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## Ecofisiología y manejo de cultivo



## Recursos naturales y ambiente

# Growth and yield of *Syagrus schizophylla* (Mart.) Glass. in response to light gradients

## Crecimiento y rendimiento de *Syagrus schizophylla* (Mart.) Glass. en respuesta a gradientes de luz

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**Abstract**

This research studied growth and yield of *Syagrus schizophylla,* an extinction-endangered ornamental palm, grown under five light gradients. The treatments were: $G_1$ - PAR=1234.10 µmol photons $m^{-2}$ $s^{-1}$, $G_2$ - PAR=913.16 µmol photons $m^{-2}$ $s^{-1}$, $G_3$ - PAR=666.34 µmol photons $m^{-2}$ $s^{-1}$, $G_4$ - PAR=419.56 µmol photons $m^{-2}$ $s^{-1}$ and $G_5$ - PAR=534.77 µmol photons $m^{-2}s^{-1}$. Before the experiment and at three, five and seven months of treatment, growth (plant height, collar diameter, number of leaves, petiole length, leaf length and width), gas exchange, chlorophyll *a*, and leaf green color intensity were assessed. The highest net photosynthetic rates were observed in plants under $G_2$, $G_3$ and $G_4$. Values of maximum quantum efficiency ($F_v/F_m$) over 0.75 were observed under $G_2$. At seven months, estimated SPAD values were 36 in $G_2$ plants and 32 in $G_1$ plants. According to the Dickson quality index (DQI), presented the highest growth and development rates. We conclude that $G_2$ suits plants to be transplanted into the field, whereas $G_3$ would be best for plants grown under shade environments.

**Keywords**
Arecaceae • gas exchange • palm tree • photosynthesis • shading

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## Resumen

*Syagrus schizophylla* es una especie de palmera ornamental en peligro de extinción. El objetivo de esta investigación fue estudiar los aspectos fisiológicos del crecimiento y la calidad de las plantas producidas bajo gradientes de luz. Los tratamientos de gradientes de luz fueron: G1 - PAR = 1234.10 µmol fotones $m^{-2}$ $s^{-1}$, G2 - PAR = 913.16 µmol fotones $m^{-2}$ $s^{-1}$, G3 - PAR = 666.34 µmol fotones $m^{-2}$ $s^{-1}$), G4 - PAR = 419,56 µmol fotones $m^{-2}$ $s^{-1}$ y G5 - PAR = 534,77 µmol fotones $m^{-2}$ $s^{-1}$. Antes de aplicar los tratamientos y a los tres, cinco y siete meses de tratamiento, se analizaron la altura de la planta, diámetro del collar, número de hojas, largo del pecíolo, largo y ancho de la hoja), intercambio gaseso, fluorescencia de la clorofila a y color verde de la hoja. Las tasas fotosintéticas netas más altas se observaron en plantas bajo G2, G3 y G4. Em G2 se observaron valores de Fv/Fm superiores a 0,75. En el séptimo mes, los valores estimados del índice SPAD fueron 36 en plantas bajo G2 y 32 en plantas bajo G1. El mejor crecimiento y desarrollo de las plantas se observó en G2 de acuerdo con DQI que muestra que este gradiente debe usarse para plantas destinadas al campo, mientras que G3 sería mejor para plantas destinadas a entornos sombríos.



**Palabras clave**

Arecaceae • intercambio gasesoso • palmera • fotosíntesis • sombreado


## Introduction

*Syagrus schizophylla* (Mart.) Glass. is an indigenous Arecaceae, naturally found in the Brazilian Sandy Coast (Restinga) and the Atlantic Rain Forest (17). Ordinarily known as *aricuriroba*, *coco-babão* and *licuriroba,* the fruits are considered an unconventional food and an alternative source of carbohydrates, proteins and lipids (23). Unfortunately, the species is considered endangered by the Flora Conservancy National Center (CNCFlora Red List, Brazil), mainly given to both urban and tourist pressures (25).

In forests, palm distribution depends on light and water availability. The Arecaceae family shows a wide diversity of genera and species, still unstudied regarding adaptive traits. Due to spatial distribution and diversity, palms are suitable models for studying biodiversity of tropical and subtropical ecosystems (17, 23, 25).

The photosynthetically active radiation (PAR, µmol fótons $m^{-2}s^{-1}$), ranges between 400 and 700 nm (10). Red light incidence (600-700 nm) on the leaf mesophyll enhances stomatal opening (1). However, exposure for long periods may cause red light syndrome, leading to low quantum yield, low photosynthetic capability and lack of stomatal response (20, 27). Light intensity and quality vary according to daytime, season, location, climate, position within the plant canopy, and even within the cell (21). Such variations can result in morphoanatomical changes affecting leaf biomass allocation, chlorophyll *a*/*b* ratio, leaf thickness, stomatal density and photochemical dissipation (27), organization of the photosynthetic apparatus, etiolation, leaf area and carbohydrate storage, among others (14, 20). Blue, red and infrared light activate specific receptors, triggering independent key physiological events (20). This research hypothesizes that using shade nets with light gradients in the blue and red ranges would result in optimized plant carbon allocation and photosynthesis. We studied the physiological response of *S. schizophilla* to different wavelengths determining the best environmental conditions for sapling yield. The experiments used different shade nets, assuming that specific wavelengths modify the intercepted energy, leading to optimized carbon fixation, growth and yield (5, 10).

Given the need for the preservation and recovery of negatively anthropized areas, this study might contribute to optimizing plant conservation, preservation and sustainable use, while offering income diversification in rural areas.

## Material and methods

**Plant material, seedling transplant and light gradients**

Seeds were obtained and germinated as described by Beltrame *et al.* (2019). Ninety days after emergence, seedlings of *S. schizophylla* were transplanted into pots of approximately 6.3 L, previously filled with a mixture of commercial substrate Plantmax® and coconut fibre (1:1; v:v) with the following physicochemical characteristics: P = 0.01 g $kg^{-1}$, K = 5.47 g $kg^{-1}$, S = 4.82 g $kg^{-1}$, Ca = 7.89 g $kg^{-1}$, Mg = 4.34 g $kg^{-1}$; B = 15.07 mg $kg^{-1}$, Fe = 1.54 mg $kg^{-1}$, Mn = 4.34 mg $kg^{-1}$, Zn = 26.46 mg $kg^{-1}$, Cu = 0.2 µg $kg^{-1}$, pH = 4.11, CE 0.8 µS $cm^{-1}$, bulk density = 0.42 g $cm^{-3}$, true density = 1.58 g $cm^{-3}$ and total porosity of 73% of container volume.

Initially, the plants were kept for 270 days under an average of 534.77 µmol photons $m^{-2}$ $s^{-1}$ (May 2016 to February 2017), in a greenhouse, with mean PAR 534.77 µmol photons $m^{-2}$ $s^{-1}$. After that period, treatments lasted 210 days (from March to October 2017). The seedlings were randomly organized and grown under tunnels (1.80x1.50x1.80 m) covered with different shade nets resulting in different light gradient treatments: plain sunlight - control ($G_1$) (PAR=1234.10 µmol photons $m^{-2}$ $s^{-1}$), red Chromatinet® 50% of shade ($G_2$) (PAR=913.16 µmol photons $m^{-2}$ $s^{-1}$), two overlapping layers of red Chromatinet® 50% of shade ($G_3$) (PAR=666.34 µmol photons $m^{-2}$ $s^{-1}$), black polyolefin 50% of shade ($G_4$) (PAR = 419.56 µmol photons $m^{-2}$ $s^{-1}$) and overlapping layers of milky plastic film and polyolefin 50% of shade ($G_5$) (PAR = 534.77 µmol photons $m^{-2}$ $s^{-1}$).

HOBO Pro v2 Data Loggers hourly monitored mean, minimum, and maximum temperatures throughout the experimental period. In addition, light spectral quality was evaluated using the USB2000+RAD Ocean Optics UV/Vis spectrum radiometer, obtaining three consecutive readings in each tunnel, at 9 am and three consecutive readings at noon, on bright sunny days.

**Growth analyses**

Before light treatments (BT), and at three, five and seven months, we measured collar diameter (DC), shoot height (SH), number of leaves (NL), petiole length (LP), leaf length (LL) and width (LW). The LP, LL and LW were measured with a ruler, on the second pair of fully expanded leaves.

After seven months, shoot (SDW) and root dry weight (RDW), and plant total leaf area (TLA) were determined. All leaves were detached, and leaf blades and petioles were separated with pruning shears for TLA determination using a Li-3100 (Li-Cor, USA) leaf area meter. For dry weight determination, plant shoot and roots were separated, paper bagged and dried in a convection oven at 70 ± 2°C for 96 hours. Root and shoot dry weights were gravimetrically determined (±0.0001 g).

Dickson quality index (DQI) was calculated according to Dickson *et al.* (1960) (eq. 1), using total dry mass (TDM), shoot height (SH), collar diameter (DC), shoot dry weight (SDW) and root dry weight (RDW).

$$\mathbf{DQI = \{TDM/[(SH/DC) + (SDW/RDW)]\}} \quad (1)$$

**Gas exchange, chlorophyll *a* fluorescence and green color intensity**

Gas exchange was evaluated between 8 am and 10 am with a portable infrared analyzer (IRGA - model Li-6400 XT - Li-Corporation/USA). Evaluation cycles correspond to cycle 1 = one month after initializing treatments (AT)], cycle 2 = three months after AT and cycle 3 = seven months after AT. Net photosynthesis ($A$), transpiration ($E$), stomatal conductance ($g_s$) and internal vs. external $CO_2$ ($C_i/C_a$), were determined on the second pair of completely developed leaves. For that purpose, a 6 $cm^2$ chamber was conditioned with 1500 µmol photons $m^{-2}$ $s^{-1}$ light intensity, 500 µmol $s^{-1}$ airflow, and 400 ppm standard $CO_2$ concentration (obtained with a $CO_2$ mixer) at room temperature, with mean temperatures varying between 25 and 30°C. Light response curves with 24 levels of PPFD from 1500 to 0 µmol $m^{-2}$ $s^{-1}$ allowed for Optimal photosynthetic photon flux density (PPFD) determination. Meanwhile, and on the same leaf used for gas exchange measurements, chlorophyll fluorescence was determined using a Pocket fluorimeter PEA (*Plant Efficiency Analyser*, Hansatech, England).

Leaves were dark-adapted for 30 minutes with leaf clips (Hansatech), avoiding leaf veins, so that all reaction centres were in the oxidized state. Maximum quantum yield of photosystem II (*Fv/Fm*) and the photosynthetic index (*PI*) were determined according to Strasser *et al.* (2004). Leaf green colour intensity (SPAD index), which correlates with chlorophyll content, was measured with a portable chlorophyll meter (model SPAD-502 Minolta, Japan). Mean values were obtained from eight measures per plant.

Gas exchange and chlorophyll fluorescence were measured one, three and seven months later. SPAD measurements were taken before treatments (AT) and three, five and seven months later.

### Statistical analysis

The experiment was conducted in a completely randomized design with five light gradient treatments and six replications, totalizing 30 plants. Data were subjected to ANOVA, and means were compared by Tukey test at 5% probability. Statistical analyses were performed with *R* (26).

## Results and discussion

### Light, gas exchange and photosynthetic capacity

Under all light gradients, air temperature and humidity were very similar, with mean temperatures varying between 25 and 30°C, and air humidity around 80%. Light spectrum varied as follows: 300 - 900 nm in $G_1$, 300 - 850 nm in $G_2$, 300 - 800 nm in $G_3$, 400 - 700 nm in $G_4$ and 400 - 750 nm in $G_5$. Net photosynthesis (A) varied independently of light gradient (figure 1A). At cycle 1, the highest A values were observed in $G_2$ and $G_3$, reaching approximately 8.0 µmol $CO_2$ $m^{-2}$ $s^{-1}$ (figure 1A).

Cycle 1 (one month of light treatment), cycle 2 (three months of light treatment) and cycle (seven months of light treatment).

Upper case letters compare evaluation cycles; lower case letters compare light gradients, by Tukey test (p<0.05).

Ciclo 1 (un mes de fototratamiento), ciclo 2 (tres meses de fototratamiento) y ciclo (siete meses de fototratamiento).

Letras mayúsculas comparan ciclos de evaluación; letras minúsculas comparan gradientes de luz, según la prueba de Tukey (p<0,05).

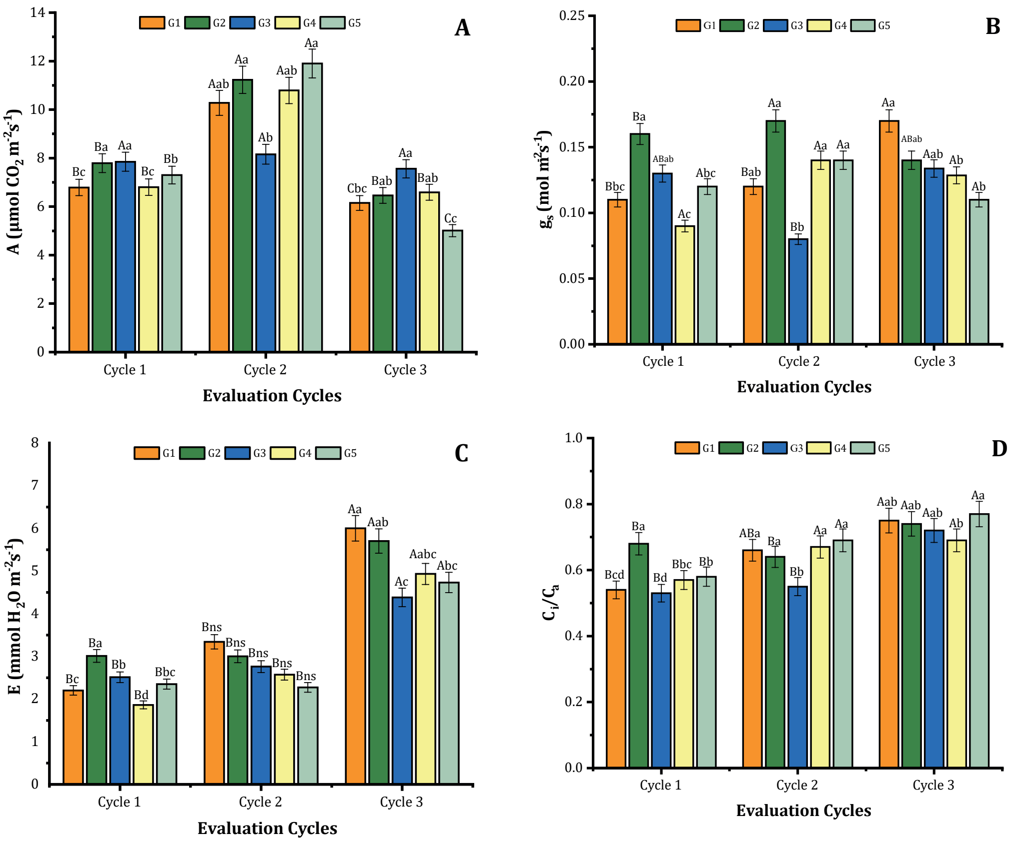



**Figure 1.** Net photosynthesis (*A*), stomatal conductance ($g_s$), transpiration (*E*) and internal vs. external concentration of $CO_2$ ($C_i/C_a$) of *Syagrus schizophylla* plants under light gradients ($G_1$, $G_2$, $G_3$, $G_4$ and $G_5$) and evaluation cycles.

**Figura 1.** Fotosíntesis neta (A), conductancia estomática (gs), transpiración (E) y concentración interna *vs.* externa de $CO_2$ (Ci/Ca) de plantas de *Syagrus schizophylla* bajo gradientes de luz (G1, G2, G3, G4 and G5) y ciclos de evaluación.

At cycle 2, that is, after three months of light treatment, mean A values were different among light gradients. G2 and G5 were different from G3, showing the highest rates of net photosynthesis (figure 1A, page 4). At seven months (cycle 3), higher mean values were observed in plants from G2, G3 and G4, varying between 6 and 7.5 µmol CO2 $m^{-2}$ $s^{-1}$ (figure 1A, page 4). Gas exchange measurements, particularly net photosynthesis, allows understanding genotype x environment photosynthetic patterns (15), constituting a reliable indicator of plant physiological status (24).

Stomatal conductance ($g_s$) showed statistical differences among treatments. At evaluation cycle 1, the highest mean $g_s$ were observed in plants from $G_2$ and $G_3$ (figure 1B, page 4). At cycle 2, no significant differences were observed in $G_2$, $G_4$ and $G_5$. The lowest $g_s$ was observed in $G_3$ (figure 1B, page 4). However, at evaluation cycle 3, mean $g_s$ exceeded 0.11 mol $m^{-2}$ $s^{-1}$ regardless of light gradient (figure 1B, page 4).

While studying *S. schizophylla* palm plants, the highest $g_s$ observed in G2, G3 (cycles 1 and 3) and G2 (cycle 2), suggested that red light somehow influences the stomatal opening. Our results might be related to light incidence and quality, resulting in fast $g_s$ increases and responses to white light components (1). Dumont *et al.* (2013) reported that stomata are especially sensitive to blue light. In this sense, a weak but significant linear correlation between A and $g_s$ (R = 0.45), corroborated our results. On the other hand, Lavinsky *et al.* (2014) reported that A increased 3.5 fold in *Euterpe edulis* saplings acclimated at 25.0 mols photons $m^{-2}$ $d^{-1}$, in relation to understory saplings, acclimated at 1.3 mol photons $m^{-2}$ $d^{-1}$, and presenting low respiration rate and rapid gas response.

Transpiration rate (*E*) of *S. schizophylla* plants showed statistical differences among light gradients at evaluation cycles 1 and 3. At evaluation cycle 1, E was highest in $G_2$ (figure 1C, page 4). No significant differences in E under light gradients were observed in cycle 2. At cycle 3, the highest *E* values were close to 6.0 mmol $H_2O$ $m^{-2}$ $s^{-1}$ in $G_1$ and $G_2$ plants (figure 1C, page 4). Assimilation of $CO_2$ inevitably requires water loss (*E*), as gas diffusion rates increase with stomatal conductance (18).

Mean internal *vs.* external $CO_2$ ratio ($C_i/C_a$) of *S. schizophylla* plants showed statistical differences, with the highest $C_i/C_a$ in $G_2$ plants (figure 1D, page 4). At evaluation cycle 2, mean $C_i/C_a$ ratios in $G_1$, $G_2$, $G_4$ and $G_5$ showed no differences (figure 1D, page 4). In cycles 2 and 3, at three and seven months, mean $C_i/C_a$ ratio increased significantly under light treatments, probably related to plant growth and development, and light quality, since gas exchange is maximized under blue (400-500 nm) and red (600-700 nm) lights (25). In this study, *S. schizophylla* plants showed different responses under different shading, stating a species-dependent need to use shade nets.

Cycles 1 and 2 showed no statistical differences among $F_v/F_m$ (figure 2A, page 6). In contrast, cycle 3 resulted in $F_v/F_m$ significant differences among $G_2$ (lower value) and $G_5$ (higher value) plants, all exceeding 0.75 (figure 2A, page 6). These results suggest that despite the light treatment, *S. schizophylla* did not suffer photoinhibition, in accordance with Bolhàr-Nordenkampf *et al.* (1989) who reported that $F_v/F_m$ varied from 0.75 to 0.85 in plants with intact photosynthetic apparatus. These authors state that photoinhibitory damage on PSII reaction centres causes decreased $F_v/F_m$. Lavinsky *et al.* (2014) reported that *E. edulis* acclimated to understory conditions showed a steep drop in $F_v/F_m$, from 0.8 to 0.5, during the first week after being transferred to a more luminous environment. After 21 days of re-acclimation, $F_v/F_m$ increased, and at 110 days it was almost re-established to the initial value (0.7). Another study also observed that stressed plants tend to decrease $F_v/F_m$ values, dissipating the exceeding energy and preventing photoinhibition (12). In accordance with our findings, other authors found decreased $F_v/F_m$ and photosynthetic index (*PI*) in cucumber plants exposed to red light (19, 20, 27). Osório *et al.* (2012) also state low $F_v/F_m$ for photosynthesis, photochemical efficiency and photoinhibition studies.

Regarding photochemical efficiency, the photosynthetic index (*PI*) showed interaction among treatments. The highest *PI* value at cycle 1 exceeding 6, was observed in plants from $G_1$, while other treatments had *PI* values under 4.5 (figure 2B, page 6). At evaluation cycle 2, the highest values were observed in $G_1$, $G_3$ and $G_5$ (figure 2B, page 6). Cycle 3, showed no significant differences among *PI* in $G_1$, $G_3$, $G_4$ and $G_5$, varying from 3.5 to 4.5 (figure 2B, page 6). Finally, excepting $G_5$ plants, *PI* of *S. schizophylla* plants decreased along cycles, probably given by light stress (13).

Upper case letters compare evaluation cycles; lower case letters compare gradients by Tukey test ($p < 0.05$).

*ns = non-significant ($p < 0.05$).

Las letras mayúsculas comparan los ciclos de evaluación; las letras minúsculas comparan gradientes mediante la prueba de Tukey ($p < 0,05$).

* ns = no significativo ($p < 0,05$).

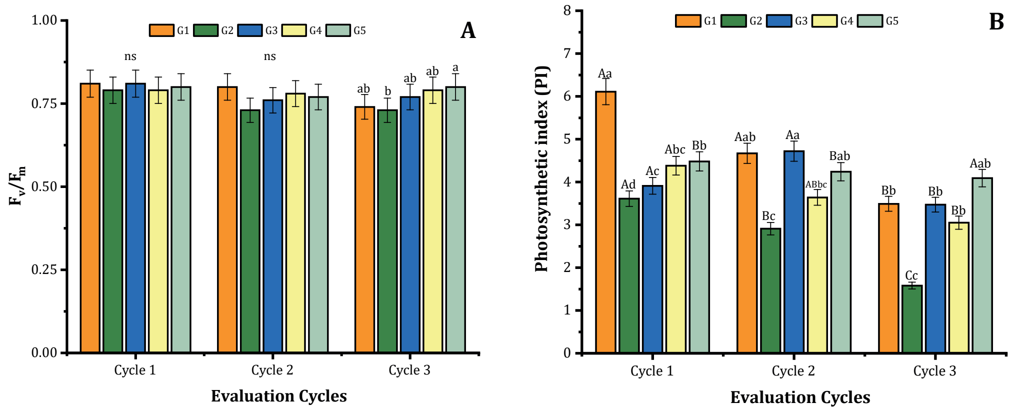



**Figure 2.** Maximum quantum yield of photosystem II ($F_v/F_m$) and photosynthetic index (*PI*) of *Syagrus schizophylla* plants under light gradients ($G_1$, $G_2$, $G_3$, $G_4$ e $G_5$) at evaluation cycles 1 (one month of light treatment ), 2 (three months of light treatment) and 3 (seven months of light treatment).

**Figura 2.** Rendimiento cuántico máximo del fotosistema II (Fv/Fm) e índice fotosintético (PI) de plantas de *Syagrus schizophylla* bajo gradientes lumínicos (G1, G2, G3, G4 y G5) en los ciclos de evaluación 1 (a un mes de tratamiento con luz), 2 (a los tres meses de tratamiento con luz) y 3 (a los siete meses de tratamiento con luz).

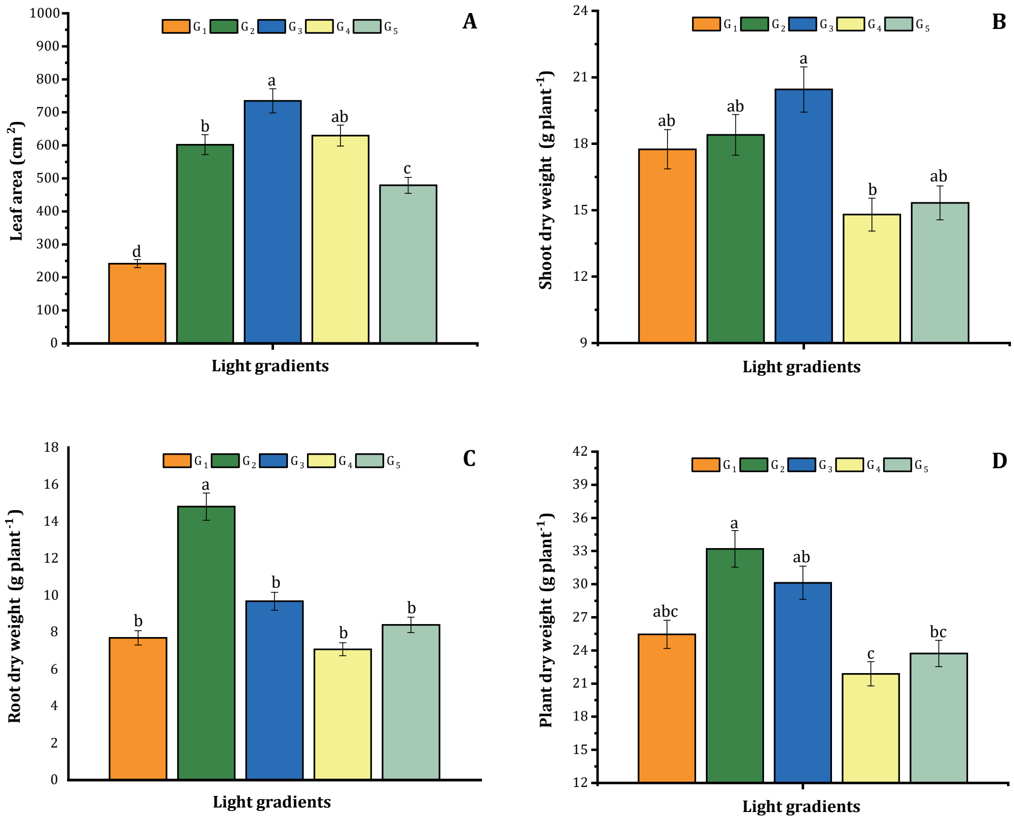
**Plant growth and development as a function of light**

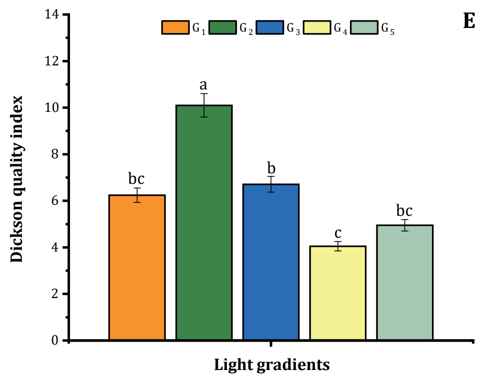
Total leaf area was higher in $G_2$, $G_3$ and $G_4$, followed by $G_5$ and $G_1$ plants; the latter presenting the smallest leaf area, 250 $cm^2$ (figure 3A, page 7). Similarly, Gatti *et al.* (2011) observed decreasing leaf area of *E. edulis* plants as light intensity increased.

Significant differences were only observed among shoot dry weights in $G_3$ and $G_4$ (figure 3B, page 7). However, the highest root dry weight, 14.5 g $plant^{-1}$, was observed in $G_2$ (figure 3C, page 7). Mean plant dry weight under $G_2$ exceeded 33 g $plant^{-1}$ (the highest mean), while the lowest mean was 22.5 g $plant^{-1}$, in $G_4$ (figure 3D, page 7).

The highest Dickson quality index (DQI) was observed under $G_2$ (above 10.0); followed by $G_3$, $G_1$, $G_5$ and $G_4$ plants, in decreasing order and ranging from 7.0 to 4.0 (figure 3E, page 7). DQI is considered a good parameter for quality assessment in nursery plants, considering the amount and even the distribution of phytomass, pondering different parameters that help estimate plant performance in the field (9). De Oliveira *et al.* (2009) observed DQI ranging from 2.29 to 2.77 for *Copernicia hospita* seedlings after three months of growth, and values from 15.78 to 20.70 after nine months of growing in different-size containers under plain sunlight. For *B. capitata,* Costa *et al.* (2018) recommended red shade nets, inducing stomatal and leaf anatomical changes enhancing light harvesting, photosynthesis and plant growth and development. *B. capitata* plants grown under red shade net (50%) showed higher biomass than plants from other shade treatments (4).

The present research suggests that red shade nets contribute to increased plant growth rates and vigour of *S. schizophylla* by enhancing photosynthetic efficiency. Moreover, gas exchange, growth and development of *S. schizophylla* plants throughout the experiment with light gradients generated valuable information in relation to productivity. Plants exposed to a greater amount of light had greater biomass. Additionally, in $G_2$, a higher DQI suggested this index may also predict plant survival after field transplanting and/or shade environments. The results not only confirm that this species is tolerant to light variations but also suggest it undergoes acclimation, optimizing resource allocation through structural and physiological adaptations.




Different letters indicate significant differences for Tukey test ($p < 0.05$).

*ns = non-significant ($p < 0.05$).

Diferentes letras indican diferencias significativas según la prueba de Tukey ($p < 0,05$).

* ns = no significativo ($p < 0,05$).

**Figure 3.** Leaf area (A), shoot dry weight (B), root dry weight (C), plant dry weight (D), Dickson quality index (E) of *Syagrus schizophylla* plants at seven months under different light gradients ($G_1$, $G_2$, $G_3$, $G_4$ and $G_5$).

**Figura 3.** Área foliar (A), peso seco del brote (B), peso seco de la raíz (C), peso seco de la planta (D), índice de calidad de Dickson (E) de plantas de *Syagrus schizophylla* bajo diferentes gradientes de luz (G1, G2, G3, G4 y G5) a los siete meses de tratamiento.

## Conclusions

One layer of Red Chromatinet® 50% should be recommended for the first year of growth of *S. schizophylla* plants to be later transplanted to the field. Two Red Chromatinet® 50% layers would be best for plants meant to grow under shade environments.

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# Correlations between physical and chemical characteristics of Cortibel guava (*Psidium guajava* L.) fruits grown in the Brazilian Cerrado

## Correlaciones entre las características físicas y químicas de los frutos de guayaba (*Psidium guajava* L.) Cortibel cultivados en el Cerrado brasileño

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**Abstract**

The correlation between physical and chemical attributes of fruits can serve as indicators for the ideal harvest time and function as selection criteria to enhance the management and productivity of crops. This study aimed to investigate the correlations among physical and chemical properties of Cortibel guava fruits grown in the Brazilian Cerrado. Parameters assessed included skin and pulp color, weight, diameter, length, total soluble solids, titratable acidity, and the ratio of these characteristics. Data were analyzed using Pearson's linear correlation with a significance level of P < 0.05. Several physical and chemical properties of the fruits exhibited significant correlations. The highest correlation coefficients were observed between weight and fruit diameter, as well as between hue angle of the skin and skin lightness. The properties of Cortibel guava fruits cultivated in the Brazilian Cerrado exhibit significant correlations. These findings enable the utilization of straightforward parameters in the selection processes of Cortibel guava for breeding objectives.

**Keywords**
*Psidium guajava* (L.) • fruit quality • indirect selection • physicochemical properties

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**Resumen**

La correlación entre los atributos físicos y químicos de las frutas puede servir como indicador del momento ideal de cosecha y funcionar como criterio de selección para mejorar el manejo y la productividad de los cultivos. Este estudio tuvo como objetivo investigar las correlaciones entre las propiedades físicas y químicas de los frutos de guayaba Cortibel cultivados en el Cerrado brasileño. Los parámetros evaluados incluyeron color de cáscara y pulpa, peso, diámetro, longitud, sólidos solubles totales, acidez titulable y la relación de estas características. Los datos se analizaron mediante la correlación lineal de Pearson con un nivel de significancia de $P < 0,05$. Varias propiedades físicas y químicas de los frutos exhibieron correlaciones significativas. Los mayores coeficientes de correlación se observaron entre el peso y el diámetro del fruto, así como entre el ángulo de tonalidad de la cáscara y su luminosidad. Las propiedades de los frutos de guayaba Cortibel cultivados en el Cerrado brasileño exhiben correlaciones significativas. Estos hallazgos permiten la utilización de parámetros sencillos en los procesos de selección de guayaba Cortibel para objetivos de mejoramiento.

**Palabras clave**

*Psidium guajava* (L.) • calidad de la fruta • selección indirecta • propiedades fisicoquímicas

## Introduction

Native to South America, the guava tree (*Psidium guajava* L.) has adapted to tropical and subtropical regions and is widely cultivated in these areas (28). The fruits hold high economic value due to their quality, favorable consumer acceptance, and rapid revenue, thereby enhancing their productive potential (3, 28). Guava is a significant crop in numerous countries, with fruits rich in minerals, flavonoid compounds, antioxidants, and vitamins, particularly vitamin C, which can be up to four times higher than in oranges (10, 26).

First bred in Santa Teresa - ES, Brazil, Cortibel guava production has since increased in the Brazilian Southwest because of its fungal resistance. This variety yields firm, fleshy fruits with an appealing exterior, high productivity, and extended shelf life, making them suitable for export. The fruits exhibit a wrinkled appearance with red pulp and are well-received in the fresh fruit market (1, 27).

Fruit characterization and the correlation between their attributes enable the study of genetic diversity among accessions or populations and the identification of potential parents or even genotypes with superior qualities (24). The discovery of high-quality materials in vegetatively propagated fruit species, such as the guava tree, facilitates the replication of superior quality fruits (26).

Characterization based on the physical and chemical properties of cultivars determines the intended market segment for the fruits, whether for processing or fresh fruit consumption. Fruits intended for processing must have high titratable acidity and soluble solid content in the pulp, while those for fresh consumption require low acidity and high soluble solids. Assessing the characterization and correlations of these attributes enables the production of high-potential fruits for both segments (5, 28).

Variables such as weight, length, and equatorial diameter are important attributes in breeding programs. These factors assist in selecting genotypes with desirable commercial properties for the fresh fruit market (8). Understanding physical and chemical attributes enables maintaining fruit quality and developing new products (15), as well as offering cost-effective methods for selecting species with high potential for use in breeding programs (16). Studying the correlations between these attributes is promising, as it helps determine the harvest point, functions as a selection criterion, and provides techniques to enhance crop management and productivity (2, 19).

Numerous authors have evaluated the correlations between physical and chemical attributes of guava fruits in different regions, including characteristics of four guava varieties in Colombia (22), fruits from 122 accessions across Pakistan (17), fruits of 22 genotypes in

New Delhi, India (23), fruits from nine genotypes in Punjab, India (13), fruits of six localities in the Sumapaz province of Colombia (6), fruits of 128 accessions in four different regions of Kenya (4), and fruits from eight indigenous guava cultivars in Pakistan (28).

Agronomic evaluations suggest that the Southwest region of Goiás, Brazil, holds promising potential for fruit tree cultivation. The region's climatic conditions and high-quality soil are conducive to the establishment of various crops, particularly those suited to tropical climates. However, research on the correlations between physical and chemical attributes of Cortibel guava fruits in this Brazilian state remains limited. Understanding these correlations and how an increase in one attribute can influence others is crucial, as it can aid breeding program managers in making more efficient decisions during selection to achieve desired outcomes.

Considering the above, the hypothesis is that Cortibel guava fruits produced in the Brazilian Cerrado exhibit significant correlations between their attributes, allowing for selection in future crosses. Consequently, this study aimed to evaluate the correlations between the physical and chemical properties of Cortibel guava grown in the Brazilian Cerrado.

## Material and methods

The experiment was conducted in an experimental orchard in Jataí, Goiás, Brazil, situated at 17°53'08'' S and 51°40'12'' W, at an altitude of 696 m, from October to January. During this period, the relative humidity ranged from 64% to 80%. The region's climate is of the tropical savannah type (Aw), with a rainy season from October to April and a dry season from May to September. The average annual rainfall and temperature are 1,541 mm and 23.3°C, respectively (18).

The study sample consisted of 120 fruits with red pulp and smooth skin, collected from the middle third of 10 four-year-old plants. Fully ripe fruits were hand-harvested using visual evaluation to determine the ideal harvest point. All fruits with yellow skin were considered mature. After harvesting, the fruits were washed, air-dried, and weighed in the laboratory. A completely randomized experimental design was employed, with ten plants and 12 replications (fruits) per plant. The fruits were assessed for length, diameter, weight, skin and pulp color as determined by the CIELAB color space, total soluble solids (TSS), titratable acidity (TA), and the ratio between total soluble solids and titratable acidity (TSS/TA).

The length and diameter of the fruits (mm) were measured using a digital caliper (Mitutoyo®, Japan). The weight (g) was determined by individually weighing the fruits on a digital scale with a precision of 0.01 g (Marte Científica®, Brazil). Skin and pulp color were evaluated using the coordinates L* (degree of lightness), a* (red), b* (yellow), C* (Chroma), and h° (hue angle [arctan b*/a*]), measured by a digital colorimeter at two spots (Konica Minolta® CR-10, Japan). The L* value represents the brightness of the sample, ranging from 0 (least luminous) to 100 (most luminous); a* represents green (from 0 to -60) and red (from 0 to +60) colors; b* represents the yellow color (from 0 to +60); C* represents color saturation; and h° is the hue angle (from 0 to 360°), indicating the quadrant in which the sample color is located (11).

The pulp was processed in an electric blender without adding water and filtered through a nylon sieve. The total soluble solids content (°Brix) was measured by placing two drops of pulp onto a digital refractometer (Atago® model Palette PR-101, Japan). Titratable acidity (%) was obtained by titration using an NaOH solution and 1% phenolphthalein as an indicator (14). The ratio was calculated as the quotient of total soluble solids to titratable acidity. Data were analyzed for Pearson's linear correlation ($P < 0.05$) between the physical and chemical characteristics of the fruits using SAS software.

## Results and discussion

Most of the evaluated characteristics exhibited a positive and significant correlation. A positive correlation was observed between fruit weight and diameter (r = 0.890) and between length and diameter (r = 0.560), indicating that these variables increase at the same rate (table 1).

**Table 1.** Pearson's correlation coefficients for the weight (g), diameter and length (mm), L*, a*, b*, C* and hue angle of the skin and pulp, total soluble solids (°Brix), titratable acidity (%), and the ratio of Cortibel guava (*Psidium guajava*) grown in the Brazilian Cerrado.

**Tabla 1.** Coeficientes de correlación de Pearson para el peso (g), diámetro y longitud (mm), L*, a*, b*, C* y tonalidad de la cáscara y pulpa, sólidos solubles totales (°Brix), acidez titulable (%), y proporción de guayaba Cortibel (*Psidium guajava*) cultivadas en el Cerrado brasileño.

| | W | D | L | L*S | a*S | b*S | C*S | h°S |
|---|---|---|---|---|---|---|---|---|
| **W** | 1.00 | | | | | | | |
| **D** | 0.890** | 1.00 | | | | | | |
| **L** | 0.747** | 0.560** | 1.00 | | | | | |
| **L*S** | 0.098$^{ns}$ | 0.132$^{ns}$ | 0.018$^{ns}$ | 1.00 | | | | |
| **a*S** | 0.180** | 0.195** | 0.042$^{ns}$ | 0.359** | 1.00 | | | |
| **b*S** | -0.009$^{ns}$ | 0.017$^{ns}$ | -0.019$^{ns}$ | 0.233** | 0.163$^{ns}$ | 1.00 | | |
| **C*S** | 0.048$^{ns}$ | 0.064$^{ns}$ | 0.016$^{ns}$ | 0.491** | 0.298** | 0.489** | 1.00 | |
| **h°S** | -0.084$^{ns}$ | -0.108$^{ns}$ | -0.022$^{ns}$ | -0.349** | -0.867** | -0.192** | -0.349** | 1.00 |
| **L*P** | 0.190** | 0.157$^{ns}$ | 0.202** | 0.085$^{ns}$ | -0.306** | -0.070$^{ns}$ | -0.128$^{ns}$ | 0.267** |
| **a*P** | -0.022$^{ns}$ | -0.008$^{ns}$ | -0.143$^{ns}$ | -0.073$^{ns}$ | 0.235** | 0.005$^{ns}$ | 0.119$^{ns}$ | -0.207** |
| **b*P** | 0.098$^{ns}$ | 0.070$^{ns}$ | 0.031$^{ns}$ | 0.023$^{ns}$ | -0.249** | -0.093$^{ns}$ | -0.126$^{ns}$ | 0.202** |
| **C*P** | 0.118$^{ns}$ | 0.085$^{ns}$ | -0.044$^{ns}$ | -0.109$^{ns}$ | -0.015$^{ns}$ | -0.072$^{ns}$ | -0.014$^{ns}$ | -0.009$^{ns}$ |
| **h°P** | 0.063$^{ns}$ | 0.024$^{ns}$ | 0.171$^{ns}$ | 0.053$^{ns}$ | -0.274** | -0.032$^{ns}$ | -0.152$^{ns}$ | 0.233** |
| **TSS** | 0.037$^{ns}$ | 0.040$^{ns}$ | -0.009$^{ns}$ | 0.096$^{ns}$ | -0.089$^{ns}$ | 0.028$^{ns}$ | 0.024$^{ns}$ | 0.113$^{ns}$ |
| **TA** | -0.181** | -0.145$^{ns}$ | -0.181** | 0.101$^{ns}$ | -0.159$^{ns}$ | 0.021$^{ns}$ | 0.146$^{ns}$ | 0.104$^{ns}$ |
| **TSS/TA** | 0.033$^{ns}$ | -0.003$^{ns}$ | 0.008$^{ns}$ | -0.013$^{ns}$ | 0.175$^{ns}$ | 0.033$^{ns}$ | -0.115$^{ns}$ | -0.091$^{ns}$ |

| | L*P | a*P | b*P | C*P | h°P | TSS | TA | TSS/TA |
|---|---|---|---|---|---|---|---|---|
| **W** | | | | | | | | |
| **D** | | | | | | | | |
| **L** | | | | | | | | |
| **L*S** | | | | | | | | |
| **a*S** | | | | | | | | |
| **b*S** | | | | | | | | |
| **C*S** | | | | | | | | |
| **h°S** | | | | | | | | |
| **L*P** | 1.00 | | | | | | | |
| **a*P** | -0.591** | 1.00 | | | | | | |
| **b*P** | 0.644** | -0.248** | 1.00 | | | | | |
| **C*P** | 0.060$^{ns}$ | 0.615** | 0.555** | 1.00 | | | | |
| **h°P** | 0.723** | -0.948** | 0.473** | -0.401** | 1.00 | | | |
| **TSS** | 0.175$^{ns}$ | 0.014$^{ns}$ | 0.109$^{ns}$ | 0.089$^{ns}$ | 0.003$^{ns}$ | 1.00 | | |
| **TA** | -0.047$^{ns}$ | 0.096$^{ns}$ | -0.002$^{ns}$ | 0.029$^{ns}$ | -0.093$^{ns}$ | 0.106$^{ns}$ | 1.00 | |
| **TSS/TA** | -0.023$^{ns}$ | -0.029$^{ns}$ | -0.010$^{ns}$ | -0.033$^{ns}$ | -0.008$^{ns}$ | 0.359** | -0.734** | 1.00 |

** $P < 0.05$, ns = not significant. W: weight; D: diameter; L: length; L*S: lightness of the skin; a*S: red of the skin; b*S: yellow of the skin; C*S: chromaticity of the skin; h°S: hue angle of the skin; L*P: lightness of the pulp; a*P: red of the pulp; b*P: yellow of the pulp; C*P: chromaticity of the pulp; h°P: hue angle of the pulp; TSS: total soluble solids; TA: titratable acidity; TSS/TA: total soluble solids content and titratable acidity ratio.

** $P < 0,05$, ns = no significativo. W: peso; D: diámetro; L: longitud; L*S: luminosidad de la cáscara; a*S: enrojecimiento de la cáscara; b*S: color amarillo de la cáscara; C*S: cromaticidad de la cáscara; h°S: tonalidad de la cáscara; L*P: luminosidad de la pulpa; a*P: enrojecimiento de la pulpa; b*P: color amarillo de la pulpa; C*P: cromaticidad de la pulpa; h°P: tonalidad de la pulpa; TSS: sólidos solubles totales; TA: acidez titulable; TSS/TA: relación de contenido de sólidos solubles totales y de acidez titulable.

Positive correlations are common among such characteristics in fruit species because larger fruits typically have a greater weight and amount of pulp, making them more appealing to consumers (9). Fruit species, such as passion fruit grown in Viçosa, Minas Gerais, Brazil (21), and guava cultivated in Colombia (6), India (13), and Pakistan (17), yielded similar results.

Correlation coefficients can be classified as very strong when R-values range from ± 0.91 to ± 1.00, strong for values ranging from ± 0.71 to ± 0.90, average when they range from ± 0.51 to ±0.70, and weak when R-values range from ± 0.31 to ± 0.50 (12). In the present study, there is a very strong correlation between fruit weight and diameter and a strong correlation between fruit length and diameter. The positive association between weight, length, and diameter underscores the importance of these characteristics in the selection of fruits for breeding purposes (13).

The correlations between h° and L* of the pulp (r = 0.723), C* and a* of the skin (r = 0.489), C* and a* of the pulp (r = 0.615), L* and b* of the pulp (r = 0.64), and between h° and a* of the pulp (r = 0.473) were positive and significant (table 1, page 13).

The positive and significant correlation between h° and L* of the pulp in the present study indicates that these fruits have the potential to exhibit brighter red pulp. There was also a positive correlation for C* with b* of the skin and for C* with a* of the pulp, indicating that these fruits display skin of a more pronounced yellow color and pulp of an intense red color. The external appearance of fruits, including color, is the first quality parameter that consumers evaluate when selecting fruits (9).

Negative correlations were observed between the h° of the skin and a* of the pulp (r = -0.207), L* and a* of the pulp (r = -0.591), h° and a* of the pulp (r = -0.948), and between C* and h° of the pulp (r = -0.401), indicating that lightness and hue angle fluctuate during fruit ripening. This occurs due to increased pigment concentrations and oxidative reactions during ripening, which results in a change in dark colors and elevated luminosity values (20).

Negative correlations were found for titratable acidity with weight (r = -0.181) and length (r = -0.181) (table 1, page 13), suggesting that acidity content decreases as fruit size increases. Acidity content in fruits diminishes with their maturation and development (25). The negative correlations obtained in the present study support this information, indicating that fully ripe and developed fruits exhibit lower titratable acidity.

The ratio exhibited a positive correlation with total soluble solids (r = 0.359) (table 1, page 13), signifying that an increase in soluble solid content in the pulp enhances this characteristic. The ratio serves as a maturity indicator, playing a crucial role in fruits for both fresh consumption and processing industries. It also indicates flavor, as fully ripe fruits with higher ratio values have a balance between soluble solid content and titratable acidity. As observed in the present study, the ratio may also indicate physiological maturity for many other fruit species (25).

No significant correlation was found between total soluble solid content and titratable acidity (r = 0.106) (table 1, page 13), which suggests that these two characteristics do not depend on each other. Selection criteria for breeding purposes stipulate that fruits must have high levels of acidity and soluble solids to meet the requirements of processing industries (7). Given that processing industries require fruits with higher titratable acidity and soluble solid content (28), the Cortibel guava fruits produced in the present study can satisfy the demand of this segment.

## Conclusion

Some physical and chemical characteristics of the Cortibel guava fruits grown in the Brazilian Cerrado show significant correlations. These results allow using simple evaluations, such as fruit size or skin and pulp color, to estimate productive parameters during the selection of Cortibel guava and to detect materials of interest. The correlation of these characteristics directs the appropriate commercial use of the fruits.

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# Seed treatments with salicylic acid and *Azospirillum brasilense* enhance growth and yield of maize plants (*Zea mays* L.) under field conditions

## Tratamientos de semillas con ácido salicílico y *Azospirillum brasilense* aumentan el crecimiento y el rendimiento de las plantas de maíz (*Zea mays* L.) en condiciones de campo

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### Abstract

Salicylic acid and *Azospirillum brasilense* stimulate plant growth and productivity. In some environments, plant physiology similarly responds to both bioactive products. Considering this, a field experiment was conducted to study the physiological effect of Salicilic acid and *A. brasilense* on growth and grain yield of maize plants. The experiment involved three treatments consisting of imbibed seeds in an aqueous solution of SA (0.01 mM), inoculated seeds with *A. brasilense* and a control treatment. Seed imbibition in SA and inoculation with *A. brasilense* improved vegetative growth in the early stages of crop ontogeny, increasing leaf growth, plant height, stem diameter and biomass accumulation. Spikelet length and weight were greater in plants first inoculated with *A. brasilense* and then treated with SA. Results indicated that SA stimulated biomass partitioning towards leaves, root and stem, while *A. brasilense* mainly affected leaf growth, plant height, ear dimensions and grain yield. Such results turn crucial for biological fertilization strategies aimed at reducing pollutant loads that accompany chemical fertilizers. Both products can be part of maize management practices given competitive economic advantages and sustainability.

**Keywords**
bioactive products • biofertilizers • plant hormones

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**Resumen**

El ácido salicílico y *Azospirillum brasilense* estimulan el crecimiento y la productividad de las plantas y, en algunos entornos, las respuestas fisiológicas de las plantas a la aplicación de ambos productos bioactivos parecen ser similares. Con el objetivo de estudiar su efecto fisiológico en el crecimiento y la producción de granos de plantas de maíz se diseñó un experimento de campo. Los tratamientos consistieron en embeber las semillas en una solución acuosa de SA (0,01 mM) e inocularlas con *A. brasilense* y un tratamiento control. La imbibición de las semillas en SA y la inoculación con *A. brasilense* mejoraron el crecimiento vegetativo en las primeras etapas de la ontogenia del cultivo, aumentando el crecimiento foliar, la altura de la planta, el diámetro del tallo y la acumulación de biomasa. La longitud y el peso de las espigas fueron mayores en las plantas inoculadas con *A. brasilense* y tratadas con SA, en ese orden. Los resultados indicaron que el SA estimuló la acumulación de biomasa hacia las hojas, la raíz y el tallo, mientras que *A. brasilense* tuvo más influencia en el crecimiento de las hojas, la altura de la planta, las dimensiones de la mazorca y el rendimiento del grano. Tales resultados son relevantes para el diseño de estrategias de fertilización biológica con la respectiva reducción de la carga contaminante que acompaña a los fertilizantes químicos. Ambos productos pueden formar parte de las prácticas agronómicas del cultivo de maíz por sus ventajas económicas competitivas y por ser ambientalmente sostenibles.

**Palabras clave**
productos bioactivos • biofertilizantes • hormonas vegetales

## Introduction

Maize constitutes an essential nutrient for the human diet. Despite a low protein content compared to other vegetables, it provides, on average, 39% of overall protein intake, and 59% of human energy requirements (38).

Mexico is the largest market for maize, consuming 11% of world production (29). However, the low-yielding domestic production does not meet the growing demand, and importation reaches approximately 11.3 and 0.145 million tons of yellow and white maize, respectively (36). Hence, there is an evident need to explore technological alternatives for environmentally friendly production and increasing yields.

The indiscriminate use of ammonia fertilizers on some bedrock types of soil, causes serious acidification problems. Soils in the Frailesca region of Chiapas are undergoing an acidification process (11) and consequent increasing toxic aluminium contents in maize plants, particularly at pH levels below 5.0 (6). Faced with such a situation, finding alternatives entailing reduced agrochemical applications may contribute to reduced soil degradation. In this sense, several biofertilizers based on nitrogen-fixing microorganisms have shown positive effects on certain cultivated plants, such as maize (3, 25, 32), sorghum (19), soybeans (10), and rice (12).

Among nitrogen-fixing bacteria, *A. brasilense* is considered one of the most important plant growth-promoting bacteria (8). On one hand, this bacterium promotes growth and increases plant production, after promoting phytohormone synthesis (5) and biological nitrogen fixation. On the other hand, *A. brasilense* restricts certain plant pathogens through antibiosis and siderophores (14). In addition, *Azospirillum* may associate with more than 100 plant species, 14 of which are grasses (30). As stated by Gavilanes *et al.* (2020), *A. brasilense* (Ab-V5 and Ab-V6) has been widely used as a commercial inoculant in Brazil (14), with positive effects on grain dry matter and nitrogen accumulation in plants, especially in grain crops such as maize and wheat (13, 24).

Given the mentioned phytohormone-producing capacity of this bacterium, inoculation effects could be related to the role played by salicylic acid. Several studies performed with rhizosphere bacteria show that most strains produced metabolites of the AIA type, siderophores and salicylic acid (22). Salicylic acid (SA) is a phenolic secondary metabolite (20) present in plant tissues (33, 41), regulating plant growth and increasing crop yield when supplied in low exogenous concentrations (34). In maize, the application of SA increases grain production per plant, total dry biomass, and N, P, K contents (41).

Some hypotheses about the effect of SA and *A. brasilense* consider the role of SA as a plant hormone and the ability of *A. brasilense* to fix atmospheric nitrogen and increase nutrient uptake by promoting increased stem growth and grain production (15). The effect of both bioactive products (SA and *A. brasilense*), effective biomolecules and plant growth regulators (26), depended on the genotype and environmental conditions. In this sense, given the agronomic implications on possible biological fertilization strategies in maize, comparing the effect of both products turns interesting. Thus, this research aimed to study the physiological effect of *A. brasilense* and SA on growth and grain production of maize plants under field conditions.

