SYLWAN 168 (1): 62-69, January 2024
https://doi.org/10.26202/sylwan.2023048
Journal homepage: https://sylwan-journal.pl

SHORT COMMUNICATION

The effect of bio-stimulants containing *Bacillus* bacteria on the development and protection of Scots pine seedlings

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ABSTRACT

Bacillus bacteria belong to the genus of gram-positive aerobes and spore-forming organisms that have the potential to produce diverse biochemical compounds. *Bacillus* bacteria are useful for a variety of biological preparations including a group that can aid in plant growth and development. Biological preparations containing *Bacillus* bacteria have been effectively utilized in agriculture, horticulture and orchards for many years. The use of these preparations has resulted in fruitful outcomes. The purpose of this study was to assess the efficacy of the two AGRARIUS-produced bio-stimulants, *Bi fosfor*® and *Bi protect*® on the growth of pine trees in semi-open fields. The preparations used contained *Bacillus megaterium* (*Bi fosfor*®) and *Bacillus subtilis* (*Bi protect*®). The preparations were tested for the improvement of seedling biometric characteristics and also for their potential protection against pathogens causing damping-off disease. The study took place in a forest nursery located in the Radziwiłłów Forest District. Before the pine was sown, an experimental plot was established. The first soil spraying was conducted using the recommended dose by the producer of 1 kg of the preparation per 1000 liters of water per hectare. The procedure was repeated twice. About a month after the pine seeds had germinated, the severity of the seedlings fungal infection causing damping-off disease was assessed. Several seedlings were simultaneously analyzed to confirm that the disease was caused by pathogens. Biometric parameters were evaluated after a five-month interval from the last treatment. The length of the aerial part was measured and the root systems were scanned. The collected data was analyzed using WinRhizo software to obtain results. This enabled comparison of parameters such as the total length of the roots, surface area, average diameter and number of tips. Fungi belonging to the genus *Fusarium* was identified as the causal agent of pathogenic damping-off disease. Our analysis identified no statistically significant variations in the level of seedling infection. The assessment of biometric traits revealed that the seedlings grown on the plots treated with aforementioned preparations exhibited markedly superior root system characteristics (especially those on the plot treated with *Bi-protect*). Conversely, no notable differences were observed in the development of the above-ground part of the seedlings.

KEY WORDS

Bacillus, bacteria, forest nurseries, pine, plant bio-stimulants

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Received: 31 July 2023; Revised: 15 February 2024; Accepted: 16 February 2024; Available online: 15 March 2024

Introduction

The effectiveness of bacteria in promoting plant growth has been established through stimulation of their nutrition and growth. Biofertilization and direct plant growth promotion are the mechanisms utilized by the *Bacillus* species to achieve this. Through biofertilization, *Bacillus* populations enhance the bioavailability of essential compounds and increase the supply of minerals to the host plant (Pérez-Garcia, 2011). It is important to note that the adoption of these practices can lead to an increase in agricultural productivity. In soil, organic phosphorus primarily exists in an insoluble form, and several *Bacillus* species, such as *B. amyloliquefaciens* (ex Fukomoto 1943) Priest 1987, contribute to soil biofertilization by producing specialized extracellular phosphatases. These enzymes enable the sequential hydrolysis of phytate to less phosphorylated derivatives and ultimately inorganic phosphate (Jorquera *et al*., 2008). A comparable process occurs with iron. In soil, the most abundant form of iron is Fe^{3+} which is relatively insoluble compared to the more reduced form Fe^{2+} and taken up less by plants and microorganisms. *Bacillus* species such as *B. megaterium* can reduce metals potentially increasing the bioavailability of iron (Valencia-Cantero *et al*., 2007). Such properties of bacteria in the genus *Bacillus* can be successfully used in plant development and health improvement in various applications including forestry. Most certainly, they will not replace chemical preparations, but they can become an important element of pest insect and disease integrated management. The *Bacillus* genus bacteria can be successfully used as biological preparations which are regulators of plant growth and development or as biostimulators (Maciejewska *et al*., 2007; Gawrońska and Przybysz, 2011; Matyjaszczyk, 2015) as well as plant growth and nutrition stimulants (Joubert and Lefranc, 2008), or preparations containing effective microorganisms (Boligłowa and Gleń, 2008; Kaczmarek *et al*., 2008; Derkowska *et al*., 2015).

