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Enhancement of Tomato Seed Germination and Growth Parameters through Seed Priming with Auxin-Producing Plant Growth Promoting Bacteria Strains

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Abstract: The use of microbial seed priming may be a promising tool to improve the first stages of seed germination of several herbaceous species. In tomatoes (*Solanum lycopersicum* L.), enhanced germination and vigor, and biotic and abiotic stress control, with a reduction in chemicals, have been reported. In this study, seeds from two Italian tomato varieties (Canestrino di Lucca and Pisanello) were primed with seven different strains of plant growth-promoting rhizobacteria (PGPB) belonging to *Azospirillum baldaniorum*, *A. brasilense*, *Methylobacterium symbioticum*, *Bacillus amyloliquefaciens*, *B. licheniformis*, and *B. subtilis*. They were selected for their ability to produce auxin. The germination test was carried out on treated seeds and the germination percentage was calculated. The obtained seedlings were transplanted and kept in greenhouse conditions. After 60 d, fresh and dry weight, root number, and length of plantlets were recorded. A general and significant improvement in the growth parameters was observed in the treated plants. All microbial strains proved to be indolacetic acid (IAA) producers using the Salkowsky method. A positive relationship between root number and length, and amount of IAA was found. The overall results suggest that the microbial priming of tomato seed could be useful for advancing organic farming, sustainable agriculture, and environmental protection.

Keywords: PGPB; seed priming; IAA; *Solanum lycopersicum*; tomato varieties; *Azospirillum* spp.; *Bacillus* spp.; *Methylobacterium* spp.

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1. Introduction

Agronomic practices with low environmental impact are becoming essential for meeting the increasing demand for food with high nutritional value [1]. Thus, a decrease in agricultural inputs, represented by synthetic fertilizers, is imperative. In soil-based systems, plant growth-promoting rhizobacteria (PGPB) have been demonstrated to exert positive influences on plants due to their ability to reduce abiotic stresses and diseases [2]. Plants can more easily intake key nutrients, including iron, phosphorus, potassium, and fixed nitrogen, from the soil or atmosphere. Furthermore, PGPB may directly affect plant growth as a consequence of producing phytohormones such as gibberellins, cytokinins, and auxins [3].

There are several ways to apply PGPB to crops for obtaining positive outcomes [4]. Common biological approaches include seed treatment, root dipping, and foliar and soil application [5–8]. Concerning species that are usually gamic propagated, improving seed quality is crucial for achieving a rapid and uniform emergence. 'Priming' is an array of

methodologies that improves seed germination rates, resilience to biotic and abiotic stresses, and crop yields [7]. Specifically, priming methods include nutripriming, hydropriming, thermopriming, solid matrix priming, chemopriming, nanopriming, osmopriming, and biopriming [2,7,9,10]. By controlling seed rehydration, priming triggered the metabolic processes which are activated during the early stages of germination [11,12]. Primed seeds exhibit a greater range of germination temperatures, synchronous and rapid emergence, decreased photo- and thermo-dormancy, improved ability to compete with diseases and weeds, and water use [11,12]. However, the success of seed priming may strongly depend on plant genotype and physiology, seed lot and vigor, and the applied technique [13].

Studies on tomato seeds treated with liquid microbial cultures, including *Azospirillum*, *Bacillus,* and *Methylobacterium* sp., revealed significant increases in germination and vigor [14,15]. Moreover, Bashan and de-Bashan [16] have showed that tomato seeds primed with *A. brasilense* combined with streptomycin and foliar bactericide significantly reduced disease severity caused by *Pseudomonas syringae*. Significant results have been obtained in *Catharanthus roseus,* where *Azospirillum* and *Azotobacter* also improved antioxidant enzyme activities [17]. Saber et al. [18] have found better wheat agro-morphological parameters as a consequence of treatments with commercial biofertilizers containing different bacterial species, such as *Bacillus lentus*, *B. subtilis*, *Pseudomonas fluorescens*, *P. putida*, and *Azospirillum* spp. Priming of maize seeds with *Azotobacter* spp. and *Azospirillum* spp. have induced a considerable dry-weight increase and grain production [19]. Under saline stress conditions, two different strains of *B. subtilis* have been able to improve seed germination and plant growth of *Phaseolus vulgaris* L. [20]. Parinith et al. [15] confirmed the efficacy of biopriming tomato seeds with *Bacillus paralicheniformis* under salinity stress.

PGPB have been also employed in the soil as biofertilizers by plant inoculation. Several interesting findings emerged [21]. *Azotobacter* sp. and *Azospirillum* sp. have had positive influences on root and shoot growth parameters [21–24]. On tomato seedlings, *B. subtilis* and *B. pumilus* have enhanced phosphate solubilization, indole acetic acid (IAA) production, germination, stem growth, and root growth [25]. *B. amyloliquefaciens* strains have exhibited strong biocontrol against *Ralstonia solanacearum* maintaining high CFU (colony-forming unit) densities in the rhizosphere, and ammonia, IAA, and siderophore production [26]. *Methylobacterium* spp. highlight their potential in promoting tomato plant development, with IAA production, enhanced seed vigor index, root growth, and biomass [27].

Nevertheless, employing PGPB cell formulations might sometimes also present a significant drawback: maintaining the original population of the inoculated PGPB in the soil is the biggest challenge. Indeed, they need to compete with the established native microbial community and survive predation by soil microfauna [28]. PGPB strains facing these challenges can be utilized to create cell-free supernatants (CFSs). These are produced from broth cultures through mechanical and physical processes that eliminate cells. CFSs are typically obtained via centrifugation and various filtration methods, which can be used alone or in combination [29]. The CFS derived from *A. brasilense* Cd strain has been shown to promote growth in *M. polymorpha* seedlings, leading to early nodulation and changes in root morphology and function through ethylene production [30]. In another study, a CFS formulation from the *A. brasilense* Cd strain also significantly enhanced in vitro growth of *O. sativa*, improving lateral root development, root elongation, surface area, and dry matter [31]. Furthermore, the cytokinin-rich ethyl acetate extract of *Methylobacterium* spp. CFSs showed beneficial effects on *Triticum aestivum* L. seed germination and growth. Under salinity stress, canola and soybean seed germination has been improved by CFS from *Deviosa* sp. [32].

