ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Stimulatory effects of defective and effective 3-indoleacetic acid-producing bacterial strains on rice in an advanced stage of its vegetative cycle

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Abstract

The production of 3-indoleacetic acid (IAA) by plant growth-promoting bacteria (PGPR) stimulates root development and plant growth. In addition, morphological changes such as an increased root ramification and root hair production improves nutrient absorption and biomass accumulation. The objective of this work was to evaluate the effect of IAA-producing strains on rice in an advanced stage of its vegetative cycle. Rice was inoculated with *Gluconacetobacter diazotrophicus* PAL 5 and its lao- mutant, deficient in auxin production, *Azospirillum baldaniorum* Sp 245, and *Escherichia coli* DH10b. Both the mutant and wild-type *G. diazotrophicus* stimulated root elongation, area, volume, and diameter. However, the lao- mutant strain was the only one capable of increasing the number of roots. In turn, inoculation with *A. baldaniorum* had no significant effect on plant development. The inoculation with *E. coli* led to changes in root volume, area, and diameter, and a response that may be related to the stress caused by its presence. We conclude that the inoculation with *G. diazotrophicus* stimulates the root system's growth independently of their IAA production ability, suggesting that a metabolite other than IAA is responsible for this effect at advanced stages of the rice's vegetative cycle.

Keywords *Oryza sativa*, *G. diazotrophicus* PAL 5, *Gluconacetobacter diazotrophicus* lao-, *Auxin*

Introduction

Plant roots release various organic substances and molecular signals that attract microbial populations, especially those capable of metabolizing root exudates and proliferating in the rhizosphere [1]. Root metabolism is closely associated with these microorganisms [2] and, among them, there are several plant growth-promoting rhizobacteria (PGPR) that benefit plant growth and development.

One of the most commonly used mechanisms to explain the beneficial effects of PGPR on plants is the production of phytohormones, such as auxin [3]. This hormone controls physiological processes in plants, such as cell growth and division, tissue differentiation, and responses to light and gravity [4]. Indole-3-acetic acid (IAA) is the most prevalent and essential auxin for plants [3]. Inoculation studies support the hypothesis that the synthesis of IAA by PGPRs increases plant rooting. The consequent root growth allows plants to explore the soil better, thus benefiting the absorption of water and nutrients. Furthermore, IAA may significantly

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increase root exudation, which increases root colonization by PGPR, improving the inoculation effect [3, 4].

Among these microorganisms are *Gluconacetobacter diazotrophicus* [5] and *Azospirillum* sp. [6], which are nitrogen-fixing and phytohormone-producing bacteria [7]. Although initially isolated from sugarcane plants grown in Brazil [8], *G. diazotrophicus* colonizes other species endophytically, such as rice [9]. In fact, rice has been used as a plant model to study interactions with *G diazotrophicus* since it is easy to manipulate and cultivate in the laboratory [10–12]. Furthermore, rice inoculation experiments with variety IAC4440 and the low IAA-producing *G. diazotrophicus* lao- strain demonstrated the importance of auxin in altering root architecture. Under these conditions, root length has been stimulated by the wild-type strain if compared to lao-inoculated plants [13].

Nevertheless, these evaluations were made within an initial period of 3 and 7 days [13]. Similarly, the inoculation of *Azospirillum baldaniorum* in *Arabidopsis* increased the number of root hairs and lateral roots compared to the inoculation of a mutant strain deficient in auxin biosynthesis. This effect has been evaluated only seven days after inoculation [14]. It is possible to infer that the evaluation time is a restrictive factor to observe the inoculation's actual effectiveness since the PGPR effect may not be expressed throughout the plant cycle. In fact, a study has shown that the population of *G. diazotrophicus* has decreased throughout the commercial cycle of different sugarcane genotypes [15]. Possibly, the manifestation of growth promotion by *G. diazotrophicus* was more accentuated in the initial stages.

Through these observations, the hypothesis of our work is that bacterial auxin may have an expressive effect beyond the initial stages of interaction between the plant and bacteria. To test this hypothesis, we evaluated whether IAA-producing bacteria affects root morphology in a more advanced stage of rice's vegetative cycle. *Azospirillum baldaniorum* Sp 245 [14], *G. diazotrophicus* PAL 5, and its lao- IAA-deficient production mutant strain [13] were used as model organisms, allowing us to access the effect of this hormone on root development.

