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Initial development of upland rice inoculated and co-inoculated with multifunctional rhizobacteria

Desenvolvimento inicial de arroz de terras altas inoculado e coinoculado com rizobactérias multifuncionais

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Highlights

Multifunctional rhizobacteria can promote the growth of roots.
We investigated how rhizobacteria affect the growth of upland rice.
Multifunctional rhizobacteria promoted greater initial development of the culture.

Abstract

Inoculation and co-inoculation of upland rice with multifunctional rhizobacteria can promote plant growth, especially the root system. Thus, this study aimed to evaluate the effect of inoculation and co-inoculation with *Azospirillum* sp. and *Bacillus* sp. in the early development of upland rice. The experiment was conducted using a completely randomized design with 4 treatments and 10 replications, totaling 40 plots. The treatments were: 1) Ab-V5 (*Azospirillum brasilense*), 2) BRM 63573 (*Bacillus* sp.), 3) co-inoculation of Ab-V5 + BRM 63573, and 4) control (without rhizobacteria). Inoculation and co-inoculation with the multifunctional rhizobacteria Ab-V5 and BRM 63573 provided positive effects on the initial development of upland rice. Inoculation with isolate BRM 63573 had significant effects on root length, shoot, and total biomass, while inoculation with isolate Ab-V5 had significant effects on root length and production of root and total biomass. Co-inoculation treatment had significant effects on variables such as diameter, volume, total surface, root biomass, and total biomass. The control treatment (without multifunctional rhizobacteria)

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had the worst results for most of the analyzed variables.
Key words: *Azospirillum brasilense. Bacillus* sp. Growth promotion. *Oryza sativa.* Sustainability.

Resumo

A inoculação e coinoculação do arroz de terras altas com rizobactérias multifuncionais pode promover o crescimento das plantas, especialmente do sistema radicular. Assim, este estudo teve como objetivo avaliar o efeito da inoculação e coinoculação com *Azospirillum* sp. e *Bacillus* sp. no desenvolvimento inicial do arroz de terras altas. O experimento foi conduzido em delineamento inteiramente casualizado com 4 tratamentos e 10 repetições, totalizando 40 parcelas. Os tratamentos foram: 1) Ab-V5 (*Azospirillum brasilense*), 2) BRM 63573 (*Bacillus* sp.), 3) coinoculação de Ab-V5 + BRM 63573 e 4) controle (sem rizobactérias). A inoculação e coinoculação com as rizobactérias multifuncionais Ab-V5 e BRM 63573 proporcionaram efeitos positivos no desenvolvimento inicial do arroz de terras altas. A inoculação com o isolado BRM 63573 teve efeitos significativos no comprimento da raiz, parte aérea e biomassa total, enquanto a inoculação com o isolado Ab-V5 teve efeitos significativos no comprimento da raiz e produção de raiz e biomassa total. O tratamento com coinoculação teve efeitos significativos em variáveis como diâmetro, volume e superfície total de raiz e biomassa de raiz e total. O tratamento controle (sem rizobactérias multifuncionais) apresentou os piores resultados para a maioria das variáveis analisadas.
Palavras-chave: *Azospirillum brasilense. Bacillus* sp. Promoção de crescimento. *Oryza sativa.* Sustentabilidade.

Introduction

Rice (*Oryza sativa* L.) is one of the most produced and consumed cereals in the world. It is a staple food for a large part of the world's population (Silva et al., 2017). In Brazil, most upland rice crops are in the Cerrado biome, mainly in the Midwest region, where they are rainfed (81% of the area) instead of irrigated (Oliveira, 2015; Alok et al., 2014; Coêlho, 2021). Therefore, it is essential to seek sustainable agricultural practices to increase upland rice production, especially from the perspective of population growth (Devkota et al., 2019).

Multifunctional rhizobacteria can be a safe and environmentally sound alternative to increase agricultural production. These bacteria can contribute to reducing the application of fertilizers and pesticides, which helps to reduce the environmental contamination of soil, water, and air (Santos Dias, 2022; Babalola, 2010). Rhizobacteria have different mechanisms of action, allowing them to act in various ways, such as through the production of phytohormones, the availability of nutrients, or the control of pathogens or other stresses. For this reason, they are classified as bio-stimulators, biofertilizers, or biocontrol agents (Santos Lopes et al., 2021a; Santos Lopes et al., 2021b; A. P. da Silva & Dourado, 2022a). These mechanisms mainly benefit the greater root development, providing conditions for the plant to explore a larger volume of soil and absorb more water and nutrients (Cruz et al., 2022).

