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Application rates of *Azospirillum brasilense* in cucumber seeds

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Abstract

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The use of high-quality seedlings is a key factor for the success of the vegetable production system, and the use of plant growth-promoting bacteria (PGPB) can be decisive for the production of quality cucumber seedlings. Seeds from two cucumber cultivars (Aodai Melhorado and Marketmore76) were used to investigate the effectiveness of applying inoculant rates containing *Azospirillum brasilense* to improve germination rate and initial seedling growth. Seeds previously inoculated with inoculant containing the strains AbV5 and AbV6 of *A. brasilense* [0 (control), 4, 8, and 16 mL kg^{-1} of seed] were placed to germinate in a germination chamber at 25°C for 8 days. The experimental design used was completely randomized in a 2 × 4 factorial scheme, with four replications of 50 seeds. The results reported that the inoculant application containing the strains AbV5 and AbV6 of *A. brasilense* reduced germination percentage and germination rate index, whereas the inoculation of *A. brasilense* resulted in an increase in the mean germination time, especially for the Marketmore76 cultivar. The inoculation of seeds with *A. brasilense* has little effect on the shoot and root growth of cucumber seedlings. The negative and positive effects of the application of *A. brasilense* on the growth of cucumber seedlings are dependent on the genotype used. These results indicate that further studies should be conducted to investigate other application methods and use of other strains of *A. brasilense* to prove the beneficial effects of using these PGPB in improving the quality of cucumber seedlings.

Keywords: Cucumis sativus L.; plant growth-promoting bacteria; seed inoculation

Introduction

In Brazil, cucumber (*Cucumis sativus* L. – Cucurbitaceae) is one of the main vegetables grown primarily in the southeastern states. The major cucumber producing states in the Brazil are São Paulo, Minas Gerais, Rio de Janeiro, and Goiás. Total area under production in the Brazil in 2016 was 10,000 ha, with a total production of 308.0 tons (IBGE, 2016). Cucumber crop has been considered an excellent alternative for cultivation by small growers in Brazil, especially due to its high profitability and ease of commercialization. However, the quality of seeds or seedlings used is a key factor for the success of the Brazilian cucumber production system.

An alternative management technique that can be used to improve seed germination and seedling growth rate and, consequently, the quality and vigor of cucumber seedlings would be the use of plant growth promoting bacteria (PGPB). The PGPB may improve plant growth through a broad range of processes, i.e., biological nitrogen fixation, plant hormone production, siderophore production, antioxidant and antifungal activity, phosphate solubilization, systemic resistance induction and plant-microbe symbiosis promotion (Bashan et al., 2014; Fukami et al., 2018; Helaly et al., 2020).

Bacteria of the *Azospirillum* genus are, certainly, the most employed and studied PGPB in Brazil and worldwide (Marks et al., 2013; Hungria et al., 2015; Bulegon et al., 2017; Silva et al., 2019). *Azospirillum brasilense* is a gram-negative endophytic bacterium, capable of fixing N and colonizing all plant parts, especially the roots (Bashan & Holguin, 1997). In addition to atmospheric N_2 fixation capacity, this bacterium contributes to greater plant growth and development through various mechanisms, such as the synthesis of growth-promoting substances, especially auxin, gibberellin and cytokinin, increased activity of the enzyme nitrate reductase, induction of plant resistance to abiotic and biotic stresses, siderophore synthesis and phosphate solubilization (Fukami et al., 2018; Domenico, 2019).

Some studies have proven the effectiveness of using *A. brasilense* to improve the growth and production of some vegetable crops (Lima et al., 2018; Domenico, 2019; Helaly et al., 2020). However, the effect of *A. brasilense* inoculation is dependent on the vegetable species, inoculation method, bacteria strain, inoculum concentration and plant's growing environment (Mangmang et al., 2015).

