

# Evaluation of phosphate solubilization from phosphoric rock via *Aspergillus niger* and *Penicillium sp*

## Evaluación de solubilidad del fosfato de la roca fosfórica vía *Aspergillus niger* y *Penicillium sp*

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### ARTICLE DATA

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Ejemplo de cultivo de hongo *Aspergillus niger* en vinazas de café y roca fosfórica

### ABSTRACT

Phosphoric rock is the main source of phosphorus used in fertilizers. In most cases, it is applied alone or mixed with other nutrients in water; however, its low solubility means that it is not used efficiently, causing excess phosphorus to be wasted. As a result, it generates economic expenses to the farmer, or causing problems of eutrophication in water sources. In this article, different mixtures of solubilizing fungi with coffee pulp stillage were studied to improve phosphates' solubility in phosphoric rock. Despite that in different studies is clear the fungi's role in P solubilization, its application on soils is still made without any additional treatment. Phosphoric rock samples were obtained from the municipality of Aipe (Huila) and were treated for 6 weeks with coffee pulp stillage (*Coffea arabica*) and inoculums of *Aspergillus niger* and *Penicillium sp*, pH and temperature controlled. The fungus species were obtained from environmental media on PDA culture, then it was isolated and identified on MEA culture according to standard morphologic identification methods. *Aspergillus niger* showed greater mycelial growth in the stillage and a higher concentration of phosphates dissolved in the aqueous phase ( $72 \pm 31$  M) compared to *Penicillium sp*. The mixture of the two fungi in the treatments allowed to obtain the highest percentage of phosphates (17.8% of the phosphate soluble in  $H_2SO_4$  40% v / v).

**Key words:** sustainable agriculture; land resources; liquid fertilizers; Phosphate solubilizing microorganisms; fungi.

### RESUMEN

La roca fosfórica es la principal fuente de fósforo usada en los fertilizantes. En la mayoría de los casos, se aplica sola o mezclada con otros nutrientes en agua; sin embargo, su baja solubilidad no permite su total aprovechamiento, haciendo que el exceso de fósforo se desaproveche, genere gastos económicos para al agricultor o produzca problemas de eutrofización en fuentes de agua. En este artículo se estudiaron diferentes mezclas de hongos solubilizadores (MSP) con vinaza de pulpa de café para mejorar la solubilidad de los fosfatos de la roca fosfórica, ya que a pesar de que en diferentes estudios se ha

evidenciado su rol en la solubilización de P, su aplicación en suelos se sigue haciendo sin ningún tratamiento adicional. Las muestras de roca fosfórica fueron obtenidas del municipio de Aipe (Huila) y se trataron durante seis semanas con vinazas de pulpa de café (*Coffea arabica* L.) e inóculos de *Aspergillus niger* y *Penicillium sp.*, pH y temperatura controlados. Los hongos fueron obtenidos en Agar Papa Dextrosa (PDA), y aislados e identificados en Agar Extracto de Malta (MEA) de acuerdo con métodos estándar de identificación morfológica. El *Aspergillus niger* mostró mayor crecimiento micelial en las vinazas y obtuvo una mayor concentración de fosfatos disueltos en fase acuosa ( $72 \pm 31$  M) en comparación con *Penicillium sp.* La mezcla de los dos hongos en los tratamientos permitió obtener el mayor porcentaje de fosfatos (17,8% del fosfato soluble en  $H_2SO_4$  40%v/v).

**Palabras clave:** Agricultura sostenible; recurso suelo; fertilizantes líquidos, microorganismos solubilizadores de fosfatos; hongos.

## INTRODUCTION

Agriculture consumes large amounts of phosphorus for soil fertilization using phosphate rock extracted from deposits. According to the 2017 reports from the USGS (United States Geological Surveys) (USGS, 2017) and the FAO (Zapata and Roy, 2007), it is estimated that in about 50 years, between 2040 and 2060, the conventional sources of phosphate rock (PR) will begin to be scarce. As well in about 100 years, the mines with easy access to this mineral would be exhausted (Gilbert, 2009).

Thus, promoting the exploitation of less profitable mines (Restrepo *et al.*, 2015). However, current fertilization practices that have a phosphorus application efficiency of less than 30% (Goldstein *et al.*, 1993) could affect a possible crisis scenario in agricultural production (Cordell, 2010; Cordell *et al.*, 2009; Walan *et al.* 2014; Edixhoven *et al.*, 2013).

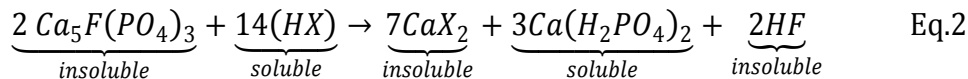
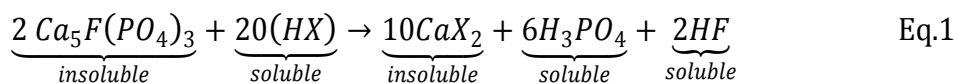
Microorganisms secrete various metabolites within their metabolic cycle as organic acids capable of solubilizing phosphorus from insoluble forms of phosphorus minerals as PR (Rashid *et al.*, 2004). These acids (D-malic, D-lactic, L-malic, L-lactic, acetic, citric, oxalic, gluconic, Iyyappan *et al.*, 2018; Li *et al.*, 2015) act as ligands of sequestering cations bound to phosphate groups in a mechanism called chelation.

In fact, these acid metabolites play determining roles in the rhizosphere dynamics and jointly influence the solubilization of phosphates from soil minerals (Wakelin *et al.*, 2004). The fungi *Aspergillus niger* and *Penicillium Sp* are potentially usable as

phosphate solubilizing microorganisms (PSM) on an agro-industrial scale (Yin *et al.*, 2015; Mendes *et al.*, 2015) because these fungi are practically ubiquitous species in the natural environment, and they are characteristic of Colombian soils (Pérez *et al.*, 2012). However, there is a great variety of species around the world.

In addition, these fungi have the ability to withstand environmental conditions and difficult substrates for other species, which implies that they have adaptive mechanisms to feed on unconventional sources of nutrients, and they generate specialized substances capable of carrying out these metabolic processes (Kaur and Reddy, 2017). For example, studies of deficient phosphorus soils (as Amazon basin soils and moor's acid soils) several phosphorus-solubilizing microorganisms, that have allowed the conservation of biota have been isolated (Vera *et al.*, 2002; Lizarazo and Vásquez, 2015).

A well-studied reaction scheme in the solubilization of phosphate, using microorganisms via the formation of metal complexes of  $Ca^{+2}$ , is presented in equations 1 and 2 (Ivanova *et al.*, 2006). In these reactions,  $Ca^{+2}$  is presented as a main component of apatites (or phosphoric rocks), and organic acids such as oxalic acid and citric acid that form calcium oxalate and calcium citrate, respectively. These acids are common metabolites produced by species of the fungi *Penicillium sp* and *Aspergillus niger* (Visagie *et al.*, 2014) and are recognized as PSM (Posso and De Prager, 2017).



Where XH can be citric ( ) or oxalic acid ( )

Equations 1 and 2 show the organic acid's action in forming poorly soluble compounds namely as calcium chelates,  $\text{CaX}_2$ , and soluble compounds; calcium diphosphate as an intermediate product (superphosphate) and phosphoric acid ( $\text{H}_3\text{PO}_4$ ). In these processes, the phosphate ion is released from the solid substrate by the chelating action of citric or oxalic acids on the  $\text{Ca}^{+2}$  cation (Mai *et al.*, 2016). The power of complex-forming acids (chelates) consists in the action of oxygens (more electronegative) of the carboxyl groups that surround the calcium cation (Harvey, 2000). In this way, they can solubilize insoluble compounds as found in apatite.

The global trend is oriented towards scientific advancement for the development of innovative technologies that make agribusiness sustainable (Zapata and Roy, 2007), and the use of microorganisms to improve the solubility of phosphates in phosphate rock has been suggested in different works (Restrepo *et al.*, 2015; Stutter *et al.*, 2012; Li *et al.*, 2015). For this reason, there are different advances in soil bioremediation (Pineda, 2015) and in biofertilization (Pérez *et al.*, 2012).

Therefore, the *Aspergillus niger* and *Penicillium sp* species were used in the formulation, application and evaluation of treatments that allow solubilizing phosphates from phosphate rock samples taken from the Media Luna mine in Aipe- Huila (Colombia), one of the places where phosphate rock is extracted.

## MATERIALS AND METHODS

**Phosphoric Rock.** The ground phosphoric rock samples were collected from the Media Luna mine in Aipe, Huila, with coordinates: Latitude  $3^\circ 54' 0.50184''$ N, Longitude  $75^\circ 19' 32.0592''$ , 20km from the municipal seat. The samples were sieved in the laboratory and the fractions with particle diameters between 125 - 500 $\mu\text{m}$  were worked on.

**Coffee pulp substrate (stillage).** The biological treatment was carried out in an aqueous medium, with aeration, and coffee pulp (*Coffea arabiga*) was used as substrate; material selected for its content of carbohydrates and essential nutrients (Silva *et al.*, 2013; Serrat *et al.*, 2018) due to the recognized problem as a residue in coffee production (Beyene *et al.*, 2012). Fresh coffee pulp from the rural area of San Agustín was obtained with coordinates: Latitude  $1^\circ 54' 05.9760''$  N, Longitude:  $75^\circ 17' 06.9828''$ W.

The coffee pulp was collected from the coffee pulping process, part of the coffee mill in which the pulp is generally discarded as waste. Later, the coffee pulp was stored under refrigeration and transferred to laboratory. In laboratory, the pulps were macerated with distilled water, filtered, sterilized in an autoclave (model NO75X) for 20min at  $120^\circ\text{C}$  and 1.0MPa.

No additional nutrient was added. This preparation will be referred to as vinasse due to its richness in fermentable sugars and its easy biodegradation by microorganisms. The pH measured in the stillage was 4.0, which was not modified to sow of the inocula.

**Colonies of *Aspergillus niger* and *Penicillium sp.*** 20mL of PDA (Potato Dextrose Agar) were poured into 90mm Petri dishes. The boxes were then exposed to the environment for 30 minutes to collect the microorganisms presented. The mushrooms's capture was made in the urban area of Neiva-Huila with coordinates (2°55'50.85"N, 75°15'54.503"W), with a warm tropical climate and average daytime temperatures of 28°C.

Then, the Petri dishes were sealed and incubated in the dark for 7 days at an average temperature of 29°C. After this time, the Petri dishes were selected in which, possible colonies of *Aspergillus niger* and *Penicillium sp* were identified according to the Visagie (2014) methodology by observing their macroscopic characteristics. Then, under aseptic conditions with a controlled environment in a laminar flow cabinet, colonies of individual species were sown from the cultures previously obtained and identified in Malt Extract Agar

(MEA). Finally, the macroscopic and microscopic identification of the species was made using an Olympus model microscope by comparing their morphological characteristics, observing the outcrop of conidiophores, and the formation of hyphae inoculated fungi.

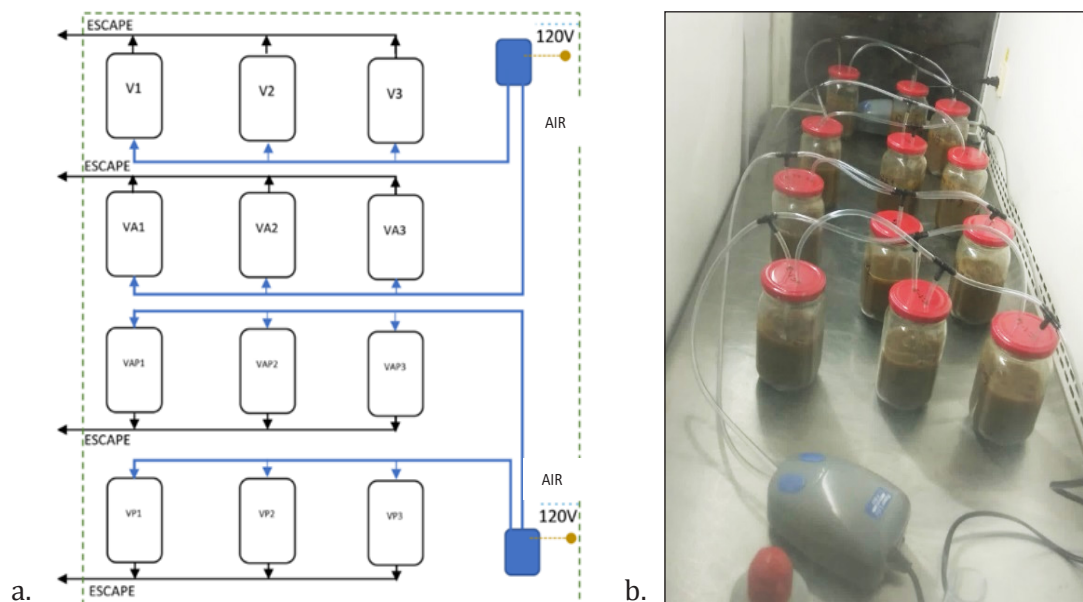
**Evaluation of the treatments with stillage, *Aspergillus niger*, and *Penicillium sp.*** The applied treatments V, VA, VP, VAP, and H40 are described in Table 1 for a 2x2 factorial design, for a total of 15 experimental units where the composition of the treatment was varied in a controlled temperature of 30.6°C. The variable evaluated was the composition of the mixture, conserving the proportions of RF and stillage used, but changing the mass ratio of the fungi, and the observable was the dissolved phosphate for several days of incubation.

The change in pH was controlled with the addition of CaCO<sub>3</sub> and all experiments were done by triplicate. In the treatments, the coffee pulp (*Coffea arabica*) was used as a substrate due to its easy obtaining as a by-product in the Coffee production (Puerta and Ríos, 2014; Serrat *et al.*, 2018). The treatments were carried out in a controlled environment (Figure 1) with aeration during intervals of two hours per day.

**Table 1.** Description of the biological treatments used for the solubilization of phosphorus.

No.	Treatment (B)	Descripción	Mass ratio of <i>Aspergillus niger</i> : <i>Penicillium sp.</i>
1	V	PR + stillage	(0:0)
2	VA	PR + stillage + <i>Aspergillus niger</i>	(1:0)
3	VP	PR + stillage + <i>Penicillium sp.</i>	(0:1)
4	VAP	PR + stillage+ <i>Penicillium sp.</i> + <i>Aspergillus niger</i>	(1:1)
5	H40*	PR+ (Reference)	----

\* Biological treatment.



**Figure 1.** Configuration of the treatment aeration system a) Scheme b) Photograph of an aeration system.

**Dissolved phosphate was determined by colorimetry.** 7mL samples were taken. They were centrifuged for 30min at 5000rpm to eliminate the high turbidity and the solids particles. Then, 5mL aliquots were taken and diluted to 50mL with distilled water. These solutions were treated with aluminum sulfate crystals to remove the color caused by the biological remnant and were centrifuged for 15 min to remove the flocs formed.

Finally, 10mL of the clarified solution was mixed with 2.5mL of vanadate-molybdate reagent (RVM), and the absorbance at 400nm wavelength was measured in a SPECTROQUANT model PHARO 300 spectrophotometer.

The results were correlated with the curve of monopotassium phosphate standards, previously prepared and valid for a range of 0 to 50 ppm of phosphate in the aqueous phase.

**Data analysis.** The Bartlett test was performed to establish the normality of the data ( $p > 0.05$ )

checking the assumptions of normality and homoscedasticity, and performing an analysis of variance by ranges for non-parametric distributions with the Kruskal-Wallis test. The null hypothesis was evaluated (for each case of interest) with a significance level of 5%. Matlab R2016a, Inc. software was used for statistical analysis (Mathworks, 2019a; Mathworks, 2019b; Mathworks, 2019c; Mathworks, 2019d).

## RESULTS AND DISCUSSION

Table 2 shows the percentage of total dissolved phosphorus achieved with the treatments and  $H_2SO_4$  (40% v/v). The V, VA, VP, and VAP treatments were positive in the phosphate dissolution of the phosphate rock, the VAP treatment being the most prominent, 7.8% of the phosphorus solution obtained with  $H_2SO_4$  (40% v/v). All treatments showed significant differences among themselves for 42 days of incubation.

**Table 2.** Dissolved PO<sub>4</sub> values obtained for each biological treatment for 42 days of incubation at 30°C.

No.	Treatment	Descripción	PO <sub>4</sub> dissolved (%)*	$\frac{P_t}{P_{H40}} \times 100$ (%)
1	V*	PR + stillage	0,15 ± 0,01	2,4
2	VA*	PR + stillage + <i>Aspergillus niger</i>	0,33 ± 0,03	5,3
3	VP*	PR + stillage + <i>Penicillium sp.</i>	0,19 ± 0,01	2,9
4	VAP*	PR + stillage + <i>Penicillium sp.</i> + <i>Aspergillus niger</i>	1,13 ± 0,03	17,8
5	H <sub>2</sub> SO <sub>4</sub> 40%	PR+ (Authors)	6,34 ± 0,10	100

\*x ± σ, (n = 5)

Whereas the dissolved phosphate concentrations (Table 3) for the VA and VAP treatments increased significantly as the incubation days passed, the phosphorus concentrations with the V and VP treatments decreased from the first week of

incubation. The highest phosphate concentration was for the VAP treatment (140 ± 17.1mg/L) and the lowest concentration was with the V treatment (13 ± 1.5mg/L).

**Table 3.** Average concentrations of soluble P (mg/mL) for each of the biological treatments according to the days of incubation.

Treatments	Concentration of P (mg/mL)/Days of incubation*					
	0	5	12	18	25	31
V	28 ± 0,9	19 ± 12,9	16 ± 2,6	13 ± 1,5	18 ± 3,0	19 ± 4,8
VA	23 ± 1,8	29 ± 3,4	47 ± 7,0	41 ± 4,7	34 ± 1,0	35 ± 2,5
VP	26 ± 2,0	22 ± 6,1	25 ± 2,7	21 ± 3,3	19 ± 2,6	18 ± 1,6
VAP	31 ± 5,1	103 ± 31,6	127 ± 21,2	140 ± 17,1	134 ± 25,0	127 ± 17,2

\*n = 5, x ± σ

The phosphate concentrations are similar to other values (201.3-283.7mg/L) reported in similar works (Chunquiao *et al.*, 2015) with solubilizing fungi e.g *Aspergillus niger* and *Aspergillus carbonarius*. The authors incorporated ammonium and nitrate to the culture medium as additional nutrients or to the soluble phosphate concentrations (284.7 - 366.6mg/L) reported in

a work carried out with the genetically modified *Aspergillus niger* fungus (Cássia *et al.*, 2014).

Significant differences were found between the data groups of each treatment for each week of study with a significance level of 0.05, applying the Kruskal-Wallis test (Table 4).

**Table 4.** Kruskal –Wallis hypothesis test for a significance level of 0.05 for the treatments during different days of incubation.

Caso	Assumed hypothesis	H < X <sup>2</sup>
i.	H <sub>a</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments for day 1.	0,119<5,87
ii.	H <sub>a</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments by day 5.	0,0415<8,23
iii.	H <sub>0</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments by day 12.	0,01530<10,42
iv.	H <sub>0</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments by day 18.	0,01560<10,38
v.	H <sub>a</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments by day 25.	0,0227<9,56
vi.	H <sub>a</sub> : There is a significant difference in PO <sub>4</sub> <sup>-3</sup> concentration between different treatments by day 31.	0,0230<9,53

The increase of the micellar diameter was a result of the fungi's growth. Aeration allowed constant mixing which contributed to abundant hyphal growth and sporulation in the second week. In all treatments, the formation of supernatant gelatinous masses, or also submerged in the substrate, was observed mainly composed of mycelia of fungi and organic matter. In some treatments, changes are observed in the color of the liquid phase, which turned dark. In addition, samples of the treatments were periodically observed under the microscope, and the presence of inoculated fungi was verified.

In the VP treatment, there was little growth of *Penicillium sp* colonies, in the form of thin superficial films (approx. 1mm) that showed slight sporulation. In VA, there was a growth of *Aspergillus niger* colonies, in the form of superficial films (1-2mm), and abundant sporulation with a darker coloration. In VAP, the majority growth of *Aspergillus niger* colonies (over those of *Penicillium sp*), was observed covering almost the entire surface in the form of superficial films of great thickness (10-30mm) with abundant sporulation and with small islands of colonies of *Penicillium sp*; also, sporulated (less than 10mm in diameter). On a microscopic scale, the growth of the characteristic conidiophores inoculated in VA, VP, and VAP was periodically observed, as well as the gelatinous films formed in which an abundant growth of hyphae was identified.

The metabolism the *Aspergillus niger* and *Penicillium sp.* fungus excretes organic acids. The increase in pH can be explained by different causes including the dissolution of carbonates and / or hydroxyl present in RF. There is also the probability that there was ammonia production because of the unwanted presence of bacteria (Beyenne *et al.*, 2012; Peterson *et al.*, 2005).

According to the results, it can be inferred that *Aspergillus niger* presented a better adaptation to the substrate in the process conditions than *Penicillium sp.* Interestingly. The highest dissolution values were reached in the combined VAP treatment, which may a possible synergistic activity between the two species. Nevertheless, the predominant advance of *Aspergillus niger* was evidenced macroscopically. An interesting feature in the tests where *Aspergillus niger* thrived was the black staining of the aqueous phase, possibly due to the high production of spores; this characteristic coincided with a higher dissolution of phosphates. Finally, it was found that these are a suitable medium for the growth of the fungi *Aspergillus niger* and *Penicillium sp* concerning stillage; following what is suggested by other authors (Ivanova *et al.*, 2006), even at low pH values.

## CONCLUSIONS

All the biological treatments applied in this study contributed to the solubilization of phosphates in phosphate rock with significant differences between them. The treatment that included the mixture of fungi *Aspergillus niger* - *Penicillium sp* reached the highest concentration of soluble phosphate ( $1.13 \pm 0.03$ ), achieving 17.8% of the solution obtained with concentrated sulfuric acid ( $H_2SO_4$ -40%).

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**Conflict of interests:** The authors declare that there is no conflict of interest.

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# Biofertilizer potential of digestates from small-scale biogas plants in the Cuban context

Potencial biofertilizante del digestato obtenido en plantas de biogás a pequeña escala en el contexto cubano.

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## ABSTRACT

The residual sludge from anaerobic digestion known as digestate has been used as a valuable biofertilizer, but the effect of the substrate, the configuration of the reactor and the operating parameter changes the quality and amounts of nutrients in it. Therefore, it is necessary to know its potential characteristic of fertilizer to apply it correctly in crops of national interest. The aim of this work was to characterize the digestate generated from three biodigester technologies (fixed dome, floating drum and tubular) and three substrates: swine manure, cow manure, and co-digestion of swine and cow manure obtained in the province of Sancti Spiritus, Cuba, in terms of nutrient and matter content. For this purpose, stratified statistical sampling was carried out to ensure representative samples and descriptive statistical techniques were used to process the analyses. The digestate was divided into liquid and solid fractions according to the dry matter content (15%). The content of organic matter and ash represented around 50% for both fractions, which enhances its value as a soil conditioner. The nutrient content of both fractions showed good fertilizing properties, having a nutrient ratio ( $\text{NH}_4^+:\text{PO}_4^{3-}:\text{K}^+:\text{SO}_4^{2-}:\text{Mg}^{2+}:\text{Ca}^{2+}$ ) in the liquid (0.002:0.80:0.10:1.00:0.89:0.93) and solid (0.0003:0.96:0.002:1.00:0.52:0.50) fractions, that would contribute to the return nutrients to the soil. The quality of the liquid fraction as irrigation water was assessed as good, according to the relationship between the concentration of the nutrients (Ca, Mg, Na and K) and hardness. Further research is needed on the appropriate dosage for the different crops, and its contribution to sustainable agriculture in the Cuban context.

**Keywords:** biodigester; digestate; fertilizer; irrigation water; nutrient; organic matter.

## RESUMEN

El lodo residual de la digestión anaerobia conocido como digestato ha sido utilizado como un valioso biofertilizante, pero el efecto del sustrato, la configuración del reactor y los parámetros operacionales cambian la calidad y las cantidades de nutrientes en él. Por lo tanto, es necesario conocer su característica potencial de fertilizante para aplicarlo correctamente en

cultivos de interés nacional. El objetivo de este trabajo fue caracterizar el digestato generado a partir de tres tecnologías de biodigestores (campana fija, campana flotante y tubular) y tres sustratos (estiércol: cerdo, vaca y codigestión de ambos) obtenidos en la provincia de Sancti Spíritus, Cuba, en términos de nutrientes y contenido de materia. Para ello, fue realizado un muestreo estadístico estratificado para asegurar muestras representativas y se usaron técnicas de estadística descriptiva para procesar los análisis. El digestato se dividió en fracciones líquidas y sólidas de acuerdo al contenido de materia seca (15%). El contenido de materia orgánica y cenizas representó alrededor del 50% en ambas fracciones, lo que mejora su valor como acondicionador del suelo. El contenido de nutrientes mostró buenas propiedades fertilizantes, con una relación de nutrientes ( $\text{NH}_4^+:\text{PO}_4^{3-}:\text{K}^+:\text{SO}_4^{2-}:\text{Mg}^{2+}:\text{Ca}^{2+}$ ) en las fracciones líquidas (0.002: 0.80: 0.10: 1.00: 0.89: 0.93) y sólidas (0.0003: 0.96: 0.002: 1.00: 0.52: 0.50), que contribuirían al retorno de nutrientes al suelo. La calidad de la fracción líquida como agua de riego se evaluó como buena, de acuerdo con la relación entre la concentración de los nutrientes (Ca, Mg, Na y K) y la dureza. Futuras investigaciones evaluarán la dosis adecuada de digestato para los diferentes cultivos y su contribución a la agricultura sostenible en el contexto cubano.

**Palabras clave:** agua de riego; biodigestor; digestato; fertilizante; materia orgánica; nutriente.

## INTRODUCTION

Anaerobic digestion (AD) has proven to be an economically feasible technology for the cyclic use of large-scale and small-scales organic waste (Huang *et al.*, 2016; Alkhalidi *et al.*, 2019). It is a widely used to convert organic material into energy-rich biogas and the residual sludge known digestate (Albuquerque *et al.*, 2012). Digestate can be used as a biofertilizer for arable land, enabling recirculation of plant nutrients, and thus reducing the need for fossil fuel-dependent inorganic fertilizers (Alkhalidi *et al.*, 2019). The rapid development of biogas plants in European countries has increased the recycling of waste for energy generation, while producing large quantities of digestate (Franchino *et al.*, 2016). There has been a tendency in the last decades of increased emphasis on improved sustainability in agriculture and preservation of natural resources, thus changing the focus of digestate processing from nutrient removal and disposal, to integrated nutrient recovery and recycling (Drosg *et al.*, 2015). This contributes to promoting the circular economy in the agroindustrial sector, relevant aspect at present (Suárez *et al.*, 2019). The use of digestate has great importance, especially in developing countries, not only because inorganic fertilizers are often too expensive, but also

because recycling nutrients from organic sources is essential.

The concentration variability of macronutrients, micronutrients and matter in digestate depends on substrate type, the anaerobic digestion performance and the digestate posttreatment like type of solid-liquid separation (Makádi *et al.*, 2012; Akhiar *et al.*, 2017). When digestate is obtained from different manures, the nutrient composition is greatly dependent on several factors such as digestion type (omnivore, ruminant), sex, species, age and diet of the animals, as well as the geographical and climatic conditions of the regions (Lukehurst *et al.*, 2010; Risberg *et al.*, 2017).

