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TESTING NEW STRAIN BENEFICIAL SOIL MICROBES TO IMPROVE GERMINATION, PLANT GROWTH AND PROTECTION AGAINST FUNGAL SOIL-BORNE PHYTOPATHOGENS IN *PHASEOLUS VULGARIS L.*

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Abstract

This paper presents the results obtained in the research conducted for the case study included in the Bachelor's Thesis. All experiments underlying this study were performed in the laboratory of Ecology and Environmental Microbiology within the Faculty of Land Reclamation and Environmental Engineering within the U.S.A.M.V. Bucharest. In this paper we present the results obtained by testing the microorganisms from the consortium present in the soil sample taken from the vegetable garden of the Zurini family from Crucea de Piatră, Călugăreni commune, Giurgiu county and the new strain of Bacillus sp. isolated. This new edaphic bacterial strain of the genus Bacillus isolated and identified by us has the ability to produce biofilms and inhibitory compounds (like antibiotics and organic volatile compounds) that inhibited the development of phytopathogenic fungi in the complex that creates the fall of seedlings in beans (Phaseolus vulgaris), root rot and hypocotyl (Pythium sp., Rhizoctonia sp., Fusarium sp.,) that are naturally present in the soil or on the beans. Also, the new edaphic bacterial strain Bacillus sp. has the ability to stimulate the germination and development of bean plants (Phaseolus vulgaris), in addition to the ability to provide protection against infection with phytopathogenic fungi from the soil.

Key words*: beneficial soil microbes, improve germination, Phaseolus vulgaris L plant growth, protection against fungal soil-borne phytopathogens.*

INTRODUCTION

Many soil-borne fungal pathogens are widespread throughout dry bean and snap bean growing areas around the world. Yield losses range from a trace to 100 percent, especially when adverse environmental conditions persist after planting and through flowering. The most common diseases and their pathogens are Rhizoctonia root and pod rots (*Rhizoctonia solani*), Pythium damping off, wilt and pod rot (various *Pythium* species) and Fusarium root rot or dry rot (*Fusarium solani* f. sp. *phaseoli*), Fusarium wilt or yellows (*Fusarium oxysporum* f. sp. *phaseoli*).

Root rots are favored by moderate to high soil moisture, various soil temperature regimes, soil compaction, poor drainage, continuous or frequent cropping to beans, and other factors that cause plant stress.

Pathogens survive for years in infected debris and infested soil. Root rot fungi can persist for many years in previously infected bean debris and infested soil by producing overwintering structures. These may be thick-walled spores (*Pythium* oospores and *Fusarium* chlamydospores), hyphae (fungal threads of *Rhizoctonia*), or small dark sclerotia (*Rhizoctonia*).

Overwintering structures are stimulated to germinate by plant exudates from developing susceptible tissue such as bean roots. Structures also may be stimulated by non-host roots to germinate harmlessly or maintain and reproduce themselves until susceptible plant tissue becomes available. The inoculum densities (numbers of survival structures per unit of soil) of these pathogens can be reduced by naturally-occurring soil-borne organisms that are antagonistic to them (Schwartz et al, 2005).

The incidence and severity of each root rot fungus and the disease complex they cause are affected by many environmental, host and cultural factors, such as: soil moisture, temperature, compaction, organic matter, fertility, bean rotation, other crops, plant density, seed quality, cultivation, irrigation runoff.

Rhizoctonia root rot symptoms may occur on scattered plants in a somewhat circular to irregular field pattern. The fungus can cause seedling death (damping off), root and hypocotyl rot, stem cankers and pod rot.

Initial symptoms appear on roots or hypocotyls soon after planting as linear or circular reddish-brown sunken lesions or cankers delimited by a brown to reddish-brown margin. Cankers can enlarge with age and become darker and rough-textured and retard plant growth. The pathogen can invade the central part of the lower stem and produce a brick-red discoloration of older seedlings.

Severely infected seedlings or young plants may be killed or break off at the infected and weakened portions of the hypocotyl. Lesions also can develop on pods in contact with the moist soil surface, and cause pod rotting and seed (Schwartz et al, 2011).

