The possibilities of biologically protecting plants against diseases in nurseries, with special consideration of Oomycetes and *Fusarium* fungi

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Abstract. Achieving high quality propagative material is difficult today due to the limited number of pesticides recommended for use. Simultaneously, EU regulations on Integrated Pest Management (IPM) in forest nurseries came into force, requiring a search for alternative plant protection methods that are safe for humans, animals and the environment. In this paper, we present the possibilities of using bio-fungicides against diseases in forest nurseries, their mechanisms of action, as well as the direction of their development (according to IPM rules). We reviewed the results achieved by different research teams presenting the possibilities and trends in combatting Oomycetes and *Fusarium* spp. pathogens currently having the most important economic impact.

Key words: forest protection, forest nurseries, biological control, Oomycetes, *Fusarium*

1. Introduction

Ecosystems constitute highly complex system of relations and connections between environmental factors and living organisms. Plant tissues colonize numerous microorganisms (fungi, bacteria, actinobacteria, protozoans, viruses, etc.), which use the plants as the source of nutrition, living environment or means of transport to new habitats. Great diversity and number of microorganisms living in different ecosystems provide grounds for assuming that they are the reservoirs of an unfulfilled potential in various aspects of human activity, including biological control of plant diseases. Biological diversity of microorganisms in forest environment frequently determines the plant health, since the use of chemical plant protection agents has been more and more limited (Głowacka et al. 2012).

Major diseases encountered in forest nurseries include seedling blight and root blight occurring a few weeks after sprouting. They are caused by the large group of fungal pathogens including mainly *Phytophthora* and *Pythium* (Oomycetes) and *Fusarium* genus of fungi (Mańka 2005; de Vasconcellos and Cardoso 2009, Lefort et al. 2013).

Polish forest nursery is currently contending with limited availability of fungicides, what results in difficulties in reducing the disease of forest reproductive material. Consequently, an urgent necessity to use alternative methods of plant protection, including biological control, has arisen in forestry. The results of the research presented in this paper and concerning the use of biological control factors in eliminating forest nurseries pathogens, tree stands and natural ecosystems indicate a great potential for adopting biological methods in for-
Biological control – definition and mechanisms

The basis of biological plant control formulated by Baker and Cook (1974) defined its principles as controlling pathogenic organisms with the help of other living organisms. Currently, the notion of biological plant protection is more complex and defined in terms of the use of biopesticides, that is the plant control agents containing the biotic factor or factors (Biological Control Agents – BCA) in order to reduce pathogenic organisms through one or more mechanisms of action. They have either direct or indirect impact on pathogens or on pathogens and plants. The biotic microbiological factors are represented by living organisms: bacteria, fungi and pathogen – antagonistic viruses or the viruses inducing the mechanisms of plant disease resistance (Cook 1993, Schouten et al. 2008). The mechanism of action of microbiological BCA is based on the use of their competitive abilities (quick growth, intensive sporulation and high adaptive capacities), which allow for colonization of ecological niche and reduction in the size of pathogenic organisms population, either in the soil or on the plant. BCA, apart from competing with pathogens for living space, fight for nutrients in the soil (Okorski 2007).

The BCA directly affects pathogenic microorganisms through the synthesis of lytic enzymes and antibiotics hindering their growth and development as well as through establishing direct parasitic contact with the pathogen (hyperparasitism) (Whipps, 2001). In relationship with plants, the BCA induce resistance through the activation of the systemic acquired resistance (SAR) (Kozłowska, Konieczny 2003) or the induced systemic resistance (ISR). In the first case (SAR), both biotic and abiotic factors are the triggers of the plant resistance reaction, with salicylic acid as an intermediate (Salas-Marina et al. 2011). The ISR is activated by saprotrophic fungi and bacteria, ethylene is a signal molecule and jasmonic acid (JA) plays a key role (Pieterse et al., 2011).

The consequence of the plant resistance response is the accumulation of pathogenesis-related proteins (PR), phytoalexins (FA), chitinases, glucanases and peroxidases as well as the synthesis of phenolic compounds (Khan et al. 2004).

The fungi, which are the most frequently used in biological control, belong to the following genera: Trichoderma, Gliocladium, Ampelomyces, Candida and Coniothyrium (Favel 2005). Fungal BCA form secondary metabolites with antibiotic properties (Vinale et al., 2008), synthesize the following enzymes: chitinases, cellulases, glucanases and proteases allowing for developing mycoparasitic relationship (Harman et al. 2004) and induce the SAR and ISR mechanisms in plants (Salas-Marina et al., 2011).

The bacteria classified as biological control factors belong to PGPR group (Plant Growth Promoting Rhizobacteria), which apart from having the antagonistic effects on pathogen exert a positive impact on the plants. The PGPR bacteria make difficult to obtain forms of minerals available to the plants, improve the structure of the soil, produce the analogues of plant growth regulators, as well as bind toxic heavy metals (Gutierrez-Manero et al., 2001). The PGPR bacteria are mostly the representatives of the following genera: Acetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Derxia, Enterobacter, Gluconacetobacter, Klebsiella, Ochrobactrum, Pseudomonas, Rhodococcus, Serratia and Zoogloea (Singh et al., 2011). Since the PGPR bacteria produce siderophores, synthesize antibiotics and induce the ISR resistance, they enter into severe competition with pathogens (Figueiredo et al. 2010). The mycophagous bacteria are also the biological control factors, which with the help of active mechanisms parasitizes fungal hyphae (de Boer et al. 2005; Fritsche et al. 2006).

Particularly important BCA group are actinobacteria, which synthesize approximately 70–80% of all familiar secondary metabolites produced by the microorganisms (Berdy 2005; Golńska and Dahm 2013). The greatest potential for controlling plant diseases have the representatives of Streptomyces and Micromonospora genera (Lehr et al. 2008; El-Tarabily et al. 2010). Actinomycetes affect the pathogen through the production of antibiotics, extracellular polysaccharides inducing the plant resistance responses (exopolysaccharides – EPS) and enzymes hydrolysing fungal cell wall: β-1,3-, β-1,4-, β-1,6-glucanases, siderophores and phytohormones (Gohar et al., 2006; Valois et al. 1996; Ma and Berkowitz 2007; Khamna et al. 2009).

