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Scientific Article

Quality of floral stems of lisianthus (*Eustoma grandiflorum* Raf.) inoculated with *Bacillus subtilis* and *Glomus intraradices*

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Abstract

Lisianthus (*Eustoma grandiflorum*) is an ornamental species used as a potted plant or cut flower, its popularity is due to the diversity of colors, number of flower buds, and shelf life. Nevertheless, during the first phases of development, problems such as foliar chlorosis and root diseases affects most cultivars, causing poor growth, thin stems, and few flowers. The use of plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) improved plant growth as these microbes colonize the plant system root. Therefore, in order to provide better conditions for the stem development, the aim of this work was to evaluate the individual and combined effect of *Bacillus subtilis* (PGPR) and *Glomus intraradices* (AMF) on the growth and postharvest quality of the stems of lisianthus cv. Mariachi. Then commercial product Alubión-X (*Bacillus subtilis* (PGPR) and mycorrhizal fungus (*Glomus intraradices*) were used. The variables evaluated were stem height and diameter, foliar area, leaves number and in postharvest, buds number, open and diameter of flowers and stem dry weight. The results showed a significant effect of the inoculation of *G. intraradices* on the size (66.92 cm) of the stem, as well as the combination of *B. subtilis* + *G. intraradices* (65.51 cm) compared to the control (36.9 cm). The number of buds and open flowers of the stems treated with *G. intraradices* were 33.35 and 23.9 respectively significantly higher than the control. *G. intraradices* alone is the best option for applying to lisianthus, when compared to applying with *B. Subtilis.*

Keywords: arbuscular mycorrhizal fungi, ornamental plant, plant development, plant growth-promoting rhizobacteria, postharvest quality.

Resumo

Qualidade de hastes florais de lisianthus (*Eustoma grandiflorum* Raf.) inoculadas com *Bacillus subtilis* e *Glomus intraradices*

Lisianthus (*Eustoma grandiflorum*) *é uma espécie ornamental utilizada como planta em vaso ou flor de corte***,** cuja popularidade se deve à diversidade de cores, número de botões florais e vida útil. No entanto, durante as primeiras fases de desenvolvimento, problemas como clorose foliar e doenças radiculares afetam a maioria das cultivares, causando baixo crescimento, hastes finas e poucas flores. O uso de rizobactérias promotoras de crescimento de plantas (PGPR) e fungos micorrízicos arbusculares (FMA) melhora o crescimento das plantas, pois esses microrganismos colonizam a raiz do sistema vegetal. Portanto, a fim de proporcionar melhores condições para o desenvolvimento da haste, o objetivo deste trabalho foi avaliar o efeito individual e combinado de *Bacillus subtilis* (PGPR) e *Glomus intraradices* (FMA) sobre o crescimento e a qualidade pós-colheita em hastes de lisianthus cv . Mariachi. Utilizando-se o produto comercial Alubión-X (*Bacillus subtilis,* PGPR) e o fungo micorrízico *Glomus intraradices*. Os resultados mostraram um efeito significativo da inoculação de *G. intraradices* no tamanho (66,92 cm) da haste, bem como a combinação de *B. subtilis* + *G. intraradices* (65,51 cm) em relação ao controle (36,9 cm). O número médio de botões e flores abertas das hastes tratadas com *G. intraradices* foram 33,35 e 23,9 respectivamente, significativamente maiores que o controle n***ão*** tratado. *G. intraradices* sozinho ***é a*** melhor opção para aplicar em lisianthus*,* quando comparado a aplicação isolada de *B. Subtilis.*

Palavras-chave: desenvolvimento vegetal, fungos micorrízicos arbusculares, planta ornamental, qualidade pós-colheita, rizobactérias promotoras de crescimento vegetal.

