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Growth of pecan seedlings in response to inoculation with beneficial microorganisms

Crescimento de mudas de noz-pecã em resposta à inoculação com microrganismos benéficos

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Highlights

Inoculation with *Azospirillum brasilense* increases dry mass in pecan seedlings.
Trichoderma harzianum increases chlorophyll content in pecan leaves.
Beneficial microorganisms stimulate enzymatic activity associated with plant defense.

Abstract

Global pecan production is estimated at 320,000 tons, with the United States and Mexico responsible for approximately 90% of this output. Brazil ranks fourth worldwide, producing 7,200 tons, mainly in the states of Rio Grande do Sul (6,600 tons, 91,6%) and Paraná (300 tons, 5%). Since production is concentrated in southern Brazil, harvesting takes place during the off-season in the USA and Mexico, enabling Brazilian pecans to reach higher market values. This study aimed to evaluate the growth of potted pecan seedlings (cv. Barton) inoculated with *Trichoderma harzianum* and *Azospirillum brasilense.* Seedlings were inoculated in the soil on the day of planting, using manufacturer-recommended doses. Results demonstrated that microbial inoculation produced distinct beneficial effects on seedling development. *A. brasilense* increased plant dry mass, peroxidase activity, and phosphorus uptake, while *Trichoderma harzianum* enhanced leaf chlorophyll content, peroxidase activity, and phosphorus content. By contrast, co-inoculation produced no significant effects across the measured variables. Overall, inoculation with beneficial microorganisms improved seedling performance by promoting

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greater growth, reducing oxidative stress enzyme activity, and ensuring adequate absorption of all essential macronutrients.
Key words: *Azospirillum brasilense.* Macronutrients. Rootstock. *Trichoderma harzianum.*

Resumo

A produção global de noz-pecã é estimada em 320.000 toneladas, sendo os Estados Unidos e o México responsáveis por aproximadamente 90% dessa produção. O Brasil ocupa o quarto lugar no ranking mundial, produzindo 7.200 toneladas, principalmente nos estados do Rio Grande do Sul (6.600 toneladas, 91,6%) e Paraná (300 toneladas, 5%). Como a produção está concentrada no sul do Brasil, a colheita ocorre durante a entressafra nos EUA e no México, permitindo que as noz-pecã brasileiras atinjam maiores valores de mercado. Este estudo teve como objetivo avaliar o crescimento de mudas de noz-pecã em vasos (cv. Barton) inoculadas com *Trichoderma harzianum* e *Azospirillum brasilense.* As mudas foram inoculadas no solo no dia do plantio, usando as doses recomendadas pelo fabricante. Os resultados demonstraram que a inoculação microbiana produziu efeitos benéficos distintos no desenvolvimento das mudas. *A. brasilense* aumentou a massa seca da planta, a atividade da peroxidase e a absorção de fósforo, enquanto *Trichoderma harzianum* aumentou o teor de clorofila foliar, a atividade da peroxidase e o teor de fósforo. Em contraste, a coinoculação não produziu efeitos significativos nas variáveis medidas. De modo geral, a inoculação com microrganismos benéficos melhorou o desempenho das mudas, promovendo maior crescimento, reduzindo a atividade das enzimas do estresse oxidativo e garantindo a absorção adequada de todos os macronutrientes essenciais.
Palavras-chave: *Azospirillum brasilense.* Macronutrientes. Porta-enxerto. *Trichoderma harzianum.*

Introduction

The pecan tree (*Carya illinoinensis*) was first introduced to Brazil in the early 1900s by North American immigrants, with the initial seedlings established in São Paulo state. Commercial-scale plantations, however, were only established in southern Brazil (Fronza et al., 2018).