Mycorrhizal fungi, as the biological control factors, play a key role in the protection of forest ecosystems. It
is estimated that approximately 5000–6000 fungi species can be involved in ectomycorrhiza (Molina et al. 1992). The beneficial effects the mycorrhizal fungi have on plants include increasing the root system capacities for the absorption of minerals and the water (Read and Perez-Moreno 2003), therefore applying mycorrhizal fungi to container nursery seedlings before their transfer to the environment considerably improves their survival rates (Domenech et al. 2004). There are reports indicating that mycorrhizal fungi can increase plant resistance to infections caused by soil pathogens (Azcón-Aguilar and Barea 1996; Graham 2001). Some ectomycorrhizal fungi produce siderophores, which bind iron in the soil (Renshaw et al. 2002), whereas the others synthesize antibiotics (Tsantrizos et al. 1991). The underlying impact mechanism of these fungi on the pathogen is the competition and creation of physical barrier preventing infection (Graham 2001). The most important mycorrhizal fungi used in biological control belong to Glomus genus, and they include the following species: Gigaspora margarita, Hebeloma crustuliniforme and Sclerocystis dussi (Kowalski and Wujnowski 2009; Ozgonen et al. 2009; Kavatagi and Lakshman 2012).

Another group of biological control factors are organic and nonorganic chemical compounds, which can be applied both to the soil and directly to the plants and seedlings to reduce the diseases caused by pathogens. The organic chemical compounds include plant extracts, essential oils, glucosinolates, chitosan and synthetic compounds, such as salicylic acid, benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester, benzothi-adiazole (BTH) and β-aminobutyric acid (Oostendorp et al. 2001, Alabouvette et al. 2006, Muthukumar et al. 2010; Abdel-Monaim 2013). These compounds suppress the growth of pathogens and like antagonistic microorganisms, they activate plant resistance mechanisms (Alabouvette et al. 2006).

3. Biological control in forest nursery

The literature on the methods of biological control of plant diseases provides many examples of beneficial BCA application with respect to agricultural and horticultural crops, while there are only few examples of the use of biological control factors in reducing the diseases occurring in forest nurseries. The authors of the research works have reported that biological control of tree diseases is very difficult, what stems from the production specificity (Reglinski and Dick 2005; Hill et al. 2007). In New Zealand (where container nursery provides approximately fifty million seedlings per annum), an important support for the production is the use of biological control factors, in order to develop disease resistance and exert beneficial effects on the plant growth (Hohmann et al. 2011). The BCA can be applied to forest nurseries in two ways: through seed treatment and sprayings (Hohmann et al. 2011). It is worth emphasising that seed treatment is more economical and at the same time very effective in terms of diseases caused by soil fungi (Mousseaux et al. 1998; Bell et al. 2000). According to Bent et al. (2001), an important element of the nursery production technology is the inoculation of tree seedlings with the PGPR bacteria, because it improves the plants’ condition and increases their adaptive capacities after replanting. In the opinion of Reglinski and Dick (2005), antagonistic microorganisms, which include the representatives of Trichoderma genus, have a considerable potential for reducing pathogens occurring in forestry. It has been confirmed by the results of the studies conducted by Hill et al. (2007), who showed that applying Trichoderma genera improved the health of Pinus radiata seedlings in the container nursery. Other studies revealed that Trichoderma fungi have positive effects on different arborescent plants (Paderes et al. 2005, Adams et al. 2007, Grodnitskaya and Sorokin 2007), whereas T. harzianum genus which was used in Kelley’s studies (1970) reduced Pinus echinata seedling blight. The same BCA genus applied in container nurseries reduced the Douglas fir seedling mortality due to plant infection caused by F. oxysporum (Mousseaux et al. 1998). Another studies conducted by Hill et al. (2007) proved that both seeds treatment and spraying with preparations containing antagonistic Trichoderma strains improved seeds sprouting and the health of P. radiata seedlings in container nurseries.

According to Bent et al. (2001), optimization of the use of microorganisms in forest nurseries requires detailed knowledge of the PGPR interaction mechanisms with the plants and determination of environmental conditions affecting the colonisation of the niche by specific microorganisms. Kelley (1976) showed that the Trichoderma representative was not able to prevent Pinus echinata seedling blight caused by P. cinnamomi under conditions of the soil moisture remaining close to the saturation point. Other research works have not found any impact of Trichoderma fungi and calcium compounds on Phytophthora suppression; however, their authors when summarising of the obtained results arrived at the conclusion that controlling Phytophthora requires the integration of all the available methods, including the biological one (Reglinski et al. 2008).
Correspondent to the Reglinski’s studies are the results obtained by Minchin et al. (2012), who have not observed any beneficial effects of the commercial biopreparation containing *Trichoderma atroviride* (five isolates) and *T. harzianum* (one isolate) on the plant growth. The authors demonstrated the lack of negative impact of BCA on the colonization of seedlings by *Pinus radiata* ectomycorrhizal fungi in container nurseries. Other studies related to combined application of *Paenibacillus polymyxa* and *Pseudomonas fluorescens* bacteria showed adverse impact on the growth and the root mass produced by *Pinus contorta* in comparison with individual BCA application. At the same time, it has been found that the level of rhizosphere colonization by the bacteria does not correlate with bacteria’s beneficial impact on the plant growth (Bent et al. 2001). However, the studies conducted by Hohmann et al. (2011) showed the increase in the seedlings growth in comparison with the control, as a result of applying *Trichoderma* fungi isolates coming from indigenous ecosystems to the con- tainer nursery. The beneficial effects of the indigenous population of BCA microorganisms on the health of the beech and oak seedlings were also confirmed by the results of the most recent studies carried out by Lefort et al. (2013). Their in vivo experiments showed a considerable reduction in the seedling infections caused by *P. cambivora* and *P. cinnamoni* oomycetes.

