Biopreparation growth stimulator for conifer seedlings and obtaining procedure

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Abstract. In recent years, the need to define and adopt more sustainable and environmentally friendly forestry practices has been well recognized. The interest in ecological solutions in modern forestry arises from the search for natural strategies to reduce the application of chemical substances in forestry. Today, it is accepted that microorganisms thrive in various natural environments within complex microbial communities. Therefore, the use of beneficial microorganisms combined in consortia is auspicious for improving the yield and quality of crops, representing a reliable and ecological solution that can address the challenges of modern forestry. A well-designed application of natural microorganisms and organic amendments can greatly increase the yield of conifer seedlings or the control of seedling pathogens in an ecologically sustainable manner. The microbial biopreparation, is composed of the strains *Brevibacillus borstelensis* GyA23 + *Pseudomonas putida* VfC23, isolated from soil (the strains of GyA23 and VfC23 was isolated and named by authors, and belong to them). This study focuses on the development of a bacterial biopreparation used as a biostimulant. This consortium can promote the growth of *Picea abies* seedlings through nutrient mobilization, phytohormone production, and plant protection by direct and indirect antagonism against phytopathogens. This was followed by in vitro analyses and testing of the biostimulatory effect of the biopreparation both in the laboratory and in the greenhouse. During testing, the development of conifer seedling cultures was monitored comparatively, focusing on seedlings inoculated with the biopreparation consortium *B. borstelensis* GyA23 + *P. putida* VfC23. The obtained data were processed using the methods of mathematical statistics. Analyzing the results obtained from the in vitro testing of the biopreparation, the authors conclude that the bacterial consortium *B. borstelensis* GyA23 + *P. putida* VfC23 had a beneficial effect on the growth and development of *Picea abies* seedlings, visibly stimulating the total weight of the seedlings.
Key Words: microbial biopreparation, growth stimulator, conifer seedlings, greenhouse.

Introduction. The tasks of gradually transferring forestry management to continuous and sustainable forest management, increasing the productivity of forest stands, and improving their qualitative composition have not lost their relevance today. One of the ways of solving them is artificial reforestation. In forestry management, a method has been introduced in recent decades that has positively proven itself in agriculture and experimental work at forest nurseries, namely, the method of growing the planting material with the use of growth stimulants. Literature studies highlight the significant role of plant growth-promoting bacteria (PGPR) in sustainable agriculture due to their beneficial characteristics, which contribute to nutrient mobilization for plants and plant protection (Pathania et al. 2020). Some bacterial strains found in soil participate in nutrient cycling between soil and plants. These bacteria are widely used in agriculture for plant growth promotion and soil biofertilization. Beneficial bacteria can be categorized as organic matter decomposers, plant growth-promoting rhizobacteria (PGPR), and antagonists of plant pathogens.

The most well-known PGPR bacteria belong to the *Pseudomonas* and *Bacillus* genus. Fluorescent *Pseudomonas* species, such as *Pseudomonas putida*, not only promote plant growth by producing indole-3-acetic acid (IAA), siderophores, and antibiotics but also mineralize organic materials in the soil. This mineralization occurs through the hydrolytic activity of extracellular enzymes released by bacteria, including cellulases, phytases, acid or alkaline phosphatases, alkaline or neutral proteases, peptidases, and esterase's. Morphologically, these bacteria are Gram-negative, non-sporulating, straight or curved rods measuring 0.5–1.0 μm by 1.5–5.0 μm. *P. putida* can also function as a biocontrol

agent. Its presence in the rhizosphere induces systemic resistance in plants, protecting the host plant against infection and pathogen proliferation (Matilla et al 2010) It has shown potential biocontrol properties, effectively antagonizing plant pathogens such as *Pythium aphanidermatum* and *Fusarium oxysporum* (Postma et al 2013), which cause damping-off and root rot in conifer seedlings.

Brevibacillus species are considered PGPR and are prominent in soil and sediments. They have been exploited for their beneficial use in agriculture and environmental remediation due to their numerous potential functions. Morphologically, *Brevibacillus* is a Gram-positive/negative, aerobic, mobile, spore-forming, rod-shaped bacterium commonly found in soil, air, water, and decomposing matter. Several researchers have explored this bacterium as a PGPR, applying it to various crops such as tomatoes, cotton, maize, and tea (Girish & Umesha 2005; Nehra et al 2016; Dutta & Thakur 2017). *Brevibacillus* species exhibit antagonistic properties against soil-borne phytopathogens (Khan et al 2001) and stimulate the development of endomycorrhizae on plant roots (Grichko et al 2000; Zhou & Leul 1999; Burd et al 2003).

Various microorganism-based biopreparations exist, including bacterial consortia with biostimulant effects and multifunctional actions on crops and soil. Currently, synthetic fertilizers are widely applied to crops to meet the global food demand, leading to high health, economic, and environmental costs (Madhurankhi & Deka 2020). A well-studied and sustainable alternative for improving plant growth and soil fertility is the application of plant growth-promoting bacteria (PGPB). These bacteria possess functional traits that regulate the growth, development, and productivity of crops. These growth-promoting effects are due to the enhanced availability and biosynthesis of several limiting macro- and micronutrients, as well as crop protection against stressful environmental conditions (Rojas-Solis et al 2020; Morales-Cedeno et al 2021). It has been reported that bacterial consortia enhance beneficial plant traits compared to individual strains by covering a diverse set of plant growth-promoting and biocontrol mechanisms (Ju et al 2019). Utilizing these consortia is a feasible strategy for mitigating drought (Joshi et al 2020), salinity (Nawaz et al 2020), nutrient absorption (Rana et al 2012), pests, and phytopathogenic infections (Villa-Rodriguez et al 2019) in agricultural crops. Moreover, some bacterial consortia can fix nitrogen, transform certain unavailable nutrients into an assimilable form, produce phytohormones, and chelate iron, which is crucial for maintaining soil quality and health. They can also mitigate the negative effects of unsustainable conventional agricultural practices (Gosal & Kaur 2017).

