

1 **Heavy metal tolerant *Pseudomonas protegens* isolates from agricultural well water in**
2 **northeastern Algeria with plant growth promoting, insecticidal and antifungal activities**

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6 Bensidhoum Leila¹, Elhafid Nabti¹, Nassira Tabli¹, Kupferschmied Peter², Angelo Weiss³,
7 Michael Rothballer³, Michael Schmid³, Christoph Keel², and Anton Hartmann³

8

9 ¹ Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la Nature et de
10 la Vie, Université de Bejaia, 06000 Bejaia, Algeria

11 ² Université de Lausanne, Département de Microbiologie Fondamentale (DMF), Quartier
12 UNIL-Sorge, Batiment Biophore, CH-1015 Lausanne, Switzerland

13 ³ Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH),
14 Research Unit Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D-85764 Neuherberg,
15 Germany

16

17 Corresponding author:

18 Prof. Dr. Anton Hartmann, Helmholtz Zentrum München, German Research Center for
19 Environmental Health (GmbH), Department for Environmental Sciences, Research Unit
20 Microbe-Plant Interactions, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

21 Phone: 0049 89 3187 2852

22 Fax: 0049 89 3187 3382

23 e-mail: a.hartmann@helmholtz-muenchen.de

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26 Abstract

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28 The application of plant growth promoting bacteria (PGPB) with biocontrol activities as
29 inoculants of crop plants against phytopathogenic fungi and insect pests provide a biological
30 alternative to the use of agrochemicals. Two *Pseudomonas protegens* strains were isolated
31 from agricultural well water in a heavy metal contaminated area near Bejaia, northeastern
32 Algeria. The isolates S4LiBe and S5LiBe had 16S rRNA gene sequence similarities of 99.4%
33 to 99.7% with *P. protegens* CHA0^T and other *P. protegens* strains. The phenotypic profiles
34 tested with BIOLOG-GN2-microplates showed differences in 12 of 95 carbon sources tested,
35 as compared to the type strain *P. protegens* CHA0^T. The isolates S4LiBe and S5LiBe showed
36 plant growth promoting potential which is commonly associated with the production of the
37 phytohormone indole acetic acid and siderophores and the solubilization of insoluble
38 phosphate. In addition, they produce chitinase and other polymer degrading enzymes. As the
39 strains S4LiBe and S5LiBe were isolated from heavy metal polluted well water, they are
40 resistant against several heavy metals (2.0mM K₂Cr₂O₇ and 3.0mM CoSO₄, HgSO₄, CdSO₄
41 8H₂O and PbCl₂), while the reference strain *P. protegens* CHA0^T was very sensitive to Hg²⁺
42 and Cd²⁺ and had lower tolerance towards Co²⁺ and Pb²⁺. The isolates S4LiBe and S5LiBe
43 were active in mycelial growth inhibition assays against *Botrytis cinerea*, *Verticillium dahlia*,
44 *Fusarium graminearum*, *Aspergillus niger* and *A. flavus* (growth inhibition between 88% and
45 48%). Furthermore, S4LiBe and S5LiBe showed effective insecticidal activities, when tested
46 in the *Galleria* injection assay and they were tested positive for the insect toxin gene *fitD*
47 alike the reference strain CHA0^T. Finally, inoculation of barley seeds with S5LiBe in non-
48 polluted agricultural soil significantly stimulated the germination rate and growth of
49 seedlings, with increased shoot length (11.96cm ± 0.59), shoot and root fresh weight
50 (0.10g±0.009, 0.04g±0.006), shoot and root dry weight (0.075g±0.003, 0.03g±0.007) as

51 compared to non-inoculated plants ($10.23\text{cm}\pm 0.84$, $0.06\text{g}\pm 0.007$, $0.025\text{g}\pm 0.006$,
52 $0.047\text{g}\pm 0.006$, and $0.016\text{g}\pm 0.004$, respectively). In heavy metal contaminated soil, inoculation
53 with strain S5LiBe resulted in similar increase of germination rate and growth parameters of
54 barley like in the non-polluted soil, while *P. protegens* CHA0^T inoculated plants were not
55 stimulated. Thus, the heavy metal tolerant isolates S4LiBe and S5LiBe have a potential as
56 beneficial bacteria for agricultural application even in heavy metal polluted soils, e.g. for the
57 stimulation of biomass crops. The demonstration of successful isolation from agricultural well
58 water may open more ready access for a wide variety of this kind of beneficial bacteria for
59 agricultural application.

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61 **Keywords:** *Pseudomonas protegens*, heavy metal tolerance, plant growth promoting bacteria,
62 biocontrol and insecticidal activity, enzymatic activities, barley

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1. Introduction

Inoculation with plant growth promoting bacteria (PGPB) represents an agro-biotechnology designed to improve growth, yield and health of agricultural crops [53, 55]. The use of these beneficial microorganisms appears to be cost-effective, ecosystem friendly and a healthy alternative to the extensive use of chemicals such as fungicides, herbicides and insecticides, which have negative impacts on the environment and human health.

The potential of PGPB to increase crop production involves the solubilization of inorganic phosphate and ferric iron minerals, the increased uptake of mineral nutrients [37], the ability to reduce stress ethylene production in plants [21], N₂ fixation [35], and the production of plant hormones such as auxins, gibberellins and cytokinins [20]. PGPB are able to control pathogens by several mechanisms: competitive root colonization, production of antimicrobial compounds [13], production of hydrolytic enzymes, siderophores, HCN, ammonia, and by inducing systemic resistance of plants towards phytopathogens [12, 53]. In addition, insecticidal activities were reported for some PGPB in addition to the existing biocontrol repertoire with perspectives for application against insect crop pests [52]. Recent reports suggest that PGPB also enhance the tolerance of plants towards abiotic stresses such as drought [58, 62], chilling injury [3], salinity [25], metal toxicity [16] and elevated temperature stress [1, 2].

A high diversity of PGPB have been identified which enhance plant growth by several mechanisms. These PGPB belong for example to the bacterial genera *Pseudomonas*, *Azospirillum*, *Cellulosimicrobium*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* [11, 20, 24, 30, 39, 40, 43].

Among PGPB, *Pseudomonas* spp. have been broadly studied for their roles in plant growth promotion and biological control [18, 19, 24, 28, 34, 50, 63]. Plant growth promoting

activities of *Pseudomonas* spp. include production of indole acetic acid (IAA) [18], phosphate solubilization [64], degradation of toxic compounds [42] and production of bioactive metabolites against phytopathogens like the production of siderophores [8] and antibiotics [24, 27] and of insect toxins against insect pests [32, 45].

Due to industrialization, environmental pollution by heavy metals is a common problem all over the world. However, heavy metal contamination of soils and water may also originate from natural occurring geominerals. Nevertheless, in many sites anthropogenic activities have enriched or deposited huge amounts of heavy metal containing rocks, soil and debris, which are the sources of pollution plumes into the surrounding soil and water bodies. In heavy metal-polluted sites, heavy metal-resistant bacteria have been found, which face the pollution and even have the ability to reduce it by bioremediation activities, partly in combination with plants [42].

In the present investigation, heavy metal contaminated agricultural well water was tested for the presence of plant growth promoting bacteria, because this water was fed from heavy metal polluted soil. The contamination originated from a lead mineral containing rock deposit in some distance, origination from lead mining, which is no longer in operation. The hypothesis was that rhizosphere colonizing PGPB should be part of the bacterial community of the well water. A special emphasis was laid on the *Pseudomonas* group of bacteria, because these have a wide spectrum of plant beneficial activities ranging from antagonistic activities against a wide range of phytopathogenic fungi, insecticidal activity, and phytohormone, siderophore and extracellular enzyme production. The aim was to get PGPB-isolates which could be demonstrated to stimulate plant growth in non-polluted and heavy metal polluted soil.

2. Material and Methods

2.1 Isolation of bacteria from heavy metal polluted well water

Plant growth promoting bacteria were isolated from heavy metal polluted well water (receiving effluents from agricultural soil) in the region of Bejaia (northern Algeria). The well water had the following contamination levels (ppm): Cr 65.65 $\pm$ 0.22; Cd 8.6 $\pm$ 0.23; Hg 3.87 $\pm$ 0.12; Co 21.0 $\pm$ 0.42; Pb 212.45 $\pm$ 0.85; As 2.3 $\pm$ 0.1; and Zn 133.80 $\pm$ 0.88. The soil in this region is contaminated with heavy metals as follows (ppm): Cr 52.0 $\pm$ 0.43; Cd 8.2 $\pm$ 0.12; Hg 3.54 $\pm$ 0.12; Co 19.19 $\pm$ 0.62; Pb 199.51 $\pm$ 1.3; As 3.12 $\pm$ 0.08; Zn 125.12 $\pm$ 0.96. (Laboratory of physico-chemical soil analysis, PROFER-company, Mostaganem-Bejaia, Algeria). Water samples were serially diluted in sterile distilled water and 0.1 ml of each dilution was seeded in triplicates onto nutrient broth (NB) agar plates containing in g/l: trypton 10g, meat extract 5g, NaCl 5g from bioKar diagnostics and agar-agar 18g from Liofilchem. The agar plates were incubated at 28°C for 1 week and colonies with different morphologies were selected, re-streaked on nutrient agar medium, and checked for purity. Originally 11 isolates were randomly picked and on the basis of antifungal and enzymatic activities as well as molecular phylogenetic analysis the *Pseudomonas protegens* isolates were obtained.

2.2 Phenotypic and molecular phylogenetic characterization:

The isolates were tested for the Gram-staining following the standard protocol [5]. The motility test was carried out on mannitol mobility gels according to Guiraud and Galzy [23]. Selected isolates and reference strains were phenotypically characterized carbon source utilization using the Biolog GN2 MicroPlate™ with 95 different carbon sources according to

the manufacturer's instruction. For the phylogenetic characterization, genomic DNA extraction from pure bacterial colonies was carried out using the FastDNA® SPIN kit in conjunction with the FastPrep FP120 instrument (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. The genomic DNA was further PCR amplified for 16S rDNA gene sequencing using the flanking primer pair 616F (5'AGA GTT TGA TYM TGG CTCAG 3') and 630R (5' CAK AAA GGA GGT GAT CC 3') resulting in the amplification of the whole 16S rRNA-sequence (1500 nucleotides). The amplification program was performed in a thermocycler peqSTAR 96 Universal, with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of 45s denaturation at 94°C, 45s annealing at 55°C, 1 min extension at 72°C, and a final extension step at 72°C for 10 min. Correct amplification was tested with standard horizontal agarose gel electrophoresis followed by ethidium bromide staining. Amplification products were cloned using the StrataClone PCR cloning system. Clones were then sequenced using the Big Dye Terminator Labeling Kit (Applied Biosystems, Foster City, USA). For phylogenetic analyses the obtained 16S rDNA-sequences were aligned with the *Sina Aligner V1.2.11* on the Silva website (www.arb-silva.de) and phylogenetical allocated with the software package ARB [36]. Phylogenetic tree construction was performed by using the Maximum-Likelihood [44].

