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USE OF MICROORGANISMS IN PLANT PROTECTION AGAINST FUNGAL DISEASES

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Summary. Environmental degradation due to the widespread use of fungicides has forced scientists to look for alternative methods of plant protection. Biological plant protection, which uses the antagonistic activity of microorganisms (bacteria, yeasts, filamentous fungi), has attracted considerable interest. Antagonism of microorganisms towards pathogens consists of several mechanisms, including competition for space and nutrients, mycoparasitism, secretion of compounds with antifungal activity such as antibiotics or killer toxins, induction of resistance in plants, as well as production of volatile compounds that can play a major role in biocontrol. Studies on biological plant protection show more and more positive aspects, e.g. reduction of pathogenic microorganisms development, but also possibility of plants quality improvement, thus improving the quantity and quality of plant production, and soil quality, i.e. its fertilization, enrichment in nutrients or shaping of soil tuberous structure.

Key words: plant protection, microorganisms, antagonism, biocontrol

INTRODUCTION

During their growth, crops are exposed to numerous diseases caused by pathogenic microorganisms, which may contribute to reduced global annual level of food production. Fungi are among the most common causes for plant diseases. The most dangerous fungi include those classified in genera *Penicillium*, *Botrytis*, *Monilinia*, *Rhizopus*, *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum*, *Geosporium* and *Mucor* [Grzegorczyk 2015]. Thus far, chemical compounds referred as fungicides have been commonly used for the

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purpose of crop protection. In spite of the high selectivity of such preparations, they may exhibit toxic effect towards environment, and their residues in foods are a threat to the health safety of consumers. Their misuse may result in reduced soil fertility, surface water eutrophication and groundwater pollution. Microorganism immunization to fungicides is also becoming an issue in the use of these products [Krzyśko-Łupicka and Walkowiak 2014].

In view of this situation, scientists have been searching for new, safer plant protection methods. Biological plant protection with the use of saprophytic or low pathogenic bacteria and fungi strains is gathering an increasing interest. The focus is mainly on the use of antagonistic effect of different microorganisms or products of their metabolism against phytopathogens [Krzyśko-Łupicka 2010]. Application of biological plant protection products is also beneficial due to their positive impact on plants themselves. Microorganisms contained in such a preparation may, via their activity, lead to enrichment of cultures with nutrients, thus stimulating growth and development of plants and other beneficial soil microorganisms [Krupa et al. 2014]. Certain studies demonstrated that the use of specific yeast strains in such products may contribute to reduced amounts or even degradation of mycotoxins in soil [Castoria et al. 2011, Pfliegler et al. 2015].

Thus far, only some of the microorganisms exhibiting antagonistic activities have been used for commercial production of biological plant protection products. They include, but are not limited to, bacteria of the genus *Bacillus* and *Pseudomonas*, actinomycetes of the genus *Streptomyces*, yeasts and mold fungi of the genus *Trichoderma* [Grzegorczyk et al. 2015, Carmona-Hernandez et al. 2019]. Despite limited numbers of specific biofungicides, the demand for these products in agriculture as alternatives to synthetic pesticides was increased over the last few years. The adoption and widespread use of biofungicide will make it possible to produce food that are exempt from or have low values of chemical residues. This will contribute to the consumption of more natural, healthy, and safe foods with respect to fungicide usage [Souza et al. 2018, Abbey et al. 2019, Bazioli et al. 2019]. The global biofungicides market is estimated for a value of USD 1.6 billion in 2020 and is projected to grow at a CAGR (Compound Annual Growth Rate) 16.1%, to reach a value of USD 3.4 billion by 2025 [Biofungicides Market by Type…].