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Introduction

Lisianthus (*Eustoma grandiflorum*) is a non-traditional floral species that has gained popularity in recent years, due to the diversity of colors, number of flower buds, and durability of the flower stem (Castillo-González et al., 2017). During the first phases of development, problems such as foliar chlorosis, growth retardation, or an under developed root system may occur, as a result of the conditions of the culture medium, pH, incidence of root diseases caused by *Fusarium avenaceum*, *F. solani*, or presence of root galls associated with the nematode *Meloidogyne* sp. (Neves et al., 2017; Xiao et al., 2018). The incidence of root diseases caused by *Fusarium* spp., *Pythium* spp, *Rhizoctonia* sp. among others, affects most cultivars of this species, causing poor growth, thin stems, and few flowers (Mcgovern, 2018).

In the rhizosphere, there is a wide variety of microorganisms with high beneficial microbial activity (plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF)) that can improve plant growth as these microbes colonize the plant system root increasing its yield (Sagar et al., 2021). The predominant bacteria identified as PGPR include the genera *Bacillus* and *Pseudomonas*. The mechanisms they use to promote plant growth are: (1) biofertilization (for example, biological fixation of atmospheric nitrogen, phosphate solubilization, siderophore production, and exopolysaccharide production); (2) phytostimulation (production of indole acetic acid, gibberellins, cytokinins, and ethylene); and (3) biological control (induction of systemic resistance, competition for iron, nutrients, and space, production of antibiotics, lytic enzymes, hydrogen cyanide, and volatile compounds) as well as resistance to heavy-metal, drought or temperature stress (Noumavo et al., 2016; Xie et al., 2020).

Several important species of the genus *Bacillus* (*B. cereus, B. pocheonensis, B. circulans, B. amyloliquefaciens*, and *B. subtilis*) have shown the ability to increase crop yields, quality, and plant health. Also, *Bacillus* genus can stimulate plant growth through the synthesis and secretion of phytohormones (auxins or cytokinins), organic volatiles, and even the activation of the compounds production that reinforce plant immunity (*e.g.*, jasmonic acid, salicylic acid, and phytoalexins); additionally, improves the bioavailability of Fe and P and increases tolerance to water stress in ornamental plants modifying hormone levels (Nordstedt and Jones, 2020; Barros et al., 2018). On the other hand, this bacterium synthesizes and secrete antibiotics or other compounds (belong to polyketides, heterocyclic nitrogenous compounds, and lipopeptides) which have broad-spectrum action by the inhibition of phytopathogenic organisms or, by the activation of the induced systemic response (ISR), a mechanism by which the plant activates its defense systems against pathogen infection (Sohrabi et al., 2020; Kenawy et al., 2019).

Meanwhile, the arbuscular mycorrhizal fungi (AMF) - *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, and *G. intraradices* (Schenck & Smith) - have proven their effectiveness in different ornamental species. For example, Soroa et al. (2003) studied the effect of the application of arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) on some growth and yield variables of *Gerbera jamesonii* cv. Bolus, their results showed that *Glomus fasciculatum* inoculation which increased the diameter of the flowers by 27.9%, accelerated the beginning of flowering (50 days before), and increased yield compared to the control. Khandan-Mirkohi et al. (2015) evaluated the inoculation of lisanthus with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and *G. intraradices*, showing a significant reduction in the number of days to flowering, an increase in the length and number of floral stems and flowers, diameter and fresh weight of flowers per plant, and a reduction in the requirements of external phosphorus.

The combination of PGPR and FMA also has a positive effect. When *B. subtilis* and *P. fluorescens* were combined with AMF in bean roots, Mohamed et al. (2019) noticed an increase in the activity of chitinase, peroxidase, and polyphenol oxidase enzymes, reducing the *Sclerotium rolfsii* infection, and showing their capacity as disease control bioagents, possibly as a consequence of the production of antifungal compounds, the increase of root cell lignification, and the removal of high Fe concentrations from the medium, as well as the limitation of fungal growth. Nevertheless, Cai et al., (2021) evaluated the effect of plant symbiotic microbes in tomato plants, and the results showed that depending of the combination, the microbes may or may not promote the plant development, for instance the height of the plant inoculated with *Bacillus subtilis* was 50.5 cm, or with *Trichoderma harzianum* 53.2 cm, and combined the height remained in 50.5 cm, nevertheless with *Rhizophagus intraradices* + *B. subtilis* + *Trichoderma harzianum* the plant height was significantly higher (60.5 cm).

