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ABSTRACT

of a Ph.D. thesis for awarding the educational and scientific degree
"Philosophy Doctor"
Professional area 4.3 Biological sciences
Scientific specialty "Microbiology"

Physiological and biochemical characteristics of the plant - microbial symbiosis of representatives of the genus *Pseudomonas*

GLORIA BISEROVA GEORGIEVA

Scientific supervisor:
Assoc. Prof. Dr. Trayana Nedeva

Head of Department:
Prof. Dr. Petya Hristova

Scientific consultant:
Prof. Dr. Petya Hristova

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The Ph.D. thesis is written on standard A4 pages and contains the following sections: **Introduction** – **1 page**; **Literature review** – **45 pages; Objective and tasks - 2 pages**; **Materials and methods – 20 pages**; **Results and discussion – 86 pages**; **Conclusions – 2 pages**; **Contributions – 1 page**; **69 figures** and **10 tables** are included. The **Reference** section includes **347 titles**. The experimental work was carried out in the **Department of General and Industrial Microbiology** and the microbiological laboratory of the company **ROMB Ltd.**

The Ph.D. thesis was discussed at a meeting of the Department of General and Industrial Microbiology, Faculty of Biology, SU "St. Kliment Ohridski" held on 27.06.2024, 10:30 a.m. and scheduled for defense in front of a scientific jury, formed by Order …….. of the Rector of SU "St. Kliment Ohridski".

Scientific jury:

1.

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The Ph.D. thesis defense will take place at ……., Faculty of Biology of the SU "St. Kliment Ohridski".

The documents regarding the Ph.D. thesis can be found in the Department of General and Industrial Microbiology and on the website of the Faculty of Biology.

Abbreviations used:

- ADIC - 2-Amino-2-deoxyisochorismic acid
- AOCHC - 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid
- ABA – Abscisic acid
- ACC - 1-Aminocyclopropane-1-Carboxylate Deaminase
- AHLs - N-Acyl homoserine lactones
- BAS – Biologically Active Substances
- BGCs - Biosynthetic gene clusters
- DHHA - Trans-2,3-dihydro-3-hydroxyanthranilic acid
- HHPDC - Hexahydrophenazine-1,6-dicarboxylic acid
- IAA – Indole Acetic Acid
- PAMP - Pathogen Associated Molecular Patterns
- PCA – Phenazine-1-carboxylic acid
- PGP – Plant Growth Promoting
- PGPF – Plant Growth Promoting Fungi
- PGPM - Plant Growth Promoting Microorganisms
- PGPR – Plant Growth Promoting Rhizobacteria
- PRRs - Pattern Recognition Receptors
- ROS – Reactive Oxygen species
- THPCA - Tetrahydrophenazine-1,6-carboxylic acid
- T3SS – Type III Secretion System
- VOC – Volatile Organic Compounds

INTRODUCTION

Our times have emphasized caring for nature and soil as one of its five components. The continuous increase in human population and proportionately increasing industrialization threatens our planet's global ecosystem with a scarcity of food resources and progressively enlarges the harmful effects on the environment. New strategic solutions and their widespread implementation are needed to improve agricultural yields and sustainability, thus the needs of the human population to be met with as little environmental impact as possible.

Part of the solution may be the plant-microbial symbiont system, and particularly the specialized microbial component - bacteria that stimulate plant growth and development as an essential component in ecosystem functioning. Plant growth and development-promoting bacteria colonize the rhizosphere and exert their positive effect on the plant counterpart through various mechanisms. Among these, representatives of the genus *Pseudomonas* have become a research focus for improving overall plant development and biocontrol of plant bio-fertilization (Singh *et al.*, 2013). Bacteria belonging to the g. *Pseudomonas* has potential applications in biotechnological production, plant growth and development promotion, bioremediation, and biological control (Dimkic *et al*., 2022). However, this broad set of characteristics is species-(even strain-) specific. Searching for strains with the pronounced potential to promote plant growth and development and demonstrating this potential on model rhizosphere systems with economic relevance is a good alternative for targeted rhizosphere habitat modeling in the search for ecologically sound solutions for sustainable agricultural practices.

OBJECTIVE AND TASKS

The main objective of the Ph.D. thesis is to select and study bacterial strains belonging to the genus *Pseudomonas* to reveal the phenomenon of plant-microbe symbiosis and demonstrate their PGP potential.

The objective is achieved through the performance of the following tasks:

1. Screening of *Pseudomonas* isolates for PGP potential:

   1.1. Complex biochemical characterization of five isolates by multienzyme assays (API 20NE and API ZYM systems);

   1.2. Analysis of key PGP characteristics: biosynthesis of lytic and antioxidant enzymes, production of iron-binding proteins (siderophores) and phytohormones, biological N_2-fixation.

2. Demonstration of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 PGP potential:

   2.1. Optimization of the cultivation process in terms of C, N, and P source, induction of IAA biosynthesis, and fermentation process scale-up;

   2.2. Investigation of phenazine-1-carboxylic acid and siderophore production;

   2.3. Evaluation of the strains' applicability as biocontrol agents - determination of antifungal activity;

   2.4. Optimization of the strains' preservation and stability of their bioactive fermentation products during lyophilization and spray drying.

3. Study of the plant-microbe symbiosis of the selected strains with model plant systems of cereals and ornamental plants in different growing phases:

   3.1. Seed germination efficiency monitoring: evaluation of the strains' effect on root system biometrics of the cereals maize, soybean, and wheat;

   3.2. Evaluation of plant-microbe symbiosis with potted ornamental plants primrose, chrysanthemum, and cyclamen: investigation of the effect of fresh and dried cultures of the strains (individually and in combination) on plant roots (by watering) and on their phyllosphere (by foliar spraying).

MATERIALS AND METHODS

1. Microorganisms

The *Pseudomonas* strains were provided by "ROMB Ltd" (company microbiological collection): *Pseudomonas chlororaphis* 1S4, *Pseudomonas yamanorum* 1046, *Pseudomonas yamanorum* Or5, *Pseudomonas yamanorum* G-52, and *Pseudomonas yamanorum* R6. *Fusarium proliferatum* C1, *Fusarium oxysporum* F6, and *Fusarium solani* F7 strains were used to evaluate antifungal activity.

2. Nutrient media

2.1 Nutrient media for maintenance of the strains and determination of their biochemical characteristics: MPA (Nutrient agar) HiMedia, KDA (Potato dextrose agar) HiMedia, PDG - peptone - 5 g/L, yeast extract - 5 g/L, glucose - 6 g/L, and agar - 2%;

2.2 Solidified media used for analytical purposes - Starch agar, Milk agar, CAS agar, LB agar, Pikowska's medium;

2.3 Liquid media used for analytical purposes - NFb medium;

2.4 Media used for batch cultivation:

- *Pseudomonas* fermentation medium – glucose - 5 g/l, $(NH_4)_2SO_4$ - 3 g/l, KH_2PO_4 - 1 g/l, $MgSO_4$ - 0.5 g/l, peptone - 5 g/l, yeast extract - 1 g/l; MPB HiMedia;
- Modified fermentation media for *Pseudomonas* with added L-tryptophan (HiMedia) - 0.01%; 0.02%;0.04%;
- Fermentation media for studying the optimum ratio of carbon, nitrogen, and phosphorus sources, designed according to the following scheme (3 x 3 = 9 variants; see page 8).

3. Batch cultivation in flasks and bioreactor.

4. Methods for monitoring the cultivation processes:

4.1 Methods for monitoring the growth dynamics of bacterial cultures;

4.2 Soluble protein quantification method;

4.3 Method for quantification of residual sugars;

4.4 Method for the determination of colony forming units (CFU).

C↓	N →		1		2		4	P↓
1		I.	$\frac{1}{2}$N:$\frac{1}{2}$C:$\frac{1}{2}$P = 1:1:1	IV.	N:$\frac{1}{2}$C:$\frac{1}{2}$P = 2:1:1	VII.	2N:$\frac{1}{2}$C:$\frac{1}{2}$P=4:1:1	**1**
2		II.	$\frac{1}{2}$N:C:P = 1:2:2	V.	N:C:P = 2:2:2	VIII.	2N:C:P = 4:2:2	**2**
4		III.	$\frac{1}{2}$N:2C:2P = 1:4:4	VI.	N:C:P = 2:4:4	IX.	2N:2C:2P=4:4:4	**4**

5. Methods for cell morphology assessment:
   5.1. Proof of Gram affiliation.

6. Enzymological analyses:
   6.1. Determination of main enzyme characteristics by the ApiZYM system;
   6.2. Determination of assimilation characteristics by the API 20 NE system;
   6.3. Proteolytic activity;
   6.4. Amylolytic activity (ASM, 2012);
   6.5. Catalase activity (ASM, 2016);
   6.6. Biosynthesis of siderophores (Arora & Verma, 2017);
   6.7. Determination of phosphorus solubilization (Shahid *et al*., 2015).

7. Determination of indole acetic acid (Gusmiaty and Payangan, 2019).
8. Determination of phenazine-1-carboxylic acid (Raio *et al.*, 2017).
9. Performance of biological N_2 fixation.
10. HPLC assay for quantification of phytohormones (ThermoScientific Co, USA).
11. Genetic analyses for identification (Macrogen Europe - 16sRNA; ITS regions).
12. Plant seed germination tests:
    12.1. Seed outgrowth in rolls (Draves *et al.*, 2022);
    12.2. Plant tests - pot experiments.
13. Methods to determine antifungal activity.
14. Methods for obtaining dry preparation of g. *Pseudomonas* strains:
    14.1. Spray drying;
    14.2. Lyophilization.

RESULTS AND DISCUSSION

Plant-microbe symbiosis is the basis of microbial application in agriculture. Plants secrete exudates through their root system, which contain a wide range of inorganic and organic compounds that attract PGPR and mycorrhizal species, promote nutrient access, and provide protection against biotic and abiotic stresses. This type of coordinated communication between plants and rhizosphere microorganisms through which they exert their PGP effect is the subject of study in the present work. Herein, the potential capabilities of five strains of g. *Pseudomonas* to exert a PGP effect are analyzed.

1. Screening of *Pseudomonas* isolates for plant growth-promoting potential (PGP)

Five strains of the genus *Pseudomonas* were included in the biochemical screening. Three of them were provided by the microbiological collection of ROMB Ltd - *Pseudomonas chlororaphis* 1S4, *Pseudomonas putida* Or5, and *Pseudomonas putida* 1046, and *Pseudomonas yamanorum* R6 and *Pseudomonas yamanorum* G-52 were isolated in the course of the present work by applying a standard protocol for microbial isolation from soil.

A series of analyses was conducted to construct the PGP profile of the investigated strains, which included morphological characterization, essential biochemical parameters generally accepted to demonstrate PGP potential, and genetic analyses.

1.1 Morphological characterization of the isolates

All isolates showed the typical morphological characteristics of representatives of the genus *Pseudomonas*: Gram-negative status, obligate aerobic metabolism, and rod-shaped with a size of 0.5 - 3.0 μm. They move by a single polar flagellum, in some cases more than two flagella may be observed. They form opaque, shiny, flat, irregular-edged colonies. One of them produces a pigment, which is a species-specific feature (Fig. 1).

Using a modified Gram affiliation technique (Buck, 1982), all five strains were shown to be Gram-negative (Fig. 2).

1.2 Assimilation characteristics

The assimilation characteristics of the strains were determined by biochemical tests using the API 20 NE system. Three strains were found to reduce nitrate to molecular nitrogen - 1S4, 1046, and G-52. Two strains (G-52 and 1046) tested positive for L-arginine dihydrolase. Only strain 1S4 carried out hydrolysis of esculin by β-glucosidase. Regarding the spectrum of carbon sources, all five strains assimilated most of them, except maltose, and strain 1S4 did not assimilate mannitol and adipic acid but was the only one to assimilate gelatin (Fig. 3).

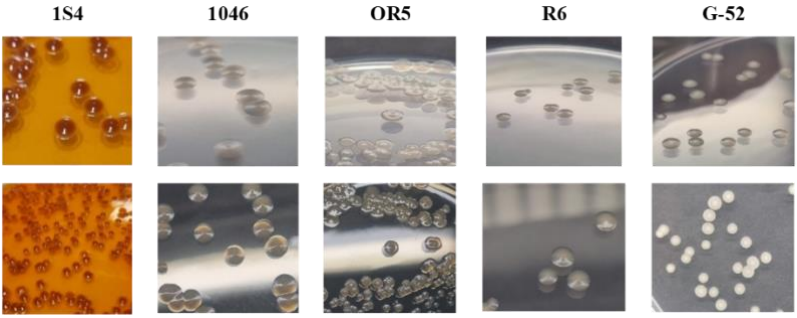


Figure 1. Macromorphology of the studied microorganisms.

