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Promotion of plant growth and rooting of stem cuttings by endophytic bacteria from black pepper roots

Abstract – The objective of this work was to investigate three endophytic bacterial strains as to their ability to promote the growth and rooting of black pepper stem cuttings, as well as to determine the mechanisms involved in the promoting activity through molecular methods. The strains were identified by 16S sequencing as belonging to the genera *Bacillus*, *Priestia*, and *Lysinibacillus*. They were characterized for the production of indoleacetic acid (IAA), phosphate solubilization, and siderophore production. In two assays, the roots of the cuttings were immersed in bacterial suspensions to evaluate growth promotion through plant height, stem diameter, and root and shoot dry mass. The *Bacillus* sp. C1.4 and *Priestia* sp. T2.2 strains were able to produce siderophores, whereas *Priestia* sp. T2.2 and *Lysinibacillus* sp. C5.11 produced IAA. In addition, *Priestia* sp. T2.2 significantly increased plant height and dry mass, whereas *Lysinibacillus* sp. C5.11 significantly increased root dry mass. Therefore, *Priestia* sp. T2.2 and *Lysinibacillus* sp. C5.11 are able to promote the growth and rooting of black pepper stem cuttings, respectively. This growth promotion is linked directly to the production of IAA and siderophores.

Index terms: *Lysinibacillus* sp., *Piper nigrum*, *Priestia* sp., beneficial microorganisms, 16S rDNA sequencing, phytohormones.

Promoção de crescimento e enraizamento de estacas por bactérias endofíticas de raízes de pimenta-do-reino

Resumo – O objetivo deste trabalho foi investigar três isolados de bactérias endofíticas quanto à sua capacidade de promover o crescimento e o enraizamento de estacas de pimenta-do-reino, bem como determinar os mecanismos envolvidos na atividade promotora por métodos moleculares. Os isolados foram identificados por sequenciamento 16S como pertencentes aos gêneros *Bacillus*, *Priestia* e *Lysinibacillus*. Foram caracterizados quanto à produção de ácido indolacético (IAA), à solubilização de fosfato e à produção de sideróforos. Em dois ensaios, as raízes das estacas foram imersas em suspensões bacterianas para avaliar a promoção do crescimento por meio de altura da planta, diâmetro do caule e massa seca de raízes e brotos. Os isolados *Bacillus* sp. C1.4 e *Priestia* sp. T2.2 foram capazes de produzir sideróforos, enquanto *Priestia* sp. T2.2 e *Lysinibacillus* sp. C5.11 produziram IAA. Além disso, o isolado *Priestia* sp. T2.2 aumentou significativamente a altura e a massa seca da planta, enquanto *Lysinibacillus* sp. C5.11 aumentou significativamente a massa seca da raiz. Portanto, *Priestia* sp. T2.2 e *Lysinibacillus* sp. C5.11 são capazes de promover o crescimento e o enraizamento de estacas de pimenta-do-reino, respectivamente. Esta promoção de crescimento está diretamente ligada à produção de IAA e sideróforos.

Termos para indexação: *Lysinibacillus* sp., *Piper nigrum*, *Priestia* sp., microrganismos benéficos, sequenciamento de 16S rDNA, fitormônios.

Luana Cardoso de Oliveira
Universidade Federal do Sul e Sudeste do Pará, Marabá, PA, Brazil.
E-mail: luanaoliveira.qi@gmail.com

Alessandra Keiko Nakasone✉
Embrapa Florestas, Colombo, PR, Brazil.
E-mail: alessandra.nakasone@embrapa.br

Sílvia Mara Coelho do Nascimento
Universidade de Lavras, Lavras, MG, Brazil.
E-mail: silviacoelho266@gmail.com

Deyse Ribeiro Silvino de Jesus
Universidade Federal Rural de Pernambuco, Recife, PE, Brazil.
E-mail: silvinodeise@gmail.com

Walkymário de Paulo Lemos
Embrapa Amazônia Oriental, Belém, PA, Brazil.
E-mail: walkymario.lemos@embrapa.br

Patrícia Santana Barbosa Marinho
Universidade Federal do Pará, Belém, PA, Brazil.
E-mail: pat@ufpa.br