**Biological control of blight diseases caused by the Oomycetes and Fusarium**

Many research groups focused their works on the analysis of antagonistic microorganisms impact on the pathogens responsible for blight diseases (the *Oomycetes* and *Fusarium*) occurring in agricultural, horticultural and forest tree crops. Most of the available research analysed the activity of antagonistic organisms in comparison with the plants, fungi and oomycetes pathogens with the help of Petri dish. These experiments frequently constituted the introduction to the vase experiments, and in some case for the field experiments (Table 1).

The example of such analysis is the research conducted by Paul and Sarma (2006), who assessed the efficacy of IISR-6 *P. fluorescens* strain with strong antibiotic properties (pyoluteorin, pyrrolnitrin, HCH) in terms of controlling *Phytophthora capsici*. The authors proved severe hindering of mycelium growth (at approximately 70%), reduction of the sporangia production and spores sprouting. Another biological control factor was analysed by the group led by Picard (Picard et al. 2000b). The Petri dish experiment showed the antagonistic impact of *Pythium oligandrum* (1010) strain on *Phytophthora parasitica*. The authors have suggested that high affinity of *Py. oligandrum* to host cells was triggered by chemical stimuli or chemotropism, and the cells damage ensued from the synthesis of hydrolytic enzymes: β-1,3-glucanase and cellulase. In further research works, the authors proved that secondary metabolite synthesised by *Py. oligandrum* suppressed the symptoms of the tomato plant disease caused by *P. parasitica*. The application of oligandrin reduced the number of sick plants displaying most severe disease symptoms (Picard et al. 2000b). In subsequent analyses, the same group of researchers showed that applying *Py. oligandrum* spores reduced by approximately 60% the symptoms of tomato plant diseases (Picard et al. 2000a). According to the authors, *Py. oligandrum* has a direct impact on fungi pathogenic cells and oligandrin is the elicitor of the plant resistance response. In the authors’ opinion, the tomato plants are equipped with functional receptors of oligandrin intermediating in specific signal path leading to resistance response, manifested by the synthesis of phytoalexin and phenolic compounds (Picard et al. 2000a).

The analysis of the data provided by the subject literature indicates that bacteria and actinobacteria with antibiotic properties are the most frequently used microorganisms in the biological control of plant blight diseases (30 out of 47 works) (Table 1).

The example of the role of antibiosis in the biological control of plant diseases is the use of *Serratia plymuthica* (A21-4) species to counteract *P. capsici* on pepper plants (*Capsicum annuum* L.) (Shen et al. 2005). The authors conducted the research under in vitro conditions and observed that zoosporangium and zoospores growth was hindered by A21-4 strain synthesising macrocyclic lactone. The laboratory results were verified in vase and greenhouse experiments, which showed high effectiveness of the colonization of plant roots by antagonistic strain. Population of *S. plymuthica* steadily remained in rhizosphere as well as on grafted and newly grown pepper roots. A month after replanting the plants to the medium infected by *P. capsici*, the damages of control plants reached 75%, whereas the plants under biological control were damaged only at 12.6% (Shen et al. 2005). The antibiosis was also investigated by Logeshwaran et al. (2011), who showed that antagonistic strains of *Glucanacetobacter diazotrophicus* (L5 and PAL5) synthesising secondary metabolite with antibiotic properties (pyoluteorin) hindered the mycelium growth of *F. oxysporum* and *F. solani*. The growth of mycelium of the
abovementioned species was hindered, respectively, by 53.49% and 60.0% in case of PAL5, and by 43.48% and 46.66% in case of L5.

However, it should be noted that large part of research works was conducted only under laboratory conditions or in vase experiments using sterilised soil or artificial mediums (Gilbert et al. 1990; Smith et al. 1993; Chen et al. 1996; Okamoto et al. 1998; Picard et al. 2000a, b; Jung and Kim 2005; Timmusk et al. 2009; Logeshwaran et al. 2011). The results obtained under laboratory conditions are not always identical with the ones obtained under in vivo conditions, which was illustrated by the studies carried out by Devaki et al. (1992). The researchers proved high antagonistic effects of Trichoderma harzianum synthesising β-1,3-glucanases on the growth of mycelium of Py. aphanidermatum and Py. myriotylum. On Petri dish, in the area of interaction of BCA mycelium with phytopathogen they observed the autofluorescence, which explicitly indicated cell deaths. In case of vase experiments, the antagonist efficacy was confirmed only in sterilised soil, while the protective effect in unsterilised lised soil was relatively small (Devaki et al. 1992).

Different research groups also conducted the extensive and multilayered studies concerning the implementation of biological control to counteract various plant pathogenic microorganisms. The examples of such studies are multifaceted works related to the application of Bacillus cereus strain (UW85) (synthesising zwittermicin A and kanosamine) to suppress the species belonging to mycetozoa of Phytophthora and Pythium genera: Py. torulosum (Shang et al. 1999), Py. aphanidermatum (Chen et al. 1996), P. cactorum (Gilbert et al. 1990), P. parasitica (Handelsman et al. 1990) and P. megasperma f. sp. megasperma (Handelsman et al. 1990).

First studies were conducted under in vitro conditions to counteract P. cactorum and they found the lysis of zoospores affected by antibiotic metabolites of UW85 strain (Gilbert et al. 1990). The studies were continued using quick mortality test of medick seedlings from P. megasperma f. sp. medicaginis (Handelsman et al., 1990) and under controlled conditions to control tobacco plant against seedling blight (Py. torulosum). The studies showed complete suppression of disease progression (Shang et al. 1999).

Yuan and Crawford (1995), having conducted the antagonism tests with the help of the Petri dishes, proved the hindered growth of selected plant pathogenic fungi, including Py. ultimum, Aphanomyces euteiches, F. oxysporum and R. solani przez Streptomyces lydicus (WYEC108) (Yuan and Crawford 1995). The authors observed the growth disorder and the lysis of mycelium hyphae, and due to scanning technique of electron microscopy, the damage of sprouting oospores, as well as the damages of mycelium cell wall of Py. ultimum. Further studies by the group of researchers (Yuan and Crawford 1995) included the protection of pea seeds against pre-emergence infection (Py. ultimum). In consequence of seeds inoculation with WYEC108, the 40% damage was reported, while 100% of control seeds displayed the disease symptoms. The studies also showed that the population of S. lydicus remained stable and at a high level of both sterilised and unsterilised soil resulted in high protective effect aimed at reducing the pea seedlings blight (Yuan and Crawford 1995).