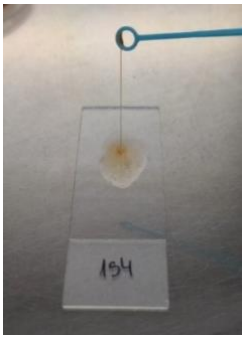
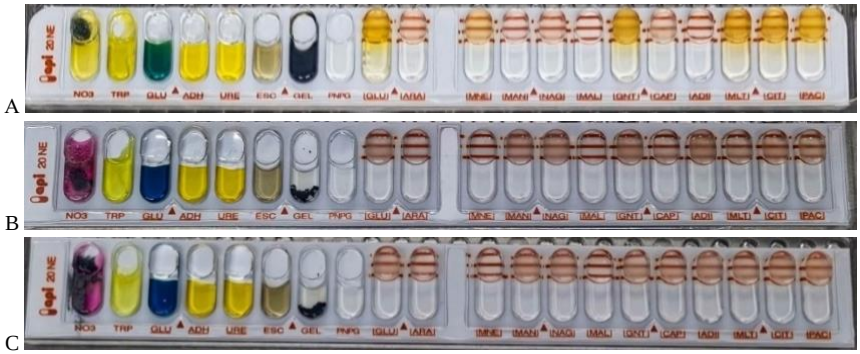


Figure 2. Demonstration of Gram affiliation of *Pseudomonas chlororaphis* 1S4. Illustration of a positive reaction for a Gram-negative culture by 3% KOH test.



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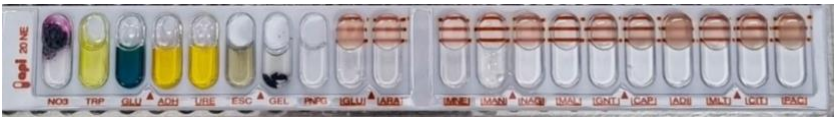

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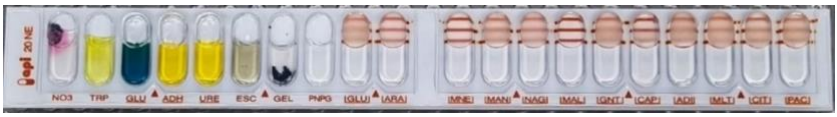


Figure 3. Biochemical assay to determine assimilation characteristics using the API 20 NE system. A - strain 1S4, B - strain 1046, C - strain G-52, D - strain R6, E - strain Or5.

1.3. Generation of enzyme profile

The multienzyme profile of the isolates was determined by a semiquantitative microarray method using the ApiZYM system (BioMerieux, Inc.).

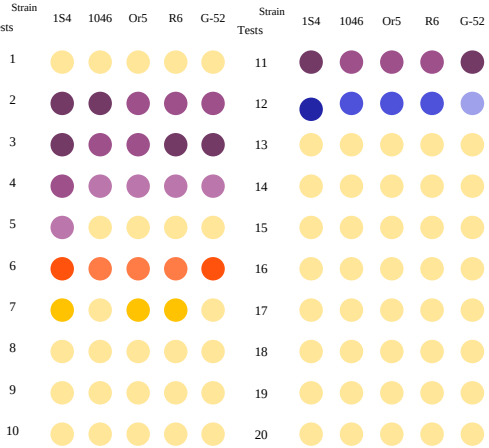


Figure 4. Multienzyme analysis (microtest) of *Pseudomonas* representatives by the ApiZYM system.

The generated enzymatic profile showed that all five isolates produced alkaline and acid phosphatase, esterase, and esterase-lipase, in different concentrations.

1.4. Production of iron-binding proteins - siderophores

All five strains were found to form a yellowish halo zone (Fig. 5), an indicator of siderophore synthesis, with the capacity to do so most pronounced in isolate 1S4.

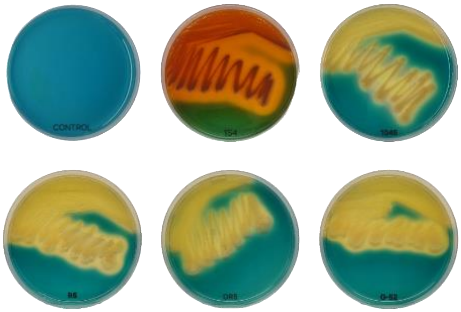


Figure 5. CAS analysis for the determination of siderophores.

1.5. Biosynthesis of lytic enzymes

The results of the proteolytic activity assay (Fig. 6) indicated the presence of active proteases in isolates 1S4, R6, Or5, and G-52, visualized by the distinct halo zone around the growth zone. Quantitative differences were observed, with the largest size of the hydrolytic zone again in isolate 1S4.

Figure 6. Proteolytic activity assay.

Amylolytic activity is another essential PGP characteristic that complements the PGP profile. Amylolytic activity was observed in three isolates - 1S4, 1046, and R6.

1.6. Catalase activity

Catalases are involved in the antioxidant defense of cells and also in the processes of cell development and differentiation. Four of the five isolates (1046, R6, Or5, and G-52) showed a clear positive response (Fig. 7).

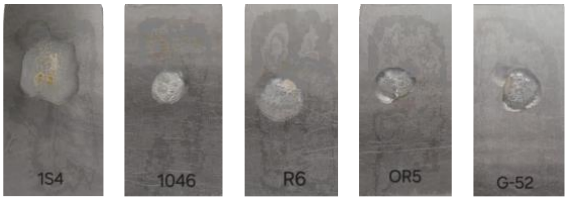


Figure 7. Analysis of catalase activity

1.7. Solubilization of inorganic phosphorus-containing compounds

Phosphorus is an essential element for plant growth and development and a component of nutrient cycles in the soil. Although the total amount of phosphorus in soils is high, most of it is in a form not assimilable by plants (Wang *et al.*, 2022). A halo zone around the growth stretch was observed in all five isolates, a phosphatase activity indicator (Fig. 8).

Figure 8. Phosphorus solubilization.

1.8. Performance of biological nitrogen fixation

Members of the genus *Pseudomonas* have essential PGP properties, among which is the ability to fix nitrogen, found in many of them (Singh *et al.*, 2023). The five members of the genus *Pseudomonas* demonstrated the ability to fix nitrogen to varying degrees (Table 1).

Table 1. Nitrogen fixation capacity of the strains studied.

Strain	N (ppm)
1S4	133,488
1046	50,126
Or5	40,008
R6	39,448
G-52	36,648

The strain *Pseudomonas chlororahis* 1S4 demonstrated the highest potential for nitrogen fixation (133,488 ppm), followed by *Pseudomonas yamanorum* 1046 (50,126 ppm).

1.9. Biosynthesis of phytohormones

The phytohormone profile of the five studied microorganisms and the production of retardants influencing plant growth and development are presented in Table 2. In the HPLC analysis performed, it was found that all five isolates produced the majority of the studied regulators, and the main difference between them was quantitative: aminocyclopropane-1-carboxylic acid (ACC) - *Ps. yamanorum* 1046 - 393.39 ng/ml, trans-zeatin - *Ps. chlororaphis* 1S4 - 89,89 ng/ml, gibberellin A8 - *Ps. yamanorum* 1046 - 106.02 ng/ml, indole acetic acid (IOA) - *Ps. yamanorum* 1046 - 105,31 ng/ml. Analyses of the presence of retardants showed that the highest amount was observed in *Ps. chlororaphis* 1S4, followed by *Ps. yamanorum* 1046.

Table 2. HPLC analysis for quantification of the phytohormones produced by the strains.

Strain / Phytohormones	1S4	1046	Or5	R6	G-52
	ng/ml				
ACC,ACPC	149,56	396,39	132,19	82,46	32,76
DAMINOZIDE	208,95	1,29	1,57	0,34	0,28
MALEIC HYDRAZIDE	3,43	4,44	11,50	6,62	1,90
trans-ZEATIN	89,89	30,37	7,80	5,86	47,38
GIBBERELLIN A8	10,57	106,02	17,45	69,57	11,56
GR5	25,75	5,92	13,09	24,08	1,28
IAA	13,99	105,31	10,34	11,62	80,71
THIDIAZURON	0,63	1,46	1,35	0,02	0,02
ANCYMIDOL	0,55	0,52	0,04	0,02	0,06
JASMONIC ACID	12,23	19,62	28,82	9,39	14,02
STRIGOL	0,55	0,66	3,10	-	-
OROBANCHOL	0,43	0,51	0,43	-	-
PACLOBUTRAZOL	0,62	1,74	9,74	0,28	0,10
FLURIDONE	2,16	4,17	2,38	1,04	0,46
FLURPRIMIDOL	0,78	1,64	1,86	0,27	0,22
NAA	75,72	63,20	278,72	19,28	237,64
UNICONAZOLE	1,10	2,80	1,41	0,39	0,10

ABCISIC ACID	7,65	3,07	5,76	4,65	1,63

The summarized biochemical characteristics of the studied isolates are presented in Table 3.

Table 3. Biochemical characterization of the investigated strains of the genus *Pseudomonas*.

ApiZYM system						
№	**Strain** / **Enzyme**	***Ps. chlororaphis* 1S4**	***Ps. yamanorum* Or5**	***Ps. yamanorum* R6**	***Ps. yamanorum* G-52**	***Ps. yamanorum* 1046**
1	**Control**					
2	**Alkaline phosphatase**	+++	++	++	++	+++
3	**Esterase (C 4)**	+++	++	+++	+++	++
4	**Esterase Lipase (C 8)**	++	+	+	+	+
5	**Lipase (C 14)**	+	-	-	-	-
6	**Leucine arylamidase**	+++	++	++	+++	++
7	**Valine arylamidase**	+	+	+	-	-
8	**Cystine arylamidase**	-	-	-	-	-
9	**Trypsin**	-	-	-	-	-
10	**α-chimotrypsin**	-	-	-	-	-
11	**Acid phosphatase**	+++	++	++	+++	++

12	**Naphtol-AS-BI-phosphohydrolase**	+++	++	++	+	++
13	**α-galactosidase**	-	-	-	-	-
14	**β- galactosidase**	-	-	-	-	-
15	**β-glucuronidase**	-	-	-	-	-
16	**α-glucuronidase**	-	-	-	-	-
17	**β-glucosidase**	-	-	-	-	-
18	**N-acetyl-β-glucosaminidase**	-	-	-	-	-
19	**α-mannosidase**	-	-	-	-	-
20	**α-fucosidase**	-	-	-	-	-
Api 20 NE system						
1	**NO_3 Reduction of nitrates to nitrites** **Reduction of nitrates to nitrogen**	- +	- -	- -	- +	- +
2	**TRP Indole production (tryptophan)**	-	-	-	-	-
3	**GLU Fermentation (glucose)**	-	-	-	-	-
4	**ADH Arginine dihydrolase**	-	-	-	+	+
5	**URE Urease**	-	-	-	-	-

6	**ESC Hydrolysis (β-glucosidase) (esculin)**	+	-	-	-	-
7	**GEL Hydrolysis (protease) (gelatin)**	+	-	-	+	-
8	**PNGP β-Galactosidase (paranitrophenyl-ß-D-galactopyranosidase)**	-	-	-	-	-
9	**|GLU| Assimilation (glucose)**	+	+	+	+	+
10	**|ARA| Assimilation (arabinose)**	+	+	+	+	+
11	**|MNE| Assimilation (mannose)** **|MAN| Assimilation (mannitol)**	+ -	+ +	+ +	+ +	+ +
12	**|NAG| Assimilation (N-acetylglucosamine)**	+	+	+	+	+
13	**|MAL| Assimilation (maltose)**	-	-	-	-	-
14	**|GNT| Assimilation (potassium gluconate)**	+	+	+	+	+
15	**|CAP| Assimilation (capric acid)**	+	+	+	+	+
16	**|ADI| Assimilation (adipic acid)**	-	+	+	+	+
17	**|MLT| Assimilation (malate)**	+	+	+	+	+
18	**|CIT| Assimilation (trisodium citrate)**	+	+	+	+	+
19	**|PAC| Assimilation (phenylacetic acid)**	+	+	+	+	+

20	**OX Cytochrome oxidase**	+	+	+	+	+
Enzymatic activities						
1	**Amylolytic activity**	+	-	+	-	+
2	**Proteolytic activity**	+	+	+	+	-
3	**Catalase activity**	+/-	+	+	+	+
Other PGP properties						
1	**Siderophore synthesis**	+	+	+	+	+
2	**Phosphorus solubilization**	+	+	+	+	+
3	**Pigment synthesis**	+	-	-	-	-
4	**N_2fixation**	+	+	+	+	+
Temperature profile						
1	**Growth at 4°C**	+	+	+	+	+
2	**Growth at 37°C**	+ (no pigmentation)	-	-	-	-

The biochemical characterization results were processed using ABIS online software (https://www.tgw1916.net/bacteria_logare_desktop.html) designed for microorganisms' biochemical identification. According to the ABIS results, all the strains were identified as g. *Pseudomonas* representatives. Species identification varied between 80% and 99% due to the need to upgrade the biochemical profile with additional analyses. To confirm the identification based on biochemical data, a genetic analysis of the isolates was also performed (see Section 1.10).

1.10. Genetic analysis

Cultures from the five isolates were subjected to genetic analysis by Macrogen and identified as: *Pseudomonas chlororaphis* 1S4 - 99%; *Pseudomonas yamanorum* 1046 - 99%; *Pseudomonas yamanorum* Or5 - 99%; *Pseudomonas yamanorum* R6 - 99% and *Pseudomonas yamanorum* G-52 - 99%. The species *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 were selected for further studies.

2. Demonstration of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 PGP potential

To confirm and further investigate the two selected strains, *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046, they were subjected to additional tests to study their behavior under different conditions of the cultivation process and, subsequently, to study their interaction with model test plants.

2.1. Optimizing the cultivation process

Optimization of C, N, and P sources concentrations

An experiment was conducted to optimize the basal concentrations of C, N, and P sources in a batch system of flask cultures, followed by scale-up in a 5 L bioreactor to select the most suitable culture medium. Data were recorded after a 48-h culture period and presented in Fig. 9 and Fig. 10.

