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LAYOUT

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Review paper

Sclerotinia species in Serbia and possibilities of their control

Milica Mihajlović[1]*, Jovana Hrustić[1], Maja Živanović[1], Mladen Petreš[2] and Brankica Pešić[1]

[1]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia
[2]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia
**Corresponding author: milica.mihajlovic@pesting.org.rs*

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SUMMARY

Sclerotinia species are economically important, necrotrophic and aggressive plant pathogens with a broad host range and worldwide distribution. They act as airborne or soilborne pathogens, and can be transmitted by seed. These pathogens can affect crops both during the growing season and after harvest. Yield losses due to Sclerotinia diseases in susceptible crops vary and may be as high as 100%. The most common pathogen from the genus *Sclerotinia* in Serbia is *S. sclerotiorum*. It occurs regularly on sunflower and its incidence may exceed 50% in some years, thus causing economically important crop losses in Vojvodina. Recently, two new species were detected in Serbia: *S. trifolium* in alfalfa and *S. minor* in lettuce plants. Diseases caused by *Sclerotinia* spp. are difficult to control due to the long-term survival of sclerotia in the soil and development of airborne ascospores. As with many other diseases, there is no single treatment that can completely control these pathogens. Implementation of multiple strategies, such as cultural practices (sanitation, crop rotation and tillage), physical, chemical and biological protection, as well as deployment of resistant cultivars, is necessary for effective disease management.

Keywords: plant-pathogenic fungi, *Sclerotinia sclerotiorum*, *S. trifoliorum, S. minor*, identification, plant disease control

INTRODUCTION

Sclerotinia species are some of the most destructive, necrotrophic and aggressive plant pathogens with a broad host range and worldwide distribution (Rothmann & McLaren, 2018). Three closely related *Sclerotinia* species - *Sclerotinia sclerotiorum, Sclerotinia minor* and *Sclerotinia trifoliorum,* are considered the most important species of this genus. Depending on their hosts, species in this genus cause diseases called rot, white mold, Sclerotinia drop rot, late blight or watery soft rot (Farr et al., 1989). *Sclerotinia* spp. cause a wide range of symptoms on above-ground plants parts. Symptoms include yellowing, leaf collapse, and water-soaked lesions with the appearance of white, fluffy fungal threads and black, dormant structures, the sclerotia (Kim & Cho, 2002; Morrall et al., 1972).

Sclerotinia species are capable of airborne or soilborne spread, acting as airborne or soilborne pathogens. In addition, the pathogens can be transmitted by seed, which is an important route of dissemination. Accordingly, there are significant differences in the epidemiology of these two types of diseases caused by *Sclerotinia* spp. regarding the effects of weather conditions on their occurrence (Purdy, 1979). Sclerotia have a major role in disease cycles since they are able to remain dormant in soil for up to 10 years (Adams & Ayers, 1979; Willetts & Wong, 1980). These structures allow the pathogen to persist in the absence of a host and act as a source of infection in following crops. The depth at which sclerotia are buried in soil has a significant impact on their ability to survive. Sclerotia situated deeper in the soil, 10-30 cm, remain alive longer than those in the top 5 cm soil layer. Also, the viability of sclerotia depends on their size - smaller sclerotia have less food resources and are more easily destroyed by soil organisms than larger sclerotia (Ćosić et al., 2012).

Sclerotinia diseases are one of the major causes of agricultural crop losses, despite regular applications of fungicides (Saharan & Mehta, 2008). These pathogens can affect crops both during the growing season and after harvest, causing significant losses to commercially valuable products. Economic losses come from entirely unmarketable collapsed vegetable crops and from decreased grain or oilseed harvests resulting from a decline in seed weight or quality. Yield losses due to Sclerotinia diseases in susceptible crops vary and may be as high as 100% (Purdy, 1979). Every year, Sclerotinia diseases cause hundreds of millions of dollars' worth of yield losses worldwide. *S. sclerotiorum* causes annual crop losses of over $200 million in the United States while an outbreak of Sclerotinia head rot in sunflower in 1999 resulted in crop losses of $100 million (Bolton et al., 2006). Sclerotinia stem rot of soybean has been recently ranked in the top 10 yield-reducing diseases in the USA (Willbur et al., 2017). Losses in peanut production in North Carolina due to Sclerotinia blight have been estimated to reach between $1 and $4 million per year (Smith et al., 2008). Lettuce is also highly susceptible to *Sclerotinia* species. In the United Kingdom, losses in field-grown lettuce are typically 10%, but they may reach 50% under wet conditions (Young et al., 2004). Despite the use of fungicides, losses caused by *S. minor* on lettuce in intensive lettuce-growing areas of Australia are estimated to range from 10 to 45% (Porter et al., 2002).

The most common Sclerotinia disease in Serbia is Sclerotinia wilt, caused by *S. sclerotiorum*. It occurs on sunflower at a rate of 15-20% on average, although the incidence may exceed 50% in some years (Marić et al., 1988). According to Tančić et al. (2011), Sclerotinia wilt causes economically important crop losses in Vojvodina. Recently, two new species have been described in Serbia: *S. trifolium* in alfalfa and *S. minor* in lettuce plants (Mihajlović et al., 2016a; 2022a). There is a lack of data on yield losses caused by *S. trifoliorum* in our country. *S. minor* was described in Serbia in 2020, and up to our knowledge, this pathogen has appeared in three distinct lettuce-producing regions, with an incidence of up to 50% (Mihajlović et al., 2023a).

Sclerotinia sclerotiorum

Taxonomic group: Fungi, Ascomycota, Leotiomycetes, Sclerotiniaceae
Teleomorph: *Sclerotinia sclerotiorum* (Lib.) de Bary 1884

The ascomycete fungus *S. sclerotiorum* is one of the most damaging soilborne fungal pathogens, affecting more than 600 plant species, including many commercially important crops (Liang & Rollins 2018). It is the most important *Sclerotinia* species that has a tremendous economic impact on crop production worldwide (Hao & Subbarao, 2005).

Hosts - *S. sclerotiorum* is a nonspecific plant pathogen. The host range of this pathogen comprises more than 600 plant species, including almost all dicotyledonous and some monocotyledonous plants (Liang & Rollins 2018). It can infect many crops of economic importance, such as sunflowers, beans, soybeans, canola, cotton, potatoes, peas, tomatoes, lettuce and rapeseed, as well as monocotyledonous plants such as tulips and onions (Xu et al., 2015; Liang & Rollins, 2018). Although *S. sclerotiorum* is a widespread, destructive necrotrophic pathogen of dicot plants that causes enormous economic losses every year, it has recently been discovered that not only could it grow as a beneficial endophyte in wheat, rice, barley, corn and other cereal plants, but it also provided protection against Fusarium head blight and wheat rust. To describe microorganisms that may behave as destructive pathogens on one set of plants, while still living mutualistically as endophytes on another group of plants (split nutritional strategy), Tian et al. (2020) coined the term "schizotrophism". Their study indicated that a broad-spectrum pathogen of one group of plants may serve as a biocontrol agent in another group of plants, where they can be utilized as biocontrol agents.

Symptoms - Vegetable crops are susceptible to *S. sclerotiorum* infection at any stage of development. Symptoms typically develop on stems, lower leaves, or

the tops of densely grown crops (Willbur et al., 2019). They include browning, water-soaking lesions, wilting, bleaching, accompanied by distinctive white cotton-like mycelium on infected leaves, stems, fruits, and petioles. Under wet and cold conditions, this fungus rapidly grows inside the infected host tissue and produces symptoms of browning, bleaching, and wilting, which results in necrosis, stunting, premature ripening, and wilting of the host. Later the diseased host tissue becomes soft and watery, which finally results in the crop's complete failure (Figure 1). After destroying the host plant, this fungus develops inside diseased tissue, forming black sclerotia (Liang & Rollins, 2018; Kim & Cho, 2002).

Disease cycle - Sclerotia, the long-lasting survival structures of the pathogen, have a major role in the disease cycle. They have an ability to remain dormant in soil for up to 10 years (Adams & Ayers, 1979; Willetts & Wong, 1980). Depending on environmental conditions, sclerotia can germinate directly by developing mycelium (myceliogenic - asexually) or indirectly (carpogenically - sexually) by producing ascospores that can be released into the air from apothecia and asci and attack the above-ground parts of host plants (Aldrich-Wolfe et al., 2015).

Aerial infection, also known as sclerotial **carpogenic germination**, occurs when sclerotia produce apothecia, fungal structures containing ascospores that are required for dissemination and infection. Carpogenic germination causes most above-ground infections in the field, where 1–20 apothecia can produce up to 2,000,000 ascospores on average (Wu et al., 2007). When ascospores land on susceptible host tissue, they can germinate under favourable conditions and start a new cycle of infection. Sclerotia should be maintained at low temperatures in order to break dormancy and germinate carpogenically, and the most optimal temperature range is between 10 and 20°C. It has been demonstrated that the production of apothecia stopped at temperatures higher than 26°C and the optimum temperature for their production was 21°C (Clarkson et al., 2004, 2007). Viable ascospores have the ability to spread over long distances. Ungerminated ascospores can survive in crop canopy for up to 12 days, depending on location and environmental factors (Willbur et al., 2019). Temperatures over 21°C and exposure to UV light both enhance ascospore mortality. Once the ascosporic mycelium makes contact with a susceptible tissue, it produces an appressorium, and penetration occurs either by mechanical disruption of host cuticle or through natural openings (Johnson & Atallah, 2014).

Unlike carpogenic germination, **myceliogenic sclerotia germination** results in the germination of mycelium directly from sclerotia (O'Sullivan et al., 2021). High humidity is required for germination of *S. sclerotiorum* sclerotia (Huang, 1985). Mycelium can penetrate enzymatically or mechanically by producing appressoria, unless appressorial penetration occurs through stomata (Lumsden, 1979). During myceliogenic germination, sclerotia produce mycelia in the presence of exogenous nutritients. However, in the absence of exogenous nutrients, germination occurs only when the sclerotia are devoid of black pigments, as in the case of immature sclerotia (Smith et al., 2008). Myceliogenic germination is also triggered when normal black sclerotia with crusts are damaged by mechanical means, desiccant treatments or freezing (Bardin & Huang, 2001).

Sclerotinia trifoliorum

Taxonomic group: Fungi, Ascomycota, Leotiomycetes, Sclerotiniaceae
Teleomorph: *Sclerotinia trifoliorum* Eriksson, 1880

S. trifoliorum is a fungal pathogen that primarily affects leguminous crops, and other forage legumes worldwide in countries with temperate climates. This pathogen can cause significant yield losses and impact the productivity and quality of these crops.

