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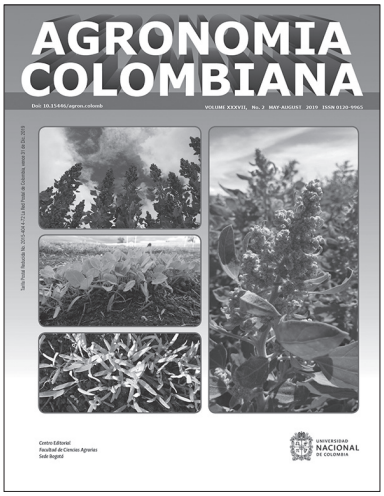

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Article on pages: 144-152

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Diseño de casetes de expresión para variedades de papa (*Solanum tuberosum* L.), basados en el gen *Cry*1Ba1

Natalyth Erira[1], Alejandro Chaparro-Giraldo[1]*, and Silvio López-Pazos[2]

ABSTRACT

The most serious insect pest problem in the potato crop in Colombia is the lepidopteran *Tecia solanivora* that causes significant economic losses. In this research, we designed expression cassettes based on the *cry*1Ba1 gene of *Bacillus thuringiensis* that could confer resistance to *T. solanivora* via the variety Pastusa Suprema. We selected the elements of the designed expression cassettes through an analysis of scientific literature and patent databases; the considered factors were the proteolytic activation of the *Cry*1Ba1 protoxin, modification of codonic use, polyadenylation signals, and cryptic splicing sites. We used a tissue-specific patatine promoter to reduce potential biosafety risks, because it is expressed only in the tuber. The freedom to operate analysis suggests that the commercial use of the designed expression cassettes in transgenic potato plants does not affect the rights of third parties in Colombia.

Key words: *Bacillus thuringiensis,* freedom to operate analysis, patatin promoter, *Tecia solanivora*.

RESUMEN

El más grave problema de insectos plaga en el cultivo de la papa en Colombia, es el lepidóptero *Tecia solanivora*, que causa importantes pérdidas económicas. En este trabajo se diseñaron casetes de expresión basados en el gen *cry*1Ba1 de *Bacillus thuringiensis* que pudiesen conferir resistencia a *T. solanivora* para la variedad Pastusa Suprema. Los elementos de los casetes de expresión diseñados fueron seleccionados mediante un análisis de literatura científica y de bases de datos de patentes; se tuvieron en cuenta los siguientes factores: activación proteolítica de la protoxina *Cry*1Ba1, modificación de uso codónico, señales de poliadenilación y sitios crípticos de splicing. Se usó un promotor tejido específico de patatina que puede disminuir potenciales riesgos de bioseguridad, debido a que se expresa solo en el tubérculo. El análisis de libertad de operación sugiere que el uso comercial de los casetes de expresión diseñados en plantas transgénicas de papa no afecta los derechos de terceros en el territorio de Colombia.

Palabras clave: *Bacillus thuringiensis,* análisis de libertad de operación, promotor de patatina, *Tecia solanivora*.

Introduction

In Colombia, potato is the fourth most important crop; in colder climates, it is the main agricultural production system with a planted area of 125,660 ha (ICA, 2011; FEDEPAPA, 2017). Worldwide, the development of genetically modified (GM) plants using proteins for insect resistance has occurred in several prominent crops, including potatoes (ISAAA, 2017). Their use has demonstrated advantages such as the reduced use of pesticides and protection of the crop throughout the farming season, while averting damages to the associated entomofauna and the natural environment. This, in turn, has improved production and the economies of farmers that have adopted this technology (Sauka *et al.,* 2008; Klümper and Qaim, 2014).

The Guatemalan potato moth, *Tecia solanivora* (Lepidóptera: Gelechiidae), is one of the main agricultural pests in potato farming in Colombia (ICA, 2015). Due to the fact that it affects the tuber's quality, it can cause direct losses of up to 35% (Vargas *et al*., 2004). When this insect undergoes complete metamorphosis, the larval stage inflicts the most damage. After the larvae emerge and following the female's oviposition in the rifts of the plant stems or under the soil adhering to the potato tubers (Vargas *et al*., 2004; López-Pazos and Cerón, 2013), microorganisms enter the plant through the vertical and horizontal galleries created by the larvae as they feed. Food residues, excrement, and mold left by the larvae rot and become compacted, causing the tubers to acquire a dark tonality in the field or during storage. This decreases the quality

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and commercial value of the tubers, causing in some cases their rejection (Niño, 2004).

For over 100 years the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) has been used for the biological control of insect pests. This microorganism produces various virulent factors that enable it to colonize its host. The most widely studied proteins from this bacterium are Cry proteins. These proteins are active against pest insects of the orders Lepidoptera, Coleoptera and Diptera, and others (Soberón and Bravo, 2007). Cry proteins have high specificity because their action mechanism depends on various stages, which include their solubilization and proteolytic activation in the insect. In addition, these proteins must be recognized by the protein receptors of the susceptible insect's intestinal membrane. Then, active oligomers must form to develop lithic pores that destroy the intestinal tissue's integrity, eventually causing the death of the host (Soberón and Bravo, 2007).

Important developments have been made on the biological activity of *cry*1Ba on *T. solanivora* as well as on the lethality of other Cry proteins. These developments make them a worthy alternative for consideration in biological plans for the control of this insect.

In order to obtain potato plants with resistance to the Guatemalan potato moth through the use of the *cry*1Ba1 gene, the gene must be included in an expression cassette. Therefore, in this research we designed five expression cassettes *in silico*, delving into certain elements inherent to the context of cassette design. The designed expression cassettes are preliminary for the development of future transgenic lines of the Pastusa Suprema potato. Using the tissue-specific promoter patatin B33 diminishes potential biosafety risks, since the plants would only express the transgene in the tuber. The tuber is the tissue affected by *T. solanivora*, avoiding exposure of the entomofauna associated with the crop or gene flow (Vanegas *et al*., 2010).

Research on GM crops for insect resistance has promoted the development for the expression of different *cry* genes. Having several options is valuable considering 1) biological scenarios that imply adaptation to diverse agronomic environments; 2) biosafety contexts for commercial release; 3) alternatives for possible cases of resistance development to a specific Cry protein of a GM crop in the field; and 4) marketing contexts such as those that include intellectual property rights, besides other developments, suggesting the need for viable alternatives for generating new events with other cry1 genes. The development of transgenic lines with gene stacking using a cry gene that is different from cry1Ac is an alternative in scenarios such as the appearance of resistant pest insects (Pitre *et al*., 2008; López-Pazos *et al*., 2010).

Although genetic engineering programs have been initiated in Colombia to control *T. solanivora* using the cry1Ac gene, our research demonstrates the possibility of obtaining transgenic potato plants evaluated with other genes; the cry1B gene is a good candidate for developing plants that are resistant to *T. solanivora*.

Materials and methods

Background studies

We conducted two studies using recombinant Cry proteins on *T. solanivora* larvae. In the first study, higher toxicity results were observed for Cry1Ac protoxin compared to the other proteins evaluated (20% mortality at a concentration of 4.37 µg cm^{-2}) by using protoxins Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, and Cry1E expressed by recombinant strains of *Escherichia coli*. These protoxins were purified and assessed on the first instar of *T. solanivora* larvae at a concentration of 4 µg cm^{-2} (corresponding to the LC50 of the positive control strain Bt *kurstaki* HD1). Cry1Aa, Cry1Ab, Cry1B, Cry1C protoxins showed mortality percentages of 6.24%, 6.76%, 4.16%, and 5.72% respectively, whereas protoxins Cry1E and Cry1D were the least toxic with an average mortality of 3.12% (Martínez *et al*., 2003).

In the second study, the recombinant toxins Cry1Aa, Cry1Ac, Cry1B, and Cry1C were activated with trypsin and purified by ion exchange column chromatography before their biological activity was assessed on the first instar of the *T. solanivora* larvae. This experience yielded an LC50 of 0.103 µg cm^{-2} for Cry1Aa, 0.107 µg cm^{-2} for Cry1Ac, 0.085 µg cm^{-2} for Cry1B, and 0.112 µg cm^{-2} for Cry1C.

These data were subjected to an analysis of variance that found no significant differences between the recombinant proteins. The similar average mortalities demonstrated that *T. solanivora* was highly susceptible to Cry1 proteins. The proteins used in this work had a high level of purity (chromatography) and conferred greater lethality because the proteolysis of the protoxin had been carried out *in vitro,* favoring their recognition by the receptor. The use of the activated toxins produced a faster effect on the larvae. The Cry1B protein showed the best results in a subsequent trial evaluating a Cry1B-Cry1I hybrid protein in domain

II (Pitre *et al*., 2008). This protein was obtained from a plasmid in *E. coli*, which encoded the hybrid protein that was constructed by replacing a section of domain II of the *cry*1B gene with the corresponding fragment of the *cry*1I gene. The protoxins produced were treated with trypsin and purified. The *Cry*1Ba-activated toxin evaluated individually has high activity towards *T. solanivora* with 60% mortality at a dose of 0.5 µg cm^{-2} (López-Pazos *et al*., 2010).

Valderrama *et al*. (2007) and Villanueva *et al*. (2014) examined the use of Cry proteins in potato crops by plant transgenesis. They found that the developed varieties have a high level of expression of the *cry*1Ac gene, and because they were expressed constitutively, they can pose biosafety issues with commercial release of the plant (Vanegas *et al*., 2010). Therefore, a study was carried out in which López and Chaparro-Giraldo (2007) propose an *Agrobacterium tumefaciens* transformation system of potato explants of the androsterile variety Pastusa Suprema. Androsterility is an important feature in the environmental biosafety of GM plants for their release in centers of origin or in crop areas of high biodiversity. In Colombia, Pastusa Suprema is the only potato variety with this characteristic obtained by conventional breeding processes. Torres *et al*. (2012) developed transgenic lines using the variety Pastusa Suprema which was transformed using the *cry*1Ac gene. These lines were transformed with the plasmid p1AcPRD, which contained the *cry*1Ac gene that was donated by the University of Ottawa under a material transfer agreement (MTA). These lines were subjected to a freedom to operate (FTO) analysis, in which it was concluded that the FTO was affected by the MTA, because it restricted its use to research only and did not allow commercialization (Hincapié and Chaparro-Giraldo, 2014).

Analysis of the biological activity of the *Cry*1Ba1 protein

An exhaustive literature review of the available data evaluating the *Cry*1Ba protein on larvae of the Guatemalan potato moth led to a determination of the key parameters validating the selection of the *cry*1Ba1 gene as an option for potato transgenesis to confer resistance to *T. solanivora*, within the context of its toxic activity towards the insect.

In silico design of expression cassettes

We designed five expression cassettes *in silico*. We obtained the sequences from the nucleotide database of the National Center for Biotechnology Information (NCBI) as *cry*1Ba1 gene access number X06711.1, promoter B33 (NCBI A08215.1), promoter CaMV35S (NCBI AF234316.1) and T-nos (NCBI AET75772.1). We used the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/) to determine the *cry*1Ba1 gene open reading frame (ORF). We also used Visual Gene Developer 1.3 software (Jung and McDondald, 2011) to produce the codon use modification. We made modifications of codon usage to the *cry1Ba1* gene sequences to ensure their closer resemblance to the potato gene sequences using the Kasuza database (http: //www.kazusa.or.jp/codon/) considering splicing sites, polyadenylation signals, and the removal of restriction sites that could interfere with transcription. Then we assembled the sequences with ApE-A plasmid Editor 2.0 software. We placed restriction enzyme recognition sequences present in the polylinker region of the pCAMBIA2300 vector that did not cut anywhere in the gene sequence at the ends of the construct. With this software, we verified that the inserted sites did not alter the reading frame. Generay Biotech Co. Ltd. (China) synthesized the cloning of the five expression cassettes designed in the pCAMBIA2300 vector.

Genetic transformation of *Escherichia coli*

The genetic transformation of the commercial strain DH5alpha of *E. coli* was carried out through electroporation, to clone each expression cassette. The bacterial strains were phenotypically selected by their growth in the presence of kanamycin, considering that the pCAMBIA2300 plasmid contains a resistance gene for this antibiotic; strains were confirmed using Polymerase Chain Reaction (PCR) assays. We performed plasmid DNA extraction using the GeneJET Plasmid Miniprep Kit (Termofisher, Waltham, MA, USA). The PCR reaction was carried out with 1X Buffer PCR, 0.5 uM dNTP MIX, 2.5 mM Mg, and 1 µM of the primers that amplified the expression cassette and specifically the *cry*1Ba1 gene, and 0.3 U Taq polymerase. The temperature cycle had the following stages: denaturation (95°C for 5 min), synthesis (95°C for 1 min, 57°C for 2 min, 72°C for 1 min, for 30 cycles), and elongation (72°C for 5 min).

Obtaining and characterizing *Agrobacterium tumefaciens* LBA4404

We transformed the strain LBA4404 of *A. tumefaciens* by electroporation with the plasmid pCAMBIA2300 containing each expression cassette. To this end, we incubated 25 µl of electrocompetent cells and 1 µl of vector (100 ng μl^{-1}) in SOC medium (Triptein 2%, 8.56 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$ heptahydrate) for three hours at 28°C and planted in Luria Bertani growth (LB) medium with kanamycin (50 mg L^{-1}) and rifampicin (50 mg L^{-1}). These media were incubated at 28°C for 48 h. We selected bacterial strains by their growth in solid LB medium with kanamycin (50 mg L^{-1}) and rifampicin

(50 mg L^{-1}) by PCR assays using primers that amplify the expression cassette and the *cry*1Ba1 gene.

Plant transformation

We carried out the phenotypic characterization of *A. tumefaciens* LBA4404, *in vitro* regeneration and transformation of 3 to 4-week-old potato seedlings following the protocols by Trujillo *et al.* (2001) and Jiménez *et al.* (2009). Regeneration occurred using internodal stem segments (5-6 mm length) with the expression cassette 5 (containing the CaMV35S promoter with the *cry*1Ba1 gene without C-terminal fragment and codon usage adjustment). The regeneration system allowed the formation of callus and the regeneration of the explants. The efficiency of the regeneration system was evaluated through the induction of callus, percentage of regeneration, and the number of regenerants per explant. The transformation of the seedlings proceeded based on a method proposed by López and Chaparro-Giraldo (2007) by taking groups of 40 explants and placing them in the co-culture medium, which consisted of liquid MS medium supplemented with vitamins and 20 mg L^{-1} of acetosyringone. The explants were placed in contact with the bacterial suspension for 8 min, using as a control a group of 40 explants not subjected to cocultivation and transferred directly to MS solid culture medium without the addition of hormones. We transferred the co-cultivated explants to Petri dishes with solid coculture medium and incubated them in the dark at a temperature of 20 ± 3°C for 18 h. Then, we transferred the explants to a regeneration medium supplemented with 250 mg L^{-1} of cefotaxime for 7 d, and transferred to the same medium supplemented with 50 mg L^{-1} of kanamycin as the selection concentration that has been used for explants of Pastusa Suprema (López and Chaparro-Giraldo, 2007; Torres *et al.*, 2012) and other varieties (Andersson *et al.*, 2003; Khan *et al.*, 2006; Meiyalaghan *et al.*, 2011; Molla *et al.*, 2012).

Freedom to operate analysis

We performed a search for requests and conceded patents in the public access international database The Lens (https://www.lens.org/), using keywords relating to the elements of the designed cassettes. The analysis was centered on the claims of the patent requests. The Lens locates patents granted by the United States Patent Office and its European counterpart, as well as the records in the Patent Cooperation Treaty (PCT) that are in the World Intellectual Property Organization (WIPO) database. Later, we performed the search in the patent database of the Superintendencia de Industria y Comercio (SIC) (Colombian National Superintendence of Industry and Commerce).

Results and discussion

Selection of the different *Cry1Ba1* sequences

We considered the mechanism of action of the Cry1 proteins in Lepidoptera in order to define the characteristics of the coding region for the *Cry1Ba1* protein (Pigott and Ellar, 2007). The protoxins must be processed by insect proteases that cleave the protoxin polypeptide into specific sequences (Deist *et al.*, 2014), and this is crucial for determining the mode of action of these proteins after ingestion of the crystal and the pH-dependent solubilization of the protoxin (for the orders Lepidoptera and Diptera this happens at basic pH, while, for the orders Coleoptera and Hemiptera, the pH is neutral to acidic). In the case of the Cry1A protein (~130-135 kDa), the cleavage results in an active toxin of 65 kDa, caused by trypsin (or similar to trypsin) and chymotrypsin-type proteases, where enzymatic digestion eliminates a sequence of ~600 amino acids at the C-terminus and the first 28 amino acids at the N-terminus. For the orders Lepidoptera and Diptera, the main intestinal proteases are of the serine type, while in Coleoptera, the main proteases are cysteine and aspartic acid proteases, although some use cathepsin G and also trypsin and chymotrypsin (serine protease). In the intestinal fluid of various insects, other proteases (elastase, pronase, thermolysin) have been identified and recognized for their possible interactions with Bt protoxins. The cleavage of the protoxin is a prerequisite for its insertion into the membrane. These interactions are complex, and the activation process *in vivo* is unclear and could vary because of differences in proteases in the intestines of insects (Miranda *et al.*, 2001). The secondary structure of the toxin and the toxin receptors in the epithelial cells of the insect's midgut are important determinants of toxin specificity, and it is likely that the differential proteolysis of the protoxin by the intestinal juice of the different larvae may affect toxicity; this has been demonstrated in different species (Bah *et al.*, 2004).

Considering the importance of the proteolytic activation of the *Cry1Ba1* protoxin, determining the structure of the respective active toxin was vital. Exactly where each type of protease performs the cleavage depends on the type of Cry protein and the specific proteinase population of each insect (Pigott and Ellar, 2007). Since the mechanism of action of Cry1 proteins in *T. solanivora* is not completely understood, in this research we designed five expression cassettes that contemplate possible scenarios of the mechanism of action of the *Cry1Ba1* protein in this species. To this end, we identified the functional domains of

the *Cry1Ba1* protein according to the NCBI database of conserved domains (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), which corresponds to A) N-terminal I domain-amino acid 89, B) domain II-amino acid 267, and C) domain III-amino acid 489 (Fig. 1). This analysis contemplates putative processing by proteases that have been identified in other Cry proteins.

In silico design

The *in silico* design of expression cassettes permits changes in the nucleotide sequences that can improve the expression of the gene. The codon modifications are one of the most critical factors for achieving adequate levels of expression (Christey *et al*., 2006).

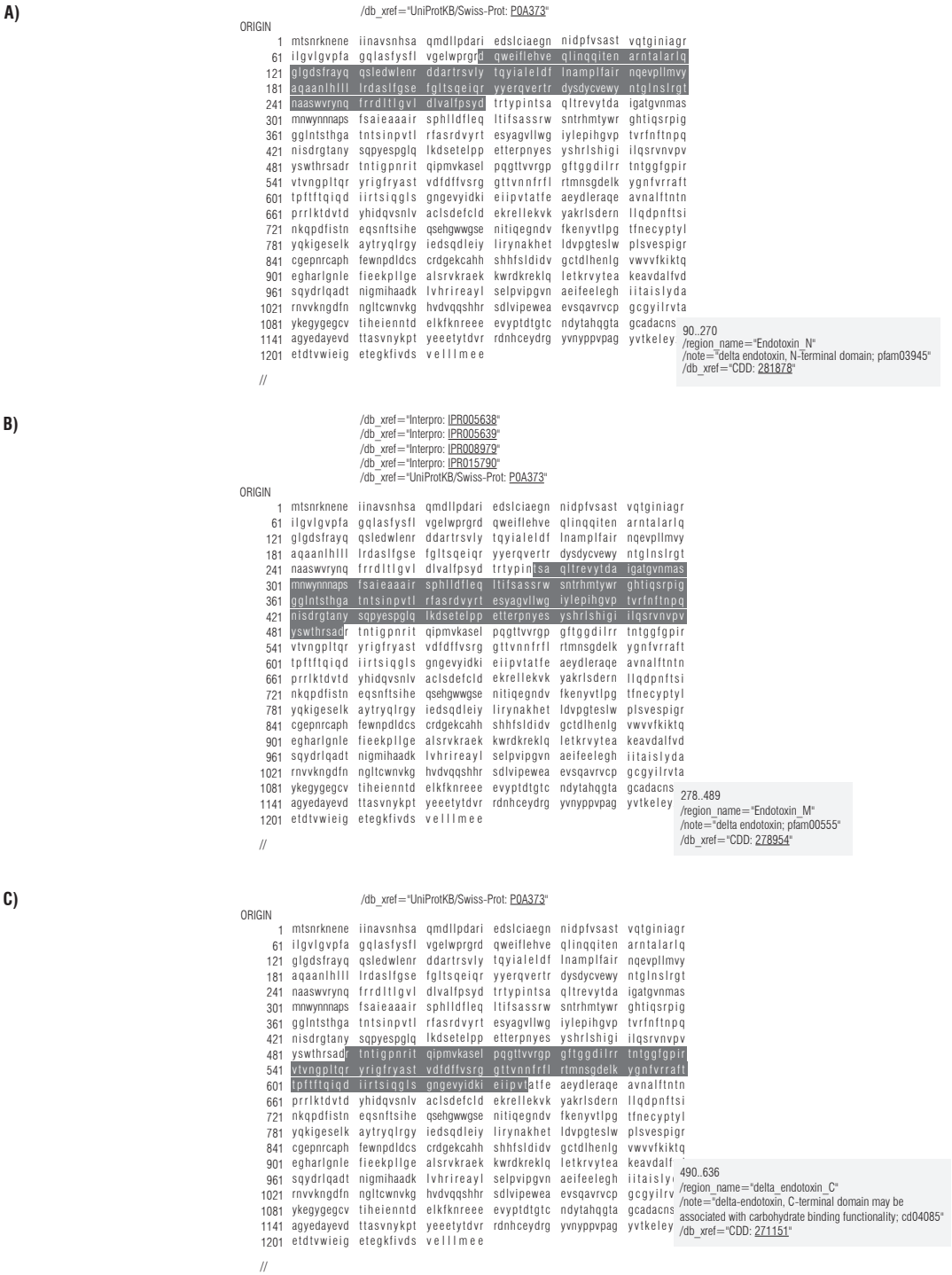

FIGURE 1. Functional domains of protoxin *Cry1Ba1*: A) domain I, B) domain II, C) domain III.

We describe the five expression cassettes designed (Fig. 2) in this work as follows: 1) Contains the *cry*1Ba1 gene without the C-terminal fragment and codon usage adjustment, it is formed by the first 1947 bp of the *cry*1Ba1 gene; this construct contains the B33 promoter; 2) Contains the *cry*1Ba1 gene without C-terminal fragment and codon usage adjustment in the first 87 aa, is composed of 1947 bp; 3) Contains the *cry*1Ba1 gene coding for protoxin with codon usage adjustment, conformed by 3687pb; 4) Contains the *cry*1Ba1 gene encoding the active toxin cleaved at the N-terminus and C-terminal with codon usage adjustment, conformed by 1647 bp that start at nucleotide 267 and ends at nucleotide 1908 of the *cry*1Ba1 gene; 5) Contains the *cry*1Ba1 gene without the C-terminal fragment and codon usage adjustment, it is made up by the first 1947 bp of the *cry*1Ba1 gene, it contains the constitutive promoter CaMV35S. The first four constructs contain the B33 promoter that has a high degree of specificity for expression in the tuber (Fig. 2).

All the designed expression cassettes contain the T-nos terminator. The sequence of the *cry*1Ba1 gene is of bacterial origin and has a lower content of guanine and cytosine (GC) than that reported for plant genomes (Li *et al*., 2016). The modifications of codon usage allowed changing the content of GC to 42%, which is a value closer to the potato genome (42.4%). Cryptic splicing sequences and premature polyadenylation sites of the ORF were eliminated in each of the cassettes.

Phenotypic and molecular characterization of recombinant strains

After the transformation of bacteria with expression cassettes 1, 2 and 5 to establish the characterization protocol presented below, we planted by exhaustion in the respective selection medium. As a phenotypic characteristic, the non-transformed colonies showed no growth and the colonies positive for PCR were subcultured in a selection medium for subsequent cryopreservation at -80°C. We performed a PCR assay on the positive colonies transformed with expression cassettes 1 and 2 using the forward primer for the B33 promoter and the reverse primer for the T-nos terminator. We considered the colonies that had an amplicon of 3917 bp positive. We considered positive the PCR assay using the primer pair consisting of the forward primer for the promoter CaMV35S and reverse primer for the terminator T-nos with an amplicon of 2602 bp. We confirmed the *A. tumefaciens* and

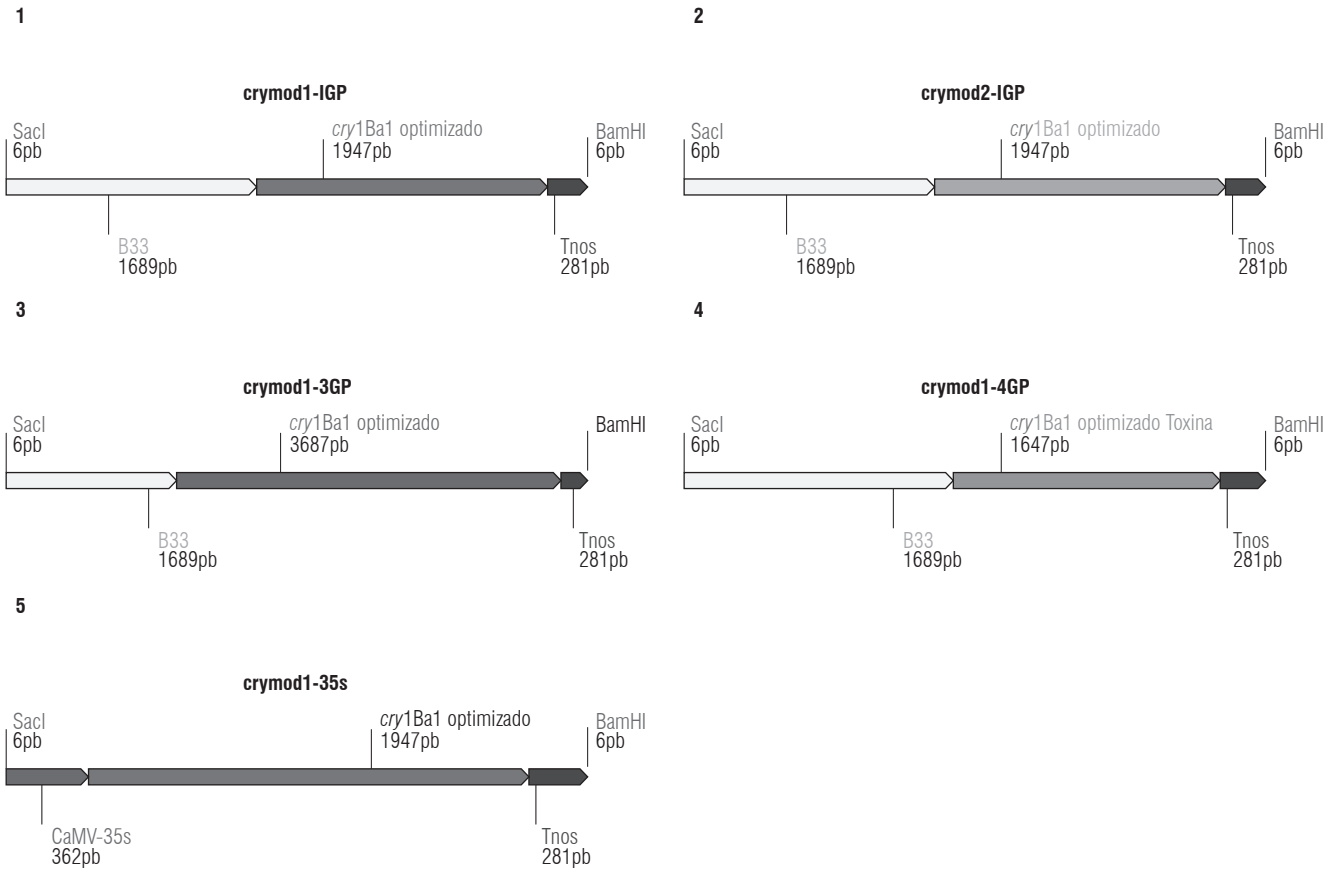

FIGURE 2. Expression cassettes.

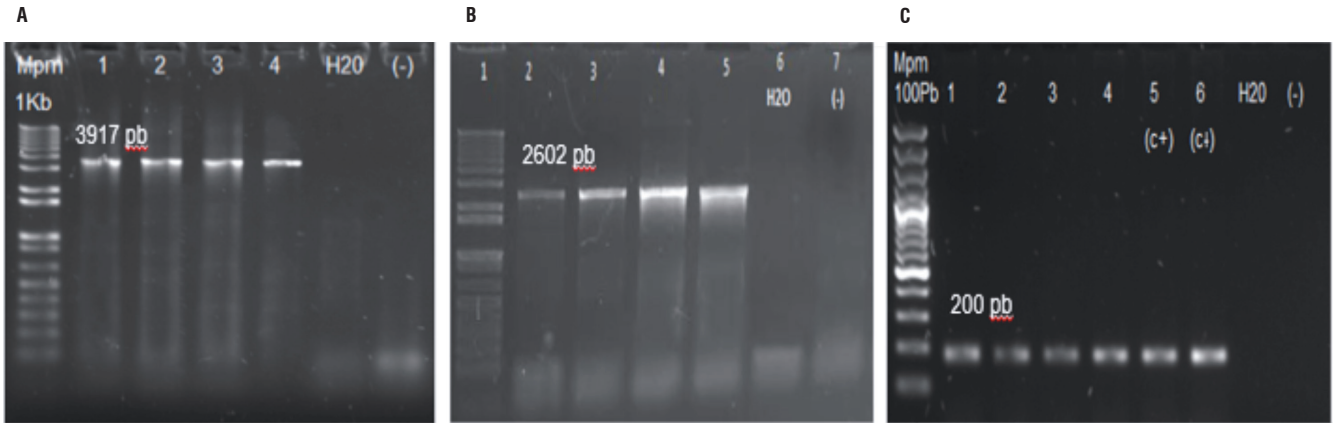

FIGURE 3. Molecular characterization of the recombinant strains of *E. coli* and *A. tumefaciens*. A) Amplification of expression cassettes 1 and 2, with the B33 promoter; B) Amplification cassette 5 with the CaMV 35S promoter; C) *cry*1Ba1 gene from the cassettes, negative controls (plasmid pCAMBIA 2300).

E. coli colonies that showed growth by a PCR assay for a region of the *cry*1Ba1 gene, and subcultured in selection medium. The PCR result was considered positive when 200 bp amplicons were obtained (Fig. 3).

Transformation tests

We used the application of the transformation protocol to determine the infective capacity of *A. tumefaciens* LBA4404 on internodal segments of potato, leading to the subsequent introduction of the T-DNA with the gene of interest and its insertion in the genome of the plant. We evaluated the growth of regenerants in the presence of kanamycin with cassette 5 containing the nptII gene as a plant selection marker. The control group of regenerated explants without transformation with the expression cassette exhibited callus formation in the second week with a percentage of callus induction of 96%. That is, approximately 38 internodal explants of the 40 cultivated in regeneration medium, and a percentage of regeneration of 90%; approximately 36 explants regenerated with numerous shoots per explant of 4 ± 1. The untransformed explants cultivated in regeneration medium with selection antibiotic began to show oxidation at week one; during week two they presented oxidation and chlorosis in medium with kanamycin (50 mg L^{-1}). Twenty percent of the cocultivated explants initiated the process of callogenesis, and by the second week they began to lose viability and exhibited complete oxidation. Eighty percent of the explants did not display callogenesis or regeneration; this could be associated with the effects on the plant genome at the time of coculture, physical damage, and the presence of the antibiotic. The explants transformed with the recombinant strain LBA4404 showed a behavior similar to that of the previous group, with the difference being that from the fourth week on, 10% persisted in the selection medium and produced regenerants. The resistance presented by the explants in the selection medium could be associated with the insertion of the nptII gene transferred by the recombinant strain LBA4404 containing the expression cassette 5 (Fig. 4).

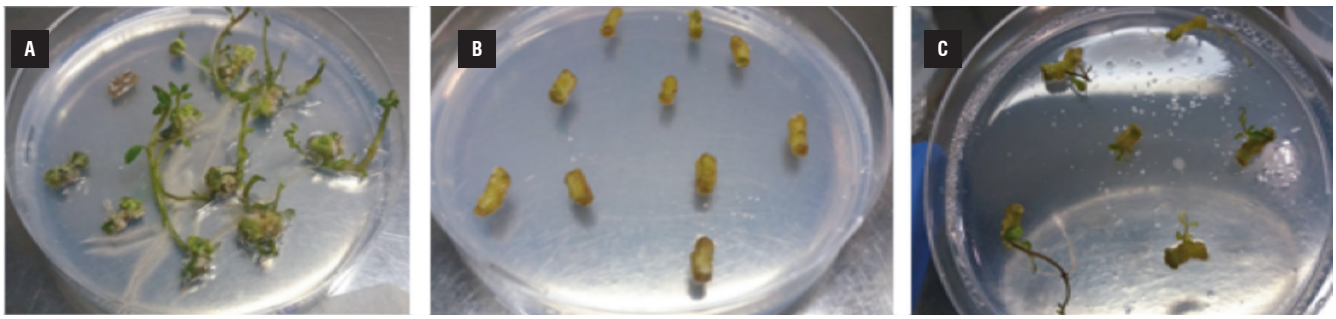

FIGURE 4. Application of the transformation protocol in potato explants using the recombinant strain of *A. tumefaciens* LBA4404. A) Untransformed explants in regeneration medium without kanamycin, showing processes of callogenesis and regeneration; B) Untransformed explants in regeneration medium with 50 mg L^{-1} of kanamycin, no regeneration was observed; C) Explants transformed with the strain LBA4404 in regeneration medium with kanamycin (50 mg L^{-1}), regenerants were evident.

Employing a regeneration medium containing 50 mg L^{-1} of kanamycin as a selection agent has proven useful for the preliminary selection of possible transformants when using potato explants; they avoid selection pressure and loss of possible transformed explants by oxidation, chlorosis or tissue necrosis when higher concentrations of the antibiotic are used. This is demonstrated by some authors that have used concentrations of 100 mg L^{-1} (Andersson *et al*., 2003; Khan *et al*., 2006; López and Chaparro, 2007; Meiyalaghan *et al*., 2011; Molla *et al*., 2012; Torres *et al*., 2012).

Freedom to operate analysis

We identified patents relating to each of the genetic elements of the constructs beginning from the deconstruction of the product. The patent EP2482639A2 is within the patents relating to the *cry*1B gene, which is part of the application by PCT number WO2011041256A2 (Syngenta Participations AG). This protects nucleic acid molecules, which encode the Cry1Ba protein as well as modifications in its amino acid sequences, and the insertion of this with promoters such as patatin. Patent US20100235951A1 (Bayer Bioscience) includes plant cells or plants comprising a *cry*1C chimeric gene with *cry*1B or *cry*1D. The patent US8772577B2 (Pioneer Hi-Bred International, Inc.) protects a sequence that possesses 88% identity with the *cry*1Ba gene. In the search of the Colombian national database, we found no granted patents or patent requests for the *cry*1Ba1 gene. We found patent applications involving *cry*1B sequences, such as WO2011060009A2, whose claims protect a sequence possessing 88% identity with the *cry*1Ba, *cry*1Bc and *cry*1Bb genes. Another patent application is US20120324606A1 that includes plants that express *cry*1Ab sequences and *cry*1Be, has been requested in Colombia with the number CO6561804A2. However, this does not correspond to the variant of the gene used in this work (*Cry*1Ba1). Similarly, patent application US 20110277184A1 claims the *cry*1Bf sequence, a protein with an identity of at least 95% in plants. During the search for patents requested and approved in Colombia, we found patent CO6561804A2. This patent was requested nationally on June 16, 2012; it was denied because patenting of genes is not allowed in Colombia.

Concerning the promoter B33, patent EP0375092B1 was granted but it expired in 2009. The patent protected the use of this promoter in the production of potato plants. Patent US8334429 concerning the strain of *A. tumefaciens* was found (Pioneer Hi-Bred International, Inc.). The patent US 20090075358A1 frames within its claims the general structure of the vector pCAMBIA. We found no patents protecting the use of the T-nos terminator sequence. Therefore, to date when the review was carried out, no intellectual property rights relating to the selected sequences in the design of the expression cassettes were registered in Colombia.

Conclusions

We selected the *cry*1Ba1 gene as a viable option for obtaining GM potato lines, according to the biological context of the mechanism of action of Cry1 proteins.

The *in silico* design of expression cassettes enabled the attainment of modified versions of the *cry*1Ba1 gene for transformation of potato plants, with modifications that allowed the desired levels of expression and lethal effects on the target insects. These studies should be carried out on the transgenic lines that we obtained.

To date, the freedom to operate analysis of the expression cassettes in this work, suggested that their use does not affect the rights of third parties in Colombia.

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Cross-species transfer of SSR markers in *Setaria sphacelata* and *Trichloris crinita* sp.

Transferencia cruzada de marcadores SSR en *Setaria sphacelata* y *Trichloris crinita* sp.

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ABSTRACT

Setaria sphacelata and *Trichloris crinita* are subtropical forage species that are important for livestock breeding in Argentina. Genomic information is scarce for these species, and there are no molecular markers designed for them; this limits the development of genetic improvement programs. We performed a cross-species transfer of SSR markers from several Poaceae species. In *S. sphacelata*, 8 SSR markers were transferred from *Setaria italica* (40% transfer rate), exhibiting 83% polymorphism. Kazungula, Splenda and Narok cultivars were genetically differentiated and the experimental material "Selección INTA" was separated from Narok, from which it was derived. For *T. crinita*, 19 microsatellites were transferred from 5 Poaceae species (7.3% transfer rate), with 69% polymorphism. The results obtained in this study show the potential of the transferred SSR markers for assessing genetic variation and for expanding the genetic resources available for these species.

Key words: microsatellites, polymorphism, feed crops, genetic variation, plant genetic resources.

RESUMEN

Setaria sphacelata y *Trichloris crinita* son especies forrajeras subtropicales, estratégicas para el desarrollo de la actividad ganadera argentina. Para estas especies, la información genómica es escasa y no existen marcadores moleculares desarrollados en las mismas, por lo cual el desarrollo de programas de mejoramiento genético se ve limitado. En este contexto, realizamos una transferencia de marcadores SSR de varias especies de poáceas. En *S. sphacelata*, se transfirieron 8 marcadores desarrollados en *Setaria italica* (tasa de transferencia del 40%), mostrando un 83% de polimorfismo. Los cultivares Kazungula, Splenda y Narok se diferenciaron genéticamente y el material experimental "Selección INTA" se separó de Narok, del cual se deriva. Para *T. crinita*, se transfirieron 19 microsatélites de 5 especies poáceas (tasa de transferencia del 7.3%), con 69% de polimorfismo. Todos los individuos se pudieron difrenciar genéticamente. Los resultados obtenidos en este trabajo muestran la capacidad de los marcadores SSR transferidos para evaluar la variabilidad genética, expandiendo los recursos genéticos disponibles para estas especies.

Palabras clave: microsatélites, polimorfismo, plantas forrajeras, variación genética, recursos genéticos vegetales.

Introduction

Setaria sphacelata (Poaceae) is a perennial pasture grass native to tropical Africa (Hacker, 1991). It is a cross-pollinating, tetraploid species (2n=4x=36) (Hacker and Jones, 1969; Le Thierry d´Ennequin *et al*., 1998) of summer growth. It is cultivated in regions with rainfall exceeding 750 mm; but it also exhibits resistance to drought, flooding, and nutrient-deficient soils (Borrajo and Pizzio, 2006). In Argentina, *S. sphacelata* is cultivated in the northern regions, and the most agronomically important cultivars are Splenda, Kazungula, and Narok (Pensiero, 1999; Borrajo *et al*., 2006; Burghi *et al*., 2014).

Trichloris crinita (Lag.) Parodi (Poaceae) is a perennial grass species native to arid regions of the American continent (Kozub *et al.,* 2017). It is a warm-season species with good forage quality due to its protein content and palatability (Nicora and Rúgolo de Agrasar, 1987). It is an autogamous tetraploid (2n=4x=40) (Fedorov, 1969), tolerant to drought and low-salinity (Aronson, 1989; Greco and Cavagnaro, 2003), which is used for protection against soil erosion (Dalmasso, 1994). In Argentina, *T. crinita* integrates the grasslands of arid and semi-arid regions (Cano, 1988). In 2012, the genus *Trichloris* was embedded in *Leptochloa* and the species *T. crinita* was renamed *L. crinita* (Snow and Peterson, 2012).

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Both species are used as forage in Argentina and are strategic for the development of regional and national livestock breeding. The accessibility of molecular markers could complement the genetic improvement programs for these species. Although there are no microsatellites specifically designed for these grass species to date, a few studies have been reported using molecular markers. On the one hand, Li *et al.* (1998) applied random amplification of polymorphic DNA (RAPD) analysis to assess the intraspecific and interspecific variation in several species of genus *Setaria*, including *S. sphacelata*. Moreover, simple sequence repeat (SSR) markers were developed in *S. italica* and transferred to *S. sphacelata* (Gupta *et al*., 2012; Gupta *et al.,* 2013; Kumari *et al*., 2013; Pandey *et al*., 2013). Cavagnaro *et al.* (2006) assessed the genetic diversity in *T. crinita* varieties using amplified fragment length polymorphism (AFLP) markers. More recently, SSR markers were developed in *T. crinita* using sequence data from related grass species (Kozub *et al*., 2018).

In order to expand the set of molecular markers available for genetic improvement programs of *S. sphacelata* and *T. crinita*, we conducted an SSR cross-amplification from related species of the Poaceae family. For this, we first optimized the DNA extraction for *T. crinita*.

Materials and methods

Plant materials

We studied three *S. sphacelata* cultivars in this research: Narok, Splenda, and Kazungula. We also included an experimentally improved population derived from Narok, called "Selección INTA" (EEA INTA Mercedes, Corrientes), in the analysis. Twenty individuals from each cultivar were analyzed in bulk.

We collected *Trichloris crinita* material from four sites in Argentina: one individual from La Pampa (36°35'34.4" S and 64°41'47.6" W), eight individuals from Catamarca (29°18'22" S and 65°08'40" W), seven individuals from Cordoba (three individuals from 29°57-59' S, 64°28-29' W, and 300 m a.s.l., and four individuals from 29°49-54' S, 64°27-28' W, and 310 m a.s.l.), and seven individuals from La Rioja (three individuals from 31°24-25' S, 66°46-47' W, and 490 m a.s.l. and four individuals from 31°30-32' S, 66°48-49' W, and 450 m a.s.l.) (Quiroga *et al*., 2010).