Material and methods

Strains of bacteria and cultivation conditions

The bacterial strains used in the experiment were *Gluconacetobacter diazotrophicus* PAL 5 (= BR11281), which is the type strain of its species [16]; *Gluconacetobacter diazotrophicus* lao-, which is derived from PAL 5 and deficient in the production of IAA due to a mutation in the L-amino acid oxidase (lao) gene [13]; *Azospirillum baldaniorum* Sp 245 (= BR11005), formerly classified as *A.*

brasilense [17], isolated from wheat, and an IAA producer [18, 19]; and *Escherichia coli* DH10b, a strain used for cloning in molecular biology assays [20]. *E. coli* was added as a negative control because it is not a classic PGPR. *G. diazotrophicus* strain PAL 5 and lao-inocula were prepared in liquid DYGS medium at 30 ºC for 48 h [21], with a final concentration greater than 10^8 CFU.mL^{-1}. Additionally, the medium used for lao- received kanamycin at a concentration of 200 μg mL^{-1}. *E. coli* DH10b was grown in LB medium [22], at 37 ºC for 24 h, and the *A. baldaniorum* Sp 245 inoculum was prepared in DYGS medium for 48 h at 28ºC.

Rice experiment in nutrient solution

The experiment was carried out in a completely randomized design, with five treatments and six replicates. The treatments consisted of inoculation with *G. diazotrophicus* PAL 5, *G. diazotrophicus* lao-, *A. baldaniorum* Sp 245, *E. coli* DH10b, and a control without inoculation.

The Piauí variety, which has been used as a model for plant nutrition [23–27] and microbiology studies [28], was chosen for experimentation. The seeds were initially disinfected in 2% sodium hypochlorite, for 30 min, under orbital agitation. Then, the seeds were washed ten times in distilled water and allowed to germinate on wet gauze. Ten days after germination (DAG), the seedlings were transferred to 700-mL pots filled with Hoagland's solution [29] modified at ½ ionic strength. Each pot received four seedlings. Three days later, the seedlings were inoculated with 5 mL of each strain according to their respective treatments. The inocula were applied to the roots any time the nutrient solution was renewed. The plants were cultivated in a growth chamber under a 12 h/12 h (light/dark) photoperiod at 25 °C and sampled 21 DAG.

The following variables were measured: shoot and root's fresh and dry weights, root length (mm), surface area (mm^2), average diameter (mm), and volume (mm^3), and the number of root tips. The root variables were measured with the software WinRHIZO Arabidopsis coupled to an image scanner Epson Expression 11000XL LA2400, using the method described by [30, 31]. The rice roots were evenly distributed under a layer of water in a transparent tray (20 cm wide and 30 cm long). Subsequently, the images were digitized, and the mentioned variables were calculated.

Data analysis

Data normality and homoscedasticity of the variances were verified by the Shapiro–Wilk and Bartlett tests, respectively. Outliers were identified based on the boxplot analysis. One outlier was identified per treatment and removed. Thus, five replicates were used for subsequent

statistical analyses. Each of the measured variables were subjected to an analysis of variance (ANOVA) with the F test ($p < 0.05$), and the means were compared to the control by the t-test ($p < 0.05$). The data was also submitted to principal component analysis (PCA) on the correlation matrix. All analyzes were performed using the R software [32].

Results

The plants inoculated with either *G. diazotrophicus* lao- or PAL 5 showed significantly greater root length (Fig. 1A), surface area (Fig. 1B), volume (Fig. 1C), and average diameter (Fig. 1E) compared to the uninoculated plants (Fig. 2). In addition, lao- also stimulated an increase in the number of root tips (Fig. 1d), pointing out to the existence of an

