# Characterization and application of native Patagonian rhizobacteria to improve clonal performance and growth in Cannabis sativa L

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## Abstract

### Background

The increasing global demand for medicinal *Cannabis sativa L.* has intensified the need for sustainable agronomic practices capable of supporting high yields and consistent phytochemical profiles. Plant growth-promoting rhizobacteria (PGPR) represent a promising alternative to chemical fertilizers, yet their application in cannabis cultivation remains largely unexplored. In this study, 51 bacterial isolates obtained from Patagonian soils were characterized for key plant growth–promoting (PGP) traits, including nitrogen fixation, phosphate solubilization, siderophore production, indole-3-acetic acid (IAA) synthesis, and amylolytic and proteolytic activities. Then, ten representative strains were selected for *in vivo* assays to evaluate their ability to increase *C. sativa* clone survival, root colonization, and plant growth. Clones of 21 days were randomly selected from each treatment for optical and electron microscopic examination of roots. Finally, Seeds of *Cannabis sativa* were inoculated with four selected plant growth-promoting (PGP) bacterial.

### Results

The isolates exhibited substantial inter- and intraspecies variability in their PGP profiles, as revealed by multivariate analysis. Several strains—including *Pantoea agglomerans* M4C1, *Bacillus* sp. AM10, *Pseudomonas* sp. M4C3', and *Microbacterium* sp. M5C1—significantly improved clone establishment and root development. Moreover, compared with no inoculation, inoculation of seeds with selected isolates increased plant height by 12–16%, confirming their biofertilizer potential. Notably, both endophytic and nonendophytic strains effectively promoted plant growth, indicating multiple modes of action.

### Conclusions

Overall, our findings demonstrate that native Patagonian rhizobacteria can increase early propagation and vegetative growth of *C. sativa*, offering an environmentally sustainable strategy to reduce reliance on synthetic fertilizers. This work provides a foundation for the development of microbial inoculants tailored to cannabis cultivation and highlights the importance of integrating microbial biotechnology into the expanding medicinal cannabis sector.

## Introduction

*Cannabis sativa* L. is a plant of significant medicinal value that is widely used for treating conditions such as anxiety, depression, neurological disorders, and Alzheimer's disease, as well as for alleviating symptoms associated with HIV and cancer [1]. Currently, cannabis legislation is rapidly evolving worldwide, with the medical use of cannabis being legalized in many countries. This trend highlights the substantial potential of medicinal cannabis cultivation as a driver of regional economic development [1, 2]. However, large-scale cultivation presents several challenges, particularly from an agronomic perspective, where effective fertilization is crucial for achieving high yields and desirable metabolic profiles [2,3]. Chemical fertilizers are commonly used to increase crop yield and quality; however, their extensive use increases production costs and raises environmental concerns, including air, water, and soil pollution [4, 5].

An alternative and more sustainable approach is to use plant growth-promoting rhizobacteria (PGPR) to reduce or replace agrochemicals. These bacteria increase crop productivity and ecosystem functioning through various mechanisms, including nitrogen fixation, phosphate solubilization, siderophore production, and indole-3-acetic acid (IAA) synthesis. Additionally, some beneficial bacteria exhibit amylolytic and proteolytic activities, which further contribute to plant nutrition.

Nitrogen (N) and phosphorus (P) are essential macronutrients for plant growth. Certain nonsymbiotic nitrogen-fixing bacteria can colonize the rhizosphere and convert atmospheric N□ into ammonium, which serves as a precursor for amino acid synthesis and subsequent IAA production—a key hormone in plant growth promotion [7]. Conversely, while phosphorus is vital for plant development, its limited availability in soils often restricts plant growth. Typically, only approximately 75% of the P added to the soil is absorbed by plants because it binds strongly to iron and aluminum oxides in acidic soils or precipites as calcium phosphate in alkaline soils. Several soil microorganisms can mineralize organic phosphates and solubilize inorganic phosphates, thereby increasing phosphorus bioavailability. This process is primarily mediated by microbial phosphatases and the production of organic and inorganic acids [8]. Furthermore, PGPR that produce amylolytic and proteolytic enzymes facilitate the release of carbon and nitrogen from soil organic matter, increasing nutrient uptake by plants [9].

Iron (Fe) is another essential micronutrient for all living organisms, playing a critical role in redox reactions, adenosine triphosphate (ATP) synthesis, electron transport, and other vital biochemical processes [10]. Although iron is abundant in soils, its bioavailability is limited because it is oxidized to the ferric (Fe□□) state, which is insoluble in water. Certain microorganisms produce siderophores—low-molecular-weight ligands capable of chelating ferric iron, solubilizing it, and transporting it into cells [10, 11]. Siderophore-producing bacteria can stimulate plant growth by supplying bioavailable Fe, either directly through interaction with roots or via endocytosis of siderophore–Fe complexes. In addition to promoting plant nutrition, these bacteria can inhibit pathogenic microorganisms by

sequestering available iron, thereby exhibiting a biocontrol effect. These properties have been documented in bacterial genera, including *Pseudomonas* and *Bacillus* [11].

The study of PGPR in relation to *Cannabis sativa* remains an emerging research field. Therefore, greater attention must be given to elucidating the roles of soil bacteria in cannabis cultivation and plant nutrition. Understanding these microbial interactions will facilitate the development of microbial biostimulants that increase plant growth and health while reducing dependency on agrochemical fertilizers. Consequently, this study aimed to evaluate the plant growth-promoting traits of Patagonian bacterial strains and to assess their beneficial effects during different stages of *C. sativa* cultivation.

## Materials and methods

## Bacterial strains

Fifty-one strains isolated from Parque Nacional Los Alerces (Chubut, Argentina), stored at -20°C in 20% (v/v) glycerol, and belonging to the CIEFAP culture collection were used in this study. Before performing the assays, the cells were transferred to fresh Luria–Bertani (LB) broth (Britania) and cultured overnight (16 h) at 30°C.

**In vitro characterization of the plant growth-promoting traits of the strains**

To select bacteria with plant growth-promoting activity, their amylolytic and proteolytic activities, nitrogen-fixing ability, phosphate-solubilizing capacity, and siderophore and indole-3-acetic acid (IAA) production were evaluated.

Bacterial isolates were screened for their amylolytic potential by inoculating 10 µl of the bacterial cultures on a starch agar plate (0.5 g/L beef peptone, 0.1 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.1 g/L $(NH_4)_2SO_4$, 0.1 g/L $NaH_2PO_4$, 10 g/L soluble starch, 15 g/L agar, pH 7.0). The α-amylase-producing bacteria were detected by adding Lugol's iodine to the plates after 48 h of incubation [12]. Protease activity was tested on skim milk media (100 g/L skim milk, 10 g/L yeast extract, 15 g/L agar) by inoculating 10 µL of bacterial culture on agar plates. Plates were incubated for 72 h, and a clear zone around the bacterial colonies indicated protease activity [13].