Andrey Moacir do Rosario Marinho
Universidade Federal do Pará, Belém, PA, Brazil.
E-mail: andrey@ufpa.br

Kátia de Lima Nechet
Embrapa Meio Ambiente, Jaguariúna, SP, Brazil.
E-mail: katia.nechet@embrapa.br

Bernardo de Almeida Halfeld-Vieira
Embrapa Meio Ambiente, Jaguariúna, SP, Brazil.
E-mail: bernardo.halfeld@embrapa.br

Jorge Teodoro de Souza
Universidade de Lavras, Lavras, MG, Brazil.
E-mail: jorge.souza@ufla.br

✉ Corresponding author

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Introduction

The endophytic microorganisms, mainly bacteria and fungi, found in the internal tissues of all plant species examined to date are indispensable for the development of healthy plants and their adaptation to the environment (Kandel et al., 2017). This occurs because plants and endophytes are co-evolved and depend on each other for several vital physiological processes, in such a way that plants devoid of endophytes may be unable to survive (Abreu-Tarazi et al., 2010; Kandel et al., 2017). For this reason, in the last few years, there has been an increasing number of research in endophytic microorganisms due to their promising roles in the environment and in promoting more sustainable agricultural practices (Anand et al., 2023).

Endophytic bacteria may be used to decrease the amounts of applied chemical pesticides and fertilizers (Aravind et al., 2012; Tran et al., 2019; Ngo et al., 2020; Nguyen et al., 2021). In addition, these bacteria may promote plant growth through direct and indirect mechanisms (Pandey et al., 2017; Oliveira et al., 2021). According to these same authors, the direct mechanisms include the synthesis and regulation of phytohormones (auxins, gibberellins, and cytokinins, for example), the secretion of enzymes such as ACC deaminase, nitrogen fixation, phosphate solubilization, and iron sequestration by siderophore production, whereas indirect mechanisms cover improved nutrient availability, biocontrol of pathogens and pests, and increased tolerance to environmental stresses.

In the literature, the potential use of bacterial endophytes has been reported for several crops, such as black pepper (*Piper nigrum* L.) in the studies of Aravind et al. (2012), Tran et al. (2019), Ngo et al. (2020), Oliveira et al. (2020), and Nguyen et al. (2021). This species is a crop of global significance, being economically and socially important in Brazil, its second largest producer (FAO, 2025). In the country, most black pepper-growing areas are owned by smallholder farmers, who cultivate the crop to supplement their income (Dalazen et al., 2022).

Black pepper is currently propagated by rooted stem cuttings, which maintain the genetic characteristics of selected mother plants and reduce the time for fruiting (Secundino et al., 2014). Therefore, to obtain productive and healthy plantations with a low cost and greater financial return, the production of vigorous and uniform stem cuttings is essential (Veloso & Albuquerque, 1989). The inoculation with beneficial microorganisms is a management practice that could be incorporated for this purpose.

Among endophytic microorganisms, *Bacillus* species were reported to be capable of increasing the height and fresh mass of black pepper stem cuttings (Ngo et al., 2020). Similarly, *Pseudomonas aeruginosa* and *Bacillus megaterium* had a positive effect on the rooting of black pepper stem cuttings, increasing root mass and total mass (Aravind et al., 2012). In a previous study, three endophytic bacterial strains from black pepper roots were selected from a total of 164 strains as promising candidates to be used, as they increased the height of black pepper stem cuttings by 26.8 to 38.0% and dry mass by 10.0% (Oliveira et al., 2020).

The objective of this work was to investigate three endophytic bacterial strains as to their ability to promote the growth and rooting of black pepper stem cuttings, as well as to determine the mechanisms involved in the promoting activity through molecular methods.

Materials and Methods

The evaluated bacterial strains were T2.2, C5.11, and C1.4, of which some were previously selected as promising growth promoters to increase the height and dry mass of black pepper stem cuttings (Oliveira et al., 2020). These strains were obtained from internal tissues of three- and four-year-old roots of the Singapura black pepper cultivar, cultivated in the municipalities of Tomé-Açu and Castanhal, in the state of Pará, Brazil.