Plants were grown at 25°C in 20 cm pots filled with a 3:1 mixture of fertilized soil and vermiculite in a greenhouse at the Ewald A. Favret Institute of Genetics, National Institute of Agricultural Technology (IGEAF-INTA). Pots were watered approximately every 3 d. The photoperiod was 16 h light and 8 h dark.

DNA extraction

Different protocols were attempted for DNA extraction from *S. sphacelata* without successful results. Therefore, in order to obtain high-purity genomic DNA, we modified the extraction protocol described by Dellaporta *et al.* (1983). Fresh young leaves were ground in liquid nitrogen and 30-40 mg were resuspended in 800 µl of extraction buffer (50 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 10% SDS, 10 mM β-mercaptoethanol, pH 8.0). Tubes were incubated at 65°C for 30 min. Then, 200 µl of potassium acetate (5 M) was added, and the tubes were incubated in ice for 20 min. The suspension was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatants were transferred into clean tubes. Next, 800 µl of chloroform:isoamyl alcohol 24:1 (v/v) was added, and the tubes were centrifuged again at 12,000 rpm for 10 min. Isopropanol (1 volume) was added to the supernatants for DNA precipitation, and the tubes were spun at 12,000 rpm for 30 min at 4°C. The supernatants were discarded and the DNA pellets resuspended in 250 µl of TE1X buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Then, 2.5 µl of RNase (10 mg/ml) was added, and the tubes were incubated for 30 min at 37°C. Later, phenol (1 volume) was added, and the tubes were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were washed with TE1X buffer and spun. One volume of chloroform:isoamyl alcohol 24:1 (v/v) was added to the supernatants, and the tubes were centrifuged again. The supernatants were transferred to new tubes, and the DNA was precipitated with isopropanol (1 volume). The tubes were incubated for 30 min at 4°C and then spun at 12,000 rpm for 40 min at 4°C. The supernatants were discarded, and the pellets were washed with 500 µl of 70% (v/v) ethanol. Finally, the tubes were centrifuged at 12,000 rpm for 2 min, and the DNA pellets were resuspended in HPLC water.

Genomic DNA from *T. crinita* was extracted using the Saghai-Maroof method, modified by Pérez de la Torre *et al.* (2008). The DNA extracted from both species was verified by 0.8% agarose gel electrophoresis.

SSR markers

We selected a set of 29 SSR primers for cross-amplification in *S. sphacelata*: 20 pairs from *S. italica* (www.ncbi.nlm.nih.gov, Jia *et al*., 2007), two from *Bromus tectorum* (Ramakrishnan *et al*., 2004), one from *Lolium perenne* (Jones *et al*., 2001), three from *Triticum aestivum* (Röder *et al*.,

1998), and three from *Zea mays* selected from http//www.agron.missouri.edu. We labeled all forward primers for *S. sphacelata* with FAM (6-carboxyfluorescein) and HEX (hexachloro-6-carboxyfluorescein) fluorescent dyes (Alpha DNA).

For cross-amplification in *T. crinita*, we selected a set of 260 SSR primers: six pairs from *B. tectorum* (Ramakrishnan *et al*., 2004), 50 from *Cenchrus ciliaris* (Jessup, 2005), seven from *Eleusine coracana* (Dida *et al*., 2007; Arya *et al*., 2009), 20 from *Festuca arundinacea* (Saha *et al*., 2003), 10 from *L. perenne* (Jones *et al*., 2001), 12 from *Panicum maximum* (Ebina *et al*., 2007; Chandra and Tiwari 2010), 20 from *S. italica* (Jia *et al*., 2007; www.ncbi.nlm.nih.gov), 66 from *T. aestivum* (Röder *et al*., 1998; http://wheat.pw.usda.gov), and 69 designed in *Z. mays* (http//www.agron.missouri.edu).

PCR amplification

We performed PCR reactions in a total volume of 20 µl, which contained 75 ng of DNA, 1X PCR buffer (Invitrogen), 0.5 U of Taq DNA polymerase, 0.125 mM of each primer, 0.15 mM of dNTPs, and 2.5 mM of $MgCl_2$. The amplification reactions were performed in a thermal cycler (Mastercycler Epgradient S, Eppendorf), under the following conditions: initial denaturation temperature of 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 30 s, and amplification at 72°C for 2 min, with a final extension at 72°C for 10 min. We applied each PCR four times.

In *S. sphacelata*, we analyzed the individuals from each cultivar in bulk. We detected SSR fragments by ABI PRISM 3130 Genetic Analyzer using Genemapper 3.4 software (Applied Biosystems). In *T. crinita*, we separated PCR products by 6% non-denaturing polyacrylamide gels and stained them with ethidium bromide. The fragment size for each product was determined by 100, 50, and 10 bp standard size markers (Invitrogen).

Data analysis

We estimated genetic distances among the materials using Dice and Jaccard coefficients for *S. sphacelata* and *T. crinita*, respectively. We conducted clustering between species using the Unweighted Pair-Group Method with the arithmetic Average (UPGMA). In the dendrogram, we used *S. italica* and *C. ciliaris*as external controls (outgroups) for *S. sphacelata* and *T. crinita*, respectively. We quantified the level of polymorphism for each SSR with the Polymorphism Index Content (PIC) (Botstein *et al*., 1980). We performed Principal Coordinates Analysis, along with the rest of the statistical analyses described in this section, using InfoGen software (Balzarini and Di Rienzo, 2016). We estimated genetic variability within and among species via an analysis of molecular variance (AMOVA) using Gen AIEx 6.2 software (Peakall and Smouse, 2006).

Results and discussion

Eight out of the 20 markers designed in *S. italica* were transferable to *S. sphacelata*. None of the primer pairs from the other species, *B. tectorum* (2), *L. perenne* (1), *T. aestivum* (3) and *Z. mays* (3), generated amplicons.

We obtained 21 different PCR products with an average of 2.62 bands per marker and with sizes ranging from 120 to 411 bp. The average transfer rate of SSR markers from *S. italica* to *S. sphacelata* was 40% (Tab. 1). Six of the eight transferred microsatellites could amplify polymorphic bands, which represents a 75% level of polymorphism. The polymorphism index content (PIC) varied from 0.27 to 0.36.

Transferability between *S. italica* and *S. sphacelata* was consistent with the results obtained by Barbará *et al*. (2007) for amplification within genera in monocots (40%). However, the polymorphism obtained in this study for the

TABLE 1. Characteristics of the SSR markers transferred from *Setaria italica* to *Setaria sphacelata*. bp: base pairs; nd: not determined.

SSR	Forward primers (5´- 3´)	Reverse primers (5´- 3´)	Size (bp)		Repeat motif	Reference
			Expected	Observed		
2	AGAAAGTTGTAGATTGGGAAGA	AATAATGTGGAAAGACCCTGT	346	346	nd	GI: 29123369
3	ACTATGTGGTGGAGGGCGGT	GATGATAAGAGGCAGGAGTG	279	186- 393	nd	GI: 30351051
4	TACTTGACTGCTCACACCTTC	TTGTCCTTGTAATCCACTCC	376	375- 411	nd	GI: 22002467
9	TATGCCTCAAACAACATCC	ACTCCCTTCCAATGATACAAC	333	273-357	nd	GI: 62318483
15	AGAAAGTTGTAGATTGGGAAGA	GAGAGCGACTGAGACACC	286	270- 285	nd	GI: 15558946
P2	CCAACACGCAATCGCAGAA	AGGCAGTGGGTTTGAGCAT	120-127	120	CT	Jia *et al.*, 2007
P5	TTGCCTTGAGCTCTTTGATG	GCTGATACTGATATGTCTGATGAGGA	300-307	300-310	CAT	Jia *et al.*, 2007
P13	GGAGAGATTCCGGGCTCTAGT	ACGGTTCCGACATTTTAACG	166-170	160- 170	CA	Jia *et al.*, 2007

transferred markers to *S. sphacelata* (75%) was higher than the 26% observed by Jia *et al.* (2007) with microsatellites developed and evaluated in *S. italica*. It is also higher than the polymorphism obtained by Wang *et al.* (2005) between Poaceae species (67%) and between self-incompatible species (57%).

In order to assess the applicability of the transferred SSR markers, we performed a genetic diversity analysis in *S. sphacelata* cultivars (Fig. 1). The genetic distance of materials ranged from 0.18 to 0.33. The dataset computed a high cophenetic correlation between matrixes (0.892). "Selección INTA" was separated from Narok cultivar at a genetic distance of 0.18, which is expected considering that the former derives from the latter (Borrajo *et al.*, 2009). The genetic distance between *S. sphacelata* and *S. italica* obtained in this study was similar to that reported by Kumari *et al.* (2013).

Previous research on *S. sphacelata* reports significant variability in agronomically relevant, morphological characteristics among populations, lines, and cultivars (Hacker and Cuany, 1997; Jank *et al.*, 2007). In this context, the availability of molecular markers for the species could contribute to a production of more proficient genetic improvement programs. Furthermore, Hacker and Cuany (1997) studied variation in seed production between cultivars of *S. sphacelata* and found that Kazungula and Narok are the most contrasting ones. Similar results are reported by Hacker (1991), Hacker and Cuany (1997), Jank and Hacker (2004) and Jank *et al.* (2007), coinciding with the molecular classification obtained in this study (Fig. 1).

For *T. crinita*, 19 (out of the 260 evaluated markers) SSR were successfully transferred from five Poaceae species (a 7.3% transfer rate). Microsatellites from *C. ciliaris* and *Z. mays* showed the highest transfer rates of 18% and 10%, respectively (Tab. 2). These results are in full agreement with reports by Barbará *et al.* (2007) on transfer rates between genera of approximately 10% for eudicots and even lower for monocots.

PIC varied from 0.11 to 0.36. Sixty-one different PCR products were obtained with an average of 3.21 bands per marker and sizes ranging from 85 to 450 bp. In total, 69% of the transferred SSR were polymorphic. These are high levels of polymorphism in comparison with the 34-46% obtained by Saha *et al.* (2006) with transferred SSR markers from *F. arundinacea* to Poaceae species. Our results are similar to those obtained by Wang *et al. (*2005) for *Cynodon* with SSR transferred from major cereal crops.

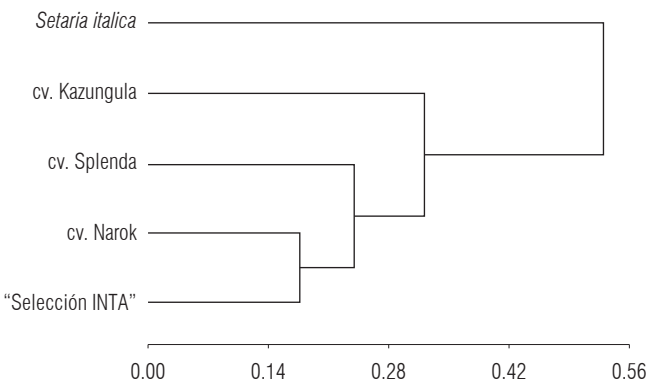

FIGURE 1. Genetic variability among *Setaria sphacelata* cultivars based on the 8 SSR markers transferred from *S. italica*. The clustering analysis was performed using the UPGMA and Dice coefficient. *S. italica* was used as an outgroup.

Kozub *et al.* (2018) also transferred SSR markers to *T. crinita*. Whereas the level of polymorphism obtained in our work (69%) is higher than that obtained by the authors (37.5%), our transfer rate (7.3%) is lower than theirs (15.2%). This could be explained by the use of highly phylogenetically related species by Kozub *et al.* (2018).

We assessed genetic diversity among *T. crinita* individuals through cluster analysis. We grouped materials in four clusters, with genetic distances ranging from 0.13 to 0.37 and a cophenetic correlation coefficient of 0.905 (Fig. 2). Although the distribution of the individuals into the four clusters was not completely consistent with the collection sites, the SSR markers used for this study exhibited a potential for discriminating the individuals from Catamarca and Cordoba. Individuals from La Rioja were scattered between the groups. The sample collected in La Pampa (cluster A) was the first to separate, with a genetic distance of 0.37. Cluster B, formed by four individuals collected in Cordoba, separated at a genetic distance of 0.34. Finally, clusters C and D separated at 0.31. The first was composed of three individuals from Cordoba and two from La Rioja, and the latter consisted mainly of individuals from Catamarca.

Additionally, we performed a principal coordinates analysis (PCoA) to better visualize the variability among the individuals (Fig. 3). The distribution obtained with the PCoA is analogous to the classification obtained with the cluster analysis. Moreover, an AMOVA was computed, and we observed that 79% of the molecular variance belonged to differences within populations, whereas 21% belonged to differences among them.

TABLE 2. Summary of the SSR markers transferred to *Trichloris crinita* from five Poaceae species.

SSR	Forward primers (5´- 3´)	Reverse primers (5´- 3´)	Size (bp)		Repeat motif	Donor species	References
			Expected	Observed			
LPSS-RH01A07	TGGAGGGCTCGTGGAGAAGT	CGGTTCCCACGCCTTGC	350	225-400	nd	*L. perenne*	Jones *et al*., 2001
NFFa019	GCTCGTGTATGGCCTTCAAT	TGGATTTGCAATTAGCCTCA	190	180-350	nd	*F. arundinacea*	Saha *et al*., 2003
2D03b	CAATGGGAGCTCAAATTAGCA	CGGGGAAGAAGTTTGTTCTTT	250	160-200	AT	*C. ciliaris*	Jessup, 2005
2D09	CAAATCGGAGCAAATCGG	AGGAAAGCCTCGGGAAAC	358	115-205	AAC	*C. ciliaris*	Jessup, 2005
6E10	ACTCCACTGCTGCCTCCT	CTTCCACCACCATACCCT	389	180-200	GCC	*C. ciliaris*	Jessup, 2005
7B11	CTCCATTCGCCTCCCTAC	GTTTCGTCTCTCCCATCAG	391	200	GA	*C. ciliaris*	Jessup, 2005
7E09	GGAGGTAGATGTTGATGTTGA	CCCTTTGTCCCGCCATAC	360	85-320	CGG	*C. ciliaris*	Jessup, 2005
7H12	TCTTATTCCTCCGAGCCGTA	GGAAAATTGGGACCCTTTGT	182	170-450	CT	*C. ciliaris*	Jessup, 2005
10E12	CTCTTGAACCCCGAGGCTAT	ATCTCGCGTCATCGTTTAGG	196	105-350	GT	*C. ciliaris*	Jessup, 2005
10G10a	AAGAAGAAGAAGAAGAAGAAGGA	GGAAGAGGAGACCAACAAA	193	350-380	TGAC	*C. ciliaris*	Jessup, 2005
10H10	CGACTCAGACCACCTCTC	GGCTCCCAGTTCTTCATC	307	160	CGA	*C. ciliaris*	Jessup, 2005
xgwm190	GTGCTTGCTGAGCTATGAGTC	GTGCCACGTGGTACCTTTG	201-253	165-350	CT	*T. aestivum*	Röder *et al*., 1998
bnlg0439	TTGACATCGCCATCTTGGTGACCA	TCTTAATGCGATCGTACGAAGTTGTGGAA	nd	200-220	nd	*Z. mays*	http//www.agron.missouri.edu
bnlg1014	CACGCTGTTTCAGACAGGAA	CGCCTGTGATTGCACTACAC	nd	160-190	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg1016	CCGACTGACTCGAGCTAACC	CCGTAACTTCCAAGAACCGA	nd	120-180	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg1811	ACACAAGCCGACCAAAAAAC	GTAGTAGGAACGGGCGATGA	nd	165-185	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg2057	CAGCAGAACCTGTGGACAGA	TGCATACTTGAGGATCGGAG	nd	125-195	AG	*Z. mays*	http//www.agron.missouri.edu
umc1220	ATCTTTTTCCTTCCGAGCTGTACG	GTGGACGAGTCCGTGCTCAG	nd	130-140	AG	*Z. mays*	http//www.agron.missouri.edu
umc1331	TTATGAACGTGGTCGTGACTATGG	ATATCTGTCCCTCTCCCACCATC	nd	145-170	GGT	*Z. mays*	http//www.agron.missouri.edu

bp: base pairs; nd: not determined.

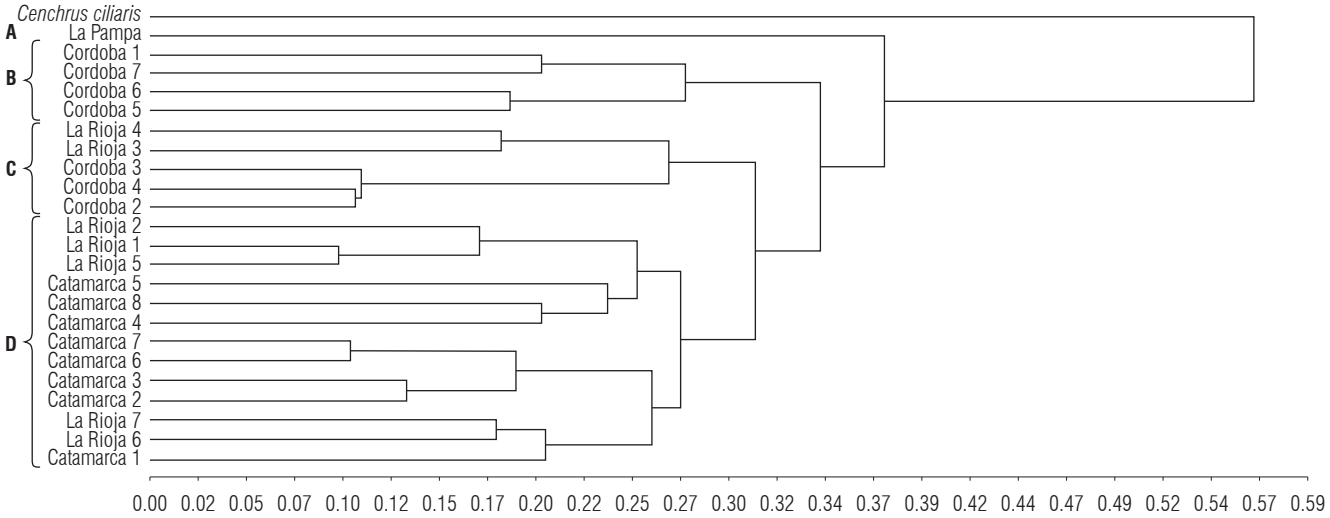

FIGURE 2. Dendrogram showing the genetic relationships among 23 *Trichloris crinita* individuals based on the transferred SSR markers. The clustering analysis was performed using the UPGMA and Jaccard coefficient. Letters indicate groups discriminated at 50% of the maximum estimated genetic distance. We used *C. ciliaris* as an outgroup.

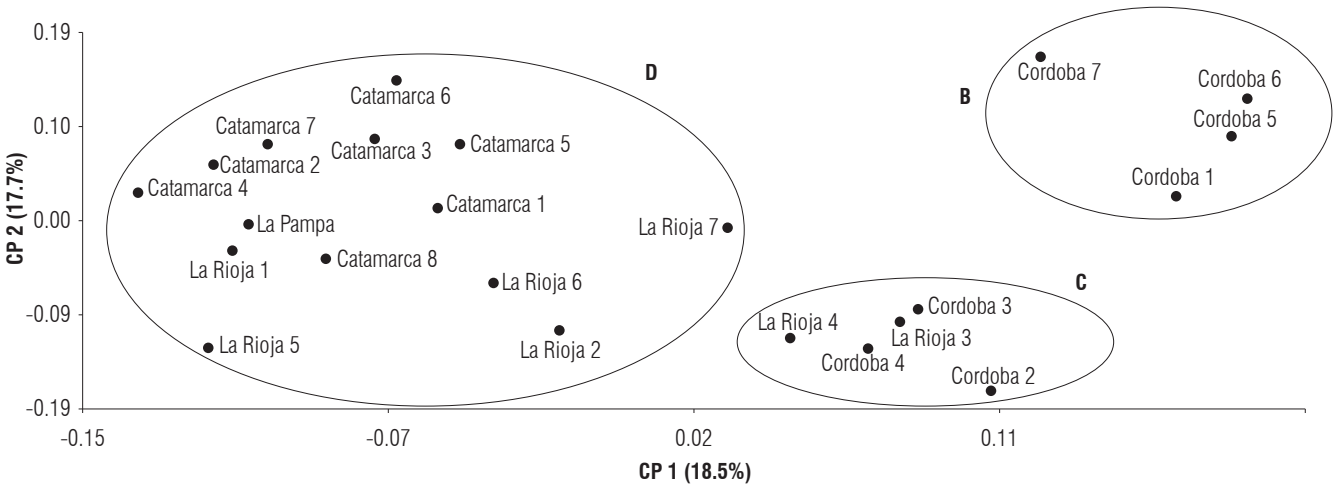

FIGURE 3. Principal coordinates analysis plot. Variability among 23 *Trichloris crinita* individuals based on the transferred SSR markers. Circles delimit the individuals grouped into clusters B, C, and D according to the clustering analysis showed in Figure 2.

Conclusions

The goal of this study was to generate a molecular tool for *S. sphacelata* and *T. crinita* that could be used to make more efficient genetic improvement programs in these species. The strategy was to exploit the available genetic resources of agronomically relevant crops to identify markers in grass species with limited genomic information.

We successfully transferred eight polymorphic SSR markers to *S. sphacelata* and 19 to *T. crinita*. These markers widen the available molecular resources for these forage crops, especially in *T. crinita* for which this study constituted the second report of transferred microsatellites in the species. As for *S. sphacelata*, these findings not only expanded the available genetic resources but also increased the number of methodological tools for the species with the optimization of the DNA extraction protocol. We expect the results obtained in this study will be valuable for the development of new molecular breeding programs and novel strategies to assess genetic diversity in the species.

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Yield and physicochemical quality of *Physalis peruviana* L. fruit related to the resistance response against *Fusarium oxysporum f.* sp. *physali*

Producción y calidad fisicoquímica del fruto de *Physalis peruviana* L. en relación con respuesta de resistencia a *Fusarium oxysporum f.* sp. *physali*

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ABSTRACT

Cape gooseberry (*Physalis peruviana* L.) is a fruit of great interest, due to its high nutritional and potential medicinal value. Vascular wilt disease caused by the fungus *Fusarium oxysporum* f. sp. *Physali* (*Foph*) is responsible for crop losses of up to 100% which makes necessary to identify resistant cultivars. To contribute to crop improvement processes, a physicochemical characterization was performed on fruits of 33 cape gooseberry genotypes using 18 quantitative descriptors. The genotypes were planted in the field under high and no pressure of *Foph*. The Student's *t* test detected statistically significant differences ($P<0.05$) between the two conditions for yield, fruit cracking (%) and fruit juice pH. The principal component analysis explained in five factors 84.96% of the total variance, in which the fruit physical variables were the major contributor to the first component (41.65%). Cluster analysis grouped the genotypes under high and no pressure in seven and eight clusters, respectively. Two contrasting genotypes showing differential resistance response to the pathogen were analyzed for fruit antioxidant capacity, in which DPPH and ORAC methods presented significant differences ($P<0.05$) between the two genotypes with greater antioxidant activity in the susceptible material.

Key words: fruit quality, antioxidant capacity, promising genotypes, vascular wilt.

RESUMEN

La uchuva (*Physalis peruviana* L.*)* es una fruta de gran interés por su alto contenido nutricional y potencial medicinal. En el cultivo, la marchitez vascular, enfermedad ocasionada por el hongo *Fusarium oxysporum* f. sp. *physali* (*Foph*), genera pérdidas hasta del 100%, siendo necesario identificar cultivares resistentes. Para contribuir con procesos de fitomejoramiento, se caracterizaron fisicoquímicamente frutos de 33 genotipos de uchuva con 18 descriptores cuantitativos. Los genotipos fueron sembrados en campo, bajo condiciones de alta presión del patógeno *Foph* y sin presencia de este. El test de Student detectó diferencias estadísticas ($P<0.05$) entre las dos condiciones para rendimiento, rajado del fruto y pH. El análisis de componentes principales explicó con cinco factores el 84.96% de la varianza total, donde las variables físicas del fruto fueron las de mayor contribución para el primer componente (41.65%). Mediante un análisis de conglomerados, se clasificaron genotipos con y sin presencia del patógeno, en siete y ocho grupos, respectivamente. Sobre dos genotipos constrastantes por su respuesta diferencial al patógeno, se realizó un análisis de capacidad antioxidante de frutos, en donde los métodos DPPH y ORAC presentaron diferencias ($P<0.05$) entre los dos genotipos siendo mayor la actividad antioxidante en el material suceptible.

Palabras clave: calidad de fruto, capacidad antioxidante, genotipos promisorios, marchitez vascular.

Introduction

Cape gooseberry, *Physalis peruviana* L. is native to the Andes, grows between 1,500 and 3,000 m a.s.l. as a wild plant and as a crop between 1,800 and 2,700 m a.s.l. (Fischer *et al*., 2005). Colombia is the largest producer in the world, generating revenues of up to 31.7 million dollars per year and concentrating 90% of world production (Agronet, 2016). The extracts of the plant and its fruits are attributed with several anti-cancer and anti-inflammatory properties, due to the high content of antioxidants, vitamins, minerals, and fiber and its low-calorie content (Ramadana, 2015). Recently, in Colombia, two outstanding cultivars were registered for their fruit quality (ICA, 2016). However, cape gooseberry still requires improved cultivars to accurately control diseases. At the national level, the greatest limitation is vascular wilt (Enciso-Rodríguez *et al*., 2013; Osorio-Guarín *et al*., 2016), a disease caused by *Fusarium oxysporum* f. sp. *physali* (Simbaqueba *et al*., 2018), causing losses of up to 100% of production and yield (González and Barrero, 2011). In terms of quality, the cracking of the fruit stands out as the greatest limitation (Fischer *et al*., 2011; Valdenegro *et al*., 2013).

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Several studies of phenotypic characterization of *P. peruviana* and related species from Colombian germplasm have found variability in the fruit descriptors. Among these, the research of Bonilla (2008) with 24 accessions from the provinces of Nariño, Valle del Cauca, Caldas, and Cundinamarca stands out. Morevoer, Trillos *et al.* (2008) identified that fruit cracking, calyx weight, and fruit number per plant were useful descriptors for the differentiation of 46 cape gooseberry accessions conserved in the Germplasm Bank for Food and Agriculture, Plant Subsystem, located at the Research Center "La Selva" of AGROSAVIA. Madriñan *et al.* (2011) determined that the Brix degrees, weight and fruit diameter allowed the differentiation of 29 introductions from the work collection of the Universidad Nacional de Colombia (Palmira campus). On the other hand, Herrera *et al.* (2012) identified diversity by weight, size, pH and maturity index in 54 accessions of the Center and North-East regions of Colombia.

Regarding the resistance response to *Fusarium oxysporum* f. sp. *physali* (*Foph*), González and Barrero (2011) used the highly virulent strain of the pathogen (MAP5) to evaluate 58 accessions of cape gooseberry and 12 related species from the collections of the Universidad Nacional de Colombia (Bogota campus), the Universidad de Nariño and AGROSAVIA. The authors identified accessions with different levels of phenotypic resistance response to the pathogen in plants under controlled conditions (Enciso-Rodríguez *et al.*, 2013). From the accessions that showed different levels of phenotypic response comprising resistant (R), moderately resistant (MR) or susceptible (MS); seed multiplication was performed for further evaluation of field response with a high and no inoculum pressure of the *Foph* MAP5 strain (Rodríguez, 2013). Moreover, Osorio-Guarín *et al.* (2016) identified promising accessions with different degrees of resistance, as well as sixteen Single Nucleotide Polymorphisms (SNPs) markers associated with the resistance response.

The main objective of the present research was to evaluate fruit traits of commercial interest, i.e. yield, quality, physicochemical and antioxidant capacity in 14 accessions previously identified by carrying traits of interest, and investigate their relationship to the resistance response to *Foph* in order to identify promising genotypes for their direct use or as parentals for genetic improvement processes.

Materials and methods

The study was carried out at the Tibaitata Research Center of the Colombian Corporation for Agricultural Research, AGROSAVIA, located in the municipality of Mosquera (Cundinamarca), at 4°42'00" N and 74°12'00" W, at 2,543 m a.s.l., cold thermal floor (Fa). The experiment was carried out in the field between September 2010 and September 2011 under the following climatic conditions: average temperature between 13 and 14°C, relative air humidity between 80 and 85%, precipitation between 34 and 198 mm/month and effective solar brightness between 2.6 and 4 h. The plants used in the experiment were previously germinated or hardened under greenhouse conditions in 1 kg bags with 50% husk: 50% soil substrate. They were grown and maintained in the greenhouse during 45 d. After that, they were moved to the field and planted in solarized soil contained in No.6 plastic bags with a capacity of approximately 25 kg (Rodriguez, 2013). Once planted, the plants were located on the ground and later positioned at a planting distance of 2 m between rows and 2 m between streets. The experiment comprised two lots (500 m^2 each), one with high inoculum pressure of the MAP5 strain of *Foph* ($1x10^5$ cfu ml^{-1}) sprayed directly to the soil, and a second one without pathogen inoculation. For each case, a completely randomized experimental design was implemented (Rodríguez, 2013). The inoculum for the experiment was produced according to the methodology proposed for Namiki *et al.* (1994) with some modifications. MAP5 isolate was grown in 2L Erlenmeyer flasks containing 500 mL of PDB medium during 10 d at 28°C in constant shaking. The culture was filtered using three layers of sterile gauze and conidial suspension was adjusted to the concentration mentioned above. This concentration was tested previously in greenhouse experiments under controlled conditions (Enciso *et al.*, 2013).

In each lot, 30-31 genotypes from 14 *P. peruviana* accessions were evaluated. The accessions were provided by the Colombian Germplasm Bank for Food and Agriculture, Plant System (Tab. 1). Fertilization, cultural practices and pest and disease management were carried out as described in the Technical Manual for the management of the cape gooseberry crop (Zapata *et al.*, 2002).

The phenotypic response of the accessions evaluated under field conditions was recorded considering the disease severity variable. The symptoms monitoring was carried out monthly, adapting the scale of evaluation of vascular wilt symptoms in the greenhouse (Enciso *et al.*, 2013). This scale is composed of 10 categories, where 0 groups the plants that show no symptoms of the disease and 9 those that have died from the infection caused by *Foph*. For this study, plants with values ≤4 on the severity scale were considered resistant and plants with values >4 were considered

TABLE 1. Response of accessions from the cape gooseberry collection (*P. peruviana*) evaluated after the infection process by *Foph* MAP5 strain under greenhouse and field conditions.

Accession code[a]	Number of genotypes per accession evaluated in field	Genotype code per accession evaluated in field[b]	Country of collection (province)	Phenotypic response in greenhouse[c]	Resistance response in field[d]
09U047	7	1-7	Colombia (Boyaca)	MR	S
09U086	1	33	Ecuador	S	S
09U089	3	34-36	Colombia (Antioquia)	AS	S
09U099	3	37-39	Colombia (Caldas)	AS	S
09U116	1	40	Colombia (Antioquia)	AS	R
09U128	1	41	Colombia (Cundinamarca)	S	S
09U136	1	43	Colombia (Cundinamarca)	AS	S
09U138	3	44-46	Francia	MS	S
09U140	1	55	South Africa	AS	S
09U210	2	68-69	Colombia (Nariño)	S	S
09U216	3	70-72	Colombia (Nariño)	MS	S
09U274	3	73-75	Colombia (Cundinamarca)	S	S
09U279	3	76-78	Colombia (Nariño)	R	R
09U261	1	100	Colombia (Cundinamarca)	MS	S

[a]Code assigned by the Germplasm Bank and plant subsystem to the accessions evaluated under conditions of high and no presence of *Foph*. [b]Code assigned to each genotype obtained by *in vitro* multiplication or self-pollination of accessions evaluated in greenhouse and later in the field.

[c]Phenotypic response in greenhouse according to Pulido (2010). AS: highly susceptible, S: susceptible, MS: moderately susceptible, MR: moderately resistant, R: resistant. [d]Phenotypic response in the field according to Rodríguez (2013).

susceptible. Statistical analyzes were carried out using SAS software version 6.1 using a Chi-square test (X^2) in order to determine the percentage of resistance of the evaluated accessions (Rodriguez, 2013). At 281 d after the establishment in the field, fruit harvest was started at an optimum degree of maturation, during three biweekly samplings. The fruits were classified according to the Colombian Technical Standard NTC 4580 (Icontec, 1999). Eighteen quantitative descriptors, with 5 fruits per descriptor, related to quality and yield were used (Herrera *et al*., 2012) (Tab. 2). The physicochemical variables were analyzed on export-type fruits (Icontec, 1999).

The fruit antioxidant capacity was evaluated on the genotypes 09U274-74 and 09U279-76 selected for their differential resistance response to *Foph* (Tab. 1). This variable was quantified using the methods ABTS (Antioxidant capacity of Trolox equivalents), DPPH (1,1 diphenyl-2-pyridylhydrazyl) according to Rufino *et al*. (2007), FRAP (Antioxidant Power of Ferric Reduction) according to Benzie and Strain (1996), and ORAC (Absorbance Capacity of Oxygen Radicals) and total phenols according to Andre *et al.* (2007).

Data from the 18 descriptors were analyzed using the statistical software SAS version 9.4 (SAS Inst., Inc., Cary, NC). The antioxidant capacity was analyzed with the GLIMMIX procedure of the mentioned software, under a completely randomized design with a 2x2 factorial arrangement (two genotypes x two types of pathogen pressure). The mean comparison was performed using the Tukey test (P<0.05).

Results and discussion

Comparison of means of the variables analyzed

The *t-Student* test showed statistically significant differences (P<0.05) between the conditions of high pressure of the pathogen and no presence of the same, for the variables Yield (Y), Percentage of cracked fruit (%CF) and pH (Tab. 2). Regarding the comparison of means, genotypes with high pressure of the pathogen showed a decrease in Y of 723 g/plant, an increase in %CF of 4.12% and an increase in pH of 0.05. The above agrees with previous publications related to production losses caused by *Foph* (Zapata *et al*., 2002; González and Barrero, 2011).

For the materials without pathogen pressure, it was observed that the values for fruit equatorial diameter (FEd), fruit polar diameter (FPd), total soluble solids (TSS), total titratable acidity (TTA), fruit weight with calyx (FWC) and Y were placed within the standard ranges according to NTC 4580 (Icontec, 1999).

TABLE 2. Description of quantitative variables evaluated in the work collection of *P. peruviana*.

Variable	Unit	Average*		SD		CV		
		P	NP	P	NP	P	NP	
Fruit weight (FW)	Grams (g)	5.61	5.20	0.97	0.76	17.73	14.28	
Fruit weight with calyx (FWC)	Grams (g)	6.04	5.70	0.99	0.72	24.90	15.74	
Dry fruit weight (DFW)	Grams (g)	1.03	1.00	0.14	0.12	13.96	12.28	
Dry fruit weight with calyx (DFWC)	Grams (g)	1.23	1.19	0.17	0.14	13.80	11.39	
Fruit volume (V)	mL	5.4	5.1	0.96	0.73	17.86	14.18	
Fruit equatorial diameter (FEd)	Mm	21.2	20.8	1.55	1.05	7.51	5.03	
Fruit polar diameter (FPd)	Mm	19.7	19.4	1.12	0.89	6.09	4.53	
Yield (Y) (3 harvests)	g/plant	1026.7*	1749.7*	370.9	567.9	36.9	36.4	**
Weight of cracked fruit (WCF) (3 harvests)	g/plant	74.63	47.06	109.1	61.2	1497	130.4	
Percentage of cracked fruit (%CF)	Percentage	6.95	2.83	7.73	3.36	114.4	116.2	**
pH	pH	3.53	3.48	0.05	0.05	1.66	1.38	**
Maturity index (MI)	SST/ATT	7.88	8.03	0.96	0.95	11.99	11.62	
Total soluble solids (TSS)	Brix degrees	15.03	14.75	0.85	0.88	5.56	5.85	
Total titratable acidity (TTA)	% citric acid	1.94	1.87	0.18	0.20	9.36	10.58	
Firmness (F)	Pounds/in^2	3.50	3.21	0.62	0.68	17.86	22.75	
Weight of 100 seeds (WS)	Grams (g)	0.10	0.10	0.01	0.01	7.13	8.42	
Number of seeds per fruit (NSF)	Number	221.82	214.71	36.48	47.58	18.57	21,99	
Weight of seeds of 5 fruits (WS5)	Grams (g)	1.11	1.06	0.18	0.24	18.07	22.32	

*Average of three harvests. **Statistically significant differences ($P<0.05$). SD: Standard deviation. CV: Coefficient of variation. P: high pressure of the pathogen. NP: no presence of the pathogen.

Principal component analysis (PCA)

The PCA for materials under high and no pathogen pressure accumulated 85.45% and 85.27% of the total variance, respectively, at the first five main components. For materials under high pathogen pressure, component one accumulated 40.5% of the variance, in which FEd, FPd, Fruit volume (V), FWC, Dry fruit weight with calyx (DFWC), Fruit weight (FW) and Dry fruit weight (DFW) were the variables of greatest importance to define this component. The component two that participated with 18.13% of the total variance was explained mainly by the variables: Weight of cracked fruit (WCF), %CF, Weight of seeds of 5 fruits (WS5), Number of seeds per fruit (NSF) and Y. The third component accumulated 11.45% of the variance, in which Firmness (F) and TTA were the variables with the highest contribution, while factors 4 and 5 contributed 8.98% and 8.64% of the variance, respectively (Tab. 3).

TABLE 3. Contribution in variance for each of the main components selected in materials under high and no pressure of the pathogen.

Principal Component	Percentage of total variance explained		Descriptor	Genotype number of higher contributions per component
	Absolute	Accumulated		
Under high pathogen pressure				
1	40.50	40.50	FEd, FPd, V, FWC, DFWC, FW and DFW	1,2,4,5,35,38, 44,77,100
2	18.13	58.63	WCF, %CF, WS5, NSF and Y	1,4,6, 35,36,37,38,40,44,45, 46
3	11.45	70.08	F and TTA,	3,5,33, 34, 35,36,39,55,68,69,71,73,74,75,76,77
4	8.98	79.06	WCF, %CF and pH	2,5,36, 73,100
5	6.39	85.45	F, TSS and MI	4,5,33,36,41,44
Without pathogen pressure				
1	40.58	40.58	FEd, FPd, V, FWC, DFWC, FW and DFW	1,2,7,35,44,68,70,75,78,100
2	13.19	53.77	WCF, %CF and TTA	,3,6,36,37,40,43,44, 46, 68, 73. 75
3	11.53	65.30	TSS, NSF and WS5	3,33, 34, 35,36,39,55,68,74,75,76,77
4	11.33	76.63	F, WCF, %CF, MI and pH	1,2, 6, 35,36, 41, 40, 43, 44, 69 71,76
5	8.64	85.27	Y	1,2,3,36,37, 55, 70,71,72,73, 74, 75

In the materials without the pressure of the pathogen, component number one included variables related to fruit diameter, volume, and weight, accumulating 40.58% of the total variability. Component two, which accumulated 13.19% of variability, was explained by the variables WCF, %CF, and TTA; therefore, it grouped genotypes of high fruit cracking and a high percentage of citric acid. The third and fourth components, with 11.53 and 11.33% respectively, were explained by variables related to fruit seeds, fruit cracking, maturity index (MI) and pH.

Criollo *et al*. (2001) worked with the cape gooseberry collection of the Universidad de Nariño and found that the fruit diameter, weight, and Brix degrees descriptors were the most important. These results also agree with those reported by Bonilla *et al*. (2008), Trillos *et al*. (2008), Herrera *et al*. (2012) and García-Arias *et al*. (2018). This indicates that national collections from different sources agree on the variables of greatest importance to discriminate the phenotypic variability associated with fruit cape gooseberry traits.

We found significant positive Pearson correlations for MI and TSS (0.54), as well as for V/FW and DFWC/DFW (0.98). Also, a negative correlation (-0.87) between MI and TTA was found. The above agrees with studies published by Herrera *et al.* (2012). Although these correlations are barely affected by the presence or absence of the pathogen in the plant (Toledo-Souza *et al.,* 2012), they are of great help for future selection processes or morpho-agronomic characterization processes, since the positive relationships between traits may allow reducing the number of study variables such as the case of fruit volume (V) and fresh fruit weight (FP).

Cluster analysis

A cluster analysis was carried out based on the main components selected. Based on the dendrogram corresponding to the genotypes under high pathogen pressure (Fig. 1A), seven homogeneous groups of materials were generated. Group one was comprised by genotypes 1, 3, 34, 77, and 5, with materials 77 with high resistance response and material 1 with the highest Y (1975.40 g/plant) being the ones that stood out. The mean values of DFWC (1.31 g) and pH (3.56) were higher compared to the other groups. In group two, consisting of genotype 4, the variables Y (1543.10 g/plant), FEd (23.40 mm) and FPd (20.80 mm) presented means above the general average of all materials (Tab. 4). Therefore, it is possible to propose the use of genotypes 1, 4 and 77 in breeding programs focused on yield, fruit size, and fruit quality.

Group three comprised genotypes 2, 44, 100, 33, 36, 72, 70, 39, 43, and 55, with high MI (8.51), which is higher than the general average (7.88), and low TTA (1.82%) (Tab. 4). Group four showed the lowest mean for Y with 647.33 (g/plant). Group five harbored genotypes 35, 38, 71, 74, 75, and 76, the latter with high resistance response in the field (Tab. 1). These materials also had high TSS (15.42 °Brix), a feature of great interest for industrial processes.

Group six, composed of genotypes 46, 68 and 73, included materials with high CF (83.64 g/plant), compared to the general average (74.63 g/plant). On the other hand, the values for FPd (17.86 mm) and FEd (18.99 mm) were the lowest within the materials under high *Foph* pressure. Group seven gathered materials 37, 69, 78, and 40, the latter standing out for its resistance to the pathogen in the field.