In Cuba, researchers have been mainly focused on the direct application of digestate on beans (Negrin Brito and Jiménez Peña, 2012), tomatoes (Utria-Borges *et al.*, 2008; López Dávila *et al.*, 2017a), carrot, radish (Hernández *et al.*, 2008) and onion (López Dávila *et al.*, 2017b). These authors focused on digestate application to crops and its effect on plants. In all cases, they obtain benefits in terms of crop yield. However, very few information was found on the characteristics of the digestate applied (considering the type of biodigester and the substrate used for AD) in the Cuban context; which

leads to excessive application of nutrients in crops becoming toxic for plants. The characterization of Cuban digestate in terms of nutrient and matter contents can enable to assess it as biofertilizer and use it safely, with possible positive impacts on crop yields and reduction in the use of inorganic fertilizer. Therefore, the aim of this work is to characterize the digestate obtained in the province of Sancti Spíritus, Cuba, in terms of nutrient and matter content, considering the type of biodigester and the substrate used during AD processes. To this end, the objectives were (i) to classify the digestate in terms of liquid and solid fraction, (ii) to determine the content of matter and nutrients using classical and instrumental analytical techniques, as well as (iii) to evaluate the liquid fraction as irrigation water. The characterization of the digestate can determinate its biofertilizer potential, which has positive environmental impact due to the recirculation of nutrients from substrates considered waste and the decrease in the demand of inorganic fertilizer. Which help reduces the exploitation of natural reserves and also the high pollution that is generated in the industrial manufacture of these inorganic fertilizer.

## MATERIALS AND METHODS

The methods applied to fulfill the objective of this research are described in this section. Firstly, overviews of the existing biodigester technologies in Sancti Spíritus (21°56'02"N 79°26'38"W), Cuba are briefly described. Later, the methods for the sampling procedure, the physical-chemical analysis and the characterization as irrigation water are also explained.

**Biodigester technologies.** The most widespread biodigesters in Cuba are: fixed dome biodigester, floating drum biodigester and tubular biodigester (Suárez-Hernández *et al.*, 2018).

**Fixed dome biodigesters.** Consist of a closed system, usually built of masonry and under ground

level. These biodigesters have a fixed dome-shaped cover that contains the biogas inside, an inlet to feed the substrate, and a digestate outlet that allow solid and liquid phases separation to produce digestate in both states, without mechanical process. The lack of agitation in this technology allows the solids to form flocs and precipitate to the bottom of the reactor (increasing the solid retention time), while in the compensation tank the liquid digestate comes out by overflow. The sludge, together with other non-degraded materials that precipitate, is extracted every 15 or 30 days and forms the solid digestate.

**Floating drum biodigesters.** are formed by a masonry cylinder in its lower part (with a stop to support the bell), and a floating bell storing the gas in the upper part. Inside the reactor, the substrate flows uniformly allowing a better contact of the microorganism and the substrate, and a higher degradation of the organic matter with respect to fixed dome biodigesters. The digestate is collected as sludge.

**Tubular biodigesters.** are formed by a bag resistant to environmental conditions. The substrates are fed by the inlet pipe and occupy the bottom of the bag while the upper part serves as a container for the biogas generated during the operation. Once the fed substrate has been digested, the digestate leaves the bag through the outlet pipe, coming out in the form of sludge, similar to the floating drum biodigester.

**Digestate collection.** The number of samples was calculated using the methodology established by Vivanco (2005) through equation 1:

$$n = \frac{NZ^2 * pq}{d^2 (N-1) + Z^2 * pq} \quad (1)$$

Where:

n: number of digesters to be sampled

- $p$ : proportion of digesters in operation ( $p = 0.8$ )  
 $q$ : proportion of digesters out of operation ( $1 - p$ ) ( $q = 0.2$ )  
 $N$ : size of the population (number of digesters)  
 $Z$ : deviation from the mean value accepted to achieve the desired level of confidence, based on the 95% confidence level, with  $Z = 1.96$ .  
 $d$ : level of absolute precision, referring to the amplitude of the confidence interval desired in the determination of the average value of the variable under study. This value was taken as 0.16.

Taking into account the existence of several types of digesters in the province of Sancti Spíritus and the different types of substrates, it is necessary to carry out a random stratified sampling procedure (descriptive analysis), to ensure that each technology and substrate are proportionally represented in the final sample. For that aim, equation 2 was used (Vivanco, 2005):

$$fh = \frac{n}{N} \quad (2)$$

Where:

fh: fraction of the stratum; n: sample size;

N: population size.

The following operating parameters: temperature range, hydraulic retention time, reactor volume, feeding substrate and methane productivity were considered in the descriptive analysis and evaluation in the equation 1 and 2.

The digestate was taken in all cases at the biodigesters outlet. The collection of samples in tubular and floating drum biodigesters was carried out by using a valve located in the lower part of the biodigesters. In the fixed dome biodigesters, the liquid and solid fractions were collected separately as the technology allows that. The liquid fraction was taken from the compensation tank, prior to the exit of the pos-treatment system (lagoons); while the solid fraction was collected from the sludge

outlet at the biodigester bottom. All samples were stored in sterile glass bottles and kept in freezing at  $-20^{\circ}\text{C}$  to avoid biodegradation until their analysis.

**Physical - chemical analyses.** As the liquid digestate is highly colored and turbid due to the presence of suspended matter, all digestate were converted to ashes. The ashes were used for determining sodium, potassium, sulfate, phosphate, calcium and magnesium, matters content, electrical conductivity and ammonium nitrogen were determinate in fresh samples.

For the physical-chemical characterization of digestate, *The Standard Methods for the Examination of Water and Wastewater* (APHA 2012) were used. The content of dry matter (DM, %) (section 2540-B), fixed matter (ash, %) (section 2540-E) and organic matter (OM, %) (section 2540-E), were determined by gravimetric methods. Calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) were determined by complexometric titration (sections 3500-Ca-B and 3500-Mg-B, respectively); Orthophosphate (section 4500- $\text{PO}_4^{3-}$ -C) and sulfate (section 4500- $\text{SO}_4^{2-}$ -E), by spectrophotometry; and ammonium nitrogen by (Kjendalh) (section 4500- $\text{NH}_4^+$ -C). In addition, it was determined Potassium  $\text{K}_2\text{O}$ -K (section 3500-K-B) and sodium  $\text{Na}_2\text{O}$ -Na (section 3500-Na-B), by flame photometry, whereas the electrical conductivity (EC) were determined by conductimetry (section 2520-Salinity-B).

**Characterization as irrigation water.** To assess the quality of the liquid fraction of digestate as water for irrigation, the sodium adsorption ratio (SAR) (Del Valle, 1992) and the Kelly ratio (KR) (Quispe Mamani, 2016) were used (Equations 3 and 4, respectively). In addition, the relationship of both parameters with the hardness and electrical conductivity was taken into account to classify the quality of digestate as irrigation water.

$$\text{SAR} = \frac{c(\text{Na})}{\sqrt{\frac{c(\text{Ca})+c(\text{Mg})}{2}}} \quad (3)$$

$$\text{KR} = \frac{c(\text{Ca})}{c(\text{Ca})+c(\text{Mg})+c(\text{Na}) \times 100} \quad (4)$$

Where  $c(\text{Na})$  is the sodium concentration ( $\text{meq L}^{-1}$ ),  $c(\text{Ca})$  is the calcium concentration ( $\text{meq L}^{-1}$ ) and  $c(\text{Mg})$  is the magnesium concentration ( $\text{meq L}^{-1}$ ).

**Statistical analysis.** All statistical descriptive analysis (biogas technology and substrate used frequency as well as predominant nutrient and matter contents per substrate) performed in the selection and evaluation of technologies and samples were done with SPSS software, version 23,0 (SPSS, 2013). The analyses were performed in triplicate, the central tendency and dispersion statistics (mean, standard error of the mean, and ranges) were calculated as shown in the figures and tables of the study.

## RESULTS AND DISCUSSION

Digestates were collected from 20 biogas plants (according to the results from equation 1), located 11 in Cabaiguán municipality ( $22^{\circ}05'02''\text{N } 79^{\circ}29'43''\text{W}$ ) and 9 in Banao town ( $21^{\circ}82'86''\text{N } 79^{\circ}56'86''\text{W}$ ). To ensure representative samples, three strata were formed in accordance with the type of biogas plant (Table 1), which consists of 13 fixed dome, 1 floating

drum and 6 tubular biogas plants, (according to equation 2). In addition, in accordance with the type of substrates, three strata were also considered consisting of 13 swine manure (SM), 2 cow manure (CM) and 5 codigestion of swine and cow manure (SCM) (equation 2).

Table 1 shows, the operating parameters: temperature range (mesophilic) and hydraulic retention time (HRT) (15–50 days), were equal for all plants; while reactor volume ( $1.8\text{--}50 \text{ m}^3$ ) and feeding ( $2\text{--}4 \text{ kg of volatile solid/day/m}^3$ ) were different. The methane productivity of all plants was  $0.5 \text{ m}^3 \text{ reactor/day}$ .

As 50% of the digestate produced in Sancti Spiritus, corresponds to fixed dome biogas plants treating SM, they were the higher strata (13 biogas plants). This can be attributed to the decentralized pig production in Cuba, the necessity for SM treatment in situ and the high dissemination of fixed dome biogas plants (Sosa *et al.*, 2014). For that reason, this substrate and this technology were chosen for a more detailed analysis in the present study.

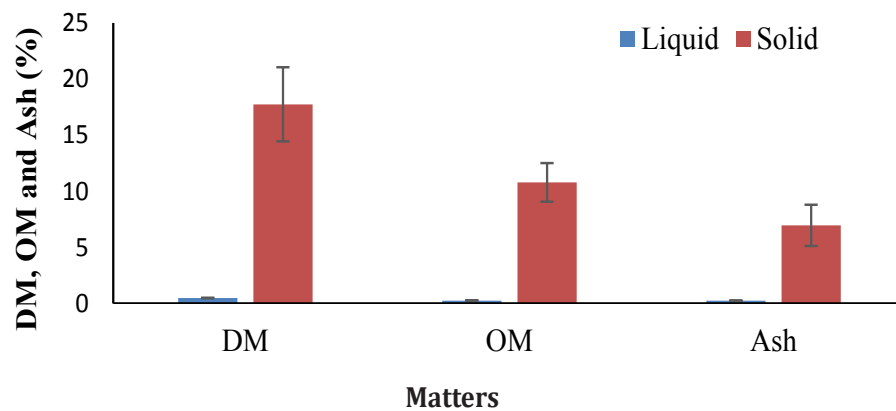
### Digestate characterization

**Dry matter.** The content of dry matter (DM), organic matter (OM) and ash for SM digestate (solid and liquid fractions) obtained from fixed dome biogas plants is shown in Figure 1.

**Table 1.** Operating parameters and methane productivity for small-scale biogas plants which produced the digestates studied.

Biogas plant types	Reactor Volume ( $\text{m}^3$ )	Temperature range	Feeding ( $\text{kg/day/m}^3$ )	HRT (days)	Methane productivity ( $\text{m}^3 \text{ reactor/day}$ )
Fixed dome	20-50	Mesophilic	2	15-50	0.5
Floating drum	1,5	Mesophilic	2	15-50	0.5
Tubular	3	Mesophilic	2-4	15-50	0.5

HRT: hydraulic retention time.



**Figure 1.** Contents of dry matter (DM), organic matter (OM) and ash in swine manure (SM) digestate (solid and liquid fractions) obtained from fixed dome biodigesters. The bars indicate the standard deviation.

The solid fraction of the digestate showed a DM content of  $17.77 \pm 3.30\%$  (Figure 1), being considered as a solid digestate, following the approach of Makádi *et al.* (2012), who suggested this classification for DM content in digestate above 15%. For that reason, SM digestate (solid fraction) obtained from fixed dome biodigesters can be considered as biofertilizer rich in stable and fibrous material which should have a positive effect on soils by minimizing erosion and improving water-holding capacity and soil structure, especially when they are used on sandy soils (Bermejo *et al.*, 2010). The liquid fraction of this digestate presented a very low DM content (average  $0.48 \pm 0.03\%$ ), due to the large dilution of the SM occurring when more than 40 L of water per animal head are used during the cleaning of piggeries (Sosa *et al.*, 2014).

**Organic matter.** The efficiency of OM conversion through mesophilic (i.e.  $35^{\circ}\text{C}$ ) or thermophilic (i.e.  $55^{\circ}\text{C}$ ) AD is generally in the range of 13–65%, and depends on the type of substrate fed to the biodigester, as well as on anaerobic reactor parameters, such as the Organic Loading Rate (OLR) and the HRT (Menardo *et al.*, 2011). In the solid and liquid fractions of the SM digestate, OM represented more than 50% of DM, agreeing

with the values reported by Marcato *et al.* (2008). These high OM values can be attributed to several aspects: 1) the high OLR and short HRT applied to fixed dome biodigesters in piggeries; as the number of pigs increases during the time for the same biodigester volume; and 2) the low removal efficiency (<50%) of organic matter reported for fixed dome biodigesters due to its cylindrical shape that does not ensure a uniform path of the substrate inside the biodigester (Montalvo and Guerrero, 2003).

These aspects induce a rise of the more recalcitrant molecules in digestates, such as lignin, cutin, humic acids, steroids, complex proteins. These non-degraded stable carbon compounds are beneficial for the soil, being potential humus precursors with high biological stability (Kalakodio *et al.*, 2017; Diacono *et al.*, 2019) "plainCitation": "(Kalakodio *et al.* 2017; Diacono *et al.* 2019. This allows increase fertility, functionality, microbial activity, aeration, and water storage capacity in the soil (Kalakodio *et al.*, 2017).

**Ash.** The ash content in the solid and liquid fractions was 39% and 50%, respectively. During AD, organic compounds are broken down by bacteria resulting in the production of methane and



carbon dioxide. Thus, as a result of the digestion process, a number of changes in the solid content can be expected; including a substantial reduction (up to 25%) in the total solids and a consequent increase in the ash content (as %DM), due to the conservation of minerals and the organic matter removal (Fageria and Moreira, 2011).

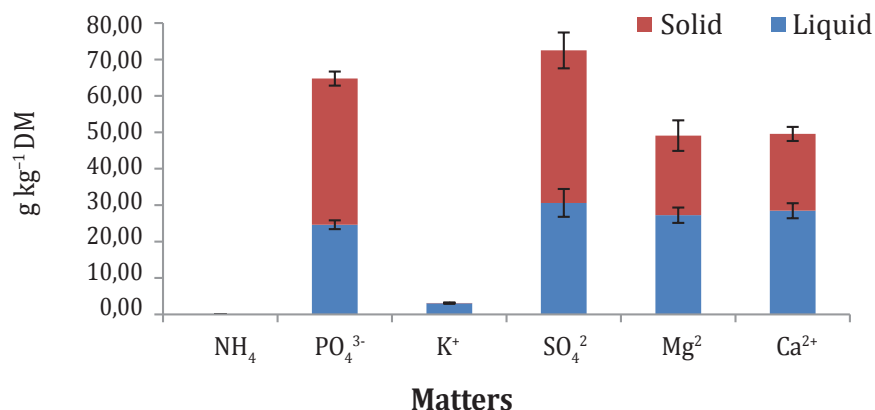
**Matter content of the remaining digestates.** A summary of DM, OM and Ash for the remaining digestates available in Sancti Spíritus is provided in Appendix A. The DM content of the digestate solid fraction obtained from fixed dome biodigester during the treatment of SCM, rendered similar values ( $17.50 \pm 1.89\%$ ) with respect to fixed dome biodigester using SM only. However, a lower DM content was obtained in tubular and floating drum biodigesters ( $7.08 \pm 0.10\%$  and  $4.42 \pm 0.20\%$ , respectively) treating CM. Tubular biodigesters using SM and SCM reported a DM content of  $1.02 \pm 0.03\%$  and  $6.10 \pm 0.30\%$ , respectively. Organic matter and ash represented from 40-50% and from 20-40% of DM, respectively. These values make the digestates available in Sancti Spiritus, a suitable soil amendment, based on its matter content.

**Nutrient content.** Figure 2 shows the nutrient content of SM digestate in term of nitrogen ( $\text{NH}_4^+\text{-N}$ ),

phosphorous ( $\text{PO}_4^{3-}\text{-P}$ ), sulfates ( $\text{SO}_4^{2-}\text{-S}$ ), calcium ( $\text{Ca}^{2+}\text{-Ca}$ ), magnesium ( $\text{Mg}^{2+}\text{-Mg}$ ) and potassium ( $\text{K}^+\text{-K}$ ) concentrations ( $\text{g kg}^{-1}\text{DM}$ ).

**Nitrogen.** The  $\text{NH}_4^+\text{-N}$  concentration in biofertilizers is of great importance as it is immediately available to the plant. The concentrations of  $\text{NH}_4^+\text{-N}$  in the liquid and solid fractions were  $0.06 \pm 0.00$  and  $0.01 \pm 0.00 \text{g NH}_4^+ \text{kg}^{-1} \text{DM}$ , respectively. The N content of the digestate was a consequence of its preservation during AD (Tambone *et al.*, 2010; Drosig *et al.*, 2015). During organic matter degradation, part of the organically bound nitrogen is reduced to the  $\text{NH}_4$  form, (Kuusik *et al.*, 2017). The  $\text{NH}_4^+\text{-N}$  is concentrated in the digestate by the degradation of the proteins available in the fed substrate (Kryvoruchko *et al.*, 2009). Typically, pigs include a high protein content in their diet (e.g., animal feed, food waste and slaughterhouse waste), as part of these proteins are not assimilated by the pigs, being expelled in the excreta; they are degraded to  $\text{NH}_4^+$  during the AD process and released in the liquid digestate mainly.

For that reason, values between 1.00 and 1.80g of  $\text{NH}_4^+ \text{kg}^{-1} \text{DM}$  has been considered as typical for the liquid digestate obtained from biogas plants treating SM (Rossi and Mantovi, 2012; Tigrini



**Figure 2.** Nutrient content expressed in g per kg of dry matter (DM), in the swine manure (SM) digestate (solid and liquid fractions). The bars indicate the standard deviation.

*et al.*, 2016). However, in this study the  $\text{NH}_4^+$ -N concentration in the liquid fraction was 0.06 g of  $\text{NH}_4^+$   $\text{kg}^{-1}$  DM, being 60% of the typical values reported (Rossi and Mantovi, 2012; Tigrini *et al.*, 2016). The higher concentration of N for the liquid fraction with respect to the solid fraction, agreed with Tampio, Marttinen and Rintala (2016), who stated that solid-liquid separation is increasing in application during digestate treatment for the extraction of nitrogen from the liquid fraction.

**Phosphorous.** The phosphorus content of digestate is expressed as total phosphorus or as phosphate equivalents. The AD process does not affect the content of phosphate in digestate, which is mostly dependent on the content in the substrate (Drosg *et al.*, 2015). The concentration of  $\text{PO}_4^{3-}$ -P obtained in this study for the liquid fraction digestate, was  $14.63 \pm 1.20$  g  $\text{kg}^{-1}$  DM, agreeing with the values reported in Albuquerque *et al.* (2012), being 7 times higher than the values (1 to 2 g  $\text{PO}_4^{3-}$   $\text{kg}^{-1}$  DM) reported by Rossi and Mantovi (2012).

The concentration of  $\text{PO}_4^{3-}$ -P for the solid fraction digestate was  $40.14 \pm 1.93$  g  $\text{kg}^{-1}$  DM, showing that phosphorous is mainly accumulated in the solid fraction of digestates, which agree with the values reported by Tampio *et al.* (2016) (Figure 2). Phosphates are a limited non-renewable resource, which is as an essential plant nutrient that cannot be replaced by other substances (Shepherd *et al.*, 2016). Therefore, the use of this digestate for the Cuban agriculture can generate economic and environmental benefits, by substituting P-rich commercial biofertilizers; which is of prime importance in the recycling of limiting nutrients like phosphorous.

**Potassium.** Potassium concentrations of  $0.09 \pm 0.00$  g  $\text{kg}^{-1}$  and  $3.06 \pm 0.17$  g  $\text{kg}^{-1}$  DM were obtained for the solid and liquid fractions of the

digestate, respectively. Albuquerque *et al.* (2012) reported values from 50 to 1000 times higher for the liquid and solid fractions, respectively. As potassium degradation during AD is negligible, the differences found in potassium concentration can be attributed to the pig diet in the different regions. For example, pigs Cuban farms eats cereals but also home-made food such as cassava and sweet potato yogurt (Almaguel *et al.*, 2016) castrated males and females in equal proportion of 85 days old with an average initial weight of 30.0 kg. The pigs were distributed according to a random blocks design in four experimental treatments and 12 replicates per treatment (position inside the pen).

**Sulfates.** A high sulfate concentration of the solid and liquid fractions was observed, with average concentrations of  $41.89 \pm 4.92$  and  $30.61 \pm 3.82$  g  $\text{kg}^{-1}$  DM, respectively (Figure 2). These values were similar to those reported by Chen *et al.* (2010), who states that this is also related to the proteins consumption in the pigs diet.

**Calcium and Magnesium.** In the solid and liquid fractions, the Ca:Mg ratio was around 1:1; being these values lower than those reported by (Negrin Brito and Jiménez Peña, 2012) for a digestate obtained from the AD of agricultural waste and manure (88 Ca% and 12 Mg%DM ). A further discussion will be provided in the next section about Ca and Mg concentrations in the liquid fraction of the SM digestate.

In general, AD enables the attainment of a final product (digestate) with good fertilizing properties, having a nutrient ratio ( $\text{NH}_4^+:\text{PO}_4^{3-}:\text{K}^+:\text{SO}_4^{2-}:\text{Mg}^{2+}:\text{Ca}^{2+}$ ) in the liquid (0.002:0.80:0.10 :1.00:0.89:0.93) and solid (0.0003:0.96:0.002:1.00:0.52:0.50) fractions, that would contribute to return macronutrients to the soil after AD of SM. A summary of the nutrient content for the digestates available in Sancti Spíritus is provided in Table 2.

**Table 2.** Nutrient and matter content of the digestate taken from fixed dome, tubular and floating drum digesters when using different substrates (n=3).

Characteristic	Fixed dome						Tubular		Floating drum	
	SM		SCM		SM	SCM	SCM	CM	CM	CM
	Liquid	Solid	Liquid	Solid	Solid	Liquid	Liquid	Liquid	Solid	CM
DM (%)	0.48 ± 0.03	17.77 ± 3.30	0.55 ± 0.01	17.50 ± 1.89	1.02 ± 0.03	6.10 ± 0.30	7.08 ± 0.01	4.42 ± 0.02		
OM (%)	0.24 ± 0.04	10.78 ± 1.72	0.17 ± 0.01	11.69 ± 0.82	0.58 ± 0.07	2.39 ± 0.17	4.99 ± 0.76	3.05 ± 0.42		
Ash (%)	0.24 ± 0.03	6.96 ± 1.84	0.18 ± 0.01	5.48 ± 0.57	0.43 ± 0.05	1.20 ± 0.19	2.09 ± 0.23	1.37 ± 0.01		
NH <sub>4</sub> <sup>+</sup> - N (g kg <sup>-1</sup> DM)	0.06 ± 0.00	0.01 ± 0.00	0.04 ± 0.00	0.003 ± 0.00	0.04 ± 0.00	0.002 ± 0.00	0.003 ± 0.00	0.001 ± 0.00		
PO <sub>4</sub> <sup>3-</sup> P (g kg <sup>-1</sup> DM)	24.63 ± 1.20	40.14 ± 1.93	11.93 ± 0.81	17.03 ± 0.34	13.29 ± 1.32	7.23 ± 0.22	6.64 ± 0.02	12 ± 0.06		
K <sup>+</sup> - K (g kg <sup>-1</sup> DM)	3.06 ± 0.17	0.09 ± 0.00	2.65 ± 0.31	0.06 ± 0.02	1.45 ± 0.27	0.25 ± 0.02	0.16 ± 0.01	0.36 ± 0.16		
SO <sub>4</sub> <sup>2-</sup> -S (g kg <sup>-1</sup> DM)	30.61 ± 3.82	41.89 ± 4.92	18.67 ± 1.51	45.00 ± 1.29	29.92 ± 1.55	68.13 ± 4.80	12.82 ± 0.02	56.32 ± 0.23		
Ca <sup>2+</sup> - Ca (g kg <sup>-1</sup> DM)	28.47 ± 2.06	21.09 ± 1.94	18.50 ± 1.70	31.54 ± 2.83	15.82 ± 1.68	4.47 ± 0.30	12.23 ± 0.15	7.80 ± 0.26		
Mg <sup>2+</sup> - Mg (g kg <sup>-1</sup> DM)	27.24 ± 2.10	21.87 ± 4.42	20.27 ± 1.00	7.79 ± 1.08	10.74 ± 0.93	1.27 ± 0.38	5.51 ± 0.17	4.48 ± 0.21		
pH	7.30 ± 0.02	7.34 ± 0.03	7.24 ± 0.21	7.49 ± 0.29	7.20 ± 0.09	7.11 ± 0.08	7.18 ± 0.08	7.09 ± 0.01		
EC (μS cm <sup>-1</sup> )	1.04 ± 0.01	1.31 ± 0.02	1.15 ± 0.04	1.05 ± 0.04	1.37 ± 0.09	1.46 ± 0.11	1.33 ± 0.01	1.47 ± 0.01		

SM: swine manure, SCM: cow manure, SCM: Co-digestion of swine and cow manure, DM: Dry matter, OM: Organic matter, EC: Electrical conductivity.

**Liquid fraction of digestate as irrigation water.** The liquid fraction generated from SM was assessed as water for irrigation based on its content of interchangeable ions, EC and hardness. Table 3 shows the experimental measurements, the calculations of these parameters and their acceptance criteria.

SAR and KR were calculated according to equations described by Del Valle (1992) and Quispe Mamani (2016), respectively. The SAR value was 0.03 for the liquid digestate, indicating its low sodification power. In general, the higher the SAR, the less suitable the water is for irrigation (Aboukarima *et al.*, 2018) in the order  $305 > 240 > 137 > 104 > 65$  mm/h for SAR of 3.34, 3.52, 4.14, 4.18, and 7.60, respectively. The results showed that 180 min after the initial time of measurement in the sandy-loam soil, the final infiltration rates were in the range of 21.1–44.0mm/h for the different qualities of water considered in this study, with an average value of 33.8mm/h. Hence, the infiltration rate is sensitive to the SAR of the applied water. The final infiltration rate (IRf). That is either diminish the soil permeability, reducing the formation of crusts that can modify the physical-chemical properties of the soil with the consequent deterioration of the crop yield (Lesch and Suarez, 2009). Moreover, the KR value for the liquid digestate (<28%) was lower than

the optimum value (higher than 35%) for plant growing (Quispe Mamani, 2016), because of the higher proportion of Mg with respect to Ca, and the low contribution of Na concentration (see Eq. 4). In terms of hardness, the digestate was considered as very hard water (78.5°F) because its values were higher than the acceptance criterion of 54°F.

According to the diagram for the interpretation of the irrigation water value (Riverside Standards) (Olías *et al.*, 2005), digestate was classified as C3S1. That is, digestate has highly saline waters ( $EC\ 1320\ \mu S\ cm^{-1}$ ); therefore, there must be good drainage conditions, salinity must be controlled and only plants that are resistant to salinity should be cultivated. On the other hand, it presents a low alkalization hazard (SAR=0.03), so it can be used without serious damage to plant development.

Besides, according to the Wilcox Standard (Olías *et al.*, 2005), which takes into account the percentage of Na over the other cations (Ca, Mg and K) and the EC, it was obtained that the digestate of Sancti Spiritus is in the category from “good” to “admissible”, representing a potential alternative to increase agriculture sustainability in the Cuban context. Further studies are needed on the appropriate dosage for the different crops.

