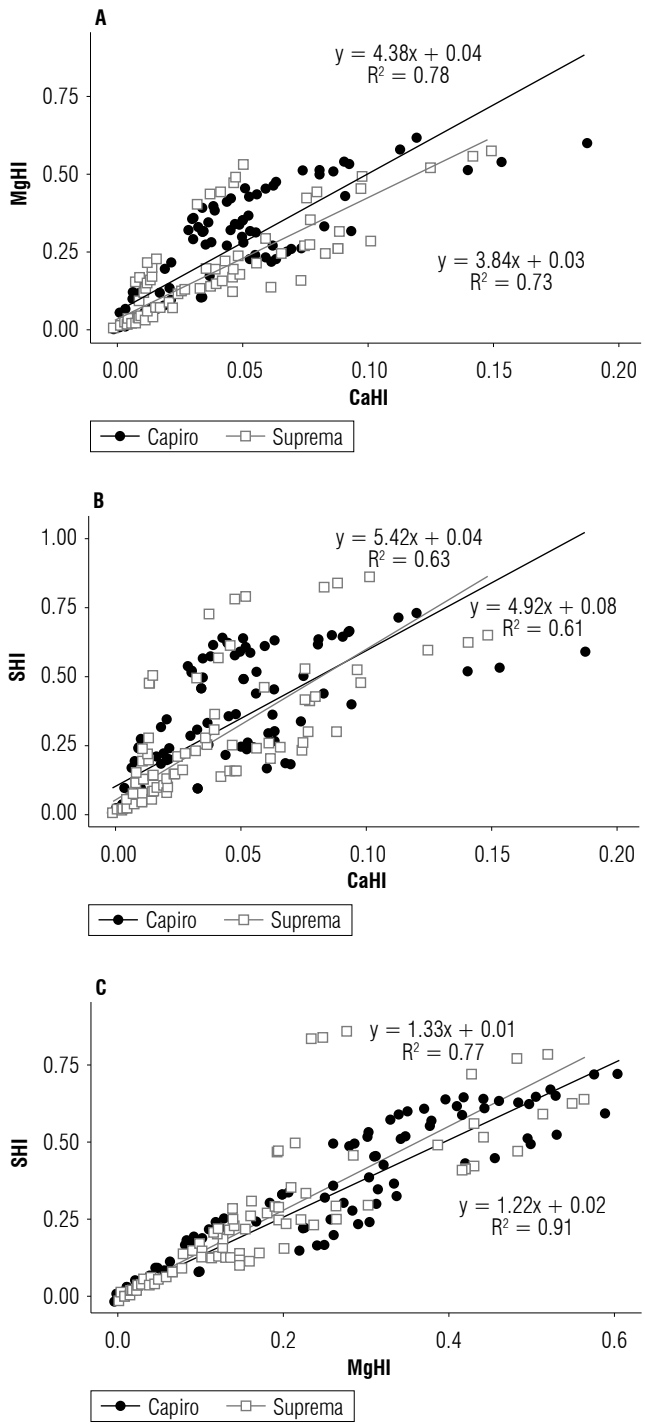

FIGURE 3. Relationships between the harvest indices of A) magnesium (MgHI) and calcium (CaHI), B) sulfur (SHI) and calcium (CaHI), and C) sulfur (SHI) and magnesium (MgHI) for Diacol Capiro and Pastusa Suprema (n = 130 for each cultivar). The lines represent the fitted model for each cv. R^2: coefficient of determination.

The models followed a positive linear behavior (Tab. 6) with an adjustment level greater than 0.7 (R^2) (Fig. 4). There were significant differences between cultivars for

the total Ca-S and total Mg-S ratio. For each unit of Mg, there was an increase of 0.55 and 0.66 units of S in Capiro and Suprema, respectively. Each unit of Ca increased 0.40 and 0.36 units of Mg and of S 0.29 and 0.20 for Capiro and Suprema, respectively.

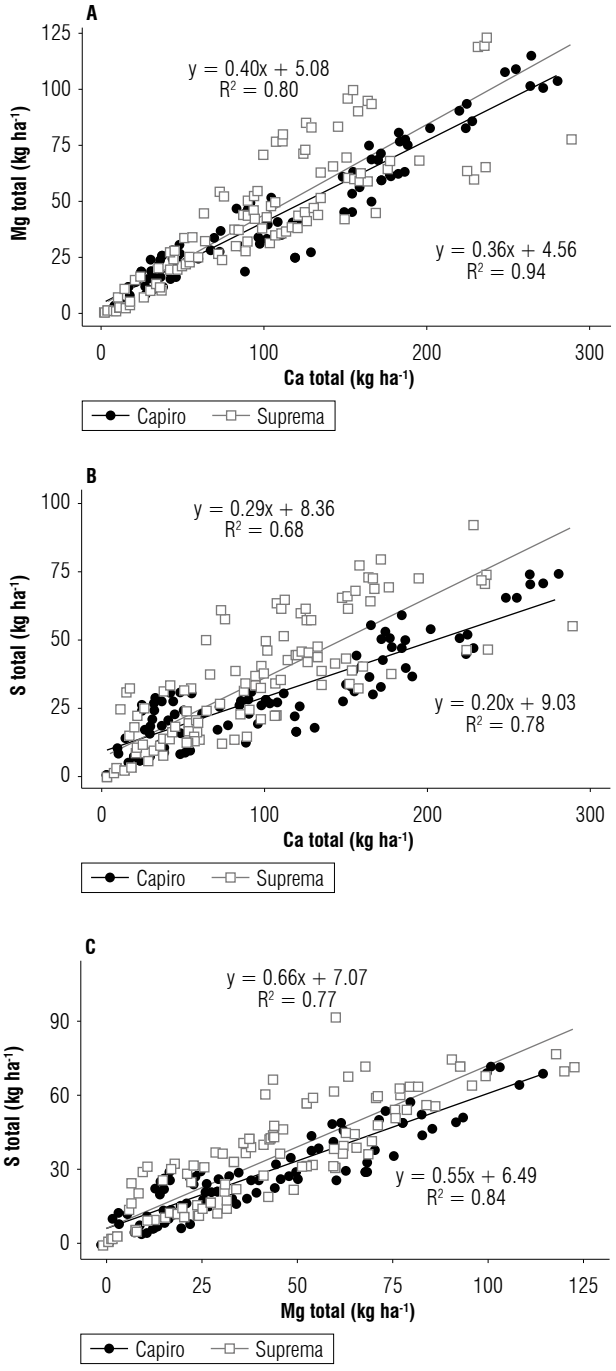

FIGURE 4. Relationship between the total content of A) Ca, B) Mg, and C) S for Diacol Capiro and Pastusa Suprema (n=130 for each cultivar). The lines represent the fitted model of each cv. R^2: coefficient of determination.

The Ca:S ratio was the highest one, followed by Ca:Mg and S:Mg, with Capiro having higher values than Suprema (Tab. 5). The correlation (r) between the yield (fresh weight of the tubers) and the content of Ca, Mg and S in the tubers was 0.94, 0.99 and 0.95, respectively (Tab. 6). The models had a positive linear behavior and an R^2 greater than 0.6. All the models showed significant differences between cultivars. For each t ha^{-1} increase in yield, Ca, Mg, and S increased 0.08, 0.28, and 0.27 kg ha^{-1} in the tubers for Capiro and 0.07, 0.25, and 0.29 kg ha^{-1} in the tubers for Suprema (Fig. 5, Tab. 6).

TABLE 5. Proportion between the total contents of Ca, Mg, and S in potato cultivars Diacol Capiro and Pastusa Suprema.

Relationship	Capiro	Suprema
Ca:Mg	2.8:1	2.5:1
Ca:S	5.0:1	3.4:1
Mg:S	1.8:1	1.5:1

TABLE 6. Confidence intervals for the linear models of the relationship between Ca, Mg, and S in cultivars Diacol Capiro and Pastusa Suprema.

Correlation	Cultivar	CI (m) 95%	CI (b) 95%
CaHI-MgHI	Capiro	3.98 - 4.78 ns	0.02 - 0.06 ns
	Suprema	3.43 - 4.25 ns	0.01 - 0.04 ns
CaHI-SHI	Capiro	4.22 - 5.61 ns	0.05 - 0.12 *
	Suprema	4.70 - 6.14 ns	0.01 - 0.07 *
MgHI-SHI	Capiro	1.15 – 1.28 ns	0.003 - 0.04 ns
	Suprema	1.21 – 1.46 ns	(-0.01) - 0.04 ns
Ca total-Mg total	Capiro	0.34 - 0.38 ns	2.68 - 6.44 ns
	Suprema	0.36 - 0.44 ns	0.81 - 9.35 ns
Ca total-S total	Capiro	0.18 - 0.22 **	6.90 - 11.16 ns
	Suprema	0.25 - 0.32 **	4.66 - 12.07 ns
Mg total-S total	Capiro	0.51 - 0.60 *	4.52 - 8.46 ns
	Suprema	0.59 - 0.72 *	3.87 - 10.28 ns
FTubW-Catub	Capiro	0.07-0.08 ns	(-0.34) – 0.12 **
	Suprema	0.06 - 0.08 ns	0.18 - 0.68 **
FTubW-Mgtub	Capiro	0.26 - 0.29 **	(-2.12) - (-0.73) **
	Suprema	0.24 - 0.26 **	(-0.66) - 0.001 **
FTubW-Stub	Capiro	0.27 - 0.30 ns	(-1.79) - (-0.53) *
	Suprema	0.24 - 0.31 ns	(-0.62) - 1.58 *

CI: confidence interval; CaHI: calcium harvest index; MgHI: magnesium harvest index; SHI: sulfur harvest index; FTubW: fresh weight of tubers; Catub: calcium content in the tubers; Mgtub: magnesium content in the tubers; Stub: sulfur content in the tubers.

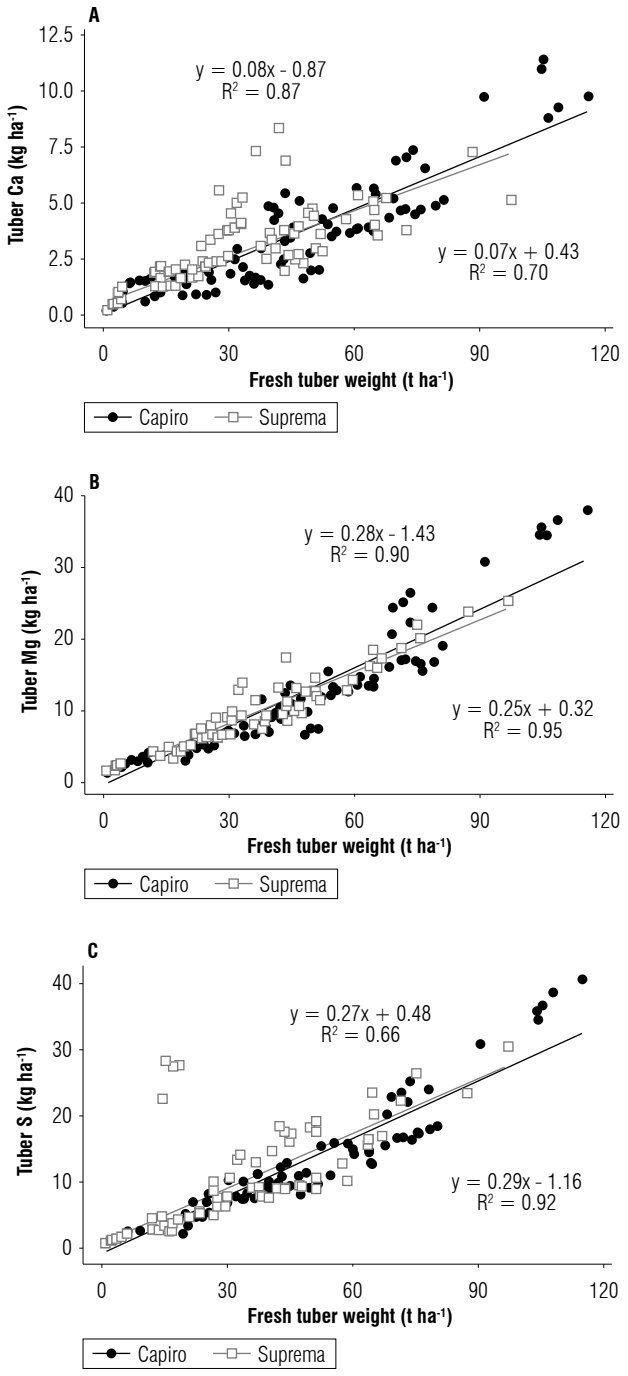

FIGURE 5. Relationship between the content of A) Ca, B) Mg, and C) S in the tubers with the fresh weight of the tubers (yield) for Diacol Capiro and Pastusa Suprema (n=130 for each cultivar). The lines represent the fitted model of each cultivar. R^2: coefficient of determination.

Discussion

The effect of "dilution" of Ca observed in potato agrees with Addiscott (1974), who reports a possible effect of dilution of a fixed amount of Ca in potato, contrasting with Walworth and Muniz (1993) who argue that the concentration of Ca tends to increase with age in the plants and to decrease in the tubers. The concentration of Ca required in the cytoplasm is less than 1 µM and the unused Ca stored in the vacuoles allows correct cellular activity (Koch *et al*., 2020). In this case, the Ca stored in the vacuole would not have a significant effect on the change in its concentration in the plant when considering a stable scenario for the availability of the nutrient. Although the concentration of Ca could be expected to increase as the plant grows due to its structural function, a greater and faster accumulation of other nutrients, proteins, carbohydrates, and other molecules explains the dilution effect (Koch *et al*., 2020).

The concentration of Ca in tubers during tuber maturation (0.68%) was lower than that reported by Jahanzad *et al*. (2017) for tubers of *S. tuberosum* "Dark Red Norland", (0.8%) and "Superior" (1.4%) at harvest time. The Mg concentration in tubers during the productive cycle (0.30-0.65%) was higher than that reported by Hauer-Jákli and Tränkner (2019) in leaves (0.14%) and by Walworth and Muniz (1993) in tubers (0.25%). This is because most studies do not establish the concentration in the whole plant and do not consider the variation of critical concentration during the development of the crop, instead proposing a single critical value. Likewise, the natural variation in nutrient consumption between cultivars must be considered. The concept of critical Mg concentration in plants has been poorly studied, so the establishment of values by phenological stage was a new proposal that complements what was previously published by Walworth and Muniz (1993).

The value of Sc for the flowering stage and maximum filling (0.25% and 0.28%) agrees with that reported by Walworth and Muniz (1993) in the potato. The Sc curve had a high dilution coefficient (b=0.39), higher than that established for other C3 crops such as wheat (b=0.17; Reussi *et al*., 2012), rape (b=0.18; Ferreira & Ernst, 2014), and soybean (b=0.11; Divito *et al*., 2016). The high coefficient *a* in potato (0.6-0.7) could indicate that the crop has a high requirement of S in the initial stages of development (<1 t ha^{-1}), explained by the rapid and high generation of foliage where the S is needed for the synthesis of proteins and sulfolipids.

The rapid decrease in the concentration of mineral nutrients observed for biomass of 1-6 t ha^{-1} is caused by the beginning of tuberization and filling of tubers. Where the total biomass in potato increases significantly, demand is high for photoassimilates and starch accumulates in harvestable organs (Gómez *et al*., 2019b), diluting the concentration of mineral nutrients. The nutrient with the

highest dilution was S, possibly because the requirements of Ca and Mg remain higher during growth and development due to their structural functions in cell walls, chlorophylls, and energy metabolism (Maathuis, 2009) and during the end of the cycle due to its structural role and the filling of tubers. On the other hand, S is mainly found in amino acids, proteins, and as part of glutathione, whose need and synthesis rate may be lower than the growth rate (Moussa *et al*., 2018). The greater slope of Sc for Capiro may indicate that this variety is more efficient in the use of S than Suprema (Santana *et al*., 2020). The dilution in the Sc curve was similar to that previously established for N (Gómez *et al*., 2019b), confirming the interaction between the assimilation of S and N in relation to their function in protein formation (Kopriva & Rennenberg, 2004).

The higher Ca and Mg concentrations in Capiro throughout the cycle are consistent with its higher quality for industrial processing. The higher proportion and concentration of S in Suprema is related to its indeterminate growth habit which demands higher protein synthesis. The nutrient differences in the potato seed between cultivars that directly affect the concentration of nutrients in the early stages must also be considered. For future research, it would be of interest to establish the nutrient content of the potato seed.

The dilution curves of Cac, Mgc and Sc are a useful tool to carry out the diagnosis of these nutrients in critical stages of potato cultivation from a sample of plant tissue. Concentration values in the sampling of plants above or below the dilution curve (in the corresponding phenological stage) indicate that the plants are growing with an excess or deficiency of the nutrient. Concentration values close to or fitting the curve indicate sufficiency of the nutrient (Marouani *et al*., 2014; Carciochi *et al*., 2019).

The low harvest indexes of Ca-Mg-S show that their mobility to the tuber is low and is lower than what was previously reported for the N-P-K nutrients (CI greater than 0.6) (Gómez *et al*., 2019a). The higher indexes for Capiro and homogeneity between localities indicate that this cultivar has a better accumulation capacity and phenotypic plasticity in different environments, favoring its productive potential and agreeing with the results in yield previously published by Gómez *et al*. (2019a). The higher CaHI value of Capiro in the first Chocontá cycle could be related to the higher recorded evapotranspiration, which favors the movement of Ca. On the other hand, Suprema showed better adaptation to the conditions of Chocontá (acid soils and low fertility), possibly because the environmental conditions of higher precipitation and lower temperature favored the solubility of nutrients in the soil and decreased the respiration rate of the plants. In contrast, in Facatativá, with soils of high fertility and higher temperatures, Suprema had a high consumption of N (Gómez *et al*., 2019a), which affected tuberization, translocation, and accumulation of Ca-Mg-S.

The greater translocation of Mg and S from Capiro in Facatativá could have an indirect effect on their accumulation in the tubers by favoring a greater formation of roots on the tubers and stolons that participate in uptake of nutrients directly from the soil for their later accumulation in these organs (Kratzke & Palta, 1985; Palta, 1996), positively affecting the harvest indices in the cultivar. These results are related to the higher yield of this cultivar and its quality for industrial uses (Gómez *et al*., 2018), because these nutrients participate in the translocation of photoassimilates and protein synthesis. Additionally, the positive effect of the bearing of the Capiro plant must be considered, since its leaves have larger leaflets and are parallel to the ground, while Suprema has a more perpendicular arrangement. This characteristic favors the incidence of radiation and, therefore, the photosynthetic rate, yield, and nutrient translocation in Capiro.

For the nutrients evaluated, the accumulation in the tuber followed the order of Ca<Mg<S, a result consistent with their mobility in the phloem in potato (Subramanian *et al*., 2011). The mobility of Ca by transpiration stream affects its translocation to organs of low transpiration rate, such as tubers (Schabow & Palta, 2019). Compared to other crops, the CaHI in potato (0.05) was lower than that reported for wheat (0.09) (Shen *et al*., 2019) and similar for corn (0.05) (Szczepaniak, 2016). The high harvest rates of Mg and S (greater than 0.5 for S and 0.3-0.4 for Mg), confirm the high mobility and accumulation in the tuber proposed by Silva *et al*. (2020) and Subramanian *et al*. (2011). The high accumulation of S in the tubers is due to its role in the partition of photoassimilates towards the tubers and as a structural element in amino acids (methionine and cysteine) and proteins (Dhakad *et al*., 2019). On the other hand, the MgHI of potato was lower compared to cereal crops such as rice (0.52) (Sánchez *et al*., 2019) or wheat (0.46) (Shen *et al*., 2019).

From these models, the value of SHI can be estimated with a good level of reliability from the MgHI; it has greater predictive confidence than CaHI. Likewise, the S content in the entire plant can be established from the total Ca or Mg content. The higher proportion between nutrients (total contents) in Capiro could be related to the fact that its growth is determined with respect to Suprema; with

Capiro having a more rapid tuberization and accumulation of reserves. Likewise, the lower Ca:S ratio in Suprema may be related to a higher S requirement due to its indeterminate growth habit. Future research should establish the relationship between these results and qualities in terms of mechanical resistance, disease incidence, and occurrence of pathophysiologies in both cultivars. Differences between cultivars may indicate variation in the processes of accumulation or utilization, confirming that Capiro and Suprema present different requirements for Ca, Mg and S. The models established for the interaction between nutrients are valid for the experimental conditions evaluated for Diacol Capiro cultivars and Pastusa Suprema from the Andigenum Group.

The interaction between Ca, Mg, and S in their total content and Ca-Mg in the tuber is explained by the participation of these nutrients in linked processes during crop growth. Magnesium participates in photosynthesis, energy metabolism, synthesis of proteins, enzymatic activity, and the transport of photoassimilates. Calcium participates in signaling processes and structuring of cell membranes and walls, and S is part of sulfolipids, proteins, and participates in oxidation-reduction processes (Koch *et al*., 2020). The results of the Mg-S interaction agree and those of Ca-S and Ca-Mg contrast as reported by Subramanian *et al*. (2011). The results of this research should not be confused with nutrient interaction within the soil, where antagonism between Ca-Mg, synergism between S-Mg and lack of correlation between S-Ca can occur (Klikocka & Głowacka, 2013; Barczak & Nowak, 2015; Rietra *et al*., 2017; Rhodes *et al*., 2018).

The low correlation between the content of nutrients in the aerial part and in the tubers could indicate a low dependence on the accumulation of nutrients in the aerial parts with respect to the tuber growth, which agrees with what was proposed by Gómez *et al*. (2019a). On the other hand, the correlation between the fresh weight of the tubers and the content of Ca-Mg-S confirms the importance of these nutrients for crop yield (Hamdi *et al*., 2015; Helal & AbdElhady, 2015; Muthanna *et al*., 2017; Seifu & Deneke, 2017; Wang *et al*., 2020). The models proposed for this relationship are a first approach to estimating the content of Ca-Mg-S in the tubers, according to the tuber growth.

Conclusions

The critical dilution curves of calcium, magnesium, and sulfur established are the first reported for two potato cultivars of the Andigenum Group. These are a first approach and provide a guide to improved nutritional diagnosis and adjustments based on the growth and development of the crop. The tool is valid for the evaluated genotypes. The results in harvest indexes expand the information on the Ca-Mg-S accumulation dynamics of the tubers. The study confirms that Capiro has greater plasticity under contrasting edaphoclimatic conditions, while Suprema shows greater adaptation to low fertility soils. The Ca-Mg-S nutrients show a high correlation in the plant, making it possible to estimate the total content or the tuber content. The proposed linear models are a first step to make estimates; this should be expanded in future research. As well, it would be of interest to evaluate a range of fertilization doses ranging from moderate deficiency to excess of Ca-Mg-S.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

KC developed the methodology, prepared, created, and presented the published work and oversaw its visualization, and wrote of the original draft; MIG developed the conceptualization, provided funding acquisition, and conducted the research process; LER carried out the supervision and validation. All authors reviewed the manuscript.

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Altitude as a determinant of fruit quality with emphasis on the Andean tropics of Colombia. A review

La altitud como determinante de la calidad del fruto con énfasis en el trópico andino de Colombia. Una revisión

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ABSTRACT

Due to global warming, the highlands of the tropics have become more important for planting fruit trees. The climate at high altitudes is mainly characterized by decreased temperatures and increased solar radiation. A systematic literature review using four bibliographic databases revealed 22 studies that determined fruit quality at two altitudes. With increasing altitude, duration of fruit development was prolonged, and, in most cases, higher fresh weights and sizes were found; however, fruit firmness decreased. The intensity of the fruit color increased because of greater radiation in high areas. Mostly, the total soluble solids of the fleshy fruits augmented with altitude, probably because of an increase in photosynthesis with higher solar radiation. The total acidity did not show a clear trend with increasing elevations. At higher altitudes, the content of antioxidants (mainly phenolics) increased in the fruits, especially in the epidermis as a reaction to the increasing ultraviolet (UV) light. Physiological disorders in the fruits included sunburn and damage caused by low temperatures. Different species and varieties react differently to the conditions of highlands, depending on their origin and whether climatic conditions are optimal for a specific fruit tree. There are no positive effects on fruit quality when altitude is at the limit or above the recommended range for the fruit species.

Key words: UV radiation, temperature, fruit development, physical quality, chemical quality, physiological disorder.

RESUMEN

Debido al calentamiento global, las zonas altas de los trópicos han ganado importancia para la siembra de los frutales. El clima en estas áreas se caracteriza principalmente por tener bajas temperaturas y mayor radiación solar. Mediante una revisión de literatura sistemática en cuatro bases de datos bibliográficas se encontraron 22 estudios que determinaron la calidad de los frutos evaluada en mínimo dos altitudes. A mayor altitud el desarrollo del fruto se prolongó y en la mayoría de los casos, se encontró un mayor peso fresco y tamaño, sin embargo, la firmeza del fruto disminuyó. La intensidad del color del fruto aumentó debido a la mayor radiación en zonas altas. En la mayoría de los casos, los sólidos solubles totales de frutos jugosos se incrementaron con la altitud, probablemente, por el aumento de la fotosíntesis debido a la mayor radiación solar, mientras que la acidez total no mostró una tendencia clara con el incremento de la elevación. Con la altitud ascendente aumentó el contenido de los antioxidantes (principalmente compuestos fenólicos) en los frutos y, especialmente, en su epidermis, como reacción al aumento de luz ultravioleta (UV). Dentro de los desórdenes fisiológicos en los frutos se destacan los golpes de sol y los daños por bajas temperaturas. Las especies y variedades reaccionan de forma diferente a las condiciones de las zonas altas, dependiendo de su origen, y si las condiciones climáticas están dentro de las óptimas para este frutal. No se detectan efectos positivos sobre la calidad del fruto cuando la altitud está en el límite o por encima del rango recomendado para la especie frutal.

Palabras clave: radiación UV, temperatura, desarrollo del fruto, calidad física, calidad química, desorden fisiológico.

Introduction

The tropics have thermal uniformity, without marked temperature seasons. A reduction in temperature with increasing altitudes means that these zones have altitudinal thermal "floors" (Fischer & Orduz-Rodríguez, 2012). Since plants can grow satisfactorily only in certain temperature ranges (Das, 2012), they can only grow in a certain altitude range where the main component is temperature.

Under the colder conditions of the Andean highlands, fruit development is prolonged, and the time to harvest is increased (Mayorga *et al*., 2020), which is why larger and better quality fruits can be generated, as compared to lower

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altitudes, as seen in feijoa (Parra-Coronado *et al*., 2015, 2016, 2017a), banana passion fruit (Mayorga *et al*., 2020), and apples (Fischer *et al*., 2016; Gutiérrez-Villamil *et al*., 2022). In the tropics, each fruit crop has its optimal altitude range for meeting ecophysiological demands (Fischer & Orduz-Rodríguez, 2012).

The phytochemical compounds of fruits are considerably affected by climatic site conditions and the interaction between environmental conditions and varieties (Zeng *et al*., 2020). The environmental conditions of a particular site are crucial for fruit quality and are the basis for the development of commercial crops in a region or country (Fischer & Orduz-Rodríguez, 2012). Fruits and vegetables, which are key parts of a healthy diet, are highly exposed to the magnitude of climate change, where increased temperatures reduce production and quality, especially in the tropics and subtropics (Shukla *et al*., 2019).

However, global warming may also increase fruit production in some areas. Fischer and Melgarejo (2021) and Tito *et al*. (2018) pointed out that an overly high increase in temperature in the Andes, which would affect the growth and development of fruit trees, could be avoided by planting crops in higher altitude areas. In other parts of the world, such as India, the optimum altitudes for apple cultivation of 1,200-1,500 m a.s.l. in the 1980s shifted to 1,500-2,500 m a.s.l. in this century, with maximum elevations even exceeding 3,500 m a.s.l. (Sahu *et al*., 2020). However, Van Leeuven and Darriet (2016) stated that, in the case of wine grapes, the movement of vineyards to higher altitudes as the result of global warming has a high economic and social cost. Of course, there are also the possibilities of choosing species and varieties more adapted to the phenomenon of climate change (Yohannes, 2016) without moving to higher altitudes, apart from plant breeding programs such as the combination of genetic modification of high yield germplasm with proper crop management (Fischer *et al*., 2022). But these topics were not the objective of this review.

Ecophysiological studies are very important for finding adaptation strategies for fruit trees given changing environmental conditions (Sánchez-Reinoso *et al*., 2019). Given the fact that highlands over 1,500 m a.s.l. comprise almost a quarter of the planet's land surface (Mengist *et al*., 2020) and those tropical altitudes can be "escape zones" for global warming, the objective of this review was to characterize the effect of increasing altitudes, with an emphasis on the Andean tropics, on fruit quality to facilitate decisions for future research and for more adaptive production.

Methods

Information from different databases was used following the PRISMA guide (Preferred Reporting Items for Systematic Reviews and Meta-Analysis), applying the modified methodology of Page *et al*. (2021). The keywords "altitude" and "fruit quality" were used, in English and Spanish, which generated 500 article titles in the "Google Academic" database, of which 74 were used (14.8%). The SciELO database only had 19, with 3 (15.8%) used. ScienceDirect listed 565 titles, of which 28 (15.8%) were used, and the Redalyc had 4,268 articles, with 57 (1.3%) useful for this review. To ensure better topicality, greater emphasis was placed on publications from 2015 onwards; however, some studies from previous years were included because of their importance.

In a second filter, research conducted at only a single altitude was excluded, with a final total of 22 studies that compared fruit quality at least two different altitudes.

Tropical altitude and climatic characteristics

Since the tropics have thermal uniformity, the largest fluctuation occurs in the highlands during the 24 h cycle of a day, where daytime can be described as summer, and night can be described as winter (Fischer, 2000).

Climatic changes with increasing tropical altitudes that affect the growth, development, and quality of fruit trees include a reduction in temperature, about 0.6 to 0.7°C per 100 m (Benavides *et al*., 2017), and partial pressure of gases such as CO_2, O_2, and N_2 and water vapor (Fischer & Orduz-Rodríguez, 2012). There is also reduced precipitation which has an inverse relationship with radiation (Benavides *et al*., 2017), while the visible UV, infrared radiation, and wind increase with altitude (Fischer & Orduz-Rodríguez, 2012). Since the atmospheric layer that filters solar rays is thinner in high areas, UV radiation increases between 10 to 12% with each increase of 1,000 m in altitude (Benavides *et al*., 2017). This greater incidence of solar radiation at high elevations increases soil temperature (Fischer *et al*., 2022), favoring plant growth. In addition, the microclimate of a particular site can vary because of other factors, *e.g*., the gradient of decreasing temperature with altitude can be modified by location, time of day (Benavides *et al*., 2017), and cloudiness (Paull & Duarte, 2011).

This reduction in temperature with increasing altitude leads to zones that are suitable for fruit cultivation, classified in altitudinal thermal floors as described by Paull and

Duarte (2011) in the equatorial zone as: (1) the hot zone (0-1,000 m a.s.l.; "warm climate altitudinal zone"); (2) the temperate zone (1,000-2,000 m a.s.l.; "temperate climate altitudinal zone"); and (3) the cold zone (>2,000 m a.s.l.; "cold climate altitudinal zone"). These authors pointed out that, in these zones, the temperature depends on latitude, wind pattern, and precipitation, among other factors.

For a specific area such as Colombia, there are recommended altitude ranges for fruit trees (Tab. 1). These ranges can even be specific to the varieties of a certain fruit species, *e.g.*, the optimal altitude ranges in the Department of Boyacá in Colombia for the 'Anna' apple are 1,700 to 2,800 m a.s.l. (Fig. 1) (Gutiérrez-Villamil *et al.*, 2022), for the 'Triunfo de Viena' pear 2,400 to 2,800 m a.s.l., for the Japanese plum 'Beauty' 2,600 to 2,800 m a.s.l. (Fischer, 2000), and for feijoa 1,800 to 2,700 m a.s.l. (Parra-Coronado *et al.*, 2019). Likewise, there are recommendations for a narrower range, such as those in Table 1, which may have better production results, for example, cape gooseberry at 2,200 to 2,400 m a.s.l. (Fischer & Orduz-Rodríguez, 2012), purple passion fruit at 1,700 to 2,000 m a.s.l. (Ocampo *et al.*, 2020), fig at 2,400 to 2,500 m a.s.l. (Fischer, Almanza-Merchán & Piedrahíta, 2012), and feijoa at 2,100 to 2,600 m a.s.l. (Duarte & Paull, 2015). On the other hand, at greater proximity to the equator, temperatures increase, which is why fruit trees grow at higher altitudes there. For example, in Ecuador, there are cape gooseberry, tree tomato, and Andean blackberry crops up to an altitude of 3,300; 3,000, and 3,200 m a.s.l., respectively (Carrillo-Perdomo *et al.*, 2015).

It is important that crops are located within altitude limits because this guarantees good physiological and productive performance, *e.g.*, in an ecophysiological study on

TABLE 1. Recommended altitude ranges for Colombian Andean fruit cultivation.

Plant family	Species	Altitudinal range (m a.s.l.)
		0 500 1000 1500 2000 2500 3000 3500
Passifloraceae	Yellow passionfruit[1]	0–1300
	Purple passionfruit[1]	1,600–2,300
	Sweet granadilla[1]	1,800–2,600
	Banana passionfruit[1]	1,800–3,200
Solanaceae	Lulo[2]	1,600–2,400
	Tree tomato[3]	1,700–2,600
	Cape gooseberry[4]	1,800–2,800
Myrtaceae	Guava[5]	0–2,000
	Feijoa[6]	1,800–2,700
	Champa[7]	800–1,600
Ericaceae	Blueberry[8]	2,200–2,800
	Andean blueberry[9]	2,200–3,200
Rosaceae	Apple[2]	1,700–2,800
	Pear[2]	1,800–2,800
	Peach[2]	2,000–2,600
	Japanese plum[2]	1,800–2,800
	Strawberry[2]	1,800–2,700
	Andean blackberry[2]	1,500–2,600
Annonaceae	Cherimoya[2]	1,500–2,200
Lauraceae	Avocado[10]	1,200–2,600
Moraceae	Fig[11]	800–2,500
Cactaceae	Pitaya[12]	1,500–1,900

[1]Fischer and Miranda (2021); [2]Fischer and Orduz-Rodríguez (2012); [3]Bonnet and Cárdenas (2012); [4]Fischer and Melgarejo (2020); [5]Fischer and Melgarejo (2021); [6]Parra-Coronado *et al.* (2019); [7]Balaguera-López *et al.* (2022); [8]Cleves (2021); [9]Medina *et al.* (2015); [10]Carvalho *et al.* (2015); [11]Fischer, Almanza-Merchán and Piedrahíta (2012); [12]Corredor (2012).

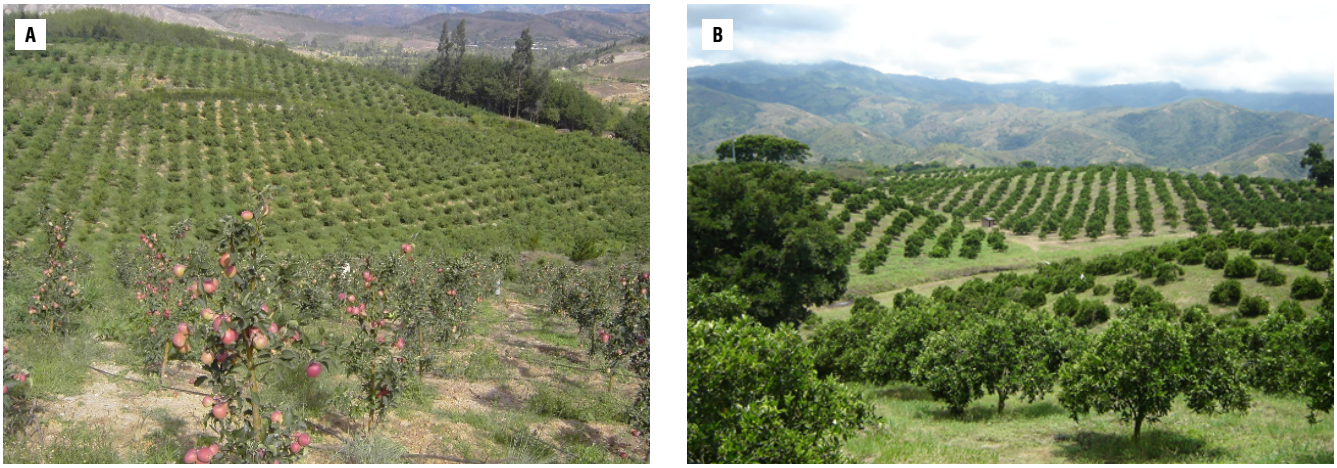

FIGURE 1. Apple orchards in the A) Boyacá highlands and B) citrus fields in the Valle department of Colombia.

passion fruit in the Department of Huila (Colombia) grown at 2,060 and 2,270 m a.s.l., the predawn values of the maximum photochemical efficiency of photosystem II (*Fv/Fm* >0.86) confirmed that the plants were not exposed to stress conditions and the two altitudes are suitable for the cultivation of passion fruit in this region (Fernández *et al*., 2014). Fruit cultivars outside their optimal altitude range do not generate economic profitability, and plants at a higher altitude grow too slowly, with lower yields and qualities. At too low altitudes, they grow too fast, without developing their typical and stable post-harvest quality (Fischer & Parra-Coronado, 2020).

Adaptation of fruit trees to highland conditions

Cultivars originating from regions near the equator and/or higher altitudes, as the case of Andean fruit crops, may have developed a greater tolerance to UV-B radiation (Caldwell *et al*., 1980). A good example of the adaptation of fruit trees to higher altitudes is the study by Voronkov *et al*. (2019), who found that the levels of phenolic substances and polyunsaturated fatty acids increased in the skin of apples with altitudes increasing from 300 to 1,200 m a.s.l. in the Caucasus. This increase in phenols protects fruits against high UV radiation because of their powerful antioxidant effects, while unsaturated fatty acids retain the fluidity of the fruit's cell membranes within the physiological range (Voronkov *et al*., 2019).

In cape gooseberry plants, Fischer *et al*. (2007) found that plants formed a more superficial root system at a higher altitude (2,690 m a.s.l.) in Boyacá (Colombia) than at 2,300 m a.s.l. to take better advantage of soil warming by the midday sun. In addition, this species, which originated in the Andean highlands, can increase the number of leaf stomata at higher altitudes to compensate for the lower concentrations of CO_2 and O_2 at these conditions (Fischer & Melgarejo, 2020), along with dense pubescence in all aerial organs to counteract the high UV radiation and nocturnal cooling of the atmosphere (Fischer *et al*., 2021).

Plants at high elevations develop a lower height because UV light decreases auxin production (Fischer & Melgarejo, 2014) and affects the synthesis of gibberellins in the internodes (Buchanan *et al*., 2015). In addition, fruit trees develop a smaller leaf area because of the greater solar radiation and increased temperature resulting from climate change (Fischer, Almanza-Merchán & Ramírez, 2012). Moreover, the cuticle as well as the leaves becomes thicker because of the increased number of parenchyma layers that better resist UV light (Fischer & Miranda, 2021). Within this context, varieties with higher photosynthetic performance and lower susceptibility to photoinhibition are better adapted to high elevation belts (Fischer *et al*., 2016).

For plant breeders, climatic adaptation is the requirement that a new variety must meet, and fruit trees are continuously modified through genetic selection to adapt to new environments, such as light intensity, photoperiod, cold and heat, type of soil, and moisture condition. Many of these requirements change with altitude and latitude (Sherman & Beckman, 2003). It is noteworthy to mention the existence of a genotype-by-environment interaction, as shown in the case of guava fruits, where a significant genotype × environment interaction was found for sugar and organic acid content in four genotypes at different altitudes in the department of Santander in Colombia (Solarte *et al*., 2014).

Effect of altitude on fruit quality

In general, for the comparison of different altitudes, the intrinsic characteristics of each site must be considered, *e.g*.,

slope, acidity, and organic matter content of the soil, among others, along with the climatic factors (Brenes-Gamboa, 2017). Table 2 shows some studies (22) that compare the effect of different altitudes on the physical and chemical components of fruits. Pérez and Melgarejo (2015) confirmed that the environmental conditions must be very close to the optimum to achieve the highest possible yields and qualities on fruit plantations, which are determined by the genetic potential of the species.

The physical characteristics of fruits

Physical features, such as weight, size, shape, firmness, and color, along with the duration of the reproductive phenological phase, depend first of all on the genetics of the species and variety; the potential is expressed to the maximum under optimal climatic conditions of the crop (Fischer, Ramírez & Almanza-Merchán, 2012), which favor physiological processes, such as photosynthesis, transpiration, translocation of photoassimilates, respiration, and metabolism, which are crucial to the internal and external quality and postharvest longevity (Ladaniya, 2008).

The duration of fruit development until physiological maturity depends to a high degree on the temperature of the cultivation site; the lower the temperature during the growing season, the later the fruit will ripen (Moretti *et al*., 2010). Thus, feijoas at 1,800 m a.s.l. and an average temperature of 18.3°C took 155 d, while at 2,580 m a.s.l., (12.3°C) they took 180 d (Parra-Coronado *et al*., 2015); cape gooseberries took 66 and 75 d at 2,300 (17.4°C) and 2,690 m a.s.l. (12.5°C), respectively (Fischer *et al*., 2007). Posnette (1980) reported that the growth cycle of 'Lacatan' banana fruits in Jamaica increased by 1 month with each 100 m increase in altitude. Temperature, as one of the most important climate variables, affects the growth and development of fruit trees by regulating the length of the different phenological stages, *i.e*., fruit development is prolonged at higher altitudes because of decreased temperatures (Parra-Coronado *et al*., 2015; Mayorga *et al*., 2020). Ramírez *et al*. (2018) stated that the length of fruit development is highly dependent on climatic differences and particularly on the temperature. These authors mentioned that higher mean temperatures enhance fruit growth rates and decrease the time to maturity, whereas lower average temperatures tend to extend time necessary for fruit growth and maturity. Fischer, Ramírez and Almanza-Merchán (2012) emphasized that carbohydrate transport to the fruits and the rate of biochemical reactions catalyzed by enzymes (Moretti *et al*., 2010) are highly temperature-dependent.

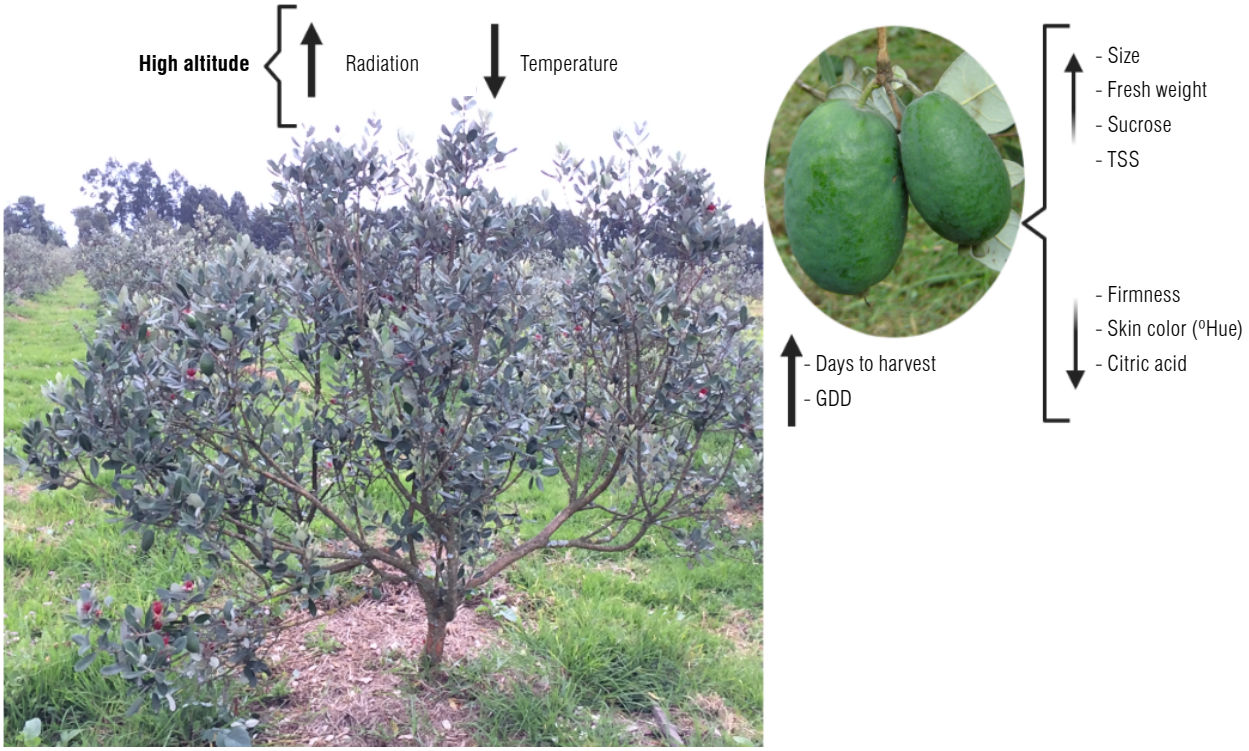

FIGURE 2. Representation of the effect of a higher altitude (2,580 *vs.* 1,800 m a.s.l.) on development and quality characteristics of feijoa (*Acca sellowiana*) fruits (Parra-Coronado, Fischer & Camacho, 2015; Parra-Coronado *et al*., 2017a, 2019, 2022). GDD: growing degree days; TSS: total soluble solids.

Several researchers compared growing degree days (GDD) during fruit development at different altitudes, with more physiological data and better adaptation to the temperature of the site, *e.g.*, feijoa at 1,800 m a.s.l. requires 2,965±113 GDD (167.5 ± 4.97 calendar days) from flower bud to harvest and, at 2,580 m a.s.l., requires 2,337±124 GDD (210.25 ± 10.08 calendar days) (Parra-Coronado *et al*., 2015) (Fig. 2). Bugaud *et al.* (2006) found that for bananas, at higher elevations (300 m a.s.l. *vs.* 50 m a.s.l.), the sum of temperatures (measured in GDD) was higher, which is why fruit fingers developed with greater thickness and density (Tab. 2), and the green life was shorter.

Of the 22 studies in Table 2, the fresh weight (FW) and size of the fruit increased in 12 studies (*e.g.*, feijoa, banana passion fruit, Andean blackberry, lime, cactus pear) with increasing altitude, while in five studies, these properties decreased (*e.g.*, cape gooseberry, guava, kiwi); the conditions of the sites must be within the optimal range required by these fruit species (Fischer *et al*., 2016), especially for temperature, precipitation and solar radiation (Fischer, Ramírez & Almanza-Merchán, 2012). In this regard, Viera *et al*. (2019) indicated that the effect of location on fruit weight and blackberry production was due to climate and soil characteristics.

Possibly, the development of fruits with a smaller size and lower weight at lower elevations occurs because of accelerated development driven by high temperatures, as compared to higher elevations of the same species. Therefore, there is a shorter time for accumulation of photoassimilates, along with a smaller daylight integral, as observed by Mayorga *et al*. (2020) in banana passion fruit. The increase in fruit weight may be highly related to cooler nights at high altitudes (Fischer *et al*., 2016) which reduce maintenance respiration in fruits and energy costs, favoring carbon balance and dry weight (DW) accumulation in these organs (Gariglio *et al*., 2007).