Therefore, in order to provide better conditions for improving growth, the aim of this work was to evaluate the individual and combined effect of *Bacillus subtilis* (PGPR) and *Glomus intraradices* (FMA) on the development and postharvest quality of the stems of lisianthus cv. Mariachi.

Materials and methods

Experiment conditions

The experiment was established in a greenhouse with a milky plastic cover (25% shade), located at 19° 28'- 19° 36' N and 98° 47'- 98° 55' W, and an altitude of 2,250 m. The climate is temperate with rains in summer, the average annual temperature is 15 °C, and the average annual precipitation is 772 mm. The conditions inside the greenhouse during the period of plant growth (November-July) were monitored with temperature and humidity sensors (HOBO® Data logger Onset® U12-012). The minimum and maximum monthly temperatures were 10.6 and 33.1 °C, respectively; relative humidity ranged from 33.1% to 91.1%; light conditions ranged from 900 to 8,000 µmol m^2 s^{-1} of photosynthetically active radiation (PAR), between noon and 4 pm at the plant level.

Plant material

Lisianthus var. Mariachi Blue (Sakata®) were transplanted when they had 7-8 leaves. Then, they were planted in 3500-cm^3 plastic pots with 11 kg of a mixture of agrolite and soil (1:2); three seedlings were placed per pot. Before transplanting, the substrate was treated with the fungicide Terrazole 35% (i.a. etridiazole 35% WP Arysta Lifescience®) (3.3 mL L^{-1} water). Five days after transplanting, another application of the same concentration was made at the base of the stem. The soil was disinfected with the fungicide Bunema®55 GE ([Metam sodium N-methyl dithiocarbamate], Buckman, Mexico) (18.5 mL m^{-2}) to control *Fusarium* spp. and *Phytophthora* spp. The soil had a sandy loam texture and the following characteristics: pH 6.5, electrical conductivity 0.56 dS m^{-1}, organic matter (2.7%), total nitrogen (0.03%), Olsen phosphorus (3 mg L^{-1}), and potassium (0.16 meq 100 g^{-1}).

The commercial product Alubión-X (*Bacillus subtilis* (PGPR)) was used as biofertilizer, while the mycorrhizal fungus (*Glomus intraradices*) was isolated from the rhizosphere of *Dahlia* x *hybrida* and multiplied in wheat (*Triticum aestivum* L.) seedlings. The harvest was carried out after eight months and the root staining (0.05% trypan blue) was determined using the Phillips and Hayman (1970) method, while the percentage of the root mycorrhizal colonization was measured by the intersection of quadrants and the values were expressed as a percentage. The number of spores was evaluated by separating the spores with 44-, 325- and 400-µm sieves by the method of Gerdemann and Nicolson (1963). The stained root longitudinal segments were examined with a microscope (American Optical, USA) at 100X.

Biofertilizers inoculation*:* The treatments were: T1=*B. subtilis*; T2=*G. intraradices*; T3= *B. subtilis* + *G. intraradices*; and T4=Control. The commercial product Alubión-X (*Bacillus subtilis*) was applied manually at $1x10^7$ CFU at the base of the stem of the plants using an atomizer, 15 and 30 days after transplantation (ddt). Meanwhile the inoculation of *G. mosseae* was carried out by transplanting the plants and adding 350 spores (10 g of substrate) to the root system of each plant, in order to assure the inoculation.

Variables evaluated

Plant height was measured every two months with a graduated ruler from the base of the stem to the apical part. Stem diameter was measured every two months with a digital vernier (Caldi-6MO, Truper, U. S. A.) at 10 cm from the base. Leaf number and leaf area were determined every two months with a leaf area integrator (Licor, Model 3100 Area Meter®).

The floral stems were harvested after the second flower was completely open and they were randomly placed in glasses with 300 mL of tap water for postharvest evaluation. The postharvest variables evaluated were: number of flower buds, open flowers, and the diameter of the third flower. After harvest, the dry weights of the root, stem, and leaves were recorded by drying the tissues at 70 °C in an oven (Thermo Scientific Model No. 3471-1), until a constant weight was reached.