ANTAGONISTIC ACTIVITY OF MICROORGANISMS

Antagonism of microorganisms is determined by the presence of several types of mechanism that cooperate within a phenomenon of biocontrol. These include, i.a. competition for habitat and nutrients, production and secretion of antifungal and antibacterial metabolites and proteins, direct mycoparasitism and induction of defensive and immune mechanisms in plants [Liu et al. 2013]. Antagonistic activity is typically found in yeasts classified in genera *Pichia*, *Candida*, *Aureobasidium*, *Metschnikowia*, *Debaryomyces*, *Saccharomyces*, *Pseudozyma*, *Tilletiopsis* etc. Bacteria are second largest group of antagonistic microorganisms, and are represented by such species as: *Pseudomonas fluorescens*, *Pseudomonas siringae*, *Agrobacterium radiobacter*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Pantoea agglomerans*. Only several mold fungi species have been found to exhibit antagonistic mechanisms, and those are i.a.: *Coniothy-*

rium minitans, *Ampelomyces quisqualis* and numerous *Trichoderma* species [Ippolito and Nigro 2000, Grzegorczyk et al. 2015].

The most important mechanism in biocontrol observed in every antagonist is the competition for habitat and nutrients. This phenomenon consists in the capacity for rapid propagation at low levels of nutrients in soil, as well as better adaptation to environmental conditions than pathogenic microorganisms. Bacteria are often observed to produce biofilm. Biofilm is a multicellular structure encapsulated with a layer of organic and inorganic substances and with energetically favorable cell metabolism. Biofilm increases the chances for bacteria survival in the given environment, as compared to those found outside of it. It further increases their resistance towards unfavorable environmental conditions and toxic substances [Maserti et al. 2015]. As an alternative way to inhibit pathogen growth, microorganisms may produce siderophores, compounds that chelate iron ions. Iron is necessary for microorganisms for the correct cell functioning. This mechanism has been observed in certain yeast strains and *Trichoderma* mold fungi. As a result of siderophore activity, iron in the form of chelates may remain unavailable to other microorganisms, including pathogens. Evidence has been presented for particular significance of this mechanism in inhibiting the growth of fungi of the genus *Fusarium* and *Gaeumanomyces* [Barabasz et al. 1999, Saravanakumar et al. 2008].

Many genera of bacteria and fungi are characterized by the capacity to produce antibiotics, that is substances with antibacterial and/or antifungal activity. This phenomenon is known as antibiosis. Antibiotics are secondary metabolites of microorganisms, which affect cellular structures, typically cell wall and membrane or metabolic processes of other microorganisms, leading to inhibition of their growth and replication. Commonly used antibiotics produced by bacteria include streptomycin produced by *Streptomyces griseus*, syringomycin synthesized by *Pseudomonas syringae.* The phenomenon of antibiosis is also observed in yeasts *Pseudozyma* spp. and *Tilletiopsis* spp. [Liu et al. 2013, Vinale et al. 2014].

Mycoparasitism is another highly important mechanism observed among antagonists. Many types of beneficial fungi have specialized in the synthesis of enzymes resulting in disturbances of the fungal cell morphology of a phytopathogen, including reduced turgor, decomposition of cellular structures. These enzymes include: glucanases, chitinases, proteinases [Druzhinina et al. 2011]. The most important aspect of hydrolytic enzymes is the decomposition of cell walls in filamentous fungi, thus preventing mycelium growth and penetration of plant cells is prevented. Mycoparasitism depends on the capacity of antagonists to adhere to the cell walls of pathogenic filamentous fungi [Howell 2003, Grzegorczyk et al. 2015].

Saprophytic fungi and bacteria, via synthesis of phytohormones and vitamins, increase availability of biogenic elements, release of nutrients from soil and organic matter may stimulate growth and development of plants. They are known as Plant Growth Promoting Fungi (PGPF) and Plant Growth Promoting Rhizobacteria (PGPR). Specific genera of microorganisms from these groups are used to produce yield quality increasing products [Altomare et al. 1999]. The above mentioned groups of microorganism may also participate in Inducing Systemic Resistance (ISR) in plants. Such compounds as jasmonic acid and ethylene fulfill the main role of signaling transmitters. They modulate plant reactions to stress by metabolic changes, i.a. intensified production of peroxidases and phytoalexins, accumulation of hydrolase and structural polymer deposition [Han et al. 2000].