The study aimed to assess the effectiveness of *Bacillus megaterium* de Bary 1884 (Approved Lists 1980) and *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 in promoting the growth of Scots pine *Pinus sylvestris* L. seedlings and reducing damage caused by fungi responsible for damping-off disease under semi-open field conditions.

Materials and methods

Basic information about the tested products. Bacteria were used in the form of biopreparations *Bi fosfor*® *B. megaterium* and *Bi protect*® *B. subtilis* produced by the Agrarius company. Both preparations contain single, carefully selected bacteria. *Bi fosfor*® is a high-quality microbial soil fertilizer that helps enrich the soil with plant-available phosphorus derived from the decomposition of its complex and insoluble forms. It stimulates plant growth which increases yield and improves quality. The second tested preparation *Bi protect*® is a microbial soil fertilizer that accelerates the decomposition of organic matter, participates in the creation of soil structure, improves plant health and makes nutrients available to plants.

Establishment of the experimental area. The evaluation of the effectiveness of *Bi fosfor*® and *Bi protect*® preparations was carried out in central Poland in the forest nursery of the Radziwiłłów Forest District (coordinates 51.9916 N, 20.3374 E) on one-year-old Scots pine *Pinus sylvestris* seedlings. For this purpose, plots of 1 m^2 were staked in an unused part of the production line, and the soil was sprayed according to the manufacturer's label instructions. A total of 3 plots were designated for each of the preparations and control plots with no applied treatments. In addition, a 50 cm buffer zone was established between plots (Tkaczyk *et al*., 2022).

The experimental plot in the nursery was established on April 11, 2022. At the same time, the first soil sprayings with the bacterial preparations *Bi fosfor*® and *Bi protect*® were carried out. The second spraying with these preparations was carried out on April 20, 2022. Both preparations in each treatment were applied according to the dose recommended by producer of 1 kg of the powder per 1000 l ha^{-1} of water. On May 6, 2022, the Scots pine seeds were sown (about 10 g of seed per square meter) in the designated production line. The preliminary information about the emergence of pine on the experimental plots was obtained on May 9.

Evaluation in terms of limiting the damage caused by damping-off disease. Within a month of pine germination, a visual assessment was made of the number of seedlings affected by damping-off. Damping-off is caused by several soil-borne fungi, most commonly including *Pythium* spp., *Rhizoctonia solani* J.G. Kühn and *Fusarium* spp. (Lamichhane *et al*., 2017). For this purpose, in all plots (both control and treatment plots) all seedlings that showed symptoms of damping-off disease (necrosis and constriction of root necks) and all healthy seedlings were counted, and then the percentage of seedlings with disease symptoms was determined. In addition, 5 seedlings with symptoms were taken from each experimental plot to confirm the causes of infection. These seedlings were first sterilized in 1% sodium hypochlorite solution for one minute, then rinsed in sterile distilled water and dried with a paper towel. Tissue fragments with visible necrosis were placed on potato dextrose agar (PDA) medium. The prepared plates were incubated at room temperature (approximately 20°C) and observed daily under a ZEISS axiolab microscope at 20× magnification. The first emerging mycelial hyphae were immediately transplanted onto new plates. The prepared plates were incubated for one week. Afterwards, microscopic observations were made to identify the causative agent of the necrosis.

Evaluation of the biometric characteristics of the pine seedlings. Five months after the last treatment, 10 cuttings were taken from each plot (a total of 30 cuttings for each experimental variant) in which the length of the aerial part was first measured. Subsequently, all roots were scanned using EPSON Perfection V700 Photo Scanner software and analyzed using WinRhizo® software (Regent Instruments, Canada). As a result of this analysis, a number of parameters were determined such as total root length (TRL), root area (SA), average root thickness (AD), and number of root tips (NoT) (Bouma *et al*., 2000). These parameters were used to compare the development of root systems between the control variants and the variants where bacteria-based preparations were applied.