2.3 Characterization of plant growth promoting properties:

2.3.1 Production of indole acetic acid (IAA)

IAA production was measured using the method described by Bric et al. [6]. The isolates were grown in LB liquid medium (containing in g/l: NaCl 4, trypton 10, yeast extract 5) supplemented with 0.5% glucose and 500 µg/ml L-tryptophan using 100 rpm shaking

conditions at 30°C for 72h. 5 ml of culture was centrifuged at 9000 rpm for 20 min and 2 ml of supernatant was transferred to a fresh tube to which 100µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0, 5 M FeCl₃ in 50 ml of 35% HClO₄) were added. The mixture was incubated at room temperature for 25 min and the absorbance of pink color developed was read at 530 nm.

2.3.2 Phosphate solubilization

The isolates were tested for inorganic phosphate solubilization. Freshly grown bacterial culture was inoculated to Pitkovskaya agar containing inorganic phosphate and incubated at 30°C for 5 days. A clear halo around the bacterial colony indicates solubilization of mineral phosphate [57].

2.3.3 Cellulase Activity

Bacteria were inoculated in CMC (Carboxy Methyl Cellulose) agar containing (g/l): Na₂HPO₄ 6, KH₂PO₄ 3, NaCl 0.5, NH₄Cl 1, yeast extract 3, CMC 7, agar-agar 15 and incubated at 30°C for 8 days. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was inundated with solution of Congo red (1% w/v) for 20 min. Congo red solution was then poured off, and the plates were further treated with 1 M NaCl [9]. Clear zones around the colony indicated the production of extracellular cellulase.

2.3.4 Esterase activity

The isolates were inoculated in the media which is described by Sierra [60], containing (g/l) peptone 10, NaCl 5, CaCl₂ 2H₂O 0.1, agar-agar 18, and 1% of sterilized tween 80. After incubation at 30°C for 48h a clear halo around the colonies demonstrated esterase activity.

2.3.5 *Lipolytic activity*

Lipolytic activity was determined as described above for the determination of esterase activity [60]. In this experiment, tween 80 was replaced by tween 20. Clear halos around the colonies indicate lipolytic activity.

2.3.6 *Protease activity*

Protease activity tests were carried out in skim milk agar plate medium, containing (g/l) casein hydrolysate 10, yeast extract 5, NaCl 4, skim milk powder 20, agar-agar 15. After incubation at 30°C for 48h, the presence of a clear zone around colony was the indication of protease production [29].

2.3.7 *Urease activity*

Urease activity was determined as described by Christensen [14]. The agar-medium contains (g/l) peptone 1, glucose 1, NaCl 5, Na₂HPO₄ 1.2, KH₂PO₄ 0.8, phenol red 0.012, and agar-agar 15. After autoclaving, 50 ml of urea solution (40%) were added. Urease production was indicated by a bright pink color around colony.

2.3.8 *Siderophore production*

The experiments were carried out in Chrome Azurol S agar, according to the method of Schwyn and Neilands [59]. CAS agar was prepared from four solutions, which were sterilized separately before mixing: the Fe-CAS indicator solution, buffer solution, nutrient solution and casamino acid solution. After autoclaving and at a temperature of about 50°C, nutrient solution and casamino acid solution were added to the buffer solution. The indicator solution was added last with sufficient stirring to mix the ingredients. The isolates were streaked on

the surface of the blue agar plates, incubated at 30°C for 3 days, and examined for growth and production of red to orange halos surrounding the colonies, indicating siderophore production.

2.3.9 Chitinase Activity

Chitinase activity was determined as described by Kopečný et al. [31]. Bacterial isolates were inoculated in minimal salt medium containing 0,8% of colloidal chitin as sole carbon and energy source and the following ingredients (g/l): K₂HPO₄ 2.7, KH₂PO₄ 0.3, MgSO₄ 7H₂O 0.7, NaCl 0.5, KCl 0.5, yeast extract 0.13, agar-agar 15. The plates were incubated at 30°C for 7 days. Clear zone around the colonies indicate extracellular chitinase activity.

2.4 Characterization of biocontrol activities

2.4.1 Antifungal activity

The isolates were assayed for antifungal activities against *Botrytis cinerea*, *Verticillium dahlia* and *Fusarium graminearum* as described by Sagahón et al. [56]. 1cm² fungal plug was inoculated in the center of Luria Bertani (LB) plate and each bacterial isolate was inoculated at a distance of 2.5 cm from the fungal inoculum. Plates without potential bacterial antagonist served as negative control. Three replicates were performed for each confrontation experiment. The plates were then incubated at 25±2°C for 5 days and verified every day. The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula: $PGI (\%) = \frac{KR - R1}{KR} \times 100$ where KR corresponds to the distance from the point of inoculation to the colony margin on the control dish (mm). R1 represents the distance (mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes.

2.4.2 Insecticidal activity

As biological insecticidal activity test, the *Galleria mellonella* virulence assay was performed. The injection assays for virulence determination used last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) as described before by Péchy-Tarr et al., [45] with 18 larvae per tested bacterial strain.

To test the presence of the *fitD* toxin gene *fitD* specific primers were applied with DNA extracted from the bacteria. The polymerase chain reaction was performed using the GoTaq DNA Polymerase kit (Promega) according to the manufacturer's instructions and the primer pairs *fitD*-screen-F: 5'-CCTGCTCAATACCCTGATCG-3' and *fitD*-screen-R: 5'-GTGGTTGGCGAAGTACTGCTC-3' (P. Kupferschmied, unpublished).

2.5 Determination of heavy metal tolerance

Heavy metals incorporated media were used to examine the ability of the isolates to resist heavy metals. Cells of overnight grown cultures were inoculated on nutrient agar plates supplemented with different heavy metals (K_2CrO_7 , $HgSO_4$, $CdSO_4 \cdot 8H_2O$, $CoSO_4$, $PbCl_2$). The concentration of each heavy metal solution (0.5, 1, 1.5, 2, 2.5 and 3 mM) was prepared in sterile deionized water and sterilized by autoclaving at 121°C for 15 min. After incubation for 24-48 hrs at 30°C, the plates were examined for cell growth [26].

2.6 Plant growth stimulation tests

2.6.1 Surface sterilization of barley seeds

Barley (*Hordeum vulgare* L.) seeds were surface-sterilized as described by Götz et al. [22]. First, the seeds were treated with 70% ethanol for 1 min and then with 12% acidified

hypochlorite for 15min. The seeds were washed thoroughly in sterile water. Then the seeds were germinated on LB agar plates in the dark at room temperature for 2 days.

2.6.2 Inoculation and growth of barley seedlings

For seed inoculation, S5LiBe was grown in LB medium overnight at 30°C. The bacterial culture was pelleted by centrifugation and the supernatant was discarded. Cell pellet was washed twice with 20 ml phosphate buffered saline (PBS, pH 7.2), and suspended in PBS. The optical density of bacterial suspension was 0.1 at 620nm, corresponding to a cell density of 10^8 cells/ml [17]. Surface sterilized barley seeds were incubated with bacterial suspension for 1 h at room temperature. Control seeds were incubated in sterile distilled water under the same conditions [39]. The seeds were planted in pots filled with unpolluted agricultural soil with following characteristics: pH (7.04), granulometry (clay 17.1%, fine silt 23.0%, coarse silt 7.1%, fine sand 12.4%, coarse sand 28.9%), active limestone 0.38%, conductivity 200 μ S/cm, organic carbon 6.38%, exchangeable K₂O 0.11 g/kg, exchangeable CaO 3.99 g/kg, exchangeable MgO 5.24 g/kg.

The experiment consisted of 7 lots and each lot was composed of 7 seeds, inoculated or non-inoculated with bacterial suspension. The seeds were sown at a depth of approximately 1 cm. The experiment was performed under natural dark/light cycles (16h of light and 8h of dark) at temperature of 25-35°C for two weeks [54]. Seed germination was determined by counting germinated seeds at 3 and 7 day after sowing the seeds. Results corresponding to final counts were reported as percentage of germination [29]. Plant growth response parameters were measured after 15 days including shoot length, fresh and dry weight of shoot, fresh and dry weight of root. Fresh weight was determined by measuring the weight of shoots and roots in the fresh state directly after harvest. For dry weight measurements, harvested roots and shoots were dried in a hot oven at 45°C until stabilization of the weight.

In order to show the ability of the strain S5LiBe to stimulate barley growth in heavy metal contaminated soil, a similarly designed experiment as above (but with 5 lots instead of 7 lots) was carried out using soil with heavy metal contamination (see the composition above). In this experiment, a heavy metal sensitive reference strain *P. protegens* CHA0^T was included as well as a control without any inoculation.

2.7 Statistical analysis

Data obtained for plant growth parameters were subjected to multi-analysis of variance (MANOVA) by the least significant difference (LSD) test at $p \leq 0.05$ with statistical software XLSTAT version 2009.1.02.

3. Results

3.1 Phylogenetic and phenotypic characterization of the isolates

Two bacterial strains (S4LiBe and S5LiBe) were selected among the originally tested 11 isolates from the well water. The colonies had a greenish yellow color, resembling *Pseudomonas* spp. Bacterial cells stained Gram-negative and were motile.

For molecular phylogenetic characterization of the isolates, 16S rDNA sequence analysis was performed. PCR-amplified 16S rDNA of the bacterial strains was sequenced and blasted with the NCBI database. Comparative analysis with whole 16S rDNA data base sequences suggested that the isolates S4LiBe and S5LiBe were most closely related to *Pseudomonas protegens*. The 16S rDNA sequences of the two isolates were 99.8% similar to each other. S4LiBe showed 99.8% 16S rRNA similarity to *Pseudomonas* sp. AF521651, 99.7% to *P. protegens* PF-5 (sequence AJ417073), 99.6% to *P. protegens* PGNR1 (sequence AJ417071), and 99.5% to *P. protegens* CHA0^T (sequence AJ278812). The 16S rDNA of S5LiBe had 99.4% similarity with *Pseudomonas* sp. AF521651, 99.6% with *Pseudomonas protegens* AJ417073 and 99.5% with *P. protegens* CHA0^T (sequence AJ278812) (see Table S2). The phylogenetic position of the isolates based on 16S rDNA similarity is also shown in the dendrograms based on maximum likelihood tree calculation (Figure 1).