There are two known types of bacterial consortia—simple and complex. The differences lie in the fermentation strategy or protocol (producing a large bacterial population that will later be formulated into an inoculant), where strains are cultivated individually or in combination with other species/strains in a medium suitable for all PGPB species (de-Bashan et al 2020). This is a crucial step, as a higher number of species generally leads to a greater number of interactions between strains, generating differences in metabolite secretions. On the other hand, the success of bacterial consortia in field conditions depends on the type and function of the strains used, where certain aspects require special attention, including adaptation to unfavorable climatic conditions, survival, and persistence in the soil after inoculation (Gosal & Kaur 2017; Verbruggen et al 2013). Some consortia include strains belonging to the genus *Pseudomonas* and consortia that include strains belonging to the genus *Brevibacillus*. This study describes a consortium of bacterial strains, specifically *Brevibacillus* sp. *and Pseudomonas putida*, and their biostimulatory effect on the growth of conifer seedlings. Different consortia/biopreparations based on inoculated microorganisms have been described, with biostimulatory effects and antagonism against phytopathogens, applicable in agriculture, but not in forestry. These biopreparations have the disadvantage that they do not exhibit the expected effects due to the specificity of ecological niches, the plant-microorganism relationship, and the influence of region-specific pedological and climatic conditions.

The objective of this research was to develop a biopreparation containing two bacterial strains—*Pseudomonas putida* and *Brevibacillus borstelensis*—which have various beneficial characteristics for the growth of conifer seedlings and show antagonistic activities

against different phytopathogenic fungi. Based on these strains, a consortium was formed, and its beneficial effects were tested on *Picea abies* seedlings.

Material and Method. The microbial biopreparation resulting from this research is composed of the strains *Brevibacillus borstelensis* GyA23 + *Pseudomonas putida* VfC23 (name given by authors), which promote the growth and protection of conifer seedlings of the *Picea abies* species. The procedure for obtaining beneficial complex-effect biopreparations with the help of the *B. borstelensis* GyA23 and *P. putida* VfC23 bacterial strains, isolated from soil and litter samples (where the density of naturally regenerated seedlings is high), involves obtaining an inoculum by cultivating the *B. borstelensis* GyA23 strain in media containing glucose as a carbon source, corn extract, ammonium sulfate as nitrogen sources, and mineral salts at a temperature of 28°C for 24 hours with agitation at 140 rpm. The *P. putida* VfC23 strain is cultivated in media containing sucrose as a carbon source, soy flour as a nitrogen source, and mineral salts at a temperature of 28°C for 24 hours with agitation at 200 rpm. The two obtained biomasses are then mixed in a gravimetric ratio of 1:1 and applied to seeds, seedlings, or soil. The bacterial strains *B. borstelensis* GyA23 and *P. putida* VfC23 used in the biotechnological process are isolated from soil in the Gurghiu Depression: the Joseni area and the Vârful Caprei Remetea peak, where the natural regeneration of *Picea abies* seedlings had a high density.

The microbial biopreparation with a beneficial complex effect on *Picea abies* seedlings was obtained by mixing the *B. borstelensis* GyA23 (name given by authors) and *P. putida* VfC23 (name given by authors) bacterial strains isolated from soil in the forest, where natural regeneration had a high density. For the cultivation media, glucose and sucrose were used as carbon sources, while soy flour, corn extract, and ammonium sulfate were used as nitrogen sources. In addition to these raw materials, mineral salts containing calcium, iron, potassium, phosphorus, and magnesium were used. The effect of the obtained microbial biopreparation was studied in vitro on conifer seedlings, with favorable results in terms of beneficial traits and growth promotion of *Picea abies* seedlings.

Procedure for obtaining the microbial biopreparation by biosynthesis. The procedure for obtaining microbial biopreparations involves the following steps:

Phase 1. Obtaining the pre-inoculum culture. In the first phase, a pre-inoculum culture is obtained by cultivating the *B. borstelensis* GyA23 and *P. putida* VfC23 bacterial strains in shaken flasks with suitable media for each strain, taking into account the biochemical characteristics of bacterial strains (Table 1). These media contain glucose and sucrose as carbon sources and soy flour, corn extract, ammonium sulfate, and mineral salts as nitrogen sources. The development of the microbial strains occurs at a temperature of 28°C for 24 hours. The inoculum phase takes place under the same conditions as the pre-inoculum, but in a larger volume of the culture medium. In the bioprocess phase, the bacterial strains are developed and cultured using a cultivation medium different from the inoculum. The culture media differ based on the bacterial strains used: *B. borstelensis* GyA23 and *P. putida* VfC23, but the working conditions are nearly identical. For obtaining the *B. borstelensis* GyA23 biomass, the incubation temperature is 28°C for 24 hours with agitation at 140 rpm, while for the *P. putida* VfC23 strains, the incubation temperature is 28°C for 24 hours with agitation at 200 rpm.

Table 1

Biochemical characteristics of bacterial strains

Characteristics	*Brevibacillus borstelensis GyA23*	*Pseudomonas putida VfC23*
Gram staining	±	-
Spore formation	+	-
Glucose oxidation	+	+
Lactose oxidation	-	-
Nitrate reduction	+	+
Type of respiration	Aerobic	Aerobic

The identification of the *B. borstelensis* GyA23 and *P. putida* VfC23 strains was based on the 16S rDNA sequence (SME SeqOmics Biotechnology Ltd) (Table 2).

Table 2

Identification of the strains based on 16S rDNA sequences

Strain	*Length of 16S rDNA sequence (base pairs)*	*Nucleotide composition of the 16S rDNA sequence*	*Strains based on 16S rDNA sequence similarity in GenBank (Reference number; similarity percentage)*
GyA23	1486	CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGA GCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGA GTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATA ACATGGGGAAACTCATGCTAATACCGGATAGGGTCTT CTCTCGCATGAGAGGAGACGGAAAGGTGGCGCAAGC TACCACTTACGGATGGGCCTGCGGCGCATTAGCTAGT TGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTA GCCGACCTGAGAGGGTGACCGGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAACCAGCAGTAG GGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAA CGCCGCGTGAACGATGAAGGTCTTCGGATTGTAAAG TTCTGTTGTCAGAGACGAACAAGTACCGTTCGAACAG GGCGGTACCTTGACGGTACCTGACGAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG CGCGCAGGCGGCTATGTAAGTCTGGTGTTAAAGCCC GGGGCTCAACCCCGGTTCGCATCGGAAACTGTGTAG CTTGAGTGCAGAAGAGGAAATCGGTATTCCACGTGTA GCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT GCCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGA GGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGG GTTGGGGGTTTCAATACCCTCAGTGCCGCAGCTAACG CAATAAGCACTCCGCCTGGGGAGTACGCTCGCAAGA GTGAAACTCAAAGCGATTGACGGGGGCCCGCACAAG CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA GAACCTTACCAGGTCTTGACATCCCGCTGACCGTCCT AGAGATAGGGCTTCCCTTCGGGGCAGCGGTGACAGG TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT TGGGTTAAGTCCCGACGCGAGCGCAACCCTTATCTTT AGTTGCCAGCATTCAGTTGGGCACTCTAGAGAGACTG CCGTCGACAAGACGGAGGAAGGCGGGGATGACGTC AAATCATCATGCCCCTTATGACCTGGGCTACACACGT GCTACAATGGCTGGTAGTACGGGAAGCTAGCTCGCG AGAGTATGCCAATCTCTTAAAACCAGTCTCAGTTCGG ATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATC GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC GTTCCCGGGCCTTGTACACACCGCCCGTCACACCAC GGGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCG CAAGGAGCCAGCCGCCGAAGGTGGGGTAGATGACTG GGGTGAAGTCGTAACAAGGTATCCGTACCGGAAGG	*Brevibacillus borstelensis* - similarity 99%