## Materials and methods

### Location

The research was conducted from June 2016 to January 2017 in the locality of Calzada Larga, municipality of Villaflores, Chiapas, México, at 16°21'08.5" N and 93°18'58.2" W and 713 m a. s. l. (8). The predominant climates are warm and semi-warm, with an average temperature of 24.5 °C, an average rainfall of 1200 mm per year, and the following predominant soil types: lithosols, luvisols, cambisols and vertisols.

### Growth conditions and plant material

Seeds of the CLTHW11002 hybrid, yellow maize from the International Maize and Wheat Improvement Center (CIMMYT), were manually sowed at 0.8 m between rows and 0.35 m between plants on a luvisol soil with loamy texture and enough water content.

According to the Mexican Official Standard (27), the soil is strongly acidic, free of carbonates and salts, low in potassium, moderately low in organic matter, medium phosphorus (P-Bray) and nitrogen (N-$NO_3$), moderately high in magnesium (Mg) and very high in iron (Fe). Both aluminium content and proportion *vs.* total soil cations (4.61%) were low, despite the extreme soil acidity (table 1).

**Table 1.** Soil chemical properties.

**Tabla 1.** Caracterización química del suelo del área experimental.

| pH | O.M (g kg$^{-1}$) | N-$NO_3$ | P-Bray | K | Ca | Mg | Fe | Al | CEC (Meq/100g) |
|---|---|---|---|---|---|---|---|---|---|
| | | mg kg$^{-1}$ | | | | | | | |
| 4.49 | 14.4 | 16.6 | 25.4 | 1.03 | 2402 | 655 | 48.4 | 95.2 | 23.0 |

O. M: organic matter, CEC: cation exchange capacity.

M. O: Materia Orgánica, CEC: Capacidad de intercambio catiónico.

Two nitrogen fertilizations were applied at 15 days and 30 days after sowing, both at a rate of 75 kg ha$^{-1}$ of N, for a total dose of 150 kg ha$^{-1}$ (46-00-00). Weed chemical control was carried out with Velquat 1.5 L ha$^{-1}$ at 15 days after sowing, and 1.5 L ha$^{-1}$ Tacsaquat at 60 days after sowing. The fall armyworm (*Spodoptera frugiperda*) was controlled with Cipermetrine 21.12% EC at a dose of 1.0 L ha$^{-1}$.

### Experimental design and treatments

The randomized block experimental design with three treatments and three replicates consisted of T1: salicylic acid (SA), T2: *A. brasilense* (5 x 108 CFU), T3: control treatment without SA and not inoculated. Each experimental unit measured 25 m$^2$ (5 x 5 m), leaving one border plant on each side of the plot.

**Seed inoculation with *A. brasilense***

The commercial product tested was Azofer®, composed of *A. brasilense* (50%), peat (37.5%) and calcium carbonate (12.5%). Calcium carbonate allows achieving the ideal pH (6.8-7.0) for the inoculant (6), while not affecting plant growth or development. The concentration of *A. brasilense* was 5 x 108 CFU's per gram of commercial product.

For seed inoculation, an inert solution was prepared with 60 g of carbosil-methyl-cellulose adhesive powder dissolved in 1.5 L of distilled water and left to stand for two hours. Subsequently, mointened seeds with the adherent powder were homogeneously covered with the biofertilizer and left to dry in the shade, at room temperature, before sowing.

**Imbibition of seeds with salicylic acid**

A solution with distilled water and salicylic acid at a concentration of 0.01 mM, was prepared according to Hayat and Ahmad (2007), Gordillo-Curiel *et al*. (2020) and Rodríguez-Larramendi *et al.* (2017). The seeds were soaked for 2 hours and left to dry at room temperature before sowing.

**Analytical evaluations**

For growth measurements at 30 and 60 days after sowing, five plants per replicate were selected, number of leaves per plant (LP) was counted, and plant leaf area was determined in $cm^2$ (LA) with a CI-202 (Bioscience ®) portable leaf area meter. Plant height (PH) was measured with a millimetre ruler. Leaves, stem, roots of the five selected plants were separated, and oven dried at 80°C for 72 hours. Finally, leaves dry weight (LDW), shoot dry weight (SDW) and root dry weight (RDW) were obtained using a Sartorius® analytical balance. Plant dry weight was calculated.

**Growth rates**

Based on leaf area and dry weight, the leaf area index (LAI) was calculated by dividing plant leaf area (LA) by soil coverage. Root weight fraction (RWF, $g\ g^{-1}$) was calculated as the root dry weight *vs.* plant dry weight ratio. This indicator expresses the "root's investment" or biomass gain (4). Leaf weight fraction (LWF, $g\ g^{-1}$) was calculated as leaf dry weight *vs.* plant dry weight, estimating leaf partitioning (42). The root mass/leaf mass ratio (dimensionless) estimated phenotypic plasticity in biomass allocation (4). Specific leaf area (SLA, $cm^2\ g^{-1}$) was calculated as leaf area/leaf dry weight, reflecting functional traits of leaf morphology such as thickness and density (31) and vegetative vigor (23). Leaf area ratio (LAR, $cm^2\ g^{-1}$), one important determinant of plant relative growth rate (42), was calculated as leaf area by plant dry weight.

**Yield components**

When the crop reached physiological maturity, five plants per treatment were selected in each replicate and ear length (EL, cm), ear diameter (ED, cm), 100-grain dry weight, and dry grain yield per plant (Yield, g $plant^{-1}$) at 14% grain moisture, were assessed.

**Statistical analysis**

ANOVA with a randomized block design followed by LSD mean comparison test was conducted considering $p \leq 0.05$. ANOVA assumptions were verified by the Cochran and Barttlet tests. A multivariate Principal Component Analysis (PCA) searched for a possible relationship between plant growth, yield, ear weight and ear size. All analyses were performed with the statistical package STATISTICA® release 8.0 (39).

## Results

The number of leaves per plant was significantly higher in inoculated plants, compared to those treated with SA and control, the latter of which produced fewer leaves. Despite a tendency towards more leaves per plant on both sampling dates (at 30 and 60 das), at 60 das, this difference was not significant (table 2, page 21).

**Table 2.** Effect of salicylic acid (SA) and *A. brasilense* on growth of maize plants at 30 and 60 days after sowing (das).

**Tabla 2.** Efecto del ácido salicílico (AS) y *A. brasilense* en el crecimiento de plantas de maíz a los 30 y 60 días después de la siembra (dds).

| Treatments | Leaves per plant (LP) | Plant height (PH, cm) | Stem diameter (SD, cm) | Leaf area (LA, $m^2$ $plant^{-1}$) | Leaf dry weight (LDW, g) | Stem dry weight (SDW, g) | Root dry weight (RDW, g) | Plant dry weight (PDW, g) |
|---|---|---|---|---|---|---|---|---|
| | 30 das | | | | | | | |
| SA | 8.75 b | 48.04 a | 1.96 a | 0.14 a | 5.01 a | 3.11 | 2.12 a | 10.24 a |
| *A. brasilense* | 9.42 a | 44.29 b | 1.77 b | 0.12 ab | 5.02 a | 3.26 | 1.67 ab | 9.96 ab |
| Control | 8.00 c | 42.93 b | 1.58 c | 0.10 b | 3.96 b | 2.86 | 1.40 b | 8.21 b |
| Standard error | 0.11 | 0.50 | 0.04 | 0.01 | 0.23 | 0.25 | 0.15 | 0.52 |
| | 60 das | | | | | | | |
| SA | 10.08 | 168.00 a | 1.98 a | 0.37 | 33.04 a | 56.83 a | 37.88 a | 127.75 a |
| *A. brasilense* | 10.17 | 165.00 a | 1.80 b | 0.34 | 29.42 b | 53.63 b | 32.21 b | 115.25 b |
| Control | 9.00 | 155.83 b | 1.63 c | 0.32 | 24.96 c | 43.21 c | 25.92 c | 94.08 c |
| Standard error | 0.36 | 2.17 | 0.04 | 0.02 | 0.83 | 2.02 | 1.96 | 3.55 |

ns: Not significant; * Statistically significant for p ≤ 0.05, das: days after sowing

Ns: sin diferencias significativas, * Diferencias estadísticas significativas para p ≤ 0,05, dds: días después de la siembra.

However, plant height and stem diameter were significantly higher in plants grown from SA-treated seed, and even higher than those inoculated with A. brasilense at 30 das. At 60 das, no significant difference in plant height was observed between SA and A. brasilense-treated plants (table 2).

Leaf area was larger in SA-treated plants at 30 das than control plants. The latter exhibited no significant differences concerning those inoculated with A. brasilense. At 60 das, no difference in leaf area was observed among treatments (table 2).

Leaf dry weight was statistically higher in plants treated with SA and A. brasilense than in control plants at 30 das. At 60 das this difference was even higher for SA-treated plants followed by *A. brasilense*, compared to the control. Similar results were observed for root and stem dry weight and, therefore, for total plant dry weight. However, stem dry weight was statistically different at 60 das (table 2).

Table 3 (page 22) shows the effect of bioactive products on growth ratios, only significant at 30 das, when an increasing leaf area index was observed for the SA treatment. No difference was observed between A. brasilense and control. At 60 das, such growth-stimulating effects were not significant (table 3, page 22).

The higher leaf growth values observed with SA and A. brasilense at early stages of maize plant development (table 2) was similar to the increase detected in ear length and weight, and yield per plant (figure 1, page 22).

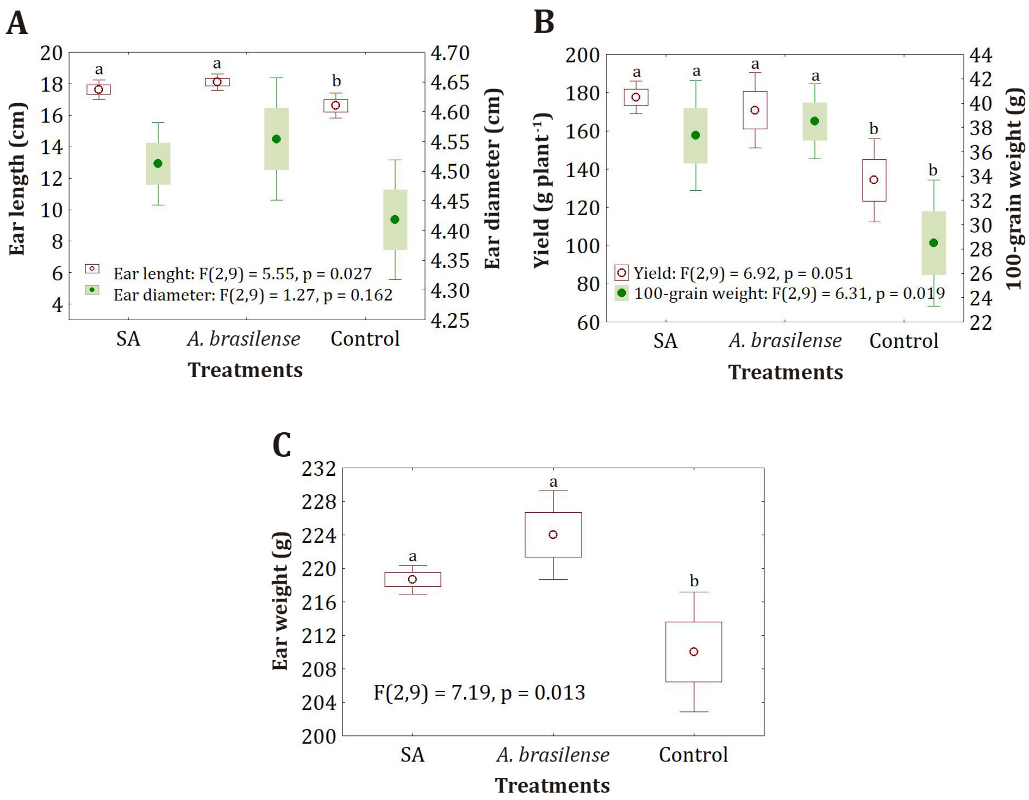
**Table 3.** Effect of the application of salicylic acid (SA) and *A. brasilense* on growth rates of maize plants at 30 and 60 days after sowing (das).

**Tabla 3.** Efecto de la aplicación de ácido salicílico (AS) y *A. brasilense* en los índices de crecimiento de plantas de maíz a los 30 y 60 días despues de la siembra (dds).

| Treatments | Leaf area index (LAI) | Specific leaf area ($cm^2\ g^{-1}$) | Leaf area ratio ($g\ g^{-1}$) | Leaf weight ratio ($g\ g^{-1}$) | Leaf weight fraction ($g\ g^{-1}$) | Root/stem ratio ($g\ g^{-1}$) | Root/leaf weight ratio ($g\ g^{-1}$) |
|---|---|---|---|---|---|---|---|
| 30 das | | | | | | | |
| SA | 0.50 a | 285.16 | 138.19 | 0.21 a | 0.49 | 0.27 a | 0.43 |
| *A. brasilense* | 0.44 ab | 248.21 | 124.89 | 0.17 b | 0.50 | 0.20 b | 0.34 |
| Control | 0.37 b | 262.77 | 127.58 | 0.17 b | 0.48 | 0.21 b | 0.36 |
| Standard error | 0.03 | 14.46 | 8.53 | 0.01 | 0.01 | 0.02 | 0.03 |
| 60 das | | | | | | | |
| SA | 1.31 | 112.24 | 29.11 | 0.29 | 0.26 | 0.42 | 1.14 |
| *A. brasilense* | 1.23 | 116.73 | 29.77 | 0.28 | 0.25 | 0.39 | 1.10 |
| Control | 1.15 | 130.03 | 34.68 | 0.27 | 0.27 | 0.38 | 1.04 |
| Standard error | 0.06 | 5.22 | 1.99 | 0.01 | 0.01 | 0.02 | 0.04 |

Ns: Not significant; * Statistically significant for p ≤ 0.05.

Ns: sin diferencias significativas, * Diferencias estadísticas significativas para p ≤ 0,05 dds: días después de la siembra.




Box and vertical lines indicate the standard error and standard deviation, respectively.

Los cuadros y las líneas verticales indican el error etándar y la desviación estándar de la media respectivamente.

**Figure 1.** Effect of salicylic acid and *A. brasilense* on A) ear length and diameter, B) yield and 100-grain weight and C) ear weight.

**Figura 1.** Efecto del tratamento con ácido salicílico y *A. brasilense* en: A) longitud y diámetro de la mazorca, B) rendimiento y peso de 100 granos y C) peso de la mazorca.

Plants treated with SA and *A. brasilense* developed longer ears, consistently with the higher 100-grain weight (figure 1 B, page 22), ear weight and yield per plant (figure 1 B, C, page 22).

Considering vegetative growth, ear size and grain production, a PCA allowed establishing the effects of both bioactive products as a function of the evaluated variables (figure 2). Component 1 (48.58%) discriminates control plants from both bioactive treatments, and component 2 (16.20%) discriminates both bioactive treatments. Variables related to biomass accumulation and stem diameter, discriminate in favour of SA treatment, while variables related to leaf growth, plant height, ear dimensions and grain yield, separate the *A. brasilense* treatment (figure 2).

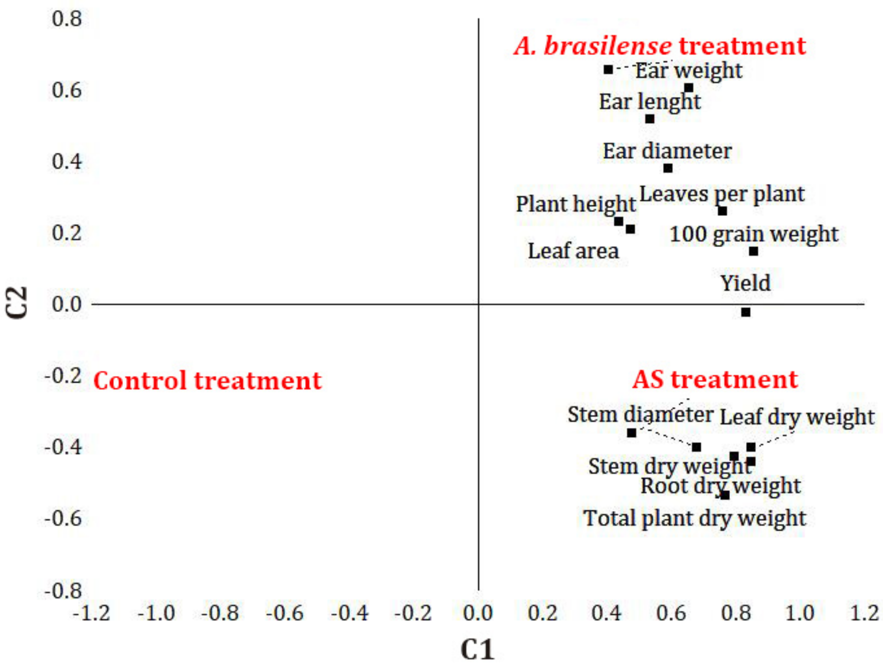



**Figure 2.** PCA biplot of growth variables, ear dimensions and grain yield of corn plants treated with SA and *A. brasilense*, according to components C1 (48.) % and C2 (16.20%).

**Figura 2.** Biplot de las variables de crecimiento, dimensiones de la mazorca y producción de granos de plantas de maíz tratadas con AS y *A. brasilense*, en el plano formado por las componetes C1 (48.) % y C2 (16.20%).

## Discussion

Maize growth response to seed inoculation with *A. brasilense* and imbibition with SA on two sampling dates suggests a possible relationship with plant physiological stage and ontogeny, something to be further addressed in future research. The effect of both bioactive products resulted organ dependent.

The results further demonstrate that SA and *A. brasilense* have similar effects, promoting vegetative growth of maize plants at the early stages. However, these effects depend on sampling date (34) and particularly, on plant organ. This effect is probably given by SA and *A. brasilense* sharing an analogous property, stimulating hormone synthesis and interacting with cytokinins. SA constitutes a plant hormone exceeding plant immunity and abiotic stress (34). In coordination with cytokinins, ethylene, auxins, gibberellins, jasmonic acid and abscisic acid, SA significantly contributes to growth and development regulation, although through unknown mechanisms (34). In this sense, it has been shown that the growth-promoting effects of SA could be related to hormone modulation (1, 37) or gas exchange improvement (39).

The PCA showed correlations between leaf growth *vs.* plant height, and ear size *vs.* increased grain production. These aspects are related to the *A. brasilense* enhanced nitrogen absorption, even in fertile soils (15), and enhanced hormone synthesis, including auxin, gibberellin, and cytokinin (2, 17).

The effect of SA on grain production in maize plants is also supported by other studies. Low doses of SA induce increased ear length and maize grain yield (41). However, in our research, SA was applied to leaves (41) and not seeds, opening a new field of research.

Positive plant responses to inoculation with *A. brasilense* might be given by biological nitrogen fixation, probably overshadowed by the nitrogen fertilization carried out in the experiment, and by plant hormonal production. In this regard, inoculations with *A. brasilense* had improved root growth and development of *Setaria viridis* grass after increased $CO_2$ fixation and reduced accumulation of photo-assimilated carbon in leaves, resulting in greater canopy growth, increased water content in plant tissues, and reduced stress (24, 28). In addition, increased production of indoleacetic acid may improve nutrient uptake by augmenting root growth (24, 28). Zeffa *et al.* (2019) studied *A. brasilense* inoculated seeds, finding intensified plant growth, improved biochemical traits and raised NUE under nitrogen deficit. Other authors have found that regardless of nitrogen source and dosage, *A. brasilense* increased maize grain yield (16).

SA effects on plant dry weight are probably related to SA ability to increase N, $P_2O5$ and $K_2O$ contents in plant tissues (41). On the other hand, seed inoculation with *A. brasilense* stimulates root growth, increases root exploration capacity and promotes biological nitrogen fixation, which may be related to higher grain production and ear length in inoculated plants. Specifically, in maize, other studies have shown that inoculations with nitrogen-fixing bacteria result in a 9% increase in maize grain production (16).

Our results show that SA and *A. brasilense* had similar stimulating effects on growth and yield of maize plants, from early stages of plant ontogeny. This physiological effect may be related to the ability to interact with other hormones (37) or promote hormone synthesis (16). However, we suggest caution before issuing a hypothesis on this matter as, apart from the above-mentioned properties, *A. brasilense* contributes to plant nutrition through biological nitrogen fixation, a fact that may mask the effects of both bioactive products on plant growth and development.

## Conclusions

We consider that the results obtained show robust evidence supporting theoretical and methodological bases, as well as sufficient evidence on the physiological effect of S.A and the bacterium *A. brasilense* on growth and grain production of maize under field conditions. Such results are relevant to the design of biological fertilization strategies with the respective reduction of pollutants. Considering economic and environmental sustainability, both products can be considered within the agronomic practices of maize cultivation.

The results indicated that SA and *A. brasilense* exhibited similar stimulating effects on growth and yield of maize plants, from early stages. SA induced greater plant biomass accumulation, with a tendency to maximize root dry mass, while *A. brasilense* stimulated greater leaf growth and plant height, consequently inducing increased ear growth and grain production.

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# Conventional to organic transition of *Citrus* x *sinensis* (L.) Osbeck (pro. sp.) orchards in municipalities of northern Veracruz state

## Transición de huertos de *Citrus* x *sinensis* (L.) Osbeck (pro. sp.) convencionales a orgánicos en municipios de la zona norte de Veracruz

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**Abstract**

This research determined the degree of transition towards organic production and management of Valencia orange in the municipalities of northern Veracruz State. One hundred and six surveys were administered to producers grouped into four consolidated civil associations. A constructed transition index considering six variables revealed an average of 0.768. Citrus grower/institution positively correlated with intercropping ($p = 0.0281$) and with internal factors ($p = 0.0257$). Determinant factors for transition index were intercropping ($p = 0.00009$), and citrus grower/institution interaction ($p = 0.0000$). These internal and external factors together with years of conversion, averaging six years, resulted in an intermediate transition degree towards organic Valencia orange production. Municipalities of northern Veracruz State stand on varying degrees of transition towards organic agriculture. Yet, many growers still conserve some management practices not consistent with organic production.

**Keywords**
organic agriculture • transition index • citrus

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### Resumen

El objetivo de la investigación fue determinar el grado de transición hacia la producción orgánica y el manejo de naranja Valencia de los municipios de la zona norte de Veracruz. Se aplicaron 106 encuestas a productores agrupados en cuatro asociaciones consolidadas y se construyó un índice de transición con seis variables. El índice promedio de transición fue 0,768. Se hallaron interacciones positivas entre citricultor/institución *vs*. intercalado de cultivos (p = 0,0281) y *vs*. factores internos (p = 0,0257). Los factores determinantes para el índice de transición son el intercalado del cultivo (p = 0,00009) y la interacción citricultor/institución (p = 0,0000). Estos factores internos y externos junto con los años en trabajo de conversión, con un promedio de seis años, resultan en un grado intermedio de transición orgánica de los productores de naranja Valencia. En conclusión, aun cuando la zona norte del estado de Veracruz se encuentra en transición hacia la agricultura orgánica, algunos productores mantienen prácticas de manejo que no siguen las guías de este tipo de producción.



**Palabras clave**
agricultura orgánica • índice de transición • cítricos


## Introduction

Organic agriculture (OA) produces chemical-free food for human consumption and under safer environmental conditions than conventional agriculture. OA considers minimum external inputs while avoiding synthetic fertilizers and pesticides (1, 17, 22, 30, 31). Globally, approximately 2.7 million producers distributed mainly in India, Uganda, and Mexico (7), cultivate under OA. The North American continent represents 16% of worldwide OA, with 11.2 million hectares (ha) of organic production (7). OA in Mexico began in the 1980s. During 2018, the Servicio de Información Agroalimentaria y Pesquera (SIAP) (Fisheries Agrifood Information Service) reported 1,126,000 ha organically managed, 162,386 ha planted and certified as organic, and 11,380 ha of orchards in transition from conventional to organic (6, 24). In 2019, Mexico ranked sixth globally for Valencia orange [*Citrus* x *sinensis* (L.) Osbeck (pro. sp.)] production, contributing 6.3% of global volume (10, 25).

Due to global demand for citrus, Mexico is investing in citriculture, primarily in Veracruz and Michoacan states. In Veracruz, different citrus industries have invested in organic Valencia orange production (12). Within the OA scope, organizations establish requirements and standards. At the beginning of 2001, the Research Institute of Organic Agriculture (FiBL) developed an integrated organic management strategy to control and regulate the vector of Citrus Huanglongbing disease, *Diaphorina citri* Kuayama in Mexico (3, 21). This infection, previously called Citrus Greening Disease is caused by unculturable phloem-limited bacteria. In February 2006, the Organic Products Law was issued in Mexico, then followed by specific regulations in 2010. In 2013, an agreement was signed on guidelines for the organic operation of agricultural activities (26). The National Institute of Forestry, Agricultural and Livestock Research (INIFAP), together with the Consejo de Productores y exportadores de limon Persa (COPELP) (Council of Producers and Exporters of Persian Lemon A.C.) in 2017, compiled a list of bioinsecticides against *D. citri* allowed within organic citriculture (8).

### Conceptual framework addressing OA

Agricultural sustainability is defined/limited by the effects of conventional or intensive agriculture. The Food and Agriculture Organization of the United Nations (1991) conceptualizes sustainable agriculture as:

"The management and conservation of natural resources and the orientation of technological and institutional changes in order to ensure the continuous satisfaction of human needs for present and future generations. Such sustainable development conserves soil, water, and animal and plant genetic resources; does not degrade the environment; and is technically appropriate, economically viable and socially acceptable."

Thus, sustainability is an interaction between society and nature over time, including social progress towards agroecosystem sustainability (4).

In a three-dimensional approach considering broad agroecology, Toledo (2012) emphasized the relationship between scientific research, new practices, and social movements in Latin America (including the Caribbean), describing the five regional kernels of agroecology: Brazil, the Andean Region, Central America, Mexico, and Cuba. He found a tripartite and interrelated innovation process (knowledge, technology, and sociopolitics) interacting with recent political and cultural events (*e.g.* the emergence of governments with social perspective, and indigenous and peasant resistance). Contemporary agroecology encompasses the relationship between scientific paradigms and technological changes with social and political movements, providing important transformational gains toward building sustainable societies (27). Agroecology enhances perspectives for researchers, growers, and industries seeking more environmentally friendly production practices to protect soil, flora, fauna, and ecosystems.

Among management methods for improving agroecosystems, OA (which focuses on agroecology) uses efficient technologies combined with ecological knowledge to reduce negative environmental impact (30). Niggli (2015) states that OA is a practical way of developing sustainable agriculture.

There are four fundamental pillars in agroecology (28). The first pillar is "to build biodiverse agroecosystems such that the composition (species richness, with high genetic variability) and structure (abundance and spatial/temporal distribution of species) acquire complexity, achieving the objectives sought by farmers to maintain ecosystem functionality…" The second pillar consists of "capturing as much solar energy as possible through photosynthesis. This energy supports a complex food chain and agroecosystemic productivity". The third pillar refers to "promoting the greatest recycling of materials introduced into the production process, the incorporation of organic fertilizers compensating for harvest output, the least physical soil intervention, and the use of green manures and ground cover for erosion prevention..." The fourth pillar states the "efficient use of water, a scarce resource". These pillars help build resilient, sustainable, and socially accepted agroecosystems for communities internationally.

According to IFOAM basic standards (13), "Organic Agriculture is a production system that sustains the health of soils, ecosystems, and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic Agriculture combines tradition, innovation, and science to benefit the shared environment and promote fair relationships and good quality of life for all involved."

In Mexico, the Secretary of Agriculture and Rural Development defined OA as an activity prescinding from agrochemicals and developed using natural inputs (no artificial fertilizers or pesticides) aiming to obtain vegetables and animal products or subproducts free of toxic residues (23). According to Niggli (2015), the objective is to support farm ecological and social quality, increasing food production. OA development can be characterized by comprehensive social, ecological, and technological innovations, where dynamic interactions among farmers and scientists are included. According to Kodirekkala (2017), social internal factors interact with external factors, affecting traditional ecological knowledge. Internal factors include socioeconomic and environmental conditions determining decision-making processes, which together with external factors, such as local economy and society, are affected by markets and regulations.

Our research studied the transition degree of Valencia orange orchards towards OA in northern Veracruz State. To this end, the approach described by Niggli (2015) was complemented by the internal and external factors proposed by Kodirekkala (2017). Our research also followed the principles and practices of OA promoted by the International Federation of Organic Agriculture (13, 22), considering production *vs*. environment (16). This included the law and regulations of organic products (15, 20) and the Guidelines for the Organic Operation of agricultural activities (11), describing all regulations for OA transition in Mexico.

The hypothesis stated that Valencia orange producers in northern Veracruz State are transitioning towards organic production, resulting from the dynamic temporal interactions between farmers and institutions strengthening this producing model. Therefore, the objective was to determine the degree of transition towards organic production and management of Valencia orange in the municipalities of northern Veracruz State.

## Materials and methods

### Sample collecting

Our investigation was performed in northern Veracruz State, in the municipalities of Álamo Temapache, Tantoyuca, Chalma, Chicontepec, and Ixhuatlán de Madero, where Valencia orange producers are transitioning from conventional to organic management. To determine management transition, structured interviews were conducted on 106 organic producers of Valencia orange [(*Citrus* x *sinensis* (L.) Osbeck (pro. sp.)]. These producers were grouped into four established civil associations (table 1). In the absence of an official register of organic Valencia orange producers in northern Veracruz State, we implemented a non-probabilistic sampling protocol using the snowball method (5), carried out in March 2021.

**Table 1.** Number of interviewed producers per Association and Municipality in northern Veracruz State.

**Tabla 1.** Número de productores entrevistados en cada asociación de cítricos orgánicos de municipios de la zona norte de Veracruz.

| Associations | Sample | Municipalities |
|---|---|---|
| Productores Ecológicos del Norte de Veracruz S.C. de R.L. de C.V. | 13 | Álamo Temapache |
| Organización Cítricos Hermanos del Ángel S.C. de R.L. de C.V. | 18 | Álamo Temapache |
| Sociedad de Productores Orgánicos S.C. de R.L. de C.V. | 60 | Álamo Temapache - Tantoyuca - Chalma - Chicontepec - Ixhuatlán de Madero |
| Asociación de Citricultores de Chicontepec S.C. de R.L. | 15 | Chicontepec |

### Operationalization, variable weighting, and transition index construction

Table 2 (page 31) lists all variables and indicators. Based on the theoretical framework, the variables and indicators were defined and operationalized. The questionnaire comprised dichotomous answers. Variable and indicator weighting was carried out according to the professional experience of the researchers (29). Relative weights were normalized by multiplying each indicator in the questionnaire with the corresponding weighted value. Then their sum was multiplied by the weighted value of each variable (29). The sum of the normalized relative weights for all the indicators must equal 1 (table 3, page 32). Therefore, the transition index is the sum of the results of the weighted variables and indicators. Coded and tabulated data in an Excel® spreadsheet allowed variables and index calculation. Then, correlation analyses were carried out with all the variables dedicated to OA practice using Statistica software (9). Statistically different variables were classified into lower, intermediate, and upper by Line Plot. An ANOVA compared these values with the transition index. Although surveys started in different years with OA as an empirical division, they were divided into incipient, intermediate, advanced, and organic, from 1 to 6 years, 7 to 12 years, 13 to 18 years, and 19 to 26 years, respectively. The years dedicated to OA were plotted against the transition index.

*According to the Guidelines for the Organic Operation of agricultural activities (11), in the process and organic operation towards general conversion, "the machinery has to be different from that of conventional...". ** The Organic integrity, that is, the quality of an organic product obtained in accordance with the Law, which must be maintained during production and handling until the final point of sale... (20).

* De acuerdo con los Lineamientos para la Operación orgánica de las actividades agropecuarias (11) en la operación orgánica y sus procesos hacia la conversión general, "la herramienta y maquinaria utilizada en la operación orgánica deberá diferenciarse de la utilizada en la actividad agropecuaria convencional...". **La integridad orgánica es la cualidad de un producto orgánico obtenido de acuerdo con la Ley, la cual deberá ser mantenida durante la producción y manejo hasta el punto final de venta... (20).

**Table 2.** Variables and indicators contributing to the Transition Index.
**Tabla 2.** Definición de variables e indicadores que componen el Índice de Transición.

| Index | Variables | Indicators | Definition |
|---|---|---|---|
| **Transition to organic agriculture:** Production systems that make the most use of orchard resources, emphasizing soil fertility and minimizing the use of non-renewable resources. In addition, not using synthetic fertilizers and pesticides to protect agroecosystems and human health. | **Organic fertilizer:** Use of recycled materials and the incorporation of other organic materials, obtained from decomposition of animal/plant organic waste. | **Reuse of pruning waste** | Use of pruning waste for decomposition and mineralization to fertilize orchard soils. |
| | | **Utilization of rotten fruit waste** | Decomposed fruit material used as natural compost. |
| | | **Vermicompost application** | Use of vermicompost for water retention and nutrient release. |
| | | **Manure application** | Use of decomposed animal feces as soil fertilizer. |
| | | **Ash broth application** | Use of mineral mixtures for soil fertilization. |
| | **Intercropping:** Simultaneous sowing of two or more plant species in the same orchard to help optimize cultivation systems by increasing diversification. | **Wind-break barriers** | Trees and shrubs reducing wind speed and insect introductions. |
| | | **Repellent crops** | Plants with repellent or insectistatic effects. |
| | | **Legume cultivation** | Sowing legumes to fix soil nitrogen. |
| | **Pesticide use:** Synthetic and natural compounds used to control pest insects, herbs, rodents, mold, germs. | **Organic** | Organic pesticides obtained from natural sources to control or prevent pests affecting citrus. |
| | | **Synthetic** | Inorganic pesticides or chemical substances controlling or preventing citrus pests. |
| | | **Mineral oils** | Products obtained from petroleum distillation. |
| | | **Paraffinic oils** | Complex mixtures of paraffinic hydrocarbons produced by petroleum distillation. |
| | | **Fatty acids** | Sodium and potassium salts for pest control. |
| | **Citrus grower-institution interactions:** Interaction of citrus growers with public institutions for knowledge transfer. | **Consultancies** | Receiving institutional advice. |
| | | **Courses** | Private or governmental training courses on cultivation. |
| | | **Hiring technical assistance** | Contracted assistance for production process improvement. |
| | | **Willingness to receive advice** | Will to receive technical advice for crop development. |
| | **Internal factors:** Socioeconomic and environmental conditions influencing decision-making. | **Management decision-making** | Grower´s decision to improve management and generate changes. |
| | | **Access to organic inputs** | Direct access to acquire organic inputs. |
| | | **Willingness to use organic products** | Will to use organic products. |
| | | **Possession of machinery** | Equipment and machinery for organic orchard management are owned*. |
| | **External factors:** Effects from outside the local economy, such as the local, national, or international market, and regulations. | **Market** | National or international certification is the same**. |
| | | **Marketing route** | Organic integrity maintained whether the product is sold directly or through an association**. |
| | | **Certification** | A certification title is possessed. |
| | | **Regulations** | Type of regulation that applies to an orchard for USDA, LOOAA, CERTIMEX certification. |

**Table 3.** Operationalization and weighting of variables and indicators for determining the Transition Index.

**Tabla 3.** Operacionalización y ponderación de variables e indicadores para determinar el Índice de Transformación.

| Variables (weighting) | Indicator (weighting) | Definition (response value measurement) |
|---|---|---|
| Organic fertilizer (0.3) | Reuse of pruning waste (0.15) | Use of pruning waste as compost to fertilize orchard soils (Yes: 1; No: 0) |
| | Utilization of rotten fruit (0.2) | Use of rotten fruits as a natural compost (Yes: 1; No: 0) |
| | Vermicompost application (0.3) | Use of vermicompost for water retention nutrient release (Yes: 1; No: 0) |
| | Manure application (0.1) | Use of decomposed animal feces as soil fertilizer (Yes: 1; No: 0) |
| | Ash broth application (0.25) | Use of mineral mixtures for soil fertilization (Yes: 1; No: 0) |
| Intercropping (0.1) | Wind-break barriers (0.2) | Plant trees and shrubs to reduce wind speed and insect immigration (Yes: 1; No: 0) |
| | Repellent crops (0.3) | Plants in orchards have repellent or insecticidal effects (Yes: 1; No: 0) |
| | Legume cultivation (0.5) | Nitrogen-fixing legumes are available (Yes: 1; No: 0) |
| Pesticide use (0.1) | Organic (0.4) | Application of organic pesticides to control or prevent pests affecting citrus (Yes: 1; No: 0) |
| | Synthetic (0.1) | Use of inorganic pesticides or chemical substances controlling or preventing citrus pests (Yes: 0; No: 1) |
| | Mineral oils (0.15) | Use of products obtained from petroleum distillation (Yes: 1; No: 0) |
| | Paraffinic oils (0.15) | Use of complex mixtures of paraffin hydrocarbons produced by petroleum distillation (Yes: 1; No: 0) |
| | Fatty acids (soaps) (0.2) | Use of sodium and potassium salts for pest control (Yes: 1; No: 0) |
| Interaction among citrus growers and institutions (0.1) | Consultancies (0.35) | Receive institutional advice (Yes: 1; No: 0) |
| | Courses (0.2) | Attend private or governmental training courses on cultivation, (Yes: 1; No: 0) |
| | Hiring technical assistance (0.35) | Use contracted assistance for production process improvement (Yes: 1; No: 0) |
| | Willingness to receive advice (0.1) | Willing to receive technical advice for crop development (Yes: 1; No: 0) |
| Internal factors (0.15) | Managing decision-making (0.1) | Growers' decision to improve management and generate changes (Yes: 1; No: 0) |
| | Access to organic inputs (0.3) | Direct access to acquire organic inputs (Yes: 1; No: 0) |
| | Willingness to use organic products (0.35) | Willing to use organic products (Yes: 1; No: 0) |
| | Personal machinery (0.25) | Possession of personal equipment and machinery for organic orchard management (Yes: 1; No: 0) |
| External factors (0.25) | Market (0.37) | Harvest destination (Local: 0; national or international: 1) |
| | Marketing route (0.25) | Harvest sale (self-consumption: 0; direct or through an association: 1) |
| | Certification (0.3) | Possession of certification (Yes: 1; No: 0) |
| | Regulated (0.08) | OA regulations or certification by USDA, LOOAA, CERTIMEX (Yes: 1; No: 0) |

## Results and Discussion

Table 4 shows minimum and maximum values for each indicator, meaning some growers are implementing more organic management than others. The average transition index was 0.768, indicating an average value of transition towards OA, consistent with the number of years dedicated to OA.

A: organic fertilizer. B: intercropping. C: pesticide use. D: interaction between citrus growers and institutions, E: internal factors, F: external factors.

A: fertilizante orgánico. B: intercalado de cultivo. C: uso de pesticidas. D: interacción citricultores-instituciones, E: factores internos. F: factores externos.

**Table 4.** Maximum, minimum, and mean values of variables A to F, and the transition index.

**Tabla 4.** Valores máximos, mínimos y media de las variables e índice de transformación. Cada una de las letras identifica las distintas variables.

| | A (0.3) | B (0.1) | C (0.1) | D (0.1) | E (0.15) | F (0.25) | Transition Index (1) |
|---|---|---|---|---|---|---|---|
| **Mean** | 0.1833 | 0.0727 | 0.0659 | 0.0525 | 0.1450 | 0.2497 | 0.7687 |
| **Maximum** | 0.3000 | 0.1000 | 0.1000 | 0.1000 | 0.1500 | 0.2500 | 0.9500 |
| **Minimum** | 0.0750 | 0.0200 | 0.0250 | 0.0100 | 0.0675 | 0.2300 | 0.5950 |

Figure 1 (page 34) shows biplots and consequent correlations between variables. Significant positive correlations existed between the citrus grower/institution interaction and intercropping ($p = 0.0281$; $r = 0.2133$), and the citrus grower/institution interaction with internal factors ($p = 0.0257$; $r = 0.2167$). A weak and negative correlation between pesticide use and citrus grower/institution interaction ($p = 0.0369$; $r = -0.2030$) was given by producers buying pesticides from agrochemical suppliers (based on seller recommendations) without seeking advice from institutions. Thus, the citrus grower/institution interaction with intercropping constitutes a factor providing recent information in Veracruz State. Production of organic *C. sinensis* is gaining importance given a favourable international market demand and different processing industries generating products such as orange juice, essential oils, and cosmetics. The International Copper Chemistry (IQC), Cítricos ex S.A. of C.V. (Citrex), Citrusper, and Procitris, S.A de C.V. industries are sought by countries such as the United States, Canada, the European Union, and Asia (12). According to Bigaran and Ramos (2022), it is also important to measure the logistical performance of distribution channels of different lengths (km) based on criteria supported by the concept of food miles and in the main logistical practices of distribution of fruits and vegetables, contribute to the definition of strategies to mitigate food losses.

Figure 2 (page 34) shows the categorized transition index with the statistically different factors according to Line plot. Growers with higher transition indices use intercropping and have more interaction with institutions, while increased activities help growers obtain certification for organic production. Yet, given all growers applied organic fertilizers and organic pesticides, other variables were not different within the categorized transition index. Plant nutrition with organic fertilizers such as pruning residues, animal feces and mixtures of mineral products promotes vigorous and healthy crops. Additionally, only organic pesticides, and sodium/potassium salts are used to control or prevent pests affecting citrus. In this sense, organic citriculture results complex. Changes in scientific-technological paradigms are built in constant association with social movements, where sustainable citriculture is in constant transition and growers must interact with all agents related to this industry. Thus, organic citriculture is an alternative for researchers, producers and industrialists seeking less environmental impact and human well-being (27). Improving organic practices requires social, ecological, and technological innovation in dynamic interaction among farmers and scientists strengthening system resilience and exploiting research from diverse scientific disciplines (18).

A: organic fertilizer.
B: intercropping.
C: pesticide use.
D: citrus grower institution interaction.
E: internal factors.
F: external factors.

A: fertilizante orgánico.
B: intercalado de cultivo.
C: uso de pesticidas.
D: interacción citricultores-instituciones. E: factores internos. F: factores externos.

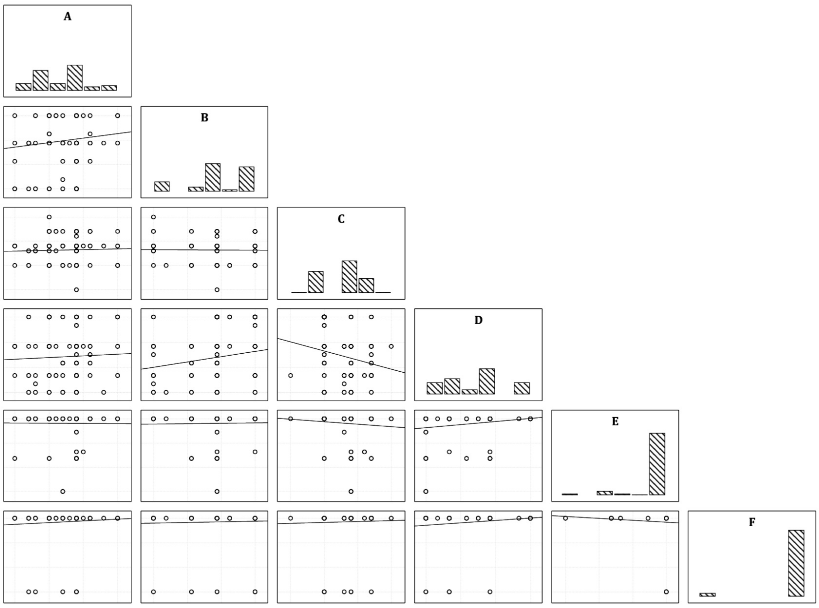


**Figure 1.** Correlation matrix.
**Figura 1.** Matriz de correlación.

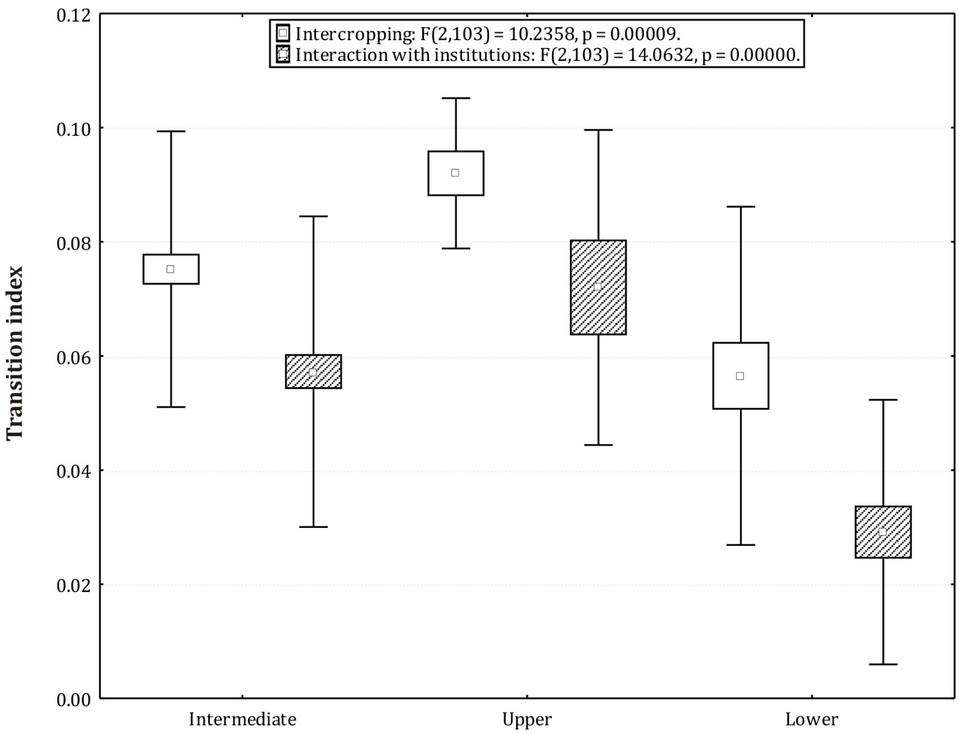


**Figure 2.** Analysis of the transition index and variables with statistical differences, heuristically divided by Line plot.
**Figura 2.** Análisis del índice de transición con las variables divididas heurísticamente por Line plot que presentaron diferencia estadística.

The citrus grower/institution interaction evidences how OA is achieved through a dynamic interaction among farmers and scientists promoting a comprehensive culture including social, ecological, technological, and environmental innovation (18). This is achieved because producers are willing to receive advice, control, and supervision in different cultivation activities, while also getting involved in training activities.

Figure 3, shows a highly significant difference (p= 0.0000) between the transition index and the degree of transition to OA, indicating that as conversion time increased, more practices approved by the International Federation of Organic Agriculture Movements (2005) were implemented. Importantly, several growers with less time under OA could apply all practices as advanced or organic according to the weighting of variables and indicators, and all had their orchards certified as organic. Current challenges should satisfy the production demand for Valencia orange while reducing waste, improving the production of healthy oranges for consumption, conserving natural resources, mitigating and adapting to climate change and reducing social and cultural injustice and cultural erosion (*i.e.* the loss of traditional knowledge). Improvement of most agricultural activities is strongly needed (14).

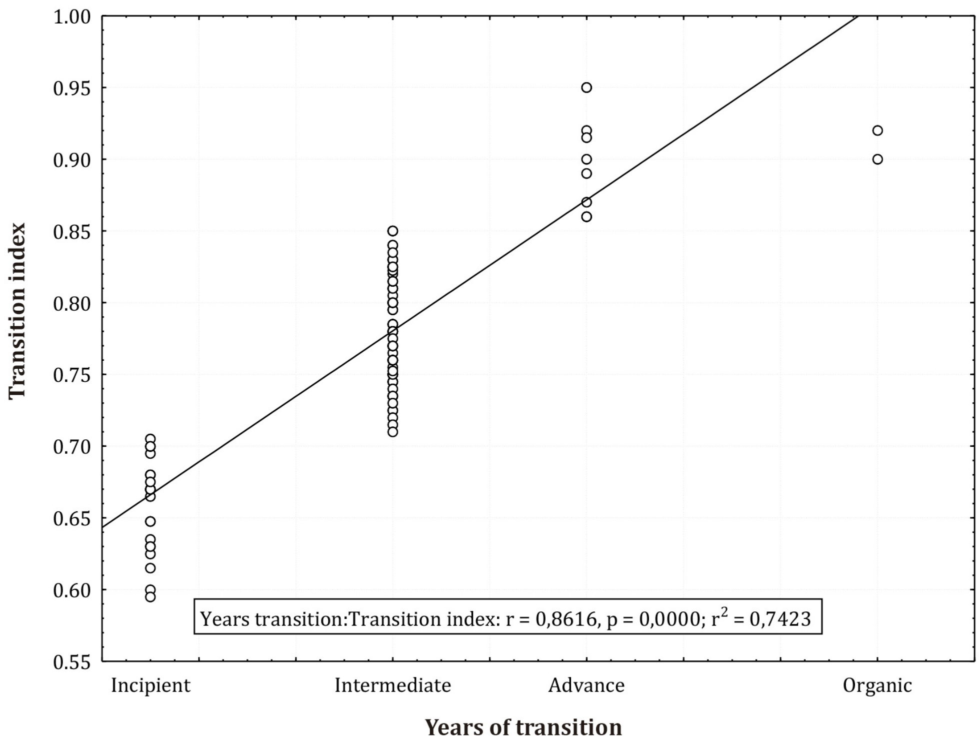


**Figure 3.** Relationship between the transition index and years of conversion to organic agriculture.

**Figura 3.** Relación entre el índice de transformación y los años de conversión hacia la agricultura orgánica.

## Conclusions

Even considering all interviewed producers had orchards certified as organic, Valencia orange producers in northern Veracruz State are still transitioning to organic production above an average transition index (0.7687). Producers with more years in organic production showed a higher transition index. This transition degree is primarily influenced by the organic matter recycling practices employed, the non-use of synthetic pesticides and fertilizers, and the dynamic interactions between producers and institutions. Yet, internal and external factors did not significantly influence the process of transition towards an organic model for citrus production.

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# Quality of Tanzania grass (*Panicum maximum*) haylage in relation to plant dry matter content

## Calidad del pasto Tanzania (*Panicum maximum*) almacenada como henolaje según la materia seca de la planta

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### Abstract

This study aimed to evaluate the quality of Tanzania grass (*Panicum maximum*) haylage with varying contents of dry matter (DM) and stored for 90 days. The quality of this grass was evaluated through the lens of a variety of physiochemical properties (*e.g.*, chemical composition, aerobic stability, pH, microbial profile, etc.). A completely randomized design was used with four treatments (*in natura*, 400, 500, and 600 g $kg^{-1}$ DM) and five replicates. Treatment with 600 g $kg^{-1}$ DM yielded the highest DM haylage ($p < 0.01$) and soluble carbohydrate content ($p < 0.01$). Treatment *in natura* resulted in the highest $O_2$ concentration inside the bales ($p < 0.01$), whereas treatments with 500 and 600 g $kg^{-1}$ DM resulted in the highest $CO_2$ values. The highest acetic acid concentrations of 36.4 ± 1.6, 38.2±1.6, and 48.9 ± 1.6 g $kg^{-1}$ DM ($p < 0.01$) were observed post the *in natura*, 500 g $kg^{-1}$ DM, and 600 g $kg^{-1}$ DM treatments, respectively. Treatment with 600 g $kg^{-1}$ DM yielded the highest pH value at hour zero ($p < 0.01$). Tanzania grass with 500 and 600 g $kg^{-1}$ DM produced the highest quality haylage.

**Keywords**
conservation • haylage • grasses • microbiology • moisture • Tanzania grass

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## Resumen

El objetivo fue evaluar la calidad del henolaje del pasto Tanzania (*Panicum maximum*) con diferentes contenidos de materia seca y un almacenamiento de 90 días, a través de la composición química, cuantificación de gases, ácidos grasos volátiles, perfil microbiológico, estabilidad aeróbica, pH y nitrógeno amoniacal. El diseño experimental utilizado fue completamente al azar con cuatro tratamientos y cinco repeticiones. Los tratamientos consistían de cuatro contenidos de materia seca (MS) de la planta en el momento de la producción del henolaje del pasto Tanzania siendo: en materia fresca (sin deshidratación), 400, 500 y 600 g $kg^{-1}$ de MS (deshidratados en pleno sol). El tratamiento con 600 g $kg^{-1}$ de MS de la planta proporcionó la mayor estimación ($p < 0,01$) de MS en el henolaje con 581,6 ± 15,4 g/kg, y el mayor ($p < 0,01$) contenido de carbohidratos solubles con 45,4 ± 1,24 g/kg MS. Después de 90 días de almacenamiento, el tratamiento en materia fresca presentó mayores ($p < 0,01$) cantidades de $O_2$ en el interior de los fardos. En relación al $CO_2$ los mayores índices fueron observados para los tratamientos con 500 y 600 g $kg^{-1}$ de MS. También se observó el mayor ($p < 0,01$) contenido de ácido acético en los tratamientos materia fresca y con 500 y 600 g $kg^{-1}$ de MS de la planta, con 36,4 ± 1,6, 38,2 ± 1,6 y 48,9 ± 1,6 g $kg^{-1}$ de MS, respectivamente. Para el ácido butírico se obtuvo la mejor ($p < 0,01$) valoración de 27,0 ± 0,5 g $kg^{-1}$ de MS en el tratamiento en materia fresca. El tratamiento con 600 g $kg^{-1}$ de MS mostró mayor ($p < 0,01$) valor de pH en la hora cero con 6,36 ± 0,03. El tratamiento en materia fresca presentó mayor valor de $N_{-NH3}$ en la hora cero de exposición al aire con 4,65 ± 0,12. El pasto Tanzania con 500 y 600 g $kg^{-1}$ de MS, presenta el henolaje de mejor calidad.