**Table 3.** Evaluation of swine manure liquid fraction digestate as water irrigation.

Parameters	EC ( $\mu S \cdot cm^{-1}$ )	Hardness (°F)	SAR	KR (%)
Liquid digestate	1320.00	78.50	0.03	28.22
Acceptance requirements	< 750 Excellent 750 – 3000 Good >3000 Unacceptable	0-22 Sweet 32-54 Hard > 54 Very hard	< 10 Optimum	> 35 Optimum

SAR: Sodium Adsorption Ratio, KR: Kelly's ratio, EC: Electrical conductivity.

## CONCLUSION

The digestates obtained in biodigesters in the province of Sancti Spíritus, Cuba were characterized in terms of nutrient and matter content. Most of the digestate samples (50%) were taken from fixed dome biodigesters treating SM because they were widely available in Sancti Spíritus.

The highest dry matter content (DM) was obtained for the solid digestate sampled from fixed dome biodigesters, with values above 17%, being considered as stable and fibrous material which should have a positive effect on soils, by minimizing erosion and improving water-holding capacity and soil structure.

The nutrient content of both digestates showed good fertilizing properties, having a nutrient ratio ( $\text{NH}_4^+:\text{PO}_4^{3-}:\text{K}^+:\text{SO}_4^{2-}:\text{Mg}^{2+}:\text{Ca}^{2+}$ ) in the liquid (0.002:0.80:0.10:1.00: 0.89:0.93) and solid (0.000 3:0.96:0.002:1.00:0.52:0.50) fractions, that would contribute to the return of organic matter and nutrients to the soil in a short-term period.

Swain manure treated in fixe dome biodigester was the one that produced the most nutrient-rich digestate, therefore, it was the one with the highest biofertilizing potential.

The quality of the liquid fraction as irrigation water was assessed as good, according to the relationship between the concentration of the nutrients (Ca, Mg, Na and K) and hardness, agreeing with the water classification for irrigation of the Wilcox and Riverside standards.

**Conflict of interest:** The authors declare that there is no conflict of interest.

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# Quality parameters of pink pepper seeds as sustainability indicators

## Parámetros de calidad de las semillas de pimienta-rosa como indicadores de sostenibilidad.

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### ABSTRACT

The poor management and marketing of rose pepper (*Schinus terebinthifolius* Raddi) in São Pedro D'Aldeia - RJ called the local governments' attention to adopt measures that favor the value chain, increase income, train rural producers, and launch the first Simplified Forest Management Plan. All of this, to authorize the sustainable management of this species. This study aimed to evaluate the quality parameters of pink pepper as sustainability indicators. The following was measured: the moisture content, purity, uniformity in the sieve, and the weight of one thousand seeds in samples obtained from three different batches. The germination test was only carried out for samples from lot 3. The results showed that the high moisture content of the seeds (22.99%) has a negative effect on their quality, facilitating the action of spoilage organisms. The degree of purity was satisfactory (97.1%). The seeds from lot 1 were larger and more uniform than the seeds from lots 2 and 3. Only lot 1 presented the weight of seeds compatible with that required by MAPA. The germination rate was 72%, which did not vary between seeds of different sizes. The settlement's extractive activity is moving towards sustainability, which can be improved by incorporating good seed collection, processing and storage practices.

**Keywords:** *S. terebinthifolius*; seed analysis; rural settlement; sustainable development; extractivism.

### RESUMEN

El inadecuado manejo y la comercialización de la pimienta-rosa (*Schinus terebinthifolius* Raddi) en São Pedro D'Aldeia - RJ, llamó la atención de los gobiernos locales sobre la adopción de medidas que favorezcan la organización de la cadena de valor; generación de ingreso; capacitación de productores rurales y la elaboración del primer Plan de Manejo Forestal Simplificado para autorizar el manejo sustentable de la especie. Este estudio tuvo como objetivo evaluar los parámetros de calidad de la pimienta-rosa como indicadores de sostenibilidad. Para ello se determinó: el contenido de humedad, pureza,



uniformidad en tamiz y peso de mil semillas en muestras obtenidas de tres lotes diferentes. La prueba de germinación solo se realizó para muestras del lote 3. Los resultados mostraron que el alto contenido de humedad de las semillas (22,99%) tiene un efecto negativo en su calidad, facilitando la acción de organismos deterioradores. El grado de pureza fue satisfactorio (97,1%). Las semillas del lote 1 fueron más grandes y uniformes en comparación con las semillas de los lotes 2 y 3. Solo el lote 1 presentó un peso de semillas compatibles con lo requerido por MAPA. La tasa de germinación fue del 72%, la cual no varió entre semillas de diferentes tamaños. La actividad extractivista en el asentamiento avanza hacia la sostenibilidad, la cual se puede mejorar mediante la incorporación de buenas prácticas de recolección, procesamiento y almacenamiento de semillas.

**Palabras clave:** *S. terebinthifolius*; análisis de semillas; asentamiento rural; desarrollo sostenible; extractivismo.

## INTRODUCTION

Pink pepper (*Schinus terebinthifolius* Raddi) is an Anacardiaceae native to Brazil. It is a helophyte plant that also grows in dry and poor soils. Its crown is rounded, and its trunk is tortuous with thick and cracked bark. It occurs from Pernambuco to Santa Catarina and Mato Grosso do Sul and, it is found in several vegetation formations, from the sandbank to the high semi-deciduous and rain forests (Neves *et al.*, 2016).

The pepper exploitation in Rio de Janeiro began to intensify in late 1990's and has been growing in recent years with the main extractive activity in the region. The informal pink pepper market was evidenced around the 2000s due to the increase in the cargo seizures occurrences. In the state's scale of participation in exploring the species, Rio de Janeiro occupied first place, representing 42% of the market with 640 tons per harvest (Gomes *et al.*, 2013).

Due to lack of legislation knowledge, management techniques for exploration and security, the collectors of Baixada Litorânea, especially those from the Ademar Moreira settlement farmers, acted without structuring the productive segment without knowledge about pruning techniques, fruit selection, processing, and storage.

Currently, the legal basis for the exploitation of pink pepper is provided by Federal Law n. 11.428/2006

(Presidência da República de Brasil, 2006) which requires an authorization instrument from the competent state or federal agency for the cutting, suppression, and commercial exploitation of trees in the Mata Atlântica biome, and the Resolution No. 124/2015 from INEA ch. V, art. 26 (INEA, 2015) which state the norms for Non-Timber Forest Products. If their exploration is carried out for commercial purposes, it will be considered Sustainable Forest Management and will depend on the presentation and approval of a Simplified Forest Management Plan for the forest species that will be exploited commercially.

In 2017, the SENAR-RJ (Serviço Nacional de Aprendizagem Rural-RJ) promoted the settlement farmers involved in extractives courses on pink pepper management and acceptable practices. Moreover, INEA contemplated the Settlement's Farmers Association with the first environmental authorization for species' sustainable forest management in May 2018, developed by the working team Aroeira (INEA, 2018). The three spheres of government acted together and actively elaborated the management plan, training and organizing of farmers and giving technical assistance.

The non-timber simplified forest management plan was first developed in the RJ state. It includes the exploitation of pink pepper inside the Ademar Moreira Settlement in collective legal reserve,

regularizing this farmer's traditional activity. The authorization recognizes the Natural resources, sustainable economic exploitation importance for income generation in rural communities, and environmental conservation.

Inadequate harvesting and processing can result in a rapid deterioration and fruit quality loss, negatively affecting production sustainability. The lack of seeds quality can be expressed in the low uniformity index, degree of maturity, insects and fungi that hinder better yield in harvesting and marketing with a loss of market value for sale. The problem can be solved through better dissemination and technology transfer to harvest fruits appropriately and apply conservation or pre-processing techniques (Gomes *et al.*, 2013).

In this way, the incentive for rural producers' technical qualification concerning acceptable pink pepper management practices is in accordance with two Sustainable Development Goals (SDG) established by United Nations (2015):

Goal n. 12: Ensure sustainable production and consumption patterns, aiming to contribute to 2030 agenda, achieving sustainable management and efficient use of natural resources and ensuring that people have relevant information for developing sustainable eco-friendly lifestyles; and

Goal n. 15: To protect, recover and promote the use of terrestrial ecosystems, managing forests, combating desertification, reserving land degradation and stopping biodiversity loss.

The low quality of the product directly interferes with market competitiveness and profit, making extractive activity unsustainable. Therefore, this work aims to analyze the quality of pink pepper seeds and guide producers to adopt appropriate techniques for collecting, conserving and, storing seeds; to increase the extractive activity's quality and sustainability.

## MATERIALS AND METHODS

**Characterization of the study area.** Seed collection was carried out at the federal settlement Ademar Moreira (coordinates -22.713908, -42.108458), São Pedro D'Aldeia / RJ. The climate is tropical, with an average annual temperature ranging between 24 and 28°C. November and December are the months with the highest rainfall, while June and July are the driest.

The settlement consists of 21 families. Sixteen are involved in the extraction of pink pepper, and part of the average family income comes from the sale of pink pepper.

The settlement comprises 487.68ha inserted in the Atlantic Forest biome. Of them, 180.37ha are intended to harvest pink pepper. However, each lot has an average of 20 individuals in management's potential (INEA, 2018).

**Seed collection and analysis.** The collection of seed samples from Lots 1 and 2 was carried out *in loco*. The samples were acquired from a bouquet of seeds from different pink pepper trees, harvested in May/2018 and June/2018. The sample from Lot 3, taken in May 2019, was acquired at a bio-economy event (Green Rio), directly from Ademar Moreira settlement producers.

From each lot, samples composed of pink pepper seeds of adequate size for the tests and in similar proportions were obtained to guarantee uniform and accurate results in the analyses. The composed samples were taken at random and manually removed from the lots, each weighing approximately 1.000g.

The samples were stored in individual packages of transparent and impermeable plastic, identified;

according to the lot they were collected. Subsequently, they were adequately packaged to avoid damage during transport, free of excess heat, humidity, and contamination. They were immediately sent to the Laboratory of Reproductive Biology and Conservation of Tree Species (LACON) in the Department of Silviculture/Forest Institute/UFRRJ.

The procedures for analyzing the quality of pink pepper seeds were performed based on the "RAS - Rules for Seed Analysis" (MAPA, 2009 and MAPA, 2013), and the parameters determined were purity analysis; moisture content; uniformity in a sieve, the weight of a thousand seeds and germination and vigor test.

**Purity analysis.** Working samples of approximately 100g were used; after weighing were placed on a smooth white surface. Then, the pure seeds. Other seeds and inert materials were separated manually and weighed individually on a scale with a precision of 0.0001 as described in the RAS (MAPA, 2009). The percentages were based on the sum of the component weights.

**Determination of moisture content.** The moisture content of the seeds was determined by the greenhouse method at  $105 \pm 3^\circ\text{C}$ . Initially, from each lot, two 5g subsamples were extracted from the 100g working sample used in the purity test. The subsamples were initially placed in an aluminum crucible and weighed on a 0.001g precision scale to determine the seeds' moisture content. Subsequently, they were sent to the greenhouse at  $105 \pm 3^\circ\text{C}$  and maintained for 24 hours. After the drying period, the subsamples were removed from the greenhouse, covered, and placed in a desiccator until cool. After being cold, they were weighed again, and the moisture content was determined based on the formula described in the RAS (MAPA, 2009).

**Uniformity test (sieve retention).** Two working samples of approximately 100g of seeds from each lot were weighed, placed on a set of analysis sieves (tyler 3.5 = 5.6mm, tyler 4 = 4.75mm and tyler 5.0 = 4.0mm), and the whole was shaken for one minute. The seeds retained in the sieves were separated, weighed and had their percentage calculated according to RAS (MAPA, 2009). Due to the ripe fruits of the pink pepper having an average of 4.0 to 5.5mm in diameter, the seeds' percentages retained in the 5.66, 4.75 and 4.00mm mesh sieves were considered.

**Thousand seed weight.** Eight repetitions of 100 seeds each, from each lot, were counted manually and at random. Then, the seeds of each repetition were placed in a petri dish and weighed. The result of the thousand seed weight test is the average weight of the repetitions of 100 seeds, multiplied by ten, according to RAS (MAPA, 2009).

**Germination and vigor test.** For the test, only the seed samples from Lot 3 (collected in May/2019) were used as they had shorter storage times. From this, 400 seeds retained in each sieve (Tyler 4 - 4.75mm and Tyler 5 - 4.0mm) were selected and divided into four repetitions of 100. These seeds were soaked for 10 minutes in a neutral detergent solution at 5% (5 drops of detergent per 100ml of distilled water); subsequently, the seeds were rinsed with distilled water and immersed in a 2% sodium hypochlorite solution for two minutes and washed again with distilled water. After asepsis, the seeds were distributed inside gearbox boxes, on two sheets of germinet paper previously sterilized in an autoclave. According to RAS, the paper was moistened with 4.5mL of distilled water, an amount established based on the leaves' weight (paper weight x 3.0). Then, the gearbox boxes with the seeds were placed in germination chambers (BOD) at a constant temperature of  $25^\circ\text{C}$  for 21 days.

The germination test was evaluated three times, every 7 days until the 21<sup>st</sup> day, by counting the formation of normal, abnormal seedlings, primary root emission, dormant and dead seeds as proposed by MAPA (2009) and MAPA (2013). Germinated seedlings were considered normal seedlings. The result of the germinated seedlings was expressed as a percentage (G%), and the count of the number of seedlings germinated in the first seven days was used to analyze the vigor of the seeds.

**Sustainability assessment.** A scale of 1 to 3 was used to classify the seeds' quality parameters; based on scientific research results (bibliographic review) and normative instructions from

MAPA. Grade 3 is related to high sustainability, grade 2 to medium sustainability and grade 1 to low sustainability (Table 1).

**Statistical analysis.** The data obtained were analyzed using a statistical package STATISTICA 7.0. The experimental design used was completely randomized. The t-test was performed for the germination and vigor test to check if the differences between treatments are significant at the level of 5% significance (depending on the mesh); moreover, analysis of variance (ANOVA) and comparison between means by the Tukey test for the parameters moisture content, thousand seeds weight and uniformity in a sieve.

**Table 1.** Degree of sustainability based on seed quality parameters.

Parameters	Sustainability Degree	Bibliographic references for assigning grades
<b>Moisture Content</b>	< 12%	3
	12% a 20%	2
	> 20%	1
<b>Purity</b>	> 97%	3
	97% a 96%	2
	< 96%	1
<b>Uniformity in Sieve</b>	High*	3
	Medium**	2
	Low***	1
<b>Thousand Seeds Weight (seeds/Kg)</b>	37,000 a 44,000	3
	44,001 a 45,599	2
	> 46,000	1
<b>Germination and Vigor</b>	>80%	3
	80% a 60%	2
	< 60%	1

\* High uniformity refers to 100% of the seeds retained in the 5.66 mm and 4.75mm mesh sieve. \*\* Medium uniformity refers to more than 40% of the seeds retained in the 4.75mm mesh sieve. \*\*\* Low uniformity refers to more than 40% of the seeds retained in the 4.0mm sieve.

## RESULTS AND DISCUSSION

**Purity analysis.** According to the Normative Instruction 45 MAPA (2013), which establishes identity and quality standards for the production and commercialization of seeds, the analyzes for pink pepper seeds must present a satisfactory result of a minimum purity of 97%. Thus, it can be seen that Lots 2 and 3 presented a high percentage of purity according to the determined standards. It is greater than 97%, while Lot 1 presented 94.2% purity (Table 2).

The presence; and the number of impurities present in a seed lot are related to post-harvest procedures. The reduced amount of impurities in the samples corresponding to Lots 2 and 3

indicates that the producers performed a good manual cleaning (scavenging), adding value to the product and increasing the production sustainability.

**Moisture content.** The average values of moisture content obtained for Lots 1, 2 and 3 were 21.63%, 20.75% and 26.60%, respectively (Table 3).

The high moisture content observed in the lots of pink pepper seeds studied it may have occurred due to its inadequate drying, in the post-harvest phase. However, for future harvests, the seeds will be dehydrated in a greenhouse at 45 °C, until the moisture content of 12% is reached, allowing the maintenance of their quality for a longer period.

**Table 2.** Percentage of purity of pink pepper seeds (Lots 1, 2 and 3).

Lot	Initial weight (g)	Pure seeds (g)	Other seeds (g)	Inert material (g)	Impurities (%)	Purity (%)
1	128.1	123.5	0.0	4.5	5.8	94.2
2	113.0	110.7	0.0	2.4	2.7	97.3
3	115.4	114.4	0.0	0.0	1	99.9

**Table 3.** Moisture content of pink pepper seeds (Lots 1, 2 and 3).

Crucible	Lot	Tare (g)	Pu (g)	Ps (g)	U (%)	Mean moisture content (%)	Total means moisture content (%) / CV*
19	1	27.04	32.46	31.27	21.92	21.63 A	22.99 12.78*
25	1	25.96	31.02	29.94	21.33		
6	2	28.55	33.75	32.74	19.47	20.75 A	
40	2	36.80	41.97	40.83	22.04		
44	3	43.7	49.30	47.8	26.8	26.60 B	
16	3	39.9	45.20	43.8	26.4		

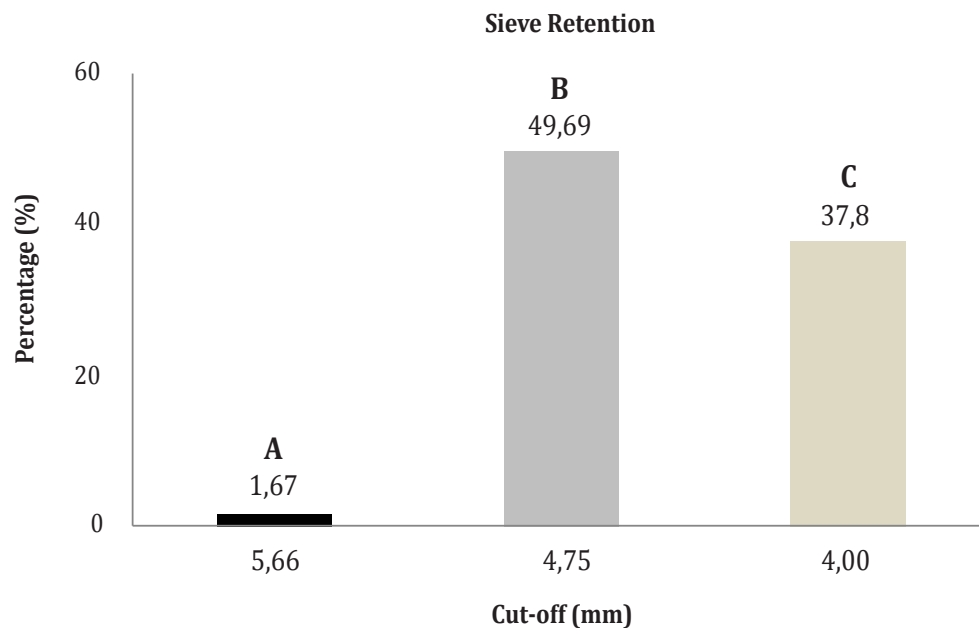
Pu (Wet weight) Ps (Dry weight) U (Moisture percentage) and CV\* (Coefficient of variation). Averages followed by the same letter do not differ statistically from each other by the Tukey test at 5% significance.

Neves *et al.* (2016) considers that pink pepper seeds can be stored at 12.6% humidity for up to five months in the environment without affecting their quality as long as they are packed in a paper bag. However, pink pepper seeds have an orthodox behavior; they resist drying to low values, around 5 to 7%, and can be stored with a low moisture and temperature content, maintaining their viability for a longer period.

**Uniformity test (sieve retention).** The result shows that 5.66, 4.75 and 4.00mm mesh sieve

retained 1.67, 49.69 and 37.80% of the pink pepper seeds, respectively (Figure 1).

Although there is less retention in the sieve with a mesh of 5.66mm (1.67%) and 4.0 mm (37.80%), the differences were significant and may assist in decision-making about product marketing. On the other hand, larger and more uniform seeds may be sold *in natura* at a higher price. Instead of being sold at a lower price, the smaller seeds may be used to produce essential oils, adding value to the product.

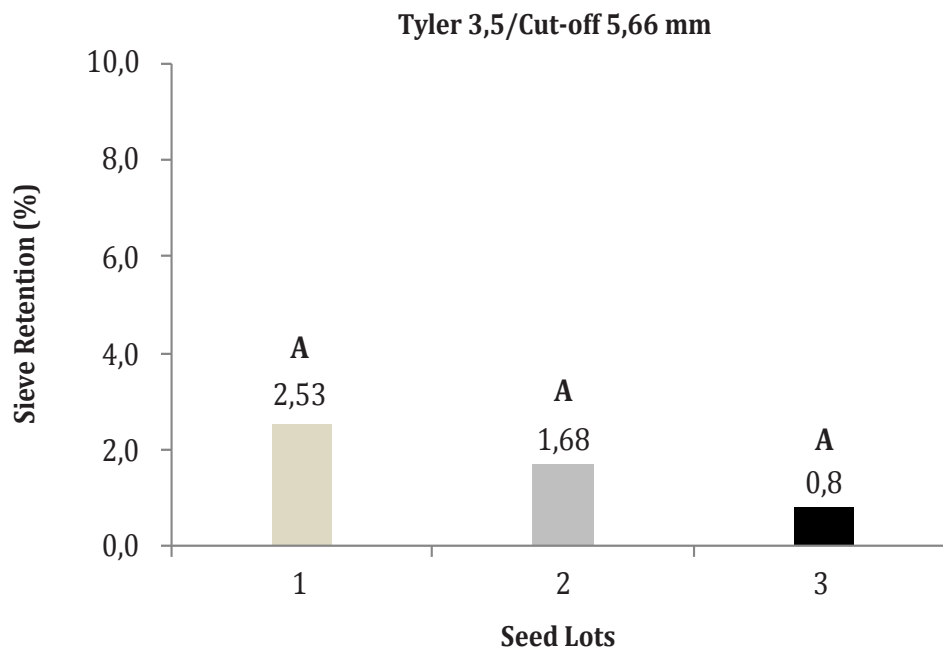


**Figure 1.** Percentage of seeds retained in a sieve of different meshes (mm). Averages followed by the same letter do not differ at 5% significance using the Tukey test.

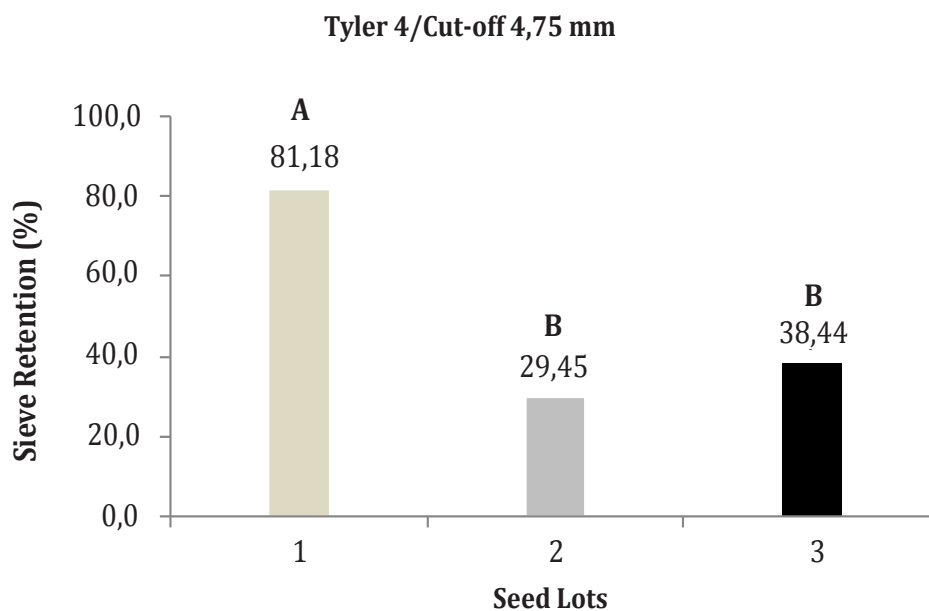
No statistical differences were observed between lots for retention in 3.5 tyler sieves (5.66mm). It is observed that the values found for Lots 1, 2 and 3 are similar (Figure 2).

For the 4 Tyler sieve (4.75mm), it is observed that there were differences between lots, with

greater retention of seeds from Lot 1 when compared to Lots 2 and 3. The results obtained indicate Lot 1 presents more uniform seeds, bigger dimensions and better appearance, achieving a better quality standard; than Lots 2 and 3 (Figure 3).



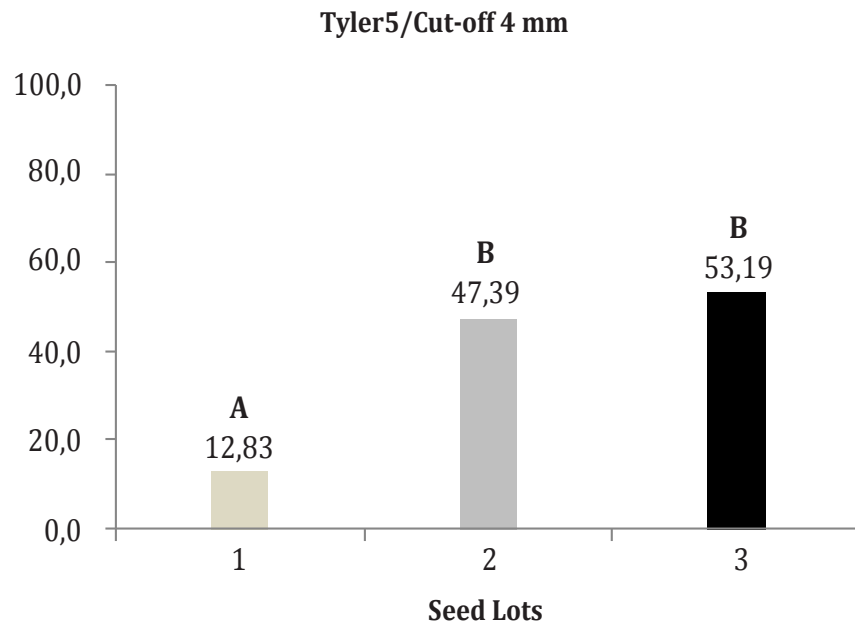
**Figure 2.** Percentage of seeds retained between lots, for 3.5 Tyler sieve, 5.66mm mesh. Averages followed by the same letter do not differ at 5% significance using the Tukey test.



**Figure 3.** Percentage of seeds retained between lots, for 4 Tyler sieve, 4.75mm mesh. Averages followed by the same letter do not differ at 5% significance using the Tukey test.

As for the 5 Tyler sieve (4.00mm), there was greater retention of seeds from Lots 2 and 3, indicating that these lots are smaller and can be classified for commercialization in a lower quality standard than Lot 1, (Figure 4).

**Thousand seed weight.** It is observed that there are significant differences at the level of 5% of significance between the lots for the average weight of a thousand seeds (Table 4).



**Figure 4.** Percentage of seeds retained between lots, for 5 Tyler sieve, 4.0mm mesh. Averages followed by the same letter do not differ at 5% significance using the Tukey test.

**Table 4.** Results of thousand seed weight tests (Lots 1, 2 and 3).

Repetition	Lot 1	Lot 2	Lot 3
	Net weight (g)	Net weight (g)	Net weight (g)
1	2.68	2.23	2.32
2	2.66	2.17	2.29
3	2.72	2.34	2.15
4	2.62	2.05	2.30
5	2.20	2.01	2.32
6	2.38	2.11	2.29
7	2.51	2.16	2.30
8	2.45	2.13	2.30
<b>Seed average</b>	<b>2.53</b>	<b>2.15</b>	<b>2.28</b>
<b>Thousand seed weight</b>	<b>25.27 A</b>	<b>21.51 B</b>	<b>22.83 B</b>

Averages followed by the same letter do not differ at 5% significance using the Tukey test.