Global pecan production is approximately 320,000 tons, with the United States and Mexico accounting for 90% of this total. Brazil ranks fourth worldwide, producing 7,200 tons, mainly in the states of Rio Grande do Sul (6,600 tons; 91,6%) and Paraná (300 tons; 5%). Since production is concentrated in southern Brazil, harvesting takes place during the off-season in USA and Mexico, enabling Brazilian pecans to reach higher market values (International Nut and Dried Fruit Council Foundation [INC], 2021; Instituto Brasileiro de Geografia e Estatística [IBGE], 2024; Instituto Brasileiro de Pecanicultura [IBPECAN], 2024). With technological innovation and the introduction of new cultivars, Brazil has considerable potential to expand production in the coming years, (Saretta, 2021).

A key limitation to pecan cultivation is the quality of nursery trees. As a long-lived perennial plant, the pecan tree can remain in vegetative growth for several years, reaching reproductive maturity and initiating nut production (Fronza et al., 2018). Currently, most nursery trees are produced by grafting onto seedling rootstocks. However, this

process is slow and may take several months, especially when rootstock germination and growth are suboptimal (Poletto et al., 2016; Liu et al., 2022).

The use of beneficial microorganisms may accelerate early development and improve nutrient and water uptake, thereby enhancing the production of high-quality nursery trees (Huang et al., 2021). Among these microorganisms, *Azospirillum* is one of the most studied genera. This free-living diazotrophic bacterium is widespread in Brazilian soils and forms beneficial associations with a variety of plant species (A. J. de Oliveira et al., 2020).

Research has shown that *Azospirillum brasilense* not only fixes atmospheric nitrogen, increasing plant productivity and dry mass, but also contributes to biochemical processes linked to oxidative stress and phytohormone synthesis, including jasmonates and salicylic acid. These mechanisms may enhance plant resistance to biotic and abiotic stress (Cruz-Hernández et al., 2022; Fukami et al., 2018; Roberto & Colombo, 2020; Sáenz-Hidalgo et al., 2023).

Fungi of the genus *Trichoderma* are also widely used in agriculture, particularly for biological control of phytopathogens. These microorganisms also promote enzymatic activity and improve nutrient uptake, including phosphorus, calcium, and micronutrients (El-Hasan et al., 2018; Yan & Khan, 2021).

Given this background, the present study aimed to evaluate the growth of potted pecan seedlings (cv. Barton) inoculated with *Trichoderma harzianum* and *Azospirillum brasilense.*

Materials and Methods

The study was conducted in a greenhouse in Guarapuava, Paraná State, southern Brazil (25º23'26"S and 51º27'15"W, altitude 1,100 m). The region has a humid subtropical climate (Cfb, according to Köppen´s classification), with mean annual precipitation and temperature of 1,800 mm and 18.2ºC, respectively (Dubreuil et al., 2018).

Pecan seeds (cv. Barton) were harvested and dried in a forced air oven for four days until constant mass. They were then placed on trays, covered with a 5 cm layer of sand, and subjected to cold stratification at 4ºC for 70 days to overcome dormancy. The sowing substrate, a 1:1 mixture of soil and sand, was autoclaved twice (1 h, 121º C). Seeds were sown at 3 cm depth in 3.6 L pots filled with the sterilized substrate.

A randomized block design was used, with four treatments, four replicates and experimental plots of four pots. Treatments were: 1) Control (no inoculation), 2) *Azospirillum brasilense*; 3) *Trichoderma harzianum*; and 3) *Azospirillum brasilense* + *Trichoderma harzianum.* Inoculants included Biomax Azum® (BioSoja, 3 x 10^8 CFU of *A. brasilense*) and Trichodermil 1306 SC®, (KOPPERT, *T. harzianum* Rifai, strain ESALQ-1306, ≥2.0 x 10^9 conidia mL^{-1}).

Both inoculants were applied at 3.6 L m^3 of soil, according to the manufacturer's recommendation. Inoculation was performed at sowing by pipetting the solution at 3 cm depth.