Microorganisms inducing the plant resistance mechanisms and exhibiting competitive properties towards pathogens were also used to suppress blight diseases. The method was, for instance, adopted by Benhamou et al. (2000). In their studies, they assessed the usefulness of Serratia plymuthica bacteria (RIGC strain) for controlling the blight of cucumber seedlings. The cucumber seeds were being soaked for 24 hours in suspension containing the bacteria cells. After 5 days since the plant inoculation with the Py. ultimum spores, the control object showed severe root damage and plant withering. The biologically controlled plants were not completely healthy, but the disease symptoms mainly appeared on the side roots (Benhamou et al. 2000). According to the authors, the obtained results indicate that the reduction of the disease symptoms not only stems from the decrease in pathogen growth rate and colonisation of tissues but also from the induction of structural and biochemical barriers in a host plant. This approach has been confirmed by the results achieved by van Peer et al., (2001), who demonstrated the increased accumulation of phytoalexins occurring in carnation roots treated with Pseudomonas bacteria at the beginning of the attack caused by Fusarium fungi.

The induction of plant resistance mechanism was also confirmed by the studies on the suppression of pepper plant phytophosphiosis through the application of antagonistic species of Paenibacillus illinoiensis (KJA-424) (Jung et al. 2005). The vase experiment showed high efficacy of pepper roots inoculation with KJA-424 and reported approximately 84% decrease in plant damages caused by P. capsici species compared with the control variant (Jung et al. 2005). Analogically, Cordier et al. (1998) studies on the use of mycorrhizal species of Glomus mosseae (BEG 12) in biological control of tomato against P. parasitica found that ISR mechanism
Table 1. Biological Control Agent (BCA) used to eradicate diseases caused by pathogens of the genera *Phytophthora*, *Pythium* and *Fusarium*.

<table>
<thead>
<tr>
<th>Biological Control Agent</th>
<th>Mechanism</th>
<th>Organism to be eradicated</th>
<th>Plant</th>
<th>Scope of the research</th>
<th>Effectiveness/results</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Bacillus megaterium</strong> KL39 (purified antibiotic)</td>
<td>Antibiosis</td>
<td><em>P. capsici</em></td>
<td>Chilli pepper</td>
<td><em>In vitro, in vivo</em></td>
<td>Medium</td>
<td>Jung i Kim (2005)</td>
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<td><strong>Burkholderia cepacia</strong> strain ASPB2D</td>
<td>Induction of ISR</td>
<td><em>P. nicotianae</em></td>
<td>Tobacco</td>
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<td>High</td>
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<td><strong>G. mosseae</strong></td>
<td>Competition</td>
<td><em>P. nicotianae</em> var. <em>parasitica</em></td>
<td>Tomato</td>
<td><em>In vivo</em></td>
<td>Medium to high</td>
<td>Trotta et al. (1996)</td>
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<td><strong>Glomus macrocarpum, Glomus fasciculatum</strong></td>
<td>SAR/ISR, competition</td>
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<td>Tomato</td>
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<td>Chili pepper</td>
<td><em>In vivo</em></td>
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<td>Organism</td>
<td>Type</td>
<td>Competition</td>
<td>Plant</td>
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<td><strong>Gluconacetobacter diazotrophicus PAL5, L5</strong></td>
<td>Antibiosis (pyoluteorin)</td>
<td><em>F. oxysporum, F. solani</em></td>
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<td><em>In vitro</em></td>
<td>Limitation of mycelia growth</td>
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<td><strong>Hebeloma crustuliniforme, Hebeloma sinapizans, Laccaria laccata, Paxillus involutus</strong></td>
<td>Competition</td>
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<td>-</td>
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<td><strong>P. fluorescens 89B61</strong></td>
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<td><strong>Paenibacillus illinoisensis KJA-424</strong></td>
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<td><em>In vitro</em></td>
<td>Protection against colonization by zoospores</td>
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<td><strong>Paxillus involutus 0262</strong></td>
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<td><strong>Penicillium striatisporum Pst10</strong></td>
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<td>Ph. oligandrum 1010</td>
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<td>Pseudomonas spp. 3A17</td>
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<td>Antibiosis</td>
<td>P. capsici</td>
<td>Cucumber</td>
<td>In Vitro</td>
<td>Inhibition of zoospore germination</td>
<td>Okamoto et al. (1998)</td>
</tr>
<tr>
<td>Serratia plymuthica A21-4</td>
<td>Macrocyclic lactone (A21-4) (inhibition of mycelia growth, creation of zoospores and sporangia)</td>
<td>P. capsici</td>
<td>1-year-old paprika</td>
<td>Potted, greenhouse</td>
<td>High</td>
<td>Shen et al. (2005)</td>
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<tr>
<td>Serratia plymuthica strain R1GC4</td>
<td>ISR</td>
<td>Ph. ultimum</td>
<td>Cucumber</td>
<td>Potted</td>
<td>Limitation of disease symptoms</td>
<td>Benhamou et al. (2000)</td>
</tr>
<tr>
<td>Streptomyces griseus H7602</td>
<td>Chitinase, β-1,3-glucanase, lipase, protease</td>
<td>P. capsici</td>
<td>1-year-old paprika</td>
<td>In vitro, potted</td>
<td>Medium</td>
<td>Nguyen et al. (2012)</td>
</tr>
<tr>
<td><strong>Streptomyces lydicus</strong> WYEC 108</td>
<td>Antibiosis, chitinase, production of siderophores</td>
<td><strong>Ph. ultimum</strong></td>
<td>Cotton, peas</td>
<td>In vitro, potted</td>
<td>Elimination of oospores, mycelium lysis, high protection effect</td>
<td>Yuan and Crawford (1995)</td>
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<tr>
<td><strong>Streptocyes sp. (15, 32, 93, PonSSII, GS93-23, GS43-5, GS2-21, GS8-22)</strong></td>
<td>Antibiosis</td>
<td><strong>P. medicaginis, P. sojae</strong></td>
<td>Lucerne, soy</td>
<td>In vitro, potted,</td>
<td>Reduction of disease symptoms</td>
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<tr>
<td><strong>T. asperellum PR11</strong></td>
<td>Competition, hyperpathogenicity</td>
<td><strong>P. megakarya</strong></td>
<td>Theobroma cacao</td>
<td>Field</td>
<td>Low</td>
<td>Deberdt et al. (2008)</td>
</tr>
<tr>
<td><strong>BCA</strong></td>
<td>Mechanism</td>
<td>Organism being eradicated</td>
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<td>References</td>
</tr>
<tr>
<td><strong>T. hamatum s382</strong></td>
<td>SAR</td>
<td><strong>P. capsici</strong></td>
<td>Cucumber</td>
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<td>High</td>
<td>Khan et al. (2004)</td>
</tr>
<tr>
<td><strong>T. harzianum</strong></td>
<td>β-(1,3)-glucanase</td>
<td><strong>Py. aphanidermatum, Ph. myriotylum</strong></td>
<td>Tobacco</td>
<td>In vitro, potted</td>
<td>Limitation of disease symptoms in sterile soil</td>
<td>Devaki et al. (1992)</td>
</tr>
<tr>
<td>11 isolates of Actinomycetes</td>
<td>β-1,3-, β-1,4- and β-1,6-glucanase</td>
<td><strong>P. fragariae var. rubi</strong></td>
<td>Raspberry</td>
<td>In vitro, potted</td>
<td>Reduction of root rot of raspberry</td>
<td>Valois et al. (1996)</td>
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</table>
is activated in mycorrhized plants, which leads to the increase in phenolic compounds concentration. The studies showed the reduction of root necrosis as a result of plants inoculation with mycorrhizal fungus, but the protective effect depended on the effectiveness of plant mycorrhization (Cordier et al. 1998). The authors stated that the mycorrhization of tomato plants triggered not only a local reaction but also a systemic immunity in the plant roots. Additionally, callose development was observed in mycorrhized cells, which built a barrier against infection.

