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Portada: Unimedios
Contraportada: Klara Torres Restrepo
Dirección postal: Apartado Aéreo 568, Medellín, Colombia
Dirección electrónica: rfnagron_med@unal.edu.co
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EVALUADORES

El Comité Editorial dentro de sus políticas, envía los artículos a especialistas, con el fin de que sean revisados. Sus observaciones en adición a las que hacen los editores, contribuyen a la obtención de una publicación de reconocida calidad en el ámbito de las Ciencias Agrarias. Sus nombres son mencionados como una expresión de agradecimiento.

Alexander Calero Hurtado. Universidad Estadual Paulista "Júlio de Mesquita Filho". São Paulo, Brasil. alexcalero34@gmail.com

Alfonso Parra Coronado. Universidad Nacional de Colombia. Bogotá, Colombia. aparrac@unal.edu.co

Benigno Estrada Drouaillet. Universidad Autónoma de Tamaulipas. Victoria, México. benestrada@uat.edu.mx

Celia María Fernández Chávez. Universidad Mayor de San Andrés. La Paz, Bolivia. cmfch3311@hotmail.com

Claudio Sánchez Jáuregui. Universidad del Azuay. Cuenca, Ecuador. csanchez@uazuay.edu.ec

Fermín J. Chamorro. Universidade Federal do Ceará. Benfica, Fortaleza, Brasil. ferminchamorro@gmail.com

Gelber Rosas Patiño. Universidad de la Amazonia. Florencia, Colombia. g.rosas@udla.edu.co

Giovanni Orlando Cancino Escalante. Universidad de Pamplona. Pamplona, Colombia. gcancino@unipamplona.edu.co

Hipolito Cortez Madrigal. Instituto Politécnico Nacional. Ciudad de México, México. hcortezm@ipn.mx

Ingeborg Zenner De Polania. Universidad de Ciencias Aplicadas y Ambientales U.D.C.A. Bogotá, Colombia. izenner@udca.edu.co

John Cristhian Fernández L. Universidad de La Salle. Yopal, Colombia. jofernandez@unisalle.edu.co

Karla Muñoz Páez. Universidad Nacional Autónoma de México. México D.F., México. KMunozP@iingen.unam.mx

Leneidys Pérez Pelea. Universidad de la Habana. La Habana, Cuba. lene@fbio.uh.cu

Manuel Sandoval Villa. Colegio de Postgraduados. Montecillo, México. msandoval@colpos.mx

María Eugenia López Arellano. Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Tamaulipas, México. mlopez_arellano@hotmail.com

María J. Pascualone. Universidad Tecnológica Nacional. Buenos Aires, Argentina. mariapascualone@gmail.com

María Margarita Ramírez Gómez. Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA). Bogotá, Colombia. mmramirez@agrosavia.co

María Mercedes Pérez Trujillo. Universidad Militar Nueva Granada. Cajicá-Zipaquirá, Colombia. maria.perez@unimilitar.edu.co

Miriam Elisabet Arena. Universidad de Morón. Buenos Aires, Argentina. miarena@unimoron.edu.ar

Nelson Rodríguez. Centro Nacional de Investigaciones de Café (Cenicafé). Manizales, Colombia. nelson.rodriguez@cafedecolombia.com

Oscar Rordríguez. Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA). Espinal-Chicoral, Colombia. orodriguezr@agrosavia.co

Rafaela Soto Ortiz. Universidad Cienfuegos. Cienfuegos, Cuba. rsoto@ucf.edu.cu

Ramona Cecilia Párraga Álava. Universidad Técnica de Manabí. Manabí, Ecuador. ingceciliaparraga@hotmail.com

Shahzadi Mahpara. Ghazi University. Dera Ghazi Khan, Pakistan. mahpara.gudgk@gmail.com

Silvia Radice. Universidad de Morón. Buenos Aires, Argentina. siradice@yahoo.com

Victor Adolfo Gómez López. Universidad Nacional de Asunción. San Lorenzo, Paraguay. vgomez98@hotmail.com

William Viera. Instituto Nacional de Investigaciones Agropecuarias INIAP. Ecuador. william.viera@iniap.gob.ec

EDITORIAL

Bodas de Roble

Por esta época nos sorprendemos de tantos logros académicos para festejar. Pareciera como si después de una siembra responsable y lejana, nos hubiera llegado finalmente el tiempo para recoger una buena cosecha que nos anima a celebrar y compartir. Particularmente nos alegramos de la abundancia de celebraciones que realiza la Universidad Nacional de Colombia y que trascienden al país entero. Hace apenas dos años tuvimos la oportunidad de festejar el sesquicentenario de la Universidad con múltiples actividades académicas, culturales y lúdicas; pero ante todo con la publicación de la obra conmemorativa que recogió en doce volúmenes la historia de las diferentes áreas del conocimiento que ha cultivado la institución a lo largo de 150 años. Ese sello recordatorio del sesquicentenario a través de una publicación será el más importante que se conservará para que las próximas generaciones no pierdan de vista el trabajo que hemos realizado en siglo y medio de labores.

Las publicaciones conmemorativas son ante todo la oportunidad para describir la cosecha, para contarla, para registrarla y para dejar plasmado ese recuerdo que se conserva de una celebración, como una fotografía que se expone en un lugar visible para que todos los visitantes futuros la vean y sepan de qué se trató ese acto que representa la imagen.

Por eso, me siento muy honrado de hacer parte de esa fotografía, de haber sido invitado a escribir estas líneas para la publicación de la Revista Facultad Nacional de Agronomía Medellín, que cumple 80 años de fundada y que se ha convertido en el instrumento principal de difusión de los resultados de investigación del área. Se trata de una feliz coincidencia que se denomine “Bodas de Roble” al cumpleaños 80 de un acontecimiento, porque es precisamente el roble un símbolo de fortaleza, pero además porque es objeto de estudio de las ciencias agropecuarias.

También es una afortunada coincidencia que se celebre en este mes de septiembre el natalicio 250 de Alexander von Humboldt, quien realizó la primera expedición científica a través de nuestro territorio para descubrir, describir y maravillar al mundo con la riqueza y diversidad de nuestra flora y fauna, presente en la geografía americana.

A través de las revistas, de los libros y en general, de los documentos de divulgación científica, se comunican los expertos, los estudiantes, los investigadores, contribuyendo a la formación de las futuras generaciones en un área del conocimiento, brindando soporte al alcance de técnicos y expertos que abordan autónomamente nuevas investigaciones. Ese trabajo iniciado por Humboldt nos enseñó que todo aquello que se descubre, si no se comparte y se cuenta, si no se publica, permanecerá oculto como estaba antes de descubrirse.

Justamente ese propósito general lo ha venido cumpliendo a cabalidad la Revista Facultad Nacional de Agronomía Medellín durante estos 80 años que hoy celebramos, y encuentro otros importantes aportes específicos que debo resaltar de esta octogenaria publicación, como son:

1. Un papel destacado en el sector agropecuario, no solo para el desarrollo del país, sino para la construcción y la consolidación de la paz, especialmente con la divulgación de calificados argumentos, continuamente publicados, sobre la protección de la seguridad y soberanía agropecuaria.

2. La Revista Facultad Nacional de Agronomía Medellín ha sido un motor de la divulgación del conocimiento generado en el sector agrario. Especialmente el logrado a través de alianzas estratégicas entre la Facultad de Ciencias Agrarias de Medellín con otros centros de investigación a nivel nacional e internacional, que ha hecho posible un enriquecimiento científico histórico.

3. La Revista Facultad Nacional de Agronomía Medellín ha sido testigo de los cambios conceptuales y tecnológicos en el sector. Del paso de la Revolución Verde a los conceptos de sostenibilidad económica, social y ambiental.

4. La Revista Facultad Nacional de Agronomía Medellín ha sido herramienta de profesores, estudiantes e investigadores con la que han dejado la huella de sus resultados más importantes.

La historia de las facultades y de los programas curriculares en los que se han formado los profesionales del sector agrario, está contada a lo largo de los 80 años de vida de la Revista; así que es inevitable hacer un reconocimiento especial a todos los editores y comités editoriales que han tenido a cargo la publicación.

Finalmente, deseo felicitar muy sinceramente a todos los profesores, estudiantes, egresados, empleados administrativos y trabajadores de la Facultad de Ciencias Agrarias de la Sede Medellín de la Universidad Nacional de Colombia por su trabajo y contribuciones para que la divulgación de sus logros sea compartida y conocida a través de la Revista. Ustedes, todos, son una familia que hoy aparece en esta fotografía de “Bodas de Roble” que verán expuesta las próximas generaciones que sigan sus acertados pasos.

IGNACIO MANTILLA PRADA,
Dr. Rer. Nat.
EXRECTOR
Universidad Nacional De Colombia

Research article

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Revista Facultad Nacional de Agronomía

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Phenological scale for the mortiño or agraz (*Vaccinium meridionale* Swartz) in the high Colombian Andean area

Escala fenológica para el mortiño o agraz (*Vaccinium meridionale* Swartz) en la zona altoandina colombiana

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Clara I. Medina Cano[1,*], Enrique Martínez Bustamante[2] and Carlos A. López Orozco[3]

Keywords:
Andean blueberry
Branching
Flowering
Fructification
Promising species
Sprouting

ABSTRACT

Mortiño, *Vaccinium meridionale* Swartz, represents a viable alternative for fruit growing because of the presence of appropriate ecological niches and spontaneous populations in the Colombian Andean zone. The knowledge of plants' phenology is useful to identify the response to critical periods (stages and phases) to different biotic or abiotic factors and to define agronomic practices adjusted to their requirements. Only the different phenological stages have been recognized in the mortiño; therefore, it is necessary to detail the phases within each one of them. The identification of the phenological stages and phases of the mortiño's canopy evolution was based on the scale of the blueberries *Vaccinium corymbosum*. It was adjusted between 2008 and 2011 to describe in detail the phenological stages of mortiño through monthly photographic records in five natural populations of three Colombian departments; where 48 individuals were randomly identified in each one. The purpose of the elaboration of this scale was to describe and visually identify the phenological phases of natural populations in similar climatic conditions. Four stages were found, the first one comprised the vegetative button formation (VB) with 5 phases, which ends with the formation of shoots. The second stage was the development of the inflorescence (ID) distributed in 5 phases as well, from floral bud to floral anthesis. In the third stage, the floral development (FD) took place, also with 5 phases, from flowering to the beginning of berry formation. The last stage, the berries were developed (BD) through 4 phases, from fruit formation until harvest maturity.

Palabras clave:
Arándano andino
Ramificación
Floración
Fructificación
Especie promisoria
Brotación

RESUMEN

El mortiño, *Vaccinium meridionale* Swartz es una alternativa frutícola viable por la presencia de nichos ecológicos apropiados y poblaciones espontáneas en la zona andina colombiana. El conocimiento de la fenología de las plantas es útil para identificar la respuesta a épocas críticas (etapas y fases) a distintos factores bióticos o abióticos y definir prácticas agronómicas ajustadas a sus requerimientos. En el mortiño solo se reconocen las distintas etapas fenológicas; por tanto, se requiere detallar las fases de cada una de aquellas. La identificación de las etapas y fases fenológicas de evolución del dosel del mortiño se fundamentó en la escala de los arándanos, *Vaccinium corymbosum*; la que se ajustó entre 2008 y 2011 para describir, detalladamente, los estados fenológicos del mortiño, a través de un registro fotográfico, con observaciones mensuales, en cinco poblaciones naturales de cuatro departamentos colombianos; donde se identificaron, al azar, 48 individuos en cada una. La elaboración de esta escala tuvo como objetivo describir e identificar, visualmente, las fases fenológicas de poblaciones naturales, en condiciones climáticas similares. Se encontraron 4 etapas: la primera comprendió formación del botón vegetativo (VB) con 5 fases, la que finaliza con la formación de brotes; la segunda fue el desarrollo de la inflorescencia (ID) distribuida en 5 fases, de botón floral hasta antesis floral; en la tercera sucedió el desarrollo floral (FD), con 5 fases, desde florescencia hasta inicio de formación de bayas; en la última se desarrollaron las bayas (BD), a través de 4 fases, de formación de frutos hasta madurez de cosecha.

[1] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA). C. I. La Selva. Km 7, vía Las Palmas, vereda Llanogrande, Rionegro, Colombia.

[2] Facultad de Ciencias Agrarias. Universidad Nacional de Colombia. AA 1779, Medellín, Colombia.

[3] Empresa Comestibles Ricos S.A. Calle 17 D N° 116-15. Fontibón, Colombia.

* Corresponding author: <cmedina@corpoica.org.co>

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The word *mortiño* is described by Castilian language as a term applied to plants of the genus *Vaccinium*, name imposed by the Spanish in America; the first reference of the expression *mortiño* dates from 1548, which was used in the region of Guaca, current province of Carchi, Ecuador, a village inhabited by the "Pastos" indigenous(Patiño, 2002).

The agraz, mortiño or vichachá, *Vaccinium meridionale* Swartz, is a clonal plant belonging to the tribe Vaccinieae Rehb. and Ericaceae Juss. family; it comprises about 35 genera and more than 1000 species. Also, the genus *Vaccinium* has registered between 400 and 450 species, distributed in the northern hemisphere and in the mountains of the tropical regions of the Andes, South Africa and Madagascar (Luteyn, 2002; Smith *et al*., 2004). On the other hand, the mountains of northwestern South America and especially on the Pacific slope are rich in this group of plants (Salinas and Betancur, 2007). In the High Andean Area, there is information available on five species: *V. corymbodendrom* Klotzsch*, V. floribundum* Kunth, *V. meridionale* Swartz, *V. euryanthum* A.C. Smith, and *V. singularis* Salinas & Betancur; the last mentioned is only known in the Pacific slope of the department of Nariño (Salinas and Betancur, 2007). According to Ligarreto (2009), the species of the genus *Vaccinium* are distributed in the Departments of Antioquia, Boyacá, Cauca, Chocó, Cundinamarca, Magdalena, Meta, Nariño, Norte de Santander, Putumayo, Quindío, Santander, and Tolima; with the highest number of reports in Antioquia, Boyacá and Cundinamarca.

The mortiño represents a viable alternative for fruit growing with possibilities of development in the country in similar climatic conditions. This is based on a series of aspects such as international and national demand for berries, the presence of ecological niches suitable for their production, spontaneous populations in the Andean area with various forms of agro-industrial use. It is also a plant of great agro-industrial interest for the antioxidant activity of its berries, which includes this species in the category of nutraceutical products. Additionally, the taxon provides ecosystem services in wild environments (Medina *et al*., 2005; Zapata *et al*., 2015).

Secondary metabolites, including flavonoids, play an important role in the physiology of these plants because they are synthesized in response to stress to defend against biotic and abiotic agents (Zakaryan *et al*., 2017). Dróżdż *et al.* (2017) found different phenolic compounds in the diversity of species of the genus *Vaccinium*, as well as in the different varieties, phenological stages and in the post-harvest of the berries. *Vaccinium* species are recognized as a functional food because its high content and structural diversity of secondary metabolites. They have an important content of phenolic compounds, flavonoids, and anthocyanins (Gaviria *et al*., 2012; Lopera *et al*., 2013; Maldonado *et.al*., 2018; Tian *et al*., 2017). Likewise, the previous authors, with Krikorian *et al.* (2010) and Liu *et al.* (2011), stated that these substances have beneficial effects on health; for their action at ocular level, against degenerative diseases; certain types of cancer; memory impairment; and protection of the cardiovascular system. Additionally, they act as reducing agents for donating hydrogen that inhibits *singlet* oxygen, metal ion chelators; preventing the formation of free radicals (Galleano *et al*., 2010; Ghasemzadeh and Ghasemzadeh, 2011).

Phenology is defined as the study of the periodic and repetitive phases or activities of the life cycle of plants, both in their development and in their growth, due to the diurnal and annual temporal variations (Cook *et al*., 2012). The knowledge of the phenology of plants is useful to identify critical periods where plants develop appropriate strategies to face the effect of biotic or abiotic factors, stressors or not (Valbuena *et al.,* 2009). Phenology is useful to know the differences among plant's genotypes and develop agronomic practices adjusted to plant's requirements (Martínez-Adriano *et al*., 2016). Additionally, it helps to recognize the plasticity between plants and identify the different environments to which they have adapted (Harder and Johnson, 2005).

It must be considered that climate is the main factor controlling and regulating phenological events (Menzel *et al*., 2006) since plants are affected by environmental changes, within and between years, such as temperature, relative and soil humidity, nutrient availability, light and CO_2 increase (Nord and Lynch, 2009; Nijlanda *et al*., 2014; Martínez-Adriano *et al*., 2016). The phenology responds to the climate for the following reasons: for gene flow to occur between individuals for the same population and to avoid damage due to unfavorable changes over time (Jianwu *et al*., 2016). Besides, to face this climatic and genetic

variability, plants adopt different phenological strategies (Körner *et al.*, 2016). Rainfall is generally considered as an environmental signal for the variation of phenological events in tropical regions (Morellato *et al.*, 2013, 2006). It is also related to the interpretation of the variability of these phases and their events, which originates the adaptation of plants to diverse environments (Martínez-Adriano *et al.*, 2013, 2016).

For certain species of the *Vaccinium* group, Antunes *et al.* (2008) suggested that the choice of cultivars is based on the identification of the species' phenological phases. This description can allow obtaining a staggering production and an increase of the same in strategic periods of supply and demand of the fruit. Also, Kron *et al.* (2002), in a study of the phylogenetic relationships carried out with the tribe of blueberry species (Vaccineae, Ericaceae) concluded that in the genus *Vaccinium* there is not a very clear grouping among taxa from different continents, or between those of tropical and temperate climate origin. Also, these authors tend to recognize several small groups (clades), rather than a large set of *Vaccinium* species, which would be essentially redundant to the Vaccinieae tribe.

Studies carried out by Ligarreto *et al.* (2011) revealed a wide morphological variation in the metapopulation of *V. meridionale* by quantitative and qualitative factors of the species, highly influenced by the environment. There is also an important wild genetic resource for *V. meridionale* that could cement the introduction of this species in the agricultural world. In a commercial cultivation of blueberries in Guasca (Colombia), with the Biloxi and Sharpblue cultivars, follow-ups were advanced in batches of 20 and 36 months, where it was identified that this last clone presented a superior yield because of a greater number of fruits and an increase in total soluble solids (Cortés-Rojas *et al.*, 2014).

For this species, information on phenological studies has been obtained, such as the one described by Gómez (2004), where certain development events are detailed; among them, the sprouting of leaves, floral button, open flower, flowering, fruiting, and green and ripe fruits. Other works describe six stages of maturity of the berries (Buitrago *et al.*, 2015). The peasant communities of Guarne, Antioquia, have expressed that there occurs biannual fruiting, which is a product of the bimodal rainfall of this area. For the area of the Antilles, Berazaín (2006) has identified that flowering occurs from the beginning of winter in the Ericaceae family. This phenological phase extends throughout the spring, until the beginning of summer; likewise, fruiting lasts from the beginning of autumn, continues throughout the winter, with a maximum in the spring-summer period. These are plants with abundant flowering and fruiting throughout the year.

Consequently, because the mortiño belongs to a group of species of the genus *Vaccinium*, it presents phenological stages and phases similar to blueberries, but not evaluated in conditions of the high Andean zone. Therefore, the objective of developing this scale was designed to describe and visually identify in the stages already known, the phenological phases that natural populations present in climatic conditions similar to the high Andean area studied.

MATERIALS AND METHODS

Locality

The monitoring in the different phenological stages of the mortiño was carried out between 2008 - 2011, in five natural populations selected in the Departments of Antioquia (Santa Rosa de Osos and Medellín, township of Santa Elena), Cundinamarca (Guachetá) and Santander (California), whose geographic coordinates and climatic characteristics are detailed in Table 1. It should be noted that precipitation is bimodal in all locations, and they are located between 2400 and 2900 masl, except California (Santander), where a natural population was identified at almost 3500 m of altitude. These locations are characterized by fluctuating minimum temperatures between 7.0 and 13.2 °C, maximum temperature from 20 to 25 °C, and relative humidity between 43 and 91%.

Identification of the phases and phenological phases of the canopy

The categorization of the growth states of *Vaccinium meridionale* was based on the phenological scale of blueberries, *Vaccinium corymbosum* (Michigan State University, 2003), which describes and includes graphs on the development of foliar, floral, flower and fruit buttons. Additionally, a scale was constructed with the description of the phenological stages of this Andean species, through a photographic record and monthly observations for 26 months in the five natural populations previously described,

and on 48 individuals, between 100 to 150 buds were marked randomly. The phases of development of the berries were identified based on the photographic scale achieved by Hernández *et al*. (2012).

Table 1. Geographic location and climatic conditions of the natural populations of mortiño *Vaccinium meridionale* evaluated in Antioquia, Cundinamarca, and Santander.

Locality		Geographic location	Altitude (masl)	Temperature (°C)		Relative Humidity (%)	
Department	Town			Mín.	Máx.	Mín.	Máx.
Antioquia	Medellín (Santa Elena)	6°15'57"N, 75°29'47" W	2.475	11.5	24.7	53.0	86.5
	Santa Rosa de Osos	6°4'30"N, 75°25'22"W	2.555	13.2	22.9	63.5	85.8
Cundinamarca	Guachetá	5°27'47"N, 73°39'52"W	2.872	8.75	20.5	48.8	89.9
Santander	California	7°22'39"N, 72°54'49"W	2.737	7.70	22.8	42.6	90.7
		7°23'16"N, 72°53'22"W	3.439				

RESULTS AND DISCUSSION

According to Halle *et al*. (1978), the mortiño presents a simplified branch, by abortion of the terminal buds at the end of each growth period. This model was called George Mangenot, which is initially orthotropic. Then, it leads to a plagiotropic development of the branches by replacing the lateral buds, which simultaneously show different phenological stages (Medina, 2010). Consequently, in this set of taxa, during the growth periods, reproductive and vegetative stems are observed simultaneously (Kawamura and Takeda, 2002).

The growth periods were continuous; so that in the dry season 75% of the structures were in the vegetative stage (VB), and the rainy season they increased up to 80% (Figure 1). On the other hand, in the dry season,

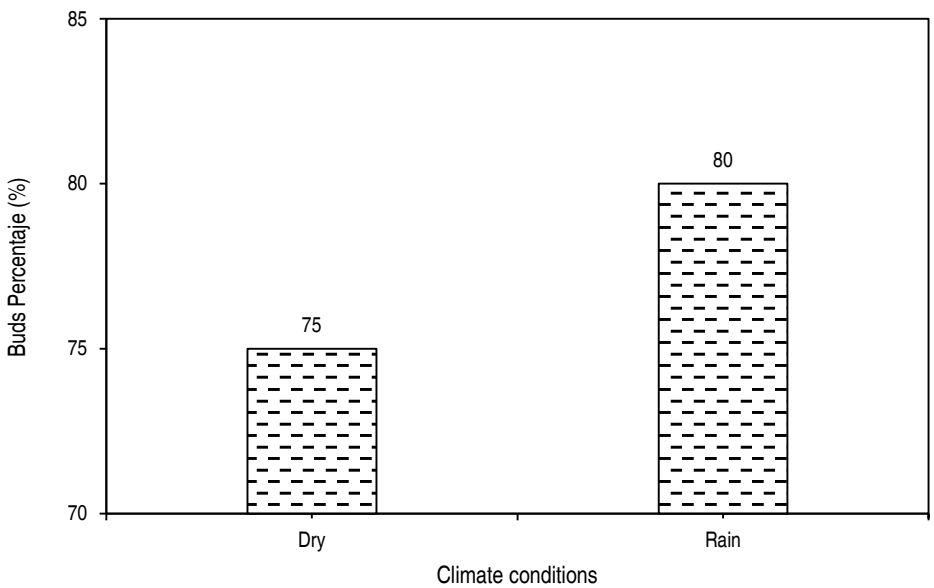

Figure 1. Percentage of mortiño's structures in vegetative stage (VB) according to both dry and rainy seasons.

25% were reproductive structures, of which 15% were in the development of the inflorescence (ID), 7% were floral development (FD), and 8% were the development of the berries (BD). Besides, in the rainy season, the proportion of these was 20%, of which 6% was DI, 7% FD and 7% BD (Figure 2).

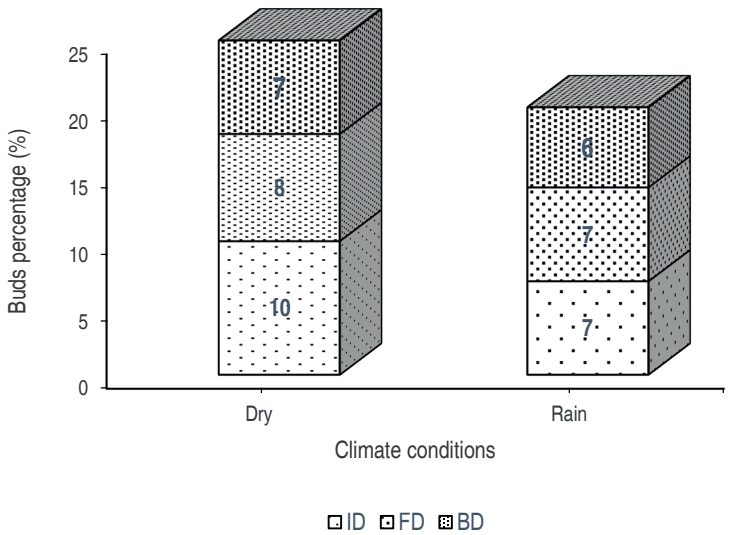

Figure 2. Percentage of moritño's reproductive structures according to both dry and rainy seasons. BD: development of the berries; FD: floral development; ID: inflorescence.

The canopy of the *Vaccinium* has the property of being evergreen (Kawamura and Takeda, 2002), and to last all year long (Gómez, 2004); as foliage, its main characteristic is the predominance of mature leaves in all phases of tree development, which last around one to four years in tropical forests (Coley, 1988).

On the other hand, the role of leaves, besides carrying out the photosynthetic process, is the storage of nutrients and photo-assimilates and as a source of nutrients in the process of remobilization during senescence (Severino and Auld, 2013). It is also important to identify the period of foliage formation since this process has consequences in the interactions between plants and herbivores (Novotny *et al.*, 2006); it is the case between the natural populations and the phytophagous insects where the present study was carried out.

For the mortiño, a detailed description of the evolution of the canopy is not known. In research carried out in the municipality of Guarne, Antioquia, it was found that the growth and development of the foliage are permanent. However, it increases up to a maximum in the rainy season and high relative humidity, which coincides with the decrease in flowering and the formation of new berries (Gómez, 2004); unlike the mortiño, cranberry in Chile has two marked periods in leaf development, one for induction of leaf shoots and another for vegetative development (Bañados *et al.*, 2007).

Stages and phases of canopy development

Under the climatic conditions of this study (Table 1), the phenological development of mortiño's canopy extended from the sprouting of the meristems, with the later formation of the vegetative structures until the maturity of the fruits. The first stage included the development of vegetative bud (VB) or foliar shoots and the formation of leaves and branches, which was subdivided into 5 phases. The second was related to the evolution of the inflorescence (ID), also distributed in 5 phases. In the third, floral development (FD) occurred, which extended into 5 phases. Finally, the berries were formed through 4 phases (BD).

Vegetative bud (VB) or foliar sprouts. The development phases of the vegetative buds originated from the point

of non-visible growth (VB0), which is characterized by being dormant axillary buds (meristems) and ended with the formation of young buds (YB), not lignified, located in the terminal part of the branches (Figure 3). They do not possess dominant apical bud, with anthocyanin pigments in the leaves, very sensitive to the attack of foliage-eating insects and the damage caused by frost; from these young buds, reproductive shoots will be formed (Medina, 2010).

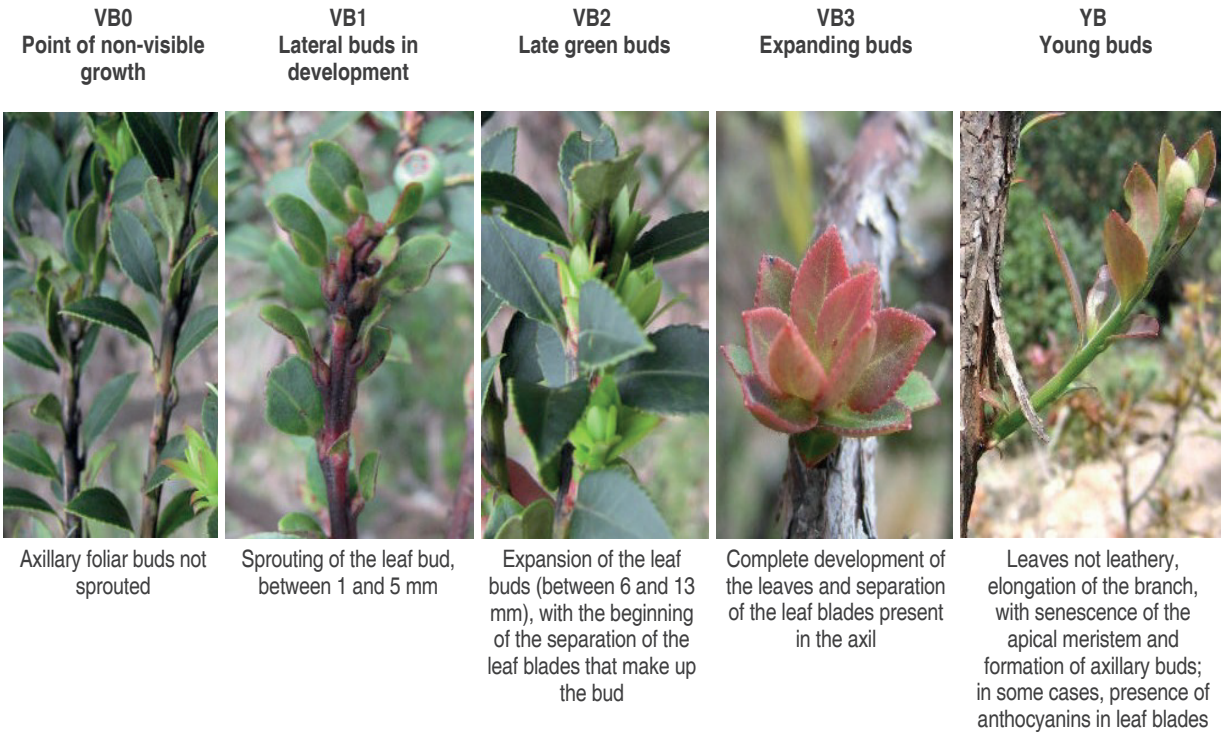

Figure 3. Stage of development of vegetative bud (VB) or foliar sprouts, where the leaves and branches are formed subdivided into 5 phases.

The growth phases of the VB were characterized by the budding of the lateral buds (VB1), which can reach up to 5 mm in length. The late green buds (VB2) expanded with the corresponding separation of the leaf blades that formed the buds. It continues with the bud's expansion (VB3), leaves development, and the separation of the leaf blades present in the axils. Finally, they became young buds (YB), characterized by having non-leathery leaves, prominent elongation of the branch and leaves, and formation of other axillary buds that can be differentiated into vegetative or reproductive. YB leads to the senescence of the apical meristem; in some cases, anthocyanin pigments were present in the leaf blades from the VB3. Feng *et al*. (2017) and Mazza and Miniati (1993) stated that these secondary metabolites are important in plants because they serve as attractants, protect against ultraviolet rays. They are correlated with an increase in the concentration of them regarding altitude, are associated with resistance of pathogens, are enhancers of photosynthesis in plants of tropical forests, and they regulate gas exchange in woody plants.

In the southern hemisphere, in *Vaccinium corymbosum* L., Rivadeneira and Carlazara (2011) described four stages of growth of the vegetative buds: One with short internodes, later the lengthening of these and leaf expansion occurs. Finally, the branches are fully formed. The vegetative growth is usually by periods, and it stops to start the development of inflorescences and subsequently, the flowers (Bañados *et al*., 2007).

In other evergreen *Vaccinium* spp., as in the case of *V. bracteatum*, the branches do not necessarily grow every year (Kawamura and Takeda, 2002), an aspect that also occurs in *V. meridionale*. It may be affected by climatic events such as the global warming that has affected

both the distribution of species and leaf senescence and reproduction (Menzel *et al*., 2006). The most critical factor that affects plant cycles for high-latitude areas is temperature. However, in the tropics, it is the periodicity between dry and rainy seasons (Mendoza *et al*., 2017).

Development of the inflorescence (ID). As seen in Figure 4, in this stage, the inflorescence develops. It extends from the budding of the floral bud (ID1) to a fully developed elongated structure (ID5), to start flowering later.

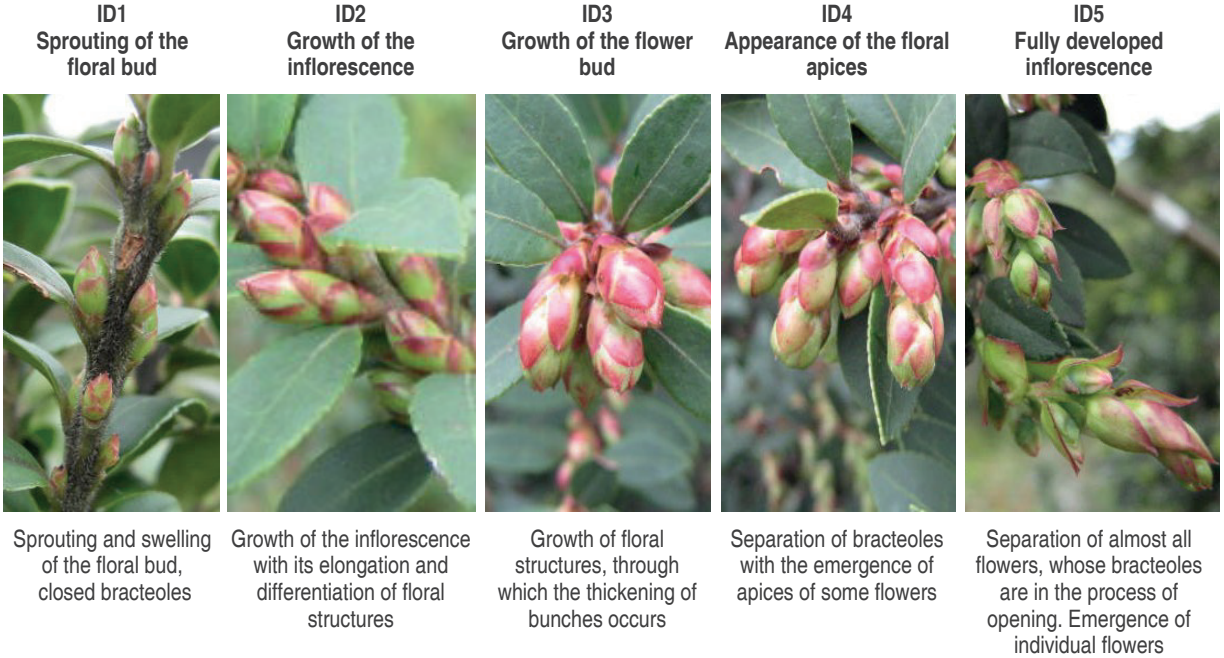

Figure 4. Stage of development of the inflorescence (ID) where the emergence of individual flowers occurs subdivided into 5 phases.

During inflorescence's growth and development, the budding of the floral bud (ID1) occurred because of its hydration and swelling. Subsequently, the elongated and differentiated inflorescence was observed in the different floral structures (ID2); after which, the development of the buds (ID3) and all floral structures was triggered, causing the thickening of the bunches. Once this happened, the floral apices emerged (ID4), with the separation of the bracteoles and emergence of some flowers. In the fully developed inflorescence (ID5) appeared the separated flowers, whose bracteoles were in the process of opening with the corresponding emergence of individual flowers.

In other *Vaccinium*, it has been expressed that the periods of flowering and ripening of fruits may vary according to the year and the location (Antunes *et al*., 2008). In the zone of San Miguel de Sema (Boyacá), there are two periods of flowering, the first from February to April and the second from July to September; likewise, the individuals that bloom in each period are different (Chamorro, 2014). In *V. corymbosum* a swollen bud that will give rise to the flowers was described as the beginning of the inflorescence formation (Meyer and Prinsloo, 2003). These structures are bunches of simple lateral buds that are found in the ending part of the branch; an inflorescence is formed by a knot, but in thick buds, it can be up to two (Gil, 2006).

Floral development (FD). In this stage, the evolution of flower development was described. It elapses from florescence (FD1) to corollas fall, and the beginning of the berries formation (FD5).

During the floral development (FD) (Figure 5), the closed corollas were observed white with slight reddish tints and grouped in bunches (FD1). Later, the flowers separated from the main axis, and reddish tones appeared on the

upper half of the floral apices (FD2). Then, the opening of some flowers of the bunches (FD3) began; followed by a number of open flowers in most of the inflorescences, whose apices are curved upwards (FD4). In the last phase, the fall of the senescent corollas was observed, whose rudimentary pistils remained adhered to the pedicel, and the green fruit exhibition occurred (FD5). Although, Chamorro (2014) described ten floral states that range from its opening to floral senescence, under the conditions of this research, only 5 phases were seen in the FD.

Figure 5. Stage of floral development (FD) subdivided into 5 phases, which end with the formation of the berries.

The flowers' colors are signals given by plants to interact with pollinators and seed dispersers. In addition, another of these colors functions is the protection against abiotic stressors that could interfere with their signaling function to pollinate and disperse animals (Stournaras and Schaefer, 2017).

For the area of eastern Antioquia, Corantioquia (2003) reported that the mortiño flowers twice a year, from February to May and from August to November, which coincides with what was found in this research. According to Gómez (2004), the flowering coincides with the dry seasons and is inversely related to the foliage sprouting and expansion in this region. In San Miguel de Sema (Boyacá) and Guachetá (Cundinamarca), the presence of mortino's blooms is identified between January to March and June to August (Chamorro and Nates-Parra, 2015). Studies conducted in Japan suggested that individuals with early blooms are disadvantaged by the availability of pollinators (Suzuki, 2002).

Regarding the flowering registered in the present study, it was observed that it occurred between February to May and August to November in Santa Elena (Antioquia), and from November to January and April to June in Santa Rosa de Osos (Antioquia). Likewise, from January to April and from June to August in Guachetá (Cundinamarca), and from March to May in California (Santander).

Development of berries (BD). The phases of this stage (Figure 6) were exhibited from the beginning of the fructification (BD1) to the harvest maturity of the berries (BD4). It began with the elongation of the green fruits (BD1) of variable size. Later, the berries began to develop anthocyanins (BD2), which were identified by their reddish coloration from the apical to the basal part of the fruit. They reached their physiological maturity when 25% of the bunch berries had developed reddish, anthocyanin colorations; covering 75 to 100% of the epicarp (BD3).

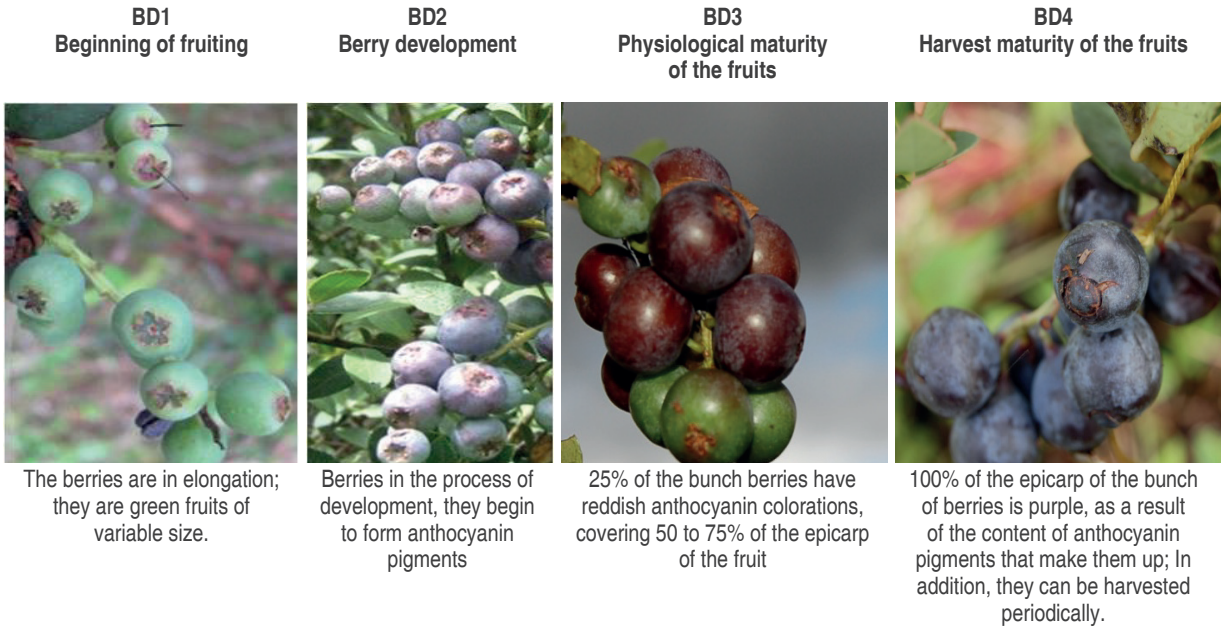

Figure 6. Stage of development of the berries (BD) subdivided into 4 phases, from the beginning of the fructification to the harvest. Adjusted by Hernández *et al.* (2012).

This phase is considered by Hernández *et al.* (2012) as the state III of fruit development.

The last phenological phase is harvest maturity of the fruits (BD4), which is characterized by the purple color throughout the epicarp and indicates the appropriate time for collection and consumption. The harvest is carried out periodically in the months of production because of formation of the reproductive structures of this perennial plant. According to these ripeness states of the berry, Hernández *et al.* (2012) identified that the seeds increase their weight as they develop, and the BD4 phase is the most appropriate time for their extraction.

According to Maldonado *et al.* (2018) and Gaviria *et al.* (2012), the berries in BD4 have high anthocyanin content and antioxidant activity. They also have a content of total phenols and natural colors comparable or superior to other *Vaccinium*; therefore, it is a promising fruit for its production and sale as a nutraceutical source to develop functional foods, or for the fresh fruit market.

The fruit is a rounded-shape and fleshy berry of 8-14 mm, with peduncle of 1 cm long. The fruits are green in the immature state (BD1) and purple or black when ripe (BD4). In certain occasions, they are covered by a waxy layer, they conserve rudiments of the calyx in the apex, and they possess numerous small seeds (Toro, 2012). The size of the berries is also related to the increase of seeds per fruit (Retamales and Hancock, 2012). Fruiting occurs in eastern Antioquia in two main periods, from April to June and September to December (Corantioquia, 2003); however, Gómez (2004) found that in natural populations, fruiting occurred throughout the year, with percentages ranging from 17% to 39%.

Regarding the reproductive phase of this plant (FD and BD), Chamorro and Nates-Parra (2015), in Guachetá (Cundinamarca) and San Miguel de Sema (Boyacá), found that a bud develops completely when its flowers bloom, 18 days after beginning its formation and six days later the senescence of this organ occurs. However, Chamorro (2014) stated that the flowers' duration was only six to ten days, which it is considered a long floral longevity characteristic of the Ericaceae family (Primack, 1985), and these are mechanisms to increase the attraction of pollinators. Rathcke (2003), Torres-Díaz *et al.* (2011), Chamorro (2014) and Chamorro and Nates-Parra (2015) found that the species produces a high quantity of flowers and low fruit production, which has been evidenced by selective abortion of self-pollinated fruits.

The growth stages in *V. corymbosum* were identified in leaves, inflorescences, flowers, and fruits. Thus, the development of the vegetative or foliar bud presented four phases, the floral button four, flowering five, and the development of the fruits five phases as well (Michigan State University, 2003). This scale served as the basis for the categorization of the growth stages of *Vaccinium meridionale*.

CONCLUSIONS

The growth periods were continuous, such that in the dry season the proportion of vegetative structures was 75% and the other 25% were reproductive organs distributed in the following proportions: 15% ID, 7% FD, 8% BD. In the rainy season, the vegetative organs reached 80% and 20% the reproductive organs, whose proportions were 6% ID, 7% FD and 7% BD. *V. meriodionale* Swartz expressed four phenological stages similar to *V. corymbosum*: development of vegetative buds, basic tissues for the formation of leaves and branches, development of the inflorescence, floral and berry development. Each of these stages was made up of five phases, except the last one that only exhibited four phases. The *V. meridionale* expressed a growth by continuous periods, in such a way that different vegetative and reproductive phases were appreciated, simultaneously. The phenological scale achieved in the five evaluated Colombian high Andean natural populations can be applied to other regions with similar climatic characteristics.

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Ana María López Gutiérez [1]*, Marta Leonor Marulanda Ángel[1], Lina María Gómez López[1] and Carlos Felipe Barrera Sánchez[2]

ABSTRACT

Rubus glaucus is widely distributed throughout the three mountain ranges of Colombia, where the blackberry growers have highlighted the need to standardize the supply of planting material, starting with plant breeding schemes that lead to more productive varieties with morphological characteristics that ease agricultural activities. Plant breeding activities have improved by considering the pollination mechanisms of plants. The implementation of controlled sexual hybridization depends on these pollination mechanisms, and several plant breeding methods have been adapted to crop pollination patterns. Morphological characteristics and studies on the floral biology of *R. glaucus* Benth were conducted to improve plant breeding processes. In addition, a study on pollen viability and stigma receptivity were performed. The reported morphological characteristics of *R. glaucus* enabled characterization of its flowers as complete and perfect with a regular, actinomorphic, perianth heterochlamydeous, dialipetalous, and dialisepalous structure. Meanwhile, the evaluation of different collection times for pollen viability revealed significant differences. The highest pollen viability occurred at 10:00 am, followed by 9:00 am. Qualitative evaluation of stigma receptivity led to the conclusion that the highest stigma receptivity is at anthesis at 12:00 m.

Keywords:
Andean blackberry
Androecium
Gynoecium
Viability

RESUMEN

La especie *Rubus glaucus*, está distribuida en las tres cordilleras de Colombia, donde los productores asociados de mora en Colombia, han destacado la necesidad de formalizar la oferta de materiales de siembra, iniciando con esquemas de fitomejoramiento que conduzcan a obtener variedades más productivas, con características morfológicas que faciliten las actividades agrícolas. El tener en cuenta los mecanismos de polinización en plantas, ha hecho más eficiente las actividades de fitomejoramiento. La implementación de una hibridación sexual controlada depende de los mecanismos de polinización, incluso varios métodos de mejoramiento en plantas se han ajustado a los patrones de polinización del cultivo. Con el fin de avanzar en un futuro, en procesos de fitomejoramiento para la especie *Rubus glaucus* Benth, se realizaron las descripciones morfológicas y estudios sobre la biología floral de la especie mencionada, además del estudio de viabilidad del polen y la receptividad del estigma. Las descripciones morfológicas realizadas para *R. glaucus* en esta investigación, permitieron caracterizar la flor de *R. glaucus*, como completa y perfecta, con una estructura actinomorfa regular y periantada, heteroclamídea, dialipétala y dialisépala. Entre tanto, se encontraron diferencias significativas al evaluar diferentes horas de viabilidad polínica. Encontrando que, la mayor viabilidad del polen se obtenía a las 10:00 horas, seguido por el tratamiento de las 9:00 horas. Las evaluaciones cualitativas de receptividad del estigma permitieron concluir que, el estado de mayor receptividad del estigma es el de antesis y la hora de mayor receptividad es a las 12 horas del día.

Palabras clave:
Mora de los Andes
Androceo
Gineceo
Viabilidad

[1] Facultad de Ciencias Ambientales. Universidad Tecnológica de Pereira. CP 660003, Pereira, Colombia.
[2] Facultad de Ciencias Agrarias. Universidad Nacional de Colombia. AA 1779, Medellín, Colombia.
* Corresponding author: <alopez@utp.edu.co>

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Rubus is a widely distributed genus. Its species are found on all types of cultivable soils ranging from tropical to subarctic regions. However, the exact number of species remains unknown because its classification was last done by Focke (1910-1914), and since then, many species have been described. It is estimated that the number of species of this genus ranges from 600 to 800. Difficulties in its taxonomic classification, particularly of *Rubus* subgenus in Europe and North America, are due to the prevalence of interspecific hybridizations, polyploidy, and various forms of apomixis (Thompson, 1995). The Andean blackberry (*Rubus glaucus* Benth.) is distributed in the three mountain ranges of Colombia and has combined characteristics of *Idaeobatus* and *Rubus* subgenera. It is a fertile amphiploid or allotetraploid that has resulted from the genome fusion of two species (Jennings, 1988).

In Colombia, blackberry production and cultivated area, but not yield, have increased, and it is considered a fruit with cultivation opportunities in Colombia, both for supply in the domestic market and export. The Colombian National Fruit Plan (PFN, Spanish acronym; 2006–2026) expects a 94.1% increase in cultivated area in Colombia by 2026 (MADR, 2006), i.e., from 10,743 ha in 2008 to 20,631 ha in 2026, which would generate approximately 6,917 direct jobs. This species exhibits great variability in terms of size, color, and fruit quality, which was possibly produced through selection breeding from wild plants in ancient times (Rativa *et al.,* 2016).

The associated producers of blackberry in Colombia have highlighted the need to standardize the supply of planting material, starting with plant breeding schemes that lead to more productive varieties with morphological characteristics that ease agricultural activities; the desired characteristics include absence of spines, tolerance to diseases, and increase in productivity. Therefore, it is necessary to collect, multiply, and characterize possible parent plants and conduct crossing. The latter step warrants studies on the morphology and floral biology of this species for improvement.

In plant breeding activities, the desired characteristics are obtained using existing variability. The understanding of pollination mechanisms in plants is critical for improvement processes. Sexual hybridization, essential in plant breeding, constitutes controlled pollination and implementation of such pollination mechanisms (Frankel and Galun, 2012).

In this study, for advancements in breeding processes for *R. glaucus*, morphological characteristics and studies on floral biology of this species were conducted as well as pollen viability and stigma receptivity were studied.

MATERIALS AND METHODS

Vegetal material

Experimental plant material (*R. glaucus* flowers) was obtained in August 2017 from the Botanical Garden of Universidad Tecnológica de Pereira, located at 1,467 masl (4°47'28.2" N 75°41'24.5" W).

Floral morphology

Flowers collected at anthesis (fully opened flowers) were inspected using a Leica EZ4 stereoscope. The morphological characteristics of the flowers, as suggested by Strasburger (1994), were evaluated and classified for external (chalice and corolla) and internal verticils (androecium and gynoecium). Finally, the floral formula of *R. glaucus* was constructed.

Pollen viability

A total of 25 flowers were collected at anthesis from different plants, and their anthers were detached under a stereoscope to collect pollen grains. To obtain a random sample, 100 pollen grains per sheet were counted for evaluation at each collection time. Pollen collection started at 8:00 am and continued until 1:00 pm with periodic collection conducted every hour (6-hour collection, treatments 1 to 6).

Pollen viability analysis

Pollen viability analysis was conducted using coloration with 2% acetocarmine glycerol as described by Alexander (1969). This procedure can detect non-aborted pollens and indirectly evaluate their viability. Pollens were visualized under a Leica DM750 optical microscope with a photographic head. Pollens were considered viable when they presented reddish coloration and a hyaline halo (Figure 1A). Viable pollens were quantified in percentage by determining viability percentage in 100 pollen grains from 5 flowers;

this procedure was performed in triplicates. Statistical analysis was performed using ANOVA after determining normal distribution of data using Shapiro–Wilk test with at 5% significance. Differences among treatments were determined using Tukey test. All statistical analyses were performed using the R3.4.4 software.