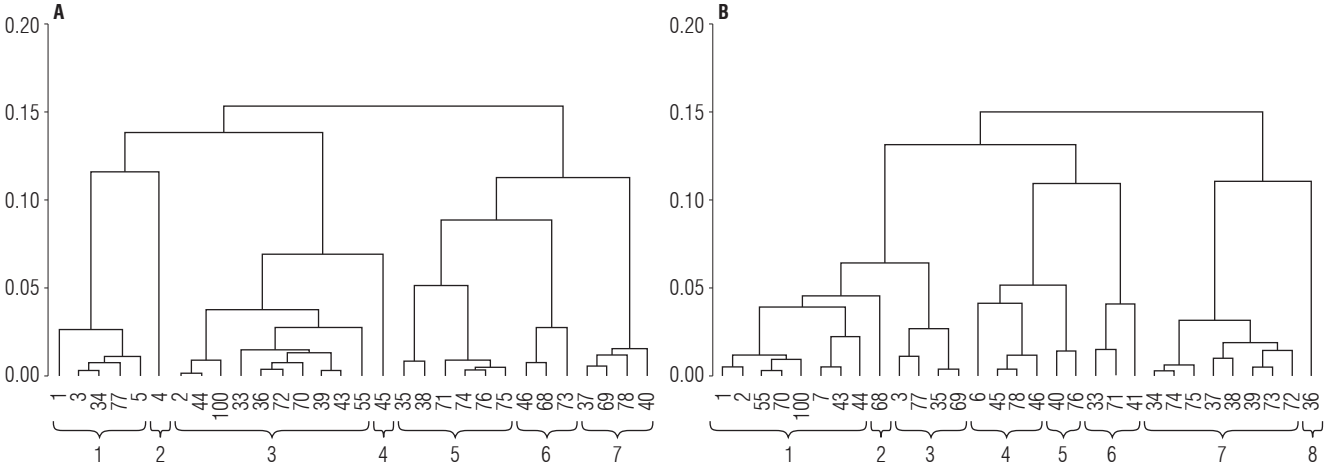

FIGURE 1. Dendrogram for materials under high pressure (A) and without the pressure of the pathogen (B). Coding: Field code (internal numbers of each group) according to Tab. 1. External horizontal numbers of the brackets indicate the number of clusters.

TABLE 4. Mean of the variables for each group conformed by cluster analyses, for genotypes with high pressure of the pathogen.

Variable	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
FW	6.32	6.83	6.00	5.47	5.74	4.28	4.26
FWC	6.82	7.10	6.41	6.14	6.16	4.62	4.67
DFW	1.12	1.03	1.07	0.94	1.09	0.78	0.90
DFWC	1.31	1.22	1.28	1.16	1.30	0.95	1.06
V	6.26	6.40	5.87	5.40	5.58	4.16	4.15
FEd	22.57	23.40	21.77	20.85	21.41	18.99	19.09
FPd	20.44	20.80	20.20	19.00	19.91	17.86	18.48
Y	1518.12	1543.10	905.51	647.33	1010.00	681.59	965.14
WCF	65.09	60,10	54.10	50.83	58.62	83.64	29.19
%CF	4.41	39.01	5.94	7.85	5.88	10.38	3.41
pH	3.56	3.54	3.55	3.51	3.50	3.53	3.42
MI	8.28	6.48	8.51	6.01	7.73	7.71	6.97
TSS	15.00	14.43	15.32	13.33	15.42	15.06	14.25
TTA	1.83	2.24	1.82	2.22	2.02	1.95	2.05
F	3.26	4,31	3.47	3.86	3.68	4.00	2.94
WS	0.09	0.10	0.10	0.11	0.10	0.09	0.09
NSF	221.55	208.00	200.02	147.72	264.08	244.33	218.31
WS5	1.07	1.04	1.06	0.84	1.34	1.10	0.97

For materials under no pathogen pressure, eight groups were identified (Fig. 1B). Group 1 comprised eight genotypes (1, 2, 55, 70, 100, 7, 43, and 44). Group two was made up of genotype 68, in which the variables FEd (22.20 mm), FPd (21.00 mm), V (μ=6.6 6 ml), FWC (μ=7.11 g) and FW (μ=6.87 g) presented higher values, representing characteristics of interest for selection of parents, in relation to increasing in fruit size (Tab. 5). In group three, genotypes 3, 77, 35 and 69 characterized by high concentrations of total soluble solids TSS (15.42 °Brix) and high maturity index MI (8.99) were located. Genotypes 6, 45, 78, and 46 formed group 4, in which the percentage of citric acid TTA (2.08) was the most important variable. Group 5 (40 and 76) was characterized by a low number of NSF seeds (159.86), low

TABLE 5. Mean of variables for each group conformed by cluster analyses, for genotypes with no pressure of the pathogen.

Variable	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
FW	5.85	6.87	5.40	4.94	3.85	4.83	4.87	4.97
FWC	6.35	7.11	5.88	5.33	4.45	5.35	5.39	5.65
DFW	1.08	1.23	1.04	0.89	0.80	0.92	0.98	1.11
DFWC	1.28	1.46	1.22	1.08	0.94	1.09	1.18	1.30
V	5.70	6.66	5.30	4.78	3.73	4.75	4.85	5.00
Fed	21.81	22.20	20.79	20.53	18.31	20.58	20.42	20.93
FPd	20.25	21.00	19.62	18.92	17.63	19.31	19.11	18.56
Y	1825.77	1549.13	1821.49	1024.16	1259.00	1488.07	2174.44	2324.17
WCF	56.03	50.0	20.02	40.08	53.95	21.01	34.05	3266
%CF	3.66	0.32	1.10	3.59	4.49	1.38	1.55	13.84
pH	3.48	3.37	3.46	3.46	3.44	3.54	3.46	3.56
MI	8.26	7.09	8.99	6.75	7.52	8.91	7.81	8.50
TSS	14.74	14.60	15.42	13.77	14.73	14.07	15.07	15.59
TTA	1.80	2.06	1.75	2.08	1.98	1.58	1.95	1.84
F	3.16	3.19	3.88	3.48	4.21	2.69	2.75	3.08
WS	0.10	0.10	0.09	0.09	0.08	0.10	0.09	0.09
NSF	219.50	244.66	186.66	180.43	159.86	173.07	257.34	289.25
WS5	1.12	1.22	0.86	0.88	0.70	0.94	1.25	1.40

equatorial diameter and polar fruit FEd (17.63 mm), FPd (18.31 mm) and high firmness F (2.10 kg/in^2). Therefore, they can be considered as promising materials to improve tolerance to physical postharvest damage.

Materials 33, 71 and 41, included in group 6, showed low contents of citric acid (1.58). Group 7 assembled genotypes 34, 74, 75, 37, 38, 39, 73, and 72, harboring materials with high Y on average (2174.44 g/plant), in which genotype 73 had the highest average production (2843.12 g/plant). Genotype 74 showed the highest concentration of TSS solutes (16.14 °Brix). In group 8, there were high Y materials (2324.17 g/plant) and the highest average value for TSS (15.59), highlighting genotype 36. Thus, groups 2 and 8 represent a genetic source of interest to improve fruit quality and production (Tab. 5).

Antioxidant capacity in fruits

The antioxidant capacity analysis was carried out on genotypes 74 (from accession 09U274) and 76 (from accession 09U279), which presented differential resistance responses to *Foph* (Tab. 1).

The concentration of total phenols did not show statistically significant differences between the evaluated materials 09U279 (resistant) and 09U274 (susceptible) (P<0.05), and it was not affected by the high pathogen pressure (P<0.05) (Tab. 6). On the other hand, the DPPH activity was higher in the susceptible material (309.0 µmoltrolox/100 g BS), compared with the resistant one (213.4 µmoltrolox/100 g BS) (P<0.001), while the high pressure of the fungus did not affect the DPPH antioxidant activity (P<0.05) (Tab. 6). For the oxygen radical absorbance capacity (ORAC), there were differences (P<0.05) between the materials with resistant and susceptible responses, with a greater antioxidant activity in the susceptible material (75.3 µmol trolox/g BS) compared with the resistant one (64.4 µmoltrolox/g BS) (Tab. 6).

The antioxidant activity measured by ABTS and FRAP showed significant differences in the interaction between the resistance response (susceptible and resistant) and the pathogen pressure (high and no pathogen (P<0.05, Tab. 6). Therefore, the material with higher resistance response (09U279) showed an increase for these two activities when it was challenged to a high pressure of the pathogen, while for the susceptible material the pathogen pressure was related to the decrease of the antioxidant capacity (Fig. 2).

The results of antioxidant activity are higher compared to those reported by Lopez *et al*. (2013), with values in FRAP of 99.70 mg AA/100 g of sample and DPPH of 53.97 µmol trolox/100 g BS. Likewise, they are superior when compared with studies by Botero (2008) with results for FRAP of 54.98 mg AA/100 g sample, total phenols with 39.15 mg AG/100 g of sample and DPPH of 192.51 µmoltrolox/100 g BS for the Colombia ecotype (La Union, Antioquia). This is possibly due to differences in the genotypes and environments studied.

The antioxidant activity could be related to the differential response of the cape gooseberry genotypes against the high inoculum pressure of the *Foph* pathogen, possibly associated with the expression of an important group of enzymes related to antioxidant activities, signaling, and plant defense. It has been reported that enzymes such as lipoxygenases (LOX), catalases, superoxide dismutases, peroxidases, glutathione reductases, polyphenol oxidases, phenylalanine ammonia lyases, chitinases, and β-1,3-glucanases are induced in response to attack (Peteira and León, 2011). Antioxidant activity measure by ORAC and DPPH in susceptible genotypes could be a defense mechanism of the plant.

TABLE 6. Combined analysis of variance under high and no pathogen pressure for genotypes 76 (09U279) and 74 (09U274) with differential resistance response.

Factor	ABTS (µmol trolox/g BS)	FRAP (mg AiiiA/100 g BS)	ORAC (µmoltrolox/g BS)	DPPH (µmoltrolox/100 g BS)	Total phenols (mg AG/g BS)
Pathogen pressure					
High	19.8	181.6	71.4	267.1	0.80
No	20.3	186.5	68.3	255.3	0.81
P value	0.8656	0.4057	0.5352	0.5815	0.6859
Genotypes					
09U279 -76 *	20.5	184.3	64.4 b	213.4 b	0.82
09U274 -74 **	19.5	183.8	75.3 a	309.0 a	0.79
P value	0.7272	0.9299	0.0425	0.0003	0.4068
P value of interaction	0.0485	0.0247	0.5318	0.1952	0.1982

*Genotypes with resistance * and susceptible response **. Averages with different letters indicate significant differences between means (P<0.05).

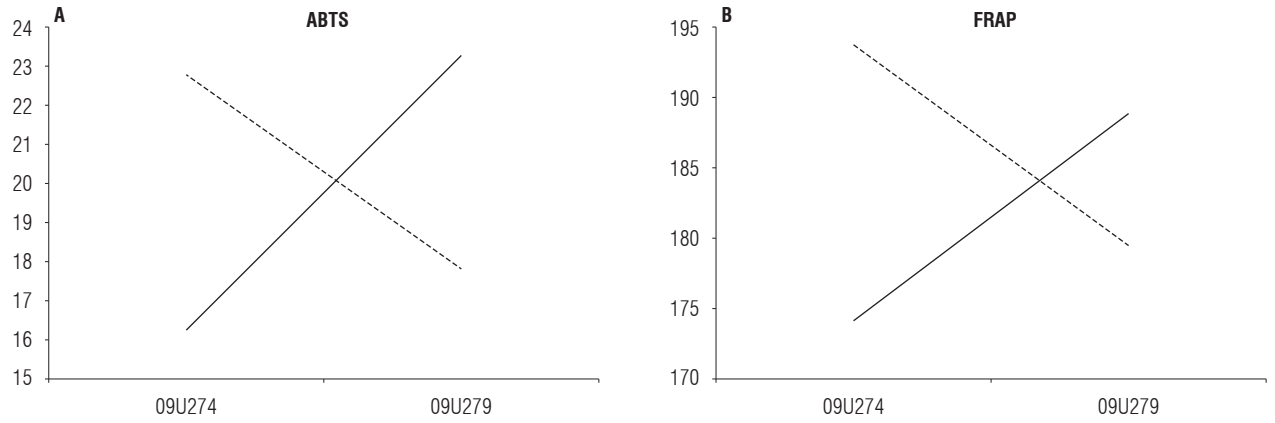

FIGURE 2. Interaction response of resistance x inoculum pressure on A) the antioxidant capacity of Trolox equivalents (ABTS) and B) the antioxidant power of iron reduction (FRAP) according to Pearson's correlation. Material with resistant response (09U279 -76). Susceptible material (09U274-74). Solid line: High pathogen pressure. Dashed line: No pathogen pressure.

Conclusions

The study allowed the identification of materials of interest for the genetic improvement of the cape gooseberry crop focused on fruit quality and yield, in relation to the resistance response to the most limiting pathogen of its production in Colombia, *Foph*. Privileging the resistance response to *Foph* and the statistical analyzes of this study, genotypes 1, 36, 68, 73, 74, and 77 are recommended as promising materials for subsequent genetic improvement schemes. In this context, material 36 has been selected for evaluation in different environments for the generation of direct cultivars or as a future parent.

The study, in turn, allowed identifying differential expression in the antioxidant activity of resistant and susceptible genotypes, possibly associated with a mechanism of defense of cape gooseberry against the *Foph* attack. Future studies will require identifying the defense genes associated with these mechanisms to open possibilities for new approaches in the protection of cape gooseberry against the pathogen.

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Variability of P26 and P10 genes in Colombian isolates of *Potato yellow vein virus* (PYVV)

Variabilidad de los genes P26 y P10 en aislamientos colombianos del *Potato yellow vein virus* (PYVV)

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ABSTRACT

Potato yellow vein virus (PYVV) is the causal agent of the potato yellow vein disease and can reduce potato production up to 50%. This virus also infects tomatoes and can remain asymptomatic in plants. PYVV transmission is mediated by vegetative seed, the vector *Trialeurodes vaporariorum,* and grafts. Its genome has the P26 and P10 genes that are orthologues in the *Crinivirus* genus, which have been characterized as pathogenic factors and have not been studied in PYVV. We analyzed the variability of P26 and P10 from 45 and 48 sequences, which were obtained by RT-PCR amplification of the total RNA of symptomatic potato leaves from the provinces of Nariño, Cundinamarca, and Boyaca (Colombia). We included sequences of each gene of the PYVV genome of potato and tomato isolates from GenBank. The variability in these genes is influenced by the flow and uncontrolled use of vegetative seed between different provinces, that favor the dispersion of viral variants. In addition, the variability analysis based on maximum likelihood trees, haplotypes, and diversity indices showed that P26 is more variable than P10 and both are more variable in Andigena than in Phureja potatoes. The Tajima and Fu and Li tests revealed that these genes are subject to negative selection.

Key words: tripartite genome, *Crinivirus, Solanum tuberosum,* negative selection.

RESUMEN

El virus *Potato yellow vein virus* (PYVV), en español virus del amarillamiento de las venas de la papa, es el agente causal de la enfermedad conocida como amarillamiento de venas de la papa y puede reducir la producción hasta un 50%. Este virus también infecta tomate y puede permanecer en plantas asintomáticas. Su transmisión está mediada por semilla vegetativa, el vector *Trialeurodes vaporariorum* e injertos. Su genoma codifica los genes P26 y P10 que son ortólogos en el género *Crinivirus,* en el cual se han caracterizado como factores de patogenicidad y no han sido estudiados en PYVV. Se analizó la variabilidad de P26 y P10 a partir de 45 y 48 secuencias respectivamente, obtenidas de la amplificación por RT-PCR del RNA total de hojas sintomáticas de papa de los departamentos de Nariño, Cundinamarca y Boyacá (Colombia), incluyendo tres secuencias de cada gen de los genomas de PYVV de aislamientos de papa y tomate reportados en GenBank. La variabilidad en estos genes está influenciada por el flujo y uso no controlado de semilla vegetativa entre diferentes departamentos, lo que favorece la dispersión de variantes virales. Además, los análisis de variabilidad basados en árboles de máxima verosimilitud, haplotipos e índices de diversidad mostraron que P26 es más variable que P10 y que ambos son más variables en papa Andígena que en Phureja. Las pruebas de Tajima y Fu and Li revelaron que estos genes están sometidos a la selección negativa.

Palabras clave: genoma tripartito, *Crinivirus, Solanum tuberosum,* selección negativa.

Introduction

Potato (*Solanum tuberosum*) is cultivated in Colombia, especially in the provinces of Antioquia, Boyaca, Cundinamarca and Nariño, where Cundinamarca and Boyaca are the most important areas for potato production (Ñústez, 2011). According to Fedepapa (2014), 250 potato cultivars are produced in Colombia; but the most important are: Criolla Colombia, Criolla Dorada, Criolla Ocarina, Pastusa Suprema, Superior, Diacol Capiro, Ica Puracé, Tuquerreña, and Roja Nariño.

The *Crinivirus* genus comprises an important group of emergent plant viruses widely distributed in the world belonging to the Closteroviridae family (Martelli *et al*., 2013). Within their genomes two proteins are coded that oscillate between 26 and 28 KDa (group P26) and 8 and 10 KDa (group P10). These proteins are unique in the genus and are orthologous in the 15 species sequenced and reported in the GenBank (Kiss *et al*., 2013; Genbank, 2019). These proteins have been characterized in some criniviruses, in which their participation in infectious processes, cell movement

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(Grimsley *et al*., 1986; Medina *et al*., 2003; Medina *et al*., 2005; Wang *et al*., 2009; Kiss *et al*., 2013), and physical self-interaction (Stewart *et a*l., 2009) have been demonstrated.

Potato yellow vein virus (PYVV) is a re-emergent *Crinivirus* that causes potato yellow vein disease (PYVD), which is characterized by chlorosis in veins and can remain in asymptomatic plants. It also infects *Solanum lycopersicum*, *Rumex* sp., *Catharanthus* sp. and *Polygonium* sp. plants (Salazar *et al*., 2000; Muñóz *et al*., 2017). The virus is transmitted by infected tubers, grafts, and by the whitefly *Trialeurodes vaporariorum* in a semi-persistent way (Salazar *et al*., 2000; Gamarra *et al*., 2006; Wale *et al*., 2008; Barragán and Guzmán-Barney, 2014; Hernández and Guzmán-Barney, 2014).

PYVV was first reported in Antioquia (Colombia) in 1943 in plants of the *S. tuberosum* group Phureja (Diploid) (Salazar *et al*., 2000), and it was later reported in *S. tuberosum* group Andigena (Tetraploid). In 1996 the PYVV virus dispersed to Ecuador, Peru and in 1998 to Venezuela (Salazar *et al*., 2000; EEPO, 2019).

PYVV is a virus limited to the phloem and accompanying cells. It has a tripartite genome composed by three molecules of single-stranded RNA, positive polarity and two defective RNAs (Eliasco *et al*., 2006; Muñoz *et al*., 2017). RNA 1 (8,035 kb) has three open reading frames (ORFs), RNA 2 (5,339 kb) and has five ORFs, and RNA 3 (3,892 Kb) has three ORFs. The P10 and P26 proteins of PYVV are present in both RNA 2 and RNA 3.

The *Closteroviridae* family is considered to be invariable, but there are differences among the genera. For example, *Ampelovirus* and *Closterovirus* genera are the most variable, and *Crinivirus* the least variable (Rubio *et al*., 2013; Erkiş-Güngör and Bayram, 2019). Low variability and genetic diversity have been reported for PYVV through single strand conformation polymorphisms (SSCP), restriction fragment length polymorphisms (RFLP) and sequence analysis of major capsid protein gene (CP), minor capsid gene (CPm) and heat shock protein homologue gene (HSP70h), although CPm is more variable and has a tendency for recombination (Offei *et al*., 2004; Guzmán *et al*., 2006; Rubio *et al*., 2013; Chaves-Bedoya *et al*., 2014; Cubillos and Guzmán-Barney, 2015).

Due to the importance of P10 and P26 gene orthologues group in pathogenicity, its viral cycle in *Crinivirus*, and the fact that these genes have not been studied in PYVV, it is necessary to know about the variability, evolution and presence of molecular viral variants in potato plants generated by selection pressure, mutations, genetic drift, migration and also inter and intraspecific recombination events. These factors may impact the PYVV infectious capacity within its host and the ability to colonize other plant species, increasing the hosts' range as in tomato plants (Ruiz *et al*., 2018). Our objective in this study was to analyze the P26 and P10 genes of PYVV isolates from two contrasting geographic regions of Colombia to determine their genetic variability, evolution, and possible relationships with the host.

Materials and methods

Plant material

Sampling was performed in potato plots with a high incidence of plants affected by PYVD symptoms in Nariño, Cundinamarca, and Boyaca (Colombia). From each crop plot leaf samples from the middle third of different plants of the potato *S. tuberosum* Andigena group (StA) and Phureja group (StP) were collected. The samples were covered with absorbent paper towels, stored in perforated plastic bags, and transported to the Biotechnology Laboratory of the Facultad de Ciencias Agrarias of the Universidad Nacional de Colombia, Bogota campus for the analysis.

Primer design

To obtain a set of primers for specific amplification of P10 and P26 genes of PYVV, we used the Primer 3 program v.0.4.0 (Koressaar and Remm, 2007; Untergasser *et al*., 2012) and Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). As templates, we used accessions AJ557129.1 (for P10 gene) and AJ508757.2 (for P26 gene) of PYVV Peruvian genome sequences from GenBank. The two primer pairs with the best characteristics in terms of length (20 to 25 bp), > 40% guanine-cytosine percentage, minimum dimerization and temperature delta between primers, flanking location for covering 100% of each gene, and melting temperature between 50 and 60°C were selected. The AmplifX program version 1.5.4 (http://ifrjr.nord.univ-mrs.fr/AmplifX) was used to simulate the migration of fragments of the P10 and P26 genes with the selected primers in a virtual agarose gel.

Total RNA extraction and verification of PYVV presence

Total RNA was extracted with the RNeasy Plant Minikit (Qiagen™) following the manufacturer's instructions, except for the use of the QIAshredder® column. The same column was used to purify and concentrate two replicates of each sample, and these were unified. RNA was diluted

in 30 µL of RNAase free water (Qiagen™) and maintained at -20°C until use. To verify the presence of PYVV in the samples, the CP gene was amplified as a control, according to the protocol by Hernández and Guzman-Barney (2014).

Primer verification, RT-PCR and sequencing

All the reagents used for the reverse transcription (RT) and amplification (PCR) process were from the Invitrogen™ brand (Thermo Fisher Scientific, MA, USA), and RT-PCR was performed in a thermal cycler (C1000, BioRad®, Berkeley, CA).

Two samples from Nariño and Cundinamarca were selected for primer verification. SuperScript® III kit (First-Strand Synthesis System for RT-PCR®) was used for P10 and P26 RT in positive samples for amplification of PYVV-CP, following the manufacturer's instructions. In separated reactions we mixed 3 µL of total RNA, 0.5 µM of P10 and P26 primer reverse (designed in this investigation, Tab. 2), 1 mM of dNTPs, 1X of buffer, 10 mM of ditioeritrol (DTT), 1.6 u μL^{-1} of RNaseOut®, 5 mM of $MgCl_2$, 8 u μL^{-1} of reverse transcriptase (Superscript®III), and RNAse free water to complete the final volume of 10 µL. After RT was performed, PCR for P10 and P26 genes were carried out in separated reactions with PCR® kit, 1X of buffer, 2.5 mM of $MgCl_2$, 0.4 µM of forward and reverse primers (Tab. 2), 0.4 mM of dNTPs, 1 u μL^{-1} of high fidelity Taq Polymerase Platinum®, 0.6 µL of cDNA, and diethyl pyrocarbonate (DEPC) treated water to complete a final volume of 15 µL. The amplification cycle was as follows: 1 min at 94°C, followed by 34 cycles of 1 min at 94°C, 30 s at 58°C for annealing and 1 min at 72°C for extension, and 10 min at 72°C for final extension. For the amplicon visualization, 2 µL of amplified product was loaded in 1% agarose gel in TAE buffer. The gel was stained with 0.02 mg mL^{-1} of ethidium bromide. The kit Pure Link PCR Purification was used to purify P10 and P26 gene PCR products. Purified products were diluted in 30 µL DNAse free water and sent to Macrogen® for forward and reverse sequencing.

Variability and diversity of P26 and P10 genes of PYVV

Forward and reverse sequences obtained from each amplicon were assembled and a consensus sequence was generated using CAP3 of the PRABI-Doua- program (Huang and Madan, 1999). Complete P26 and P10 gene sequences were submitted to the EMBL/GenBank to verify the identity using the P26 and P10 genes of PYVV. After verifying the genes' identity, the best primers were selected for RT-PCR performance and sequencing of all the samples in the same way as described above.

For the variability analysis, the contigs of P26 and P10 genes of PYVV were grouped as follows: (1) potato genotype: Andigena (Tetraploid) and Phureja (Diploid); (2) geographical origin: Nariño (N), Cundinamarca (C) and Boyaca (B): and (3) total contigs. The Sequence Demarcation Tool was used for evaluating nucleotide identity through the pairwise matrix (Muhire *et al.,* 2014). The Mega 7 Program (Kumar *et al.,* 2016) was used for multiple alignments by codons with Clustal algorithm and to estimate the best-fit nucleotide substitution model by the Akaike Information Criterion (AIC). The best model was used for the construction of the maximum likelihood tree for each gene with the bootstrap test with 1000 replicas for generating the consensus tree (Nei and Kumar, 2000). The Mega 7 program was used to estimate the average of non-synonymous (dN) and synonymous (dS) mutations and their ratio (dN/dS). The program DNaSP 5.0 (Librado *et al.,* 2009) was used for genetic variability in which we estimated total variable sites (s), total mutations (η), nucleotide diversity (π), total haplotypes (h), and haplotype diversity (dH). The neutrality test of Tajima's D (Tajima, 1989) based on the difference between segregating sites and the average of nucleotide differences, and the Fu and Li's F* test (Fu and Li, 1993) based on the differences between singletons and the average of the number of pairwise nucleotide differences were also used. The program Network 5 (Bandelt *et al*., 1999) was used for graphic representation of haplotypes for the visualization of phylogenetic networks through the Median-Joining (MJ) algorithm and the statistical parsimony method as described by Templeton *et al*. (1992).

Results

Plant material

A total of 46 samples from symptomatic *S. tuberosum* Phureja and Andigena groups (Fig. 1), from six varieties (Tetraploids: Pastusa Suprema, Diacol Capiro, and Superior. Diploids: Criolla Colombia, Criolla Guaneña and Mambera) were sampled in eight municipalities from Nariño, six from Cundinamarca and one from Boyaca (Tab. 1).

Primer selection for amplification of P10 and P26 genes of PYVV

P26 and P10 genes were amplified during the preliminary test (Fig. 2) from samples CT0426 and NT1926 (Tab. 1) (positive to the PYVV-CP gene) through four pairs of primers (Tab. 2). We decided to select P10F and P10R primers for the P10 gene amplification and P26F gene and P26R for the P26 gene.

FIGURE 1. Potato plants affected by Potato Yellow Vein Disease (PYVD). The symptoms in the potato crop are observed as yellow plants distributed in foci. A) potato crop with high incidence of plants affected by PYVD, B) potato plant with initial PYVD symptoms, C) potato plant with advanced PYVD symptoms, D) detail of a potato leaf affected by PYVD.

TABLE 1. Isolate codification of P26 and P10 genes of *Potato yellow vein virus* (PYVV) obtained from Cundinamarca, Nariño and Boyaca.

P26 gene code	P10 gene code	Province	Municipality	Host	Variety
CT0426	CT0410	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1126	CT1110	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1226	CT1210	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1326	CT1310	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1426	CT1410	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1526	CT1510	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1626	-----	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1726	CT1710	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
CT1826	CT1810	Cundinamarca	Subachoque	Andigena	Pastusa Suprema
NT1926	NT1910	Nariño	Carlosama	Andigena	Diacol Capiro
NT2026	-----	Nariño	Carlosama	Andigena	Diacol Capiro
NT2126	NT2110	Nariño	Carlosama	Andigena	Diacol Capiro
NT2226	NT2210	Nariño	Ipiales	Andigena	Diacol Capiro
NT2326	NT2310	Nariño	Cumbal	Andigena	Diacol Capiro
NT2426	NT2410	Nariño	Cumbal	Andigena	Diacol Capiro

Continue

P26 gene code	P10 gene code	Province	Municipality	Host	Variety
NT2526	NT2510	Nariño	Pupiales	Andigena	Diacol Capiro
NT2626	NT2610	Nariño	Gualmatan	Andigena	Diacol Capiro
NT2726	NT2710	Nariño	Pasto	Andigena	Diacol Capiro
NT2826	NT2810	Nariño	Pasto	Andigena	Diacol Capiro
ND2926	ND2910	Nariño	Ipiales	Phureja	Guaneña
ND3026	ND3010	Nariño	Ipiales	Phureja	Guaneña
-----	ND3110	Nariño	Carlosama	Phureja	C. Colombia*
-----	ND3210	Nariño	Carlosama	Phureja	C. Colombia*
ND3326	ND3310	Nariño	Tuquerres	Phureja	Guaneña
ND3426	ND3410	Nariño	Tuquerres	Phureja	Guaneña
ND3526	ND3510	Nariño	Guaitarrilla	Phureja	Mambera
ND3626	ND3610	Nariño	Guaitarrilla	Phureja	Mambera
ND3726	ND3710	Nariño	Tuquerres	Phureja	C. Colombia*
ND3826	ND3810	Nariño	Tuquerres	Phureja	C. Colombia*
CT3926	-----	Cundinamarca	Villa Pinzon	Andigena	Diacol Capiro
CT4026	CT4010	Cundinamarca	Villa Pinzon	Andigena	Diacol Capiro
CT4126	CT4110	Cundinamarca	Guasca	Andigena	Diacol Capiro
CT4226	CT4210	Cundinamarca	Villa Pinzon	Andigena	Diacol Capiro
CT4326	CT4310	Cundinamarca	Suesca	Andigena	Diacol Capiro
CT4426	CT4410	Cundinamarca	Suesca	Andigena	Diacol Capiro
-----	CT4510	Cundinamarca	Usme	Andigena	Diacol Capiro
CT4726	CT4710	Cundinamarca	Guasca	Andigena	Diacol Capiro
-----	CT4610	Cundinamarca	Suesca	Andigena	Diacol Capiro
CT4926	CT4910	Cundinamarca	Suesca	Andigena	Diacol Capiro
-----	CT5010	Cundinamarca	Usme	Andigena	Diacol Capiro
CT5126	CT5110	Cundinamarca	Usme	Andigena	Diacol Capiro
CT5226	CT5210	Cundinamarca	Usme	Andigena	Diacol Capiro
CT5326	CT5310	Cundinamarca	El Rosal	Andigena	Pastusa Suprema
CT6226	-----	Cundinamarca	Usme	Andigena	Diacol Capiro
-----	BT6710	Boyaca	Ventaquemada	Andigena	Pastusa Suprema
-----	BT7010	Boyaca	Ventaquemada	Andigena	Pastusa Suprema

*Criolla Colombia.

TABLE 2. Sequences of primers obtained for P10 and P26 genes amplification of *Potato yellow vein virus* (PYVV).

Primer	Sequence	%GC	3'	ΔT°	MT	Amp	AT
P10F	GAAAGACATGACAGATGAGGAAGTG	44	0	0	52°C	628	55°C
P10R	CTGTCTGCTCTAACCTGAATCTTTG	44	0				
P10F2	AAGGTTACACACTGAGAAGAGAA	39	0	0	50°C	367	55°C
P10R2	GGATCCATTGTTCTAGTACCTCA	43	1				
P26F	GGCATTGAACAGTCCGAACAC	52	0	0	53°C	900	55°C
P26R	ATCACTCGTACTAGACCTCGGG	54	0				
P26F2	CGAAGACACATGCCAACAAG	50	0	1	51°C	998	55°C
P26R2	TCGTACTAGACCTCGGGTAAATAA	42	0				

%GC: Guanine-Cytosine percentage; 3´:3´ complementarity; *ΔT°*: temperature delta between primers; *MT*: melting temperature; *Amp*: expected amplicon size in base pairs (bp); *AT*: annealing Temperature. Grey highlight: selected primer sequences for amplification of all samples.

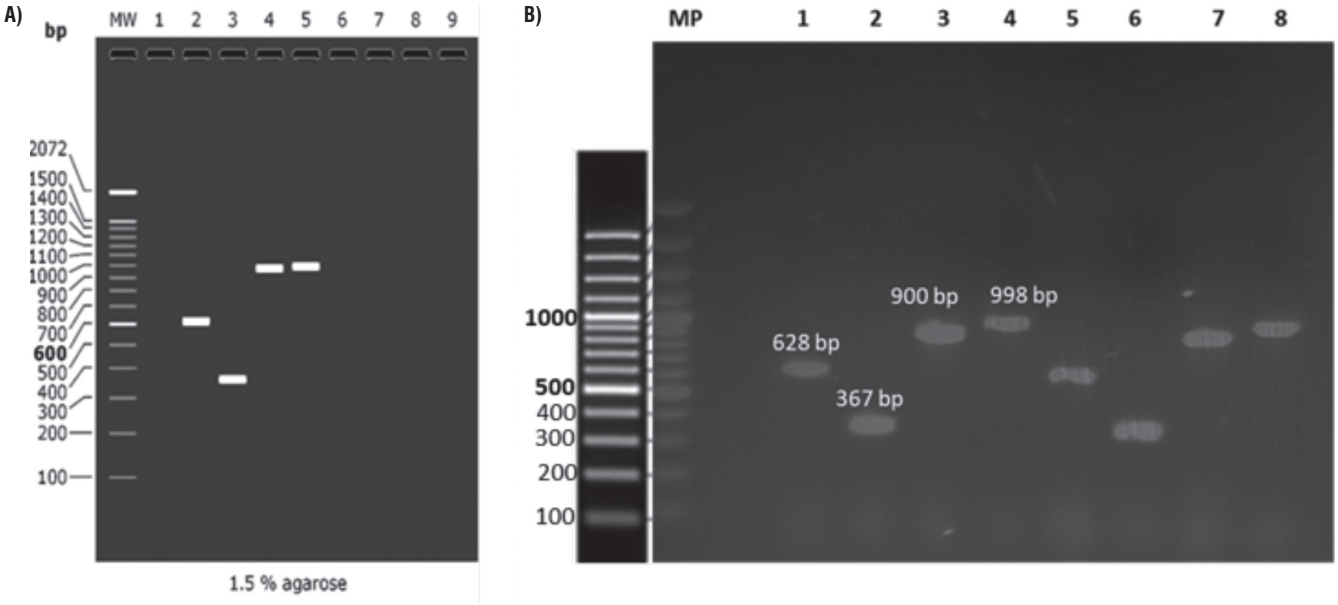

FIGURE 2. Representative amplification gel of P10 and P26 genes of *Potato yellow vein virus* (PYVV) by RT-PCR and amplicon migration comparison A) in silico and B) experimental amplification. A) In silico migration obtained by AmpliFx: lane 1: molecular weight marker, 2 and 3: P10 gene primers set, 4 and 5: P26 gene primers set. B) Representative experimental gel of amplification for P10 gene (Lanes 1, 2 and 5, 6) and P26 gene (lanes 3, 4 and 7, 8), CT0426 sample (lanes 1 to 4) and NT1926 sample (lanes 5 to 8).

Analysis of sequences

Forty-two contig sequences of P26 gene and 45 of the P10 gene of PYVV from potato leaflets were obtained (Tab. 1). Three reference sequence accessions for P26 and P10 genes of PYVV genomes were included. The first one was obtained from the *S. tuberosum* Andigena group (Cajamarca, Peru) (Livieratos *et al*., 2002), the second one from the *S. tuberosum* Phureja group (Antioquia) (Muñoz *et al*., 2016), and the third one from tomatoes (Antioquia) (Gutiérrez *et al*., 2017). In addition, the sequences of the P26 and P10 genes of the *Crinivirus* LIYV were used as an out-group in the phylogenetic analysis (Tab. 3).

Haplotype diversity of P26 and P10 genes of PYVV and networks

Eleven haplotypes (haplotypes 1 to 11) for the P10 gene of PYVV were obtained. These haplotypes had frequencies between 6.6% and 62.3% (Tab. 4). Haplotype 4 was the most frequently formed with 28 isolates from Cundinamarca and Nariño. This haplotype was the most ancestral, according to the haplotype network (Fig. 3). For the P26 gene of PYVV, 23 haplotypes (Haplotypes 1 to 23) were obtained with frequencies between 2.4% and 16.7% (Tab. 5) in which haplotypes 4, 7 and 9 were the most frequent and haplotype 4 was formed by four isolates from

TABLE 3. P10 and P26 gene reference accessions of *Potato yellow vein virus* (PYVV) and lettuce infectious yellow virus orthologues.

P26 gene Accession	P10 gene Accession	Province/country	Municipality	Host	Reference
AJ508757.2	AJ557129.1	Cajamarca/Peru	Chota	Potato Andigena	Livieratos *et al.,* 2002
KX573903.1	KX573902.1	Antioquia/Colombia	Marinilla	*S. lycopersicum*	Gutiérrez *et al.,* 2017
KR998195.1	KR998194.1	Antioquia/ Colombia	La Union	Group Phureja	Muñoz *et al.,* 2016
U15441.1	U15441.1	Maryland/USA	-	*Nicotiana clevelandii*	Klassen *et al.,*1994

TABLE 4. Haplotypes for the P10 gene of *Potato yellow vein virus* (PYVV).

P10 gene haplotypes	Quantity of sequences	Codes of P10 and P26 gene sequences	Frequency (%)
Hap 1	6	KR998194.1, AJ557129.1, CT1310, CT1810, CT4110, CT4210	13.3
Hap 2	1	KX573902.1	2.2
Hap 3	3	CT0410, NT2210, NT2410	6.6
Hap 4	28	CT1210, CT1110, CT1410, CT1510, CT1710, NT2110, NT2510, NT2610, NT2710, NT2810, ND3010, ND3110, ND3310, ND3410, ND3510, ND3610, ND3710 ND3810, CT4010, CT4310, CT4410, CT4510, CT4610, CT4710, CT5010, CT5110 CT5210, CT5310.	62.3
Hap 5	1	NT1910	6.6
Hap 6	1	NT2310	6.6
Hap 7	1	ND2910	6.6
Hap 8	1	ND3210	6.6
Hap 9	1	CT4910	6.6
Hap 10	1	BT6710	6.6
Hap 11	1	BT7010	6.6

TABLE 5. Haplotypes for the P26 gene of *Potato yellow vein virus* (PYVV).

Haplotype P26	Quantity of sequences	Sequence code	Frequency (%)
Hap 1	1	AJ508757.2	2.38
Hap 2	1	KX573903.1	2.38
Hap 3	1	KR998195.1	2.38
Hap 4	4	CT0426, NT2326, NT2426, CT4226	9.52
Hap 5	1	CT1126	2.38
Hap 6	1	CT1226	2.38
Hap 7	4	CT1326, CT1526, CT4726, CT5326.	9.52
Hap 8	3	CT1426, CT1626, CT1826.	7.14
Hap 9	4	CT1726, CT4126, CT4326, CT4426	9.52
Hap 10	2	NT1926, NT2026.	4.76
Hap 11	1	NT2126	2.38
Hap 12	1	NT2226	2.38
Hap 13	1	NT2526	2.38
Hap 14	2	NT2626 NT2826.	4.76
Hap 15	1	NT2726	2.38
Hap 16	2	ND2926; ND3026	4.76
Hap 17	2	ND3326; ND3426	4.76
Hap 18	1	ND3526	2.38
Hap 19	2	ND3626; ND3726	4.76
Hap 20	1	ND3826	2.38
Hap 21	1	CT3926	2.38
Hap 22	1	CT4026	2.38
Hap 23	1	CT4926	2.38
Hap 24	1	CT5126	2.38
Hap 25	1	BoT5226	2.38
Hap 26	1	CT6226	2.38

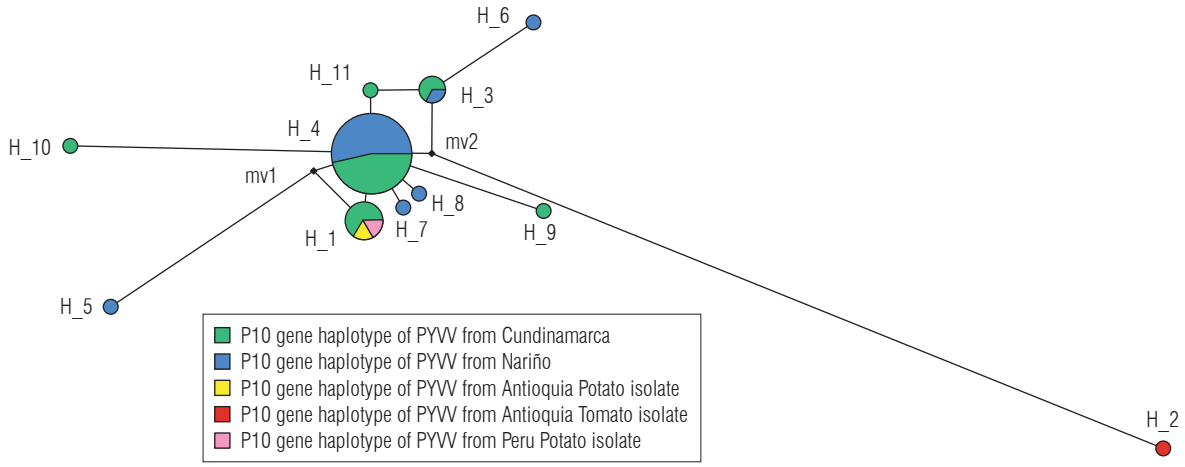

FIGURE 3. Haplotype network for the P10 gene of *Potato yellow vein virus* (PYVV).

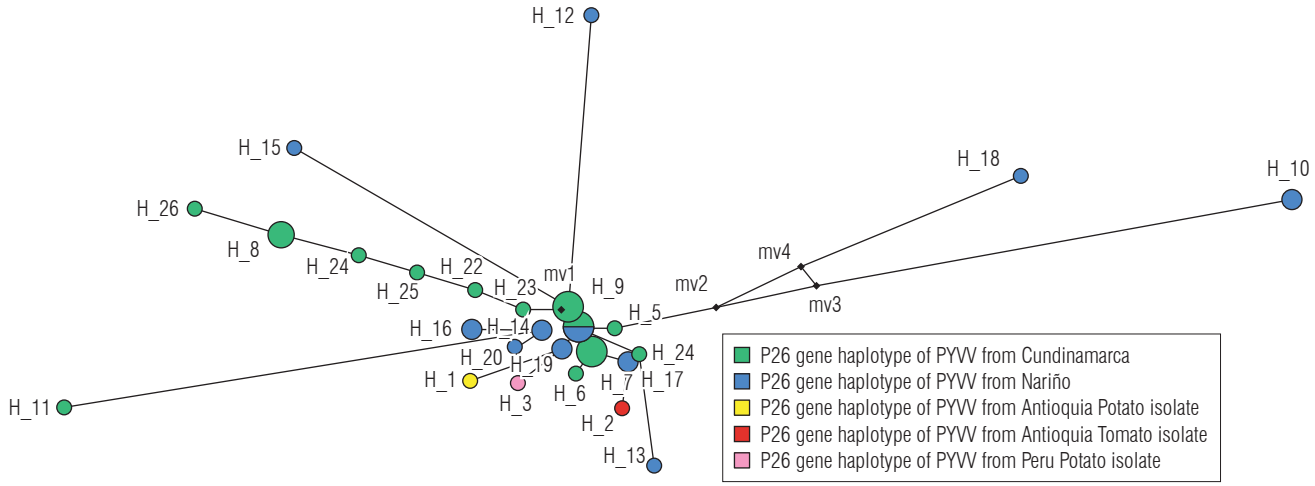

FIGURE 4. Haplotype network for the P26 gene of *Potato yellow vein virus* (PYVV).