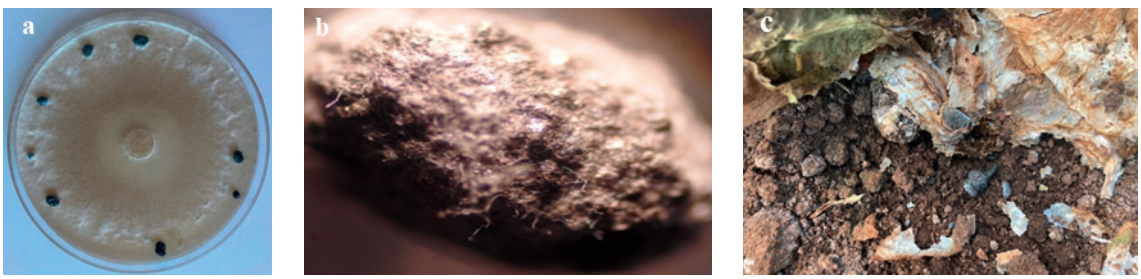

Figure 1. *Sclerotinia sclerotiorum*: a) colony grown on potato dextrose agar for 7 days, b) black sclerotia of *Sclerotinia sclerotiorum* obtained from infected lettuce plants (x10), c) lettuce drop sympthoms in the field

It is the causal agent of Sclerotinia crown and stem rot disease, also known as white mold or Sclerotinia clover rot, which causes one of the major problems in European red clover production (Purdy, 1979). This pathogen was first described in Scandinavia by Eriksson (1880), and there were reports in the early 1880s of a clover rot in England which was in 1897 attributed to *S. trifoliorum*.

Host - The fungus *S. trifoliorum* has a worldwide distribution with a narrow range of hosts mainly limited to species in the family Leguminosae and particularly to forage legumes (Kohn, 1979). In comparison to *S. sclerotiorum* and *S. minor*, *S. trifoliorum* has a relatively limited host range, comprising mostly cool season legumes. The reason for such a limited host range is unknown. *S. trifoliorum* is reported to cause diseases in 21 genera, with major losses occurring mainly in legumes, particularly forage legumes like *Medicago* and *Trifolium* spp. (Willetts & Wong, 1980).

Symptoms - The first symptoms of this disease are yellowing leaves, and hollowed out and collapsed stem. Forage legume rot caused by *S. trifoliorum* occurs as a soft rot of the crown and roots, beginning with brown leaf lesions that progress to stems and shoots (Figure 2). New growth of infected plants starts to collapse and finally dies. White mycelium often covers dead tissues, especially during rainy periods (Barbetti & You, 2014). Even though plants can be infected at any stage of development, losses are typically the highest when infection occurs at the seedling stage. This disease is most commonly observed under cool and humid conditions, often during periods of extended rains or high soil moisture. The fungus produces sclerotia, compact resting structures that can survive in the soil or plant debris for several years, contributing to the persistence of disease in fields (Kanbe et al., 2002).

Disease cycle - *S. trifoliorum* survives in the soil or plant debris as sclerotia (Willetts & Wong, 1980). Precise details of the disease cycle can vary depending on host plant, environmental conditions, and geographic location. In autumn, cool and moist conditions usually induce the formation of apothecia on sclerotia in the soil, which, near the end of October, release large quantities of ascospores into the atmosphere. Ascospores are dispersed by wind and can infect red clover leaves by direct penetration or via stomata. Ascospores are assumed to spread over large distances (Delclos & Raynal, 1995), but no exact data are available.

During winter, when plants are more susceptible due to winter stress, the fungus colonizes the entire plant. New sclerotia start appearing on diseased plants,in early spring. The next fall, sclerotia can generate new ascospore inoculum or they can stay latent in the soil for up to seven years. The mycelium of *S. trifoliorum* can infect various plant organs, including stems, leaves, and crowns. It can enter the plant through wounds, natural openings, or directly penetrate plant tissue. The development of appressoria, which directly penetrate the cuticle with or without enzymatic activity, allows *S. trifoliorum* to infect healthy tissues (Lumsden, 1979). Instances of host stomatal penetration have been documented rarely (Prior & Owen, 1964).

Depending on the weather, the disease can be nearly non-existent or completely absent, with the best conditions being a humid fall, which is required for ascospore germination, and a warm, humid winter with brief episodes of frost. Winters that are cold and dry decrease mycelial development, which stops the disease from progressing. The mycelium of *S. trifoliorum* can infect various plant organs, including stems, leaves, and crowns. It can enter the plant through wounds, natural openings or directly penetrate plant tissues. Once inside the plant, the fungus continues to grow, producing white mycelial mats on the infected tissue. The mycelium can spread within the plant, causing rotting, wilting, and decline of the affected plant parts.

Figure 2. *Sclerotinia trifoliorum*: a) colony grown on potato dextrose agar for 7 days, b) white wilt symptoms on alfalfa, c) irregular patches in alfaalfa field caused by *Sclerotinia trifoliorum*

The fungus may also produce sclerotia within infected tissues. As the disease progresses, the fungus forms sclerotia that can be found on or within infected plant tissues. They serve as survival structures, allowing the fungus to persist in soil or plant debris for future infection cycles (Marum et al., 1994).

Sclerotinia minor

Taxonomic group: Fungi, Ascomycota, Leotiomycetes, Sclerotiniaceae
Teleomorph: *Sclerotinia minor* (Jagger) 1920

S. minor is a widely distributed plant-pathogenic fungus, closely related to *S. sclerotiorum* and *S. trifoliorum* (Melzer et al., 1997). In contrast to the other two species of the genus *Sclerotinia, S. minor* mainly infects plants by myceliogenic germination to produce hyphae which attack plant tissues directly. Apothecia are seen in the field very rarely (Abawi & Grogan, 1979).

Host range – *S. minor* is less common than *S. sclerotiorum* and has narrower host range (Willetts & Wong, 1980). The most susceptible species are dicotyledonous with only three monocotyledonous plant species, namely asparagus, tulip and banana, being reported as hosts (Watson, 2007). *S. minor* primarily infects lettuce crops and is known to cause lettuce drop. However, the host range of *S. minor* is not limited to lettuce and other plant species within the Asteraceae family (endive, chicory, and radicchio). *S. minor* is a pathogen of many economically important crops including soybean, sunflower, common bean, cucumber, lettuce, spinach, cabbage, sweet potato, Irish potato, pepper, tomato, peanut, and many other (Melzer et al., 1997).

Symptoms - Disease symptoms caused by *S. minor* are similar to those caused by *S. sclerotiorum*. Initially, water-soaked lesions appear on the infected stems or leaves (Figure 3). As the disease progresses, fluffy white mycelium may become visible, lesions become bleached and necrotic, while infected stems become shredded and die (Smith et al., 2008). Only plant stems and leaves that are in close contact with soil are susceptible to infection with *S. minor,* whereas *S. sclerotiorum* can also affect upper leaves of the plant by airborne ascospores. After infection establishment, *S. minor* causes a brown, soft decay that will eventually contribute to the destruction of the plant crown tissue, resulting in wilting and collapse of entire plants, making them unsuitable for harvesting.

Disease cycle – *S. minor* has myceliogenic germination, resulting in the development of mycelium directly from sclerotia (O'Sullivan et al., 2021). The mycelium of *S. minor* attacks lower branches and rapidly invades tissue, decomposing plant cells. Plants may be infected at any growth stage from seedling to maturity. Under moist and cool conditions, the fungus rapidly invades host tissues, in which a light brown, watery rot develops, and a white, fluffy mycelial mass forms on tissue surface. Disease is often in aggregated distribution patterns within infested fields. Dispersal and transmission of the pathogen is exclusively by direct contact with germinating sclerotia in order to produce infective hyphae, which colonize plants and eventually produce more sclerotia to recur in the soil. Plant-to-plant spread between diseased and healthy plants can occur by direct contact with infected tissue (Subbarao, 1998). Sclerotia are produced abundantly on dead tissue of the plant as it dies. Some sclerotia may remain on dead plant cells as overwintering inoculum or be excreted from plant tissue into the soil. At the mycelial stage, the fungus infects a healthy plant, and the cycle begins again. After harvest, plant debris infested with *S. minor*, including sclerotia already formed, get into the soil where the inoculum remains dormant until the next planting (Purdy, 1979).

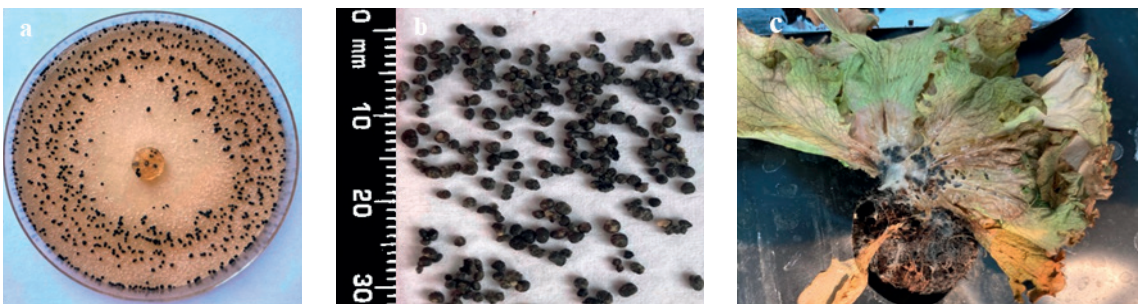

Figure 3. *Sclerotinia minor*: a) colony grown on potato dextrose agar for 7 days b) sclerotia of *Sclerotinia minor* obtained from infected lettuce plants, c) sympthoms on lettuce plant cultivated under greenhouse conditions

IDENTIFICATION

Morphological identification

Traditional morphological traits, such as cultural characteristics, sclerotial size, ascus and ascospore dimensions, and timing of apothecial development in the field, host association, Mycelial Compatibility Test (MCT) and disease symptoms are not always accurate and rapid in differentiating *S. sclerotiorum, S. trifoliorum*, and *S. minor* as distinct species. Furthermore, no differences in hyphal structures of these species have been reported (Kohn, 1979; Willetts & Wong, 1980).

Isolates within a species vary in colony colour, type, and mycelial development. Most isolates have been found to generate white colonies, while the others produced off-white colonies (Rather et al., 2022). Colonies of *S. sclerotiorum* and *S. trifoliorum* on potato dextrose agar (PDA) media consist of white to grey mycelium. The mycelium of *S. minor* is also white to grey, indistinguishable from *S. sclerotiorum* and *S. trifoliorum*, with a lot of sclerotia that are small, globose to irregular and black (Kim & Cho, 2002). *All Sclerotinia species readily produce sclerotia on infected plant material and in culture. Sclerotia dimension could be a preliminary criterion for separating Sclerotinia species (Ekins et al., 2005).* Sclerotia size is one of the differentiating traits, although it is especially unreliable for species identification because of overlapping sclerotia dimensions and shape under certain conditions. This might make it difficult to distinguish between species (Willetts & Wong, 1980). In spite of that, these characters are often used for diagnostic purposes. *S. sclerotiorum* and *S. trifoliorum* produce large sclerotia - the size of peas, whereas *S. minor*, on the other hand, produces small, sesame-sized sclerotia (Sharma et al., 2015). Moreover, compared to *S. minor*, *S. sclerotiorum and S. trifoliorum* produce fewer sclerotia (Morrall et al., 1972).

Morphological differentiation of *S. sclerotiorum* and *S. trifoliorum* relies on the size of ascospores within the ascus. *S. trifoliorum* shows dimorphism in ascospore size (two different-sized ascospores within a single ascus), whereas the ascospores of *S. sclerotiorum* and *S. minor* are monomorphic (Kohn, 1979; Uhm & Fujii, 1983).