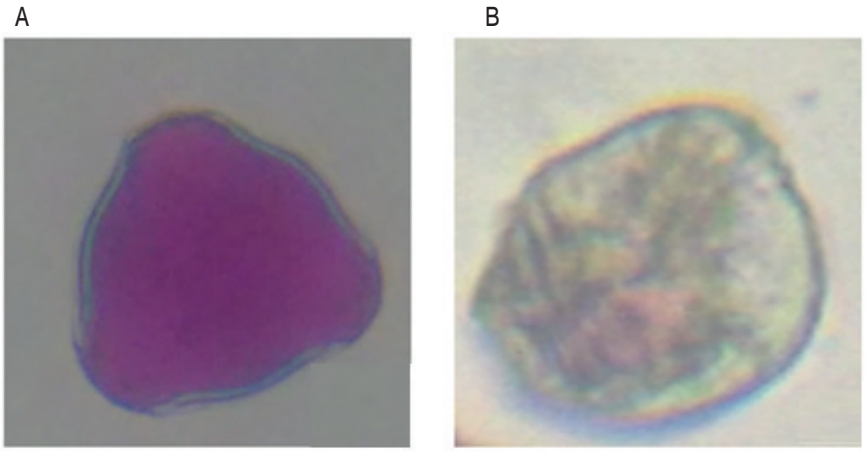

Figure 1. Pollen grains of *R. glaucus*. A. Viable pollen grain; B. Non-viable pollen grain.

Scanning electron microscopy (SEM)

To visualize pollen grains using SEM, flowers were collected at anthesis, and their anthers were detached under a stereoscope. The detached anthers were fixed in a 3% glutaraldehyde solution overnight at 4 °C. Subsequently, the samples were washed three times for 10 min in a 0.1 M PIPES solution (pH 7.2), followed by serial washes in ethanol solutions (50%, 60%, 70%, 80%, 90%, 95%, and 100%; v/v). Subsequently, the samples were transferred into a drying equipment. The specimens, previously visualized by optical microscopy, were observed under a FEI scanning electron microscope (version Quanta 250; Thermo Fisher Scientific).

Stigma receptivity

Stigma receptivity was chemically evaluated with 3% hydrogen peroxide (H_2O_2). This evaluation involved depositing H_2O_2 in the stigma of flowers by considering receptive stigmas that rapidly formed bubbles (Zambon *et al*., 2018). Evaluation of stigma receptivity was performed at pre-anthesis and anthesis stages using a periodicity schedule, which started at 7:00 am and continued until 2:00 pm.

RESULTS AND DISCUSSION

Floral morphology

External verticils. *R. glaucus* flowers were complete and perfect and had a regular and perianth, actinomorphic structure, with 5 petals and 5 differentiated sepals (heterochlamydeous). The corolla had free petals and sepals (dialipetal and dialisepal, respectively; Figure 2).

Internal verticils. The flowers were hypogynous with pluricarpelar and apocarpous gynoecium and numerous stigmas and intrusive anthers (Figure 2). Based on the previously described characteristics, the following floral formula was constructed:

Floral formula for *R. glaucus* = * K_5 C_5 A_αG5 (1)

The morphological characteristics of *R. glaucus* reported in this study are consistent with those described by Monasterio-Huelin (1992), who reported the taxonomic characteristics of *Rubus* in the Iberian Peninsula and Balearic Islands. Monasterio-Huelin (1992) described *Rubus* species as heterochlamydeous and hermaphrodites wherein the chalice is formed by 5 imbricate pieces with whole lanceolate sepals. *Rubus* from the Iberian Peninsula and Balearic Islands have white, pink, or red petals depending on the species; *R. glaucus* petal is white. On the other hand, Cancino-Escalante *et al.* (2011) described *Rubus* flowers in Colombia as pentamer flowers with welded sepals at the base; deltoid to ovate-acuminate, usually with obovate; and free; and white, pink, or red petal trichomes. These characteristics completely coincide with those found in the present study.

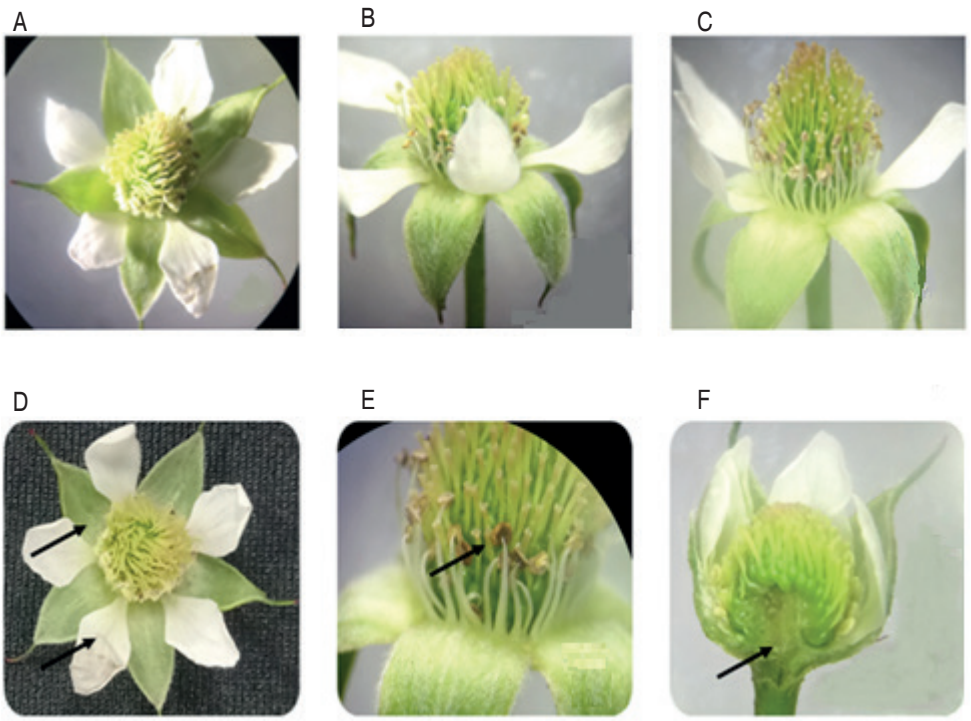

Figure 2. Floral morphology of *Rubus glaucus*. A. Regular actinomorphic flower; B. Perianth flower; C. Heterochlamydeous; D. Dialisepal–Dialipetal; E. Intrusive anther; F. Hypogynous ovary

Pollen viability

Pollen viability data showed a normal distribution and coefficient of variation of 6.77%. Analysis of variance showed highly significant differences in terms of pollen viability according to the collection time. Regarding differences in pollen viability according to the collection time, Tukey's test showed that the highest pollen viability was obtained at 10:00 am, followed by at 9:00 am and then 11:00 am. Pollen viability percentages at 9:00 and 10:00 am were significantly different from each other and different from other collection time. However, pollen viability was similar at 8:00 and 11:00 am. Likewise, pollen viability at 12:00 and 1:00 pm were similar, with the latter having the lowest pollen viability percentage (Table 1).

Table 1. Tukey test for setting differences among treatments.

Treatment (Pollen collection time)	Viability percentage (average)
8:00	0.3784 c
9:00	0.4901667 b
10:00	0.5769 a
11:00	0.3803667 c
12:00	0.2913333 d
13:00	0.2693333 d

Different letters indicate significant differences among treatments (P<0.05).

Evaluation of other *Rubus* species (Nybom, 1985) has shown 8% viability for triploid species and 54% for tetraploid species, compared with 81% for apomictic diploid species. These results coincide with those found in the present study for *R. glaucus*, which is a tetraploid species. Other studies, which evaluated pollen viability percentages from first-generation progeny individuals of interspecific crosses of *Rubus* genus, have reported values between 66.7% and 28.8%, with most frequently reported values being 45% and 60% depending on the parents of each progeny (Nybom, 1995). The previously reported results coincide with those found in the present study.

The relationship between pollens and fruits and seed formation has been recognized since the beginning of agriculture. This information has allowed for artificial pollination and double pollination in angiosperms. Darwin's contribution is also known, and his theory of evolution states that cross-fertilization is not only beneficial in evolutionary terms but is also necessary for maintaining a species' vigor and fertility. Understanding the viability of these gametes is vital for plant breeding.

SEM

R. glaucus pollen grains showed a tricolored structure (Sáenz Laín, 2004), with equatorial diameters ranging between 20 and 25 µm (Figure 3). According to Soejarto and Fonnegra (1972), this pollen size is small; they have reported that most species have a diameter between 15 and 50 µm and that almost all plant species have pollen diameters between 8 to 150 µm. In general, there is a correlation between pollen size and pollinating agents. Similar studies conducted on pollen grains from 9 European *Rubus* species have described pollen grains to be small, isopolar, and tricolporate (Tomlik-Wyremblewska, 1995) such as those found in *R. glaucus*.

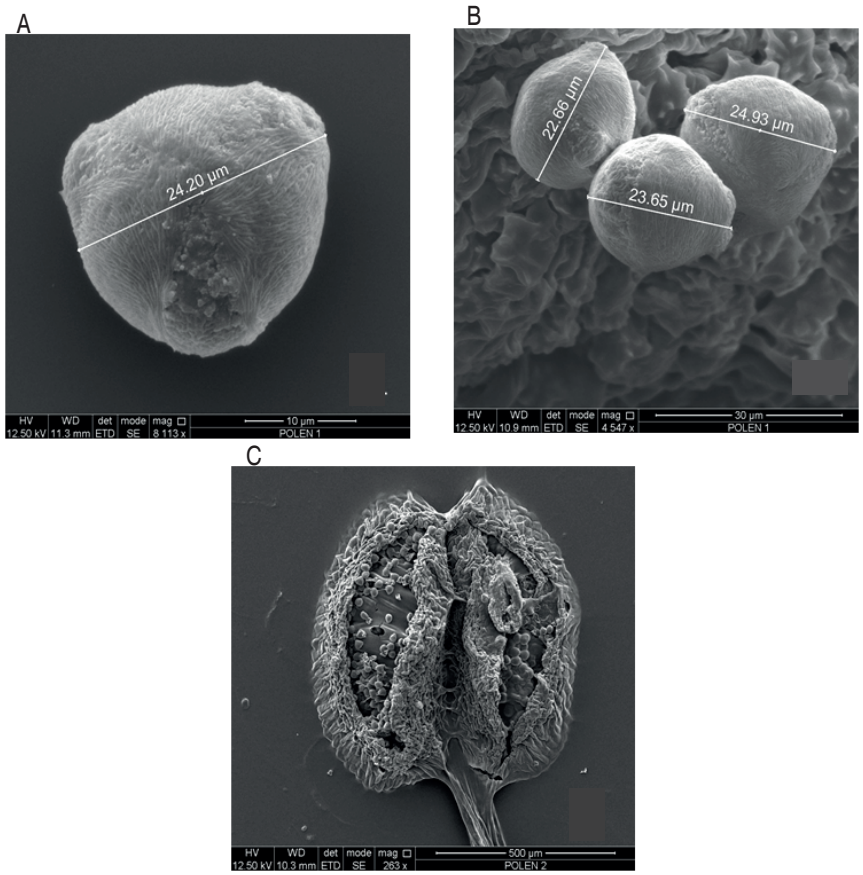

Figure 3. Scanning electron microscopy of pollen grains and *Rubus glaucus* anthers. A. Tricolpored pollen grain; B. Pollen grains; C. Anther.

Stigma viability

Qualitative assessments of stigma receptivity led to the conclusion that most stigmas were receptive at anthesis (fully open flower; Figure 4) and the time of highest stigma receptivity was 12:00 m when the highest and most frequent bubble production on stigma exposure to H_2O_2 (3%) was observed.

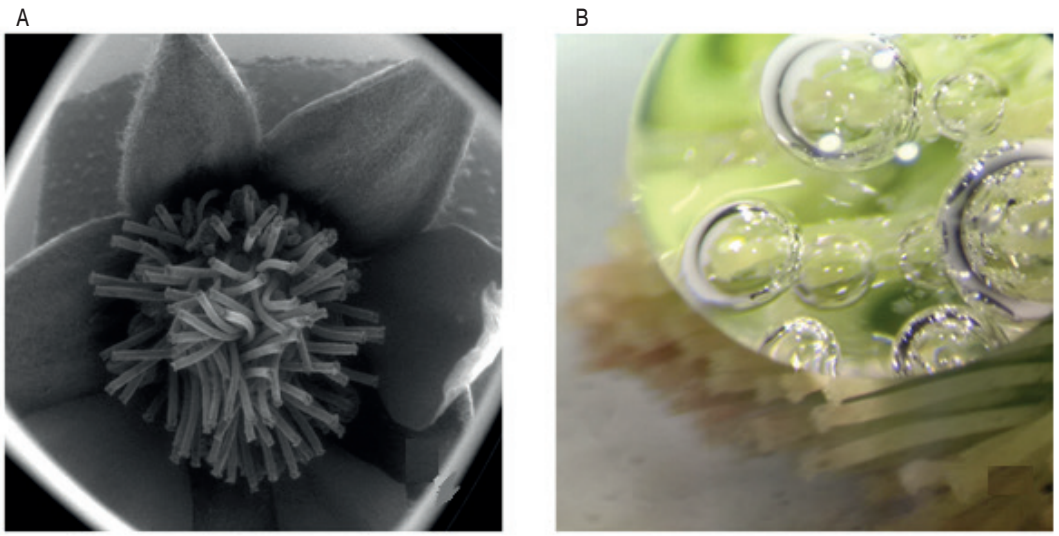

Figure 4. Appearance of stigma and production of bubbles during stigma receptivity test. A. *Rubus glaucus* multiple stigma (30×); B. Production of bubbles in *R. glaucus* stigma on reaction with hydrogen peroxide.

after being emasculated. This study led to the conclusion that flowers do not change their stigma receptivity as opposed to being emasculated or not.

A previous study (MacPhail and Kevan, 2005) on ecological aspects related to the frequency of insect visits to the wild species of *Rosa* genus, a species highly related to *Rubus*, found that the largest period of insect visits to flowers occurs between 9:00 am and 12:00 m, consistent with the findings of the present study. For *R. glaucus*, the highest insect activity is observed in the morning, with the highest frequency of *Apis mellifera* (Hymenoptera) in commercial crops; this denoted that stigma is more receptive at 12:00 m.

Understanding *R. glaucus* pollination mechanisms is vital to improve breeding processes with controlled pollination. Floral morphology indicates pollination mechanisms of this species and helps to infer its most frequent type of pollinators. In addition, determination of the fertility behavior of internal verticils (androecium and gynoecium) allows improved protocol designing for crosses that formally initiate an improvement program for this species. The last aspect has not been previously reported for *R. glaucus* and would allow establishment of a new variety, leading the productive sector to be more competitive.

Studies on the fertility aspects of other *Rubus* species have been conducted on American "blackberries" in breeding programs (Ruple *et al.,* 2010), which aimed to evaluate the effects of self-pollination and cross-pollination and the subsequent fruiting in different genotypes of *Rubus*. They have evaluated different fertility components of these genotypes in controlled conditions, particularly before and

CONCLUSIONS

Regarding floral morphology, *R. glaucus* is an actinomorphic, perfect, and complete pentamer flower, similar to other flowers of *Rubus*. Evaluation of its pollen grains led to the conclusion that the highest pollen viability occurs at 10:00 am. Meanwhile, the highest stigma receptivity occurs at 12:00 m, i.e., at anthesis. Based on gamete behavior, controlled pollination protocols can be standardized to initiate breeding programs for this species.

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Accumulation of degree days and their effect on the potential yield of 15 eggplant (*Solanum melongena* L.) accessions in the Colombian Caribbean

Acumulación de grados días y su efecto sobre el potencial de rendimiento de 15 accesiones de berenjena (*Solanum melongena* L.) en el Caribe Colombiano

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Rommel Igor León Pacheco[1]*, Ender Manuel Correa Álvarez[1], Jorge Luis Romero Ferrer[2], Heriberto Arias Bonilla[1], Juan Camilo Gómez-Correa[1], Marlon José Yacomelo Hernández[1] and Lumey Pérez Artiles[1]

ABSTRACT

The growing degree-days (GDD) provide an estimate of the accumulated thermal energy available for the development of a crop. The use of GDD allows measuring the heat requirements associated with the phenological stages of the crop, which allows in turn, to predict when a certain plant stage will occur knowing the daily temperatures. The aim of this study was to establish relationships among the effect of degree days (DD) to vegetative growth, first flowering and fructification (VG, Fl and Fr), on total yield per plant (TY/P) of eggplant grown under open-field conditions employing a randomized complete block design with 15 genotypes and four replicates. The results showed that: 1) The genotypes that initiated fruit production in less time required fewer GDD (892.14-1,077.71 °C) for this phenological phase, obtaining higher productivity. 2) The genotypes C035 and C040 had an average yield higher than the national average with values of 83.75 and 84.86 t ha^{-1}, being identified as future varieties to be produced in the Caribbean region. 3) The Caribbean region is suitable for the establishment of the crop as there were no events with limiting temperatures for this species (higher than 35 °C and lower than 15 °C). 4) The principal component analysis showed associations among the variable YT/P with the genotypes C011, C042, and C015; meanwhile, C032, C025, and C028 were associated with the variables DD to VG, Fl, and Fr. These results would be useful in developing a model to estimate yield with DD.

Keywords:
Degree days
Solanum melongena
Temperature
Yield

RESUMEN

Los grados días de desarrollo (GDD) proporcionan una estimación de la energía térmica acumulada disponible para el desarrollo de un cultivo. El uso de los GDD permite medir los requerimientos de calor asociados a las etapas fenológicas del cultivo, lo que a su vez permite predecir cuándo ocurrirá una determinada etapa de la planta conociendo las temperaturas diarias. Este estudio tuvo como objetivo determinar las relaciones entre el efecto de los grados días (GD) hasta el crecimiento vegetativo, la primera floración y fructificación (CV, Fl y Fr) sobre el rendimiento total por planta (RT/P) de berenjena cultivada en campo abierto bajo un diseño de bloques completos al azar con 15 genotipos y cuatro repeticiones. Los resultados mostraron que: 1) Los genotipos que inician producción de frutos en menor tiempo requieren menos grados días (892,14-1.077,71 °C) para esta fase fenológica, obteniendo una mayor producción. 2) Los genotipos C035 y C040 tuvieron un rendimiento promedio superior al promedio nacional con valores de 83,75 y 84,86 t ha^{-1}, identificándose como futuras variedades a producir en la región del Caribe. 3) La región Caribe es apta para el establecimiento del cultivo, debido a que no hubo eventos limitantes de temperatura para la especie (temperaturas mayores a 35 °C y menores a 15 °C). 4) El análisis de componentes principales mostro asociación entre los genotipos C011, C042 y C015 con las variables RT/P, y los genotipos C011, C025 y C028 con los GD a CV, Fl y Fr. Estos resultados serían útiles para desarrollar un modelo para estimar el rendimiento con base en los GD.

Palabras clave:
Grados día
Solanum melongena
Temperatura
Rendimiento

[1] Corporación Colombiana de Investigación Agropecuaria - AGROSAVIA. CI. Caribia, km 6 vía Sevilla-Guacamayal. Zona Bananera Magdalena, Colombia.
[2] Corporación Colombiana de Investigación Agropecuaria - AGROSAVIA. CI. Turipaná, km 13 vía Montería-Cereté. Cereté, Córdoba, Colombia.
* Corresponding author: <rleon@agrosavia.co>

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In Colombia eggplant, aubergine or brinjal (*Solanum melongena* L.) is one of the species belonging to the Solanaceae family with great importance after tomato, potato and chili pepper. Eggplant is characterized by having a typical external appearance, taste, and the presence of seeds, presenting a great diversity of genotypes that varies according to the country, the region and the target market (Arguedas-García and Monge-Pérez, 2017). In the Colombian Caribbean region, mainly in the departments of Córdoba, Bolívar, Sucre and Atlántico, it is cultivated by small producers in areas ranging between 1,000 and 2,500 m^2. This vegetable has positioned itself as necessary in the farmer economy of the region because of factors such as the short cultivation cycle, short-term economic returns, intensive production in relatively small areas, and it requires a large amount of labor generating promotion of rural employment (Araméndiz *et al*., 2008a). At a global level, the crop shows a growing demand with production figures of 46.9 million t in 2012, increasing to 51.3 million t in 2016 (FAOSTAT, 2018). This demand, among other factors, is deeply rooted in the gastronomy of the population of many countries and the benefits attributed to human health, especially regarding antioxidants and minerals, as well as healing properties for diabetes and cholesterol reduction (Sadilova *et al*., 2014).

Aubergine plants show a good adaptation in dry regions and hot climates. According to Baixauli (2001), its optimum development occurs at average temperatures between 20 and 25 °C, with an optimum diurnal temperature between 22 and 26 °C and a nocturnal temperature between 15 and 18 °C. The crop is susceptible to frost, so in regions where the temperature is below 18 °C, it is advisable to cultivate the crop under greenhouse conditions. At low temperatures (11-12 °C), the plants stop their vegetative development, and deformations along with flower and fruit abortion can occur (Concellón *et al*., 2007). At temperatures higher than 32 °C fruit maturation accelerates, and pollen becomes unfeasible and prevents full fertilization when the temperature exceeds 35 °C for prolonged periods, causing deformations in fruits (Araméndiz *et al*., 2008b). Likewise, the crop supports relatively high temperatures provided that the humidity is adequate, tolerating up to 40-45 °C (Araméndiz *et al*., 2008a). According to Lieth (1974), phenology in crops examines the different stages of plant growth and development, clearly distinguishable and observable in chronological order, including the study of the biological phenomena linked to certain periodic rhythms or phases and the relationship with the environment where these occur (Moreno-Pérez *et al*., 2011). In the tropics, the temperature is the environmental variable with the most significant influence on crop growth and development; in this sense, the reactions that directly or indirectly intervene in most of the physiological, biochemical and metabolic processes are strongly linked to temperature (Baker and Reddy, 2001).

The unit that combines time and temperature to estimate the development of an organism from one point to another in its life cycle is commonly referred to as degree days (DD), growing degree day (GDD), heat units (HU) (López *et al*., 2011 and Díaz-Lopez *et al*., 2013) or physiological time (Parra-Coronado *et al*., 2015). In agronomy, its application mainly lies in estimating how long it takes for a particular crop to reach a phenological stage of interest such as anthesis, flowering, fruiting, harvesting, senescence, among others (Orduz *et al*., 2010; Hoyos *et al*., 2012). Other applications are to establish optimum conditions for the growth and development of pests, sowing dates, crop irrigation, and fertilization, among others (Flores-Gallardo *et al*., 2012; Ferrer *et al*., 2014; Ramírez *et al*., 2015). Worldwide, many efforts have been made to study the optimum days and temperatures for eggplant cultivation (Maynard and Hochmuth, 2007; Fealy and Fealy, 2008; Rouphael *et al*., 2010; Sadek *et al*., 2013). However, despite the socio-economic and cultural importance of eggplant in the Colombian Caribbean region, there are no recent studies that evaluate the accumulation of degree days and the productive response of this species. Therefore, this study aimed to establish the number of accumulated degree-days along the crop cycle (from transplant to harvest), and its effect on the productivity of 15 eggplant varieties in the Magdalena state, the Colombian Caribbean region.

MATERIALS AND METHODS

Plant material

Fifteen eggplant accessions belonging to the genebank of Corporación Colombiana de Investigación Agropecuaria (Agrosavia) were evaluated as follows: C003, C006, C011, C014, C015, C025, C026, C027, C028, C032, C035, C036, C040, C042, and C049. The accessions were planted in an experimental plot of the Caribia Research Center (CI) of Agrosavia located in Zona Bananera [banana and plantain production zone] of Magdalena State, Colombia, from June to November 2017.

The CI Caribia is located according to the geographic coordinates at 10° 47' N latitude and 74° 10' W longitude at an elevation of 18 m. The region has an average annual temperature of 28 °C, with a relative humidity of 82% and an average annual rainfall of 1,280 mm, placing it according to Holdridge's climate classification (1967) in the Tropical Dry Forest (Bs-T) life zone.

The accessions were planted in the field under a completely randomized design with four repetitions. The experimental unit comprised 40 plants in a plot of 1××1 m. A drip irrigation system was used, using drip tapes with a discharge of 0.8 L h^{-1} and irrigation until the soil reached field capacity. The CropWat 8.0 program was used to calculate water needs. For this, the amounts of water lost by the crop or crop evapotranspiration (ETc) were estimated. The ETc was estimated using Equation 1.

$$ETc - ETo \times Kc \quad (1)$$

Where *Kc* is the crop coefficient; for the current study, the *Kc* values used were the ones reported by Allen *et al*. (2006). Reference evapotranspiration (ETo) was calculated using the FAO Penman-Monteith method (Allen *et al*., 2006), with climatic data obtained from the Davis-Vantage2plus6162 automated climatological station of CI Caribia.

The calculation of degree days was estimated based on the standard equation or simple method (López *et al*., 2011) according to Equation 2:

$$Tm = \left(\frac{T\,max + T\,min}{2}\right); \quad GD = \sum_{i=1}^{n}(Tm - Tb) \quad (2)$$

Where:
Tm: mean temperature (°C)
Tmin: minimum temperature (°C)
Tmax: maximum temperature (°C)
n: number of days
Tb: base temperature (°C)
DD: degree days

The Tb used was the one reported by Maynard and Hochmuth (2007), i.e., 15.6 °C. Four phenological stages established for Solanaceae by Meier (2001) were used with adaptations (Table 1) based on the number of days in which each phenological stage was completed in 50% of the plants sown in each plot.

Table 1. List of stages established for 15 *Solanum melongena* L. accessions.

Stage	Description
19	Development of leaves (main stem). Nine or more unfolded leaves of the main stem.
51	Appearance of the floral organ. First visible floral button.
61	Flowering. First opened flower.
71	Fruit formation. First fruit reaches the typical shape and size.

The yield was expressed in tons per hectares (t ha^{-1}) during 14 harvests. Fruits that had not reached physiological maturity (unripe state) but that had the typical shape and size of their respective cultivar were considered fruits suitable for harvest. For data analysis, descriptive statistics and multivariate principal components analysis were used, using the Infostat program version 2017.

RESULTS AND DISCUSSION

Climatic characterization of the experimental location

Because the municipality of Zona Bananera is in a low altitude region, the temperatures vary according to the altitude of the territory (temperature zones), and this municipality, having heights of less than 1,000 m.a.s.l., has a temperature behavior corresponding to a warm temperature zone, in which the average annual temperature is higher than 24 °C. According to IDEAM, the maximum average temperature of the municipality of Zona Bananera is 28 °C, remaining almost constant throughout the municipality, as well as a minimum of 24 °C (Municipio Zona Bananera, Plan básico de ordenamiento territorial, 2001).

As it can be seen in Figure 1, in the first vegetative growth stage of eggplant, which lasted approximately one month, temperatures were above 15 °C (the temperature that is limiting the crop); therefore, no event caused growth

retardation. Likewise, the same occurred with the maximum temperatures, which did not exceed 35 °C, being optimal for the crop.

Similarly, this also occurred with the relative humidity showing values between 60 and 100%, typical of the humid Caribbean region.

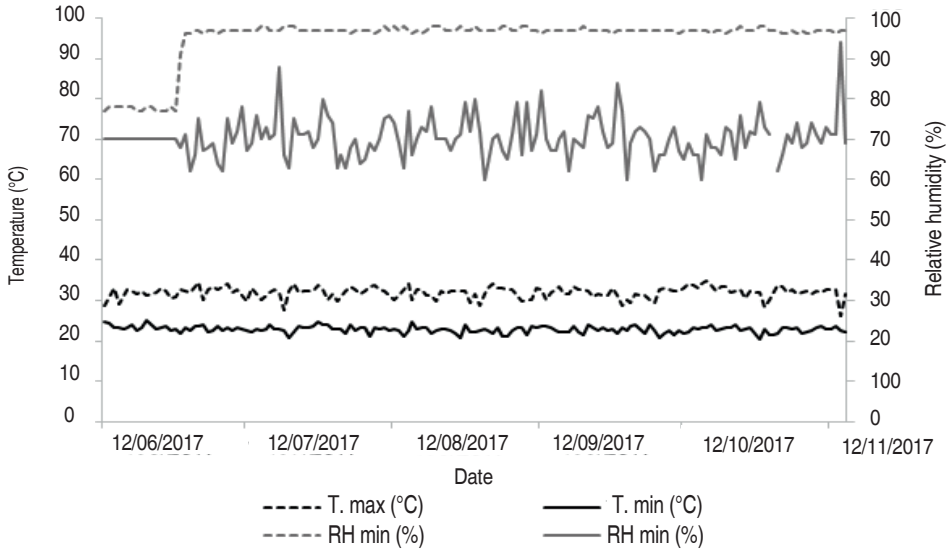

Figure 1. Characterization of temperature (°C) and relative humidity (%) during the phenological cycle of eggplant in Zona Bananera, the Caribbean region, Colombia.

Regarding the presence of diseases, even though the environment was suitable for the establishment of some limiting pathogens for eggplant such as *Sclerotium rolfsii*, its incidence was very low with 0.04% of infected plants (17 plants).

In Figure 2 and Figure 3, precipitation throughout crop phenology was higher than the reference evapotranspiration (Eto) and much higher than the Eto/2, which indicates that there were no critical drought scenarios.

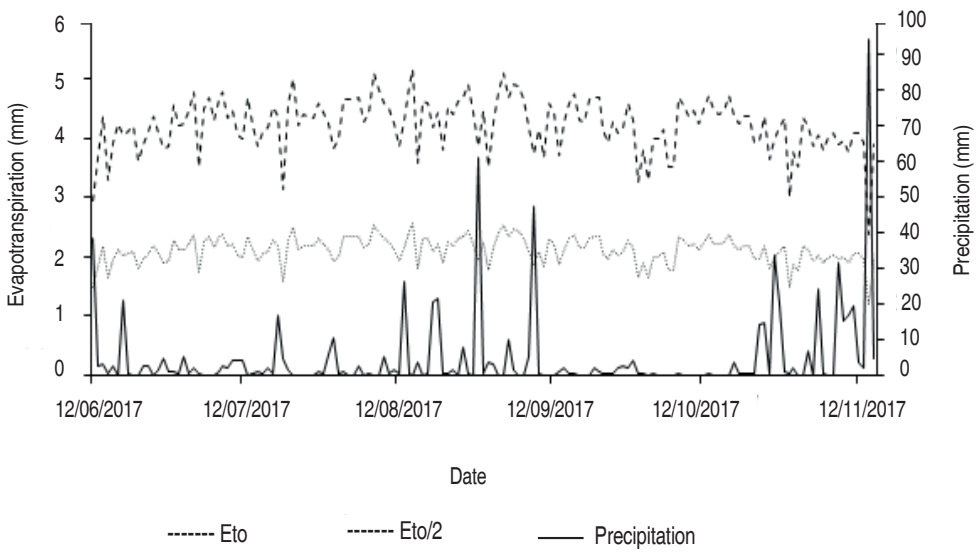

Figure 2. Precipitation and reference evapotranspiration during the phenological cycle of eggplant in Zona Bananera, the Caribbean region, Colombia.

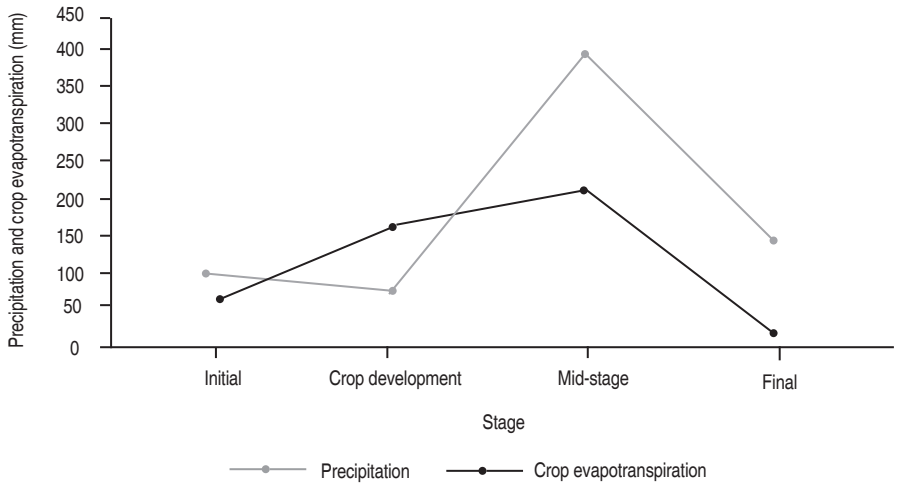

Figure 3. Precipitation and crop evapotranspiration during the phenological cycle of eggplant in Zona Bananera, Caribbean region, Colombia.

In the same way, during the four phenological crop stages, total precipitation was similar to crop evapotranspiration, which indicates that, in general terms, the water requirements were supplied through precipitation in the area and the shortage was obtained through irrigation. Moreover, the evapotranspirative demand of the crop during the four phases was 560 mm. However, the soil was monitored with tensiometers, one of 0-20 cm and another of 20-40 cm, to maintain soil humidity at field capacity.

Accumulation of degree days in 15 eggplant accessions

The accumulation of degree days for 15 eggplant accessions reached values from 892.14 to 1,077.71 °C (Table 2), with which we could select early or late accessions for these climatic conditions.

Table 2. Accumulation of degree days in 15 eggplant accessions for the phenological stages of leaf development, appearance of the floral organ, flowering, and fruit formation.

Accession	Degree days (°C)			
	Leaf development	Appearance of the floral organ	Flowering	Fruit formation
C003	399.56±42.85	478.97±79.24	733.89±0	952.88±0
C006	408.06±29.68	458.61±28.54	674.20±82.31	931.64±42.47
C011	384.73±42.35	447.19±48.58	733.89±0	931.64±42.47
C014	363.55±0	452.80±38.77	679.44±38.16	910.40±49.05
C015	372.58±18.05	431.78±35.01	695.51±45.48	931.64±42.47
C025	408.06±29.68	452.80±38.77	752.24±36.72	1,077.71±97.77
C026	363.55±0	411.28±13.42	720.48±53.29	955.91±74.39
C027	402.25±28.03	423.43±19.85	733.89±0	952.88±0
C028	399.56±42.85	444.45±33.76	733.89±0	1,019.86±77.35
C032	393.23±34.27	426.00±48.11	733.89±0	1,019.86±77.35
C035	387.41±29.14	516.02±93.47	695.51±45.48	910.40±49.05
C036	408.59±35.99	473.16±85.34	711.57±44.63	952.88±0
C040	381.60±20.84	423.43±19.85	667.95±83.71	892.14±76.21
C042	378.39±29.68	417.61±23.18	711.57±44.63	931.64±42.47
C049	378.39±29.68	463.89±67.67	733.89±0	952.88±0

On the other hand, accessions C040, C014, and C035 were those that produced fruits in less time with accumulated values in degree days of 892.14±76.21 and 910.4±49.05, respectively. This behavior is of great importance since these accessions were the ones that had the highest yields with values above 8,000 gram per plant, for a planting density of 10,000 plants per hectare, 80 t ha^{-1} were obtained, i.e., a significantly higher value than the national yield, which is around 16 t ha^{-1} (Araméndiz *et al* 2008b,c) (Figure 4). The opposite occurred with genotypes C015, C011, C032, and C025; the last two are the latest to start producing fruits suitable for harvesting, as observed in the degree days accumulation for this phenological phase (Table 2). However, no significant differences (P>0.05) were found between accession for any of the phenological stages evaluated, which gives indications that those that begin to produce earlier fruits will be those that will have the highest final yields.

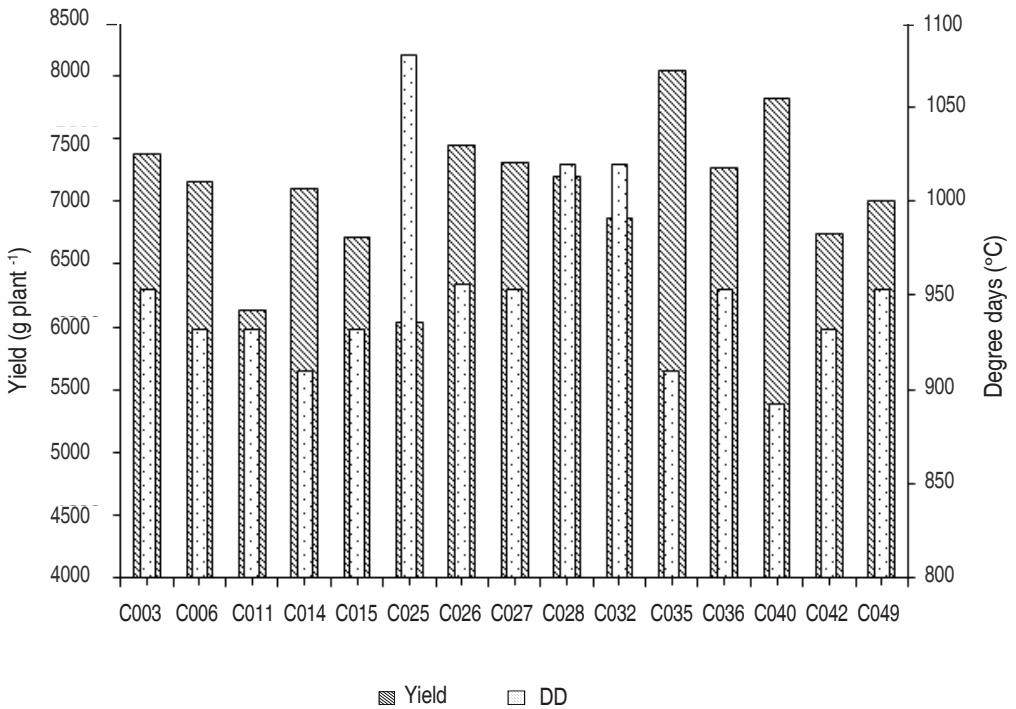

Figure 4. Yield (grams per plant) and degree days (°C) to fruits development suitable for harvesting in 15 eggplant accessions.

Accessions C028, C032, and C025, were the latest ones with more than 1,000 °C accumulated to produce fruits suitable for first harvest (Table 2); moreover, these had a strong correlation with degree days to flowering, as observed in the multivariate biplot with the largest and most significant vectors for component 1 (Figure 5).

The accumulation of degrees days was similar for the 15 accessions in the first phenological stage (vegetative growth) with average values of 388.66 °C (Table 2); this effect was because this phenological stage was measured until the plants had produced nine leaves. Furthermore, it should be noted that the maximum vegetative development rate in the crop is reached when flowering begins.

An opposite behavior was reported by Rouphael *et al.* (2010) with a highly positive relationship between the number of leaves and the thermal time during the experiment in three aubergine cultivars. The response of the numbers of the leaves to the thermal time was curvilinear, with values 450 degree days. After 450 degree days, the increase in the number of leaves per plant was linearly related to the thermal time. However, Maynard and Hochmuth (2007) report for aubergine a base temperature of 15.6°C and a maximum

temperature of 35 °C. These results could be useful to generate a model for the leaf area development and finally, a cultivation growth model for this crop.

This behavior is since crop development depends to a large extent on the temperature and the photoperiod; meanwhile, in the tropics, the temperature is the environmental variable with the greatest influence on crop development. The regulating role of temperature is through its action on the enzymatic reactions that directly or indirectly intervene in development processes (Baker and Reddy, 2001).

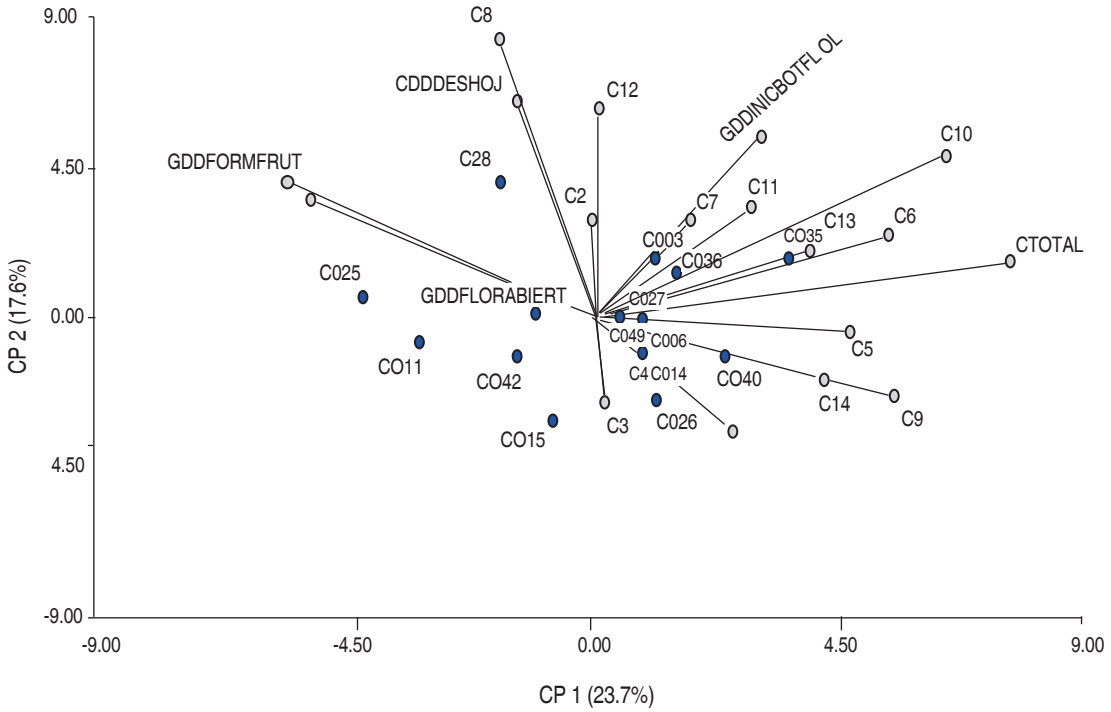

Figure 5. Double representation of 15 eggplant accessions with the variables evaluated.

This behaviour is explained by Martín and Jerez (2017), who mentions that temperature is a basic factor that influences the development rate, particularly from emergence to flowering and maturity. Many species are adapted to particular temperature ranges; thus, as the environmental temperature or mean temperature (Tm) decreases, its development rates are reduced to such an extent, that if the temperature falls to the limit, the development stops. This temperature limit is called the base temperature (Tb). On the contrary, as the temperature increases, development rates increase to an optimum temperature (To), from which they decrease and eventually stop at their maximum development temperature (Tmax).

For eggplant cultivation limiting temperatures are those below 15.6 °C and above 32 °C, finding the optimum at 22 °C (Maynard and Hochmuth, 2007; Uzun, 2007). Hence, the climatic offer of the region did not have stressful temperatures for the crop, and it had a rather additive effect, causing some accessions to begin their production in less thermal time or days.

In agriculture, the combination of time and temperature comprises the so-called thermal time (TT) or also known as the sum of heat, degree day, growing degree days, or physiological time. This approach has been used in numerous studies to describe the phenology of crops like corn (*Zea mays* L.), sugar cane (*Saccharum* spp.), melon (*Cucumis melo* L.), chickpea (*Cicer arietinum* L.) and cotton (*Gossypium hirsutum* L.) (Baker and Ready, 2001).

Likewise, Sadek *et al.* (2013) and Fealy and Fealy (2008), specify the importance of heat requirements on eggplant cultivation since these can be quantified and associated

with the time when the fructification stage begins. This crop will not continue growing if the average temperature is below the base (12 °C), and the degrees of heat provides a reasonable estimate of the heat energy available for plant growth representing an important factor during the crop cycle.

On this aspect, Uzun (2007) points out a behavior similar to the one reported in this research, in which the heat requirements were different between varieties of eggplant. Meanwhile, these authors show a curvilinear effect of the variables associating yield with the temperature factors and light intensity, the optimum being 17-20 °C and 7-17 MJ m^{-2} d^{-1} for temperature and radiation, respectively; above these values number of fruits per plant as well as average fruit weight decreased, and therefore, yield.

Nevertheless, Kürklü *et al.* (1995) conducted a study to define the response of eggplant at different temperatures in controlled greenhouse environments. The temperatures established in the greenhouses were 14, 18, 22, 26, and 30 °C. Vegetative growth was much higher as the temperature increased to a point where the growth decreased, meanwhile fruit development (size, fruit weight, and yield) had an opposite behavior, increasing their values as the temperature increased to 30 °C. The total harvested yield of eggplant at different temperatures had the highest fruit yield at 19, and 16 °C (approximately 33, 32, and 28 kg, respectively), and plants that grew at 26 and 30 °C produced much less than plants grown at lower temperatures (18 and 15.5 kg, respectively). Therefore, the optimum temperature for fruit production in eggplant is approximately 22 °C.

Multivariate analysis by principal components

Authors, as Pla (1986) mentions that it is not valid to evaluate only one component to explain data variability. Because of, it was found that only 23.7% synthesize the variability in the first component; further, applying the criterion of selection of principal components of Kaiser (Pla, 1986), the first three components were used obtaining a value of 54%.

In Figure 5 accessions C032, C025 and C028 have similar behavior and are associated with the variables degree days to leaf development, floral bud starting and flowering; meanwhile, the latter was more associated with the eighth harvest and degree days to leaf development. Similarly, a second group was formed with accessions C011, C042, and C015. In the opposite axes, three large groups are observed, accessions C003, and C036 associated mainly with harvests 6, 7, 10, 11 and 13; whereas, accessions C027, C049, C006, and C014, were associated with harvests 3, 4, 5, 9 and 14; and finally, accessions C040, C035 and C026 showed a strong association to the total variable yield.

García and López (2002) explain that this behavior occurs when plants are exposed to thermal variations on the physical environment, and these have great influence on the different physiological, biochemical and metabolic processes leading to their growth and development; furthermore, these variations determine the leaf area and the accumulation of dry matter during the biological cycle of the plant. Additionally, growth and development are undoubtedly affected by factors other than temperatures, such as the flow and duration of photosynthetically active radiation, the availability of nutrients and water, and the loss of photosynthetic tissue.

In a production system, which is developed under protected environments and where there is permanent availability of nutrients and water, other bioclimatic factors (i.e., temperature, luminosity, and CO_2 concentration) can be exploited to increase yield or improve the quality of the final product. Optimizing the conditions that determine maximum crop yield with a minimum expenditure of energy in the systems cultivated in open fields, are fundamental to generate economic and environmentally sustainable technology.

In general terms, eggplant is a plant that requires an adequate temperature during its cultivation to reach yields above the national average (16 t ha^{-1}) (Araméndiz, 2008b, c) and fruit quality. Eggplant has non-climacteric fruits (Arguedas-García, 2017). Its optimum vegetative growth occurs with temperatures between 27 and 32 °C during the day, and between 21 and 27 °C during the night, although fruit growth is favored between 22 and 26 °C (Passam and Karapanos, 2008).

Day temperature should oscillate between 25 and 30 °C together with good luminosity to satisfy the needs of the plant, and with it, greater efficiency in the photosynthesis. It should be carried out in a way that its products, when being

sent to other parts of the plant, are not used entirety for respiration. It is achievable with better night temperatures (14-16 °C), which are favorable for plant and fruit growth.

CONCLUSIONS

The accessions that initiate fruit production suitable for harvest in less time require fewer days to initiate this phenological phase, and therefore, generate higher productivity so that some early accessions with high yields can be identified based on degree days. The Caribbean region is suitable for the establishment of eggplant since there were no events with limiting temperatures for this species, which may cause growth retardation, obtaining for all accessions a density of 10,000 plants per hectare and yields above 64 t ha^{-1}. The accessions C035 and C040 had an average yield higher than the national average with values of 83.75 and 84.86 t ha^{-1}, so these have been identified as future varieties to be produced in the Caribbean region, given that additionally their fruit color, size, and shape characteristics are widely accepted in regional markets.

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Effect of different application forms of efficient microorganisms on the agricultural productive of two bean cultivars

Efecto de diferentes formas de aplicación de microorganismos eficientes en la productividad agronómica de dos cultivares de fríjol

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Alexander Calero Hurtado[1]*, Yanery Pérez Díaz[2], Dilier Olivera Viciedo[1], Elieni Quintero Rodríguez[3], Kolima Peña Calzada[1], Luke Leroy Theodore Nedd[4] and Janet Jiménez Hernández[2]

ABSTRACT

The use of Efficient Microorganisms (EM) can be an effective alternative to improve plant growth and yield in the bean cultivation. Therefore, different forms of application of efficient microorganisms were evaluated in the production of two cultivars of the common bean from November of 2013 to March of 2014. Two factors were studied; the first one was comprised of the two cultivars, Velazco Largo (VL) and Cuba Cueto (CC-25-9-N). The second factor consisted of four treatments with EM; without EM (control), soil inoculation (100 mL L^{-1}), foliage applications (100 mL L^{-1}), and the combined soil inoculation (100 mL L^{-1}) plus foliar applications (100 mL L^{-1}). The experiment was carried out in a randomized block design, in factorial outline 2×4, with three repetitions. The agronomic indicators were evaluated as the number of leaves per plant, the height of plants, number of pods per plant, number of seeds per pod, the mass of 100 seeds (g) and the yield (t ha^{-1}). The results showed that the different forms of application of efficient microorganisms stimulated the agronomic indicators evaluated in both crops. The associated applications between the inoculation of the soil and foliage applications of efficient microorganisms provided better results, producing increments in the yield of 1.13 t ha^{-1} in VL and 2.15 t ha^{-1} in CC-25-9-N.

Keywords:
Biofertilizer
Foliar application
Soil inoculation
Yield

RESUMEN

El uso de Microorganismos Eficientes (ME) puede ser una alternativa efectiva para mejorar el crecimiento y el rendimiento del cultivo de fríjol. Por lo tanto, fueron evaluadas diferentes formas de aplicación de microorganismos eficientes en la producción de dos cultivares de fríjol común, de noviembre de 2013 a marzo de 2014. Se estudiaron dos factores, el primero conformado por dos cultivares de fríjol común, Velazco Largo (VL) y Cuba Cueto (CC-25-9-N), y el segundo compuesto por cuatro tratamientos con ME; sin ME (0), inoculación al suelo (100 mL L^{-1}), aplicaciones foliares (100 mL L^{-1}) y la inoculación al suelo (100 mL L^{-1}) más aplicaciones foliares (100 mL L^{-1}), distribuidos en un diseño en bloques al azar, en esquema factorial 2×4, con tres repeticiones. Fueron evaluados los siguientes indicadores agronómicos, número de hojas por planta, altura de las plantas, número vainas por planta, número de semillas por vaina, masa de 100 semillas (g) y el rendimiento (t ha^{-1}). Los resultados mostraron que las diferentes formas de aplicación de microorganismos eficientes estimularon los indicadores agronómicos evaluados en ambos cultivares, siendo la aplicación asociada entre la inoculación al suelo y aplicaciones foliares de microorganismos eficientes la que proporcionó mayores resultados al producir incrementos en el rendimiento de 1,13 t ha^{-1} en VL y 2,15 t ha^{-1} en CC-25-9-N.

Palabras clave:
Biofertilizante
Aplicación foliar
Inoculación al suelo
Rendimiento

[1] Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP). Via de Acesso Prof. Paulo Donato Castellane s/n CP 14884-900 - Jaboticabal, São Paulo.
[2] Universidad de Sancti Spiritus "José Martí Pérez" (UNISS). Comandante Fajardo, Esquina Lepanto S/N, Olivos I, CP 60100, Sancti Spíritus, Cuba.
[3] Empresa Agropecuaria Agroindustrial "Melanio Hernández". Calle s/n, CP 62300, Tuínucu . Taguasco, Sancti Spíritus, Cuba.
[4] Instituto Nacional de Ciencias Agrarias (INCA). Carretera Tapaste km 3 1/2, San José de las Lajas, Mayabeque, Cuba.
* Corresponding author: <alexcalero34@gmail.com>

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For the next 40 years, food security will be a great challenge for the world because of the continuous increase of the population and the high consumption index growth (Ma *et al.*, 2016). The bean *Phaseolus vulgaris* L. (Fabaceae) is the most important grain consumed by humans in the world. In nutritional terms, these seeds are a source of protein, minerals, and vitamins (García-Fraile *et al.*, 2012).

Many agricultural soils are lacking enough quantities of one or more essential nutrient for these plants; as a result, their growth and development can be limited. The farmers become dependent on chemical products, as sources of fertilizers, to avoid this problem and to obtain better yields (Glick, 2012). Although the chemical fertilizers help the plants to grow, their environmental effect can be adverse, relating to fertilizers rich in phosphorus, potassium, and nitrogen (Adesemoye and Kloepper, 2009).

A less aggressive practice to the environment used to improve the nutrition of the plants has been the use of microorganisms as biofertilizers in the plants' cultivation. The rhizobacteria, which are plant growth promotors (PGPR), stood out as biofertilizers because these microorganisms adapt and grow quickly around the roots of the plants (Ahirwar *et al.*, 2015; Ul Hassan and Bano, 2015).