**Palabras clave**

conservación • henolaje • gramíneas • microbiología • humedad • pasto Tanzania


## Introduction

The storage of forage plants in the form of haylage is in line with the sustainable use of leguminous and grass forage species. For example, oats (*Avena sativa*) and ryegrass (*Lolium multiflorum*) are suitable for the production of haylage in temperate regions, whereas species in the genera *Brachiaria*, *Cynodon*, *Panicum*, and *Pennisetum* are better suited for the production of haylage in tropical regions (27). Tanzania grass (*Panicum maximum*) has shown great potential within the context of haylage production, as it has a high yield, a large number of leaves, and a high nutritional value (9, 54).

Haylage can be defined as stored pre-dried forage with a dry matter (DM) content of approximately 400 to 800 g/kg (7, 43). It is stored in the form of bales wrapped in a plastic cover, providing ideal conditions for the growth of lactic acid bacteria (LAB) that are beneficial for the conservation and storage of forage. This forage would then be used as animal feed; this is especially important when resources are scarce (*e.g.*, during droughts) (22).

The preservation of grass in the form of haylage is an option for forage grasses with high moisture content because dehydration of the material increases DM content, which reduces proteolysis, secondary fermentation, and pH buffering in the stored material (23). The moisture content of the forage plant is one factor that influences the microbial profile of the forage mass preserved by fermentation (61). When harvested, tropical grasses have a high moisture content accompanied with low levels of soluble carbohydrates (CHO) (49), which favors the occurrence of undesirable fermentation as the grass is preserved.

An alternative to adjusting the DM content in tropical grasses is dehydration in the field after cutting (10). This process increases the DM content of the forage mass, facilitating the preparation of the material for undergoing preservation via fermentation. The DM percentage of haylage influences the quality of the stored material (40).

No exact recommendations are available for the DM content of tropical grasses for conservation as haylage, and no studies have been conducted within the context of determining the DM content of Tanzania grass. Therefore, this study aimed to evaluate the quality of Tanzania grass haylage stored with different DM contents based on its chemical composition, gas quantification, volatile fatty acids, microbiological profile, and aerobic stability.

## Material and methods

### Study Area

A pasture area established in 2013 was used for haylage production. The study area is in Alvorada do Gurgueia, Piauí, Brazil, at latitude 08°25'28" South, longitude 43°46'38" West, and an altitude of 281 m. According to the Köppen classification (1936), the climate of the region is classified as BSh, hot semi-arid, with rainy summers and dry winters, as described Medeiros *et al.* (2013) and Alvares *et al.*(2013).

The area of the pasture was determined to be 0.5 hectares, and it had no artificial irrigation systems. A standardization cut was made 30 cm from the ground at the beginning of the experimental period for haylage production, according to the recommendation of Braz *et al.* (2017). Fertilization was performed according to the soil analysis and recommendations for highly demanding species (30).

### Experimental design

To assess the chemical composition and volatile fatty acids of the Tanzania grass haylage, a completely randomized design with four treatments and five replicates was adopted. The treatments consisted of four groups of haylage that varied in terms of DM content as follows: *in natura* plant (not dehydrated), 400, 500, and 600 g $kg^{-1}$ DM (dehydrated in the field until reaching the DM content of the treatment).

A completely randomized design in a 4 × 6 factorial scheme, with five replications, was adopted for the gas assessment of the Tanzania grass haylage. The factors were four levels of DM of the plant for haylage production and six gas evaluation times: 0, 7, 15, 30, 45, and 60 d after wrapping the haylage bales.

To assess the aerobic stability of Tanzania grass haylage, a completely randomized design in a 4 × 6 factorial scheme, with five replications was adopted. There were four levels of plant DM for haylage production and six evaluation times: 0, 24, 48, 72, 96, and 120 h after opening the bales.

### Haylage production

Tanzania grass was harvested right before it flowered; at this point, the pasture had a height of 90 cm (30 days), as recommended by Euclides *et al.* (2014). The extracted material was left in the field for pre-drying until it reached the determined DM content (400, 500, and 600 g $kg^{-1}$ DM), except for the material of the *in natura* treatment, which was not dehydrated and immediately baled. For treatments with pre-dried forage, the forage mass was revolved to standardize dehydration. The forage was collected and sealed when it reached the predetermined DM level. The DM content was determined using the microwave method as previously described (55).

The bales were made in manual balers and then manually wrapped in plastic film (SSFILM SSilage Xtreme®), with eight rounds per bale, as recommended previously (38), to minimize gas exchange. The haylage bales weighed approximately 3 kg and were stored for 90 d in a ventilated shed with no sunlight exposure.

To characterize the quality of the haylage, both *in natura* forage and haylage were assessed using the following variables: chemical composition, gas quantification, volatile fatty acids, microbiological profile, aerobic stability, pH, and ammonia ($N\text{-}NH_3$). The analyses were conducted in the Animal Nutrition Laboratory and Microbiology Laboratory of the Federal University of Piauí, located in the Bom Jesus, Piauí, Brazil.

### Determination of chemical composition and gases

The samples used for the chemical composition analysis of Tanzania grass before the production of the haylage (table 1, page 41) (*i.e.*, after 90 days of storage) were dried in a circulation and air renewal oven, at a maximum temperature of 55 °C, until they reached a constant weight. They were then ground in a Thomas Willey stationary mill through a 1-mm-mesh sieve. The contents of DM (n°. 934.01), crude protein (CP n°. 981.10), mineral matter (MM n°. 934.05), and organic matter (OM nº. 934.05) were determined using the methods described previously (4), whereas neutral detergent fiber (NDF) was determined using the methodology proposed by Van Soest *et al.* (1991).

**Table 1.** Chemical composition of Tanzania grass across varying dry matter (DM) contents, expressed as g $kg^{-1}$ DM, prior to the production of haylage.

**Tabla 1.** Composición química de la planta de pasto Tanzania de acuerdo con la deshidratación, expresada como g $kg^{-1}$ DM antes de la producción de henolaje.

| Variables | DM Content (g $kg^{-1}$) | | | |
|---|---|---|---|---|
| | *in natura* | 400 | 500 | 600 |
| Dry matter (g $kg^{-1}$) | 238.7 | 381.2 | 486.0 | 575.3 |
| Crude protein (g $kg^{-1}$ DM) | 106.2 | 121.2 | 123.1 | 130.2 |
| NDF | 724.3 | 749.0 | 657.8 | 613.0 |
| MM | 60.0 | 69.4 | 65.0 | 67.4 |
| OM | 939.9 | 930.6 | 934.9 | 946.2 |
| CHO | 80.9 | 73.8 | 67.7 | 54.1 |

NDF: neutral detergent-insoluble fiber. MM: Mineral matter. OM: Organic matter. CHO: Soluble carbohydrate.

NDF: Fibra insoluble en detergente neutro. MM: Materia mineral. OM: Materia orgánica. CHO: Carbohidratos solubles.

The total CHO (TCHO) content was determined using the concentrated sulfuric acid method described previously (17) with adaptations of Corsato *et al.* (2008). The TCHO content was calculated as g 100 $ml^{-1}$ based on the solution and subsequently adjusted based on the DM of each sample used.

To evaluate the gases produced in the haylage, the levels of $O_2$ and $CO_2$ were measured. Assessments were performed on the haylage on days 0, 7, 15, 30, 45, and 90, after it was wrapped. Haylage was assessed on day 0, immediately after wrapping. The readings were acquired through two valves (PVC pipes) that were inserted into each bale and sealed for the duration of the established days'. For the gas analysis, an $O_2$ meter Instrutherm® (model MO-900) was used, which also measured the internal temperature of the bales, while $CO_2$ was measured by a $CO_2$ analyzer Testoryt® (White).

**Quantification of volatile fatty acids and microorganisms**

To quantify the contents of volatile fatty acids (*i.e.*, acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids) of Tanzania grass haylage after 90 days of storage, only portions of each sample were used for analysis through the method mentioned by Kung Jr and Ranjit (2001), where the juice was extracted using a manual press. The samples were centrifuged, and subsequently, the analysis of organic acids was performed using high-resolution liquid chromatography using a high-performance liquid chromatograph (HPLC) detector model SPD-10ª VP, coupled to the ultraviolet detector (UV), using a wavelength of 210 nm. The boiling alcohol content was determined using an ebulliometer, as recommended previously (28). Analyses were performed at the Laboratory of the Luís de Queiroz College of Agriculture.

Microbiological evaluation was performed according to the recommendations of González *et al.* (2003) by collecting 25 g of fresh sample, adding 225 mL of distilled water, and processing in a blender for approximately 1 min. One milliliter of the mixture was pipetted at the appropriate dilution ($10^{-1}$ $10^{-9}$). Plating was performed in duplicates for each culture medium. The populations were determined by the selective technique of culturing in anaerobic media. Rogosa Agar medium was used for counting lactobacilli (after incubation of 48 hours in an oven at 37°C); BDA Agar medium (Potato Dextrose Agar) acidified with 1% tartaric acid, for the counting yeasts and molds (after 3-7 days of incubation at room temperature); and Brilliant Green Bile Agar medium, for counting the enterobacteria (after incubation of 24 hours at 35°C).

Plates with values between 30 and 300 colony-forming units (CFU) in the Petri dish were considered acceptable for counting. Plaque averages of the selected dilutions were considered.

**Evaluation of aerobic stability, pH, and ammonia nitrogen**

When the haylage bales were opened, the forage mass was exposed to air under a controlled room temperature (25°C); this approach was similar to that applied in evaluations conducted in Johnson *et al.* (2002). Room temperature was controlled using an INCOTERM® room thermometer. The internal temperature of the haylage was measured using an

INCOTERM® digital skewer thermometer, and the surface temperature was measured using a BENETECH® infrared digital thermometer with laser aim (-50 to 420°C). Temperature was measured at 0, 24, 48, 72, 96, and 120 h. The aerobic stability break was defined as an increase of 2°C in the temperature of the haylage in relation to room temperature after opening the bales (35). During the evaluation period, samples from each treatment were collected (approximately 100 g) at different time points (0, 24, 48, 72, 96, and 120 h) to assess pH and ammonia ($N\text{-}NH_3$) levels, as per a previously described methodology (33).

**Statistical analysis**

The data were subjected to an analysis of variance. Means were compared using Tukey's test and linear regression, and all analyses were performed at a significance level of $p < 0.05$. The data were analyzed using the SISVAR software (version 5.0; 19).

Tukey's test was used to analyze the chemical composition and volatile fatty acid data. The adopted statistical model was:

$$\mathbf{Y_{ij} = \mu + T_i + \varepsilon_{ij}}$$

where:
$Y_{ij}$ = record of the DM content i
μ = general constant
$T_i$ = effect of the DM content i
with i = 1-4; $\varepsilon_{ij}$ = random error associated with each DM content $Y_{ij}$

Gas data were analyzed using Tukey's test for plant DM and evaluation times. The following statistical model was adopted:

$$\mathbf{Y_{ijk} = \mu + A_i + T_j + AT_{ij} + \varepsilon_{ijk}}$$

where:
$Y_{ijk}$ = record k, referring to the DM content i evaluated at time j
μ = general constant
$A_i$ = effect of DM content i, i = 1-4
$T_j$ = gas evaluation time j, j = 0-120
$AT_{ij}$ = interaction between DM content i and gas evaluation time j
$\varepsilon_{ijk}$ = random error associated with each $Y_{ijk}$ record

To evaluate the aerobic stability data, Tukey's test was used for plant DM, and linear regression analysis was used for the evaluation times. The following statistical model was adopted:

$$\mathbf{Y_{ijk} = \mu + A_i + T_j + AT_{ij} + \varepsilon_{ijk}}$$

where:
$Y_{ijk}$ = record k, referring to the DM content i, evaluated at time j
μ = general constant
$A_i$ = effect of the DM content i, i = 1-4
$T_j$ = stability evaluation time j, j = 0,..., 120
$AT_{ij}$ = interaction between the DM content i and stability evaluation time j
$\varepsilon_{ijk}$ = random error associated with each $Y_{ijk}$ record

Data referring to the quantification of microbial groups ( logarithmic units, log 10) were analyzed descriptively.

## Results

### Chemical composition and gases

The chemical composition of Tanzania grass haylage according to plant DM and CHO contents was affected ($p < 0.01$) after 90 days of storage (table 2).

Means followed by different letters in a row indicate statistical differences according to Tukey's test at $p$ <0.05; NDF: Neutral Detergent Insoluble Fiber. MM: Mineral matter. OM: Organic matter. CHO: Soluble carbohydrate. SEM: standard error of the mean.

Medias seguidas de letras diferentes en la fila son estadísticamente diferentes según la prueba de Tukey con $p$ < 0,05. NDF: Fibra insoluble en detergente neutro. MM: Materia mineral. OM: Materia orgánica. CHO: Carbohidratos solubles. SEM: error estándar de la media.

**Table 2.** Chemical composition of Tanzania grass haylage according to the dry matter content.

**Tabla 2.** Composición química del henolaje de pasto Tanzania según el contenido de materia seca (MS) de la planta.

| Variables | DM Content (g kg$^{-1}$) | | | | Mean | SEM | *–p-value* |
|---|---|---|---|---|---|---|---|
| | *in natura* | 400 | 500 | 600 | | | |
| Dry matter (g kg$^{-1}$) | 267.6$^{c}$ | 397.7$^{b}$ | 480.3$^{b}$ | 581.6$^{a}$ | 406.5 | 15.4 | < 0.01 |
| Crude protein (g kg$^{-1}$ DM) | 96.9 | 87.5 | 94.8 | 99.4 | 94.6 | 3.58 | 0.18 |
| NDF | 692.0 | 650.5 | 721.3 | 653.9 | 679.5 | 54.7 | 0.77 |
| MM | 74.1 | 74.2 | 73.8 | 71.0 | 59.3 | 3.71 | 0.83 |
| OM | 925.8 | 925.7 | 926.1 | 943.6 | 920.3 | 53.6 | 0.09 |
| CHO | 35.0$^{b}$ | 36.2$^{b}$ | 42.6$^{a}$ | 45.4$^{a}$ | 39.8 | 1.24 | < 0.01 |

The highest DM and CHO contents were observed in the haylage treated with 600 gDM/kg (581.6 ± 15.4 gDM/kg and 45.4 ± 1.24 gCHO /kg DM, respectively). The other chemical composition variables were not significantly different among the treatments, yielding mean values of 94.6 ± 3.58 gCP/kg DM, 679.5 ± 54.7 gNDF/kg DM, 59.3 ± 3.71 gMM/kg DM, and 920 ± 53.6 gOM/kg DM. The desired DM contents after the 400, 500, and 600 gDM/kg treatments were very similar between plants (381.2, 486.0, and 575.3 g/kg, respectively; table 1, page 41) and haylage (397.7, 480.3, and 581.6 g/kg; table 2).

The quantification of $O_2$ and $CO_2$ gases in the Tanzania grass haylage revealed a significant interaction effect ($p < 0.01$) between the plant DM and number of days during which the gas composition of the grass was evaluated during storage (table 3, page 44). Treatment with 400 g kg$^{-1}$ DM resulted in the lowest value of $O_2$ on day 0, whereas the treatment *in natura* resulted in the highest amount of $O_2$ inside the bales when they were opened after 90 days of storage. There was a reduction in the amount of $O_2$ inside the Tanzania grass haylage bales after 7 days of storage after all the treatments, and, after 90 days of storage, the $O_2$ content was found to be less than 2.5% inside all the bales.

The lowest $CO_2$ values were observed on day 0. $CO_2$ increased between days 7$^{th}$ and 15$^{th}$ days of storage and after 90 days of storage. The treatments 400 and 500 g kg$^{-1}$ DM resulted in the highest $CO_2$ concentrations, which were 16.7 ± 1.0% and 16.2 ± 1.0%, respectively. There was a significant effect ($p < 0.05$) of the storage period on the internal temperature of the haylage, which reduced to 7.3 ± 0.21°C after 45 days, and the highest temperatures were observed on days 0 and 15.

### Volatile fatty acids and microorganisms

The highest acetic acid value of 36.4 ± 1.6 g kg$^{-1}$ DM was obtained for the 600 g kg$^{-1}$ DM treatment, followed by the values of 38.2 ± 1.6 and 48.9 ± 1.6 g kg$^{-1}$ DM for the *in natura* and 500 g kg$^{-1}$ DM treatments, respectively. As for butyric acid, the highest value (27.0 ± 0.5 g kg$^{-1}$ DM) was observed for the *in natura* treatment (table 4, page 44).

**Table 3.** Gas and temperature quantification in Tanzania grass haylage based on the plant dry matter (DM) during the various storage times

**Tabla 3.** Cuantificación de los gases y la temperatura de henolaje de hierba de Tanzania según la materia seca de la planta a través de los tiempos de almacenamiento.

| DM Content (g kg$^{-1}$) | Days | | | | | | Mean |
|---|---|---|---|---|---|---|---|
| | 0 | 7 | 15 | 30 | 45 | 90 | |
| **Oxygen ($O_2$)** | | | | | | | |
| *in natura* | 21.7aA | 1.1cdB | 1.1cdA | 1.0dA | 1.9bcA | 2.4bA | 4.9 |
| 400 | 20.8aB | 1.4AbcB | 0.8cA | 1.0bcA | 1.6bcA | 1.9bAB | 4.6 |
| 500 | 21.5aAB | 2.2bA | 0.7cA | 0.8cA | 1.8bA | 1.4bcB | 4.7 |
| 600 | 22.1aA | 1.3bB | 1.0bA | 0.8bA | 1.7bA | 1.3bB | 4.7 |
| Mean | 21.5 | 1.5 | 0.9 | 0.9 | 1.7 | 1.7 | |
| **$CO_2$** | | | | | | | |
| *in natura* | 1.8bA | 18.7aC | 20.7aB | 16.7aA | 13.5aA | 12.5AaB | 14.0 |
| 400 | 1.2cA | 29.5aB | 20.0bB | 15.0bA | 14.2bA | 14.6bB | 15.7 |
| 500 | 1.5dA | 29.5aB | 16.0bC | 13.2bcA | 12.7bcA | 16.7cA | 13.3 |
| 600 | 0.0cA | 37.2aA | 29.5aA | 18.5bA | 16.5bA | 16.2bA | 19.6 |
| Mean | 1.1 | 28.7 | 21.5 | 15.8 | 14.2 | 12.5 | |
| **Internal temperature** | | | | | | | |
| *in natura* | 34.4 | 29.0 | 33.5 | 26.8 | 26.3 | 26.5 | 29.4 |
| 400 | 32.1 | 28.6 | 33.6 | 27.0 | 26.3 | 27.2 | 29.1 |
| 500 | 34.2 | 29.0 | 33.8 | 27.2 | 26.5 | 27.6 | 29.7 |
| 600 | 34.1 | 29.4 | 33.9 | 27.4 | 26.6 | 27.9 | 29.9 |
| Mean | 33.7a | 29.0b | 33.7a | 27.1c | 26.4c | 27.3c | |

| Analysis of variance | *-p-value* | | | | |
|---|---|---|---|---|---|
| | **DM Content** | **Days** | **DM Content × Days** | | **SEM** |
| Oxygen | 0.08 | < 0.01 | < 0.01 | | 0.10 |
| $CO_2$ | < 0.01 | < 0.01 | < 0.01 | | 1.00 |
| Temperature | 0.07 | < 0.01 | 0.70 | | 0.21 |

Means followed by different letters in a column and row indicate statistical differences according to Tukey's test at $p < 0.05$. SEM: Mean Standard error.

Medias seguidas de letras diferentes en la columna y en la fila son estadísticamente diferentes según la prueba de Tukey con p <0,05. SEM: Error estándar de la media.

**Table 4.** Concentration of volatile fatty acids in Tanzania grass haylage based on the plant dry matter (DM) content, expressed as g/kg DM.

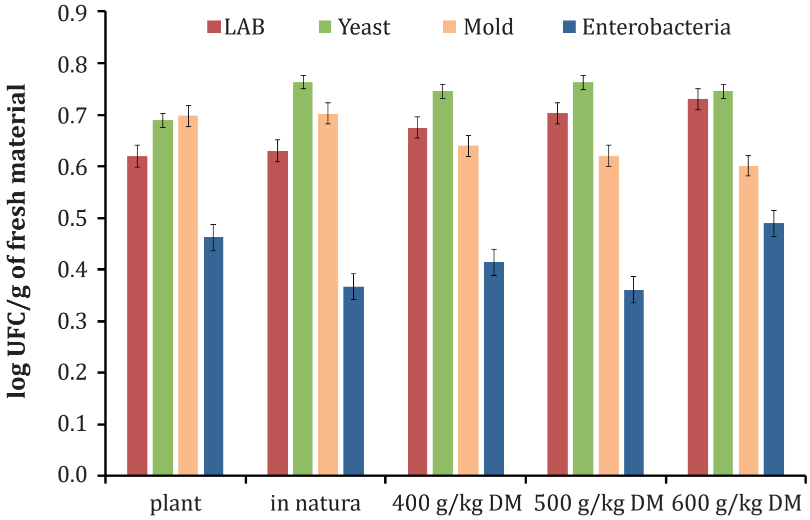
**Tabla 4.** Concentración de ácidos grasos volátiles en henolaje de pasto Tanzania según la materia seca de la planta, expresada como g/kg MS.

| DM Content (g kg$^{-1}$) | Acetic | Propionic | Isobutyric | Butyric | Isovaleric | Valeric |
|---|---|---|---|---|---|---|
| *in natura* | 36.4$^{b}$ | 3.2 | 0.5 | 27.0$^{a}$ | 9.1 | 9.6 |
| 400 | 11.1$^{c}$ | 2.7 | 0.0 | 1.4$^{b}$ | 0.0 | 0.0 |
| 500 | 38.2$^{b}$ | 0.7 | 0.0 | 2.5$^{b}$ | 3.0 | 4.5 |
| 600 | 48.9$^{a}$ | 4.1 | 2.0 | 3.2$^{b}$ | 6.1 | 1.2 |
| Mean | 38.2 | 2.7 | 0.6 | 8.59 | 4.5 | 3.8 |
| SEM | 1.5 | 1.8 | 0.4 | 0.5 | 2.4 | 2.4 |
| *P - value* | <0.01 | 0.65 | 0.12 | <0.01 | 0.22 | 0.15 |

Means followed by different letters in a column indicate statistical differences according to Tukey's test at $p < 0.05$. SEM: Mean Standard error.

Medias seguidas de letras diferentes en la columna son estadísticamente diferentes según la prueba de Tukey con p <0,05. SEM: Error estándar de la media.

In the assessment of the microbial composition of Tanzania grass and its resultant haylage (figure 1), an increase in the concentration of LAB was shown to be associated with an increase in the DM content. LAB populations were found in the haylage at 6.9, 7.0 and 7.5 log CFU/g for the 400, 500 and 600 g $kg^{-1}$ DM treatments, respectively.




LAB: lactic acid bacteria. FM: fresh material.

LAB: bacteria del ácido láctico. FM: material fresco.

**Figure 1.** Microbial composition of the plant and haylage of Tanzania grass stored with different levels of dry matter.

**Figura 1.** Población de microorganismos de la planta y henolaje de pasto Tanzania almacenado con diferentes niveles de materia seca.

No difference was found in the yeast population at the different plant DM contents used for haylage production. Tanzania haylage had the smallest yeast population of 6.9 log CFU/g. The 400, 500, and 600 g $kg^{-1}$ DM treatments yielded yeast populations of 7.0, 7.6, and 7.2 log CFU/g in the haylage, respectively.

The lowest amounts of mold, at 6.2 and 6.0 log CFU/g, were observed in the haylage for the 500 and 600 g $kg^{-1}$ DM treatments, respectively. While the smallest amounts of enterobacteria, at 4.5 and 3.5 log CFU/g, were found in haylages of the *in natura* and 500 g $kg^{-1}$ DM treatments, respectively.

**Aerobic stability, *pH*, and ammonia nitrogen**

Aerobic stability was affected by the interaction between the different plant DM contents and hours of exposure of the Tanzania grass haylage to air after opening the bales; this process was largely driven by surface temperature, internal temperature, pH, and $N\text{-}NH_3$ (table 5, page 46).

Haylage surface temperature had a linear relationship ($p < 0.01$) with the length of time the materials were exposed to air. Specifically, between 0 and 120 hours of exposure to air, increases of 2.7 ± 0.06°C, 2.4 ± 0.06°C, 1.7 ± 0.06°C, and 1.6 ± 0.06°C were observed for the *in natura*, 400 g $kg^{-1}$ DM, 500 g $kg^{-1}$ DM, and 600 g $kg^{-1}$ DM treatments, respectively. The 600 g $kg^{-1}$ DM treatment yielded the highest surface temperatures of the haylage, which were 21.9, 23.4, 21.5, 22.4, and 23.8 ± 0.06°C for the exposure to air times of 0, 24, 48, 72, 96, and 120 hours, respectively. The in natura, 400 g $kg^{-1}$ DM, and 600 treatments had an increasing linear effect ($p < 0.01$) on internal temperature over the hours of haylage exposure to air.

The in natura treatment yielded the highest temperature of 25 ± 0.82°C in the haylage after 48 hours of exposure to air, and the highest room temperature recorded was 24.6°C. During exposure to air, there was no increase of 2% in the surface and internal temperatures of the haylage compared to the room temperature (table 5, page 46).

**Table 5.** Aerobic stability of Tanzania grass haylage based on the plant dry matter (DM).
**Tabla 5.** Estabilidad aeróbica de henolaje de pasto Tanzania según la materia seca de la planta.

| DM Content (g $kg^{-1}$) | Hours | | | | | | Mean | -p-value | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 24 | 48 | 72 | 96 | 120 | | x | |
| **Room temperature (°C)** | | | | | | | | | |
| | 24.0 | 24.2 | 24.1 | 24.2 | 24.6 | 24.5 | | | |
| **Surface temperature (°C)** | | | | | | | | | |
| *In natura* | 21.0B | 22.7B | 21.1A | 21.9AB | 23.1B | 23.7A | 22.3 | < 0.01* | 56.2 |
| 400 | 21.4AB | 23.0AB | 21.0A | 21.4B | 23.2AB | 23.8A | 22.3 | < 0.01* | 35.0 |
| 500 | 21.7A | 23.2AB | 21.2A | 21.7B | 23.2AB | 23.4A | 22.4 | < 0.01* | 21.2 |
| 600 | 21.9A | 23.4A | 21.5A | 22.4A | 23.8A | 23.5A | 22.7 | < 0.01* | 37.4 |
| Mean | 21.6 | 21.9 | 22.3 | 22.6 | 22.9 | 23.3 | | | |
| **Internal temperature (°C)** | | | | | | | | | |
| *In natura* | 22.2A | 23.5A | 25.0A | 22.1A | 23.2A | 24.7A | 23.5 | 0.03* | 19.6 |
| 400 | 22.5A | 23.0A | 21.3B | 22.0A | 23.6A | 24.2A | 22.7 | 0.01* | 31.1 |
| 500 | 23.0A | 23.2A | 21.5B | 21.7A | 23.5A | 24.1A | 22.8 | $0.11^{ns}$ | - |
| 600 | 23.2A | 23.1A | 22.0B | 22.2A | 24.5A | 24.5A | 23.2 | 0.01* | 30.0 |
| Mean | 22.4 | 22.6 | 22.9 | 23.2 | 23.5 | 23.8 | | | |
| **pH** | | | | | | | | | |
| *In natura* | 5.97B | 6.16A | 6.19A | 6.17A | 6.96A | 6.95A | 6.40 | < 0.01* | 79.3 |
| 400 | 5.91B | 5.98A | 6.24A | 6.06A | 6.20A | 6.30B | 6.11 | < 0.01* | 70.2 |
| 500 | 6.00B | 6.28A | 6.23A | 6.15A | 6.42A | 6.51B | 6.26 | < 0.01* | 70.4 |
| 600 | 6.36A | 6.23A | 6.42A | 6.23A | 6.52A | 6.48B | 6.37 | $0.12^{ns}$ | - |
| Mean | 6.04 | 6.1 | 4 | 6.34 | 6.44 | 6.54 | | | |
| **N-$NH_3$ (%)** | | | | | | | | | |
| *In natura* | 4.65A | 1.60A | 2.25A | 1.60A | 1.10A | 0.20A | 1.90 | < 0.01* | 74.6 |
| 400 | 2.40AB | 2.25A | 1.60A | 1.50A | 0.90A | 0.70A | 1.55 | < 0.01* | 96.7 |
| 500 | 3.35B | 1.40A | 1.90A | 2.25A | 1.00A | 0.65A | 1.75 | < 0.01* | 62.2 |
| 600 | 1.85C | 1.15A | 1.50A | 1.55A | 0.90A | 0.35A | 1.21 | < 0.01* | 66.3 |
| Mean | 2.67 | 2.24 | 1.82 | 1.39 | 0.96 | 0.54 | | | |

| Analysis of variance | -p-value | | | |
|---|---|---|---|---|
| | DM Content | Hours | DM Content × Hours | SEM |
| Surface temperature of the haylage | < 0.01 | < 0.01 | < 0.01 | 0.06 |
| Internal temperature of the haylage | 0.06 | < 0.01 | 0.03 | 0.82 |
| pH | < 0.01 | < 0.01 | < 0.01 | 0.03 |
| $NH_3$ | < 0.01 | < 0.01 | < 0.01 | 0.12 |

Means followed by different letters in a column indicate statistical differences according to Tukey's test at $p < 0.05$. * Significant at $P < 0.05$. $^{ns}$ not significant at $p > 0.05$. x: linear effect; SEM: Mean Standard error.

Medias seguidas de letras diferentes en la columna son estadísticamente diferentes según la prueba de Tukey con p < 0,05. * significativo a p < 0,05. $^{ns}$no significativo a pb> 0,05. x: efecto lineal; SEM: Error estándar de la media.

The *in natura*, 400 g $kg^{-1}$ DM, and 500 g $kg^{-1}$ DM treatments had an increasing linear effect ($p < 0.01$) on the pH of haylage during air exposure. In all treatments, the highest pH values were recorded after 120 hours of exposure of the haylage to air, and these values were 6.95 ± 0.03, 6.30 ± 0.03, 6.51 ± 0.03, and 6.48 ± 0.03 for the *in natura*, 400 g $kg^{-1}$ DM, 500 g $kg^{-1}$ DM, and 600 g $kg^{-1}$ DM treatments, respectively. The 600 g $kg^{-1}$ DM treatment showed the highest pH value of 6.36 ± 0.03 at hour 0, while the *in natura* treatment showed the highest pH value of 6.95 ± 0.03 after 120 hours of exposure to air.

There was a decreasing linear effect ($p < 0.01$) of the hours of exposure to air on the $N\text{-}NH_3$ of Tanzania grass haylage in all treatments at the time of baling. The *in natura* treatment showed the highest $N\text{-}NH_3$ value at hour 0 of exposure to air (4.65 ± 0.12%).

## Discussion

### Chemical composition and gases

DM increased as the dehydration of the Tanzania grass continued in the field; thus, the higher DM content of the haylage obtained in the 600 g $kg^{-1}$ DM treatment, as compared to that obtained after the other treatments, was due to the grass being dehydrated to a greater extent during this treatment before it was baled (table 2, page 43). The higher DM content in the stored material optimizes the fermentation of the forage, shaping its preservation as haylage. Nath *et al.* (2018) obtained DM values for Tifton 85 grass haylage with different additives and storage times, with an average of 531.10 g/kg. Haylage is a technique that can be used for the storage of grasses because dehydration reduces the probability of secondary fermentation, which causes DM loss (31, 44, 58).

The DM values obtained for each treatment through dehydration, both before and after the production of haylage, were nearly adequate according to each treatment (400, 500, and 600 g $kg^{-1}$ DM), demonstrating that the method used to determine the DM content through a microwave is a viable alternative to quickly obtain the DM value of forage plants on the farm (55) and can be used to determine the plant DM for haylage production. The plant DM at harvest directly influences haylage fermentation (26, 40).

The haylage in the 600 g $kg^{-1}$ DM treatment group had the highest crude protein (CP) content; however, all haylage groups had a CP content greater than 70 g $kg^{-1}$ DM, which is suggested by Van Soest (1994) as the ideal amount for the growth of rumen microorganisms. The high CP content in the haylage was due to the high CP content of the Tanzania grass before storage (table 1, page 41). Castro *et al.* (2010) evaluated the chemical composition of Tanzania grass at day 42 of storage and obtained a CP content of 97.7 g/kg DM.

The results also indicated that the storage of haylage preserved the CP content at levels suitable for animal feeding. The high CP content indicates that when haylage is stored with adequate amounts of DM, it produces conditions suitable for the growth of LAB (53) and inhibits the growth of undesirable microorganisms (44) that deteriorate CP.

The content of neutral detergent fiber (NDF) was higher than the maximum limit of 550 g/kg DM recommended for good digestibility of the mass, which occurs in silages with NDF levels as described previously (58). High NDF content may be related to the loss of cellular content during the fermentation period (31), which negatively influences feed intake due to rumen filling (8). The NDF content obtained in the haylage of the 500 g $kg^{-1}$ DM treatment group was lower than that found by (6) in haylage of Tifton 85 grass, which was 723.6 g/kg DM, indicating the high quality of Tanzania grass when harvested before flowering. Since NDF constitutes the cell wall of plants (58), having haylages with NDF content similar to that of the original plant suggests the adequate preservation of nutrients (table 2, page 43).

Lower contents of mineral matter (MM) were observed in the Tanzania grass haylages in all treatments (table 1, page 41) than in the material before storage (table 2, page 43). Low MM content is an indicator of better forage conservation because when inadequate fermentation occurs, the loss of organic material increases the amount of MM in the DM. The values obtained for MM in this study were lower than those found by AOAC (1990) in the haylage of Tifton 85 grass containing a bacterial inoculant.

The higher DM content treatments (500 and 600 g $kg^{-1}$ DM) also yielded higher CHO content, which is an important substrate for the fermentation and conservation of forage in the form of haylage. The increase in dehydration of Tanzania grass increased its CHO content, indicating that in treatments with higher moisture, there was a greater use of CHO by microorganisms responsible for driving fermentation (50). In a previous study (14), it was observed that the haylages of two cultivars of perennial ryegrass (*i.e.*, AberDart and Fennema) fermented better when the DM had a higher concentration of CHO.

Most tropical forage grasses do not have adequate levels of DM, CHO, or buffering capacity to allow for fermentation to occur efficiently, resulting in losses due to secondary fermentation, effluent production, and aerobic deterioration, which are obstacles in the conservation of tropical grasses (10). Thus, the high levels of CHO in the haylage after 90 days of storage, as obtained in the 500 and 600 g $kg^{-1}$ DM treatments, showed that fermentation was well controlled, resulting in good-quality forage for animal feeding.

A high amount of oxygen was observed at the time of storage (0 days), which was subsequently reduced after the 7$^{th}$ day of storage. The presence of oxygen during storage favors the growth of microorganisms that release energy in the form of heat, and fermentation by this microbial mass results in the degradation of the roughage. Therefore, oxygen must be eliminated before fermentation; in its absence, there is a decrease in fungal and yeast growth, as anaerobic conditions are not optimal for the growth of these organisms (20).

The increase in $CO_2$ after the first days of storage was due to it being released by aerobic microorganisms inside the bales during fermentation. According to Paula *et al.* (2016), the respiration of aerobic microorganisms occurs in the aerobic phase. These microorganisms use some of the desirable substrates for energy production, causing DM consumption and $CO_2$ production, which can be considered as one of the main factors that influence the quality of haylage.

The low levels of oxygen observed in the 500 and 600 g $kg^{-1}$ DM treatments with 90 d of storage were indicative of higher levels of anaerobic fermentation inside the haylage bales, especially if it was associated with high amounts of $CO_2$, as was observed in the haylage with 600 g $kg^{-1}$ DM. Low amounts of oxygen and high amounts of $CO_2$ are desirable parameters that guarantee adequate anaerobic fermentation and yield products of good nutritional quality. The activity of certain microorganisms can be controlled using a controlled atmosphere or packaging in a modified atmosphere (52). According to Müller (2005), the greater the number of wrapping layers in the haylage bales, the greater the $CO_2$ concentration. According to Mantilla *et al.* (2010), the increase in food conservation time was due to the inhibitory effect of carbon dioxide ($CO_2$) on different microbial types and the reduction or removal of oxygen ($O_2$) from inside the bale.

It was observed that during storage, the low $CO_2$ concentration increased from the 7$^{th}$ day, and this occurred because the aerobic microorganisms and optional aerobes began to consume the available CHO, increasing the production of gases through respiration and fermentation (carbon dioxide and ethanol). After the 30$^{th}$ day, it was observed that the microbial activity stabilized, decreasing respiration, and consequently, the production of gas, as previously noted (60).

The internal temperature of the haylages increased during the first few days of storage (0, 7, and 15 days), whereas the amount of $O_2$ decreased and that of $CO_2$ increased in this time period. According to Mcdonald *et al.* (1991), in the first few days of storage until the end of the aerobic phase, it is common to observe heating of the material, which can last from 48 to 144 h.

**Volatile fatty acids and microorganisms**

A greater amount of acetic acid was observed in the 600 g $kg^{-1}$ DM treatment as compared to the other treatments, indicating that the lower moisture content caused an increase in the activity of acetic acid-producing microorganisms in the haylage of Tanzania grass. The presence of acetic acid is indicative of the action of heterofermentative LAB and enterobacteria. High levels of this acid promote greater aerobic stability of haylage after prolonged storage because it can inhibit yeast growth (1). Low concentrations of strong acids in haylage do not imply poor fermentation (38), which may be due to the high DM content of the material.

The higher concentration of butyric acid in the *in natura* treatment as compared to the other treatments is due to the higher moisture content of the plant, which favors the growth of bacteria of the genus *Clostridium* (49). No difference was observed for the other acids, and this indicates that the DM content greatly influences the production of haylage within the context of Tanzania, and its presence is shaped by the action of heterofermentative LAB, enterobacteria, and clostridia.

LAB concentrations increased according to increasing DM content of the plants used for haylage production (figure 1, page 45). The larger population of homofermentative LAB tends to reduce pH more quickly, reducing the action of undesirable microorganisms and preserving a greater amount of carbohydrates, with the increase in the DM content (36); this phenomenon was not observed in this experiment that obtained high pH values. The haylages produced had LAB populations greater than the minimum limit of 5 log CFU $g^{-1}$ recommended by Pahlow (1986) and Muck *et al.* (1991) and t required for a good fermentation process.

The increased presence of yeast populations is concerning because of their potential to rapidly multiply, but no difference was observed in the yeast population with respect to the different DM contents of the plant used for haylage-making. Notably, several types of yeasts may predominate during the haylage-making process, and the yeast species present are not necessarily aerobic. This may explain the absence of a difference in the counts of these microorganisms between treatments. Low yeast populations are desirable for preserving the material during fermentation and after bales opening (45). The yeast count of the Tanzania grass haylages was higher than that found by Müller and Johansen (2020) in reallocated haylage (5.31 log CFU $g^{-1}$) and that observed by Müller *et al.* (2011) in the haylages of horse farms (4.57 log CFU $g^{-1}$).

A lower count of molds was observed in the haylage of the 500 and 600 g $kg^{-1}$ DM treatments, which is related to the plant DM during storage. Generally, the population of microorganisms is strongly affected by the moisture content and temperature recorded during storage (37). The presence of fungi causes a reduction in nutritional value and palatability due to the associated protein degradation (16). Some mycotoxin-producing molds were observed in the haylages, but they only occurred at low concentrations; this was in line with the results of a study by Müller *et al.* (2011). The presence of such molds can be reduced through the use of additives (38).

The 600 g $kg^{-1}$ DM treatment yielded the highest amount of enterobacteria in the haylage of Tanzania grass. The large number and prevalence of these microorganisms are undesirable, as they cause protein degradation by performing secondary fermentation and producing compounds such as acetic and butyric acids, impairing conservation (51). However, these bacteria produce acetic acid, which, in the absence of lactic acid, can help conserve the material and increase its aerobic stability. Enterobacteria compete for water-soluble carbohydrates with LAB, and the component with the highest concentration at the end of this process is acetic acid, which has a positive effect on aerobic stability. Haylage with poor aerobic stability has high levels of residual sugar and lactic acid (14).

**Aerobic stability, *pH,* and ammonia nitrogen**

The temperature increase observed in Tanzania grass haylages with different DM levels throughout exposure to air was not sufficient to negatively impact aerobic stability across the evaluation period of 120 h. The aerobic stability of the haylage, regardless of treatment, was likely maintained because of the high acetic acid concentration in the 600 g $kg^{-1}$ DM treatment and the low amount of CHO in the other treatments. These characteristics inhibit the growth of deteriorating microorganisms (16). Müller (2009) did not observe a change in the aerobic stability of the haylage of plants harvested at different times.

Haylage treated with 600 g/kg DM had the highest surface temperature, probably because of the high amounts of CHO. Better haylage fermentation patterns with higher DM content provide a greater number of available substrates for the consumption of microorganisms in the aerobic phase (61).

The variation in the internal temperature of the Tanzania grass haylage was not enough to overcome the room temperature at 2°C in all treatments and times. Neres *et al.* (2013) assessed the aerobic stability of Tifton 85 grass silage and observed that the room temperature was lower than the ensiled mass temperature during the seven days of aeration, which contributed to good preservation of the roughage and inhibition of the growth of undesirable microorganisms. The aerobic stability of haylage can also be influenced by the production of acetic acid, which varies according to pH and temperature increases in the respective pre-dried forage masses (56).

Haylage stored with 600 g $kg^{-1}$ DM showed no difference in pH during exposure to air. This occurred because of the lower moisture content, which provided greater resistance to the pH drop because of the lower activity of microorganisms. Belém *et al.* (2016) reported that the limited activity of bacteria owing to moisture has a direct effect on aerobic fermentation.

The pH values observed in the Tanzania grass haylages were higher than those observed by Coblentz *et al.* (2016) in the alfalfa haylage (5.1), which was almost similar to those observed by Nath *et al.* (2018) in the haylage of Tifton 85 grass (5.72) and lower than those obtained by Weirich *et al.* (2018) in the Tifton 85 haylage (7.38). The high pH values observed in the haylage of tropical grasses may be due to the low concentration of organic acids in the masses of these species (3). Müller *et al.* (2007) compared silage to haylage and observed that haylage had a higher pH owing to lower concentrations of fermentative products.

The $N\text{-}NH_3$ content of Tanzania grass haylage decreased as its exposure to air progressed. As the times of aerobic exposure advanced, the Tanzania grass haylages showed a reduction in the average levels of ammoniacal nitrogen, probably due to evaporative processes and a decrease in the enterobacteria population (48).

The *in natura* treatment produced the highest amount of $N\text{-}NH_3$, indicating the high intensity of proteolysis during the fermentation process. However, it is important to note that all haylages were classified as those of good quality. Monteiro *et al.* (2011) classified haylages as good quality haylages when the fermented materials had levels of $N\text{-}NH_3$ below 12%. This was also indicative of low proteolysis intensity during fermentation (57).

## Conclusions

Higher plant DM yields Tanzania grass haylage of high quality. Tanzania grass with 500 and 600 g $kg^{-1}$ DM for haylage production had a high content of CHO, a better concentration of gases, and a greater amount of volatile fatty acids and beneficial microorganisms that facilitate preservation. Additionally, these haylages showed sustained aerobic stabilities.

It is necessary to conduct further studies on plant DM using other tropical grasses to produce high-quality haylage.

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# Indicators of restoration strategies in land uses: metallic and non-metallic elements

## Indicadores de estrategias de restauración en usos de suelo: elementos metálicos y no metálicos

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**Abstract**

Land management practices can have an impact on the environmental quality of soil and contribute to identifying the source of its pollution. The objective of this study was to determine presence of metallic and non-metallic elements as indicators of land use impact (livestock management, restoration strategies and without management practices) in the Monte Caldera communal lands located in Cerro de San Pedro, San Luis Potosi, Mexico. Eighteen samples were collected at depths of 0-10 cm and 10-20 cm for each land use. Total concentrations of Zr, Sr, U, Th, Pb, As, Rb, Cr, V, Ti, Zn, and Cu were determined by X-ray fluorescence. Mean concentrations ranged in the following order: Ti>Zr>Rb>V>Sr>Zn>Cr>Pb>Cu>Th>U>As, with concentrations for Ti, Cr, Th, U and As exceeding technical reference values for phytotoxic soils. Significant differences were evidenced by ANOVA between land use (Th, Pb, Rb, Cu) and soil depth (U, Pb, and As). Land use practices associated with restoration resulted in a positive environmental impact. These findings underscore the need to conduct follow-up studies in the area and further examine the relationship of such practices with other environmental factors.

**Keywords**
soil organic matter • soil depth • land use • phytotoxic

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**Resumen**

Las prácticas de manejo del suelo pueden impactar la calidad ambiental de este recurso y pueden ayudar a diagnosticar la fuente de su contaminación. El objetivo de este estudio fue determinar la presencia de elementos metálicos y no metálicos como indicadores de impacto en el uso del suelo (manejo ganadero, estrategias de restauración y prácticas sin manejo de suelo) en el Ejido Monte Caldera (Cerro de San Pedro) San Luis Potosí, México. En cada uso de suelo se recolectaron 18 muestras por uso de suelo entre 0-10 cm y 10-20 cm de profundidad. Aplicando la Técnica de Rayos X de Fluorescencia se determinaron las concentraciones totales de Zr, Sr, U, Th, Pb, As, Rb, Cr, V, Ti, Zn y Cu. Las concentraciones medias obtenidas fluctuaron a razón de Ti>Zr>Rb>V>Sr>Zn>Cr>Pb>Cu>Th>U>As. En Ti, Cr, Th, U y As se superaron las referencias técnicas de concentraciones en suelo consideradas a nivel fitotóxico. La prueba de ANOVA reveló una diferencia significativa entre uso de suelo (Th, Pb, Rb, Cu) y profundidad (U, Pb y As). El uso de suelo con prácticas asociadas a la restauración indica impacto ambiental positivo. Se identifica la necesidad de realizar estudios de seguimiento en la zona y su asociación con otros factores ambientales.

**Palabras clave**

materia orgánica del suelo • profundidad del suelo • uso de suelo • fitotóxico

## Introduction

By assessing the presence of pollutants, land management strategies can reflect soil environmental quality. Unsustainable land management has led to critical levels of physical, chemical, biological, and ecological degradation of soil, at times reducing or eliminating the quality and primary functions of this resource (24, 32). Pollution stands out among the forms of chemical degradation, considering the concentration of heavy metals in soil as an indicator of its chemical conditions (11, 24, 38). The term "heavy metal" refers to a range of metals and metalloids with an atomic density greater than 4 g $cm^{-3}$; some with concentrations ranging from 0.1 to 18 mg $kg^{-1}$ (7, 25, 30). Over 20 million hectares of soil worldwide are polluted with metallic and non-metallic elements, most notably As, Cd, Cr, Hg, Pb, Co, Cu, Ni, Zn, and Se (9, 33). X-ray fluorescence (XRF) was among the approaches and devices used in this study to facilitate determinations. XRF spectrometry is an analytical method for determining the elemental composition of various materials. In soils, portable X-ray fluorescence (pXRF) spectrometry provides a wide range of pedagogical, environmental, and agronomic applications. Specifically, soil characterization by pXRF involves a comprehensive determination of its elemental composition (nutrients, trace elements, and rare-earth elements). XRF provides a rapid, cost-effective, and residue-free assessment of soil properties that allows for assessment of a more significant number of samples and a more in-depth characterization for different purposes (22, 33, 36). A number of studies in the literature have reported on the determination of Ti, V, Cr, Mn, Ni, Cu, Zn, Zr, Sr, Rb, Pb, and Th contents using this technique (33, 35, 36, 41, 42).

In Mexico, the toxicity and abundance of some of these elements, primarily due to increasing mining activities, have turned heavy metal pollution into a growing concern, particularly in the states of Zacatecas, Queretaro, Hidalgo, and San Luis Potosi (15). Sierra de Alvarez is critical to San Luis Potosi due to its diverse climate and vast variety of vegetation in its temperate, arid, mountainous, and sub-humid landscapes. Predominant soil types include Eutric Lithosol, Haplic Luvisol, Rendzina, and Luvic Phaeozem. The intrinsic effects of agriculture, livestock production, and mining activities practiced (20) there have had a dramatic impact on the state of the environment and are reflected in the decline in ecosystem services (29). Given the human influence on land use, this area has been of interest for soil restoration plans since 2004 under the Mexican Environmental Compensation Program (CONAFOR) (14). Our study was conducted in the communal lands of Monte Caldera, within the municipality of Cerro de San Pedro, San Luis Potosi, to assess presence of metallic and non-metallic elements as indicators of the environmental impact of land uses and evaluate remediation strategies for polluted soil. Approaches to the restoration of soil quality include conservation agriculture, integrated nutrients, continuous management of vegetation cover, cover crops, diversified land use, controlled grazing, and optimal seeding rates, among others (24). To assess the impact on soil quality and determine the degree of restoration in the area under study, we examined the characteristics of land use, *i.e.* livestock production, restoration practices (sites with mechanical practices, specifically the formation of terraced reforestation with *Pinus greggii*, and livestock exclusion), and overgrazed land without management or conservation practices.

## Materials and methods

The research was conducted in the communal land of Monte Caldera, which is part of the Cerro de San Pedro municipality in the state of San Luis Potosi, Mexico. Monte Caldera is located at 100°44'4" west longitude; 22°12'31" north latitude; at 2080 meters altitude (17, 21).

### Biotic Characterization

The predominant climate in Monte Caldera is temperate semi-arid. Average annual temperature in the study area is 16.8 °C with an annual rainfall of 304 mm. The municipality of Cerro de San Pedro is located in El Salado hydrological region (HR 37), which essentially consists of a series of small endorheic basins (Cuenca San Jose, Los Pilares, and others). The region is bordered by a massif of sedimentary rocks with primarily pine-oak vegetation, induced pasture, and grassland use in the upper part (21). The soil types present are Lithosol, Chromic Luvisol, Calcareous Regosol, and Mollic Planosol (26).

### Site Selection

The assessment was conducted consecutively in July 2018 with a completely random sampling of three land uses: 1) Land use with managed livestock production; 2) Land use with restoration practices, *i.e.*, land implementing soil rehabilitation measures established in 2004 under CONAFOR, with reforestation with *Pinus greggii* and fencing (14), and 3) Land without livestock management or conservation activities, corresponding to continuous grazing. Each land use was georeferenced in the field with a global positioning system, considering a total area of 1,124 ha (figure 1).

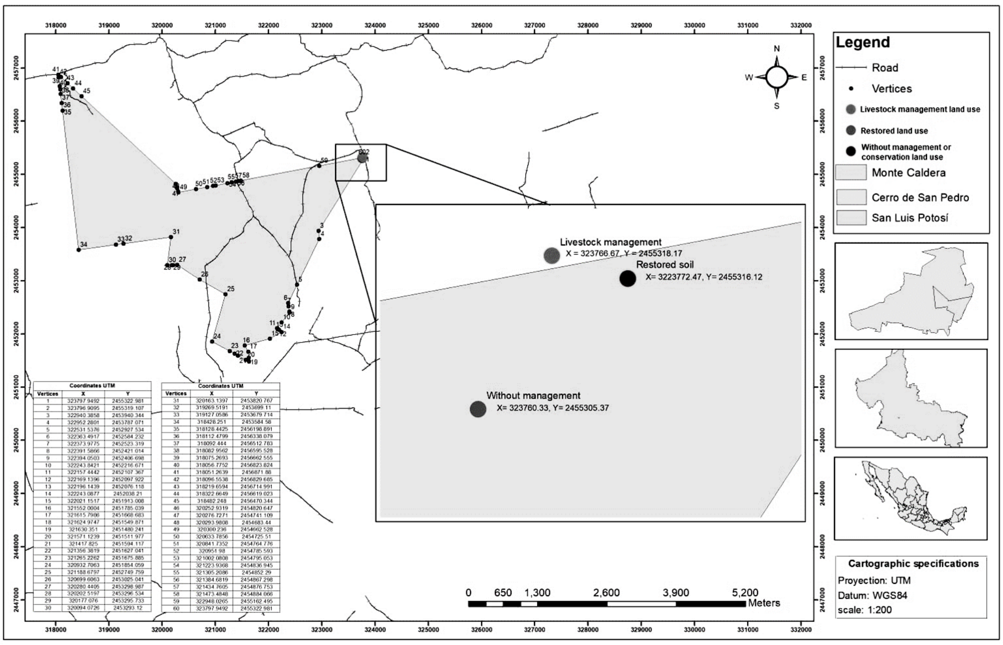



**Figure 1.** Study area.

**Figura 1.** Área de estudio.

**Soil physicochemical characteristics**

Six sampling points were selected for all three land-use practices studied, 15 to 20 meters apart from one another and completely randomly collected. Six samples and three replicates were collected for each land use at each sampling point using two depth criteria: 0-10 cm and 10-20 cm, reaching 36 samples in total. Soil properties were analyzed using the techniques established in the Mexican Official Standard NOM-021-SEMARNAT-2000 (13, 18). Analyses of pH, texture, soil organic matter (SOM), electrical conductivity (EC), and cation exchange capacity (CEC) were performed in triplicate for each sampling site. Total concentrations of Zr, Sr, U, Th, Pb, As, Rb, Cr, V, Ti, Zn, and Cu were calculated using X-ray fluorescence with a Thermo Scientific Niton TM FXL instrument (Thermo Fisher Scientific, Waltham, MA., USA). The results for each sample were based on the average of three replicates with a 60-second analysis time. An internal calibration of the instrument was performed before analyzing the samples, using standard reference soil material from the National Institute of Standards and Technology (NIST). The procedure was performed in accordance with manufacturer's instructions and recommendations of the U.S. Environmental Protection Agency (EPA) SW-846 Test Method 6200 (39, 40, 41).