The carbon source utilization patterns of the isolates S4LiBe and S5LiBe were assessed using the Biolog GN2 Microplates (Table S1) and compared with the type strain of *P. protegens* CHA0^T and *P. protegens* Pf-5. From 95 carbon sources tested with the isolates S4LiBe and S5LiBe, 89% were identical with the type strain CHA0^T, while 84% were identical with the strain Pf-5. Differences in the utilization pattern between CHA0^T and the isolates were found for m-inositol, D-psicose, sucrose, formic acid, hydroxybutyric acid, itaconic acid, succinamic

acid, D-alanine, L-alanine, L-alanylglycine, hydroxy-L-proline and L-threonine (for more information see Table S1).

Figure 1: Phylogenetic relationship of the 16S rDNA sequences of S4LiBe and S5LiBe and different reference strains. The calculated dendrogram is based on maximum likelihood tree calculation.

3.2 Plant Growth promotion traits

Table 1 shows the PGP traits of the isolates in comparison with the *P. protegens* type strain CHA0^T. The IAA-production ranged from 3.1 to 4.0 µg ml⁻¹ in the presence of 500 µg/ml L-tryptophan. The isolates and the reference strain produced urease, lipase, protease, esterase and cellulase at different levels. However, CHA0^T failed to produce chitinase under the applied test conditions. The abilities to solubilize precipitated phosphate and to produce siderophores were common in S4LiBe, S5LiBe and CHA0^T.

3.3 Heavy Metal Tolerance

Among the heavy metals, lead and cobalt were less toxic to all isolates. S4LiBe, S5LiBe and CHA0 were able to grow in the presence of K₂CrO₇ at concentrations up to 2 mM. The isolates S4LiBe and S5LiBe showed very high degree of tolerance up to 3 mM HgSO₄, CdSO₄, and PbCl₂. In contrast, CHA0 was very sensitive to HgSO₄ and less tolerant towards CdSO₄ and PbCl₂ (Table 2).

3.4 Biological control activities

3.4.1 Fungal antagonistic activity

Figure 2 shows the antifungal activity of the isolates against the pathogenic fungi *F. graminearum*, *V. dahliae* and *B. cinerea* as well as *Aspergillus niger* and *A. flavus* tested in an agar plate confrontation assays. All five fungi were inhibited to different extent by the *P. protegens* isolates S5LiBe and S4LiBe.

Figure 2: Percentage Growth Inhibition (PGI %) of plant pathogens in the presence of the isolates S4LiBe and S5LiBe

3.4.2 Insecticidal activity

The *Galleria mellonella* virulence assay of the isolates S4LiBe and S5LiBe as well as the reference strain CHA0^T clearly demonstrated that the isolates were equally effective as the *P. protegens* reference strain (Figure 3). In both isolates and the CHA0^T reference strain the *fitD* gene for the Fit insecticide toxin could be detected, while in the FitD deficient mutant F113, identified by band at approximately 900bp, was missing.

Figure 3: Insecticidal activity of the isolates S4LiBe and S5LiBe compared to the reference strain CHA0^T.

3.5 Plant growth promoting effect of isolate S5LiBe

The germination of barley seeds was significantly stimulated at 3 days after inoculation with S5LiBe over non-inoculated controls in unpolluted soil (Figure 4). Moreover, S5LiBe significantly increased germination percentage in comparison to the reference strain CHA0

and non-inoculated control seedlings at 3 and 7 days after inoculation in polluted soil (Figure 6). Concerning the plant growth parameters measured in unpolluted soil, S5LiBe increased significantly the shoot length compared to the control (Figure 5a). Bacterial inoculation also had a significant effect on fresh and dry weight (g) of shoot and root (0.1 and 0.075; 0.04 and 0.03) respectively compared to the controls (0.06 and 0.047; 0.025 and 0.016) (Figures 5b and c). For polluted soil, S5LiBe improved significantly the following parameters: shoot and root length (Figures 7a and 7b); fresh and dry weight (g) of shoot and root (Figures 7c and 7d), compared to the reference strain CHA0^T and the control.

Figure 4: Germination percentage in non-inoculated and S5LiBe-inoculated barley seeds after 3 and 7 days.

Figure 5a, b, c: Stimulation of growth of barley by inoculation with strain S5BiLe after 15 days of growth.

Figure 6: Germination percentage in non-inoculated, S5LiBe-inoculated and CHA0-inoculated barley seeds in contaminated soil after 3 and 7 days.

Figures 7a, b, c, d: Stimulation of growth of barley by inoculation with strain S5BiLe after 15 days of growth in contaminated soil.

4. Discussion

Fluorescent pseudomonads and other rhizobacteria are well known for their abilities to successfully colonize plant roots and to promote plant growth by biological control [24] and plant growth promotion activities [46]. In the present study, two *Pseudomonas protegens* isolates from agricultural well water indeed showed a wide variety of different features of plant growth promoting traits. The isolates S4LiBe and S5LiBe are within the *P. protegens* subspecies of the *P. fluorescens* species based on 16S rDNA gene similarity analysis (Figures 1 and Table S2) and carbon utilization pattern (Table S1). The isolates S4LiBe and S5LiBe are very similar (99.8% 16SrRNA gene similarity) to each other and are very closely related (99.4 to 99.7%) to the *P. protegens* strains CHA0^T and Pf-5 [48, 34].

Biological control of phytopathogens by fluorescent pseudomonads reduces the severity of many plant diseases [53]. In this study, both isolates showed clearly *in vitro* antagonistic potential against three plant pathogenic fungi *Botrytis cinerea*, *Verticillium dahliae* and *Fusarium graminearum* (88%, 80% and 82% respectively) as well as towards two *Aspergillus* spp. (Figure 2). Srinivasan et al. [61] had characterized five members of the genus *Pseudomonas*, *P. putida* FC-6B, *P. sp.* FC-7B, *P. putida* FC-8B, *P. sp.* FC-9B and *P. sp.* FC-24B, which showed antifungal activity against *F. oxysporum* and *F. sp. lycopersici*. Sagahón et al. [56] reported that *Pseudomonas* spp. 11 inhibited up to 70% of *Stenocarpella maydis* and *Stenocarpella macrospora* and the filtrates obtained in logarithmic growth phase from the *P. fluorescens* 16 inhibited 54% of the growth of *Stenocarpella maydis*. In addition to siderophore and chitinase production, the observed biological control activity of the isolates is probably also due to the production of several antibiotic compounds, like 2,4-diacetylphloroglucinol and pyoluteorin, as is well known for fluorescent pseudomonads [24, 48]. Currently, genomic analysis of the two isolates is in progress, which will demonstrate

which biosynthetic gene clusters for antibiotic production and other antagonistic activities are present in the two well water isolates from Algeria. Like the reference strain *P. protegens* CHA0^T and related strains [45, 51], the isolates S4LiBe and S5LiBe also harbor insecticidal activities, as could be demonstrated in the positive *Galleria mellonella* injection assay and the presence of the *fitD* gene (Figure 3). Since the infection and growth within insect larvae constitutes a second growth cycle of these bacteria - apart from plant roots - it may be hypothesized, that these types of bacteria were enriched in the well water also harboring insect larvae. Therefore, the isolation of PGPB with insecticidal activities from agricultural well water may be a quite straight forward approach, which may be useful in future isolation attempts.

S5LiBe-inoculated plants significantly increased germination rate, shoot length, fresh and dry biomass of barley comparing with non-inoculated after two weeks. A plant growth promoting strain of *Pseudomonas* spp. has been already described by Rosas et al. [50], who reported that the strain *P. aurantiaca* SR1, when applied on maize and wheat seeds showed a significant plant growth promoting effect. The beneficial effect of *Pseudomonas* was also confirmed by Egamberdieva et al. [17] who tested the co-inoculation of *Pseudomonas* spp. with *Rhizobium* on the growth of fodder galega (*Galega orientalis* Lam.). Co-inoculation of plants showed increased shoot and root dry matter compared to the inoculation with *R. galega* HAMBI 540 alone. The isolates S4LiBe and S5LiBe as well as the reference strain CHA0 had IAA production activity in the presence of L-tryptophan in the range of 3-4 µg/ml, which is in the range of other PGPBs. Naik and Sakthivel [41] suggested that the plant growth promoting ability of *Pseudomonas* sp. PUP6 could be based on the production of phytohormone IAA, siderophores and phosphate-solubilizing enzymes. Wahyudiet al. [63] reported that the capability to increase plant growth parameters in germination seed bioassays was highly related to the IAA level produced by *Pseudomonas* spp. applied. The growth promoting effect

of S5LiBe can also be due to another mechanism, which is linked to its phosphate-solubilizing activity. Phosphorus (phosphate) is one of the major essential macronutrients for biological growth and development and the release of insoluble forms of phosphorus increase soil phosphorus accessibility. Rodriguez and Fraga [49] reported that *Pseudomonas* and other Phosphate Solubilizing Bacteria (PSB) are capable to increase the availability of phosphate in soil. Siderophore production is another important characteristic of efficient plant growth promoting which was observed with the isolates as well as the applied reference strain CHA0. Siderophores are widely studied as one biocontrol mechanism against plant pathogenic microbes, but they also may support iron nutrition of plants. Siderophores can stimulate plant growth indirectly by the inhibition of phytopathogenic microorganisms competing for the growth limiting mostly insoluble ferric iron resource in soils [17, 33, 54].

Furthermore, the isolates S4LiBe and S5LiBe produce several exo-cellular enzymes (protease, cellulose, chitinase and urease) which are potentially relevant for soil fertility. The degradation of protein by microbial peptidases is important in N-cycling in soils by making organically bound nitrogen accessible for plants [4]. Chitinases are well known to lyse the fungal cell wall [10] and thus could effectively contribute to control plant-pathogenic fungi. As reported by Egamberdieva et al. [17], cellulose production of bacteria can enhance nodule formation. Co-inoculation of the cellulose-producing strain *Pseudomonas trivialis* 3Re27 with *Rhizobium galegae* HAMBI 540 significantly increased nodulation and nitrogen content of fodder Galega, whereas cellulose-negative *Pseudomonas extremorientalis* TSAU20 showed no significant stimulation.