Lima et al. (2018) reported that the inoculation of *A. brasilense* improved the growth rate, chlorophyll content and fruit production of the tomato plants (*Solanum lycopersiconum* L.). Besides, these authors reported that the use of 5.0 mL kg^{-1} of inoculant containing *A. brasilense* resulted in the highest production of tomato fruits. Similarly, Andrade-Sifuentes et al. (2020) showed that the inoculation of *A. brasilense* resulted in greater seed germination, plant growth and fruit production of the tomato plants. Mangmang et al. (2015) showed that the seed soaking in inoculant containing *A. brasilense* (Sp7-S and Sp245 strains) strongly enhanced root and shoot growth, germination rate and vigor of tomato seedlings. However, these authors reported that the use of *A. brasilense* had little effect on the germination rate and growth of lettuce (*Lactuca sativa* L.) and cucumber seedlings. On the other hand, Pereyra et al. (2010) also showed that inoculation of *A. brasilense* resulted in a greater length of the shoots and roots of cucumber plants. These contrary results show that other studies should be carried out, not only to investigate the effects of the inoculation of *A. brasilense* in Brazilian cucumber genotypes but also to establish what is the optimal inoculant rate to be applied.

This study was conducted to investigate the effectiveness of seed inoculation with *Azospirillum brasilense* and to establish the optimal rate of inoculant application on germination rate and initial seedling growth of two cucumber (*Cucumis sativus* L.) genotypes.

Material and Methods

Plant material and treatments

Seeds of two commercial cucumber cultivars (Aodai Melhorado and Marketmore76) were purchased from the local seed market in the municipality of Cassilândia, MS, Brazil. These two cucumber cultivars were chosen because they are widely grown by growers in the central-west region of Brazil. Seeds were previously disinfected by immersion in a sodium hypochlorite solution containing 2% (v/v) of active chlorine, for 5 minutes. Seeds were then washed three times with distilled water, and used in this study.

Treatments were arranged in a completely randomized design in a 2 × 4 factorial scheme: two cucumber cultivars (Aodai Melhorado and Marketmore76) and four inoculant application rates containing strains AbV5 and AbV6 of *A. brasilense* [0 (uninoculated seeds), 4, 8 and 16 mL kg^{-1} of seeds]. The seed inoculation with *A. brasilense* was performed with the commercial liquid inoculant AzoTotal® (Total Bio: Biosolutions for agriculture, Vinhedo, SP, Brazil), which contains the strains AbV5 and AbV6 [minimum concentration of 2.0×10^8 colony-forming units (CFU) per mL].

Germination and growth conditions

Four replicates of 30 seeds per treatment were evenly distributed in plastic boxes (11.0 × 11.0 × 3.5 cm) of the type Gerbox® containing one sheet of blotter paper, properly moistened with distilled water, in a volume equivalent to three times the weight of dry paper. The boxes were then closed with lids to prevent evaporation and maintain the relative humidity close to 100%. Germination was carried out in a germination chamber under 12/12 h photoperiod (light/darkness), photosynthetic photon flux density (PPFD) of 120 μmol m^{-2} s^{-1} and temperature of 25°C for 8 days. Seeds were considered germinated when radicle were longer than 10.0 mm. Germinated seeds were recorded every 24 h for 8 days.

Measurements of germination and seedling growth

The number of germinated seeds was recorded daily, and the final germination rate was determined after 8 days. The germination rate index (GRI) was calculated using Maguire's equation (Maguire 1962): GRI = Σ (n_i / t_i), where n_i is the number of germinated seeds on a given day, and t_i is the time in days from the starting/sowing day (0). The mean germination time (MGT) was calculated using the equation of Labouriau (1983): MGT = ($\Sigma n_i t_i$) / Σn_i, where n_i is the number of germinated seeds on a given day, and t_i is the time in days from the starting/sowing day (0).

The hypocotyl length (HL) and radicle length (RL) was measured in ten normal seedlings randomly obtained after count of the total germination (8^{th} day) using meter scale. Root volume (RV) was determined by water displacement using a calibrated cylinder of 10 mL. The dry matter partitioning into shoots and roots was determined at the end of the germination test (8 days). The shoots and roots were separated, dried in oven at 65ºC for three days, and then weighed. To determine root: shoot ratio (RSR), root dry matter obtained was divided by the shoot dry matter.