The response of the plant to the inoculation of PGPR varies considerably according to the rhizobacteria's species; the host, the soil type, and the density of the inoculum, the environmental conditions, and the inoculation method could affect such response (Shah *et al.*, 2017). The method of PGPR incorporation has an influence on the establishment and the permanency of bacterial populations in the rhizosphere and indirectly affects the growth promotors (He *et al.*, 2016).

On the other hand, in soils managed by organic inputs, exudates from bacteria, fungi, decomposed cells as well as plant and animal residues boost the soil's organic matter, which in turn improve the soil structure, function, and quality (Vejan *et al.*, 2016). The process of colonization, for some bacteria, is slow and not very effective because their growth and distribution through the rhizosphere depend on the soil's humidity, pH, temperature, microbial antagonism, competition space, perspired radicals, as well as the bacteria's physiological state. According to these factors, solo cells can proliferate quickly and invade the roots. A great number of bacteria will be able to promote the growth of the plants in an effective way (Gupta *et al.*, 2015; Pathak *et al.*, 2017).

The efficient microorganisms (EM) is a discovered and developed technology by professor Teuro Higa (Higa and Parr, 1994). They found that the success of its effect was in its mixture. From then on, this technology has been investigated, developed, and applied in a multitude of agricultural and environmental areas. It is used in more than 80 countries worldwide (Arias, 2010). Authors like Pedraza *et al.* (2010) state that the fundamental principle of this technology consists of the introduction of a group of beneficent microorganisms to improve the physical-chemical conditions of the soil.

The use of EM has been favorable for agriculture. Diverse studies have reported beneficial effects when they are introduced to common bean production (Calero *et al.*, 2016, 2017, 2018). They also have improved and benefitted the farmers (Luna and Mesa, 2016). It is well-known that bean production is low and is essential in the diet of the Cuban residents. Therefore, it was considered convenient to test the following hypotheses. Firstly, the application of EM could stimulate and increase the morphometric and productive parameters in two cultivars of common bean; and secondly, it is possible to maximize the beneficial effect of EM on increasing yields by the combined soil inoculation and foliar spraying. It was evaluated whether EM supplied in different forms increases the productivity of two cultivars of common bean, Velazco Largo and Cuba Cueto.

MATERIALS AND METHODS

Plant grow conditions

The experiment was carried out at the Collective farmer "Martires de Taguasco" (22°6'17.588'' N; 79°22'33.544'' W) in Sancti Spíritus, Cuba. The varieties, Velazco Largo (VL) and Cuba Cueto (CC-25-9-N) were donated by the Provincial Company of Seeds of Sancti Spíritus, with 96 to 97% germination, respectively. VL presented red-colored grains, a potential yield of 2.3 t ha^{-1}, a habit of growth type I, and a cycle of 72 to 77 days. CC-25-

9-N presented black colored grains, a yield of 2.7 t ha^{-1}, a habit of growth type III, and a cycle between 75 and 80 days. The cultivation was carried out in November of 2013 and the harvest in March of 2014. The cultivation was done manually at 0.60 m between rows and 0.07 m between plants. The climatic variables during the development of the research were registered by the Municipal Station of Hydraulic Resources of Cabaiguan, Sancti Spiritus, Cuba. The daily average temperature was 23.22 °C, the relative humidity 77.65% and the accumulated precipitation of 98.56 mm. The soil was classified as Brown Carbonated Sialitic by following the method by Hernández *et al.* (2015), being denominated as Cambisol (FAO, 2015).

Experimental design and treatments

The experimental design adopted was randomized blocks, factorial outline at 2×4. Two factors were studied; the first was comprised of two cultivars of common bean, VL and CC-25-9-N. The second consisted of four treatments with EM: control absence of EM (0), 100 mL L^{-1} of EM via soil inoculation (I), 100 mL L^{-1} of EM via foliar applications (F) and the combined application of via soil inoculation (100 mL L^{-1}) and foliar spraying (100 mL L^{-1}) (I+F). They were repeated three times, which formed 24 experimental parcels of 9.60 m^2. The useful area was 3.36 m^2, and the total area was 0.23 ha. The inoculation to the soil with EM was carried out before depositing the seeds, subsequently proceeded by the sowing. The foliate application was carried out with the support of a manual sprayer (ECHO MS-21H) of 7.60 liters of capacity, applying 40 L ha^{-1} of both application mode via soil and foliar spraying, in the vegetative (V4) and reproductive (R5 and R6) stages.

Bioproduct characteristics

The inoculation of efficient microorganisms EM-50 was acquired at the Labiofam of Sancti Spíritus, composed by *Bacillus subtilis* nato B/23-45-10 (5.40×10^4 colony-forming units (CFU) mL^{-1}), *Lactobacillus bulgaricum* B/103-4-1 (3.60×10^4 CFU mL^{-1}), and *Saccharomyces cerevisiae* L-25-7-12 (22.30×10^5 CFU mL^{-1}), with certificate of quality emitted by Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar (ICIDCA), code R-ID-B-Prot-01-01. The means used to obtain this bio preparation were industrial waste obtained from molasses, whey milk, rice waste mixed with leaf litter collected from a bamboo area. The methodology of Olivera *et al.* (2014) was followed.

Agro-technical management

The soil preparation, pest management, irrigation (by spraying), among other agro-technical cultivation practices were carried out following the recommendations and instructions from Faure *et al.* (2014), highlighting that no mineral or organic fertilizers were applied at any moment of the cultivation, and the practices of cleaning/ weeding were carried out manually.

Plant growth parameters

The evaluated variables corresponded to the recommended descriptors for the growth and development stages of the crop (CIAT, 1987). The samplings were carried out on the effective area of the plots, and 45 plants per treatments (15 replications) were evaluated. The indicators were the number of leaves per plant (LP) and the plant height (PH): from the cultivation date until evaluation, up to 50% of the plants presented the floral clusters (R5). All the trifoliate leaves per plant were counted (the support of a calibrated ruler was used). The number of pods per plant (PP) was evaluated when concluding the crop cycle (R9): the resultant pod count per parcel carried out on all plants was used to determine the average. The number of seeds per pod (SP) was determined by counting all the seeds contained within. The mass of 100 grains (M100) (g) was the result of the average of four samples of 100 grains in each experimental unit. The yield (t ha^{-1}) was obtained by converting the production of each parcel (kg) to t ha^{-1}.

Statistical analysis

It was used a factorial analysis to test the main effects of the four levels of EM application (EM) and two bean cultivars (VL and CC-25-9-N) and their interactions (EM × CV). Data obtained for the applied treatments were analyzed assuming normality and significance of variance with the Shapiro-Wilk and Fisher tests (P<0.05), respectively. Once these assumptions were verified, data were subjected to a two-way ANOVA. Mean values were compared using the test of Multiple Ranges of Tukey (P<0.05). All analysis was performed using the statistical software AgroEstat® (Barbosa and Maldonado, 2015).

RESULTS AND DISCUSSION

Effect of EM applications in the morphological parameters

There was a significant (*P*<0.05) and interactive effect of EM on the plant height of the two cultivars of common bean VL and CC-25-9-N (Figure 1). CC-25-9-N was superior to VL in the absence or presence of EM treatments. In both cultivars, all the EM treatments increased PH compared with the control (EM (0)). Especially, the EM (I+F) treatment surpassed the cv. VL by 5.12 cm and in the CC-25-9-N by 4.15 cm concerning the EM (I) and EM (F) treatments, and it increased both cultivars' height up to 14.11 and 8.86 cm, respectively, compared with control (EM (0)). Positive effects were achieved with the EM (I+F) treatment because it increased PH in the cv. VL by 66%, while the cv. CC-25-9-N by 59%, compared with that of the EM (0) treatment in both crops.

It is well-known that the soil inoculation with microorganism solubilizers/dissolvers of phosphate (bacteria, fungi, and actinomycetes with the capacity to break down minerals) can be fixed in the soil and can be used by the plants for their nutrition (Beltrán, 2014). These microorganisms show other activities that promote the vegetable growth such as supplying gibberellins, cytokines, ethylene, symbiotic nitrogen fixation; which are considered a potential and efficient bioagents to improve plant growth (Banerjee *et al.*, 2010). In both cultivars, all EM application was effective to increase the plant height compared with the control (EM (0)), especially the EM (I+F) treatment (Figure 1). This beneficial effect of the application of EM via soil or foliar spraying increasing the PH in beans plants as previous reported (Calero *et al.*, 2016, 2017).

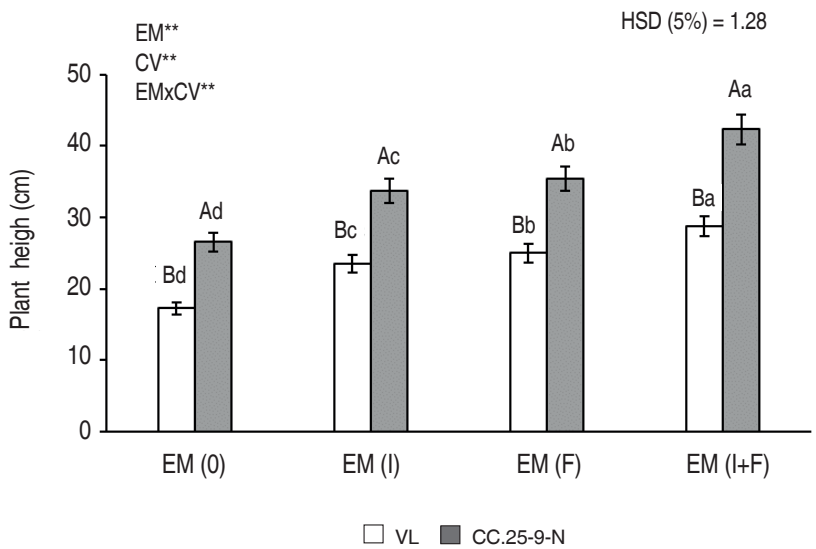

Figure 1. Plant height average of two bean cultivars (VL and CC-25-9-N), in function of EM treatments, without EM (0), soil inoculation EM (L), foliar applications EM (F) and the combined soil inoculation and foliar root applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM (*P*<0.05). ***P*< 0.01; EM × CV, efficient microorganism–cultivar interaction; HSD, honest significant difference.

The comparison of means revealed that the interaction between EM and CV was significant (*P*<0.05) on the number of leaves per plant in both bean cultivars (Figure 2). The cv. CC-25-9-N had a better response than the cv. VL in the production of the LP in absence or presence of EM.

The favorable response in the crops, under the influence of different applications of EM on the number of leaves per plant (Figure 2), suggests that the cv. CC-25-9-N showed a better genetic expression for this character regarding the cv. VL on the physiologic stage R6. The form of application of EM (I+F) surpassed the individual treatments and the control (without application).

The results demonstrated the positive effects attained in the increment of the production of leaves per plant with the application of the different forms of efficient microorganisms. Consistently with other studies on

beans, it was observed that the addition of EM (F) or EM (I) improved the number of trifoliate leaves, thus increasing yield production (Calero *et al*., 2017, 2018, 2019a). This benefit of EM increasing the leaf production is known in different species grown under different conditions, such as onion (Liriano *et al*., 2015), tomato (Olivera *et al*., 2015), strawberry (Álvarez *et al*., 2018) and tobacco (Calero 2019 c). On the other hand, it may be possible to increase the PP by the inoculation of soil or seeds with beneficial microorganisms. It also improves plant architecture by producing some substances that help in plant growth (Banerjee *et al*., 2010).

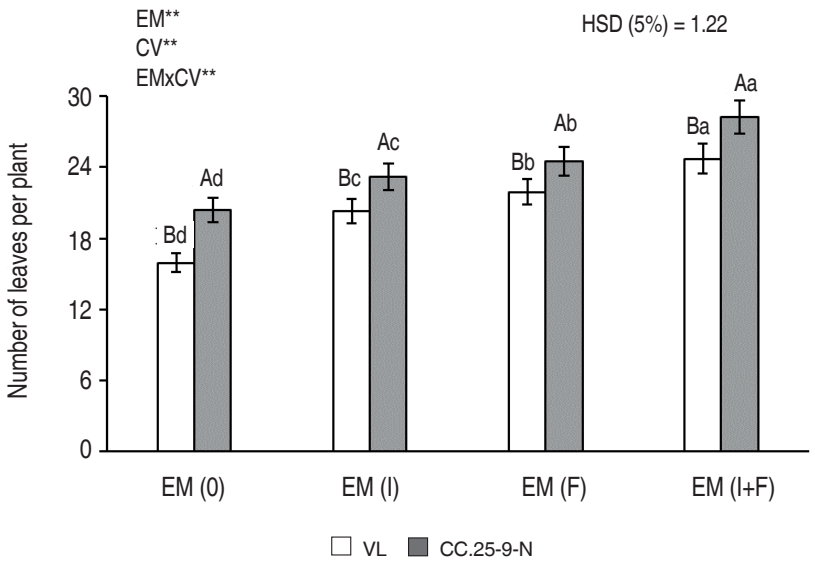

Figure 2. Number of leaves per plant of two bean cultivar (VL and CC-25-9-N), in function of EM treatments, without EM (0), soil inoculation EM (I), foliar applications EM (F) and the combined soil inoculation and foliar root applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM (P<0.05). ** P< 0.01; EM × CV, efficient microorganism–cultivar interaction; HSD: honest significant difference.

Effect of EM applications in the agro-productive parameters

There were significant differences (P<0.05) and interactive effects of EM and CV on the number of PP, in both cultivars (Figure 3). The cv. Cuba Cueto was superior in the production of PP relative to the cv. Velazco Largo in absence or presence of EM. In both cultivars, the three forms of EM application (I, F, and I+F) increased the PP, compared with the absence of EM (Figure 3). The application of EM (I+F) treatment increased PP in the cv. VL by 110% and the cv. CC-25-9-N by 97% compared to the EM (0) treatment. These findings highlighted the superiority of the EM (I+F) treatment relative to the exclusive application of either the EM (I) or EM (F) treatments. However, EM (F) by itself was superior to the control (Figure 3).

Positive results with the application EM in the cultivation of the bean was achieved by Calero *et al.* (2017), where there was an increase of 22% of the PP concerning the absence of microorganism. Furthermore, Calero *et al.* (2016) increase the average of pods per plant by 30% with the soil inoculation and foliar applications of EM with the same bio-preparation. Similar results were obtained by Abdel-Fattah *et al.* (2016), who reported significant increments in the number of legumes per plant with the inoculation of seeds.

The number of seeds per pod was significantly different (P<0.05) in the individual effects and its interaction, being greater the production in the cv. CC-25-9-N than cv. VL. All the form of application of EM were significant (P<0.05) and superior in the production of seeds per pod concerning the absence of application (Figure 4). It existed optimum temperature, and good humidity for the development of the vegetables, and the treatment with the combined application of EM stimulated the production of this component, increasing the yield.

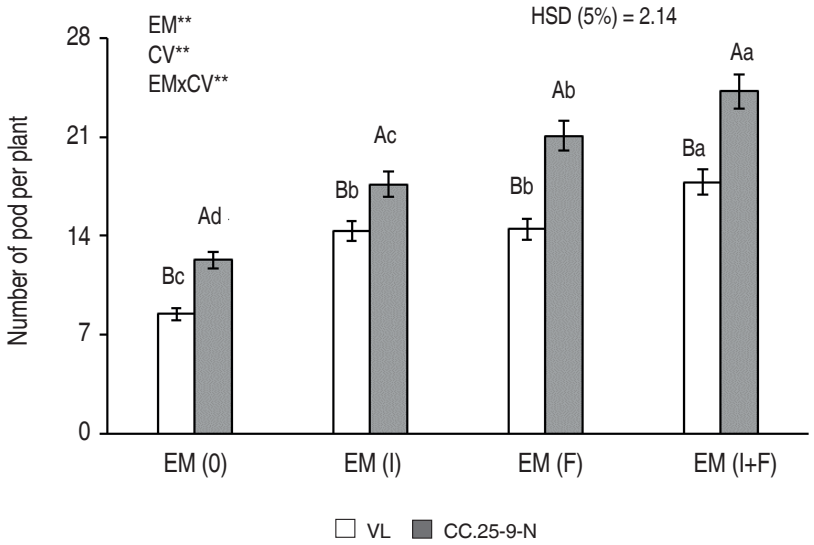

Figure 3. Number of pods per plant of two bean cultivars (VL and CC-25-9-N), in function of EM treatments, without EM (0), soil inoculation EM (I), foliar applications EM (F) and the combined soil inoculation and foliar applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM ($P<0.05$). **$P< 0.01$; EM × CV, efficient microorganism–cultivar interaction; HSD, honest significant difference.

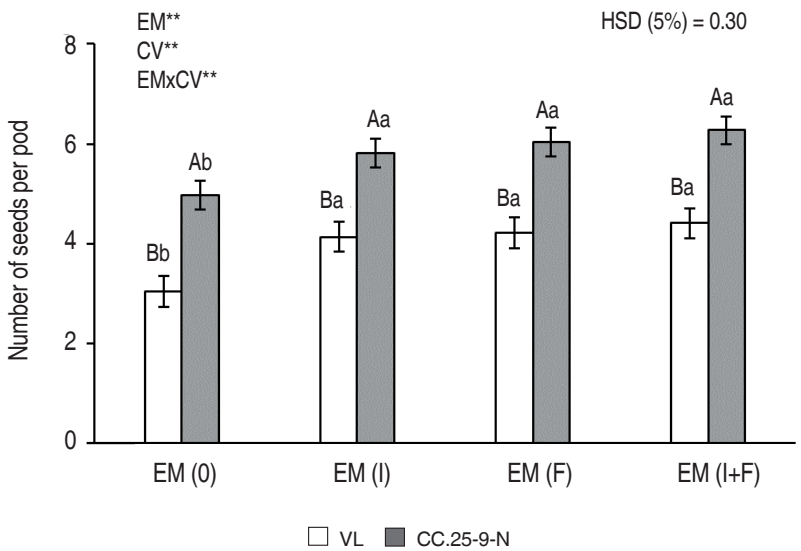

Figure 4. Number of seeds per pod of two bean cultivars (VL and CC-25-9-N), in function of EM treatments; without EM (0), soil inoculation EM (I), foliar applications EM (F) and the combined soil inoculation and foliar root applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM ($P<0.05$). **$P< 0.01$; EM × CV, efficient microorganism–cultivar interaction; HSD, honest significant difference.

Singh *et al.* (2011) indicate that the co-inoculation of bacteria and fungi with organic amendments could be an eloquent approach for sustainable management of soil fertility and crop production because they promote nitrogen fixation, the acquisition of main nutrients, and the development of branches and roots, and the improvement

of the crops' yield and quality. It is demonstrated by the increments of the morphological indicators of the beans with the individual application of EM (Calero *et al.*, 2017). }

The mass of 100 seeds was significantly different (P<0.05) in the factors and its interaction. The cv. VL was superior compared with the cv. CC-25-9-N (Figure 5). The greatest value averages were reached with the application associated with EM (I+F), concerning the individual form of EM (I) and EM (F); there were increments of 14.11 g in the cv. VL and of 8.86 g in the CC-25-9-N compared with no application. It was demonstrated that the mass of 100 grains is closely related with the agricultural yield; in this sense, Ponce *et al.* (2002) indicated that this parameter contributes to defining crop norms, and it shows the number of seeds and the number of plants possible to achieve depending on the mass.

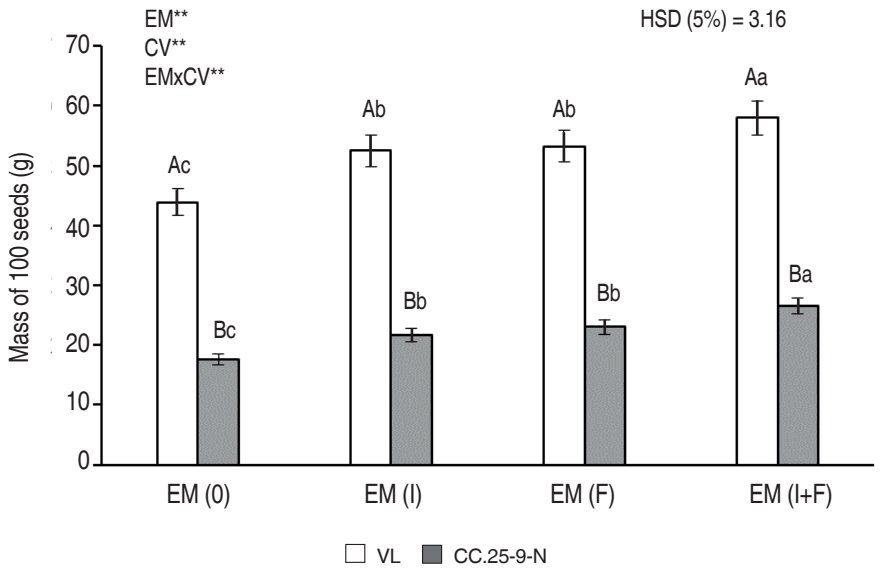

Figure 5. Mass of 100 seeds of two bean cultivars (VL and CC-25-9-N), in function of EM treatments, without EM (0), soil inoculation EM (I), foliar applications EM (F) and the combined soil inoculation and foliar root applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM (P<0.05). **P< 0.01; EM × CV, efficient microorganism–cultivar interaction; HSD, honest significant difference.

This variable is decisive for the yield because it characterizes and clarifies the production of grains. According to the CIAT (1987), the cv. VL yields big grains because the mass average of 100 grains was superior to 40 g, while the cv. CC-25-9-N presented small grains because the mass of 100 seeds was inferior to 25 g. In this sense, Calero *et al.* (2017) obtained with the application of foliate singular EM a mass average of 100 seeds superior to the control. On the other hand, Calero *et al.* (2016) evaluated the application of several foliate bio-preparations of EM with the addition of biostimulants, reaching increments in a mass of 100 grains concerning the control.

Different microorganisms can generate growth regulators that help plants to increase the growth of their upperparts. The inoculation of the soil and the seeds with bacterial promoters of vegetable growth can be a competent instrument for the management of cultivation systems (Benedetto *et al.*, 2017).

The different forms of EM application increased the yield of the cv. CC-25-9-N regarding cv. VL (Figure 6), with significant increments (P<0.05) for the factors EM and CV, as well as their interaction. The best results were reached with the application of associated EM (I+F) in both cultivars concerning the individual forms of EM (I) and (F). The mixture increases the yield up to 1.13 t ha^{-1} in the cv. VL and 2.15 t ha^{-1} in CC-25-9-N concerning the control, reaching increments of 145% in the cv. VL and 239% in the cv. CC-25-9-N.

Productivity is an important aspect to validate the investigation. The use of Plant Growth Promotors Microorganism (PGPM) helps to increase the yields of

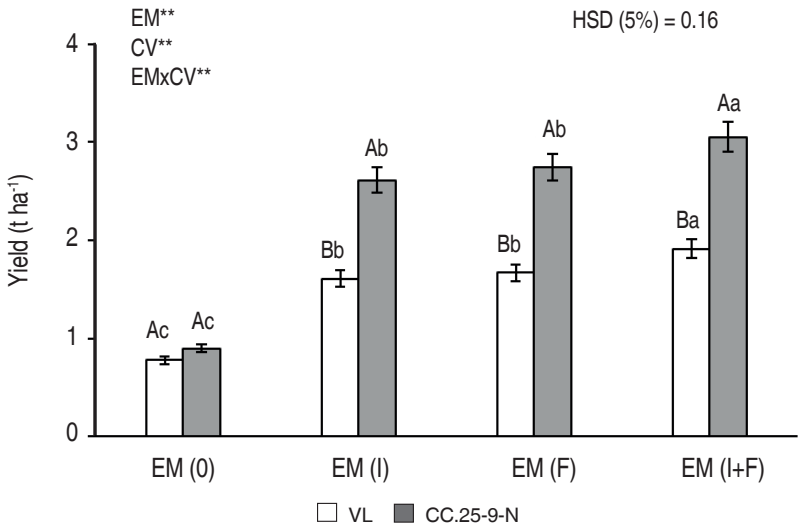

Figure 6. Yield average of two bean cultivars (VL and CC-25-9-N), in function of EM treatments, without EM (0), soil inoculation EM (I), foliar applications EM (F) and the combined soil inoculation and foliar root applications EM (I+F).
Different small letters indicate significant differences between EM treatments at the same cultivar; different capital letters indicate significant differences between cultivars at the same level of EM ($P<0.05$). ** $P< 0.01$; EM × CV, efficient microorganism–cultivar interaction; HSD, honest significant difference.

crops (Zahedi *et al*., 2016; Rashid *et al*., 2016). Similar effects in the increment of the yield were achieved with the application of efficient microorganisms compared with the control (Calero *et al*., 2017). In this respect, the application of foliate EM blended with bio-stimulants increased the yield to 78.90% regarding the yields obtained without EM application (Calero *et al*., 2016).

CONCLUSION

The different forms of application of efficient microorganisms stimulated the agronomic indicators evaluated in both cultivars. The associate application between the inoculation of the soil and foliate applications of efficient microorganisms providing the best results, producing increments in the yield of 1.13 t ha^{-1} in the cv. Velazco Largo and 2.15 t ha^{-1} in the Cuba Cueto. It was found that the beneficial effects of EM application on improved bean productivity were amplified with the soil inoculation and foliar application of EM. This study indicates that further research on the methods of EM supply should be extended to other plant species.

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Improvement of the agricultural productivity of lettuce and radish by using efficient microorganisms

Mejoramiento de la productividad agrícola de la lechuga y el rábano con el uso de microorganismos eficientes

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Ramón Liriano González[1]*, Jovana Pérez Ramos[1], Yunel Pérez Hernández[1], Iraní Placeres Espinosa[1], Sergio Luis Rodríguez Jiménez[1] and Daniela Peña Alonso[1]

ABSTRACT

The aim of this work was to evaluate the effect of the application of efficient microorganisms (EM) on the productive performance of lettuce and radish. The experiment was carried out in an organoponic culture in the municipality of Matanzas, Cuba, from December 2017 to January 2018. Five treatments were studied: control (0), EM 8 mL m^{-2} at 0 days after the transplant (DAT) (EM 8-0), EM 8 mL m^{-2} at 15 DAT (EM 8-15), EM 10 mL m^{-2} at 0 DAT (EM 10-0), and EM 10 mL m^{-2} at 15 DAT (EM 10-15). A randomized complete block design was set with four replications per treatment. An ANOVA was applied to perform the statistical data analysis, and the Duncan's Multiple Range Test (P<0.05) was used for the comparison of means. The statistic program used was STATISTICA, version 6.0 over Windows. The parameters evaluated in lettuce were the total number of leaves, number of commercial leaves, the diameter of leaf rosette, and yield. The evaluated parameters for radish were fleshy root's diameter and weight, and yield. The results indicated a positive effect on growth-response with the application of the bio-product, which can be considered a promissory alternative for vegetable production in organoponic garden conditions. The application of EM 10 mL m^{-2} at 0 and 15 DAT showed the best productive behavior for both crops.

Keywords:
Biofertilizer
Lactuca sativa
Organoponic garden
Raphanus sativus

RESUMEN

El objetivo de este trabajo fue evaluar el efecto de la aplicación de microorganismos eficientes (EM) en el rendimiento productivo de cultivos de lechuga y rábano. El experimento se llevó a cabo en un cultivo organopónico en el municipio de Matanzas, Cuba, desde diciembre de 2017 hasta enero de 2018. Se estudiaron cinco tratamientos: control (0), EM 8 mL m^{-2} a los 0 días después del trasplante (DAT) (EM 8-0), EM 8 mL m^{-2} a 15 DAT (EM 8-15), EM 10 mL m^{-2} a 0 DAT (EM 10-0) y EM 10 mL m^{-2} a 15 DAT (EM 10-15). Se estableció un diseño de bloques completos al azar con cuatro repeticiones por tratamiento. Se aplicó un ANOVA para realizar el análisis estadístico de datos, y se utilizó la prueba de rango múltiple de Duncan (P<0.05) para comparar las medias. El programa estadístico utilizado fue STATISTICA, versión 6.0 en Windows. Los parámetros evaluados en lechuga fueron el número total de hojas, el número de hojas comerciales, el diámetro de la roseta y el rendimiento. Los parámetros evaluados para el rábano fueron el diámetro y peso de la raíz carnosa y el rendimiento. Los resultados indicaron un efecto positivo en la respuesta de crecimiento con la aplicación del bio-producto, que puede considerarse una alternativa promisoria para la producción de vegetales en condiciones organopónicas de jardín. La aplicación de EM 10 mL m^{-2} a 0 y 15 DAT mostró el mejor comportamiento productivo para ambos cultivos.

Palabras clave:
Biofertilizante
Lactuca sativa
Jardín organopónico
Raphanus sativus

[1] Universidad de Matanzas. Autopista a Varadero, km 31/2. Matanzas, Cuba.
* Corresponding author: <ramon.liriano@umcc.cu>

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The use of agrochemicals has grown considerably since the "Green Revolution," which has caused a reduction in the productivity of soils due to several degrading processes, such as soil compaction, salinization, desertification, and contamination with heavy metal. It provokes severe environmental problems such as eutrophication of water bodies and diseases in humans and animals (Ge *et al*., 2016; Katiyar *et al*., 2016). Therefore, the excessive production and application of non-organic synthetic fertilizers cannot be the solution to satisfy the growing need for food to supply the world population.

Several researchers have been focused on friendly, sustainable, and organic agricultural practices; which allow to reducing the costs of production, contributing to the sustainability of agricultural systems and maintaining stable the yield and crop quality (Mesa, 2016). In Cuba, at the end of the 80s, the vegetable production began to develop on a large scale in urban areas, which grew every year to reach 4,200 t in 1994, 480,000 t in 1998 and more than 1,158,452 t in 2015, without considering the productions in gardens and plots, mainly for family consumption (GNAUS, 2015).

This intensive method for vegetable production on an organic substrate has favored obtaining high yields of crops, but at the same time, it requires adequate technological discipline, where the exploitation and management of the substrates result in a vital issue. It is because plants constantly take nutrients from the soil, being removed after harvest; therefore, the productivity of the agricultural systems depend on the different initial nutrient organic resources to guarantee high yields and multiple harvests, at least for two years. At longer periods, nutrients become limited and in consequence, yield and quality decrease.

In this context, organic fertilization should be complemented with biological products based on beneficial microorganisms (the so-called plant probiotics), which stimulate growth and development of plants throughout different mechanisms such as: the synthesis of bioactive compounds like auxin, cytokinin and gibberellin (Khatab *et al*., 2015; Damam *et al*., 2016); the solubilization of phosphates (Basu *et al*., 2017; Ramírez-Gil, 2019), the production of siderophores (Stamenković *et al*., 2018), the production of lytic enzymes and antibiotics, which play an important role in the organic matter decomposition and bio-control of phytopathogens (Sabaté *et al*., 2018; Thakur *et al*. 2017).

Those biofertilizers have been pointed out as a viable alternative for achieving an agriculture development with ecological sustainability, based on low costs of production and a minimal impact on the environment (Menendez and Garcia-Fraile, 2017). For this reason, more investigations need to be done in order to study the effect of natural products on crops' growth. The aim of the present work was to evaluate the effect of the application of efficient microorganisms on the productive response of lettuce (*Lactuca sativa* L.) and radish (*Raphanus sativus* L.) at organoponic conditions.

MATERIAL AND METHODS

Study area and EM applied

The experiment was carried out in an organoponic system at the municipality of Matanzas, Cuba, from December 2017 to January 2018. Lettuce (*Lactuca sativa* L. variety Riza-15) and radish (*Raphanus sativus* L. variety PS-9) were used in association. In the case of lettuce, the transplanting was used as sowing method, with six rows on the plot at a distance of 15 cm between rows and 15 cm between each plant. Direct sowing method was used for radish, which was planted in one row in the center of the bed with 3 cm between each plant. The average temperature during the experiment (dry season) was 21.7 °C, the relative humidity was 80.9%, and the average rain precipitation was 7.6 mm.

The efficient microorganisms (bio-product) was obtained from the bio-pesticide production laboratory "LABIOFAM" at the province of Matanzas. The microbiological composition analysis of the EM bio-product used was conducted at the Laboratory of Microbiology of the Faculty of Agronomy Sciences of Universidad de Matanzas, Cuba. The isolation of the microbial groups (bacteria, fungi, and yeast) from EM bio-preparation was carried out using 1 mL of the sample following the serial dilutions methodology (Stanier, 1996). Dilutions up to 10^{-6} were prepared in a sterile saline solution (0.9% of NaCl). 0.5 mL of the dilutions was inoculated on Nutrient Agar Medium for bacteria (10^{-6}), Saborout Agar Medium for fungi (10^{-5}) and Potato Dextrose Agar Medium for yeast (10^{-4}). Petri dishes for bacteria were incubated at 37 °C for 24 h, whereas fungi and yeast were grown at 30 °C for 72 h. Table 1 shows the concentration of the main microbial groups found in the initial EM preparation.

Tabla 1. Microbiological composition of EM bio-product.

Microbial groups	Concentration (CFU mL^{-1})
Bacteria	13×10^8
Fungi	18×10^5
Yeast	21×10^6

Experimental design

The experiment was conducted in a randomized complete block design with four replications; each one consists of an organoponic bed of 30.0 m length and 1.20 m width. With five treatments, the experimental units were of 6.0 m^2. The substrate consisted of 50% soil and 50% organic matter.

The treatments studied were:
0 = Control (without EM application).
EM 8-0 = EM (8 mL m^{-2}) at 0 days of transplanting.
EM 8-15 = EM (10 mL m^{-2}) at 0 days of transplanting.
EM 10-0 = EM (8 mL m^{-2}) at 0 and 15 days of transplanting.
EM 10-15 = EM (10 mL m^{-2}) at 0 and 15 days of transplanting.

The application of the EM bio-product was carried out early in the morning (7:00-7:30 am) using manual fumigation equipment (MATABI) of 16 liters of capacity. The application on radish was subordinated to the main crop. Insects, diseases, and weeds were intensively controlled, according to GNAUS (2007). At harvesting (28 days after seed germination in radish and 45 days after transplantation in the case of lettuce), 25 plants were randomly hand-taken in each experimental plot, and it was recorded the following morphometric and yield parameters in each crop:

Lettuce: Number of leaves per plant (by direct counting), number of commercial leaves per plant, rejecting those not suitable for commercialization; leaf rosette diameter using a measuring tape (morphometric parameters) and the yield (kg m^{-2}). Plants were weighted on with scale (Sartorius, ALC-110.4).

Radish: Fleshy root diameter (using a Vernier caliper), the weight of fleshy root and the yield, being the latter represented as kilogram per linear meter (kg m^{-1}) because of its sowing location.

Statistical analysis

The data were statistically evaluated by the Kolmogorov-Smirnov's and Bartlett's Test to verify normality and variance homogeneity, respectively. An Analysis of Variance (ANOVA) and Duncan's Multiple Ranges Test (P<0.05) (Duncan, 1955) were used for the comparison of means. STATISTICA program version 6.0 was utilized to process the experimental data.

RESULTS AND DISCUSSION

Effect the EM in lettuce

The application of EM increased the number of total leaves and the number of commercial leaves of lettuce (Table 2). Regarding the total leaves number, all the treatments with the application of efficient microorganisms displayed higher values compared with the control. Similarly, the number of commercial leaves in 0 (control) was lower than the variants with different dose of EM and times of application.

Table 2. Effect of the application of efficient microorganisms on the number of total and commercial leaves of lettuce variety Riza-15.

Treatments	Total leaves per plant	Commercial leaves per plant
0	13.29 b	12.31 b
EM 8-0	17.31 a	16.23 a
EM 8-15	17.59 a	16.59 a
EM 10-0	17.72 a	16.74 a
EM 10-15	18.05 a	17.16 a
± SE x	0.015	0.018

Different letters mean differences among treatments for each parameter (P<0.05) (Duncan, 1955). ± SE x: Standard Error of the Mean.

The higher production of commercial leaves in lettuce after EM inoculation should be positively influenced by growth parameters of the crop, which in turn determined the achieved yield. The beneficial effect of EM on the development of plant morphometric and productivity characters was also reported by several authors in different plant species such as *Spinacia oleracea* L. (Hauka *et al*., 2016), *Phaseolus vulgaris* L. (Estrada *et al*., 2017), *Oryza sativa* L. (Ghaffari *et al*., 2018) and *Rubus glaucus* Benth. cv. Thornless (Robledo-Buriticá *et al*., 2018).

The EM application increased the diameter of leaf rosette and yield (Table 3). The treatment with EM inoculation of 10 mL m^{-2} at 0 and 15 days after transplanting recorded the maximum diameter among the different variants of EM applications, which values ranged between 29.33 up to 36.56 cm. The diameter of leaf rosette in plants control was lower in comparison with all the assayed treatments.

Regarding the yield parameter in the lettuce, the treatments with the higher dose of EM (10 mL m^{-2}) and the variant EM 8 mL m^{-2} at both 0 and 15 days of transplanting promoted higher yield concerning the control; whereas the variant EM 8 mL m^{-2} at 0 days of transplanting (2.29 kg m^{-2}) showed no statistical difference with the control (1.85 kg m^{-2}).

Table 3. Effect of the application of efficient microorganisms on the diameter of leaf rosette and yield of lettuce variety Riza-15.

Treatments	Diameter of leaf rosette (cm)	Yield (kg m^{-2})
0	29.33 c	1.85 b
EM 8-0	33.08 b	2.29 ab
EM 8-15	33.43 b	2.58 a
EM 10-0	34.11 b	2.61 a
EM 10-15	36.56 a	2.67 a
± SE x	0.58	0.31

Different letters mean differences among treatments for each parameter (P<0.05) (Duncan, 1955). ± SE x: Standard Error of the Mean.

The positive response observed on lettuce yield might be related with the increment of the microbiota diversity in the substrate after the application of beneficial microorganisms, which in turn could improve several physiological processes such as photosynthetic activity, growth and productivity of the crops (Pedarza *et al*., 2010). They pointed out that efficient microorganisms consist of beneficial microorganism mixture (mainly acid lactic-producer and photosynthetic bacteria, yeast, actinomycetes, and fermenting fungi), which can be applied as an inoculant to increase the microbial diversity of soils, followed by an improvement of their quality and health. This EM application allows to enhancing growth, quality, and yield of the crops.

Several researchers have reported good results in plant growth, biochemical parameters, and crop productivity by using efficient microorganisms. Arismendi (2010) observed an enhancement of the weight in lettuce variety Great Lakes 659 after the inoculation with EM. Similarly, the application of fermented organic matter, along with commercial EM on the lettuce var. Iceberg, improved the plant height, weight, and diameter of the rosette (Pomboza-Tamaquiza *et al*., 2016). Szczech *et al*. (2016) found a better behavior of germination, fresh mass of transplants, and nitrogen content in lettuce after the application of a combination of beneficial bacterial strains.

Effect the EM on radish

The effect of the inoculation of efficient microorganisms on the diameter, weight, and yield of radish fleshy root are shown in Table 4. The application of EM revealed significant root growth-promoting effects, increasing productivity. Regarding the diameter of root and yield, all the treatments with EM showed higher results than the control without statistical difference among them. Regarding the root weight, EM 10-0 (3.89 g) and EM 10-15 (3.95 g) showed the best results in comparison with EM 8-0 and EM 8-15. The control registered the

minimum yield with an average of 0.51 kg m^{-1}. The obtained yield with the application of EM was higher than the reported by the National Group of Urban Agricultural in 2007 ranged between 0.5 to 0.8 kg m^{-1}.

Table 4. Effect of the application of efficient microorganisms on diameter, fresh weight and yield of feshy root of radish variety PS-9.

Treatments	Diameter of the root (cm)	Weight of root (g)	Yield (kg m^{-1})
0	2.41 b	2.01 c	0.51 b
EM 8-0	3.24 a	2.38 b	1.16 a
EM 8-15	3.29 a	2.41 b	1.31 a
EM 10-0	3.64 a	3.89 a	1.40 a
EM 10-15	3.68 a	3.95 a	1.49 a
± SE x	0.029	0.017	0.024

Different letters mean differences among treatments for each parameter (P<0.05) (Duncan, 1955). ± SE x: Standard Error of the Mean.

The results observed in the present investigation are in agreement with those reported by Mali *et al*. (2018), who recorded an increment in root weight and yield of radish cv. Japanese white, when applied a combination of organic manure and biofertilizer. Similar trials with *Raphanus sativus* L. reported better results on leaf area, root length, fresh and dry weight, and yield; after supplying vermicompost 12.5 t ha^{-1} and a microbial consortium (Pathak *et al*., 2017). In addition, the positive effect of native microorganisms on vegetable yield have also been reported by Núñez *et al.* (2017) in carrot (*Daucus carota* L.) under organoponic conditions. Those authors observed a good response in yield and its attributes with the inoculation of a biofertilizer, mainly with a dose of 10 mL m^{-2} which produced an increment of 0.72 kg m^{-2}.

These findings could be explained due to the effect of the efficient microorganisms on plant growth in different ways, for example throughout the production of phytohormones such as auxin and cytokine-like compounds (Berger *et al*., 2015; Nghia *et al*., 2017), the solubilization of minerals such as phosphate and nitrogen (Changas-Junior *et al*., 2015) and indirectly by the production of substances with antibiotic activity which reduce the number of phytopathogen microorganisms (Grosu *et al*., 2015). Moreover, when EM interact with the organic matter, other beneficial compounds are also released, such as vitamins, organic acids, minerals, and antioxidants (IICA, 2013).

The inoculation of the substrates with efficiency microorganisms may have enhanced the soil microbiological status and the rate of decomposition of the organic matter as well, which in turn could improve the physicochemical and biological properties of soil and the content of humus (Navia-Cuetia *et al*., 2013; Campo-Martínez *et al.* 2014). These processes release minerals available for plant nutrition, which may stimulate the cellular metabolism, photosynthesis, growth, and development of crops (Kumar *et al*., 2016; Mani and Anburani, 2018).

CONCLUSIONS

The application of EM in lettuce-radish association under organoponic conditions improved morphometric parameters and yield. The dose of 10 mL m^{-2} at 0 and 15 days of transplanting, showed the best productive performance in both crops. The results obtained indicate the potential of this technology to increase the yield and quality of lettuce and radish.

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Short article

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Revista Facultad Nacional de Agronomía

In vitro study on the nematicidal effect of different plant extracts on *Pratylenchus penetrans* and *Meloidogyne chitwoodi*

Estudio *in vitro* sobre el efecto nematicida de diferentes extractos de plantas en *Pratylenchus penetrans* y *Meloidogyne chitwoodi*

doi: 10.15446/rfnam.v72n3.76070

Aníbal Franco Cóndor Golec[1]*

ABSTRACT

The purpose of this *in vitro* study was to evaluate the nematicidal effect of different glucosinolates and other secondary metabolites extracted from several plant species on the plant-parasitic nematodes *Pratylenchus penetrans* and *Meloidogyne chitwoodi*. Glucosinolate extracts from 16 species of genera *Brassica*, seven *Lolium* species and one species of *Lupinus* were used to investigate their nematicidal effect *in vitro*. From the tested extracts, the one obtained from *Brassica juncea* (oriental) showed the most promising results, controlling both nematode species. *Lupinus* sp. also showed positive results when tested against *P. penetrans*.

Keywords:
Brassica spp.
Glucosinolates
Lolium spp.
Lupinus sp.

RESUMEN

El propósito de este estudio *in vitro* fue evaluar el efecto nematicida de diferentes glucosinolatos y otros metabolitos secundarios extraídos de varias especies de plantas, sobre los nemátodos *Pratylenchus penetrans y Meloidogyne chitwoodi* que afectan negativamente diversas plantas. Extractos de glucosinolatos provenientes de 16 especies del género *Brassica,* siete especies de *Lolium* y una especie de *Lupinus* fueron usados para investigar su efecto nematicida *in vitro*. De los extractos probados, el que proviene de *Brassica juncea* (oriental) mostró los resultados más promisorios para el control de las dos especies de nemátodos en estudio. *Lupinus* sp. también mostró resultados positivos para el control de *P. penetrans*.

Palabras clave:
Brassica spp.
Glucosinolatos
Lolium spp.
Lupinus sp.

[1] Independent Research. Plant Science. 100-4155 Sheppard Avenue East, Scarborough, Ontario. M1S 1T4. Canada.
* Corresponding author: <anibalfcg@yahoo.com>

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The identification of phytochemical based strategies for the control of plant pathogens is important since it can be used in sustainable production systems where there are not many possibilities to manage plant-parasitic nematodes. It can also be essential for the development of new nematicides in traditional agriculture (Chitwood, 2002). Nowadays, further research on environmentally friendly biofumigants is suggested (Devi, 2018).

Meloidogyne chitwoodi Golden, O'Bannon, Santo, & Finley is an important pathogen of potato and other crops in the western part of Europe and is also a major pest of potato in the Northwestern states of the United States (Castagnone-Sereno *et al*., 1999). *Pratylenchus penetrans* is an obligate plant parasite of a wide range of hosts, mainly in temperate climates. It is one of the principal nematodes infesting ornamental plants and causes serious losses in different crops (Peng and Moens, 2002).

Plant compounds may act as a repellent, attractant, hatching stimulants or inhibitors and nematotoxicants. They can be used as fumigants or introduced in crop rotation programs for nematode control (Chitwood, 2002).

Glucosinolates are a group of allelochemicals that occur in all plants of the order Brassicales or Capparales (Cronquist, 1981), being the Brassicaceae family the most numerous and important group (Fahley *et al*., 2001). Glucosinolate (GLSs) have sulfur, and that explains its strong flavor (Avato *et al*., 2013). More than 130 glucosinolates have been identified (Fahley *et al*., 2001; Kirkegaard *et al*., 1999) and may be divided into three subclasses comprising aliphatic, phenyl, and indol-3-ylmethyl glucosinolates (Buskov *et al*., 2002). Isothiocyanates (ITC) and other plant compounds such as nitriles and thiocyanates (Buskov *et al*., 2002) are released from Brassicaceae when glucosinolates are hydrolyzed by the action of the enzyme myrosinase (Figure 1) (Kirkegaard *et al*., 1999). These hydrolysis products have shown various bioactive effects against some soilborne diseases and nematodes (Rosa *et al*., 1997).

The glucosinolate content average is higher on field-grown *B. juncea*, *B. napus*, *B. campestris*, and *Eruca sativa*, compared to the greenhouse and high tunnel cultivation (Antonious *et al*., 2009).

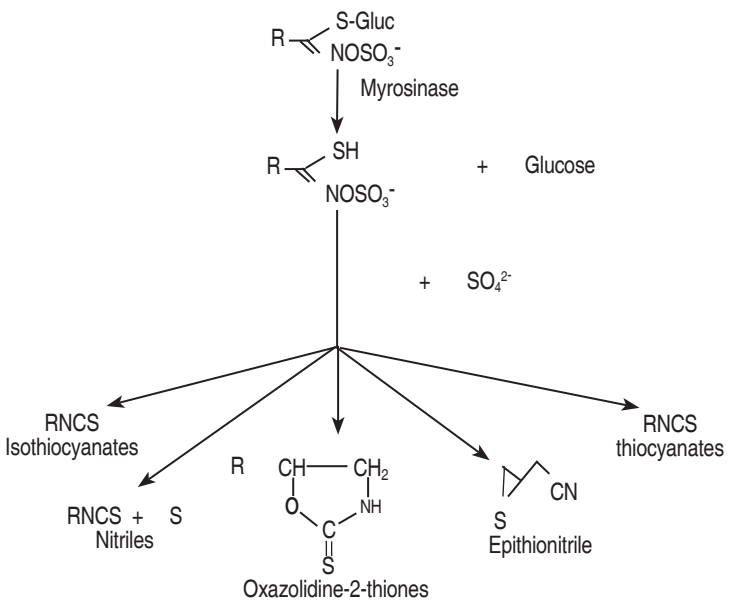

Figure 1. Enzymatic hydrolysis of glucosinolates via myrosinase activity (Kirkegaard *et al*.,1999).

Brassica species have shown a significant reduction in nematode numbers of *P. neglectus* (Potter *et al*., 1998) and *P. penetrans* (McFadden *et al*., 1992) when used as green manures. The same has been observed for *M. chitwoodi* (Mojtahedi *et al*., 1993). Other nematode species have shown mortality up to 100% when low doses of glucosinolates from Brassicaceaes have been applied, *in vitro*; such as in the case of *Globodera rostochiensis* (Serra *et al.,* 2002), *G. pallida* (Lord *et al*., 2011) and *M. incognita* (Oliveira *et al*., 2010). There is also a biocidal property with the use of GLSs on *Xiphinema index* and *Heterodera carotae* (Avato *et al*., 2013). Width control spectrum has been found by using these species (Björkman *et al*., 2011). Other plant compounds as the ones released from *Lupinus* sp. and several kinds of grass have proved to have a nematicidal effect; mulching with *Pennisetum purpureum* has been used for the control of *M. javanica* (Matsumoto *et al*., 2002) and the application of Sudan's grass extracts has reduced *M. hapla* juveniles that penetrate lettuce roots (Wildmer and Abawi, 2007). Anastasiadis and Karanastasi (2011) also presented the effectivity of brassica and ryegrass soil amendments on *M. incognit*a and *javanica*. The Fabaceae *Medicago sativa* has been found a good control against *G. rostochiensis* (D'Addabbo *et al*., 2011).

Considering the potential of these species as possible biocides, the purpose of this trial was to determine the nematicidal effects of plant extracts on plant-parasitic nematodes.

MATERIALS AND METHODS

Extracts

Brassicaceous accessions from a local field were tested: *B. fruticulosa* subsp*. mauritanica*, *B. fruticulosa*, *B. tournefortii*, *B. tournefortii*, *Sinapis arvensis* subsp. *arvensis*, *B. carinata*, *Raphanus sativus*, *R. sativus*, *B. juncea*, *B. juncea* (oriental), *B. tournefortii*, *B. oxyrrhina*, *B. napus* subsp. *oleifera*, *Sinapis alba*, *Crambe abbyssinica* and *Crambe hispanica* (identified as extract: 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 13, 15, 17, 18, 20 and 21, respectively, showed in Figure 2). The extracts of Brassicaceae and one extract of *Lupinus* sp. were obtained with a blend of 3 g of fresh shoot material in 6 mL of phosphate buffer (25 mM, pH 7, with 15 mg L^{-1} streptomycin), later sieved, centrifuged and used directly to make dilutions. Seven grasses, belonging to *Lolium* spp., were collected from dried meadows, and these samples were roasted for 3 hours from 160 to 260 °C before extracting the active components. Grasses were identified by letters (A, B, C, D, E, F, and G), as shown in Table 2. Grass A was not heat-treated while G was the most exposed to high temperatures. Grass extracts were made by blending 10 g of dried grass for one hour in 40 mL of extraction buffer.

Dilutions

Extract dilutions were made to test the dose-response of nematode migration activity. The dilutions show doses that could be obtained by practical quantities of crop residue on field. 6 mL from each extract of the stock-solution was used. This volume was diluted to 1/3, where 6 mL was used to make the dilutions 1/4, 1/16, and 1/64. The final dilutions were prepared with the same phosphate buffer (pH 7) as the original extracts. These dilutions were used for the filter plate experiments.

Filter plate experiments

The filter plate experiment was used to test the dose-response of nematode migration activity. There were two plates placed one on top of each other. The upper plate was a 96-wells high filter plate with a small tube underneath. The lower plate was a standard 96-wells plate. The upper plates were filled with cigarette filters. It was pipetted 700 µL of the plant extract solution on these filters, then 200 µL of the nematode suspension (200 *P. penetrans* and 100 J2 *M. chitwoodi* nematodes). This proportion was selected because *Meloidogyne* migrates faster, so there would be more nematodes per well. The plates were covered and stored at 20 °C for 24 hours. After 24 hours, the upper plate was transferred to a new lower plate, and 200 µL of buffer was added. The whole set was place at 20 °C for 24 hours again, so the rest of the nematodes could migrate. The plates were counted again after 48 hours. Four replications per dilution were scored, and four wells with 900 µL of buffer per plate were used as a control. For each well, the number of nematodes was scored. Juveniles and adults *P. penetrans* were counted separately.