Emergence speed index (ESI)

Seedling emergence per pot was recorded every two days from 25 to 50 days after sowing (DAS). The emergence speed index (ESI) was calculated according to Manguire (1962) as follows: ESI= (G_1/N_1) + (G_2/N_2... + (G_n / N_n), where G_1= number of normal seedlings emerged at the first count; N_1= number of days after sowing at the first count, and G_2, N_2,...,G_n, N_n correspond to the subsequent counts.

Dry mass, stem diameter and plant height

To determine dry mass (shoot, root and total), plants were cut close to the soil surface, separating the shoots and roots. Roots were washed and both fractions were dried in a forced-air oven at 65ºC until constant mass. Stem diameter was measured with a digital caliper and shoot height with a ruler.

Photosynthetic pigment analysis

Chlorophyll content was determined following Lichtenthaler and Wellburn (1983), with modifications by Porra et al. (1989).

From each plant, a fully expanded leaflet was collected, wrapped in foil to prevent light-induced pigment degradation, and stored in darkness. Extraction was performed in a dark room using a mortar and pestle, with calcium carbonate and 8 mL of 80% acetone. The homogenate was centrifuged (20 min, 4000 rpm), and supernatant absorbance was measured at 470, 663.6, and 646.6 nm with a UV-VIS spectrophotometer (Spectrum 1800, Shimadzu, Kagoshima, Japan).

Protein and enzyme activity

Leaflets were frozen in liquid nitrogen (-196 °C) and stored at -20 °C for analysis of protein content and enzyme activity (peroxidase, polyphenol oxidase, and superoxide dismutase). Samples were macerated in a chilled mortar with 2 mL of 0.01 M phosphate buffer (pH 6.0) and 1% PVP (polyvinyl-pyrrolidone) and then centrifuged (13,500 rpm, 35 min, 4 ºC). The supernatant was used as the enzyme extract.

Protein content was determined by the Bradford (1976) method, using 600 µL of 0.01 M phosphate buffer (pH 6.0), 200 µL of enzymatic extract, and 200 µL of Bradford reagent (250 mg Coomassie Brilliant Blue Dye G-250, 125 mL phosphoric acid, 125 mL distilled water). Absorbance was measured at 595 nm in a spectrophotometer, with three replicates, and the results were expressed as mg protein mL^{-1}.

Peroxidase activity (POD, EC. 1.11.1.7) was measured according to Teisseire & Guy (2000), using a reaction medium containing 30 µL enzyme extract, 50 mM potassium phosphate buffer (pH 6.5), 20 mM pyrogallol, and 5 mM hydrogen peroxide, incubated at 25 ºC for 5 min.

Superoxide dismutase (SOD, EC1.15.1.1) activity was determined following Giannopolitis and Reis (1977). The reaction solution was prepared with 250 µL of 0.1 mM EDTA 13ml of 0.25 M methionine (pH 7.8), 25ml of 0.105 M potassium phosphate buffer (pH 7.8), 15mg of nitro blue tetrazolium (NBT), and 1 mL of 0.01M riboflavin, adjusted to 250ml with distilled water. Next, 20 µL of enzyme extract and 3ml of this solution were placed in 24-well culture plates (3.5 mL capacity). One plate

was exposed to light for 10 minutes and the other kept in the dark (control). Absorbance was read at 560 nm, and SOD activity (U µg protein^{-1}) was calculated relative to 50% inhibition of NBT reduction, normalized by protein concentration (µg µL^{-1}).

Catalase (CAT) activity (EC 1.11.1.6) was determined according to Góth (1991), as adapted by Tomanková et al. (2006). For each sample, 100 µL of enzyme extract was incubated in a water bath at 38°C for 2 minutes with 500 µL of a solution containing 60 mM hydrogen peroxide in 60 mM potassium phosphate buffer (pH 7.4). The reaction was stopped by adding 500 µL of 32.4 mM ammonium molybdate. Blanks were prepared for each sample by adding ammonium molybdate without incubation. Absorbance was measured at 405 nm., and the amount of hydrogen peroxide (H_2O_2) consumed by catalase was calculated by subtracting the blank. Concentrations were determined using the extinction coefficient ϵ = 0.0655 mM^{-1} cm^{-1}.