Cundinamarca and Nariño. This represents the ancestral haplotype for the P26 gene (Fig. 4), and haplotypes 7 and 9 were formed by sequences only from Cundinamarca.

Phylogenetic relationships

The consensus maximum likelihood tree (MLT) for the P10 gene of PYVV (Fig. 5) showed three clades; GI, GII and GIII related to the host (StA or StP). The GI clade was formed by viral sequences from StP plants. The GII and GIII clades were formed by viral sequences from StA plants. The GII was formed by sequences from Cundinamarca and Peruvian and Colombian accessions, and the GIII was formed by viral sequences from Cundinamarca and Nariño and a viral isolate accession from the tomato.

The MLT for the P26 gene of PYVV (Fig. 6) showed seven clades (GI to GVII). In contrast to the P10 gene, there is no grouping by host. Additionally, there is no specific relationship by geographical origin. Nevertheless, the viral sequences from Nariño are in groups GIV, GVI, and GVII; and the Cundinamarca sequences are in groups GIII and GV. Groups GI and GII are formed by sequences from the three provinces. The three reference accessions from PYVV-P26 gene were also in this group.

Genetic distances between sequences of the P10 and P26 genes from PYVV isolates

The similarity percentage for the P10 gene was between 96 and 100% except for sequences KX573902.1 and NT1910,

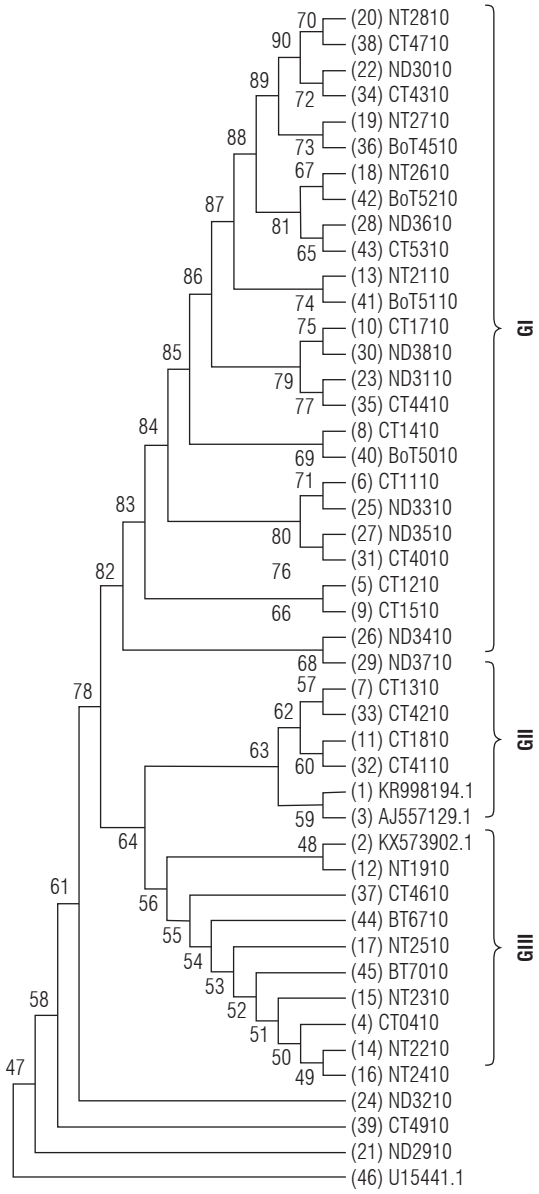

FIGURE 5. Consensus maximum likelihood tree of the P10 gene of *Potato yellow vein virus* (PYVV).

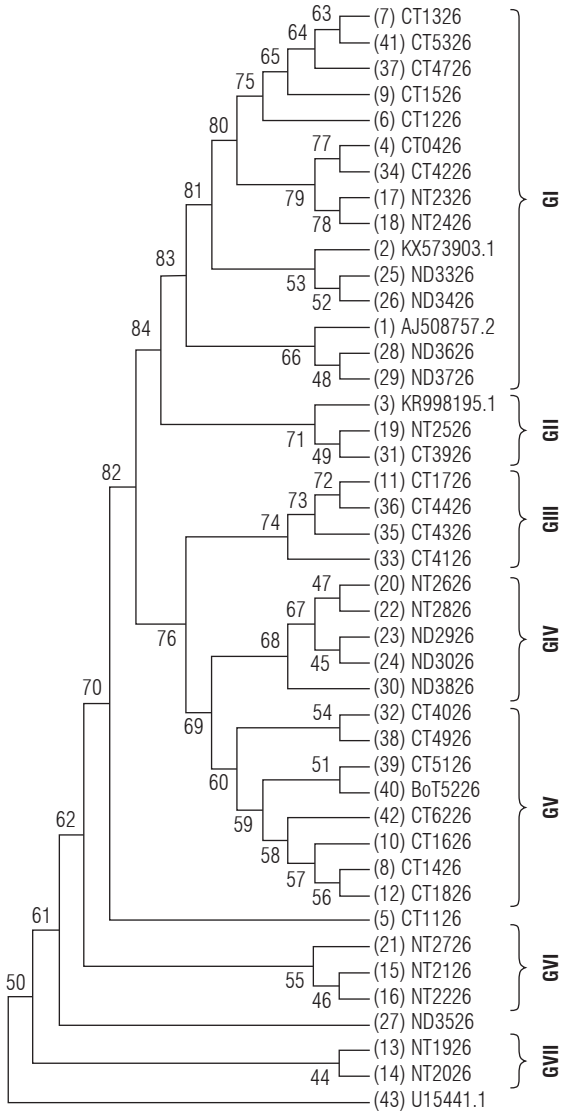

FIGURE 6. Consensus maximum likelihood tree of the P26 gene of the Potato yellow vein virus (PYVV).

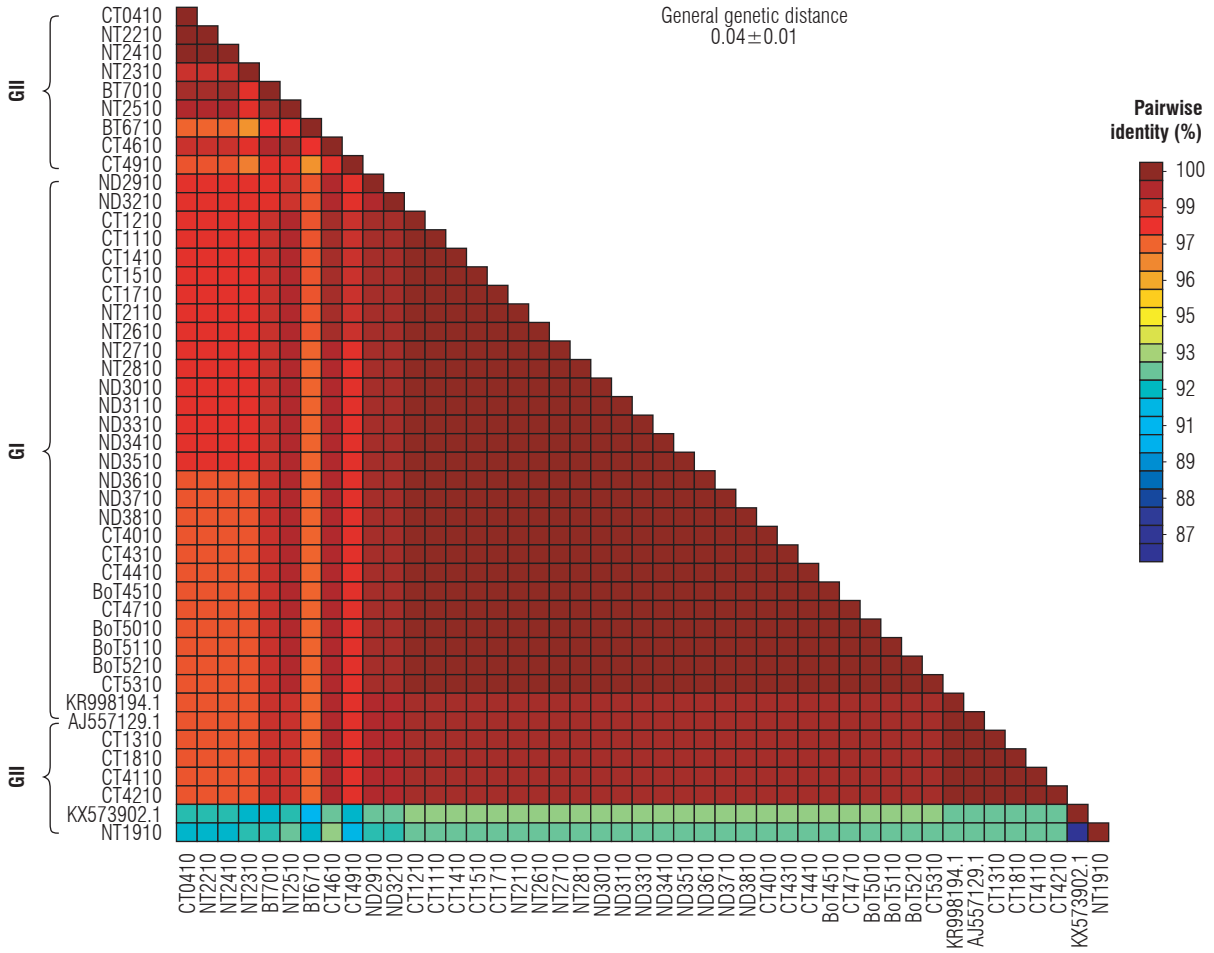

FIGURE 7. Matrix of genetic distances between sequences for the P10 gene isolates of *Potato yellow vein virus* (PYVV).

which show a similarity of between 87 and 93% respectively. In addition, the general average of genetic distances for the complete sequences was 0.04±0.01 (Fig. 7). Similarly, the matrix for the P26 gene was between 93 and 100% except for sequences NT1926, NT2026, NT2126, NT2226, ND3526, and NT2726 with percentages of similarity between 76 and 93%. The general mean of genetic distances for this gene was 0.09 ± 0.01 (Fig. 8).

Variability, diversity and neutrality tests of the P10 and P26 genes of PYVV

In general, the P26 gene of PYVV showed 252 variable sites (S) from 693 total sites (36.4% variable sites in the gene), 333 total mutations (η), a nucleotide and haplotype diversity of 0.0603 and 0.97, and a rate between non-synonymous and synonymous mutations (ω(dN/dS)) of 0.18. In the Tajima's D and Fu and Li tests significant negative values of -2.27 (<0.01) and -1.07 were found (Tab. 6, grey highlight).

The P10 gene of PYVV had 30 S of 255 total sites (11.8% variable sites in the gene), 31 η, nucleotide and haplotype diversity of 0.028 and 0.60, and a rate between non-synonymous and synonymous mutations (ω(dN/dS)) of 0.35. In the Tajima's D and Fu and Li tests, significant negative values of -2.45 (<0.001) and -4.70 were found (Tab. 6).

Internally in the groups generated for the P10 and P26 genes, greater variability was observed in isolates from tetraploid (StA) plants than in diploid (StP).

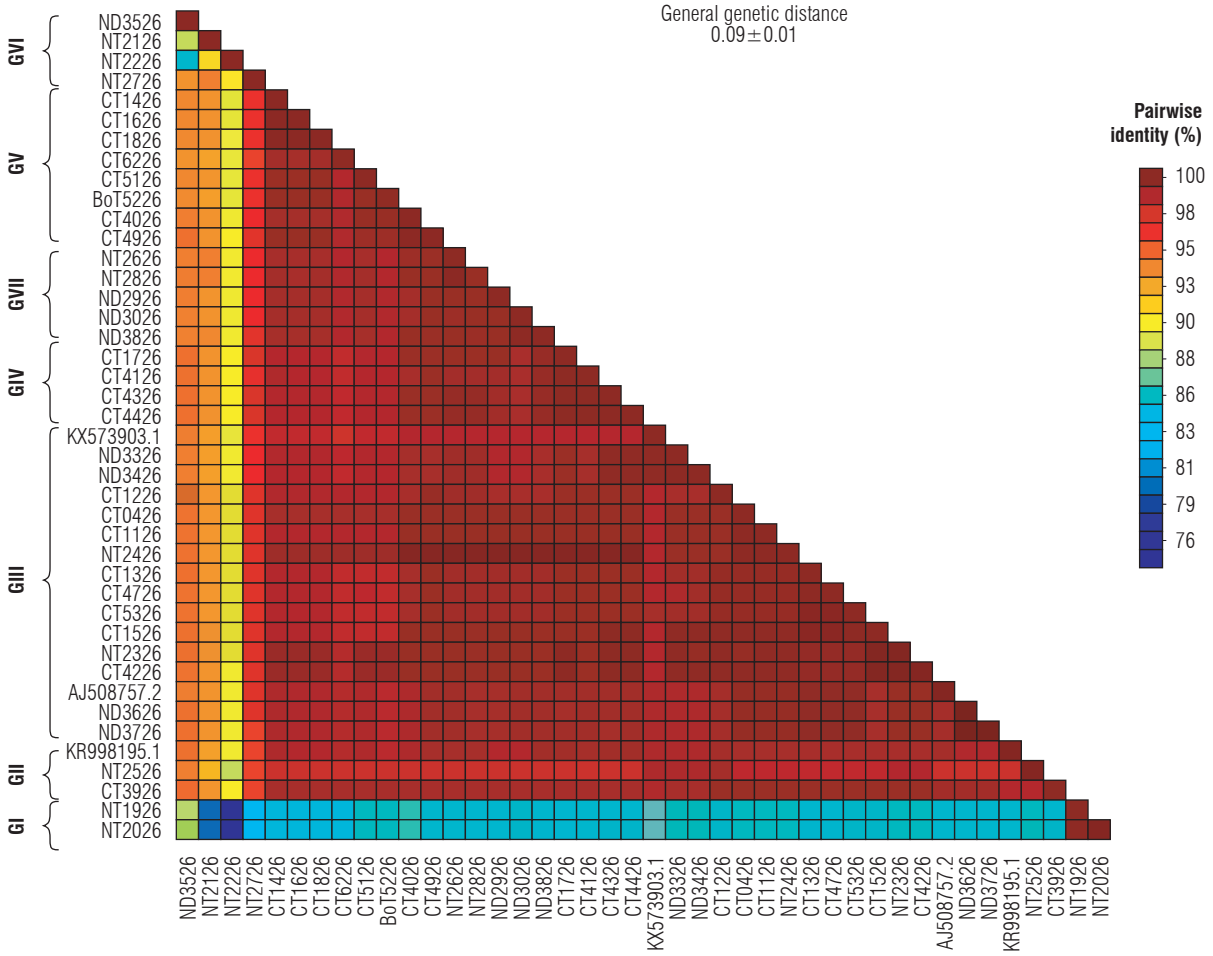

FIGURE 8. Matrix of genetic distances between sequences for the P26 gene isolates of *Potato yellow vein virus* (PYVV).

TABLE 6. Estimated parameters of genetic variability for the P26 and P10 genes of *Potato yellow vein virus* (PYVV).

Gene	Genotype	P	S	η	π	h	Hd	dN	dS	ω(dN/dS)	TD	FD
	StA	C+N	252	333	0.0926	16	0.95	0.025	0.0894	0.28	-2.30 *	-2.02
	StP	C+N	48	50	0.0199	6	0.92	0.017	0.0428	0.40	-1.53	-1.52
P26	StA+StP	C	236	239	0.0440	11	0.95	0.004	0.0253	0.17	-2.61***	-4.14
	StA+StP	N	253	290	0.124	12	0.96	0.054	0.1824	0.29	-1.50	-0.11
	StA+StP	C+N	132	138	0.0603	26	0.97	0.064	0.3501	0.18	-2.27**	-4.63
	StA	C+N	93	107	0.0437	9	0.56	0.154	0.3845	0.40	-2.55 ***	-4.15
	StP	C+N	3	3	0.0021	4	0.49	0.005	0.0197	0.25	-1.600	-1.87
P10	StA+StP	C	89	95	0.053	7	0.45	0.243	0.4571	0.53	-2.35**	-2.93
	StA+StP	N	19	20	0.012	7	0.61	0.026	0.0783	0.33	-1.86*	-2.34
	StA+StP	C+N	30	31	0.028	11	0.60	0.143	0.358	0.35	-2.45***	-4.70

Genotype: StA: *S. tuberosum* Andigena group; StP: *S. tuberosum* Phureja group. C: Cundinamarca; N: Nariño; P: Geographical origin; S: total variable sites; η: number of total sites; π: nucleotide diversity, average of nucleotide differences per site; h: total haplotype number; Hd: haplotype diversity; dN: average number of even differences by synonymous; dS: average number of even differences by nonsynonymous sites; ω(dN/dS): dN/dS ratio; TD: Tajima's D test; FD: Fu and Li D test. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Discussion

RNA viruses can accumulate mutations in their genomes because their RNA-dependent RNA polymerases have weak corrective activity, unlike DNA polymerases. Consequently, some viral species can have greater genetic variability (Domingo and Holland 1997; Sanjuán *et al*., 2010; Sanjuán and Domingo, 2016). This makes viruses with RNA genomes more variable than those with a DNA genome. Therefore, populations of RNA viruses tend to evolve rapidly and adapt to the environmental conditions derived from the interaction with their vectors (García-Arenal and Fraile, 2001; García-Arenal and Fraile, 2011). Mutation and recombination are processes that influence genetic diversity since the first one introduces specific changes in the sequences generating new variants, and the second one is a process by which segments of different genetic variants are crossed during the replication process that allows the movement of variants to produce new haplotypes. In this sense, recombination does not create mutations but creates new combinations between pre-existing mutations (Pérez-Losada *et al*., 2015). The characterization of the genetic variability of viral populations supplies important information about the processes involved in the evolution of the virus and its dispersion, where epidemiology is crucial for the design of reliable diagnostic tools that allow selecting those genes that are more conserved in time and space, as well as for the development of efficient and long-lasting disease control strategies.

According to Rubio *et al*. (2013), the *Closteroviridae* family is not very variable because it is subject to negative selection, long-distance migrations, recombination processes, interaction between viral strains, and host-virus and virus-vector interaction. These are the main forces that exert pressure in the *Closteroviridae,* according to analyses carried out on structural genes such as CP. Moncef (2010) performed an analysis of the complete genomes for some species of genera in the *Closteroviridae* and reported that these viruses are subject to positive selection processes with dN/dS values ranging from 1.508 to 2.599. In addition, that author observed that only the *Closterovirus* (Citrus Tristeza Virus or CTV) and the *Ampelovirus* (Grapevine Leafroll-associated Virus 3 or GLRaV-3) are the only ones that have recombination processes; but this author did not detect any recombination processes for criniviruses.

In this study, we reported for the first time the amplification by RT-PCR and the acquirement of nucleotide sequences of the P26 and P10 genes of PYVV, from symptomatic and non-symptomatic samples of different potato varieties from Nariño, Cundinamarca, and Boyacá in Colombia for the variability study.

The haplotype analysis showed that both the P26 and P10 genes have high haplotype numbers with high diversity and low frequency (haplotype 4 with a frequency of 62.3% for the P10 gene and haplotypes 4, 7, and 9 with a frequency of 9.5% for P26 gene). Haplotype 4 for both genes corresponds to the most ancestral gene, and diversification has been generated from it in the different provinces evaluated in this study. In a study conducted by Chávez *et al.* (2013), 33 different haplotypes for the CP gene of PYVV were found, in which haplotype 28 was the most frequent with a value of 16.7. The most frequent haplotype groups for P10 and P26 genes of PYVV do not group sequences by host genotype or by geographic origin for the P10 gene, except for haplotypes with a single sequence. However, the P26 gene haplotypes do discriminate both by origin and genotype (except for the ancestral haplotype). This is supported by the maximum likelihood tree that shows the clades to be associated with the sequences of the specific haplotypes. This situation is not seen in the P10 gene tree, since no clear differentiation between the groups was observed by genotypes or geographical origin. This could be due to the increase in the use of vegetative seeds infected by the PYVV virus of different geographical origins, which allowed the spread of the virus in the potato-producing regions and, therefore, the distribution of variants in the geographical areas analyzed in this study. This, in turn, favors the development of new variants and recombinants when subjected to pressure in different environments. It is worth mentioning that the sequences NT1910 of the P10 gene and NT1926, NT2026, NT2126, NT2226, ND3526, NT2726 of the P26 gene showed differences between 87% and 93% for the P10 gene and 76% and 93% for P26 gene. This indicates that speciation processes are being developed in PYVV both in diploid and tetraploid plants from Nariño (Townsend, 2014; Koloniuk *et al*., 2018).

The Tajima's D and Fu and Li global tests showed a significant negative value for both genes, indicating that in the PYVV populations for these two genes, there was an excess of low-frequency haplotypes (rare alleles in high frequency). This low frequency could indicate that it is a population that is diversifying after a bottleneck or that there was a selection process that decreased the frequency of abundant haplotypes, increasing those that were rare at the time and that could be explained again by the flow of infected material between geographic regions. How-

ever, when analyzing the isolates of the StP host, negative values (but not significant) were observed for the Tajima test, indicating that this subpopulation of the PYVV was undergoing a neutral process or contraction period, or that the number of sequences obtained from StP was limited to offer enough statistical robustness.

When observing the estimated parameters of genetic variability and diversity, we can affirm that the P26 gene has a global nucleotide diversity of 0.0603, but when the diversity of the hosts is observed, it can be seen that in plants of the species StA is much higher than 0.0926 compared to the global and plants of the StP species. This indicates that in this study the sequences obtained from the StA potato plants of the P26 gene provide most of the diversity. This result is repeated with the P10 gene, in which the global diversity is 0.028 and that provided by the isolates from the StA and the StP is 0.0437 and 0.0021, respectively. Similar results were obtained by Chávez-Bedoya *et al.* (2014), who reported the diversity of the CP, Hsp70 and CPm genes of the PYVV, which are higher for StA hosts in all cases (0.010, 0.016 and 0.046, respectively) and lower in the StP (0.008, 0.005 and 0.084, respectively) except for the CPm gene. This may occur since the StP group is diploid and the StA group is tetraploid, which implies that the virus could be subjected to higher selection pressure by having to evade the silencing machinery in a larger genome. On the other hand, it is possible to state that the nucleotide diversity is greater in the P26 gene than in the P10 gene. However, that diversity is generated by a larger number of synonymous mutations (dS) that neither affects the composition of amino acids nor the three-dimensional structure of the protein. This maintains protein function and consequently takes the populations of the PYVV to a process of negative selection. The fact that the P26 and P10 genes are more diverse than the CP, CPm, and HSP70 of the PYVV defines the functions that each one offers to the virus, since the P26 and P10 are associated with pathogenicity in *Crinivirus*, while those studied by Chávez-Bedoya *et al.* (2014) are structural genes that must be highly conserved to guarantee the correct assembly of the virion and the transmission through the vector.

This research allows the determination of the variability of the P10 and P26 genes, which is higher in isolates from the *S. tuberosum* Andigena group than in the Phureja group infected with PYVV. In addition, the influence of humans as a vector for the dispersion of the virus among potato producing regions in Colombia was established. This research also allows the planning of new studies aimed at evaluating the functionality of PYVV proteins P10 and P26 and the relationship of the variants to the presence or absence of symptoms in the field, the degree of severity, and the impact on the production and colonization of new hosts.

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Physiological performance of quinoa (*Chenopodium quinoa* Willd.) under agricultural climatic conditions in Boyaca, Colombia

Comportamiento fisiológico de la quinua (*Chenopodium quinoa* Willd.) bajo condiciones agroclimáticas de Boyacá, Colombia

Miguel García-Parra[1, 3]*, José García-Molano[2, 3], and Yuli Deaquiz-Oyola[2, 3]

ABSTRACT

Quinoa (*Chenopodium quinoa* Willd.) is native to South America; it is characterized by its high nutrient contents and high adaptation capacity to diverse edapho-climatic conditions, which highlights it´s genetic variability expressed as multiple physiological and phenological responses. The objective of this research was to evaluate the physiological response and proximal composition of the grain to three types of fertilization under the environmental conditions of the municipality of Oicata (Boyaca, Colombia) located at 2,875 m a.s.l. The white Soracá variety was planted using a completely randomized design with four treatments and four replicates. It was observed that the fertilization sources have an effect on the physiological and phenological behavior, mainly on the number of leaves, length of stem and chlorophyll content. The reproductive stage and the proximal composition of seeds changed, which is attributed to the application of mineral organic fertilizer that improves the production of quinoa grains, while N-P-K contribution shows greater growth and vegetable development, but less yield.

Key words: chlorophyll, fertilizer, phenology, protein content.

RESUMEN

La quinua (*Chenopodium quinoa* Willd.) es originaria de América del Sur, y está caracterizada por la alta composición nutricional y su fácil adaptabilidad a condiciones edafoclimáticas, lo que resalta su amplia variabilidad genética que se expresa en múltiples respuestas fisiologías y fenológicas. El objetivo de este estudio fue evaluar la respuesta fisiológica y la composición proximal del grano a tres tipos de fertilización bajo condiciones de clima y suelo propias del municipio de Oicatá (Boyacá, Colombia), localizado a 2.875 msnm. Se sembró quinua variedad blanca de Soracá, utilizando un diseño completamente al azar con cuatro tratamientos y cuatro repeticiones. Se observó que las fuentes de fertilización tienen efecto sobre el comportamiento fisiológico y fenológico, principalmente en el número de hojas, longitud de tallo y contenido de clorofila. Las etapas reproductivas y la composición proximal de las semillas también mostraron cambios, lo que se atribuye al aporte de abono orgánico-mineral que mejora la producción de granos de quinua, mientras que el aporte de N-P-K muestra mayor crecimiento y desarrollo vegetativo, pero menor rendimiento.

Palabras clave: clorofila, abono, fenología, contenido de proteína.

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is considered a crop with great potential because of its high agronomic characteristics and nutritional value, and especially for its inclusion in children and elderly people's diets (Valcárcel-Yamany and Silva, 2012). According to Escuredo *et al.* (2014), this plant has the capacity to produce grains of high quality and protein content. Additionally, it contains amino acids such as lysine, threonine and methionine, which are considered as essential.

These nutritional characteristics are the result of environmental conditions, such as temperature, light intensity, relative humidity and precipitation. These conditions are key factors in the quality and number of grains per panicle (Morales *et al.,* 2017), as well as in the phenological and physiological performance of the plants related to the adaptive capacity to diverse environmental conditions (Winkel *et al.,* 2016). The plant has adaptive advantages that allow it to express a great productive potential. Quinoa (*Chenopodium quinoa*) has undergone diverse evolutionary

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processes, but it has been bred from crosses with *Chenopodium carnosulum* to acquire resistance to salinity problems, *Chenopodium petiolare* to obtain adaptability to droughts, and *Chenopodium pallidicaule* to receive tolerance to frosts (Jarvis *et al.,* 2017).

The quinoa plant has the capacity to develop alternative metabolic plasticity (Bazile *et al.,* 2014). This change is induced by conditions such as temperature, light intensity, nutritional status, relative humidity, and water availability (Morales *et al.,* 2017). In addition, it is able to undergo phenological, morphological, and physiological changes known as phenotypic plasticity.

On the one hand, the soil conditions, weather, and nutrient availability are important factors in the morpho-agronomic performance of the crop. However, the physical, chemical, and microbiological characteristics are specific to each place and mark plant development and the composition, quality and quantity of the quinoa grain (Veloza *et al.,* 2016).

Moreover, in Colombia, the most cultivated varieties of quinoa are Piartal and Tunkahuan which come from Ecuador, SL47 from Nariño, White from Jerico and White from Soraca and Boyaca (Ardila *et al.*, 2006). These quinoa genotypes have an average productivity of 1.5 and 2.6 t ha^{-1} depending on the variety the fertilization plan (Delgado *et al.,* 2009) and the environmental conditions (García-Parra *et al.,* 2017).

Furthermore, the quinoa is planted at a small scale in the provinces of Nariño, Cundinamarca, Cauca and Boyaca. In Boyaca, there are reports of crops in the central zone (2,538-3,031 m a.s.l.) grown from a mix of seeds that affects crop productivity and grain quality (Veloza *et al.*, 2016).

In that order, it is necessary to evaluate the physiological performance and the composition of the quinoa grain in three types of fertilization and under the environmental conditions of the municipality of Oicata.

Materials and methods

The research was carried out from June 2016 to May 2017 in the municipality of Oicata (Boyaca, Colombia) with coordinates 5°22'48" N and 73°30'09" W and an elevation of 2,875 m a.s.l. The average temperature was 12°C, with 74.1% relative humidity and an average precipitation of 1018.9 mm per year (Tab. 1). The soil in which the experiment was established had Andic Dystrustepts and Vertic Haplustalf association (IGAC and UPTC, 2005).

White quinoa seeds from Soraca were used as plant material, which were stored by the Research Group Agricultura, Organizaciones y Frutos (AOF) for six months. Some of the fertilizers used were: Paz del Rio Fertilizer (PRF, Escoria Thomas, Colombia), Urea (U) and an organic fertilizer from The Agro-Ecological Farm Victoria (AEFV, Colombia).

The experiment area for the research was of 460 m^2, where four fertilization treatments with four replicates were established. These trials were performed based on the result of the soil analysis carried out by the Chemistry Laboratory of Soil, Water and Plants from Agrosavia (Tab. 2). The treatment T0 was the control, T1 was for the application of 6 kg of AEFV fertilizer, T2 was for the application of 3 kg of AEFV fertilizer plus 100 g of U and 50 g of PRF, and T3 was for the application of 200 g of U and 100 g of PRF (Tab. 3).

The response variables were: number of leaves, height of plants (rigid flex meter), days to reach six true leaves, and days to 50% flowering. In addition, days to milky grain and days to pasty grain state, chlorophyll (SPAD 502 plus, Konica-Minolta, Japan), dry and fresh weights of the plant (Drying oven HSY-75, 24 h at 104°C), grain productivity, protein amount (Kjeldahl Technique, NTC370), neutral detergent fiber in grain (Gravimetric determination, Van Soest AOAC 2002.4) and acid detergent fiber in grain (Gravimetric determination, Van Soest H_2SO_4) were measured every 15 d.

TABLE 1. Climatic conditions at the experimental plot.

Climate variation	June	July	August	September	October	November	December
Precipitation (mm)*	22.9	60.7	32	60.3	99.6	42.1	38.6
Solar brightness (h)**	119.7	108.8	161.7	151.8	163.7	151.8	162.3
Relative humidity (%)	77.1	78.6	75.1	74.7	74.2	74.7	72.3

*Pluviometer data of Oicata at an altitude of 2,645 m a.s.l. Code 24030450 IDEAM.

**Data supplied by the weather station from IDEAM (Universidad Pedagogica y Tecnologica de Colombia).

TABLE 2. Soil characteristics at the study site.

pH		Organic matter (%)		CECe (cmol/kg)		
5.52		3.85		4.83		
Interchangeable bases (cmol/kg)						
Ca		Mg		K	Na	
2.5		1.12		1.07	0.14	
Microelements (mg/kg)						
P	Fe	Mn	Zn	Cu	B	S
10.06	282.4	9.86	3.1	3.66	0.13	8.28

TABLE 3. Chemical characteristics of fertilizers.

	PRF (%)	U (%)	AEFV (%)
Total Nitrogen	-	46	1.86
Total phosphorus (P_2O_5)	11	-	2.44
Potassium (K)	-	-	2.45
Sodium (Na)	-	-	0.27
Calcium (CaO)	40	-	1.23
Magnesium (MgO)	1.5	-	0.86
Silicon (SiO_2)	6	-	-
Zinc (Zn)	0.001	-	0.01
Copper (Cu)	0.001	-	-
Cobalt (Co)	0.0002	-	-
Boron (B)	0.0002	-	-
Molybdenum (Mo)	0.0013	-	-
Sulfur (S)	-	-	0.36
pH	12	7.2	7.8
Humidity (%)	0.3	1	17.9

PRF: Paz del Rio fertilizer; U: Urea; AEFV: Agro-Ecological Farm Victoria fertilizer.

Statistical design

A completely randomized design with four replicates and 16 separate experimental units was established. The data obtained in the experiment were tabulated using Excel® program; a test of homogeneity of variety with the Bartlett method and a normality test with the Shapiro-Wilk method were performed. An analysis of variance (ANOVA) and a Tukey test of comparison of means with a 0.05 significance level were performed using the program R version 3.3.0.

Results and discussion

Number of leaves

As shown in Figure 1, significant changes, such as an increase in the number of leaves, were observed after day 75. However, after the start of the flowering stage (105 d), the plant showed a loss of leaves to its senescence. The highest average was found in the T3 treatment (90.75), which presented significant differences compared to the others, whereas T0 had the lowest average (32.75). All the treatments displayed a sigmoid performance. This performance was expressed in the exponentially development of the plant regarding the time (Taiz and Zeiger, 2007) taking into account that the plant induces, in the vegetative stages, the greatest number of foliar buds capable of capturing and housing the assimilates that will influence the reproductive stages and the formation of grains (Atencio *et al*., 2014).

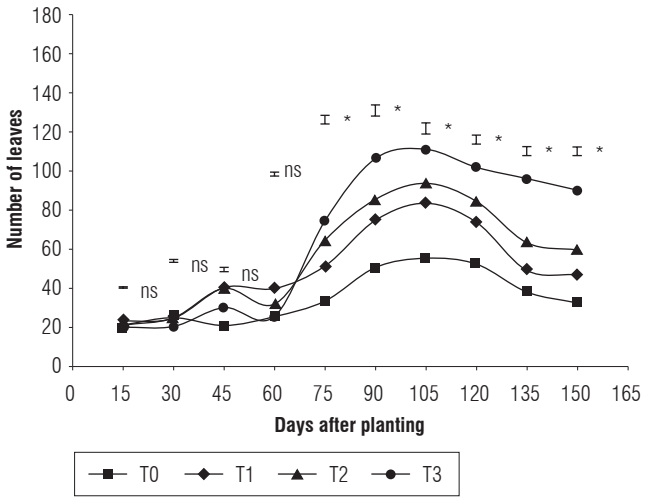

FIGURE 1. Number of quinoa leaves under different fertilization protocols in Oicata - Boyaca. T0: absolute control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer according to the Tukey test ($P \leq 0.05$). ns: not significant, *: significant for the day of sampling.

Although the number of leaves is a determining variable in the production of quinoa, it is not a key factor to crop yield. Similarly, the application of elements such as nitrogen stimulated the excessive development of fodder, which could affect the grain productivity (Kakabouki *et al*., 2018). Correspondingly, García *et al.* (2017) stated that the application of an increasing dose of N-P-K in quinoa enhanced leaf area but not productivity. Nevertheless, the opposite occurs when organic-mineral fertilizer is used.

This kind of amendment facilitates the absorption of important elements for energetic, metabolic and enzymatic activities of the plant due to the microorganism content (Ramzani *et al.,* 2017).

Plant height

Statistical differences (P<0.05) in height were recorded from day 75 for the T3 treatment. This contrast resulted in the highest average during the days 75, 90, 105, 135 and 150. The final average was 172.25 cm, compared to the absolute control treatment T0, which resulted in 93.25 cm (Fig. 2). Following day 120, the T2 treatment increased its growth due to the fertilization plan that was provided to each treatment. This increase could be due to the fact that nitrogen can stimulate plant elongation and cellular division, which allows the synthesis of auxin and cytokinins that have an effect on stem growth (Basra *et al.,* 2014).

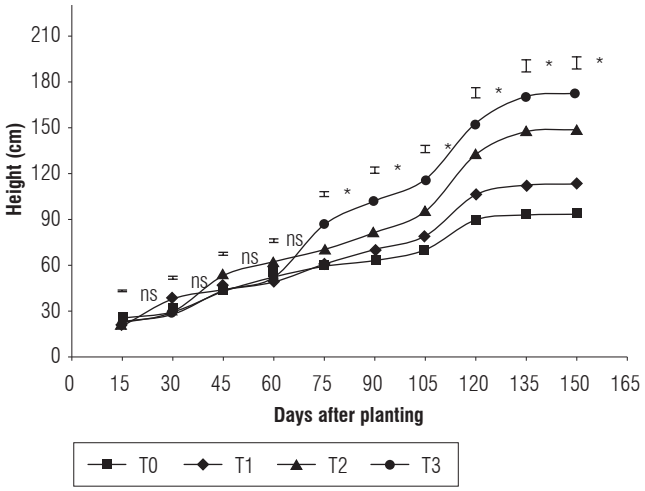

FIGURE 2. Height of quinoa plants under different fertilization protocols in Oicata - Boyaca. T0: control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer according to the Tukey test ($P \leq 0.05$). ns: not significant, *: significant for the day of sampling.

As a result, when more nitrogen is applied and there is more precipitation, an increase of the stem elongation is evident. According to Fghire *et al.* (2015), the continuous cell division and elongation increase the permeability of the root meristems and facilitates the absorption of structural minerals such as calcium, considered as fundamental in tissue rigidness and an active agent in the manifestation of plant hormones that stimulate the elongation and cellular division.

Chlorophyll content

This variable presented important statistical differences among days 90, 105, 120 and 130. T3 showed the highest average (66.4 SPAD units) in the closest phase to the flowering, at the moment in which the plant displayed the highest number of leaves. However, at 150 d, the treatments did not show significant differences (Fig. 3). This could be due to the fact that nitrogen application and chlorophyll content are directly proportional to each other (Liu *et al.,* 2015), along with the application urea in treatments T2 and T3.

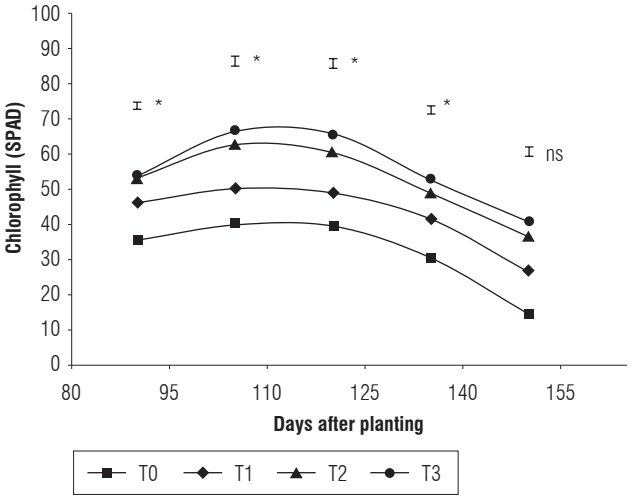

FIGURE 3. Chlorophyll content of quinoa under different fertilization protocols in Oicata - Boyaca. T0: Absolute control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer according to the Tukey test ($P \leq 0.05$). ns: not significant, *: significant for the day of sampling.

In addition, nitrogen determines the content of assimilates existing in the leaves, and consequently, the color of the leaf through which the chlorophyll meters act on the wave capture. Then, the high nitrogen applications result in high chlorophyll contents (Fghire *et al.,* 2015), as observed in the experiment. For this reason, it is important to measure the chlorophyll content to evaluate the nutritional status of plants at foliar level (Rincón and Ligarreto, 2010).

Therefore, it is evident that the highest chlorophyll content is equivalent to vegetative phases and it is reduced in the reproductive phases (Fig. 3). According to García-Parra *et al.* (2018), the plant captures the highest amount of mineral elements in these phases, while in the filling and maturation phases, the nutrients are finally accumulated in the seeds.

Fresh weight

Each one of the evaluated organs presented significant statistical differences (P<0.05), which evidences that the T2 treatment had greater fresh weight in roots and leaves. In contrast, T3 displayed the highest average in the weight of

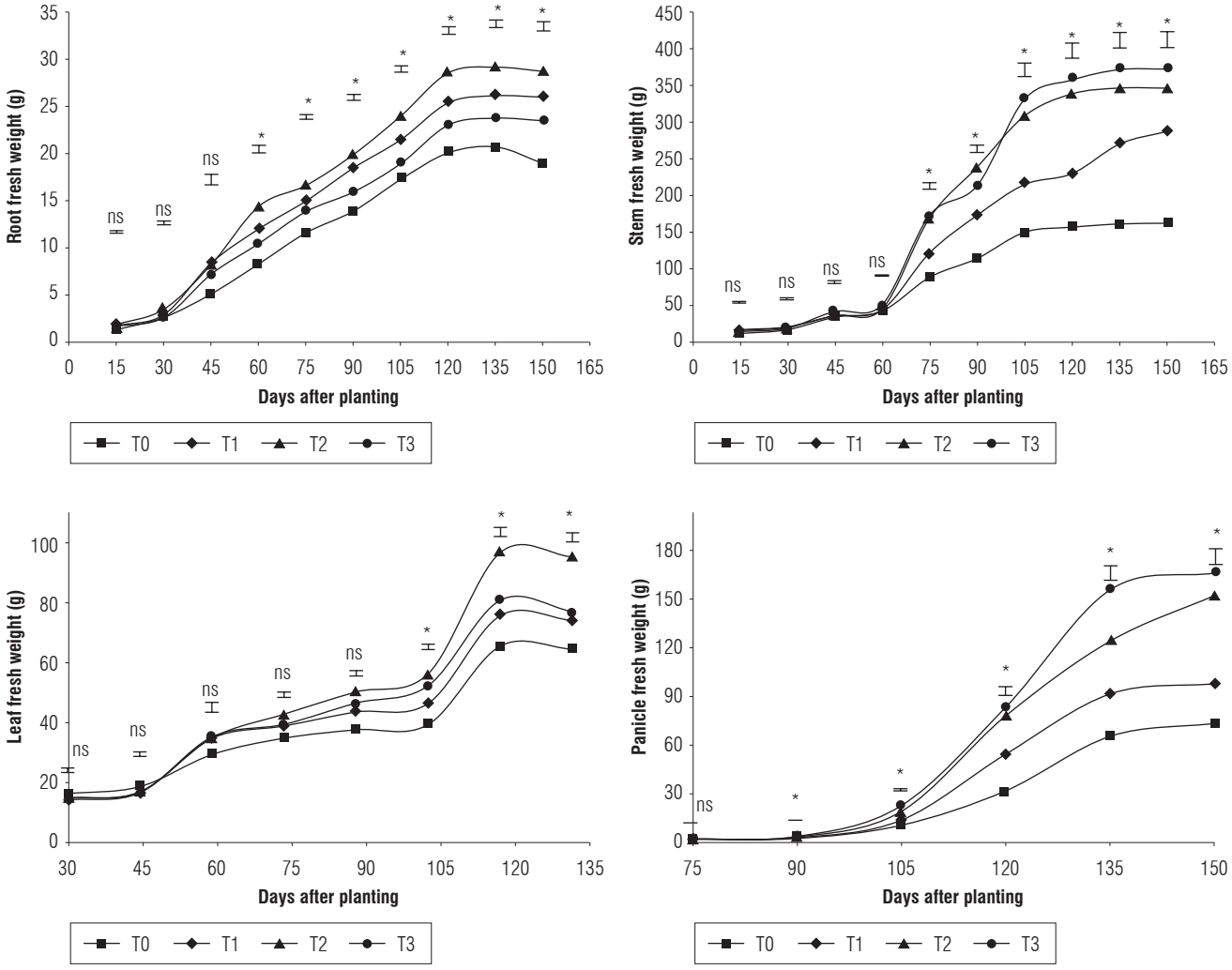

FIGURE 4. Fresh weight of stems, roots, leaves and panicles of quinoa under different fertilization protocols in Oicata - Boyaca. T0: control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer according to the Tukey test ($P \leq 0.05$). ns: not significant, *: significant for the day of sampling.

the stem and panicle (Fig. 4). These results were obtained as a response to the implementation of mineral organic fertilizers (Garcia-Parra *et al.,* 2017). Moreover, the high precipitations during the final phase of the vegetative stages and at the beginning of the reproductive stages generated the dilution of the phosphate fertilizer, which is indispensable for the development of diverse energetic activities. Similarly, the contribution of calcium is fundamental in the expression of plant hormones, which stimulate the development of leaves, stems and roots.