The aim of this research was to investigate the efficacy of seven IAA-producing microbial strains from the genera *Azospirillum, Bacillus,* and *Methylobacterium* on two tomato varieties (*Solanum lycopersicum* L.), to evaluate their potential role as seed priming and/or biostimulants on plant growth. The strains were used in two different forms: as cell suspensions (CSs) and cell-free supernatants (CFSs).

2. Materials and Methods

2.1. Microbial Strain and Treatment Preparation

Seven bacterial strains were used as listed in Table 1. Stock cultures were stored at −80 °C in 20% glycerol, and before use, they were grown overnight at 27 °C at 120 rpm in liquid nutrient medium (Oxoid).

Table 1. Microbial strains employed in this research.

Species	Strain	Reference/Source	Isolated from
Azospirillum baldaniorum	Sp245	Baldani et al., 1986 [33]; Dobbelaere et al., 1999 [34]; dos Santos Ferreira 2020 [35]	*Triticum aestivum*—Brazil
Azospirillum brasilense	Sp7	DSMZ	*Digitaria decumbens* roots—Brazil
Azospirillum brasilense	Cd	DSMZ	*Cynodon dactylon* roots—USA
Bacillus amyloliquefaciens	Fukumoto strain F	DSMZ	Soil—unknown county
Bacillus licheniformis	Gibson 46	DSMZ	Country of unknown origin
Bacillus subtilis	101BS	Filippi et al., 1984 [36]; Citernesi et al., 1994 [37]	Rhizosphere of *Dianthus caryophyllus* L.
Methylobacterium symbioticum	SB0023/3 T	Pascual et al., 2020 [38]; Symborg Inc. (EP Application No. EP3747267A1)	Spores of *Glomus iranicum* var. *tenuihypharum*

For each microbial strain, two sets of treatments were performed:

(i) Cell suspension (CS): broth culture of bacterial cells, with an initial population 10^8 CFU/mL;

(ii) Cell-free supernatant (CFS): obtained via centrifugation (5000 rpm for 15 min) and filtration of the suspension of 10^8 CFU/mL microbial cultures.

This resulted in 14 distinct treatments (7 CSs and 7 CFSs), each derived from the respective bacterial strain. The main steps of experimental trials are summarized in Table 2.

Table 2. Main steps of the experimental trials.

STEP 1	STEP 2	STEP 3	STEP 4
Seven microbial strains as (i) Cell suspension (CS)	(i) Microbial strain cultures	(i) Priming of tomato seeds with CS and CFS	(i) Repeated treatments of tomato seedlings with CS and CFS
(ii) Cell-free supernatant (CFS)	(ii) IAA quantification	(ii) Seed germination test	(ii) Plantlets growth parameters

2.2. Analysis of Indole-3-Acetic Acid (IAA) Production

The quantification of auxins by every strain was determined by colorimetry using the Salkowski reagent method, as reported by Gang et al. [39] with modifications. Each microbial strain was cultured in 100 mL of liquid nutrient medium (Oxoid) until it reached a concentration of 10^8 CFUs, both in the presence (1.5 g/L) and absence of L-TRP (Millipore).

Quantification was performed using the Infinite® M Nano microplate reader (Tecan, Switzerland) with a 96-well plate. Auxin levels expressed as mg/L were determined by spectrophotometric assays at 530 nm. All determinations were performed in triplicate, including the control groups and the calibration curve.

2.3. Germination of Tomato Seeds

Two tomato Tuscan varieties, Canestrino di Lucca (named Canestrino) and Pisanello, were selected for their pomological traits which are highly appreciated by consumers. These varieties are included in the list of 'Traditional Products of Tuscan Region' (https://www.regione.toscana.it—accessed on: 2 February 2023). Seeds (N = 750 per variety) were provided by Gargini Sementi di Toscana S.N.C., (Lucca, Italy). They were

surface-sterilized by immersion in 70% ethanol (Sigma Aldrich, Merk, Darmstadt, Germany) for 1 min, followed by treatment with 10% hypochlorite (Sigma Aldrich, Merk, Darmstadt, Germany) for 5 min, and subsequently rinsed five times in sterile distilled water. Then, the seeds were treated with the 14 bacterial preparations previously mentioned. For each treatment, 50 seeds were used, organized into 5 replicates of 10 seeds each. Seed priming was conducted by dipping seeds in 10 mL of the respective treatment solution for 30 min. A standard protocol routinely employed in our lab (unpublished data) was followed to ensure thorough exposure to the bacterial preparations and adequate seed imbibition. Water was used as control.

Seed germination was assessed as follows: seeds were placed between moistened paper towels in sealed Petri dishes and maintained at 23 ± 1 °C in dark conditions. Ten days after sowing (10 DAS), the germination percentage was determined, and the root length was measured using ImageJ software (Version 1.54g—Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/, 1997–2018—accessed on 5 June 2024). For the calculation of germination percentage, seeds that exhibited almost a radicle primordium were included. For the measurement of root length, only seeds with roots longer than 1 mm were considered.

Lastly, several seedlings were chosen from each treatment to be transplanted in pots for the greenhouse experimental phase.

2.4. Greenhouse Experiments on Tomato Plantlets

Greenhouse experiments were set up at the Department of Agriculture, Food and Environment of Pisa University, Italy (lat. 43°42′ N, long. 10°25′ E). At the beginning of May, seedlings (N = 9 per treatment, per 2 varieties) were transplanted in pots (15 × 15 × 15 cm) containing a non-sterile substrate constituted by a 1:1 mix of perlite and repotting soil. To test the potential ability of treatments to act as biostimulants, the seven bacterial strains, as CS and CFS (1×10^8 CFU) both at 100 mL final volume, were applied every 20 days. As control, water was used. After 60 days, the number and length of roots and the fresh and dry weight of plantlets were measured.