Surfactant compounds called biosurfactants also play a large role in the induction of plant resistance against plant pathogens. They are produced by many different types of microorganisms, including the genus *Bacillus, Pseudomonas*, and *Burkholderia*. Specifically, lipopeptides and rhamnolipids have been studied in the context of plant protection and show a good balance between industrial production and environmental efficacy and protection [Burketova et al. 2015, Schellenberger et al. 2019]. Rhamnolipids are classified as glycolipids. They show mainly antifungal activity against pathogens attacking crop plants. These include *Botrytis* spp., *Rhizoctonia* spp., *Fusarium* spp., *Alternaria* spp. They can stimulate plant innate immunity by activating early signaling events such as ROS accumulation, activation of defense genes. Lipopeptides (e.g. surfactin, bacilliomycin), on the other hand, occurring in cyclized form, show antibacterial, antifungal, cytotoxic, and anticancer properties. Activation of defense mechanisms with their participation occurs by inducing disruption in the plant cell membrane leading to activation of molecular events [Crouzet et al. 2020].

The antagonistic activity of microorganisms may also be related to the synthesis of nanoparticles. Nanotechnology is a new fast-growing field of science that is increasingly used in biology, medicine, electronics, and agriculture [Rana et al. 2020]. Nanoparticles range in size from 1 to 100 nanometers and can be naturally formed or intentionally produced. Nanoparticles can exhibit different physical and chemical properties from their larger equivalents. The biological method of obtaining nanoparticles using microorganisms is much more cost-effective and largely more environmentally friendly, compared to standard physical and chemical methods. Researchers are focusing on discovering, among bacteria, fungi, and viruses, those microorganisms that will be able to synthesize nanoparticles that support plant growth and metabolic functions, and protect plants from pathogens [Duhan et al. 2017, Ghosh et al. 2021]. Microbial synthesis of nanoparticles can occur intracellularly or extracellularly. Various microorganisms (*Pseudomonas proteolytic, Bacillus licheniformis, Aspergillus niger, Fusarium oxysporum*) can reduce Ag^{+} ions, resulting in the formation of silver nanoparticles (AgNPs). Biosynthesis of gold nanoparticles (AuNPs) can occur with *Fusarium oxysporum, Nocardia* spp., *Streptomyces* spp.. Soil microorganisms can also reduce other heavy metals such as U(VI), Tc(VII), Cr(VI), Co(III), and Mn(IV) [Ghosh et al. 2021]. The previously mentioned nanoparticles exhibit antimicrobial properties by disrupting the cell membrane structure of pathogens, inhibiting biofilm formation, creating pores in the cell wall, and producing reactive oxygen species. Their activity is aimed against pathogens such as: *Fusarium* spp., *Alternaria brassicae, Colletotrichum* spp., *Bipolaris sorokiniana* [Kaushal 2018, Fatima et al. 2021].

TRICHODERMA SPP. AND OTHER MICROORGANISMS IN PLANT PROTECTION

Mold fungi classified in the genus *Trichoderma* are considered to be some of the most beneficial and positive microorganisms enhancing arable quality of soils. They colonize rotting wood, soil and in particular the rhizosphere layer. First works presenting the capacity for using *Trichoderma* spp. in plant protection were first published in the early

1930s. Research on antagonistic properties of these fungi is still conducted [Benitez et al. 2004]. Characteristic trait of the genus *Trichoderma* is their capability for rapid growth and propagation. They adapt well to the environment and exhibit resistance to different chemicals, toxins synthesized by *Fusarium* spp., heavy metals. They are capable of modifying rhizosphere properties. Their antagonistic effect, such as competition for environment and nutrients, mycoparasitism, antibiosis enable their use in agri-food industry and in agriculture [Wiest et al. 2002].

Fungi of the genus *Trichoderma* aggressively compete with phytopathogens for nutrients and habitat. An undisputed advantage of these microorganisms is their high capacity to release and absorb nutrients from soil, as compared with other microorganisms. They were also observed to exhibit ability to decompose metabolites produced by fungal pathogens [Bélanger et al. 1995]. The phenomenon of mycoparasitism have been well studied for fungi of this genus. *Trichoderma* spp. can be a parasite of i.a. *Fusarium*, *Rhizoctonia*, *Phytium*. First stage of the effect of a fungi on a pathogen occurs contactless. It consists in identifying the pathogen hyphae, growth in its direction and release of enzymes that initially decompose the cell wall. Subsequently, *Trichoderma* attaches around the pathogen with the use of clamping organs. Mechanical lesions occur at the contact points on the pathogen mycelium, through which enzymes and antibiotics produced by the antagonist are introduced. These substances result in death of the pathogenic microorganism, whereas the decayed components of the host's cell wall become additional source of food for the parasitic microorganism [Howell 2003].