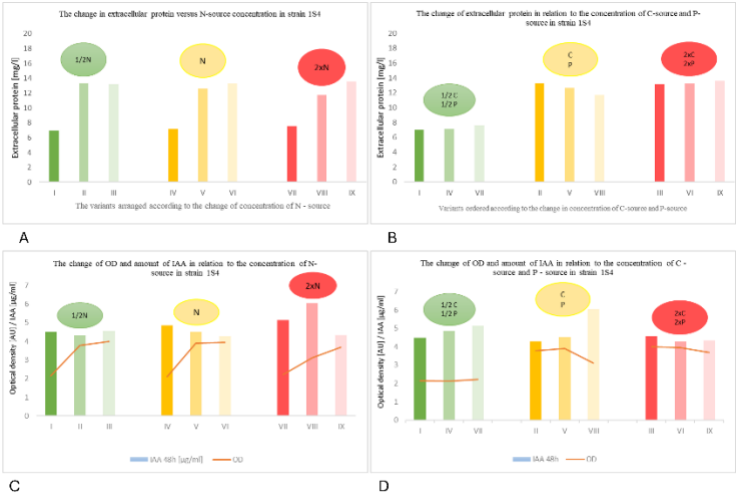


Figure 9. Batch cultivation of *Pseudomonas chlororaphis* strain 1S4; A: Variation of the amount of exocellular protein relative to the N source; B: Variation of the amount of exocellular protein relative to the C and P sources; C: Variation of the optical density and the amount of IAA produced relative to the N source; D: Variation of the optical density and the amount of IAA produced relative to the C and P sources.

Fig. 9 A and B show the accumulated amount of exocellular protein from strain 1S4 in the different treatments over a 48-h period. The results are presented in three groups according to the working N source concentrations: 25% or 1/2 part, 50% (1 part), and 100% (2 parts), respectively. The highest value of this indicator was 13.6 mg/ml in variant IX.

For the optical density parameter (Fig. 9, C and D), the lowest values were observed for ½C and ½P, with the other variants having higher optical densities and comparable values of 4 OE.

For the IAA amount indicator (µg/ml), only one variant (VIII) stood out with higher values of this parameter, 6.05 µg/ml.

The best results for all parameters were achieved in the variants with double concentrations of the three sources. However, the difference between these and the variant with the so-called baseline concentration (V) does not economically justify their industrial application. For this reason, in strain 1S4, the V variant, calculated relative to the N, C, and P source inputs and maximum values of the measured indicators, stands out as the optimal variant.

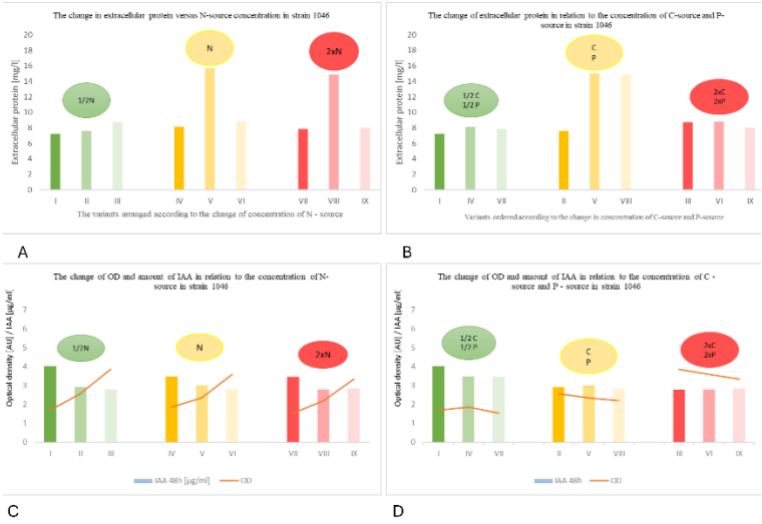


Figure 10. Batch cultivation of *Pseudomonas yamanorum strain 1046*; A: Variation of the amount of exocellular protein relative to the N source; B: Variation of the amount of exocellular protein relative to the C and P sources; C: Variation of the optical density and the amount of IAA produced relative to the N source; D: Variation of the optical density and the amount of IAA produced relative to the C and P sources. Figure 10. Periodic deep culturing of *Pseudomonas*; A: Variation of the amount of exocellular protein

For *Ps. yamanorum* 1046, maximum values were recorded at variant V - 15.0 mg/ml and VIII - 14.8 mg/ml. The highest IAA values were observed in the ½C and ½P variants, where the optical density was the lowest - the IAA ranged between 3.0 and 4.0 µg/ml.

The selected fermentation medium variant V was scaled up in a 5 L bioreactor. When cultivating *Ps. chlororaphis* 1S4, a lag phase of about 4th h was observed, followed by a phase of intense growth (log phase), and in the period from about 8th h to 24th - 28th h, the culture was in a steady state. Next, the die-off-lysis phase is observed after 40 h of the culture process, when the amount of exocellular protein rises sharply (Fig. 11).

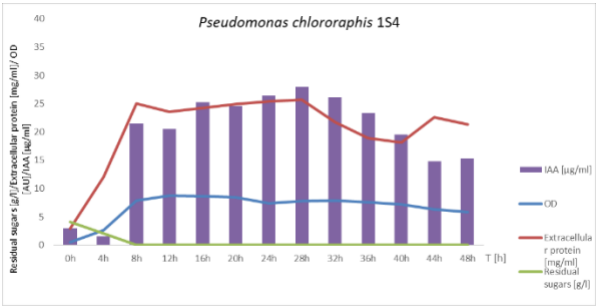


Figure 11. Batch cultivation of *Pseudomonas chlororaphis* 1S4 in a 5 L bioreactor.

Monitoring the parameter amount of IAA produced showed a significant difference during the process scaling-up, with maximum IAA values from the same producer reaching 27.9 µg/ml at 28 h.

The growth pattern of *Ps. yamanorum* 1046 batch culture exhibits some differences with respect to *Ps. chlororaphis* 1S4. In the case of *Ps. yamanorum* 1046, a prolonged lag phase was observed, lasting about 8 hours. The exponential phase is short, and the carbon source is depleted in about 4 hours (between 8th and 12th). The stationary phase is also shorter (about 12 hours), after which the culture begins to lyse. Regarding IAA production, the same pattern of increase is observed as the process scales up but at the end of the stationary phase. Maximum IAA values are reached at 44 hours - 20 µg/ml.

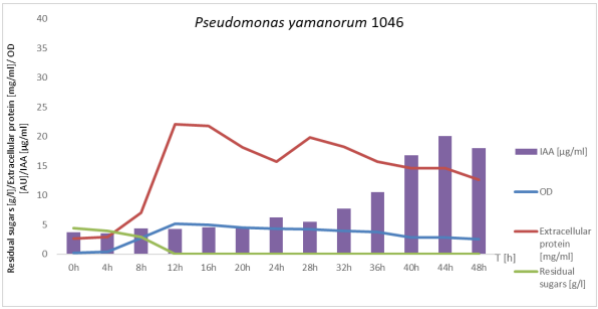


Figure 12. Batch cultivation of *Pseudomonas yamanorum* 1046 in a 5 L bioreactor.

Optimization of phytohormone biosynthesis

Phytohormones are essential for promoting plant growth and development. When the physiological precursor of IAA, L-tryptophan, is present in the medium, bacteria increase the amounts of IAA they produce (Raut *et al.*, 2017).

The L-tryptophan effectiveness was monitored in both strains at different concentrations of the inducer. Three concentrations were selected based on literature reference - 0.01%, 0.02%, and 0.04% (Bharucha *et al.*, 2013) and added to the basal fermentation medium. The effect of these concentrations was evaluated concerning control variants without added inducer. The duration of the cultivation process was 168 h (7 days), given that the target product of the cultivation was the IAA by-product. Two different inducer sources were used in the studies: synthetic L-tryptophan and L-tryptophan of microbiological origin.

The experimental results are shown in Fig. 13 and Fig. 14. It is evident that both strains show similar trends in the dynamics of OD_{600} parameters, pH, and amount of exocellular protein, regardless of the type of inducer. There was a steady increase in OD_{600} (2 to 4 AU) up to 24 h of cultivation, maintaining pH values in the range of 4 - 6 and extracellular protein concentration of about 8 - 10 mg/ml for the variants with the synthetic inducer and up to 20 mg/ml for the complex, protein-rich mixture of microbially produced L-tryptophan. These data indicate that a cultivation period of 168 h results in viable cultures with good potential for IAA biosynthesis during the stationary growth phase.

The dynamics of target IAA production were monitored over the same 168-hour period. The experimental results for *Pseudomonas chlororaphis* 1S4 with synthetic L-tryptophan inducer are illustrated in Fig. 13. It is noteworthy that in the control variant, the maximum amount of IAA of 4.2 µg/ml was recorded at the end of the process, at 168 h. As the inducer was incorporated into the medium and its concentration increased, a trend towards increased yields was observed. In the 0.01% L-tryptophan variant, the maximum amount of product was 4.6 g/ml at 0.02%, just above 5.0 µg/ml, and at 0.04%, it reached 6.5 µg/ml.

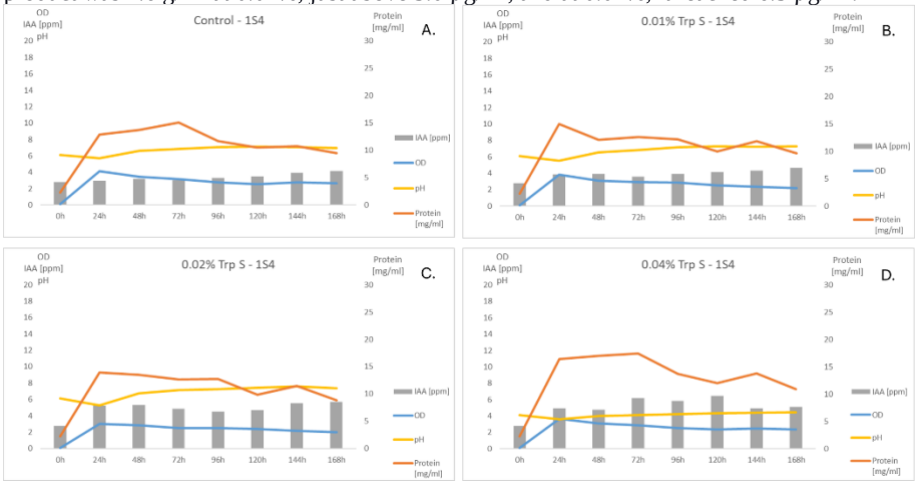


Figure 13. Batch cultivation of *Pseudomonas chlororaphis* 1S4 with added inducer synthetic L-tryptophan at concentrations of 0.01%, 0.02%, and 0.04%.

The second strain, *Pseudomonas yamanorum* 1046 was cultured under similar conditions and showed the following dependencies on the addition of increasing concentrations of the inducer L-tryptophan. At a concentration of 0.01% synthetic L-tryptophan, the amount of IAA increased as the process progressed, reaching its maximum value of 5.7 µg/ml at 168 h. In the variant induced with 0.02% synthetic L-tryptophan, there was a tendency to maintain a relatively constant IAA concentration after 72 h, with a maximum at 168 h of 8.0 µg/ml. At the maximum inducer concentration, maximum IAA yields of 12.3 µg/ml were reached.

As a general trend for the effect of synthetic L-tryptophan on IAA biosynthesis, an increase in IAA yields was observed with increasing synthetic L-tryptophan concentration in the culture medium, regardless of the producer strain. This observation indicates that synthetic L-tryptophan is an effective inducer for increasing the production of the studied BAC.

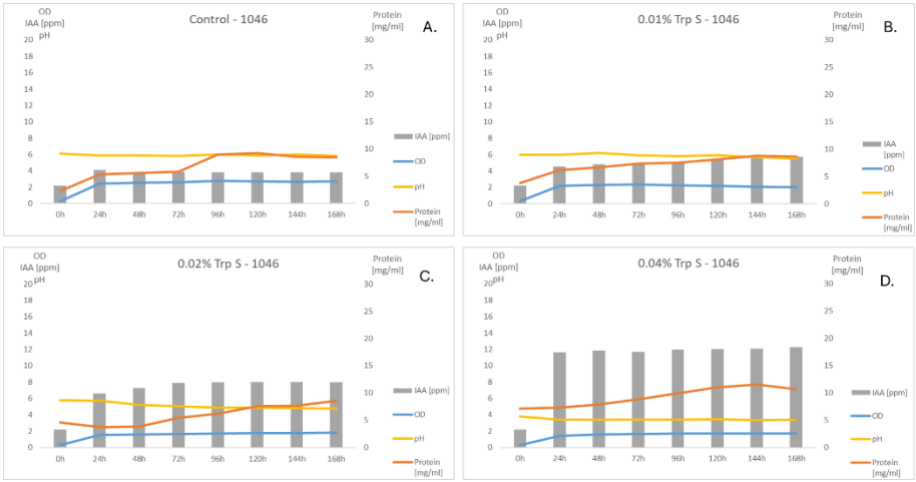


Figure 14. Batch cultivation of *Pseudomonas yamanorum* 1046 with added inducer synthetic L-tryptophan at concentrations of 0.01%, 0.02%, and 0.04%.

With the strain of *Ps. chlororaphis 1S4*, the trend of increasing the amount of the target product while increasing the inducer concentration was maintained with the microbial tryptophan. The maximum IAA yields for the 0.01% inducer variant were 10.8 µg/ml. In the

0.02% microbial tryptophan variant, the amount of IAA at 168 h was just over 13.0 µg/ml. The highest yields (14.4 µg/ml) resulted again with the highest concentration of the 0.04% inducer.

