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Plant Genetics and Biodiversity Scientific Research

Biostimulant Effect of Microorganisms on *in vitro* Germination of Hybrid Pepper Seeds

Efecto bioestimulante de los microorganismos sobre la germinación *in vitro* de semillas híbridas de pimiento

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Abstract: In Ecuador, pepper cultivation plays a vital role at the socio-economic level since it represents a significant income for several farmers. This work aims to evaluate the biostimulant effect of microorganisms on the *in vitro* germination of hybrid Yolo Wonder and Marcono Dulce Largo pepper seeds. The experiment was conducted under a completely randomized design through five treatments with five repetitions and fifteen experimental units per repetition. The treatments used were T0 (Control), T1 (*Trichoderma harzianum),* T2 (*Azotobacter vinelandii*), T3 (*Bacillus polymyxa*), and T4 (*Azospirillum brasilense*) inoculated in Petri dishes with an absorbent polystyrene pad at 1 x 10^9 CFU/ml, which was dosed with 35 mg/10 ml in distilled water both at sowing and on the seventh day after. In addition, the variables evaluated were germination percentage, radicle and coleoptile emergence, seedling weight, and radicle and coleoptile length. The data obtained were processed by an analysis of variance (ANOVA) and Tukey's comparison of means at a 5 % error probability. Noticeable stimuli were observed on the variables studied with the inoculation of different microorganism strains, the T4 treatment showing the best results, and no differences were obtained when using various pepper genotypes. Importantly, this research was carried out under specific conditions and with particular genotypes of pepper seeds. Therefore, the results may vary in other conditions and with different genotypes under the biostimulant effect of microorganisms on the *in vitro* germination of hybrid pepper seeds.

Keywords: *Azospirillum brasilense, Azotobacter vinelandii, Bacillus polymyxa,* plant growth substances*, in vitro* experimentation, *Trichoderm harzianum.*

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Resumen: En Ecuador, el cultivo de pimiento es de gran importancia a nivel socioeconómico, ya que representa una fuente de ingresos para los agricultores. El propósito de esta investigación fue evaluar el efecto bioestimulante de algunos microorganismos sobre la germinación *in vitro* de genotipos híbridos de semillas de pimiento Yolo Wonder y Marcono dulce largo. El experimento se realizó con un diseño completamente aleatorizado mediante cinco tratamientos, cinco repeticiones y quince unidades experimentales por repetición. Los tratamientos T0 (Control), T1 (*Trichoderma harzianum*), T2 (*Azotobacter vinelandii*), T3 (*Bacillus polymyxa*) y T4 (*Azospirillum brasilense*) se inocularon en cajas Petri con una almohadilla absorbente de poliestireno en concentración 1 x 10^9 UFC/ml con dosis de 35 mg/10 ml en agua destilada a la siembra y al séptimo día. Se evaluaron las variables porcentaje de germinación, emergencia de la radícula y el coleóptilo, peso de la plántula y longitud de la radícula y el coleóptilo. El análisis de datos se realizó mediante un análisis de varianza ANOVA y la comparación de medias de Tukey al 5 % de probabilidad de error. Se observaron efectos significativos sobre las variables estudiadas con la inoculación de las diferentes cepas de microorganismos, siendo el tratamiento T4 el que mostró mejores resultados, y no se obtuvieron diferencias al utilizar diversos genotipos de pimiento. Es importante destacar que esta investigación se realizó en condiciones específicas y con genotipos particulares de semillas de pimiento. Por lo tanto, los resultados pueden variar en otras condiciones y con diferentes genotipos bajo el efecto bioestimulante de los microorganismos sobre la germinación in vitro de semillas híbridas de pimiento.

Palabras clave: *Azotobacter vinelandii, Bacillus polymyxa, Azospirillum brasilense*, experimentación *in vitro*, promotor del crecimiento vegetal, *Trichoderm harzianum.*

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Introducción

The fruits of the pepper plant (*Capsicum annuum* L.) are highly valued worldwide due to their nutritional content and excellent properties. They are used as a medicine and a condiment in the food industry (Guerra et al., 2020). Therefore, seed germination is a fundamental process in plant reproduction since it determines success in crop production.

In Ecuador, the pepper is cultivated in several provinces, mainly tropical areas. Farmers have had to incorporate new improvement techniques to increase their production rates and obtain greater profitability from the crop (Delgado et al., 2022). In this context, using microorganisms as biostimulants has proven an effective strategy to improve seed germination and promote seedling growth.