Nematode counting for each dilution was compared and statistically analyzed with STATISTIX for Windows. All data were *log(x+1)* transformed for an appropriate analysis. Tukey tests were used to compare treatments with significant differences (P<0.05).

RESULTS AND DISCUSSION

All the Brassicaseous and grass extracts showed nematicidal or repellent effect at a high concentration (1/4 dilution), whereas at lower concentrations (dilutions 1/16 and 1/64) differences were not always observed (Figures 2, 3 and 4). This nematicidal effect was a function of the dilution and time of exposure.

Glucosinolates relative slight structural differences could confer deeply different nematicidal effects, confirming that

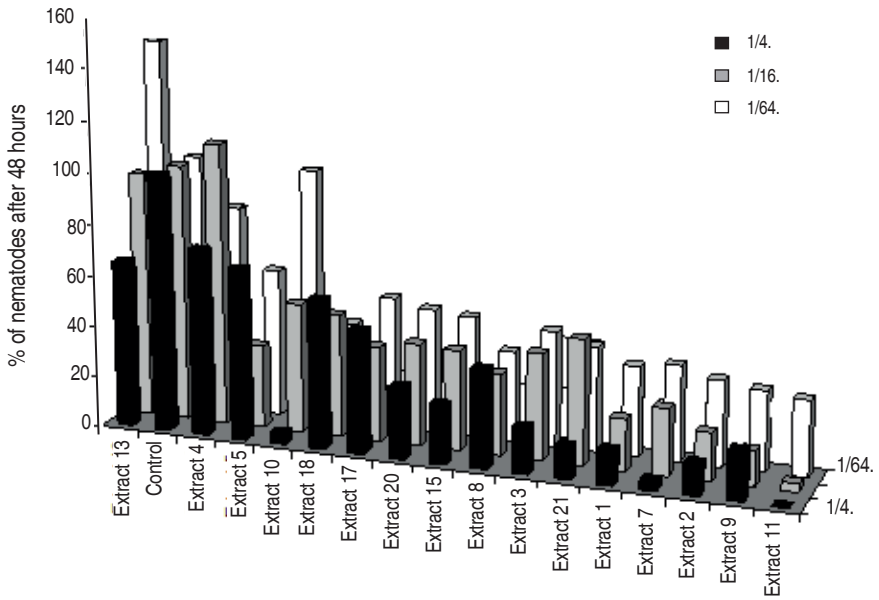

Figure 2. Percentage over the control of total *P. penetrans* found after 48 hours for different Brassicaceae extracts at different concentrations.

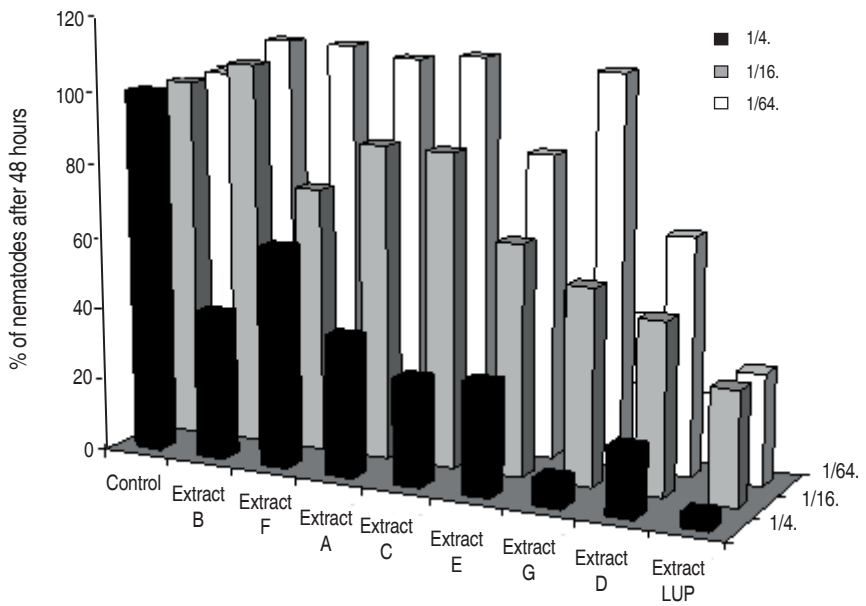

Figure 3. Percentage over the control of total *P. penetrans* found after 48 hours for the different grass extracts and *Lupinus* sp. at different concentrations.

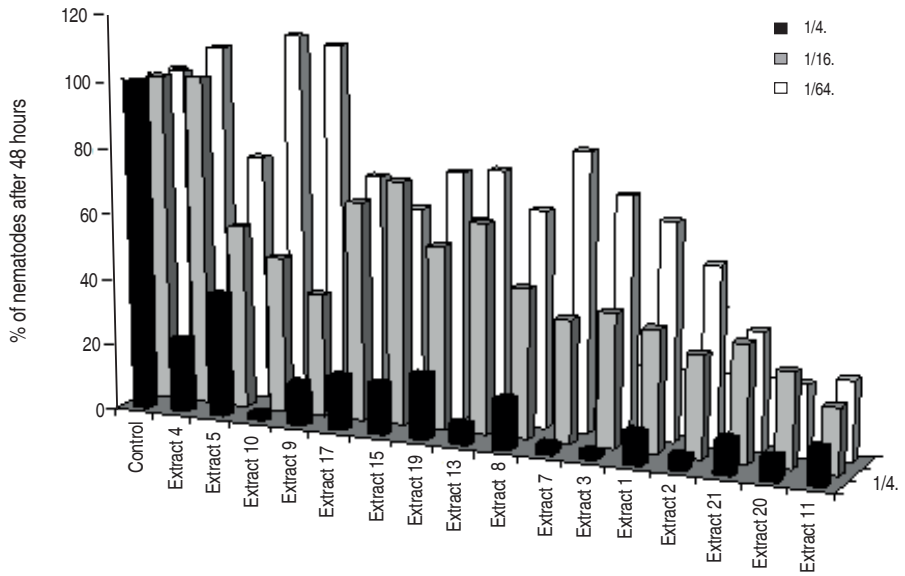

Figure 4. Percentage over the control of total *M. chitwoodi* found after 48 hours for different Brassicaceae extracts at different concentrations.

biological activity was a function of the concentration of the product and the chemical properties of the R chain (Serra *et al*., 2002; Zasada and Ferris, 2003).

Best nematicidal effects at 1/4 dilution were observed with Extracts 11, grass G and *Lupinus* for *P. penetrans* (Tables 1 and 2), and with extracts 10, 3, and 7 for *M. chitwoodi* (Table 3). At 1/16 dilution the extracts with higher effect were 11, 9, and *Lupinus* sp. for *P. penetrans* and extract 11 for *M. chitwoodi*. At the lower concentration of 1/64 most effective extracts were 2, 11, 9, and *Lupinus* for *P. penetrans*, and 20 and 11 for *M. chitwoodi*.

Table 1. Mean of total nematodes present after 48 hours for different Brassicaceae extracts at different concentrations in *P. penetrans* corrected over the control.

Extract id.	Plant extract	Dilution		
		1/4	1/16	1/64
1	*Brassica fruticulosa* subsp. *mauritanica*	12.7 cdef	19.7 de	35.0 cd
2	*B. fruticulosa*	12.1 cdef	18.5 de	30.3 d
3	*B. tournefortii*	14.8 cdef	40.5 cd	44.5 bcd
4	*B. tournefortii*	70.6 a	103.2 a	78.1 abc
5	*Sinapis arvensis* subsp. *Arvensis*	66.8 a	31.8 cd	56.7 bcd
7	*B. carinata*	3.6 fg	23.6 cde	35.4 cd
8	*Raphanus sativus*	38.4 abcd	29.8 cde	32.9 cd
9	*R. sativus*	18.3 bcde	13.5 e	27.9 d
10	*B. juncea*	4.1 efg	48.6 abc	96.9 ab
11	*B. juncea-oriental*	0.0 h	3.2 e	28.4 d
13	*B. tournefortii*	63.5 a	90.4 ab	141.7 a
15	*B. oxyrrhina*	22.7 bcde	38.6 cd	43.1 bcd
17	*B. napus* subsp. *Oleifera*	45.9 abc	36.4 cd	50.2 bcd
18	*Sinapis alba*	57.6 ab	47.1 bc	39.7 bcd
20	*Crambe abbyssinica*	276 bcde	38.1 cd	44.6 bcd
21	*Crambe hispanica*	7.7 defg	48.5 abc	40.4 bcd

Means with the same letter within dilution are not significantly different ($P>0.05$)

Table 2. Mean of total nematodes present after 48 hours for different plant grass extracts at different concentrations in *P. penetrans* corrected over the control.

Plant extract	Extract id.	Dilution		
		1/4	1/16	1/64
Lolium spp.	A	36.2 a	85.2 ab	105.9 a
	B	38.5 a	105.8 a	108.6 a
	C	27.9 a	86.1 ab	96.5 a
	D	18.3 ab	46.8 d	63.1 a
	E	30.4 a	63.6 bcd	84.1 a
	F	59.7 a	72.5 bc	110.1 a
	G	3.0 c	53.2 cd	106.4 a
Lupinus sp.	LUP	3.6 bc	30.6 e	30.6 b

Means with the same letter within dilution are not significantly different ($P>0.05$)

Table 3. Mean of total nematodes present after 48 hours for different Brassicaceae extracts at different concentrations in *M. chitwoodi* corrected over the control.

Extract id.	Plant extract	Dilution		
		1/4	1/16	1/64
1	*Brassica fruticulosa* subsp. *mauritanica*	5.1 abc	35.3 bcd	58.4 abc
2	*B. fruticulosa*	3.9 bc	27.6 cd	45.5 abcd
3	*B. tournefortii*	2.0 c	39.8 bcd	71.0 ab
4	*B. tournefortii*	21.2 ab	99.7a	107.8 a
5	*Sinapis arvensis* subsp. *arvensis*	37.1 a	54.9 abc	69.9 ab
7	*B. carinata*	2.2 c	36.5 bcd	81.5 ab
8	*Raphanus sativus*	12.8 abc	42.0 abcd	61.9 ab
9	*R. sativus*	10.1 abc	36.3 bcd	109.0 a
10	*B. juncea*	1.9 c	42.0 abcd	112.1 a
11	*B. juncea, oriental*	11.2 abc	17.7 d	23.8 cd
13	*B. tournefortii*	4.0 bc	55.6 abc	64.4 ab
15	*B. oxyrrhina*	10.5 abc	73.0 ab	59.9 ab
17	*B. napus* subsp. *oleifera*	16.0 abc	65.6 abc	70.6 ab
19	*Sinapis alba*	16.6 abc	52.1 abc	72.8 ab
20	*Crambe abbyssinica*	6.9 abc	27.0 cd	20.5 d
21	*Crambe hispanica*	10.2 abc	34.2 bcd	32.7 bcd

Means with the same letter within dilution are not significantly different ($P>0.05$)

In cases where the metabolite had a better effect when used in a lower concentration (e.g., 1/64) could be assumed that its effect did not only depend on the concentration of the plant extract, but it was also related to the optimal dilution at which the compound gave positive results for nematode management.

For *P. penetrans* control the principal compounds involved are sinigrine and gluconapin according to preliminary studies performed by the Plant Research International in Wageningen. The first one was found in high concentrations in extracts 7 and 11 (*Raphanus sativus* and *Brassica juncea* oriental, respectively), while the latter was in excess in

extracts 1 and 2 (*Brassica fructiculosa* both species) (data not shown). In the experience of Lazzeri *et al.* (1993) with a concentration of 0.05%, sinigrin and gluconapin showed nematicidal effect after 96 hours and 114 hours, respectively, for the control of *Heterodera schachtii*. *Brassica juncea* products have also shown a reduction in *Globodera pallida* (Lord *et al.*, 2011), *P. penetrans* and *M. incognita* (Zasada *et al.*, 2009). Ngala *et al.* (2014) also found nematicidal effects for the same nematode with *B. juncea* and *Raphanus sativus* deterred its multiplication.

For *M. chitwoodi* control showed a clear correlation between the type of glucosinolate so its control effectiveness could not be done easily because most of the extracts controlled the nematode effectively, at least for the lowest concentration (Table 3). However, it could be assumed that several isothiocyanates or the combination of them have detrimental effects on *M. chitwoodi* populations; therefore, its use could be highly appreciated in crops where this nematode causes economic losses. Further chemical studies of the most promissory plant species are necessary in order to determine specific compounds that have nematicidal or nematostatic effects.

CONCLUSIONS

Glucosinolates present in *Brassica juncea* and *Lupinus* sp. showed the most significant nematicidal effect with the lowest dilution (1/4). It seemed that the high concentration of these glucosinolates in the plant tissue is the direct responsible for the biocidal effect of these plants. Given the high effectiveness of most of the tested *Brassica* spp. for the control of *M. chitwoodi* and *P. penetrans*.

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Revista Facultad Nacional de Agronomía

Populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) cause significant damage to genetically modified corn crops

UNIVERSIDAD NACIONAL DE COLOMBIA

Poblaciones de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) causan notables daños en cultivos de maíz genéticamente modificados

doi: 10.15446/rfnam.v72n3.75730

Camilo Ignacio Jaramillo-Barrios[1] *, Eduardo Barragán Quijano[1] and Buenaventura Monje Andrade[1]

ABSTRACT

The fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) is an important harmful pest for corn crops in Colombia. Knowing its population's fluctuation regarding genetically modified plants allows the implementation of monitoring plans and time-effective management actions. The objective of this study was to establish the population's fluctuation of *S. frugiperda* during 2014-2016 in the hybrids 30F35R and 30F35HR (genetically modified with the Cry1F endotoxin) in El Espinal, Tolima, Colombia. Accumulations in five growing cycles were carried out until 20, 40, 60, 80, and 104 days with the number of larvae per linear meter after emergence per year and per hybrid. Results were compared statistically using linear mixed models. On the other hand, two dummy variables that reckon the presence of larvae and damage were calculated. With the indicators of presence (one) and absence (zero), a longitudinal logistic prediction model was constructed. Larger accumulation of larvae was registered in the hybrid 30F35R (6.79±0.20); however, the genetically modified genotype 30F35HR also registered the presence of larvae (4.24±0.20), inferring that the endotoxin did not exercise total control over the populations. The vegetative stage showed a higher larval population. However, when this stage is not managed, the crop can show damage up to 52% and 72% in hybrid plants with and without Cry1F, respectively. This behavior suggests that if refuge areas and strategies such as pest monitoring are not established, these insects could generate higher resistances to the plants with the endotoxin Cry1F.

Keywords:
Fall armyworm
Larvae
Pest insects
Population dynamics
Transgenic

RESUMEN

El gusano cogollero (*Spodoptera frugiperda* (J.E. Smith)) es una importante plaga del maíz en Colombia. Conocer su fluctuación poblacional en plantas genéticamente modificadas permite implementar planes de monitoreo y acciones de manejo oportunas. El objetivo de este estudio fue determinar la fluctuación poblacional de *S. frugiperda* durante los años 2014 a 2016 en los híbridos 30F35R y 30F35HR (modificado genéticamente con la endotoxina Cry1F) en el Espinal, Tolima, Colombia. Con el número de larvas por metro lineal se realizaron acumulaciones en cinco períodos de cultivo hasta los 20, 40, 60, 80 y 104 días después de emergencia por año y por híbrido. Los resultados se compararon estadísticamente mediante modelos lineales mixtos. Por otro lado, se calcularon dos variables dicotómicas que miden la presencia de larvas y daño. Con los indicadores de presencia (uno) y ausencia (cero), fue construido un modelo de predicción logístico longitudinal. Se registró mayor acumulación de larvas en el híbrido 30F35R (6.79±0.20), sin embargo, el genotipo genéticamente modificado con Cry1F 30F35HR también registró la presencia de larvas (4.24±0.20), deduciendo que la endotoxina no ejerció un control total sobre las poblaciones. La etapa vegetativa presentó mayor población de larvas que, de no manejarse, se pueden presentar áreas con daño en un 52% y 72% de las plantas en el híbrido con y sin Cry1F respectivamente. Esto sugiere que, si las áreas de refugio y estrategias tal como un monitoreo de plagas no son establecidas, estos insectos podrían generar un alta resistencia a las plantas con la endotoxina Cry1F.

Palabras clave:
Gusano cogollero
Larvas
Insectos dañinos
Dinámica de poblaciones
Transgénicos

[1] Corporación Colombiana de Investigación Agropecuaria. AGROSAVIA. Centro de Investigación Nataima. km 9 vía Espinal-Chicoral, Tolima, Colombia.
* Corresponding author: <cijaramillo@agrosavia.co>

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The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is one of the most harmful pests for commercial crops in South America. This species has a polyphagous feeding behavior in around 80 plant species including cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.) and soybean (*Glycine max* L.) (Barros *et al*., 2010; Flagel *et al*., 2018). Because of its diversity in food niches, it is considered sporadic and long-distance migratory, with adult moths that can fly more than 100 km in a single night (Johnson, 1987).

Depending on the growth stage of corn, the larvae of the fall armyworm feed on young leaves, spiral leaves, cobs, husks, and spikes; causing substantial damage to crop that occasionally results in total yield loss. Larger larvae can completely damage the stem base of corn seedlings, acting as cutworms (Sarmento *et al*., 2002; Goergen *et al*., 2016).

The extent of the damage caused by this pest depends on the time of planting, the geographic region, and the variety planted, as well as inherent cultural practices in and around the crop plot (Sarmento *et al*., 2002). The abiotic factors also have a great effect on eggs and mortality of initial larval stages, especially in rainy seasons, although predators also reduce considerably the number of small larvae that normally are present during dry seasons (Varella *et al*., 2015).

In order to control the insect without using chemical pesticides, insecticidal proteins obtained from the bacteria *Bacillus thuringiensis* (Bt) and expressed by genetically modified (GM) plants have been used as important field methods since 1996 (Tabashnik *et al*., 2013). Among these, the Cry1F corn protein that was registered for the first time in 2001 in the United States to control stalk borers and some Noctuidae moths including *S. frugiperda* (Storer *et al*., 2010). Moreover, studies carried out by several authors have demonstrated that the gene Cry1F confers resistance to corn (Hardke *et al*., 2011; Chandrasena *et al*., 2018). However, currently, Bt proteins are subject to higher selection pressure, and there is a higher risk of target insects, including *S. frugiperda*, generate resistance because of the high use of Bt crops and the lack of implementation of refuge areas (Storer *et al*., 2010; Huang *et al*., 2014; Horikoshi *et al*., 2016).

As an alternative to this problem, resistance induction experiments have attributed a synergistic effect to the interaction between fertilization with calcium and magnesium silicate and transgenic crops, suggesting a viable alternative for the control of *S. frugiperda* (De Castro Lourenco *et al*., 2017). However, there is no record of the population's assessment in genetically modified corn plants in Espinal. Therefore, the current study was conducted with a first objective of establishing the population fluctuation of *S. frugiperda* larvae with and without Cry1F endotoxin. The second objective was to generate a probability prediction model of larvae and damage presence employing a longitudinal logistic regression during three cultivation cycles.

MATERIALS AND METHODS

Location and plant material

This study was carried out at Nataima Research Center (CI Nataima) of Agrosavia, located in the municipality of El Espinal, department of Tolima, Colombia (4°11'40.48" N, 74°58'04.15" W). Meteorological conditions during the study showed a daily average temperature of 27.6 °C±0.15, a daily average relative humidity of 77.78%±0.68, and daily average precipitation of 3.13±1.11 mm.

Plantings were carried out from May 8th to August 12th, 2014 (first year), from April 17th to July 22nd, 2015 (second year), and from April 12th to July 14th, 2016 (third year). The plant materials used were the corn hybrids 30F35R (with CP4 protein) and 30F35HR (with CP4 and Cry1F endotoxin). The first has not been modified genetically for Lepidoptera resistance, and the second has been genetically modified with Cry1F gene of *Bacillus thuringiensis* employed for Lepidoptera control.

An experimental design was established with two treatments (without and with transgenesis), in paired plots (blocks) with three replicates and an experimental area of 800 m^2 each one. The distance between plants was 0.15 m and 0.85 m between rows, for a total area of 2,500 m^2 per treatment including non-experimental areas.

Evaluation of population fluctuation

Evaluations were conducted 8 days after emergence (DAE) to establish the population fluctuation of the insect pest. A transect of one linear meter was taken as a sampling unit. In each replicate, ten sites were selected randomly,

where the sampling frequency was four days, ending at 104 DAE. The response variables evaluated at each site were: total number of plants, number of plants with damage, and the total number of live larvae (Piñango *et al.*, 2007). Crop management did not include insecticide application for *S. frugiperda*. The fertilization plan was applied based on soil analysis results as following: two applications during the vegetative stage and one application at the beginning of the flowering stage, adding the sources N-P-K with grades 46-0-0, 18-46-0, 0-0-60, and minor elements. Weed control was carried out with direct applications of glyphosate on both varieties.

During the crop growth, the weather station of C.I. Nataima (Ref. Watchdog series 2000), registered the average, maximum and minimum temperatures (°C), average relative humidity (%), solar radiation (W m^{-2}) and accumulated precipitation (mm).

Statistical analysis

An exploratory and descriptive analysis of the fluctuation was carried out for each variable in order to identify population peaks and descents of the highest pest presence periods. An accumulation curve was calculated throughout the entire cycle using the "insect days or cumulative damage" to compare years and hybrids in function of the number of larvae, a technique based on the area under the progression stairs of the curves (AUDPSC) (Castro *et al.*, 2005). This procedure was done in five periods, i.e., at 20, 40, 60, 80, and 104 DAE per year and per hybrid.

According to these results, comparisons were made employing mixed linear models using a randomized complete block design with the combinatorial arrangement of fixed effects (hybrid, periods, and year) and random effects (block). Assumptions were analyzed using graphic diagnostic tests (Q-Q plot and studentized residuals vs. predicted) and the Shapiro-Wilks tests for normality and Levene for homogeneity of variances. In case of non-compliance with homoscedasticity, heteroscedastic variance models were used. This selection of the correct model was made by using the Akaike (AIC) and the Bayesian (BIC) information criteria, and the maximum likelihood value (logLik). Fisher's minimum least significant difference (LSD) test at 5% was used to indicate statistical differences.

From the variables number of larvae and percentage of plants with damage (expressed as the number of affected plants over the total evaluated plants), two indicator variables were calculated, being 1 the indicator of presence and 0 of absence. In each one, a longitudinal logistic model was defined and the probabilities (πi) of larvae or damage in site *i* were obtained in order to define in which periods the pest is more susceptible to damage. The logit link function was used, and the stepwise, forward, and backward methods were used to select the best model. Moreover, the different models proposed were compared with the likelihood ratio test and the AIC.

Finally, to establish the relationship between weather and the number of larvae, Pearson's correlations were calculated between the number of total larvae and the average, maximum and minimum temperatures (°C), average relative humidity (%), solar radiation (W m^{-2}) and accumulated precipitation (mm). The statistical programs R v.3.4.1 (R Core Team, 2017) and Infostat (Di Rienzo *et al.*, 2016) with the help of the R v.3.4.1 platform for mixed general linear models were used to adjust the models described.

RESULTS AND DISCUSSION

Population fluctuation of *S. frugiperda* in corn genotypes with and without Cry1F

The population fluctuation of *S. frugiperda* showed similar behavior for the three evaluation years, finding more larvae in the vegetative stage than in the reproductive stage, with two overlapping generations between both stages (Figure 1). Both genotypes in all assessed years showed the highest peaks at the same time after emergence. In 2014 at 28 DAE there were 5.4±0.47 and 2.6±0.35 average number of larvae in the genotype without and with the Cry1F endotoxin, respectively. Furthermore, in 2015 at 40 DAE there were values of 6.0±0.53 and 2.9±0.31, respectively, and in 2016 at 36 DAE there were values of 2.9±0.45 and 2.9±0.45, respectively. A lower larval population density was observed in the genotype with the Cry1F endotoxin; however, this was not constant for all assessments.

The number of larval generations of *S. frugiperda* was similar to the ones found in the study of Valdez-Torres *et al.* (2012), who found that the fall armyworm showed two generations during corn cultivation. Larvae distribution and

eggs of *S. frugiperda* varied according to the phenological stage of corn (Beserra *et al*., 2002). The vegetative stage showed greater damage because the first stages of growth are more susceptible to pest attack where the greatest amount of damage, due to *S. frugiperda*, occurred (Willink *et al*., 1993). Furthermore, larval populations are more stable throughout this phase and decrease during the beginning of the reproductive phase of corn (Murúa *et al*., 2006).

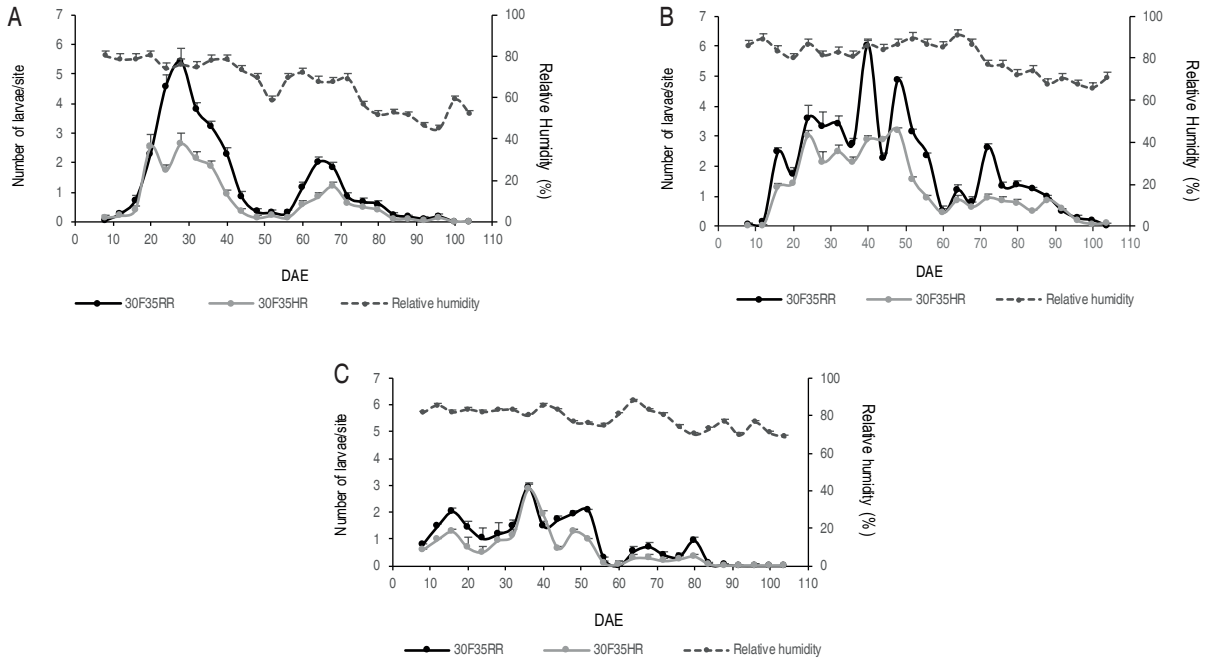

Figure 1. Population fluctuation of *S. frugiperda* in the corn hybrid 30F35R and the genetically modified hybrid 30F35HR (Cry1F Bt gene). A. Average number of larvae per site in 2014; B. Average number of larvae per site in 2015; C. Average number of larvae per site in 2016.

The populations found during the reproductive stage usually change their habit towards the reproductive structures of the plant, feeding on tassels and/or boring into the ears (Midega *et al*., 2018). It generates a positive effect on larval feeding and survival during this stage, being this choice made most probably in the first instars. Besides, corn leaves in this period are not suitable for the development of larvae in the first instars (Pannuti *et al*., 2015).

The percentage of damage expressed as the number of affected plants per linear meter over the total number of plants (Figure 2) shows that there was a gradual increase until 28 DAE in 2014. This tendency remained until 44 DAE where it descended to 60 DAE. Subsequently, damage expansion was observed, although it was not as high as the one previously seen; and a decrease in the damage was recorded at the end of the cycle. In 2015, fluctuations were observed with a linear upward trend up to 52 DAE where it decreased to 68 DAE, and then, a new increase occurred until 98 DAE, but at the end of the cycle, it declined again. Finally, for 2016, there was a continuous increase until 12 DAE, decreasing notably until 28 DAE where it increased to 40 DAE. From this point onwards, it began to fall until 60 DAE, showing a slight increase at 80 DAE, but then there was a decrease at the end of the cycle.

Concerning climatic factors, a direct linear relationship was observed for both genotypes between the number of larvae and the relative humidity in the three years assessed. In 2014, correlation coefficients of R=0.52 (*P*=0.0076) and R=0.55 (*P*=0.0043) were obtained for conventional and transgenic genotypes, respectively. In 2015, R=0.44 (*P*=0.0280) and R=0.42 (*P*=0.0348) were obtained, respectively. And in 2016, R=0.44 (*P*=0.0261) and R=0.43 (*P*=0.0335) were attained in conventional and transgenic genotypes, respectively.

Regarding the correlation coefficients obtained of larval populations with direct relative humidity found in this study, Clavijo and Notz (1978) reported a correlation coefficient

of -0.56. However, that research differs from the current study because the increase of humidity matched the upward population trend of the pest, as well as its relationship with the phenological state (Figure 4). In this regard, Murúa *et al.* (2006) noted that the fluctuation of the average number of larvae is related to the age and development of the plant.

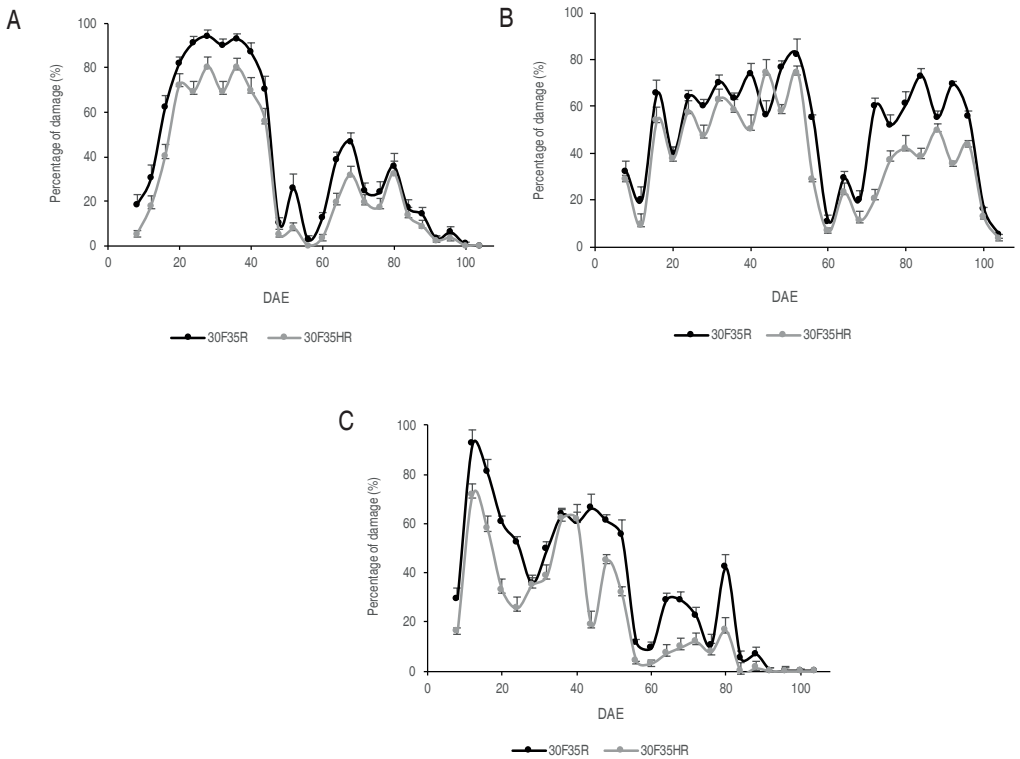

Figure 2. Percentage of *Spodoptera frugiperda* damage in corn non-genetically modified (30F35R) and modified genetically with the Cry1F Bt gene (30F35HR). A. Percentage of damage in 2014; B. Percentage of damage in 2015; C. Percentage of damage in 2016.

Comparison of larval accumulation for the periods, year and hybrid

Results showed statistically significant differences between years (F=119.66, df=2, *P*<0.0001), time periods (F=183.66, df=4, *P*<0.0001), and hybrid (F=65.08, df=1, *P*<0.0001) (Figure 3). The accumulation of larvae in the 30F35R hybrid was higher than in the 30F35HR hybrid. Moreover, the period from 21 to 40 DAE showed a higher accumulation of larvae, followed by the period from 41 to 60 DAE. Periods from 0 to 20 DAE and from 61 to 80 DAE did not show differences in accumulation of larvae, and finally, the period from 81 to 104 DAE showed the lowest accumulation of larvae compared to all assessed periods.

The differences between populations with and without the Cry1F protein gene agrees with what was reported by Hardke *et al.* (2011), who indicated that the Cry1F endotoxin showed significant reductions in leaf lesions and lower survival compared to corn tissue without Bt. Likewise, Araujo *et al.* (2012) concluded that the hybrid P 3041YG showed lower damage from *S. frugiperda*, showing a higher biomass and grain yield compared to the conventional hybrid P 3041.

Other studies using the Cry1Ab protein found that the population growth rate was 50-70% lower for the insects that consumed this corn. Other research also showed that the corn hybrids Agrisure3000 GT, Agrisure Viptera3110, and Agrisure Viptera3111c, which contain the insertion of the Bt gene, were not affected by the fall armyworm compared to the controls (Aguirre *et al.*, 2016; Sousa *et al.*, 2016). In contrast, the results obtained differ from the ones published by Murúa *et al.* (2009) who reported that Bt and conventional corns in association did not show statistically significant differences against *S. frugiperda* populations.

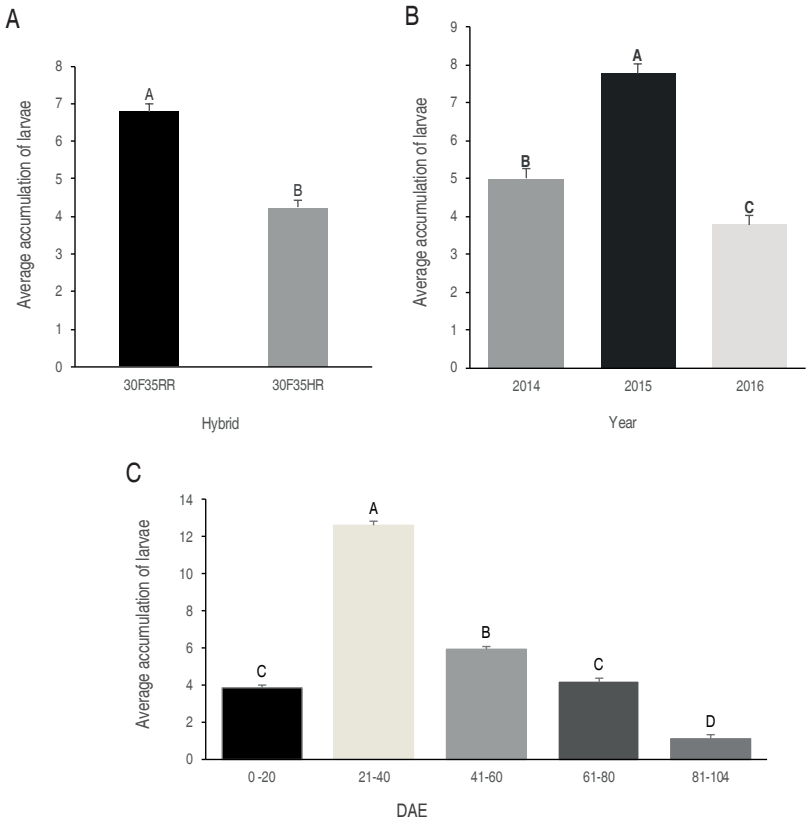

Figure 3. Comparison of cumulative area of *S. frugiperda* larvae. A. Comparison by hybrid; B. Comparison by year; C. DAE periods. Different letters indicate significant differences, according to Fischer's LSD test.

Finding populations of *S. frugiperda* in genetically modified corn could be caused by the sublethal effect and the acquired resistance (Sousa *et al*., 2016). Niu *et al.* (2014) reported that in corn plants with the Cry1F endotoxin, resistant larvae survived in 72.9% of the plants after 12-15 days and caused a leaf lesion index of 5.7 (measured according to the Davis scale of 1 to 9). In this case, larvae survival was not significantly different from the observed in non-Bt corn hybrids. It suggests that the developed resistance of RR corn hybrid with Cry1F is handled by a possible combination of biologic, geographic, and operational factors. These factors probably allowed that *S. frugiperda* evolving resistance to Cry1F (Tabashnik and Carrière, 2007; Storer *et al*., 2010).

The interaction of larval accumulation overtime per hybrid (F=12.05, df=4, *P*<0.0001), showed that the 30F35R hybrid in all periods had a higher accumulation of larvae compared to the 30F35HR, and a decrease was observed from the third period onwards (Figure 4). The interaction between time and larval populations is explained by Ayala *et al.* (2013), who pointed out that the sowing dates affected the infestation levels of *S. frugiperda,* and early planting avoided high densities of the fall armyworm that develops later in the season. As observed by these same authors, the levels of damage to corn plants were higher after stage V4, which agrees with the 20 to 40 DAE period evaluated in the current study, where there was a higher accumulation of damage. Similarly, Murúa *et al*. (2006) recorded higher larval densities at the end of the vegetative period.

Longitudinal logistic regression model to establish the probability of larvae and damage presence during the crop cycle

An initial correlation between the presence of larvae and damage was made employing the Spearman's correlation coefficient, which showed an R=0.6740584 (*P*<0.0001), which indicates a directly proportional relationship between

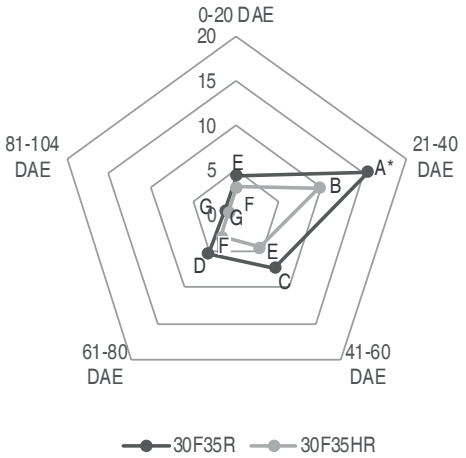

Figure 4. Comparison of cumulative area of the *S. frugiperda* larvae in the hybrid and period interaction. Different letters indicate statistically significant differences.

the damage caused by *S. frugiperda* and the presence of larvae. In order to select the best model for the presence of larvae or damage, the comparison of model 1 that included all the covariables (hybrid, DAE, year) with model 2 (without covariables) was carried out. Employing a likelihood ratio test, these two models were compared showing statistically significant differences (χ^2=589.43, *P*<0.0001), interpreting that it is necessary to include covariables inside the model. Then, model selection was carried out, and all variables were significant making necessary to include them in the model. Afterward, a comparison was made with a third model (saturated), which included all interactions compared to the model without interactions (model 1). Differences between both hybrids were found in the end; therefore, it was fundamental to include the interaction between DAE and year. Thus, model 4 was proposed (Table 1).

Table 1. Comparison and selection of the best model for the presence of *S. frugiperda* larvae and damage in corn.

Models	Comparison of larval presence models	Comparison of damage presence models
	AIC	AIC
Model 1. *Larvae(1/0)~Hybrid+DAE+Year*	5,655.8	5,009.1
Model 2. *Larvae(1/0)~1*	6,239.2	5,915.3
Model 3. *Larvae(1/0)~Hybrid×DAE×Year*	5,640.9	4,979.0
Model 4. *Larvae(1/0)~Hybrid+DAE+Year+DAE×Year*	**5,635.6**	**4,976.0**

When comparing the saturated model 3 and the proposed model 4 using the likelihood ratio test, no differences were found (χ^2=0.6783, *P*=0.8781). Finally, the models with the lowest AIC and with the lowest deviance residual were selected. The model selected for the presence of larvae and damage is as follows:

$$Ln\frac{(\pi)}{(1-\pi)} = \beta 0 + \beta 1\, Hybrid_i + \beta 2\, DAE_i + \beta 3\, Year_i + \beta 4\, DAE \times Year$$

Thereafter, the probabilities of the presence of *Spodoptera frugiperda* larvae and damage were calculated as the probability of finding larvae or any damage in the hybrid *i*, in the days after emergence, and in the year *i*. For interpretative effects of the highest pest incidence periods, the estimated average probabilities for hybrid and days after emergence in the crop cycle were calculated (Figure 5).

$$\pi_i = \frac{exp(\beta 0 + \beta 1\, Hybrid_i + \beta 2\, DAE_i + \beta 3\, year_i + \beta 4\, DAE \times Year)}{1 + exp(\beta 0 + \beta 1\, Hybrid_i + \beta 2\, DAE_i + \beta 3\, year_i + \beta 4\, DAE \times Year)}$$

Estimated probabilities show that it is more likely to find larvae and damage in conventional genotypes than in transgenic hybrids. Regarding the number of larvae during the vegetative stage, an increase in probability occurred when reaching 36 DAE that continues until 40 DAE, where it begins to decrease up to 60 DAE. These larvae were found in the vegetative bud and in the leaves. In the reproductive stage, the presence of *S. frugiperda* larvae increased during flowering, mostly found in the cob insertion axis to the stem, and sometimes in the stigmas (Pannuti *et al.*, 2015).

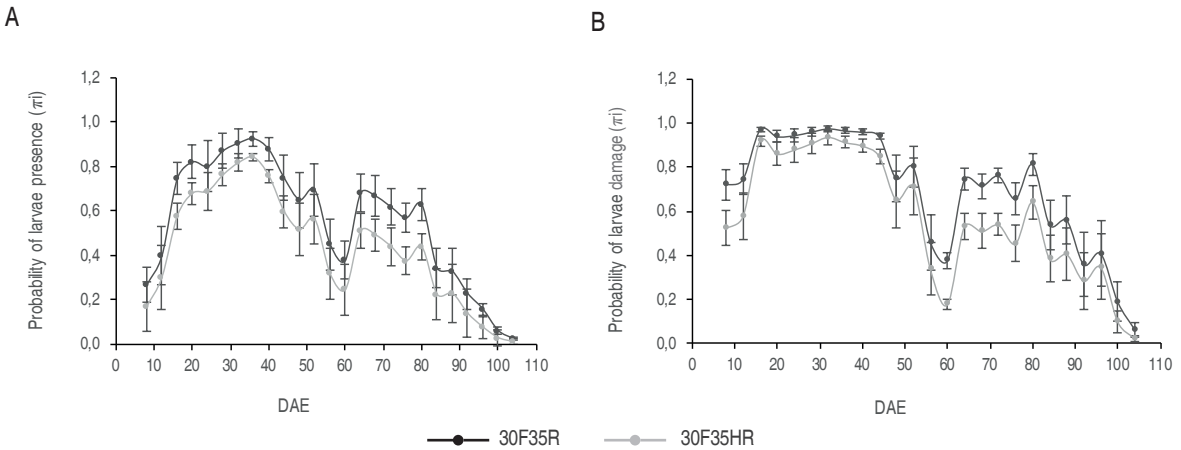

Figure 5. Estimated probability (π_i) with standard error obtained with the longitudinal logistic regression model for the genetically modified (Bt) hybrid and the non-modified with Bt hybrid. A. Presence of larvae; B. Presence of damage.

Regarding damage, when there is no pest management from the initial crop stages, areas with damage can be found in 52% and 72% of the hybrid plants with and without the Cry1F endotoxin, respectively. The damage is maintained to its full potential by the new generations of larvae that are produced up to 40 DAE. From this point onwards, the damage descends and reappears in a lower proportion during the reproductive stage of the crop, affecting stigmas and sometimes, areas inside the cob. A decrease was observed for both larvae and damage presence when reaching 80 DAE due to crop maturation and senescence.

Knowing the probability of larvae and damage presence allows incorporating this result into management strategies. The fall armyworm is a key pest in corn, and its larvae are active during the night as well as during the day, causing continuous damage to corn plants. This insect acts as a cutter, defoliator, and even damages buds. According to their development stage, It produces direct damages when they feed on spike grains (Willink *et al.*, 1993). Genetically modified corn hybrids with the Cry1F endotoxin in this study had a lower probability of larvae and damage presence, because they have shown less damage due to fall armyworm aggressiveness, and there are significant differences compared to the damages shown by the controls (Aguirre *et al.*, 2015).

CONCLUSIONS

Larvae and damage were found in corn plants with the Cry1F endotoxin, and insects show a certain range of resistance to plants with this endotoxin. The presence of larval populations and damage of the fall armyworm (*Spodoptera frugiperda*) in genetically modified corn plant genotypes needs to increase their monitoring and evaluations; besides, it is necessary to establishing refuge areas. Moreover, optimum sowing dates and phenological stages of the crop are key in order to establish adequate management actions. It is important to develop action plans that consider a baseline of resistance in populations which are not subject to Cry1F in Tolima, evaluating that in the vegetative stage

the of corn the susceptibility to *S. frugiperda* is higher, especially from 20 to 40 DAE.

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Efficient use of nutrients in fine aroma cacao in the province of Los Ríos-Ecuador

Uso eficiente de nutrientes en cacao fino de aroma en la provincia de Los Ríos-Ecuador

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Edisson W. Cuenca-Cuenca[1]*, Yina J. Puentes-Páramo[2] and Juan C. Menjivar-Flores[3]

ABSTRACT

Soil nutrients are vital to high production of cacao (*Theobroma cacao* L.); however, excessive use of fertilizers affects the environment and profitability of this crop. This research aimed to determine the efficient use of nutrients (EUN) for nitrogen (N), phosphorus (P) and potassium (K) in four fine aroma cacao clones (EET-576, EET-575, EET-103, and CCN-51), which are economically important in Ecuador. The experiment design used split plots, with four treatments and four repetitions. The treatments consisted of applications of different doses of N-P-K (kg ha^{-1}): TR (control), T1 (N_{59}-P_{29}-K_{118}), T2 (N_{74}-P_{37}-K_{148}), and T3 (N_{88}-P_{44}-K_{176}). The results showed highly significant differences for the agronomic (AE) and recovery (RE) efficiency of N-P-K in terms of the clone and treatment. The highest efficiencies were presented for clone CCN-51 and the lowest in clone EET-103. The treatment that obtained the highest efficiency was T2 for clones CCN-51, EET-575 and EET-576, except for EET-103 that reached the highest efficiency with T1. The lowest efficiencies were obtained in T3. The highest yields were presented in clone CCN-51, followed by EET-575, later EET-576, and finally EET-103. Influence of the genotypes on the maximum limit of nutrient absorption was evident under the studied conditions, suggesting that it determines the efficient use of nutrients for each clone in interaction with this specific cultivation area.

Keywords:
Agronomic efficiency
Essential nutrient
Fertilization
Nutrient recovery efficiency

RESUMEN

Los nutrientes en el suelo son importantes para que exista una alta producción en cacao (*Theobroma cacao* L.), sin embargo, el uso excesivo de fertilizantes afecta el medio ambiente y la rentabilidad del cultivo. Por ello, esta investigación tuvo como objetivo determinar el uso eficiente de nutrientes (EUN) para nitrógeno (N), fósforo (P) y potasio (K) en cuatro clones de cacao fino de aroma (EET-576, EET-575, EET-103 y CCN-51), con importancia económica en Ecuador. El diseño experimental fue de parcelas divididas, con cuatro tratamientos y cuatro repeticiones. Los tratamientos consistieron en aplicaciones de diferentes dosis de N-P-K (kg ha^{-1}): TR (control), T1 (N_{59}-P_{29}-K_{118}), T2 (N_{74}-P_{37}-K_{148}) y T3 (N_{88}-P_{44}-K_{176}). Los resultados mostraron diferencias altamente significativas para la eficiencia agronómica (AE) y de recuperación (RE) de N-P-K por efecto del clon y del tratamiento; las eficiencias más altas se presentaron para el clon CCN-51 y las más bajas en el clon EET-103. El tratamiento que obtuvo los mayores valores en las eficiencias fue el T2 para los clones CCN-51, EET-575 y EET-576, excepto el EET-103 que alcanzó la eficiencia más alta con T1; por otra parte, las eficiencias más bajas se obtuvieron con T3. Los mayores rendimientos se presentaron en el clon CCN-51, seguido por el EET-575, posteriormente el EET-576, y por último el EET-103. Siendo evidente la influencia del genotipo en el límite máximo de absorción de nutrientes bajo las condiciones de estudio, sugiriendo determinar el uso eficiente de nutrientes para cada clon en interacción con la zona específica de cultivo.

Palabras clave:
Eficiencia agronómica
Nutriente esencial
Fertilización
Eficiencia de recuperación del nutriente

[1] Departamento de Ciencias Agronómicas. Facultad de Ingeniería Agronómica. Universidad Técnica de Manabí. Av. Urbina/Che Guevara. CP 130105 Portoviejo, Manabí, Ecuador.
[2] Consultor. Palmira, Colombia.
[3] Facultad de Ciencias Agropecuarias. Universidad Nacional de Colombia. AA 237 Palmira, Colombia.
* Corresponding author: <ecuenca@utm.edu.ec>

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Cacao (*Theobroma cacao* L.) is a perennial crop of great socio-economic importance in Ecuador, providing a source of income for 100,000 families (Pino, 2017); in addition, the country also ranks third in world exports after Côte d'Ivoire and Ghana, contributing 6% to the world production of cacao beans (Fedecacao, 2018).

The cacao that is cultivated in Ecuador is more than 60% fine aroma; however, the yields are lower than 0.25 t ha^{-1} (Noboa, 2018), resulting from the absence of technological management in farms (Amores *et al*., 2009), physical and chemical factors of the soil, and the use of clones of low genetic potential (Nakayama, 2010). On the other hand, the lack or excess of fertilization contributes significantly to the yield decrease (Mite, 2016).

Therefore, several researchers have studied the importance of fertilization in cacao (*T. cacao* L.) crops in order to increase yield. For example, Morais (1998) determined that phosphorus is the nutrient that most limits production in Brazil. Rosas *et al.* (2019) determined that the acidity of the soil of the Colombian Amazon is an adverse condition to reach maximum performance; however, they found that CCN-51 clone has greater ability in the use of nutrients. The same authors (Rosas *et al*. 2017) pointed out that applications 7 Mg ha^{-1} of $Mg(CO_3)_2$ and $Ca(CO_3)_2$ allow a change in the pH of 4.36 to 6.0 in approximately two months, allowing an increase in the availability of nutrients. Uribe *et al*. (1998) observed a high response to nitrogen and potassium fertilization, while Mora *et al*. (2011) reported an increase in yield with different doses of fertilization. Regarding Ecuador, Recalde *et al*. (2012) showed that the influence of fertilization on yield is evident. However, establishing optimal nutritional management requires efficient use of nutrients, as suggested by Romero *et al*. (2016), since fertilizing with more nutrients does not guarantee a higher yield (Puentes *et al*. 2014a).

In that order of ideas, determining the efficient use of nutrients (EUN) will allow the selection of genotypes based on the ability of absorbing nutrients, which could increase crop yields (Baligar *et al*., 2001), contributing to the mitigation of environmental impacts by the indiscriminate use of fertilizers (FAO, 1999). Stewart (2007) stated that deficit or excess of fertilization causes low yield and quality in cacao crops. Ribeiro *et al*. (2008) showed that cacao genotypes respond differently to the efficient use of nitrogen. Whereas Puentes *et al*. (2014b) determined the agronomic and recovery efficiency for N-P-K, as well as the physiological efficiency of the crop (Puentes *et al*. 2016), suggesting that a low or high amount of fertilizer limits the EUN.