This analysis indicates that Lot 1 seeds are larger and have better physical quality than Lots 2 and 3. This difference may be related to the seeds' maturity, health, and water content (MAPA, 2009), or the endosperm's size, and consequently the number of reserves in the seeds (Ponce *et al.*, 2019).

**Germination and vigor test.** It is observed that the average germination index is similar between the seeds retained in the 4.75mm and 4.0mm mesh sieve (Table 5).

The performance of seedlings in the first germination count allows determining the seed's lot quality as performed in this study, by comparing

and identifying seeds with a greater or lesser probability of establishing themselves in the field (Guedes *et al.*, 2015). Thus, the results obtained in this study indicate that the seeds present, on average, a high total germinative index ( $G = 72\%$ ). However, in the first seven days, this value was low, around 20%, which can be an obstacle in establishing seedlings in the field.

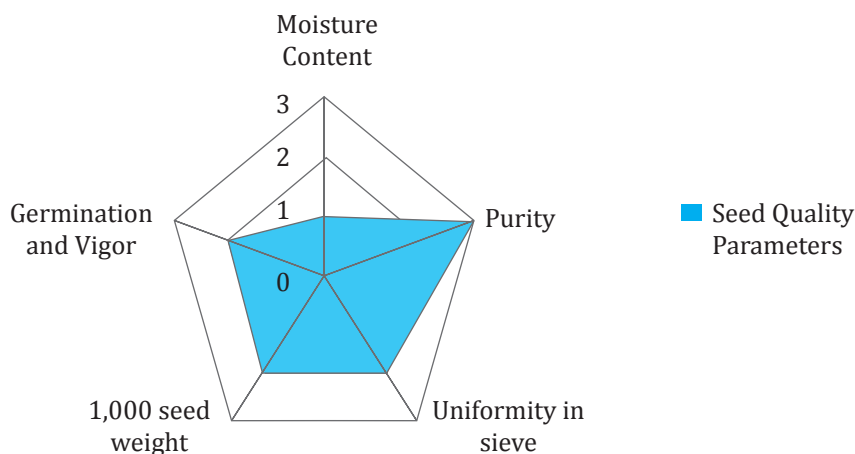
**Sustainability assessment.** The purity indicator reached an average of grade 3 - highly sustainable; the indicators insect incidence, uniformity in sieve, germination, and vigor reached grade 2 - medium sustainability; and the moisture content indicator reached grade 1 - not very sustainable, (Figure 5).

**Table 5.** The first, second, and third seedling count, in days, and total germination index (G) of pink pepper seeds retained in the 4.75 and 4.0mm sieves.

Tyler/Mesh	Repetition	Number of normal seedlings			
		7 Days	14 Days	21 Days	G (%)
4/4.75mm	1	19	48	2	69
	2	19	45	1	65
	3	25	49	2	76
	4	24	41	1	66
	<b>Average</b>	<b>22</b>	<b>46</b>	<b>2</b>	<b>69 A</b>
5/4.0mm	1	7	68	3	78
	2	26	54	1	81
	3	22	52	2	76
	4	20	42	1	63
	<b>Average</b>	<b>19</b>	<b>54</b>	<b>2</b>	<b>75 A</b>
<b>Total average</b>		<b>20</b>	<b>50</b>	<b>2</b>	<b>72</b>

G% (Total Germination Index). Averages followed by the same letter do not differ at 5% significance using the t test.

### Sustainability assessment



**Figure 5.** Sustainability assessment chart.

According to the sustainability assessment chart, it can be seen that the purity quality parameter was assigned a grade of 3. The impurity removal procedure is performed efficiently in the post-harvest, contributing to raising the physical and adding value to commercialized seeds.

Only one of the lots presented a satisfactory result in the weight test of a thousand seeds, as indicated by Normative Instruction #45 of MAPA (2013). Therefore, in the sustainability assessment, this parameter was assigned to grade 2. The results presented point to the need to observe the seeds' health, the moisture content, and the favorable period for the harvest (degree of maturity) since the thousand seed weight may vary to these factors (MAPA, 2009).

Grade 2 was assigned to the uniformity parameter. This parameter varies widely, suggesting that the seeds should be separated by size. Degradation due to humidity and delay in the harvest period are factors to be observed. It may be related to the small size and low uniformity of the seeds. The classification of seeds by size will benefit the commercialization of the product as they can be commercialized by variable size and price.

The high moisture content found in pink pepper seeds is a weak point in the environmental, social, and economic sustainability of the Ademar Moreira settlement's extractivist activity. This characteristic leads to loss of seed quality as it interferes with the increase in the incidence of fungi and deterioration by insects; in addition, it contributes to the reduction of germination and vigor. The moisture content is a parameter that needs to be improved as it can decrease the natural longevity of the seeds. According to Souza *et al.* (2016), the environmental conditions affect the viability of the seeds; however, the moisture content is one of the factors that most interfere in the conservation of the physiological potential of the seeds. This parameter was assigned to grade 1.

Regarding germination and vigor, Gomes *et al.* (2013) highlight that one of the biggest problems associated with the pink pepper stems extraction from the difficulty in maintaining genetically diverse populations to ensure good fruit production for use in both ecological and socioeconomic aspects. For the authors, the adequate storage of seeds contributes to the maintenance of germplasm banks and ensures the rescue of important genotypes for the seedlings

production for commercial plantations and use in the recovery or restoration of degraded areas. Thus, the high moisture content interferes with germination and causes genetic losses for pink pepper.

Although pink pepper is a product that mostly affects the foreign market, because its production is practically exported to Europe, Asia, and the United States of America (Sodré, 2016). The seeds' collectors are not well paid; because most of the income goes to traders. Thus, the adoption of adequate practices for harvesting, processing and storing of seeds and new processing technologies can add value to the product *in natura*. For example, essential oils can guarantee higher income for collectors and increase the sustainability of the pink pepper value chain in the Ademar Moreira settlement.

**Conflict of interest:** The authors declare that there is no conflict of interest.

## CONCLUSIONS

Among the quality parameters analyzed, the degree of purity was high, but it is necessary to pay attention to the period of fruit maturation, seed size, and moisture content as they affect products' quality.

The best germinative index of the species occurs at 14 days after sowing, and it does not vary between seeds of different sizes. However, the high moisture content of seeds sold by producers (22.99% on average) interferes with their quality and compromises production. In this way, the Ademar Moreira Settlement is moving towards the sustainability of the pink pepper extraction activity, but it is necessary to incorporate better practices for collecting, processing and storing seeds.

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## Physicochemical properties and acceptability of three formulations containing fava bean, quinoa and corn flour extrudates

### Propiedades fisicoquímicas y aceptabilidad de tres mezclas alimenticias a base de harinas extruidas de haba, quinua y maíz.

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Extruded quinoa



Extruded bean



Extruded corn

#### ABSTRACT

Andean grains, widely distributed in the high areas of the Andean Highlands, can help to reduce malnutrition owing to their high content of protein and balance of essential amino acids. Recently, their consumption has changed as they are increasingly used for instant food mixtures. However, a better design and formulation are necessary to maintain Andean grains' nutritional value and characteristics. In this study, we evaluated the physicochemical properties and acceptability of three formulations containing extruded corn (*Zea mays* L.), fava bean (*Vicia faba* L.), and quinoa (*Chenopodium quinoa* Willd) flours. Grain samples were collected from Choclococha, Acobamba, Huancavelica, Peru, and extruded at 95°C. Subsequently, three flour formulations were prepared. The formulation comprising 30% fava bean flour, 58% quinoa flour, and 12% corn flour consisted of 18.64% protein, 4.87% fat, 8.4% humidity, 2.99% ash, and 62.44% carbohydrates. This formulation with the highest quinoa flour content presented the highest acceptability in terms of color odor and flavor, and the highest protein content and digestibility (72.6%) of all analyzed formulations. Thermal analysis and pasting testing indicated that the formulation with the highest acceptability did not contain native starch; moreover, the proteins in this formulation were denatured.

**Keywords:** *Chenopodium*; acceptability; Andean foods; instant foods; food formulations.

#### RESUMEN

Los granos andinos se encuentran ampliamente distribuidos en las zonas altas de la cordillera de los andes y pueden ayudar a enfrentar la desnutrición debido a su alto contenido de proteínas y a su balance de aminoácidos. Actualmente, el consumo de estos granos ha cambiado y se ha incrementado el uso en forma de mezclas alimenticias instantáneas. Sin embargo, un buen diseño y formulación son necesarios con el fin de mantener su valor nutricional y características sensoriales de los granos andinos. En este estudio, se evaluó las propiedades fisicoquímicas aceptabilidad de tres formulaciones alimenticias a base de

harinas extruidas de maíz (*Zea mays* L.), haba (*Vicia faba* L.) y quinua (*Chenopodium quinoa* Willd). Los granos fueron recolectados del centro poblado de Choclococha, Acobamba, Huancavelica, Perú y fueron extruidas a 95°C. Posteriormente, tres formulaciones fueron preparadas. La mejor formulación (30% de harina de haba, 58% de harina de quinua y 12% de harina de maíz) presentó un contenido de proteínas de 18,64%; grasa de 4,87%; humedad de 8,4%; cenizas de 2,99%; carbohidratos de 62,44%. Esta formulación con el más alto contenido en quinua mostró la mayor aceptabilidad en relación al color, olor y sabor; además un alto contenido de proteína y digestibilidad (72,6%). El análisis térmico y la prueba pasting indicaron que la formulación con la mayor aceptabilidad no mostró la presencia de almidón nativo, así mismo, las proteínas de esta formulación fueron desnaturalizadas.

**Palabras clave:** *Chenopodium*; aceptabilidad; alimentos andinos; comidas instantáneas; formulaciones alimenticias.

## INTRODUCTION

Several new nutritional trends have focused on products with health benefits such as Andean legumes and seeds (Mendoza-Jiménez *et al.*, 2018). Recent studies have confirmed that Andean products are benefit for health because they contain soluble and insoluble fibers, resistant starch, proteins with bioactive properties, and phenolic compounds. (Delgado-Andrade *et al.*, 2016). Quinoa (*Chenopodium quinoa* Willd), an Andean grain from Peru and Bolivia's Andean areas, is an ancestral food owing to its nutritional qualities (Tovar-Hernández *et al.*, 2017).

Quinoa is consumed as raw grain, cooked, or processed into flour, bakery products, porridges, drinks, and fermented products. The quinoa's nutritional value depends on its protein contents, dietary fiber, minerals, and polyunsaturated fats. The protein content of dry quinoa varies between 13.8 and 16.5%; however, it has been reported to be 15% on average (USDA, 2015). Moreover, quinoa is a valuable food source (Filho *et al.*, 2017). Corn (*Zea mays* L.) supplements a significant amount of sulfur amino acids to the human diet (Estrada *et al.*, 2015). Miranda *et al.* (2015) reported that the corn's protein content was 8.80%; therefore, corn is an important food source. Corn is used for bakery products and represents an alternative for gluten-intolerant consumers. Therefore, it is necessary to expand the quantity and quality of corn-based products for consumers

who adopt a gluten-free diet (Estévez and Araya, 2016).

Fava beans (*Vicia faba* L.) are a significant crop and income source in Peru's Andean areas. Fava beans are rich in protein, carbohydrates, dietary fiber, lecithin, minerals, and secondary metabolites (Etemadi *et al.*, 2018). The protein content of fava beans ranges between 20 and 41%, and the main proteins in fava beans are globulins (79%), albumins (7%), and glutelins (7%) (Sathya and Devi, 2018). Fava beans have traces of several antinutritional factors, such as phytates, tannins, proanthocyanidins, sucrose, condensed tannins, protease inhibitors, and alkaloids. They are easily degraded via heat treatment (Coda *et al.*, 2015).

Extrusion is a thermomechanical process that can alter the structure of starch, cause gelatinization and change the structure of native starch, because of the pressure and temperature used during the process, increasing starch digestibility. Moreover, other polymers, such as proteins, can change their structural architecture and become denatured during extrusion, facilitating their rapid degradation by digestive enzymes (Jiangping *et al.*, 2017).

A large number of extruded products are processed and sold; as example there are pasta, breakfast cereals, breadcrumbs, cookies, croutons, baby food, snacks, confectionery, chewing gum, textured vegetable proteins, modified starch, pet food, dry

soups, and dry drink mixes. Flours or grains, are subjected to extrusion to obtain products with particular sensory characteristics and functional properties; such as water absorption, water solubility, oil absorption indices, expansion indices, apparent density, and mass viscosity (Alama *et al.*, 2015).

Numerous Andean grains (legumes and cereals) can help to reduce malnutrition in impoverished Andean areas of Peru. For this, the Andean grains' adequate industrial transformation with high nutritional value and optimization of instant flour formulations (food mixes) is required to improve Andean residents' nutritional status. Therefore, in this study, we evaluated the physicochemical characteristics and acceptability of three food formulations comprising extruded corn, fava bean, and quinoa flours.

## MATERIALS AND METHODS

**Characterization of raw materials.** Fava beans, quinoa, and corn were collected from Choclococha, Acobamba, Huancavelica, Peru located at 3 383 masl. The following methods were used to analyze the chemical composition of raw materials and food formulations: UNE-EN ISO 5983-2 for protein, UNE 64021-1970 for fat, UNE 64015-1971 for humidity, UNE 64019-1971 for ashes, and NMX-F-090-1978 for fiber; carbohydrates were determined by difference. The pepsin digestibility of the extruded mixtures was determined using the NMX-Y-085-SCFI-2006 standard method.

**Extrusion.** Extrusion was performed at the Technological Experimental Center of the National University of Callao. First, 500g of sample (fava beans and corn) were milled in a stainless-steel disc mill to an average size of 5-6mm. The quinoa grains were not ground owing to their small size. The milled samples were conditioned to a humidity of 9.32%. Extrusion was performed in

a single screw extruder at 95°C, and a retention time of 15s was used for all runs. Subsequently, the extruded samples were milled again using a hammer mill and were sieved using a Tyler N° 35 mesh, and stored on Kraft paper.

**Formulation.** The extruded formulations met the standard for infants recommended by the Food and Agriculture Organization/ World Health Organization (FAO/WHO) (2016). The following formulations were prepared: formulation 1 (F<sub>1</sub>): 38% extruded fava bean flour, 50% extruded quinoa flour, and 12% extruded corn flour; formulation 2 (F<sub>2</sub>): 36% extruded fava bean flour, 52% extruded quinoa flour, and 12% extruded corn flour; and formulation 3 (F<sub>3</sub>): 30% extruded fava bean flour, 58% extruded quinoa flour, and 12% extruded corn flour.

**Sensory analysis.** Sensory analysis at the San Francisco de Asís Educational Institution, Acobamba was carried out based on the guidelines by the Peruvian Technical Standards (2008) (ISO 4121, 2008). For this, samples of the three extruded formulations were added to hot water, and equal amounts of sugar were added to each formulation. The color, taste, and odor acceptability of the formulations were evaluated by 30 panelists using a three-point hedonic scale (1 - I like it a lot, 2 - I like it, and 3 - I don't like it).

**Thermal analysis.** The Paredes-López *et al.* (1994) method was used to determine The flours' thermal properties and best extruded formulation (F<sub>3</sub>). In brief, after 2mg of starch was weighed in an aluminum cell and 7 µL of water was added to it, the cell was closed. Subsequently, the cell was placed in a differential scanning calorimeter (Q20, TA Instruments, New Castle, NJ, USA) and was heated in the temperature range of 30-120 °C (heating rate of 10°C/min). The data and graphs were obtained using the calorimeter software.

**Pasting.** The flours' pasting propeand best extruded formulation (F<sub>3</sub>) were analyzed using a rheometer (Ar-1500ex, TA Instruments, New Castle, NJ, USA) equipped with a starch cell. Suspension of the flours and best extruded formulation (F<sub>3</sub>) with concentrations of 12% (w/w) were prepared using the method of Pérez-Gallardo *et al.* (2012). The prepared suspensions were stirred at 500s<sup>-1</sup>. Next, the suspensions were warmed and maintained at 40°C for 600s followed by heating to 95°C (heating rate of 7.5°C/min). Subsequently, the suspensions were maintained at 95°C for 600s followed by cooling to 50°C (cooling rate of 7.5°C/min). Lastly, the suspensions were maintained at 50°C for 120s.

**Statistical analysis.** A completely randomized design with five repetitions was used to evaluate The extruded formulations' effect on their chemical composition parameters. Analysis of variance was performed to detect the differences between the formulations' average chemical composition parameters ( $p \leq 0.05$ ). The least significant difference test was used to compare the chemical composition parameters of the grains and formulations. The acceptability of the extruded formulations was analyzed using the Friedman test, and statistical analysis of the data was performed using the SPSS 18.0 software.

## RESULTS Y DISCUSSION

**Chemical composition of raw materials.** Table 1 summarize the chemical compositions of fava beans, corn, and quinoa. The protein content of fava beans (32.46%) was higher than those of corn and quinoa and higher than that reported by Hernández-Bolívar *et al.* (2015), confirming that fava beans are an important source of protein (Kotue *et al.*, 2018). Quinoa presented a high content of carbohydrates (74.26%), it was higher than those of fava beans and corn; this value was slightly higher than those reported by Cerón-Fernandez *et al.* (2016) (70.30%) and Bergesse *et al.* (2015) (68 - 74%).

The fat content of corn (5.98%) was higher than fava beans and quinoa. This value was lower than that reported by Miranda *et al.* (2015) (6.58%) but was in the range reported by Ai and Jane (2016) (3 - 6%). The difference was attributed to the difference in the size of the corn germs (Ai and Jane, 2016). The ash contents of quinoa (5.49%) and corn (4.96%) were higher than fava beans. The ash content of quinoa was higher than reported by Arzapalo *et al.* (2015) for white quinoa (4.40%) but was in the range reported by Bergesse *et al.* (2015) (2.22 - 9.80%).

**Table 1.** Energy content and chemical composition of raw materials.

Parameter	Fava beans	Corn	Quinoa
Energy (kcal/100 g)	369.49 ± 0.94 <sup>a</sup>	369.98 ± 0.23 <sup>a</sup>	355.06 ± 0.42 <sup>b</sup>
Humidity (%)	7.73 ± 0.47 <sup>b</sup>	10.02 ± 0.31 <sup>a</sup>	8.92 ± 0.53 <sup>c</sup>
Protein (%)	32.46 ± 1.12 <sup>b</sup>	9.76 ± 0.93 <sup>a</sup>	8.79 ± 1.32 <sup>c</sup>
Fat (%)	2.13 ± 0.65 <sup>b</sup>	5.98 ± 0.24 <sup>a</sup>	2.54 ± 0.33 <sup>c</sup>
Carbohydrates (%)	55.12 ± 2.36 <sup>b</sup>	69.28 ± 2.04 <sup>a</sup>	74.26 ± 1.23 <sup>c</sup>
Ash (%)	2.56 ± 0.65 <sup>b</sup>	4.96 ± 0.37 <sup>a</sup>	5.49 ± 0.74 <sup>a</sup>

Data presented as means ± standard deviations. Different letters in the same row indicate significant differences ( $p < 0.05$ ).



### Chemical compositions of extruded formulations.

The chemical compositions of the extruded formulations showed the variability for the characteristics studied (Table 2). Formulation F<sub>2</sub> presented the highest energy content, which agrees with the energy recommendations of the FAO/WHO (2016) of 60-70 kcal/100mL. However, no significant differences were observed in the formulations' energy contents. The protein content of formulation F<sub>2</sub> was higher than formulations F<sub>1</sub> and F<sub>3</sub>, and was attributed to its fava bean content being the highest of all formulations. Cereals, legumes, and grains are vegetal protein sources. Moreover, extrusion was reported to increase the protein content of legume-containing formulations by 1-1.5% (Patil *et al.*, 2016).

The formulation of F<sub>1</sub>'s carbohydrate content was the highest (62.69%) of all analyzed formulations. The water content of the formulations was low (Table 2), guaranteeing the stability of extruded products during storage (Tovar-Hernández *et al.*, 2017). The fat contents of

the extruded formulations showed no significant differences. This behavior agreed with Cerón-Fernández *et al.* (2016). However, extrusion was reported to increase legume-based products' digestibility by 37-62%, depending on the extrusion technique (Patil *et al.*, 2016).

**Sensory analysis of extrudates.** Table 3 shows outcomes of the characteristics analyzed. The panelists reported significant differences in the taste and odor acceptability of the samples, and the sample with the highest odor and taste acceptability was formulation F<sub>3</sub>. No significant differences were observed in the color acceptability of the formulations. This was attributed to the formulations' quinoa content". Jiangping (2017) mentioned that extrusion significantly affected the organoleptic characteristics of color and bouquet because of the high temperature used during the process. However, to maintain these characteristics is advisable to add colorant additives to formulations.

**Table 2.** Energy content, chemical composition, and digestibility of extruded formulations.

Parameter	Formulation		
	F <sub>1</sub>	F <sub>2</sub>	F <sub>3</sub>
Energy (kcal/100 g)	366.74 ± 3.40 <sup>a</sup>	369.88 ± 2.80 <sup>a</sup>	368.15 ± 3.90 <sup>a</sup>
Proteins (%)	18.24 ± 0.03 <sup>a</sup>	19.17 ± 0.04 <sup>b</sup>	18.64 ± 0.08 <sup>c</sup>
Fat (%)	4.78 ± 0.21 <sup>a</sup>	4.88 ± 0.19 <sup>a</sup>	4.87 ± 0.32 <sup>a</sup>
Humidity (%)	7.80 ± 0.04 <sup>a</sup>	8.00 ± 0.13 <sup>b</sup>	8.40 ± 0.05 <sup>c</sup>
Ash (%)	3.15 ± 0.01 <sup>a</sup>	3.11 ± 0.06 <sup>a</sup>	2.99 ± 0.01 <sup>b</sup>
Fiber (%)	3.34 ± 0.01 <sup>a</sup>	2.52 ± 0.02 <sup>b</sup>	2.66 ± 0.03 <sup>c</sup>
Carbohydrates (%)	62.69 ± 0.08 <sup>b</sup>	62.32 ± 0.23 <sup>a</sup>	62.44 ± 0.16 <sup>a</sup>
Digestibility (%)	72.80 ± 1.28 <sup>a</sup>	71.9 ± 0.98 <sup>a</sup>	72.60 ± 1.08 <sup>a</sup>

F<sub>1</sub>: 38% extruded fava bean flour, 50% extruded quinoa flour, and 12% extruded corn flour; F<sub>2</sub>: 36% extruded fava bean flour, 52% extruded quinoa flour, and 12% extruded corn flour; F<sub>3</sub>: 30% extruded fava bean flour, 50% extruded quinoa flour, and 12% extruded corn flour.

Data presented as means ± standard deviations. Different letter in the same row indicate significant differences (p < 0.05).

**Table 3.** Sensory acceptability of the extruded formulations.

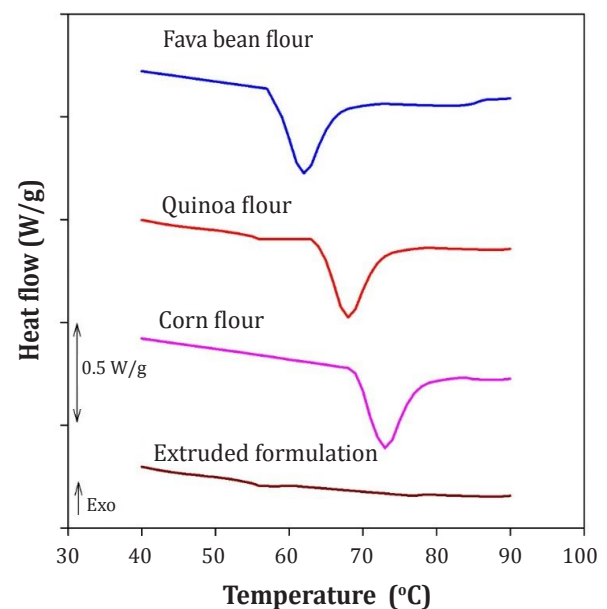
Formulation	Color	Taste	Odor
F <sub>1</sub>	1.90 ± 0.71 <sup>a</sup>	2.70 ± 0.46 <sup>c</sup>	2.43 ± 0.57 <sup>b</sup>
F <sub>2</sub>	1.93 ± 0.69 <sup>a</sup>	2.53 ± 0.68 <sup>bc</sup>	2.33 ± 0.71 <sup>b</sup>
F <sub>3</sub>	1.47 ± 0.51 <sup>a</sup>	1.73 ± 0.52 <sup>a</sup>	1.63 ± 0.61 <sup>a</sup>

Data presented as means ± standard deviations. Different letters in the same column indicate significant differences ( $p < 0.05$ ).

The extruded formulation with the highest odor and taste acceptability was F<sub>3</sub>. It was attributed to the quinoa content because Cerón-Fernandez *et al.* (2016) reported that quinoa grains contributed to the sweetness of extruded formulations. The taste acceptability of the formulations was attributed to the extrusion process, affecting the characteristics of fava beans, quinoa, and corn; improving the texture and reducing the quinoa and fava beans' bitter taste (Patil *et al.*, 2016). Formulation F<sub>3</sub> presented a better sweet taste in the final product. The panelists favored formulation F<sub>3</sub> and indicated that this sample presented a pleasant color and sweet taste; Cerón-Fernandez *et al.* (2016) reported similar results. The extruded formulations' quinoa content did not affect the color or appearance of the formulations.

**Thermal properties.** The thermograms of the non-extruded fava beans, quinoa, corn flours and extruded formulation with the highest acceptability (F<sub>3</sub>) are in Figure 1. The gelatinization temperatures of the non-extruded flours were higher than the extruded formulation. These differences were attributed to the non-extruded flours containing native starch (Velásquez-Barreto *et al.*, 2019). This did not occur for the extruded formulation because the starch had already been gelatinized during extrusion. Ye *et al.* (2018) mentioned that, starch granules gelatinized and proteins were denatured during extrusion, improving their digestibility, texture, and organoleptic properties.

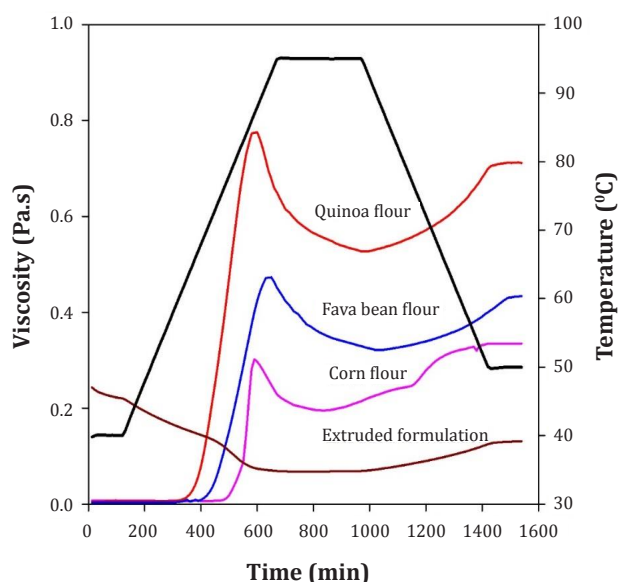
Moreover, the drinks prepared for sensory analysis presented a good dispersion due to starch gelatinization. Conversely, the extruded products presented high or complete gelatinization of native starch, indicating that extrusion was efficient, due to the absence of a gelatinization temperature from the thermogram of formulation F<sub>3</sub> (Figure 1).