Leaf nutrient analysis

Leaflets (one per pot, four per plot) were collected, washed with neutral detergent, oven dried at 70ºC to constant weight, ground in a mill and stored in polyethylene bags for analysis.

Leaf concentrations of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), zinc (Zn), and copper (Cu) were determined following nitro-perchloric digestion, and the nitrogen (N) content after sulfur digestion, using a block digester (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2009).

Soil enzyme activities

Soil enzyme activities were assessed for β-glucosidase, acid phosphatase, and urease. β-glucosidase and acid phosphatase activities were determined according to Tabatabai (1994) with modifications, and urease activity following Kandeler and Gerber (1988).

For β-glucosidase activity, 0.5 g of soil, 2.0 ml of MUB buffer (pH 6.0), and 0.5 ml of p-nitrophenyl-D-glucopyranoside (PNG) solution were added to test tubes. After shaking, samples were incubated in a water bath at 37 ºC for 1 hour. The reaction was stopped by adding 0.5 ml of 0.5 M $CaCl_2$ and 2.0 ml of 0.1 M (pH 12). The test blank was prepared in the same way, but with the PNG solution added only after incubation, thereby immediately stopping the reaction. After shaking and centrifugation (5 min, 4000 rpm), the supernatant was analyzed spectrophotometrically at 410 nm to quantify p-nitrophenol released. A calibration curve with 0-100 µg standards was used, and activity was expressed as µg of p-nitrophenol produced per hour per gram of soil (µg p-nitrophenol h^{-1} g^{-1} soil).

For acid phosphatase, 0.5 g of soil, 2.0 mL of MUB buffer (pH 6.5), and 0.5 mL of 50 mM PNP solution were incubated as described above. Activity was expressed as µg p-nitrophenol h^{-1} g^{-1} soil.

For urease, 5 g of fresh soil were mixed with 20 ml of 75 mM borate buffer (pH 10) and 2.5 ml of 80 mM urea solution. Samples were incubated at 37 ºC with shaking (200 rpm) for 2 hours. The reaction was stopped with 30ml of 1 M acidified KCl (0.01 M HCl) and shaken for 30 minutes. Aliquots (1.4 mL) were centrifuged (10 min, 4,000 rpm) and 1 mL of

supernatant was removed and mixed with 9 mL of distilled water, 5 mL of basic salicylate/sodium nitroprusside solution, and 2 mL of 0.1% dichloroisocyanuric acid solution.

After one hour at room temperature, absorbance was read at 690 nm. Negative controls were prepared simultaneously to verify non-enzymatic ammonia formation. The procedure was the same as that adopted for the samples, except that urea was added only at the end of the incubation and immediately after the addition of 1 mol L^{-1} KCl/HCl solution.Urease activity was expressed as mg of $N\text{-}NH4^{+}$ produced per hour per kilogram of soil (mg $N\text{-}NH4^{+}$ h^{-1} kg^{-1} soil).

Statistical analysis

Data were tested for normality and subjected to analysis of variance (ANOVA). Means were compared using Tukey's test at a significance level of 0.05 (Cruz, 1998).

Results and Discussion

Emergence speed index

There were no significant differences among treatments for the percentage of emergence or ESI (Table 1).

Table 1
Percentage of emergence and emergence speed index (ESI) of pecan seedlings (c. Barton) inoculated with *Azospirillum brasilense* and/or *Trichoderma harzianum*

Treatments	Emergence (%)	ESI
Control	56.2^{ns}	12.9^{ns}
Azospirillum brasilense	60.4	14.4
Trichoderma harzianum	60.2	14.6
Co-inoculation (*Trichoderma Harzianum + Azospirillum basilense*)	54.3	13.8
CV (%)	13.1	6.6

ns: Differences not statistically significant (Tukey's test, $p > 0.05$).