For the growth promotion and rooting experiments, four-month-old rooted cuttings of the 'Singapura' black pepper, at the commercial standard age, were acquired from the ProMudas commercial supplier (Castanhal, PA, Brazil). The assays were carried out from January 2023 to July 2024.

The tested strains were preserved in sterile water at 26°C and grown on medium 523 (Kado & Heskett, 1970) at 28°C, in the dark, for 48 hours. The identification of the isolates at the genus level was done by sequencing the 16S region of the ribosomal RNA (Table 1). For this, the genomic DNA of the bacterial strains was obtained by using

the hexadecyltrimethylammonium bromide method (Ausubel et al., 2003) with adaptations, and polymerase chain reaction was performed to amplify a fragment of the 16S gene of the rDNA using the universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). After amplification, sequencing was performed by the Sanger method in the ACTGene Análises Moleculares DNA sequencer (Alvorada, RS, Brazil). The sequences under study were compared via the BlastN algorithm with 16S sequences of bacterial type species accepted and listed in the List of Prokaryotic Names with Standing in Nomenclature (Parte et al., 2020). The phylogenetic tree was constructed with the MEGA, version 7.0, software (Kumar et al., 2016).

The physiological characterization of the strains was carried out by determining if they could produce indoleacetic acid, solubilize phosphate, and produce siderophores. Indoleacetic acid production was evaluated using a qualitative and a quantitative method. For the qualitative method, strains were spread on plates containing 1/10 tryptic soy agar (TSA) medium enriched with 0.204 g L^{-1} L-tryptophan, which was overlaid with a nitrocellulose membrane and incubated at 28°C, in the dark, for 24 hours. Subsequently, the membrane was transferred to another plate containing a saturated Salkowski solution composed of 1.0 mL $FeCl_3.6H_2O$ 0.5 mol L^{-1} + 50 mL $HClO_4$ 35%. Strains that produced a reddish halo on the membrane between 30 min and 2 hours of incubation, at 26°C, were considered producers of indoleacetic acid. For the quantitative evaluation, the colorimetric method was used. A portion of the bacterial colony was transferred to microplates containing 1.0 mL modified LGI-P broth, without bromothymol blue and with 10 mmol L^{-1} $(NH_4)_2SO_4$ as a nitrogen source, with or without 100 µg mL^{-1} L-tryptophan. After 48 hours of incubation, the culture was centrifuged, and 150 µL of the supernatant were mixed with 100 µL of the Salkowski solution. The indoleacetic acid concentration was recorded based on a previously determined standard curve.

For phosphate solubilization, the strains were grown on 1/10 TSA medium containing $CaHPO_4$ and K_2HPO_4 at 0.57 mol L^{-1} and $CaCl_2$ at 0.9 mol L^{-1}, being incubated at 28°C, in the dark, for seven days. Strains that produced a transparent halo around the colonies were considered phosphate solubilizers.

Siderophore production was evaluated by growing the bacterial strains in liquid King's B medium at 25°C, in the dark, for 48 hours under agitation at 100 rpm. The negative controls were the bacterial strains cultivated in the same medium, containing 2.0 µmol L^{-1} Fe^{2+} mL^{-1} prepared with autoclaved $FeSO_4.7H_2O$. Bacterial cells were centrifuged at 10.000 *g* for 20 min, and 1.0 mL of each supernatant was mixed with 1.0 mL of the chromeazurol (CAS) indicator solution. The coloration of the medium without the bacterial strains was used as an additional control. Strains that produced siderophores changed the color of the mixture of the plus CAS supernatant from blue to yellow/orange in up to 15 min.

For the growth promotion assay, roots of the cuttings were trimmed with scissors to approximately 3.0 cm and immersed for 5 min in bacterial suspensions adjusted to an optical density of 540 nm = 0.25 (Oliveira et al., 2020). Subsequently, they were planted in 3.0 kg pots with sterile coconut fiber. Every 15 days, the cuttings received 100 mL of the nutrient solution used in the Murashige & Skoog (MS) medium. To evaluate growth promotion, plant height from the soil line to the top of the cuttings and stem diameter were measured six

Table 1. Identification of the endophytic growth-promoting bacteria selected from black pepper (*Piper nigrum*) through 16S rRNA gene sequencing.