Agriculture generally exhibits problems that include phytopathogenic microorganisms (viruses, bacteria, and fungi), which cause damage to crops (Albarracin-Gomez et al., 2023). Farmers have widely used chemicals to prevent damage, but they cause soil desertification and environmental pollution. Thus, a sustainable solution is using biological products (Camelo et al., 2011; Mora et al., 2023).

Agricultural bioproducts offer numerous advantages, with one notable benefit being the application of efficient microorganisms for crop inoculation. This practice enhances crop growth, development, and protection effectively and sustainably (Chávez-García et al., 2023; Liriano et al., 2021; Lobo, 2008). Microbial seed coating is used to apply beneficial microorganisms, such as plant growth-promoting bacteria (PGPB), rhizobia, and fungi, onto seeds. It aims to enhance crop growth and yield by improving nutrient availability and protecting against diseases and pathogens (Rocha et al., 2019). Coating seeds with these beneficial microbes offers an efficient way to deliver them and shows promise as a method for inoculating various crop seeds with reduced inoculant usage compared to traditional seed treatments. The formulation of a typical inoculant includes the selection of a specific microorganism, an appropriate carrier, and relevant additives (Vassilev et al., 2020). Each microorganism has various mechanisms of action, which can be widely used in agriculture, providing significant benefits to them (Quispe-Quispe et al., 2022). Therefore, PGPB promotes plant nutrient absorption and homeostasis and enhances antioxidant activities during saline stress (Kumar et al., 2020).

Moreover, efficient microorganisms allow the production of vegetables at a low cost (Calero et al., 2019; Quispe-Quispe & Salas-Macías, 2022). Thus, this work aims to evaluate the biostimulant effect of microorganisms on the *in vitro* germination of hybrid genotypes of *Capsicum annuum* L. Experiments will be carried out under controlled laboratory conditions using different concentrations and combinations of microorganisms. Germination rate, time, percentage, and other parameters related to seedling growth will be measured. The results of this study are expected to provide valuable information on the ability of microorganisms to improve the germination of hybrid pepper seeds. This may have significant implications for agricultural production, as it could allow for the development of more efficient and sustainable methods for crop production.

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Materials and Methods

Location of the Experimental Site

The present investigation was conducted in the Plant Biotechnology laboratory at the Faculty of Sciences and Engineering of the Universidad Estatal de Milagro (UNEMI), located in the University Citadel Km 1.5 via Km 26, Milagro, Guayas, Ecuador.

Plant Material

Two hybrid genotypes of *Capsicum annuum* L. pepper seeds (Yolo Wonder and Marcono dulce largo) were used. The seeds were washed with water and liquid soap for 10 minutes (Salgado-Escobar et al., 2020). Subsequently, they were submerged (2.5 g l^{-1}) in a solution with 50 % Captan for 3 min and immediately rinsed with distilled water to remove all residues (Uribe et al., 2008). Afterward, the seeds were washed with 5 % sodium hypochlorite solution for every 100 ml of water for 10 min (Cárdenas-Burgos et al., 2019) and then rinsed sufficiently with distilled water. Finally, they were submerged in a 3 % (v/v) hydrogen peroxide solution in water for 10 min (Flores García et al., 2008).

Experiment Design and Statistical Analysis

The experiment was established under a completely randomized design (CRD), considering five treatments with five repetitions and fifteen experimental units per repetition. The treatments were distributed as follows: T0 (Control); T1 (*Trichoderma harzianum);* T2 (*Azotobacter vinelandii*); T3 (*Bacillus polymyxa*); T4 (*Azospirillum brasilense*). The strains come from the Microbiolab S.A. laboratory in Quito, Ecuador. The data obtained from all the parameters studied was analyzed using analysis of variance (ANOVA) with Tukey's test with 0.05 % as the significance level through the statistical software IBM SPSS Statistics® version 23.0.

Treatment

Sterile polystyrene absorbent pads were placed into glass Petri dishes (100 x 20 mm) to inoculate the lyophilized microorganisms at a concentration of 1 x 10^9 CFU/ml and a dose of 35 mg/10 ml in distilled water, both at seeding and the seventh day after.
Once the Petri dishes were sealed, all the treatments were placed in a compact climatic chamber for plant growth (ICH-FDA Brand, Model MP-BILC-600-C, Barcelona) with a total capacity of 600 liters. They were placed at a constant temperature of 26 °C with 75 % humidity and total darkness for 24 hours. Then, the conditions were modified with 70 % humidity, a constant temperature of 25 °C, and alternating eight hours with a light intensity of 1,500 lux and 16 hours of darkness.