In Ecuador, despite research on cacao nutrition is scarce, Carrillo *et al*. (2010) reported that balanced fertilization has benefits on the physiology and architecture of the plant. Recalde *et al*. (2012) stated the beneficial effect of fertilization on the crop. Amores *et al*. (2010) reported that the beginning of the productive stage of the crop requires 212, 23, and 321 kg ha^{-1} of nitrogen, phosphorus, and potassium, respectively; as well as 140, 71, 7.1, and 0.9 kg ha^{-1} of calcium, magnesium, manganese, and zinc, respectively. However, studies in cocoa have not evaluated the efficient use of nutrients, and the fertilizations that are carried out do not consider nutrient recovery efficiency results to determine the doses to be applied. This current practice can lead to underdosing or overdosing without correlating the amount of fertilizer used with crop production. Puentes' *et al.* (2014a) research becomes very important in that sense; however, it must be clarified that they worked in soils of very good fertility and with different clones. In their investigation, clones EET-576, EET-575, EET-103, and CCN-51 were used because Amores *et al*. (2006), determined, in a study conducted over five years, that those clones had the best yields in the area of the Ecuadorian coast (Los Ríos). Therefore, this research aimed to determine the efficient use of N-P-K in terms of agronomic efficiency and fertilizer recovery in the fine aroma clones EET-576, EET-575, EET-103, and CCN-51 in the Province of Los Ríos-Ecuador.

MATERIALS AND METHODS

The research was conducted in 2016 and 2017 at the Mocache cacao farm located in Pajarito, Province of Los Ríos (1°03'18"S and 79°25'24"W), at 73 m.a.s.l. The climatic conditions on average are 77.4% relative humidity, 24.2 °C annual temperature, 1,537 mm annual precipitation, and solar brightness of 823 light hours per year (INIAP, 2015). The soil, classified as Typic hapludalfs (Sevillano *et al*., 2012), was sampled two

months before the application of fertilizers, collecting 15 subsamples at a 0.3m depth and 1.5m from the trunk of the cacao plants for a composite sample. The physical and chemical analysis of the soil showed no limitations for the development of the crop (Amores, 1992); indicating loamy textural class, low apparent density, moderately acidic pH, low organic matter content, medium cation exchange capacity (CIC), high P and K^+ content and low levels for exchangeable Al^{3+} (Table 1).

Table 1. Main characteristics of the soil at the experimental site.

Parameter	pH	M.O (%)	P mg kg^{-1}	K^+. Cmol kg^{-1}	CIC Cmol kg^{-1}	Al^{3+} Cmol kg^{-1}	DA g cm^{-3}	Texture
Value	5.3	1.7	24	0.81	17.3	0.36	1.20	Loamy

The experiment design consisted of randomized complete blocks with an arrangement of divided plots (main plot: cocoa clones; subplots: treatments) with four treatments and four repetitions; the experimental unit was made up of four trees. Self-compatible clones of fine flavor and aroma cocoa with six years of age, three from the Tropical Experimental Station (EET-576, EET-575, EET-103) and one from the Castro Naranjal Collection (CCN-51) were used, sown at 3 m between plants and 3 m between rows for a planting density of 1,111 plants per hectare. The test area was 2,304 m^2 with 256 plants (64 for each clone).

The evaluated treatments included different doses of N-P-K, they were designed using the treatment with the highest yield obtained by Puentes *et al.* (2014a) and the nutrient ratios of 2:1 for N/P and 1:2 for N/K. Meanwhile, T2 was similar to the one evaluated by Puentes *et al.* (2014a), and, according to this treatment, T1 was 20% less, TR was 40% less (natural N-P-K concentration of soil), and T3 was 20% more than T2 (Table 2).

The application period of fertilizers was divided into three times, 33% in each part: the first part at the beginning of the rainy season (January), the second part at the end of the rainy season (May) and the third part in the dry season (September). Before starting the experiment, the fruits that had more than three months were eliminated to achieve uniform production (Puentes *et al.*, 2014b).

Table 2. Description of the treatments and amount of fertilizer per plant.

Treatments	Nutrients (kg ha^{-1})			Source (g per plant)		
	N	P_2O_5	K_2O	Urea	$(NH_4)_2HPO_4$	KNO_3
TR	44	22	88	0	0	0
T1	59	29	118	21.7	143.3	393.4
T2	74	37	148	26.0	182.2	493.4
T3	88	44	176	30.9	217.4	586.7

The foliar sampling consisted of taking the fourth leaf of branches located in the middle third of the cacao plants. It was collected a total of 60 leaves per sample (INIAP, 2016) to determine N, through Kjeldahl method; P, through colorimetry; and K, through atomic absorption. Also, the content of N, P, and K in cocoa beans was determined. The coca beans were obtained from ten completely mature pods, which were selected from each clone in each treatment. This sampling was done from the third semester of study to ensure the plant response to the treatments.

The yield was calculated, as suggested by Puentes *et al.* (2014a), considering the number of pods per tree, the

grain index (GI), the number of grains per pod, and the planting density. The GI was determined with Equation 1, proposed by Allen (1987)

$$GI = \frac{0.38 \times Total\ grain\ weight\ per\ pod}{Number\ of\ grains\ per\ pod} \quad (1)$$

The efficient use of N-P-K as a function of the recovery efficiency of fertilizer (RE) indicates the ability of the plant to absorb the nutrients applied to the soil, which was determined with Equation 2, proposed by Baligar *et al.* (2001).

$$RE(\%) = \frac{EN(f) - EN(t)}{can} \times 100 \quad (2)$$

Where:
RE: Recovery efficiency of fertilizer
EN(f): Extraction of nutrients in grains of the fertilized treatment
EN(t): Extraction of nutrients in grains from the control treatment
CNA: Amount of nutrient applied with the fertilizer

The agronomic efficiency indicated the yield increase in kilograms of dry grain for each kilogram of applied nutrient, which was determined with Equation 3, proposed by Baligar *et al.* (2001).

$$AE(kg\ kg^{-1}) = \frac{R(f) - R(t)}{CNA} \quad (3)$$

Where:
AE: Agronomic efficiency
R(f): Grain yield of the fertilized treatment
R(t): Yield of the control treatment grain
CNA: Amount of nutrient applied with the fertilizer

The data of efficiencies in the use of N-P-K and yield were subjected to Analysis of Variance (ANOVA) and Tukey's HSD (honestly significant difference) test at a probability level of 5%, and correlations and regressions using the Statistical Analysis System (SAS) program.

RESULTS AND DISCUSSION

Agronomic efficiency of N-P-K in the cacao clones

The agronomic efficiency of N-P-K showed highly significant differences (P<0.01) because of the clone and treatments according to the Tukey's test.

Meanwhile, the highest nitrogen agronomic efficiency (AEN) was seen in clone CCN-51 in the T2 treatment with 21.19 kg kg^{-1}, which means that, for each kilogram of nitrogen applied, the yield increased by 21.19 kg of dry cacao, followed by clone EET-576 in T2 (12.06 kg kg^{-1}), EET-103 in T1 (9.5 kg kg^{-1}), and EET-575 in T2 with 9.39 kg kg^{-1} (Figure 1). These AEN values exceeded those reported by Puentes *et al.* (2014a); considering that there was greater fractionation (three times) concerning the mentioned study. CCN-51 excelled in terms of the AEN in the edaphoclimatic conditions of this experiment, surpassing the other clones.

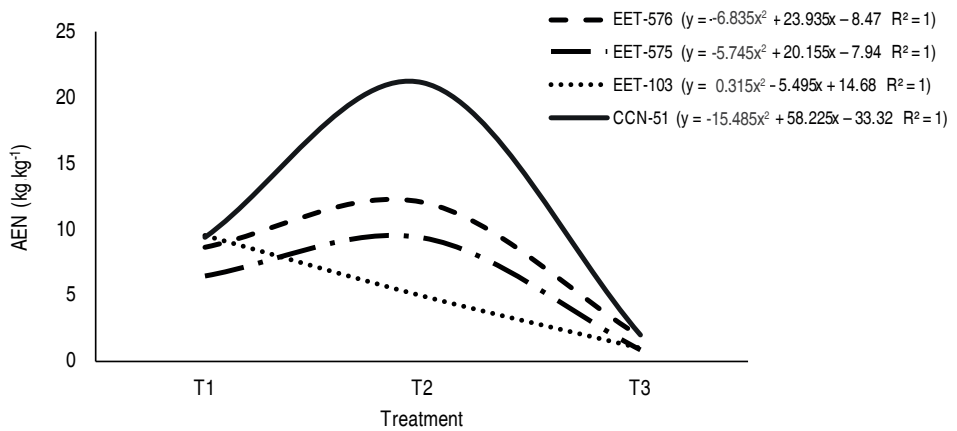

Figure 1. Agronomic nitrogen efficiency in the evaluated cacao clones.

T3 treatment presented the lower values for all the clones, which coincides with the results of Apáez *et al*. (2013), who reported a greater amount of fertilizer decreases the efficiency in the absorption of nitrogen. This efficiency decrease can be attributed to the fact that higher doses of fertilizers modify the nutrient balance in the soil solution (Ferraris *et al*., 2016). A negative effect can be caused, an antagonism, which would reduce the absorption of other nutrients. For example, if N is added as ammonia, the absorption of other cations such as Ca^{2+}, Mg^{2+} or K^{+} could be reduced.

In phosphorus (Figure 2), clone CCN-51 showed the highest agronomic efficiency in T2 (42.39 kg kg^{-1}), followed by clone EET-576 in T2 (24.11 kg kg^{-1}), EET-103 in T1 (19.33 kg kg^{-1}), and clone EET-575 in T2 (18.78 kg kg^{-1}), and the lowest efficiency was seen in T3 for the four clones. Similar results were obtained by Puentes *et al.* (2014a); however, the AEP values were higher because of the low availability of phosphorus in the experiment soil, making the response to fertilization more efficient, as suggested by Díaz *et al*. (2014) and Covarrubias *et al*. (2005).

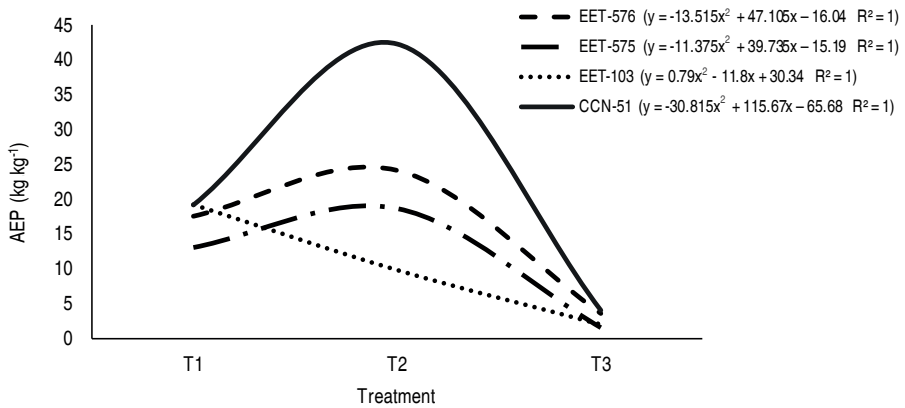

Figure 2. Agronomic efficiency of phosphorus in the evaluated cacao clones.

Figure 3 shows that the higher AEK values were in T2 for clones CCN-51, EET-576, and EET-575, with an increase of 10.6 kg, 6.03 kg, and 4.7 kg, respectively, for each kilogram of potassium applied with the fertilizer, while EET-103 presented its highest value in T1 (4.75 kg kg^{-1}). These results are higher than those reported by Puentes *et al*. (2014a), which is considered normal since the plants do not respond efficiently when K concentrations are high in the soil (Marschner, 1995), as happened with the potassium in the experiment soil.

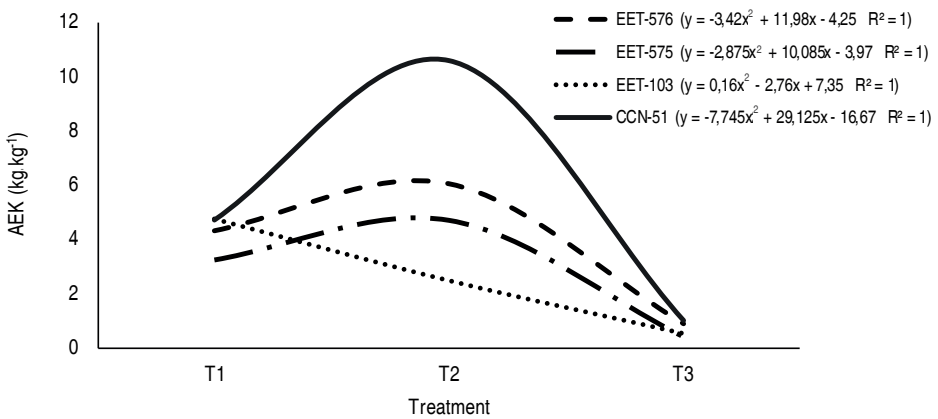

Figure 3. Agronomic efficiency of potassium in the evaluated cacao clones.

Efficiency recovery of NPK in the cacao clones

The recovery efficiency of N, P, and K showed highly significant differences (P<0.01) because of the clone and treatments according to the Tukey test (Table 3).

The recovery efficiency of nitrogen (REN) showed the highest efficiency in the CCN-51 clone in the T2, followed by EET-576 in T2, EET-103 in T1, and EET-575 in T2 and the lowest efficiency in T3 for all clones (Table 3). These values are higher than those reported by Puentes *et al*. (2014a); in the CCN-51 clone of 100% of the fertilizer applied, more than 50% was recovered, exceeding the report by Dass *et al.* (2015), who indicated that the plant recovers 50% of what was added, and the rest is exposed that can be lost by leaching, volatilization and runoff. However, according to Baligar *et al.* (2001), the REN below 50% for the remaining clones was considered normal.

Table 3. Recovery efficiency (%) of nitrogen, phosphorus, and potassium for cocoa clones.

Clone	REN			REP			REP		
	Interaction among nutrient doses per clones[1]								
	T1	T2	T3	T1	T2	T3	T1	T2	T3
EET-576	22.6 b	31.8 a	5.1 c	10.5 b	12.5 a	2.3 c	4.7 b	7.0 a	1.2 c
EET-575	19.4 a	21.4 a	3.5 b	6.6 b	9.2 a	0.7 c	3.5 b	5.6 a	0.6 c
EET-103	27.5 a	15.2 b	3.3 c	9.8 a	5.0 b	1.0 c	4.8 a	2.9 b	0.6 c
CCN-51	26.7 b	61.9 a	8.3 c	10.0 c	22.5 a	1.8 c	5.6 b	11.3 a	1.2 c
	Nutrient recovery effect among clones[2]								
EET-576	19.72 b			8.43 ab			4.30 b		
EET-575	14.77 b			5.50 bc			3.23 bc		
EET-103	15.33 b			5.27 c			2.77 c		
CCN-51	32.30 a			11.43 a			6.03 a		

[1] Values with the same letter within the row do not differ statistically (P<0.05).
[2] Values with the same letter within the column do not differ statistically (P<0.05).
REN: recovery efficiency of nitrogen; REP: recovery efficiency of phosphorus; REP: recovery efficiency of potassium.

The cacao clones showed different percentages of nitrogen recovery, CCN-51 being the one that obtained the highest recovery efficiency of N, for the remaining clones the value was lower, similar to results found by Ribeiro *et al*. (2008), indicating that the cacao genotypes, although they have a close genetic relationship, differ in their ability to extract nitrogen. Likewise, the interaction of plants with environmental factors such as solar radiation, rainfall, temperature, and microorganisms present in the soil greatly influence the efficient use of nitrogen (Baligar *et al*. 2001). Some conditions that could affect the investigation in the rainy season (January to May), since the high rainfall (1537 mm) lead to a greater loss of nutrients and that there is little aeration for the activity that microorganisms must meet.

For the recovery efficiency of phosphorus (REP), it is observed that the highest recovery for clones CCN-51, EET-576, and EET-575 was seen in T2, while for EET-103 was in T1, attributing itself, that with the application of the lowest dose of nutrients reached its maximum yield potential. Besides, concerning the other clones, it was the one that obtained the lowest yield, therefore the lowest extraction of nutrients. Lower REP values were observed in T3 for all the clones. These REP values are greater than the values reported by Puentes *et al*. (2014a) and Baligar *et al*. (2001). In general, there were differences in REP between the clones, obtaining the highest value CCN-51, followed by EET-576, EET575, and EET-103, this behavior may be directly related to the climatic conditions of the area under study and the interaction genotype by the environment.

For the recovery efficiency of potassium (REK), it was observed that, as with REN and REP, clones CCN-51, EET-576 and EET-575 obtained their highest REK in

T2, while for EET- 103, it was in T1 (Table 2). These values are low when compared with Baligar *et al*. (2001), but greater than those obtained by Puentes *et al*. (2014a); these low REK values can be explained by the presence of illite clays in the soil, which have great affinity for K. It is noted that the higher level of fertilization (T3) decreased the efficiency of recovery for the four clones, indicating a limit in the absorption of nutrients for each clone, as suggested by Puentes *et al*. (2014b).

Yield

The yield showed highly significant differences between clones and treatments; CCN-51 presented the highest yield, with 41.25% more yield than EET-576 obtained in T2, followed by EET- 575 in T2 and EET-103 in T1 (Table 4).

Table 4. Yield of dry cacao in kg ha^{-1} (cacao year 2016-2017).

Clone	Interaction between nutrient doses per clone[1]				Interaction of cocoa yield among clones[2]
	TR	T1	T2	T3	
EET-576	511.8 d	1020 b	1403.3 a	671.0 c	901 b
EET-575	640.3 d	1049 b	1363.0 a	662.8 c	928 b
EET-103	572.0 d	1097 a	902.0 b	617.0 c	797 b
CCN-51	820.0 d	1376 b	2388.0 a	995.0 c	1394 a

[1] Values with the same letter within the row do not differ statistically (P<0.05).
[2] Values with the same letter within the column do not differ statistically (P<0.05).

The obtained yields exceeded the values reported by Amores *et al.* (2009) for clones EET-576 and CCN-51, by 16.6% and 81.9%, respectively, while EET-575 presented a 9.8% lower yield. These values also surpassed those reported by Sánchez *et al.* (2015), by 127% for CCN-51 and 150% for EET-103. These results differ with the previous authors because the soil of the studied area presented adequate conditions for the development of cocoa (Amores, 1992). Therefore, it is suggested to perform this type of research in all cocoa-producing areas at the national level.

The lower yields were seen in TR (the treatment without fertilization), in the following order: EET-576, EET-575, EET-103 and CCN-51, similar to results obtained by Remache *et al*. (2017), suggesting that the fertilizer dose in TR was not sufficient to obtain high yields; likewise, the treatment with the highest dose of nutrients (T3) obtained the lowest yield, in comparison with the treatments T1 and T2. According to Puentes *et al*. (2014a), yield can be affected by excess fertilization, which causes an imbalance between nutrients and, consequently, low yield.

CONCLUSIONS

Most of the evaluated clones showed the highest NPK use efficiency with the dose: nitrogen: 74 kg ha^{-1}, phosphorus: 37 kg ha^{-1} and potassium: 148 kg ha^{-1} under the conditions of the experimental zone. CCN-51 had an evident, greater ability for the efficient use of nutrients, allowing to obtain the maximum yields, which suggests a strong influence of its genotype. It is necessary to determine the efficient use of nutrients for each cacao cultivation area in order to obtain better income and mitigate environmental pollution.

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Generation means analysis of physiological and agronomical targeted traits in durum wheat (*Triticum durum* Desf.) cross

Análisis de medias generacionales de rasgos fisiológicos y agronómicos específicos en trigo duro (*Triticum durum* Desf.) cruzado

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Manel Salmi[1,2]*, Amar Benmahammed[3], Laid Benderradji[4], Zine El Abidine Fellahi[5], Hamenna Bouzerzour[3], Abdelmalek Oulmi[3] and Abdelkader Benbelkacem[6]

ABSTRACT

Genetic parameters such as leaf relative water content, leaf chlorophyll content, plant height, above-ground biomass, harvest index, grain yield, and grain yield components of Bousselam/Mrb5 durum wheat (*Triticum durum* Desf.) cross were estimated based on generation means analysis. A, B, C, and D scaling and Chi-square (χ^2) tests revealed the inadequacy of the simple additive-dominance model. This result suggests the presence of digenic epistasis for most studied traits; the duplicate epistasis was present for relative water content, above-ground biomass, and grain yield; and complementary epistasis was observed for chlorophyll content. Significant genotypic correlation coefficients appeared among grain yield, relative water content, and above-ground biomass. This suggests useful indirect selection criteria to improve simultaneously these traits, which showed significant heritability. It can be concluded that the traits investigated show a complex genetic behavior, which implies that early selection would be less efficient; therefore, it is recommended delaying the selection to advanced generations to benefit from the reduction of non-fixable genetic variation and exploit transgressive segregators due to the significant interaction additivity×additivity (i) of the gene and duplicated epistasis.

Keywords:
Generation means analysis
Genotypic correlation
Heritability
Non-allelic interaction
Physio-agronomical traits
Triticum durum

RESUMEN

Los parámetros genéticos como el contenido relativo de agua en la hoja, el contenido de clorofila en la hoja, la altura de la planta, la biomasa sobre el suelo, el índice de cosecha, el rendimiento de grano y los componentes de rendimiento de grano del cruce de trigo duro Bousselam/Mrb5 (*Triticum durum* Desf.) se estimaron con base al análisis de medias generacionales. Las pruebas de escala A, B, C, D y Chi-cuadrado (χ^2) revelaron la insuficiencia del modelo simple de dominio aditivo. Este resultado sugiere la presencia de epistasis digénica para los rasgos más estudiados; la epistasis duplicada estuvo presente para el contenido relativo de agua, la biomasa aérea y el rendimiento de grano; y se observó epistasis complementaria para el contenido de clorofila. Aparecieron coeficientes genotípicos de correlación significativos entre rendimiento de grano, contenido relativo de agua y biomasa sobre el suelo. Esto sugiere criterios útiles de selección indirecta para mejorar simultáneamente estos rasgos, que mostraron una heredabilidad significativa. Se puede concluir que los rasgos investigados muestran un comportamiento genético complejo, lo que implica que la selección temprana sería menos eficiente; por lo tanto, se recomendaría retrasar la selección a generaciones avanzadas para beneficiarse de la reducción de la variación genética no reparable y explotar los segregadores transgresores debido a la interacción significativa aditividad×aditividad (i) del gen y la epistasis duplicada.

Palabras clave:
Análisis de medias generacionales
Correlación genotípica
Heredabilidad
Interacción no alélica
Rasgos fisio-agronómicos
Triticum durum

[1] Ecology and Environment Department, Faculty of Life and Natural Sciences, University of Mostefa Ben Boulaid, Batna, Algeria
[2] VRBN Laboratory, University of Ferhat Abbas, Setif, Algeria
[3] Ecology and Plant Biology Department, Faculty of Life and Natural Sciences, VRBN Lab, University of Ferhat Abbas, Setif, Algeria
[4] Faculty of Sciences, University of Mohamed Boudiaf, M'sila, Algeria.
[5] Faculty of Life and Natural Sciences and Earth Sciences and the Universe. University of Mohamed El Bachir El Ibrahimi, Bordj Bou Arreridj, Algeria
[6] Algerian National Institute of Agronomic Research (INRAA), Research Unit of Constantine, Algeria
* Corresponding author: <salmi.mnl@hotmail.fr>

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Durum wheat (*Triticum durum* Desf.) is an important field crop in Algeria. It is mainly grown on the high plateaus area, which belongs to a vast geographical region where agriculture, because of climatic changes, has been forecast to be at higher risk due to an increase in the frequency and severity of drought episodes (Sahnoune *et al*., 2013). Selection of drought-tolerant cultivars is sought to minimize the effects of water scarcity and sustain crop production. The release of improved cultivars that require lower amounts of water per unit yield and characterized by high yield potential, is essential for more sustainable agricultural practices, particularly in rainfed, drought-prone areas (Belagrouz *et al*., 2018). Durum wheat breeding program aims to select genotypes with improved biotic and abiotic stresses tolerance and high grain yield potential (Annicchiarico *et al*., 2005). Crop end cycle drought and high temperatures induce changes in plant physiological functions, causing damages, inhibiting growth, and thereby reducing grain yield.

Relative water content reflects a balance between water supply to the leaf and leaf transpiration rate. This trait is seen as an important indicator of plant water status under drought stress conditions (Dhanda and Sethi, 2002). Chlorophyll content or stay green is positively associated with photosynthetic rate, grain yield improvement, and transpiration efficiency under drought stress conditions (Benbella and Paulsen, 1998). Concomitant improvement of both harvest index and biological yield increase yield potential and enhance water use efficiency (Quarrie *et al*., 1999; Belagrouz *et al*., 2018). These traits could serve as selection criteria to improve both stress tolerance and yield potential indirectly. Little information is available on the inheritance of these characters, under rainfed durum wheat cropping systems, it is, therefore, necessary to assess the estimates of gene effects to ensure better selection gain.

Knowledge of genetic behavior and type of gene action controlling physiological and agronomical targeted traits is a fundamental principle for designing an appropriate breeding procedure for genetic improvement (Fellahi *et al*., 2016; Hannachi *et al*., 2017). Inheritance of quantitative traits has been described as a moving target because these traits are affected not only by the action of multiple genes, with small individual effect, but also by the interaction between genes and between genotype and environment. Genetic statistical models have been devised to investigate the inheritance of such traits (Mather and Jinks, 1982; Kearsey and Pooni, 1996). Generation means analysis is, among such models, a useful technique to estimate variance components, heritability, and gene effects governing traits of interest (Mather and Jinks, 1982; Kearsey and Pooni, 1996). This technic helps to understand the performance of selected parents and the potential of the resulting population to employ for either heterosis exploitation or pedigree selection (Singh *et al.,* 1985; Singh and Chaudahry 1985).

Results of various studies, investigating the genetic basis of quantitative traits related to stress tolerance (yield and yield components) reported that dominance effects and epistasis were more important and predominant than additive effects (Bhutta and Mishra, 1995; Golparvar *et al*., 2006; Mahpara *et al*., 2018). However, Dhanda and Sethi (1998) reported significant additive gene effects and high narrow-sense heritability for harvest index, biological yield, and relative water content concluding that possibilities existed to improve these traits under drought stress condition. The present study aims to assess the nature and magnitude of additive, dominance, and epistatic gene effects for some physiological and agronomical targeted traits in rainfed durum wheat through generation means analysis.

MATERIALS AND METHODS

Site, plant material, and experimental design

Two phenotypically divergent durum wheat (*Triticum durum* Desf.) varieties, namely, Bousselam, derived from Heider/Martes//Huevos de Oro cross, and Mrb5, derived from JoriC69/Haurani (Adjabi *et al*., 2014) were hybridized during 2015-2016 cropping season at the Field Crop Institute, Agricultural Experimental Station of Setif (ITGC, AES, 36°12' N 05°24'E, 1080 m.a.s.l., Setif, Algeria). The F1 generation was grown the following season (2016-2017), along with parental genotypes which were crossed again to obtain F1 generation, and the seeded F1 was crossed to each parent to obtain backcross generations.

The next season (2017-2018), the six basic-generation, that is parents (P1, P2), first and second filial (F1, F2) and backcrosses (BC1, BC2) generations were sown

in a randomized completed block design, with five replications. Parents, F1, and backcross generations were sown in one row, 2 m long, 20 cm inter-row spacing and 10 cm plant-plant spacing in the row. The F2 generation was sown in thirty rows 2 m long. Recommended cultural practices for the area were followed to raise a good crop. 80 kg ha^{-1} of mono-ammonium phosphate (52% P_2O_5 + 12% N) was applied just before sowing, and 80 kg ha^{-1} of urea (46% N) was broadcasted at the tillering stage. Weeds were controlled chemically by application of 150 g ha^{-1} of Zoom (Dicamba 66% Triasulfuron 4%) and 1.2 L ha^{-1} of Traxos (22.5 g L^{-1} of Pinoxaden, 22.5 g L^{-1} of Clodinafop-propargyl, 6.5 g L^{-1} of Cloquintocet-mexyl) herbicides.

Data collection

Data were collected from 5, 5, 10, and 30 plants per replication of parents, F1, backcrosses, and F2 generation, respectively. The small sample size was used for generations whose variability is only from the environmental origin (homogeneous generations. i.e., Parents and F1), while the large sample size was used for generations whose variability was both environmental and genetic origin (heterogeneous generations, i.e., BCs and F2). Relative water content (RWC) was determined according to Barrs and Weartherly (1962) as described by Pask *et al.* (2012). Fresh leaves were collected, at the anthesis, from each generation per replication and weighted immediately to record fresh weight (FW). Leaf samples were placed in distilled water for 24 h and weighed again to record turgid weight (TW). Leaf samples were then subjected to oven drying at 72 °C for 24 h to record dry weight (DW). RWC was calculated as follow: RWC=[(FW-DW)/(TW-DW)]×100. Flag leaf chlorophyll content (Chl, CCI) was determined with a CCM-200 chlorophyll meter (Opti-Sciences, Tyngsboro, MA, USA) at the anthesis growth stage. Chlorophyll measurements were taken from the middle of the flag leaf. The following measurements, plant height (PH, cm), plant dry weight (BIO, g), number of spikes (NS), number of grains per spike (NGS), grain yield (GY, g), 1000-kernel weight (TKW, g), and harvest index (HI, %), were also determined on a plant basis at crop maturity.

Data analysis

Collected data were subjected to a simple parametric analysis of variance using Cropstat software (IRRI, 2007) to test generation effect. Whenever this effect, tested against the residual mean square, was significant, genetic analysis for the specific trait was undertaken. Traits' mean, maximum, minimum and coefficient of variation values were calculated to describe the distribution of generated generations relatively to the crossed parents, and mean values were separated using Duncan Multiple Range Test at 5% probability level. Contrast method was employed to test differences between homogeneous (non-segregating) and heterogeneous (segregating) generations and within homogeneous generations; between F1 and mid-parent, and between parents, and within heterogeneous generations; between F2 and average backcrosses, and between backcrosses (Steel and Torrie, 1982). The ANOVA F-test determines the significance of generation effect, which is a prerequisite to proceed for the generation means analysis, while contrasts test the presence of additive vs. dominance effects.

In the generation means analysis, notations adopted for gene effects were (m), (d), (h), (i), (j), and (l) representing main, additive, dominance gene effects, additive×additive, additive×dominance, and dominance×dominance epistatic gene effects, respectively. Additive-dominance model adequacy was tested using Chi-square (χ^2) test as proposed by Cavalli (1952). Three-parameter model was employed to determine (m), (d), (h) gene effects in the absence of epistasis. Whereas in the presence of non-allelic interaction six-parameter genetic model ((m), (d), (h), (i), (j), and (l)) was adopted (Mather and Jinks, 1982). The most appropriate genetic model (three vs. six parameters) was also determined using the ABCD scaling test. This test provides information regarding absence or presence of gene interactions, and when the scale is adequate, the values of A, B, C, and D tests should be zero within the limit of their respective standard errors (Mather and Jinks, 1982). Significance of any one of these scaling tests indicates the presence of genes interaction, suggesting the inadequacy of the additive – dominance model. Gene effects were tested for significance using the t-test (Kearsey and Pooni, 1996). Three and six-parameter analyses were performed using GENMEANS subroutine implemented in Tnaustat software (Manivannan, 2014).

Genotypic and environmental variance components, of the measured traits, were estimated by equating the

observed values of the different generations as follows: $\sigma^2_E=¼(\sigma^2_{P1}+\sigma^2_{P2}+2\sigma^2_{F1})$, $\sigma^2_D=(2\sigma^2_{F2}-\sigma^2_{BC1}-\sigma^2_{BC2})$, $\sigma^2_H=4(\sigma^2_{F2}-½\sigma^2_D-\sigma^2_E)$ (Mather and Jinks, 1982). Standard errors were calculated with $\sigma x/\sqrt{n}$, where σx is the standard deviation of the parameter examined. The significance of the mean value of a particular parameter was tested against its corresponding standard error, via a Student's t-test, as suggested by Mather and Jinks (1982) and Uzokwe *et al.* (2017).

Broad-sense heritability (h^2_{bs}) was calculated as follow: $h^2_{bs}=(\sigma^2_D+\sigma^2_H)/(\sigma^2_D+\sigma^2_H+\sigma^2_E)=(\sigma^2_G)/(\sigma^2_P)$ (Kearsey and Pooni, 1996), where σ^2_D, σ^2_H, σ^2_E, σ^2_G, and σ^2_P stand for the additive, dominance, environmental variance components, genetic, and phenotypic variances, respectively. When σ^2_D, σ^2_H, or σ^2_E estimates were negative or zero, h^2_{bs} was calculated as σ^2_G/σ^2_P where σ^2_G is the genetic variance and σ^2_P is equal to two times the variance of the F2 progeny ($2\sigma^2_{F2}$) (Kearsey and Pooni, 1996). Narrow-sense heritability (h^2_{ns}) was estimated as follow: $h^2_{ns}=\sigma^2_D/(\sigma^2_D+\sigma^2_H+\sigma^2_E)=(\sigma^2_D)/(\sigma^2_P)$, standard errors (SE) of these estimates were calculated as: $SE(h^2_{bs})=[SE(\sigma^2_G)]/(\sigma^2_P)$ and $SE(h^2_{ns})=[SE(\sigma^2_D)]/(\sigma^2_P)$ (Hallauer and Miranda Filho, 1989). Significance of these parameters, h^2_{bs} and h^2_{ns} was tested using a t-test equals to the ratio of heritability over its standard error (Halloran *et al*., 1979). Heritability was considered as low (<30%), moderate (31-60%) and high (>61%) as proposed by Robinson *et al*. (1949), Johnson *et al.* (1955), as reported by Azimi *et al.* (2017).

The average degree of dominance was estimated as $\sqrt{H/D}=\sqrt{(\sigma^2_H/\sigma^2_D)}$ and expected a response to selection, absolute value (R) and relative to the grand mean (R%), was derived according to Sing and Chaudhary (1999) as follows: $R=2.06h^2_{bs}\sqrt{\sigma^2_{F2}}$ and R%=100R/overall trait mean. Relationship between studied traits and grain yield was inspected through genotypic correlation coefficient (rg), which was derived as the ratio of covariance to the square root of the product of the corresponding variances of the two traits considered. Genotypic covariance was determined using the property of the analysis of variance of the sum of two variables as suggested by Kwon and Torrie (1964) and described by Mansouri *et al*. (2018), using Past software (Hammer *et al*., 2001). The standard error of rg was derived using the formulae of Reeves (1955), reported by Koots and Gibson (1996), as follows. The Student's t-test was used to determinate the significance of the correlation coefficient:

$$SE_{rg}=\left(\frac{1-rg^2}{\sqrt{2}}\right)\left(\sqrt{\frac{SE_{h_i^2}SE_{hj}}{h_i^2h_j^2}}\right)$$

Where h_i^2 and h_j^2 are the trait's heritabilities considered.

RESULTS AND DISCUSSION

Mean performances

The analysis of variance (Table 1) revealed significant differences among generations' traits; RWC, CHL, PHT, BIO, NS, NGS, TKW, GY, and HI. Significant generation effect is a prerequisite to perform generation means analysis to study the inheritance of the targeted traits. The mean of traits varied among generations; from 92.37 to 97.91% for RWC, from 42.52 to 60.13 CCI for CHL, from 92.88 to 116.95 cm for PHT, from 21.27 to 53.85 g for BIO, from 7.22 to 11.16 spikes for NS, from 37.89 to 46.63 kernels for NGS, from 39.22 to 61.57 g for TKW, from 10.20 to 24.76 g for GY, and from 43.85 to 51.68% for HI. Because of segregation effects, range and CV% were higher in F2 and BCs than in parents and F1, for all traits considered (Table 2).

Application of generation means analysis procedure is based on the hypothesis that the studied generations must arise from a cross, involving two contrasting genotypes. The differences expressed during this cropping season, between the parents involved in the present cross, are shown in Figure 1. DMRT test showed that differences between crossed parents were only significant for GY, HI, TKW and CHL, for which Bousselam had an advantage, except CHL which was at the advantage of Mrb5 (Figure 1). This is confirmed by the contrast analysis (Tables 1 and 2). Dvojković *et al.* (2010) found that the crossed parents differed significantly for some traits but not for others. Hence, the choice of Bousselam and Mrb5 varieties as parents to develop breeding material for conducting genetic studies and improve both stress tolerance and yield potential appears appealing for some traits.

Significant differences existed among the means of the non-segregating generations for RWC, CHL, PHT, TKW, HI, and NGS but not for BIO, NS and GY, as shown by the analysis of variance. Contrast analysis showed that F1 means differed significantly from mid-

parent average for RWC, CHL, PHT, NGS, and HI, and non-significantly for BIO, NS, TKW, and GY (Table 1). These results suggested that partial dominance was involved in the genetic control of RWC, CHL, PHT, NGS, and HI; while additive genetic control was expressed for BIO, NS, TKW, and GY. In this context, García-Navarro *et al.* (2016) reported for two characters that the average values of F1 were higher than the mid parent values, indicating incomplete dominance of the alleles controlling these characteristics.

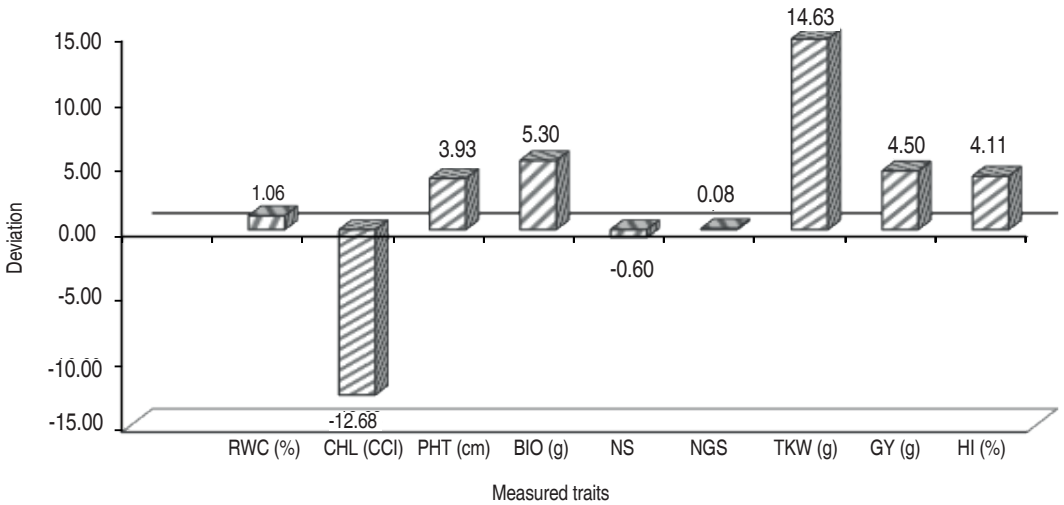

Figure 1. Mean deviations ($\bar{x}$Bousselam- $\bar{x}Mrb_5$) between the parents for the nine studied traits. RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

Table 1. Mean square deviations of the analysis of variance for nine physio-agronomical traits in durum wheat cross.

Source	DF	RWC (%)	CHL (CCI)	PHT (cm)	BIO (g)	NS	NGS	TKW (g)	GY (g)	HI (%)
Generation	5	23.84 **	218.84 **	427.20 **	523.28 **	4.42 *	45.40 **	226.50 **	119.85 **	47.95 **
Replication	4	0.54	1.12	14.55	9.26	0.88	2.12	2.73	2.24	22.91
Homogeneous (Hom)	2	7.55 *	412.47 **	428.11 **	36.85 ns	0.60 ns	29.13 **	267.80 **	25.81 ns	28.96 *
F1 vs ½(P1+P2)	1	12.29 *	423.38 **	817.45 **	3.54 ns	0.30 ns	58.24 **	0.74 ns	1.01 ns	33.39 *
P1 vs P2	1	2.81 ns	401.96 **	38.42 ns	70.23 ns	0.90 ns	0.02 ns	534.36 **	50.63 *	93.33 **
Heterogeneous (Het)	2	26.82 **	46.97 **	457.91 **	1169.64 **	6.90 **	72.23 **	166.06 **	137.73 **	2.25 ns
F2 vs ½(BC1+BC2)	1	51.35 **	1.26 ns	7.15 **	1481.92 **	0.97 ns	144.10 **	50.96 **	221.14 **	36.19 *
BC1 vs BC2	1	2.45 ns	92.72 **	909.16 **	858.40 **	12.77 **	0.46 ns	280.90 **	161.20 **	6.81 ns
Hom vs Het	1	50.57 **	175.45 **	363.66 **	203.84 **	7.06 *	24.30 *	264.63 **	165.21 **	99.01 **
Residual	20	1.78	0.96	12.01	26.00	0.91	3.94	3.32	7.23	5.16

ns: non-significant effect; *: significant effect at a 5% probability level; **: significant effect at a 1% probability level
RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

The sign of the deviation of the mid-parent mean value from F1 mean value suggested that dominance acted in the direction of the increased trait value for RWC, CHL, PHT, and HI, and in the direction of decreased trait values for NGS (Table 2). This suggested that dominant alleles control high values of the first cited traits, while recessive alleles control high values of NGS. Based on contrast analysis, differences between parents were significant for CHL, TKW, GY, and HI but not for RWC, PHT, BIO, NS, and NGS (Tables 1 and 2). Significant

differences existed among segregating generations for all studied traits except for HI. Mean values of the F2 generation deviated significantly from the BCs generations mean values for all studied traits except for CHL and NS (Table 1). Position of the F2 mean among the segregating generations (F2 and BCs) varied among traits. According to the DMRT test, F2 average was significantly higher than both BCs' means for TRE, BIO, NGS, and GY. It was significantly higher than BC1 and significantly lower than BC2 for CHL. It was significantly higher than BC2 and significantly lower than BC1 for PHT, there was non-significantly differences between F2 and both BC1 and BC2's means for NS, but significantly different from BC2 and non-significant different from BC1 means for TKW. F2 mean was significantly different from BC2 but not from BC1 mean for TKW. Harvest index F2 average was non-significantly different from BC1 but significantly different from BC2 means (Table 1 and 2). These results indicated that the mean values of the generated populations fell within the parental range for some traits and outside of this range for other traits suggesting dominance to over-dominance gene expressions. According to Dvojković *et al.* (2010), differences between F2 and BC generation mean values arise from different parental allelic contributions.

Table 2. Mean performances, range and CV% values of the 6-basic generations for nine physio-agronomical traits in durum wheat cross

Parameter	Generation	RWC (%)	CHL (CCI)	PHT (cm)	BIO (g)	NS	NGS	TKW (g)	GY (g)	HI (%)
Mean	Bou	96.52 abc	42.52 f	96.80 c	48.00 ab	8.40 b	45.48 ab	61.57 a	24.76 a	51.68 a
	Mrb5	95.46 bc	55.20 b	92.88 c	42.70 b	9.00 b	45.40 b	46.95 d	20.26 b	47.57 c
	F1	97.91 a	60.13 a	110.50 b	44.32 b	8.40 b	41.26 c	53.79 bc	23.06 ab	51.79 a
	F2	96.65 a	47.37 d	108.00 b	53.85 a	9.93 ab	46.63 a	50.77 c	23.43 ab	43.85 d
	BC1	92.37 e	44.57 e	116.95 a	35.97 c	11.16 a	37.89 cd	51.97 c	17.16 b	47.88 cd
	Bc2	93.79 de	51.35 c	93.75 c	21.27 d	7.22 b	40.41 c	39.22 e	10.20 c	48.40 bc
Range	Bou	2.14	0.60	10.20	8.11	2.00	7.22	2.16	3.30	6.81
	Mrb5	2.29	1.10	7.00	9.51	2.00	1.30	3.34	3.50	4.92
	F1	1.76	0.30	6.00	7.36	1.00	5.34	3.35	9.14	13.93
	F2	13.10	18.20	45.00	70.42	10.00	27.50	22.71	30.00	20.15
	BC1	6.89	11.00	9.50	17.60	7.00	15.86	14.82	9.70	17.80
	Bc2	6.23	9.10	25.00	9.00	5.22	14.54	12.79	2.80	16.06
CV%	Bou	0.91	0.51	4.53	7.17	10.65	6.06	1.33	5.74	5.36
	Mrb5	0.88	0.84	2.67	10.37	11.11	1.27	2.90	7.67	3.86
	F1	0.68	0.22	2.35	8.67	6.52	5.25	2.69	16.98	10.59
	F2	3.37	10.09	9.40	33.29	27.21	14.34	9.32	33.43	8.24
	BC1	2.19	8.93	2.34	14.84	16.21	13.78	8.41	16.92	12.94
	BC2	2.24	6.66	8.70	14.68	20.92	11.42	9.78	10.83	11.16

Means within a column followed by the same letter are not significantly different.
RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

Gene effects

Application of the additive-dominance model with three-parameter revealed that (m) and (d) components were significant for all traits, excepted (d) effect for NS. The (h) gene effect was non-significant for all traits (Table 3), suggesting that almost all studied traits were under the genetic control of additive nature. However, the deviation of the observed from the expected generation means was highly significant for all traits, as shown by the Chi-squared test. This result suggests that the three-parameter model was not adequate to explain the observed genetic variation for the traits under study because of the presence of non-allelic interaction. The inadequacy of the three-parameter model was also shown by the significance of at least one of the A, B, C, and D scaling tests (Table 3).

Table 3. Joint scaling and scaling tests for adequacy of the additive-dominance model for nine physio-agronomical traits in durum wheat cross.

Trait	(m)	(d)	(h)	X^2	A	B	C	D
RWC (%)	95.99±3.02 **	1.53±0.27 *	1.92±7.35 ns	86.11 **	-9.64±1.38 **	-5.74±1.42 **	-1.20±2.51 ns	7.09±1.51**
CHL (CCI)	48.86±4.82 **	-6.34±0.11 **	11.27±12.16 ns	20934.31 **	-13.50±2.52 **	-12.62±3.84 **	- 28.49±3.50 **	-1.19±2.88 ns
PHT (cm)	94.84±9.26 **	1.96±0.13 **	15.66±0.70 ns	190.98 **	26.40±7.03 **	-1.98±10.11 ns	25.32±9.77 **	0.45±6.78 ns
BIO (g)	45.35±13.72 *	2.65±1.25 *	-1.03±28.99 ns	302.30 **	-4.38±11.20 ns	-28.60±10.20 **	35.04±14.07 **	34.01±9.74 **
NS	8.7±2.49 *	-0.3±0.30 ns	-0.3±6.04 ns	42.70 **	5.52±1.24 **	-2.96±1.08 *	5.53±2.12 *	1.49±1.24 ns
NGS	4 5.44±6.61**	0.64±0.13 *	-4.18±16.57 ns	24.40 **	-9.42±17.60 ns	-6.42±13.13 ns	-15.99±7.51 **	-0.07±9.29 **
TKW (g)	54.26±5.06 **	7.31±0.36 **	-0.47±13.08 ns	47346.65 **	-25.04±11.09 **	-24.64±12.58 **	-40.85±21.77 ns	4.42±3.93 ns
GY (g)	22.51±6.07 *	2.25±0.47 *	0.55±13.06 ns	481.32 **	-11.26±6.50 ns	-19.84±5.71 **	-1.07±9.45 ns	15.01±4.29 **
HI (%)	49.63±5.88 **	2.06±0.74 *	2.17±16.79 ns	148.43 **	-14.36±14.88 ns	-14.08±8.20 ns	-31.29±15.18 **	-1.42±7.06 ns

ns: non-significant effect; *: significant effect at a 5% probability level; **: significant effect at a 1% probability level
RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

Estimates of the effect of genes are derived from the six-parameter model given in Table 4. Gene main effect (m) was significant for all analyzed traits, indicating that these traits were quantitatively inherited. These results are in accordance with Ninghot *et al.* (2016) and Bilgin *et al.* (2016). Additive (d) and dominance (h) gene effects, additive×additive (i), additive×dominance (j) and dominance×dominance (l) non-allelic interactions were significant for BIO and GY. Additive (d) gene effect, additive×additive (i), additive×dominance (j) and dominance×dominance (l) non-allelic interactions were significant for TKW. Dominance (h) gene effect, additive×additive (i), additive×dominance (j) and dominance×dominance (l) non-allelic interactions were significant for RWC. Additive (d) gene effect and additive×dominance (j) non-allelic interactions were significant for PHT and NS. Additive (d) and dominance gene effects (h), and dominance×dominance (l) non-allelic interaction were significant for CHL, while dominance gene effect (h), and additive×additive (i) non-allelic interaction were significant for HI.

Table 4. Estimates of gene effects of the six-parameter genetic model for nine physio-agronomical traits in durum wheat cross.

Trait	(m)	(d)	(h)	(i)	(i)	[l]	Epistasis
RWC (%)	96.65±0.59 **	1.41±0.93 ns	-12.35±3.04 *	-14.27±3.02 *	-3.89±0.67 *	29.74±4.48 **	Duplicate
CHL (CCI)	47.37±0.87 **	6.78±2.29 *	13.64±5.77 *	2.37±5.76 ns	-0.88±2.30 ns	23.76±9.82 *	Complementary
PHT (cm)	108±1.85 **	- 23.2±5.68 *	5.06±13.92 ns	-10.6±13.56 ns	42.48±5.79 **	-0.12±24.71 ns	
BIO (g)	53.85±3.27 **	-14.7±7.22 *	-101.94±19.66 *	-100.91±1.49 **	24.1±7.48 *	165.77±32.13 **	Duplicate
NS	9.93±0.49 **	-3.94±1.33 *	-3.27±3.33 ns	-2.97±3.31 ns	8.48±1.36 **	0.41±5.72 ns	
NGS	46.63±1.22 **	2.53±8.96 ns	-34.08±20.61 ns	-29.90±15.58 ns	-5.14±9.01 ns	46.68±40.34 ns	
TKW (g)	50.77±0.86 **	-12.74±3.52 *	-21.18±13.31 ns	-20.7±7.85 *	10.86±3.57 *	54.42±25.94 *	
GY (g)	23.43±1.38 **	-6.97±3.29 *	-38.45±9.40 ns	-39±8.58 *	9.43±3.45 *	75.42±16.19 *	Duplicate
HI (%)	43.85±1.06 **	0.52±6.74 ns	19.31±5.23 ns	17.14±4.13 *	-5.16±6.78 ns	-6.85±30.92 ns	

ns: non-significant effect; *: significant effect at a 5% probability level; **: significant effect at a 1% probability level
RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

Additive (d) and dominance gene effects (h), additive×additive (i), additive×dominance (j) and dominance×dominance (l) non-allelic interactions were non-significant for NGS. The 6-parameter model was not

adequate for this trait, suggesting that higher than digenic interaction should be tested (Table 4) Either (h) and (l) and (j) had higher values than (d) and (i), suggesting that dominance played a major role in the inheritance of RWC, CHL, PHT, BIO, NS, TKW, GY, and HI (Table 4). According to Kearsey and Pooni (1996), a greater magnitude of dominance compared to additive gene effects arises when genes are dispersed in the parents. In this case, the estimate of the additive component is reduced compared to the dominance component estimate. Results of the present study indicated that genes controlling the studied traits are predominantly dispersed in the parents. Kearsey and Pooni (1996) mentioned that epistasis is determined when dominance (h) and dominance×dominance (l) effects were significant. When these effects had the same sign, epistasis is of complementary type, while different signs indicated duplicate epistasis. In the present study, the presence of duplicate epistasis controlling the inheritance of RWC, BIO, and GY is suspected because (h) and (l) components had opposite signs. This type of epistasis limits the variability range and early selection efficiency (Kearsey and Pooni, 1996). Thus, selection of RWC, BIO, and GY must be delayed to advanced generations to benefit from the reduction of non-fixable genetic variation and exploit transgressive segregants due to significant additive×additive (i) gene effects and duplicate type epistasis.

Dominance (h) and dominance×dominance (l) epistatic gene effects had a similar sign; which suggested complementary epistatic effects for CHL. Compared to what is reported in the literature, the results of the present study corroborate findings of Ferrari *et al.* (2018), who observed that for GY additive (d) gene effect was not significant, but dominance (h) and dominance×dominance (l) epistatic effects made the higher contribution to the inheritance of this trait. For this character, Ljubičić *et al*. (2016) reported duplicate type epistasis, proposing to delay selection for this trait because of the narrow range of variability and high probability of low selection success in an early generation. Analysis of the nature and magnitude of the gene effects of quantitative traits helps to design an efficient improvement program (Shekhawat *et al*., 2000). Goldringer *et al*. (1997) found that PHT was inherited additively, while GY showed larger epistatic than additive effects. The six-parameter model revealed that non-additive (h) and epistatic genetic effects, and some additive (d) gene effects played a significant role in the inheritance of the studied traits. Dominance (h) and dominance×dominance (l) gene effects were relatively higher compared to additive (d) gene effects for all traits except NGS, revealing the low importance of additive gene effects in the genetic control of the studied traits. It seems that the genetic control of a given trait cannot be definitively characterized because it depends on the genetic material studied and the environmental conditions. Based on the findings of the present research, it can be concluded that the investigated traits show complex genetic behavior, which implies that early selection would be less efficient. Selection in advanced generations is recommended for the improvement of the above-cited traits.

