

## Article

# Effects of Individual and Consortium Inoculation of *Bacillus velezensis*, *Burkholderia pyrrocinia*, and *Streptomyces* sp. on Acclimatization and Tuberization of Micropropagated Potato

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## Highlights

### What are the main findings?

- Native *Bacillus velezensis*, *Burkholderia pyrrocinia*, and *Streptomyces* sp. exhibit strong growth-promoting and antagonistic traits.
- Isolates significantly improved potato acclimatization and tuberization; the triple-consortium maximized yield.

### What are the implications of the main findings?

- PGPB-consortia offer an effective solution for strengthening ex-vitro acclimatization and tuber yield.
- Integrating PGPB applications warrants testing for climate-resilient horticulture with reduced agrochemical inputs.

## Abstract

Potato (*Solanum tuberosum* L.), the third most important food crop worldwide, is constrained by phytopathogens, environmental stress, and heavy agrochemical use. Potato micropropagation is a biotechnological strategy for producing genetically uniform, pathogen-free plantlets. However, the acclimatization phase remains a major bottleneck in micropropagation, often resulting in poor survival and stunted growth. In this context, plant growth-promoting bacteria (PGPB) represent a sustainable strategy to improve plantlet establishment and reduce reliance on synthetic inputs. This study evaluated the biofertilization and biocontrol potential of three Douro vineyard-associated bacterial strains, *Bacillus velezensis* Nb1, *Burkholderia pyrrocinia* Gs3, and *Streptomyces* sp. 42s5, identified using 16S rRNA and gyrB sequence analysis. In dual-culture assays, the isolates showed antagonistic activity against *Fusarium oxysporum*, *F. solani*, *Botrytis cinerea*, and *Alternaria alternata*. All strains also exhibited key plant growth-promoting traits, including indole-3-acetic acid and siderophore production, and phosphate solubilization. The isolates were tested individually and in consortia on micropropagated potato plantlets during acclimatization under greenhouse conditions. Eight treatments (six replicates each), including a non-inoculated control, were evaluated. The results indicated that inoculations significantly improved vegetative growth and yield-related parameters. Notably, the Gs3 + 42s5 combination produced the highest shoot biomass, while the three-strain consortium achieved the greatest total tuber weight (36.91 g/plant) compared to the control (7.75 g/plant). These findings highlight the potential of these native PGPB, particularly when applied in multi-strain combinations, to



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enhance acclimatization efficiency and support sustainable potato production by reducing dependence on chemical inputs and promoting eco-friendly agricultural practices.

**Keywords:** potato micropropagation; acclimatization; plant growth-promoting bacteria (PGPB); *Bacillus*; *Burkholderia*; *Streptomyces*

## 1. Introduction

Potato (*Solanum tuberosum* L.), a dicotyledonous species belonging to the Solanaceae family, is one of the most widely cultivated food crops worldwide, ranking third in global importance after rice and wheat [1,2]. Originally from the Andean region of South America and serving as a dietary staple for over one billion people, it plays a crucial role in global food security and economic stability [1,3]. However, potato production is extremely vulnerable to climate change variability, as increasing heat and drought episodes, altered rainfall patterns, and changing pest and disease pressures threaten both yield and tuber quality. These stresses particularly affect early vegetative and reproductive stages, leading to yield instability and defects in tuber development [4–6]. To ensure high-quality planting material, micropropagation is widely adopted for its ability to rapidly produce genetically uniform and pathogen-free plantlets [7–9]. The acclimatization phase is a major factor limiting the success of micropropagation, as it involves the challenging transition from in vitro to greenhouse or field conditions. During this phase, plantlets face drastic shifts in humidity, light, microbial exposure, and nutrient availability, often leading to reduced photosynthetic capacity, weak root development, and high mortality rates [10–12]. Consequently, improving acclimatization success is critical for ensuring plant survival and long-term field performance.

In parallel, potato production is challenged by various biotic and abiotic factors and remains heavily dependent on chemical fertilizers and pesticides, which can negatively affect soil health, disrupt beneficial microbial communities, and compromise environmental sustainability [13]. These challenges have intensified interest in bio-based alternatives, particularly plant growth-promoting bacteria (PGPB), as tools to support sustainable and climate-resilient horticulture. PGPB colonize the rhizosphere or internal plant tissues and enhance plant growth, development, and stress tolerance through multiple direct and indirect mechanisms, including phytohormone production (e.g., indole-3-acetic acid), nutrient mobilization via phosphate solubilization and siderophore-mediated iron acquisition, nitrogen fixation, and modulation of plant stress responses and induction of stress tolerance pathways [14–18]. In addition, many PGPB exhibit biocontrol activity against phytopathogens through the production of antimicrobial compounds, lytic enzymes, and competitive exclusion, thereby contributing to improved plant health and disease suppression [2,18,19].

The application of PGPB during the acclimatization stage of micropropagated plants has received increasing attention, as this phase represents a critical window in which beneficial microbial associations can enhance root development, biomass accumulation, and stress tolerance under ex vitro conditions [20,21]. Nevertheless, there are limited studies that have explored the benefits of PGPB during the critical acclimatization phase of micropropagated plants. While numerous bacterial genera, including *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, and *Rhizobium*, have been reported to be successful in inoculating PGPB in several crops, including maize, rice, soybeans, canola, and wheat, in greenhouse and field-scale experiments [16–18,20,22–27], their targeted use, particularly *Bacillus*, *Burkholderia*, and *Streptomyces*, during the acclimatization of micropropagated potato remains notably underexplored. Most studies in potato systems have focused primarily on *Bacillus* and *Pseudomonas* species,

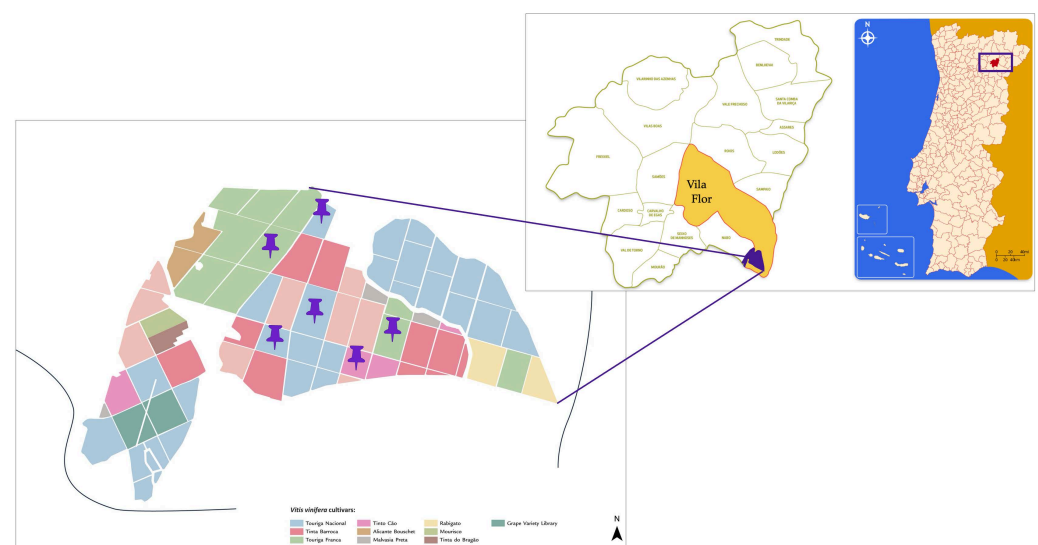
which are well known for their growth-promoting and biocontrol properties [17], whereas *Streptomyces* species, despite their recognized capacity to produce bioactive metabolites and promote plant growth, have been scarcely investigated in potato acclimatization systems. In light of current climate-change scenarios, there is a clear need for developing acclimatization protocols that improve survival, early vigor, and tuberization of micropropagated plants, while reducing agrochemicals.

To our knowledge, no study has systematically evaluated the individual and combined effects of *Bacillus velezensis*, *Burkholderia pyrrocinia*, and *Streptomyces* sp. on the acclimatization and subsequent tuberization of micropropagated potato plants. Furthermore, although each of these genera has demonstrated strong growth-promoting and bioprotective properties, the effects of their combined application during the critical ex vitro transition phase remain largely unexplored. In this context, the present study aimed to isolate native bacterial strains from Portuguese vineyard samples, characterize their in vitro antagonistic and plant growth-promoting properties, and assess their individual and consortium-based effects on vegetative growth, physiological performance, and yield-related traits of micropropagated potato plants during greenhouse acclimatization, thereby contributing to climate-adaptive strategies for producing high-quality potatoes while minimizing the need for synthetic fertilizers and pesticides.

## 2. Materials and Methods

### 2.1. Isolation of Beneficial Bacteria from Douro Vineyards

The present study evaluated three bacterial isolates from the laboratory collection, designated Nb1, Gs3, and 42s5. These isolates were originally obtained from soil and grape berry samples collected from vineyard plots located at Quinta do Ataíde (Symington), Vila Flor, northern Portugal (Figure 1). For each sample type, approximately 1 g of material was suspended in 1.5 mL of sterile distilled water in sterile Eppendorf tubes and vortexed vigorously to obtain a homogeneous suspension. Aliquots of each suspension were streaked onto Tryptone Soy Agar (TSA; HiMedia Laboratories, Mumbai, India) and Potato Dextrose Agar (PDA; HiMedia Laboratories, Mumbai, India) plates using sterile inoculation loops. Plates were incubated at 25–28 °C for 24–72 h. Morphologically distinct colonies were sub-cultured repeatedly to obtain pure isolates, which were preserved and subsequently evaluated for antagonistic activity, plant growth-promoting traits, and their effects on micropropagated potato plants under greenhouse conditions.



**Figure 1.** Geographical location of the study area: Quinta do Ataíde, Vila Flor, Portugal.

## 2.2. Selection and Characterization of Bacteria with Biopesticide and Biofertilizer Potential

### 2.2.1. In Vitro Antagonism Assay Against Phytopathogenic Fungi

The antagonistic activity of bacterial isolates was evaluated in vitro against four phytopathogenic fungi, namely *Fusarium oxysporum*, *F. solani*, *Botrytis cinerea*, and *Alternaria alternata*. Dual-culture assays were performed on PDA plates following a standard methodology. A mycelial disc from an actively growing fungal culture was placed at the center of each Petri dish, while bacterial isolates were streaked at equidistant points around the fungal plug. Plates were incubated at 25–28 °C for 8 days. The inhibition zone between the bacterial colony and the fungal mycelium was recorded in millimetres (mm). The percentage inhibition of fungal growth was calculated relative to the untreated fungal control using the following equation:

$$\text{Inhibition of fungal growth (\%)} = \frac{C - T}{C} \times 100 \quad (1)$$

where C represents fungal colony growth in the untreated control plates, and T represents fungal colony growth in plates inoculated with the bacterial isolate.