In studies conducted under controlled conditions (greenhouse) with multifunctional rhizobacteria, the isolates Ab-V5 (*Azospirillum brasilense*) and BRM 63573 (*Bacillus* sp.) have shown promising results.

They can provide greater root diameter of seedlings (J. P. T. Fernandes et al., 2021a) and higher biomass production (J. Fernandes et al., 2021b) in upland rice plants, compared to the control treatment (without rhizobacteria). Other studies have also shown that more than one multifunctional rhizobacterium can be inoculated into plants using a technique called co-inoculation (M. A. Silva et al., 2020). Co-inoculation is an agricultural practice that aims to optimize and improve plant development by associating different multifunctional microorganisms (Korber et al., 2021). As a result, the plant may experience greater initial development with positive effects on the crop's grain yield (Silva et al., 2022a). Therefore, this work aimed to evaluate the effect of inoculation and co-inoculation with multifunctional rhizobacteria of the genera *Azospirillum* sp. and *Bacillus* sp. on the early development of upland rice.

Material and Methods

The experiment was carried out in a greenhouse, under controlled conditions, at the headquarters of *Embrapa Arroz e Feijão,* in Santo Antônio de Goiás, GO - Brazil (16º28'00" S, 49º17'00" W, and 823-m altitude). A completely randomized design was adopted, with 4 treatments and 10 replications, totaling 40 plots. The treatments consisted of inoculation or co-inoculation of upland rice seeds (cultivar BRS 501 CL) plus a control, as follows: 1) Ab-V5 (*Azospirillum brasilense*), 2) BRM 63573 (*Bacillus* sp.; previously named 1301), 3) co-inoculation of Ab-V5 + BRM 63573, and 4) control (without rhizobacteria). The microorganisms were identified and stored in the collection of Multifunctional Microorganisms at Embrapa Rice and Beans.

Bacterial suspensions for seed inoculation and co-inoculation were prepared using bacteria grown in a solid medium (nutrient agar) and then grown in a liquid medium (Broth nutrient) (Kado & Heskett, 1970) in an incubator with constant stirring for 24 hours at 28°C. Bacterial concentration was fixed at 108 CFU (colony forming units) per mL, which was determined in a spectrophotometer at A540 = 0.5. Inoculation and co-inoculation were performed by soaking upland rice seeds in each rhizobacteria suspension and control seeds in water for 24 hours under constant agitation at 25 °C (Filippi et al., 2011). Twenty seeds from each treatment were suspended in 100 mL of liquid. In the co-inoculation treatment, 50 mL of suspension from each rhizobacterium was combined and mixed thoroughly to obtain a homogeneous solution before adding the rice seeds. The seeds were neither sterilized nor treated with agrochemicals.

Each experimental unit comprised a 500 mL plastic cup filled with unfertilized surface soil (0.00-0.20 m) of medium texture, in which two rice seeds were sown. Fifteen days after sowing, the seedlings were carefully removed from the cups and the root systems were washed. Subsequently, the seedlings were photographed using a digital camera and the images were stored in the WinRHIZO 2012 software from Regent Instruments, Inc., located in Quebec City, QC, Canada (Arsenault et al., 1996). The following analyses were performed: total root length (LengR, cm), root diameter (DiamR, mm), total root surface area (AreaS, cm^2), and root volume (VolR, cm^3).

After photographing, both shoot and roots were placed in separate kraft paper envelopes, labeled, and dried in a forced ventilation oven at 65 ºC until a constant mass was achieved. The material was then weighed using a precision scale with four decimal places (0.0001) to determine the root (RDM), shoot (SDM), and total (TDM) dry masses.

Data were analyzed by analysis of variance, and means were compared using Fisher's LSD test with a 5% significance level. Principal component analysis (PCA) was performed to describe correlations between response variables and treatments. Principal components (PCs) were used as response variables when the correlation coefficient was r ≥ 0.50. The isolated, co-inoculated, and control bacteria and the response variables were plotted on a biplot (two-dimensional graph). All analyses were performed using the R software version 3.5.0 (R CORE Team [R], 2016).