Strain	*Length of 16S rDNA sequence (base pairs)*	*Nucleotide composition of the 16S rDNA sequence*	*Strains based on 16S rDNA sequence similarity in GenBank (Reference number; similarity percentage)*
VfC23	1459	ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTC GAGCGGATGAGAAGAGCTTGCTCTTCGATTCAGCGG CGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTA GTGGGGGACAACGTTTCGAAAGGAACGCTAATACCG CATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGG CCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTA GTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCG TAACTGGTCTGAGAGGATGATCAGTCACACTGGAACT GAGACACGGTCCAGACTCCTACGGGAGGCAGCATTG GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGC CATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAA GCACTTTAAGTTGGGAGGAAGGGCAGTAAGCGAATA CCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCG GCTAACTCTGTGCCGGCAGCCGCGGTAATACAGAGG GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CGCGTAGGTGGTTTGTTAAGTTGAATGTGAAAGCCCC GGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAG CTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGT AGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG TGGCGAAGGCGACCACCTGGACTGATACTGACACTG AGGTGCGAAAGCGTGGGGAGCAAACAGGTTTAGATA CCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCC GTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGC ATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGT TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC GGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAGGCCTTGACATGCAGAGAACTTTCTAG AGATAGATTGGTGCCTTCGGGAACTCTGACACAGGT GCTGCATGGCTGTCGTCAGCTCGTGTCCTGAGATGTT GGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTA GTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACT GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT CAAGTCATCATGGCCCTTACGGCCTGGGCTACACACG TGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGC GAGGTGGAGCTTATCTCACAAAACCGATCGTAGTCCG GATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAA TCGCTAGTAATCGCGATCCAGAATGTCGCGGTGAATA CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT GGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCT TCGGGAGGACGGTTACCACGGTGTGATTCATGACTG GGGTG	*Pseudomonas putida* - similarity 99%

Phase 2. Determination of inorganic and organic phosphate solubilization capacity in bacterial strains with phosphate mobilization characteristics. Bacteria capable of solubilizing inorganic phosphates were selected by inoculating them on Pikovskaya nutrient media containing tricalcium phosphate. For microbes with phosphate mobilization capacity, the medium becomes transparent around the developed colonies due

to the production of organic acids. The 24-hour-old *B. borstelensis* GyA23 and *P. putida* VfC23 strains were inoculated on Pikovskaya media and incubated at 28°C for 48 hours. In this medium, the color around the bacterial colony became transparent, indicating phosphate mineral solubilization. The 24-hour-old *B. borstelensis* GyA23 and *P. putida* VfC23 strains were inoculated on phytate screening medium (PSM) to test the solubilization of organic phosphates from phytates. On this medium, the bacteria of the *B. borstelensis* GyA23 and *P. putida* VfC23 strains produced halos around the colonies, indicating the solubilization of organic phosphorus from phytates.

Phase 3. Determination of the capacity to produce auxin phytohormone (indole-3-acetic acid). The production of indole-3-acetic acid (IAA) was analyzed both qualitatively and quantitatively. The qualitative determination of IAA production was carried out on inoculated agar plates. A nitrocellulose membrane (1x1 cm) was placed over the bacterial colonies after 24-hour incubation at 28°C. The nitrocellulose membrane was then immersed in Salkowski's reagent. The appearance of a red color indicated the capability to produce indole-3-acetic acid. For the quantitative determination of IAA, the *P. putida* VfC23 strain was inoculated in liquid Tryptone-Soy Broth (TSB) medium containing 10 mg mL^{-1} tryptophan and incubated at 28°C for 72 hours at 140 rpm. After incubation, the bacterial suspension was centrifuged and the supernatant was added to Salkowski's reagent and incubated at room temperature for 25-30 minutes. The absorbance of the solution was measured at 530 nm. A calibration curve was created by measuring the absorbance at 530 nm of solutions containing indole-3-acetic acid in different concentrations. The quantity of IAA produced by the studied bacterial strains was calculated based on the calibration curve. The amount of auxin produced by the *P. putida* VfC23 strain was 16.5 mg mL^{-1}.

Phase 4. Determination of siderophore production capacity in Brevibacillus borstelensis GyA23 and Pseudomonas putida VfC23 strains. To determine the siderophore production capacity of the tested bacterial strains, solid culture media containing Chrome Azurol S (CAS) were used as described by Oldal et al (2002). The culture medium contains: piperazine, protease peptone, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, glycerin, agar, pH = 6.5. The inoculated Petri dishes were incubated at 28°C for 48 hours. The results demonstrated that the tested bacterial strains are capable of producing siderophores. Around the inoculated *B. borstelensis* GyA23 and *P. putida* VfC23 colonies, the initial blue color of the medium turned yellow, indicating that these bacterial strains can mobilize iron.

