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Review

Pseudomonas spp. in biological plant protection and growth promotion

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Abstract: Nowadays in worldwide agriculture, sustainable strategies are implemented to reduce negative effects on ecosystems created by conventional practice, mainly environmental pollution caused by intensive use of fertilizers and chemical plant protection products. Bacteria from the genus *Pseudomonas* can be considered biocontrol and plant growth-promoting agents due to their various plant beneficial traits e.g., siderophores production, phytohormones synthesis, antagonism against phytopathogenic fungi. This is a reason for increasing researchers' interest in improving of existing or elaborating new technologies that enable the effective application of these bacteria in agriculture. Pseudomonads are non-sporulating bacteria and it is a major constraint for creating bioformulation for commercial use with a sufficiently high stable number of viable cells during shelf-life. Therefore, scientists are making efforts to improve techniques of bioformulations to enable large-scale production and use of pseudomonads under field conditions. The aim of this review is to describe traits of *Pseudomonas* spp. which are useful in plant protection and growth-promotion and to highlight examined techniques for preparing bioformulations containing pseudomonads with sufficiently long shelf life.

Keywords: *Pseudomonas*; plant growth promotion; biocontrol; bioformulation

1. Introduction

Using chemical fertilizers and pesticides is common practice in agriculture to protect crops against pathogens and improve the quality and quantity of yield or prevent postharvest losses caused by pests [1]. Currently, approximately 890 synthetic chemical substances are approved to use as

pesticides and the number of products based on these substances is estimated to be 21 thousand [2]. Unfortunately, pesticides can cause detrimental effects on both human health and the environment [3,4]. At the same time, human population growth is a factor that forces to improve yields of crops to meet the nutritional requirements of people [4,5]. Therefore, there is a need to elaborate novel, environmentally and health-friendly methods of protecting crops against pathogens. One of the solutions which can be implemented is using beneficial microorganisms which gained popularity in agriculture and horticulture during the last decades. Integrated usage of chemical and biological agents can contribute to reducing the degradation of the environment and therefore improve sustainability in agriculture [6]. Microorganisms that are being applied in biological plant protection are filamentous fungi, yeasts, bacteria and viruses [7]. Within bacteria—the genus *Pseudomonas* deserves attention because of its numerous plant growth-promoting traits [8].

2. Characteristics of genus *Pseudomonas* and its major plant beneficial traits

Genus *Pseudomonas* is represented by Gram-negative, aerobic, motile rods with cells size 0.5–1.0 μm × 1.5–5.0 μm. They have low nutritional requirements which makes them ubiquitous. A lot of strains are isolated from the rhizosphere of plants [9] where they exhibit various traits which participate in plant growth promotion.

Among the features of pseudomonads that may be beneficial for plants, siderophores and antibiotic compounds synthesis are noteworthy. Iron is a very important microelement for all living cells, involved in crucial metabolic processes whereas its availability in soil is limited. Therefore, many soil microorganisms can synthesize low-molecular-weight molecules—siderophores. These molecules have a high affinity to Fe (III) ions and provide microbial cells with this microelement [10,11]. Radzki et al. [12] demonstrated that siderophores of rhizobacteria can be utilized by plants and therefore provide them an appropriate dose of iron. Moreover, siderophores excreted into the rhizosphere by genus *Pseudomonas* can be involved in inducing systemic resistance in plants [13] or compete with soil-borne phytopathogens for iron [14]. The most frequently synthesized siderophore of fluorescent pseudomonads is pyoverdine but there are also known others, e.g., pyochelin, pseudomonine, quinolobactin, PDTC (pyridine-2,6-dithiocarboxylic acid), achromobactin [15,16]. Not only siderophores produced by pseudomonads may act as antibiotics but also other antimicrobial compounds including pyrrolnitrin and phenazine. These antimicrobial compounds can take part in controlling soil-borne pathogens [17]. It was demonstrated that *Pseudomonas fluorescens* strain which inhibited the growth of potato pathogens (*Phytophthora infestans*, *Streptomyces scabies*, and *Verticillium dahliae*) was able to produce phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxphenazine [18]. Also, the inhibitory activity of *Pseudomonas protegens* FD6 against *Botrytis cinerea* and *Monilinia fructicola* was correlated with the synthesis of antimicrobial compounds—2,4-diacetylphloroglucinol and pyoluteorin [19].