Heritabilities, degree of dominance, expected response to selection, and genotypic correlation

Estimates of heritability are useful for a breeder to weigh the proportion of variation which is inheritable from that which is non-heritable. Results of the present study indicated that broad sense heritability values varied from almost 1.00 for CHL to 0.59 for HI. Based on the Student's t-test, these values were significant for RWC, CHL, PHT, BIO, NS, NGS, and GY and non-significant for TKW and HI. Narrow senses heritability values were somewhat lower than their counterpart broad sense heritabilities, varying from 0.93 for NGS to 0.35 for TKW, but they were significant for all trait, except HI (Table 5). The fact that h^2_{bs} of TKW was non-significant, while its counterpart h^2_{ns} was; it arises from the size of the standard error of each parameter, low for h^2_{ns} and large for h^2_{bs}. Globally these heritability values corroborated those reported by Dvojković *et al*. (2010), who found that narrow sense heritability values ranged among crosses, from 0.35 to 0.42 for NGS, from 0.50 to 0.50 for GY, and from 0.29 to 0.41 for TKW. Novoselovic *et al*. (2004) reported for the same parameters, values ranging, among cross combinations, from 0.54 to 0.81 for PHT, from 0.09 to 0.76 for NS, from 0.11 to 0.99 for NGS, from 0.21 to 0.78 for GY, and from 0.49 to 0.72 for TKW. High heritability values indicate that the environment least influences the characters studied in their expression, which suggests that selection for these traits would be effective owing to their high genetic transmissibility.

The average degree of dominance values was less than unity: 0.15, 0.65, 0.71, 0.72, and 0.77 for GY, RWC, CHL, NS, and PHT, respectively; it suggested partial dominance. Complete dominance was observed for BIO and GY, whose average degree of dominance was equal to unity, while super-dominance was involved in the genetic control of TKW and HI (Table 5). Thus, the preponderance of non-additive genetic control and low to intermediate heritability values suggested delaying selection to later advanced generation for the studied traits. These results agree with those reported by Dorri *et al*. (2014) and Fellahi *et al*. (2016).

Mohamed *et al*. (2013) reported predominance of additive gene effect in the inheritance of TKW while the predominance of non-additive gene effect was involved in the inheritance of PHT, NS, and GY per plant. These authors also noted that the average degree of dominance indicated overdominance for TKW, and partial to complete dominance for PHT, NS, and GY. Madic *et al*. (2002) found that the average degree of dominance indicated over-dominance in the inheritance of HI, which showed a low, narrow-sense heritability of 0.26 because of a strong environmental effect on the expression of this trait. However, Mohamed (2014) reported significant additive genetic effects and greater influence of epistasis.

Selection is concerned with changes of two or more characters simultaneously; thus, this study of the relationships between agronomic traits becomes important. This information helps to identify useful characters which are an indicator of grain yield potential. In this context, phenotypic correlation is less important than genetic correlation because environmental effects usually inflate it. Genetic correlation provides a measurement of genetic association between characters and is used in selection to target another character genetically more complex. Genotypic correlation coefficients, relating GY to the other studied traits, found in this study, were non-significant for CHL (-0.114ns), PHT (0.227ns), NS (0.143ns), NGS (0.114ns) and HI (0.182ns) and significant for RWC (0.780*), BIO (0.948*) and TWK (0.668*) (Table5). These results suggested that delayed selection based on RWC, BIO, or TKW may improve GY substantially. Expected response to selection is based on phenotypic variability, broad-sense heritability, and selection intensity (5% selection intensity). Based on the classification of this parameter, attributed to Johnson *et al*. (1955); values found in the present study were low, being less than 10% for RWC, moderate, in the 10 - 20% range, for PHT, TKW and HI, and high, above 20%, for CHL, BIO, NS, NGS, and GY (Table 5). These results corroborated findings of Majumder *et al*. (2008) who noted that PHT, NS, NGS, TKW, HI, and GY showed an appreciable

Table 5. Broad (h^2_{bs}) and narrow sense (h^2_{ns}) heritabilities, average degree of dominance ($\sqrt{H/D}$), genotypic correlation between GY (r_g) and the studied traits, and expected response to selection expressed as % of the trait overall mean (R%).

Trait	h^2_{bs}±SE	h^2_{ns}±SE	$\sqrt{H/D}$	r_g ± SE	R%
RWC (%)	0.94±0.30 *	0.69±0.14 *	0.65	0.780±0.237 *	06.55
CHL (CCI)	1.00±0.45 **	0.66±0.11*	0.71	-0.114±0.215 ns	20.71
PHT (cm)	0.91±0.29 **	0.62±0.14 *	0.77	0.227±0.250 ns	17.53
BIO (g)	0.95±0.05 **	0.50±0.17 *	0.99	0.948±0.307 *	65.32
NS	0.92±0.28 **	0.64±0.15 *	0.72	0.143±0.255 ns	51.45
NGS	0.90±0.40 **	0.93±0.10 *	0.15	0.114±0.172 ns	26.67
TKW (g)	0.93±0.56 ns	0.35±0.17 *	1.33	0.868±0.267 *	17.76
GY (g)	0.86±0.16 **	0.47±0.13 *	1.03	-	59.03
HI (%)	0.69±0.83 ns	0.19±0.10 ns	2.77	0.182±0.382 ns	17.82

ns: non-significant effect; *: significant effect at a 5% probability level; **: significant effect at a 1% probability level
RWC: relative water content; CHL: chlorophyll index; PHT: plant height; BIO: above-ground biomass; NS: number of spikes; NGS: number of grains per spike; TKW: thousand-kernel weight; GY: Grain yield; HI: harvest index.

genetic advance of 23.39, 13.31, 39.06, 33.88, 31.90, and 32.82%, respectively. Fellahi *et al*. (2015) reported estimates of the expected response to selection ranging from 10.58% for TKW to 63.25% for GY.

CONCLUSION

Results of the present investigation indicated that gene main (m) effects, derived from the six-parameter model, was significant for all studied traits; while significance of additive (d), dominance (h), additive×additive (i), additive×dominance (j) and dominance×dominance (l) gene effects and allelic interactions varied among traits. These results suggested that dominance played a major role in the inheritance of the studied traits and that the genes involved in the inheritance of these traits are predominantly dispersed in the parents with the presence of duplicate epistasis for RWC, BIO, and GY, and complementary epistasis for CHL. Narrow sense heritability values varied from 0.93 for NGS to 0.35 for TKW and significant for all trait except HI. The average degree of dominance values was less than unity for GY, RWC, CHL, NS, and PHT, suggesting partial dominance. Complete dominance was observed for BIO and GY, while super-dominance was involved in the genetic control of TKW and HI. High genotypic correlation coefficients were found between GY and RWC, BIO, and TWK. Expected responses to selection were low for RWC, moderate for PHT, TKW, and HI, and high for CHL, BIO, NS, NGS, and GY. Based on these findings it can be concluded that the investigated traits show complex genetic behavior, which implies that early selection would be less efficient; therefore, it is recommended delaying the selection to advanced generations to benefit from the reduction of non-fixable genetic variation and exploit transgressive segregators due to the significant interaction additivity×additivity (i) of the gene and duplicated epistasis.

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Combining ability as a function of inbreeding for fruit traits in *Cucurbita moschata* Duch. ex Poir.

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Habilidad combinatoria en función de la endogamia para caracteres del fruto en *Cucurbita moschata* Duch. ex Poir.

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Javier Alonso Restrepo-Salazar[1]*, Franco Alirio Vallejo-Cabrera[1] and Edwin Fernando Restrepo-Salazar[2]

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ABSTRACT

Three diallel crosses of butternut squash (*Cucurbita moschata*), each consisting of six parents with S_0, S_1, and S_2 levels of inbreeding, were evaluated to estimate the effect of inbreeding on both general (GCA) and specific combining abilities (SCA) for the following traits: fruit pulp thickness, number of seeds per fruit, fruit pulp color, dry matter, 100-seed weight, diameter of placental cavity, polar diameter of fruits, and equatorial diameter of fruits. A randomized complete block experimental design was used with four replicates, arranged in split plots, with the main plot corresponding to the diallel cross (level of inbreeding) and the subplot for the evaluated genotypes (six parents and 15 F_1 hybrids, in each of the diallel crosses). Additive effects (GCA) were responsible for the genetic control of most of the traits in the three diallel crosses, whereas non-additive effects (SCA) were also responsible for the genetic expression of most of the traits, but almost exclusively in crosses between S_1 and S_2 inbred lines. Recommended genotypes for the simultaneous genetic improvement of fruit pulp thickness and color for the fresh consumption market, which is formed by consumers who prefer whole, non-sliced fruit, were the S_0 parents (P3 and P4) as well as the between S_2 lines hybrid (P1×P6). On the other hand, a genotype recommended for the improvement of the same traits but intended either for agro-industrial use or for the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase (large fruits), was the S_2 parent (P2).

Palabras clave:
Agroindustria
Consumidores
Análisis dialélico
Híbridos
Líneas endogámicas
Ahuyama

RESUMEN

Se evaluaron tres cruzamientos dialélicos de zapallo *Cucurbita moschata*, conformados cada uno por seis progenitores con niveles de endogamia S_0, S_1 y S_2, para estimar el efecto de la endogamia en la habilidad combinatoria general (HCG) y específica (HCE) para las siguientes variables: grosor de la pulpa del fruto, número de semillas por fruto, color de la pulpa del fruto, materia seca, peso de cien semillas, diámetro de la cavidad placentaria, diámetro polar del fruto y diámetro ecuatorial del fruto. Se utilizó un diseño experimental de bloques completos al azar con cuatro repeticiones y arreglo en parcelas divididas, donde la parcela principal estuvo conformada por los cruzamientos dialélicos (tres niveles de endogamia) y la subparcela por los genotipos analizados en cada uno de los cruzamientos dialélicos (seis padres y 15 híbridos F_1). Los efectos aditivos (HCG) fueron los responsables del control genético de la mayoría de las variables en los tres cruzamientos dialélicos, mientras que los efectos no aditivos (HCE) fueron también los responsables de la expresión genética de la mayoría de las variables, pero casi exclusivamente en los cruzamientos realizados entre líneas endogámicas S_1 y S_2. Los genotipos recomendados para el mejoramiento genético simultáneo del grosor y color de la pulpa del fruto, con destino al mercado de consumo en fresco, constituido por consumidores que prefieren frutos enteros y no en rodajas, fueron los progenitores S_0 (P3 y P4) y el híbrido entre líneas S_2 (P1×P6). Por otro lado, el genotipo recomendado para el mejoramiento de las mismas variables mencionadas previamente, pero dirigido al uso agroindustrial o al mercado de consumo en fresco conformado por consumidores en los cuales el peso del fruto no es una característica limitante para su adquisición (frutos grandes), fue el progenitor S_2 (P2).

[1] Facultad de Ciencias Agropecuarias. Universidad Nacional de Colombia. AA 237 Palmira, Colombia.
[2] Escuela de Ingeniería. Universidad Pontificia Bolivariana. km 1 vía Tienda Nueva, Palmira, Colombia.
* Corresponding author: <jarestrepos@unal.edu.co>

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The butternut squash *Cucurbita moschata* (Duch. ex Lam.) Duch ex Poir. is an important species for food safety in the world due to its high nutritional value (Restrepo-Salazar *et al*., 2018a) or for providing medicinal benefits such as improved immune response through β-carotene (Kim *et al*., 2016). It also presents other medical benefits, such as anti-inflammatory, antioxidant, antidiabetic, antimicrobial, hypotensive, hepatoprotective, antiparasitic, and anticancer properties (Yadav *et al*., 2010). In addition, this species is used for agro-industrial purposes as food production for humans and animals, and biodiesel production from seed oil (Restrepo *et al*., 2018b).

Very few studies have been published about the effect of inbreeding on the genetic expression and control of different plant traits in *Cucurbita moschata*. Espitia (2004) evaluated two diallel crosses of *C. moschata* (between S_0 varieties and between S_1 inbred lines) and reported that additive effects played an important role in the expression of the following traits in the two generations evaluated: fruit production per plant, average fruit weight, number of fruits per plant, and 100-seed weight. Non-additive effects were only important in diallel crosses between S_1 inbred lines. Similar results were obtained by Ortiz *et al*. (2013) when evaluating the fruit production per plant in Candelaria (Valle del Cauca, Colombia) and by Restrepo-Salazar *et al.* (2018a), when evaluating the fruit production per plant and the average fruit weight in the same area. The above-mentioned researchers found, after evaluating three diallel crosses of *C. moschata* (between S_0 parents, between S_1 inbred lines, and between S_2 lines), that additive effects played an important role in the genetic control of the traits in all inbreeding generations, whereas non-additive effects were only important in crosses between S_1 and S_2 inbred lines (Ortiz *et al*., 2013; Restrepo-Salazar *et al*., 2018a). In other crops, such as maize, records also indicate that non-additive effects are more important in diallel crosses between inbred lines than in crosses between S_0 parents (Crossa *et al*., 1990; Rezende and Souza-Junior, 2000).

According to published literature on diallel crosses of *C. moschata,* there is no consensus about the type of gene action that predominates in the expression and genetic control of the traits: fruit pulp thickness, number of seeds per fruit, and 100-seed weight. In the case of diallel crosses between S_0 parents of *C. moschata*, some authors reported that both additive and non-additive effects was important in the genetic expression of the fruit pulp thickness (Espitia, 2004; Nisha and Veeraragavathatham, 2014; Abdein *et al*., 2017), the number of seeds per fruit (Marxmathi *et al*., 2018; Darrudi *et al*., 2018), and 100-seed weight (Nisha and Veeraragavathatham, 2014). Other authors like Espitia (2004) and Valdés *et al*. (2014) found that only additive gene effect was important for 100-seed weight, while Darrudi *et al*. (2018) reported that only non-additive gene effect was important for the same trait. Espitia (2004), for the number of seeds per fruit, and Marxmathi *et al*. (2018), for the fruit pulp thickness, found that neither of those effects was important in the expression and genetic control. On the other hand, in the specific case of diallel crosses between S_1 inbred lines of *C. moschata*, studies conducted by Mohanty (2000), Pandey *et al.* (2010), El-Tahawey *et al.* (2015), Ahmed *et al.* (2017), Singh *et al.* (2018), and Hatwal *et al*. (2018) reported the importance of both additive and non-additive effects in the expression and genetic control of the fruit pulp thickness. Following the impact of these effects, Mohsin *et al.* (2017) reported that only the non-additive gene effect was important, whereas other study found that neither of the effects was important (Begum *et al*., 2016).

According to the background in this field, this study aimed to evaluate the effect of inbreeding on the combining ability for eight traits of butternut squash fruit (*C. moschata*) and identify parents or F_1 hybrids that are outstanding; not only in terms of their combining ability but in terms of the fruit traits.

MATERIALS AND METHODS

Three diallel crosses, each involving six *C. moschata* parents with three levels of inbreeding (S_0 parents, S_1 and S_2 inbred lines derived from S_0 parents), were evaluated at the Experimental Center of the Universidad Nacional de Colombia–Palmira Campus. Table 1 presents the fruit traits of the six S_0 parents. A randomized complete block experimental design with four replicates was used. Field treatments were arranged in split plots, with the main plot corresponding to the diallel cross (level of inbreeding), and the subplot was used to evaluate

genotypes (six parents and fifteen direct crosses in each of the diallel crosses). Each experimental plot consisted of a five-plant furrow. A weighted selection index composed of traits such as average fruit weight (2.0–4.0 kg), fruit pulp thickness (3.5–5.0 cm) and salmon-colored pulp was used to select the fruits.

Table 1. Fruit traits of the six *Cucurbita moschata* S_0 parents used in the study.

Parent	Name	Geographic origin	Fruit traits[1]							
			FPT (cm)	FPC	DM (%)	DPC (cm)	PDF (cm)	EDF (cm)	NSF	100-seed weight (g)
P1	UNAPAL-Abanico-75-1	Atlantic Coast (Colombia)	3.97	Bright yellow (10)	12.53	12.21	17.64	20.15	301	11.12
P2	UNAPAL-Abanico-75-2	Atlantic Coast (Colombia)	3.33	Medium orange (12)	15.43	10.96	15.79	17.19	283	16.02
P3	UNAPAL-Dorado	Patía, Cauca (Colombia)	4.00	Medium orange (12)	13.00	17.50	15.80	21.50	372	11.40
P4	IC3A	Costa Rica (Central America)	4.38	Medium orange (12)	9.35	10.44	20.48	16.53	345	10.25
P5	UNAPAL-Llanogrande-1	Patía, Cauca (Colombia)	3.80	Medium orange (12)	11.31	10.00	15.74	16.58	263	8.91
P6	UNAPAL-Llanogrande-2	Patía, Cauca (Colombia)	4.00	Medium orange (12)	9.89	11.90	16.81	18.35	336	10.68

[1] FPT: fruit pulp thickness; FPC: fruit pulp color; DM: dry matter; DPC: diameter of the placental cavity; PDF: polar diameter of fruits; EDF: equatorial diameter of fruits; NSF number of seeds per fruit.

The following traits were evaluated: fruit pulp thickness (FPT) measured in cm; fruit pulp color (FPC) ranked from 1 to 15 based on the Roche Yolk Color Fan scale (Vuilleumier, 1969); dry matter (DM) measured as %; diameter of placental cavity (DPC), polar diameter of fruits (PDF), and equatorial diameter of fruits (EDF), all three measured in cm; number of seeds per fruit (NSF); and 100-seed weight measured in g. DM was determined by measuring the fresh weight of fruits and then oven-drying the fruits at 105 °C for 24 hours (Leterme and Estrada, 2012).

Genetic and statistical analyses were performed to estimate the combining ability of the different genotypes, using the method proposed by Hallauer and Miranda (1981), which partitions variation among genotypes (entries) into three components: parents, crosses and the parents vs. crosses contrast. Variance analysis and estimation of genetic effects were performed using the SAS/STAT® package, version 9.4 (SAS system for Windows, SAS Institute Inc©, 2012) and GENES (version 2.1 for Windows©, 2004) developed by Cruz (2013). The F-test was used for several sources of variation during the analysis of variance, and the Student's t-test was used to estimate genetic effects.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA)

Statistical differences were detected in the source of generational variation for all evaluated traits: FPT, FPC, DM, DPC, PDF, EDF, NSF, and 100-seed weight (Table 2), indicating that at least one of the inbred generations presented a mean value significantly different from the rest. Ortiz *et al.* (2013) reported similar results in *C. moschata* for FPC. On the other hand, significant differences were observed in the source of genotype variation in the three generations analyzed for all the traits. It can be inferred that there is at least one parent or hybrid that recorded an average value of FPT, FPC, DM, DPC, PDF, EDF, NSF, and 100-seed weight differed statistically from other averages in each of the generations (Table 2). Espitia (2004) also recorded statistical differences in the source of *C. moschata* genotypes for FPT, NSF, and 100-seed weight in the two inbreeding generations studied (S_0 and S_1). Ortiz *et al.* (2013) observed similar results in *C. moschata* for FPC, finding differences among genotypes in the three diallel crosses evaluated (S_0, S_1, and S_2).

For all evaluated traits, in most of the cases the parents and crosses variation sources presented statistical significance in all three generations, which confirmed that in general terms at least one of the S_0, S_1, or S_2 parents, or at least one of the crosses between said parents, showed an average performance of FPT, FPC, DM, DPC, PDF, EDF, NSF, and 100-seed weight that differed significantly from the others (Table 2). Based on these results, it is inferred that regardless of the inbreeding level, it is possible to identify at least one parent or a hybrid with

a mean value, in any of the traits, that differs statistically from the others. Significant differences have also been reported in *C. moschata* by Espitia (2004) regarding FPT, NSF, and 100-seed weight as well as by Ortiz *et al.* (2013) regarding FPC in both parents and crosses as sources in the different inbred generations under study.

Table 2. Mean squares of ANOVA for fruit pulp thickness (FPT), fruit pulp color (FPC), dry matter (DM), diameter of placental cavity (DPC), polar diameter of fruits (PDF), equatorial diameter of fruits (EDF), number of seeds per fruit (NSF), and 100-seed weight in three inbred generations of *Cucurbita moschata*.

Sources of variation	Traits								
	DF	FPT	FPC	DM	DPC	PDF	EDF	NSF	100-seed weight
Replicates (R)	3	0.89 **	1.81 **	84.28 **	9.68 **	3.83	3.30	2,387.35	3.17 *
Generations (D)	2	1.48 **	1.03 **	12.66 **	3.96 *	17.13 **	12.15 **	13,200.79 **	37.03 **
R×D	6	0.51 **	1.03 **	1.79	0.52	3.30	2.75	3,113.44	2.79 **
Genotypes (G)	60	0.62 **	0.83 **	17.25 **	12.38 **	11.94 **	22.74 **	11,317.94 **	9.51 **
Genotypes (S_0 G)	20	0.47 **	0.91 **	14.17 **	5.12 **	5.39 **	10.51 **	4,173.42 **	10.88 **
Parents (P_0)	5	0.57 *	1.74 **	19.59 **	5.18 **	3.08	10.28 *	6,842.67 *	25.91 **
Crosses (C_0)	14	0.43 **	0.66 **	13.24 **	3.64 **	5.87 **	7.96 **	2,299.35	5.29 **
GCA[1]	5	1.00 **	1.04 **	32.61 **	6.87 **	9.68 **	17.03 **	2,298.02	10.58 **
SCA[2]	9	0.11	0.45 *	2.48	1.86	3.75	2.92	2,300.09	2.35 **
P_0 vs. C_0	1	0.55	0.20	0.01	25.55 **	10.14	47.52 **	17,064.04 **	14.07 *
Genotypes (S_1 G)	20	0.50 **	0.84 **	19.82 **	16.16 **	15.57 **	25.83 **	11,946.26 **	9.23 **
Parents (P_1)	5	0.03	1.57 **	28.27 **	17.66 **	5.71	21.25 **	8,003.38 **	12.52 **
Crosses (C_1)	14	0.43 **	0.35	16.67 **	11.96 **	13.01 **	17.56 **	7,342.93 **	5.57 **
GCA	5	0.55 **	0.35	37.44 **	24.20 **	26.52 **	29.88 **	10,339.30 **	6.32 **
SCA	9	0.36 **	0.35	5.15 *	5.16 **	5.50 **	10.74 **	5,678.28 **	5.13 **
P_1 vs. C_1	1	3.75 **	4.00 **	21.61	67.43 **	100.67 **	164.48 **	96,107.41 **	44.07 **
Genotypes (S_2 G)	20	0.88 **	0.73 **	17.76 **	15.85 **	14.85 **	31.86 **	17,834.13 **	8.43 **
Parents (P_2)	5	0.26	1.57 **	22.69 **	9.46 **	14.80 **	14.65 **	18,812.67 **	3.19 *
Crosses (C_2)	14	0.63 **	0.45 *	17.18 **	10.27 **	3.39	20.00 **	11,093.89 **	3.93 **
GCA	5	1.32 **	0.44	39.92 **	24.19 **	4.03	48.18 **	9,523.44 **	6.90 **
SCA	9	0.24 *	0.46 *	4.55 *	2.54 **	3.03	4.37 *	11,966.36 **	2.27 **
P_2 vs. C_2	1	7.49 **	0.48	1.35	125.98 **	175.63 **	284.09 **	107,304.80 **	97.53 **
Error	180	0.13	0.21	2.15	0.96	2.11	1.92	1,666.37	0.87
Means		4.23	12.34	11.42	12.16	17.48	20.21	353.47	10.95
CV[3](%)		8.40	3.68	12.83	8.07	8.31	6.85	11.55	8.52

* Significant at a probability level of 0.05; ** Significant at a probability level of 0.01.
[1] General combining ability. [2] Specific combining ability. [3] Coefficient of variation

In the source of variation corresponding to the contrast between parents vs. crosses (P vs. C), significant differences were detected for most of the traits of the inbred generations, S_1 (FPT, FPC, DPC, PDF, EDF, NSF, 100-seed weight) and S_2 (FPT, DPC, PDF, EDF, NSF, 100 seed-weight), indicating that, overall, the average performance of all F_1 crosses (between S_1 or S_2 inbred lines) was higher than the average performance of parents as a whole (Table 2). Similar results were found by Espitia (2004) in S_1 for FPT, NSF, and 100-seed weight. On the other hand, in the S_0 inbred generation, statistical differences were only observed in the P vs. C contrast for DPC, EDF, NSF, and 100-seed weight (Table 2). Espitia (2004) reported similar results in S_0 for NSF but did not record differences for 100-seed weight in the S_0 generation in the contrast P vs. C.

In the diallel cross between S_0 parents only, the additive effects (GCA) were important in the genetic expression and control of FPT (Table 2). In contrast, other authors have reported in S_0 parents that in *C. moschata* both additive and non-additive effects (SCA) are important in the genetic expression of FPT (Espitia, 2004; Nisha and Veeraragavathatham, 2014; Abdein *et al*., 2017), while other authors have published that neither of the effects was important for FPT (Marxmathi *et al*., 2018). Regarding diallel crosses between S_1 and S_2 inbred lines, results indicated the importance of the additive and non-additive gene effect in the genetic control of FPT (Table 2). Mohanty (2000), Pandey *et al.* (2010), El-Tahawey *et al.* (2015), Ahmed *et al.* (2017), Singh *et al*. (2018), and Hatwal *et al*. (2018) reported similar results in the cross between S_1 inbred lines of *C. moschata*. In contrast, a study conducted by Begum *et al.* (2016) involving crosses between S_1 inbred lines of *C. moschata* indicate that neither of the effects was important in the expression of FPT, whereas Mohsin *et al.* (2017) only reported the importance of non-additive effects.

In the diallel cross between S_0 parents, neither of the two types of effects was important in the genetic expression and control of NSF (Table 2). This result indicates, on the one hand, that there is not enough statistical evidence to conclude that some parents differ in its ability to transmit genes that allow its progeny to increase or decrease its NSF; on the other hand, it suggests that there is not enough evidence to conclude that some of the hybrids had a different behavior than expected based on combining ability of their parents and general mean. Espitia (2004) reported similar results in *C. moschata*, finding that the additive and non-additive effects did not contribute in statistical terms to the genetic expression of NSF. In contrast, Marxmathi *et al*. (2018) and Darrudi *et al.* (2018) found that both types of effects were important for NSF. The importance of additive and non-additive gene action in the control of NSF was observed in the case of diallel crosses between S_1 and S_2 inbred lines (Table 2). Espitia (2004), El-Tahawey *et al.* (2015), and Mohsin *et al.* (2017) also found a significant contribution of both types of effects on the genetic expression of NSF in diallel crosses between S_1 inbred lines of *C. moschata*.

Both additive and non-additive effects were important in the genetic control of the 100-seed weight in the three diallel crosses evaluated (Table 2). These results agree with those found by Nisha and Veeraragavathatham (2014) in diallel crosses of *C. moschata* between S_0 parents, and by Espitia (2004), Mohsin *et al.* (2017) and Hatwal *et al*. (2018) in diallel crosses between S_1 inbred lines. On the other hand, Espitia (2004) reported that only additive effects were important in the expression of 100-seed weight in diallel crosses between S_0 parents of *C. moschata*, while Darrudi *et al*. (2018) reported that only non-additive effects were important. Valdés *et al.* (2014) recorded a differential response in diallel crosses between S_0 parents of *C. moschata* evaluated during two different planting seasons, finding that both additive and non-additive effects were important in the genetic expression of 100-seed weight during one season, while only additive effects were responsible for its expression in the same genotypes during another season.

In the diallel cross between S_0 parents, only additive effects were important in the genetic expression and control of PDF and EDF (Table 2). In contrast, Abdein *et al.* (2017), Kakamari and Jagadeesha (2017), and Marxmathi *et al*. (2018) have reported that both additive and non-additive effects are important in the genetic expression of PDF and EDF in *C. moschata*. In the case of diallel crosses between S_1 inbred lines, this study indicates that both types of effects were important in the genetic control of both traits. These results are similar to those reported by Jha *et al.* (2009), Ahmed *et al.* (2017), Mohsin *et al.* (2017), and Singh *et al*. (2018) for diallel crosses between S_1 parents in *C. moschata* and by Rana *et al.* (2015) in diallel crosses between advanced inbred lines.

Additive effects were the only component of important variation in the genetic control of DPC and DM in the diallel cross between S_0 parents (Table 2). Marxmathi *et al*. (2018), on the other hand, reported that no effect was important for DM. Both additive and non-additive gene actions were observed to be important in the genetic control of DPC and DM in diallel crosses between S_1 and S_2 inbred lines (Table 2). Similar results were recorded by Rana *et al.* (2015) for DPC in diallel crosses between advanced lines of *C. moschata*; both types of effects were found to control these traits. Regarding DM, these same authors reported that only additive effects contributed significantly to its genetic expression. On the

other hand, the analysis of data for FPC indicated that additive effects were important in its genetic expression and control only in diallel crosses between S_0 parents, whereas non-additive effects were important in its control in diallel crosses between S_0 parents and between S_2 inbred lines (Table 2). In contrast, Ortiz *et al.* (2013) reported the importance of additive effects in all generations evaluated (S_0, S_1, S_2), while non-additive effects were only important in the expression of FPC in the S_0 generation.

The joint analysis of the results of the three diallel crosses indicates that, in general terms, additive effects were responsible for the genetic expression and control for most of the traits in crosses made between the different inbred generations evaluated. Non-additive effects, on the other hand, were also responsible for the genetic control for most of the traits, but almost exclusively in the crosses made between S_1 and S_2 inbred lines. This could be attributed to the greater genetic divergence occurring in crosses between parents with a narrow genetic base in contrast to crosses between broad-based parents. Espitia (2004) observed similar results in *C. moschata* for 100-seed weight, yield, and yield components in diallel crosses between S_0 parents and between S_1 lines.

General combining ability (GCA) effects

A differential response was observed in parents in their general combining ability (GCA) effects for FPT, indicating the highly significant differences in additive effects detected by ANOVA in the three diallel crosses (Table 2). The S_0 parents (P3 and P4) and the S_2 inbred lines (P1, P2, and P3) presented significant GCA effects values as well as highest FPT values (Table 3). Espitia (2004), Nisha and Veeraragavathatham (2014), and Abdein *et al.* (2017) also recorded at least one S_0 parent with significant GCA effects values. Other authors (Mohanty, 2000; Espitia, 2004; Pandey *et al.*, 2010; El-Tahawey *et al.*; 2015, Ahmed *et al.*, 2017; Singh *et al.*, 2018; Hatwal *et al.*, 2018) have also reported the existence of at least one S_1 line with significant GCA effects values. However, Rana *et al.* (2015) and Begum *et al.* (2016) did not find any inbred line of *C. moschata* with significant GCA effects values. Of the outstanding genotypes mentioned previously, S_0 parents (P3 and P4) are recommended to genetically improve FPT for the fresh consumption market formed by consumers who prefer whole, non-sliced fruits, taking advantage of the additive effects of intrapopulation recurrent selection (IRS). The S_2 inbred line (P2) is recommended for the improvement of FPT for agro-industrial use or the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase (Table 3).

When evaluating FPC, almost all the S_0 parents and S_1 and S_2 inbred lines recorded significant GCA effects values

Table 3. *Cucurbita moschata* parents showing general combining ability (GCA) effects that are significant for fruit pulp thickness (FPT), fruit pulp color (FPC), polar diameter of fruits (PDF), and 100-seed weight, obtained in diallel crosses between S_0 parents and between S_1 and S_2 inbred lines.

Trait	Generation								
	S_0			S_1			S_2		
FPT	P2	**-0.21** **	*3.96*	P6×P6	**-0.28** *	*4.17*	P1×P1	**0.21** *	*4.69*
	P3	**0.38** **	*4.44*				P2×P2	**0.22** *	*4.70*
	P4	**0.21** **	*4.30*				P3×P3	**0.30** **	*4.78*
	P6	**-0.25** **	*3.94*				P5×P5	**-0.37** **	*4.22*
							P6×P6	**-0.28** **	*4.29*
FPC	P2	**-0.21** **	*12.17*	P1×P1	**-0.13** **	*12.27*	P2×P2	**-0.08** *	*12.47*
	P3	**-0.15** *	*12.09*	P2×P2	**0.18** **	*12.50*	P3×P3	**-0.27** **	*12.32*
	P4	**-0.21** **	*12.23*	P3×P3	**-0.19** **	*12.31*	P4×P4	**0.16** **	*12.59*
	P5	**0.29** **	*12.48*	P4×P4	**0.06** *	*12.49*	P5×P5	**0.16** **	*12.56*
	P6	**0.35** **	*12.51*	P5×P5	**-0.06** *	*12.41*			
				P6×P6	**0.12** **	*12.55*			
PDF							P5×P5	**0.88** **	*18.52*
100-seed weight				P2×P2	**0.98** *	*11.73*	P5×P5	**-0.99** *	*10.54*

* Significant at a probability level of 0.05. ** Significant at a probability level of 0.01
Values in bold: GCA effects values. Values in italics: average values of hybrids in diallel crosses between S_0 parents and S_1 and S_2 inbred lines.

and a medium orange color according to the Roche Yolk Color Fan scale (Vuilleumier, 1969) (Table 3). Of these genotypes, S_0 parents (P3 and P4) are recommended for the genetic improvement of FPC destined for the fresh consumption market. This market is formed by consumers who prefer whole, non-sliced, fruits. The S_2 inbred line (P2) is recommended for agro-industrial use or the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase. Ortiz *et al.* (2013) also identified in *C. moschata* at least one S_0 parent or S_1 and S_2 lines with significant GCA effects values for FPC.

In the case of 100-seed weight, the only genotypes that reported significant GCA effects values were the S_1 inbred line (P2) and the S_2 inbred line (P5) (Table 3). These genotypes, however, did not show high 100-seed weight values. Other authors haver reported similar results for 100-seed weight in S_1 lines (Espitia, 2004; Mohsin, 2017; Hatwal *et al*., 2018). Espitia (2004) found at least one S_0 parent of *C. moschata* with significant GCA effects values for 100-seed weight, whereas Valdés *et al.* (2014) did not find any S_0 parent with significant GCA effects values.

Only the S_2 line (P5) recorded significant GCA effects value for PDF (Table 3). Similar results have been reported in *C. moschata* by several authors (Jha *et al*., 2009; Rana *et al*., 2015; Ahmed *et al*., 2017; Mohsin *et al*., 2017; Singh *et al*., 2018), who found at least one inbred line with significant GCA effects values, indicating that for this specific trait, only a few genotypes evaluated had the ability to transmit favorable genes to their progenies. Kakamari and Jagadeesha (2017) and Marxmathi *et al*. (2018) reported at least one S_0 parent with significant GCA effects values for PDF. Finally, in the case of DM, DPC, EDF, and NSF, no parent was genetically superior to the other parents under this study (Table 3). Rana *et al.* (2015) did not find any inbred line of *C. moschata* with significant GCA effects values for DPC and EDF; however, they did record at least one inbred line with a significant GCA effects value for DM. Jha *et al.* (2009) also reported the non-existence of *C. moschata* lines with significant GCA effects values for EDF. However, Ahmed *et al.* (2017), Mohsin *et al.* (2017), and Singh *et al.* (2018) recorded significant GCA effects for this trait in inbred lines, whereas Kakamari and Jagadeesha (2017), and Marxmathi *et al*. (2018) reported at least one S_0 parent with significant GCA effects values for EDF. Furthermore, other authors (Espitia, 2004; El-Tahawey *et al*., 2015; Mohsin *et al*., 2017) identified at least one inbred line of *C. moschata* with significant GCA effects values for NSF, differing from the results found in the current study.

The joint analysis of all the traits evaluated in this study indicated that, for butternut squash destined to the fresh consumption market formed by consumers who prefer whole, non-sliced, fruits, S_0 parents (P3 and P4) can be suggested as genotypes to improve FPT and FPC genetically, taking advantage of the additive effects of IRS. These parents presented significant GCA effects values for both traits, with P3 presenting an FPT of 4.40 cm and P4, one of 4.30 cm; both presented a medium orange FPC (Table 3). In addition, they presented acceptable average values for the other studied traits. On the other hand, in the case of butternut squash for agro-industrial use or the fresh consumption market consisting of consumers for whom fruit weight is not a limiting characteristic for purchase, S_2 parent (P2) can be recommended for the simultaneous improvement of FPT and FPC, taking advantage of both additive and non-additive effects by IRS. This genotype reported significant GCA effects values for both traits, with an FPT of 4.70 cm and a medium orange FPC (Table 3). It also presented acceptable average values for the other traits under study.

Specific combining ability (SCA) effects

Several crosses between S_0 parents or between S_1 and S_2 inbred lines presented significant SCA effects for FPT (Table 4), presenting values above the expected average based on the GCA effects values of parents and the overall average. Similar results were observed in *C. moschata* by Espitia (2004), who reported at least one cross between S_0 parents with significant SCA effects values. Other authors have also reported the existence of at least one inbred line with significant SCA effects values for FPT in *C. moschata* (Mohanty, 2000; Pandey *et al*., 2010; El-Tahawey *et al*., 2015; Rana *et al*., 2015; Ahmed *et al*., 2017; Mohsin *et al*., 2017; Singh *et al*. 2018; Hatwal *et al*., 2018). However, Espitia (2004) and Begum *et al.* (2016) did not find any S_1 inbred line of *C. moschata* with significant SCA effects values for FPT. Of the outstanding hybrids mentioned; in the case of the fresh consumption market formed by consumers who prefer whole, non-sliced fruit. The hybrids between S_0 parents (P1×P4) and (P2×P3) are the genotypes

recommended to improve FPT, taking advantage of additive effects and allowing superior-performance varieties or lines obtained by transgressive segregation. The S_2 hybrid (P1×P6) is recommended to improve FPT for the same market, using reciprocal recurrent selection (RRS) to take advantage of both types of effects. In the case of genotypes for agricultural use or for the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase, the between S_1 inbred line hybrids (P1×P5), the S_2 inbred line hybrids (P1×P3), and (P2×P6) are recommended for improving FPT by RRS (Table 4).

Table 4. Hybrids of *Cucurbita moschata* obtained by diallel crosses between S_0 parents and between S_1 and S_2 inbred lines, showing significant combining ability effects (SCA effects) for fruit pulp thickness (FPT), fruit pulp color (FPC), dry matter (DM), diameter of placental cavity (DPC), polar diameter of fruits (PDF), equatorial diameter of fruits (EDF) and 100-seed weight.

Trait	Generation								
	S_0			S_1			S_2		
FPT	P1×P2	**-0.19** **	*3.69*	P1×P2	**-0.40** **	*4.07*	P1×P3	**0.30** **	*5.36*
	P1×P4	**0.23** **	*4.54*	P1×P4	**0.17** *	*4.54*	P1×P4	**0.13** *	*4.79*
	P1×P6	**-0.15** **	*3.71*	P1×P5	**0.41** **	*5.09*	P1×P6	**-0.43** **	*4.01*
	P2×P3	**0.07** **	*4.37*	P1×P6	**-0.18** *	*4.02*	P2×P3	**0.24** **	*5.09*
	P2×P4	**-0.16** **	*3.99*	P2×P3	**0.23** **	*4.77*	P2×P5	**-0.15** **	*4.23*
	P2×P5	**0.06** *	*3.89*	P2×P4	**-0.16** *	*4.07*	P2×P6	**0.17** **	*4.62*
	P2×P6	**0.20** **	*3.87*	P2×P5	**0.18** *	*4.76*	P3×P4	**-0.34** **	*4.41*
	P3×P4	**-0.10** **	*4.60*	P3×P4	**-0.19** *	*4.20*	P3×P5	**-0.13** *	*4.33*
	P3×P5	**-0.13** **	*4.28*	P3×P5	**-0.15** *	*4.61*	P3×P6	**0.14** **	*4.70*
	P4×P5	**0.09** **	*4.32*	P4×P6	**0.27** **	*4.26*	P4×P5	**0.18** **	*4.24*
	P4×P6	**-0.07** **	*4.05*	P5×P6	**-0.35** **	*3.98*			
FPC	P1×P2	**0.22** *	*12.22*	P1×P3	**0.16** *	*12.25*	P1×P2	**-0.31** **	*12.18*
	P1×P3	**-0.59** **	*11.36*	P1×P4	**-0.59** **	*11.78*	P1×P4	**-0.31** **	*12.31*
	P1×P5	**0.22** *	*12.58*	P1×P5	**0.28** **	*12.53*	P1×P5	**0.43** **	*12.86*
	P2×P6	**-0.21***	*12.31*	P2×P3	**-0.15** *	*12.35*	P1×P6	**0.31** **	*12.64*
	P3×P4	**0.53** **	*12.44*	P2×P4	**0.35** **	*13.00*	P2×P4	**0.25** **	*12.81*
	P4×P5	**-0.40** **	*12.19*	P2×P5	**-0.28** **	*12.32*	P3×P4	**0.43** **	*12.64*
				P3×P4	**0.22** **	*12.51*	P3×P5	**-0.31** **	*12.02*
				P3×P5	**-0.15** *	*12.11*	P4×P6	**-0.38** **	*12.39*
DM	P3×P6	**-1.29** *	*10.96*	P1×P3	**2.19** *	*17.55*			
DPC	P1×P2	**-0.83** *	*12.26*				P1×P4	**1.09** *	*14.24*
	P2×P6	**1.12** **	*13.06*				P2×P6	**1.27** *	*15.03*
	P5×P6	**-1.04** *	*9.71*						
PDF							P5×P6	**-1.46** *	*17.52*
EDF	P2×P6	**1.67** *	*20.53*						
	P5×P6	**-1.25** *	*16.77*						
100-seed weight	P1×P2	**-1.15** *	*12.48*	P1×P2	**-2.43***	*9.93*	P3×P4	**1.53****	*13.24*
	P3×P4	**0.98** *	*12.89*						

* Significant at a probability level of 0.05. ** Significant at a probability level of 0.01
Values in boldface: SCA effects values. Values in italics: Average values of hybrids.

The analysis of FPC indicated that several crosses between S_0 parents or between S_1 and S_2 inbred lines were identified with significant SCA effects values (Table 4). Of these crosses, the hybrid between S_2 inbred line (P1×P6) is recommended to genetically improve FPC for the fresh consumption market formed by consumers who prefer whole, non-sliced fruits. Ortiz *et al.* (2013) had also reported the existence of at least one cross between S_0 parents in *C. moschata* with a significant SCA effects value.

In the case of DPC, three hybrids between S_0 parents and two between S_2 inbred lines presented significant SCA effects values (Table 4). Of these, the hybrid S_2 inbred line (P2×P6) is recommended to improve DPC by RRS for agro-industrial use or for the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase. Rana *et al.* (2015) also reported the existence of at least one hybrid between advanced inbred lines of *C. moschata* with significant SCA effects values for DPC.

In the case of 100-seed weight, two hybrids between S_0 parents, one between S_1 inbred lines and another between S_2 inbred lines showed significant SCA effects values (Table 4). Similar results were reported in *C. moschata* by several authors (Espitia, 2004; Valdés *et al*., 2014; Nisha and Veeraragavathatham, 2014), who observed at least one cross between S_0 parents with significant SCA effects values. Other authors have also reported the existence of at least one inbred line of *C. moschata* with significant SCA effects values for 100-seed weight (Espitia, 2004; El-Tahawey *et al*., 2015; Mohsin *et al*., 2017; Hatwal *et al*., 2018).

One hybrid between S_0 parents and another between S_1 inbred lines presented significant SCA effects values for DM (-1.29* and 2.19*, respectively) (Table 4), indicating that their DM contents were lower in the first hybrid and higher in the second one, with respect to expected mean with base in GCA effects of its parents and general mean. Rana *et al.* (2015) had also observed the existence of at least one hybrid between advanced inbred lines of *C. moschata* with significant SCA effects values for DM.

Only two crosses with significant SCA effects values were identified in the case of EDF. These corresponded to hybrids between the S_0 parents (P2×P6) and (P5×P6) (Table 4). Kakamari and Jagadeesha (2017) and Marxmathi *et al*. (2018) found similar results in hybrids between S_0 parents for EDF. On the other hand, the existence of at least one hybrid between inbred lines of *C. moschata*, with significant SCA effects values for EDF, has been reported by other authors (Jha *et al*., 2009; Rana *et al*., 2015; Ahmed *et al*., 2017; Mohsin *et al*., 2017; Singh *et al*., 2018).

This study only revealed one cross with a significant SCA effects value for PDF, the hybrid between S_2 inbred lines (P5×P6) (Table 4). Jha *et al.* (2009), Ahmed *et al.* (2017), Mohsin *et al.* (2017) and Singh et al. (2018) had also recorded the presence of at least one hybrid between inbred lines with significant SCA effects values for PDF. Kakamari and Jagadeesha (2017) and Marxmathi *et al*. (2018), on the other hand, reported at least one hybrid between S_0 parents with significant SCA effects values for PDF.

No crosses presented significant SCA effects values for NSF. Espitia (2004) had not reported the existence in *C. moschata* of crosses between S_1 inbred lines with significant SCA effects values. However, the presence of at least one cross between inbred lines with significant SCA effects values has been observed by other authors (El-Tahawey *et al*., 2015; Mohsin *et al*., 2017). Espitia (2004), Marxmathi *et al*. (2018), and Darrudi *et al*. (2018), on the other hand, reported at least one cross between S_0 parents with significant SCA effects values for NSF.

The joint evaluation these results indicated that in the case of the fresh consumption market formed by consumers who prefer whole, non-sliced fruits; the hybrid between S_2 inbred lines (P1×P6) is the cross recommended for the simultaneous genetic improvement of FPT and FPC. It takes advantage of both additive and non-additive effects by RRS and records a significant SCA effects value for both traits, presenting an FPT of 4.01 cm and a medium orange FPC (Table 4). In addition, this hybrid presented acceptable average values for the other traits studied.

CONCLUSIONS

Additive effects were responsible for the genetic expression and control for most of the fruit traits evaluated in the three diallel crosses. Non-additive effects were also responsible for the genetic control of most of the

traits, but almost exclusively in crosses between S_1 and S_2 inbred lines. In the case of the fresh consumption market formed by consumers who prefer whole, non-sliced fruits, the S_0 parents UNAPAL-Dorado and IC3A (P3 and P4) were recommended for the simultaneous genetic improvement of traits fruit pulp thickness and fruit pulp color, taking advantage of additive effects. In the case of genotypes destined for agro-industrial use or for the fresh consumption market formed by consumers for whom fruit weight is not a limiting characteristic for purchase, the S_2 parent UNAPAL-Abanico-75-2 (P2) is recommended for the simultaneous improvement of traits fruit pulp thickness and fruit pulp color, taking advantage of additive effects. In the case of the fresh consumption market formed by consumers who prefer whole, non-sliced fruits, the hybrid between S_2 inbred lines UNAPAL-Abanico-75-1×UNAPAL-Llanogrande-2 (P1×P6) is recommended for the simultaneous genetic improvement of traits FPT and FPC, taking advantage of both additive and non-additive effects. After evaluating the effect of inbreeding on the genetic expression and control of fruit traits analyzed in this study, it was found that non-additive effects are important in diallel crosses between inbred lines than in those between S_0 parents.

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Physicochemical, sensory and stability properties of a milk caramel spread sweetened with a glucose-galactose syrup from sweet whey

Propiedades fisicoquímicas, sensoriales y de estabilidad de un dulce de leche con adición de sirope glucosa-galactosa de lactosuero dulce

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Sandra Vargas-Díaz[1]*, José U. Sepúlveda-V[1], Héctor J. Ciro-V[1], Ana Juleza Mosquera[1] and Edinson Bejarano[1]

Keywords:
Milk caramel spread
Sensory analysis
Sweeteners
Texture
Whey

ABSTRACT

Whey is a dairy industry by-product with an adverse environmental impact; therefore, obtaining sweeteners from it promote a circular economy and is an alternative to mitigate the environmental problems. The aim of this research was to evaluate the effect of different inclusions (10%, 20%, and 30%) of Glucose-Galactose syrup (GGS), obtained from whey, on the physicochemical, sensory, and stability properties of a milk caramel spread. Results showed that the syrup has a significant effect on the techno-functional properties of the final product since it turns into a darker color when the concentrations of syrup increased. Besides, the yield of the product was higher (41.4%), providing a higher volume. In a replacement of 30% GGS, the milk caramel spread at a lower amount of soluble solids achieved a texture similar to the other inclusions. At a sensory level, consumers accepted all formulations with an acceptance higher than 90%. During the storage time (60 d), different evaluated parameters increased.

Palabras clave:
Dulce de leche
Análisis sensorial
Edulcorantes
Textura
Lactosuero

RESUMEN

El lactosuero es un subproducto de la industria láctea con alto impacto ambiental, por ello, obtener edulcorantes a partir de este promueve una economía circular y es una alternativa para mitigar el problema ambiental. El objetivo de esta investigación fue evaluar el efecto de diferentes inclusiones (10%, 20% y 30%) del sirope glucosa-galactosa (SGG), obtenido a partir de lactosuero, sobre las propiedades fisicoquímicas, sensoriales y de estabilidad en un dulce de leche. Los resultados mostraron que el sirope tiene un efecto significativo en las propiedades tecno-funcionales del producto final, el cual presenta un color más oscuro a medida que se aumenta su concentración. Además, el rendimiento del producto fue mayor (41,4%) proporcionando un mayor volumen. Cuando se logra un reemplazo del 30% de SGG, se obtiene una característica de textura similar a las demás inclusiones a menos sólidos solubles. A nivel sensorial, los consumidores aprobaron todas las formulaciones con una aceptación superior al 90%. Durante el tiempo de almacenamiento (60 d) los diferentes parámetros evaluados aumentaron.

[1] Facultad de Ciencias Agrarias. Universidad Nacional de Colombia. AA 3840, Medellín, Colombia.
* Corresponding author: <salvargasdi@unal.edu.co>

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Milk caramel spread is a dairy product obtained generally by evaporation of milk at atmospheric pressure with the addition of sucrose until reaching 70% of total solids (Berwerth, 2006). In this process, sucrose is partially replaced usually by glucose syrup to avoid its crystallization, besides it participates as reducing sugar in the Maillard reactions (Moro and Hough, 1985). Besides, sodium bicarbonate is used as an ingredient to prevent the coagulation of proteins and help to get the golden color characteristic of this product. Milk caramel spread represents an important market to explore and promote because the increase in the average world consumption has been higher than 40% since 1990 (SENATI, 2011).

Usually, glucose syrup is used as a partial substitute for sugar during the preparation of milk caramel spread; being essential for its sweet flavor, contributing to the total solids of the product, and providing shine and texture (Garitta e*t al*., 2004).

Whey is a dairy industry by-product produced during cheese making, 90% of the total volume of milk used for its manufacturing becomes whey (Prazeres *et al*., 2012). Additionally, it has nutrients such as serum proteins, lactose, and minerals; where the lactose is present in greater quantity and has a greater polluting level (Beltran and Acosta, 2012). A sweetener like glucose-galactose syrup (GGS) could be obtained from sweet whey; it has lactose, glucose, and galactose in its composition, being able to provide characteristics similar to the glucose syrup (Somov *et al*., 2015). The GGS is used as a sweetener that could substitute sucrose, not only in the manufacture of milk caramel spread but also as an ingredient in other products, reducing the whey environmental impacts.

Different authors have reported the use of sweetening agents in milk caramel spread, highlighting the addition of sucrose, invert sugar, polydextrose, fructose, and sorbitol, mainly because of their sweetness attributes (Queiroz *et al*., 2009; Valencia *et al*., 2008; Valencia and Millán, 2008). Other studies have evaluated the effect of incorporating flours (Lopes *et al*., 2015) for the functionality and stability they can confer to this product. The addition or modification of raw materials in this type of products has different effects on their quality characteristics, the most important being texture, pH, moisture content, viscosity, and general acceptability of the product over time (Andrade *et al*., 2009).