2.5. Statistical Analyses

Statistical analyses were performed using both R Core Team (2021) and the package GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Prior to analyses, data were log-, square-root- and square-transformed to satisfy normality and homoscedasticity assumptions. The standard errors (±SE) of the means were calculated for each parameter measured, considering $p \leq 0.05$. Data were compared using analysis of variance (ANOVA) and Tukey's multiple range test was assessed to compare the differences among means. Principal component analysis (PCA) was also conducted using R Core Team (2021) to identify key factors affecting variation in seed germination percentage, root length of the germinated seeds, root length, number of roots of potted plantlets, and plant dry weight. Data were standardized before analysis. Principal components were selected based on the Kaiser criterion (eigenvalues > 1) and the scree plot. Component loadings and scores were analyzed to understand variable contributions and to visualize treatment and variety differentiation.

3. Results

3.1. Analysis of Indole-3-Acetic Acid (IAA) Production

In Figure 1, results on the quantification of IAA (indole-3-acetic acid) using the Salkowski method are reported. Quantification was performed on cultures (adjusted at 1×10^8 CFU) grown in controlled conditions, both in the absence or presence of L-tryptophan (TRP—1.5 g/L). As expected, no positive reaction to the Salkowsky reagent was obtained when the L-tryptophan was tested alone.

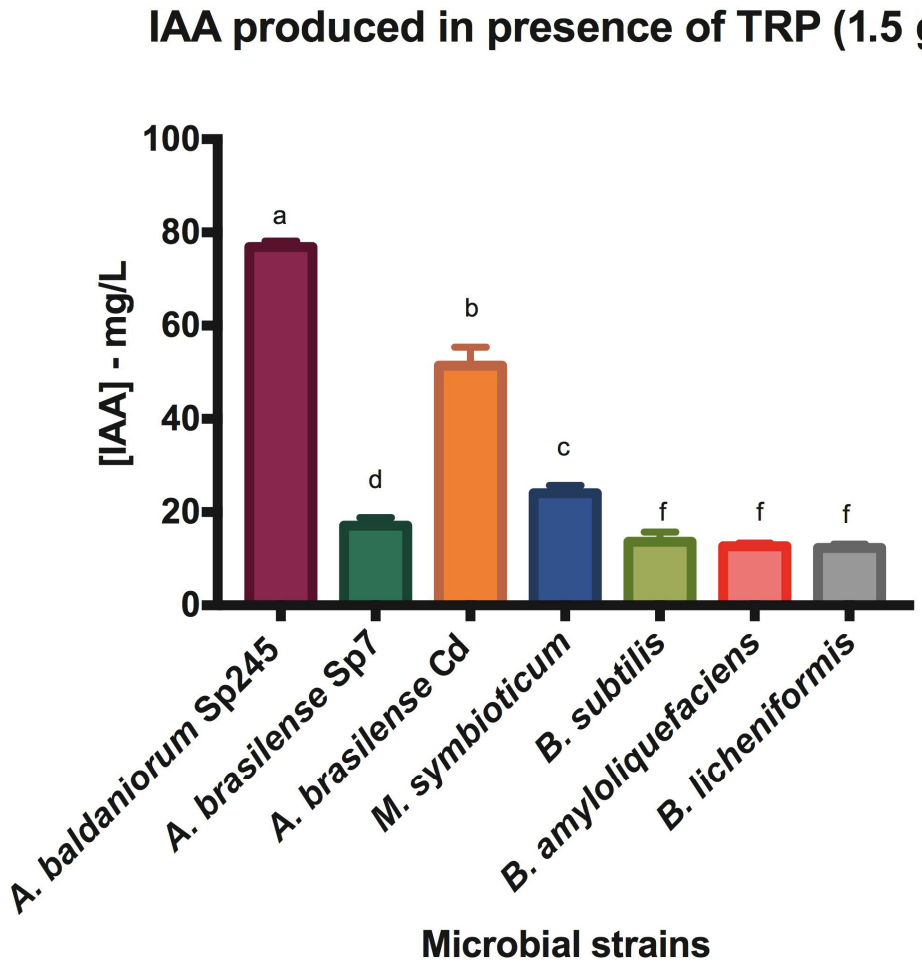

Figure 1. Mean IAA (mg/L) concentration (±SE) synthetized by different microbial strains in presence of L-tryptophan (1.5 g/L). Different letters indicate significant differences at $p \leq 0.05$.

All the microbial strains employed in this essay displayed positive reactions to the Salkowski's reagent test. Indeed, the development of a pinkish or deep red coloration indicated that the tested bacteria have the ability to metabolize L-tryptophan (TRP) into IAA or similar compounds. However, among them, significant differences were found. In particular, *A. baldaniorum* Sp245 and *A. brasilense* Cd showed the highest IAA concentrations at 79 mg/L and 58 mg/L, respectively. The IAA level detected in the other bacteria did not exceed 25 mg/L.

3.2. Germination of Tomato Seeds and Root Development

The treatments with liquid cultures of the different bacterial strains, both CS and CFS, had advantageous results with the germination process of tomato seeds. As shown in Figure 2, most treatments improved the germination percentage in both varieties in comparison with the control (+40–80% for Canestrino, +20–40% for Pisanello). In Canestrino, germination values for the treated seeds with CFS ranged from more than 70% (*B. amyloliquefaciens*) to more than 90% for the other strains. These data significantly differed from control seeds at about 50%. The germination of CS-treated seeds differed from the control in *A. baldaniorum* Sp245, *A. brasilense* Sp7 and Cd, *M. symbioticum*, and *B. licheniformis* with values at nearly 70–80%, while percentages in *B. subtilis* and *B. amyloliquefaciens* were similar to the control.