Apart from temperature, precipitation and soil moisture play the most important role in fruit filling because of their direct effect on the rate of fruit growth and translocation of carbohydrates to the fruit (Fischer, Ramírez & Almanza-Merchán, 2012). According to Mendoza *et al*. (2017), precipitation and soil moisture promote the reproductive phase of crops in the Andes to a greater degree (73.4%), as compared to temperature (19.3%) and solar radiation or photoperiod (3.2%). In cape gooseberries, a decrease in precipitation (837 *vs.* 302 mm year^{-1}) with the increase of altitude (2,300 *vs.* 2,690 m), could also have contributed to smaller fruits, number of fruits, and yield per plant (Tab. 2), although there was additional irrigation (Fischer *et al*., 2007). In the case of feijoa, Parra-Coronado *et al*. (2017a) found an opposite behavior for the size and quality of fruits, which were greater at higher altitudes (1,800 *vs.* 2,580 m a.s.l.) and with less rainfall (1,493 *vs.* 765 mm year^{-1}) although plants were near the upper recommended altitude limit at 2,700 m (Tab. 1).

TABLE 2. Effect of increasing altitude on the physical and chemical properties of fruits grown in various countries at the time of harvest.

Species	Common name / variety	Country (region) / altitude (m a.s.l.)	Physical property	Chemical property	Author
Acca sellowiana	Feijoa / clone 'Quimba'	Colombia (Cundinamarca) / 1800, 2580.	Increase of FW and size; decrease of firmness and hue angle.	Increase of TSS and sucrose; decrease of citric acid; without effect on TTA, malic acid, glucose and fructose.	Parra-Coronado, Fischer and Camacho (2015); Parra-Coronado *et al.* (2017a, 2019, 2022)
Actinidia chinensis	Kiwi / 'Hayward'	Turkey (Ardesen district of Rize) / 20, 210, 446, 610.	Decrease of FW, diameter, length and firmness.	Decrease of TSS and pH.	Zenginbal and Ozcan (2018)
Citrus latifolia	Lime / 'Tahití'	Colombia (Espinal, Villavicencio, Lebrija) / 335, 336, 1038.	Increase of size, color intensity and green coloration; decrease of firmness.	Increase of TTA; decrease of juice content.	García-Muñoz *et al.* (2021)
Citrus reticulata	Mandarine / 'Fremont'	Indonesia (West Java) / 500, 650, 800.	Increase of orange color; without effect on firmness.	Increase of vitamin C and TTA; without effect on TSS and TSS/TTA ratio.	Susanto *et al.* (2013)
Citrus sinensis	Orange / Mousambi	Nepal (Dadeldhura district) / 1400, 1700, 2000.	Increased peel thickness; decreased FW and diameter.	Increase of vitamin C, but TSS only at 1,700 m a.s.l.; decrease of juice content.	Ayer and Shrestha (2018)

to be continued

Species	Common name / variety	Country (region) / altitude (m a.s.l.)	Physical property	Chemical property	Author
Fragaria × *ananassa*	Strawberry / 'Capitola'	Venezuela (Lara, Trujillo and Aragua) / 1200, 1800, 2800.	Increase of size and dark red color; decrease of firmness.	Increase of TSS, vitamin C and anthocyanins; decrease of TTA and total polyphenols.	Pérez de Camacaro *et al.* (2017)
*Malus domestic*a	Apple / 'Red Gold'	India (Himachal Pradesh) / 1400, 1800.	Increase of FW, length and diameter; without effect on firmness and color (−L*, −a*, +b*).	Increase of TTA, glucose, total phenols and antioxidant activity; decrease of fructose, sucrose, ascorbic, malic and citric acids; without effect of TSS.	Kumar *et al.* (2019)
Musa acuminata	Banana / 'Grande Naine'	French West Indies (Martinique) / 50, 300.	Increase of diameter, firmness, % FW, density and peel hardness.	Increase TSS, citrate and Zn; decrease of P; without effect on K, Mg, Ca, Fe and Mn.	Bugaud *et al.* (2006)
Olea europaea	Olive/ 'Nabali'	Jordan (Soum and Om Al-Dananeer) / 400, 700.		Decrease of oil, oil acidity, peroxide and unsaturated fat/saturated fat ratio.	Freihat *et al.* (2008)
Opuntia ficus-indica	Cactus pear / 'Gialla', 'Rossa'	Italy (Sicily) / 301-350, 351-450, 451-550, 551-650	Increase of FW.	Increase of TSS; without effect on TTA, pH and % pulp.	Inglese *et al.* (2010)
Passiflora tripartita	Banana passion-fruit / var. *mollissima*	Colombia (Cundinamarca) / 2006, 2498.	Increase of FW, diameter, length, color: tone (°H), brightness and saturation (chroma); without effect on firmness.	Increase of TTA, ascorbic and citric acid; decrease of malic and oxalic acids.	Mayorga *et al.* (2020)
Persea americana	Avocado / 'Hass'	Colombia (Antioquia) / 1340-2420.	Increase of FW, length, diameter and % DW; decrease of % humidity.	Increase of % of oil, oleic oil, % palmitoleic and linoleic acids.	Carvalho *et al.* (2015)
Physalis peruviana	Cape gooseberry / ecotypes 'Colombia', 'Kenya', 'South-Africa'	Colombia (Boyacá) / 2300, 2690.	Decrease of accumulated DW, diameter, fruit number/ plant, and yield/plant; without effect on fruit FW.	Decrease of sucrose and β-carotene; without effect on glucose, fructose, total carbohydrates, citric and ascorbic acids.	Fischer *et al.* (2000, 2007)
Prunus armeniaca	Apricot / 162 genotypes	India (Trans-Himalayan Ladakh) / 3006-3346.	Decrease of FW, diameter, length, humidity; without effect on blush area.	Increase of TSS.	Naryal *et al.* (2020)
Prunus avium	Cherry / 'Tragana'	Greece (Prefectures of Pieria, Imathia, and Pella) / 59, 216, 490.	Increase of FW and color at 216 m a.s.l. (L*, a*, b*).	Increase of total phenols antioxidant capacity; decrease of TTA; without effect on TSS.	Faniadis *et al.* (2010)
Prunus persica	Peach / 'June Gold'	Greece (Imathia and Kozani) / 72, 495.	Increase of % red blush surface, redness (a*) and lightness (L*); without effect on firmness.	Increase of anthocyanins and in fruit epidermis: antioxidant capacity, total phenols, flavonoids and carotenoids; without effect on TSS and TTA.	Karagiannis *et al.* (2016)
Psidium guajava	Guava / 'Reg. Blanca', 'Guavatá Victoria', 'Ráquira Blanca'	Colombia (Santander) / 1570, 1720, 1890.	Decrease of FW, color change from green to yellow.	Increase of glucose and organic acids; without effects on sucrose.	Solarte *et al.* (2014)

to be continued

Species	Common name / variety	Country (region) / altitude (m a.s.l.)	Physical property	Chemical property	Author
Punica granatum	Pomegranate / 'Helow'	Omán (Al-Hajar Mountains) / 1540, 1876, 2019.	Increase of FW, length, diameter and red color of aril and peel.	Increase of TSS, SST/TTA ratio and juice volume.	Al-Kalbani *et al.* (2021)
Rubus glaucus	Andean blackberry	Colombia (Cundinamarca) / 1882, 2200, 2410, 2647.	Increase of FW, DW, firmness, humidity and consistency; Decrease of L* and chroma.	Increase of TSS; decrease of TTA.	Vergara *et al.* (2016)
Rubus idaeus	Raspberry / 'Golden Bliss', 'Heritage'	Brazil (Mantiqueira Mountains) / 918, 1628.	Increase of FW, length, diameter and L*; without effect on ^{0}hue and humidity.	Increase of total sugars and TSS/TTA ratio; decrease of TSS and TTA.	Maro *et al.* (2014)
Vaccinium ashei	Rabbiteye blueberry / 'Brightwell'	China (Provinces of Zhejiang, Jiangsu, Hubei, Guizhou, Yunnan) / 37-2010.		Increase of TSS, TSS/ATT ratio, flavonoids, phenols and anthocyanins; decrease of TTA.	Zeng *et al.* (2020)
Vitis vinifera	Grape / 'Syrah'	Brazil (Pernambuco and Bahia) / 350, 1100.		Increase of TSS, malic and succinic acids; decrease of glucose, fructose, citric and tartaric acids; in peel increase of anthocyanins and tannins and decrease of trans-resveratrol.	De Oliveira *et al.* (2019)

FW: fresh weight; DW: dry weight; TSS: total soluble solids; TTA: total titratable acidity.

An increase in altitude only increased fruit firmness in two studies, *e.g.*, in blackberry (Vergara *et al.*, 2016), while in four studies, firmness decreased (*e.g.*, in strawberry; Pérez de Camacaro *et al.*, 2017). Four authors observed no effect on this property (*e.g.*, in mandarin; Susanto *et al.*, 2013). This non-uniform effect of altitude on firmness could be because this quality characteristic depends on various factors, such as fruit morphology, cell wall composition, Ca concentration, starch concentration, ethylene production, respiratory intensity, and enzymatic activity related to softening, among others (Yahia & Carillo-López, 2019), factors that can be differentially regulated depending on altitude and crop management (fertilization, irrigation).

Two studies looked at the thickness of the epidermis, as in the case of oranges (Ayer & Shrestha, 2018) and the hardness of the epidermis in bananas (Bugaud *et al.*, 2006), which increased with higher altitude (Tab. 2), possibly promoted by higher solar radiation in these sites, which increases the number of parenchyma layers and cuticle for greater resistance against UV light (Fischer & Miranda, 2021). Additionally, Osterloh *et al.* (1996) pointed out an increase in the firmness of deciduous fruits growing in mountainous regions, which shortens the ripening processes and leads to a longer shelf-life and better aroma than those from valleys. In the case of the 'Kristal' guava in Indonesia, fruits from altitude (550 m a.s.l.) stood out as being "crispier", while those from a valley (200 m a.s.l.) were softer and heavier (Musyarofah *et al.*, 2020).

These observations coincide with the increase in the quality of the 'Anna' apple at 2,500 m a.s.l. *vs.* lower elevations in Duitama (Colombia), with a thicker cuticle and epidermis, making the fruits less susceptible to pathogens and insect pests, along with a better red coloration because of increased anthocyanin synthesis (Fischer *et al.*, 2016). This reaction agrees with the report by Campos and Quintero (2012) for banana passion fruit grown in the Colombian highlands (Tab. 1), which developed a thicker epidermis and was more resistant to anthracnose than fruits from lower sites.

Fruit color changes with increases in solar radiation at higher altitudes (Fischer & Orduz-Rodríguez, 2012); a greater intensity of color and green coloration were found in Lima Tahiti (Tab. 2) (García-Muñoz *et al.*, 2021), probably because of the role of light in the biosynthesis of chlorophylls. In another citrus, mandarin cv. Fremont, the orange color increased (Ayer & Shrestha, 2018) because of the mentioned parameter. The higher altitude was also responsible for a greater increase in the intensity of red color in strawberries (Pérez de Camacaro *et al.*, 2017) and pomegranates (Al-Kalbani *et al.*, 2021) because of the increase in the concentrations of anthocyanins that, apart

from these two crops mentioned, have also been found in peaches (Karagiannis *et al*., 2016), blueberries (Zeng *et al*., 2020) and grapes (Oliveira *et al*., 2019) (Tab. 2). Light is a determining factor in the accumulation of pigments (carotenoids and anthocyanins) in fruits (Yahia & Carrillo-López, 2019). It has been shown that light is an important component that affects the expression of genes related to the synthesis of pigments and also regulates their accumulation by controlling the light signaling apparatus (Azari *et al*., 2010; Ruiz-Sola & Rodríguez-Concepción, 2012).

Apart from light, temperature is a key factor for the red color of fruits; day temperatures around 18-24°C promote fruit growth (Fischer & Orduz-Rodríguez, 2012), depending on the species and variety, while cool night temperatures (*e.g*., 10°C) promote red coloration by anthocyanins in the fruit epidermis, as reported by Musacchi and Serra (2018) for the case of red apples, while Fischer *et al*. (2016) mentioned that cool nights promote the coloration of wine grape berries. These temperature contrasts can easily be found at tropical altitudes.

Parra-Coronado *et al*. (2017a) measured color during the growth of feijoa (cv. Quimba) fruits as a function of hue angle (H°), which varies from 180° for the pure green color to 0° for the pure red color, and found that feijoa remained a green fruit, with small decreases in H° for the two altitudes (1,800 and 2,580 m a.s.l.), with values at the time of harvest of 122.9 ± 2.0 and 125.0 ± 2.1 H°, respectively. Although an increase in temperature with a decrease in altitude accelerates the ripening process, degradation of chlorophyll and reduction of H° in the skin of feijoa fruits do not change its color because of the genetics of the fruit, which only varies within a color tonality.

Chemical characteristics of fruits

Total soluble solids (TSS), mainly soluble sugars, organic acids, and vitamins, increased in nine fruit species (*e.g*., feijoa, Andean blackberry, and cactus pear) at increased altitudes, as seen in Table 2. In two fruit species, kiwi (Zenginbal & Ozcan, 2018) and raspberry (Maro *et al*., 2014), they decreased with increasing altitude. The increase in TSS may be related to the increase in photosynthesis with altitude because of the higher luminosity (Mayorga *et al*., 2020) if the temperature is still in the optimal range of the crop and/or with the lower respiratory carbohydrate loss as a result of the lower night temperature at high elevations (Fischer *et al*., 2016). For the reduction of total soluble solids (TSS) in kiwi, Zenginbal and Ozcan (2018) found that the conversion of starch into sugar decreased due to lower temperatures at altitude of 610 m a.s.l. Temperature can influence total sugar content in yellow and purple passion fruit that are grown at different altitudes and temperature conditions (Viera *et al*., 2022). In addition, low night temperatures can limit the transport of carbohydrates to fruits (Fischer, Almanza-Merchán & Ramírez, 2012), as seen by Tombesi *et al.* (2019) in grapevines exposed to a night temperature of 15°C as compared to 25°C.

The total titratable acidity (TTA), which is the quantification of the organic acids contained in the cell juice, decreased in five species at increased altitude, especially in the berries (blackberry, raspberry, blueberry) (Tab. 2). In four species (lime, mandarine, apple, banana passionfruit), the TTA increased with elevation, possibly because the lower temperature limited the respiration rates of fruits that use organic acids as a substrate (Batista-Silva *et al*., 2018). In strawberries, blackberries, and blueberries, increases in TSS were recorded in association with a decrease in TTA with increased altitude, and the TSS/TTA ratio (maturity index) increased (Tab. 2), which could be related to the conversion of organic acids into sugars by the gluconeogenesis process (Famiani *et al*., 2015).

In apple, cherry, peach, and blueberry, increases in phenols were detect at higher altitude; in the latter three species, an increase in flavonoids was also reported (Tab. 2), which is a very important protection mechanism against UV radiation (Cheynier *et al*., 2013). On the other hand, total phenol contents decreased with higher altitude only in strawberries (Pérez de Camacaro *et al*., 2017). These secondary metabolites are essential phytochemical substances in the plant, which are activated by the light spectrum, acting as defense compounds and protectors against UV radiation (Salazar-García *et al*., 2016).

Likewise, the antioxidant capacity and activity increased with increased altitude in peaches (Karagiannis *et al*., 2016) and apples (Kumar *et al*., 2019), possibly generated by the increase in phenols, flavonoids, and ascorbic acid (especially in citrus fruits, strawberries, and banana passion fruit) (Tab. 2). These secondary metabolism substances are powerful antioxidants (Ouzounis *et al*., 2015).

In two (Tahiti lime and orange) of the three citrus species studied (Tab. 2), a decrease in juice content was found with increasing altitude, *e.g*., a 14.8% lower juice content was produced in Tahiti lime fruits at 1,038 m as compared to 336 m altitude (García-Muñoz *et al*., 2021). Possibly, this occurred because the climatic conditions were not adequate for the juiciness of the fruit, taking into account the multidimensionality of climatic factors in a given site (Zandalinas *et al*.,

2021). The volume of juice also increased in pomegranates, according to the increase in weight and size of the fruits with altitude (Al-Kalbani *et al*., 2021) (Tab. 2).

In fruits with high lipid content, such as the olive tree, the oil content decreased (Freihat *et al*., 2008), while it increased in avocados (Carvalho *et al*., 2015) with rising altitude (Tab. 2). In the latter fruits, the contents of oleic, palmitoleic, and linoleic fatty acids increased at higher elevations. Environmental factors at different altitudes influence the respiration pattern of avocados during ripening on the tree, affecting the metabolism of the fruit that is responsible for the composition and quantity of fatty acids (Carvalho *et al*., 2015). The increase in altitude to values above 2,300 m a.s.l. was reported by Henao-Rojas *et al*. (2019) as a favorable one for the fruit quality of this species, while Ramírez-Gil *et al*. (2018) mentioned the altitude range for avocado is between 1,400 and 2,600 m a.s.l.

There are very few studies that reported a lack of an effect from increasing altitude on fruit quality; one on highbush blueberries, comparing elevations of 217 and 636 m a.s.l. in Portugal, found no statistical differences for the concentrations of free sugars, organic acids and vitamin C (Correia *et al*., 2016).

In the two studies that evaluated the content of antioxidants in the epidermis of fruits, such as total phenols, flavonoids, and carotenoids in peaches (Karagiannis *et al*., 2016) and anthocyanin and tannins in grapes (Oliveira *et al*., 2019), antioxidants increased with increasing altitude, confirming the importance of these protective substances in the skin of fruits that attenuate excessive UV radiation (Caldwell *et al*., 1998). Interestingly, in bilberries (*Vaccinium myrtillus*), a direct relationship was found between altitude and antioxidant activity; in the range between 900 and 1,450 m a.s.l., the relationship between altitude and total anthocyanins was inversely proportional, while the dependence became proportional from 1,500 m a.s.l. (Papanov *et al*., 2021).

In general, if the highest altitude of studied sites was very close to or above that recommended for the crop (Tab. 1), the physical and chemical components of the fruit quality decreased or there were no differences between the sites, as observed in the case of cape gooseberries at 2,690 *vs*. 2,300 m a.s.l. in Colombia (Tab. 2) (Fischer *et al*., 2000, 2007).

In feijoa, the accumulated solar radiation during fruit growth (8,918 *vs*. 11,082 W m^{-2}) at the two altitudes (1,800 *vs*. 2,580 m a.s.l.) affected its size and quality at harvest (Fig. 2) (Parra-Coronado *et al*., 2017a). At higher altitudes (greater accumulated solar radiation), the feijoa fruits presented a larger size, higher content of TSS and less firmness of the epidermis. According to Parra-Coronado *et al*. (2017a), the higher content of TSS, and greater FW produced at higher altitudes could be explained by "the higher rate of transpiration related to greater irradiance, which would provide a prolonged entry of water and nutrients to the fruit", indicating that the greater availability of light increases and extends the transport stream from the xylem to the fruits. Furthermore, photosynthesis in adjacent and well-illuminated leaves near the fruit is promoted by the attraction of photoassimilates from the fruit (Fischer, Almanza-Merchán & Ramírez, 2012).

Physiological disorders of fruits at high altitudes

Fruit trees grown at altitudes above their optimal range can suffer abiotic stress, especially from low temperatures (chilling and freezing stress, Fig. 3A), and oxidative stress from high UV radiation, which can cause large production losses (Madani *et al*., 2019). On clear nights, the surface of the outer tissues of plants and fruits cool below air temperature because of heat exchange, leading to damage to cell membranes and intracellular compartments (Voronkov *et al*., 2019). In addition, sudden changes in temperature between day and night at altitudes can generate physiological disorders, such as fruit cracking (Fischer, Balaguera-López & Álvarez-Herrera, 2021), as reported by Miranda (2020) for sweet granadilla.

During recent decades, radiation has increased steadily, especially UV-B (280-320 nm) (Van Leeuwen & Dariet, 2016). Fruit trees, growing at higher altitudes and especially near the equator, suffer from prolonged solar radiation affecting the epidermis of the fruit, as observed in crops such as mango, kiwi, pineapple, avocado, sweet granadilla (Fig. 3B) and deciduous fruit trees (Fischer, 2000; Fischer & Orduz-Rodríguez, 2012), and from a higher concentration of potentially harmful UV-B (Benavides *et al*., 2017).

Fruit sunburns which are common at altitude sites are further promoted by prolonged dry periods due to climate change and the effect of "El Niño" in northern South America (Fischer *et al*., 2016). Damage caused by photoinhibition results when the chlorophylls in the thylakoid membrane of the chloroplasts absorb more light energy than the photosynthesis process can use and induce damage to the photosystem II and degradation of D1 protein (Casierra-Posada, 2007).

Direct solar radiation increases not only the temperature but also evapotranspiration through the fruit surface,

FIGURE 3. A) Chilling injury in feijoa (*Acca sellowiana)* and B) sunburn in sweet granadilla (*Passiflora ligularis*) in Colombian highlands.

causing an accelerated loss of moisture and increasing susceptibility to fruit cracking (Ikram *et al*., 2020; Fischer, Balaguera-López & Álvarez-Herrera, 2021). For example, in the case of Japanese plums (*Prunus salicina*), excessive radiation manifests itself as a brown to yellow discoloration on the surface of the fruits, which, in severe cases, can result in necrotic spots and cracking of the fruit epidermis (Makeredza *et al*., 2018).

In sensitive plants, prolonged UV-B radiation affects photosynthetic activity and plant growth by damaging DNA, membranes, proteins, and lipids (Hideg *et al*., 2013). However, normal (natural) levels of UV-B radiation can reduce abundant vegetative growth and the incidence of pathogens, and favor secondary metabolism from greater synthesis of anthocyanins, carotenoids, and flavonoids, improving flavor, aroma and color in the case of grapes (Fischer *et al*., 2016).

Fruits are capable of developing protection mechanisms against high UV-B radiation, such as increased synthesis of phenylpropanoids (flavonoids, *e.g*., anthocyanins) in the epidermis that can absorb this radiation and serve as antioxidants (Caldwell *et al*., 1998).

Conclusions

In this review of most information from Colombia, higher altitudes are characterized by decreased temperatures and increased solar radiation. Because of global warming, highlands will become important for planting fruit trees. The effect of altitude on the quality components of fruit trees depends, firstly, on the species and variety and, secondly on whether the climatic conditions are within the range of optimal conditions for the fruit crop growth.

In the 22 studies found in the systematic review of four bibliographic databases that compared fruit quality at least two altitudes, increased altitudes showed prolonged fruit development in most cases, and increased fresh weight and size, while, in some fruit species, fruit firmness decreased. In general, the higher radiation in the upper zone promoted the intensity of the fruit color. The total soluble solids content of the fruits increased with altitude in most cases, probably because of the increase in photosynthesis resulting from higher solar radiation. For total titratable acids, there was no clear trend for altitude, and it depended more on the fruit species and the site conditions. In various fruit species, increasing altitude means the content of antioxidants (mainly phenolics) is increased in the fruit flesh and epidermis as a reaction to increasing UV light. Physiological disorders in the highlands include stress caused by high UV radiation (oxidative stress) and too low temperatures (chilling and freezing stress).

In general, if altitude is at or above the recommended range for a fruit species, there are no positive effects on fruit quality. Varieties with higher photosynthetic performance and lower susceptibility to photoinhibition are better adapted to high altitudes.

The authors suggest undertaking more studies in different altitudes of the Andean region with all fruit species and varieties in correlation with the climatic conditions for a better understanding of fruit production and quality. These results, in the face of climate change, would serve as

an input for new plant breeding programs and improved management of these crops.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

GF wrote the initial draft and carried out the final revision of the manuscript. APC wrote and carried out the revision of the manuscript. HEB wrote and carried out the revision of the manuscript.

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Soil, climate, and management practices associated with the prevalence of clubroot in Colombia

Suelo, clima y prácticas de manejo asociadas a la prevalencia de la hernia de las crucíferas en Colombia

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ABSTRACT

Clubroot disease caused by *Plasmodiophora brassicae* is a major constraint for cruciferous crops in Colombia; however, information regarding its spread and the relationship between environmental and crop management practices with its occurrence in the country is scarce. This research established clubroot prevalence in the main cruciferous productive areas in Colombia and the relation of its occurrence with crop management practices, soil, and climatic characteristics. In total, 127 fields were visited along eight departments. Clubroot infestation was determined either by direct inspection of roots of host plants for clubroot symptoms or by report of previous observation of the disease symptoms by the farmers. Soil samples were collected for physical and chemical analysis, climatic information was obtained, and farmers were surveyed on the management practices of the production systems. The survey confirmed the presence of the disease in 53.6% of the visited fields. The only department where the disease symptoms were not observed nor reported was Nariño. A negative correlation was found between the disease occurrence and the content of aluminum in the soil, the number of days with rain per year, and the cultivation of clubroot-resistant hybrids. Moreover, a positive correlation was observed with the inclusion of cruciferous crops in the rotation scheme, the effective cation exchange capacity of the soil, soil pH, and the content of phosphorus, calcium, boron, and copper in the soil.

Key words: pathogen spread, point biserial correlation, soilborne disease, epidemiology, *Plasmodiophora brassicae*.

RESUMEN

La hernia de las crucíferas, causada por *Plasmodiophora brassicae,* es una de las mayores limitantes para la producción de crucíferas en Colombia; no obstante, la información sobre su prevalencia, y la relación entre las condiciones ambientales y prácticas de manejo con su ocurrencia es escasa. Esta investigación estableció la prevalencia de la hernia de las crucíferas en las principales zonas productoras de crucíferas en Colombia y la relación entre su ocurrencia, las prácticas de manejo y algunas condiciones edafo-climáticas. En total se visitaron 127 lotes en ocho departamentos. La infestación por hernia de las crucíferas se determinó por inspección directa de raíces de hospederos susceptibles y por reporte de observación de síntomas por parte del agricultor. Se colectaron muestras de suelo para análisis fisicoquímicos; además se obtuvo información climática y se encuestó a los agricultores con respecto a sus sistemas de producción. La investigación determinó que el 53.6% de los lotes visitados estaban infestados. El único departamento donde no se observaron ni reportaron síntomas de la enfermedad fue Nariño. Se encontró una correlación negativa entre la presencia de la enfermedad y el contenido de aluminio en suelo, el número de días con lluvia al año y el cultivo de híbridos resistentes a la enfermedad. Además, se encontró una correlación positiva entre la presencia de la enfermedad y la inclusión de especies crucíferas en el esquema de rotación, la capacidad de intercambio catiónico, el pH del suelo y el contenido de fósforo, calcio, boro y cobre en el suelo.

Palabras clave: dispersión de patógenos, correlación biserial puntual, patógeno de suelo, epidemiología, *Plasmodiophora brassicae*.

Introduction

Clubroot disease is caused by *Plasmodiophora brassicae* Woronin, a soilborne protozoan. In Latin America, this disease has been reported from Mexico, Costa Rica, Guatemala, Bolivia, Venezuela, Ecuador, Peru, Chile, Brazil, and Colombia; however, studies reporting the disease severity and economic losses it causes are not available (Botero *et al.,* 2019). Clubroot reduces yield in vegetable crops of the *Brassicaceae* family that, in 2017 occupied 2600 ha in Colombia (3.5% of the cultivation area in vegetable crops in the country) (MADR, 2018). Disease symptoms are observed as galls in the plant roots that impede nutrients and water uptake and cause growth delay,

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wilting, chlorosis, and even plant death when symptoms are severe (Dixon, 2009).

The disease cycle is divided into three main stages. During the primary infection, resting spores in soil detect root exudates of a host plant and induces their germination into a primary zoospore that swims towards the plant root hairs. Once in the root hairs, penetration occurs; and a primary plasmodium develops. This plasmodium will later cleave into a zoosporangium that produces secondary zoospores that are responsible for the secondary infection. During the secondary stage, cells of the root cortex are infected; and the disease symptoms become visible. The last stage happens when resting spores are produced in the root galls to be later released into the soil. Those resting spores will serve as primary inoculum for future infections (Kageyama & Asano, 2009).

Clubroot management is quite difficult, mainly because of the production of resting spores that can survive in soil in the absence of a host for up to 17 years (Wallenhammar, 1996). However, their average lifespan is around five to six years (Hwang *et al*., 2015). This is one of the main features that makes clubroot disease one of the major threats to cruciferous crop production around the world, including Colombia (Jaramillo & Díaz, 2006; Dixon, 2009).

Incidence and severity of diseases are modulated by the disease triangle components (host, environment, and pathogen) (Agrios, 2005). Among the environmental conditions affecting clubroot development, the most important are temperature, soil moisture, and soil properties. The most studied are soil pH, boron, and calcium contents. The optimum pH range for clubroot development has previously been determined to be between 5 and 6.5 (Webster & Dixon, 1991b; Narisawa *et al*., 2005; Niwa *et al*., 2007, 2008; Gossen *et al*., 2013; Rashid *et al*., 2013). An increase of concentration of both the nutrients calcium and boron is related to a reduction in the primary infection of the pathogen and plasmodia dehiscence (Webster & Dixon, 1991a, 1991b). Soil moisture is regarded as one of the most important factors affecting clubroot development, with disease incidence and severity increasing together with moisture levels (Samuel & Garrett, 1945; Hamilton & Crête, 1978; Dobson *et al*., 1982; Narisawa *et al*., 2005). Finally, previous reports have found that the optimal conditions for clubroot development include a temperature between 20 and 25°C, a soil pH between 5 and 5.6, and an inoculum density of 10^6 resting spores per plant (Sharma *et al*., 2011; Gossen *et al*., 2012, 2013).

While the planting of resistant cultivars is the most efficient and convenient method for clubroot management, intensive cropping of clubroot resistant cultivars (CR) exerts significant selection pressure on the pathogen (Holtz *et al*., 2018). This pressure can result in shifts in the virulence of pathogen populations, favoring the emergence of pathotypes that can break or overcome resistance, as has already been observed in canola and Chinese cabbage in Japan, Canada, and Europe (Kuginuki *et al*., 1999; Diederichsen *et al*., 2014; Orgeur *et al*., 2016; Strelkov *et al*., 2016).

Understanding the spatial patterns of pathogen populations or diseased plants is crucial to design disease management strategies (Madden *et al*., 2007). In Colombia, clubroot research is scarce and has been focused mainly on disease management (Velandia *et al*., 1998; Botero *et al*., 2015; Botero-Ramírez *et al*., 2016). Furthermore, currently, clubroot prevalence in Colombia and the relationship of its occurrence in the main cruciferous crops (cabbage (*Brassica oleracea* var. *capitata)*, broccoli (*B. oleracea* var. *italica)* and cauliflower (*B. oleracea* var. *botrytis*)) with field management practices, soil properties and climatic characteristics are unknown.

Given the lack of knowledge on clubroot prevalence in Colombia and the relationship of the disease occurrence with soil properties, climate, and crop management strategies, this research sought to achieve two main objectives: i) to determine the disease prevalence in the most important regions where cruciferous crops are grown in Colombia; ii) to evaluate the correlation between the soil properties, climate characteristics, and crop management strategies with the disease occurrence. This knowledge is needed to understand the impact of production practices of cruciferous crops on clubroot disease to outline more accurate disease management strategies.

Materials and methods

In total, 127 fields were visited in February and March of 2017 to establish the prevalence of clubroot throughout the main productive regions of cruciferous crops in Colombia. The fields were located in the departments of Cundinamarca, Antioquia, Nariño, Boyacá, Valle del Cauca, Norte de Santander, Caldas, and Cauca. Caldas was included because it was the first department where clubroot was reported in 1969 (Torres, 1969). The number of samples collected in each department was defined based on the cropped area in cabbage, broccoli, and cauliflower in 2016 (MADR, 2016) (Tab. 1).

TABLE 1. Cultivated area of cruciferous crops in the most productive departments of Colombia and the number of fields visited in each department.

Department	Area in cruciferous crops in 2016* (ha)	Number of fields visited
Cundinamarca	731.7	33
Antioquia	831.3	29
Nariño	522.4	28
Boyacá	117.2	10
Valle del Cauca	221.9	10
Norte de Santander	233.4	9
Cauca	37.6	3
Caldas	51.9	3

*The data presented are based on statistics of production from the Ministry of Agriculture and Rural Development (MADR, 2018).

Clubroot infestation

A field was determined as clubroot infested either by direct observation of typical symptoms (galling on roots) in cruciferous crops or weeds or after being reported by the farmer. When the field was with cruciferous crops at the time of the visit, plants were evaluated for the presence of symptoms; when a different crop was grown, cruciferous weeds were assessed.

When cruciferous crops were growing, twenty plants were extracted and assessed for the presence of root galls, ten were evaluated at the field entrance, and ten more following the "W" pattern sampling. When a different crop was growing, nine points were assessed following the "W" pattern sampling for the presence of cruciferous weeds, and when present, those were removed and evaluated for the presence of typical clubroot symptoms. In either case, once the disease symptoms were observed the sampling was stopped, and the field was set as clubroot infested. In those cases, were the farmer confirmed previous observations of the disease symptoms, plants were also evaluated at the patches where clubroot had been observed before.

Soil samples

At each sampling site, a composite soil sample of 500 g was collected from the top 20 cm of the soil profile. At the central point of the "W" a metal cylinder with unperturbed soil was collected for bulk and particle density estimation. Chemical and physical analyzes of the samples were performed at the Soil and Water Laboratory of the Faculty of Agricultural Sciences at the Universidad Nacional de Colombia.

Crop management information

Information regarding the management of the fields and clubroot disease was obtained by surveying the farmers in the visited fields. The farmers were interviewed if they were familiar with clubroot symptoms; if they were not familiar with clubroot symptoms, photographs of typical symptoms of the disease were shown, and they were asked again if they had observed them before.

On management strategies, farmers were asked about the period during which the farmer had been growing the field, the cultivated area, the rotation scheme, the cruciferous cultivars planted, the propagation strategy, the machinery used and its provenance, the type and application frequency of liming materials and compost, and harvest residue management. In total, 98 farmers were surveyed, since at some places it was impossible to contact the field owner or worker.

Climatic information

Climatic information was obtained from the closest IDEAM weather station to the sampling point. The dataset consisted in the historical normalized data from 1982 to 2010 (IDEAM, 2014). Analysis included average, maximum and minimum temperature, relative humidity, monthly precipitation, and number of rainy days per year.

Statistical analysis

Data analysis was performed using the SAS software (Version 9.4 for Windows, SAS Institute Inc., Cary, NC, USA). Point biserial correlation analysis were done to correlate continuous (climatic variables, soil properties) and dichotomic variables (crop management practices and disease infestation) (Kornbrot, 2014) using the CORR procedure.

Results

Description of cruciferous crops in Colombia

Cruciferous crops were grown in 80 of the 127 surveyed fields. In those the main identified crops included green cabbage (64 fields representing 80% of the fields grown in cruciferous crops), red cabbage (5 fields representing 6.25% of the fields grown in cruciferous crops), broccoli (6 fields representing 7.5% of the fields grown in cruciferous crops), and cauliflower (5 fields representing 6.25 % of the fields).

The most commonly grown green cabbage cultivars were the susceptible hybrids 'Delus' (Semillas Arroyave, 2006), and 'Globe Master' (Agroglobal S.A, 2022a), and the CR hybrid 'Tekila' (Syngenta, 2016). Those hybrids were grown in different regions, 'Delus' was cultivated mainly in Cundinamarca and Boyacá, while 'Globe Master' was mostly grown in Caldas and Norte de Santander. On the other hand, the CR hybrid 'Tekila' predominated in Antioquia.

In all other departments, farmers do not know the name of the variety they were growing.

None of the other cruciferous species showed a clear pattern in the department where they were grown. Of the 25% of farmers cultivating red cabbage, 67% of the farmers growing broccoli, and 60% of those growing cauliflower did not know the name of the variety they grew.

Half of the farmers cultivating red cabbage grew the hybrid 'Ruby King' (Agroglobal S.A, 2022b) and 25% of them grew the hybrid 'Sombrero' (Bejo Eurosemillas, 2022). For broccoli, 33% of the farmers cultivated the hybrid 'Legacy' (Bayer, 2022), and, for cauliflower, 40% of the farmers employed the hybrid 'Skywalker-F1' (Bejo, 2022). It must be pointed out that none of the cropped varieties of red cabbage, broccoli or cauliflower are clubroot resistant.

Cruciferous crops are grown in small areas; the national average size of the fields was 3 ha. The size of the fields was different among departments. Antioquia, Cundinamarca, Valle del Cauca, and Nariño were the only departments with fields larger than 1 ha with average sizes of 7.1, 3.3, 1.8 and 1.6 ha, respectively.

Production of cruciferous crops in the country had high agroclimatic variability and these are allocated at altitudes between 1600 and 3000 m a.s.l. From the surveyed field, 10% of the fields were at altitudes between 1600 and 2000 m a.s.l., 20% were between 2000 and 2500 m a.s.l., and the remaining 30% were between 2500 and 3100 m a.s.l. In Cundinamarca, Boyacá, Nariño, and Norte de Santander (half of the visited crops) cruciferous crops were allocated at altitudes between 2500 to 3600 m a.s.l. In Antioquia and the Norte de Santander cruciferous crops were at altitudes between 2000 and 2500 m a.s.l. In Cauca, Valle del Cauca and Caldas, the fields were at altitudes between 1674 and 2343 m a.s.l.

Clubroot prevalence in Colombia

The prevalence of clubroot was established within the departments where most of the cruciferous crops are grown in the country (Fig. 1). Those fields where the disease symptoms were observed in any host plant or where the farmer reported its occurrence in previous cycles of cruciferous crops were reported as clubroot infested. Clubroot was present in 53.6% of the sampled fields; from these, 48.8% were fields where the disease was observed by the researchers. The remaining 4.8% were assumed as positives as the farmer reported to have observed the disease in previous crop cycles. The disease was observed in all visited departments with the exception of Nariño (Figs. 1 and 2).

In Nariño, Boyacá, and Caldas, most of the farmers were not familiar with the disease symptoms (data not shown). In the municipalities of Sogamoso (Boyacá) and Popayán (Cauca), plants with typical clubroot symptoms were observed in fields of farmers who were not familiar with the disease and could not recognize its symptoms. Furthermore, they attributed yield losses, and symptoms such as wilting and reduction in the crop (caused by plant death) to different stresses such as water deficit and nutritional deficiencies. In Antioquia, the disease symptoms were not observed in green cabbage.

According to the farmers, clubroot was observed the first time around 2001 in Cundinamarca, 2011 in Boyacá, and 2013 in Norte de Santander. It was impossible to determine the approximate year when the disease was observed for the first time in Antioquia, Caldas, Valle del Cauca, and Cauca.

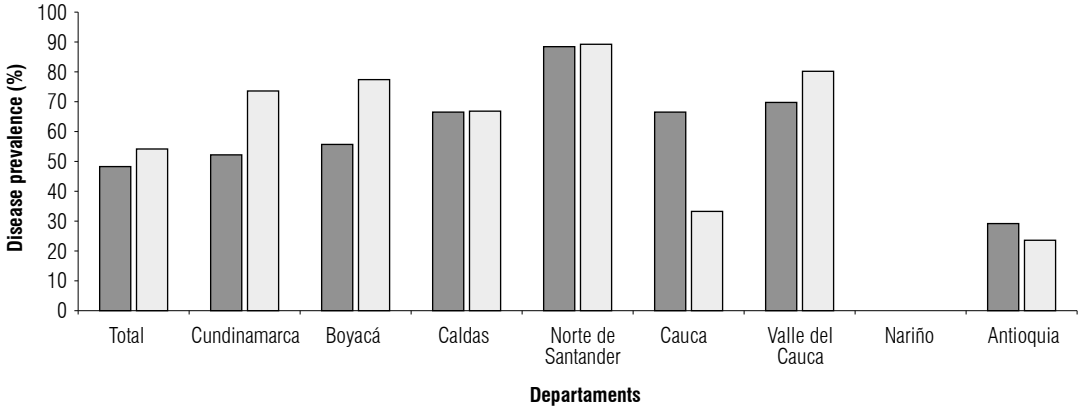

FIGURE 1. Clubroot prevalence in the main productive departments of cruciferous crops in Colombia. The graph shows the percentage of fields where clubroot symptoms were observed in any susceptible host (dark bars) or where farmers reported observation of clubroot symptoms in previous cycles of cruciferous crops (white bars). Percentages were estimated using a total of 127 fields visited in 2017.

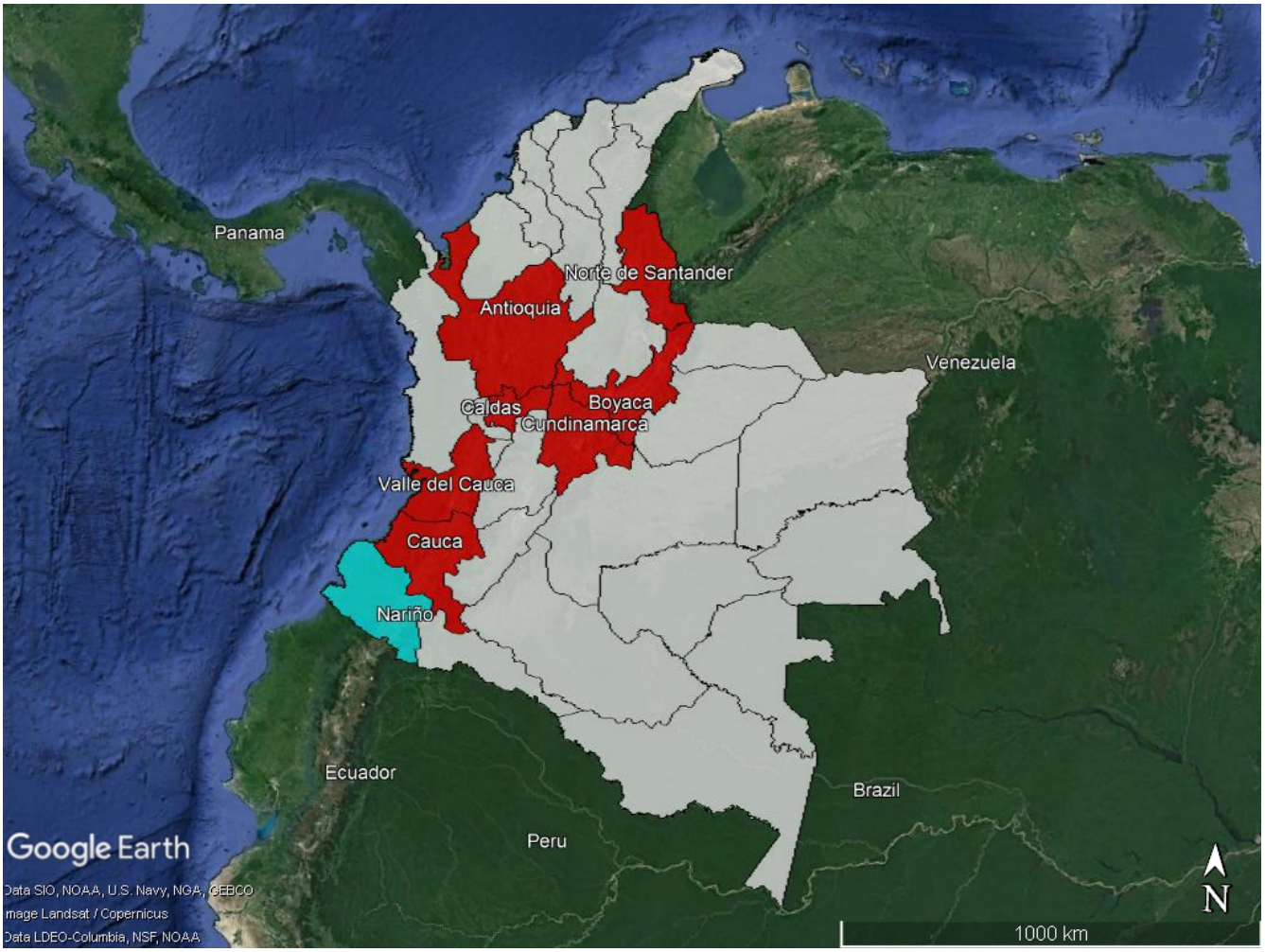

FIGURE 2. Clubroot prevalence in the main productive areas of cruciferous crops in Colombia. The map presents the departments were a survey for clubroot disease was conducted in 127 fields; the color cyan shows the only department where clubroot symptoms were not observed nor reported; in red the departments where clubroot symptoms were observed and/or reported.

Relationship between the soil, environmental characteristics, and clubroot infestation

A correlation between clubroot infestation, soil characteristics, and environmental variables was established (Tab. 2). From the evaluated variables, calcium, phosphorus, boron, copper, soil pH and the effective cation exchange capacity (ECEC) were positively correlated with the disease infestation (Tab. 2). Those results show that as levels in these variables increase, the odds of observing the disease in a field increases as well. However, aluminium content in soil and the average of rainy days per year showed a negative correlation with clubroot infestation. This implies that the odds of finding the disease in a field are reduced as the aluminium content in soil and the number of rainy days per year increase.

Relationship between crop management and clubroot infestation

Analysis showed that the probability of finding a clubroot-infested field is higher whenever cruciferous crops are included in the rotation scheme, and these chances are reduced when CR cultivars are included in the rotation scheme (Tab. 3).

Soil and climatic characteristics and their relationship with the clubroot infestation

Only the soil pH, the ECEC, the aluminium, phosphorous, calcium, boron and copper contents in soil and the number of rainy days per year correlated with clubroot infestation. The pH of the sampled soils was between 4.45 and 7.75. In Cundinamarca, soil pH was between 4.66 and 7.07, in

TABLE 2. Point biserial correlation of clubroot infestation and soil properties or environmental characteristics in the main productive areas of cruciferous crops in Colombia.

Variable	Point biserial correlation coefficient	*P*-value
Soil chemical properties		
pH	0.272	0.0037*
ECEC[a]	0.259	0.0058*
Organic carbon (%)	-0.056	0.556
Nitrogen (%)	-0.057	0.550
Calcium (meq/100 g)	0.268	0.004*
Potassium (meq/100 g)	0.084	0.375
Magnesium (meq/100 g)	0.168	0.074
Sodium (meq/100 g)	0.140	0.139
Aluminum (meq/100 g)	-0.259	0.030*
Phosphorus (mg kg^{-1})	0.413	<0.0001**
Copper (mg kg^{-1})	0.268	0.0042*
Iron (mg kg^{-1})	-0.141	0.137
Manganese (mg kg^{-1})	0.129	0.176
Zinc (mg kg^{-1})	0.112	0.241
Boron (mg kg^{-1})	0.289	0.002*
Environmental characteristics		
Altitude (m a.s.l.)	-0.165	0.092
Rainfall[b] (mm)	-0.188	0.060
Number of rainy days per year[b]	-0.297	0.002*
Average temperature[b] (°C)	0.070	0.620
Maximum temperature[b] (°C)	0.133	0.370
Minimum temperature [b](°C)	0.102	0.491

[a] Effective Cation Exchange Capacity.