Pythium problems usually are scattered throughout a field, thus the affected sectors do not form a pattern. The pathogen may affect seeds, seedlings, young and older plants, and pods. The fungus can cause seed decay and seedling death. Initial root symptoms appear as elongated water-soaked areas on the hypocotyl and roots. Symptoms usually occur within one to three weeks after planting. Initially, the infected outer tissue of the stem becomes slimy and can easily slip from the central core at this stage. However, eventually it dries out, becomes sunken, and turns tan to brown in color.

Severely infected plants commonly wilt and die. Pods in contact with moist soil also may become infected and exhibit a watery soft rot and mass of white fungal mycelia (but without forming the hard black sclerotia associated with white mold disease). The pathogen can extensively prune roots, reduce overall plant growth, and destroy much of the hypocotyl and main root system. A water-soaked region on infected seedlings or plants may extend several inches above the soil line with little, if any, visible evidence of the fungus discoloration (Schwartz et al, 2011).

Soil-borne pathogens of dry beans and other crops can be managed to reduce but not eliminate damage. Integrated and carefully implemented approaches to crop production reduce disease pressure and plant stress. This enables vigorous plants to more successfully obtain nutrients and moisture during critical vegetative and reproductive periods.

In nature, bacteria form complex and differentiated multicellular communities, known as biofilms. The coordinated actions of many cells, communicating and dividing labour, improve the ability of the biofilm community to resist antibiotics and environmental assaults. In many instances, biofilms can be beneficial in agricultural ecosystems. One example is the biocontrol agents that form biofilms on the surface of plant roots, producing antibiotics that prevent the growth of bacterial and fungal pathogens and inducing the plant systemic response (Hou et al., 2021).

The soil bacterium *Bacillus subtilis* forms beneficial biofilms that induce plant defences and prevent the growth of pathogens. It is naturally found in the rhizosphere, where microorganisms coexist in an extremely competitive environment, and thus have evolved a diverse arsenal of defence mechanisms. The main organic components of its biofilm extracellular matrix (ECM) are exopolysaccharides (EPS), BslA, a protein forming a hydrophobic coat protecting the biofilm and the amyloid-like protein TasA. Amyloid-like proteins such as TasA are extremely common in bacterial biofilms, and their assembly into fibres is important for the integrity and structure of biofilms. In addition to its structural role, the ECM is essential for *B. subtilis* spreading (Hou et al., 2021).

Like other bacteria, *B. subtilis* produces a wide repertoire of volatile compounds (VCs)—biologically active airborne molecules. VCs are used by bacteria to interact with their environment, and were first identified as cross-kingdom signals influencing survival and behaviour of fungi, plants and vertebrates. However, VCs are also used as chemical signals during bacteria–bacteria interactions, altering motility, growth and differentiation, affecting virulence and boosting antibiotic and stress

resistance of various bacterial species. Recent evidence suggests that VCs may also modulate the development of bacterial communities. In nature, biofilms exist in an extremely competitive environment, and thus engage in both positive and negative interaction. While the ability to coordinate biofilm development within a community is beneficial in some cases; the ability to inhibit competing biofilm development is no less significant. In a systematic study of biological activity of VCs on four bacterial species, several VCs (including 1-butanol, ethanol, indole and others) were found to affect biofilm formation as judged by bacterial adhesion to a microtiter plate, but the effects were highly compound- and species-specific. For *B. subtilis*, it has been reported that ammonia and acetic acid produced by *B. subtilis* pellicles (floating biofilms) stimulate neighbouring pellicle formation. On the other hand, one study has shown that biocontrol strain *Bacillus amyloliquefaciens* SQR-9 produced volatiles inhibiting the growth of plant pathogen *Ralstonia solanacearum*. In addition to the effect of VCs on the growth of the pathogen, the VCs also reduced colony spreading, motility, production of exopolysaccharides and surface attachment of their own producers. Those results suggest that in nature, the role of VCs is highly context-dependent, and that additional studies are needed to understand the mechanisms mediating the effects of VCs produced by biofilms during ecological microbial interactions (Hou et al., 2021).

This paper presents the results obtained in the research conducted for the case study included in the Bachelor's Thesis. All experiments underlying this study were performed in the laboratory of Ecology and Environmental Microbiology within the Faculty of Land Reclamation and Environmental Engineering within the U.S.A.M.V. Bucharest.