Phase 5. Analysis of the biocontrol activity of bacterial strains. The antagonistic activity of *B. borstelensis* GyA23 and *P. putida* VfC23 strains against various phytopathogenic fungi, such as *Pythium aphanidermatum and Fusarium oxysporum*, was analyzed in vitro. The bacterial biomass was spotted onto the culture medium at a maximum distance of 1.5 cm from the fungal mycelium. The cultures of phytopathogenic fungi were refreshed on SNA (Synthetischer Nährstoffarmer Agar, Nirenberg 1976) medium and incubated at 28°C for 5 days. The *B. borstelensis* GyA23 strain was refreshed on a Luria-Bertani agarized medium. The inoculated Petri dishes were incubated at a temperature of 28°C for 24 hours. The *P. putida* VfC23 strain was refreshed on King's B medium. The Petri dishes were incubated at 28°C for 48 hours. Petri dishes seeded with the bacterial strains were incubated at 28°C, and the inhibition zones caused by the *B. borstelensis* GyA23 and *P. putida* VfC23 strains were analyzed at 48 and 72 hours. The results showed that both tested bacterial strains produced antifungal metabolites. The *B. borstelensis* GyA23 strain produced a larger inhibition zone against *F. oxysporum*, while the inhibition of *P. aphanidermatum* was greater with the *P. putida* VfC23 strain.

Phase 6. Obtaining bacterial biomass through fermentation. The liquid industrial medium contained corn extract, K_2HPO_4, and sucrose. From the bacterial cultures incubated on a solid Nutrient medium at 28ºC for 24 hours, a base suspension was prepared using a sterile physiological solution. The bacterial count in this base suspension was set to 10^8 CFU mL^{-1}. This base suspension was used to inoculate a pre-inoculum in a 100 mL Erlenmeyer flask containing a liquid industrial medium. The liquid industrial

medium contained corn extract, K_2HPO_4, and sucrose. The inoculation was performed at a ratio of 1:80, meaning that the bacterial suspension was added at a rate of 1.30%. The pre-inoculum was then incubated at 28°C for 24 hours at 200 rpm. The resulting culture was further used to inoculate 200 ml of the industrial medium in a 1 L Erlenmeyer flask, using an inoculum-to-medium ratio of 1:80. The culture was maintained with process parameters of 28°C and agitation at 200 rpm for 24 hours. After 24 hours, 1 mL samples of bacterial culture were taken to determine the colony-forming unit (CFU mL^{-1}) count by creating decimal dilution series and inoculating these onto Nutrient agarized medium. The Petri dishes were incubated at 28°C for 24 hours to determine the number of viable cells. The *P. putida* VfC23 bacterial strain reached a value of 1.87 * 10^8 CFU mL^{-1} in the liquid industrial medium after 24 hours. For obtaining the *B. borstelensis* GyA23 biomass, the bacterial strain was cultivated in liquid Nutrient medium for 24 hours at 28°C with agitation at 140 rpm in 250 mL flasks. This 24-hour bacterial suspension was used to prepare the pre-inoculum. The pre-inoculum was prepared by inoculating the culture medium with the bacterial culture at an inoculation rate of 1% obtained in the Nutrient medium and incubated for 24 hours at 28°C with agitation at 140 rpm. The culture media for obtaining the pre-inoculum and the inoculant were sterilized at 121°C for 25 minutes, with a pH value of 7.5. The cell density of the pre-inoculum and the inoculant was determined by spreading on Nutrient agarized plates at 12 and 24 hours. The cell density of the *B. borstelensis* GyA23 biostimulant strain was 2.12 * 10^9 CFU mL^{-1} in the pre-inoculum after 24-hour fermentation.

In vitro biotesting of the beneficial effect on Picea abies seedlings. Biotesting was conducted in two phases: in the first phase (I), the beneficial effect of the biopreparation was tested in the laboratory, and in the second phase (II), tests were carried out in the nursery at the Gheorgheni Forest District Nursery.

Methodology for laboratory testing. For seedling growth, polypropylene containers of 11.1 x 8.2 cm (0.5 L) were used, which can be closed with a lid and are resistant to autoclaving. The containers were filled with soil, which was sterilized by autoclaving at 105ºC for 30 minutes, repeated three times on consecutive days. In the process of testing the beneficial effect of the biopreparation on *Picea abies* seedlings, 500 forest seeds from the Gheorgheni Forest District, UP I Comp. Joseni, ua.148 were used for sowing. In the containers, germinated spruce (*Picea abies*) seeds were sown, and at the base of each seedling, bacterial consortium *B. borstelensis* GyA23 + *P. putida* VfC23 (10^8 CFU/mL/plant) was added. Control samples were also included, where no biopreparation inoculants were used. The conifer seedlings were grown for 12 weeks at 25°C with 85% relative humidity, with a 12-hour/daylight period in a growth chamber (Panasonic MLR-352 PE Climate (Plant Growth) Chamber). Soil moisture was maintained at a minimum of 30% of the total dry weight. After the growth period, the total fresh biomass of the plants, as well as the fresh and dry biomass of the roots and stems (dried at 70°C for 2-3 hours), and stem length were measured. During testing, the development of the conifer seedling cultures was monitored comparatively, focusing on the growth of seedlings inoculated with the biopreparation consortium *B. borstelensis* GyA23 + *P. putida* VfC23. Measurements included stem length (cm), root fresh weight (g), and stem fresh weight (g).

Methodology for nursery-level testing in a greenhouse. For seedling growth, forest trays with 126 cells were used, with dimensions of 577 x 375 mm, cell diameter of 35 mm, and cell height of 67 mm. Before use, these forest trays were sterilized with steam at 170°C for one hour. The trays were filled with minicontainers seeded with 1500 forest seeds (*P. abies*). The seeds used for sowing were the same as those used in the laboratory testing, originating from OSR Gheorgheni S.A. UP I Comp. Joseni ua.148. The equipment used to prepare the minicontainers was the Ellepot H112 and Ellepot Turbo Multiflex production line. The minicontainers had a biodegradable paper casing, with a length of 10 cm and a diameter of 35 mm, each containing approximately 0.08 liters of soil. Temperature and humidity were continuously monitored in the greenhouse to maintain optimal conditions for the development of *P. abies* seedlings.

Statistical analysis. The research materials were statistically processed using the application software "Excel Statistics". The accuracy of the research varies within 1-3%. Based on the results, the main biometric indicators of spruce seedling growth were calculated. The graphs were plotted using Microsoft Office Excel 2021.

Results and Discussion. A significant difference was observed between treated and untreated seedlings, under laboratory testing conditions. The average stem length of seedlings treated with the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 was 33.08% (10.54 ± 0.5 cm) higher compared to the control seedlings (7.92 ± 0.5 cm), with the difference being statistically significant (Figure 1).

