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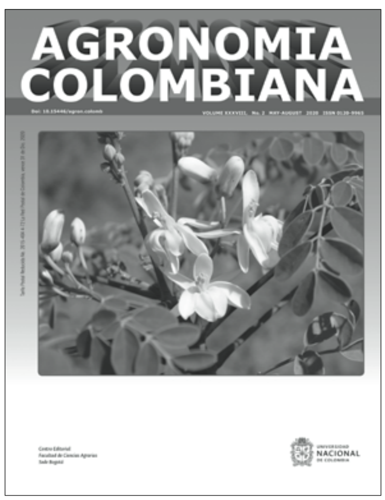

Our cover:

Nutritional characterization of Moringa oleifera leaves, seeds, husks and flowers from two regions of Mexico. Article on pages: 287-297

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Freedom to operate analysis, design and evaluation of expression cassettes that confer tolerance to glyphosate

Análisis de libertad de operación, diseño y evaluación de casetes de expresión que confieren tolerancia a glifosato

Jenny Jiménez-Barreto[1], Julián Mora-Oberlaender[1], and Alejandro Chaparro-Giraldo[1]*

ABSTRACT

Tolerance to the herbicide glyphosate is the most extended feature in commercial transgenic events released worldwide and it is an example of the successful use of genetic modification to improve weed control in crops. Glyphosate-tolerant genotypes have been developed by multinational corporations using patent-protected technologies. For some of these events, all associated patents have expired and, therefore, have become a good target for the development of herbicide-tolerant agbiogenerics by national research institutions, using local crop varieties. As a first step in this process, we present the design (*in silico*) of three expression cassettes with the purpose of using them in the transformation of Colombian soybean (*Glycine max*) varieties to confer them tolerance to glyphosate. We transformed *Nicotiana benthamiana* as a model to validate the functionality of the expression cassettes and detected the expression of the transgene by RT-PCR. Additionally, a Freedom to Operate analysis of the sequences used in the expression cassettes suggests that their commercial use in Colombia does not infringe third party rights. This analysis must be updated and validated by intellectual property experts prior to commercialization.

Key words: herbicide tolerance, codon usage, gene design.

RESUMEN

La tolerancia al herbicida glifosato es la característica predominante en los eventos transgénicos liberados comercialmente en el mundo, siendo un ejemplo del uso exitoso que ha tenido la transgénesis para mejorar el control de malezas en diferentes cultivos. Los genotipos tolerantes al glifosato han sido desarrollados por compañías multinacionales usando tecnologías protegidas por patentes. Para algunos de estos eventos todas las patentes asociadas finalizaron recientemente, y por lo tanto se han convertido en candidatos para desarrollar agrobiogenéricos resistentes a herbicidas derivados de genotipos locales a partir de investigaciones nacionales. Como primer paso del proceso, presentamos el diseño *in silico* de tres casetes de expresión con el fin de utilizarlos en la transformación genética de variedades colombianas de soya (*Glycine max*) para conferirles tolerancia al glifosato. Se transformó genéticamente la planta modelo *Nicotiana benthamiana* para validar la funcionalidad de los casetes y se detectó la expresión del transgén mediante RT-PCR. Adicionalmente un estudio de libertad de operación de las secuencias utilizadas en los casetes sugiere que su uso comercial en Colombia no viola derechos de terceros. Este análisis debe ser actualizado y validado por expertos en propiedad intelectual antes de la comercialización.

Palabras clave: tolerancia a herbicidas, uso codónico, diseño de genes.

Introduction

Glyphosate is the most used herbicide worldwide. It is a broad-spectrum herbicide that acts by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase or EPSPS (EC 2.5.1.19) (Duke, 2018). This enzyme is a key element of the shikimate pathway, by which plants produce metabolites derived from chorismate, including the aromatic amino acids tyrosine, phenylalanine, tryptophan, and physiologically important compounds such as pABA and Coenzyme Q10 (Tzin and Galili, 2010). Specifically, EPSPS catalyzes the synthesis of 5-enolpyruvyl-shikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) (Maeda and Dudareva, 2012). Given the structural similarity between glyphosate and PEP, the first acts as a competitive inhibitor of EPSPS, impeding the reaction it catalyzes (Funke *et al*., 2006), and therefore, hindering plant growth and eventually causing its death.

EPSPS is present in plants, bacteria and fungi, but not in animals, which do not synthesize their own aromatic amino acids (Padgette *et al*., 1995). There are several natural variants of EPSPS that are not affected by glyphosate; most

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are of bacterial origin (Barry *et al.*,1997; Yan *et al.*, 2011; Cao *et al.*, 2012), and some have been reported in plants (Mao *et al.*, 2016). Some of these variants have been used to generate glyphosate-tolerant transgenic plants, and the EPSPS from the *Agrobacterium tumefaciens* strain CP4 (Barry *et al.*, 1997) is the most commonly used in commercial transgenic crops.

CP4:EPSPS is a class II EPSPS enzyme with high enzymatic efficiency and tolerance to inhibition by glyphosate. It also shows a low K_m (Michaelis constant) for PEP [12 μM] (Barry *et al.*, 1997), indicative of a high affinity for this substrate. Barry *et al.* (1997) identified several class II EPSPSs, of which CP4:EPSPS showed the best kinetic parameters. Its tolerance to glyphosate is partly explained by the presence of an A100G residue in the enzyme's active site. Funke *et al.* (2006) restored CP4:EPSPS glyphosate susceptibility by means of an Ala100-Gly100 substitution which generates a slight change in its three-dimensional structure that allows the binding of glyphosate in an extended conformation that inhibits the enzyme. The presence of an A100G residue generates a narrower active site that only allows the binding of glyphosate in a condensed conformation that does not inhibit the enzyme (Funke *et al.*, 2006).

A transgenic plant with a functional *cp4:epsps* gene in its genome is glyphosate-tolerant because the presence of CP4:EPSPS is enough to ensure the synthesis of the required aromatic amino acids. In contrast, conventional plants when exposed to commercial doses of the herbicide show a marked reduction in growth and eventually they die (Padgette *et al.*, 1995).

One of the most important issues related to the production of a glyphosate-tolerant transgenic plant is to ensure good expression levels of the transgene. Gene sequence modifications as codon usage or guanine-cytosine (GC) content may improve transcription levels and protein production (Chou and Moyle, 2014; Jeong *et al.*, 2017; Sivamani *et al.*, 2019).

In addition to biologically relevant aspects for the design of the expression cassette, our work also considers that the genetic elements used have freedom to operate in Colombia. A Freedom to Operate (FTO) analysis is an evaluation of the intellectual property (IP) aspects involved in a research project or in the development of a product to make sure it can be carried out with a low or tolerable probability of infringing current patents or other IP rights (Bennett *et al.*, 2008). Performing this analysis is vital during the first stages of the research process if it involves commercial interests (Mora-Oberlaender *et al.*, 2018) such as in the present study.

Here we present the *in silico* design of three expression cassettes for conferring glyphosate tolerance to plants. The sequence of the *cp4:epsps* gene was optimized towards soybean codon usage. In our approach, the *cp4:epsps* gene is both the gene of interest and the selection marker. The objective of this study was to assess the functionality of the expression cassettes by transforming the model plant *Nicotiana benthamiana*. Additionally, in order to advance the development of herbicide tolerance agbiogenerics, we carried out an FTO analysis for the elements of the expression cassettes in order to establish if their eventual commercial use would affect third party rights.

Materials and methods

In silico design of expression cassettes

Three different expression cassettes that would confer glyphosate tolerance were designed and denominated as E-IGP, E-IGP2, and E2. The coding sequence used in the three cassettes corresponds to that of the *cp4:epsps* gene. As promoters we used either the soybean polyubiquitin, Gmubi, (Chiera *et al.*, 2007), the single (Odell *et al.*, 1985) or the 1 duplicated (Kay *et al.*, 1987) version of the CaMV35 promoter (Fig. 1).

In plants, the *epsps* gene is found in the nuclear genome. When expressed, EPSPS is transported to the chloroplast by means of a transit peptide, CTP, which is then removed by a site-specific metalloprotease (Della-Cioppa *et al.*, 1986). Therefore, we included a signal sequence, CTP, in the 5' end of the coding sequence.

The sequences for the coding regions of the expression cassettes were obtained from published patents. Specifically, the *cp4:epsps* gene and CTP transit peptide from *Petunia hybrida* correspond to SEQ ID 9 (gi:2469099) and SEQ ID 14 (gi:2469102), respectively, from patent US 5633435 (Barry *et al.*, 1997). The sequences of the promoter regions were downloaded from NCBI, where the Gmubi promoter corresponds to gi:162280984 and the CaMV35S promoter corresponds to gb|HQ698853.1|:1967-2514.

The designed expression cassettes were based on a previous study (Jiménez and Chaparro-Giraldo, 2016). The coding sequence or open reading frame (ORF) of the *cp4:epsps* gene was optimized according to the following criteria using the software Visual Gene Developer-VGD 1.3 (Jung and McDonald, 2011): modifications for soybean codon usage, removal of cryptic splice sites and of premature polyA sites. The final optimized sequence was selected according to parameters such as the codon adaptation index (CAI) (Sharp and Li, 1987), effective number of codons (Nc) (Wright, 1990), and GC content. All modifications of the nucleotide sequence were synonymous, leaving the original amino acid sequence of the coded protein unchanged. Gene and regulatory sequences were assembled into expression cassettes, and restriction sites were introduced using the software Gene Designer 2.0 (Villalobos *et al.*, 2006). We carried out an *in silico* translation analysis on a primary structure level. NCBI's ORF Finder and Blast-x were used to predict the expressed amino acid sequence.

Each of the three expression cassettes designed and evaluated *in silico* were synthesized and cloned into a pCAMBIA 1301 vector from which the plant-selection gene and reporter gene were removed. They were then used to transform cells of *A. tumefaciens*, strain LBA4404, by electroporation. Transformed bacterial cells were selected phenotypically and evaluated by PCR.

Genetic transformation of *Nicotiana benthamiana*

In order to use glyphosate as a selection agent for transformed plants, we measured the sensitivity of *Nicotiana benthamiana* to different concentrations of the herbicide. Leaf explants were placed on R media (1X MS salts, 1X Gamborg vitamins, 30 g L^{-1} sucrose 1 mg L^{-1} BAP, 7 g L^{-1} agar PTC, and pH 5.8) with increasing concentrations of glyphosate: 0, 10, 25, 50, 100, 250, 500, 1000, and 2000 μM. These concentrations were chosen accordingly to what has been reported for the closely related species *Nicotiana tabacum* (Wang *et al.*, 2003; Fathi-Roudsari *et al.*, 2009; Akbarzadeh *et al.*, 2010; Yan *et al.*, 2011). The herbicide (tissue culture grade N-(Phosphonomethyl) glycine, PhytoTechnology®) was added to the growth medium prior to pH adjustment.

Transformation of *N. benthamiana* was mediated by *A. tumefaciens*. Bacteria were grown on liquid LB medium with acetosyringone (200 μM), kept at 28°C and shaken at 200 rpm until they reached an optical density (OD) of 0.6. Square-shaped (approximately 1 cm^2) leaf explants were used for infection by placing them in liquid coculture medium for two minutes with a bacterial suspension and then transferred to solid coculture medium for 24 h (R medium with 200 μM acetosyringone). Explants were then washed five consecutive times at room temperature and 100 rpm in order to remove bacteria. They were then dried on sterile paper towels and placed on selective medium 1 (R medium with 500 mg L^{-1} carbenicillin). After two weeks, explants were transferred to selection medium 2 (R medium with 500 mg L^{-1} carbenicillin and 10 μM glyphosate). Regenerants were placed in propagation medium (1X MS salts, 1X Gamborg vitamins, 30 g L^{-1} sucrose, 2.5 g L^{-1} Gelzan, and pH 5.8) and kept *in vitro* under controlled photoperiod (16/8) and temperature (28°C) until they were used for molecular tests. Untreated explants were used as controls. Absolute control explants were placed on R medium, and negative control explants were placed on selective medium. All assays were carried out with four-week-old plants.

Molecular tests

Potential transformants of *N. benthamiana* were evaluated by conventional PCR. We designed specific primers for the version of the *cp4:epsps* gene in each expression cassette. Plants potentially transformed with cassettes E-IGP or E-IGP2 were tested with primers E-IGP1FW: TCACCATGGGGCTTGTAG and E-IGP1RV: GCTATACGGTGATCGAGATGC. PCR conditions used were an initial denaturation cycle (95°C x 10 min) followed by 35 amplification cycles (95°C x 60 sec, 61°C x 90 sec, 72°C x 90 sec) and a final elongation (72°C x 5 min). For the E-2 cassette we used primers E-2FW: ATATCCGATTCTCGCTGTCG and E-2RV: CCATCAGGTCCATGAACTCC. Here, we used Kapa Biosystems polymerase and the PCR conditions were one initial denaturation cycle (95°C x 6 min) followed by 55 amplification cycles (95°C x 20 sec, 66 °C x 15 sec, 72°C x 40 sec) and a final elongation (72°C x 30 sec). Amplification products were visualized by agarose gel electrophoresis stained with Ethidium bromide

Plants that showed a PCR product of the expected band size were selected for RT-PCR analysis. Total leaf RNA was extracted using Norgen Bioteck Corp. RNA/DNA/protein purification kit (Thorold, Canada). DNA contamination was eliminated with the DNaseI RNA free kit from ThermoScientific (Waltham, Massachusetts, USA), and its effectiveness in removing DNA was tested by conventional PCR for the constitutive actin gene using primers FW:TGGTACAAGGGTCCATAGCG and RV: GCCGTCCTCTCTCTGTATGC. These primers generate a 518 bp amplicon. cDNA synthesis was performed using the First strand cDNA synthesis kit from ThermoScientific (Waltham, Massachusetts, USA), and its quality was checked using the actin primers in a PCR assay. Expression of the genes in E-IGP, E-IGP2 and E-2 was evaluated by PCR assays using the primers described above.

For all PCR assays, plasmid DNA was used as positive control, DNA from a non-transformed plant was used as negative control, and the reaction mixture without DNA was used as an absolute control.

PCR positive individual plants (4-5-week-old) were hardened for phenotypic evaluation. They were transferred to a 3:1 peat:soil mixture and kept under controlled growth conditions as above. Plants kept under these conditions for one month were used for evaluation by applying 0.2% glyphosate on the entire aerial plant surface. The assay was performed in duplicate. The outcome of the test was evaluated 15 and 30 d after herbicide application.

Freedom to Operate Analysis (FTO)

In order to establish the potential for the eventual commercial use of transgenic plants transformed with the expression cassettes described here, we performed an FTO analysis of the genetic elements they included. This analysis was limited to Colombia. First, a patent search was carried out in three international patent databases, The Lens (https://www.lens.org/lens), Patentscope (http://www.wipo.int/patentscope/en/), and Spacenet (http://www.epo.org/). The search was then performed in the database of the Colombian Superintendence of Industry and Commerce (SIC), which is the national authority for patents (http://www.sic.gov.co/es/banco-patentes). All these databases are publicly available at no cost. In the international patent databases, we used key terms to search within the claims to identify patents related to the genes and regulatory sequences used. Once identified, the national database was queried using key terms to search within the relevant fields (inventor, assignee and title). All retrieved documents were analyzed to determine if the gene sequences or regulatory elements are protected by IP rights in Colombia. The analysis was updated to November 2019.

Results and discussion

In silico design of expression cassettes

Previous research has shown the use of glyphosate as a selection agent in the genetic transformation of plants from several species like rice, maize, cotton and soybean, using concentrations in a range from 0.5 mM to 10 mM of the growth medium (Latif *et al*., 2015; Ren *et al*., 2015; Soto *et al*., 2017). Most reports of genetic transformation of *N. tabacum* to confer tolerance to glyphosate use antibiotics to select transformed plants (Wang *et al*., 2003; Yan *et al*., 2011; Peng *et al*., 2012), but there is evidence of the use of glyphosate to select transformants in this species (Akbarzadeh *et al*., 2010). Here, we designed synthetic versions of the *cp4:epsps* gene in order to use it as both the gene of interest and a selectable marker. The different versions of the gene were included in three expression cassettes, E-IGP, E-IGP2 and E-2. The first two included the gene with modifications for codon-usage optimization in soybean in order to enhance its expression in that species. The third expression cassette carried the native sequence of the gene.

Codon-usage modification has been shown to be one of the most important factors for obtaining good levels of expression for a heterologous gene (Yan *et al*., 2011; Kucho *et al*., 2013; Sivamani *et al*., 2019). Codon bias is usually measured by CAI (Sharp and Li, 1987). Most genes used in genetic transformation in published research are modified to favor the codon usage of the transformed species, but a few studies give the CAI or the value of some other parameter for the modified sequence. Kucho *et al.* (2013) improved the translation efficiency of the gene for gentamicin tolerance using a modified version with a CAI of 0.835. A similar effect was reported for the expression of the *Toxoplasma gondii* SAG1 antigen in tobacco, in which a CAI of 0.83 was obtained (Laguía-Becher *et al*., 2010). Yan *et al.* (2011) found that modified sequences of the *Pparo1* gene with CAI values of 0.7 and 0.9 conferred good tolerance to glyphosate in transgenic tobacco plants. Accordingly, we modified the coding sequence of the *cp4:epsps* gene until a CAI of approximately 0.8 was achieved. In total, 325 codons (71.42%) from 455 codons of *cp4:epsps* coding sequence were optimized, where the synthetic gene sequence was 74% identical to the native gene sequence.

Genetic expression is a complex process and codon usage is not the only factor determining its efficiency (Jung and McDonald, 2011). During gene design it is also important to consider the GC percentage of a sequence, a factor that is related to codon usage. In plants, GC content is higher in monocots than in dicots, especially when considering the third base of each codon [GC3] (Clément *et al*., 2014; Singh *et al*., 2016). By optimizing the coding sequence, Li *et al.* (2013) were able to improve expression levels of the gene *cry1Ah* and resistance to insects in plants. Through codon-usage modifications they changed the GC content from 37-48% (native gene) to 55-63% (designed genes) and found greater expression of the designed genes both at the mRNA and protein levels, which led to a better resistance to insects. The unmodified *cp4:epsps* sequence has a high GC content (66%), especially in GC3 (84.21%). This value is notably higher than that reported for plant genomes (Singh *et al*., 2016). Using Visual Gene Developer 1.3, we changed the total GC content of the gene sequence to a final value of 51.78% and we changed the GC3 content to 48.21%. The percentage of GC3 is the most notable factor in discriminating codon usage between monocots and dicots. In the first group, 16 out of 18 amino acids favor G or C in the third position, while in dicots this number is 7 out of 18 amino acids (Murray *et al*., 1989). A high GC3 content in mRNA codons also leads to a higher potential for the formation of hairpin structures, which can affect the expression and stability of mRNA (Barry *et al*., 1997). Given this, reaching a GC3 content of 48.21% of *cp4:epsps* seems appropriate for its expression in soybean and other dicots. The total GC content in cassette E-2 (unmodified *cp4:epsps* gene) is 54.58%, while the cassettes E-IGP and E-IGP2 reached 44.64% and 47.36%, respectively, closer to the value reported for soybean (46%).

The effective number of codons (Nc) (Wright, 1990) measures the degree of synonymous codon usage bias with a number ranging between 20 and 61. If Nc is 20, codon usage is extremely biased, while an Nc of 61 corresponds to a homogeneous use of synonymous codons. The original *cp4:epsps* sequence has an Nc of 27, which is a relatively biased codon usage. In the modified version, we obtained an Nc of 49.5.

Every change made in a DNA sequence, even if it is to make it closer to the codon usage pattern of the host plant, can lead to new unexpected signals, such as cryptic splice sites or premature polyA sites. Any such sites were eliminated by synonymous codon changes that did not alter the reading frame. Figure 1 shows a diagram of the expression cassettes designed as described above. By *in silico* translation, using ORF finder and Blast-x, we checked that these cassettes effectively code for the CP4:EPSPS protein.

Genetic transformation of *Nicotiana benthamiana*

We evaluated the effect of glyphosate on the *in vitro* regeneration of *N. benthamiana*. In a non-treated control, the first sprouts were visible four weeks after planting and they were present in 98% of explants (Fig. 2). Explants planted under the lowest evaluated glyphosate concentration (10 µM) had a similar regeneration percentage, while at 25 µM there was a drastic reduction (11.4%). In a concentration of glyphosate equal or above 50 µM we observed no regeneration. In the selection of transgenic *N. tabacum* plants, Akbarzadeh *et al*. (2010) use 0.1 mM glyphosate. Our results show that for *N. benthamiana* this concentration not only inhibited regeneration but was lethal for the explants.

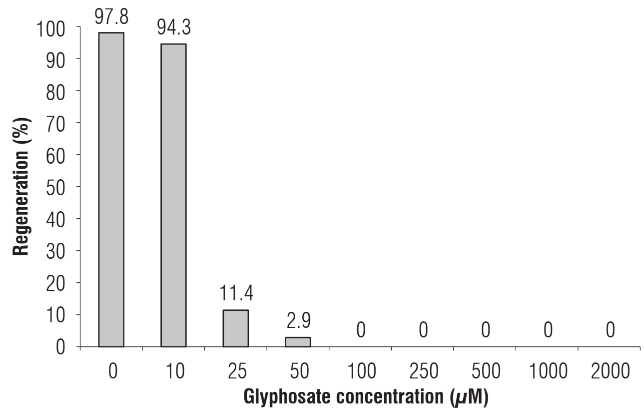

FIGURE 2. Regenerative response of *N. benthamiana* explants under different glyphosate concentrations.

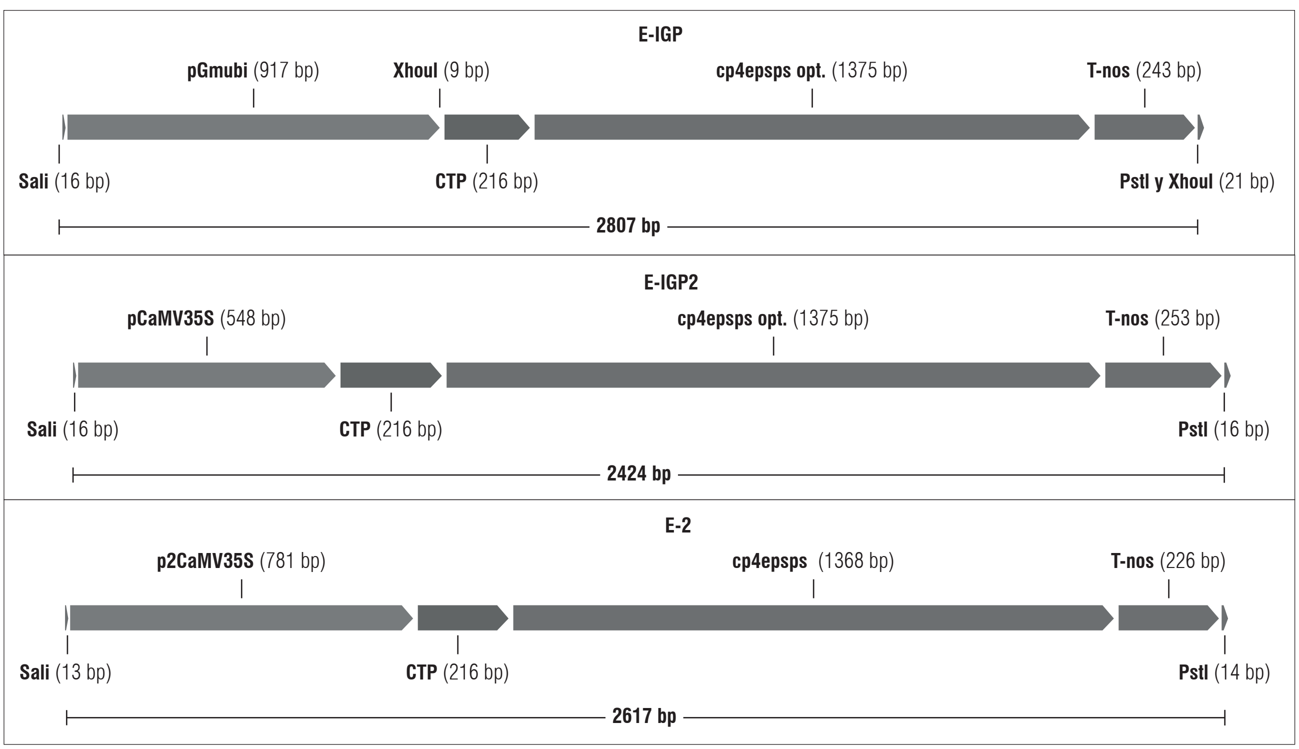

FIGURE 1. Schematic diagram of the three expression cassettes designed using Gene Designer 2.0 (Villalobos *et al*., 2006).

The three expression cassettes we designed were used for independent genetic transformation assays of *N. benthamiana*. Initially we used 25 μM glyphosate for the selection of possible transformants, but most regenerants had an abnormal phenotype (data not shown). Fathi-Roudsari *et al.* (2009) compared two different selection strategies using glyphosate in *N. tabacum*. The authors found better regeneration results when using a glyphosate-free medium for two weeks and then an initial selection concentration of 5 mM, which they doubled every two weeks up to a lethal dose of 50 mM. Accordingly, we used the two-week incubation period with no herbicide present and then transferred the explants to a medium with 10 μM glyphosate. We observed the first regenerants between weeks three and four.

Transformation assays with the expression cassettes E-IGP, E-IGP2 and E2 were set up with 80 explants each. We obtained 18 (22.5%), 37 (46.25%) and 25 (31.25%) regenerants, respectively. These values are notably lower than the regeneration percentage (95% average) for control explants not subjected to transformation or selection with the herbicide, showing the negative effects of the transformation and selection process on regeneration. The two-week incubation period without the selective agent was effective in reducing the amount of abnormal regenerants. Most of the co-cultured explants produced normal regenerants, while those used as negative control (non-transformed explants placed in identical selection conditions) generated mostly abnormal regenerants.

Molecular tests

Plants that were phenotypically selected as possible transformants were evaluated by PCR. The expected size of the amplified fragment generated by the primers designed to detect the *cp4:epsps* gene in the expression cassettes E-IGP and E-IGP2 is 907 bp. This fragment size was effectively detected in three plants transformed with E-IGP and six with E-IGP2 (Fig. 3). For those plants transformed with E-2, the expected fragment of 341 bp was detected in seven individuals. Despite losing some of the initial explants due to *A. tumefaciens* contamination, the transformation efficiency for each of the expression cassettes was 16.6% (E-IGP), 16.2% (E-IGP2) and 28% (E2). Glyphosate has been used successfully as a selective agent for transformed plants in cotton (Latif *et al.*, 2015), soybean (Soto *et al.*, 2017), maize (Ren *et al.*, 2015), and other species. We obtained transformed *N. benthamiana* plants using this selection approach, but it was with a low transformation efficiency, possibly due to the effect of the herbicide on the regeneration capacity of the explants.

In most plants for which a positive PCR result was obtained, we also detected the presence of a primary transcript by RT-PCR (Fig. 4). This suggests that the expression cassettes in these plants are functional. In two plants in which we detected the presence of the transgene by PCR (each one from cassettes E-IGP2 and E-2), there was no evidence of *cp4:epsps* mRNA by RT-PCR. Different factors may account for this result such as a position effect, since the transgene may have integrated into a heterochromatic region of the genome or in highly repetitive regions that may hinder expression (Kohli *et al.*, 2006). Other factors include the possible silencing of the transgene due to a multiple number of copies integrated into the genome (Velten *et al.*, 2012; Khuong *et al.*, 2013) or methylation processes (Rajeevkumar *et al.*, 2015). Regarding the last issue, the 35S sequence

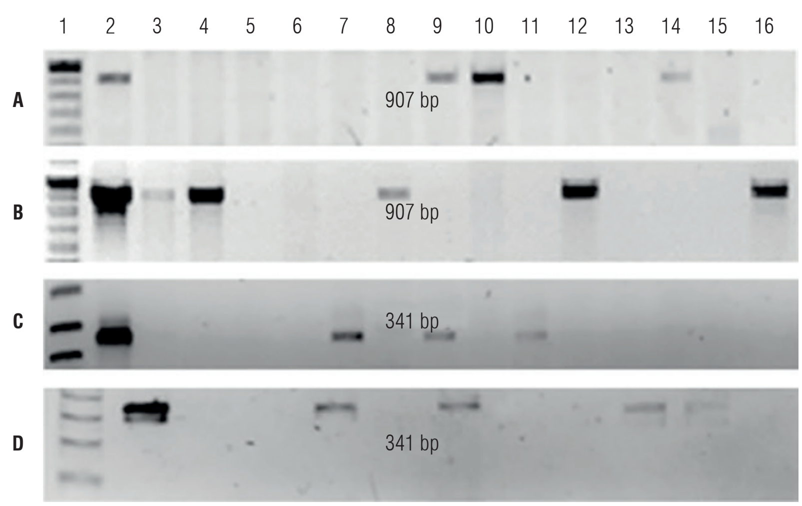

FIGURE 3. PCR identification of transformed plants. PCR product from plants transformed with A) E-IGP cassette, B) E-IGP2 cassette, and C and D) E-2 cassette. 1, molecular weight marker; 2, positive control; 3-16, PCR product from evaluated plants.

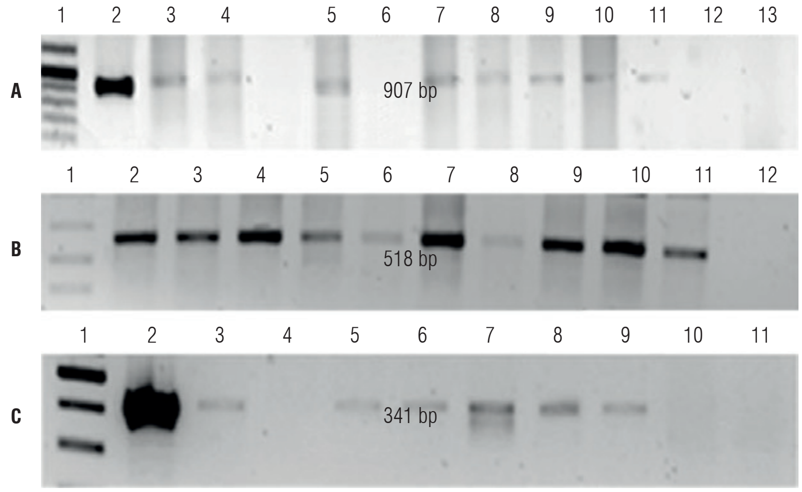

FIGURE 4. RT-PCR assay of transformed plants. A) Plants transformed with cassettes E-IGP and E-IGP2, primers: E-IGP. 1, molecular weight marker (MW); 2, positive control; 3-5, plants transformed with cassette E-IGP; 6-11, plants transformed with cassette E-IGP2; 12, negative control; 13, absolute control. B) Amplification of endogenous gene with Actine primers. 1, MW; 2-11, evaluated plants; 12, absolute control. C) Plants transformed with cassette E-2, primers E-2. 1, MW; 2, positive control; 3-9, evaluated plants; 10, negative control; 11, absolute control.

used in two of the expression cassettes has been associated with silencing by methylation (Okumura *et al*., 2016; Shimada *et al*., 2017; Wang *et al*., 2017). Additional research would be needed in order to determine the cause of the absence of *cp4:epsps* mRNA in these two plants.

Phenotypical evaluation

PCR-positive plants that were successfully hardened and established were evaluated phenotypically to further test the functionality of the expression cassettes. Glyphosate (0.2%) was applied on all the shoots. In non-transformed controls, wilting was evident 8 d after treatment and death occurred 15 d after treatment. Transformed plants showed no negative effects during this period (Fig. 5). These results, together with the molecular evidence of the presence and transcription of the transgene, indicate that the cassettes we designed effectively confer glyphosate tolerance to transformed plants.

Freedom to Operate Analysis

An FTO analysis is a valuable tool which could be used as a strategy towards the development of agbiogenerics. An FTO analysis of a genetically modified crop should include all the elements involved in its obtention, such as expression cassettes, vectors, plant material, laboratory protocols, etc. (Mora-Oberlaender *et al*., 2018). As a first step in this process, an FTO analysis of the designed expression cassettes was performed. The patents related to the sequences of the different genetic elements were identified and analyzed. The most relevant ones are summarized in Table 1. Our search yielded several patents in the United States, most of which have already expired. The sequence of

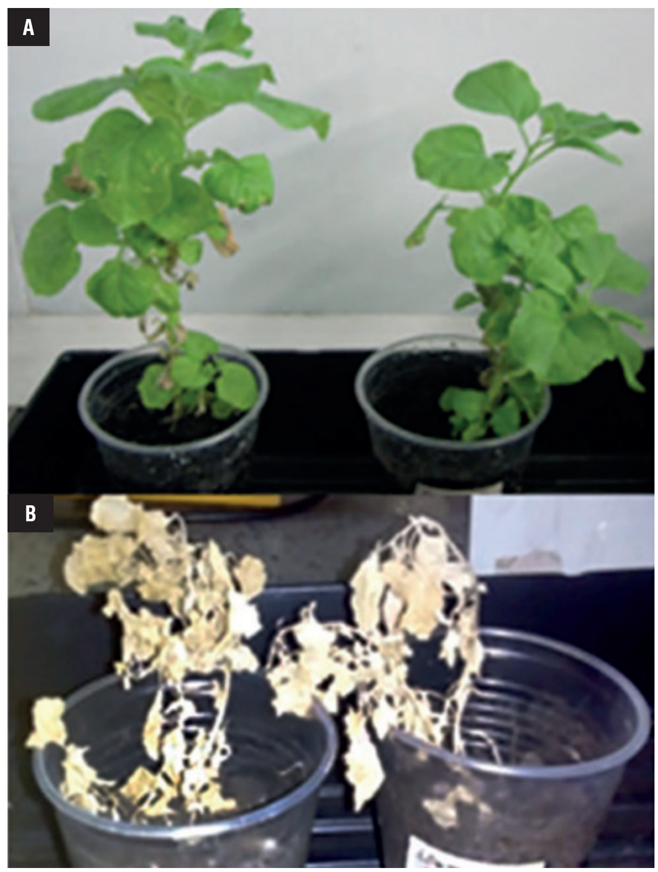

FIGURE 5. Glyphosate-treated *N. benthamiana* plants. A) Transformed plants; B) Untransformed control plants.

the gene *cp4:epsps* (patent US 5633435) became part of the public domain in 2014 and its patent was never requested in Colombia. No requested or assigned patents for the

TABLE 1. Patents and patent requests related to the sequences used in the expression cassettes.

Element	Requests/ patents	Title/subject of protection	Expiration year	Requested in Colombia
CaMV35S promoter	US 5352605 US 5530196	Use of p35S and/or p19S in genetic constructs. Use of such constructs in dicots.	2013	No
CaMV35S promoter	US 5196525	Enhancer of p35S that increases transcription products.	2011	No
	US 5322938	DNA construct with a duplicated p35S enhancer. Plant cells with such a construct.	2011	No
Duplicated Gmubi promoter	WO2008140766A2US8395021B2	Use of various soybean promoters, including Gmubi. Use of such promoters in genetic constructs, transgenic plants and products from such plants.	2028	No
***cp4:epsps* gene**	WO92/04449 US5633435 US5627061	DNA sequence of the gene, its use in genetic constructs for plant transformation and constructs with specific promoters, terminator sequences and transit peptides. Process to produce glyphosate tolerant plants in different crops. Method to control weeds.	2014	No
***cp4:epsps* gene**	WO06/130436US 7632985 US 8053184 US 7608761	Glyphosate tolerant soybean event MON89788. The protection includes the construct, flanking sequences of the insertion site, the method of detecting the event, seeds and other derived products of the event.	2026	Yes
	07136194	Event MON89788, the construct used and flanking sequences of the insertion site.	2026	Yes
	US7608761	Method for the control of pathogen caused diseases in soybean event MON89788, using glyphosate.	2026	No
	07026332	Promoters and regulator sequences of *N. tabacum, Arabidopsis thaliana,* and *Medicago truncata*. Their use in constructs to confer herbicide tolerance.	2025	Yes
***P. hybrida* transit peptide**	US 5633435 USRE039247E1	Use of the transit peptide in a sequence including a promoter, a sequence coding EPSPS and a terminator region.	2014	No
pCambia vector	US6641996B1 US7176006B2	Use of pCAMBIA vectors containing the *gus* plus cassette	2019	No
	US 7148407	Use of expression vectors containing a *gus* gene of fungal origin	2024	No

promoter regions we used were found in the Colombian jurisdiction. The only patent directly related to the subject of interest is in the SIC database under file number 07136194. This patent protects the use of a sequence that includes all or part of the expression cassette in soybean event MON89788 and its flanking sequences, which, in other words, protects the event itself. Therefore, this patent does not affect the use of the sequences used in the expression cassettes we designed and described here.

Conclusions

We designed three expression cassettes (E-IGP, E-IGP2 and E2) that included the gene *cp4:epsps* and conferred tolerance to glyphosate to transformed plants. Two of these cassettes (E-IGP and E-IGP2) included a codon-usage modification to favor expression in soybean. The functionality of these cassettes was evaluated by genetic transformation of the model plant *N. benthamiana* and the phenotypical testing of transformed lines as well as molecular assays such as PCR (to determine presence or absence of the transgene) and RT-PCR (to detect transcription). We detected the presence and transcription of the transgene as well as tolerance to the herbicide in plants transformed with each of the three expression cassettes. This suggests that they are all functional and could be used further in genetic transformation of plants. The FTO analysis we performed suggested that the potential commercial use of these cassettes does not infringe third-party rights in Colombia. This analysis, however, must be validated periodically before commercial use.

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Selection of soybean (*Glycine max* (L.) Merril) genotypes for cultivation in the Brazilian Brejo Paraibano

Selección de genotipos de soya (*Glycine max* (L.) Merril) para su cultivo en el Brejo Paraibano brasileño

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ABSTRACT

Soybeans have great commercial value and there are still regions with no indications of promising genotypes, such as Brejo Paraibano region. The objective of this study was to analyze the adaptability of 20 soybean genotypes selected with a relative maturity degree above 9. The genotypes used were: M8867RR, M8766RR, M9144RR, SYN1059 RR, M-SOY 9350, ST 920 RR, TMG1175RR, M8372IPRO, M8527RR, M8644IPRO, TOPAZIO RR, UFV - 16 (Capinópolis) and UFVS: Berilo RR, 2013, 2012, 2010, 2005, QUARTZO, 2001, and 2008. A completely randomized experimental design was used with five replicates. The data were subjected to analysis of variance and Tukey test to compare the means. From the Mahalanobis generalized distance, it was possible to apply the Tocher and UPGMA tests. In addition, using the Singh method and canonical correlation, the quantification of the characteristics that contributed most to genetic divergence was evaluated. Only ten genotypes reached the physiological maturity, and the following variables were evaluated: hypocotyl length, leaf width and length, plant height, flower diameter, and seed width and length. The characteristics leaf width, flower diameter, and seed width were significant (P<0.05) and obtained values above 1 for the ratio between genotypic variation and environmental variation (CV_G/CV_E). The higher means for the significant characteristics were observed in the genotypes SYN1059 RR, UFVS: Berilo and 2013. The genotypes SYN1059 RR, M8766RR, M8867RR, UFVS: Berilo, 2012 and 2013 were selected as the most suitable parental material to use in breeding programs.

Key words: characterization, genetic diversity, genetic breeding, adaptability, superior genotypes.

RESUMEN

La soya tiene un gran valor comercial y todavía hay regiones sin indicaciones de genotipos tolerantes, como la región del Brejo Paraibano. El objetivo de este estudio fue analizar la adaptabilidad de 20 genotipos de soya seleccionados con grado de maduración relativo superior a 9. Los genótipos utilizados fueron: M8867RR, M8766RR, M9144RR, SYN1059 RR, M-SOY 9350, ST 920 RR, TMG1175RR, M8372IPRO, M8527RR, M8644IPRO, TOPAZIO RR, UFV-16 (Capinópolis), UFVS: Berilo RR, 2013, 2012, 2010, 2005, QUARTZO, 2001 y 2008. El experimento fue conducido bajo diseño completamente al azar con cinco repeticiones. Los datos fueron sometidos a análisis de varianza y test de Tukey para comparación de medias. Por medio de la distancia generalizada de Mahalanobis fue posible aplicar las pruebas de Tocher y UPGMA. Además, con el método Singh y la correlación canónica evaluamos la cuantificación de las características que más contribuyeron a la divergencia genética. Sólo diez genotipos alcanzaron la madurez fisiológica de los cuales se evaluaron las siguientes características: longitud del hipocótilo, longitud y ancho de la hoja, altura de planta, diámetro de la flor, y longitud y ancho de la semilla. Las características del ancho de la hoja, diámetro de la flor y ancho de la semilla fueron significativas (P<0.05) y obtuvieron entre sí valores superiores a 1 para la relación entre el coeficiente de variación genética y el coeficiente de variación ambiental (CV_G/CV_A). Los promedios más altos de las características significativas se observaron en los genotipos SYN1059 RR, UFVS: Berilo RR y 2013. Los genotipos SYN1059RR, M8766RR, M8867RR, UFVS: Berilo RR, 2012 y 2013 fueron seleccionados como los materiales parentales más apropiados para usar en programas de mejoramiento.

Palabras clave: caracterización, diversidad genética, mejoramiento genético, adaptabilidad, genotipos superiores.

Introduction

Soybean (*Glycine max* (L.) Merril) is one of the main oleaginous crops in the world (Cruz *et al*., 2016). This quality relates to its productive potential, chemical composition, and nutritive value, which allows it to have multiple uses, for both human and animal feeding. This crop is also of socio-economic importance since it is a raw material that

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is indispensable to boost several agro-industrial complexes (Mauad *et al*., 2010).

Brazil is the second-largest producer of soybeans; in the 2017/2018 harvest, it produced 116,996 million t, being only behind the United States, which produced 119,518 million t of this grain (EMBRAPA, 2018). After the 1970s, soybean production became of high relevance to agribusiness. Currently the Brazilian territory has the availability of natural resources that can be favorable for the maintenance and expansion of its production (Silva *et al*., 2011). However, some regions with production potential do not have recommended and tested genotypes, such as the Brejo da Paraiba region.

Plant breeding programs can overcome some limitations, given that soybean cultivars have a broad genetic diversity related to adaptation, especially for photoperiod sensitivity and temperature, which are indispensable factors for crop expansion (Viana *et al*., 2009). It is necessary to access the genetic variability available for future uses in breeding programs since this will make possible the selection of parent plants that will form the population with the highest proportion of desirable recombinants (Vasconcelos *et al*., 2015).

In addition to an adequate genotype selection, the success of soybean cultivation depends on several factors. The most important one is the use of high-quality seeds, resulting in high vigor plants that will perform better in the field. The use of high-quality seed allows access to genetic advances, quality assurance, and adaptation technologies in different regions, ensuring higher yields (França-Neto *et al*., 2016).

Soybean is considered a short-day plant; for this reason, most of the world's cultivated area is located at latitudes greater than 30. The adaptation of soybean to the latitude conditions of the Brazilian Midwest, North, and Northeast regions was one of the major challenges faced by soybean breeding programs (Almeida *et al*., 2011). In the Northeast region, there is a great demand for soybean for animal feeding in farms of almost all states. As a highly valued crop, it has great potential for gains for producers in the region, meeting regional and global market demands (Campos *et al*., 2016).

The precipitation of the Brejo da Paraiba region allows the cultivation of many vegetable crops of short and perennial cycle, including some species that require high amounts of water (Costa *et al*., 2015). However, there is a lack of practical and scientific information in the literature regarding the recommendation of genotypes for this region.

The objective of this study was to analyze the genetic diversity of soybean genotypes provided by the germplasm bank of the Universidade Federal Viçosa through uni and multivariate techniques and recommend the superior genotypes for the analyzed characteristics.

Materials and methods

The experiment was conducted at the Departamento de Ciências Biológicas (DCB), Centro de Ciências Agrárias (CCA) of the Universidade Federal da Paraíba (UFPB), campus II, located in the municipality of Areia, in the Brejo Paraibano Microregion at an altitude of 623 m a.s.l. and with coordinates 06°57'48'' S and 35°41'30'' W. The annual average temperature was 24.0°C with a relative average humidity of approximately 80% and annual average precipitation of 1,400 mm (Costa *et al*., 2015). The climate, according to Köppen (1936), is classified as As, a tropical climate, semi-humid, with rains during the autumn-winter season.

The seeds were donated by the plant breeding program of the Universidade Federal de Viçosa. The following genotypes with a relative maturity degree above 9 were pre-selected: M8867RR, M8766RR, M9144RR, SYN1059 RR, M-SOY 9350, ST 920 RR, TMG1175RR, M8372IPRO, M8527RR, M8644IPRO, TOPAZIO RR, UFV - 16 (Capinópolis) and UFVS: Berilo RR, 2013, 2012, 2010, 2005, QUARTZO, 2001, and 2008. The seeds were sown in a commercial substrate (Basaplant®) and arranged to place three seeds of the genotypes per cell. Fifteen days after germinated, plants were transferred to 5 L plastic pots maintained under greenhouse conditions.

The harvest was performed continuously; when the pods reached the point of physiological maturity and presented predominance of a brown color, they were harvested to avoid future losses, considering that soybean has a dehiscent characteristic. The experiment was located in a region with a high level of humidity (close to 80%) with regular rainfall during short periods of drought. For this reason, the seeds were harvested because they presented a decrease in humidity.

A ruler and pachymeter were used for quantitative evaluations such as hypocotyl length, leaf width and length, flower diameter, plant height, seed length and width. To perform the germination index analysis, we divided the number of germinated seeds by the total number of seeds and multiplied by 100 to obtain the result as a percentage.

A completely randomized design with five replicates was used. Data were previously subjected to analysis of variance, with a subsequent Tukey test ($P \leq 0.05$) to compare the means. For the multivariate analysis, a residual covariance matrix was obtained and as a measure of dissimilarity, the generalized distance of Mahalanobis (D^2) was estimated; based on this, it was possible to apply the Tocher and UPGMA grouping method. The cut-off point of the dendrograms generated by the hierarchical methods, as well as the number of groups were estimated based on the method of Mojena (1977) based on the relative size of the levels of fusions (distances) in the dendrogram. The Singh criterion was also applied to quantify the relative contribution of these characteristics to the genetic divergence. Finally, the canonical correlation was also used. The analyses were performed using the Genes computational program (Cruz, 2013).

TABLE 1. Seed size and germination index of 20 soybean genotypes.

Genotypes	Seed size	% germination
UFVS Berilo RR	Big	26.60
UFVS 2013	Big	20.00
M8867RR	Big	33.30
UFVS 2012	Medium	33.30
UFVS 2010	Big	20.00
M8766RR	Medium	33.30
M9144RR	Big	20.00
UFVS 2005	Big	33.30
SYN1059 RR	Big	33.30
UFVS QUARTZO	Big	20.00
M-SOY 9350	Big	13.30
ST 920 RR	-	13.30
UFV - 16 (Capinópolis)	-	13.30
UFVS 2001	-	13.30
UFVS 2008	-	20.00
TMG1175RR	Medium	33.30
M8372IPRO	-	13.30
M8527RR	-	6.60
M8644IPRO	Medium	6.60
TOPAZIO RR	-	13.30

Fields filled with “-” did not reach the breeding stage and had no seeds to be quantified.

Results

From the 20 genotypes used, only 10 reached physiological maturity. The germination index of the seeds is shown in Table. 1. The genotypes that had the highest germination percentage were M8867RR, M8766RR, SYN1059 RR, TMG1175RR, UFVS: 2012 and 2005. The genotypes M8527RR and M8644IPRO had the lowest percentage of germination (6%).

According to the analysis of variance by the F test at 5% probability, a significant difference was observed for leaf width, flower diameter, and seed width (Tab. 2). For hypocotyl length, leaf length, plant height, and seed length, no significant difference was observed.

The characters that had heritability values (h^2) above 60% were leaf width, flower diameter, and seed width, which indicates that phenotypic selection can be performed with

TABLE 2. Summary of the ANOVA for the seven traits evaluated in ten soybean genotypes.

SV	MQ							
	DF	HL	LW	LH	PH	FD	SL	SW
Treatments	9	2.82^{ns}	1.41*	1.54^{ns}	331.16^{ns}	1.72*	0.67^{ns}	0.74*
Residues	20	2.25	0.43	0.81	147.91	0.50	0.39	0.29
CV	-	17.25	25.94	28.06	26.04	13.65	9.06	12.33
CV_F	-	0.93	0.47	0.51	110.38	0.57	0.22	0.24
CV_E	-	0.75	0.14	0.27	49.30	0.16	0.12	0.09
CV_G	-	0.18	0.32	0.24	61.08	0.40	0.09	0.15
h^2	-	19.95	70.00	47.21	55.33	70.69	42.24	61.18
CVg (%)	-	4.97	22.70	15.32	16.73	12.24	4.47	8.93
CV_G/CV_E (%)	-	0.24	2.28	0.88	1.24	2.50	0.75	1.66

SV - source of variation; MQ - medium square; DF - degree of freedom; HL - hypocotyl length; LW - leaf width; LH - leaf length; PH - plant height; FD - flower diameter; SL - seed length; SW - seed width; CV - coefficient of variation; CV_F - phenotypic variation; CV_E - environmental variation; CV_G - genotypic variation; h^2 - heritability coefficient; CVg - genetic coefficient of variation; CV_G/CV_E - ratio between genotypic variation and environmental variation; ns - not significant and ** significant at 5% probability by the F test.

higher accuracy in relation to the other analyzed variables. This can be confirmed by the values of CV_G/CV_E, above 1 for the same characters.

The means of the characteristics were analyzed by the Tukey test ($P \leq 0.05$) (Tab. 3). The highest averages in these characteristics were obtained by five genotypes, M8867RR, SYN1059 RR, UFVS: Berilo RR, 2013 and 2012. The values obtained for leaf width ranged from 1.83 to 3.63 cm. Based on this characteristic the most recommended genotype was SYN1059 RR. For flower diameter, the values ranged from 3.91 to 6.26 cm, and the superior genotypes were M8867RR, UFVS: Berilo RR and 2012. For seed width, the values ranged from 3.71 to 5.27 cm, and the genotype that obtained the highest value was UFVS 2013.

TABLE 3. Mean of three traits evaluated in ten soybean genotypes according to the Tukey test.

Genotypes	Traits		
	LW	FD	SW
UFVS Berilo RR	1.83b	6.26a	4.25ab
UFVS 2013	1.86b	4.74ab	5.27a
M8867RR	2.20ab	6.24a	4.76ab
UFVS 2012	2.60ab	5.74a	3.71b
UFVS 2010	3.50ab	5.14ab	4.22ab
M8766RR	1.93b	4.53ab	3.73b
M9144RR	2.00b	5.18ab	3.92ab
UFVS 2005	3.16ab	4.75ab	4.40ab
SYN1059 RR	3.63a	3.91b	4.77ab
UFVS QUARTZO	2.50ab	5.57ab	4.47ab

LW - leaf width; FD - flower diameter; SW - seed width. Means followed by the same letter in the column do not differ at 5% probability according to the Tukey test.

The Tocher's clustering method, from the generalized distance of Mahalanobis (1936), makes it possible to divide the genotypes into three genetically distinct groups (Tab. 4).

TABLE 4. Grouping of ten soybean genotypes evaluated for seven traits established by the Tocher method, using the generalized Mahalanobis distance as a measure of dissimilarity.

Groups	Genotypes
1	UFVS 2012; UFVS 2005; UFVS 2010; M9144RR; UFVS QUARTZO; M8867RR
2	UFVS Berilo RR; UFVS 2013; M8766RR
3	SYN1059 RR

In group 1, six genotypes that show genetic similarity (60% of the total) were included. Group 2 was composed of three genotypes with genetic similarity (30% of the total). Group 3 comprised only one genotype (10% of the total), indicating that it has genetic dissimilarity with the others.

A co-phenotype correlation coefficient of 0.76 was obtained using the Mahalanobis dissimilarity matrix to generate the dendrogram by the UPGMA method. This correlation confirms an adequate relation between the distance matrix and the generated dendrogram (Fig. 1). Using the Mojena method, it was possible to obtain two groups: one with the genotypes M8766RR, M9144RR, M8867RR, UFVS: Berilo RR, 2010, 2012, 2005, 2013 and QUARTZO; and another with only the SYN1059 RR genotype.

In both the Tocher and UPGMA methods, the SYN1059 RR genotype was isolated in a different group from the others, which indicates a significant genetic dissimilarity when compared to the other genotypes tested.

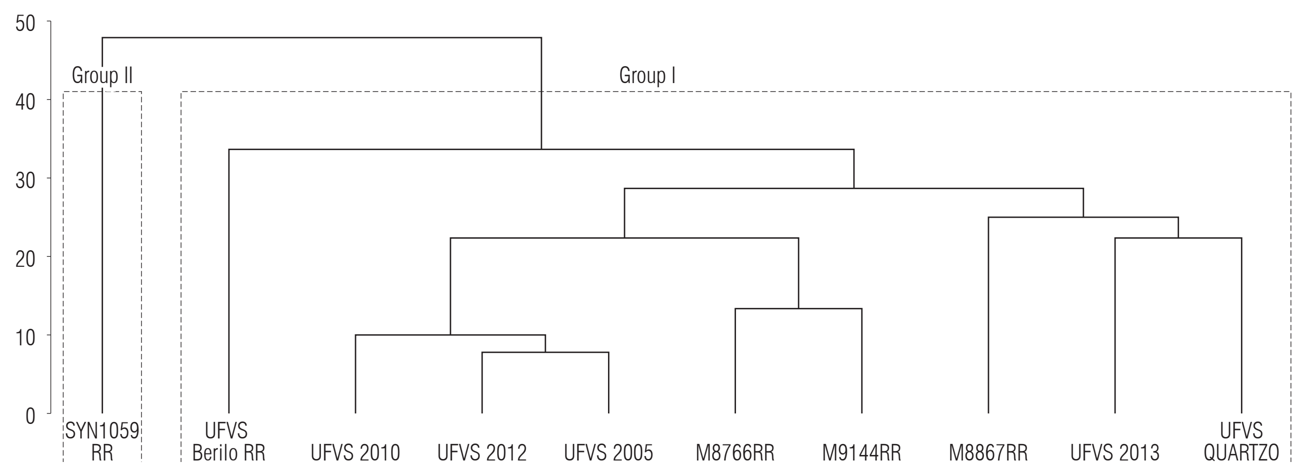

FIGURE 1. Dendrogram of the analysis of ten soybean genotypes by the UPGMA method obtained from the generalized distance of Mahalanobis, generated with seven characteristics. Coefficient of correlation (r) = 0.76.

From the Singh method (1981), which consists of the relative contribution of the characters to the diversity (value in %), based on the generalized distance of Mahalanobis, it was observed that the leaf width (35.45%) was the characteristic that contributed the most to the data variability, followed by the characteristics flower diameter (19.13%) and plant height (18.34%) (Tab. 5).

TABLE 5. Relative contribution of seven characteristics to the genetic dissimilarity of 10 soybean genotypes according to the method proposed by Singh (1981).

Traits	Value in %
HL	8.08
LW	35.45
LH	2.08
PH	18.34
FD	19.13
SL	8.51
SW	8.41

HL - hypocotyl length; LW - leaf width; LH - leaf length; PH - plant height; FD - flower diameter; SL - seed length; SW - seed width.

In order to analyze the canonical correlation, the first three canonical pairs had a genetic contribution of 90.14% (Tab. 6), thus permitting the presentation of the genotypes in a graphical dimension, where six groups were observed (Fig. 2), different from the cluster analyses mentioned above.

TABLE 6. Canonical correlation of the relative characteristics evaluated in the 10 soybean genotypes.

Traits	Eigenvalues %	Accumulated %
HL	48.02	48.02
LW	21.95	69.97
LH	20.17	90.14
PH	4.92	95.07
FD	2.92	98.02
SL	1.59	99.61
SW	0.38	100.00

HL - hypocotyl length; LW - leaf width; LH - leaf length; PH - plant height; FD - flower diameter; SL - seed length; SW - seed width.

Discussion

For the selection of promising soybean genotypes, those with significant values of leaf width, flower diameter, and seed size are of interest since they contribute to a higher photosynthetic surface, pod size, and physiological quality of the plant, which are associated with higher yields.

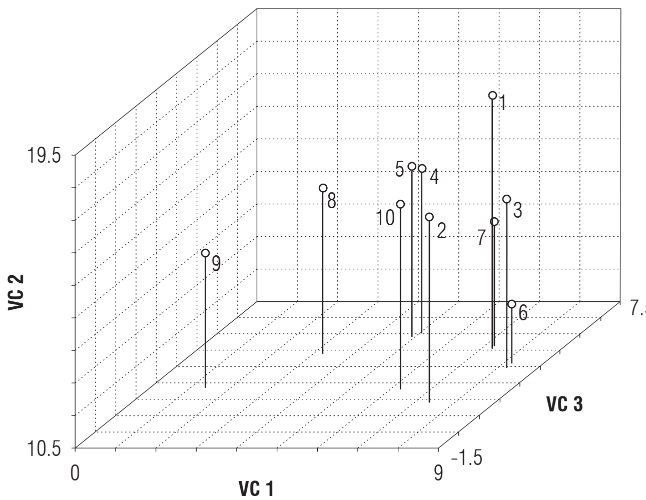

FIGURE 2. Analysis of the canonical correlation by the graphic dispersion. 1) UFVS Berilo RR; 2) UFVS 2013; 3) M8867RR; 4) UFVS 2012; 5) UFVS 2010; 6) M8766RR; 7) M9144RR; 8) UFVS 2005; 9) SYN1059 RR; 10) UFVS QUARTZO.

Given that the main commercial product of soybeans is the grain, it should be pointed out that the size of the seed affects its physiological quality, as mentioned by Pádua *et al.* (2010). Thus, the selection of larger seeds is indicated, because these will theoretically originate more vigorous plants. Therefore, the genotypes M8867RR, SYN1059 RR and UFVS 2005 are recommended based on the superiority of this characteristic (Tab. 1).

Another important attribute for selection is the existence of genetic variability. According to Martins *et al.* (2012), the characteristics that have significant differences according to the F test indicate the existence of genetic variability (Tab. 2). According to Domiciano *et al.* (2015), the values of the CV_G/CV_E ratio above 1 indicate that the plant breeding of the species through morphological characteristics is possible to be carried out through phenotypic selection of promising genotypes.

The multivariate analysis is a tool widely used in genetic divergence studies, as evidenced by Santos *et al.* (2011) and Almeida *et al.* (2011) in studies with soybean. This analysis offers the possibility of using various techniques, such as the Tocher grouping, or UPGMA dendogram the Singh method, and the canonical correlation. Viana *et al.* (2009) mentioned that the characters used in these analyzes allow the identification of genotypes with higher agronomic limits, identifying superior materials for breeding programs.

According to Martins *et al.* (2012), it is not viable to cross varieties contained in the same group as in the Tocher method since they present significant genetic similarity, which theoretically reduces the obtaining of superior materials. It would be recommended to cross genotypes that are in different groups, which increases the genetic dissimilarity and facilitates obtaining superior materials. However, besides the genetic divergence, it is necessary to observe mainly the values of heritability and variances.

For the Tocher grouping, the UPGMA method and the graphic dispersion, showed a difference in the formation and distribution of the genotypes within the groups. According to Azevedo *et al.* (2013), it is possible that this occurs since there is a difference between the accuracy and grouping criteria of the methods.

Based on the Singh test (Tab. 5), it is possible to notice the importance of the leaf width characteristic, since it contributes to genetic divergence and is closely related to the photosynthetic capacity of assimilatory tissues; thus, a higher leaf width may increase energy synthesis and finally improve the productive use (Petter *et al*., 2016)

Conclusions

The genotypes M8766RR, M8867RR, SYN1059 RR, UFVS Berilo RR, and UFVS 2012 showed the best values for leaf width, plant height, and flower diameter, having great potential to be cultivated in the target region, and are indicated for the continuity of the soybean plant breeding program.