The Mycelial Compatibility Test is a technique used to evaluate the genetic relationship of fungal isolates by examining the behaviour of their mycelia upon coming into contact with each other (Aldrich-Wolfe et al., 2015). Incompatibility occurs between mycelia when there is a clean space that has not been colonized between them. In between these two extremes, there are numerous other types of intermediate responses that can occasionally be observed macroscopically, but very often, microscopic studies are required to determine the interaction between the hyphae of various mycelial types. Wong and Willetts (1975) investigated the effects of mycelial interactions between *S. minor*, *S. trifoliorum*, and *S. sclerotiorum*. Based on these findings, it was proposed that *S. minor*, *S. trifoliorum*, and *S. sclerotiorum* be classified as three distinct species. *S. trifoliorum* was first distinguished from the other two by incompatibility line (Loveless, 1951). According to Tariq et al. (1985), the mentioned classification of mycelial interactions is in agreement with their findings. However, they asserted that the only incompatible interaction was a reaction which resulted in a visible zone of separation between the mycelia of different species. The authors additionally verified that mycelial interactions can serve as a means of distinguishing the three species, although only when combined with some other criteria.

Molecular identification

For fast, reliable and accurate identification of these species, the use of molecular methods, primarily the PCR technique, is necessary. Different genes and regions are used for *Sclerotinia* species identification (Table 1). Analyses based on 18S rDNA or the ITS region, the common methods for fungi identification, have revealed that species belonging to the family Sclerotiniaceae have almost identical sequences, so that the methods are not reliable for species identification (Freeman et al., 2002).

For identification of *Sclerotinia* spp., genes for β-tubulin, calmodulin, aspartyl protease, and glyceraldehyde 3-phosphate dehydrogenase are used in standard PCR protocols (White et al., 1990; Njambere et al., 2010; Powers et al., 2001; Cho et al., 2013; Staats et al., 2005).

Based on the sequence variation of laccase 2 (*Lcc2*), aspartyl protease (*Aspr*) and calmodulin (*Cad*) genes, Abd-Elmagid et al. (2013) developed specific primers to rapidly identify *Sclerotinia* species. A multiplex PCR assay developed for specific detection of *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and *S. homoeocarpa* is a useful, fast and reliable technique that ensures precise identification of isolates in only one reaction (Abd-Elmagid et al., 2013).

Table 1. Targets, primer codes, primer sequence, expected size of polymerase chain reaction products for *Sclerotinia* spp. identification

Target region	Primer code	Primer sequence (5' - 3')	Expected size (bp)
ITS[a]	ITS1/ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	540 *S. sclerotiorum* *S. trifoliorum* *S. minor*
ITS[a]	ITS5/ITS4	GGAAGTAAAAGTCGTAACAAGG TCCTCCGCTTATTGATATGC	560 *S. sclerotiorum* *S. minor* 1000 *S. trifoliorum*
18 S[a]	NS5/NS6	AACTTAAAGGAATTGACGGAAG GCATCACAGACCTGTTATTGCCTC	250 *S. sclerotiorum* 600 *S. trifoliorum*
18 S[b]	NS1/NS8	GTAGTCATATGCTTGTCTC TCCGCAGGTTCACCTACGGA	1750 *S. sclerotiorum* >1750 *S. trifoliorum*
β-tubulin[c]	TU1/TU2	CCTGAAAAGCACCCCACTAT ACGGCACGAGGAACATACTT	494 *S. sclerotiorum* *S. trifoliorum*
	TU2/TU3	ACGGCACGAGGAACATACTT AACTCAACTCGACCGATGCT	390 *S. trifoliorum*
β-tubulin[d]	Bt2a/Bt2b	GGTAACCAAATCGGTGCTGCTTTC ACCCTCAGTGTAGTGACCCTTGGC	452 *S. sclerotiorum* *S. trifoliorum* *S. minor*
Calmodulin[e]	STCadF/STCadR	TCCTAGATCGACTCT CCTCCTTT TCAAACGCCAAAGCT GTATG	97 *S. trifoliorum*
Aspartyl Protease[e]	SSasprF/ SSasprR	CATTGGAAGTCTCGTCGTCA TCAAACGCCAAAGCTGTATG	171 *S. sclerotiorum*
Laccase2[e]	SMLcc2F/SMLcc2R	CCCTCCTATCTCTCTTCCAAACA TGACCAATACCAATGAGGAGAG	264 *S. minor*
Glyceraldehyde 3-phosphate dehydrogenase[f]	G3PDHfor/G3PDHrev	ATTGACATCGTCGCTGTCAACGA ACCCCACTCGTTGTCGTACCA	985 *S. sclerotiorum* *S. trifoliorum* S. minor

[a]Njambere et al. (2008) and White et al. (1990)
[b]Powers et al. (2001) and White et al. (1990)
[c]Vleugels et al. (2012)
[d]Cho et al.(2013)
[e]Abd-Elmagid et al. (2013)
[f]Staat et al. (2005)

DISEASE MANAGEMENT

Diseases caused by *Sclerotinia* spp. are difficult to control due to the long-term survival of sclerotia in soil and development of airborne ascospores (Bolton et al., 2006). As with many other diseases, there is no single treatment that can completely control these pathogens. Growers therefore need to consider environmental variables, disease pressure, and risks when planning their management strategy. Preventing the spread of Sclerotinia diseases to other crops and regions requires early detection and diagnosis. Regular field inspections and monitoring for symptoms can aid in early detection, whereas laboratory examination may verify the pathogen's presence (Saharan & Mehta, 2008; Mihajlović, 2014; Mazumdar, 2021).

Implementation of multiple strategies, such as cultural practices (sanitation, crop rotation, and tillage), physical, chemical and biological protection, and deployment of resistant cultivars, would likely be necessary for effective disease management (Peltier et al., 2012; Mihajlović et al., 2015, 2017a).

Cultural Practices

Cultural and agronomic practices are an important factor in disease management because they reduce the amount of sclerotia in soil and mitigate disease severity. However, they are not sufficient by themselves to control the disease effectively. Cultural practices include crop rotation, plant density reduction, and practices to reduce ascospore production and release (Saharan & Mehta, 2008; et al., 2016c).

Crop rotation is not always an effective control practice against soilborne pathogens such as *Sclerotinia,* which has a wide host range and produces overwintering sclerotia that survive in soil for a long time (Rothmann & McLaren, 2018). Crop rotation with less susceptible crops will help reduce *S. minor*, but may not reduce *S. sclerotiorum* population. Small grain cereals, such as corn, wheat, barley, oats and sorghum, are not susceptible to infection with *Sclerotinia* spp. and are therefore acceptable for rotation. However, a break of two to three years may be required to decrease the amount of sclerotia in the soil (Peltier et al., 2012). Even though these small grain crops are still susceptible to *Sclerotinia* infection (Tian et al., 2020), avoiding infected or nearby fields for 1-4 years may be the best management strategy, provided that it is economically feasible. Lettuce drop, caused by *S. minor* and *S. sclerotiorum*, is an important disease of lettuce. It has already been demonstrated that rotating broccoli and lettuce reduces the amount of sclerotia in the field in the instance of *S. minor* infection of lettuce. Crop rotation with broccoli could be an effective strategy for reducing lettuce drop incidence caused by *S. minor*, particularly on farms with higher inoculum levels (Hao & Subbarao, 2005).

Resistant varieties - The use of Sclerotinia-resistant varieties may be the most effective way to reduce pesticide use. However, due to the specific nature of diseases caused by this pathogen, breeding programs have so far had limited success (Uloth et al., 2014; Lin et al., 2022). Resistant varieties are not yet available, although less severe lettuce drop may occur in varieties with upright growth, where leaves are more or less off the ground (Barbetti et al., 2014).

Sanitary measures - Sclerotia and mycelium can also be spread from infested to clean fields by contaminated agricultural equipment and footwear, contaminated and diseased seedlings and contaminated soil. Infected plants need to be found and destroyed as soon as possible prior to sclerotia development. To the greatest extent feasible, all debris and contaminated plant materials should be collected and destroyed (Saharan & Mehta, 2008).

Tillage operations have both positive and negative effects in reducing the sclerotia population in soil. While sclerotia can persist for several years in the plow layer, only those near the soil surface germinate and generate apothecia and ascospores. As a result, burying infected residues with a moldboard plow can prevent sclerotia from germinating. However, repeated plowing in another season might bring those sclerotia back to the surface, and any tillage activity can therefore contribute to sclerotia dissemination (Purdy, 1979).

Reducing fertilizers and delaying planting to control vegetative growth should also be taken into consideration, as overfertilization and early planting can produce tall, bulky plants during flowering that form a denser plant mass and increase the chance of disease incidence during periods of heavy rain (Webster et al., 2023).

Managing irrigation and moisture level might prevent soils from becoming excessively moist, and so promoting the development of *Sclerotinia* spp. The beds should be as high as possible to provide adequate drainage. Watering the soil while it is not actively in production might also help to decrease the survival of sclerotia, especially *S. minor*. Flooding soils with irrigation for 2-3 weeks during summer, drastically minimizes sclerotia viability. Nevertheless, this approach may not be relevant to all crops and producing regions (Matheron & Porchas, 2018). Keeping the optimum plant row spacing will help to produce a microclimate that will make it challenging for the fungus to survive. In addition, varietal selection is crucial to minimize excessive overlap of the leaves of nearby plants. By providing adequate ventilation, excessive moisture during vegetable cultivation will be reduced. *S. sclerotiorum* spreads primarily during the flowering or early stages of plant development, when ascospores can easily colonize the plant due to high amounts of water on leaves. White mold development is strongly induced by moisture content on leaves, and using sprinkler irrigation systems in vegetable production should therefore be minimized. Subsurface drip irrigation is less favourable for disease development than furrow irrigation, and high temperatures, soil moisture, and low oxygen levels can reduce the development of lettuce drop caused by *S. minor*. This can be done by starting an early-morning irrigation schedule that allows leaves enough time to dry during the day (Webster et al., 2023).

Weed control

Many broadleaf weeds serve as hosts for the pathogens and aid in their transmission between crops. When rotating with a non-host crop, the effect of tillage (or lack of it) on weed control is an additional consideration, as poor suppression of broadleaf weeds may lessen the benefit of crop rotation. If these weeds occur in a field, they may provide inoculum for a host crop (O'Sullivan et al., 2021).

Biological control

Environmentally friendly methods for eradicating microorganisms from soils by using bacteria, fungi or actinomycetes have become a replacement for chemical control of Sclerotinia disease (Mihajlović et al., 2012; Mihajlović et al., 2016b). Pathogen suppression biocontrol strategies include mycoparasitism, antagonism, competition for resources and space to enhance plant resistance and antibiosis (Ahemad & Kibret, 2014; Mihajlović et al., 2023b).