**Figure 1.** Thermograms of non-extruded fava bean, quinoa, and corn flours, and extruded formulation (F<sub>3</sub>).

**Pasting.** Pasta obtained from non-extruded fava beans, quinoa, and corn presented similar compartments (Figure 2), and differed from the extruded formulation with the highest acceptability (F<sub>3</sub>). This behavior was attributed to the native starch swelling upon heating non-

extruded flours owing to heating and mechanical agitation (Velásquez-Barreto *et al.*, 2019). But it was not observed for the  $F_3$  formulation because the starch had already been gelatinized and lost its integrity. These results were consistent with the thermograms. Quinoa flour presented the highest peak viscosity followed by fava bean and corn flours. The gelatinization temperatures of the non-extruded corn, quinoa, and fava bean flours were 72.1, 64.5 and 68.7°C, respectively. These results were similar to those reported by Bustillos-Rodríguez *et al.* (2018), ranging between 70.6 and 72.2°C. Moreover, Arzapalo *et al.* (2015) reported that the gelatinization temperature of quinoa starch was 66°C, and Wani *et al.* (2016) indicated that fava beans' starch gelatinization temperature ranged between 66.6 and 70.59°C. Formulation  $F_3$  did not present a pasting temperature or peak viscosity, indicating that the native starch in this formulation was highly or completely gelatinized and confirmed that extrusion was efficient. These results were in agreement with the thermal properties of the analyzed flour samples.



**Figure 2.** Behavior of non-extruded fava bean, quinoa, and corn flours and extruded formulation ( $F_3$ ).

## CONCLUSIONS

The chemical composition and energy of fava beans, corn, and quinoa in this study agreed with the data reported in the literature. Extruded formulations of corn, fava beans, and quinoa presented the following chemical compositions: protein (18.24 - 19.17%), fat (4.78 - 4.88%), humidity (7.8 - 8.4%), ash (2.99 - 3.15%), fiber (2.52 - 3.34%), carbohydrates (62.32 - 62.69%) and digestibility (71.9 - 72.8 %).

The extruded formulation with the highest acceptability consisted of fava bean (30%), quinoa (58%) and corn (12%) flours; the odor, taste, and, color parameters of this formulation were 1.63, 1.73, and 1.47, respectively. The extruded formulation's thermal and pasting properties with the highest acceptability indicated that the entire amount of native starch present in the flours was gelatinized during extrusion.

This extruded formulation could be used as instant breakfast food for children and adults owing to its chemical composition and acceptability. Future studies should focus on the *in vivo* digestibility of these formulations and determining their amino acid balance to obtaining optimal formulations of extruded corn, fava bean, and quinoa flours.

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**Conflict of interest:** The authors declare that there is no conflict of interest.

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# Low-temperature applied to rice seed storage: an efficient protection method against fungal contamination

## Temperatura baja aplicada al almacenamiento de semillas de arroz: Eficaz método de protección contra la contaminación por hongos

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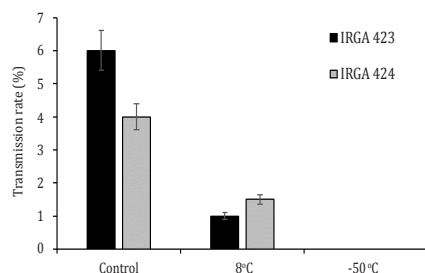
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The effect of storage temperatures on fungal seed-to-seedling transmission for rice (*Oryza sativa* L.) IRGA 423 and 424 seeds after 90-days of storage.

### ABSTRACT

Low-temperatures have long been thought to enhance seed health during storage. Here, we tested the effect of low-temperature on the mycoflora associated with rice seeds. Seeds of the IRGA 423 and 424 cultivars were stored in refrigeration (8 and -50°C) or at room temperature (25 ± 10°C). Following storage (1, 45, and 90 days) was investigated the fungi associated with the seeds. We found that low-temperature stored seeds had a lower fungal load than the seeds at room temperature for both cultivars. After 90 days of storage, there is a decrease in its incidence rate average of more than 85% in the lower temperature (-50°C). All mycoflora was eliminated at 90 days except for *Fusarium* sp. The *Trichoderma* sp. was detected only in IRGA 423 seeds, whereas all other fungi were detected in the treatments in both cultivars. Only *Bipolaris* sp. was observed in seed-to-seedlings transmission analyses, in both cultivars, at 8°C, and was not detected at -50°C. Based on our results, we recommend low-temperature storage (at -50°C) of rice seeds.

**Keywords:** disinfection method; mycoflora phytopathology; refrigeration; seed protection; *Oryza sativa* L.

### RESUMEN

Las bajas temperaturas han sido pensadas para mejorar la calidad de las semillas durante el almacenamiento desde hace mucho tiempo. Nosotros hicimos un test sobre el efecto que tienen las bajas temperaturas en la micoflora asociada con las semillas de arroz. Las semillas de los cultivos IRGA 423 y 424 se almacenaron en refrigeración (8 y -50°C), o en temperatura ambiente (25 ± 10°C). Con el seguimiento del almacenamiento (1, 45 y 90 días), se investigó el hongo asociado con las semillas. Encontramos que, para ambos cultivos, las semillas almacenadas a bajas temperaturas presentaron más baja carga de hongos que las almacenadas en temperatura ambiente. Después de 90 días, en almacenamiento a baja temperatura hay una disminución en el promedio de su tasa de incidencia de más del 85% (-50°C). Toda la micoflora fue eliminada

después de 9<sup>o</sup> días exceptuando por *Fusarium* sp. La *Trichoderma* sp. Se detectó únicamente en las semillas del IRGA 423, mientras que todos los demás hongos se detectaron en los tratamientos de ambos cultivos. Sólo se observó *Bipolaris* sp. en los análisis de transmisión entre semillas en ambos cultivos, a 8°C, y no se detectó a -50°C. Basándonos en nuestros resultados, recomendamos el almacenamiento a baja temperatura (a -50°C) de las semillas de arroz.

**Palabras clave:** método de desinfección; fitopatología de la microflora; refrigeración; protección de semillas; *Oryza sativa* L.

## INTRODUCTION

Rice (*Oryza sativa* L.) is one of more important commodities of the world with more than 760 Million Tons produced in 2017, highlight Asian countries' production and Brazil, one of the top 10 producers (FAOSTAT, 2019). Once this is a cereal that feeds more than half of the world's population, many efforts have been made to food security of this crop (Godfray *et al.*, 2010). In this sense, fungal contamination is the one of main problems related to seed production and storage; therefore, the elimination or minimization of contamination by mycotoxins and fungi is crucial for reducing economic loss in agriculture (Adeyeye, 2016).

Seed storage is complicated by climatic conditions between regions and the potential reduction of quality. However, treatments as chemical disinfection, heat and/or ionizing radiation have been developed aimed to reduce the number of microorganisms infesting the surface and inner tissue of seeds (Khamsen *et al.*, 2016). In this context, low-temperature emerges as an interesting process of storage and sterilization of seeds (Cardoso *et al.*, 2004), yet during this time, and temperature variation, stored rice seeds may lose physiological quality (Marini *et al.*, 2012). However, we previously demonstrated that rice seeds can be stored at low-temperature without losing physiological quality (Aguiar *et al.*, 2012; Aguilar *et al.*, 2015). Besides that, low-temperature technology has proven effectiveness in reducing the microbial population on the food surface not permitting the invasion by storage fungi, reduce the potential for attack by pathogenic

microorganisms, and minimizing the loss of quality (Zuchi and Bevilacqua, 2012).

Once seed storage at elevated-temperature can lead to deterioration despite sterilization, and there is a clear need for studies aimed at establishing suitable storage conditions for rice seeds, here we evaluate the effect of low-temperature storage on the sanitary quality of rice seeds, testing different temperatures of storage and if occurs some seed-to-seedling transmission.

## MATERIAL AND METHODS

**Plant material and seed treatments.** Seeds used in this study, cultivars IRGA 423 and 424 (Irga ®), were obtained from the rice seed agro-industries from Formoso do Araguaia and Lagoa da Confusão, in the state of Tocantins - Brazil. Collected seeds were placed in a forced airflow dryer (BKIA, Carlos Becker®) until reaching 11% of humidity. The cooling process of seeds was realized in two chambers with temperature control (8 and -50°C) and a capacity of 20L. Seeds were then packed (1.5kg) in sealed chambers with a volumetric capacity of 2L and stored at 8±1°C or -50±1°C. The controls were kept in a paper bag (2kg) at room temperature (uncontrolled conditions) with an average temperature of around 25±10°C.

**Microbiological analysis and fungal identification.** To analyze the effect of low-temperature storage on rice seeds' protection against fungal contaminations, the samples were transferred to sterile stomacher bags containing 50mL Butterfield's Phosphate Buffer after each

storage time. The bags were kneaded for 3 min to obtain a homogenized spore solution. Serial dilutions were operated, and 0.5mL of solution was spread on each plate with Dichloran Rose-Bengal Chloramphenicol medium. After 3 days of culturing at 30°C, the colonies were labelled. Two replicate in triplicate were performed for each sample, and the mean of six replicates  $\pm$  standard deviation was reported. The initial spore population of untreated samples was  $1.3 \times 10^7$  CFU g<sup>-1</sup>. The fungal identity was confirmed based on colony morphology and spore characteristics, according to Kimati *et al.* (1997). Single spore cultures of these fungus strains were maintained on Potato Dextrose Agar medium at 27°C. Inoculum was prepared by using the conidia of two-week-old cultures of the fungi. The conidia were removed from the medium's surface by flooding with sterile distilled water and gentle rubbing with a sterilized glass rod. The suspensions were filtered through cotton wool removing mycelial fragments and adjusted to  $10^5$  conidia mL<sup>-1</sup>. Following this, all conidial and spore suspensions were used for inoculation in PDA culture medium. The results were expressed in the percentage of incidence of each fungus.

**Pathogen transmission via seed-to-seedling test.** Seed-to-seedling pathogen transmission analyses were done as described by Lucca Filho (1991), using seeds stored for 90 days. One-hundred seeds of each treatment were sown in sterilized recipients containing 10% bacteriological agar substrate and sterile water. Inoculum was prepared by using the conidia of two-week-old cultures of the fungi. The conidia were dislodged from the medium's surface by flooding with sterile distilled water and gentle rubbing with a sterile glass rod. The suspensions were filtered through cotton wool removing mycelial fragments and adjusted to  $10^5$  conidia mL<sup>-1</sup>. The percentage of transmission was calculated according to the

seed-associated mycoflora's transport rate and the corresponding number of seedlings that developed disease symptoms.

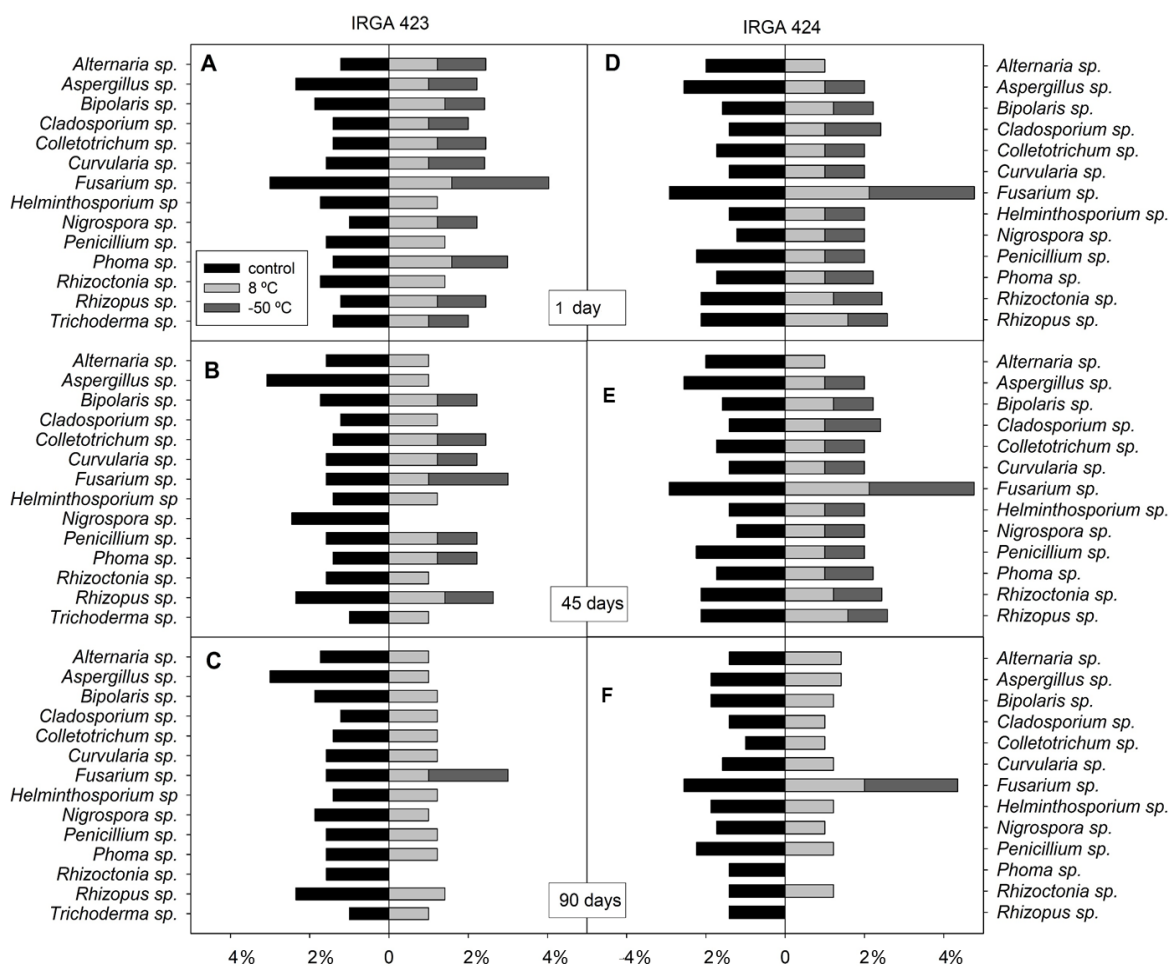
#### **Experimental design and statistical analysis.**

The experiment was conducted in a factorial arrangement of 3 x 3 x 2, with three storage periods (1, 45 and 90 days), three temperatures (25 $\pm$ 10°C, 8°C, and -50°C), and two cultivars (IRGA 423 and 424). A completely randomized experimental design was used, with four replications (100 seeds per treatment, 25 seeds per plate), following the description in the Rules for Seed Testing - RAS (Ministério da Agricultura, Pecuária e Abastecimento, 2009). The data, obtained in percentage, were submitted to analysis of variance (ANOVA) and Tukey's test ( $p \leq 0.05$ ), using statistical software R (R Core Team, 2019).

## **RESULTS AND DISCUSSION**

In total, we found 14 fungal genera in the IRGA 423 seeds and 13 in the IRGA 424 seeds (Figure 1). The incidence of pathogenic fungi was higher for control seeds than those stored at low temperatures (Figure 1 and Table 1). It was verified that at -50°C, 45 days of storage was a protection treatment, inhibiting the fungi's development with a reduction of more than 60% of incidence average, and 90 days, the reduction was more than 85% (Table 1). Low-temperature storage massively reduces fungal infection on rice seeds, and almost all fungi were eliminated, except for *Fusarium sp.*, which only reduced its incidence rate. The *Trichoderma sp.* was detected only in IRGA 423 seeds, whereas all other fungi detected were found in both cultivars (Figure 1). However, during the storage, when subjected to -50°C, it was eliminated from the rice seed.





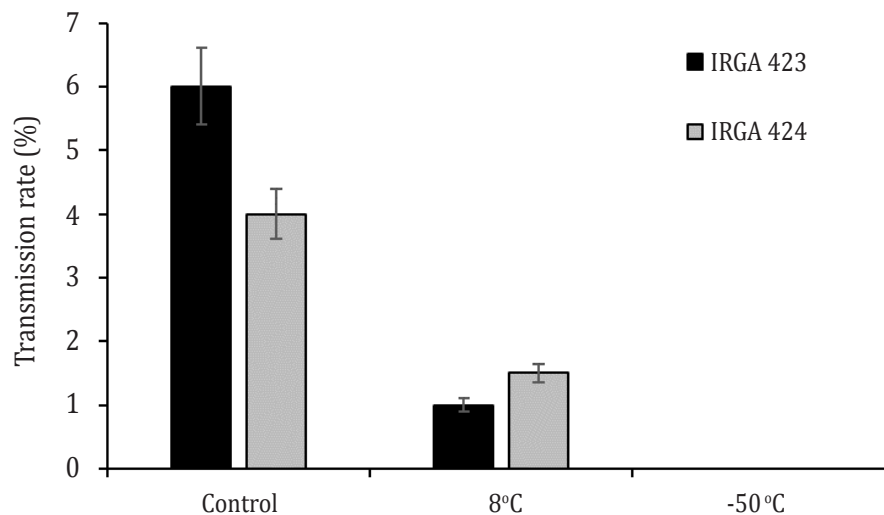
**Figure 1.** The microflora (percentage of infestation) of IRGA 423 (A, B, and C) and 424 (D, E, and F) rice seeds (*Oryza sativa* L.) after 1 (A and D), 45 (B and E), and 90 (C and F) days of storage at different temperatures.

**Table 1.** Average of fungi incidence rate per treatment calculated as a percentage of occurrence.

	1 day			45 days			90 days		
	control	08 °C	-50 °C	control	08 °C	-50 °C	control	08 °C	-50 °C
IRGA 423	1.64±0.14	1.25±0.06	1.01±0.18	1.71±0.15	1.07±0.09	0.60±0.18	1.70±0.14	1.07±0.09	0.14±0.15
IRGA 424	1.88±0.14	1.17±0.10	1.12±0.16	1.69±0.11	1.13±0.12	0.64±0.20	1.67±0.11	1.07±0.15	0.18±0.18

In our seed-to-seedlings transmission analyses, only *Bipolaris* sp. was observed. For IRGA 423, *Bipolaris* sp. was detected in 6% of the control seedlings but only 1% of the 8°C stored seedlings

(Figure 2). For IRGA 424, *Bipolaris* sp. was detected in 4% of the control seedlings and 1.5% of those stored at 8°C. No pathogen transmission was detected in the -50°C treatment groups (Figure 2).



**Figure 2.** The effect of storage temperatures on fungal seed-to-seedling transmission for rice (*Oryza sativa* L.) IRGA 423 and 424 seeds after 90-days of storage.

For rice seeds, temperatures above 26°C are considered harmful to seed quality due to gradual biochemical or physiological changes (Marini *et al.*, 2012). Medina *et al.* (2009) reported poor germination and viability for triticale seeds stored under uncontrolled conditions and an increasing level of storage fungi, mainly *Penicillium* spp. Our findings are in line with those of Tanaka *et al.* (2001) that evaluated the incidence of fungi associated with corn seeds during 12 months of storage, comparing cold room (14°C) storage against an uncontrolled environment. Compared to the cold chamber, the authors observed a higher frequency of the fungi *Alternaria alternata*, *Bipolaris maydis*, *Cephalosporium acremonium*, *Cladosporium herbarum*, and *Rhizoctonia solani* under the uncontrolled environment; in addition, *Rhizopus* spp. and *Trichoderma* spp. *Aspergillus* spp. and *Penicillium* spp. were also more prevalent under uncontrolled conditions.

Seed-associated fungi often damage plants during their initial development, causing them to wilt or die (Malavolta *et al.*, 2002; Maciel *et al.*, 2012).

According to Rey *et al.* (2009) and Maciel *et al.* (2012), seeds can serve as an inoculum source for the aerial part of seedlings. Therefore, seeds are considered as means of dissemination that might introduce pathogens into pathogen-free areas or accumulate them in already infested areas through consecutive plantings of infected seeds. Improved sanitary conditions can help reducing seed-to-seedlings pathogen transmission (Maciel *et al.*, 2012).

In the present study, although at 8°C storage temperature was able reducing the transmission of *Bipolaris* sp. to seedlings, the fungus was not eliminated. Control of temperature of storage is an important factor for conservation of seeds in tropical regions, directly affecting the biochemical and physiological processes in rice seeds and based on our data here demonstrated and previously reported (Aguiar *et al.*, 2012), we recommend that rice seeds be stored low-temperature, preferably at -50°C. Obviously, any methodologies that require a large temperature change will need a great energy cost. So, this treatment could not be

done anywhere. Another pertinent thing is that we believe that the germination rate is maintained in these conditions however further experiments are necessary.

## CONCLUSIONS

Low-temperature storage massively reduces fungal association with rice seeds.

All fungi were eliminated, except for *Aspergillus* sp. that reported a decrease in its incidence rate.

The *Trichoderma* sp. was detected only in IRGA 423 seeds, whereas all other fungi detected were found in both cultivars.

In our seed-to-seedlings transmission analyses, only *Bipolaris* sp. was observed in both cultivars at 8°C, while -50°C was not detected.

Based on our data, we recommend that rice seeds be stored low-temperature preferably at -50°C.

**Conflict of interest:** The authors declare that there is no conflict of interest.

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# Evaluation of pre-germination treatments in *Carapa guianensis* AUBL.

## Evaluación de tratamientos pre-germinativos en *Carapa guianensis* AUBL.

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### ABSTRACT

Understanding seed germination of native forest species of the Tropical wet forest is essential for ecosystem regeneration and conservation, which have been affected by the exploitation of wood and non-wood forest products. *Carapa guianensis* Aubl. (Meliaceae) has been exploited due to the high quality of its wood and seeds. Particularly, the latter is a source of a medicinal oil of high value to the pharmaceutical industry. The indiscriminate use of this species has reduced its population size; therefore, scientific knowledge of its sexual propagation in nurseries is vital. This research aimed to evaluate seed germination of *C. guianensis* under different pre-germination treatments and substrates at the Centro de Investigación El Mira of AGROSAVIA. Pre-germination assays were conducted in a split-plot design with a random distribution of four repetitions. The cumulative germination percentage (CGP) and mean germination time ( $t_{50}$ ) were calculated and analyzed through an ANOVA, and Tukey's multiple comparison test using InfoStat V.2016. Significant differences for CGP among treatments ( $p<0.0001$ ) and interactions ( $p<0.0096$ ) were found. The highest germination percentage (61%) was achieved in S1+T4; however, the  $T_{50}$  did not show significant differences ( $p=0.24$ ). This research demonstrates that the highest germination percentage is achieved through a scarification treatment with imbibition in sand substrate under the agroclimatic conditions of Tumaco.

**Keywords:** Andiroba; tropical wet forest; scarification; propagation; seeds.

### RESUMEN

El estudio de la germinación de especies forestales nativas del bosque húmedo tropical es fundamental en la regeneración y conservación de este ecosistema, el cual se ha visto afectado por la explotación de los productos maderables y no maderables. En este sentido *Carapa guianensis* Aubl. (Meliaceae), ha sido aprovechada por su madera de alta calidad y semillas; de donde se obtiene aceite medicinal de alto valor para la industria farmacéutica. El uso indiscriminado ha disminuido su población, por cuanto generar conocimiento científico sobre

su propagación sexual en vivero es de vital importancia. La presente investigación tuvo como objetivo evaluar la germinación de semillas de *C. guianensis* bajo diferentes tratamientos pre-germinativos y sustratos en el C.I El Mira de AGROSAVIA. Las pruebas pre-germinativas se realizaron mediante un diseño de parcelas divididas con cuatro repeticiones distribuidas al azar. Para el procesamiento de la información se calculó el porcentaje de germinación acumulada (PGA) y tiempo medio de germinación (t50). Los resultados se analizaron a través del ANOVA y de pruebas de comparación de Tukey mediante el programa estadístico InfoStat V.2016. Se obtuvieron diferencias significativas en PGA para los tratamientos ( $p < 0.0001$ ) y las interacciones ( $p < 0.0096$ ); en S1+T4, se alcanzó el mayor porcentaje de germinación (61%). T50 no presentó diferencias significativas ( $p = 0.24$ ). La investigación demostró que, bajo las condiciones agroclimáticas del municipio de Tumaco, el tratamiento escarificación con imbibición en el sustrato arena proporcionó el mayor valor en la germinación.

**Palabras clave:** Andiroba; bosque húmedo tropical; escarificación; propagación; semillas.

## INTRODUCTION

Tropical forests provide goods and ecosystem services that benefit the communities inhabiting these ecosystems; however, uncontrolled exploitation of wood and non-wood forest products drastically affects biodiversity and the abundance of certain native species (Palacios and Jaramillo, 2016). Indiscriminate deforestation reduces the number of seed trees; therefore, risking the future genetic base and seed propagation (Rojas, 2015). Furthermore, the scarcity of information about native forest species' propagation greatly adds to this problematic (Amaral *et al.*, 2017).

Most tropical forest species propagate sexually; therefore, phytosanitary and genetic aspects are important to obtain quality seedlings that meet commercial expectations (Nascimento *et al.*, 2019). However, many native species are not included in forest production plans and assessments of pre-germination treatments, which is likely due to limited information in rural settings or restricted access to seeds. Furthermore, several industries, environmental authorities, and nursery growers require information about forest seeds' adequate management to achieve efficient and low-cost propagation (Nascimento *et al.*, 2019).

*Carapa guianensis* Aubl. (Meliaceae), known as andiroba in Brazil (Gonçalves *et al.*, 2018) and tangare in the Pacific coast of Nariño in

Colombia, is a promising native species of rapid growth, reaching a maximum height of 55 m. It is distributed in South America and is native to the Amazon rainforest (Leite and Lima, 2018). This species displays important ecological characteristics, and high commercial value due to its wood and medicinal properties (Gonçalves *et al.*, 2018; Reis *et al.*, 2018) since the oil extracted from its seeds has pharmaceutical applications (Reis *et al.*, 2018). Reproduction is mainly sexual (Lobo and Furtado, 2015); the fruit is a spherical capsule composed of eight seeds that animals disperse (May, 2016; Gonçalves *et al.*, 2018; Leite and Lima, 2018). Seed harvesting is generally done by local communities who lack training regarding seed management, collection methods, or the plant population's analysis or successional structure (Guarino *et al.*, 2014).

The exceptional characteristics of *C. guianensis* promote its high use; however, this exploitation considerably affects its population size and natural areas. Therefore, information about germination dynamics and seed morphology are important to establish seed collection and management guidelines to successfully propagate this species (Abbade and Takaki, 2014; Peixoto *et al.*, 2019). For *C. guianensis*, germination behavior is variable; for instance, 24% to 90% germination can be reached in different periods ranging from days to months (Kossmann *et al.*, 2002; Amaral *et al.*, 2017; Hernández-Coronado *et al.*, 2018). Therefore,

nursery propagation alternatives for this species have led to the use of different pre-germination treatments such as mechanical scarification, sunlight exposure, and seed imbibition in sand and soil substrates (Torres *et al.*, 2017). These methods provide adequate conditions to accelerate and increase germination of the embryo (Viveros *et al.*, 2015).

In this context, and as part of the project entitled “Planification and management strategies for forest plantations in agroecosystems of Colombia”, Corporación Colombiana de Investigación Agropecuaria (Colombian Corporation for Agricultural Research) – AGROSAVIA developed this study to evaluate to evaluate seed germination of *C. guianensis* under different pre-germination treatments and substrates in nursery conditions at the Centro de Investigación El Mira of AGROSAVIA.