The data of germination percentage, seedling length and dry matter accumulation were used to calculate the seedling vigor indexes. Seedling length (SLVI) and weight vigor index (SWVI) in each treatment was calculated using following equations, as suggested by Abdul-Baki & Anderson (1973): SLVI = [total seedling length (cm) × seed germination (%)] and SWVI = [total seedling dry weight (mg) × seed germination (%)].

Statistical analysis

The data were previously submitted to the tests of verification of the statistical hypotheses of homoscedasticity of variances (Levene test; $p > 0.05$) and normality of residues (Shapiro-Wilk test; $p > 0.05$). Then data were submitted to analysis of variance (ANOVA), and means of cucumber genotypes and *A. brasilense* inoculation were compared by Fisher's Least Significant Difference (LSD) test at the 0.05 level of confidence. The analyses were performed using the Sisvar® software, version 5.6 for Windows (Statistical Analysis Software, UFLA, Lavras, MG, BRA).

Results and Discussion

The results of the analysis of variance reported that the effect of cucumber cultivars was significant on all seed germination and plant growth traits, while the effect of *A. brasilense* inoculation rates was significant for most of the measured traits, except for dry matter of shoots, roots, total and root: shoot ratio. Interaction between cucumber cultivars and *A. brasilense* application showed significant effect on all germination and plant growth traits (Table 1). The significant interaction between the effects of genotypes and *A. brasilense* application indicates that cucumber cultivars have a distinct response to the inoculant application rates containing strains AbV5 and AbV6 of the *A. brasilense*.

The germination rate of uninoculated cucumber seeds (control) was higher than the standard value used as reference for the commercialization of cucumber seeds in the Brazil (i.e., 80%) (Figure 1A). This indicates that seeds used in this study were of high physiological quality.

The germination capacity of cucumber seeds was negatively affected by the application of *A. brasilense*, especially for the cultivar Marketmore 76 (Figure 1A). The germination rate of uninoculated seeds was 93% and 98%, respectively for

Table 1. Summary of the analysis of variance for the measurements of germination, growth, dry matter partitioning, and vigor indices of cucumber seedlings as affected by rates of inoculant containing *Azospirillum brasilense*

Causes of variation	Probability > F											
	G[1]	GRI	MGT	HL	RL	RV	SDM	RDM	TDM	RSR	SLVI	SWVI
Block	0.505	0.434	0.095	0.008	0.628	<0.000	0.425	0.007	0.876	0.002	0.268	0.631
Cultivars (C)	0.001	0.003	0.004	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	0.041	<0.000
Inoculant (I)	<0.000	<0.000	<0.000	0.042	0.044	0.005	0.629	0.490	0.512	0.712	<0.000	<0.000
C × I	0.003	<0.000	0.001	<0.000	<0.000	<0.000	0.046	0.008	0.047	0.050	0.023	<0.000
CV (%)	10.01	11.94	7.83	6.73	8.69	12.95	7.52	9.48	6.83	10.18	13.39	13.20

[1]G: germination. GRI: germination rate index. MGT: mean germination time. HL: hypocotyl length. RL: radicle length. RV: root volume. SDM: shoot dry matter. RDM: root dry matter. TDM: total dry matter. RSR: root: shoot ratio. SLVI: seedling length vigor index. SWVI: seedling weight vigor index.