Since heavy metals cannot be biologically degraded to harmless products and hence persist in the environment indefinitely, heavy metal contamination of agriculture soil is a significant environmental problem and has several disadvantages on human health and agriculture [65]. The selection of metal-tolerant plant growth promoting microorganisms can be advantageous

to speed up the recolonization of the plant rhizosphere in the polluted soil [15]. Both isolates in this study were characterized by considerable tolerance to rather high levels (up to 3mM) of heavy metals Co, Pb, Hg and Cd and up to 2mM to chromium (Table 3). Dell' Amico et al. [16] showed that inoculation with cadmium-resistant strains *Pseudomonas tolaasi* and *Pseudomonas fluorescens* enabled *Brassica napus* to grow under cadmium stress because of the production of indole acetic acid (IAA), siderophores and ACC (1-aminocyclopropane-1-carboxylate) deaminase, which protected the plants against cadmium stress. Since the *P. protegens* reference strain CHA0^T (like related PGPB) is rather sensitive to these heavy metals (Table 3), the heavy metal tolerance of S4LiBe and S5LiBe can be regarded as unique among this group of bacteria. Probably, the vicinity of the heavy metal deposit had resulted in soil contamination which fostered the distribution of heavy metal tolerance. It is well known, that metal resistance transposons or plasmids are shared between Gram-negative bacteria [38]. Further detailed investigation of the actual contamination in the area of isolation and the well water as well as about the genetic basis in the isolated bacteria are necessary.

In conclusion, the isolates S5LiBe and S4LiBe revealed plant growth promoting potentials, as shown by the presence of many key features of plant growth promotion and by the stimulation of germination and growth barley seedlings upon inoculation. Furthermore, the estimation of the antagonistic effect against pathogenic fungi and also insecticidal activity add further valuable activities for possible plant beneficial bacterial inoculants. To find out most efficient biological control agents against plant diseases under given application conditions, an increasing number of isolates is still to be investigated [7]. The observed heavy metal tolerance of the new isolates towards mercury, cadmium, cobalt and lead may enable the newly isolated *P. protegens* strains as superior candidates for inoculation of biomass crops in heavy metal contaminated soils.

503

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Tables

Table 1: Plant growth promoting traits of the isolates S4LiBe, S5LiBe, and P. protegens CHA0T. Zones of clearance or indicator color changes around colonies were measured; “+”: diameter between 7 and 10mm; “++”: diameter between 10 and 20mm; “+++”: diameter above 20mm

Traits	S4LiBe	S5LiBe	CHA0 ^T
IAA (µg/ml)	4.0	3.1	3.5
Chitinase	+	++	-
Protease	+	+	++
Esterase	+	+	+
Cellulase	+	+	+
Lipase	+	+	++
Urease	++	+++	+
Phosphate solubilization	++	++	++
Siderophore production	++	+++	++

Table 2: Heavy metal tolerance of the isolates S4LiBe and S5LiBe and *P. protegens* CHA0^T;
“+++”: High resistance (more than 100 colonies per plate); “++”: average resistance (30-50 colonies per plate); “+”: resistance (very few colonies per plate); “-”: sensitive (complete growth inhibition)

		Isolates		
		S4LiBe	S5LiBe	CHA0 ^T
K ₂ CrO ₇ (mM)	0.5	+++	+++	+++
	1	+++	+++	++
	1.5	++	++	++
	2	+	+	+
	2.5	-	-	-
	3	-	-	-
HgSO ₄ (mM)	0.5	+++	+++	-
	1	+++	+++	-
	1.5	++	++	-
	2	++	++	-
	2.5	++	++	-
	3	++	++	-
CdSO ₄ ·8H ₂ O (mM)	0.5	+++	+++	++
	1	++	++	+
	1.5	+	++	-
	2	+	+	-
	2.5	+	+	-
	3	+	+	-
CoSO ₄ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	++	++	++
	2.5	+	+	+
	3	+	+	-
PbCl ₂ (mM)	0.5	+++	+++	+++
	1	+++	+++	+++
	1.5	+++	+++	++
	2	+++	+++	++
	2.5	+++	+++	+
	3	+++	+++	+

Legends to Figures

Figure 1: 16S rRNA gene similarity tree, based on maximum likelihood analysis

Figure 2: Percentage Growth Inhibition (PGI %) of plant pathogenic fungi (plate confrontation assays) by the isolates S4LiBe and S5LiBe

Figure 3: Insecticidal activity of the isolates S4LiBe and S5LiBe compared to the reference strain CHA0^T.

A) PCR-analysis of the *fitD* gene: In lane F113, a *fitD* deletion mutant of CHA0 was applied; dH₂O: blank control

B) *Galleria mellonella* virulence assay, according to Péchy-Tarr et al. [41];

Black circle: *P. protegens* CHA0^T (positive control)

Blue diamonds: *Pseudomonas* sp. S4LiBe;

Red squares: *Pseudomonas* sp. S5LiBe;

Gray triangles: 0.9% NaCl solution (negative control)

Figure 4: Germination percentage of non-inoculated and S5LiBe-inoculated barley seeds in unpolluted soil after 3 and 7 days. Different letters indicate significant differences obtained by the Fischer LSD test ($p \leq 0.05$).

Figure 5: Stimulation of growth of barley seedlings in unpolluted soil due to inoculation with strain S5BiLe after 15 days of growth as compared to non-inoculated control plants. Different letters indicate significant differences obtained by the Fischer LSD test ($p \leq 0.05$).

A) Effect on shoot length,

B) Effect on fresh and dry weight of shoots,

C) Effect on fresh and dry weight of roots.

740 Figure 6: Germination percentage of non-inoculated, S5LiBe- or CHA0-inoculated barley
741 seeds in heavy metal polluted soil after 3 and 7 days. Different letters indicate
742 significant differences obtained by the Fischer LSD test ($p \leq 0.05$).

743 Figure 7: Stimulation of growth of barley seedlings in heavy metal polluted soil due to
744 inoculation with strain S5BiLe or CHA0 after 15 days of growth in comparison
745 with non-inoculated (control) seedlings. Different letters indicate significant
746 differences obtained by the Fischer LSD test ($p \leq 0.05$).

- 747 A) Effect on shoot length
- 748 B) Effect on root growth
- 749 C) Effect on fresh weight of shoots and roots
- 750 D) Effect on dry weight of shoots and roots

Table S2: Carbon source utilization patterns (BIOLOG GN2 microplate tests) of S4LiBe, S5LiBe and *P. protegens* CHA0^T and Pf-5; “+”: Positive reaction, “(+)”: Weak positive reaction, “-”: Negative reaction

Metabolite	S5LiBe	S4LiBe	CHAO	Pf5
α -Cyclodextrin	-	-	-	-
Dextrin	+	+	+	+
Glycogen	-	-	-	-
Tween 40	+	+	+	+
Tween 80	+	+	+	+
N-Acetyl-D-Galactosamine	-	-	-	-
N-Acetyl-D-Glucosamine	+	+	+	-
Adonitol	-	-	-	-
L-Arabinose	-	-	-	-
D-Arabitol	-	-	+	-
D-Cellobiose	-	-	-	-
i-Erythritol	-	-	-	-
D-Fructose	+	+	+	+
L-Fucose	-	-	-	-
D-Galactose	-	-	-	-
Gentiobiose	-	-	-	-
α -D-Glucose	+	+	+	+
m-Inositol	+	+	-	-
α -D-Lactose	-	-	-	-
Lactulose	-	-	-	-
Maltose	-	-	-	-
D-Mannitol	+	+	+	-
D-Mannose	+	+	+	-
D-Melibiose	-	-	-	-
β -Methyl-D-Glucoside	-	-	-	-
D-Psicose	-	+	+	-
D-Raffinose	-	-	-	-
L-Rhamnose	-	-	-	-
D-Sorbitol	-	-	-	-
Sucrose	+	+	-	+
D-Trehalose	+	+	+	-
Turanose	-	-	-	-
Xylitol	-	-	-	-
Methyl pyruvate	+	+	+	-
Mono-Methyl-succinate	-	-	-	-
Acetic Acid	+	+	+	+
Cis-Aconitic Acid	+	+	+	+
Citric Acid	+	+	+	+
Formic Acid	+	-	+	-
D-Galactonic Acid Lactone	-	-	-	-
D-Galacturonic Acid	-	-	-	-
D-Gluconic Acid	+	+	+	+
D-Glucosaminic Acid	-	-	-	-
D-Glucuronic Acid	-	-	-	-
α -Hydroxybutyric Acid	-	-	-	-
β -Hydroxybutyric Acid	+	+	+	+
γ -Hydroxybutyric Acid	-	-	+	+
p-HydroxyPhenylacetic Acid	+	+	+	-
Itaconic Acid	-	-	+	+

α -Keto Butyric Acid	-	-	-	-
α -KetoGlutaric Acid	+	+	+	+
α -KetoValeric Acid	-	-	-	-
D,L-Lactic Acid	+	+	+	+
Malonic Acid	+	+	+	+
Propionic Acid	+	+	+	+
Quinic Acid	+	+	+	+
D-Saccharic Acid	-	-	-	-
Sebacic Acid	-	-	-	-
Succinic Acid	+	+	+	+
Bromosuccinic Acid	+	+	+	+
Succinamic Acid	(+)	-	+	-
Glucuronamide	-	-	-	-
L-Alaninamide	-	-	-	-
D-Alanine	-	(+)	-	-
L-Alanine	+	(+)	-	-
L-Alanylglycine	+	-	-	-
L-Asparagine	+	+	+	+
L-Aspartic Acid	+	+	+	+
L-Glutamic Acid	+	+	+	+
Glycyl-L-Aspartic Acid	-	-	-	-
Glycyl-L-Glutamic Acid	+	+	+	-
L-Histidine	+	+	+	+
Hydroxy-L-Proline	+	+	+	-
L-Leucine	+	+	-	-
L-Ornithine	-	-	-	-
L-Phenylalanine	-	-	-	-
L-Proline	+	+	+	+
L-Pyroglutamic Acid	+	+	+	-
D-Serine	-	-	-	-
L-Serine	+	+	+	+
L-Threonine	-	-	+	-
D,L-Carnitine	-	-	-	-
γ -Amino Butyric Acid	+	+	+	+
Urocanic Acid	+	+	+	+
Inosine	+	+	+	-
Uridine	-	-	-	-
Thymidine	-	-	-	-
Phenyethylamine	-	-	-	-
Putrescine	+	(+)	+	+
2-Aminoethanol	+	+	+	+
2,3-Butanediol	-	-	-	-
Glycerol	+	+	+	+
D,L- α -Glycerol Phosphate	+	+	+	-
Glucose-1-phosphate	-	-	-	-
Glucose-6-phosphate	+	+	+	+