Statistical analysis

Five monitorings were carried out (November 5, January 5, March 5, May 5, and July 5) and 20 repetitions were taken for each variable (1 plant=1 repetition). The generalized additive model (GAM) was applied to analyze the possible non-linear behavior between each growth variable and time. The model does not subject the data to the torture of transformation to meet a requirement of a linear relationship between the response variable and an explanatory variable (time). The model divides the data into fragments defined by several points (knots) and fits a polynomial regression in each data subset that, to analyze the effect of treatments, is defined as: $g(\mu)=Ti+f(x)+\epsilon\_ij$; i=1,2,…,k;j=1,2,…,rep.

Where Ti is the i-th treatment of a total of k treatments, f is a smoothing function (*i.e.*, the polynomial by parts), and compare the curves of growth variables, comparing all pairs of curves, through a permutation test of the difference between two growth curves. The quality variables were subject to the Kruskal and Wallis model which analyzes the effect of the treatments.

Results and Discussion

G. intraradices had high percentage of colonization and sporulation (87.5 %) in lisianthus, structures typical of plant-AMF symbioses such as hyphae, vesicles and arbuscules were present in all screened roots at each location (Figure 1).

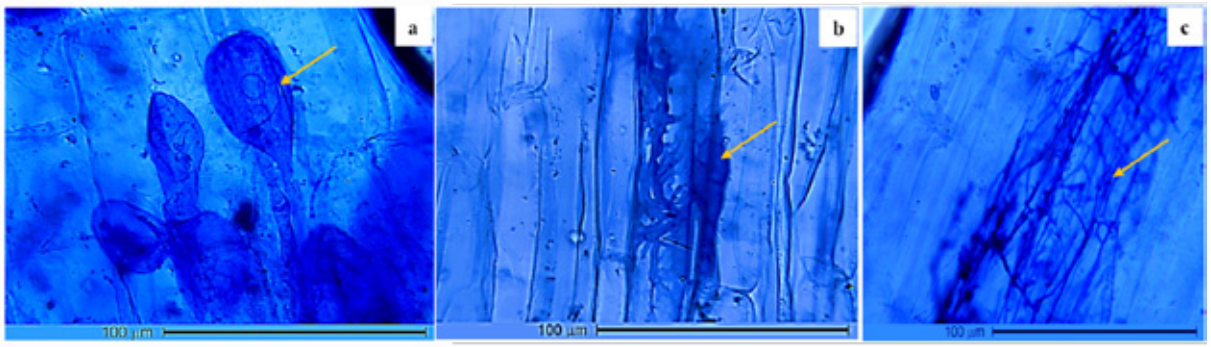

Figure 1. a) Typical vesicles of mycorrhizal fungi, b) Example of arbuscule of arbuscular mycorrhizal fungi, and c) Root colonization of *G. intraradices* in lisianthus. The roots were stained with trypan blue (100X).