The purpose of this study was to evaluate different inclusions of GGS regarding the physicochemical, sensory, and stability properties of a milk caramel spread during storage at environmental conditions.

MATERIALS AND METHODS

Raw material

GGS (68-72 °Brix) was obtained by evaporating an enzymatic hydrolyzate from a lactose concentrate. The concentrated lactose was obtained employing the membrane filtering process (ultrafiltration and nanofiltration) of sweet whey, in a 100 L capacity filter pilot plant with a ceramic membrane and cut-off size of 100 Da. The operating conditions used for the nanofiltration process were: Temperature of 23 °C, concentration factor of 5, an inlet pressure of 31.5 bar and stage pressure of 2 bar. This lactose concentrate was hydrolyzed with β-galactosidase (1.2 mL L^{-1}) at a temperature of 36 °C for 3 hours (Perez-Escobar *et al*., 2020). This product was stored under refrigeration temperature (4 °C) until preparation of the milk caramel spread. Sucrose was purchased from a local supplier.

Characterization of raw materials

The GGS was characterized by measuring the following properties: pH (AOAC 945.10), acidity for lactic acid (AOAC 945.64), moisture content (AOAC 925.45), total solids (AOAC 925.45), soluble solids (AOAC 932.14), proteins (AOAC 991.20), minerals (AOAC 985.35), and ashes (AOAC 923.03). The apparent viscosity measurement was stablished at 4 °C using a Brookfield DV-III rheometer and concentric cylinder geometry. Lactose, glucose, galactose, and lactic acid were quantified by High-Performance Liquid Chromatography (HPLC), using an AGILENT 1200 series chromatograph, with an AMINEX HPX-87H ion-exchange column (300×7.8 mm), the concentration of the standards (lactose, glucose, galactose, and lactic acid) were from 1 to 5 g L^{-1}, and as mobile phase a solution of H_2SO_4 0.008 N at a constant flow of 0.6 mL min^{-1}. The sweetening power was determined through a sensory test per multidimensional approach with a three-trained panel. This test was performed according to Colombian Technical Standards NTC 3501 and NTC 3915.

Whole milk was used for making milk caramel spread and the following characteristics were determined: Content of total solids (NTC 4979), proteins (AOAC 972.16), fat (AOAC 989.04), acidity as a percentage of lactic acid (NTC 4623), density (Quevenne lactodensimeter), cryoscopic point (DE568) and pH (NTC 4592).

Milk caramel spread preparation

A final sweetness of 17% was proposed for the milk caramel spread during its formulation. Sodium citrate, at 0.1% of the total mixture, was added to the previously filtered milk. Milk heating was started with continuous agitation when it reached a temperature of 30 °C, sugar and powder milk were added. The sweetener was neutralized with sodium bicarbonate ($NaHCO_3$) to achieve 0.12% lactic acid since, without this pre-treatment, the milk caramel spread generated a sandy texture. Immediately, when this mixture reached a concentration of 40 °Brix, the previously treated sweetener (GGS) was added, and the evaporation processing was continuously running until reaching a concentration of 70 to 75 °Brix. The milk caramel spread was poured into the containers, allowed to cooling at room temperature; finally, it was stored.

Physicochemical analysis of milk caramel spread

The pH was determined using an OHAUS STARTER 3100 pH-meter potentiometer (NTC 4592). The color was evaluated through the tristimulus colorimetry technique with the Konica Minolta CR-400 series colorimeter. Total solids (measured as degrees Brix, °Brix) were measured with a gravimetric method (AOAC 925.45); determination of viscosity was achieved using a Brookfield DV-III rheometer with an RV7 spindle, measurements were performed at 25 °C and 10 rpm (Andrade *et al*., 2009). Fat content was measured through the modified Babcock method. Protein was determined by the Kjeldahl method (AOAC 991.20-23). Soluble solids were evaluated by means of a digital refractometer (HI 96801) (Baldasso *et al*., 2011). Water activity was measured by the dew point method with the Aqualab 4TE series equipment (AOAC, 2000, 2007). Moisture content was obtained using a Memmert vacuum stove (AOAC 977.21). Minerals were determined through atomic absorption spectrophotometry (NTC 5151). The spreadability analysis was carried out with a conical perspex probe 30P/30C in a texturometer TA-TX2, with a pre-test speed of 10 mm s^{-1}, test speed of 3 mm s^{-1} and a distance of 30 mm. The yield of the process was determined as follows where W_i is the initial weight and W_f is final weight:

$$Y(\%) = \frac{W_i - W_f}{W_i} \times 100$$

Sensory evaluation of milk caramel spread

The intensity attributes of milk caramel spread were evaluated following the Colombian technical standard NTC 2680. This panel was comprised of 20 expert technicians belonging to the Laboratory of Dairy Products of the Universidad Nacional de Colombia - Sede Medellín. In the sensory testing, the intensity of characteristics as texture, color, aroma, crystallization, and spreadability on a structured scale of 10 points was considered. Acceptability tests were carried out with 100 consumers for the different samples of milk caramel spread 24 hours after its preparation. Consumer perception regarding the general acceptability was evaluated in a structured hedonic scale of 9 points.

Product stability

The pH, apparent viscosity, color, water activity, and moisture content were evaluated over a period of 60 days under storage conditions (Temperature of 25 °C and relative humidity of 60%) using a Memmert ICH 260 climatic chamber. Milk caramel spreads were packaged in polypropylene bags.

Statistical analysis

A randomized experimental factorial design with four factors determined by the substitution (in relation to sweetness) of 0%, 10%, 20%, and 30% of sucrose for GGS (72 °Brix) was established, for a total of 12 experiments. The experimental data of the response variables were analyzed by ANOVA and Fisher's LSD method at a 5% significance level.

RESULTS AND DISCUSSIONS

Table 1 shows the physicochemical properties of GGS, indicating an acid raw material, dense with low humidity, yellow-green color due to the content of vitamin B2 (Arndt and Wehling, 1989), together with a high amount of minerals and a sweetening power of 50% regarding the sucrose value. In addition, the GGS is a non-Newtonian pseudoplastic fluid according to its rheological properties. Milk showed high-quality characteristics according to the NTC 399.

Table 1. Physicochemical characterization of the GGS and cow's milk used as a raw material for the preparation of milk caramel spread.

Property	Mean Value
GGS	
Acidity (% Lactic acid)	1.250±0.088
pH	4.850±0.073
Density (Kg m^{-3})	1370±12
Moisture (%)	24.24±2.62
Water activity	0.73±0.026
Soluble Solids (°Brix)	72.51±1.85
n-Fluency index	0.848±0.071
k- consistency index (Pas^n)	1.416±0.224
Color	L*: 35.18±1.85 a*: -3.35±0.95 b*: 16.89±1.96
Protein (% w/w)	<2.5%
Calcium (% w/w)	0.26±0.04
Phosphorus (% w/w)	0.38±0.04
Magnesium (mg Kg^{-1})	834±60
Potassium (% w/w)	0.98±0.13
Ashes (% w /w)	3.45±0.26
Lactose (Kg m^{-3})	192.85±4.97
Glucose (Kg m^{-3})	392.86±10.84
Galactose (Kg m^{-3})	297.51±11.62
Lactic acid (Kg m^{-3})	52.80±18.68
Sweetening power	0.5
MILK	
Density (Kg m^{-3})	1031.3±0. 4
pH	6.68±0.04
Acidity (% Lactic acid)	0.17±0.01
Fat (%)	3.7±0.1
Protein (Kg m^{-3})	3.25±0.06
Non-fatty solids (%)	8.70±0.08
Total solids (%)	12.37±0.13
Cryoscopic point	0.514±0.003

Physicochemical properties of milk caramel spread

The pH value showed a statistical significance (P<0.05) among samples with addition of GGS, where pH decreased at the highest level of GGS inclusion even after neutralization, due to the presence of syrup, with values close to the reported by Novoa and Ramírez-Navas (2012) (Figure 1A). Hence, as a result of this low sweetening power, it should be added in greater quantity

than sugar. This decrease in pH by increasing the GGS level was also related to the Maillard reactions because there is an interaction between the amino groups of milk proteins and reducing sugars such as glucose, galactose, and lactose. As there are a greater number of molecules of nitrogen interacting with sugars, the pH will be lower since these amino groups help to have alkaline pH and when reacting, acid groups predominate causing a decrease in pH (Belitz *et al.*, 2012).

The moisture content was similar for 10 and 20% of GGS inclusion, whereas, for 30%, there was significantly different (P<0.05). This change is due to the process was stopped at lower soluble solids because it achieved the appropriate texture at a shorter evaporation time. Since the evaporation time was shorter than the other treatments, more water remained inside the product. The moisture content was close to the defined in the Colombian technical standard NTC 3757. Also, these values coincided with the reported by Andrade *et al.* (2009), where values of 22% of moisture content for a milk caramel spread from buffalo milk were found.

For soluble solids, results indicated a statistically significant effect (P<0.05) for GGS inclusion (Figure 1B).

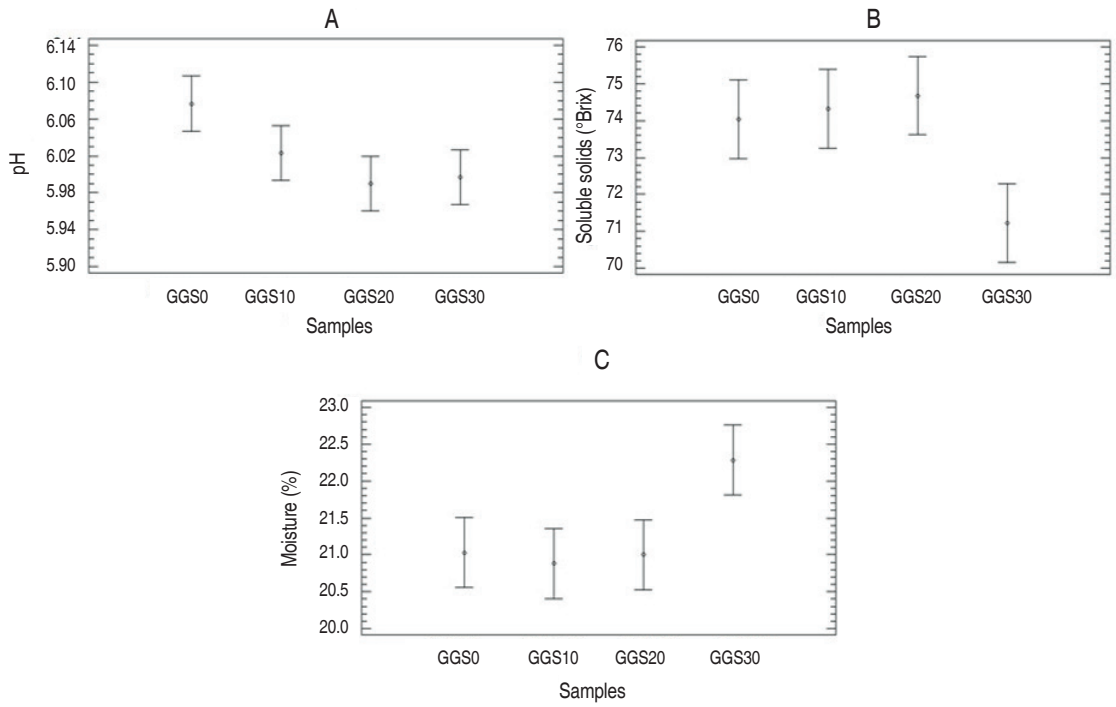

Figure 1. Physicochemical parameters of milk caramel spread with different contents of GGS. A. pH; B. soluble solids; C. moisture content.

Thus, to reach the desired texture, about 74 and 75 °Brix were required when adding up to 20% of sweetener; while when 30% of the syrup was added, this texture was achieved at soluble solids of 70.5 °Brix. This result could be explained by the cause of the contribution of soluble solids of GGS in such quantity. Additionally, soluble solids were within the ranges reported by Novoa and Ramírez-Navas (2012) for commercial milk caramel and milk caramel spread from buffalo milk reported by Andrade *et al.* (2009).

The GGS did not present statistically significant differences in the viscosity of the milk caramel spread (Table 2). However, it is a type of product that diminishes the apparent viscosity as deformation rate increases, presenting characteristics of a pseudoplastic or thixotropic fluid (Andrade *et al.*, 2009; Pauletti *et al.*, 1990; Rovedo *et al.*, 1991).

Textural parameters as adhesiveness, maximum force, and energy did not show statistically significant differences

(*P*>0.05) with the inclusion of GGS (Table 2). The differences in texture are due to the variations of pH in the different sweetened milk caramel spread, when by varying the pH, the electrical charges of the proteins are distributed heterogeneously, improving interactions between them (Valencia and Millán, 2008). The experimental values reported by Valencia *et al.* (2008) in terms of adhesiveness were lower than those of the present research. This fact was due to the soluble solids were lower (60 °Brix) and moisture content greater than 30% providing softer textures in the final product. However, the study reported by Valencia *et al.* (2008) coincided with those reported in the present work.

Table 2. Average values (± standard deviation) of the physicochemical parameters of the milk caramel spread with GGS in different concentrations.

Parameter	GGS (%)			
	0	10	20	30
Adhesiveness (J)	-1.423 (±2.150)	-2.654 (±1.557)	-2.410 (±0.462)	-1.228 (±0.737)
Force (N)	3,981 (±0.135) a	4,419 (± 0,117) a	4,306 (±0.146) a	4,218 (±0,165) a
Energy (J)	19.051 (±0.189) a	19.012 (±0.153) a	19.032 (±0.195) a	19.023 (±0.119) a
Apparent viscosity (mPa s)	55,167 (±1579) a	44,187 (±1719) a	66,700 (±1258) a	53,613 (± 2458) a
Water activity (A_w)	0.7993 (±0.0073) a	0.7923 (±0.0048) ab	0.7897 (±0.0240) ab	0.8223 (±0.0153) a
Fat (%)	8.1 (±0.3) a	7.6 (±0.4) a	7.6 (±0.5) a	7.6 (±0.5) a
Total Solids (%)	78.9713 (±1.370) a	78.502 (±0.2451) a	79.0007 (±0.6252) a	77.7167 (±0.4753) a
Protein (% w/w)	7.8 (±0.5) a	7.3 (±0.9) a	8.0 (±0.2) a	7.3 (±0.4) a
Calcium (% w/w)	0.25 (±0.02) a	0.26 (±0.01) a	0.27 (±0.02) a	0.25 (±0.04) a
Phosphorus (% w/w)	0.23 (±0.02) a	0.25 (±0.02) a	0.26 (±0.01) a	0.25 (±0.03) a
Magnesium (mg kg^{-1})	262.3 (±7.8) a	342.3 (±13.0) a	381.3 (±12.8) c	401.3 (±12.7) a
Potassium (% w/w)	0.48 (±0.03) a	0.63 (±0.07) a	0.63 (±0.02) c	0.72 (±0.04) c
Ashes (% w/w)	2.16 (±0.07) a	2.39 (±0.06) a	2.63 (±0.08)	2.66 (±0.06) c
Yield (%)	37.4 (±0.11) a	38.5 (0.057) a	39.6 (±0.26) c	41.4 (±0.45) d

Average values with the same lowercase letters in the same row denote that there were no significant differences according to Fisher's test (*P*<0.05).

Concerning water activity (A_w) and fat content, Table 2 shows that the GGS incorporation level did not show a significant effect (*P*>0,05). This result could be explained because of the water activity of the product is very similar to the syrup, both subjected to evaporation. Additionally, previous research reports that in the whey permeate there is less than 1 g L^{-1} of fat (Atra *et al.*, 2004). Therefore, this sweetener obtained a low fat due not only to the previous processing by which fat globules should be removed before subjecting the whey to ultrafiltration but also by the ultrafiltration and nanofiltration technologies, to which it was subjected. The values were within the range allowed by the NTC 3757, that mentions it should be between 6 and 9%, and it is validated by Andrade *et al.* (2009), who reported values of 7.95 for a caramel made of buffalo milk.

Protein did not present statistically significant differences between samples; it ranges from 7.3 to 8.0% (Table 2) values analogous to those reported by Garitta *et al.* (2004) and Sousa *et al.* (2002) who found values between 7.81 and 10%.

Two minerals (Magnesium and potassium) were evaluated in the milk caramel spread showing a statistically significant effect (*P*<0.05) by including GGS (Table 2). The results showed an increase in these minerals when more syrup was added; this same tendency was presented in ashes content. Besides, Calcium and Phosphorus minerals did not present statistically significant differences reaching values from 0.25 to 0.27% and between 0.23 to 0.26% w/w, respectively.

The CIELab color space in the final product indicated that GGS addition had a statistically significant effect (*P*<0.05). The lightness (L*) decreased as GGS content increased (Figure 2A), while value a* showed an adverse

effect, as seen in Figure 2B. In consequence, the product turns darker brown as the syrup percentage increases. This color changes could be related to Maillard reactions because syrup containing reducing sugars such as lactose, glucose, and galactose. Besides, a higher level of GGS (monosaccharides) inclusion in addition to low pH favors this reaction producing a caramel color. Novoa and Ramírez-Navas (2012) reported the color of different milk caramel where L* ranged from 42 to 45, a* between 13.7 and 15.8, and b* ranges from 31.3 to 37.06, which according to the CIELab coordinates shows a lighter color than the milk caramel spread produced in this research. The ranges of CIELab values for Argentine milk caramel found by Castañeda *et al.* (2004) were L* (26.36 to 41.31), a* (14.72 to 17.09), and b* (26.37 to 31.49); being the color of milk caramel spread analyzed in the current work close to these values.

Nutritionally, the milk caramel spread with GGS contains a greater amount of minerals necessary for the proper functioning of the human body. Additionally, the GGS is understood to have galactooligosaccharide from the enzymatic hydrolysis of lactose (Neri *et al.*, 2009; Rodriguez-Colinas *et al.*, 2014), providing a prebiotic function at the nutritional level.

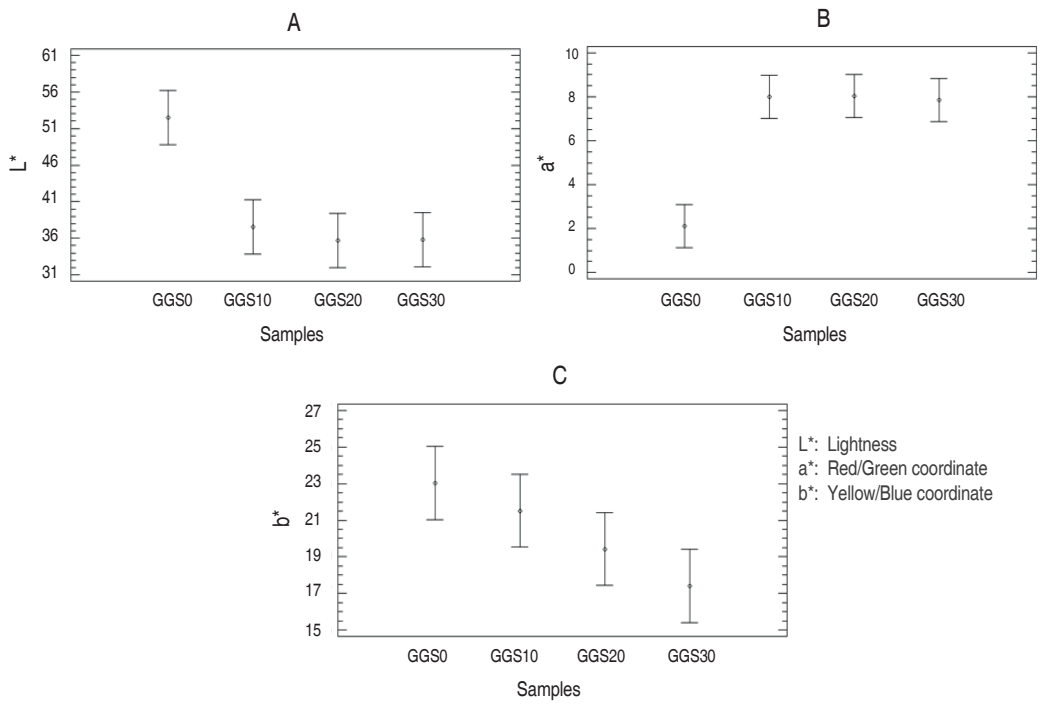

Figure 2. Color parameters of the milk caramel spread with different contents of GGS. A. lightness (L*); B. red to green (a*); C. yellow to blue (b*).

Sensory evaluation of milk caramel spread

Different factors were evaluated in the sensory test: Texture, color, aroma, crystallization, and spreadability (Figure 3A). The texture perception was qualified more fluid for the 10% GGS inclusion, while the other two inclusions (20% and 30%) perceived them a little dense. The color had the same tendency observed in the physicochemical analysis when increasing the addition of GGS, the darker the rating. The three inclusions were qualifying close to 5 (amber color), but the tendency demonstrated that when darker, the expert technicians could perceive this condition in every sample. In the perception of aromas, the evaluators rated them as "very nice." Additionally, spreadability was evaluated in the central line, but tending a little towards intense. Crystallization had a low rating, which means they did not perceive the crystals, sugar, or sandiness in any case.

Sensory analysis of the products through the acceptance test with 100 consumers for each sample is shown in Figure 3B. All the inclusions had a very good acceptance,

where more than 90% of the consumers described them from "I like it lightly" to "I like it very much", having the highest acceptance the 10% of inclusion, which did not have an unfavorable rating, nevertheless replacements of 20% and 30% remained close to the replacement of 10%.

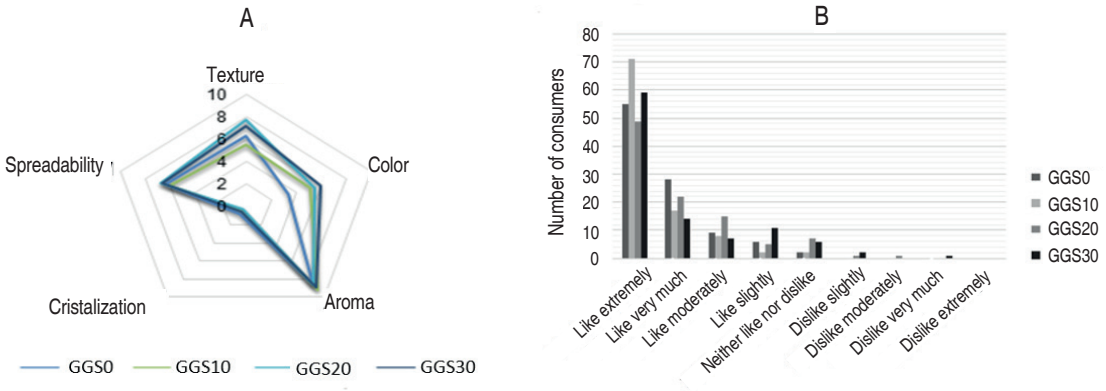

Figure 3. Sensory tests for milk caramel spread: Sensory descriptors (A); General consumer acceptance test for the different inclusions of GGS (B).

Stability of milk caramel spread

The pH presented statistically significant differences ($P<0.05$) for all samples during storage time; this value rose gradually with time and was greater at the end of the storage time (Figure 4). This behavior was also reported by other authors (Valencia and Millán, 2008). The apparent viscosity had the same behavior related to the interaction of the proteins during the stability study (Valencia and Millán, 2008).

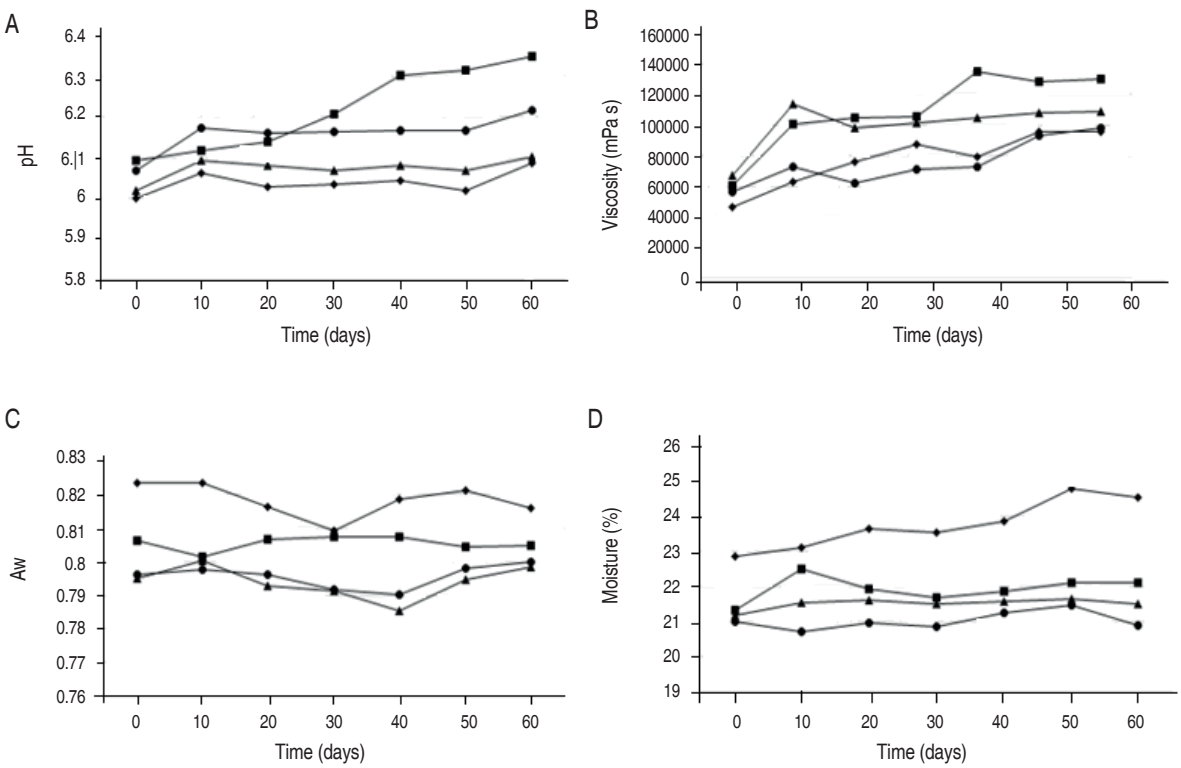

Figure 4. Stability properties of milk caramel spread sweetened with GGS at different concentrations during 60 days of storage under environmental conditions. 0% GGS (■), 10% GGS (●), 20% GGS (▲), 30% GGS (♦). A. pH; B. Apparent Viscosity; C. Water Activity; D. Moisture Content.

It was observed that the values of water activity (A_w) changed from 0.8013 to 0.8072 for the sample without GGS, 0.7904 to 0.8000 for the addition of 10% GGS; in the case of 20% GGS, it was from 0.7855 to 0.8002, and finally between 0.8091 and 0.8233 for the 30% substitution. Although in several cases, there were significant differences (P<0.05), the results did not show a defined behavior. The moisture content increased with time (P<0.05); that is, during the storage time at room temperature, the product was able to capture moisture from the environment because the milk caramel spread was not hermetically sealed with an aluminum film although it is noted that on day 60, it did not reach the maximum value allowed by NTC 3757 reported at 30%.

The average values for the CIELab color parameters in different formulations of milk caramel spread with GGS are given in Table 3. The L* value significantly increased in formulations of milk caramel spread of 10% GGS and 20% GGS at the end of the storage period, while 0% GGS and 30% GGS decreased. The values a* differ significantly (P<0.05) among the different formulas of milk caramel spread, a measure that elapsed the storage time; there was an increase in this parameter for all samples, becoming browner (Table 3). This characteristic of color in milk caramel spread could be associated with the possible presence of compounds derived from the Maillard reaction. The b* value also presented statistically significant differences (P<0.05) at the end of the 60 days of storage, this value is lower on the last day for the sample of 0% GGS and 30% GGS; on the other hand the samples with 10 and 20% GGS showed an increase, this phenomenon is also explained by the possible presence of derivatives of the Maillard reaction because this sweetener has reducing sugars and undergoes high temperatures during its preparation.

Table 3. CIELab color parameters of the milk caramel spread containing GGS at different concentrations for 60 days of storage at 25 °C and 60% of relative humidity.

CIELab Parameter	Time (d)	Sample			
		0GGS	10GGS	20GGS	30GGS
L*	0	54.66 (±0.90) a	38.28 (±0.02) a	34.85 (±0.05) a	36.24 (±0.03) a
	10	46.81 (±1.97) b	40.05 (±0.19) ab	37.71 (±2.74) b	33.96 (±1.35) ab
	20	45.99 (±0.84) bc	35.27 (±0.39) c	32.55 (±1.36) ac	30.48 (±2.11) c
	30	51.39 (±1.08) a	41.90 (±1.40) b	34.17 (±1.43) Acd	31.64 (±1.57) bcd
	40	46.69 (±1.79) bcd	38.31 (±0.22) abd	33.20 (±2.17) acde	31.48 (±1.20) bcde
	50	39.50 (±2.60) e	36.23 (±0.72) c	34.05 (±0.59) acdef	34.24 (±3.24) abdef
	60	47.04 (±3.04) bcd	39.34 (±2.27) abd	35.63 (±1.12) abdef	34.01 (±0.86) abdef
a*	0	1.98 (±0.05) a	6.84 (±0.02) a	7.47 (±0.02) a	7.94 (±0.05) a
	10	2.28 (±0.08) b	7.78 (±0.09) b	8.35 (±0.50) b	8.81 (±0.24) b
	20	2.50 (±0.04) c	6.85 (±0.06) a	7.66 (±0.17) ac	7.58 (±0.36) ac
	30	2.66 (±0.07) d	8.61 (±0.37) c	8.14 (±0.05) acd	8.09 (±0.04) acd
	40	2.85 (±0.09) e	8.05 (±0.19) bd	8.48 (±0.38) bde	8.00 (±0.32) acde
	50	3.26 (±0.03) f	7.61 (±0.17) b	8.72 (±0.25) be	7.28 (±0.54) c
	60	3.04 (±0.15) g	8.18 (±0.23) d	8.11 (±0.33) bcd	8.36 (±0.17) abde
b*	0	24.15 (±0.80) a	19.62 (±0.01) a	17.25 (±0.02) a	16.22 (±0.02) a
	10	19.20 (±0.17) b	21.92 (±0.19) b	19.01 (±1.95) ab	18.30 (±1.03) b
	20	19.28 (±0.42) bc	17.39 (±0.25) c	16.40 (±0.63) ac	14.32 (±0.89) c
	30	21.58 (±0.77) d	24.13 (±1.37) d	18.29 (±0.08) abd	15.94 (±0.24) ad
	40	19.46 (±0.48) bc	21.33 (±0.62) be	18.49 (±0.84) abde	15.05 (±0.43) acde
	50	17.82 (±0.89) e	19.37 (±0.65) a	19.12 (±0.82) bde	13.46 (±1.04) c
	60	21.35 (±1.17) d	21.26 (±0.23) be	17.25 (±1.37) abcde	15.67 (±0.38) ade

Average values with the same lowercase letters in the same column indicate that there are no significant differences between different formulations based on Fisher's test (P<0.05).

CONCLUSIONS

The glucose-galactose syrup can be used as a sweetener for the elaboration of milk caramel spreads considering the sensory, physicochemical, and stability analysis. The maximum recommended level is 30% of inclusion concerning the sweetness. GGS syrup influences the increase in the levels of magnesium and potassium. The texture was also achieved at lower soluble solids, increasing the product yield as the percentage of inclusion increases; therefore, it is important for cost reduction. In GGS, the content of reducing sugars significantly influenced the Maillard reactions altering the color and pH of the product, causing a darker brown color. During the storage period, milk caramel spreads have a shelf life of at least 60 days.

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Biohydrogen production by co-digestion of fruits and vegetable waste and coffee mucilage

Producción de biohidrógeno por co-digestión de residuos de frutas, verduras y mucílago de café

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Edilson León Moreno Cárdenas[1]* and Arley David Zapata Zapata[2]

ABSTRACT

In the present investigation, the effects of the substrate composition, organic load, medium acidification time, operation pH, and temperature on the production of hydrogen by anaerobic fermentation with fruits and vegetable waste, and fresh mucilage of coffee was evaluated. For this purpose, tests were carried out in a 20-liter bioreactor operated in batch mode, under a central composite experimental design (CCD). The fermentations were conducted under mesophilic conditions, without adding inoculum, and without sterilizing the substrate. The results for maximum daily hydrogen production (MDP), the maximum hydrogen content in the gas (MHC) and cumulative production (CHP) showed an acceptable fit to second-order polynomial models. All the independent variables were significant, especially the operation pH and the acidification time. Also, the premises for a model obtained by regression, according to an error analysis, were fulfilled. In the same way, it was possible to optimize the response variables. The maximum specific production concerning the organic load was 5511 mL H_2 $g_{CODremoved}^{-1}$, and regarding the volatile solids was of 670 mL H_2 $g_{VSadded}^{-1}$. These values are higher than those reported with similar substrates in continuous fermentation, with cell retention, use of inoculum, and substrate pretreatment.

Keywords:
Biofuels
Coffee mucilage
Fermentation
Vegetable waste

RESUMEN

En la presente investigación se evaluó el efecto de la composición del sustrato, carga orgánica, tiempo de acidificación del medio, pH de operación y temperatura sobre la producción de hidrógeno por fermentación anaerobia de residuos de frutas, verduras y mucílago fresco de café. Para ello se realizaron pruebas en un bioreactor de 20 L operado en modo *batch*, bajo un diseño experimental de composición central (CCD). Las fermentaciones fueron realizadas en condiciones mesofílicas, sin adición de inóculo y sin esterilizar el sustrato. Los resultados en la producción diaria máxima de hidrógeno (MDP), contenido máximo de hidrógeno en el gas (MHC) y producción acumulada (CHP), presentaron ajuste aceptable a modelos polinomiales de orden dos. Todas las variables independientes fueron significativas, destacándose el pH de operación y el tiempo bajo condiciones ácidas, además se cumplieron las premisas para un modelo obtenido por regresión, según un análisis del error. De igual manera fue posible optimizar las variables de respuesta. La máxima producción específica respecto a la carga orgánica fue 5511 mLH_2 $g_{DQOremovido}^{-1}$ y frente a los sólidos volátiles fue 670 mL H_2 $g_{VSadicionado}^{-1}$, valores superiores a los reportados con sustratos similares en fermentaciones continuas, con retención de células, uso de inóculo y pretratamiento del sustrato.

Palabras clave:
Biocombustible
Mucílago de café
Fermentación
Residuos vegetales

[1] Facultad de Ciencias Agrarias. Universidad Nacional de Colombia. AA 1779, Medellín, Colombia.
[2] Facultad de Ciencias. Universidad Nacional de Colombia. AA 3840, Medellín, Colombia.
* Corresponding author: <elmorenoc@unal.edu.co>

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Molecular hydrogen (H_2) as an energy vector has aroused great interest given its high energy capacity, the diversity of applications, and the low environmental impact during its use. It can be obtained through microorganisms in microbial electrolysis cells, photo-fermentation, and dark fermentation. In the first case, the cost of the electrodes is high; and in the second one, it is necessary to supply light, creating a negative energy balance (Show *et al*., 2011; Liu *et al*., 2015). Dark fermentation does not depend on the light supply, it is considered more sustainable, and it allows the use of complex substrates such as organic waste (Laxman *et al*., 2015, Ortigueira *et al*., 2015). These complex substrates are discarded in agricultural, livestock, agro-industrial processes, and human activities; making them abundant and inexpensive (Ghimire *et al*., 2015). Fermentations with simple substrates composed of monosaccharide such as glucose, fructose, xylose or mannose generated high yields in hydrogen production; however, their cost and limited availability restrict their use.

Some researchers have proposed the co-digestion of several types of organic waste, previously sterilized and with the addition of inoculum, in order to reach and even exceed the yields obtained when using simple substrates. This approach could make it possible to achieve a better balance of the Carbon/Nitrogen ratio, a better dilution of toxic components, an increase in the loading of biodegradable organic matter, an improvement the nutrient balance, and the production of synergistic effects among microorganisms (Angeriz-Campoy *et al*., 2015). Besides, the co-digestion helps to regulate the increase in the volatile organic acid concentrations, which are produced when biohydrogen is generated and can limit the gas generation. It has been documented that for concentrations higher than 9 g L^{-1} of butyric acid, the growth of several hydrogen-producing bacteria is inhibited (Chong *et al*., 2009).

In the biohydrogen production by fermentation, the use of pure cultures has prevailed. However, its main limitation lies in the availability of the substrate and in guaranteeing its sterilization (Lee *et al*., 2011). When using organic waste, the simultaneous presence of obligate and facultative anaerobic bacteria can contribute to improving the yields (Elsharnouby *et al*., 2013). In these cases, some bacteria can facilitate the hydrolysis of complex carbohydrates, leaving them available for the hydrogen-producing bacteria; besides, different types of bacteria can consume a particular component without competing with others (Chong *et al*., 2009; Mthethwa *et al*., 2019). Fermentations that use substrates with a low cost, high-availability, rapid assimilation, and that do not require pretreatment and sterilization, are considered the most desired (Elsharnouby *et al*., 2013). Furthermore, when a low-cost acidification pretreatment is applied, some bacteria that consume hydrogen will die, which could improve hydrogen production (Prabakar *et al*., 2019). A fermentation based on an indigenous consortium could satisfy these demands because the bacteria would be adapted to thrive in a non-sterile environment and would adjust better to sudden changes during fermentation (Show *et al*., 2011).

Few reports are available in Colombia about the generation of hydrogen by fermentation, Hernández *et al*. (2014) used coffee mucilage in co-digestion with pig manure to generate hydrogen, obtaining a maximum production rate of 7.6 $NLH_2\ L_{mucilage\text{-}day}^{-1}$, and hydrogen content in the gas of up to 39%. They also identified that said gas was generated through two metabolic pathways, butyric and acetic. Cano (2015) studied the production of biohydrogen from urban organic waste, achieving yields of 1.9 $LH_2\ L_{waste\text{-}day}^{-1}$ and 132.9 mL of H_2 per gram of Volatile Solids (VS) added, finding that the hydrogen content and its production were fitted to second-order polynomial models. Their subsequent optimization indicated that the maximum values were reached at a pH of 6.2, an agitation of 41 rpm and an organic load between 45,000 and 75,000 mg $O_2\ L^{-1}$.

Considering the requirements (inoculum and susbtrate) of hydrogen production nowadays, the objective of the present work was to evaluate the effect of substrate composition, organic load, acidification time, operation pH, and temperature on the hydrogen production obtained by anaerobic fermentation of a complex substrate, composed of fruit and vegetable wastes with coffee mucilage, in mesophilic conditions without sterilization of the substrate and using its native microorganisms.

MATERIALS AND METHODS

The tests were performed in the Agricultural Mechanization Laboratory of the Faculty of Agricultural Sciences at Universidad Nacional de Colombia, Medellín campus. Their execution was done under a central composite experimental design (CCD), with five factors, two levels

in each factor, and three dependent variables, which was implemented in the Design Expert® software version 9.0 from State-easy. The central composite experimental design was partial to reduce the number of trials and cost. The study factors were substrate composition (SC, %), organic load (ORL, $mgO_2\ L^{-1}$), acidification time (AT, days), operation pH (pHo) and temperature (Tf, °C). The design produced 26 tests, and the factor values were normalized between cero and one (Myers *et al*., 2009).

SC was a proportion by volume of fruit and vegetable waste (lettuce, Tommy Atkins mango, Valencia orange, guava, and papaya), and fresh coffee mucilage. The Central Mayorista de Antioquia supplied the fruit and vegetable wastes, and the fresh mucilage by Casa de Sabaneta farm in Sabaneta (Antioquia), using a mechanical demucilager for Castillo coffee variety. At the beginning of each test, the fruits and vegetable waste were crushed and mixed with the mucilage, and then deposited in a 20-liter stainless steel bioreactor operated in batch mode with a working volume of 14 L. Then, nothing was done to the process during the AT. This process was natural, no chemical compound was added, and when this time ended, agricultural lime was added (with 95% of calcium carbonate, $CaCO_3$, 2% humidity and 54% soluble CaO), until reaching the operation pH of the test, with a range of ±0.2. Simultaneously, the temperature was increased until reaching the value corresponding to the test, and the mixture was stirred at 45 rpm for 5 minutes every hour. This agitation was the same for all the tests.

The SC levels, fresh mucilage:fruit and vegetable ratio, were 80:20, 20:80, 50:50, 100:0, and 0:100. ORL was defined as the chemical oxygen demand of the substrate (COD), with a lower level of 20,000 $mgO_2\ L^{-1}$. It was calculated at the beginning and the end of the tests (iCOD and fCOD respectively, $mgO_2\ L^{-1}$), the same as the total volatile solids (iTVS and fTVS, $mg\ L^{-1}$). For each trial, the organic load was obtained by diluting the substrate in water. In both cases, samples of 250 mL were taken and analyzed according to methods 5220D and 2540E (APHA, 2012). AT levels were of 1, 2, and 3 days. pHo was recorded with a YK-21PH device, with a resolution of 0.02 and an accuracy of ±0.2, and an S450CD Sensorex electrode. The lowest level of pHo was of 5.5, as recommended by Moreno *et al*. (2013), the same as the COD levels. The temperature of the tests (Tf) was measured in the bioreactor with an analogous sensor with a resolution of 1 °C and an accuracy of ±1 °C. The levels were of 30, 35 and 40 °C, according to preliminary tests.

The dependent variables were the maximum hydrogen content in the gas (MHC, %), maximum daily hydrogen production (MDP, $LH_2\ d^{-1}$) and the cumulative hydrogen production (CHP, LH_2). Samples were taken using Tedlar bags with a capacity of 1 L in order to measure the fraction of hydrogen present in the gas (Restek 22950). These samples were analyzed by gas chromatography in an Agilent 3000 Micro-GC instrument, equipped with a thermal conductivity detector (TCD), a molecular-sieve 5A column of 10 m×0.32 mm with Argon 5.0 carrier gas, and a column PLOT U of 8 m×0.32 mm with Helium 5.0 carrier gas. In the samples, the hydrogen (H_2), oxygen (O_2), nitrogen (N_2), methane (CH_4), monoxide, and carbon dioxide (CO, CO_2) concentrations were quantified. The temperature of the injector was of 60 °C and 80 °C for the column, and the pressure was of 206.8 kPa.

The MDP was obtained as the product between the hydrogen content and the gas volume per day, which was recorded with a Metrex G2.5 gas meter with a precision of 0.040 $m^3\ h^{-1}$ and a maximum pressure of 40 kPa. CHP was the sum of the daily hydrogen production. In addition, four process indicators were defined as follows: productivity (HP, $LH_2\ L_{waste}^{-1}\ d^{-1}$), specific production regarding the substrate (SPS, $LH_2\ kg_{waste}^{-1}$), specific production regarding the volatile solids (SPVS, $mL\ H_2\ g_{VSadded}^{-1}$), and specific production according to the decrease of contaminant load (SPC, $mL\ H_2\ g_{CODremoved}^{-1}$). For these calculations, the maximum daily hydrogen production was taken and divided between the volume of the substrate used, the mass of the substrate, the volatile solids added and the difference between the iCOD and the fCOD, to obtain the respective indicators.

For MHC, MDP and CHP non-linear models were obtained using multiple regression, whose coefficients were determined by least squares. The Stepwise method was used to reduce the models by eliminating some of the low significance terms (Myers *et al*., 2009). Then an analysis of variance (ANOVA) was performed, where an F test was used to determine the significance of the models and the *P*-value (<0.05) to obtain the statistical significance of

their terms. The coefficients of determination, R^2, $R^2_{adjusted}$, and $R^2_{prediction}$ were obtained to analyze the fit of the models and its predictive capacity. Additionally, the assumptions of the models were checked through a residue analysis where their normal distribution, the constant variance of the error, the identification of outliers, the influence of the tests through Cook's distances, Leverage values, difference in the fit (DFFITS), and in the coefficients (DFBETAS) were verified.

The Design Expert 9.0® software was used to obtain the models and then optimize them using two methods. One was a numerical method of step-by-step ascent, which progress is a function of the regression coefficients, and the other was a graphic method based on the maximization of the desirability function (Equation 1). The graphic optimization included the superposition of multiple response surfaces to find a region where the three dependent variables are optimized (Myers *et al*., 2009).

$$D = (d_1 \times d_2 \times ... \times d_n)^{1/n} = (\prod_{i=1}^{n} d_i)^{1/n} \qquad with\ 0 \leq D \leq 1 \qquad (1)$$

Where:
D: desirability function for the variable (hydrogen content, daily or cumulative production), if D=0 indicates that it is undesirable and if D=1, the value obtained as a response is desirable.
d: desirability for each response variable.
n: number of response variables.

RESULTS AND DISCUSSION

Hydrogen production in experimental tests

Table 1 shows the results for the dependent variables. Test 22 stands out because in it the maximum daily and cumulative hydrogen production is twice as high as the next value. However, the highest hydrogen content in the gas was recorded in test 24.

Table 1. Results in the dependent variables for the experimental tests.

Test	SC (mu:fru-veg)[1]	ORL ($mgO_2\ L^{-1}$)	TVS ($mg\ L^{-1}$)	AT (d)	pHo	Tf (°C)	MDP ($LH_2\ d^{-1}$)	MHC ($\%H_2$)	CHP (LH_2)
1	100:0	37,125	10,760	2	5.9	35	0.0	0.0	0.0
2	80:20	33,750	16,300	3	5.6	30	0.0	0.0	0.0
3	80:20	17,125	11,400	3	5.3	40	0.0	0.0	0.0
4	80:20	50,875	33,600	1	5.3	40	0.0	0.0	0.0
5	80:20	22,125	10,460	1	5.4	30	0.0	0.0	0.0
6	80:20	52,875	21,160	3	5.9	40	0.0	0.0	0.0
7	20:80	63,750	64,140	1	6.6	40	3.0	30.3	3.0
8	20:80	53,000	22,120	1	5.6	40	0.0	0.0	0.0
9	20:80	45,875	26,440	3	5.3	30	0.0	0.0	0.0
10	20:80	83,375	56,660	3	5.3	40	0.0	0.0	0.0
11	20:80	67,500	53,380	1	5.3	30	0.0	0.0	0.0
12	20:80	26,650	27,480	1	5.8	30	1.9	20.9	1.9
13	20:80	28,000	24,060	3	6.0	40	0.0	0.0	0.0
14	0:100	47,500	38,160	2	6.1	35	0.4	14.4	0.4
15	50:50	43,840	59,629	2	6.4	35	3.4	34.2	6.8
16	50:50	37,440	57,125	2	6.2	35	1.3	30.9	2.6
17	50:50	37,120	58,750	2	6.3	35	5.7	37.9	8.3
18	50:50	32,140	65,000	2	6.3	35	3.4	34.3	7.0
19	50:50	35,900	52,300	2	6.1	35	2.7	26.5	5.0
20	50:50	37,660	52,000	2	6.3	35	7.8	31.1	11.4
21	80:20	22,500	37,500	1	6.3	40	0.0	0.0	0.0
22	80:20	54,000	38,660	1	6.5	30	13.3	35.9	25.9
23	80:20	28,000	15,700	3	6.0	30	2.3	32.5	3.4
24	20:80	59,700	43,500	3	6.5	30	5.6	40.0	10.1
25	50:50	72,000	70,900	2	6.1	35	5.9	24.7	8.9
26	50:50	42,500	35,000	0	6.1	35	0.0	0.0	0

[1] Mucilage:Fruits and vegetables ratio.

Mathematical models obtained by regression

Regression models and analysis of variance. Table 2 shows the variance analysis for the three dependent variables with the significant terms, their coefficients, the significance of the models, and the coefficients of determination. All the models were significant; the variation around the mean explained by the models was acceptable with values of determination between 81.8% and 90.8%. The variation described by the models, keeping in mind the number of terms, ranged between 67.6% and 81.2% ($R^2_{adjusted}$). Meanwhile, the variation explained by the models for new data ($R^2_{prediction}$) varied between 41.5 and 66.9%, which could indicate that there is a difficulty to estimate some values accurately, in sectors of the analysis space.

Table 2. Variance analysis for the models in the dependent variables.

Component	MDP ($LH_2\ d^{-1}$)	*P*-value	MHC ($\%H_2$)	*P*-value	CHP (LH_2)	*P*-value
Intercept	-149.94		-370.39		-137.74	
SC	0.27	0.0136	0.79	0.5729	-0.93	0.0088
ORL	2.31×10^{-4}	0.0026	-1.81×10^{-3}	0.1056	-1.24×10^{-3}	0.0042
AT	20.32	0.6826	31.01	0.0013	7.65	0.5197
pHo	23.65	0.0006	64.28	<0.0001	23.79	<0.0001
Tf	2.56	0.0031	8.56	0.0025	5.88	0.0011
SC×AT	-0.05	0.0239	-	-	-0.09	0.0156
SC×pHo	-	-	-	-	0.22	0.0103
ORL×AT	-7.99×10^{-5}	0.0163	-	-	-9.88×10^{-5}	0.0476
ORL×pHo	-	-	3.30×10^{-4}	0.0895	2.58×10^{-4}	0.0162
AT×pHo	-1.62	0.1253	-	-	-	-
AT×Tf	-	-	-	-	0.20	0.1536
pHo×Tf	-0.49	0.0246	-1.65	0.0372	-1.14	0.0017
SC^2	-1.32×10^{-3}	0.0432	-8.27×10^{-3}	0.0022	-1.82×10^{-3}	0.0507
AT^2	-1.23	0.0341	-7.72	0.0010	-1.70	0.0332
R^2	0.8186		0.8802		0.9081	
$R^2_{adjusted}$	0.6760		0.8128		0.8085	
$R^2_{prediction}$	0.4151		0.6694		0.5837	
P-value	0.0015		<0.0001		0.0003	

In MDP and CHP, the variables SC, ORL, pHo, and T showed a significant effect. In the MDP the interactions between SC and AT, ORL and AT, and pHo and Tf were significant with negative coefficients, these indicate that if both of them increases, the daily hydrogen production will decrease. In the CHP, the interactions between SC and AT, SC and pHo, ORL and AT, ORL and pHo, and pHo with Tf were equally significant. The positive coefficients for the interactions SC-pHo and ORL-pHo indicate that an increase in them generates an increase in the cumulative hydrogen production. In MHC, AT, pHo, Tf, and the interaction between pHo and Tf had a significant effect. The latter with a negative coefficient, therefore if both increase, the MHC decreases. In the three dependent variables, the quadratic terms of the SC and the AT are significant with negative coefficients.

Regarding the independent variables, when the substrate was composed of a single type of waste, the hydrogen production was a very low or nothing at all (test 1 and 14), in turn, the highest values recorded were in test 22, with an SC of 80:20. Hernández *et al*. (2014) reported the generation of hydrogen with fresh coffee mucilage in co-digestion with pig manure in a 50:50 ratio. Angeriz-Campoy *et al*. (2015) found that hydrogen production improved when organic municipal solid waste and food waste were used. Tawfik and El-Qelish, (2014) indicated that in co-digestion when passing from a mixture of 1:2 to 1:3, the hydrogen production doubled. The highest hydrogen production under co-digestion can be associated with: a better balance of nutrients, a better carbon/nitrogen ratio, an increased in the biodegradable load, the dilution of toxic substances, the synergistic effects of microorganisms, and a better buffering capacity of the pH (Sreela-or *et al*., 2011; Zhou *et al*., 2013; Tyagi *et al*., 2014; Elsamadony and Tawfik, 2015).

The production of hydrogen decreased when ORL was lower than 30,000 and higher than 70,000 mgO_2 L^{-1} and increased for values between 37,660 and 59,700 mgO_2 L^{-1}. These results are consistent with the ones reported by Moreno *et al*. (2013) and Cano (2015), using fruits and vegetable waste. Srikanth and Venkata Mohan (2014) and Elsamadony and Tawfik (2015) stated that if the ORL decreases, there is a smaller amount of food for bacterial growth; however, an excessive increase can generate inhibition by the accumulation of volatile fatty acids, lowering the pH and interrupting the hydrogen production. Additionally, the inhibition may occur due to high concentrations of ammonium, generated by compounds present in the municipal organic solid waste (Jiang *et al*., 2013, Zahedi *et al*., 2013, Hidaka *et al*., 2015).