**Statistical analysis**

Minitab 16 Statistical Software (Minitab Inc., State College, PA., USA) was used for data analysis. A model was designed considering the effect of land use factors and soil depth on 12 metallic and non-metallic elements, determining the SOM, EC, and CEC soil indicators with Pearson's correlation coefficient, ANOVA (Tukey's test, $p \le 0.05$) and a Principal Component Analysis (PCA) for mean comparisons. To minimize error, readings of the soil samples were made in triplicate.

## Results and discussion

The average SOM behavior of all samples assessed was 4.14%, EC was 0.33 mS $cm^{-1}$, and CEC was 15.86 Cmol $kg^{-1}$. The pH ranged from 6.5 to 7. The soil textures found ranged from loam (Livestock management) to silt and clay loam (Restored soil), and to loam and clay loam (Land without management or conservation). Overall mean values for the 12 elements were as follows: Ti (3,839.1 mg $kg^{-1}$) > Zr (518.2 mg $kg^{-1}$) > Rb (111.19 mg $kg^{-1}$) > V (79.83 mg $kg^{-1}$) > Sr (72.33 mg $kg^{-1}$) > Zn (60.84 mg $kg^{-1}$) > Cr (42.60 mg $kg^{-1}$) > Pb (19.64 mg $kg^{-1}$) Cu > (17.13 mg $kg^{-1}$) > Th (11.51 mg $kg^{-1}$) > U (6.94 mg $kg^{-1}$) > As (6.13 mg $kg^{-1}$). Table 1 (page 57) illustrates the mean soil concentrations of these elements. Cr, U, and Th values exceeding the technical reference values for concentrations considered phytotoxic are marked as relevant (1, 19, 31). Several works in the literature have reported varying concentrations of U and Th in soils in countries such as the United States, Canada, Germany, Jamaica, Cuba, India, and Egypt (17). Considering the values presented in table 1 (page 57), it is worth noting that the Th contents measured in our study were within the means reported for soils in the United States (2.2-21 mg $kg^{-1}$), Canada (4.2-14.1 mg $kg^{-1}$), Germany (0.4-15 mg $kg^{-1}$), Jamaica (0.9-25 mg $kg^{-1}$), and Cuba (5-12.3 mg $kg^{-1}$). The mean values for U in soil obtained in this study were consistent with those obtained for soil from the United States (0.3-10.7 mg $kg^{-1}$), Germany (0.42-11 mg $kg^{-1}$), and Jamaica (0.7-14 mg $kg^{-1}$). The presence of any element at higher or lower concentrations in a given soil is strictly contingent on its mineral composition, nature of the original sediments, and depositional environment (18). There were 113 interactions, 20 of which were significant according to Pearson's correlation coefficient ($p \le 0.05$, table 2, page 57).

**Table 1.** Mean concentrations of metallic and non-metallic elements in soils and reference values (n=36).

**Tabla 1.** Relación de concentraciones medias de elementos metálicos y no metálicos en suelos y niveles de referencia (n=36).

Note: Mean metal concentrations refer to the total samples from the different land uses evaluated. NA=Data is not available in the technical reference. * Mean soil concentration of Th is 6 mg $kg^{-1}$ (1).

Nota. Los niveles de concentraciones medias de metales refieren el total de muestras de los diferentes usos de suelo evaluados. NA. Dato no disponible en la referencia técnica. La concentración media de Th en suelo es de 6 mg $kg^{-1}$(1).

| | **Mean concentration in samples (n=36)** | **USEPA (19)** | **NOM-147-SEMARNAT-SSA/1996 (17) (agricultural use)** | **The handbook of trace elements (31)** |
|---|---|---|---|---|
| **Element** | **mg $kg^{-1}$** | **mg $kg^{-1}$** | **mg $kg^{-1}$** | **mg $kg^{-1}$** |
| Ti | **3839,1** | NA | NA | 1800-3600 |
| Zr | 518,2 | NA | NA | 30-2000 |
| Rb | 111,19 | NA | NA | 50-120 |
| V | 79,19 | NA | 78 | 3-230 |
| Sr | 72,83 | NA | NA | 50-1000 |
| Zn | 60,84 | 200-400 | NA | 10-300 |
| Cr | **42,6** | 2-10 | NA | 5-1000 |
| Pb | 19,64 | 50-100 | 400 | 3-189 |
| Cu | 17,13 | 20-100 | NA | 2-100 |
| Th | **11.51*** | NA | NA | NA |
| U | **6,94** | NA | NA | 0.79-3.70 |
| As | **6,13** | 100-1000 | 22 | 0.1-48 |

**Table 2.** Relationship between metallic and non-metallic elements and soil physicochemical characteristics.

**Tabla 2.** Relación entre elementos metálicos y no metálicos y propiedades fisicoquímicas del suelo.

* Indicates statistically significant relationships (p≤0.05).

Note: Values with ** refer to highly significant r values, those with * to significant r values.

* Indica relaciones estadísticamente significativas (p≤0,05).

Nota: Valores con ** se refieren a r altamente significativa, valores con * se refiere a r significativa.

| **Elements** | **r value** | **Elements** | **r value** |
|---|---|---|---|
| Sr-Zr | -0.463** | Ti-Sr | -0.439** |
| Th-Sr | -0.327* | Ti-Pb | -0.328* |
| Pb-Sr | 0.351* | Ti-Cr | -0.335* |
| Pb-U | -0.330* | Ti-V | 0,303 |
| As-Pb | 0.591** | Zn-Zr | -0.554** |
| Rb-Sr | -0.402* | Zn-Sr | 0.344* |
| Rb-Th | 0.450** | Zn-Pb | 0.696** |
| Cr-Th | 0.347* | Zn-As | 0.419** |
| Ti-Zr | 0.780** | Zn-Ti | -0.593** |
| Ti-Sr | -0.439** | Cu-Pb | 0.447** |
| Ti-Pb | -0.328* | Cu-Cr | 0.526** |
| Ti-Cr | -0.335* | Cu-Zn | 0.431** |
| Ti-V | 0.303 | SOM-Ti | -0.378* |
| Ti-Zr | 0.780** | SOM-Zn | 0.413* |

Clay, calcium carbonate, SOM, and pH are among the constituents or properties that contribute to the presence of these elements in soil (39). Heavy metals exist in most soils as carbonates, sulfides, oxides, or salts, and their concentrations may vary from soil to soil (35). In our study, however, significant correlations (p≤0.05) with positive or negative signs were observed for the SOM, EC, and CEC soil parameters, with the highest values being those recorded between SOM-Zn (r=0.41) and SOM-Ti (r=-0.37). In turn, pH and organic carbon had the most significant influence on the solubility of metals (34). In other studies, soil chemical properties such as pH and EC have been found to vary depending on practices such as forest use and conservation. Their effect can be explained by an increase in soil cover, which decreases with the intensity of agricultural use (10). In addition, positive relationships have been observed among elements such as Fe, Cr, Mn, Ni, As, Cu, and Zn, As, and Cr and between these elements and soil constituents or properties such as clay, calcium carbonate, organic matter and pH (39).

Tukey's test ($p \leq 0.05$) showed significant differences in Th, Pb, Rb, and Cu among the means for the different land uses analyzed by our study (figure 2). The samples corresponding to livestock management had the most significant mean values for Th (12.97 mg $kg^{-1}$), Pb (22.0 mg $kg^{-1}$), Rb (119.12 mg $kg^{-1}$), and Cu (19.21 mg $kg^{-1}$). In like manner, SOM concentrations were significant at a rate of 4.74% in restored soil, 3.97% in livestock management, and 3.31% for areas without management or conservation. Specifically, SOM (humic and fulvic acids) in soil is a component with a high sorption capacity for heavy metals, impacting their immobilization (23, 28).

Different letters indicate significant differences.

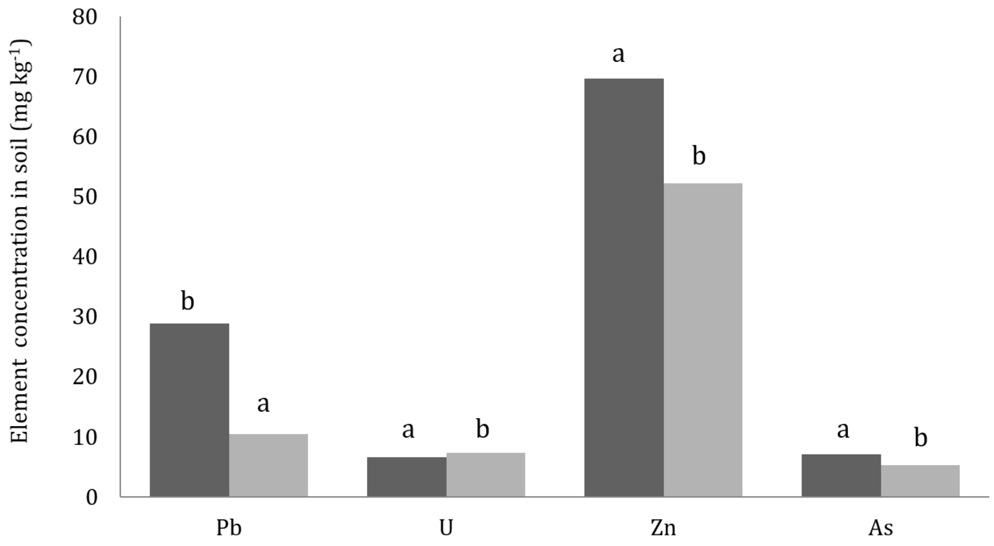
Medias que no comparten una letra son significativamente diferentes.


**Figure 2.** Mean values among significant element concentrations in relation to land use ($p \leq 0.05$, n=36).

**Figura 2.** Medias de los elementos con concentraciones significativas según usos de suelo (p≤0,05, n=36).

A difference of 4 mg $kg^{-1}$ in copper (Cu) content was observed between land use with livestock management and land use with restored soils. Cu is a metal that occurs naturally in rocks, soil, water, and air. Agricultural activities and wastewater discharge into rivers and lakes also contribute to its release (3). In the Handbook of Trace Elements, Pais and Jones (1997) indicate that the total content of Cu in soil ranges from 2 to 100 mg $kg^{-1}$; Cu content exceeding phytotoxic levels ranges from 20 to 100 mg $kg^{-1}$ (14, 19). The Canadian Soil Quality Guidelines (CSQG) for the Protection of Environmental and Human Health assign a soil quality guideline of 63 mg $kg^{-1}$ as necessary to protect the environment (12). As for thorium (Th), small amounts of this element occur naturally in the environment in rocks, soil, water, plants and animals; soil contains an average of 6 mg $kg^{-1}$ (1). Lead (Pb) levels detected by our analysis were below the limit for agricultural use, which is 400 mg $kg^{-1}$ according to the Mexican Official Standard NOM-147-SEMARNAT/SSA1-2004 (2007). Its total concentration in the soil ranges from 3 to 189 mg $kg^{-1}$ (31); therefore, the value found for this element does not exceed technical reference values for concentrations considered phytotoxic, which are 50 to 100 mg $kg^{-1}$. In terms of Rubidium (Rb), the Handbook of Trace Elements reports that the total content of this element in soil ranges between 50 and 120 mg $kg^{-1}$ (31). Finally, it should be noted that some metals such as Cu, Pb, Zn, and As are absorbed by grassland. It has been found that cattle can involuntarily ingest from 1% to nearly 18% of soil within dry matter, which can vary depending on season and farm management. Heavy metals may be found in their feces, contributing to their spread (37).

A significant effect of the soil depth factor was found in relation to U, Pb, As, and Zn concentrations (figure 3). Pb, As, and Zn values were notably higher at a depth of 0-10 cm, and only U values were higher at a depth of 10-20 cm. According to the Handbook of Trace Elements, the total amount of uranium in the soil ranges from 0.10 to 11.2 mg $kg^{-1}$ (30). Uranium is released into the soil when rocks are eroded by wind or water (2, 6). As an element widely distributed in soils around the world, total soil concentrations of Arsenic (As) range from 0.1 to 48 mg $kg^{-1}$ (5, 13, 27). The concentration found in this study is below the value of 12 mg $kg^{-1}$ established by the CSQG, therefore it does not affect soil quality (12). The total concentration of Zinc (Zn) in soil ranges from 10 to 300 mg $kg^{-1}$. Natural processes release a small amount of Zn, whereas human activities such as mining and steel production account for its total amount (4). Significant concentrations of Zn were observed in our study, ranging from 52.13 to 69.54 mg $kg^{-1}$, and were higher in the soil samples collected at 10 cm depth, with a difference of 17.41 mg $kg^{-1}$ between the two studied depths. According to EPA guidelines (1992), these levels of Zn do not exceed reference values for concentrations considered phytotoxic.




Different letters indicate significant differences.

Medias que no comparten una letra son significativamente diferentes.

**Figure 3.** Mean values among significant element concentrations in relation to land use ($p \leq 0.05$, n=36).

**Figura 3.** Medias de los elementos con concentraciones significativas según usos de suelo ($p \leq 0,05$, n=36).

It is essential to relate the presence of SOM to these results, since concentrations obtained considering the depth factor were significant. Compared to the value established in NOM-021-SEMARNAT-2000 (2002), SOM concentrations were high, ranging from 3.6 to 6.0%, for all land uses and soil depths applied in this study. Soil samples had an average of 4.5% SOM content at 0-10 cm, and 3.73% at 10-20 cm, representing a difference of 0.82% between both soil depths. In terms of land use, SOM concentration was 4.74% in restored land, representing the highest value, 1.03% more than that for managed livestock production activities, which was 3.71%. A decrease in SOM with agricultural and livestock production practices may be due to changes in stocking capacity, removal of crop residues, and more rapid decomposition, oxidation, and soil erosion processes. In contrast, an increase in organic matter has been observed in areas where soil conservation practices are developed (10). Organic carbon and CEC are the two main factors that suppress the effect of toxicity on soil respiration in heavy metal-polluted soils, with Zn, Cu, and Pb being the most prominent elements (33). Humic and fulvic acids derived from organic matter are beneficial because of their high absorption capacity for various contaminants, including heavy metals, which can result in their immobilization (22).

According to the guidelines set by NOM-147-SEMARNAT/SSA1-2004 (2007), the Pb and As contents we found do not exceed the limits for agricultural use, which are 400 mg $kg^{-1}$ and 22 mg $kg^{-1}$, respectively. The presence of these elements may be influenced by the origin and composition of soil, as well as by the impact of human activities such as livestock production and agricultural uses and external environmental factors.

The highest and lowest concentrations considering land use (for Th, Pb, Rb, and Cu) and soil depth (for Pb, U, Zn, and As) may be related to the natural composition and mineral structure of soil and human activities. In this study, agricultural and livestock production activities affected metal concentrations; however, except for Pb concentrations, our findings were influenced by both land use and soil depth. In soils where restoration and reforestation activities occurred, in addition to a higher concentration of organic matter, there were lower concentrations of these elements. Higher organic matter content implies greater nutrient availability from mineralization of organic compounds, allowing for a higher rate of microbial development, and consequently lower metal stress in polluted soils (34).

Two data groups were identified with component analysis, which may explain the variation in the significant presence of metallic and non-metallic elements based on the land uses and soil depths assessed (table 2, page 57 and figure 4, page 61). This variation may be associated with the physicochemical dynamics, composition, and loam-clay texture of the soil in the different land uses, with Pb, Cu, Th, Zn, As and SOM being strongly related to this variation. This may have an environmental impact on homogeneity, primarily due to land use with livestock production and agricultural activities, soil restoration practices, and soil conservation. The percent SOM was significant in land with restoration practices such as reforestation, rehabilitation of gullies, and regeneration of vegetation, among others. This could be an essential factor in the oxidation, adsorption, or retention of metals in soil. Assefa *et al.* (2020) indicate that assessment of the physicochemical properties of soil associated with overgrazing, absence or limitation of fallow periods, steep slopes, and land-use management practices, is related to an understanding of soil status and quality (10). In the results presented in table 3 (page 61) and figure 4 (page 61), Factor 1 illustrates the data relationship explaining 38% of the variance in our study, related to the soil samples with the highest concentration of Pb, Zn, As, and Sr (positive charges) and of Ti and Zr (negative charges). Factor 2 is associated with Th, Rb, and Cr (positive charges) and with Sr and EC (negative charges), explaining 22% of the accumulated variation. Factor 3 explains 19% of such variation and was more closely related to CEC, V, and Ti. Regarding the analysis of factors, the three determining factors account for 80% of the variation, indicating a relationship among soil properties (EC, CEC, and SOM), land use and soil depth (table 2, page 57 and figure 4, page 61). The similarity analysis found relationships between metallic and non-metallic elements and soil parameters of 58.64% to 88.98%, with a particularly notable relationship between Zr-Ti and Pb-Zn (positive charges). The lowest percentage, namely 58.64%, was observed between EC and the rest of the elements and properties, 64.92% for CEC and V-Zr-Ti; and the closest interactions were those between SOM with Pb-Zn-As and Cr-Cu (figure 5, page 62). This result is associated with the correlation analysis presented in table 1 (page 57). Our results are consistent with similar PCA results and correlations, indicating that elements such as As, Cr, Fe, Mn, and Ni could be of lithogenic origin, while Cu, Pb, and Zn may be due to human influence (8, 39).

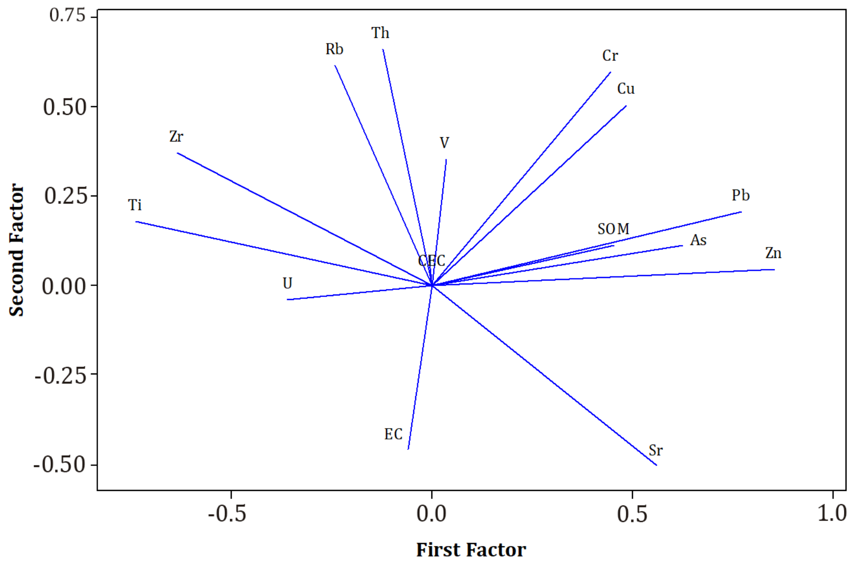



**Figure 4.** Loading plot of metallic elements, non-metallic elements and soil properties derived from the factor analysis.

**Figura 4.** Diagrama carga de los elementos metálicos, no metálicos y propiedades del suelo derivado del análisis de factores.

**Table 3.** Variable loading coefficient (vectors) of the first three factors using 15 variables related to land use (livestock management, restoration, and land use without management or conservation).

**Tabla 3.** Coeficiente de carga de variables (Vectores), de los tres primeros factores utilizando 15 variables de acuerdo con los usos de suelo (manejo de ganado, restaurado y sin manejo o conservación).

| Variable | Factor 1 | Factor 2 | Factor 3 | Communality |
|---|---|---|---|---|
| Zr | -0.636 | 0.373 | 0.431 | 0.729 |
| Sr | 0.560 | -0.505 | 0.074 | 0.574 |
| U | -0.361 | -0.038 | -0.308 | 0.226 |
| Th | -0.125 | 0.661 | -0.454 | 0.659 |
| Pb | **0.770** | 0.208 | 0.298 | 0.726 |
| As | 0.621 | 0.114 | 0.270 | 0.472 |
| Rb | -0.243 | 0.618 | -0.347 | 0.562 |
| Cr | 0.444 | 0.599 | -0.180 | 0.588 |
| V | 0.034 | 0.354 | 0.585 | 0.469 |
| Ti | **-0.739** | 0.180 | 0.543 | 0.873 |
| Zn | **0.853** | 0.047 | -0.551 | 0.732 |
| Cu | 0.484 | 0.506 | 0.321 | 0.593 |
| SOM | 0.453 | 0.114 | -0.108 | 0.230 |
| EC | -0.059 | -0.461 | 0.230 | 0.269 |
| CEC | -0.008 | 0.081 | 0.593 | 0.358 |
| Variance | 3.816 | 2.277 | 1.965 | 8.059 |
| % Var | 0.254 | 0.254 | 0.131 | 0.537 |

Factor loads in bold (> 0.70) are considered highly weighted. SOM=Soil Organic Matter, EC= Electrical Conductivity, and CEC=Cation Exchange Capacity.

Las cargas de factores en negrita (>0,70) se consideran altamente ponderadas. MOS (Materia Orgánica del Suelo), CE (Conductividad eléctrica), CIC (Capacidad de Intercambio Catiónico).

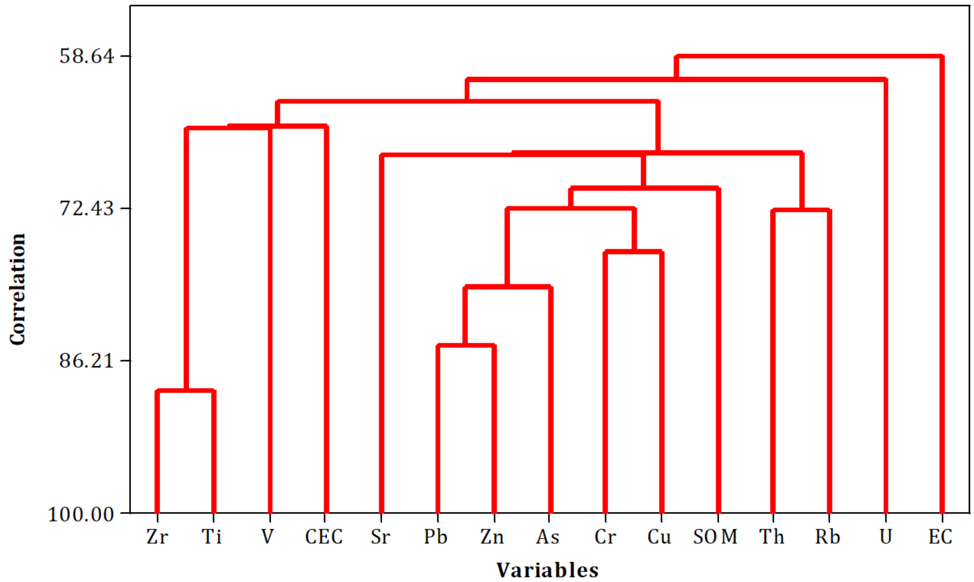



**Figure 5.** Dendrogram derived from the correlation coefficient between metallic and non-metallic elements and soil properties.

**Figura 5.** Dendrograma derivado del coeficiente de correlación entre elementos metálicos y no metálicos y propiedades del suelo.

## Conclusion

The land use factor related to livestock production, restoration and conservation confirmed the presence of 12 metallic and non-metallic elements, which may be attributed to the natural origin of soil and the dynamics of human activities in the region studied. The SOM value was classified as high in accordance with Mexican regulations for soils, principally due to soil remediation practices that have had a positive environmental impact on modulating heavy metals. Notably, As, Pb, and Zn levels did not exceed the limits set by Mexican regulations and international references in soils with these land uses. Based on technical considerations, the total concentrations of Th, Pb, Rb, Cu, Sr, As, and Zn in soil were within permissible limits. However, Ti, Cr, Th, U and As exceeded the technical references for soil concentrations considered phytotoxic. Livestock production practices had a notable impact on the concentrations of elements such as Th, Pb, Rb, and Cu. Land use activities with remediation practices and without management contribute to the low availability of metallic and non-metallic elements in the studied region. These findings underscore the need to continue technical research and monitor environmental impact as related to other natural resources and land uses.

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# About identification of features that affect the estimation of citrus harvest

## Sobre la identificación de factores que afectan la estimación de la cosecha de cítricos

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**Abstract**

Accurate models for early harvest estimation in citrus production generally involve expensive variables. The goal of this research work was to develop a model to provide early and accurate estimations of harvest using low-cost features. Given the original data may derive from tree measurements, meteorological stations, or satellites, they have varied costs. The studied orchards included tangerines (*Citrus reticulata* x *C. sinensis*) and sweet oranges (*C. sinensis*) located in northeastern Argentina. Machine learning methods combined with different datasets were tested to obtain the most accurate harvest estimation. The final model is based on support vector machines with low-cost variables like species, age, irrigation, red and near-infrared reflectance in February and December, NDVI in December, rain during ripening, and humidity during fruit growth.

**Keywords**

MODIS • SVM • selection of variables • machine learning • sweet orange • Murcott tangor

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### Resumen

En la producción de cítricos, los modelos precisos para estimación temprana de producción involucran variables de alto costo. El objetivo de este trabajo fue desarrollar un modelo que proporcione estimaciones tempranas y precisas utilizando características de bajo costo. Los datos iniciales considerados tienen diferentes costos, ya que provienen de mediciones en los árboles, de las estaciones meteorológicas o de satélite. Los huertos de cítricos estudiados correspondieron a mandarino (*Citrus reticulata* x *C. sinensis*) y dos naranjas dulces (*C. sinensis*); ubicados en el noreste argentino. Se han probado varios métodos de aprendizaje automático junto con diferentes conjuntos de datos, con el objetivo de obtener la mejor estimación de producción. El modelo final se basa en máquinas de vectores soporte con las siguientes variables de bajo costo: especie, edad de los árboles, irrigación, reflectancia roja e infrarroja cercana en febrero y diciembre, NDVI en diciembre, lluvia durante madurez y humedad en periodo de crecimiento de frutos.



**Palabras clave**

MODIS • SVM • selección de variables • aprendizaje automático • naranja dulce • tangor Murcott


## Introduction

According to Federcitrus (2022), citrus production in Argentina amounts to approximately 3.5 million tons, with sweet oranges roughly contributing 1 million tons and Valencia late being the most important variety. The cultivation area for Salustiana is increasing, and mandarins contribute around 500,000 tons, with Tangor Murcott as one major type.

Estimating citrus yield is challenging due to interannual and individual variations in productive traits. Typically, estimation relies on agronomic conditions, tree characteristics, historical orchard yield, and subjective observations, leading to estimation errors ranging from 15% to 25% (Apolo-Apolo *et al.*, 2020). Recently, precision agriculture incorporating computing, robotics, artificial intelligence, and remote sensing, has improved yield estimation accuracy.

Several researchers have explored remote sensing and machine learning methods to predict crop yield. Córdoba *et al.* (2012) employed PCA (principal component analysis) to assess spatial covariation of soil properties and crop yield. Teixidó *et al.* (2018) developed semi-automated methods using different image capture systems and segmentation techniques. Wang *et al.* (2021) successfully tested various image capture methods by developing target image detection technology for remote sensing images based on deep learning.

Remote sensing data captured by civilian satellite-borne sensors enables monitoring Earth surface at different temporal and spatial scales. Begué *et al.* (2018) highlighted the convenience of using these images, which offer low costs per unit area while providing consistent spatial and temporal comparisons of vegetation conditions. Various vegetation indices have been developed, including the Standard Vegetation Difference Index (NDVI) for monitoring vegetation biomass. Arango *et al.* (2016a, 2016b, 2017) employed MODIS sensor images and associated variables such as soil properties, biophysical characteristics of crop sites, cultural treatments, and production, identifying arable land.

Machine learning techniques, including support vector machines (SVM), random forest (RF), and artificial neural networks (ANN), have proven effective in estimating agricultural variables of interest. Díaz *et al.* (2017) and Bóbeda *et al.* (2018) used machine learning systems to predict citrus production and load, respectively. Taghizadeh *et al.* (2020) employed SVM and RF algorithms to forecast land suitability for rain-fed wheat and barley. Numerous studies have explored the use of machine learning algorithms to predict crop yield for maize, and potato tuber, among other crops.

The objectives of this study are to identify low-cost and accessible variables for estimating citrus harvest while developing a methodology for early estimation of fruit number per tree using remote sensing and machine learning techniques.

## Material and Methods

### Area and Material of Study

The study collected empirical data from citrus-producing orchards located in the Corrientes and Entre Rios provinces, northeastern Argentina, with geographical coordinates 27°39´39” to 31°23´59” S and 57°00´01” to 58°58´59” W. Orchard age ranged from 7 to 30 years and varietal composition included 44% Murcott tangor (*Citrus reticulata* x *C. sinensis*), 52% Valencia late, and 4% Salustiana sweet oranges (*C. sinensis*). Among the orchards, only 40% were irrigated, 78% of the trees were planted in sandy soil, and 22% were planted in clayey soil. Salustiana orchards were included in the dataset to increase variability, but further research is needed to develop a yield estimation model for this variety.

The dataset comprised three types of variables: tree and orchard characteristics, climatic variables, and satellite information. Field data were collected using a systematic random sampling method during the 2005/06 to 2015/16 seasons. The sample included 2-3% of trees from each orchard, and the following information was gathered:

Harvest: The target variable is the average count of fruits per tree recorded during harvest in each orchard.

Orchard characteristics: This category includes species (tangerine, sweet orange); variety (Murcott, Salustiana, Valencia late); soil type (sandy, clayey); irrigation (presence, absence); and age.

Tree traits: Canopy height and trunk diameter in meters. To estimate harvest time, fruits were counted in a sampling frame of 0.125 cubic meters at 1.5 meters from the ground and at the four cardinal points of the canopy. Then, fruits were manually counted 60 and 30 days before the estimated harvest time. Average number of fruits was calculated.

Climatic variables: This category included total rainfall, average temperature, and humidity during full bloom (September), fruit growth (December to March), and ripening (April to July). These data were obtained from weather stations located 5 to 45 km from the orchards.

Satellite information: MODIS data were used to obtain near-infrared reflectance, red reflectance, and NDVI during full bloom (September), fruit growth and ripening (December to June). Two monthly records allowed average value calculations for each month. NDVI is defined as

$$NDVI = \frac{(REF_{nir} - REF_{red})}{(REF_{nir} + REF_{red})}$$

where *REFnir* is Reflectance in the infrared spectrum and *REFred*, in the red spectrum.

MODIS is aboard the Terra and Aqua satellites. The primary product used in this study was MOD091, which provides reflectance data for terrestrial coverage assessment with daily temporal resolution and a spatial resolution of 250 m. NDVI and reflectance values, as well as database organization related to orchards, followed an automated extraction process outlined in a four-stage workflow depicted in figure 1 (a) (page 68): (1) Orchards location, and centroids calculation. (2) The MODIS sensor time series product MOD09GQ1 download using R Statistics routine (Arango *et al.*, 2016a). (3) NDVI estimation based on seasons, specific time points, and orchard locations. (4) Database construction.

### Data analysis

The cost of gathering data depends on multiple factors. The most expensive aspect involves the on-site laborious measurement of each tree. Climatic variables are obtained from closely located weather stations. Satellite data is freely available. Considering the costs and difficulties associated with measuring these variables, three distinct datasets were created to examine prediction performance based on information-collecting costs (refer to table 1, page 68). Noteworthy is that the variables in dataset d1 are the cheapest, while, conversely, certain features in d3 are quite expensive as they rely on human resources.

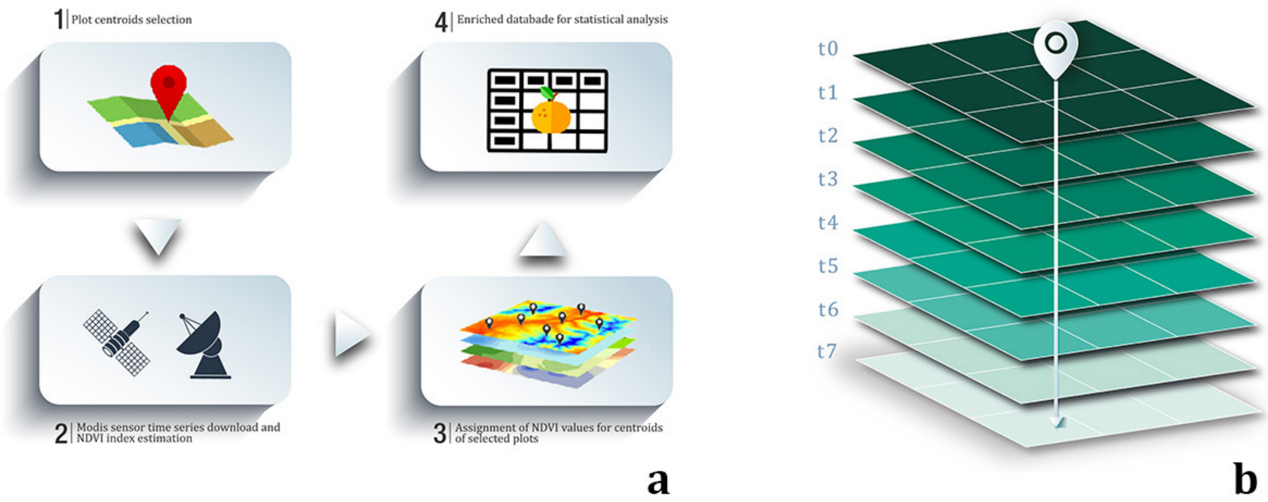


**Figure 1(a).** Steps for data extraction from MODIS sensor.
**Figure 1(b).** Maps t-layer containing monthly NDVI summary for each moment (0 to t7).
**Figura 1(a).** Etapas del proceso de extracción data del sensor MODIS.
**Figura 1(b).** t-capas de mapas con los resúmenes mensuales de NDVI por momento (0 a 7).

**Table 1.** Description of variables in each dataset. Harvest is the target variable.
**Tabla 1.** Descripción de variables en cada conjunto de datos. Cosecha es el valor de comparación.

| Type of variables | Set | Variables |
|---|---|---|
| **Satellite** | d1 | Near-infrared reflectance, red reflectance and NDVI at full bloom (September) and fruit growth and ripening (December to June). Harvest. |
| **Orchard + climatic + satellite** | d2 | Specie, season, soil type, irrigation, and tree age.<br><br>Total rainfall, average temperature, and humidity at full bloom (September), during fruit growth (December to March) and ripening (April to July).<br><br>Near-infrared reflectance, red reflectance and NDVI at full bloom (September) and fruit growth and ripening (December to June). Harvest. |
| **Tree + orchard + climatic + satellite** | d3 | Canopy height and larger diameter, fruit number at the four cardinal points and average value.<br>Specie, season, soil type, irrigation, and tree age.<br><br>Total rainfall, average temperature, and humidity at full bloom (September), during fruit growth (December to March) and ripening (April to July).<br><br>Near-infrared reflectance, red reflectance and NDVI at full bloom (September) and fruit growth and ripening (December to June). Harvest. |

**Methods to estimate orchard production**

ANNs are machine learning algorithms inspired by brain neural networks. They are widely used for both classification and regression tasks across various domains, including agriculture (9). One type of ANN is the multilayer perceptron (MLP), which consists of multiple layers of neurons. Each neuron receives input solely from neurons in the previous layer and provides output exclusively to neurons in the next layer. The first layer represents dataset input features, while the last layer represents the output. The number of hidden layers in between is typically determined through experimentation. During the training process, weights between adjacent neurons are adjusted to minimize prediction error. MLP has been applied in agricultural studies (27).

SVMs transform input data into a high-dimensional feature space using a predefined kernel function, wherein a hyperplane is derived to capture nonlinear relationships. SVM discovers this hyperplane by utilizing support vectors (essential training tuples) and margins (defined by the support vectors). Even though SVMs interpretation can be complex, they have been applied in agriculture with high accuracy (15, 35).

RT adopt a divide-and-conquer strategy to construct a tree. Each path from root to leaf determines a region representing a more homogeneous subset of the input data. Various existing regression tree-based models are characterized by different splitting criteria, prune rules, and methods for estimating leaf values. CART uses variance as the splitting criterion, M5 employs standard deviation reduction, and conditional trees utilize covariance. In CART and conditional trees, the estimated value for a leaf remains constant, while M5 approximates it using linear regression models (21). In general, M5 outperforms CART and conditional trees in terms of accuracy and simplicity. These models have been extensively used in agriculture (7, 20).

Random Forest (RF) constructs decision trees by repeatedly sampling the original training data through bootstrapping. Each decision tree is trained on a different random sample, resulting in trees trained on slightly different data subsets. RF combines the individual decision trees by averaging their predictions, reducing variance in predictions and improving overall accuracy. By assembling a collection of decision trees, RF mitigates the risk of overfitting and enhances model generalization performance on unseen data (16).

Lazy methods (as KNN) are distance-based learning methods that predict output values based on the nearest neighbors in the training set, assuming all features used to describe the dataset are relevant, and that close examples are likely to have the same output value. It computes distances (Euclidean or other) between examples to classify each training example by selecting the k closest neighbors. Since based on distances, KNN is quite sensitive to sliding scale but can be useful when interpretability is not a requirement for modelling a prediction problem (12).

**Training and testing**

Each dataset was divided into training and test sets, with a split ratio of 75% for training and 25% for testing. This process was repeated 50 times, ensuring unbiased results. The training phase followed a cross-validation model with 10 folds. The tested methods included M5, conditional trees (ctree), CART (implemented as rpart and rpart2), SVM with polynomial kernel (svm1) or radial kernel (svm2), perceptron with one layer (mlp) or two layers (mlpMP), k-nearest neighbors (knn), and random forest (RF).

Model performance was assessed through various metrics, including the root mean square error (RMSE), commonly used for validating physical system models (6). It is defined as follows:

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - \hat{u_i})^2}$$

where:

*n* = the sample size

*i* = the output value and *u* is the prediction

The mean absolute error (MAE) quantifies the average difference between the measured data and the estimated data (17), quantifying error magnitude without considering direction. A lower MAE indicates a better model fit, and can be calculated using the following formula:

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|y_i - \hat{u}_i|$$

## Results

### Machine learning + datasets comparison

Different machine learning methods were assessed for prediction performance. Graph analysis indicated that random forest (rf) and SVM with polynomial kernel (svm1) had the lowest MAE and RMSE values. Across all datasets, svm1 consistently outperformed the other methods. Statistical significance was determined after conducting one-tailed t-tests to compare average MAE and RMSE differences for svm1 against all other methods. All comparisons showed significant values ($p \leq 0.05$), confirming that, for citrus production, svm1 had lower MAE and RMSE errors than other methods. The only exception was the RF comparison using dataset d1, showing no statistically significant difference in RMSE compared to svm1 ($p=0.486$). SVM with polynomial kernel (svm1) showed the best performance in terms of MAE and RMSE across all input datasets. Therefore, the analysis focused on evaluating svm1 performance.

Figure 2 shows the MAE *vs.* RMSE comparison obtained with svm1 using d1, d2 and d3 as inputs. Note that the worst performance was obtained with d1 dataset. A paired t-test compared d1 and d2 results and observed significant differences in MAE ($p=1.757206\text{-}07$) and RMSE ($p=1.007665\text{-}06$). Thus, d2 resulted the best dataset. On the other hand, d2 and d3 show small, non-significant differences (MAE ($p=8.356207\text{-}01$), RMSE ($p=1.339823\text{-}01$). Dataset selection was based on the variables used, considering measurement difficulties and costs. Given tree variables were the most difficult and expensive to collect, dataset d2 was chosen for not including these variables. This combination method-dataset threw a prediction average error of 3.99% with 3.7 % standard deviation for fruit number estimation. This error results much smaller than the 10% and 46% obtained in maize yield estimation (20).

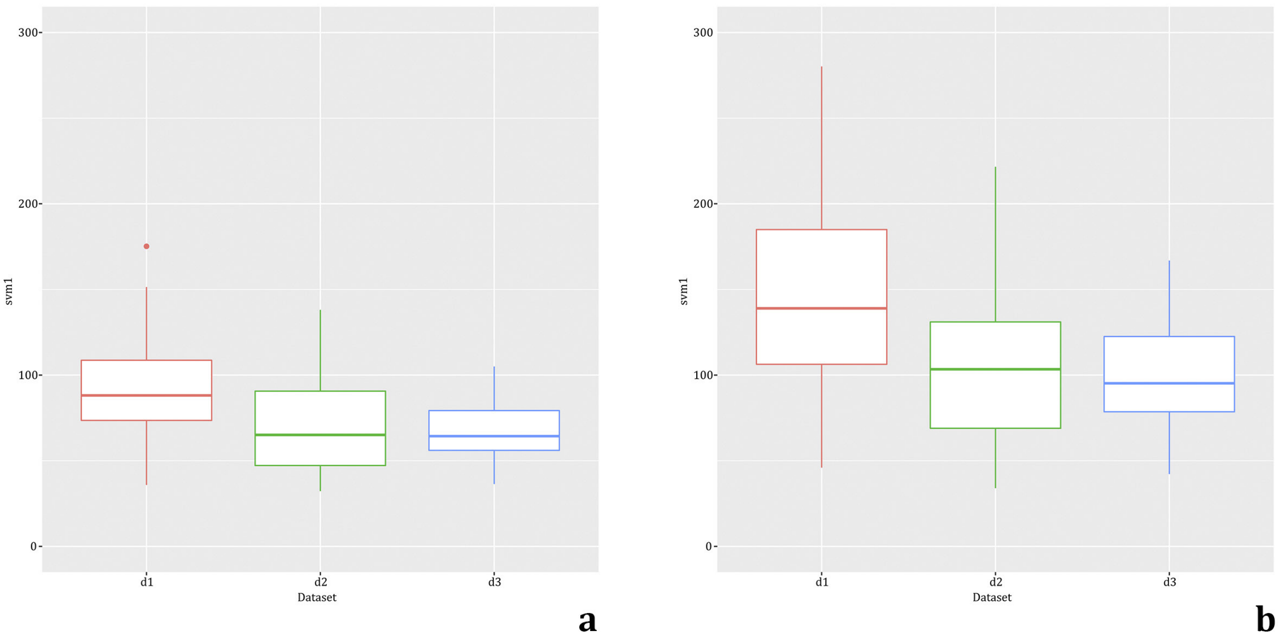


**Figure 2.** MAE (a) and RMSE (b) values obtained with svm1 for datasets d1, d2, d3.
**Figura 2.** Valores de MAE (a) y RMSE (B) obtenidos con svm1 para los conjuntos de datos d1, d2 y d3.

**Analysis of relevant features**

As previously demonstrated, the optimal combination for harvest prediction involves using dataset d2 and the machine learning method svm1. However, one SVM drawback is the complicated assessment of feature relative importance in model construction, besides the fact that there is no standardized approach for evaluating variable importance in SVM-based classification models.

Despite this limitation, investigating the most relevant variables in this context remains important. To this end, this research assumed that if SVM performance weakened when all variables except one were used for training, then that excluded variable was significant for model construction. To check this assumption, the training used all variables except the one being considered, obtaining the associated error (ei). Afterwards, each variable was ranked according to this errors, obtaining a ranking, ri. This process was repeated 50 times, obtaining 50 different rankings, then aggregated using scoring ranking rules and assigning each candidate with a score, finally obtaining variable importance. Although many different ways may obtain a consensus ranking (28, 29, 30, 31, 32), the Borda count is a quite simple convex-ranking-rule (8), already successfully applied similarly by Rúa *et al.* (2023).

The 10 more important variables were species, age, irrigation, red reflectance in February and December, near-infrared reflectance in February and December, NDVI in December, rain during ripening, and humidity during fruit growth.

To check this "variable importance estimation", svm1 was trained with a new dataset called d2-filtered, using only the 10 most important variables selected above. Figure 3 compares svm1 trained with d2 and with d2-filtered. Note that training svm1 with d2-filtered seems to reduce MAE and RMSE, although not significantly (MAE, p=0.05455, RMSE, p=0.2808). Thus, by using only these 10 most relevant variables, performance is not affected, and costs are reduced.

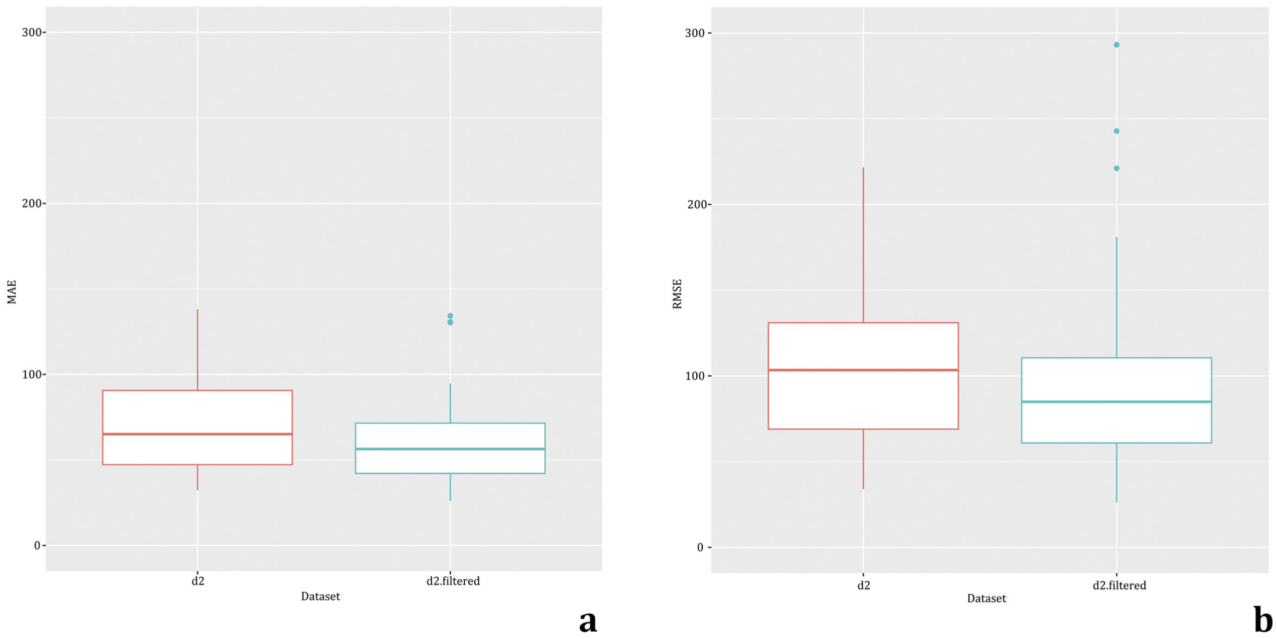


**Figure 3.** Comparison of MAE and RMSE using svm1 with d2 and d2 - filtered.
**Figura 3.** Comparación de MAE y RMSE empleando svm1 con d2 y d2 - filtrado.

## Discussion

This work evaluated several machine learning methods for low-cost orchard production estimation. These previously tested models determined volume, fruit number to harvest, or crop yield, using different remote sensors and yielding results in agreement with our research. RF and SVM resulted the best performance methods (14, 15).

Leroux *et al.* (2019) compared a linear regression model with RF and found that RF outperformed the linear model and estimated maize yield two months before harvest using only data from the vegetative period. Han *et al.* (2019) explored four machine-learning regression methods (linear regression, SVM, ANN, and RF) modelling maize above-ground biomass using remote-sensing data.

ANN and SVM were considered difficult to interpret while the RF model gave the most balanced results, with low error and a high ratio of explained variance for the training and tested set. Feng *et al.* (2020) used machine learning-based integration with remotely sensed data to improve capabilities in monitoring agricultural drought.

Maya Gopal & Bharghavi (2019) evaluated features for accurate crop yield prediction and demonstrated that the RF model performed better. The variables used were planting area, number of tanks, number of tube wells and open wells, canal length for irrigation, amount of fertilizers consumed, seed quantity, cumulative rainfall, cumulative global solar radiation, and maximum, average and minimum temperatures.

Nyalala *et al.* (2019) developed a computer vision system for tomato volume and mass estimation based on depth images and several regression models. SVM showed significant advantages over other supervised learning algorithms. Kurtulmus *et al.* (2013) investigated various techniques for peach number estimation in a canopy, including SVM, ANN, and discriminant analysis. SVM demonstrated superior performance in certain scenarios, consistent with our findings. After evaluating multiple methods, RF and SVM with a polynomial kernel resulted the most effective, with the latter performing significantly better than other approaches across all datasets.

Figure 3 (page 71), shows harvest estimation using low-cost information related to species, season, tree age, soil type, irrigation, temperature, rain, and humidity, as well as satellite data at different moments. Begué *et al.* (2018) found similar results. Available literature on remote sensing for mapping cropping practices, concludes that testing at local scale is highly dependent on ground data. Robson *et al.* (2017) found a consistent positive correlation between vegetation index using near-infrared band 1 and red edge band with total fruit weight and average fruit size, concluding that orchard location and growing season influence this relationship. In the same line, Rahman & Zhang (2017) evaluated high-resolution satellite imagery for mango yield estimation by integrating tree crown area and spectral vegetation indices. They used ANN models, considering that the combination of these types of data allows estimating total fruit yield and fruit number with high accuracy. In addition, our estimation with almost 4% error for fruit number per tree, resulted in better fittings than those obtained by Leroux *et al.*(2019). The method presented in this study represents an improvement over Bóbeda *et al.* (2018), who relied on on-field information and the RT procedure to estimate fruit number in sweet orange and tangerine, with 29% error.

The 10 finally selected variables agree with previous research. Genotype and tree-age effects on citrus production are well-known and significant traits (25) for fruit number estimation in citrus. Concerning humidity, rainfall, and irrigation, plant optimal water intake is necessary for optimal plant growth and development. Kern *et al.* (2018) found an association between rain and yield in winter crops. NDVI and reflectance values for yield estimation resulted as previous yield predictors, based on the conclusions of Kern *et al.* (2018) and Lopresti *et al.* (2015). In addition, noteworthy is that several of the most important features are measured during early crop stages.

## Conclusions

This study presents a methodology using SVM for accurate estimations of fruit count per tree in Murcott tangor and Valencia late sweet oranges. The SVM model employs a polynomial kernel and considers several variables, such as species, tree age, irrigation conditions, rainfall during fruit maturation (April to July), humidity during fruit growth (December to March), red and near-infrared reflectance in February, and NDVI, near-infrared, and red reflectance in December. Easily obtainable ground variables, including species, tree age, and irrigation conditions, were recorded in each orchard. Meteorological stations provided rainfall and humidity data, while civilian satellites offered information. Estimations rely on low-cost variables obtained early in the determination process. The proposed estimation method enables safe and accurate anticipation of harvests at a reduced cost, demonstrating practicality and applicability.

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# Does harmonization reduce the impact of SPS measures on agricultural exports? An assessment from the Chilean fruit sector

## ¿La armonización reduce el impacto de las MSF en las exportaciones agrícolas? Una evaluación desde el sector frutícola chileno

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**Abstract**

Non-tariff measures (NTMs) are relevant to agricultural trade policies, especially since trade negotiations have significantly decreased tariffs. Countries impose Sanitary and Phytosanitary Measures (SPS), a technical NTM, to protect human, animal, and plant health by regulating specific food quality and safety aspects. This article aims to assess the impact of SPS measures imposed by Chile's main trading partners on agricultural trade, specifically on the value of fruit exports. It also seeks to determine the effects of harmonizing technical regulations between Chile and its partners. We estimated a gravity equation as a negative binomial regression model with Chilean fruit exports to main destination markets from 2010 to 2019 as the dependent variable. Our results confirm a negative impact of foreign SPS measures on Chilean fruit exports. However, that impact is mitigated if Chile has a harmonized SPS measure. Thus, we can conclude that harmonization reduces the negative effects of foreign SPS measures on exports. Our results suggest that trade agreements, which often contain a chapter on SPS, positively contribute to SPS harmonization and mitigate SPS's negative impacts on trade flows.