In this paper we present the results obtained by testing the microorganisms from the consortium present in the soil sample taken from the vegetable garden of the Zurini family from Crucea de Piatră, Călugăreni commune, Giurgiu county and the new strain of *Bacillus* sp. isolated.

This new edaphic bacterial strain of the genus *Bacillus* isolated and identified by us has the ability to produce biofilms and inhibitory compounds (like antibiotics and organic volatile compounds) that inhibited the development of phytopathogenic fungi in the complex that creates the fall of seedlings in beans (*Phaseolus vulgaris*), root rot and hypocotyl (*Pythium sp., Rhizoctonia sp., Fusarium sp.,*) that are naturally present in the soil or on the beans. Also, the new edaphic bacterial strain *Bacillus* sp. has the ability to stimulate the germination and development of bean plants (*Phaseolus vulgaris*), in addition to the ability to provide protection against infection with phytopathogenic fungi from the soil.

MATERIALS AND METHODS

1. Determining the physicochemical parameters of the soil.

We determined N, P, K and pH with the Hanna Instruments soil analysis kit.

The nitrogen, phosphorus, and pH tests are colorimetric tests. During the tests, a colour is developed which corresponds with the fertility of the soil. To determine the fertility, the colour developed has to be compared with one of the supplied colour cards.

The reagents for nitrogen follow the Ned method to determine concentration as nitrate-nitrogen (NO_3--N). These reagents are designed to be used with samples to generally indicate the nitrogen amount in trace, low, medium, and high quantities.

The reagents for phosphorus follow the ascorbic acid method to determine concentration as phosphorus pentoxide (P_2O_5). These reagents are designed to be used with samples to generally indicate the phosphorus amount in trace, low, medium, and high quantities.

The reagents for pH follow the colorimetric indicator method to determine soil pH. These reagents are designed to be used with samples that have an expected pH range of 4 to 9 pH.

The potassium (K_2O) test is a turbidimetric test. If potassium is present in a soil sample, turbidity is formed. A blue colour will also develop to help in reading the test result. To determine the amount of potassium present, the colour developed has to be compared with the supplied colour card.

The reagents for potassium follow the tetraphenylborate method to determine concentration as potassium oxide (K_2O). These

reagents are designed to be used with samples to generally indicate the potassium amount in trace, low, medium, and high quantities.

2. In vitro culture in the laboratory.

Experiment to test the capacity of the new soil isolate *Bacillus* sp., to germinate and stimulate the growth of bean plants (*Phaseolus vulgaris*) and to inhibit the phytopathogenic fungi *Pythium* sp., *Fusarium* sp. and *Rizoctonia* sp. took place in March 2022.

In order to carry out the experiment to stimulate the germination and growth of *Phaseolus vulgaris* bean plants and protection against phytopathogenic fungi in the soil, we went through the following stages:

a) We weighed 600 g of soil and distributed it evenly, 50 g in each container for each of the three replicas of the control, marked M, sample 1, marked P1, sample 2, marked P2 and sample 3 marked P3.

b) The control (M) contained only beans, sample 1 (P1) contained beans and microorganisms from the soil extract with the consortium of microorganisms, sample 2 (P2) contained beans and bacterial suspension from the newly isolated bacterium Bacillus from the soil, microorganisms, and sample 3 (P3) contained soil extract with the consortium of microorganisms and bacterial suspension from the pure culture of the new bacterial isolate Bacillus sp.

After planting the bean seeds in containers containing 50 g of unsterilized soil, 5 mL of suspension was added from the soil extract containing the consortia of microorganisms in sample P1, 5 mL of *Bacillus* bacterium suspension containing 123 x 10^5 cells/ mL in sample P2 and 2.5 mL of suspension from the soil extract containing the consortia of microorganisms plus 2.5 mL of suspension *Bacillus* bacterium containing 123 x 10^5 cells/ mL in sample P3.

c) All samples were kept at room temperature throughout the experiment. During this period, periodic observations were made of the containers containing the control and the three samples, each in three replicas.