Mycorrhizal species of *Glomus macrocarpum* and *Glomus fasciculatum* were used to counteract fusaric withering of tomato (*F. oxysporum* f. sp. lycopersici) (Kapoor 2008). In vase experiment, mycorrhized tomato plants have been reported to show twice as high phenolic compounds concentration, six times higher activity of PAL enzyme and over 18 times higher JA content (in fresh plant mass) in comparison with control plants. The studies showed also increased efficacy of biological control. *Macarocarpum* and *G. fasciculatum* species suppressed the disease symptoms on the tomato plants by, respectively, 75% and 78%.

Furthermore, promising results were obtained based on ectomycorrhizal species – waxy laccaria (*Laccaria lacata*). Chakravarty and Hwang (1991) proved that the species strongly suppressed the *F. oxysporum* mycelium growth on the Jack pine seedlings (*Pinus banksiana*) compared with other fungi. As a consequence of plant mycorrhization, the decrease in the number of isolated colonies of *Fusarium* fungi in the rhizosphere was observed, while culture filtrates of waxy laccaria applied to in vitro studies considerably suppressed sprouting of spores and hyphae of *Fusarium oxysporum*. Mycorrhizaded pine seedlings exhibited increased phenolic compounds content compared with the control plants, indicating the activation of resistance mechanisms (Chakravarty and Hwang 1991). The studies involving *L. lacata* species were continued focused on the protection of the stone pine (*Pinus pinea* L.) against the seedling blight caused by *F. oxysporum* and *F. verticilloides* (Machón et al., 2009). The experiment with plant pots filled with autoclaved a peat-vermiculite medium reported no substantial impact of antagonistic species on plant survival in terms of preemergence blight. Intensification of postemergence blight symptoms depended on the level of the mycorrhization of pine seedlings (Machón et al. 2009). Other ectomycorrhizal species - such as brown roll-rim (*Paxillus involutus*) was used to control the red pine (*Pinus resina* Ait.) root blight (*F. oxysporum*). After 14 days since the infection, the pine seedlings mortality reduced by 55% and *F. oxysporum* sporing by approximately 80% (Machón et al. 2009).

The results presented in the above subsection and related to the studies on the use of individual BCA microorganisms in biological control were obtained in the course of laboratory researches as well as the vase and greenhouse experiments. These experiments mainly involved artificial medium or sterilised soil. Under such conditions, introduced microorganisms competed only with the populations of pathogens inoculated to the plants, while high and medium effectiveness of blight pathogens control, which was achieved by the authors, is extremely difficult to obtain under field conditions. The above assumption is confirmed by the results of Deberdt et al., (2008), who applied PR11 *Trichoderma asperellum* strain to counteract black spot leaf diseases of cacao tree (*P. megakarya*). The studies showed statistically significant, over 20% reduction in disease symptoms compared with the control variant; however, much higher efficacy was measured when applying chemical protection, reporting approximately 2% damage within the research period.

The recipe for improving low efficacy of biological preparations observed under production conditions is a combined use of several compatible microorganisms in one treatment or in subsequent treatments conducted at different stages of the plant growth (Table 2). Since the most frequently applied biological control factors can synthesize secondary metabolites with antibiotic properties, in case of combined use of antagonistic microorganisms, it is necessary to do conformance tests under in vitro conditions.

The example of efficacious combination of microorganism to control *P. parasitica* and *Fusarium* fungi was the use of *Paenibacillus* sp. (B2) bacteria with antibiotic properties together with mycorrhizal species of *G. mosseeae*. Petri dish experiments confirmed that the bacteria do not show antagonistic properties towards *G. mosseeae*, but it reduces the growth of plant pathogens (Budi et al., 2000). The experiment under in vitro conditions showed the suppression of spores sprouting, as well as the hindered growth of *P. parasitica* hyphae and the mycelium of *F. oxysporum* and *F. culmorum*. The in vivo experiment demonstrated the decrease in the necrosis symptoms on tomato plants due to individual and combined application of antagonistic microorganisms. However, the most remarkable reduction in the disease symptoms resulted from the combined application of BCA microorganisms (63%) (Budi et al. 2000).
Other studies showed the effectiveness of the combined use of ectomycorrhizal species of *Paxillus involutus* and the *B. subtilis* – the bacteria strain with antibiotic properties (Hwang et al. 1995). *In vivo* experiment conducted in Erlenmeyer flask containing sterilised vermiculite proved that combined application of antagonists reduces by 16% the jack pine (*P. banksiana*) mortality from the infection caused by *F. moniliforme*.