Figure 1 revised
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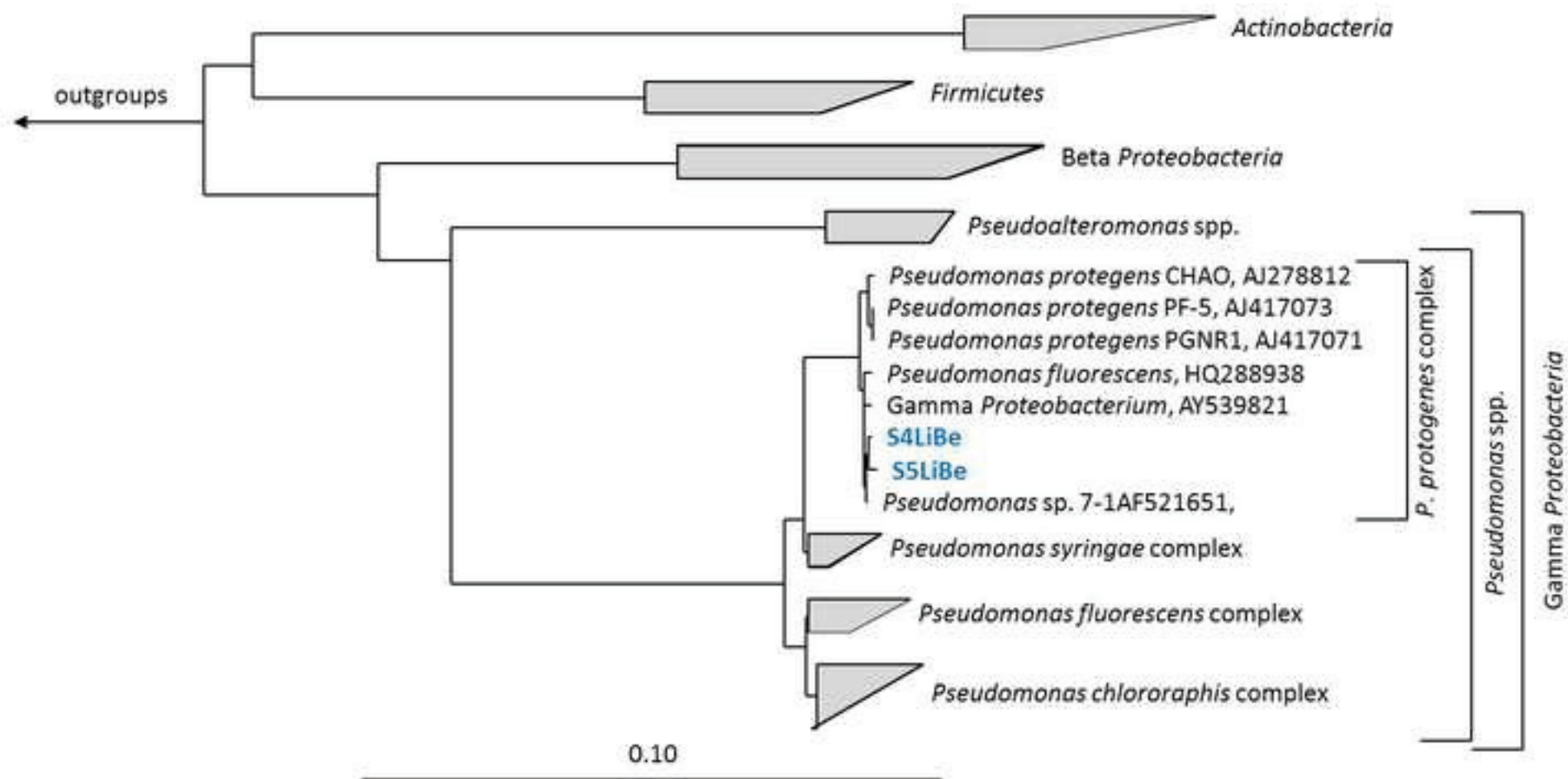


Figure 2
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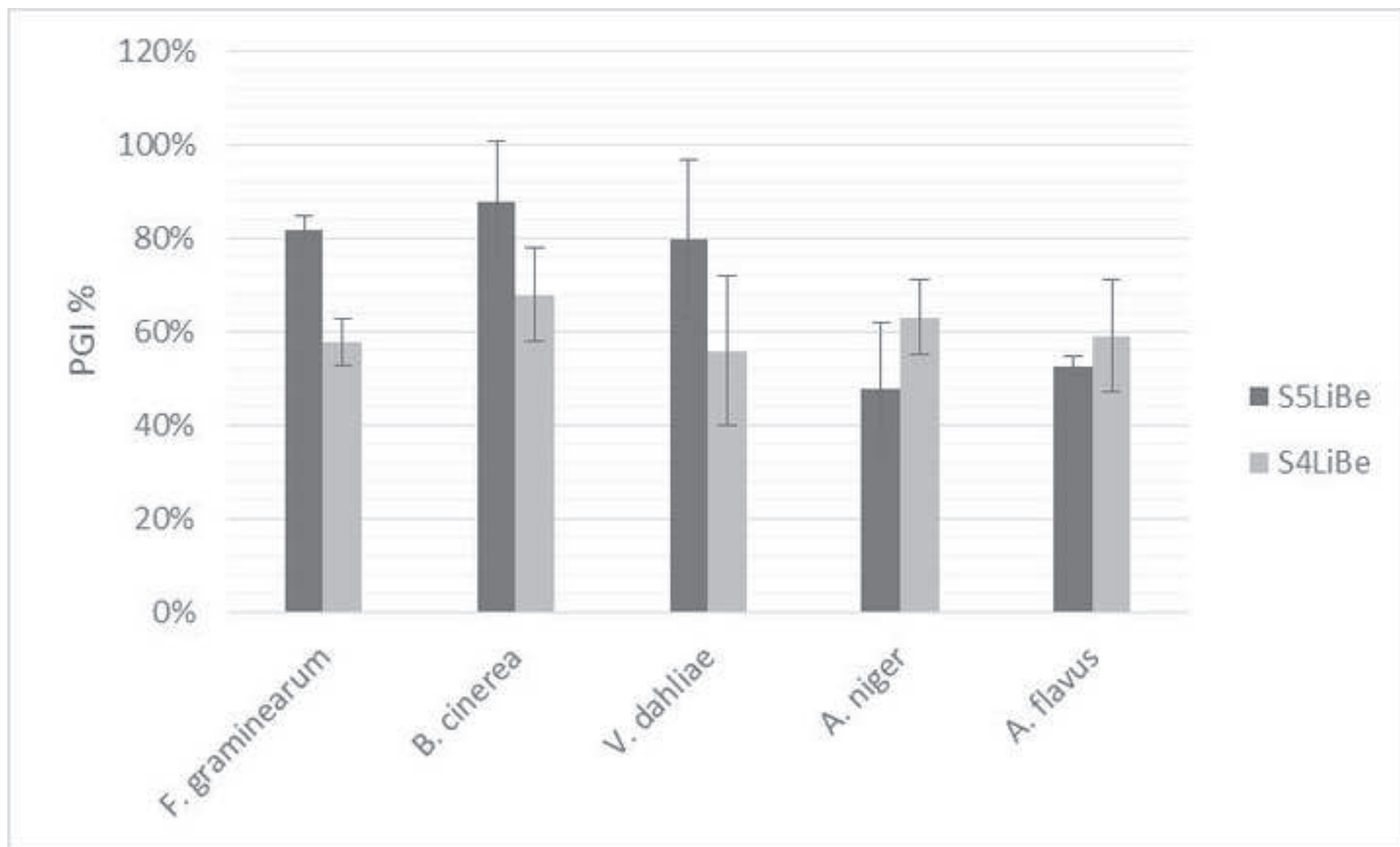


Figure 3
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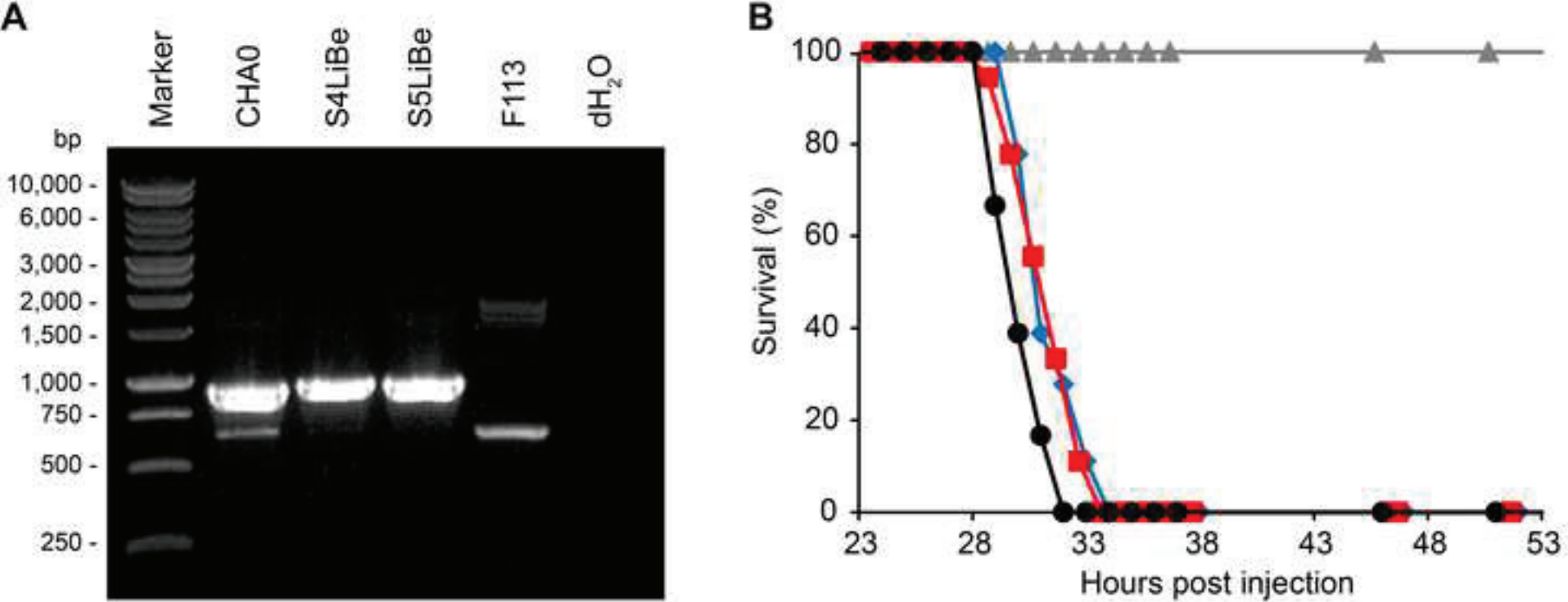