The crosses between these genotypes are indicated as promising to obtain hybrids, since they have genetic variability and higher averages in the characteristics that contributed the most to the genetic divergence, such as leaf width, flower diameter, and seed width.

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Effect of gibberellic acid-3 and 6-benzylaminopurine on dormancy and sprouting of potato (*Solanum tuberosum* L.) tubers cv. Diacol Capiro

Efecto del ácido giberélico-3 y 6-bencilaminopurina sobre el reposo y brotación de tubérculos de papa (*Solanum tuberosum* L.) cultivar Diacol Capíro

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ABSTRACT

The duration of the dormancy period in tubers is a determining factor in the planning of potato planting and production. The effect of two plant growth regulators on the dormancy period and sprouting of cv. Diacol Capiro tubers was evaluated in this study. The experiment was carried out under storage conditions (15°C and 75% RH) using a completely randomized block design with 3×3×3 factorial arrangement. The factors were: gibberellic acid-3 (GA_3) and 6-benzylaminopurine (6BAP) (0, 25, and 50 mg L^{-1}) and the immersion time (iT) (10, 60, and 120 min). The application of GA_3 and iT had an effect on dormancy breakage; the treatments with 25 mg L^{-1} GA_3 and 60 min of immersion were enough to reduce dormancy by 18 d (35%) compared to untreated tubers. The factor GA_3 increased tuber weight loss (10.2%), generated sprouts with higher weight (25.6-28.4%), higher length growth rate (42.3%), and lower dry matter content (21.8-28.4%), and it increased secondary sprouting (36.2-57.9%) in comparison with untreated tubers. This way, despite the treatments with 25 mg L^{-1} GA_3 reduced the dormancy period, this dose generated sprouts more susceptible to mechanical damage. The treatments with 6BAP did not significantly affect the evaluated variables.

Key words: tuber potato seed, cytokinins, gibberellins, growth regulators, sprouts.

RESUMEN

La duración del periodo de reposo en tubérculos es un factor determinante en la planeación de las siembras y la producción de papa. En este estudio se evaluó el efecto de dos reguladores de crecimiento sobre el periodo de reposo y la brotación de tubérculos de papa cultivar Diacol Capíro. El experimento se realizó en condiciones de almacenamiento (15°C y 75% HR) con un diseño de bloques completamente al azar en arreglo factorial 3×3×3. Los factores fueron: ácido giberélico-3 (AG_3) y 6-bencilaminopurina (6BAP) (0, 25 y 50 mg L^{-1}) y el tiempo de inmersión (Ti) (10, 60 y 120 min). La aplicación de AG_3 y Ti tuvo efecto en interrumpir el periodo de reposo y tratamientos con 25 mg L^{-1} de AG_3 y 60 min de inmersión fueron suficientes para disminuir el reposo en 18 días (35%) con respecto a los tubérculos no tratados. El factor AG_3 aumentó la pérdida de peso de los tubérculos (10.2%), generó brotes de mayor peso (25.6-28.4%), con mayor tasa de crecimiento en longitud (42.3%), con menor contenido de masa seca (21.8-28.4%) y aumentó la brotación secundaria (36.2-57.9%) con respecto a tubérculos no tratados. De esta forma, a pesar de que los tratamientos con 25 mg L^{-1}de AG_3 redujeron el periodo de reposo, esta dosis generó brotes más susceptibles al daño mecánico. Los tratamientos con 6BAP no afectaron significativamente las variables evaluadas.

Palabras clave: tubérculo semilla de papa, citoquininas, giberelinas, reguladores de crecimiento, brotes.

Introduction

The potato (*Solanum tuberosum* L.) is one of the most important horticultural crops in the world and the fourth most consumed source of carbohydrates for humans after rice, wheat, and corn. Potatoes have high nutritional value and they are consumed in both fresh and processed form (Devaux *et al*., 2014; Navarre and Pavek, 2015). In Colombia, Diacol Capiro is one of the cultivars with the largest planted areas. It is also the main cultivar (cv.) used for processing due to its high productivity and frying quality (Ñústez, 2011). Dormancy is a factor that can limit seed availability when establishing new crops (Mustefa *et al*., 2017; Deligios *et al*., 2020). The most common strategy for breaking dormancy is storage in warehouses (Jansky and Hamernik, 2015). The dormancy period for cv. Diacol Capiro could last up to three months at 15°C and 75% RH (Ñústez, 2011). This affects the costs for seed producers.

The most common potato propagation method is the vegetative one with seed tubers. The quality of seed tubers is linked to different parameters, such as the physiological

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age of the tuber, its dormancy state and sprouting ability. These are variables that affect the yield potential of the crop (Mikitzel, 1993; Knowles and Knowles, 2006; Struik, 2007; Blauer *et al.,* 2013). Dormancy is the absence of visible sprout growth, even under adequate conditions for it to occur (Suttle, 2007). It is a period that begins simultaneously with the tuber filling at the mother plant and ends with sprouting (Claassens and Vreugdenhil, 2000; Nambara and Marion-Poll, 2005). Dormancy is a plant strategy used to escape from hostile conditions, and this is very important in sessile organisms like plants to ensure their survival (Henis, 1987; Suttle, 2007). In tubers, dormancy is present in the meristems, i.e. buds (Lang *et al*., 1987; Sonnewald and Sonnewald, 2014), while the rest of the tuber remains metabolically active (Viola *et al*., 2007; Sergeeva *et al*., 2012). Viola *et al*. (2007) establish the presence of at least one sprout of 2 mm length or greater as a criterion to determine the end of the tuber dormancy period. Dormancy breakage begins with the reactivation of the symplastic pathway in the tuber that regulates the distribution of energy reserves towards the meristematic tissues, allowing their activation and sprout development (Viola *et al*., 2007).

The sprouting process begins with the development of the apical sprout that becomes dominant and inhibits the development of lateral sprouts (Carli *et al*., 2010). This state is called "apical dominance" or "paradormancy" (Lang *et al*., 1987; Sonnewald and Sonnewald, 2014). Dominance decreases gradually with the increase of the tuber's physiological age and ends in a state of multiple sprouting that is considered adequate for seed sowing (Krijthe, 1962; Teper-Bamnolker *et al*., 2012; Blauer *et al.,* 2013; Eshel, 2015). The dormancy period varies according to components, such as genotype, environmental conditions, agronomic management of the crop, storage, and endogenous hormonal dynamics of the tuber (Carli *et al*., 2010; Sonnewald and Sonnewald, 2014; Eshel, 2015; Christensen *et al*., 2019; Deligios *et al*., 2020).

During dormancy the levels of plant growth inhibitors, such as abscisic acid and ethylene, are high. These are the main hormones involved in dormancy initiation and maintenance (Carli *et al*., 2010; Mani *et al*., 2014; Sonnewald and Sonnewald, 2014; Deligios *et al*., 2020). When dormancy is overcome and sprouting begins, the hormonal dynamics change and the concentrations of growth promoters such as cytokinins, gibberellins (Mani *et al*., 2014) and auxins increase, although the role of auxins is less clear than the other two (Suttle, 2004; Carli *et al*., 2010).

Different studies have shown that the exogenous contribution of plant growth regulators can increase efficiency of dormancy breakage and potato seed tuber sprouting. The use of exogenous regulators, such as gibberellins (Jansky and Hamernik, 2015; Mustefa *et al*., 2017; Chindi and Tsegaw, 2019; Christensen *et al*., 2019; Deligios *et al*., 2020), cytokinins (Turnbull and Hanke, 1985; Suttle, 2008; Campbell *et al.,* 2014) or their combination (Alexopoulos *et al.,* 2007) have generated positive responses. However, most of the available information is limited to cultivars of the Group Tuberosum, which is not a group of potatoes planted in Colombia (Huaman and Spooner, 2002). The objective of this research was to evaluate the effect of the application of gibberellic acid-3 and 6-benzylaminopurine on tuber dormancy and sprouting in potato cv. Diacol Capiro seed tubers at different immersion times.

Materials and methods

Plant material and experiment establishment

The experiment was carried out at the Universidad Nacional de Colombia Bogota campus, using seed tubers under storage conditions with diffuse light, an average temperature of 16°C and 40-80% relative humidity. Potato cv. Diacol Capiro tubers were used 10 d after harvest. The tubers were washed and classified into three categories (I: 90-110 g, II: 70-89 g, and III: 50-69 g). The tubers that showed morphological changes or some type of damage were discarded.

A completely randomized block design with factorial arrangement (3×3×3) was used. The factors were: gibberellic acid-3 (GA_3), 6-benzylaminopurine (6BPA), at concentrations of 0 mg L^{-1}, 25 mg L^{-1}, and 50 mg L^{-1}, and immersion times (iT) of tubers in the solutions at 10, 60, and 120 min. A control without treatment was used for the variable "dormancy period". The experimental unit (EU) included 20 tubers for the variables: dormancy period, weight loss, number of sprouts and secondary sprouting, and five tubers for the variables apical sprout growth, sprout weight and dry matter. Tuber size was used as a blocking factor.

Seed tubers were placed on plastic meshes and submerged in solutions with the different hormone treatments. The tubers were then removed at different immersion times. Subsequently, the tubers were allowed to dry, randomly arranged inside plastic baskets that were stacked in towers per block. The baskets were randomized per height twice a week and stored until the end of the trial, 100 d after applying the treatments (DAT).

Evaluated variables

Dormancy period

The tubers that showed at least one bud with a sprout of 2 mm length or greater were counted and recorded twice a week. With this information, the number of days necessary to obtain 80% of sprouting tubers was determined. This number was considered as the breakage of the dormancy period.

Weight loss and number of sprouts

Tuber weight was determined using a milligram precision balance (XB220A, Precisa, Switzerland) at 8 DAT (initial weight) and 100 DAT (final weight), and the percentage of weight loss was calculated with these data. The number of sprouts was estimated as the total number of sprouts with sufficient vigor to survive in the field at 100 DAT.

Apical sprout growth

Five tubers were randomly selected and the sprouts of the apical zone were individually marked, and their length and diameter measured at 16, 24, 32, 39, 52, 60, 73, and 90 DAT. The tuber growth rate in terms of length and diameter was estimated using the obtained data.

Sprout weight and dry matter percentage

At 100 DAT, all sprouts from five tubers were removed, weighed (fresh weight), and then brought to constant weight in an oven (Thelco Model 16, Precision Scientific Company, Chicago, USA) at 70°C (dry weight). The dry matter percentage was then determined.

Secondary sprouting

The number of tubers that showed secondary sprouting was quantified. The secondary sprouts are characterized by the presence of small and thin sprouts in the primary sprout (Krijthe, 1962).

Statistical analysis

The data were analyzed with the R statistical package (version 3.5.1), using an ANOVA to determine the effect of the factors on parametric variables and the Tukey test for a comparison of means ($P \leq 0.05$). For the variable sprout growth (length and width), a generalized linear gamma model with repeated measures was performed based on the methodology of Dobson and Barnett (2008).

Results and discussion

Dormancy period

For this variable, statistically significant differences were observed for GA_3, iT and the $GA_3 \times iT$ interaction (Tab. 1). The response in the dormancy period was different between GA_3 concentrations. Without GA_3 application, the dormancy period was 42.2 d on average, whereas with 25 mg L^{-1} GA_3 it was 32.8 d and with 50 mg L^{-1} GA_3 it was 30.1 d. The three levels were statistically different from each other (Fig. 1A). The data showed that GA_3 promoted the breakage of tuber dormancy in less time, with the dose of 50 mg L^{-1} as the most effective.

TABLE 1. Significance levels of ANOVA results of the variables dormancy period (DP), weight loss (WL), number of sprouts (NS), fresh weight of sprouts (FW), dry weight of sprouts (DW), percentage of dry matter of sprouts (% DM), and secondary sprouting (SS) with the applications of different concentrations of gibberellic acid-3 (GA_3), 6-benzylaminopurine (6BAP), and immersion time (iT) in *S. tuberosum* cv. Diacol Capiro tubers.

	DP	WL	NS	FW	DW	%DM	SS
Block	*	ns	ns	***	***	***	*
GA_3	***	***	ns	***	***	***	***
6BAP	ns	ns	ns	ns	ns	ns	ns
iT	**	ns	ns	ns	ns	ns	ns
$GA_3 \times$6BAP	ns	ns	ns	ns	ns	ns	ns
$GA_3 \times$iT	*	ns	ns	ns	ns	ns	ns
6BAP×iT	ns	ns	ns	ns	ns	ns	ns
$GA_3 \times$6BAP×iT	ns	ns	ns	ns	ns	ns	ns
Variation coefficient	17.3%	6.7%	18.5%	35.1%	32.5%	8.6%	32.1%

*** $P \leq 0.1\%$; ** 1%; * 5%; ns: non-significant differences.

The dormancy breakage response in the tubers due to the exogenous application of GA_3 has been reported in other studies with different cultivars of the Group Tuberosum, such as 'Red Pontiac' (Guzmán, 1963), 'Agria' (Salimi *et al.*, 2010; Barani *et al.*, 2013; Mohammadi *et al.*, 2014), 'Marfona' (Salimi *et al.*, 2010; Barani *et al.*, 2013), 'Solara' (Hartmann *et al.*, 2011), 'Draga' (Barani *et al.*, 2013), 'Born' (Mohammadi *et al.*, 2014), 'Bubu', 'Bate' (Mustefa *et al.*, 2017), 'Pasat', 'Dorota' (Wróbel *et al.,* 2017), 'Shepody' (Dean *et al.,* 2018a), 'Spunta', 'Monalisa', 'Europe', 'Arinda' (Deligios *et al.*, 2020) and 'Payette', 'Russet' and 'Alturas' (Dean *et al.,* 2018b). However, the available information in Andigenum cultivars is scarce. Alexopoulos *et al.* (2007) evaluated treatments with GA_3, 6BAP and their combination at different temperatures in the Andigenum cv. Chacasina, applying the solution directly to the tuber-stolon junction. The authors found that GA_3 treatments and the combination of GA_3 and 6BAP reduced the dormancy period and showed the synergic effect of the two plant growth regulators.

Treatment with iT resulted in statistically significant responses between all the evaluated times as compared to the untreated control (49 d), showing the effect of humidity at the beginning of the sprouting processes (Fig. 1B). The 10 min iT generated a dormancy reduction of 12 d compared to the untreated control. It also differed statistically from 60 and 120 min (14 d), which did not differ from each other (Fig. 1B). These results suggested that, for the cv. Diacol Capiro, 60 min were enough to generate the dormancy breakage response in the evaluated treatments. This result was similar to that reported by Alexopoulos *et al*. (2008) in the Andigenum cv. Chacasina, in which 120 min was the optimum time of the treatment. It also underlines that the iT has more influence than the plant growth regulator dose, possibly due to the higher amount of solution that enters the tuber. There were interactions between GA_3 and iT (Fig. 1C) in which GA_3-iT combinations 25-60, 25-120, 50-10, 50-60 and 50-120 reduced the dormancy period by 18 d on average compared to non-treated tubers, and 11 d compared to tubers immersed in water (Fig. 1C).

There were no effects of the treatments with 6BAP on the tuber dormancy period. Different studies have shown that the effectiveness of treatments with cytokinins as dormancy disrupters in potato varies with conditions such as tissue sensitivity that is greater in less deep stages of dormancy (Turnbull and Hanke, 1985; Suttle, 2001; Rodríguez and Moreno, 2010; Hartmann *et al.,* 2011), genetic component, and type of cytokinins used (Suttle, 2008).

Weight loss

Only the GA_3 treatment showed significant differences in the tuber weight loss (Tab. 1). At 100 d of storage, the treatments with 50 mg L^{-1} GA_3 showed a lower weight loss percentage (4.4%) than the one obtained with 25 and 50 mg L^{-1} (4.9%). The last two had no differences between them (Fig. 3). Weight loss is a response associated with the degree of tuber longevity that increases when sprouting is higher, due to the greater permeability of the cell membranes of the sprouts compared to other tuber tissues (Carli *et al*., 2010). The response is similar to that reported in cultivars Andigena of the subspecies *andigena*, such as 'Chacasina' (Alexopoulos *et al*., 2007; Alexopoulos *et al*., 2008) and the Group Tuberosum, such as 'Agria' (Rentzsch *et al.,* 2012), and 'Bubu' and 'Bate' (Mustefa *et al*., 2017), in which weight loss is related to an increase in the respiratory rate and sprout development in GA-treated tubers.

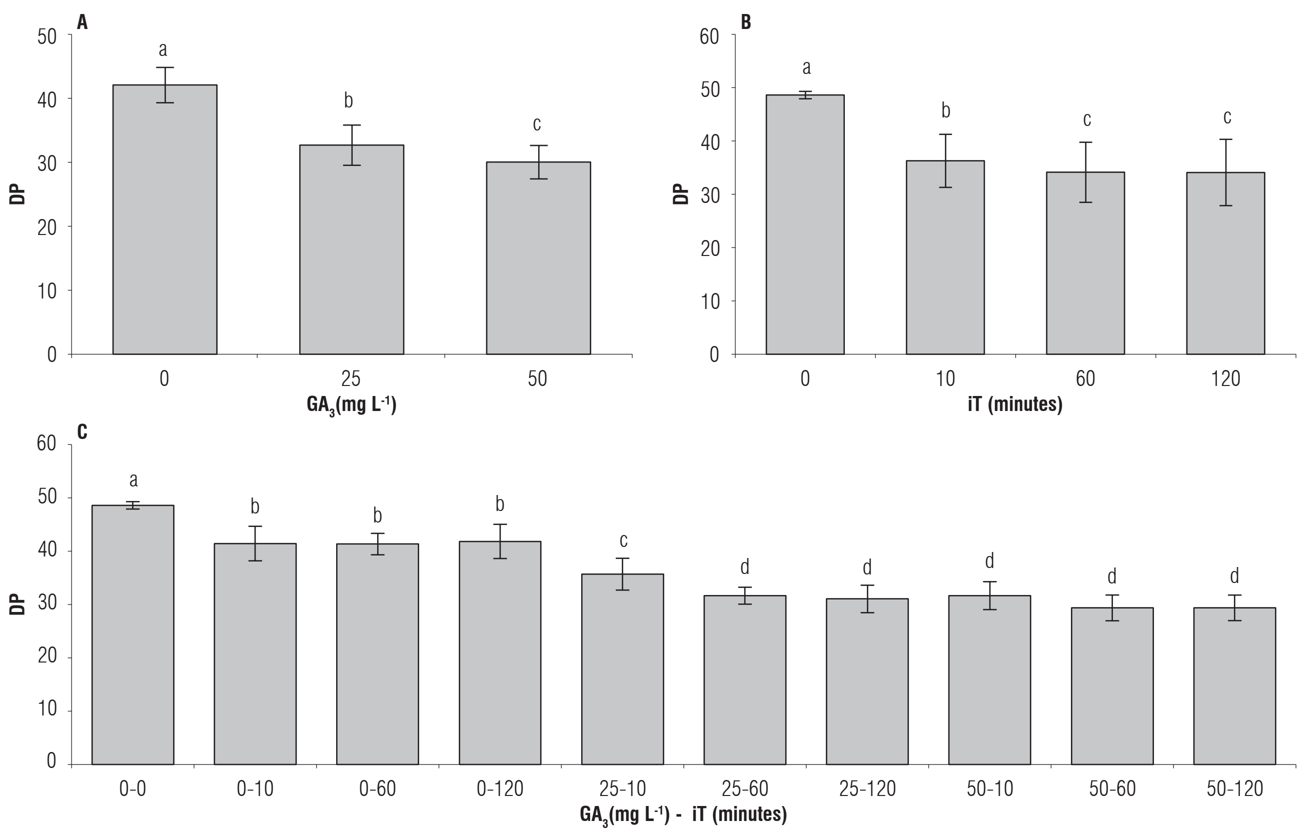

FIGURE 1. Duration of the tuber dormancy period in days (DP) in response to A) gibberellic acid-3 (GA_3), B) immersion time (iT), and C) the interaction $GA_3 \times$iT, in the cv. Diacol Capiro. The data shown is the number of days needed to reach breakage in 80% of the tubers after the application of the treatments. Standard deviations are indicated by vertical bars. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N: A/B = 27; C = 9.

FIGURE 2. Sprout development in A) untreated, B) treated with water for 10 min, C) treated with a 25 mg L^{-1} gibberellic acid-3 (GA_3) solution for 10 min, and D) treated with a 50 mg L^{-1} GA_3 solution for 10 min potato (*S. tuberosum*) cv. Diacol Capiro tubers 39 days after treatment. Arrows show the buds that have sprouts.

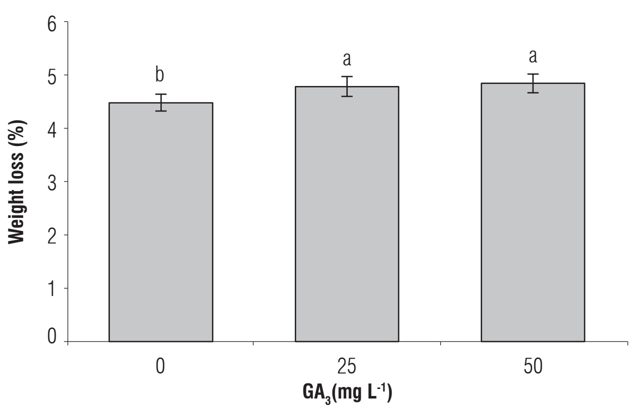

FIGURE 3. Effect of different of gibberellic acid-3 (GA_3) concentrations on the weight loss percentage in potato cv. Diacol Capiro tubers at 100 DAT. Standard deviations are indicated by the vertical bars. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N = 27.

Number of sprouts

In this study, none of the treatments affected the number of sprouts per tuber (Tab. 1), confirming Alexopoulos *et al.* (2007). Studies with GA_3 applications at low concentrations (20 mg L^{-1}) in cultivars of the subspecies show an increase in the number of vigorous sprouts per tuber (Mustefa *et al.*, 2017). Concentrations between 1 and 2 mg L^{-1} GA_3 in tuber seeds increase the number of stems and the percentage distribution of tubers harvested by size in the crop (Herman *et al.*, 2016; Wróbel *et al.*, 2017; Dean *et al.*, 2018a; Dean *et al.*, 2018b).

Growth in apical sprout length

The applied treatments affected the length of sprouts (Tab. 2). Taking into account the DEVIANCE model used, the differential effect of the DAT (time factor) and their interactions with each of the evaluated factors were identified as follow: GA_3×DAT, 6BAP×DAT and Ti×DAT (Tab. 2). For this reason, the analysis is presented as repeated measures over time.

TABLE 2. Significance levels of DEVIANCE analysis of growth variables in apical sprouts expressed in terms of growth rate in length (L) and diameter (D), treated with different concentrations of gibberellic acid-3 (GA_3) and 6-benzylaminopurine (6BAP) and immersion time (iT), in *S. tuberosum* cv. Diacol Capiro tubers.

	L	D		L	D
Size (Block)	ns	***	DAT	***	***
GA_3	***	***	GA_3×DAT	***	***
6BAP	ns	ns	6BAP×DAT	*	ns
iT	***	ns	iT×DAT	***	ns
GA_3×6BAP	*	ns	GA_3×6BAP×DAT	*	**
GA_3×iT	ns	***	GA_3×iT×DAT	*	ns
6BAP×iT	ns	*	6BAP×iT×DAT	ns	ns
GA_3×6BAP×iT	ns	ns	GA_3×6BAP×iT×DAT	ns	ns

GA_3 (Factor): Gibberellic acid-3; 6BAP (Factor): 6-benzylaminopurine; It: Immersion time; DAT (Time factor): Days after treatment; *** $P \leq 0.1\%$; ** 1%; * 5%; ns: non-significant differences.

The GA_3 treatments affected the growth rate of sprouts (Fig. 4A). Treatments with 0 mg L^{-1} had the lowest growth rate (0.15 mm/d) compared to treatments with 25 mg L^{-1} (0.26 mm/d) and 50 mg L^{-1} (0.26 mm/d) without statistically significant differences between the last two. The sprout length at 90 DAT for 50 mg L^{-1} (2.2 cm) was 83% higher than that in treatments without GA_3 (1.2 cm), while in treatments with 25 mg L^{-1} (2.0 cm) sprout length increased by 66% (Fig. 4B). When observing the effect of 6BAP and iT, GA_3 had the greatest effect on the growth of apical sprouts results (Alexopoulos *et al*., 2008) in the cv. Chacasina.

The treatment with 6BPA did not affect the growth rate or length of sprouts during the evaluation period (Figs. 4C and 4D). The factor iT showed differences in sprout length throughout the evaluation time (iT×DAT) (Tab. 2). The growth rate was higher with 60 min (0.25 mm/day) and differed from treatments of 10 min (0.22 mm/d), whereas treatments of 120 min (0.24 mm/d) did not differ from the previous ones (Fig. 4E). However, the length values at 90 DAT were not very different from each other. They showed the lowest value at 10 min (17 mm), followed by 120 min (18 mm) and 60 min (19 mm), with maximum differences of 11% in length (Fig. 4F).

The interaction between GA_3xiTxDAT and GA_3x6BAPx-DAT affected the length and growth rate of tuber sprouts (Tab. 2). These data suggest that the factor that had the greatest influence was GA_3, since treatments with 50 mg L^{-1} GA_3, regardless of their combination with other treatments, generated 3 of the 4 highest sprout lengths (22.6 mm). The highest GA_3 concentrations together with the combination 25-60 (GA_3-iT) showed no differences in growth rate (Fig. 5A). The other treatments with 25 mg L^{-1} GA_3 (25-120, 25-10) resulted in higher sprout length averages (18.5 mm) and higher growth rates than the treatments with 0 mg L^{-1} GA_3 (Fig. 5B). There were no differences in sprout length at 90 DAT or in the growth rate of the treatments with 0 mg L^{-1} GA_3, indicating that the effect of iT requires the presence of GA_3 (Fig. 5B).

The GA_3×6BAP×DAT interaction showed differences in sprout length at 90 DAT. Only the 50-0 treatment (GA_3-6BAP) differed statistically in the growth rate (Fig. 5C) and showed the greatest length (24.5 mm), 49% higher than the average of the treatments with 0 mg L^{-1} GA_3 (12.1 mm) (Fig. 5D).

Growth in diameter of the apical sprout

With the statistical model used, the effects of DAT and its interaction with GA_3 and the interaction GA_3×6BPA (Tab. 2) were identified. The GA_3 affected the diameter growth rate of the sprouts. The treatments with 0 mg L^{-1} had the highest growth rate (0.073 mm/d), significantly higher compared to 50 mg L^{-1} (0.045 mm/d) (Fig. 6A). At the end of the evaluation (90 d), the application of 50 mg L^{-1} GA3 generated smaller sprout diameter at levels 25 and 0 mg L^{-1}, respectively (Fig. 6B). This coincides with Alexopoulos *et al.* (2008).

We saw no interaction between GA_3 and 6BAP in the sprout diameter, but we observed the growth rate as a product of the interaction of the factors GA_3×6BAP×DAT (Tab. 2, Fig. 6C). The treatment with 0 mg L^{-1} GA_3 and with 6BAP did not differ from the treatment with 0 mg L^{-1} GA_3 and without 6BAP in the diameter growth rate (Fig. 6C), but the diameter in length was superior to the other treatments with different GA_3 and 6BAP combinations. This result suggests a negative effect of GA_3 on sprout diameter (Fig. 6D), but a positive effect on length.

Jansky and Hamernik (2015) observed similar results in the growth of sprouts in tubers treated with GA_3. They found increases in sprout elongation with doses of up to 1000 mg L^{-1} GA_3 and attributed this effect to the role this plant growth regulator plays in cellular elongation processes. The development of long and/or thin sprouts is a characteristic that can affect the quality of the tuber as seed by making sprouts more susceptible to damage by handling during transport and planting (Virtanen *et al*., 2013).

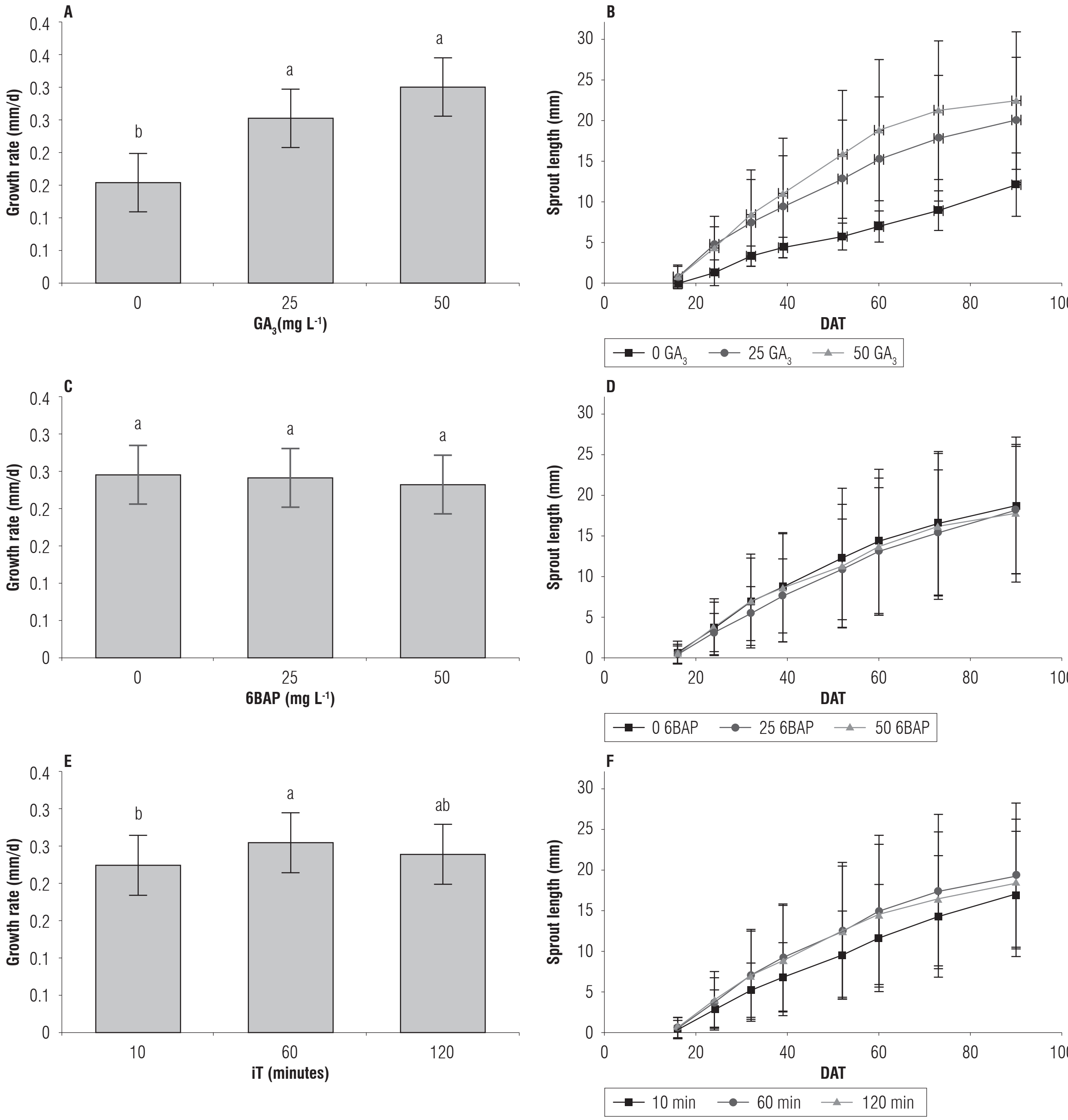

FIGURE 4. Growth rate (A, C, E) and length (B, D, F) of apical sprouts in cv. Diacol Capiro in response to factors gibberellic acid-3 (GA_3) (A, B), 6-benzylaminopurine (6BAP) (C, D) and immersion time (iT) (E, F) 90 days after treatment (DAT). Data shown in A, C and E correspond to the growth rate during the evaluation period. Vertical bars correspond to the standard deviation. Different letters above the bars indicate a significant difference at 95% confidence, according to the Tukey test. N: A/C/E = 135 per point, B/D/F = 27.

Sprout weight and dry matter

Significant differences were only found for the growth of sprouts (Tab. 1, Fig. 7) represented by fresh weight (FW), dry weight (DW) and dry matter percentage (DM) in response to GA_3 application. The FW and DW of the sprouts were significantly lower for treatments with 0 mg L^{-1} GA_3 (3.02 g, 0.68 g) compared to 25 mg L^{-1} (4.06 g, 0.87 g) and 50 mg L^{-1} (4.54 g, 0.95 g), but there were no significant differences between these last two (Fig. 8A, Fig. 8B). These results are similar to those observed by other authors in different cultivars (Alexopoulos *et al*., 2007; Salimi *et al.*, 2010; Mustefa *et al*., 2017).

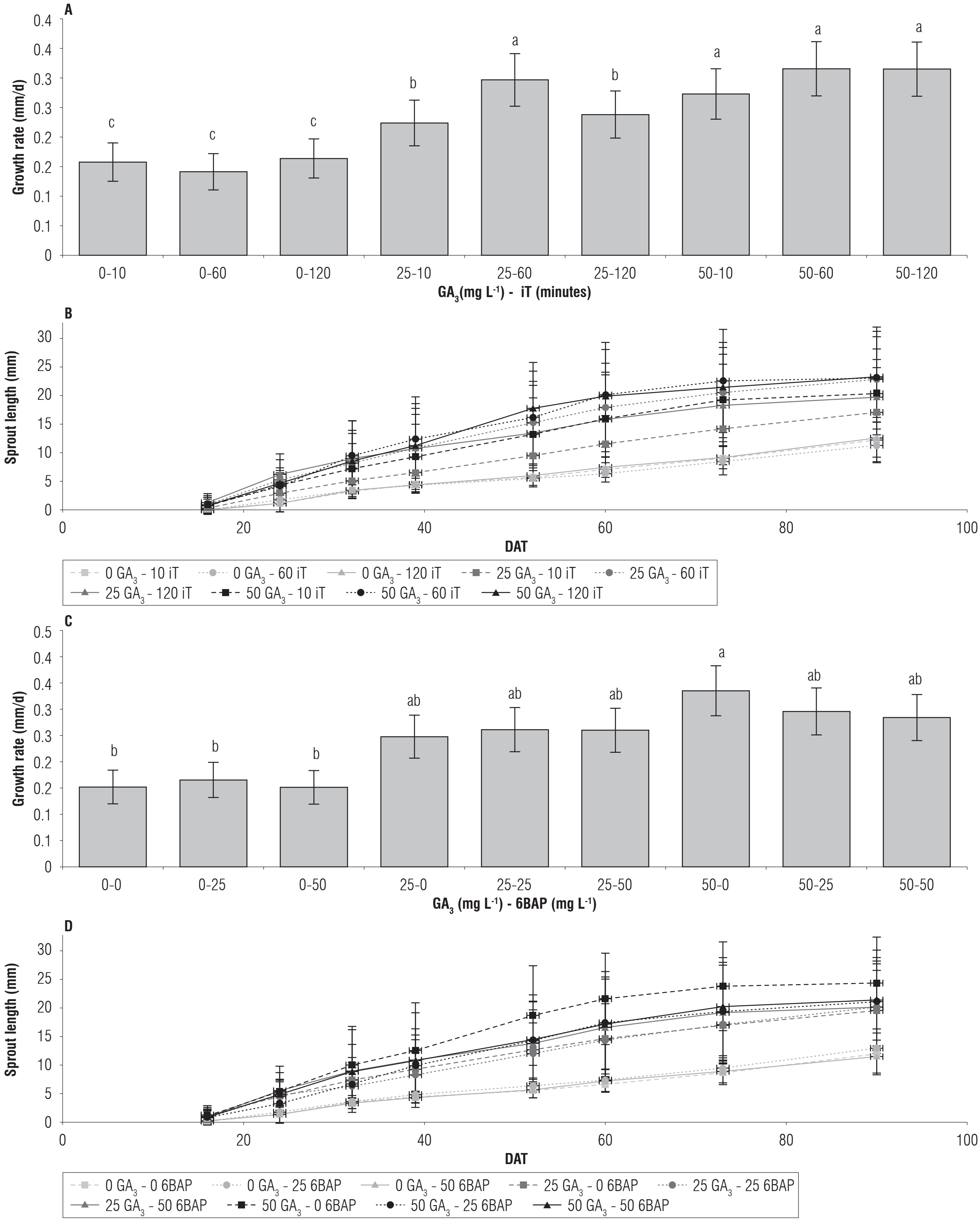

FIGURE 5. Growth rate (A, C) and length (B, D) of apical sprouts in cv. Diacol Capiro in response to interactions $GA_3 \times iT$ (A, B) and $GA_3 \times 6BAP$ (C, D) 90 days after treatment (DAT). Data shown in A and C correspond to the growth rate during the evaluation period. Vertical bars correspond to the standard deviation. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N: A/C/E = 45 per point; B/D/F = 9.

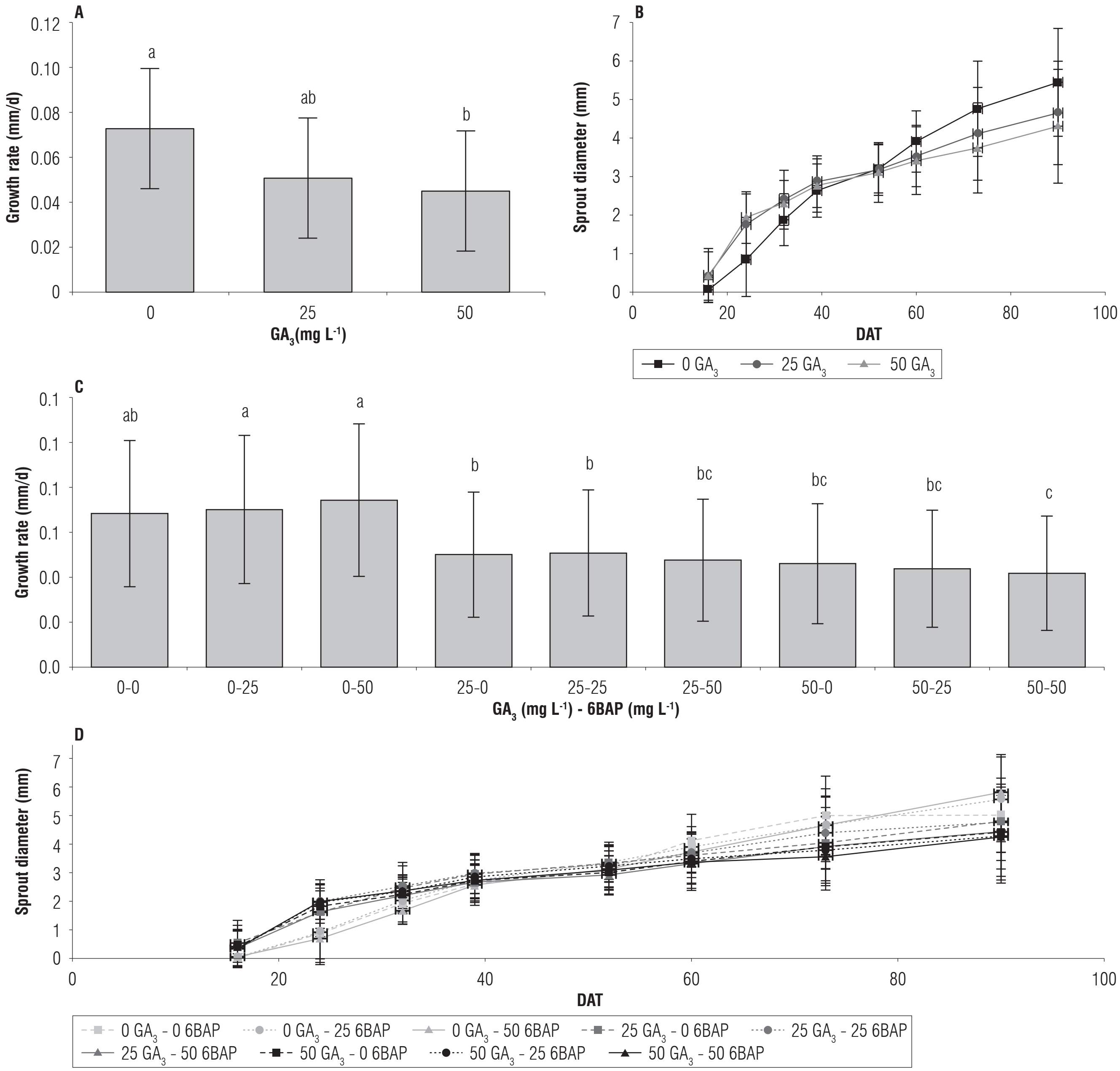

FIGURE 6. Growth rate (A, C) and diameter (B, D) of apical sprouts in the cv. Diacol Capiro in response to gibberellic acid-3 (GA_3) (A, B) and the GA_3×6BAP interaction (C, D) 90 days after treatment (DAT). Data shown in A and C correspond to the growth rate during the evaluation period. Vertical bars correspond to the standard deviation. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N: A = 135 per point, B = 27; C = 45 per point; D = 9.

The dry matter percentage in the sprouts showed significant differences for the treatments with GA_3 (Fig. 8C). Treatments with 0 mg L^{-1} GA_3 obtained greater dry matter percentages (23%) than the treatments with 25 mg L^{-1} and 50 mg L^{-1} (21%) with no differences between the last two. These results suggest that sprout growth promoted by GA_3 was caused by cellular elongation rather than by division. Salimi *et al*. (2010) find similar results in response to GA_3 treatments. However, partially opposite results are reported by Alexopoulos *et al*. (2007), who find that tuber sprouts treated with GA_3 and 6BAP are larger, with greater dry mass percentages than in untreated tubers.

Secondary sprouting

Secondary sprouting corresponds to the formation of thin sprouts that grow on the main sprout (Krijthe, 1962). A

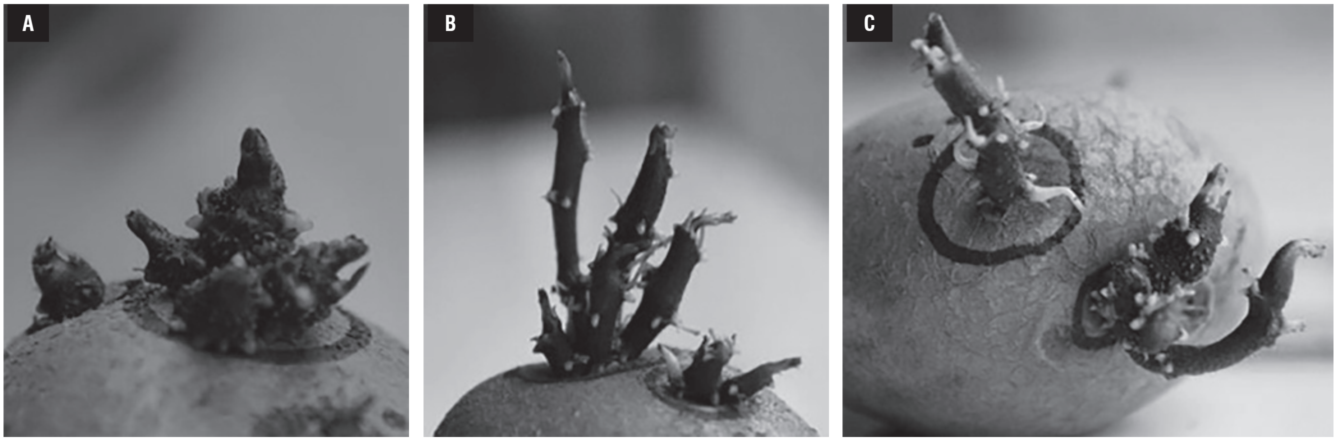

FIGURE 7. Apical sprouts of potato (*S. tuberosum*) tubers cv. Diacol Capiro A) immersed in pure water for 10 min, B) treated with 25 mg L^{-1} gibberellic acid-3 (GA_3) for 60 min, and C) treated with 50 mg L^{-1} GA_3 for 120 min, 90 d after treatment.

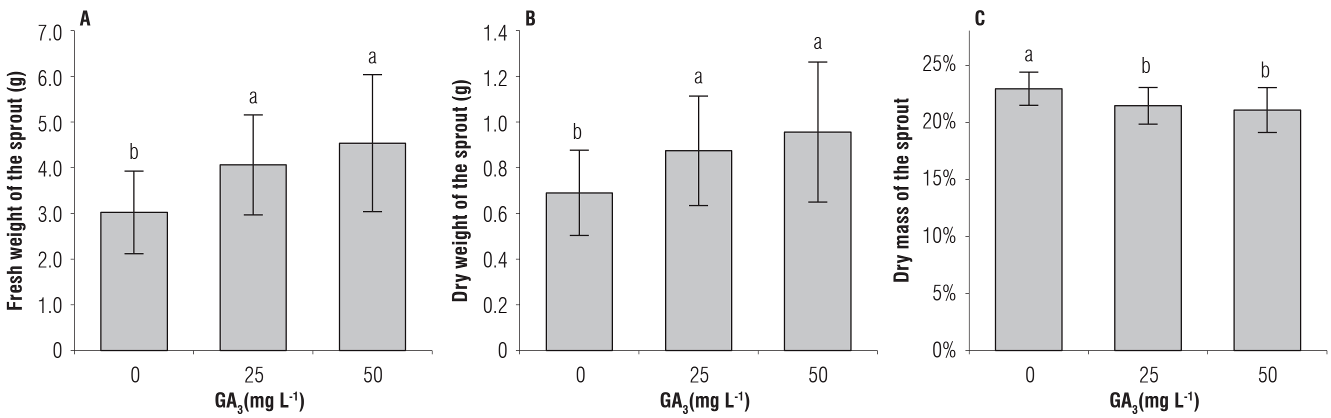

FIGURE 8. A) Fresh weight, B) dry weight, and C) percentage of dry matter of tuber sprouts of *S. tuberosum* L. cv. Diacol Capiro in response to different doses of gibberellic acid-3 (GA_3). Vertical bars correspond to the standard deviation. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N: A/B/C = 27.

significant effect of GA_3 was observed in our research (Tab. 1), increasing the percentage of occurrence when the dose was higher, with results of 51% for treatments with 0 mg L^{-1}, 80% for 25 mg L^{-1} and 88% for 50 mg L^{-1} (Fig. 9). Secondary sprouting takes place in tubers with very long periods of storage time and this is associated with tuber senescence (Struik, 2007; Carli *et al*., 2010) or high storage humidity.

Blocking in the experiment was performed by tuber size and this influenced most of the variables, because size is associated with the physiological age of the tubers (Struik, 2007; Salimi *et al*., 2010; Mohammadi *et al*., 2014; Christensen *et al*., 2019). The dormancy period decreases when the tuber is larger (Knowles and Knowles, 2006). In our research, the largest tubers developed larger sprouts with a higher dry matter content and increased secondary sprouting. These data show that there is a differential response to treatments with growth regulators due to the size of the tubers, which highlights the importance of taking this factor into account to apply GA_3 as a seed treatment.

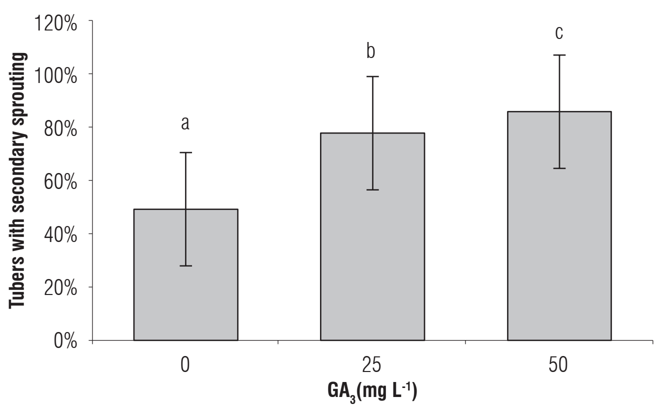

FIGURE 9. Percentage of tubers with secondary sprouting in *S. tuberosum* L. cv. Diacol Capiro treated with different concentrations of gibberellic acid-3 (GA_3). Standard deviations are indicated by vertical bars. Different letters above the bars indicate significant difference at 95% confidence, according to the Tukey test. N = 27.

Conclusions

The results showed that exogenous GA_3 applications decrease the dormancy period of cv. Diacol Capiro tubers and their effect increases as an effect of prolonged immersion times (60 and 120 min). The treatments with 25 or 50 mg L^{-1} GA_3 and iT 60 min reduced the dormancy period by 35% (18 d). However, 50 mg L^{-1} GA_3 generated long sprouts with smaller diameter and lower DM content. This makes them more susceptible to mechanical damage and increases the occurrence of characteristics associated with advanced physiological age and affects the quality of the tuber. In this study, 6BAP showed no clear effect on dormancy breakage.

Acknowledgments

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Effects of pre-sowing treatments with allelopathic plant extracts on tree tomato (*Solanum betaceum* Cav.) seedling emergence and performance

Efectos de tratamientos previos a la siembra con extractos de plantas alelopáticas sobre la emergencia y el rendimiento de plántulas de tomate de árbol (*Solanum betaceum* Cav.)

Kazım Mavi[1]* and Fulya Uzunoğlu[1]

ABSTRACT

Seeds of the tree tomato (*Solanum betaceum* Cav.) were treated with allelopathic plant extracts: ferula gum (FER, 0.2 g gum L^{-1}, 25°C, 24 h), lantana petal extract (LAN, 4 g dried petal L^{-1}, 25°C, 24 h) and marigold petal extract (TAG, 4 g dried petal L^{-1}, 25°C, 24 h) along with an untreated control to determine the effect of these extracts on seedling emergence and performance. The pre-sowing influence of the allelopathic extracts (FER, LAN and TAG) on the emergence percentage (%), mean emergence time (days), emergence index, coefficient of velocity of emergence (%), seedling length (cm), seedling fresh weight (mg), seedling dry weight (mg), and stimulatory allelopathic index (%) was investigated in tree tomato seeds. The results revealed that pre-sowing treatment with the studied allelopathic plant extracts showed higher seedling emergence and performance. The greatest advantage of the pre-sowing treatment was observed in the TAG treatment. Allelopathic TAG treatment resulted in 15% higher seedling emergence rates, 3.3 d faster mean emergence time, 153 mg heavier seedling weights, and higher emergence index compared to untreated seeds. In addition, the effect of allelopathic FER and LAN treatments was better than the control.

Key words: organic treatment, tamarillo seedlings, stimulatory effect, allelochemicals.

RESUMEN

Las semillas de tomate de árbol (*Solanum betaceum* Cav.) fueron tratadas con extractos de plantas alelopáticas: resina de férula (FER, 0.2 g de resina L^{-1}, 25°C, 24 h), extracto de pétalos lantana (LAN, 4 g de pétalos secos L^{-1}, 25°C, 24 h) y extracto de pétalos caléndula (TAG, 4 g de pétalos secos L^{-1}, 25°C, 24 h) junto con un control sin tratamiento para determinar el efecto de estos extractos sobre la emergencia y desempeño de las plántulas. Se estudiaron la influencia previa a la siembra de los extractos alelopáticos (FER, LAN y TAG) sobre el porcentaje de emergencia (%), tiempo medio de emergencia (días), índice de emergencia, coeficiente de velocidad de emergencia (%), altura de la plántula (cm), peso fresco de la plántula (mg), peso seco de la plántula (mg) e índice alelopático estimulante (%) en semillas de tomate de árbol. Los resultados revelaron que el tratamiento previo a la siembra con los extractos de plantas alelopáticas estudiados mostró una mayor emergencia y rendimiento de las plántulas. La mayor ventaja del tratamiento previo a la siembra se observó en el tratamiento con TAG. El tratamiento con TAG alelopático dio como resultado tasas de emergencia de plántulas 15% más altas, tiempo de emergencia promedio 3.3 d más rápido, pesos de plántulas 153 mg superiores e índice de emergencia más alto con respecto a las semillas no tratadas. Además, el tratamiento alelopático de FER y LAN fue mejor que el control.

Palabras clave: tratamiento orgánico, plántulas de tamarillo, efecto estimulante, aleloquímicos.

Introduction

The tree tomato (*Solanum betaceum*) is one of the most important members of the Solanaceae family and the genus *Solanum* cultivated in South America. The tree tomato is native to tropical and subtropical climates, is found from Colombia to Peru (Albornoz, 1992), and grows between 1600 and 2400 m a.s.l. It is a semi-shade and perennial plant species that can grow to between 3.0 m and 5.5 m in height. Plant development from seeds to fruiting occurs after 18 months, and its commercial life takes seven to eight years. The flowers are pink and lavender and are grouped in terminal clusters that bloom in stages between May and June (Ramírez and Kallarackal, 2019). The fruits are berries whose colors vary from yellow to red, are ovoid with pointed apices and contain approximately 150-300 small seeds (Vasco *et al.*, 2009).

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Although it is a lesser known species, the tree tomato has great economic potential thanks to its fruits or some ornamental species. However, the development of technologies for seedling production is necessary to reach full potential. Dormancy in freshly extracted seeds is thought to reduce the germination of tree tomato seeds. Tree tomato propagation is mainly by seed (Neto *et al*., 2015), and seedling emergence of tree tomato seeds is low and uneven, deterring the formation of quality seedlings. Dormancy is also a problem directly related to germination and storage of tree tomato seeds (Neto *et al*., 2015).

Allelopathy is defined as the effects of a plant or organism on another through the emission of chemicals with stimulating or inhibitory effects (Rice, 1979). Allelopathy more commonly refers to the inhibitory effects of one species on another. All of the allelochemicals present may not always be harmful, as beneficial interactions have also been reported (Mavi, 2014). Allelochemicals that inhibit the growth of some species at certain concentrations can stimulate the growth of the same or of different species at lower concentrations.

Bioherbicides are used as an alternative to the intensive use of synthetic herbicides that cause serious environmental hazards and increase production costs (Atak *et al*., 2016). Bioherbicides have also found use as antifungal, antimicrobial and antibacterial coating material in fruit storage (Kotan *et al*., 2013). However, studies on their positive effects on germination, emergence and seedling development are limited. Hence, positive effects of these natural herbicides on seedling development and quality should be studied in different fields, particularly organic farming. Fungal infections during seed germination are one of the important factors preventing seedling development from seeds. The antifungal properties of such allelochemicals can be useful for the development of healthy and quality seedlings (Mavi, 2018).

Moringa oleifera leaf extract is the most studied allelopathic species for stimulation of germination and emergence (Basra *et al*., 2011). The allelopathic effect of marigold extract as an organic material in priming applications has been used successfully in seed applications in ornamental plants (Mavi, 2013), eggplant (Mavi, 2014), watermelon (Mavi and Atak, 2016), mulberry (Gündüz *et al*., 2019) and different pepper species (Mavi, 2016; Mavi, 2018). Different species of wild fennel (Turkyilmaz-Unal *et al*., 2019) and lantana (Achhireddy and Singh, 1984) are also known as allelopathic species, and their stimulatory effects have not been studied. Whereas in previous research the leaves and roots of plants were used to assess the negative allelopathic effect of these genera, this experiment was designed to determine the stimulatory effect of the gum of ferula (*Ferula elaeochytris* Korovin) and petals of lantana (*Lantana camara* L.). These two species, along with the marigold (*Tagetes patula* L.) whose allelopathic effect on different species was known, were used to study their effect on the seedling emergence and performance of the tree tomato.

Materials and methods

Seeds taken from the mature fruits of a tree tomato genotype found in the genetic collection of exotic vegetables belonging to the Faculty of Agriculture, Department of Horticulture at Mustafa Kemal University in Turkey were used in this research.

Marigold flowers were collected, and the petals were extracted from the flowers and dried for 10 d in the shade at room temperature (25±3°C). The dried petals were stored in glass jars in the laboratory until the treatment was carried out. The dried petals (4 g) were brewed in boiling distilled water (1 L) and after cooling, this herbal tea was used as an allelopathic priming material. Application of marigold is referred to in this paper as TAG.

Lantana flowers were collected, and petals of the flower were dried; the extracts (4 g L^{-1} dried petal) were prepared in a similar manner as that of marigold. Lantana treatment is termed LAN in this paper.

Wild fennel is a species used for medicinal purposes with roots locally known as *Çakşır* (Hatay -Turkey) or *hinojo* in Spanish. This species is collected by removing the roots. In the observations we made, pure sap flowed from the regions close to the roots of the species, and the sap became amber-colored when it dried (Fig. 1A-B). The use of this wild fennel gum, instead of removing the plant completely, prevents damage to natural flora. Determining the stimulatory effect of this wild fennel gum on germination and emergence is important for organic farming. The gum was dissolved in boiling water (0.2 g gum L^{-1}) and was used for seed treatment after cooled. This dose was determined after preliminary studies (data not shown). Wild fennel gum treatment is called FER in this article.

The seeds of the tree tomato were treated on filter paper moistened with 30 ml of each treatment and kept in 15 cm petri dishes in the dark at 25°C for 72 h. Seeds were covered with plastic film during the priming treatment to ensure

FIGURE 1. A) General view of a wild fennel (*Ferula elaeochytris*) plant; B) Pure sap flowing from the root that turns amber when dried.

that there was no loss of moisture. Once the treatment was completed, the seeds were rinsed with tap water. All conditions (temperature, duration, etc.) were the same for all treatments. All treated seeds were used in emergence tests after surface drying. Untreated dry seeds were kept as controls (CON).

After the treatments, emergence tests were conducted after seeds were surface-dried and three replicates of 25 seeds per treatment were set up. To determine the effects of pre-sowing treatments on seedling emergence, seeds were sown at a depth of 1 cm in plastic boxes with peat moss (pH 5.5-6.5, electrical conductivity 40 mS m^{-1}, Potgrond P, Klasman, Germany). Seedlings were grown under laboratory conditions at 23±2°C for 24 d. The appearance of the hypocotyl hook on the peat moss surface was used as an emergence criterion and emerged seedlings were recorded daily. To measure the effect of the pre-sowing treatments on emergence uniformity, the emergence percentage, mean emergence time (Orchard, 1977), emergence index (Maguire, 1962), and coefficient of velocity of emergence (Kader, 2005) were calculated.

Following the stabilization of the number of emerged seedlings, 21 normally developed seedlings of each treatment were randomly harvested to determine the seedling growth rate. The length (cm) and fresh and subsequently dry weights of harvested seedlings were recorded by drying at 80°C for 24 h in a laboratory oven (M3025P, Elektro-mag, Istanbul, Turkey). Fresh and dry weights were expressed as mg/plant.

The stimulatory allelopathy index (SAI) was obtained using the formula by Williamson and Richardson (1988).

$$SAI\ (\%) = (T/C - 1) \times 100 \quad (1)$$

where T is the treatment value and C is the control value. SAI was used to indicate the stimulatory effects of aqueous extracts on the emergence index (EI), coefficient of velocity of emergence (CVE), seedling length (SL), seedling fresh weight (SFW) and seedling dry weight (SDW).

Mean data for each character were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. When the *P* value was less than 0.05 it was considered as significant. Percentages were arcsine-transformed prior to analysis and the untransformed data were shown in the figures.

Results and discussion

The percentage of cumulative emergence was determined as a significant ($P \leq 0.05$) difference between pre-sowing treatments and control seeds (Fig. 2). TAG, FER and LAN-treated seeds reached at 54%, 52% and 43%, respectively, after 10 d, while control seeds had a seedling emergence of 11% for the same time (Fig. 3). The TAG pre-sowing allelopathic treatment was superior throughout the duration of the experiment. There was little difference among all pre-sowing treatments, but a significant difference between treated and control seeds was observed. Finally, TAG-treated seeds reached 96% emergence after 18 d, while control seeds had 72% seedling emergence at that time (Fig. 2).