Nitrogen fixation was evaluated on N-free malate (Nfb) medium [14]: 5.0 g malic acid, 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2.2H_2O$, 4.5 g KOH, 2 ml micronutrient solution (0.04g/L $CuSO_4.5H_2O$, 0.12g/L $ZnSO_4.7H_2O$, 1.4 g/L $H_3BO_3$, 1.0 g/L $Na_2MoO_4.2H_2O$, 1.175 g/L $MnSO_4$. H2O), bromothymol blue solution (5 g/L in 2 N KOH), 2 mL FeEDTA solution (16.5 g/L), 1 ml of vitamin solution (0.1 g/L Biotin, 0.2 g/L pyridoxine), and 7 g/L agar. The medium pH was adjusted to 5.5, and the volume was adjusted with distilled water to obtain 1 L of culture medium. Ten microliters of the bacterial cultures were inoculated on agar plates and incubated for 72 h at 30°C. The colonies that fixed $N_2$ alkalinized the culture medium by producing ammonia which was visualized as a swerve of the color media from light green to blue around the colony.

Phosphorus solubilization was assessed by using the Pikovskaia's basal medium [15, 16] (0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L KCl, 0.3 g/L $MgSO_4.7H_2O$, 0.04 g/L $MnSO_4$ x $H_2O$, 0.002 g/L $FeSO_4.7H_2O$, 0.2 g/L NaCl, 10 g/L glucose, 0.5 g/L yeast extract, and 5 g/L $CaH_4P_2O_8$. H2O, 0.1 g/L bromocresol purple, 15 g/L agar, pH 7.2) Ten microliters of each isolate was inoculated on agar plates and incubated at 30°C for 72 h. Phosphate solubilization was visualized as a halo that swerved from violet to yellow around the colonies.

The production of siderophores by the bacterial isolates was assessed via the universal chrome azurol S (CAS) assay [17]. CAS agar plates were spot inoculated with the bacterial strain suspensions and incubated at 30°C for 48 h. Siderophore production was confirmed by the presence of yellow−orange halos around the bacterial colonies after incubation.

Differences in the amylolytic and proteolytic activities, nitrogen fixation and phosphate solubilization capacities, and siderophore production of the strains were determined by measuring the halo zone sizes around each colony (in millimeters).

Indole acetic acid (IAA) production by the isolated strains was determined via the method of [18]. The bacterial strains were cultured in LB broth containing tryptophan (1 g/L) at 30°C in the dark for 5 days. Then, the bacterial suspensions were centrifuged at 13000 × *g* for 15 min, and 0.1 ml of the supernatant was mixed with 0.1 ml of Salkowski's reagent (50 ml of 35% perchloric acid: 1 ml of 0.5 N FeCl3) and incubated in the dark at room temperature for 20 min. For quantification, an IAA calibration curve was used (10–100 µg/ml). The optical density was measured at 536 nm.

Measurements were performed in triplicate from two independent assays and were expressed as the means ± SDs. A Two-way cluster analysis was conducted to compare strains on the basis of their PGP attributes using a categorical matrix to obtain different groups to be tested in *C. sativa* plants. The analysis clustered bacterial isolates according to their amylolytic and protease activities, nitrogen fixation capacity, phosphorus solubilization ability, and indole-3-acetic acid (IAA) and siderophore production. To perform the analysis, the categorical matrix was constructed by classifying the results for each isolate into four categories: high, medium, low, or no activity. The Sørensen (Bray–Curtis) coefficient was used as the distance measure, and the group-average algorithm was applied as the linkage method. All analyses were performed using PC-ORD version 6 [19].

## Molecular identification of bacterial strains

The bacterial isolates selected in the previous assay were grown overnight (16 h) at 30°C in Luria–Bertani (LB) broth (Britania). Genomic DNA was extracted by heating the bacterial suspension at 95°C for 10 min, followed by the CTAB method [20]. The DNA was subsequently resuspended in 50 µl of Tris-EDTA buffer (TE/10) (10 mM Tris-HCl pH 7.5; 0.1 mM EDTA). The DNA concentration was quantified using a Nanodrop 2000 (Thermo Scientific), and the integrity was verified by electrophoresis on 0.8% agarose gels.

For molecular identification, the 16S rRNA gene region was amplified using primers 27f (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). Amplifications were carried out in 50 µl containing 1×Taq Buffer (PBL company), 0.2 mM of each dNTP (PBL company), 1 U of Taq Pegasus (PBL company), 0.2 µM of each primer (PBL company) and 75–100 ng of DNA. The thermal cycling conditions were as follows: 1 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 48°C and 5 min at 72°C; and a final step of 10 min at 72°C. The PCR products were purified and sequenced by the sequencing service of the CERELA Institute (Tucumán, Argentina). The sequence data were analyzed via the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the megablast algorithm and using the EzBioCloud platform (https://www.ezbiocloud.net/). Bacterial identification was further confirmed on the basis of cell morphology and Gram staining [21].

**Cannabis sativa cloning improvement**

To evaluate whether the selected bacterial isolates could enhance *Cannabis sativa* cloning, each strain was grown in standard medium 1 (ST1) broth (15 g/L meat peptone, 3 g/L yeast extract, 6 g/L NaCl, and 1 g/L glucose) for 48 h at 28°C and diluted in sterile water in order to obtain a cell density of 3×10□ CFU mL□□.

Plant assays were conducted at CCT CONICET–CENPAT, a licensed growth facility operating under the Interdisciplinary Cannabis Program. Two *C. sativa* varieties—Ballena Franca (BF) and Mariquita Sánchez (MS) (registered by the National Council for Scientific and Technical Research-CONICET in the INASE- Seeds National Institute), which were provided by the Interdisciplinary Cannabis Program from CCT CONICET-CENPAT located in Puerto Madryn, Chubut, Argentina, were used in this assay. Uniform cuttings (height: 8 cm; dry weight: $0.25 \pm 0.05$ g) were obtained from mother plants ($N = 6$ per variety and treatment). The upper three fully expanded leaves were retained, and all other foliage was removed.

Stems were gently scraped and dipped into one of twelve treatments: sterile water (control), α-naphthaleneacetic acid rooting hormone (Fertifox), or the bacterial isolates M4C15, B7, M4C1, B1, AM01, AM10, M5C1, M4C3', M5C7', and AM05. Treated cuttings were then placed in 250 mL pots containing MGgrow commercial soil (Minas Magri S.A.) preinoculated with 10 mL of the corresponding bacterial suspension and thoroughly mixed with water.

Clones were maintained in transparent plastic boxes with lids to preserve humidity and placed one day in obscurity and under LED illumination (~ 150 µmol m□□ s□□; 18 h light/6 h dark photoperiod). After 21 days, the survival rate was recorded. The surviving plants from each treatment were carefully removed, washed, and measured for stem and root length. Samples were then dried at 35°C until a constant weight was reached to determine the stem and root dry biomasses.

Clone survival was statistically analyzed using Goodman–Kruskal's lambda test and the uncertainty coefficient test to determine the degree of dependence between survival and treatments. A Kruskal–Wallis nonparametric test ($\alpha = 0.005$, assuming a chi-square distribution with 11 degrees of freedom) was applied to compare root and stem dry weights, as well as root and stem lengths, among treatments. Post hoc pairwise comparisons were performed using the Conover–Inman test. Statistical analyses were conducted using SYSTAT 13 software, version 13.00.005 (Systat Software, Inc.).