The period from planting to germination was noted, observations were made regarding the difference between each sample and control, the degree of development of each seedling, and at the end of the experiment the plants were measured and weighed. Weighing the bean seedlings was done with a Precisa balance. The degree of protection of the plants against the attack of the phytopathogenic fungi Pythium sp., *Fusarium* sp. and *Rizoctonia* sp. was also noted.

RESULTS AND DISCUSSIONS

1. Determining the physicochemical parameters of the soil.

The assessment of the physicochemical results achieved with the help of the soil test from Hanna Instruments is made qualitatively compared to the standard cards provided by the manufacturer. Thus, this type of colorimetric test based on color reactions, as described in the section Materials and Methods for nitrogen, phosphorus and pH, the following values were obtained: nitrogen a medium concentration, phosphorus a high concentration, and the pH value of former 5.

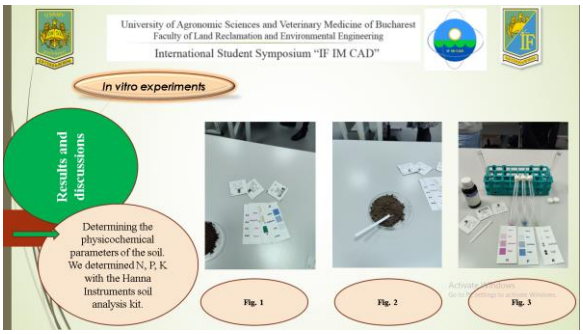

Figure 1. Determining the physicochemical parameters of the soil

The amount of nitrogen and phosphorus is directly proportional to the color intensity obtained in the test tubes. The stronger the coloration, from pale pink to dark pink fuchsia, the more nitrogen there is in the soil sample. The soil sample analyzed by us showed a pink color at level three intensity, which is equivalent to an average nitrogen concentration. In the same way, the concentration of phosphorus in the soil sample is interpreted with the difference that in this color reaction results a blue colored compound.

The soil sample we analyzed for phosphorus showed a blue color at level four (maximum)

intensity, which is equivalent to a high concentration of phosphorus.

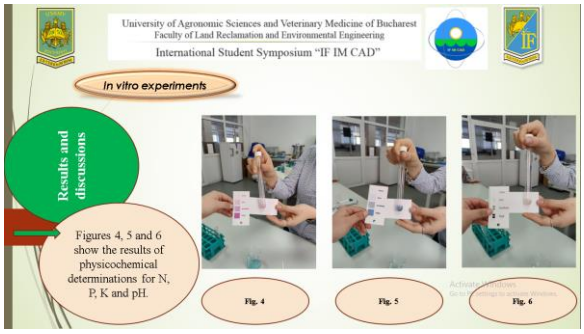

Figure 2. Results of physicochemical determinations for N, P, K

The pH value obtained with the Hanna Instruments colorimetric test was 5, so the analyzed soil has a weakly acidic value.

The analysis for the potassium present in the soil sample is a turbidimetric analysis, which means that the potassium concentration is assessed by observing the test tube with the cardboard provided by the manufacturer and observing how cloudy or transparent the sample is. The more cloudy the soil sample, the higher the potassium concentration in the soil. In the case of the soil sample analyzed by us, we obtained a low to medium potassium concentration.

2. In vitro culture in the laboratory.

Experiment to test the capacity of the new soil isolate *Bacillus* sp., to germinate and stimulate the growth of bean plants (*Phaseolus vulgaris*) and to inhibit the phytopathogenic fungi *Pythium* sp., *Fusarium* sp. and *Rizoctonia* sp. took place in March 2022 in the laboratory of Ecology and Environmental Microbiology.

Five days after planting, the beans began to germinate only in samples P1, P2 and P3 with small differences in rhythm, in sample P1 the least (slowly), in sample P2 the most (fast) and in the intermediate P3 sample between the two samples. In the control containers, the beans did not germinate. The beans planted in the three replicas of the control (M) did not germinate at all, due to contamination with phytopathogenic fungi *Pythium* sp., *Fusaium* sp. and *Rizoctonia* sp.