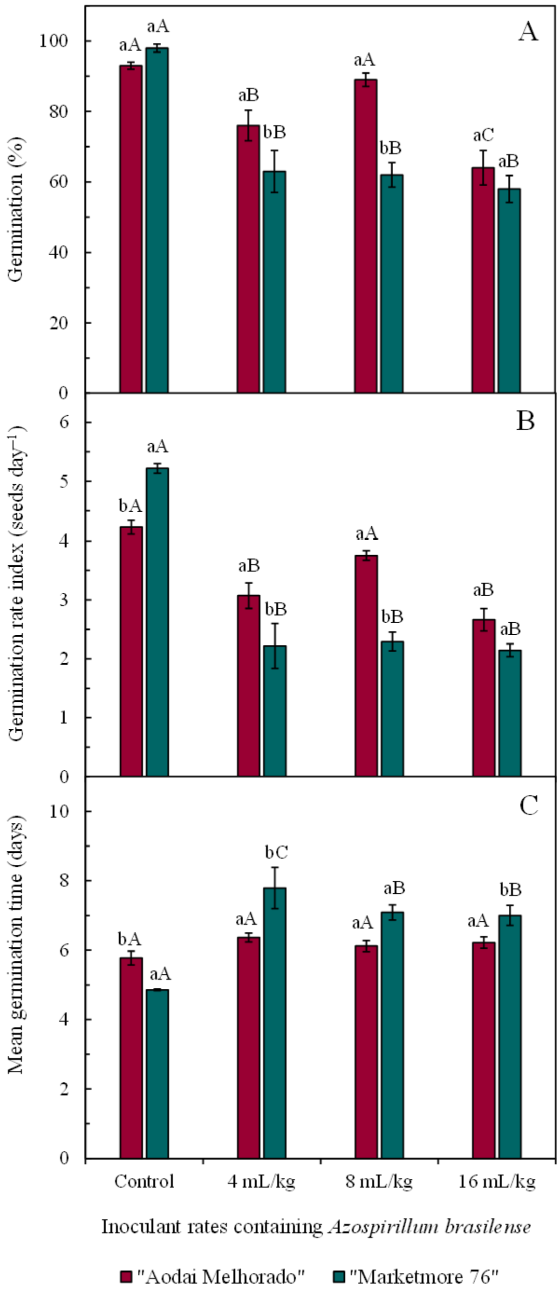

Fig. 1. Effects of inoculant rates containing *Azospirillum brasilense* on germination (A), germination rate index (B), and mean germination time (C) of two cucumber cultivars (*Cucumis sativus* L.). Bars followed by the same lower-case letters, between the cucumber cultivars or same upper-case letters, for the rates of inoculant are not significantly different by LSD test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± mean standard error

Aodai Melhorado and Marketmore76 cultivars, whereas when the seeds were inoculated with *A. brasilense* the germination rate ranged from 64% to 89% and from 58% to 63%, respectively, for Aodai Melhorado and Marketmore76 cultivars.

The germination rate index (GRI) of uninoculated seeds was 4.2 and 5.2 seed day^{-1}, respectively for Aodai Melhorado and Marketmore76 cultivars, whereas for seeds inoculated with *A. brasilense* the GRI ranged from 2.7 to 3.7 seed day^{-1} and from 2.1 to 2.3 seed day^{-1}, respectively for Aodai Melhorado and Marketmore76 cultivars (Figure 1B).

The mean germination time (MGT) of the seeds of cultivar Marketmore76 was delayed with the inoculation of *A. brasilense*, whereas the application of *A. brasilense* had no significant effect ($p > 0.05$) on the MGT of the seeds of Aodai Melhorado (Figure 1C). The inoculation of *A. brasilense* resulted in an average delay of 2.2 days (4.8 to 7.2 days) when compared to uninoculated seeds. The MGT is a measure of the rate and time-spread of germination (Bewley et al., 2013). A delay in the mean time to germination may be disadvantageous for successful establishment, since the delayed germination leaving the seeds more vulnerable to attack from predators (pests and pathogens) and unfavorable environmental conditions (temperature, substrate moisture, among others) and, therefore, compromise the establishment of a uniform stand.

In general, these results indicate that when cucumber seeds were inoculated with *A. brasilense*, the germination percentage and germination rate index were drastically inhibited, which led to a delay in the seed germination process, especially in the cultivar Marketmore 76 (Figure 1). Pinto et al. (2017) also showed that inoculation with *A. brasilense* reduced the germination rate of wheat seeds when compared to uninoculated seeds. However, most studies that have investigated the effect of *Azospirillum* inoculation on seed germination have reported that there are no negative impacts of the use of this bacterium on the seed germination process, as reported in tomatoes (Lima et al., 2018; Romagna et al., 2019), lettuce (Romagna et al., 2019) and wheat (Rampim et al., 2012). The lower germination percentage of seeds inoculated with *A. brasilense* may have occurred due to the endogenous content of GA_3 in cucumber seeds plus the exogenous input of this plant hormone released by these bacteria to have caused an excessive concentration of GA_3 in the seeds, thus resulting, in the inhibition and delay of the seed germination process (Taiz et al., 2017). The *A. brasilense* has the potential to promote plant growth and development through the synthesis of plant hormones, mainly auxins (AX), gibberellins (GA) and cytokinins (CK) (Fukami et al., 2018).