[b] Annual average of the historical normalized data from 1981-2010.

The asterisks (*) show the variables which are correlated with clubroot infestation ($P<0.05$).

Boyacá it was between 5.11 and 7.7, between 4.75 and 6.93 in Caldas, between 6.12 and 7.75 in Norte de Santander, between 4.68 and 7.72 in Valle del Cauca, between 4.45 and 6.47 in Cauca, between 4.85 and 7.33 in Nariño, and between 4.85 and 7.33 in Antioquia. The ECEC was highly variable, ranging from 1.03 to 85 meq 100 g^{-1}, and Cundinamarca and Boyacá had the highest averages (33 and 30 meq 100 g^{-1}, respectively). Calcium, aluminium, phosphorus, copper, and boron contents in soil had average values of 17.22 meq 100 g^{-1}, 0.76 meq 100 g^{-1}, 21.99 mg kg^{-1}, 2.23 mg kg^{-1}, and 0.59 mg kg^{-1}, respectively. The national average of number of rainy days per year was 149, and departmental averages were 135 d in Boyacá, 180 d in Caldas, 171 d in Norte de Santander, 213 d in Cauca, 161 d in Valle del Cauca, 195 d in Nariño, and 217 d in Antioquia.

TABLE 3. Point biserial correlation of clubroot presence and crop management variables in the productive areas of cruciferous crops in Colombia.

Variable	Point biserial correlation coefficient	*P*-value
Use of seedlings instead of direct sowing	-0.069	0.535
Inclusion of cruciferous crops in rotation scheme	0.763	<0.0001*
Cruciferous cultivars included in rotation scheme	-0.489	0.0006*
Property of the mechanisation equipment	-0.007	0.949
Application of liming materials	-0.211	0.053
Application of organic matter	0.042	0.701
Application of fresh organic matter	-0.031	0.823
Incorporation of harvest residues	0.110	0.311

The asterisks (*) show the variables that are correlated with clubroot infestation ($P<0.05$).

Discussion

This research reports on the first clubroot survey conducted in Colombia, and to our knowledge in Latin America. The research allowed the estimation of the disease prevalence in the main productive areas of cruciferous crops in Colombia. It also assessed the correlation between clubroot infestation and soil and climatic characteristics, and crop management strategies.

This survey confirmed that clubroot is present in all departments where cruciferous crops are grown in Colombia with the exception of Nariño. These results expand previous reports from Torres (1969) and Jaramillo and Díaz (2006), who confirmed the disease presence in Cundinamarca, Antioquia and Caldas, therefore, confirming for the first time clubroot infestation of fields in Norte de Santander, Cauca, Valle del Cauca and Boyacá. Though in Nariño disease symptoms were not observed, the department cannot be declared 'clubroot free' yet; further confirmation is required by the application of molecular techniques for *P. brassicae* detection in soil such as endpoint PCR and/or qPCR (Cao *et al*., 2007; Rennie *et al*., 2011). Given the widespread presence of the pathogen in the country, it is likely that some inoculum of *P. brassicae* is already present in the fields of Nariño, but the inoculum densities are below the required threshold to cause visible disease symptoms under the predominant environmental conditions. Hwang *et al*. (2011) reported that for consistent symptoms of development under highly conductive conditions, a minimum

inoculum density of $1x10^3$ resting spores per gram of soil is required. The department either has lower inoculum densities or the environmental conditions are not conducive for symptom development.

Our results show that about half of the fields where cruciferous crops are grown in Colombia are infested with clubroot (national prevalence 53.6%). However, this estimation is likely skewed, and the percentage of infested fields is even higher. Such skewing might have been caused by Antioquia, since in this department clubroot symptoms were not found in fields grown with white cabbage since all of them were grown with the cultivar 'Tekila'. Therefore, it is likely that *P. brassicae* inoculum is already present in those fields, but they were reported as non-infested since disease symptoms were not observed by the researchers nor by the farmers in that department. The fields where clubroot was reported and/or observed were grown with red cabbage, broccoli, or cauliflower, given that resistant cultivars with highly commercial acceptance are not available.

A positive relationship was observed between the disease´s presence and some edaphic conditions such as pH, ECEC, and aluminium, phosphorus, calcium, boron, and copper contents in soil. Calcium content in soil and pH are crucial for clubroot development (Webster & Dixon, 1991b). Previous research shows a weak correlation between clubroot and soil pH, where alkaline soils (with pH higher than 7.2) can reduce disease levels even under highly conductive scenarios (Wallenhammar, 1996; Gossen *et al*., 2013). These results at first glance would indicate a disagreement of previous reports with ours, since our results show that as the pH increases so does the likelihood of finding an infested field.

Conducive pH levels for clubroot development are between 5.0 and 6.5 (Webster & Dixon, 1991a; Narisawa *et al*., 2005; Niwa *et al*., 2007, 2008; Ruaro *et al*., 2010; Gossen *et al*., 2013; Rashid *et al*., 2013). From the collected soil samples 83.6% were acidic, from those, 76% were in the range between 5.0 and 6.5 the most favourable pH for the disease development. This might indicate that the observed pH values in this research are below or very close to the optimum required for disease development. For that reason, as the pH levels are increased the conditions for the disease development are improved, explaining the observed correlation.

As calcium and boron content in soil increased, the chance of finding clubroot in a field increased. These results differ from previous research reporting that an increment in calcium and boron concentrations cause a reduction in the number of infections and expression of the disease symptoms (Webster & Dixon, 1991a, 1991b). The effect of these nutrients over the disease symptoms is negatively correlated with the inoculum levels; and thus, under high inoculum pressure, disease reduction is diminished (Webster & Dixon, 1991a, 1991b; Gossen *et al*., 2014). In most of the infested fields, disease was very severe (data not shown); as disease severity is positively corelated with high inoculum densities (Murakami *et al*., 2002) we can presume that inoculum levels in the infested fields is high, hindering the calcium and boron effect on it.

Bhering *et al*. (2017) reported that low pH values and high aluminium contents in soil are favourable for clubroot development; however, our results show the opposite pattern. The negative correlation between clubroot infestation and aluminium contents in soil might be explained mainly by the negative effect of aluminium over the reduction of root branching in the host roots (Marschner, 1995); it can lower the infection probability and along with it the chance of finding the disease in a field. However, that hypothesis needs to be proved by further research.

The largest correlation coefficient with disease occurrence was for the inclusion of cruciferous crops in the rotation scheme; that agrees with reports from Gossen *et al*. (2014) and Dixon (2009), who state that clubroot incidence and severity increases as cruciferous crops are intensified.

Finally, a negative correlation was found in the number of rainy days per year and clubroot infestation. Bhering *et al*. (2017) indicates a lower chance of observing clubroot disease in areas with low precipitation and well drained soils, but fluctuations in the amount of precipitation (rainy seasons, with precipitation levels over the average, followed by drought seasons) can cause epidemics. It is also important to dig deeper into this observation, since it might be a spurious result; the departments with more rainy days per year were Antioquia and Nariño that also are the ones with the lowest number of infested fields, the first one because mostly CR cabbage is grown and the second because it might be clubroot free or the inoculum densities are low.

This research confirmed the presence of clubroot in the main productive departments of cruciferous crops in Colombia with the exception of Nariño. Based in the current methodology, Nariño appears to be clubroot-free; however, further confirmation is required. This is the first research that attempts to establish clubroot disease status

in Colombia, and it becomes a starting point for the design and implementation of integrated management practices for the disease.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

ABR, CGD, and FLPH contributed to development of the research concept. ABR and CGD secured funding support and supervised and administered the project. ABR and FLPH designed and directed sample collection and processing, ABR performed statistical analyses and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Fusarium species that cause corn stalk rot in the Ubaté valley of Cundinamarca, Colombia

Especies de *Fusarium* que causan la pudrición del tallo del maíz en el valle de Ubaté en Cundinamarca, Colombia

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ABSTRACT

In recent years, corn (*Zea mays* L.) crops in the Colombian cold tropics located in the Ubaté valley in Cundinamarca have been affected by stalk rot with incidences up to 40%. Despite the importance of this disease, accurate diagnosis has not been conducted. The objectives of the study were to determine the causal agents of corn lodging associated with stalk rot in this corn-producing region and describe the symptoms of the disease. Two plots with stalk rot reported in the municipality of Simijaca in July 2016 were sown with the regional varieties Simijaca and Sogamoso. Plants were randomly inspected on a monthly basis for describing disease symptoms and isolating the pathogen. The *Fusarium* species isolated were morphologically and molecularly identified and pathogenicity tests were conducted. The disease was detected at early plant developmental stages with the combination of chlorosis, leaf anthocyanosis, and dwarfism as the main symptoms in the two corn varieties evaluated. Crown and node necrosis in longitudinal sections of the stalk and purple colorations in the crown, nodes and internodes of plants were observed 90 d after sowing. Finally, lodging occurred at any phenological stage of the crop. *Fusarium* spp. were isolated in all stages of plant development. *Fusarium* species were identified as *F. graminearum* in the *Fusarium graminearum* species complex and *F. subglutinans* in the *Fusarium fujikuroi* species complex, which have cold-climate production zones as their ecological niche. Pathogenicity tests confirmed *F. graminearum* and *F. subglutinans* as the causal agents of stalk rot in the regional corn variety Simijaca in the Ubate valley in Cundinamarca.

Key words: *Zea mays* L., *Fusarium graminearum* species complex (FGSC), *Fusarium fujikuroi* species complex (FFSC), corn lodging, cold-climate corn.

RESUMEN

En los últimos años, los cultivos de maíz (*Zea mays* L.) en el trópico frío colombiano localizados en el valle de Ubaté en Cundinamarca han sido afectados por una pudrición del tallo con incidencias hasta del 40%. A pesar de la importancia de esta enfermedad, no se ha realizado un diagnóstico preciso. El objetivo de este estudio fue determinar los agentes causales del volcamiento de maíz asociado a la pudrición del tallo en esta región productora y describir los síntomas de la enfermedad. Dos lotes con registro de pudrición de tallo en el municipio de Simijaca en el valle de Ubaté en julio de 2016 fueron sembrados con las variedades regionales Simijaca y Sogamoso. Las plantas fueron inspeccionadas aleatoriamente de forma mensual para describir los síntomas de la enfermedad y aislar el patógeno. Las especies de *Fusarium* aisladas fueron morfológicamente y molecularmente identificadas y se realizaron las pruebas de patogenicidad en maíz. La enfermedad fue detectada en estados tempranos de desarrollo de la planta como la combinación de clorosis, antocianosis de las hojas y enanismo de la planta; estos como los principales síntomas en las dos variedades de maíz evaluadas. La necrosis de cuello y nudos fue observada en cortes longitudinales del tallo y coloraciones púrpura en cuello, nudos y entrenudos de la planta fueron observados 90 d después de la siembra. Finalmente, el volcamiento ocurrió en cualquier estado fenológico del cultivo. *Fusarium* spp. fue aislado en todos los estados de desarrollo de la planta. Las especies de *Fusarium* fueron identificadas como *F. graminearum* perteneciente al complejo de especies *Fusarium graminearum* y *F. subglutinans* perteneciente al complejo de especies *Fusarium fujikuroi,* las cuales tienen las zonas de producción de clima frío como su nicho ecológico. Las pruebas de patogenicidad confirmaron a *F. graminearum* y *F. subglutinans* como los agentes causales de la pudrición del tallo en la variedad regional de maíz Simijaca en el valle de Ubaté en Cundinamarca.

Palabras clave: *Zea mays* L., complejo de especies de *Fusarium graminearum* (FGSC), complejo de especies de *Fusarium fujikuroi* (FFSC), volcamiento del maíz, maíz de clima frío.

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Introduction

Corn is one of the bases of planetary food security. It is the second most important crop after wheat (Paliwal, 2016). Colombia is the fourth largest corn producer in South America, and this crop is the third most sown in the country (CIAT & CIMMYT, 2019). Simijaca is a regional corn variety of free pollination. It is adapted to cold climate production zones in Colombia. This variety was widely cultivated by the Muisca, the indigenous inhabitants of the Ubaté valley region who selected it for its culinary quality. Therefore, this corn variety has been highly accepted in Andean cities (Ligarreto, 2017). In recent years, corn crops in the municipality of Simijaca at the Ubaté valley have been affected by stalk rot but without accurate diagnosis (Gómez *et al*., 2017). This is a complex disease and its cause is difficult to determine since many fungi, bacteria, and oomycetes may appear in affected plants as secondary invaders and saprophytes (Wicklow *et al*., 2005).

Stalk rot caused by *Fusarium* spp. typically reduces yield by 10% or 30%-50% in severely affected areas (Gai *et al*., 2018). Although *Fusarium* stalk rot (FSR) is among the most economically important diseases of corn around the world (Yang *et al*., 2010; Wang *et al*., 2017), it is not frequently seen in Colombia and had not been reported in Cundinamarca until now (Buritica, 1999). In production areas around the world, at least 22 *Fusarium* species can be found in corn causing diseases (Munkvold *et al*., 2018). Two species, *Fusarium graminearum* in the *Fusarium graminearum* species complex (FGSC) and *Fusarium verticillioides* within the *Fusarium fujikuroi* species complex (FFSC) are the main causes of FSR in corn. The first species is more common in cold regions and is one of the most damaging causal agents of stalk rot, while the second *Fusarium* species is more common in hot, dry climates and is particularly damaging if it attacks before flowering (CIMMYT, 2004). In Colombia, the ancient *F. fujikuroi* has been reported causing pink rot in seeds and ears in Antioquia, Cordoba, Cundinamarca, Santander, Tolima, and Valle del Cauca, whereas *F. graminearum* has been reported as associated with stalk and ear rot in Antioquia, Cordoba, Nariño, and Valle del Cauca (Buritica, 1999).

Fusarium sambucinum species complex (FSAMSC) includes the FGSC, *F. cerealis*, and *F. culmorum* (Laraba *et al.,* 2021). Several taxa have been identified within the FGSC, and attempts have been made to divide this complex into phylogenetically separated species. Some of them are present in particular continents. However, it is arguable whether these taxa should all be defined as species, or if they reflect populations or lineages within a broader concept of *F. graminearum sensu lato* (Summerell, 2019). In the last 30 years, reviews of FFSC have reported 45 phylogenetic species, 10 biological species and 34 morphospecies. This complicates the identification of new isolates based only on morphological characters, generating misclassification and underestimation of species diversity (Leyva-Madrigal *et al*., 2015). The taxonomy within FFSC and FGSC is mainly based on DNA sequence analysis of *calmodulin*, elongation factor 1-α (*EF1*), and β-tubulin genes identifying most of the species in these complexes (Leyva-Madrigal *et al*., 2015). Currently EF1 is the most widely used molecular marker in phylogenetic and taxonomic studies within the *Fusarium* genus (Stakheev *et al*., 2018).

Fusarium spp. produce different kinds of spores that may be transported and disseminated by air, raindrops, insects and seeds and that are infected through the pistils (Windels *et al*., 1976; Ooka & Kommedahl, 1977; Munkvold *et al*., 1997; Duncan & Howard, 2010). *F. graminearum, F. verticillioides*, and *Fusarium proliferatum* (the last two in the FFSC) enter the corn plant through trichomes, leaves, xylem and stems (Nguyen *et al*., 2015; Nguyen *et al*., 2016). There is evidence of *Fusarium* spp. endophytes in wild and cultivated plants and the best example is *F. verticillioides*. In this case, *F. verticillioides* is associated with corn plants along the complete crop cycle, where plant responses to the infection depend on several factors related to plants, fungi, and the environment (Kuldau & Yates, 2000). This ancient and evolutive relationship can promote plant growth, protect the seed from infection by other 10 genera of fungi while the plant serves as a source of carbon and a pathway of vertical and horizontal transmission of the fungus (Van Wyck *et al*., 1988; Wicklow, 1988; Yates *et al*., 1997; Schulz *et al*., 1999; Kuldau & Yates, 2000).

The endophyte state is transient and *F. verticillioides* switches from an asymptomatic and biotrophic lifestyle to an hemibiotrophic one causing disease (Schulz *et al*., 1999). Stress conditions promote the disease onset and a range of virulence can be observed among different strains. However, strains that can be pathogenic are known to be asymptomatic under optimal plant growth conditions (Kuldau & Yates, 2000). Thermal stress (cold and heat), drought, high sowing density, shadow, pest attacks and the use of fertilizers with high nitrogen and low potassium content are examples of stress conditions that can promote diseases on a very well-balanced association between the plant and *F. verticillioides* (Dodd, 1980; Schulz *et al*., 1999; Kuldau & Yates, 2000; Blandino *et al*., 2009).

Given the recent problem of corn lodging caused by stalk rot in high-altitude corn-producing regions of the Ubaté

valley of Colombia, the objectives of this study were to determine the causal agents of corn lodging associated with stalk rot in this corn-producing region and describe the symptoms of the disease. Our research describes the symptoms and signs associated with stalk rot in corn plants of the regional varieties Simijaca and Sogamoso (var. Simijca and var. Sogamoso) under field conditions. The associated causal agents were morphologically and molecularly identified, and their pathogenicity was determined in corn plants of the regional variety Simijaca (var. Simijaca).

Materials and methods

Description of symptoms and signs

During 2016, corn seeds of the regional varieties Simijaca and Sogamoso were sown (22000 plants ha^{-1}) in two plots with stalk rot reports located in the municipality of Simijaca (Cundinamarca, Colombia) (5°29′49′′N; 73°49′55′′W and 5°33′11′′N; 73° 47′29′′W). These plots were selected due to the fact that the disease has been reported since 2016 and symptoms of stalk rot have been observed in corn crop cycles during recent epidemics in the region. Plants were randomly inspected on a monthly basis from sowing to tasseling to describe disease symptoms and signs of the pathogen. The inspection dates matched the developmental stages of three, six, nine true leaves, tasseling, silking, and grain with 40% of dry weight (V3, V6, V9, VT, R1, and R4, respectively) according to the scale proposed by Hanway *et al*. (1966). Diseased and healthy plants of both corn varieties in each development stage of the crop were collected and transported to the laboratory of plant pathology (Universidad Nacional de Colombia, Bogotá campus) for detailed inspection under stereoscope, processing and pathogen isolation to identify the causal agent of the disease. Data of precipitation (mm), relative humidity (%), wind speed (m s^{-1}), maximum, minimum and average temperatures (°C) were registered using an iMETOS® 300 climatic station (Pessl instruments, Weiz, Austria) at a 10 min frequency. Data were registered in the two experimental plots and inspected in real time. At the end of the trials, the climatic data obtained were compared with a 30-year database (1986-2016) for the municipality of Simijaca provided by the Corporación Autónoma Regional de Cundinamarca (CAR).

Fusarium isolation from corn plants affected by stalk rot

Fusarium spp. was isolated from symptomatic plants following the Murillo-Williams and Munkvold (2008) protocol. For this purpose, tissue from roots, crown and stalk was collected, disinfected, and sown in Petri dishes with potato dextrose agar (PDA) medium (Oxoid®) acidified at 0.1% (v/v) with lactic acid. The dishes were incubated under dark conditions at 25°C for 10 d (Model FD 23, Binder®, Germany). Afterwards, the frequency of *Fusarium* isolation per corn variety and the plant's explants origin were recorded. The most representative *Fusarium* colonies were purified in PDA and monosporic cultures were obtained in 3% agar (30 g L^{-1}) (WA) (Oxoid®) amended with 12 ml L^{-1} chloramphenicol and 20 ml L^{-1} streptomycin sulfate (Leslie & Summerell, 2006). These cultures were then incubated on PDA at 25°C with a 12:12 h light/dark photoperiod for 15 d in growth chambers (MLR- 351H, Sanyo®, Japan). The resulting pure isolates were stored at -70°C in 15% glycerol. *Fusarium* frequencies, according to the corn variety and part of the plant used for isolation of the pathogen, were analyzed under a completely randomized design (n=24) and subjected to normality and variance tests; means were compared using the Tukey`s test (P=0.05).

Morphological identification of *Fusarium* spp.

Morphological identification of the produced *Fusarium* isolates was performed according to Leslie and Summerell (2006). Carnation leaf piece agar (CLA), Spezieller Nährstoffarmer Agar (SNA), WA and PDA media were used and incubated in growth chambers (MLR- 351H, Sanyo®, Japan) at 25°C with a 12:12 h light/dark photoperiod for 15 d. The color of sporodochia, shape and size of macroconidia and microconidia, number of septa, type of conidiogenesis, formation of chlamydospores and perithecia were determined by light microscopy (CX 31, Olympus®, Japan). Additionally, pigmentation, appearance of the colony, and rate of mycelial growth were evaluated in PDA medium under the same incubation conditions previously described.

Molecular identification of *Fusarium* spp.

Molecular identification was performed by sequencing the *elongation factor* 1-α (EF1) following the methodology of Stakheev *et al*. (2018). For this purpose, 100 mg samples of fresh mycelium per *Fusarium* isolate were taken from seven-day-old colonies grown on PDA and mechanically lysed with 3 mm diameter tungsten beads in a TissueLyser (Qiagen®, Hilden, Germany) (30 Hz/5 min). DNA extraction was performed using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corporation®, Canada) following the manufacturer recommendations. Species were identified using the polymerase chain reaction (PCR) of the elongation factor 1-α (EF1) using the oligonucleotides EF50Fw: 5' CGACTCTGGCAAGTCGACCAC 3' and EF590R: 5' CTCGGCTTTGAGCTTGTCAAG 3' following the methodology of Stakheev *et al.* (2018). The phylogenetic analysis was performed using the CLASSIFIER algorithm from the package MEGA version 7.0 (MacOS), using the

neighbor-joining methodology with 1000 bootstrap replicates. The phylogenetic tree for EF1 was built using the T92+G model described by Tamura (1992).

Pathogenicity test of *Fusarium graminearum* and *Fusarium subglutinans* on corn regional variety Simijaca and their effect on plant growth

For the pathogenicity test, *F. graminearum* (26B) was multiplied following the chaff-grain methodology (Leslie & Summerell, 2006) and *F. subglutinans* (45D) was multiplied in liquid Czapek medium while stirring (Inkubator 1000 - Unimax 1010, Heidolph, Germany) at 150 rpm for 15 d at room temperature (± 20°C) (Leslie & Summerell, 2006). Conidia of each *Fusarium* species were harvested, centrifuged at 2500 rpm for 15 min (MIKRO22R, Hettich® UK), and the inoculum suspensions were adjusted to 1.0 x 10^5 conidia ml^{-1} by hemocytometer counting (Neubauer, VWR, Darmstadt, Germany).

Seeds of the regional variety Simijaca were used and treated with hot water at 52°C for 5 min in an evaporator (Water B-480, BÜCHI Labortechnik®, AG, Switzerland) following the methodology of Daniels (1983). Seeds were then inoculated with 100 ml of the previously prepared conidia suspension of *F. subglutinans* and *F. graminearum* by shaking the mixture vigorously (Wilke *et al*., 2007). Additionally, the combined inoculation with both *F. subglutinans* and *F. graminearum* was conducted using 50 ml of a suspension of each species. The inoculation of the *Fusarium* species obtained (*F. subglutinans, F. graminearum*, and the mixture) was evaluated and considered as treatments (Reid *et al*., 1999). Seeds without treatment and seeds treated with heat were used as absolute control and thermal control, respectively, and were mock inoculated with sterile distilled water (SDW).

After inoculation, the seeds were placed on plastic trays with a 5 cm layer of soil (Warham *et al.,* 1997) from a non-agricultural area from which the presence of *Fusarium* was previously ruled out according to Leslie and Summerell (2006). Once the seeds germinated, 40 seedlings were selected per treatment. These seedlings were then individually transferred to bags with 1.5 kg of soil of the same origin and taken to a greenhouse (± 25°C, ~75% relative humidity). Three months after sowing (V9), longitudinal cuts of plant stems were taken to evaluate the presence of symptoms associated with stalk rot. Plants showing apical chlorosis, leaf anthocyanosis, and dwarfism were considered diseased plants. Incidence of the disease and internal rot stalk (I) were determined using Equation 1 according to Madden *et al*. (2007).

$$I = \left(\frac{Pd}{Pt}\right) \times 100 \quad (1)$$

where Pd represents the number of plants showing the characteristic, and Pt is the total number of plants per treatment. Pathogen isolation was conducted 90 d after sowing (DAS) on PDA medium at 25°C as described above. Plant height (cm) from the stem base until the tip of the third true leaf and stem diameter at the base (mm) were also registered. Data analysis was conducted using a completely randomized design (n=40) and subjected to normality and variance tests; mean comparisons between treatments were performed using the Tukey's test (P= 0.05).

Results

Symptoms of stalk rot (FSR) and signs of the pathogen

The external symptoms of FSR in plant grown in the municipality of Simijaca at the Ubaté valley corresponded to apical chlorosis, leaf anthocyanosis, and plant dwarfism (Fig. 1A-C). Intense necrosis of the crown and plant nodes was detected in longitudinal stalk sections, and progressed towards the internodes (Fig. 1G) causing basal disintegration (Fig. 1I).

In longitudinal sections of healthy plants, the pith was cream-colored and the pith of diseased plants had occasional purple coloration (Fig. 1H-I). Lodging, as the final manifestation of corn stalk rot, occurred at any phenological stages of the crop (Fig. 1D). Initial infection was contained in the crown of the plant and then spread to all the plant levels through the nodes colonized by the pathogen at initial stages of plant development, causing a systemic infection (Fig. 1F).

Fusarium isolation and morphological identification

Although there was no statistical difference of *Fusarium* frequency of isolation between corn varieties (P=0.091) or between the different plant organs analyzed (P=0.112), isolation was 44%, 58%, 78%, and 87% for developmental stages of three, six, and nine true leaves and tasseling, respectively (V3, V6, V9, and VT). Progressive colonization of the stem was observed, with *Fusarium* spp. detected at low frequencies at V3 increasing at V6, V9, and VT. Similar frequencies were observed on the crown and roots (data not shown). Two representative morphotypes of *Fusarium* were isolated from symptomatic plants, purified and morphologically identified.

F. graminearum (isolate 26B) showed white colonies that changed to ocher and reddish tones with a feathery appearance (Fig. 2A) on PDA medium. Brown sporodochia

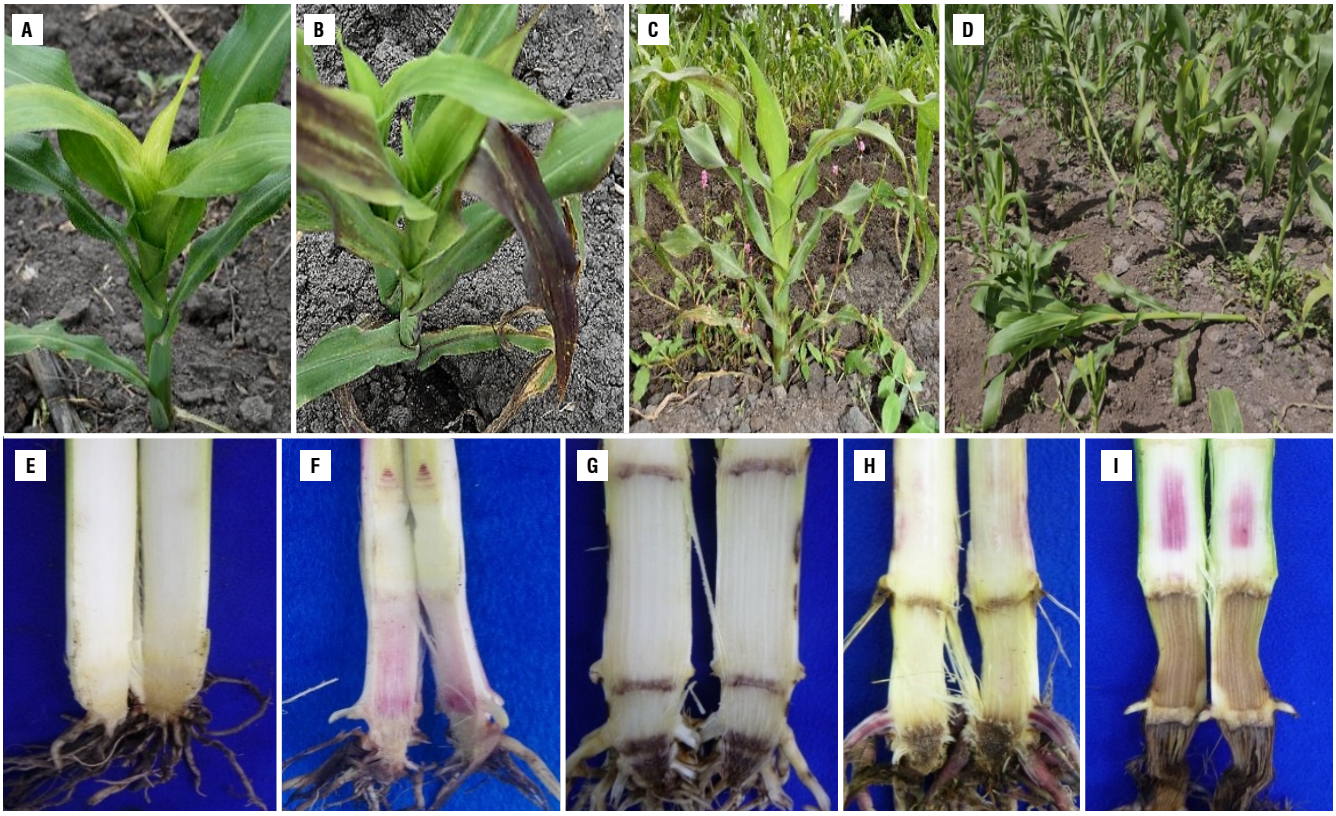

FIGURE 1. External and internal symptoms of stalk rot in corn plants of the regional variety Simijaca three to five months after planting (V6-R1) in the municipality of Simijaca (Cundinamarca, Colombia). A) Apical chlorosis of leaves (V9), B) leaf anthocyanosis (V9), C) plant dwarfism (V9), D) plant lodging (R1), E) healthy plant (V9), F) dissemination of the disease through nodes (V6), G) crown and node necrosis (VT), H) progress of the lesion from nodes to internodes (VT), and I) rot and stalk base disintegration (R1).

were observed on CLA medium (Fig. 2B). Macroconidia were slightly swollen in the middle, 40-50 µm x 4.5-5.5 µm in size with five or six septa moderately curved, with the ventral side straight and the dorsal side arched. The basal cell was foot-shaped, and the apical cell straight with a narrow hook or beak (Fig. 2C). Superficial perithecia of *F. graminearum* were observed on the crop debris of the regional corn varieties Simijaca and Sogamoso, mainly on the stalk nodes of diseased plants. These structures were red under lactophenol blue staining with asci containing usually eight trisected-ascospores of approximately 38 µm in length (Fig. 2D-E).

Fusarium subglutinans (isolate 45D) showed white cottony colonies with orange and purple colorations (Fig. 3A) on PDA medium. On CLA medium, orange sporodochia

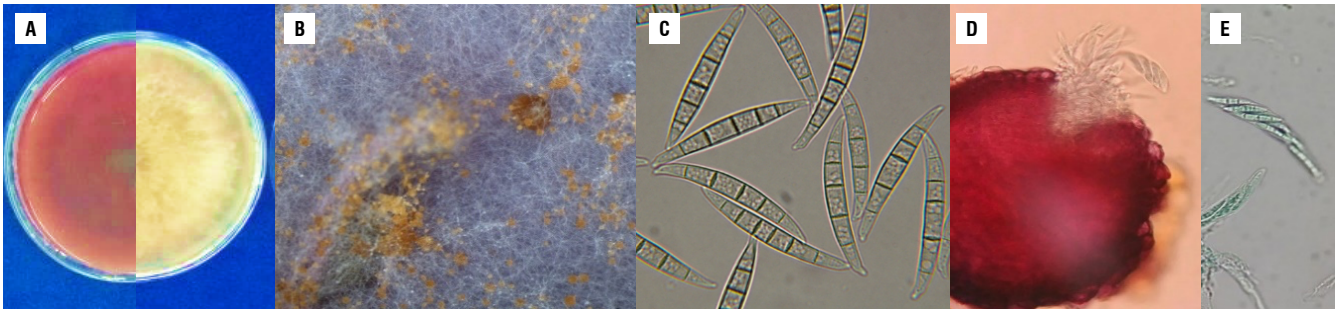

FIGURE 2. Morphological characteristics of *Fusarium graminearum* isolate 26B (in the *Fusarium graminearum* species complex FGSC), isolated from corn plants with stalk rot. A) Characteristics of the colonies, right: appearance of the colony and left: pigmentation on the back of the Petri dish on PDA medium 15 d after culture, B) brown sporodochia on CLA medium, C) macroconidia on CLA medium, slightly swollen in the middle, with five or six septa, moderately curved, with the ventral side straight and the dorsal side arched. The basal cell was foot-shaped, and the apical cell straight with a narrow hook or beak, D) *In situ* perithecia on CLA medium, E) asci and ascospores. PDA - Potato Dextrose Agar; CLA - Carnation Leaf Agar.

were formed (Fig. 2B) and macroconidia were typical of the FFSC, 50-60 µm x 3-4 µm in size, three to four septa, straight with curved apical cell and poorly developed basal cell. Microconidia were predominantly oval, usually without septa and (Fig. 3C) forming pseudo-heads over polyphialids (Fig. 3D). Chlamydospores were not formed on CLA, WA or SNA media. On PDA medium, the growth rate of *F. subglutinans* was less than 1.0 cm per day, whereas *F. graminearum* showed a growth rate over this value.

Molecular identification of *Fusarium* spp. from corn plants with stalk rot

The phylogenetic trees of *EF1* is shown in Figures 4 and 5 in which isolate 26B (MT598159) was grouped with the graminearum clade containing *F. culmorum, F. cerealis* and two species belonging to the FGSC (*F. graminearum sensu stricto* and *F. ussurianum*) (72%) within the FSAMSC (Fig. 4). Isolate 45D (MT598158) was grouped with species of the FFSC (98%) and the species *F. subglutinans* (100%)

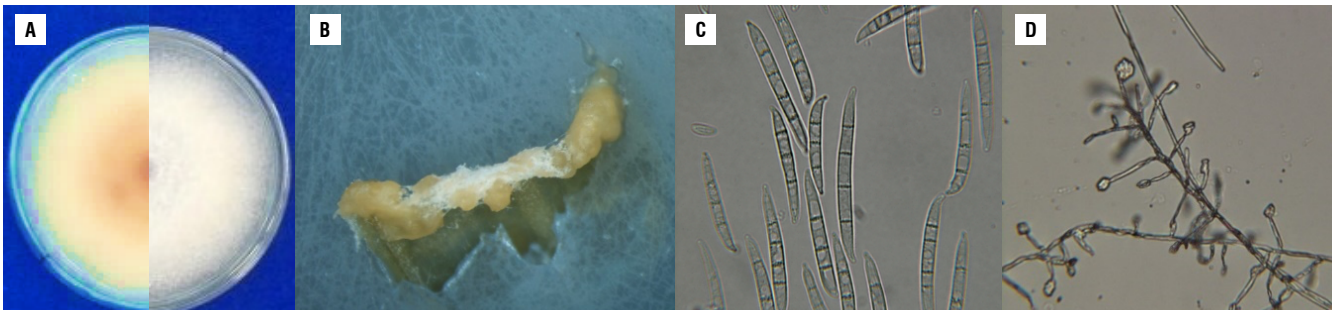

FIGURE 3. Morphological characteristics of *Fusarium subglutinans* isolate 45D (in the *Fusarium fujikuroi* species complex FFSC), isolated from corn plants with stalk rot. A) Characteristics of the colonies, appearance of the colony (right) and pigmentation on the back of the Petri dish on PDA medium 15 d after culture (left), B) orange sporodochia on CLA medium, C) macroconidia typical of the *fujikuroi* complex with three to four septa, straight with curved apical cell and poorly developed basal cell and microconidia predominantly oval, usually without septa, D) microconidia *in situ* on CLA medium forming pseudoheads. PDA - Potato Dextrose Agar; CLA - Carnation Leaf Agar.

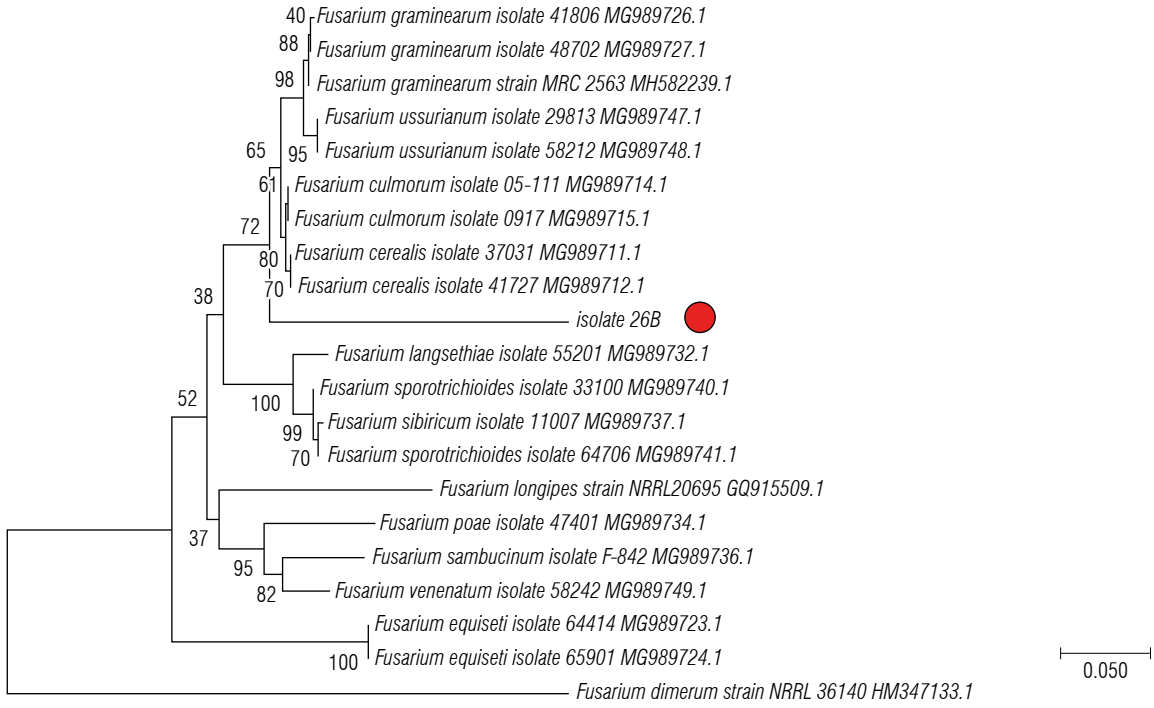

FIGURE 4. Phylogenetic tree of fungal isolates 26B obtained from corn plants with stalk rot symptoms and obtained by the Elongation factor 1-α (EF1). The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used (0.05) to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter=3).

(Fig. 5). Finally, *EF1* sequences were annotated in the National Center for Biotechnology Information (NCBI).

Pathogenicity of *Fusarium graminearum* and *Fusarium subglutinans* on corn regional variety Simijaca and their effect on plant growth

Isolates 26B of *F. graminearum* and 45D of *F. subglutinans* were pathogenic in corn plants of the regional variety Simijaca. At 90 d after sowing (DAS), the inoculated plants showed necrosis and reddish to purple colorations in the basal internal part of the stalk (Fig. 6). Incidence of internal symptoms was observed in 40% of the plants inoculated with *F. graminearum* and 25% of plants inoculated with *F. subglutinans*, whereas these symptoms were observed in 20% of plants inoculated with both species. *Fusarium graminearum* showed a tendency to be located mainly in

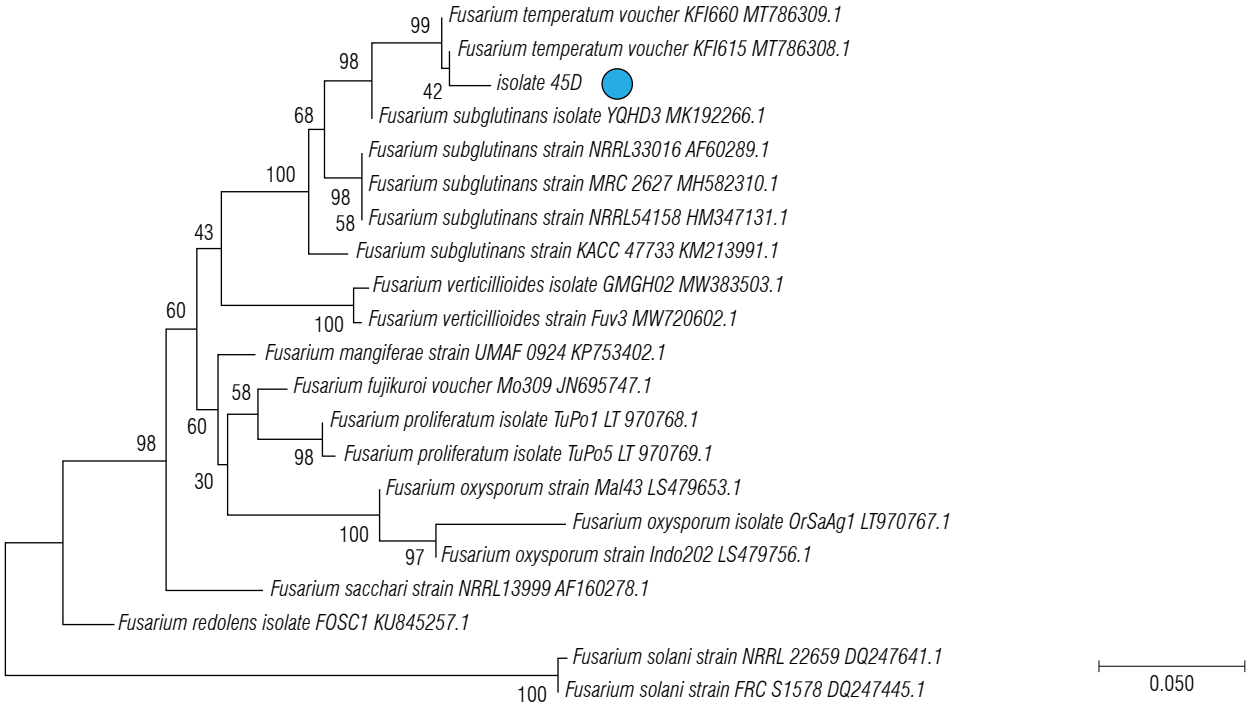

FIGURE 5. Phylogenetic tree of fungal isolates 45D obtained from corn plants with stalk rot symptoms and obtained by the Elongation factor 1-α (EF1). The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (0.05) used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter=3).

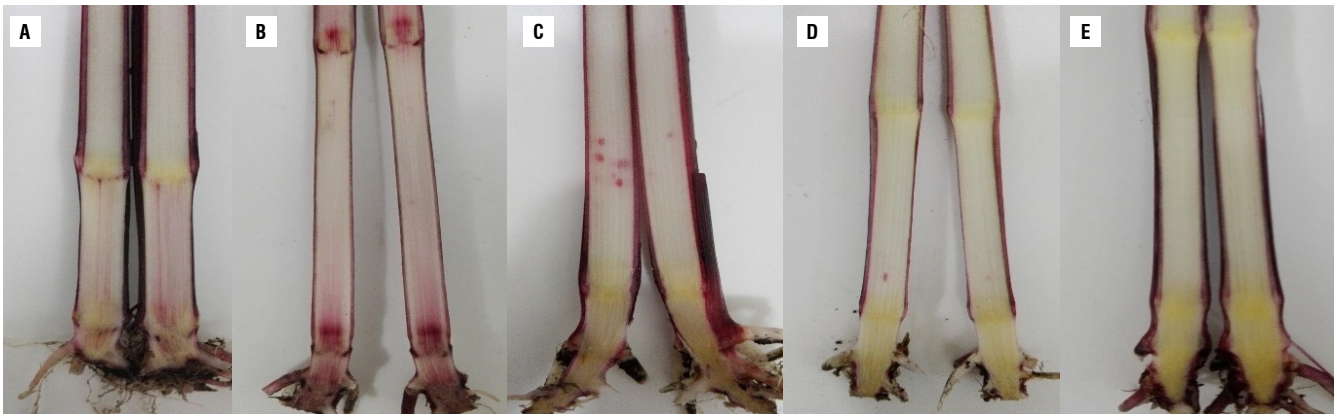

FIGURE 6. Pathogenicity tests of *Fusarium* spp. in corn variety Simijaca three months after sowing (V9) under greenhouse conditions. A) Longitudinal sections of plants inoculated with the mixture of *F. subglutinans* and *F. graminearum*, B) *F. graminearum*, C) *F. subglutinans*, D) absolute control (seeds without treatment), and E) thermal control (seeds treated with hot water at 52°C).

plant nodes (Fig. 6B), whereas *F. subglutinans* was located in the internodes (Fig. 6C). Necrosis of nodes and internodes occurred with the inoculation of the combination of both *Fusarium* species (Fig. 6A). The thermal control did not show symptoms (Fig. 6E), and absolute control showed a low degree of affectation (Fig. 6D). From their respective treatment, *F. subglutinans* and *F. graminearum* were isolated on PDA medium and morphologically identified fulfilling Koch postulates. *Fusarium* was not isolated from the thermal control.

Regarding plant height, the individual inoculation of *F. subglutinans* and *F. graminearum* had a significant effect generating the tallest plants (98 cm) ($P<0.0001$) at 90 DAS, whereas the joint inoculation of *Fusarium* species and the absolute control (seeds without thermal treatment) had the shortest plants (90 cm), and intermediate values were obtained on the thermal control ($P<0.0001$). Although stem diameter data were not adjusted to normality, the joint inoculation of *F. subglutinans* and *F. graminearum* suggests a higher stem diameter compared to the other evaluated treatments.

Discussion

Pathogenicity tests carried out in corn seeds of the regional variety Simijaca developed symptoms similar to those initially observed in the field after inoculation of isolates 26B of *F. graminearum* and 45D of *F. subglutinans*. *Fusarium graminearum* appears to cause necrosis mainly in the nodes and crown of the plant, whereas *F. subglutinans* caused necrosis in the pith and crown. In this study, the joint inoculation of both *Fusarium* species caused symptoms in the crown, nodes and internodes. Therefore, the obtained results may be considered as the first report of *F. graminearum* in the FGSC and *F. subglutinans* within the FFSC causing corn stalk rot in corn crops in Cundinamarca (Colombia), specifically in the municipality of Simijaca. Internal symptoms observed in the pathogenicity tests conducted with thermal treated seeds showed the positive effect of the treatment on the reduction of symptoms. The effect of the treatment was clear in plants of thermal control (seeds treated with hot water at 52°C) that showed the normal cream-white color of the inner stem. In contrast, an internal purple color of the pith of plants was observed in plants of the absolute control (seeds without treatment). This is according to the report for contaminated seeds, as natural source of *Fusarium* spp. inoculum (Duncan & Howard, 2010). These observations are also in contrast to the more severe symptoms observed in the field study, that used untreated corn seeds. Similar positive results have been reported for the thermal treatment of corn seeds and other cereals (Clear *et al*., 2002; Coutinho *et al*., 2007; Bennett & Colyer, 2010; Piñeros-Guerrero *et al*., 2019). The lack of continuity of the necrotic area in nodes and internodes can be explained by the morphology and development of corn plants, which is used by *Fusarium* spp. for its plant colonization and dissemination. The nodes formed by the apical meristem are initially contained in the crown of the plant (Nielsen, 2008). Once the stem elongation begins, these nodes may cause a systemic infection of the plant if infected by the pathogen, as observed in this study. This is consistent with the histological observations conducted by Lawrence *et al*. (1981), who find that the fungus initially confined to the basal parts of the stalk had a rapid spread along the plant at the time of flowering.