The ERI reflects the germination and establishment capacity of seedlings. In this study, inoculation with *Azospirillum brasilense* and *Trichoderma harzianum* did not alter ESI compared to the control.

These findings contrast with those of Junges et al. (2016), who reported that *Tricoderma* spp. increased emergence and leaf production in *Peltophorum dubium* seedlings. Similarly, C. F. Oliveira et al. (2021) demonstrated that inoculation with *A. brasilense* and *T. harzianum* enhanced root development in arugula (*Eruca vesicaria* ssp. *Sativa*), improving nutrient and water uptake and accelerating seedling growth. In pecan, however, the hard shell and seed morphology pose barriers to germination, which reduces seedling vigor (Wang et al., 2021).

Dry mass, stem diameter and plant height

Plant height, stem diameter, and shoot dry mass did not differ among treatments. Root dry mass was higher in seedlings inoculated with *A. brasilense,* although this was not statistically significant compared with the control. The total dry mass of *A. brasilense*-treated plants exceeded that of *T. harzianum* but was similar to that of the co-inoculation and control treatments (Table 2).

Table 2
Height, stem diameter, shoot, root, and total dry mass (g) of potted pecan seedling (cv. Barton) inoculated with *A. brasilense* and/or *T. harzianum*

Treatment	Height (g)	Diameter (mm)	Dry mass (g)		
			Shoot	Root	Total
Control	14.66^{ns}	3.37^{ns}	5.47^{ns}	17.82 a	23.29 ab
A. brasilense	16.94	3.38	5.78	18.06 a	23.84 a
T. harzianum	15.84	3.34	5.07	14.16 b	7.23 b
Co-inoculation (*A. brasilense* + *T. harzianum*)	14.93	3.53	5.53	16.74 ab	10.28 ab
CV (%)	13.78	8.92	15.42	9.69	8.61

* Means followed by the same letter do not differ significantly according to Tukey's test at 5% significance. ns: Differences not statistically significant (Tukey's test, $p > 0.05$)

These results are consistent with previous reports that A. brasilense enhances biomass accumulation in corn by increasing chlorophyll production, photosynthesis, and nutrient uptake (Quadros et al., 2014; Ribeiro et al., 2022).

With respect to chlorophyll, inoculation with T. harzianum, and co-inoculation significantly increased Chl a and Chl b contents, while A. brasilense alone only increased Chl a and did not differ from the control (Table 3).

According to Silva Oliveira et al. (2023), Gaspareto et al. (2023), Moreira et al. (2022), and C. F. Oliveira et al. (2021), inoculation generally enhances leaf chlorophyll content, although the effect depends on inoculum concentration and species, and may decline if the optimal dosage is exceeded (Silva Oliveira et al., 2023).

Table 3
Chlorophyll a, b, and total contents (mg g^{-1}) of potted pecan seedling (cv. Barton) inoculated with *A. brasilense* and/or *T. harzianum*

Treatment	Chlorophyll a	Chlorophyll b	Chlorophyll Total
Control	0.60 b*	0.23 b	0.84 b
A. brasilense	0.70 ab	0.21 b	0.91 b
T. harzianum	0.77 a	0.38 a	1.15 a
Co-inoculation (*A. brasilense* + *T. harzianum*)	0.74 a	0.29 b	1.03 b
CV (%)	6.24	7.42	4.43

* Means followed by the same letter do not differ significantly according to Tukey's test at 5% significance.

Soil enzyme activity did not differ significantly among treatments (Table 4). By contrast, plant enzyme responses varied. Catalase activity was highest in the control and lowest in A. brasilense. Peroxidase activity was lowest in the control and co-inoculation treatments, and elevated in *A. brasilense* and *T. harzianum,* while SOD activity did not differ significantly (Table 5).