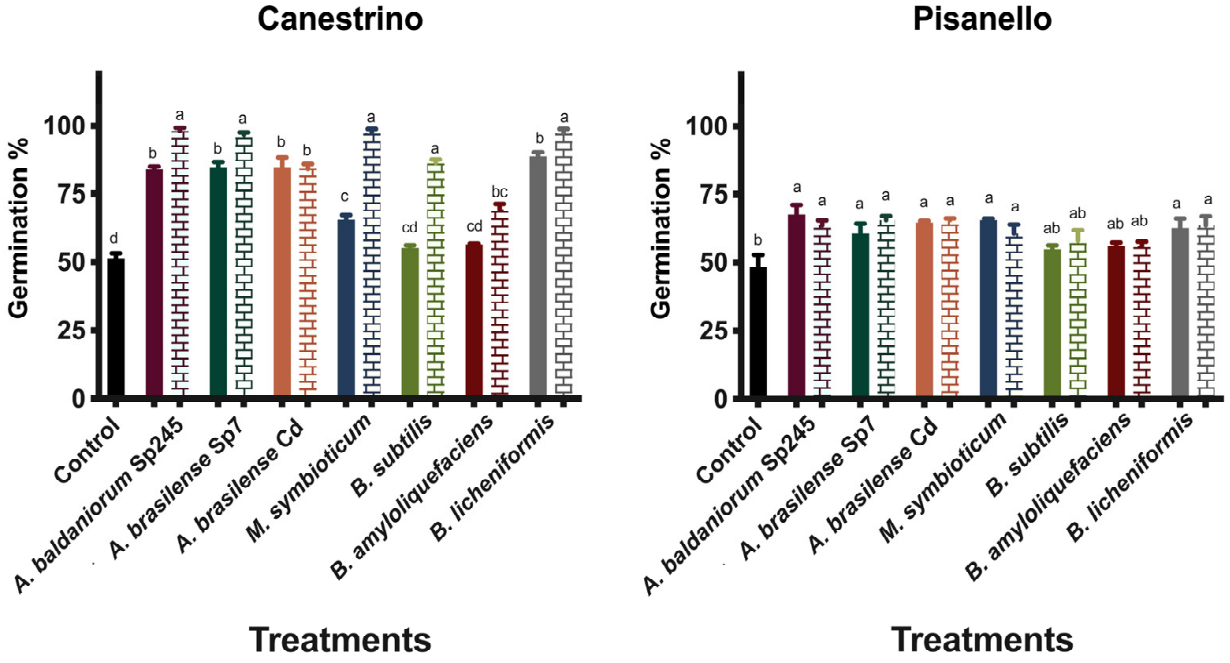

Figure 2. Effect of cell suspension (CS, full bars) and cell-free supernatant (CFS, shaded bars) from different bacterial strains on seed germination of Canestrino and Pisanello tomato varieties. Data are means (±SE). Different letters indicate significant differences at $p \leq 0.05$.

For the Pisanello variety, the treatments induced smaller germination increases than Canestrino. In comparison with untreated seeds (49.5%), significant differences were found for *A. baldaniorum* Sp245, *A. brasilense* Sp7 and Cd, *M. symbioticum*, and *B. licheniformis* (about 60–70%). CS and CFS treatments did not show significant differences.

Similarly to what was observed for the seed germination, all bacterial suspensions, both CS and CFS, determined a beneficial effect on root development in comparison with the control group for Canestrino and Pisanello varieties (Figures 3a,b and 4).

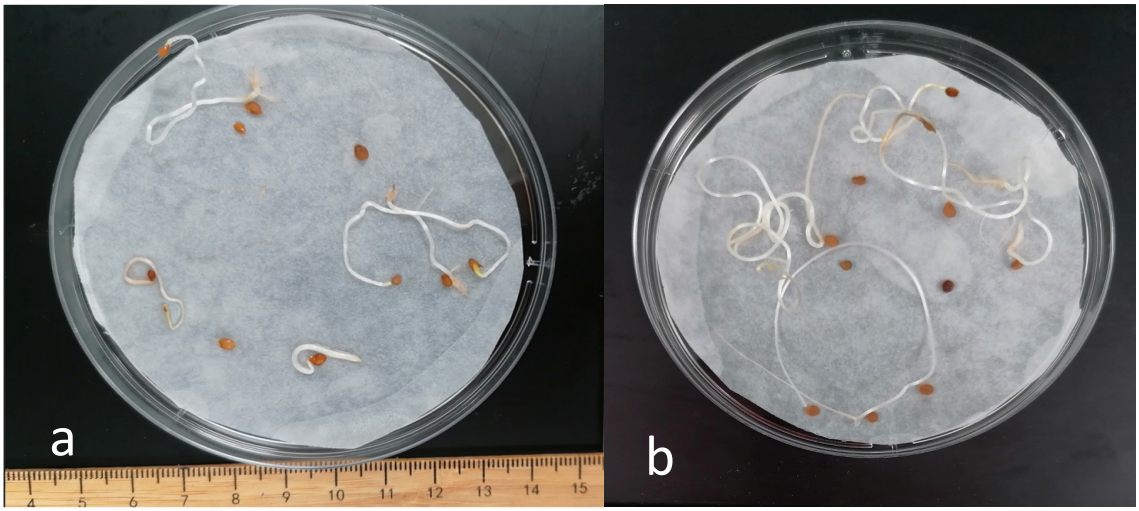

Figure 3. Canestrino seeds treated with water (**a**) and with CFS from *A. baldaniorum* Sp245 (**b**) at 10 DAS. CFS = cell-free supernatant; DAS = days after sowing.