The performance of the seeds was evaluated for 12 days using the positive germination criteria (radicle and coleoptile emergence), taking into account the following variables: germination percentage (%), radicle emergence (d), seed emergence coleoptile (d), seedling weight (g), radicle length (mm), and coleoptile length (mm). The weights were taken using a 220 g analytical balance

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(BOECO Brand, Model BAS 31 PLUS, Poland) with a precision of 0.0001 g. In contrast, the longitudinal measurements were taken with a rectified stainless-steel caliper (BEST VALUE Brand, Mechanic, Ecuador) with a measuring range of 0.000–6 in.

Results and Discussion

Table 1 presents the results obtained from the biostimulant effect of microorganisms on the *in vitro* germination of hybrid pepper seeds during 12 days of evaluation.

Table 1. Biostimulant effect of microorganisms on seed germination of hybrid pepper seeds in the laboratory

Genotype	Variable	Treatments					SE (±)	*p*-value
		T0	T1	T2	T3	T4		
Yolo Wonder	Germination percentage (%)	84.67[b]	87.00[ab]	84.67[b]	91.00[ab]	93.00[a]	1.73	0.020
	Radicle emergence (d)	6.00[b]	5.00[a]	5.33[ab]	5.00[a]	5.00[a]	0.15	0.002
	Coleoptile emergence (d)	9.33[b]	8.00[a]	9.00[b]	8.00[a]	8.00[a]	0.15	0.000
	Seedling weight (g)	0.22[c]	0.48[ab]	0.45[b]	0.54[a]	0.55[a]	0.02	0.000
	Radicle length (mm)	10.27[b]	17.83[a]	16.86[ab]	18.64[a]	19.47[a]	1.51	0.010
	Coleoptile length (mm)	12.79[b]	16.96[a]	15.45[ab]	18.01[a]	18.07[a]	0.76	0.003
Marcono Dulce Largo	Germination percentage (%)	80.00[a]	84.67[a]	89.00[a]	89.00[a]	91.00[a]	2.60	0.079
	Radicle emergence (d)	6.00[b]	5.00[a]	5.33[ab]	5.00[a]	5.00[a]	0.15	0.002
	Coleoptile emergence (d)	9.33[b]	8.00[a]	9.00[b]	8.00[a]	8.00[a]	0.15	0.000
	Seedling weight (g)	0.23[b]	0.47[a]	0.46[a]	0.48[a]	0.49[a]	0.03	0.000
	Radicle length (mm)	9.67[b]	17.67[ab]	16.33[ab]	18.87[a]	19.67[a]	1.96	0.031
	Coleoptile length (mm)	10.07[b]	18.52[a]	17.39[a]	20.18[a]	20.42[a]	0.98	0.000

[a, b] Different letters within a row indicate significant differences in Tukey's test at $P < 0.05$. SE: Standard Error. T0 (Control); T1 (*Trichoderma harzianum*); T2 (*Azotobacter vinelandii*); T3 (*Bacillus polymyxa*); T4 (*Azospirillum brasilense*).
Source: Prepared by the authors.

The comparison of means between treatments shows statistically different groups for all the variables studied (germination percentage, radicle and coleoptile emergence, seedling weight,

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radicle and coleoptile length) using microorganisms in both pepper genotypes studied, i.e., Yolo Wonder and Marcono Dulce Largo. However, no difference was found in the percentage of germination obtained in the latter. This data was corroborated by Liriano et al.'s (2021) study. They observed a positive effect on the growth and quality of pepper plants in the seedbed when using microorganisms such as *Bacillus* spp., *Pseudomonas* spp., *Trichoderma, Aspergillus, Rhizopus, Mucor, Penicillium,* and *Saccharomyces* spp., thus achieving plants with a height of 17.19 cm, root lengths of 8.82 cm, and increased weight. These findings demonstrate the potential of the microorganisms studied to stimulate root growth and development and aerial organs in pepper plants.

The benefits of efficient microorganisms include restoring the microbiological balance of the soil. In addition, the diversity of interrelationships between microorganism species in the environment could be synergistic or antagonistic, and abiotic and biotic factors could influence physical-chemical and biological competition. They affect the plant-soil-microbiota-environment interaction, directly impacting plant growth and development (Cano, 2011).