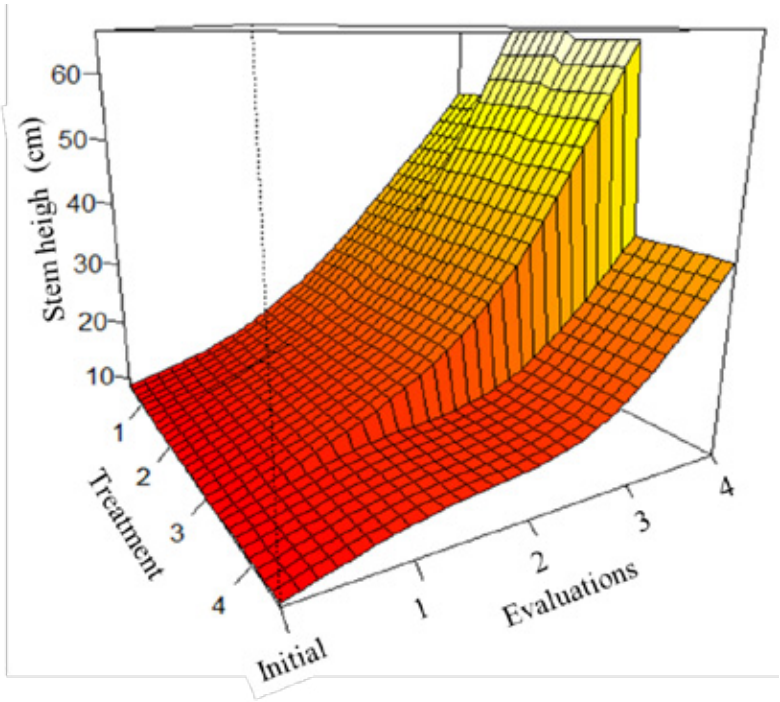

Figure 2. Stem height growth curves of lisianthus (*Eustoma grandiflorum* Raf.) inoculated with *B. subtilis* (T1), *G. intraradices* (T2), *B. subtilis* + *G. intraradices* (T3), and Control (T4).

According to the analysis of growth curves based on estimates derived from the GAM model, the treatments with *G. intraradices* and the *B. subtilis* + *G. intraradices* combination significantly increased plant height (Figure 2).

The control treatment plants were the smallest. The symbiosis established by arbuscular mycorrhizal fungi (AMF) with most plant species manages to increase their development, because it facilitates the absorption of nutrients (mainly phosphorus) and increases plant vigor. Factors such as the species of mycorrhizae, dose, and time of inoculation are important to obtain greater benefits. If the AMF infection units are sufficient, they can contribute to guarantee an appropriate colonization rate. In addition to the development stage of the infection, the time when plants are inoculated can be critical: the earlier the inoculation, the greater the benefits for the plant (Meir et al., 2010). For example, Rubí-Arriaga et al. (2012) inoculated *G. fasciculatum* and *B. subtilis* in *Lilium* sp. which resulted in an increase in height and diameter of the stem, as well as greater dry weight of the vegetative aerial part of the inoculated plants, in relation to the control plants. Adding phosphorus produced higher quality flowers than control and improved plant growth. While some studies suggest that mycorrhizal fungi and soil bacteria may compete for carbon in the rhizosphere, other studies indicate that plant growth promotion by mycorrhizal fungi may counteract this effect and actually stimulate bacterial activity in the soil rhizosphere (Sagar et al., 2021).

Depending on the strain, PGPR improve plant growth through the production of phytohormone precursors; however, they also facilitate the absorption of nutrients or produce substances that repel pathogens. The colonization of PGPR plants can be highly specific to certain plant species, which could explain why, in this case, *B. subtilis* alone only had a lower effect than *G. intraradices* (Table 1).

Table 1. Stem diameter (mm) of lisianthus (*Eustoma grandiflora* Raf.) inoculated with *B. subtilis* (T1), *G. intraradices* (T2), *B. subtilis* + *G. intraradices* (T3), and Control (T4).

Treatment/ Evaluations	Stem Diameter (mm)				
	Initial	1	2	3	4
1	0.20 a	0.41 a	0.92 a	2.00 c	4.32 c
2	0.19 a	0.39 b	0.99 a	2.18 b	5.68 b
3	0.19 a	0.41 a	1.01 a	2.42 a	6.78 a
4	0.20 a	0.38 b	0.66 b	1.35 d	2.99 d
HSD	0.017	0.02	0.12	0.08	0.12
CV(%)	10.58	6.0	16.16	5.03	3.02

*Different letters in each column show significant differences. HSD= honest significance difference and CV: coefficient of variation (%). n=20. Evaluations performed every two months: November (initial), January (1), March (2), May (3), and July (4).

The root colonization and symbiotic interaction formation depends on several factors such as the composition of the exudates, the development stage, environment (such as soil type, temperature, moisture, pH, and nutrient availability) and biofilm formation (Lucke et al., 2020).

The differences between the diameter and the leaf area of the stems become more evident from the third evaluation onwards; the combined treatment of *B. subtilis* + *G. intraradices* results in thicker stems and a greater leaf area (Figure 3, Table 1).