**Keywords**

Non-tariff measures • sanitary and phytosanitary measures • harmonization • food safety • agricultural trade • fruit exports

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### Resumen

Las medidas no arancelarias (MNA) son relevantes para las políticas comerciales agrícolas, especialmente porque las negociaciones comerciales han reducido significativamente los aranceles. Los países imponen Medidas Sanitarias y Fitosanitarias (MSF), una MNA técnica, para proteger la salud humana, animal y vegetal mediante la regulación de aspectos específicos de calidad e inocuidad de los alimentos. Este artículo tiene como objetivo evaluar el impacto de las MSF impuestas por los principales socios comerciales de Chile en el comercio agrícola, específicamente en el valor de las exportaciones de frutas. También busca determinar los efectos de la armonización de normas técnicas entre Chile y sus socios. Estimamos una ecuación gravitacional como un modelo de regresión binomial negativo con las exportaciones de frutas chilenas a los principales mercados de destino de 2010 a 2019 como variable dependiente. Nuestros resultados confirman un impacto negativo de las medidas sanitarias y fitosanitarias extranjeras en las exportaciones de frutas chilenas. Sin embargo, ese impacto se mitiga si Chile cuenta con una MSF armonizada. Por lo tanto, podemos concluir que la armonización reduce los efectos negativos de las medidas sanitarias y fitosanitarias extranjeras sobre las exportaciones. Nuestros resultados sugieren que los acuerdos comerciales, que a menudo contienen un capítulo sobre MSF, contribuyen positivamente a la armonización de MSF y mitigan los impactos negativos de MSF en los flujos comerciales.



**Palabras clave**

medidas no arancelarias • medidas sanitarias y fitosanitarias • armonización • inocuidad • comercio agrícola • exportaciones frutícolas


## Introduction

Non-tariff measures (NTMs) are increasingly present in international trade regulation (20). Following the United Nations Conference on Trade and Development, NTMs were defined as policy measures separate from standard customs tariffs that may economically impact international trade in goods, specifically in quantities, prices, or both (52). The literature cites that, in some cases, NTMs are replaced by Non-tariff barriers (NTBs). Both concepts are very close, almost synonyms; however, the term "barrier" implies a higher probability of negative impact on trade than "measure." Deardorff and Stern (1997) state formal and informal NTBs. Formal NTBs appear in official legislation and governmental mandates. On the other hand, informal NTBs arise from administrative procedures and unpublished regulations and policies, market structure, and institutional framework. Moreover, informal NTBs are often disguised to protect the national industry from foreign competition (18).

NTMs are classified into three general groups: import technical measures, import non-technical measures, and export measures. Within the technical NTMs are sanitary and phytosanitary (SPS) measures, technical barriers to trade, pre-shipment inspections, and other formalities (53). SPS measures protect human, animal, and plant health by regulating specific quality and safety aspects for domestic and imported products. They are subject to multilateral regulation through the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary Measures (WTO SPS Agreement). The objective of the SPS Agreement is to ensure that countries can adopt and enforce legitimate SPS measures and to prevent those measures that are real trade barriers disguised as SPS measures (34). The SPS Agreement requires that countries justify their measures through a risk assessment based on scientific evidence (59). It also encourages countries to use international SPS regulations when possible and accept the regulations of other countries as equivalent if they reach an appropriate level of protection. Countries must notify the WTO of initiating or modifying SPS measures to promote transparency. However, there are often informal NTBs related to SPS, especially administrative procedures such as unannounced inspections or excessive bureaucracy at customs, also known as "red tape." The literature shows that red tape affects variable trade costs for exporting companies and consequently impacts the extensive trade margin (37).

The analysis of the impact of SPS and technical NTMs has generally focused on their effects on trade. The standard approach in literature has been to model commercial flows through gravity equations. SPS measures are often introduced in gravity models by a dummy variable (presence/absence) and less frequently by "coverage" and "frequency" ratios or by SPS *ad valorem* equivalence (8, 21).

The heterogeneous conclusions on the impact of SPS measures on trade reached by this research have depended on: the type of measure (2, 16, 17, 38, 55, 56); producer characteristics (24, 25, 32, 49, 57); the trading partners' economic levels (31, 43, 45, 54); and particularly, the level of harmonization of technical regulations between trading partners (4, 22, 33, 42, 43). In this, harmonization can be understood as the imposition of equivalent technical measures directed at the same product (same tariff line) by two countries, for instance, an alike regulation on the labeling of a product.

The literature has shown, first, that low-income and developing countries' exports, specifically those from China and African countries, are negatively affected by SPS measures. This especially occurs when countries have a lot of small, national, or inexperienced companies, and their regulation is not harmonized with that of the importing countries. In contrast, high-income countries are the ones that impose the most SPS measures (10, 11). As far as we know, there is no specific research on the trade effects of the "red tape," or unofficial NTBs, related to SPS measures. However, assessing the effective impact of SPS measures on trade should also absorb that of the procedures associated with their compliance. There, exporters from countries with a history of SPS non-compliance may be subject to more recurrent and severe border inspections (48). It is worth mentioning that the impact of SPS measures not reported to the WTO is impossible to measure with the usual method. However, it is expected that given the adherence of the WTO members to the SPS Agreement, this percentage will be negligible.

As is generally the case in Latin American countries, in Chile, the case study for this article, agriculture and food are critical to the national economic strategy. Agricultural, food and forestry exports represented over half the Chilean non-copper trade revenue in 2020, totaling USD 15.9 billion FOB. Despite this, aspects of the implications of technical NTMs and SPS measures on its trade have not been thoroughly explored. The first investigations on technical NTMs in Chile took a descriptive approach, with exporters as their source of information. They concluded that food and agricultural trade was especially subject to NTMs, with Latin American partners being the most stringent markets (41, 52, 58). Later, Engler *et al.* (2012) compiled the managers' opinions of fruit exporting companies to evaluate the stringency and harmonization levels of the SPS measures imposed by Chile's main markets. Melo *et al.* (2014) used this information to estimate a gravity model where the relative weight of Chilean fruits compared to the importing countries' consumption and production was the dependent variable. They showed that more stringent regulations have a significant negative impact. More recently, De María *et al.* (2018) identified the SPS measures faced by Chilean and French apple exporters and scored their complexity. The authors concluded that the Chilean exporters were more prepared for stringent markets than the French. They suggested that the reason might be that Chilean technical regulations are also demanding. Chile stands out in terms of food control capacity compared with other countries in Latin America, especially in regulatory quality (12). However, throughout Latin America we can see how farmers are concerned in producing in a more responsible way, as well as research is focusing on ilustrate more sustainable value chains (7, 40).

This article aims to assess the impact of the SPS measures imposed by Chile's main trading partners on the value of Chilean agricultural exports and determine the effect of harmonizing these technical regulations. In this regard, we hypothesize that harmonization contributes to mitigating SPS's negative effects on agricultural exports. We will specifically focus on fruit exports to increase the homogeneity of our analysis; also, they represent 65% of the value of Chilean agricultural exports. Chile is the fifth fruit exporter in the world and the leader in the South Hemisphere. In addition, Chile is a developing country, which has been - except for China and some African countries - scarcely considered a case study on the existing SPS research.

## Materials and methods

### Collection of SPS measures data and descriptive analysis

The data on SPS measures were collected from the WTO SPS Information Management System database. This is the most comprehensive global database on SPS measures available today. It contains an updated inventory with open access to all SPS notifications reported to the WTO by its members, disaggregated by members imposing the measure and partners and products (identified by Harmonized System (HS) codes) affected by the action. A link to the relevant official documents is also provided for each notification.

The importing markets in this study were China, the United States, the European Union, Japan, Mexico, South Korea, Brazil, Venezuela, Peru, and Taiwan, representing the top 10 destination markets for Chilean agricultural products. The SPS measures considered are their submissions to the WTO secretariat from the beginning of 2010 to the end of 2019.

A descriptive analysis of all the SPS notifications compiled will be carried out once the final version of the database is completed. That analysis will focus on characterizing the SPS measures by country imposing, year of submission, measure type determined by the objective and instrument used, and products involved. The country states the explicit SPS goals in submitting the measure to the WTO. Countries can declare one or more explicit objectives for an SPS. The possible objectives are food safety, which refers to "handling, preparing and storing food in a way to reduce best the risk of individuals becoming sick from foodborne illnesses" (5); plant protection, which is "the ability to anticipate the emergence and spread of noxious organisms and to prevent their introduction and spread before they become agricultural pests in specific crops and regions" (6); protecting humans from animal/plant pests or diseases, which could also be interpreted as biosecurity or "trying to prevent new pests and diseases from arriving, and helping to control outbreaks when they do occur" (30); and protecting the territory from pest damage. The researchers assigned each measure an instrument following the Crivelli and Gröschl (2012) methodology. Those instruments relate to the type of requirements that the SPS asks. They are associated with product characteristics such as pesticides, labeling, additives, phytosanitary requirements, geographically protected zones, and quarantine requirements, or with conformity assessment such as certificate requirements, testing, inspection, approval procedures, pest risk analysis, systems approach, and regulations. The objectives and instruments are shown in table 1.

**Table 1.** Objectives and instruments commonly found in SPS measures.

**Tabla 1.** Objetivos e instrumentos encontrados comúnmente en las MSF.

| Objective | Instrument | Reason |
|---|---|---|
| | | Pesticides |
| | | Labeling |
| Food safety | Product characteristics | Additives |
| | | Phytosanitary requirements |
| Plant protection | | Geographical protected zones |
| | | Quarantine requirements |
| | | Certificate requirements |
| Protect humans from animal/plant pests or diseases | | Testing |
| | | Inspection |
| | Conformity assessment | Approval procedures |
| Protect territory from pest damage | | Pest risk analysis |
| | | Systems approach |
| | | Regulations |

Source: Compiled by authors.

Fuente: Elaborado por los autores.

The products subject to each SPS measure and the exported value data were collected considering the tariff lines in chapter 08 from the harmonized system (HS 08): "Edible fruit and nuts, peel of citrus fruits or melons." It considered six digits codes, *i.e.*, the most detailed international disaggregation level.

**Methodological approach and empirical model for impact assessment**

The use of gravity equations to explain international trade flows was first developed by Tinbergen (1962), who enunciated that the exports from country *i* to country *j* depend on the gross national product (GNP) of country *i*; the GNP of country *j* and the geographic distance between country *i* and country *j*. The author stated that additional variables could be added to the model, such as common borders or trade agreements between countries.

There have been significant adjustments in the theoretical foundations and application of the gravity equation model. It was shown that log-linear models by ordinary least squares (OLS) have some associated problems when estimating, such as selection bias. Heckman (1979) proposed using the estimation of a sample selection equation (Probit) before the gravity model by OLS. He also suggested using joint maximum likelihood estimation to avoid efficiency problems, which was later supported by Amemiya (1981) and Maddala (1983). However, Santos Silva and Tenreyro (2006, 2011) criticized the fact that Heckman's model assumes normality and homoscedasticity of error terms and ignores the effects of Jensen's inequality ($E(In_y) \neq InE(y)$ being any random variable). The authors proposed using a Poisson model by pseudo maximum likelihood (PML). Later, Burger *et al.* (2009) adapted Santos Silva and Tenreyro's model when problems of overdispersion appear - as Poisson assumes equi-dispersion - using a negative binomial regression.

Negative binomial regression specifies the variance as a function not just of the mean but also of a particular scattering parameter (14). According to Greene (2018), for mathematical convenience, the parameter $u_i$ assumes a gamma distribution $\left(g\,(u_i) = \frac{\theta^{\theta}}{\Gamma(\theta)} e^{-\theta\, u_i}\, u_i^{\,\theta-1}\right)$ so the expression for the density of $y_i$ is:

$$f(y_i \mid \mathbf{x}_\mathrm{i}) = \frac{\Gamma(\theta + y_i)}{\Gamma(y_i + 1)\Gamma(\theta)}\, r_i^{\,y_i}\, (1 - r_i)^{\theta}, \text{where } r_i = \frac{\lambda_i}{\lambda_i + \theta'}$$

Considering this framework, the empirical model in this research is a negative binomial gravity equation regression, generally specified as:

$$Y_{ijt}^{k} = \exp\big(\beta_0 + \beta_1\, GDP_{it} + \beta_2\, GDP_{jt} + \beta_3\, Dist_{ij} + \beta_4\, Border_{ij} + \beta_5\, Lang_{ij} + \beta_6\, Tariff_{ijt}^{k} + \beta_7\, SPS_{jt}^{k} + \beta_8\, ER_{ijt} + \beta_9\, HI_{ijt}^{k} + \delta_t + \varepsilon_{it}\big)$$

where:
$\beta_1 \dots \beta_9$ = the parameters to be estimated
$\delta_t$ = vector for year dummies
$\varepsilon_{it}$ = the error term of the model

To alleviate the assumption of independence of the observations, we will estimate the model clustering by HS codes, as we can suppose similarities between comparable products. The independent variables in the model are defined in table 2 (page 80).

Data for the export value (US$ FOB) of each tariff line (HS 08) were obtained from the World Bank WITS facility, except for Taiwan, whose data were collected from the database of the Chilean Office of Agricultural Studies and Policies (ODEPA). Most macroeconomic information on countries' gross domestic product was obtained from the World Bank World Development Indicators database. In the case of Taiwan, the data was obtained from the National Statistics of the Republic of China database, and Venezuela's information between 2015 and 2019 was collected from the Economic Commission for Latin America and the Caribbean (CEPALSTAT). The geographic distances between countries (as the sum of distances between major cities weighted by their population), the existence of a common border, and a language linkage were obtained, besides national sources, from the databases of the Centre d'Etudes Prospectives et d'Informations Internationales (CEPII). Tariff data was obtained from the WTO's Tariff Analysis Online (TAO) database when available and from the texts of the FTAs between Chile and each partner.

**Table 2.** Definition of the independent variables in the model.
**Tabla 2.** Definición de las variables independientes en el modelo.

| Variable | Definition |
|---|---|
| $Y_{ijt}^{k}$ | Exports from country *i* to country *j* (US$ FOB) for product *k* in the year *t* |
| $GDP_{it}$ | Gross domestic product of country *i* in the year *t* |
| $GDP_{jt}$ | Gross domestic product of country *j* in the year *t* |
| $Dist_{ij}$ | Geographic distance between country *i* and country *j* |
| $Border_{ij}$ | Dummy variable with a value of 1 if country *i* and country *j* share a border (0 otherwise) |
| $Lang_{ij}$ | Dummy variable with a value of 1 if country *i* and country *j* have the same official language (0 otherwise) |
| $Tariff_{ijt}^{k}$ | *Ad valorem* tariff imposed by country *j* on a country *i* for product *k* in the year *t* |
| $SPS_{jt}^{k}$ | Dummy variable with a value of 1 if there is at least one SPS measure imposed by country *j* on the imports of product *k* in the year *t* (0 otherwise) |
| $ER_{ijt}$ | The average exchange rate between country *i* and country *j* in the year *t* |
| $HI_{ijt}^{k}$ | Dummy variable with a value of 1 if there is at least one SPS measure imposed by country *i* on the imports of product k in effect in the year *t* with the same objective and instrument (0 otherwise) |

Source: Compiled by authors.
Fuente: Elaborado por los autores.

In some cases, the equivalent *ad-valorem* tariff was considered when there was no *ad-valorem* tariff information in TAO. The information was obtained from the Market Access Map from the International Trade Centre. Exchange rates were collected from the Central Bank of Chile database. Details on importing countries' SPS measures came from the database on notifications previously created and detailed in the preceding subsection. However, for inclusion in the gravity equation, we aggregated the observations of all the measures that affect a tariff line *k*. First, information on Chilean SPS measures was collected from the WTO SPS Information Management System for the harmonization dummy. Then it was compared with our SPS database.

## Results

### Descriptive analysis of the SPS measures

In total, 424 SPS notifications were reported for fresh fruits by Chile's main export destinations between 2010 to 2019. The number of measures reported each year increased throughout the period. From 2010 to 2013, the annual measure notification average was 17.5; from 2014 to 2016, it was 35.6; from 2017 to 2019, it was 82.3. Japan imposed the most measures, followed by Brazil and the United States. The other countries imposed much fewer and in this descending order: Taiwan, European Union, China, Korea, Peru, and Mexico.

The most common objective of the measures was "food safety" and, to a lesser extent, "plant protection." The main instrument used by the SPS measures was "product characteristics," with 375 notifications, while "conformity assessment" was in only 49 notifications. There were 325 that gave Maximum Residues Levels (MRLs) on Pesticides as a reason, and all other reasons were sporadic. A great diversity of products were involved since a notification can cover several tariff lines. When disaggregating the 424 notifications by tariff lines in each case, they covered 75 different HS-08 codes. The most affected products by the SPS measures under study were tropical fruits, berries, citrus, melons, and apples.

### SPS impact assessment

The estimation results of the specified model are detailed in table 3. The second column contains the estimated coefficients, four statistically significant. The third column contains the related standard deviation.

**Table 3.** Negative binomial gravity model: estimation results.

**Tabla 3.** Modelo gravitacional binomial negativo: resultados de la estimación.

| Variables | Parameter | SD |
|---|---|---|
| Constant | 13.35 | 0.65 |
| $GDP_{it}$ | $6.32(10^{12})$*** | $2.05(10^{12})$ |
| $GDP_{jt}$ | $1.51(10^{12})$*** | $4.36(10^{12})$ |
| $Dist_{ij}$ | $0.02(10^{-3})$ | $0.18(10^{-4})$ |
| $Border_{ij}$ | -0.31 | 0.46 |
| $Lang_{ij}$ | 0.15 | 0.34 |
| $Tariff_{ijt}^{k}$ | $0.95(10^{-2})$ | 0.02 |
| $SPS_{jt}^{k}$ | -0.23*** | 0.08 |
| $ER_{ijt}$ | $-0.59(10^{-3})$ | $0.10(10^{-2})$ |
| $HI_{ijt}$ | 0.37* | 0.20 |
| Log-likelihood | -37,620.07 | |
| Observations | 2315 | |

Dependent variable: Value of exports from country *i* (Chile) to country *j* (US$ FOB) for product *k* in the year *t*. The dummy variable for the years 2018 and 2019 was omitted for collinearity. *Significant at 10%; ** Significant at 5%; *** Significant at 1%.

Source: Compiled by authors.

Variable dependiente: Valor de las exportaciones del país *i* (Chile) al país *j* (Dólares americanos FOB) para el product *k* en el año *t*. La variable dummy para los años 2018 y 2019 fue omitida por colinealidad. * Significativo al 10%; ** Significativo al 5%; *** Significativo al 1%.

Fuente: Elaboración propia.

On the specific results, the variables $GDP_{it}$ ($p<0.01$), $GDP_{jt}$ ($p<0.01$) and $HI_{ijt}$ ($p<0.1$) have a positive association with the value of fruit exports from Chile to its main destination markets, while $SPS_{jt}^{k}$ ($p<0.01$) has a negative impact. Finally, the variables $Dist_{ij}$, $Border_{ij}$, $Lang_{ij}$, $Tariff_{ijt}^{k}$ and $ER_{ijt}$ are non-significant for the value of fruit exports.

## Discussion

The number of SPS measures notified to the WTO grew during the period under study. This is consistent with Correa and Moreira's (2021) results from reviewing the evolution in SPS measure notifications since 2000, showing an increasing trend. The authors highlight the role of large commodity exporters like Brazil and developed countries in generating SPS notifications. Our findings for characterizing SPS measures on fruits by notifying countries are also coherent with those results. Boza and Muñoz (2017) evidenced that high-income countries' legal and technical capabilities are key factors that justify their outsized participation in SPS notifications.

Most of the identified SPS measures address food safety by regulating MRLs for pesticides. This is consistent with Grübler and Reiter (2021), who compiled and analyzed a dataset on NTM notifications from 1995 to 2019. They showed that the most common keyword for SPS notifications was "food safety," and the fourth was "maximum residue level." Tiu (2021) describes MRLs as a "never-ending challenge" since pesticide technology advances so fast that there are always new issues.

Our gravity model showed a negative impact of SPS measures on the value of Chilean fruit exports. As Orefice (2017) points out, even though SPS measures are imposed to protect consumers' health, they *de facto* increase trade costs, which would also help explain our results. The high presence of MRLs in the SPS measures imposed by Chile's main markets might also be related to our findings. Hejazi *et al*. (2018) used U.S. exports to show that MRLs constrain international fruit and vegetable trade, decreasing the export probability and intensity. Xiong and Beghin (2017) presented some results that qualify those of Hejazi *et al.* (2018). After applying a gravity model for MRLs imposed by high-income countries, the authors showed that they negatively affect export supply but positively impact import demand, which they suggest is related to risk mitigation.

Hejazi *et al.* (2018) also demonstrated that the negative impact of SPS measures on trade increases when there is a more significant difference between the MRLs mandated by each trading partner for a given pesticide and commodity. Our results aligned as our proxy variable to harmonization ($HI_{ijt}$), has a significant and positive relation to Chilean fruit exports. This specific outcome is coherent with our hypothesis that harmonization mitigates the adverse effects of SPS on agricultural exports and with existing literature that supports the idea (*e.g.*, 4, 22, 33, 42, 43). The 26 Free Trade Agreements (FTAs) that Chile has in effect, with a chapter on SPS measures that enhances communication and coordination between the parties, might have contributed to improving harmonization. Also, Chile has produced many SPS notifications (11, 15). This may help Chilean exporters meet foreign SPS measures, especially when those measures are comparable to national requirements.

Tariffs were not significant to Chilean fruit export values. This might also be related to the large number of FTAs that Chile has in effect. The chapters on market access in those agreements present a list of tariff reduction commitments. As a result, the tariffs faced by Chilean companies when exporting fruit to its main destination markets should not represent a significant barrier. By 2010, Chile had signed FTAs with all destinations but Brazil, Taiwan, and Venezuela; however, these three countries represented only 7.3% of Chilean fruit exports to the selected destinations in the timeframe studied. Thus, most destinations had reduced or eliminated tariffs on Chilean fruits in the analyzed period.

Additionally, the main three markets for Chilean fruit from 2010 to 2019 were China, the European Union, and the United States, representing 84% of the fruit exports. Their main imported fruits were cherries, fresh grapes, blueberries, avocados, and apples. Most products entered those markets with zero tariffs or, in the case of China, significantly reduced tariffs compared to suppliers with no trade agreements, which may also explain our model's result.

The exchange rate was also non-significant to Chilean fruit export values. Chile has been distinguished by its stability even in the face of external shocks (1). Distance from the importing country and sharing a common language or border were also non-significant. The advantage of the counter-season with the Northern Hemisphere is one factor that justifies the expansion of Chilean fruit exports (35), as opposed to targeting countries by geographical or cultural proximity. China, Chilean's current main market for agricultural products, is in its antipodes. This strategy might also explain the significant and positive coefficient for the importer GDP, *i.e.*, Chile has privileged the market size.

## Conclusion

This article aimed to assess the impact of SPS measures imposed by Chile's main trading partners on the value of Chilean agricultural exports and determine the effect of harmonization of these technical regulations. We hypothesized that harmonization mitigates SPS's negative impact on agricultural exports. The gravity equation estimates confirm our hypothesis, as the presence of an SPS measure imposed by the importing country has a significant and negative relationship with the export value for a given fruit product. Meanwhile, the existence of an SPS measure imposed by Chile for the same product and objective (our proxy of a harmonized SPS) has a significant and positive effect.

Then, how to ease the consequences of SPS measures on trade? Harmonization reduces the effects of SPS measures on exports. The extensive list of trade agreements Chile has signed might positively contribute to SPS harmonization, as most have an SPS chapter that encourages coordination. In this regard, Chile recently initiated the ratification process for the Agreement for Transpacific Partnership (TPP). This agreement is a paradigmatic example of a mega-trade deal. It contains a chapter on SPS that looks for higher integration between partners, eventually limited by their technical differences. The effects of the TPP on SPS harmonization are to be seen; however, in Chile, they might be marginal, as the country already has previous FTAs with every signatory member.

On the other hand, trade facilitation simplifies procedural and administrative impediments to trade - *i.e.*, "red tape," and today, is an essential part of international negotiations. According to OECD Trade Facilitation Indicators, Chile is among the highest-ranked countries, especially in governance, procedures, and information availability. Since 2019, Chile has had a National Trade Facilitation Committee as part of its Ministry of Foreign Affairs.

Thus, Chile - and other similar economies - should continue relating in a fluid and transparent way with its trading partners, with the aim of cooperation. Given the off-season, it is crucial to consider that Chilean fruit exports do not compete directly in its main markets (US, China, EU) with the national fruit industry, which might encourage maintaining and increasing cooperation.

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# Selectivity of latifolicides associated with glyphosate applied in postemergence on soybean (*Glycine max*) cultivars

## Selectividad de latifolicidas asociados con glifosato aplicados en post-emergencia en cultivares de soja (*Glycine max*)

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**Abstract**

The genetic improvement of soybean cultivars over the years has focused on increasing the yield potential and tolerance to some abiotic and biotic factors. However, during the transfer of genes of interest, some genes responsible for a lower tolerance to herbicides can be integrated into the genome of the new cultivar. Thus, the objective of this study was to evaluate the selectivity of herbicide associations applied in the postemergence period of three soybean cultivars. The experiment was conducted in a randomized completely block design, with four replications. The selected cultivars were M7110 IPRO®, Foco IPRO®, and Bônus IPRO®. The herbicides and the respective doses (g a.i. $ha^{-1}$) used were glyphosate (1176), glyphosate + bentazon (1176 + 600), glyphosate + fomesafen (1176 + 175), glyphosate + lactofen (1176 + 120), glyphosate + imazethapyr (1176 + 100), glyphosate + chlorimuron (1176 + 10), glyphosate + cloransulam (1176 + 39.5), and a control without herbicide application. The visual note of intoxication was evaluated for each treatment. The components of growth and yield evaluated were height, stand, weight of one hundred grains, and yield. The application of postherbicide herbicides did not alter the plant stands of soybean cultivars. Additionally, these herbicides did not reduce the yield of the M7110 IPRO® and Foco IPRO® cultivars. Glyphosate isolated and in association with lactofen or imazethapyr reduced the grain yield of the Bônus IPRO® cultivar.

**Keywords**

Chemical control • *Glycine max* • herbicides • phytointoxication

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## Resumen

El mejoramiento genético de los cultivares de soja a lo largo de los años se ha centrado en aumentar la capacidad productiva y la tolerancia a algunos factores abióticos y bióticos. Sin embargo, durante la transferencia de genes de interés, algunos genes responsables de una menor tolerancia a los herbicidas pueden integrarse en el genoma del nuevo cultivar. El objetivo de este estudio fue evaluar la selectividad de las asociaciones de herbicidas aplicadas en la post-emergencia de tres cultivares de soja. El diseño experimental utilizado fue un bloque completamente al azar, con cuatro repeticiones. Los cultivares seleccionados fueron M7110 IPRO®, Foco IPRO® y Bônus IPRO®. Los herbicidas y las respectivas dosis (g a.i. $ha^{-1}$) utilizados fueron: glyphosate (1176), glyphosate + bentazon (1176 + 600), glyphosate + fomesafen (1176 + 175), glyphosate + lactofen (1176 + 120), glyphosate + imazethapyr (1176 + 100), glyphosate + chlorimuron (1176 + 10), glyphosate + cloransulam (1176 + 39,5), y un control sin aplicación de herbicida. Se hizo una nota visual de intoxicación para cada tratamiento. Los componentes de crecimiento y rendimiento evaluados fueron: altura, rodal, peso de cien granos y rendimiento. La aplicación de herbicidas en post-emergencia no alteró la masa vegetal de los cultivares de soja. Además, estos herbicidas no redujeron el rendimiento de los cultivares M7110 IPRO® y Foco IPRO®. Tanto el glifosato aislado como mezclado con lactofen o imazethapyr redujeron el rendimiento del cultivar Bônus IPRO®.



**Palabras clave**

Control químico • *Glycine max* • herbicidas • fitointoxicación


## Introduction

The repetitive use of the same herbicide can generate risks to the sustainability of agricultural systems, as it causes important changes in the composition of the weed community present in agricultural areas. The emergence of herbicide-resistant weed biotypes is among the main concerns related to plant protection globally (5, 14).

Due to the large-scale adoption of Roundup Ready® (RR®) technology, which gives plants tolerance to glyphosate, postemergence applications of this herbicide have become quite frequent in large crops such as soybeans (17). This fact significantly contributed to the increase in the population of weed species tolerant (13) to this herbicide in the cultivated areas, in addition to accelerating the selection of resistant biotypes. This change in the weed community has led to the need to complement chemical control, especially with the application of latifolicides in soybean crops (1).

The association among herbicides with different modes of action has become an important strategy for the control of a wider spectrum of weed species (21), in addition to mitigating the occurrence of weed resistance to herbicides (18). Currently, ALS (*e.g.*, imazethapyr, chlorimuron) and PPO (*e.g.*, fomesafen, lactofen) inhibitors are among the most widely used herbicides in postemergence soybean crops and are used mainly in association with glyphosate. Despite showing satisfactory control for several weed species, especially broadleaves, these herbicides can cause injuries to soybean plants, with internal chlorosis and leaf tissue necrosis as the most frequent visual symptoms, which can negatively affect crop yield (2).

In a study by Alonso *et al.* (2011), it was observed that the use of glyphosate isolated and in association with other postemergence herbicides provided visual symptoms of injuries in RR® soybean plants, causing a reduction in crop yield components. The most common symptom caused by the isolated application of glyphosate on RR® soybean is the chlorosis of the trifoliated leaves positioned in the upper portion of the plants; however, other negative effects were observed in studies with this herbicide, such as reduced nutrient absorption and plant growth (6, 22).

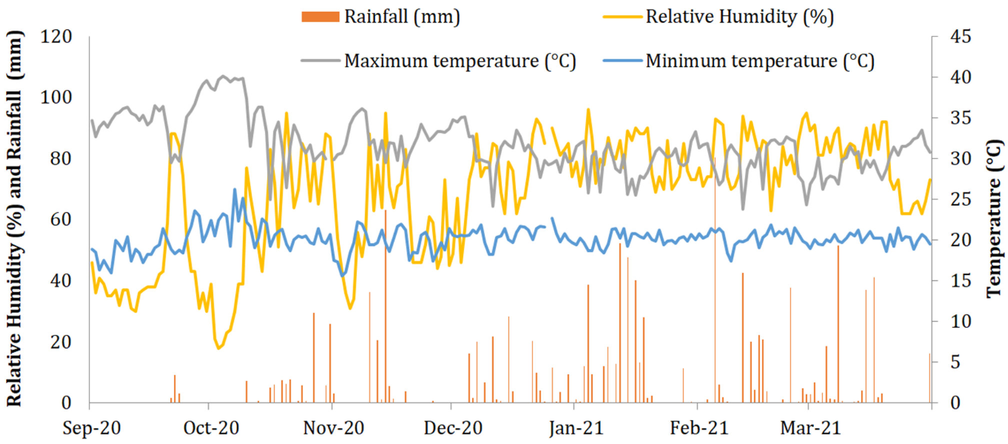
The postemergence herbicide applications in soybean crops are more effective in weed control when carried out at the early stages of development. Fornazza *et al.* (2011) reported that some combinations of herbicides applied in the initial postemergence of soybean can affect the grain yield due to the low selectivity observed for some cultivars. The selectivity of herbicides for soybean cultivars, mainly related to the use of these products in associations, is an aspect to be carefully observed, mainly due to the current use in Brazil of genotypes with great phenotypic variation, highlighting relevant aspects, such as maturation groups, growth types, leaflet area, leaf inclination angle, and pubescence. The correct choice of soybean cultivars can avoid losses in grain yield resulting from low tolerance to herbicides.

In this context, the objective was to study the selectivity of latifolicides in association with glyphosate applied in the postemergence period of three RR® soybean cultivars in the Midwest region of Brazil.

## Materials and methods

Three experiments were carried out in the field in the same plot located in the municipality of Rio Verde (Goiás State), Brazil (17°52'05"S and 50°55'36"W; altitude: 741 m), from November 18th, 2020 to March 18th, 2021. In each experiment, the selectivity of herbicides applied postemergence to a soybean cultivar of indeterminate growth type and with great representation in terms of cultivated area in the Midwest region of Brazil was evaluated. The evaluated cultivars were M7110 IPRO® (maturity group - MG: 6.8), Focus IPRO® (MG: 7.2), and Bônus IPRO® (MG: 7.9).

According to Köppen's classification, the climate of the municipality where the experiments were carried out is of the Aw type, which is called "tropical with the dry season", characterized by more intense rainfall in summer than in winter. In figure 1, there are climatological data related to temperature and relative humidity of the air, luminosity, and rainfall during the period of conducting the experiments.




Source: INMET - Instituto Nacional de Meteorologia. Collection station: Rio Verde (Goiás State).

Fuente: INMET - Instituto Nacional de Meteorología. Estación de recolección: Rio Verde (Estado de Goiás).

**Figure 1.** Data on temperature and relative air humidity, luminosity and rainfall during the period of conducting the experiments. Rio Verde (Brazil), 2020/2021.

**Figura 1.** Datos sobre temperatura y humedad relativa del aire, luminosidad y precipitación durante el período de realización de los experimentos. Rio Verde (Brasil), 2020/2021.

Before the installation of the experiments, the analysis of soil samples collected at depths from 0 to 20 cm was carried out, which revealed the following physicochemical properties: pH in $CaCl^2$ of 4.8; 5.0 $cmol_c\ dm^{-3}$ of $H^+ + Al^{+3}$; 2.78 $cmol_c\ dm^{-3}$ of $Ca^{2+}$; 1.09 $cmol_c\ dm^{-3}$ of $Mg^{+2}$; 0.11 $cmol_c\ dm^{-3}$ of $K^+$; 4.3 mg $dm^{-3}$ of P; 30.2 g $dm^{-3}$ organic matter; 42% sand; 7% silt and 51% clay (sandy clay texture). Before sowing, the weeds present in the experimental area received two herbicide applications (burndown desiccation), the first being carried out ten days before sowing (November 8th, 2020) with the application of glyphosate (720 g a.e. $ha^{-1}$) and the second on the day of sowing (November 18th, 2020), with the application of glyphosate + flumioxazin (900 + 20 g a.i. $ha^{-1}$) in association with the addition of Joint Oil® (0.5% V/V).

Soybean sowing was carried out mechanically, adopting a spacing of 0.5 m between rows. Twenty-two, 16, and 10 seeds of soybean were distributed per linear meter for the cultivars M7110 IPRO®, Foco IPRO®, and Bônus IPRO®, respectively. The seeds used in the experiments received industrial treatment with fungicides and insecticides. Fertilization was carried out at sowing time, with application in the furrow of the equivalent of 400 kg ha$^{-1}$ of 02-20-28 (N-P-K). The emergence of soybean seedlings of the three cultivars occurred on November 25th, 2020.

In all experiments, a randomized complete block design was used, evaluating eight treatments with four replications. The treatments consisted of the evaluation of herbicide associations applied in the postemergence period of soybean (table 1). It is worth noting that no adjuvants were added to the application of any of the treatments; this criterion was adopted because all the associations contained a glyphosate-based product in their composition. The experimental units consisted of six sowing lines, with a length of 5.0 m (15.0 m$^2$). Only the four central lines of each experimental unit were considered useful areas for the evaluations, excluding 0.5 m from each end.

**Table 1.** Treatments evaluated in post-emergence applications of soybean cultivars. Rio Verde (Brazil), 2020/2021.

**Tabla 1.** Tratamientos evaluados en aplicaciones de post-emergencia de cultivares de soja. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$)* | Mode of action** |
|---|---|---|
| Check without herbicide | - | - |
| Glyphosate | 1176 | EPSPs inhibitor |
| Glyphosate + bentazon | 1176 + 600 | EPSPs inhibitor + PSII inhibitor |
| Glyphosate + fomesafen | 1176 + 175 | EPSPs inhibitor + PPO inhibitor |
| Glyphosate + lactofen | 1176 + 120 | |
| Glyphosate + imazethapyr | 1176 + 100 | EPSPs inhibitor + ALS inhibitor |
| Glyphosate + chlorimuron | 1176 + 10 | |
| Glyphosate + cloransulam | 1176 + 39.5 | |

1/ For glyphosate 1176 g of active ingredient (a.i.) ha$^{-1}$ corresponds to 960 g of acid equivalent (a.e.) ha$^{-1}$. 2/ EPSPs = 5-enolpyruvylshikimate-3-phosphate synthase; PSII = photosystem II; PPO = protoporphyrinogen oxidase; ALS = acetolactate synthase.

1/ Para el glifosato 1176 g de ingrediente activo (i.a.) ha$^{-1}$ corresponde a 960 g de ácido equivalente (e.a.) ha$^{-1}$. 2/ EPSPs = 5-enolpiruvilshikimato-3-fosfato sintase; PSII = fotosistema II; PPO = protoporfirinógeno oxidase; ALS = acetolactato sintase.

The treatments application in the three experiments was carried out on December 18th. 2020 (23 days after emergence- DAE). On this occasion, the soybean plants were at stage V5 (5 trifoliated leaves) for the cultivars M7110 IPRO® and Foco IPRO® and at V4 (4 trifoliated leaves) for the Bônus IPRO® cultivar, with plant height varying between 15 and 18 cm. At the time of application, the soil was wet, the temperature and relative humidity, minimum and maximum, were 23.1 and 25.1 °C and 60 and 64%, respectively, and the sky had the presence of few clouds and wind speed at values close to 1.2 km h$^{-1}$. The applications were carried out using a $CO_2$ pressurized back sprayer equipped with a boom fitted with 6 fan-type spray tips XR-110.015 spaced 50 cm apart and regulated to a pressure of 0.24 MPa. These application conditions provided an application rate equivalent to 150 l ha$^{-1}$.

To ensure that the soybean plants were only exposed to the effect of herbicide treatments, manual weeding of the species that made up the weed community of all experimental units was carried out throughout the entire crop cycle. In addition, during the development of soybean, cultural practices were carried out following the recommendations of Embrapa (2013), to control pests and diseases without letting them negatively influence the development of the crop. All phytosanitary applications, except for the herbicide treatments that were the object of evaluation, were carried out using a tractor sprayer machine, adopting an application rate equivalent to 150 l ha$^{-1}$.

Injury level evaluations of soybean cultivars were carried out at 7 and 28 days after herbicide application (DAA), using for this evaluation the scale proposed by the SBCPD (1995), which presents grades ranging from 0% to 100%, where 0% means the absence of symptoms and 100% represents the death of all plants present in the useful area. Plant height assessments (cm) were carried out at 50 DAE and at harvest time with the aid of a graduated measuring tape, measuring the distance from the soil surface to the apex in 5 plants per experimental unit. In addition, at the time of harvest, an evaluation of the plant stand was carried out, counting the number of plants present in a 3 m row, with the data for this variable presented as the number of plants per linear meter (plants m$^{-1}$).

Additionally, in harvest, the yield components, number of pods per plant, and mass of 100 grains were evaluated. To evaluate the number of pods per plant, the count of pods present in 5 plants per experimental unit was performed. In the evaluation of 100 grain masses, 100 grains were counted and weighed on a precision scale, correcting the humidity content to 13%. To determine the grain yield, all plants present in the useful area of each experimental unit were plucked manually (M7110 IPRO® and Foco IPRO®, harvested on March 13th, 2021; Bônus IPRO®, harvested on March 18th, 2021), where this material was later submitted to threshing, packaging, identification, weighing and grain humidity correction processes to 13%.

Statistical analyses were performed using SISVAR software (2011). Data from all experiments were submitted to analysis of variance by the F test (*p≤0.05*), and when there was a significant effect, the Scott–Knott mean grouping criterion (*p≤0.05*) was applied.

## Results and discussion

### Experiment I: Selectivity of herbicide associations applied postemergence to the soybean cultivar M7110 IPRO®

Observing the phytotoxicity results for the cultivar M7110 IPRO® at 7 DAA, it can be seen that the levels of injuries caused by the herbicides varied from 11.25 and 14.50%, with no significant difference among the herbicide treatments; however, all differed from the control without herbicide application (table 2). Furthermore, it is worth emphasizing that, in this evaluation, the latifolicides associated with glyphosate postemergence did not enhance injury levels compared to the application of glyphosate isolated.

**Table 2.** Injury level of soybean (cultivar: M7110 IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 2.** Fitointoxicación de la soja (cultivar: M7110 IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | Injury level (%) | | | |
|---|---|---|---|---|---|
| | | 7 DAA | | 28 DAA | |
| Control without herbicide | - | 0.00 | a | 0.00 | a |
| Glyphosate | 1176 | 11.25 | b | 2.50 | a |
| Glyphosate + bentazon | 1176 + 600 | 11.25 | b | 3.75 | a |
| Glyphosate + fomesafen | 1176 + 175 | 12.50 | b | 5.00 | a |
| Glyphosate + lactofen | 1176 + 120 | 14.50 | b | 7.50 | b |
| Glyphosate + imazethapyr | 1176 + 100 | 11.25 | b | 3.75 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 13.25 | b | 8.25 | b |
| Glyphosate + cloransulam | 1176 + 39.5 | 11.25 | b | 2.50 | a |
| $F_{Value}$ | | 14.80* | | 3.85* | |
| CV (%) | | 21.81 | | 66.60 | |

a.i. = active ingredient; DAA = days after application. * Significant by F test (*p≤0.05*). Means followed by different letters in the column differ from each other by the Scott-Knott test (*p≤0.05*).

i.a. = ingrediente activo; DAA = días después de la aplicación. * Significativo por prueba F (*p≤0,05*). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott (*p≤0,05*).

In the evaluation carried out at 28 DAA, the highest percentages of injury levels were seen in soybean plants that received postemergence applications of the association's glyphosate + chlorimuron and glyphosate + lactofen, which presented values of 8.25% and 7.50%, respectively (table 2). In a study by Alonso *et al.* (2010), it was observed that the association glyphosate + lactofen did not present selectivity for the soybean cultivar CD 214 RR®. In this final evaluation, except for the treatments mentioned above, no differences were observed between the other treatments and the control without herbicide application in terms of injury levels, which demonstrates the crop's ability to recover from the negative effects caused by these herbicides.

The evaluation of plant height showed a direct relationship with the results of injury levels, since the treatments that provided higher percentages of injuries promoted a reduction in soybean size at 50 DAE (table 3, page 91). On this occasion, the combination of glyphosate + chlorimuron and glyphosate + lactofen directly affected the plant size of this cultivar, providing reductions of 9.66% and 6.02%, respectively, for the height values measured in the control without herbicides. The low levels of foliar injuries found in the last phytotoxicity evaluation in the other treatments were not able to induce a reduction in the growth of soybean plants, since the height values were similar to the control without herbicide application.

**Table 3.** Height and stand of soybean plants (cultivar: M7110 IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 3.** Altura y soporte de plantas de soja (cultivar: M7110 IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | Plant height (cm) | | | | Stand (plants m$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | 50 DAE | | Harvest | | 50 DAE | |
| Control without herbicide | - | 79.70 | a | 103.95 | a | 19.24 | a |
| Glyphosate | 1176 | 79.85 | a | 103.50 | a | 19.33 | a |
| Glyphosate + bentazon | 1176 + 600 | 78.70 | a | 103.05 | a | 19.16 | a |
| Glyphosate + fomesafen | 1176 + 175 | 78.75 | a | 100.00 | b | 19.24 | a |
| Glyphosate + lactofen | 1176 + 120 | 72.00 | b | 98.50 | b | 20.58 | a |
| Glyphosate + imazethapyr | 1176 + 100 | 76.55 | a | 103.20 | a | 18.24 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 69.10 | b | 98.35 | b | 20.41 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 74.90 | a | 102.75 | a | 19.41 | a |
| $F_{Value}$ | | 6.02* | | 3.14* | | 0.73$^{ns}$ | |
| CV (%) | | 4.19 | | 2.58 | | 8.88 | |

a.i. = active ingredient; DAE = Days after emergence. * and $^{ns}$ Significant and non-significant, respectively, by the F test (*p≤0.05*). Means followed by different letters in the column differ from each other by the Scott-Knott test (*p≤0.05*).

i.a. = ingrediente activo; DAE = Días después de la emergencia. * y $^{ns}$ Significativo y no significativo, respectivamente, por la prueba F (*p≤0,05*). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott (*p≤0,05*).

At harvest, the PPO inhibitor herbicides fomesafen and lactofen and the ALS inhibitor chlorimuron, all in association with glyphosate, promoted significant reductions in plant height compared to the control without herbicide (table 3). The average reduction in height value imposed by these treatments reached values of 4.81%, with no differences among these three herbicide treatments. The other herbicide treatments did not differ from the control regarding the final plant height. It is noteworthy that the height of plants can influence certain parameters of the soybean crop, such as the potential for plant lodging or yield losses in the mechanized harvesting operation due to the presence of pods at lower heights concerning the height work of the harvester cutting deck (8).

None of the herbicide treatments applied postemergence resulted in decreases in the range of plants evaluated at harvest (table 3). In general, when the herbicide is registered for use in the crop and its positioning is followed correctly, it is unlikely that the plants will die, causing reductions in the final population of the crop. Regarding the evaluations of yield components, as well as soybean grain yield, there were no significant differences among treatments (table 4). In this sense, it is observed that the occurrence of foliar injuries associated with the reduction in the size of plants caused by some herbicide treatments was not enough to affect the productive response of soybean cultivar M7110 IPRO®.

**Table 4.** Number of pods per plant (NPP), the mass of 100 grains (M100G), and soybean grain yield (cultivar: M7110 IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 4.** Número de vainas por planta (NPP), masa de 100 granos (M100G) y rendimiento de grano de soja (cultivar: M7110 IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | NPP | | M100G (g) | | Yield (kg ha$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| Control without herbicide | - | 30.90 | a | 18.62 | a | 4177 | a |
| Glyphosate | 1176 | 27.10 | a | 18.85 | a | 4390 | a |
| Glyphosate + bentazon | 1176 + 600 | 27.50 | a | 18.51 | a | 3776 | a |
| Glyphosate + fomesafen | 1176 + 175 | 28.67 | a | 18.71 | a | 4075 | a |
| Glyphosate + lactofen | 1176 + 120 | 25.60 | a | 18.95 | a | 4314 | a |
| Glyphosate + imazethapyr | 1176 + 100 | 32.50 | a | 18.78 | a | 4008 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 30.90 | a | 18.62 | a | 4238 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 29.05 | a | 18.89 | a | 4135 | a |
| $F_{Value}$ | | 1.84$^{ns}$ | | 0.10$^{ns}$ | | 0.86$^{ns}$ | |
| CV (%) | | 11.65 | | 4.95 | | 9.98 | |

a.i. = active ingredient. $^{ns}$ Not significant by the F test (*p≤0.05*).

i.a. = ingrediente activo. $^{ns}$ No significativo por la prueba F (*p≤0,05*).

This fact can be explained by the regular rainfall in the months after spraying the treatments (figure 1, page 88), which may have promoted greater crop recovery capacity after light foliar stresses caused by the herbicides used in the experiment, preserving the yield components (number of pods per plant and mass of 100 grains), as well as grain yield. Work carried out by Alonso *et al.* (2010, 2011, 2013) also demonstrated the selectivity of these herbicides to soybean crops. Despite this, selectivity studies are always necessary, since genetic variations of cultivars are one of the main factors influencing the greater or lesser tolerance of plants to a particular active ingredient (19).

Based on the results obtained in the study with this cultivar, it is evident that all herbicide associations applied postemergence showed selectivity for M7110 IPRO® at the doses and application stage in which they were used. In addition, it appears that the cultivar M7110 IPRO® has good adaptability to cultivation in the region where the experiment was conducted since it presented high yield levels.

### Experiment II: Selectivity of herbicide associations applied postemergence to the soybean cultivar Foco IPRO®

Cultivar Foco IPRO® showed differential susceptibility to the variable injury level, with significant differences among herbicide treatments and the control without application (table 5). In the evaluation carried out at 7 DAA, glyphosate in association with lactofen, as well as in association with ALS-inhibitors herbicides, imazethapyr, chlorimuron, and cloransulam, provided the highest percentage of injuries to soybean plants, at levels ranging from 8,75 to 12.50%. The application of glyphosate isolated and the associations of this herbicide with bentazon or fomesafen at 7 DAA resulted in lower levels of intoxication than the other herbicide treatments but were still higher than the control.

**Table 5.** Injury level of soybean (cultivar: Foco IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 5.** Fitointoxicación de la soja (cultivar: Foco IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | Injury level (%) | | | |
|---|---|---|---|---|---|
| | | 7 DAA | | 28 DAA | |
| Control without herbicide | - | 0.00 | a | 0.00 | a |
| Glyphosate | 1176 | 3.75 | b | 1.25 | a |
| Glyphosate + bentazon | 1176 + 600 | 5.00 | b | 0.00 | a |
| Glyphosate + fomesafen | 1176 + 175 | 6.25 | b | 2.50 | a |
| Glyphosate + lactofen | 1176 + 120 | 10.25 | c | 6.25 | b |
| Glyphosate + imazethapyr | 1176 + 100 | 12.50 | c | 10.00 | c |
| Glyphosate + chlorimuron | 1176 + 10 | 10.75 | c | 5.75 | b |
| Glyphosate + cloransulam | 1176 + 39.5 | 8.75 | c | 5.75 | b |
| $F_{Value}$ | | 12.98* | | 14.75* | |
| CV (%) | | 32.37 | | 47.15 | |

a.i. = active ingredient; DAA = Days after application. * Significant by F test ($p\leq0.05$). Means followed by different letters in the column differ from each other by the Scott-Knott test ($p\leq0.05$).

i.a. = ingrediente activo; DAA = días después de la aplicación. * Significativo por prueba F ($p\leq0,05$). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott ($p\leq0,05$).

Regarding the results obtained in the evaluation carried out at 28 DAA, the association of glyphosate with imazethapyr provided the highest level of injury for this cultivar (10.00%) (table 5), with leaf chlorosis being the main observed visual symptom. Following the treatments with higher levels of injuries, lactofen, chlorimuron, and cloransulam were all applied in association with glyphosate. Furthermore, in this evaluation, the ability of the cultivar Foco IPRO® to recover from the phytotoxicity caused by these previously mentioned herbicides inhibiting PPO and ALS was evident. According to Oliveira Jr. *et al.* (2008), the effects of applying glyphosate to soybeans with RR® technology may vary according to the genotype, the time of application, and the dose of herbicide used.

The data referring to the evaluations of plant height (50 DAE and harvest) and plant stand at harvest are presented in table 6 (page 93). Regarding the evaluation of plant height, the herbicide treatments did not cause changes in the plant height of soybean (cultivar Focus IPRO®) in both evaluations, with no significant differences from the control without application. With the above, there is a quick recovery of the stresses promoted by the herbicides applied postemergence, detected in the visual evaluations of injury levels, allowing that the vegetative growth of the plants was not negatively affected.

**Table 6.** Height and stand of soybean plants (cultivar: Foco IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 6.** Altura y soporte de plantas de soja (cultivar: Foco IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | Plant height (cm) | | | | Stand (plants m$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | 50 DAE | | Harvest | | 50 DAE | |
| Control without herbicide | - | 69.35 | a | 105.10 | a | 13.61 | a |
| Glyphosate | 1176 | 67.20 | a | 104.45 | a | 13.38 | a |
| Glyphosate + bentazon | 1176 + 600 | 66.35 | a | 104.20 | a | 13.88 | a |
| Glyphosate + fomesafen | 1176 + 175 | 66.00 | a | 102.80 | a | 13.46 | a |
| Glyphosate + lactofen | 1176 + 120 | 63.45 | a | 102.20 | a | 13.08 | a |
| Glyphosate + imazethapyr | 1176 + 100 | 60.85 | a | 101.35 | a | 13.77 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 63.80 | a | 104.00 | a | 14.10 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 66.75 | a | 104.35 | a | 13.16 | a |
| $F_{Value}$ | | 2.53* | | 0.64$^{ns}$ | | 0.44$^{ns}$ | |
| CV (%) | | 5.07 | | 3.11 | | 7.79 | |

a.i. = active ingredient; DAE = Days after emergence. * and $^{ns}$ Significant and non-significant, respectively, by the F test ($p≤0.05$). Means followed by different letters in the column differ from each other by the Scott-Knott test ($p≤0.05$).

i.a. = ingrediente activo; DAE = Días después de la emergencia. * y $^{ns}$ Significativo y no significativo, respectivamente, por la prueba F ($p≤0,05$). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott ($p≤0,05$).