The control (M) contained only beans, sample 1 (P1) contained beans and microorganisms from the soil extract with the consortium of microorganisms, sample 2 (P2) contained beans and bacterial suspension from the newly isolated bacterium *Bacillus* from the soil, microorganisms, and sample 3 (P3) contained soil extract with the consortium of microorganisms and bacterial suspension from the pure culture of the new bacterial isolate *Bacillus* sp.

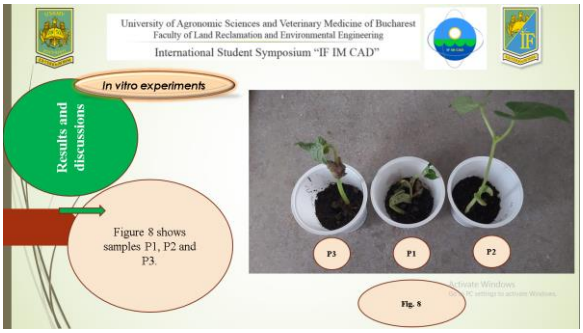

Figure 3. Samples P1, P2 and P3

In the P1 sample containers, we noticed that the seedlings, after they started to grow, were attacked by phytopathogenic fungi present in the soil and on the beans naturally.

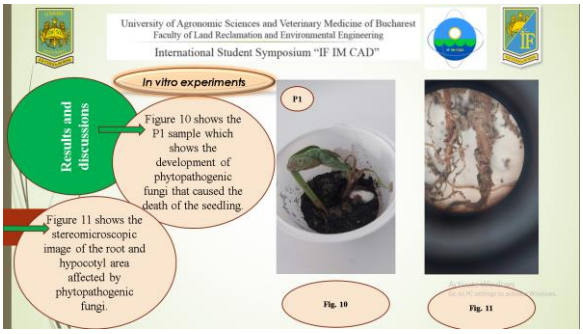

Figure 4. Stereomicroscopic image of the root and hypocotyl area affected by phytopathogenic fungi

In the P2 sample containers we noticed that the seedlings had the fastest growth rate, vigor and showed no signs of infection with the phytopathogenic fungi *Pythium* sp., *Fusarium* sp. and *Rhizoctonia* sp., which means that the applied suspension contained suspension of newly isolated bacteria by us, *Bacillus* sp. stimulated the germination of beans and the development of seedlings. Also due to the antifungal substances that these bacteria can produce in the soil in the niche competition with phytopathogenic fungi.

In the containers with sample 3 (P3) we noticed that the beans germinated, the seedlings

developed, but had smaller dimensions and less weight than those developed in sample 2 (P2).
At the end of the experiment, when the seedlings were removed from containers and weighed, measured and observed under a stereomicroscope in the hypocotyl and root area, we observed that in sample 1 (P1) the seedlings showed signs of damage caused by fungal infection, staining in reddish brown and softening of the hypocotyl area.
The seedlings in sample 1 (P1) had the lowest weight (1.89 g), those in sample 2 (P2) had the highest weight (2.49 g), and those in sample 3 (P3) had an average weight (2.38 g).
Regarding the size of the seedlings, those in sample 1 (P1) were 11 cm without root and 15 cm with root, those in sample 2 (P2) were 36 cm without root and 43.5 cm with root, and those in sample 3 (P3) had 23 cm without root and 30 cm with root.

CONCLUSIONS

In this paper we present the results obtained by testing the microorganisms from the consortium present in the soil sample taken from the vegetable garden of the Zurini family from Crucea de Piatră, Călugăreni commune, Giurgiu county and the new strain of *Bacillus* sp. isolated.
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This new edaphic bacterial strain of the genus *Bacillus* isolated and identified by us has the ability to produce biofilms and inhibitory compounds (like antibiotics and organic volatile compounds) that inhibited the development of phytopathogenic fungi in the complex that creates the fall of seedlings in beans (*Phaseolus vulgaris*), root rot and hypocotyl (*Pythium* sp., *Rhizoctonia* sp., *Fusarium* sp.,) that are naturally present in the soil or on the beans. Also, the new edaphic bacterial strain *Bacillus* sp. has the ability to stimulate the germination and development of bean plants (*Phaseolus vulgaris*), in addition to the ability to provide protection against infection with phytopathogenic fungi from the soil.
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All experiments were performed in triplicate.

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