The fresh weight of plants is an indicator of efficiency in the uptake of nutrients and water (Torres *et al.,* 2000), which can be influenced by environmental conditions. Thus, the biggest part of fresh biomass is found in the panicles followed by the stems, because the panicles support the leaves, glomerulus, and seeds, and are the duct for important substances in the nutrition of quinoa (Al-Naggar *et al.,* 2017).

Dry weights

The plants treated with different fertilization protocols under the edaphoclimatic conditions of the municipality of Oicata presented significant statistical differences ($P<0.05$) regarding the dry weight of the roots, stems, leaves, and panicles. These results showed that the treatments T2 and T3 had the highest average during the entire test (Fig. 5) because of the implementation of the organic material that acts as a retainer of moisture (García, 2006). This facilitates the dilution of urea, Paz del Rio fertilizer, and minerals that are present in the AEF fertilizer, all expressed as assimilates in dry matter. In the case of T3, the increment occurred because of the application of soluble fertilizer and the increase of precipitation during the experiment.

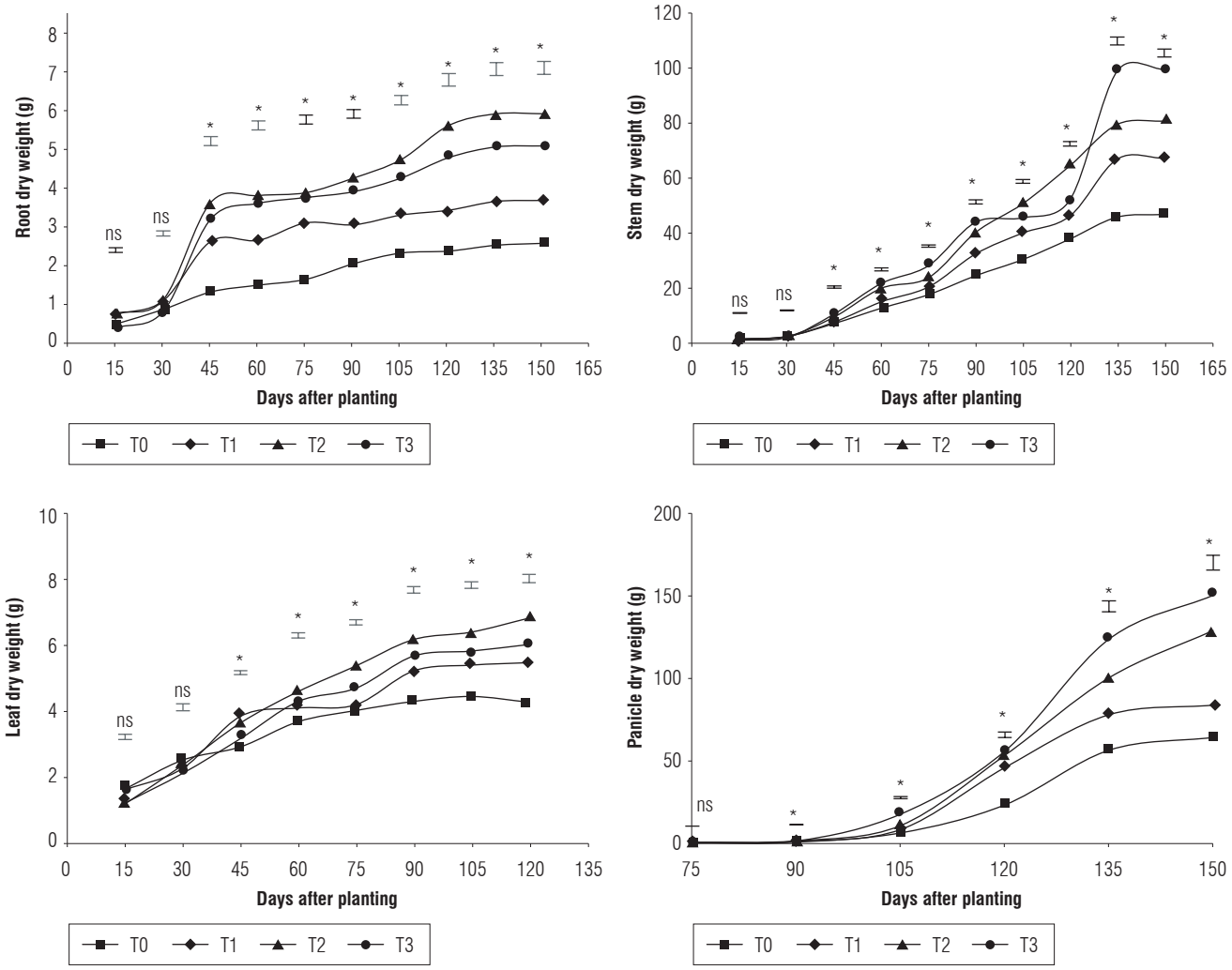

FIGURE 5. Dry weights of quinoa stems, roots, leaves and panicles under different fertilization protocols in Oicata - Boyaca. T0: Absolute control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer according to the Tukey test ($P \leq 0.05$). ns: not significant, *: significant for the day of sampling.

Furthermore, the dry matter is an indicator of the capacity that plants have to absorb nutrients (Magolbo *et al.,* 2015). Aliro *et al.* (2011) stated that in the vegetative stages the highest dry matter accumulation occurs in leaves, while in grain formation the elements, minerals, and photo-assimilated compounds are transported from the source to the sink organs.

Nevertheless, the high indexes of dry weight do not certainly indicate either the productivity of the plant or the quality of its composition. It is due to the fact that quinoa expresses diverse potentials according to its variety and origin; the nutrients absorbed not only reach sink organs like seeds, but they are also stored in source organs as reserve, which are determining in the dry weight of leaves, roots and stems (Jayme-Oliveira *et al.,* 2017).

Phenological performance

According to Table 4, the differentiation of the phenological performance started at flowering stage when treatments T1 and T2 took less time to get into their reproductive stages. However, after reaching the stages of milky and pasty grain, T0 presented a shorter productive cycle compared to T1, T2 and T3. This effect was due to the availability of nitrogen, which stimulated the longevity of the plant tissue, generating a constant production of new cells that are expressed in longer productive cycles.

The phenological response by the crop occurs through determining factors such as the soil and weather (Vargas *et al.,* 2015). For this reason, quinoa plants allow to display longer productive cycles when fertilization plans are carried out with N-P-K in excess (Gómez and Aguilar, 2016).

TABLE 4. Phenological performance of quinoa according to the treatments.

Treatment	Days to six true leaves	Days to 50% flowering	Days to milky grain	Days to pasty grain
T0	27±0.0 a	119.5±3.3 a	142.2±1.5 c	171.5±1.0 a
T1	25.7±0.9 a	118.5±1.7 ab	143±0.0 c	173±0.8 b
T2	26.5±0.5 a	118.7±3.5 ab	150±0.0 b	188±1.6 c
T3	26.7±0.5 a	124±0.0 b	154.2±3.4 a	202±2.1 d

Different letters in the same column indicate significant differences according to the Tukey test ($P \leq 0.05$). T0: absolute control; T1: organic fertilizer; T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: urea + Paz del Rio fertilizer.

TABLE 5. Production and composition of the quinoa grain.

Treatment	Grain productivity (g/m^2)	Protein (%)	NDF (%)	ADF (%)
T0	243±1.6 a	13.9±1.6 a	15± 1.1 a	10.79±0.9 a
T1	389±3.9 a	14.7±0.5 a	16.11± 0.2 a	10.24±0.3 a
T2	298.2±1.7 a	14.9±2.8 a	14.08±0.8 a	9.72±0.2 b
T3	374.2±1.7 a	13.6±0.5 a	15.69±0.3 a	11.64±1.2 c

NDF: Neutral Detergent Fiber. ADF: Acid Detergent Fiber. Different letters in the same column indicate significant differences according to the Tukey test ($P \leq 0.05$). T0: absolute control, T1: organic fertilizer, T2: organic fertilizer + urea + Paz del Rio fertilizer, and T3: Urea + Paz del Rio Fertilizer.

Moreover, and regarding the climatic factors, the activity of the Rubisco activase (enzyme) declines as temperatures increase above the thermal optimum of photosynthesis. This loss of activity causes Rubisco deactivation, which in turn is proposed to reduce photosynthetic capacity at elevated temperatures (Raines 2011).

In addition, elements such as nitrogen determine how long the quinoa plant can be harvested (Geren, 2015). This growth effect is triggered when this element stimulates plant hormones that generate the production of foliar buds, which are indispensable in the photosynthesis process. It also composes the chlorophyll molecule that is found between 40-52 SPAD units in the vegetative phases (García-Parra *et al.,* 2017).

Another determining factor is the edapho-climatic conditions during the establishment and development of the crop. The metabolic and phenotypical plasticity processes adapt to the water levels, sun radiation and relative humidity, which are all variables that intervene in the metabolic activities of the plant. These variables have allowed the plant to adapt to diverse regions of the world (Tabaglio *et al*., 2015).

Production and composition of the grain

Grain production did not show significant statistical differences ($P<0.05$). However, T1 showed a greater productivity per square meter, while protein, neutral detergent fiber (NDF) and acidic detergent fiber (ADF) had the best results in T2, T1 and T3, respectively (Tab. 5). The protein of T1 could be higher because it does not contribute to the available nitrogen despite of the organic fertilizer supply; the population of diazotrophic bacterium existing in this kind of fertilizer influences its availability in the plant (Parra-Cota *et al.,* 2014). Additionally, a balance in elements like phosphorus, magnesium and other microelements contribute to the synthesis of protein (Marschner, 2012).

On the one hand, quinoa production is determined by factors such as the availability of elements in the soil. For this reason, even though no significant differences were observed in the grain productivity, treatments T3 and T2 outweigh the amount reported by Delgado *et al.* (2009). Moreover, the amount of protein obtained is between the ranges established by Jacobsen (2003), which are between 13 and 18%. These ranges are higher in comparison to cereals such as rice (7.5%) and corn (13.4%) (Elsohaimy *et al*., 2015).

Regarding NDF content, the amount was higher in T1 seeds. However, the values for every treatment are higher in comparison to what was obtained by Peiretti *et al.* (2013), who reported values of 12.75%. The amount of ADF in the test was low compared to studies by Simranpreet *et al.* (2017), who reported 77.73%. These results were obtained because the composition and presence of fiber are influenced by the maturity stage of the seed (Reguera *et al.,* 2018). The results in this test are related to the fact that when plants started the grain phenological stage, precipitations caused the elongation of the panicle that formed new seeds. This prolonged the phenological period of the plant

to seven months; in other words, two more months than the phenological average of the variety in previous tests.

It is noteworthy that reports of ADF and NDF are very diverse in different tests reported in around the world. This could be happening because of the variety, maturity stage of the grain and fertilization, as referenced by Peiretti *et al*. (2013), whose results were 12.75% of ADF and 5.49% of NDF. On the other hand, Marmouzi *et al*. (2015) reported ADF of 72.03% and ADF of 27.06%, similar to the results of Simranpreet *et al*. (2017), with values of 77.73% and 27.4% for NDF and ADF, respectively.

Conclusions

Quinoa under the high Andean tropic conditions has a better reaction and performance reflected on grain productivity and quality when it is amended with organic and mineral fertilizers. Nonetheless, from a physiological activity perspective, the plant responded better to mineral fertilization. Even though the organic fertilizer did not contribute to the nitrogen available to the plant, the population of diazotrophic bacteria native to this environment is efficient in the contribution of nitrogen for plant development.

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Effect of nematicide rotation on banana (*Musa* AAA cv. Williams) root nematode control and crop yield

Efecto de la rotación de nematicida en el control de nematodos y la producción de banano (*Musa* AAA cv. Williams)

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ABSTRACT

The effects of nematicide rotation on banana (*Musa* AAA cv. Williams) root weight, root nematode control, and crop yield were compared in a commercial banana plantation in Ecuador, testing six treatments in a randomized complete block design with six replicates. Treatments consisted of two, three and four different nematicide cycles per year plus the untreated control. Regarding the untreated plants and averaging the 24 root nematode samplings after treatment application, the nematicide applications reduced significantly *R. similis* ($P<0.0001$) between 20 and 49%, *Helicotylenchus* spp. ($P<0.0001$) between 31 and 51%, and total nematode populations ($P<0.0001$) between 29 and 49%. Accordingly, in the treated plants, there was an increase between 16 and 21% in living root weight ($P=0.0003$), and its percentage ($P<0.0001$) reached between 74.5 and 81.7% in the follower suckers. In addition, the death of roots by nematodes decreased ($P=0.0009$) between 20 and 46%. At harvest, nematicide applications increased bunch weight ($P=0.0002$; $P=0.0467$), ratio ($P=0.0003$ at 12 months), ratooning ($P<0.0001$; $P<0.0001$) and the number of boxes of 18.14 kg ($P<0.0001$; $P=0.0005$) per hectare per year at 12 and 24 months after treatment application, respectively. Plants treated with nematicides increased yield between 671 and 1,158 (12.2 - 21 t) and the number of boxes of 18.14 kg per hectare per year also increased between 545 and 1,046 (9.9 - 19.0 t), which resulted in a net profit between US $3,266 - $5,750 and between US $2,587 and $5,144 per hectare per year at 12 and 24 months after treatment application, respectively.

Key words: Cavendish subgroup, *Helicotylenchus* spp., nematicide, *Radopholus similis*, root weight, total number of nematodes.

RESUMEN

El efecto de la rotación de nematicida en peso de raíces, control de nematodos y la producción se comparó en una plantación comercial de banano (*Musa* AAA cv. Williams) en Ecuador, evaluando seis tratamientos en un diseño de bloques al azar con 6 repeticiones. Los tratamientos consistieron en dos, tres y cuatro aplicaciones de diferentes nematicidas por año más el testigo sin aplicación. El promedio de los 24 muestreos realizados después de la aplicación de los tratamientos mostró que la aplicación de nematicidas redujo la población de *R. similis* ($P<0.0001$) entre un 20 y un 49%, *Helicotylenchus* spp. ($P<0.0001$) entre un 31 y un 51% y del total de nematodos ($P<0.0001$) entre un 29 y un 49%. En congruencia, en las plantas tratadas con nematicida se encontró un aumento entre 16 y 21% en el peso de raíces vivas ($P=0.0003$) y en su porcentaje ($P<0.0001$) en los hijos de sucesión, que alcanzó entre 74.5 y 81.7%. También se observó una reducción ($P=0.0009$) en la muerte de raíces por nematodos de entre un 20 y un 46%. A la cosecha, las aplicaciones de nematicida aumentaron el peso de racimo ($P=0.0002$; $P=0.0467$), ratio ($P=0.0003$ a los 12 meses), retorno ($P<0.0001$; $P<0.0001$) y el número de cajas de 18.14 kg ($P<0.0001$; $P=0.0005$) por hectárea por año a los 12 y 24 meses después de la aplicación de los tratamientos, respectivamente. En las plantas tratadas con nematicida el aumento varió de 671 a 1,158 (12.2 a 21.0 t) y de 545 a 1,046 (9.9 a 19.0 t) cajas de 18.14 kg por hectárea por año, lo que resultó en una ganancia neta entre US $3,266 y US $5,750 y entre US $2,587 y US $5,144 por hectárea por año, a los 12 y 24 meses después de la aplicación de los tratamientos, respectivamente.

Palabras clave: subgrupo Cavendish, *Helicotylenchus* spp., nematicida, *Radopholus similis*, peso de raíces, total de nematodos.

Introduction

Banana (*Musa* AAA) is the most important crop in Ecuador, which generates two million jobs and accounts for almost 10% of the total exports (Clúster Banano, 2018). In 2018, 345 million boxes of 18.14 kg were exported to the European Union, Russia, USA, Japan, China, and others, with a production area of 200,000 ha, which gave a total income of about US $2,700 million (Salazar, 2019).

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Besides the requirements and demands of the banana market, there are other limiting factors to the production of this crop. Abiotic factors affecting yield, such as the edaphic soil condition, radiation, rain distribution, and temperature, are constraints to banana production in Ecuador. Among the biotic factors, banana-root nematodes are second after black Sigatoka caused by the fungi *Pseudocercospora fijiensis*. Banana nematodes live within the roots, where they weaken the plant anchorage and restrict water and nutrients uptake, retard leaf emission, and reduce photosynthesis, bunch weight, ratio, ratooning, and plant longevity (Gowen *et al*., 2005; Quénéhervé, 2008).

Plant-parasitic nematodes are common in the five provinces where banana is produced in Ecuador (Cañar, El Oro, Guayas, Los Ríos, and Santo Domingo) (Aguirre *et al*., 2016a; 2016b) and usually only polyspecific communities occur, consisting of a mixture mainly of *Radopholus similis* and *Helicotylenchus* spp. To avoid or reduce nematode damage, the only alternative management strategy currently available is the regular application of non-fumigant nematicides, due to its low cost for growers. Nematicide application is recommended when the total plant-parasitic nematode population exceeds the economic threshold of 2,500 individuals per 100 g of fresh roots (Instituto Nacional Autónomo de Investigaciones Agropecuarias INIAP, 2018).

The nematicides registered for bananas are rotated according to their physical-chemical characteristics and weather conditions to prevent their biodegradation. However, in Ecuadorian conditions, most banana growers stopped applying nematicides which resulted in high nematode populations, root damage, and severe yield reduction. In young plantations of less than 5 years old, the vigor of plants and their production, generally are higher than 3,000 boxes of 18.14 kg per hectare per year, make technicians and growers think that the attack of nematodes is negligible and that its effect on production is not of economic importance. Therefore, the objective of this study was to evaluate the effect of nematicide rotation in different applications per year on banana plant-parasitic nematode control and crop yield and to determine the net profit of the chemical nematode control in the crop.

Materials and methods

The field experiment was carried out in a 6-year-old renovated commercial banana (*Musa* AAA cv. Williams) farm infected by plant-parasitic nematodes located in the Milagro county, province of Guayas, Ecuador. The soil was taxonomically classified as an Inceptisol and it had a loamy texture (33% sand, 49% silt and 18% clay) with a pH of 7.1 and 1.8% organic matter. The following concentrations of extractable bases were found using Mehlich 3 (Mehlich, 1984) as the extractant: Ca 16.5, Mg 5.7, and K 2.4 cmol L^{-1}, and P 29, S 16, Zn 3.1, Cu 7.6, Fe 60 and Mn 9 μg ml^{-1}. The area where the experiment was established had an average production in 2015 of 3,600 boxes of 18.14 kg ha^{-1}. The evaluation period was performed from October 2015 to October 2017.

Plant density was about 1,450 plants ha^{-1}. De-suckering was carried out every eight weeks, leaving the production unit with a bearing mother plant, a large daughter sucker (follower) and a small grand-daughter (pepper) when possible. Bunching plants were propped with double polypropylene twine to the bottom of two well-developed adjacent plants, reason why plant toppling was not considered as a variable in the experiment. The follower sucker of each production unit was fertilized every 15 d with a mixture of nutrients at 100 kg ha^{-1}, adapted to the soil and crop requirements, consisting of 15-4-36 (N-P_2O_5-K_2O) fertilizers.

During the rainy season, from January to May each year, water requirements were supplied by rainfall. Annual precipitation was 1,771, 2,190 and 1,656 mm per year, for 2015, 2016 and 2017, respectively. A complex system of primary, secondary and tertiary drains was provided to disperse excess rainfall and prevent waterlogging during heavy rains. From June to December each year, water was supplied by sprinkling irrigation. Mean daily maximum/minimum temperatures were 29-31/25-22°C during the studied period.

Leaf fungi, especially black Sigatoka (*Pseudocercospora fijiensis*), was managed by deleafing weekly to reduce the pressure of black Sigatoka inoculum and by aerial spraying of alternate fungicides which resulted in 24 sprayings per year with 8 to 14 d intervals. The fungicides applied were: difenoconazole, fenpropimorph, epoxiconazole, tebuconazole, isopyrazam+azoxystrobin, pyrimethanil, spiroxamine, metiram, mancozeb, *Bacillus subtilis* in emulsion with miscible oil (Spraytex) and water or in a water solution of 19 L ha^{-1}. Weeds were controlled spraying every 12 weeks a glyphosate solution of 1.5 L in 200 L of water with a manual knapsack sprayer. Before the beginning of the experiment, nematodes were controlled every year by the rotation of one nematicide application (Verango® 50SC-fluopyram-Bayer, Vydate® 24SL oxamyl-DuPont, Counter® 15GR-terbufos-AMVAC).

Six treatments were evaluated: 1 and 2 consisted of two different nematicide rotations per year; 3 and 4 consisted of three different nematicide applications per year; 5: nematicide application based on nematode economic threshold of 2,500 plant-parasitic nematodes per 100 g of roots (INIAP, 2018), and 6: the untreated control. The applied nematicides were those available in Ecuador, including Counter® 15GR (terbufos-AMVAC), Verango® 50SC (fluopyram-Bayer), Vydate® 24SL (oxamyl-DuPont), Mocap® 15GR (ethoprophos-AMVAC) and Rugby® 10GR (cadusaphos-FMC) (Tab. 1).

The rectangular plots for each treatment consisted of 150-175 production units. Plots were arranged in a randomized complete block design with six replicates. The application was performed by spreading the products in a banded arc with a radius of approximately 0.40 m around each follower sucker pseudostem sprouting from the base of the sucker. The Swissmex backpack equipment specific for Counter®, Rugby®, and Mocap® and the spotgun for Vydate® were used for the application. The rates used per follower sucker were the recommended by the manufacturer: 3 g a.i. for Counter® and Mocap®, 2.4 g a.i. for Vydate®, 2 g a.i. for Rugby® and 0.3 ml a.i. for Verango®. Verango® was applied in a water solution adding 1 L of the product to 150 L of water plus 200 g of blue coloring, and 100 ml of this solution was spread onto the soil surface with a manual dosing snack pack. Plant debris was removed from the soil surface prior to distributing the nematicides onto moist soil as directed by the product label. During the development of the experiment, no rooting or organic matter was applied in the experimental area.

One day before the nematicide application, and then every 30 d for 24 months (total time of the experiment), root samples were collected in each repetition. Each sample consisted of the roots of three follower suckers between 1.5 and 2.5 m high from recently flowered plants or prompt to bearing. In front of each follower sucker, a hole of 26 cm long, 13 cm wide and 30 cm deep (soil volume of 10.14 L) was dug at the plant base using a shovel. All the roots found were collected and placed in labeled plastic bags and delivered to the INIAP (Instituto Nacional Autónomo de Investigaciones Agropecuarias) laboratory in coolers.

In the laboratory, the root samples were registered and processed as soon as possible, and when it was necessary, stored in a refrigerator (General Electric) at 6-8ºC until being processed. The roots were rinsed to remove the soil, separated into living roots (white or cream-colored roots), dead roots by plant-parasitic nematodes (with symptoms of nematode damage, with necrosis, but without root decay), and dead roots by other causes (rotten roots by excess water, snapping). Then the roots were left to dry off the surface moisture and weighed (Cas computing scale precision 5 kg ± 1 g). During the root separation process, in some roots, it was necessary to cut some damaged parts, which were classified accordingly. The total root weight corresponds to the sum of living roots, dead roots by plant-parasitic nematodes, and dead roots by other causes.

The living and dead roots by nematodes were cut into 1-2 cm length pieces, and after homogenization, 10 g were randomly selected. These roots were macerated (Taylor and Loegering, 1953) in a kitchen blender (Osterizer; Sunbeam-Oster, USA) for two periods of 10 s at medium speed with a resting period of 4 s in between, and nematodes were recovered in a 0.038 mm (400 mesh) sieve. The nematodes were identified at the genus and species level when possible, based on the morphological characteristics under a light microscope, following the key of Siddiqi (2000). The population densities of all plant-parasitic root nematodes were recorded, and the values were converted to numbers per 100 g of roots.

TABLE 1. Description of the treatments evaluated with the sequence of nematicides and date of application.

Treatment	Months of evaluation																								
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 (2c per year)	Vy						Co						Ru						Co						Ru
2 (2c per year)	Ve						Co						Ve						Ru						Ve
3 (3c per year)	Co				Ru				Vy				Co				Ru				Co				Mo
4 (3c per year)	Ve				Co				Ru				Co				Ve				Co				Ru
5 (ET)	Ru			Co			Vy			Ru			Co			Ru			Co			Ru			Co
6 (UTC)																									

Note: month 0= October, 2015 and 24= October 2017. c per year= number of nematicide cycles per year. ET= Application based on the economic threshold of 2,500 nematodes per 100 g roots. UTC= Untreated control. Vy= 2.4 g a.i. Vydate® 24SL, Co= 3 g a.i. Counter®15GR, Ru= 2.0 g a.i. Rugby®10GR, Ve= 0.3 g a.i. Verango®50SC, and Mo= 3 g a.i. Mocap®15GR.

At the beginning of the experiment, and 12 and 24 months after the first nematicide application, 90 randomly selected bunches of each treatment (15 per useful replicate) without plot edges, edge drains, cable edges or dompings were evaluated. Bunches were harvested by calibration starting when bunches reached 10 weeks of age. The bunch was harvested when in the second hand, the central fruit of the outer whorl had a diameter of at least a grade of 45 (35.5 mm-diameter). If in week 13 fruits did not reach the required minimum grade of 45, they were harvested with the grade they had. The harvest age, date of harvest, number of hands, applied dehanding, bunch weight (Tru-Test electronic scale XR3000 Kg ± 1g) and calibration of the central fruit of the outer whorl of the second hand were registered. To calculate the ratio, which is the number of boxes of 18.14 kg given by each bunch, a reduction of 20% was considered because it is the average of the farm, which includes 12% of rachis and 8% of non-marketable fruit. With the data of the number of bunches harvested in 2015 in the area where the experiment was located and the number of plants per hectare, the initial ratoon was estimated. In addition, with the age of bunches and harvest dates, the ratooning (number of bunches per production unit per year) was estimated at 12 and 24 months.

Root and nematode data were averaged by experimental plot across the 24 months, excluding the first evaluation pre-treatment application. The composition of the plant-parasitic nematode population was determined before treatment application, and then for the average of the 24 root samplings. Data of root weights before treatment application, and thereafter for the average of the 24 root samplings, were subjected to ANOVA by Proc GLM of SAS and mean separation by LSD-test. The number of nematodes was analyzed with generalized linear models, using the log transformation as link function and negative binomial distribution of the errors for the first nematode sampling alone, and thereafter for the average of the 24 nematode samplings together after the application. Bunch weight, number of hands per bunch, fruit calibration in the second hand, ratio, ratooning, and number of boxes of 18.14 kg per hectare per year (97% bunch recovery, 1,406 bunches x ratio x ratooning) were averaged for each repetition and harvest, and subjected to ANOVA and mean separation using LSD-test in PC-SAS® version 9.4.

Results

In the root sampling carried out before treatment application, no differences were found in the content of living roots (P=0.7148), dead roots by nematodes (P=0.2897), dead roots by other causes (P=0.4873), total roots (P=0.9799) and living root percentage (P=0.3373). The contents varied between 27.0 and 38.6 g for living roots; the dead roots by nematodes ranged from 3.9 to 10.4 g; the dead roots by other causes oscillated from 1.3 to 9.8 g, and the total roots from 40.3 to 45.8 g per follower sucker (Fig. 1A-D). The percentages of living roots in the sucker ranged between 66.0 and 87.8% (Fig. 1E). Similarly, in this sampling, no difference was detected among treatments in the populations of *R. similis* (P=0.8674), *Helicotylenchus* spp. (P=0.5294) and total nematodes (P=0.2458), which corresponds to the sum of the plant-parasitic nematode species detected (Fig. 2A-C). Nematode populations among treatments varied: *R. similis* between 7,267 and 21,200, *Helicotylenchus* spp. between 17,333 and 31,633, and total nematodes between 27,733 and 42,167 individuals per 100 g of roots. The composition of the nematode population before treatments application was: 31.5% of *R. similis*, 68.2% of *Helicotylenchus* spp. with a negligible amount of *Meloidogyne* spp. and *Pratylenchus* spp. (data not shown).

Root content and nematode populations through the 25 samplings are presented in Figures 1 and 2. Across the different samplings, root content and nematode populations followed a similar trend in all the treatments. After treatments application, when comparing the average of the 24 samplings (Fig. 3), differences were found among treatments in the contents of living roots (P=0.0003) and dead roots by nematodes (P=0.0009). The highest increase in living roots was observed in plants treated with three nematicide cycles per year with 21 and 17%, followed by the plants treated according to the economic nematode threshold of 2,500 plant-parasitic nematodes per 100 g of roots, which resulted in four applications per year with 16% (Fig. 3A) compared to untreated control. The application of nematicides reduced the dead of roots by nematodes between 20 and 46% (Fig. 3B). In agreement to the increase of roots in plants treated with nematicide and their lower dead of roots by nematodes, these plants had the highest (P<0.0001) percentage of living roots, which varied between 74.6 and 81.7%. These results contrast the 67.3% found in the untreated control plants (Fig. 3E). The grams of dead roots by other causes (P=0.0707) and total root (P=0.1570) weight were similar among treatments, ranging from 2.2 to 3.4 g, and from 41.2 to 44.9 g per sucker, respectively (Fig. 3C-D).

The biggest nematode population per 100 g of roots of *R. similis* (P<0.0001), *Helicotylenchus* spp. (P<0.0001) and total nematodes (P<0.0001) was found in the untreated plants (Fig. 2 and Fig. 4). Compared to the untreated plants,

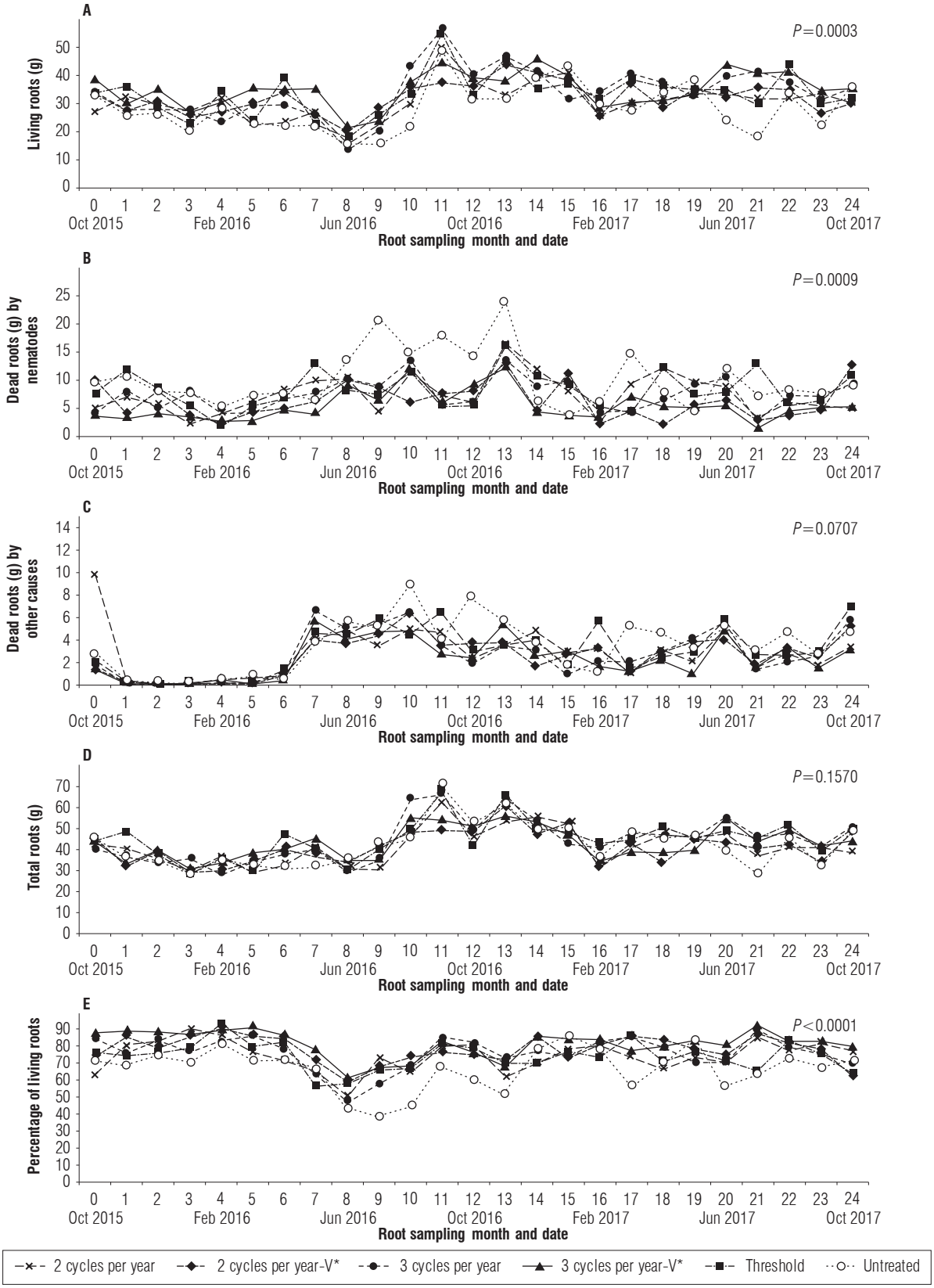

FIGURE 1. Fresh root weight (g) of living roots (A), dead roots by nematodes (B), dead roots by other causes (C), total roots (D), and percentage of living roots per sucker in banana (*Musa* AAA cv. Williams) plants treated with different number of nematicide cycles per year. Each point is the average of six repetitions. In each repetition, a hole of 26 cm long 13 cm wide, and 30 cm deep was dug at the base and in front of three follower suckers from 1.5 to 2.5 m high to collect all roots. V*= one nematicide cycle was with Verango®.

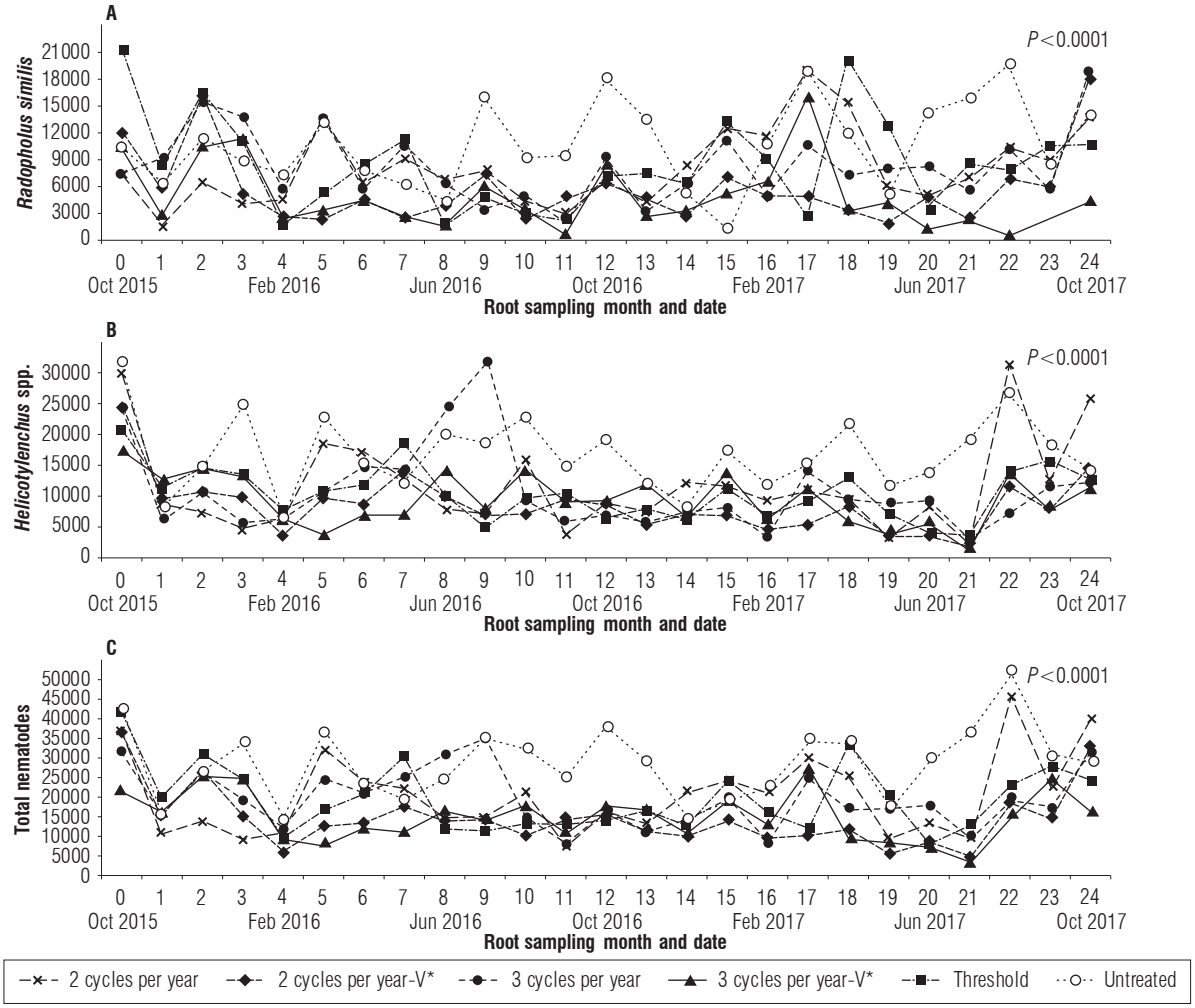

FIGURE 2. Number of *Radopholus similis* (A), *Helicotylenchus* spp. (B) and total nematodes (C) per 100 g of banana (*Musa* AAA cv. Williams) roots treated with a different number of nematicide cycles per year. Each point is the average of six repetitions. In each repetition, a 26 cm long 13 cm wide, and 30 cm deep hole was dug at the base and in front of three follower suckers from 1.5 to 2.5 m high to collect all roots. V*= one nematicide cycle was with Verango®.

nematicide treatments reduced *R. similis* between 20 and 49%, *Helicotylenchus* spp. between 31 and 50%, and the total nematode populations between 29 and 49% (Fig. 4A-C). Changes in the nematode population composition were observed by averaging the 24 samplings taken after treatment applications, with *R. similis* increasing to 45.8%, *Helicotylenchus* spp. decreasing to 52.2%, and *Meloidogyne* spp. and *Pratylenchus* spp. remaining negligible with 1.4% and 0.4%, respectively (data not shown).

The data of the three harvests carried out at the beginning of the experiment, and at 12 and 24 months after the first treatment applications are presented in Table 2. Bunch weight (P=0.1961) was similar among treatments at the initial harvest, varying between 34.8 and 37.9 kg per bunch (Tab. 2). In this initial harvest, the ratio (P=0.1926), which fluctuated between 1.53 and 1.67, and number of hands (P=0.4120), which varied between 8.9 and 9.3 hands per bunch, were also similar among treatments (Tab. 2). In congruence, the number of boxes per hectare (P=0.1922) which ranged from 3,600 to 3,923 (Tab. 2) and the calibration (P=0.1612) of the central fruit of the outer whorl of the second hand, which varied between 35.6 and 36.2 mm (data not shown), were similar among treatments. The initial ratooning in the experimental area was 1.67 bunches harvested in each banana stool per year (Tab. 2), which is equivalent to an interval between crop harvests of 218.5 d.