The final stand of the plants was also not affected by the herbicide treatments (table 6), which demonstrates, once again, the safety regarding the use of these herbicides for the soybean cultivar Foco IPRO®. The evaluated herbicides did not influence yield components (number of pods per plant and weight of 100 grains) or grain yield, not showing significant differences from the control without application (table 7). Marchi *et al.* (2013) reported that the association of glyphosate (1176 g a.i. ha$^{-1}$) with chlorimuron (10 g a.i. ha$^{-1}$) applied to soybean plants at stage V2-V3 resulted in a significant reduction in the mass of 100 grains but without an impact on the grain yield. Regular and expressive rainfall observed mainly in the months of January and February favored the cultivar Foco IPRO® in the aspect of reestablishing foliar intoxication initially promoted by the herbicides, favoring the demonstration of herbicide selectivity. The cultivar Foco IPRO® presented, regardless of the herbicide treatments, yields ranging from 3,510 to 4,067 kg ha$^{-1}$, which demonstrates its good adaptability to the southwestern region of Goiás State.

**Table 7.** Number of pods per plant (NPP), mass of 100 grains (M100G) and soybean grain yield (cultivar: Focus IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 7.** Número de vainas por planta (NPP), masa de 100 granos (M100G) y rendimiento de grano de soja (cultivar: Focus IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | NPP | | M100G (g) | | Yield (kg ha$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| Control without herbicide | - | 45.70 | a | 16.76 | a | 3879 | a |
| Glyphosate | 1176 | 47.60 | a | 16.28 | a | 3935 | a |
| Glyphosate + bentazon | 1176 + 600 | 46.70 | a | 16.02 | a | 4067 | a |
| Glyphosate + fomesafen | 1176 + 175 | 47.05 | a | 16.06 | a | 3510 | a |
| Glyphosate + lactofen | 1176 + 120 | 50.00 | a | 16.05 | a | 3715 | a |
| Glyphosate + imazethapyr | 1176 + 100 | 45.60 | a | 15.92 | a | 3889 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 48.40 | a | 16.36 | a | 3835 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 49.80 | a | 15.89 | a | 4017 | a |
| $F_{Value}$ | | 0.57$^{ns}$ | | 1.54$^{ns}$ | | 1.02$^{ns}$ | |
| CV (%) | | 9.36 | | 2.87 | | 9.05 | |

a.i. = active ingredient. $^{ns}$ Not significant by the F test ($p≤0.05$).

i.a. = ingrediente activo. $^{ns}$ No significativo por la prueba F ($p≤0,05$).

**Experiment III: Selectivity of herbicide associations applied postemergence to the soybean cultivar Bônus IPRO®**

The injury level caused by the herbicide treatments for the Bônus IPRO® soybean cultivar is shown in table 8. As seen for the other cultivars, more accentuated levels of phytotoxicity occurred in the first evaluation (7 DAA), with a partial or complete recovery of most soybean plants in the later evaluation (28 DAA).

**Table 8.** Injury level of soybean (cultivar: Bônus IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 8.** Fitointoxicación de la soja (cultivar: Bônus IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. $ha^{-1}$) | Injury level (%) | | | |
|---|---|---|---|---|---|
| | | 7 DAA | | 28 DAA | |
| Control without herbicide | - | 0.00 | a | 0.00 | a |
| Glyphosate | 1176 | 2.50 | a | 0.00 | a |
| Glyphosate + bentazon | 1176 + 600 | 6.25 | b | 1.25 | a |
| Glyphosate + fomesafen | 1176 + 175 | 12.50 | c | 3.75 | a |
| Glyphosate + lactofen | 1176 + 120 | 13.75 | d | 5.00 | b |
| Glyphosate + imazethapyr | 1176 + 100 | 11.25 | c | 2.50 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 16.25 | d | 10.00 | b |
| Glyphosate + cloransulam | 1176 + 39.5 | 10.75 | c | 7.00 | b |
| $F_{Value}$ | | 22.97* | | 6.73* | |
| CV (%) | | 25.93 | | 73.71 | |

a.i. = active ingredient; DAA = Days after application. * Significant by F test (*p≤0.05*). Means followed by different letters in the column differ from each other by the Scott-Knott test (*p≤0.05*).

i.a. = ingrediente activo; DAA = días después de la aplicación. * Significativo por prueba F (*p≤0,05*). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott (*p≤0,05*).

At 7 DAA, the PPO and ALS inhibitors lactofen and chlorimuron, respectively, both associated with glyphosate, presented the highest percentages of injury levels to the soybean cultivar Bônus IPRO®, with averages of 13.75% and 16,25%, respectively, differing significantly from the other treatments and the control without herbicide application. These data corroborate the results presented by Alonso *et al.* (2010), who verified that the association of herbicides with PPO and ALS modes of action to glyphosate present symptoms of foliar phytotoxicity to soybean plants superior to other tested mixtures in evaluations carried out at 7 and 15 DAA.

The initial leaf symptoms observed after the application of these herbicides were similar to those described by Alonso *et al.* (2013), including chlorimuron chlorosis followed by necrosis in the apical leaves and chlorosis with subsequent necrosis and wrinkling for lactofen. Even at 7 DAA, the associations of glyphosate with cloransulam, imazethapyr, or fomesafen showed intermediate levels of injuries, with phytotoxicity varying from 10.75 to 12.50%, not differing from each other. The PSII inhibitor bentazon, in association with glyphosate, promoted mild injury symptoms (6.25%), characterized by small and few chlorotic and necrotic spots on the leaves.

At 28 DAA, the soybean plants of the Bônus IPRO® cultivar already showed good recovery from the symptoms of injuries; however, higher levels of injuries were observed in treatments involving the association of glyphosate with lactofen, chlorimuron, and cloransulam, where the levels were in the range of 5.00 to 10.00%. The other herbicide treatments evaluated did not show significant differences among them, with a maximum percentage of injuries of 3.75%, and were statistically equivalent to the control, which was without application.

At 50 DAE, a significant reduction was observed in the soybean plant size of the Bônus IPRO® cultivar that received a postemergence application of the association glyphosate + chlorimuron, with an average decrease of 18.31% in plant height when compared to the control without herbicide application (table 9, page 95). The glyphosate + imazethapyr and glyphosate + lactofen treatments also resulted in a lower plant height of soybean, but at a lower magnitude, with an average reduction of 7.42%. All other herbicide treatments did not significantly affect the size of soybean plants when compared to the control without herbicide application. Of the morphological variables, plant height was the most affected by herbicides applied postemergence and is an important parameter for measurement in selectivity experiments (12).

At harvest, plants of the cultivar Bônus IPRO® recovered for the variable height, with no more differences among the glyphosate + imazethapyr and glyphosate + lactofen associations and the control without herbicide application (table 9). Only the glyphosate + chlorimuron treatment provided a lower final plant height, with an average reduction of 9.56%, compared to the average height of soybean plants present in the control treatment plots without herbicides. Correia and Durigan (2007), evaluating the selectivity of eight commercial formulations of glyphosate-based products to two RR® soybean cultivars, found that none of the herbicides influenced the plant height.

a.i. = active ingredient; DAE = Days after emergence. * and ns Significant and non-significant, respectively, by the F test (*p≤0.05*). Means followed by different letters in the column differ from each other by the Scott-Knott test (*p≤0.05*).

i.a. = ingrediente activo; DAE = Días después de la emergencia. * y ns Significativo y no significativo, respectivamente, por la prueba F (*p≤0,05*). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott (*p≤0,05*).

**Table 9.** Height and stand of soybean plants (cultivar: Bônus IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 9.** Altura y soporte de plantas de soja (cultivar: Bônus IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. ha$^{-1}$) | Plant height (cm) | | | | Stand (plants m$^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | 50 DAE | | Harvest | | Harvest | |
| Control without herbicide | - | 62.15 | a | 122.05 | a | 7.69 | a |
| Glyphosate | 1176 | 63.20 | a | 120.40 | a | 7.30 | a |
| Glyphosate + bentazon | 1176 + 600 | 61.50 | a | 117.35 | a | 8.33 | a |
| Glyphosate + fomesafen | 1176 + 175 | 61.25 | a | 122.05 | a | 7.72 | a |
| Glyphosate + lactofen | 1176 + 120 | 56.70 | b | 116.70 | a | 7.47 | a |
| Glyphosate + imazethapyr | 1176 + 100 | 57.65 | b | 115.85 | a | 6.80 | a |
| Glyphosate + chlorimuron | 1176 + 10 | 50.45 | c | 107.75 | b | 8.14 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 60.70 | a | 117.60 | a | 8.25 | a |
| $F_{Value}$ | | 9.66* | | 4.50* | | 1.43$^{ns}$ | |
| CV (%) | | 4.53 | | 3.69 | | 11.29 | |

The final stand of plants for the Bônus IPRO® cultivar remained statistically the same for all treatments, with no significant reduction occurring among the experimental units that received the herbicides and the control (table 9). This result consolidates the results found for the cultivars M7110 IPRO® and Foco IPRO®, where it is verified that the evaluated herbicide treatments did not reduce the plant stand. For the variables number of pods per plant and mass of 100 grains, negative changes were not verified in the soybean plants of the cultivar Bônus IPRO® due to the application of postemergence herbicides (table 10, page 96). This result differs from the study carried out by Alonso *et al.* (2011), where it was verified that the association of glyphosate and lactofen promoted a significant reduction in the mass of 100 grains.

Regarding grain yield, the treatments isolated glyphosate, glyphosate + lactofen, and glyphosate + imazethapyr provided significant reductions in this parameter of 8.99%, 6.62%, and 9.67%, respectively, compared to the grain yield recorded in the control without herbicide application (table 10, page 96). These results demonstrate the lack of selectivity of these herbicide treatments for use in production areas that use this cultivar. According to Constantin *et al.* (2016), the attenuation of negative effects, such as foliar injuries, caused by the herbicide glyphosate in genetically modified soybean cultivars (RR®) can be minimized, maintaining the crop's yield potential through the use of biostimulant products. The other herbicide treatments did not damage the grain yield of the cultivar Bônus IPRO®, which presented yields ranging from 3,288 to 3,903 kg ha$^{-1}$, regardless of the evaluated herbicide treatments.

**Table 10.** Number of pods per plant (NPP), mass of 100 grains (M100G) and soybean grain yield (cultivar: Bônus IPRO®) after application of post-emergence herbicide associations. Rio Verde (Brazil), 2020/2021.

**Tabla 10.** Número de vainas por planta (NPP), masa de 100 granos (M100G) y rendimiento de grano de soja (cultivar: Bônus IPRO®) después de la aplicación de asociaciones de herbicidas de postemergencia. Rio Verde (Brasil), 2020/2021.

| Treatments | Doses (g a.i. $ha^{-1}$) | NPP | | M100G (g) | | Yield (kg $ha^{-1}$) | |
|---|---|---|---|---|---|---|---|
| Control without herbicide | - | 69.70 | a | 22.00 | a | 3694 | a |
| Glyphosate | 1176 | 66.65 | a | 20.25 | a | 3288 | b |
| Glyphosate + bentazon | 1176 + 600 | 60.90 | a | 22.50 | a | 3903 | a |
| Glyphosate + fomesafen | 1176 + 175 | 60.50 | a | 20.25 | a | 3707 | a |
| Glyphosate + lactofen | 1176 + 120 | 60.40 | a | 21.00 | a | 3449 | b |
| Glyphosate + imazethapyr | 1176 + 100 | 64.80 | a | 21.25 | a | 3337 | b |
| Glyphosate + chlorimuron | 1176 + 10 | 57.85 | a | 21.75 | a | 3607 | a |
| Glyphosate + cloransulam | 1176 + 39.5 | 68.55 | a | 21.00 | a | 3746 | a |
| $F_{Value}$ | | $1.31^{ns}$ | | $0.79^{ns}$ | | 2.51* | |
| CV (%) | | 11.94 | | 8.48 | | 7.54 | |

a.i. = active ingredient. * and $^{ns}$ Significant and non-significant, respectively, by the F test ($p \leq 0.05$). Means followed by different letters in the column differ from each other by the Scott-Knott test ($p \leq 0.05$).

i.a. = ingrediente activo. * y $^{ns}$ Significativo y no significativo, respectivamente, por la prueba F ($p \leq 0,05$). Medias seguidas de letras diferentes en la columna difieren entre sí por la prueba de Scott-Knott ($p \leq 0,05$).

## Conclusion

The stand of plants at harvest of soybean cultivars M7110 IPRO®, Foco IPRO®, and Bônus IPRO® was not decreased by postemergence applications of glyphosate, either isolated or in association with bentazon, fomesafen, lactofen, imazethapyr, chlorimuron, and cloransulam.

None of the evaluated herbicide treatments caused a reduction in the grain yield of cultivars M7110 IPRO® and Foco IPRO®; however, the herbicides lactofen and chlorimuron, in association with glyphosate, were the ones that caused the highest levels of phytotoxicity to M7110 IPRO®, and the combination of glyphosate with imazethapyr caused greater phytotoxicity in the cultivar Foco IPRO®.

Post-emergence application of glyphosate was isolated, and the associations of glyphosate + lactofen and glyphosate + imazethapyr decreased the grain yield of the Bônus IPRO® soybean cultivar.

The results of the present work reinforce the need for care in choosing the soybean cultivar and the selection of the herbicides to be applied postemergence, as the genetics of the material are decisive for the response in terms of sensitivity to the applied herbicides.

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# Control capacity of the LPSc 1067 strain of *Beauveria bassiana* (Ascomycota: Hypocreales) on different species of grasshoppers (Orthoptera: Acrididae: Melanoplinae), agricultural pests in Argentina

## Capacidad de control de la cepa LPSc 1067de *Beauveria bassiana* (Ascomycota: Hypocreales) sobre diferentes especies de tucuras (Orthoptera: Acrididae: Melanoplinae), plagas del agro de Argentina

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**Abstract**

Grasshoppers affect agriculture worldwide, causing serious economic damage. Currently, the application of chemical insecticides against grasshoppers is the only effective strategy, even considering the significant environmental concern. This study aimed to test the entomopathogenic fungi *Beauveria bassiana* (LPSc 1067) as biocontrol agent on six harmful grasshopper species in Argentina. Significant differences were observed (DF= 5; F= 9.93; P<0.0001) when considering *B. bassiana* pathogenicity on third-instar nymphs of the different grasshopper species. The highest mortality (100%) was registered on *Trimerotropis pallidipennis* and *Dichroplus maculipennis* nymphs while the lowest mortality (48.6 ±3.5%) was observed on *Scotussa lemniscata* nymphs. The lowest mean survival time (MST) was recorded for *T. pallidipennis* (3.5 ±0.15 days) and the highest MST was observed on *Dichroplus pratensis* nymphs (7.48 ±0.28 days). Results suggest that *B. bassiana* LPSc 1067 may constitute an excellent candidate to be further studied as biological control agent of *T. pallidipennis* and *D. maculipennis*.

**Keywords**
entomopathogenic fungi • biocontrol • insect pests

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## Resumen

Las tucuras causan graves pérdidas económicas en la agricultura a nivel mundial. En la actualidad, los insecticidas químicos siguen siendo el único medio utilizado para el control de acridios, pero los efectos de su utilización son ambientalmente preocupantes. El objetivo de este trabajo fue probar la eficacia de la cepa *Beauveria bassiana* (LPSc 1067) sobre seis especies de tucuras consideradas plagas de Argentina. En cuanto a la patogenicidad de *B. bassiana* sobre ninfas de tercer estadio de las diferentes especies tratadas, se encontraron diferencias significativas (DF= 5; F= 9.93; P<0.0001). Los valores de mortalidad más altos (100%) se registraron en ninfas de *Trimerotropis pallidipennis* y *Dichroplus maculipennis* y la mortalidad más baja se observó en ninfas de *Scotussa lemniscata* con una mortalidad de 48,6 +3,5%. El tiempo medio de supervivencia (MST) más bajo se registró para *T. pallidipennis* (3,5 ±0,15 días) y el MST más alto se observó en ninfas de *Dichroplus pratensis* (7,48 ±0,28 días). Los resultados sugieren que *B. bassiana* LPSc 1067 constituye un excelente candidato para ser estudiado en profundidad como agente de control biológico de *T. pallidipennis* y *D. maculipennis*.



**Palabras clave**
hongos entomopatógenos • biocontrol • insectos plaga


## Introduction

In Argentina, Melanoplinae grasshoppers represent one of the most relevant (and numerous) subfamilies within the Acrididae family (Insecta Orthoptera). Several species in this subfamily are considered plagues (2, 12). These species cause serious damage to grasslands and economically important crops such as maize, soybean, and wheat, among others (1, 14). Since the mid-nineteenth century, these insects have been reported in several regions of Argentina, following the progressive agricultural development of the country. So far, synthetic insecticides are still the only alternative against grasshoppers, regardless of negative environmental consequences (5).

In this sense, entomopathogens acting as biocontrol agents have been considered excellent alternatives to chemical control. Fungi are among the most important entomopathogens, naturally regulating insect populations widely found in multiple types of environments (9, 23). More than 700 species of entomopathogenic fungi have been described worldwide. Nevertheless, only a few have been found to affect grasshoppers. *Beauveria bassiana* (Balsamo) Vuillemin, *Entomophaga grylli* (Fresenius) Batko, *Metarhizium anisopliae* (Metsch.) Sorokin and *Metarhizium flavoviridae* Gams & Rozsypal are the most frequently observed fungal species infecting acrididae (10). Furthermore, *B. bassiana* has been reported to cause natural epizootics in grasshoppers, in different geographical regions (4). However, in Argentina, only a few records mention acridids naturally infected with *B. bassiana* (16). This work aimed to test the efficacy of the strain *B. bassiana* (LPSc 1067) on six grasshopper species in Argentina.

## Materials and Methods

### Insect collecting

*Dichroplus maculipennis* (Blanchard 1851), *Dichroplus elongatus* (Giglio-Tos 1894), *Dichroplus pratensis* (Bruner 1900), *Scotussa lemniscata* (Stal 1861), *Ronderosia bergi* (Stal 1878) individuals were collected from the southern Pampas region (Laprida county, Buenos Aires province, Argentina, 37°32'60'' S, 60°49'00'' W). *Trimerotropis pallidipennis* (Burmeister 1838) individuals were sampled from the locality of Salinas de Bustos, in La Rioja province. The insects were kept in a rearing room under controlled conditions (30°C, photoperiod 14-10 h light-dark, 40% RH) as previously described (13). Different bioassays used first laboratory generations [F1].

**Pathogenicity assays**

*B. bassiana* strain LPSc 1067 (GeneBank accession number KF500409) was isolated in 2008 from a katydid (Orthoptera: Tettigoniidae), closely related to the long-horned grasshopper. The strain was collected at Salinas de Bustos, (30°18'9.4" S, 67°34'40.6" W), La Rioja province, Argentina, where high temperatures and low humidity are unfavourable for fungal development (8, 23). After isolation, the strain was deposited at the Spegazzini Institute culture collection. Conidia were obtained from cultures on potato-dextrose-agar medium after incubation for 10 days at 25°C in the dark (7). They were later harvested with disposable cell scrapers (Fisherbrand®) and placed in test tubes containing 0.01% (v/v) Tween 80 (Merck). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and concentration was adjusted to 1 x $10^8$ conidia/ml using a Neubauer hemocytometer according to Prior *et al.* (1995). Conidia viability was determined after 24 h, as described by Lane *et al.* (1988). This germination test was repeated for each stock suspension. Nine replicates (on different dates) of 10 third-instar nymphs of each grasshopper species, were sprayed with about 1 ml of conidial suspension using a 35-ml glass atomizer, according to Prior *et al.* (1995). Three additional control replicates per species, each with 10 grasshoppers, were sprayed with 1 ml 0.01% [v/v] Tween 20. Groups of 10 individuals were kept in acetate tubes of 50 x 9 cm and fed with lettuce, cabbage leaves and wheat bran (6). Treated and control insects were kept at 30°C, 60% relative humidity, and 14:10 h light:dark photoperiod. Cumulative mortality was recorded for 10 days. Dead grasshoppers with no external mycelia were surface-sterilized by successive dipping in 70% ethanol (10-15 s), 0.5% sodium hypochlorite solution (1 min), and sterile distilled water (1 min, two consecutive baths) according to Vega *et al.* (2012). Next, insects were placed in sterile culture chambers consisting of a Petri dish (60 mm diameter) with a filter-paper disk periodically moistened with sterile distilled water and incubated at 25°C in the dark. Mycosis was confirmed by microscopic examination of dead grasshoppers.

**Statistical analysis**

Mortality data were subjected to one-way ANOVA, after checking assumptions were met. Mean comparisons were assessed by the Tukey test (P = 0.05). Analyses were performed with InfoStat 2011 software (3). For mortality equal to or higher than 50%, median survival time (MST) was calculated based on the Kaplan-Meier Survival distribution function (25). Pairwise comparisons between survival curves were made by Long-rank Test (P<0.0001).

## Results

Significant differences were observed when assessing pathogenicity of *B. bassiana* (LPSc 1067) on third-instar nymphs (DF= 5; F= 9.93; P<0.0001). The highest mortality (100%) was registered in third-stage nymphs of *T. pallidipennis* and *D. maculipennis* (figure 1, page 101). The lowest mortality (50 ± 3.5%) was observed in nymphs of *S. lemniscata* (figure 1, page 101). Further mortalities ranged between 70 ± 8.3% on *D. pratensis* and 80 ± 5.7% on *R. bergi* (figure 1, pag 101). Controls recorded no mortality. Besides, significant differences in MST were observed according to the log-rank test (P<0.0001). The lowest MST was observed on *T. pallidipennis* nymphs at 3.5 ± 0.15 days while the highest MST was observed on *D. pratensis* nymphs with 7.48 ± 0.28 days MST (table 1, page 101).


Different letters denote significant differences between treatments according to the Tukey test (P<0.05).

Letras distintas indican diferencias significativas entre tratamientos de acuerdo con el test de Tukey (P<0,05).

**Figure 1.** Mean mortality (percent ± SD) on third-instar nymphs of different grasshopper species with 1x10$^{8}$ conidia/ml of *B. bassiana* (LPSc 1067) strain.

**Figura 1.** Porcentaje de mortalidad ± DS sobre ninfas de tercer estadio, de las diferentes especies de tucuras plagas cuando sobre ellas fue aplicada una concentración de 1x10$^{8}$ conidios/ml de la cepa (LPSc 1067) de *B. bassiana*.

**Table 1.** Median survival time (MST) expressed in days, on third-instar nymphs for each evaluated grasshoppers species.

**Tabla 1.** Tiempo medio de supervivencia (MST) expresado en días, sobre ninfas de tercer estadio de cada una de las especies de tucuras evaluadas.

Different letters indicate significant differences according to the Long-rank Test (P<0.0001).

Letras diferentes indican diferencias significativas según Long-rank Test (P<0,0001).

| Species | Median survival time (MST) |
|---|---|
| *Trimerotropis pallidipennis* | 3.5 ± 0.15 **a** |
| *Ronderosia bergi* | 5.13 ± 0.25 **b** |
| *Dichroplus maculipennis* | 5.96 ± 0.26 **b** |
| *Dichroplus elongatus* | 6.54 ± 0.31 **b c** |
| *Scotussa lemniscata* | 7.33 ± 0.22 **c** |
| *Dichroplus pratensis* | 7.48 ± 0.28 **c** |

## Discussion

Entomopathogenic fungi comprise important pathogens of insect pests. Some advantages to consider in control programs consist of their high specificity, contact transmission, natural dispersion, safety for non-target organisms and the ability to maintain lasting control once established in the environment (24). The present study determined pathogenicity of *B. bassiana* (LPSc 1067) strain on six harmful grasshopper species in Argentina. *T. pallidipennis* and *D. maculipennis* resulted the most susceptible, exhibiting 100% mortality, while the least affected grasshopper species was *S. lemniscata,* with 50%

mortality. These results agree with those obtained by Pelizza *et al.* (2012a), who evaluated the association between enzymatic activity and fungal virulence in 59 entomopathogenic fungal isolates native to Argentina. Isolate LPSc 1067 caused the highest mortality on *Tropidacris collaris* nymphs (97.7 ± 1.22%), nine isolates caused no mortality, while the remaining 49 caused mortalities ranging between 6.6 ± 0.3% (LPSc 770) to 91.06 ± 1.51% (LPSc 906). Furthermore, another study showed laboratory effectiveness of 26 fungal strains (isolated from insects and soil in Argentina) against *Schistocerca cancellata* (Serville) (Orthoptera: Acrididae) (18). These authors also studied the association between chitinase, protease, and lipase levels in these fungi and their insecticidal activities. They observed that *B. bassiana* (isolate LPSc 1067) caused the highest mortality (90 ± 1.03 %) while exhibiting the highest values of chitinolytic, proteolytic and lipolytic activity (6.13 ±0.05; 2.56 ± 0.11, and 2.33 ± 0.47, respectively) and the lowest median survival time (MST) (5.96 days).

The study by Schaefer *et al*. (1936) demonstrated that, in the laboratory, *B. bassiana* infects grasshoppers and all locust nymphs and adults sprayed with conidia. Mortalities caused by the fungi were registered within 5-20 days. Regarding the MST, our results agree with those obtained by Roberts and Hajek (1992), who observed MST values between 4.1 and 7.9 days when applying *B. bassiana* conidia on *Melanoplus sanguinipes* (Fabricius) adults. Also, results agree with those reported by Prior *et al*. (1995) who during various experiments concerning inoculation protocols, observed 95% mortality within 4-5 days using conidial suspension with $1 \times 10^7$ and $1 \times 10^8$ conidia/ml concentrations. On the other hand, *Uvarovistia zebra* (Uvarov) (Orthoptera: Tettigoniidae) treated with $5 \times 10^6$ conidia/ml of *B. bassiana* showed a cumulative mortality of 57.7% (15), while other authors evaluated the effect of *B. bassiana* (LPSc 1067) on nymphal developmental time, fecundity, and survival of *D. maculipennis* and *R. bergi* under laboratory conditions (19), and observed altered adult survival after infection, with a fungal concentration of $1 \times 10^3$ conidia/ml. Mortality of *D. maculipennis* during third through sixth-instar (last) was significantly higher among treated nymphs (66 ± 3.8%) than in controls (15 ± 1.7%). Similarly, mortality in *R. bergi* during third through fifth instar (last) was higher in treated nymphs (71 ± 2.8%) than in controls (19 ± 1.5%).

## Conclusions

The fungal isolate LPSc 1067of *B. bassiana*, could act as a biological controller of grasshopper pests *T. pallidipennis* and *D. maculipennis* in Argentina. Nevertheless, a greater number of laboratory and, fundamentally, field studies should confirm future investigations.

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# Serological relationships among strains of grapevine leafroll-associated virus 4 reflect the evolutive behavior of its coat protein gene

## Las relaciones serológicas y la identidad molecular de variantes de grapevine leafroll-associated virus 4 reflejan el comportamiento evolutivo del gen de su proteína de cubierta

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### Abstract

This research studied serological relationships and genetic diversity of Argentinean isolates of grapevine leafroll-associated virus-4 (GLRaV-4). Phylogenetic analysis of coat protein (CP) sequences from 19 local isolates revealed clustering with the previously described GLRaV-4 strain 5, strain 6, and strain 9 groups. Evolutionary sequence analysis of the obtained and database-available sequences showed evidence of recombination events. Additionally, both CP N- and C-terminal regions appeared to be under purifying selection, but the N-terminal region presented seven sites under positive selection, with a $d_N/d_S$ ratio 5-fold greater than that of the C-terminal region. Serological reactivity against monoclonal antibodies supports a higher occurrence probability for linear epitopes in the N-terminal region, as inferred by the sequence analysis. The obtained results reflect an unusual evolutionary behavior of the CP that, together with protein serological reactivity, suggests biological significance of the observed variability.

**Keywords**

molecular characterization and serology • grapevine leafroll disease • ampelovirus • selection pressure • antigenic properties

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### Resumen

Las relaciones serológicas y la diversidad genética de cepas argentinas de grapevine leafroll associated virus 4 (GLRaV-4) fueron analizadas. El análisis filogenético de la cápside proteica (CP) conducido sobre las secuencias obtenidas mostró un agrupamiento de las primeras con GLRaV-4 raza 5, GLRaV-4 raza 6 y GLRaV-4 raza 9. El análisis evolutivo de las secuencias locales y las disponibles en bases de datos infirió eventos de recombinación y sugirió que tanto los extremos C-terminal como N-terminal de la CP están bajo presión de selección purificante, pero la región N-terminal mostró siete sitios bajo presión de selección positiva, con una relación $d_N/d_S$ cinco veces mayor que aquellas posiciones de la región C-terminal. La reactividad serológica contra anticuerpos monoclonales sustenta la probabilidad de ocurrencia de epitopes lineales en la región N-terminal inferida en el análisis evolutivo. Los resultados obtenidos reflejan un comportamiento evolutivo inusual de la CP y junto con la reactividad serológica de dicha proteína, permiten postular una significancia biológica de dicha variabilidad.



**Palabras clave**

caracterización molecular y serología • enfermedad del enrollado de la hoja de la vid • ampelovirus • presión de selección • propiedades antigénicas


## Introduction

Grapevine Leafroll Disease (GLD) is one of the most widespread and deleterious viral diseases affecting grapevines with a particularly complex etiology. The development of serological reagents led to the identification of seven putative species belonging to the Closteroviridae family and generically named grapevine leafroll-associated virus (GLRaV) 1-7. However, during the 1990s and 2000s, the easier acquisition of genetic data arose the number of new putative species to 12 GLRaVs, tentatively or definitively assigned to the *Ampelovirus* genus (most of the species), to the *Closterovirus* genus (GLRaV-2) or the newly defined *Velarivirus* genus (GLRaV-7) in the *Closteroviridae* family. Generally, the main criteria establishing newly described viral isolates as new species were, first, the lack of serological reaction against previously developed monoclonal antibodies or polyclonal antiserum, and second, over 10% divergence in the sequence of taxonomic relevant genes (HSP70h and CP). The proliferation of taxonomic entities required a revision of the GLRaVs taxonomy, and considering available genetic information, it was established that most of the GLRaVs described at an early stage (GLRaV-4, -5, -6, -9, -De, -Pr and -Carn) should be considered as divergent isolates of a single species (GLRaV-4) (21).

In addition to the above-mentioned taxonomic issues, the serological relationships among the GLRaV-4 groups of isolates (previously known as different species) remain unclear. Gugerli (2009) performed an extensive review of the different serological reagents developed during the past 30 years and highlighted some of the arisen ambiguities concerning antibodies and antisera. However, scarce information followed this review, being a serological characterization of four isolates of GLRaV-4 (1) the most comprehensive work up to date. Western blot reactivity among those four isolates (two of GLRaV-4 strain 6 and one of each strain 4 and 5) against monoclonal antibodies (Mabs) raised against GLRaV-4 strain 4, 5 and 6, was clear and straight, without any cross-reaction among them. Meanwhile, all four isolates reacted against a monoclonal mix developed for generic detection of GLRaV-4 (2) and a commercial polyclonal antiserum anti-GLRaV-4 strain 5.

The complete genome sequencing of different GLRaV-4 strains allowed clarifying the taxonomy of this virus. Nevertheless, the significance of CP antigenic properties of the different GLRaV-4 isolates remains unclear.

The present work presents a sequence analysis of the CP gene of Argentinean GLRaV-4 isolates, and an additional serological analysis of purified virions, aiming to establish significance levels of such genetic and serological variability.

## Material and Methods

### Virus Isolates

One hundred forty-one grapevine plants of different varieties exhibiting mild to severe symptoms of grapevine leafroll disease (GLD) were selected from the ampelographic collection located in the Mendoza Research Station of the National Institute of Agronomic Technology (EEA Mendoza, INTA). In order to determine symptomatic nature, these plants were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) using commercial reagents for GLRaV-1, -2, -3, -4, -4 strain 6, and -7. The study included positive samples for GLRaV-4 or GLRaV-4 strain 6 (24 samples) and those negative for all tested viruses (13 samples).

### RNA extraction, RT-PCR, cloning, and sequencing

Double-stranded RNA (dsRNA) was extracted from cambial scrapings of mature grapevine canes, reverse-transcribed, and subsequently amplified by Polymerase Chain Reaction (PCR) using a proofreading polymerase (DeepVent DNA Polymerase, New England Biolabs, USA). The complete Coat Protein (CP) Open Reading Frame (ORF) of GLRaV-4 was amplified using specific primers (ACPF 5'-GCTGGATAGGTTYAGRTCNAAAGAYACYCC-3' and ACPR 5'-TAACCTCCATATTTTCAAACG-3') designed over the upstream and downstream sequences of such ORF (p60 and p23, respectively) based on database available nucleotide sequences of GLRaV-4. The resulting PCR products were resolved by agarose gel electrophoresis. The occurrence of multiple infections with GLRaV-4 genetic variants in a single plant was investigated by RT-PCR-Restriction Fragment Length Polymorphism (RFLP), digesting the resulting PCR products with both AluI and HinfI restriction enzymes. Restriction fragments were resolved by electrophoresis on a 2% agarose gel. Undigested PCR products were cloned into the pGEM-T Easy Vector System I, and the resulting clones were sequenced. After blue/white screening, 19 white colonies from each transformation were selected, amplified with ACPF and ACPR, and restricted as mentioned for accurate identification of clones of different genetic variants. Three colonies belonging to each restriction pattern by sample were randomly selected and sequenced using both pUC/M13 reverse and forward sequencing primers at Macrogen Inc. (Korea).

### Sequence analysis

Sequences from each clone were assembled and edited obtaining the coding sequence of the CP ORF. Codon multiple sequence alignment was performed using the aligned codons from these sequences, together with all the GLRaV-4 CP sequences available in the NCBI GenBank database. Using the HyPhy software package, evolutionary and phylogenetic analyses evaluated whether selection pressure affects viral strain evolution (16). Recombination analysis was performed using Single Breakpoint (SBP), Genetic Algorithm Recombination Detection (GARD), and confirmed by RDP software (19). Three methods, namely Single Likelihood Ancestor Counting (SLAC), Random Effects Likelihood (REL), and Fixed Effects Likelihood (FEL), allowed the identification of selection pressure. Results were integrated by integrative selection analysis. The aligned codons (with additional CP sequence of PMWaV-1 (AF414119) as outgroup) were subjected to preliminary phylogenetic reconstruction by Maximum Likelihood (ML) analysis using the PAUP software package (28) and heuristic search, with random addition sequences considering one hundred replicates. Group support was estimated by Garli program (31) generating 1000 replicates to obtain bootstrap values. Branches with bootstrap values under 70% were collapsed. From the phylogenetic tree inferred, seven clusters were defined. Sequences belonging to the different strains of GLRaV-4 allowed a clear identification of such groups: GLRaV-4 strain 5 (AF233934), strain 9 (AY297819), strain 6 (FJ467504), strain 4 (FJ467503), strain Ob (AB720874), strain Carn (FJ907331), and strain Pr (FM244690). The genetic distance within and among groups was estimated using the Tamura-Nei model of MEGA5 software by estimating the standard error from a bootstrap of 1000 replicates. Overall distance of GLRaV-4, and genetic distances for all the available CP sequences of GLRaV-1, -2 and -3 were also estimated.

The presence of putative linear epitopes over the deduced amino acid CP sequence was evaluated by BepiPred software (18). Complementary to epitope detection and through the SomeNA and SNAP2 tools implemented in the Predict Protein server, a structure prediction analysis identified a putative nucleic acid binding motif and the functional effect of a point mutation in CP (30).

### Serological characterization

After the ELISA-positive samples for GLRaV-4 or GLRaV-4 strain 6, nineteen samples were selected for further serological assays based on the restriction pattern of observed CP. Viral particles were purified from cortical scrapings of mature grapevine canes as described by Savino (1993). The resulting extracts were analyzed to determine serological characteristics of the GLRaV-4 variants. The purified virions were resolved over 30 mm wide lanes into a 14%/4% SDS PAGE, electroblotted to nitrocellulose membrane. After blocking the membrane, ten individual longitudinal strips from each lane were excised. Each strip (3 mm wide) was probed with each of seven monoclonal antibodies: Mab 36-117, Mab3-1, Mab8-2, Mab43-1, Mab3-3, Mab6-3, Mab 15-5 (12, 13) and three polyclonal antisera: ASGLRaV-5 from Biorad (Hercules, CA, USA), AS GLRaV-4 strain-6 from Bioreba AG (Switzerland) and AS GLRaV-4 I252-IL (provided by Dr. Boscia, 2006). The strips were revealed after incubation with Goat-AntiMouse AP conjugated or Goat-AntiRabbit AP conjugated (Sigma, MI, USA).

## Results

Out of the 141 ELISA-tested samples exhibiting leafroll disease symptoms, 13 samples reacted with none of the tested reagents. When considering reactions against GLRaV-4 and GLRaV-4 strain 6 reagents, 10 samples resulted positive with both antibodies, 12 only reacted with the GLRaV-4 reagents, and 2 samples only reacted with the GLRaV-4 strain 6 reagents. These 37 samples were examined by RT-PCR using the primers described above. No product was amplified from the 13 ELISA-negative samples, while a product of the expected size (1,100 bp) was generated from all 24 ELISA-positive samples.

Restriction of the RT-PCR products yielded several fragments in all cases (Supplementary Figure 1). In some cases, band number and size indicated presence of a single genetic variant, while in other cases, the digestion of multiple PCR products had the same size but different sequence. Cloning and screening of these RT-PCR products using PCR and restriction over the white colonies allowed to identify the genetic variants in the original sample. When two samples shared the same restriction pattern and serological behavior, only one was sequenced. Three colonies corresponding to each restriction pattern were sequenced from samples with multiple patterns, considering a total of 90 clones sequenced from 19 different plant samples. Generally, sequences of clones sharing the same restriction pattern obtained from the same plant were identical and considered a single sequence. Consequently, this study generated 30 sequences (Supplementary Table 1). One single ORF was identified in each sequence, sizing according to GLRaV-4 CP. Most sequences produced a 269-amino acid translation product, but some sequences exhibited minor size divergences (individual sequences of 265, 268, 271, and 272 amino acids). Codon multiple alignment revealed deletions in the first 40 amino acids of the protein (shorter sequences) or a mutation in the stop codon leading to size differences.

### Sequence analysis

The 164 sequences of GLRaV-4 used considered 30 CP sequences obtained in this study and 134 sequences available in NCBI Genbank database. The phylogenetic tree inferred by ML analysis discriminated seven monophyletic groups supported by high bootstrap values (Supplementary Figure 2). The seven GLRaV-4 described strains (strain 4, strain 5, strain 6, strain 9, strain Ob, strain Pr, strain Carn) represent the seven groups. This phylogeny agrees with the previously reported phylogeny of the HSP70 of GLRaV-4 (20). Most of the local sequences obtained in this study clustered with the reference sequences of GLRaV-4 strain 5 and GLRaV-4 strain 6, while only one sequence grouped with GLRaV-4 strain 9. No local sequence clustered with GLRaV-4 strain 4, strain Pr, strain Ob or strain Carn.

Table 1 shows the amino acid sequence identity level within and among these seven groups. Identity among sequences inside all these groups, except GLRaV-4 strain 6, exceeded 90%. Sequences belonging to GLRaV-4 strain Ob and GLRaV-4 strain Pr were the most divergent, sharing identities under the proposed 25% divergence threshold (21), with sequences from other groups. However, when the alignment was arbitrarily split into two (first 40 residues, and from residue 40 to the end of the protein), identity levels among sequences changed substantially. The C-terminal region was conserved among all analyzed sequences. Identity level in this region within the seven groups was over 95%, while identity level among sequences of the different groups was always over 80% (table 2). Variability was considerably high in the N-terminal region of CP with identity difference levels between pairs of sequences as high as 43% within the GLRaV-4 strain 5 group, and 53% within the GLRaV-4 strain 6 group (table 2). Average genetic distances estimated for the 128 CP sequences of GLRaV-4 was 0.127, higher than GLRaV-3 but in line with GLRaV-2 (0.118) and the estimated 0.106 for GLRaV-1 (7). Both GLRaV-1 and GLRaV-2 are considered highly variable viral species.

**Table 1.** Estimates of evolutionary divergence between coat protein sequences.

**Tabla 1.** Estimaciones de divergencia evolutiva entre secuencias de proteínas de cápside proteica.

Range of aminoacidic identities per site between GLRaV4 sequences of the seven groups identified and defined according to the inferred phylogeny.

Rango de identidades aminocídicas por sitio entre secuencias de GLRaV-4 de los siete grupos identificados y definidos según la filogenia inferida.

| | **GLRaV-4 strain Pr** | **GLRaV-4 strain Carn** | **GLRaV-4 strain 9** | **GLRaV-4 strain 6** | **GLRaV-4 strain 5** | **GLRaV-4 strain Ob** | **GLRaV-4 strain 4** |
|---|---|---|---|---|---|---|---|
| GLRaV-4 strain 4 | 0.75 - 0.79 | 0.77 - 0.78 | 0.80 - 0.81 | 0.79 - 0.81 | 0.79 - 0.84 | 0.73 - 0.76 | 0.94 - 0.99 |
| GLRaV-4 strain Ob | 0.72 - 0.75 | 0.75 - 0.75 | 0.73 - 0.75 | 0.73 - 0.77 | 0.73 - 0.78 | 0.93 - 0.93 | |
| GLRaV-4 strain 5 | 0.75 - 0.78 | 0.76 - 0.79 | 0.84 - 0.87 | 0.82 - 0.87 | 0.92 - 1 | | |
| GLRaV-4 strain 6 | 0.71 - 0.75 | 0.76 - 0.79 | 0.84 - 0.87 | 0.90 - 1 | | | |
| GLRaV-4 strain 9 | 0.75 - 0.77 | 0.78 - 0.78 | 0.97 - 0.99 | | | | |
| GLRaV-4 strain Carn | 0.75 - 0.75 | - - - | | | | | |
| GLRaV-4 strain Pr | 0.97 - 0.97 | | | | | | |

**Table 2.** Estimates of evolutionary divergence between coat protein sequences considering amino-terminal and carboxyl-terminal regions.

**Tabla 2.** Estimaciones de divergencia evolutiva entre las secuencias de proteínas de cápside considerando las regiones amino-terminal y carboxi-terminal.

Range of aminoacidic identities per site between GLRaV4 sequences of the seven groups identified and defined according to the phylogeny inferred. Above the diagonal, comparison of the first 40 aminoacids. Below the diagonal, comparison from residue 41 to the end of the sequences.

Rango de identidades aminocídicas por sitio entre secuencias de GLRaV-4 de los siete grupos identificados y definidos en base a la filogenia inferida. Por encima de la diagonal, la comparación de los primeros 40 aminoácidos. Por debajo de la diagonal, comparación desde el residuo 41 hasta el final de la secuencia proteica.

| | | **GLRaV-4 strain 4** | **GLRaV-4 strain Ob** | **GLRaV-4 strain 5** | **GLRaV-4 strain 6** | **GLRaV-4 strain 9** | **GLRaV-4 strain Carn** | **GLRaV-4 strain Pr** |
|---|---|---|---|---|---|---|---|---|
| | | 0.63 - 0.92 | 0.21 - 0.29 | 0.29 - 0.47 | 0.29 - 0.45 | 0.34 - 0.39 | 0.39 - 0.47 | 0.21 - 0.33 |
| GLRaV-4 strain 4 | 0.98 - 0.96 | | 0.63 - 0.63 | 0.17 - 0.39 | 0.14 - 0.37 | 0.23 - 0.31 | 0.27 - 0.27 | 0.21 - 0.31 |
| GLRaV-4 strain Ob | 0.82 - 0.84 | 0.97 - 0.97 | | 0.57 - 1 | 0.28 - 0.60 | 0.31 - 0.54 | 0.26 - 0.34 | 0.26 - 0.44 |
| GLRaV-4 strain 5 | 0.87 - 0.90 | 0.81 - 0.84 | 0.95 - 1 | | 0.47 - 1 | 0.36 - 0.51 | 0.25 - 0.43 | 0.18 - 0.36 |
| GLRaV-4 strain 6 | 0.86 - 0.89 | 0.80 - 0.84 | 0.88 - 0.93 | 0.95 - 1 | | 0.85 - 0.94 | 0.37 - 0.40 | 0.36 - 0.38 |
| GLRaV-4 strain 9 | 0.88 - 0.89 | 0.81 - 0.82 | 0.91 - 0.94 | 0.91 - 0.94 | 0.99 - 1 | | - - - | 0.28 - 0.33 |
| GLRaV-4 strain Carn | 0.84 - 0.85 | 0.82 - 0.82 | 0.84 - 0.86 | 0.82 - 0.85 | 0.85 - 0.85 | - - - | | 0.87 - 0.87 |
| GLRaV-4 strain Pr | 0.84 - 0.86 | 0.82 - 0.83 | 0.83 - 0.86 | 0.80 - 0.83 | 0.82 - 0.83 | 0.83 - 0.84 | 0.98 - 0.98 | |

### Evolutionary analysis

The recombination analysis conducted by SBP inferred a putative recombination event over the multiple sequence alignment. Such event was also detected by using the RDP software, suggesting that GLRaV-4 strain 9 sequences were recombinants between GLRaV-4 strain 5 and GLRaV-4 strain 6. This same event was also identified when the recombination analysis was performed with complete genomic sequences of GLRaV-4 strain 5, 6 and 9 available in the database. However, GARD was unable to detect the recombination event among these three complete sequences. In consequence and to avoid a biased analysis due to the recombination effect in the selection pressure analysis, the complete alignment was split into two datasets according to the inferred breakpoint (from position 1 to 120, and from 121 to the end of the codon alignment).

Consequently, selection pressure analysis was performed for two datasets. Results of overall $d_N/d_S$ ratios obtained by SLAC and REL were consistently different for both datasets (table 3), being the mean $d_N/d_S$ ratio estimated by SLAC 5 times greater in the N-terminal region. Although all obtained ratios were lower than 1, indicating a negative or purifying selection, the C-terminal region (the most conserved region) was subjected to heavier purifying selection than the N-terminal region. When the $d_N/d_S$ ratio was analyzed for the entire CP as a single dataset, the value fell in between the partial values obtained, similar to those reported by Maliogka *et al.* (2008) (mean of 0.085 by FEL). The site-by-site analysis integrating the three individual analyses (REL, FEL, and SLAC) was different between both datasets. In the first dataset, seven sites were significantly inferred as being under positive selection by REL (two of them also identified by FEL and SLAC, Supplementary Figure 3), and 23 of the 33 remaining sites resulted under negative selection pressure by at least one method. For the second dataset (position 121-end), both methods failed to detect positive selection pressure, whereas 198 sites (over 236 codons) were inferred as being under negative selection by FEL and 190 codons by SLAC. REL could not be performed due to alignment size restrictions. In general, except for the negatively selected sites at positions 1, 5, 12, 14 20 and 21, the high prevalence of negatively selected sites begins at position 24 of the multiple codon alignment (Supplementary figure 3).

**Table 3.** Estimates of selection pressure on the coat protein gene of GLRaV-4.

**Tabla 3.** Estimación de la presión de selección actuante en el gen de la cápside proteica de GLRaV-4.

| | | **Entire dataset (276 codons)** | **Splitted dataset: N-terminal region (40 codons)** | **Splitted dataset: C-terminal region (236 codons)** |
|---|---|---|---|---|
| **SLAC** | Mean $d_N/d_S$ | 0.146 | 0.524 | 0.092 |
| | Positively selected sites | 4 | 2 | 0 |
| | Negatively selected sites | 206 | 12 | 190 |
| **REL** | Mean $d_N/d_S$ | Analysis not performed due to size restriction | 0.641 | Analysis not performed due to size restriction |
| | Positively selected sites | | 7 | |
| | Negatively selected sites | | 23 | |
| **FEL** | Positively selected sites | 2 | 2 | 0 |
| | Negatively selected sites | 218 | 14 | 198 |

The linear epitope prediction analysis of all concerned sequences performed by BepiPred revealed the highest probability of occurrence of a B-cell linear epitope in the first 40 amino acids, in agreement with previous reports (6, 20). Noteworthy is that in the same positions, most of the sites under positive selection were inferred, and as previously observed, the region was the most CP variable (Supplementary Figure 3). In every protein sequence, a single polynucleotide binding site was inferred by SomeNA. All these predicted sites were located between the 175 to 182 CP residues (Supplementary Figure 3).

**Serological analysis**.

Western blot analysis of purified extracts from nineteen GLRaV-4 infected plants and a virus-free accession revealed variable specificity from the different Mabs and AS used. Two of the three AS used (AS-GLRaV-5 and AS-I-252-IL) showed nonspecific reactions. Several bands were observed in all the analyzed samples, even in the virus-free Chardonnay. However, the 35KDa GLRaV-4 CP band was clearly identified. The three AS reacted with the GLRaV-4 CP of the nineteen analyzed extracts, but the Sangiovesse Fiano sample only faintly reacted with AS-I252-IL. Two Mabs (6-3 and 15-5) did not react with any western blot sample. The five remaining Mabs showed variable reactions with the tested samples, from clear to faint bands (figure 1). Western blot results are summarized in Supplementary Table 1.

A) Reaction of three samples against five Mabs and three PAS. Healthy controls clearly show nonspecific reactions against two PAS. B) Overexposure of the same membrane reveals faint reaction against four Mabs.

A) Reacción de tres muestras contra cinco anticuerpos monoclonales y tres antisueros policlonales. El control sano muestra reacción no específica contra dos antisueros. B) Sobre exposición de la misma membrana muestra reacción débil contra cuatro anticuerpos.

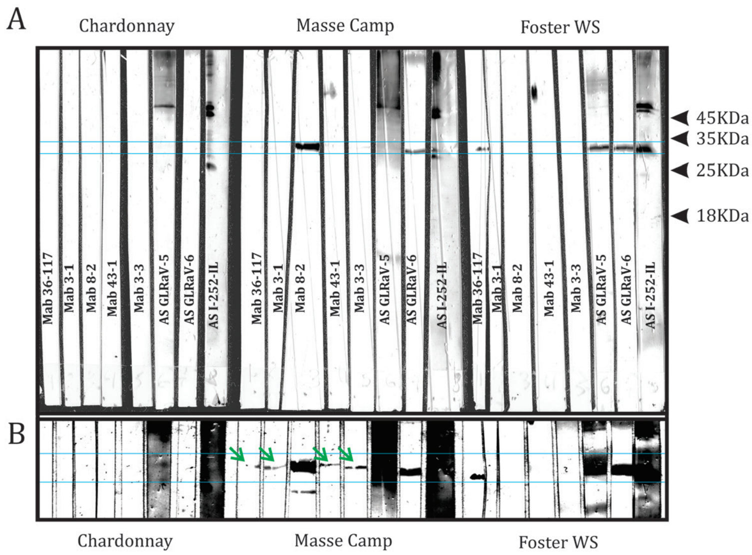


**Figure 1.** Nitrocellulose membrane after western blot analysis.

**Figura 1.** Membrana de nitrocelulosa revelada tras western blot.

## Discussion

Despite the taxonomic controversy during the early ampelovirus history, today GLRaV-4 is considered a single viral species composed of several genetic variants. In the present study, 30 CP sequences of GLRaV-4 were obtained from 19 leafroll-affected grapevine plants. All plants reacted with one or both ELISA reagents for GLRaV-4 and GLRaV-4 strain 6. The RT-PCR analysis of dsRNA extract from these plants allowed amplifying a 1.100 bp fragment in all cases, containing the entire CP ORF. Sequence analysis led to sequence identification of GLRaV-4 strain 5, strain 6 and strain 9. Serological analysis showed specific reactions of such samples against Mabs for GLRaV-4 strain 5 (Mab 8-2, 43-1, 3-3) and -6 (Mab 36-117), whereas no clear reaction was obtained against GLRaV-4-specific Mab (Mab 3-1). Mab 15-5 and 6-3 did not react against any sample in western blot (as previously recorded with the same extract used for mice immunization) (2). As these two Mabs compose the GLRaV-4 DAS ELISA reagent set used, and give positive reaction with the tested samples, both Mabs are obviously directed against a conformational epitope, dissociated during the denaturing SDS-PAGE.

Both molecular and serological analysis revealed the occurence of mixed infections, an usual behavior in grapevine-infecting closterovirids, also reported in other host species (8).

CP sequence variability of GLRaV-4 was considerably high. In fact, some CP sequences (such as GLRaV-4 strains Ob and Pr) showed higher divergence than the proposed threshold of 25% as criteria for species discrimination in the *Ampelovirus* genus (21). Given these variability levels, the proposed divergence threshold of 25% over the CP aminoacidic sequence should be closely revised and raised to 30% considering the currently available sequences belonging to GLRaV-4. A closer analysis of such variability showed an asymmetrical distribution, with the CP N-terminal region (the first 40 residues) much more variable than the C-terminal region. Besides the nucleotide substitutions leading to amino-acid changes, all insertions or deletions were concentrated in the first 120 nucleotides of the multiple sequence alignment. Furthermore, a segmented analysis of the observed variability evidenced that all available CP sequences of GLRaV-4 shared more than 80% identity over the C-terminal region (233 to 236 residues). In all cases, pair-wise similarity among sequences of the referred C-terminal region was over 90%, reflecting a high conservation degree.