Bacterial biological control agents, including *Streptomyce*s spp. (Chen et al., 2016), *Bacillus* spp. (Hu et al., 2014; Mihajlović et al., 2017b; Mihajlović et al., 2023b), and *Pseudomonas* spp. (Lee et al., 2012) have been used successfully against *S. sclerotiorum*. Several species of fungi, such as *Coniothyrium minitans* or *Trichoderma* spp. are known to be antagonistic to *Sclerotinia* spp. (Vinale et al., 2008; Druzhinina et al., 2011; Hermosa et al., 2012; Mihajlović et al., 2022b). Some insects and nematodes have also been documented to have negative effects on *S. sclerotiorum* development (Coley-Smith & Cooke, 1971). According to Anas and Reeleder (1988), dark-winged fungus gnat larvae (*Bradysiu coprophila*) damage sclerotia during feeding, which affects sclerotia survival and increases their susceptibility to mycoparasitic species. Due to the action of the enzyme chitinase in salivary secretion, larval saliva inhibits the capacity of sclerotia to germinate. Moreover, *Trichoderma viride* parasitize sclerotia injured by *B. coprophila* more severely than healthy sclerotia (Anas & Reeleder, 1988).

Mycoviruses, which are viruses that infect fungi, have the potential to be used as novel biocontrol agents for fungal diseases (Zhang et al., 2022). *Sclerotinia* spp. are capable of hosting a variety of mycoviruses, including single-stranded circular DNA viruses, double-stranded RNA viruses, and single-stranded RNA viruses (Xie & Jiang, 2014). According to Yu et al. (2013), the DNA mycovirus *S. sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1) has the ability to infect and confer hypovirulence on *S. sclerotorium*.

In Serbia, only one bioproduct based on *Bacillus amyloliquefaciens* has been registered for use against *Sclerotinia* spp. in different crops (Team of Editors, 2022).

Chemical control

Fungicide application is necessary for efficient management of Sclerotinia diseases. The number of applications depends on weather conditions, the duration of crop vegetation and sensitive plant phenophases, such as flowers or petals availability for infection by ascospores (Mihajlović, 2014). More treatments are required for plants with longer flowering periods. A range of chemical substances with registration against *Sclerotinia* spp. are available in the USA, Canada, Australia, China and Europe, including: boscalid, fluazinam, fluxapyroxad, pyraclostrobin, penthiopyrad, picoxystrobin, prothioconazole, pyraziflumid and trifloxystrobin (Wang et al., 2015; Derbyshire & Denton-Giles, 2016; Kikutake et al., 2020; Team of Editors, 2022).

In Serbia, the majority of active ingredients registered for *Sclerotinia* spp. suppression are intended for application in field crops, primarily oilseed rape and sunflower. Thus, azoxystrobin is registered for sunflower and oilseed rape, while the triazole fugicides metconazole, prothioconazole and difenoconazole are permitted in oilseed rape. Boscalid, alone or in combination with pyraclostrobin, is registered for the suppression of white mold in rapeseed, sunflower, flax, as well as soya, mustard, hazelnut and walnut. To manage Sclerotinia disease in vegetable crops only a few registered products are available in Serbia, mostly for leaf crops such as lettuce, rocket, endive, and spinach (Table 2). New active ingredients from the pyrazole-4- carboxamide group, fluxapyroxad (+ difenoconazole) and penthiopyrad, are allowed for use in leaf vegetables, while fluxapyroxad (+ difenoconazole) can be used in carrots and parsley, besides leaf vegetables. Cyprodinil in combination with fludioxonil is registered for a wide range of vegetable crops, including leaf crops, melons, zucchini, squash, and beans. The only active ingredients with low resistance risk that are available for use in vegetable crops are copper oxychloride and *B. amyloliquefaciens*-based biofungicide. All other mentioned groups of fungicides have been listed by the Fungicide Resistance Action Committee (FRAC) as medium- to high-risk for resistance development, and should be used in accordance with the resistance management strategy (Team of Editors, 2022).

Table 2. List of fungicides with FRAC risk registered against Sclerotinia diseases in Serbia

Active ingredient	Chemical group	FRAC risk[1]	Crop
Azoxystrobin	Strobilurine	High	Sunflower, oilseed rape
Bacillus amyloliquefaciens	Biological control agent	Low	Lettuce, strawberries, cucumber, zucchini, melon, watermelon, beans, oilseed rape, spinach, rocket, radish, endive, common corn salad
Boscalid	Carboximide	Medium to high	Sunflower, oilseed rape
Boscalid + piracloxystrobin	Carboximide + strobilurine	High	Sunflower, oilseed rape, sunflower, soya, flax, mustard, hazelnut, walnut
Copper oxychloride	Inorganic	Low	Lettuce
Cyprodinil + fludioxonil	Anilino-pyrimidines + phenylpyrrole	Medium	Lettuce, rocket salad, endive, radicchio, common corn salad, chard, chives, leek, melon, watermelon, bean
Difenoconazole	Triazole	Medium	Oilseed rape, sugar beet
Fludioxonil	Phenylpyrroles	Low to medium	Sunflower, soya, oilseed rape
Fludioxonil + difenoconazole	Phenylpyrroles + triazole	Medium to high	Lettuce, rocket salad, endive, radicchio, common corn salad, carrot, parsley
Fludioxonil + prothioconazole	Phenylpyrroles + triazole	Medium	Soya, oilseed rape, sunflower
Fluopyram + prothioconazole	Pyridinyl-ethylbenzamides + triazole	Medium to high	Soya, oilseed rape, sunflower
Fluxapyrad + difenoconazole	Pyrazole-4- carboxamides + triazole	Medium to high	Lettuce, rocket salad, endive, radicchio, common corn salad, carrot, parsley
Metconazole	Triazole	Medium	Oilseed rape
Penthiopyrad	Pyrazole-4-carboxamides	Medium to high	Lettuce, rocket salad, spinach,
Prothioconazole	Triazole	Medium	Oilseed rape

[1]FRAC Code List ©*2022: Fungal control agents sorted by cross-resistance pattern and mode of action

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Vrste roda *Sclerotinia* u Srbiji i mogućnosti njihovog suzbijanja

REZIME

Vrste roda *Sclerotinia* su polifagne, nekrotrofne fitopatogene gljive koje izazivaju ekonomski značajne štete u poljoprivrednoj proizvodnji širom sveta. Poseduju sposobnost širenja vazdušnim putem i putem zemljišta, a mogu se prenositi i semenskim materijalom. Vrste ovog roda ugrožavaju biljnu proizvodnju tokom perioda vegetacije, ali i nakon skladištenja, a gubici koji nastaju mogu dostići i 100%. Najzastupljenija vrsta roda *Sclerotinia* u Srbiji je *Sclerotinia sclerotiorum*. Redovno se javlja na suncokretu i u pojedinim godinama može izazvati štete i preko 50%. Nedavno su u Srbiji opisane dve nove vrste ovog roda: *Sclerotinia trifolium* na lucerki i *Sclerotinia minor* na biljkama zelene salate. Suzbijanje vrsta roda *Sclerotinia* je vrlo izazovno, zbog sklerocija, tvorevina za preživljavanje, koje se mogu održati dugi niz godina u zemljištu, ali i zbog prisustva askospora u vazduhu. Kao i kod mnogih drugih prouzrokovača bolesti, ne postoji jedinstven tretman koji može u potpunosti suzbiti ili eliminisati patogene ovog roda. Implementacijom različitih strategija, kao što su primena agrotehničkih mera, hemijska i biološka zaštita, moguće je uticati na širenje bolesti uzrokovanih *Sclerotinia* spp.

Ključne reči: patogene gljive, *Sclerotinia sclerotiorum*, *S. trifoliorum*, *S. minor*, identifikacija, suzbijanje bolesti

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Original scientific paper

The effects of medium nutritional profile on *Bacillus* sp. Par 3 plant-growth promoting and biocontrol activity against *Botrytis cinerea*

Tatjana Dujković[1]*, Ivana Pajčin[1], Vanja Vlajkov[1], Olja Šovljanski[1], Siniša Markov[1], Marta Loc[2], Mila Grahovac[2] and Jovana Grahovac[1]

[1]University of Novi Sad, Faculty of Technology Novi Sad, Bulevar cara Lazara 1, Novi Sad, Serbia
[2]University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, Novi Sad, Serbia
**Correspponding author: tatjana.dujkovic@uns.ac.rs*

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SUMMARY

Substantial agricultural losses resulting from plant diseases caused by different plant pathogens are one of the worldwide challenges today. Among these, *Botrytis cinerea*, responsible for gray mold disease, stands out for its capacity to devastate significant quantities of diverse valuable crops. Utilization of biocontrol agents for suppressing phytopathogens has become imperative, and bacteria from the genus *Bacillus* hold an immense potential due to their rapid replication rate, resistance to adverse environmental conditions, enhanced effectiveness in promoting plant growth and broad-spectrum activity. The objective of this study was to determine the best sources of carbon, nitrogen and phosphorus in cultivation media with the aim of maximizing both antimicrobial activity against *B. cinerea* and plant-growth-promoting (PGP) potential during the early stages of cucumber plant development, exhibited by *Bacillus* sp. isolate Par 3. Antimicrobial activity was tested using the well diffusion method. The influence of *Bacillus* sp. isolate Par 3 on plant germination was tested on cucumber seeds. The largest inhibition zones were achieved in two cases, with 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source. Seeds treated with a culture liquid of *Bacillus* sp. isolate Par 3 using the optimized medium exhibited the best results in terms of cucumber germination percentage (100%), root length (53.09 mm) and shoot length (13.26 mm). *Bacillus* sp. Par 3 isolate was identified as *Bacillus subtilis* using 16S rRNA gene sequencing. The results of this study underscore the significance of media optimization for the production of biocontrol agents, taking into account both antimicrobial efficacy and PGP characteristics.

Keywords: *Bacillus subtilis*, *Botrytis cinerea*, cucumber, nutrient medium optimization, plant growth promotion, antimicrobial activity

INTRODUCTION

Rising human population and a need for adequate food supplies present significant challenges for the agricultural sector. Besides the already demanding task of meeting these needs, crop damage caused by pathogens also adds to the complexity. Pathogen infections have been approximated to result in a global food crop loss of 10-16%, equivalent to an economic loss of around 200 million euros each year (Toral et al., 2020).

The necrotrophic fungus *Botrytis cinerea* is a pathogen responsible for significant economic losses, able to attack more than 200 plant species, including tomato, potato, oilseed rape, kiwi fruit, cucumber, and other high-valued and important economic crops (Yang et al., 2020). While this pathogen mainly targets dicotyledonous plants, it can also infect some monocotyledonous plants. *B. cinerea* is the most destructive on mature plant parts or senescent tissue, even though it typically gains entry to such tissues during earlier stages of crop development. After entering tissue, the pathogen remains dormant until changes in the host's environment and physiology trigger sudden rotting. Common symptoms on leaves and soft fruit include soft rot, which manifests as the collapse and water-soaking of parenchyma tissues, followed swiftly by the appearance of gray masses of conidia (Williamson et al., 2007). This phytopathogenic fungus is difficult to control because it has a broad host range, various attack modes, and both sexual and asexual stages to survive under favorable, as well as unfavorable conditions (Hua et al., 2018).

One of the plants most affected by *B. cinerea* is cucumber. Cucumber is one of the most widely used vegetables and a member of the popular Cucurbitaceae family. It holds a vital place in various human diets and is commonly consumed fresh in salads. However, the presence of cucumber gray mold as a severe disease in cucumber cultivation raises concerns about food safety (Soliman et al., 2015).

Currently, chemical fungicides are the most used agents against gray mold caused by *B. cinerea* and they represent about 8% of the global pesticide market. However, the excessive use of chemical pesticides and fertilizers in agriculture has resulted in the accumulation of harmful residues in the environment, which poses risks to human health. These concerns have spurred the exploration of alternative approaches to pest and disease management (Toral et al., 2020).