Table 4
Soil enzyme activities of potted pecan seedlings (cv. Barton) inoculated with *A. brasilense* and/or *T. harzianum*

Treatment	Soil enzymes Urease	Soil enzymes Acid Phosphatase	Soil enzymes Glucosidase
Control	0.29^{ns}	0.42^{ns}	0.69^{ns}
A. brasilense	0.31	0.35	1.02
T. harzianum	0.28	0.42	1.11
Co-inoculation (*A. brasilense* + *T. harzianum*)	0.24	0.39	0.91
CV (%)	11.24	23.50	37.74

*ns: Differences not statistically significant (Tukey's test, $p > 0.05$).

Table 5
Activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in potted pecan seedlings (cv Barton), inoculated with *A. brasilense* and/or *T. harzianum*

Treatment	Enzyme		
	CAT	POD	SOD
Control	15.49 a	13.12 b	55.52^{ns}
A. brasilense	3.30 d	15.13 a	67.31
T. harzianum	10.88 b	15.47 a	60.08
Co-inoculation (*A. brasilense* + *T. harzianum*)	7.40 c	12.81 b	51.82
CV (%)	12.18	6.40	20.03

* Means followed by the same letter do not differ significantly according to Tukey's test at 5% significance.

The higher CAT activity in the control contrasts with reports that inoculation enhances oxidative stress defense (Ribeiro et al., 2022). According to Fukami et al. (2018), this discrepancy may be due to the application method, whereby foliar inoculation more strongly activates oxidoreductases, whereas soil inoculation stimulates phytohormone synthesis. Importantly, the increase in POD activity, an enzyme associated with pathogen defense and wound healing capacity, observed in the inoculated treatments, indicates that inoculation enhances plant health. This result corroborates Fukami et al. (2018), who reported increased phytohormone defense activity in pecan inoculated with *A. brasilense*, and Oliveira et al. (2021), who observed more vigorous and healthier initial growth in pecan plants inoculated with *A. brasilense* and *T. harzianum.*

Seedlings inoculated with *T. harzianum* showed the highest phosphorus concentrations, suggesting improved root development, consistent with earlier studies (De Jaeger et al., 2011; Li et al., 2015; Mercl et al., 2020). Across treatments, N, P, K, Ca, Mg, and S concentrations were within optimal ranges, indicating healthy seedling development and adequate nutrient uptake (Table 6). These findings support the role of inoculants in enhancing nutrient uptake efficiency, even under acidic soil conditions common in Brazil (Bibi et al., 2024).

Table 6
Leaf macronutrient contents (N, P, K, Ca, Mg, S) of potted pecan seedlings (cv. Barton) inoculated with *A. brasilense* and/or *T. harzianum*

Treatment	N	P	K	Ca	Mg	S
	mg kg^{-1}					
Control	4.53^{ns}	1,72 ab	2.28^{ns}	4.41^{ns}	2.83^{ns}	2.67^{ns}
A. brasilense	3.98	1.66 b	2.26	4.34	2.87	2.46
T. harzianum	4.09	2.01 b	2.22	4.59	2.97	2.96
Co-inoculation (*A. brasilense* + *T. harzianum*)	4.02	1.83 ab	2.40	4.30	2.79	2,69
CV (%)	10.3	8.51	10.46	7.55	9.81	19.63

Means followed by the same letter do not differ significantly according to Tukey's test at 5% significance. *ns: Differences not statistically significant (Tukey's test, p > 0.05).

Conclusion

Inoculation with *A. brasilense* and *T. harzianum* enhanced the development of pecan cv. Barton seedling, promoting higher dry mass, chlorophyll content, peroxidase activity, and phosphorus content. These results highlight the potential of inoculation as a cost-effective and sustainable technology for fruit tree nurseries.

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