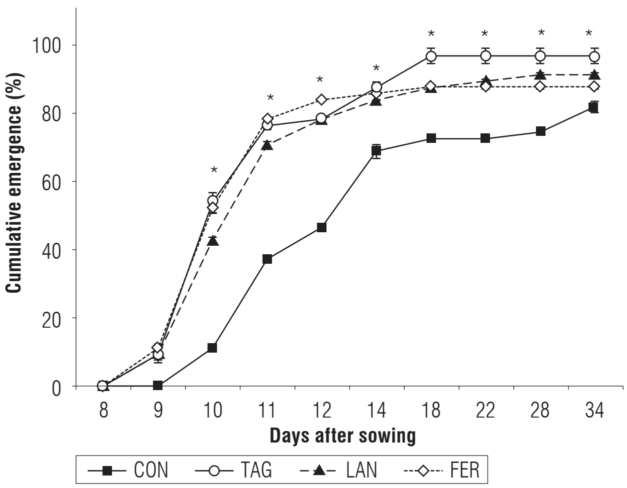

FIGURE 2. Cumulative emergence percentage of tree tomato seeds sown at 23±2°C under different extracts of allelopathic plants (TAG, LAN and FER). CON - control, TAG - marigold treatment, LAN - lantana treatment, FER - wild fennel gum treatment. Error bars represent ± standard error; n = 3. * indicates differences between untreated seeds and treatments at $P<0.05$.

Mean emergence time (MET), EI, and CVE are shown in Fig. 4. The FER treatment showed a MET value of 10.55 d. No statistically significant differences were observed among MET, TAG and LAN. The MET values of these treatments were significantly higher than those obtained by the control.

The EI and CVE increased with the effects of pre-sowing treatments (Fig. 4) and all of them were statistically different compared to the controls. While the highest CVE (9.49) was recorded in seeds treated with FER, the highest EI (3.17) was recorded in seeds treated with TAG.

The variables SL, SFW and SDW of plants with pre-sowing treatments with TAG, LAN and FER were better than in the untreated group (Fig. 5). Maximum SL (6.61 cm) was obtained under pre-sowing treatment with TAG, whereas the minimum SL (5.60 cm) was recorded in the seedlings from untreated seeds. Similarly, the TAG treatment showed the highest values of SFW (475 mg) and SDW (28.4 mg).

The five indicators, including SDW, SFW, EI, CVE and SL, were used to estimate the SAI value to know the total allelopathic intensity of the three species of pre-sowing treatment extracts on the tree tomato (Fig. 6). The SAI values of the three species of pre-sowing treatment extracts were as follows: TAG > LAN > FER.

The results of this research highlighted the potential of allelopathic pre-sowing treatments with TAG, LAN, and FER as natural seedling growth enhancers in tree tomato.

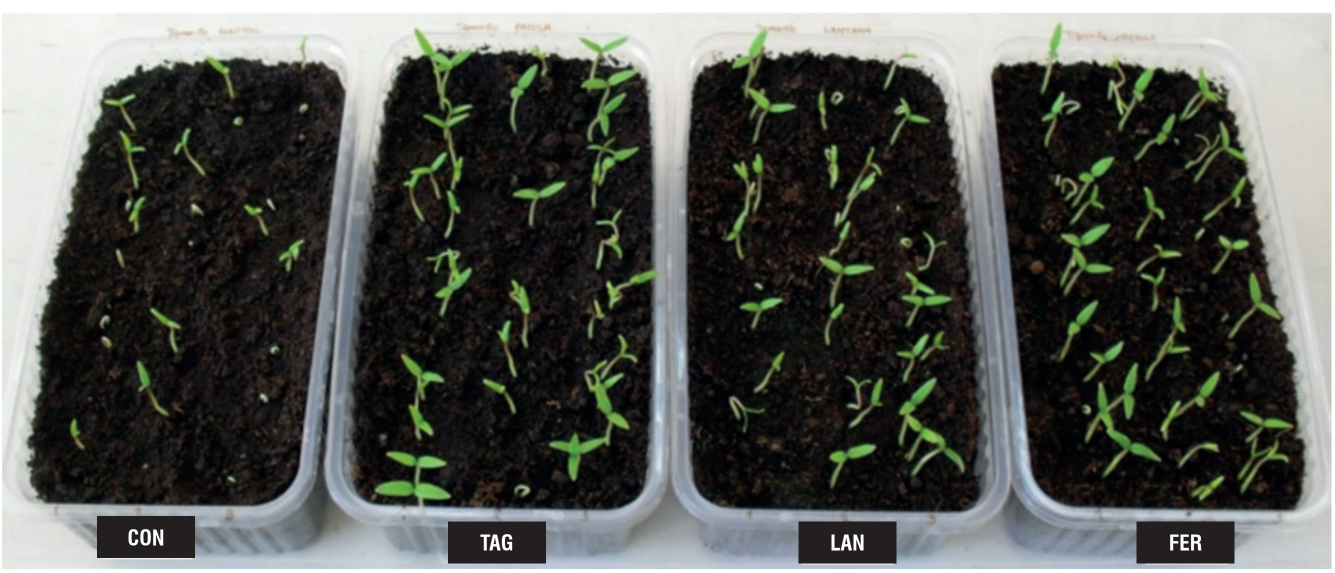

FIGURE 3. Early emerging performance of pre-sowing allelopathic treatments in tree tomato seeds 10 d after sowing. CON - control, TAG - marigold treatment, LAN - lantana treatment, FER - wild fennel gum treatment.

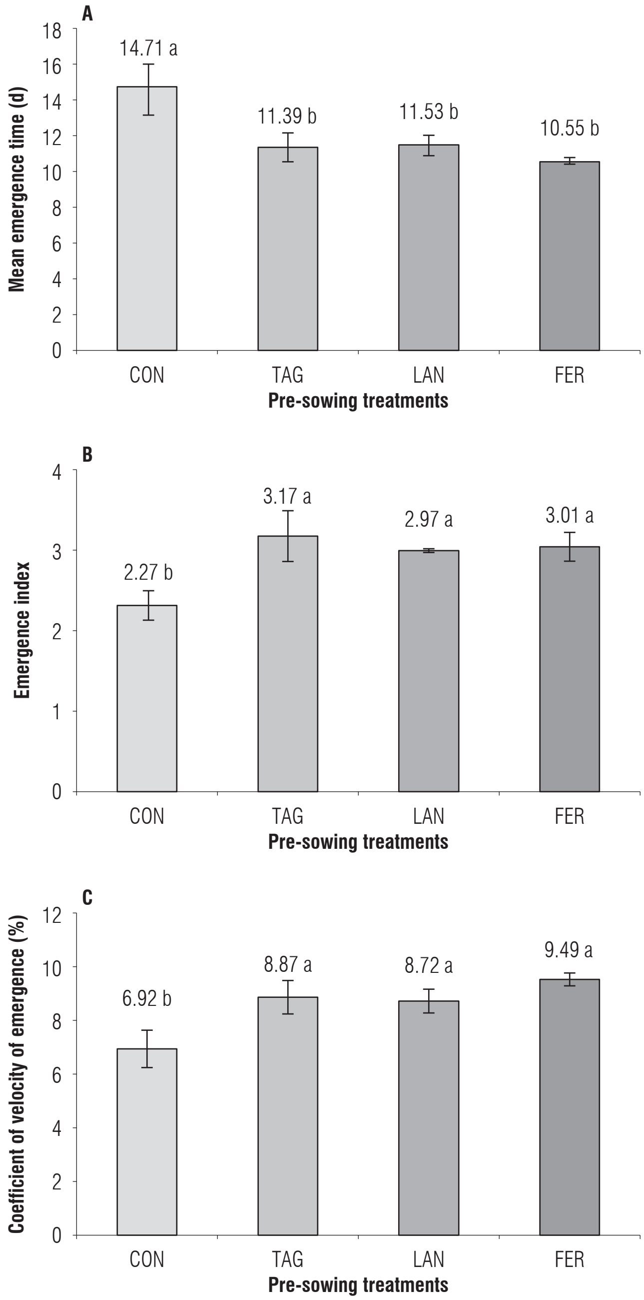

FIGURE 4. Effect of pre-sowing allelopathic treatments with TAG, LAN, and FER on A) mean emergence time, B) emergence index, and C) coefficient of velocity of emergence. CON - control, TAG - marigold treatment, LAN - lantana treatment, FER - wild fennel gum treatment. Error bars represent ± standard error; n = 3.

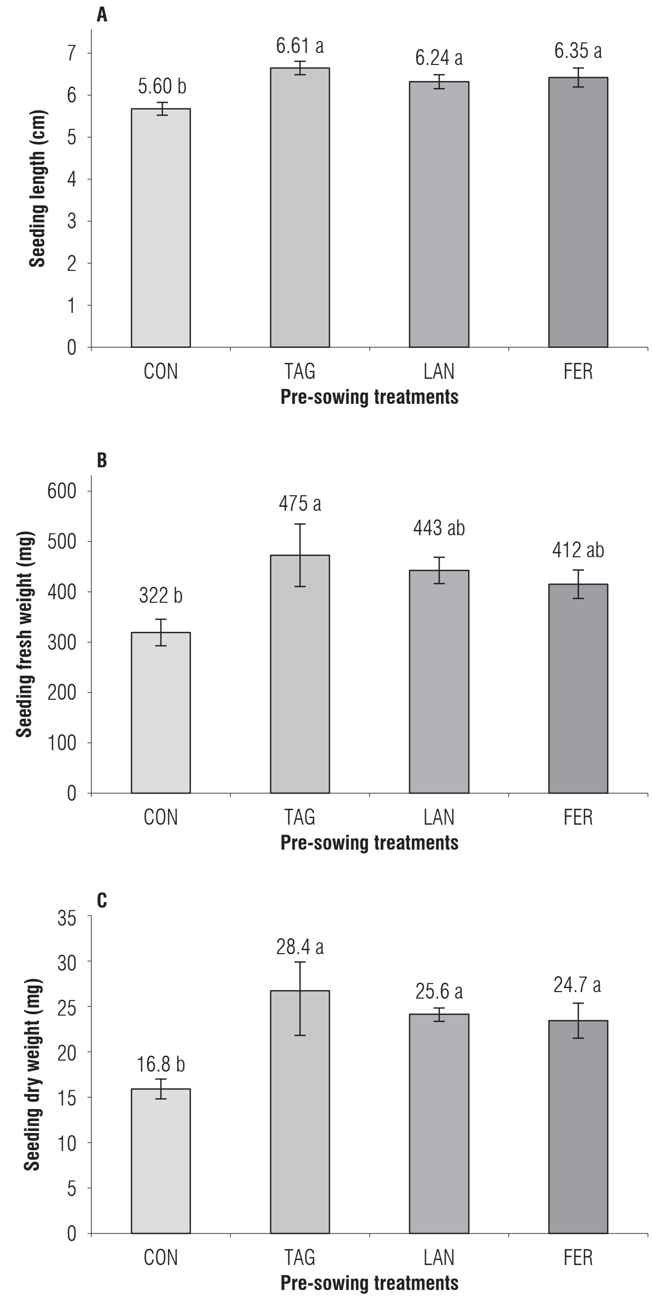

FIGURE 5. Effect of pre-sowing treatments with TAG, LAN, and FER on A) seedling length, B) seedling fresh weight, and C) seedling dry weight. CON - control, TAG - marigold treatment, LAN - lantana treatment, FER - wild fennel gum treatment. Error bars represent ± standard error; n = 3.

Allelopathic materials were mostly used for weed control because of their inhibitory effects (Atak *et al*., 2016). However, in recent years, marigold extract (TAG) had been successfully used to improve seedling emergence and performance in some different species such as ornamental plants (Mavi, 2013), eggplant (Mavi, 2014), watermelon (Mavi and Atak, 2016), mulberry (Gündüz *et al*., 2019) and different types of pepper (Mavi, 2016; Mavi, 2018). In this study, it was also determined that two different allelopathic materials (LAN and FER) can be used in a tree tomato genotype to improve seedling quality.

Typically, tree tomato is propagated by seeds that have uneven germination, which may be related to dormancy

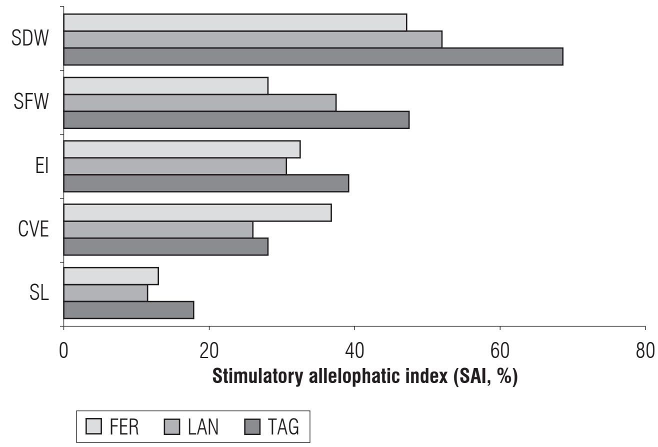

FIGURE 6. Stimulatory allelopathic index (SAI) of the three species of pre-sowing extracts of the five indicators, including seedling dry weight (SDW), seedling fresh weight (SFW), emergence index (EI), coefficient of velocity of emergence (CVE) and seedling length (SL). FER - wild fennel gum treatment, LAN - lantana treatment, TAG - marigold treatment.

(Neto *et al*., 2015). This characteristic is common in seeds of other *Solanaceae* species, such as *Solanum chenopodioides* (Cabrera *et al*., 2010), which have slow and irregular germination. The allelopathic pre-sowing treatments used in a tree tomato genotype caused an increase in the emergence performance (Figs. 2-4) and seedling quality (Figs. 5 and 6). A similar effect was observed in eggplant (Mavi, 2014), watermelon (Mavi and Atak, 2016) and different pepper species (Mavi, 2018) for the TAG pre-sowing treatment. The effectiveness of the allelopathic pre-sowing treatments FER and LAN has been demonstrated for the first time with this study.

Seed pre-sowing treatments (osmopriming, halopriming, hydropriming, hormonal priming, matrix priming, smoke priming, etc.) produce a repair effect at the stagnant stage before DNA synthesis. RNA, protein and DNA syntheses and some enzymes such as L-isoaspartylmethyltransferase, and catalase have caused changes in germinated seeds after priming (Côme, 1983; Kester *et al*., 1997; Kibinza *et al*., 2011). In this study, the allelopathic pre-sowing treatments increased the emergence percentage, which confirms that repair mechanism.

Conclusions

In conclusion, as a result of the application of these allelopathic pre-sowing treatments, there has been a clear improvement in the emergence performance and seedling quality of tree tomato seeds. LAN (flower extract) and FER (gum extract) were used as stimulants for the first time to improve seed emergence performance in tree tomato. Although it is not possible to obtain commercially available LAN, FER and TAG extracts, the potential of these materials to be used as priming agents by a simple method has been demonstrated here. Consequently, pre-sowing treatment with allelopathic plant materials should be proposed to improve seedling emergence and performance in the tree tomato since it is an inexpensive, organic, eco-friendly, effective and simple technique. Furthermore, the allelochemicals present in these plant extracts should be studied for antibacterial effects on seedlings.

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Detection of mycotoxins produced by *Fusarium* species in Colombia

Detección de micotoxinas producidas por especies de *Fusarium* en Colombia

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ABSTRACT

The genus *Fusarium* produces mycotoxins that are metabolites of low molecular weight that affect the quality of crops, and even more importantly, they affect the health of humans and animals. Among those that cause damage to health are trichothecenes, fumonisins, and zearalenones. The objective of this study was to quantify the mycotoxins produced by species of the genus *Fusarium* from a population of isolates obtained from different crops and locations in Colombia. From 206 isolates, only 14 amplified to regions associated with mycotoxins deoxynivalenol (DON) belonging to the group of trichothecenes, fumonisin (FUM) and zearalenone (ZEA) using PCR. Each isolate with the presence of mycotoxins was conserved in potato dextrose agar (PDA) medium. Eight days after seeding in corn kernel medium, the samples were processed to perform the quantitative analysis of DON, ZEA and FUM using an ELISA kit based on enzyme-linked immunosorbent assays. The results show that mycotoxins were present in the evaluated isolates and their levels were above the standards regulated by Mercosur and the European Union. The use of immunosorbent assays using the ELISA technique becomes a useful tool to detect and quantify mycotoxins of species of the genus *Fusarium* that affect different crops in Colombia.

Key words: fumonisins, trichothecenes, zearalenone, ELISA.

RESUMEN

El género *Fusarium* produce micotoxinas que son metabolitos de bajo peso molecular que afectan la calidad de los cultivos y especialmente la salud de humanos y animales. Entre las que causan daños a la salud se encuentran los tricotecenos, las fumonisinas y las zearalenonas. El objetivo de este estudio fue cuantificar las micotoxinas producidas por especies del género *Fusarium* de una población de aislamientos obtenida de diferentes cultivos y localidades de Colombia. De 206 aislamientos, sólo 14 amplificaron regiones asociadas a las micotoxinas deoxinivalenol (DON), que pertenece al grupo de los tricotecenos, fumonisina (FUM) y zearalenona (ZEA) usando PCR. Cada uno de los aislamientos con presencia de micotoxinas se conservaron en medio papa dextrosa agar (PDA). Ocho días después de la siembra en medio de granos de maíz, las muestras se procesaron para realizar el análisis cuantitativo de DON, ZEA y FUM utilizando un kit de ELISA basado en ensayos de inmunoabsorción ligados a una enzima. Los resultados revelan la presencia de micotoxinas en los aislamientos evaluados cuyos niveles se encuentran por encima de los estándares regulados por Mercosur y la Unión Europea. El uso de ensayos de inmunoabsorción por medio de la técnica de ELISA se convierte en una herramienta útil para detectar y cuantificar micotoxinas de especies del género *Fusarium* que afectan diferentes cultivos en Colombia.

Palabras clave: fumonisinas, tricotecenos, zearalenona, ELISA.

Introduction

The study of mycotoxins has been an object of interest for the world in the last twenty years, not only because of the large losses in crop and animal productivity but also because of their deleterious effects. Mycotoxins are considered the secondary metabolic product of a large number of fungi that in small amounts are capable of triggering alterations and pathological conditions in both humans and animals. The intake of food contaminated with mycotoxins may cause acute or chronic mycotoxicosis. Depending on the degree of toxicity, the central nervous, digestive, cardiovascular, and pulmonary systems can be affected. Mycotoxins can also trigger cancerous diseases, produce mutagenic and teratogenic damages and, in some cases, can behave as immunosuppressants (Bennett and Klich, 2003; Bertero *et al*., 2018).

Mycotoxins are produced by fungi capable of growing on a wide variety of substrates, so they often contaminate food. The biosynthesis is performed by a few precursors derived from intermediates of its primary metabolism. Production occurs during the stationary phase of growth with little or no production of these mycotoxins during the active growth phase (Berthiller *et al*., 2013; Pitt and Miller, 2017).

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Since mycotoxins were recognized as a public health problem, quantification by analytical techniques has continuously improved. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most used analytical techniques today. These techniques determine several trichothecenes simultaneously with low concentration levels (ng g^{-1}) of evaluated samples, requiring high stringency, especially in the cleaning processes. The ELISA technique allows a large number of samples to be measured routinely with high precision compared to CG and HPLC methods, which are expensive and take longer to get results (Escobar and Fragas, 2004; Pleadin *et al*., 2012).

One of the first reports on food contamination by *Fusarium* mycotoxins in human and animal health mentions the T-2 toxin produced by alimentary toxic aleukia (ATA), which was reported in Russia in the 1940s and fumonisins that cause equine leukoencephalomalacia, reported in several countries in the 1980s (Pitt and Miller, 2017).

Mycotoxin production is stimulated by oxidative and nutritional stress, environmental factors such as pH, temperature, water activity, fungicides and secondary metabolites produced in these environments (Martínez *et al.,* 2010). Evaluations in some regions of Europe showed that 80-100% of grains were contaminated with trichothecenes (Reverberi *et al.,* 2010). These high incidences occurred in regions where crops were affected by drought, infested by insects, or inadequately maintained using inappropriate harvesting equipment or storage facilities (Pohland, 1993).

In the case of *Fusarium*, which is essentially regarded as a field pathogen, mycotoxins are synthesized mainly during plant infection; in the case of cereals they can accumulate in the grain during the production, harvesting and storage stages (Desjardins and Hohn, 1997). Species of this genus, which are contaminants of these plants, can produce a large variety of mycotoxins. The mycotoxigenic profile may vary at inter- and intra-specific levels and often includes a wide range of mycotoxins. Fumonisins and trichothecenes are the most frequent and toxic mycotoxins produced by *Fusarium*, although this genus can produce other important mycotoxins such as zearalenone, enanthines, and moniliformina (Chakrabarti, 2013; Zhang *et al*., 2013).

Environmental conditions, susceptible hosts, and virulence of the pathogen are the factors that promote fungus growth and, consequently, the production of toxins. Therefore, agricultural products affected by these microorganisms may be susceptible to contamination in the production, harvesting, transport, and storage processes (Nelson *et al*., 1993; Desjardins and Hohn, 1997; Desjardins and Proctor, 2007; Zhang *et al*., 2013).

In Colombia, resolution 4506 of October 30 establishes the maximum levels of food contaminants intended for human consumption (Ministry of Health and Social Protection, 2013). The maximum permissible limit for deoxynivalenol (DON) is 100-1750 μg kg^{-1}, for fumonisins (FUM) it is 200-4000 μg kg^{-1} and for zearalenone (ZEA) it is 20-350 μg kg^{-1}. These ranges depend on the food type, the size of particles generated in the industrial process, and the age of the consumers. These values are remarkably similar to those managed by the European Union (EU) and Mercosur (WHO/FAO, 2003).

The overall objective of this study was to quantify the mycotoxins present in *Fusarium* isolates from a collection of samples belonging to the Universidad Nacional de Colombia located in Palmira (Valle).

Materials and methods

This study was conducted in the laboratory of Plant diagnostics of the Universidad Nacional de Colombia located in Palmira.

Fourteen isolates previously evaluated by PCR (Tab. 1) with the trichothecene (*TRI)*, fumonisin (*FUM)* and zearalenone (*PKS)* genes were selected and amplified for trichothecene (DON), fumonisin (FUM) and zearalenone (ZEA) detection (Tab. 2).

TABLE 1. Primers used to detect mycotoxins in *Fusarium* isolates from the collection of the Universidad Nacional de Colombia - Palmira campus.

Mycotoxin	Primer	Sequence 5'-3'	Amp (bp)+	°T hybridization	Genotype	Reference
Deoxynivalenol	TRI 5 F TRI 5 R	5´-AGCGACTACAGGCTTCCCTC -3´ 5´-AAACCAATCCAGTTCTCCATCT-3´	545	59	*TRI*	Li *et al*., 2005
Fumonisin	FUM 1F FUM 4R	5´-GAGGCCCGAGCGAGCACTGG-3' 5´-CCAGCCGCGGAAATTAGGGATGTG-3'	1456	58	*FUM*	Bluhm *et al*., 2004
Zearalenone	PKS13F PKS13R	5'-CCCAGCCAAGCCCAGTACGC-3´ 5´-ACAGCGGCTGACCTGGGTCA-3´	532	59	*PKS*	Stępień *et al*., 2011

Amp (bp)+ = expected amplicon size in base pairs.

TABLE 2. Mycotoxin detection present in isolates obtained by phylogenetic analysis from the collection of the Universidad Nacional de Colombia - Palmira campus.

Isolate	Species	Host	Mycotoxin	Locality
32	*F. graminearum*	Corn	TRI-ZEA	Las piedras/Tolima
38	*F. verticillioides*	Corn	FUM	Tierralta/Cordoba
40	*F. verticillioides*	Corn	FUM	San Agustin/Huila
42	*F. napiforme*	Corn	FUM	Palmira/Valle
61	*F. proliferatum*	Corn	FUM	Bolivar/Valle
67	*F. graminearum*	Corn	TRI-ZEA	Versalles/Valle
73	*F. verticillioides*	Corn	FUM	Cerrito/Valle
75	*F. verticillioides*	Corn	FUM	Guacari/Valle
80	*F. verticillioides*	Corn	FUM	Riofrio/Valle
83	*F. verticillioides*	Corn	FUM	Palmira/Valle
113	*F. equiseti*	Melon	TRI-ZEA	Roldanillo/Valle
132	*F. verticillioides*	Grape	FUM	Roldanillo/Valle
144	*F. verticillioides*	Blackberry	FUM	Ginebra/Valle
202	*F. oxysporum*	Pepper	FUM	Yumbo/Valle

*TRI: Trichothecene; FUM: Fumonisin; ZEA: Zearalenone.

Mycotoxin quantification methods

The isolates were cultured in Petri dishes with potato dextrose agar (PDA) medium. Then, to determine the amount of mycotoxin, the isolates were introduced to corn kernel medium with a concentration of 1 x 10^8 ml^{-1} conidia. These isolates were preserved and incubated with a photoperiod of 12 h of light and 12 h of darkness. Once the mycelium reached growth for 15 d, the conidia count was carried out in a Neubauer chamber at a concentration of 1 x 10^6 ml^{-1} conidia; then, it was separated with a stirring rod adding 10 ml of 70% methanol. The mix was then filtered with Watman No. 1 paper and 100 µl of the solution was added with a micropipette to the controls and samples following the protocol of the NEOGEN® Veratox commercial kit according to the established concentration for each mycotoxin. Finally, the reading was performed in a Neogen 4700 microwell reader 9303 (NEOGEN© Corporation, Lansing, MI, USA) with a length of 650 nm wave. Calibration curves for the quantification of deoxynivalenol, zearalenone and fumonisins were performed with the controls established for each kit (Tab. 3).

Statistical analysis

The absorbance values and the concentration of the samples were performed using the regression formula established by the Elisa reader program (Eq. 1) (NEOGEN© Corporation, Acumedia, Lansing, MI, USA) through the optical densities of both the controls and the samples to be evaluated.

$$Y= \log i\ t\ (Abs)\ X=\log\ (Conc) \qquad (1)$$

where X and Y are the axes.

Results and discussion

Deoxynivalenol

The results show that the evaluated isolates (32, 67, and 113) have concentrations ranging between 0.6 and 2 mg L^{-1}, corresponding to *F. graminearum* and *F. equisetti* (Fig.1). This method demonstrates there is a high sensitivity for the test that is the most used for trichothecene detection in different foods for both human and animal consumption (Meneely *et al*., 2011).

TABLE 3. Values of controls used in mycotoxin quantification assays.

Mycotoxin	Concentration	C1	C2	C3	C4	C5
Deoxynivalenol	mg L^{-1}	0	0.5	1	2.0	5.0
Fumonisin	mg L^{-1}	0	1.0	2	4.0	6.0
Zearalenone	μg L^{-1}	0	25	75	150	500

C= X=log (Conc).

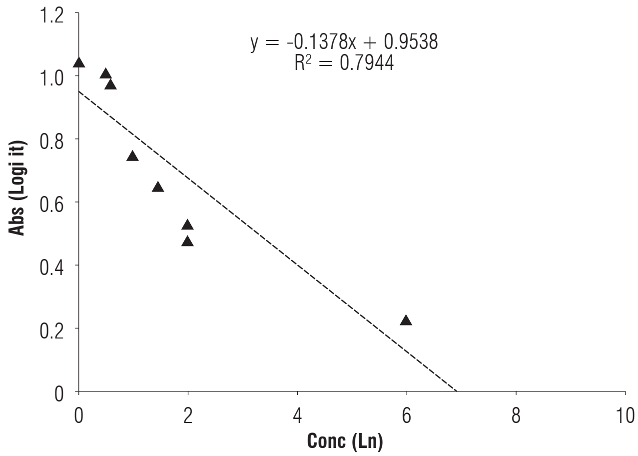

FIGURE 1. Concentration in mg L^{-1} and absorbance values of deoxynivalenol produced by isolates.

Studies of trichothecene quantification are performed using methods universally accepted by the International Official Association of Analytical Chemists (AOAC International), recognized by the Food and Drug Administration (FDA) for the determination of mycotoxins (Yoshizawa *et al*., 2004).

The results obtained in this evaluation indicate that the values for the presence of trichothecenes in food for human consumption are above the levels allowed in Colombia and the EU (Rojas and Wilches, 2011). Values greater than 750 µg kg^{-1} (0.75 mg kg^{-1}) found in *F. graminearum* isolates in corn indicate a high mycotoxin concentration in the evaluated samples. In Argentina corn samples with a high incidence of DON contamination of 0.93 mg kg^{-1} have been found (Pacin *et al*., 1997). In Brazil, the values of this toxin did not exceed 0.6 mg kg^{-1} (Furlong *et al*., 1995). There are no universal values for measuring the damage caused by DON in foodstuffs for human consumption. However, each country or group of countries, such as the EU and Mercosur, manage ranges between 300-2000 µg kg^{-1} with the lowest values being those of the EU (Pleadin *et al*., 2012).

In Colombia, studies on the detection and quantification of DON have been recorded since 1995 (Diaz and Cespedes, 1997; Duarte and Villamil, 2006; Rojas and Wilches, 2011; Rojas *et al*., 2015). These studies show a high concentration of DON in foodstuffs for human consumption, especially processed products from corn and wheat. This fact is worrying since to date controls and application of normativity are not carried out in the Colombia despite the resolution of the Ministry of Health and Social Protection issued in 2014 (Ministry of Health and Social Protection, 2013).

Risks caused by DON are initiated when it enters the food chain, and it is transmitted to humans directly through consumption of cereals and cereal products. Foods such as corn and wheat are the most susceptible to be contaminated with DON. The toxin can remain in processed foods made from contaminated cereal, such as bread, pasta, cookies, etc. Toxicity may occur by contact (causing skin, eye and throat irritation) or by direct ingestion. Depending on the amount ingested, it may cause vomiting, diarrhea, tachycardia, leucopenia (immunosuppressive effect due to leukocyte reduction), or teratogenic effects (associated with congenital diseases) (Sudakin, 2003; Pietsch *et al*., 2014; Yang *et al*., 2014).

On the other hand, it is important to highlight that trichothecenes such as DON are translocated in the plant tissues before any symptoms of the disease and signs of the fungus occur. Metabolites act by decreasing the plant protein synthesis and are able to suppress or delay the defense response. It has been found that isolates that do not produce trichothecenes are less pathogenic and affect the productive development of the plant to a lesser extent (Desjardins and Hohn, 1997; Desjardins, 2006).

Zearalenone

In isolates 32 and 67 (*F. graminearum*) and 113 (*F. equiseti*) amplified with the *PKS* gene by PCR, there was also the presence of mycotoxin zearalenone through the serological ELISA test (Fig. 3). *F. graminearum* predominates in environments with temperatures of 25°C and relative humidity greater than 88%. In contrast, *F. equiseti* develops in tropical and subtropical areas affecting several crops, appearing mainly in harvest and post-harvest time. Several studies show that zearalenone production in stored food increases with water activity, i.e., the amount of free water that is available for microorganisms' growth. The optimum amount is 0.98 mg kg^{-1} which facilitates toxin biosynthesis (Lacey and Magan, 1991, Velluti *et al*., 2000).

The maximum permissible values of this mycotoxin in human food are 0.1 mg kg^{-1} (Ministry of Health and Social Protection, 2013). However, the values found in the evaluated isolates are above the established ranges for Colombia (Fig. 2).

It is observed that the DON and ZEA mycotoxins appear simultaneously in the species *F. graminearum* and *F. equiseti*, and this condition is further aggravated due to the possible synergistic effects associated with ingestion of food contaminated with various mycotoxins. In Kenya, the simultaneous ocurrence of DON, ZEA, OTA (ochratoxin)

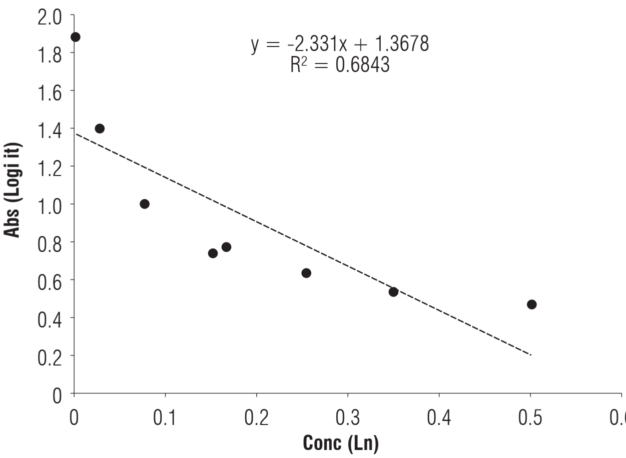

FIGURE 2. Concentration in mg L^{-1} and absorbance values of zearalenone produced by isolates.

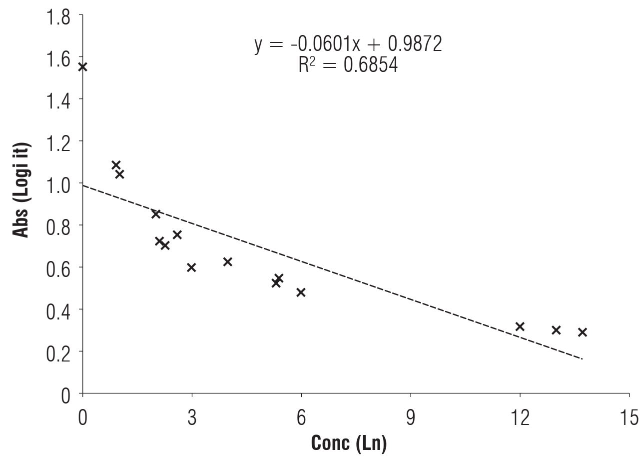

FIGURE 3. Absorbance values and mg L^{-1} concentration of isolates producing fumonisins.

and AFs (aflatoxins) obtained from wheat crops has been reported (Muthomi *et al*., 2008; Li *et al*., 2014).

In studies conducted by Rojas *et al*. (2015) on mycotoxin co-occurrence in food for infants, it was found that ZEA shows high concentrations between 421.34 and 1518.22 µg kg^{-1} (0.421-1.5 mg kg^{-1}), which exceed the legal values established for Colombia and the EU (20 µg kg^{-1}). In a study carried out in 1999 (Díaz, 2005), values ranging from 35 to 134 µg kg^{-1} (0.035-0.134 mg kg^{-1}) were found in corn, which are above the current ranges, as well as those found in this study.

The zearalenone toxin is thermostable which allows it to survive under adverse conditions. Once it enters the body it is rapidly metabolized, producing estrogenic substances such as α-zearalenol and β-zearalenol. These substances have in their structure a lactone that initiates estrogenic activity capable of competing and interacting with estrogen receptors which activate and deactivate metabolic pathways (Li *et al*., 2014).

Information about damages caused by ZEA in humans is poor. However, studies concerning high ZEA concentrations in foods and the emergence of diseases associated with estrogen such as precocious puberty and breast cancer have led to the belief that this toxin triggers a series of events that cause the formation of cancer cells and also affects the immune system (EFSA, 2011).

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) categorize ZEA and DON produced by *F. graminearum* into group 3, i.e. they are not classified as carcinogenic to humans (IARC, 2016). However, damage caused by these toxins to humans is reflected in estrogenic damage (Rojas and Wilches, 2011).

Fumonisin

High concentrations of FUM were found in *F. verticillioides* isolates 38, 40, 73 and 75 from corn exceeding the control values of 6 mg kg^{-1} (Fig. 3). In Colombia, the permitted limits for this toxin are 3 mg kg^{-1}; however, for the EU the values range from 0.2 to 4 mg kg^{-1}. For the species *F. proliferatum* values also exceed the permitted ranges. In the species *F. napiforme, F. oxysporum and F. verticillioides* (isolates 80, 83 and 144), the concentration of FUM does not exceed those ranges.

Since corn is a daily food in Colombia, concentrations found in culture medium are high compared to the estimated ranges for direct and processed food. Studies carried out in Mexico, where FUM concentration in corn both under field and controlled conditions were evaluated, a high amount of fumonisins (5 mg kg^{-1}) is found above the established standards, becoming a potential risk for human and animal health (Gallardo-Reyes *et al*., 2006). In a study conducted in Brazil by Ono *et al*. (1999) high relative humidity and temperature are key to fungus growth and subsequent fumonisin contamination in the field. The period close to cob maturity or grain filling generates the highest FUM levels (Martínez *et al*., 2010). Dry periods before and during the grain filling also promote the disease severity and increase the fumonisins accumulation (Munkvold, 2003).

Fumonisins are also produced by some species of the *F. fujikuroi* species complex (FFCS) such as *F. proliferatum* and *F. napiforme* and the *F. oxysporum* species complex, with the toxin amount playing an important role in plant pathogenicity (Winter *et al*., 1996; Braun and Wink, 2018). FUM formation is caused by the expression of the *FUM* gene (Waalwijk *et al*., 2004; Stępień et *al*., 2011). The *FUM* gene cluster is highly correlated between *F. oxysporum*, *F. verticillioides* and *F. proliferatum* given by number of genes, orientation and order (Proctor *et al*., 2008). FUM FB1 amount depends on the substrate, the genotype and the environmental conditions; these conditions play an important role in the transcription factors of the *FUM* gene (Jurado *et al*., 2008).

The species *F. verticillioides* is well known worldwide as the largest FUM producer. In addition, toxicity in corn has been documented for more than 100 years (Desjardins and Hohn, 1997). Studies about FUM toxicity were initiated in 1988 when a devastating disease occurred affecting farm animals especially horses, donkeys, mules and rabbits. Research in South Africa determined that *F. verticillioides* was the causal agent of leukoencephalomacia. This research led to conduct relevant studies with this mycotoxin, demonstrating that oral and intravenous doses lead to the disease development in horses and liver cancer in rats (Marassas, 1988).

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) categorize this mycotoxin into group 2B, i.e., possibly carcinogenic. However, it is believed that there is a relationship between the occurrence of *F. verticillioides* and esophageal cancer (IARC, 2016; WHO/FAO, 2019). Epidemiological studies conducted in Italy, Iran, Zimbawue, China, the United States and Brazil relate this type of cancer to high concentrations of FUM B1, B2 produced by *F. verticillioides*. Additionally, the high intake of corn and wheat in diets with low content of minerals such as manganese, molybdenum, selenium, folate and vitamins A, C, E and B12 may also promote disease development (Cao *et al*., 2013). In regions such as China, South Africa, and the Texas-Mexico border, neural tube defects are associated with high corn intake (Mutchinick *et al*., 1999; Guéant-Rodriguez *et al*., 2006).

For Colombia, mycotoxin detection studies are required in food for direct consumption, raw materials and processed products to generate the necessary information to determine the safety of the food consumed.

The ELISA technique used in this study allowed the quantification of mycotoxins DON, ZEA and FUM in the isolates previously detected by PCR.

The high levels of mycotoxins detected and quantified with the extraction method used and the immunoabsorption test allowed the obtention of permissibility levels above those established for Colombia.

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Effect of urea on lead absorption in corn (*Zea mays* L.), spinach (*Spinacia oleracea* L.) and cabbage (*Brassica oleracea* L.)

Efecto de la urea en la absorción de plomo en maíz (*Zea mays* L.), espinaca (*Spinacia oleracea* L.) y col (*Brassica oleracea* L.)

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ABSTRACT

Agricultural intensification has led to an elevated use of pesticides and fertilizers such as urea, without considering the negative effects these products can cause, such as acidification of the soil and the entry of lead (Pb) into the trophic chain. The objective of this research was to determine the effect of urea on the absorption of lead (Pb) in the leaf structure of corn *(Zea mays* L.), spinach (*Spinacia oleracea* L.), and cabbage (*Brassica oleracea* L.). These three plant species were exposed to five different concentrations of urea and a constant concentration of Pb in the form of Pb acetate for 93 days. The effect of urea and Pb was determined through analysis of the leaf structure. The results showed an increase in the dry weight of corn, whereas in spinach (T_4) (1.5 g urea kg^{-1} of soil plus 0.5 g Pb kg^{-1} of soil) dry weight decreased as well as the fresh weight and foliar area. An increase in the chlorophyll index in corn, spinach, and cabbage was observed in the treatments with urea, and there was an influence on soil acidification. We also observed that spinach concentrated more Pb (T_4) in its foliage at higher rates of N application. The idiosyncratic nature of the crop responses to the combined effects of Pb and N highlights the need for more research on this subject.

Key words: acidification of the soil, crops, fertilizers, heavy metals, leaf structure.

RESUMEN

La intensificación agrícola ha generado un alto uso de plaguicidas y fertilizantes como la urea, sin considerar los efectos negativos que estos productos puedan ocasionar, como la acidificación del suelo y el ingreso de plomo a la cadena trófica. El objetivo de la presente investigación fue determinar el efecto de la urea en la absorción de plomo (Pb) en la estructura foliar de maíz (*Zea mays* L.), espinaca (*Spinacia oleracea* L.) y col (*Brassica oleracea* L.). Estas tres especies fueron expuestas a cinco diferentes concentraciones de urea y a una concentración constante de Pb en forma de acetato de Pb durante 93 días. Se determinó el efecto de la urea y el Pb por medio del análisis de la estructura foliar. Los resultados mostraron un aumento en el peso seco del maíz, mientras que en la espinaca (T_4) (1.5 g urea kg^{-1} de suelo más 0.5 g Pb kg^{-1} de suelo), el peso seco disminuyó, así como el peso fresco y el área foliar. Se observó un incremento de los valores de clorofila en el maíz, espinaca y col en los tratamientos con urea, y una influencia en la acidificación del suelo. También se observó que la espinaca concentró mayor Pb foliar (T_4) con tasas de aplicación de N más altas. La naturaleza idiosincrásica de las respuestas de los cultivos a los efectos combinados de Pb y N resalta la necesidad de más investigación sobre este tema.

Palabras clave: acidificación del suelo, cultivos, fertilizantes, metales pesados, estructura de la hoja.

Introduction

The global demand for nitrogen (N) fertilizers is increasing. In the case of urea, consumption was 174.29 million t worldwide in 2016 (IFA, 2017). In Peru, the import volume of urea for agricultural purposes was 398 961 t in 2019 (AGRODATA PERÚ, 2020).

Key factors determine crop productivity and one of them is N fertilizers such as urea (Campillo *et al*., 2007). Intensive use of N fertilizers can cause negative effects such as acidification or nitrification of soils (Tong and Xu, 2012; FAO, 2014).

The existence of heavy metals, such as lead (Pb), in agricultural soils is mainly due to irrigation with contaminated water (Méndez *et al*., 2009). Additionally, the use of pesticides, such as Pb arsenate, in Peruvian agriculture (Iannacone *et al*., 2009) until its ban in 2013 (MINAM, 2013) and the atmospheric deposition of industrial and vehicular

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air particles are some of the causes of the presence of these metals in the soil (Nolasco, 2001; Bellido, 2018).

The bioaccumulation of heavy metals, such as Pb, in crops is influenced by factors such as weather, atmospheric depositions, soils with concentrations of other heavy metals, use of fertilizers, the nature of the soil in which vegetables are grown, and agricultural areas with a long-term history of treated or untreated wastewater for irrigation (Sinha *et al*., 2005).

Anthropogenic factors, such as fertilizers, can contribute to the absorption of heavy metals in crops by modifying the physical-chemical properties of the soil, such as pH, organic matter, and bioavailability of heavy metals in the soil (Sharma *et al*., 2008). According to Yu-kui *et al*. (2009), corn (*Zea mays* L.) absorbs mineral elements and heavy metals due to the application of N fertilizers. In the case of cabbage (*Brassica oleracea* L.), the plant has heavy metal hyperaccumulator characteristics (Yanqun *et al*., 2005). Also, spinach (*Spinacia oleracea* L.) has a high potential to accumulate heavy metals (Kumar *et al*., 2013).

The absorption of heavy metals by the different parts of plants (root, leaves, and fruit) is the first step for their incorporation in the trophic chain (Méndez *et al*., 2009). Therefore, it represents a health hazard due to the negative effects these heavy metals can cause (Salazar *et al*., 2012).

The objective of this research was to determine the effect of urea on the absorption of Pb in corn (*Z. mays*), spinach (*S. oleracea*) and cabbage (*B. oleracea* var. *capitata*), and to determine which of these plant species absorbs more Pb in its foliage and how urea influences soil acidification.

Materials and methods

The study was carried out in the Environmental Engineering greenhouse of the Universidad Cientifica del Sur (UCSUR), Lima, Peru, and in the Laboratory of Ecology and Animal Biodiversity of the Universidad Nacional Federico Villarreal (LEBA), Lima, Peru. A total of 112.5 kg of greenhouse substrate was prepared with the following characteristics: 40% peat moss, 30% farm soil, 10% coarse sand, 10% compost, and 10% horse manure. Two samples were analyzed for the selection of farm soil; one belonged to the district of Huachipa and the other to the district of Lurin, Lima, Peru. The samples were analyzed in the Laboratory of Soil, Plants, Water and Fertilizer Analysis (LASPAF) of the Faculty of Agronomy of the National Agrarian University La Molina (UNALM), Lima, Peru (Tab. 1). The protocol for the determination of the field capacity (FC) followed the procedure of Rai *et al.* (2017).

The soil chosen for the substrate was from the district of Lurin because the concentration of Pb was below the environmental quality standard (EQS) of Peru for agricultural land (0.07 g kg^{-1}). In contrast, the soil from Huachipa had a Pb concentration above the EQS of Peru for agricultural land. The greenhouse substrate was mixed with the respective concentration of Pb (0.5 g Pb kg^{-1} of soil) in a plastic container. The contaminant used was Pb acetate, and the urea doses were added to the substrate according to the treatments listed in Table 2.

The plant materials used for the present experiment were spinach (*S. oleracea*), and cabbage (*B. oleracea* var. *capitata*) seeds acquired at the nursery of the Universidad Nacional Agraria La Molina (UNALM), Lima, Peru. Corn (*Z. mays*) seeds were purchased from the Corn Program of UNALM, Lima, Peru. The corn cultivar was Indurata; for spinach, the cultivar was Viroflay, and for cabbage, the cultivar was Brunswick.

A completely randomized block design was established with five treatments (Tab. 2) with three replicates per crop. The experimental unit was a 15 L plastic container with 2.5 kg of greenhouse substrate. The seeds were sown directly in the substrate except for the cabbage that was transplanted after two weeks of germination. Three seeds were sown in each pot, and after three weeks of germination, only one plant was left per pot. Only a general linear model (GLM) factorial two-way ANOVA was performed to evaluate the effects of urea and Pb application on foliar Pb and N contents in corn, spinach, and cabbage.

TABLE 1. Analysis of the farm soil for the preparation of the greenhouse substrate.

Sample	Parameters									
	pH (1:1)	EC $_{(1:1)}$ dS m^{-1}	$CaCO_3$ %	OM %	P mg L^{-1}	K mg L^{-1}	Al^{+3}+H^+ meq/100 g	Pb g kg^{-1}	FC %	N %
Agricultural land (Lurin)	8.40	2.45	2.30	2.59	146	318	0	0.026	24.8	0.15
Agricultural land (Huachipa)	7.54	1.14	0.50	0.77	29	139	0	0.12	18.1	0.07

EC - electrical conductivity; $CaCO_3$ - calcium carbonate; OM - organic matter; P - Phosphorus; K - Potassium; Al^{+3} + H^+ - exchangeable acidity; Pb - Lead; FC - Field capacity; N - Nitrogen.

Plant growth and development were under greenhouse conditions. Temperature and relative humidity data were collected from the nearest SENAMHI weather Von Humboldt station located in La Molina district, Lima, Peru (12°05 S, 76°57' W, and 243.7 m a.s.l.). The following temperature (16.62°C, range = 12.0-27.9°C) and humidity parameters (82.27%, range = 75.44-84.71%) were recorded.

TABLE 2. Treatments to evaluate the effect of urea on Pb absorption in corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*).

Treatment	Urea application (g of urea kg^{-1} soil)	Pb application (g of Pb kg^{-1} soil)
Control	0 g	0 g (*)
T_1	0 g	0.5 g
T_2	0.5 g	0.5 g
T_3	1.0 g	0.5 g
T_4	1.5 g	0.5 g

(*) This value (0 g) corresponds to the application of Pb acetate on the soil and not to the value of Pb in the composition of the soil of the farm (0.026 g kg^{-1}) which is below the Environmental Quality Standard (EQS) of Peru (0.07 g Pb kg^{-1} soil) for agricultural land.

Plants were irrigated with demineralized water from the time of sowing to one week before harvest. The volume of irrigation water per pot was enough to approximate the humidity of field capacity (Rai *et al*., 2017). The field capacity of the substrate was not modified by the use of peat moss and coarse sand, which prevented leachate of excess water and, therefore, a possible loss of N, Pb, or other nutrients from the substrate. Each pot was irrigated twice a week; the first irrigation had a volume of 1000 ml, and then plants were irrigated with a volume between 200 and 500 ml according to the need of each plant (Ugáz *et al*., 2000). In total, each plant of corn utilized 7600 ml, spinach 6800 ml, and cabbage 5800 ml.

During plant growth and development, the soil pH was evaluated with a potentiometer (AAMS, MHS Iberica, Madrid, Spain) (1:1 soil:water ratio) every 7 d to determine its behavior (Collins *et al*., 1970). Biometric measurements were performed at the end of the trial, at the time of harvest at 93 d for corn, spinach, and cabbage, without considering their different periods of growth of each crop but implementing the same period of exposure to urea and Pb. A portable electronic balance (0.1 g readability, SPJ6001, Ohaus, Melrose, USA) was used to determine the fresh weight and dry weight of plants. For measuring the height of the plant, a measuring tape (±1 mm) was used. For corn, plants were measured from the ground level to the flag leaf (the last leaf that emerges from the stem), and the spinach and cabbage plants were measured from the ground level to the highest leaf.

A portable chlorophyll meter (SPAD-502, Konica Minolta, Tokyo, Japan) was used to determine the chlorophyll index. Three measurements were taken between the main nerve and the edge of the central leaflet of each plant to obtain the average chlorophyll index in SPAD units (Yllanes *et al*., 2014; Hurtado *et al*., 2017). The ImageJ program version 1.50i was used to determine the leaf area. Photographs of all the leaves of each plant were taken before sending them to the laboratory.

The plant shoot of each crop was placed in paper bags and then taken to the LASPAF of UNALM, Lima, Peru, where the concentration of Pb and N in leaves was determined. The reading or measurement of the concentration of Pb was performed on an atomic absorption spectrophotometer (AAnalyst 200, Perkin Elmer, Waltham, USA) using a wavelength of 283.3 nm. The analysis of plant tissue for the determination of total N was carried out using the method described by Kjeldahl, as described in Sáez-Plaza *et al*. (2013), which is based on transforming the entire organic fraction of N (proteins, amino acids) into the ammonium sulfate, and which can be distilled to obtain the N contained in the leaf sample.

Data were analyzed using the statistical program SPSS, version 24.00. An analysis of variance (ANOVA) with a confidence of 95% was performed to analyze the differences between the control and the treatments as well as the relationship between the parameters of the plants. Additionally, the non-parametric Kruskal-Wallis test was used for those variables that did not meet all the requirements (normality and homogeneity of variance). Posteriorly, the Tukey test was performed to determine the significant differences for each of the parameters. The observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were also determined. The negative effects of LOEC and NOEC refer to any increase or reduction for each of the evaluated variables. In addition, for NOEC and LOEC, the highest statistically divergent values were used compared to the control in the case of observing a non-linear relationship. A *general linear model* (GLM*)* factorial two-way ANOVA was performed to evaluate the effects of urea and Pb application on crops of corn, spinach, and cabbage.

The relationship between the urea dose, acidity, and soil pH were also analyzed as well as the relationship between the soil pH, and the concentration of Pb were analyzed by Pearson's correlation, with 95% confidence.

The data obtained from the LASPAF of UNALM, Lima, Peru regarding the concentration of Pb in the leaves of corn, spinach, and cabbage were compared with the maximum permissible levels (MPLs) of Codex Alimentarius and of other countries (MINCOTUR, 2017). Codex Alimentarius is the regulation that governs Peruvian legislation because there was no MPL for food until now.

The Bioconcentration factor (BCF) is the ratio of the elements contained in the foliar part of the plant concerning the soil (Vysloužilová *et al.*, 2003). If the quotient is greater than or equal to one, the plant is considered a Pb accumulator (Santoyo-Martínez *et al.*, 2020). Pb concentration in the leaf structure was estimated for the foliar parts. In the case of the control treatment, the data from the soil analysis of the used farmland (22.32 mg kg^{-1}) was considered for determining Pb concentration in the soil, and for the other treatments, the Pb acetate concentration (500 mg kg^{-1}) that was applied.

Results

Fifteen experimental units were evaluated for corn, 15 experimental units for spinach, and only nine experimental units for cabbage, because in the third week the plants of T_3 and T_4 died, possibly due to the application of dose levels of urea and Pb totaling 39 experimental units that grew and developed for 93 d.

Pb and foliar nitrogen

There were significant differences between treatments for foliar Pb in spinach, with T_4 showing the highest concentration of Pb compared to corn and cabbage. Significant differences were evident between treatments for foliar N in cabbage, with T_2 showing the highest concentration compared to the control and T_1. NOEC and LOEC had the lowest statistical values for foliar Pb and N in cabbage (Tab. 3). The GLM factorial two-way ANOVA indicated significant differences in the treatments with urea and Pb application on foliar Pb (F=4.09, P<0.05), but no differences were observed on the foliar N (F=1.70, P=0.18) of corn, spinach, and cabbage. Also, the factorial two-way ANOVA indicated significant differences between crops of foliar Pb (F=10.51, P<0.05) and foliar N (F=16.82, P<0.05). Finally, the factorial two-way ANOVA indicated significant differences in the interaction between treatments of urea and Pb application and the crops on foliar Pb (F=2.78, P<0.05) but no differences were observed on foliar N (F=0.88, P=0.52) of corn, spinach, and cabbage.

Corn

For the chlorophyll index, the treatments in which urea was applied (T_2, T_3, and T_4) had the highest SPAD values compared to the treatments without urea application (control and T_1). T_2 and T_3 showed greater fresh weight than the control, and T_1, T_2, T_3, and T_4 showed greater dry weight than the control. T_2 plants showed greater height compared to the control, and the leaf area for T_1, T_2, and T_3 was greater than in the control. NOEC and LOEC had the statistically lowest values for chlorophyll index, fresh weight, and height in corn (Tab. 4).

Spinach

There were no significant differences between treatments for dry weight, height, and leaf area. Regarding the chlorophyll index and fresh weight, T_2 was higher compared to the control. NOEC and LOEC had the statistically lowest values for fresh weight in spinach (Tab. 5).

TABLE 3. Comparison of the effects of urea and Pb application on foliar Pb and N in corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*).

Concentrations (g kg^{-1} soil)		Treatment	Corn		Spinach		Cabbage	
Pb	Urea		Pb (mg kg^{-1})	N (%)	Pb (mg kg^{-1})	N (%)	Pb (mg kg^{-1})	n (%)
0	0	Control	2.90±0.53 a	1.23±0.19 a	5.01±1.17 b	3.31±0.43 a	5.25±1.37 a	2.77±0.19 a
0.5	0	T_1	7.34±1.68 a	1.75±0.24 a	7.68±2.85 b	3.84±0.69 a	6.54±0.69 a	2.93±0.31 a
0.5	0.5	T_2	5.73±0.98 a	2.39±0.21 a	10.31±0.88 ab	4.78±0.15 a	5.13±0.57 a	2.39±0.21 b
0.5	1	T_3	6.43±1.85 a	2.97±0.12 a	11.78±0.82 ab	4.76±0.43 a		
0.5	1.5	T_4	5.17±0.78 a	2.87±0.90 a	18.07±6.66 a	4.81±0.03 a		
		F	1.72	2.87	0.1*	0.27*	0.68	11.76
		P	0.22	0.08	<0.05	>0.05	0.54	0.01
		NOEC	T_4**	T_4	T_3	T_4	T_2	T_1
		LOEC	ND	ND	T_4	ND	ND	T_2

F - Fisher statistic; *the Kruskal Wallis test was used because the data did not meet the normality test. *P*- Level of significance; NOEC - maximum concentration in which no negative effects are observed compared to the control; LOEC - minimum concentration in which negative effects are observed compared to the control; (±) Standard error; ND - not determined; **for NOEC and LOEC, the highest statistically different values were used in relation to the control in the case of a non-linear relationship; equal lowercase letters in the same column indicate that the means are statistically equal according to the Tukey test (*P*<0.05).

Cabbage

In cabbage, there were no significant changes between treatments for fresh weight, dry weight, height, and leaf area. For the chlorophyll index, T_2 was higher compared to the control. NOEC and LOEC had the statistically lowest values for the chlorophyll index in cabbage (Tab. 6).

TABLE 4. Progressive effect of urea and Pb application on the five physiological variables evaluated in leaves of corn (*Zea mays*).

Concentrations (g kg^{-1} soil)		Treatment	Chlorophyll index (SPAD)	Fresh weight (g)	Dry weight (g)	Height (cm)	Leaf area (cm^2)
Pb	Urea						
0	0	Control	23.62±1.17 b	78.55±4.48 c	13.66±0.19 b	131.00±7.33 b	4856.23±650 b
0.5	0	T_1	28.61±1.65 b	92.30±4.11 bc	23.14±3.31 a	117.33±8.08 c	7263.08±668 a
0.5	0.5	T_2	47.67±2.37 a	123.55±3.39 a	23.50±1.34 a	158.33±14.19 a	7195.30±394 a
0.5	1	T_3	43.69±0.52 a	118.78±3.25 a	22.34±1.18 a	114.33±6.96 c	7780.03±862 a
0.5	1.5	T_4	42.48± 1.97 a	102.24±4.39 b	19.24±0.81 ab	99.33± 4.10 d	4732.65± 638 b
		F	39.56	22.13	5.71	120.7	4.85
		P	0.0	0.0	0.01	0.0	0.02
		NOEC	T_1	T_1	T_4**	ND	T_4**
		LOEC	T_2	T_2	ND	T_1	ND

F - Fisher statistic; *the Kruskal Wallis test was used because the data did not meet the normality test; SPAD- chlorophyll index value; *P* - the level of significance; NOEC - maximum concentration to which no negative effects are observed concerning the control; LOEC - minimum concentration where negative effects are observed concerning the control; ± standard error; ND - not determined; ** for NOEC and LOEC, the highest statistically different values were used in relation to the control in the case of a non-linear relationship; equal lowercase letters in the same column indicate that the means are statistically equal according to the Tukey test ($P<0.05$).

TABLE 5. Progressive effect of urea and Pb application on the five physiological variables evaluated in leaves of spinach (*Spinacia oleracea*).

Concentrations (g kg^{-1} soil)		Treatment	Chlorophyll index (SPAD)	Fresh weight (g)	Dry weight (g)	Height (cm)	Leaf area (cm^2)
Pb	Urea						
0	0	Control	46.49±3.12 bc	118.77±13.66 bc	12.44±3.65 ab	42.00±4,93 a	2910.13±420 ab
0.5	0	T_1	36.45±2.40 c	128.66±14.67 ab	10.44±1.72 ab	37.67±1,86 a	4077.55±599 a
0.5	0.5	T_2	63.37±3.32 a	181.92±13.30 a	17.22±1.78 a	43.67±2,91 a	3764.13±120 a
0.5	1	T_3	50.71±3.44 b	63.85±1.45 cd	5.38±0.52 b	32.33±0,88 a	2340.59±302 ab
0.5	1.5	T_4	42.17±2.00 bc	45.57±21.87 d	4.91±2.00 b	31.00±3.51 a	1228.18±535 b
		F	12.18	13.99	5.13	3.22	7.01
		P	0.0	0.0	0.02	0.06	0.0
		NOEC	T_4	T_3	T_4	T_4	T_4
		LOEC	ND	T_4	ND	ND	ND

F - Fisher statistic; *the Kruskal Wallis test was used because the data did not meet the normality test; SPAD - chlorophyll index value; *P* Level of significance; NOEC - maximum concentration to which no negative effects are observed concerning the control; LOEC - minimum concentration where negative effects are observed concerning the control; ± standard error; ND - Not determined; **for NOEC and LOEC, the highest statistically different values were used in relation to the control in the case of a non-linear relationship; equal lowercase letters in the same column indicate that the means are statistically equal according to the Tukey test ($P<0.05$).

TABLE 6. Progressive effects of urea and Pb application on the five physiological variables evaluated in leaves of cabbage (*Brassica oleracea* var. *capitata*).