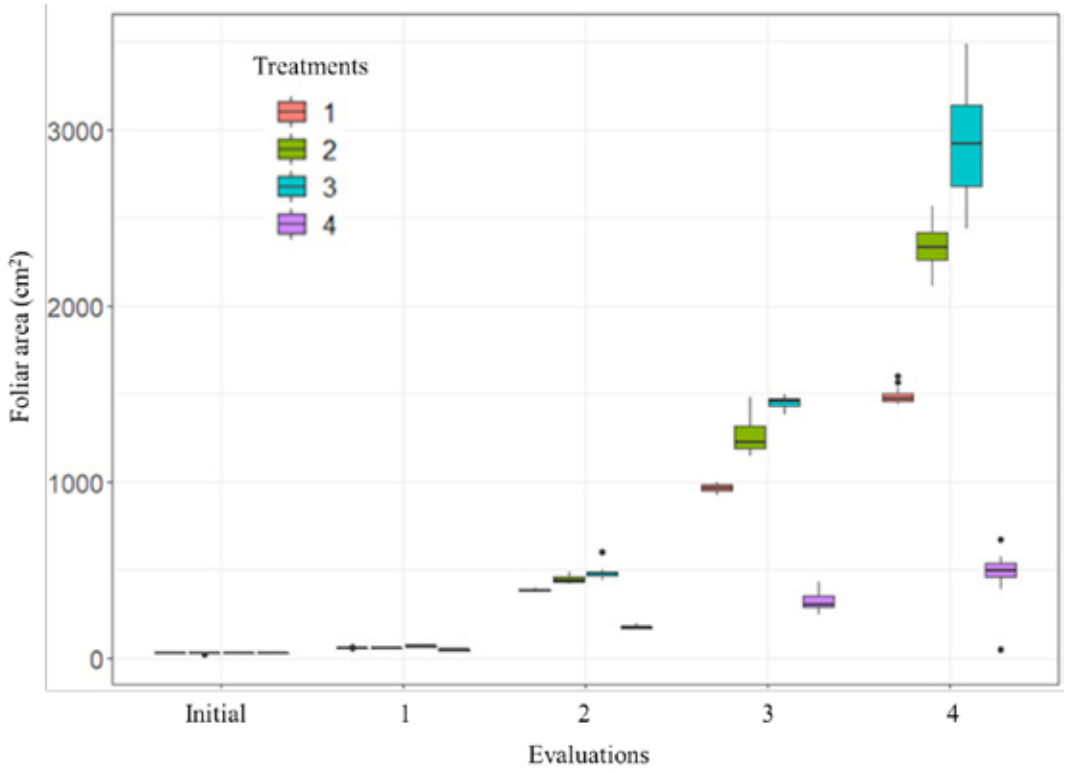

Figure 3. Leaf area (af) growth curves over time of lisianthus (*Eustoma grandiflorum* Raf.) subjected to different treatments. *B. subtilis* (T1), *G. intraradices* (T2), *B. subtilis* + *G. intraradices* (T3), and Control (T4). Evaluations performed every two months: November (initial), January (1), March (2), May (3), and July (4).

One of the functions of AMF is to improve the availability of phosphorus in the soil by solubilizing its inorganic forms or by mineralizing organic phosphorus. Therefore, the more colonized the root system is, the plant will make a more efficient use of nutrients and accumulate more dry matter (Table 2).

Table 2. Number of leaves in stems of lisianthus (*Eustoma grandiflora* Raf.) inoculated with *B. subtilis* (T1), *G. intraradices* (T2), *B. subtilis* + *G. intraradices* (T3), and Control (T4) during the experiment.

	Number of leaves				
Treatment/Evaluation	Initial	1	2	3	4
1	7.70 a	18.20 b	37.20 b	59.25 b	85.15 c
2	7.70 a	19.40 a	40.15 a	67.30 a	96.75 b
3	7.45a	19.45 a	39.70 a	72.85 a	102.25 a
4	8.00a	16.75 c	19.55 c	33.20 c	40.45 d
DHS	0.6	0.77	1.38	6.76	3.27
CV	9.33	5.0	4.85	13.99	4.84

* Different letters in each column show significant differences. HSD= honest significance difference and CV: coefficient of variation (%). n=20. Evaluations performed every two months: November (initial), January (1), March (2), May (3), and July (4).