The inoculation of *A. brasilense* resulted in a beneficial effect on some of the growth traits of cucumber seedlings, especially for cultivar Marketmore76 (Figure 2). The hypocotyl and radicle length of cultivar Aodai Melhorado was significantly greater for seedlings from uninoculated seeds and with the use of 16 mL kg^{-1} of inoculant containing *A. brasilense* (Figure 2A and 2B). For seedlings of cultivar Marketmore76, the hypocotyl length was significantly greater with the use of 8 mL kg^{-1} of inoculant when compared to uninoculated seeds or with the application of 16 mL kg^{-1} of inoculant containing *A. brasilense* (Figure 2A). The radicle length of cultivar Marketmore76 was significantly greater for seedlings from seeds inoculated with 4 and 8 mL kg^{-1} of the inoculant containing *A. brasilense* when compared to uninoculated seeds (Figure 2B). The root volume of cultivar Marketmore76 was significantly higher for seedlings from seeds inoculated with 4 mL kg^{-1} of inoculant containing *A. brasilense*, followed by the application of 8 mL kg^{-1}, and significantly lower for non-inoculated seeds. In turn, the inoculation of *A. brasilense* had no significant effect on the root volume of seedlings of cultivar Aodai Melhorado (Figure 2C).

In general, the use of inoculant containing *A. brasilense* improved the shoot and root growth of cultivar Marketmore76 seedlings, whereas the growth of Aodai Melhorado seedlings was inhibited by the application of inoculant containing *A. brasilense* (Figure 2). These results indicate that the higher plant growth of cucumber seedlings in response to inoculation with *A. brasilense* is dependent on the genotype. Lima et al. (2018) also showed that tomato cultivars Gaúcho Melhorado and San Marzano have different responses to seed inoculation with *A. brasilense*. Therefore, it is important to understand the response of cucumber genotypes to the inoculation of plant growth-promoting bacteria (PGPB).

The accumulation and partitioning of dry matter between the shoots and roots of the cucumber seedlings were little influenced by the seed inoculation with *A. brasilense* (Figure 3). The seed inoculation with *A. brasilense* did not significantly affect ($p > 0.05$) the dry matter of the shoots, roots, total and the root: shoot ratio of the cultivar Marketmore76 seedlings. The root dry matter and root: shoot ratio of cultivar Aodai Melhorado was also not significantly influenced ($p > 0.05$) by seed inoculation with *A. brasilense.* In turn, the shoot dry matter of cultivar Marketmore76 was significantly lower with the use of 4 and 8 mL kg^{-1} of inoculant containing *A. brasilense* when compared to uninoculated seeds (Figure 3A). The total dry matter of cultivar Aodai Melhorado was significantly lower with the use of 8 mL kg^{-1} of inoculant when compared to uninoculated seeds (Figure 2C).

An increase in the shoot and root growth of seedlings could be expected due to the beneficial effect of *A. brasilense* in promoting plant growth, as reported in the other