Results and Discussion

Isolated inoculations (Ab-V5 and BRM 63573) promoted root lengths 54.44 and 34.16% longer than the control, which, in turn, did not differ statistically from co-inoculation (Table 1). This is because multifunctional rhizobacteria can promote better root development through different mechanisms of action, such as the synthesis of phytohormones like AIA (indoleacetic acid) and increased nutrient availability (Taiz & Zeiger, 2009). These findings are promising, as plants with longer roots may have greater access to soil water and nutrients, leading to increased absorption capacity, improved photosynthetic capacity, and overall plant development (Dhouib et al., 2019; Caballero-Mellado et al., 2006; Taiz & Zeiger, 2009). This, in turn, can result in more vigorous and productive plants (Bashan et al., 2004; Hungria, 2011).

However, co-inoculation with Ab-V5 and BRM 63573 was the best treatment for the other variables analyzed using WinRHIZO software, including root diameter, total root surface, and root volume. This treatment was the only one that had a significant increase in root diameter compared to the control treatment (55%). For total root surface area, co-inoculation showed increments of 43.17%, and the isolated Ab-V5 showed 28% higher than the control treatment. Regarding root volume, co-inoculation showed a significantly higher value than the isolated treatments, but it did not differ statistically from the control.

Table 1
Total length (LengR), diameter (DiamR), surface area (AreaS), and volume (VolR) of roots of upland rice seedlings (cultivar BRS 501 CL) as a function of the seed inoculation or co-inoculation with *Azospirillum brasilense* and *Bacillus* sp. after 15 days of sowing. Standard deviation in parentheses

Treatment	LengR (cm)	DiamR (mm)	AreaS (cm^2)	VolR (cm^3)
Ab-V5	69.66 a (11.79)	0.1172 ab (0.030)	49.53 ab (7.434)	0.0915 b (0.011)
BRM 63573	80.19 a (12.90)	0.1031 b (0.016)	44.75 bc (7.449)	0.0924 b (0.005)
Ab-V5 + BRM 63573	63.06 bc (11.88)	0.1435 a (0.043)	55.55 a (13.752)	0.1022 a (0.009)
Control	51.92 c (12.70)	0.0927 b (0.029)	38.80 c (9.197)	0.0943 ab (0.011)

* Means followed by the same letter in the column do not differ from each other by the Tukey test (p≤0.05).

Greater initial development of root systems, including length, diameter, surface, and volume, is important since it increases efficiency in water and nutrient absorption, resistance to drought, tolerance to attack by soil pests, germination and/or sprouting capacity, plant size, tolerance to machine movement, among others. These factors can also impact crop yield (Silva et al., 2022b). In a study conducted by J. P. T. Fernandes et al. (2021a), they observed greater root length of seedlings with multifunctional rhizobacteria inoculation in upland rice culture, including an isolate of the genus *Azospirillum* spp. The authors also observed a greater root diameter with the inoculation of isolates Ab-V5 and BRM 63573, which were 13.81% and 13.06%, respectively, higher than the control treatment (without microorganisms). However, the authors did not assess the co-inoculation with the Ab-V5 and BRM 63573 isolates. This is a notable counterpoint to our study, which found co-inoculation with these isolates to be the best treatment for root diameter, total root surface, and root volume.

The isolated use of Ab-V5 and BRM 63573 (inoculation) resulted in greater root length, but their combination (co-inoculation) did not affect this parameter. However, co-inoculation performed better concerning other variables such as root diameter, total root surface, and root volume. The scientific community has increasingly turned its attention toward the use of microorganisms in crops, but there are still several interactions between microorganisms and the environment that need to be studied, understood, and disseminated. It is possible that some environmental factors may have affected root length in the co-inoculation treatment and did not provide higher values for this variable, but did so in other variables related to root development (root diameter, total root surface, and root volume). According to Silveira and Freitas (2007), the interaction between different species of multifunctional rhizobacteria with plants and the environment is not a simple process, and there is no consensus on the subject. Therefore, further studies, including those under field conditions, should be carried out to better understand the interactions between microorganisms, plants, and the environment.