### 2.2.2. Assessment of Plant Growth-Promoting Traits

The three bacterial isolates were assessed for key plant growth-promoting (PGP) traits, including indole-3-acetic acid (IAA) and siderophore production, and phosphate solubilization, following protocols previously established by our research group [28], with minor modifications.

- Estimation of IAA Production

IAA production was estimated using a colorimetric assay based on the Salkowski reagent following the described procedure by our group [28]. Fresh bacterial isolates were grown overnight in nutrient broth, after which 100 µL of culture was transferred into 10 mL of nutrient broth (Difco; Becton, Sparks, MD, USA) supplemented with 250 µg/mL L-tryptophan (Sigma-Aldrich, Saint Louis, MO, USA). Cultures were incubated at 28 °C with shaking at 150 rpm for 24, 48, and 72 h. Cells were removed by centrifugation at 16,355 × g for 5 min, and 2 mL of the supernatant was mixed with 4 mL of Salkowski reagent. After incubation for 30 min in the dark at room temperature, absorbance was measured at 350 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations were estimated using a standard curve generated with pure IAA (1–100 µg/mL; Sigma-Aldrich, St. Louis, MO, USA) and presented as IAA equivalents (IAAE).

- Siderophore Production

Siderophore production was evaluated qualitatively using Chrome Azurol S (CAS; Sigma-Aldrich, St. Louis, MO, USA) agar medium. Bacterial isolates were spot inoculated onto CAS agar plates and incubated at 28 °C for 8 days. Siderophore production was indicated by the formation of yellow-to-orange halos surrounding bacterial colonies. Results were recorded qualitatively as positive (+) when halo formation was observed, and negative (−) when no halo was present. Halo and colony diameters were measured to calculate the siderophore production index (SPI) and siderophore production efficiency (SPE) using the following equation [29]:

$$SPI = \frac{\text{Colony diameter} + \text{yellow/orange halozone diameter}}{\text{Colony diameter}} \quad (2)$$

$$SPE (\%) = \frac{\text{Siderophore production diameter}}{\text{Growth diameter}} \times 100 \quad (3)$$

- Phosphate Solubilization on Solid Medium

Phosphate solubilization was initially assessed on the National Botanical Research Institute's Phosphate (NBRIP) agar medium [28], supplemented with either tricalcium phosphate (TCP; VWR Life Science, Avantor, Radnor, PA, USA) or aluminum phosphate (AlPO<sub>4</sub>; Alfa Aesar, Thermo Fisher Scientific, Haverhill, MA, USA) as the sole phosphorus source (5 g/L). Fresh bacterial isolates were streaked onto the NBRIP agar plates and incubated at 28 °C for 8 days. Phosphorus-solubilizing ability was indicated by the formation of a clear halo surrounding the bacterial colonies, due to the localized dissolution of insoluble phosphate. Halo and colony diameters were measured to calculate the phosphate solubilization index (PSI) and solubilization efficiency (PSE) using the following equation [29]:

$$PSI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}} \quad (4)$$

$$PSE (\%) = \frac{\text{Halozone diameter}}{\text{Colony diameter}} \times 100 \quad (5)$$

- Phosphate Solubilization in Liquid Medium

The phosphate-solubilizing capacity of isolates Nb1, Gs3, and 42s5 was evaluated in liquid NBRIP broth supplemented with tricalcium phosphate (TCP), following the method described by Suleimanova et al. [30] with minor modifications. Initially, the bacterial isolates were cultivated in Tryptic Soy Broth (TSB; HiMedia Laboratories, Mumbai, India) for 48 h at 30 °C and 150 rpm, centrifuged at 6000 rpm for 10 min, washed twice with phosphate-free NBRIP medium, and inoculated into 20 mL of liquid NBRIP medium at an initial OD<sub>600</sub> of 0.1. Cultures were incubated at 30 °C with shaking for 4 and 8 days. Uninoculated NBRIP medium served as the abiotic control. After incubation, supernatants were collected to determine pH and soluble phosphate concentration using the ammonium molybdate colorimetric method. Absorbance was measured at 355 nm, and orthophosphate concentration was calculated using a KH<sub>2</sub>PO<sub>4</sub> (Merck, Darmstadt, Germany) standard curve. The phosphorus solubilization efficiency (%) was determined by comparing phosphate levels in the inoculated samples with those in the abiotic control. All assays were performed in triplicate, and the mean values were reported.

### 2.3. Molecular Identification and Phylogenetic Analysis

The molecular identification of the bacterial isolates was carried out following the protocol described by Laranjeira et al. [28], with minor modifications. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB; AppliChem, VWR, Darmstadt, Germany) method.

Amplification of 16S rDNA and *gyrB* gene regions was performed using the specific primer sets. PCR reactions were prepared in a final volume of 15 µL, containing 5.5 µL of genomic DNA, 7.5 µL of 2× MyTaq™ HS Mix (Bioline, London, UK), and 1 µL of each primer (10 µM). For 16S rDNA, the primers used were 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') under the conditions of 1 min at 95 °C denaturation, 2 min at 55 °C annealing, and 3 min at 72 °C extension for 35 cycles, with a final extension at 72 °C for 10 min.

For *gyrB* amplification, the primer pair employed was *gyrB*-UPf (5'-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3') and *gyrB*-UFr (5'-AGC AGG ATA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3') with thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min [31]. PCRs were carried out in a T100 Thermal

Cycler (Bio-Rad, Laboratories, Hercules, CA, USA), and the PCR products were analyzed by electrophoresis on a 1.5% agarose gel (VWR Life Science, Avantor, Radnor, PA, USA) in  $0.5\times$  TBE buffer (VWR Life Science, PA, USA) for 25 min at 90 V and visualized using the Gel Doc™ EZ Imaging System (Bio-Rad, Laboratories, Hercules, CA, USA). Amplicons were purified and sequenced by StabVida (Lisbon, Portugal).

Partial 16S rRNA gene sequences were first used to assign the isolates to the genera *Bacillus*, *Burkholderia*, and *Streptomyces*. The partial 16S rRNA gene sequences were compared with reference sequences in the NCBI GenBank database using BLASTn, and genus-level assignments were based on the closest sequence matches and percentage sequence identity. However, 16S rRNA provided insufficient resolution to discriminate among closely related species, mainly within the *Bacillus* and *Burkholderia* group complexes. Therefore, the housekeeping gene *gyrB* was used as an alternative phylogenetic marker [32], as it evolves faster and provides greater phylogenetic resolution and has been recommended for *Bacillus* and *Burkholderia* taxonomy [33,34]. The resulting sequences were compared with those in the NCBI GenBank database using BLASTn. Phylogenetic analyses were performed using MEGA 12 (MEGA Software, Philadelphia, PA, USA), and trees were constructed using the maximum likelihood method with 1000 bootstrap replicates.

## 2.4. Experimental Design and Plant Growth Conditions

### 2.4.1. Bacterial and Plant Materials

Three bacterial isolates (Nb1, Gs3, and 42s5) representing different genera were selected based on their antagonistic activity and PGP traits. Micropropagated potato plantlets (*Solanum tuberosum* L. cv. 'La Vie') were obtained from the Plant Genetics and Biotechnology Laboratory at the University of Trás-os-Montes and Alto Douro (UTAD). Before acclimatization, plantlets were cultured on Murashige and Skoog (MS; Sigma-Aldrich, St. Louis, MO, USA) medium supplemented with 45 g/L sucrose (Sigma-Aldrich, St. Louis, MO, USA) under controlled conditions (16-h photoperiod/8-h dark cycle at  $23 \pm 2$  °C, with a light intensity of  $45 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by cool-white, fluorescent lamps), with optimal growth obtained in the absence of BAP (6-benzylaminopurine). Healthy, 4-week-old plantlets were selected for transplantation based on uniform morphological traits, including shoot length and node count (about 5 cm and 3–5 nodes, respectively), and well-developed root systems. Before experimental assignment, plantlets were screened to confirm baseline homogeneity among groups ( $p > 0.05$ ).

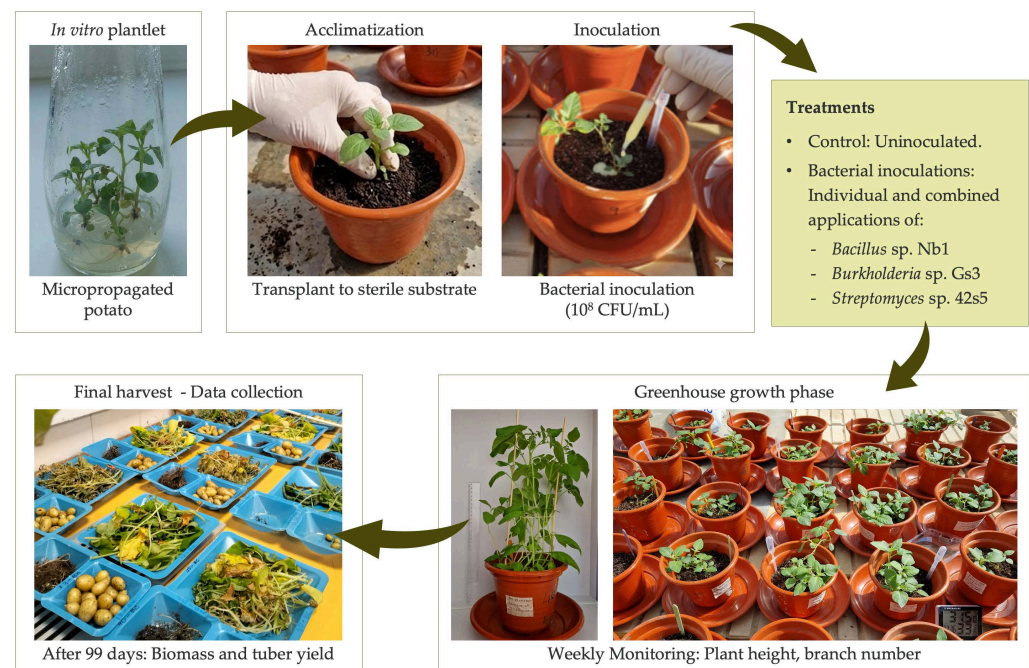
### 2.4.2. Substrate Preparation and Acclimatization Conditions

The substrate used for acclimatization consisted of a commercial Profi-substrate (Gramoflor GmbH & Co. KG, Vechta, Germany), which was sterilized by autoclaving at 121 °C for 45 min. After cooling, 1 kg of the substrate was placed into individual pots (1.5 L capacity). Micropropagated plantlets with well-developed shoots and roots were carefully removed from the culture medium, and residual agar was gently washed off using sterile distilled water. Each plantlet was then transplanted into a pot and maintained under controlled greenhouse conditions throughout the experiment. The greenhouse was operated under natural daylight conditions, with daytime and nighttime temperatures of  $25 \pm 2$  °C and  $18 \pm 2$  °C, respectively, and a relative humidity of  $80 \pm 5\%$ . Plantlets were irrigated with distilled water twice weekly (50 mL per irrigation) to maintain the substrate near field capacity, and no additional mineral fertilization was applied during the acclimatization period.