The ML analysis performed on the complete CP sequences herein generated and on the ones available in the database revealed strong clustering in seven genetic groups. Most viral strains obtained from local vineyards clustered together with GLRaV-4 strains 5 and 6, while one of the obtained sequences exhibited a close relationship with GLRaV-4 strain 9. The seven groups of sequences were compared by a genetic distance study conducted by using observed cluster distribution in the ML tree. Intragroup level of genetic distance (≤ 0.1) was different from intergroup distance (over 0.14 for the closest GLRaV-4 strain 5 and 9, and exceeding 0.2 for the remaining groups). These results are consistent with the amino acid sequence identity levels between groups (table 1, page 108). When GLRaV-4 was considered as a single species, the average genetic distance in the group was 0.127, barely higher than that in GLRaV-1 and -2, members of *Ampelovirus* and *Closterovirus* respectively, and reported as highly variable species.

In the first evolutionary analysis, a putative recombination event was inferred as related to GLRaV-4 strains 9, 5 and 6, producing a topological incongruence between the N- and C-terminal regions of CP in the phylogenetic analysis. This difference was already observed in the HSP70h and CP inferred phylogeny conducted by Maliogka *et al.* (2008), even though the authors did not record any recombination evidence. Recombination is one main force driving evolutionary history of plant viruses, with a significant impact in the *Closteroviridae* family, as observed for CTV (*Closterovirus*), GLRaV-3 and GLRaV-4 strain 5 (*Ampelovirus*). Considering GLRaV-4, the restricted host range, long host lifespan, high sequence similarity and high occurrence of mixed infections could provide a favorable environment for increasing effective recombinants. However, the recombination signal identified in GLRaV-4 could not constitute a true genetic exchange among donor and recipient viruses, but a variable rate of mutation among the different genes of the virus (29). A more detailed study with more GLRaV-4 strain 9 sequences could confirm this event.

After splitting the dataset according to putative recombination, a significant difference was revealed in the selection pressure over CP during GLRaV-4 evolution history. In fact, the entire CP sequence was subjected to a strong negative selection as indicated by the global $d_N/d_S$ value of 0.144 (in concordance with most plant viral CP), but the variable N-terminal region appeared to present sites subjected to positive selection (fairly unusual in CP of plant virus). So far, the occurrence of positively selected sites has been reported only on a few plant viruses (for instance GLRaV-1 and GLRaV-4 strain 5) but absent positively selected sites over the CP are more frequent. In fact, the comprehensive study of Chare and Holmes (2004) showed only three of 36 plant virus species with low number of sites under positive selection into the CP gene. High conservation levels of most CP (where up to 198 of 233 C-terminal residues were inferred to be under negative selection) reflect a strong purifying selection, probably maintaining some CP functions. For instance, a putative nucleotide binding site was inferred in all the analyzed sequences in the 175 to 182 positions, typically saturated of sites under strong negative selection pressure. This selective behavior may be a consequence of structural requirements.

In addition to structural functions, CP of plant viruses is involved in vector specificity. The virion-vector interaction of lettuce infectious yellow virus (LIYV, *Crinivirus*) has been thoroughly studied, and the minor coat protein (CPm) determined virions to vector binding (5). GLRaV-4, in opposite to most *Closteroviridae* family members, lacks a CPm homologue, and the viral particle appears to be completely covered by CP, as a homologous antibody uniformly decorates the entire viral particle, whereas GLRaV-2 left an undecorated tail (1). In consequence, the CP replacing the absent CPm should constitute the vector binding determinant. Generally, plant viruses are considered host generalists and vector-specific. Nearly 60% of plant virus species are transmitted by a single vector, but less than 10% of viral species infect one single host (24). GLRaV-1, -3 and -4 showed a particular behavior, given they naturally infect only *Vitis spp*, whereas they are transmitted by up to eleven different mealybugs and soft-scale insect species (9). Different isolates of GLRaV-4 have been reported as transmissible with variable efficiency, or even not transmitted by six pseudococids species belonging to two families. This biological behavior is somewhat similar to the serological reactivity against Mabs previously reported (11). In this work, we confronted serological reactivity of local isolates of GLRaV-4 to a wide panel of Mabs proved to be highly specific. Furthermore, the heterologous reaction observed may be caused by multiple strain infection, rather than *sensu stricto* heterologous serological reaction. Considering the variability and antigenicity observed across the CP of GLRaV-4, we postulate that linear epitopes reactive to Mab36-117, Mab 8-2, Mab3-1 and Mab3-3 are located in the highly immunogenic N-terminal region of the protein. Previous research demonstrated that for cucumber mosaic virus, a short epitope of five residues exposed on the surface of the virion reacts with a Mab, essential for virus transmissibility, as single residue mutations abolish both transmissibility and reactivity against Mab (3, 19). However, one single mutation can provide vector affinity advantages. If such mutation increased transmission efficiency or augmented the number of vector species having affinity for the virion, it would confer an impressive ecological advantage compared with the wild-type population. This may explain the unusual occurrence of positively selected sites in the CP of GLRaV-4. Conversely, the highly conserved C-terminal region may be the result of structural conformation of CP or strong CP interaction requirements in the virus replication cycle in the plant. The SNAP2 analysis revealed that the region comprising the first 40 residues was mostly composed of amino acids whose substitution led to a neutral function effect, whereas mutations in amino acids located around the putative polynucleotide binding site (located in the C-terminal region) could have a functional effect (Supplementary Figure 3). This suggested biological significance of the observed sequence conservation.

The same evolutionary behavior described (a variable N-terminal region with positively selected sites) has been reported for bean yellow mosaic virus (Potyvirus). In that species, Parella and Lanave demonstrated that one of the positively selected sites identified belonged to a motif involved in CP-vector interaction, crucial for transmissibility (23).

The significance behind these observations in ampeloviruses can be assessed by an exhaustive study of the transmission efficiency of different genetic variants in the presence of different mealybug species. Recently, Rivadeneira *et al.* (2022) reported differential incidence of GLRaV -3 and -4, suggesting higher levels of GLRaV-3 linked to the occurrence of *Planococcus ficus.* However, surprisingly in the presence of mealybugs, GLRaV-4 incidence remains quite low. In contrast, GLRaV-3 incidence is lower in Mendoza Province (10, 17). Consequently, assertive identification of viral strains should be considered for disease impact, like in modelization approaches of vigour components in grapevine (14, 15).

Epitope prediction conducted using BepiPred revealed high occurrence probability of a linear B-cell epitope in the N-terminal region of CP, consistent with previous observations (6, 20). In addition to the implication in the abovementioned transmissibility, some important immunological issues need discussion. Considering that most available serological reagents for characterizing GLRaV-4 isolates are monoclonal antibodies (11) with good reactivity against denatured CP in Western blots, they might be directed against a linear epitope. Moreover, given viral particles were applied in the native form during immunization, these epitopes may be located on the virion surface. Considering the most immunogenic region as a linear epitope (the most variable region), monoclonal antibodies targeting these epitopes will not be useful for taxonomic purposes at species level. This statement considers the identity level found in the present study for GLRaV-4 strain 5 in the N-terminal region ranged from 57% to 100% while for GLRaV-4 strain 6, it ranged from 47% to 100%. However, these antibodies remain useful for strain discrimination.

Conversely, the antibodies present in the commercially available reagent set for GLRaV-4 (2) appeared to target a conformational epitope (as they are nonreactive against the denatured CP in Western blots) highly conserved and probably located in the C-terminal region of CP. Since no available systems predict conformational epitopes from the primary structure of the proteins, this issue remains unresolved and warrants further research.

## Conclusions

This work first reports a linkage among the distinctive evolutionary behavior of the coat protein of GLRaV-4 and biological properties of such protein, providing an alternative point of view in the study of virus-vector interactions, transmissibility and ecology.

### Supplementary tables and figures

https://docs.google.com/document/d/1KVV5AW4Pqq7VqANYI1G5sIkCMR8B9fmA/edit?usp=sharing&ouid=111310786017351827239&rtpof=true&sd=true

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# PCR identification of lactic acid bacteria populations in corn silage inoculated with lyophilised or activated *Lactobacillus buchneri*

# Identificación por PCR de poblaciones de bacterias del ácido láctico ensilado a partir de maíz inoculado con *Lactobacillus buchneri* liofilizado o activado

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## Abstract

This study aimed to evaluate the effect of inoculation with lyophilised and/or activated *Lactobacillus buchneri* on lactic acid bacteria populations in corn silage. Experimental treatments consisted of corn silage without additives or silage with the inoculants of *L. buchneri* ($1 \times 10^5$ cfu/g) applied according to the manufacturer's recommendations (1 g/tonne fodder) in the forms of the lyophilised inoculant and pre-activated inoculant. Purified isolates from corn silage with and without the inoculant were identified, and 93% of the isolates corresponded to the lactic acid bacteria of the species *Lactobacillus plantarum*. Among the isolates, no bacteria of the species *L. buchneri* were detected. The application of lyophilised or activated *L. buchneri* improved the microbiological profile and reduced ethanol production in corn silage, even without being identified among the isolates captured 70 days after ensilage.

**Keywords**

fermentation • inoculant • lactic acid bacteria •polymerase chain reaction

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### Resumen

Este estudio tuvo como objetivo evaluar el efecto de la inoculación con *L. buchneri* liofilizado y/o activado sobre las poblaciones de bacterias de ácido láctico en ensilajes de maíz. Los tratamientos experimentales consistieron en ensilaje de maíz sin aditivo y ensilaje con los inoculantes *L. buchneri* 1 × $10^5$ ufc/g, aplicados según las recomendaciones del fabricante (1 g/tonelada de forraje) en las formas de inoculante liofilizado e inoculante preactivado. Se identificaron aislados purificados de ensilajes de maíz con y sin inoculación, y el 93% de los aislados correspondieron a bacterias de ácido láctico de la especie *L. plantarum*. Entre los aislados no se detectaron bacterias de la especie *L. buchneri*. La aplicación de *L. buchneri* liofilizado o activado en la masa ensilada promueve un aumento considerable de la población de bacterias de ácido lácticas en los ensilajes de maíz durante todo el período de fermentación.



**Palabras clave**
fermentación • inoculante • *Lactobacillus plantarum* • reacción en cadena de la polimerasa


## Introduction

Chemical characteristics associated with the roughage used, such as dry matter content, concentration of water-soluble carbohydrates, buffer substances and populations of microorganisms present in the forage, and associated with the silage process bottlenecks, *i.e.* the silo filling time, compaction and sealing, are among the main factors that modulate the silage production process and modify the process and the characteristics of the silage (23, 34).

The silage process is directly associated with the nature of the epiphytic microbial community, where bacterial diversity is the determinant factor of the fermentation pattern of silage. The microbial communities verified in forage crops before the silage process differ considerably in number and taxonomy from those quantified in silage (3, 7, 21).

Corn (*Zea mays L.*) has adequate characteristics for good fermentation in a silo when harvested with the right dry matter content (16). However, due to the high concentration of water-soluble carbohydrates (WSC), DM losses might occur during fermentation and when the silo is opened to withdraw the silage (16, 26).

Thus, several corn silage inoculants have been researched in recent years, increasing knowledge about the dynamics of the action of bacteria in silage mass (31, 32). The main additives were composed of microbial species of lactic acid bacteria (LAB), which are gram-positive, non-spore forming, strictly fermentative, anaerobic or aerobic tolerant, and acidophilic, and classified according to the type of hexose fermentation (16, 18, 28), which can be deferred. According to the species of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Lactococcus* and *Streptococcus*, classified as homolactic fermenters (*Lactobacillus plantarum*) or heterolactic fermenters (*Lactobacillus buchneri*), both producers of lactic acid and heterolactics can also produce acetic acid (17).

*Lactobacillus buchneri* has slow growth, with its effects observed from 45 to 60 days (20, 29), which increases the aerobic stability of silage (8, 24). The lactic acid and acetic acid produced are inhibitors of yeasts and moulds (20, 31).

However, the formation of a large amount of acetic acid in silage does not present an advantage. This is related to the loss of energy and the reduction of the intensity of the pH drop in the silage (14, 17). The further development of *L. buchneri* in silage mass may promote the increased aerobic stability of corn silage (30).

Through commercial products, the inoculation of silage occurs industrially from the dilution in water of the inoculant with strains of lyophilised lactic acid bacteria. The previous activation of the inoculant in reconstituted skim milk (RSM) prior to the ensiling process may increase the availability of active bacteria in the silage mass. The activation in RSM favours LAB in competition with other undesirable microorganisms and redirects the fermentation pattern and aerobic stability of the silage (27, 31), promoting increased inoculation efficiency and higher yield and quality of the final product.

Knowledge of epiphytic microbial diversity can contribute to the understanding of the fermentation pattern of silage, as well as the phenomena arising from the exposure of this material to aerobiosis. Molecular biology techniques have been used to determine changes in the microbial community, favouring the intensity of responses about microbial diversity in silage and the effects of the inoculation of different strains of isolated LAB (13, 26).

Accordingly, the aim was to evaluate the effect of inoculation with *L. buchneri* lyophilised or activated in RSM on LAB populations in corn silage.

## Material and methods

### Location and meteorological data

The experiment was conducted in the Forage Farming Sector of the Department of Animal Science of the Center of Agricultural Sciences, Federal University of Paraiba. The climate in the region is As' (hot and humid), according to the Köppen classification. According to data from the Meteorological Station of the Agricultural Sciences Center of the Federal University of Paraiba, the average annual precipitation is 1400 mm; the average annual temperature is 24.5°C; and the average relative humidity is 80%.

### Corn silage and treatments

The corn harvest was carried out at 97 days of age, when the grains were in the milky / pasty stage. The plants were harvested manually with an MS content of 26.2% and chopped in a stationary forage machine regulated to cut the forage into particles of approximately 2 cm and homogenised prior to inoculation and silage of the corn plant (table 1).

**Table 1.** Chemical composition and microbial populations of forage prior to inoculation and silage.

**Tabla 1.** Composición química y poblaciones microbianas del forraje antes de la inoculación y ensilaje.

| | |
|---|---|
| Dry Matter (g/kg) | 262.49 |
| Crude Protein (g/kg DM) | 49.05 |
| Mineral Matter (g/kg DM) | 32.34 |
| NDFcp[1] (g/kg DM) | 548.34 |
| Total Soluble Carbohydrates (g/kg DM) | 152.14 |
| LAB (log cfu/g) | 5.50 |
| Yeasts (log cfu/g) | 4.30 |

[1] Neutral Detergent Fibre. corrected for ashes and proteins.

[1] Fibra Detergente Neutra corregida para cenizas y proteínas.

Ensiling was carried out in tubular silos of PVC (15 cm diameter and 40 cm height), according to the treatments: silage without the inoculant (*in natura*), silage with the lyophilised inoculant (SLI) and silage with the activated inoculant (SAI).

The inoculant with strains of *L. buchneri* ($1 \times 10^5$cfu / g; *Lactobacillus buchneri* CNCM I-4323, Lallemand and Animal Nutrition) was applied according to the manufacturer's recommendations (1 g / tonne fodder). The lyophilised inoculant was diluted in 100 ml distilled water and applied uniformly (2 ml / kg fodder) from a spray and constant mixture.

The inoculant was preactivated in 10% RSM 24 hours prior to ensiling. Skimmed milk powder (10 g) was solubilised in 100 ml of distilled water, and two grams of sucrose was added as an energy source for microbial growth, according to the methodology of Santos *et al.* (2008). After growth, counts of lactic acid bacteria in the RSM were performed after 24 hours, as well as in the inoculant dissolved only in water, through the MRS culture medium (5) for *Lactobacillus* ssp. Populations of $2.1 \times 10^8$ and $4.5 \times 10^6$cfu / ml were grown in RSM growth medium and diluted in water, respectively. In 10 ml of RSM with reactivated *L. buchneri*, which was rediluted in 90 ml of distilled water, 2 ml / kg of natural material of this mixture was applied by spray.

The additives were mixed homogeneously to fill the experimental silos. Immediately, forage compaction was carried out in the silos, aiming to reach a specific mass of 600 kg / $m^3$ of natural matter. The silos were stored for 70 days at a mean temperature of 24°C before opening. Openings were performed at 1, 3, 7, 14 and 70 days after the silos were closed (table 2).

**Table 2.** Mean pH and concentrations of organic acids, ethanol and yeast of corn silage without inoculants and with lyophilised or activated microbial inoculants.

**Tabla 2.** Valores medios de pH, concentraciones de ácidos orgánicos, etanol y levaduras de ensilajes de maíz sin inoculantes y con inoculante microbiano liofilizado o activado.

| | Inoculation | | |
|---|---|---|---|
| | **In natura** | **SLI [1]** | **SAI [2]** |
| pH | 3.52 | 3.72 | 3.66 |
| Latic Acid (g/kg DM) | 46.61 | 34.14 | 38.56 |
| Acetic Acid (g/kg DM) | 28.50 | 21.89 | 29.67 |
| Lat:Acet Ratio[3] | 1.64 | 1.56 | 1.30 |
| Ethanol (g/kg MS) | 32.63 | 26.80 | 21.72 |
| Yeasts (log cfu/g fodder) | 4.86 | 4.65 | 5.17 |

[1] SLI = Corn silage with lyophilised microbial inoculant; [2] SAI = Corn silage with activated microbial inoculant; [3] Lat: Acet Ratio = Latic acid and Acetic acid ratio.

[1] Ensilaje de maíz con inoculante microbiano liofilizado; [2] SAI = Ensilaje de maíz con inoculante microbiano activado; [3] Relación Lat: Acet = Relación de ácido láctico y ácido acético.

**Quantification of lactic bacteria populations**

The population count of lactic acid bacteria was performed according to the recommendations of González and Rodrigues (2003). Twenty-five grams of fresh silage samples were collected according to the defined opening periods, and 225 ml of sterile ringer solution was added and processed in a blender for approximately 1 minute. One millilitre of these mixtures was removed and pipetted to an appropriate dilution ($10^{-1}$ to $10^{-9}$).

Plating was performed in duplicate for each culture medium. The populations were determined by the selective culture technique in anaerobic medium, where the culture medium MRS was used and incubated for 48 hours in an oven at 37°C, according to the methodology of De Man *et al.* (1960).

The plaques considered susceptible to counting were those in which there were values between 30 and 300 cfu in a Petri dish. The plate means of the selected dilutions were then considered.

**Lactic bacteria culture technique**

After quantification of lactic acid bacteria populations using the pour plate technique in agar MRS culture (5), cultures were purified and cultured in a Falcon tube with 5 mL of MRS broth for 24 h at 37°C, and 10 cfu of each treatment was randomly selected 70 days after ensiling. The cultures were centrifuged for 10 minutes at 3600 rpm (rotation per minute) to obtain the cell pellet. The supernatant was removed, and 1 mL of saline (Ringer's solution) was added and vortexed. Using a pipette, the entire volume of the pellet was transferred to the microtube and centrifuged at 6000 rpm for 3 minutes. The microtubes were stored in a freezer until DNA extraction was performed.

**DNA extraction**

DNA extraction from the isolates was performed using the Wizard Genomic DNA Purification Kit (Promega). The pellet was resuspended in 480 μl of 50 mM EDTA (Ethylic DiaminoTetracyclic Acid) in vortex. Fifty microlitres of lysozyme (50 mg / ml concentration) were added. The samples were incubated in a water bath at 37°C for 60 minutes and centrifuged for 2 minutes at 12,000 RPM, and the supernatant was removed.

After DNA extraction, DNA quantification procedures were carried out using Nanovue equipment (Nanodrop) at the Animal Biotechnology Laboratory of the Animal Science Department of the Federal University of Paraiba.

On average, the extraction resulted in a concentration of 1161.37 ng of DNA with 1.9 degrees of purity. After quantification, it was diluted to a concentration of 20 ng of DNA per µL.

**Polymerase chain reaction (PCR)**

Amplification of the 16S rDNA fragment of the isolates occurred with Primer 1492R (TAG G(C/T)A CCT TGT TAC GAC TT) and Primer p027F (GAG AGT TGA TCC TGG CTC AG) (Heuer *et al.*, 1997). The PCR reaction was performed in 0.2 mL tubes containing 50 µL of the reaction mixture: DNA (80 ng), 5X buffer solution (0.1 mol/L Tris-HCl, pH 8.0, 0.5 mol/L KCl), 1.5 mmol/L $MgCl_2$, pH 8.0; 0.2 mmol/L dNTP mix (Promega), Taq polymerase 2U (Promega), 0.12 µmol/L primer p027F 0.12 µmol/L, and 1429R 0.12 µmol/L (IDT Síntese Biotecnologia). The volume of the reaction mixture was filled to 50 µL with sterile ultrapure water. PCR was performed in a thermocycler 3Prime (Techne), and the reaction conditions employed in the PCR were: 94°C/5 min; 30 cycles (denaturation: 94°C for 30 seconds; 60°C for 30 seconds); polymerisation: 72°C/2 min; final extension: 72°C/5 min. An aliquot of 3 µl of the PCR product was mixed in 3 µl of the mixture: 1 µL Gel Red (Biotium) and 2 µL of pigment 6X Gel Loading (Promega) and analysed using agarose gel electrophoresis (1.2%) in buffer solution Tris-Borate-EDTA (TBE 1X). The gel was visualised under ultraviolet light, and images were captured using a gel system for photo documentation (MBS). The PCR product, a fragment of approximately 1500 bp, was sent to the Macrogen Company, Korea, for purification and sequencing.

**Sequence analysis of the isolates**

The sequences of the isolates were compared with those available in the GenBank database and aligned using the BLASTn algorithm (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST) for nucleotides. Sequences of the 16S rRNA gene that presented similarity equal to or greater than 95% were considered to belong to the same Operational Taxonomic Unit (OTU) (1).

**Experimental design and statistical analysis**

The experiment was carried out in a completely randomised design, with 3 treatments and 5 replicates per treatment, in each opening period (1, 3, 7, 14 and 70 days). The microbial counts were transformed into log10. Variance analysis and multiple comparisons of data were performed using the GLM procedures in SAS. The means were compared using the Kruskal-Wallis test.

Data on culture, DNA extraction, PCR and sequence analysis were performed and discussed through descriptive statistical analysis.

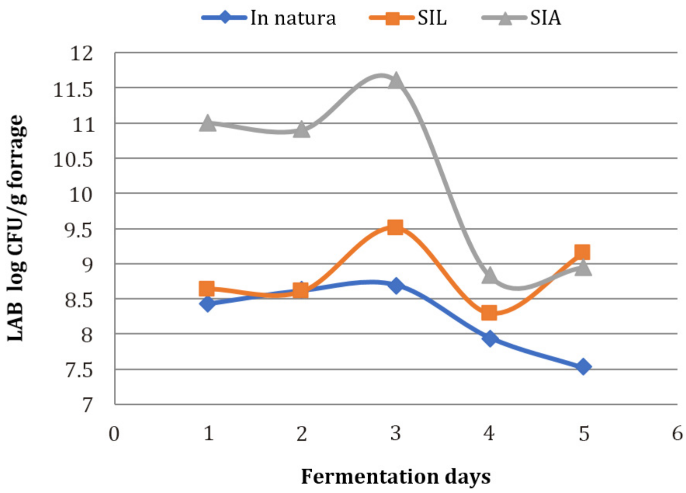
## Results

**Quantification of lactic acid bacteria in silage**

Quantification of lactic acid bacteria populations was estimated at the different opening periods of the silos (1, 3, 7, 14 and 70 days). The lactic acid bacteria showed a rapid multiplication speed, reaching their highest development at 7 days of the fermentation process (figure 1, page 120).

**Identification of lactic acid bacteria populations in silage**

It was observed in this study that a greater number of the isolates (93.34%) were formed by lactic acid-producing strains of the species *L. plantarum* (table 3, page 120).




SLI = Silage treated with the lyophilised inoculant; SAI = Silage treated with the activated inoculant.

SLI = Ensilado tratado con inoculante liofilizado; SAI = Ensilaje tratado con inoculante activado.

**Figure 1.** Mean growth values of lactic acid bacteria in corn silage *in natura* and inoculated with *Lactobacillus buchneri* during different fermentation periods.

**Figura 1.** Valores medios de crecimiento de bacterias lácticas en ensilaje de maíz *in natura* e inoculado con *Lactobacillus buchneri* durante diferentes períodos de fermentación.

**Table 3.** Molecular identification of samples of bacteria of the genus *Lactobacillus* isolated 70 days after ensilage.

**Tabla 3.** Identificación molecular de muestras de bacterias del género Lactobacillus aisladas a los 70 días del ensilaje.

| Sample | Species | Identification (%) | Gen Bank Access |
|---|---|---|---|
| Silage In natura | *Lactobacillus plantarum* | 96 | NR 042394.1 |
| Silage In natura | *Lactobacillus plantarum* | 99 | NR 042394.1 |
| Silage In natura | *Lactobacillus plantarum* | 98 | NR 042394.1 |
| Silage In natura | *Lactobacillus plantarum* | 97 | NR 042394.1 |
| Silage In natura | *Lactobacillus plantarum* | 94 | NR 117813.1 |
| Silage In natura | *Lactobacillus plantarum* | 99 | NR 117813.1 |
| Silage In natura | *Lactobacillus plantarum* | 98 | NR 117813.1 |
| Silage In natura | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage In natura | *Lactobacillus plantarum* | 99 | NR 117813.1 |
| Silage In natura | *Lactobacillus plantarum* | 98 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 98 | NR 104573.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 99 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 97 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 97 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 94 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 96 | NR 042394.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Lyophilised Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus paraplantarum* | 95 | NR 025447.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 97 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 98 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus paraplantarum* | 97 | NR 025447.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 95 | NR 104573.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 98 | NR 117813.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 95 | NR 042394.1 |
| Silage Activated Inoculant | *Lactobacillus plantarum* | 96 | NR 117813.1 |

With the sequencing of the 16S rDNA fragment produced by PCR using Primers 1492R and p027F, the isolates were closely related to *L. plantarum*. Figure 2, shows that the PCR products were visualised using agarose gel electrophoresis.

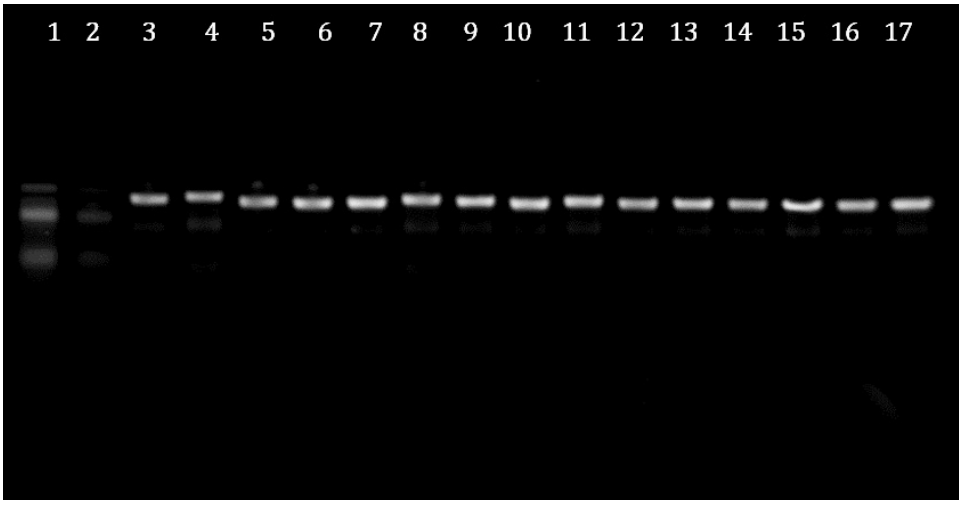



**Figure 2.** PCR amplification products for Lactobacilli. Line 1, Molecular marker (100 bp DNA ladder); Line 2, White; Lines 3 to 17, *Lactobacillus plantarum*.

**Figura 2.** Productos de amplificación por PCR para Lactobacilli. Línea 1. Marcador molecular (escalera de DNA de 100 pb); Línea 2. Blanco; Líneas 3 a 17: *Lactobacillus plantarum*.

## Discussion

The silage inoculated with preactivated *L. buchneri* had the highest values of lactic bacteria populations until the 14th day of the fermentation period, reaching 11 log CFU / g in the forage at 7 days of fermentation (figure 1, page 120). These results corroborate those observed by de Santos *et al.* (2008) since there was the addition of a larger number of active bacteria, thus facilitating their multiplication. An indication of the presence of heterofermentative bacteria was the reduction of lactic acid concentration, the increase in acetic acid concentration and the reduction of the proportion of lactic and acetic acids. Consequently, the ethanol levels were low at the end of the 70 days of silage (6).

However, the addition of a group of microorganisms as additives at the end of the silage process may not be present. These microorganisms promote changes in the pH value, and the presence of several organic compounds, such as acetic, lactic, butyric, propionic, ethanol, $CO_2$, and antimicrobial and/or bacteriostatic compounds, also affect the interaction between the various groups of microorganisms (14).

These modifications were desirable considering that the pre-silage material had a dry matter content (26.2%) below that recommended for good-quality silage (16). According to Danner *et al.* (2003), lactic acid formation is fundamental to reducing the pH of the silage, causing the selection of microorganisms in the silage mass. However, acetic acid also needs to be formed to ensure that the silage mass has an adequate pH of 3.8-4.2 (16), thus providing control of moulds and yeasts. However, when the pH value is reduced too much, it may favour the development of these microorganisms, which causes a decrease in aerobic stability (20).

Although there was no reduction in yeast in the corn silage with lyophilised or activated microbial inoculant, the presence of acetic acid has antifungal action, which reduces the production of other compounds, such as ethanol (22, 36), as observed in this study. According to Sadiq *et al.* (2019), there is a consensus from the scientific community that organic acids are the main antifungal metabolites of LAB.

Organic acids in their dissociated or undissociated form are lipophilic in nature and thus readily diffuse across the fungal cell membrane and accumulate in the cytoplasm, reducing activity or leading to yeast death. However, acetic acid has higher inhibitory activity against fungal growth compared to lactic acid. Although the LAB populations of the silages treated with the lyophilised inoculant showed lower growth compared with the activated inoculation, they stabilised at 70 days with populations above 9.0 log cfu/g forage. The values were similar in silage with the inoculant and higher than in silage without the inoculants (figure 1, page 120). This difference may be due to a peak in LAB development at 7 days of silage, when there may have been intensified substrate competition. Probably, the activation of the lyophilised LAB with RSM caused this difference in the population on the 7$^{th}$ day of silage, which remained until the 70$^{th}$.

The fermentative process of silage is complex and involves many species of LAB and their interactions. The use of specific inoculants is indicated to dominate or overcome the number of epiphytic lactic bacteria present in the forage, either to improve the fermentation process (19, 32) or to increase the aerobic stability of silage (37). However, the increase in LAB in the ensiled mass promotes the greater availability of specific microorganisms. The greater or lesser degree of development of these bacteria depends on the conditions of the medium (9).

Inoculation of the silage with activated heterofermentative LAB (*L. buchneri*), facultative aerobes, produces lactic acid and acetic acid. These characteristics cause a rapid proliferation of the same in the ensiled mass and cause an adequate reduction of the silage pH, controlling the growth of yeasts and moulds (table 2, page 118). Thus, the ensiled mass can provide greater development of epiphytic bacteria, such as *L. plantarum*, which is homofermentative (15, 28).

The pH of the ensiled mass rapidly decreased due to the homofermentative LAB multiplying rapidly, producing more lactic acid. *Lactobacillus buchneri* bacteria limit the growth and metabolism of lactic acid degradation as a strategy to maintain cell viability (table 2, page 118) (20, 29). The activity of *L. buchneri* was evidenced by higher acetic acid production. In the 70-day fermentation period, the selected isolates of inoculated corn silage were not identified (table 3, page 120). Possibly due to the greater fermentative capacity in the corn silages, *L. plantarum* dominated the fermentation of the silage. Guo *et al.* (2018) observed similar behaviour in alfalfa silage, including *L. buchneri*, which is tolerant to acidic environments and uncompetitive compared to other LAB species.

The high frequency of *L. plantarum* in corn silage promoted the effective fermentation of lactic acid, rapidly reducing the pH of the ensiled mass and preventing the development of microorganisms deleterious to silage, such as Clostridia and enterobacteria. In addition to decreasing the pH from lactic acid production, *L. plantarum* can also inhibit the growth of filamentous moulds through the production of antifungal activity (2). However, this characteristic is more likely for heterofermentative LAB species (25).

In this study, strains of *Lactobacillus paraplantarum* (6.6% of isolates) were also identified in corn silage inoculated with RSM pre-activated bacteria (table 3, page 120). Zhang *et al.* (2017) observed the presence and predominance of *L. paraplantarum* in LAB populations in corn silage with high humidity.

Wang *et al.* (2017), characterising isolated lactic acid bacteria and their effects on the fermentation of silage, verified the growth of *L. paraplantarum* limited to 10°C and pH 3.0. According to the authors, the growth of *L. paraplantarum* strains in low pH and temperature environments confirmed the resistant and acidic nature of this species. At the end of the 70-day silage period, the dynamics of the microbial population provided the prevalence of the largest population of *L. lantarum* among LAB. These findings corroborate the findings of Guo *et al.* (2018), who reported that alfalfa silage inoculated with *L. buchneri* changed the population profile of the epiphytic LAB species on the 60th day after silage. The same authors stated that the *L. plantarum* population predominated by more than 90%, but they failed to confirm the cause of this change with certainty and suggested more studies related to the metabolomics of LAB.

## Conclusions

The application of lyophilised or activated *L. buchneri* improved the microbiological profile and reduced ethanol production in corn silages, even without being identified among the isolates isolated 70 days after ensilage.

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## Conflict of interest statement

The authors had no conflict of interest.

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# Biometric genetics in Cowpea beans (*Vigna unguiculata* (L.) Walp) I: phenotypic and genotypic relations among production components

## Genética biométrica en Caupí (*Vigna unguiculata* (L.) Walp) I: relaciones fenotípicas y genotípicas entre componentes de producción

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**Abstract**

In the semi-arid region of Paraíba, cowpea has low productivity due to irregular rainfall and poor use of production technologies. An extensive study aimed at selecting more productive cultivars was conducted using biometric models. This first work had the following objectives: i. Quantify direct and indirect effects of primary and secondary components on grain production; ii. Identify variables with greater potential for cultivar selection in the semiarid region of Paraíba and iii. Determine the most appropriate selection strategies for the evaluated variables. The experiment was conducted in an experimental field. The influence of 6 primary and 6 secondary production components was evaluated on grain yield. Data were subjected to ANOVA. Genetic parameters, correlations and path analysis were estimated. Given the strong direct phenotypic and genotypic effects, pod yield results the most promising variable for higher grain yield selection. Direct and simultaneous selections are the most suitable strategies for the set of evaluated variables. However, further studies on selection indices are necessary to maximize genetic gains.

**Keywords**

Path analysis • genetic improvement • selection • productivity • relationships among characters • *Vigna unguiculata* (L.) Walp.

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## Resumen

En la región semiárida de Paraíba, el caupí es el principal producto de la agricultura familiar. El cultivo tiene baja productividad debido a lluvias irregulares y condiciones climáticas desfavorables. Además, la productividad también se ve afectada por el uso deficiente de las tecnologías de producción. Con el objetivo de superar estas limitaciones y aumentar la eficiencia de la selección de cultivares superiores, se llevó a cabo un extenso estudio utilizando modelos biométricos en caupí. Este primer trabajo tuvo los siguientes objetivos: i. Cuantificar los efectos directos e indirectos de los componentes primarios y secundarios en la producción de granos; ii. Identificar variables con mayor potencial para la selección de cultivares de caupí en la región semiárida de Paraíba y iii. Determinar las estrategias de selección más adecuadas para el conjunto de variables evaluadas. El experimento se llevó a cabo en un campo experimental en el Centro de Ciencia y Tecnología Agroalimentaria de la Universidad Federal de Campina Grande. Se evaluó la influencia de 6 componentes primarios y 6 componentes secundarios de la producción sobre la variable rendimiento de grano. Los datos se sometieron a ANAVA y se estimaron parámetros genéticos. También se realizaron correlaciones y análisis de ruta. Se identificaron variables con mayor potencial para la selección de cultivares superiores de caupí en la región semiárida de Paraíba. Se encontró que la variable rendimiento de vaina es la más prometedora para la selección de cultivares con mayor rendimiento de grano debido a la magnitud de los efectos fenotípico y genotípico. La selección directa y la selección simultánea son las estrategias más adecuadas para el conjunto de variables evaluadas. Sin embargo, para maximizar las ganancias genéticas, se continuó el estudio a través de índices de selección.



**Palabras clave**

análisis de ruta • mejoramiento genético • selección • productividad • relación entre caracteres • *Vigna unguiculata* (L.) Walp.


## Introduction

Among the annual crops traditionally cultivated by small and medium farmers in the Northeast region of Brazil, cowpea (*Vigna unguiculata*, L. *Walp*), also called macassar bean or green bean according to the location, stands out with economic, social and food importance. Cowpea is one main source of employment and income for rural population, besides being rich source of vegetable protein, daily consumed in a variety of dishes (43). Grains constitute important sources of protein, amino acids and dietary fiber, considered for public policy programs focused on improving life quality (44).

Irregular rainfall and traditional farming, highly dependent on labor and little use of agricultural inputs, have recurrently promoted low yields, with 328 kg ha$^{-1}$ on average in the Northeast and 366 kg ha$^{-1}$ in Brazil (30). For Oliveira *et al.* (2001), low productivity levels are mainly given by traditional cultivars with low agronomic quality. Measures promoting cultivar identification and greater adaptation would determine the revitalization of the culture's productive chain (33).

According to Ferreira *et al.* (2007), understanding the relationships among variables related to productivity is key for cultivar identification and selection. Besides, knowing these relationships allows the indirect selection of hard-to-measure variables with low heritability by considering another simpler-to-assess and associated variable (14).

Phenotypic correlation measures the association between two variables (21). This correlation has two known origins: genetic and environmental. When a gene conditions more than one variable, the genetic correlation is known as pleiotropy (16).

According to Nogueira *et al.* (2012), correlations are not measures of cause and effect. Determining a selection strategy based on a direct interpretation of correlation values can compromise the achievement of superior cultivars. In other words, high correlations between two variables may result in indirect effects of a third variable. In this case, other methodologies, such as partial correlations or path analysis, are better choices (25, 48, 49).

In this sense, path analysis uses regression equations to unfold the direct and indirect effects of a set of variables on a basic or main variable (11), determining the most suitable selection strategy for each variable and identifying the most promising cultivar.

Available literature mentions correlation studies and path analysis on cowpea (19, 35, 42). However, new sets of variables in path analysis with chain diagrams should be further studied in order to identify easy-to-measure yield components with high heritability (2, 47).

Given the above, this work aimed to quantify the direct and indirect effects of primary and secondary components on grain production, identifying selection strategies of greater potential for cultivar selection in the semiarid region of Paraíba.

## Material and methods

The experiment was carried out in an experimental field at the Center for Agri-food Science and Technology, Federal University of Campina Grande, CCTA/UFCG, Campus de Pombal – Paraíba. With geographic coordinates 06°46' south latitude, 37°48' west longitude of the Greenwich Meridian (3). According to Köppen's classification, the climate is Aw, semi-arid, with average annual rainfall of 800 mm, and February, March and April concentrating 60 to 80% of total annual precipitation (29).

Before the experiment, plowing was carried out 15 days before sowing, followed by cross harrowing 5 days before bean planting, providing weed control and conditions for good germination. Soon after this procedure, the plots were marked and distributed in the field. Sowing was done in manually opened holes with a hoe at approximately 5 cm depth, placing three seeds per hole. Spacing was 0.5 m with five plants per linear meter.

Fertilization was according to the Fertilization and Liming recommendations for the state of Ceará (17). Thinning occurred about 15 days after emergence, keeping two plants per hole. For pest management, Dimethoate was sprayed twice at a dosage of 1.0 liter/ha, against aphid (*Apis cracyvora* Koch) and thrips (Order Thysanoptera), Methomyl was sprayed once at 0.5 liter/ha against caterpillars (*Spodoptera frugiperda*) and Imidacloprid and Beta-cyfluthrin, once at 270 g/ha to control whitefly (Order Hemiptera).

The experimental design consisted of randomized blocks with eight treatments and four replications, totaling 32 experimental units, with 2.0 m between blocks and plots. The treatments consisted of eight cultivars of cowpea, namely: Costela de Vaca, BRS Marataoã, BRS Itaim, BR-17 Gurguéia, BRS Novaera, Paulistinha, Setentão and BRS Patativa.

Each experimental plot was 3m x 3m (9 $m^2$) with six rows of plants and a useful area of 2m x 2m (4 $m^2$). Spacing between rows was 0.5 meters, with fifteen holes and two plants. Two lateral rows were considered borders. Data collection was carried out in the third and fifth rows.

Cultivar evaluation involved the study of phenology. Precocity was evaluated by considering initial flowering (FL) and initial fruiting (DAFFF), determined by the number of days between sowing and flowering until 50% of the studied plants had at least one flower or an open pod, respectively. Precocious plants reach full flowering 70 days after sowing. Then, the number of days between flowering and fruiting (DAFFH) was calculated.

Harvest of dry pods was manually performed. At harvest time, yield components were measured: total number of pods per plant (TNP); pod unit mass (PUP), in kilograms; pod length (PL), in cm; pod diameter (PD) in mm with the aid of a caliper; pod grain number (NGP), counting the grains of a sample of 10 pods; number of pods per plant (NPP), obtained by the ratio between total pods and number of plants; grain yield (GY) in tons $ha^{-1}$; pod bark productivity (SS), in tons $ha^{-1}$; pod yield (PP), in tons $ha^{-1}$, and seed/pod ratio (PSR), as the ratio between total grain mass and pod number.

The collected data were subjected to ANOVA, and genetic parameters were estimated (10). The correlations and phenotypic, genotypic and environmental trail analysis were performed as described by Cruz *et al.* (2012). For correlations and path analyses, grain yield (GY) was classified as a basic or main variable. The variables TNP, PUP, PL, PD, NGP and NPP were classified as primary components of production, while SS, POS, PSR, FL, DAFFF and DAFFH were classified as secondary components of production. Thus, the path analysis followed a chain diagram scheme (figure 1, page 129).

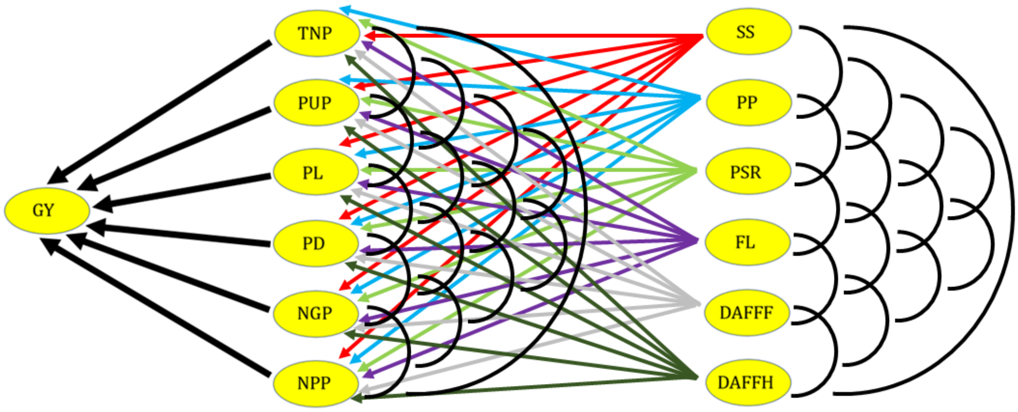


**Figure 1.** Causal diagram illustrating the direct and indirect effects of secondary components on primary components and grain yield in cowpea.

**Figura 1.** Diagrama de causas que ilustra los efectos directos e indirectos de los componentes secundarios sobre los componentes primarios y el rendimiento de grano en caupí.

Heritability coefficients and measurement allowed variable classification into primary and secondary components. More complex variables, with low heritability and selection difficulty, were classified as primary components. Those with high heritability and easy to measure were classified as secondary components.

Before the path analysis, all variables were submitted to multicollinearity diagnosis by verifying the condition number (NC) as established by Montgomery and Peck (1981). Once severe multicollinearity was verified, the crest regression analysis (8) established a constant k (figure 2).

All genetic-statistical analyses were performed by Genes software (Cruz 2012).

Todos los análisisenético-estadísticos fueron interpretadosor Genes software (Cruz, 2012).

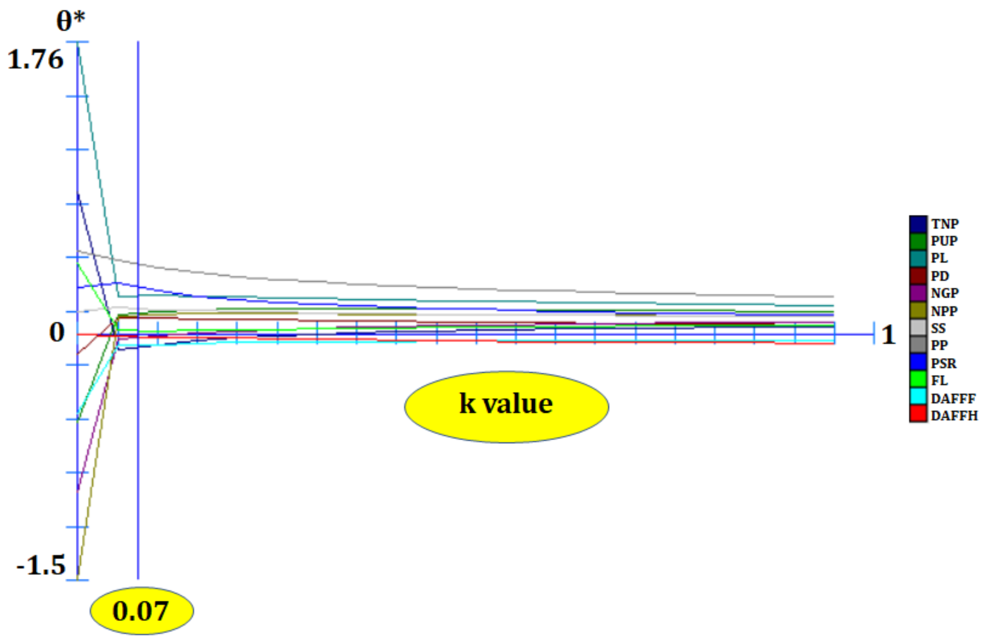


**Figure 2.** Estimates of path coefficients (θ*) as a function of k values and obtained in the analysis using grain yield as the basic variable.

**Figura 2.** Estimaciones de los coeficientes de ruta (θ *) en función de los valores k y obtenidos en el análisis utilizando el rendimiento de grano como variable básica.

## Results

The ANOVA showed significant differences between variables except for TNP, PUP and NPP. The coefficient of variation ranged between low, for FL, and very high, for TNP (table 1).

**Table 1.** ANOVA of variables evaluated in an experimental field at the Center for Agri-food Science and Technology of the Federal University of Campina Grande in the city of Pombal - Paraíba.

**Tabla 1.** ANOVA de variables evaluadas en campo experimental del Centro de Ciencia y Tecnología Agroalimentaria de la Universidad Federal de Campina Grande en la ciudad de Pombal - Paraíba.

| Mean Squares | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FV | GL | TNP | PUP | PL | PD | NGP | NPP | GY |
| Blocks | 3 | 2235.37 | 0.33 | 1.22 | 0.51 | 2.04 | 0.13 | 0.31 |
| Treatments | 7 | 5895.19$^{ns}$ | 0.67$^{ns}$ | 16.15** | 1.71* | 36.71** | 0.97$^{ns}$ | 0.61** |
| Residue | 21 | 4659.06 | 0.36 | 2.37 | 0.53 | 2.68 | 0.40 | 0.14 |
| Mean | | 227.31 | 3.31 | 18.64 | 7.97 | 14.19 | 3.18 | 1.78 |
| CV(%) | | 30.03 | 18.19 | 8.27 | 9.20 | 11.55 | 19.98 | 20.98 |
| **Mean Squares** | | | | | | | | |
| FV | GL | SS | PP | PSR | FL | DAFFF | DAFFH | - |
| Blocks | 3 | 0.02 | 0.55 | 3.59 | 2.28 | 1.08 | 2.28 | - |
| Treatments | 7 | 0.05* | 1.12** | 50.57** | 60.13** | 24.28** | 60.13** | - |
| Residue | 21 | 0.02 | 0.27 | 8.98 | 1.18 | 0.70 | 1.18 | - |
| Mean | | 0.59 | 2.29 | 75.26 | 44.91 | 6.0 | 40.09 | - |
| CV(%) | | 25.40 | 22.98 | 3.98 | 2.43 | 13.97 | 2.72 | - |

** and * significant at 1 and 5% probability; respectively; by F test; ns non-significant; by F test

** y * significativo al 1 y 5% de probabilidad; respectivamente; por prueba F; ns no significativo; por prueba F

Regarding genetic parameters, the genotypic variance exceeded the environmental variance for variables PL, NGP, PSR, FL, DAFFF and DAFFH (table 2).

**Table 2.** Estimates of genetic parameters of the evaluated variables in an experiment conducted at the Center for Food Science and Technology of the Federal University of Campina Grande in the city of Pombal - Paraíba.

**Tabla 2.** Estimaciones de parámetros genéticos de las variables evaluadas en el campo experimental del Centro de Ciencia y Tecnología de Alimentos de la Universidad Federal de Campina Grande en el municipio de Pombal - Paraíba.

| Genetic Parameters | TNP | PUP | PL | PD | NGP | NPP |
|---|---|---|---|---|---|---|
| VG | 309.03 | 0.07 | 3.45 | 0.29 | 8.50 | 0.14 |
| VE | 4659.06 | 0.36 | 2.37 | 0.53 | 2.68 | 0.40 |
| $H^2$ | 20.97 | 46.49 | 85.3 | 68.68 | 92.69 | 58.52 |
| CVg(%) | 7.73 | 8.48 | 9.96 | 6.81 | 20.56 | 11.86 |
| CVg/CVe | 0.26 | 0.47 | 1.2 | 0.74 | 1.78 | 0.59 |
| GY | SS | PP | PSR | FL | DAFFF | DAFFH |
| 0.11 | 0.01 | 0.21 | 10.39 | 14.73 | 5.89 | 14.73 |
| 0.14 | 0.02 | 0.27 | 8.98 | 1.18 | 0.70 | 1.18 |
| 77.1 | 61.52 | 75.28 | 82.23 | 98.03 | 97.11 | 98.03 |
| 19.25 | 16.06 | 20.05 | 4.28 | 8.55 | 40.47 | 9.58 |
| 0.92 | 0.63 | 0.87 | 1.08 | 3.53 | 2.9 | 3.53 |

Heritability coefficients showed high magnitude for PL, NGP, GY, PP, PSR, FL, DAFFF and DAFFH; average magnitude for PD, NPP and SS; and low magnitude for TNP and PUP.

The genotypic coefficients of variation exceeded 10 for NGP, NPP, GY, SS, PP, DAFFPF and DAFFH. Also, for PL, NGP, PSR, FL, DAFFF and DAFFH, the b index, *i.e.* the ratio between genotypic variation coefficient and experimental variation coefficient (CVg/CVe), exceeded unity.

Table 3 shows direct and indirect effects of the explanatory variables on grain yield per hectare. Even with multicollinearity, the crest regression analysis showed high precision considering determination coefficients, residual effects and the adjustment of the k constant.

**Table 3.** Phenotypic, genotypic and environmental path analysis among the explanatory variables.

**Tabla 3.** Correlaciones fenotípicas, genotípicas y ambientales entre las variables explicativas.

| Variable | Effect | Estimate | | |
|---|---|---|---|---|
| | | Phenotypic | Genotypic | Environmental |
| TNP | Direct on GY | -0.07 | 0.01 | 0.08 |
| | Indirect via PUP | 0.00 | 0.00 | 0.00 |
| | Indirect via PL | 0.05 | -0.07 | 0.00 |
| | Indirect via PD | -0.03 | 0.26 | -0.01 |
| | Indirect via NGP | 0.00 | -0.22 | 0.00 |
| | Indirect via NPP | 0.10 | -0.59 | -0.04 |
| | Indirect via SS | -0.02 | -0.09 | 0.06 |
| | Indirect via PP | 0.12 | 0.07 | 0.39 |
| | Indirect via PSR | 0.15 | 1.23 | 0.01 |
| | Indirect via FL | 0.00 | -0.30 | -0.01 |
| | Indirect via DAFFF | 0.02 | 0.22 | 0.02 |
| | Indirect via DAFFH | 0.00 | -0.30 | 0.00 |
| | Total | 0.32 | 0.24 | 0.50 |
| PUP | Direct on GY | 0.13 | 0.00 | -0.07 |
| | Indirect via TNP | 0.00 | 0.00 | 0.00 |
| | Indirect via PL | 0.16 | -0.57 | -0.03 |
| | Indirect via PD | 0.01 | -0.03 | 0.01 |
| | Indirect via NGP | -0.01 | -1.48 | 0.01 |
| | Indirect via NPP | 0.00 | 0.02 | 0.00 |
| | Indirect via SS | 0.10 | 0.16 | -0.13 |
| | Indirect via PP | 0.35 | 1.46 | -0.18 |
| | Indirect via PSR | 0.04 | 0.05 | 0.16 |
| | Indirect via FL | 0.01 | 0.69 | 0.01 |
| | Indirect via DAFFF | 0.00 | 0.00 | 0.01 |
| | Indirect via DAFFH | 0.01 | 0.69 | 0.01 |
| | Total | 0.80 | 0.99 | -0.19 |
| PL | Direct on GY | 0.23 | -0.15 | -0.05 |
| | Indirect via TNP | -0.01 | 0.00 | 0.01 |
| | Indirect via PUP | 0.09 | 0.00 | -0.03 |
| | Indirect via PD | 0.00 | 0.05 | 0.03 |
| | Indirect via NGP | -0.01 | -0.99 | 0.02 |
| | Indirect via NPP | 0.05 | -0.28 | 0.00 |
| | Indirect via SS | 0.04 | 0.04 | 0.00 |
| | Indirect via PP | 0.30 | 0.81 | 0.12 |
| | Indirect via PSR | 0.17 | 0.70 | 0.03 |
| | Indirect via FL | 0.01 | 0.46 | 0.03 |
| | Indirect via DAFFF | -0.01 | -0.10 | 0.00 |
| | Indirect via DAFFH | 0.01 | 0.46 | 0.03 |
| | Total | 0.87 | 0.99 | 0.18 |

Total number of pods (TNP), pod unit mass (PUP), pod length (PL), pod diameter (PD), pod grains number (NGP ), number of pods per plant (NPP), pod yield per hectare (SS), pod yield per hectare (PP), seed to pod ratio (PSR), flowering (FL), days after flowering to fruiting (DAFFF), days after flowering for fresh harvest (DAFFH) and the basic variable grain yield per hectare (GY), evaluated in cowpea cultivars in an experiment conducted in Pombal - PB.