Biocontrol agents are conducive to sustainable agriculture as they aid in reducing reliance on chemical pesticides. This not only minimizes negative effects on the environment and human health but also prevents the development of pathogen resistance to chemical pesticides. Species belonging to the *Bacillus* genus are widely utilized as biocontrol agents (BCAs) applied as biofertilizers or biopesticides in different crops and against a variety of soil-borne diseases. The widespread use of *Bacillus*-based bioproducts can be attributed to several distinctive traits of this genus, such as rapid replication rate, resistance to adverse environmental conditions, increased efficiency in plant growth promotion, and broad-spectrum activity (Samaras et al., 2021). The aim of this study was to determine the best sources of carbon, nitrogen and phosphorus in cultivation media in terms of maximizing the biocontrol activity against *B. cinerea* and PGP potential in the initial phases of cucumber plant development exhibited by *Bacillus* sp. isolate Par 3.

MATERIALS AND METHODS

Microorganisms

The antagonistic microorganism used in this study was *Bacillus* sp. isolate Par 3, isolated from tomato rhizosphere by using the selective medium HiCrome *Bacillus* agar (HiMedia Laboratories, India). The procedure was as follows: 1 g of soil sample was mixed with 9 ml of saline solution, subjected to heat treatment (100 °C, 8 min), serially diluted (10 and 100-fold), and placed on the surface of a selective medium (100 µl, HiCrome *Bacillus* agar, Himedia Laboratories, India), followed by incubation at 28 °C for 48 h and selection of single colonies. Colony selection and incubation steps were repeated until visually pure cultures were obtained. The isolate was kept on a nutrient agar slant (4 °C). Biochemical characterization of the isolate *Bacillus* sp. Par 3 was done using a VITEK2 device and BCL cards according to the manufacturer's instructions (Biomerieux, France). The isolate was identified by the 16S rRNA gene sequencing (Macrogen, Netherlands) of PCR (polymerase chain reaction) products obtained using the primers 27f and 1492r. The PCR procedure and genomic DNA isolation were previously described (Pajčin et al., 2020). The 16S rRNA gene sequence (1411 bp) was deposited in the NCBI GenBank database under accession number OR690892 and compared to the NCBI GenBank database sequences using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Botrytis cinerea isolate R9, which was used as a test pathogenic microorganism in this research, was isolated from cucumbers with symptoms of gray mold and stored on SMA (Sabouraud maltose agar, Himedia India) at a temperature of 4°C. Identification was confirmed by polymerase chain reaction by amplifying and sequencing

the amplified region using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White et al., 1990). Gene sequences were analyzed using the BLASTn algorithm online at http://blast.ncbi.nlm.nih.gov/ with already deposited sequences in the NCBI GenBank database. The sequence was deposited in the NCBI GenBank database under the accession number OR644880.

Screening of plant growth promotion traits

For better understanding the PGP potential of the producing strain *Bacillus* sp. isolate Par 3 PGP, parameters such as the ability to produce surfactin and indole acetic acid were screened as described below. *Bacillus* sp. Par 3 was cultivated in Erlenmeyer flasks, using nutrient broth medium (Himedia Laboratories, Mumbai, India), for 48 h on a rotary shaker (170 rpm) at 28 °C. L-Trp (1.02 g/l) was added to the media for the experiments aimed at indole components quantification. To obtain a cell-free supernatant of the cultivation broth for the mentioned experiments, bacterial biomass was separated by centrifugation (12000 × g, 10 min, 25°C, Z 326 K, Hermle LaborTechnik GmbH, Germany).

Surfactin production

Surfactin concentration in the culture supernatant of *Bacillus* sp. Par 3 was determined using the CPC-BTB (cetylpyridinium chloride-bromothymol blue) method (Yang et al., 2015). In this method, bromothymol blue (BTB) and the mediator cetylpyridinium chloride combine to form a green-colored complex. When surfactin is introduced, it forms a colorless complex with the CPC, releasing BTB molecules into the medium, resulting in a detectable color change that can be measured spectrophotometrically. The quantification process involved mixing 300 µl of supernatant sample with 2.4 ml of the CPC-BTB reagent and incubating the mixture at 25°C for 5 minutes. Finally, the absorbance at 600 nm was measured using a UV1800 spectrophotometer (Shimadzu, Japan), and surfactin concentration was determined on the basis of standard curve prepared using the surfactin standard (Sigma-Aldrich, Burlington, MA, USA) (Valenzuela-Ávila et al., 2020).

Indole acetic acid production

Determination of IAA concentration in the supernatant of *Bacillus* sp isolate Par 3 was performed with a slight modification of the colorimetric method described by Syed-Ab-Rahman et al. (2018). In brief, 1 ml of *Bacillus* sp. Par 3 cultivation broth supernatant was mixed with 2 ml of Salkowski reagent (1.2% [w/v] $FeCl_3$ in 7.9 M H_2SO_4) and incubated in a dark place at room temperature for 30 minutes. Pink color development indicates the microorganism's ability to produce IAA. After the 30-minute incubation period, spectrophotometric measurements were taken at a wavelength of 535 nm (UV 1800, Shimadzu, Japan). Distilled water was used as a blank sample, and the calibration curve was prepared using the indole acetic acid (IAA) standard (Sigma-Aldrich, Burlington, MA, USA).

Selection of carbon, nitrogen and phosphorus sources – Media composition and cultivation conditions

Substrates for cultivation were prepared according to a full experimental design by varying the basic components of the medium - sources of carbon, nitrogen and phosphorus. The effect of all components and their interactions on the outcome was investigated, where all combinations of independent variables were included in the full experimental design. Glycerol and sucrose were used as carbon sources (5 g/l). Potassium nitrate, ammonium nitrate and ammonium sulfate were used as nitrogen sources (1 g/l). Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium dihydrogen phosphate, and diammonium hydrogen phosphate were used as sources of phosphorus (1g/l). The effects of variations in medium composition were investigated by testing the *in vitro* antimicrobial effect of *Bacillus* sp. Par 3 cultivation broth on the growth of *B. cinerea* pathogen. The cultivation broth sample that exhibited the best results in terms of suppressing the phytopathogen *B. cinerea* was used to determine its effect on the germination of cucumber seeds, as well as the cultivation broth sample produced on the synthetic commercial medium (nutrient broth, Himedia Laboratories, India) under the same cultivation conditions. Cultivation of the *Bacillus* isolate was carried out on a rotary shaker at 28 °C and 170 rpm, under spontaneous aeration, during 96 h, with 10% (v/v) inoculum prepared using nutrient broth (Himedia Laboratories, India).

Antimicrobial activity assay

The suspension of the test microorganism *B. cinerea* isolate R9 was prepared by adding fungal spores into sterile saline. Sabouraud maltose agar

Table 1. Biochemical characteristics of *Bacillus* sp. isolate Par 3

BXYL	+	LysA	–	AspA	(–)	LeuA	(+)	PheA	+	proA	–
BGAL	+	PyrA	+	AGAL	+	AlaA	(–)	TyrA	+	BNAG	–
APPA	–	CDEX	–	dGAL	+	GLYG	–	INO	–	Mdg	+
ELLM	+	MdX	–	AMAN	–	MTE	+	GlyA	–	dMAN	–
dMNE	+	dMLZ	+	NAG	+	PLE	+	IRHA	–	BGLU	+
BMAN	–	PHC	–	PVATE	–	AGLU	+	dTAG	–	dTRE	+
INU	+	dGLU	–	dRlB	–	PSCNa	–	NaCl 6.5%	+	KAN	–
OLD	–	ESC	+	TTZ	+	POLYB_R	+				

media (Himedia Laboratories, India) were melted and tempered (50±1 °C) and, before pouring into Petri plates, inoculated with 1 ml of previously prepared spore suspension (10^5 spores/ml). The well diffusion method was employed in triplicate tests to evaluate the antimicrobial activity of the cultivation broth samples (100 µl) obtained after 4 days of cultivation of the producing microorganism, *Bacillus* sp. Par 3, against the phytopathogenic isolate. Incubation was performed at 26 °C for 96 h and followed by inhibition zone diameter measurements. Sterile distilled water was used as a negative control.

Plant germination assay

The influence of *Bacillus* sp. Par 3 isolate on plant germination was tested on cucumber (*Cucumis sativus* L.) seeds. Seeds used in this assay were surface sterilized by chlorine bleach solution (6% (v/v), 1 min) and thoroughly washed with sterile distilled water for 5 min. After drying, fifty cucumber seeds were placed in each Petri plate containing filter paper and then soaked with 1 ml of optimized cultivation broth and 5 ml of sterile tap water. The Petri plates were then incubated at 25 °C for 7 days. After the incubation period, the length of cucumber roots and shoots was measured and compared to the negative control, which used tap water, and cultivation broth produced by using nutrient broth as cultivation medium (Himedia Laboratories, India).

Experimental data analysis

Statistical analysis of the experimental data was performed using the Statistica 13.3 software (Dell Technologies, TX, USA). Duncan's multiple range test was performed to establish homogenous groups of variances of the dependent variables. All statistical analyses were performed at the significance level of 95%.

RESULTS

Identification and biochemical characterization of *Bacillus* sp. isolate Par 3

Biochemical characterization of the *Bacillus* sp. isolate Par 3 was conducted using VITEK2 biochemical tests, and the results are shown in Table 1. Based on the BLASTn results, *Bacillus* sp. isolate Par 3 was identified as a member of the *Bacillus subtilis* group with 97.45% sequence similarity with several other *B. subtilis* strain sequences of the 16S rRNA gene available in the NCBI GenBank database.

PGP traits of the producing strain *Bacillus* sp. Par 3

The capacity of *Bacillus* sp. isolate Par 3 for promoting plant growth was investigated in terms of its ability to produce surfactin and IAA. The results shown in Table 2 represent mean values and standard deviations of concentrations of produced surfactin and IAA obtained in 3 repeated tests.

Table 2. Screening results of PGP traits from cultivation broth of *Bacillus* sp. isolate Par 3

	Concentration (mg/l)
Surfactin production	440.67±0.67
IAA production	7.96±0.02

Analysis of effects of different nutrients on antimicrobial activity of *Bacillus* sp. Par 3 against phytopathogenic *B. cinerea*

Table 3 presents the effects of carbon, nitrogen and phosphorus sources used in the medium for *Bacillus* sp. Par 3 cultivation, and their interaction, on antimicrobial activity against *B. cinerea* based on the analysis of variance. Statistically significant effects are bolded in Table 3. It can be inferred from the data presented in Table 3 that the antimicrobial activity of *Bacillus* sp. Par 3 against the phytopathogen *B. cinerea* is influenced significantly by all nutrients provided except the carbon source. Among the combinations of independent variables tested, only the combination of carbon source and nitrogen source did not display a statistically significant effect on antimicrobial activity.