Strain	Identification	Accession number[(1)]	Fragment size (bp)	Closest match in databases/ accession number[(2)]	Identity (%)
C1.4	*Bacillus* sp.	OL362028	802	*Bacillus cereus*^{T} ATCC 14579/NR_074540.1; *Bacillus tropicus*^{T} MCCC 1A01406/NR_157736.1; other four *Bacillus* species	100.00
T2.2	*Priestia* sp.	OL362031	1,427	*Priestia aryabhattai*^{T} B8W22/NR_115953.1	100.00
C5.11	*Lysinibacillus* sp.	OL362034	1,401	*Lysinibacillus pakistanensis*^{T} NCCP-54/NR_113166.1	99.29

[(1)]Accession numbers obtained in the present study. [(2)]Curated rRNA/ITS databases with type strains were used. The closest matches were defined on the basis of identity and coverage.

times every 15 days. The dry mass of the whole plant was determined in the last evaluation. Cuttings with roots immersed in sterile distilled water were used as the control treatment. Assessments were initiated 15 days after inoculation.

The experiment was installed in a randomized complete block design with four treatments (three strains and the control), with five replicates. Each replicate consisted of one pot with one plant. Plant growth and diameter were used to calculate the area under the growth progress curve (AUGPC). The values of the AUGPC were subjected to the analysis of variance, and treatments were compared by the least significant difference t-test, at 5% probability.

Another assay was carried out as described previously, using the same design and number of replicates, but root mass and shoot dry mass were measured 60 days after inoculation to evaluate rooting. The values obtained for root and shoot dry mass were subjected to the analysis of variance, and treatments were compared by the least significant difference (LSD) t-test, also at 5% probability.

Results and Discussion

The following three bacterial genera were identified among the strains evaluated in the present study: *Bacillus*, *Priestia* (Syn. *Bacillus*), and *Lysinibacillus*, belonging to the phylum Firmicutes and class Bacilli. The identities of the 16S rRNA sequences ranged from 99.29 to 100% (Table 1). The identification of these strains is essential to safeguard their use in agricultural production since many bacterial strains obtained from the environment may be harmful to human and animal health and cannot be used in such applications (Xu & Kovács, 2024). The identified *Bacillus* strain, for example, was in the *Bacillus cereus* clade that may contain human pathogens. However, although the identified genera were once classified as *Bacillus*, they have been reclassified in many different genera due to the progress in molecular phylogeny and genomics (Gupta et al., 2020).

Species of the genera *Bacillus*, *Priestia*, and *Lysinibacillus* are capable of acting as biocontrol agents against pests and diseases and of promoting plant growth by facilitating nutrient availability and enhancing stress tolerance (Naureen et al., 2017; Fonseca et al., 2024). *Bacillus* sp. C1.4 and *Priestia* sp. T2.2 were able to produce siderophores, whereas *Priestia* sp. T2.2 and *Lysinibacillus* sp. C5.11 produced indoleacetic acid (Table 2). However, none of the strains were able to solubilize the phosphate from the $CaHPO_4$ and K_2HPO_4 sources. In the literature, there are previous reports of the production of siderophores by *Bacillus* strains (Naureen et al., 2017), indoleacetic acid by *Lysinibacillus* (Naureen et al., 2017), and of both metabolites by *Priestia* (Fonseca et al., 2024).

In the growth promoting assays, only *Priestia* sp. T2.2 significantly increased plant height and plant dry mass by 75 and 136% when compared with the control (Figure 1 A, B, and C), a growth-promoting activity considered related to the production of indoleacetic acid and siderophores. However, none of the evaluated bacterial strains were able to increase the stem diameter of the black pepper plants. As observed here, other authors also attributed the growth-promotion ability of bacterial strains directly to indoleacetic acid (Etesami et al., 2014, 2015) or to siderophore (Cen et al., 2024) production. Pandey et al. (2017) and Oliveira et al. (2021) highlighted that endophytic bacteria are able to promote plant growth through several mechanisms, including nitrogen fixation, phosphate solubilization, siderophore production, and indoleacetic acid production.