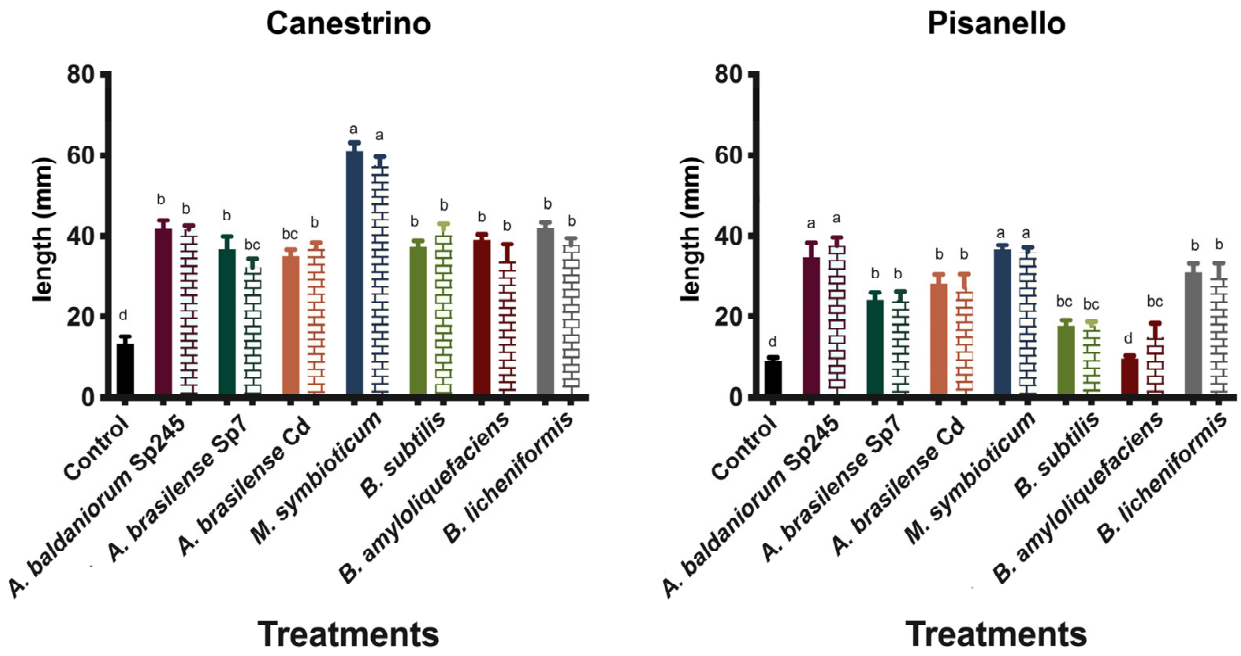

Figure 4. Effect of cell suspension (CS, full bars) and cell-free supernatant (CFS, shaded bars) from different bacterial strains on root length (mm) of tomato seeds (Canestrino and Pisanello varieties). Data are means (±SE). Different letters indicate significant differences at $p \leq 0.05$.

Based on root lengths shown in Figure 4, percentage increases in treated seeds were determined in comparison with controls. The highest values (more than +300%) were obtained in both varieties with *M. symbioticum* and in Pisanello with *A. baldaniorum* Sp245. Treatments with *B. licheniformis*, *A. brasilense* Cd, and *A. brasilense* Sp7 induced increases between +140 and +240% in both varieties. On the other hand, B. subtilis and B. amyloliquefaciens gave different responses in relation to the variety: Pisanello showed lower increments while Canestrino was characterized by higher values, ranging between +150 and +215%.

3.3. Greenhouse Experiments on Tomato Plantlets

At the end of the 60-day period in the greenhouse and after three rounds of treatments, the hypogeal and epigeal morphological parameters of plantlets were recorded.

Concerning the root apparatus, in both varieties all treatments induced an improvement in terms of the total number of roots in comparison with the control groups which showed a number of 10 and 9.3 for Canestrino and Pisanello, respectively (Figure 5). In Canestrino, the best results were given by the CS treatments that produced a root mean per plant ranging from 39.8 (*M. symbioticum*) to 58.7 (*A. baldaniorum* Sp245) with increases in percentages of +298% and +487% than the control, respectively. In *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*, the root number was just over 20, without differences between CS and CFS. This occurrence was also verified in Pisanello for all treatments. In this variety, the resulting root numbers were mostly improved by *A. brasilense* Cd and *A. baldaniorum* Sp245 by a mean of 36.8 and 39.7, with increases in percentages of +295% and +326% than the control, respectively. The other strains stimulated an average of 19.8–34.3 roots per plant.