## Microbial root colonization analysis by optical and scanning electron microscopy (dup: abstract ?)

Clones of 21 days were randomly selected from each treatment for optical and electron microscopic examination. Cannabis roots collected from the proliferation area were carefully washed in water to remove excess of substrate without wiping attached microorganisms. Tissue samples from inoculated and noninoculated seedling roots were first evaluated using an optical microscope (Leica, Model DM2500). The samples were also observed by scanning electron microscopy (Amray 1600) at 20 kV. The samples were previously fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and incubated at 8°C for 1.5 h, washed twice in the same buffer for 10 min and postfixed in 1.0% $Os0_4$ for 4 h. Dehydration was performed via incubation in increasing ethanol concentrations (30%, 50%, 70%, 85% and twice with 95% for 15 min). Finally, the samples were dried using the critical point drying (CPD) method.

## Plant growth promotion achieved through PGPR seed inoculation

Seeds of *Cannabis sativa var. Fruti* (F1 provided by Dr Gregorio Bigatti from his personal collection in Puerto Madryn City, Chubut, Argentina) were surface sterilized with NaClO 20% (v/v) for 15 min, followed by rinsing with sterile distilled water. The seeds were then incubated in 70% (v/v) ethanol for 3 min and rinsed three additional times with sterile distilled water for a total of 15 min. Sterilized seeds were subsequently transferred to pots containing 250 mL of MGgrow commercial soil (Minas Magri S.A.) and incubated in an environmentally controlled growth chamber (22°C; 16 h/8 h light/dark photoperiod; 120 µmol m□□ s□□ photosynthetically active radiation; 65% relative humidity). After 8 days, four seedlings per treatment were inoculated with 10 mL of each selected plant growth-

promoting (PGP) bacterial isolate (AM10, M4C3′, M4C15, and M5C1) at a final concentration of 1 × 10□ CFU mL□□. Control plants were inoculated with sterile water. Nondestructive measurements of plant height were recorded at 21 and 48 days post-germination. Plant height was analyzed using a Kruskal–Wallis nonparametric test ($\alpha = 0.005$, assuming a chi-square distribution with 4 degrees of freedom), followed by Conover–Inman post hoc pairwise comparisons conducted using SYSTAT 13 software, version 13.00.005 (Systat Software, Inc.).

## Results

### In vitro characterization of plant growth-promoting traits

The majority of the isolates (46) exhibited at least one plant growth-promoting (PGP) activity. However, a high degree of variability was observed among the different PGP traits. Isolates M4C13 and M4C15 presented the highest amylolytic activities, followed by isolates B1, AM8, M5C6, B5, and S15, whereas twelve isolates (M5C1, M5C7, B2, M4C14, CALA2, AM10, H4, B4, S8, S10, S12, and AM9) presented the lowest amylolytic activities.

With respect to protease activity, the largest halo diameters were observed in strains S9, M5C5, B2, M4C13, and M4C15, whereas the smallest halos were found in isolates B7, B8, S13, AM2, AM4, M4C6, AM6, M5C1, M5C7, AM10, S8, S10, S12, AM9, B1, AM8, and B5.

Among the tested bacteria, 24 isolates were capable of fixing atmospheric nitrogen. Isolates M4C3, M4C15, M5C7, M5C7′, and H4 showed the highest nitrogen fixation capacity, followed by the isolates M4C5, M4C16, M4C3′, M5C1, H3, and H9, which presented moderate fixation levels. In contrast, isolates B1, B3, B8, S9, S13, S14, CALA 2, AM1, AM2, AM4, AM5, AM8, and AM10 presented the lowest nitrogen fixation activity.

Only four bacterial isolates showed a high phosphate solubilization capacity (AM1, AM11, M4C1, and M4C3’). The remaining 25 phosphate-solubilizing isolates presented either moderate (isolates M4C5, M4C4, CALA 2, M4C3′, AM3, and AM7) or low solubilization capacity (B7, B8, S13, AM2, AM4, AM6, AM10, S8, S10, S12, AM9, B1, AM8, AM5, M4C16, S15, M5C7′, S9, B2, and H4).

The ability to produce IAA was detected in 31 isolates. Bacterial isolates B7, AM1, AM3, AM11, and SP10 produced the highest amounts of IAA, followed by isolates M5C1, B1, B4*, B8, S3, S14, AM5, AM6, AM7, AM8, and SP5. Isolates M4C1’, M4C3’, M4C5’, M4C6’, M4C9, M4C16’, M4C3b, M5C5, B3’, B5, S8’, S10’, S15, H3’, and AM10 also produced IAA but at lower concentrations.

Finally, of the 51 screened isolates, only eight were capable of producing siderophores. These included M4C16’, M4C3b, B8, S3, AM5, AM7, AM1, and AM3.

The PGP activities varied substantially among isolates, as revealed by two-way cluster analysis, which compared bacterial strains and their PGP traits. The analysis revealed 8 different clusters (Fig. 1). Cluster 1 included isolates with nitrogen fixation, and medium to high IAA production, medium to high phosphate solubilization and protease activity. This cluster was subdivided into two groups, one composed of isolates with high siderophore production and the other composed of isolates unable to produce siderophores. Cluster 2 included isolates exhibiting the highest amylolytic activity, with medium to high protease activity and low to moderate phosphate solubilization and IAA production; some isolates in this cluster were also nitrogen-fixing. Cluster 3 included isolates with high amylolytic activity, moderate protease activity, low IAA production and high phosphate solubilization, whereas Clusters 4 and 5 grouped isolates with moderate protease activity and high nitrogen fixation capacity. Cluster 5 differed from Cluster 4 due to its high amylolytic activity. Cluster 6 included isolates with high phosphate solubilization ability, as well as siderophore and IAA production. Cluster 7 consisted of isolates exhibiting one or no PGP traits. In contrast, Cluster 8 included isolates with medium to high IAA production and nitrogen fixation capacity.

On the basis of the two-way cluster analysis, ten representative strains were selected for further evaluation (Table 1): M4C1 (cluster 1), AM05 (cluster 1), B1 (cluster 2), AM10 (cluster 2), M4C3’ (cluster 4), M4C15 (cluster 5), M5C7’ (cluster 5), B7 (cluster 6), AM01 (cluster 6) and M5C1 (cluster 8).