In the second harvest, carried out 12 months after the first treatment applications, an increase between 3.6 and 6.5 kg (11-20%) was observed in bunch weight (P=0.0002) except the bunches of the untreated plants (Tab. 2). In agreement to these results, treatments with nematicide increased the number of hands (P=0.0003) between 0.3 and 0.8 (3-10%),

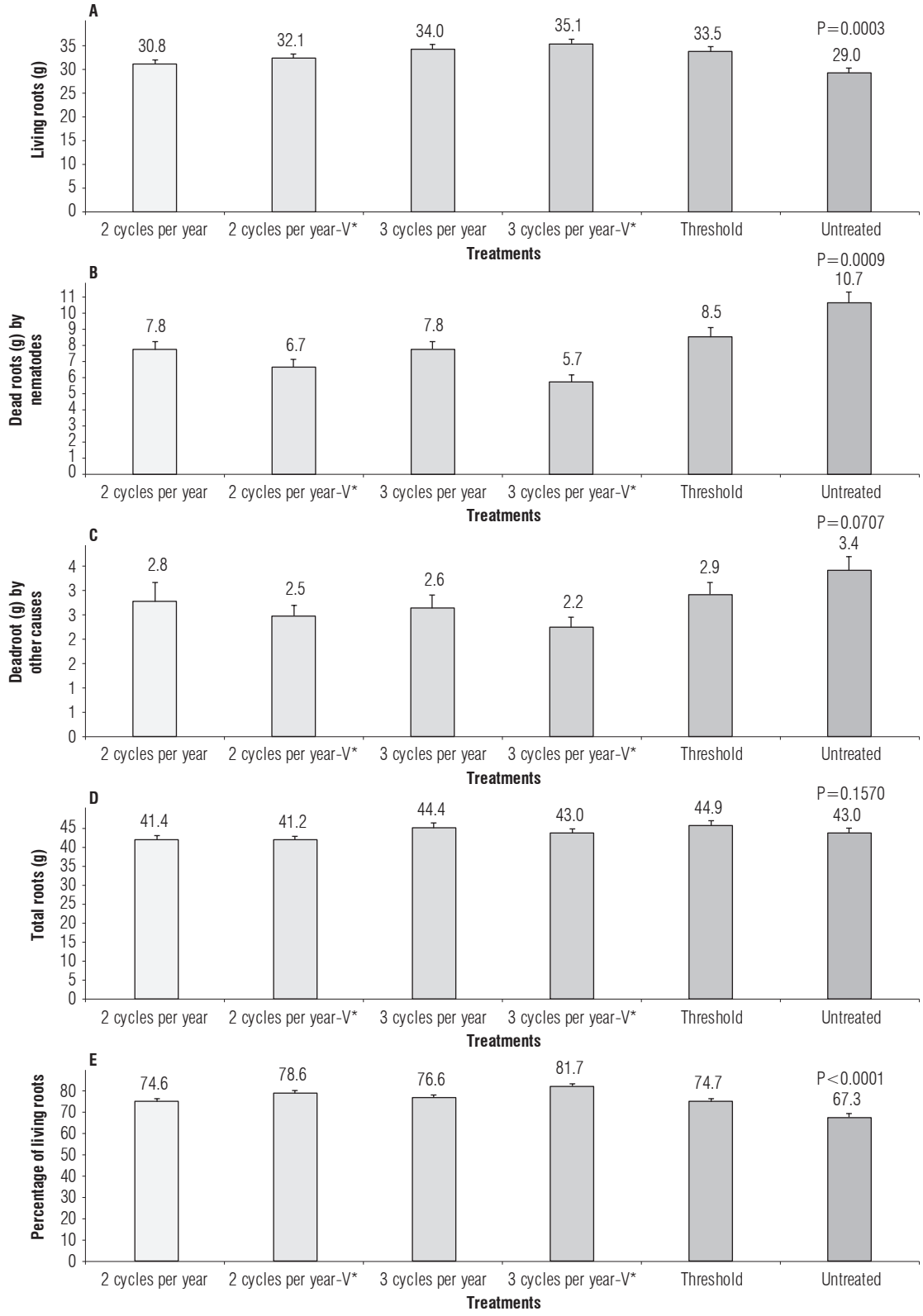

FIGURE 3. Average fresh root weight (g) of living roots (A), dead roots by nematodes (B), dead roots by other causes (C), total roots (D) and percentage of living roots (E) per follower sucker in banana plants (*Musa* AAA cv. Williams) treated with different number of nematicide cycles per year. Each bar is the average of 144 observations (24 samples per six repetitions), and in each repetition, the data are the average of three follower suckers. In front of each follower sucker, a hole of 26 cm long, 13 cm wide, and 30 cm deep was excavated at the base, and all roots were collected. V*= one nematicide cycle was with Verango®.

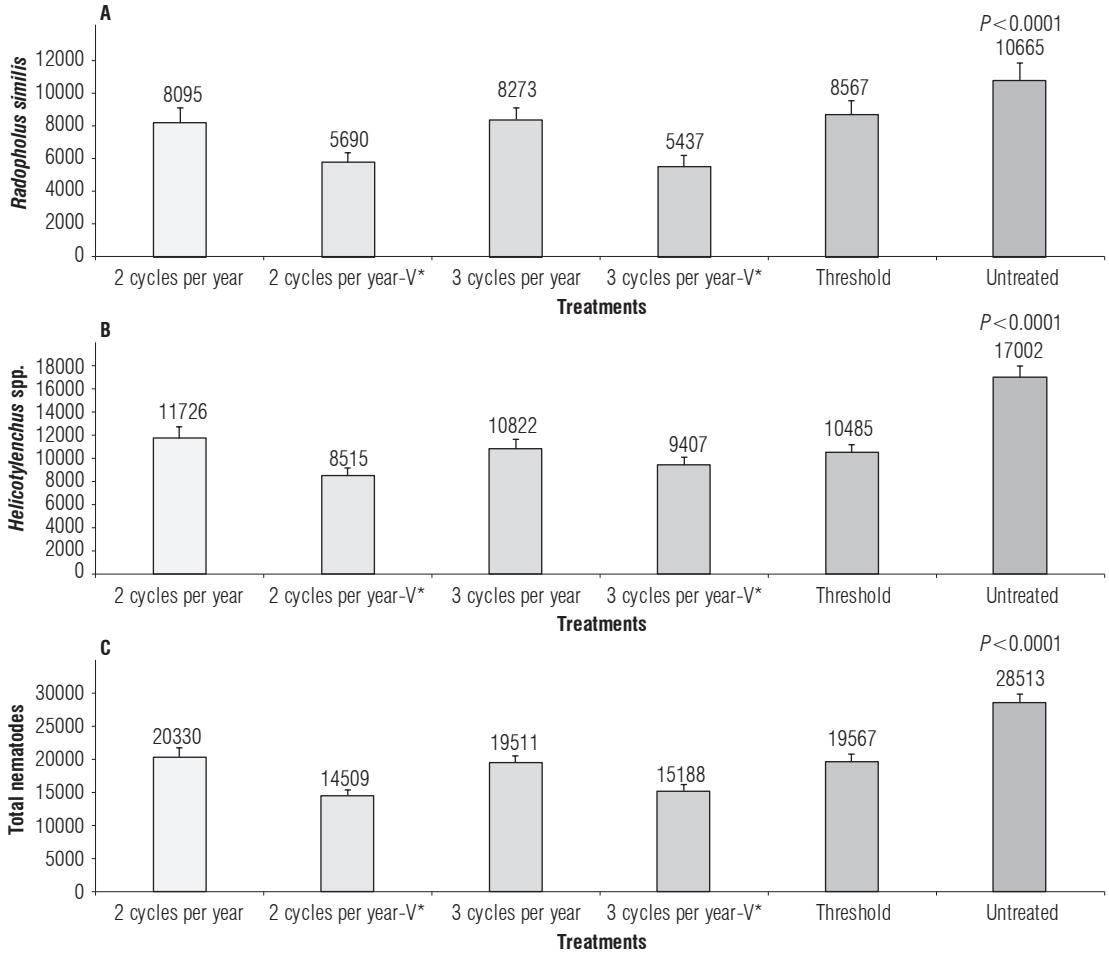

FIGURE 4. Number of *Radopholus similis* (A), *Helicotylenchus* spp. (B) and total nematodes (C) per 100 g of banana roots (*Musa* AAA cv. Williams) treated with a different number of nematicide cycles per year. Each bar is the mean ± standard error of 144 observations (24 samplings per six repetitions) and in each repetition, the data are the average of three follower suckers of 1.5-2.5 m high. A 26 cm long, 13 cm wide and 30 cm deep hole was dug in front of each follower sucker and all roots were collected. V*= one nematicide cycle was with Verango®.

ratio (P=0.0003) between 0.16 and 0.28 (11-20%), ratooning (P<0.0001) between 0.11 and 0.24 (7-15%) and number of boxes (P<0.0001) between 671 and 1,158 (20-36%) per hectare per year (Tab. 2). In comparison to the first harvest, which was performed at the time of establishing the experiment, treatments with nematicide increased the number of boxes between 190 and 510 (5-14%) per hectare per year, while in the untreated control a reduction of 408 (11%) boxes was found.

In the third harvest, 24 months after the first application of the treatments, differences in bunch weight (P=0.0467, Tab. 2) were found again. The maximum difference with the untreated plants was 4.7 kg (13.5%). The number of hands (P=0.0794) and ratio (P=0.0727) were similar among treatments, varying between 8.5 and 9.1 hands per bunch and between 1.53 and 1.73 boxes per bunch, respectively (Tab. 2). In all treatments with nematicide applications, an increase in ratooning (P<0.0001), varying between 0.14 and 0.31 (9-20%) was observed (Tab. 2). In parallel, in treatments with nematicide, an increase (P=0.0005) between 545 and 1,046 (16-31%) boxes per hectare per year was obtained compared to the untreated plants (Tab. 2).

The increases in bunch weight in the plants treated with nematicide led to an increase in the ratio (more boxes per bunch). In contrast, in the bunches of the untreated plants, a reduction of 0.11 units was observed in ratio at 12 months, which multiplied by the number of bunches per hectare per year gave a reduction of 246 boxes in the control. In the treatments with nematicide application, when the increase in their ratio in the second and third harvest was multiplied by the respective number of bunches harvested per hectare per year, an increase between 410 and 722 and between 22 and 497 boxes per hectare per year was found at 12 and 24 months, respectively (Tab. 2).

The increase in ratooning at 12 and 24 months after the treatments were applied was between 0.11 and 0.24 and between 0.14 and 0.31 units, compared to the control, respectively (Tab. 2). The initial ratooning of 1.67 from the experimental area was reduced in the untreated plants by 0.07 units at 12 months and by 0.03 additional units at 24 months (1.64 and 1.57, respectively) (Tab. 2). In contrast, in treatments with nematicide, the ratooning increased from 1.67 at the beginning of the experiment to 1.71-1.84, and from 1.71-1.88 at 12 and 24 months, respectively. This means that the interval between harvests at 12 and 24 months was reduced between 5.1 and 20.2 d and between 19.5 and 38.8 d in the nematicide treatments, while in the untreated plants, the interval was extended in 10 and in 4.7 additional d, going from 218.5 d at the beginning of the experiment to 228.6 and 232.7 d between harvests at 12 and 24 months, respectively. The planting density was 1,450 plants ha^{-1}, of which 97% of the bunches (1,406) were processed. Then, when this number of harvested plants of 1,406 bunches per hectare was multiplied by the respective ratooning in the untreated plots, 2,245 and 2,205 bunches were harvested per hectare per year at 12 and 24 months, respectively. Compared to these untreated plants, in the nematicide treatments, between 159 and 342, and between

TABLE 2. Banana (*Musa* AAA cv. Williams) yield parameters according to the number of nematicide cycles per year and cost-benefit relation at the second and third harvest. Sell price of each box of 18.14 kg was US $6.15.

Treatment	Bunch weight Kg	Number of hands / bunch	Ratio	Ratoon	Boxes ha^{-1} per year	Difference in boxes with untreated	Additional income US $	Treatment cost US $	Additional packing cost US $	Net income US $	Net profit by dollar
First harvest at the beginning of the experiment											
2 cycles per year	35.6	9.1	1.57	1.67	3,686						
2 cycles per year-V*	35.7	9.2	1.58	1.67	3,709						
3 cycles per year	36.0	9.1	1.59	1.67	3,733						
3 cycles per year-V*	37.9	9.3	1.67	1.67	3,921						
Threshold	34.8	8.9	1.53	1.67	3,592						
Untreated	35.0	9.0	1.54	1.67	3,616						
Probability	$P=0.1961$	$P=0.4120$	$P=0.1926$		$P=0.1922$						
Second harvest at 12 months after the first treatment application											
2 cycles per year	37.4	9.5	1.65	1.71	3.967	750	4,612	305	562.5	3,744	12.2
2 cycles per year-V*	36.7	9.3	1.61	1.71	3.871	654	4,022	355	490.5	3,176	8.9
3 cycles per year	37.2	9.5	1.64	1.81	4.173	956	5,879	460	717.0	4,702	10.2
3 cycles per year-V*	39.0	9.8	1.72	1.81	4.377	1,160	7,134	505	870.0	5,759	11.4
Threshold	36.1	9.4	1.59	1.84	4.113	896	5,510	620	672.0	4,218	6.8
Untreated	32.5	9.0	1.43	1.60	3.217						
Probability	$P=0.0002$	$P=0.0003$	$P=0.0003$	$P<0.0001$	$P<0.0001$						
Third harvest after 24 months of the first treatment application											
2 cycles per year	39.2	9.1	1.73	1.71	4,159	804	4,944	305	603.0	4,036	13.2
2 cycles per year-V*	36.6	9.1	1.61	1.72	3,893	538	3,308	355	403.5	2,550	7.2
3 cycles per year	36.5	8.9	1.61	1.84	4,165	810	4,981	460	607.5	3,914	8.5
3 cycles per year-V*	38.6	9.1	1.70	1.84	4,400	1,045	6,426	505	783.7	5,137	10.2
Threshold	34.6	8.5	1.53	1.88	4,044	689	4,237	620	516.7	3,100	5.0
Untreated	34.5	9.0	1.52	1.57	3,355						
Probability	$P=0.0467$	$P=0.0794$	$P=0.0727$	$P<0.0001$	$P=0.0005$						

Ratio= number of boxes of 18.14 kg per bunch (80% of the bunch weight was packed (20% rejection that includes 12% bunch stalk and 8% rejected bananas) / 18.14 kg per box). 1,450 plants per hectare from which 97% of the bunches were processed (1,406 bunches), ratoon= number of bunches harvested per each banana stool by year, boxes per hectare per year= (1,406 bunches * ratio * ratoon). Each value is the mean of six replicates, and in each replicate, 15 bunches were harvested. V* one nematicide cycle was with Verango®. Net profit= additional income - treatment control cost - banana box packing cost of US $0.75 each. Counter® 15GR $150, Verango® $200, Vydate® 24SL $150, Rugby® 10G $160, and Mocap® 15GR $170 per hectare.

202 and 441 more bunches per hectare per year were harvested. This number of bunches multiplied by the respective ratio resulted in values between 261 and 544 and between 348 and 676 more boxes per hectare per year at 12 and 24 months, respectively.

Discussion

In the sampling carried out before treatments application, no differences were found among treatments in root contents, populations of nematodes, or in the production variables evaluated at the time of establishing this experiment. This means that any difference that was found after applying the treatments was attributed to their effect. At the beginning of the experiment, the nematode population consisted mainly of *Helicotylenchus* spp. (68.2%) and *R. similis* (31.5%), reducing the proportion of *Helicotylenchus* spp. to 52.3% at the end of the experiment, while *R. similis* increased in the plant-parasitic nematode community to 45.8%.

This greater proportion of *Helicotylenchus* in banana nematode attacks has been observed in Cavendish plantations with insufficient control, as reported by Araya and Moens (2005) and Salguero *et al*. (2016). *Helicotylenchus* spp. is an ecto-endoparasite (Blake, 1966; Orion and Bar-Eyal, 1995; Gowen, 2000; Guzmán-Piedrahita, 2011a) that induces necrotic lesions on the surface of the roots. In contrast, *R. similis* is a migratory endoparasite that causes necrotic lesions along the entire root, in the epidermis, cortical parenchyma and vascular cylinder (Blake, 1966; Orton and Siddqi, 1973; Jackson *et al*., 2003; Guzmán-Piedrahita, 2011b). The high population of *Helicotylenchus* spp. and *R. similis* is favored because banana production is in perennial monoculture, although it is an annual crop.

The application of nematicide on the soil surface, in front of the follower suckers, reduced the populations of *R. similis* between 20 and 49%, of *Helicotylenchus* spp. between 31 and 50% and of total nematodes between 29 and 49% compared to the untreated control. This reduction in the population agrees with the results of Barriga *et al*. (1980) and Jaramillo and Quirós (1984), who found in average, a reduction between 49 and 82% of the plant-parasitic nematodes, respectively with different nematicide treatments. Araya and Cheves (1997a, 1997b) reported reductions of 22-63% for *R. similis* and 25-89% for *Helicotylenchus* spp. Quénéhervé *et al*. (1991a, 1991b, and 1991c) indicated reductions of 22.7-90.7% for *R. similis* and 32.5-100% for *Helicotylenchus* spp. In addition, Castillo *et al*. (2010) found drops of 38-60% for *Helicotylenchus* spp., 24% for *R. similis*, and 25-33% for total nematodes. Moens *et al*. (2004) recorded reductions of 18-59% for the total plant-parasitic nematodes and Salguero *et al*. (2016) found decreases of 33-47% for *R. similis*, 36-65% for *Helicotylenchus* spp. and 35-59% for total nematodes. In agreement with the significant reduction of nematodes in treatments with nematicide, a significant increase in living roots of up to 81.7% and in the percentage of living roots of 21% were observed. Additionally, a decrease of up to 46% of dead roots by nematodes was recorded. Comparing treatments of 2 and 3 nematicide cycles per year (in which one of the cyles was with Verango®), those plants that with Verango® application showed a lower number of *R. similis*, *Helicotylenchus* spp. and total nematodes, and lower dead roots by nematodes and a higher percentage of living roots.

In response to the increased root health in plants treated with nematicide, an increase in bunch weight of 3.6-6.5 kg (11-20%) was found in the second harvest and up to 4.7 kg (13.5%) in the third harvest. The percentages of bunch weight increase recorded in this experiment were consistent with some of those cited by Vilardebó and Guerout (1976) between 12 and 123% and by Gowen (1993) between 16 and 45%. On the other hand, these percentages were lower than those reported by Araya and Cheves (1997a; 1997b) (22.1% and 40.8%, respectively), and than the ones found by Stanton and Pattison (2000) (44%), Moens *et al*. (2004) (45%), and Quénéhervé *et al*. (1991a) (48%).

The reductions in the interval between harvests are congruent with Quénéhervé *et al*. (1991b), who found a cumulative reduction in time to harvest according to the cycle of 28 d in the first, 57 d in the second and 128 d in the third harvest cycle in plants treated with nematicides. Similarly, Quénéhervé *et al*. (1991a) and Gowen (1995) report an increase in the harvest period from 13 to 32 and from 22 to 40 d, respectively, in plants infected with nematodes that were not treated compared to those with nematicide application. In congruence with this extension in the period to harvest, Roderick *et al*. (2012) reported an increase of 13.6 more days to harvest in Mbwazirume banana plants to which they added nematodes compared to plants without the addition of nematodes.

The highest number of boxes per hectare per year was due to the application of nematicide that resulted in a significant reduction of nematodes, which led to an increase in the percentage of living roots that favored water and nutrients uptake. This, in turn, allowed a better growth of the crop, which led to higher bunch weights, ratio, and ratoon. In the second and third harvest, nematicide treatments produced

from 671 to 1,158 (12.2 to 21.0 t) and from 545 to 1,046 (9.9 to 19.0 t) more boxes of 18.14 kg per hectare per year than plants of the untreated plots, at 12 and 24 months of the treatment application, respectively. This means that nematode control increased production between 21 and 36% and between 16 and 31% at 12 and 24 months of treatment application, respectively. The smallest increases in production, 24 months after applying the treatments, probably indicate the proximity of the optimum yield; at that point, maintaining adequate control of the pest would stabilize the production, until the natural senescence of the crop begins.

The observed percentages of yield increase agreed with some of the percentages compiled by Gowen and Quénéhervé (1990), who mentioned increases of 14-263%, and Gowen (1995) who reported increases of 5-275%. However, these percentages were lower than those reported by Stanton and Pattisson (2000) of 46%. The increases in production found were in line with that reported by Cubillos *et al*. (1980), who cited increases of more than 300 boxes of 20.0 kg (6.0 mt), Quénéhervé *et al*. (1991b) who indicated increments in production of 523 to 1,157 boxes (9.5 to 21.0 mt), Pattison *et al*. (1999) who reported increases of 655 to 953 boxes of 13 kg (8.5 to 12.3 t), Araya and Lakhi (2004) who cited increments of 1,245 boxes of 18.14 kg (22.6 t), and Salguero *et al*. (2016) who found increases of 545 to 832 boxes of 18.14 kg (9.9 to 15.1 t) per hectare per year, controlling nematodes through the application of nematicides.

The highest yield (number of boxes per hectare per year) was observed in plants treated with three nematicide cycles per year. These results agree with that reported by Araya (2003), who registered higher yields as the number of nematicide cycles per year increased in Costa Rican banana plantations infected with nematodes. These increases in production as a result of nematodes control are in parallel with Guerout (1972), Charles *et al*. (1985), Quénéhervé *et al*. (1991a, 1991b), and Salguero *et al*. (2016), who cited negative and significant linear correlations between the populations of *R. similis*, *Helicotylenchus* spp. and total nematodes with bunch weight in bananas.

The high population of *Helicotylenchus* spp. and the increases in production achieved with the application of nematicides indicated that their parasitism reduces growth, development, and production. These results are in accordance with observations by McSorley and Parrado (1986), Gowen and Quénéhervé (1990), Chau *et al*. (1997), Barekye *et al*. (1998, 2000), Gowen (2000), Guzmán-Piedrahita (2011a), Coyne *et al*. (2013), and Salguero *et al*. (2016), who reported that *H. multicinctus* and *H. dihystera* damaged the banana root system and reduced yield between 19% (Speijer and Fogain, 1999) and 34% (Reddy, 1994). Additionally, Sijmons *et al*. (1994) indicated that the induction and maintenance of feeding sites of *Helicotylenchus* spp. causes physiological changes in the structure of cells. In the case of *R. similis,* it is well supported that it reduced the yield in banana (Gowen and Quénéhervé, 1990; Gowen, 1993, 1995; Araya, 1995, 2004; Guzmán-Piedrahita, 2011b; Roderick *et al*., 2012; Coyne *et al*., 2013).

The presence of nematodes with different parasitic habits, *R. similis* migratory endoparasite and *Helicotylenchus* spp. an ecto-endoparasite, most likely exacerbates root damage, since lesions can develop at feeding sites and through root tissue. In addition, plants often activate post-infection resistance mechanisms, even in cases where the population of nematodes increases over time and the nematode-plant interaction is compatible. Therefore, together these processes can represent high energy expenditure for plants which can interfere with the filling and development of the bunch. Given that both nematode genera cause damage to the crop, for the implementation of options for their management, the population of all present plant-parasitic nematodes should be considered, as has been suggested by Araya (2004), Ramclam and Araya (2006), Salguero *et al*. (2016), and Aguirre *et al*. (2016a, 2016b).

During the development of the experiment, the market price of a box of 18.14 kg of bananas was US $6.15, and of a nematicide application cycle including the application cost per hectare was US $150 for Counter® 15GR, US $200 for Verango®, US $150 for Vydate® 24SL, US $160 for Rugby® 10GR, and US $170 Mocap® 15GR. The costs of the fertilizer, control of black Sigatoka and weeds, plant propping, and other tasks were the same for the control plots and those treated with nematicide since the increase recorded was for the bunch weight, ratio and ratooning. The additional net income from the increase in yield deducted the cost of labor of US $0.75 for packing each additional box. The cost of the product and its application was from US $3,266 to $5,750 and from US $2,587 to US $5,144 per hectare per year at 12 and 24 months after treatments application, respectively. This net gain agrees with that indicated by Pattison *et al*. (1999) who reported amounts between US $2,494 to US $5,910 per hectare per year. This means, that for every dollar invested in nematode control, at 12 months, the net profit ranged from US $6.8 to $12.2, and at 24 months from $5.0 to $13.3. Despite the higher production in the plants that received three nematicide cycles per year, in which one of the cycles was with Verango®,

the highest net profit was obtained with two nematicide cycles per year, with a return of US $12.2 and US $13.3 at 12 and 24 months, respectively, of applied treatments for every dollar invested in nematode control.

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Different soaking times and niacin concentrations affect yield of upland rice under water deficit conditions

Diferentes tiempos de remojo y concentraciones de niacina afectan el rendimiento de arroz de tierras altas bajo condiciones de déficit hídrico

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ABSTRACT

Rice is an important source of energy for a large part of the world's population. The development and application of technologies that contribute to the improvement of production for this grain have great importance. The objective of this study was to evaluate the influence of rice seed immersion in a niacin solution on plant development, physiology and production. The treatments were defined by a combination of two immersion times for the seeds (12 h and 24 h) in four niacin concentrations (0.00, 100, 200 and 300 mg L^{-1}) distributed in five replicates. Characteristics relating to seedling emergence, plant vegetative development, relative indices of chlorophyll and grain yield were evaluated. We verified that the immersion of the seeds for 12 h gave higher relative indices of chlorophyll, whereas immersion for 24 h increased the speed of emergence and the number of tillers and panicles. The doses of niacin positively affected the relative chlorophyll indices and the production characteristics, up to a maximum concentration of 172.57 mg L^{-1}. We concluded that the immersion of rice seeds for 24 h increased the speed of seedling emergence, leaf number, and panicles per area. However, the relative indices of chlorophyll in leaves decreased. The use of niacin promoted the numbers of spikelets per panicle, fertility of the spikelets and the weight of 1000 grains, besides increasing the relative index of chlorophyll in the leaves of rice plants.

Key words: *Oryza sativa* L., vitamin B3, biostimulant, abiotic stress, seed priming.

RESUMEN

El arroz es una importante fuente de energía para una gran parte de la población mundial. El desarrollo y la aplicación de tecnologías que contribuyen a la mejora del sistema de producción de este grano tienen una gran importancia. Por lo tanto, el objetivo de este estudio fue evaluar el comportamiento vegetativo y productivo del arroz de tierras altas sometido la inmersión de las semillas en niacina. Los tratamientos se definieron por la combinación de dos tiempos de remojo (12 y 24 h) en cuatro concentraciones de niacina (0.00, 100, 200 y 300 mg L^{-1}), distribuidos en cinco repeticiones. Se evaluaron las características relacionadas con la emergencia de las plántulas y el desarrollo vegetativo de las plantas, los índices relativos de clorofila y el rendimiento de grano. Se verificó que la inmersión de las semillas durante 12 h proporciona índices relativos más altos de clorofila, mientras que la inmersión durante 24 horas aumenta la velocidad de emergencia, el número de macollas y panículas. Las dosis de niacina afectaron los índices relativos de clorofila y las características de producción positivamente, hasta la concentración máxima de 172.57 mg L^{-1}. Se concluyó que la inmersión de las semillas de arroz durante 24 h aumenta la velocidad de emergencia de las plántulas, el número de hojas y las panículas por área. Sin embargo, los índices relativos de clorofila en las hojas disminuyen. El uso de niacina promueve ganancias en el número de espiguillas por panícula, la fertilidad de las espiguillas y el peso de mil granos, además de aumentar el índice relativo de clorofila en las hojas de las plantas de arroz.

Palabras clave: *Oryza sativa* L., vitamina B3, bioestimulante, estrés abiótico, condicionamiento de semillas.

Introduction

About 90% of Brazilian rice production is obtained by cultivation in irrigated systems, while the rest is produced by rainfed conditions. The productivity of rainfed rice is close to 2,028 kg ha^{-1}. This is considered low and economically unprofitable and has culminated in economically unattractive production, leading to a decrease in this type of cultivation in recent years (Oliveira Neto, 2015). Rainfed production is dependent on rainfall distribution and water deficit during the flowering and grain filling phases and leads to significant reduction in productivity (Oliveira

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Neto, 2015). A lack of good production practices contributes to an increase in the detrimental effect of drought stress (Oliveira Neto, 2015).

The occurrence of more frequent extreme weather events increases the risk of low yields and creates concerns for the future of crop production and, consequently, for global food security (Wheeler and Von Braum, 2013). This is related to the effects triggered by stress factors, such as prolonged droughts and rising air temperature, that are reflected by a decrease in crop growth and productivity in several regions of the world.

Soil water deficiencies also compromise the vegetative and reproductive development of rice plants (Terra *et al*., 2015). Damage associated with soil water deficits is caused by the onset of a series of morphological and physiological changes, such as a decrease in the relative intracellular water content, leading to lower photosynthetic capacity and gas exchange reductions and culminating in lower amounts of energetic reserves used for the formation of tissues in different developmental stages (Awasthi *et al*., 2014; Zhou *et al*., 2017).

Initial plant establishment is relevant to the next stages of vegetative and productive development (Batista *et al*., 2015). The occurrence of adequate internal moisture contents in seed germination is essential for optimum development of the seedling, since water is the main factor involved in germination (Taiz *et al*., 2017). During stages I and II of hydration, the distribution and translocation of nutrient reserves present in the composition of the endosperm takes place, which will nourish the embryo and the seedling until it becomes autotrophic (Franco *et al*., 1997; Taiz *et al*., 2017). For this reason, Franzin *et al*. (2007) observes that upland rice seeds immersed in water for periods of 16 h to 24 h increases their capacity for normal plant formation with a greater developmental capacity.

In addition to the technique of soaking seeds in water, the introduction of vitamins into the solutions has potential agricultural use, especially cases of stressful plant developmental factors. This fact was demonstrated by using niacin and thiamine solutions for soaking corn seeds, in order to alleviate the deleterious effects caused by saline stress during the germination and initial developmental stages of plants (Hassanein *et al*., 2009; Kaya *et al*., 2014). In these studies, the authors observe positive responses up to the highest concentrations of 100 mg L^{-1} for both vitamins.

Despite the benefits of introducing vitamins into plant production, research on these compounds is still scarce and application techniques have not been established. Thus, we propose new research that exposes new methodologies of vitamin use in different species of economic interest, in which possible benefits or damages from the application of these substances can be demonstrated in the face of abiotic stress factors of the productive system.

From seed germination to grain production, a rice crop needs 450-700 mm of water (Rodrigues *et al*., 2004). This amount of water can be optimized with the application of good practices, such as the previous immersion of seeds in biostimulants. The objective of this research was to evaluate the influence of rice seed immersion in a niacin solution on plant development, including influences on the plant's physiological status and production.

Materials and methods

This research was conducted in an experimental zone located in the city of Goiânia, in the central region of Brazil (16°40'S, 49°15'W and 750 m a.s.l.). The region has an Aw climate in the KÖPPEN-GEIGER classification (Cardoso *et al*., 2014) and is characterized by a tropical climate with a rainy season from October to April and a dry period with less than 100 mm of monthly rainfall from May to September. Mean monthly temperature varies from 20.8°C in June and July to 25.3°C in October (Cardoso *et al*., 2014).

We calculated the water balance extract, potential and crop evapotranspiration (Fig. 1A) with the methodology of Rolim *et al*. (1998), and we used different Kc values in the different crop developmental periods according to Arf *et al*. (2012). We obtained temperature, relative humidity, and precipitation during the study through data gathered from a meteorological station located 100 m from the experimental area (Fig. 1B).

Soil at the experimental area was classified as a LATOSSOLO VERMELHO (Santos *et al*., 2013) with the following characteristics: Ca^{2+}: 4.30 $cmol_c$ dm^{-3}, Mg^{2+}: 1.80 $cmol_c$ dm^{-3}, K^{+}: 170.00 mg dm^{-3}, P (Mehlich I): 47.00 mg dm^{-3}, organic matter: 16.00 g dm^{-3}, Al^{3+}: 0.0 $cmol_c$ dm^{-3}, H+Al: 4.00 $cmol_c$ dm^{-3}, pH ($CaCl_2$): 5.40, CEC: 10.53 $cmol_c$ dm^{-3}, V: 62.00% (Donagemma *et al*., 2011). The granulometric analysis of the soil showed 4800 g kg^{-1} of clay in the 0-0.20 m layer (Silva, 2009).

The experiment was carried out in randomized blocks, with a 2x4 factorial scheme. The treatments were defined

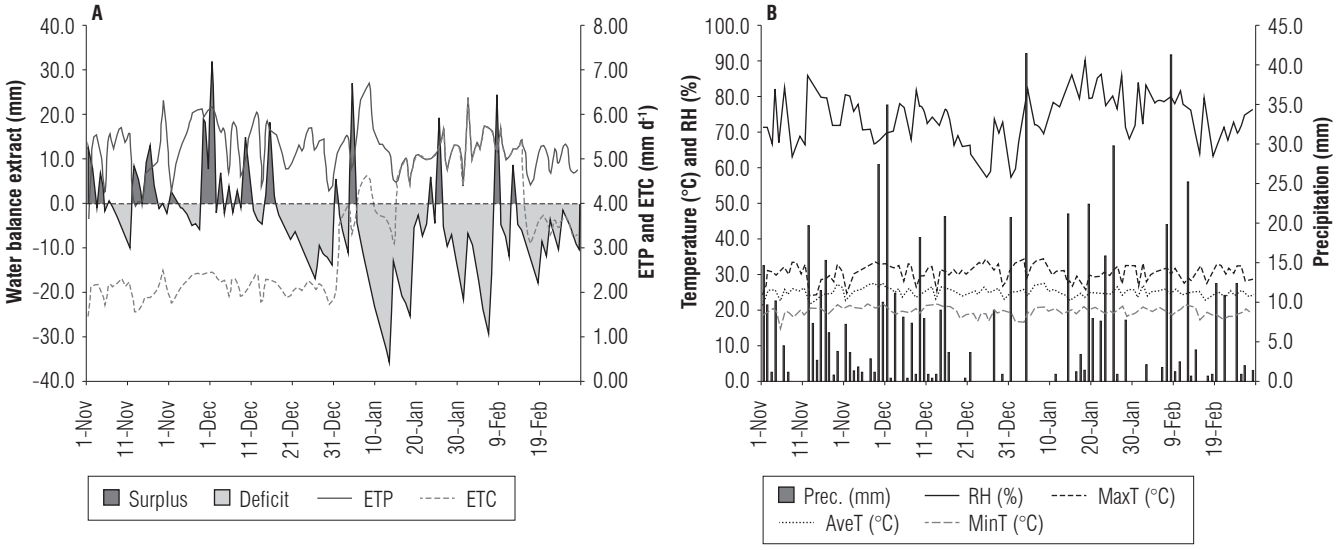

FIGURE 1. Water balance, potential evapotranspiration (ETP), crop evapotranspiration (ETC) (A), precipitation (Prec.), relative air humidity (RH), maximum (MaxT), average (AveT), and minimum (MinT) temperature (B) during the experiment.

by a combination of two seed soaking times (12 and 24 h) at four concentrations (0, 100, 200 and 300 mg L^{-1}) of niacin solutions (100% a.i. nicotinic acid) distributed in five replicates. Each experimental plot had an area of 0.20 x 0.20 m in high blue density polyethylene recipients with a capacity of 140 L and dimensions 0.21 m x 0.51 m x 2.16 m.

We applied the treatments by soaking seeds of upland rice (cv. BRS Primavera) in 100 ml solutions containing the niacin concentrations during the pre-established periods. For the control, the seeds were immersed in distilled water for the same pre-established periods. After soaking, we removed the seeds with a plastic sieve and placed them for drying on paper towels for 30 min in a shaded environment at environmental air temperature.

Sowing in the plots occurred on October 28, 2016, when we arranged twenty seeds in two lines, at a depth of 3 cm. There were no pest occurrences or pests requiring chemical control. We carried out single cover fertilization in stage V4 when we applied nitrogen using urea (46%) at a dose equivalent to 40 kg ha^{-1}. We irrigated daily up to 10 d after planting, and after this period watering occurred naturally from rains. We used natural water restriction to create a stress environment.

We performed daily counting of seedlings from the fourth day after sowing, to obtain seedling emergence rates (Eq. 1), mean emergency times (Eq. 2) (Labouriau, 1983) and the emergency speed index (Eq. 3) (Maguire, 1962), using the following equations:

$$EME = \frac{E}{S} \quad (1)$$

where:

EME: seedling emergence (%).

E: total emerged seedlings.

S: total seeds sown.

$$AET = \frac{(E1T1 + E2T2 + \cdots + EnTn)}{N} \quad (2)$$

where:

AET: average emergence time (days).

E1, E2, and En: number of emerged seedlings on the first, second and last count.

T1, T2, and Tn: first, second and last day of evaluation.

N: number of emerged seedlings during the test.

$$ESI = \frac{E1}{T1} + \frac{E2}{T2} + \cdots + \frac{En}{Tn} \quad (3)$$

where:

ESI: emergence speed index.

E1, E2, and En: number of emerged seedlings on the first, second and last count.

T1, T2, and Tn: first, second and last day of evaluation.

We performed the first evaluation of the vegetative development when the plants were in the V4 stage. We selected three plants per plot to obtain height, number of tillers,

number of leaves, and fresh and dry weight of the shoots. To obtain the dry weight of the shoots, we conditioned the plants in paper bags, followed by drying in a forced air circulation oven (SL-102/336, Solab, Piracicaba, SP, Brazil) (65°C) until we obtained a constant weight.

After the first evaluation, we removed excess plants so that only three plants per plot remained until harvest time, 100 d after sowing. On harvest day we measured the relative indices of chlorophyll a, b and total chlorophyll with a portable chlorophyll meter (CFL1030; Falker, Porto Alegre, RS, Brazil) as well as the biometric characteristics of height and dry plant weight. By obtaining the number of tillers and panicles, we estimated the values of these characteristics for an area of 1 m^2. Finally, we obtained the number of spikelets per panicle, fertility of the spikelets and the weight of 1000 grains.

We subjected the data for soaking times to an analysis of variance (test F) and when significant we used the Tukey test at 5% of probability ($P<0.05$). For the data related to the niacin concentrations, we used a polynomial regression analysis, adopting the significant regression of a greater degree.

Results and discussion

We observed three periods of marked water deficit between the intervals from December 17 to 30, January 05 to 13, and January 29 to February 06, when precipitations of 13.8 mm, 0.8 mm and 4.0 mm occurred (Fig. 1). During these periods we observed the morphological response of leaf wilting.

We observed no interaction between the two studied factors (soaking time and niacin concentration). However, we did observe different responses in plant characteristics according to the treatments applied.

During the emergence of the rice seedlings, we observed that only the emergence speed was affected by the soaking periods, regardless of the niacin concentrations (Tab. 1). The average time of emergence was lower when the seeds were submerged for a period of 24 h. We observed an inverse behavior for the emergence speed index; it was higher for submerged seeds during this same period. Thus, seed immersion for a 24 h period, regardless of niacin concentrations, decreased the average emergence time by 3.70% and increased the emergence speed index by 13.38%.

The variable factors of immersion time and niacin concentrations did not affect biometric characteristics during the initial development of the rice plants (Tab. 2). The results may be associated with good moisture conditions in the early stages of development, since we irrigated daily until 10 d after sowing; and after that period there were daily rains with a total of 244.10 mm.

TABLE 1. Mean values of characteristics related to the emergence of rice plants subjected to different seed soaking times and concentrations of niacin.

Soaking	FC	EMER	AET	ESI
	(%)	(%)	(d)	-
12 h	11.45	70.00	5.07a	2.84b
24 h	13.30	76.50	4.89b	3.22a
LSD	1.86	8.28	0.16	0.37
		Niacin (mg L^{-1})		
Control	14.00	80.00	5.01	3.27
100	11.30	71.00	5.05	2.90
200	13.40	74.00	4.83	3.13
300	10.80	68.00	5.01	2.80
L.R.	ns	ns	ns	ns
Q.R.	ns	ns	ns	ns
CV%	23.25	17.44	4.89	18.68

Common letters in a column indicate means are not significantly different at $P<0.05$ according to the Tukey test. FC: First Count; EMER: Emergence Index; AET: Average Emergence Time; ESI: Emergence Speed Index; L.R.: Linear Regression; Q.R.: Quadratic Regression; LSD: Least Significant Difference; CV: Coefficient of Variation.

TABLE 2. Mean values of biometric characteristics of rice plants in V4 stage subjected to different seed soaking times and concentrations of niacin.

Soaking	HEI	TN	LN	FW	DW
	(cm)	-	-	(g)	(g)
12 h	11.29	1.01	3.75	1.08	0.89
24 h	11.17	0.95	3.60	1.05	0.89
LSD	0.69	0.33	0.20	0.18	0.14
			Niacin (mg L^{-1})		
Control	11.39	0.95	3.70	1.02	0.90
100	11.54	1.08	3.60	1.15	0.90
200	10.66	0.80	3.65	0.90	0.81
300	11.35	1.10	3.75	1.20	0.93
L.R.	ns	ns	ns	ns	ns
Q.R.	ns	ns	ns	ns	ns
CV%	9.42	51.39	8.43	25.32	24.09

HEI: Height; TN: Tiller Number; LN: Leaf Number; FW: Fresh Weight; DW: Dry Weight; L.R.: Linear Regression; Q.R.: Quadratic Regression; LSD: Least Significant Difference; CV: Coefficient of Variation.

The good soil chemical conditions observed through laboratory analysis, in which high levels of P, K, and organic matter were found, were sufficient for the plants for proper

development up to the V4 stage. In a study carried out to verify the responses of different upland rice cultivars to nitrogen fertilization, Cancellier *et al*. (2011) verifies that the cultivar used in the present study is responsive to low levels of nitrogen in the soil.

During the final evaluation, we observed that the applied treatments did not significantly influence the biometric characteristics of height and dry mass of the plants. The averages obtained for these variables were 11.23 cm and 0.89 g, respectively, and these averages were lower than those obtained in different studies with upland rice, cv. Spring (Cancellier *et al*., 2011; Terra *et al*., 2015).

Water stress due to lack of water affects the vegetative development of upland rice plants (Terra *et al*., 2015). The partial suppression of water supply to plants can trigger a series of morphological and physiological changes. Plants subjected to this type of stress show a decrease in relative intracellular water contents resulting in a decrease in photosynthetic capacity and gas exchange. Consequently, plants have smaller amounts of energy reserves and inadequate vegetative and reproductive development (Awasthi *et al*., 2014; Zhou *et al*., 2017).

For the relative indices of chlorophyll a, b, and total chlorophyll, we observed that immersion of the seeds for a period of 12 h, regardless of the concentration of niacin, provided values of 16.57%, 15.49% and 16.39% higher than plants from seeds submerged for 24 h (Tab. 3).

TABLE 3. Mean values of the characteristics of rice plants subjected to different seed soaking times.

Soaking	RICA (FCI)	RICB (FCI)	RITC (FCI)	TN (per m^2)	PAN (per m^2)
12 h	27.02a	4.92a	31.95a	150.00b	89.58b
24 h	23.18b	4.26b	27.45b	162.92a	119.79a
LSD	1.88	0.50	2.29	9.28	16.03
CV%	11.54	16.88	11.89	9.16	23.64

Common letters in a column indicate means are not significantly different at $P<0.05$ according to the Tukey test. RICA: Relative Index of Chlorophyll a; RICB: Relative Index of Chlorophyll b; RITC: Relative Index of Total Chlorophyll; TN: Tiller Number; PAN: Panicle Number; FCI: Falker Chlorophyll Index; LSD: Least Significant Difference; CV: Coefficient of Variation.

However, for the number of tillers and the number of panicles per m^2, seeds soaked for a period of 24 h increased these characteristics to 8.61% and 33.72% respectively, compared to the plants obtained from 12 h of soaking (Tab. 3). According to Franzin *et al.* (2007), the immersion of upland rice seeds in water for periods of 16 to 24 h can significantly increase the formation of normal and more highly developed plants. This is due to the favoring of complementation between stages I and II of hydration, required for the distribution and translocation of nutrient reserves present in the endosperm of the embryo (Franco *et al*., 1997). Initial establishment of plants is important, since malformed plants will have difficulties during their development, especially in environments affected by stress factors.

The relative indices of chlorophyll a and total chlorophyll were affected by niacin concentrations (Fig. 2). We observed the increase of these contents up to the maximum concentrations of 208.00 and 205.91 mg L^{-1}, with FCI indices of 26.79 and 31.67, respectively. At that point, the increase reached 21.24% and 20.72% of the relative indices of chlorophyll a and total chlorophyll, compared to the control treatment.