Synergistic interaction between AMF and PGPR most of the times, effects positively the plant growth than to single inoculation (Nanjundappa et al., 2019; Mohamed et al., 2019), but the positive result of the positive effect will depend on the species of microorganism, host plant, and environment (Cai et al., 2021). On the other hand, Cruz-Crespo et al. (2020) showed that foliar fertilization with Humifert (N=2.0, P=1.0, and K=1.0 g L^{-1}) during the growth and development of lisianthus ´Flamenco purple´ result in significantly wider stems than the fertilization with Bayfolan Forte® (N = 0.33, P = 0.24 and K = 0.18 g L^{-1}), showing the response of lisianthus stem to higher nutrient doses. Likewise, the combined *B. subtilis* + *G. intraradices* treatment increased significantly the diameter of the stems, foliar area and number of leaves compared to the control treatment, as a probably greater absorption of macro and micronutrients (Table 2).

Arbuscular mycorrhizal fungi search for soil nutrients with higher efficiency, as a result of their greater surface-volume ratio. Therefore, they penetrate deeper into the soil and extract water with higher efficiency; consequently, plants inoculated with FMA grow faster. Azcón et al. (1992) showed that *Lactuca sativa* L. plants inoculated with *Glomus mosseae* or *G. fasciculatum* had greater leaf area, fresh weight of leaves, and photosynthetic activity than control plants, regardless of the source of nitrogen fertilization. But Garmedia and Mangas (2012) did not get a positive nutritional effect of mycorrhizal inoculation in cut roses leaves, probably due to the low root colonization percentages reached. In our study from the second evaluation, the size of the stems increases more than 40% in all treatments, except for the control (25%). Finally, as the last evaluation approached, the size of the stems increased more than 70% and consequently increasing its leaf area. Figure 3 shows a significant increase in foliar area for *G. intraradices* (2,342 cm^2) and for the *B. subtilis* + *G. intraradices* combination (2,902 cm^2), compared with the *Bacillus* treatment and control, which reached 1,487 and 479 cm^2 respectively.

The number of leaves increased significantly from the second evaluation. The lisianthus leaves are turgid with an intense green color, symbiosis indirectly stimulates photosynthesis to supply the plant energetic balance. More leaves represent greater photosynthetic activity and consequently a greater reserve of carbohydrates —which is useful to maintain the respiratory intensity of the stem, once it has been cut.

Quality evaluation

Unlike the growth variables, the mean and the median of the quality variables had very close values, which implies almost symmetrical distributions. Likewise, the quality variables of the stems responded better to the inoculation by *G. intraradices* and to the *B. subtilis* + *G. intraradices* combination. Sagar et al. (2021), describe that the dual inoculation of PGPR and AMF enhances nutrient uptake and productivity of several crops compared to a single inoculation in both normal and stressed environments. Positively interacting PGPR + AMF combination is an efficient and cost-effective recipe for improving plant growth. Lisianthus stems are characterized by thick stems and a large number of flower buds, although not all of them develop and open (Figure 4).

Figure 4. Appearance of lisianthus (*Eustoma grandiflorum* Raf.) stems and flowers, subjected to different treatments at harvest.

The number of flower buds and open flowers were 50 % greater and the flower diameter was significantly wider in the entire floral stem in the *G. intraradices* (8.23 cm) and *B. subtilis* + *G. intraradices* treatments (7.58 cm) compared to *B. subtilis* and the control treatments (5.88 and 4.17 cm respectively). In addition, the dry weight of the stems was considerably higher for *G. intraradices* (3.73 g plant^{-1}) compared to 1.62 g plant^{-1} of the control treatment, which indicates a higher carbohydrate reserve —a determining factor in the development of the bud and flower opening (Figure 5).