Número total de vainas (NTV), unidad de masa de vaina (MUV), longitud de vaina (COMPV), diámetro de vaina (DIAMV), número de granos de vaina (NGVA), número de vainas por planta (NVPL), rendimiento de corteza por hectárea (PDC), rendimiento de vaina por hectárea (PDV), proporción de semilla a vaina (RSV), floración (FL), días después de la floración para fructificación (DAFPF), días después de la floración para cosecha fresca (DAFPCF) y la variable básica rendimiento de grano por hectárea (PDG), evaluados en cultivares de frijol común en un experimento realizado en la ciudad de Pombal - PB.

| Variable | Effect | Estimate | | |
|---|---|---|---|---|
| | | Phenotypic | Genotypic | Environmental |
| PD | Direct on GY | 0.10 | -0.36 | 0.03 |
| | Indirect via TNP | 0.03 | 0.00 | -0.01 |
| | Indirect via PUP | 0.02 | 0.00 | -0.02 |
| | Indirect via PL | 0.00 | 0.02 | -0.02 |
| | Indirect via NGP | 0.00 | 0.08 | 0.01 |
| | Indirect via NPP | -0.03 | 0.16 | 0.01 |
| | Indirect via SS | 0.05 | 0.06 | -0.05 |
| | Indirect via PP | 0.07 | 0.32 | -0.14 |
| | Indirect via PSR | -0.10 | -0.49 | 0.04 |
| | Indirect via FL | 0.00 | 0.28 | -0.02 |
| | Indirect via DAFFF | 0.00 | 0.00 | -0.02 |
| | Indirect via DAFFH | 0.00 | 0.28 | -0.02 |
| | Total | 0.14 | 0.27 | -0.21 |
| NGP | Direct on GY | -0.02 | 0.29 | 0.04 |
| | Indirect via TNP | 0.00 | 0.04 | 0.00 |
| | Indirect via PUP | 0.11 | 0.45 | 0.00 |
| | Indirect via PL | 0.18 | -0.80 | -0.03 |
| | Indirect via PD | 0.00 | -0.03 | 0.01 |
| | Indirect via NPP | 0.02 | -0.05 | 0.00 |
| | Indirect via SS | 0.09 | 0.18 | 0.05 |
| | Indirect via PP | 0.27 | 0.78 | 0.14 |
| | Indirect via PSR | 0.08 | 0.43 | -0.08 |
| | Indirect via FL | 0.01 | 0.23 | 0.02 |
| | Indirect via DAFFF | 0.00 | 0.03 | 0.00 |
| | Indirect via DAFFH | 0.00 | -0.69 | 0.02 |
| | Total | 0.75 | 0.86 | 0.13 |
| NPP | Direct on GY | 0.10 | -0.38 | -0.05 |
| | Indirect via TNP | -0.06 | 0.01 | 0.05 |
| | Indirect via PUP | 0.00 | 0.00 | 0.00 |
| | Indirect via PL | 0.10 | -0.09 | 0.00 |
| | Indirect via PD | -0.03 | 0.15 | 0.00 |
| | Indirect via NGP | 0.00 | -0.15 | 0.00 |
| | Indirect via SS | -0.02 | -0.09 | 0.07 |
| | Indirect via PP | 0.16 | 0.34 | 0.29 |
| | Indirect via PSR | 0.18 | 0.89 | -0.03 |
| | Indirect via FL | 0.00 | -0.13 | 0.00 |
| | Indirect via DAFFF | 0.01 | 0.08 | 0.00 |
| | Indirect via DAFFH | 0.00 | -0.13 | 0.00 |
| | Total | 0.44 | 0.50 | 0.33 |
| SS | Direct on GY | 0.15 | 0.10 | 0.22 |
| | Indirect via TNP | 0.01 | 0.00 | 0.02 |
| | Indirect via PUP | 0.09 | 0.00 | 0.04 |
| | Indirect via PL | 0.07 | -0.05 | 0.00 |
| | Indirect via PD | 0.03 | -0.23 | -0.01 |
| | Indirect via NGP | -0.01 | -0.85 | 0.01 |
| | Indirect via NPP | -0.01 | 0.19 | -0.02 |
| | Indirect via PP | 0.32 | 0.77 | 0.46 |
| | Indirect via PSR | -0.13 | -0.50 | -0.13 |
| | Indirect via FL | 0.01 | 0.46 | 0.01 |
| | Indirect via DAFFF | 0.04 | 0.25 | -0.02 |
| | Indirect via DAFFH | 0.01 | 0.46 | 0.03 |
| | Total | 0.58 | 0.58 | 0.61 |

| Variable | Effect | Estimate | | |
|---|---|---|---|---|
| | | Phenotypic | Genotypic | Environmental |
| PP | Direct on GY | 0.42 | 0.98 | 0.62 |
| | Indirect via TNP | 0.00 | 0.00 | 0.05 |
| | Indirect via PUP | 0.10 | 0.01 | 0.08 |
| | Indirect via PL | 0.16 | -0.12 | -0.01 |
| | Indirect via PD | 0.01 | -0.12 | 0.00 |
| | Indirect via NGP | 0.00 | -0.90 | 0.00 |
| | Indirect via NPP | 0.04 | -0.13 | -0.02 |
| | Indirect via SS | 0.12 | 0.10 | 0.17 |
| | Indirect via PSR | 0.04 | 0.16 | 0.05 |
| | Indirect via FL | 0.01 | 0.40 | 0.00 |
| | Indirect via DAFFF | 0.03 | 0.17 | -0.01 |
| | Indirect via DAFFH | 0.02 | 0.40 | 0.00 |
| | Total | 0.95 | 0.95 | 0.93 |
| PSR | Direct on GY | 0.28 | 0.96 | 0.38 |
| | Indirect via TNP | 0.04 | 0.01 | 0.00 |
| | Indirect via PUP | 0.00 | 0.00 | -0.02 |
| | Indirect via PL | 0.14 | -0.10 | 0.00 |
| | Indirect via PD | 0.00 | 0.20 | 0.00 |
| | Indirect via NGP | 0.00 | -0.40 | 0.00 |
| | Indirect via NPP | 0.07 | -0.35 | 0.00 |
| | Indirect via SS | -0.07 | -0.04 | -0.08 |
| | Indirect via PP | 0.07 | 0.16 | 0.11 |
| | Indirect via FL | 0.00 | 0.10 | -0.01 |
| | Indirect via DAFFF | -0.03 | -0.17 | 0.00 |
| | Indirect via DAFFH | 0.00 | 0.06 | 0.00 |
| | Total | 0.42 | 0.43 | 0.38 |
| FL | Direct on GY | 0.02 | 0.64 | 0.09 |
| | Indirect via TNP | 0.01 | 0.00 | -0.01 |
| | Indirect via PUP | 0.10 | 0.00 | -0.01 |
| | Indirect via PL | 0.16 | -0.10 | -0.02 |
| | Indirect via PD | 0.03 | -0.16 | 0.00 |
| | Indirect via NGP | -0.02 | -1.11 | 0.01 |
| | Indirect via NPP | -0.01 | 0.08 | 0.00 |
| | Indirect via SS | 0.08 | 0.07 | 0.02 |
| | Indirect via PP | 0.21 | 0.60 | 0.01 |
| | Indirect via PSR | 0.02 | 0.11 | -0.03 |
| | Indirect via DAFFF | -0.01 | -0.08 | 0.00 |
| | Indirect via DAFFH | 0.02 | 0.64 | 0.09 |
| | Total | 0.61 | 0.69 | 0.15 |
| DAFFF | Direct on GY | -0.07 | -0.32 | -0.04 |
| | Indirect via TNP | 0.03 | -0.01 | -0.01 |
| | Indirect via PUP | 0.00 | 0.00 | 0.01 |
| | Indirect via PL | 0.07 | -0.04 | -0.01 |
| | Indirect via PD | 0.00 | -0.05 | 0.00 |
| | Indirect via NGP | 0.00 | -0.11 | 0.01 |
| | Indirect via NPP | -0.02 | 0.09 | 0.00 |
| | Indirect via SS | -0.08 | -0.07 | 0.09 |
| | Indirect via PP | -0.18 | -0.52 | 0.20 |
| | Indirect via PSR | 0.14 | 0.52 | 0.02 |
| | Indirect via FL | 0.00 | 0.17 | 0.00 |
| | Indirect via DAFFH | 0.00 | 0.17 | 0.00 |
| | Total | -0.13 | -0.17 | 0.27 |

| Variable | Effect | Estimate | | |
|---|---|---|---|---|
| | | Phenotypic | Genotypic | Environmental |
| DAFFH | Direct on GY | -0.02 | -0.64 | -0.09 |
| | Indirect via TNP | -0.02 | 0.00 | 0.01 |
| | Indirect via PUP | -0.10 | 0.00 | 0.01 |
| | Indirect via PL | -0.16 | 0.11 | 0.02 |
| | Indirect via PD | -0.03 | 0.16 | 0.00 |
| | Indirect via NGP | 0.02 | 1.11 | -0.01 |
| | Indirect via NPP | 0.02 | -0.11 | 0.00 |
| | Indirect via SS | -0.08 | -0.10 | -0.02 |
| | Indirect via PP | -0.21 | -0.58 | -0.01 |
| | Indirect via PSR | -0.02 | -0.08 | 0.03 |
| | Indirect via FL | -0.03 | -0.64 | -0.09 |
| | Indirect via DAFFF | 0.02 | 0.08 | 0.00 |
| | Total | -0.61 | -0.69 | -0.15 |
| Determination coefficient ($R^2$) | | 0.96 | 0.87 | 0.90 |
| Effect of residual variable | | 0.18 | 0.35 | 0.31 |
| k value used in the analysis | | 0.07 | 0.07 | 0.07 |

Initially, phenotypic and genotypic correlations of the explanatory variables with the basic variable GY showed the same sign and similar magnitude. The genotypic and environmental correlations between PUP x GY, PD x GY and DAFOPF x GY, showed different signs. Environmental correlations showed positive and negative values.

PUP, PL and PP showed high phenotypic and genotypic correlation with GY, while NGP, SS and FL moderately correlated with GY and TNP, PD, NPP and PSR weekly correlated with GY. Finally, GY correlated weakly and negatively with DAFOPF and moderately and negatively with DAFFH.

A high environmental correlation was observed between POS and GY, moderate environmental correlation of SS with GY and weak environmental correlation of TNP, PL, NGP, NPP, PSR, FL and DAFPOF with GY, and of PUP, PD and DAFFH with GY.

Among the variables showing high phenotypic correlation with GY, only PL and PP had direct high-magnitude effects exceeding the residual effect estimate. Despite a high correlation with GY, PUP had a direct effect, not exceeding the residual effect. Among the variables that showed moderate phenotypic correlation with GY, NGP had a direct negative effect on GY. SS and FL had a direct effect, not exceeding the residual effect. Among the variables showing weak phenotypic correlation with GY, TNP had a direct negative effect on the main variable, while PD and NPP had a direct effect not exceeding the residual effect. Despite a weak phenotypic correlation with the main variable, PSR showed a direct effect exceeding the residual effect.

Regarding the genotypic path analysis of the variables showing high correlation with GY, only PP had a direct effect exceeding 2.5 times the residual effect. PUP had no direct effect, and PL had a negative direct effect. NGP and SS had a direct effect not surpassing the residual effect, while FL had a direct effect 1.5 times higher than the residual effect. Among the variables with a weak correlation with the main variable, TNP had no direct effect. PD and NPP had a direct negative effect, and PSR showed a direct effect 2 times higher than the residual effect.

Among most variables, considering the environmental trail analyses, the direct effects did not exceed the magnitude of the residual effect. However, the direct effect of POS on GY, exceeding twice the residual effect, was noteworthy.

Table 4 (page 135) shows the direct and indirect effects of the secondary components on the primary components of grain yield in cowpea beans based on the causal diagram shown in figure 1 (page 129).

Once again, considering the coefficient of determination, there was good precision in the regression analysis. It appeared that the SS variable had a moderate correlation and a high magnitude direct effect with PUP and NGP. The variable SS, despite a weak correlation with PL, haD a direct high-magnitude effect exceeding the residual effect.

**Table 4.** Direct and indirect effects of six secondary components (SS, PP, PSR, LFL, DAFFF and DAFFH) on six primary components (TNP, PUP, PL, PD, NGP and NPP) of grain yield evaluated in cowpea cultivars in an experiment conducted in the municipality of Pombal – PB.

**Tabla 4.** Efectos directos e indirectos de seis componentes secundarios (PDC, PDV, RSV, LFL, DAFPF y DAFPCF) sobre seis componentes primarios (NTV, MUV, COMPV, DIAMV, NGVA y NVPL) del rendimiento de grano evaluado en cultivares de frijol caupí en un experimento realizado en la ciudad de Pombal - PB.

| **Effect** | | **TNP** | **PUP** | **PL** | **PD** | **NGP** | **NPP** |
|---|---|---|---|---|---|---|---|
| Direct | SS | -0.58 | 2.08 | 1.69 | 0.15 | 1.06 | 0.08 |
| Indirect | PP | -0.21 | -0.57 | -0.48 | -0.00 | -0.51 | -0.23 |
| Indirect | PSR | -0.45 | -0.51 | -0.63 | 0.18 | -0.41 | -0.53 |
| Indirect | FL | 0.17 | 0.30 | 0.32 | 0.39 | 0.83 | -0.01 |
| Indirect | DAFFF | 0.78 | -0.18 | -0.17 | -0.07 | 0.08 | 0.50 |
| Indirect | DAFFH | 0.12 | -0.41 | -0.37 | -0.19 | -0.49 | 0.08 |
| Total | | -0.17 | 0.68 | 0.30 | 0.32 | 0.57 | -0.16 |
| Effect | | TNP | PUP | PL | PD | NGP | NPP |
| Direct | PP | -0.26 | -0.75 | -0.63 | 0.00 | -0.67 | -0.31 |
| Indirect | SS | -0.44 | 1.59 | 1.29 | 0.11 | 0.81 | 0.06 |
| Indirect | PSR | 0.15 | 0.17 | 0.22 | -0.06 | 0.14 | 0.18 |
| Indirect | FL | 0.15 | 0.27 | 0.29 | 0.35 | 0.75 | -0.01 |
| Indirect | DAFFF | 0.58 | -0.14 | -0.13 | -0.05 | 0.06 | 0.37 |
| Indirect | DAFFH | 0.11 | -0.37 | -0.34 | -0.17 | -0.45 | 0.07 |
| Total | | 0.30 | 0.77 | 0.69 | 0.17 | 0.65 | 0.37 |
| Effect | | TNP | PUP | PL | PD | NGP | NPP |
| Direct | PSR | 0.96 | 1.10 | 1.37 | -0.37 | 0.90 | 1.15 |
| Indirect | SS | 0.26 | -0.96 | -0.78 | -0.07 | -0.49 | -0.03 |
| Indirect | PP | -0.04 | -0.12 | -0.10 | -0.00 | -0.10 | -0.05 |
| Indirect | FL | 0.02 | 0.04 | 0.04 | 0.05 | 0.11 | -0.00 |
| Indirect | DAFFF | -0.67 | 0.16 | 0.14 | 0.06 | -0.07 | -0.43 |
| Indirect | DAFFH | 0.01 | -0.05 | -0.05 | -0.02 | -0.06 | 0.01 |
| Total | | 0.55 | 0.16 | 0.61 | -0.39 | 0.27 | 0.63 |
| Effect | | TNP | PUP | PL | PD | NGP | NPP |
| Direct | FL | 0.30 | 0.55 | 0.57 | 0.69 | 1.49 | -0.03 |
| Indirect | SS | -0.32 | 1.16 | 0.95 | 0.08 | 0.59 | 0.04 |
| Indirect | PP | -0.13 | -0.38 | -0.32 | 0.00 | -0.34 | -0.15 |
| Indirect | PSR | 0.07 | 0.08 | 0.10 | -0.02 | 0.06 | 0.08 |
| Indirect | DAFFF | -0.34 | 0.08 | 0.07 | 0.03 | -0.03 | -0.22 |
| Indirect | DAFFH | 0.22 | -0.74 | -0.67 | -0.35 | -0.89 | 0.15 |
| Total | | -0.22 | 0.74 | 0.67 | 0.35 | 0.89 | -0.15 |
| Effect | | TNP | PUP | PL | PD | NGP | NPP |
| Direct | DAFFF | -1.38 | 0.33 | 0.30 | 0.12 | -0.15 | -0.88 |
| Indirect | SS | 0.32 | -1.17 | -0.95 | -0.08 | -0.60 | -0.04 |
| Indirect | PP | 0.11 | 0.32 | 0.27 | 0.00 | 0.28 | 0.13 |
| Indirect | PSR | 0.46 | 0.53 | 0.66 | -0.18 | 0.43 | 0.55 |
| Indirect | FL | 0.07 | 0.13 | 0.14 | 0.17 | 0.37 | 0.00 |
| Indirect | DAFFH | 0.05 | -0.18 | -0.16 | -0.08 | -0.22 | 0.03 |
| Total | | -0.33 | -0.02 | 0.28 | 0.01 | 0.10 | -0.18 |
| Effect | | TNP | PUP | PL | PD | NGP | NPP |
| Direct | DAFFH | -0.22 | 0.74 | 0.67 | 0.35 | 0.89 | -0.15 |
| Indirect | SS | 0.32 | -1.16 | -0.95 | -0.08 | -0.59 | -0.04 |
| Indirect | PP | 0.13 | 0.38 | 0.32 | 0.00 | 0.34 | 0.15 |
| Indirect | PSR | -0.07 | -0.08 | -0.10 | 0.02 | -0.06 | -0.08 |
| Indirect | FL | -0.30 | -0.55 | -0.57 | -0.69 | -1.49 | 0.03 |
| Indirect | DAFFF | 0.34 | -0.08 | -0.07 | -0.03 | 0.03 | 0.22 |
| Total | | 0.22 | -0.74 | -0.67 | -0.35 | -0.89 | 0.15 |
| $R^2$ | | 0.90 | 0.87 | 0.93 | 0.32 | 0.93 | 0.75 |
| Residual | | 0.32 | 0.36 | 0.26 | 0.82 | 0.26 | 0.50 |

The PSR variable had a moderate correlation with TNP, PL and NPP and a high-magnitude direct effect, and weak correlation with PUP and NGP, with a high-magnitude direct effect outweighing the residual effect. The FL variable had a strong correlation with PUP and NGP and a high-magnitude direct effect outweighing the residual effect, and moderate correlation with PL, but with a direct effect exceeding the residual.

Finally, DAFFH had a low correlation with PUP, PL and NGP, but with direct high-magnitude effect exceeding the residual effect.

## Discussion

The existence of genetic variability explains the significant differences between the variables evaluated in cowpea cultivars (4). Specifically, this variability is caused by different alleles and the phenotypic expression of these variables under evaluation (13). For genetic improvement, this crucial result allows the artificial selection of superior cultivars regarding these important production components (46).

The calculated coefficients of variation were heterogeneous. According to the classification proposed by Gomes (20), values were low for PL, PD, PSR, FL and DAFFH, average for NGP and DAFFF, and high for TNP, PUP, NPP, GY, SS and POS. According to Marques Júnior *et al.* (1997), the heterogeneity of the experimental material contributes to a higher coefficient of variation. This heterogeneity would explain why experiments with cowpea show higher estimates of the coefficient of variation than other cultures. It should also be noted that many of these variables were previously evaluated in other studies with coefficients of variation between similar ranges (26, 41). Thus, our experimental precision may be considered adequate (5, 45).

According to Dutra Filho *et al.* (2020), the phenotypic expression of PL, NGP, PSR, FL, DAFFF and DAFFH is mostly due to genetic effects since the genotypic variance exceeded the environmental variance. This result points to a repetition in the phenotypic expression of these important production components in the respective environment for selection of superior cultivars.

The genotypic variation coefficient (CVg) allows measuring genetic variability (40). Although the ANOVA had previously identified variability among the variables, the CVg identifies those variables with the greatest genetic variability for a breeder to practice selection and obtain greater gains. According to Oliveira *et. al.* (2008), CVg >10, is considered high; therefore, in the present work, NGP, NPP, GY, SS, PP and DAFFF showed high potential for breeding strategies.

The ratio between the genotypic coefficient of variation and the experimental coefficient of variation (CVg/CVe) was greater than unity for PL, NGP, PSR, FL and DAFFH. This genetic parameter, also called index b, identifies variables with greater genetic variability and cultivar selection potential, guiding the most suitable breeding method for the crop (6). In other words, the greater the magnitude, the simpler methods with a high probability of significant genetic gains. When index b shows heterogeneity in the variables considered production components, applying different methods of selection indices turns out important for maximizing genetic gains in each analyzed variable.

Heritability coefficients indicate high reliability of the phenotypic value as an indicator of genetic value in NGP, NPP, GY, SS, PP, DAFFPF and DAFFH, considering the estimated values were high > 75 (22). Heritability coefficients for PD, NPP and SS showed medium magnitude. Thus, ample possibilities for significant genetic gains can be inferred in cowpea selection based on variables with high-magnitude heritability (38). Variables with medium magnitude coefficients may allow genetic gains to a lesser extent.

Heritability corresponds to the heritable proportion of the total genetic variability of the variables under evaluation (7). High magnitude estimates in the present work indicate the need to study correlations among these characters, especially heritable genotypic correlations guiding an adequate selection strategy (23). This procedure, with the respective developments in path analysis, will allow defining the best selection index model maximizing genetic gains through direct and indirect selection and, thus, increasing farm productivity with new cultivars in the hinterland of Paraíba.

Regarding the correlations, according to Cruz *et al.* (2012), sampling errors are the main cause of different signals in phenotypic and genotypic correlations of a given variable. Phenotypic and genotypic correlations of explanatory variables with GY presented in table 3 (page 131-134), with same sign and similar magnitude, demonstrate an excellent experimental and analytic precision (18).

When genotypic and environmental correlations show different signs, such as PUP x GY, PD x GY and DAFOPF x GY, the causes of genetic and environmental variations influence these variables by different physiological mechanisms (1). In environmental correlations, TNP x GY, SS x GY and POS x GY present the same sign and are influenced by the same sources of environmental variations, while different signs evidence how the environment favors one character over the other (36).

As explained, the correlation coefficient measures the association between two variables, assuming that for high correlation and positive sign, a gain on one variable can be obtained through indirect selection on the other (37). However, the type of strategy, whether direct or indirect selection, should initially be designed by path analysis, carefully observing the direct and indirect effects when a large number of variables is available. The study is finally complemented by evaluating selection indices.

In the path analysis, the variable PUP had a high phenotypic and genotypic correlation with the basic variable GY, however, the direct effect is low and null (table 3, page 131-134). This means that direct selection will not provide significant genetic gains in the basic variable GY (41). In addition, PUP had a low magnitude heritability coefficient (table 2, page 130). The recommended strategy would be simultaneous selection of characters with emphasis on those with high indirect effects, such as POS (15). This emphasizes the importance of evaluating and identifying suitable models of selection indexes.

The variable PL also presented a high phenotypic and genotypic correlation with GY. However, in this case, the direct effect is low in the phenotypic correlation and negative in the genotypic correlation, given by the absence of cause and effect; *i.e.* pod length is not the main determinant of GY. Therefore, our recommendation is to identify other variables providing greater selection gain (11).

Regarding PP, high phenotypic correlation with GY and a direct effect in favor of selection defines an efficient indirect selection. This result is even more promising when observing the genotypic correlation between PP and GY. In addition to being a high genotypic correlation, and therefore heritable, the direct effect has a high magnitude exceeding 2.5 times the residual effect. These variables have a true cause-and-effect association. POS is the main determinant of GY, and since this association is directly proportional (with a positive sign in the correlation), it implies that cowpea selection with higher pod yield will be an effective indirect selection of higher grain yield (39). It should also be noted that in the present work and due to its high heritability, POS was classified as a secondary component of production.

For NGP, the simultaneous selection strategy should pay special attention to the PP variable with high-magnitude indirect effects (18).

The variables NPP, SS, FL and DAFFH showed moderate phenotypic and genotypic correlation with GY. NPP did not correlate with GY. Considering SS, simultaneous selection must be adopted, and for FL, a direct selection strategy is the most suitable, *i.e.*, the shorter the flowering time, the earlier the harvest and consequent investment return. The variable DAFFH presents an inversely proportional relationship of cause and effect with GY, meaning early cycle cultivars allow higher productivity and earlier harvests.

Variables showing weak correlations obtained costly direct and indirect selection gains. Again, we recommend selection index models verifying feasibility.

In the environmental path analysis, the POS variable highly correlated with GY with a direct and favorable effect. As this experiment was conducted under an irrigated system, we could infer that both variables benefited from the same environmental variations, in particular, fertilization, irrigation and pest control (9).

The path analysis of the secondary components for each primary component demonstrated that for SS, the direct selection strategy maximized selection gain for PUP and NGP.

The high and moderate correlation of PP with PUP, PL and NGP was determined by the indirect effect via SS. Thus, a good strategy in POS selection obtaining significant gains in PUP, PL and NGP, should consider SS through simultaneous selection. Direct PSR and FL selection strategy should maximize gains in TNP, PL and NPP, and in PUP, PL and NGP, respectively.

After carefully approaching path analysis of secondary components with primary components of production, no easy-to-measure secondary morphological components of great importance could determine the primary components of grain production (GY), with the exception of FL and DAFFH. This difficulty has already been reported by other authors working with bean crops (24). New correlations and path analyses should consider other secondary components in the semi-arid region of Paraíba.

## Conclusions

Variables with greater potential were identified for the selection of superior cultivars of cowpea in the semiarid region of Paraíba.

The pod yield variable (PP) seems promising for cultivar selection considering higher grain yield (GY).

Direct and simultaneous selection are the most suitable strategies.

Maximized genetic gains call for further studies on selection indices.

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# Biometric genetics in Cowpea beans (*Vigna unguiculata* (L.) Walp) II: estimates of genetic gains through selection indices

## Genética biométrica en Caupí (*Vigna unguiculata* (L.) Walp) II: estimaciones de ganancias genéticas a través de índices de selección

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### Abstract

Low cowpea productivity (*Vigna unguiculata* (L.) Walp) in the semi-arid region of Paraíba is due, among other factors, to poor-quality cultivars. This research tested biometric models intending to increase productivity of superior cultivars with the following objectives: i. Estimate genetic gains in production components; ii. Identify the selection index model providing the greatest gains through simultaneously selecting a set of variables, and iii. Select cultivars with higher productivity. The experiment was carried out in the experimental field of the Agrifood Science and Technology Center of the Federal University of Campina Grande. Eight cultivars and 13 variables were evaluated. Data were subjected to ANOVA and means were grouped using the Scott and Knott test. Genetic gains were estimated by correlated response, classic selection index, rank sum and index based on desired gains. Direct selection of the secondary pod yield component provides significant genetic gains in main grain yield. Among the methodologies used, the classic selection index provided greater distribution of genetic gains for main grain yield and primary production components. These results allow concluding that Costela de vaca, BRS Marataoã and Paulistinha cultivars should be selected for cultivation and commercial exploitation in the semiarid region of Paraíba.

**Keywords**
selection indices • genetic improvement • simultaneous selection • productivity • correlated response

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## Resumen

En la región semiárida de Paraíba, el caupí (*Vigna unguiculata* (L.) Walp) es el principal producto de la agricultura familiar. El cultivo tiene baja productividad debido a lluvias irregulares y al uso de cultivares de baja productividad. Con el objetivo de superar estas limitaciones y aumentar la eficiencia de la selección de cultivares superiores, se llevó a cabo un extenso estudio utilizando modelos biométricos en caupí, con los siguientes objetivos: i. Estimar las ganancias genéticas de los componentes de la producción; ii. Identificar el modelo de índice de selección que proporciona las mayores ganancias mediante la selección simultánea de un conjunto de variables y iii. Seleccionar cultivares con mayor productividad. El experimento se llevó a cabo en el campo experimental del Centro de Ciencia y Tecnología Agroalimentaria de la Universidad Federal de Campina Grande. Se evaluaron 13 variables relacionadas con la productividad. Los datos se sometieron a ANOVA y las medias se agruparon según la prueba de Scott y Knott p≤ 0,05. También se estimó el coeficiente de heredabilidad de cada variable. Las ganancias genéticas se estimaron utilizando las siguientes metodologías: respuesta correlacionada, índice de selección clásico, suma de rango e índice basado en las ganancias deseadas. Se encontró que la selección directa del componente de rendimiento de la vaina secundaria proporciona ganancias genéticas significativas en rendimiento de grano. Entre las metodologías utilizadas, el índice de selección clásico proporcionó una mayor distribución de las ganancias genéticas para rendimiento de grano y para los componentes primarios de producción. Estos resultados permiten concluir que los cultivares Costela de vaca, BRS Marataoã y Paulistinha pueden seleccionarse para cultivo y explotación en la región semiárida de Paraíba.



**Palabras clave**

índices de selección • mejoramiento genético • selección simultánea • productividad • respuesta correlacionada


## Introduction

Cowpea beans (*Vigna unguiculata* L. Walp) are one main product of family farming in Paraíba, and one major source of rural employment and income. As in other northeastern states, agricultural productivity is greatly affected by semi-arid conditions (37). However, according to Oliveira *et al.* (2001) low productivity is not only linked to unfavourable environmental conditions but also to the use of traditional, poor-quality cultivars.

Superior cultivars should meet a set of favourable agronomic and yield-related traits while satisfying both consumer and producer requirements (29). According to Cruz *et al*. (2012), selecting one or a few traits turns inefficient. While improving only a few selected variables, other undesired traits may be unintentionally selected. To overcome this limitation, breeders have used different selection methodologies (13, 16, 17).

Normally, selection indices are obtained from linear combinations of a set of variables, allowing a single value to perform an efficient selection with significant genetic gains (19). In other words, selection indices simultaneously combining several variables of economic importance, result in superior cultivars choices for this set of variables, regardless of any correlation among them (1, 17).

Existing literature mentions several Selection Index methodologies for cowpea (5, 33). However, there is strong need for new sets of variables with high heritability and ease of measurement. Additionally, these studies are still scarce in semiarid regions. Given the above, this work aimed to estimate genetic gains in production components, finding one selection index model providing greater gains through simultaneous selection of a set of variables for productive cultivars.

## Material and methods

The experiment was carried out in an experimental field at the Center for Agrifood Science and Technology, Federal University of Campina Grande, CCTA/UFCG, Campus de Pombal - Paraíba, (06°46' South latitude, 37°48' West longitude) (4). According to Köppen's classification, the climate is Aw, semi-arid, with summer and autumn rains and average annual rainfall of 800 mm, with the rainiest period between February and April, concentrating 60 to 80% of the total annual precipitation (25).

For experimental set-up, ploughing was carried out 15 days before sowing, followed by cross harrowing 5 days before bean planting, providing weed control for germination. Soon after this procedure, the plots were marked and randomly distributed in the field.

Sowing was manual and holes were opened with a hoe, at approximately 5 cm depth, placing three seeds per hole. Spacing was 0.5 m with five plants per linear meter (41).

Fertilization was carried out according to Fernandes (1993). Thinning was close to the ground, about 15 days after emergence, keeping two plants per hole. Pest management involved two sprays with Dimethoate (1.0 litre/ha$^{-1}$) against aphids (*Apis cracyvora* Koch) and thrips (Order Thysanoptera), one spray with Methomyl (0.5 litre/ ha$^{-1}$) for armyworm (*Spodoptera frugiperda*) and one with Imidacloprid and Beta-cyfluthrin (270 g/ha$^{-1}$) for whitefly (Order Hemiptera).

The experimental design was randomized blocks (18) with eight treatments and four replications, totalling 32 experimental units, with 2.0 m spacing among blocks and plots. The treatments consisted of eight cowpea cultivars: Costela de Vaca (Control), BRS Marataoã, BRS Itaim, BR-17 Gurguéia, BRS Novaera, Paulistinha, Setentão and BRS Patativa.

Each experimental unit consisted of 9 m$^2$ with six rows of plants and a useful area of 4m$^2$. Spacing between rows was 0.5 meters, with fifteen holes and two plants per hole. Two lateral rows were considered borders. Data collection was carried out in the third and fifth rows.

Cultivar evaluation included phenology. The following characteristics related to precocity were evaluated: initial flowering (FL) and initial fruiting (DAFFF), determined by number of days between sowing and 50% of the plants with at least one flower or an open pod. Precocious plants were those reaching full flowering 70 days after sowing. This helped obtaining number of days between bloom and fruiting (DAFFH).

Manual harvest was performed with completely pale brown (dry) pods. During harvest, several yield components were measured. Total number of pods (TNP); pod unit mass (PUP, kg); pod length (PL, cm); pod diameter (PD, mm) obtained with a caliper; pod grain number (NGP), counting the grains of a sample of 10 pods; number of pods per plant (NPP), as the ratio between total pods in the usable area and number of plants; grain yield (GY, kg.) later transformed into tons per hectare; pod bark productivity (PDC, kg.), later transformed into tons per hectare; pod yield (PP, kg.), later transformed into tons per hectare, and seed/ pod ratio (PSR), as the ratio between total mass of grains and pod number.

Data were submitted to ANOVA using linear additive model of randomized blocks, according to Cruz (2006a). Means were grouped by the Scott and Knott test (1974) at 5% probability.

Genetic gains were estimated through Correlated response, Classic selection index proposed by Smith (1936) and Hazel (1943), Rank-sum-based index proposed by Mulamba and Mock (1978) and the index based on gains proposed by Pesek and Baker (1969) and Cruz *et al.* (2012). For genetic gain calculation in the correlated response and classic selection index, k value was established at 0.3, according to Cruz (2006b) for multicollinearity, allowing a correlation of 0.90 between the index and the genotypic aggregate. The methodologies proposed by Mulamba and Mock (1978) and Pesek and Baker (1969) exclude multicollinear variables. All genetic-statistical analyzes were performed by Genes software (11).

## Results and discussion

The ANOVA revealed significant differences between the evaluated variables, except for TNP, PUP and NPP. Means grouping allowed the establishment of superior groups regarding the variables PL, PD, NGP, GY, PP, PSR, FL, DAFFF and DAFFH (table 1, page 143).

**Table 1.** Mean grouping for evaluated in an experiment conducted in an experimental field at the Center for Food Science and Technology of the Federal University of Campina Grande in the city of Pombal - Paraíba.

**Tabla 1.** Agrupación de los promedios de los caracteres evaluados en el campo experimental del Centro de Ciencia y Tecnología de Alimentos de la Universidad Federal de Campina Grande en la ciudad de Pombal - Paraíba.

| Cultivars | TNP | PUP(kg) | PL(cm) | PD(cm) | NGP | NPP | GY(t.ha$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Costela de vaca | 277.5a | 3.50a | 22.28a | 8.31a | 16.30a | 4.12a | 2.40ª |
| BR-17 Gurguéia | 267.2a | 3.12a | 18.07c | 7.21b | 15.85a | 3.50a | 1.65b |
| BRS Itaim | 263.7a | 3.00a | 17.12c | 7.15b | 10.67b | 3.42a | 1.40b |
| BRS Patativa | 228.7a | 3.75a | 18.91b | 8.22a | 15.67a | 2.95a | 2.12ª |
| Setentão | 208.2a | 3.50a | 17.43c | 9.05a | 14.05a | 2.75a | 1.70b |
| BRS Marataoã | 206.2a | 3.62a | 19.70b | 7.47b | 15.70a | 3.13a | 2.10ª |
| BRS Novaera | 193.5a | 2.50a | 15.76c | 8.34a | 8.42b | 2.95a | 1.25b |
| Paulistinha | 173.2a | 3.50a | 19.83b | 8.01a | 16.82a | 2.58a | 1.65b |
| F | 1.26$^{ns}$ | 1.89$^{ns}$ | 6.80** | 3.19* | 13.67** | 2.41$^{ns}$ | 4.36** |
| **Cultivars** | **PDC(t.ha$^{-1}$)** | **PP(t.ha$^{-1}$)** | **PSR(%)** | **FL** | **DAFFF** | **DAFFH** | - |
| Costela de vaca | 0.57a | 2.92a | 80.60a | 47.25b | 7.00b | 37.75c | - |
| BR-17 Gurguéia | 0.60a | 2.20b | 74.85b | 45.75b | 4.00c | 39.25c | - |
| BRS Itaim | 0.40a | 1.75b | 77.93a | 38.00d | 6.50b | 47.00a | - |
| BRS Patativa | 0.65a | 2.75a | 76.91a | 47.00b | 5.25b | 38.00c | - |
| Setentão | 0.72a | 2.42a | 69.07b | 46.25b | 3.75c | 38.75c | - |
| BRS Marataoã | 0.75a | 2.87a | 73.51b | 45.00b | 4.00c | 40.00c | - |
| BRS Novaera | 0.47a | 1.60b | 72.62b | 40.25c | 6.25b | 44.75b | - |
| Paulistinha | 0.52a | 1.82b | 76.53a | 49.75a | 11.25a | 35.25d | - |
| F | 2.59* | 4.04** | 5.62** | 50.70** | 34.57** | 50.71** | - |

Total number of pods (TNP), pod unit mass (PUP, kg), pod length (PL, cm), pod diameter (PD, mm), pod grain number (NGP), number of pods per plant (NPP), grain yield (GY, kg.), pod bark productivity (PDC, kg.), pod yield (PP, kg.), seed/pod ratio (PSR), initial flowering (FL), initial fruiting (DAFFF) and number of days between bloom and fruiting (DAFFH).

Means followed by the same letter belong to the same group by the Scott and Knott test at 5% probability. ** and * show significance at 1 and 5% probability; respectively by the F test. ns non-significant by the F test.

Número total de cápsulas (TNP); masa unitaria de la vaina (PUP, kg); longitud de la vaina (PL, cm); diámetro de la vaina (DP, mm); grano de vaina número (NGP); vainas por planta (NPP), grano rendimiento (GY, kg.); corteza de vaina productividad (PDC, kg.), rendimiento de vaina (PP, kg.), semilla/proporción de cápsulas (PSR), inicial floración (FL), inicial fructificación (DAFFF) y número de días entre florecer y fructificar (DAFH).

Las medias seguidas de la misma letra pertenecen al mismo grupo según la prueba de Scott y Knott al 5% de probabilidad. ** y * muestran significancia al 1 y 5% de probabilidad; respectivamente; por la prueba de F. ns no significativa; por prueba F.

The ANOVA showed great genetic variability among cultivars, as verified by Rocha *et al.* (2003) and Silva and Neves (2011), who also detected significant cultivar effect on grain yield (39). This result allows the application of different selection index methods with favourable perspectives of simultaneous gains in a set of variables (24). Rodrigues *et al.* (2017), verified the existence of genetic variability in cowpea cultivars under water stress conditions and successfully applied different selection index methodologies, identifying superior genotypes.

Regarding mean grouping, three groups were established for PL, emphasizing cultivar Costela de vaca, with the greatest pod length and separately allocated in group 'a'. According to Araújo (2019), pod length varies from 15 to 20 cm. This author emphasizes the importance of smaller pods for mechanized harvesting and larger pods for manual harvesting. In the present work, the results were diverse with smaller pod cultivars for mechanized harvesting and larger pods (> 18cm) for manual harvesting, suitable for small farmers without financial and technological resources to implement mechanized harvesting.

For PD, two groups are observed. Group "a" comprises cultivars Costela de vaca, BRS Patativa, Setentão, BRS Novaera and Paulistinha. For Costa *et al.* (2021), larger diameter pods would contain heavier seeds.

Two groups were also established for NGP. Cultivars Costela de vaca, BRS Marataoã, BR-17 Gurguéia, Paulistinha, Setentão and BRS patativa were allocated to group 'a'. This variable has already been stated as one primary component of production (40), Andrade *et al.* (2010) estimated genetic parameters and correlations in cowpea demonstrating that this variable must be carefully studied in selection indices models, since it is related to other primary components, including PL.

Regarding GY variables, cultivars Costela de vaca, BRS Marataoã and BRS Patativa were allocated to group 'a' while cultivars Costela de vaca, BRS Marataoã, Setentão and BRS

Patativa were allocated to group 'a' for GY and PP. According to Freire Filho *et al.* (2007), grain yield constitutes an important commercial trait for expanding consumption, industrial processing and commercialization of seeds among farmers. These authors also emphasize that high off-season production reaches high market prices. Cultivars Costela de vaca, BRS Marataoã and BRS Patativa showed better GY performance than the other cultivars due to gene recombination and possible transgressive segregation (31). However, before effectively selecting these cultivars, it must be considered that GY is determined by several genes and correlated with several other primary components. Therefore, to truly obtain superior cultivars, evaluating models of selection indices simultaneously gathering several favourable attributes, becomes necessary (5, 12).

For PSR, Costela de vaca, BRS Itaim, Paulistinha and BRS Patativa were placed in group 'a'. Regarding FL, four groups were found and Paulistinha was separately allocated to group 'a'. Noteworthy is that BRS Itaim showed higher precocity in relation to the others. As to DAFFF, three groups resulted with Paulistinha separately allocated to group 'a'.

Finally, for DAFFH, four groups separately allocated BRS Itaim in group 'a' and cultivar Paulistinha in group 'd'.

Genetic gains obtained by correlated responses, that is, by direct and indirect selection, are shown in table 2.

**Table 2.** Estimates of original means ($\overline{X}_0$), selected cultivars ($\overline{X}_s$), broad sense heritability ($h^2$) and direct and indirect selection gains (GS) for 13 traits, evaluated in 8 cowpea cultivars.

**Tabla 2.** Estimaciones de medias originales ($\overline{X}_0$), cultivares seleccionados ($\overline{X}_s$), heredabilidad en sentido amplio ($h^2$) y ganancias de selección directa e indirecta (GS) para 13 caracteres, evaluados en 8 cultivares de caupí.

| **Variables** | $\overline{X}_0$ | $\overline{X}_s$ | **h²%** | **GS** | **GS%** |
|---|---|---|---|---|---|
| TNP | 227.31 | 241.87 | 21.00 | 3.05 | 1.34 |
| PUP | 3.31 | 3.56 | 46.49 | 0.12 | 3.51 |
| PL | 18.64 | 20.99 | 85.30 | 2.01 | 10.76 |
| PD | 7.97 | 7.89 | 68.68 | -0.05 | -0.7 |
| NGP | 14.18 | 16.0 | 92.69 | 1.68 | 11.84 |
| NPP | 3.17 | 3.63 | 58.52 | 0.26 | 8.31 |
| **GY** | **1.78** | **2.25** | **77.10** | **0.36** | **20.12** |
| PDC | 0.59 | 0.66 | 61.52 | 0.04 | 7.85 |
| **PP** | **2.29** | **2.9** | **75.27** | **0.46** | **19.90** |
| PSR | 75.25 | 77.06 | 82.23 | 1.48 | 1.97 |
| FL | 44.90 | 46.12 | 98.02 | 1.19 | 2.66 |
| DAFFF | 6.0 | 5.5 | 97.10 | -0.49 | -8.09 |
| DAFFH | 40.09 | 38.90 | 98.02 | -1.19 | -2.98 |
| Total | | | | 8.92 | 76.49 |
| Selected | Costela de Vaca and BRS Marataoã | | | | |

It appears that direct selection of PP provides, for most of the studied variables, considerable genetic gains, PD, DAFFF and DAFFH.

The variable PP is the main GY determinant, given high direct phenotypic and genotypic effects. Direct selection on this easy-to-measure secondary component allows a response correlated with a high magnitude (>20%) genetic gain in the main variable GY.

Direct selection in POS also provides considerable gains in NGP, NPP, PL and PDC. For the variables TNP and PUP, whose heritability coefficients were low, it is possible to obtain genetic gains.

Falconer (1987) states that obtaining greater gains with indirect selection is possible when the auxiliary variables have higher heritability than the main variable, as for NGP and PL. Corroborating this, Gonçalves *et al.* (2007), stated that to obtain superior cultivars by simultaneously combining a series of favourable attributes and higher productivity, evaluating different selection index methodologies is important (28). Selection gains obtained by the methodology of Smith (1936) and Hazel (1943) are presented in table 3 (page 145).

**Table 3.** Estimates of original means ($\overline{X}_0$), selected cultivars ($\overline{X}_s$), heritability ($h^2$), covariances (Cov) and indirect selection gains (GS) based on the Smith (1936) and Hazel (1943) index for 7 traits, evaluated in 8 cowpea cultivars.

**Tabla 3.** Estimaciones de medias originales ($\overline{X}_0$), cultivares seleccionados ($\overline{X}_s$), heredabilidad ($h^2$), covarianzas (Cov) y ganancias de selección (GS) indirecta basadas en los índices de Smith (1936) y Hazel (1943) para 7 caracteres, evaluados en 8 cultivares de caupí.

| Variables | $\overline{X}_0$ | $\overline{X}_s$ | $h^2$% | Cov (Xj,I) | GS | GS% |
|---|---|---|---|---|---|---|
| **PP** | **2.29** | **2.37** | **75.27** | **3.67** | **0.55** | **24.12** |
| PDC | 0.59 | 0.55 | 61.52 | 0.46 | 0.07 | 11.75 |
| **GY** | **1.78** | **2.02** | **77.10** | **3.30** | **0.50** | **27.83** |
| NPP | 3.17 | 3.35 | 58.52 | 1.74 | 0.26 | 8.22 |
| NGP | 14.18 | 16.56 | 92.69 | 16.90 | 2.55 | 17.94 |
| PUP | 3.31 | 3.50 | 46.49 | -0.12 | -0.01 | -0.55 |
| TNP | 227.31 | 225.37 | 21.00 | 254.90 | 38.38 | 16.89 |
| Total | | | | | 41.8 | 105.66 |
| Index Variance | 71.23 | --- | --- | --- | --- | --- |
| Selection Intensity | 1.27 | --- | --- | --- | --- | --- |
| Selection Differential | 10.36 | --- | --- | --- | --- | --- |
| Selected | Costela de vaca and Paulistinha | | | | | |

This methodology allows obtaining significant and simultaneous genetic gains in important primary yield components, with the exception of PUP. However, this simultaneous selection only caused few changes in this variable (-0.01). Therefore, one can consider null changes in PUP and proceed with a safe selection.

In popcorn, Granate *et al.* (2002) used several selection index methodologies and obtained significant genetic gains. Gains obtained with the Smith (1936) and Hazel (1943) indices were superior to those predicted with other indices, as also obtained by Rodrigues *et al.* (2017), when selecting cowpea populations under water stress. According to Cruz *et al.* (2012), the Smith (1936) and Hazel (1943) indices are superior to direct selection because they consist of linear combinations of several economic variables, whose weighting coefficients maximize the index/genotypic aggregate correlation.

Table 4 shows genetic gains obtained according to Mulamba and Mock (1978), considering variable exclusion after multicollinearity.

**Table 4.** Original means ($\overline{X}_0$), of selected cultivars ($\overline{X}_s$), heritability ($h^2$), covariances (Cov) and indirect selection gains based on the sum of ranks for 7 traits, evaluated in 8 cowpea cultivars.

**Tabla 4.** Estimaciones de medias originales ($\overline{X}_0$), cultivares seleccionados ($\overline{X}_s$), heredabilidad ($h^2$) y ganancias de selección indirecta (GS) basadas en la suma de rangos para 7 caracteres, evaluados en 8 cultivares de caupí.

| Variables | $\overline{X}_0$ | $\overline{X}_s$ | $h^2$% | GS | GS% |
|---|---|---|---|---|---|
| **PP** | **2.29** | **2.90** | **75.27** | **0.46** | **19.90** |
| PDC | 0.59 | 0.66 | 61.52 | 0.05 | 7.85 |
| **GY** | **1.78** | **2.25** | **77.10** | **0.36** | **20.12** |
| NPP | 3.17 | 3.63 | 58.51 | 0.26 | 8.31 |
| NGP | 14.18 | 16.0 | 92.69 | 1.68 | 11.84 |
| PUP | 3.31 | 3.56 | 46.49 | 0.12 | 3.51 |
| TNP | 227.31 | 241.87 | 21.00 | 3.05 | 1.34 |
| Total | | | | 5.98 | 72.87 |
| Selected | Costela de Vaca and BRS Marataoã | | | | |

Once again, significant and simultaneous genetic gains were obtained in primary components of production. No undesired changes in PUP were obtained through the rank sum methodology. In fact, given the simplicity of result interpretation, the rank sum methodology is among the most used in genetic improvement for estimating selection gains. The significant genetic gains here obtained using the sum of ranks, although slightly lower than those obtained by Smith (1936) and Hazel (1943) methodologies, are given by an economic weight equivalent to CVg that considers all the evaluated variables as the main ones (6).

Table 5, shows genetic gain estimates based on the desired gain methodology proposed by Pesek and Baker (1969).

**Table 5.** Original averages ($\overline{X}_0$), of selected cultivars ($\overline{X}_s$), heritability ($h^2$), covariances (Cov) and indirect selection gains based on selection by the Pesek and Baker (1969) index for 7 traits evaluated in 8 cowpea cultivars.

**Tabla 5.** Estimaciones de medias originales ($\overline{X}_0$), cultivares seleccionados ($\overline{X}_s$), heredabilidad ($h^2$) y ganancias de selección indirecta (GS) basadas en la selección por médio del índice de Pesek y Baker (1969) para 7 caracteres, evaluados en 8 cultivares de caupí.

| **Variables** | $\overline{X}_0$ | $\overline{X}_s$ | **$h^2$%** | **GS** | **GS%** |
|---|---|---|---|---|---|
| **PP** | **2.29** | **2.37** | **75.27** | **0.06** | **2.67** |
| PDC | 0.59 | 0.55 | 61.52 | -0.02 | -3.93 |
| **GY** | **1.78** | **2.02** | **77.10** | **0.19** | **10.40** |
| NPP | 3.17 | 3.35 | 58.51 | 0.10 | 3.18 |
| NGP | 14.18 | 16.56 | 92.69 | 2.20 | 15.52 |
| PUP | 3.31 | 3.50 | 46.49 | 0.08 | 2.63 |
| TNP | 227.31 | 225.75 | 21.00 | -0.40 | -0.18 |
| Total | | | | 2.21 | 30.29 |
| Selected | Costela de vaca and Paulistinha | | | | |

The methodology based on desired gains provided simultaneous gains in the POS, GY, NPP, NGP and PUP variables. However, it caused undesired changes in PDC. Despite having provided significant gains in NGP (above 15%), it did not provide greater gains in POS and GY, as also evidenced in other studies (36). It should be noted that this methodology was developed after the difficulty of establishing relative economic weights to the variables, replacing them with the desired gains. These desired gains, according to Crossbie *et al.* (1980) and Vieira (1988) could be replaced by the genetic standard deviation for each variable. However, despite the use of genetic standard deviation, the results do not outweigh gains obtained with other indices. Rodrigues *et al.* (2017) also used the Pesek and Baker (1969) methodology along with the sum of ranks and the classic Smith (1936) and Hazel (1943) indices, obtaining similar results in magnitude and direction, but undesired changes in grain index when using the desired gains index. These divergent results can be explained by the limitations of the genetic structure of the breeding population (23). Thus, it is up to the breeder choosing the best methodology to find the greatest genetic gains, and practice selection with greater safety.

## Conclusions

Direct selection in the secondary component PP provides significant genetic gains in the main variable GY;

The classic selection index presents a greater distribution of genetic gains for the main variable and for the primary components of production;

Cultivars Costela de vaca, BRS Marataoã and Paulistinha are recommended for cultivation and commercial exploitation in the semiarid region of Paraíba.

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