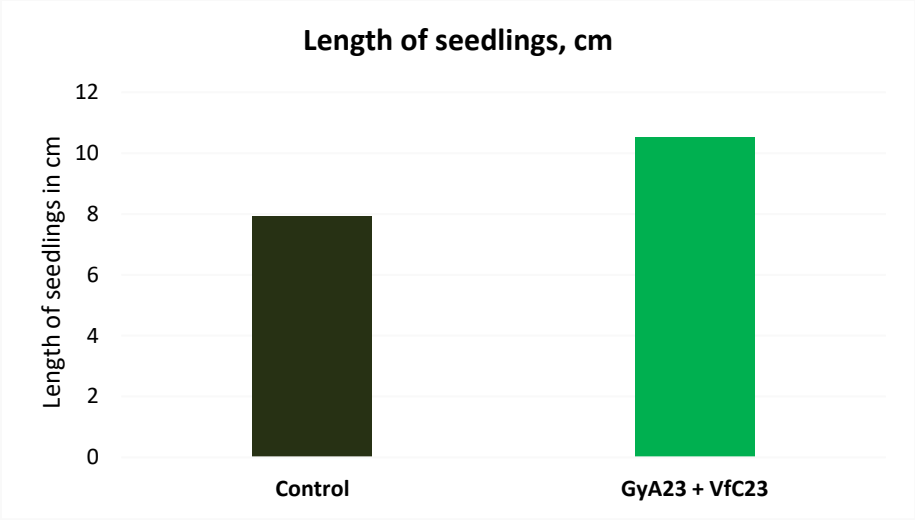

Figure 1. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

Upon analyzing the results, it was observed that the average total weight of seedlings treated with the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 was 0.94 ± 0.05 g, representing a significant increase of 62% compared to the control samples that were not treated with the microbial biopreparation (0.58 ± 0.06 g) (Figure 2).

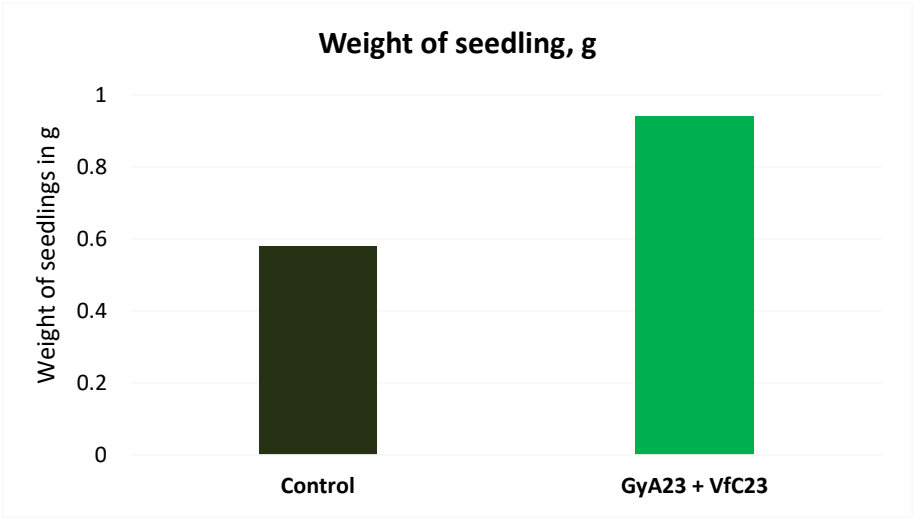

Figure 2. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

The bacterial consortium visibly stimulated the growth of root fresh weight in the case of the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 (0.54 ± 0.051 g) compared to the control (0.32 ± 0.051 g). The percentage increase was 68.75% (Figure 3).

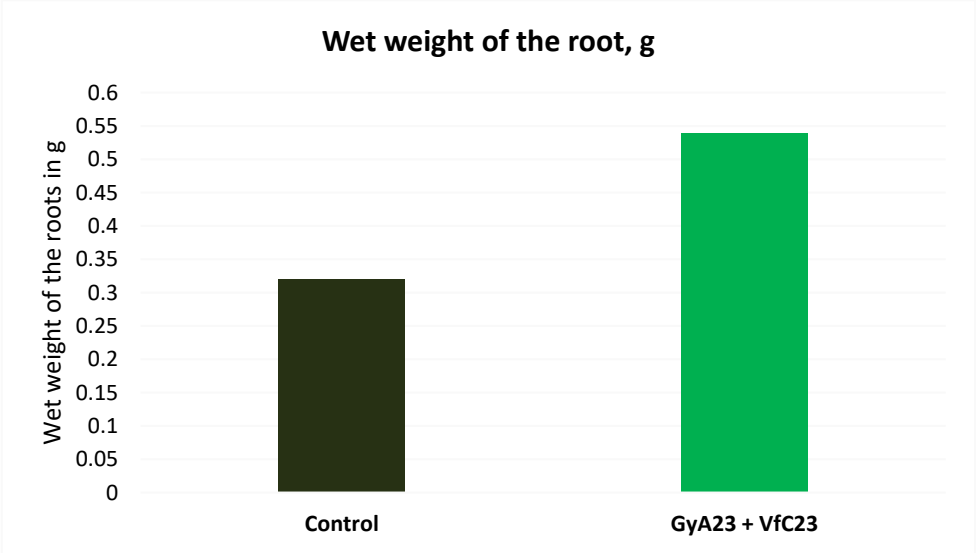

Figure 3. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

Additionally, a 104.16% increase was observed in the dry weight of the roots (0.49 ± 0.051 g) compared to the control (0.24 ± 0.051 g) (Figure 4).