The symptoms and signs found in this study were similar to those reported for stalk rot caused by *Fusarium* spp. in corn (CIMMYT, 2004). Additionally, superficial perithecia of *F. graminearum* were found in crop debris, mainly in stalk nodes. Unlike *F. graminearum* which is a homothallic fungus (Leslie & Summerell, 2006), *F. subglutinans* and *F. verticillioides* are heterothallic. This characteristic explains why perithecia of *F. verticillioides* are rarely observed in nature, although they are easily inducted under laboratory conditions. The same may occur with *F. subglutinans*. Blacutt *et al*. (2018) stated that in contrast to *F. graminearum*, where ascospores are the primary inoculum source, sexual reproduction in *F. verticillioides* and other species within FFSC contributes to their genetic diversity without being essential for their life cycle.

The *Fusarium* species associated with Simijaca and Sogamoso corn plants were morphologically and phylogenetically identified as *F. graminearum* in the FGSC and *F. subglutinans* within the FFSC that are reported worldwide as causal agents of stalk rot in corn (CIMMYT, 2004; Leslie & Summerell, 2006). The lineages defined as FGSC are morphologically indistinguishable (Yli-Mattila *et al*., 2009; Summerell, 2019). In this study, isolate 26B, morphologically and biologically identified as *F. graminearum,* was phylogenetically grouped with *F. culmorum*, *F. cerealis* and two species belonging to the FGSC (*F. graminearum s.s.* and *F. ussurianum*) within the FSAMSC. The *Fusarium* species described in this study, probably, belong to the homothallic species *F. graminearum* and not to *F. cerealis* or *F. culmorum*. This is supported by the fact that superficial perithecia were found on the nodes of the stalk of corn plants, and the sexual stage of *F. cerealis* and *F. culmorum* is unknown (Leslie & Summerell, 2006).

Fusarium boothii (in the FGSC) with the 15-acetyldeoxynivalenol (15 ADON) chemotype and *Fusarium meridionale* (in the FGSC) with the nivalenol (NIV) chemotypes are the lineages/species/chemotypes endemic to South America. However, *F. asiaticum* (in the FGSC) with its 3-acetyldeoxynivalenol (3-ADON) and NIV chemotypes has been introduced in the region. In general, *F. graminearum s.s.* 15 ADON is the most common species in Brazil and Argentina with some displacement by more aggressive 3 ADON populations (Van der Lee *et al*., 2018). Determining the species/lineages and chemotypes to which isolate 26B belongs may contribute to the knowledge of species/lineage distribution in the cold tropics of Colombia in South America. Additionally, these results may help to predict toxicological risks and aggressiveness according to the species/lineages and chemotypes present.

Isolate 45D, identified in this study as *F. subglutinans,* may represent two cryptic species distinguishable by amplified fragment length polymorphism (AFLP): *F. subglutinans s.s*. and *F. temperatum* in the FFSC (Czembor *et al*., 2015; Fumero *et al*., 2016). Determining the species to which this isolate belongs is important to predict its distribution and toxicological profile since *F. temperatum* apparently produces fumonisins (Wang *et al*., 2014), beauvericins and fusaproliferin. *Fusarium subglutinans* does not produce fumonisins, but it does produce other types of mycotoxins such as moniloformines and fusaproliferin (Fumero *et al*., 2016). *F. subglutinans* is frequent in cold areas of Peru, Mexico, and Argentina (Logrieco *et al*., 1993; Figueroa-Rivera *et al*., 2010; Reyes-Velázquez *et al*., 2011; Fumero *et al*., 2016) and *F. temperatum* has been found in Argentina and Southern Brazil (Fumero *et al*., 2016). Future studies should be carried out to document the species occurrence within the FFSC in the Colombian cold tropics of South America, where *F. verticillioides* is the most common species in warm parts of the continent (Chulze *et al*., 1996).

In this research, we found *F. subglutinans* in the FFSC and *F. graminearum* within the FGSC associated with the corn stalk rot disease on the regional varieties Simijaca and Sogamoso, which are adapted to the cold and high altitude production zones in Colombia. This result matches the effect of latitude and altitude on the distribution of *Fusarium* species reported by Munkvold *et al.* (2018) in corn. These authors observe that *F. verticillioides* prevails in warm and dry tropical and subtropical areas, whereas *F. graminearum* and *F. subglutinans* are the dominant species in cold-temperate regions as altitude increases, with reports in Europe, Asia, Oceania, and North and South America.

Although *Fusarium* stalk rot is among the most economically important diseases of corn around the world, it had not been reported in the country for at least 30 years. Therefore, corn stalk rot may be considered an emergent disease in Colombia according to our findings and the epidemics occurring in corn plots in Simijaca at the Ubaté valley in the last years. The climatic conditions observed during the period of the study (2016 and 2017) were colder, with average temperatures of 13°C (below the historical average of 14°C). Additionally, dry conditions framed on a tropical El Niño episode with accumulated precipitation of 429 mm (125 mm lower than the historical) were also registered for this period of time in the Ubaté valley. Therefore, cold stress and drought conditions could have contributed to a shift in the biotrophic and symptomless association between the Simijaca corn variety and the *Fusarium* spp. migrating toward a disease-causing condition (stalk rot) (Dodd, 1980; Schulz *et al*., 1999; Kuldau & Yates, 2000; Bacon *et al*., 2008; Blandino *et al*., 2009). Because of their importance, the findings of this study need to be expanded. Therefore, further studies should be conducted with a higher number of isolates and the potential of toxin production of the species present in the field should be evaluated. Further research regarding *Fusarium* species diversity in corn along the Andean region in the Colombian cold tropic and a more robust phylogenetic analysis must be conducted to determine the species/lineages present in the country. Evaluating the toxicological profile and aggressiveness of these species/lineages may also contribute to an understanding of FSR in corn crops under cold climate conditions.

Conclusions

Fusarium graminearum within FGSC and *Fusarium subglutinans* within the FFSC were found associated with corn stalk rot in the Ubaté valley throughout the entire crop cycle, and its pathogenicity was confirmed in the corn variety Simijaca. The initial infection of the pathogen was contained in the crown, but it spreads towards the upper part of the plant through the nodes previously colonized at initial stages of plant development, causing a systemic infection. Necrosis of the crown, nodes and internodes and the pith showing purple colors could be observed in longitudinal stalk sections. These symptoms caused basal disintegration and lodging, as the final manifestation of the corn stalk rot that may occur at any phenological stage of the crop. Although *Fusarium* stalk rot is well reported as an economically important disease in corn, it could be considered an emergent disease under conditions of the Ubaté valley in Colombia.

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Conflict of interest statement

The authors declare that there is no conflict of interests for the publication of this article.

Author's contributions

GMA conducted the research and investigation process and wrote the original draft. GMA, GLM, and SGC formulated the overarching research goals and aims, wrote, reviewed and edited the manuscript. SGC verified the overall replication/reproducibility of results. GLM and SGC obtained the financial support for the project leading to this publication. All authors reviewed the manuscript.

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Efficiency of herbicides for weed control in chickpea and effect of their residues on wheat growth

Eficacia de herbicidas para el control de malezas en garbanzo y efecto de sus residuos en el crecimiento del trigo

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ABSTRACT

In order to determine the best time to use and the adequate dose of four herbicides to control weeds in dryland chickpea (*Cicer arietinum* L.) fields, we performed the present experiment in 4 × 5 m plots. Fourteen treatments were carried out that from 1 to 9 included trifluralin. Treatments 1, 2, and 3 were with increasing doses of trifluralin (480, 720, and 960 g ai ha^{-1}) applied 30 d before planting. Treatments 4, 5, and 6 included increasing doses of trifluralin (480, 720, and 960 g ai ha^{-1}) applied 15 d before planting. Treatments 7, 8 and 9 consisted of increasing doses of trifluralin (480, 720, and 960 g ai ha^{-1}) applied at the time of planting. Treatments 10, 11, and 12 included pyroxasulfone (85 g ai ha^{-1}), flumioxazin (51 g ai ha^{-1}) and imazethapyr (100 g ai ha^{-1}), respectively. These last three treatments were carried out at the time of planting; treatments 13 and 14 were: weed-infested (without weed control) and weed-free (manual weeding during the entire season). Flumioxazin 66% and pyroxasulfone 57% (mean of two samples) reduced weed dry weight compared to uncontrolled treatment. The results showed that the treatments were significantly different for 100-seed weight, biological yield, and seed yield of chickpea. Weed-infested and weed-free plants had the lowest and highest grain yield, respectively. Herbicide treatments of flumioxazin, trifluralin 960 g ai ha^{-1}, and pyroxasulfone at planting produced 55%, 44%, and 40% higher grain yield, respectively, than the weed-infested plots. Also, none of the herbicide treatments reduced chickpea yield and biomass. The herbicide residues had no adverse effect on wheat growth in the next crop season.

Key words: dryland conditions, flumioxazin, imazethapyr, rotation, trifluralin.

RESUMEN

Con el fin de identificar el mejor momento de uso y la dosis adecuada de cuatro herbicidas para el control de malezas en campos áridos de garbanzo (*Cicer arietinum* L.), el presente experimento se realizó en parcelas de 4 × 5 m. Se realizaron 14 tratamientos donde, del 1 al 9 incluyeron trifluralina; los tratamientos 1, 2 y 3 fueron con dosis crecientes de trifluralina (480, 720, and 960 g ia ha^{-1}) aplicada 30 d antes de la siembra; los tratamientos 4, 5 y 6 incluyeron dosis crecientes de trifluralina (480, 720, y 960 g ia ha^{-1}) 15 d antes de la siembra. Los tratamientos 7, 8 y 9 consistieron en dosis crecientes de trifluralina (480, 720, y 960 g ia ha^{-1}) al momento de la siembra. Los tratamientos 10, 11 y 12, incluyeron piroxasulfona (85 g ia ha^{-1}), flumioxazina (51 g ia ha^{-1}) e imazetapir (100 g ia ha^{-1}) respectivamente. Estos tres últimos tratamientos se realizaron al momento de la siembra; los tratamientos 13 y 14 fueron: infestado de maleza (sin control de maleza) y libre de maleza (desmalezado manual durante toda la temporada). La flumioxazina al 66% y la piroxasulfona al 57% (media de dos muestras) redujeron el peso seco de las malezas en comparación con la parcela infestada de malezas. Los resultados mostraron que los tratamientos fueron significativamente diferentes para el peso de 100 semillas, el rendimiento biológico y el rendimiento de semillas de garbanzo. Las plantas infestadas de malezas y libres de malezas tuvieron el rendimiento de grano más bajo y alto respectivamente. Los tratamientos con herbicidas flumioxazina, trifluralina 960 g ia ha^{-1}, y piroxasulfona en la siembra, mostraron un rendimiento de grano 55%, 44% y 40% mayor, respectivamente, que las parcelas infestadas de malezas. Además, ninguno de los tratamientos con herbicida redujo el rendimiento y la biomasa del garbanzo. Los residuos del herbicida no tuvieron efecto adverso sobre el crecimiento del trigo en la siguiente temporada de cultivo.

Palabras clave: condiciones áridas, flumioxazina, imazetapir, rotación, trifluralina.

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Introduction

According to FAO statistics, the primary producers of chickpea worldwide are India, Turkey, and Russia, and the chickpea grain yield average is 1,038 kg ha^{-1} worldwide (FAO, 2021). The area under cultivation of chickpea in Iran is about 456 thousand ha, with an average grain yield of 439 kg ha^{-1}, which is very low compared to the global average yield.

Weeds are the biggest challenge to food production worldwide and reduce crop yields due to high competitiveness (Naghib *et al*., 2020). The competitive capacity of chickpea is lower than other crops compared to weeds, so productivity is seriously affected by weeds (Abdulahi *et al*., 2012). There are different reports of weed damage to chickpea fields under the free control of weeds. Some studies reported a 92% reduction in performance and a damage rate of up to 97% (Paolini *et al*., 2006; Mousavi *et al*., 2007). In western Iran (Kurdistan), a 77.5% reduction in yield because of weed interference is estimated (Fathi *et al*., 2017). Another research estimates the amount of damage in weed-free control as 48.3% in Kermanshah and 66.4% in Tabriz (Mohammadi *et al*., 2005). Due to the long growing season and rainfall in autumn and winter, weeds are a massive problem in winter cultivation of chickpeas and sometimes heavy weed infestations can cause 88% crop failure, while in spring cultivation with plowing before planting, a large volume of weeds are controlled (Knott & Halila, 1988). Important and dominant broadleaf weeds of chickpea fields in Kermanshah were chicory (*Cichorium intybus* L.), bindweed (*Convolvulus arvensis* L.), stickywilly (*Galium aparine* L.), Jeweled distaff thistle (*Carthamus oxyacantha* M. Bieb.), and cowcockle (*Vaccaria pyramidata* Medik.). The narrow leaf weeds were wild barley (*Hordeum spontaneum* Koch.), wild oat (*Avena ludoviciana* Durieu.), and bermuda grass (*Cynodon dactylon* L. Pers.) (Chalechale *et al*., 2015).

The dinitroaniline chemical group has the aniline construction as a basis containing NO_2 molecules. Trifluralin and pendimethalin belong to this group with more than ten different herbicides. Trifluralin has been used in agriculture since 1963 (Grover *et al*., 1997). This herbicide is registered in various countries for controling weeds separately or in mixtures, and it is used in the following crops: *Glycine max*, citrus, *Gossypium hirsutum*, *Arachis hypogaea*, *Phaseolus vulgaris*, and *Allium sativum* (Rodrigues & Almeida, 2018). Its application at pre-planting mixed alone with soil or in combination with the post-emergent herbicides is one of the standard methods to control weeds in bean crops (Rouse *et al*., 2018). Pyroxasulfone is a herbicide that inhibits the biosynthesis of very-long-chain fatty acids (VLCFAs) (Tanetani *et al*., 2009). This herbicide is a pre-emergent discovered amongst several herbicidal 3-sulfonylisoxazoline derivatives (Ito *et al*., 2015). Another pre-emergent herbicide imazethapyr belongs to the imidazolinone group, a class of herbicides that inhibits acetohydroxyacid synthase in synthesizing branched-chain amino acids in plants (Tan *et al*., 2005). Imazethapyr is used in weed control of soybeans, alfalfa, corn, rice, and peanuts (Barnett & Brundage, 2010). The pre-emergent herbicide flumioxazin is an herbicide that blocks protoporphyrinogen oxidase (PPO) activity (Iwashita *et al*., 2022). Flumioxazin is used in the Fabaceae family since it provides a wide range of protective action against weeds (Norsworthy *et al*., 2012).

Providing available, effective, low-cost control solutions for the presence of weeds has economic importance in chickpea cultivation. The number of herbicides introduced to control chickpea weeds in Iran and other countries is not comparable to cereal products. Hence, this study was conducted to estimate the appropriate dose and time of application of trifluralin and evaluates the effect of three other herbicides at planting: imazethapyr, pyroxasulfone, and flumioxazin for weed control in chickpea under dryland conditions. Also, possible residual growth effects on wheat growth and yield have been studied.

Materials and methods

The experiment was conducted in the dryland agricultural research sub-institute-Sararood in Kermanshah, Iran (34º20'N, 47º19'E, 1351 m a.s.l.) during the 2018-19 growth season. The climate at the experimental site was semi-arid and moderately cold with long-term total annual rainfall and maximum and minimum rainfall of 449 and 171 mm. The average annual temperature was 13.8°C, the absolute minimum temperature was -24°C, and the absolute maximum temperature was 44°C. Total rainfall during the experimental conduction (2018-2019) was 783 mm. Figure 1 shows the monthly precipitation of Sararood station in 2018-2019.

The experiment was performed in a randomized complete block design with four replicates. Treatments applied in 4 x 5 m plot size included trifluralin (48%) applications 30 d before planting (DBP) (480, 720, and 960 g ai ha^{-1} for treatments 1, 2, and 3); trifluralin applied 15 DBP (480, 720, and 960 g ai ha^{-1} for treatments 4, 5, and 6); trifluralin applied at planting time (480, 720, and 960 g ai ha^{-1} for treatments 7, 8, and 9); pyroxasulfone (85%) at planting time (85 ai g

ha^{-1} for treatment 10); flumioxazin (51%) at planting (51 g ai ha^{-1} for treatment 11); imazethapyr (10%) at planting time (100 g ai ha^{-1} for treatment 12); weed-infested and weed-free (treatment 13 and 14) (Tab. 1). A Matabi backpack sprayer was used to spray herbicides with calibrated nozzles based on 300 L ha^{-1} of water. At each stage, immediately after applying the herbicide, a surface disking operation was performed to mix the herbicide with the surface layer of the soil.

Chickpea seeds (cv. Mansour) were planted mechanically using an Aske 2200 (Sazeh Kesht Bukan Company, Iran) on March 19, 2019. Each plot consisted of seven rows with 35 cm row-spacing. The distance between chickpea seeds on planting rows was 8 cm, and the planting depth was 5 cm (35 plants/m^2). During two stages, one at the beginning of the growing season and another at the chickpea flowering stage, weeds were manually removed from the plots as weeding check treatment. No control operations were performed in weed-infested (WI) plots. Chickpea harvest was done manually, and seeds and straw were separated and measured manually.

Chickpea growth traits

Measurements were taken at two different chickpea growth stages, in the 8-10 leaf stage (May 8, 2019) and at the beginning of pod formation (May 24, 2019), using a quadrat (with dimensions of 70 x 50 cm) that included two rows of planting with a length of 50 cm. Biologic yield (total biomass + yield), grain yield, plant height, number of pods m^{-2}, number of seeds per plant, 100-seed weight, plant dry weight (stems+leaves) at two sampling stages, number of seeds per pod, and plant density of chickpea were measured. In order to measure weed density and dry weight, samples were taken separately from each plot in each treatment. After collecting the samples, weeds were counted per species. Then, to determine the dry weight of the weeds, the samples were dried separately in an oven at 75°C for 48 h.

Wheat traits

These consisted of the visual assessment of the effects of herbicides on wheat growth. The assessment of possible herbicidal effects on the plants was done using a scoring method with a range of 0 to 100. A score of 0 indicated no adverse effect, and a score of 100 indicated plant death. At the end of the growth season, after the complete wheat growth (growth stage 22 according to the Zadox method), the number of tillers was measured in five randomly selected plants in each plot. To measure the 100-seed weight and grain yield, the plot area was harvested and weighed by considering the marginal plot effect at the time of full ripening, and the data were registered in kg ha^{-1}.

Statistical analysis

To determine the richness, the Shannon-Wiener diversity index and their relative frequency at two chickpea growth stages (8-10 leaves) were used using a frame (70 × 50 cm) contained two rows of crop. After collecting the samples, weed plants were counted by species. The samples were then placed in an oven with a temperature of 75°C for 48 h to determine the weed dry weight. Weed species richness, Shannon-Wiener diversity index, and their relative frequency were calculated as follow:

A) Weed species richness indicates the number of weed species present in each treatment (Poggio, 2005); B) Relative frequency of weeds is the ratio of each weed in the sample to the total number of weeds multiplied by 100 (Booth *et al.*, 2003); C) Shannon-Wiener Diversity Index was calculated using the following equations:

$$H = -\Sigma[pi(lnpi)] \text{ and } pi = ni/N$$

where ni is the number of weeds (i) in the sample, and N is the total number of weeds in the sample.

One-way ANOVA procedure was applied using SAS software (Version 8.1) to assess all effects. Significant differences among treatment means were identified by least significant differences test (LSD) (P<0.05) (SAS Institute, 1998).

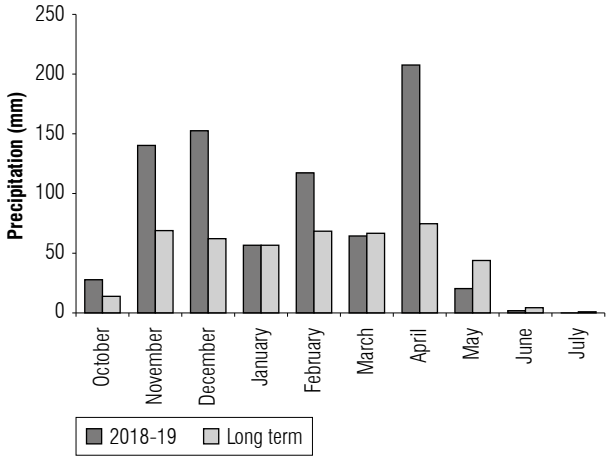

FIGURE 1. Monthly precipitation at Sararood station (Iran) in 2018-19 and comparison with monthly long-term precipitation.

TABLE 1. List of herbicides and characteristics used in the experiment.

Common name	Trade name	Chemical group	Recommended dose, g ha^{-1}	ai† and formulation	Mode of action
Trifluralin	Treflan	Dinitroanilines	720	48% EC⁑	Inhibitors of microtubule assembly
Imazethapyr	Pursuit	Imidazolinone	100	10% SL	ALS, AHAS Inhibitor of biosynthesis of amino acids
Pyroxasulfone	Sakura	Pyrazole	85	85% WG	Blocking heme and chlorophyll biosynthesis
Flumioxazin	Chateau	N-phenyl phthalimide	21	51% WDG	PPO inhibition

† Active ingredient.

⁑ EC, emulsion concentrate; SL, soluble liquid; WG, wettable granule; WDG, water dispersible granule.

Results and discussion

Weeds

Hare's ear (*Bupleurum rotundifolium* L.) had the highest relative frequencies in all treatments (average 25%), followed by bitter bean (*Sophora alopecuroides* L.) (average 11%), field bindweed (*Convolvulus arvensis* L.) (average 10%), syrian cephalaria (*Cephalaria syriaca* L.) (average 7%), chicory (*Cichorium intybus* L.) (average 8%), and prickly lettuce (*Lactuca scariola* L.) (average 7%). Moreover, other weeds with relatively low frequencies were present in some treatments (Tab. 2). Pyroxasulfone had the lowest relative frequency for hare's ear, although no significant difference was generally observed in relative frequency in different treatments. Researchers stated that the herbicide trifluralin could control lemongrass properly (Mirkamali & Maddah, 1974); also, they reported better control of lemongrass by trifluralin than imazethapyr (Moradi, 2009).

Results of analysis of variance in the first stage of weed sampling showed that the effect of treatments on the richness of weed species was insignificant (Tab. 3). Most species richness was related to weed-infested plants. On the other hand, treatments 10 and 11 (pyroxasulfone and flumioxazin) had the lowest species richness (Tab. 3). Analysis of weed species richness variance in the second sampling stage showed a significant difference (P<0.01) between treatments. The herbicide treatment of trifluralin 960 g ai ha^{-1} at planting had the loweste species richness. The pyroxasulfone and flumioxazin were in the next class, and other treatments were not different from the weed-infested (WI) plot (Tab. 4). Changes in the management of field activities may

TABLE 2. Relative frequencies of weed species in treatments 30 d after herbicide application. The data is the mean of four replicates.

	Treatments												
Weeds	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Bupleurum rotundifolium	0.28	0.29	0.40	0.22	0.35	0.21	0.24	0.36	0.38	0.00	0.15	0.08	0.32
Sophora alopecuroides	0.08	0.18	0.09	0.05	0.04	0.23	0.07	0.15	0.10	0.08	0.15	0.18	0.05
Cichorium intybus	0.09	0.20	0.09	0.15	0.05	0.08	0.03	0.08	0.05	0.08	0.00	0.00	0.17
Convolvolus arvensis	0.10	0.02	0.05	0.00	0.20	0.07	0.00	0.00	0.32	0.19	0.06	0.18	0.08
Glycyrrhiza glabra	0.06	0.09	0.01	0.06	0.00	0.03	0.08	0.13	0.00	0.23	0.00	0.27	0.00
Lactuca scariola	0.05	0.08	0.02	0.06	0.07	0.06	0.12	0.07	0.00	0.20	0.00	0.09	0.10
Triticum aestivum	0.00	0.04	0.00	0.07	0.00	0.08	0.00	0.00	0.00	0.07	0.06	0.00	0.00
Anthemis cotula	0.02	0.04	0.06	0.04	0.00	0.00	0.00	0.08	0.00	0.00	0.08	0.00	0.04
Cephalaria syriaca	0.14	0.00	0.03	0.14	0.08	0.03	0.22	0.05	0.05	0.15	0.00	0.00	0.10
Erodium multifida	0.00	0.02	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
Lathyrus sp.	0.02	0.00	0.00	0.01	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.04	0.00
Cynodon dactylon	0.00	0.00	0.07	0.11	0.00	0.00	0.00	0.00	0.08	0.00	0.38	0.04	0.00
Carthamus oxyacantha	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00
Galium aparine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Cardaria draba	0.03	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00
Euphorbia helioscopia	0.00	0.00	0.00	0.00	0.20	0.15	0.14	0.00	0.00	0.00	0.00	0.00	0.02
Tragopogon major	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Vicia sp.	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.05	0.00	0.00	0.00	0.00	0.00
Neslia apiculata	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.07
Adonis aestivalis	0.05	0.03	0.00	0.06	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.05

1, 2, and 3: trifluralin 30 d before planting (480, 720, and 960 g ai ha^{-1}), 4, 5, and 6: trifluralin 15 d before planting (480, 720, and 960 g ai ha^{-1}), 7, 8 and 9: trifluralin applied at planting time (480, 720, and 960 g ai ha^{-1}), 10, 11 and 12: pyroxasulfone, flumioxazin, and imazethapyr at planting time, and 13: weed-infested (no weed control).

change the species richness in the field. Field operations may create the conditions for the invasion of one species and make the conditions unfavorable for the presence of other species (Liebman *et al*., 2001). Managing various factors, especially the chemical management of weeds causes a change in the species richness of the field (Liebman *et al*., 2001). The combination of various weeds in the field indicates the presence of plants with different abilities in the utilization of water and nutrients that makes it more difficult for the crop to compete with the weeds and then restricts the crop growth (Mousavi *et al*., 2005).

The effect of different treatments on the Shannon diversity index at the first stage of sampling was insignificant, and vice-versa was significant in the second stage of sampling. Flumioxazin and trifluralin at 960 g ai ha^{-1} had the lowest effects at planting treatments, indicating that these treatments effectively reduced weed diversity. An investigation by examining the diversity index in imazethapyr, trifluralin, and control plots (without herbicide) reported that the value of this index in different stages of chickpea growth in check and imazethapyr was more than trifluralin herbicide treatment (Abbasian, 2011).

Weed density in both sampling stages was affected by herbicides (Tab. 5). In the first stage of sampling, pyroxasulfone, flumioxazin, and trifluralin 960 g ai ha^{-1} applications at planting produced the lowest number of weeds. These herbicides had 67, 51, and 48% reduction compared to the WI. The other treatments with WI were in the same class. Weed control in two sampling stages has no significant difference in weed density by the chemical control method (Nourbakhsh, 2013). Pyroxasulfone and flumioxazin followed by trifluralin 960 g ai ha^{-1} at planting resulted in the lowest weed dry weight (Fig. 2) and the lowest weed density (Fig. 3).

TABLE 3. ANOVA for weeds richness and Shannon's index.

Source of variation	Degree of freedom	Mean square			
		Richness1‡	Richness2⁑	Shannon1‡	Shannon2⁑
Replicate	3	1.25^{ns}	0.173^{ns}	0.123^{ns}	0.0037^{ns}
Treatment	12	1.91	3.67**	0.13	0.288**
Residual	36	1.67	0.90	0.17	0.102
Total	51	1.70	1.51	0.15	0.140
CV%		34	23	38	26

‡ First stage (8-10 leaf stage of chickpea).

⁑ Second stage (the beginning of chickpea pod formation).

ns, no significant difference; ** significant difference at $P<0.01$.

TABLE 4. Mean comparison of Richness and Shannon's index in different treatments at two sampling stages.

Treatments	Treatment	Richness 1‡	Richness 2⁑	Shannon 1‡	Shannon 2⁑
Trifluralin 480 g ai ha^{-1} 30 DBP†	1	4.3	5.3	1.1	1.6
Trifluralin 720 g ai ha^{-1} 30 DBP	2	3.8	5.0	1.1	1.4
Trifluralin 960 g ai ha^{-1} 30 DBP	3	3.3	4.5	1.0	1.2
Trifluralin 480 g ai ha^{-1} 15 DBP	4	4.3	5.5	1.2	1.6
Trifluralin 720 g ai ha^{-1} 15 DBP	5	3.8	3.3	1.1	1.0
Trifluralin 960 g ai ha^{-1} 15 DBP	6	3.8	4.3	1.3	1.3
Trifluralin 480 g ai ha^{-1} at planting	7	4.5	3.8	1.2	1.2
Trifluralin 720 g ai ha^{-1} at planting	8	3.3	3.8	0.9	1.1
Trifluralin 960 g ai ha^{-1} at planting	9	3.5	2.8	1.1	0.9
Pyroxasulfone 85 g ai ha^{-1} at planting	10	2.8	3.0	0.9	1.1
Flumioxazin 51 g ai ha^{-1} at planting	11	2.5	3.3	0.7	0.8
Imazethapyr 100 g ai ha^{-1} at planting	12	3.8	3.3	1.1	1.0
Weed-infested (WI)	13	5.0	5.3	1.3	1.6
LSD 0.05		1.9	1.4	0.6	0.5

† Days before planting.

‡ First stage (8-10 leaf stage of chickpea).

⁑ Second stage (the beginning of chickpea pod formation).

TABLE 5. ANOVA of weeds density and weeds weight in 2 sampling stages.

Source of variation	Degree of freedom	Weed density 1‡	Weed dry weight 1‡	Weed density 2⁑	Weed dry weight 2⁑
Replicate	3	0.538^{ns}	0.739^{ns}	0.974^{ns}	10.392^{ns}
Treatment	12	23.840**	3.290**	61.244**	58.547**
Error	36	6.18	0.975	6.363	9.277
CV%		27	26.7	25.1	26.4

‡ First stage (8-0 leaf stage of chickpea).

⁑ Second stage (the beginning of chickpea pod formation).

^{ns}, no significant difference; ** significant difference at $P<0.01$.

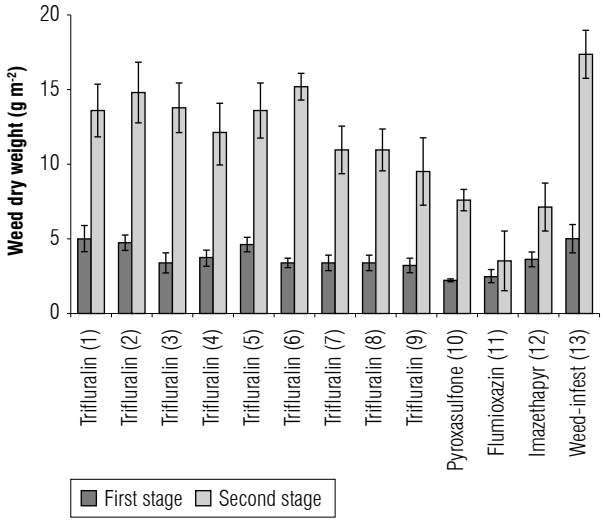

FIGURE 2. Weed dry weight (g m^{-2}) in treatments at two sampling stages (bars represent standard error). 1, 2, and 3: trifluralin 30 d before planting (480, 720, and 960 g ai ha^{-1}), 4, 5, and 6: trifluralin 15 d before planting (480, 720, and 960 g ai ha^{-1}), 7, 8 and 9: trifluralin applied at planting time (480, 720, and 960 g ai ha^{-1}), 10, 11 and 12: pyroxasulfone, flumioxazin, and imazethapyr at planting time, and 13: weed-infested (no weed control).

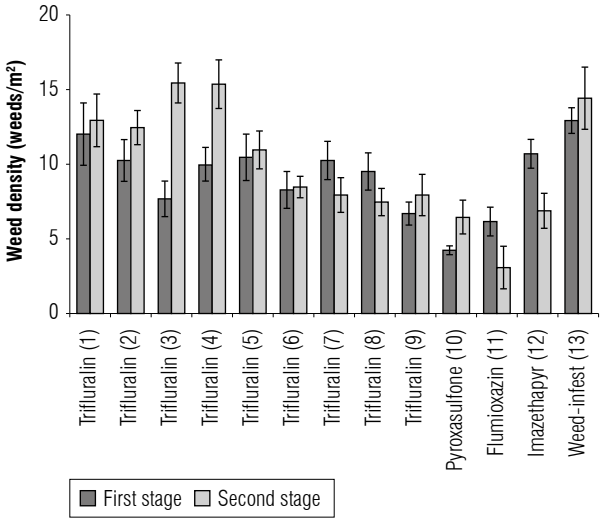

FIGURE 3. Weed density (weeds/m^2) intreatments at two sampling stages (bars represent standard error). 1, 2, and 3: trifluralin 30 d before planting (480, 720, and 960 g ai ha^{-1}), 4, 5, and 6: trifluralin 15 d before planting (480, 720, and 960 g ai ha^{-1}), 7, 8 and 9: trifluralin applied at planting (480, 720, and 960 g ai ha^{-1}), 10, 11 and 12: pyroxasulfone, flumioxazin, and imazethapyr at planting time, and 13: weed-infested (no weed control).

TABLE 6. ANOVA for biologic yield (BioY), grain yield (GY), plant height (PH), number of pods per m^2 (NSM), number of seeds per plant (NSP), 100-seed weight (100-SW), plant weight at two sampling stages (PW1, PW2), number of seeds per pod (NSpod), plant density (PD) of chickpea in different treatments.

Source of variation	DF	BioY	GY	PH	NSM	NSP	100-SW	PW1	PW2	NSpod	PD
Replication	3	30628.2^{ns}	4600.75^{ns}	13.64*	502.97^{ns}	3.089^{ns}	4.755^{ns}	0.260*	0.329^{ns}	0.010^{ns}	1.93^{ns}
Treatment	13	24811*	7497.17**	1.683^{ns}	300.72^{ns}	2.572^{ns}	5.971*	0.0053^{ns}	0.173^{ns}	0.061*	1.8^{ns}
Error	39	12536.1	1737	2.957	389.3	3.57	2.41	0.013	0.140	0.029	1.400
CV%		14.7	13.2	7.4	19.7	21.9	4.3	18.1	18.7	12.5	7.4

^{ns}, no significant difference; * significant difference at $P<0.05$. ** significant difference at $P<0.01$.

Chickpea

The effect of treatments was significant only for the 100-seed weight, biologic yield, and grain yield (Tab. 6). The one hundred seed weight is one of the characteristics related to the quality of chickpea seeds and is essential in terms of marketability and price, since the higher the seed weight, the greater is the chickpea marketability (Abdulahi *et al*., 2012). The lowest and the highest of 100-seed weight was related to WI and weed-free at 34.2 and 38.2 g. Pyroxasulfone and trifluralin 960 g ai ha^{-1} at planting were also in this class (Tab. 7). Another study showed that the highest amount of 100-seed weight of chickpea was obtained under weed-free conditions followed by pyridate herbicide, and the lowest 100-seed weight was related to WI (Shahsavari, 2017). When increasing the number of pods and consequently increasing the number of seeds

TABLE 7. Mean comparisons of biologic yield (BioY), grain yield, and 100-SW of chickpea in different treatments.

Treatments	Biologic yield (kg ha^{-1})	100-SW⁑ (g)	Grain yield (kg ha^{-1})
Trifluralin 480 g ai ha^{-1} 30 DBP	745 abcd†	36.5 abc	278 de
Trifluralin 720 g ai ha^{-1} 30 DBP	700 bcd	35.6 abc	296 cde
Trifluralin 960 g ai ha^{-1} 30 DBP	634 d	34.8 bc	284 de
Trifluralin 480 g ai ha^{-1} 15 DBP	749 abcd	35.9 abc	289 cde
Trifluralin 720 g ai ha^{-1} 15 DBP	737 abcd	36.8 abc	286 cde
Trifluralin 960 g ai ha^{-1} 15 DBP	750 abcd	36.5 abc	318 bcd
Trifluralin 480 g ai ha^{-1} at planting	818 abcd	34.9 bc	311 cde
Trifluralin 720 g ai ha^{-1} at planting	798 abcd	35.9 abc	310 cde
Trifluralin 960 g ai ha^{-1} at planting	784 abcd	37.6 a	354 abc
Pyroxasulfone 85 g ai ha^{-1} at planting	837 abc	37.7 a	344 abcd
Flumioxazin 51 g ai ha^{-1} at planting	861 ab	37.1 ab	380 ab
Imazethapyr 100 g ai ha^{-1} at planting	674 bcd	34.9 bc	315 bcd
Weed-infested	653 cd	34.2 c	245 e
Weed-free	901 a	38.2 a	408 a

† Means with the same letter in the same column are not significantly different according to test LSD ($P<0.05$).

⁑ Seed weight.

per plant, the 100-seed weight decreased (Samaei *et al*., 2006). This may be due to the limitations of photosynthetic compounds produced and stored. In this experiment, probably due to drought stress (Tab. 1), the number of seeds per plant was reduced in all treatments, but 100-seed weight of chickpeas was normal and similar to average climatic conditions, and this agrees with the results of others that the sensitivity of this trait to the number of seeds per plant and drought stress is lower (Samaei *et al*., 2006; Yousefi *et al*., 2006).

The highest grain yield was found in the weed-free plots with 408 kg ha^{-1}, and the lowest yield was found in the WI with 245 kg ha^{-1}. The flumioxazin, trifluralin 960 g ai ha^{-1} at planting, and pyroxasulfone produced 55%, 44%, and 40% higher grain yields than the WI. Many researchers have reported the decreased yield of chickpea in weed competition conditions (Nezami *et al*., 1997; Mousavi *et al*., 2007; Nasari, 2010; Abdulahi *et al*., 2012; Mahmoudi *et al*., 2012; Nourbakhsh, 2013; Shahsavari, 2017). Another study stated that no herbicide alone can achieve the same grain yield as a weed-free crop (Moradi, 2009); and, therefore, the use of herbicides in this study alone was not sufficient and could not be equivalent to grain yield in a weed-free treatment. Consequently, including a weeding step in the weed management program is necessary.

Wheat growth

Wheat plants in the tillering stage were examined by visual evaluation for residual herbicide effect. None of the herbicide treatments had any adverse effect on wheat growth. Analysis of variance of wheat tiller number per plant, 1000 grain weight, number of plants per m^2, and grain yield of wheat showed that the effect of treatments on these traits was not significant.

Conclusions

Several pre-emergent and pre-planting herbicides have been applied in chickpea crops that helped to control many broadleaf weeds. Even if pre-emergent herbicides control the initial wave of weed growth at the beginning of the growing season, the persistence period of the herbicide may not be able to control the weeds later in the season; late-emerging weeds make it especially difficult to harvest. Therefore, control of broadleaf weeds in chickpea cultivation requires pre-planting herbicides and the subsequent use of post-emergent herbicides or other management methods to control the remaining weeds. Applications of flumioxazin, trifluralin 960 g ai ha^{-1} at planting, and pyroxasulfone reduced weed number and subsequently resulted in higher grain yields in chickpea. The study of herbicide residual on wheat growth in the next cropping seasons showed no adverse effect.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

SB and SKM designed the experiments, SL and AA carried out the field and laboratory experiments, SB, PS and IT

contributed to the data analysis, SB and SL wrote the article. All authors have read and approved the final version of the manuscript.

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The oil palm cadastre in Colombia

Catastro de la palma de aceite en Colombia

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ABSTRACT

This article describes the process of constructing a model of the geographic information management for the cultivation of oil palm in Colombia. Due to the need to collect, store, update, and analyze data from planted areas in the country, it was necessary to rely on the soft systems model to propose an information system structure that would respond to the needs of accounting for planted areas and to be able to integrate such information with other strategic data for the oil palm sector. This research developed a database model on which the geographic data related to the Colombian planted area of palm oil has been stored for over ten years. The geographic model has allowed creating new information at various territorial scales, integrated with phytosanitary data important for regional crop management. The integration of a web-based platform has positioned the oil palm cadastre as a consultation service for users working in various roles in the oil palm industry, as a reliable geographical bank of information, available to other oil palm project agribusinesses.

Key words: cadastral administration, land administration domain model, monoculture, oil crops.

RESUMEN

Este artículo describe el proceso de construcción de un modelo de gestión de la información geográfica para el cultivo de palma de aceite en Colombia. Debido a la necesidad de capturar, almacenar, actualizar y analizar datos de las áreas sembradas en el país, fue necesario soportarse en el modelo de sistemas blandos para plantear una estructura de sistema de información que respondiera a las necesidades de inventario de áreas sembradas, y que estuviera en capacidad de integrar dicha información con otros datos estratégicos para el sector palmicultor. Esta investigación permitió construir un modelo de base de datos sobre el cual, durante más de diez años, se han almacenado de manera continua los datos geográficos relacionados con el área sembrada en palma de aceite en el país. El modelo geográfico ha permitido generar nueva información a diferentes escalas territoriales y la integración con datos de índole fitosanitaria, de gran importancia para el manejo regional de los cultivos. La integración de una plataforma tecnológica web ha logrado posicionar el Catastro Palmero como servicio de consulta para usuarios de diversos roles dentro del gremio palmicultor y como información geográfica base de confianza para soportar otros proyectos de agronegocio de la agroindustria palmera.

Palabras clave: administración catastral, modelo para el ámbito administrativo del territorio, monocultivo, cultivos oleaginosos.

Introduction

The oil palm crop in Colombia has achieved a significant presence, accounting for approximately 10% of the country's total planted area and 30% of the agro-industrial crops (DANE, 2016; Fedepalma, 2019). This presence calls for the development and permanent update of an inventory of the country's planted areas, since this information could be the cornerstone for adequate planning and ordering of the territories.

From its inception, the Federación Nacional de Cultivadores de Palma - Fedepalma has worked to consolidate and develop a database that provides information on aspects relevant to Colombian agribusiness that includes the delimitation, area, and spatial representation of planted areas that can assist the palmiculture sector in adequate decision-making. Therefore, as a first approach to achieving these objectives, Fedepalma, through the Oil Palm Sector Statistical Information System (SISPA), has periodically compiled data of country wide seed sales as an alternative to an estimation of the planted area. This method leads to certain inaccuracies in the calculation determining the effectively planted area requiring corrections, mainly related to discarded seeds.

Fully aware of this condition, in 2007, Fedepalma, in an agreement with Corporación Colombia Internacional

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(CCI), completed the first georeferencing study on the area planted with oil palm in the country, creating polygons for around 276,000 ha of the 301,000 ha estimated to be planted. Additionally, in 2009 the Centro de Investigación en Palma de Aceite - Cenipalma, as part of a project funded by the Servicio Nacional de Aprendizaje (SENA), made the first adjustment to the cartographic base developed by the CCI and georeferenced additional areas of the crop that added up to a total 310,000 ha in comparison to the 350,000 ha estimated for that year.

Simultaneously, Cenipalma, as part of several regional phytosanitary information management projects, developed geographic databases that used polygons to represent the planted areas in the areas of the Colombian eastern plains, the Magdalena Medio (Central region), and San Andres de Tumaco (Pacific coast). In 2012, this first stage of data collection required focusing efforts on the planning and design of an information management system that would ensure the storage, administration, and consultation of data from the crop plots, *i.e*., from a specific cadastre of the areas with palm oil. The cadastre (a public register showing the details of ownership and value of land) corresponds to a land inventory (Kaufmann & Steudler, 1998) and under the new trends of the multipurpose cadastre, the inventory transcends the delimitation of properties and is supported by subsystems that carry out inventories of land uses (Williamson *et al*., 2014; Ponvert *et al*., 2015). So, the subsystem called Oil Palm Cadastre (OPC) was conceived, its cornerstone was the "land management paradigm" (Williamson *et al*., 2014) in which high-quality territorial information was an essential component, using the property cadastre as a methodological framework.

According to Ponvert *et al*. (2015), most national cadastre information systems store general data that often cannot meet the particular needs of every user. For this reason, it is convenient to propose and create specific subsystems that allow managing information at a larger scale, leading to the creation of cadastre subsystems mainly in rural areas (which, by tradition in the countries, has been characterized for having less detailed sales). In the last decade this need has been framed as Green Cadastres (Zysk *et al.,* 2020). Some examples include the land parcel information system to record crops and manage agricultural subsidies in the European Community (Inan *et al*., 2010), the Specialized

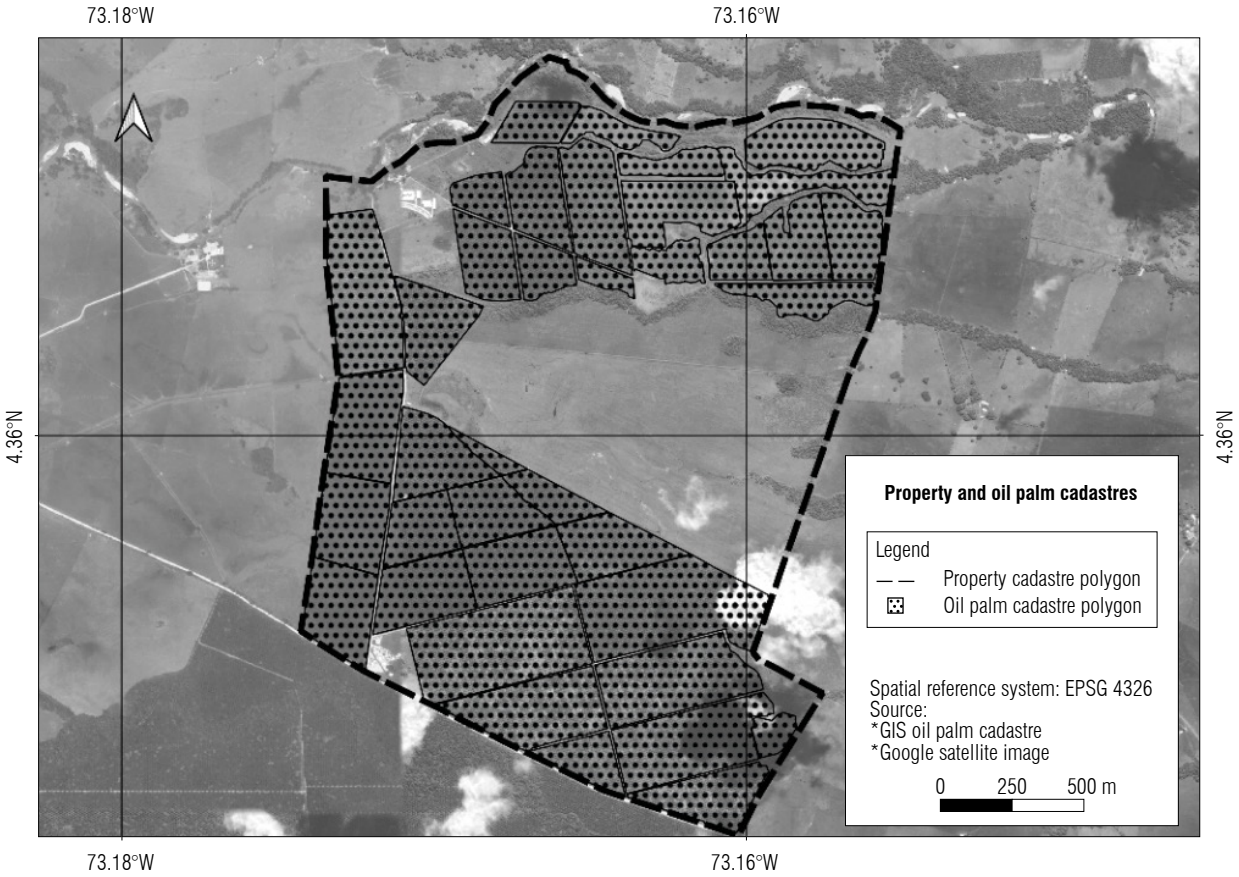

FIGURE 1. Difference between minimum units of the property cadastre and crop cadastral subsystems.