Concentrations (g kg^{-1} soil)		Treatment	Chlorophyll index (SPAD)	Fresh weight (g)	Dry weight (g)	Height (cm)	Leaf area (cm^2)
Pb	Urea						
0	0	Control	48.99±0.93 b	138.99±7.03 a	15.59±1.40 a	36.67±0,88 a	4045.16±137 a
0.5	0	T_1	49.42±0.78 b	150.53±28.28 a	16.34±1.74 a	33.00±1.16 a	4296.64±566 a
0.5	0.5	T_2	60.64±1.55 a	202.20±6.43 a	19.84±0.82 a	38.33±2.33 a	5283.28±186 a
		F	0.07*	3.81	5.13	2.96	3.43
		P	<0.05	0.09	0.02	0.13	0.10
		NOEC	T_1	T_2	T_2	T_2	T_2
		LOEC	T_2	ND	ND	ND	ND

F - Fisher statistic; *the Kruskal Wallis test was used because the data did not meet the normality test; SPAD - chlorophyll index value; *P*- level of significance; NOEC - maximum concentration to which no negative effects are observed concerning the control; (LOEC) minimum concentration where negative effects are observed for the control; ± standard error; ND - not determined; **for NOEC and LOEC, the highest statistically different values were used in relation to the control in the case of a non-linear relationship; equal lowercase letters in the same column test indicate that the means are statistically equal according to the Tukey ($P<0.05$).

Soil pH

Soil pH in the control and T_1 for corn was slightly alkaline during the time of the experiment. T_2 remained slightly acidic until day 36, and then acidity increased. Treatments T_3 and T_4 were slightly acid pH until day 50 (Fig. 1).

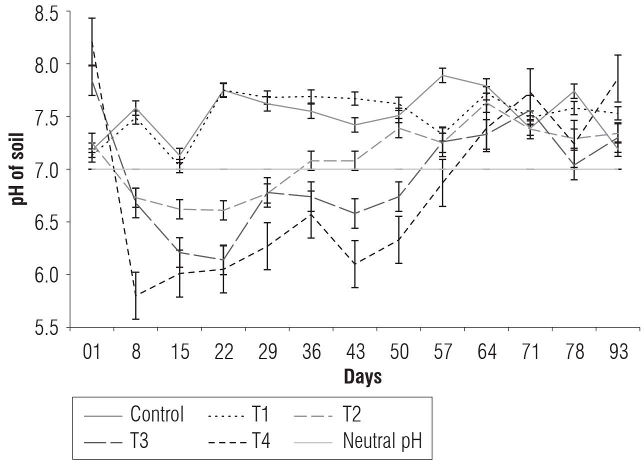

FIGURE 1. Comparison of the five treatments in corn *(Zea mays)* for soil pH behavior. Treatment 1 (T_1): urea application level (0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 2 (T_2): (0.5 urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 3 (T_3): (1.0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 4 (T_4) (1.5 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil). Bars represent the standard error.

The soil pH in spinach in control and T_1 was slightly alkaline during the 93 d. However, T_2, $T_{3,}$ and T_4 remained slightly acidic until days 29, 50, and 64, respectively (Fig. 2).

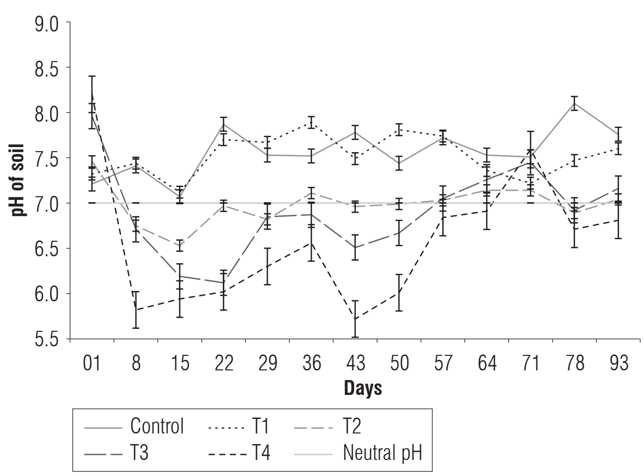

FIGURE 2. Comparison of the five treatments on spinach (*Spinacia oleracea*) for soil pH behavior. Treatment 1 (T_1): urea application level (0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 2 (T_2): (0.5 urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 3 (T_3): (1.0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 4 (T_4) (1.5 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil). Bars represent the standard error.

Regarding the soil pH in cabbage during all the assays, the control and T_1 were slightly alkalized. T_2 remained slightly acidic until day 22, and from day 29, it was somewhat alkalized; T_3 and T_4 were slightly acidified until day 15 (Fig. 3).

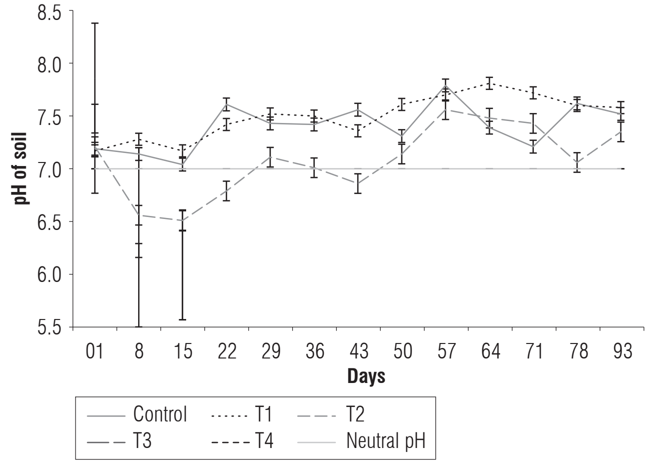

FIGURE 3. Comparison of the five treatments in cabbage (*Brassica oleracea* var. *capitata*) for soil pH behavior. Treatment 1 (T_1): urea application level (0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 2 (T_2): (0.5 urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 3 (T_3): (1.0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 4 (T_4) (1.5 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil). Bars represent the standard error.

The pH behavior among the treatments for the three plants together was similar; the control and T_1 were slightly alkalized, and T_2, $T_{3,}$ and T_4 were acidified temporarily to approach neutrality or become somewhat alkalized (Fig. 4).

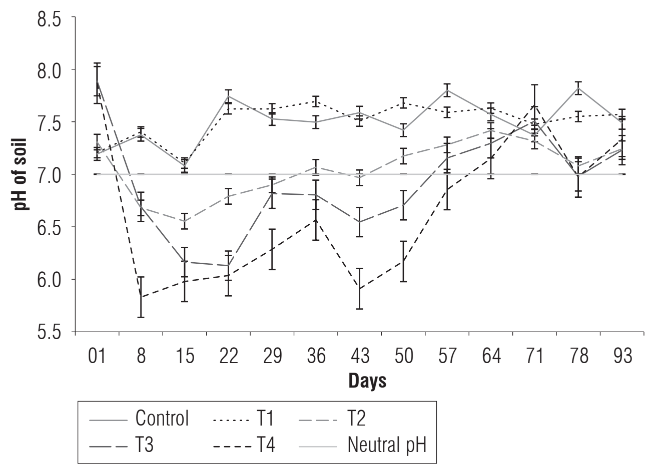

FIGURE 4. Comparison of the five treatments combined in corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*) for soil pH behavior. Treatment 1 (T_1): urea application level (0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); treatment 2 (T_2): (0.5 urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 3 (T_3): (1.0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); treatment 4 (T_4) (1.5 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil). Bars represent the standard error.

We observed that the urea dose had a direct relationship with the acidity time of the soil for corn, spinach, and cabbage, and the urea dose had a negative relationship with the soil pH for corn and spinach (Tabs. 7 and 8). We also observed that the soil pH had a negative correlation with foliar Pb contents only for spinach (Tab. 9).

TABLE 7. Pearson correlation coefficient values among the doses of urea and the acidity time in corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*).

Dose of urea (g urea kg^{-1} soil)	Soil acidity time (d)	*P*
Corn	r = 0.98	0.00
Spinach	r = 0.98	0.00
Cabbage	r = 1.00	0.00

$P<0.05$ is statistically significant, indicating a relationship between the dose of urea and the acidity time of the crops.

TABLE 8. Pearson correlation coefficient values illustrating the dose of urea and its effect on the soil pH in corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*).

Dose of urea (g urea kg^{-1} soil)	pH of the soil	*P*
Corn	r = - 0.97	0.01
Spinach	r = - 0.97	0.01
Cabbage	r = -0.98	0.14

$P<0.05$ is statistically significant, indicating a relationship between the dose of urea and the soil pH.

TABLE 9. Pearson correlation coefficient values showing the relationship between the soil pH and the foliar Pb contents in corn (*Zea mays*), spinach (*Spinacia oleracea*) and cabbage (*Brassica oleracea* var. *capitata*).

pH of the soil	Foliar Pb contents (mg kg^{-1})	*P*
Corn	r = -0.17	0.79
Spinach	r = -0.95	0.01
Cabbage	r = 0.73	0.48

$P<0.05$ is statistically significant, indicating a relationship between soil pH and foliar Pb contents of the crop.

Maximum permissible limit (MPL) of Pb in food

We observed that the concentration of Pb in the leaf structure of spinach, corn, and cabbage in all treatments and control exceeded the Pb MPL of both the Codex Alimentarius (Peruvian legislation) and the limits of other countries (Tab. 10).

The BCF values were less than 1 in all the treatments for spinach, corn, and cabbage that indicate that these plants are not considered efficient accumulators of Pb (Tab. 11).

Discussion

Effect of treatments on chlorophyll contents, fresh weight, dry weight, and leaf area

Chlorophyll contents of corn, spinach (T_2), and cabbage were higher in the treatments where urea was applied and

TABLE 10. Comparison of Pb contents in leaves of corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*) with the maximum permissible limits of the Codex Alimentarius (Peruvian legislation) and legislation of other countries.

Plant species	Treatment	Foliar content (Pb mg kg^{-1})	Codex Alimentarius (Pb mg kg^{-1})	European Union (Pb mg kg^{-1})	Australia and New Zealand (Pb mg kg^{-1})	Brazil (Pb mg kg^{-1})	Japan (Pb mg kg^{-1})
	Control	2.90	0.05	0.05	0.20	0.50	
	T_1	7.34	0.05	0.05	0.20	0.50	
Corn	T_2	5.73	0.05	0.05	0.20	0.50	
	T_3	6.43	0.05	0.05	0.20	0.50	
	T_4	5.17	0.05	0.05	0.20	0.50	
	Control	5.01		0.10	0.10	0.50	5.00
	T_1	7.68		0.10	0.10	0.50	5.00
Spinach	T_2	10.31		0.10	0.10	0.50	5.00
	T_3	11.78		0.10	0.10	0.50	5.00
	T_4	18.07		0,10	0.10	0.50	5.00
	Control	5.25	0.30	0.30	0.30	0.50	
Cabbage	T_1	6.54	0.30	0.30	0.30	0.50	
	T_2	5.13	0.30	0.30	0.30	0.50	

Treatment 1 (T_1): urea application level (0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 2 (T_2): (0.5 urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 3 (T_3): (1.0 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil); Treatment 4 (T_4) (1.5 g urea kg^{-1} soil) and Pb application level (0.5 g Pb kg^{-1} soil).

TABLE 11. Bioconcentration factor (BCF) of corn (*Zea mays*), spinach (*Spinacia oleracea*), and cabbage (*Brassica oleracea* var. *capitata*).

Treatment	Spinach			Corn			Cabbage		
	Soil Pb (mg kg^{-1})	Foliar Pb (mg kg^{-1})	BCF	Soil Pb (mg kg^{-1})	Foliar Pb (mg kg^{-1})	BCF	Soil Pb (mg kg^{-1})	Foliar Pb (mg kg^{-1})	BCF
Control	22.32	5.01	0.23	22.32	2.90	0.13	22.32	5.25	0.24
T_1	500	7.68	0.02	500	7.34	0.02	500	6.54	0.01
T_2	500	10.31	0.02	500	5.73	0.01	500	5.13	0.01
T_3	500	11.78	0.02	500	6.43	0.01			
T_4	500	18.07	0.04	500	5.17	0.01			

BCF - Bioconcentration factor = Pb concentration in foliar tissues/Pb concentration in soil.

when Pb concentration increased in the soil. T_3 and T_4 of spinach showed a decrease in chlorophyll contents, both fresh or dry weight, and leaf area, possibly due to the presence of high doses of urea and the presence of Pb taken from the soil (Tabs. 4-6). According to Sonbai *et al.* (2013), the increase in chlorophyll levels is because N from urea can be absorbed through the roots and used to form more chlorophyll (Ai and Banyo, 2011; Surya, 2013). In *Cannabis sativa,* an increase in leaf number, plant height, and root length is observed at high doses of urea with contaminated soil with Pb, when compared to a control (Hadi *et al*., 2014). Khan *et al.* (2016) noted that Pb could alter chlorophyll biosynthesis and N metabolism.

The fresh weight in corn (T_2 and T_3) was higher in the treatments where urea was applied, and in spinach the greater fresh weight was observed in T_2 (Tabs. 4-6). Nitrogen is an essential element since it is a component of proteins and chlorophyll. Therefore, it is essential for photosynthesis, vegetative (fresh weight), and reproductive growth, and often determines the yield of corn (Iqbal *et al*., 2006). According to Pangaribuan *et al.* (2018), increasing N levels can also increase the fresh weight per plant, which can be attributed to the greater availability of N with the higher rate of N fertilizer.

In spinach, T_4 had the highest concentration of foliar Pb (Tab. 3) and, at the same time, the lowest fresh weight (Tab. 5). Similar effects are observed in studies with *Plantago major* L. and spinach, where reduction in fresh and dry weight is observed in the different parts of the plant when exposed to Pb (Kosobrukhov *et al*., 2004; Lamhamdi *et al*., 2013). The specific symptoms of exposure to Pb in plants are characterized by the reduction of dry weight in the shoot since Pb causes a decrease in the protein content in tissues and an alteration in the lipid composition (Cao *et al.,* 2015). Alia *et al.* (2015) note that, in *S. oleracea,* an increase in the doses of Pb in the soil with different doses (mg kg^{-1}) reduces the growth parameters (shoot and root lengths, biomass and number of leaves). Hart *et al.* (2005) and Zhou *et al.* (2018) studied the effects of Pb doses in the soil on plant shoot and root growths and photosynthesis in various types of plants. Under Pb stress, the chloroplasts are deformed, and the thylakoid lamellae are gradually expanded, resulting in the separation from the cell wall and the eventual shrinkage of the nucleus (Zhou *et al*., 2018).

In corn, the treatments where Pb was applied (T_1, T_2, and T_3) obtained a greater dry weight compared to the control (Tab. 4). According to Hu *et al.* (2015), the reduction in dry weight in plants that are exposed to Pb shows variability depending on the species, as shown in this research for corn, spinach, and cabbage that had different Pb concentration in the leaves. This was also observed for *P. major* plants, where a decrease in dry weight was observed. This effect was also noted in *Z. mays,* where there was an apparent increase in dry weight with the increase in the synthesis of polysaccharides in the cell wall.

In cabbage, there were no significant changes between treatments for fresh weight, dry weight, height, or leaf area. Paschalidis *et al.* (2013) observed that cabbage plants do not show growth disturbance, and the plants show no symptoms of phytotoxicity as a result of Pb application. These studies note the tolerance of cabbage plants to high concentrations of Pb in the soil. These authors found that fresh weights of leaves of cabbage significantly increase in the lowest applications (10 mg Pb kg^{-1}) compared to the control treatment (Paschalidis *et al*., 2013). The stimulatory effect of Pb on chlorophyll contents in cabbage in T_2 could be attributed to the fact that metal ions can serve as activators of enzymes in cytokinin metabolism that accelerates the growth of plants. Cytokinins can delay senescence while maintaining the chlorophyll and photosynthetic activity of leaf tissues of cabbage (Paschalidis *et al.,* 2013).

Nas and Ali (2018) explain that Pb accumulation in the soil inhibits germination of seeds and retards growth of seedlings, decreases the germination percent, germination index, root/shoot length, number of leaves, tolerance index and dry mass of roots and shoots. Lead inhibits photosynthesis, decreases mineral nutrition, water balance, and enzyme activities. Lead inhibits chlorophyll synthesis by causing reduced uptake by plants of essential elements such as magnesium and iron. However, the presence of urea in this study caused a different behavior with an increase in dry and wet weight, and a decrease in leaf area with higher urea concentrations and constant Pb values according to the treatments in corn. In spinach, the highest values in all variables analyzed were seen in T_2. In cabbage, most of the variables showed no changes compared to the control, and only the chlorophyll index showed an increase in T_1 and T_2 compared to the control.

Crop response to treatments

A decrease in height was observed in corn in T_3 and T_4 that were the treatments with the highest rates of urea application (Tab. 4). On the other hand, different studies show that increasing N doses has positive increased plant height in corn (Kaplan *et al*., 2016; Chauhdary *et al*., 2017).

In cabbage, T_3 and T_4 were not successful because the plants died at 21 d after sowing. The Pb uptake may have influenced this effect under greater urea concentrations than by the Pb concentration in the soil itself when it existed at concentrations that do not exceed 1 g of Pb kg^{-1} soil. Pb accumulates in higher amounts in the different organs of the plant, and non-tolerant plants may die (Rodríguez *et al*., 2006). Likewise, the acidity of the soil may also have influenced because both treatments had the lowest pH value (5.87) during the first weeks. Cabbage has an optimum pH range between 6.2-6.5 (FAXSA, 2002) and is intolerant of acidity (Ugáz *et al*., 2000). Bioavailability of Pb in plants depends on the acidity of the soil and pH values (López *et al*., 2005). Paschalidis *et al.* (2013) observed that 500 mg Pb kg^{-1} significantly increases Pb accumulation in roots of cabbage compared to the control treatment. Pb accumulation in Pb-treated plants was not dose-related, but it significantly increased only with the greatest dose of Pb, compared to the control treatment. The lower Pb accumulation was probably due to the relatively low mobility of this metal caused by the binding of Pb to clay minerals. Pb is taken up by plants only in very low amounts, with its largest proportion being accumulated in roots, and only a small portion is transported to the shoots.

Spinach obtained higher concentrations of foliar Pb compared to corn and cabbage (Tab. 3). Spinach was the only plant species that increased Pb levels in the leaves by increasing urea concentrations and the presence of Pb in the soil. This could be explained by the fact that spinach differs from corn and cabbage in its uptake of heavy metals and its sensitivity to Pb, which is a factor that depends on certain soil conditions and root morphology (Hart *et al*., 2005). There are plant species with the capacity to store higher concentrations of specific metals in the shoots (Pilon-Smits, 2005; Callahan *et al*., 2006). In spinach, this could be related to a greater capacity to resist the oxidative stress generated by Pb (Lamhamdi *et al*., 2010). Spinach also can accumulate heavy metals, such as Pb, due to a greater translocation capacity for Pb compared to other plant species (Kumar *et al*., 2013). Thanh *et al*. (2013) and Khan *et al.* (2016) point out that the capacity of plants to absorb Pb depends on each plant species, so this was observed in the present research with spinach showing higher levels of foliar Pb than in the other two plant species.

A higher concentration of foliar Pb was observed in spinach in T_4 (Tab. 3) and, at the same time, a greater reduction in the leaf area compared to T_1 (Tab. 5) where the concentration of foliar Pb was lower (Tab. 3). This symptom may be related to the inhibitory effect of Pb on the growth of the different plant shoots (Kopittke *et al*., 2007), where a lower number of leaves, a smaller size, and a greater fragility are observed (Gupta *et al*., 2009). This can be attributed to metabolic disorders of nutrients (Kopittke *et al*., 2007; Gopal and Rizvi, 2008) and a disturbance of photosynthesis (Islam *et al*., 2008). All correlation relationships for Pb concentrations within shoot and leaves from a variety of plants were positive. The increasing concentrations of Pb within roots also correspond to increasing concentrations within the shoots (Alia *et al*., 2015). Most of the Pb ions absorbed by plants from the soil remain concentrated in the roots, and a small portion is transferred to the stems and leaves (Zhou *et al*., 2018).

Soil acidification

In corn, spinach, and cabbage, in the treatments where urea (T_2, T_3, and T_4) was applied, the soil was slightly alkalized during the first 7 d and then temporarily acidified (Figs. 1-5). This is because when urea is applied to the soil, it has two behaviors, a basic behavior during the first days when it goes from amide to ammonium carbonate, and an acidic behavior when it passes from the ammoniacal to the nitric form (releasing H^+ to the medium); therefore, urea behavior is acidic at the end (FERTIBERIA, 2002). However, the acidification of the soil can be long-lasting or temporary, and the degree or intensity with which it varies is due to the continuous applications of N fertilizers such as urea (Baldoncini, 2015). In our research, acidification was temporary because the dose of urea was only applied once. Additionally, this temporality had a direct relationship with the dose of urea, and the greater the dose of urea, the longer the soil remains acidic (Boccolini, 2016). Paschalidis *et al.* (2013) observed that the application of Pb significantly increases the total N content in the soil compared to the control treatment, perhaps because of the application of the metal in the nitrate form. In our research, the contaminant used was Pb acetate. The availability of Pb for uptake by plants is generally lower at high pH than at low pH (Paschalidis *et al.*, 2013).

Maximum permissible limit (MPL) and bioconcentration factor (BCF) of Pb

In corn, spinach and cabbage, all treatments including the control exceeded the MPL for Pb (Tab. 10) of the Codex Alimentarius (0.05 mg kg^{-1} for corn and 0.3 mg kg^{-1} for cabbage) and other countries (0.1 mg kg^{-1} for spinach for the European Union, Australia and New Zealand, 0.5 mg kg^{-1} for Brazil, and 5 mg kg^{-1} for Japan). It should be noted that, in the control, Pb was not applied in the form of Pb acetate. Still, the soil already contained 0.026 mg kg^{-1} (a concentration lower than the MPL for Pb in agricultural land for Peruvian legislation) in the soil of the farm that was used to make the greenhouse substrate.

The possible source for the high levels of Pb, even in the controls, could be related to some possible variables not evaluated in this research that could be irrigation water (although it was demineralized water), the concentration of Pb in the soil at 93 d of exposure, or even atmospheric contamination and foliar deposition of Pb; however, it is not certain which of the factors listed was the main source of Pb in the leaves of the crops (Natasha *et al.*, 2020). Some researchers point out that Pb is present in plants under natural conditions (Tóth *et al.*, 2014). These results can be observed in the crops where agrochemicals, organic fertilizers, and pesticides are frequently used (Pesca, 2015). This overdose in both the amount and frequency of application (Torres and Ramírez, 2014) increases the probability of transfer or absorption of heavy metals in food, soil, and water (CONPES, 2014). In Peru, research has been carried out where it is evident that foodstuffs such as potatoes (Luna and Rodríguez, 2016) and eggs (González, 2015) exceed the MPL for Pb in food. Therefore, the spinach analyzed in this research is not suitable for consumption and poses a real risk of Pb exposure in the human body.

The BCF of Pb is the best way to know the availability of important metals transferred from soil to grown vegetables (Ugulu *et al*., 2020). The BCF values were less than 1 in all the treatments for the three plant species, which indicated that they were not accumulators of heavy metals in the agroecosystem. Similarly, Pakchoi cabbage *(Brassica campestris* L.) shows BCF values of less than 1 for Pb with the influence of green waste compost (Liu *et al*., 2020).

Conclusions

We observed that Pb increased the dry weight in corn and decreased the fresh and dry weights and leaf area in spinach (T_4). Likewise, urea increased the values of chlorophyll in corn, spinach, and cabbage, indicating that urea influences the absorption of Pb in the leaf structure in the three plant species under study.

We determined that spinach is the only plant species that absorbed by the roots and translocated more Pb to the shoot of the plant by N fertilization (T_4). Spinach showed a greater Pb storage capacity in its leaves (10.57 mg kg^{-1} average in all treatments) compared to corn (5.51 mg kg^{-1} average in all treatments) and cabbage (5.64 mg kg^{-1} in all treatments).

In corn and spinach, we determined that there was a direct relationship between the dose of urea and the acidity time of the soil, and an indirect relationship between the dose of urea and the pH value, which means that urea influenced soil acidification.

The idiosyncratic nature of the crop responses to the combined effects of Pb and N highlights the need for more research on the subject.

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Detection and molecular characterization of the cucumber mosaic virus in chili pepper (*Capsicum* spp. L.) crops

Detección y caracterización molecular del virus del mosaico del pepino en cultivos de ají (*Capsicum* spp. L.)

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ABSTRACT

The chili pepper (*Capsicum* spp. L.) is a vegetable of economic importance that has been affected worldwide by the cucumber mosaic virus (CMV), a pathogen that causes a devastating disease in this crop. The aim of this research was the detection and characterization of CMV in chili pepper crops in Valle del Cauca, Colombia. Leaves of three chili pepper varieties (tabasco, cayenne and habanero) with viral symptoms were collected in four municipalities of Valle del Cauca. Total RNA was purified and a fragment of capsid protein (CP) from CMV was amplified by RT-PCR. Then, it was sequenced and bioinformatically analyzed, and from these sequences, specific primers were designed. From 71 chili pepper samples collected in Palmira, Yumbo, Vijes and Yotoco, 37 were positive for CMV (52.1%). The CMV chili pepper sequence analysis showed that they had their highest identity (98.5%) with a CMV isolated from bananas in Ecuador. Specific primers designed for CMV chili pepper showed greater sensitivity for detecting this virus (64.7% vs. 52.1%). The CMV chili pepper CP analysis indicated that it could be transmitted by the species *Aphis gossypii*. This study reports, for the first time, the molecular characterization of CMV in three chili pepper varieties.

Key words: *Cucumovirus,* diagnostics, habanero, PCR, Solanaceae, tabasco.

RESUMEN

El ají (*Capsicum* spp. L.) es una hortaliza de importancia económica que se ha visto afectada por el virus del mosaico del pepino (cucumber mosaic virus - CMV), un patógeno que ocasiona una enfermedad devastadora para este cultivo a nivel mundial. El objetivo de la investigación fue la detección y caracterización molecular de CMV en cultivos de ají en el Valle del Cauca, Colombia. Se recolectaron hojas de tres variedades de ají (tabasco, cayena y habanero) con síntomas virales en cuatro municipios del Valle del Cauca. Se purificó su RNA total y se amplificó un fragmento del gen de la proteína de la cápside (CP) de CMV por RT-PCR. Después se secuenció, se analizó bioinformáticamente y a partir de estas secuencias se diseñaron primers específicos. De 71 muestras de ají colectadas en Palmira, Yumbo, Vijes y Yotoco, 37 resultaron positivas para CMV (52,1%). El análisis de las secuencias de CMV ají mostró que presentaban su mayor identidad (98,5%) con un CMV aislado de banano en Ecuador. Los primers específicos diseñados para CMV ají mostraron mayor sensibilidad para detectar este virus (64,7% vs. 52,1%). El análisis de CP de CMV ají indicó que este virus podría ser transmitido por la especie *Aphis gossypii*. Este estudio reporta, por primera vez, la caracterización molecular de CMV en tres variedades de ají.

Palabras clave: *Cucumovirus*, diagnóstico, habanero, PCR, Solanaceae, tabasco.

Introduction

The chili pepper (*Capsicum* spp. L.) is one of the most important vegetables worldwide because of its culinary and medicinal uses (Green and Kim, 1991). Currently, the region of Valle del Cauca is one of the largest producers of chili pepper in Colombia, due to the climatic conditions and nutritional characteristics of the soil. However, the yield has decreased in the past ten years (Ministerio de Agricultura y Desarrollo Rural, 2018) as a result of factors such as temperature and humidity that are constantly changing due to climate change or diseases related to fungal, bacterial and viral pathogens; this last factor has become a major limitation for chili pepper production worldwide (Moury and Verdin, 2012).

In the tropics, viral diseases affect crop production and are common in plants of the Solanaceae family like chili pepper. These viruses belonging mainly to genera *Begomovirus*, *Potyvirus*, *Tospovirus* and *Cucumovirus* and affect chili pepper crops, causing more than 90% losses (Subramanya-Sastry, 2013). Although a great variety of viral genera have been reported as infectious agents, few studies have been carried out in the Valle del Cauca region or in Colombia.

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Only the presence of a potyvirus, the pepper deforming mosaic virus (PepDMV) (Morales *et al*., 2005) and recently, a begomovirus (Vaca-Vaca *et al.,* 2019) have been reported as presently affecting these crops. The cucumber mosaic virus (CMV) has only been detected using serological techniques like ELISA. However, these techniques have been displaced by molecular strategies that are more sensitive (González-Garza, 2017).

In Colombia, since 2005 no studies of RNA viruses in chili pepper have been published, so there is no accurate information on the current viral diseases that affect chili pepper crops in this biogeographic area. Therefore, it is likely that due to current global climate changes, a variation in the dynamics of populations of the viral vectors (insects) has occurred, causing changes in virus populations. So, the scenario of those last studies does not match the current situation of the dynamics of RNA viruses. This is more evident if we consider that these viruses evolve following the "Quasispecies" model (Shukla *et al*., 1994) which, through high mutation rates can generate new variants and in most cases new viral species that could emerge as new and more aggressive viral pathotypes.

CMV has a genome composed of three segments of positive single-stranded RNA (RNA1, RNA2 and RNA3). Each viral RNA fragment is encapsidated in an isometric particle and translated into important proteins responsible for the encapsidation, replication, and viral movement through the plant (International Committee on Taxonomy of Viruses, 2018). This virus is transmitted by aphids in a non-persistent manner (Scott, 2006). CMV isolates have been clustered into two groups, I and II, according to serology, nucleic acid hybridization, and gene sequences (Owen and Palukaitis, 1988; Wahyuni *et al*., 1992; Dubey *et al.,* 2008). Then, Roossinck *et al.* (1999) divided subgroup I into IA and IB based on gene sequences and phylogenetic analysis. CMV is one of the major viral diseases worldwide due to its global distribution as well as its host range covering more than 1000 plant species (International Committee on Taxonomy of Viruses, 2018). In chili pepper crops, CMV produces some symptoms like mosaics, leaf distortion, stunted growth and fruit lesions, which result in a reduction in yield (Rahman *et al*., 2016; Azizan *et al*., 2017).

The aim of this research was the detection and molecular characterization of CMV in three different chili pepper varieties in the Valle del Cauca region of Colombia. Based on this information, the producers may be able to develop strategies to control this virus as well as having the necessary information to implement improvement programs for controlling viral diseases.

Materials and methods

Plant material

Seventy-one samples of three chili pepper varieties, habanero (*Capsicum chinense)*, tabasco (*Capsicum frutescens*) and cayenne (*Capsicum annuum* var. *acuminatum*), at different phenological stages and with viral symptomatology were randomly collected in four municipalities (Palmira, Yumbo, Vijes and Yotoco) of Valle del Cauca in Colombia. Tissues collected from each plant were ground using liquid nitrogen and stored in a refrigerator at -20°C for further analysis.

RNA extraction

Total RNA extraction was performed from 100 mg of frozen or dehydrated plant tissue (with silica gel), using the TRIZOL® reagent (Invitrogen™). The quality and quantity of total RNA was verified in 0.7% agarose gels stained with ethidium bromide. These gels were visualized in a ChemiDoc™ XRS photodocument, using the Quantity One 4.6 software (BioRad™, California, USA).

Virus detection by RT-PCR

For cDNA synthesis, 5 µL of total RNA was used as a template using the RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific™, Massachusetts, USA). The cDNA was synthesized using both primers included in the kit: random hexamer and oligo dT. In order to detect CMV, a 586 bp fragment corresponding to the gene coding for the capsid protein (CP) was amplified by RT-PCR (Herrera-Vásquez *et al*., 2009) using the BIOLASE™ PCR kit DNA Polymerase (Bioline™, London, UK). For this reaction, a primer melting temperature (Tm) of 50°C was used. The amplification of the fragment was carried out in a C100 thermal cycler (BioRad™, California, USA). In order to characterize the CMV detected in chili pepper, the 586 bp fragment was purified using the Wizard® DNA Clean up system (Promega®, Wisconsin, USA) purification kit and was sent to Macrogen® (Seoul, South Korea) for Sanger sequencing.

Bioinformatics analysis

In order to know the identity of the 586 bp fragment, a sequence nucleotide analysis was performed by BlastN algorithm using the default search algorithm parameters: database: refseq genomes, organism: virus, program selection: somewhat similar sequences (blastn). The sequence was edited using the BioEdit program (North Carolina, USA); a multiple alignment was performed in the Clustal W program (Larkin *et al*., 2007). The identity values between the sequences, at nucleotide level, were calculated using the Sequence Demarcation Tool (SDT) 1.2 program (Muhire *et al*., 2014). Phylogenetic analysis was performed using the Mega 7 program (Kumar *et al*., 2016). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987) and evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*., 2004). Also, a protein alignment was carried out to manually identify the key amino acids for vector transmission (Perry *et al*., 1998).

Design of specific primers for CMV chili pepper detection

Based on the CMV sequence obtained, a new set of primers was designed using the CLC main workbench v7.0® program (Qiagen®, Hilden, Germany). These primers amplified a 229 bp fragment of the CP gene. Then, the amplification conditions were standardized by gradient PCR. To evaluate the specificity of the primers, cDNA from different plants, including the three chili pepper varieties infected with this virus, were used as a template.

Mechanical inoculation of CMV chili pepper

Using inoculum from the field (tabasco chili pepper plants infected only with CMV), mechanical inoculation of CMV into 15 healthy and young (one month) plants of tabasco chili pepper (*Capsicum frutescens*) was performed under greenhouse conditions. Carburundum powder (600 mesh) and phosphate buffer pH: 7.4 (Na_2HPO_4 + NaH_2PO_4) were used for this purpose. One month after inoculation, CMV was detected by RT-PCR using the specific primers that we previously designed (CMV chili pepper-F / CMV chili pepper-R).

Results and discussion

Detection by RT-PCR of cucumber mosaic virus (CMV) in chili pepper

Ten samples of habanero pepper (*Capsicum chinense*), 59 samples of tabasco pepper (*Capsicum frutescens*), and two samples of cayenne pepper (*Capsicum annuum* var. *acuminatum*) were collected in 2017. The collected samples showed symptoms related to the viral disease such as leaf deformation and mosaics (Fig. 1).

Detection of CMV using primers described by Herrera-Vásquez *et al.* (2009) was carried out and fragments of the expected size (~586 bp) in 52.1% of analyzed samples (37/71) were obtained: 100% (6/6) of the samples from Palmira,

FIGURE 1. Tabasco pepper with viral symptomatology: mosaics and severe leaf deformation, A) Palmira; B) Vijes: mosaic and mild leaf deformation; C) Yumbo; D) Yotoco.

50% (12/24) of the samples from Yumbo, 56.5% (17/30) of the samples from Vijes and 27.2% (3/11) of the samples from Yotoco (Fig. 2). The highest number of positive samples for CMV was found in Palmira and Vijes, where severe viral symptoms were observed, such as the presence of mosaics and leaf deformation (Fig. 1A-B). The samples collected in Yotoco had a lower viral presence, reflected in the presence of mild symptoms (Fig. 1D).

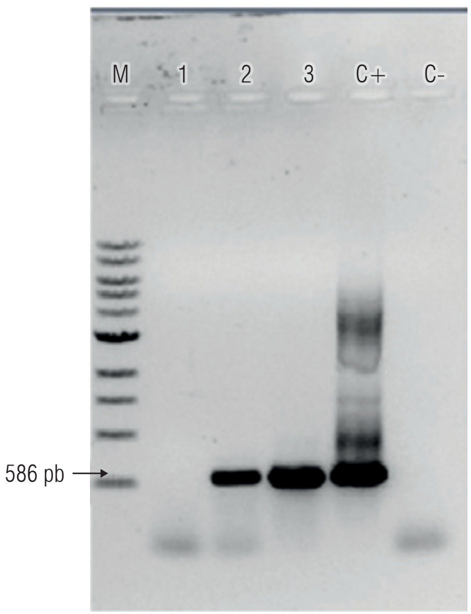

FIGURE 2. Detection of cucumber mosaic virus by RT-PCR in chili pepper. Products were analyzed through electrophoresis on 1% agarose gel. M: 1 kb DNA ladder; lane 1: A209 (Vijes), lane 2: A150 (Palmira), lane 3: A239 (Yotoco), C+: positive control (Plasmid DNA containing a CMV fragment), C-: negative control (water). The arrow indicates the expected fragment size.

Similar results have been found in other chili pepper varieties. Valadez *et al.* (2019) detected CMV by means of RT-PCR in jalapeño pepper in the Colima region of Mexico. Robles-Hernández *et al.* (2010) describe that symptoms, such as mosaics and leaf distortion, necrosis, defoliation, and flower fall, are known in jalapeño pepper crops in Mexico. These symptoms result in the decrease of fruits and can generate almost 90% of pepper crop losses.

Molecular characterization of CMV chili pepper

In order to confirm the identity of the detected virus in chili pepper crops, two CMV fragments from samples collected in each municipality were sequenced for a total of eight CMV fragments. A sequence of 549 bp of the CP gene was obtained for each fragment and an identity analysis was carried out among all the sequences of this study. First of all, both sequences from each municipality were the same. Then, a 98.5% to 100% identity was found among all the sequences: the quasispecies from Palmira and Yumbo are 100% identical, and at the same time, they are 99.45% identical to the quasispecies from Vijes and 98.54% identical to the quasispecies from Yotoco. Finally, the quasispecies of Vijes and Yotoco share 99% identity. This might indicate that the CMV quasispecies found in Palmira, Yumbo, Vijes and Yotoco are the same.

Therefore, a sequence from each municipality was chosen for subsequent analysis. The sequence of these fragments was uploaded to the GenBank database with the following accession numbers: CMV-chili pepper isolate Palmira: MK889209, CMV- chili pepper isolate Yumbo: MK889210, CMV-chili pepper isolate Vijes: MK889211, and CMV-chili pepper isolate Yotoco: MK889212.

In order to know the percentage of identity of our quasispecies with other isolates from the GenBank database, an identity analysis was carried out in SDT (Tab. 1). Our CMV quasispecies isolated from chili pepper are highly related to the CMV isolated from the banana crop in Ecuador (KU127245) with 98.5% identity, followed by the CMV isolated from lulo (*Solanum quitoense*) in Colombia (MG696856) with 98.3% identity (Gallo *et al.*, 2018). However, it is important to highlight that our CMV quasispecies have a lower identity percentage (between 93.5 and 94.3%) with CMV isolated from tomato-JX101617 in Palmira, Colombia (Uribe-Echeverry, 2012), taking into account that this isolate was found in a plant from the same botanical family and that it was located in the same biogeographic region of the samples evaluated in this study.

Considering the classification of the different CMV isolates explained above, CMV sequences of subgroups IA, IB and II were included for our bioinformatics analysis. All reference sequences were downloaded from GenBank. Multiple nucleotide alignment among the chosen sequences and the CMV sequences obtained in this study showed their highest nucleotide identity with a CMV isolated from bananas in Ecuador (KU127245.1) that is placed into subgroup IA. The phylogenetic tree (Fig. 3) confirms that the CMV quasispecies detected in this study belongs to subgroup IA and has a close phylogenetic relationship with the CMV isolate found in banana plants in Ecuador and Colombia. This result has a huge impact because banana was previously grown in some of the areas where our CMV quasispecies was detected. Therefore, it is very possible that the CMV isolate has jumped from banana plants to chili peppers and has successfully adapted to this crop through time and through different selective pressures that operate in chili

TABLE 1. Percentage identities of the 549 bp fragment from CP gene of CMV chili pepper regarding the most related CMV variants found in the GenBank database. The highest identity values are shown in bold.

CMV Isolate	Subgroup	GenBank accession	Host	Country	CMV chili pepper Palmira / Yumbo / Vijes / Yotoco
CMV Banana	IA	7KU127245	*Musa paradisiaca*	Ecuador	98.5% / 98.3% / 98.7% / 98.5%
CMV Lulo	IA	MG696856	*Solanum quitoense*	Colombia	98.3% / 98.3% /98.1% / 98.3%
CMV Tobacco	IA	AB448694	*Nicotiana tabacum*	Syria	98.1% / 98.1% / 97.9% / 97.8%
CMV Banana	IA	U32859	*Musa paradisiaca*	Ecuador	97.9% / 97.8% /98.1% / 97.9%
CMV Plantain	IA	U32858	*Musa balbisiana*	Colombia	97.6% / 97.6% / 97.8% / 97.9%
CMV Melon	IA	KP165582	*Cucumis melo*	Germany	97.6% / 97.4% / 97.4% / 97.2%
CMV Passionfruit	IA	KU976477	*Passiflora edulis*	Nigeria	97.2% / 97% / 97% / 97.2%
CMV Tomato	IB	JX101617	*Solanum lycopersicum*	Colombia	94.3% / 94.1% / 94.1% / 93.9%
CMV Pepper	IB	KU976471	*Capsicum* sp.	Nigeria	93.9% / 93.8% / 93.8% / 93.6%
CMV Pepper	IB	KU976466	*Capsicum* sp.	Iran	92.7% / 92.5% / 92.5% / 92.3%
CMV Pepper	IB	KU976467	*Capsicum* sp.	Sudan	91.9% / 91.8% / 91.8% / 91.2%
CMV Pepper	II	KU976485	*Capsicum* sp.	Australia	78.6% / 78.5% / 79% / 79%
CMV Tobacco	II	KU976486	*Nicotiana tabacum*	Germany	77.7% / 77.5% / 78.1% / 78.1%
CMV Tobacco	II	AJ810256	*Nicotiana tabacum*	USA	77.7% / 77.5% / 78.1% / 78.1%

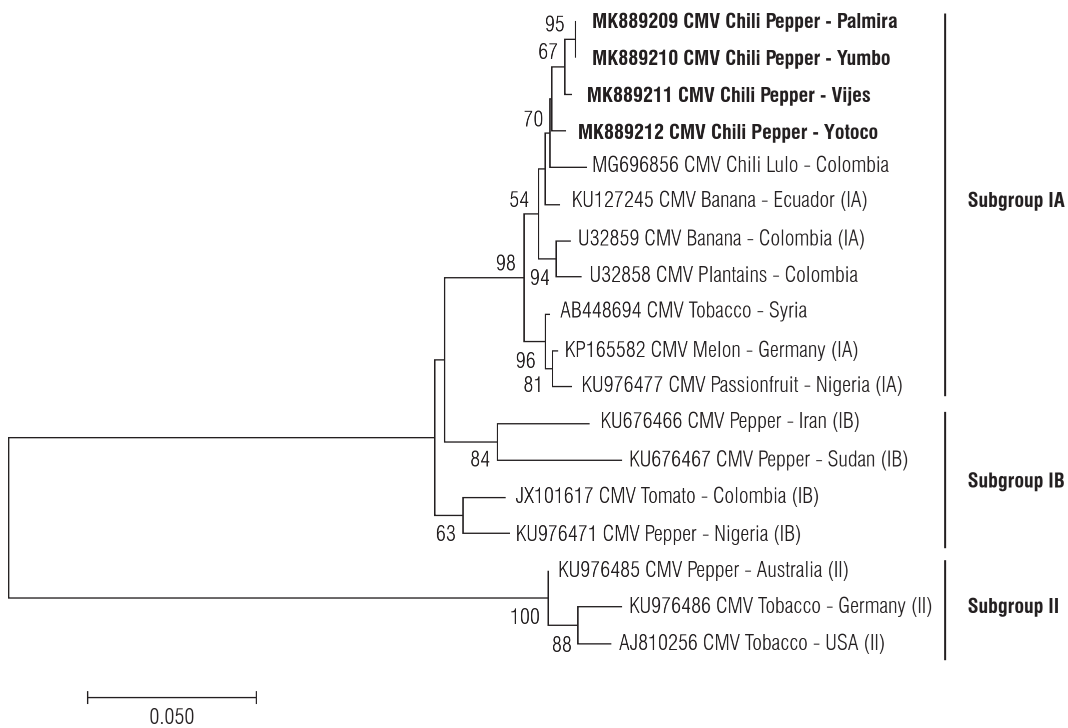

FIGURE 3. Phylogenetic relationships of the CMV chili pepper, constructed from the CP gene at the nucleotide level and its relationship with related CMV isolates. The bar below the tree indicates nucleotide substitutions per site. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances were computed using the maximum composite likelihood method. The bootstrap consensus of the tree was inferred from 2000 replicates. Only bootstrap values above 50% are shown.

peppers in relation to those that operate in banana. It is also noteworthy that, although a CMV quasispecies isolate from tomato had been previously detected in Colombia, it is not related to our isolates because the CMV isolated from tomato is clustered in subgroup IB, characterized by having "Asian strains" (Palukaitis and Zaitlin, 1997).

Despite being solanaceous, these results may indicate that the evolutionary selective pressures on the quasispecies found in these crops are different and are leading to speciation that has taken the viral variants so far that they are practically associated with different groups. We cannot discard the possibility that this event could probably happen through the exchange of contaminated seeds, due to the fact that CMV is also disseminated by seeds (Ali and Kobayashi, 2010). To our knowledge, this is the first record of the molecular identification of a CMV quasispecies affecting chili peppers in Colombia.

Specific primer design and detection of CMV chili pepper

With the previously obtained sequences, a pair of primers was designed in order to perform a more precise and sensitive detection of this CMV quasispecies: CMV chili pepper-F (CTT-TAC-GAA-CTG-TCA-CCC) and CMV chili pepper-R (AAC-TAT-TAA-CCA-CCC-AAC-C) that amplified a 229 bp fragment from the CP gene. In order to corroborate the efficacy of these primers, RT-PCR was carried out by taking cDNA from chili pepper samples positive for CMV as well as genomic chili pepper DNA. According to the results obtained in the efficacy test (data not shown), the expected fragment (229 bp) of CMV was observed in the sample that had previously been reported as positive for CMV using the primers described by Herrera-Vásquez *et al.* (2009). It was also observed that there was no amplification when genomic DNA was used as a template, so the possibility that the amplified fragment corresponds to a part of the genome of the plant is discarded.

With this pair of primers, a new detection on the same samples was carried out. We found that 64.7% (46/71) of the samples were positive for this CMV quasispecies: 100% (6/6) of the samples from Palmira, 62.5% (15/24) of the samples from Yumbo, 73.3% (22/30) of the samples from Vijes and 27.2% (3/11) of the samples from Yotoco. Besides reaffirming the results obtained with the previously described primers, new samples were detected as positive for this virus (Tab. 2). This result shows that the primers designed in this study have high sensitivity, since the number of positive samples for CMV was increased by 12% (64.7%).

The presence of CMV chili pepper was found in all (three) of the evaluated chili pepper varieties. However, we found that the varieties tabasco and habanero show symptoms associated with viral disease, which could indicate that they are susceptible to CMV chili pepper. Otherwise, the variety cayenne was phenotypically asymptomatic despite being positive for CMV chili pepper. From this result, we can suggest that this variety would serve as a virus reservoir, but it could also be a good material to look for CMV tolerance genes.

Capsid protein analysis of CMV chili pepper

Studies by Perry *et al.* (1998) have shown that in the capsid protein of CMV there are certain key amino acid positions for viral transmission mediated by vector insects (aphids): one isolate is highly transmissible by aphids (CMV-Fny), and the other one is transmitted at low frequency by aphids (CMV-M), and the amino acids and their position have been determined (Fig. 4). In this study, our CMV isolates were compared and we found that CMV chili peppers shared the amino acid arrangement with the CMV-Fny isolate (Fig. 4) indicating that our isolates most likely belong to the group of those that are highly transmissible by aphids. Also, the study by Perry *et al.* (1998) shows that some mutations in the conserved positions of the CMV-Fny isolate increase or decrease the transmission by different aphid species, such as *Aphis gossypii* or *Myzus persicae*. Our analysis indicates that the CMV chili pepper, in addition to being

TABLE 2. PCR comparison in the CMV chili pepper detection in chili pepper crops using primers reported by Herrera-Vásquez (2009) and primers designed in this work (CMV chili pepper).

Municipality	Chili pepper variety	Positive plants / plant collected (% viral infection)	
		Herrera-Vásquez's primers universal	Primers designed in this work (CMV chili pepper)
Palmira	Habanero	1/1 (100%)	1/1 (100%)
	Tabasco	5/5 (100%)	5/5 (100%)
Yumbo	Tabasco	11/22 (50%)	14/22 (63%)
	Cayena	1/2 (50%)	1/2 (50%)
Vijes	Tabasco	11/21 (52.3%)	14/21 (66.6%)
	Habanero	6/9 (66.6%)	8/9 (88.8%)
Yotoco	Tabasco	2/11 (18.1%)	3/11 (27.2%)
Four municipalities	Tabasco	29/59 (49.1%)	36/59 (61%)
	Habanero	7/10 (70%)	9/10 (90%)
	Cayena	1/2 (50%)	1/2 (50%)
	Total	**37/71 (52.1%)**	**46/71 (64.7%)**

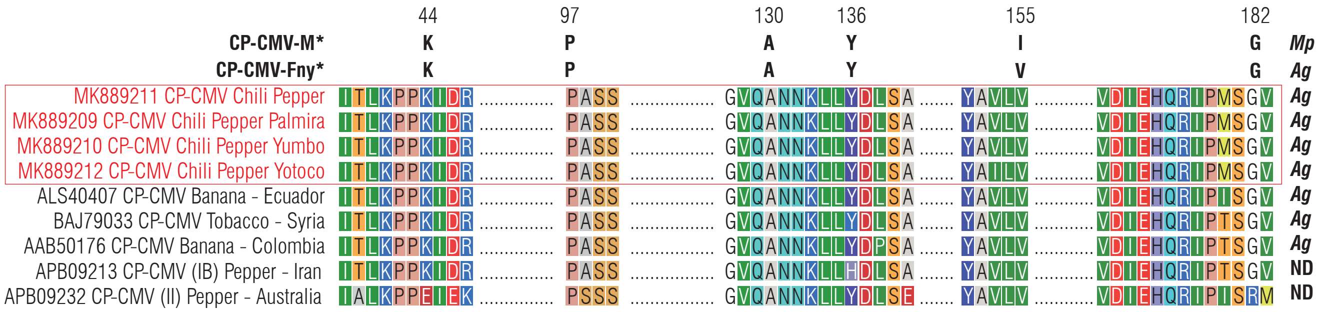

FIGURE 4. Capsid protein analysis from CMV chili pepper. Abbreviation on the right side indicates which aphid species transmits each CMV isolate: *Mp* (*Myzus persicae*), *Ag* (*Aphis gossypii*), ND (Not determined).

FIGURE 5. A) Symptoms in tabasco pepper (*Capsicum frutescens*) collected from the field in the municipality of Yumbo, positive for CMV chili pepper. B) Symptoms of CMV chili pepper in a tabasco pepper plant mechanically inoculated under greenhouse conditions. C) Healthy tabasco pepper plant.

an isolate highly transmissible by aphids, is more likely to be transmitted by *A. gossypii* and has a low probability of being transmitted by *M. persicae*.

Mechanical transmission of CMV chili pepper

At one-month post-inoculation, CMV chili pepper was detected in 86.6% (13/15) of tested plants by RT-PCR, using the specific primers that we previously designed (CMV chili pepper-F / CMV chili pepper-R). These plants showed symptoms such as leaf deformation and yellow vein (Fig. 5). This result confirms that the CMV chili pepper is an infectious agent for chili pepper plants in Colombia.

Conclusions

This study reports, for the first time, the detection and characterization of cucumber mosaic virus in chili pepper crops in four municipalities of Valle del Cauca using molecular techniques in 64.7% (46/71) of the evaluated samples. The CMV chili pepper CP analysis indicated that the virus could be transmissible by the species *Aphis gossypii*. Also, the CMV primers reported in this study can be implemented as an effective tool in the detection of CMV due to their sensitivity. Finally, this CMV isolate can be considered an infectious agent according to the observed symptoms in the mechanical transmission assay.

Acknowledgments

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Immediate and latent damages of drying temperature in the quality of black oat (*Avena strigosa* Schreb.) seeds

Daños inmediatos y latentes de la temperatura de secado en la calidad de las semillas de avena negra (*Avena strigosa* Schreb.)

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ABSTRACT

The objective of this research was to evaluate the immediate and latent drying temperature damages on the physical and physiological quality of black oat seeds. The seeds were dried in an oven with air circulation at temperatures of 30, 40 and 50ºC, in addition to the use of alternating temperatures (40-50ºC). After drying the seeds were stored under environmental conditions for 180 days, and physical and physiological analyses were performed every 45 days. It was observed that the physical quality of black oat seeds was reduced with an increase in the storage time, regardless of the drying temperature used. The physiological quality of black oat seeds increased throughout storage until dormancy was overcome, regardless of the drying temperature. Elevated drying temperatures cause immediate damages to the physical quality of black oat seeds, whereas physiological quality tends latently to suffer the negative influence of increasing the temperature of the drying air.

Key words: conservation, germination, thermal damage, vigor.

RESUMEN

El objetivo de esta investigación fue evaluar los daños inmediatos y latentes por temperatura de secado en la calidad física y fisiológica de las semillas de avena negra. Las semillas se secaron en un horno con circulación de aire a temperaturas de 30, 40 y 50ºC, además del uso de temperaturas alternas (40-50ºC). Después del secado, las semillas se almacenaron en condiciones ambientales durante 180 días, y los análisis físicos y fisiológicos se realizaron cada 45 días. Se observó que la calidad física de las semillas de avena negra se redujo con un aumento en el tiempo de almacenamiento, independientemente de la temperatura de secado utilizada. La calidad fisiológica de las semillas de avena negra aumentó durante el almacenamiento hasta que se superó la latencia, independientemente de la temperatura de secado. La elevación de la temperatura de secado causa daños inmediatos en la calidad física de las semillas de avena negra, mientras que la calidad fisiológica tiende a sufrir la influencia negativa del aumento de la temperatura del aire de secado de forma latente.

Palabras clave: conservación, germinación, daño térmico, vigor.

Introduction

Black oats (*Avena strigosa* Schreb.) is a winter grass, with good adaptability to soils with low fertility (Fontaneli *et al.*, 2012). It is used as a cover crop (Wolschick *et al.*, 2016) and as a forage crop singly or in association with other crops, showing good forage yield and higher weight gain for cattle production (Ferrazza *et al.*, 2013; Lupatini *et al.*, 2013).

The use of high-quality seeds can significantly influence black oats performance in the field, reflecting, for example, higher dry matter yield and leaf area index (Schuch *et al.*, 2008). Seed quality is characterized by physical quality, measured mainly by moisture content, 1000-seed weight, and hectoliter weight, and by physiological performance measured by the germination and vigor tests (Marcos Filho, 2015). However, seed quality can be influenced by drying and storage operations, as verified by white oat seeds (Oliveira *et al.*, 2010) and wheat seeds (Scariot *et al.*, 2018).

Black oat seeds are orthodox, i.e. they are long-lived and dessicant-tolerant and so are tolerant of a reduction in moisture content. As dessicant-tolerant seeds, drying allows them to be stored with better quality and for a longer time. Among the main factors to be considered during the seed drying process is the air temperature. The use of elevated drying air temperatures can result in damage such as cracks and fissures, reserve denaturation, cell membrane damage, germination power loss and even embryo death. Any seed sensitivity to thermal damage can vary according to the plant species, exposure time to heat and drying speed (Garcia *et al.*, 2004; Afrakhteh *et al.*, 2013).

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Seed storage aims to minimize quality losses over time. However, during storage there may be changes in seed quality due to deterioration and aging. Such modifications can be influenced by the storage conditions, mainly in relation to the temperature and relative humidity, but also by the initial physical and physiological conditions of the seeds. Seeds that are damaged or degraded at the beginning of storage are more fragile and, consequently, lose quality more quickly over storage time (McDonald, 1999; Deliberali *et al.,* 2010). Damages caused by the drying temperature can be detected immediately after drying or during storage. These kinds of damages are called immediate and latent damages (Afonso Júnior and Corrêa, 2000).

Some studies have highlighted the damage caused by the drying air temperature on the quality of oat seeds (Oliveria *et al.*, 2010). However, there are few (if any) studies on the behavior of dry oat seeds with different drying air temperatures during the storage period. The objective of this study was to evaluate the immediate and latent damages of different drying air temperatures on the physical and physiological quality of black oat seeds.

Materials and methods

This experiment was carried out at the Laboratory of the Agricultural Systems and Sustainable Management Group (MASSA) of the Universidade Federal da Fronteira Sul (UFFS), Erechim, RS. The experiment had a completely randomized design, arranged in a 4x5 factorial scheme (drying air temperatures x storage time) with four replicates.

Black oat seeds, cultivar EMBRAPA 139 (Neblina), were obtained from the experimental area of the UFFS during the 2016 crop season. The crop cultivation was accomplished under a no-tillage system with fertilization and phytosanitary management, carried out according to the technical suggestions for the crop. Harvesting was performed manually when the seeds contained approximately 25% moisture content. After that the material was subjected to threshing with the aid of a mechanical thresher.

Samples containing 500 g of seeds were packed in kraft paper bags and dried in an oven (Marconi®, model MA033/1080, Piracicaba, SP, Brazil) with forced air circulation (stationary drying) at drying air temperatures of 30°C, 40°C and 50°C until reaching approximately 11% moisture content. Additionally, a drying treatment was tested that alternated the air temperatures, i.e. the seeds were first exposed to a temperature of drying air of 40°C until reaching approximately 16% moisture content and then they were subjected to a temperature of 50°C to reach 11% moisture content.

After drying, the seeds were sent to storage. Storage began on December 2, 2016. Seeds were stored in a conventional system (kraft paper bags) and in a natural environment for 180 d. During the experiment, the temperature and relative humidity of the storage location were monitored daily. The data are shown in Fig. 1 in monthly means.

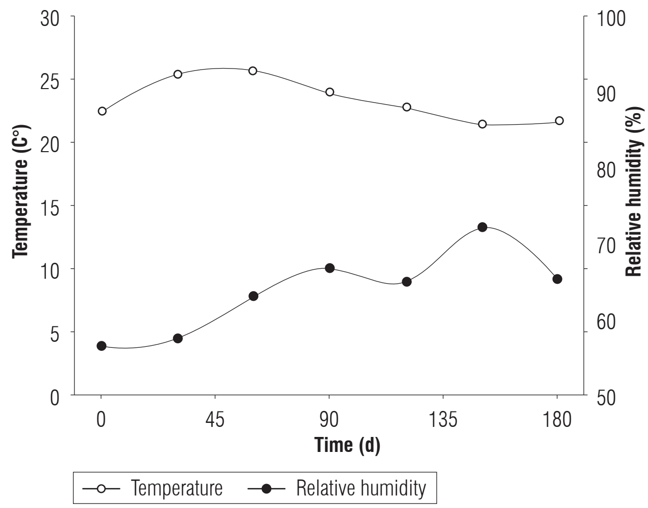

FIGURE 1. Monthly means of temperature and relative humidity of the black oat seeds storage location.

During seed storage there was a reduction in temperature and an increase in the relative humidity of the air due to the approach of the winter period. The temperatures during seed storage varied between 21.4°C and 26.8°C, with a mean temperature of 23.3°C. The relative humidity showed values between 56% and 72% with an average of 62%.

Laboratory analyses were carried out immediately after drying and later every 45 d, defining five evaluation moments (0, 45, 90, 135, and 180 d). The moisture content, 1000-seed weight, hectoliter weight, electrical conductivity, and germination were analyzed. Seed vigor was determined by the tests of the first germination count, the germination speed index, accelerated ageing and the shoot length.

The moisture content was determined in triplicate for each replicate by the oven method at 105±3°C for 24 h (Ministério da Agricultura, Pecuária e Abastecimento, 2009). The results were expressed as a percentage and wet basis (wb).

The weight of 1000 seeds was determined by weighing eight subsamples of 100 seeds for each replicate, with the results being expressed in grams (Ministério da Agricultura, Pecuária e Abastecimento, 2009). The hectoliter weight was determined with the aid of a hectoliter weight balance (Comag®, Panambi, RS, Brazil), with a quarter liter capacity. Values were obtained in triplicate for each replicate and the results expressed in kg hl^{-1} (Ministério da Agricultura, Pecuária e Abastecimento, 2009). The values of a 1000-seeds weight and hectoliter weight were adjusted to 13% moisture content.