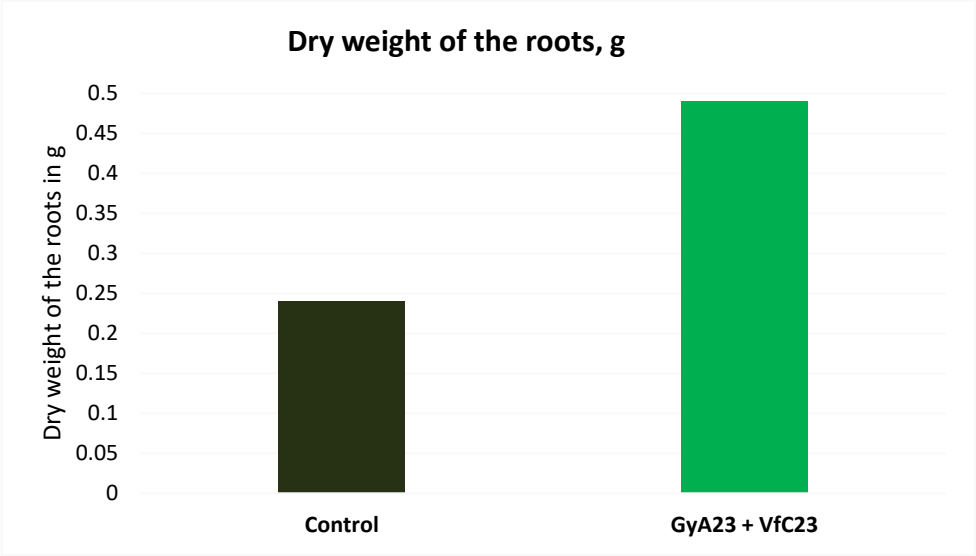

Figure 4. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

The biopreparation also had a significant beneficial effect on the fresh weight of the seedling stems. The difference was 33.33% (0.48 ± 0.051 g) compared to the control samples (0.36 ± 0.051 g) (Figure 5). Furthermore, a 55.55% increase was observed in the dry weight of the stems (0.056 ± 0.005 g) compared to the control (0.036 ± 0.004 g) (Figure 6).

The results of nursery-level testing in the greenhouse conditions were similar, as were the results measured during laboratory testing. During testing, the same parameters were monitored as in the laboratory: the comparative development of conifer seedling cultures, focusing on the growth of seedlings inoculated with the biopreparation consortium *B. borstelensis* GyA23 + *P. putida* VfC23. Measurements included stem length (cm), root fresh weight (g), and stem fresh weight (g). The average stem length of seedlings treated with the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 was 37.96% (8.25 ± 0.5 cm) higher compared to the control seedlings (5.98 ± 0.5cm), the difference being statistically significant (Figure 7).

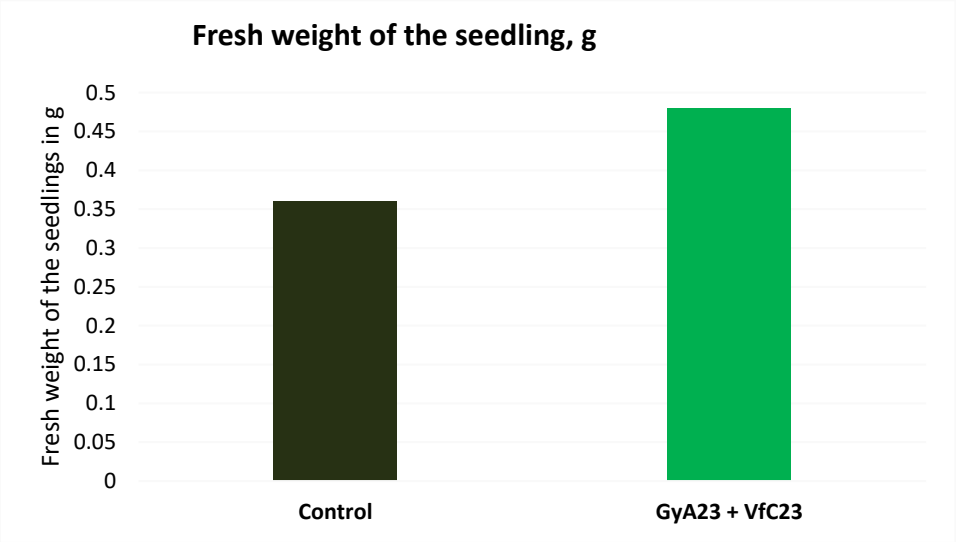

Figure 5. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

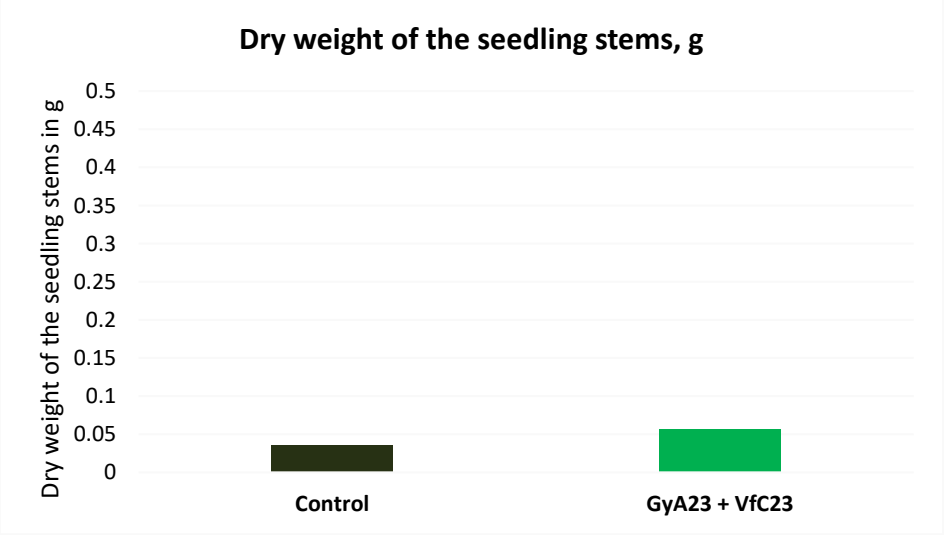

Figure 6. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings.