Regarding biomass production, all treatments provided higher values than the control for root and total biomass. However, for shoot biomass, only the isolate BRM 63573 stood out (Table 2). Co-inoculation provided a root biomass production 150% higher than the control, followed by Ab-V5 (120%) and BRM 63573 (70%) alone. In terms of shoot biomass, BRM 63573 was 450% higher than the control. For total biomass, in general, co-inoculation provided an increase of 170%, BRM 63573 of 160%, and Ab-V5 of almost 150%. J. Fernandes et al. (2021b) also recorded a higher total biomass for upland rice co-inoculated with Ab-V5 + BRM 63573. Similarly, Braga et al. (2018) showed that soybean inoculation with *Bacillus subtilis* increased shoot and root dry biomasses. In general, the benefits of using multifunctional rhizobacteria in rice plants include greater root development and increased dry matter, as observed in our study (Biswas et al., 2000; Chi et al., 2005).

Table 2
Root (RDM), shoot (SDM), and total (TDM) dry masses, in grams, of rice seedlings (cultivar BRS 501 CL) as a function of the seed inoculation or co-inoculation with *Azospirillum brasilense* and *Bacillus* sp. after 15 days of sowing. Standard deviation in parentheses

Treatment	RDM	SDM	TDM
Ab-V5	0.022 ab (0.004)	0.015 ab (0.003)	0.037 a (0.005)
BRM 63573	0.017 b (0.007)	0.022 a (0.027)	0.039 a (0.028)
Ab-V5 + 1301	0.025 a (0.025)	0.015 ab (0.002)	0.041 a (0.010)
Control	0.010 c (0.004)	0.004 b (0.002)	0.015 b (0.005)

* Means followed by the same letter in the column do not differ from each other by the Tukey test ($p \leq 0.05$).

A higher proportion of aboveground biomass can improve the efficiency of gas exchange in plants, particularly in capturing light for photosynthesis (H. Poorter et al., 2012; Alvarez et al., 2015). Conversely, greater biomass production in roots can increase water and nutrient uptake, providing better plant support and fixation in the soil (L. Poorter, 2001; H. Poorter et al., 2012). These characteristics can influence plant development and potentially increase crop yields. Therefore, our findings are crucial as the multifunctional rhizobacteria studied were effective in increasing both shoot biomass production (with isolate BRM 63573) and root biomass production (with inoculation of isolates BRM 63573 and Ab-V5, and co-inoculation) compared to the control (without rhizobacteria).

The principal component analysis showed that treatments with rhizobacteria isolated (Ab-V5 and BRM 63573) and combined (Ab-V5 + BRM 63573) had significant variability for the analyzed parameters (length, total surface, diameter, volume root and shoot, root, and total biomass), which were best described by two principal components (PCs). These two components, PC1 and PC2, accounted for 95.9% of the data variation (65.2% and 30.7%, respectively) as shown in Figure 1. The graph reinforces the effect of the multifunctional rhizobacteria evaluated on the initial development of upland rice seedlings, concerning the root system and the biomass production of the plants.

The factor map, or biplot (Figure 1), displays the parameters as arrows that indicate their positive and negative correlations with each principal component (PC). The length of each arrow represents the magnitude of response for that parameter concerning each PC. For instance, PC1 showed no positive correlation with the analyzed parameters, while PC2 displayed positive correlations with root volume and shoot and total dry masses, but negative correlations with the other parameters.