Table 3. Analysis of variance of the influence of nutrients choice in cultivation medium on antimicrobial activity of the production microorganism *Bacillus* sp. Par 3 against the phytopathogen *B. cinerea*

Effect	SS	DF	MS	F	*p*-value
Intercept	**218240.2**	**1**	**218240.2**	**5505.710**	**<0.0001**
C	72.0	1	72.0	1.816	0.1841
N	**1694.7**	**2**	**847.3**	**21.377**	**<0.0001**
P	**725.0**	**3**	**241.7**	**6.097**	**0.0013**
C*N	70.1	2	35.0	0.884	0.4197
C*P	**883.2**	**3**	**294.4**	**7.427**	**0.0003**
N*P	**2193.1**	**6**	**365.5**	**9.221**	**<0.0001**
C*N*P	**1425.0**	**6**	**237.5**	**5.992**	**0.0001**
Error	1902.7	48	39.6		

Based on the results of Duncan's multiple range test (Table 4), the largest inhibition zone diameter was achieved through nutrient combinations numbered 23 and 24.

Table 4. The results of Duncan's test for the influence of nutrient choice on antimicrobial activity of the production microorganism *Bacillus* sp. Par 3 against *B. cinerea*. Data represent mean values and standard deviations of three independent experiments.

	Carbon source	Nitrogen source	Phosphorus source	Inhibition zone diameter (mm)
1.	Glycerol	Ammonium sulfate	Potassium dihydrogen phosphate	34.00 ± 5.29^{a}
2.	Sucrose	Ammonium sulfate	Dipotassium hydrogen phosphate	36.33 ± 5.13^{ab}
3.	Sucrose	Potassium nitrate	Ammonium dihydrogen phosphate	37.00 ± 1.00^{ab}
4.	Sucrose	Ammonium sulfate	Potassium dihydrogen phosphate	37.67 ± 4.04^{ab}
5.	Glycerol	Ammonium sulfate	Ammonium dihydrogen phosphate	46.33 ± 7.77^{bc}
6.	Glycerol	Ammonium sulfate	Diammonium hydrogen phosphate	49.00 ± 11.53^{cd}
7.	Glycerol	Potassium nitrate	Diammonium hydrogen phosphate	51.00 ± 4.58^{cde}
8.	Glycerol	Ammonium nitrate	Ammonium dihydrogen phosphate	52.66 ± 12.86^{cdef}
9.	Glycerol	Potassium nitrate	Ammonium dihydrogen phosphate	53.00 ± 13.45^{cdef}
10.	Glycerol	Ammonium nitrate	Diammonium hydrogen phosphate	56.33 ± 7.09^{cdef}
11.	Sucrose	Ammonium nitrate	Potassium dihydrogen phosphate	56.33 ± 14.15^{cdef}
12.	Glycerol	Ammonium nitrate	Potassium dihydrogen phosphate	56.66 ± 6.67^{cdef}
13.	Sucrose	Ammonium sulfate	Diammonium hydrogen phosphate	59.33 ± 1.15^{def}
14.	Glycerol	Potassium nitrate	Potassium dihydrogen phosphate	59.66 ± 0.58^{def}
15.	Glycerol	Ammonium sulfate	Dipotassium hydrogen phosphate	60.33 ± 4.16^{def}
16.	Sucrose	Potassium nitrate	Potassium dihydrogen phosphate	63.00 ± 1.00^{ef}
17.	Sucrose	Ammonium nitrate	Ammonium dihydrogen phosphate	63.00 ± 1.00^{ef}
18.	Sucrose	Potassium nitrate	Dipotassium hydrogen phosphate	63.00 ± 1.00^{ef}
19.	Sucrose	Potassium nitrate	Diammonium hydrogen phosphate	63.66 ± 0.58^{f}
20.	Sucrose	Ammonium nitrate	Dipotassium hydrogen phosphate	64.00 ± 0.00^{f}
21.	Sucrose	Ammonium sulfate	Ammonium dihydrogen phosphate	64.33 ± 0.58^{f}
22.	Glycerol	Potassium nitrate	Dipotassium hydrogen phosphate	64.66 ± 0.58^{f}
23.	Glycerol	Ammonium nitrate	Dipotassium hydrogen phosphate	65.00 ± 1.00^{f}
24.	Sucrose	Ammonium nitrate	Diammonium hydrogen phosphate	65.00 ± 0.00^{f}

Notably, the final six combinations in Table 4, exhibiting the most substantial inhibition zones, showed no statistically significant distinctions. They were grouped within the same statistical significance level, forming a homogeneous cluster. Consequently, any of these combinations can be applied with equal importance and it can be assumed that they will provide approximately the same value of inhibition zone diameter against the phytopathogen *B. cinerea*. On the other hand, the smallest inhibition zone diameter against the phytopathogen *B. cinerea* was noted in the case of nutrient combination comprising glycerol as carbon source, ammonium sulfate as nitrogen source, and potassium dihydrogen phosphate as phosphorus source. The inhibition zone diameter achieved by applying the cultivation broth sample obtained using nutrient broth as a medium was 43.50±0.50 mm.

Plant germination assay

Figure 1 presents data regarding the germination and length of roots and shoots of cucumber seeds treated with the cultivation broth of *Bacillus* sp. isolate Par 3 based on an optimized medium (nutrient combination number 24), in comparison to the negative control and seeds treated with the cultivation broth of *Bacillus* sp. isolate Par 3 based on a commercial medium - nutrient broth. Seeds treated with the culture liquid of *Bacillus* sp. isolate Par 3 in nutrient broth exhibited a slightly higher germination percentage (98%) than seeds treated with tap water (96%), yet still lower than seeds treated with cultivation broth based on the optimized medium (100%). Treating seeds with cultivation broth derived from the optimized medium has proven to be highly successful, considering the following mean values for root and shoot length: 53.90±14.90 mm and 13.26±4.88 mm, respectively, with corresponding maximum values of 82 mm and 24 mm. The substantial growth enhancement, achieved by treating seeds with culture liquid from the optimized medium, is most prominently demonstrated in root growth.

DISCUSSION

Members of the genus *Bacillus* are extensively researched examples of PGP rhizobacteria. *Bacillus* species hold significant potential due to their capacity to generate

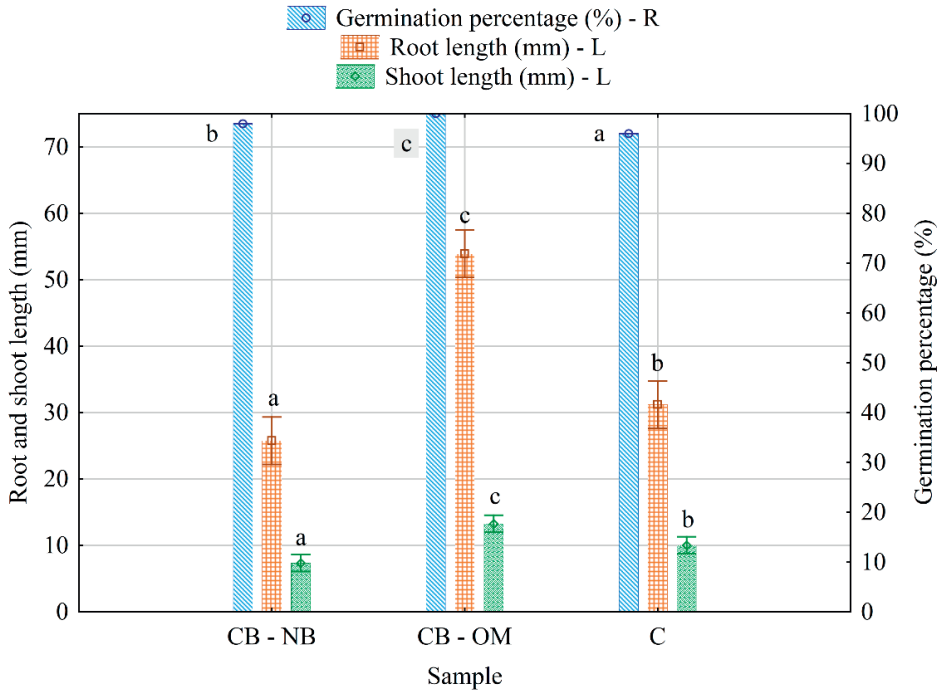

Figure 1. The effects of *Bacillus* sp. Par 3 cultivation broth based on nutrient broth (CB-NB) and optimized medium with previously selected carbon, nitrogen and phosphorus sources (CB-OM) on cucumber seed germination, root and shoot length in comparison to control (tap water - C). Means and standard deviations of root and shoot lengths are given together with homogenous group designations (a, b, c) according to Duncan's multiple range test.

various advantageous elements, including indole acetic acid (IAA), hydrocyanic acid (HCN), siderophores, hydrolytic enzymes, antimicrobial compounds, along with their capabilities for phosphate solubilization and nitrogen fixation (Vlajkov et al., 2023). The *Bacillus* isolate employed in this research was identified using 16S rRNA sequencing, revealing its affiliation with *Bacillus subtilis* species with identification accuracy of 97.45%, while biochemical characterization was done using the VITEK2 device and BCL cards (Biomericux, France). Biochemical characterization, shown in Table 1, included 46 tests, encompassing the examination of carbon source utilization, enzymatic activities, inhibition by 6.5% NaCl, and resistance to antibiotics. Previous research had proved that *B. subtilis* has a potential for use as a biocontrol agent. Bu et al. (2021) investigated the influence of *B. subtilis* L1-21 isolate on the suppression of *B. cinerea* phytopathogen in postharvest tomatoes, resulting in 86.57% control efficacy. In a study conducted by Touré et al. (2004), the investigated *B. subtilis* isolate GA1 demonstrated high effectiveness in reducing gray mold incidence within the initial 5 days after pathogen inoculation, and it maintained a protection level of 80% over the subsequent 10 days. Furthermore, it has been determined that in addition to its significant antagonistic effect, *B. subtilis* holds great importance due to its PGP properties. Strain RH5, examined by Jamali et al. (2020), exhibited a range of PGP attributes (indole acetic acid, siderophore, hydrogen cyanide production and phosphate, Zn, K solubility), hydrolytic enzymatic (chitinase, protease, cellulase, xylanase) activity, and presence of antimicrobial peptide biosynthetic genes (bacylisin, surfactin, and fengycin), which support the strain in efficient colonization of hyphae and pathogen inhibition. *B. subtilis* also enhances stress tolerance in plant hosts by inducing the expression of stress-response genes, phytohormones, and stress-related metabolites (Hashem et al., 2019).

Commercially available media, frequently utilized during preliminary investigation phases of bioprocess development for manufacturing biocontrol agents, are considered inadequate for the production scale-up stages. The rationale behind this lies in their prohibitively high cost, which constrains the potential transition to large-scale industrial production and subsequent product commercialization. This underscores a need for additional efforts to identify nutrient sources that can support particular isolates' growth and metabolic activity. The discovery of waste streams rich in suitable nutrients for microbial growth and metabolic activity is a step further in the creation of complex media based on natural components (Vlajkov et al., 2022).

It is crucial to note that the nutritional requirements of microorganisms are highly dependable on a particular strain. Among the pivotal elements shaping the traits and metabolic functioning of these strains is their ability to effectively process particular nutrients in both qualitative and quantitative terms. Carbon is the most significant component of a medium since it provides microorganisms with energy, aids their growth, and helps them produce primary and secondary metabolites. The creation of biomass and/or primary or secondary metabolites can frequently be affected by the rate at which a carbon source is digested (Singh et al., 2017). The carbon sources used in this study were picked to act as exemplars of typical industrial waste streams. These sources might be taken into account as parts of culture medium in further studies to direct research towards integration of the circular economy principles in the production of biocontrol agents.