### 2.4.3. Preparation and Application of Bacterial Inoculum

Bacterial suspensions were prepared by diluting fresh cultures in sterile 0.8% NaCl (Merck, Darmstadt, Germany) solution to an approximate density of  $1 \times 10^8$  colony-

forming units (CFU)/mL. Treatments included single inoculations with each isolate (Nb1, Gs3, and 42s5), dual combinations (Nb1 + Gs3, Nb1 + 42s5 and Gs3 + 42s5), a three-strain consortium (Nb1 + Gs3 + 42s5), and a non-inoculated control (Ctrl). For dual treatments, equal volumes of the two single-strain suspensions were mixed 1:1 (*v/v*), and for the three-strain consortium, the three suspensions were mixed in equal volumes (1:1:1, *v/v/v*). In all inoculated treatments, each plant received 1 mL of the corresponding suspension, delivering a total dose of approximately  $1 \times 10^8$  CFU per plant across all single, dual, and triple treatments. In contrast, control plants received 1 mL of sterile 0.8% NaCl solution (Figure 2).



**Figure 2.** Schematic representation of the greenhouse experimental design.

#### 2.4.4. Experimental Design and Data Collection

The experiment was conducted in a completely randomized design (CRD) with eight treatments and six biological replicates per treatment (one plant per pot). Each pot was considered an independent experimental unit. At the start of the experiment, pot positions on the benches were randomly assigned and re-randomized once per week to minimize positional effects within the greenhouse. Plant height and number of branches per plant were recorded weekly for five weeks during the acclimatization phase. At harvest (99 days after transplantation), tuber number per plant, total tuber weight per plant, shoot dry biomass, and root dry biomass were determined. Biomass samples were oven-dried to constant weight before measurement (Figure 2).

#### 2.5. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA), followed by Tukey's multiple comparison test at a significance level of  $p < 0.05$ . Specifically, *in vitro* screening assays involving a single factor were evaluated using one-way ANOVA, whereas assays with two factors were evaluated using two-way ANOVA including the interaction term. The greenhouse experiment was conducted in a randomized design consisting of 8 treatments with 6 independent biological replicates per treatment ( $n = 48$  plants in total). Non-destructive, weekly temporal parameters were analyzed using a Two-Way Repeated-Measures ANOVA, evaluating Treatment and Time as factors and individual plants ( $n = 6$  per treatment) as the repeated-measures unit. For the harvested endpoint, data were analyzed using a  $2 \times 2 \times 2$

Factorial ANOVA to evaluate the main effects of strain inclusion and their two-way and three-way interaction terms. Mean separations were performed using Tukey's honest significant difference (HSD) post-hoc test at  $\alpha = 0.05$ . Results are presented as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0.1 and 11 (GraphPad Software, San Diego, CA, USA).

### 3. Results

#### 3.1. Isolation and Identification of Beneficial Bacterial Strains

Three bacterial isolates, designated Nb1, Gs3, and 42s5, originating from vineyard soil and grape berry samples collected in the Douro region of northern Portugal, were evaluated in this study. The isolates underwent molecular identification and were subsequently assessed for antagonistic activity against phytopathogenic fungi, plant growth-promoting traits, and effects on the acclimatization, growth, and yield-related parameters of micro-propagated potato plants under greenhouse conditions.

Molecular identification using partial 16S rRNA gene sequencing assigned isolates Nb1, Gs3, and 42s5 to the genera *Bacillus*, *Burkholderia*, and *Streptomyces*, respectively (Figure S1, Table S1), without providing sufficient resolution for reliable species-level identification, particularly within the *Bacillus* and *Burkholderia* groups. Sequences were deposited in GenBank with accession numbers PZ525967, PZ525968, and PZ525969 for *Bacillus*, *Streptomyces*, and *Burkholderia* strains, respectively.

To achieve higher taxonomic resolution, the housekeeping gene *gyrB* was analyzed as a complementary marker (Figure S2, Table S1), which allowed refinement of the placement of strain Nb1 as *Bacillus velezensis*, clearly distinguished from closely related members of the *B. subtilis* complex, and confirmed strain Gs3 as *Burkholderia pyrrocinia*, clearly separated from the *B. cepacia* complex. In contrast, isolate 42s5 was clustered within the genus *Streptomyces*, but could not be confidently assigned to a described species based on both 16S rRNA and *gyrB* sequences. Therefore, the *gyrB* sequence analysis showed that isolate 42s5 shared its highest nucleotide identity (98.70%) with *S. roseovorticillatus* (GenBank accession number AB072891.1). While the data indicated a close relationship to the *S. roseovorticillatus* clade, which has been reported as a heterotypic synonym of *S. hiroshimensis*, we conservatively designated this isolate as *Streptomyces* sp. 42s5 due to the limited resolution provided by both loci and the complexity of this genus. The partial *gyrB* gene sequences obtained from isolates Nb1, Gs3, and 42s5 were used for phylogenetic analysis and were deposited in GenBank with the accession numbers PZ885215, PZ885216 and PZ885217, respectively. Regarding their isolation source, *B. velezensis* Nb1 was isolated from grape berry tissue, whereas *B. pyrrocinia* Gs3 and *Streptomyces* sp. 42s5 were obtained from vineyard soil samples.

#### 3.2. Antagonistic Activity Against Phytopathogenic Fungi

The antagonistic potential of the three bacterial isolates was evaluated in vitro against four phytopathogenic fungi (*Fusarium oxysporum*, *F. solani*, *Botrytis cinerea*, and *Alternaria alternata*) using a dual-culture assay. All isolates inhibited mycelial growth of the tested pathogens. However, the magnitude of inhibition varied among bacterial strains and fungal species, with *Streptomyces* sp. 42s5 producing clear and well-defined inhibition zones against all four fungi after 8 days of incubation (Figure 3).

Quantitative measurements of inhibition zones are presented in Table 1. The largest inhibition zone against *F. oxysporum* was observed for *Streptomyces* sp. 42s5 (10.50 mm), followed closely by *B. pyrrocinia* Gs3 (10.00 mm). Against *B. cinerea*, *B. pyrrocinia* Gs3 exhibited the greatest inhibitory effect (10.17 mm). Both *B. pyrrocinia* Gs3 and *Streptomyces* sp. 42s5 showed a comparable inhibition against *F. solani* and *A. alternata*. In contrast, *B.*

*velezensis* Nb1 exhibited moderate antagonistic activity, with the highest inhibition obtained against *A. alternata* (8.17 mm), while inhibition of *F. oxysporum*, *F. solani*, and *B. cinerea* remained below 6 mm.



**Figure 3.** *Streptomyces* sp. (42s5) showing antagonistic activities against tested phytopathogenic fungi after 8 days of incubation.

**Table 1.** In vitro antagonistic activity of the three bacterial isolates against four phytopathogenic fungi after 8 days of incubation.

| Measurement                     | Bacterial Isolate            | Phytopathogenic Fungi      |                             |                             |                            |
|---------------------------------|------------------------------|----------------------------|-----------------------------|-----------------------------|----------------------------|
|                                 |                              | <i>F. oxysporum</i>        | <i>F. solani</i>            | <i>B. cinerea</i>           | <i>A. alternata</i>        |
| Inhibition zone (mm)            | <i>B. velezensis</i> Nb1     | 05.00 ± 0.50 <sup>Bb</sup> | 04.17 ± 0.76 <sup>Bb</sup>  | 05.17 ± 1.04 <sup>Bb</sup>  | 08.17 ± 1.04 <sup>Aa</sup> |
|                                 | <i>B. pyrrocinia</i> Gs3     | 10.00 ± 1.00 <sup>Aa</sup> | 07.17 ± 0.76 <sup>Ba</sup>  | 10.17 ± 0.76 <sup>Aa</sup>  | 07.00 ± 0.50 <sup>Ba</sup> |
|                                 | <i>Streptomyces</i> sp. 42s5 | 10.50 ± 0.50 <sup>Aa</sup> | 08.83 ± 0.29 <sup>ABa</sup> | 09.33 ± 0.58 <sup>ABa</sup> | 08.00 ± 1.00 <sup>Ba</sup> |
| Inhibition of fungal growth (%) | <i>B. velezensis</i> Nb1     | 70.00 ± 1.25 <sup>Bb</sup> | 70.42 ± 0.72 <sup>Bb</sup>  | 69.17 ± 1.91 <sup>Bb</sup>  | 78.33 ± 1.91 <sup>Aa</sup> |
|                                 | <i>B. pyrrocinia</i> Gs3     | 68.21 ± 2.00 <sup>Ba</sup> | 59.29 ± 1.51 <sup>Cb</sup>  | 67.33 ± 0.40 <sup>Ba</sup>  | 61.71 ± 2.06 <sup>Cb</sup> |
|                                 | <i>Streptomyces</i> sp. 42s5 | 76.25 ± 1.25 <sup>Aa</sup> | 74.58 ± 0.72 <sup>Aa</sup>  | 75.83 ± 1.44 <sup>Aa</sup>  | 72.08 ± 1.91 <sup>Bb</sup> |

Values are expressed as mean ± standard deviation (SD) for three replicates. For each bacterium and fungus, different superscript uppercase and lowercase letters indicate statistically significant differences within rows and columns, respectively. Data were analyzed by two-way ANOVA followed by Tukey's test ( $p < 0.05$ ).