Fig. 1 Length (**a**), surface area (**b**), volume (**c**), number of tips (**d**), and average diameter (AvD) (**e**) of roots of rice plants inoculated with *A. baldaniorum*, *E. coli* DH10b, *G. diazotrophicus* lao- (mutant deficient in the production of IAA) and *G. diazotrophicus* PAL 5 (IAA-producing wild strain). "Control" stands for the uninoculated treatment. *T*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$;*****$p < 0.0001$) for the means of effects between the control and different treatments, ns = not significant
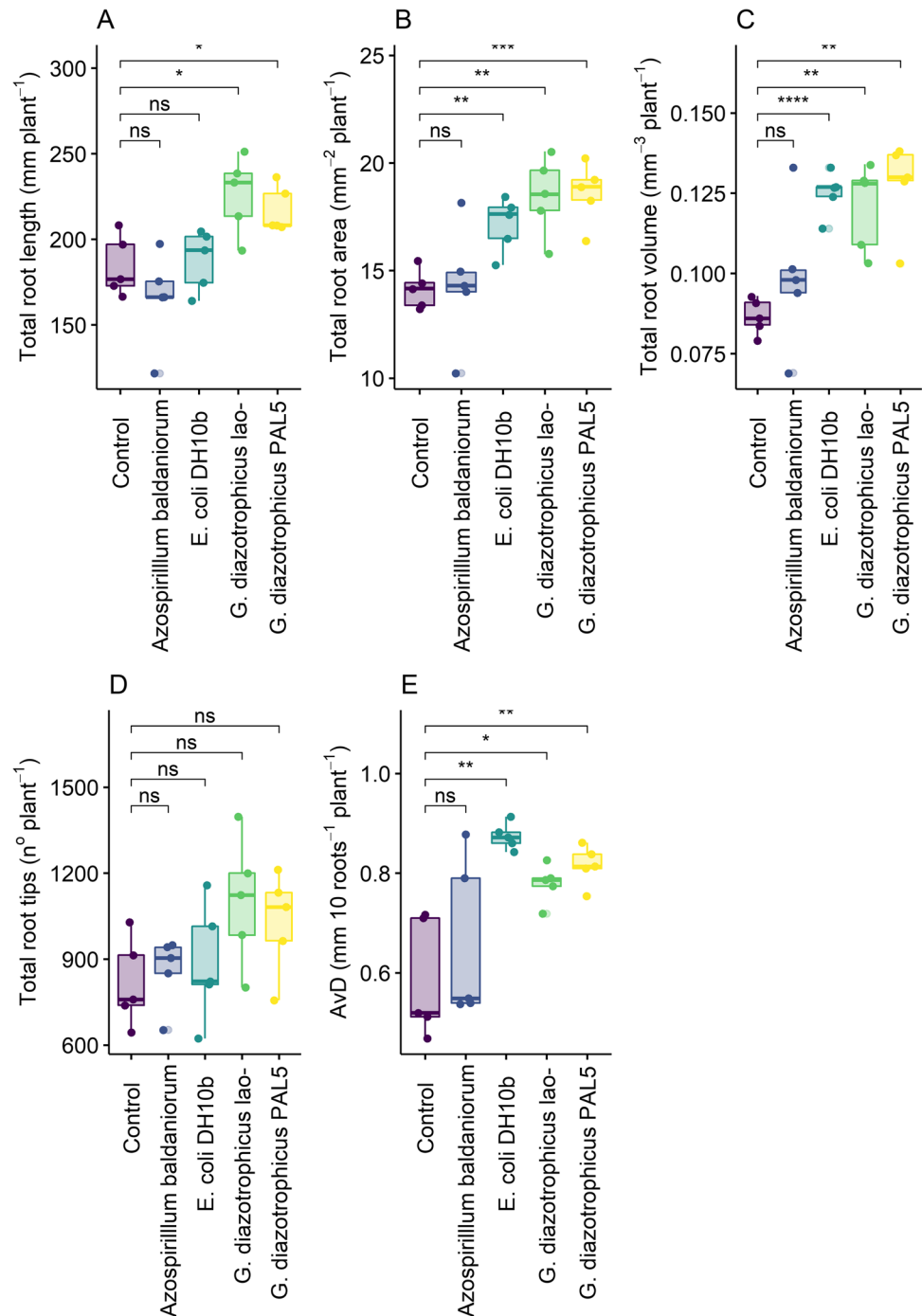
Fig. 2 Shoot (**a**) and root (**b**) fresh weight, shoot (**c**) and root (**d**) dry weight, and root/shoot ratio (**e**) of rice plants inoculated *A. baldaniorum*, *E. coli* DH10b, *G. diazotrophicus* lao- (mutant deficient in the