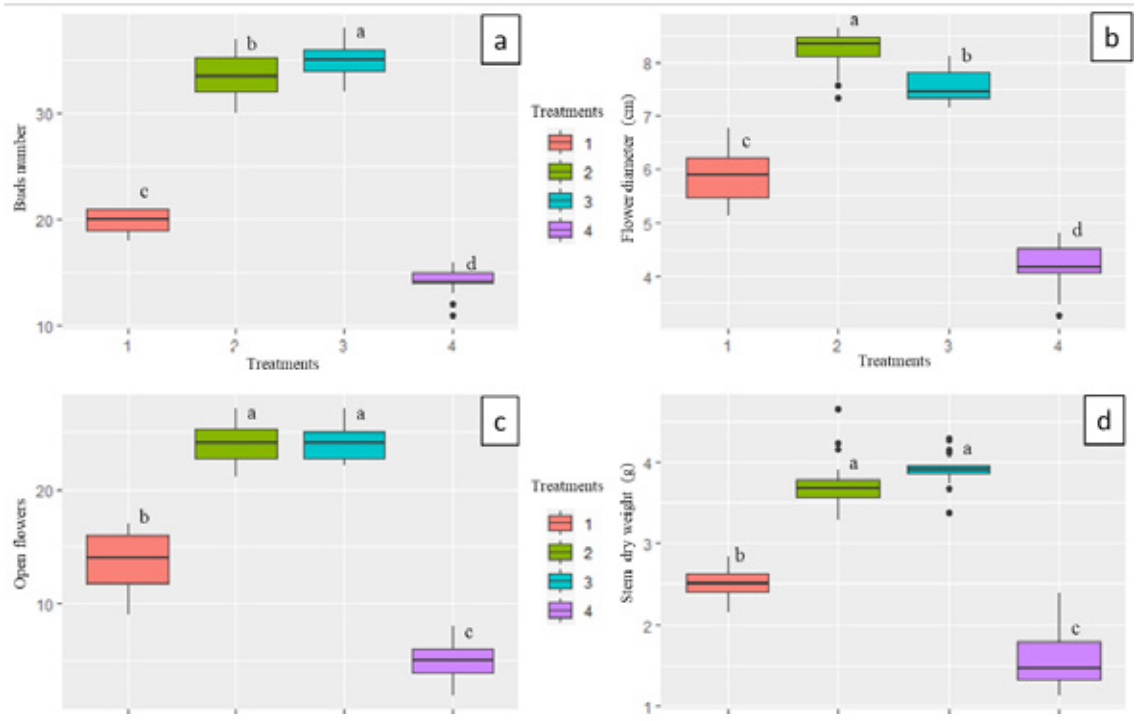

Figure 5. Quality variables for lisianthus (*Eustoma grandiflorum* Raf.) flower stems. a) Buds number; b) Flower diameter; c) Open flowers; d) Stem dry weight. Where treatments are: *B. subtilis* (T1), *G. intraradices* (T2), *B. subtilis* + *G. intraradices* (T3), and Control (T4).

Cavasini et al. (2018) recorded a >50% reduction in carbohydrate reserves in lisianthus buds, from 20.74 mg 100 g^{-1} (harvest) to 9.85 mg 100 g^{-1} (day 17), which decrease during the harvest and postharvest stage, so bigger and wider stems represent more reserves for postharvest (Norikoshi et al., 2016).

Conclusions

The growth and development of the lisianthus plants was stimulated by the inoculation of *Glomus intraradices* and the *B. subtilis* + *G. intraradices* combination, being *Glomus* by itself more efficient in stimulating the absorption of nutrients that was reflected in thicker and taller stems, more dry weight that means higher carbohydrate reserves for flower opening. So we can assume that the nutrients absorption in lisianthus was more efficient for the arbuscular mycorrhizal fungi (AMF) than for *Bacillus* a plant growth-promoting rhizobacteria. Then in order to minimize costs, applying only *Glomus intraradices* would be enough.

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Author contribution

DJC: Field work and data capture, literature review. **MLAG**: Research project coordinator, processing and data analysis, literature review and writing. **MERG**: Experiment design and data analysis. **JCI**: Literature review and English translation. **MVHV**: Field work literature review.

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