The percentage inhibition of fungal growth was significantly affected by bacterial isolate, fungal pathogen, and their interaction ( $p < 0.05$ ; Table 1). *Streptomyces* sp. 42s5 showed the highest inhibition against *F. oxysporum* (76.25%), *F. solani* (74.58%), and *B. cinerea* (75.83%). In contrast, *B. velezensis* Nb1 showed the highest inhibition against *A. alternata* (78.33%). *B. pyrrocinia* Gs3 showed the lowest inhibition against *F. solani* (59.29%) and *A. alternata* (61.71%). Within each isolate, Nb1 showed significantly greater activity against *A. alternata* than against the other fungi, whereas 42s5 showed lower inhibition of *A. alternata* than of the other tested pathogens.

### 3.3. Plant Growth-Promoting Traits of the Bacterial Strains

The three selected antagonistic bacterial isolates were evaluated for key PGP traits, including IAA production, siderophore synthesis, and phosphate solubilization.

#### 3.3.1. Production of Indole-3-Acetic Acid (IAA)

Quantitative determination of phytohormone production using the Salkowski method revealed that all bacterial isolates produced detectable levels of IAA at 24, 48, and 72 h of incubation (Table 2). IAA production increased progressively over time for all isolates. *B. pyrrocinia* Gs3 consistently produced the highest IAA concentrations at all time points, reaching a maximum of 57.56 µg IAAE/mL at 72 h, followed by *B. velezensis* Nb1, which produced intermediate IAA levels, with a maximum concentration of 31.06 µg/mL at

72 h, while *Streptomyces* sp. 42s5 showed the lowest IAA production, reaching 20.14 µg IAAE/mL at the same time point.

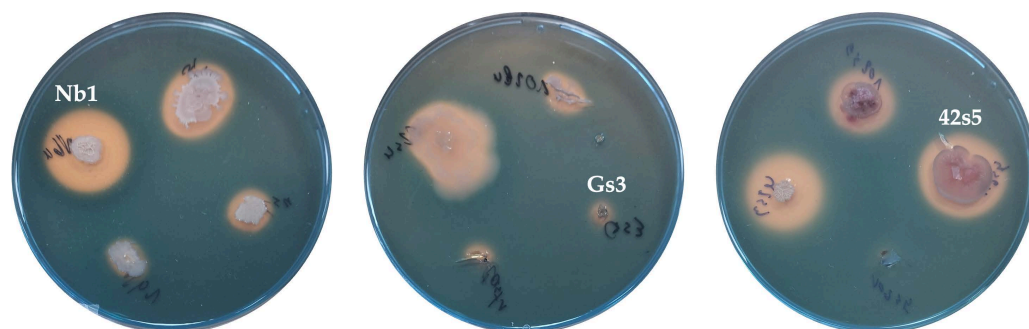
**Table 2.** Production of IAA by the three isolated bacteria after 24 h, 48 h, and 72 h of incubation.

| Bacteria Isolate             | IAA Content (µg of IAAE/mL) |                            |                            |
|------------------------------|-----------------------------|----------------------------|----------------------------|
|                              | 24 h                        | 48 h                       | 72 h                       |
| <i>B. velezensis</i> Nb1     | 11.65 ± 2.34 <sup>Cb</sup>  | 18.27 ± 1.86 <sup>Bb</sup> | 31.06 ± 1.70 <sup>Ab</sup> |
| <i>B. pyrrocinia</i> Gs3     | 17.70 ± 1.13 <sup>Ca</sup>  | 30.63 ± 0.38 <sup>Ba</sup> | 57.56 ± 4.93 <sup>Aa</sup> |
| <i>Streptomyces</i> sp. 42s5 | 08.14 ± 0.28 <sup>Bc</sup>  | 17.13 ± 1.49 <sup>Ab</sup> | 20.14 ± 0.49 <sup>Ac</sup> |

Values are expressed as mean ± SD (n = 3). For each bacterium and time point, different superscript uppercase and lowercase letters indicate statistically significant differences within rows and columns, respectively ( $p < 0.05$ ).

### 3.3.2. Siderophore Production

Siderophore production was detected in all three bacterial isolates using CAS agar medium (Figure 4). Halo zone diameters, siderophore production indices (SPIs), and siderophore production efficiencies (SPEs) are summarized in Table 3. *B. velezensis* Nb1 produced the largest halo zone (16.00 mm) and the highest SPI (3.78). In contrast, *B. pyrrocinia* Gs3 formed the smallest halo zone (1.97 mm) but showed the highest SPE (89.80%). *Streptomyces* sp. 42s5 exhibited intermediate halo diameters and the lowest SPE (17.03%).



**Figure 4.** Isolates *B. velezensis* Nb1, *B. pyrrocinia* Gs3, and *Streptomyces* sp. 42s5 showing siderophore production after 8 days of incubation.

**Table 3.** Siderophore production by the three isolated bacteria after 8 days of incubation.

| Bacteria Isolate             | Siderophore Production    |                           |                           |
|------------------------------|---------------------------|---------------------------|---------------------------|
|                              | HZD (mm)                  | SPI                       | SPE (%)                   |
| <i>B. velezensis</i> Nb1     | 16.00 ± 0.40 <sup>a</sup> | 03.78 ± 0.07 <sup>a</sup> | 42.17 ± 3.65 <sup>b</sup> |
| <i>B. pyrrocinia</i> Gs3     | 01.97 ± 0.25 <sup>c</sup> | 02.66 ± 0.05 <sup>b</sup> | 89.80 ± 2.96 <sup>a</sup> |
| <i>Streptomyces</i> sp. 42s5 | 07.67 ± 1.04 <sup>b</sup> | 02.52 ± 0.03 <sup>b</sup> | 17.03 ± 1.29 <sup>c</sup> |

Values are expressed as mean ± SD (n = 3). HZD: Halo zone diameter. SPI: Siderophore Production Index. SPE: Siderophore Production Efficiency. Different superscript lowercase letters within a column indicate statistically significant differences at  $p < 0.05$ .

### 3.3.3. Phosphate Solubilization

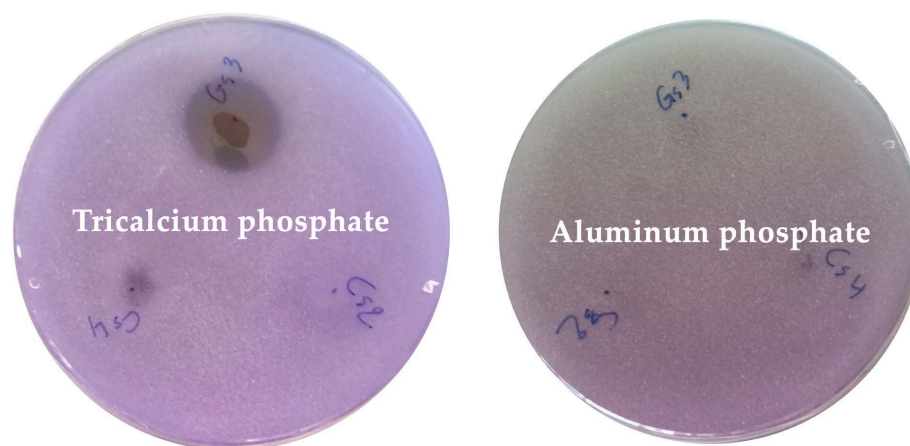
Phosphate solubilization ability of the three isolates was initially assessed using NBRIP agar supplemented with TCP and  $\text{AlPO}_4$  as insoluble P sources. On the TCP medium, all isolates produced clear solubilization halos (Table 4; Figure 5). *B. pyrrocinia* Gs3 exhibited the highest phosphate solubilization capacity, with a halo diameter of 15.33 mm, a solubilization index (PSI) of 5.17, and a solubilization efficiency (PSE) of 107.3%. *Streptomyces* sp. 42s5 showed moderate activity, whereas *B. velezensis* Nb1 exhibited the smallest halo diameter and lowest PSE. On  $\text{AlPO}_4$  medium, no clear halos were observed for any isolate;

however, medium acidification was detected by a color change, with *B. pyrrocinia* Gs3 showing the strongest response (+++), and *Streptomyces* sp. 42s5 and *B. velezensis* Nb1 showing moderate responses (+).

**Table 4.** Inorganic phosphate solubilization by the three isolated bacteria after 8 days of incubation, using NBRIP agar medium.

| Bacteria Isolate             | TCP                       |                           |                           | AlPO <sub>4</sub> * |
|------------------------------|---------------------------|---------------------------|---------------------------|---------------------|
|                              | HZD (mm)                  | PSI                       | PSE (%)                   |                     |
| <i>B. velezensis</i> Nb1     | 00.77 ± 0.25 <sup>c</sup> | 02.23 ± 0.06 <sup>b</sup> | 69.28 ± 3.57 <sup>b</sup> | +                   |
| <i>B. pyrrocinia</i> Gs3     | 15.33 ± 1.04 <sup>a</sup> | 05.17 ± 0.16 <sup>a</sup> | 107.3 ± 8.11 <sup>a</sup> | +++                 |
| <i>Streptomyces</i> sp. 42s5 | 02.67 ± 0.29 <sup>b</sup> | 02.55 ± 0.05 <sup>b</sup> | 52.93 ± 3.49 <sup>c</sup> | +                   |

Values are expressed as mean ± SD (n = 3). HZD: Halo zone diameter. PSI: Phosphate Solubilization Index. PSE: Phosphate Solubilization Efficiency. Different superscript letters within each column indicate statistically significant differences ( $p < 0.05$ ). \* For AlPO<sub>4</sub>, phosphate solubilization was evaluated qualitatively by color change around the colony in the absence of a clear halo. Solubilization was categorized as follows: (+++) high, (++) intermediate, (+) low, (−) none.



**Figure 5.** *B. pyrrocinia* Gs3 showing phosphate solubilization after 8 days of incubation.

In liquid NBRIP medium containing TCP, soluble phosphate concentration and pH changes differed significantly among isolates over time (Table 5). *B. pyrrocinia* Gs3 demonstrated the highest phosphate solubilization, with soluble phosphorus increasing from 362.50 µg/mL at day 4 to 822.50 µg/mL at day 8, accompanied by a decrease in pH (from 3.76 to 3.63). Correspondingly, its PSE increased markedly from 36.25% to 82.25%, the highest among the tested isolates. *Streptomyces* sp. 42s5 showed intermediate solubilization levels (from 188.00 µg/mL to 333.93 µg/mL), while *B. velezensis* Nb1 showed the lowest solubilization potential, producing 129.10 µg/mL and 147.40 µg/mL of soluble phosphorus on days 4 and 8, respectively.