Figure 4 (white)
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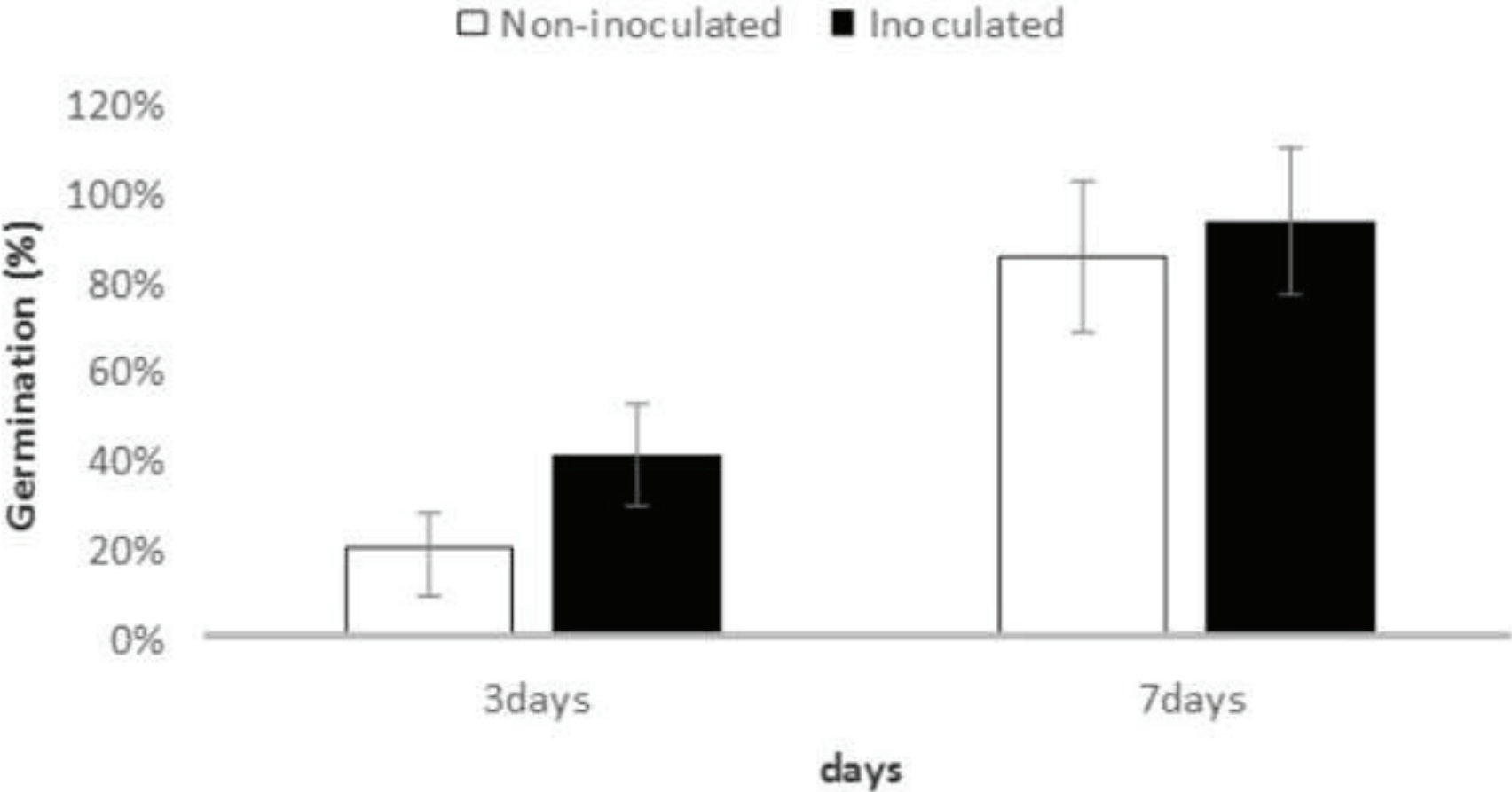


Figure 5a

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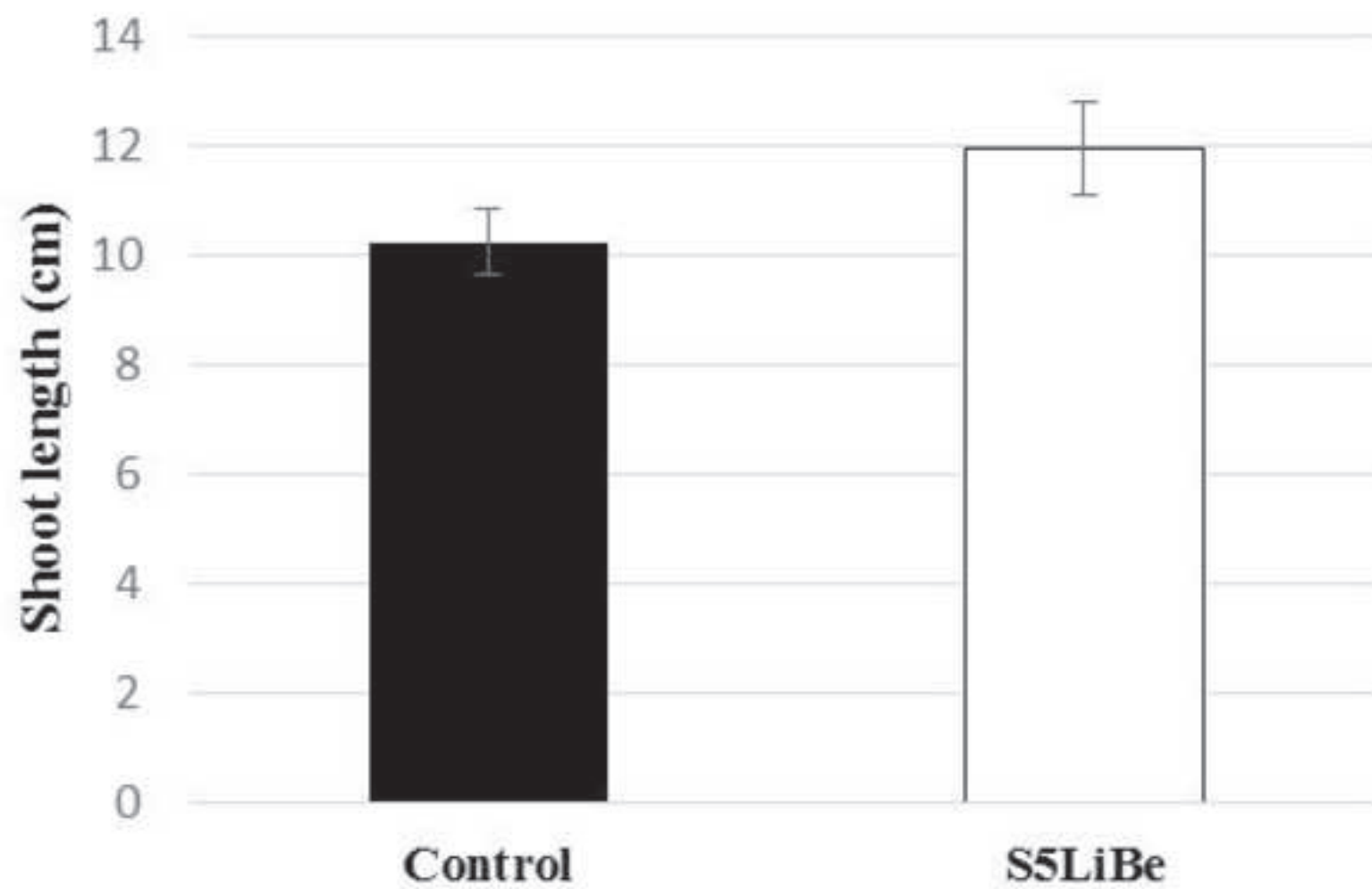


Figure 5b
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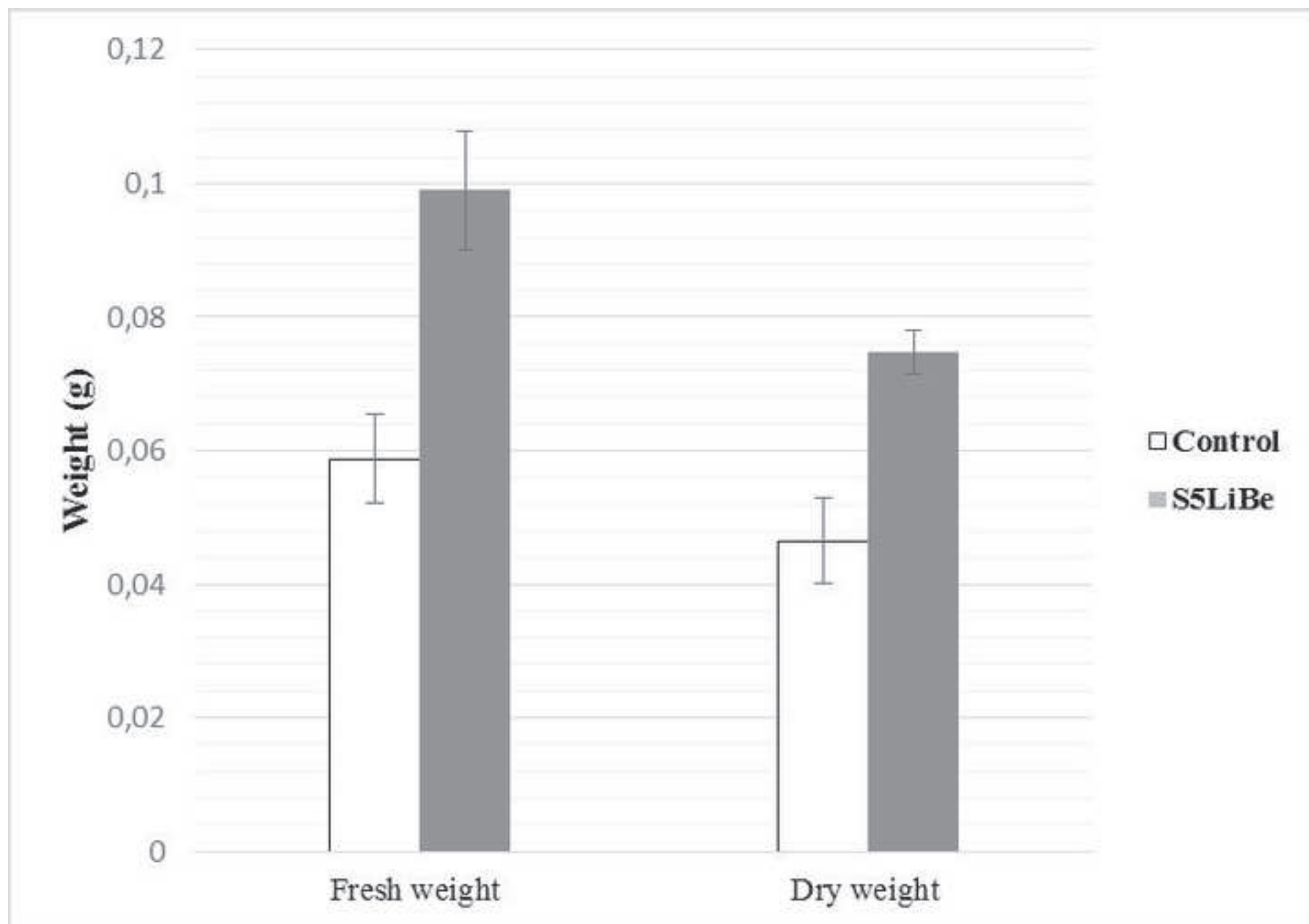