The seed germination percentage variable was slightly higher in treatment T4, with 93 % germination in the Yolo Wonder genotype. At the same time, no differences were found between treatments for the Marcono Dulce Largo genotype. However, using this same microorganism, the best results were obtained for germination (91 %) compared to the other treatments. According to Reyes et al. (2008), inoculating peppers with *Azospirillum* strains increased their germination percentage significantly. Moreover, they observed some specificity between *Azospirillum* and the crop type depending on the microorganism used. Some need mutual symbiosis with other strains to enhance their effect on plants or are specific to certain plant species.

The results of the radicle emergence were similar among treatments T1, T3, and T4, where root appearance was observed on the fifth day after sowing. In contrast, in treatments T0 and T2, it occurred the following day for both genotypes. This is probably due to certain microorganisms' specificity in coexisting with particular plant species, allowing them to develop properties that promote plant growth (Romero-García et al., 2016). The availability of the metabolic potential of microorganisms will depend on several factors, including the competitive capacity of microorganisms, temperature, humidity, pH, soil type, nutrient availability, and inoculum concentration (Hernández-Ruíz et al., 2017).

Trichoderma species are microorganisms commonly used as plant growth promoters, but their effect is limited depending on the crop type, development conditions, and concentration of microorganisms (Stewart & Hill, 2014). The *Bacillus polymyxa* bacterium is a biological control agent because it can produce antibiotics and other antibacterial and antifungal substances, preventing the development of plant pathogens (Pozo et al., 2006). In addition, *Azospirillum* allows for an increase in the plant's growth, leaf area, and root volume, where cases of double production have also been noted (Jarquín-Rosales et al., 2023).

The analysis of the coleoptile emergence begins with the appearance of its apex for T1, T3, and T4 on the eighth day after sowing; however, in treatments T0 and T2, it occurs from the ninth

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day in both pepper genotypes evaluated. Quispe-Quispe et al. (2022) argue that microorganisms such as *Trichoderma*, *Bacillus*, *Streptomyces,* and *Pseudomonas* act as biostimulants, improving some agronomic and productive components in plants. Still, they could also inhibit and reduce the growth of pathogenic microorganisms that negatively affect germination. Certain microorganisms have biofertilizer and biostimulant applications, influencing various physiological responses of crops; thus, germination and emergence can be influenced by factors such as temperature, humidity, light, and oxygen, among others.

There were differences in seedling weight of Yolo Wonder and Marcono Dulce Largo pepper genotypes between treatments. The best results were reported with treatment T4 with a weight of 0.55 and 0.49 g for each genotype, respectively. These results coincide with the data found by Cervantes-Vázquez et al. (2022), which describe a heavier weight of the plant when using *Azospirillum* and easy adaptability in the cultivation of *Capsicum annuum* L., translating into a greater yield and fruit quality unlike conventional systems (Vásconez et al., 2020). In this way, *Azospirillum* significantly promotes rooting, fresh weight of roots and foliage, stem diameter, and plant height (Palomino-Malpartida et al., 2023). The use of PGPB has shown to be a critical biotechnological alternative since it promotes the growth of plants with a relevant production of biomass after sowing (Criollo et al., 2012).

Regarding radicle length, differences between treatments are shown; however, the best results were reported with treatment T4, with 19.47 mm for Yolo Wonder and 19.67 mm for Marcono Dulce Largo. The treatment T0 achieved 10.27 mm and 9.67 mm, respectively. Associations of *Azospirillum* strains act as biofertilizers promoting root growth and plant root hairs (Rangel-Lucio et al., 2011), which may be limited *in vitro*. However, *in situ,* it could express its maximum capacity by trapping other nitrogen sources, fixing and incorporating them into plants to promote their development, as reported in the literature (Restrepo-Correa et al., 2017).

For the coleoptile length, all the treatments with microorganisms (T1, T2, T3. and T4) were superior to the control in both pepper genotypes studied. Despite this, the best averages were obtained when inoculating *Azospirillum brasilense* in pepper seeds. *Azospirillum* improves plant development due to the release of auxins, gibberellins, and cytokinins, which encourage *Azospirillum* to fix nitrogen, producing positive effects on plant height, stem diameter, and weight (Pérez-Velasco et al., 2019).

Few studies have been reported describing the benefits of *Azospirillum* on vegetables. However, some others have begun to explore the potential of these bacteria to improve the yield and quality of vegetables. These research studies have revealed that *Azospirillum* can promote root growth, increase nutrient absorption, and improve disease resistance in vegetable plants. Although more research is still needed, these findings suggest that *Azospirillum* could become a promising tool for vegetable farming.