Table 1
Plant growth promotion traits and molecular ID of selected strains.

| Isolate | ID | Cluster | Amylolytic activity | Protease activity | Phosphorus solubilisation | Nitrogen fixation | IAA production | Siderophores production |
|---|---|---|---|---|---|---|---|---|
| M4C1 | *Pantoea* sp. | 1 | - | +++ | +++ | - | + | - |
| AM05 | *Pseudomonas* sp. | 1 | - | +++ | ++ | + | ++ | + |
| B1 | *Bacillus* sp. | 2 | +++ | +++ | + | + | ++ | - |
| AM10 | *Bacillus* sp. | 2 | +++ | ++ | ++ | + | + | - |
| M4C3' | *Pseudomonas* sp. | 4 | - | +++ | - | +++ | + | - |
| M4C15 | *Chryseobacterium* sp. | 5 | +++ | +++ | - | +++ | - | - |
| M5C7' | *Arthrobacter* sp. | 5 | ++ | ++ | - | +++ | - | - |
| B7 | *Bacillus* sp. | 6 | - | ++ | ++ | - | +++ | - |
| AM01 | *Pseudomonas* sp. | 6 | - | - | +++ | + | +++ | + |
| M5C1 | *Microbacterium* sp. | 8 | +++ | - | - | ++ | ++ | - |

# Molecular identification of bacterial strains

The obtained bacterial sequences (GenBank codes: PX662079 to PX662086) were compared against the EZBioCloud and NCBI databases for taxonomic identification. Isolate M4C15 was identified as *Chryseobacterium* sp. (query cover 100%, identity 99.14%), whereas isolates AM10, B1, and B7 were identified as *Bacillus subtilis* (query cover 100%, identity 98.64%). Isolate M4C1 was identified as *Pantoea* sp. (query cover 100%, identity 97.45%), and M5C1 was identified as *Microbacterium* sp. (query cover 100%, identity 98.15%). Isolate M5C7' showed the closest match to *Arthrobacter* sp. (query cover 100%, identity 98.23%), and M4C3' was identified as *Pseudomonas* sp. (query cover 100%, Identity 100%). Finally, isolates AM01 and AM05 exhibited high similarity to *Pseudomonas* sp. (query cover 100%, identity 99.56%). In all the cases, the results of the morphological identification agreed with the molecular data.

**Cannabis sativa cloning improvement**

The ability of the bacterial isolates to increase *C. sativa* clone development was evaluated in an *in planta* assay. Inoculation with the selected microorganisms produced significant differences in clone survival among the treatments in both *C. sativa* varieties (Lambda = 1; P = 0.000 for MS and Lambda = 1; P = 0.000 for BF), demonstrating a beneficial effect on seedling establishment (Fig. 2**).**

For the MS cuttings, both the control group and the groups treated with commercial rooting hormones M5C7′, AM05, M4C15, and M5C1 showed 50% survival. Interestingly, when cuttings were inoculated with strain M4C1, the percentage of rooted seedlings increased to 67%, while inoculation with strains B1, AM01, AM10, and M4C3 resulted in 83% of rooted seedlings. In contrast, inoculation with strain B7 resulted in a lower survival rate compared to the control group (Fig. 2a).

For the BF variety, 67% of the plants in the control group survived, which was also observed in the cuttings inoculated with strains M4C5 and B7. However, the survival rate (50%) of the cuttings treated with commercial rooting hormone was lower than that of the control cuttings. In contrast, inoculation with strains M4C1, B1, AM01, AM10, M5C1, and M4C3′ resulted in greater survival (83%) relative to the control. The highest survival rate (100%) was achieved with strains M5C7′ and AM05 (Fig. 2b).

When the rooted clones were evaluated, clear differences were observed between the two cultivars. In the MS variety, uninoculated cuttings produced a moderate root system with an average root length of 9.6 cm. In contrast, control cuttings of the BF variety showed almost no root development. For both cultivars, all treatments promoted root formation to some extent.

In BF cuttings, strains AM10, AM01, B1, AM05, and M4C3′ significantly increased root length compared with the control group (KW = 23.125, df = 11, p = 0.017). Plants inoculated with bacterial strains developed roots ranging from 10.6 to 13.3 cm in length (AM10, AM01, B1, AM05), while the commercial hormone treatment produced roots averaging 9.0 cm in length. Although M4C3′ induced a smaller root system (6.85 cm), this size was still significantly greater than that in the control group (Fig. 3a).

In the MS cultivar, a positive effect on root elongation was also observed; however, due to high variability, statistical significance was not achieved (KW = 18.449, df = 11, p = 0.072). Nevertheless, cuttings inoculated with strains M4C3′ and AM10 showed a clear trend toward greater root development (average root lengths of 27.5 cm and 20.65 cm, respectively), followed by those inoculated with M4C1 and AM01 (16.85 cm and 13.95 cm, respectively), compared with control plants (9.6 cm). Interestingly, MS cuttings treated with the commercial rooting hormone presented a shorter root length (8.5 cm) than the control group (Fig. 3b).

# Microbial root colonization analysis by optical and scanning electron microscopy

The isolated bacteria were able to colonize the root tissues of 21-day-old seedlings of both Cannabis cultivars (Fig. 4b-e). All treated clones showed a high number of bacterial cells adhered to the root surface. However, isolates M4C1, M5C1 and AM10 were also capable of penetrating the root tissues (Fig. 4f). A higher number of bacterial cells (bacterial cluster) were observed when the clones were inoculated with the strains M4C1, M4C3 and M4C15, probably due to the production of a thin and transparent layer that was observed attached to the root surface (Fig. 4g-h).

### Cannabis sativa seed inoculation with PGPB

*Cannabis sativa* seeds (cultivar *Fruti*) were inoculated with four different PGP bacterial strains. After 48 days, a positive effect on plant growth, mainly reflected by increased plant height, was observed in those seeds inoculated with the strains M4C3', M5C1, and AM10 ($KW = 11.093$, $df = 4$, $p = 0.026$). Plants derived from seeds inoculated with strain AM10 were, on average, 12% taller than those in the control group, whereas those inoculated with strain M5C1 were 14% taller. The greatest increase in height was observed in seeds inoculated with strain M4C3', which were 16% taller than those in the control group; although significant differences in plant height were observed between inoculated and control plants, no significant differences were detected among the different bacterial treatments (Fig. 5).

## Discussion

This study analyzes the plant growth-promoting (PGP) properties of native Patagonian rhizobacteria and provides valuable insights into the use of plant growth-promoting rhizobacteria (PGPR) as biofertilizers for different cultivars of *Cannabis sativa.* Additionally, this study explored the potential application of PGPR at various stages of production.

*In vitro* assays revealed that bacterial isolates displayed a high degree of variability in their PGP traits. Interestingly, high variability was observed among isolates, even within the same species. For instance, *Bacillus* sp. B1 and AM10 were grouped in the cluster characterized by the highest amylolytic activity, moderate to high protease activity, and low to moderate phosphate solubilization and indole acetic acid (IAA) production. In contrast, *Bacillus* sp. B7 clustered separately (cluster 6) among the isolates with high phosphate solubilization capability as well as siderophore and IAA production. In this respect, it has already been reported that different *Bacillus* sp. strains, even those from the same species, have different PGP traits. Additionally, [22] reported that strains of *B. pumilus* have different effects on siderophore and IAA production, while [23] reported that *Bacillus* sp. strains have different capacities to produce IAA and siderophores, to solubilize phosphorous, and have different proteolytic and antifungal activities.