**Table 5.** Quantitative phosphate solubilization (content), medium acidification (pH), and phosphate solubilization efficiency (PSE) by the three bacterial isolates after 4 and 8 days of incubation in NBRIP broth supplemented with tricalcium phosphate.

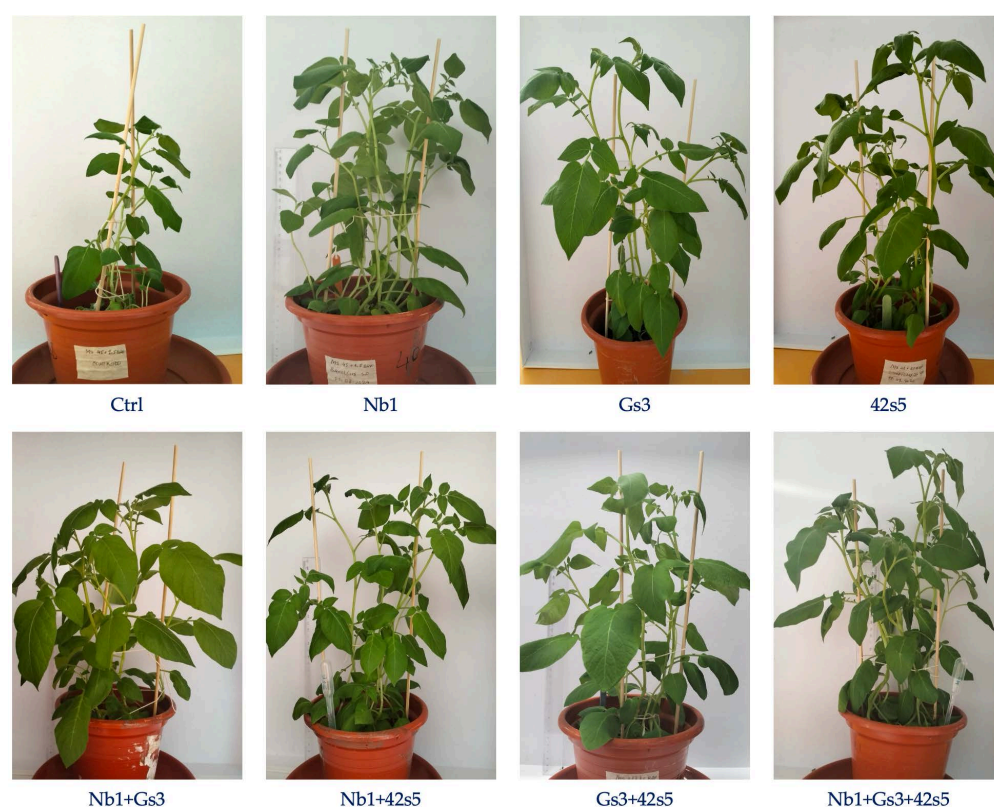
| Bacteria Isolate             | pH                        |                           | Phosphorus Content (µg/mL)  |                             | PSE (%)                    |                            |
|------------------------------|---------------------------|---------------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|
|                              | 4 Days                    | 8 Days                    | 4 Days                      | 8 Days                      | 4 Days                     | 8 Days                     |
| <i>B. velezensis</i> Nb1     | 5.31 ± 0.01 <sup>Aa</sup> | 5.06 ± 0.10 <sup>Aa</sup> | 129.10 ± 1.97 <sup>Bc</sup> | 147.40 ± 2.11 <sup>Ac</sup> | 12.91 ± 0.20 <sup>Bc</sup> | 14.74 ± 0.21 <sup>Ac</sup> |
| <i>B. pyrrocinia</i> Gs3     | 3.76 ± 0.10 <sup>Ac</sup> | 3.63 ± 0.25 <sup>Ac</sup> | 362.50 ± 1.70 <sup>Ba</sup> | 822.50 ± 6.79 <sup>Aa</sup> | 36.25 ± 0.17 <sup>Ba</sup> | 82.25 ± 0.68 <sup>Aa</sup> |
| <i>Streptomyces</i> sp. 42s5 | 4.42 ± 0.05 <sup>Ab</sup> | 4.59 ± 0.07 <sup>Ab</sup> | 188.00 ± 3.70 <sup>Bb</sup> | 333.93 ± 4.65 <sup>Ab</sup> | 18.80 ± 0.39 <sup>Bb</sup> | 33.39 ± 0.47 <sup>Ab</sup> |

Values are expressed as mean ± SD (n = 3). For each bacterium and time point, different superscript uppercase and lowercase letters indicate statistically significant differences within rows and columns, respectively ( $p < 0.05$ ).

### 3.4. Effects of Bacterial Inoculation on Potato Growth During Acclimatization and Tuber Production

#### 3.4.1. Effect of Bacterial Isolates on Vegetative Growth Parameters

The results clearly demonstrated that bacterial inoculation significantly influenced the vegetative growth of micropropagated potato plants during the acclimatization phase under greenhouse conditions. All inoculation treatments influenced potato growth parameters during the acclimatization phase. Compared with the control, plants inoculated with bacterial isolates exhibited enhanced vegetative development, as reflected by increased branch formation, plant height, and biomass accumulation (Figures 6–8; Table 6). Among the inoculations, *B. pyrrocinia* Gs3, both alone and in combination with *Streptomyces* sp. 42s5 and *B. velezensis* Nb1, produced the most pronounced effects.

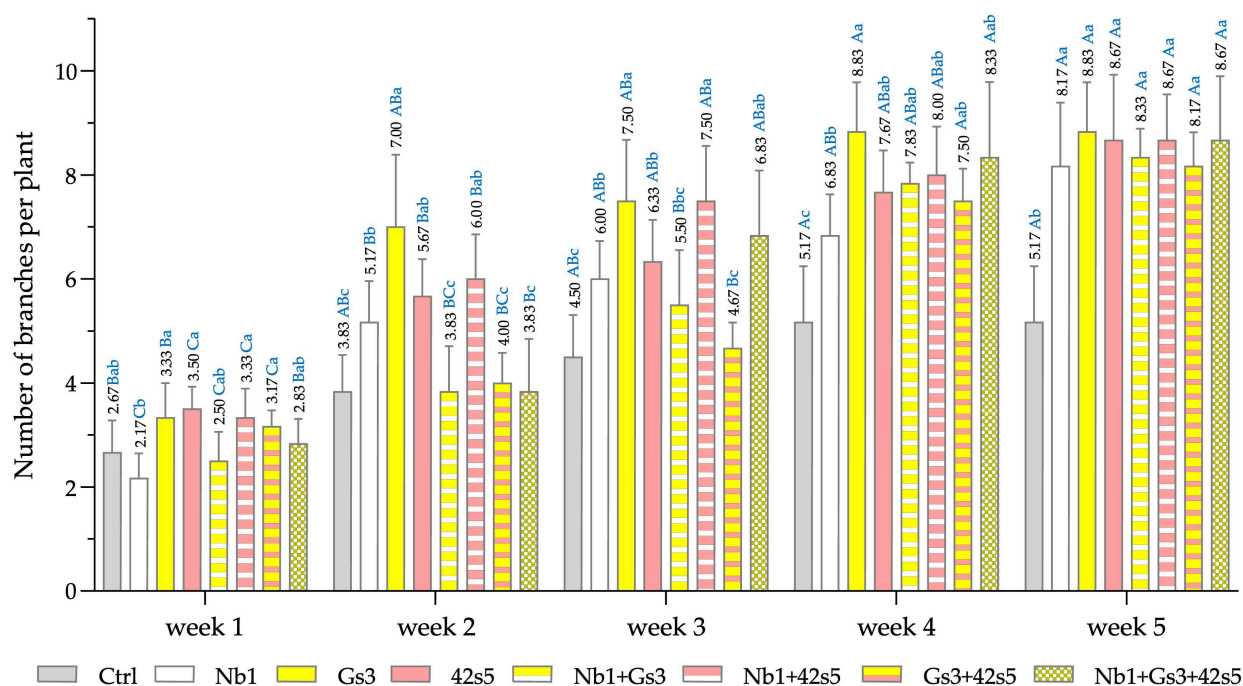


**Figure 6.** Representative micropropagated potato plants under control and bacterial inoculation treatments after 50 days of acclimatization under greenhouse conditions.

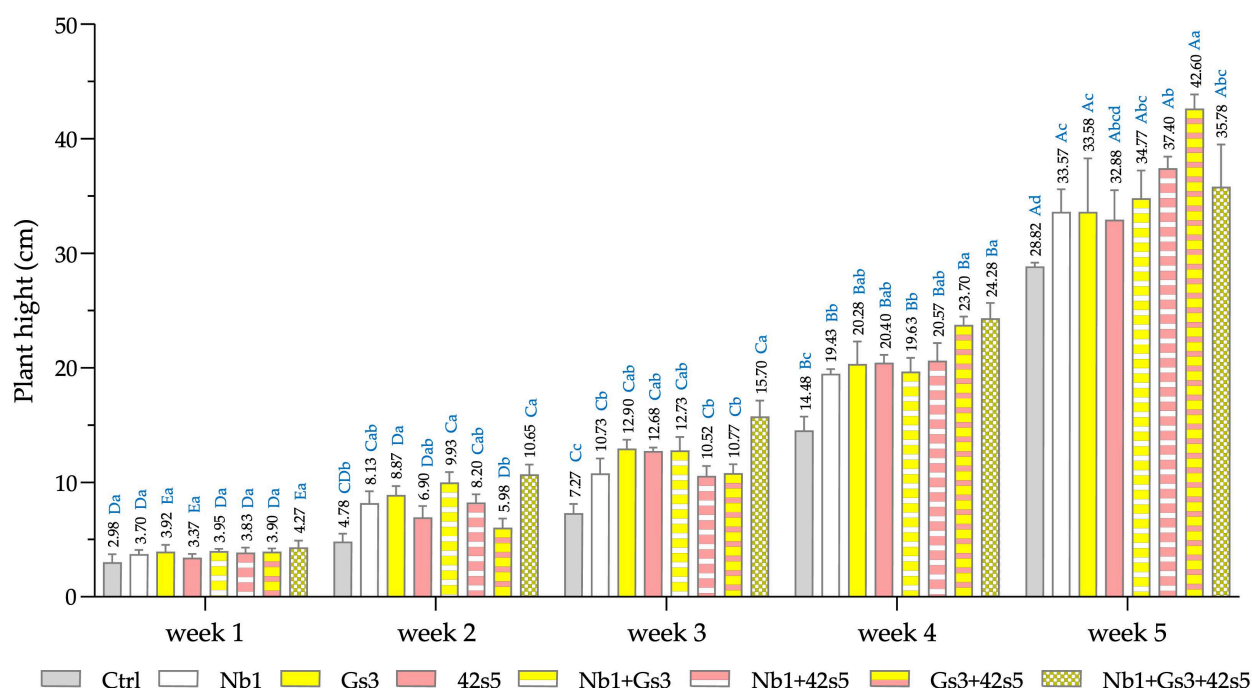
**Table 6.** Effects of bacterial inoculation on shoot and root dry biomass of micropropagated potato plants after 99 days of acclimatization under greenhouse conditions.