High efficacy of biological control was reported after applying two antagonistic organisms to counteract cucumber seedling blight (*P. ultimum*) (Roberts et al. 2005). Two independent experiments showed that the best results in terms of the disease control were obtained after applying *T. viridans* (GL21) or combining *T. viridans* (GL3) with *Burkholderia cepacia* (BC-1) (Roberts et al. 2005).

There are also reports on the attempts of combined use of commercial biological preparations to control the blight diseases. Elliott et al. (2009) carried out the assessment of five commercial biological preparations containing: *B. subtilis* GB03, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. subtilis* QST 713, *S. lydicus* WYEC 108, *T. atroviride* CHS 861 and *G. virens* GL-21 in terms of their effectiveness in *P. ramorum* control. The efficacy was analysed based on the antagonism tests and *in vivo* experiments, using the leaves of plants sensitive to infection (*Caucasian rhododendron*, the Japanese camellia). In the Petri dish, the most efficacious preparation reducing the mycelium growth of all the *P. ramorum* strains (100% growth suppression) turned out to be the Plant Helper (*Trichoderma atroviride*), whereas in *in vivo* experiments this preparation did not produce any positive effects. In this case, the most effective was the Serenade preparation (*Bacillus subtilis* QST 713) displaying the average results on Petri dish. According to the authors, the lack of relationship between the Petri dish results and the leaves tests eliminates the first method as a reliable assessment of biological preparations (Elliott et al. 2009). In the antagonism tests of individual BCA, the authors proved lack of mutual antagonism between *T. atroviride* and *S. lydicus*, which allowed for the attempt to use two preparations altogether. However, the combined use of microorganisms did not produce more beneficial protective effects as compared with individual BCA application (Elliott et al. 2009). The attempts to control the blight of sugar beet seedlings (*Py. ultimum*) showed lack of improvement in biological control efficacy with respect to combined use of antagonists, in comparison with microorganisms used individually (Fukui et al. 1994), while the studies of de Boer et al. (2003) have indicated that incompatible isolates applied together in *in vitro* test gave identical results with the strains applied separately (RS56 and RS111) (de Boer et al. 1999).

Few studies on combined use of biological preparations were conducted under field conditions. These are for instance the studies of Kim et al. (2008), who used antagonistic bacteria of *S. plymuthica* (C-1), *Chromobacterium* sp. (C-61), *Lysobacter enzymogenes* C-3 to control *P. capsici* and other pepper pathogenic species (*R. solani, F. oxysporum* f *F. solani*).

In *in vivo* experiment, the authors noticed that among individually applied bacteria (which were mentioned above), *S. plymuthica* (C-1) strain had the strongest antagonistic effect on *P. capsici*, though the separate BCA showed the average effectiveness in the control of fungal complex. In *in vivo* experiments, the applied strains when combined with one another produced high protective effects in the control of pathogen complex, and obtained results were also verified in field experiments. BCA combination was applied on three different dates, eventually obtaining high efficacy of biological control in two independent experiments (Kim et al. 2008).

The control of sugar beet seedling blight (*Pythium*) using the antagonistic organisms of *Stenotrophomonas maltophilia* (W81) and *P. fluorescens* (F113Rif) was conducted by Dunne’s team under field conditions (Dunne et al. 1998). The authors demonstrated that the combined application of antagonistic microorganisms led to the decrease in the plant infections and was at the same level as the chemical control (Dunne et al. 1998).

In the studies of Ezziyyani et al. (2007) pepper blight (*P. capsici*) was controlled with the combination of two antagonistic microorganisms applied altogether: *T. harzianum* (2413), *S. rochei* (467). The studies showed that the lowest doses of *S. rochei* applied separately did not perform their protective function, the high doses caused delayed development of disease symptoms and the highest doses reduced the plant mortality.

Individual application of *T. harzianum* species did not improve the pepper plants health. Due to combined application of microorganisms, phytophthorais symptoms decreased by 79.8% in *in vivo* experiment, and by 74.8% in field experiment (Ezziyyani et al. 2007). Slight fall in the efficacy of biological control observed in field experiment (compared with the *in vivo* one) was explained with the fact that antagonistic BCA had to compete with homogenous microorganisms in natural soil, while in sterilised vase medium such competition did not occur.