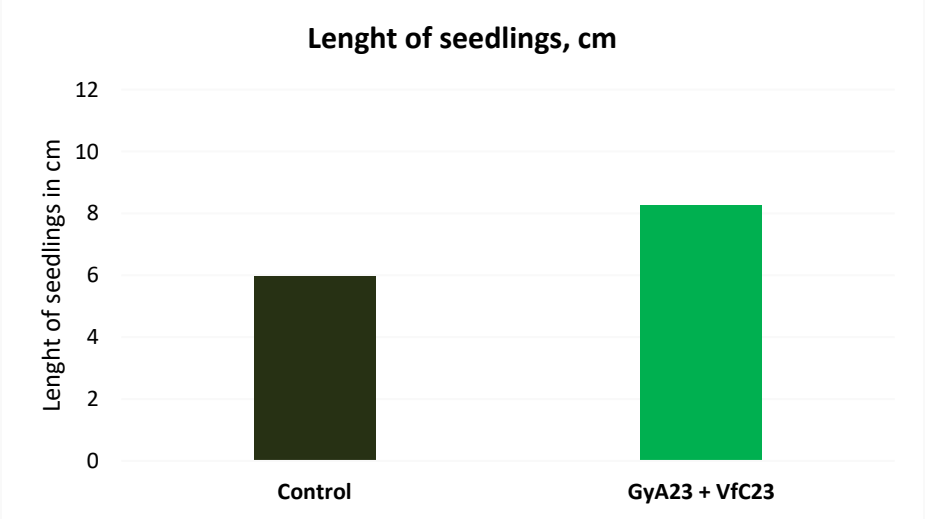

Figure 7. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

Analyzing the results, it was observed that the average total weight of seedlings treated with the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 was 0.8 ± 0.05 g, which indicates a significant increase of 56.86% compared to the control samples that were not treated with the microbial biopreparation (0.51 ± 0.06 g) (Figure 8).

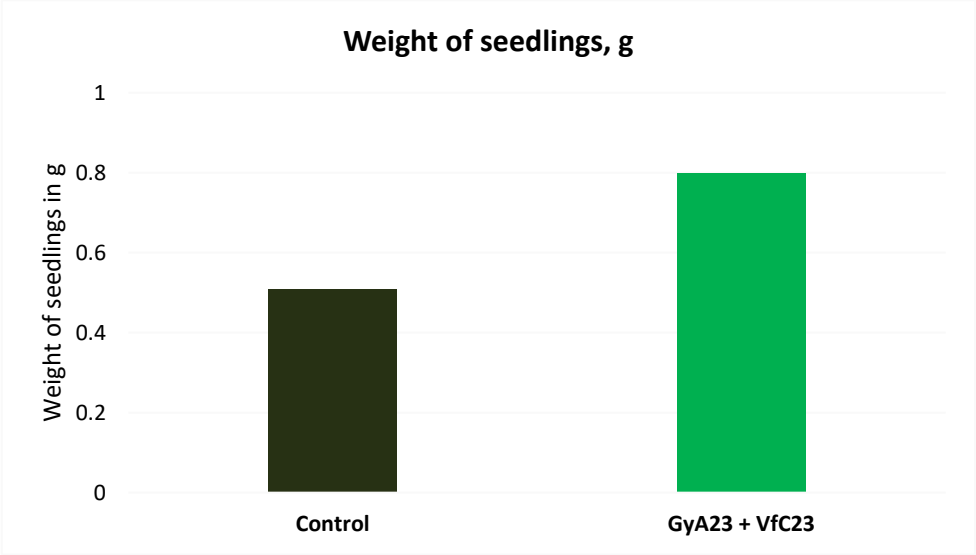

Figure 8. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

The bacterial consortium visibly stimulated the increase in root fresh weight of seedlings in the case of the consortium *B. borstelensis* GyA23 + *P. putida* VfC23 (0.45 ± 0.051 g) compared to the control (0.29 ± 0.051 g), with a percentage increase of 55.17% (Figure 9).

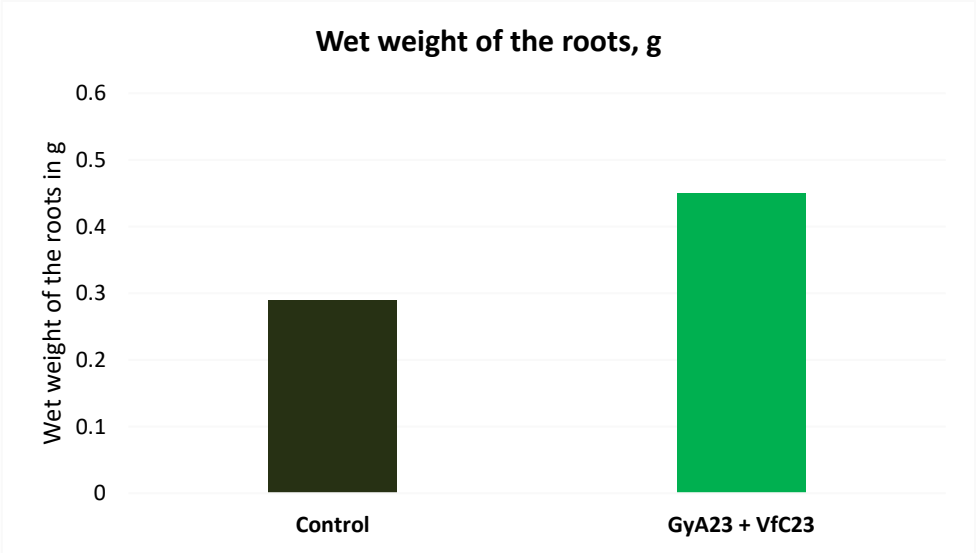

Figure 9. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

Additionally, a 94.44% increase was observed in the dry weight of the roots (0.35 ± 0.051 g) compared to the control (0.18 ± 0.051 g) (Figure 10). The biopreparation also had a significant beneficial effect on the fresh weight of the seedling stems of the *P. abies* species, with a 37.5% increase (0.44 ± 0.051 g) compared to the control samples (0.32 ± 0.051 g) (Figure 11). Furthermore, a 46.88% increase was observed in the dry weight of the seedling stems (0.047 ± 0.005 g) compared to the control (0.032 ± 0.004 g) (Figure 12).