The germination test was conducted on Germitest paper rolls, previously soaked in distilled water at a ratio 2.5 times their weight and kept in a germinator (Tecnal, model TE-405, Piracicaba, SP, Brazil) at 20±3ºC, with a photoperiod of 12 h, without the use of agents to overcome dormancy. Two hundred seeds per replicate were used, distributed in four rolls of 50 seeds that totaled 800 seeds per treatment. The first count was performed on the fifth day after sowing by counting the number of normal seedlings. The final evaluation was carried out on the 10th day after sowing. The results were expressed as a percentage (Ministério da Agricultura, Pecuária e Abastecimento, 2009).

The germination speed index was carried out simultaneously with the germination test, by counting the number of normal germinated seeds per day, from the time of sowing until the fifth day, and the index was determined as proposed by Maguire (1962).

The electrical conductivity was determined by the mass system, with two samples of 50 seeds per repetition, conditioned in a glass beaker, weighed and immersed in 50 ml of distilled water. The containers were kept in B.O.D chambers at a temperature of 25±3°C. Evaluations were carried out 24 h after seed immersion with the aid of a benchtop electrical conductivity meter (Gehaka®, model CG1800, São Paulo, SP, Brazil). Results were expressed as $\mu S\ cm^{-1}\ g^{-1}$ (Marcos Filho *et al.*, 1987).

In the accelerated ageing test, the seeds were placed in a gerbox containing 50 ml of distilled water, suspended with a grid, and conditioned in a B.O.D chamber at 41ºC for 72 h (Marcos Filho *et al.*, 1987). After this stage the test was conducted as described for the germination test, and was carried out on the fifth day after sowing, counting the number of normal seedlings, and expressing the results as percentages.

For the determination of shoot length, the seeds were sown on the same way as described for the germination test. Eighty seeds were used per replicate, distributed in four rolls of germitest paper containing 20 seeds each, totaling 320 seeds per treatment. On the 10th day after sowing, 10 seedlings of each roll were randomly chosen and measured with a ruler graded in millimeters. The results were expressed in cm (Nakagawa, 1994).

Data were submitted to analysis of variance by the F-test ($P \leq 0.05$). The drying temperatures were compared with the Tukey's test ($P \leq 0.05$) using the Statistica® 10.0 software. For storage time, regression analysis was applied through Sigma Plot® 10.0 software. The models were selected based on the significance of the model parameters by the application of the t-test ($P \leq 0.05$), by the significance of the mathematical model ($P \leq 0.05$), by the coefficient of determination ($r^2 \geq 0.60$) and by the biological phenomenon.

Results and discussion

According to the variance analysis performed using the F-test ($P \leq 0.05$), there was a significant interaction between the factors drying air temperature and storage time for all variables analyzed.

The values of seed moisture content for the different drying temperatures showed oscillations throughout the storage period, tending to the hygroscopic equilibrium as a function of the relative humidity and the air temperature in the storage environment, which showed relative humidity and average air temperature values of 62% and 23.3°C, respectively. However, no mathematical model showed a satisfactory fit for the observed data (Fig. 2A). Similar results were found by Scariot *et al.* (2018), who observed oscillations in the moisture content of wheat seeds stored for 240 d under environmental conditions. The 1000-seed weight and hectoliter weight of seeds decreased linearly over the storage time, regardless of the drying temperature used (Fig. 2B and C).

The reduction of the 1000-seed weight and hectoliter weight of seeds throughout the storage occurs because of the respiratory process that causes the consumption of reserves and, consequently, the reduction of seed weight and density (Wang *et al.*, 2018). The respiratory rate of seeds during storage can be influenced by several factors: temperature and air relative humidity, as well as thermal damage suffered in drying and pest damages (Ferrari Filho *et al.*, 2012).

Soon after drying, the drying temperature of 30ºC provided seeds with greater specific mass and a 1000-seed weight, while lower values were observed with the other temperatures. The lowest values were observed at a temperature of

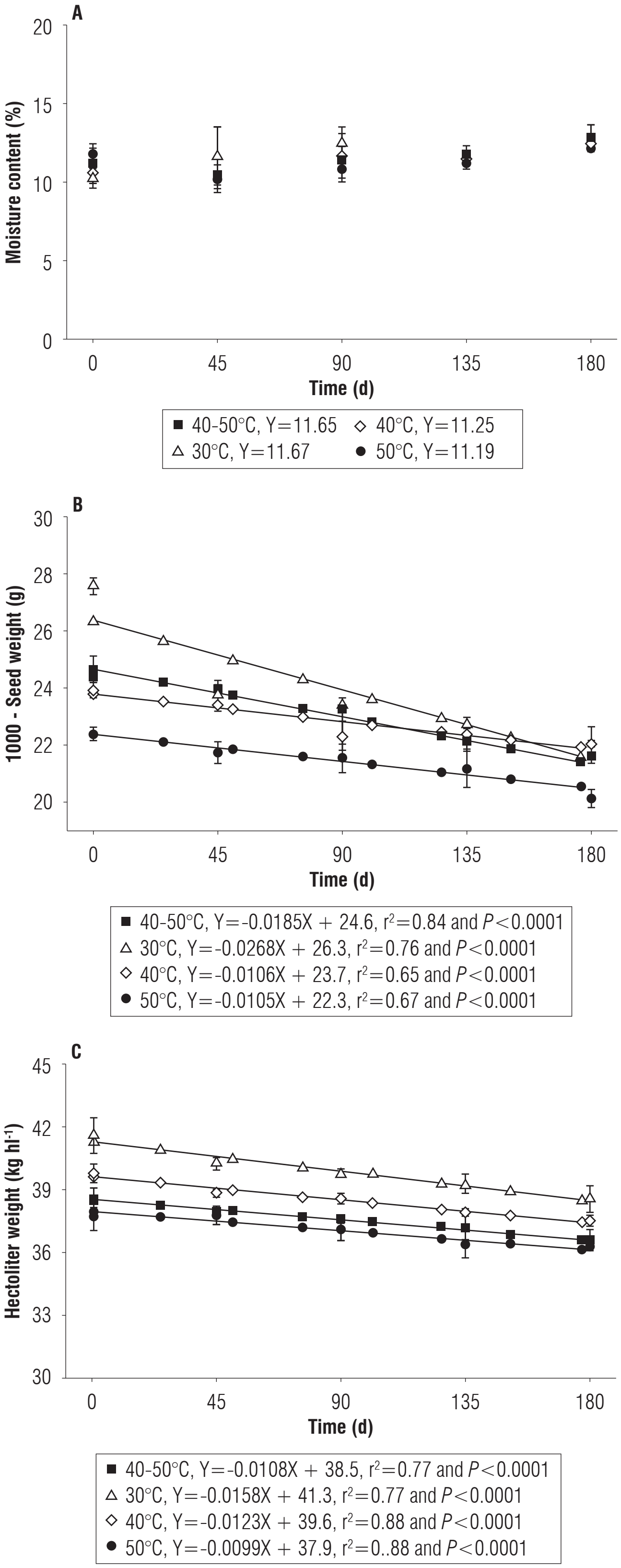

FIGURE 2. A) Moisture content, B) 1000-seed weight, and C) hectoliter weight of black oat seeds cultivar EMBRAPA 139 (Neblina), subjected to different drying temperatures and stored under ambient conditions for 180 days.

50°C, indicating immediate damage. Seeds dried at alternating temperatures showed better physical quality when compared to the ones dried only at a temperature of 50°C.

The reduction of the 1000-seed weight throughout storage was more pronounced for the lower temperatures, including the alternating temperatures. This result indicated more latent damage when compared to the temperature of 50°C, that showed a less marked reduction, but with 1000-seed weight values lower than the other temperatures during the entire experiment. For the hectoliter weight, similar behavior was observed for the reduction in the values between the temperatures and in the alternating temperature. This indicated that the latent seed density damage was similar between the tested temperatures. However, at the end of storage the seeds dried at the lower temperatures had a higher specific mass.

These results agreed with Oliveira *et al.* (2010), who verify the reduction of the 1000-seed weight and hectoliter weight of white oat seeds with the increase of drying air temperature from 50°C.

The reduction in physical quality of the seeds caused by the increase in the temperature of the drying air can also be related to thermal damages like cracks and fissures. For hectoliter weight, the appearance of cracks causes an increase in the seed volume because of the appearance of empty spaces, which leads to density reduction (Eichelberger and Portella, 2003).

Seed germination throughout storage showed similar behavior for the drying temperatures that were studied. The increase in the values was observed for up to 90 d of storage with a subsequent reduction due to the degrading and ageing of seeds (Fig. 3A). Throughout the experiment the highest germination percentages were obtained from the seeds that were dried at a temperature of 30°C.

This described behavior of seeds is characteristic of some species soon after harvesting and includes black oats, due to the process of primary or innate dormancy that is genetically programmed into the mother plant as a function of environmental conditions. Overcoming dormancy can occur over time under conditions of low seed moisture content, culminating in the increase of the germination percentage in storage (Grzybowski *et al.*, 2015).

No immediate damage was observed due to the increase of the drying temperature on seed germination. However, at the end of the storage period, a reduction in seed

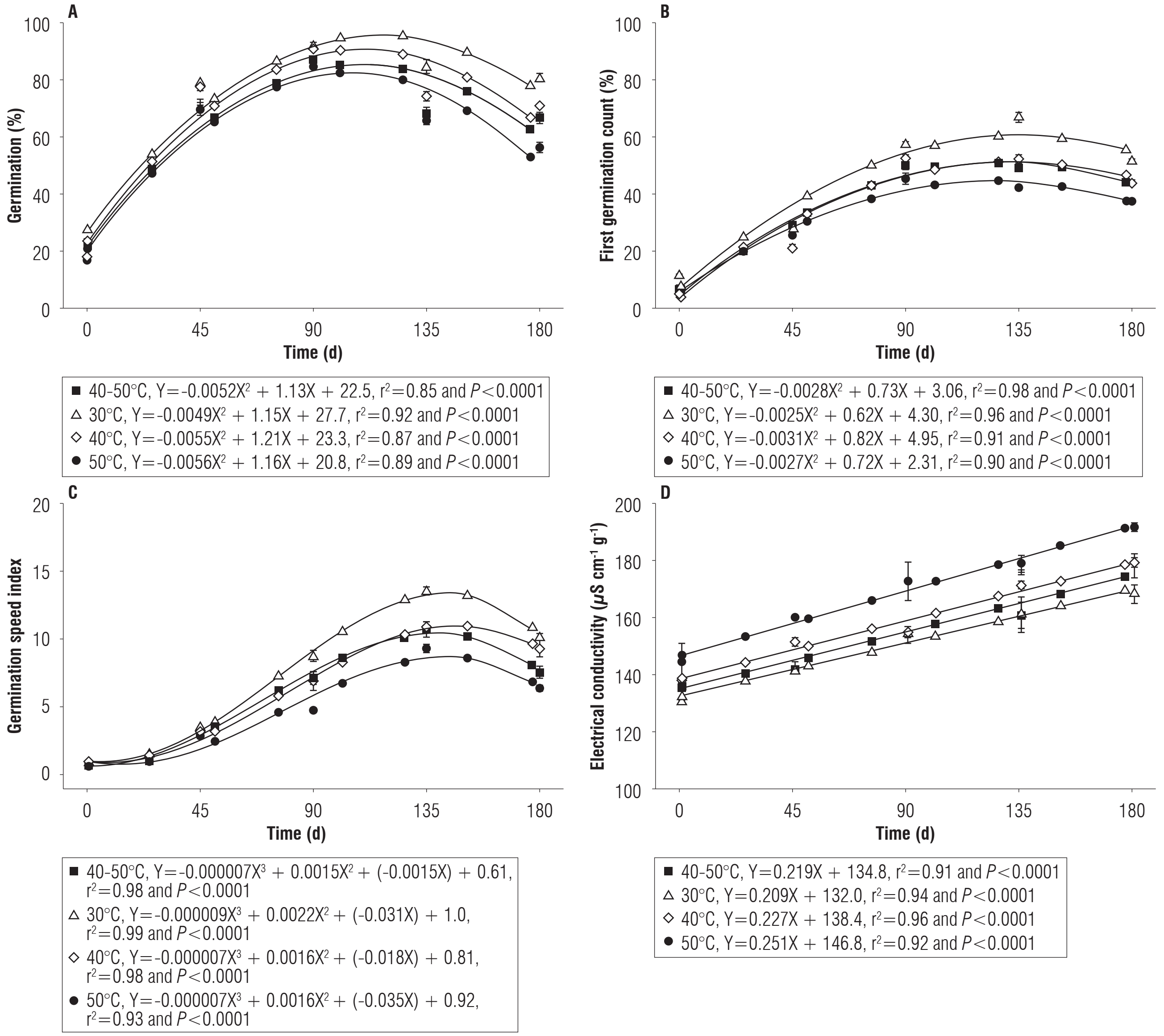

FIGURE 3. A) Germination, B) first germination count, C) germination speed index, and D) electrical conductivity of black oat seeds cultivar EMBRAPA 139 (Neblina), subjected to different drying temperatures and stored under ambient conditions for 180 days.

germination percentage was observed with increasing drying temperature, showing the latent effect of temperature on black oat seed germination. According to Nakagawa *et al.* (2004), the seed's conservability during storage is related to the factors that define its initial quality, including drying.

The results are in agreement with those obtained by Oliveira *et al.* (2010) who demonstrated that stationary drying at temperatures of 50°C, 75°C and 110°C resulted in a reduction in white oat seed germination immediately after drying when compared to a control (25°C). However, the authors used higher temperatures than those used in this study, which may have caused immediate damage.

Seed vigor, when analyzed by germination, first germination, and speed index count tests, showed similar behavior throughout the storage for all the drying temperatures, with an increase in the values until approximately 135 d, and a drop until the end the storage period (Fig. 3B and C). This behavior is due to seed dormancy that is overcome with storage time and is reflected in the increased physiological activity of the seed.

Regarding germination, no immediate damage was observed due to the increase in drying temperature on the germination speed index. For the first germination count, a higher percentage of normally-germinated seedlings were observed for the drying temperature of 30ºC, indicating a higher vigor. However, during the storage time, lower values were verified for these two variables with the increase in the drying temperature, indicating loss of quality by the latent effect.

Electrical conductivity showed a linear increase according to storage time for all drying temperatures (Fig. 3D). The increase of electrical conductivity in storage is due to the process of deterioration and seed ageing. The destabilization of cell membranes is one of the first events related to these processes that culminate with the increase of solute leaching and electrical conductivity (Mira *et al.*, 2011). These results are similar to those observed by Zucareli *et al.* (2015) and Scariot *et al.* (2018) who verified the increase in electrical conductivity of bean and wheat seeds over storage time.

The elevation of drying temperature caused immediate and latent damages in the cellular structure of black oat seeds, since the electrical conductivity values were higher at higher drying temperatures throughout the storage time. This result can be attributed to the fact that the increase in drying air temperature provides rapid removal of water from seeds. This can cause damage to the selective permeability of cell membranes, increasing solute leaching, reflected by higher conductivity values (Corrêa and Afonso, 1999). Botelho *et al.* (2015) and Scariot *et al.* (2017a) verified increased electrical conductivity of soybean and bean seeds with an increase in drying air temperature of up to 80°C and 50ºC, respectively.

Seed vigor, measured by the accelerated ageing test, increased up to approximately 135 d as a result of overcoming dormancy that decreased for all drying temperatures, but with a more pronounced reduction for seeds dried at higher temperatures (Fig. 4A).

Drying temperatures did not cause immediate damage to seed vigor, according to the accelerated ageing test. However, latent drying temperature damage was observed during storage, since seeds subjected to drying temperatures above 30ºC showed a lower percentage of normally-germinated seedlings. However, there was immediate damage to the vigor of white oat seeds due to the increase of drying air temperatures, though air temperatures above 50°C were used in that study (Oliveira *et al.,* 2010).

Shoot length showed similar behavior for all drying air temperatures during storage (Fig. 4B). The observed values increased up to approximately 135 d due to overcoming dormancy with a later drop at the end of the storage period. Tunes *et al.* (2010) and Scariot *et al.* (2017b) verified the increase in shoot length of barley and wheat seedlings up to 180 and 120 d, respectively, due to the process of overcoming dormancy.

Immediate and latent damage to the shoot length of seedlings has been found due to the increase in the drying air temperature since the seeds subjected to drying at a temperature of 30°C showed the highest values at the beginning and end of the storage period. Similarly, Scariot *et al.* (2017a) verified immediate damage of the drying

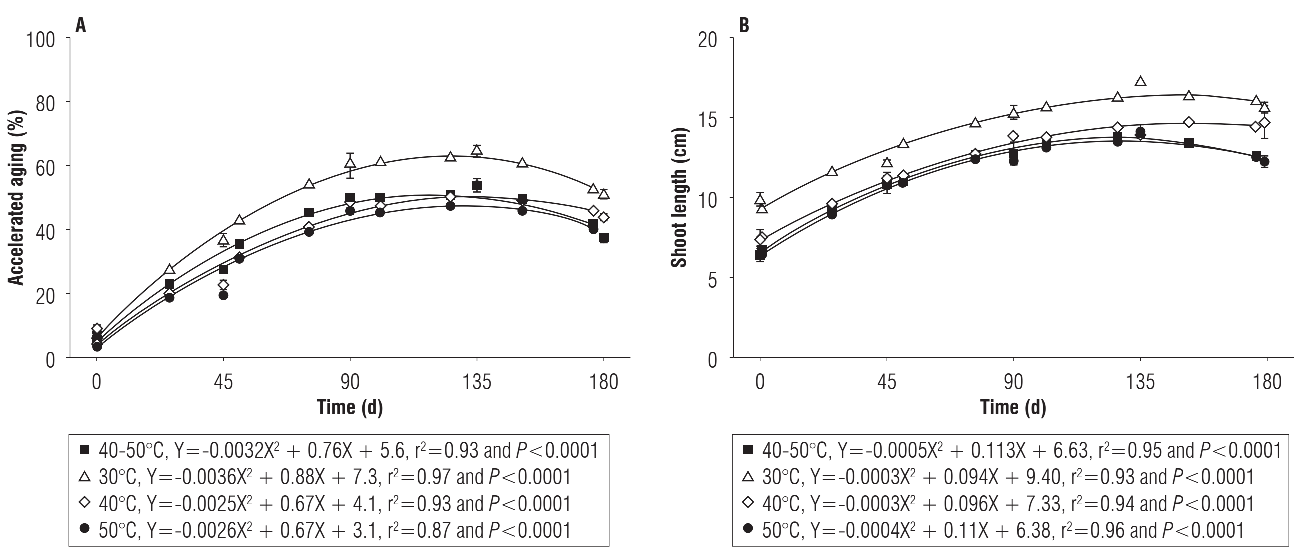

FIGURE 4. A) Accelerated ageing and B) shoot length of black oat seeds, cultivar EMBRAPA 139 (Neblina), subjected to different drying air temperatures and stored under ambient conditions for 180 days.

temperature increment of up to 50°C on the shoot length of bean seedlings.

Shoot length may be an indicator of seed vigor, since vigor is related to the seed's ability to nourish the embryo, culminating in longer seedlings (Marcos Filho, 2015). Thus, the reduction in shoot length observed with the increase in drying temperature may be related to the thermal damages caused to the embryonic axis or to the reduction of reserves that can be observed in the 1000-seed weight and hectoliter weight of seeds.

The use of the alternating temperature provided better results for germination, germination first count, and germination speed index when compared to drying using only the temperature of 50ºC. Electrical conductivity showed better results in relation to the temperatures of 40 and 50ºC, when applied individually. However, the results for vigor, measured by the accelerated ageing test and shoot length obtained with alternating temperatures were similar to those obtained when using the highest temperature individually (50ºC).

These results demonstrate that the use of high temperatures leads to a reduction in the physiological quality of black oat seeds even when they are alternated with low temperatures, since the analysis of seeds dried at a lower temperature (30°C) showed the best results.

Conclusions

The physical quality of black oat seeds reduces with storage time, regardless of the drying temperature. However, an increase in drying temperature can cause immediate damages to the physical quality of the seeds.

The physiological quality of black oat seeds increases throughout storage until dormancy is overcome, regardless of the drying temperature. However, after dormancy is overcome, the physiological quality of seeds is reduced.

The physiological quality of black oat seeds tends to suffer latently the negative influence of the rise of the drying temperature. Thus, the increase in the drying temperature intensifies the loss of quality throughout storage.

Dry black oat seeds with alternating drying temperatures (40-50ºC) showed lower physical and physiological quality than seeds dried at temperatures up to 40ºC, but they showed better quality when compared to seeds dried at a temperature of 50ºC.

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Influence of the AquaCrop soil module on the estimation of soybean and maize crop yield in the State of Parana, Brazil

Influencia del módulo suelo del AquaCrop en la estimación del rendimiento de los cultivos de soya y maíz en el Estado de Paraná, Brasil

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ABSTRACT

The values of the physical-water attributes of soils for use in agricultural simulation models are usually obtained using difficult and time-consuming methods. The objective of this study was to analyze the performance of the AquaCrop model to estimate soybean and maize crop productivity in the region of Campos Gerais (Brazil), with the option of including soil physical-water attributes in the model. Real crop productivities and input data (soil, climate, crop and soil management) were obtained from experimental stations of the ABC Foundation for the crop years 2006 to 2014. Sixty-four yield simulations were performed for soybean (four municipalities) and 42 for maize (three municipalities), evaluating input soil data scenarios of AquaCrop as follows: i) all soil physical-water attributes were measured (standard) and ii) the attributes were measured only using textural classification of the area (alternative). Real and simulated yields were verified by simple linear regression analyses and statistical indices (*r*, *d*, *c*). The standard scenario yielded performances between very good and excellent ($0.75<c\leq1.0$) for soybean and between bad and excellent ($0.40<c\leq1.0$) for maize. The alternative scenario was more variable, with performances between terrible and excellent ($0.0<c\leq1.0$) for soybean and terrible and medium ($0.0<c\leq0.65$) for maize. Using only the soil texture classification in AquaCrop indicated an easier way to estimate crop yields, but low performances may restrict estimates of soybean and maize yields in Campos Gerais.

Key words: simulation, soil attributes, *Glycine max*, *Zea mays*.

RESUMEN

Los valores de los atributos físico-hídricos del suelo para uso en modelos de simulación agrícola generalmente se obtienen usando métodos difíciles y demorados. El objetivo del presente trabajo fue analizar el desempeño del modelo AquaCrop para estimar la productividad de los cultivos soya y maíz en la región de los Campos Gerais (Brasil), de acuerdo con la opción de incluir los atributos físico-hídricos del suelo en el modelo. Las productividades reales de los cultivos y datos de entrada (suelo, clima, cultivo y manejo del suelo) se obtuvieron de las estaciones experimentales de la Fundación ABC, para los años cosecha entre 2006 y 2014. Se llevaron a cabo 64 simulaciones de productividad para soya (cuatro municipios) y 42 para maíz (tres municipios), evaluando escenarios de entrada de los datos del suelo en el AquaCrop de la siguiente manera: i) todos los atributos físico-hídricos del suelo medidos (estándar) y ii) sólo la clasificación textural del área (alternativo). Las productividades reales y simuladas se verificaron por análisis de regresión lineal simple e índices estadísticos (*r*, *d*, *c*). El escenario estándar obtuvo desempeños entre muy bueno y excelente ($0.75<c\leq1.0$) para la soya y entre malo y excelente ($0.40<c\leq 1.0$) para el maíz. El escenario alternativo fue más variable, con desempeños entre pésimo y excelente ($0.0<c\leq1.0$) para soya y entre pésimo y mediano ($0.0<c\leq0.65$) para maíz. Utilizar solamente la clasificación de textura del suelo en el AquaCrop indicó una forma más fácil de estimar los rendimientos de los cultivos, pero los bajos desempeños pueden restringir estimativas de las productividades de los cultivos soya y maíz en Campos Gerais.

Palabras clave: simulación, atributos del suelo, *Glycine max*, *Zea mays*.

Introduction

The availability of input data is one of the greatest difficulties for using agricultural simulation models. Some models require large amounts of input data that are difficult to obtain and expensive (Jones *et al*., 2017; Rosa *et al*., 2020). However, since crop growth modeling has progressed over the last decades (Siad *et al*., 2019), the evolution of simulation models combined with the simplification and robustness of the programs tends to increase precision (Steduto *et al*., 2009; Steduto *et al*., 2012; Tonitto *et al*., 2018).

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Researchers from the Food and Agriculture Organization (FAO) aiming to meet the demand for more widely usable models developed the AquaCrop model (García *et al*., 2017). AquaCrop is a crop water productivity model designed to assess the effect of management and the environment on the production of different crops, simulating yield responses to water (Issoufou *et al*., 2020; López-Urrea *et al*., 2020). Among its other advantages, AquaCrop requires a small number of input data and their insertion is easier (Steduto *et al*., 2012; Raes *et al*., 2018a).

The literature considers that analyses to obtain soil physical-water attribute values still use difficult and time-consuming methods; also, the interpretation of their measurements is not direct due to the complexity and specificity of the evaluated soils (Lin *et al*., 1999; Bünemann *et al*., 2018). The standard methodologies require sensitive and expensive equipment that does not match the structure of most laboratories in Brazil. In addition, the results often produce errors and are not reliable (Oliveira *et al*., 2002). Therefore, the physical-water attribute data for insertion into the models may not be so easy to obtain or either operational.

In this regard, AquaCrop has shown advances in its development, making it possible to indirectly obtain some parameters or attributes of the agricultural environment as, for example, the use of pedotransfer functions to obtain the main soil physical-water attributes, considering only texture (Raes *et al*., 2018a; 2018b). In order to overcome this problem, AquaCrop allows: i) the introduction into the model saturated hydraulic conductivity (K_{sat}) values, field capacity (θ_{FC}), permanent wilting point (θ_{PWP}) and saturation (θ_{sat}) volumetric values; and ii) the generation of values of soil physical-water attributes (K_{sat}, θ_{FC}, θ_{PWP} and θ_{sat}) based on soil texture (clay, silt and sand), using pedotransfer functions.

The alternative of using only the soil textural classification in AquaCrop is simple and quite interesting for carrying out analyses in places with little soil physical-water attribute data. In the literature, there are several studies with AquaCrop, but none have evaluated the performance using the input method of the physical-water attributes (measured or estimated). However, this option can lead to considerable errors that have not yet been properly studied nor evaluated. This aspect is limiting, since the soil hydraulic properties, together with hydraulic gradients, control the water flow towards the roots (Pinheiro *et al*., 2019) and the soil physical-water attributes are fundamental to the interactions in the soil-plant-atmosphere continuum.

Considering the difficulty of obtaining the main physical-water attributes required as input in some simulation models, the objective of this study was to evaluate the performance of the AquaCrop model for estimating the productivity of soybean and maize crops in the region of Campos Gerais, according to the option of entering soil physical-water attributes into the model.

Materials and methods

The present study was carried out in the region of Campos Gerais, located in the States of Parana and São Paulo, which is the reference for grain production in Brazil. Maize and soybean historical crop data (2006/07 to 2013/14 harvest) of the ABC Foundation - Research and Agricultural Development were used. The experimental plots and stations used in the study were located at the Agrometeorology sector of the ABC Foundation (Tab. 1), which includes flat to gently undulating relief typical of the region. The soil tillage system is no-tillage with homogeneous vegetable mulching. The crop rotation system used is alternated between soybean and maize in summer, and wheat and black oats in winter. Pest and disease control is performed according to the usual methods in the region, and fertilization is performed by supplying all the nutrients necessary for full crop development. The data came from a historical experimental series carried out at the experimental stations of the ABC Foundation, harvests from 2006/07 to 2013/14.

The model used in the analyses was AquaCrop version 4.0. This experiment is part of several studies in the region of Campos Gerais, located in the States of Parana and São Paulo, in which the first phase consisted of testing the AquaCrop model using the parameters recommended by the model's creators.

To verify AquaCrop performance under agricultural production conditions, 42 and 64 simulations of maize and soybean production (kg ha^{-1}), respectively, were compared to real yields (kg ha^{-1}) from previous experiments carried out in the field, using harvests from 2006/07 to 2013/14. AquaCrop uses simulated groups of conservative parameters (that do not depend on cultivation conditions and water regimen, temporal scale, management practices, climate or geographic location) and non-conservative parameters (depending on the crop under study and the environmental conditions of cultivation). The required conservative parameters were based on the recommendations of Raes *et al*. (2013), and the non-conservative parameters were adjusted according to protocol data of the ABC Foundation.

TABLE 1. Locality, soil, climate, geographic coordinates and altitudes of ABC Foundation experimental stations.

Locality/State	Soil[1]	Mean daily air temperature (°C)	Annual precipitation (mm/year)	Climate[2]	Latitude[3] (degrees)	Longitude[3] (degrees)	Altitude[3] (m)
Arapoti/Parana	Oxisol	18.8	1482.3	Cfa/Cfb[(4)]	24.18° S	49.85° W	902
Castro/Parana	Inceptisol	17.4	1368.1	Cfb	24.85° S	49.93° W	1001
Itabera/São Paulo	Ultisol	18.4	1380.1	Cfa	24.07° S	49.15° W	735
Ponta Grossa/Parana	Oxisol	17.9	1551.8	Cfb	25.01° S	50.15° W	1000
Socavão/Parana	Histosol	16.8	1397.4	Cfb	24.68° S	49.75° W	1026

[1]Classification obtained from ABC Foundation soil maps (scale 1:10000); [2]adapted from Alvares *et al.* (2013); [3]geographic coordinates measured with GPS device; [4]climate transition site according to Köppen's climate classification for Brazil: Cfa = humid subtropical, oceanic climate without dry season and with a hot summer and Cfb = humid subtropical, oceanic climate without dry season with a temperate summer.

To begin simulation, the AquaCrop model requires input data on soil, climate, crop and management (Raes *et al.*, 2018a and 2018b). To obtain data, data entry varied according to the experimental stations and agricultural crop following methodological aspects.

Soil physical-water attributes scenarios

With the soil data input, two scenarios were considered in soybean and maize crops simulations: i) Standard - input of all measured soil physical-water attributes needed in AquaCrop, corresponding to each experimental station analyzed; and ii) Alternative - generation of the soil physical-water attribute values using AquaCrop pedotransfer functions, based on soil textural classification for each experimental station.

Soil

For each experimental plot (50 × 100 m), five representative points were analyzed in the field. The soil layers considered at each point were 0.0-0.10 m, 0.10-0.25 m and 0.25-0.40 m in order to reach the effective rooting depth of crops. For each layer, data were entered into AquaCrop according to simulation scenarios (standard or alternative). At each point and soil layer disturbed and undisturbed soil samples were collected totaling 75 soil samples (5 experimental stations, 5 experimental points and 3 depths). Volumetric water content at saturation (m^3/m^3) and field capacity (m^3/m^3) were determined according to Teixeira *et al.* (2017) using undisturbed soil samples collected with volumetric rings (5 cm diameter and 3 cm height). The water content at field capacity (m^3/m^3) was determined while the water balance was kept stable in the tension table at a tension of 0.01 MPa. The water content at permanent wilting point (m^3/m^3) was estimated in the soil water retention curve, created with SPLINTEX pedotransfer software (Prevedello, 1999). The volumetric water content at 1.5 MPa tension was considered as the permanent wilting point. The saturated hydraulic conductivity (mm/d) was determined with a constant head permeameter developed and calibrated at the Department of Soil and Agricultural Engineering of the Federal University of Parana, according to Teixeira *et al.* (2017). Soil texture was measured with disturbed soil samples using the densimeter method, according to the methodology of Teixeira *et al.* (2017). The soil volumetric water content at planting time (m^3/m^3) was estimated according to Souza *et al.* (2013). Thus, it was considered that the soil reached the water content at field capacity at the moment prior to planting when there was a heavy rainfall. From this date onwards, the daily inflow and outflow water in the soil began to be accounted for until planting time.

Climate

The climate data used came from the agrometeorological stations in each experimental field. The minimum and maximum air temperature (°C) and rainfall (mm) data were obtained from the climate databases provided by the Agrometeorology sector of the ABC Foundation. The reference evapotranspiration (*ETo*; mm/d) was estimated with the Penman-Monteith method (ASCE-EWRI, 2005). The mean yearly atmospheric CO_2 concentrations (ppm) were provided by the AquaCrop program, measured at the Mauna Loa observatory in Hawaii (Raes *et al.*, 2018b).

Crop

The required data were the planting date, duration of each phenological cycle (d), plant population (plants ha^{-1}) and effective rooting depth (m). The data came from historical experiment series carried out at the experimental stations of the ABC Foundation, harvests from 2006/07 to 2013/14.

Management

A fertilization level near optimal was considered. As the areas were under a no-tillage system, soil cover of mulch was considered to be 50% of the total soil covering in all experimental stations.

The input data were inserted into AquaCrop generating a soil and climate database for each experimental field in harvests between 2006 and 2014. The management data were the same for all simulations, only requiring a change in the crop data.

Statistical analyses were performed following the recommendations of Souza (2018). The respective values of real crop (kg ha^{-1}) and estimated (kg ha^{-1}) productivities obtained in standard or alternative simulation scenarios were compared statistically using linear regression analysis, correlation coefficient (*r*; Eq. 1), concordance *d* (Willmott, 1982; Eq. 2) and performance *c* (Camargo and Sentelhas, 1997; Eq. 3) indices. The analyzed results were organized by localities to verify the possibility of relating the result to the soil type of each experimental station.

$$r = \frac{\sum_{i=1}^{n} \left[(Yr_i - \bar{Yr}) x (Ys_i - \bar{Ys}) \right]}{\sqrt{\sum_{i=1}^{n} (Yr_i - \bar{Yr})^2 \, x \sum_{i=1}^{n} (Ys_i - \bar{Ys})^2}} \quad (1)$$

$$d = 1 - \frac{\sum_{i=1}^{n} (Ys_i - Yr_i)^2}{\sum_{i=1}^{n} \left(\left| Ys_i - \bar{Yr} \right| + \left| Yr_i - \bar{Yr} \right| \right)^2} \quad (2)$$

$$c = d \, x \, r \quad (3)$$

where: r = Pearson correlation coefficient (dimensionless); d = d index (dimensionless); c = c index (dimensionless); Yr_i = real yields observed in the field at each *i*-experiment (kg ha^{-1}); $\bar{Yr}$ = real average yields from all cultivars observed in the field (kg ha^{-1}); Ys_i = estimated yield observed in the model at each *i*-experiment (kg ha^{-1}); $\bar{Ys}$ = observed average yields from all cultivars estimated in the model (kg ha^{-1}); n = number of harvests in the localities (dimensionless).

The interpretation criteria of *c* performance was classified as excellent (c>0.85); very good (0.75<c≤0.85); good (0.65<c≤0.75); medium (0.60<c≤0.65); tolerable (0.50 <c≤ 0.60); bad (0.40 <c≤ 0.50); and terrible (c≤0.40).

Results and discussion

The measured values (standard) of physical-water attributes from the soils for some experimental stations were different or close to those considered as alternative by AquaCrop (Raes *et al*. 2013), obtained according to textural classification of each soil (Tab. 2).

The measured θ_{sat} values (standard) in Arapoti were lower than the alternative values. In Castro and Socavão, the measured θ_{sat} values were higher, and in Itabera and Ponta Grossa these values were very similar. The values of θ_{sat} (considered equal to the total soil porosity) and θ_{FC} allowed calculating the porosity without water ($\beta = \theta_{sat} - \theta_{FC}$), consisting of the porous space not occupied by water (Tab. 2). The literature indicates that β should be enough for the oxygen diffusion in the soil to meet the oxygen demand of agricultural crops (Erickson, 1982; Reichardt and Timm, 2004). Thus, β values are more important than the magnitude (lower or higher) of θ_{sat} and θ_{FC} values. Studies indicate that β should be higher than or equal to 0.1 m^3/m^3 or 10% (Erickson, 1982; Reichardt and Timm, 2004). In order not to hamper the normal functioning of the plant root system, Yevtushenko *et al*. (2016) consider that β should not be below 0.15 m^3/m^3 or 15% in the surface layer.

Generally, β values calculated with the measured data (standard; Tab. 2) indicated better aeration conditions for soybean and maize crops than the estimated values (alternative; Tab. 2). The following means were verified: β = 17.0% (standard) and β = 8% (alternative) for sandy clay loam soil in Arapoti; β = 15% (standard) and β = 11% (alternative) for sandy clay in Ponta Grossa; β = 15.9% (standard) and β = 1.0% (alternative) for clay soils located in Castro, Itabera and Socavão; and β = 15.9% (standard) and β = 4.4% (alternative) for all soils analyzed from the Campos Gerais region (sandy clay loam, sandy clay and clay). Therefore, since they are considered in the calculation the values of β (alternative) can penalize the yields estimated in AquaCrop, obtaining higher errors compared to the values of real crop productivity or estimated productivity with the measured physical-water attributes (standard).

The measured values of θ_{FC} and θ_{PWP} (standard; Tab. 2) were lower compared to the estimated values (alternative; Tab. 2), except for sandy clay soil (0-10 cm) in the experimental station of Ponta Grossa, where θ_{FC} was equal to the estimated value (alternative). It is important to consider that the measured (standard) and estimated (alternative) values of θ_{FC} and θ_{PWP} showed differences between the evaluated methods. However, the measured (standard) and estimated (alternative) values of soil available water ($\theta_{AWC} = \theta_{FC} - \theta_{PWP}$) were very close, resulting in the following means: θ_{AWC} = 0.16 m^3/m^3 (standard) and θ_{AWC} = 0.21 m^3/m^3 (alternative) for sandy clay loam soil in Arapoti; θ_{AWC} = 0.15 m^3/m^3 (standard) and θ_{AWC} = 0.12 m^3/m^3 (alternative) for sandy clay soil in Ponta Grossa; θ_{AWC} = 0.14 m^3/m^3 (standard) and θ_{AWC} = 0.15 m^3/m^3 (alternative) for clay soils located in Castro, Itabera and Socavão; and θ_{AWC} = 0.15 m^3/m^3 (standard) and

TABLE 2. Estimated (alternative) and measured (standard) soil physical-water attributes in the experimental areas of the ABC Foundation in the Campos Gerais region.

Layer (m)	Textural classification	θ_{sat} [1]	θ_{FC} [1]	θ_{PWP} [1]	K_{sat} [2]	β [3]	θ_{AWC} [4]	*AWC*[5]
		(m^3/m^3)			(mm/d)	(%)	(m^3/m^3)	(mm)
Estimated (alternative) physical-water attributes obtained according to textural classification (Raes *et al*., 2013)								
0.0-0.40	Sandy clay loam	0.52	0.44	0.23	120	8.0	0.21	84.0
0.0-0.40	Sandy clay	0.50	0.39	0.27	75	11.0	0.12	48.0
0.0-0.40	Clay	0.55	0.54	0.39	2	1.0	0.15	60.0
Measured (standard) physical-water attributes in Arapoti								
0.0-0.10	Sandy clay loam	0.49	0.34	0.15	1394.9	15.0	0.19	19.0
0.10-0.25	Sandy clay loam	0.47	0.30	0.15	1268.4	17.0	0.15	22.5
0.25-0.40	Sandy clay loam	0.49	0.30	0.16	1124.5	19.0	0.14	21.0
Measured (standard) physical-water attributes in Castro								
0.0-0.10	Clay	0.63	0.50	0.36	418.3	13.0	0.14	14.0
0.10-0.25	Clay	0.60	0.47	0.33	368.2	13.0	0.14	21.0
0.25-0.40	Clay	0.62	0.45	0.32	325.7	17.0	0.13	19.5
Measured (standard) physical-water attributes in Itabera								
0.0-0.10	Clay	0.55	0.40	0.28	516.5	15.0	0.12	12.0
0.10-0.25	Clay	0.54	0.37	0.24	462.3	17.0	0.13	19.5
0.25-0.40	Clay	0.54	0.37	0.22	420.4	17.0	0.15	22.5
Measured (standard) physical-water attributes in Ponta Grossa								
0.0-0.10	Sandy clay	0.51	0.39	0.20	743.3	12.0	0.19	19.0
0.10-0.25	Sandy clay	0.50	0.35	0.20	732.6	15.0	0.15	22.5
0.25-0.40	Sandy clay	0.54	0.36	0.25	636.3	18.0	0.11	16.5
Measured (standard) physical-water attributes in Socavão								
0.0-0.10	Clay	0.57	0.43	0.28	336.0	14.0	0.15	15.0
0.10-0.25	Clay	0.59	0.41	0.27	351.1	18.0	0.14	21.0
0.25-0.40	Clay	0.59	0.40	0.24	355.5	19.0	0.16	24.0

[1]θ_{sat}, θ_{FC}, θ_{PWP} = volumetric water content at saturation, field capacity and permanent wilting point, respectively; [2]K_{sat} = saturated hydraulic conductivity; [3]β = porosity without water; [4] $\theta_{AWC} = \theta_{FC} - \theta_{PWP}$ = available water in the soil; [5]$AWC = \theta_{AWC} \times z$ = available water capacity at the *z* depth.

θ_{AWC} = 0.16 m^3/m^3 (alternative) for all soils analyzed from the Campos Gerais region (sandy clay loam, sandy clay and clay). Therefore, in terms of soil available water capacity, it is important to note that the measured (standard) and estimated (alternative) θ_{FC} and θ_{PWP} values showed no differences in the simulations.

Considering the available water values ($\theta_{AWC} = \theta_{FC} - \theta_{PWP}$) and the root system depth (z; 0-10 cm, 10-25 cm and 25-40 cm, respectively) of each layer, calculations of water capacity ($AWC = \theta_{AWC} \times z$) to z = 40 cm with the measured (standard) and estimated (alternative) soil physical attributes indicated the following values (Tab. 2): *AWC* = 62.5 mm (standard) and *AWC* = 84.0 mm (alternative) for sandy clay loam soil in Arapoti; *AWC* = 54.5 mm (standard) and *AWC* = 60.0 mm (alternative) for clay soils in Castro; *AWC* = 54.0 mm (standard) and *AWC* = 60.0 mm (alternative) for clay soil in Itabera; *AWC* = 58.0 mm (standard) and *AWC* = 48.0 mm (alternative) for sandy clay soil in Ponta Grossa; *AWC* = 60.0 mm (standard) and *AWC* = 60.0 mm (alternative) for clay soil in Socavão; and AWC_{mean} = 57.8 mm (standard) and AWC_{mean} = 62.4 mm (alternative) for all soils analyzed from the Campos Gerais region (sandy clay loam, sandy clay and clay). The occurrence of water deficiency in the simulations is unlikely in the Campos Gerais region. In a study in Ponta Grossa region, Scheraiber (2012) verified that the occurrence of water deficiency (mm/decade) is practically zero in soil simulations with water balance containing: *AWC* = 60 mm, available water fraction (p) between 0.3 and 0.7, and crop coefficient (kc) from 0.75 to 1.1. Thus, it is believed that simulated water storage values of the harvests will not be responsible for probable differences that may occur between values of estimated productivity with the measured (standard) and estimated (alternative) physical-water attributes.

The measured K_{sat} (standard) values were much larger than the estimated (alternative) values for all textural classifications studied. Therefore, the studied soils (standard) have drainage characteristics (τ) different from the alternative ones. According to Raes *et al.* (2018c), the drainage characteristic is a less constant unit responsible for the size given to the potential water depletion curve of a soil layer, originally at saturation, at the end of the first day of free drainage in the soil. The drainage (τ) is a variable that depends on the saturated hydraulic conductivity. Thus, the use of estimated attributes (alternative) considers that water depletion of a soil layer would occur more slowly than the measured attributes (standard).

The measured (standard) and estimated (alternative) K_{sat} values had the following means (Tab. 2): 1263 mm/d (standard) and K_{sat} = 120 mm/d (alternative) for the sandy clay loam in Arapoti; K_{sat} = 704 mm/d (standard) and K_{sat} = 75 mm/d (alternative) for sandy clay soil in Ponta Grossa; K_{sat} = 395 mm/d (standard) and K_{sat} = 2 mm/d (alternative) for clay soils in Castro, Itabera and Socavão; and K_{sat} = 630 mm/d (standard) and K_{sat} = 40 mm/d (alternative) for all soils analyzed from the Campos Gerais region (sandy clay loam, sandy clay and clay). The amplitude of measured values (standard) is acceptable for the evaluated areas since the K_{sat} has high variability in different soil types.

The correlation coefficients indicated that the associations between real crop yield and estimated (*Yr* vs. *Ys*) in AquaCrop are less accurate when using the alternative (estimated) scenario of soil physical-water attributes in the simulations (Tab. 3 and Fig. 1). In the simulations with the standard (measured) scenario, $r \geq 0.88$ was obtained for soybean (Castro) and $r \geq 0.81$ for maize (Castro). In simulations with the alternative scenario, the associations indicated a higher dispersion with lower *r* occurring in Castro (*r* = 0.41 for soybean) and Socavão (*r* = 0.56 for maize). When analyzing the soil physical-water attributes (Tab. 2) it was not possible to identify the reason for this higher dispersion. Castro and Socavão have clay soil that limits the aeration porosity (β) to 1% and K_{sat} = 2 mm/d. The attribute values could have interfered in the yield estimation results in AquaCrop; however, Itabera showed the same values of β and K_{sat} and did not exhibit a reduction of *r*.

The *r* values of the association *Yr* vs. *Ys* were lower in the alternative scenario. However, the drop in performance was mainly due to the *d* index values (Tab. 3). The use of estimated (alternative) soil physical-water attributes, based only on the soil textural class, somehow altered the simulated yield values (*Ys*) and data dispersion distanced from 1:1 line (Fig. 1). Castro and Socavão again were the locations that had the highest reduction in *d* index values. Considering maize and soybean and maize crops, the *Yr* vs. *Ys* conjunction analyses for Campos Gerais also drew attention to a reduction in *d* index values.

The performance of soybean and maize crop yield estimates with AquaCrop using the input data from the alternative scenario varied between terrible and very good, with a predominance of terrible (Tab. 3). Only Ponta Grossa (excellent) and Itabera (very good) showed satisfactory performances for the soybean crop. With the exception for Ponta Grossa, all performances obtained in AquaCrop were lower in the alternative scenario simulations than those obtained in the standard (measured) scenarios.

TABLE 3. Performance of the AquaCrop model, according to standard and alternative simulation scenarios, for soybean and maize crops in Campos Gerais region.

Crop	Locality	Standard scenario				Alternative scenario			
		r	*d*	*c*	Performance	*r*	*d*	*c*	Performance
Soybean	Arapoti	0.94	0.87	0.82	Very good	0.84	0.69	0.58	Tolerable
Soybean	Castro	0.88	0.86	0.76	Very good	0.41	0.59	0.24	Terrible
Soybean	Itabera	0.91	0.84	0.77	Very good	0.94	0.89	0.83	Very good
Soybean	Ponta Grossa	0.99	0.99	0.98	Excellent	0.97	0.94	0.92	Excellent
Soybean	Campos Gerais[1]	0.93	0.89	0.83	Very good	0.85	0.80	0.68	Good
Maize	Castro	0.81	0.52	0.42	Bad	0.77	0.46	0.36	Terrible
Maize	Socavão	0.92	0.77	0.71	Good	0.56	0.44	0.25	Terrible
Maize	Ponta Grossa	0.98	0.97	0.95	Excellent	0.92	0.69	0.63	Terrible
Maize	Campos Gerais[2]	0.88	0.79	0.69	Good	0.76	0.49	0.37	Terrible
Soybean and maize	Campos Gerais[3]	1.00	0.84	0.84	Very good	0.99	0.66	0.65	Medium

[1]Experiments with the soybean crop in Arapoti, Castro, Itabera and Ponta Grossa; [2]Experiments with maize crops in Castro, Socavão and Ponta Grossa; [3]Experiments with soybean and maize crops in Arapoti, Castro, Itabera, Ponta Grossa and Socavão.

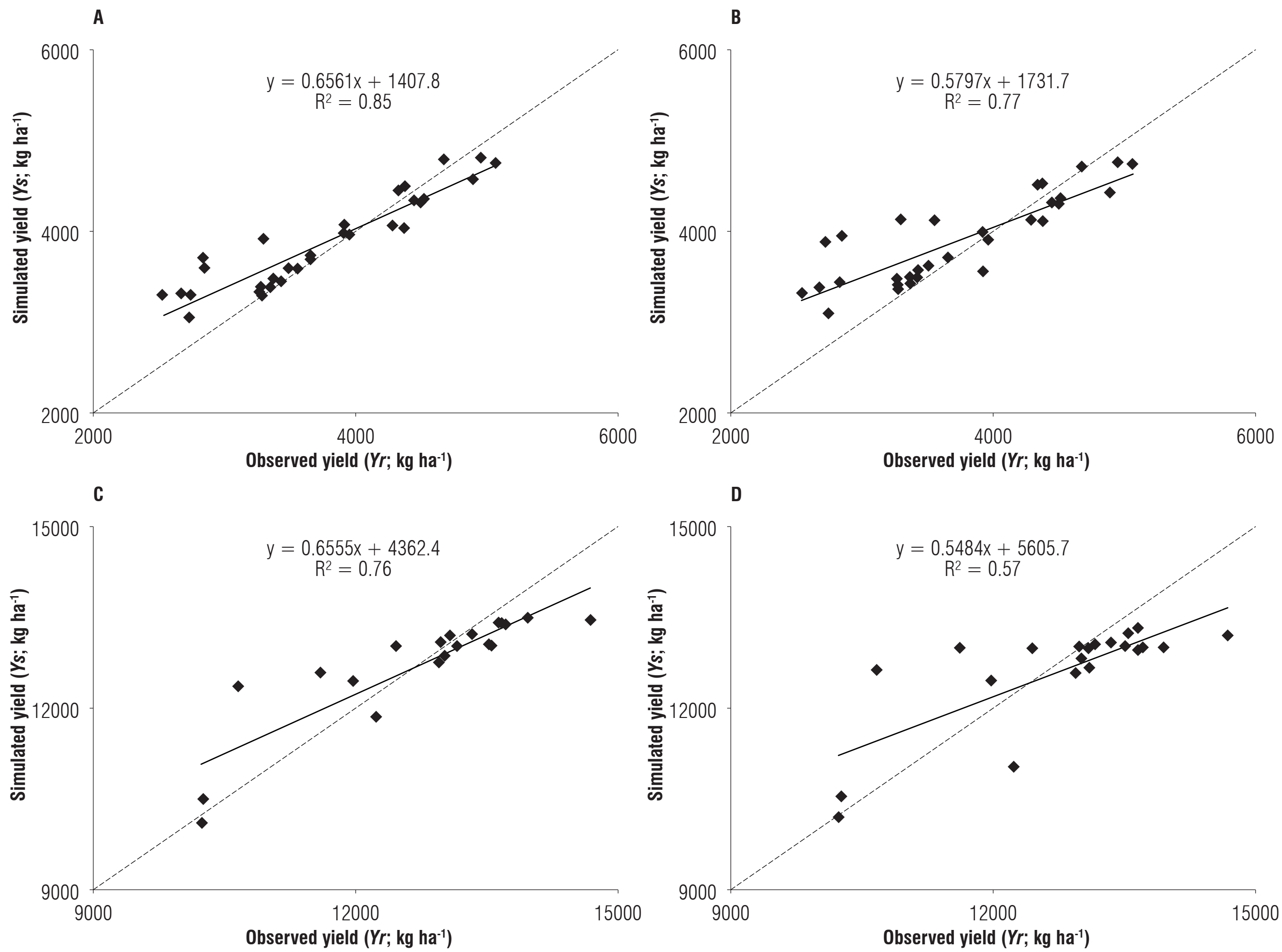

FIGURE 1. Linear regression and corresponding determination coefficient between the real crop and estimated mean productivity with AquaCrop in the Campos Gerais region, in the following simulation scenarios with soil physical-water attributes: A) measured (standard) simulation for soybean; B) estimated (alternative) simulation for soybean; C) measured (standard) simulation for maize; and D) estimated (alternative) simulation for maize.

The alternative scenario in AquaCrop should be used with much restriction. Using soil texture to predict the values of soil physical-water attributes proved to be an easier way to estimate the productivity of soybean and maize crops; however, it resulted in low performance for estimating soybean and maize yields in Campos Gerais. The positive results for the soybean crop in Ponta Grossa (excellent) and in Itabera (very good) were insufficient, since they did not allow the identification of cause and effect relationships with the measured physical-water attribute values.

Considering the results obtained in this study, we believe that AquaCrop has great potential to be used for soybean and maize crops in the region of Campos Gerais, with all soil physical-water attributes measured (standard) and, the ability to achieve reliable results for scientific studies in the Brazilian scenario.

Conclusions

The simulation scenario in AquaCrop using measured soil physical-water attributes (standard) provided better results (very good to excellent), indicating the need of inserting the real soil physical-water attribute values for more accurate productivity simulation results.

The simulation scenario with estimated soil physical-water attributes (alternative) in AquaCrop should be used restrictively. Using only soil texture indicated an easier method for estimating the productivity of soybean and maize crops, but with low performance for estimating soybean and maize yields in Campos Gerais.

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Impact of dry sludges and sludge biochar on height and dry matter of *Solanum lycopersicum* L.

Impacto de lodos secos y biocarbón de lodos sobre la altura y materia seca de *Solanum lycopersicum* L.

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ABSTRACT

The generation of sludge as anthropic waste is a fundamental pollution problem. However, its conversion to biochar can be an alternative to conventional fertilization for its management and use in agriculture. In this research, we evaluated the effect of the application of different doses of dry sludge (DS) and biochar of pyrolyzed sludge (PS) on the height and dry matter of a tomato (*Solanum lycopersicum* L.) crop and the nutrient content in the substrate. The biochar was made by rapid pyrolysis, and the substrate and the dry matter of plants were analyzed by different physical and chemical methods. An evaluation of 11 treatments was carried out in allometric measurements of plants and foliar dry matter, in three replicates with two materials (DS and PS) added to the substrate at different levels. The plant height and dry weight were evaluated using an incomplete factorial design in a completely randomized arrangement by performing statistical analysis of multivariate variance. An increase in plant height and dry weight was observed when the doses of DS and PS were increased; however, there were no statistical differences between the two materials. The amount of carbon, organic matter, and Ca concentrations in the dry leaf weight were increased with the addition of DS and PS. Likewise, the use of these materials as conditioners or amendments to agricultural soil at doses of 10-15 t ha^{-1} may be viable and can contribute to reducing environmental externalities through the use of these anthropic waste materials.

Key words: substrate, tomato, pyrolysis, soil fertility.

RESUMEN

La generación de lodos como residuos antrópicos es un problema fundamental de contaminación. Sin embargo, su conversión a biocarbón puede ser una alternativa a la fertilización convencional para su manejo y uso en la agricultura. En este trabajo se evaluó el efecto de la aplicación de diferentes dosis de lodos secos (LS) y biocarbón de lodos pirolizados (LP) sobre la altura y materia seca de un cultivo de tomate (*Solanum lycopersicum* L.) y sobre el contenido de nutrientes en el sustrato. El biocarbón se elaboró por pirolisis rápida, y el sustrato y la materia seca de plantas se analizaron mediante diferentes métodos físicos y químicos. Se evaluaron 11 tratamientos en medidas alométricas de plantas, sustratos y materia seca foliar, en tres réplicas con dos materiales (LS y LP) adicionados en diferentes niveles al sustrato. La altura y materia seca de la planta se evaluaron bajo un diseño factorial incompleto en arreglo completamente al azar, realizando un análisis estadístico de varianza multivariante. Se observó un incremento en la altura y materia seca en las plantas cuando aumentaron las dosis de LS y LP; sin embargo, no existieron diferencias estadísticas entre los dos materiales evaluados. La cantidad de carbono, la materia orgánica y las concentraciones de Ca en el sustrato aumentaron con la adición de LS y LP. Así mismo, el uso de estos materiales como acondicionadores o enmiendas del suelo agrícola puede ser viable en dosis de 10-15 t ha^{-1} aportando en la disminución de externalidades ambientales mediante el uso de estos materiales de desecho antrópico.

Palabras clave: sustrato, tomate, pirólisis, fertilidad del suelo.

Introduction

The accelerated process in urbanization and industry has generated an increase in the production of waste from industrial, domestic, and agricultural activities. Simultaneously to this development, protection policies have been established for the different ecosystems. However, the associated problems persist, particularly in water ecosystems. The construction of alternatives that mitigate pollution, such as aqueducts, sewers, and industrial and municipal treatment plants (wastewater treatment plants (WWTP)) has been encouraged. Although these plants manage to control water pollution problems to a large extent, some by-products, such as sludge, are generated.

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These by-products are produced from the accumulation of solids found in the effluent (primary sludge) or from the suspension of solids (activated sludge) that results from dissolved solids in wastewater (Morales, 2005).

For the first decade of this century, the average sludge production worldwide was estimated at approximately 30 kg per person/year (Hospido *et al*., 2010). In Colombia, approximately 247 t of these sludges are generated (94 t dry weight), of which 97% are produced in three treatment plants: Cañaveralejo, San Fernando, and Salitre (PTAR, 2009). Specifically, Salitre treats wastewater from the Salitre river and the Torca and Conejera wetlands, generating about 150 t/d of sludge that could be used in the reforestation of degraded soils (EAAB, 2009). These sludges are rich in macronutrients such as N and P, micronutrients such as Zn and Mo, and organic matter. However, they also contain some pathogens and heavy metals (Hartman *et al*., 2003); in consequence, the disposition of these materials must be done carefully, both for the volumes that are generated and for the environmental risks they represent.

An alternative for improving fertility indicators and the quality of degraded soils is the inclusion of exogenous organic materials. Through the application of organic amendments, the nutrient status in the soil is improved, and these amendments can serve as a source of macro and microelements to improve the functions and physical-chemical processes of the soil (Lal, 2016). So, the use of sludge in the soil or as an agricultural substrate can serve as an option for increasing yields of different crops. However, it should be noted that many of these materials must be stabilized before application because of potentially toxic elements and pathogens. Through the composting process, it is possible to obtain dry sludge (DS) for uniform and biologically stable products that can act as a source of nutrients for plants (Sullivan *et al*., 2002).

One of the strategies reported in the literature for improving soils and reducing or neutralizing these adverse elements is the incineration of these materials through pyrolysis, which consists of the burning of organic materials in the absence or low levels of oxygen. One of the products obtained under this process is biochar, also known as pyrolyses or biocarbon. These are solid products of fine and porous grain obtained from the thermal conversion of biomass in a limited oxygen environment. Biochar is similar to coal, which is produced by natural burning, and that reaches great stability in the soil compared to the nitric Nitrogen from which it comes (Lehmann and Joseph, 2009; IBI, 2012). According to Amonette (2009), biochar has a high content of organic carbon, with high resistance to decomposition and high residence time in soils. These characteristics prevent the transformation of biochar and its early release of CO_2 into the atmosphere. Biochar creates a recalcitrant carbon deposit (carbon-negative) that functions as a carbon sequestration network (FAO, 2004). Likewise, this material, acting as an amendment or soil conditioner, can improve the soil's physical condition towards better nutrients adsorption, increased cationic retention, and reduced N_2O emissions (Lal, 2004; Lehmann *et al*., 2005). In addition, biochar can promote the sorption of organic compounds such as pesticides, herbicides, and enzymes, retain pollutants such as cadmium and chlorinated compounds (Escalante-Rebolledo *et al*., 2016), toxic organic compounds such as dioxins, furans, esters, polycyclic aromatic hydrocarbons and other organic pollutants (Agrafioti *et al*., 2013).

Both the direct application of dry sludge (DS) in agricultural systems and the conversion of DS to pyrolyzed sludge (PS) for later incorporation can represent an economically and environmentally viable alternative for the disposal of these materials reducing their polluting potential. Kistler *et al.* (1987), Paterson *et al.* (2008), and Hossain *et al.* (2010) showed that the addition of biochar from sludge to tomato crops increased the agrological properties of the soil, which increased crop yield and production. Likewise, these authors claim that the heavy metals concentrated in the solid product become more chemically and biologically inert, decreasing transport or bioaccumulation in the plant and in soils when biochar is used as an amendment.

Nzanza *et al.* (2012) report that no benefits were found for fruit yield and nutrient absorption with the addition of PS and tree mycorrhizae to the soil of a tomato crop. Because of contrasting results like these found in the literature, more research is needed for plant species of great agricultural importance regarding the effect of biochar application to the soil/substrate, particularly when DS is used.