production of IAA) and *G. diazotrophicus* PAL 5 (IAA-producing wild strain). "Control" stands for the uninoculated treatment. *T*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$;****$p < 0.0001$) for the means of effects between the control and different treatments, ns = not significant
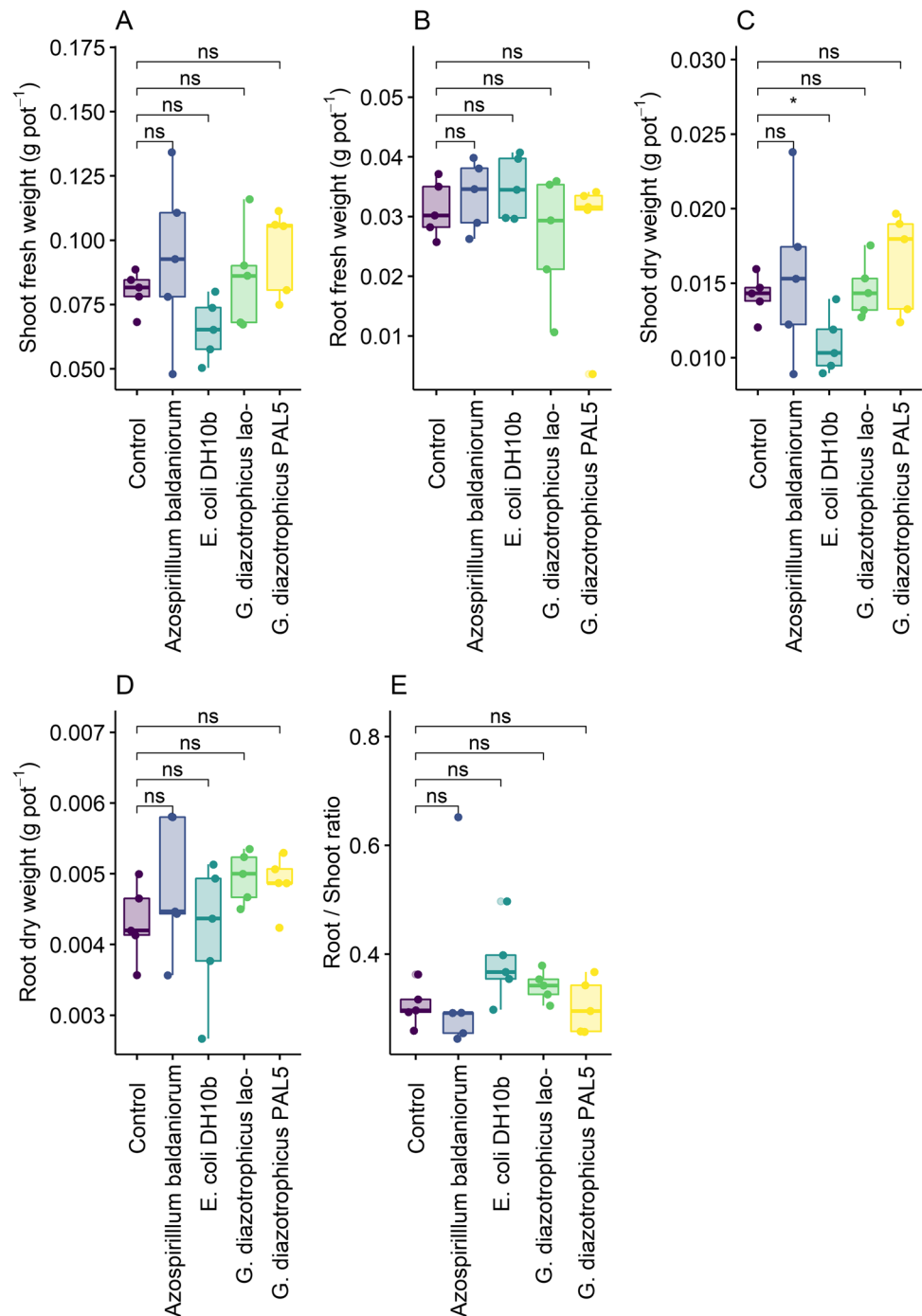
IAA-independent mechanism stimulating root growth. The *E. coli* DH10b inoculation stimulated an increase in root diameter, volume, and surface area (Fig. 1E, C, B), indicating that rice plants can respond to the stimulation of a non-PGPR species. Finally, none of the root morphological variables were affected by *A. baldaniorum* Sp 245 inoculation (Figs. 1 and 2). No differences were observed among all treatments for root and shoot biomasses and root:shoot ratio (Fig. 2A–E).