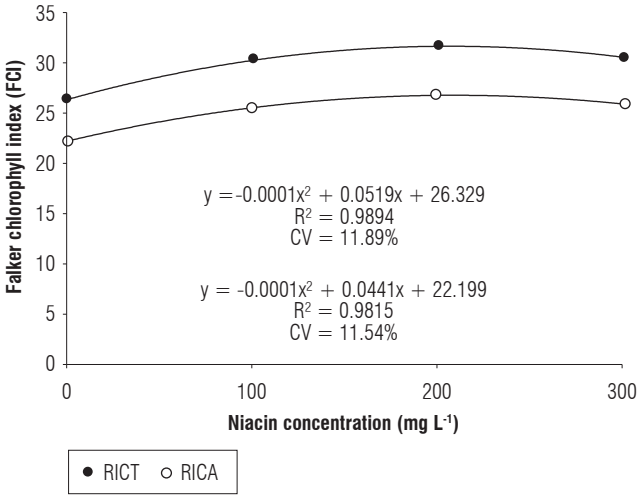

FIGURE 2. Relative chlorophyll a (RICA) and total (RICT) index in rice plants subjected to different concentrations of niacin by soaking seeds.

The results are connected to the exogenous application of niacin, which can favor plant development in environments under the influence of abiotic factors. This can be due to the fact that this vitamin has interactions with plant hormones, acting as a factor in enzymatic activation and participating in the composition of elements of the photosystem, such as NADP, used in atmospheric carbon fixation (Oertli, 1987; Hassanein *et al*., 2009; Taiz *et al*., 2017). The use of this vitamin may culminate in an increase of energetic and nutritional reserves and also contributes to vegetative and productive developmental characteristics, as in the case of wheat plants (El-Bassiouny *et al*., 2014).

We also observed the effect of niacin for the productive elements of upland rice, in which the increase of niacin concentrations increased the number of spikelets per

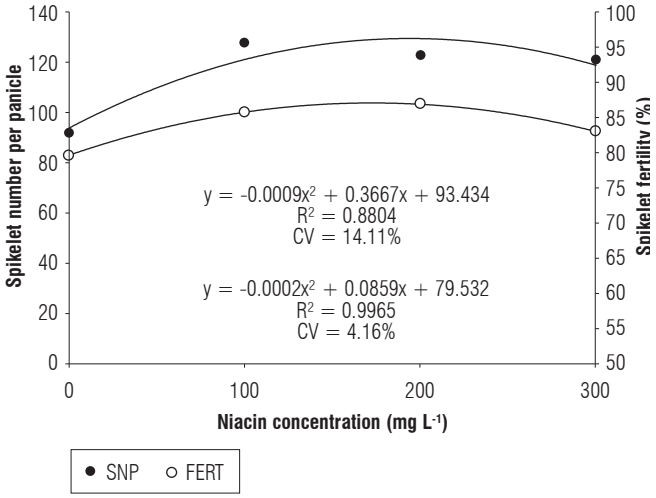

FIGURE 3. Spikelet number (SNP) and fertility (FERT) per panicle in rice plants subjected to different concentrations of niacin by soaking seeds.

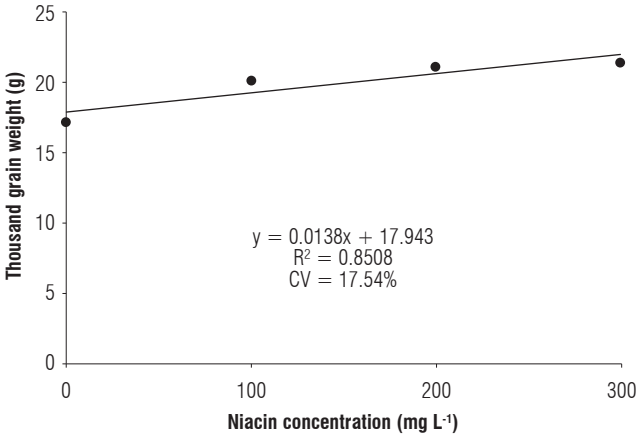

FIGURE 4. Weight of 1000 grains of rice plants subjected to different concentrations niacin by soaking seeds.

panicle as well as the fertility of the spikelets (Fig. 3), up to maximum values of 129.00 grains and 86.95% of spikelet fertility, obtained with the concentrations of 194.03 and 172.57 mg L^{-1}, respectively. The increase in relation to the control treatment was 41.37% of grains per panicle and 7.49% of fertile spikelets.

We found that increasing concentrations of niacin provided linear and positive responses on the weight of 1000 grains (Fig. 4). Thus, in the highest concentrations used during the study, we obtained with 300 mg L^{-1} an increase of 4.24 g/1000 grains or 24.58% compared to the control treatment, with rice seed immersion in water. The results might be caused by hormonal action, since the application of niacin stimulates an increase in the amounts of these compounds in the plant tissues (Hassanein *et al*., 2009; El-Bassiouny *et al*., 2014). The hormones have the capacity to mobilize different nutrients that can be used in the formation of new tissues of rice plants, besides contributing to normal functioning of the photosynthetic mechanism and the synthesis of proteins (Taiz *et al*., 2017).

We obtained similar results in studies with fava beans (Abdelhamid *et al*., 2013) and quinoa (Abdallah *et al*., 2016), where we obtained productive increments by increasing concentrations of niacin up to 400 and 100 mg L^{-1}, respectively, mainly in plants under the influence of abiotic stresses. These results demonstrate that, besides the use of niacin as a potential tool to combat the effects of abiotic stresses, the species factor and the form of application should be taken into account. Thus, we recommend further studies in order to obtain specific information to different growing conditions.

Conclusions

Soaking rice seeds for 24 h, regardless of the concentration of niacin, increases the speed of emergence of seedlings and the number of tillers and panicles per area. However, it decreases the relative indices of chlorophyll in leaves.

The use of niacin promotes gains in the number of spikelets per panicle, fertility of the spikelets and the weight of 1000 grains, besides increasing the relative index of chlorophyll in the leaves of the rice plants.

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An analytical model to evaluate the performance of associative seed producer organizations in the framework of Plan Semilla - Agrosavia (Colombia)

Un modelo analítico para evaluar el desempeño de las empresas asociativas productoras de semilla en el marco del Plan Semilla - Agrosavia (Colombia)

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ABSTRACT

Low access and use of quality seeds limit agricultural competitiveness. Since 2013, the Corporacion Colombiana de Investigacion Agropecuaria -Agrosavia- initiated "Plan Semilla" with the aim of consolidating nuclei of quality seed producers under associative schemes that guarantee quality seed supply in the regions where the seeds will be used. Between 2013 and 2016, we undertook characterizations of the organizations participating within the framework of Plan Semilla using various qualitative tools for their diagnostics. However, it was not possible to specify the actions that needed to be taken in order to strengthen these organizations. The aim of this research was to generate an analytical model to evaluate the performance of participating organizations that would establish quality seed production nuclei and to validate the model's use in those organizations that produce cocoa seed in the Plan Semilla framework. An analytic hierarchy process (AHP) was used to construct the model, which is composed of 4 dimensions (technical capacities, environmental resources, organizational capacities, and management capacities) that are related to criteria that are considered decisive for the consolidation of nuclei of quality seed producers. The model was assessed by 11 experts who identified the importance weight of the elements. In the validation, we used indicators from 30 cocoa seed producer organizations participating in Plan Semilla. We calculated additive utility functions and used a cluster analysis to define the thresholds and to establish the level of performance of the organizations. The results have improved the procedural rationality for the classification of organizations that seek to consolidate quality seed production nuclei.

Key words: quality seed production nuclei, analytical hierarchy process (AHP), organizational performance, organizational typification, cocoa.

RESUMEN

El bajo acceso y uso de semillas de calidad limita la competitividad agrícola. Desde 2013, la Corporación Colombiana de Investigación Agropecuaria inició el Plan Semilla, buscando la consolidación de núcleos de productores de semillas de calidad bajo esquemas asociativos, a fin de garantizar la oferta de semilla de calidad en los territorios para ser empleada principalmente por productores de agricultura campesina y familiar. Entre 2013 y 2016 en el marco del plan se realizaron caracterizaciones usando diversas herramientas cualitativas para el diagnóstico de las organizaciones, sin lograr precisar las acciones a emprender para el fortalecimiento de las mismas. El objetivo de esta investigación fue generar un modelo analítico para evaluar el desempeño de las organizaciones asociativas que establecen núcleos de producción de semillas de calidad y validar su uso en aquellas que producen semillas de cacao en el marco de Plan Semilla. Se usó el proceso de análisis jerárquico (AHP) para construir el modelo, el cual está compuesto por cuatro dimensiones (capacidades técnicas, recursos ambientales, capacidades organizativas y capacidades de gestión) relacionadas con criterios que se consideran determinantes para la consolidación de los núcleos; para la estimación del modelo se sometió a consulta de 11 expertos logrando identificar el peso de la importancia que se atribuye a sus elementos. En su validación, se usaron indicadores de 30 organizaciones de productores de semilla de cacao participantes en el Plan Semilla; se calcularon funciones de utilidad aditiva y se realizó un análisis clúster para definir los umbrales, lo que permitió establecer el nivel de desempeño de las organizaciones. Los resultados obtenidos han permitido mejorar la racionalidad procedimental para la tipificación de organizaciones que aspiran a consolidar núcleos de producción de semilla de calidad.

Palabras clave: núcleos de producción de semilla de calidad, proceso de análisis jerárquico (AHP), desempeño organizacional, tipificación de organizaciones, cacao.

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Introduction

In Colombia, small agricultural producers face low-quality seed availability for the development of their productive activity (Arenas *et al*., 2015). This, together with a reduction of agrobiodiversity, a dependence on improved genetic resources with spontaneous flows, the existence of informal seed markets as well as the low adoption of technologies, besides other factors, have created increased risk and uncertainty in national agricultural production (Martín, 2001).

Since 2013, the Corporacion Colombiana de Investigacion Agropecuaria (Agrosavia, formerly known as Corpoica) signed a proposal called "Plan Semilla" (Seed Plan) within the framework of a public policy. This proposal seeks the consolidation of seed producer nuclei under associative schemes in different regions of Colombia that will guarantee a supply of quality seed and the adequate and timely availability of seeds and plant materials with genetic, physiological and sanitary quality. The aforementioned will allow, in the short term, the strengthening and competitiveness of agricultural production systems in Colombia, with the aim of supplying seeds to farmer families and community agricultural producers who traditionally do not have easy access to these necessities (Corpoica, 2015).

In the implementation of the Plan, which seeks to establish formal systems that guarantee the production of certified or selected quality seed, provisions of the Resolution 3168 of 2015 of the Colombian Agricultural Institute (ICA, for its acronym in Spanish) have been followed. ICA regulates and controls the production, import and export of genetically improved seeds. ICA also supervises seed commercialization and planting and maintains a registry of agronomic evaluation and/or research units in plant breeding, for the country.

Since its inception, about 291 producer organizations in various regions of the country (which are related to the production of 22 species important in Colombian agriculture) have participated in some of the phases of the plan (Corpoica, 2015). Among the actions carried out within the framework of the plan between 2013 and 2016, has been the collection of information from participating organizations for carrying out an organizational diagnosis. To carry out this analysis technical-productive, social-business, commercial and organizational variables were used. The main approach used for the diagnosis was qualitative-descriptive and tools were designed incorporating various elements proposed in other methodologies. Among the elements that stand out are the Link methodology of inclusive business models for small farmers of the International Center for Tropical Agriculture (Lundy *et al*., 2014), the Canvas business model (Osterwalder and Pigneu, 2010), the organizational capacities index (ICO, for its acronym in Spanish) (MADR, IICA, BM, 2003; Rivas, 2013) and the organizational state assessment (VEO, for its acronym in Spanish) (USAID, 2008). These methodologies are important as variables related to the business model, the value chain, the prototype cycle, and inclusive business (Lundy *et al*., 2014). They are also related to services offered within the organization, participation, and democracy in the decision-making process, the economic and financial situation, the management and administrative capacities, and human development capabilities (MADR, IICA, BM, 2003; USAID, 2008; Rivas, 2013). These methodologies are also linked to productive, commercial, financial, administrative, and organizational capacities (MADR, 2011) and to the criteria related to the specific environment in which business deals are established (Báez, 2010). The above-mentioned elements were incorporated into the information collection instruments that were applied to the organizations participating in the seed plan. In addition, criteria related to technical variables that demand the production of quality seeds were included (Corpoica, 2013, 2014, 2015). These criteria were subsequently used in the methodology implemented for categorizing these organizations using qualitative scales designed for that purpose (Corpoica, 2015). However, during the progress made within the framework of Plan Semilla for the characterization of organizations, it has not been possible to find a robust and complete methodology that allows identifying the weaknesses of the organizations for achieving their classification according to their degree of performance.

Due to the fact that the models used by Corpoica incorporated generic criteria to promote business in organizations of agricultural producers, and because they were not designed specifically to establish the degree of capacities or performance of the associative organizations dedicated to specialized quality seed production, the scales and procedures used for their weighting and prioritization tend to be subjective and are based on the interpretation of the data obtained from the information collection stages. The obtained results have a limit for clearly determining the necessary actions to be carried out in order to strengthen areas with low performance in these organizations. Consequently, the models face the risk that the associative organizations might fail and that the quality seed production nuclei could have low sustainability, which could compromise the success of the plan.

The benefits attributed to associations in rural areas are numerous. Some of these benefits include the contribution associations make to improve product prices, achieve scale economies that allow the reduction of production costs, and to facilitate market entry and access to the support granted by sectoral public policies (training, technical assistance, value addition to the product), among other things (Machado, 2000; Moyano, 2006; Pérez *et al*., 2014).

In Colombia, agricultural producers, in their eagerness to access aid and support incentives granted by public policies, usually are organizations that, in many cases, do not turn out to be functional (Aristizabal, 2017; Gómez *et al*., 2017). Among these small-farmer organizations, the low empowerment of their members as well as the low developmental capabilities of collective actions stand out (Parrado *et al*., 2009). This situation means that the impact and contributions attributed to organizations for the improvement of living conditions of rural societies are low (Rondot and Collion, 2001), thus, perpetuating the dependence of rural producers on the institutional system (Mora and Sumpsi, 2004).

From specialized literature, some of the elements that explain the low success of producer organizations in the agricultural sector can be highlighted. These elements include, among others, the low capacity for collective action development (Machado, 2000). This situation does not contribute to the execution of plans or programs developed by groups (Olson, 1971) that prevent small-farmer organizations from reaching their outlined objectives. Ostrom and Ahn (2003) explain that the success of collective action depends on social capital, defining it as "the sum of real or potential resources that are linked to the possession of a lasting network of knowledge relations and mutual recognition - affiliation to a more or less institutionalized group that provides each of its members with the support of socially acquired capital". Bourdieu (1986) states that collective action is facilitated by "the existence of a set of shared norms and values", which delimit the social structure and determine the networks that exist by actors that cooperate and share their resources to pursue common interests (Coleman, 1990). In recent years, the production of cocoa in Colombia has gained considerable importance. It has been promoted by different institutions as a productive alternative for the national territories. Cocoa has a soil use vocation and there are adequate biophysical conditions for its development as a substitution of illicit crops. This could contribute to improve the quality of life and generate a decent income for the agricultural producers of these areas (Abbott *et al*., 2018).

A recent study of zoning for commercial cocoa cultivation performed by the Unit of planning of rural lands, land adaptation and agricultural uses (UPRA, 2016) identified 21.3 million ha with an aptitude for cocoa crops in the country. In addition, the national government and the cocoa producers' union -Fedecacao- have planned to increase the area planted with cocoa crops (Nuñez *et al*., 2017). In the last years, 57% of producers have renewed their crops through public policy actions executed principally in post-conflict territories (Abbott *et al*., 2018).

In 2017, cocoa production reached 60.535 t (Agronet - MADR, 2018). This production volume has been obtained because producers are implementing technological packages and materials that have been the outcome of research for several years that have resulted in more productive and healthy crops (tolerant to main pests and diseases) (Fedecacao, 2017).

It is important to be aware that quality seeds are required to achieve the goals set in the Plan Semilla. Ensuring an adequate seed supply (available and timely volumes of plant material) with physiological, genetic and phytosanitary quality will allow the strengthening of seed production systems by small-farmer organizations.

The aim of this research was to generate an analytical model to evaluate the performance of associative organizations that establish quality seed production nuclei and to validate its use in those organizations that produce cocoa seed within the Plan Semilla framework.

Materials and methods

In the first phase, we carried out a literature review in order to identify the theoretical approaches, methodologies, and tools used for the diagnostics of small-farmer associative organizations in the agrarian sector; through this review, we identified some elements that affect the success of the agrarian organizations. The collective action logic (Olson, 1971; Ostrom and Ahn, 2003; Gordon, 2005; Payne, 2016) and some tools used for organizational diagnoses such as IDEO, ICO, Canvas, and RUTA were highlighted. We analyzed the tools that Corpoica (2015) used for characterization of the organizations that participated in the previous phases of Plan Semilla and we reviewed the variables and indicators implemented by the producer organizations that have participated in the plan.

In the second phase, for an analytical model that evaluates the performance of small-farmer organizations, the

Analytical Hierarchy Process -AHP- (Saaty, 1980) and the most commonly used multi-criteria decision making methods -MCDM- (Velasquez and Hester, 2013), related with the Multi-Attribute Utility Theory methods -MAUT- (Fishburn, 1967; Keeney and Fishburn, 1974; Keeney, 1977) are useable methods. These methods are widely used to analyze and resolve problems of multiple scenarios, characters, and criteria providing alternatives (Moreno-Jiménez, 2002). The AHP has been frequently used in recent years (Ho, 2008; Sipahi and Timor, 2010). Its main applications for agricultural issues include: various assessments of sustainability of agricultural systems (Parra-López *et al*., 2008; Rezaei-Moghaddam and Karami, 2008; Veisi *et al*., 2016), an analysis of extension systems (Allahyari, 2009), and decision-making related with agriculture in the developing countries (Alphonce, 1997) and producer organizations in the agricultural sector (Aranda-Camacho *et al*., 2014).

The AHP allows modeling the problem by recognizing hierarchical relationships of a set of elements (dimensions, criteria, and subcriteria) of a qualitative and quantitative nature. These are logically arranged allowing different people or groups of interest to participate in analysis and assessment (Forman and Selly, 2001). To assess a model, the consulted participants comparatively judge each pair of elements belonging to the same node and level of the hierarchy using a common scale (Saaty, 1980). Through the aggregation of individual judgments (AIJ) (Forman and Peniwati, 1998) it is possible to estimate local weights attributed by the members of the evaluating group. This evaluation defines the relative importance of the elements belonging to the same node and level in the hierarchy of the model. Then, after the synthetic process, it is possible to estimate global weights that show the relevance of each element compared to all the others that comprise the model. Likewise, by using the AHP method, it is possible to verify the consistency of judgments and perform sensitivity analyses (Pacheco and Contreras, 2008). This improves the procedural rationality used for the analysis of the decisions of the studied problem (Velasquez and Hester, 2013).

The model incorporating determinant elements in the design of inclusive businesses and competencies of organizations of the agrarian sector (previously commented in the introduction) was designed following proposals of the AHP. This first model was submitted to seven experts (academics and researchers with experience in technical issues of seed production, agrarian economics, innovation and organizations of agricultural producers) to verify the pertinence of the model's elements. The recommendations of the experts were incorporated into the model for its adjustment. Elements were subsumed, debugged and organized, complying with the properties of completeness, operability, decomposability, non-redundancy and minimality that are required for modeling (Saaty, 1980; Aranda, 2015).

The goal at the first level of the analytical model was to establish the capacities and resources available for the organization in order to consolidate itself as a quality seed production nucleus. In the second level of the model's hierarchy, there are four dimensions (technical capacities, environmental resources, organizational capacities, and management capacities) that condition the consolidation of seed production nuclei of associative organizations, and these have 12 criteria and 39 sub-criteria relating to these four dimensions (Fig. 1).

Phase 3 consisted of the evaluation stage: we designed a questionnaire and a glossary with the definitions of the elements of the model and their respective graphic representation. For the estimations of the model, we consulted a group of 11 experts with specific knowledge of seed production, the agricultural and rural organizations, and agrarian technical assistance (managers, academics, researchers and professionals who provide technical and organizational assistance). These experts provided comparative value judgments between pairs of elements belonging to the same node and model level, using the Saaty (1980) scale. Before prioritization and synthesis, we used the AIJ technique (Forman and Penniwati, 1998) to build matrices of group judgments, from which we estimated the importance weights of each of the elements of the model. We first estimated the local importance weights, and after their synthesis, we obtained weights of global importance. Then, we verified the inconsistency index. We processed the data using the Expert Choice software version 11.

In the fourth phase, we used information from 30 cocoa seed producer organizations participating in Plan Semilla in order to validate the application of the model. These organizations are located in the provinces of Santander (8 organizations), Antioquia (4 organizations), Huila (6 organizations), Tolima (1 organization), Nariño (1 organization), Norte de Santander (3 organizations), Cesar (1 organization) and Boyaca (6 organizations). We obtained information of small-farmer organizations that implemented cocoa seed quality productive nuclei through the compilation of the instruments applied by Agrosavia between 2013 and 2015. Based on this information, we obtained indicators relating to the sub-criteria of the last model level.

To establish the degree of performance of organizations, we estimated the additive utility functions (Keeney and Raiffa, 1976; Fishburn, 1982) using the obtained information. First, we calculated a partial utility for each dimension, which was obtained as the product of the estimated global weight for each element of the last level (sub-criteria) and the value of the indicator related to these sub-criteria for each organization. Secondly, we calculated the total utility by adding the partial profits obtained previously. We repeated this procedure for each of the 30 organizations used as cases. These utility functions reflected the degree of performance of each of the evaluated organizations related to each one of the dimensions and the global performance.

To establish the ranges of the performance thresholds of the organizations as model decision alternatives, we performed a cluster analysis using the Ward method for the conglomeration. We used the Euclidean squared distance metric for both the partial utility of each dimension and also for the total utility. We used the value of the 5% cut-off average to obtain whisker plots with the respective upper and lower limits from which we established the ranges of the cut-off thresholds for three performance levels: high performance, average performance, and low performance. Once the performance levels with the utility values were established, we classified the organizations according to their performance. We analyzed the data with the software Statgraphics Centurion XVI and Excel.

Results and discussion

The AHP model for evaluating the performance of associative organizations that produce quality seeds

Based on the literature of various diagnostic methods of organizations and after a thorough analysis of the tools used within the framework of Plan Semilla (Corpoica, 2015), we identified the main criteria for characterizing and diagnosing seed producer organizations. Summarizing the above, it was possible to establish three dimensions constituted by 214 criteria and sub-criteria. Then, an exhaustive purification of these criteria was carried out, incorporating the comments and suggestions of the experts consulted in the first and second stages. Finally, we generated an analytical model including a main objective, four dimensions, 12 criteria and 39 sub-criteria (Fig. 1).

The first level of the model is the general objective to establish the level of capacities and resources available for the organization wishing to consolidate as a nucleus of quality seed production.

In the second level, four dimensions were found: (1) *Technical capacities* refering to the organizations linked to Plan Semilla that have the necessary knowledge and experience for the use, production, and management of good quality seed; (2) *Environmental resources,* the entire environmental conditions (soil, water, biophysical offer, etc.) that the territory in which the organizations are located have available for its use; (3) *Organizational capacities*, includes all the aspects that contribute to the personal and collective growth of the members of the association, highlighting characteristics that lead to the success of an organization. This also involves all the necessary actions for achieving a better position, including being more competitive, achieving its objectives, goals, fulfilling commitments, ensuring its continuity and projecting the organization into the future; (4) *Management capacities*, those abilities of the organizations relating to the administration of their financial, administrative, and commercial talents. This also includes all those relationships that occur with external entities, for the purpose of creating partnerships that are beneficial to the organization, and knowing the capacities that they have to obtain resources that can be used as capital, projects dealing with production, among others.

In the third level of the model are found the criteria relating to each of the dimensions. Regarding the technical capacities dimension, the criteria are as follow: a) *Production process,* which covers the main activities carried out by organizations at the technical and environmental levels to be able to produce and manage seeds that meet quality standards; and b) *Production resources*, refering to all the physical and human resources available in organizations to carry out seed production processes.

The criteria related to the environmental resources dimension are as follow: c) *Environmental offering*, which corresponds to all natural or biophysical resources that are part of the territory where the organization is located (such as soil, water, humidity, temperature, among others), that comprise seed production activity; d) *Threats due to natural phenomena* defining natural threats as those elements of the environment that are dangerous to humans and that are caused by external forces.

The organizational capacities dimension includes criteria such as e) *Social and relational capital,* that refers in particular to the degree of trust between members including the unlimited exchange of information within the group; f) *Experience in the development of collective actions,* which starts from the recognition that as individuals, members do not have all the necessary information, resources, and

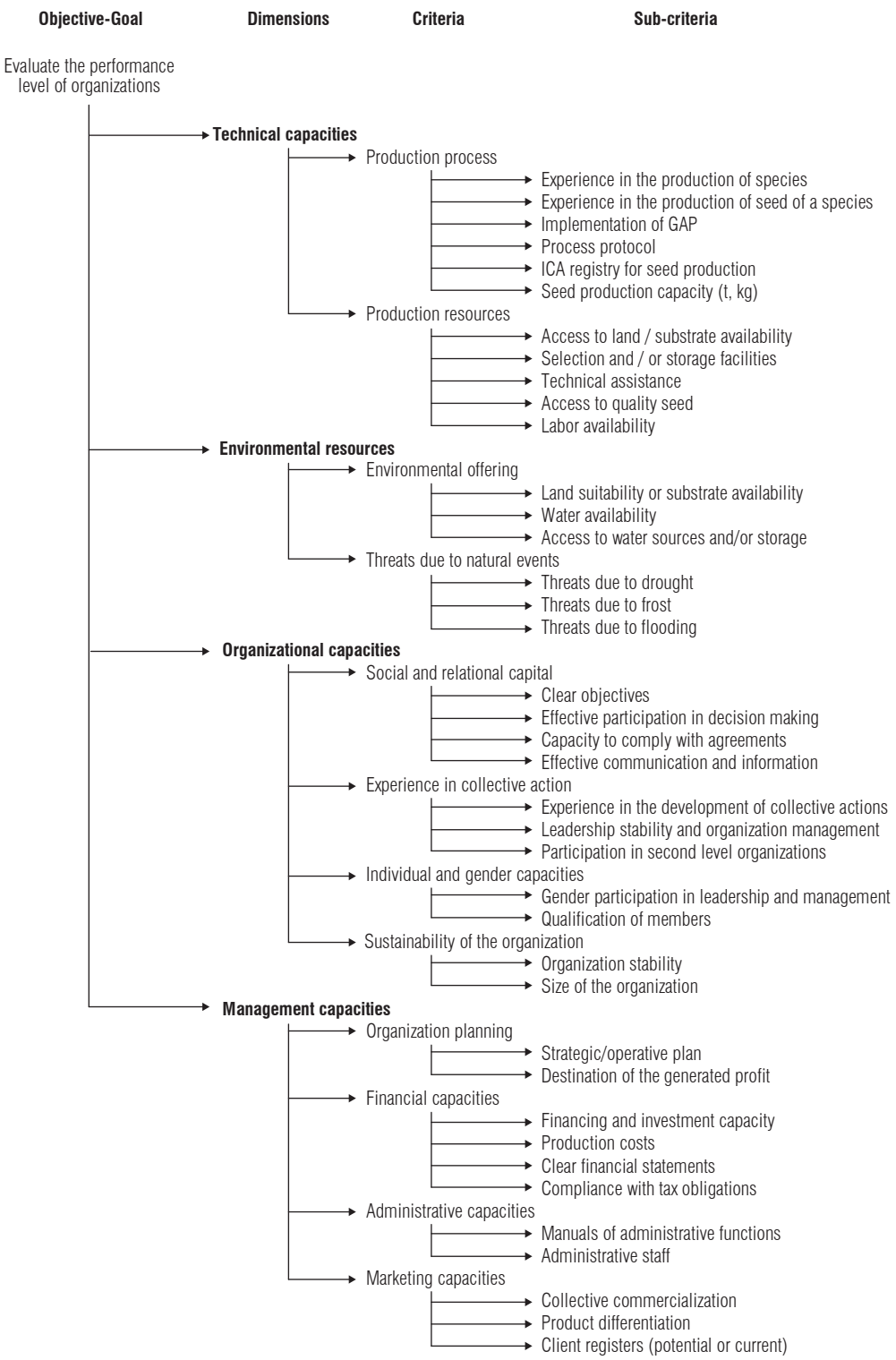

FIGURE 1. AHP model for establishing the performance of an organization to consolidate quality seed production nuclei.

competencies required to meet a specific demand; g) *Individual capacities and gender*, which identifies if rural organizations apply the gender approach to recognize the capacities of the organization through the educational level of its members; h) *Sustainability of the organization,* the composition and functioning of the organization, in which the participation of the associates in decision-making processes and the way these processes will be conducted are identified.

Finally, management capacities criteria include: i) *Organization planning,* the organizations linked to Plan Semilla establish goals and stipulate the steps that must be followed to achieve these aims; j) *Financial capacities:* possibilities that organizations have to perform payments and investments (caused by good profit margins) in the short, medium and long terms, for their development and growth; k) *Administrative capacities,* related to the management staff that the organization has, as well as the way in which economic resources are managed and invested; l) *Marketing capacities:* strategies that will be used to sell a certain product, and in the end, the desired profitability that is achieved.

Nonetheless, sub-criteria are included in the fourth and final level (Fig. 1), and verifiable indicators are related to each of these sub-criteria, which allow the establishment of the performance of the organizations.

Estimation of priorities of the elements of the model based on the preferences of the consulted experts

Table 1 summarizes the local weights (W_L) and global weights (W_G) associated with the dimensions, criteria, and sub-criteria of the hierarchical model. In all cases, the consistency index was less than 10%, showing the logical consistency of the weights obtained in the estimation. Likewise, the ranking of the weights of each element is presented in relation to the level to which it belongs.

At a second level, the group of experts expressed that the dimension that had greater importance was *Environmental Resources* (W_G 0.27); second, *Technical Capacities* (W_G 0.25); third, *Organizational Capacities* (W_G 0.24), and, finally, the *Management Capacities* dimension (W_G 0.23) (Tab. 1). The group of experts has assigned a degree of similar importance to the four dimensions of the model, and in most cases, the difference is only 2 percentage points.

It is important to emphasize that in the previous methodological proposals used by Agrosavia to characterize organizations in Plan Semilla there was no dimension relating to environmental issues. In this research, the *Environmental Dimension* was incorporated in a novel way following the recommendation of the experts who evaluated the relevance of the model. The environmental component is fundamental for the development of productive activities in rural areas, due to conflicts in the access and use of natural resources such as soil and water; rural populations face vulnerability to the development of productive activities due to phenomena such as climate change, natural disasters and the risks inherent to agriculture (Muñoz *et al*., 2012).

TABLE 1. Weights of importance and ranking of priorities of the model elements based on the preferences declared by the consulted experts.

ELEMENTS OR NODES OF THE AHP MODEL	Priorities by node		Ranking of priorities		
Level 1 (Objective)					
Level 2 (Dimensions)					
Level 3 (Criteria)					
Level 4 (Sub-criteria)	**W_L**	**W_G**	**Dimension**	**Criteria**	**Sub-criteria**
Technical capacities	**0.253**	**0.253**	**2**		
Production process	0.537	0.136		2	
Experience in the production of the species	0.192	0.026			13
Experience in the production of seed of the species	0.218	0.03			9
Implementation of good agricultural practices - GAP	0.037	0.019			22
Process protocol	0.159	0.022			18
ICA registry for seed production	0.156	0.021			19
Seed production capacity	0.138	0.019			23
Production resources	0.463	0.117		4	
Access to land	0.179	0.021			20
Selection and/or storage facilities	0.165	0.019			24
Technical assistance	0.204	0.024			16
Access to quality seed	0.222	0.026			14
Labor availability	0.231	0.027			12

Continue...

Table 1. *Continuation*

ELEMENTS OR NODES OF THE AHP MODEL	Priorities by node		Ranking of priorities		
Level 1 (Objective)					
Level 2 (Dimensions)					
Level 3 (Criteria)					
Level 4 (Sub-criteria)	W_L	W_G	Dimension	Criteria	Sub-criteria
Environmental resources	**0.270**	**0.27**	**1**		
Environmental offering	0.488	0.132		3	
Land use suitability - TUT or substrate availability	0.311	0.041			7
Water availability	0.329	0.044			6
Access to water sources and/or storage	0.359	0.047			3
Threats due to natural events	0.512	0.138		1	
Threat due to drought	0.521	0.072			2
Threat due to frost	0.142	0.02			21
Threat due to flooding	0.337	0.047			4
Organizational capacities	**0.242**	**0.242**	**3**		
Social and relational capitals	0.232	0.056		10	
Clear objectives	0.412	0.023			17
Effective participation in decision making	0.156	0.009			38
Capacity to comply with agreements	0.274	0.015			30
Effective communication and information	0.158	0.009			39
Experience in collective action	0.239	0.058		9	
Experience in the development of collective actions	0.517	0.03			10
Leadership stability and organization management	0.298	0.017			27
Participation in second level organizations	0.185	0.011			37
Individual and gender capacities	0.170	0.041		12	
Gender participation in leadership and management positions	0.310	0.013			35
Qualification of members	0.690	0.028			11
Sustainability of the organization	0.359	0.087		5	
Organization stability	0.837	0.073			1
Size of the organization	0.163	0.014			33
Management capacities	**0.234**	**0.234**	**4**		
Organization planning	0.255	0.06		8	
Strategic/operative plan	0.749	0.045			5
Destination of the generated profit	0.251	0.015			31
Financial capacities	0.272	0.064		7	
Financing and investment capacity	0.270	0.017			28
Cost production registry	0.208	0.013			36
Clear financial statements	0.303	0.019			25
Compliance with tax obligations	0.219	0.014			34
Administrative capacities	0.187	0.044		11	
Manuals of administrative functions	0.425	0.019			26
Administrative staff	0.575	0.025			15
Marketing capacities	0.287	0.067		6	
Collective commercialization	0.526	0.035			8
Product differentiation	0.247	0.017			29
Client registers (potential or current)	0.227	0.015			32

Local weights (W_L) - Global weights (W_G).

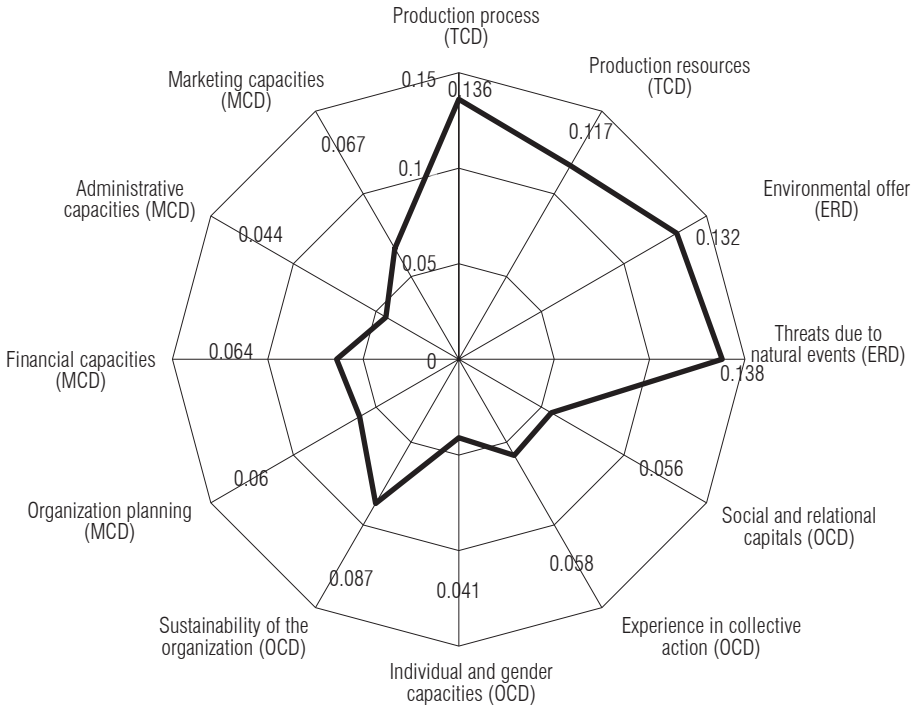

FIGURE 2. Results of Global priorities (W_L) at the level of criteria estimated in the AHP model. (TCD) - Technical capacities dimension; (ERD) - Environmental resources dimension; (OCD) - Organizational capacities dimension; (MCD) - Management capacities dimension.

With relation to the priorities obtained in the *Technical Capacities* dimension, the *production process* criterion (W_L 0.537) is considered the most important, whereas *production resources* obtained a W_L of 0.463. After the synthesis of the model at the level of the general objective, the *production process* ranked second taking into account all the model criteria (W_G 0.136), whereas *production resources* ranked fourth among 12 criteria in the third level of the model. Therefore, the organizations that focus on the production of quality seed must have adequate knowledge and experience in the production of both the species and the seed in order to ensure product quality.

Regarding the *Environmental Resources* dimension, the *threat due to natural phenomena* occupied the first place in the ranking of all criteria of the model (W_L 0.512; W_G 0.138). On the other hand, the *environmental offer* criterion was ranked, after the synthesis at the general objective level of the model, as the third criterion in order of importance (W_L 0.488, W_G 0.132). According to the consulted experts, the threat that agricultural producers face due to natural phenomena turns out to be an element of transcendental knowledge, in order to anticipate the occurrence of these phenomena and minimize the risk to which they are exposed through the implementation of measures for their mitigation. In this regard, FAO (2000) states that the vulnerability of production systems increases when agricultural activities use unfit areas or lands, or places that are at risk, or when natural resources are mismanaged. This is especially due to marginality, poverty, the absence of social organization, and above all to the lack of policies for the management of the environment. Additionally, territorial planning and the lack of education of the population is unable to prevent and face the risks surely plays an important role. Therefore, besides having access to adequate information on agroclimatic variables, producer organizations that establish nuclei of quality seed production should strive to training their members on issues relating to water management and, in particular, they should comply with soil use zoning according to their suitability for the species that they are producing in the particular locality (FAO, 2000).

At the *Organizational Capacities* dimension, the *sustainability of the organization* criterion stands out in fifth place among all criteria (W_G 0.087), whereas the *collective action experience* criterion ranked second in this group (W_L 0.239) and it ranked ninth (W_G 0.058) when performing the synthesis among all criteria. The foregoing indicates that one of the success factors of an organization is the strength it has to maintain itself through time under different circumstances (Lundy *et al.*, 2014). According to the preferences declared by the experts, it is strange that

collective action, *social and relational capital*, and *individual capacities and gender* have been classified among the last elements at the criteria level. This may be due to the technical profile of some of the consulted experts who did not completely categorize the importance of these elements for establishing collective initiatives around the associated companies producing quality seeds. The *social and relational capital* criterion (W_L 0.232, W_G 0.056) was ranked tenth in the ranking of all criteria of the model. This result contrasts with what was stated by Serageldin (1998), who presents empirical evidence on the relevance that social capital acquires for the development of collective actions by small agricultural producers. However, the results of the estimation of this model by the consulted experts reached no agreement about the particular ways in which social capital contributes to development and how it can be operationalized. The *individual capacities and gender* criterion is located in the fourth place (W_L 0.17, W_G 0.041) of the criteria associated with the *organizational capacities* dimension. This is the last criterion among all models that indicates low recognition of the importance of female participation in managerial roles and also low recognition of the importance of the capacities of the associates in general.

Finally, in the criteria related to the *Management Capacities* dimension, the experts attributed a greater weight to *marketing capacities* (W_L 0.287, W_G 0.067) and this places it at sixth place in the general level of the ranking of the criteria. This position acknowledges the importance attributed by experts for organizations to strengthen their management capacities to implement value-added strategies that help to clearly identify current and potential clients, for whom the products will be oriented. Nonetheless, the *financial capacities* criterion (W_L 0.272, W_G 0.064) ranked seventh among all criteria. The *Organization planning* criteria (W_L 0.06, W_G 0.044) was ranked eighth among all the criteria of the model. In the dimension of *management capacities*, the group attributed the least importance to the *administrative capacities* criterion (W_L 0.187, W_G 0.044) and it was classified in tenth place among the twelve criteria of the model. Among the previous criteria, *marketing capacities* stands out; in this respect Knickel *et al.* (2008), Schermer *et al.* (2010) and Anderson *et al.* (2014) showed empirical evidence on the importance of skills, knowledge and market competencies for agrarian producer organizations that seek to articulate agri-food systems and how these skills are becoming tools that facilitate community development. Additionally, these skills also ensure that producers have access to markets and participate in them in a more democratic way at multiple scales.

In Colombia, Aranda-Camacho and Parrado (2016) showed the importance of these capacities so that agricultural producers achieve the development of markets that favor food and nutritional security. This could apply to the specific field of quality seed production. Aranda-Camacho *et al.* (2017) detail some specific actions developed to strengthen capabilities, both individual and collective. These actions were implemented in a scaling-up project of technical innovations (three new cultivars of more nutritive yellow potatoes). The project had the participation of small-farmer organizations that established nuclei of quality seed production and developed inclusive businesses in localities in the south and center of the Andean region of Colombia (Cuéllar *et al.*, 2018).

The above-mentioned empirical evidence shows the importance of the fact that organizations must strengthen their management skills and knowledge of the market so that they can undertake the development of strategies that encourage added value to their products, directing specific promotion activities for current and potential customers. In order to guarantee the development of small-farmer organizations in the marketplace, it is important that seed producer organizations have adequate capabilities that allow the development of mutually beneficial collective actions (Cuéllar *et al.*, 2018). In spite of this, in our national context, the producers and their organizations have few capacities to successfully develop their commercial functions. In general, this conditions the efficiency of the commercialization of their products (which are sometimes excluded) or presents limitations for small producers to achieve associative ventures.

The priorities assigned by the consulted experts indicate that, in any organization, it is important to strengthen marketing and commercialization capacities. In order to do so, knowledge of the dynamics and structure of the market is fundamental, so that organizations can participate effectively in it. However, in the context of this article, commercialization is the part of the commercial process in which producers and their organizations are less trained. This affects the efficiency with which products are linked to the market. Thus, products are excluded and limitations arise for small producers to achieve sustainable associative ventures (Rodríguez and Fernandez, 1996).

To summarize, after estimating the model based on the preferences declared by the expert consultants, the most important criteria for establishing nuclei of quality seed production are the threats due to natural phenomena, the previous experience in the production of the species,

an adequate environmental offering, the availability of production resources and the sustainability of the organization. Conversely, the least important criteria are those related to organizational and management capacities such as social and related capital, administrative capacities, and individual capacities and gender.

In the fourth level of the model, the following sub-criteria stand out due to their placement in the ranking: the *stability of the organization*, *threats due to drought*, *access to water sources and/or storage*, *threats due to flooding* and the *strategic/operational plan*, all of which are located in the first five places, respectively. It is worth mentioning that three of the five most important sub-criteria are related to the *Environmental Resources* dimension, which was the one that obtained the highest degree of importance within the dimensions consulted by the experts.