Trichoderma spp. produce numerous bioactive compounds, such as: enzymes, antibiotics, volatiles and plant hormones. With them, they are able to promote plant growth and development, as well as inhibit the growth of pathogenic microorganisms. Different *Trichoderma* strains can synthesize one or several substances of antibiotic nature, which inhibit growth of different phytopathogens. The known antibiotics are: trichoviridin, peptide antibiotics (peptaibols), sesquiterpenoids, isonitriles and other. They may also induce IRS in monocotyledonous and dicotyledonous plants, as a result of biotic and abiotic stress. Thanks to these properties they are classified as Biological Control Agents (BCA) and are commercially used for their production [Naher et al. 2014].

During study on a *T. harzianum* mutant induced with UV rays, an isonitrile antibiotic was isolated, labeled as homothalin II. This compound exhibited strong antifungal and antibacterial activity against i.a. *Pythium ultimum*, *Rhizoctonia solani, Escherichia coli, Bacillus cereus, Pseudomonas putida*, *Micrococcus luteus* [Faull et al. 1994]. The following compounds were isolated from *T. koningii* cultures: dermadin, exhibiting bioactivity towards Gram-positive and Gram-negative bacteria and numerous fungi, and trichoviridin, which limited the growth of *Escherichia coli* and *Trichophyton asteroides* [Pyke and Dietz 1966, Yamano et al. 1970, Tamura et al. 1975].

Bioproducts containing *Trichoderma* fungi are available on the Polish, as well as foreign market. These include, but are not limited to Trianum (contains spores of *Trichoderma harzianum* fungi, strain T-22) by Koppert Biological Systems, TRICHODERMA (*Trichoderma atrobruneum* and *Trichoderma koningii*), BACTIM VIGOR by Intermag (*Trichoderma harzianum* strain TH01 and *Trichoderma viride* strain TV03). They are intended for inhibiting the growth and development of phytopathogenic microorganisms, as well as improving the quality and size of yield. Products based on *T. harzianum* T-22 isolate have been successfully used to combat *Fusarium, Phytium, Rhizoctonia, Sclerotinia homeocarpa* in the USA and Europe in field and covered cultures for the production of i.a. cotton, corn, sugar beets, beans, sorghum, cabbage, tomatoes, cucumbers [Pietr 2000]. Among biopreparations based on fungi, it is worth mentioning Polyversum by Biopreparaty Sp. z o.o., which has been used for years in Poland and other countries. This is an agent containing *Pythium oligandrum*, designed to protect against pathogens occurring on ornamental plants, fruit, vegetables and lawns.

The largest group of biological plant protection products comprises of preparations based on *Bacillus* bacteria. Their characteristic trait consists in the capacity to synthesize numerous fungistatic metabolites, i.a. antibiotics, enzymatic proteins, chitinases, glucanases. *Bacillus subtilis* can form a biofilm around plant roots. In this manner it feeds on root secretions [Liu et al. 2010]. In the study of Zhao et al. of 2010 the *Bacillus vallismortis* ZZ185 strain was isolated, which by means of produced bacilliomycin D efficiently inhibited the development of *Alternaria alternata*, *F. graminearum*, *R. solani*, *Cryphonectria parasitica* and *Phytophthora capsici*. The article of Liu et al. [2010] described the capabilities of *B. subtilis* EDR4 strain, isolated from wheat roots, to inhibit pathogen growth. Its activity was based on the secretion of a protein with pronounced fungistatic activity towards *Macrophoma kuwatsukai*, *Rhizoctonia cerealis*, *F. oxysporum* f.sp. *vasinfectum*, *F. graminearum*, *B. cinerea*, *Gaeumannomyces graminis* var. *tritici*. In addition, *B. polymyxa* VLB16 was isolated from rice rhizosphere. It synthesized a protein that inhibited growth of *Puricularia grisea* and *R. solani*, pathogens producing greatest losses of rice crops. The products, such as Serenade, Rhio-Plus (based on the *Bacillus subtilis*) are available on the market.