Similarly, *Pseudomonas* sp. AM05, AM01, and M4C3´ were classified into clusters 1, 6, and 4, respectively, highlighting the intragenus and intraspecific diversity in the PGP profiles. However, the strains AM05 and AM01 were the only *Pseudomonas* strains studied that could produce siderophores. Consistently, strains AM05 and AM01 had higher identity with *P. caricapapayae*, while the M4C3´ DNA sequence was assigned to a different species. *Pseudomonas* aerobic growth is highly dependent on the presence of Fe; thus, most *Pseudomonas* species produce siderophores; moreover, it has been reported that *Pseudomonas* species belonging to the same genomic group (likely the fluorescent *P. syringae* group) produce a specific pyoverdine [24].

Ten representative strains were selected to assess their ability to enhance *C. sativa* cloning and promote plant growth: *Pantoea* sp. M4C1 (cluster 1), *Pseudomonas* sp. AM05 (cluster 1), *Bacillus* sp. B1 (cluster 2), *Bacillus* sp. AM10 (cluster 2), *Pseudomonas* sp. *s* M4C3' (cluster 4), *Chryseobacterium* sp. M4C15 (cluster 5), *Arthrobacter* sp. M5C7' (cluster 5), *Bacillus* sp. B7 (cluster 6), *Pseudomonas* sp. AM01 (cluster 6), and *Microbacterium* sp. M5C1 (cluster 8). Inoculation with the selected microorganisms resulted in significant differences in clone survival among treatments, with variable effects depending on both the *C. sativa* variety and the bacterial isolate. However, for both Cannabis varieties, *Pantoea* sp. M4C1, *Pseudomonas* sp. M4C3' and AM01, and *Bacillus* sp. AM10 and B1 showed the higheest improvement in clone survival.

*Pantoea* sp. have been previously described as a species that may contribute to plant growth by improving nutrient availability, producing phytohormones, and biocontrol, reducing harmful microbiological populations by competence or through the production of biocontrol agents. This rod-shaped, non-spore-forming, gram-negative bacterium is a diverse and versatile species that has been isolated from different environments and has a high ability to adapt to different conditions [25]. Luziatelli et al. [25] stated that *P. agglomerans* C1 produces molecules which positively affect the *de novo* root formation and their morphology and efficiency. Interestingly, bacteria from this species are known to have PGP traits and also to inhibit plant pathogens by producing pantocins and other antimicrobial compounds, as well as being able to prime plant defense mechanisms. *Pantoea* sp. M4C1 showed phosphate solubility ability and the capacity to produce IAA. Similarly, Luziatelli et al. [25, 26] reported that *P. agglomerans* C1 (isolated from the phyllosphere of lettuce) was also able to solubilize phosphate and produce IAA. It has already been stated that IAA and auxin-related compounds produced by bacteria can induce modifications in root tissues and their system architecture. Similarly, *Pseudomonas* spp. and *Bacillus* spp. strains are well known for their PGP capabilities and their possible use as bioinoculants. Several horticultural crops inoculated with *Pseudomonas* spp. strain have shown increased root and shoot fresh weight and simultaneous suppression of deleterious pathogenic microflora. Additionally, *Pseudomonas* spp. strains may increase peanut seed germination under field conditions [27]. Similarly, strains of *Bacillus* spp. can also increase the growth, yield, and biomass of plants by increasing the availability of nutrients, the synthesis of indole-3-acetic acid, nitrogen fixation, and phosphorous solubilization [28]. Additionally, *Bacillus* strains were shown to induce lateral root formation in pine seedlings and to colonize their roots [29].

On the other hand, the strains M5C7´ and M4C15 were classified as belonging to cluster 5 and identified as *Arthrobacter* sp. and *Chrisobacterium* sp., respectively. Members of the *Arthrobacter* genus are known to be endophytic and to utilize organic compounds as substrates of metabolism (proteolytic and amylolytic activity), facilitating resource acquisition by plants and thus promoting their growth [30]; however, in our case, *Arthrobacter* sp. M5C7´ did not show an endophytic behavior, suggesting that it can also colonize roots as rhizobacteria. Members of *Chrisobacterium* are known to harbor genes that commonly aid in nitrogen cycling. Indeed, it has been reported that genes controlling nitrogen-related gene expression under nitrogen-limiting conditions are conserved in all species, whereas genes related to nitrogen fixation are strain specific [31]. Strain M5C1 was classified in cluster 8 and belongs to the genus *Mycobacterium*; members of these genera are known to produce IAA, and some of the species in the genus, such as *Microbacterium kunmingense*, have been shown to be able to fix nitrogen [32, 33]. The M5C1 strain has high similarity (98.15%) with *Microbacterium keratanolyticum* and is able to produce IAA and fix nitrogen. Nevertheless, in our assays, strains M5C7´, M4C15, and M5C1 had moderate effects on improving clone survival or promoting root development.

One of the factors that defines the ability of any bacteria to promote plant growth is their efficacy to colonize plant roots, which is known to be influenced by root exudate signals for colonization by beneficial microbes [34]. The studied strains were observed to be associated with the root surface of inoculated clones. Notably, some isolates—particularly *Pantoea* sp. M4C1, *Microbacterium* sp. M5C1, and *Pseudomonas* AM10—were also capable of colonizing internal root tissues, suggesting an endophytic lifestyle. In this respect, M4C1 was identified as *Pantoea* sp. with high identity with the endophytic species *Pantoea agglomerans*. Indeed, it has been stated that the endophytic strain *P. agglomerans* 33.1 was able to form biofilms on the root surface of sugarcane and form aggregates around the roots, infecting it through radicular fissures [35]. The fact that this strain was isolated from *Eucalyptus* plants suggests a lack of specificity of *P. agglomerans* to a particular host [35]. This could be the case for *Pantoea* sp. M4C1, which was isolated from the *Austrocedrus chilensis* rhizosphere and colonized the roots of *C. sativa*. On the other hand, members of *Microbacterum* and *Bacillus* have also been reported to have an endophytic lifestyle. Nevertheless, endophytic colonization was not associated with enhanced plant growth-promoting effects with respect to nonendophytic strains since B1 and M4C3’, which were not detected to be present inside the roots, but also showed to promote plant growth of *C. sativa* clones to a similar or higher extent than the endophytic strains did.

When these isolates were applied as seed inoculants, a positive impact on plant growth was also observed, confirming their potential as bioinoculants. On average, plants derived from inoculated seeds were 12–16% taller than those from the control group. Although all isolates promoted plant growth to some degree, their effects were variable.

In general, the most promising and effective isolates—*Bacillus* sp. B1 and AM10 (cluster 2), *Pseudomonas* sp. M4C3’ (cluster 4) and AM01 (cluster 6)—showed consistent benefits across all the tested *C. sativa* cultivars, improving clone survival, root development, and plant height (Table 2). These strains presented nitrogen fixation capacity and the ability to produce IAA. N promotes vegetative growth and plant health in *C. sativa*. Indeed, in the vegetative stage, *C. sativa* requires 160 mg/L N for optimal biomass production and physiological function [36]. On the other hand, IAA can induce seed germination, root development, and early seedling growth [37].