The number of bacterial strains capable of promoting plant growth varies greatly among different plant species. For citrus (*Citrus* spp.) rootstocks, for example, the percentages of growth-promoting strains, from different origins and ecology, varied from 6 to 44% (Giassi et al., 2016). For maize (*Zea mays* L.) and sorghum [*Sorghum bicolor* (L.) Moench], the percentages of endophytic strains from sugarcane (*Saccharum officinarum* L.) leaves and stalks with the capacity to promote plant growth ranged from 53 to 100% (Aquino et al., 2019). In comparison, the reported

Table 2. Production of siderophores and indole acetic acid (IAA) and phosphate solubilization by endophytic bacteria from black pepper (*Piper nigrum*).

Strain	Siderophores[(1)]	IAA (μmol L^{-1} mL^{-1})	Phosphatase
Bacillus sp. C1.4	+	0.00	-
Priestia sp. T2.2	+	45.19	-
Lysinibacillus sp. C5.11	-	52.43	-

[(1)] +, positive reaction indicated by a color change; and -, negative reaction, without color change.

percentage of endophytic strains from black pepper roots able to promote the growth of stem cuttings of the species seems to be relatively low since it was only 2% (Oliveira et al., 2020). Similarly, in the first round of screening that resulted in the three strains used in the present study, only four (2.4%) out of the total 164 were able to promote plant growth (Oliveira et al., 2020); however, this apparently low number is explained by the fact this is a follow up of a previous work, in which 164 strains were evaluated (Oliveira