Abo-Elyoussr et al. (2009) to control the cotton seedling blight caused by *P. debaryanum* and *F. oxysporum*
<table>
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<tr>
<th>Biological Control Agent</th>
<th>Mechanism</th>
<th>Organism to be eradicated</th>
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<tbody>
<tr>
<td>1<em>B. subtilis</em> GB03, 2<em>B. subtilis</em> QST 713, 3<em>Streptomyces lydicus</em> WYEC 108</td>
<td>1<em>Itrine (antibiotic), 1</em>ISR, 2<em>Antibiosis, 2</em>ISR, 3<em>Antibiosis, 3</em>chitinase, 3*production of siderophores</td>
<td><em>P. ramorum</em></td>
<td>Rhododendron caucasicum, Camellia japonica</td>
<td><em>In vitro</em> (detached leaves)</td>
<td>No improvement in the overall efficiency of the application of the BCA</td>
<td>Elliott et al. (2009)</td>
</tr>
<tr>
<td>LS213 (1<em>B. subtilis</em> GB03, B. amyloliquefaciens IN937a, chitosan), B. licheniformis CECT 5106, P. fluorescens CECT 5398, Chryseobacterium balustinum CECT 5399</td>
<td>Production of siderophores, SAR/ISR</td>
<td><em>F. oxysporum</em></td>
<td>Pepper, tomato</td>
<td><em>In vivo</em> (cartridge)</td>
<td>Medium to high</td>
<td>Domenech et al. (2006)</td>
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<th>Biological Control Agent</th>
<th>Mechanism</th>
<th>Organism to be eradicated</th>
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<tbody>
<tr>
<td><em>P. putida</em> 32-2,GR12-2, <em>P. fluorescens</em> A1, ML5, <em>P. aureofaciens</em> PGS12</td>
<td>Competition, production of siderophores, antibiosis</td>
<td><em>Ph. ultimum</em></td>
<td>Sugar beet</td>
<td><em>In vitro</em>, potted</td>
<td>Low in inclusive application of the BCA</td>
<td>Fukui et al. (1994)</td>
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<tr>
<td>Organism</td>
<td>Model System</td>
<td>Competition, Production</td>
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<td>Scope of the research</td>
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<td><em>P. putida</em> RE8, <em>P. fluorescens</em> RS111</td>
<td>Radish, Potted</td>
<td>Low</td>
<td>de Boer et al. (1999)</td>
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<td><em>P. putida</em> WCS358 i RE8</td>
<td>Radish, Potted</td>
<td>Low</td>
<td>de Boer et al. (2003)</td>
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<td><em>Serratia plymuthica</em> C-1, <em>Chromobacterium</em> sp. C-61, <em>Lysobacter</em> enzymogenes C-3</td>
<td>Pepper, Potted, greenhouse, field</td>
<td>High</td>
<td>Kim et al. (2008)</td>
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<td><em>Stenotrophomonas maltophilia</em> W81, <em>P. fluorescens</em> F113</td>
<td>Sugar beet, Pythium spp., In vitro, potted, field</td>
<td>High</td>
<td>Dunne et al. (1998)</td>
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<tr>
<td>Biological Control Agent</td>
<td>Mechanism</td>
<td>Induction of SAR, increase in the content of phenolic compounds in plants</td>
<td>Medium</td>
<td>Abo-Elyousr et al. (2009)</td>
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<tr>
<td><em>Trichoderma harzianum</em> AUSB-26328, AUSB-26330, <em>Paecilomyces lilacinus</em> AUSB-26336, RIS (Bion, SA)</td>
<td>Cotton, Greenhouse, field</td>
<td>Medium to high</td>
<td>Roberts et al. (2005)</td>
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<tr>
<td><em>Trichoderma harzianum</em> 2413, <em>Streptomyces rochei</em> 467</td>
<td>Pepper, Potted, field</td>
<td>High</td>
<td>Ezziyyani et al. (2007)</td>
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used *T. hamatum* (AUSB-26328), *T. harzianum* (AUSB-26330), *Paecilomyces lilacinus* (AUSB-26336) and synthetic resistance inducers (RIs): Bion (BTH) and salicylic acid (SA). In field studies, the most positive results concerning the reduction of both plant pathogenic species of *P. debaryanum* and *F. oxysporum* were obtained after applying the following variant: *P. lilacinus*, *T. harzianum*, SA and Bion. In consequence, the disease index of both pathogens decreased by 50% (Abo-Elyoussef et al. 2009).

4. Biological control – current status and development perspectives

Application of biological plant control agents in the EU countries is regulated by the Directive 91/414/EEC (EU 1991), the Directives: 2001/36/EC (EU 2001) and 2005/25/EC (EU 2005), as well as by the Regulation EC No. 1107/2009 (EU 2009). The European Commission precisely defines the rules for the producers registering for the use of BCA preparations. Diverse interpretation of law provisions among individual EU countries results in prolonged registration process, which is also different in each member state. Currently, Poland is one of the six member states, which do not have detailed law regulations on the use and registration of biological control agents. Expensive registration procedures and a great deal of time they involve result in 16 biofungicides currently available in Europe (www.rebeca-net.de).

Commercial biological preparations available on the market contain single strains of antagonistic microorganisms with a very specific mechanism of impact on pathogens. They are mainly used for emergency purposes in terms of particular lifecycle phase of pathogenic factor (Jumaid et al., 2013, www.rebeca-net.de). Most of the soil fungi species are considered cosmopolitan, which means that they can easily move to other environment (Gams 2007). Comparative analyses of soil fungi population conducted in various parts of the world indicate that both the number and the qualitative content of soil fungi taxa isolated from different environments are similar (Hawksworth 2001); therefore, according to Bae et al. (2011), the BCA species can be used in various environmental conditions.

A fundamental issue, which should be considered in terms of biological control, is achieving satisfactory effectiveness of biopreparations and repeatability of results in soil and on the plants under production conditions. To obtain a good effect, the biological factors must first of all colonise the plant tissues. Mutual interactions mainly concern competition for living space and nutrients. They developed in the coevolution process in the soil and on the plants where competition between the microorganisms is based solely on the colonization rate (growth rate, lifecycle rate, adaptation capacities or reproductive potential), but it is connected with dynamic competition between the microorganisms using all the possible means of defence and aggression. Microorganisms existing in specific ecological niche developed various strategies to fight off the competition. They include detoxification of secondary metabolites of other organisms, repression of genes responsible for the synthesis of metabolites, synthesis of antibiotics harmful for competitive organisms and resistance to antibiotics synthesised by other microorganisms (Duffy et al. 2003). The above listed interactions may result in lower effectiveness of biological control in natural ecosystems. In addition, mutual relations between microorganisms are affected by many factors not subject to control and determining the efficacy of protective activities. They include: environmental conditions, tem-perature, soil pH, presence of growth hindering factors and types of microorganism species living in particular niche (Benhamou 2004). For this reason, the studies on BCA application under *in vitro* conditions are not fully relevant to (there is no linear relationship) the results obtained under *in vivo* conditions (Elliot et al. 2009). Mathematical models used for the analysis of the course of disease taking into account the biological control mechanisms showed that the BCA efficacy depends on the colonization level of the plant tissues or the soil and on the BCA span of activity (Jeger et al. 2009), while the studies conducted by Zeng et al. (2012) proved that the effectiveness of the biological control depends on steady presence of BCA population introduced into the environment. In view of the above, it can be deduced that the BCA efficacy does not hinge on a short-term impact of antagonistic microorganisms, and breaking down the pathogen resistance mechanism does not guarantee a satisfactory protective effect. For this reason, the biological control efficacy in terms of the field crops is limited and the most successful use of BCA relates to greenhouse crops (Paulitz and Belanger 2001). Small effectiveness of biological control observed in some field experiments may also stem from the use of the antagonistic organisms with narrow mechanism of impact on pathogens; therefore, it is recommended to use the microorganisms with wide range of antagonistic capacities (Cook 1993). Specialised mechanism of impact on pathogenic microorganisms is associated with very
small probability of the host change, as for example, hyperparasite species of *Ampelomences quisqualis* – parasitising only the fungi responsible for powdery mildew (Angeli et al., 2012). According to some researchers, high host specialisation can be correlated with evolutionary liability (Parker and Gilbert 2004), while in the opinion of Heydari and Pessarakli (2010), hyperparasitising organisms introduced into the environment do not provide sufficient protection, since they exhibit an aggressive behaviour towards other organisms only under conditions of limited availability of nutrients.