Sucrose was chosen as one of carbon sources, considering that it is the main component of molasses, the by-products of sugar beet and sugar cane processing. Molasses have been mainly used as cultivation media for biosurfactant production by different *Bacillus* strains (Saimmai et al., 2011; Al-Bahry et al., 2013). Furthermore, it was found that *Bacillus* cultivation on molasses could lead to the production of diverse types of enzymes and other valuable products (Gojgic-Cvijovic et al., 2019; Shikha et al., 2007; Chaijamrus & Udpuay, 2008). The main factors behind extensive use of molasses as substrates are their affordable pricing, as compared to other carbon sources, and the existence of various additional substances besides sucrose. These include vitamins, minerals, and organic substances, all of which are crucial for the fermentation process (Saimmai et al., 2011). Glycerol was used as the second carbon source. A notable volume of glycerol is obtained as a by-product from the continually expanding biodiesel sector. This has garnered considerable scientific attention for its potential conversion into various value-added products through microorganisms. For example, crude glycerol derived from biodiesel production was employed as a carbon source in the cultivation medium for biosurfactant production by *Bacillus* strains (Sousa et al., 2012; de Sousa et al., 2014). Potassium nitrate, ammonium nitrate, and ammonium sulfate were observed as the most promising nitrogen sources to promote antifungal activity against *B. cinerea*. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium dihydrogen phosphate, and diammonium hydrogen phosphate were observed as the most potent sources of phosphorus. The selection of these nutrient sources was conducted not only to

support growth, antifungal activity and PGP traits of the producing strain *Bacillus* sp. Par 3, but also to be additional plant nutrient sources, considering that cultivation broth usually contains small residual amounts of initially added nutrients which were not used by the producing microorganism during cultivation (Pajčin et al., 2020).

Factorial ANOVA was employed to determine whether there were significant differences between inhibition zone diameters obtained by assaying cultivation broth samples of *Bacillus* sp. Par 3 against *B. cinerea*, depending on different carbon, nitrogen and phosphorus sources in cultivation medium. ANOVA results showed that the effect of phosphorus inorganic compounds from phosphorus and nitrogen sources, as well as their interaction, were significant (p-values less than 0.05), while the effect of carbon source selection showed less effect even in combination with nitrogen and phosphorus sources.

In order to determine which combination of carbon, nitrogen and phosphorus sources is the most suitable for producting bioactive agents effective against *B. cinerea* by *Bacillus* sp. Par 3 isolate, the well diffusion method was applied. The largest inhibition zones were achieved in two cases, with 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source, and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source (Table 4). Based on the findings presented in Tables 3 and 4, it can be deduced that biocontrol agents that play crucial roles in suppressing the phytopathogen *B. cinerea* mainly depend on the source of phosphorus, followed by the source of nitrogen. However, carbon source did not show a statistically significant effect on diameters of inhibition zones against the phytopathogen *B. cinerea*. According to the results shown in Table 4, it can be inferred that ammonium nitrate demonstrated superior performance as nitrogen source, possibly due to two different nitrogenous ions as separate nitrogen sources for microbial growth. Antimicrobial activity of *Bacillus* species is largely attributed to the action of a wide spectrum of secondary metabolites, among which metabolites of lipopeptide nature have a significant impact. Production of these secondary metabolites with antimicrobial activity is considered as an indirect mechanism of plant growth promotion due to an ability to suppress pathogens, thus providing more favorable conditions for plant growth and development (Soni & Keharia, 2021). Surfactin, a cyclic lipopeptide, is one of the most important biosurfactants because of its strong activity as a surfactant and its antimicrobial activity (Janek et al., 2021). In a study conducted by Zhou et al. (2023), the concentration of produced surfactin under optimized fermentation conditions was 1.82 g/l. Our research showed that *Bacillus* sp isolate Par 3 has the ability to produce surfactin in a concentration of 440.67±0.67 mg/l, which might be accelerated through optimization of the medium and process parameters. According to previous research, the possibility of producing surfactin by cultivating *Bacillus* strains on waste streams of biodiesel, dairy and wine industries has been proven (Sousa et al., 2012; De Andrade et al., 2016; Dmitrović et al., 2022). Previous studies have also shown an important role of surfactins produced by *Bacillus* species in facilitating biofilm formation and plant root colonization (Debois et al., 2014; Aleti et al., 2016).

To investigate the effect of cultivation broth of *Bacillus* sp. isolate Par 3 on cucumber seed germination, the medium with sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source was chosen for further research due to a wider availability of molasses in these areas.

Besides antimicrobial activity, species belonging to the *Bacillus* genus have numerous benefits in stimulating plant growth. They synthesize a multitude of secondary metabolites that influence the environment and enhance the availability of nutrients to plants. Some species within this genus produce plant hormones such as cytokinins, gibberellins or indole acetic acid. Additionally, they can generate siderophores, ACC deaminase, various enzymes (proteases, pectinases, cellulases, lipases, etc.), and have an ability to decompose diverse organic and inorganic compounds of phosphorus, potassium and zinc, thereby augmenting the accessibility of these crucial elements to plants (Hajnal Jafari et al., 2020). The production of indole acetic acid (IAA), which falls under the category of phytohormones, is one of the most significant benefits for plants. It influences various physiological activities in plants, including cell enlargement, cell division, root growth initiation, growth rate, phototropism, geotropism, and apical dominance (Chrouqi et al., 2017). Previous research had confirmed that three root-colonizing *Bacillus* strains, including *B. amyloliquefaciens, B. subtilis* and *B. tequilensis* possess the capacity to produce IAA, and *in vivo* experiments have substantiated the role of IAA-producing strains in promoting plant growth (Shahid et al., 2021; Khan et al., 2021). The concentration of IAA produced by the isolate *Bacillus* sp. BioSol021 was 15 mg/l (Vlajkov et

al., 2023), while *Bacillus* sp. E25 and *Bacillus* sp. CR71, investigated by Rojas-Solis et al. (2020), produced IAA in concentrations ranging from 20.46±1 to 31.18±1.5 μg/ml, depending on saline stress. In the present study, the demonstrated capability of IAA production by the tested *Bacillus* sp. isolate Par 3 in a concentration of 7.96±0.02 mg/l makes it a promising candidate for development of biotechnological products for agricultural applications, with a possibility to direct medium optimization towards maximization of IAA production. In a study conducted by Kumar et al. (2012), all seven tested *Bacillus* isolates from the rhizosphere showed IAA production, while maximal IAA production was recorded in *Bacillus* sp. BPR7 cultivation broth (17 μg/ml).

Through the production of enzymes, such as cellulases, proteases, pectinases, lipases and other, the breakdown of complex compounds into simpler forms metabolizable by bacteria is facilitated. This leads to an increase in the diversity/biodiversity of soil microflora, which enhances soil fertility and availability of easily accessible nutrients (Hashem et al., 2019; Mohandas et al., 2018). In this study, the influence of the cultivation broth of *Bacillus* sp. Par 3 isolate was investigated on cucumber seeds. It was found that medium optimization for *Bacillus* cultivation has a significant effect on the improvement of germination, i.e. elongation of cucumber roots and stems, which underscores the importance of medium optimization in the production of biological preparations. This is a significant finding in comparison with the commercial medium (nutrient broth), considering the possibility of reducing significantly the production cost related to cultivation medium by using formulated medium with widely available and cost-efficient components, which also affects market price of the biocontrol product and its market competitiveness (Ortiz & Sansinenea, 2023). Further techno-economical analyses will be required to assess the cost-effectiveness of the proposed medium composition on a large scale, which is possible to achieve by using bioprocess simulation tools. Further research is also required to determine the specific PGP traits of the isolate *Bacillus* sp. Par 3, as well as mechanisms involved in cucumber growth promotion.

CONCLUSION

Based on the results obtained in this study, it can be concluded that *Bacillus* sp. isolate Par 3, identified as a member of the *Bacillus subtilis* group, has a significant potential for application as a biocontrol and PGP agent. Proven production of surfactin and IAA in significant concentrations is crucial for its use as a biocontrol and PGP agent in agriculture. By selecting suitable sources of carbon, nitrogen and phosphorus, antimicrobial activity of the isolate was notably enhanced, along with its capacity to promote plant growth. The highest antimicrobial activity with inhibition zones of 65 mm was observed when using media with the following composition: 1) sucrose as carbon source, ammonium nitrate as nitrogen source, and diammonium hydrogen phosphate as phosphorus source, and 2) glycerol as carbon source, ammonium nitrate as nitrogen source and dipotassium hydrogen phosphate as phosphorus source. Treating cucumber seeds with the isolate multiplied on the sucrose-based optimized medium resulted in higher germination rate and significantly faster and greater root and shoot development, compared to the cultivation broth based on commercial medium and negative control. The ability of *Bacillus* sp. Par 3 isolate to utilize sucrose and glycerol as carbon sources opens a possibility for utilization of waste streams from the sugar industry and biodiesel production as substrates for producing this biocontrol agent. Subsequent research would focus on using these waste streams for *Bacillus* sp. Par 3 cultivation and determining optimal process conditions to maximize the beneficial properties of this biocontrol and PGP agent.

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Uticaj sastava medijuma na sposobnost podsticanja rasta biljaka i biokontrolna svojstva izolata *Bacillus* sp. Par 3 protiv fitopatogena *Botrytis cinerea*

REZIME

Jedan od globalnih problema današnjice predstavljaju značajani gubici u poljoprivredi usled biljnih bolesti koje izazivaju različiti patogeni. Među ovim patogenima, bitnu ulogu ima plasan *Botrytis cinerea*, koja kao uzročnik sive truleži, nanosi veliku štetu različitim važnim usevima. Upotreba biokontrolnih agenasa za suzbijanje fitopatogena postala je imperativ, pri čemu bakterije iz roda *Bacillus* imaju veliki potencijal zbog brzog razmnožavanja, otpornosti na nepovoljne uslove okoline, sposobnosti promovisanja rasta biljaka i širokog spektra delovanja. Cilj ovog istraživanja bio je da se utvrde najbolji izvori ugljenika, azota i fosfora u medijumu za kultivaciju *Bacillus* bakterija, sa namerom da se postigne što veća antimikrobna aktivnost protiv fitopatogena *B. cinerea* i podstakne brži rast krastavca u različitim fazama razvoja, primenom izolata *Bacillus* sp. Par 3. Da bi se utvrdila najpogodnija kombinacija izvora ugljenika, azota i fosfora za proizvodnju bioaktivnih agenasa koji efikasno deluju protiv *B. cinerea* od strane izolata *Bacillus* sp. Par 3, primenjena je metoda bunarića. Uticaj izolata *Bacillus* sp. Par 3 na klijanje biljaka testiran je na semenkama krastavca. Najveće zone inhibicije postignute su pri upotrebi sledeća 2 medijuma: 1) saharoza kao izvor ugljenika, amonijum nitrat kao izvor azota i diamonijum hidrogenfosfat kao izvor fosfora, i 2) glicerol kao izvor ugljenika, amonijum nitrat kao izvor azota i dikalijum hidrogenfosfat kao izvor fosfora. Semena tretirana kultivacionom tečnošću izolata *Bacillus* sp. Par 3 korišćenjem optimizovanog medijuma pokazala su najbolje rezultate u pogledu procenta klijanja (100%), dužine korena (53,09 mm) i dužine izdanka (13,26 mm) krastavca. *Bacillus* sp. Par 3 izolat je identifikovan kao *Bacillus subtilis* metodom sekvenciranja 16S rRNA gena. Rezultati ovog istraživanja ističu značaj optimizacije medijuma za proizvodnju biokontrolnih agenasa, uzimajući u obzir kako antimikrobnu efikasnost, tako i karakteristike promocije rasta biljaka.