Statistical analysis. All obtained results were compared using the nonparametric Kruskal-Wallis test at a significance level of α=0.05. This procedure was chosen because of the unequal numbers in the groups and the lack of normal distribution which was confirmed by the Shapiro-Wilk test. The multiple rank comparison test was used to compare the pairs of means between the variants. All calculations were performed using the STATISTICA 13.1 package (TIBCO, 2017).

Results

Comparison of biometric parameters. The comparison of biometric characteristics of pine seedlings between the control variants and the experimental variants in which spraying with *Bi fosfor*® and *Bi protect*® preparations was carried out is shown in Table 1. Statistically significant differences were found in all analyzed parameters of the root systems. In the area where *Bi protect*® was applied, statistically significant higher values for total root length, surface area and number of tips were observed five months after treatment. All analyzed parameters for these seedlings

had values almost twice as high as those of the seedlings in the control variant. Further, the seedlings from the area where *Bi fosfor*® was applied had statistically significantly higher values for parameters such as average diameter and number of tips. These values were lower than those of the seedlings growing on soil sprayed with *Bi protect*® but significantly higher than those of the control variant.

Comparison of infected seedling numbers. The analysis showed the presence of fungi of the genus *Fusarium* spp. in the infected tissues in all plots where the study was carried out. Symptoms of pathogenic seedling rot were observed in all plots. The results of the analysis of the plants affected by damping-off disease are presented in Table 2. A comparison between groups showed no significant differences between the experimental variants with an assumed significance level α=0.05 (p-value = 0.224). Despite the lack of statistically significant differences, most infected seedlings were observed in the control variant. On average, about 13% of infected pines were observed in all plots. Slightly fewer infected seedlings were observed in variants where the soil was sprayed with preparations containing bacteria. In plots sprayed with the *Bi fosfor*® preparation, the percentage was 12.7% while in the *Bi protect*® variant it was 12.5%.

Discussion

The results presented in relation to the use of growth-promoting preparations of *Bi fosfor*® and *Bi protect*® showed a positive effect on the development of biometric characteristics of one-year-old pine seedlings after five months following spray application. Both preparations are designed to enrich the soil and improve its fertility by providing beneficial bacteria of the genus *Bacillus* (*B. megaterium* – *Bi fosfor*®, *B. subtillis* – *Bi protect*®) which are saprophytes responsible for the decomposition of organic compounds with a plant origin. The results showed that the plants that grew on the plots where the above preparations were applied to the soil almost doubled the parameters of the root systems during one growing season. However, better results were obtained

Table 1.

Comparison of biometric features of the collected pine seedlings (summary of the median ± quartile and the results of the multiple comparison test of mean ranks); significant differences are marked with an asterisk

	p-value	Control	*Bi fosfor*®	*Bi protect*®
The height of the aboveground part [cm]	0.218	7.01 ±1.34	7.41 ±1.32	8.09 ±0.98
Total root length [cm]	0.005	40.23 ±16.22	60.87 ±28.86	87.37 ±27.98*
Surface area [cm^2]	0.012	6.77 ±2.91	12.86 ±6.72	14.35 ±4.33*
Average diameter [mm]	0.011	0.53 ±0.08	0.65 ±0.09*	0.53 ±0.08
Number of tips	0.000	59.25 ±27.62	141.88 ±47.51*	162.8 ±55.15*

Table 2.