For *Pseudomonas yamanorum* 1046, a similar relationship was observed. IAA product amounts increased with increasing inducer concentration. At a concentration of 0.01%, 6.5 µg/ml of IAA was reported at 0.02% - 9.7 µg/ml, and 16.9 µg/ml at the highest concentration of the microbial tryptophan.

IAA synthesis depends on the exogenous concentration of L-tryptophan and is regulated positively by the presence of this inducer. The induction of IAA production with tryptophan of microbial origin is much more efficient compared to the synthetic option: IAA concentrations of cultures with microbial tryptophan exceed those in cultures with the synthetic inducer by 34 %.

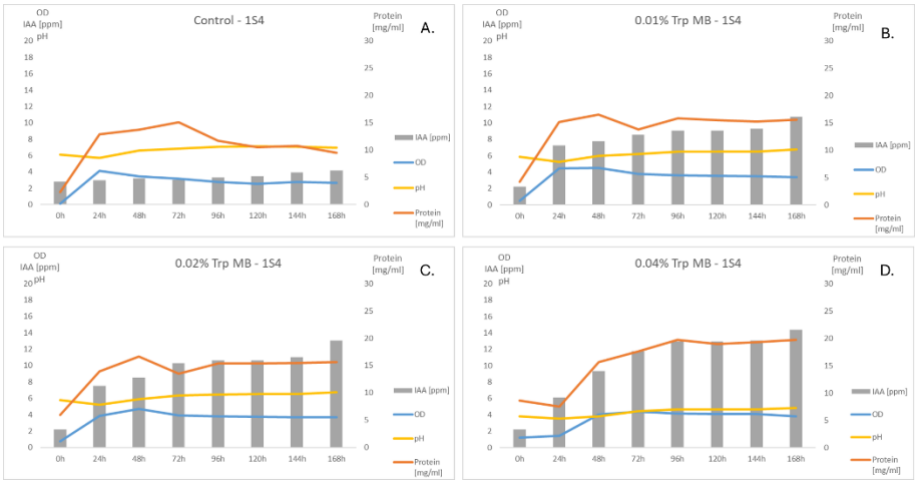


Figure 15. Batch cultivation of *Pseudomonas chlororaphis* 1S4 with added microbially derived tryptophan at concentrations of 0.01%, 0.02%, and 0.04%.

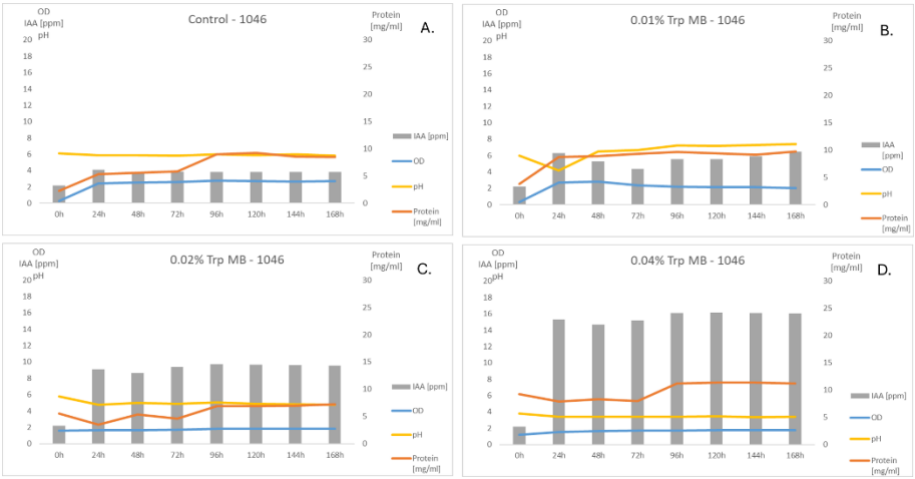


Figure 16. Batch cultivation of *Pseudomonas yamanorum* 1046 with added inducer microbially derived tryptophan at concentrations of 0.01%, 0.02%, and 0.04%.

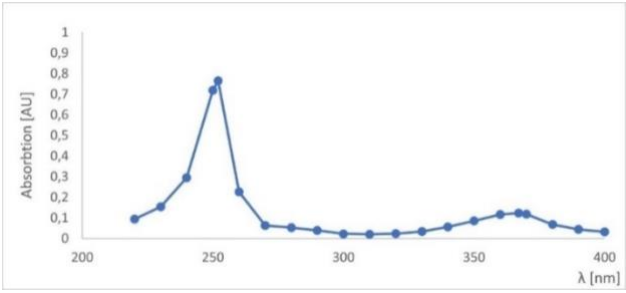
2.2. Production of phenazine-1-carboxylic acid

The biocontrol properties exhibited by the *Pseudomonas chlororaphis* 1S4 against plant pathogens of the *Fusarium* genus raise questions about the potential factors contributing to this effect. The production of phenazine pigments is a characteristic associated with antifungal activity due to their antibiotic functions. The study of phenazine-1-carboxylic acid (PCA) production by the *Ps. chlororaphis* 1S4 was conducted on 24-hour cultures. During the extraction process of the product from the dried sediment, crystalline structures typical of phenazine derivatives were observed directly with the naked eye and under a microscope (40x magnification) (Figure 17).

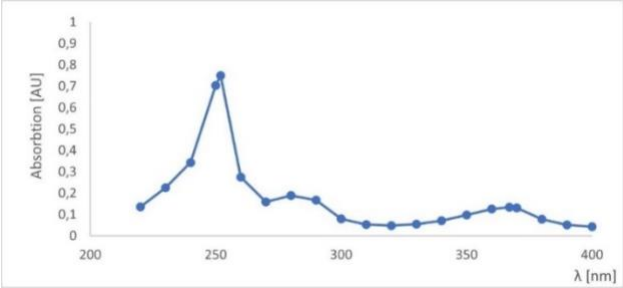
A) B)

Figure 17. Crystalline structure of phenazine-1-carboxylic acid isolated from a 24-hour culture of *Pseudomonas chlororaphis* 1S4. A) Direct observation B) Observation under a light microscope at 40x magnification.

The spectral curve of PCA in 0.1 N NaOH reveals two absorption maxima – at 252 nm and 367 nm (Figure 18-A). Spectral curves were plotted for both native and sterilized 24-hour cultures. The sterilization aimed to assess the retention of the spectral profile (i.e., the quality/quantity of PCA), which is thermally stable due to the three conjugated aromatic rings in its structure. The absorption spectrum of PCA remains entirely preserved (Figure 18-B), confirming its thermal stability.



A



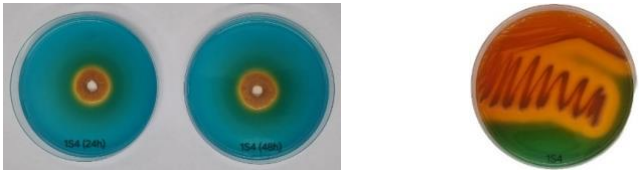
B

Figure 18. UV spectral curve of phenazine-1-carboxylic acid isolated from a 24-hour culture of *Pseudomonas chlororaphis* 1S4. A) Fresh/filtered culture broth, B) Sterilized culture broth.

Based on the spectral curves, it can be inferred that the batch cultivation of the *Ps. chlororaphis* 1S4 for 24 to 48 hours in a suitable nutrient medium stimulates the production of PCA. This compound is responsible for the significant inhibitory effect of the strain against fungal plant pathogens.

2.3. Production of siderophores

The ability of *Ps. chlororaphis* 1S4 to produce siderophores was demonstrated during the biochemical screening. In addition to this analysis, two culture broth variants (24 and 48 hours) were tested to assess the production dynamics. It was found that the production was identical in both variants (Figure 19 A).



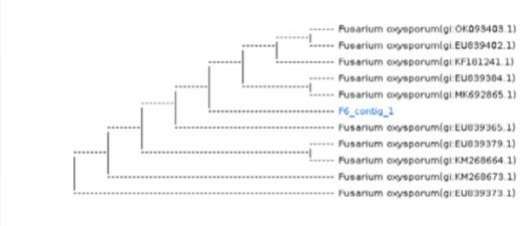
A) B)

Figure 1. Production of siderophores by *Pseudomonas chlororaphis* 1S4. A) Test with 24-hour culture (left) and 48-hour culture (right); B) 24-hour culture streaked on CAS agar.

2.4. Antifungal activity of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046

Representatives of the g. *Pseudomonas* are able to produce biocontrol agents with fungicidal effects against economically significant phytopathogens (Leon *et al.,* 2009). The antagonistic effect of *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 against economically important *Fusarium* pathogens was investigated. Bath cultures of each strain were tested at 24 hours and 48 hours, evaluating their inhibitory effects on the growth and development of three *Fusarium* species: *Fusarium proliferatum* C1 (provided by the microbiological collection of ROMB Ltd.), *Fusarium oxysporum* F6, and *Fusarium solani* F7, isolated during this study from a severely diseased cornfield in Northern Bulgaria in the summer of 2022. These isolates underwent genetic analysis of the ITS regions of the 18S rRNA genes. The results of this genetic analysis, morphologically identified as *Fusarium sp*., showed a confidence level of 99.0%, as demonstrated in Fig. 20.

- *Fusarium oxysporum* F6;



- *Fusarium solani* F7;

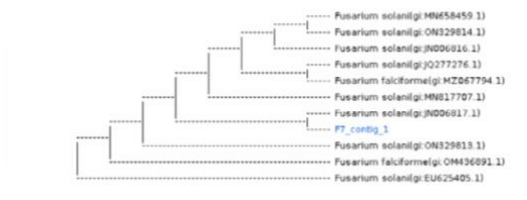


Figure 20. Genetic analysis of the isolates belonging to the g. *Fusarium.*

To monitor the effect of the two *Pseudomonas* strains against the *Fusarium* representatives a study was conducted for their antifungal activity in vitro. The assessment of the inhibitory capacity was formed based on tracking the parameter radial growth rate of the

colonies of the pathogenic representatives and calculating the inhibition effect (in %). The results are presented in Figs. 21 - 27.

Maximum inhibitory effect against *F. proliferatum* C1 was observed with variant *Ps. chlororaphis* 1S4 (48 h) during the first 72 h of the experiment, reporting over 80% growth inhibition. At 24 h culture, the time dependence of the effect was maintained, with a maximum value of 56.31% in the first 72 h (Fig. 21A).

In the radial growth rate indicator, over a period of 168 h, a more than 5-fold delay was observed when treated with a 48-h culture of *Ps. chlororaphis* 1S4 in the first 72 hours and 9-fold between 72 and 120 hours (Fig. 21B).

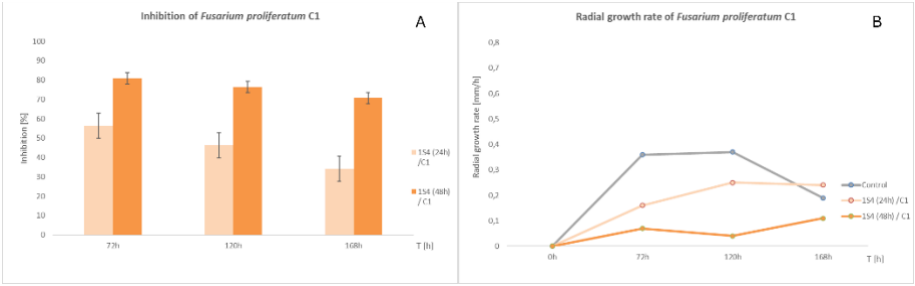


Figure 21. Growth inhibition (%) of *Fusarium proliferatum* C1 by *Pseudomonas* chlororaphis 1S4 (A) and radial growth rate dynamics of *Fusarium proliferatum* C1 (B).

The inhibitory effect of *Pseudomonas yamanorum* 1046 against *Fusarium proliferatum* C1 was weaker compared to *Ps. chlororaphis* 1S4. Maximal inhibition values (30.56%) were recorded at 72 h in the 24-hour culture. In the radial growth rate, no significant changes were observed in the development dynamics of the treated fungal culture (< 2 times) compared to the untreated control.

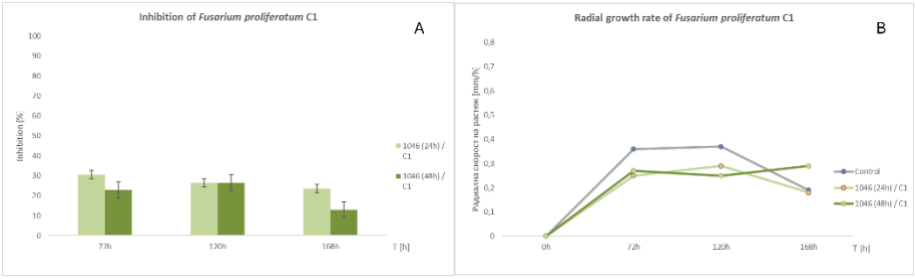


Figure 22. Growth inhibition (%) of *Fusarium proliferatum* C1 by *Pseudomonas* yamanorum 1046 (A) and radial growth rate dynamics of *Fusarium proliferatum* C1 (B).

The inhibitory effect of the two strains against the wild strain *F. oxysporum* F6 was evaluated in an experimental setup such as for the *F. proliferatum* C1. The obtained results are presented in Figs. 23 and 24.