Genus *Pseudomonas* can produce hydrogen cyanide (HCN) and volatile organic compounds (VOCs). HCN is a secondary metabolite of pseudomonads involved in pathogen suppression [20]. Its role in antifungal activity was proven, among others, by Nandi et al. [21]. They demonstrated that HCN is involved in antifungal activity of *Pseudomonas chlororaphis* strain PA23 against *Sclerotinia sclerotiorum* (phytopathogenic fungus causing infection of numerous plants, for example—lettuce, carrots, rapeseed) [22]. There is also described nematicidal activity of HCN produced by *Pseudomonas*

fluorescens CHA0 which suppressed plant-pathogenic root-knot nematode *Meloidogyne javanica* [23]. Also, VOCs-producing strain—*Pseudomonas chlororaphis* PA23 showed nematicidal activity against a model nematode—*Caenorhabditis elegans* [16]. VOCs are a large group of compounds including, among others, alkanes, aldehydes, ketones, and aromatic compounds involved in antagonistic actions, also against phytopathogenic fungi [24]. Dimethyl sulfide, S-methyl thioacetate, methyl thiocyanate, dimethyl trisulfide and 1-undecan—VOCs synthesized by *Pseudomonas donghuensis* P482 inhibited the growth of plant pathogenic fungi *Rhizoctonia solani* and *Fusarium culmorum* [25]. Hernandez-Leon et al. [26] identified VOCs such as methanethiol, dimethyl sulfide, dimethyl disulfide, and N,N-dimethylhexadecylamine, synthesized by different *Pseudomonas fluorescens* strains. There is also a proven antifungal activity of pseudomonads VOCs against *Botrytis cinerea* [24,26] and *Penicillium italicum* [27].

Some soil microorganisms, including *Pseudomonas* strains, can convert insoluble forms of phosphorus [28,29], potassium [30], or zinc [30,31] to soluble forms which can be easily absorbed by plants. These abilities of pseudomonads can contribute to the reduction of chemical fertilizers usage when bacteria from this genus are applied under field conditions.

Genus *Pseudomonas* has the ability to produce phytohormones which play a crucial role in plants' growth and development—mainly auxin—IAA (indole-3-acetic acid) [32], gibberellins [33], cytokinins [34] and salicylic acid [35]. By modulating phytohormones levels, pseudomonads can reduce the negative effects of stress factors and thereby indirectly take part in improving the yield of crop plants. Moreover, a lot of plant growth-promoting *Pseudomonas* strains produce the enzyme ACC deaminase which decomposes ethylene precursor (1-aminocyclopropane-1-carboxylic acid) in plants. The activity of bacterial ACC deaminase contributes to lowering ethylene levels and as an effect—increases plants' resistance to different stress conditions, for instance—high salinity [36] or drought [37].

Pseudomonads also synthesize lytic enzymes involved in the suppression of plant diseases caused by soil-borne pathogens [35]. Antagonistic activity of *Pseudomonas fluorescens* against *Rhizoctonia solani* was correlated with high levels of β-1,3-glucanase and chitinase [35,38]. These enzymes are involved in fungal cell walls degradation. Moreover, *Pseudomonas aeruginosa* strain FG106 was able to produce extracellular proteases and lipases which may contribute to its antagonistic activity against *Alternaria alternata*, *Botrytis cinerea*, and *Rhizoctonia solani* [39]. Similarly, *Pseudomonas chlororaphis* GBPI_507 also produced extracellular enzymes—protease and lipase. Strain GBPI_507 inhibited the growth of *Alternaria alternata*, *Phytophthora* spp, *Fusarium solani*, and *Fusarium oxysporum* [40]. Secretion of hydrolytic enzymes such as chitinases, glucanases, lipases, and proteases enables hydrolysis of cell walls of pathogenic fungi [41]. Products of mycelium lysis can be used as a source of nutrients [42]. Not only fungal pathogens are a target for pseudomonads' lytic enzymes. A study conducted by Siddiqui et al. [43] showed that extracellular AprA protease produced by *Pseudomonas fluorescens* CHA0 contributed to the suppression of root-knot nematode *Meloidogyne incognita*. AprA protease caused an inhibitory effect on egg hatching and contributed to killing juvenile nematodes.