Sugar Cane Registry in Cuba (Samuel & Reyes, 2007; Rojas-Martínez *et al*., 2014), the Forest Cadastre in Greece (Drosos, 2014), the vineyards cadastre in Romania (Hutanu & Moca, 2010; Hutanu *et al*., 2016), and the inventory of tea areas in Turkey (Ozcelik & Nisanci, 2016), etc.

From these initiatives, and taking into account that the cadastre of the property was historically proposed to determine the value of such properties for tax purposes and/or to create a register for the legitimacy of the owners (Alcázar, 2000; Hopfer, 2003), the difference between these subsystems and the cadastre of the property was defined as the fact that the delimitation of the polygons specifically marks the minimum constituent unit of the cadastral subsystem (Samuel & Reyes, 2007; Ozcelik & Nisanci, 2016) and is not carried out considering the property's boundaries (Fig. 1). Therefore, the cadastral subsystems are a complement to this type of cadastre, with the spatial representation as an integrating component (Ponvert *et al*., 2015).

Therefore, it was important to create a system to store the georeferenced inventory of areas planted with oil palm at a plot scale in Colombia. The primary purpose of this system was to support decision-making in the oil palm industry and to provide updated information about planted areas. The system was developed as a technological tool under the guidelines of a cadastre information system (Çağdaş & Stubkjær, 2011); therefore, this paper describes the OPC conceptualization and the development of a continuously updated system. This tool was proposed as a benchmark for consultation about oil palm plantations in Colombia.

Materials and methods

The system was planned based on "soft systems" theory (Çağdaş & Stubkjær, 2011) to define the elements and actors of the OPC and to define the technical aspects that allowed implementing the OPC based on the system's conceptual framework.

System conceptualization

The OPC system is based on the uncertainty about the actual planted areas and their characteristics and geographic distribution. Therefore, the purpose of elucidating them is to create a theoretical framework through practice (Roux & Barry, 2009).

For this reason, the OPC is conceived as a system and, as such, it has the following characteristics (Wastell, 2012):

- **Purpose:** to provide georeferenced information on areas planted with oil palm as well as associated information in order to make decisions related to the oil palm sector;
- **Transformation:** a collection of geographical data for areas planted with oil palm and data related to the characteristics and management of the crops in order to create information for research, extension, and decision-making;
- **Feedback cycles:** the purpose is to identify the entire planted area of the country; therefore, measurements ensure the fulfillment of its purpose.

However, this is understood as a soft system under systems theory, considering that its purpose as a registry storage system transcends the inclusion of the complexity of human decision-making processes in all its components (Çağdaş & Stubkjær, 2011).

From its inception, the OPC was conceived as a tool whose main actors are Fedepalma, Cenipalma, and palm-growers, interrelated through the collection, storage, and consultation of data on the areas planted with oil palm and the information products generated from them.

A first activity carried out was the definition of the constituent elements of OPC, to break down each of the elements that participate in the definition of the areas with oil palm cultivation. Such a definition is considered a hierarchy determined by spatial units mainly associated with the functionalities and interests of the actors; each unit was assigned characteristics and in turn topological relationships.

Driving the conception of the system towards the operational component, the aspects in which the OPC is transformed from an inventory of areas to functional data were conceived that can be integrated with different sources to consolidate multipurpose characteristics of the inventory.

Technical aspects

Geographical data sources were classified into three categories: products of photointerpretation, a direct survey with GPS devices, or third-party information available at the plantations. Therefore, a data processing scheme had to be defined to monitor the technical operation of the geographical database construct (Fig. 2). These data structuring aspects were designed considering a detailed revision, correction, and adjustment for the geographical information, based on the Colombian Technical Standard NTC 5043 (ICONTEC, 2010):

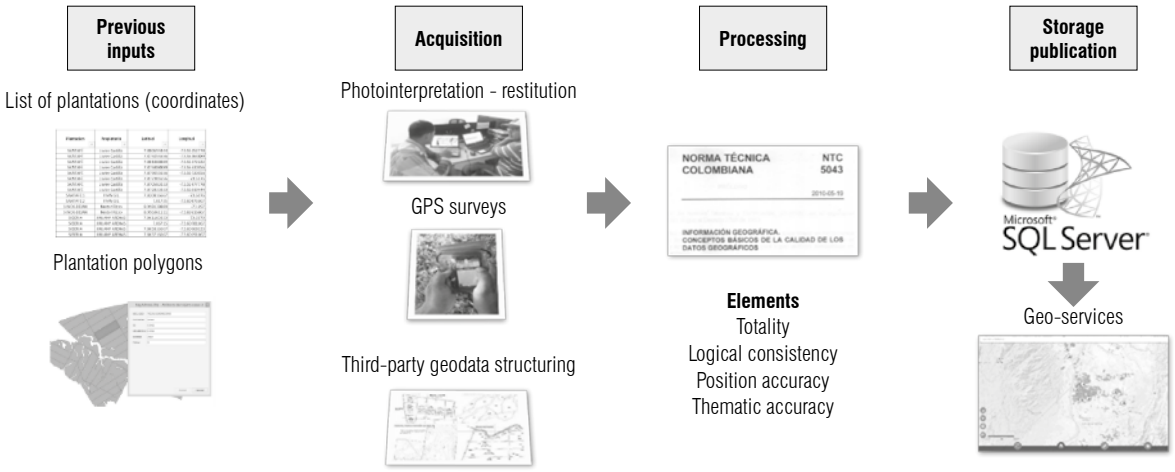

FIGURE 2. Data processing scheme.

- **Totality**

  The list of plots per plantation provided at the beginning of the project was used as a source, understanding that the total polygons must be equal to the number of plots reported for the plantation;

- **Logical consistency**

  The following elements were evaluated:

  » Domain consistency, for the name, area, palms, and material attributes.

  » Topological consistency that evaluates compliance with topological rules such as overlapping, invalid geometries, gaps, and non-repeating geometries;

- **Position accuracy**

  The data provided by the plantations were reviewed and the actual location of the polygons was validated through satellite images.

Results and discussion

Elements of the oil palm cadastre

The oil palm crop in Colombia is distributed throughout more than 120 municipalities. The geographic delimitation of what is known in the palm-growing sector as palm-growing areas from an administrative and logistics perspective, were named as follow: north, central, east, and southwest (Fedepalma, 2019) (Fig. 3). These were established based on the surrounding regions that are part of the same geographical region. Similarly, for operationality and accessibility, a group of sub-zones was established within each zone, defined mainly as plantation aggregates. The boundaries of the sub-zones may or may not coincide with the boundaries of municipalities. These sub-zones contained plantations in various rural properties used mainly to grow oil palm, and they are legally owned by one or several people/companies.

Depending on their size, these plantations cover one or several plots (plot being the minimum spatial and administrative division within a plantation) that are usually defined based on homogeneous characteristics of its constituent elements (cultivar, planting date, type of soil, etc.), geographical accidents, or infrastructure works developed inside the plantation. Therefore, it was important to emphasize that an oil palm plantation was a group of plots, which in turn had diverse geographical elements and/or characteristics inherent to them (palms, roads, channels, type of cover, etc.). However, there is no absolute relationship between the property and the plantation, or the property and the plot, because the latter may conform to the entire property, a fraction of it, or the sum of several properties (Sagris & Devos, 2008).

Below are the definitions of the elements that comprise the OPC.

- **Plot:** minimum spatial unit planted with oil palm within a plantation that generally has similar characteristics regarding the planting year and cultivar;
- **Plantation:** set of plots —not necessarily adjacent— grouped under the property and administered by one or several persons (natural or legal);
- **Sub-zone:** Geographical sub-division of the oil palm zones that allow for adequately monitoring the logistics and extension. The boundaries of one sub-zone may or may not coincide with those of the municipalities;

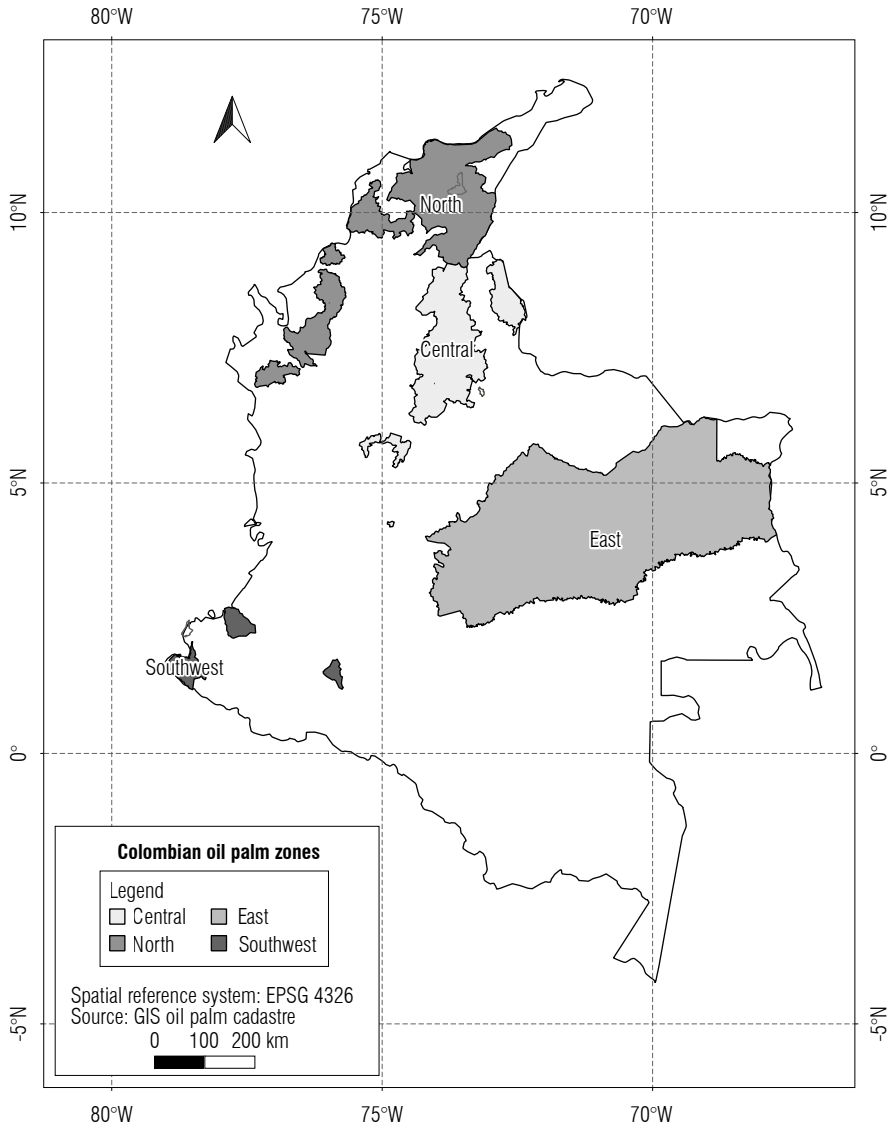

FIGURE 3. Distribution of oil palm zones in Colombia.

- **Zone:** Geographical division created by Fedepalma to adequately manage the palm productive areas in Colombia. It covers one or more municipalities in one or more departments.

Features of the OPC

The cadastre of the property generally refers to the spatial and legal features (Çağdaş& Stubkjær, 2011). In the case of the OPC, the physical feature of the property refers to identifying and representing geographical elements (plots, plantations, sub-zones, and zones). The agronomic feature refers explicitly to the specification of the cultivar, species, age, phytosanitary status, etc., to describe crop's particularities found in the smallest mappable unit (plot).

Topological relationships

Considering that the fundamental elements of the OPC have geographical representation, topological relationships that establish the hierarchical connections between them had to be defined; *i.e.*, the zone (the element with the highest hierarchy) is formed by various sub-zones that in turn are formed by a certain number of oil palm plantations that cover one or several plots. Figure 4 is a graphical representation of the hierarchical distribution of the elements of the OPC.

Furthermore, defining the relationship of the elements of the OPC with the cadastre of the property allowed integrating new actors to the s ystem. The territorial entities

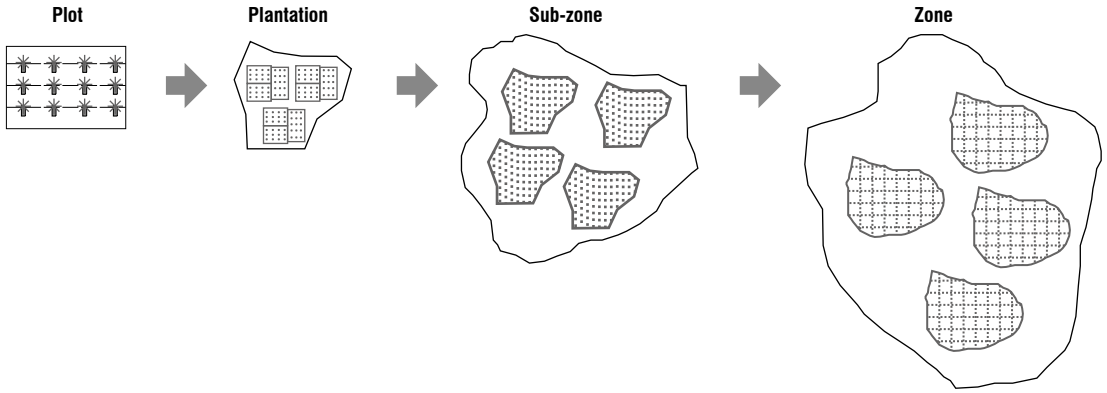

FIGURE 4. Hierarchical distribution of elements of the OPC.

and institutions related to the ordering of the territories considered the information products created by the OPC as inputs. Integrated to the property, the inputs are fundamental for planning and proposing strategies and policies in the territories.

Multi-purpose structure

In a first instance, the OPC was conceived as a solution to quantify the total planted area in Colombia. However, having the geographical conceptualization and representation of the spatial objects allowed integrating another type of strategic information for representation. Therefore, the OPC is currently conceived under a multi-purpose approach that facilitates the integration of agronomic information derived fro crop activities that enhance decision-making criteria.

Therefore, the OPC elements were redefined, considering the possibility of representing phytosanitary and productive variables and the management of crops at a regional scale.

Defining plot boundaries

Considering cadastres of property, an accuracy to the centimeter is essential for urban areas. However, in Colombia, rural cartography has been developed at a maximum scale of 1:10,000, substantially reducing accuracy requirements. However, the material definition of property boundaries in rural and urban spaces is indicative and accurate, *e.g.,* fences of rural properties. However, oil palm plots are generally known for having no explicit physical delimitation. Therefore, the conceptualization included a definition of the plot boundaries, understood as the projection to the ground of the canopies of the palms planted on the edge of the plot.

In practice, under the above definition, the boundary would change as the palm grows; hence, the above concept was complemented with the projection to the ground of the canopies of adult palms, which in practical terms can be extrapolated to half the planting distance between palms.

After defining the concept of plot boundary, it was necessary to specify the minimum accuracy for surveying it. Hence, it was decided that the maximum tolerable error to define the perimeter of a plot would be 10 m, thus tolerating the variation found in the definition of the boundary based on the canopy projection. This allowed including tools such as low-accuracy GPS devices and satellite images with a 5-m resolution and optimized the area surveying works without affecting data quality. As a result, and considering the guidelines set by Tobler (1987), a scale of 1:20,000 was established for the OPC.

Considering that the OPC development aims to provide an approximate quantification of the country's planted area and coordination of the multi-purpose approach, two fundamental components were defined that established the system's objectives and scope. First, approximation to the physical aspect (quantification and geographical representation), and the second inclusion of the agronomic aspect (crop characterization).

Physical aspect

With the first approach, the areas planted with oil palm crops were identified via photointerpretation of satellite images without leading to a definitive demarcation of the plots and plantations (Kelly *et al*., 1999; Rodríguez *et al*., 1999; Johansen *et al*., 2009). The sensors used as data sources were Sentinel 2 and Landsat 8, considering the free access to their data, the multi-temporality, and characteristics for crop discrimination (Sarvia *et al*., 2020; Jayanth *et al*., 2022). A future alternative would be support in the delimitation of the plots from satellite image processing techniques, such as fully convoluted networks (KC *et al.,* 2021; Taravat *et al*., 2021). However, these techniques are

still in the research stage and in order to guarantee the quality of the data, human photointerpretation was included in the processing.

The following properties of a plot were established to define the rules of photointerpretation (Stone, 1955): shape, size, structure, texture, and color.

- **Shape:** the plots planted with oil palm are generally known for having regular shapes; however, there are also irregular shapes or shapes defined by administrative considerations that are not related to geographical boundaries (Fig. 5);
- **Size:** generally, an area with oil palm may vary between 1 and 20 ha without identifiable discontinuity like roads or water bodies. However, there may be unusual cases where there are plantations with a larger area;
- **Structure:** the structure inside an oil palm plot must include the presence of staggered formations or planting patterns that form equilateral triangles between palms. However, there may be cases where the plantations do not meet this rule, such as terraced plantations. The presence of irrigation channel infrastructure within the crop may be an indicator of new or young plantations;
- **Texture and color:** In this case, two closely related properties were addressed, since both are mainly subject to variation in planting age. In high-resolution spatial images, it is possible to distinguish the staggered formation. However, images with a spatial resolution equal to or higher than 5 m required defining a series of keys that facilitate identifying the crops (Fig. 6). However, the similarity between oil palm crops' reflectance with other crops like banana and forests implies field verification.

To establish orderly photointerpretation, a grid of 5 x 5 km^2 was defined to cover all the palm areas. Each grid was assigned tracking information to develop the process This series of data traced the area update process. A revision was established for each square, manually employing lateral and vertical displacement, where each of the areas identified as oil palms were restored.

Since the process of updating areas planted with oil palm is carried out every six months, management of the grid data was established as an additional component in the OPC database, making it possible to keep track of the revisions made in the same quadrant over time and identify potential areas for updating.

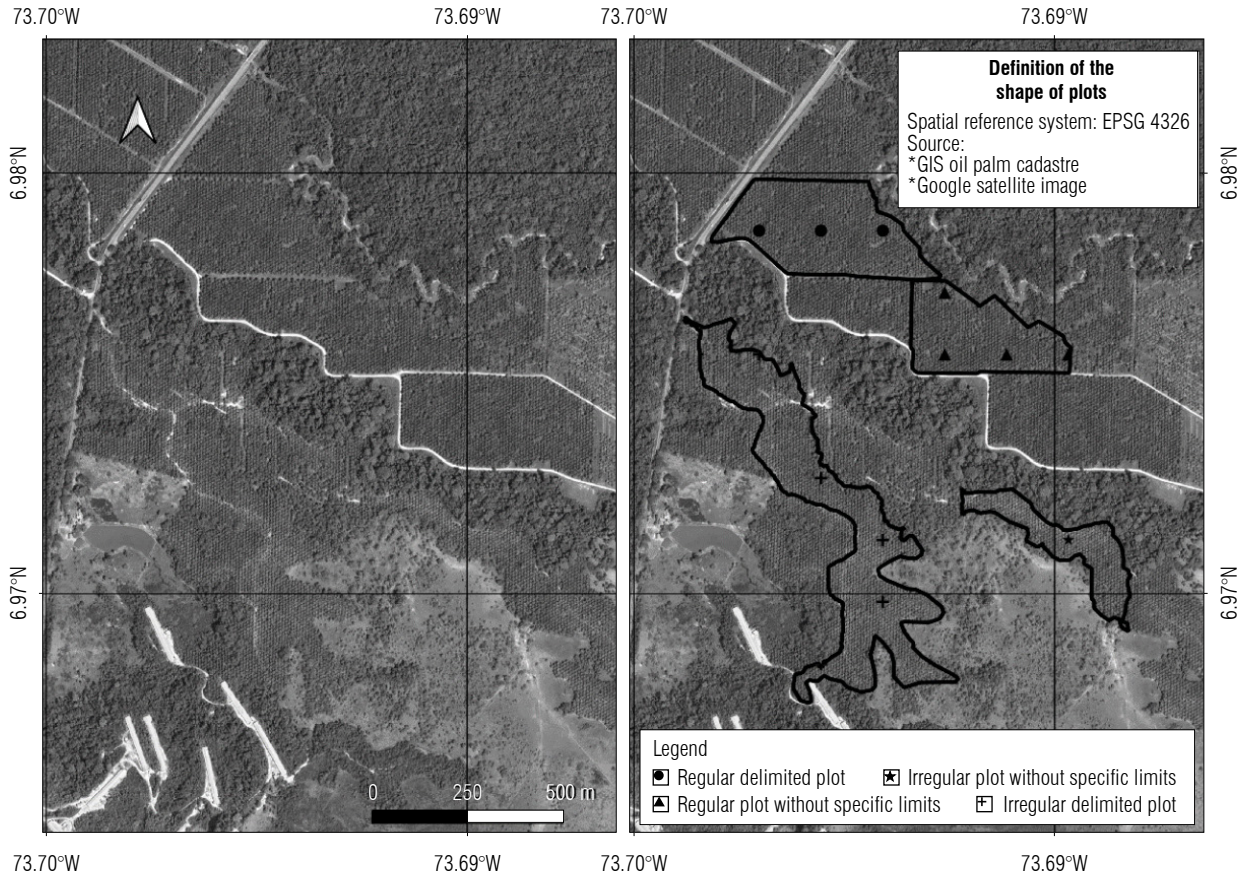

FIGURE 5. Definition of the shape of plots in oil palm crops.

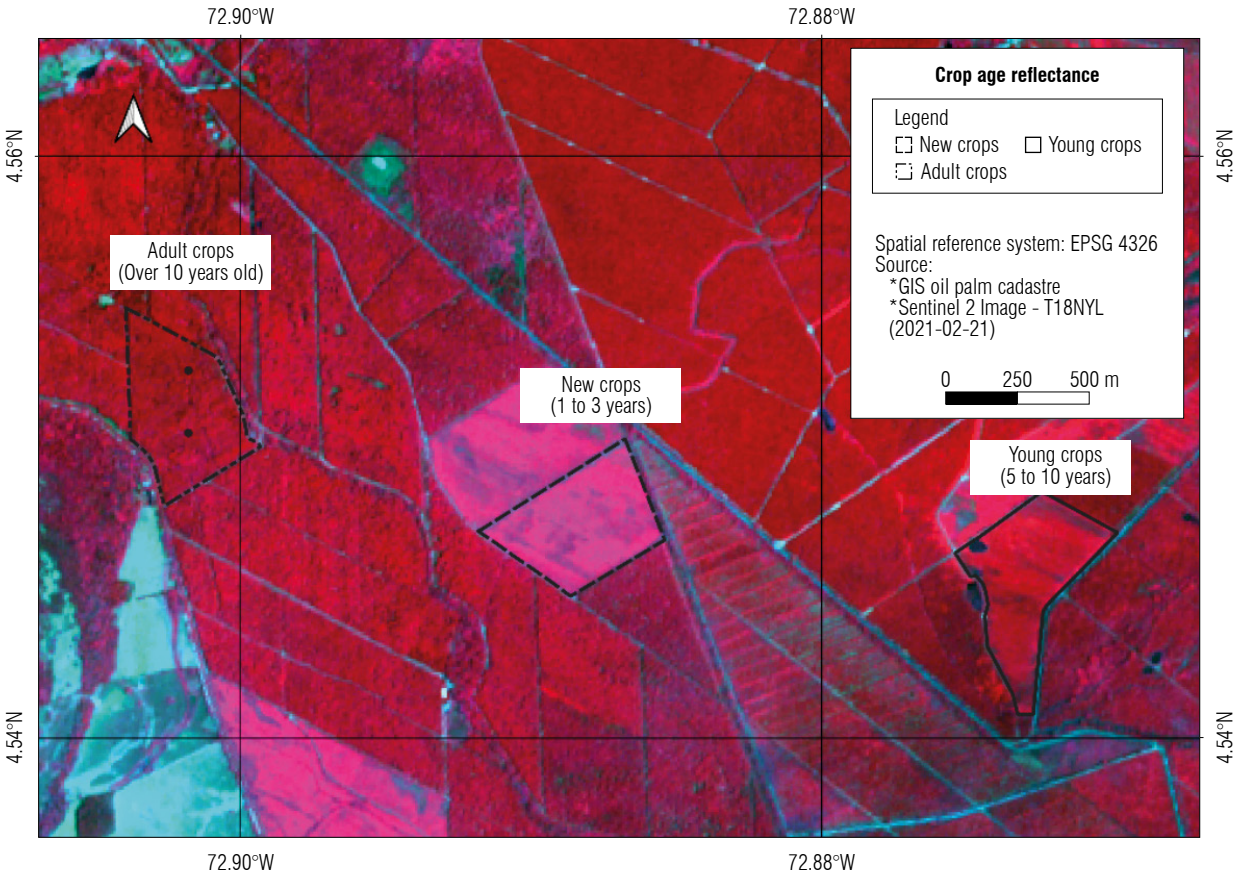

FIGURE 6. Interpretation of palm age in combination with false color infrared in LandSat 8 image.

Agronomic feature

This corresponded to characterization of the minimum agronomic administration unit of the plantations, commonly called a plot. In the absence of a standard for the definition of plot limits, each plantation was autonomous to define its plots, and it was necessary to define the plot as the minimum grouping of palms to which production data, phytosanitary, etc., were recorded. In most cases, plots contained palms from the same planting year, the same cultivar, and had physical limits. In this way, the agronomic feature defined the geometry of the plots in accordance with the administration of the plantation and complemented the data on the following: number of palms, planting density, cultivar(s), planting year and net area.

This feature required greater efforts and dedication, as it demanded establishing direct contact with the owners, managers, or directors of the plantation through palm clusters (oil palm mills). The bonds of trust between clusters and palm growers allowed access to the plantations and access to truthful data.

The contact with the commercial liaison between the clusters and the palm growers, the Technical, Environmental and Social Assistance Units (UAATAS) adopted in 2008 (Fedepalma, 2009) enhanced the palm-grower contact strategy because the clusters were receptive to considering the importance of georeferencing planted areas at a plot scale. Below is the process scheme designed for the correct management and collection of plantation cadastral information (Fig. 7).

Finally, the National Oil Palm Cadastre Database is integrated in two stages:

- Inclusion of geographical data (physical feature), with semi-annual update;
- Association of the agronomic information with each plot (agronomic features), which is a permanent process.

With the inclusion of technical considerations, a new actor became evident in the OPC system that had not been contemplated in the initial formulation. Hence, the palm tree clusters appeared as a determining factor in the process of formation and as a consumer of the information. Thus, finally, the formalization of the processes oriented to management and processing of the data finally consolidated the model of the Oil Palm Cadastre System (Fig. 8).

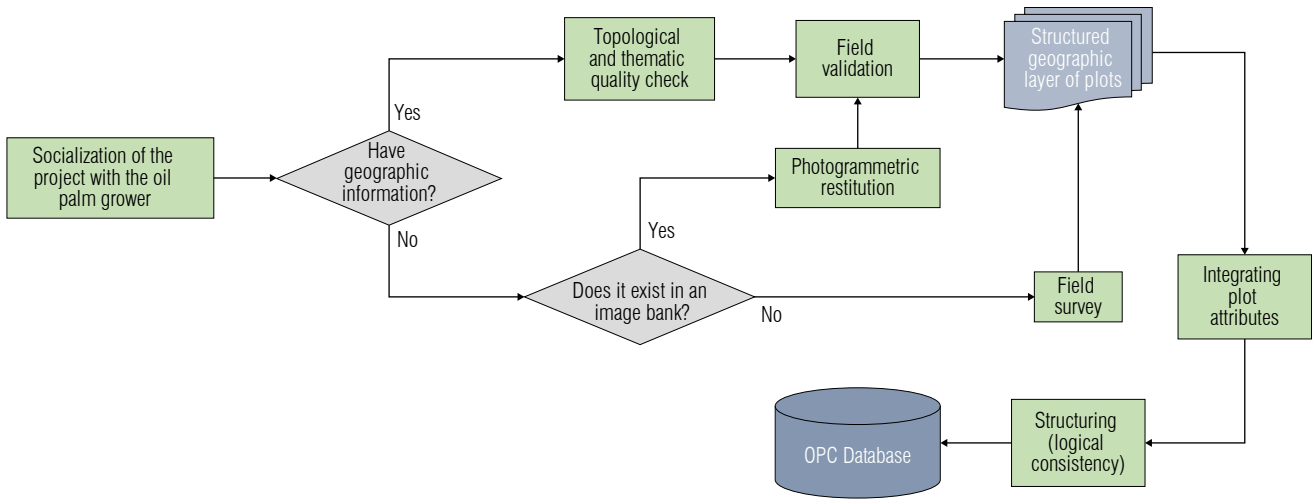

FIGURE 7. Data management scheme of plantation cadastral information.

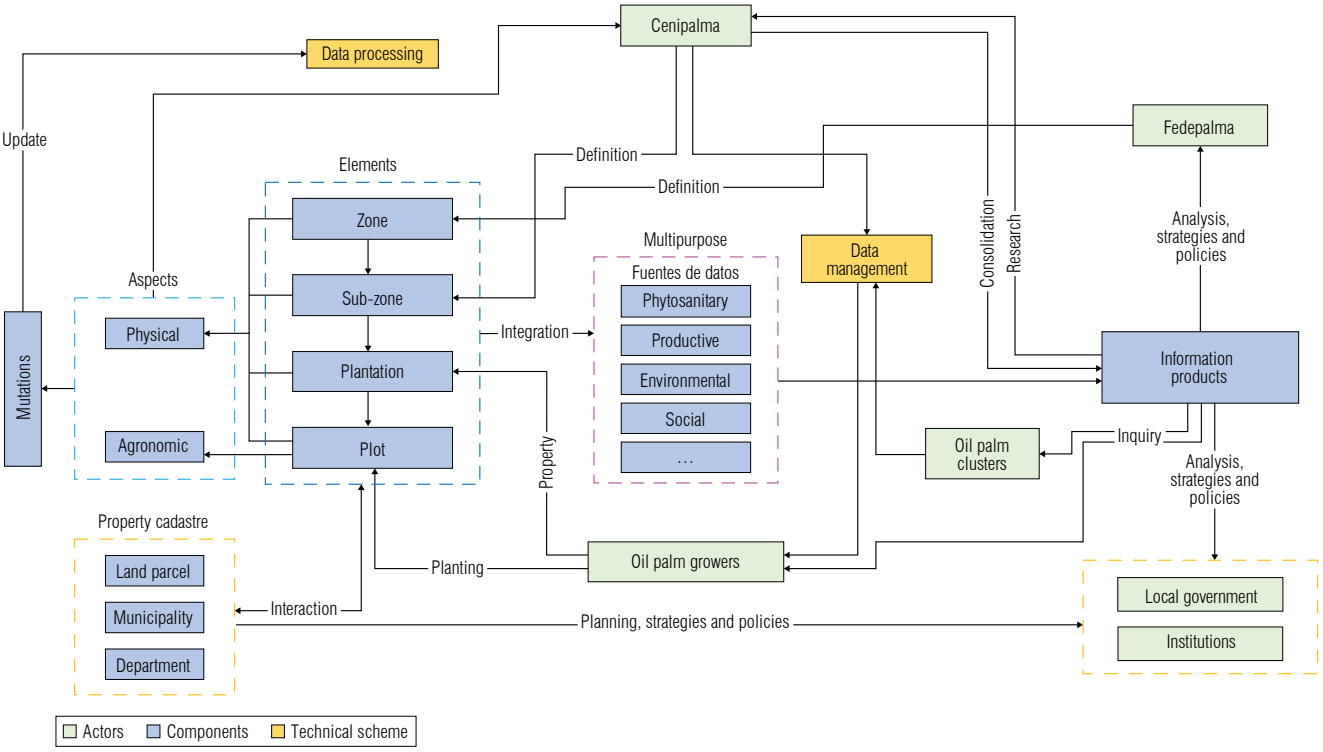

FIGURE 8. Oil palm cadastre system model.

Information technologies artifacts

A spatial database was designed and implemented in Microsoft SQL Server Database Management System to store spatial and alphanumeric data. This allowed elimination of redundancy, allowing capability for data processing, analysis, visualization and exchange (Dawidowicz *et al.*, 2020). The spatial database on the SQL Server was integrated into the ArcServer platform to develop geoservices aimed at publishing the OPC information on the internet: https://geopalma.cenipalma.org

The main developed geoservice consolidates the values of planted areas at a municipality scale and was an example of the interaction between the OPC and territorial units such as municipalities (Fig. 9).

Progress in the consolidation of information

As mentioned above, the work of the Corporación Colombia Internacional (CCI) in 2007 is considered the foundation of the OPC. Throughout 10 years, about 100% of the estimated area planted with oil palm in the country

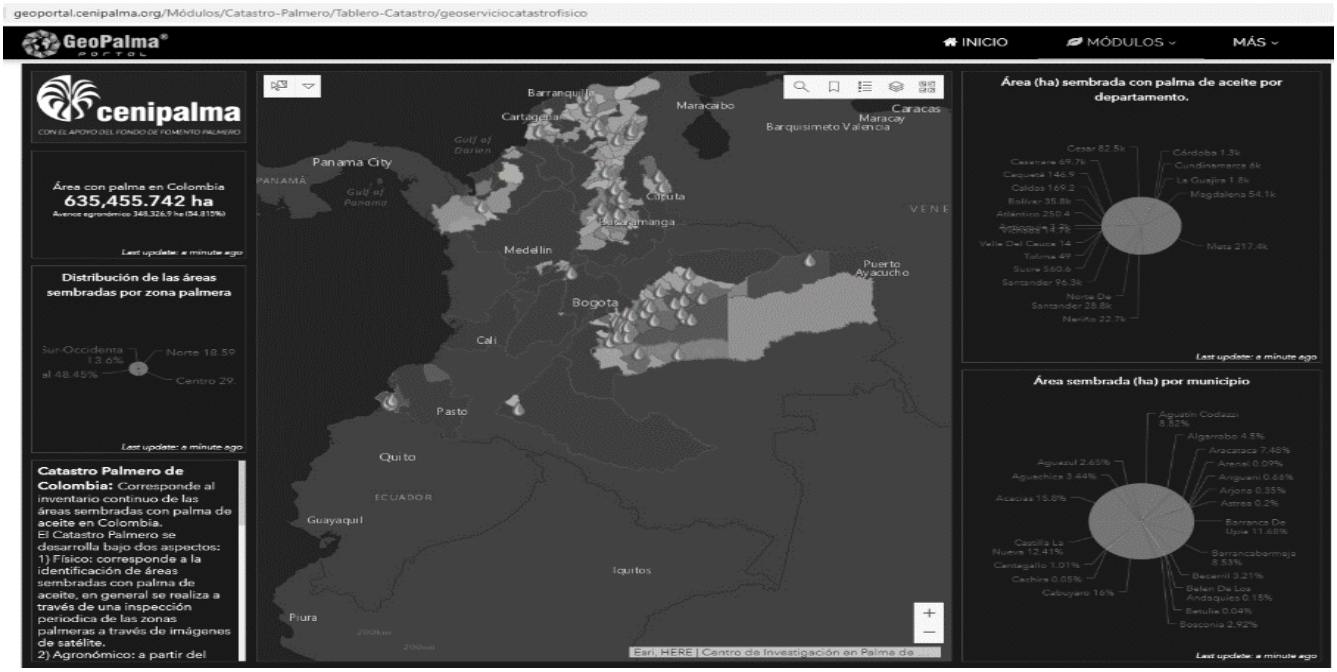

FIGURE 9. Geoservice developed for consultation of data for the oil palm cadastre (in Spanish; https://geopalma.cenipalma.org/M%C3%B3dulos/Catastro-Palmero/Tablero-Catastro/geoserviciocatastrofisico).

has been georeferenced. This physical feature has had continuous revision (a review of the areas is carried out every two years) parallel to the expansion of the crop, beginning from about 276,000 ha georeferenced in 2007 to 625,066 ha in 2020, exceeding the area reported by the SISPA. Regarding the progress of the agronomic feature, though it is slower it increased from zero hectares in 2007 to 160,000 ha in 2017. Thus, the speed of this (agronomic) feature increased during the last three years, mainly due to the active participation and strong interest of palm growers during OPC development (Fig. 10). As of 2020, there were a total of 625,066 ha in physical terms, of which 345,600 ha are fully characterized physically and agronomically.

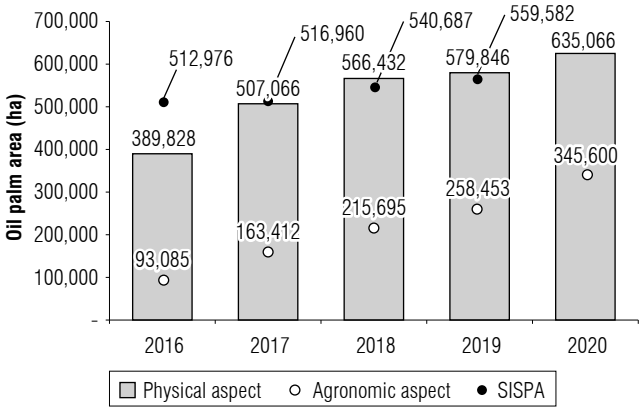

FIGURE 10. Evolution of the oil palm cadastre.

Progress in the above features depended on the conditions of each region, with sub-zones such as South Cesar, South Bolívar, Acacías or Fundación mostly able to consolidate their agronomic aspect due to the integration and participation of the oil palm clusters of the sub-zones.

The physical feature has enabled accurately identification of the extent of oil palm trees in each of the palm-growing areas (Fig. 11). To date, the eastern zone has about 46% of the country's total.

One of the most interesting results has been the possibility of consolidating data at a territorial unit scale, such as departments (Fig. 12). This data was previously estimated without the OPC. As of 2017, the department with the

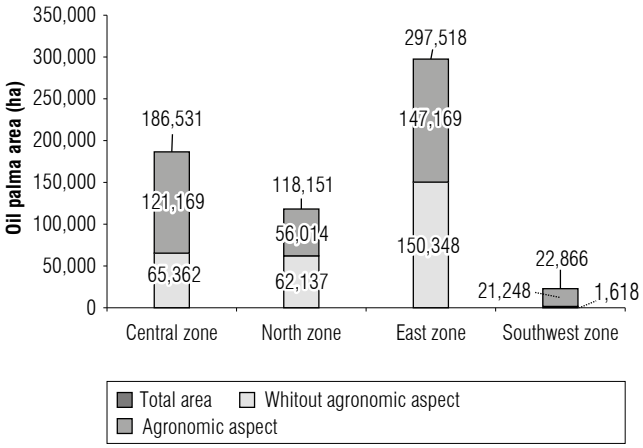

FIGURE 11. Distribution of planted area of oil palm per zone.

largest planted area of the country is Meta, followed by Santander and Cesar, reiterating that the Eastern and Central palm growing areas have the country's most extensive planted areas.

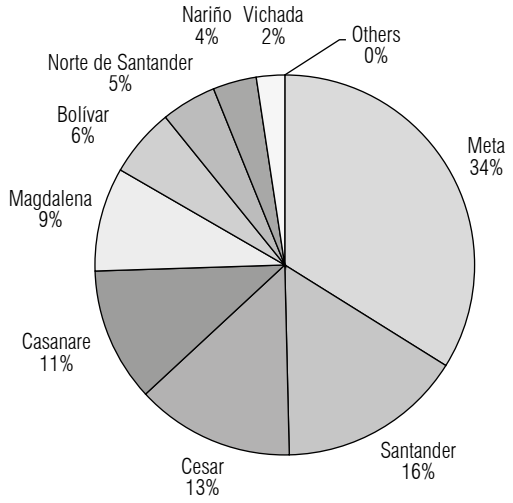

FIGURE 12. Planted area of oil palm by department.

Conclusions

From a soft systems perspective, the OPC system model allowed including the complex interactions between actors. This was translated into a significant advance in the consolidation of the system's information. Incorporating the palm clusters and growers as active actors accelerated data collection and guaranteed continuous feedback for the project's results.

The OPC's conceptualization was made "along the way," allowing adjustments that were mainly due to the inclusion of new actors in the process. With this, soft systems theory is confirmed as an alternative to conceptualize cadastral systems and subsystems.

The creation of a record of planted areas by delineating each of their perimeters is an effort that has involved many years and resources. However, this effort has been worthwhile, due to the complexity of the crop and its setting. From this experience, we highlight the integration of the system to the pre-established processes of the extension program (UAATAS), without which, it would not have been possible to consolidate the agronomic features, and the system would have been limited to only the physical features.

After several years of work, the OPC is considered to have reached maturity, consolidating processes that guarantee the quality of the data, which are increasingly closer to representing the country's total planted areas. Therefore, upcoming efforts should focus on the system's communication phase. Although with some progress, this phase must be mainly aimed at the use of the information produced, in order to transcend the processes of the palm-sector and to connect with the governmental territorial organizations. It is worth mentioning that the oil palm agroindustry is an essential part of the development of the regions where it exists.

Acknowledgments

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

VRR wrote the initial draft. VRR and AMV formulated de overarching research goals and aims. VRR, AMV and AZQ designed the methodology. OBA and VRR implemented the computer code and supporting software. JTL managed and coordinated the project and reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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Dual-purpose production of forage and seeds in maize by detopping and defoliation

Producción doble propósito de forraje y semillas en maíz mediante una poda apical y defoliación

Hassan Heidari[1]* and Mozhgan Amiriani[1]

ABSTRACT

Maize is one of the most productive crops whose seeds are used in the poultry sector as one of the main ingredients in the diet; it is also important forage for ruminants as silage. The aim of this research was to assess the effect of defoliation and detopping on dual-purpose maize production in field (Kermanshah, Iran, Mediterranean climate conditions) and laboratory experiments. The study included a control (intact plant), removal of leaves at the top of the ear, removal of leaves under the ear, removal of all leaves, detopping (stem removal at the top of the ear), and detopping including removal of leaves under the ear. In the laboratory experiment, germination traits were assessed in seeds obtained from the mother plants in the field experiment. The field and laboratory experiments were conducted with a randomized complete block design and completely randomized design, respectively. The data were analyzed using a general linear model. The removal of leaves under the ear produced an increased seed number per row compared to the removal of leaves at the top of the ear. Intact plants (control) and the plants defoliated under the ear had a higher 100-seed weight than other treatments. There was no difference between detopping and control plants in seed yield. Removal of leaves under the ear of mother plants produced a lower seed germination percentage (83%), radicle length (11.3 cm), and seed vigor than in other treatments. The results show that maize can be cultivated as a dual-purpose crop for forage and seed production.

Key words: dual-purpose crop, germination, mother plant.

RESUMEN

El maíz es uno de los cultivos más productivos cuyas semillas se utilizan en el sector avícola como uno de los principales ingredientes de la dieta; además, es un forraje importante para la alimentación de rumiantes en forma de silo. El objetivo del estudio fue evaluar el efecto de la defoliación y poda apical en la producción de maíz de doble propósito en campo (Kermanshah, Irán, condiciones de clima mediterráneo) y laboratorio. El estudio incluyó un control (planta intacta), eliminación de hojas en la parte superior de la mazorca, eliminación de hojas debajo de la mazorca, eliminación de todas las hojas, poda apical (eliminación del tallo en la parte superior de la mazorca), y poda apical más eliminación de hojas debajo de la mazorca. En el experimento de laboratorio, se evaluaron parámetros de germinación de las semillas producidas de plantas madre del experimento en campo. El experimento de campo y de laboratorio se llevaron a cabo con un diseño en bloques completos al azar y un diseño completamente al azar, respectivamente. Los datos se analizaron utilizando un modelo lineal generalizado. La remoción de hojas debajo de la mazorca produjo un mayor número de semillas por fila que la remoción de hojas en la parte superior de la mazorca. Las plantas intactas (control) y las plantas defoliadas debajo de la mazorca tuvieron un peso de 100 semillas más alto que otros tratamientos. No hubo diferencia entre la poda apical y el control en el rendimiento de semillas. La eliminación de las hojas debajo de la mazorca de las plantas madre produjo un porcentaje de germinación de semillas (83%), longitud de radícula (11.3 cm) y vigor de semilla más bajos que otros tratamientos. Los resultados muestran que el maíz puede ser cultivado como un cultivo de doble propósito, con producción de forraje y de semillas.

Palabras clave: cultivo de doble propósito, germinación, planta madre.

Introduction

One of the major problems of agriculture is insufficient nutrition of livestock and poultry. Maize is one of the most productive crops whose seeds are used in the poultry industry as one of the main ingredients in the diet (Singh & Ravindran, 2019). Also, it is an important fodder for ruminant nutrition in the form of silage (Horst *et al*., 2020). In maize for grain, plant residues are usually dried and are not suitable for livestock. In maize crops, defoliation is a

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removal of plant leaves, and detopping is the removal of the plant stem just above the maize ear.

Defoliation is the subject of much research (Rua *et al*., 2020; Song *et al*., 2020; Donovan *et al*., 2021; Sánchez-Cuesta *et al*., 2021; Wang *et al*., 2021; Yang *et al*., 2021). In maize with increasing defoliation at the ear initiation stage, the grain yield decreases (Iledun & Rufus, 2017). Removal of two leaves from the top of maize plants increases the biomass in maize and soybeans in a maize-soybean intercropping system (Raza *et al*., 2019). In intercropping of maize and legume forage as the following crops, the appropriate rate of maize defoliation is 25% to 50% without a negative effect on dry matter production of maize (Hassen & Chauhan, 2003). In a study on maize, the treatments consisted of four stages of source restriction: including defoliation at the middle of silking and three consecutive 10 d intervals from the middle of the silking as well as three defoliation intensities (zero, half, and total leaf removal) (Emam *et al*., 2013). Delay in source restriction was found to be associated with reduced grain weight (Emam *et al*., 2013). The highest mean grain weight was obtained by defoliation at the middle of silking, which also resulted in the lowest reduction in grain yield compared to the non-defoliation treatment. Increased defoliation intensity was associated with decreased grain yield. However, delay in defoliation after the middle of silking had no significant effect on grain yield (Emam *et al*., 2013). Defoliation reduces stem biomass, grain yield, and the maize harvest index. Decreasing the height of the maize plants increases the stability of grain yield by reducing lodging and increasing stress tolerance during the flowering stage (Edmeades & Lafitte, 1993).