The principal component analysis (PCA) explained 68.1% of the total data variability in the first two components (Fig. 3). The first component explained 40.6% of the variation and represents a combination of root growth variables (root length, volume, diameter, and area, number of tips, and root dry weight), which had the highest weight in this component. The control was negatively correlated to these variables while the inoculations with *G. diazotrophicus* PAL 5 and lao- were positively correlated (Fig. 3). The second

Fig. 3 Principal component analysis carried out on rice growth variables. Shoot (FWs) and root (FWr) fresh weight; shoot (DWs) and root (DWr) dry weight; number of roots (tips); root length (length); root surface area (SA); root volume (volume); average root diameter (AD); root/shoot ratio (RS). The circles with a larger diameter represent the centroids for each of the treatments
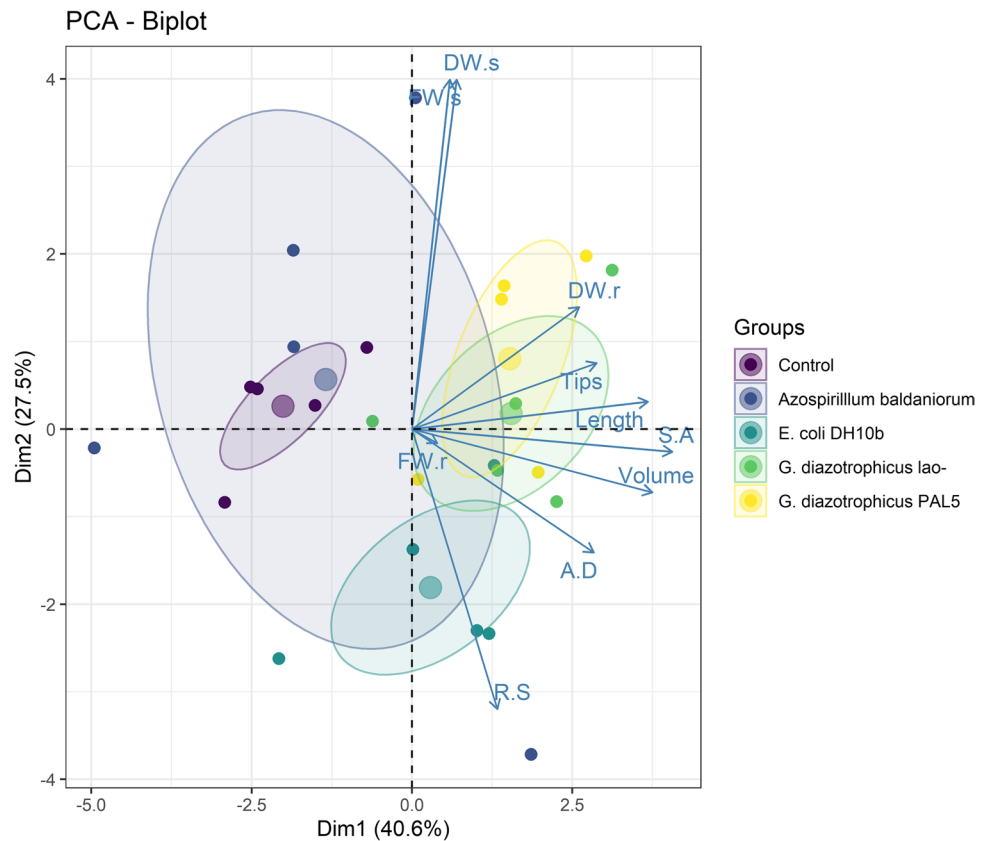
component explained 27.5% of the variation and represented a contrast between the shoot biomass and root: shoot ratio. *E. coli* DH10b inoculation was negatively correlated with shoot wet and dry weights, but positively correlated with the root:shoot ratio (RS), showing that the plants invested proportionally in root than in shoot growth. Finally, the plant response to *A. baldaniorum* inoculation was highly variable, as indicated by the high dispersion of the points in the PCA. In general, this treatment showed lower root growth than the inoculation of the other two PGPR.

Discussion

Microorganisms have marked effects on plant establishment in the environment, influencing root growth and development and improving nutrient uptake [33]. These effects are relevant at the beginning of a plant's growth cycle since they can give plants an advantage to establish in the field. Nevertheless, plants can also benefit from microbial stimulation throughout their growth cycle, which may be relevant for their metabolism, resistance to biotic and abiotic stresses, and improved crop productivity. In the present work, we evaluated the effects of some of these microorganisms during advanced stages of rice's vegetative growth. We also

took advantage of an IAA production-deficient *G. diazotrophicus* strain to evaluate the role of this hormone.