The highest hydrogen generation was reached at a pHo of 6.5, 30 °C, and 1 day under acidic conditions. There was no production at a pHo lower than 5.8, a similar situation to the reported under mesophilic conditions and with organic solid waste by Choi and Ahn (2014), Dareioti *et al*. (2014), and Cano (2015). When the temperature was set at 40 °C, only there was hydrogen production for an organic load higher than 60,000 mgO_2 L^{-1}. In fermentations with organic solid waste, temperatures higher than 39 °C produce enzymes denaturation and higher than 41 °C, produce the death of microorganisms (Laothanachareon *et al*., 2014; Arimi *et al*., 2015; Ghimire *et al*., 2015).

In general, all the independent variables presented significance in the models; however, pHo and AT had the highest coefficients, especially the pHo. The pHo is very important to hydrogen production by fermentation; variations in the extracellular pH can damage the plasma membrane of microorganisms and inhibit the enzymatic activity of hydrogenase (Robledo-Narváez *et al*., 2013, Dareioti *et al*., 2014).

Residual analysis

In MDP, MHC, and CHP, the normal probability compared to the internally studentized residuals showed a straight-line trend, verifying that the models fulfilled the assumption of the normal distribution of residuals. Regarding the verification of the constant variance of the error, the externally studentized residual compared to the estimated values did not show a defined pattern, with randomly distributed estimates and verifying the constant variance in the error. In the verification of atypical results (outliers), the externally studentized residual showed that only the hydrogen content in test 23 was considered atypical. This result prompted its verification, but no anomalies were found in it, preserving its value.

In the influence analysis of the dependent variables of the tests, the Leverage distance value was lower than one (1) in all the variables; thus, all the tests were relevant in the fit of the models without any of them

Table 3. Residual analysis of the maximum daily hydrogen production (MDP), maximum hydrogen content (MHC), and the cumulative hydrogen production (CHP).

Test	MDP (LH_2 d^{-1})		MHC (%H_2)		CHP (LH_2)	
	DFFITS	DFBETAS	DFFITS	DFBETAS	DFFITS	DFBETAS
1	-1.210	0.188	-0.282	-0.051	-1.787 •	0.320
4	-1.743 •	-0.551•	0.480	0.192	0.646	0.174
5	1.555 •	0.229	-0.343	-0.120	0.708	0.177
7	-0.044	-0.011	2.156 •	0.561 •	1.494 •	0.359
10	-0.248	-0.025	0.967	0.014	1.528 •	0.014
12	-1.568 •	-0.134	-1.990 •	-0.064	-0.244	-0.005
16	-0.633	-0.506 •	0.091	0.071	-0.723	-0.563 •
20	1.120	0.932 •	0.206	0.171	0.987	0.811 •
22	3.181 •	0.962 •	-0.200	-0.057	4.676 •	1.014 •
23	-0.044	0.006	2.009 •	-0.367	-0.072	0.011
25	-0.086	-0.063	-1.231	-0.916 •	-0.668	-0.490 •

• A value that exceeds the limits on the residual analysis.

been highly influential. In Cook's distance, only the CHP variable in test 22 was superior to one (1), indicating that it could be an atypical case; however, it was not influential since its Leverage was lower than one (1). Regarding the DFFITS indicator, eight tests were outside the desired range, having a greater incidence in the prediction of the models. Likewise, six tests had a DFBETAS value outside the desired range, having a higher incidence in the regression coefficients (Table 3).

Analysis of process indicators

The process indicators show that hydrogen production was associated with the removal of the organic load (reduction of COD and TVS). The high values obtained in HP, SPS, SPVS, and SPC, especially in test 22, indicate that a significant amount of the substrate was used in the production of hydrogen. This implies greater use of the carbon and energy sources, contributing to the efficiency of the process (Table 4). The high transformation of organic matter into hydrogen in a mixed culture, like the one used in the present work, may be due to the presence of high-performance bacteria on the appropriate conditions, or to the synergic work of bacteria that consume the different organic fractions of the substrate (Mohanakrishna *et al*., 2011; Robledo-Narváez *et al*., 2013; Chinellato *et al*., 2013; Reungsang *et al*., 2013; Choi and Ahn, 2014; Yang *et al*., 2019).

Table 4. Results of the process indicators for the experimental tests where there was hydrogen production.

Test	HP ($LH_2\ L^{-1}\ d^{-1}$)	SPS ($LH_2\ kg^{-1}$)	SPVS ($mLH_2\ g_{VSadded}^{-1}$)	SPC ($mLH_2\ g_{CODremoved}^{-1}$)
7	0.3	0.3	47	92
12	0.4	0.4	69	353
14	0.1	0.1	10	160
15	0.3	0.6	114	562
16	0.1	0.2	46	726
17	0.6	0.8	141	1515
18	0.3	0.6	108	4217
19	0.3	0.5	96	1462
20	0.8	1.0	219	1676
22	1.2	1.7	670	5511
23	0.2	0.4	217	3400
24	0.6	1.0	232	4208
25	0.5	0.6	126	989

The maximum values obtained in the present work, for the four indicators analyzed, are comparable to those reported when organic waste has been used in thermophilic conditions (between 55 and 60 °C, with negative energy balance), in reactors in semi-continuous mode, continuous, use of inoculum and pre-treatment (Table 5). In the current research, no inoculum was added, and no sterilization process was performed; this means that the microorganisms native to the waste were used (substrate without pretreatment). This implies that this type of bioprocess is simpler, faster, and with a lower energy requirement (operation at 30 °C), thus increasing its potential as a hydrogen generation method.

Optimization of the models obtained by regression

Numerical optimization. The three dependent variables were optimized, reaching higher values than those obtained during the experimental phase (Table 6). The MDP went from 13.3 to 16.1 $LH_2\ d^{-1}$, the MHC from 40% to 50.4% and the CHP from 25.9 to 31.3 LH_2. The optimal values for the MDP and CHP are reached for an organic load around 80,000 $mgO_2\ L^{-1}$, a substrate composition close to 70:30, a pHo of 6.5, a temperature of 31 °C and

Table 5. Indicators in the present work and some reported by other authors.

Fermentation characteristics Substrate / Type / Inoculum / Pre-treatment	Value	Source
Productivity (HP, LH_2 L^{-1} d^{-1})		
Food waste / Batch / Yes / No	0.9	Yasin *et al.* (2013)
Urban waste / Batch, 55 °C / Yes / Yes	2.9	Elsamadony and Tawfik (2015)
Urban waste-mucilage / Batch / No / No	1.2	This work
Specific production regarding the substrate (SPS, LH_2 kg^{-1})		
Urban waste / Semi-continuous 55 °C / Yes / No	0.8	Escamilla-Alvarado *et al.* (2013)
Vegetable waste / Batch, 37 °C/ No / No	1.9	Marone *et al.* (2014)
Urban waste-mucilage / Batch / No / No	1.7	This work
Specific production regarding TVS (SPVS, mLH_2 $g_{VSadded}^{-1}$)		
Urban waste / Batch / Yes / Yes	120	Dong *et al.* (2010)
Vegetable waste / Batch, 55 °C / Yes / Yes	171	Ghimire *et al.* (2015)
Sucrose / Batch / No / Yes cells retained	488	Singh and Wahid (2015)
Urban waste-mucilage / Batch / No / No	670	This work
Specific production according to COD (SPC, mLH_2 $g_{CODremoved}^{-1}$).		
Urban waste-wastewater / Batch, 37 °C / Yes / No	145	Tawfik and El-Qelish (2014)
Glucose-wastewater / Batch / Yes / Yes	448	Bundhoo *et al.* (2015)
Urban waste / Batch, 30 °C / No / No	1598	Cano (2015)
Urban waste-mucilage / Batch / No / No	5511	This work

Table 6. Numerical optimization of the dependent variables and their corresponding value in the independent variables.

SC (mu:fru-veg)[1]	ORL (mgO_2 L^{-1})	AT (d)	pHo	Tf (°C)	Dependent variable		
					MDP (LH_2 d^{-1})	MHC (%H_2)	CHP (LH_2)
72:28	80,605	0.3	6.5	31	16.1	-	-
50:50	73,424	2.1	6.5	33	-	50.4	-
71:29	82,416	0.5	6.4	30	-	-	31.3

[1] Mucilage:Fruits and vegetables ratio.

an AT between 6 and 12 hours. These conditions, except for the ORL, are close to the ones in test 22, where the highest values for MDP and CHP were obtained. To reach the maximum hydrogen content in the gas, the temperature, pHo, and ORL values must be similar to the aforementioned for MDP and CHP, while the AT is of 2 days, and the substrate composition must be 50:50. The response surfaces for the three optimized variables are presented in Figures 1, 2, and 3. The graphs correspond to the interactions that were significant in the variance analysis. The figures show that the MDP and CHP increase as the AT decreases and the amount of mucilage becomes higher than the amounts of fruits and vegetables (SC). Meanwhile, the hydrogen content in the gas increases when the temperature decreases and the pHo rises up to 6.5.

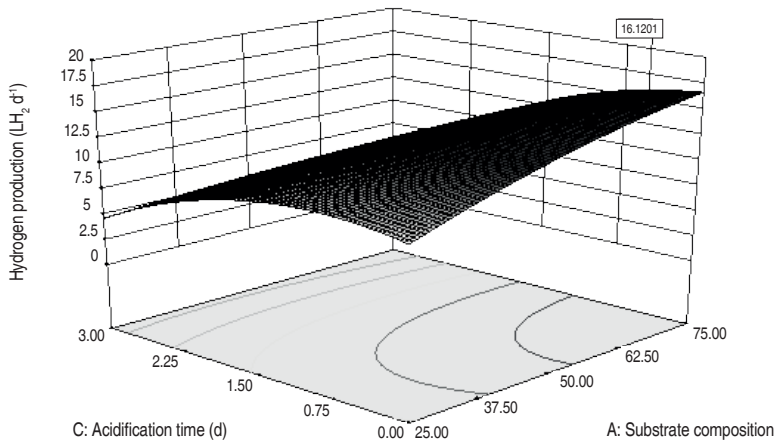

Figure 1. Response surface for the optimized maximum daily hydrogen production (MDP).

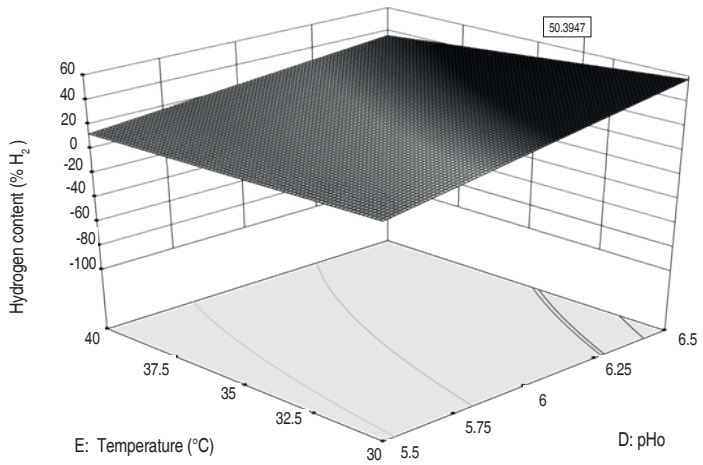

Figure 2. Response surface for the optimized maximum hydrogen content (MHC).

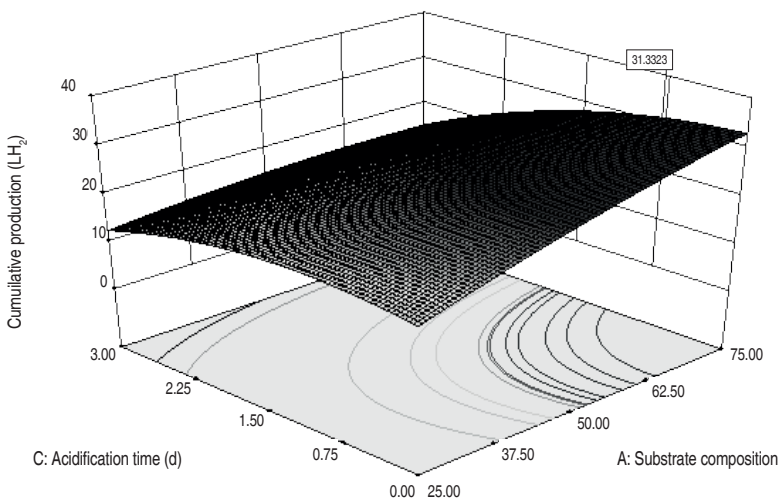

Figure 3. Response surface for the cumulative production (CHP).

Graphical optimization. In the study space, the area that provided the highest values simultaneously for all three-response variables was identified (hatched zone in Figure 4).

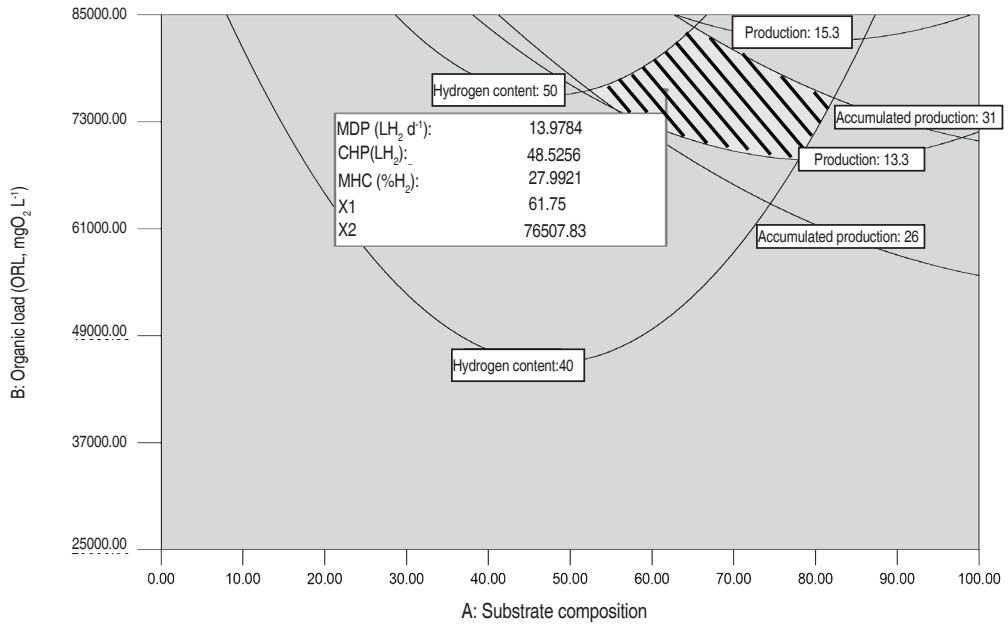

Figure 4. Superposition of the multiple response surfaces.

In this region, the MDP ranges between 13.3 and 15.3 L H_2 d^{-1}, the MHC between 40% and 50% and the CHP between 26 and 31 LH_2. The area was obtained for an AT of one day, a pHo of 6.5, a temperature of 30.4 °C, an ORL between 68,000 and 83,000 mgO_2 L^{-1}, and an SC between 52:48 and 82:18. The values for the variables ORL, SC, AT, pHo, and Tf, are in accordance with those obtained in the numerical optimization for the three dependent variables. Both optimization methods show that for the three dependent variables the highest values are reached under mesophilic conditions (temperature of 30 °C), with a pHo of 6.5 and an AT of less than a day, which reduces the startup time of the production. The results are in accordance with the values reached in the experimental phase, and with those reported in other studies with organic waste in mixed culture, where the optimum production is reached with a temperature between 30.3 and 38 °C and a pH between 5.5 and 7 (Sreela-or *et al*., 2011; Nath and Das, 2011; Sekoai and Gueguim-Kana, 2013). Additionally, the results indicate that hydrogen production increases when the substrate includes both types of waste (co-digestion), instead of just one type. This is similar to that reported by other authors such as Hernández *et al*. (2014), Gomez-Romero *et al*. (2014), Angeriz-Campoy *et al*. (2015) and Elsamadony and Tawfik (2015).

CONCLUSIONS

Given the results obtained, it was concluded that it is possible to produce hydrogen and achieve high yields (1.2 L H_2 L^{-1} d^{-1}, 1.7 L H_2 kg^{-1} of waste, 670 mL H_2 $g_{VSadded}^{-1}$ and 5511 mL H_2 $g_{CODremoved}^{-1}$) without using inoculum, without pre-treating the substrate, and under mesophilic conditions. Also, all the variables analyzed showed a significant effect on hydrogen production, being the operational pH (pHo) the most significant variable. The variables under study were adjusted to second-order polynomial models with coefficients of determination higher than 0.8186, and according to residual analysis, all the assumptions of the regression were fulfilled. Hydrogen production was successfully optimized when the substrate composition (SC) was between 68:32 and 83:17, the organic load (ORL) between 68,000 and 82,000 mgO_2 L^{-1}, the acidification time (AT) between 12 and 24 hours, the pHo of 6.5 and the temperature between 30 °C and 33 °C.

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POLÍTICA EDITORIAL

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La Revista recibe y publica, sin ningún costo, artículos en idioma inglés de investigación, revisión, reseñas, cartas al editor y editoriales.

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Se debe utilizar el Sistema Internacional de Unidades (SIU), y aquellas unidades específicas de mayor uso por parte de la comunidad científica. Las unidades combinadas deben usar la forma exponencial. Ejemplo: kg ha^{-1}. El significado de las abreviaturas debe citarse por extenso cuando se mencionan por primera vez en el manuscrito. El estilo de escritura debe ser absolutamente impersonal, en tiempo gramatical pasado para la introducción, los procedimientos y los resultados y presente para la discusión, evitando la conjugación de verbos en primera o tercera persona del singular o el plural.

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El título del artículo no debe incluir abreviaturas y es obligatoria su respectiva traducción al idioma español. En lo posible, el título no debe exceder de 15 palabras y debe reflejar con precisión el contenido del documento. Cuando contenga nombres científicos de especies vegetales o animales, éstos se deben escribir con letra cursiva (itálica) en minúsculas, sólo con mayúsculas la primera letra del género y del clasificador. Debajo del título en inglés se escribe el nombre(s) y apellido(s) de los autores, sin sus respectivos títulos académicos, ni cargos laborales, en una línea horizontal y de acuerdo con su contribución en la investigación y/o preparación del artículo.

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El resumen no debe exceder de 250 palabras escritas en un único párrafo. Se debe escribir en inglés y español. Debe contener en forma breve la justificación, los objetivos, los métodos utilizados, los resultados obtenidos más relevantes y las conclusiones. Es obligatorio acompañar el resumen con un máximo de seis palabras clave distintas a las utilizadas en el título. Se aceptan como palabras clave no sólo las palabras simples, sino también términos compuestos hasta de tres palabras. Deben ir escritas en minúsculas y separadas por comas.

Introducción

Puede tener o no título. Define el problema e informa sobre el estado del arte respecto al tema principal del artículo; además, señala las razones que justifican la investigación y plantea los objetivos de la misma. Es obligatorio acompañar los nombres vulgares con el nombre(s) científico(s) y la abreviatura(s) del clasificador en la primera mención dentro del texto. No se deben mencionar marcas de productos, sino su nombre genérico o químico

Materiales y métodos

En este apartado se deben describir en forma clara, concisa y secuencial, los materiales (vegetales, animales, implementos agrícolas o de laboratorio) utilizados en el desarrollo del trabajo; además, se mencionan los aspectos relacionados con la ubicación, preparación y ejecución de los experimentos. Se debe indicar el diseño seleccionado, las variables registradas, las transformaciones hechas a los datos, los modelos estadísticos usados y el nivel de significancia empleado. Evitar detallar procedimientos previamente publicados.

Resultados y discusión

Son la parte central del artículo, deben estar respaldados por métodos y análisis estadísticos apropiados. Se deben presentar de manera lógica, objetiva y secuencial mediante textos, tablas y figuras; estos dos últimos apoyos deben ser fáciles de leer, autoexplicativos y estar siempre citados en el texto. Las tablas se deben elaborar con pocas columnas y renglones. Se debe tener la precaución de incluir el nivel de significancia estadística representado por letras minúsculas del comienzo del alfabeto (a, b, c, d,...), un asterisco simple (*) para $P<0,05$, doble asterisco (**) para $P<0,01$ o triple asterisco (***) para $P<0,001$. Las investigaciones que no siguen un diseño estadístico, deben mostrar la información de manera descriptiva. Use subíndices para modificaciones, reserve superíndices para potencias o notas al pie en tablas y figuras.

La discusión: Se refiere al análisis e interpretación objetiva de los resultados, confrontándolos con los obtenidos en otras investigaciones, o con los hechos o teorías conocidos sobre el tema. Explica los resultados en particular cuando difieren de la hipótesis planteada. Destaca la aplicación práctica o teórica de los resultados obtenidos y

las limitaciones encontradas. Resalta la contribución que se hace a una determinada área del conocimiento y el aporte a la solución del problema que justifica la investigación. Finalmente, proporciona elementos que permitan proponer recomendaciones o lanzar nuevas hipótesis. No se deben hacer afirmaciones que van más allá de lo que los resultados pueden apoyar.

Conclusiones

Son las afirmaciones originadas a partir de los resultados obtenidos, deben ser coherentes con los objetivos planteados y la metodología empleada; además, expresar el aporte al conocimiento en el área temática estudiada y proponer directrices para nuevas investigaciones.

Agradecimientos

Si se considera necesario, se incluyen los agradecimientos o reconocimientos a personas, instituciones, fondos y becas de investigación, que hicieron contribuciones importantes en la concepción, financiación o realización de la investigación.

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García Rodríguez JL, Giménez Suarez MC, Ortega Pérez E, Martín Ramos B, Calderón Guerrero C. 2014. Operaciones auxiliares en repoblaciones e infraestructuras forestales. Ediciones Paraninfo SA, Madrid. 208 p.

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Bertoft E and Blennow A. 2016. Chapter 3 - Structure of potato starch. pp 57-73. In: Singh J and Kaur L. (eds.). Advances in potato chemistry and technology. Second edition. Academic Press, London. 752 p.

Artículos de revistas: Autor(es). Año. Título del artículo. Nombre completo de la revista volumen(número de fascículo): página inicial–página final. doi. Ejemplo: García S, Clinton W, Arreaza L and Thibaud R. 2004. Inhibitory effect of flowering and early fruit growth on leaf photosynthesis in mango. Tree Physiology 24(3): 387-399. doi: 10.1093/treephys/24.4.387

Ponencias en memorias de congresos, seminarios, simposios: García M. 1998. La ingeniería geotécnica y la protección del medio ambiente. pp. 65-94. En: Memorias IX Congreso Colombiano de la Ciencia del Suelo. Sociedad Colombiana de la Ciencia del Suelo, Bogotá.

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Tesis, trabajos de grado. Gómez C. 2004. Autoecología del Mortiño (*Vaccinium meriodinale* Swartz Ericaceae) (Tesis de maestría). Universidad Nacional de Colombia. Medellín, Colombia. 78 p.

Adam M. 1992. The Impact of the Common Agricultural Policy on Agriculture in Greece (Master's thesis). Cambridge University. Cambridge, United Kingdom. 80 p.

Cita de cita, sólo se referencia la fuente consultada. Ejemplo: Gómez C. 2004. Autoecología del Mortiño (*Vaccinium meriodinale* Swartz Ericaceae) (Tesis de maestría). Universidad Nacional de Colombia. Medellín, Colombia.

Suplemento de revista: Silva AM y Carrillo NN. 2004. El manglar de piruja, Golfito, Costa Rica: un modelo para su manejo. Revista de Biología Tropical 52 Suppl. 2: 195-201.

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Patentes: Autor(es). Año. Título. País de la patente y número. Fuente. Ejemplo: Glenn RW. 1996. Liquid personal cleansing compositions which contain soluble oils and soluble synthetic surfactants. U.S. Patent No. 6194364. Retrieved from: https://patents.google.com/patent/US6194364B1/en

PUBLISHING POLICY

REVISTA FACULTAD NACIONAL DE AGRONOMÍA MEDELLÍN

The Journal *Revista Facultad Nacional de Agronomía Medellín* (RFNA) is published by the Faculty of Agricultural Sciences of Universidad Nacional de Colombia – Medellín. It is aimed at professors, researchers and students in agronomy, animal, and forestry sciences, food and agricultural engineering, agricultural advisers and at all those professionals who create knowledge and articulate science and technology to make the field more productive at business and rural economy levels.

The Journal receives and publishes, without any cost, research articles, reviews, revisions, letters to the editor and editorials written in the English language.

The Journal is a four-monthly publication at national and international level. Its aim is to publish original, unpublished, and peer-reviewed articles of a scientific nature which respond to specific questions and provide support and testing of a hypothesis, related to agronomy, animal husbandry, forestry engineering, food and agricultural engineering, and related areas that contribute to the solution of the agricultural constraints in the tropics.

Taking into account Colciencias (Administrative Department of Science, Technology and Innovation of Colombia) criteria, the journal welcomes papers of the following types:

Research papers in science and technology: A document presenting in detail the original results of completed research projects. The structure generally used contains four main parts: Introduction, methodology (materials and methods), results and discussion, and conclusions. The maximum extension must be 5200 words; excluding figures, tables, references. The maximum number of bibliographic references suggested is 30. This type of article is peer-reviewed and indexed.

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Articles must be submitted in accordance with the guidelines set forth in “Instructions to Authors”; those who violate the rules will not initiate the basic editorial process. Shall be filled the form “Authorization for Release of Works and Economic Rights Assignment”, which will be provided by the Journal. This document is explicit in mentioning that all authors are informed and agree with article submitted for consideration to the Journal, that there is no conflict of interest between them, and also state that the manuscript has not been and will not be submitted for publication to another Journal.

The Editorial Board, supported by a team of associate editors, will evaluate the scientific merit of the paper and will then submit it for evaluation under double-blind method- that is to say, strict anonymity in the review is kept- by two arbitrators specialized in the area, preferably one national and one international, who will give their report on the format provided by the Journal. The Editorial Board reserves the right to accept collaborations. The report, after the review process, can be: accepted for publication with no or few modifications; accepted for publication with major changes according to the comments of the evaluators; reconsidered for publication if it is substantially modified - in this case, it will be deemed as new material; rejected for publication. If articles are accepted, they will be returned to authors for correction and sent again to the Director of the Journal within 30 calendar days.

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INSTRUCTIONS TO AUTHORS

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Papers must be sent b through the Open Journal System in the Universidad Nacional de Colombia journals web side http://www.revistas.unal.edu.co/, Will be considered only papers written in English. The four following formats must be submitted with the manuscript: (1) Editorial Criteria Checklist for Paper Submission; (2) Paper Publishing Authorization for the Revista Facultad Nacional de Agronomía Medellín, which accepts no simultaneous nomination of the article to other journals or editorial bodies, and the rights are given to the Journal for its release by the signature of all the manuscript's authors; (3) Personal information of each author; (4) Suggestion of possible peer reviewers. Publishing forms are: scientific and technological research articles, review articles, reflection articles, and short articles. Articles can be developed by professors and/or researchers at the Universidad Nacional de Colombia, or other related national or international institution, on Agricultural, Forestry, Food and Agricultural Engineering matters. Article extension must not exceed 5,200 words for research articles and 6,000 words for reviews. The manuscript must be letter-size sheets, line spacing double, continuous line number 12 point Times New Roman or Verdana font, 3 cm margin at the upper, 2 cm in the lower, 2.5 cm on the left and right side margins. Tables and figures (i.e, graphics, drawings, diagrams, flowcharts, photographs and maps) should be shown on separate sheets and numbered consecutively (Table 1 ... Table n, Figure 1…Figure n, etc.). Texts and tables should be submitted in MS-Word® word processor, original tables and diagrams of frequency (bar charts and pie charts) must be supplied in manuscript file and in its original MS-Excel®; other figures, such as photographs on paper and drawings, can be sent in original or scanned and sent in digital format compression JPG (or JPEG), preferably with a resolution of 600 x 600 dpi (300 dpi at least); original photographs are suggested to be sent as slides. As a general rule, tables and figures are only accepted in black and white. Color figures will be exceptionally accepted when strictly necessary and under discretion of the Editorial Board.

Units, abbreviations and style

International System of Units (SI), and those specific units of greater use by the scientific community must be used. When required must be used the exponential form. Example: kg ha^{-1}. The meaning of abbreviations should be cited in full when first mentioned in the manuscript. The writing style should be totally impersonal. Introduction, procedures and results should be written in grammatical past tense. Discussion should be written in grammatical present tense, avoiding the conjugation of verbs in first or third person singular or plural.

The numbers from 1 to 9 are written in words, except when they include units of measure or several numbers are listed. Example: “eight treatments”, “3,7 and 9 readings”, “15 kg”. Use zero before the decimal point. To separate numbers in intervals of one to two years, use the letter “a” and hyphen for growing seasons. Example period 2002 to 2005, growing seasons 1999-2000, 2000-2001.

Title and authors

The article should not include abbreviations and its translation into English is required. As far as possible, the title should not exceed 15 words and must accurately reflect the paper content. When the article contains scientific names of plants or animals, they should be written in italics in lower case, only the first letter of gender and classifier should be capital. Under the title in English the author or authors' name (s) and surname (s) is /are written, without academic degrees or job positions, in a horizontal line according to the contribution to research and / or preparation of the article.

As a footnote on the first page, write the title of undergraduate, authors' job positions, the name and city location of the entity to which they serve, or the sponsors for the research work and their respective email address. In addition, a summarized authors' résumé including reference to the articles published in other magazines should be attached.

Abstract and key words

The abstract should not exceed 250 words written in a single paragraph. It must be written in English and Spanish. It should contain in brief the justification, aims, methods used, the most relevant results, and conclusions. It is required to accompany the abstract with a maximum of six key words, translated into English, different from those used in the title. Single words as well as compound terms of up to three words are accepted as key words. They must be written in lowercase, separated by commas.

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It may or not have a title. It defines the problem and reports on the state of the art on the main subject of the article, it also points out the reasons for the research and sets out its aims. It is required to accompany common names with the corresponding scientific name (s) name and abbreviation (s) of the classifier at the first mention in the text. Brands must not be mentioned but the generic or chemical name.

Materials and methods

In this section, materials (crops, livestock, agricultural or laboratory implements) used in the development of work should be clearly, concisely and sequentially described. Aspects related to the location, preparation and execution of experiments should also be mentioned. The selected design, the recorded variables, the changes made to data, the statistical models used and the significance level used should be indicated. Authors must avoid detailing procedures previously published.

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They are the central part of the article and must be supported by appropriate statistical methods and analysis. They should be presented in a logical, objective and sequential way through texts, tables and figures; the latter two supports should be easy to read, self- explanatory and always quoted in the text. The tables should be composed by few columns and rows. Care should be taken to include the statistical significance level represented by lowercase letters of the beginning of the alphabet (a, b, c, d,…), a single asterisk (*) for $P<0.05$, double asterisk (**) for $P<0.01$ or triple asterisk (***) for $P<0.001$. Researches that do not follow a statistical design should display the information in a descriptive way. Use subscripts to modifications, reserve superscripts for potencials or footnotes in tables and figures.

Discussion

It refers to the analysis and objective interpretation of results, comparing them with those obtained in other research, or with known facts or theories on the subject. It explains the results, especially when they differ from the stated hypothesis. It emphasizes the practical or theoretical application of the obtained results and constraints encountered. Discussion also highlights the contribution that is made to a particular area of knowledge and to the solution of the problem that justifies the research. Finally, it provides elements that allow making recommendations or launching new hypotheses. Statements that go beyond what the results may support should be avoided.

Conclusions

Conclusions are assertions arising from the obtained results. They should be consistent with the objectives stated and the methodology used. They should also express the contribution to knowledge in the studied subject area and propose guidelines for further researches.

Acknowledgements

If necessary, acknowledgements or recognitions to individuals, institutions, funds and research grants that made important contributions in the design, financing or carrying out of the research are included.

Citing in-text format

- Citations in the text should be in parenthesis and include author's surname and year, with comma in-between. Example: (Pérez, 1995).

- If more than one date, they are separated by commas: Example: (Pérez, 1995, 1998, 2001).

- If there are two authors, they will be separated by the conjunction and. Example: (Gil and Ortega, 1993)

- If there are several works of an author published in the same year, they will be cited with a letter in alphabetical sequence of titles, adjacent to year. Example: (Gómez, 2000a, 2000b, 2000c)

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Beral H. 1996. Capítulo 6: Evapotranspiración. pp. 112-125. En: Agrios G. (ed.). Fitopatología. Segunda edición. Editorial Limusa, México D.F. 400 p.

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Patents: Author(s). Year. Title. Patent country and number. Retrieved from. Example: Glenn RW. 1996. Liquid personal cleansing compositions which contain soluble oils and soluble synthetic surfactants. U.S. Patent No. 6194364. Retrieved from: https://patents.google.com/patent/US6194364B1/en

ÉTICA EN LA PUBLICACIÓN CIENTÍFICA Y ACUERDO SOBRE POSIBLES MALAS PRÁCTICAS

La revista Facultad Nacional de Agronomía espera y verificará que los autores, revisores, editores y en general la comunidad académica y científica involucrada en nuestro proceso editorial, sigan estrictamente las normas éticas internacionales requeridas en el proceso de edición.

La revista Facultad Nacional de Agronomía sigue las normas éticas presentes en el COPE Best Practice Guidelines for Journal Editors y por el International Standars for Editors and Authors publicado por Committee on Publication Ethics.

Los autores deben evitar incurrir al plagio de la información. La revista define los siguientes lineamientos, criterios y recomendaciones sobre la ética en la publicación científica:

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1.1. Los artículos deben contener suficiente detalle y referencias que permitan replicar o rebatir el estudio.

1.2.Declaraciones fraudulentas o deliberadamente inexactas constituyen un comportamiento poco ético.

1.3. Si el estudio incluye productos químicos, procedimientos o equipos que tienen cualquier riesgo inusual inherente a su uso, el autor debe identificar claramente estos en el artículo.

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1.5. Se deben respetar los derechos de privacidad de los seres humanos.

2. Autoría[2]

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b) Redacción o revisión del contenido intelectual.

c) Aprobación de la versión final.

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Recomendaciones:

2.6. Antes de iniciar la investigación se recomienda documentar la función y la forma como se reconocerá la autoría de cada investigador.

2.7. No se debe mentir sobre la participación de una persona en la investigación o publicación, si su contribución se considerada "sustancial" se justifica la autoría, bien sea como coautor o colaborador.

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3.1. Hace referencia a la adición, supresión o reorganización de los nombres de autor en la autoría de un artículo aceptado.

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4. Conflicto de intereses[4]

Criterios:

4.1. Cuando un investigador o autor, editor tenga alguna opinión o interés financiero/personal que pueda afectar su objetividad o influir de manera inapropiada en sus actos, existe un posible conflicto de intereses. Este tipo de conflictos pueden ser reales o potenciales.

4.2. Los conflictos de intereses más evidentes son las relaciones financieras, como:

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b) Indirectas: honorarios, asesorías a organizaciones promotoras, la propiedad de fondos de inversión, testimonio experto pagado.

4.3. Los conflictos también pueden existir como resultado de relaciones personales, la competencia académica y la pasión intelectual. Por ejemplo, un investigador que tenga:

a) Algún tipo de interés personal en los resultados de la investigación.

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Recomendaciones:

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4.5. Revelar el papel de un promotor (o promotores) del estudio, si los hubiere, en el diseño del estudio, en la recopilación, análisis e interpretación de los datos, en la redacción del informe y en la decisión de presentar el documento para su publicación.

4.6. Los investigadores no deben entrar en acuerdos que interfieran con su acceso a todos los datos y su capacidad de analizarlos de forma independiente, y de preparar y publicar los manuscritos.

4.7. Al presentar un documento, se debe hacer una declaración (con el encabezamiento "Papel que ha tenido la fuente de financiación") en una sección separada del texto y colocarse antes de la sección "Referencias".

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4.9. Todas las fuentes de apoyo financiero para el proyecto deben ser revelados.

4.10. Se debe describir el papel del patrocinador del estudio.

5. Publicación duplicada[5]

Criterios:

5.1. Los autores tienen la obligación de comprobar que su artículo sea basado en una investigación original (nunca publicada anteriormente). El envío o reenvío intencional de su trabajo para una publicación duplicada se considera un incumplimiento de la ética editorial.

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Criterios:

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7. Fraude científico[6]

Criterios:

7.1. El fraude en la publicación científica hace referencia a la presentación de datos o conclusiones falsas que no fueron generados a través de un proceso riguroso de investigación.
7.2. Existen los siguientes tipos de fraude en la publicación de resultados de investigación:
a) Fabricación de datos. Inventar datos y resultados de investigación para después comunicarlos.
b) Falsificación de datos. La manipulación de materiales de investigación, imágenes, datos, equipo o procesos.
La falsificación incluye la modificación u omisión de datos o resultados de tal forma que la investigación no se representa de manera precisa. Una persona podría falsificar datos para adecuarla al resultado final deseado de un estudio.

Recomendaciones:

7.3. Antes de enviar un artículo, lea cuidadosamente las políticas editoriales y de datos de la revista.
7.4. Nunca modifique, cambie u omita datos de forma intencional. Esto incluye materiales de investigación, procesos, equipos, tablas, citas y referencias bibliográficas.
7.5. Tanto la fabricación como la falsificación de datos son formas de conducta incorrecta graves porque ambas resultan en publicaciones científicas que no reflejan con precisión la verdad observada.
7.6. El autor debe hacer una gestión adecuada de los datos que soportan la investigación, teniendo especial cuidado en la recopilación, producción, conservación, análisis y comunicación de los datos.
7.7. Mantenga registros minuciosos de los datos en bruto, los cuales deberán ser accesibles en caso de que un editor los solicite incluso después de publicado el artículo.

8. Plagio[7]

Criterios:

8.1. El plagio es una de las formas más comunes de conducta incorrecta en las publicaciones, sucede cuando uno de los autores hace pasar como propio el trabajo de otros sin permiso, mención o reconocimiento. El plagio se presenta bajo formas diferentes, desde la copia literal hasta el parafraseado del trabajo de otra persona, incluyendo: datos, ideas, conceptos, palabras y frases.
8.2. El plagio tiene diferentes niveles de gravedad, como por ejemplo:
a) Qué cantidad del trabajo de otra persona se tomó (varias líneas, párrafos, páginas, todo el artículo)
b) Qué es lo que se copió (resultados, métodos o sección de introducción).
8.3. El plagio en todas sus formas constituye una conducta no ética editorial y es inaceptable.
8.4. La copia literal solo es aceptable si indica la fuente e incluye el texto copiado entre comillas.

Recomendaciones:

8.5. Recuerde siempre que es esencial reconocer el trabajo de otros (incluidos el trabajo de su asesor o su propio trabajo previo) como parte del proceso.
8.6. No reproduzca un trabajo palabra por palabra, en su totalidad o en parte, sin permiso y mención de la fuente original.
8.7. Mantenga un registro de las fuentes que utiliza al investigar y dónde las utilizó en su artículo.
8.8. Asegúrese de reconocer completamente y citar de forma adecuada la fuente original en su artículo.
8.9. Incluso cuando haga referencia a la fuente, evite utilizar el trabajo de otras personas palabra por palabra salvo que lo haga entre comillas.
8.10. El parafraseado solo es aceptable si indica correctamente la fuente y se asegura de no cambiar el significado de la intención de la fuente.
8.11. Incluya entre comillas y cite todo el contenido que haya tomado de una fuente publicada anteriormente, incluso si lo está diciendo con sus propias palabras.

9. Fragmentación[8]

Criterios:

9.1. La fragmentación consiste en dividir o segmentar un estudio grande en dos o más publicaciones.
9.2. Como norma general, con tal de que los "fragmentos" de un estudio dividido compartan las mismas hipótesis, población y métodos, no se considera una práctica aceptable.
9.3. El mismo "fragmento" no se debe publicar nunca másde una vez. El motivo es que la fragmentación puede dar lugar a una distorsión de la literatura haciendo creer equivocadamente a los lectores que los datos presentados en cada fragmento (es decir, artículo de revista) se derivan de una muestra de sujetos diferente. Esto no solamente sesga la "base de datos científica", sino que crea repetición que hace perder el tiempo de los editores y revisores, que deben ocuparse de cada trabajo por separado. Además, se infla injustamente el número de referencias donde aparece citado el autor.

Recomendaciones:

9.4. Evite dividir inapropiadamente los datos de un solo estudio en dos o más trabajos.
9.5. Cuando presente un trabajo, sea transparente. Envíe copias de los manuscritos estrechamente relacionados al manuscrito en

cuestión. Esto incluye manuscritos publicados, enviados recientemente o ya aceptados.

10. Consentimiento informado

Criterios:

10.1. Los estudios sobre pacientes o voluntarios requieren la aprobación de un comité de ética.

10.2. El consentimiento informado debe estar debidamente documentado.

10.3. Los permisos y las liberaciones deben ser obtenidos, cuando un autor desea incluir detalles de caso u otra información personal o imágenes de los pacientes y cualquier otra persona.

10.4. Especial cuidado debe tenerse con la obtención del consentimiento respecto a los niños (en particular cuando un niño tiene necesidades especiales o problemas de aprendizaje), donde aparece la cabeza o la cara de una persona, o cuando se hace referencia al nombre de un individuo u otros datos personales.

11. Corrección de artículos publicados[9]

Criterio:

Cuando un autor descubre un error o inexactitud significativa en el trabajo publicado, es obligación del autor notificar de inmediato a la revista y cooperar en el proceso de corrección.

Referencias

Black, William, Rodolfo Russo, y David Turton. «The Supergravity Fields for a D-Brane with a Travelling Wave from String Amplitudes». Physics Letters B 694, n.º 3 (noviembre de 2010): 246-51.

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[3] William Black, Rodolfo Russo, y David Turton, «The Supergravity Fields for a D-Brane with a Travelling Wave from String Amplitudes», Physics Letters B 694, n.º 3 (noviembre de 2010): 246-51.

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[7] Elsevier, «Plagio. Ethics in research & publication», accedido 8 de agosto de 2014, http://www.elsevier.com/__data/assets/pdf_file/0016/183400/ETHICS_ES_PLA01a_updatedURL.pdf.

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PUBLICATION ETHICS AND PUBLICATION MALPRACTICE STATEMENT

The journal Revista Facultad Nacional de Agronomia follows the COPE Code of Conduct and Best Practice Guidelines for Journal Editors and the International Standards For Editors and Authors, published by Committe on Publication Ethics.
The journal puts forth the following criteria and recommendations for ethical scientific publications:

1. General criteria[1]

1.1. Articles must contain sufficient details and references that allow the study to be replicable or refutable.
1.2. Fraudulent or deliberately inexact statements constitute unethical behavior.
1.3. If a study includes the use of chemical products, procedures, or equipment that presents an inherent risk, the author must state so in the article.
1.4. If the study involves the use of animals or human beings, the article must contain a clear statement that all of the procedures were carried out in strict compliance with laws and institutional directives.
1.5. The privacy of the human beings must be respected.

2. Authorship[2]

Criteria:
2.1. An "author" is a person that has made a significant intellectual contribution to an article; all of the individuals that are named as authors must fulfill the requirements for authorship and all of those individuals that do so must be explicitly named.
2.2. Three basic criteria must be met in order to be considered an author:
a) Substantial contribution to the study concept, design, and data collection, analysis and interpretation.
b) Revision of the intellectual content.
c) Approval of the final version.
2.3. The order of the author list must be a joint decision of the coauthors.
2.4. The individuals that participate in a study but that do not meet the criteria for authorship must be listed as an "Assistant" or "recognized person."
2.5. There are three types of unacceptable authorship: "ghost" authors, who make a substantial contribution but are not recognized (often paid by commercial promoters); "guest" authors, who do not make a discernable contribution but are named in order to increase the probability of publication; and "honorary" authors, who only have a tenuous connection to the study.
Recommendations:
2.6. Before starting the research, establish the function of each researcher and the manner in which they will be recognized.
2.7. It is not necessary to mention an individual's participation in a study or publication, but if their contribution is substantial, than authorship would be justified, either as an author or assistant.
2.8. Authorship cannot be bestowed on an individual without their consent.
2.9. All of the individuals that are named as authors must meet the requirements for authorship and all of those that meet the requirements must appear as authors or assistants.
2.10. Some groups list the authors alphabetically, sometimes with a notation that indicates that all of the authors contributed equally to the study and the publication.

3. Changes in the authorship[3]

Criteria:
3.1. Additions to, removals from, and reorganization of the author names in accepted articles must be noted.
3.2. Petitions to add to, remove from, or reorganize the authors must be sent by the corresponding author of the accepted articles and must include:
a) The reason for the addition, elimination, or reorganization.
b) A written statement (e-mail) from all of the authors that confirms their agreement with the addition, elimination, or reorganization. In the case of an addition or elimination, a confirmation is also required from the author to be added or removed.

4. Conflict of interest[4]

Criteria:
4.1. When a researcher or author has a financial/personal opinion or interest that could affect their objectivity or improperly influence their actions, there exists a possible conflict of interest. Conflicts can be actual or potential.
4.2. The most evident conflicts of interest are financial, such as:
a) Direct: employment, stocks, scholarships, patents.
b) Indirect: assistantship to promoting organizations, investment funds, paid expert testimony.
4.3. Conflicts can also arise from personal relationships, academic competition, and intellectual passion. For example, an author could have:
a) Some personal interest in the results of the research.
b) Personal opinions that are in direct conflict with the research topic.
Recommendations:
4.4. Disclose all conflicts of interest, actual or potential, that inappropriately influence the findings or results of a study, including any that arise within the three (3) years after the start of said study if they could unduly (bias) influence the study.
4.5. Disclose the role of any promoter (or promoters) in the study, if any, in the design, in the collection, analysis or interpretation of the data, in the document review, or in the decision to present the document for publication.
4.6. The researchers must not enter into agreements that interfere with their access to all of the data or with their ability to independently analyze the data or to prepare and publish the manuscript.
4.7. The document must contain a statement (with the heading "Role of the financial source") in a section that is separate from the text and before the References section.
4.8. Some examples of conflicts of interest that must be revealed include: employment, consulting, stocks, honorariums, paid expert testimony, patent requests or registration, and subsidies or other financing.
4.9. All of the sources of financial support for the project must be revealed.
4.10. The role of any study sponsors must be described.

5. Duplicate publication[5]

Criteria:
5.1. Authors have the obligation of proving that their article is based on original research (never before published). The intentional submission or resubmission of a manuscript for duplicate publication is considered a breach of editorial ethics.
5.2. A duplication publication, or multiple publication, results when two or more articles, without any reference to each other, essentially share the same hypothesis, data, discussion points, and/or conclusions. This can occur to different degrees: literal duplication, partial but substantial duplication or paraphrasal duplication.
5.3. One of the main reasons that duplicate publications are considered unethical is that they can result in the "inappropriate weighting or unwitting double counting" of results from just one study, which distorts the available evidence.
Recommendations:
5.4. Articles sent for publication must be original and not sent to other editors. When sent, the authors must reveal the details of related articles (even when in another language) and similar articles being printed or translated.

5.5. Even though a submitted article is being reviewed and the final decision is not known, wait to receive notification from the editors before contacting other journals and then only do so if the editors decline to publish the article.
5.6. Avoid submitting a previously published article to another journal.
5.7. Avoid submitting articles that essentially describe the same research to more than one journal.
5.8. Always indicate previous submissions (including presentations and recorded results) that could be considered duplicate results.
5.9. Avoid writing about your research in two or more articles from different angles or on different aspects of the research without mentioning the original article.
5.10. Creating various publications based on the same research is considered a type of manipulation.
5.11. If an author wishes to send an article to a journal that is published in a different country or a different language, ask for permission from the editors first.
5.12. When submitting an article, indicate all of the details of the article that were presented in a different language along with the relevant translations.

6. Acknowledging sources

Criteria:

6.1. Authors must cite the publications that had an influence on the determination of the nature of the offered study.
6.2. Privately obtained information cannot be used without the express written consent of the source.
6.3. Republishing tables or figures requires the permission of the author or editor, who must be appropriately cited in the table or figure legend.
6.4. Information obtained through confidential services, such as arbitration articles or subsidy applications, cannot be used without the express written consent of the author of the work involved in said services.

7. Scientific fraud[6]

Criteria:

7.1. Fraud in scientific publications refers to the presentation of false data or conclusions that were not obtained through a rigorous research process.
7.2. The following types of fraud exist for the publication of research results:
a) Fabricating data. Inventing research data and results for later dissemination.
b) Falsification of data. The manipulation of research material, images, data, equipment or processes. Falsification includes the modification or omission of data or results in such a way that the research is not represented in a precise manner. A person may falsify data in order to obtain the desired final results of a study.

Recommendations:

7.3. Before submitting an article, carefully read the editorial and data policies of the journal.
7.4. Never modify, change or omit data intentionally. This includes research material, processes, equipment, tables, citations, and bibliographical references.
7.5. Fabricating and falsifying data constitute grave misconduct because both result in scientific publications that do not precisely reflect the actual observations.
7.6. Authors must appropriately manage the data that supports the research, taking special care in the compilation, production, preservation, analysis and presentation of the data.
7.7. Maintain precise records of the raw data, which must be assessable in case the editors request them after publication of the article.

8. Plagiarism[7]

Criteria:

8.1. Plagiarism is one of the more common types of misconduct in publications; it occurs when an author passes the work of others off as their own without permission, citations, or acknowledgment. Plagiarism can occur in different forms, from literally copying to paraphrasing the work of another person, including data, ideas, concepts, paragraphs, and phrases.
8.2. Plagiarism has different degrees of severity; for example:
a) The quantity of work taken from another person (various lines, paragraphs, pages, or the entire article).
b) What is copied (results, methods, or introduction section).
8.3. Plagiarism, in all of its forms, constitutes unethical behavior and is unacceptable.
8.4. Literal copying is acceptable if the source is indicated and the text is placed in quotation marks.

Recommendations:

8.5. Always remember that it is vital to recognize the work of others (including the work of your assistants or your previous studies).
8.6. Do not reproduce the work of others word for word, in totality or partially, without the permission and recognition of the original source.
8.7. Maintain a record of the sources that are used in the research and where they are used in the article.
8.8. Be sure to accurately acknowledge and cite the original source in your article.
8.9. Even when referencing the source, avoid using the work of others word for word unless it is placed in quotations.
8.10. Paraphrasing is only acceptable if the source is correctly indicated and the source's intended meaning is not changed.
8.11. Use quotations, and cite all of the content that is taken from a previously published source even when using your own words.

9. Fragmentation[8]

Criteria:

9.1.Fragmentation occurs when a large study is divided or segmented into two or more publications.
9.2. As a general rule, as long as the "fragments" of a divided study share the same hypothesis, populations, and methods, this not considered an acceptable practice.
9.3. The same "fragment" can never be published more than one time. Fragmentation can result in distortion of the literature, creating the mistaken belief in readers that the data presented in each fragment (i.e. journal article) are derived from different subject samplings. This not only distorts the "scientific database", but creates repetition that results in a loss of time for editors and evaluators that must work on each article separately. Furthermore, the cited author receives an unfair increase in their number of references.

Recommendations:

9.4. Avoid inappropriately dividing the data of one study into two or more articles.
9.5. When presenting your work, be transparent. Send copies of the manuscripts that are closely related to the manuscript in question, including published, recently submitted and accepted manuscripts.

10. Informed consent

Criteria:

10.1. Studies on patients and volunteers require the approval of the ethics committee.
10.2. The informed consent must be duly documented.
10.3. Permission and waivers must be obtained when an author wishes to include details of a case or other personal information or images of the patients or any other person.
10.4. Special care should be taken when obtaining the consent

of children (especially when a child has special needs or learning disabilities) when their head or face is displayed or when reference is made to the name of an individual or other personal data.

11. Correction of published articles[9]

Criterion:

When an author discovers a significant inexactitude or error in a published article, they must immediately notify the journal and cooperate in the correction process.

References

Black, William, Rodolfo Russo, y David Turton. «The Supergravity Fields for a D-Brane with a Travelling Wave from String Amplitudes». *Physics Letters B* 694, n.º 3 (noviembre de 2010): 246-51.

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