Removal of the tassel increases maize yield by 11% to 32% (Mashingaidze *et al*., 2010). Defoliation at the anthesis (50% tasselling) increases yield by 16% to 28%, while defoliation three to four weeks before or after anthesis has no significant effect on yield. Removal of the tassel increases solar radiation absorption by leaves below the tassel and ear leaves. In addition, removal of the tassel may reduce the terminal dominance that aids in grain filling (Mashingaidze *et al*., 2010). Among detopping (removal of the upper ear stem) of maize at 10, 20, and 30 d after silking, detopping at 20 d after silking results in the higher grain yield (Amanullah, 2020). Detopping 30 d after silking, removal of the top 6 leaves after physiological maturity, removal of all leaves above the ear, or detopping above the tenth internode produces the highest forage yield and net yield with partial or no reduction in grain yield (Rajkumara *et al*., 2020). In a study of three detopping levels including complete removal of the shoot from above the ear, leaving one or two leaves above the ear, and four detopping times (different times after pollination) in maize, detopping reduces grain yield by 18% and lowers the 1000-seed weight. Complete removal of shoots from above the ear produces the highest forage yield and leaving two leaves above the ear produces the least forage. Detopping is not recommended if the purpose of the crop is seed production, but if the purpose is forage plus seed production, the best detopping time is at the end of pollination or 10 d after pollination (Afarinesh, 2005). In maize, detopping at different stages does not produce a significant effect on the studied parameters; however, the highest dry matter yield is with detopping 30 d after silking, and the lowest dry matter yield is 10 d after silking. The highest plant height, leaf number, leaf area index, dry matter, and yield are by detopping up to two upper leaves and the lowest of these traits is found by detopping up to six upper leaves (Bhargavi *et al*., 2017).

The environment of the mother plant affects the germination traits of the produced seeds. Different levels of defoliation in vetch (*Vicia sativa*) produce seeds with the same germination percentage and germination time (Koptur *et al*., 1996). Artificial defoliation of wheat (*Triticum aestivum* L.) has little effect on the germination traits of produced seeds (Heidari *et al*., 2013). Defoliation caused by *Cameraria ohridella* (Lepidoptera: Gracillariidae) in *Aesculus hippocastanum* L. causes a decrease in shoot weight, root weight, total biomass, root length, and root diameter; the seeds from infested trees have higher germination than non-infested ones (Takos *et al*., 2008). We studied the effect of defoliation and detopping (removal of the upper ear stem) in the mother plant and their effect on the germination traits of the produced seeds. Therefore, this study was designed to determine the best defoliation or detopping treatment for dual-purpose production of maize as fresh forage and seeds in the field conditions of Kermanshah, Iran.

Materials and methods

Site description

A field experiment was conducted in the arable lands of the Chamchamal plain (34° N, 47° E, and altitude 1300 m a.s.l.) with an average annual rainfall of 442 mm (IMO, 2012) located 47 km from Kermanshah, Iran. The Chamchamal plain has fertile agricultural lands and is one of the production areas in the west of the country. The average monthly temperature, relative humidity and rainfall of the region are shown in Figure 1. Soil texture was silty clay loam, and pH and electrical conductivity were 7.2, and 1.6 dS m^{-1}, respectively.

To evaluate the effect of field treatments on germination traits of produced seeds, a laboratory experiment was conducted in the Crop Physiology Laboratory, Faculty of Agricultural Science and Engineering, Razi University (Iran).

Treatments and experimental design

We conducted a field experiment in a randomized complete block design with three replicates. The plots were 3 m x 3 m. The distance between the plots was 2 m. Treatments included the control (intact plants), removal of leaves above the ear, removal of leaves below the ear, removal of all leaves, detopping (removal of stem above the ear), and detopping in addition to removal of leaves below the ear. In the defoliation treatments, the leaf blade was cut with a sharp cutter from the point where it was attached to the stem. In the detopping treatment, the plant stem was cut from the top of the ear. In order to evaluate the effect of field treatments on germination of produced seeds, a laboratory experiment was conducted in a completely randomized design with three replicates.

Plant management in field and laboratory

At the beginning of March 2014, the land was plowed using a moldboard plow. Triple superphosphate fertilizer was mixed with soil at the rate of 333 kg ha^{-1}. On April 7, 2014, maize (cv. single cross 704) seeds were sown at a rate of 27 kg ha^{-1} using a maize pneumatic machine. Planting row spacing was 75 cm. The most important weeds were *Amaranthus retroflexus* L., *Chenopodium album* L., *Setaria viridis* L., *Sorghum halepense* L., *Phragmites australis* Cav., and *Cynodon dactylon* L.. The 2,4-D (2,4-dichlorophenoxyacetic acid) and nicosulfuron (2-[(4,6-dimethoxypyrimidin-2-yl) carbamoylsulfamoyl]-*N*,*N*-dimethylpyridine-3-carboxamide) herbicides were used to control the weeds. The cultivator was used to earth up and open irrigation ditches. Urea fertilizer at the rate of 367 kg ha^{-1} was applied at two moments (May 31 and June 22). The plants were irrigated eight times by the surface method until the end of the growth season.

After field measurements, a laboratory study was developed. The seeds of each treatment were first sterilized with sodium hypochlorite (1% active chlorine) for 10 min and then 10 seeds were placed on filter paper in sterile Petri dishes. Eight ml of distilled water was added to each Petri dish to prevent evaporation, and the Petri dishes were placed in a plastic bag. The Petri dishes were stored in a germination chamber at 25°C for one week.

Sampling and measurements

Due to the size of the plot, which was 3 x 3 m^2, there were four planting lines at a distance of 75 cm in each plot. At the time of harvest (August 29, 2014), three plants were randomly selected from the two middle lines of the plot and the desired ears were harvested. The selection criteria were plants that represented the plot. Sampling was not performed from the two side lines of the plot to remove the margin effect. After drying the ears, the ear husks were first separated from the ears and the ear husks were weighed. Each ear was then weighed without husk. Seed numbers per column and row of ear, length and weight of cobs, seed yield, and weights per 100 seeds were determined.

One week after the seed germination test, seed germination percentages, coleoptile and radicle lengths, and seed vigor were measured. Two millimeters of coleoptile growth was the germination criterion. The seed vigor estimate was calculated by multiplying the germination percentage by the seedling length (coleoptile length plus radicle length) (Heidari, 2013).

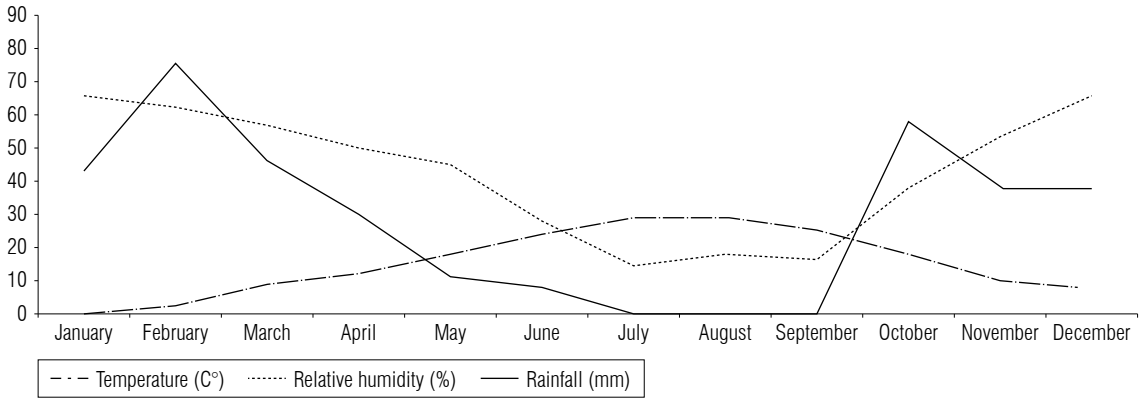

FIGURE 1. The average monthly temperature, relative air humidity, and rainfall of the region in 2014 (IMO, 2014).

Data analysis

Before analyzing the data, normality was checked and the data were analyzed by variance. Data were then compared by Duncan's multiple range test at a 5% probability level. Correlation between traits was also calculated. SAS, MINITAB, and SPSS statistical software were used (Soltani, 2007).

Results and discussion

Field experiment

Ear husk weight and ear weight

Analysis of variance showed that detopping and defoliation did not affect maize ear husk weight (Tab. 1). A mean comparison with Duncan's test also showed that there was no difference between detopping and defoliation treatments in ear husk weight (Tab. 2). The ear husk weight was, probably, relatively complete by the beginning of the seed filling period (milking stage). Maize ear husks contain chloroplasts and can photosynthesize as leaves. The ear husk begins to develop earlier than the seeds and due to its proximity to the seeds has an effective role in seed filling (Koocheki & Sarmadnia, 2011). Data analysis of variance showed that detopping and defoliation had no significant effect on maize ear weight (Tab. 1). The mean comparison showed that the detopping with the removal of the lower leaves of the ear had a lower ear weight than the control (no leaf removal and no detopping) (Tab. 2). But there was no difference between detopping and the control. These results indicated that the upper leaves and stem of the ear could be harvested at the seed milking stage for livestock use, without affecting the ear weight. The lack of weight loss of the ear at the milking stage is, probably, due to the compensatory properties of other photosynthetic organs such as the lower leaves of the ear (Sun *et al*., 2021). Because in these conditions the lower leaves of the ear are not shaded by the upper leaves, they receive more light and their photosynthesis increases (Liu *et al*., 2020). The removal of all leaves from the plants was no different from the control in terms of ear weight. But detopping is easier than removing all leaves from the plants because it can be mechanized. However, during the milking stage the leaves and stems of the plants are still green and palatable to livestock, while less residue remains on the soil surface that interferes with the tillage operation for the next crop. Ear weight had a positive and significant correlation with all studied traits except the seed number per column (Tab. 3).

Seed number per column and row of the ear

Analysis of variance showed that detopping and defoliation did not affect seed number per ear column (Tab. 1). The mean comparison revealed no differences between the studied treatments in terms of seed number per ear column (Tab. 2). Considering that the time of application of treatments was at the seed milking stage, it is clear that if the treatments were applied at the pollination stage, it would have a greater effect on the seed number per column.

TABLE 1. Analysis of variance of the effect of detopping and defoliation on maize traits.

Source of variation	df	Ear husk weight		Ear weight		Seed number per column		Seed number per row		Cob length		Cob weight		Seed yield		100-seed weight	
		MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F
Block	2	0.82^{ns}	0.97	68.8^{ns}	0.89	17.9^{ns}	0.64	0.819^{ns}	0.10	0.862^{ns}	0.72	23.5^{ns}	0.28	39.8^{ns}	0.91	13.4^{ns}	0.138
Treatment	5	6.07^{ns}	0.49	1293.7^{ns}	0.15	16.3^{ns}	0.82	0.659^{ns}	0.12	0.755^{ns}	0.90	18.7^{ns}	0.40	1164.7^{ns}	0.09	53.9^{**}	0.001
Error	9	6.39		610.4		37.9		0.286		2.577		16.2		444.6		5.5	

^{ns} and (**) are non-significant and significant at the probability level of 1%, respectively. df= degree of freedom, MS= mean square, and Pr>F= the *P*-value to determine whether to reject the null hypothesis.

TABLE 2. Mean comparison of effect of detopping and defoliation on maize traits.

Treatments	Ear husk weight (g/plant)	Ear weight (g/plant)	Seed number per column	Seed number per row	Cob length (cm)	Cob weight (g m^2)	Seed yield (g m^2)	100-seed weight (g)
T1	8.0a	123.3a	36a	13ab	17.5a	16.6a	105.9a	22.0a
T2	10.3a	87.9ab	39a	12b	17.3a	15.5a	72.3ab	15.6b
T3	5.7a	99.5ab	42a	14a	17.8a	18.5a	84.9ab	21.0a
T4	9.5a	83.1ab	43a	13ab	18.1a	19.9a	65.2ab	12.3b
T5	9.5a	98.9ab	40a	13ab	17.4a	17.4a	81.2ab	15.6b
T6	8.3a	60.6b	39.4a	12.8ab	16.6a	13.2a	47.3b	12.0b

T1, T2, T3, T4, T5, and T6 are control (intact plants), removal of leaves above the ear, removal of leaves below the ear, removal of all leaves, detopping (removal of stem above the ear), and detopping plus removal of leaves below the ear, respectively. Means followed by a different lowercase letter in the column are different at a 5% probability level by the Duncan test.

At this stage, the seed number is fixed and only the weight of the seeds changes, *i.e.*, the seeds remain small (Tollenaar & Daynard, 1987). Analysis of variance showed that detopping and defoliation did not affect seed number per ear row (Tab. 1). The mean comparison showed that removal of leaves below the ear produced more seeds per row than removal of leaves above the ear and there was no difference between other treatments (Tab. 2). The importance of upper leaves of the ear in grain filling is known (Xue *et al*., 2017). Because the lower leaves of the ear are older and in the shade, they may even act as a sink for photosynthetic compounds. Therefore, removing the lower leaves of the ear can eliminate the respiration of this part and provide the plant with more photosynthetic substances for seed filling.

Weight and length of cob

Analysis of variance showed that detopping and defoliation had no significant effect on weight and length of cob (Tab. 1). The mean comparison showed that there was no difference between defoliation and detopping treatments in cob length and weight (Tab. 2). The cob, which is the necessary element for seed growth, must be formed before seed formation. Apparently, at the seed milking stage, the cob reaches relatively full growth. Therefore, this trait should have fewer changes than other studied traits. Defoliation at the ear initiation stage reduced the weight and length of maize cob so that the highest defoliation intensity provides the lowest cob weight and length (Iledun & Rufus, 2017). The difference between the results of the present study and the results of Iledun and Rufus (2017) is related to the time of defoliation.

One hundred seed weight and seed yield

Analysis of variance of data showed that detopping and defoliation had a significant effect on the 100-seed weight of maize (Tab. 1). The mean comparison showed that intact plants (control) and plants with lower leaves removed had higher 100-seed weight than other treatments (Tab. 2). These results indicated the importance of the upper leaves of the ear in production of coarse grains. The lower leaves of the ear were of little importance in the production of photosynthetic materials at the seed milking stage due to their age and being in the shade, and even their removal at this stage does not have an adverse effect on the seed weight. But the upper leaves of the ear had a great effect on the seed filling because they are younger and exposed to more light. Maize is a C4 plant and its light requirement is high. The data of this study showed that the current photosynthesis of the leaf at the seed milking stage was still very important in seed filling. Grain weight loss due to the removal of upper leaves of the ear is shown in previous studies (Umashankara, 2007). Analysis of variance showed that detopping and defoliation had no significant effect on maize seed yield (Tab. 1). Mean comparisons showed that the detopping treatment with removal of lower leaves of the ear had lower seed yields than the control treatment and no difference was obtained between the other treatments (Tab. 2). These results showed that maize can be grown for both seed and forage production. In other words, harvesting green forage of the upper stem and leaves of the ear is possible without reducing the seed yield of maize. This can also be mechanized because removing the stem along with the upper leaves of the ear is easier than removing the leaves alone and produces less damage to the plants. Maize leaves and stems are green and palatable to livestock during the milking stage. At the same time, less maize residue remains on the field, which hinders tilling for the next crop. Considering that the detopping treatment with the removal of the lower leaves of the ear had lower seed yield than the control, it can be inferred that in a situation where only the upper leaves of the cob are removed during detopping, photosynthesis of other photosynthetic organs such as the lower leaves of the cob increases. On the other hand, by removing the upper stem and leaves of the ear, more light reaches the lower leaves of the ear, and these leaves increase photosynthesis. Therefore, detopping with removal of lower leaves of the ear had lower seed yield than the control, but the removal of lower leaves alone was not different from the control. The timing of the treatment is very important: if these treatments were applied at the time of pollination or earlier, detopping may also reduce the seed yield of the plants. Therefore, in areas that are faced with a shortage of forage at the milking stage, the upper stem and leaves of the ear can be harvested for livestock; and the dry seed can be harvested later with a combine. Studies show a more severe decrease in grain yield with defoliation at the early stages of plant growth than at the late stages (Ibrahim *et al*., 2010). Seed yield had a positive and significant correlation with all studied traits except seed number per row and ear husk weight (Tab. 3).

TABLE 3. Pearson correlation coefficients between studied traits in maize under detopping and defoliation.

	Ear husk weight (EHW)	Ear weight (EW)	Seed number per column (SNC)	Seed number per row (SNR)	Cob length (CL)	Cob weight (CW)	Seed yield (SY)	100-seed weight (100-SW)
EHW	1							
EW	0.485*	1						
SNC	0.677**	0.584*	1					
SNR	-0.150	0.295	0.021	1				
CL	0.833**	0.726**	0.817**	0.006	1			
CW	0.774**	0.827**	0.745**	0.167	0.905**	1		
SY	0.438	0.998**	0.548*	0.289	0.688**	0.792**	1	
100-SW	0.164	0.886**	0.204	0.316	0.393	0.544*	0.91**	1

* and ** are significant correlations at the probability level of 5% and 1%, respectively.

Laboratory experiment

Analysis of variance showed that detopping and defoliation had a significant effect on the germination percentage, radicle length, and vigor of maize seeds (Tab. 4). Mean comparisons showed that removal of leaves under the ear of mother plants produced lower seed germination percentages, radicle lengths, and seed vigor than other treatments (Tab. 5). Analysis of variance showed that leaf removal and detopping had no significant effect on coleoptile length (Tab. 4). Mean comparisons also showed that there was no difference between detopping and leaf removal treatments in terms of coleoptile length (Tab. 5). The results of the field experiment showed that the removal of the lower leaves of the ear had a 100 seed weight equal to 100 seed weight of control and more than other treatments. Therefore, in proportion to seed weight, these large seeds may not have received the nutrients or hormones necessary for germination on the mother plant through the lower leaves of the plant. The lower leaves usually mature earlier and send their nutrients to the seeds through re-mobilization. Because the only difference between removing the lower leaves of the ear to complete removal of the leaves or removing the lower leaves of the ear with detopping is the same difference in the 100 seed weight. The last two treatments have lost their lower leaves, but their 100-seed weight is less than that of removing the lower leaves of the plant. Although the treatment of removing the lower leaves of the ear had larger seeds than other treatments except for the control, its radicle was shorter. Therefore, the seeds of this treatment probably have dormancy, which may be due to hormonal imbalance. Seed germination is influenced by the relationship between stimulants and inhibitors of germination (Koocheki & Sarmadnia, 2011). Defoliation stress may have increased the production of seed germination

TABLE 4. Analysis of variance of the effect of detopping and defoliation of the mother plants on maize seed traits.

Source of variation	df	Germination		Coleoptile length		Radicle length		Seed vigor	
		MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F
Treatment	5	133.3*	0.049	0.905^{ns}	0.49	26.5*	0.02	61.04**	0.002
Error	12	44.4		0.981		6.8		7.61	

*, ** and ^{ns} are significant at the probability level of 5% and 1% and non-significant, respectively. df= degree of freedom, MS= mean square, and Pr>F= the *P*-value to determine whether to reject the null hypothesis.

TABLE 5. Mean comparison of effect of detopping and defoliation of the mother plants on maize seed traits.

[a] Treatments	Germination (%)	Coleoptile length (cm)	Radicle length (cm)	Seed vigor (% cm)
T1	100 a	7.2a	16.8a	2400a
T2	100 a	8.4a	19.9a	2830a
T3	83 b	7.6a	11.3b	1540b
T4	100 a	7.8a	16.1a	2390a
T5	100 a	8.4a	17.7a	2610a
T6	96 a	8.5a	18.7a	2630a

[a] T1, T2, T3, T4, T5, and T6 are control (intact plant), removal of leaves above the ear, removal of leaves below the ear, removal of all leaves, detopping (removal of stem above the ear), and detopping plus removal of leaves below the ear, respectively. [b] Means followed by a different lowercase letter in the column are different at a 5% probability level by the Duncan test.

stimulants during seed development on the mother plant, but because in most treatments the seed weight has also decreased, the proportion of these substances decreased. However, when removing the lower leaves of the ear, the seed weight did not decrease, so the ratio of germination stimulants to germination inhibitors decreased and seed dormancy increased. Some studies indicate that the defoliation of the mother plant does not affect the germination percentage of produced seeds (Koptur *et al*., 1996; Heidari *et al*., 2013). Part of the difference between the results of others and our results can be attributed to the application time of the treatments. Maize seed vigor had a positive and significant correlation with coleoptile and radicle length and germination percentages (Tab. 6). Coleoptile length, radicle length, and germination percentages could be regarded as the components of seed vigor.

TABLE 6. Pearson correlation coefficients between studied traits in maize seeds after detopping and defoliation of the mother plants.

	Germination percent (GP)	Radicle length (RL)	Coleoptile length (CL)	Seed vigor (SV)
GP	1			
RL	0.333	1		
CL	-0.068	0.489*	1	
SV	0.616**	0.925**	0.526*	1

* and ** are significant correlations at the probability level of 5% and 1%, respectively.

Conclusion

Maize detopping at the milking stage of the seed formation to produce green forage from the upper stem and leaves of the ear was not different in seed yield from intact plants. Therefore, the plants can be allowed to reach physiological maturity and also produce seeds, and the farmer can benefit from the seed production. Therefore, a suitable device could be designed to harvest the upper parts of the maize ear without damaging the plant. However, this could also be done manually. This type of dual-purpose crop can be a good way to feed livestock in times of critical need for fodder. At the same time, less maize residue remains on the soil surface, hindering the tillage operation for the next crop.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

MA conducted the experiment. HH designed and conducted the experiment and wrote the manuscript. All authors reviewed the manuscript.

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Taxonomic identification and diversity of effective soil microorganisms: towards a better understanding of this microbiome

Identificación taxonómica y diversidad de microorganismos efectivos del suelo: hacia un mejor entendimiento de este microbioma

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ABSTRACT

Soil microorganisms found in agricultural residues and the so-called efficient microorganisms (EM) are attractive for their potential applications and benefits in the bioremediation of complex ecosystems. However, the knowledge about *Who is doing what*?, as well as the trophic interaction in those communities that explain its benefits are limited; a better understanding of this microbiome is needed to explain its benefits. The objective of this research was to characterize the microorganisms isolated from two soil communities and the efficient microorganisms obtained in laboratory (EM16 consortium), taking into account physico-chemical characteristics, diversity, quantification, and taxonomic identification through microbiological and molecular techniques. A microbiological analysis was performed according to the morphological characteristics of the colonies as well as the study of the dynamics and taxonomic identification of the microbial populations through the TRFLP and Ion Torrent techniques. The diversity, dynamics, and taxonomic identification achieved in these studies showed the prospects for using these soil EM in bioremediation, considering the diverse metabolic pathways that these species have and their symbiotic interactive potential for biodegradation of lignocellulosic-resilient compounds. This study provides the first molecular characterization of the EM (EM16 consortium) and soil isolates from agricultural residues (sugarcane crop and bamboo field). The results suggest that the use of microbiological and molecular tools in a polyphasic approach allows the complete characterization of non-cultivable microorganisms that could contribute to sustainable environmental management and crop production.

Key words: molecular methods, soil microorganisms, agricultural residues, polyphasic approach.

RESUMEN

Los microorganismos del suelo que se encuentran en los residuos agrícolas y los llamados microorganismos eficientes (ME) son atractivos por su potencial aplicación y beneficios en la biorremediación de ecosistemas complejos. Sin embargo, el conocimiento sobre *¿Quién hace qué?*, así como la interacción trófica en esas comunidades que explican sus beneficios son limitados; se necesita una mejor comprensión de este microbioma que explique sus beneficios. El objetivo de esta investigación fue caracterizar los microorganismos aislados de dos comunidades de suelo y los ME obtenidos en el laboratorio (consorcio EM16), teniendo en cuenta las características físico-químicas, la diversidad, la cuantificación y la identificación taxonómica mediante técnicas microbiológicas y moleculares. Se realizó un análisis microbiológico según las características morfológicas de las colonias, así como el estudio de la dinámica e identificación taxonómica de las poblaciones microbianas mediante las técnicas TRFLP e Ion Torrent. La diversidad, dinámica e identificación taxonómica logradas en este estudio mostraron las perspectivas para uso de estos ME del suelo para la biorremediación, considerando las posibles rutas metabólicas que tienen estas especies y su potencial de interacción simbiótica para la biodegradación de compuestos lignocelulósicos resistentes. Este estudio proporciona la primera caracterización molecular de los ME (consorcio EM16) y de aislados del suelo procedentes de residuos agrícolas (cultivo de caña de azúcar y campo de bambú). Los resultados sugieren que el uso de herramientas microbiológicas y moleculares en un enfoque polifásico permite la caracterización completa de microorganismos no cultivables que podrían contribuir a la gestión ambiental sostenible y a la producción de cultivos.

Palabras clave: métodos moleculares, microorganismos del suelo, residuos agrícolas, enfoque polifásico.

Introduction

A wide diversity of microbial species interacts in natural ecosystems, contributing to waste degradation (Sun *et al*., 2013; Azman *et al*., 2015; Raja *et al*., 2017). The use of agricultural crop residues and agro-industrial waste would be attractive alternatives as a source of renewable energy

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because they close production cycles in a circular economy approach. However, despite the diversity of microorganisms involved in the decomposition of organic matter, the efficient biodegradation of these residues remains a challenge for bioethanol and biogas production due to the high percentages of lignocellulosic material, whose polymers are difficult to decompose (Cortés, 2016; Widjaja *et al*., 2016; Amin *et al*., 2017). The search continues, therefore, for microorganisms that naturally contain this lignocellulolytic capacity that can be exploited biotechnologically.

Teuro Higa developed in 1986 the technology of so-called efficient microorganisms (EM) (Allahverdiyev, Kırdar *et al*., 2011; Higa & Parr, 2013) that are isolated from undisturbed soils and ecosystems. EM technology has been applied in agriculture and forestry in more than 80 countries worldwide (Calero Hurtado, Pérez Díaz *et al*., 2019; Tanya & Leiva-Mora, 2019) with beneficial impacts. Microbial community compositions related to anaerobic EM consortia have not been characterized, but previous studies have identified phylogenetic groups, including Actinomycetales and fermenting fungi, photosynthetic bacteria, yeasts, and lactic acid bacteria (Namasivayam *et al*., 2014; Joshi *et al*., 2019).

Soil microorganisms, including EM consortia, play a crucial role in agricultural production and environmental decontamination, as they allow the availability of assimilable nutrients for plants during the biodegradation of organic matter, where phytohormones and secondary metabolites are released (López-Dávila, Gil Unday *et al*., 2017; Calero Hurtado, Quintero Rodríguez *et al*., 2019; Joshi *et al*., 2019; Tanya & Leiva-Mora, 2019; Castro *et al*., 2022). However, physico-chemical characteristics and microbiological composition of EM consortia should be clarified in order to understand their beneficial effects on agricultural production (Calero Hurtado *et al*., 2020). The beneficial uses of EM consortia include restoration of damaged soils after application of chemical fertilizers and pesticides (Alvarez *et al*., 2018), manufacturing of fermented fertilizers (biofertilizers) (Calero Hurtado, Quintero Rodríguez *et al*., 2019), processing of organic waste, and wastewater treatment (Allahverdiyev, Atilla *et al*., 2011). It has been widely reported that *Lactobacillus* species are prevalent in the EM consortia that is the basis for their use as a biofertilizer to enhance agricultural production (Blainski *et al*., 2018; Daranas *et al*., 2018; Quattrini *et al*., 2018; Naik *et al*., 2019; Abd El-Mageed *et al*., 2020; Muhialdin *et al*., 2020).

Therefore, an important strategy would be to determine not only the main physico-chemical characteristics but also which of them contribute to the improvement of soil properties, growth of plants, and other important environmental processes (Higa & Parr, 2013). The EM consortia works synergistically to release beneficial substances such as vitamins, hormones, enzymes, organic acids, bioactive minerals, and various antioxidants when they come into contact with the soil organic matter (Allahverdiyev, Kırdar *et al*., 2011). In addition, the EM consortia helped to improve the soil pH and increase the action of mineral nutrients, hormones, and other metabolites that accelerate decomposition of organic wastes and increase formation of biogas (López-Dávila, Gil Unday *et al*., 2017). Therefore, the hydrolytic and fermentative activities of the EM consortia could be potentially used as a pre-treatment or biostimulant in the agricultural waste bioconversion processes into bioenergy, for instance in bioethanol or biogas production.

The microbial dynamics of natural ecosystems (soils) as well as artificial ecosystems (anaerobic reactors), can be very complex due to the wide diversity of species and metabolic interactions required to degrade the organic matter. Microbiological characterization of these ecosystems using culture-dependent techniques are insufficient since only 0.1-10% of the bacteria in the environment can be cultivated (Nobu *et al*., 2015; Saw *et al*., 2015; Jiménez-Hernández *et al*., 2021). Hence, it is necessary to apply molecular methods that allow the analysis of taxonomic diversity and spatial structure of complex microbial communities to identify specific microbial populations in their natural habitat as well as to predict existing metabolic interactions.

Previous studies for microbiome monitoring employed molecular techniques based on 16S rRNA gene analysis such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (TRFLP), single-strand conformation polymorphism (SSCP) or capillary sequencing of selected genes by the Sanger method (Zoetendal *et al*., 2008). In complex microbial ecosystems, these methods provide an incomplete composition of the microbial community, showing only the existence of certain groups. The development of metagenomic approaches based on next-generation sequencing (NGS) has been one of the great advances in molecular biology (Zhou *et al*., 2015; Sanz & Köchling, 2019). The combination of different technologies seems to be imperative to reach the goals. Techniques complement each other, being the weaknesses of some and the strengths of others, especially when the study requires the identification of species to elucidate predominant metabolic pathways (Carabeo-Pérez *et al*., 2019).

Since 2014, in the Microbiology Laboratory of the University of Sancti Spíritus (Universidad de Sancti Spíritus "José Martí Pérez" - UNISS, Cuba), EM consortia have been developed, taking into account the methodology proposed by Teuro Higa and modified by Olivera-Viciedo *et al*. (2014). These studies were focused on the application of EM as a biostimulant to soils and plants. However, a detailed characterization of this microbial consortium has not been performed.

Agricultural soils in Cuba are currently in unfavorable conditions due to intensive cultivation and inadequate management (Febles-González *et al*., 2014). Most of the agricultural soils in this overexploited condition are located in the central region of the country that is a major producer of sugarcane (ONEI, 2020). These are brown carbonate soils with a pH between 5.0 and 8.5. In addition, they have high N, P and K content due to fertirrigation with wastewater from sugar factories and distilleries (Crespo *et al*., 2018). However, the diversity and dynamics of the microbial populations present in this soil and how they contribute to the degradation of agricultural residues were not found in previous studies.

There are also extensive areas of soil in Cuba that have very little anthropogenic action and are of special interest. These are generally forested areas covered by bamboo species (*Phyllostachys reticulata* [Ruprecht] Koch) and marabu (*Dichrostachys cinerea* [L.] Wight & Arnott). In the case of bamboo, they are generally found in forested areas near rivers or canals with alluvial soils (non-carbonate) (Oca-Risco *et al*., 2014), and marabu species can germinate in any type of soil. No further molecular characterization of these virgin ecosystems was done in this study.

The objective of this research was to characterize the microorganisms isolated from two soil communities (sugar cane crop and bamboo forest) and the efficient microorganisms obtained in the laboratory (EM16 consortium), taking into account physico-chemical characteristics, diversity, quantification, and taxonomic identification through molecular techniques.

Materials and methods

Obtaining isolates of EM consortium and agricultural soils

The EM consortium was produced in the Microbiology Laboratory of the University of Sancti Spíritus (UNISS, Cuba) pilot plant (20 L fermenter), following a methodology similar to that proposed by Olivera-Viciedo *et al*. (2014). Production was carried out as follows: 5 L of whey was mixed with 5 L of "C molasses" from a sugarcane factory. This mixture was added in layers to 10 kg of decomposing foliage and soil obtained from virgin forests undisturbed by human activity in the vicinities of the Zaza River of the Southern region of Sancti Spiritus province. Nine kg of corn flour was added to the mixture which was homogenized, compacted, and sealed in an anaerobic tank. The mixture (solid EM material) was kept at 28°C in the dark for 21-25 d for fermentation. After this time, 2 kg of this solid EM material was diluted in 18 L of distilled water containing 1 L of whey and 1 L of "C molasses" to obtain a total volume of 20 L of liquid bioproduct. The culture was kept hermetically sealed for 7 d under the same conditions described above, to avoid disturbing the fermentation process. The consortium obtained was called EM16.

To obtain the culture of soil microorganisms, 10 samples of 20 g were collected from two different agroecosystems: i) 10 cm of soil beneath the leaf floor of sugarcane field 7 d after harvest and ii) 10 cm of soil beneath the leaf floor of 5-year-old bamboo (*Bambusa vulgaris* Schrader) field (brown soils with carbonates and alluvial). Each soil sample was individually suspended in 350 ml of peptone cellulose solution (PCS medium: 0.1% yeast extract, 0.5% peptone, 0.2% $CaCO_3$, 0.5% NaCl, 0.5% cellulose, pH 7.0) for 7 d at 30°C in static culture flasks to isolate microbial strains capable of degrading lignocellulosic substrates.

Physico-chemical characterization of EM

The EM16 consortium and the soil isolates grown for 7 d in PCS were characterized by the following physico-chemical parameters: dry matter (DM), volatile solids (VS), ash, and pH, according to the 23rd Standard Methods for the Examination of Water and Wastewater (Baird *et al*., 2017).

Morphological analysis (growth in plates)

To identify the cultural bacteria, NA (nutrient agar) medium was used, employing surface spreading plate technique, with 0.1 ml of original samples and dilutions 10^{-1}, 10^{-2} and 10^{-3} as inoculum. Petri plates were incubated at 37°C for 96 h, after which it was possible to differentiate the colonies by their morphological characteristics in the culture.

DNA extraction

Genomic DNA was extracted from each sample (triplicate aliquots of 200 mg) using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, USA) according to the manufacturer's guidelines. The quantity and purity of DNA were determined photometrically using a NanoDrop 2000/2000c (Thermo Scientific, USA)

according to the manufacturer's guidelines. Isolated DNA was stored at -20°C until further processing.

Amplification of bacterial 16S rRNA gene sequences

Genes encoding for bacterial 16S rRNA (*rrs*) were amplified using the PCR primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') (Lane, 1991; Sipos *et al*., 2007) and 1492r (5'- TACGGYTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991; Després *et al*., 2007). The PCR reaction mix contained 1X *Taq* buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.4 µM of each primer, 1 µl (10 ng approximately) of template DNA and 1 U of *Taq* DNA polymerase for a final volume of 25 µl. The amplification was performed with an initial denaturation step for 1 min at 94°C, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 2 min and a final extension stage at 72°C for 3 min.

Chemicals and enzymes were provided by Fermentas (St. Leon-Rot, Germany), Promega (USA) Corporation and Molecular Biology (Thermo Scientific, USA). The correct length of the amplicons was verified by 1% agarose gel electrophoresis, using the GeneRuler® 1kb Plus DNA Ladder (Thermo Scientific, USA). To minimize the risk of any PCR bias, three parallel PCR reactions were performed for each of the three parallel DNA extracts obtained from each environmental sample. The concentration of the amplified 16S rRNA gene fragments was measured using the Qubit4® fluorometer (Invitrogen, Thermo Scientific, USA).

Terminal Restriction Fragment Length Polymorphism analysis (TRFLP)

For the bacteria diversity study, TRFLP analysis was carried out following the protocol proposed before by Rademacher *et al*. (2012). The primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') (Lane, 1991; Sipos *et al*., 2007), labeled at the 5' terminal end with Indodicarbocianin (Cy5) and 926r (5'-CCGTCAATTCMTTTRAGTTT-3') (Weisburg *et al*., 1991; Després *et al*., 2007) were used. The three independent amplification products, based on the same DNA template, were pooled and purified by applying the PureLink® PCR Purification Kit (Invitrogen, Thermo Scientific, USA). The concentration of purified products was measured using the Qubit4® fluorometer (Invitrogen, Thermo Scientific, USA).

After purification, the PCR products were digested with MspI and Hin6I following the manufacturer guidelines for each restriction enzyme. The digestion fragments were electrophoretically separated and detected by fluorescence using a GenomeLab® GeXP Genetic Analysis System (Beckman Coulter, Krefeld, Germany). The data obtained were analyzed using the DataConnect® software (Applied Biosystems interface-Thermo Fisher) considering the size calculation of the detected terminal restriction fragments (TRFs) based on the migration time of the applied size standard. All fragments with a sequence length between 60 and 640 bp were used for further analyses with the T-Rex software package, available online (http://trex.biohpc.org). The identification of "true" peaks by distinguishing baseline "noise" from signals of fluorescently labeled fragments, as well as the alignment of TRFs with a threshold of 0.5, was based on the evaluation of the peak height. In the last evaluation step, TRFs were visualized by their relative distribution, considering that TRFs with a relative abundance lower than 2% were removed from the analyses.

Bacterial 16S RNAr gene sequencing by Ion Torrent

To amplify the 16S hypervariable regions, the Ion 16S® Metagenomics Kit (Life Technologies, Thermo Scientific, USA) was used, on the Ion Torrent Personal Genome Machine® (PGM, Ion Torrent) platform, following the manufacturer guidelines. The remaining PCR reaction was purified using the PureLink PCR Purification Kit (Life Technologies, Thermo Scientific), and the concentration of the purified amplicons was assessed with the Qubit4 fluorometer and Qubit® dsDNA High Sensitivity Assay Kit (Life Technologies, Thermo Scientific). The DNA library construction, quantification, template preparation, and sequencing were developed according to the standard protocol, well-described by Adamiak *et al*. (2018). Metagenomic data was evaluated using the Ion Reporter® software (Invitrogen, Thermo Scientific, USA), showing the taxonomic distribution of the target microbial community, based on the software database. The raw data of sequences have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the Bioproject accession number PRJNA764521 (http://www.ncbi.nlm.nih.gov/bioproject/764521).

Analysis of the diversity and organization of the microbial communities

The bacteria richness (Rr) considering more than 1% of abundance was calculated by the methodology proposed by Marzorati *et al*. (2008), based on the number of OTUs obtained in each electropherogram or cluster. The Shannon-Weaver diversity index (H) (Shannon & Weaver, 1963) was used to evaluate and compare this diversity, and the evenness index (Pielou index, H/Hmax) of the microbial community in each sample was calculated as the quotient of the Shannon index and the potential number of species

in the sample (Hmax). This index describes the uniformity of the distribution of individuals in the community (Pielou, 1966).

Results and discussion

Physico-chemical characterization of EM16

The EM16 consortium presented low contents of dry matter (DM), which ranged from 0.90% to 1.57% for fresh matter (FM). Also, more than 50% of DM are volatile solids (VS) (0.51-0.95 of VS (% FM)), and 56.8-60.67 VS (% DM) is attributable to the biomass content. During the 7 d anaerobic fermentation process, biomass production is limited due to the anaerobic condition (Madigan *et al*., 2019). In addition, the dilution during the EM preparation described above (EM and agricultural soils isolates obtention) could contribute to this measure. Dai *et al*. (2016) also described a low organic matter content in EM bioproducts. The EM16 consortium had acid properties (pH = 4.31-4.46), agreeing with other reports where the pH ranged from 3.1 to 4.7 (López-Dávila, Calero Hurtado *et al*., 2017; Calero Hurtado *et al*., 2020). In the EM16 consortium, the low pH might be due to: i) the use of substrates with low pH like whey and molasses (Núñez-Caraballo *et al*., 2019; González-Herrera *et al*., 2021) and ii) the volatile organic acids formed during the fermentation processes (Xiong *et al*., 2012; Dai *et al*., 2016). In addition, the low pH reported by Calero *et al*. (2020) could be linked to the assimilation of some important mineral nutrients, such as N, P, K, and Ca that help to improve soil properties and plant growth. Also, knowledge of this characterization could help to identify substances important for use in agricultural production and other environmental practices.

Morphological analysis

The EM16 sample did not showed colony formation on plate growth (in aerobic condition). This result may be related to the lower relative abundance of the bacterial species in this sample with respect to the other isolated soil or that these microorganisms were not capable of growing under these conditions. EM consortium is frequently composed mainly of *Lactobacillus* species (Tanya & Leiva-Mora, 2019) that are facultative anaerobic bacteria difficult to grow in a Petri dish. On this basis, Calero *et al*. (2020) reported that the EM consortium is composed of some species of *Lactobacillus bulgaricum, Bacillus subtilis* and *Saccharomyces cerevisiae* that represent microbial diversity. Therefore, in the EM16 consortium, some of these species should also be identified.

In contrast, in the undiluted soil isolate samples, bacterial growth exceeded 300 colonies after 24 h of incubation; those plates are reported as uncountable. However, the diluted samples (10^{-2} and 10^{-3}) showed colony growth. After the incubation period, it was possible to differentiate the bacteria colonies according to the different morphological characteristics in the culture.

In sugarcane soil isolates, bacterial colonies were observed mostly in a circular, irregular, or rhizoid shape, with rounded, wavy, or lobed edges and different surfaces such as flat, convex, accumulated, and umbilical. Bamboo soil isolated colonies with irregular or circular shapes with flat surfaces and wavy edges predominated.

The observed morphological diversity demonstrated that a great variety of metabolic pathways may also exist in soil microorganisms. This physiological diversity allows the microbial community to maintain the balance of agroecosystems. To guarantee soil health, decomposition of organic matter, crop yields, availability of the nutrients, etc., the microorganisms establish a range of ecological interactions that make the microbial populations develop or not (Vilatuña, 2019). These relationships are poorly studied and should be considered in research on soil microbiota (Goncharov & Tiunov, 2014; Jacoby *et al*., 2017; Erktan *et al*., 2020). When the microbial culture is performed, the growth of the entire community will not be observed because, perhaps, the growth of some species depends on others and once isolated in a different culture medium it does not grow; perhaps the new culture medium does not provide the necessary nutrients or perhaps the dilution factor used affected growth.

Traditional phenotypic identification schemes based on "observable" colony characteristics, such as morphology, development, and biochemical and metabolic properties, are essential as diagnostic methods for microbial community studies. However, these are insufficient since, from these methods, it is only possible to observe the growth of those facultative anaerobic microorganisms; but when it comes to environmental samples or samples taken from anaerobic systems, the use of other molecular techniques that contribute to quantification and microbial identification is recommended. It is important to determine exactly the relationship of the form and physiology of those microorganisms because it would help to understand their importance and function within the processes and how to manage them better.

Bacterial community structure and dynamics

In EM16 samples, 49 terminal fragments belonging to the Bacteria Domain were detected by TRFLP analysis.

Terminal fragments (TFs) with 62, 181, 404, 476, 546, 571, 572, 575, 579, 582, 585, 597, 600, and 610 bp showed high relative abundance of more than 2% (Fig. 1). From the sugarcane straw soil isolated samples, 94 TFs were retrieved, where the fragments of 192, 194, 204, 225, 276, 282, 451, 453, 472, 505, 508, 525, 546, 571, 575, 585, and 597 bp showed more than 2% relative abundance. From the bamboo soil isolated samples, 83 TFs were obtained, where the TFs of 152, 192, 194, 204, 225, 276, 281, 282, 451, 453, 472, 505, 508, 525, 546, and 579 bp showed a relative abundance more than 2% (Fig. 1). When the total communities are explored, including the TFs with a relative abundance below 2%, 13 TFs were found in both isolated soil samples (*i.e.*, 192, 194, 204, 225, 276, 282, 451, 453, 472, 505, 508, 525, and 546 bp). That is, probably, because they are endemic microbiota from these niches (brown carbonate soils).

According to Ion Torrent identification results from the Bacteria Domain (Tab. 1), five phyla were detected in the samples studied.

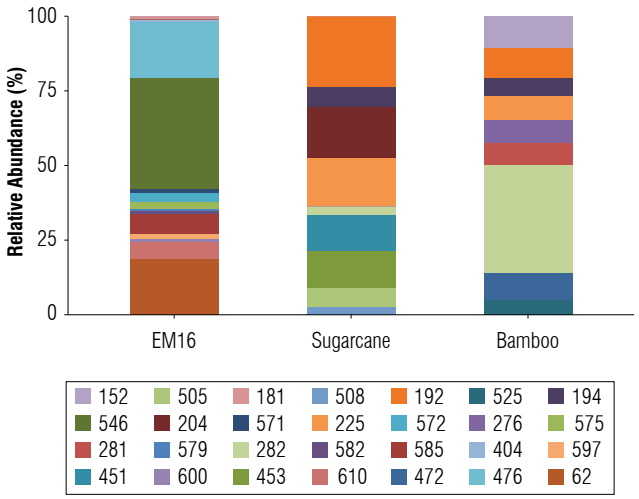

FIGURE 1. Relative abundance of bacteria terminal restriction fragments and their sizes (bp) in samples of EM16 and soil isolates (sugarcane crop and bamboo field). The relative abundance of each fragment was determined based on the height of its maximum of fluorescence in relation to the total of the heights of all the peaks detected in the sample.

TABLE 1. Taxonomic distribution of the bacteria domain in samples of EM16 consortium and soil isolates identified by Ion Torrent technique.

Phylum	Class	Order	Family	% of mapped reads in EM16	% of mapped reads in sugarcane	% of mapped reads in bamboo
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	0.09	0	0
			Propionibacteriaceae	0.16	0	0
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	0	0	0.36
			Porphyromonadaceae	0	0	15.53
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	0	1.86	0.42
	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	0.11	0	0
			Sphingobacteriaceae	0	0.2	1.68
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	0	0.38	0
			Bacillaceae	0	1.59	10.62
			Sporolactobacillaceae	92.25	0	0
			Paenibacillaceae	0	2.98	2.75
		Lactobacillales	Lactobacillaceae	3.25	0	0
	Clostridia	Clostridiales	Christensenellaceae	0	0	0.21
			Clostridiaceae	1.62	22.13	16.22
			Clostridiales Family XI. Incertae Sedis	0	4.36	3.17
			Eubacteriaceae	0	0.63	0
			Lachnospiraceae	0	1.68	0.78
			Oscillospiraceae	0	0	0.1
			Peptococcaceae	0	2.36	2.69
			Peptostreptococcaceae	0	12.76	9.05
			Ruminococcaceae	0.08	17.79	8.09
			unclassified Clostridiales	0	0	0.91
	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	0	0	0.33

to be continued

Phylum	Class	Order	Family	% of mapped reads in EM16	% of mapped reads in sugarcane	% of mapped reads in bamboo
Planctomycetes	Planctomycetia	Candidatus Brocadiales	Candidatus Brocadiaceae	0.05	0	0
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	0.08	9.41	0.47
		Rhizobiales	Bradyrhizobiaceae	0.1	0	0
		Rhodospirillales	Acetobacteraceae	0.32	0.45	0.92
			Rhodospirillaceae	0	5.44	5.87
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	0	0	0
			Burkholderiaceae	1.46	0	0
			Comamonadaceae	0.16	0.8	2.34
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	0.06	0	0
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	0	0	0
		Enterobacteriales	Enterobacteriaceae	0.1	7.77	6.39
		Pseudomonadales	Moraxellaceae	0	0.93	1.5
			Pseudomonadaceae	0.04	4.39	8.3
		Xanthomonadales	Xanthomonadaceae	0.08	0.22	0
Total				100.00	98.13	98.7

Almost 97% of the microbial community in EM16 was represented by the Firmicutes phylum. Other species were detected belonging to the phyla Proteobacteria (2.4%), Actinobacteria (0.25%), Bacteroidetes (0.11%), Planctomycetes (0.05%) (Tab. 1 and Fig. 2). A similar composition of microbial structure was identified in a thermoacidophilic EM consortium with six predominant phyla (Proteobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Actinobacteria, and Acidobacteria) (Henry *et al*., 2020). The result of this study showed that the microbial community in the EM16 consortium is favored by the formation of *Sporolactobacillus* species (family Sporolactobacillaceae 92.25%) (Fig. 2).