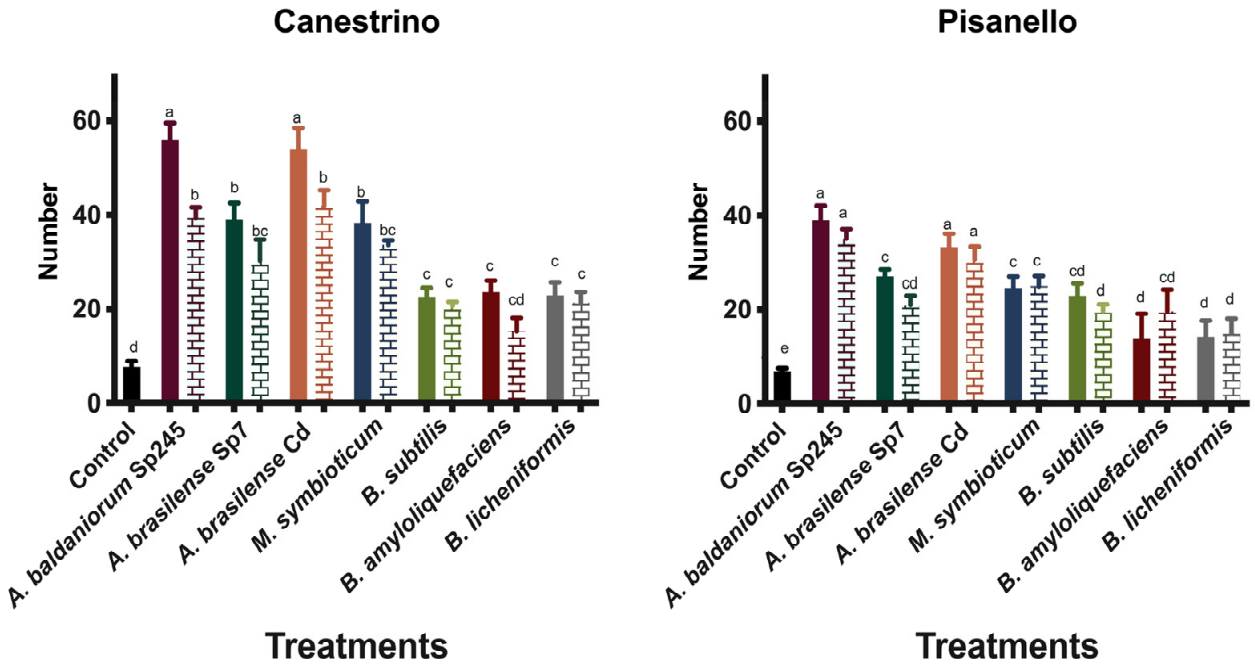

Figure 5. Effect of cell suspension (CS, full bars) and cell-free supernatant (CFS, shaded bars) from different bacterial strains on root number of tomato plantlets (Canestrino and Pisanello varieties). Data are means (±SE). Different letters indicate significant differences at $p \leq 0.05$.

Data regarding the root length are presented in Figure 6. In both varieties, CS and CFS treatments gave similar results for all strains. The root growth was markedly improved when compared with control plants that showed a mean length of about 50 mm. The highest values were ascribed to *A. baldaniorum* Sp245 and *A. brasilense* Cd by more than 100 mm in Canestrino (about +180% compared to the control). An analogous effect was obtained for the Pisanello variety where the root mean length reached a maximum of 94.7 mm (about +110% compared to the control). Although lower increases were recorded for *A. brasilense* Sp7, *M. symbioticum*, *B. subtilis*, and *B. licheniformis*, they differed statistically from the controls.

A regression analysis was conducted between root length (Figure 7a,b) and number (Figure 7c,d) of Canestrino and Pisanello tomato varieties and IAA produced by the bacteria. A highly significant and positive relationship between variables was found. Coefficients were attested at R^2 = 0.79–0.80 for root length and at R^2 = 0.58–0.61 for root number. This occurrence suggests that the auxin levels produced by microorganisms present in the treatments influenced the root growth.

Concerning the evaluation of plant growth, the dry weights of hypogeal and epigeal organs were recorded. Differences between varieties were observed (Figure 8). Treated Canestrino plantlets showed a significant dry-weight increase, ranging from 1.8 to 2.7 g, in comparison with the control. For Pisanello, the weight increases were more modest, statistically differing from the control in the plants treated with both CS and CFS of *A. brasilense* Cd, *B. subtilis*, *B. licheniformis*, and *M. symbioticum* only for CS.

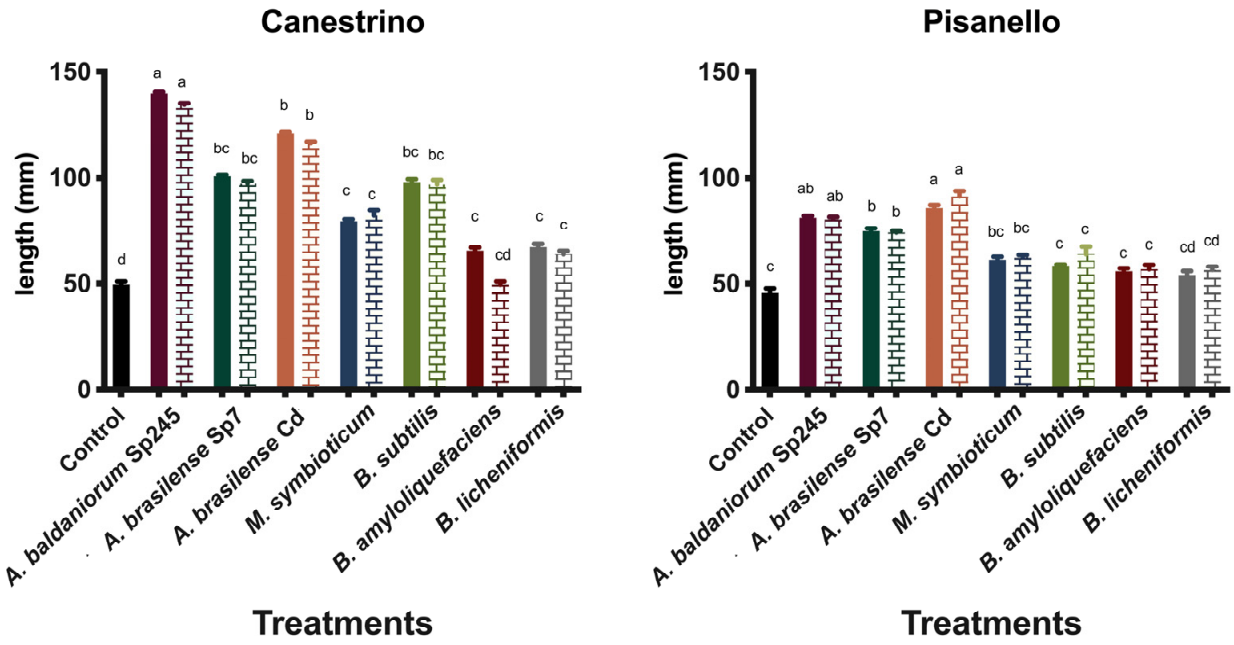

Figure 6. Effect of cell suspension (CS, full bars) and cell-free supernatant (CFS, shaded bars) from different bacterial strains on root length (mm) of tomato plantlets (Canestrino and Pisanello varieties). Data are means (±SE). Different letters indicate significant differences at $p \leq 0.05$.