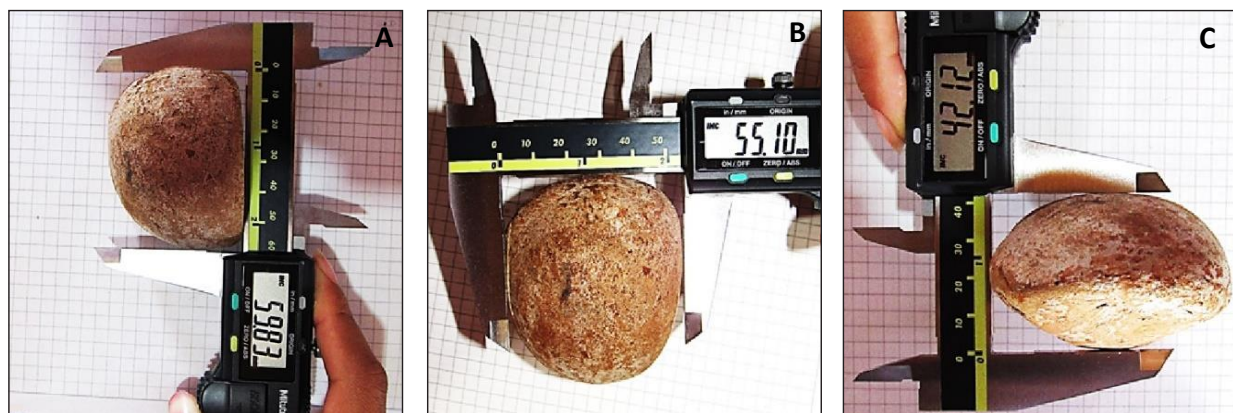
## MATERIALS AND METHODS

**Location.** This study was conducted at the Centro de Investigación el Mira of the Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), which is geographically located at 78°41'22"N and 1°32'58"W, in Tumaco, Department of Nariño (Colombia). The study site is

situated at an elevation of 16 m a.s.l., with a mean precipitation of 3067 mm/year, mean temperature of 25.5°C, and relative humidity of 88% (Reyes *et al.*, 2008).

**Seed collection.** This study used seeds from Salahonda in the municipality of Francisco Pizarro (Nariño) at 2°02'30"N and 78°39'40"W. The seeds were collected from 10 trees according to the methodology described by Espitia-Camacho *et al.* (2018). The selected trees were visibly healthy and vigorous, with straight, long and accessible trunks, as Torres *et al.* (2017) recommended.

**Physical description of the seed.** A general description was made of the total seed lot based on eight samples composed of 100 units each, which were individually weighed on a Mettler Toledo tenth of a gram digital scale (Asociación Internacional de Pruebas de Semillas-ISTA, 2005; Espitia-Camacho *et al.* 2018). Next, the biometric properties of the seeds from each repetition were determined based on the following variables: width (mm), length (mm), and thickness (mm), measured using a Mitutoyo digital caliper. Diseased and infected seeds were discarded (Hernández-Coronado *et al.*, 2018) (Figure 1).



**Figure 1.** Seeds of *C. guianensis*. A: length (mm), B: width (mm), C: thickness (mm).

**Pre-germination treatments and substrates.**

Three pre-germination treatments and a control were performed (Table 1). According to the study by García *et al.* (2019), the pre-germination

treatments were selected for tropical forest species. The substrates were selected based on Lobo and Furtado (2015), with modified proportions.

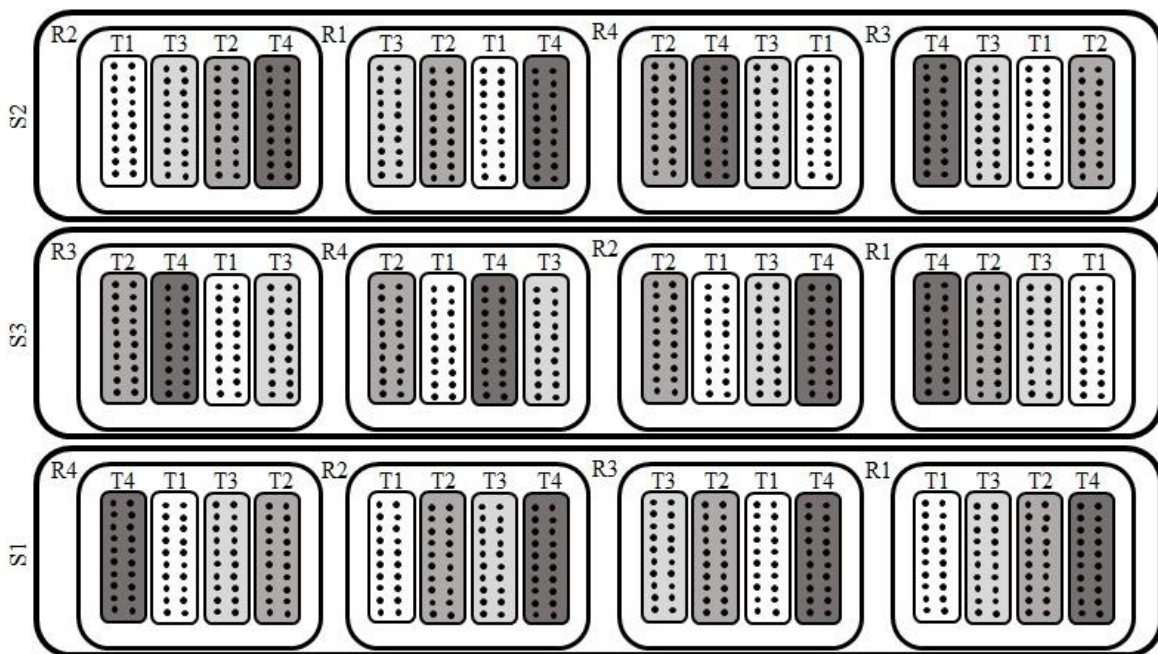
**Table 1.** Pre-germination treatments and substrates used for propagating *C. guianensis*.

Pre-germination treatments	Substrate
T1: control without treatment	S1: sand (100%)
T2: sunlight exposure for 24h + imbibition for 24h	S2: sand and soil (50%-50%)
T3: mechanical scarification	S3: soil (100%)
T4: mechanical scarification + imbibition for 24h	

The substrates were disinfected with dazomet (Theaux, 2015) at a dose of 40g per m<sup>2</sup>; then, the substrate was covered with plastic for five days and left to settle for 12 more days.

**Experimental design.** A randomized blocks design with split-plots and four repetitions was implemented. The large plots corresponded to the substrates, namely sand (S1), sand and soil (S2),

and soil (S3) (Santiago *et al.*, 2015). Furthermore, the subplots corresponded to the seed treatments: control without treatment (T1), T2: sunlight exposure for 24h + imbibition for 24h (T2), mechanical scarification (T3), and T4: mechanical scarification + imbibition for 24h (T4). In total, 12 combinations were obtained, and the experimental unit was 20 seeds/pre-germination treatment for a total of 960 seeds (ISTA, 2005) (Figure 2).



**Figure 2.** Split-plots design: the main plot = substrates (S1, S2, S3), the subplots = treatments (T1, T2, T3, T4) and four repetitions (R1, R2, R3, R4).



The effects of the pre-germination treatments and substrates were measured as the cumulative germination percentage (CGP) and mean germination time ( $t_{50}$ ) (Lobo and Furtado, 2015). Furthermore, the initial and final germination percentages were determined (Torres *et al.*, 2017). After planting, the number of germinated seeds was recorded daily until day 42 (i.e., when germination remained constant). A seed was considered to have germinated when the epicotyl grew to at least 1cm (Céspedes, 2018).

The CGP was calculated per experimental unit for each substrate, according to equation 1:

$$\text{CGP} = (\text{Sg}/\text{Ss}) * 100\% \quad [1]$$

Where:

Sg = Number of germinated seeds and

Ss = Number of planted seeds.

The  $t_{50}$  was calculated according to equation 2 proposed by Ellis and Roberts (1978). This equation establishes the time needed to reach 50% seed germination (Sánchez-Soto *et al.*, 2017).

$$t_{50} = \frac{[(N/2) - N_1] * (T_2 - T_1)}{N_2 - N_1} + T_1 \quad [2]$$

Where:

N = Final % of germinated seeds.

$N_1$  = % of germinated seeds prior to N/2.

$N_2$  = % of germinated seeds after to N/2.

$T_1$  = Number of days for  $N_1$ .

$T_2$  = Number of days for  $N_2$ .

$t_{50}$  = Number of days for N/2.

**Statistical analysis.** The statistical tests were performed using InfoStat version V. 2016. The variables were tested for normality, using the Shapiro-Wilk test and Q-Q plots, as well as homoscedasticity. The treatments, substrates, and their interaction (treatments with substrates)

were analyzed by a variance analysis (ANOVA), followed by Tukey's multiple comparison test. This same analysis was performed for the time of onset of germination and meantime of germination. *P*-values less than 0.05 were considered significant ( $P < 0.05$ ) (Zar, 2010).

## RESULTS AND DISCUSSION

**Physical description of the seed.** The seed weight of *C. guianensis* varied within a broad range (i.e., from 23.36g to 49.60g), with an average of 35.7g. This value is higher than reported by Hernández-Coronado *et al.* (2018), who found an average weight of 22.1g based on 100 seeds. Similarly, Kossmann *et al.* (2002) reported weights between 25 and 32g and established that 30-50 seeds are required to obtain 1kg. In this study, the data indicates that approximately 28 seeds are required per kg. Peixoto *et al.* (2019) found different weights for *C. guianensis*, even seeds from the same fruit, and determined that this variability could be associated with the non-domestication of this species.

The other biometric parameters, such as length, width, and thickness of *C. guianensis* seeds, (Table 2) also showed higher variation than in Teixeira *et al.* (2019), who reported mean values 38.21, 33.56, and 32.95mm, respectively. Several researchers indicate that the agroclimatic conditions of the site can affect seed development, leading to variations in seed size, weight, and morphology (Peixoto *et al.*, 2019). Based on the results shown in Table 2, this seed can be considered extremely large compared to other forest seeds, according to the manual of tropical and subtropical seeds of ISTA (2005).

**Table 2.** Biometric data of the seed of *C. guianensis*.

Variable	Mean value ± SD
Weight (g)	35.7± 6.07
Length (mm)	59.1±3.59
Width (mm)	54.8±3.28
Thickness (mm)	43.2±4.16

SD: Standard deviation.

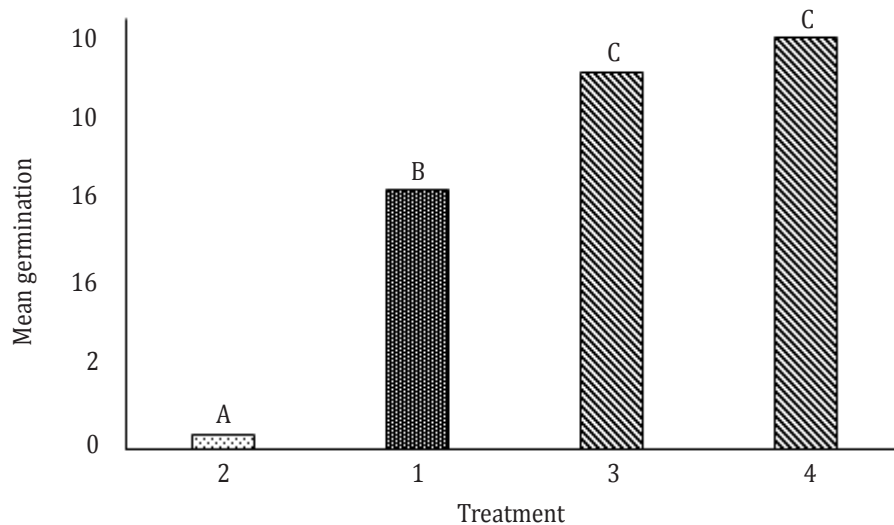
**Evaluation of germination.** The CGP did not show significant differences in response to the substrate ( $P=0.9453$ ); therefore, this variable did not affect germination. These results differ from reports by Severino *et al.* (2005) indicated that forest seeds' germination depends on the substrate since it provides different physical factors such as aeration and humidity. The pre-germination treatments showed significant differences in CGP ( $P<0.0001$ ), as well as the interaction between substrates and pre-germination treatments ( $P<0.0096$ ) (Table 3).

**Table 3.** Results of the analysis of variance (ANOVA) for the split-plots.

F of V	DF	P-value
Model	20	<0.0001***
Substrates	2	0.9453 ns
Error Factor A	9	0.0018
Treatments	3	<0.0001***
Interaction	6	0.0096**
Error Factor B	27	-
Total	47	-

F of V; factor of the variable. DF; degrees of freedom. p: probability limits of the ANOVA. \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ; ns:  $P > 0.05$ . R squared = 0.93. CV= 24.25 Factors: factor A: substrates on germination % and factor B: treatments on germination %.

For pre-germination treatments, Tukey's multiple comparison test indicated that treatments T3, and T4 had the highest values with similar means; therefore, these treatments do not differ and either pre-germination method is suitable (Figure 3).



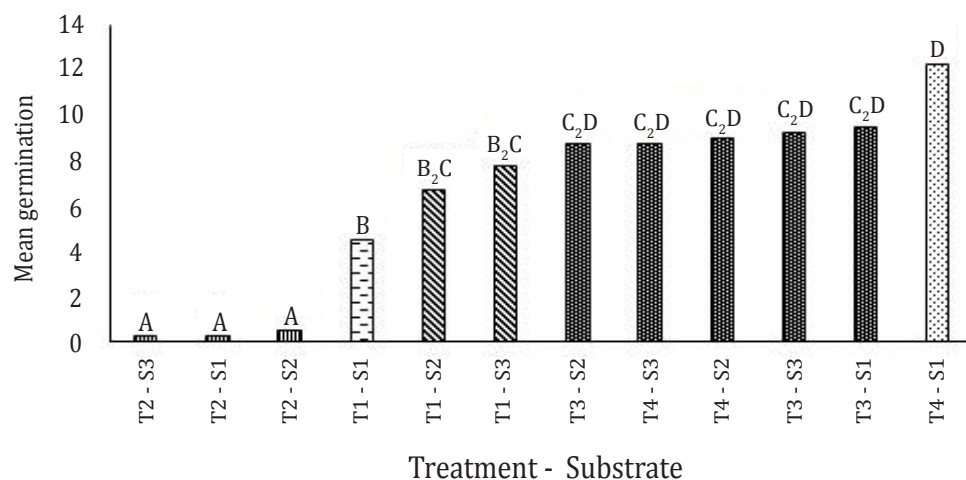
**Figure 3.** Cumulative germination per pre-germination treatment. Tukey's multiple comparison test. Different letters above the columns indicate a significant difference (Tukey.  $P < 0.05$ ).

According to Figure 3, T2 does not promote germination; this finding differs from Lobo and Furtado (2015) reports. These authors assessed the effect of different levels of sunlight on *C. guianensis* and established that germination is significantly promoted under all light intensity levels. Bewley *et al.* (2013) mention that sunlight exposure can cause water deficits in seeds, affecting the velocity and final germination percentage and germination variability. The results found here suggest that sunlight exposure likely induces photoinhibition of germination; thus, negatively affecting propagation. Several authors suggest that this type of seeds should be planted immediately after collection (Sánchez *et al.*, 2015).

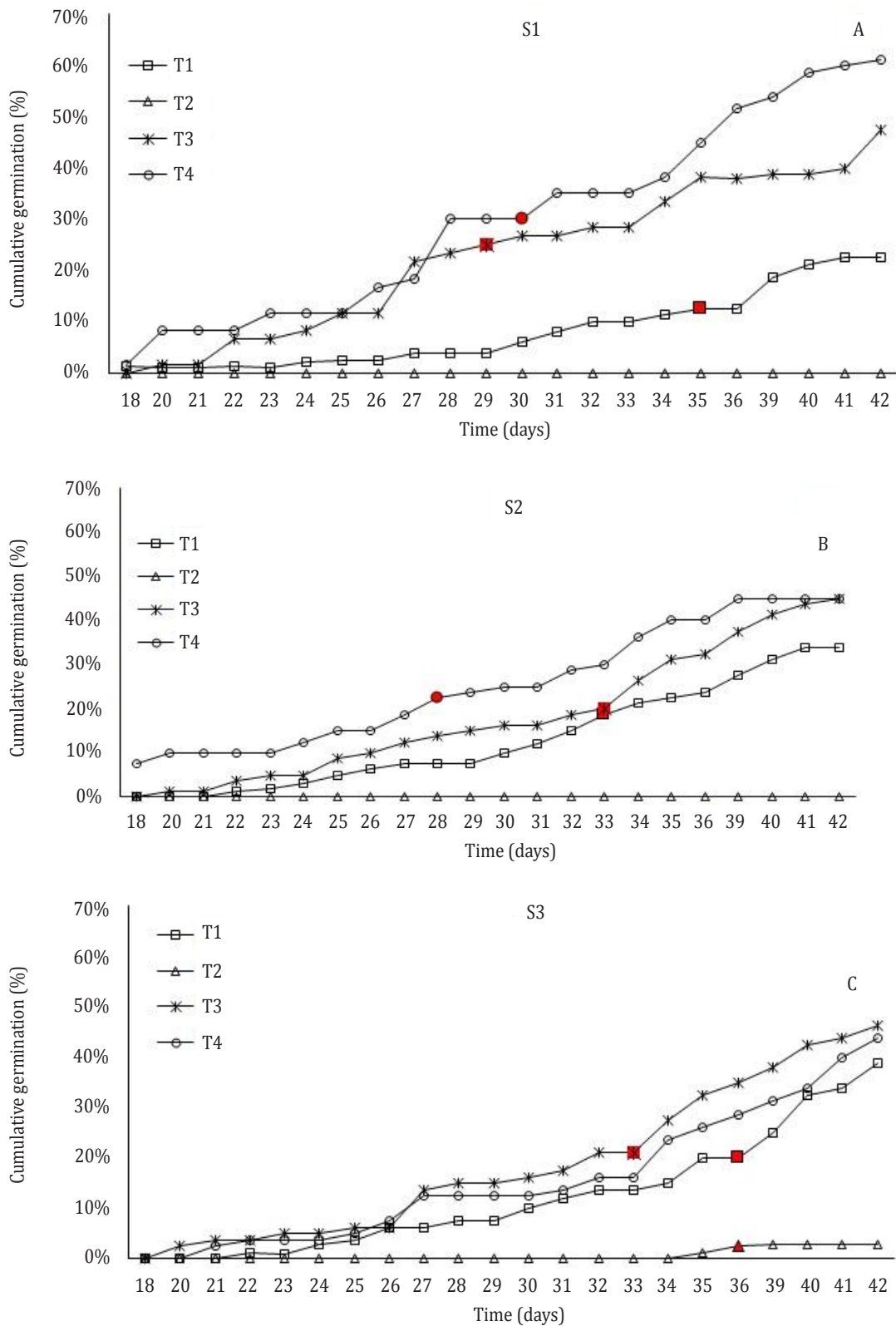
Tukey's multiple comparison test for the interaction between treatments and substrates (Figure 4) indicated five groups (A, B, BC, CD, D). Furthermore, T4 in S1 shows the highest mean germination ( $\bar{x}=12.25$ ), equal to 61% germination. The cumulative germination percentage (Figure 5) was congruent with this result; accordingly, T4 in S1 showed the highest germination percentage than other substrates and treatments. Overall,

*C. guianensis* was found to germinate gradually, which agrees with reports by Villota (2016) and Hernández-Coronado *et al.* (2018).

No significant differences ( $P=0.32$ ) were found for the initial germination percentage, as shown in Figure 5. However, the highest value was found for T4 in S2, it reached 8% germination on day 18 of the experiment (Figure 5B). Villacorta (2010) reported the same results and determined that this species' onset of germination occurred on day 18 after planting, based on a previous imbibition treatment for 48 hours at room temperature. However, these results differ from those found by Vianna (1982), who assessed the behavior of *Andiroba* seeds in Tapajos National Forest (Santarém, PA). The author reported that onset of germination occurred at day 1, without applying pre-germination treatments. Conversely, Villota (2016) reported that the onset of *Carapa amorphocarpa* W., a species of the same family, occurred on day 43 in sand + soil substrate. Sanchez *et al.* (2019) indicated that *C. guianensis* develops better in very humid substrates, such as soil, storing and retaining moisture.



**Figure 4.** Cumulative germination for pre-germination treatments and substrates interaction. Tukey's multiple comparison test. Different letters above the columns indicate significant differences (Tukey.  $P < 0.05$ ).



**Figure 5.** The Behavior of the cumulative germination percentage of *C. guianensis*. A: S1; B: S2; C: S3 under pre-germination treatments: T1; T2; T3; T4. Shapes in red: mean germination time ( $t_{50}$ ).

Regarding the final germination percentage (Figure 5), T4 in S1 reached 61% after 42 days (Figure 5A), while T2 showed 0% to 3% germination in the same period. These results exceed those reported by Kossmann *et al.* (2002). He determined that *C. guianensis* only reached 30% germination in 40 days without any treatment. Scarano *et al.* (2003) a hard wood tree from the Brazilian Amazon, has large recalcitrant seeds that can germinate and establish in both flood-free (terra-firme) mention that 88% of seeds germinated after 30 days, 82% after 2 months, and 70% after 90 days after a flotation technique applied to different seed lots of *C. guianensis*.

There were no significant differences ( $P=0.24$ ) in  $t_{50}$  for the total number of germinated seeds. The mean germination time ranged from 28 to 36 days. The lowest value was obtained for T4 in S2 ( $t_{50}=28$  days with 23% germination), indicating that less time was required to reach 50% germination than in other treatments and substrates. Meanwhile, T2 in S3 ( $t_{50}=36$  days with 3%) showed the highest mean germination time. Nevertheless, this result is lower than Myers (2013) reported because he found a mean germination time of 65 days of *C. guianensis*. The results differ from those reported for *Swietenia macrophylla* King, a species of the same family (Meliaceae) since it displays a germination time ranging from 8 to 14 days (Céspedes, 2018). Therefore, seed germination and establishment requirements are species-specific and are influenced by the characteristics of the region that the species has adapted to (Sánchez *et al.*, 2019).

## CONCLUSIONS

The *C. guianensis* seeds' biometric characteristics from Tumaco-Nariño allow classifying the seeds as relatively large (weight: 35.7g, length: 59.1mm, width: 54.8mm, and thickness: 43.2mm).

A comparison of the CGP of *C. guianensis* reported in different studies, including this study, shows that

germination is highly variable and likely depends on agroecological conditions.

By comparing the CGP with other studies, we determined that germination of *C. guianensis* is highly variable and it likely depends on the agroecological conditions of the site. Therefore, in ecosystems similar to the one found in Tumaco-Nariño, the interaction of mechanical scarification + imbibition for 24h in sand substrate allows cumulative germination of 61%.

The mean germination time ranged from 28 to 36 days; however, it was not affected by the pre-germination treatments and substrates used, indicated by non-significant differences.

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**Conflict of interest:** The authors declare that there is no conflict of interest.

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# Simultaneous selection indices for yield and stability in sugarcane

## Índices de selección simultánea de rendimiento y estabilidad en caña de azúcar

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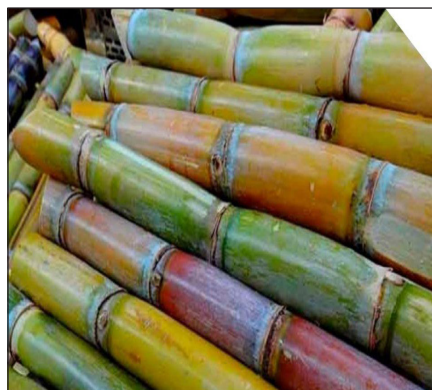
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### ABSTRACT

In Venezuela, sugarcane is grown in distinct environments, including soil categories and cultural practices. This research aimed to identify high-yield and stable sugarcane (*Saccharum* spp., hybrid) genotypes using selection indices. The experimental material consisted of sixteen genotypes and two commercial varieties as control. The genotypes were evaluated in five locations and by two harvest cycles. The yield in Ton Pol per hectare (TPH) was subjected to an analysis of variance according to the AMMI model. Methodologies of adaptability, stability, and indices that combine both stability and yield were determined. It was possible to distinguish the indices in three groups in the biplot. The superiority index (Pi), geometric adaptability index (GEI), and reliability index (Ii) were located in group 1 (G1). This group coincided in classifying genotypes CR87-339, C323-68, and V98-76 as the most stable and the best yield. The second group (G2) consisted of Kang's rank-sum (RS), Stability index (I), Sustainability index (SI), and geometric adaptability index (GDI) and located the genotypes CR87-339 and C323-68 as the most desired. The third group (G3) was constituted by the Ecovalence statistics (W), Shukla's unbiased stability variance ( $\sigma^2i$ ) and the AMMI value (ASV) and classified the genotypes CP87-1762, V90-6 and CP 92-1641 as the best. The indices that best discriminated the genotypes and most associated with yield were PI, GAI and Ii.

**Key words:** adaptability; AMMI; biplot; hybrid; *Saccharum* spp.; stability index.

### RESUMEN

En Venezuela, la caña de azúcar es cultivada en disímiles ambientes, incluyendo tipos de suelos y prácticas culturales. El objetivo de este trabajo fue identificar genotipos de caña de azúcar (*Saccharum* spp., híbrido) estables y de altos rendimientos usando índices de selección. El material experimental consistió de dieciséis genotipos experimentales y dos testigos comerciales. Los genotipos fueron evaluados en cinco localidades y dos ciclos de cosecha. Los datos de rendimiento en Tonelaje de Pol por hectárea (TPH) fueron analizados mediante el modelo AMMI. Metodologías de adaptabilidad, estabilidad e



índices que combinan tanto estabilidad y rendimiento fueron determinadas. En el biplot se logró distinguir los índices en tres grupos. En el grupo G1 se ubicaron los índices de superioridad (Pi), adaptabilidad geométrica (GAI) y confiabilidad (Ii). Este grupo coincidió en clasificar a los genotipos CR87-339, C323-68 y V98-76 como los más estables y de mejor rendimiento. El segundo grupo (G2) estuvo conformado por los Índices de estabilidad (I), suma de rangos (RS), sostenibilidad (IS) y estabilidad genotípica (GDI) y ubicaron a los genotipos CR87-339 y C323-68 como los más promisorios. El tercer grupo (G3) fue constituido por los estadísticos Ecovalencia (W), Varianza estabilizante de Shukla ( $\sigma^2$ ) y el valor de AMMI (ASV) y clasificaron a los genotipos CP88-1762, V90-6 y CP92-1641 como los mejores. Los índices que mejor discriminaron y estuvieron más asociados al rendimiento fueron PI, GAI e Ii.

**Palabras clave:** adaptabilidad; AMMI; biplot; híbrido; índices de estabilidad; *Saccharum* spp.

## INTRODUCTION

Sugarcane (*Saccharum* spp hybrid), grows in tropical and sub-tropical areas and produces approximately 70% of the world's sugar (Scortecchi *et al.*, 2012). In Venezuela, sugarcane grows up in diverse environments. One of the main goals of sugarcane breeding programs is to obtain high-yielding cultivars adapted to different agro-ecological regions (Rea *et al.*, 2014). Therefore, it is essential to establish selection indices that simultaneously combine yield and stability. However, Genotype by Environment Interaction (GEI) reduces the association between genotypic and phenotypic values and affects the selection progress (Rea *et al.*, 2014). The importance of GEI in genotype evaluations and breeding programs has been demonstrated in many crops, including sugarcane. Several statistical methods (parametric and non-parametric) have been proposed to study the GEI (Lin *et al.*, 1986, Mohammadi and Amri, 2008). Becker and León (1988) proposed two concepts for these models: the biological or static, where the ideal genotype will be one that presents minimal variation across environments, showing a constant performance in any area of production (minimum statistical variance), and the agronomic or dynamic that represents an exiguous GEI and is associated with the pretension to obtain an increase in yield in response to environmental improvements.

The most commonly used univariate parametric methods to estimate stability are regression

(Eberhart and Russel, 1966), stability variance (Shukla, 1972) and ecovalence (Wricke, 1962). The multivariate model of the Principal Additive Effects and Multiplicative Interactions (AMMI) is based on a linear-bilinear statistical model (Cossa *et al.*, 2004), in which the main effects of genotypes and environments are considered linear terms and are explained by conventional analysis of variance; the bilinear component (non-additive) is imputed to the GEI, and analyzed by the principal component technique. Purchase *et al.* (2000) developed the AMMI stability value (ASV) using the first two principal components (CP1 and CP2) of the AMMI model.