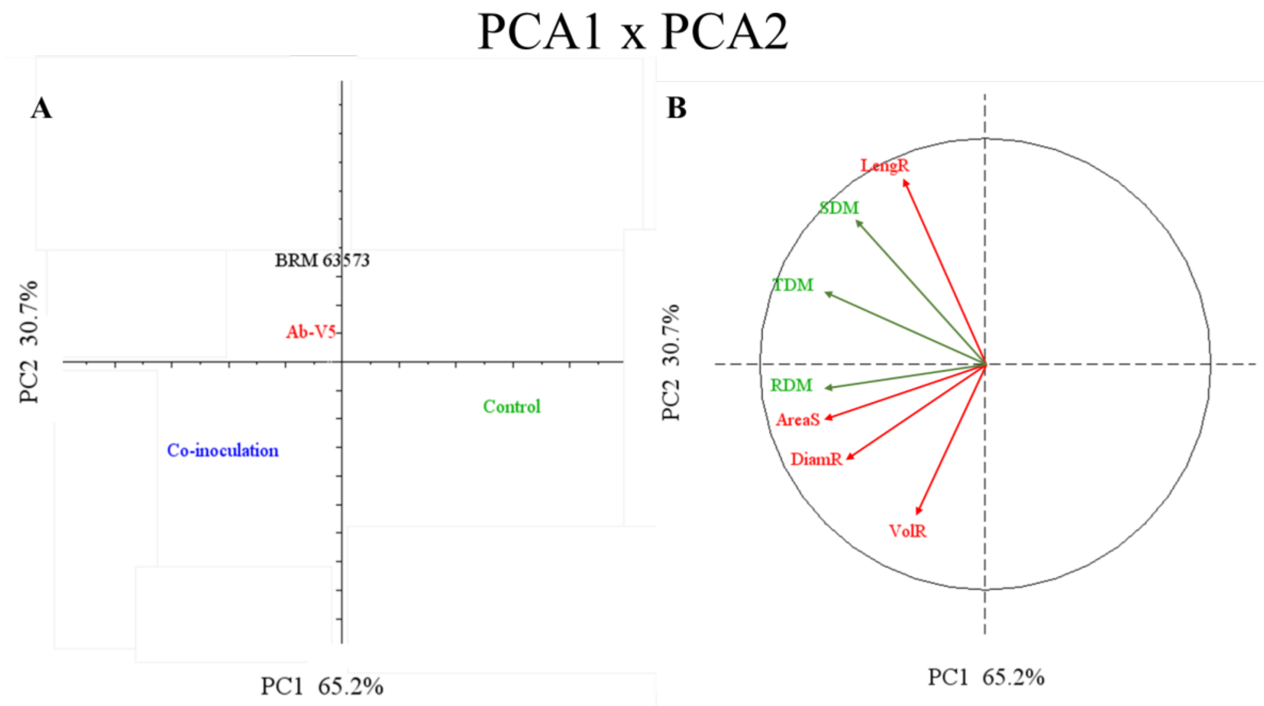

Figure 1. Principal component analysis - PCA (PC1 × PC2) explaining the correlations between the evaluated variables and the treatments with rhizobacteria isolates and the control (without the microorganism). A. Biplot graph of treatments: BRM 63573 (*Bacillus* spp.), Ab-V5 (*Azospirillum brasilense*), Co-inoculation (Ab-V5+ BRM 63573), and Control (without microbiolization). B. Chart of Parameter Correlations.

The quality of the treatments with isolated and combined multifunctional rhizobacteria, as well as the analyzed parameters, showed that the isolates BRM 63573 and Ab-V5 were positively correlated with the variables root length and shoot and total biomass. Co-inoculation (Ab-V5 + BRM 63573) correlated positively with total surface area, diameter, volume and root biomass (Figure 1), while the control (without rhizobacteria) did not correlate positively with any of the analyzed variables. Both inoculation and co-inoculation of rice seeds with multifunctional rhizobacteria isolates (Ab-V5 and BRM 63573) efficiently enhanced the development of seedling roots, leading to the increased lenght, diameter, total surface, and volume, and consequently, biomass production (Figure 1).

Our results highlight that adding two types of bacteria, Ab-V5 and BRM 63573, to rice plants improved their growth and development in the early stages (Figure 2). This is important because better plant growth leads to more efficient absorption of nutrients and water from the soil, increases tolerance to drought and pest and disease attacks, improves plant support and fixation in the soil, and hence increases productivity. J. P. T. Fernandes et al. (2020) also found that rice plants treated with multifunctional microorganisms had greater root development, photosynthetic rates, carboxylation efficiency, number of tillers, shoot and root dry biomasses, and absorption of nutrients, reflecting higher grain yields.

Co-inoculation of rice plants with Ab-V5 + BRM 63573 was clearly more effective than using these isolates separately in terms of root diameter, volume, total surface, and root biomass. The use of BRM 63573 alone had a significant impact on root length and shoot and total biomass, while the use of Ab-V5 alone had a significant impact on root length and root biomass production. Still, further research is needed to better understand the interactions between microorganisms, plants, and the environment.