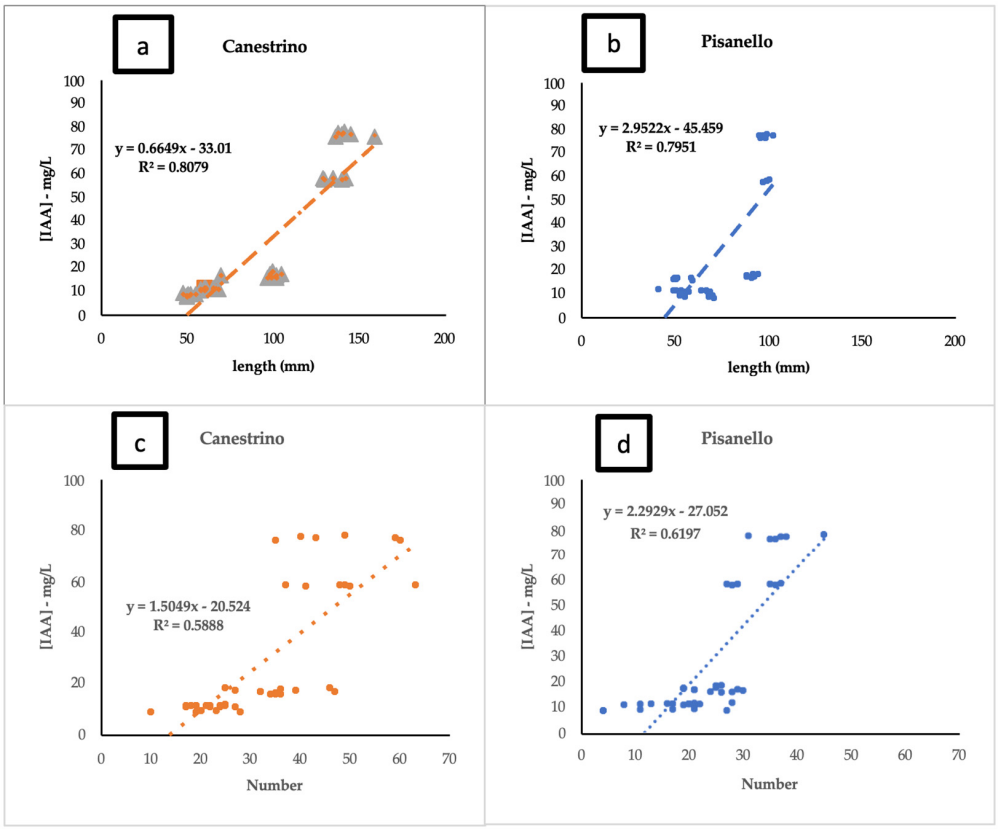

Figure 7. Linear regression between root length (mm) of Canestrino (**a**) and Pisanello (**b**) and root number of Canestrino (**c**) and Pisanello (**d**) tomato varieties and IAA (mg/L) produced by the seven bacterial strains.

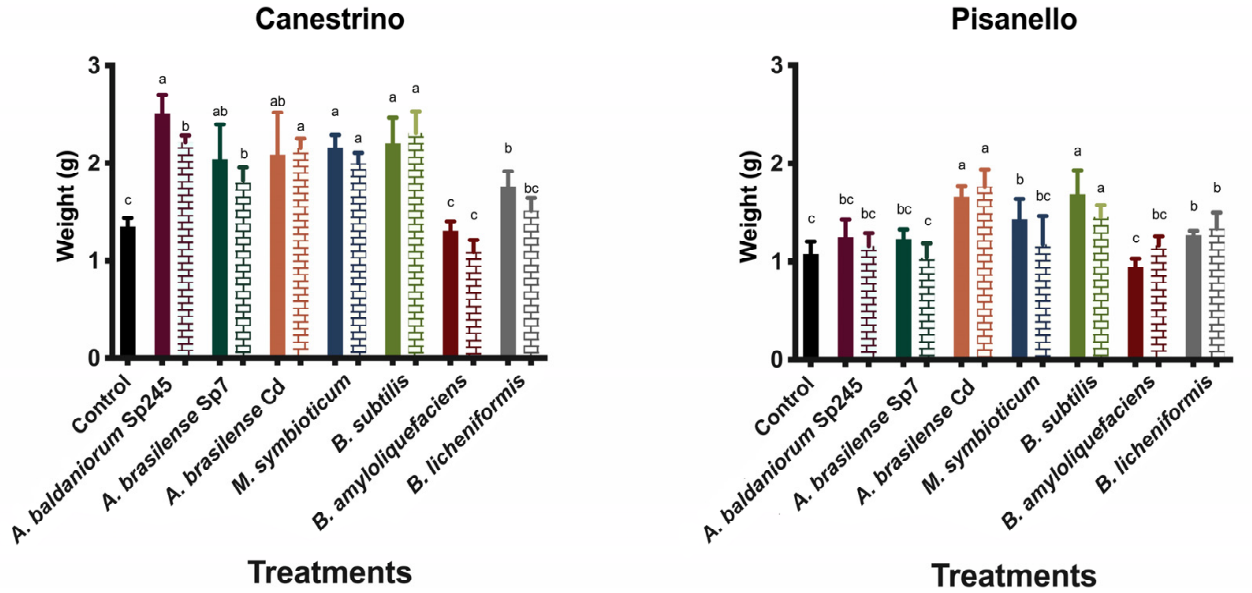

Figure 8. Effect of cell suspension (CS, full bars) and cell-free supernatant (CFS, shaded bars) from different bacterial strains on dry weight of tomato plantlets (Canestrino and Pisanello varieties). Data are means (±SE). Different letters indicate significant differences at $p \leq 0.05$.

The PCA results highlighted that *A. baldaniorum* Sp245 and *A. brasilense* Sp7 had the greatest impact on Pisanello and Canestrino varieties (Figure 9). Conversely, treatments with *B. amyloliquefaciens* and *B. subtilis*, positioned close to the control, were the least effective on both varieties.