| Treatment                    | Dry Mass (g/Plant)        |                           |
|------------------------------|---------------------------|---------------------------|
|                              | Shoot                     | Root                      |
| Control                      | 1.02 ± 0.38 <sup>c</sup>  | 0.11 ± 0.06 <sup>c</sup>  |
| <i>B. velezensis</i> Nb1     | 1.93 ± 0.74 <sup>a</sup>  | 0.54 ± 0.25 <sup>a</sup>  |
| <i>B. pyrrocinia</i> Gs3     | 1.74 ± 0.48 <sup>ab</sup> | 0.48 ± 0.21 <sup>ab</sup> |
| <i>Streptomyces</i> sp. 42s5 | 1.84 ± 0.58 <sup>a</sup>  | 0.42 ± 0.12 <sup>ab</sup> |
| Nb1 + Gs3                    | 1.95 ± 0.26 <sup>a</sup>  | 0.46 ± 0.21 <sup>ab</sup> |
| Nb1 + 42s5                   | 1.89 ± 0.37 <sup>a</sup>  | 0.39 ± 0.18 <sup>ab</sup> |
| Gs3 + 42s5                   | 2.36 ± 0.27 <sup>a</sup>  | 0.33 ± 0.14 <sup>b</sup>  |
| Nb1 + Gs3 + 42s5             | 1.92 ± 0.49 <sup>a</sup>  | 0.38 ± 0.16 <sup>ab</sup> |

Values are expressed as mean ± SD (n = 6). Different superscript letters within each column indicate statistically significant differences ( $p < 0.05$ ).



**Figure 7.** Effect of bacterial inoculation on the number of branches of micropropagated potato during five weeks of acclimatization under greenhouse conditions. Different lowercase letters within the same week indicate significant differences among treatments; different uppercase letters indicate significant differences in branch numbers across weeks within the same treatment ( $p < 0.05$ ).



**Figure 8.** Effect of bacterial inoculation on plant height of micropropagated potato during five weeks of acclimatization under greenhouse conditions. Different lowercase letters within the same week indicate significant differences among treatments; different uppercase letters indicate significant differences in plant height across weeks within the same treatment ( $p < 0.05$ ).

Several inoculation treatments enhanced plant growth parameters during acclimatization relative to the control.

Bacterial inoculation influenced plant growth during the acclimatization phase.

- Branch Formation and Plant Height Progression

The number of branches per plant increased progressively over the five-week acclimatization period in all treatments (Figure 7). From the second week onward, inoculated plants consistently developed a higher number of branches than the non-inoculated control. Statistically significant differences between control and inoculated plants became more pronounced during weeks 4 and 5. Plants inoculated with *B. pyrrocinia* Gs3, either alone or in combination with other isolates, generally exhibited the highest number of branches during the later stages of acclimatization.

Similarly, plant height was affected by bacterial inoculation (Figure 8). No significant differences among treatments were observed during the first week after transplantation. From week 2 onward, all inoculated treatments showed significantly greater plant height compared with the control. By week 5, the tallest plants were observed in the treatment combining *B. pyrrocinia* Gs3 and *Streptomyces* sp. 42s5, while all bacterial treatments maintained significantly greater height than the non-inoculated plants.

- Shoot and Root Dry Biomass Accumulation

In this study, the impact of bacterial inoculation on biomass accumulation was evaluated after 99 days of acclimatization, with measurements taken after drying to determine shoot and root dry mass. The results demonstrated that all bacterial treatments significantly improved both shoot and root dry biomass compared to the non-inoculated control (Table 6). Shoot dry biomass ranged from 1.74 to 2.36 g per plant in inoculated treatments, compared with 1.02 g per plant in the control. The highest shoot biomass was obtained in plants inoculated with the combination of *B. pyrrocinia* Gs3 and *Streptomyces* sp. 42s5 (2.36 g per plant), while *B. velezensis* Nb1 produced the highest root dry biomass (0.54 g/plant), followed by *B. pyrrocinia* Gs3 (0.48 g per plant) and *Streptomyces* sp. 42s5 (0.42 g per plant). Combined inoculations resulted in intermediate root biomass values, all of which remained significantly higher than the non-inoculated control.

### 3.4.2. Effect of Bacterial Inoculation on Tuberization and Yield Traits

The effects of bacterial inoculation on tuberization and yield of micropropagated potato plants were evaluated 99 days after transplantation under greenhouse conditions. Tuber number and total tuber weight per plant were significantly influenced by bacterial treatments compared with the non-inoculated control (Figure 9; Table 7).

**Table 7.** Effects of bacterial inoculation on tuber numbers and yield production after 99 days of acclimatization.

| Treatment                    | Tuber Production per Plant |                             | Mean Tuber Weight (g/Tuber) |
|------------------------------|----------------------------|-----------------------------|-----------------------------|
|                              | Number of Tubers           | Weight of Tubers (g)        |                             |
| Control                      | 07.17 ± 2.48 <sup>d</sup>  | 07.75 ± 2.53 <sup>d</sup>   | 0.95 ± 0.53 <sup>c</sup>    |
| <i>B. velezensis</i> Nb1     | 16.00 ± 4.10 <sup>bc</sup> | 28.61 ± 3.87 <sup>c</sup>   | 1.84 ± 0.29 <sup>abc</sup>  |
| <i>B. pyrrocinia</i> Gs3     | 20.33 ± 6.62 <sup>a</sup>  | 34.43 ± 4.39 <sup>ab</sup>  | 2.00 ± 0.85 <sup>abc</sup>  |
| <i>Streptomyces</i> sp. 42s5 | 16.33 ± 4.50 <sup>bc</sup> | 32.23 ± 3.00 <sup>abc</sup> | 2.31 ± 0.94 <sup>ab</sup>   |
| Nb1 + Gs3                    | 14.00 ± 3.03 <sup>c</sup>  | 36.21 ± 4.05 <sup>a</sup>   | 3.56 ± 1.86 <sup>a</sup>    |
| Nb1 + 42s5                   | 17.67 ± 3.01 <sup>b</sup>  | 31.68 ± 4.84 <sup>b</sup>   | 2.01 ± 0.77 <sup>abc</sup>  |
| Gs3 + 42s5                   | 13.00 ± 2.90 <sup>c</sup>  | 34.31 ± 5.67 <sup>ab</sup>  | 2.94 ± 1.11 <sup>b</sup>    |
| Nb1 + Gs3 + 42s5             | 13.50 ± 1.64 <sup>c</sup>  | 36.91 ± 5.04 <sup>a</sup>   | 2.77 ± 0.49 <sup>b</sup>    |

Values are expressed as mean ± SD (n = 6). Different superscript letters within each column indicate statistically significant differences ( $p < 0.05$ ).

Regarding the number of tubers, all inoculated treatments produced significantly higher numbers of tubers per plant than the control (Table 7). The highest mean tuber number was observed in plants treated with *B. pyrrocinia* Gs3, which produced 20.33 tubers

per plant. Plants inoculated with *Streptomyces* sp. 42s5 and *B. velezensis* Nb1 produced 16.33 and 16.00 tubers per plant, respectively, whereas the control plants produced an average of 7.17 tubers per plant. Dual and triple inoculations resulted in intermediate tuber numbers. Among these treatments, the combination Nb1 + 42s5 produced the highest tuber number (17.67 tubers per plant), followed by Nb1 + Gs3 (14.00), Nb1 + Gs3 + 42s5 (13.50), and Gs3 + 42s5 (13.00). Although combined treatments produced fewer tubers than some single-strain inoculations, all combinations remained significantly superior to the non-inoculated control (07.17).



**Figure 9.** Visual morphological comparison of the influence of bacterial inoculation on tuber number and size in micropropagated potato plants during acclimatization under greenhouse conditions.

Concerning total tuber weight per plant, it was significantly enhanced by bacterial inoculation (Table 7). The lowest tuber weight was observed in the control treatment (7.75 g/plant). Among single inoculations, *B. pyrrocinia* Gs3 resulted in the highest tuber weight (34.43 g/plant), followed by *Streptomyces* sp. 42s5 (32.23 g/plant) and *B. velezensis* Nb1 (28.61/plant). Combined inoculations produced variable tuber-weight responses. Although some consortium treatments showed numerically higher mean tuber weights than single-strain treatments, some of these comparisons were not statistically significant ( $p > 0.05$ ). This contrasted with the results for tuber number. The highest tuber weight was measured in plants inoculated with the three-strain consortium (Nb1 + Gs3 + 42s5), yielding 36.91 g/plant. This was closely followed by the dual combination Nb1 + Gs3 (36.21 g/plant) and Gs3 + 42s5 (34.31 g/plant). The Nb1 + 42s5 treatment produced a mean tuber weight of 31.68 g per plant. All combined treatments resulted in significantly greater tuber weight than the non-inoculated control, with an average of only 7.75 g per plant.

Mean tuber weight was also affected by bacterial inoculation (Table 7). The control treatment produced the lowest mean tuber weight ( $0.95 \pm 0.53$  g/tuber). The highest mean tuber weight was recorded in the Nb1 + Gs3 treatment (3.56 g/tuber), which was significantly higher than that of the control. The *Streptomyces* sp. 42s5 and Nb1 + Gs3 treatments also produced significantly heavier tubers than the control. Although the

remaining treatments showed numerically higher mean tuber weights than the control, these differences were not statistically significant.