Due to the results obtained in this work and the importance of *Azospirillum* as a biostimulant in pepper cultivation at an *in vitro* level, the findings by Domingues Duarte et al. (2020) indicate that *Azospirillum brasilense* is a group of PGPB found in the soil in different territories. It also has the ability to associate with plant roots, stimulating root and aerial development and increasing production and economic benefits to farmers, which come from the production of auxin-type growth-promoting phytonutrients. Thus, efficient microorganisms capable of promoting plant development are considered an alternative to biofertilization (Pedraza et al., 2010) to help reduce the application of synthetic fertilizers (Torres Pérez et al., 2022).

In the case of cucumber seeds, a commercial preparation of *T. harzianum* was used as a seed coating agent, resulting in higher seedling emergence and increased fresh shoot weight than untreated seeds (Pill et al., 2009). Additionally, *Trichoderma* spp. proved to be effective in enhancing germination and seedling growth of *Cucurbita pepo* under various salinity stress conditions (50 and 100 mM NaCl solution; Afzal et al., 2020), as well as for *Cuminum cyminum* under drought conditions. In a separate study, different strains of *T. harzianum* and *Pseudomonas fluorescence* bacteria were applied to the seeds, leading to an increase in soluble protein and antioxidant enzyme activity in the inoculated seeds compared to a control group without inoculation, ultimately resulting in improvements in some morphological indicators (Piri et al., 2019).

This demonstrates the effectiveness of additional *ex-situ and in-situ* preservation techniques, giving preference to the type of genetic material used (Arias & Cano, 2009). The adequate inoculation of efficient microorganisms could be a viable strategy within the processes of plant micropropagation in space during the acclimatization phase, transferring the plants to the field with an already established microbiota and contributing to a better adaptation under the conditions established in the field (Pérez Moncada et al., 2015).

Conclusions

The biostimulant effect of microorganisms was demonstrated on the *in vitro* germination of hybrid *Capsicum annuum* L. pepper seeds at a dose of 35 mg/10 ml of distilled water with a concentration of 1 x 10^9 CFU/ml, inoculated at sowing and the seventh day after. The impact of the different strains of microorganisms used (*Trichoderma harzianum, Azotobacter vinelandii, Bacillus polymyxa,* and *Brazilian Azospirillum*) on the germination percentage, radicle emergence, coleoptile emergence, seedling weight, radicle length and coleoptile length is notable.The treatment with *Azospirillum brasilense* showed the best results, and no difference was observed with different pepper genotypes.

Despite the promising results obtained in the present study on the biostimulant effect of microorganisms on the *in vitro* germination of hybrid pepper seeds, some limitations that could have affected the results stand out. First, the sample used in this study was relatively small, which could limit the generalizability of the results to a broader population. Furthermore, the monitoring time was short, which does not allow us to evaluate the long-term impact of the microorganisms on the growth and development of pepper seedlings in subsequent phenological stages. Finally, although efforts were made to control the experimental conditions, environmental variables could have influenced the results and were not considered in this study. Despite the limitations above, the results obtained in this study open new perspectives in the investigation of the biostimulant effect of microorganisms on the *in vitro* germination of hybrid pepper seeds. These findings could be used as a basis for future research, in which the sample size can be expanded, follow-up time prolonged, and other relevant environmental variables considered. Furthermore, additional studies could be conducted to determine the mechanisms underlying this biostimulant effect and explore the possibility of practically applying these microorganisms in large-scale pepper seedling production.

In summary, this study on the biostimulant effect of microorganisms on the *in vitro* germination of hybrid pepper seeds is promising. Despite the limitations above, a significant increase in the germination rate and vigor of the seedlings treated with the microorganisms was observed. These findings suggest that using microorganisms can effectively improve germination and initial development of pepper seedlings. However, more research is required to fully understand the mechanisms involved and evaluate the long-term impact on adult plant growth and performance.

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Authors' Contributions

All authors made writing contributions and corrections to the manuscript. The contributions made by the authors were as follows: José Humberto Vera Rodríguez: design and development of the project, methodology, data collection and analysis, and writing of the manuscript; Mónica del Rocío Villamar Aveiga: conduct of the experiment; Diego Barzallo: translation of the manuscript into English; Johnny Xavier Barcia-Anchundia: database construction.

Ethical Implications

This article has no ethical implications.

Conflict of Interest

The authors declare no conflicts of interest in this study.

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