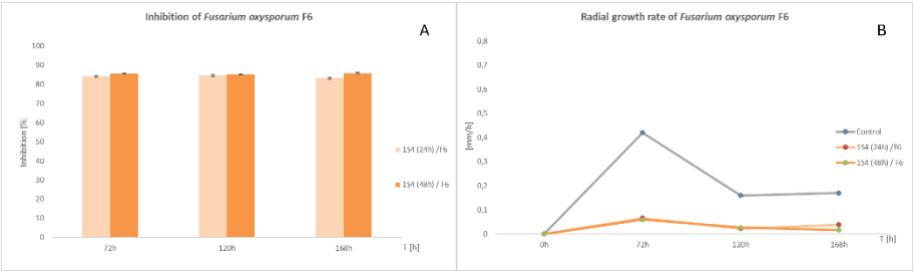


Figure 23. Inhibition (%) of the growth of *Fusarium oxysporum* F6 by *Pseudomonas chlororaphis* 1S4 (A) and radial growth rate dynamics of *Fusarium oxysporum* C1 (B).

Ps. chlororaphis 1S4 demonstrated a strong inhibitory effect against *F.* oxysporum F6. An inhibition of over 85% was observed at 48 h of culture. However, it is noteworthy that the difference with 24-hour culture is only 1%, which makes it economically unjustified to apply the variant *Ps. chlororaphis*1S4 (48 h). In terms of radial growth rate, 48 h culture retarded the development of *F. oxysporum* F6 between 7- and 10-fold. In the variant of *Ps. chlororaphis* 1S4 (24 h), the maximum growth retardation was seven times.

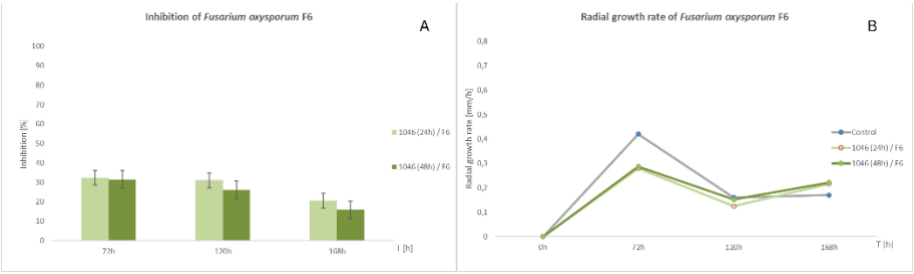


Figure 24. Inhibition (%) of *Fusarium oxysporum* F6 growth by *Pseudomonas yamanorum* 1046 (A) and radial growth rate dynamics of *Fusarium oxysporum* F6 (B).

The effect of *Ps. yamanorum* 1046 versus *F. oxysporum* F6 is presented in Fig. 24. *Ps. yamanorum* 1046 had a weaker inhibitory effect against *F.* oxysporum F6 isolate compared to *Ps. chlororaphis* 1S4. Here, the dependence observed in *F. proliferatum* C1 is preserved; *Ps. yamanorum* 1046 (24 h) is more active, with the maximum values of this indicator being 32.38% during the first 72 hours of the process. The variant *Ps. yamanorum* 1046 (48 h) reached a

maximum inhibition of 31.49%. In both variants, a decrease in the inhibition effect was observed over time.

Regarding the second indicator - the radial growth rate, *Ps. yamanorum* 1046 affected this indicator most strongly during the first 72 hours of the experiment, delaying the development of the pathogen less than twice.

The results of the antifungal activity test against *Fusarium solani* F7 are presented in Figs. 25 and 26.

Ps. chlororaphis 1S4 demonstrated an inhibitory effect against *F. solani* F7. Again, a better inhibitory effect was presented by the culture of *Ps.* chlororaphis 1S4 (48 h) – 72.31%, an effect that persists up to 120 h of the experiment. *Ps. chlororaphis* 1S4 (24 h) exhibits a maximum suppression effect of 69.54%. Within the first 72 hours of the process, the radial growth rate of the treated cultures was four times lower in both variants of *Ps. chlororaphis* 1S4 – 24 h and 48 h.

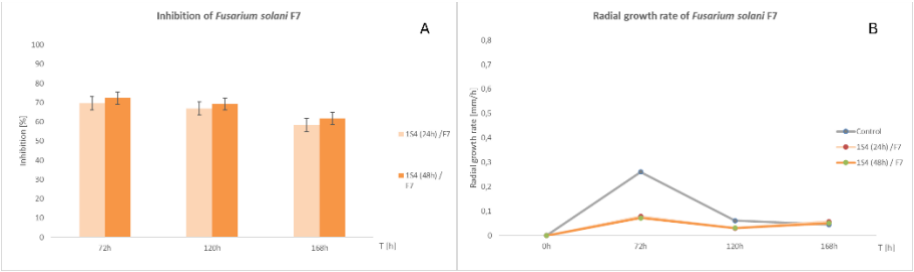


Figure 25. Inhibition (%) of *Fusarium solani* F7 growth by *Pseudomonas chlororaphis* 1S4 (A) and radial growth rate dynamics of *Fusarium solani* F7 (B).

Ps. yamanorum 1046 retained its tendency for better inhibitory activity in the younger culture. The maximum inhibition of *F. solani* F7 was observed with the variant *Ps. yamanorum* 1046 (24 h) – 41.43%. The radial growth rate was not significantly different from the control variant, with values relative to the control being 35% lower than the maximal inhibited variant.

When screening for the presence of antifungal activity of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046, higher inhibition values were reported for *Ps. Chlororaphis* 1S4. The maximum inhibition values of *F. proliferatum* C1 were 80.85%, F. *oxysporum* F6 – 85.92%, and *F. solani* F7 – 72.31%. The difference between the cultures *Ps. chlororaphis* 1S4 (24 h) and *Ps. chlororaphis* 1S4 (48 h) are practically insignificant in the context of their biotechnological application.

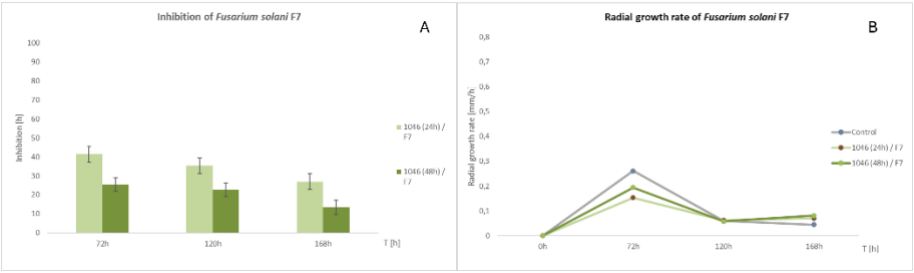
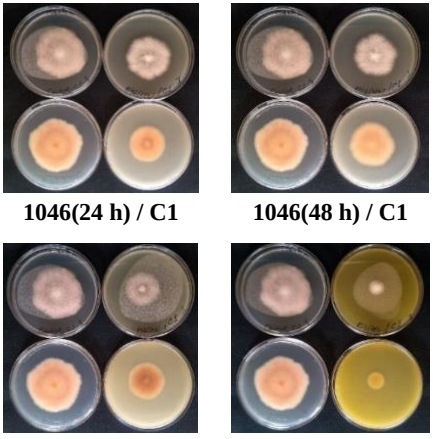


Figure 26. Inhibition (%) of *Fusarium solani* F7 growth by *Pseudomonas yamanorum* 1046 (A) and radial growth rate dynamics of *Fusarium solani* F7 (B).

A significant difference in the development rate of the phytopathogenic representatives was reported in the test and control variants treated with *Ps. chlororaphis* 1S4. It reaches up to 10 times slower development in strain *F. oxysporum* F6 and varies between 4 and 7 times in the other two fungal representatives. This strain demonstrated potential for application as a biocontrol agent with a fungicidal effect. *Ps. yamanorum* 1046 showed weaker antifungal activity, reaching a maximum value of 41.43% in *the F. solani* F7. The radial growth rate was little affected in all test variants. In *Ps. yamanorum* 1046, a clearly pronounced dependence of higher efficiency was observed in the 24-hour hourly culture, which is the economically more appropriate option for treatment. An illustration of the dependencies discussed above can also be seen in Fig. 27.



1046(24 h) / C1 **1046(48 h) / C1**

1S4(24 h) / C1 **1S4(48 h) / C1**

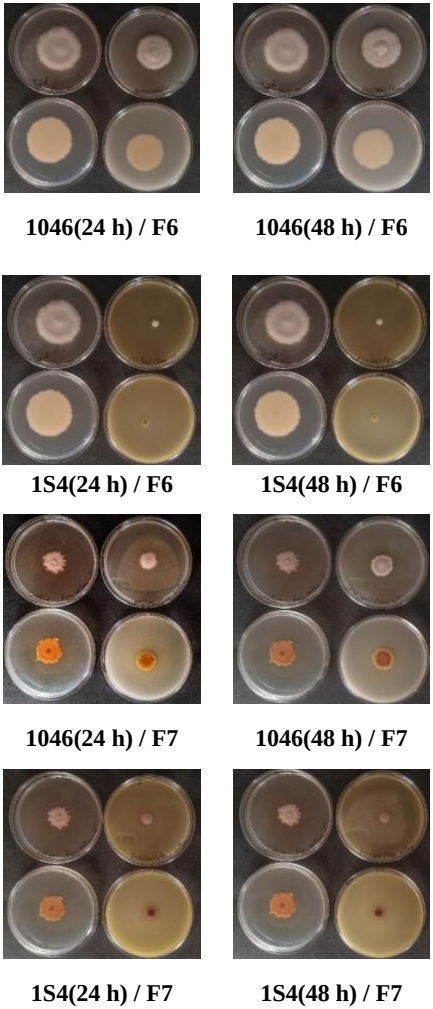
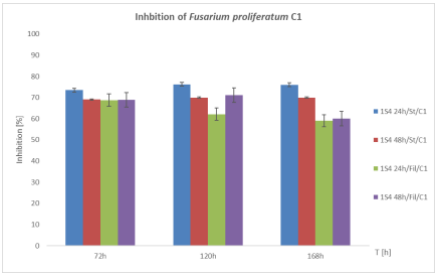


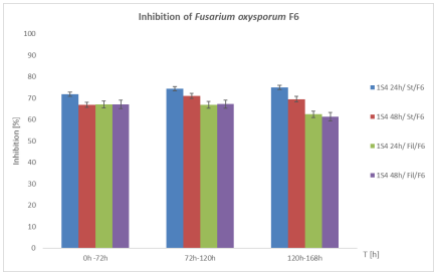
Figure 27. Photographic visualization of the antifungal activity of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 against plant pathogens of g. *Fusarium*.

The clear inhibitory effect against the representatives of the g. *Fusarium* underlines the potential of *Ps. chlororaphis* 1S4 to be integrated into sustainable agricultural practices.

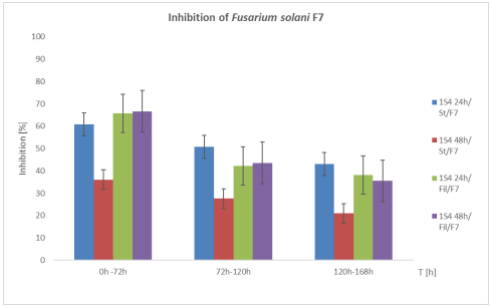
Since PCA and siderophores are exocellular metabolites, the two variants of culture fluid – 24 h and 48 h – were subjected to processes to eliminate the bacterial biomass - filtration/sterilization of culture fluid. The cultures treated in this way were tested for fungicidal effect against representatives of the genus *Fusarium*. The results of the experiment are presented in Fig. 28



A



B



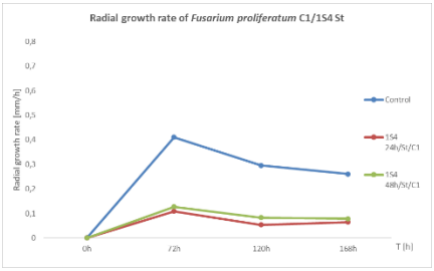
C

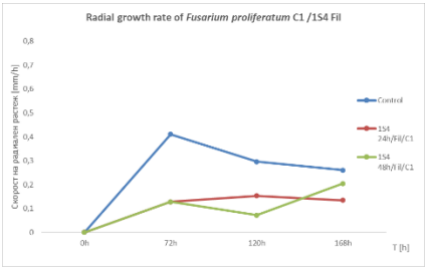
Figure 28. Inhibitory effect of *Pseudomonas chlororaphis* 1S4 – sterilized (St) and filtered (Fil) culture against: A - *Fusarium proliferatum* C1, B - *Fusarium oxysporum* F6, and C - *Fusarium solani* F7. The inhibitory effect was calculated relative to the growth of an untreated control.

Inhibition of the growth of *Fusarium proliferatum* C1 was maintained using the filtrate from the culture liquid with an efficiency in the range of 60.06 – 71.25%. In the experiments with *Fusarium oxysporum* F6, the maximum inhibitory effect was achieved with a 48-h culture broth filtrate - 67.18%. The growth of *Fusarium* solani F7 was inhibited most effectively by the 48-h filtrate of *Ps. chlororaphis* 1S4 (with 66.62%). In the first 72 hours of the process, the radial growth rate was three times lower than the control.