Bioproducts with *Pseudomonas* strains are also available on the market. In 2008, Mikani et al. investigated over a dozen strains of this genus, isolated from leaves and fruit of apple for its combat of *Botrytis mali*. Ten of those exhibited strong fungistatic properties, which were mainly based on the production of numerous secondary metabolites inhibiting the growth of a pathogen, e.g. pyrrolintrin. Bioproducts containing *P. fluorescens* are used to combat pathogens of the genus *Fusarium* and *Rhizoctonia*. They are most frequently used to protect apples, pears, cotton and many types of vegetables. Their another characteristic property is counteracting plant organ frosting. Bioproducts with *Pseudomonas syringae* (Biosave 10LP and 110 by Eco Science Corporation, USA) strains are also used to protect stored post-harvest material.

Another interesting group of microorganisms exhibiting antifungal effect are actinomycetes, primarily the genus *Streptomyces.* These are Gram-positive bacteria with high biotechnological potential. They are characterized by the capacity to produce numerous widely known antibiotics: streptomycin, nystatin and other. In 2008, Wan et al. isolated the *Streptomyces platensis* F-1 strain, which synthesized volatile compounds exhibiting fungistatic effect towards *B. cinerea, S. sclerotiorum* and *R. solani*. Actinomycetes also synthesize highly active antifungal enzymes from the group of chitinases and glucanases. In 2005, Joo isolated *Streptomyces halstedii* AJ-7 strain, which produced a chitinolytic enzyme with pronounced effect towards *Fusarium oxysporum*. To a lesser extent it inhibited the growth of *Alternaria alternata, Colletotrichum gloeosporioides* and *Septoria lucopersici*.

POTENTIAL AND LIMITATIONS OF BIOPRODUCTS

Research on biological plant protection products exhibit an increasing number of positive aspects of their usage in agriculture instead of fungicides used globally. The main advantage of their application is not only limitation of the development of pathogenic microorganisms, but also the possibility for enhancing plant quality, and thus improved quantity and quality of plant production. By introducing appropriate microorganism species in the plant protection products, soil quality can be improved i.e. its fertilization, enrichment in nutrients or shaping of soil tuberous structure. Bioproducts can be used to protect plants during growth, as well as after harvest and during storage [Harman 2000].

However, biological plant protection products must meet rather strict criteria. It is assumed that they should inhibit disease development at the level of 95–98%, which unfortunately can only be obtained by using them with a low dose of synthetic fungicides. In addition, production of antagonistic microorganism biomass should be efficient and cost-effective, and the microorganisms should exhibit high survival rate during processing [Tomalak and Sosnowska 2010, Martyniuk 2011]. The process of bioproduct manufacturing is however time-consuming and has several stages. Initially, the pure ‘mother culture’ is prepared. subsequently, microorganisms are propagated in fermenters until specified volume is obtained, and then the biomass is separated from post-culture liquid via filtration or centrifuging. The formed antagonist suspension is mixed with a sterile carrier, which ensure survival conditions for the microorganisms, e.g. lignite dust, peat, talc. It is very important to develop such a formulation of the end product that will provide convenient conditions guaranteeing good viability and activity of microorganisms. The produced biopreparations can be typically purchased in the form of solid, lyophilized and liquid products [Martyniuk 2011].

Another important aspect related to biopreparation production is that the microorganisms used for this purpose are characterized by suitable mechanisms of action, genetic stability and purity. They should also maintain properties of laboratory cultures independently of the production scale [Kardowska-Wiater 2011].