Figure 5c
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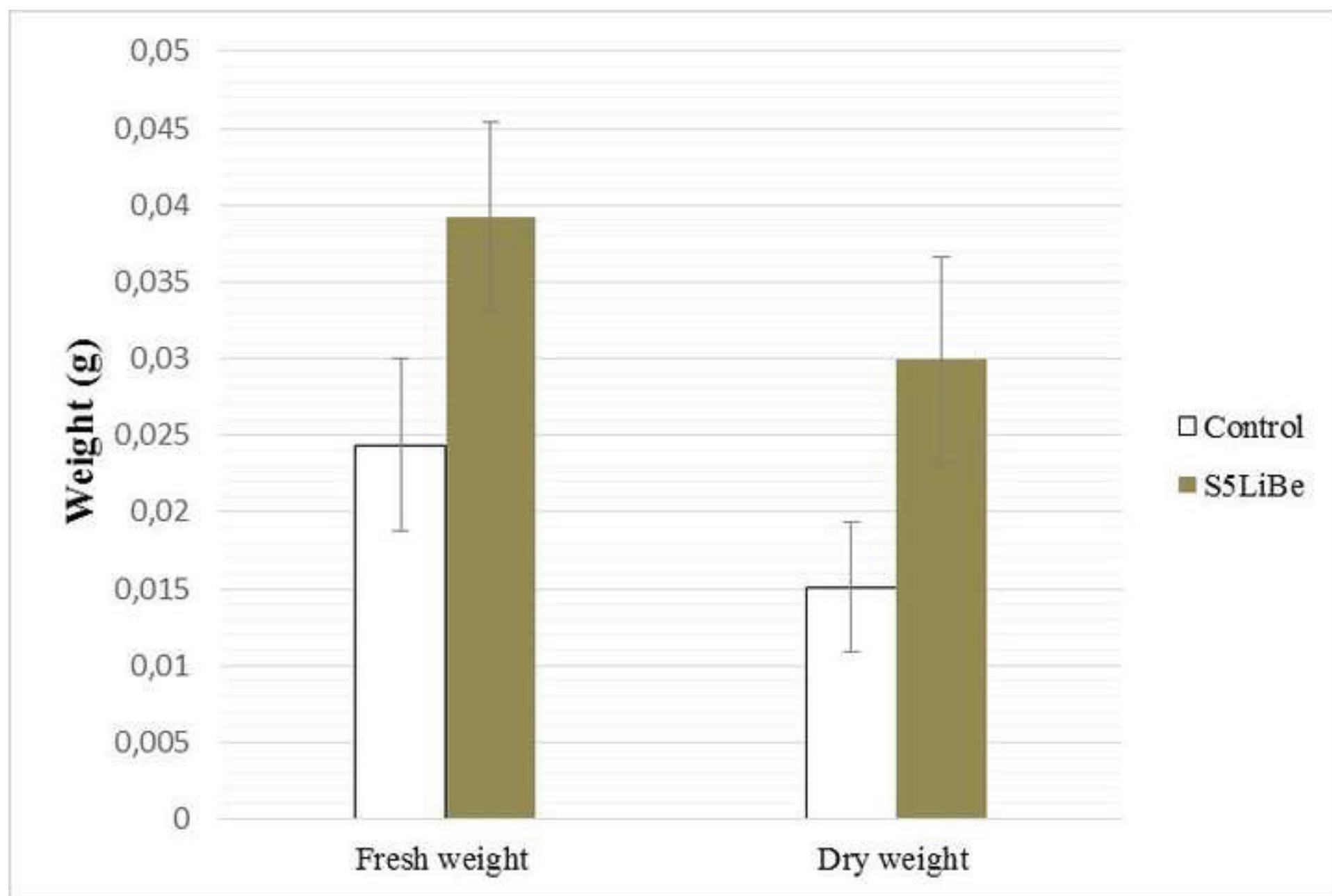


Figure 6 new
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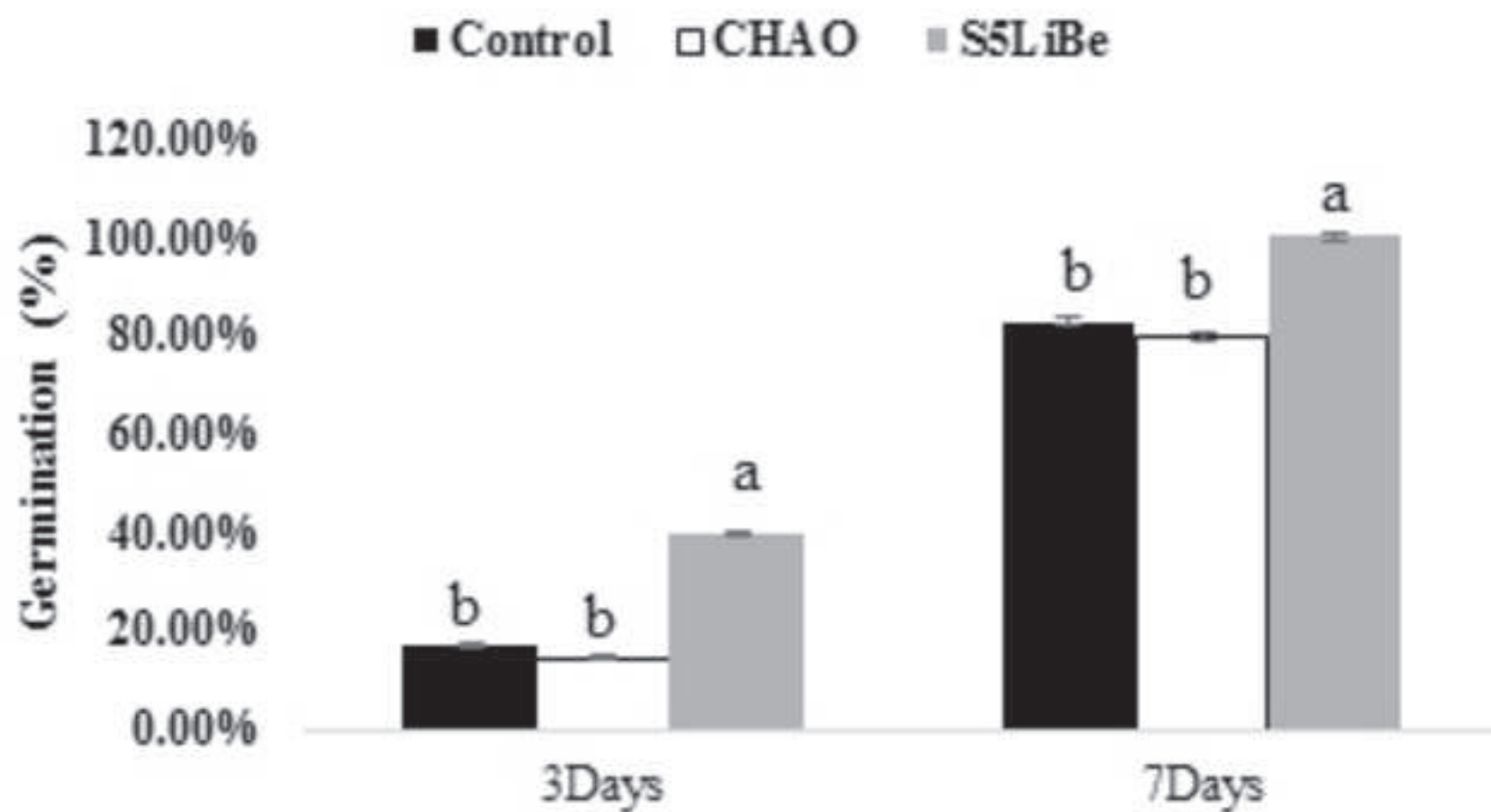