3. Bioformulations of *Pseudomonas* strains to use in field conditions

Due to numerous traits of pseudomonads that can be beneficial for plants—scientists and industry are still making efforts to optimize processes of creating *Pseudomonas*-bioformulations with an

optimal shelf life for commercial use. In contrast to the genus *Bacillus* which can form spores that could help to survive in harsh environmental conditions, *Pseudomonas* does not have that ability. It makes bacteria from this genus more sensitive to unfavorable conditions during technological processes. This is the disadvantage of using non-sporulating bacteria in bioformulations for commercial use [44]. Moreover, the shelf-life of the product designed to use under field conditions, containing living microorganisms, is suggested to be not shorter than 18 months of storage at room temperature [45]. Commercial formulations available on the market, used for *Pseudomonas* storage are in liquid or wettable powder form (mainly talc-based).

3.1. Formulations containing freeze-dried cells

Researchers are working on the improvement of formulation technology and such parameters which could contribute to obtaining long-term survival of pseudomonads during storage. One of the techniques which are used to preserve pseudomonads is freeze-drying. Parameters that are supposed to affect bacterial viability are growth medium and temperature, the addition of protective compounds, biomass harvesting time, and pretreatment of cells with some stress factors before lyophilization. Bisutti et al. [46] demonstrated that *Pseudomonas fluorescens* Pf153 had the highest survival rate when cultivated on King B medium with glycerol and when cells were harvested at the end of a log phase or at the early stationary phase. Results obtained by Mputu Kanyinda et al. [47] also confirm that the addition of glycerol or maltodextrin positively affected the survival rate of freeze-dried *Pseudomonas fluorescens* BTP1 during storage. Stephan et al. [48] conducted a study to determine freeze-drying process parameters for four *Pseudomonas* strains used as biocontrol agents against *Botrytis cinerea*. They tested 20 cryoprotective agents and demonstrated that the highest survival rate in comparison to control (without protective substances) was obtained with the use of saccharose, lactose, glucose ligninosulfonic acid and skimmed milk (1% fat content). Cabrefiga et al. [49] examined also the effects of other cryoprotectants and additionally—osmoadaptation of bacterial cells—to improve a dry formulation of *Pseudomonas fluorescens* EPS62e. Results of the examination showed that the highest viability was obtained by this strain when lactose was used as cryoprotectant and bacterial cells were osmoadapted by cultivation in GMM (glucose minimum medium) containing NaCl and glycine betaine. Preadaptation of bacterial cells with such stress factors as treatment with 3 wt% NaCl and/or 0.05 wt% H_2O_2 was also examined by Wu et al. [50]. They demonstrated that this kind of bacterial culture pretreatment resulted in an approximately 5.6 times higher survival rate of *Pseudomonas fluorescens* ATCC 13525 after cold-air drying. Bisutti and Stephan [51] conducted a study that confirmed the influence of cultivation temperature and cells harvesting time on the survival rate after freeze-drying. Cells fermented at 20 °C showed a better survival rate than those fermented at 28 °C. Moreover, reduction of the survival rate of cells harvested in the mid-log phase was lower in contrast to cells harvested at the beginning of the stationary phase. Mputu Kanyinda et al. [52] demonstrated that during the process of freeze-drying and storage of freeze-dried *Pseudomonas fluorescens* BTP1 cells there had taken place processes resulted in cell membranes damage. They demonstrated that low temperature of storage (4 °C) of freeze-dried *P. fluorescens* BTP1 resulted in higher survivability of bacterial cells in comparison to those stored at 20 °C [52, 53]. It is noteworthy that the authors also showed using flow cytometry that after 7 months of storage at 20 °C more than half of the cells were viable but not culturable due to their deteriorated physiological state.