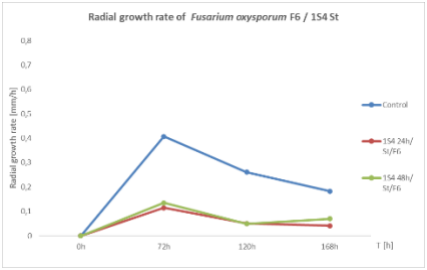
When applying sterilized culture *Ps. chlororaphis*1S4 against *F. proliferatum* C1, an inhibitory effect was achieved in the range of 69.05% - 76.25%, with the highest value reported

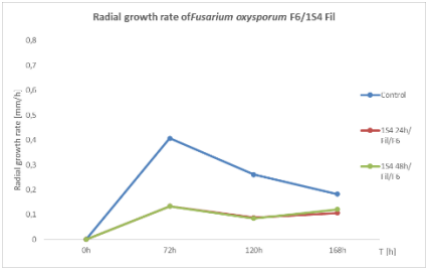
for the variant *Ps. chlororaphis* 1S4 (24 h) St. The radial growth rate was 4-fold lower than the control, results that were comparable to those of the unsterilized culture fluid of *Ps. chlororaphis* 1S4. An interesting phenomenon was observed with this fungal species – at 24 h culture of *Ps. chlororaphis* 1S4 inhibition of the fungal pathogen increased after treatment that included removal of cell biomass: in *Ps. chlororaphis*1S4 (24 h) – 56.31%, *Ps. chlororaphis* 1S4 (24 h) Fil – 68.81% and *Ps. chlororaphis* 1S4 (24 h) St – 76.25%. In *F. oxysporum* F6, the maximum inhibitory effect was reported for the variant *Ps. chlororaphis* 1S4 (24 h) St – 74.97%, varying between 66.86% - 74.97%. The antifungal activity of *Ps. chlororaphis* 1S4 against *F. solani* strain F7 was also retained. A maximal inhibitory effect was observed at 24 h sterilized culture, achieving 60.80% suppression. The radial growth rate of *F. solani* F7 was little affected, with a 37% reduction compared to the control.



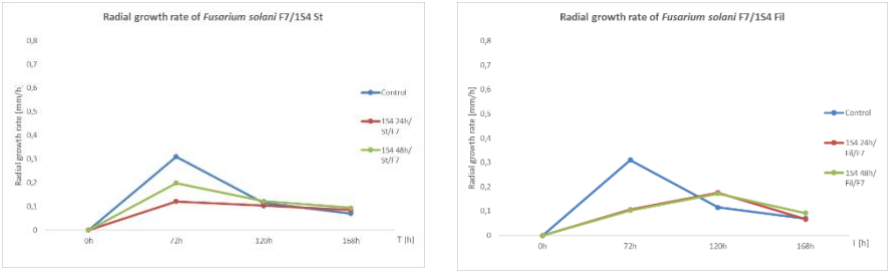


A





B



C

Figure 29. Radial growth rate of A - *Fusarium proliferatum* C1, B - *Fusarium oxysporum* F6, and C - *Fusarium solani* F7 treated with 24 h and 48 h *Pseudomonas chlororaphis* 1S4 filtrated (Fil) and sterilized cultures (St).

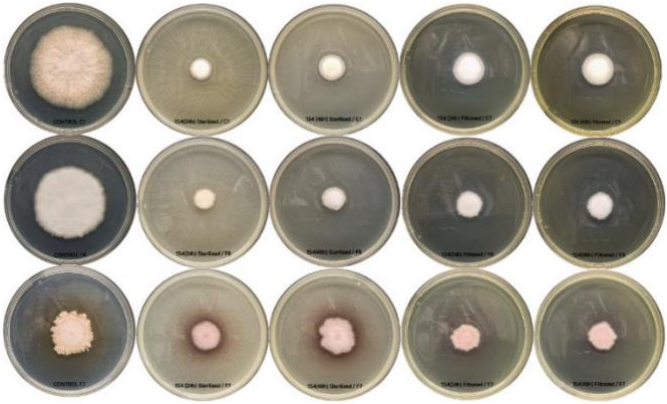


Figure 30. Visual presentation of the inhibition effect of *Pseudomonas chlororaphis* 1S4 - sterilized culture and filtered culture broth on A - *Fusarium proliferatum* C1, B - *Fusarium oxysporum* F6, and C - *Fusarium solani* F7.

Table 5 shows the correlation between the age variables of the bacterial cultures and the growth inhibition of the *Fusarium* fungi treated with different variants of the bacterial cultures.

Table 5. Correlation coefficients demonstrating the relationship between the age of the *Pseudomonas chlororaphis* 1S4 culture and the growth inhibition of plant pathogens of the g. *Fusarium*.

Age of bacterial culture / Inhibition of fungal strains	24 h			48 h		
	Fresh culture	Sterilized culture	Filtered culture	Fresh culture	Sterilized culture	Filtered culture
F. proliferatum C1	0,832306	0,855726	0,804308	0,83894	0,853242	0,795369
F. oxysporum F6	0,750935	0,705175	0,630442	0,760982	0,658245	0,600843
F. solani F7	0,749637	0,851647	0,761761	0,773195	0,842985	0,770122

Correlation coefficients in the range corresponding from strong to very strong correlation were reported, which prove the relationship between the age of the culture, the type of its processing, and its effect on inhibiting fungal growth.

2.5. Optimization of the strains' preservation and stability of their bioactive fermentation products during lyophilization and spray drying

Lyophilization

To carry out the lyophilization process *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 were batch cultivated for 24 hours in a fermentation medium optimal for the strains with added cryoprotectant 10% dry milk. The efficiency of the process was monitored according to the criterion: preservation of bacterial viability. The results of the lyophilization process of *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 are presented in Table. 6.

During lyophilization of the *Pseudomonas* strains, a decrease in the number of viable cells was observed in both samples. The *Ps. chlororaphis* 1S4 activity decreases only by one and a half orders of magnitude, and in *Ps. yamanorum*1046, with two orders.

Spray drying

The tested strains demonstrated a high degree of survival after the spray drying process, which was within two orders of magnitude lower than the CFU before the drying process (Table 7). These data show a high efficiency of this process as an approach to obtain powdered bioproducts.

Table 6. Results of the lyophilization process of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046

Strain	Culture broth [ml]	Lyophilizate mass [g]	Dry weight [%]	CFU before lyophilization	CFU after lyophilization
1S4	320 ml	11,97 g	91,61%	1*10^9	6,4*10^7
1046	320 ml	11,80 g	92,04 %	3,2*10^8	1,5*10^6

Table 7. Results of the spray drying process of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046.

Strain	Culture broth [L]	Dry product mass [kg]	Dry weight [%]	CFU before drying	CFU after drying
1S4	200 L	7,8 kg	89,24%	4*10^9	7*10^7
1046	200 L	6,8 kg	87,14%	2*10^8	4*10^6

3. Study of the plant-microbe symbiosis of the selected strains with model plant systems of cereals and ornamental plants in different growing phases

To evaluate the PGP potential of the studied bacterial strains, tests were carried out with model plant systems in different vegetation phases.

3.1. Germination of cereal seeds

Germination of maize (*Zea mays*) seeds in coils

Experiments were carried out with a model system corn (*Zea mays*) seeds (Dekalb – DKC 5830 HD (Hybrid 101)) treated with bacterial cultures of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4.

The assessment of the PGP effect was performed based on the plant biometric indicators, the vigor index, and the degree of interaction presented by correlation analysis (Figs. 31 - 35).

Root length: the values of the growth parameter “root length” are presented in Fig. 31. They visualize the positive effect of bacterial cultures on plant physiology. This effect was

best represented in *Ps. yamanorum* 1046 when treated with a 0.2% solution. An increase of 22.87% was observed for 16 h culture and 28.33% for 48 h.

The highest values for this indicator in *Ps. chlororaphis* 1S4 were observed in the variants treated with 0.1% bacterial suspension (the increase was 17.70% for 16 h culture and 4.44% for 48 h one).

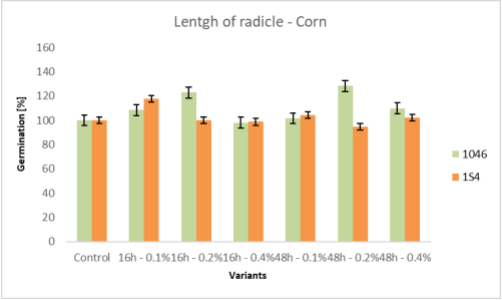


Figure 31: PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on maize seed germination as assessed by root length index.

Coleoptile length: the data showed that in *Ps. yamanorum* 1046 the three tested concentrations demonstrated a positive effect on its growth (Fig. 47). The values indicated an increase in this growth indicator between 17.4% and 49.6%. The trend for the best stimulatory effect of 0.2% concentration observed for root length was also observed for this parameter. An increase of 35.96% was found for the 0.2% and 49.56% for the 0.4% suspension. In *Ps. chlororaphis* 1S4 maximum effect was observed at 0.1% concentration of 16 h suspension – 33.72%, a result that was also observed for the root length indicator.

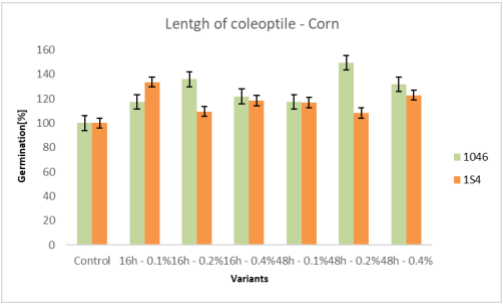


Figure 32: PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on maize seed germination as assessed by coleoptile length index.

Number of lateral roots: the third index used to evaluate the germination of maize seeds showed a relatively lower stimulatory effect 2.78% - 18.60%, when treated with *Ps. yamanorum* 1046. In *Ps. chlororaphis* 1S4 the highest number of lateral roots was reported in the variant 16 h - 0.1% - 20.45% more compared to the control variant.

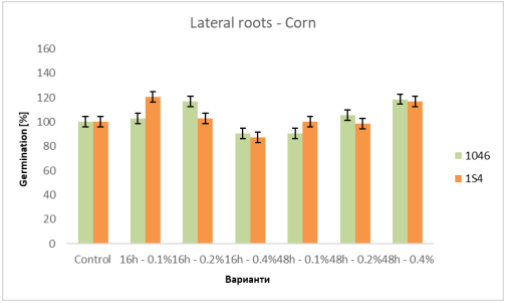


Figure 33. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on maize seed germination as assessed by the number of lateral roots.

Vigor index: The highest values for the vigor index in *Ps. chlororaphis* 1S4 were for 16 h variants at a concentration of 0.1% - 1584.58 and for the 48 h, at a concentration of 0.4% - 1138.02.

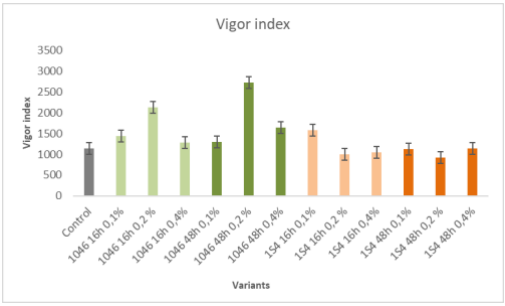


Figure 34: PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on maize seed germination as assessed by the vigor index.

Correlation analysis: Quantitatively, the correlation coefficient can be assessed as very strong, as its values fall within the range of 0.9 – 1.0 for 16 h cultures, and from 0.4 to 1.0 for the 48 h cultures (Table 8).

Table 8. Data for the correlation analysis of 16 h and 48 h cultures of the bacterial strains in a corn model system.

Correlation	Radicle	Coleoptile	Lateral roots	Vigor index
1046 16h	0,98927	0,99982	0,95572	0,975104
1046 48h	0,89168	0,99194	0,36147	0,907166
1S4 16h	0,78429	0,77401	0,79312	0,736771
1S4 48h	0,60912	0,65450	0,34091	0,543061

Germination of soybean (*Glycine max* L.) seeds in coils

Soybean (*Glycine max* L.) seeds were used as a second plant model system. The plant biometrics for assessing seed germination monitored in the process were root length, epicotyl length, and hypocotyl length, all reported as a percentage of the untreated control. A vigor index was also calculated.

Root length: A positive effect of both strains was observed in all treatment options. In *Ps. chlororaphis* 1S4, an increase of this indicator between 31.20% - 50.34% was observed, with the highest effect at a concentration of 0.4% for 48 h culture. In *Ps. yamanorum* 1046, a positive effect was also observed in all treatment variants. This effect varies between 20.97% and 47.23%. The best effect of 16 h culture was observed at 0.2% concentration – 47.23% increase in root length.

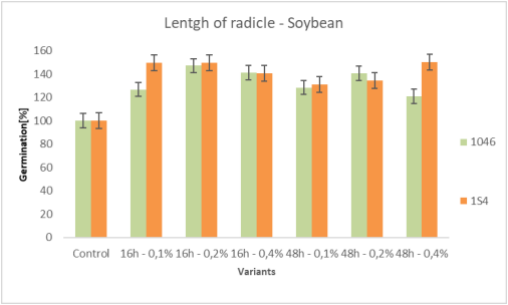


Figure 35: PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 strains on soybean seed germination as assessed by root length index.

Epicotyl length: *Ps. chlororaphis* 1S4 has an effect in the range of 24.69% - 45.54%. The best result was observed in the variant – 0.4%, 48 h – 45.54%. *Ps. yamanorum* 1046 showed the best effect for the variant 0.2%, 16 h – 41.90%.