Figure 7a new
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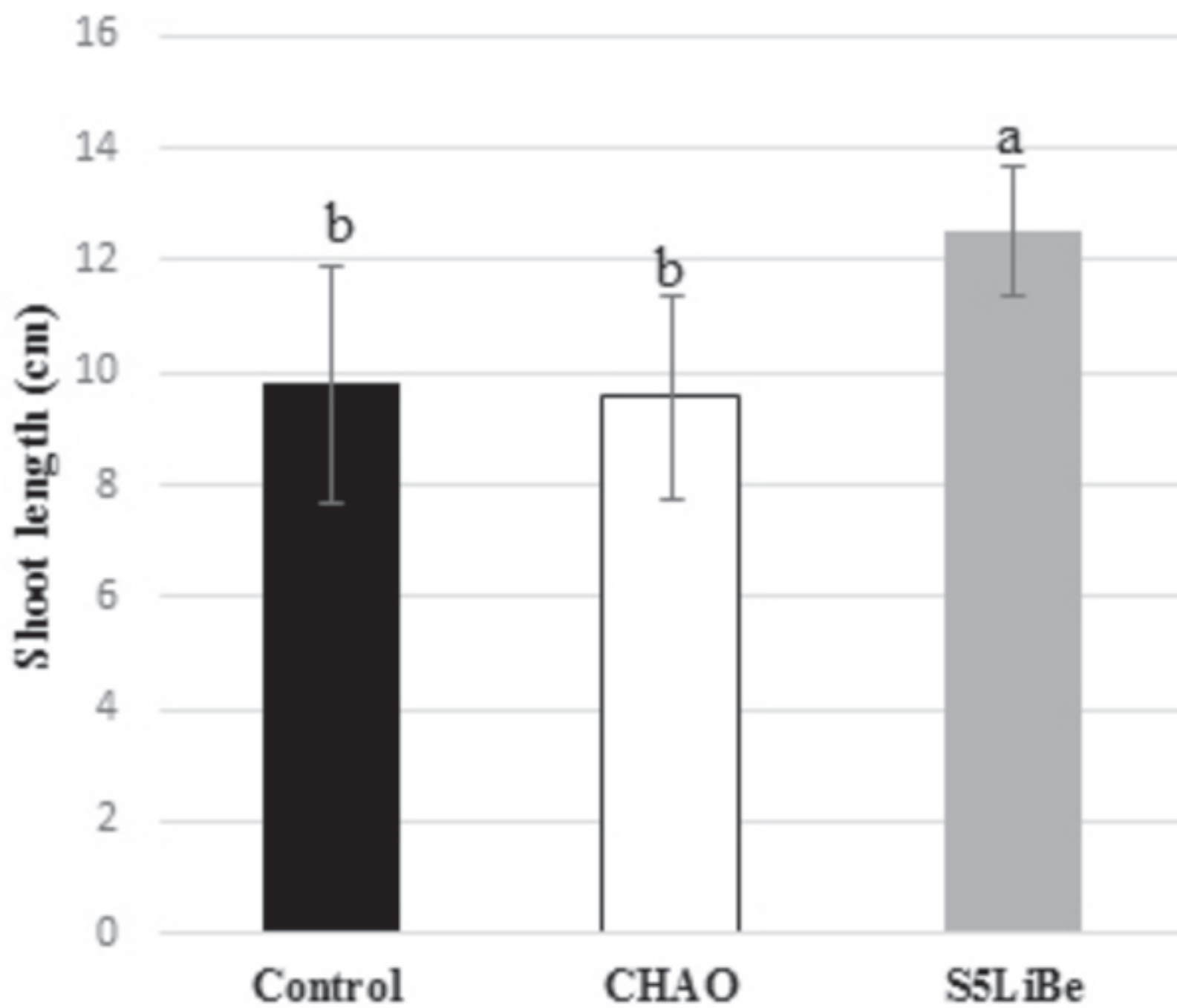


Figure 7b new
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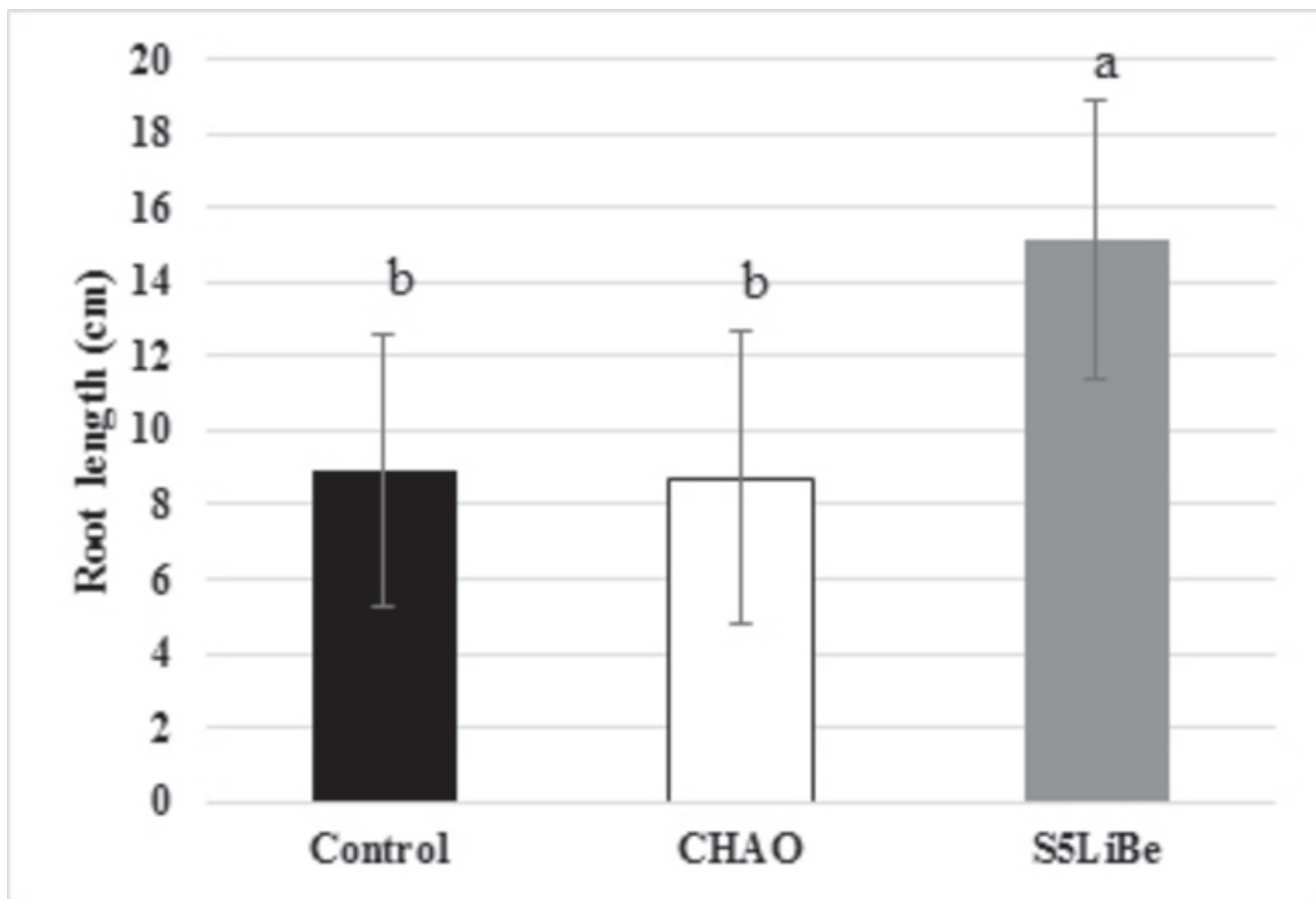


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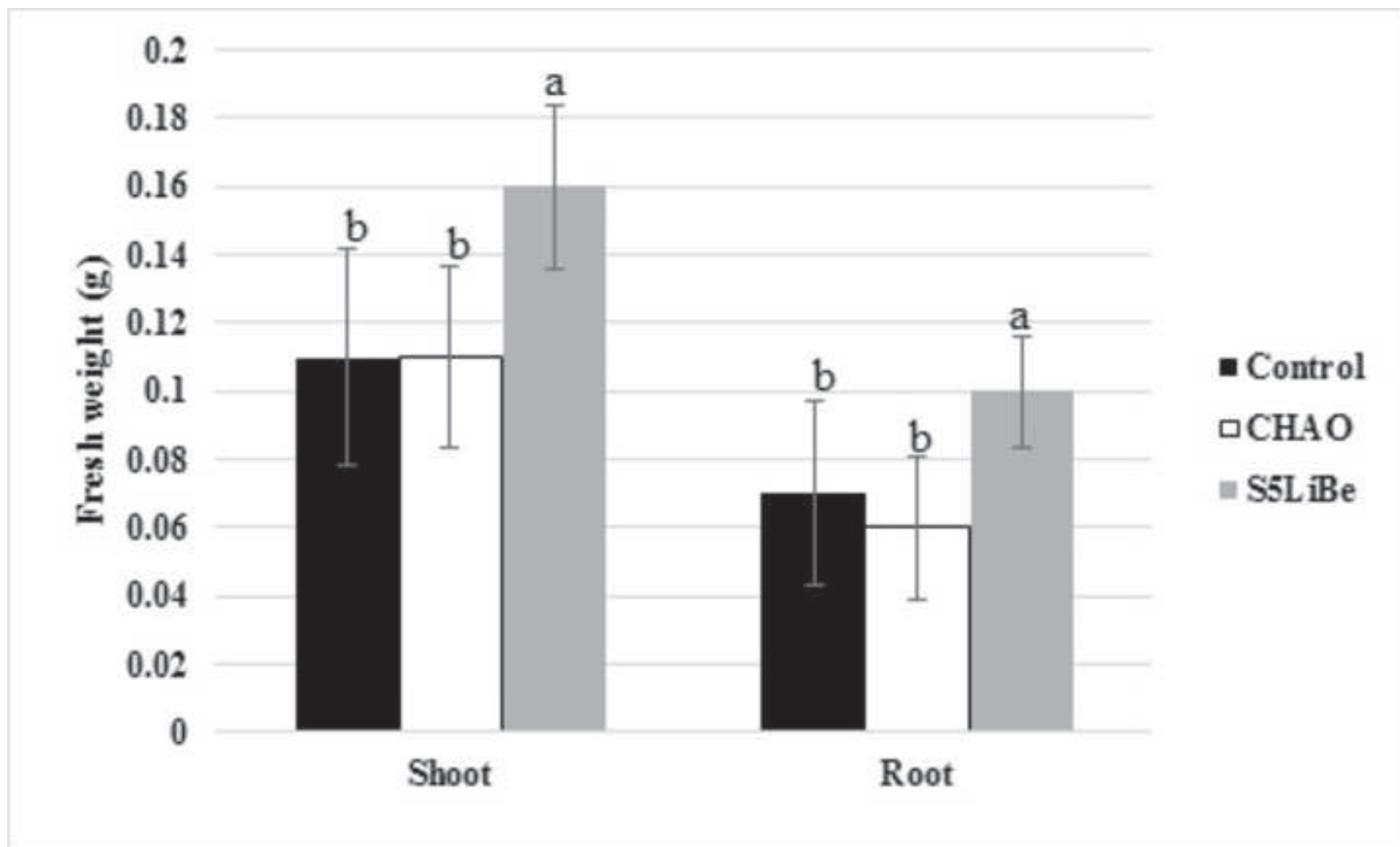


Figure 7d new
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