To evaluate individual strain effects and combined-strain interactions on harvest traits, a three-way factorial ANOVA ( $2 \times 2 \times 2$ ) was conducted at 99 days after transplanting (Table S2). For total tuber weight per plant, significant main effects were detected for the strain *B. pyrrocinia* Gs3 ( $F_{1,40} = 22.18, p < 0.001$ ) and strain *Streptomyces* sp. 42s5 ( $F_{1,40} = 5.82, p = 0.021$ ). Importantly, a statistically significant three-way interaction was confirmed (Nb1  $\times$  Gs3  $\times$  42s5;  $F_{1,40} = 5.12, p = 0.029$ ), indicating that co-inoculation with the three-strain consortium established a cooperative effect that significantly enhanced overall tuber yield. Similarly, shoot dry biomass showed significant main effects for Gs3 ( $F_{1,40} = 8.16, p = 0.007$ ) and 42s5 ( $F_{1,40} = 4.30, p = 0.044$ ), alongside significant two-way (Gs3  $\times$  42s5;  $F_{1,40} = 8.00, p = 0.007$ ), and three-way interaction (Nb1  $\times$  Gs3  $\times$  42s5;  $F_{1,40} = 6.84, p = 0.012$ ). In contrast, root dry weight and tuber number per plant were not significantly affected by any main or interaction term.

Following harvest, tubers were surface-sterilized, and internal tissue samples were plated on PDA for *Streptomyces* sp. 42s5 and on TSA for strains Nb1 and Gs3. Colonies consistent with *B. velezensis* Nb1 were recovered from tuber tissues obtained from Nb1-inoculated plants. No colonies consistent with *B. pyrrocinia* Gs3 or *Streptomyces* sp. 42s5 were recovered under the conditions tested. No colonies were recovered from uninoculated control tubers.

## 4. Discussion

Beneficial microorganisms, particularly PGPB, are increasingly recognized as sustainable tools to enhance plant growth, suppress phytopathogens, and improve crop productivity [35]. Vineyard ecosystems, especially in Portugal's Douro region, shaped by long-term management and distinct edaphoclimatic conditions, represent valuable yet underexplored reservoirs of such bacteria. In this study, three strains from different genera, *B. velezensis* Nb1, *B. pyrrocinia* Gs3, and *Streptomyces* sp. 42s5, selected from more than 30 isolates obtained from Portuguese vineyards, combined antagonistic activity against major phytopathogenic fungi with key PGP traits. These strains were therefore evaluated, individually and in consortia, for their ability to improve acclimatization success, enhance the growth of micropropagated potato plants under greenhouse conditions, and increase tuber yield and quality.

### 4.1. Molecular Identification of the PGPB Strains

Attaining species-level identification of PGPB is considered essential for their application in sustainable horticulture. Initial identification was based on 16S rRNA sequencing, which allowed us to assign the isolate to only the genus level due to its limited resolution, particularly when handling the *Bacillus* and *Burkholderia* complex [34,36]. To enhance taxonomic resolution, we used the housekeeping gene *gyrB* to discriminate among closely related groups, as it has been recently recommended to be significantly faster and to provide the higher phylogenetic resolution needed to separate these groups [33,34]. Through *gyrB* sequencing, the isolate Nb1 was identified as *Bacillus velezensis*, and Gs3 as *Burkholderia pyrrocinia*, whereas isolate 42s5 showed the highest similarity to *S. roseovorticillatus* strain IFO 14694 (GenBank accession AB072891.1), with 98.7% nucleotide identity. Although these data indicate a close relationship to the *S. roseovorticillatus* clade, we could only be assigned as *Streptomyces* sp., since neither 16S rRNA nor *gyrB* sequences supported a confident placement within a described species, reflecting a well-known challenge in the actinomycete group [37,38]. *S. roseovorticillatus* has been reported to possess effective antifungal activity in combination with PGP effects [39,40]. Species-level identification is particularly important because the species *B. velezensis* and *B. pyrrocinia*, and several

*Streptomyces* spp., are recognised as key plant-associated taxa with strong biocontrol and growth-promoting potential in agricultural systems [41–43].

#### 4.2. Antagonistic and Plant Growth-Promoting Traits of Bacterial Isolates

All three selected isolates demonstrated significant antagonistic effects against *F. oxysporum*, *F. solani*, *B. cinerea*, and *A. alternata*. The observed antifungal activity is likely due to multiple mechanisms, including the synthesis of antimicrobial metabolites, the production of hydrolytic enzymes, siderophore-mediated iron competition, and competitive exclusion for nutrients and space [2,19,35,44]. Among the isolates, *Streptomyces* sp. 42s5 and *B. pyrrocinia* Gs3 exhibited the strongest antagonistic effects, whereas *B. velezensis* Nb1 showed moderate but consistent inhibition. Among the tested fungi, *F. solani* showed the highest tolerance, while *F. oxysporum* was the most sensitive.

These findings are consistent with previous studies that reported the broad-spectrum antifungal activity of *Streptomyces* spp., which are known producers of diverse secondary metabolites, lytic enzymes, and siderophores [23,41]. Similarly, members of the genus *Burkholderia* have been widely reported as effective biocontrol agents against *Fusarium* spp. and other soil-borne pathogens affecting potato and other crops [22,45–48]. Although the antagonistic activity of *B. velezensis* Nb1 was comparatively lower, species within this genus are widely regarded as key PGP with antagonistic activity against several phytopathogenic fungi, particularly *Fusarium* species, due to their ability to produce antimicrobial lipopeptides and enzymes that contribute to disease suppression [35,44,45,49–54].

In addition to their biocontrol capacity, the three isolates expressed multiple PGP traits relevant to early plant establishment. The production of IAA, a major auxin phytohormone, by all three bacteria highlights their potential to influence root architecture, cell elongation, and lateral root formation, which are particularly important during the transition from in vitro to ex vitro conditions [55,56]. Among the tested isolates, *B. pyrrocinia* Gs3 produced the highest IAA levels, in agreement with previous reports describing *Burkholderia* species as efficient auxin producers [22,45,57,58]. *B. velezensis* Nb1 ranked second in IAA production, which aligns with reports describing members of the *Bacillus* genus as strong auxin synthesizers [45,49,53,56], while *Streptomyces* sp. 42s5 showed the lowest concentration but sustained IAA production, possibly due to its slower growth compared to the other isolates; however, *Streptomyces* species remain notable IAA producers, as reported in several studies [41,59–62]. Siderophore production was detected in all isolates, although quantitative differences were observed among genera. *B. velezensis* Nb1 exhibited the largest halo zones, indicating strong iron-chelating activity, whereas *B. pyrrocinia* Gs3 showed the highest siderophore production efficiency relative to colony size. Siderophores play a dual role in PGP by enhancing iron availability for the host plant and limiting iron access to competing phytopathogens [63]. The siderophore-producing capacity observed in this study aligns with previous findings for *Bacillus* [14,53], *Burkholderia* [22,64,65], and *Streptomyces* [59–61,66].

Regarding phosphate solubilization, all isolates were able to produce clear halos on solid medium containing TCP as the inorganic phosphate source. In contrast, on  $\text{AlPO}_4$  medium, only a color change around the colonies was observed without halo formation, suggesting acidification of the medium without detectable phosphate diffusion. Based on these observations, TCP was selected as the sole phosphate source for the quantitative determination of solubilized phosphorus in liquid medium. *B. pyrrocinia* Gs3 consistently exhibited the highest phosphate solubilization capacity on both solid and liquid media, accompanied by a marked reduction in medium pH, indicating organic acid-mediated solubilization. *Streptomyces* sp. 42s5 showed moderate activity, while *B. velezensis* Nb1 displayed lower solubilization efficiency.

Similar patterns have been reported for these genera of *Bacillus* [53,67,68], *Burkholderia* [22,47,57], and *Streptomyces* [41,59,61,69–72], where phosphate solubilization is largely driven by the secretion of organic acids, which chelate and dissolve phosphate compounds, and by the production of phosphatase enzymes that mineralize organic phosphorus sources, thereby improving phosphorus availability [59,73]. Given the central role of phosphorus in early plant growth and tuber initiation, this trait is particularly relevant for potato acclimatization and subsequent yield formation [63,74].

Overall, the results demonstrated that the selected isolates possess complementary antagonistic and PGP traits. *B. pyrrocinia* Gs3 emerged as the most functionally versatile isolate, combining strong antifungal activity, high IAA production, and superior phosphate solubilization, while *B. velezensis* Nb1 and *Streptomyces* sp. 42s5 contributed distinct but supportive functional attributes. This functional diversity provides a mechanistic basis for their subsequent performance during plant acclimatization and supports their evaluation as individual inoculants and as components of multi-strain consortia.

#### 4.3. Effect of Bacterial Inoculation on Micropropagated Potato Growth

The acclimatization phase is a critical step in potato micropropagation, during which plantlets adapt to the more variable ex vitro environment after growing under aseptic in vitro conditions. This stage is typically associated with physiological stress, limited root functionality, and reduced growth, often resulting in poor establishment and sub-optimal plant performance [10–12]. Unfavorable temperature shifts can further impair shoot growth and accelerate physiological decline in sensitive potato cultivars [6]. In the present study, inoculation with native PGPB significantly enhanced vegetative growth parameters of micropropagated potato plants during acclimatization, indicating that early microbial association can support plant adaptation under greenhouse conditions. These findings suggest that native PGPB may support stabilizing plant growth under fluctuating greenhouse microclimates.

All bacterial treatments resulted in improved vegetative performance compared with the non-inoculated control, as reflected by increased branch formation, plant height, and shoot and root biomass accumulation. Notably, plant survival reached 100% across all treatments, indicating that the strains not only promoted growth but also improved acclimatization efficiency. Our findings are consistent with similar improvements in vegetative development during acclimatization that have been reported in potato and other crops following inoculation with PGPB, including *Azospirillum*, *Ochrobactrum*, and *Bacillus* species [56,75–80]. Enhanced shoot and root growth during this phase is considered a key indicator of successful acclimatization, as it reflects the plant's ability to re-establish functional water and nutrient uptake systems under ex vitro conditions [11,20]. Among the tested isolates, *B. pyrrocinia* Gs3 consistently promoted greater branch formation and plant height, both as a single inoculant and in combination with other strains. These effects are consistent with the high IAA production and phosphate-solubilizing capacity observed for this isolate, traits that are closely associated with shoot elongation, lateral branching, and early biomass accumulation [22,45,57,58]. Previous studies have similarly reported that *Burkholderia* species can enhance vegetative growth and acclimatization efficiency in micropropagated plants by stimulating hormonal balance and improving nutrient availability [25,57,81,82].