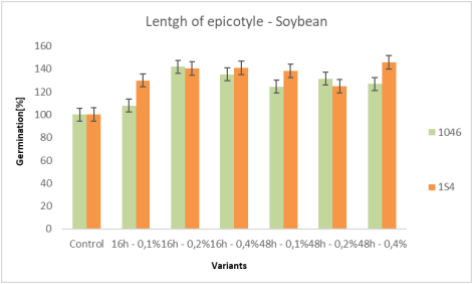


Figure 36. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on soybean seed germination as assessed by epicotyl length index.

Hypocotyl length: the third metric was the hypocotyl length. When treated with *Ps. chlororaphis* 1S4, the best effect was observed at 0.4% for 48 h – 48.64%. When treated with *Ps. yamanorum* 1046, the best results were recorded at a concentration of 0.2%, 16h – 45.34%.

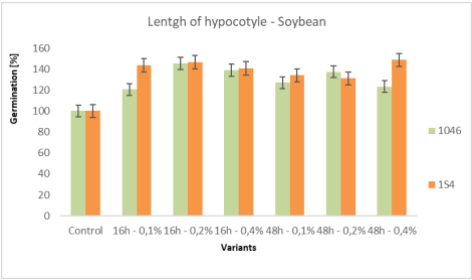


Figure 37. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on soybean seed germination as assessed by hypocotyl length index.

Vigor index: The highest value of the index in *Ps. chlororaphis* 1S4 was registered for the variant 0.4%, 48 h – 3838.88, followed by the variant 0.2%, 16h – 3533.73 at a control index of 1013. For *Ps. yamanorum* 1046, the maximum value of this indicator was observed for the variant 0.2%, 16 h – 3385.89, followed by 0.4%, 16 h – 2730.08.

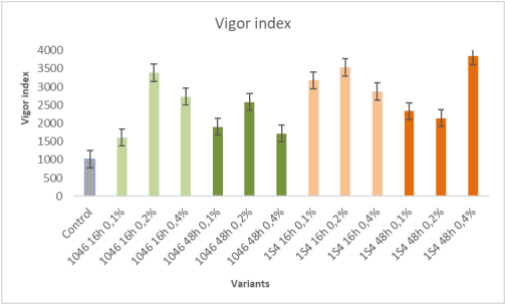


Figure 38. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on soybean seed germination as assessed by the vigor index.

Correlation analysis: based on the data for the biometric indicators of the culture, a correlation analysis was conducted. The results presented in Table 9 prove the positive relationship between the two microorganisms, the growth parameters of the plant root system, and the vigor index. In a quantitative aspect, the correlation coefficient can be assessed as very strong, as its values fall within the range of 0.86 – 0.99 for the 16 h cultures and from 0.63 to 0.99 for the 48 h cultures.

Table 9. Data from the correlation analysis for the effects of the two bacterial strains in a soybean model system.

Correlation / Strain	Radicle	Epicotyl	Hypocotyl	Vigor index
1046 16h	0,99716	0,94062	0,99859	0,96090
1046 48h	0,97399	0,95186	0,96877	0,99714
1S4 16h	0,86691	0,96391	0,89292	0,92395
1S4 48h	0,90701	0,63465	0,82493	0,79099

Germination of wheat in agar

As a third plant model system, wheat seeds (*Triticum aestivum*) were used, placed in an agar medium (Maeda *et al.*, 2021), and treated with solutions of the two bacterial strains at 0.1%, 0.2%, and 0.4%. Seeds soaked in water were used. The results of the experiment are shown in Figs. 39 – 42.

In the treatment with *Ps. yamanorum* 1046, maximum values for the root length parameter were achieved with the variant 16 h, 0.2% - 46%, exceeding the control variant. As

for the number of the lateral roots, a significant increase was observed with the same treatment variant - 250%. The third indicator, the aerial part of the plant, indicated that maximal size was registered with *Ps. yamanorum* 1046 at a concentration of 0.2%, 16 h culture – 15% over the control.

The *Ps. chlororaphis* 1S4 results also demonstrated its positive effect on wheat seed germination. The first indicator - average root length reaches a maximum value when treated with 0.2%, 16 h - 39% over the control. The maximum result for the number of lateral roots was reported in the variant– 0.4%, 16 h - 313%. The difference in the values between the higher and the lower concentrations is 13%, which does not economically justify the application of the higher concentration of the product. In the third indicator - aerial part, no significant differences were observed compared to the control variant, and the highest value of this indicator was recorded for 0.2%, 16 h - 13% over the control.

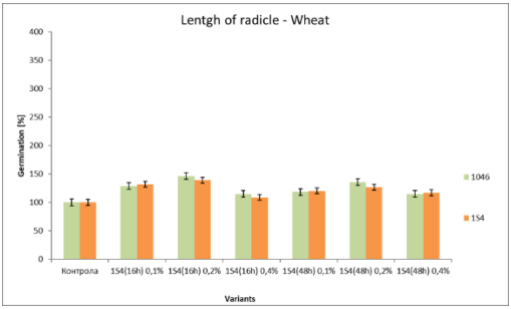


Figure 39. PGP - effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 strains on wheat seed germination as assessed by root length index.

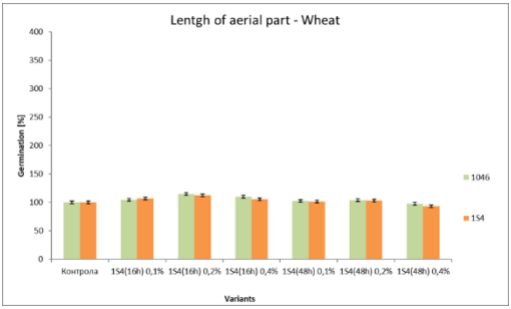


Figure 40. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on wheat seed germination as assessed by aerial part index.

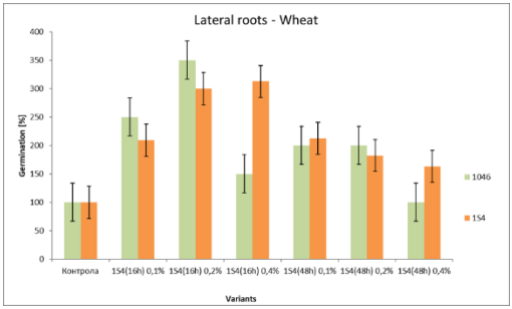


Figure 41. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on wheat seed germination as assessed by the number of lateral roots.

The next indicator is the vigor index, which confirms the positive effect of the studied microorganisms on the model system (Fig. 42). The highest value of the index for *Ps. chlororaphis* 1S4 was registered for the variant 0.2%, 16h – 1503.12, compared with a control index 1090. *Ps. yamanorum* 1046 reached the maximum value of this indicator in the variant 0.2%, 16 h – 1662.59.

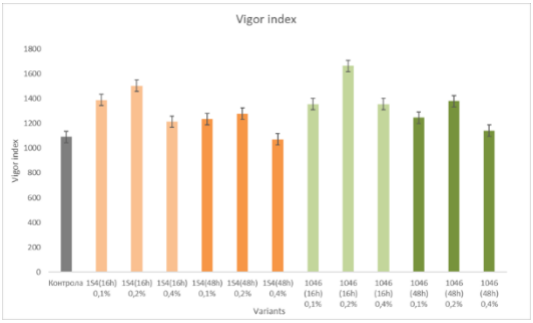


Figure 42. PGP effect of *Pseudomonas yamanorum* 1046 and *Pseudomonas chlororaphis* 1S4 on wheat seed germination as assessed by vigor index.

The correlation analysis performed shows a very high degree of correlation with values between 0.9 and 1.0 (Table 10).

Table 10. Data of the correlation analysis for the effects of the two strains in a wheat model system.

Correlation	Radicle	Lateral roots	Aerial part	Vigor index
1046 16h	0,98885	0,99339	0,98198	0,99913
1046 48h	1	0,86603	0,96077	0,99887
1S4 16h	0,93783	0,99865	0,99902	0,96887
1S4 48h	0,96347	0,70898	0,98198	0,95439

Based on the seed germination experiments in different substrates, the biometric indicators, and the correlation analyses, it can be concluded that the most suitable for use are the variants of 16 h cultures for both strains with an optimal concentration for treatment – 0.02%.

3.2. Evaluation of plant-microbe symbiosis with potted ornamental plants

Common primrose (*Primula vulgaris*) experiment

Fresh 16 h cultures of *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 were used. Plant treatments started in the seedling phase (5-6 leaves) and were repeated every 20 days until the end of the vegetation. The following treatment options have been investigated:

- Control – treated with mineral nutrition solution (NPK);
- 1046 – 0.2% - watering;
- 1046 – 0.2% - foliar spraying;
- 1046 – 0.1% - watering + leaf spraying;
- 1046 – 0.2% - watering + leaf spraying;
- 1S4 – 0.2% - watering;
- 1S4 – 0.2% - foliar spraying;
- 1S4 – 0.1% - watering + leaf spraying;
- 1S4 – 0.2% - watering + leaf spraying;

The following plant biometric parameters were measured to determine the effect of the studied microorganisms - plant diameter, crown circumference, flower weight, green mass weight, and fresh/dry plant root weight.

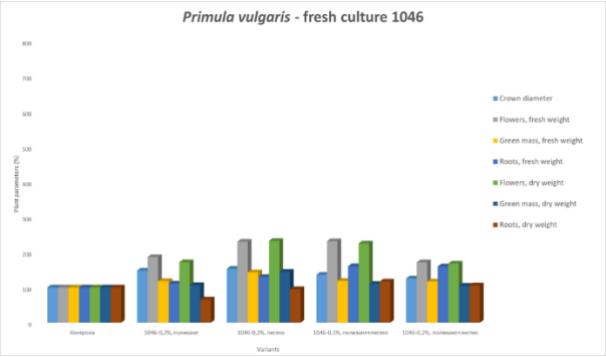


Figure 43. Treatment of *Primula vulgaris* with a fresh culture of *Pseudomonas yamanorum* 1046.

The following results were obtained: the highest values for the crown circumference were measured in the variant treated foliarly with 0.2% *Ps. yamanorum* 1046 – 53%. Regarding the flower weight, the heaviest were those in the *Ps. yamanorum* 1046 variant 0.1% - irrigation + foliar treatment –32% over the control. The highest weight of the green parts was recorded in the plants treated foliarly with a 0.2% solution - 43%, and the highest weight of the roots was recorded in the combined treatment - watering and spraying, with a 1% difference between the two concentrations. The maximum increase of this indicator is 60%.

All these results show the influence of *Pseudomonas yamanorum* 1046 on the plant phyllosphere and its potential for application as a foliar fertilizer.

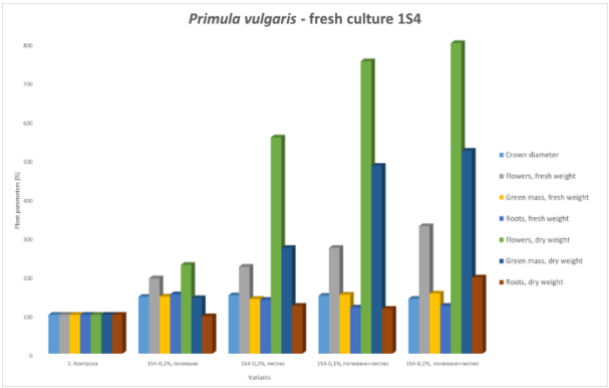


Figure 44. Treatment of *Primula vulgaris* with a fresh culture of *Pseudomonas chlororaphis* 1S4.

The *Pseudomonas chlororaphis* 1S4 effect was tested under the same experimental conditions. In the first indicator, the crown circumference, there was no significant difference between the different variants. Substantial variation compared to the control was observed in the fresh flowers' weight, which in the variant *Ps. chlororaphis* 1S4 – 0.2% - watering + foliar spray, reached a weight of 228% over the control. The weight of the green mass was the highest in plants from the 0.2% watering + spraying option – 54%. A maximum root weight of 52% over the control was observed in the irrigation-only treatment.

Chrysanthemums (*Chrysanthemum indicum*) experiment

Fresh 16 h cultures of *Ps. chlororaphis* 1S4 and *Ps.* yamanorum 1046 were used for treatment of *Chrysanthemum indicum* seedling that was repeated every 20 days until the end of the vegetation.

To account for the effect of the studied strains, biometric indicators suitable for the plants and the process were tracked - number of branches, plant circumference, number and mass of the flowers, number and mass of the buds, weight of the plant green mass, weight of the dried green mass, and weight of fresh and dry roots. The results are presented in Figs. 45 – Fig. 47.

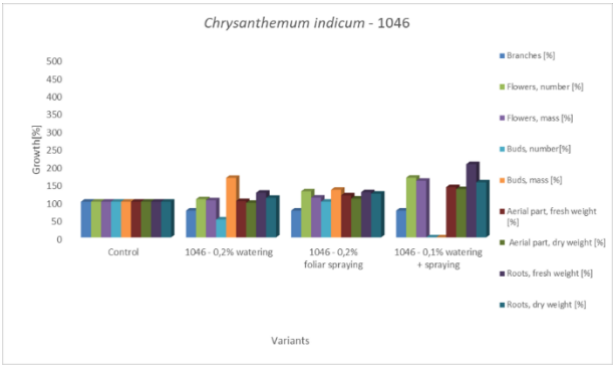


Figure 45. Treatment of *Chrysanthemum indicum* with *Pseudomonas yamanorum* 1046.