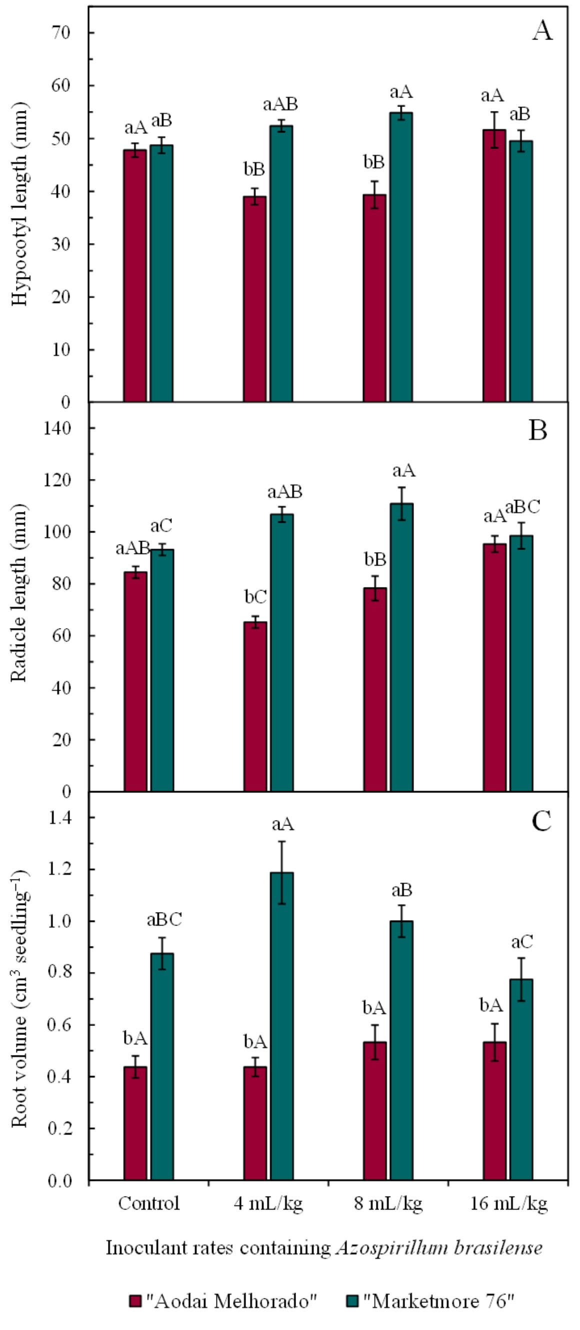

Fig. 2. Effects of inoculant rates containing *Azospirillum brasilense* on hypocotyl length (A), radicle length (B), and root volume (C) of two cucumber cultivars (*Cucumis sativus* L.). Bars followed by the same lower-case letters, between the cucumber cultivars or same upper-case letters, for the rates of inoculant are not significantly different by LSD test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± mean standard error

crops. The seed inoculation with *A. brasilense* improved the growth and dry matter accumulation of the shoots and roots of the tomato seedlings, which was attributed to the production of plant hormones such as auxin, gibberellin and cytokinin by the bacteria, improving the shoot and root growth (Lima et al., 2018). Similarly, Mangmang et al. (2015) showed that the seed soaking in inoculant containing *A. brasilense* strongly enhanced growth and vigor of tomato seedlings. The results presented here suggest that the other studies should be proposed to investigate the interaction between *Azospirillum* and cucumber genotypes to determine the ability of this bacterium in improve the plant growth rate.

The seedling length vigor index (SLVI) of the two cucumber cultivars was significantly higher for uninoculated seeds when compared to seeds inoculated with *A. brasilense* (Figure 4A). The seedling weight vigor index (SWVI) for cultivar Aodai Melhorado was significantly higher for uninoculated seeds when compared to seeds inoculated with *A. brasilense*, whereas for seedlings of cultivar Marketmore76, the SWVI was significantly greater for uninoculated seeds and with the use of 8 mL kg^{-1} of inoculant when compared with the application of 16 mL kg^{-1} of inoculant containing *A. brasilense* (Figure 4B). The lower seedling vigor index obtained with the application of the inoculant containing *A. brasilense* was due to the negative impact of its application