The objective of this research was to evaluate the variables (height (cm) and dry matter (g)) of tomato plants in pots, by analyzing the characterization of DS and PS and dry leaf weight, after adding DS and PS to the substrates at different doses. The results showed that the plant height and dry weight increased when these doses of sludge increased. However, no significant differences were found when the treatments between the two types of materials were compared.

Materials and methods

Analysis of soil samples

The soil used in the experiment was a degraded soil from the municipality of La Vega (Colombia). The soil samples were used as a substrate and were placed in each of the experimental units: pots of 15.5 cm for the upper diameter, 11.5 cm for the lower diameter, with heights of 11.0 cm and two-liter capacity. Previously, the samples were dried at room temperature for 10 d and then placed in a REDLINE RF 115 mechanical convection drying oven (General Lab & Cleanroom Supply, California, USA) at 70°C for 72 h. The samples were then ground and passed through a 2 mm sieve and sent to the Soil and water laboratory at Universidad Jorge Tadeo Lozano, Bogota, Colombia, for analysis.

The samples were evaluated for the following characteristics: texture, organic matter, pH, cation exchange capacity (CEC), macro and micronutrients, among which heavy metals (totals) were also analyzed. We determined the percentage of clays, silt, and sand using the Bouyucos method. It is important to indicate that the characterization of the chemical elements of the soil used in the research is represented by the T_0.

We quantified organic matter according to the methodology of Walkley and Black (Kumada, 1987) and SSL (Soil Survey Laboratory) (1995). N was determined using Kjedahl digestion. We measured the pH in water in a 1:1 ratio (You *et al*., 1999) in 20 g of soil/20 g of H_2O. The CEC was determined based on the sum of the bases, extracted with NH_4O Ac 1 N at pH 7 (BT). We determined the exchangeable acidity (EA) with KCl 1 N (Motta, 1990) and phosphorus availability with lactate (Egner, 1941). The B was analyzed with azomectin H (Wolf, 1974), the S was determined with turbidimetry (Combatt *et al*., 2014), and the Fe, Mn, Cu and Zn were analyzed using the diethylene triamine-pentacetic acid (DTPA) method.

Foliar analysis in the different treatments

Destructive sampling was carried out using six-month-old plants, which were dried at 70°C for 72 h and then incinerated. The incinerated material was analyzed to determine different macro and microelement values, using the following techniques: N by Kjeldahl method, P and B by colorimetry, K, Ca, Mg, Na, Fe, Mn, Cu and Zn by atomic absorption, and S by turbidimetry (Combatt *et al*., 2014).

Preparation of dry sludge (DS) and biochar (PS)

The DS was obtained from the saltpeter PTAR located in Bogota D.C. (Colombia). DS was then dried outdoors for 30 d at room temperature (30ºC) in the greenhouses of the Faculty of Agricultural Sciences of the Universidad Nacional de Colombia. Approximately 5 kg of DS were used for the production of the PS biochar. A proportion of this material was pyrolyzed in the rotary kiln of the Department of Mechatronics of the Faculty of Engineering of the Universidad Nacional de Colombia, Bogota campus. The PS was obtained under rapid pyrolysis with a temperature of 850°C for 40 min and a heating rate of 80-100°K/min. The PSs were prepared according to the methodology proposed by Jeffery *et al*. (2011).

The obtained PS (1.5 kg) was packed in bags and stored at room temperature at 15°C as average temperature and then was taken to the Soil biology laboratory at the Faculty of Agricultural Sciences of the Universidad Nacional de Colombia, for chemical and physical characterization. Before applying the different treatments, a ball hammer and two sieves of number 10 and 20 with openings of 2.0 and 0.85 mm, respectively, were used to reduce the particle size of the two materials. For the PS, an approximate grain size of 2 mm was used. These assessments were performed considering the International Biochar Initiative and the set of characteristics that define the quality of biochar for use in agriculture. Those parameters include particle size distribution of these materials, pH, specific area, porosity, C, and nutrient content, as well as pollutant content (heavy metals and polycyclic aromatic hydrocarbons) (IBI, 2012). The chemical analysis of both PS and DS was determined under the same parameters of the soil samples. The chemical characterization of the two materials is shown in Table 1.

We performed a laboratory characterization of the two sludges of the treatments with the following variables: bulk density, cation exchange capacity (CEC), organic carbon, organic matter, ash, carbon/nitrogen ratio, pH (acidity reaction), total nitrogen, ammoniacal nitrogen, nitric nitrogen, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), sodium (Na), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) and boron (B) (Tab. 1).

Experimental design

We carried out the test in the greenhouses of the Faculty of Agricultural Sciences of the Universidad Nacional de Colombia between February and July 2015, under average daytime temperatures of 24°C and average nighttime temperatures of 18°C. The experiment was carried out in the pots. The soil mixed with the biocarbon and DS (substrate) was placed in the pots. We planted five seeds of the tomato variety Miland at a depth of 3 cm. We previously treated the seeds with hot water to counteract possible pathogens. Once

TABLE 1. Evaluated properties of dry and pyrolyzed sludge.

Variables		Dry sludge	Pyrolyzed sludge	Relative change (%)
	Units	Dry base	Dry base	
Bulk density	g cm^{-3}	0.88	0.79	-10.23(+)
Cation exchange capacity (CEC)	cmol kg^{-1}	28.17	64.45	128.79
Organic carbon	%	20.4	19.31	-5.34
Organic matter	%	44.26	41.9	-5.33
Ashes	%	55.74	58.1	4.23
Carbon/Nitrogen ratio	w/w	8.5	8.05	-5.29
pH (acidity reaction)		5.8	5.9	1.72
Total Nitrogen	%	2.4	2.4	0.00
Ammoniacal Nitrogen	%	0.024	0.084	250.00
Nitric Nitrogen	%	0.075	0.123	64.00
Phosphorus (P)	%	0.93	1.1	18.28
Potassium (K)	%	0.21	0.35	66.67
Calcium (Ca)	%	1.900	2.8	-99.85
Magnesium (Mg)	%	0.24	0.27	12.50
Sulfur (S)	%	0.62	2.1	238.71
Sodium (Na)	%	0.056	0.062	10.71
Iron (Fe)	ppm	4183	17372	315.30
Manganese (Mn)	ppm	179	286	59.78
Copper (Cu)	ppm	29.6	37.2	25.68
Zinc (Zn)	ppm	360	777	115.83
Boron (B)	ppm	14	13.5	-3.57

(+) Values given by 100*(end _ value-start _ value)/start _ value). The sign (-) means a decrease.

the seeds germinated after 10 d, we transplanted those with the most vigor into the center of each pot, which were randomly arranged on the greenhouse tables. We irrigated the plants by sprinkling with a flow rate of 2 L/h, three times a week, based on the needs of the crop. We subsequently sampled the plants destructively six months after sowing when they had grown eight branches; the biomass on the soil surface was collected by cutting at the base of the stem. No chemical and organic synthesis products were applied during the test. In the absence of reference levels for the doses established for both DS and PS, we based the amounts that we applied in this research on studies by Chan *et al*. (2007) and Van Zwieten *et al*. (2009), who determined benefits of biochars produced from paper sludge and poultry feces with an average dose of 10-15 t ha^{-1}.

The experimental design was associated with an incomplete factorial in a completely randomized arrangement, since the doses were nested for each material, with DS and PS materials. The nested doses were 0.0, 2.5, 5.0, 7.5, 10.0, and 15.0 t ha^{-1} consisting of 12 randomized treatments on the experimental units (pot with a tomato plant), using three repetitions per treatment. The treatments were labeled as follows: (T_0) control (only soil for both materials); (DS_1) 2.5 t ha^{-1}; (DS_2) 5 t ha^{-1}; (DS_3) 7.5 t ha^{-1}; (DS_4) 10 t ha^{-1}; (DS_5) 15 t ha^{-1}; (PS_1) 2.5 t ha^{-1}; (PS_2) 5 t ha^{-1}; (PS_3) 7.5 t ha^{-1}; (PS_4) 10 t ha^{-1}; and (PS_5) 15 t ha^{-1}. The response variables evaluated simultaneously were dry matter (g), and height (cm) of the plants measured six months after planting. Finally, with the same treatment structure, we evaluated the macro and microelements (N, P, K, Ca, Mg, Cl, Fe, Mn, Cu, and Zn) in leaves 6 months after sowing.

Statistical analysis

The relative change was analyzed taking into account the final values of the variables of PS compared to the initial values of DS, represented by a radial diagram. An exploratory analysis of the data was carried out to describe the variables involved in the study statistically. The analysis included calculations of the mean, standard deviation, and coefficient of variation of the two response variables (height and plant weight), discriminated by treatment. A conditional graph was constructed to show the relationships of all the variables involved.

A bivariate analysis of variance was used for the evaluation of the effects of the added material and its nested doses on the height and weight of the plant, for the incomplete factorial experimental design in a completely randomized arrangement. We validated the assumptions of bivariate normality and equality of matrices of variances and covariances by treatments using the Roystone test and the Box M test, respectively. The statistical package R was used for all analyses. For the analysis of the macro and micro nutritional elements of the leaf, the relative percentage change of the treatment was measured with a higher average compared to the control.

Results and discussion

Under the conditions of this study, the apparent density showed a 10.23% reduction in the conversion of DS to biochar or PS, which could be caused by the micropores that are generated when the temperature in the pyrolysis process increases (Verheijen *et al*., 2009). The pH level remained stable in the conversion from DS to PS, despite rapid pyrolization (high temperature and short residence times), which is thought to increase alkalinity due to the loss of carboxylic acid groups by temperature (Harris and Tsang, 1997). This situation could be due to the fact that there was no significant change in the amounts of organic carbon in this conversion, i.e., there would not be a noticeable loss of these functional groups. In addition, higher levels in the contents of P and K were found in treatments with PS as well as a greater positive percentage difference for these treatments (Tab. 1).

There could be a significant contribution of P from protein materials and polyphosphates (which were not lost in the pyrolysis process) from detergents that might be constituents of the evaluated DS (Korboulewsky *et al*., 2002; Esteller *et al*., 2009). However, the levels of total N, Mg, and Na were similar for the two materials (with increases not greater than 13% in the percentage change in the conversion from DS to PS). The permanence of the levels of these elements after pyrolysis in the PSs could be due to the fact that the temperatures did not reach the point of volatilization. In contrast, Gaskin *et al*. (2008) found the volatilization of N in sludge when the temperature was increased from 300 to 700°C.

In general, the percentages of micronutrients were higher in the PS, while organic matter showed higher levels in the DS. In contrast, authors such as Okuno *et al*. (2005) consider that Ca begins to decrease in the biochar matrix in pyrolysis with temperatures above 600°C. The higher values of Ca in the PSs in this study may be associated with higher levels in the height and dry matter of the plant. With the addition of PS, there is an increase in water retention capacity and a greater uptake of nutrients due to an increase of the CEC (Fig. 1).

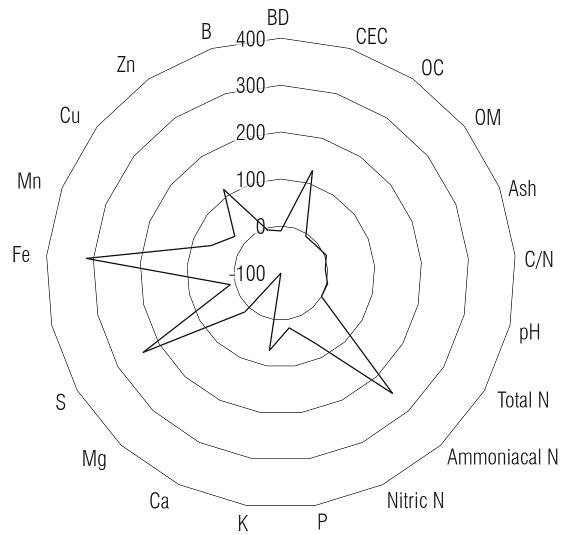

FIGURE 1. Relative changes in physical and chemical properties of biochar from sludge to dry sludge. BD - bulk density, CEC - cation exchange capacity, OC - organic carbon, OM - organic matter, Ash - ashes, C/N - carbon/nitrogen ratio, pH - potential reduction, Total N - total nitrogen, Ammoniacal N - ammoniacal nitrogen, Nitric N - nitric nitrogen, P - phosphorus, K - potassium, Ca - calcium, Mg - magnesium, S - sulfur, Fe - iron, Mn - manganese, Cu - copper, Zn - zinc, B - boron.

Fe, Cu, and Zn increased with the conversion from DS to PS in percentages of 315.35%, 25.6%, and 115.8%, respectively (Tab. 1). Some studies corroborate the results of our research, in which authors such as Lehmann *et al*. (2006) establish that, although nutrients such as N and P in the sludge may decrease due to volatilization during pyrolysis, heavy metals, such as Cu and Zn, can increase their concentration. In studies by Hossain *et al*. (2010), different heavy metals such as Zn, Pb, and Ni increased their contents in sludge pyrolysis at temperatures greater than 500°C. This implies that the increase of these elements with PS in the substrate can affect the chemical properties of the sludge, so that there is a competition of nutrients with a high amount of Fe.

Regarding the CEC, a relative change (increase) of 128.8% was found for the PS with reference to DS. This result is contrary to the expected decrease in the values of this variable, as reported by Lin *et al*. (2012) and Rajkovich *et al*. (2012), and could happen due to the removal of functional groups that increase ion retention with their load.

The higher level of CEC in the PSs in this research could be due to a high number of these groups and also to the conservation of heteroatoms within them (Cantrell and Martin, 2012).

With increasing doses of both PS and DS in each treatment, there was a tendency to increase the dry weight and height of the plants (Fig. 2). The bivariate analysis of variance showed no significant effect attributable to the PS and DS materials on the height or the dry matter (P=0.788) of the plants but rather between doses of each material (P<0.001) (Tabs. 2 and 3).

TABLE 2. In-treatment dose test statistics.

Statistics	Value	F-Value	Num DF	Den DF	Pr>F
Wilk'Lamda	0.979	0.24	2	23	0.788

TABLE 3. Inter-material dose test statistics.

Statistics	Value	F-Value	Num DF	Den DF	Pr>F
Wilk'Lamda	0.003	9.21	20	46	<.0001

In both DS and PS, the control was below average compared to the other treatments. Taking as reference the treatments with higher plant heights (DS_5 and PS_5), the control plants were found to be 27.63% and 30.92% shorter than PS_5 and DS_5 plants, respectively (Tab. 4). As in the present study, Hossain *et al*. (2001) reported significant effects on the height of cherry tomato plants with applications of biochar made with sludge from treatment plants in the Sydney area (Australia). In that study, applications of biochar were evaluated in the greenhouse, both alone and in mixtures with inorganic fertilizers, using doses of 10 t ha^{-1}. Likewise, Silva *et al*. (2017) found similar results in seedlings of *Eucalyptus grandis* W. evaluating DS and PS.

In accordance with the present research, Cabrera *et al*. (2007) also found a positive effect on the height of tomato plants with the addition of DS to the soil from a WWTP, compared to soils fertilized with urea and soils without fertilization in the greenhouse. Such results can be attributed to improvements in some chemical properties of soils

TABLE 4. Descriptive statistics for response variables height (cm) and dry weight (g) of plants.

		Dry sludge						Pyrolyzed sludge					
		Dose											
		0	2.5	5	7.5	10	15	0	2.5	5	7.5	10	15
Variables													
Height	Ẋ*	16.100	20.900	28.733	31.066	39.200	58.266	16.100	20.766	29.566	35.800	42.466	52.066
	S**	2.080	4.300	3.611	3.827	6.148	3.295	2.080	3.234	2.274	6.791	6.143	5.772
	CV***	12.924	20.574	12.569	12.321	15.686	5.656	12.924	15.576	7.692	18.969	14.466	11.087
Dry weight	Ẋ*	6.217	9.260	16.715	21.071	30.678	49.717	6.217	7.913	15.922	25.221	30.478	46.175
	S**	2.883	2.065	2.692	2.316	3.397	5.767	2.883	1.012	1.060	7.519	5.719	7.839
	CV***	46.37	22.30	16.109	10.991	11.074	11.600	46.378	12.792	6.662	29.812	18.766	16.977

*Ẋ: average, **S: standard deviation, ***CV: coefficient of variation.

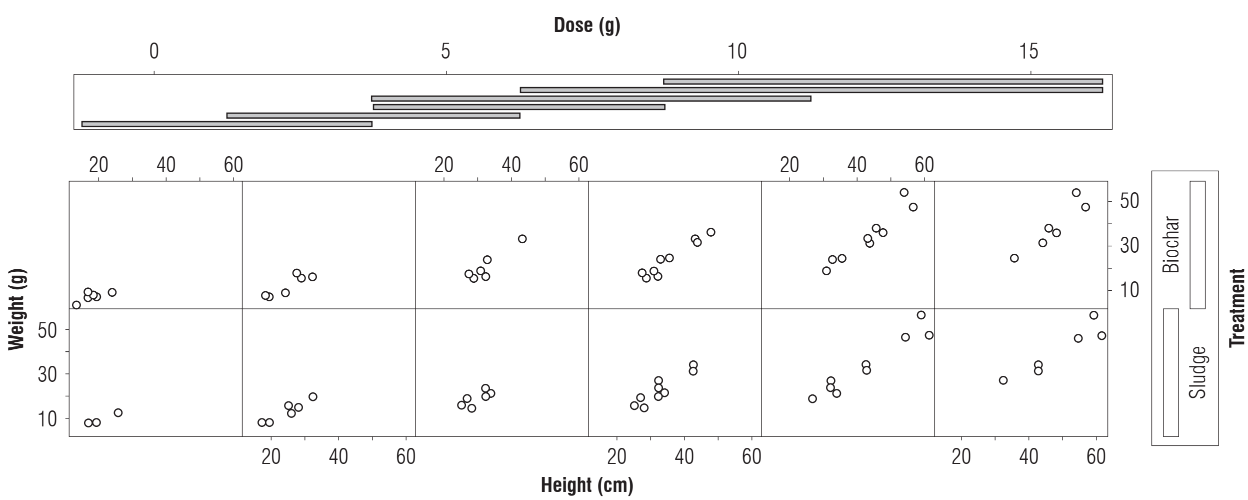

FIGURE 2: Dry weight distribution as a function of plant height per nested dose in each experimental treatment.

produced by an application of these materials, due to the contribution of immediate and gradual inorganic forms of organic molecules.

In this research, dry matter and plant height increased with the addition of higher doses of both LS and LP. The control plants (with an average of 6.2 g of dry matter) were found to have 12.5% and 13.2% less dry matter than the DS_5 and PS_5 plants, respectively (Tab. 4). This could be due to the fact that these types of sludge contain high amounts of nutrients and organic matter that could have a positive effect and contribute to a greater gain of dry matter given higher doses of these materials (Melo *et al*., 2007). In this regard, Moral *et al*. (2005) also state that higher production of plants may be due to a greater amount of organic matter present in the biochar. These organic elements are composed of proteins, simple sugars, organic acids, amino acids, and peptides that are easily degraded by microorganisms, increasing those populations that contribute to a greater dynamic of transformation from non-soluble elements to elements available to plants. The addition of PS could cause greater production and growth in the plant by the reduction in the leaching of nutrients and the increase in the union of organic matter and nutrients through the cation exchange capacity (Amonette, 2009; Granatstein *et al*., 2009; Atkinson *et al*., 2010; Lehmann *et al*., 2011).

In other studies, in tomato, Hossain *et al*. (2010) found no significant differences with the addition of PS at doses of 10 t ha^{-1} and inorganic fertilizers. However, the researchers reported higher plant growth with amendments added to sludge biochar mixtures and chemical fertilizers. In the present research, with the comparative analysis of the application of the two materials, the profiles and effects were similar to the response in dry weight (g) and height (cm) of the evaluated tomato plants (Fig. 2).

The increase in dry matter and height in the plants with the addition of PS in this study could be related to the contribution of these materials to generate better conditions and dynamics of the different properties of the substrates and, therefore, improve plant growth. In this sense, studies such as those by Glaser *et al*. (2002) describe improvements in the different properties of the soil with the addition of biochar or pyrolysis and the increase of dry matter in plants. The researchers argue that *terra preta* soils have approximately 18% higher water retention values, compared to adjacent soils. Thus, the addition of any organic amendment produces an increase in water retention, which will be represented as a positive increase in the different properties of the soil, and consequently, in improved nutrient uptake by plants. On the other hand, Hue and Ranjith (1994), McBride *et al*. (1997), and Shuman (1998) state that dry sludge can increase nutrient availability due to the low molecular weight of its aliphatic components and the increase of CEC in the soil.

Comparing the application of the DS and PS carried out according to the norms of this study, the use of biochar from PS could be a better option because of the stabilization of this material that occurs in its conversion to biochar through the pyrolization process. For example, the use of PS can reduce the availability of heavy metals included in the soil, which pass into the trophic chain through the root absorption of plants (Mtshali *et al*., 2014). Although present at low values, the increase of metals such as Fe and Cu in the conversion of dry sludge to biochar in percentages of 3.1 and 0.2%, respectively, was evident in this research (Tab. 1).

Foliar analysis

According to the different percentages of nutrients from the foliar samples found in the various treatments compared to the control, generally, higher levels of micronutrients were found in the PS treatments. However, higher values were found in the macronutrient levels of the DS (except for K) (Tab. 5).

The absorption of N by tomato plants was present at higher levels within the PS treatments for treatments with higher doses of this material. For example, treatment LP_5, with the highest dose of PS in the substrate, showed the highest concentration of N in plant tissues in the pyrolyzed treatments. This could be due to the concentration of N in the ashes of the biochar adsorbed by the plant from the substrate. In this sense, authors such as Steiner *et al*. (2008), Zimmerman (2011), Lehmann *et al*. (2010) and Rajkovich *et al*. (2012) suggest that the uptake of nutrients such as N increases in biochar that comes from high temperatures because they contain a lower labile fraction. This means that there is less immobilization by microorganisms and, therefore, there is a greater amount of N available to be absorbed by the plant. Among the PS treatments, the PS_5 showed the highest level of Ca in the leaf tissue of the plants (Tab. 5), which could be correlated with a higher level of biochar for this treatment, due to the initial high concentration of Ca in the added PS material (Tab. 1).

However, the function of K is associated with root growth, tolerance to water stress, cellulose formation, enzymatic activity, and photosynthesis (Thomson, 2008). Although the treatments with higher doses of the two materials in this study had the highest yields (dry matter) and heights (DS_5 and PS_5), the same was not found regarding K uptake

TABLE 5. Foliar analysis of nutritional elements in different treatments with applications of dry sludge and sludge biochar in *Solanum lycopersicum* L.

Treatments	Macro elements (g*100 g^{-1})						Micro elements (g*100 g^{-1})					
	N	P	K	Ca	Mg	S	Na	Fe	Mn	Cu	Zn	B
T_0	0.16	6.51	0.72	4.86	1.20	-	-	42.8	1.93	0.31	0.36	0.51
DS_1	2.25	0.19	3.50	1.90	0.63	0.52	0.129	3793.0	193.0	9.7	67.9	17.7
DS_2	1.49	0.20	2.80	1.40	0.34	0.41	0.059	14036.0	150.0	5.2	44.7	15.3
DS_3	1.11	0.20	2.40	1.50	0.25	0.39	0.055	3693.0	121.0	8.1	39.4	16.1
DS_4	2.26	0.24	2.70	1.60	0.42	0.41	0.073	7608.0	184.0	8.9	71.6	19.1
DS_5	1.68	0.22	3.10	1.90	0.45	0.48	0.0071	4448.0	164.0	8.8.	58.4	20.2
PS_1	1.46	0.24	3.30	1.70	0.55	0.39	0.150	4786.0	178.0	7.4	62.1	16.0
PS_2	1.86	0.24	3.00	1.80	0.52	0.31	0.081	6135.0	213.0	7.6	62.3	17.4
PS_3	1.87	0.22	3.80	1.40	0.53	0.35	0.094	3769.0	170.0	12.8	86.8	28.6
PS_4	1.33	0.24	3.40	1.40	0.32	0.38	0.056	2164.0	93.3	8.1	65.5	21.2
PS_5	2.03	0.24	2.80	1.90	0.45	0.38	0.077	3675.0	170.0	12.4	93.5	33.1
Minimum optimal level	2.80	0.30	3.50	1.60	0.36	0.64	0.05	84.0	55.0	6.0	40.0	54.0
Maximum optimal level	4.20	0.45	5.00	3.20	0.49	1.94	0.2	112.0	65.0	15.0	60.0	76.0

TABLE 6. Relative changes for different elements in foliar tissue samples for treatments and evaluated doses.

	N	P	K	Ca	Mg	Cl	Fe	Mn	Cu	Zn
DS_1	1306.3	-97.1	386.1	-60.9	-47.5	8762.1	9900	3029.0	18761.1	3370.5
DS_2	831.3	-96.9	288.9	-71.2	-71.7	32694.4	7672.0	1577.4	12316.7	2900.0
DS_3	593.8	-96.9	233.3	-69.1	-79.2	8528.5	6169.4	2512.9	10844.4	3056.9
DS_4	1312.5	-96.3	275	-67.1	-65	17675.7	9433.7	2771.0	19788.9	3645.1
DS_5	950	-96.6	330.6	-60.9	-178.6	10292.5	8397.4	2738.7	16122.2	3860.8
PS_1	812.5	-96.3	358.3	-65.0	-54.2	11082.2	9122.8	2287.1	17150	3037.3
PS_2	1062.5	-96.3	316.7	-63.0	-56.7	14234.1	10936.3	2351.6	17205.6	3311.8
PS_3	1068.8	-96.6	427.8	-71.2	1.9	8706.1	8708.3	4029.0	24011.1	5507.8
PS_4	731.3	-96.3	372.2	-71.2	-73.3	4956.1	4734.2	2512.9	18094.4	4056.9
PS_5	1168.8	-96.3	288.9	-60.9	-62.5	8486.4	8708.3	3900	25872.2	6390.2

in plant tissues in these same treatments. In fact, the lowest amounts of K absorbed were found in treatments DS_5 and PS_5 (Tab. 6). This reaction can be possibly due to the long-term action of biochar, as described by Major *et al.* (2010) in studies conducted in corn crops between 2003 and 2006, where there were increases in elements such as K, Ca and Mg until the third year, with the addition of biochar made from wood residues. However, it is expected that the amounts of Ca and Mg contributed by biochar in the soil will increase in the long term, which may have an impact on leaf tissues through the uptake of the elements by the plant, as established by authors such as Major *et al.* (2010).

It is known that P is usually conserved during the volatilization of associated organic molecules and is present as ashes within the biochar, which when solubilized, becomes available to plants (Mašek and Brownsort, 2011; Escalante-Rebolledo *et al.*, 2016). However, McLaughlin *et al.* (2009) demonstrated that P in the sludge acts as a slow-release fertilizer in soils deficient in this element (Hossain *et al.*, 2010). In addition, the concentration of P in the sludge can be reduced by the conditions of pyrolysis through the evolution of volatile compounds of greater molecular weight, while the concentration of metals present in the biochar is expected to increase (Hossain *et al.*, 2010).

This could explain the results of this study regarding the lower content of P in both DS and PS compared to the control and the higher Fe content found in these two materials. In this research, although the amounts of P are significantly lower in the two sludge materials compared to the control (Tab. 5), it could be inferred that in T_0 the element is neutralized (due to high amounts of bases like K). For this reason, it is not available for the plant and could not provide a contribution to its production (interpreted in terms of height and dry matter).

Na ions in large quantities can affect the seeds of *S. lycopersicum* by decreasing the water potential in the substrate (El-Habbasha *et al*., 1996; Cuartero and Fernández-Muñoz, 1998). These concentrations also affect the growth of the roots of the plant by altering the absorption of water (an osmotic component) (Shannon and Grieve, 1999). The above could be reflected in this research given the results obtained regarding the high content of Na since higher levels of this element were found in the leaf samples of the treatments with lower height and dry matter (DS_1 and PS_1), and lower levels of Na in treatments with higher height (DS_5 and PS_5) (Tab. 4).

In the analysis of Mn, Zn, and B, there was no clear trend that could relate a higher dose of treatments to an increase in the content of these elements in plant tissues. Foliar analyses revealed significant amounts of Fe, particularly in DS in treatments DS_2 and DS_4. The PS treatments that were found with greater amounts of this element were the PS_1 and PS_2. In relation to this, Pérez-Sanz *et al*. (2002) investigated the efficiency in the growth of citrus crops with the application of sludge enriched with Fe without finding significant improvements in this growth parameter. These results are in line with what was obtained in our study, particularly with the application of treatments that include biochar, since it was found that treatments with high amounts of Fe such as PS_1 and PS_2 had the lowest plant height and dry matter values.

Conclusions

The results obtained indicated that the applications of DS and PS on sludges with tomato plants under greenhouse conditions generated an effect on the height and dry matter of the plants, which was proportional to the increase in the doses of these materials to the substrate. Although no significant differences were found in the application of the two materials, there were statistical differences found in the nested doses for each material, and greater response in the evaluated variables at higher doses of each material.

With the addition of PS to the substrate, an improvement in the availability of nutritional elements of the soil, such as K was observed. Additionally, an increase in the absorption of these elements by tomato plants (particularly in the PS_3) was observed, which positively influenced greater heights and values of dry matter obtained in the plants. Essential elements such as Ca increased in the substrate with the application of DS and PS. However, increased amounts of Fe were also found in the PS, which could have a negative impact on the chemical conditions of the soil and plants. When higher levels of nutrients (particularly nitrogen) are found in the plants under treatment with PS, a contribution of nutritional elements by this material was inferred. This is a very important aspect that must be investigated in depth due to its potential benefits in the nutrition and production of cash crops.

The application of DS and PS could be a viable proposal for agricultural systems as an alternative to the disposal of sewage sludge and as an amendment or soil conditioner. Although greater benefits were found in some variables measured in this study with the application of some DS, there could be a greater benefit in environmental terms with the use of PSs given that the heavy metal load is reduced when pyrolysis is applied to the soil. This benefit may mean a lower impact in terms of bioaccumulation of these metals in the plant.

However, for future studies, it is necessary to analyze the content of toxic organic compounds such as dioxins, furans, phthalic acid esters, polycyclic aromatic hydrocarbons, and other organic contaminants that may be present in DS and PSs. This information can be important to define criteria for the selection of one of these materials to be used as a soil improver.

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Carbon-nitrogen ratio in soils with fertilizer applications and nutrient absorption in banana (*Musa* spp.) cv. Williams

Relación carbono-nitrógeno en suelos con aplicación de fertilizantes y absorción de nutrientes en banano (*Musa* spp.) cv. Williams

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ABSTRACT

This research took place in Uraba, Antioquia, in the CENIBANANO-AUGURA experimental field, where a research program on nutrition and fertilization in bananas is carried out. This crop requires high amounts of nitrogen for production, so it is indispensable to evaluate the impact of these applications on the carbon-nitrogen ratio (C/N ratio) in soil. Published literature is scarce for this problem. This research evaluated the C/N ratio in areas with fertilizer applications and nutrient uptake, along with the interaction with production in a banana crop of the AAA group giant Cavendish subgroup, Williams clone, sixth generation in two production cycles. A randomized complete block design was used with five treatments that consisted of differential doses of nitrogen (161, 321.8, and 483 kg ha^{-1}), and an omission and absolute control distributed in four replicates. The treatments with nitrogen doses generated statistical differences for the interactions between the two study zones for the percentages of carbon and total soil nitrogen and C/N ratios; the highest values were found in the fertilization zone during the first production cycle (2.47% C, 0.33% N, and 7.7 C/N ratio). The treatment with 483 kg ha^{-1} of N obtained the greatest increases in the values for these variables that are attributed to the highest dose of nitrogen and the residual acidity of urea that was able to release non-free carbon from the soil. For this reason, the correlation analysis for the C/N ratio and production was significant for the study areas (absorption and fertilization), inferring that higher C/N ratio values tend to increase production.

Key words: crop nutrition, plant development, organic matter, edaphogenesis, soil moisture.

RESUMEN

La presente investigación se desarrolló en el Urabá antioqueño, en el campo experimental CENIBANANO-AUGURA, donde se lleva a cabo el programa de investigación en nutrición y fertilización en banano. Este cultivo requiere altas cantidades de nitrógeno en su proceso productivo, haciendo indispensable el evaluar el impacto de dichas aplicaciones en la relación carbono-nitrógeno (relación C/N) en el suelo. La literatura publicada es escasa para esta problemática. Esta investigación evaluó la relación C/N en las zonas de aplicación de fertilizantes y absorción de nutrientes, junto con la interacción con la producción en un cultivo de banano del grupo AAA, subgrupo Cavendish gigante, clon Williams de sexta generación en dos ciclos de producción. Se usó un diseño experimental en bloques completos al azar con cinco tratamientos que consistieron en dosis diferenciales de nitrógeno (161, 321,8 y 483 kg ha^{-1}), un testigo de omisión y uno absoluto distribuidos en cuatro repeticiones. Los tratamientos con dosis de nitrógeno generaron diferencias estadísticas para la interacción entre las dos zonas de estudio para los porcentajes de carbono y nitrógeno total del suelo y la relación C/N; los mayores valores se encontraron para la zona de fertilización durante el primer ciclo de producción (2.47% C, 0.33% N y relación C/N 7.7). El tratamiento con 483 kg ha^{-1} N obtuvo los mayores incrementos en los valores para estas variables, los cuales se atribuyen a la mayor dosis de nitrógeno y a la residualidad ácida de la urea que pudo liberar carbono no libre del suelo. Por esta razón, el análisis de correlación para la relación C/N y la producción fue significativo para las zonas de estudio (absorción y fertilización), infiriendo que a mayores valores de la relación C/N la producción tiende a incrementarse.

Palabras clave: nutrición de cultivos, desarrollo vegetal, materia orgánica, edafogénesis, humedad del suelo.

Introduction

Bananas are one of the most cultivated fruit crops in the world; production exceeds 70 million t, making them one of the most important crops (Robinson and Galán, 2012). For 2017, the main producing countries were the Philippines (251.792 t), Guatemala (236.571 t), Ecuador (158.991 t), Dominican Republic (143.744 t) and Colombia (117.797 t) (Carvajal *et al.*, 2019). About 20% of global banana production goes to international markets with 95% of the *Musa*

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acuminata cultivars from the Cavendish group (Robinson and Galán, 2012).

One of the most important agronomic practices for this crop is fertilization management, traditionally carried out with frequent applications in a small area. Fertilizers, especially nitrogen-based ones, are concentrated, causing the soil to have increased acidification and salinity. This leads to product loss through volatilization, runoff, and leaching, and generates changes in organic matter that can affect soil quality (Flores, 1991; López and Espinosa, 1998).

Nitrogen is one of the most important elements in crop nutrition because of the functions it performs and its high mobility in soil and plants (Havlin *et al*., 2014). It is associated with soil carbon, as seen in the carbon-nitrogen ratio (C/N ratio), indicating the availability of nitrogen for plants and whose ideal value is between 10 and 20 reaching a value of 30 in extreme cases. Soils with a high content of organic matter have high nitrogen content (Fassbender, 1993), depending on the mineralization rates that are higher in cultivated and fertilized soils (Piccolo, 2001; Mosquera *et al*., 2007).

The C/N ratio is a parameter that is related to the decomposition of organic matter, indicating the availability of nitrogen in a soil (Torres *et al.,* 2017). When organic matter has high nitrogen content, microorganisms have greater mineralization since the microflora (bacteria, fungi and actinomycetes) satisfy the nitrogen requirements so that it is not a limiting factor. On the other hand, if the nitrogen content is low, the rate of decomposition of organic matter decreases, and the rate of carbon mineralization will depend on the addition of nitrogenous sources (Ferrera and Alarcón, 2011).

The literature reports that 11 is the average value for the C/N ratio, which can vary from 4 to 30, depending on the soil, content, and type of organic matter, environmental conditions, and the use and management of the soil (Torres *et al.,* 2017). Therefore, organic matter with a C/N ratio of 30 (30:1) favors immobilization; lower ratios (20:1) have faster mineralization, so both processes increase soil fertility (Navarro, 2003).

The economic conditions seen in the global agricultural sector and the need to preserve soil resources require the efficient use of nutrients. Soil dedicated to the cultivation of bananas currently sees decreases in organic matter because of improper agronomic management, including fertilization. Therefore, soil recovery through management practices reduces the negative impact of this activity on soil. Organic matter has a large influence on the chemical and physical properties of soils (Robert, 1996); although this fact is well known in banana-producing zones, its application is deficient.

Advances in nutrition and fertilization management in bananas are of crucial importance because of the high costs of this activity, representing between 30% and 40% of total costs. Additionally, improvements in the technologies and practices of this crop for increasing productivity have resulted in the need for more efficient fertilization management. For this reason, the objective of this study was to evaluate and compare the C/N ratio in areas with fertilizer applications and nutrient absorption for sixth-generation banana crops during two harvest cycles.

Materials and methods

Study area

This research was conducted in lots 3 and 4 of the experimental field at the AUGURA Tulenapa Research Center (Carepa-Antioquia), located at 7°46’46” N and 76°40’20” W, with an average altitude of 20 m a.s.l., an average temperature of 27°C, a solar brightness of 1,800 h/year, 87% relative humidity, and rainfall of 844.9 mm distributed in the first production cycle and 2.088 mm in the second cycle.

The soils included fine Fluventic Eutrudepts, fine loam over clay Fluvaquentic Eutrudepts and fine loam Vertic Endoaquepts. These soils were developed from deep, poorly drained, and poorly filtrated alluvial sediments with a moderately slow hydraulic conductivity, high cation exchange capacity (CEC) and low carbon content (Gutiérrez, 2007). Table 1 shows the data of the chemical analysis of the soils used in this study.

Plant material

The crop belonged to the AAA group, giant Cavendish subgroup, Williams clone, sixth generation, with a planting density of 1600 plants ha^{-1}.

Experimental management

For the development of the experiment, 20 experimental units were selected, corresponding to sections called “botalon” with 1563 m^2 (250 plants). Fertilization cycles began with the selection of the ratoon plant (September), which coincided with the periods of maximum flowering of mother plants. The experimental lots had the same agronomic management as the other productive units, except for fertilization through the application of the treatments.

TABLE 1. Chemical analysis of the soil.

Soils	Fine Fluventic Eutrudepts	Fine loam over clay Fluvaquentic Eutrudepts	Fine loam Vertic Endoaquepts
Organic Matter %	3.08	2.84	3.16
Total Nitrogen %	0.17	0.22	0.26
Total Carbon %	1.27	1.30	1.65
P (mg kg^{-1})	9.18	8.21	6.06
K (cmol kg^{-1})	0.39	0.40	0.43
Ca (cmol kg^{-1})	15.58	15.38	16.64
Mg (cmol kg^{-1})	5.83	6.24	5.76
Al (cmol kg^{-1})	0.16	0.33	0.14
N-NH_4 (mg kg^{-1})	3.44	6.60	6.85
N-NO_3 (mg kg^{-1})	34.93	25.14	33.64
pH	5.77	5.57	5.68
CEC (cmol kg^{-1})	23.64	25.28	25.18

CEC - cation exchange capacity.

Samplings were carried out in the fertilizer application area at 30 cm on the pseudostem, at depths between 5 and 15 cm and in the nutrient absorption zone at a space between 5 cm and 15 cm from the region of the soil bordering the corm that had the highest proportion of roots (López and Espinosa, 1998). Samples were collected at different phenological stages (vegetative phase, floral differentiation, flowering, fruit filling, and harvesting). Fertilization was carried out every three weeks, and sampling was performed one week before the fertilization cycles, 17 applications per year, spaced out at approximately three weeks between applications. The commercial fertilizer source used was urea (46%).

Evaluated variables

Total nitrogen and carbon were determined with the methodology of Dumas (Batjes, 1996), with dry combustion and 0.2 g of soil. Measurements were taken with a Tru Spec elemental analyzer (Laboratory Equipment Company LECO, Otario, Canada) calibrated with standardized samples in the Soil analysis laboratory at the Universidad Nacional de Colombia. Productivity was measured by subtracting the weight of the rachis before taking the samples from the fresh weight of the cluster. This value was presented in kg per plant and transformed to ha^{-1}.

Experimental design and statistical analysis

A randomized complete block design was used with five treatments and four replicates. The treatments were adjusted from soil analyses performed before the start of the research and from the balanced dose proposed by CENIBANANO (321.8 kg ha^{-1} of N, 87.1 kg ha^{-1} of P_2O_5, 678.8 kg ha^{-1} of K_2O, 50.5 kg ha^{-1} of CaO, 117.5 kg ha^{-1} of MgO, 64.2 kg ha^{-1} of S, 1.4 of kg ha^{-1} of B, and 9.3 kg ha^{-1} of Zn). Treatments included an absolute control, a control without nitrogen, and doses of 161, 321 and 483 kg ha^{-1} of N.

Multivariate analyses of variance that met the criteria of homogeneity and normality of matrices were used, and the interactions between the study areas (application and fertilization) and treatments were evaluated by applying the Tukey test. The analyses were complemented with a correlation analysis to determine the best functional relationship between the production and C/N ratio (IBM SPSS Program).

Results and discussion

Total soil carbon and nitrogen content

The total nitrogen content of the soil showed significant statistical differences in the interaction zone of the study between the treatments. The treatment with 483 kg ha^{-1} of N had the highest contents in the fertilization zone with an average of 0.33%. However, there were no significant differences with the other treatments (Tab. 2). In the absorption zone, the treatments did not show significant differences; the total nitrogen contents oscillating in the soil were between 0.25% and 0.27% (Tab. 2).

Nitrogen fertilizers can be lost through volatilization (44.1%), leaching (14.8%) and denitrification (4.4%). Fertilizers, along with the decomposition of roots, leaves or other contributions of organic matter that favor nitrogen fixation by microorganisms cause significant increases in

TABLE 2. Contents of total nitrogen (%) in soil of the study area and treatment interaction in two production cycles.

Treatments	Production cycles			
	1		2	
	Absorption zone	Fertilization zone	Absorption zone	Fertilization zone
Control	0.28 ns	0.28 ns	0.28 ns	0.28 ab
0 kg ha^{-1} N	0.29 ns	0.27 ns	0.27 ns	0.24 b
161 kg ha^{-1} N	0.29 ns	0.29 ns	0.29 ns	0.28 ab
321.8 kg ha^{-1} N	0.28 ns	0.28 ns	0.28 ns	0.29 ab
483 kg ha^{-1} N	0.27 ns	0.32 ns	0.29 ns	0.33 a

Values with the same letter within the column do not statistically differ according to the Tukey test ($P<0.05$). ns: not significant.

the total nitrogen content of soil (Ju *et al.,* 2009) that was not observed in the present study.

A significant result for the fertilization zone was observed with the treatment of 483 kg ha^{-1} of N, which had an increase of 27% in the N content, as compared to the control without nitrogen, in which the total nitrogen was 0.24%. This increase was due to the application of urea, whose dissolution allows nitrogen to take different routes. This could have possibly favored the incorporation of ammonium into the soil, along with other action pathways, such as being absorbed by the plants or by microorganisms, oxidized to a nitrate form, leached or denitrified (Gasser *et al.,* 1967) (Tab. 2).

The NH_4^+ from urea can easily adhere to the cation exchange sites in inorganic and organic colloids in the soil, and its proportion depends on the soil's edaphogenetic characteristics (Fuentes and González, 2007). Specifically, the treatment with 483 kg ha^{-1} of N showed a tendency to increase the total nitrogen in the fertilization zone and establish significant statistical differences in the second production cycle. These effects were attributed to the precipitation conditions (near the normal ranges for the period) and to the type of clay present in the studied soils (vermiculite), which corroborates the proposal by Fuertes and González (2007).

The response to the nitrogen doses may have been related to the ammonium released by the urea in the soil that can be directly associated with the interaction between the clay present in the studied soil (vermiculite, illite, and montmorillonite). This interaction causes ammonium fixation through expandable clays and loss through runoff, an effect attributed to increases in the total nitrogen for the high doses (483 kg ha^{-1} of N), as observed in the second production cycle with increases in precipitation, compared to the first cycle.

Total soil carbon showed significant differences between the treatments for the fertilization zone, where the treatment of 483 kg ha^{-1} of N had the higher values (2.47 and 2.45%). No significant differences were found in the absorption zone although the observed values tended to be lower than those of the fertilization zone; however, the treatment with 483 kg ha^{-1} showed the best response (Tab. 3).

Increase in total carbon was associated with the residual acid effect generated by urea that could have acted on the calcium carbonate applied to the crop and the possible

TABLE 3. Contents of total carbon (%) in soil of the study area and treatment interaction in two production cycles.

Treatments	Production cycles			
	1		2	
	Absorption zone	Fertilization zone	Absorption zone	Fertilization zone
Control	1.57 a	1.92 a	1.56 ns	2.05 ab
0 kg ha^{-1} N	1.92 b	2.25 ab	1.69 ns	2.02 a
161 kg ha^{-1} N	1.70 ab	1.97 a	1.83 ns	1.86 a
321.8 kg ha^{-1} N	1.74 ab	1.98 a	1.72 ns	2.11 ab
483 kg ha^{-1} N	1.79 ab	2.47 b	1.81 ns	2.45 b

Values with the same letter within the column do not statistically differ according to the Tukey test ($P<0.05$). ns: not significant.

release of mineral carbon present in the parental materials (carbonates) of the soil (Guerrero, 2004; Zapata, 2004).

Urea reacts with moisture, $CO(NH_2)_2$ (urea) + H^+ + $2H_2O$; this reaction releases 2 NH_4^+ (ammonium) and HCO_3^- (bicarbonate) (Guerrero, 2004). The latter was able to react with elements of the soil since the morphogenesis of the soils where the research was developed indicated that there was a high load of suspended sediments, sand, and some silt with calcium and magnesium contents. The calcium had a saturation over 70%, allowing carbonate and calcium to form calcium bicarbonate that affected the increases in the total carbon of the soil causing an increase in the total carbon in the fertilization zone. Liu *et al.* (2008) evaluated four treatments with fertilization and found that an increase in the inorganic carbon of the soil resulted from fertilization because of carbonate stability. Therefore, further studies in which organic carbon is dissociated from inorganic components are needed.

Carbon-nitrogen ratio (C/N ratio)

The C/N ratio displayed an interaction between the treatments and study areas. The treatments resulted in an increase in the C/N ratio values for the fertilization zone. The control and 0 kg ha^{-1} of N showed values in the fertilization area similar to the dose with high N. These results are associated with the nitrogen and carbon present in the soil that was released from organic matter. This can also be influenced by the recycling process between a mother plant and its successors due to nitrogen-free fertilization (Robinson and Galán 2012). In the absorption zone, the behavior was similar, but with small variations between the treatments and values lower than in the fertilization zone (Tab. 4).

The highest carbon contents were observed for the fertilization zone with the treatment with 483 kg ha^{-1} of N. These values are the product of the acidifying effect of urea accompanied by conditions of high humidity in the second production cycle. This condition helped the movement of these elements to the absorption zone and impacted the C/N ratio (Jones and Jacobsen, 2005). For this reason, the values seen in the present study, all less than or equal to 8, were below the levels considered normal or ideal for agricultural land (Moreno and Moral, 2008; Osorio, 2014) (Tab. 4).

Torres *et al.* (2017) stated that, for the doses 161 and 321.8 kg ha^{-1} of N, there was low agronomic efficiency, corroborating the low response in the nitrogen application as reflected in the C/N ratio with values oscillating between 6 and 6.4 for the absorption zone (Tab. 3). Moreno and Moral (2008) confirmed that low C/N ratios can be associated with an excess of nitrogen that is lost, a situation that can be associated with the low agronomic efficiency observed by Torres *et al.* (2017).

The C/N ratio values during the stages of development varied between 6.0 and 8.2, indicating a high nitrogen content compared to the carbon in the soil, but the values can also be interpreted as a rapid release of the mineral and organic carbon (Osorio, 2014) that occurred in the present study.

The variation of total carbon contents during the development of the banana crop tended to decrease; this can be attributed to the soil and climatic conditions and the agronomic management that implied a gradual loss of soil quality (Rosales *et al.,* 2006). It is necessary to establish a management plan for organic matter that maintains and improves carbon content and, therefore, nitrogen in order to proportionally reduce nitrogen doses for the benefit of producers and the environment (Torres *et al.,* 2017).

Carbon-nitrogen ratio and production

Production during the two cultivation cycles showed positive responses to the treatments of 321.8 and 483 kg ha^{-1} of N, with values of 41.3 and 42.0 t ha^{-1} for the first cycle and 40.9 and 46.4 t ha^{-1} for the second cycle (Fig. 1), values within the production range reported by Robinson and Galán (2012) for cv. Williams (24 to 64 t ha^{-1}). The effect of

TABLE 4. Carbon-nitrogen ratio in soil for the treatment and study area interaction.

Treatments	Production cycles			
	1		2	
	Absorption zone	Fertilization zone	Absorption zone	Fertilization zone
Control	6.0 ns	7.0 ab	6.2 ns	7.2 ab
0 kg ha^{-1} N	6.8 ns	8.2 b	6.4 ns	8.2 b
161 kg ha^{-1} N	6.0 ns	7.1 a	6.5 ns	7.1 a
321.8 kg ha^{-1} N	6.4 ns	7.3 ab	6.3 ns	7.3 ab
483 kg ha^{-1} N	6.6 ns	7.7 ab	6.4 ns	7.6 b

Values with the same letter within the column do not statistically differ according to the Tukey test (P<0.05). ns: not significant.

low production of absolute control in cycle two was associated with the loss of the nutritional recycling effect of the mother-ratoon relationship (Robinson and Galán, 2012).

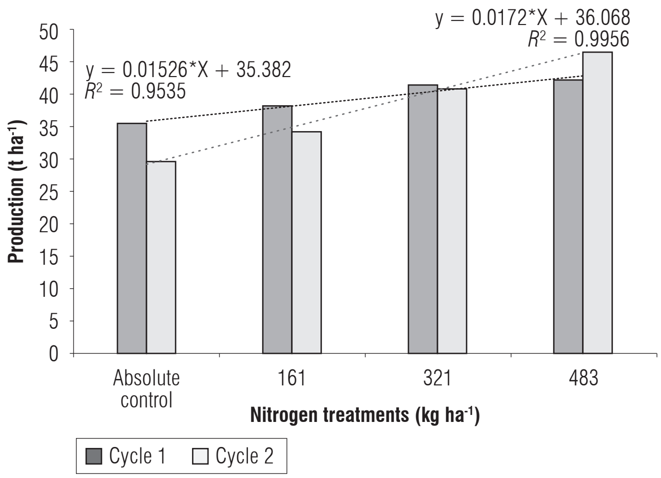

FIGURE 1. Production in t ha^{-1} in the treatments and productive cycle.

Responses in production to high doses of nitrogen were associated with low levels of total carbon (<2%) in the study areas (Gutiérrez, 2007). It should be remembered that no applications of organic matter were performed on any treatment in the production cycles that accentuated the effect of the nitrogen doses, an effect similar to that found by Srikul and Turner (1995), Orozco and Pérez (2006) and Nyomby *et al.* (2010).

To evaluate the association between the production variable and the C/N ratio, a correlation analysis was performed that obtained coefficients of 0.44 and 0.42 in the absorption and fertilization zones, respectively. These coefficients were positive, implying that, as the C/N ratio increased, production increased.

The regression analysis for the study areas between the production variable and the C/N ratio was significant (R^2 = 0.2). This led to the inference that, although the C/N ratio values were low, the best response in production was associated with the high C/N ratio. The low correlation coefficient that can be attributed to production did not depend on the C/N ratio alone.

The correlation analysis for the study areas during the two production cycles did not show significant results between the production and C/N ratio values. However, for the first production cycle, the best fit was seen for the treatment with 321 kg ha^{-1} of N (r = 0.95). In the second cycle, the treatment with 483 kg ha^{-1} of N (r = 0.86) had the best fit, leading to the inference that, even though the C/N ratio values were low, the treatments with the best response in production were associated with the higher C/N ratios. Robinson and Galán (2012) confirmed that the behavior of a cultivar is associated with its genetic characteristics, agronomic management, and climatic and soil characteristics (Tab. 5).

When analyzing the response by treatment, the control in the correlation between production and C/N ratio showed correlation coefficients of -0.49 and -0.37 for the absorption and fertilization zones, respectively (Tab. 5). This negative value means that, as production increased, the C/N ratio decreased; this could be attributed to the fact that the nitrogen extracted by the plants for production

TABLE 5. Correlation analysis of the carbon-nitrogen (C/N) ratio in soil and production by treatment.

Production cycle	Treatments	Correlation analysis	
		Absorption zone	Fertilization zone
		C/N ratio	C/N ratio
1	Control	0.37	0.78
	0 kg ha^{-1} N	0.22	0.66
	161 kg ha^{-1} N	0.87	0.78
	321.8 kg ha^{-1} N	0.78	0.95**
	483 kg ha^{-1} N	0.82*	0.8*
2	Control	-0.49*	-0.37**
	0 kg ha^{-1} N	0.91	0.18
	161 kg ha^{-1} N	0.97	0.85
	321.8 kg ha^{-1} N	0.95*	0.63
	483 kg ha^{-1} N	0.75*	0.86**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

was available in the soil and carbon decreased before the application of organic matter. Torres *et al.* (2017) confirm that the low productivity of the absolute control during the second production cycle is associated with the loss of the effect of nutrient recycling from the mother and ratoon relationship and the impoverishment of the soil without fertilizer, as corroborated by Robinson and Galán (2012).

The correlation values in the nutrient absorption zone for the two cycles reached coefficients higher than 0.7, reflecting the influence of nitrogen fertilization. Nave *et al.* (2009) stated that nitrogen inputs help meet the requirements of crops, improving productivity, and increasing carbon storage in the soil, as observed in the treatment with 483 kg ha^{-1} of N with the best response in production and its interaction with the C/N ratio (Tab. 5).

For cycle two, the treatment with 483 kg ha^{-1} of N obtained a correlation of 0.86 between production and the C/N ratio and 0.75 for the absorption zone. By taking into account the low correlation coefficient from one area to another, it could be inferred that the plants absorbed the nitrogen and the fertilizer was not immobilized; it passed from the fertilization zone to the absorption zone (Tab. 5) and was not lost through leaching or runoff processes. It is worth mentioning that for the second cycle the treatment with 321.8 kg ha^{-1} of N showed a significant adjustment in the absorption zone for the production correlation and C/N ratio ($r = 0.95$). This demonstrates that the contributions of nitrogen through fertilizer had an impact on the production and, in turn, on the C/N ratio.

The high doses of nitrogen in the form of urea had an important impact on the carbon and nitrogen contents despite the possible losses of the latter through leaching and volatilization (Mengel and Kirkby, 2000).

Torres *et al.* (2017) concluded that production can be increased by improving the efficiency of fertilization that includes organic matter. This requires the development and promotion of technological models that take into account the observations of Robinson and Galán (2012) for interactions among soil, plants, climate, and agronomic management.

Conclusions

The residual acidity generated by the urea and the mineral composition of the parent material constituted possible causes for the atypical responses of the variables soil total carbon and C/N ratio for the studied areas and demonstrated the effect of increasing doses of nitrogen in the form of urea, especially for the fertilization zone.

Higher production was associated with the higher C/N ratios and nitrogen doses, demonstrating the need to adjust nitrogen fertilization plans and taking into account the C/N ratio. So balancing doses of organic products with doses of synthetic nitrogen sources is important to improve the soils of banana crops and to make the process of nitrogen fertilization more efficient and friendly.

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ERRATUM

The authors of the article "Carbon-nitrogen ratio in soils with fertilizer applications and nutrient absorption in banana (Musa spp.) cv. Williams" report an erratum in the equations of Figure 1 of the article published in Agronomía Colombiana, vol. 38 No. 2 (2020), pp. 253–260.

The correct equations in Figure 1 should be as follows:

Equation for Cycle 1: $Y = 0.01526*X + 35.382$

Equation for Cycle 2: $Y = 0.0172*X + 36.068$

April 30, 2024

Agricultural infrastructure as the driver of emerging farmers' income in South Africa. A stochastic frontier approach

La infraestructura agrícola como factor clave en los ingresos de los agricultores emergentes en Sudáfrica. Una aproximación de frontera estocástica

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ABSTRACT

A stochastic frontier model was applied to cross-sectional data to examine whether availability and accessibility of agricultural infrastructure for emerging farmers enhance their agricultural income through efficiency gains. Using a stratified sampling approach, the study grouped the farmers into two; those who had agricultural infrastructure and those who did not have it. Through a survey, data collected from a sample of 150 smallholder farmers in the study area were analyzed using the frontier model. The explanatory variables that were statistically significant and which influenced the agricultural income of the emerging farmers in the study area included the following: equipment, social, institutional availability and physical accessibility indices, education, access to agricultural extension services, age of farmers, assistance of household members in farming, membership in farmers' organizations, and marital status of the farmers. Informed policies aimed at improving the income of smallholder farmers might consider the results of the explanatory variables included in this study.

Key words: agricultural income, smallholder farmer, infrastructure availability, infrastructure accessibility.

RESUMEN

Se aplicó un modelo de frontera estocástica a los datos de corte transversal para examinar si la disponibilidad y accesibilidad de la infraestructura agrícola para los agricultores emergentes incrementa su ingreso agrícola a través del aumento de la eficiencia. Utilizando un enfoque de muestreo estratificado, el estudio agrupó a los agricultores en dos; los que tenían la infraestructura agrícola y los que no la tenían. A través de una encuesta, los datos recolectados de una muestra de 150 pequeños agricultores en el área de estudio fueron analizados utilizando el modelo de frontera. Las variables explicativas que fueron estadísticamente significativas y que influyeron en el ingreso agrícola de los agricultores emergentes en el área de estudio incluyen: equipo, índices de disponibilidad social, institucional y de accesibilidad física, educación, acceso a servicios de extensión agrícola, edad de los agricultores, asistencia de los miembros del hogar en agricultura, membrecía de organizaciones de agricultores y estado civil de los agricultores. Las políticas informadas dirigidas a mejorar los ingresos de los pequeños agricultores pueden considerar los resultados de las variables explicativas incluidas en el estudio.

Palabras clave: renta agraria, pequeño agricultor, disponibilidad de infraestructura, accesibilidad a la infraestructura.

Introduction

It is clear that in developing countries agricultural growth is an instrument of economic growth and poverty reduction (Headey *et al*., 2005; Byerlee *et al*., 2009). Long-term agricultural growth has been associated with growth in yields, which is encouraged by investment in research, extension, human capital and infrastructure (Rosegrant and Hazell, 2000; Jayne *et al*., 2010). The importance of good infrastructure for agricultural development is recognized in its ability to enhance productivity and reduce transactional costs in agricultural marketing (Andersen and Shimokawa, 2007; Munyanyi, 2013; Khapayi and Celliers, 2016). The three types of agricultural infrastructure are road networks, irrigation technology, and post-harvest storage technology (Munyanyi, 2013). Ghosal (2014) provides two categories of infrastructure services in agriculture: soft and hard infrastructure. 'Soft infrastructure' includes transportation services, financial services, animal husbandry, input distribution, and marketing; 'hard infrastructure' includes roads, telecommunications, electrification, and irrigation (Ghosal, 2014). Infrastructure could also be disaggregated as physical (for example transport, storage, and irrigation infrastructure), equipment (tractors, ploughs, and sprayers), institutional (cooperatives and financial institutions), and social infrastructure-consisting of health and education services (Andersen and Shimokawa, 2007).

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According to Stilwell and Makhura (2004), infrastructure can also be classified as either economic or physical (for example, roads, storage, bridges, and railways), social or soft (e.g. health and education), or institutional (farmers' cooperatives and agricultural institutions). Economic infrastructure is that component of an economy's capital stock that generates services for enabling economic production or for assisting as input in production (Stilwell and Makhura, 2004). It is important to note that institutional and social infrastructures are equally important as they both play a key role in an economy. In agriculture, institutional infrastructure such as cooperatives, lending, and marketing institutions play a significant role in linking farmers to the markets and thus increasing productivity (Kumar *et al.,* 2015). Social infrastructure impacts the quality of life directly and indirectly since it is the principal foundation that provides services in agriculture, health, education, and recreation (Stilwell and Makhura, 2004). Equipment infrastructure entails all the farm level machinery and small equipment needed to improve labor productivity including tractors, ploughs, and sprayers (Andersen and Shimokawa, 2007; Munyanyi, 2013). Therefore, agricultural infrastructure entails all the basic services, facilities, institutions and equipment that are needed for efficient production and marketing of agricultural commodities (Munyanyi, 2013). This definition was adopted for this study.