Share of seedlings [%] with damping-off disease symptoms and the number of infected seedlings

Variant	Seedling category	Median number of seedlings ± quartiles	% of infected seedlings
Bi fosfor®	Healthy	291 ±285	12.73
	Infected	43 ±42	
Bi protect®	Healthy	296 ±286	12.50
	Infected	53 ±18	
Control	Healthy	205 ±203	13.00
	Infected	42 ±23	

with the *Bi protect*® preparation. This is of particular importance for the subsequent survivability of the plant, especially under unfavorable conditions. Even if the above-ground part is small but has a well-developed root system, it can produce trees that develop better later. Although these preparations do not act as pesticides, they can increase resistance to biotic factors (Jayaraman *et al*., 2011; Tkaczyk *et al*., 2016, 2022). However, the research conducted did not show any tested preparations that had any effect on reducing damage to seedlings caused by damping-off disease. The percentage of seedlings infected with fungi of the genus *Fusarium* was similar between the variants.

A similar stimulatory effect on plant growth elongation using beneficial bacterial strains of *B. subtilis* was previously observed in similar studies (Oszako *et al*., 2019). In the study by Oszako *et al*. (2019) a positive effect was described not only on an increase of biometric characteristics of birch seedlings, but also on the reduction of damage caused by pathogens of the genus *Phytophthora* [especially *P. plurivora* (T. Jung & T.I Burgess)]. Bacilli of the genus *Bacillus* represent a group of rhizobacteria and are referred to as plant growth promoting rhizobacteria (PGPR) (Hussain *et al*., 2017; Hashem and Abd Allah, 2019). The peptide antibiotics synthesized by *B. subtilis*, such as polymyxin B and subtilin, have amphiphilic properties that contribute to the decomposition of the cell wall of pathogens which is a promising strategy in the fight against microbial infections of plants.

Direct stimulation of plant growth by *Bacillus* consists of an improvement in the parameters of plant growth and development through the production of phytohormones (Tsavkelova *et al*., 2006). For example, several *Bacillus* species are capable of producing auxin which can stimulate root proliferation and nutrient uptake (Spaepen *et al*., 2007). For example, in *B. amyloliquefaciens* indole-3-acetic acid (IAA) biosynthesis is responsible for stimulating plant growth, which in turn is highly dependent on the presence of one of the most important compounds in plant root secretions, tryptophan (Idris *et al*., 2007). Similarly, inoculation of plants with cytokinin-producing strains of *B. subtilis* or *B. megaterium* has a positive effect on plant growth (Arkhipova *et al*., 2007; Ortíz-Castro *et al*., 2008). These are the same bacteria present in the preparations tested in the current study. In addition, VOCs from *B. subtilis* have been shown to induce growth promotion of Arabidopsis by regulating auxin homeostasis providing a new paradigm for plant growth promotion by these bacteria (Zhang *et al*., 2007).

In forestry, testing of preparations to promote plant growth is still a new topic. There are reports of testing preparations containing algae extract *Ascophyllum nodosum* L. to promote root growth of dune pine *Pinus contorta* Dougl. ex Loud. during spring (MacDonald *et al*., 2012). Preparations based on the same algae extract were also tested by Tkaczyk *et al*. (2022) for the development of English oak seedlings. A large group of mineral-containing bio-stimulants was also tested for the development of species such as pine *Pinus* spp., oak *Quercus* spp., alder *Alnus* spp., beech *Fagus* spp., and spruce *Picea* spp. (Tkaczyk *et al*., 2015). In all the studies described above, a positive effect was observed in relation to the use of preparations to stimulate plant growth and development. In a study published by Buraczyk *et al*. (2020), the use of preparations based on humic acids and chitosan polymers reduced oak powdery mildew infestation but did not affect the growth and development of the seedlings themselves. In another study, Tkaczyk *et al*. (2014) confirmed that the use of phosphite-based preparations also reduced infection of oak seedlings with *Erisyphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam., although this was a side effect of the research. Finally, Solla *et al*. (2021) presented the positive effect of the use of phosphites on the protection of adult oak stands from the harmful effects of pathogens of the genus *Phytophthora*. The reports described above indicate the need for further research

on the use of bio-stimulants and other preparations that promote both the development and resistance of woody plants.

Authors' contributions

All authors substantially conceived of the ideas, contributed to conceptualization, resources, writing the original draft as well reviewing and editing the text.