Empirical validation of the model in associative organizations that establish nuclei of quality cocoa seed production

Using the estimated weights defined by the expert consultants of each sub-criteria related to the analyzed dimensions, we proceeded to calculate utility functions for each of the 30 associative organizations that implemented nuclei of quality cocoa seeds. We selected verifiable indicators related to the elements of the last level (sub-criteria), which we used to estimate utility functions as the Multi-Attribute Utility Theory (MAUT) (Fishburn, 1967; Keeney and Fishburn, 1974; Keeney and Raiffa, 1976; Keeney, 1977).

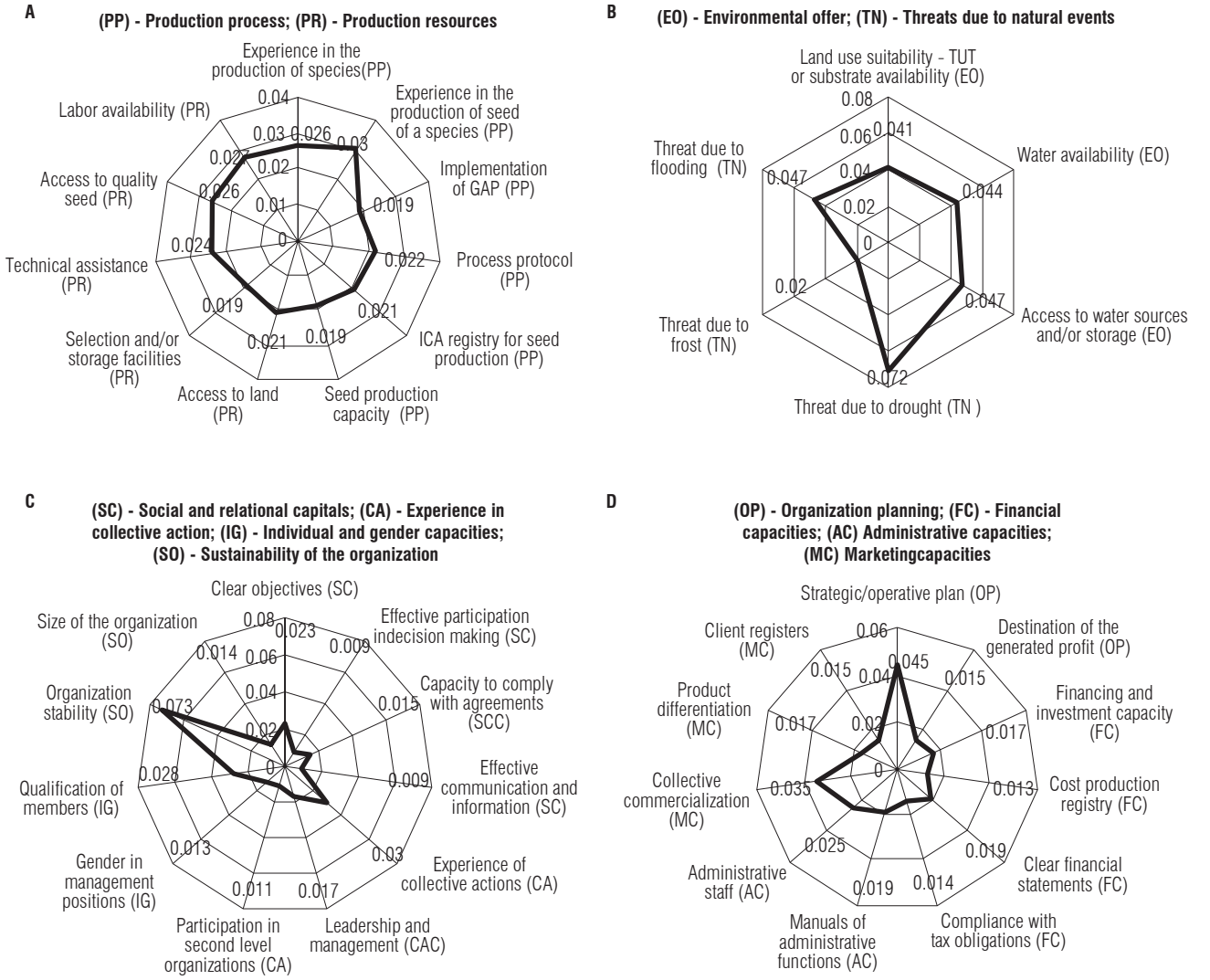

FIGURE 3. Results of Global priorities (W_G) at the sub-criteria level estimated for the AHP model. a) W_G of sub-criteria of the Technical Capacities Dimension; b) W_G of sub-criteria of the Environmental Resources Dimension; c) W_G of sub-criteria of the Organizational Capacities Dimension; d) W_G of sub-criteria of the Management Capacities Dimension.

The scale used to qualify each indicator in the 30 cases of cocoa seed producer organizations that had information was 0, 1 and 2 (i.e. low level (0) to a high level (2)).

Once the functions of partial and global utility were obtained for each organization, we performed a cluster analysis, and with these results, we proposed three performance levels: high performance, average performance, and low performance. We determined the cut-off thresholds using whisker plots with a value of 5% for constructing the respective upper and lower limits (Tab. 2).

Table 3 shows the performance of the 30 associative organizations that participated in the Plan Semilla framework and that were analyzed in this research. The results presented allow distinguishing the level of performance achieved by each organization based on the partial utility for each analyzed dimension, and also the level of global performance of each of the organizations.

The results show that four organizations stand out for their global performance: Coopercacao (Santander), Aprocafrum (Santander), Aprocampa (Boyaca) and Asocati

TABLE 2. Total profit thresholds defined to establish the levels of performance of cocoa seed producer organizations.

Dimensions	W_G of the dimension	Maximum dimension utility	Cutting thresholds* Low performance		Cutting thresholds* Medium performance		Cutting thresholds* High performance	
			Lower limit	Upper limit	Lower limit	Upper limit	Lower limit	Upper limit
Technical dimension	0.254	0.598	0	0.315	0.316	0.400	0.401	0.598
Environmental dimension	0.270	0.542	0	0.105	0.106	0.19	0.191	0.542
Organizational dimension	0.242	0.640	0	0.308	0.309	0.454	0.455	0.64
Management dimension	0.234	0.594	0	0.235	0.236	0.351	0.352	0.594
Total utility	1.000	2.374	0	0.997	0.998	1.306	1.307	2.374

*Thresholds cluster dimension obtained with truncated mean of 5%.

TABLE 3. Levels of performance of cocoa producer organizations - Plan Semilla.

Case	Organization	Partial Utility Performance Technical dimension		Partial Utility Performance Environmental dimension		Partial Utility Performance Organizational dimension		Partial Utility Performance Management dimension		Total Utility Global performance	
1	APROCAMPO 27	0.168	L.P.	0	L.P.	0.151	L.P.	0.117	L.P.	0.436	L.P.
2	ASOPECA	0.168	L.P.	0.144	M.P.	0.164	L.P.	0.187	L.P.	0.663	L.P.
3	APROCAPAL	0.288	L.P.	0.144	M.P.	0.138	L.P.	0.152	L.P.	0.722	L.P.
4	MUZCACAO	0.329	M.P.	0.094	L.P.	0.194	L.P.	0.168	L.P.	0.785	L.P.
5	ASOPAICOL -ASOCACAO	0.28	L.P.	0.144	M.P.	0.232	L.P.	0.152	L.P.	0.808	L.P.
6	ASOHUPAR	0.288	L.P.	0.144	M.P.	0.178	L.P.	0.221	L.P.	0.831	L.P.
7	ASAPA	0.28	L.P.	0.144	M.P.	0.229	L.P.	0.202	L.P.	0.855	L.P.
8	ASOPROCAL	0.31	L.P.	0.144	M.P.	0.215	L.P.	0.187	L.P.	0.856	L.P.
9	ASOCAT	0.384	M.P.	0.094	L.P.	0.249	L.P.	0.149	L.P.	0.876	L.P.
10	ASOPROCAMU	0.282	L.P.	0.144	M.P.	0.274	L.P.	0.206	L.P.	0.906	L.P.
11	ASOPROLAN	0.456	H.P.	0.094	L.P.	0.193	L.P.	0.168	L.P.	0.911	L.P.
12	ASOCAVAL	0.378	M.P.	0.072	L.P.	0.218	L.P.	0.269	M.P.	0.937	L.P.
13	APRASEF	0.336	M.P.	0	L.P.	0.418	M.P.	0.186	L.P.	0.94	L.P.
14	ASOPROCAR	0.331	M.P.	0.144	M.P.	0.283	L.P.	0.197	L.P.	0.955	L.P.
15	ASOCASAR	0.274	L.P.	0.144	M.P.	0.33	M.P.	0.221	L.P.	0.969	L.P.
16	ASOCATIGRA	0.349	M.P.	0.144	M.P.	0.356	M.P.	0.212	L.P.	1.061	M.P.
17	Consejo Comunitario Tablon Dulce	0.345	M.P.	0.166	M.P.	0.436	M.P.	0.173	L.P.	1.12	M.P.

Continue...

Table 3. *Continuation*

Case	Organization	Partial Utility Performance Technical dimension		Partial Utility Performance Environmental dimension		Partial Utility Performance Organizational dimension		Partial Utility Performance Management dimension		Total Utility Global performance	
18	ASOMUCARI	0.271	L.P.	0.144	M.P.	0.504		0.202	L.P.	1.121	M.P.
19	APROCASUR	0.376	M.P.	0.072	L.P.	0.435	M.P.	0.238	M.P.	1.121	M.P.
20	APROCESU	0.427	H.P.	0.072	L.P.	0.356	M.P.	0.272	M.P.	1.127	M.P.
21	EL MANANTIAL	0.3	L.P.	0.144	M.P.	0.402	M.P.	0.282	M.P.	1.128	M.P.
22	ASOCAM	0.308	L.P.	0.144	M.P.	0.434	M.P.	0.247	M.P.	1.133	M.P.
23	CIPAOTANCHE	0.279	L.P.	0.144	M.P.	0.457		0.264	M.P.	1.144	M.P.
24	ASOCACABO	0.324	M.P.	0.144	M.P.	0.434	M.P.	0,316	M.P.	1.218	M.P.
25	CORTIPAZ	0.442	H.P.	0.072	L.P.	0.499	H.P.	0.218	L.P.	1.231	M.P.
26	ASOCAP	0.305	L.P.	0.238	H.P.	0.513	H.P.	0.197	L.P.	1.253	M.P.
27	COOPERCACAO	0.343	M.P.	0.144	M.P.	0.49	H.P.	0.434	H.P.	1.411	H.P.
28	APROCAFRUM	0.379	M.P.	0.238	H.P.	0.522	H.P.	0.283	M.P.	1.422	H.P.
29	APROCAMPA	0.464	H.P.	0.238	H.P.	0.495	H.P.	0.278	M.P.	1.475	H.P.
30	ASOCATI	0.397	M.P.	0.238	H.P.	0.5	H.P.	0.417	H.P.	1.552	H.P.

H.P. High performance; M.P. Medium performance; L.P. Low performance.

(Norte de Santander); these showed a high level of performance (13%). Eleven organizations corresponding to 37% showed an average level of performance, whereas 15 organizations (50%) showed a low level of performance.

The analytical model developed in this research has allowed the characterization of the organizations based on the level of performance of the analyzed dimensions, improving the procedural rationality used until now within the framework of Plan Semilla. The obtained results have allowed identifying with greater clarity and certainty the areas in which organizations are considered to have a low degree of performance. Given the attributes of the hierarchical composition of the designed model, the model can be used to propose specific actions to improve those criteria that are considered strategic in order to contribute to correcting and improving the capacities for the consolidation of quality seed production nuclei.

Some of the actions that could be implemented to design plans to strengthen the capacities of organizations may be the following: organizations with low performance in the environmental resources dimension should prioritize training actions to develop capacities to identify the agroclimatological risks they might face and that could affect the procurement of quality seed. They must also acquire skills and knowledge to take appropriate measures for both the prevention and mitigation. In organizations with low performance in the organizational dimension, actions that lead to the strengthening of individual and collective capacities to comply with agreements and norms should be implemented. Leadership issues should be considered so as to improve the participation of the associates and their capacities so that they assume management positions. This could improve the probability of success of the undertakings carried out by rural organizations. In order to strengthen management capabilities in those organizations that show low performance, specific actions could be taken to lead organizations and their members to acquire skills and abilities for strategic planning, monitoring, and analysis of production costs. In addition, these organizations should implement the use of records to determine the unit cost and, in turn, the profitability of the business.

Conclusions

The identification of the variables relating to technical, economic, social, organizational, managerial and regulatory components, among others, should influence the adequate consolidation of nuclei of quality seed production by collective organizations of small agricultural producers. It has been an expensive and complex process that has required a great capacity of synthesis to select those elements that could be more representative for the purpose of this research.

The environmental resources dimension, considered the most important after estimating the model in this research,

has been incorporated in a novel way compared to other methodologies used to characterize organizations in the agricultural sector. This dimension had not been taken into account in the characterizations made by Plan Semilla, due to the specificity of the resources and dimensions required for the production of quality seed.

The AHP has been used in a novel way as a discrete method of multicriteria decisions, which allows improving the procedural rationality required to analyze complex scenarios influenced by multiple variables like agricultural producer organizations. Nonetheless, the developed methodology and the results obtained have allowed a reduction in the level of subjectivity with which agricultural sector organizations are traditionally defined.

Using the Multi-Attribute Utility Theory (MAUT), it was possible to calculate additional utility functions that incorporate both the weight of importance associated with the elements of the model in each of its levels as well as the performance of organizations based on verifiable indicators.

Based on calculated utility values and cluster analysis, it has been possible to establish cut-off thresholds that have allowed the construction of relative performance ranges (low, medium and high) that allow rating and ranking performances (partial and total) of producer organizations currently seeking to consolidate nuclei of quality seed production. This allows the identification and selection of those elements that are considered as weaknesses in an organization and that requires the development of specific actions to strengthen them to achieve sustainability as associative enterprises that produce cocoa seeds.

We consider that the selected elements are universal for organizations that produce seeds; nevertheless, it is necessary to establish new thresholds to define the levels of performance if the model is used for other species.

It is important that institutions that work with organizations of agricultural producers develop research projects with prior implementation of appropriate intervention actions in the processes of characterization. It is necessary to have, from the beginning, conceptual and methodological clarity about the scope of the project, and for the use of the appropriate tools for the exercises of organizational typification. This should be done in order to propose pertinent actions according to the needs that the organizations face as well as to strengthen their capacities of collective action and social capital at technical and social-business levels, which are key for the development of any joint initiative.

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SCIENTIFIC NOTE

Crude protein content in hybrids of *Paspalum* evaluated in the Pampa Biome of Southern Brazil

Contenido de proteína bruta en híbridos de *Paspalum* evaluados en el Bioma de Pampa en el sur de Brasil

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ABSTRACT

The use of forage species adapted to the local environment allows an easier management, and greater production and stability. It also allows the conservation of the natural genetic resources, and the reduction of the costs and risks of production that further result in higher sustainability of the system. Forage quality may not be considered as important as biomass production in many forage systems. However, when forage plants constitute most or all of the ruminant diet it assumes substantially greater importance. Therefore, the goal of this study was to evaluate the crude protein (CP) content of hybrids of *P. plicatulum* x *P. guenoarum*, in a region of Southern Brazil in the Pampa biome. The hybrids evaluated were: 10202, 1020104, 102084, 102080, 1020133, 102058, 102069 (*P. plicatulum* "4PT" x *P. guenoarum* "Azulão"), 103063, 10308, 103042, 103040, 103061, 103077, 103087, 103093, 103031, 103020, 103084, and 103037 (*P. plicatulum* "4PT" x *P. guenoarum* "Baio"). The CP analyzes were performed on the leaves of the genotypes in each harvest. The hybrid 102069 "Azulão" presented the best CP content (16.4%) compared to the other genotypes in both years. These results are encouraging for forage breeding studies with species of the genus *Paspalum*.

Key words: forage plants, forage quality, forage breeding.

RESUMEN

El uso de especies forrajeras adaptadas al entorno local permite un manejo más sencillo y una mayor producción y estabilidad. También permite la conservación de los recursos genéticos naturales, la reducción de los costos y riesgos de producción, lo que resulta en una mayor sostenibilidad del sistema. La calidad del forraje no puede considerarse tan importante como la producción de biomasa en muchos sistemas forrajeros. Sin embargo, cuando las plantas forrajeras constituyen la mayor parte o la totalidad de la dieta para rumiantes, asume una importancia sustancialmente mayor. Por lo tanto, el objetivo de este estudio fue evaluar el contenido de proteína cruda (PC) de los híbridos de *P. plicatulum* x *P. guenoarum*, en una región del sur de Brasil en el bioma de Pampa. Los híbridos evaluados fueron: 10202, 1020104, 102084, 102080, 1020133, 102058, 102069 (*P. plicatulum* "4PT" x *P. guenoarum* "Azulão"), 103063, 10308, 103042, 103040, 103061, 103087, 103093, 103031, 103020, 103084 y 103037 (*P. plicatulum* "4PT" x *P. guenoarum* "Baio"). Los análisis de PC se realizaron en las hojas de los genotipos en cada cosecha. El híbrido 102069 presentó el mejor contenido de PC en comparación con los otros genotipos en ambos años. Estos resultados son alentadores para los estudios de cría de forrajes con especies del género *Paspalum*.

Palabras clave: plantas forrajeras, calidad del forraje, mejoramiento de forrajeras.

Introduction

Worldwide, forage plants are the cheapest way to produce and provide food to animals, enabling meat and milk production at low costs (Follett *et al*., 2001). Species of the genus *Paspalum* are the most important forage constituents of the natural grasslands in South America (Novo *et al*., 2016). The greatest diversity of species is found in the Central and Southern regions of Brazil and Paraguay, Eastern Bolivia and Northeastern Argentina; therefore, intraspecific and interspecific variability is high (Sartor *et al*., 2009). *Paspalum* species occur in most of the herbaceous communities in the distinct Brazilian ecosystems. It has suitable characteristics to be used for grazing as well as beneficial chemical composition for ruminant production (Valls, 2000).

The use of forage species adapted to the local environment allows an easier management, and greater production and stability. It also allows the conservation of the natural

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genetic resources and the reduction of the costs and risks of production, resulting in higher sustainability of the system (Townsend, 2008).

The quality of forage plants is a factor that greatly affects the productivity of grazing ruminants. Therefore, evaluation of the crude protein (CP) content plays a very important role in the qualitative analysis of forages. In diets that do not provide a minimum of 7% of CP in the dry matter, the recycled urea is not enough to meet the nitrogen demand of rumen microorganisms, resulting in a decreased feed intake and digestibility (Van Soest, 1994). These factors determine the amount of ingested nutrients, which are necessary to meet the maintenance and production requirements of the animals (Gomide, 1993). The selection of genotypes with greater forage production, forage quality and wide adaptability to diverse environments is one of the main goals in forage breeding programs.

Steiner *et al*. (2017) obtained higher forage production with two native ecotypes of *P. guenoarum* and two ecotypes of *P. notatum* compared to the cultivar "Pensacola" (*P. notatum)* which demonstrates the possibility to use native ecotypes as cultivated pastures. Forage quality may not be considered as important as biomass production in many forage systems. However, when forage plants constitute most or all of the ruminants' diet it assumes substantially greater importance (Brink *et al*., 2015). Therefore, the goal of this study was to evaluate the CP content of hybrids of *P. plicatulum* x *P. guenoarum* in a region of Southern Brazil in the Pampa biome.

Materials and methods

The experiment was carried out in the state of Rio Grande do Sul, in the municipality of El dorado do Sul, Depressão Central region, Brazil (30°05' S, 51°39' W, 40 m a.s.l.) during two growing seasons, from February to April 2013 and from February to April 2014. The average annual temperature varied from 8.5-8.7°C (June and July, coldest months) to 29.4-30.2°C (January and February, hottest months). The average annual rainfall was approximately 1445 mm (Bergamaschi *et al*., 2013). Nitrogen fertilizer (as urea) was applied in the amounts of 180 and 130 kg ha^{-1} of N in 2013 and 2014, respectively.

Artificial hybridizations were performed in greenhouse by the Forage Plants Breeding Group at the Department of Forage Plants and Agrometeorology (DPFA) of the Federal University of Rio Grande do Sul (UFRGS). Hybrids obtained from these crosses were previously evaluated in the field (individual plants) during the summer of 2010 and throughout the year 2011. The genotypes with the highest forage production were selected to compose the present study (Forage Plants Breeding Group, 2013). The hybrids evaluated were: 10202, 1020104, 102084, 102080, 1020133, 102058, and 102069, which resulted from crosses between *Paspalum plicatulum* "4PT" x *P. guenoarum* "Azulão", and the hybrids 103063, 10308, 103042, 103040, 103061, 103077, 103087, 103093, 103031, 103020, 103084, and 103037, which resulted from crosses between *P. plicatulum* "4PT" x *P. guenoarum* "Baio". The parents ("4PT", "Azulão" and "Baio") and the cultivar "Aruana" (*Megathyrsus maximus)* were used as controls. "Azulão" and "Baio" are native to the state of Rio Grande do Sul, Southern Brazil, while ecotype "4PT" is native to Corrientes, Argentina. "Aruana" is a warm-season grass used in animal production systems in Brazil.

Five clonal plants of each genotype were planted, spaced by 20 cm, in a 1 m row. Each row was planted 50 cm apart. The experimental design was a randomized complete block (RCBD) with four replicates. The plants were harvested when 80% of the genotypes reached 35 cm height, leaving a 15 cm stubble height. The harvests were carried out on February and March 2013 and February and April 2014. The plant samples were separated into leaves, stems and inflorescences. Subsequently, the samples of the plant components were placed to dry in a forced air circulation oven at 55°C for 72 h to obtain the dry matter weights, which were used to determine the CP content.

The CP analyzes were performed on the leaves of the genotypes in each harvest. The CP content was determined according to the methods proposed by AOAC (1984) in the Animal Nutrition Laboratory of Embrapa Pecuária Sul. The data were submitted to analysis of variance (ANOVA) and an F test at 5% probability using SAS software (SAS Institute, 2002). When differences between genotypes were observed, a means comparison was performed using Scott-Knott test at 5% probability.

Results and discussion

There was significant genotype x harvest interaction ($P<0.05$) for CP content in both years. At the harvest performed in February 2013, the CP content ranged from 11.4% (102084 and 103031) to 16.3% for hybrid 102069 (Tab. 1). Higher CP content values were observed with the hybrids 102069, 102080 and 10308 compared to the ecotypes "4PT", "Azulão" and "Baio" and cultivar "Aruana". In April 2013,

the CP content ranged from 11.1% for hybrid 102080, to 18.5% (103042). In this harvest, hybrids 103042 and 102069 had higher CP content than the ecotypes "4PT", "Azulão" and "Baio" and cultivar "Aruana" (control). At the harvest performed in February 2014, the CP content ranged from 10.9% (103084) to 17.8% for hybrid 102069. Higher values for CP content were observed at the second harvest in 2014 (April) ranging from 12.8% (103087) to 20.4% (102069). In both harvests of 2014, the hybrid 102069 showed higher CP content than ecotypes "4PT", "Azulão" and "Baio" and cultivar "Aruana".

The CP content observed in some *Paspalum* interspecific hybrids was higher compared to other studies reported in the literature. Echevarria *et al.* (2016) evaluated *Urochloa* interspecific hybrid BRS RB331 Ipyporã and observed a CP content of 13.8%. Evaluating species of *Megathyrsus maximus*, *Urochloa brizantha* and *Urochloa decumbens*, Lima *et al.* (2018) reported CP contents of 10.2, 4.1 and 10.8% respectively. In a study with acessions of *Paspalum atratum* Swallen and *Paspalum lenticulare* Kunth, Marcon *et al.* (2018) observed CP content of 10.1 and 10.8% respectively.

The CP content is an important trait for the selection and improvement of nutritional characteristics in forage plants. Steiner *et al.* (2017) described mean values of 14.7% and 14.2% for CP content from "Azulão" and "Baio", respectively. The findings in this research revealed that there were hybrids with higher CP content than the ecotypes used as parents and the commercial cultivar, especially hybrid 102069 that had superior CP content in all harvests and years. The National Academies of Sciences, Engineering, and Medicine (2016) recommends 12% of CP for finishing cattle. Therefore, it can be considered that CP values found for some hybrids and its progenitors are satisfactory for cattle weight gain even in the most demanding classes.

Average across genotypes and higher CP content values were observed on April 2014 when compared to the other harvests (Tab. 1).

TABLE 1. Crude protein content (%) of leaf blade samples of interspecific hybrids and ecotypes of the genus *Paspalum*, and the cultivar "Aruana" evaluated in Eldorado do Sul, RS, Brazil.

Genotypes	Harvest				
	February 13	April 13	February 14	April 14	Mean
102069	16.3 C-a	15.7 C-b	17.8 B-a	20.4 A-a	17.6
102080	15.4 A-b	11.1 C-f	13.5 B-c	15.4 A-c	13.9
10308	15.1 A-b	13.3 B-d	13.3 B-c	15.0 A-c	14.2
102058	14,5 B-c	12.9 C-d	14.0 B-b	16.2 A-b	14.4
103042	14.4 C-c	18.5 A-a	13.7 D-c	16.3 B-b	15.7
10104	14.1 B-c	12.6 C-e	14.8 A-b	14.8 A-c	14.1
103087	12.7 A-e	12.3 A-e	11.6 B-e	12.8 A-e	12.4
103077	13.9 B-c	12.8 C-d	13.9 B-c	16.2 A-b	14.2
"4PT"	13.8 B-d	12.9 C-d	13.6 B-c	15.0 A-c	13.8
"Azulão"	13.7 C-d	12.8 D-d	14.4 B-b	16.4 A-b	14.3
103061	13.6 A-d	12.3 B-e	12.3 B-d	13.6 A-d	13.0
10202	13.5 C-d	12.6 D-e	14.1 B-b	14.8 A-c	13.8
103093	13.3 C-d	14.2 B-c	14.4 B-b	15.2 A-c	14.3
103037	13.3 B-d	12.4 C-e	13.4 B-c	15.8 A-b	13.7
103063	13.1 B-e	13.3 B-d	12.7 B-d	14.9 A-c	13.5
"Baio"	14.0 B-c	14.0 B-c	14.2 B-b	15.3 A-c	14.4
1020133	12.5 C-e	12.3 C-e	13.6 B-c	14.3 A-d	13.2
103040	12.3 C-e	13.1 B-d	13.0 B-c	14.8 A-c	13.3
103084	12.3 B-e	11.5 C-f	10.9 C-f	13.3 A-e	12.0
103020	11.9 B-f	12.1 B-e	12.6 B-d	13.7 A-d	12.6
"Aruana"	11.9 B-f	12.5 B-e	12.4 B-d	13.2 A-e	12.5
102084	11.4 C-f	12.6 B-e	13.7 A-c	13.4 A-e	12.8
103031	11.4 D-f	12.2 C-e	13.6 B-c	14.5 A-c	12.9
Mean	13.4	13.0	13.5	15.0	13.8

Means followed by the same lowercase letters in rows and uppercase letters in columns do not differ by the Scott-Knott test at 5% probability.

This result can be attributed to cumulative effect of the nitrogen application in the second year, which may have provided benefits to increase CP content of the genotypes. Pereira *et al*. (2011), evaluating ecotypes of *P. guenoarum* and *P. lepton* (ex-*nicorae*), also observed the variation in the CP content between harvests and related it to the effect of nitrogen fertilization to increase CP.

The improvement of *Paspalum* species through artificial hybridization can be an important tool to obtain novel genetic resources with higher CP for pasture-based livestock production.

Conclusions

The hybrid 102069 (*Paspalum plicatulum* "4PT" x *P. guenoarum* "Azulão") presented the best CP content compared to the other genotypes in both years. These results are encouraging for forage breeding studies with species of the genus *Paspalum*. Promising hybrids can be used in new crossings in order to continue to increase forage nutritional characteristics or to be released as new cultivars. The hybrid 102069 will be used in coming evaluations within the Forage Plants Breeding Program of UFRGS. Since the hybrid progeny may include individuals for the sexual or apomictic type of reproduction, it is important to evaluate the reproductive biology of these hybrids. This analysis is already in progress.

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SCIENTIFIC NOTE

Leptostylus hilaris Bates, 1872 (Coleoptera: Cerambycidae) on Tahiti lime (*Citrus latifolia* Tanaka, Rutaceae) in Colombia

Leptostylus hilaris Bates, 1872 (Coleoptera: Cerambycidae) en lima Tahití (*Citrus latifolia* Tanaka, Rutaceae) en Colombia

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ABSTRACT

Leptostylus hilaris Bates, 1872 (Coleoptera: Cerambycidae) or stem borer larvae were found and collected in Espinal, Tolima province (Colombia) in Tahiti lime (*Citrus latifolia* Tanaka-Rutaceae) tree crops. This is the first record for this species infesting this host plant in Colombia.

Key words: xylophagous beetle, crop, new record.

RESUMEN

La larva del taladrador *Leptostylus hilaris* Bates, 1872 (Coleoptera: Cerambycidae) fue colectado en Espinal, departamento de Tolima (Colombia) en plantaciones de árboles de lima ácida Tahití (*Citrus latifolia* Tanaka-Rutaceae). Este es el primer registro para esta especie en Colombia, y en este hospedante.

Palabras clave: escarabajo xilófago, cultivo, nuevo registro.

Introduction

Citrus fruits are perennial and commonly have high adaptability to diverse climatic conditions. Some varieties of Citrus adapt well because Colombian geography has favorable characteristics for their growth and development (Orduz-Rodríguez and Mateus, 2012). In Colombia, 96,000 ha were reported with citrus crops in the year 2017 (MADR, 2017), and, according to the National Agricultural Survey (DANE, 2016), the area cultivated with Tahiti limes included 23,638 ha distributed in 26 provinces. The main producer and exporter provinces of Tahiti lime are Santander, Tolima and Valle del Cauca. For the year 2017, Tahiti lime exports included 12.56 thousand t, which represented about 4% of the exportable horticultural products (Asohofrucol, 2017). The Tahiti lime has constant production throughout the year and, like other fruit trees, has technification limitations resulting from poor planning and fertilization (Rodríguez *et al*., 2018). In addition, phytosanitary problems, especially insect emerging pests, are present in this crop. This is the case for the stem borer *Leptostylus hilaris,* which is being reported for the first time for Colombia and for the Tahiti lime.

Given constant production throughout the year and the population dynamics of insects, the phytosanitary management of production is complex. The presence of a new insect associated with the cultivation of citrus fruits makes it necessary and vital to elaborate and disseminate this record.

In a one ha Tahiti lime (*Citrus latifolia* (Rutaceae)) crop located at the Nataima research center in the municipality of Espinal, province of Tolima, the presence and damage caused by the insect *L. hilaris* were detected. To quantify the presence of larvae, pupae and adults of this insect and the damage (perforations) caused by the larvae in the trunks and branches of the trees (Fig. 1), samples were taken monthly during the period from July, 2016 to March, 2017. At the end of this period, 58 adult, 26 pupae and 60 larvae were collected. The insect samples were analyzed in the Entomology Laboratory of this center and stored in the "Luis María Murillo" Taxonomic Collection of Insects [Colección Taxonómica Nacional de Insectos Luis María Murillo] (CTNI), with the following information (Spanish in quotation marks):

"**COLOMBIA: Tolima:** Espinal, Vda. San Francisco, C.I. Nataima, 410 m, 4°5' N, 74°56' O, ix-2016, B. Monje, Cap. Manual, "Taladrador de *Citrus latifolia* (Rutaceae) - Lima ácida Tahití". Three adult beetles and a pupa were deposited in the collection [CTNI 182] Det: J.P. Botero 2016.

The identification of the specimens was performed by consulting the original description and the photo of a holotype

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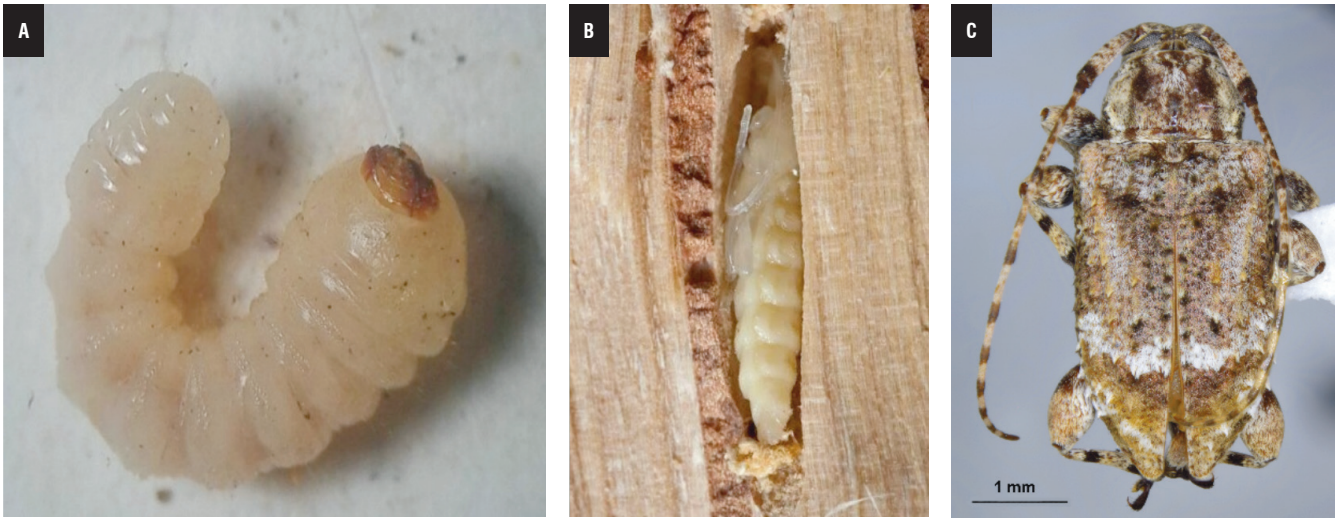

FIGURE 1. *Leptostylus hilaris* individuals at different stages of development: a) larvae; b) pupae inside the stem; c) adult.

specimen. Additionally, specimens were compared with the ones in the collection of the Museu de Zoologia of the Universidade de São Paulo (São Paulo, Brazil).

In consecutive evaluations of Tahiti lime plants at the Nataima research center, it was demonstrated that the presence of *L. hilaris* is a new phytosanitary problem in the province of Tolima. This species is currently found in 60% of the crops. Populations of this stem borer are found inside stems and branches located in weak or dead organs. Sanitary pruning of branches and dead stems is a cultivation task that reduces the presence of this insect.

Several species of the *Leptostylus* genus are recorded as pests and are usually found on timber plantations with different feeding habits. Some examples are the xylophagy in *Pinus oocarpa* (Jiménez and Maes, 2005) or the spermophagy in *L. gibbulosus* on *Manilkara zapota* (Sapotaceae) and *Sapindus saponaria* (Sapindaceae) (Vogt, 1949; Arguedas, 2007; Romero *et al*., 2007; Hernández-Jaramillo *et al*., 2012), *L. terraecolor* on *Rhizophora mangle* (Rhizophoraceae) (Craighead, 1923), *L. gundlachi* in *Erythrina glauca* (Fabaceae) (Wolcott, 1948), and *L. spermovoratis* in *Dyospiros* sp. fruits (Ebenaceae) (Chemsak, 1972).

Damage

Leptostylus hilaris larvae bore stems and branches that have previously been affected mechanically or by disease. Therefore, the presence of this insect in affected Tahiti lime plants is secondary. After completing its organ development, the larva makes a cell in the terminal part of a gallery that was previously perforated while feeding; the larva then prepares a chamber inside and closes the entrance of this chamber with a fiber plug cut from stem tissues; the larva then transforms into a pupa of a white or creamy color. After that, the adult emerges from the pupa, crawls through the galleries prepared when it was a larva, and perforates the tree bark until it reaches the exterior. It generates an elliptical contour hole whose diameter matches the thickness of the body (Fig. 2).

Morphological characteristics of the subfamily, tribe, genus and species of the stem borer *Leptostylus hilaris* are shown below.

Subfamily Lamiinae Latreille, 1825

It is the largest subfamily of Cerambycidae, extremely variable in form and size. It can be differentiated by the large and vertical frons and mouthparts oriented ventrally to posteroventrally; apical palpomere acuminated at the apex, and an anterior tibiae with a ventral obliquus sulcus.

Tribe Acanthocinini Blanchard, 1845

Eyes emarginated, finely granulated; antennae generally longer than the body; scape cylindrical and elongated; sides of prothorax with or without tubercles or spines; elytra long, and wider than the base of the prothorax; femora pedunculated; mesotibiae with sulcus; tarsal claws forked and simple.

Genus *Leptostylus* LeConte, 1852

Body convex; pronotum with evident tubercles; relatively short antennae, slightly longer than body; elytra with basal gibbosities and longitudinal rows of hairs.

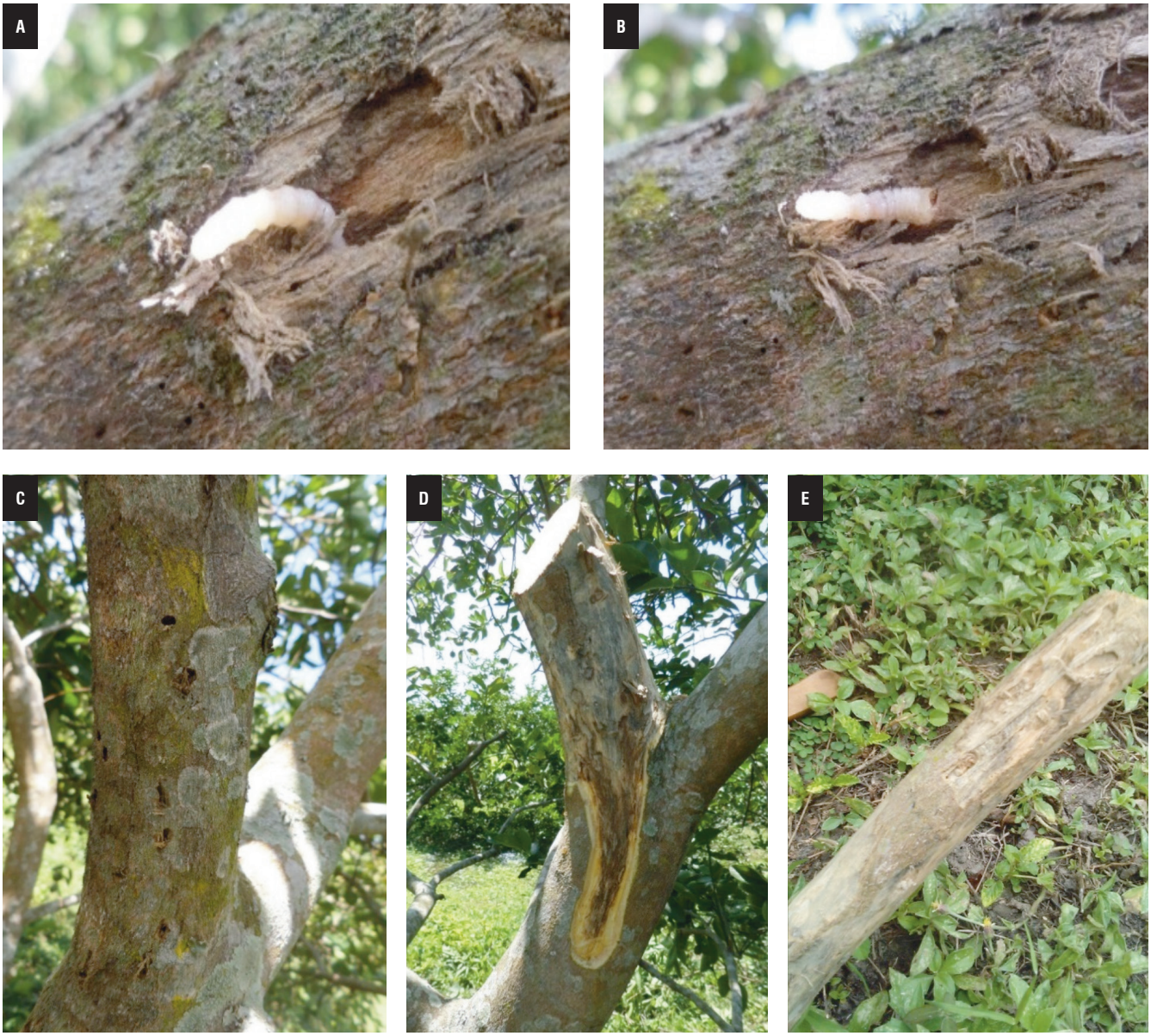

FIGURE 2. *Leptostylus hilaris* larvae (a, b) and the damage they cause in Tahiti lime trees (c, d, e). Photographs by Guzmán, 2016.

Leptostylus hilaris Bates, 1872

Prothorax with lateral tubercles clearly evident, rounded at apex; pronotum with five tubercles; elytra without lateral carina; apex of elytra obliquely truncated; femora apex unarmed.

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Research article length should not exceed 5,200 words, whereas scientific notes should not have more than 4,000 words. Tables and figures, that is to say, diagrams, drawings, schematic and flow diagrams, pictures and maps, should be consecutively numbered (Table 1 ...Table n; Figure 1... Figure n, etc).

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Results and discussion can be displayed in two different sections or in a single section at the authors convenience. The results shall be presented in a logical, objective, and sequential order, using text, tables (abbreviated as Tab.) and figures (abbreviated as Fig.). The latter two should be easily understandable and self-explaining, in spite of having been thoroughly explained in the text. The charts should be two-dimensional and prepared in black and white, resorting to a tone intensity degradation to illustrate variations between columns. Diagram curves must be prepared in black, dashed or continuous lines (- - - - or ———), using the following conventions: ▪, ▲, ◆, ●, □, △, ◇, ○. The tables should contain few columns and lines.

Averages should be accompanied by their corresponding Standard Error (SE) values. The discussion shall be complete and exhaustive, emphasizing the highlights and comparing them to the literature.

This section should briefly and concisely summarize the most important findings of the research.

Conclusion (optional)

A short conclusion section is useful for long or complex discussion. It should provide readers with a brief summary of the main achievements from the results of the study. It also can contain final remarks and a brief description of future complementary studies which should be addressed.

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Illustrative cases:

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  E.g., Dubey, N.K. (ed.). 2015. Plants as a source of natural antioxidants. CABI, Wallingford, UK. Doi: 10.1079/9781780642666.0000.

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- ***Reference to a website:*** Author(s). Year. Title of the document. Retrieved from: URL; consulted date. E.g.: FAO. 1994. Neglected crops 1492 from a different perspective. Retrieved from: http://www.fao.org/docrep/t0646e/T0646E00.htm#Contents; consulted: August, 2017.

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