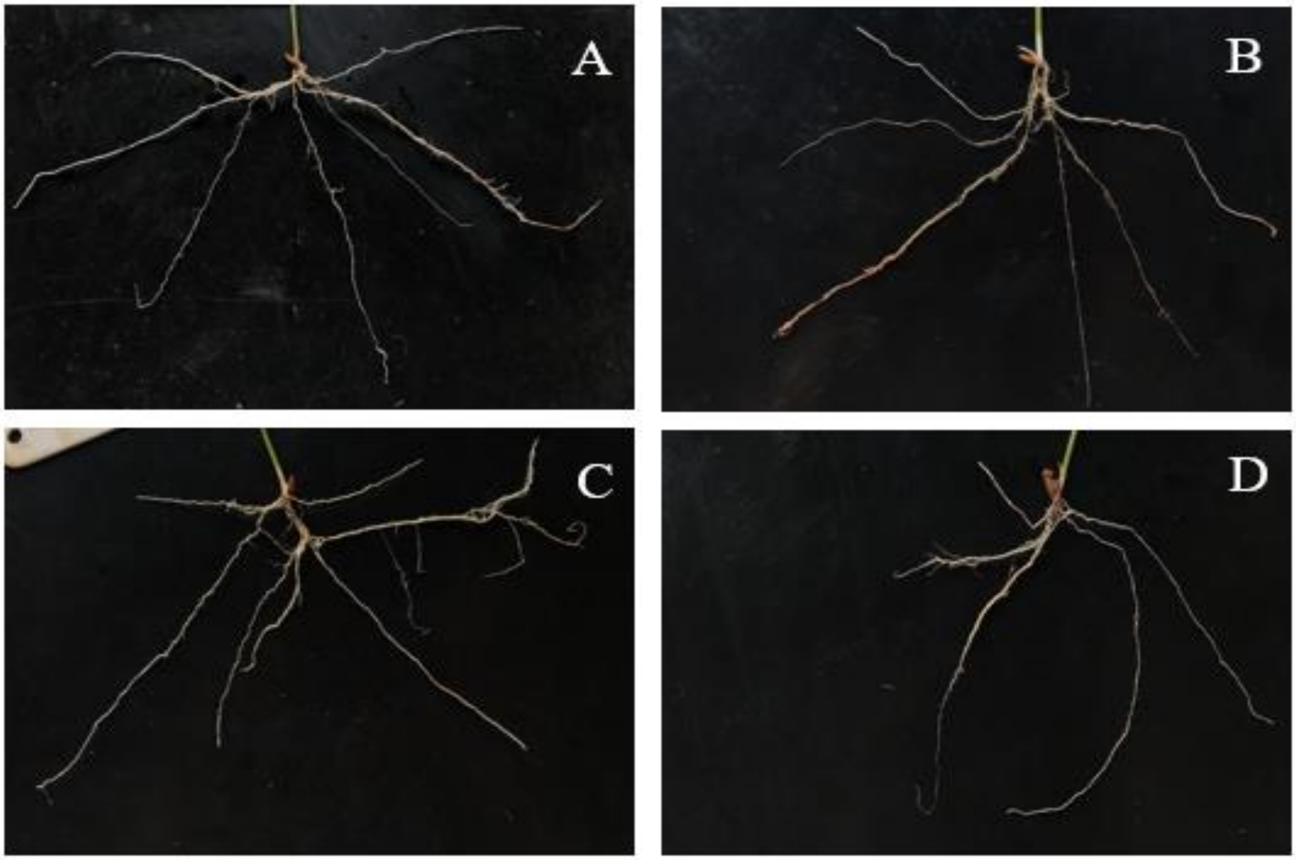

Figure 2. Root system of upland rice seedlings subjected to microbiolization with multifunctional rhizobacteria:
A. BRM 63573 (*Bacillus* spp.), B. Ab-V5 (*Azospirillum brasilense*), and C. Co-inoculation (Ab-V5+ BRM 63573), D. Control (no microbiolization). Rice seedlings were photographed 15 days after sowing with a digital camera. The images were analyzed using the WinRHIZO 2012 software.

The results obtained in this study were relevant and showed that using multifunctional rhizobacteria, either isolated or combined, provides greater initial development of rice plants. This can significantly influence the increase in the productivity of rice grains and other crops, in addition to being a common practice in current agriculture (Figueiredo et al., 2010). This is due to the search for decreased dependence on synthetic inputs, reduced environmental contamination, and lower production costs, all aimed at sustainable agriculture (Moreira & Siqueira, 2006).

Conclusions

The multifunctional rhizobacteria Ab-V5 and BRM 63573, whether inoculated alone or co-inoculated, positively influenced the initial development of upland rice seedlings. Isolate BRM 63573 significantly affected root length and shoot and total biomass, while isolate Ab-V5 significantly affected root length and production of root and total biomass. Co-inoculation provided significant effects on diameter, volume, total surface, root biomass, and total biomass. The control treatment had the worst results for most of the analyzed variables.

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