Table 2
Effect in clone survival, clone root length, type of root colonization and plant growth promotion of selected strains.

| Species | Strain | Clade | Clone survival MS | Clone survival BF | Clone root length MS | Clone root length BF | PGP through seedling inoculation | Root adherence | Inner colonization | Adherence layer |
|---|---|---|---|---|---|---|---|---|---|---|
| *Pantoea* sp. | M4C1 | 1 | ++ | ++ | - | +++ | | Yes | Yes | Yes |
| *Pseudomonas* sp. | AM05 | 1 | + | +++ | +++ | - | | Yes | No | No |
| **Bacillus** sp. | **B1** | **2** | **+++** | **++** | **+++** | **++** | | Yes | No | No |
| **Bacillus** sp. | **AM10** | **2** | **+++** | **++** | **+++** | **+++** | **++** | Yes | **Yes** | No |
| **Pseudomonas** sp. | **M4C3'** | **4** | **+++** | **++** | **++** | **+++** | **++** | Yes | No | **Yes** |
| *Chryseobacterium* sp. | M4C15 | 5 | + | + | - | ++ | - | Yes | No | Yes |
| *Arthrobacter* sp. | M5C7' | 5 | + | +++ | - | ++ | | Yes | No | No |
| *Bacillus* sp. | B7 | 6 | - | + | + | ++ | | Yes | No | No |
| **Pseudomonas** sp. | **AM01** | **6** | **+++** | **++** | **+++** | **++** | | Yes | No | No |
| *Microbacterium* sp. | M5C1 | 8 | + | ++ | + | - | ++ | Yes | Yes | No |

## Conclusions

Overall, the *in vivo* results highlight that bacterial inoculation not only enhances clone rooting and establishment but also promotes general plant growth in the varieties of *C. sativa* studied. These findings suggest that carefully selected PGPR strains can play crucial roles in improving propagation efficiency and plant vigor in medicinal cannabis cultivation.

From a broader perspective, this study underscores the potential of harnessing native Patagonian PGPR as sustainable biofertilizers for high-value crops such as *C. sativa*. By reducing the dependence on synthetic agrochemicals, these microbial inoculants contribute to environmentally friendly cultivation practices while supporting plant productivity and health. Furthermore, the demonstrated variability in PGP mechanisms among isolates provides a foundation for future work focused on optimizing bacterial consortia tailored to specific cultivars or growth stages. This work, therefore, represents an important step toward integrating microbial biotechnology into the emerging medicinal cannabis industry, promoting both economic and ecological sustainability.

## Abbreviations

ATP
Adenosine triphosphate
BF
Ballena Franca
CAS
chrome azurol S
CPD
Critical point drying (CPD)
dNTP
Deoxynucleotide Triphosphates
IAA
Indole-3-acetic acid
LB
Luria–Bertani
MS
Mariquita Sánchez
NCBI
The National Center for Biotechnology Information
Nfb
N-free malate
PCR
Polymerase Chain Reaction
PGP
Plant growth–promoting
PGPR
Plant growth-promoting rhizobacteria
ST1
Standard medium 1

## Declarations

**Ethics approval and consent to participate:** Not applicable

**Consent for publication:** Not applicable

**Availability of data and materials:** Data is provided within the manuscript. The bacterial sequences obtained were deposited on GenBank under the codes PX662079 to PX662086.

**Competing Interests:** The authors declare no competing interests.

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**Authors' contributions:**

**Jorge A. Marfetán:** Molecular identification of bacterial strains, *Cannabis sativa* cloning improvement, Microbial root colonization analysis by optical and scanning electron microscopy, Plant growth promotion achieved through PGPR seed inoculation, Conceptualization, Formal and statistical analysis, Project administration, Writing—original draft, Writing—review and editing, Funding acquisition.

**Leila Joaquin:** *In vitro* characterization of the plant growth-promoting traits of the strains, *Cannabis sativa* cloning improvement, Writing—review and editing.

**Francisco Fernandez Torne:** *Cannabis sativa* cloning improvement, Writing—review and editing.

**Romina Cangelosi:** *Cannabis sativa* cloning improvement, Plant growth promotion achieved through PGPR seed inoculation, Writing—review and editing.

**María L. Vélez:** Funding acquisition, Project administration, Writing—review and editing.

**Yanina L Idaszkin:** *Cannabis sativa* cloning improvement, Conceptualization, Writing—review and editing.

**Micaela Pescuma:** Funding acquisition, Project administration, *In vitro* characterization of the plant growth-promoting traits of the strains, Conceptualization, Project administration, Writing—original draft, Writing—review and editing.

**Gregorio Bigatti:** *Cannabis sativa* cloning improvement, Microbial root colonization analysis by optical and scanning electron microscopy, Conceptualization, Writing—review and editing.

**Omar F. Ordoñez:** *In vitro* characterization of the plant growth-promoting traits of the strains, Molecular identification of bacterial strains, Plant growth promotion achieved through PGPR seed inoculation, Conceptualization, Funding acquisition, Project administration, Writing—review and editing.