The use of BCA affecting solely the specific type of pathogen has frequently no impact on other organisms inhabiting the niche, which can lead to the colonization of plants by other pathogenic organisms. This phenomenon is of paramount importance in terms of soil-borne pathogens producing similar or the same disease symptoms on the plants. In such case, lack of precise diagnostic procedures to be adapted by forest nurseries does not provide the answer for the question about efficacious elimination of specific organism, and as long as the plant is colonised by other pathogenic organisms the issue of effective disease control remains unresolved. Additional impediments are numerous endospore forms and pathogenic spores, which are dispersed in the groundwater and the soil. In the situation of severe competition between microorganisms, these forms enable the survival of the species, while the size of the fungi and hyperparasite bacteria population introduced into ecosystem is subject to reduction (Lourenco et al. 2006). To conclude, it should be stressed that the use of biological plant control is very difficult in comparison with chemical methods, since it requires extremely precise procedures for BCA application to specific pathogens and plant species as well as the biological knowledge of both target organisms and biological control factors. Due to this fact, the researchers should either search for new microorganisms with antagonistic properties to pathogens to extend the BCA arsenal, or they should focus on using local strains with the properties allowing for their use in particular habitats. Production of such biopreparations is obviously very difficult; however, the devoted effort will be compensated by the improvement of biopreparations efficacy as well as it will help to dispel many doubts concerning the microorganisms coming from distant parts of the world and their introduction into the environment. The recipe for enhancing the BCA efficacy is the combined use of synergistic factors of biological control, which improves the protective effect and enables to fight off larger number of pathogens (Dunne et al. 1998; Jetiyanon and Kloepper 2002). There are many reports on the application of several BCA, compatible in terms of mechanism of action (Table 2).

However, their interaction in the environment may raise some doubts. The BCA populations used simultaneously or in short time intervals may compete with each other (Xu et al., 2011), that is why the combined use of several BCA can generate beneficial, neutral or adverse protective effect. This effect depends on the target organism, the size of its population and selection of specific BCA (Kessel et al. 2002; Lourenco et al. 2006; Ezziyyani et al. 2007; Xu et al., 2010). Xu et al. (2011) when analysing the BCA activity model of various mechanisms of impact on the pathogen, arrived at the conclusion that the application of individual BCA with multiple mechanism of impact is more effective than using the combination of several BCA with various yet isolated antagonistic capacities. At the same time, Xu et al. (2011) did not exclude the increase in efficacy of combined BCA but stressed that synergistic impact is determined by mutual compatibility of microorganisms, which has to be confirmed by prior laboratory analyses. The above assumption has been substantiated with the newest results presented by Xu and Jeger (2013), who have showed that combined use of BCA of competitive and hyperparasitic properties resulted in the delayed epidemic progression.

It seems beneficial to preventatively introduce to this system the microorganisms stimulating the plant resistance response (SAR, ISR), that is before the period of the greatest sensitivity of plants or the highest activity of pathogen. It obviously requires thorough studies determining the procedures for the combined use of specific BCA with regard to particular pathogen, plant and habitat. A successful method for reducing Oomycetes is to mix biological control with standard plant protection products (Silva et al. 2004) and to combine BCA with synthetic resistance inducers (*RIs*) (Abo-Elyour et al. 2009). The use of mycorrhizal fungi, which improve the plant growth and are the antagonist of pathogenic fungi, is important for the development of biological control methods in forest nurseries (Pozo et al. 1999). It is beneficial due to the lack of negative impact on the environment and the financial considerations (Harrier and Watson 2004). The effectiveness of the biological fungi preparations can be improved by enriching their composition with calcium salts (CaCO₃), which stimulates fungi sporulation and increases the enzyme synthesis (Saxena et al., 2001, Wuyep et al., 2003). Calcium
included in biopreparations enhances their quality and stimulates the antagonist activity after biofungicide application (Spadaro and Gullino 2004). The studies conducted by Sugimoto et al. (2008) showed that calcium ions hindered both the mycelium growth and *P. sojae* zoospore release, hence a deliberate enrichment of BCA preparations for countering oomycetes of *Phytophthora* species. This thesis has also been confirmed by von Broembesen and Deacon (1997), who proved the efficacy of CaCl$_2$ and Ca(NO$_3$)$_2$ in reducing *P. parasitica*, and by the studies on the use of ions Ca$^{2+}$ to protect *Quercus ilex* against *P. cinnamomi* (Serrano et al. 2012).

5. Conclusions

The literature data indicate a high potential of biological control for suppressing blight diseases in forestry, particularly with regard to the container nursery.

Application of biological control under production conditions requires formulation of precise procedures with respect to specific biofungicides, environmental conditions, the host and the target organisms.

The effectiveness of biological preparations can be improved by combining the treatment with chemical control as well as by using several antagonist organisms exhibiting tolerance towards each other and variously affecting the pathogen.

References


Kim H.S., Sang M.K., Jeun Y.C., Hwang B.K., Kim K.D. 2008. Sequential selection and efficacy of antagonistic rhizobacteria for controlling Phytophthora blight of pep-


Authors’ contribution

Translated by: Małgorzata Oszako