These parameters are effectively assessing adaptability, but there are cases in which the most adapted genotype does not show good yield. Due to these failures, efforts have been made to develop indices that incorporate both stability and yield in only one criterion to select new cultivars (Rea *et al.*, 2014). Kang and Pham (1991) evaluated several methods for simultaneous selection of yield and stability and proposed the rank-sum that combines the Shukla stability variance and yield in a single value. Bajpai and Prabhakaran (2000) proposed a modified method to rank-sum termed stability index (I). The superiority index (P) of Lin and Binn (1988) integrates yield and stability and has been used in selection programs in corn (*Zea mays* L.), sugarcane, rice (*Oryza sativa* L.), and soybean (*Glycine max* L.).

The practical interest in combining high yields and stability led to the reliability index's development (Annicchiarico, 2002). Farshadfar (2008), which adds the ASV and yield in a single criterion, suggested an approach known as the genotypic selection index (GSI). The sustainability index (SI) has been used to select stable and high yield in wheat genotypes (*Triticum aestivum* L.) (Farshadfar *et al.*, 2011).

The objectives of this research were: a) to identify high-yield and stable sugarcane (*Saccharum* spp., hybrid) genotypes and b) to evaluate the interrelation of stability index and yield.

## MATERIALS AND METHODS

**Experimental clones.** Yield data were obtained from the sugarcane-breeding program at the National Institute for Agricultural Research (INIA), Yaracuy State-Venezuela. The evaluated material consisted of sixteen experimental genotypes: CP80-1743, CP80-1827, CP88-176, CP89-2143, CP89-2377, CP92-1213, CP92-1641, CR83-323, CR87-339, LCP85-384, V90-11, V90-14, V90-2, V90-3, V90-6 and two commercial varieties as control: C323-68 (T) and CP74-2005 (T).

**Locations.** The genotypes were evaluated in five locations for two years (plant and ratoon crops) in Carora, La Pastora, Turbio I (early growing season), and Turbio II (late growing season) located in Lara state, El Palmar in Aragua state, and Matilde in Yaracuy state. Table 1 shows some agroclimatic characteristics of the localities. Each genotype was planted in three-row plots of 10 m long and 1.5 m wide with three replications in a completely randomized block design. Cultural and agronomic practices prevalent for each environment were applied.

**Table 1.** Some agroclimatic characteristics of the evaluated localities.

Locations	rainfall (mm)	Texture	pH
Matilde	1189	Silt loam	8.1
Carora	1146	Loam	7.3
The Palmar	1115	Sandy loam	6.7
Rio Turbio	700	Clay loam	8.0

**Variable evaluated.** The sugarcane was burned, and the plots of each genotype were harvested by hand. A random sample of 10 stems was taken from each plot and weighed. The samples were pressed, and the juice was analyzed to determine the apparent sugar content (Pol% cane).

The evaluated variable was cane yield expressed in TPH, which was calculated as a ratio of TCH and Pol% cane by the formula (1) by (Pérez-Guerra *et al.*, 2009):

$$\text{TPH} = (\text{TCH} \times \text{Pol\% Cane}) / 100 \quad (1)$$

**Statistical analysis.** An analysis of variance using the AMMI model was performed to access the variables. Methodologies of adaptability, stability and indices that combine both stability and yield were determined: Genotypic stability index (GSI), stability index (I), reliability index (Ii), geometric adaptability index, rank-sum (RS) and sustainability index (SI). A principal components analysis was performed using the rank of the statistics and indices. The AMMI analysis was carried out according to the model suggested by Crossa *et al.* (2004) equation 2.

$$Y_{ij} = \mu + g_i + \varepsilon_j + \sum_{k=1}^n \lambda_k a_{ik} \gamma_{jk} + \varepsilon_{ij} \quad (2)$$

Where:

$Y_{ij}$  = Mean yield of *i*th genotype in the *j*th environment

$\mu$  = the general mean

$g_i$  = The *i*th genotypic effect

$\varepsilon_j$  = the *j*th environment effect  
 $n$  = the number of PCA axes retained in the model  
 $\lambda_k$  = eigen value of the PCA axis  
 $a_{ik}$  = the *i*th genotype and *j*th environment PCA scores for the PCA axis *n*  
 $\epsilon^{ij}$  = is the residual

### Stability methods

**Ecovalencia.** Wricke (1962) suggested using the GEI for each genotype as a measure of stability which was denominated ecovalence (*W*).

$$W_i = \sum_j^e (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..})^2 \quad (3)$$

Where:  $Y_{ij}$  = Observed response of the genotype ;  
 $\bar{Y}_{i.}$  = Average of a genotype through environments;  
 $\bar{Y}_{.j}$  = mean yield in an environment;  $\bar{Y}_{..}$  = grand mean; *e* = number of environments; a genotype with a low *W* value is considered high stability.

The stability variance ( $\sigma_1^2$ ) is based on the decomposition of the GEI into *g* genotypes. It is equal to environmental variance within each environment ( $\sigma_0^2$ ) more environmental variance for each genotype, corrected for additive effects of environments, If  $\sigma_1^2 = \sigma_0^2$  implies that if  $\sigma_0^2 = 0$ ; then the genotype will be stable.

Variance of Shukla (4)

$$\hat{\sigma}_1^2 = \frac{1}{(G-1)(G-2)(E-1)} \left[ G(G-1) \sum_j (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..})^2 - \sum_i \sum_j (Y_{ij} - \bar{Y}_{i.} - \bar{Y}_{.j} + \bar{Y}_{..}) \right]$$

Where:  $Y_{ij}$  = Observed response of the genotype;  
 $\bar{Y}_{i.}$  = average of a genotype through environments;  
 $\bar{Y}_{.j}$  = mean yield in an environment;  $\bar{Y}_{..}$  = Grand mean; *G* = number of genotypes; *E* = Number of environments; low values of variance indicates that a genotype is stable.

**AMMI value (ASV).** The AMMI Model does not foresee a quantitative measure to estimate stability. Purchase *et al.*, (2000) proposed the

value of AMMI (AMMI stability value-ASV). The ASV is the distance from the origin in a dispersion diagram of a two-dimensional system towards the scores of CP1 vs.CP2.

$$ASV = \sqrt{\left[ \frac{SSCP1}{SSCP2} (ScoreCP1) \right]^2 + [ScoreCP2]^2} \quad (5)$$

Where: SSCP1 = is the sum of squares of the first principal component (CP1); SSCP2 = is the square sum of the second principal component (CP2); Score CP1 and Score CP2 are the scores of the first two principal components. Low values of ASV in the genotypes are considered widely adapted.

### Simultaneous selection for stability and yield

**Rank sum (RS).** One of the advantages of nonparametric measures is their ease of calculating and interpreting (Rea *et al.*, 2015). This statistic gives equal weight to the rank of yield and variance of Shukla in a single measure. The average genotype's yield was assigned the rank of 1, and the genotype with the lowest variance of Shukla was imputed the rank of 1. Low values of the rank-sum (RS) indicate better behavior of the genotype (Kang and Pham, 1991).

$$RS = \text{Shukla variance rank} + \text{yield rank in TPH} \quad (6)$$

**Genotypic stability index (GSI).** This index was recommended by Farshadfar *et al.* (2011) and is defined as the rank sum of ASV value plus the rank sum of the genotypic mean across the environment.

$$GSI = RASV_j + RY_i; \quad (7)$$

**Where:** GSI is the stability index for the *i*<sup>th</sup> genotype through the environment for TPH; RASV<sub>*j*</sub> is the rank of the *i*<sup>th</sup> genotype across environment based on the ASV value (AMMI Value); RY<sub>*i*</sub> is the rank of the *i*<sup>th</sup> genotype based on the average in each environment. Genotypes with low GSI values were considered the best across environments.

**Stability index (I).** This index was proposed by Bajpai and Prabhakaran (2000) and was computed according to the following equation:

$$I = \left[ \frac{\bar{Y}_i}{\bar{Y}} + \frac{1}{\sigma_i^2} \right] / \left[ \frac{1}{n} \sum_i \left( \frac{1}{\sigma_i^2} \right) \right] \quad (8)$$

Where:

$\bar{Y}_i$  = Average of  $i^{\text{th}}$  genotype;  $\bar{Y}$  = General mean,  $\sigma_i^2$  = Shukla variance of the  $i^{\text{th}}$  genotype. Genotypes with high values of stability indexes are the best.

**Superiority index (P<sub>i</sub>).** The index was calculated from the sum of squares of the differences between the genotype of interest for the genotype of higher yield in each of the environments, so it represents the mean square of the joint effect of genotypes and GEI. In addition, it determines the adaptability in a broad sense (Lin and Binn, 1988) calculated about the maximum response. A small value of  $P_i$  implies general adaptation of a genotype. The calculation formula was the following:

$$P_i = \frac{\sum_{j=1}^n (Y_{ij} - \bar{M}_j)^2}{2n} \quad (9)$$

Where

$Y_{ij}$  = is the mean yield of the  $i^{\text{th}}$  cultivar in the  $j^{\text{th}}$  environment

$\bar{M}_j$  = is the maximum response observed among all the cultivars in environment  $j$ .

$n$  = is the number of environments.

**Reliability index (I<sub>r</sub>).** This index was determined following the procedure described by Annichiriaco (2002), based on the distribution of the observed means of TPH across the test environments. The equation used was the following:

$$I_r = m_i - Z(P)S_i \quad (10)$$

Where:  $m_i$  = mean yield;  $S_i$  = square root of the environmental variance and  $Z_{(P)}$  = Percentile of the

normal distribution for a probability value (P). Depending on the level of probability (P),  $Z_{(P)}$  can assume the following values: 0.675 to  $P = 0.75$ ; 0.840 to  $P = 0.80$ ; 1.040. P values may vary between 0.95 (for subsistence agriculture in unfavorable cropping regions) to 0.70 for modern agriculture in most favorable regions.

**Geometric adaptability index (GAI).** The geometric mean can be used as a measure of adaptability:

$$GAI = \sqrt[E]{(\bar{X}_1)(\bar{X}_2)\dots(\bar{X}_i)} \quad (11)$$

Where  $X_1$ ,  $X_2$  are the mean yields of the first, second and  $i^{\text{th}}$  genotype through environments and  $E$  is the number of environments. Genotypes with high GEI are the chosen ones (Pourdad, 2011).

**Sustainability Index (SI).** The Sustainability Index was estimated according to the equation used by Babarmanzoor et al. (2009).

$$SI = \left[ \frac{(Y - \sigma n)}{YM} \right] \times 100 \quad (12)$$

Where

$Y$  = TPH mean of a genotype through environments

$\sigma n$  = standard deviation

$YM$  = Better yield of a genotype in any year or location.

The sustainability index values were divided arbitrarily inside of 5 groups: Very low (until 20 %), Low (21-40 %), moderate (41-60 %), high (61-80%) and very high (plus of 80 %).

**Association between statistics, indices and yield.** A principal components analysis was performed using the rank matrix to determine the degree of association between indices, statistics and the mean yield in TPH.

## RESULTS AND DISCUSSION

**AMMI model.** The mean yield in TPH was significantly affected by environmental and genotypic effects. The first two axes of the principal components (CP1 and CP2) of the interaction of the AMMI model were significant ( $p < 0.01$ ).

The TPH mean of the locations varied between 14.80 (Turbio I) and 20.56 (Matilde). The yields of the clones ranged from 9.82 (CP92-1641) to 27.92 (CR87-339) (Table 2). Significant differences for the GEI show that the response of the varieties was distinct in diverse environments. Rea *et al.* (2014) note that the GEI reduces the correlation between genotypic and phenotypic values, affecting selection's progress. One of the methods to minimize the GEI is to select stable cultivars using stability analysis.

**Yield stability in TPH.** Table 4 shows the results of several statistics and stability indices. For the ecovalence and variance of Shukla, the most stable genotypes were CP88-1762, V90-6 and CP92-1641. This similarity in the results of both methods has been demonstrated by Becker and León (1988). The AMMI stability value was estimated according to Purchase *et al.* (2000). The results showed that the sum of squares of the first two principal components explained 67% of the data variance. According to the ASV, the genotypes V90-6, CP88-1762 and CP92-1641 are the most stable, coinciding with the genotypes selected by the ecovalence and Shukla variance methods. Purchase *et al.* (2000) point out that ASV can be compared with  $W$  and  $\sigma_i^2$ .

**Table 2.** Average yield in TPH of eighteen sugarcane genotypes at six environments in two cycles of harvest.

Variety	Carora	Palmar	Matilde	La Pastora	Turbio I	Turbio II	Mean
C 323-68 (T)	19.90	23.61	22.74	22.88	17.35	17.72	20.70
CP74-2005 (T)	15.39	21.85	23.22	15.99	15.71	15.91	18.01
CP80-1743	13.62	16.13	22.90	19.22	15.22	17.27	17.39
CP80-1827	10.54	15.49	18.47	21.86	10.92	14.13	15.23
CP88-1762	13.32	15.01	19.36	17.37	12.64	13.43	15.19
CP89-2143	18.13	16.59	21.50	20.77	15.38	12.98	17.56
CP89-2377	13.64	16.14	16.99	20.88	13.97	10.38	15.33
CP92-1213	11.31	16.06	20.21	21.21	10.75	13.72	15.54
CP92-1641	9.82	14.56	14.90	13.52	11.02	10.67	12.41
CR83-323	18.30	17.34	19.70	18.27	15.64	17.20	17.74
CR87-339	25.42	27.92	27.56	25.91	24.15	23.52	25.75
LCP85-384	16.10	18.52	20.05	18.27	15.85	13.11	16.98
V90-11	14.57	16.74	24.19	19.65	13.78	15.83	17.46
V90-14	12.19	14.59	16.91	16.75	14.26	11.07	14.30
V90-2	13.63	17.24	19.91	14.08	12.27	20.75	16.31
V90-3	18.63	15.51	20.57	17.92	13.93	16.85	17.23
V90-6	14.54	15.70	19.70	17.98	14.20	14.39	16.09
V98 76	15.94	16.45	21.14	18.01	19.44	16.31	17.88
<b>Mean</b>	15.28	17.52	20.56	18.92	14.80	15.29	17.06

(T)= Checks

### Indices for stability and yield

**Kang's (1988) rank-sum.** The results of six stability indices are presented in Table 3. According to Kang's rank-sum, genotypes with low values are considered the most desirable. This index revealed that the genotypes CR 87-339 and C32-368 (T) had the lowest values and therefore were the most stable and yielding, while those with high values such as the genotypes: CP80-1827, V90-2, CP89-2377, CP92-1213 were the most unstable.

**Stability index (I).** The basic element in the index construction is that the genotypes' achievement

levels and their stability are quantified by the individual achievement expression relative to the average behavior of the group of genotypes evaluated. Bajpai and Prabhakaran (2000) proposed this index arguing that the rank sum method has its weaknesses due to the heavyweight given to yield. The ranks assigned by the stability index were in increasing order, the genotype with the highest stability index (I) received the rank 1, and the lowest index received the rank 14 of the 18 genotypes evaluated. According to this index, the CP88-1762, V90-6, CR87-339 and C323-68 genotypes were the most stable and CP80-1827, CP89-2377 and CP92-1213 the adapted less.

**Table 3.** Indices for simultaneous selection of stability and yield in eighteen sugarcane genotypes evaluated in six environments.

Genotipos	TPH	(W)	$\sigma_i^2$	Score CP1	Score CP2	ASV	RS	I	$P_i$	$I_i$	GAI	SI (%)	GSI
C 323-68 (T)	20.7	35.5	7.3	0.7	1.38	1.5	9	0.04	161.2	16.6	20.7	66.8	6
CP 74-2005 (T)	18.0	90.6	19.7	-3.2	0.62	4.5	19	0.03	383.9	13.5	17.7	54.2	17
CP 80-1743	17.4	41.1	8.5	-0.7	-2.8	2.9	17	0.03	463.6	13.1	17.2	53.6	19
CP 80-1827	15.2	107.9	23.6	3.9	-4.1	6.8	35	0.03	738.2	10.2	14.7	42.5	31
CP 88-1762	15.2	3.2	0.01	0.3	-0.61	1.0	17	5.66	703.4	10.3	15.0	48.2	18
CP 89-2143	17.6	52.6	11.1	2.4	2.02	3.9	17	0.03	432.4	13.5	17.3	61.1	19
CP 89-2377	15.3	84.1	18.2	4.8	1.84	6.9	28	0.03	690.7	11.3	14.9	50.3	31
CP 92-1213	15.5	84.9	18.4	3.5	-3.93	6.2	28	0.03	691.5	10.7	15.3	46.2	28
CP 92-1641	12.4	17.5	3.2	-0.6	0.77	1.1	21	0.03	1073.1	8.6	12.3	52.5	21
CR 83-323	17.7	35.7	7.3	-1.9	1.44	2.9	13	0.03	394.9	14.6	17.7	71.2	15
CR 87-339	25.8	34.2	6.9	-1.3	2.61	3.2	7	0.05	0.0	21.4	25.7	73.6	13
LCP 85-384	16.9	24.5	4.8	0.5	2.57	2.7	14	0.03	467.2	12.7	16.8	54.1	16
V 90-11	17.5	45.7	9.6	0.3	-2.74	2.8	17	0.03	459.7	12.8	17.1	49.3	15
V 90-14	14.3	24.9	4.9	1.3	1.8	2.6	22	0.03	802.5	11.4	14.8	64.3	22
V 90-2	16.3	178.5	39.4	-6.7	-3.43	9.8	29	0.03	600.7	11.5	16.0	51.1	29
V 90-3	17.2	57.9	12.3	-1.9	0.51	2.7	21	0.03	461.6	12.6	17.1	56.9	16
V 90-6	16.1	2.8	0.09	0	0.1	0.1	14	0.34	574.5	12.1	15.9	57.6	13
V 98-76	17.9	63.8	13.6	-1.5	1.96	2.8	17	0.03	399.2	14.1	17.8	63.4	13

(T)= Checks

**Linn and Binn superiority index ( $P_i$ ).** This index is a unique measure of stability and yield. Based on this method, the selected genotypes were those with low indices and yields above the general average (Kilic *et al.*, 2010). In this case, the best genotypes were C87-339, C323-68, CP74-2005 (T), CR83-323 and V98-78.

**Geometric adaptability index (GAI).** This index is based on the geometric mean of the genotype. Genotypes with high GAI values are acceptable (Pourdad, 2011). In this case, the genotypes CR 87-339, C323-68 (T), V98-76 and CR 83-323 were those with the best yields and stability.

**Reliability index ( $I_i$ ).** To estimate this index, it was assumed that the technological level for agriculture of Venezuela falls between subsistence levels and modern agriculture, where it was taken ( $P$ ) = 0.8 corresponding to a value of  $Z(p) = 0.84$ . This value was inserted in the equation of  $I_i$ ; a genotype is selected if it has high values of  $I_i$ . In this way, the genotypes: CR87-339, C323-68, CR83-323 and V98-76 were the most outstanding. Gómez-Becerra *et al.* (2007) selected genotypes of wheat (*T.Aestivum*) based on this method.

**Sustainability Index (SI).** The values of this index were arbitrarily divided into five groups, from very low and very high. The genotypes in the high group (61-80%) of sustainability were CR87-339, CR83-323, C323-68, V90-14, V98-76, CP89-2143. The rest of the genotypes were grouped as of moderate sustainability. This index was used successfully by Koli and Prakash (2013) in the selection of rice varieties.

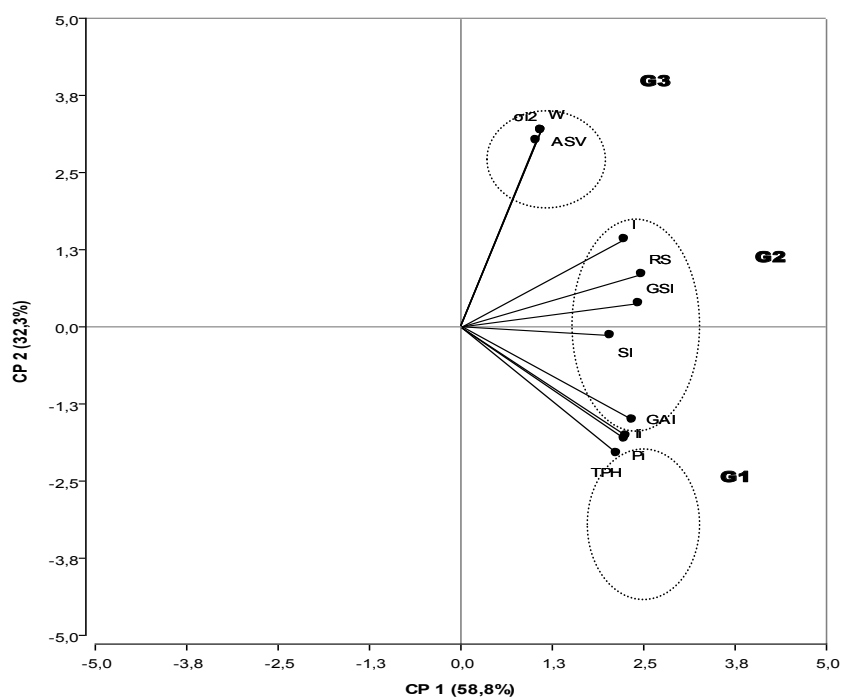
**Genotypic stability index (GSI).** This index integrates both the AMMI value and the yield in a single selection criterion proposed by Fardshafar (2008). The genotypes with low values of this index are those selected. According to this criterion, the genotypes with the best behavior were C323-68, CR87-339, V98-78 and V90-6.

In the results obtained, it can be observed that when only the stability methods are used, the selected genotypes are not the ones with the best yields; On the other hand, when the selection indices are used, the most yielding genotypes appear among those selected: CR87-339 and C32-362. This demonstrates the importance of considering both stability and yield selecting genotypes when they are evaluated in diverse environments.

**Association between different indexes using main components.** An analysis of the principal components of the matrix of ranks of statistics and stability indexes was made to observe the associations between them (Table 4). A biplot with the values of CP1 and CP2 is shown in Figure 1. The two principal components represented 90.8% of the total variation, distinguishing the indices in three different groups. In group 1 (G1) were located  $P_i$ , GAI,  $I_i$  and the yield in TPH. This group coincided in classifying genotypes CR87-339, C323-68 and V98-76 as the most stable and best performing. The second group (G2) was composed of RS,  $I_i$ , SI and GDI and identified the genotypes CR87-339 and C323-68 as the best. The genotype V98-76 was selected by the GDI and SI indices. The W statistics, Shukla and the AMMI value constituted the third group (G3). According to the statistics, the best genotypes were CP87-1762, V90-6 and CP 9216-41. Group 3 differed in the classification of genotypes to the groups 1 and 2. This is a clear indication of the utility of using indices where both yield and stability are considered in a single criterion. The statistics of Group 3 only measure stability, and there are cases in which the most stable genotype is not the most yield (Mohammadi and Amri, 2008). These results corroborate the findings of other authors searching for approaches that include both stability and yield in a single index for cultivars selection in different environments (Babarmanzoor *et al.*, 2009; Farshadfar *et al.*, 2011; Rao and Prabhakaran, 2005).

**Table 4.** Ranks of sugarcane genotypes according to the mean of TPH, stability and stability indexes.

Genotipos	TPH	W	$\sigma^2$	ASV	RS	I	$P_i$	$I_i$	GEI	SI	GSI
C323-68	2	7	7	4	2	4	2	2	2	3	1
CP74-2005	3	16	16	14	11	11	3	6	4	9	9
CP80-1743	8	9	9	11	6	7	8	7	7	11	11
CP80-1827	15	17	17	16	18	18	16	17	17	18	17
CP88-1762	16	1	1	2	6	1	15	16	14	16	10
CP89-2143	6	11	11	13	6	7	6	5	6	6	11
CP89-2377	14	14	14	17	15	16	13	14	15	14	17
CP92-1213	13	15	15	15	15	16	14	15	13	17	15
CP92-1641	18	3	3	3	12	13	18	18	18	12	13
CR83-323	5	8	8	10	3	6	4	3	5	2	5
CR87-339	1	6	6	12	1	3	1	1	1	1	2
LCP85-384	10	4	4	6	4	5	10	9	10	10	7
V90-11	7	10	10	8	6	7	7	8	8	15	5
V90-14	17	5	5	5	14	13	17	13	16	4	14
V90-2	11	18	18	18	17	15	12	12	11	13	16
V90-3	9	12	12	7	12	11	9	10	9	8	7
V90-6	12	2	2	1	4	2	11	11	12	7	2
V98-76	4	13	13	9	6	7	5	4	3	5	2

**Figure 1.** Biplot of ranks for TPH, stability and stability indexes in sugarcane genotypes.



## CONCLUSIONS

The selection of cultivars for high yield and stability is an important component of the sugarcane-breeding program, where environments are variable and unpredictable. According to this work, the genotypes most stable and best yields were CR87-339, C323-68 and V98-76.

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# Environmental offering and coffee cup quality (*Coffea arabica* L.) Var. Castillo in southern Colombia

## Oferta ambiental y calidad de taza de café (*Coffea arabica* L.) Var. Castillo en el sur de Colombia

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### ABSTRACT

The determinant factors of coffee cup quality are highly variable and depend on their interaction with coffee production and benefit. This study aimed to analyze soil and climatic factors and their association with the cup quality of Castillo coffee variety of three to five years of age from production units in ecotypes 220A and 221A of the Department of Nariño. The study farms were located in three different altitudinal ranges:  $\leq 1500\text{m}$ , between 1501 and 1700m, and  $>1700\text{m}$ . Soil, climate, and coffee cup quality variables were analyzed through principal component analysis and cluster analysis. A low level of association was found between climatic and soil nutritional factors and coffee cup quality. Soil Mn, Fe, and Cu contents showed the highest association levels with cup quality, indicated by an average score of 80.89. The highest values of photosynthetically active radiation -PAR- and thermal amplitude were found in La Unión - Nariño, and these variables were associated with the group that obtained the highest cup quality score (82.58). Cup quality was not associated with elevation since the highest scores (85.5 and 82.33) were obtained from production units located at  $\leq 1500\text{m.a.s.l.}$  and  $>1700\text{m.a.s.l.}$ , respectively.

**Keywords:** score; quality; ecotypes; soil; climate; multivariate analysis.

### RESUMEN

Los factores determinantes de la calidad de taza de café son muy variables y dependen de la interacción de los relacionados con su producción y beneficio. El presente trabajo se orientó al análisis de los factores de suelo y clima y su relación con la calidad de taza de café variedad Castillo de tres a cinco años de edad en unidades productivas de los ecotopos 220A y 221A del departamento de Nariño. Las fincas de estudio se ubicaron en tres diferentes rangos altitudinales:  $\leq 1500\text{m}$ , entre 1501 y 1700m y  $>1700\text{m}$ . Las variables de suelo, clima y calidad de taza se sometieron a los Análisis de

Componentes Principales y Agrupamiento. Se detectó un bajo nivel de asociación de los factores contenidos nutricionales del suelo y climáticos con la calidad de taza. Los contenidos de Mn, Fe y Cu en el suelo fueron los que mostraron un mayor nivel de asociación con la calidad de taza con un promedio de 80,89. En el municipio de La Unión se presentaron los mayores valores de radiación fotosintéticamente activa-RFA y amplitud térmica los cuales estuvieron relacionados con el grupo que se caracterizó por tener altos puntajes de calidad de taza (82,58). Los puntajes de calidad de taza no presentaron una tendencia a asociarse con la altitud; es así como los mayores puntajes (85,5 y 82,33) se encontraron en unidades productivas ubicadas a  $\leq 1500$ msnm y  $> 1700$ msnm respectivamente.