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## Figures

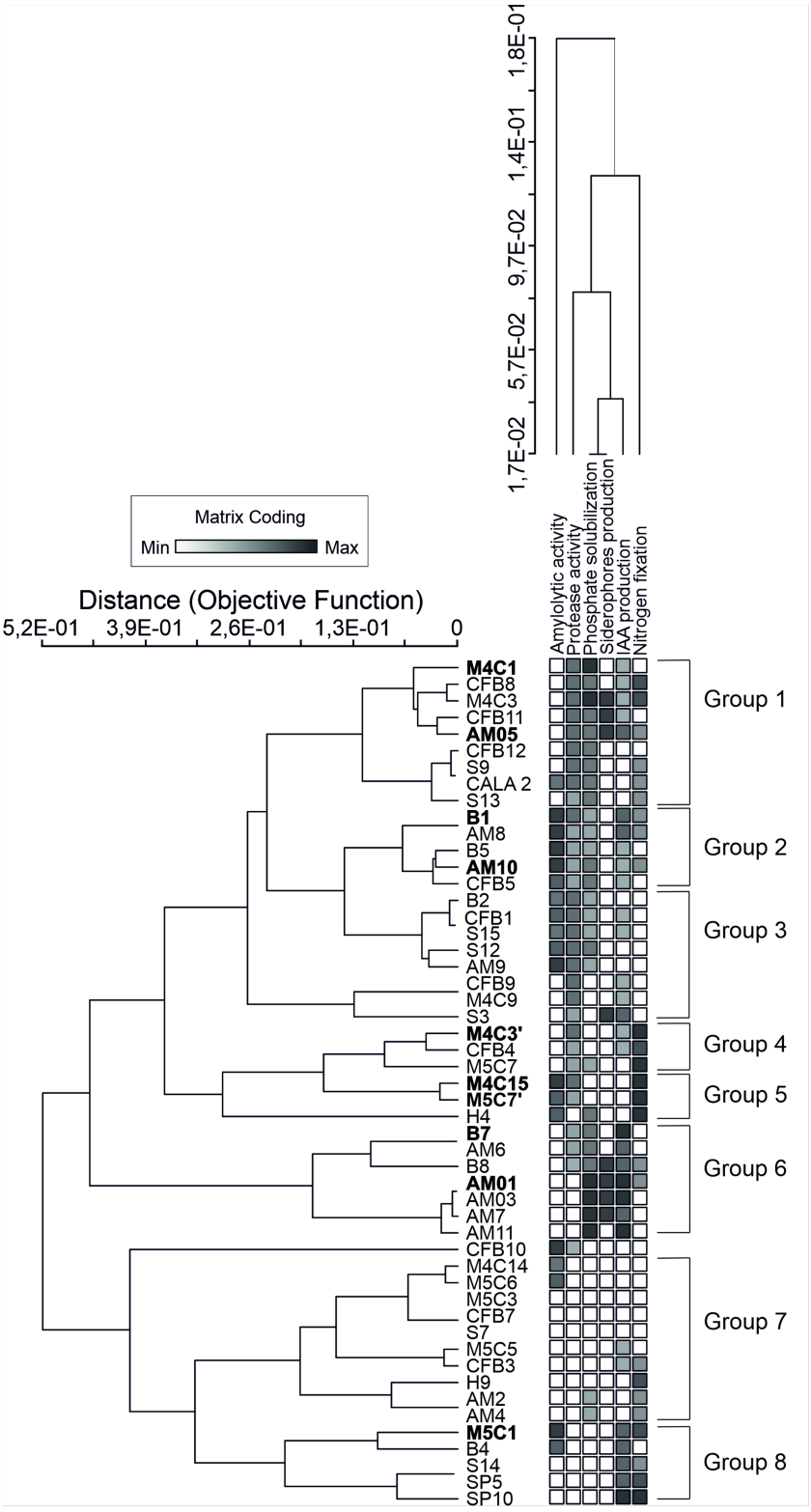


Figure 1

Two-way cluster analysis evaluating differences in the PGP traits among different bacterial isolates.

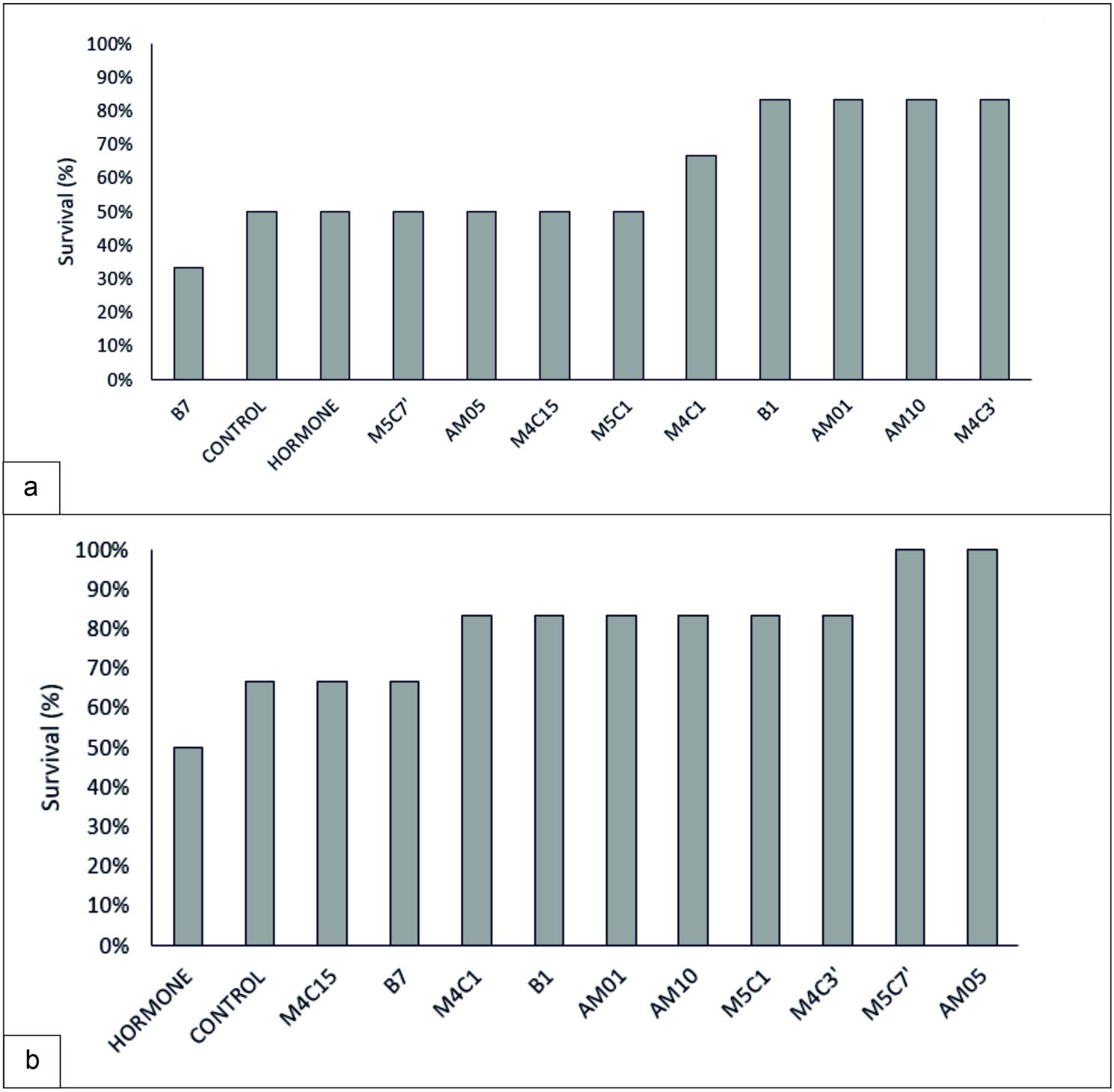


Figure 2

Clone survival, expressed as a percentage, among the different treatments in both *C. sativa* varieties. A-Results in Mariquita Sanchez clones. B- Results for Ballena Franca clones.