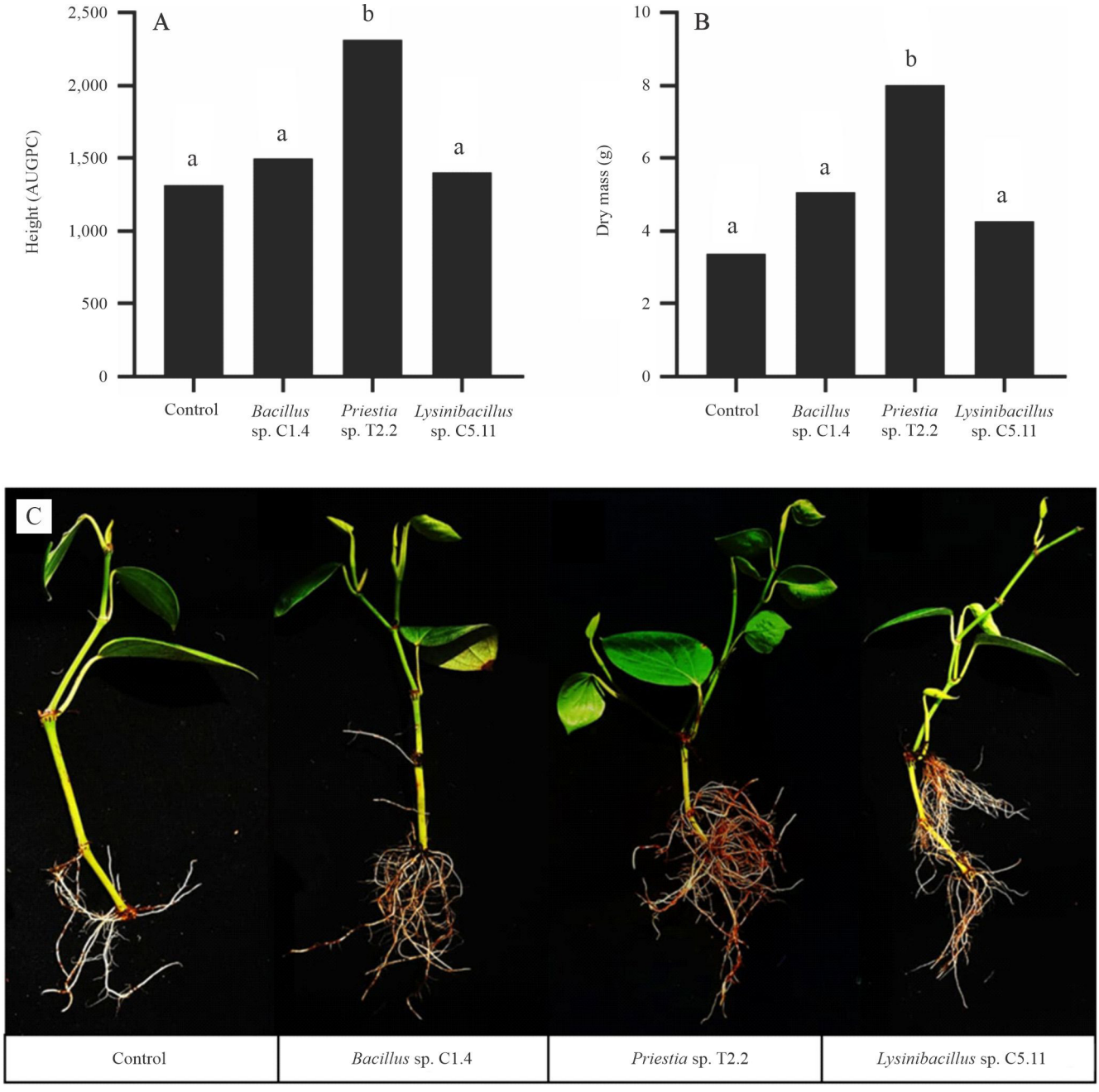

Figure 1. Growth of black pepper (*Piper nigrum*) cuttings influenced by endophytic bacterial strains, showing: plant height (A), plant dry mass (B), and stem cuttings 90 days after inoculation (C). Averages followed by different letters differ significantly according to the least significant difference t-test, at ($p \leq 0.05$). AUGPC, area under the growth progress curve.

et al., 2020). To further elucidate the mode of action of these strains, future researches should analyze the influence of limiting factors in the growth-promoting activity of these strains, such as associations with hormone-producing bacteria in nutrient-rich soils and with nutrient solubilizers in poor soils, which was not done here.

In the rooting experiment, only *Lysinibacillus* sp. C5.11 significantly increased root dry mass by 333% when compared with the control (Figure 2 A and C).