**Palabras clave:** puntaje; calidad; ecotopos; suelo; clima; análisis multivariado.

## INTRODUCTION

Colombian coffee transitioned from being unique due to its overall characteristics to have quality attributes determined by agroecological conditions associated with the coffee variety, benefit process, and growing region. Additionally, the coffee origin is important for positioning on national and international markets (Raynolds, 2009; Sepúlveda *et al.*, 2013). The factors that affect cup quality are highly variable and represent a bottleneck for creating protocols to obtain quality products. Accordingly, Orozco *et al.* (2011) determined an unclear association between variety, elevation, soil, and cup quality. The characteristics that define cup quality are determined by a series of physical and chemical variables, such as color, characteristic diameter, equatorial and polar firmness, fresh and dry weight, moisture content, fresh, dry weight ratio, titratable acidity, soluble solids, and pH of the pulp, as well as organoleptic traits such as aromatic intensity, bitterness, body, aroma, and overall standard (Moreno, 2007).

Coffee grain yield, chemical composition, and physical characteristics can be modified or limited by factors such as temperature (i.e., as a result of altitudinal effects) that prolong maturity of the coffee berry, thus favoring grain-filling and cup quality. Furthermore, other relevant factors include the origin of the soil and fertility, atmospheric conditions, harvest time and distribution, moisture content of the coffee berry, and collection methods

and quality (Montilla *et al.*, 2008; Suárez *et al.*, 2015).

The selection of the components of the Castillo variety involved an assessment of the physical and organoleptic beverage's attributes concerning the genotype. A descriptive analysis of its components was used to identifying the genotypic groups that contribute to its sensory characteristics (Alvarado *et al.*, 2009).

The maturation process involves physico-chemical changes in the coffee berry. Marín *et al.* (2003) determined that the physical changes most associated with fruit maturation were removal force and equatorial and polar firmness since these variables decreased as the berries matured. The best-scored cup was obtained from semi-ripe, ripe, and over-ripe berries, while unripe green and dry berries received low quality scores. The best coffee cup quality is derived from adequately processed ripe berries, reflected by the flavor and aroma (Puerta-Quintero, 2000a).

A comparison between grains developed under shade and sun exposure indicated that the former display greater development of the periplasm, which contributes to grain size and reduced sucrose and sugar contents that delay fruit maturation (Geromel *et al.*, 2008).

Quality is also associated with specialty; therefore, the coffee must meet characteristics associated

with a particular flavor and be originated from outstanding ecological niches, where crops are subjected to strict management standards. For these reasons, the consumer is willing to pay a higher price (Castro *et al.*, 2004). According to Farfán (2007), specialty Colombian specialty are characterized by their excellent quality, consistency in their physical and sensory properties, benefit, and preparation.

Ramos and Criollo (2017) state that cup quality is a multifactorial variable. These authors determined that elevation does not directly affect quality, although it is inversely related to the presence of the coffee berry borer, low phosphorous content, and reduced fermentation (16-17h) and drying (20-25h sunlight) times, which were associated with higher quality scores.

In the international market, coffee quality is defined by physical attributes such as size, shape, color, and uniformity, as well as organoleptic traits such as acidity, body, aroma, flavor, roasting, and visual features (i.e., color and clarity of the infusion) (Estrella and Chaves, 2008; Céspedes, 2012). These attributes and characteristics are unique to each variety and, along with benefit, are responsible for the final quality of the coffee beverage (Alvarado and Puerta, 2002). The Castillo variety displays grain and quality traits similar to or better than other crop varieties traditionally grown. Furthermore, the quality characteristics of Caturra, the Timor hybrid, and Castillo are highly homogenous. The beverages show body, slight bitterness, pronounced aroma, and acidity for medium degrees of roasting. Coffee processing under similar and optimal conditions during benefit, roasting, and beverage preparation do not show significant differences in cup quality (Alvarado *et al.*, 2009; Alvarado *et al.*, 2005).

The contrasting results among different authors has not allowed clearly establishing the environmental requirements and optimal pre and post-harvest managements to achieve long-term high-quality standards. Therefore, coffee-growers who produce high-quality coffee are currently hesitant about the factors that contribute to this attribute and, thus, struggle when attempting to maintain the high-quality levels required by specialty markets.

This research aimed to characterize the edaphoclimatic offer for Castillo coffee variety in ecotypes 220A and 221A of the Department of Nariño and to establish the association between environmental offer and cup quality.

## MATERIALS AND METHODS

This study was conducted in farms growing coffee trees of the Castillo variety in ecotypes 220A and 221A of the Department of Nariño. Ecotype 220A is located in the Patía river basin and the sub-basins of Juanambú and Mayo rivers between 1°21' and 1°42'N at 1300 to 1800m.a.s.l., with an annual precipitation ranging from 1700 to 1900mm. This zone is characterized by shallow soils of volcanic origin (i.e., from Doña Juana volcano), with low organic matter content, medium natural fertility, and susceptibility to erosion. The coffee crops are characterized by scattered shade trees. Moreover, ecotype 221A is found in the Guitara river basin located between 1°05' and 1°36'N at 1400 to 2100m.a.s.l., with annual precipitation ranging from 1400 to 1700mm. It is characterized by shallow soils derived from volcanic ash, with low moisture retention capacity and susceptibility to erosion (Gómez *et al.*, 1991). Traditional crop management is related to family economy (Burgos, 2015).

In this study, 82 coffee growers with young coffee crops (3-5 years) of the Castillo variety were selected, who were located at three altitudinal strata: high (H), medium (M), and low (L). Among these coffee growers, 12 were chosen as representatives of the two ecotypes (six per ecotype as quota) using non-probabilistic quota sampling (Otzen and Manterola, 2017). In each ecotype, two coffee farms were selected per altitudinal strata (Table 1).

Each sampling unit consisted of crops with different agronomic practices and conventional (chemical) fertilization. Furthermore, at each unit, passport information was recorded and the location was georeferenced using a Garmin global positioning system device.

The edaphoclimatic variables analyzed were maximum and minimum temperatures, relative

humidity, photosynthetically active radiation, and precipitation. These data were obtained from records for a single year from meteorological stations (Spectrum Technologies, Inc. Model: 2900ET Weather Station), located at the experimental farms objective of the SGR project entitled "Evaluation of the effect of shade by different tree species on the agronomic behavior and quality of coffee, Consacá, Nariño, Occidente".

Soil trait data related to pH, cation exchange capacity (CEC), organic matter (OM), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), boron (B), iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), magnesium (Mg), carbon (C) and sulfur (S) were obtained from the analyses carried out by specialized laboratories of the Universidad de Nariño, which determined.

**Table 1.** Ecotype and altitudinal distribution of the farms selected in this research.

Ecotype	Local	Municipality	Farm	Elevation (m a.s.l.)
220A	1	La Unión	La Onda	>1700 (H)
	2	La Unión	El Oasis	>1700 (H)
	3	La Unión	I.E.D.R. El Sauce	1501-1700 (M)
	4	La Unión	San José	1501-1700 (M)
	5	La Unión	El Retiro	≤1500 (L)
	6	La Unión	El Porvenir	≤1500 (L)
221A	7	Sandoná	Las delicias	>1700 (H)
	8	La Florida	Altos del Carmen	>1700 (H)
	9	Sandoná	Villa María	1500-1700 (M)
	10	Consacá	El Paraíso	1500-1700 (M)
	11	Consacá	El Fantasio	≤1500 (L)
	12	La Florida	La joya	≤1500 (L)

The traits for cup quality were determined based on physical and sensory analyses. They included: fragrance/aroma (Fr/Ar), flavor (Fl), residual flavor (Fr), acidity (Ac), body (Bd), uniformity (Un), balance (Bl), pH, score assigned by the taster (St), and total score (TS). The Specialty Coffee Association of America-SCAA (2015) established a classification according to the score obtained: <80 below the score for a specialty coffee, 80 - 84.99 very good, 85- 89.99 excellent, and 90 - 100 exceptional. The analyses were conducted according to the norms and procedures of Juancafé private laboratory in the city of Pasto, Nariño, which follows the standard protocols of the Specialty Coffee Association (SCAA) and the norms of the Federación Nacional de Cafeteros de Colombia, and it has Q grader tasters certified by the Coffee Quality Institute.

During the main harvest, between April and June, 10 kilograms of coffee cherry were manually collected at each farm and processed through traditional methods. The beans were sun dried at each farm. When the grains reached 10-12% humidity, three random samples of 500g of dry parchment coffee- DPC were taken to Juancafé private laboratory for their physical and sensory analyses (i.e., tasting process).

All of the variables were organized and categorized for the multivariate principal component analysis (PCA), which provides a summarized multivariate description of study conditions, individuals, or sites and allows

comprehending the structure of the associations across the variables (Ávila *et al.*, 2015). Next, a clustering analysis was conducted based on criterion Ward (1963), which is an appropriate statistical method to group variables so that the inertia within groups is minimum and between groups is maximum. The multivariate and cluster analyses were done with the SPAD- 6.3 software.

## RESULTS AND DISCUSSION

The cup quality scores varied between 76.50 and 85.50 (Table 2). Puerta-Quintero *et al.* (2016) indicate that lower temperatures and elevations above 1600m promote better conditions for healthy coffee production. Furthermore, low temperatures allow for natural and controlled fermentation; however, this is not enough to determine cup quality. In contrast, this study found that the highest cup quality was obtained at low elevation ( $\leq 1500$ m.a.s.l.) in El Retiro farm in ecotype 220A. In this regard, Lara and Vaast (2007) state that elevation can significantly influence the biochemical composition and physical and sensory quality of the coffee cup. Ramos and Criollo (2017) found a low correlation (-0.42) between elevation and cup quality. Similarly, Cruz *et al.* (2017) did not find differences in cup quality in response to the altitudinal range (1300 and 1800m.a.s.l.). Therefore, coffee cup quality is not determined by one or a few variables but is rather the result of the entire production and benefits processes (Aristizabal and Duque, 2006), as confirmed by Orozco *et al.* (2011).

**Table 2.** Behavior of sensory variables: fragrance/aroma (Fr/Ar), flavor (Fl), residual flavor (Fr), acidity (Ac), body (Bd), uniformity (Un), balance (Bl), pH, score assigned by the taster (St), and total score (TS), assessed for determining coffee cup quality in Nariño.

Ecotype	Farm	Altitudinal range	pH	Fr/-Ar	Fl	Fr	Ac	Bd	Un	Bl	St	TS
220A	La Onda	A	4.43	7.67	7.50	7.33	7.50	7.50	7.50	7.67	7.67	80.33
	El Oasis	A	4.67	7.17	7.00	7.00	7.00	7.00	7.00	7.17	7.17	76.50
	I.E.D.R.	M	4.47	7.50	7.00	7.17	8.17	7.50	7.50	7.83	7.83	80.50
	San José	M	4.43	7.67	7.17	7.17	8.33	7.33	7.33	7.83	7.83	80.67
	El Retiro	B	4.43	8.17	8.00	7.67	9.17	8.00	7.83	8.33	8.33	85.50
	El Porvenir	B	4.47	7.00	7.00	7.17	9.17	7.33	7.33	7.33	7.33	79.67
221A	Las delicias	A	4.60	7.50	7.50	7.33	9.17	7.50	7.67	7.83	7.83	82.33
	A. del Carmen	A	4.57	7.67	7.50	7.33	7.67	7.50	7.67	7.83	8.00	81.17
	Villa María	M	4.47	7.83	7.50	7.50	7.83	7.50	7.50	8.00	8.00	81.67
	El Paraíso	M	4.70	7.50	7.50	7.50	7.50	7.33	7.33	7.50	7.50	79.67
	El Fantasio	B	4.47	7.67	7.50	7.33	7.83	7.33	7.33	7.83	7.83	80.67
	La Trasyoya	B	4.47	7.17	7.50	7.00	7.17	7.17	7.00	7.17	7.17	77.33
<b>Max</b>			4.70	8.17	8.00	7.67	9.17	8.00	7.83	8.33	8.33	85.50
<b>Min</b>			4.43	7.00	7.00	7.00	7.00	7.00	7.00	7.17	7.17	76.50
<b>SD</b>			0.09	0.31	0.28	0.19	0.74	0.23	0.24	0.33	0.34	2.19

SD= standard deviation.

The PCA of the soil and cup quality variables (Table 3) showed that the first three components explain 77.24% of the total data variability. The first component, accounting for 35.70% of the variability, comprised variables P, Ca, Zn, and B with correlation factors (CF) between -0.81 and -0.89. The second component, contributing to 27.19% of the variance, was mainly composed of variables N and C, with CF of 0.84 and 0.82, respectively. The highest CF for component 3 was obtained for the variable S (sulfur content), indicated by a value of -0.81.

**Table 3.** Factor-variable correlations and contribution to variability of the first three principal components associated with soil variables and cup quality.

Variable	Factor		
	1	2	3
Soil pH	-0.66	-0.06	-0.65
Organic matter	-0.51	0.73	0.33
Phosphorous	-0.89	0.13	0.14
CEC	-0.43	0.52	0.38
Calcium	-0.81	-0.08	-0.51
Magnesium	-0.66	-0.12	-0.70
Potassium	-0.77	0.16	0.18
Iron	-0.59	-0.71	0.20
Manganese	-0.22	-0.62	-0.16
Copper	-0.61	-0.72	0.06



Table 4 continued

Variable	Factor		
	1	2	3
Zinc	-0.89	-0.30	0.12
Boron	-0.82	-0.11	0.42
Nitrogen	-0.48	0.84	0.02
Carbon	-0.43	0.82	-0.15
Sulfur	0.22	0.35	-0.81
Density	0.08	-0.74	-0.01
Cup quality	-0.07	-0.45	0.19
Eigenvalue	6.07	4.62	2.44
PV (%)	35.7	27.19	14.36
CPV (%)	35.7	62.89	77.24

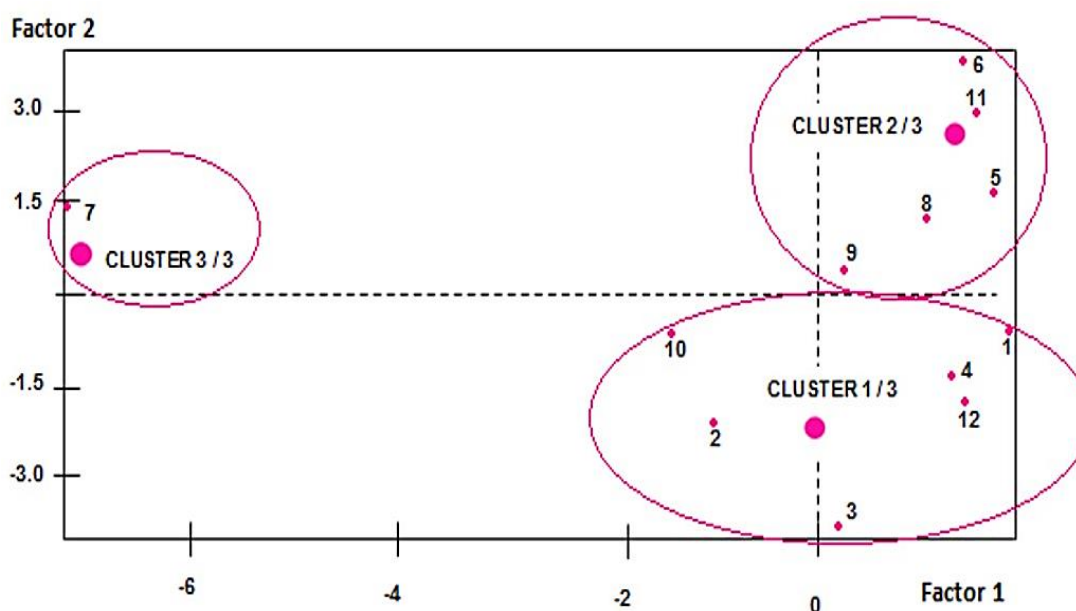
PV= percentage of the variance; CPV= cumulative percentage of the variance.

The highest CF for cup quality was observed in component 2 with a negative direction, and it was shared by iron and soil density, which contributed most to this component. This negative direction and high contribution of iron (Table 3) confirm the findings reported in Mexico by Rosas *et al.*

(2008), who demonstrated a positive influence of this element on the fragrance of the infusion, being able to identify subgroups, including nuts, chocolates, florals, caramels, fruits, and phenolics. Furthermore, they reported a negative contribution of Cu on the intensity of the acidity.

The classification analysis identified three groups. The first group comprised estates 1, 4, 2, 3, 12, and 10; the second group was composed of estates 5, 6, 8, 9, and 11; and group 3 comprised estate 7, which was far away from the others (Figure 1).

The soils of the first group of estates showed significant differences ( $p < 0.05$ ) in Mn content (30mg/kg), higher density (1.07g/cc), higher Cu content (3.72mg/kg), and higher Fe content (138.67mg/kg) than the overall means of the study population (16.97mg Mn/kg, density of 1.06mg/kg, 2.66mg Cu/kg, and 109.51mg Fe/kg). Additionally, these soils showed organic matter (3.44%), nitrogen (0.13%), and carbon (1.87cmol/kg) contents below the overall means (4.63%, 0.19%, and 2.81cmol/kg, in the same order).



**Figure 1.** Distribution of the first two principal components for the estates analyzed based on the soil factor and the three groups identified in the classification analysis.

Group 2 was characterized by higher nitrogen (0.23%) and carbon (3.61cmol/kg) contents than the overall means (0.19% and 2.81cmol/Kg, in the same order). Furthermore, this group showed lower density values (0.92g/cc) and Zn (0.65mg/Kg), Mn (2.29mg/Kg), Cu (0.91mg/Kg), and Fe (58.02mg/Kg) contents in contrast to the overall means (1.00 g/cc, 2.56mg Zn/Kg, 16.97mg Mn/Kg, 2.66mg Cu/Kg, and 109.51mg Fe/Kg).

Group 3 comprises estate 7 and showed values greater than the overall mean for most variables analyzed, except for Mn (12.2mg/Kg), S (2.4mg/Kg), density (0.94g/Kg), and cup quality score (79.67%); this last variable was very similar to the overall mean of the population analyzed (80.5%). Suárez *et al.* (2015) indicated a high association between the sensory attributes of cup quality and the chemical characteristics of the soil; furthermore, the authors determined that acid soils with high Fe and Al contents lead to intermediate and high-quality coffee cups. The same study reported that low cup quality was associated with moderately acid soils with high Cu, Zn, S contents, and low Ca and Mg contents. Alternatively, they reported a negative relationship between cup acidity and Ca content and a positive correlation between fragrance/aroma and soil K, Na, and Al contents.

The first two PC groups, which account for 91.6% of the population, arrange the estates close to the origin. This reflects the few differences in the edaphic characteristics of these soils compared with the overall mean. The remaining 8.33% of the population comprised estate 7, or group 3, which was distant from the origin and showed clear differences in its edaphic traits compared to the other estates assessed.

However, despite the differences between groups, cup quality was similar across groups (80.89, 80.2, and 79.6 for groups 1, 2 and 3) and the overall mean (80.5%). Therefore, this study could not

establish the effect of certain soil variables on the physical and organoleptic quality of the coffee beverage. Accordingly, cup quality should be considered a multifactorial variable that depends on the genotype, environment, and the interaction between these two components (Suárez *et al.*, 2015; Ramos and Criollo, 2017).

The PCA of the climatic variables allowed explaining 84.64% of the total variance in three large components. The first component accounted for 51.5% of the variability, the second component explained 17.62%, and the third contributed to 15.44% of the variance (Table 4). Precipitation, photosynthetically active radiation (PAR), and solar radiation were most variable across the estates analyzed.

**Table 4.** Factor-trait correlations and contribution to variability of the first three principal components associated with climatic variables and coffee cup quality.

Trait	Factor		
	1	2	3
Solar radiation-V1	-0.92	0.10	-0.01
PAR-V2	-0.94	0.22	0.09
Relative humidity-V3	0.40	-0.54	-0.61
Temperature-V4	-0.83	-0.31	0.22
Precipitation-V5	-0.39	0.38	-0.77
Temperature amplitude-V6	-0.87	-0.12	-0.23
Cup quality-V7	-0.37	-0.79	0.02
Eigenvalue	3.613	1.23	1.00
PV (%)	51.58	17.62	15.44
CPV (%)	51.58	69.28	84.64

PV= percentage of the variance; CPV= cumulative percentage of the variance.

The first component mainly included: solar radiation, PAR, temperature, and temperature amplitude with CF between 0.83 and 0.94. Similarly, component 2 comprised the variables

relative humidity (-0.54) and cup quality score (-0.79). Lastly, relative humidity and precipitation were determinant variables of component 3, with CF of -0.61 and -0.77, respectively.

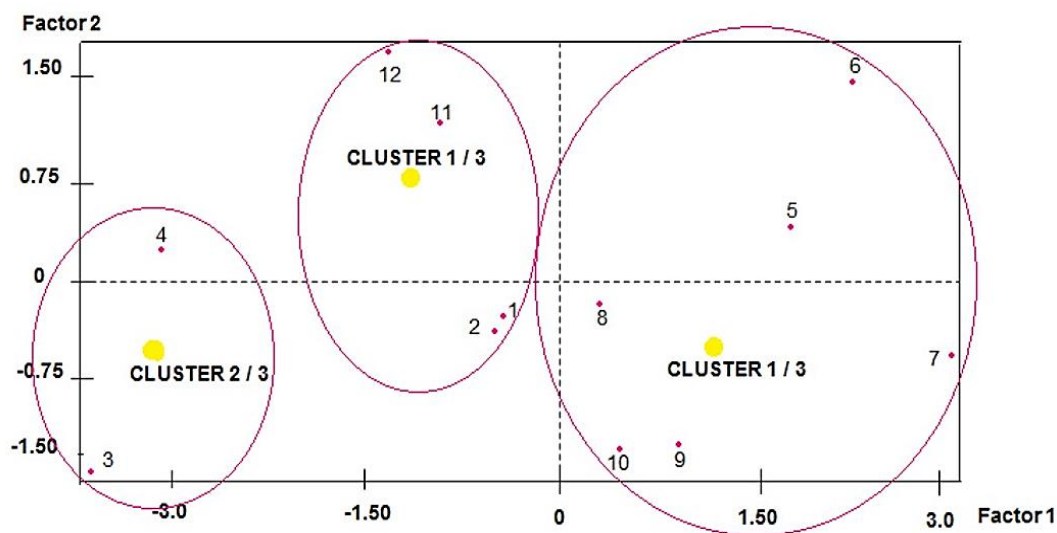
The classification analyses allowed identifying three groups. The first group encompassed estates 1, 2, 11 and 12 that represent 33.3% of the total estates. The first two coffee farm estates are located in the municipality of La Unión at elevations between 1501 and 1700m, while the last two are located in the municipality of La Florida at  $\leq 1500\text{m}$  and  $>1700\text{m.a.s.l.}$ , respectively. The second group included estates 3 and 4, which account for 16.6% of the estates and are located in La Unión between 1501 and 1700m.a.s.l. The third group comprised estates 5, 6, 9, 10, 7, and 8, representing 50% of the estates (Figure 2).

The coffee farm estates of the first group are characterized by greater precipitation (721.1mm) compared with the overall mean (572.88mm). Furthermore, other variables that showed higher values than the overall mean were thermal amplitude ( $5.26^{\circ}\text{C}$ ) and PAR (412.98nm). The cup quality score of the coffee produced in these lands was 79.92%, while the overall mean was 80.5%.

Group 2 (Figure 2) comprised farms that received higher solar radiation ( $256.44\text{W}/\text{m}^2$ ), greater PAR (509.51nm), and showed greater diurnal and nocturnal temperature amplitude ( $5.95^{\circ}\text{C}$ ) in comparison to the overall means ( $202.77\text{W}/\text{m}^2$ , 401.06 nm, and  $4.86^{\circ}\text{C}$ , in the same order). The cup quality score of these farms was 82.85.

The farms in group 3 were characterized by higher relative humidity (73.41%) than the overall mean (72.73%). Additionally, they showed lower temperature amplitude ( $4.23^{\circ}\text{C}$ ), precipitation (457.13mm), PAR (356.96nm), solar radiation ( $184.75\text{W}/\text{m}^2$ ), and environmental temperature ( $18.94^{\circ}\text{C}$ ) compared with the overall means ( $4.86^{\circ}\text{C}$ , 572.88mm, 401.06nm,  $202.77\text{W}/\text{m}^2$ , and  $19.64^{\circ}\text{C}$ , respectively). Coffee cup quality (80.2) for this group was very similar to the overall mean (80.5).

Several studies found that the highest scores for the sensory attributes of the cup are obtained from shade-grown coffee at higher elevations. This demonstrates the need to adequately manage tree cover along with elevation (Suárez *et al.*, 2015).



**Figure 2.** Distribution of the estates analyzed in the first two principal components based on the environmental factors and the three groups identified through the classification analysis.

The effects of shade trees on coffee production are difficult to characterize since studies often omit important data or do not quantify them; for instance, intensity and quality of the radiation that reaches the tree canopy, daily and seasonal fluctuations in temperature and relative humidity, nutrition, and overlooked crop management conditions (DaMatta and Ramalho, 2006; DaMatta and Rodríguez, 2007). In this study, no tendency was observed regarding quality in response to the environments in ecotypes 220A and 221A.

Coffee benefit comprises all the processes aimed to transform the coffee berry into dry parchment coffee without affecting its organoleptic, sanitary, and physical characteristics (Gómez, 2010). The analysis of variables associated with this process showed that 41.6% of the farms mix coffee from different harvest days before the fermentation process, and 50% select the coffee after washing. Furthermore, 75% use a motorized coffee pulper, 16.6% use a mucilaginator, and the remaining farms use a manual pulper. After drying, only 16.6% of coffee producers obtain humidity between 10 and 12%, while 83.3% package the coffee grain with more than 12% humidity. Low humidity contents provide the coffee grain with greater stability of its organoleptic characteristics; thus, better conserving its quality (Puerta-Quintero, 2006).

Regarding the pH of the fermenting mass, 75% of producers begin with a pH between 5.0 and 6.5. This data agrees with Córdoba-Castro and Guerrero-Fajardo (2016), who found an initial fermenting pH between 5.5 and 5.6 for Castillo variety. Puerta-Quintero (2013) reported an initial pH of 5.6 and indicates that these values depend on the maturity of the grain, the time between collection and pulping, and fruit manipulation. Moreover, 8.3% of the farms begin the process with a pH > 6.5, while 16.6% do not ferment the coffee grain. Additionally, 25% of the producers terminate the process when the pH is between 3.0

and 3.5, while 58.3% end it at a pH > 3.5. According to Jackels and Jackels (2005), a final pH of 4.6 may be optimal to terminate the fermentation process without risking over-ferment of the grain.

During fermentation, the pH decreases due to the increased acidity of the mass. Generally, pH values between 3.7 and 4.1 for the fermented mucilage are adequate and safe to interrupt the fermentation process (Puerta-Quintero, 2012). Only 8.3% of the producers achieved 'excellent' cup quality scores, 41.6% produced coffee with a 'very good' qualification, and 50% did not achieve a specialty coffee qualification due to scores below 80 points.

## CONCLUSIONS

Coffee cup quality scores did not show an association with elevation. The highest score was achieved for coffee grown at  $\leq 1500$  m.a.s.l. Low levels of association were found between the soil variables and cup quality; furthermore, Fe and Cu contents showed the highest association levels among the variables assessed.

The farms in La Unión showed the highest radiation, PAR, and thermal amplitude and conformed a group with the highest cup qualities. The characteristics associated with higher cup quality were found La Unión and Consacá in production units located at elevations  $\leq 1500$  m.a.s.l.

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**Conflict of interest:** The authors declare that there is no conflict of interest.

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