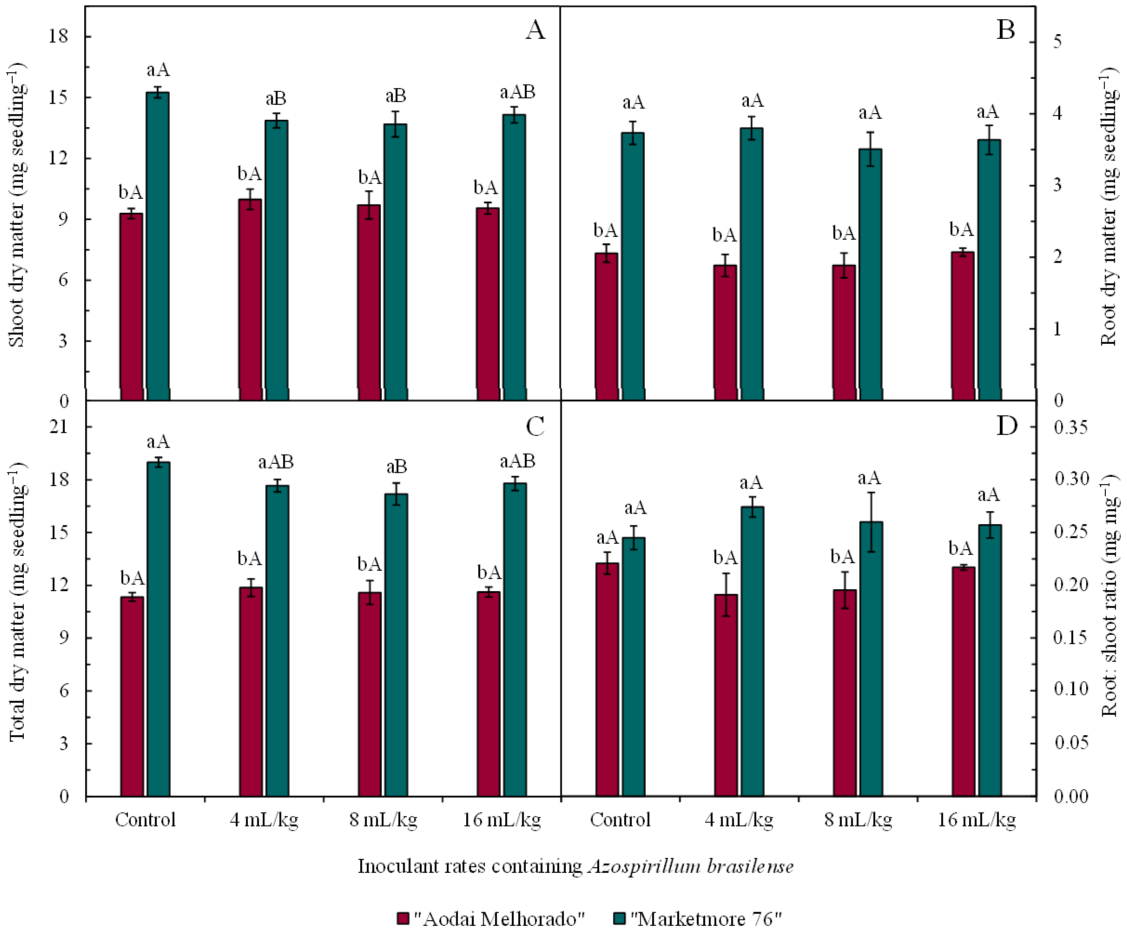

Fig. 3. Effects of inoculant rates containing *Azospirillum brasilense* on shoot dry matter (A), root dry matter (B), total dry matter (C), and root: shoot dry matter ratio (D) of two cucumber cultivars (*Cucumis sativus* L.). Bars followed by the same lower-case letters, between the cucumber cultivars or same upper-case letters, for the rates of inoculant are not significantly different by LSD test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± mean standard error

on the seed germination rate, especially for the cultivar Marketmore76 (Figure 1).