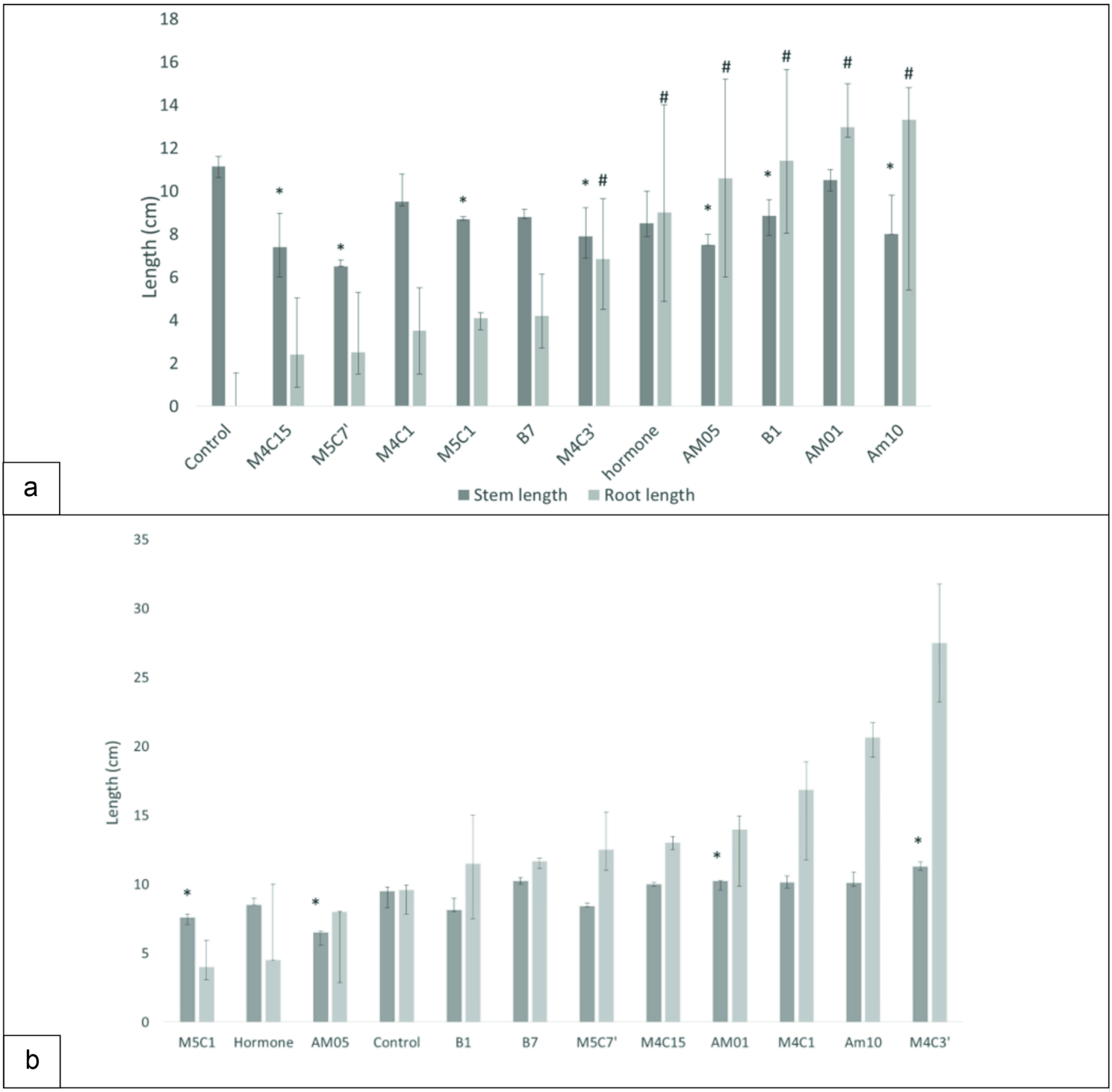


Figure 3

Stem and root length in plants inoculated with different PGP strains, expressed as medians and quartiles. A- Plants of the Ballena Franca cultivar, B- Plants of *C. sativa* var. Mariquita Sanchez. Symbols refer to significant differences compared with the control group.

Image not available with this version

Figure 4

Optical microscopy and SEM images of fresh roots obtained from Cannabis clones inoculated with PGPB. The figure shows Cannabis seedling roots with and without bacteria. A- Control group. B−E- Bacterial adherence to the root surface (arrow). F- Internal Colonization. G−H- Bacteria actively colonize the plant on the root surface in the presence of a thin transparent layer, forming clusters of bacterial cells (arrow).

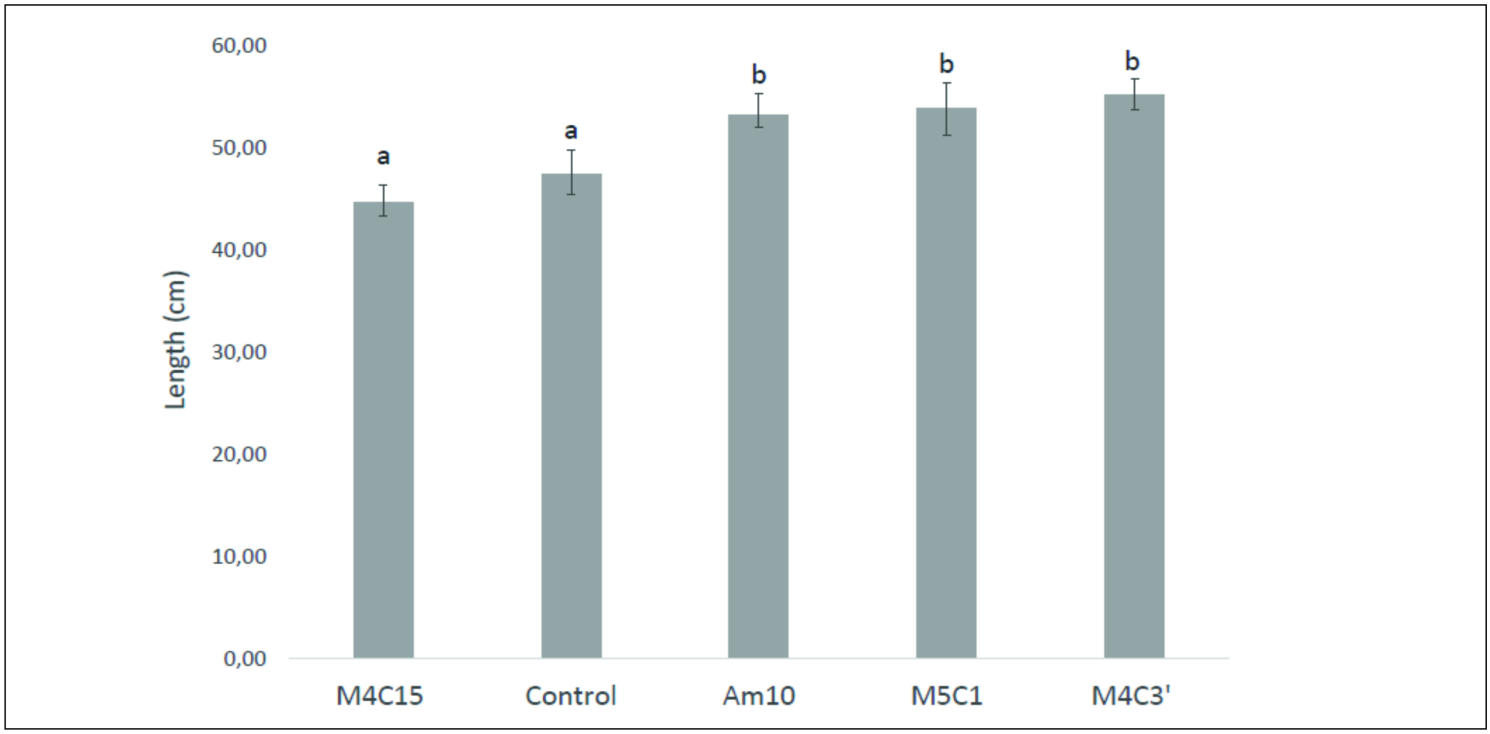


Figure 5

Plant-growth promotion of selected strains, measured as plant height, in *C. sativa,* expressed as medians and quartiles. Different letters indicate significant differences within groups.