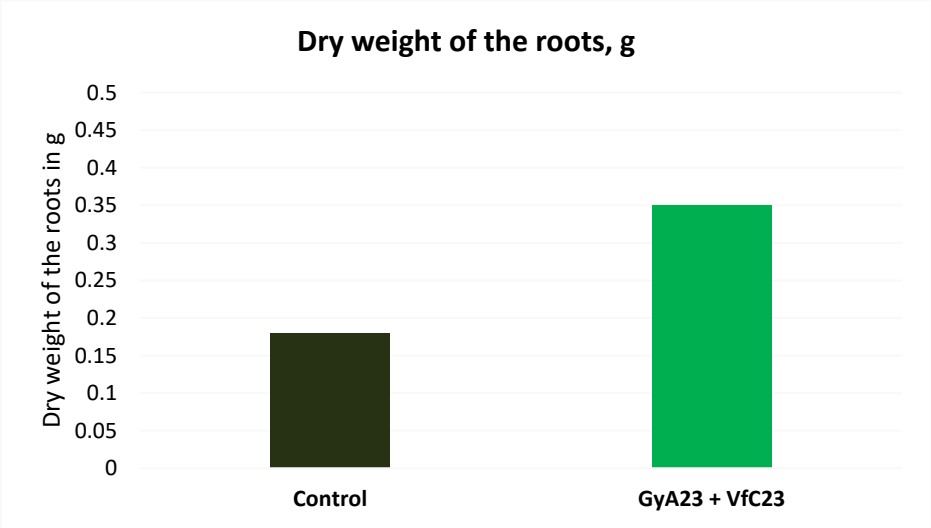

Figure 10. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

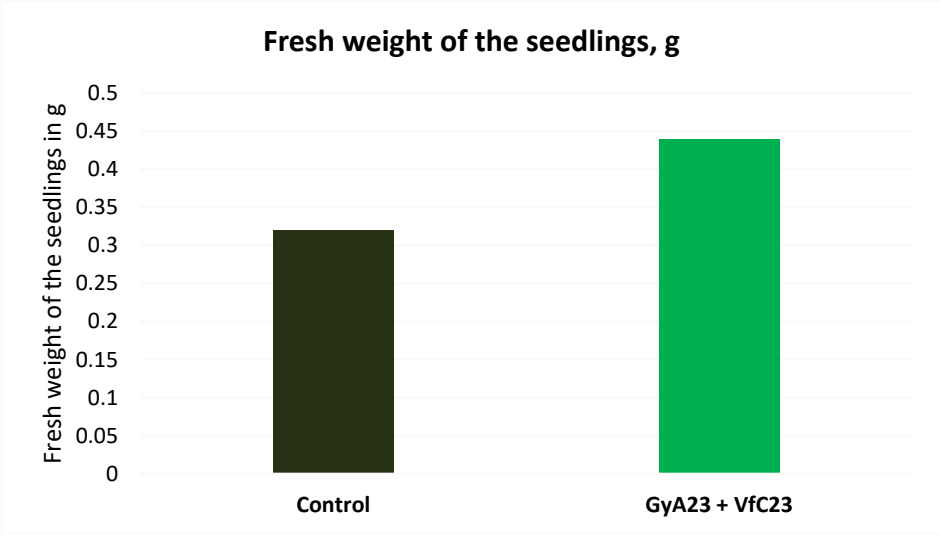

Figure 11. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

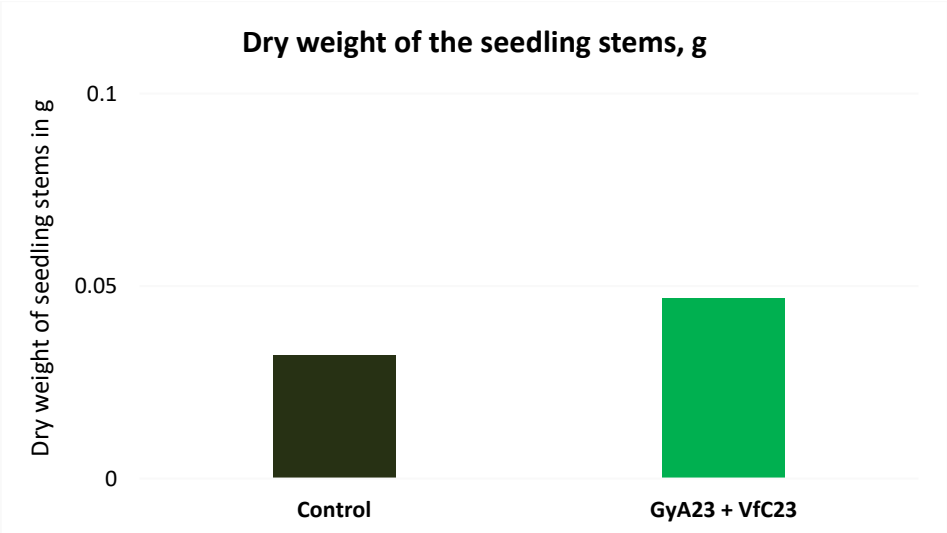

Figure 12. Effect of biopreparation inoculation on biometric parameters compared to control in *Picea abies* seedlings tested in vitro in the greenhouse.

By analyzing the results obtained from the in vitro testing of the biopreparation, which is the subject of this study, we can conclude that the bacterial consortium *B. borstelensis* GyA23 + *P. putida* VfC23 had a beneficial effect on the growth and development of *P. abies* seedlings, visibly stimulating the total weight of the seedlings.

Conclusions. In this study, an approach composed of two compatible species was considered, resulting in a mixed microbial biopreparation composed of two bacterial strains: *B. borstelensis* GyA23 + *P. putida* VfC23. This consortium can promote the growth of *Picea abies* seedlings through nutrient mobilization, phytohormone production, and plant protection by direct and indirect antagonism against phytopathogens. This was followed by in vitro analyses and testing of the biostimulatory effect of the biopreparation both in the laboratory and in the greenhouse. During testing, the development of conifer seedling cultures was monitored comparatively, focusing on seedlings inoculated with the biopreparation consortium *B. borstelensis* GyA23 + *P. putida* VfC23. Analyzing the results obtained from the in vitro testing of the biopreparation, we can affirm that the bacterial consortium *B. borstelensis* GyA23 + *P. putida* VfC23 had a beneficial effect on the growth and development of *P. abies* seedlings, visibly stimulating the total weight of the seedlings. The findings presented in this study indicate that the compounds of the biostimulatory biopreparation with organic compounds in the soil can enhance the growth and development of conifer seedlings. Testing the efficacy of the biostimulatory biopreparation in laboratory and greenhouse settings, as well as its general ecological impact on the native soil microbiome, will allow for the definition of new biostimulators for more sustainable and resilient forestry.

Acknowledgements. This article is the result of the project activities "Development of Innovative Processes and Products in the Forestry Sector for Increasing Competitiveness and Quality of Life" funded by The Ministry of European Funds.

Conflict of interest. The authors declare no conflict of interest.

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Received: 04 November 2024. Accepted: 20 December 2024. Published online: 30 December 2024.
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How to cite this article:
Melles E., Csergő Z., Horváth A., Korpos A., Benedek A., Domokos E., 2024 Biopreparation growth stimulator for conifer seedlings and obtaining procedure. AAB Bioflux 16(1):59-73.