Infrastructure has a huge impact on economic growth. According to the Development Bank of Southern Africa (DBSA, 1997), it is important to invest sufficiently in infrastructure as it allows diversification of trade and the country benefits more from globalization. Governments and donors invest heavily in the development of rural roads and transport corridors because investments in rural infrastructure have an important positive effect on agricultural production and trade (Tripathi and Prasad, 2009; Jouanjean, 2013; Manjunath and Kannan, 2017). Adequate and reliable infrastructure provides the physical linkages between different inputs needed for the economy to be functional (Cloete, 2010).

The South African Government introduced the Land Reform to ensure restitution, redistributions and tenure reform of land especially for the previously disadvantaged black South African smallholder farmers from 2002 (Antwi and Oladele, 2013). In order to expedite the development of such farmers into commercialized farmers, the government is investing in infrastructure for smallholder farmers in the North West Province, through programs such as the Comprehensive Agriculture Support Programme (CASP) (DAFF, 2017). Farmers who have received governmental support through the Land Reform and CASP are termed emerging farmers, and they will be referred to as such throughout this paper. The CASP identifies five types of infrastructure as follows: resource management infrastructure, production infrastructure, mechanization (tractors), water storage infrastructure for household food, and marketing infrastructure. This program has continued to fund smallholder farmers since 2005 in terms of agricultural infrastructure and support initiatives for farmers (DAFF, 2017).

There is little formal evidence to show how and under what conditions such infrastructure benefits rural smallholder farming households and improves agricultural income (Jouanjean, 2013). This study used the physical institutional, social, and equipment infrastructure availability and accessibility indices with their components already mentioned to analyze the effect of infrastructure on agricultural income. According to Idiong (2007), other socioeconomic factors such as age, educational level of household head, and access to extension were also included in the analysis as they were expected to have a direct influence on efficiency. Therefore, the objective of the study was to examine whether accessibility and availability of agricultural infrastructure enhance farmers' agricultural income through efficiency gains.

Materials and methods

Data sources and analyses

A structured questionnaire subject to reliability and validity tests was employed to collect data through a survey of 150 smallholder farmers in the North-West Province (NWP). The NWP covers an estimated surface area of 116,320 km^2, with a population density of approximately 30 people per km^2 (Statistics South Africa, 2012). The Province is located in the western part of South Africa (26.6639° S, 25.2838° E), sharing borders with Limpopo Province to the north, Gauteng to the east, the Free State to the east and south, the Northern Cape to the south, and Botswana to the west and north (DAFF, 2017). The NWP was selected as it is mostly rural and agricultural as the main contributor to the provincial gross domestic product (DAFF, 2017).

Data was collected from March to July in 2016 and questionnaires were administered through face to face interviews. The research used stratified random sampling to group farmers receiving infrastructure support and those who did not receive support from the government. Under the CASP of the South African Government, emerging farmers received financial support in form of subsidies to purchase farm infrastructure. However, not all intended beneficiaries of the program received the financial support and the infrastructure (DAFF, 2017). Therefore, the population consisted of 114 smallholder farmers receiving infrastructural support and 72 without support (DAFF, 2017). Using the Raosoft sample size calculator, the sample frame consisted of 89 farmers with infrastructure support and 69 without support. Farmers from each stratum were randomly selected to come up with a total of 150 farmers.

Captured data from completed questionnaires on STATA version 14.0 were analyzed using descriptive statistics and the Stochastic Frontier Model (Battese and Coelli, 1995). The study used the model to assess factors that influence smallholder farmers' agricultural income with respect to availability and accessibility to agricultural infrastructure. Furthermore, factors that influence the agricultural income of the smallholder farmers without access and agricultural infrastructure were analyzed.

Principal component analysis (PCA) was used to compute composite indices of availability and accessibility of infrastructure in terms of physical, institutional, social and equipment infrastructure. The selection of indicators was guided by insights drawn from the literature on agricultural infrastructure (Munyanyi, 2013; Stilwell and Makhura, 2014) as well as data availability. All the major dimensions of agricultural infrastructure were represented by at least one indicator. PCA is a data reduction method used to re-express multivariate data in fewer dimensions. The procedure transforms selected indicators into smaller components that capture most of the information (variation) in the original indicators. A detailed account of the use of PCA for constructing socio-economic status indices has been outlined by Vyas and Kumaranayake (2006). The variables selected for constructing the indices were the agricultural infrastructure categories stated in the questionnaire, which were coded as 1 if the answer was yes and 0 if it was no. These major infrastructure groups were in accordance with the literature on agricultural infrastructure in South Africa, (physical, social, institutional and equipment infrastructure). STATA software was used to provide a simple measure of the aggregation of the agricultural infrastructure indices and PCA, after which the same STATA command was used to predict the availability index, the accessibility index and the satisfaction index used in the study.

A stochastic frontier model for agricultural income for farmers with agricultural infrastructure (availability)

This study follows the modern economic theory based on the assumption of optimizing behavior for producers both technically and economically (Kokkinou, 2010). Agricultural infrastructure and advanced agricultural production methods are synonymous with farmers who can produce efficiently and increase agricultural income. Parametric or non-parametric methods can be used to determine efficiency.

The stochastic frontier production function proposed for this study is a parametric model that makes use of econometric methods to analyze agricultural income for smallholder farmers (Masunda and Chiweshe, 2015). The model was selected due to its extensive use in productivity estimation for smallholder agriculture in previous studies (Binam *et al*., 2004; Mango *et al*., 2015; Abdul-Hanan and Abdul-Rahaman, 2017). The Stochastic Frontier Model was originally proposed for panel data analysis by Battese and Coelli (1995). However, the model was adopted for cross sectional data in which the parameters of agricultural income (production function) were specified to be a function of variables associated with a socio-economic variable, such as access to extension services, land tenure, number of years involved in farming, etc. (Mango *et al*., 2015). The different factors of production involved may affect the responsiveness of the produce (agricultural income). The models are presented in terms of production functions in which the former can only allow a constant return to scale and the latter has more flexibility. As adopted from Abdul-Hanan and Abdul-Rahaman (2017), two functional forms for the stochastic frontier production function that were estimated are described by:

$$LogY_i = \beta_0 + \Sigma^7 \beta_j LogX_{ji} + (V_i - U_i) \quad (1)$$

$$LogY_i = \beta_0 + \Sigma^7 \beta_j LogX_{ji} + \Sigma \Sigma^6 \beta_j (LogX_{ji})(LogX_{ji}) + (Vi - Ui) \quad (2)$$

where: *Log* represents the logarithm base 10; the subscript i represents the i^{th} sample of smallholder farmers; the subscript j represents the number of socio-economic and farm-specific variables;

- Y represents the seasonal agricultural income by smallholder farmers in Rands;
- X_1 represents the physical infrastructure availability index on the i^{th} farm;
- X_2 represents the social infrastructure availability index on the i^{th} farm;
- X_3 represents the institutional infrastructure availability index on the i^{th} farm;
- X_4 represents the equipment infrastructure availability index on the i^{th} farm;
- X_5 represents the farmer's level of education on the i^{th} farm;

X_6 represents the extension access on the i[th] farm;

X_7 represents the non-farming activities on the i[th] farm;

X_8 represents the farmer's membership in an organization on the i[th] farm;

X_9 represents the age of the farmer on the i[th] farm;

X_{10} represents the marital status of the farmer on the i[th] farm;

X_{11} represents the land tenure on the i[th] farm;

X_{12} represents the farmer's number of years involved in farming on the i[th] farm;

X_{13} represents assistance of the household member n farming on the i[th] farm;

β_j j = 0, 1, ..., 13 are parameters to be estimated;

V_i's are assumed to be independent and identically distributed N (0, σ^2) random variables; and

U_i's are assumed to be independent and identically distributed non-negative truncations of the N (μ, σ 2) distribution.

These models were separately considered for two categories: availability of agricultural infrastructure and non-availability of agricultural infrastructure. In the first case of farmers with the availability of agricultural infrastructure, factors affecting agricultural income were analyzed to understand the efficiency of farmers with agricultural infrastructure on agricultural income. In the latter case, farmers without agricultural infrastructure were taken into consideration. The above models (1 and 2) are production functions, in which the inefficiency effect is subtracted because observed outputs are no larger than their corresponding stochastic frontier, due to the presence of inefficient use in producing the involved outputs. The non-negative random variables U_i in Equation 1 imply that the observed socio-economic (input) variables for a given level of output and quasi-fixed inputs are not as small as possible if the farmers were fully efficient in their use of inputs. Following Abdul-Hanan and Abdul-Rahaman (2017), the translog production function was used in this study. This is specified in Equation 2, so that more general technologies could be accounted for than with the Cobb-Douglas model.

The test of the hypothesis for the parameters of the frontier model was conducted using the generalized likelihood-ratio statistics, λ, defined by:

$$\lambda = -2 \log[L(H_1) - L(H_0)] \quad (3)$$

where $L(H_0)$ is the value of the likelihood function for the frontier model in which parameter restrictions are specified by the null hypothesis; H_0 is imposed, and $L(H_1)$ is the value of the likelihood function for the general frontier model (Battese and Coelli (1995). If the null hypothesis is true, then λ has approximately a chi-square (or mixed square) distribution with degrees of freedom equal to the difference between the parameters estimated under H_1 and H_0, respectively.

Stochastic frontier model for agricultural income for farmers with access to agricultural infrastructure

Similarly, two separate models were used for the agricultural farmers with and without access to agricultural infrastructure. The dependent variable was agricultural income and the independent variables used were the same as the ones used for farmers with available infrastructure. However, the infrastructural accessibility indices were used instead of the availability indices.

Results and discussion

Descriptive statistics for the demographic and socio-economic characteristics of emerging farmers

The socio-economic and demographic characteristics of emerging farmers in the North West Province are presented in Table 1. According to Stilwell and Makhura (2004), these characteristics are important because the key household activities are coordinated by the head of the household and decisions of the head are most likely influenced by such demographic characteristics. The results show that the majority (65%) of farmers were males, 61% were married, and 57% had no formal education. The analysis above reveals that participation of women in smallholder farming still remains a challenge in the North West Province. This could be attributed to the fact that most households sampled for the study were made up of more males than females. This finding is consistent with those of Antwi and Oladele (2013) who found that agriculture is mostly reserved for males while women are expected to perform domestic activities in the household. The majority of smallholder farmers in the North West Province are aged between 41 and 60 years, an indication of a paucity of involvement of the youth in smallholder farming agricultural activities in the Province. Smallholder farmers do not have a strong educational background and most of them have no formal education; this could limit their adaptation to new farming agricultural innovations and agricultural infrastructure. This finding is consistent with the results obtained by Montshwe (2006), who found that people with higher levels of education are able to better interpret information. This revelation could be linked to labor costs that have risen in previous years and to the fact that children are being statutorily compelled

TABLE 1. Demographics and socio-economic data.

Discrete variables			
Variables		Frequency (N = 150)	% Frequency (N = 150)
Gender	Male	97	65
	Female	53	35
Land tenure system	Private	9	6
	Communal	4	3
	Renting	54	36
	Allocated through land reform	83	55
Level of education	At least secondary	65	43
	Primary or none	85	57
Marital status	Married	91	61
	Otherwise	59	39
Non-farming activities	Yes	85	57
	No	65	43
Membership of farmers' organizations	Yes	39	26
	No	111	74
Assistance of household members in farming	Yes	62	41
	No	88	59

Continuous variables				
	Mean	Standard deviation	Maximum value	Minimum value
Age	54.51	11.13	30	79
Number of years involved in farming	9.467	4.515	3	20

to be at school during the day. This is also in line with the finding of Harding *et al*. (2005), who highlighted insufficient family labor as a production constraint of smallholder farming. There is a low number (26%) of people considered as members of farmer groups in the area, posing a threat to organized lending opportunities for farmers.

Factors affecting agricultural income in relation to availability and accessibility of infrastructure following the stochastic models

The results of the stochastic frontier models on factors affecting agricultural income with regards to availability and accessibility of infrastructure are presented in Tables 2 and 3, respectively. The models are appropriate given their significant chi-square (P<0.01) and Log likelihood ratios (for both models in Tabs. 2 and 3).

Physical infrastructure availability index

The results in Table 2 show that the relationship between the physical infrastructure availability index and agricultural income is positive and statistically significant for farmers who have the available infrastructure and those without infrastructure (P<0.01). Physical infrastructure includes roads, railway lines, fences and irrigation that is infrastructure required for production and marketing of agricultural products. According to Fakayode *et al*. (2008), Llanto (2012) and Eke and Effiong (2016), provision of efficient infrastructure is widely acknowledged as indispensable to agricultural progress. Increased availability of physical infrastructure would assist farmers to produce efficiently and generate more agricultural income. However, some of the available infrastructure such as railway corridors have become obsolete due to lack of proper maintenance, as people opt for other models of transport. Where irrigation infrastructure is available, lack of government advisory services on the use and maintenance of the infrastructure results in farmers not optimally utilizing the available infrastructure (Khapayi and Celliers, 2016).

Social infrastructure availability index

The influence of the social infrastructure availability index on agricultural income was positive and statistically significant (P<0.01) for both groups of farmers. The positive relationship between the social infrastructure availability index and agricultural income could be attributed to the fact that social infrastructure such as culture, health, and education plays a critical role in terms of maintaining farmers' physical and mental wellbeing, thus, maximizing the labor productivity of the farmers (Nadeem *et al.,* 2011; Kumar *et al.*, 2015). Moreover, the positive relationship between the social infrastructure availability index and the agricultural income of farmers without available infrastructure could be attributed to the fact that farmers could access social infrastructure even though it is not available in their area (for example, health services through mobile clinics and education through distance learning) (Kumar *et al.*, 2015). Social infrastructure plays a strategic role in producing large multiplier effects within the economy, thus, leading to agricultural growth (Bom and Ligthart, 2014). In South Africa, the standards of primary health care facilities are relatively poor and residents in rural areas usually forget their appointments or they miss them due to transportation constraints (Frost *et al.,* 2017). Therefore, low levels of social infrastructure, such as primary health care in rural areas, jeopardize the health of farmers and in turn they impact labor productivity.

Institutional infrastructure availability index

This study revealed a positive and statistically significant relationship between the availability of infrastructure and agricultural income for both groups of farmers (P<0.01). The institutional infrastructure availability index is associated with infrastructure such as financial institutions, farmers' unions, cooperative societies, and agricultural markets. Availability of this infrastructure is most likely to reduce transactional costs of marketing, thus increasing agricultural income. According to Satish (2006), rural infrastructure in the form of farmers' access to markets and availability of institutional finance leads to agricultural expansion and increased technical efficiency. However, in South Africa the collapse of the development corporations in former homelands and the increasing inability of the government to provide agricultural support services have limited productivity of smallholder farmers (Willemse, 2000).

Level of education of smallholder farmers

The study revealed that as the level of education increases, agricultural income was likely to increase significantly for both groups of farmers (P<0.01). This was expected as the higher the farmer's level of educational, the better the agricultural practices of farmers, thus, leading to higher agricultural income for smallholder farmers (farmers with and without agricultural infrastructure). According to Ferreira (2015), educated farmers are more likely to adopt new technologies, and this, in turn, leads to the diffusion of technology to other less educated farmers within the community. The descriptive statistics show that most (57%) of the farmers had no formal education despite its importance in sustainable agriculture.

Access to agricultural extension services

Access to extension services for farmers with available agricultural infrastructure and those without it was found to be statistically significant and positively affecting agricultural income (P<0.01). This implies that any relative increase in access to extension services caused agricultural income to respond positively. Farmers' access to extension services plays a very critical role in the production activities of smallholder farmers, and extension officers are often the ones who share information with smallholder famers on programs to finance infrastructure (Anderson and Masters, 2007).

Membership of farmers' organizations

The study revealed a positive and statistically significant relationship between agricultural income and membership in farmers' organizations (P<0.05) for farmers with infrastructure. The results highlight the importance of grassroot farmers' organizations such as Grain South Africa (Grain-SA) and Agri South Africa (Agri-SA). Grain South Africa is a voluntary organization for grain producers in South Africa that allows sharing information on various commodity markets and the adoption of technology. Agri South Africa is a coalition of all smallholder farmers groups with the overall aim of improving farmers' access to information, finance, and markets for sustainable agricultural production. Farmers' organizations provide the platform for smallholder farmers to discuss issues for increasing the availability of infrastructure and engaging governments to fund projects on infrastructure development (DAFF, 2017). Similarly, Kumar *et al.* (2015) emphasized the role of cooperatives in ensuring efficient delivery of financial support in smallholder farmers.

Age of smallholder farmers

The study revealed a positive statistically significant increase in agricultural income in relation to age for both groups of farmers (P<0.01). Age can be used as a proxy for experience, assuming that farmers are able to utilize infrastructure efficiently and have a better understanding of production practices with experience (Mango *et al.,* 2015). In this study, the small number of young people involved in agriculture is a cause for concern as this jeopardizes the future of smallholder agriculture.

Marital status of emerging farmers in the study area

A positive and statistically significant relationship was observed between marital status and agricultural income

(P<0.01) for farmers without infrastructure. This could be attributed to the fact that when one gets married, one's assets may increase and his or her partner could bring some income into the house. Such income could be used to procure more inputs, which could be used in the production activities of farmers, thus increasing agricultural income, even without available infrastructure.

Household members in farming activities

A positive and statistically significant relationship was observed between agricultural income and assistance for household members in farming for farmers with infrastructure (P<0.05) and farmers without infrastructure (P<0.01). The assistance of household members in farming enterprises is very critical for the productivity of smallholder farmers. Labor from household members is relatively affordable since in most cases household members have shares in the business and strive to make them as productive and efficient as possible. This is in line with the findings of Adepoju and Salman (2013), who asserted that household labor is statistically significant and positively influences productivity.

Physical infrastructure access index

The results in Table 3 show that access to physical infrastructure in relation to agricultural income is likely to cause a positive and statistically significant increase in agricultural income for both groups of farmers (P<0.01). This implies that any relative change in access to physical infrastructure in the study area caused agricultural income to respond positively. This is an indication that as farmers in the study area increase their access to physical infrastructure such as roads, fencing and irrigation equipment, they become technically efficient in their agricultural production,

TABLE 2. Stochastic frontier for factors affecting agricultural income in relation to availability of agricultural infrastructure.

	With infrastructure				Without infrastructure			
Independent variables	**Coefficient**	**Standard error**	**Z value**	***P*>|Z|**	**Coefficient**	**Standard error**	**Z value**	***P*>|Z|**
Constant	-1.176	0.406	-2.90	0.004***				
Physical infrastructure availability index	0.782	0.143	5.46	0.000***	0.737	0.148	4.97	0.000***
Social infrastructure availability index	0.617	0.200	3.07	0.002***	0.772	0.201	3.84	0.000***
Institutional infrastructure availability index	1.051	0.256	4.11	0.000***	0.614	0.215	2.85	0.004***
Equipment availability index	0.163	0.195	0.84	0.403	0.001	0.194	-0.00	0.996
Level of education	0.960	0.174	5.53	0.000***	0.891	0.179	4.98	0.000***
Access to extension services	1.355	0.293	4.62	0.000***	1.242	0.302	4.11	0.000***
Non-farm activities	0.123	0.304	0.41	0.685	0.286	0.311	0.92	0.358
Membership of farmers' organizations	0.591	0.295	2.01	0.045**	0.478	0.303	1.57	0.116
Age of farmers	0.054	0.011	4.78	0.000***	0.058	0.012	4.87	0.000***
Marital status of farmers	0.210	0.145	1.45	0.147	0.282	0.148	4.97	0.000***
Land tenure	-0.029	0.096	-0.30	0.765	0.030	0.098	0.31	0.755
Number of years in farming	-0.009	0.028	-0.03	0.761	-0.010	0.030	-0.33	0.738
Household members in farming	0.237	0.116	2.03	0.042**	0.326	0.117	2.79	0.005***
/lnsig2v	0.344	0.141	2.44	0.015**	0.423	0.141	3.01	0.003***
/lnsig2u	30.131	0.179	-0.02	0.987	-32.104	2.827	-0.01	0.991
Sigma _ v	1.188	0.084			1.236	0.087		
Sigma _ u	2.86e-07	0.000			1.07e07	0.000		
Sigma squared	1.410	0.198			1.526,995	0.215		
Lambda	2.41e-07	0.084			8.65e-08	0.087		
Log likelihood = -160.670					Log likelihood = -164.700			
Prob>chi2 = 0.000***					Prob>chi2 = 0.000***			
Wald chi2(13) = 9088.630					Wald chi2 (13) = 8386.04			
Number of observations = 89					Number of observations = 61			

***, ** and * represent significance at 1%, 5% and 10% level, respectively.

thus increasing their income. As stated by Fakayode *et al*. (2008), access is key to the efficient use of infrastructure; for instance, if there are communal grain silos in the area but the road networks are poor, farmers would not be able to access them, thus resulting in post-harvest losses.

Social infrastructure access index

Regarding access to social infrastructure in relation to agricultural income for farmers with access to infrastructure, any relative change in access to social infrastructure within the study area caused agricultural income to respond positively ($P<0.1$). This shows that with increased access to recreation, health and educational facilities for smallholder farmers in the study, farmers are likely to be healthier and make economically rational farming decisions, thus, improving their labor productivity. Kumar *et al*. (2015) asserted that, for social infrastructure, it is the accessibility of the services rather than their availability that is more important.

Equipment infrastructure access index

A positive and statistically significant relationship was observed between agricultural income and access to equipment infrastructure for farmers with ($P<0.01$) and those without ($P<0.05$) infrastructure. The reason for the increase in agricultural income is because agricultural equipment constitutes very important input in a farming enterprise. Farmers with access to inputs are likely to produce sufficiently and are able to harvest efficiently and yield more output, thus contributing positively to their farm income.

Level of education of smallholder farmers

Education had a positive significant influence on the agricultural income of smallholder farmers for both groups ($P<0.01$). As the level of education of smallholder farmers with access to infrastructure increased, the agricultural income of selected farmers also increased. This is due to the fact that educated farmers easily adapt to innovation and understand the fundamentals of production easier, thus implying that educated farmers were able to better utilize infrastructure that is made available to them. With better utilization of infrastructure, farmers in the study area were able to increase their agricultural income. In a similar study, Oduro-Ofori *et al*. (2014) indicated that a formal secondary education level was adequate for famers to comprehend technology used in agriculture, and extension education had more returns to agricultural productivity in Ghana. This result acknowledges the importance of education for the conception of the principles of basic production.

Access to agricultural extension services

The relationship between access to extension services and agricultural income was positive and statistically significant for both groups of farmers ($P<0.01$). Farmers with access to extension services received services such as technical support and training from extension officers. These services could assist farmers to expand their production activities, thus contributing to an increase in farm income. To deal with these challenges and to foster agricultural growth, especially in rural areas, agricultural advisory services (AASs) are brought to the center of today's international development discourse (Birner *et al*., 2009).

Membership of farmers' organizations

The results in Table 2 show a positive statistically significant relationship between agricultural income and membership in farmers' organizations with ($P<0.05$) and without infrastructure ($P<0.01$). Farmers who are members of farmers' organizations are able to share information on the utilization of agricultural infrastructure. Organizational training could be arranged to train farmers on the use of infrastructure. This finding concurs with that of Adepoju and Salman (2013) who found that membership in farmers' cooperatives also significantly increased agricultural productivity in a study conducted in Nigeria. Furthermore, Akankwasa *et al*. (2015) found that farmers who participated more in community-based organizations were likely to engage in social learning about technology, hence raising their likelihood to adopt the technology.

Age of smallholder farmers

This study revealed a positive statistically significant relationship between agricultural income and age for both groups of farmers ($P<0.01$). This could be attributed to the fact that as farmers become older, the greater their experience and exposure to the utilization of infrastructure, the more this contributes to the efficient utilization of infrastructure by farmers. The positive reaction of agricultural income to farmers without infrastructure is due to the fact that as farmers' age increases, they become more knowledgeable and gain skills in farming efficiently. The experience of farmers also contributes to efficient farming using indigenous knowledge even without sufficient access to infrastructure. Although Saiyut *et al*. (2018) obtained similar results in Thailand where farmers aged 15-19 were inefficient as compared to farmers over 60, farmers who have aged and subsequently become less productive, should also be considered.

TABLE 3. Stochastic frontier for factors affecting income in relation to accessibility to agricultural infrastructure.

	With infrastructure				Without infrastructure			
Independent variables	**Coefficient**	**Standard error**	**Z value**	***P*>|Z|**	**Coefficient**	**Standard error**	**Z value**	***P*>|Z|**
Constant	-1.187	0.359	-3.30	0.001***				
Physical infrastructure access index	1.288	0.191	6.72	0.000***	0.922	0.165	5.60	0.000***
Social infrastructure access index	0.385	0.228	1.69	0.091*	0.126	0.225	0.56	0.575
Institutional access index	-0.195	0.191	-1.02	0.306	-0.094	0.198	-0.48	0.635
Equipment infrastructure access index	0.623	0.209	2.98	0.003***	0.433	0.211	2.05	0.041**
Level of education	1.207	0.170	7.12	0.000***	1.246	0.178	7.00	0.000***
Access to extension services	1.640	0.310	5.30	0.000***	1.630	0.326	5.00	0.000***
Non-farm activities	0.259	0.321	0.81	0.420	0.180	0.337	0.53	0.593
Membership of farmers' organization	0.773	0.302	2.56	0.010**	0.860	0.316	2.72	0.007***
Age of farmers	0.059	0.012	4.93	0.000***	0.070	0.012	5.78	0.000***
Marital status of farmers	0.097	0.148	0.65	0.515	0.110	0.156	0.70	0.483
Land tenure	-0.029	0.110	0.26	0.794	0.038	0.114	0.33	0.739
Number of years in farming	-0.046	0.034	-1.37	0.171	-0.054	0.035	-1.54	0.123
Household members in farming	0.394	0.131	3.01	0.003***	0.338	0.136	2.48	0.013**
/lnsig2v	0.457	0.140	3.25	0.001***	0.560	0.141	3.98	0.000***
/lnsig2u	31.696	2.586	-0.01	0.99	28.538	0.113	-0.03	0.980
Sigma _ v	1.257	0.088			1.323	0.093		
Sigma _ u	1.31e-07	0.000			6.35e-07	0.000		
Sigma2	1.579	0.222			1.749	0.246		
Lambda	1.04e-07	0.088			4.80e-07	0.093		
Log likelihood = -166.386					Log likelihood = -171.568			
Prob>chi2 = 0.000***					Prob>chi2 = 0.000***			
Number of observations = 89					Number of observations = 61			

***, ** and * represent significance at 1%, 5% and 10% level, respectively.

The assistance of household members in farming activities

The study revealed that agricultural income was positively associated with a change in the number of household members assisting the farming enterprises for both groups of farmers ($P<0.01$). Barro (2001) stated that labor is a major factor of production in traditional farming systems and as such, laborers are key elements in increasing productivity. When household members assist in farming activities, their experience is retained on the farm, thereby increasing labor productivity which ultimately increases technical efficiency of farmers and agricultural income in the long run.

Conclusion

The Government of South Africa has rolled out different agricultural developmental programs including the Comprehensive Agricultural Support that enabled access to infrastructure for smallholder farmers so that they progress towards commercialization. There has been a lack of farm level information on how availability and accessibility of infrastructure affect the productivity of farmers. Therefore, this research was aimed at assessing the effects of availability and accessibility of agricultural infrastructure on emerging farmers' incomes. Stratified sampling was used to identify 89 farmers with infrastructure and 61 without infrastructure in the North West Province of South Africa. Data were collected using semi-structured questionnaires administered through face to face interviews and using a stochastic frontier model for data analysis.

It can be concluded that variables that have a greater influence on agricultural income regarding infrastructure availability include: physical infrastructure availability index, social infrastructure availability index, institutional infrastructure availability index, level of education, access to extension services, membership of farming organizations, age of farmers, and assistance of household members in farming enterprises. Regarding infrastructure accessibility, the variables that statistically significantly influenced agricultural income positively are physical infrastructure accessibility index, social infrastructure accessibility index, equipment accessibility index, level of education, access to extension services, and membership to farming organizations, age and number of household members in farming enterprises. The results show that infrastructure plays a critical role in assisting smallholder farmers to produce more efficiently, thereby improving agricultural income. The majority of smallholder farmers were over 35 years of age, indicating low participation of young people in farming. This is currently the trend for most areas in South Africa, and in most agricultural enterprises. There is a need for government to prioritize programs that will increase the participation of the youth in agricultural activities, particularly in commercial farming since younger farmers may find it easier to utilize infrastructure efficiently and optimally. There is a need to promote greater membership in farmers' organizations among smallholder farmers in the study area. This would assist in sharing information by smallholder farmers in the study area. Farmers who are part of a farmers' organization could also come together to lobby the government, development agencies, and commercial funders to assist with infrastructure funding.

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SCIENTIFIC NOTE

Climatic characterization and evaluation of the need for supplementary irrigation for cacao in southern Bahia, Brazil

Caracterización climática y evaluación de la necesidad de riego suplementario para el cacao en el sur de Bahía, Brasil

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ABSTRACT

Cacao is a typical plant of a tropical climate and demands ample water. Thus, in periods of water deficit, the need for supplementary irrigation can influence crop development. This study aimed to characterize the climate and evaluate the need for supplementary irrigation for cacao crops in the south of the state of Bahia (Brazil). For this research, historical data (2001-2017) for climate variables were obtained that are available at the weather station of the Executive Commission for Cacao Cultivation Planning (CEPLAC), located in southern Bahia. With the complete database, the monthly values for each variable were calculated and subjected to descriptive statistical analyses. Subsequently, the mean values for rainfall and air temperature were used to calculate potential evapotranspiration. The climatological water balance was then calculated, defining the periods of water deficit and surplus and whether supplementary irrigation was necessary or not, based on the total monthly water deficit. Overall, the region had a favorable climate for the development of cacao. However, this pattern did not continue throughout the historical series of climatic data, which showed temporal discontinuity, especially in February. The main months requiring supplementary irrigation for the cultivation of cacao grown in southern Bahia were February and December.

Key words: climate favorability, climatological water balance, *Theobroma cacao*, water deficit.

RESUMEN

El cacao es una planta típica del clima tropical y requiere abundante agua. Por lo tanto, en períodos de déficit hídrico, la necesidad de riego suplementario puede influir en el desarrollo de los cultivos. Este estudio tuvo como objetivo caracterizar el clima y evaluar la necesidad de riego suplementario para los cultivos de cacao en la región sur del estado de Bahía (Brasil). Para esta investigación, se obtuvieron datos históricos (2001-2017) de las variables climáticas, que están disponibles en la estación meteorológica de la Comisión Ejecutiva para la Planificación del Cultivo del Cacao (CEPLAC), ubicada en el sur de Bahía. Con la base de datos completa, se calculó el valor mensual de cada variable y se sometió a un análisis estadístico descriptivo. Posteriormente, los valores medios de lluvia y temperatura del aire se usaron para calcular la evapotranspiración potencial. Luego, se calculó el balance hídrico climatológico, definiendo los períodos de déficit y exceso de agua y si el riego suplementario era necesario o no, con base en el déficit hídrico mensual total. En general, la región tenía un clima favorable para el desarrollo del cacao. Sin embargo, este patrón no continuó a lo largo de la serie histórica de datos climatológicos, que mostró discontinuidad temporal, especialmente en febrero. Los principales meses que requirieron riego suplementario para el cultivo de cacao cultivado en el sur de Bahía fueron febrero y diciembre.

Palabras clave: favorabilidad climática, balance hídrico climatológico, *Theobroma cacao*, déficit hídrico.

Introduction

A higher water demand coupled with water supply constraints influence water availability for various sectors including agriculture. The rational and optimized use of water in the agricultural sector requires planning within the various systems of production, ensuring a continuous supply and increased water productivity for each crop (Souza *et al*., 2013).

Conducting a survey on the climatic characteristics of the place where one intends to begin or expand agricultural production is extremely important, since it aims to describe atmospheric phenomena on a temporal scale, identifying critical periods for decision-making in crop management. Based on this, the water availability of a region can be quantified by the climatological water balance (CWB) (Santos *et al*., 2010) that shows seasonal variations through relationships between the inputs and outputs of a control

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condition, mainly rainfall and potential evapotranspiration (Castro *et al.*, 2010).

Thus, CWB is an appropriate methodology for classifying dry and rainy periods of a locality or region, since it assumes the indication of water deficit or surplus in the soil and may consider other climatic variables besides the total rainfall regimen (Lopes *et al.*, 2017). This knowledge is important for the management of crops located in the region, especially cacao grown under shade trees, *cabruca cocoa*, which is the most significant and potential cacao in southern Bahia (Chiapetti, 2018).

Cacao is a typical plant of tropical climate and demands ample water. It is very sensitive to the lack of soil moisture (Souza *et al.*, 2009). Additionally, studies on the requirements for supplementary irrigation for this crop in humid regions are still incipient. Thus, in periods of water deficit the implementation of a supplementary irrigation system might have benefits ensuring the sustainability of the system of production over the years. However, the decision to irrigate must also consider other technical and socioeconomic aspects.

The aim of this study was to perform a climatic characterization and to evaluate the need for supplementary irrigation for cacao crops in the southern region of the state of Bahia (Brazil).

Materials and methods

For this study, historical data (2001-2017) on rainfall, mean, maximum and minimum air temperatures, relative humidity, wind speed at 10 m height, and maximum wind speed were obtained. These are available at the weather station of the Executive Commission for Cacao Cultivation Planning (CEPLAC), located in southern Bahia between the municipalities of Ilheus and Itabuna. The climate of the region is tropical rainforest Af, according to the Köppen classification (Alvares *et al.*, 2013), with a mean annual rainfall of 1,830 mm, mean annual relative humidity of 80%, and mean air temperature of 23.5°C. The daily potential evapotranspiration estimated by the modified method of Penman is 3.42 mm/d (Tagliaferre *et al.*, 2012).

To develop the database of this study, analyses were performed considering the values of the accumulated monthly variables of the historical series of climatic data. The accumulated monthly totals were divided by the number of years of observation, to obtain the mean monthly values of the variables, including daily rainfall. Results were subjected to descriptive statistical analyses, aiming to establish the mean values and the variation around the mean score (standard deviation) of all variables for each month during the period from 2001 to 2017.

Mean rainfall and mean air temperature values were used to calculate the standard potential evapotranspiration (ETPp) for each month by means of the Thornthwaite method (Thornthwaite and Mather, 1955) (Eq.1 to 3).

$$ETPp = 16\left(10 \times \frac{Ti}{I}\right)^{a} \qquad Ti > 0°C \qquad (1)$$

$$I = \sum_{i=1}^{12} (0.2 \times Ti)\ 1.514 \qquad Ti > 0°C \qquad (2)$$

$$a = 6.75 \times 10^{-7} \times I^{3} - 7.71 \times 10^{-5} \times I^{2} + 1.7912 \times 10^{-2} \times I + 0.49239 \quad (3)$$

where: Ti is the mean monthly temperature (°C), I is an index that expresses the heat level of the region, and exponent "a", in function of I, is also a regional thermal index.

To convert ETPp to potential evapotranspiration (ETP), a correction for days of the month and photoperiod was performed. The photoperiod was calculated as a function of the site's latitude and solar declination (Eq. 4).

$$ETP = ETPp \times \frac{N}{12} \times \frac{1}{30} \qquad (4)$$

where: N is the photoperiod during the hours of a given day.

CWB for the historical series was calculated according to a methodology proposed by Thornthwaite and Mather (1955), assuming a storage capacity of water available in the soil of 50 mm. The need for supplementary irrigation for cacao cultivation was based on the total monthly water deficit. We estimated that the number of months needed for supplementary irrigation would be conditioned on the total monthly water deficit being equal to or higher than 250 mm - a range defined by Despontin (2018) with a potential risk that could harm cacao crops. Considering the 17-year historical series, when converting this information into mean values, the main condition was that the value for monthly water deficit was equal to or higher than 14.7 mm.

Results and discussion

The mean values and standard deviation for monthly and daily rainfall from the CEPLAC weather station for 2001 to 2017 are presented in Table 1.

TABLE 1. Descriptive statistics of monthly and daily rainfall for the period from 2001 to 2017.

	Monthly rainfall (mm)		Daily rainfall (mm)	
Month	**Mean**	**SD**	**Mean**	**SD**
January	151.6	76.3	4.4	11.71
February	133.4	82.93	4.1	11.77
March	153.0	69.15	5.7	18.2
April	137.6	69.1	5.0	33.77
May	112.3	51.38	3.8	21.01
June	145.1	82.77	3.3	15.93
July	145.5	54.13	4.4	16.79
August	107.9	55.33	3.5	20.28
September	75.9	46.7	2.7	21.78
October	120.4	68.76	3.5	17.42
November	143.5	77.47	4.1	19.1
December	137.2	115.9	5.3	22.66
Mean	130.3	70.82	4.1	19.88
Total	1563.4		49.8	

SD - standard deviation.

Regarding the annual distribution, the climate of the region showed well-distributed rainfall throughout the year. The months with the highest and lowest rainfalls were March (late rainy season and September (late dry season, having 153.0 and 75.9 mm of precipitation, respectively. Thus, using this data interpretation, by adding up the rainfall volumes for the rainy season months (December, January, February, and March), a higher value (17.5%) was observed in relation to the dry season months (June, July, August, and September).

Regarding the standard deviation of the mean daily rainfall, it is possible to observe that April showed a higher value, indicating that this month showed a larger amplitude of data in relation to the mean, and was different from January and February, which showed the lowest values. These results corroborate those found by Correa and Galvani (2017), who also found higher standard deviation values for the transition period between the rainy and dry seasons.

Rainfall is the meteorological element that shows the greatest variability both in quantity and in monthly and annual distribution, especially in tropical regions. Rainfall variations occur due to the behavior of regional atmospheric circulation throughout the year, along with local or regional geographic factors (Almeida *et al*., 2017). When analyzing the spatiality of rainfalls in southeastern Bahia from 1978 to 2008, Santos and França (2011) found an east-west variation of quite distinct rainfall values, in the range of 1,000 to 2,000 mm per year. The coastal range had the highest values, confirmed by the present study.

Table 1 shows that the region has high annual rainfall, benefiting cacao tree culture since the crop has a minimum water demand of 1,200 mm. In addition to needing high annual rainfall, cacao trees require the water supply to be distributed throughout the year in order to meet its needs at different phenological stages, especially from anthesis to fruit maturation, with a required monthly rainfall of approximately 100 mm (Souza *et al*., 2009). Based on this assumption, only September showed a rainfall distribution lower than the ideal one. This requires more accurate monitoring of cacao crops in the southern region of the

TABLE 2. Descriptive statistics for mean, maximum and minimum air temperatures for the period from 2001 to 2017.

	Mean air temperature (°C)		Maximum air temperature (°C)		Minimum air temperature (°C)	
Month	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
January	24.7	0.65	30.5	0.83	21.6	0.73
February	24.7	0.74	30.5	1.08	21.5	0.69
March	24.8	0.64	30.7	0.96	21.7	0.62
April	24.1	0.55	30.1	0.66	21.4	0.76
May	22.9	0.96	28.6	1.14	20.3	0.96
June	21.5	0.44	27.2	0.63	18.9	0.51
July	20.9	1.01	26.9	1.81	17.9	0.73
August	20.8	0.36	26.6	0.38	17.7	0.54
September	21.9	0.75	27.6	0.76	18.8	1.20
October	23.0	0.56	28.7	0.86	19.8	0.77
November	23.9	0.81	29.1	1.32	21.0	0.57
December	24.6	0.70	30.2	1.38	21.2	0.60
Mean	23.1	0.68	28.9	0.98	20.1	0.72

SD - standard deviation.

state regarding plant disturbances. In addition, specific studies related to the presence of water deficit are also needed, since deficits can damage productivity.

Table 2 shows the mean monthly values for mean, maximum and minimum air temperatures. The mean temperatures were below 22°C from June to September (dry season period), and August was the month with the lowest maximum and minimum temperature, - 26.6 and 17.7°C, respectively.

The rainy season had the highest mean temperatures of the year, especially in March, which had the highest mean rainfall (Tab. 1). This result is associated with a lack of seasonality of the phenomenon of southern Bahia with no well-defined seasons, especially in the late rainy season, which can have torrential rain on sunny days (with short intervals). A torrential rain increases soil saturation and consequently causes a delay or suppression of cacao flowering. This is a common occurrence in the region (Santos and Sodré, 2017).

Temperature and rainfall are the climatic elements that most influence the growth and production of cacao trees. Since rainfall can be supplemented by irrigation, several researchers claim that temperature, among all climatic elements, is the most critical for cacao growth and production, and this affects vegetative growth, flowering, and fruit development (Almeida and Valle, 2007).

The ideal mean temperature for good development and production of cacao is up to 25°C, and the absolute minimum temperature should not be below 10°C with a minimum mean above 15.5°C in the coldest month (Souza *et al*., 2009). The values observed in Table 2 are within the range recommended for cacao cultivation, confirming an aptitude for cacao cultivation in the region (Almeida and Valle, 2007).

Table 3 shows the mean monthly values and standard deviation for the variables of atmospheric relative humidity, wind speed at 10 m height, and maximum wind speed. It was obvious that the atmospheric relative humidity showed little variation for the means between months. The month with the highest mean value for humidity was June (early dry season), and the one with the lowest mean value was February (mid-wet season) with values of 92.8 and 88.9%, respectively, and a difference of 3.93%.

There is no scientific evidence for direct effects of relative humidity on the physiology of cacao plants. However, this climatic variable directly influences the development of fungal diseases, such as brown rot (*Phytophthora spp.*) and witches' broom (*Moniliophthora perniciosa*) disease (Bridgemohan and Mohammed, 2019). During rainy years, these diseases occur frequently in several cacao-producing cities in the southern region of the state, but particularly when the relative humidity of the air exceeds 80%, the diseases are regularly observed during all months of the year.

TABLE 3. Descriptive statistics for atmospheric relative humidity, wind speed at 10 m height, and maximum wind speed for the period from 2001 to 2017.

	Relative humidity (%)		Wind speed at 10 m height (m/s)		Maximum wind speed (m/s)	
Month	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
January	89.3	6.25	2.4	2.64	3.6	1.23
February	88.9	7.65	2.0	1.72	3.8	1.33
March	91.1	5.39	1.8	1.98	3.0	1.02
April	92.2	5.18	1.8	2.39	2.7	1.17
May	92.7	5.39	1.6	2.12	2.7	1.14
June	92.8	5.28	1.6	2.25	2.7	0.96
July	92.6	4.93	1.7	2.17	2.8	1.08
August	91.8	4.71	2.5	3.25	3.2	1.1
September	91.2	4.57	3.0	3.88	3.3	1.96
October	90.4	5.8	2.7	3.57	3.2	1.29
November	90.8	5.48	1.6	1.5	3.7	1.24
December	90.2	5.46	1.5	1.38	3.8	1.03
Mean	91.2	5.5	2.0	2.4	3.2	1.21

SD - standard deviation.

This shows that the region has an environment favorable to the development of such diseases.

The mean values for the maximum wind speeds were high during the rainy season and February and December were the months with the highest value (3.8 m s^{-1}). April, May and June were the months with the lowest values (2.7 m s^{-1}). This result confirms the study of Pereira *et al*. (2016), who found that June showed the lowest value for maximum wind speed when evaluating the mean monthly data of climatic variables.

Amongst the climatic variables, winds can be problematic for cacao trees as they can cause great damage to crops, resembling what happens with most cultivated plants. The action of high wind speeds hinders the formation and maintenance of cacao trees, since the young plants are the most sensitive. Serra (2004) states that cold winds can cause cacao leaves to burn, thus reducing the photosynthetic area of the plant and its yield. Furthermore, according to this author, the occurrence of strong winds causes the leaves to be torn and flowers to fall. The installation of wind-breaks is the most appropriate way to control such damage.

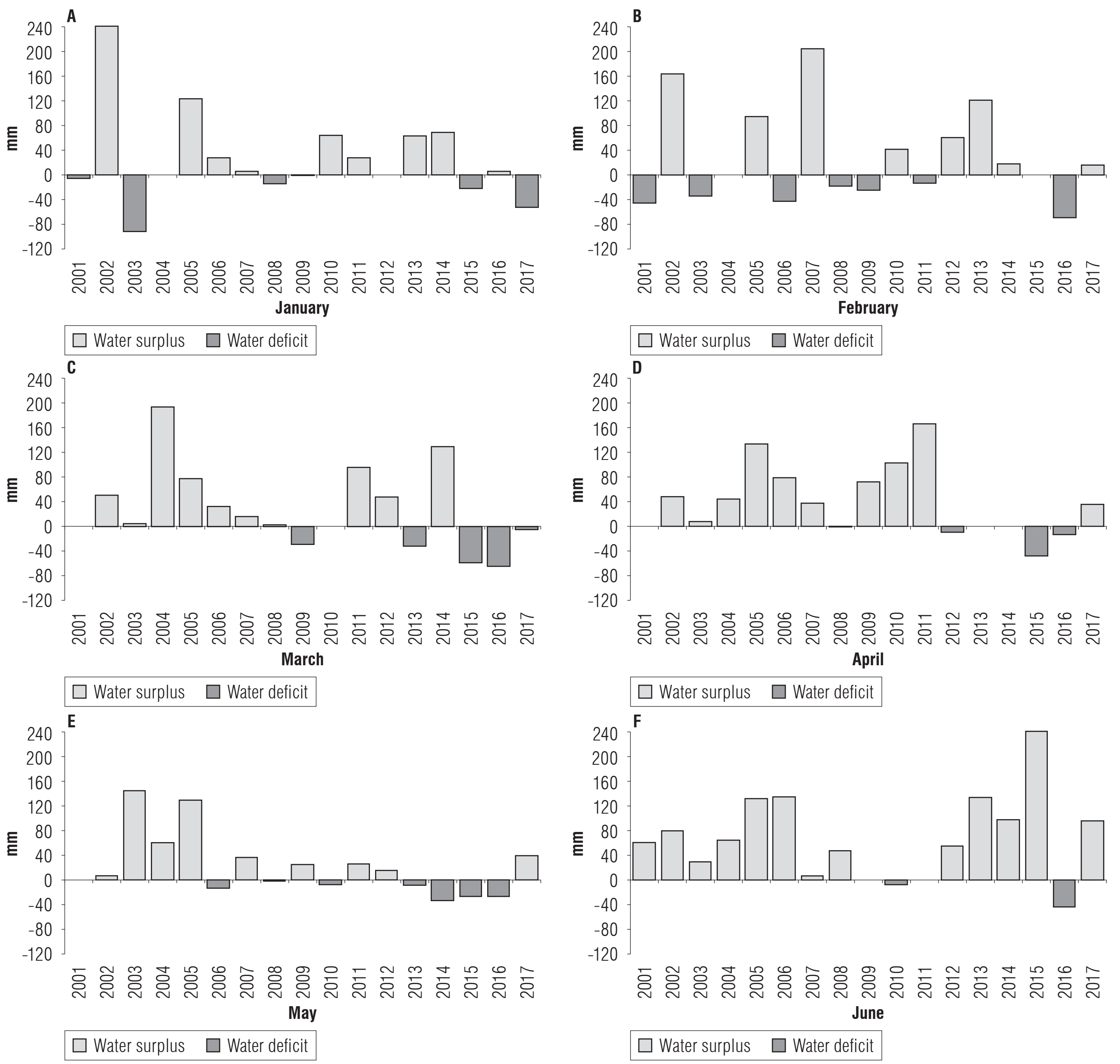

FIGURE 1. Histograms for water deficit and surpluses obtained by the CWB from January to June for the period from 2001 to 2017.

Windbreaks are recommended when the wind speed at 10 m height is greater than 2.5 m s^{-1}, a situation that occurred only in October (2.7 m s^{-1}).

However, in the cacao system of production that prevailed in the southern part of the state, winds are not usually a problem for crop development. Cacao trees are grown in a system commonly called *cabruca*, where they are planted under large trees remnant of the Atlantic Forest, and these large trees act as natural windbreaks (Guimarães *et al.*, 2017).

Figures 1 and 2 show histograms for water deficit and surplus obtained by the CWB month by month for the historical series from 2001 to 2017. Only the year 2007 had no deficit for any month, whereas 2015 had the highest number of months when water loss was higher than the precipitation with seven months of deficit (average of 95.0 mm), especially in November. The year 2015 can be considered an atypical period in the historical series, since southern Bahia had a prolonged rainfall shortage that generated high water stress in plants, especially during the early rainy season (fundamental for fruit picking)

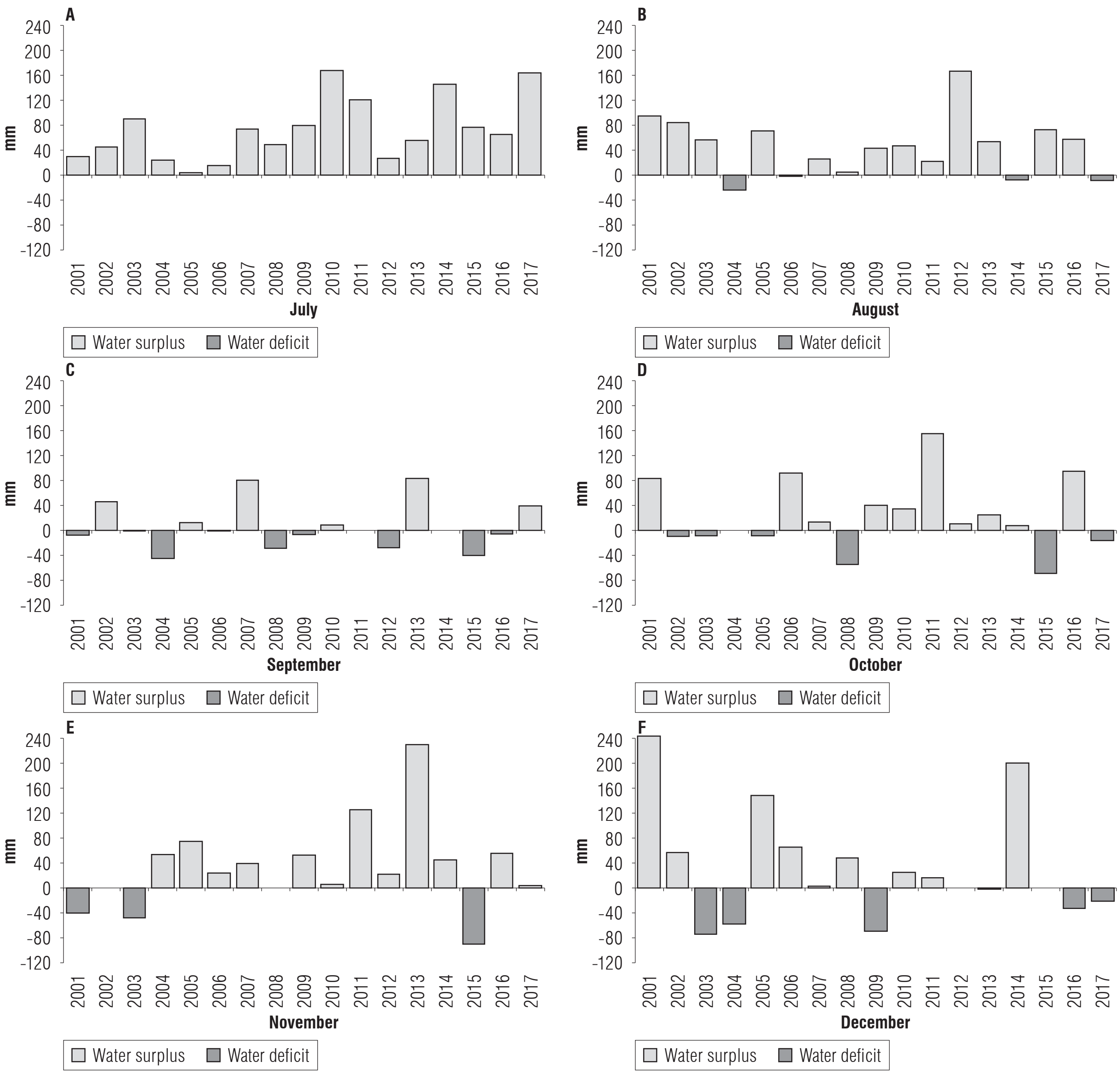

FIGURE 2. Histograms for water deficits and surpluses obtained by the CWB from July to December for the period from 2001 to 2017.

and directly compromising the production planning for cacao crops.

All histograms exhibited periods of water deficit and surplus throughout the evaluated years (except for July), demonstrating a non-homogeneous tendency of the climate of the region. One year a month could show high water deficit, and the next it could be quite rainy. This fact stands out in February, as it showed the largest deficit between months in the mean of the years, with values of 47.0 mm (2001) and 43.5 mm (2006). In the subsequent years (2002 and 2007), this was the month that displayed the highest mean values of water surplus (166.4 and 207.5 mm, respectively).

When this behavior is observed in a historical series, there is an indication that the distribution of the phenomenon is discontinuous, deviating from an expected pattern. Temporal discontinuous behavior in an historical series of climatic data may be associated with the influence of atypical periods of distribution and/or with a natural condition of the phenomenon under study. When the second condition is the predominant one, special attention should be paid to February since cacao trees under conditions of low water storage in the soil show a reduction in fruit production during this period, providing fewer fruits at the end of the main harvest (Santos and Sodré, 2017). A reduction in fruit production in cacao is associated with physiological and metabolic changes suffered by plants under water stress conditions. Water stress affects the internal availability of water in plants, translocation of assimilates, source-sink relationships and the development of flowers and fruits (Bridgemohan and Mohammed, 2019).

Throughout the historical series, February and December are the months indicated for supplementary irrigation, since they were the only ones with total water deficit above the recommended limit, 253.2 mm (Fig. 1) and 255.4 mm (Fig. 2), as well as mean water deficits, 14.9 mm and 15.0 mm, respectively. As observed in Table 1, these months showed on average low to medium rainfall with relation to the other months. They also displayed an absence of seasonality, i.e., a behavior of high variation between years (mainly February). This impairs crop development at fundamental phenological phases and highlights the importance of the implementation of a supplementary irrigation system for cacao trees in order to provide water during periods of high-water deficit.

February corresponds to the end of the harvest and the period when the cacao tree is producing new leaves and flowers and is physiologically preparing to produce the second crop of the year (known as *safra temporã*). Thus, soil water deficit during this month may reduce the total yield and cause young fruit mummification in adult plants, premature leaf fall, basal leaf yellowing, and reduced growth (Carr and Lockwood, 2011).

Santos and Sodré (2017) state that the constant water deficit during the early part of the rainy season (which involves a large period in December) over the years makes the cacao fruit vulnerable to a disorder known as "cherelle wilt". According to the authors, this disorder usually occurs in cacao plantations in southern Bahia in the first 90 d after fruit picking and reaches its maximum state between 40 and 70 d after flower fertilization. The disorder causes a nutritional imbalance in the plant. The authors also state that during months of high-water deficit (December), soil water storage may remain close to or below 5% of the water available to cacao plants and may result in fruits with disturbances.

It is important to consider that investment in a supplementary irrigation system in humid and subhumid regions, such as the cacao plantations considered in this study, may be economically viable for applying the fertigation technique, allowing a greater technological level for the crop production to be reached. This could result in increased productivity and fertilizer application efficiency and reduced labor costs. Additionally, the use of this technique may ensure production stability over the years.

Overall, CWB proved to be a very important technique for planning cacao cultivation. This is relevant for indicating the need for supplementary irrigation in some periods of water deficit in the region. However, further studies are recommended to estimate the necessary water level and how it will be distributed to fill this deficit during dry periods. Such studies will generate pioneering and interpretable information on the different phenological stages of cacao tree cultivated under the *cabruca* system for the various producers in the southern region of Bahia.

Conclusions

In general, southern Bahia has a favorable climate for the cultivation of cacao. However, this pattern does not continue throughout the historical series of climatic data, rather the series shows a temporal discontinuity during some months, especially in February. The main months requiring supplementary irrigation for cacao cultivation grown in southern Bahia were February and December.

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SCIENTIFIC NOTE

Effect of the use of pre- and post-emergence herbicides on nodulation and production of cowpea (*Vigna unguiculata* L.) in the Amazonian savannah

Efecto del uso de herbicidas pre- y post-emergentes en la nodulación y productividad del frijol caupí (*Vigna unguiculata* L.) en la sabana amazónica

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ABSTRACT

As a control measure against weeds, the use of herbicides is an effective and inexpensive alternative. However, there are no products recommended for the cultivation of cowpea in Brazil, making it necessary to search for alternative solutions. The objective of this study was to evaluate the effect of herbicides applied in the pre- and post-emergence on cowpea nodulation and production under conditions of the Amazonian savannah. Two experiments were carried out in a randomized block design with four replicates, using the cowpea cultivar BRS Aracê subjected to the pre-emergence herbicides: Metribuzin, Sulfentrazone, S-metolachlor, Pendimethalin, Oxadiazon, Alachlor, Metribuzin + Pendimethalin, Metribuzin + Alachlor and Quizalofop-p-ethyl, Bentazon, Fomesafen, Imazethapyr, Imazamox + Bentazon, Quizalofop-p-ethyl + Imazethapyr, Quizalofop-p-ethyl + Imazamox and Quizalofop-p-ethyl + Bentazon, and post-emergence herbicides: Quizalofop-p-ethyl, Bentazon, Fomesafen, Imazethapyr, Imazamox + Bentazon, Quizalofop-p-ethyl + Imazethapyr, Quizalofop-p-ethyl + Imazamox, and Quizalofop-p-ethyl + Bentazon. The number of nodules in each plant, the dry matter of nodules, dry matter of roots and the grain yield were evaluated. According to the results obtained, the management of weeds in pre- or post-emergence according to the herbicide used affects the nodulation and productivity of cowpea under the conditions of the Amazonian savannah. The herbicides Metribuzin in pre-emergence, and Fomesafen and the mixture of Quizalofop-p-ethyl + Imazethapyr in post-emergence are not recommended for weed control in cowpea. The application of Oxadiazon, Alachlor, and Pendimethalin in pre-emergence can be considered interesting because they do not inhibit the development of the root system or the nodulation of cowpea which provides a greater grain yield. Regarding weed control strategies at post-emergence, the application of the herbicide Imazethapyr and the combination of the herbicides quizalofop-p-ethyl + imazamox, Quizalofop-p-ethyl + Bentazon and Imazamox + Bentazon allow satisfactory levels of grain yield, root system development and nodulation of cowpea.

Key words: chemical control, nitrogen fixation, weeds.