Conflicts of interest

Authors declare no personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of the reported research results.

Funding source

This paper was partially supported by the State Forest Holding in Poland (Project No. 500 488).

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STRESZCZENIE

Wpływ biostymulatorów zawierających bakterie z rodzaju *Bacillus* na rozwój i ochronę sadzonek sosny zwyczajnej

Bakterie z rodzaju *Bacillus* są coraz powszechniej stosowane jako baza różnego rodzaju preparatów biologicznych, w tym stymulujących wzrost i rozwój roślin. Od wielu lat preparaty zawierające bakterie z rodzaju *Bacillus* są z powodzeniem wykorzystywane w rolnictwie, ogrodnictwie i sadow-

nictwie. Celem przedstawionych badań było sprawdzenie skuteczności dwóch biostymulatorów produkowanych przez firmę Agrarius – *Bi fosfor*® i *Bi protect*® – na rozwój sosny zwyczajnej *Pinus sylvestris* L. w warunkach polowych półotwartych. Preparaty te zawierają *Bacillus megaterium* (*Bi fosfor*®) i *Bacillus subtilis* (*Bi protect*®). Ich skuteczność sprawdzano pod kątem poprawy cech biometrycznych sadzonek, ale również potencjalnej ochrony przed patogenami wywołującymi patogeniczną zgorzel siewek.

Doświadczenie realizowano w szkółce leśnej znajdującej się na terenie Nadleśnictwa Radziwiłłów (RDLP w Łodzi), gdzie jeszcze przed wysiewem sosny *P. sylvestris* wyznaczono dla każdego z preparatów po 3 poletka oraz 3 poletka kontrolne. W tym samym czasie wykonano pierwsze opryski doglebowe tymi preparatami. Zabieg wykonano dwukrotnie w kwietniu 2022 r. zgodnie z dawką zalecaną przez producenta, tj. 1 kg ha^{-1}. Na początku maja na wyznaczonej powierzchni wysiano sosnę zwyczajną. Około miesiąca po skiełkowaniu sosny dokonano oceny stopnia porażenia siewek przez grzyby powodujące zgorzel. Jednocześnie pobrano kilka siewek do analiz, aby potwierdzić, czy choroba była faktycznie wywołana przez patogeny. Ocenę parametrów biometrycznych przeprowadzono po upływie 5 miesięcy od daty wykonania ostatniego zabiegu. W tym celu zmierzono długość części nadziemnej oraz zeskanowano systemy korzeniowe, które następnie poddano analizie z użyciem oprogramowania WinRhizo. Dzięki temu możliwe było porównanie takich parametrów korzeni jak całkowita długość, powierzchnia, średnia grubość oraz liczba zakończeń.

Jako sprawcę patogenicznej zgorzeli siewek zdiagnozowano grzyby z rodzaju *Fusarium*. Przeprowadzone analizy nie wykazały istotnych statystycznie różnic pomiędzy stopniem porażenia siewek między wariantami testowymi a wariantem kontrolnym (tab. 1). Ocena cech biometrycznych wskazała, że siewki, które rosły na poletkach, gdzie zastosowano preparaty, wykazywały o wiele lepsze cechy systemów korzeniowych (w szczególności siewki rosnące na poletku, gdzie zastosowano preparat *Bi protect*®). Nie obserwowano istotnych różnic w rozwoju części nadziemnej sadzonek (tab. 2). Z przeprowadzonej obserwacji wynika, że zastosowanie preparatów na bazie *B. megaterium* (*Bi fosfor*®) i *B. subtilis* (*Bi protect*®) znacząco poprawiło cechy biometryczne systemów korzeniowych jednorocznych siewek już po zaledwie 5 miesiącach. Jest to o tyle istotne, że sadzonki z prawidłowo rozwiniętym systemem korzeniowym, nawet jeżeli nie będą się wyróżniały wielkością części nadziemnej, będą o wiele lepiej odżywione, co wpływa bezpośrednio na podniesienie odporności roślin na dalszych etapach rozwoju.