The members of *Sporolactobacillus* genus are catalase-negative, microaerophilic, spore-forming, homofermentative, lactic acid-producing species and require mainly carbohydrate for growth (Chang *et al*., 2008). Although *Sporolactobacillus* species are typically isolated from soil, they are occasionally isolated from fermented or spoiled foods (Yanagida *et al*., 1987; Fujita *et al*., 2010). The presence of these species was expected in the EM16 consortium due to the composition of the substrate (made in part by whey) considering that *Sporolactobacillus*, as well as *Lactobacillus*, are common in all types of dairy products (Madigan *et al*., 2019). The presence of lactic acid bacteria related to the breakdown of cellulolytic and lignified organic materials has also been reported in EM (Salminen & von Wright, 2004; Joshi *et al*., 2019).

The families Lactobacillaceae (3.14%) and Clostridiaceae (1.62%) were less abundant in this consortium (Fig. 2). The presence of *Clostridium* species is essential for cellulose degradation (Madigan *et al*., 2019), therefore they could be used to improve the degradation of lignocellulosic residues. On the other hand, *Lactobacillus* spp. have potential effects for agricultural needs, so they can enhance soil properties since they show an antagonistic effect against different phytopathogenic agents in the soil, mainly due to the decrease in pH, production of peptides with antimicrobial activity such as class I bacteriocins, and nisin that is very active against gram-positive bacteria (Tanya & Leiva-Mora, 2019). In addition, *Lactobacillus* spp. can enhance the trophic interactions (Quattrini *et al*., 2018; Naik *et al*., 2019), biotic and abiotic responses (Tsuda *et al*., 2016; Blainski *et al*., 2018; Abd El-Mageed *et al*., 2020; Muhialdin *et al*., 2020), and plant growth and productivity (Daranas *et al*., 2018; Quattrini *et al*., 2018). The presence of these species is, therefore, essential in the EM16 consortium to enrich the soil microbiome and its functionality during the cycle of assimilation and de-assimilation of nutrients.

The taxonomic groups identified in EM16 are similar to other EM consortia (Alvarez *et al*., 2018; Naik *et al*., 2019); however, the diversity and richness of species depend on the environmental conditions and the substrate from which they come. The EM16 consortium could be an efficient alternative to use in sustainable agriculture since it can promote microbial consortia interaction in the soil and

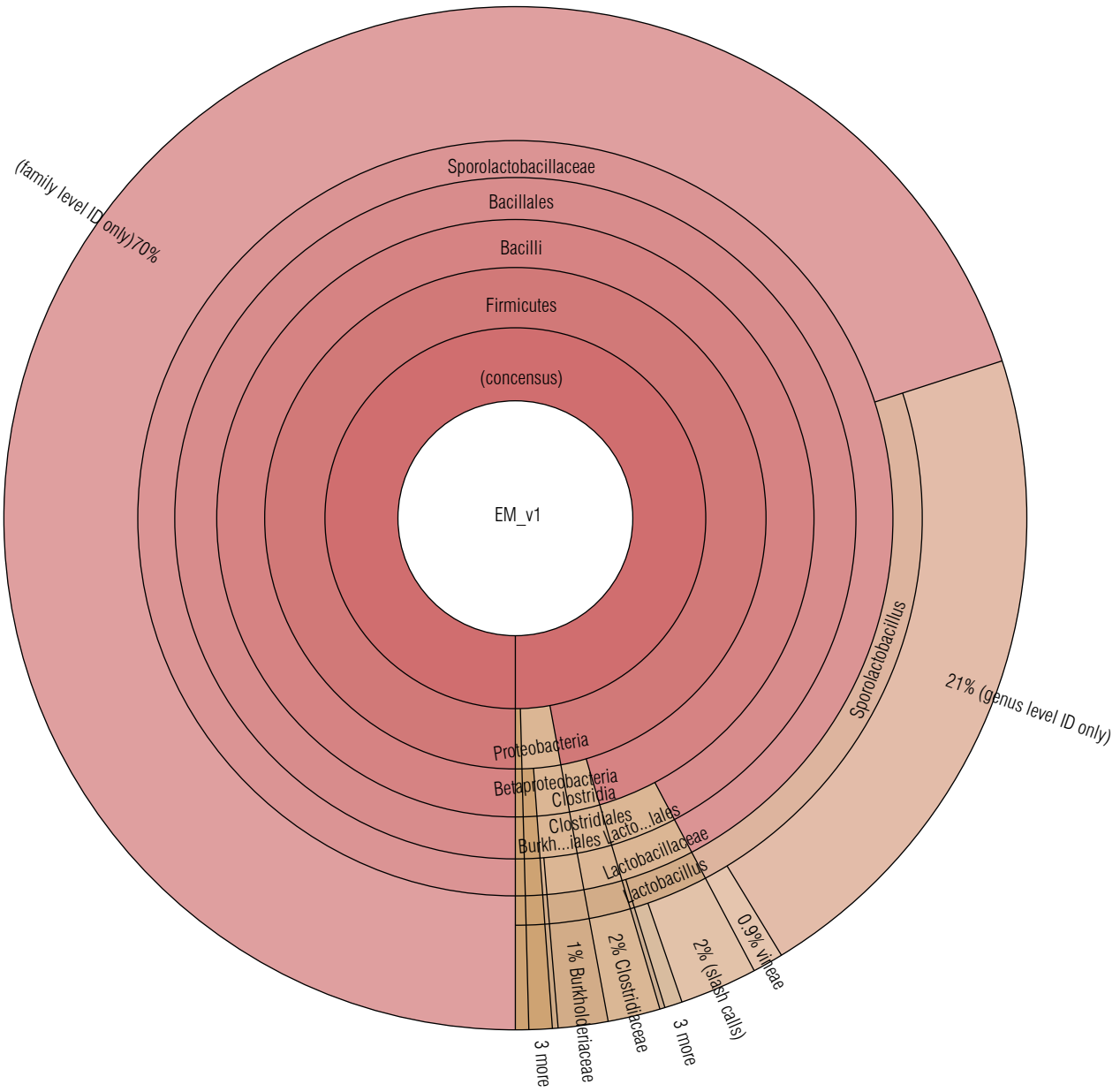

FIGURE 2. Krona plot of identified bacteria in EM16 sample, through the Ion Torrent technique, by using the Ion Reporter® software. The Krona plot shows the taxonomic distribution from phyllum to species level and the associated abundance based on the percentage of mapped reads taking into acount the NGS repositories based on NCBI database.

degrades organic components at the same time it supports a better micro or macronutrient assimilation by roots.

The sugarcane soil sample was represented by the Firmicutes (67.06%), Proteobacteria (29.64%), and Bacteroidetes (3.38%) phyla (Tab. 1). The most abundant families were Clostridiaceae (22.13%), Ruminococcaceae (17.59%), and Peptostreptococcaceae (12.76%) (Fig. 3).

A similar distribution of phyla was detected in bamboo soil samples, represented by Firmicutes (55.52%), Proteobacteria (26.28%), and Bacteroidetes (18.17%) (Tab. 1). The most abundant families were Clostridiaceae (16.22%), Porphyromonadaceae (15.53%), and Bacillaceae (10.62%) (Fig. 4).

In the sugarcane and bamboo soil samples (Figs. 3-4), where bacterial species belonging to the phylum Firmicutes

FIGURE 3. Krona plot of identified bacteria in sugacane isolated soil sample, through the Ion Torrent technique, by using the Ion Reporter® software. The Krona plot shows the taxonomic distribution from phyllum to species level and the associated abundance based on the percentage of mapped reads taking into acount the NGS repositories based on the NCBI database.

predominated, more than 10 different species of the *Clostridium* genus were identified to the species level. These are fermentative bacteria, strictly anaerobic with great metabolic diversity, gram-positive, endospore-forming, generally fermenting sugars or amino acids usually found in soil samples (Madigan *et al*., 2019). Besides, in the Clostridiales order, with an abundance greater than 1.5% the genera *Ruminococcus* and *Sedimentibacter* have been identified in both soil samples.

Ruminococcus species are defined as strictly anaerobic, gram-positive, non-motile cocci that do not produce endospores and require fermentable carbohydrates for growth (Goodfellow *et al*., 2009). They have been detected

FIGURE 4. Krona plot of identified bacteria in bamboo isolated soil samples using the Ion Torrent technique, using the Ion Reporter® software. The Krona plot shows the taxonomic distribution from the phyllum to species level and the associated abundance based on the percentage of mapped reads taking into acount the NGS repositories based on the NCBI database.

in greatest abundance in samples taken from herbivorous and omnivorous animal hosts, but a few have been found in environmental sources, generally in host-associated samples (La Reau *et al*., 2016). Some species are cellulolytic, playing an important role in the degradation of particulate substrates (Flint *et al*., 2008). Other members of family Ruminococcaceae are numerically abundant in the human intestinal tract, like bacterial species capable of degrading crystalline cellulose (Chassard *et al*., 2012; Morais *et al*., 2016). Others are non-cellulolytic and use polysaccharides like resistant starches (Ze *et al*., 2012) or selectively use various plant hemicelluloses (Wegmann *et al*., 2014).

Species of the genus *Sedimentibacter* have been commonly isolated from anaerobic microbial communities (Breitenstein *et al*., 2002; Woo *et al*., 2004; Imachi *et al*., 2016). Cells are slightly curved rods, motile, gram-positive and spores might be formed. Growth is strictly anaerobic and

requires yeast extract and is supported by the fermentation of pyruvate or amino acids in a Stickland-type reaction. Hydrogen is not produced and carbohydrates are not fermented (Breitenstein *et al*., 2002).

Members of the genus *Pseudomonas* (class Gammaproteobacteria) were also identified in soil samples from sugarcane and bamboo. These species show great metabolic diversity and consequently are widely distributed in nature. Some species are pathogenic for humans, animals, or plants. The metabolism of *Pseudomonas* is typically respiratory with oxygen as the terminal electron acceptor; but some species can also use nitrate as an alternative electron acceptor and carry out oxygen-repressible denitrification (dissimilatory reduction of nitrate to N_2O or N_2), allowing growth to occur anaerobically (Palleroni, 2015). They are typically oxidase and catalase-positive, do not form spores, with no gas formation from glucose, and perform catabolism of the carbohydrates by the alternative Entner-Doudoroff pathway and the cycle of tricarboxylic acids (Madigan *et al*., 2019).

The microbial diversity found in bamboo and sugarcane soil isolates could be used to enrich the EM16 consortium to achieve a more complete bioproduct (with a broader metabolic capacity).

The phylogenetic analysis coincides with the results of the TRFLP, where some fragments were detected in both soil samples. In general, species richness, diversity and the evenness index allows a comparison of isolated soil and EM16 consortium samples as shown in Table 2, considering the Ion Torrent and TRFLP analyses results. As expected, the richness, diversity, and Pielou index were higher in isolated soil samples compared to EM16 samples (Tab. 2) due to the dynamic conditions and the diversity of nutrients in these natural ecosystems. Despite the low diversity in the EM16 samples, it is worth noting the uniformity of the community with a coefficient of over 0.5.

TABLE 2. Richness, diversity, and evenness indexes calculated for each sample considering the Ion Torrent and TRFLP (in parenthesis) analysis results. Species with a relative abundance more than 1% were considered.

Index	EM16	Sugarcane	Bamboo
Rr	4 (8)	25 (9)	22 (9)
H	0.73 (1.64)	2.89 (2.01)	2.86 (1.96)
(H/Hmax)	0.52 (0.79)	0.90 (0.92)	0.92 (0.89)

Rr=species richness, H=diversity index, H/Hmax=evenness index.

Despite the differences among the indices with respect to the richness, the tendency with respect to diversity and the evenness is similar for both techniques. That is why the authors consider that TRFLP is more useful for diversity and dynamic evaluation and Ion Torrent is used for deeper identification until species level.

Conclusions

This study provides a comprehensive morphological and molecular characterization of the efficient microorganisms (EM16) and sugarcane and bamboo soil samples. The diversity, dynamic and taxonomic identification achieved in this study for the EM16 showed the perspective for using these consortia for bioremediation, considering the wide metabolic pathway including the presence of key species like *Lactobacillus* sp. with high potentials for biodegradation of lignocellulosic resilient compounds. Future studies may be aimed at evaluating the mixture of these microorganisms to obtain a microbial consortium with a higher metabolic capacity and increased effectivity in its agricultural or environmental use compared to EM already studied. The use of microbiological and molecular tools under polyphasic approaches allows the completed characterization of non-cultivable microorganisms reported for the first time from an efficient microorganism consortium.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

JJ and CH formulated the overarching research goals and coordinated the research activity planning and execution. DH and PA provided the study materials, reagents, laboratory samples, instrumentation, computing resources, and other analysis tools. ACP carried out the laboratory activities, applied statistical, mathematical, computational, and other formal techniques to analyze study data. ACP and JJ conducted the research and investigation process, specifically performing the experiments. ZG verified the overall replication/reproducibility of results. All authors

contributed to the writing of the manuscript and carried out the revision of the manuscript.

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Use of reduced Bokashi doses is similar to NPK fertilization in iceberg lettuce production

El uso de dosis reducidas de Bocashi es similar a la fertilización NPK en la producción de lechuga iceberg

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ABSTRACT

The aim of the study was to compare horticultural variables of iceberg lettuce using two Bokashi compost doses, alone and in combination, with the bioactivator Penergetic (Penergetic International AG®) against standard mineral fertilization (NPK) in three crop cycles. Experiments were conducted in a plastic greenhouse at the Universidade Estadual de Londrina, Brazil (548 m a.s.l.). The treatments were: negative control (water only); positive control (NPK, 4-14-8); Penergetic alone; Bokashi doses 5 g and 10 g/plant with or without a combination with Penergetic. Lettuce plants were grown in pots filled with soil. Commercial biomass (CM), head diameter (HD), plant height (PH) and chlorophyll index (CI) were evaluated. In the three cycles tested, the Bokashi 10 g/plant, Bokashi 10 g/plant + Penergetic, and NPK treatments surpassed the control. The studied variables (CM, HD, PH and CI) in the lower Bokashi dose treatment were also higher than controls but lower than higher Bokashi doses and NPK. Penergetic increased the CM in the lower Bokashi dose treatment just in the first production cycle, probably due to the poor organic matter content in the soil. The treatment Bokashi 10 g/plant improved significantly the lettuce horticultural variables vs. control treatments and was similar to chemical fertilization in two of three crop cycles.

Key words: chemical fertilizer, *Lactuca sativa*, organic fertilizer, Penergetic.

RESUMEN

El objetivo de este trabajo fue comparar las variables hortícolas de la lechuga iceberg utilizando dos dosis de compost Bokashi, solo y en combinación, con el bioactivador Penergetic (Penergetic International AG®) frente a la fertilización mineral estándar (NPK) en tres ciclos de cultivo. Los experimentos se realizaron en un invernadero de plástico en la Universidade Estadual de Londrina, Brasil (548 msnm). Los tratamientos fueron: control negativo (agua solamente); control positivo (NPK, 4-14-8); Penergetic solo; Bokashi en dosis de 5 y 10 g/planta con o sin combinación con Penergetic. Las plantas de lechuga se cultivaron en macetas llenas de tierra. Se evaluaron biomasa comercial (BC), diámetro de cabeza (DC), altura de planta (AP) e índice de clorofila (IC). En los tres ciclos evaluados, los tratamientos Bokashi 10 g/planta, Bokashi 10 g/planta + Penergetic y NPK superaron al testigo. En general, las variables estudiadas (BC, DC, AP y CI) en el tratamiento de menor dosis de Bokashi también fueron más altas que el control pero menores que con la dosis más alta de Bokashi y NPK. Penergetic aumentó la BC en el tratamiento con dosis más bajas de Bokashi solo en el primer ciclo de producción, probablemente debido al bajo contenido de materia orgánica en el suelo. El tratamiento Bokashi 10 g/planta mejoró significativamente las variables hortícolas de la lechuga frente al tratamiento de control y fue similar a la fertilización química en dos de los tres ciclos de cultivo.

Palabras clave: fertilizante químico, *Lactuca sativa*, abono orgánico, Penergetic.

Introduction

Leafy vegetables are important in the human diet, providing vitamins and mineral nutrients (Kumar *et al.,* 2020). In 2019, lettuce (*Lactuca sativa* L.) and chicory (*Cichorium intybus* L.), another important leafy vegetable, reached 29 million t of production in 1.31 million ha worldwide; the top five producers were China (16.3 million t), United States (3.7 million t), India (1.3 million t), Spain (1.01 million t), and Italy (0.76 million t) (Faostat, 2021).

In high input agriculture, the risk of environmental contamination by overuse of pesticides and fertilizers and soil salinity is high. In greenhouses, the nitrogen losses are mostly by leaching and nitrous oxide; the main cause is the excessive use of nitrogen-based fertilizers (Qasim *et al.,* 2021). This previous meta-analysis suggests that fertilization may be adjusted because the current proposed doses, often over N 1,500 kg ha^{-1}, could be replaced by about N 762 kg ha^{-1} without reducing yields (Qasim *et al.,* 2021).

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Studies regarding adjusting fertilizer use as well as applying integrated fertilization management are important for increasing agriculture/horticulture sustainability.

Bokashi compost is a soil amendment that provides organic matter rich in microorganisms and mineral nutrients (Scotton *et al.,* 2017; Hata *et al.,* 2020). The use of Bokashi together with other amendments in organic agriculture should be tested to verify possible additive and even synergetic effects (Quiroz & Céspedes, 2019). Several formulae and inoculum sources have been proposed according to local availability of the materials. These variations reflect the results of the performance of the compost as a fertilizer and soil conditioner (Scotton *et al.,* 2017). A variety of studies demonstrated an increase of plant yield and fresh or dry biomass by using Bokashi in vegetables such as arugula (*Eruca sativa* L.), cabbage (*Brassica oleracea* L. var. Capitata), lettuce, radish (*Raphanus sativus* L.), strawberry (*Fragaria* × *ananassa* Duch.), and tomato (*Solanum lycopersicum* L.) (Goulart *et al.,* 2018; Hata *et al.,* 2019; Sarmiento *et al.,* 2019; Xavier *et al.,* 2019; Hata, Paula *et al.,* 2021; Hata, Ventura *et al.,* 2021). Approximately 20 g/plant of Bokashi compost is suggested for lettuce and other leafy vegetable production (Trani *et al.,* 2014).

The combined use of organic fertilizers may reduce the amount of fertilizer use by a synergistic effect between the sources and an increase of microbial community composition and, thus, increase plant development (Gao *et al.,* 2020). Instead of increasing the fertilizer dose, a lower dose of mixed fertilizers might produce a similar result of plant yields. The association of Bokashi with rock phosphate increases chlorophyll index and dry matter in parsley (*Petroselinum crispum* [Mill.] Fuss) (Maass *et al.,* 2020). Penergetic-K and Penergetic-P activators are produced based on bentonite and molasses that are processed by a technology in which bentonite clays are subjected to the application of electric and magnetic fields (Artyszak & Gozdowski, 2020). The manufacturer states that these products increase the photosynthetic efficiency of plants (Penergetic-P) and improve the performance of organic matter from the decomposing organisms of the soil (Penergetic-K) (Artyszak & Gozdowski, 2020). A series of experiments demonstrate an increase in the production of snapbeans (*Phaseolus vulgaris* L.), soybean (*Glycine max* L. Merr.), and tomato by Penergetic application (Brito *et al.,* 2012; Souza *et al.,* 2017; Hata, Ventura *et al.,* 2021).

To date, we have not found reports on the comparisons of dosages and mixtures with other inputs for lettuce. Hence, this research deals with the evaluation of reduced Bokashi compost doses, alone or in association with the bioactivators Penergetic P and K, compared with the standard NPK treatment on lettuce production variables for three consecutive production cycles.

Materials and methods

The bioassay was carried out in a greenhouse at the Universidade Estadual de Londrina, Brazil (23°20'28''S, 51°12'34''W at 548 m a.s.l.) at an average temperature of 28°C. Three experiments were conducted in pots with a capacity of 5 dm^3, filled with soil classified as very clayey Ferralsol (Santos *et al.,* 2018), collected in the 0-20 cm soil layer and mixed with sand in the proportion (v:v) of 3:1. The chemical analysis of the soil obtained was pH_{H2O} = 5.10, P = 6.00 mg dm^{-3}, K^+ = 0.75 $cmol_c$ dm^{-3}, Ca^{+2} = 1.35 $cmol_c$ dm^{-3}, Mg^{+2} = 1.20 $cmol_c$ dm^{-3}, Al^{+3} = 0.0, H^+Al^{+3} = 2.10 $cmol_c$ dm^{-3}, and organic matter (%) = 1.80. A dose of 10 g per pot of calcitic limestone (lime) was used to elevate the base saturation percentage to 70% one month before the experiments began.

The recommended dose of Bokashi previously suggested was 20 g/plant (Trani *et al.,* 2014). The treatments used in the present experiments had a reduction of 50% and 75% of the Bokashi recommended dose. Hence, the treatments were as follow: control (water only); Penergetic (Pen); Bokashi 5 g/plant (Bok 5 g); Bokashi 5 g/plant + Penergetic (Bok 5 g+Pen); Bokashi 10 g/plant (Bok 10 g); Bokashi 10 g/plant + Penergetic (Bok 10 g+Pen), and NPK on formula 4-14-8 (Heringer, Paulinia, Brazil) at 6 g/plant (3 g applied one week before and 3 g applied two weeks after transplanting). Treatments were applied in the pot, before and after transplanting.

We prepared Bokashi using maize, wheat, rice, and soybean brans and the composting accelerator Embiotic® (Korin Meio Ambiente e Agropecuária, Brazil) that contains a mixture of *Lactobacillus plantarum* 104 UFC/mL and *Saccharomyces cerevisiae* (Korin, 2020). The previous materials were mixed every day in the shade under ambient conditions (25°C). When there was no odor and the appearance of the mix was homogeneous, the Bokashi was ready to use.

After complete preparation, Bokashi chemical analyses showed N as 37.67 g kg^{-1}; P as 14.36 g kg^{-1}; K as 21.01 g kg^{-1}; Ca as 12.00 g kg^{-1}; and Mg as 8.8 g kg^{-1}. Bokashi was applied over the soil one week before transplanting.

Penergetic-K and Penergetic-P (Penergetic International AG Company) are produced from bentonite clays subjected to the application of electric and magnetic fields (Artyszak & Gozdowski, 2020). One week before transplanting,

Penergetic K (1.5 g L^{-1}) was applied over the soil by drenching. Two weeks after transplanting, Penergetic P (1.5 g L^{-1}) was foliar sprayed.

Lettuce seedlings, cv. Amelia, were purchased from a commercial nursery. Transplant and harvest were achieved on March 02 and April 10, 2018 (39 d after transplanting - DAT); April 25, 2018 and June 07, 2018 (43 DAT); and June 20, 2018 and August 21, 2018 (61 DAT) for first, second, and third cycles. Phytosanitary measures were not necessary during the three plant cycles. Irrigation was performed with drippers for 10 min, three times a day.

All the experiments were conducted in accordance with the organic vegetable production Rule 10.831/2003 with inputs allowed by Normative proceeding 46/2011, regulated by Normative proceeding 17/2014 (Brazil-Mapa, 2014). The only exception was the NPK fertilizer that was used as a positive control.

The measured variables included commercial mass (CM in grams), head diameter (HD cm), plant height (PH cm), and a chlorophyll index (CI = Falker Index). CM was determined by weighing fresh biomass after discarding external leaves. HD and PH were also measured after discarding external leaves. CI was determined by using an indirect chlorophyll measure, Falker ClorofiLOG® 1030 by using three records on the head of the plant 25 DAT.

The experimental design was completely randomized with five replicates. One plant per pot was used as an experimental unit.

Means obtained were submitted to the variance homogeneity test (Bartlett test) and normality test (Shapiro-Wilk test). Once the assumptions were met, data were submitted for analysis of variance and the means compared by the Scott-Knott test ($P<0.05$) using the *R* software "package Easyanova". A Pearson correlation was performed between the lettuce commercial mass and the Falker Index ($P<0.05$).

Results

The treatments Bok 5 g, Bok 5 g+Pen; Bok 10 g, Bok 10 g+Pen, and NPK, with the exception of Penergetic alone, influenced at least one variable, when compared to the control (Fig. 1A-B).

FIGURE 1. Experiments of iceberg lettuce with reduced Bokashi compost doses, alone or in association to the bioactivators Penergetic, compared with the standard NPK (4-14-8) treatment. A = first cycle of growth; B = second cycle of growth.

In the first production cycle, higher values of CM were obtained in the treatments Bok 5 g+Pen, Bok 10 g, Bok 10 g+Pen, and NPK than in Bok 5 g. The Bok 5 g values were higher than those obtained in the control and Pen (Tab. 1). For HC, H, and CI in the treatments Bok 5 g, Bok 5 g+Pen, Bok 10 g, Bok 10 g+Pen, and NPK, higher values were obtained than in the control and Pen. Penergetic, when used alone, did not lead to significant increments in the assessed variables. The Penergetic application associated with Bokashi in the lower dose increased CM on 26.9%, when compared to the Bokashi applied alone. CI was lower in the control and in the Penergetic treatments than in the other treatments.

In the second production cycle, higher CM means were obtained for the Bok 10 g, Bok 10 g+Pen, and NPK treatments (Tab. 2). Higher HD was obtained for Bok 5 g, Bok 5 g+Pen, Bok 10 g, Bok 10 g+Pen, and NPK than for the control and Penergetic alone. Increments of 10.52% were estimated in Bok 10 g+Pen (19 cm) when compared to NPK (17 cm). Higher PH values were obtained for the treatments Bok 10 g, Bok 10 g+Pen, and NPK than in the others. The Bok 5 g and Bok 5 g+Pen treatments also produced higher PH than the control and treatment with Penergetic but lower than Bok 10 g, Bok 10 g+Pen and NPK. The lowest CI values were obtained for control, Pen and NPK; intermediate values were found for Bok 5 g and Bok 5 g+Pen; and the highest values were found for Bok 10 g and Bok 10 g+Pen.

For the third cultivation cycle, the CM from the NPK was higher than in the other treatments and 35.68% higher than the second-best treatment Bok 10 g+Pen (Tab. 3). The Bok 10 g and Bok 10 g+Pen CM means were higher than Bok 5 g and Bok 5 g+Pen. The lowest values were obtained from the control and Penergetic alone. Higher PH means were recorded for treatments Bok 10 g, Bok 10 g+Pen and

TABLE 1. Means (± standard error of the mean) of commercial mass (CM), head diameter (HD), plant height (PH), and chlorophyll index (CI) in iceberg lettuce fertilized with Bokashi, 4-14-8 NPK formulation, and Penergetic, for the first growth cycle. Londrina, Brazil.

Treatment	CM (g)	HD (cm)	PH (cm)	CI
Control	25.33±5.03 c	7.17±0.76 b	11.67±2.36 b	10.27±1.80 b
Pen	26.00±10.39 c	9.00±1.73 b	11.33±2.75 b	8.73±0.56 b
Bok 5 g	70.67±8.33 b	16.33±4.73 a	18.33±2.89 a	14.16±0.58 a
Bok 5 g+Pen	96.67±8.08 a	17.50±4.82 a	19.17±1.61 a	13.30±1.11 a
Bok 10 g	122.00±17.09 a	16.33±2.08 a	18.00±0.87 a	13.35±1.04 a
Bok 10 g+Pen	115.33±17.47 a	16.33±2.08 a	18.67±2.08 a	15.88±1.50 a
NPK	132.00±27.06 a	20.33±3.79 a	20.67±0.76 a	13.83±2.61 a
CV (%)	17.98	21.82	12.25	11.55
F	26.01	6.63	9.88	8.22

Pen: Penergetic; Bok 5 g = Bokashi (5 g/plant); Bok 5 g+Pen = Bokashi (5 g/plant) + Penergetic; Bok 10 g = Bokashi (10 g/plant); Bok 10 g+Pen = Bokashi (10 g/plant) + Penergetic; and NPK (6 g/plant).

CV: coefficient of variation. Means followed by the same letter in the columns did not differ with the Scott-Knott test, $P>0.05$.

TABLE 2. Means (± standard error of the mean) of commercial mass (CM), head diameter (HD), plant height (PH) and chlorophyll index (CI) in iceberg lettuce fertilized with Bokashi, 4-14-8 NPK formulation and Penergetic, for the second growth cycle. Londrina, Brazil.

Treatment	CM (g)	HD (cm)	PH (cm)	CI
Control	41.20±5.59 c	13.40±0.89 b	13.60±1.34 c	13.58±1.55 c
Pen	40.80±5.40 c	13.20±1.64 b	13.60±1.14 c	13.87±2.01 c
Bok 5 g	140.80±21.00 b	17.40±2.07 a	16.90±0.55 b	25.16±1.48 b
Bok 5 g Pen	146.40±28.40 b	17.20±1.30 a	17.60±0.55 b	27.07±2.11 b
Bok 10 g	176.00±9.38 a	17.40±1.95 a	18.70±1.20 a	31.42±3.42 a
Bok 10 g+Pen	178.00±10.49 a	19.00±1.41 a	19.60±1.52 a	33.29±4.22 a
NPK	174.80±12.46 a	17.00±1.87 a	19.20±1.30 a	14.49±2.15 c
CV (%)	17.73	10.01	6.71	11.44
F	36.45	8.99	24.20	54.62

Pen: Penergetic; Bok 5 g = Bokashi (5 g/plant); Bok 5 g+Pen = Bokashi (5 g/plant) + Penergetic; Bok 10 g = Bokashi (10 g/plant); Bok 10 g+Pen = Bokashi (10 g/plant) + Penergetic; and NPK (6 g per plant).

CV = coefficient of variation. Means followed by the same letter in the columns did not differ by Scott-Knott test, $P>0.05$.

TABLE 3. Means (± standard error of the mean) of commercial mass (CM), head diameter (HD), plant height (PH), and chlorophyll index (CI) in iceberg lettuce fertilized with Bokashi (4-14-8 NPK formulation and Penergetic) for the third growth cycle. Londrina, Paraná, Brazil.

Treatment	CM (g)	HD (cm)	PH (cm)	CI
Control	35.60±6.07 d	6.84±2.19 c	12.60±1.14 c	18.88±4.55 b
Pen	40.40±6.54 d	8.82±0.47 b	13.42±0.68 c	15.92±2.83 b
Bok 5 g	115.20±13.61 c	9.80±0.91 b	16.86±1.09 b	26.94±4.09 a
Bok 5 g+Pen	110.80±23.22 c	9.70±0.84 b	16.50±1.32 b	20.98±2.28 b
Bok 10 g	154.60±20.97 b	10.50±1.00 b	17.70±0.57 a	25.82±3.63 a
Bok 10 g+Pen	164.00±29.93 b	10.50±0.79 b	17.90±1.43 a	20.98±2.92 b
NPK	255.20±27.44 a	14.70±1.25 a	18.90±0.74 a	24.32±5.18 a
CV (%)	16.25	11.66	6.42	16.88
F	70.16	20.35	25.69	5.64

Pen: Penergetic; Bok 5 g: Bokashi (5 g/plant); Bok 5 g + Pen: Bokashi (5 g/plant) + Pen; Bok 10 g: Bokashi (10 g/plant); Bok 10 g + Pen: Bokashi (10 g/plant) + Pen; and NPK (6 g/plant).

NPK and the lowest PH values were from control and Pen. The HD mean from NPK was higher than the other ones and the control showed the lowest mean compared to all of other treatments. CI from Bok 5 g, Bok 10 g, and NPK were higher than in other treatments.

Discussion

The Bok 10 g and Bok 10 g+Pen treatments (Bokashi higher doses with or without Penergetic) showed similar results when compared to NPK. The only exception was for CM and HD in the third cycle, when NPK showed higher means than Bok 10 g and Bok 10 g+Pen for this variable. Though the higher dose of Bokashi provided greater amounts of nitrogen (37 g/plant) compared to NPK (24 g/plant), in general, the plants had similar means for the horticultural variables. This can be explained by the other nutrients. The phosphorus content of NPK was higher (84 g/plant) than Bokashi (14.4 g/plant) and the potassium content of NPK was also higher (47 g/plant) than Bokashi (21 g/plant). In addition to the nutrient balance, the microorganisms found in Bokashi may have solubilized nutrients for the plants, as discussed below.

In a previous study realized in a greenhouse, higher doses of Bokashi (about 45 g/plant) are needed to provide similar lettuce cv. Elba yields to NPK (about 3 g/plant) (Souza *et al.,* 2016). In general, production in this previous study is higher than what we obtained in the present study. Also, the yield (290 g/plant of lettuce) found by Goulart *et al.* (2018) was higher from those obtained by our study and can be explained by the higher Bokashi dose used (31 g/plant). For the present study, we used 5 L pots, and these limited root development. The previous studies cited were performed in the field providing a better condition for ideal root development and consequently higher yields.

Bokashi is a suitable fertilizer for organic production. Besides the relative richness in nutrients, the microorganisms supplied by inoculum enable higher mineralization and availability of nutrients from the organic matter (Quiroz & Céspedes, 2019). Treatments with Bokashi show 50% to 216% higher microbial carbon biomass than the control (water only) in microcosms experiments and lettuce production (Scotton *et al.,* 2017; Hata *et al.,* 2020). This indicates higher microorganism's activity that may be reflected in a mean of 53% increase in the lettuce production experiments (Hata *et al.,* 2020). The Bokashi microorganism community depends on its raw materials and the inoculum used. The main fungi genera found in these treated soils are *Aspergillus*, *Dactylium,* and *Rhizopus* (Magrini *et al.,* 2011). One of the inocula used for Bokashi preparation is the "EM" that are effective microorganisms composed mainly by photosynthetic bacteria (*Rhodopseudomonas palustris* and *Rhodobacter sphaeroides*), lactobacilli (*Lactobacillus plantarum*, *Lactobacillus casei*, and *Streptococcus lactis*), yeasts (*Saccharomyces* spp.), and Actinomycetes (*Streptomyces* spp.) (Javaid, 2010). Phytohormones such as auxins, gibberellins, and cytokinins can be synthesized by specific species of fungi (*Aspergillus* sp. and *Rhizopus* sp.), heterotrophs and phototrophs Prokaryotes (Tsavkelova *et al.,* 2006). Inoculation with *R. sphaeroides*, L. *plantarum*, and *Saccharomyces cerevisiae* (a similar composition of microorganisms found in Bokashi) on cucumber (*Cucumis sativus* L.) plants altered the plant metabolic pathways by increasing the contents of amino acids, chlorophyll and phytohormones (gibberellic acid and abscisic acid) (Kang *et al.,* 2015). Similarly, the increase of these substances and

phytohormones may have increased lettuce development observed in the present study.

In general, the Bokashi and NPK treatments increased the chlorophyll indexes. Chlorophyll indirect measurements with a hand-held device are an important tool for rapid and real-time estimation of chlorophyll content. In lettuce, the correlations between the device readings and the leaf nitrogen concentration or chlorophyll content were highly significant (Mendoza-Tafolla *et al.,* 2019). That means that using these devices can accurately predict the agronomic variables tested. In the present study, there was a positive and significant moderate correlation ($P<0.01$) between the chlorophyll index and CM: r^2 of 0.36, 0.46 and 0.22, for first, second and third growth cycles, respectively.

The Penergetic bioactivator was effective in improving lettuce CM only with the 5 g of Bokashi per plant dose in the first cycle. However, the lower organic matter content (1.8%) in the soil did not enable the maximum effect of Penergetic, as discussed below. Planting iceberg lettuce with Penergetic that is used with no association provided a yield increment in one out of two cycles in greenhouse summer cultivation (Hata *et al.,* 2020). In contrast to these findings, tomato fruit production is improved in the two-cycle experiment by using Penergetic (Hata, Ventura *et al.,* 2021). In general, effective bioactivators demand a suitable threshold of organic matter (Franco *et al.,* 2018). In a future study, incorporation of additional organic matter between production cycles could be evaluated to assess if Penergetic effects maintain throughout the sequential cultivation. Another combination that could be tested is the NPK and Bokashi in field conditions or in hydroponics systems adapted to the farmers' reality.

Bokashi significantly improved lettuce horticultural variables when compared with the control treatment. In general, the higher dose (10 g) generated higher commercial mass and head diameter and were similar to those in the standard treatment (NPK).

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

DPL: Acquisition of data, analysis and interpretation of data, and draft preparation; GAFF: Conception and design, draft preparation, and critical review of the manuscript; FTH.: Acquisition of data, analysis and interpretation of data, and draft preparation; MUV and JTVR: Conception and design, draft preparation, and critical review of the manuscript; CSW and AF: Acquisition of data, draft preparation, and critical review of the manuscript. All authors have read and approved the final version of the manuscript.

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Total polyphenolics, antioxidants, and cytotoxic activity of infusions from soursop (*Annona muricata*) leaves from two Mexican regions

Polifenoles totales, antioxidantes y actividad citotóxica de infusiones de hojas de guanábana (*Annona muricata*) de dos regiones de México

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ABSTRACT

Infusions of soursop or graviola (*Annona muricata* L.) leaves have been used as alternative medicine for their phytochemical composition, pharmacological and cytotoxic activity that are related with antibacterial, antioxidant, and anticancer activities. Infusions of soursop leaves were obtained at 5, 10, and 15 min in samples collected from the Chiapas and Nayarit regions of Mexico. Total soluble phenols (TSP), flavonoids (FC), condensed tannins (CT), total anthocyanins (AC), antioxidant activity, attenuated total reflectance – Fourier transform infrared spectroscopy analysis (FTIR-TRA), cytotoxic activity in MCF7 and HT-29 cell lines were evaluated. The average contents of TSP, FC, CT and AC were as follows: 0.229±0.006 mg gallic acid equivalents ml^{-1}, 0.177±0.003 mg catechin equivalents ml^{-1}, 0.298±0.012 mg cyanidin 3-glucoside equivalents ml^{-1}, and 0.189±0.003 mg catechin equivalents ml^{-1}, respectively. The FTIR-ATR analysis determined carbonyl, hydroxyl, ester, and carboxylic acid groups. The antioxidant activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were 0.446±0.014 and 3.53±0.515 mM Trolox equivalents ml^{-1}, respectively. The extraction yields of TSP, FC, CT, and AC by infusion were 88.41, 66.12, 34.93, and 56.09%, respectively. The antioxidant activity was 50.77% for ABTS and 21.44% for DPPH. Cytotoxic activity was present against the MCF7 (80%) cell line. The infusions had high polyphenol contents, antioxidant and cytotoxic activities.

Key words: antioxidant capacity, cell viability, tannins, flavonoids, anthocyanins.

RESUMEN

Las infusiones de hojas de guanábana (*Annona muricata* L.) se han utilizado como medicina alternativa por su composición fitoquímica, actividad farmacológica y citotóxica, que les confieren actividad antibacteriana, antioxidante y anticancerígena. Las infusiones de hojas de guanábana se obtuvieron a 5, 10 y 15 min en muestras colectadas de las regiones de Chiapas y Nayarit en México; se evaluaron fenoles solubles totales (FST), flavonoides (F), taninos condensados (TC), antocianinas totales (AT), actividad antioxidante, reflectancia total atenuada por análisis de espectroscopia infrarroja transformada de Fourier (FTIR-ATR) y actividad citotóxica en líneas celulares MCF7 y HT-29. El contenido promedio de FST, F, TC y AT fue de 0.229±0.006 mg equivalentes de ácido gálico ml^{-1}, 0.177±0.003 mg equivalentes de catequina ml^{-1}, 0.298±0.012 mg equivalentes de cianidina 3-glucósido ml^{-1} y 0.189±0.003 mg equivalentes de catequina ml^{-1} respectivamente. El análisis FTIR-ATR determinó grupos carbonilo, hidroxilo, éster y ácidos carboxílicos. La actividad antioxidante por 2,2-difenil-1-picrilhidracilo (DPPH) y 2,2'-azino-bis-3-etilbenzotiazolina-6-ácido sulfónico (ABTS) fue de 0.446±0.014 y 3.53±0.515 mM equivalentes de Trolox ml^{-1} respectivamente. Los rendimientos de extracción de FST, F, TC y AT mediante infusión fueron 88.41, 66.12, 34.93 y 56.09 respectivamente. La actividad antioxidante fue 50.77% de ABTS y 21.44% de DPPH. Se presentó actividad citotóxica contra la línea celular MCF7 (80%). Las infusiones presentaron un alto contenido de polifenoles, actividad antioxidante y citotóxica.

Palabras clave: capacidad antioxidante, viabilidad celular, taninos, flavonoides, antocianinas.

Introduction

Soursop belongs to the *Annona* genus, which contains more than 160 species. The soursop tree is distributed in tropical regions around the world, most of the year it tends to bloom and is evergreen (Terán-Erazo *et al.,* 2019). Nowadays, soursop is an important economic crop in Mexico, Venezuela, Brazil, and Colombia because of the fruits. The main growing regions in Mexico are the tropical areas, located in Nayarit, Chiapas, Colima, Michoacán, and Veracruz states (Jiménez-Zurita *et al.,* 2016; Escobedo-López *et al.,* 2018; SADER, 2020). In 2019, the Mexican government reported

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a national production of 30,790 t of soursop, with a commercial value of 248,170 million Mexican peso (SIAP, 2019).

Many plant species are rich sources of natural bioactive compounds and could have a diverse variety of biological effects; they have long been employed in alternative medicine because of their therapeutic potential and health advantages (Etheridge & Derbyshire, 2019). Due to the pharmacological, phytochemical, and toxicological effects as well as for their pharmacokinetics, most therapies in Mexican folk medicine are administrated in the form of infusions. These beverages are the first alternative usually suggested by "yerberos" or traditional healers for health care (Alonso-Castro *et al.,* 2017). The infusions consist of an aqueous extraction prepared by pouring boiling water on the leaves and then letting them steep for 5-15 min (Coz-Bolaños *et al.,* 2018).

Several studies report the phenolic characterization of methanolic (Saklar *et al.,* 2015; Nam *et al.,* 2017; Olugbuyiro *et al.,* 2018) and ethanolic (Roduan *et al.,* 2019) infusions of the leaves of soursop; however, due to their easy preparation, infusions are extensively used to treat various diseases and this nutraceutical potential is associated with their phenolic compounds including flavonoids, tannins, and anthocyanins (Coria-Téllez *et al.,* 2019; Balderrama-Carmona *et al.,* 2020; Nguyen *et al.,* 2020). An analytical method used to study important bioactive compounds, such as phenols, is the Fourier transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) (Daud *et al.,* 2016; Hidalgo *et al.,* 2019; Ibrahim *et al.,* 2021) that allows identifying functional groups that absorb in the mid-infrared and produce particular signal vibrations of the molecular bonds. The resulting spectrum is known as the sample fingerprint (Grijalva-Verdugo *et al.,* 2018).

Soursop leaf infusions show antibacterial (Iyanda-Joel *et al.,* 2019), antifungal (Folorunso *et al.,* 2019), antiprotozoal (Calzada *et al.,* 2020), and antiviral activity (Balderrama-Carmona *et al.,* 2020); and they protect against oxidative stress that could prevent hypertension (Ola-Davies *et al.,* 2019), diabetes (Rahayu *et al.,* 2019), inflammation (Cercato *et al.,* 2021), obesity (Sasso *et al.,* 2019), and cancer (Coria-Téllez *et al.,* 2019; Hassan *et al.,* 2019).

Some cytotoxic studies of *A. muricata* extract have been conducted against HeLa (Coria-Téllez *et al.,* 2019), MDA, and SKBR3 (Gavamukulya *et al.,* 2014) cell lines. The anticancer activity of *A. muricata* is attributed to acetogenin content that are fatty acid metabolic derivatives of long chains (35 to 37 carbon atoms). These compounds contain a terminal α-β-unsaturated or saturated γ-lactone group, together with their central regions with one to three rings of tetrahydrofuran or tetrahydropyran (Aguilar-Hernández *et al.,* 2020; Grba *et al.,* 2022).

The concentrations of phenolic compounds in leaves are influenced by the time of harvest, the growth environment, the soil type, the solar exposure, and the site of production (Guzmán-Maldonado *et al.,* 2020). These factors influence the content of phenolic compounds in the infusions; however, the main factor is the amount of leaves used and the steeping time of the aqueous preparation (Pérez-Burillo *et al.,* 2018).

Many studies approached the relationship of steeping time and migration of polyphenolic compounds into herbal infusions (Rusak *et al.,* 2008; Ramalho *et al.,* 2013; Pérez-Burillo *et al.,* 2018). In this sense, Hardoko *et al.* (2015) and Hardoko *et al.* (2018) assayed the effect of soursop leaves processed as black and green tea with times of extraction for 15, 30, and 45 min; and they characterized the content of phenols, tannins, and flavonoids. Several studies report the phenols and antioxidants in soursop leaf infusions using 10 min of steeping time and 90°C temperature (Innocent-Ukachi & Onukwugha, 2019). Irawan and Mahmudiono (2018) used 70°C water and a steeping time of 4 min, and Cercato *et al.* (2021) used water at 100°C and 15 min of steeping. But few studies have reported the polyphenolic compounds and antioxidant activity of the aqueous extracts of soursop leaves in Mexico (Coria-Téllez *et al.,* 2019; Balderrama-Carmona *et al.,* 2020).

However, there are limited reports about the relationship between production site, phenolic content, antioxidant capacity, and steeping time for soursop leaf infusions from Mexico. The aim of this study was to evaluate the polyphenolic composition, antioxidant activity, cytotoxic activity, and the effect of steeping time of infusions from Mexican soursop leaves collected from the regions of Chiapas and Nayarit.

Materials and methods

Plant samples

Annona muricata leaves were collected from two Mexican states, Nayarit (Tepic, 21°30'0" N, 104°54'0" W) and Chiapas (Cantón el Carmen, 14°46'24" N, 92°13'24" W), in July-August 2020. The selection of the plant material was made considering their healthy phytosanitary characteristics. The leaves were freeze-dried (Labconco, LYPH Lock 4.5, USA), milled (electric mill, NutriBullet, Los Angeles,

USA) and stored (two weeks) in closed plastic bags (Ziploc) at room temperature in the absence of light until analyses.