RESUMEN

Como medida de control contra las malezas, el uso de herbicidas es una alternativa efectiva y asequible. Sin embargo, no hay productos recomendados para el cultivo de frijol caupí en Brasil, por lo que es necesario buscar soluciones alternativas. El objetivo de este estudio fue evaluar el efecto de herbicidas aplicados en pre- y post-emergencia sobre la nodulación y productividad del frijol caupí bajo las condiciones de la sabana amazónica. Se realizaron dos experimentos en bloques al azar con cuatro repeticiones, utilizando el cultivar de frijol caupí BRS Aracê sometido a los herbicidas pre-emergentes: Metribuzina, Sulfentrazona, S-metolaclor, Pendimetalina, Oxadiazón, Alaclor, Metribuzina + Pendimetalina, Metribuzina + Alaclor y Quizalofop-p-etil, Bentazona, Fomesafen, Imazetapir, Imazamox + Bentazona, Quizalofop-p-etil + Imazetapir, Quizalofop-p-etil + Imazamox y Quizalofop-p-etil + Bentazona, y herbicidas post-emergentes: Quizalofop-p-etil, Bentazona, Fomesafen, Imazetapir, Imazamox + Bentazona, Quizalofop-p-etil + Imazetapir, Quizalofop-p-etil + Imazamox, Quizalofop-p-etil + Bentazona. Se evaluaron el número de nódulos por planta, la masa seca de nódulos, la masa seca de raíces y el rendimiento de grano. Según los resultados obtenidos en este estudio, el manejo de malezas en pre o post-emergencia de acuerdo con el herbicida utilizado afecta la nodulación y el rendimiento de grano de frijol caupí bajo condiciones de la sabana amazónica. Los herbicidas Metribuzina en pre-emergencia, y Fomesafen y la mezcla de Quizalofop-p-etil + Imazetapir en post-emergencia no se recomiendan para el control de malezas en frijol caupí. La aplicación de Oxadiazón, Alaclor y Pendimetalina en la pre-emergencia puede considerarse interesante porque no inhiben el desarrollo del sistema radicular o la nodulación de frijol caupí lo que proporciona un mayor rendimiento de grano. En lo relacionado con las estrategias de control de malezas posteriores a la emergencia, la aplicación del herbicida Imazetapir y la combinación de los herbicidas Quizalofop-p-etil + Imazamox, Quizalofop-p-etil + Bentazona e Imazamox + Bentazona permiten niveles satisfactorios de rendimiento de grano, desarrollo del sistema de raíces y nodulación de frijol caupí.

Palabras clave: control químico, fijación de nitrógeno, malezas.

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Introduction

Cowpea (*Vigna unguiculata* L. Walp.; Fabaceae) is a widely adapted legume tolerant to water stress (Yadav *et al*., 2017; Oparaeke *et al*., 2018). Because of this, this crop has become very important socioeconomically and is widely cultivated in the northern, northeastern and mid-western regions of Brazil (Dias *et al*., 2019).

Historically, in Brazil, cowpea has been cultivated in areas of insufficient and poorly distributed rainfall and low fertility soils. It is mainly cultivated by small producers using low technology such as manual harvesting, low quality seeds, and inefficient control of pests, diseases and weeds (Costa *et al*., 2017). Because of this, average crop productivity in Brazil is between 300 and 400 kg ha^{-1}, considered low compared to its productive potential of at least 1000 kg ha^{-1} when grown with advanced technologies (Mancuso *et al*., 2016).

One of the main problems faced by producers in the northern parts of Brazil is weed interference (Costa *et al*., 2017). Since sowing is carried out using wider spacing, crops show slow initial growth, allowing weeds to compete directly with cowpea for space, water, light and nutrients (Lamego *et al*., 2011; Linhares *et al*., 2014), and consequently, reduce the crop yield by more than 66% (Gonzaga *et al*., 2018).

Although manual weed removal is a proven effective method of weed control, labor unavailability and its very high cost limit its use. Thus, integrated weed management could be a better approach for reducing yield losses in cowpea because of weeds (Gupta *et al*., 2016). Chemical weed control (i.e. use of herbicides) has proven to be the most practical, effective and economical way to control accessible or inaccessible plants or harmful weeds (Yadav *et al*., 2017). There are no registered herbicides for cowpea in Brazil, which prevents recommendations; however, 27 and 55 herbicides are registered for crops belonging to the same botanical family (Fabaceae) such as the common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.), respectively (Gonzaga *et al*., 2018).

Herbicides are products that interfere in the biochemical and physiological processes of weeds and can kill or significantly retard their growth (Fontes *et al*., 2010; Monteiro *et al*., 2012). However, their effects on soil microorganisms, especially nitrifying bacteria, can be adverse due to the large number of environmental factors involved in this process, which can be harmful, beneficial or null (Reis *et al*., 2010). Diseases usually occur due to nitrifying microorganisms that share biosynthetic routes similar to those of the host plant (Santos *et al*., 2006).

The action of herbicides on microorganisms can occur mainly by the indirect absorption of endosymbionts in plants treated with these substances. Absorption via soil solution is less expressive, since the inactivation of some molecules in this environment is fast (Reis *et al*., 2010). However, the main mechanism for dissipating herbicides in the soil is through microbial degradation. Procópio *et al*. (2015) report that herbicides can affect the formation and growth of root hairs and this affects the process of infection by nitrifying bacteria.

The mechanisms of dissipation, persistence and transformation of herbicides in the environment and in the plant are complex and deserve special attention since they affect directly and indirectly the nodulation of plants of the family Fabaceae. Thus, knowledge about the tolerance of bacteria to herbicides and the effects of these substances on cowpea nodulation should be studied to support the use of efficient weed control techniques in this crop (Fontes *et al*., 2010; Rodrigues and Almeida, 2017).

Therefore, the objective of this study was to evaluate the effect of herbicides applied in the pre- and post-emergence on cowpea nodulation and production under the conditions of the Amazonian savannah.

Materials and methods

Two experiments were carried out at the Agricultural Sciences Center of the Federal University of Roraima, Cauame Campus, Boa Vista, Roraima, Brazil (2°52'15.49" N, 60°42'39.89" W, and 85 m a.s.l., with annual mean precipitation of 1678 mm). One of the experiments used pre-emergence herbicides and the other used post-emergence herbicides.

The soil of the experimental area was classified as Yellow Udox Soil (EMBRAPA, 2018), clayey texture, with the following physical and chemical attributes (depth 0 to 0.2 m): pH (H_2O) = 5.4; Ca^{2+} = 0.50 $cmol_c$ dm^{-3}; Mg^{2+} = 0.3 $cmol_c$ dm^{-3}; K^+ = 0.02 $cmol_c$ dm^{-3}; Al^{3+} = 0.8 $cmol_c$ dm^{-3}; (H + Al) = 2.62 $cmol_c$ dm^{-3}; P_2O_5 = 30 mg dm^{-3}; sum of bases (SB) = 0.40 $cmol_c$ dm^{-3}; base saturation (V) = 14%; clay = 185 g kg^{-1} (18.5%); silt = 50 g kg^{-1} (5.0%), and sand = 765 g kg^{-1} (76.5%). Based on the interpretation of the chemical analysis, the soil was amended using 1,500 kg ha^{-1} of limestone, 50 kg ha^{-1} of FTE BR 12 (Nutriplant®, Barueri, Brazil), 90 kg ha^{-1} of P_2O_5 in the form of simple superphosphate and 60 kg ha^{-1} of K_2O in the form of potassium chloride.

The experiment was carried out in a randomized design with four replicates. The treatments consisted of ten weed control strategies, with the application of eight pre-emergence herbicides or eight post-emergence herbicides on cowpea, in addition to the plots with and without weed control (Tab. 1). Each experimental plot consisted of six rows of 5 m of length, spaced 0.5 m apart, in which the ten central rows were considered as the useful area.

The cowpea cultivar used in the experiments was BRS Aracê, indicated for cultivation in the State of Roraima. It shows a semi-pruned growth, with an average yield of 1,246 kg ha^{-1}. A mechanized planter was used for sowing in a no-tillage system, adjusting the planting density to 10 bean seeds per linear meter. Before seeding, the seeds were inoculated with 500 g of inoculum for 50 kg of seeds moistened with a sugar solution (10%), with a minimum concentration of 108 cells g^{-1} of *Bradyrhizobium* BR 3262, a strain recommended for the State of Roraima by Zilli *et al.* (2006).

The pre-emergence herbicides were applied one day after planting and the post-emergence herbicides were applied 14 d after sowing when the cowpea plants had formed the third clover and the dicotyledon weeds had three to five pairs of leaves. Applications were performed using two Turbo TeeJet® (TT) 110.02 nozzles spaced 0.5 m with a pressure of 2 bars and a syringe volume of 170 L ha^{-1}. Both herbicides were applied in the evening, between 17:00 and 18:00 h, due to the lower temperature and higher humidity of the air and soil at that time. Weeding was performed in the weed-free plots, while the control plots with weeds (weedy check) were maintained in cohabitation with weeds throughout the cycle.

TABLE 1. Herbicides used in pre-emergence as a strategy to control weeds in cowpea, their modes of action, and the crops for which they are registered (MAPA, 2019).

	Mode of action	Crop	Dose (g ha^{-1} a.i.)
	Pre-emergence herbicides		
Metribuzin (M)	Inhibition of photosynthesis - photosystem II	Soybean	360
Sulfentrazone (S)	Cell membrane disruption - protoporphyrinogen oxidase (PPO) inhibitor	Soybean	600
S-metolachlor (S-m)	Inhibition of very-long-chain fatty acid (VLCFA) (inhibition of cell division)	Soybean, common bean	1200
Pendimethalin (P)	Inhibition of mitosis and cell division, inhibition of microtubule assembly	Soybean	750
Oxadiazon (O)	Inhibition of protoporphyrinogen oxidase (PPO), leading to irreversible cell membrane damage	-	1000
Alachlor (A)	Inhibition of very-long-chain fatty acid (VLCFA) (inhibition of cell division)	Soybean, peanut	2400
Metribuzin + Pendimethalin (M + P)	Inhibition of Photosystem II + Microtubule assembly	Soybean	360 + 750
Metribuzin + Alachlor (M + A)	Inhibition of Photosystem II + Inhibition of very-long-chain fatty acid (VLCFA)	Soybean	360 + 2400
	Post-emergence herbicides		
Quizalofop-p-ethyl (Q)	Inhibition of acetyl CoA carboxylase (ACCase)	Soybean, common bean, peanut	100
Bentazon (B)	Inhibition of photosynthesis (photosystem II)	Soybean, common bean, peanut	720
Fomesafen (F)	Protoporphyrinogen oxidase (PPO) inhibitor	Soybean, common bean	225
Imazethapyr (Ir)	Inhibition of acetolactate synthase (ALS), acetohydroxyacid synthase (AHAS)	Soybean, common bean	100
Imazamox + Bentazon (Ix + B)	Inhibition of acetolactate synthase (ALS), acetohydroxyacid synthase (AHAS) + inhibition of photosynthesis (photosystem II)	Soybean, common bean, peanut	50
Quizalofop-p-ethyl + Imazethapyr (Q+Ir)	Inhibition of acetyl CoA carboxylase (ACCase) + inhibition of acetolactate synthase (ALS) (acetohydroxyacid synthase (AHAS))	Soybean, common bean, peanut	100 + 100
Quizalofop-p-ethyl + Imazamox (Q+Ix)	Inhibition of acetyl CoA carboxylase (ACCase) + inhibition of acetolactate synthase (ALS) (acetohydroxyacid synthase (AHAS))	Soybean, common bean, peanut	100 + 168
Quizalofop-p-ethyl + Bentazon (Q+B)	Inhibition of acetyl CoA carboxylase (ACCase) + inhibition of photosynthesis (photosystem II)	Soybean, common bean, peanut	100 + 720
	Non herbicide treatments		
Weed-free (manual weeding)	-	-	-
Weedy check	-	-	-

The evaluation of the effect of herbicides on nodulation was performed 30 d after planting (DAP). At the time, the plants were harvested with the roots using a straight blade and then the roots were washed using a stream of water. After that, the roots were separated from the shoot by a cut at the base of the stem; the nodules were removed and counted to determine the number of nodules. The nodules and roots were dried in a forced air circulation oven (TE-394/5-MP, Tecnal®, Piracicaba, Brazil) for 72 h at 65°C until reaching a constant mass for the determination of the dry matter of nodules and roots.

To estimate grain yield, the pods were harvested and threshed manually according to maturity; then, the dry matter of the beans was weighed to determine the yield, expressing the values in kg ha^{-1}.

The results were subjected to the Shapiro-Wilk normality test; the visual control evaluation data were arcsine square root-transformed and presented with mean separation based on transformed values. The data were subjected to an ANOVA and when the variances were significant, the averages were compared by the Tukey's test at $P \leq 0.05$. The Pearson correlation matrix was also applied using the R software (R Development Core Team, 2018).

Results and discussion

The use of pre-emergence and post-emergence herbicides in the cowpea crop significantly interferes with the characteristics evaluated in this research.

In the case of pre-emergence herbicides, we observed a higher mean number of nodules in the weed-free and weedy check treatments, and with alachlor, pendimethalin and oxadiazon application which did not differ statistically from each other (Tab. 2). All the plants died in the metribuzin treated plots and, therefore, did not show a number and dry matter of nodules, dry matter of roots or yield (Tab. 2). According to Souza Cruz *et al.* (2018), metribuzin and

TABLE 2. Mean values of number of nodules (Nn) per plant, dry matter of nodules (DMn), dry matter of roots (DMr) and grain yield (Y) in response to the application of pre- and post-emergence herbicides in cowpea plants with weed-free and weedy check treatments.

Pre-emergence herbicides	**Nn**	**DMn (g)**	**DMr (g)**	**Y (kg ha^{-1})**
Metribuzin	0.00 d	0.00 f	0.00 f	0.0 d
Sulfentrazone	128.75 c	0.53 e	6.76 cde	617.0 c
S-metolachlor	234.50 b	1.53 cd	5.79 e	1,315.0 b
Pendimethalin	351.50 a	2.32 b	8.68 ab	1,437.0 b
Oxadiazon	336.50 a	1.71 c	7.91 bc	1,669.0 a
Alachlor	360.50 a	2.44 ab	7.85 bcd	1,377.0 b
Metribuzin + Pendimethalin	266.75 b	0.47 e	6.35 e	396.0 c
Metribuzin + Alachlor	262.25 b	1.63 c	5.89 e	560.0 c
Weed-free	366.00 a	2.72 a	9.36 a	1,682.0 a
Weedy check	364.50 a	1.16 d	6.60 de	566.0 c
VC (%)	8.01	11.03	7.98	21.93
Post-emergence herbicides	Nn	DMn (g)	DMr (g)	Y (kg ha^{-1})
Quizalofop-p-ethyl	339.25 ab	1.60 bcd	7.18 bc	1,405.0 b
Bentazon	347.00 ab	1.97 b	5.96 c	1,139.0 cd
Fomesafen	323.00 ab	1.32 d	6.39 c	1,001.0 cde
Imazethapyr	159.25 c	1.91 bc	11.78 a	1,256.0 bc
Imazamox + Bentazon	364.75 ab	1.74 bcd	11.76 a	1,210.0 bcd
Quizalofop-p-ethyl + Imazethapyr	192.75 c	1.71 bcd	7.15 bc	949.0 de
Quizalofop-p-ethyl + Imazamox	315.25 b	1.93 b	6.63 c	1,453.0 ab
Quizalofop-p-ethyl + Bentazon	199.25 c	1.46 cd	11.29 a	1,404.0 b
Weed-free	375.50 a	2.60 a	8.33 b	1,711.0 a
Weedy check	366.50 ab	2.05 b	6.54 c	916.0 e
VC%	7.91	10.39	7.11	17.10

(VC%) = variation coefficient; means followed by the same letter in the column do not differ from each other according to the Tukey test at 5% probability ($P \leq 0.05$).

all its mixtures are not recommended as pre-emergence weed control strategies in cowpea because they are not selective to this crop. However, Costa *et al*. (2017) report that metribuzin and sulfentrazone are promising for cowpea selectivity; such disagreements may be related to the cowpea cultivars used in the cited works and their differential behavior in relation to the herbicide molecule.

As for the effect of post-emergence herbicides, we also observed the highest number of nodules in the weed-free and weedy check treatments, but these treatments did not differ from the plots with the application of imazamox + bentazon, bentazon, quizalofop-p-ethyl and fomesafen. These results corroborate the studies of Santos *et al*. (2006) in which no harmful effects on nodulation were observed after the application of the herbicides bentazon (14.19 mg L^{-1}), imazamox (0.69 mg L^{-1}) and fomesafen (4.92 mg L^{-1}). Monteiro *et al*. (2012), when evaluating the effect of bentazon on the number of nodules, observed that the herbicide shows low phytotoxicity to cowpea and has little influence on the nodulation of the crop. However, these authors observed a decrease in the number of nodules by the application of the herbicide fomesafen, which was not observed in our study. The lowest numbers of nodules were observed in the plots treated with the herbicides sulfentrazone and metribuzin.

The application of metribuzin resulted in the death of all the plants of the plot, as observed by Fernandes *et al*. (2012) in common bean at the dose of 1440 g ha^{-1}. The effect of the use of the herbicide metribuzin mixed with the herbicides pendimethalin and alachlor was also harmful to cowpea. Herbicide mixtures are common in crops; however, few farmers are aware of the negative effects of this management on microorganisms, especially in cowpea.

Table 2 shows that the weed-free treatment, and alachlor and pendimethalin applied in pre-emergence showed the highest mean dry matter of nodules. Opposite results were obtained with the application of sulfentrazone and metribuzin + pendimethalin that negatively affected nodulation in cowpea. Nodule dry matter was significantly impaired by both weed coexistence and the use of any herbicide in the post-emergence period, since the weed-free treatment was better than all the other treatments.

The highest mean dry matter of roots was verified in the weed-free treatment compared to the pre-emergence herbicides and weedy check treatments. On the other hand, the plots treated with imazethapyr, imazamox + bentazon and quizalofop-p-ethyl + bentazon in post-emergence showed mean dry matter of roots larger than in the weed-free treatment (Tab. 2). This indicates that pre-emergence herbicides possibly caused antagonistic effects, leading to root death. However, the use of herbicides in post-emergence may even inhibit the healthy development of roots, causing them to thicken and to increase their dry mass. However, low yield was still observed due to the decrease of nutrient absorption by root hairs.

The most damaging effects for the dry mass of nodules and roots in post-emergence herbicide applications were verified for fomesafen and bentazon, respectively. Monteiro *et al*. (2012) evaluated the effect of bentazon on dry mass of roots and nodules and observed that the herbicide showed low phytotoxicity to cowpea and had little influence on nodulation and biomass of the crop, while the herbicide fomesafen caused significant loss of dry matter of nodules, corroborating the results in our study.

The highest grain yields were obtained with the application of the herbicide oxadiazon in pre-emergence and with the post-emergence mixture of quizalofop-p-ethyl + imazamox. However, neither treatment differed from the weed-free treatment (Tab. 2). Similar yield results were obtained by Simplício *et al*. (2016), but the results in this study were significantly higher than in all herbicide-free weeds.

The results for grain yield showed that all treatments were satisfactory when compared to the coexistence of the crop with the weeds that significantly reduced productivity. Similar results were obtained by Gonzaga *et al*. (2018), who observed a 67% reduction productivity in cowpea yield with weeds present. According to Lamego *et al*. (2011), the low yield in some treatments can be explained by the phytotoxicity exhibited by the crop, which reduces growth and causes the death of some plants, affecting productivity.

Bandeira *et al*. (2017) conclude that cowpea cv. BRS Aracê shows tolerance to the herbicide quizalofop-p-ethyl and moderate tolerance to imazethapyr and the formulated bentazon + imazamox. However, in our study, the isolated application of imazetapyr reduced productivity by 26.56% compared to the weed-free treatment and the application of quizalofop-p-ethyl reduced productivity by 17.85%. The application of the herbicide mixture quizalofop-p-ethyl + imazethapyr promoted more severe phytotoxicity effects, reducing yield at the same level as the weedy check treatment and the application of fomesafen.

According to Gupta *et al*. (2016), the mixture of the herbicides quizalofop-p-ethyl + imazethapyr promotes a lower

TABLE 3. Pearson correlation matrix for the number of nodules (Nn), dry matter of nodules (DMn), dry matter of roots (DMr) and grain yield (Y) in cowpea plants with herbicide application in pre- and post-emergency.

	Pre-emergence herbicides				Post-emergence herbicides			
	Nn	DMn	DMr	Y	Nn	DMn	DMr	Y
Nn	-	0.354 ns	-0.448 ns	0.129 ns	-	0.775 **	0.864 **	0.700 *
DMn	-	-	-0.082 ns	0.442 ns	-	-	0.924 **	0.549 ns
DMr	-	-	-	0.280 ns	-	-	-	0.781 **
Y	-	-	-	-	-	-	-	-

** Significant at $P \leq 0.01$, * significant at $P \leq 0.05$, and ns not significant according to the t test.

dry mass of weeds and is efficient in its control (84%). However, the isolated use of the herbicide imazethapyr shows higher production of cowpea. Nevertheless, there is a lack of information that can assist farmers in the proper use of these mixtures.

The low yields in the fomesafen-treated plots reflect the symptoms of severe phytotoxicity presented by them. These results are similar to those of Linhares *et al.* (2014) in which the herbicide causes intoxication of the cowpea crop, foliar necrosis and consequent loss of productivity. According to Estorninos *et al.* (2010), fomesafen delays cowpea growth and shows low efficiency in the control of weeds in the long term. However, according to Mancuso *et al.* (2016) fomesafen applied at the initial stage of crop establishment is efficient in controlling weeds. In the present study, the phytotoxicity of fomesafen provided yields of grains similar to those obtained with the weedy check treatment.

Pre-emergence mixtures of metribuzin + pendimethalin and metribuzin + alachlor provided better results than the single application of metribuzin, suggesting the need for further studies on the interaction between these active principles.

Although there was no direct correlation between the variables with the pre-emergence application of the herbicides (Tab. 3), there were positive correlations among all variables in the post-emergence application except for the dry mass of nodules and yield. This suggests that the increase of one of the variables directly and positively affected the others, so that the increase in the number of nodules increased the dry matter of nodules and roots and the yield of the crop. The issue of nodules, although directly affecting the matter of the roots, does not affect the yield, and the matter of the roots directly affects the grain yield of cowpea, as observed by Simplício *et al.* (2016), who cited that a larger number of nodules, but of smaller size (matter) is more important for grain yield than a smaller number of nodules with a larger size.

Conclusion

The management of weeds in pre- or post-emergence according to the herbicide used affects the nodulation and productivity of cowpea under the conditions of the Amazonian savannah.

The herbicide metribuzin in pre-emergence, and fomesafen and the mixture of quizalofop-p-ethyl + imazethapyr in post-emergence are not recommended for weed control for cowpea.

The application of oxadiazon, alachlor, and pendimethalin in pre-emergence is interesting because they do not inhibit the development of the root system or the nodulation of cowpea, which provides greater grain yield.

In post-emergence weed control strategies, the application of the herbicide imazethapyr and the combination of the herbicides quizalofop-p-ethyl + imazamox, quizalofop-p-ethyl + bentazon and imazamox + bentazon allow satisfactory levels of yield, roots system development and nodulation of cowpea.

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Nutritional characterization of *Moringa oleifera* leaves, seeds, husks and flowers from two regions of Mexico

Caracterización nutricional de hojas, semillas, cáscara y flores de *Moringa oleifera* de dos regiones de México

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ABSTRACT

Moringa oleifera (MO) is a native tree species found in the south of the Himalayas, India, Bangladesh, Afghanistan and Pakistan that is used commercially to produce tea, animal fodder and herbal medicines. The aim of the present study was to determine the nutritional characteristics of different parts of the MO plant from two regions of Mexico (States of Guerrero and Sonora). The proximal composition analyses (protein, lipids, ashes and fiber) were carried out according to the methods of the Association of Official Analytical Chemists (AOAC), and the antioxidant capacity and phenolic compounds were determined by spectrophotometric methods. Protein (38.5%) and lipid contents (42.2%) were higher in MO seeds compared to other plant parts. These results were similar between the two regions of Mexico that were evaluated. MO flowers from Guerrero showed the highest content of phenols and tannins (1908.71 mg Gallic Acid Equivalents (GAE) 100 g^{-1}, 220.27 mg Catechin Equivalents (CE) 100 g^{-1}, respectively) while MO leaves from Sonora showed the highest flavonoid content (2859 mg GAE 100 g^{-1}). The Trolox Equivalent Antioxidant Capacity (TEAC) analysis showed that MO husks obtained from Guerrero had the highest antioxidant capacity (224.45 µmol Trolox Equivalents (TE) g^{-1}). The Oxygen Radical Absorbance Capacity (ORAC) test showed that MO flowers from Guerrero had the highest antioxidant capacity (382 µmol (TE) g^{-1}). The differences between MO from the two different regions reported in this study could be attributed to factors such as soil type, climatic conditions and the growing region.

Key words: Trolox Equivalent Antioxidant Capacity, Oxygen Radical Absorbance Capacity, phenols, flavonoids, proximate composition.

RESUMEN

La *Moringa oleifera* (MO) es una especie arbórea nativa del sur del Himalaya, India, Bangladesh, Afganistán y Pakistán, utilizada comercialmente para producir té, forraje para animales y medicinas herbales. El objetivo del presente estudio fue determinar las características nutricionales de diferentes partes de la planta de MO provenientes de dos regiones de México (Estados de Guerrero y Sonora). Los análisis de composición proximal (lípidos, proteínas, cenizas y fibra) se realizaron en base a las metodologías establecidas por la Asociación de Químicos Analíticos Oficiales (AOAC), y la determinación de capacidad antioxidante y compuestos fenólicos se realizó por métodos espectrofotométricos. Los contenidos de proteínas (38.5%) y lípidos (42.2%) de las semillas de MO fueron los más altos en comparación con otras partes de la planta. Estos resultados fueron similares entre las dos regiones de México que fueron evaluadas. Las flores de MO de Guerrero mostraron el mayor contenido de fenoles y taninos (1908.71 mg Equivalentes de Ácido Gálico (EAG) 100 g^{-1}, 220.27 mg Equivalentes de Catequina (EC) 100 g^{-1}, respectivamente) mientras que las hojas MO de Sonora mostraron el mayor contenido de flavonoides (2859 mg EAG 100 g^{-1}). El análisis de Capacidad Antioxidante Equivalente de Trolox (CAET) mostró que las cáscaras de MO obtenidas en la región de Guerrero tienen la mayor capacidad antioxidante (224.45 µmol Equivalentes de Trolox (ET) g^{-1}). La prueba de Capacidad de Absorción de Radicales de Oxígeno (CARO) señaló que las flores de MO de Guerrero tienen la mayor capacidad antioxidante (382 µmol ET g^{-1}). La diferencia entre el MO de las dos regiones diferentes reportadas en este estudio podría atribuirse a factores como el tipo de suelo, las condiciones climáticas y la región de siembra.

Palabras clave: Capacidad Antioxidante Equivalente de Trolox, Capacidad Absorción Radicales de Oxígeno, fenoles, flavonoides, composición proximal.

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Introduction

Moringa oleifera (MO) belongs to the Moringaceae family and is mainly distributed in Latin America, Asia, and Africa (Tao-Bin *et al.*, 2018). The high concentration of nutrients in leaves, pods and seeds, as well as its rapid growth in poor soils with a pH of 5.0 to 9.0 and resistance to droughts, have transformed this plant into an attractive partial solution to problems such as malnutrition in the most vulnerable sectors of the population (Rashid *et al.*, 2008).

Fresh MO leaves have 7 times more vitamin C than oranges, 10 times more vitamin A than carrots, 17 times more calcium than milk, 9 times more protein than yogurt, 15 times more potassium than banana and 25 times more iron than spinach. Therefore, MO has been widely used for human and animal consumption (Fahey, 2005; Anwar *et al.*, 2007; Moyo *et al.*, 2011; Bonal *et al.*, 2012; Gopalakrishnan *et al.*, 2016). MO seeds have around 33-41% oil, containing high concentrations of oleic acid (>70%). MO oil is commercially known as "ben oil" due to its content of behenic acid (docosanoic), which shows high resistance to oxidation, has various medicinal uses, and high nutritional value. In addition, seed residues generated during oil extraction contain a peptide that has proved to be one of the best naturally occurring coagulating agents (Rashid *et al.*, 2008; Sánchez-Machado *et al.*, 2010).

Some parts of the MO plant, such as leaves, pods and seeds, have several medicinal properties. Therefore, these plant parts have been used to treat diseases such as hypertension, diabetes, anemia, fertility problems, neurodegenerative, liver and kidney diseases as well as skin disorders and cancer (Gowrishankar *et al.*, 2010). In 2007, MO was named the "Botanical of the Year" by the National Institute of Health (NIH) due to its nutraceutical and medical properties (Gupta *et al.*, 2018).

In recent years, Mexico has increased MO production, mainly in the states of Sonora and Guerrero, supporting the commercialization of the edible parts of the plant and the development of new products from them (Sánchez-Machado *et al.*, 2010). However, factors such as growing conditions, climatic change, location of crops and soil type promote different nutritional components in the plant. Moreover, when high temperatures are reached, enzyme denaturation occurs (Gopalakrishnan *et al.*, 2016). The aim of this work was to evaluate the nutritional quality (proximate composition, phenolic compounds, and antioxidant capacity) of different parts of the MO plant from two different regions of the Mexican Republic and to offer reliable information to the consumer and producer who use parts of MO in their products or as a nutritional supplement.

Materials and methods

Sample collection

Leaves, seeds and husks were collected from places in two Mexican states (Etchojoa, Sonora and Acapulco, Guerrero) in November, 2016 (Fig. 1). Due to the collection date,

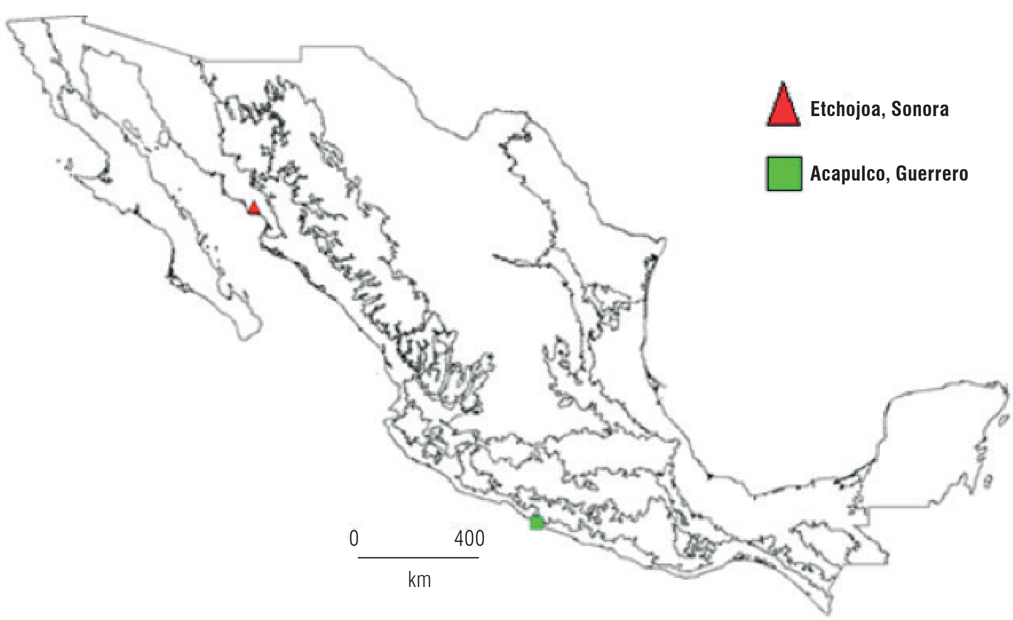

FIGURE 1. Geographic regions of *Moringa oleifera* collecting points.

flowers could only be collected from Guerrero. Samples were dried at 60°C for 12 h in an electric oven (DP43C, Yamato Scientific America, Santa Clara, USA). The dry samples were milled (electric mill, NutriBullet, Los Angeles, USA) and collected in closed plastic bags (Ziploc®) at room temperature, and then stored in a cabinet to avoid light, until their analysis.

Proximate composition

The proximal analysis of the samples was performed following the AOAC methods for protein (960.52), fat (920.85) and ash (923.03) (AOAC, 2000). Total dietary fiber was calculated based on the gravimetric enzymatic method according to Prosky *et al.* (1998), using a thermostable α-amylase and amyloglucosidase (Merck, Darmstadt, Germany). Insoluble dietary fiber was determined by the same procedure, but methanol (Merck, Darmstadt, Germany) was not added after the enzymatic treatment. Finally, soluble dietary fiber was calculated by subtracting the total dietary fiber from the insoluble dietary fiber. Carbohydrates were estimated as the remaining percentage reached 100% of the proximal composition (FAO/WHO, 2004).

Phenolic compounds

Total phenol determination was carried out following the Folin-Ciocalteu spectrophotometric method described by Singleton and Rossi (1965), using gallic acid as a standard for the calibration curve, with an optimal concentration range of 0.05 to 0.2 mg ml^{-1}. The crude extract was obtained by adding 100 mg of the sample in 10 ml of methanol (30%) in deionized water. The sample was stirred for 10 min at 8000 rpm, then centrifuged for 10 min (5000 rpm), and the supernatant was recovered. An aliquot of 125 µl of the crude extract was added to 500 µl of deionized water and stirred for 15 seconds (3000 rpm). Afterwards, 125 µl of Folin-Ciocalteu reagent was added to the sample which was maintained for 6 min in the dark; then 1.25 ml of Na_2CO_3 (7%) + 1 ml of deionized water were added and the sample was left in the dark for 90 min. Finally, the absorbance at 750 nm was registered using a spectrophotometer (6405 UV/Vis, JENWAY, San Diego, USA). The results were expressed as mg of Gallic Acid Equivalents (GAE) 100 g^{-1} of sample (mg GAE 100 g^{-1}) (R^2 = 0.9991).

Condensed tannins were determined following the vainillin method proposed by Hagerman *et al.* (1989), using (+) catechin as a standard in concentrations from 0.0039 to 1 mg ml^{-1}. A crude extract was obtained from 200 mg of dry sample in 10 ml of absolute methanol and stirred at 8000 rpm for 20 min. Later, 1 ml of the crude extract was mixed with 5 ml of freshly prepared vanillin reagent (1% vanillin in methanol and 8% hydrochloric acid in methanol 1:1) in a falcon tube. The tube was kept in a water bath for 20 min at 30°C. Finally, the absorbance at 500 nm was read in a spectrophotometer. At the same time, a correction factor with 1 ml of the crude extract mixed with 5 ml of hydrochloric acid (4%) in methanol (instead of vanillin reagent) was evaluated. The final concentration was expressed as mg (+) Catechin Equivalents (CE) 100 g^{-1} of the sample (mg CE 100 g^{-1}) (R^2 = 0.9997).

Total flavonoids were determined according to the method proposed by Borrás-Linares *et al.* (2015), using a standard curve of (+) catechin in a range of concentration from 0.015 mg ml^{-1} to 0.5 mg ml^{-1}. The crude extract was obtained from 200 mg of dry sample in 5 ml of aqueous methanol (60:40) mixed in a falcon tube and stirred for 10 min using a vortex. Later, the tube was sonicated for 30 min at room temperature and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was recovered and 250 µl of this supernant + 75 µl of $NaNO_2$ (5%) was poured into a test tube and stirred. After, 150 µl of $AlCl_3$ (10%) + 500 µl of NaOH (1M) + 1.52 ml of deionized water were added to the tube to total 2.5 ml which was placed in repose for 5 min. The final concentration of flavonoids was expressed in mg (+) catechin equivalents 100 g^{-1} of the sample (mg CE 100 g^{-1}).

Antioxidant capacity (TEAC and ORAC)

Trolox equivalent antioxidant capacity (TEAC) or $ABTS^+$ antioxidant capacity was determined following the method described by Van der Berg *et al.* (1999). The crude extract was obtained with 20 mg of sample in 5 ml of acetone/water (1:1 v/v) mixed in a test tube. The mix was then sonicated for 10 min and centrifuged at 5000 rpm for 5 min. The radical $ABTS^+$ was prepared dissolving 38 mg of the reactive ABTS (Merck, Darmstadt, USA) in 1 ml of potassium persulphate 2.45 mM in deionized water. To determine the antioxidant capacity, 0.15 ml of the $ABTS^+$ solution was diluted in 14 ml of phosphate buffer at pH 7.4. The resultant absorbance was 0.7±0.020 at 734 nm. Then, the absorbance at initial time (t=0) was measured in 990 µl of the radical $ABTS^+$ solution, and 10 µL of the sample was immediately added. The mix was left to rest for 6 min and the absorbance at 734 nm (t=6) was measured. Antioxidant capacity was calculated by comparing the absorbance values of the sample to a Trolox standard (TS) curve (100 µM TS g^{-1} to 3000 µM TS g^{-1}) (R^2 = 0.9903).

The ability to trap peroxyl radicals (ROO°) or oxygen radical absorbance capacity (ORAC) was determined according to Ou *et al.* (2001). The crude extract was obtained with 50 mg of sample in 5 ml of acetone/water (1:1 v/v) mixed in a test tube. The mix was then sonicated for 10 min and centrifuged at 5000 rpm for 5 min and the supernant was recovered. To standardize the crude extract, 15 µl was taken and diluted with 1.98 ml of phosphate buffer solution (pH 7.4). Afterwards, 1.5 ml of fluorescein solution was collected (7.7 µl of stock fluorescein solution, 0.5315 mM in 50 ml of crude extract) and placed in a quartz cell.

In the same quartz cell, 0.75 ml of diluted crude extract was added. The cell was placed in a water bath for 5 min at 37°C and 0.75 ml of AAPH (2,2'-azobis (2-amidinopropane) HCl) (0.415 g of AAPH in 10 ml of phosphate buffer solution) was added. The first reading was made in the fluorometer (TBS-380, Turner Biosystems, Massachusetts, USA) (λ excitation= 493 and λ emission= 515) and the sample was returned to the water bath. The sample was read every minute until the value corresponded to 10% of the initial value. Results were expressed in Trolox equivalent micromoles per gram of dry sample (µmol Trolox g^{-1}) using a standard curve (R^2 = 0.9971).

Statistical analysis

All analyses were performed in triplicate (n = 3) and standard deviation was determined. Additionally, a principal component analysis was carried out using NTSYSpc, version 2.20f, to elucidate main traits that intervened in the differences of MO parts and origins. This multivariate statistical analysis factorized the standardized variance and covariance matrix in their Eigenvalues and Eigenvectors to show the total variability of all experimental units, MO parts and origins. The reduced Euclidean planes allow comparing the means of the distance of the projection of each experimental unit in the three principal components three Eigenvectors with the highest Eigenvalues (Jonhson, 1998; Núñez-Colín and Escobedo-López, 2014).

Results and discussion

Proximal composition

Ashes

There was a difference between ash contents from MO leaves obtained in Sonora and Guerrero (Tab. 1). Guzmán-Maldonado and Fuentes (2015) analyzed samples from Celaya and Guanajuato, Mexico, reporting an ash content of 12.3%. Moreover, Sánchez-Machado *et al.* (2010) studied samples from Sonora, Mexico reporting ash contents of 14.60% in MO leaves. It is possible that Guerrero's environmental conditions (the type of weather and soil, sowing region, and crop conditions) may have affected the ash content. MO seeds showed an ash content of 4.48% for Sonora and 4.31% for Guerrero. These values were lower than those reported by Abdulkarim *et al.* (2005) in samples from Selangor, Malaysia (6.5%) and Sánchez-Machado *et al.* (2010) in Sonora, Mexico (7.62%). The analysis of the seed shell showed ash values of 2.96% and 4.92% for Sonora and Guerrero, respectively. The ash content of MO flowers collected in Guerrero was 8.86%, which is similar to the 9.68% reported by Sánchez-Machado *et al.* (2010).

The results suggested that there is an influence on the ash contents according to the sowing region, as can be observed in the results obtained from MO leaves and seed shells. Faizi *et al.* (1998) report that it is normal to find these types of differences in the results due to the sowing region. Lalas and Tsaknis (2002) mention that factors such as the soil

TABLE 1. Proximal composition (%, dry base) of the different structures of *Moringa oleifera**.

Samples	Ashes	Ethereal extracts	Proteins	Soluble fiber	Insoluble fiber	Carbohydrates
			Seeds			
Etchojoa, Sonora	4.48 ± 0.07	41.70 ± 0.71	38.55 ± 0.30	1.40 ± 0.08	5.41 ± 0.07	8.45 ± 1.19
Acapulco, Guerrero	4.31 ± 0.28	42.27 ± 0.018	38.57 ± 1.31	5.67 ± 0.02	3.35 ± 0.01	5.82 ± 0.81
			Husks			
Etchojoa, Sonora	2.96 ± 0.01	11.94 ± 0.02	7.59 ± 1.29	17.33 ± 1.29	28.51 ± 0.45	31.28 ± 0.04
Acapulco, Guerrero	4.92 ± 0.01	9.42 ± 0.88	8.69 ± 0.88	14.38 ± 0.51	35.65 ± 17.45	26.93 ± 2.70
			Leaves			
Etchojoa, Sonora	13.38 ± 1.10	10.76 ± 1.46	18.34 ± 0.02	8.31 ± 0.65	7.66 ± 0.68	41.58 ± 2.19
Acapulco, Guerrero	6.83 ± 0.03	6.95 ± 0.10	21.79 ± 0.34	9.01 ± 0.36	7.45 ± 0.05	48.45 ± 0.06
			Flowers			
Acapulco, Guerrero	8.86 ± 0.057	6.10 ± 0.23	14.94 ± 0.92	4.34 ± 0.18	7.91 ± 0.07	57.88 ± 1.17

*The values reported are the average of n = 3 ± standard deviation.

* The total fiber results are not shown in the table, but they represent the sum of soluble fiber + insoluble fiber.

type, water stress, plant age and genetic factors are reflected in the proximal composition of MO plants.

Proteins

The content of proteins in MO leaves was 18.34% for Sonora and 21.79% for samples from Guerrero. Makkar and Becker (1996) and Sena *et al.* (1998) report similar values to the ones obtained for MO leaves from Guerrero (25.10% and 22.90% from Nicaragua and Balayera, Nigeria, respectively). On the other hand, Reyes *et al.* (2006) report protein contents of 17.80% in samples from Managua, Nicaragua, resembling the percentage of protein observed in samples from Sonora. Previous studies have shown a variation in protein contents in MO leaves according to the planting area. Moyo *et al.* (2011) report protein contents of 30.3% in MO leaves from Africa, while Dhakar *et al.* (2011) report 27.1% in Pakistan. It is noteworthy that the protein content of MO leaves from Sonora and Guerrero is higher than the values reported in chard *(Beta vulgaris subsp.*) (2.9%) (Macías de Costa *et al*., 2003) or edible algae (5 to 13%) (Sánchez-Machado *et al*., 2004).

Moringa oleifera seeds showed a protein content of 38.55% (Sonora) and 38.57% (Guerrero); these values are similar to those reported by Amaglo *et al.* (2010) (35.7%) and Vaknin and Mishal (2017) (37.59%). However, the protein content of MO seeds from this study differs from the 20.66% value reported by Gidamis *et al.* (2003).

MO seed shells resulted in the lowest values of proteins compared to the other analyzed parts of the plant (7.59% for Sonora and 8.69% for Guerrero). The main applications of seed shells are for obtaining bioadsorbents (Do Carmo *et al*., 2013) and biofuels due to their high carbohydrate content (Raman *et al*., 2017). That is the reason why there are fewer reports related to the protein content of this MO part.

Moringa oleifera flowers showed a protein content of 14.94%. This value is lower than the one reported by Sánchez-Machado *et al.* (2010) (18.92%) in samples from Sonora, Mexico. It is not possible to make a broader comparison of the total protein content in MO flowers due to a lack of references. However, a related study found 9,443 peptides with molecular weights between 40-70 kDa, suggesting the possible use of these proteins in the fermentation industry as well as in the development of functional peptides (Shi *et al*., 2018).

Lipids

The lipid content in MO leaves showed differences between the evaluated regions (Tab. 1). Samples from Sonora showed a lipid content of 10.76%, whereas samples from Guerrero showed a content of 6.95%. Sánchez-Machado *et al.* (2010) report a value of 4.60% for lipid contents in MO leaves collected in Sonora, Mexico. Besides, Makkar and Becker (1996) report a percentage of 5.40% of lipids in MO leaves; this is lower than the lipid contents obtained in the present research. This variation could be attributed to the specific area of sowing, harvest season, the age of the plant, and the climate conditions of the region (Sánchez-Machado *et al*., 2010).

MO seeds from Sonora showed 41.70% lipids and the ones obtained from Guerrero showed 42.27% (Tab. 1). These values are similar to the ones reported by Anhwange *et al.* (2004) and Anwar and Bhanger (2003), which are 40.39% and 41.58%, respectively. In this research, no significant differences were found in lipid contents between the sowing areas. However, authors such as Bhutada *et al.* (2016) state that these contents may vary from 16% to 47% depending on the sowing regions.

The lipid content of MO seeds is a critical factor since it is one of the most interesting products of this plant, and the oil is currently accepted as a substitute for olive oil. Additionally, its lipid content is higher than the lipid content of soybean, which contributes to its nutritional importance (Vaknin and Mishal, 2017).

Seed shells collected in Sonora had a lipid content of 11.94% and those obtained from Guerrero showed values of 9.42%. It is noteworthy that seed shells contain significant amounts of lipids, but currently there are no reports related to the use of these parts. Nowadays, seed shells are mainly used for bioethanol production due to their high content of carbohydrates (39.8%) that can be converted into simple sugars (Martín *et al*., 2010).

The lipid content in MO flowers from Guerrero (6.10%) was higher than the 2.91% reported by Sánchez-Machado *et al.* (2010) and the contents reported by FAO (2003) (4.1%). It is important to mention that oil from MO flowers is a plentiful source of linoleic, oleic and linolenic acids (Sánchez-Machado *et al*., 2010), which gives the MO flower the potential for extraction of this oil, in addition to the acids extracted from the seeds.

Fiber

Total fiber contents (soluble fiber + insoluble fiber, Tab. 1) of leaf samples were 15.97% and 16.46% for the states of Sonora and Guerrero, respectively. The results are within the range (16% to 24%) reported by Aree and Jongrungruangchok

(2016) in MO leaves from Thailand, and they are also similar to the 19.2% reported by Dhakar *et al.* (2011) in samples from Pakistan. However, fiber contents in MO leaves were lower than the percentages reported by Guzmán-Maldonado and Fuentes (2015) and Sánchez-Machado *et al.* (2010), who mention values of 27.8% and 30.97%, respectively in MO leaves from the regions of Sonora and Guanajuato, Mexico. The percentage of fiber in MO leaves is high when compared to foods such as spinach (2.2%), cabbage (2.5%), chard (1.6%) and artichoke (5.4%) that are commonly recommended due to their high fiber content (Titchenal and Dobbs, 2005).

The fiber content in MO seeds was 6.81% in samples from Sonora and 9.02% in those obtained from Guerrero. These results are similar to the 9.0% reported by Anwar *et al.* (2006), and they are also within the range of 5.6% to 7.5% mentioned by Anwar and Bhanger (2003). Anudeep *et al.* (2016) point out that MO seeds have a high potential for food applications due to their high content of total dietary fiber (33%) and a high percentage of soluble fiber (6.5%). The parts with the highest fiber percentage were the seed shells with values of 45.84% for Sonora and 50.03% for Guerrero. Seeds have higher fiber contents compared to MO pods (39.2%) and cortex (33.1%), according to the data reported by Abdulkadir *et al.* (2016). The seed fiber contents are also similar to the fiber content (46.78%) reported by Sánchez-Machado *et al.* (2010) for MO pods in samples from Sonora, Mexico. Ali and Kemat (2017) used MO seed shells to obtain bioethanol due to their high content of lignocellulosic material. Warhurst *et al.* (1997) obtained activated charcoal with MO seed shells because of their high content of lignocellulose. These are the only applications performed to MO seed shells, and there are not many studies proposing an integral use of them.

The flower samples from the state of Guerrero showed a fiber content of 12.25%. This value is lower than that reported by Sánchez-Machado *et al.* (2010) of 32.45%. There are only a few reports that show the percentage of fiber in MO flowers. However, the percentages of fiber present in the Jamaican flower (8.5-12.0%) are similar to those obtained in the MO flowers, which makes MO a feasible alternative for adding crude fiber in the diet (Sáyago-Ayerdi and Goñi, 2010).

Carbohydrates

Moringa oleifera leaves had carbohydrate contents of 41.58% and 48.45% in the regions of Sonora and Guerrero, respectively (Tab. 1). Sánchez-Machado *et al.* (2010) report 35.3% in leaves collected from Sonora, Mexico and Guzmán-Maldonado and Fuentes (2015) report 27.05% in leaves obtained from Celaya, Mexico. Also, Gopalakrishnan *et al.* (2016) show an average carbohydrate content of 38.2% in MO leaves in their research. All these studies report lower values than those obtained in the present research. However, Toro *et al.* (2016) mention values of 52.10% of carbohydrate content for MO leaves grown in the region of Bolivar, Colombia.

The percentage of carbohydrates in MO seeds obtained from Sonora showed a value of 8.45%, while those obtained from Guerrero showed a value of 5.82%. These results are similar to the ones obtained by Olagbemide and Alikwe (2014) (8.67%) in seeds of MO cultivated in Ibadan, Nigeria. However, the results differ from the values of carbohydrate percentages reported by Aja *et al.* (2013) (18%) in seeds collected from Ebonyi, Nigeria. The highest percentage of carbohydrates reported for MO seeds were 40% in samples collected in the region of Irene, Pretoria, South Africa (Mabusela *et al.*, 2018), which differs from that obtained in the present work. These differences could be attributed to the different planting regions and crop conditions.

On the other hand, MO seed shells show carbohydrate contents of 31.28% in samples from Sonora and 26.93% in samples from Guerrero in Mexico. Abdulkadir *et al.* (2016) mention values of 26.9% of carbohydrates in the cortex and Sánchez-Machado *et al.* (2010) report carbohydrate values of 24.98% in MO seed pods. It is important to mention that there are no previous reports of carbohydrate analysis for MO seed shells; however, these results are similar to the ones obtained for seed pods and cortex.

For the percentage of carbohydrates in flowers, samples from Guerrero showed 57.88%. This value is higher than the one reported by Sánchez-Machado *et al.* (2010) (36.04%) and lower than the one indicated by Arise *et al.* (2014) (66.45-73.25%) in MO flowers collected in Kwara, Nigeria. MO flowers show a higher content of carbohydrates compared to the leaves, consistent with that reported in previous studies by Sánchez-Machado *et al.* (2010). A variation in the carbohydrate content related to the sowing region is notable in all the analyzed parts of the plant, and this is attributable to the geographical area of sowing, the time of cultivation, and the availability of water and fertilizer, since they are the main factors affecting the nutritional characteristics of the plant (Velázquez-Zavala *et al.*, 2016).

Phenolic compounds

Results obtained for total phenols, tannins and flavonoids of the different parts of the MO plant are shown in Table 2.

TABLE 2. Content of phenolic, tannins, and flavonoids in the different structures of *Moringa oleifera*.

Samples	Phenols (mg GAE 100 g^{-1})	Tannins (mg CE 100 g^{-1})	Flavonoids (mg GAE 100 g^{-1})
Seeds			
Etchojoa, Sonora	160.87 ± 14.13	ND	249.86 ± 22.08
Acapulco, Guerrero	184.05 ± 13.52	ND	149.47 ± 6.30
Husks			
Etchojoa, Sonora	3.65 ± 0.20	52.49 ± 3.56	273.85 ± 25.18
Acapulco, Guerrero	4.50 ± 0.42	57.46 ± 3.58	202.81 ± 18.91
Leaves			
Etchojoa, Sonora	20.25 ± 0.02	13.81 ± 0.004	1427.27 ± 53.44
Acapulco, Guerrero	26.14 ± 0.06	13.78 ± 0.009	2859.44 ± 220.63
Flowers			
Acapulco, Guerrero	1908.71 ± 110.74	220.27 ± 16.30	936.44 ± 88.03

GAE - Gallic Acid Equivalents; CE - Catechin Equivalents.

Moringa oleifera flowers showed the highest concentration of total phenols (1908.71 mg GAE 100 g^{-1}). The results were similar to the value reported by Alhakmani *et al.* (2013) (1931 mg GAE 100 g^{-1}) in MO flowers from Al Batinah, Oman. However, the obtained values differ from the results reported by Pakade *et al.* (2013) (2970 mg GAE 100 g^{-1}) for MO flowers from Limpopo and Atteridgeville, South Africa.

When comparing these results to other edible flowers, values are similar to those found in *Calendula officinalis* (120-150 mg GAE 100 ml^{-1}) (Alhakmani *et al.*, 2013) and *Hibiscus sabdariffa* (2400 to 10,000 mg GAE 100 g^{-1}) (Fernandes *et al.*, 2017). Likewise, MO flowers showed the highest concentration of condensed tannins (220.27 mg CE 100 g^{-1}); however, contents are lower than those reported for linden flowers (19.5 g GAE 100 g^{-1} of extract) and *Crataegus monogyna* flowers (11.5 g GAE 100 g^{-1} of extract), which are consumed as medicinal plants (Ropiak *et al.*, 2016). The anti-nutritional nature of tannins due to their capacity to form insoluble complexes with proteins and carbohydrates (Chaparro *et al.*, 2009) does not impede the consumption of MO flowers as food or herbal remedies since flowers do not provide important quantities of these compounds.

Regarding the results of flavonoids, MO leaves collected in Guerrero showed the highest values (2859.44 mg GAE 100 g^{-1}). They were twice as high as those obtained from leaves from Sonora (1427.27 mg GAE 100 g^{-1}). Cabrera-Carrión *et al.* (2017) studied the effect of age and height on the concentration of flavonoids in MO leaves and found values from 1183 to 3485 mg 100 g^{-1}, which are similar to the values obtained in leaf samples from both states. The flavonoid differences between the evaluated states can be attributed to conditions such as age, height and geographical region. Furthermore, the results are comparable to previous studies reporting a high content of phenolic compounds and flavonoids in MO leaves (Gopalakrishnan *et al.*, 2016; Cabrera-Carrión *et al.*, 2017).

Antioxidant capacity

TEAC and ORAC

These methods were used to quantify the antioxidant capacity of different MO parts and their results are shown in Table 3. TEAC showed the highest antioxidant capacity in MO husk collected in Guerrero (224.45 µmol Trolox Equivalents (TE) g^{-1}), while ORAC indicated that MO leaves from the region of Sonora showed the highest antioxidant capacity (721.29 µmol TE g^{-1}). These results suggested that there is no correlation between the TEAC and ORAC methods. Variations in results between these two methods are attributed to the different antioxidant compounds contained in samples since they have different kinetics and reaction mechanisms (Zulueta *et al.*, 2009). The antioxidant capacity of MO leaves is related to that reported by Guzmán-Maldonado and Fuentes (2015) and Cuellar-Núñez *et al.* (2018) who obtained values of 500.02 µmol TE g^{-1} and 636 µmol TE g^{-1}, respectively. MO leaves show higher antioxidant capacity compared to epazote (31.5 µmol TE g^{-1}), oregano (25 µmol TE g^{-1}) and parsley (60 µmol TE g^{-1}) (Mercado-Mercado *et al.*, 2013).

TABLE 3. Antioxidant capacity of the different structures of *Moringa oleifera.*

Samples	TEAC (μmol TE g^{-1})	ORAC (μmol TE g^{-1})
	Seeds	
Etchojoa, Sonora	107.99 ± 6.88	64.35 ± 6.06
Acapulco, Guerrero	152.62 ± 9.34	61.82 ± 1.89
	Husks	
Etchojoa, Sonora	154.87 ± 11.95	102.91 ± 4.59
Acapulco, Guerrero	224.45 ± 9.07	93.47 ± 9.15
	Leaves	
Etchojoa, Sonora	122.05 ± 1.54	721.29 ± 71.77
Acapulco, Guerrero	183.36 ± 13.97	645.33 ± 23.82
	Flowers	
Acapulco, Guerrero	124.13 ± 2.33	382.60 ± 33.54

TEAC - Trolox Equivalent Antioxidant Capacity; ORAC - Oxygen Radical Absorbance Capacity; TE - Trolox Equivalents.

Principal component analysis (PCA)

The three principal components (Eigenvalues) explain 90.3% of the total variation; the first component represents a separate variance of 37.84%, while the second component represents 34.79%, and the third one a total of 17.67%. These main components contributed with the greatest influence (Tab. 4).

TABLE 4. Eigenvalues and explained variance of the principal component analysis (PCA) using 13 biochemical variables on different structures of *Moringa oleifera.*

PCA	Eigenvalue	Explained variance (%)	Cumulative variance (%)
1	4.9204	37.8490	37.8490
2	4.5233	34.7948	72.6438
3	2.2974	17.6722	**90.3160**
4	0.7866	6.0506	96.3666
5	0.4010	3.0848	99.4515
6	0.0713	0.5485	100.0000

The value in bold represents the cumulive variance (%) ≥90%.

Moreover, each of the main components refers to different variables (Eigenvectors); component one (main) is composed of the ethereal extract variables with -0.9860, carbohydrates 0.9142 and protein -0.8746, whereas component two is represented by dietary fiber which showed a value of -0.8849, insoluble fiber -0.790 and soluble fiber -0.895 (Fig. 2). Component three is made up of up to four variables representing the highest phenols with 0.7021, tannins 0.7083, flavonoids -0.6598 and antioxidant capacity determined by the ORAC method with -0.6319 (Tab. 5). There was a difference between the different parts of the plant; nevertheless, there are only minor changes between regions. Seeds are the part that showed greater differences compared to leaves, husks and flowers.

TABLE 5. Eigenvectors and cumulative variance (%) of the analysis of the principal component analysis (PCA) using 13 biochemical variables on different structures of *Moringa oleifera*.

Variable	PC1	PC2	PC3
Phenols	0.3736	0.5984	**0.7021**
Tannins	0.6231	0.3100	**0.7083**
Flavonoids	0.4567	0.4309	**-0.6598**
Ashes	0.4382	0.6312	-0.3554
Moisture	0.6420	0.4449	0.4715
Proteins	**-0.8746**	0.4142	-0.0429
Ethereal extract	**-0.9860**	0.0692	0.1325
Total dietary fiber	0.4393	**-0.8849**	0.0630
Insoluble fiber	0.4012	**-0.8790**	0.1780
Soluble fiber	0.4876	**-0.8095**	-0.2117
Carbohydrates	**0.9142**	0.3618	-0.0739
TEAC	0.3467	-0.6849	-0.1189
ORAC	0.5432	0.5477	**-0.6319**

TEAC - Trolox Equivalent Antioxidant Capacity; ORAC - Oxygen Radical Absorbance Capacity.

Values in bold represent the significant correlations between the principal components and the variables.

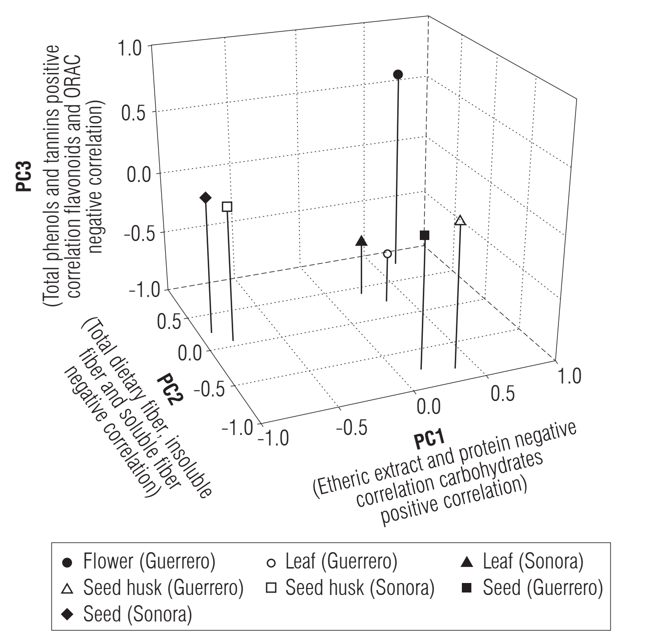

FIGURE 2. Projection on the three first principal components of different structures of *Moringa oleifera.*

Conclusions

The different parts of MO analyzed showed differences in the proximal composition and functional compounds as phenols, flavonoids and tanins. However, the comparative analysis (principal component analysis, PCA) did not show important differences between the samples obtained from the States of Guerrero and Sonora. The companies in Mexico that market products of the MO plant can get it from both regions and so they treat them as similar. MO seeds and flowers could be an alternative to enriched foods (wheat flour or cereals) due to their high protein values. The use of MO flowers could be recommended as an antioxidant supplement due to their high values of phenols and flavonoids. These values are even higher than the ones observed in leaves, which are currently the most commercialized part of the MO plant.

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Acknowledgements

When considered necessary, the authors may acknowledge the researchers or entities that contributed - conceptually, financially or practically - to the research: specialists, commercial organizations, governmental or private entities, and associations of professionals or technicians.

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