PGPR, such as *Gluconacetobacter* and *Azospirillum*, are associated with plants and positively impact their growth [34]. This stimulatory effect may be related to IAA production, and it has been demonstrated for the first days after root inoculation [13, 14]. Nevertheless, in the present work, both *G. diazotrophicus* wild-type and mutant strains similarly stimulated rice root growth, indicating that auxins' production was not the primary factor promoting root growth at the advanced stage of rice's vegetative cycle. In fact, lao- was the only strain positively affecting the number of lateral rice roots ($p < 0.1$) compared to the uninoculated treatment (Fig. 1D). This result contrasts with data from Rodrigues et al. [13], who observed a positive effect of *G. diazotrophicus* PAL 5 three and seven days after inoculation and related this result to auxin production. This finding suggests that the auxin produced by the wild-type strain has an initial effect if we think mainly of this hormone's function as a signaling molecule among plant bacteria [35]. However, it may no longer be essential for root development at an advanced growth stage.

The genus *Azospirillum* has been reported to stimulate the growth of different crops, and for this reason, it has been used in agricultural inoculants [36, 37]. Despite *Azospirillum*'s ability to fix nitrogen, this process has

not been considered as its primary plant-growth promotion mechanism since it does not provide significant N amounts for the crops. Therefore, their phytostimulatory effect has been considered the main factor responsible for plant growth promotion [36]. Species of this genus colonize cereals, including rice [38–40]. Unexpectedly, no changes in root morphology were observed upon *A. baldaniorum* Sp 245 inoculation. A possible explanation may be that the Piauí variety is unresponsive to inoculation with the tested strain. The genetic differences between cultivars are relevant factors and determine, at least in part, the plant response to inoculation [41]. Another possibility may be that environmental conditions affected the plant-bacterial interaction. In vitro tests showed that the concentration of indoles produced by *Azospirillum* changes in the presence of specific N sources [42]. The presence of these sources also decreased cell multiplication. Therefore, we cannot rule out that N might have affected the stability of the Sp 245 strain. Besides, if we assume that the auxin production is involved in the plant-bacterium interaction [4], it is plausible to suggest that variations on its levels as influenced by the environment affect the plant-bacterium communication and, consequently, bacterial colonization.

E. coli has been used here as a neutral control since it has not been recognized as PGPR. *E. coli* is generally studied as a human pathogen [43], but the *E. coli* DH10b strain is non-pathogenic and commonly used in molecular biology procedures [20]. Even so, some lineages have been reported as adapted to the soil and able to colonize plant roots [44]. Unexpectedly, the inoculation of *E. coli* DH10b affected the root diameter, leading to a greater volume and area (Fig. 2). The phytostimulatory effect of clinical *E. coli* has been reported primarily by Walker and collaborators [45]. These authors observed that *E. coli* K-12 increased the maize's root system, and that this increase was higher than the increase observed upon *Azospirillum* inoculation. Although inoculation with *E. coli* DH10b altered the rice's root system, plant growth was limited (Fig. 1A, C). *E. coli* DH10b-inoculated plants had lower shoot biomass and higher root/shoot ratio, indicating root thickening at the expense of shoot growth (Fig. 3). We can assume that this strain potentially stressed the root cells, which can compromise the transport of water, nutrients, and, consequently, shoot growth. However, further studies are needed to confirm this hypothesis since the statistical analysis have not detect a significant inoculation effect on shoot growth. These results show that plants can respond to the inoculation of microorganisms that are not PGPR. This effect may have been enhanced by the axenic conditions and the high bacterial cell concentration. It remains to be seen whether the same effects would be observed under natural soil conditions.

Conclusions

The inoculation with *G. diazotrophicus* stimulates the root system independently of their 3-indoleacetic acid production ability, suggesting that a metabolite other than 3-indoleacetic acid is responsible for this effect at advanced stages of the rice's vegetative cycle. *A. baldaniorum* Sp 245 was not effective with rice cultivar Piauí, suggesting a possible genotypic effect. *E. coli* DH10b inoculation promoted root thickening, indicating a possible stress and compromising shoot biomass accumulation.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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