In general, our results showed that the inoculation of cucumber seeds with *A. brasilense* has a negative effect on the germination rate and vigor index of the seedlings, and a lesser impact on the length and dry matter accumulation of the seedlings. Contrary results have been reported by Mangmang et al. (2015), which showed that soaking cucumber seeds in inoculant containing *A. brasilense* (strains Sp7-S and Sp245) had no effect on the germination rate and initial seedling growth. On the other hand, Pereyra et al. (2010) reported that inoculation of *A. brasilense* resulted in a greater length of the shoots and roots of cucumber plants. The increase in the germination rate and greater initial seedling growth with the inoculation of *A. brasilense* has been commonly reported for tomato crop (Mangmang et al., 2015; Lima et al., 2018; Andrade-Sifuentes et al., 2020). These results indicate that further studies should be conducted to investigate the effects of the application of *A. brasilense* strains AbV5 and AbV6 on other cucumber genotypes. In addition, other methods of inoculant application should be studied, such as soaking and drenching, as well as the use of other strains of *A. brasilense*.

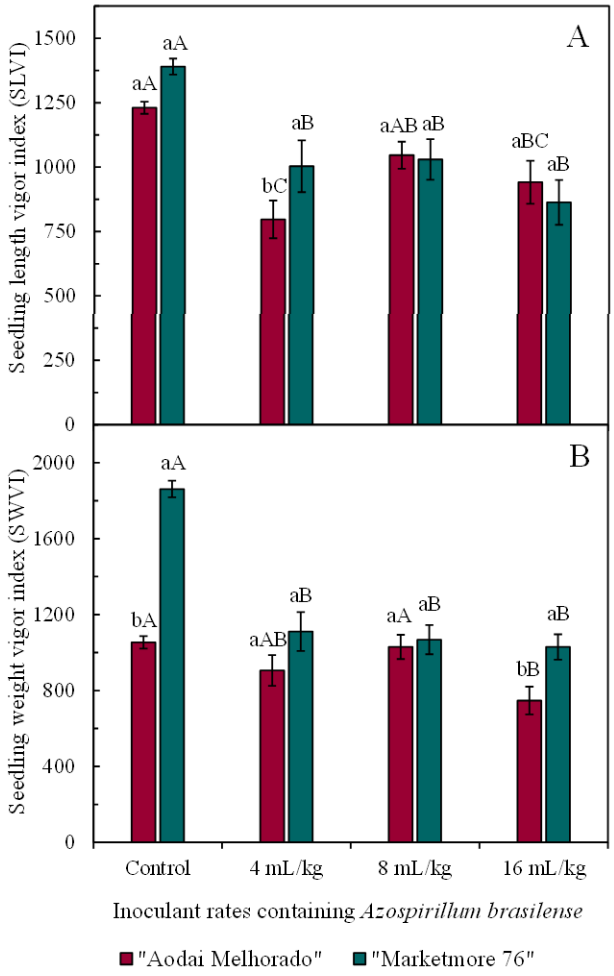

Fig. 4. Effects of inoculant rates containing *Azospirillum brasilense* on length vigor index (A), and weight vigor index (B) of seedlings from the two cucumber genotypes (*Cucumis sativus* L.). Bars followed by the same lower-case letters, between the cucumber cultivars or same upper-case letters, for the rates of inoculant are not significantly different by LSD test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± mean standard error

Currently, there is great interest and high investment in research for the production of soil microbial inoculants, however, the results are still fickle. Although there is a wide range of plant growth-promoting bacterial strains, few strains are highly efficient for a wide range of plant species (Li et al., 2020). Such inference may be related to the different environments of origin of these microorganisms, which may have their survival compromised by external factors of the growth medium and the specificity of plant species (Huang et al., 2015), among others. In addition, Li et al. (2020) reported that cucumber seedling growth was improved by inoculating the seeds with other species of plant growth-promoting bacteria (*Providencia rettgeri*, *Advenella incenata*, *Acinetobacter calcoaceticus* and *Serratia plymuthica*), indicating that other studies should also be performed to test the effect of other species of plant growth promoting bacteria on the growth of cucumber crop.

Conclusions

Inoculation of the seeds with strains AbV5 and AbV6 of *Azospirillum brasilense* reduced the germination rate and the vigor index of the cucumber seedlings, especially of cultivar ‘Marketmore 76’.

The inoculation of seeds with *A. brasilense* has little effect on the shoot and root growth of cucumber seedlings, and the beneficial and/or adverse effects of the application of *A. brasilense* on initial seedling growth are dependent on the cucumber genotype used.

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