Root biomass accumulation was particularly enhanced in plants inoculated with *B. velezensis* Nb1, which produced the highest root dry mass among all treatments. These findings are aligned with numerous studies reporting the positive impact of *Bacillus* strains, particularly *B. cereus*, on potato growth and performance through their capacity to stimulate root development by producing phytohormones, siderophores, and other bioactive

compounds, as well as their ability to persist in the rhizosphere and endophytic compartments [53,68,83–87]. Improved root system development during acclimatization is especially important for potato, as it facilitates nutrient uptake and supports subsequent tuber initiation and growth. *Streptomyces* sp. 42s5 also contributed to enhanced vegetative growth, particularly when applied in combination with *B. pyrrocinia* Gs3. Although *Streptomyces* species have been less frequently studied in the context of potato acclimatization, they are known to produce a wide range of secondary metabolites and phytohormones that can positively influence plant growth and stress tolerance [41,59–62]. The observed improvements in shoot biomass and plant height in co-inoculated treatments suggest that *Streptomyces* may play a complementary role by supporting overall plant vigor during early establishment.

Overall, these results indicate that bacterial inoculation during acclimatization enhances vegetative growth by improving key morphological traits associated with plant vigor and early development. The differential responses observed among isolates further suggest that distinct functional traits, such as auxin production, phosphate solubilization, and root stimulation, contribute to growth promotion in a strain-specific manner. These findings highlight the importance of selecting functionally compatible bacterial strains to support the successful acclimatization of micropropagated potato plants under greenhouse conditions.

#### 4.4. Effect of Bacterial Inoculation on Tuberization and Colonization

Tuberization represents the final and economically most relevant outcome of successful potato establishment, integrating the cumulative effects of early vegetative growth, nutrient acquisition, and physiological status throughout the growth cycle. In the present study, bacterial inoculation during the acclimatization phase had a clear and lasting impact on tuberization, as reflected by significant increases in both tuber number and total tuber weight compared with the non-inoculated control.

Individual inoculations, particularly with *B. pyrrocinia* Gs3, resulted in the highest number of tubers per plant. This response is consistent with the strong vegetative growth observed in plants treated with this isolate. It may reflect the role of auxin production and enhanced phosphorus availability in promoting stolon initiation and tuber formation. Previous studies have reported that PGPB capable of producing IAA and solubilizing phosphate can positively influence tuber initiation by modulating hormonal balance and improving nutrient uptake during early developmental stages [57,68,80,88]. The increased tuber number observed with *B. pyrrocinia* Gs3 aligns with reports highlighting the capacity of *Burkholderia* species to enhance reproductive and storage organ development in several crops [57,88].

In contrast, combined inoculations tended to favor tuber biomass accumulation rather than tuber number. The highest total tuber weight was obtained with the three-strain consortium (Nb1 + Gs3 + 42s5), followed closely by the dual combination Nb1 + Gs3. These results suggest that while individual strains may preferentially stimulate tuber initiation, multi-strain consortia may enhance overall assimilate allocation and tuber bulking. Similar trends have been reported in potato and other crops, where microbial consortia outperform single-strain inoculants in terms of yield and biomass production due to functional complementarity among strains [53,56,80,89].

The differential effects observed between single and combined inoculations likely reflect the integration of complementary microbial functions. *B. pyrrocinia* Gs3 contributed strong auxin production and phosphate solubilization, *B. velezensis* Nb1 enhanced root biomass development, and *Streptomyces* sp. 42s5 provided additional growth-supporting and protective functions. Together, these traits may promote more efficient nutrient uptake

and carbon allocation during later developmental stages, resulting in increased tuber weight rather than a greater number of tubers. Such functional complementarity is a key factor underlying the improved performance of microbial consortia reported in previous studies [18,53,90]. Furthermore, because potato plants are highly sensitive to thermal stress, which can depress tuber yield and distribution [6], the ability of the triple consortium to enhance total tuber weight under variable greenhouse conditions represents a crucial step toward more climate-resilient potato farming.

Endophytic colonization assays further revealed differences among the tested isolates. Only *B. velezensis* Nb1 was successfully re-isolated from internal tuber tissues, indicating its ability to persist within the endophytic compartment of potato plants. This finding is consistent with numerous reports describing *Bacillus* species as effective endophytes capable of colonizing diverse plant tissues, including roots, stems, and storage organs, and maintaining stable populations over time [50,85,91]. The absence of detectable endophytic colonization by *Burkholderia* and *Streptomyces* does not preclude their contribution to plant growth and yield. Rhizosphere-associated bacteria can exert substantial indirect effects through nutrient mobilization, hormonal signaling, and pathogen suppression, ultimately influencing plant performance and productivity [22,41,57]. The observed increases in tuber number and weight in treatments containing these isolates support the relevance of such mechanisms.

Overall, the results demonstrated that bacterial inoculation during the acclimatization phase not only enhances early vegetative development but also exerts lasting effects on tuberization and yield. The contrasting responses observed between single-strain and consortium treatments underscore the importance of strain selection and functional compatibility when designing bioinoculant formulations for potato production.

#### 4.5. Benefits of Multi-Strain Bacterial Combinations

The present study demonstrated that multi-strain bacterial inoculations can improve the acclimatization and subsequent development of micropropagated potato plants. While individual bacterial isolates promoted specific growth and yield traits, the combined application of functionally distinct strains resulted in more consistent improvements, particularly with respect to shoot biomass accumulation and tuber weight. Generally, plants inoculated with consortia outperformed those receiving individual treatments in terms of overall productivity, despite producing fewer tubers in some cases. This pattern suggests that microbial combinations primarily enhanced assimilate partitioning and tuber bulking rather than tuber initiation. These findings align with numerous reports that have reported in potato and other crops, where multi-strain inoculants improved yield stability and biomass accumulation by integrating complementary microbial functions, including nutrient solubilization, phytohormone production, root stimulation, and pathogen suppression [18,53,62,90].

These enhanced effects are likely associated with complementary mechanisms, including phosphate solubilization, siderophore production, IAA production, and antimicrobial activity, which act together, leading to improved nutrient uptake, hormone regulation, and pathogen suppression during the critical acclimatization stage. Such cooperative interactions not only strengthen shoot and root development but also enhance the final yields. Furthermore, co-inoculation of bacterial combinations may stimulate the synthesis of novel biologically active molecules or increase the concentration of bioactive metabolites, which could have beneficial effects not only in promoting plant growth and defense but also be beneficial for human health [53]. However, the overall success of microbial combinations strongly depends on the compatibility and ecological balance among strains, as antagonistic interactions may reduce their effectiveness and compromise plant growth

promotion [92,93]. Based on these findings, it can be suggested that carefully designed bacterial combinations are essential for further optimizing plantlet adaptation and early establishment under greenhouse conditions, offering a promising bio-based strategy to enhance acclimatization success and maximize final yield production [20,94].

Overall, to the best of our knowledge, this study is the first to investigate the individual and combined effects of three bacterial strains from distinct genera, *Bacillus*, *Burkholderia*, and *Streptomyces*, on the acclimatization success of micropropagated potato plants. The results clearly demonstrated that both single and combined inoculations significantly enhanced plantlet establishment, vegetative growth, and final tuber yield under greenhouse conditions.

## 5. Conclusions

This study demonstrated that the native strains *B. velezensis* Nb1, *B. pyrrocinia* Gs3, and *Streptomyces* sp. 42s5 possess relevant PGP and antagonistic traits, including IAA and siderophore production, phosphate solubilization, and inhibition of well-known phytopathogenic fungi. Inoculation with these isolates, applied individually and in combination, significantly improved the acclimatization of micropropagated potato plants by enhancing vegetative growth, biomass accumulation, and final tuber yield under greenhouse conditions. Distinct responses were observed between single and combined inoculations. While individual strains, particularly *B. pyrrocinia* Gs3, favored tuber number, multi-strain consortia, especially the three-strain combination, maximized total tuber weight, highlighting the advantage of functional complementarity among strains. The successful endophytic colonization of potato tubers by *B. velezensis* Nb1 further underscores its versatility and potential contribution to sustained plant performance. Overall, these findings support the use of native PGPB as effective biofertilizers and biocontrol agents to enhance the transition of micropropagated plants to ex vitro conditions while reducing reliance on synthetic agrochemical inputs. Furthermore, designing potato propagation systems around native PGPB consortia may contribute to climate-resilient production by improving crop performance under variable greenhouse environments and enabling low-input management. Further field-based validation across different cultivars and environmental conditions, including strain-level safety assessments, is required to assess their practical and commercial applicability in sustainable agricultural systems.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae12091134/s1>. Figure S1: Maximum-likelihood phylogenetic tree based on partial 16S rRNA gene sequences showing the relationships of isolates Nb1, Gs3, and 42s5 with closely related *Bacillus*, *Burkholderia*, and *Streptomyces* reference strains retrieved from the NCBI GenBank database. Isolates investigated in this study are shown in bold. Superscript “T” indicates a type strain. The Tamura–Nei model with a proportion of invariant sites (TN93+I) was selected as the best-fit nucleotide-substitution model and implemented in MEGA 12. Bootstrap support values were calculated from 1,000 replicates and are shown at the nodes. The scale bar represents the number of nucleotide substitutions per site; Figure S2: Maximum-likelihood phylogenetic tree based on partial *gyrB* gene sequences showing the phylogenetic positions of isolates Nb1, Gs3, and 42s5 among closely related *Bacillus*, *Burkholderia*, and *Streptomyces* reference strains retrieved from the NCBI GenBank database. Isolates investigated in this study are shown in bold. Superscript “T” indicates a type strain. The General Time Reversible model with a proportion of invariant sites (GTR+I) was selected as the best-fit nucleotide-substitution model and implemented in MEGA 12. Bootstrap support values were calculated from 1,000 replicates and are shown at the nodes. The scale bar represents the number of nucleotide substitutions per site; Table S1: Strains and GenBank accession numbers of the sequences used in the 16S rRNA and *gyrB* gene phylogenetic analyses;

Table S2: Three-Way Factorial ANOVA ( $2 \times 2 \times 2$ ) evaluating main strain effects and two-way and three-way interactions for plant biomass and tuber yield parameters at 99 days post-transplanting.

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