The effectiveness of most biopreparations approved for practical use is lower compared with chemical agents, and their use is more difficult and troublesome [Martyniuk 2012]. The main factor limiting production and use of plant protection bioproducts on a large scale is the variability of the efficiency of antagonist microorganism action, which depends on environmental conditions. Both physicochemical properties of soils, as well as effect of plants on microorganisms via secreting bioactive substances have an immense effect on the counts and properties of microorganisms [Droby et al. 2009]. The production process of biological plant protection products is highly expensive and time-consuming. It requires many years of in vitro and in vivo study conducted in laboratory, as well as in field conditions. Also the registration procedure of such products is difficult and is subject to strict legal control. In Poland, bioproduct registration is regulated by the Ministry of Agriculture and Rural Development [Grzegorczyk et al. 2015].

MICROORGANISM ANTAGONISM STUDY METHODS

Antagonistic properties of microorganisms can be investigated via several methods. Two methods are best known and widely used due to their highest efficiency: bar and well method. They form the basis for the research on antagonism of both bacteria and fungi.

Bar method is the most commonly used in this type of research. In this method, first a specific medium inoculated with suitable volume of 24-hour culture of antagonistic microorganisms is poured on Petri dishes. Incubation is conducted in conditions suitable for the growth of antagonists for 24 hours. After this time, 3–4 bars with constant diameter (8–11 mm) are cut from the culture and moved onto a medium containing specified volume of 24-hour culture of indicator strains, previously prepared and poured on Petri dishes. Incubation is conducted in conditions depending on the requirements of indicator microbes. The result is the value of the diameter of indicator strain growth inhibition, from which the bar diameter is deducted [Spelhaug and Harlander 1989, Strus 1998, Kraszewska et al. 2005].

The second commonly used method is the well method. It has a similar course to the bar method. Medium inoculated with 24-hour culture of indicator strains is poured onto Petri dishes. Then, 3–4 wells of specified diameter (10–11 mm) are cute in the cultures. A specified amount of 24-hour culture of antagonist strain, suspension containing fungal spores or conidia, supernatant containing extracted microbial metabolites can be introduced to the formed well. Incubation is conducted in specified conditions for 24 hours. Also here the result is the diameter value of the indicator strain growth inhibition zone from which bar diameter has been deducted [Strus 1998, Piegza and Stempniewicz 2002].

CONCLUSIONS

Biological plant protection methods undoubtedly constitute a highly interesting alternative to chemical fungicides. It seems particularly advantageous to use the antagonistic properties of saprophythic or low pathogenic microorganisms. The use of such preparations may contribute not only to inhibiting the growth of pathogens, but also improving the quality of crops and soil properties, thus increasing the level of food production. However, due to the complexity of the phenomenon of biocontrol, and taking into account the fact that these are living organisms whose growth, development, life and biochemical features depend on habitat conditions, biopreparations require further research on their specificity and the possibility for practical application.

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WYKORZYSTANIE MIKROORGANIZMÓW W OCHRONIE ROŚLIN PRZED CHOROBAMI POWODOWANYMI PRZEZ GRZYBY

Streszczenie. Degradacja środowiska w wyniku powszechnego stosowania fungicydów zmusiła naukowców do szukania alternatywnych metod ochrony roślin. Duże zainteresowanie skupia na sobie biologiczna ochrona roślin wykorzystująca działalność antagonistyczną mikroorganizmów (bakterii, drożdży, grzybów strzępkowych). Na antagonizm drobnoustrojów względem patogenów składa się kilka mechanizmów, w tym: konkurencja o przestrzeń życiową i składniki pokarmowe, mykoparazytyzm (mykopasożytnictwo), wydzielanie związków o aktywności przeciwgrzybicznej, takich jak antybiotyki, indukcja odporności w roślinach, a także produkcja związków lotnych, mogących odgrywać dużą rolę w zjawisku biokontroli. Badania nad biologicznymi środkami ochrony roślin wykazują coraz więcej pozytywnych aspektów, takich jak możliwość poprawy jakości roślin, co za tym idzie poprawy ilości i jakości produkcji roślinnej oraz jakości gleby, tj. użyźnienia jej, wzbogacenia w składniki odżywcze, czy kształtowanie struktury gruzełkowatej gleby.

Słowa kluczowe: ochrona roślin, mikroorganizmy, antagonizm, biokontrola