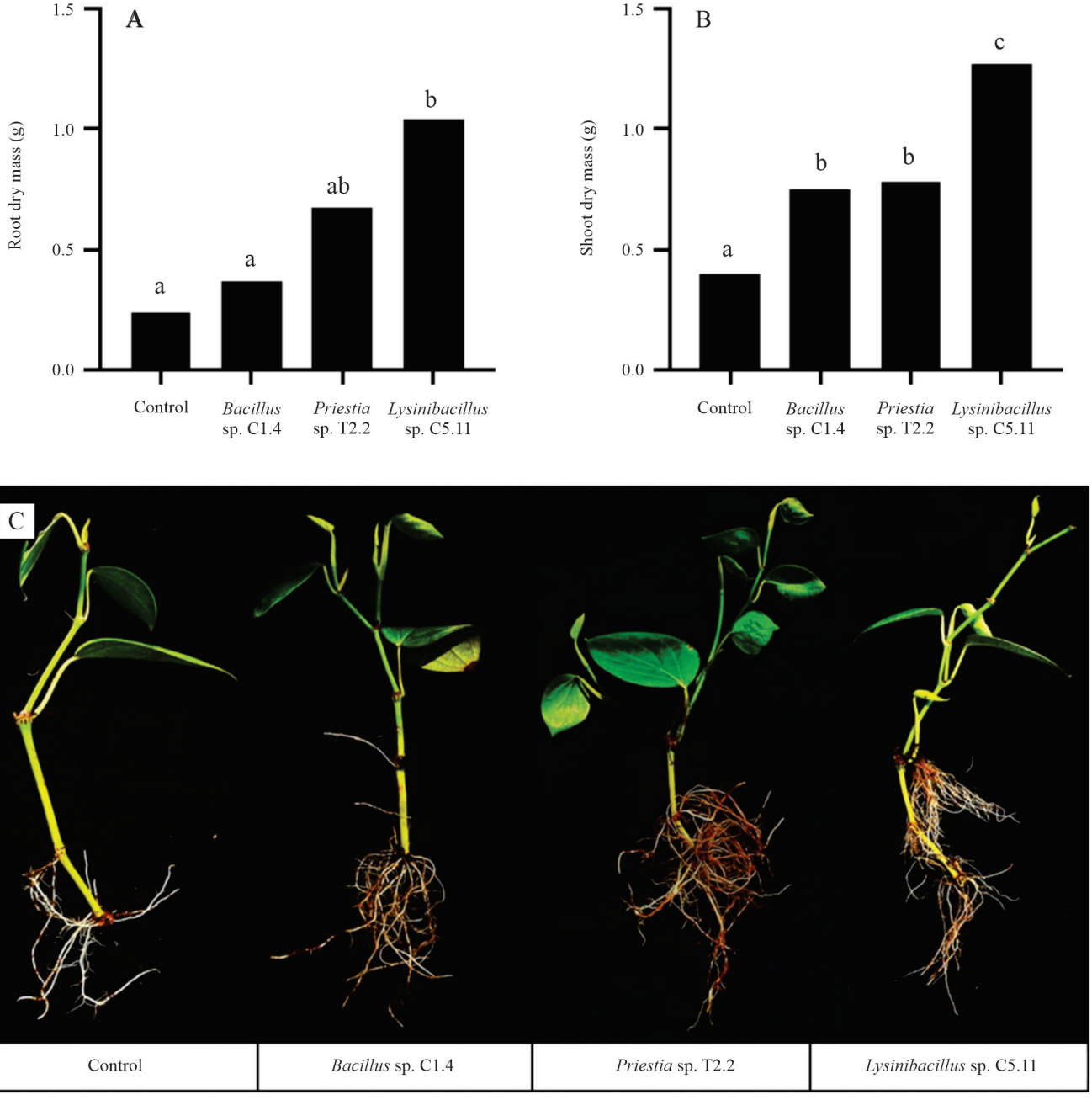

Figure 2. Rooting of black pepper (*Piper nigrum*) cuttings influenced by endophytic bacterial strains, showing: root dry mass (A), shoot dry mass (B), and stem cuttings 60 days after inoculation (C). Averages followed by different letters differ significantly according to the least significant difference t-test, at $p \leq 0.05$.

However, the three selected strains significantly increased shoot dry mass compared with the control (Figure 2 B and C), as follows: *Lysinibacillus* sp. C5.11 by 217%, *Bacillus* sp. C1.4 by 87%, and *Priestia* sp. T2.2 by 95%.

The rooting activity of *Lysinibacillus* sp. C5.11 was probably related directly to its ability to produce indoleacetic acid. Lau et al. (2020) also found that bacterial isolates were efficient in stimulating the rooting of black pepper stem cuttings through the production of indoleacetic acid. In this line, Shahzad et al. (2019) concluded that auxins are essential for plant propagation through cuttings, considering the important role indoleacetic acid plays in rooting promotion (Rocha et al., 2019; Shahzad et al., 2019). However, as also observed for *Priestia* sp. T2.2, further studies need to be conducted to confirm the presented hypothesis.

Considering that *Priestia* sp. T2.2 is related to *Priestia aryabhattai* (Syn. *Bacillus aryabhattai*) and *Lysinibacillus* sp. C5.11 to *Lysinibacillus pakistanensis*, these strains may be safely used in plant growth promotion and may be further developed into formulated products to improve the growth of black pepper cuttings. Since 2024, biological products have been regulated by Brazilian Federal Law number 15.070/2024, according to which bioinputs are not classified as pesticides and are authorized to be used on on-farm production (Brasil, 2024). This change in regulatory requirements is an important step that allows of overcoming obstacles in developing biological products based on the selected strains.

In this context, the study of endophytic bacteria as biological promoters of plant growth represents an environmentally-friendly alternative, with the prospect of reducing the use of chemical fertilizers in the cultivation of black pepper and better establishing the crop in production areas, especially considering the low rooting percentage of its cuttings, currently used as propagation units. Therefore, further studies should focus on testing these promising strains under field conditions and in formulations to manage, for example, *Fusarium*-induced diseases on black pepper.

Conclusion

Priestia sp. T2.2 and *Lysinibacillus* sp. C5.11 are able to promote the growth and rooting of black pepper (*Piper nigrum*) stem cuttings, respectively, which is likely linked to their ability of producing indoleacetic acid and siderophores.

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