Mputu Kanyinda and Thonart [53] conducted an experiment to optimize the production of freeze-

dried *P. fluorescens* BTP1. Their results show that increasing the pressure in the bioreactor to 0.3 bar contributed to the fact that *Pseudomonas* culture gained stationary phase 6 hours faster and also reached higher cell density than culture under 0.1 bar pressure. Authors also demonstrated that the survival rate during storage of bacterial cells after freeze-drying was higher when there was added a combination of protective compounds (1%–2% glycerol, 5%–10% maltodextrin, 0.2% ascorbic acid). However, the 26% survivability value indicates the high sensitivity of *Pseudomonas fluorescens* to freeze-drying. Results obtained by Palmfeldt et al. [54] show that carbon starvation of cells before freeze-drying and addition of protective compounds (best effect observed for 100 g/L sucrose) determined cells survival rate after drying at 24% (without protective compounds and pretreatment survivability rate was 0.1%). During 50 days of storage, the number of viable cells decreased to 6% of an initial number of cells and after 200 days only 2% of freeze-dried cells were viable. Due to high costs generated by high energy consumption, freeze-drying is not a common commercially used method of microorganisms preservation [55]. Pseudomonads are sensitive to freeze-drying—even if protective compounds are added their survivability rate is low [53].

3.2. Liquid formulations

There are also documented attempts to produce a liquid formulation of plant growth-promoting pseudomonads strains to provide stable viability during storage of bioformulations to obtain the optimal length of formulation's shelf-life. Manikandan et al. [56] demonstrated the positive impact of glycerol or trehalose addition which maintained viability during storage of *Pseudomonas fluorescens* Pf1 strain in nutrient broth. The formulation was stored in sealed plastic containers. After 120 days of storage at room temperature, there were detected 10^8 CFU/mL for trehalose and glycerol amended formulation. Whereas in control formulation without any amendments population of tested strain decreased gradually during storage and after 90 days no viable cells were detected. Selvaraj et al. [57] also conducted a study where glycerol addition to liquid formulation resulted in increased viability of *P. fluorescens*. Agricultural waste products also are investigated to be a liquid carrier for pseudomonads to minimize the production costs of the formulation. Anith et al. [58] used coconut water amended with 2% glycerol and 2% polyvinylpyrrolidone to obtain 6 months of stability of *P. fluorescens* formulation with a bacterial population at a range of 10^7 CFU/mL after storage. There were also studied field efficacy against phytopathogens and other plant growth-promoting traits during storage of bioformulations. Results demonstrated that storage in liquid formulation did not have negative effects on plant beneficial features of tested strains.

3.3. Formulations containing immobilized bacterial cells

Another technique that is becoming popular to preserve biological control agents is encapsulation. There are many reports of the use of this technique to obtain optimal viability and thus sufficiently long shelf-life of bioformulations. Different microencapsulation techniques and carrier or shell materials are still being tested to provide survivability and preserve bacterial plant growth-promoting traits. Fathi et al. [59] demonstrated that microencapsulation by extrusion technique using alginate and addition of whey protein concentrate allowed to obtain high efficacy of encapsulation (84.23%) of *Pseudomonas fluorescens* VUPF506. Furthermore, this combination of components for microencapsulation contributed to the controlled gradual release from microcapsules of immobilized