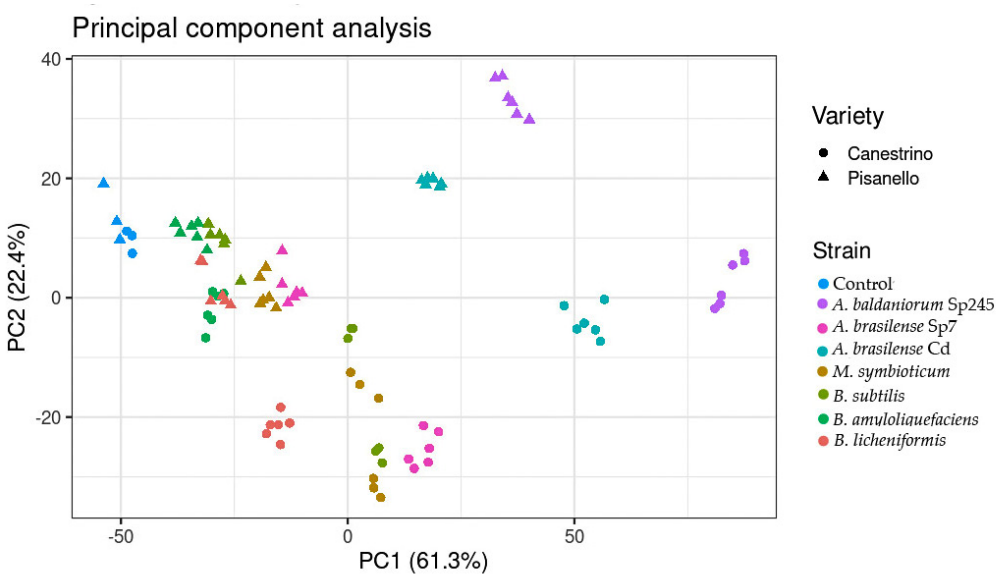

Figure 9. The PCA biplot illustrates the variation in seed germination percentage and plantlets parameters (shoot and root length, root number, and dry weight) across Canestrino and Pisanello tomato varieties and strain treatments. Principal Component 1 (PC1) explains 61.3% of the total variance, and Principal Component 2 (PC2) accounts for 22.4%.

4. Discussion

A crucial phase of a tomato plant's life cycle is seed germination, and this could be positively influenced using the priming technique. The employment of effective microorganisms in a pre-sowing seed treatment is currently considered an environmentally friendly

system [2]. The seven microbial strains tested, both as CS and CFS, influenced the seed performance of two Italian tomato varieties, Canestrino di Lucca and Pisanello.

As a general trend, all CS and CFS improved the seed germination process when compared with the untreated seeds. In some cases, the CFS treatment was more effective than CS.

Azospirillum strains induced noteworthy amelioration on seed germination and improved root growth parameters such as number and length. These results are consistent with research carried out on tomato seeds: when priming was performed with different strains of *A. brasilense*, better germination and root growth were achieved, in addition to an increase in plantlet development [22,40,41].

Bacillus strains also gave interesting results with seeds and plantlets: *B. subtilis* increased the dry weight in both tomato varieties. Moreover, in Canestrino, an improvement in root length was recorded. *B. amyloliquefacies* increased germination percentage and *B. licheniformis* favored seed vigor. The results are coherent with other studies involving different *Bacillus* species on tomato plants. In particular, *B. licheniformis* significantly affected plant height, leaf area, and fruit production, also inducing less disease and higher competitive ability [42]. Co-inoculation of *B. pumilus*, *B. amyloliquefaciens*, and *B. mojavensis*, at specific plant growth stages, have maximized biomass, yield, and fruit nutrient content [43]. *B. licheniformis* FMCH001 and *B. subtilis* FMCH002, alone or in combination, have enhanced fresh and dry mass, root volume, and root length, showing persistent colonization in the rhizosphere [44].

M. symbioticum, a nitrogen-fixing strain isolated in 2020 [38], has shown successful results with strawberry, maize, and lettuce [45–47]. On these species, it has been able to exert a positive influence on seeds, root growth, and dry weight of plantlets as well. *M. symbioticum*, employed on Canestrino and Pisanello varieties as a priming agent (both as CS and CFS), resulted in an enhancement of seed germination, which was perfectly comparable or better to other bacteria. Findings on this bacterium appeared promising and innovative, suggesting its potential use as a priming agent and inoculant during the early growth stages of seedlings.

The significant improvement in growth parameters obtained by the considered microbial treatments may be due to the ability of the strains to produce hormones, including IAA. It is a key phytohormone-like substance that plays a vital role in root–microbe interactions and that improves the quality of the root system architecture [48]. It has been proven that IAA-producing bacteria enhance root elongation and branching, increasing the hair formation [49]. This occurrence, leading to a better water and nutrient uptake, could allow a reduction in chemical fertilizers [50,51]. This possibility was supported the regression analysis between the IAA amount produced by the microbial strains and root growth parameters (number and length) recorded for the treated tomato plantlets. The Salkowski's reagent test, designed to detect IAA and its precursor compounds and commonly employed as an initial screening method for identifying IAA-producing rhizobacteria [39], performed well in the described experimental trials. After extensive bibliographic research, it can be stated that this analysis was applied for the first time on *Bacillus* and *Methylobacterium* strains considered in this research.

5. Conclusions

The findings of this study further support the conclusions of previous research about the positive influence of liquid cultures from *Azospirillum*, *Bacillus*, and *Methylobacterium* strains. These bacteria were found to exert benefits on seeds and plantlets of Canestrino and Pisanello tomato varieties. Noteworthy enhancements in germination percentage and growth were observed.

The innovative inoculation method with cell-free supernatants (CFSs) was successfully applied, yielding comparable results obtained to those obtained with cell suspensions (CSs).

A plant genotype effect was highlighted, confirming that the success of microbial priming depends on the interactions between bacterial strain and plant genotypes.

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