Infusion preparation

Tea bags for herbal teas were used for the infusion preparations. Each bag of tea, containing 3 g of lyophilized soursop leaves, were infused in 240 ml of boiling water and allowed to steep for 5 min, 10 min, and 15 min; afterwards the infusions were allowed to cool to room temperature and TSP, FC, CT, AC and antioxidant activity were determined (Coz-Bolaños *et al.,* 2018).

Methanolic extraction yields

The polyphenolic compounds from soursop leaves were extracted using solutions of water/methanol (ME), ratio 70:30 and 40:60 (v:v) for total soluble phenols and flavonoids, and absolute methanol was used for tannins. The mixtures were prepared in a ratio 10:1 (m:v), then stirred for 10 min, centrifuged at 5000 rpm for 10 min, and the supernatant were filtered using a Whatman filter paper and the same analyses as for infusions were made (Deshpande & Cheryan, 1985; Singleton *et al.,* 1999; Dewanto *et al.,* 2002).

Total soluble phenols

Total soluble phenols (TSP) were determined by spectrophotometric methods using Folin-Ciocalteu's reagent (Hycel), according to Singleton *et al.* (1999) with some modifications as follow: an aliquot (60 µl) of soursop infusion was mixed with 240 µl of distilled water and 60 µl of Folin-Ciocalteu reagent were added. The samples were then stirred and left to steep for 6 min. Finally, 600 µl of 7% (w/v) Na_2CO_3 (Meyer) solution and 480 µl of distilled water were added and maintained in dark conditions for 90 min at room temperature. Absorbance was read at 750 nm using a Multiskan GO (Thermo Fisher Scientific, 51119200, USA). A gallic acid (Fermont) standard curve (acid gallic = $0.2643(Abs_{750})$ - 0.006, R^2=0.9898) was elaborated using known concentrations (0.200 – 0.020 mg ml^{-1}). The results were expressed as milligrams of gallic acid equivalents per milliliter (mg GAE ml^{-1}).

Flavonoid content

The flavonoid content (FC) was determined following the procedure described by Dewanto *et al.* (2002) with some modifications: 150 µl of soursop infusion was mixed with 45 µl of 5% (w/v) $NaNO_2$ (J. T. Baker) solution, 90 µl of recent prepared 10% (w/v) $AlCl_3$ (J. T. Baker) and 300 µl of 1 M NaOH (J. T. Baker) were added. The mixture was brought to 1.5 ml with distilled water, mixed and left to repose for 5 min. Absorbance was measured against the blank at 510 nm using a Multiskan GO. Catechin (Sigma-Aldrich®, USA) was used as a standard (catechin=$0.5803(Abs_{510})$ + 0.0538, R^2=0.9817), and the solutions were prepared at 0.300-0.062 mg ml^{-1}. The results were expressed as milligrams of catechin equivalents per milliliter (mg CE ml^{-1}).

Condensed tannins

The methodology reported by Deshpande and Cheryan (1985) was used with some modifications to determine condensed tannins (CT) in the infusions. A total of 200 µl of sample was reacted with 1 ml of a solution prepared in proportion 1:1 (v/v) from 1% vanillin (Sigma-Aldrich®, USA) (w/v, dissolved in methanol) and 8% HCl (J. T. Baker) (v/v, dissolved in methanol) and incubated at a temperature of 30°C for 20 min. Absorbance was read at 500 nm against the blank using a Multiskan GO. To estimate the concentration of tannins, a calibration curve with catechin (Sigma-Aldrich®, USA) (catechin=$5.5325(Abs_{500})$ - 0.1577, R^2 =0.9866) was obtained using known concentrations of catechin (0.750-0.031 mg ml^{-1}). Condensed tannin contents were expressed as milligrams of catechin equivalents per milliliter (mg CE ml^{-1}).

Total anthocyanins

The anthocyanin contents (AC) were studied according to the Abdel-Aal and Hucl (1999) method with modifications. The infusion was measured and adjusted to pH 1 with 4N HCl. The sample was read in a Multiskan GO at 535 nm against the blank. Total anthocyanin content per sample (mg ml^{-1}) was calculated as cyanidin 3-glucoside:

$$C = \left(\frac{A}{\varepsilon}\right) \times \left(\frac{vol}{1000}\right) \times MW \times \left(\frac{1}{sample\ wt}\right) \times 10^6$$

where C is the concentration of total anthocyanin (mg ml^{-1}), A is the absorbance reading, ε is the molar absorptivity (cyanidin 3-glucoside=25,965 cm^{-1}), *vol* is the total volume of infusion, and MW is molecular weight of cyanidin 3-glucoside=449.

Antioxidant capacity (ABTS and DPPH)

The method of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) to evaluate the antioxidant capacity was carried out according to Re *et al.* (1999). The radical $ABTS^+$ was prepared dissolving 3.8 mg of the reactive ABTS (Sigma-Aldrich®, USA) in 1 ml of 2.45 mM potassium persulphate (Fermont), and the mixture was kept in the dark at 4°C for 12 h before use. Then 0.15 ml of the $ABTS^+$ solution was diluted in 14 ml of phosphate buffer at pH 7.4 to obtain an absorbance of 0.7+0.020 at 734 nm using a spectrophotometer (Hach DR3900, USA). The

phosphate buffer was prepared by adding 8 g NaCl, 0.2 g KCl and 1.44 g KH_2PO_4 in 1 L of distilled water. First, the absorbance at initial time (t_0) was measured in 990 µl of the diluted radical $ABTS^+$ solution, and 10 µl of the soursop infusion was immediately added. The mix was kept in the dark at room temperature for 6 min; after that time the absorbance was measured at 734 nm (t_6). The antioxidant capacity was calculated by comparing the absorbance values of the infusion to a Trolox (Sigma-Aldrich®, USA) standard curve (Trolox equivalent=0.0253($Abs_{(t0-t6)}$) + 0.3864, R^2=0.9878). The curve was obtained using known concentrations of Trolox (3000-500 mM). The result was expressed as the equivalent Trolox per milliliter (mmol TE ml^{-1}).

The DPPH antioxidant activity of infusions was determined using Brand-Williams *et al.* (1995) with some modifications using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich®, USA) as a free radical. A 200 µM DPPH solution in methanol (Reasol) was prepared. The solution was prepared on the same day of the analysis and the mixture was kept in the dark. A 180 µl aliquot of the DPPH radical solution was mixed with 20 µl of the infusion in wells of a 96-well plate and kept in the dark for 30 min. Absorbance was read at 515 nm in a Multiskan GO. The antioxidant activity was calculated using a Trolox calibration curve (Trolox equivalent= 0.0117($Abs_{(t0-t30)}$) – 0.0286, R^2=0.9748). The antioxidant capacity value was expressed as Trolox equivalents per milliliter (mmol TE ml^{-1}).

FTIR-ATR spectroscopy analysis

Two g of milled leaves from each region were mixed with 40 ml of absolute methanol; the mix was sonicated for 10 min, and was allowed to rest for 24 h, after which it was filtered. This procedure was repeated twice, and the filtrates were mixed, then evaporated using a rotary evaporator (Yamato Scientific, RE201) until a dark brown syrup was obtained. A small amount of ME syrup was analyzed using FTIR (PerkinElmer, Dynascan® brand spectrum 100 model) equipped with an attenuated total reflectance interferometer (ATR) at a temperature of 25±2°C. The spectra were obtained from 16 points with a resolution of 4 cm^{-1} in a region of 4000-500 cm^{-1} (Ramírez-Hernández *et al.,* 2020).

Preparation of leaf extract for cytotoxic activity assay

The procedure of Coria-Téllez *et al.* (2019) was used with some modifications; specifically, 630 mg of lyophilized and crushed soursop leaves were mixed with 15 ml of boiling water and the mixture was ground for 10 min, then allowed to steep until reaching room temperature. The mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a 0.45 µm membrane.

Cytotoxic activity assay

The tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay was used to measure the cytotoxic activity of the extracts against MCF7, and HT-29 cells line according to the procedure described by Coria-Téllez *et al.* (2019). MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich); and the HT-29 cells were cultured in McCoy's Medium (Gibco), both containing 10% (v/v) fetal bovine serum, 1% penicillin–streptomycin antibiotic solution, and 5 ml trypsin. The cells were cultured in a CO_2 incubator with 5% CO_2 at 37°C. The cells were seeded at a density of $1x10^4$ cells per well, incubated for 24 h in darkness and exposed to various concentrations of aqueous concentrated extract (21, 10.5, and 5.25 mg ml^{-1}). The cells were also treated with 50 µl of dimethylsulfoxide (DMSO) (negative control). At the end of the treatment, the supernatant was removed in each well and replaced with 150 µl of MTT (3.33 mg ml^{-1}) followed by incubation at the same conditions for 4 h. The reaction was stopped by adding 100 µl DMSO to each well. The absorbance of each solution was read in a microplate spectrophotometer at 595 nm.

Statistical analysis

All experiments were conducted in triplicate and data were presented as the means ± standard deviations. Statistical analysis was performed using Minitab 17 for Windows (Minitab Statistical Software, USA). Significant differences between the samples were tested using analysis of variance (ANOVA) followed by Tukey's test comparison (P<0.05).

Results and discussion

Total soluble phenols (TSP)

TSP of samples ranged from 0.182 to 0.246 mg GAE ml^{-1} (Tab. 1). TSP in the extracts from Nayarit region correlated with extraction time, the longer infusion time the higher the phenolic content, but not for the Chiapas region. In leaves recollected in Chiapas, at 10 min of extraction the phenolic content decreased; and it increased at 15 min of extraction, even though the infusion from Chiapas reposed for 15 min and had the highest TSP (0.246±0.009). Hardoko *et al.* (2015) analyzed soursop leaf samples at different time extractions and reported the highest concentration of TSP at 45 min of extraction (0.321 mg GAE ml^{-1}), while the lowest concentrations were at 15 min of extraction (0.200 mg GAE ml^{-1}). In accordance with the results obtained in this study, Pérez-Burillo *et al.* (2018) reported for white tea

that increasing the extraction time in the infusions also improved the phenolic extraction (about 10 min).

The increase in TSP with respect to extraction time is related to the polarity and hydrophilicity of the bioactive compounds (Kelebek, 2016). In addition, the water temperature used in the infusions (100°C) influences the extraction efficiency, because the heating process opens the vacuoles of the cells (Hardoko *et al.,* 2015) facilitating the migrations and solubility of polyphenols due to diffusion coefficients of the components to be extracted (Saklar *et al.,* 2015).

Flavonoid content (FC)

For Chiapas, the FC of soursop leaf infusion increased with time, reaching the highest content at 15 min, while Nayarit had the highest concentration at 10 min (0.178 mg CE ml^{-1}). After this time the content decreased, but it was not significant. Several authors found the same effect for long infusion times. For example, Saklar *et al.* (2015) report for green tea elevated catechin concentrations at the beginning of the infusion using water at 75°C and after this time the concentration increased. However, using water at 95°C the catechin content did not change significantly during steep time. Therefore, increasing the steeping time of the infusion beyond 20 min would not result in a greater extraction of flavonoids (Saklar *et al.*, 2015). Ramalho *et al.* (2013) report that the catechin flavonoid content decreased after 10 and 15 min for Indian and British brands of black teas, but the Brazilian brand tea continued increasing up to 30 min. The levels of flavonoids in infusions are liable to change depending on the sample preparation conditions (Jin *et al.*, 2019). For example, long infusion times suggest that the flavonoids as catechins can suffer epimerization (the flavonoids are converted to their corresponding non-epi isomers) (Saklar *et al.,* 2015); and the high temperatures used in the extraction process could induce the flavonoid oxidation resulting in damage to the flavonoid compounds (Hardoko *et al.*, 2015).

Anthocyanin content (AC)

The AC in Chiapas infusions increased with the infusion time (Tab. 1), meanwhile, Nayarit infusions did not show the same behavior because at 10 min of infusion the AC decreased and increased at 15 min. The AC of infusions for the two regions ranged from 0.148 to 0.330 mg C3GE ml^{-1}, and Nayarit showed the highest content of AC at the three-repose time. A wide range of AC values has been reported for different infusion types, ranging from 0.126 to 1.645 mg C3GE ml^{-1} for *Hibiscus sabdariffa* infusion (Salmerón-Ruiz *et al.*, 2019), 0.119 mg C3GE ml^{-1} for *Hibiscus sabdariffa* mixed with bilberry tea that contained berry fruits of aronia, black currant, rose hip, raspberry, bilberry, and blueberry (Bratu *et al.*, 2018). In this sense, the variables that have the greatest impact on anthocyanin extraction are the number of leaves, volume of water, water temperature, and repose time used in the infusion preparation. Therefore, fewer leaves and a lower proportion of water allow for greater extraction efficiency, but it may take longer (Talib *et al.*, 2020). In addition, the use of high temperature water increases the extraction process that improves both anthocyanin solubility and diffusion coefficients (Paraíso *et al.*, 2021). However, high temperatures may not be suitable for anthocyanins, since they are susceptible to thermal degradations and could cause them to break down and lose their antioxidant activity and, consequently, their bioactivity (Salmerón-Ruiz *et al.*, 2019).

Condensed tannins (CT)

The CT content was increased according to the infusion time (Tab. 1) that ranged from 0.131 mg to 0.219 mg CE ml^{-1}. Previous research reports the concentrations ranging

TABLE 1. Phenolic contents and antioxidant activity of infusions from soursop leaves harvested in two Mexican states.

Region	Time (min)	TSP (mg GAE ml^{-1})	FC (mg CE ml^{-1})	AC (mg C3GE ml^{-1})	CT (mg CE ml^{-1})	ABTS (mM TE ml^{-1})	DPPH (mM TE ml^{-1})
Nayarit	5	0.182 ± 0.020^{b}	0.176 ± 0.011^{ab}	0.318 ± 0.028^{a}	0.151 ± 0.013^{de}	1.21 ± 0.065^{b}	0.405 ± 0.042^{ab}
	10	0.205 ± 0.024^{ab}	0.178 ± 0.002^{a}	0.247 ± 0.030^{ab}	0.199 ± 0.006^{ab}	1.33 ± 0.075^{ab}	0.440 ± 0.041^{ab}
	15	0.221 ± 0.008^{ab}	0.175 ± 0.019^{ab}	0.330 ± 0.052^{a}	0.219 ± 0.009^{a}	1.24 ± 0.073^{b}	0.492 ± 0.006^{a}
	Mean	0.202 ± 0.006^{B}	0.177 ± 0.003^{A}	0.298 ± 0.012^{A}	0.189 ± 0.003^{A}	1.26 ± 0.031^{A}	0.446 ± 0.014^{A}
Chiapas	5	0.235 ± 0.012^{a}	0.148 ± 0.006^{b}	0.148 ± 0.022^{c}	0.131 ± 0.011^{e}	1.19 ± 0.007^{b}	0.377 ± 0.051^{b}
	10	0.207 ± 0.012^{ab}	0.168 ± 0.005^{ab}	0.175 ± 0.027^{bc}	0.166 ± 0.011^{cd}	1.28 ± 0.060^{b}	0.381 ± 0.018^{b}
	15	0.246 ± 0.009^{a}	0.171 ± 0.007^{ab}	0.219 ± 0.029^{bc}	0.185 ± 0.003^{bc}	1.47 ± 0.082^{a}	0.357 ± 0.043^{b}
	Mean	0.229 ± 0.006^{A}	0.162 ± 0.003^{B}	0.181 ± 0.012^{B}	0.161 ± 0.003^{B}	1.31 ± 0.031^{A}	0.372 ± 0.019^{B}

Total soluble phenols (TSP); Flavonoid content (FC); Anthocyanin content (AC); Condensed tannins (CT); Antioxidant capacity (ABTS and DPPH). Mean is the average of the three repose times for each region for each phenolic and antioxidant activity. For each column, different lowercase letters indicate significant differences ($P<0.05$) between times measured by Tukey's multiple range test. For each column, different capital letters indicate significant differences ($P<0.05$) measured by Tukey's multiple range test.

from 0.199 to 0.519 mg CE ml^{-1} (Hardoko *et al*., 2015; Hardoko *et al*., 2018). The variations in the concentrations reported in the literature can be attributed to the maturity and treatment of leaves and the temperature and time used for drying (Jimenez-Garcia *et al*., 2020). The time, water temperature, and type of extraction are also related to extraction conditions. This may explain the prevention of tannin oxidation by increasing the concentration of these compounds in the extract (Castiglioni *et al*., 2015; Silva-Ramírez *et al*., 2020).

Antioxidant activity

The results of ABTS assays show that the infusions of Chiapas have the higher antioxidant activity (1.47 mmol TE ml^{-1}) (Tab. 1). The infusion time of 5 min has the lowest antioxidant activity with a significant increase only after 15 min from Chiapas. This behavior is similar to that observed for TSP, AC, and CT. The ABTS results obtained in this study are lower than those obtained by Kelebek (2016) for black tea (1.96 mmol TE ml^{-1}). Almajano *et al*. (2008) report that the antioxidant activity of peppermint infusion and red tea was equal to 0.315 and 0.825 mmol TE ml^{-1}, respectively. This indicates that the antioxidant capacity of the soursop infusion is similar to that of the commercial teas.

The DPPH assays found that Nayarit leaves infused for 15 min produce the highest DPPH values (0.492 mmol TE ml^{-1}). There were significant statistical differences ($P<0.05$) in antioxidant activity depending on infusion time for Nayarit, but not for Chiapas. The behavior of antioxidant activity with respect to time of extraction for Chiapas and Nayarit was similar to TSP and CT behavior. Jin *et al*. (2019) point out the influence of temperature and time of extraction on the antioxidant activity through DPPH method, and they report a maximum at 10 min and 95°C, while after 30 min the activity decreased; however, at 65°C after 10 min the value increased significantly.

It is important to note that no one method determines all the antioxidant compounds of a sample. For this reason, in this study, two methodologies were used to evaluate the antioxidant activity of the infusions obtained. The difference in the results between the ABTS and DPPH was explained by the different compounds with antioxidant activity in the samples. These compounds have different reaction mechanisms and kinetics, due to their chemical structure and functional groups. However, both methods show a decrease in absorbance due to the reduction of radicals. On the one hand, the cationic form of the ABTS radical is reduced by the interaction with hydrogen or electron donor species, while the DPPH radical is reduced in the presence of hydrogen donor antioxidants (Brand-Williams *et al*., 1995; Re *et al*., 1999; Grijalva-Verdugo *et al*., 2018; Guzmán-Maldonado *et al*., 2020).

The high antioxidant activity of the infusions is due to the presence of phenolic compounds and secondary metabolites, such as alkaloids, vitamins, terpenoids, saponins, and essential oils produced for the high metabolic activity of the leaves; and all these compounds in the infusions of soursop leaves play an important role over their antioxidant activity (Kelebek *et al*., 2016; Menezes *et al*., 2019; Roduan *et al*., 2019; Balderrama-Carmona *et al*., 2020; Mannino *et al*., 2020; Silva-Ramírez *et al*., 2020).

The reports about the antioxidant activity shows variations between the different authors, because the results are reported using different units (mg L^{-1} or μmol L^{-1}). However, the use of these practices makes it difficult to understand the real contribution in terms of consumption by portion size (Urías *et al*., 2020). In this research, the consumption of a portion (240 ml) of soursop leaf infusion contributes to antioxidant activity of 229 mM TE per portion. The same activity is proportioned by drinking the same portion of apple juice. Meanwhile, the same portion of red wine gives 1.8 times more antioxidant activity than soursop leaf infusion (Park *et al*., 2018).

Methanolic extraction yields

Table 2 shows the polyphenolic contents and antioxidant activity of methanolic extract of soursop leaves. The results of methanolic extracts did not show significant differences between regions in the variables tested. However, the infusions showed significant differences (Tab. 1) for TSP, FC, AC, CT and DPPH, where a higher content of FC, AC, CT and DPPH in the infusions was also observed

TABLE 2. Phenolic contents and antioxidant activity of methanolic extracts from soursop leaves harvested in two Mexican states.

Region	TSP (mg GAE ml^{-1})	FC (mg CE ml^{-1})	AC (mg C3GE ml^{-1})	CT (mg CE ml^{-1})	ABTS (mM TE ml^{-1})	DPPH (mM TE ml^{-1})
Nayarit	0.336 ± 0.076^{a}	0.290 ± 0.072^{a}	0.853 ± 0.255^{a}	0.423 ± 0.266^{a}	3.53 ± 0.515^{a}	2.08 ± 0.256^{a}
Chiapas	0.259 ± 0.041^{a}	$0.245\pm.053^{a}$	1.077 ± 0.065^{a}	0.287 ± 0.163^{a}	2.58 ± 0.540^{a}	2.56 ± 0.164^{a}

Total soluble phenols (TSP); Flavonoid content (FC); Anthocyanin content (AC); Condensed tannins (CT); Antioxidant capacity (ABTS and DPPH). The results are the mean ± standard deviations of n=3. For each column, different letters indicate significant differences at $P<0.05$ as measured by Tukey's multiple range test.

with Nayarit leaves, while TSP was higher for infusions with Chiapas leaves. The ABTS assays of the infusions did not show significant differences between Chiapas and Nayarit. Chiapas and Nayarit have different climatic and geographical conditions (temperatures, air humidity, and soil composition), inducing differences in the biochemical and physiological responses of the plants (Syed-Najmuddin *et al*., 2017; Ovando-Domínguez *et al*., 2019).

The infusion extraction yields for Chiapas at 15 min showed values of 91.7%, 70%, and 64.5% for ME, TSP, FC and CT, respectively. The yield for the Nayarit region showed results of 34.7% and 23.6% for AC and DPPH, respectively. Coz-Bolaños *et al*. (2018) report yields of 62.4% and 40% for TSP and FC for moringa infusions. Balderrama-Carmona *et al*. (2020) point out yields of 71.97% and 12.57% of antioxidant activity of ABTS and DPPH in soursop leaf infusions, compared to the yield of the extractions with acidified alcohol. Also, Nam *et al*. (2007) report yields of 20% in extractions for TSP in soursop leaves using water, in comparison to the methanol extractions. An accurate comparison could be difficult between the results previously reported by different authors. Due to a lack of uniformity in the conditions of infusion preparation and the properties of the leaves such as leaf age, leaf size and harvesting season, it is difficult to compare accurately the results obtained in the present work with other studies (Saklar *et al*., 2015).

FTIR-ATR analysis of *A. muricata* extracts

The FTIR spectrometry is a physico-chemical analytical technique that reveals the functional groups of the components separated based on their peak ratio. Figure 1 provides the functional groups present in *A. muricata* leaves extract.

In the FTIR, the broad and strong band between 3700 and 3000 cm^{-1} is due to overlapping stretches of vibrations of functional -OH groups, showing the presence of phenolic compounds and the methanol residual in the extracts (Daud *et al*., 2016). The peak at 2,924 cm^{-1} is due to the carboxylic acid group (Ibrahim *et al*., 2021). The peaks observed at 2,853 cm^{-1} are assigned to asymmetric stretching of the -CH_3 groups of the lactone rings, meanwhile the 1,740 cm^{-1} band is attributed to the -C=O stretch vibrations of the γ-lactone ring of acetogenins (Hidalgo *et al*., 2019). The absorptions at 1,656, 1,513, and 1,450 cm^{-1} correspond to the C=C stretch vibration characteristics of the flavonoid bonds (Grijalva-Verdugo *et al*., 2018). The bands between 1,376-1,320 cm^{-1} are assigned to the -CH_3 groups of the alkanes and alkenes (Ibrahim *et al*., 2021). The peaks observed at 1,286, 1,248, and 1,205 cm^{-1} are attributed to the functional group -OH out of plane torsion of carboxylic acids, C-O stretching vibration and C-O-C asymmetric stretching, respectively, belonging to the pyran ring structure of tannins (Daud *et al*., 2016; Grijalva-Verdugo *et al*., 2018). The signals observed at 1,161, 1,071, and 1,027 cm^{-1} correspond to the O=C-O ester functional group attributed to coumarins (Ibrahim *et al*., 2021). Strong absorptions are observed in the region 828-719 cm^{-1}, assigned to the C-O stretching vibrations of α and β pyranose compounds present in anthocyanins (Grijalva-Verdugo *et al*., 2018).

The FTIR spectra of the infusion from the two regions showed slight differences in the intensity and width of the bands and peaks; the differences may be due to the variation in the number of functional groups that produce particular vibrational signals of molecular bonds corresponding to that specific wavenumber. The resulting spectrum is a fingerprint of the *A. muricata* leaf extract (Farooq & Sehgal, 2019).

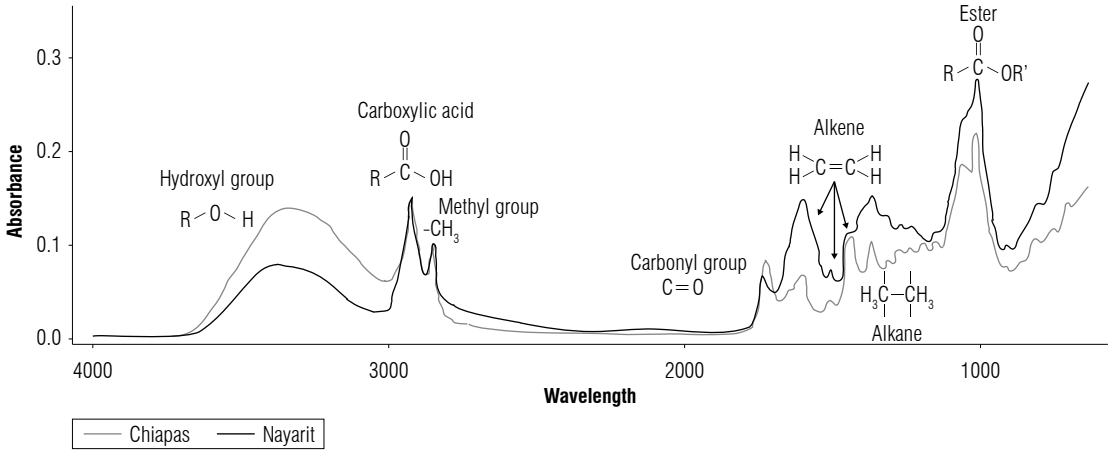

FIGURE 1. FTIR spectra in the region of 4000-850 cm^{-1} of *A. muricata* leaves extracts from Chiapas and Nayarit.

Cytotoxic activity assays

Figure 2 shows the results of cytotoxic activity assays of aqueous extract from Nayarit and Chiapas regions against MCF7 and HT-29 cells. The high cytotoxic activity was found for MCF7 cells with aqueous extract from Chiapas. Also, the results for HT-29 cells did not show statistical differences.

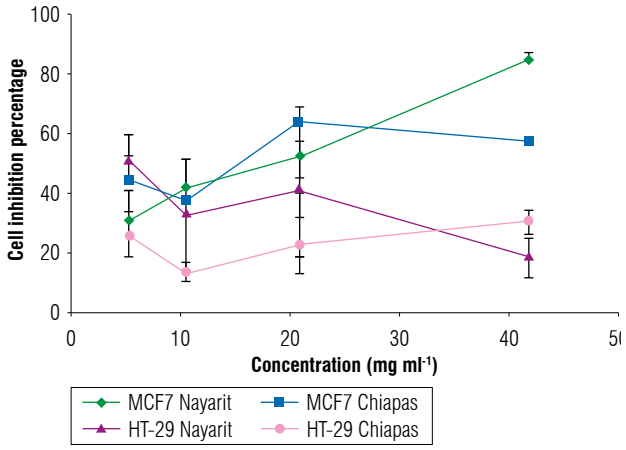

FIGURE 2. Cytotoxic effect of the aqueous extracts from Nayarit and Chiapas against MCF7 and HT-29 cells. Vertical bars correspond to the standard deviation.

Coria-Téllez *et al*. (2019) report that aqueous extracts of *A. muricata* from Nayarit reached a viability of 50% against HeLa cells with a dose extract of 2.42 mg ml^{-1}. Hadisaputri *et al*. (2021) mention a viability cell of 20% against MCF7 cell line using an aqueous fraction of *A. muricata* leaves at 250 µg ml^{-1}; the results show that the survival rate of the cells decreased with increased concentration. In the same way, Syed Najmuddin *et al*. (2016) evaluated the cytotoxic activity of water extracts of leaves of *A. muricata* against breast cells lines, MDA-MB231, MCF7, MCF-10A and 4T1 and reports a varied anti-breast cancer activity, around 50% for MCF-10A, 40% for MCF7, 30% for the cell lines MDA-MB231 and 4T1. The cytotoxic activity has been attributed to acetogenins in the leaves of *A. muricata*, from which more than 45 have been identified (Hadisaputri *et al*., 2021). These secondary metabolites are responsible for the cytotoxicity activity because they inhibit the complex I of the mitochondrial respiratory chain, reducing the production of ATP, bringing on cellular apoptosis (Aguilar-Hernández *et al*., 2020; Grba *et al*., 2022).

Conclusions

The soursop leaves from the Chiapas region showed the highest TSP at 15 min of infusion. The highest TF value appeared in the leaves from Nayarit at 10 min of infusion. For AC and CT, the leaves from Nayarit region at 15 min had the highest values. The higher antioxidant activity of ABTS was obtained for Chiapas and DPPH was highest for the Nayarit region, both at 15 min. The comparative analysis showed significate differences between the regions for TSP, FC, AC, CT and DPPH, but not for ABTS and methanolic extracts. The infusions had the best cytotoxicity activity against MCF7 compared to HT-29 cells. The FTIR-ATR analysis showed important functional groups as carbonyl, hydroxyl, ester, and carboxylic acid, related to the active compounds in the extracts. The use of soursop leaves as infusions can be recommended as a source of antioxidants due to their composition in polyphenolics; furthermore, its secondary metabolites offer cytotoxic activity against breast cancer cell lines. However, the presence and biological activity of its molecules from soursop leaves must be analyzed more deeply.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

CGV, DMA and MGRC worked in the formal analysis, research, and methodology. JRRN and CLAM worked in the conceptualization, project administration, funding acquisition, writing, and preparing of the original draft. JMVF and RBM contributed with the sampling in the Chiapas and Nayarit regions. CANC was responsible for data curation and formal analysis. All authors have read and approved the final version of the manuscript.

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SCIENTIFIC NOTE

Assessment of physicochemical characteristics of biofertilizers and their role in the rooting capacity of plants

Evaluación de las características fisicoquímicas de biofertilizantes y su papel sobre la capacidad de enraizamiento de plantas

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ABSTRACT

The concentration of supplied mineral nutrients is one of the most important and limiting factors for enhancing the efficiency of plant nutrition. Optimal concentration of nutrient solutions (NS) provide plants with the necessary amount of nutrients. From this point of view, research on several physicochemical parameters that characterize concentrations of NS and uptake of nutrients by the plants remains an actual problem. The changes of electrical conductivity (EC) and total dissolved solids (TDS) depending on the concentration of biofertilizer as well as the role of biofertilizer solutions in the rooting capacity of cuttings (lateral sprouts) of *Callisia fragrans* are presented here. The EC and TDS of the biofertilizer-water mixture changed gradually according to the biofertilizer concentration. The biofertilizer solution was a good medium for the rooting of *C. fragrans* cuttings. The results could help to provide the crops with the necessary amount of mineral nutrients and regulate the suitability of irrigation during the entire vegetation period.

Key words: electrical conductivity, total dissolved solids, nutrient solution, sustainable agriculture, hydroponics, *Callisia fragrans*.

RESUMEN

La concentración de los nutrientes minerales suministrados es uno de los factores más importantes y limitantes para mejorar la eficiencia nutritiva de las plantas. La concentración óptima de la disolución nutritiva (DN) proporciona a las plantas la cantidad necesaria de nutrientes. Desde este punto de vista, la investigación sobre varios parámetros fisicoquímicos que caracterizan la concentración de la DN y la absorción de nutrientes por parte de las plantas sigue siendo un problema en la actualidad. Se muestran aquí los cambios de conductividad eléctrica (CE) y de los sólidos disueltos totales (SDT) en función de la concentración del biofertilizante, así como el papel de la disolución del biofertilizante sobre la capacidad de enraizamiento de las estacas (brotes laterales) de *Callisia fragran*s. Ambos parámetros CE y SDT de la mezcla biofertilizante con agua, cambian gradualmente dependiendo de la concentración del biofertilizante. La solución del biofertilizante fue un buen medio para el enraizamiento de estacas de *C. fragrans*. Los resultados podrían ayudar a proporcionar a los cultivos la cantidad necesaria de nutrientes y regular la idoneidad del riego durante todo el periodo vegetativo.

Palabras clave: conductividad eléctrica, sólidos disueltos totales, solución nutritiva, agricultura sustentable, hidroponía, *Callisia fragrans*.

Introduction

Biofertilizers are formulations composed of living latent cells of efficient strains of various microorganisms that help plants take up nutrients during the interaction in the rhizosphere. Due to the number of advantages these eco-friendly products are very popular in modern agriculture. Application of biofertilizers reduces and/or totally excludes the amount not only of chemical fertilizers, but also pesticides, insecticides, fungicides, and other chemicals and decrease the dangerous impact of chemicals on the environment. Due to special compositions, biofertilizers improve soil fertility and enhance crop productivity by providing healthy and ecologically safe bioproducts. Application of biofertilizers is an effective approach to sustainable agriculture. Biofertilizers are actively used for rooting cuttings, seed germination, and foliar nutrition (Wong *et al*., 2015; Alori & Babalola, 2018; González-Díaz *et al*., 2019). Nowadays, there are various biofertilizers in the global market labeled under different trademarks. The microorganisms of biofertilizers mostly include the N-fixing, P-solubilizing, P-mobilizing, K-solubilizing, S-oxidizing, Zn-solubilizing species, and plant growth promoting Rhizobacteria (PGPR) (Antoun & Prevost, 2005; Fuentes-Ramirez & Caballero, 2005; Vessey, 2015; Anli *et al*., 2020; Fasusi *et al*., 2021).

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Agriculture in regions with a dry climate may benefit from biofertilizers (Schütz *et al.*, 2018). González-Díaz *et al.* (2019) observe that biofertilizers inoculated with nitrogen fixing bacteria of the genera *Azotobacter* and *Azospirillum* contribute to the crop yield of *Eucalyptus grandis*. In another study (Onyia *et al.*, 2020), growth and yield of maize significantly improve when treated with biofertilizer. Moreover, the applied biofertilizer protect the plants from pathogen/insect attack (Onyia *et al.*, 2020).

Biofertilizers improve soil chemical and physical characteristics including with long-term action (Demir, 2020; Wang *et al.*, 2021). Biofertilizers are effective for application in both open and protected crops (Wu *et al.*, 2005; El-Ghandour *et al.*, 2009; González-Díaz *et al.*, 2019; Demir, 2020; Bergstrand, 2022).

The concentration of supplied nutrients is one of the most important and limiting factors for enhancing the efficiency of plant nutrition. If the concentration of nutrients is extremely low, plant growth is lowered. Extremely high concentrations of nutrients lead to osmotic stress, ionic toxicity, and growth restrictions (Sakamoto & Suzuki, 2020). Optimal concentrations of nutrient solutions (NS) will provide the plants with the necessary amount of nutrients. Two main parameters that best characterize the concentration of NS are electrical conductivity (EC) and total dissolved solids (TDS). Optimization of the concentration of NS is becoming an urgent issue, especially in hydroponics, as the nutrients in the supplied solution remain the main source for the plant nutrition. The EC of NS in hydroponics is in the range 0.8–4.0 dS m^{-1} (Sambo *et al.*, 2019).

The requirement for a concentration of NS, and therefore of an optimal EC and TDS of the NS, may be different for each plant. For example, Ding *et al.* (2018) showed that, for the hydroponic production of pakchoi, the optimal EC treatment should be 1.8 or 2.4 dS m^{-1}.

In conventional hydroponic systems, inorganic fertilizers are very common, as organic compounds in the NS inhibit plant growth and have been regarded as phytotoxic (Shinohara *et al.*, 2011). On the other hand, the use of organic fertilizers in hydroponics is important, as it will allow recycling organic compounds. Therefore, the application of biofertilizers in hydroponics remains the focus of the active study (Lee & Lee, 2015; Mendes *et al.*, 2017; Dewi *et al.*, 2021).

Research on several physicochemical parameters characterizing concentrations of NS and uptake of nutrients by the plants remains an actual problem.

In the frame of this study, changes were considered for the two most important parameters: EC and TDS in organic solutions of biofertilizer, depending on the concentration, as well as the role of the solution of the biofertilizer on the rooting capacity of the valuable medicinal plant *Callisia fragrans*.

Materials and methods

The experiments were carried out at the Laboratory of Plant Nutrition and Productivity of the G.S. Davtyan Institute of Hydroponic Problems (National Academy of Sciences, Republic of Armenia).

In the study, a biofertilizer Ecobiofeed+® was used. It was developed by the "Armbiotechnology" Scientific and Production Center (National Academy of Sciences, Republic of Armenia). This ecologically safe bioproduct, based on natural raw material, contains zeolites and a complex of nitrogen-fixing microorganisms: *Azotobacter vinelandii* (strain AV1) (Avetisova *et al.*, 2021) and *Rhizobium pusense* (strain RP1). This biofertilizer provides plants with macro- and microelements, vitamins, and protein amino acids.

Preparation of nutrient solution

The nutrient solution (NS) was prepared according to the following steps:

A) Ten ml of the biofertilizer were added to the glass container that contains 1000 ml of water (solution A). After the measurements, 10 ml of the biofertilizer were added to the solution A. This process was repeated by adding 10 ml of the biofertilizer each time, until content of the biofertilizer in solution A became 100 ml (solution B). The following ratios (v/v) of the biofertilizer and water were in this prepared solution: 0.01:1, 0.02:1, 0.03:1, 0.04:1, 0.05:1, 0.06:1, 0.07:1, 0.08:1, 0.09:1, and 0.1:1.

B) One hundred ml of the biofertilizer were added to the solution B. After the measurements, 100 ml of the biofertilizer were added to the solution B. This process repeated by adding for 100 ml of the biofertilizer each time, until the volume of the biofertilizer in solution B became 1000 ml. The following ratios (v/v) of the biofertilizer and water were in the obtained solution: 0.1:1, 0.2:1; 0.3:1, 0.4:1, 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, and 1.0:1.

Electrical conductivity and total dissolved solids

Electrical conductivity (EC) and total dissolved solids (TDS) of biofertilizer solution were measured depending on the concentration. Measurements were done with the

nutrient meter (Bluelab Truncheon Nutrient Meter, New Zealand). Resolution of the equipment was 50 mg L^{-1}, 0.1 mS cm^{-1}, and equipment accuracy: ± 50 mg L^{-1}, ± 0.1 mS cm^{-1}.

Rooting capacity

The lateral sprouts (cuttings without leaf rosette) of valuable medicinal plant *Callisia fragrans* were used. The plants were grown under open-air hydroponic conditions of the Ararat Valley (Karapetyan, 2020). Standard (10–15 cm length) cuttings of lateral sprouts were taken from the plants and immediately placed in plastic cups with a volume of 150 ml (5 cuttings per cup). The cups were filled up with a 120 ml solution of the biofertilizer-water and placed in a laboratory-controlled condition (18–20°C). Rooting was checked up daily. Along with the reduction of the volume of the solution, fresh solution of the biofertilizer-water was added, keeping the total volume at 120 ml. The solution of biofertilizer-water was prepared according to the following ratio: 10 ml of the biofertilizer was added to the 1000 ml of water.

Data analysis

Data were presented as means ± standard deviation SD (n= 4) that were calculated using GraphPad Prism 8 Software Package. The graphs were created with Microsoft Excel 2016.

Results and discussion

Electrical conductivity

In nutrient solutions (NS) containing 100 ml of the biofertilizer, electrical conductivity (EC) reached up to 1.05 mS cm^{-1}. Moreover, each 20 ml of the biofertilizer increased the value by 0.1 mS cm^{-1} (Fig. 1A). Upon increasing the concentration, the EC changed accordingly: the addition of each 100 ml of biofertilizer increased EC from 0.1:1 to 0.2:1 ratio by 0.5 mS cm^{-1}, from 0.2:1 to 0.5:1 ratio by 0.3 mS cm^{-1}, from 0.5:1 to 0.7:1 by 0.2 mS cm^{-1}, 0.7:1 to 1.0:1 by 0.15 mS cm^{-1} (Fig. 1B). EC of water was 0.5 mS cm^{-1}.

Total dissolved solids

In NS containing 100 ml of the biofertilizer, TDS reached up to 525 mg L^{-1}. Moreover, each 20 ml of the biofertilizer increased the value by 50 mg L^{-1} (Fig. 2A). Upon increasing the concentration, TDS changed according in the following: each 100 ml of biofertilizer added TDS: from 0.1:1 to 0.2:1 by 250 mg L^{-1}, 0.2:1 to 0.5:1 by 150 mg L^{-1}, 0.5:1 to 0.7:1 by 100 mg L^{-1}, 0.7:1 to 1.0:1 by 75 mg L^{-1} (Fig. 2B). TDS of water was 250 mg L^{-1}.

The changes of the above values were faster in comparatively diluted solutions. Upon increases of the concentration the changes became less. This could be explained by the fact that saturated solutions were created, and further addition of the fertilizer did not play a significant role on the strength level of the solution.

Rooting capacity

After 2-3 d from the beginning of the experiments the cuttings of *C. fragrans* that were placed in the solution of the biofertilizer-water developed green sprouts. Moreover, within two weeks all cuttings developed roots. In other studies, the efficiency of biofertilizers on the rooting of the plants has also been confirmed. Gortari *et al.* (2019) prove that mini-cuttings of yerba mate inoculated with *Trichoderma asperelloides* is distinguished by high rooting capacity as well as a great number and length of the roots. Efficiency of plant growth promoting microorganisms (PGPM) on rooting in plant tissue culture is also approved (Soumare *et al.*, 2021). The rooting percentage of *Eucalyptus* cuttings

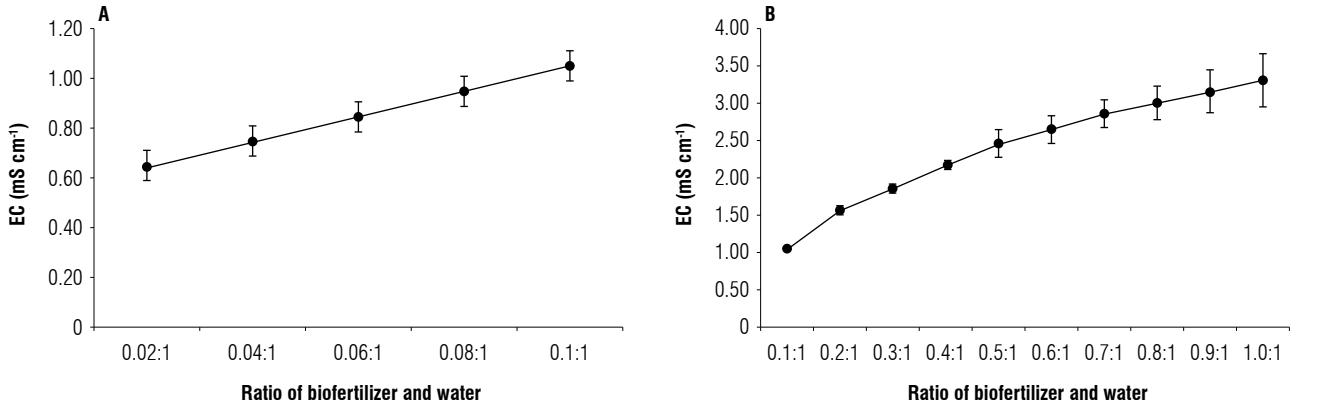

FIGURE 1. Electrical conductivity (EC) of biofertilizer solution depending on the ratio (v/v) of biofertilizer and water: A) from 0.01:1 to 0.1:1, B) from 0.1:1 to 1.0:1. Data are the mean of four replicates ± standard deviation.

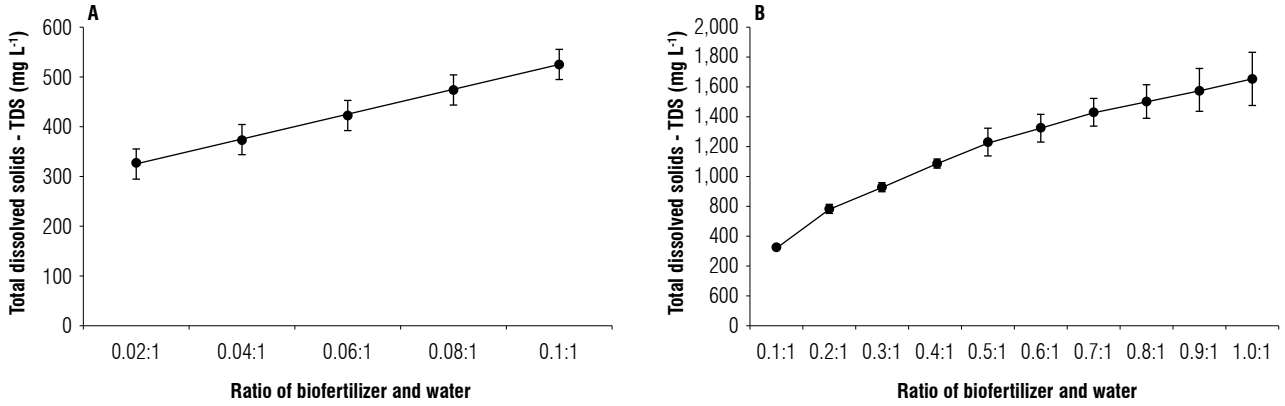

FIGURE 2. Total dissolved solids (TDS) of biofertilizer solution depending on the ratio (v/v) of biofertilizer and water: A) from 0.01:1 to 0.1:1, B) from 0.1:1 to 1.0:1. Data are the mean of four replicates ± standard deviation.

increases during interaction between indole-3-butyric acid and biofertilizer (Rajabi *et al*., 2015).

Conclusion

The EC and TDS of the biofertilizer-water solution changed gradually depending on the biofertilizer concentration. Moreover, the changes were faster in comparatively diluted solutions, upon increasing the concentration the changes became less. Within two weeks all cuttings developed roots. The measurement of EC and TDS of the applied NS could be important for evaluating the suitability of irrigation. The findings of the present paper are important for the application of biofertilizers in agriculture and provide valuable information.

Conflict of interest statement

The author declares that there is no conflict of interests regarding the publication of this article.

Author's contributions

AK developed and conducted the experiments, carried out the statistical analysis, interpreted the study data and wrote the scientific note.

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Acknowledgments

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Published dissertation or thesis references

Example: Franco, C. V. (2012). *Efecto de la colchicina sobre el número cromosómico, número de cloroplastos y características morfológicas del fruto en ecotipos de uchuva (*Physalis peruviana *L.) Colombia, Kenia y Perú* [Undergraduate thesis, Universidad Francisco de Paula Santander]. UFPS Library. http://alejandria.ufps.edu.co/descargas/tesis/1610259.pdf

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