In *Chrysanthemum indicum* plants, *Ps. yamanorum* 1046, in the variant of watering with 0.2% solution of culture broth, higher buds' mass and roots' weight were observed. These phenomena are due to the phytohormones produced by the microorganism.

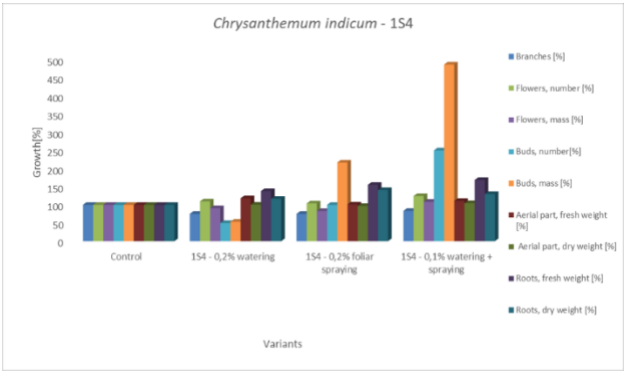


Figure 46. Treatment of *Chrysanthemum indicum* with *Pseudomonas chlororaphis* 1S4.

In *Ps. chlororaphis* 1S4, the combination of watering and spraying with a 0.1% solution again stands out - the number of buds was 140%, and their mass - was nearly 400% higher than the control. This indicator is essential for flowering ornamental plants because it determines their commercial appearance, an important factor in this type of production.

Before treatment	After first treatment	After second treatment
		
Control		
		
Pseudomonas yamanorum 1046 0,2% - watering		

Pseudomonas yamanorum 1046 0,2% - foliar spraying

Pseudomonas yamanorum 1046 0,1% - combination of watering and spraying

Pseudomonas chlororaphis 1S4 0,2% - watering

Pseudomonas chlororaphis 1S4 0,2% - foliar spraying

Pseudomonas chlororaphis 1S4 0,1% - combination of watering and spraying

Figure 47. Photographies of *Chrysanthemum indicum* plants treated with *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046.

Cyclamen (Wadiv cyclamen) experiment

In the cyclamen (*Wadiv cyclamen*) model system, a variant with a combination of the two strains at a concentration of 0.2% in soil treatment, was tested. The plants treatment started in the seedling phase, watering with the test solutions was carried out during 14 days until the end of the plant's vegetation. The parameters plant diameter, number and weight of the flowers, number and weight of the buds, average weight of 3 leaves, weight of the aerial part, the tuber diameter, and the weight of the dry aerial part were monitored to follow the combinatory effect of the two strains. The experimental results are presented in Figs. 66 – 67.

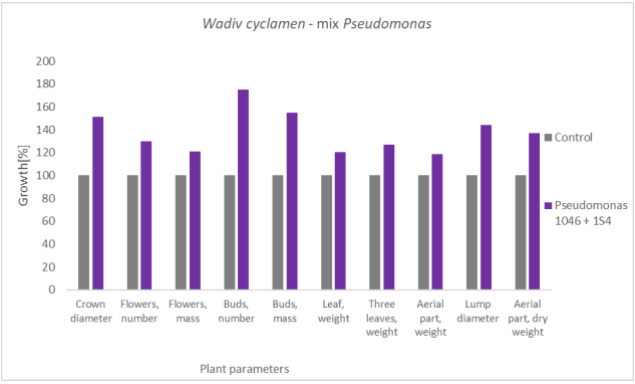


Figure 66. Treatment of *Wadiv cyclamen* with a combination of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046.

The combination of the two strains demonstrated a positive effect on all monitored plant biometrics. The crown diameter of the plant was 51% larger in the treated variants. The number of colors and their weight are also increased, by 30% and 21% respectively. The number of buds increases by 75% and their weight by 55%. Average leaf weight was also increased by 26%. Tuber diameter was 45% over the control, and total dry biomass was 37% heavier. All these indicators and their positive values indicate the overall better development of the treated plants and the ability of the two strains to work as a team.

Figure 49. Photography of the *Wadiv cyclamen*, treated with a combination of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046.

Primrose (*Primula vulgaris*) and dried cultures of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 strains experiment

The PGP effect of the dried powdered forms of the studied microorganisms was tested on *Primula vulgaris* - primrose. The treatment variants included fresh and dried powdered cultures to compare the outcome between the two forms.

Solutions of the cultures were prepared by rehydrating the powder form to the initial volume (stock solution), from which the necessary concentrations - 0.1% and 0.2% were prepared. Plant treatment began at the seedling phase - 5-6 leaves and was carried out every 20 days until the end of the plants' vegetation.

The biometric parameters of the plants were monitored – the plant diameter, circumference, weight of the fresh and dry green mass, and weight of the roots - fresh and dried to evaluate the effect of treatment with the dry variants of the studied strains. The results are presented in Figs. 50 and 51.

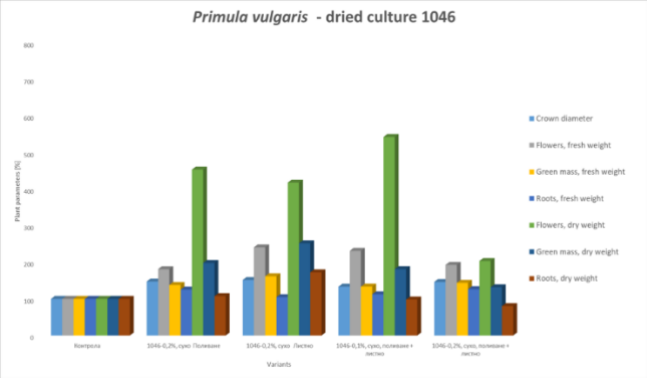


Figure 50. Treatment of *Primula vulgaris* with a preparation of *Pseudomonas yamanorum* 1046 in a dry form.

When treating the test subjects with the dry form of *Ps. yamanorum* 1046, positive effects were observed with the 0.1% watering + foliar treatment. The dry flower weight was 450% greater compared to the control variant.

When treated with the dry form of *Pseudomonas chlororaphis* 1S4, maximal results were achieved at 0.2% - *Ps. chlororaphis* 1S4 dry - watering + foliar treatment - over 300% increase in the weight of the flowers in their fresh form, and over 700% in the dried flowers.

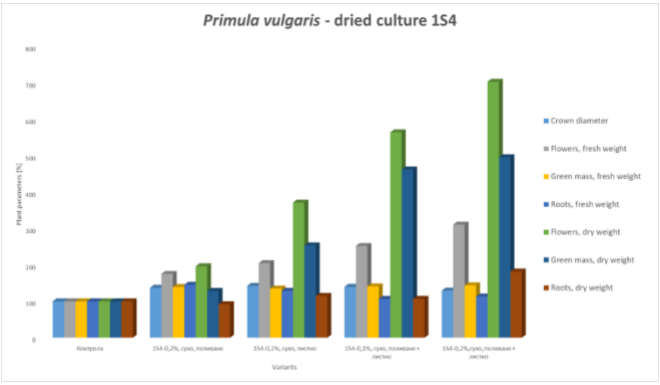


Figure 51. Treatment of *Primula vulgaris* with *Pseudomonas chlororaphis* 1S4 preparation in a dry form.

All these plant indicators demonstrate the PGP effect of the investigated microorganisms after undergoing a spray drying.

CONCLUSIONS

The following conclusions were formulated as a result of the conducted experiments and obtained results:

1. Five strains belonging to g. *Pseudomonas* were isolated and taxonomically identified. Based on their physiological and biochemical characteristics (synthesis of phenazine pigments, proteolytic and antioxidant activity) as indicators of their PGP potential, the strains *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 were selected for further work.

2. A nutrient medium, with optimal for the two strains N, C, and P concentrations was established for carrying out a highly productive process for the target product IAA (in the range of 4.0 – 6.6 µg/ml). In process scaling up, IOC's output of *Ps. chlororaphis* 1S4 reached 27.9 µg/ml at 28 h of the process.

3. The influence of the inducer synthetic L-tryptophan and tryptophan of microbial origin on the biosynthesis of IAA by *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 was investigated. The inducer L-tryptophan of microbial origin was 34% more effective than the synthetic variant.

4. The production of phenazine-1-carboxylic acid (PCA) by *Pseudomonas chlororaphis* 1S4 and the production of siderophores by the two studied strains were determined.

5. The antagonistic effect of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 against economically significant pathogens of g. *Fusarium* (*F. proliferatum* C1, *F. oxysporum* F6, and *F. solani* F7) has been proven. Maximal inhibition effect for *Ps. chlororaphis* 1S4 was reported against *F. oxysporum* F6 - 85.92%, and for *Ps. yamanorum* 1046 - 41.43% against *F. solani* F7.

6. The biological activity of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 in the germination of cereal seeds was proven and evaluated by the biometric indicators of the root system of the model plants. The highest values for the both strains were recorded in the treatment regimen of 0.2%, 16 h.

7. The analysis of the plant-microbial symbiosis of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum*1046 and potted ornamental plants proves the positive effect of fresh and dried cultures (individually and in combination) of the microbial component applied to different vegetative parts of the plant models (roots and leaves) through various approaches (watering and foliar spraying).

8. The most effecti option in terms of concentration and form of application for the different model systems was determined - 0.2% foliar spray for the primrose model, 0.1% foliar spray + watering for the chrysanthemum model, and a combination of the two microbial representatives applied by watering, for the cyclamen model.

CONTRIBUTIONS

Original contribution:

- The PGP profile of the rhizosphere strains *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 was proven, a prerequisite for their practical application in agricultural practices.
- An economically profitable approach was applied to replace the inducer synthetic L-tryptophan with a tryptophan of microbial origin. A synergistic effect was observed regarding the induction of IAA production between the microbial tryptophan and the other biocomponents in the complex mixture applied.
- A pronounced inhibitory effect of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 was demonstrated about the mycopathogenic species belonging to the g. *Fusarium*, and their possible use as components of biocontrol preparations.
- A prototype series of fresh and dry cultures of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum*1046 was constructed with the potential to be applied as an environmentally sound alternative to conventional fertilizers.

Confirmatory contributions

- A scheme was aprobated for tracking plant-microbial symbiosis between bacterial representatives and model plant systems of cereals and ornamental plants. Indicators have been introduced to track the process regarding:
    - **The microbial component**: age, form, concentration, and single/joint application;
    - **The plant component**: efficiency of seed germination and change of biometric indicators of different vegetative parts of the plant models during different vegetation stages;
    - **The application technology**: foliar spraying, watering, and their combination.

PUBLICATIONS

1. **Georgieva G.**, Nedeva T., Badalova M., Deleva V., Savov V., (2023). Study of the plant growth-promoting capacity of *Pseudomonas* putida 1046 in a model plant system. In: Chankova S, Danova K, Beltcheva M, Radeva G, Petrova V, Vassilev K (Eds) Actual problems of Ecology. BioRisk 20: 115–128. https://doi.org/10.3897/biorisk.20.97581
2. **Georgieva G.**, Badalova M., Feyzula *F.*, Nedeva T., (2024). Antifungal Activity of *Pseudomonas chlororaphis* 1S4. *(Accepted)*
3. **Georgieva G.**, Badalova M., Nedeva T., (2024). Induction of Two *Pseudomonas* Strains Indole Acetic Acid Production By Synthetic And Biologically-Produced L-Tryptophane: A Comparative Study. *(Accepted)*

PARTICIPATION IN SCIENTIFIC FORUMS

1. Posters:
   - **Georgieva G**., Nedeva T., Badalova M., Deleva V., Savov V. Study of the plant growth-promoting capacity of *Pseudomonas putida* 1046 in a model plant system. INTERNATIONAL SEMINAR OF ECOLOGY- 2022. (29/09 2022)
   - **Georgieva G**., Badalova M., Nedeva T. Examination of the antifungal activity of *Pseudomonas chlororaphis* 1S4. INTERNATIONAL CONFERENCE KLIMENT'S DAYS 2023. (9/11 2023)
   - **Georgieva G**., Hristova P., Nedeva T. Evaluating the biochemical properties of *Pseudomonas* strains to explore their potential. Youth Scientific Conference "60 years Department of General and Industrial Microbiology". (17/11 2023)
   - **Georgieva G**., Badalova M., Nedeva T. PGP impact of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 on technical and ornamental plants. INTERNATIONAL SEMINAR OF ECOLOGY- 2024. (26/09 2024) *(Accepted)*

NOTED CITATIONS

Article:

1. **Georgieva G.**, Nedeva T., Badalova M., Deleva V., Savov V., (2023). Study of the plant growth promoting capacity of *Pseudomonas putida* 1046 in a model plant system. In: Chankova S, Danova K, Beltcheva M, Radeva G, Petrova V, Vassilev K (Eds) Actual problems of Ecology. BioRisk 20: 115–128. https://doi.org/10.3897/biorisk.20.97581

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1. Ghosoun Habib Sabri Al-Rubaie and Saba Abd Al-Hadi Kadhim Al-Fallooji. Chemical and Molecular Detection of New Strains of Xanthomonas Campestris pv. Vesicatoria and *Pseudomonas* putida Associated with Tomato Crop 2023 *IOP ConF. Ser.: Earth Environ. Sci.* 1262 032031 DOI 10.1088/1755-1315/1262/3/032031
2. Maity P. Enhancement of rice seed vigor to mitigate cold stress by using indigenous microbes. M.Sc. Thesis, 2023 https://krishikosh.egranth.ac.in/server/api/core/bitstreams/300b269d-2b32-4a98-8c6b-6f842450fdd1/content