strain within 60 days and the reduction of symptoms caused by *Rhizoctonia solani* on tested potato plants. Also, using oxidized sodium alginate and starch to form microcapsules of *Pseudomonas protegens* SN15-2 by extrusion technique led to obtaining high efficacy of encapsulation and increased biodegradability of microcapsules [60]. There are also reports showing an increase in plant growth-promoting traits of immobilized bacteria in comparison with free cells. Minaxi and Saxena [61] revealed that *Pseudomonas fluorescens* BAM-4 when immobilized using alginate and skim milk as carrier materials had demonstrated better phosphorus solubilization capabilities. Similar to preculture treatment before lyophilization Wang et al. [60] applied cultivation of *Pseudomonas protegens* SN15-2 in hyperosmotic conditions to make bacterial cells less susceptible to harsh conditions during microcapsules production. Results demonstrated that hyperosmotic cultivation and the use of fluidized-bed drying technique with skimmed milk as a protective substance helped to minimize bacterial viability losses.

Another possibility to obtain bacterial formulation for agricultural use is immobilization in the porous carrier material. For this purpose, there are often used such carriers as peat, talc, coal, clay minerals, and even plant waste materials [62]. Correa et al. [62] tested talc, peat, and coconut fiber with different amendments—carboxymethylcellulose, xanthan gum, or both to improve the shelf-life of *Pseudomonas chlororaphis* 63-28 formulation. The authors gained expected results—stable viability of examined strain at the range 10^8 CFU/g during 120 days of storage but it is noteworthy that the formulation was stored under refrigeration (3±1 ℃). Moreover, their results indicate that there were no significant differences in bacteria viability between formulations with amendments or without them. Results of the study of Vidhyasekaran et al. [64] demonstrated that the viability of *Pseudomonas fluorescens* Pf1 in talc-based formulation amended with carboxymethylcellulose was stable only during 1 month of storage at room temperature.

3.4. Commercially available formulations

Despite the difficulties in developing technology to make the stable formulation of pseudomonads containing a sufficient number of viable bacterial cells during storage, in the marketplace there are some products containing *Pseudomonas* strains to use in agriculture. For instance—Cerall® and Cedomon® produced by Koppert. Both are liquid formulations for cereal seed treatment whose active ingredient is *Pseudomonas chlororaphis* strain MA342 with declared concentration 10^9–10^{10} CFU/mL. The main difference is that Cerall® is a water-based suspension and Cedomon® is an oil-based suspension. These products are biofungicides to combat such pathogens as *Fusarium* spp., *Tilletia caries,* and *Septoria nodorum*. There are also available some solid-based formulations—for example, Bio-Save® 10 LP (JET Harvest Solutions) containing *Pseudomonas syringae* strain ESC-10 (9×10^{10} CFU/g) formulated into water-soluble powder. It is designed to post-harvest control of fungal diseases of citrus and pome fruits, cherries, potatoes, and sugar beets. Proradix® also is a wettable powder fungicide containing *Pseudomonas* sp. strain DSMZ13134 (6.6×10^{10} CFU/g) which is used to protect potatoes against *Rhizoctonia solani*. Another product in wettable powder form is Salavida® (Sourcon Padena)—a biostimulant that contains *Pseudomonas trivialis* (1.6×10^{10} CFU/g).

4. Summary

There is an unabated interest of scientists and pesticide producers to explore *Pseudomonas* spp. potential to be used as a biological control agent. There are still made efforts to isolate new strains with beneficial traits, especially antagonism against phytopathogenic fungi which are a major cause of yield losses. Methods to preserve pseudomonads in formulations of biological plant protection products are still being optimized because of viability losses of these bacteria during processing and storage. A promising way of pseudomonads storage and delivery is the encapsulation of bacterial cells. This technique could protect bacterial cells against biotic and abiotic stresses and enable long-term survival of bacteria during storage. Encapsulation can also provide controlled release of microorganisms in the soil after application and thereby prolong the time of their plant growth promoting activity. An increasing number of biological plant protection products on the market including these containing pseudomonads indicates that there is a demand for them and farmers are increasingly willing to use this form of plant protection. Therefore, there is a justified need to further studies on the biocontrol potential of pseudomonads, bioformulation technologies, and also long-term effects of application in agriculture.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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