Ključne reči: *Bacillus subtilis*, *Botrytis cinerea*, krastavac, optimizacija medijuma, podsticanje rasta biljaka, antimikrobna aktivnost

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Impact of neem cake amendment in the casing soil on control of *Trichoderma aggressivum* Samuels & W. Gams and *Lycoriella ingenua* (Dufour) and mushroom yield

Tanja Drobnjaković, Jelena Luković, Svetlana Milijašević-Marčić, Biljana Todorović, Miloš Stepanović, Ivana Potočnik* and Emil Rekanović

Institute of Pesticides and Environmental Protection, Banatska 31B, 11080, Belgrade, Serbia
**Corresponding author: ivana.potocnik@pesting.org.rs*

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SUMMARY

The study was focused on improvement of the integrated management strategy against green mould disease agent *Trichoderma aggressivum* Samuels & Gams and mushroom fly *Lycoriella ingenua* (Dufour) as pests of the white button mushroom *Agaricus bisporus* (Lange) Imbach. The impact of neem cake amendment in casing soil on regulation of the abundance of mushroom sciarid fly adults, efficacy in controlling the green mould disease agent, and mushroom yield was evaluated. Casing soil was supplemented with different concentrations of neem cake: 1, 2.5, 5, 10 and 15%. Neem cake added as a supplement to casing soil at a rate of 2.5% reduced the number of mushroom fly adults by 83.93% and green mould disease incidence by 59.6% in comparison to the control. No adverse effect on mushroom formation, yield and quality of fruiting bodies was observed at that concentration. Amendment of 2.5% neem cake in the casing soil could be recommended for application in mushroom production to control *L. ingenua* and symptoms of green mould disease without negative impact on mushroom yield.

Keywords: *Agaricus bisporus*, *Azadirachta indica*, green mould disease, mushroom fly

INTRODUCTION

Substrate preparation is the most important process in the production of white button mushroom [*Agaricus bisporus* (Lange) Imbach]. Mushroom substrate is made from fermented and pasteurized mixture of plant material and animal manure. Application of pesticides during wheat production and the use of antibiotics in poultry farming results in reduced compost quality. Chemical residues disrupt the genuine microbiome of substrate in favour of harmful organisms. After completing spawn-run of mushroom mycelia, compost is cased with a 3-5 cm thick layer of casing soil. Casing soil is made mainly of black peat, neutralized with limestone and sterilized by various disinfectants. Casing layer is essential for mushroom development and fructification, enabling suitable microbiome, humidity and aeration (Jarial et al., 2005). Preventive disinfection and treatment of casing soil with chemical pesticides is a common practice in disease and pest control.

The prevailing mycosis of cultivated mushrooms in Serbia and worldwide is green mould disease caused by *Trichoderma aggressivum* Samuels & W. Gams, which results in crop losses exceeding 60% (Kosanović et al., 2013). The most significant mushroom pest in Serbia and globally is the mushroom fly (fungus gnat), *Lycoriella ingenua* (Dufour) (Sciaridae: Diptera), which causes great economic losses in commercial mushroom production (Rinker, 2017; Drobnjaković et al., 2019). Fletcher and Ganney (1968) recorded that mushroom pests and diseases were mostly transmitted by casing soil, and suggested formaldehyde as a control method. Other widely used casing soil disinfectants are also: sodium hypochlorite, potassium permanganate, sulphur, calcium chloride and chlorinated compounds (Sharma & Guleria, 1999). Recently, casing soil disinfection based on the eco-friendly substance peracetic acid was implemented (Potočnik et al., 2014).

Chemicals induce harmful effects on mushroom mycelia, causing losses in quality and yield (Shamshad, 2010), and resulting in residues presence in harvested mushrooms (Gea et al., 2021). Lately, many pesticides have been withdrawn from the market. Only two chemical fungicides (prochloraz and metrafenone) and bioinsecticides based on pyrethrin and entomopathogenic nematodes have been officialy recommended for use in mushroom cultivation by the OEPPO (Carrasco et al., 2017; Navarro et al., 2021). Pyrethrin-based bioinsecticides, which are very effective in the control of mushroom flies, have appeared to be highly toxic to non-target organisms. On the other side, the use of bioinsectides based on entomopathogenic nematodes has shown variable success in the control of mushroom flies (Navarro et al., 2021; Ruchika et al., 2021). However, none of the mentioned formulated bioinsecticides is registered in Serbia.

Natural products of the Indian neem tree [*Azadirachta indica* A. Juss. (Meliaceae)] have several uses in agriculture, industry, medicine, and the environment (Adusei & Azupio, 2022). The limonoid-based azadirahtin, the primary and most important active ingredient in all neem tree derivates (plant extracts, essential oils, neem cake, etc.) (Gupta et al., 2019), accounts for over 90% of the pest-control actions. It acts as a natural fertilizer with pesticidal properties (Schmutterer, 1995).

In agriculture, neem products are used as pesticides, pest fumigants, fertilizers, manures, compost, urea coating agents, and soil conditioners. Neem-based pesticides are effective against many plant pests and disease agents, such as insects, nematodes, fungi and bacteria (Lokanadhan et al., 2012). Neem cake is the by-product obtained in the process of cold pressing of whole neem tree fruits, depulped seed/kernels, and either by expeller or solvent extraction process.

Azadirachtin acts as antifeedant, repellent, feeding inhibitor, oviposition deterrent, growth regulator in insects (Isman, 1993), and growth-inhibitor in fungal pathogens (Adusei & Azupio, 2022). Due to the complex mode of action of azadirachtin, there is no risk of cross-resistance (Siegwart et al., 2015). Neem components affect the insect endocrine system, rather than neurological or digestive system like chemical insecticides [Mordue (Luntz) & Nisbet, 2000]. It has demonstrated typical insect growth regulating actions in the larval stages of insects (Koul, 2007). More than 105 insect pests from 10 orders are controlled with neem kernels, among which are numerous pests in the order Diptera (Roychoudhury, 2016). Besides, neem cake organic manure protects plant roots not only from soil insects but from nematodes as well (Alam, 1993; Abbasi et al., 2005). We are not aware of any earlier study conducted to determine the impact of neem cake on control of *L. ingenua* populations.

Neem and its constituents are effective in inhibiting the growth of a wide range of microorganisms, including viruses, bacteria, and fungi. Extracts and aerosols of seeds and leaves of neem have been found to suppress the growth of many plant pathogenic fungi: *Alternaria alternata* (Fr.) Keissl. (Chaudhary et al., 2003), *Fusarium solani* (mart.) Sacc. and *Rhizoctonia solani* J.G. Kühn (Darwish & Shaker, 2005), *Verticillium dahliae* Kleb. and *Pythium aphanidermatum* (Edson) Fitzp. (Abbasi et al., 2005), *Alternaria solani* Sorauer (Moslem & El-Kholie, 2009; Jabeen et al., 2013), *Cercospora canesens* Ellis. & Martin (Trivedi et al., 2014). Neem powder inhibited the growth of fungal species *Fusarium oxysporum* Schlecht (Hadian et al., 2011). Neem seed extracts were efficient against the bacteria *Pseudomonas syringae* pv. *syringae* Van Hall, *Xanthomonas arboricola* pv. *corylina* Dye, and *Agrobacterium tumefaciens* Smith & Townsend (Goel et al., 2016).

Neem as a biopesticide has no adverse effects on plants or soil (Mukhopadhyay et al., 1992). Neem products serve as nitrification inhibitors as they block soil bacteria from converting nitrogenous compounds into nitrogen gas, and prolong the availability of nitrogen to both short and long duration crops (Puri, 2004). They reduce alkalinity in soil as they produce organic acids upon decomposition. Their application is compatible with soil microbes and rhizosphere microflora, and ensures soil fertility. They improve the organic matter content in soil, soil texture and its aeration, as well as water holding capacity (Puri, 2004).

In view of a large knowledge gap regarding azadirachtin and neem activity against harmful mushroom pests and diseases, neem oil has already been evaluated in control of the mushroom sciarid fly, *L. ingenua* (Drobnjaković et al., 2019). The aim of this study was to determine the impact

of neem cake amendment in casing soil on regulation of the abundance of *L. ingenua* adults. Moreover, its effect on the control of *T. aggressivum*, the green mould disease agent, and impact on yield of white button mushroom (*A. bisporus*) were also evaluated. The study was focused on improving mushroom fly and green mould disease integrated management strategies.

MATERIAL AND METHODS

Tests in mushroom growing room

Mushroom substrate was provided by the compost producer Uča, Vranovo, Serbia. Plastic boxes sized 0.340 x 0.215 x 0.130 m (*l* x *w* x *h*) were filled with 1.5 kg of compost mixed with 15 g of grain spawn of *A.bisporus* A15 (Sylvan, Hungária zRt) to prepare 1% spawned substrate. The boxes were incubated at 25°C (spawn-run) for 18 days. Compost was cased with 1.2 kg of black peat casing soil Wokas Casing Soil - Typ S (Wokas S.A., Łosice, Poland), and disinfected with peracetic acid 0.02% (Peral-S 15%, Vetprom, Belgrade, Serbia), equaling 90 ml per m^2 of casing soil. Neem cake – »Azadiroko« (BioGenesis d.o.o., Serbia) was tested as a potential casing soil amendment in the range of: 1, 2.5, 5, 10, and 15% of casing soil, and placed on mushroom compost. Casing soil was cased in a 50 mm layer and incubated at 22°C for 8 days (case-run). The day of casing was regarded as day one. Over the following seven days air temperature was reduced in stages to 17°C. The trial consisted of two groups, uninoculated plots and inoculated with *T. aggressivum* f. *europaeum* T77. Control plots within both groups were sprayed with tap water. Efficacy of the neem cake was evaluated against the green mould disease agent *T. aggressivum* f. *europaeum* T77 (artificial infection) and the mushroom fly *L. ingenua* (natural infection). The pathogenic fungus *T. aggressivum* f. *europaeum* T77, isolated in 2010 from a mushroom farm in Lisovići, Barajevo, was identified previously based on morpho-physiological characteristics and ITS1/ITS4 sequence analyses (Kosanović et al., 2013). Inoculation with *T. aggressivum* f. *europaeum* T77 was arranged from a culture grown on PDA at 25°C for three days. Mycelia of the pathogenic fungi were scraped from the surface of PDA plates, mixed with water and Tween 20 (v/v 0.01%) (REANAL Finomvegyszergyár Rt., Hungary, No. 805383) and filtered through sterile gauze. Spore concentration was determined by counting on a hemocytometer and the suspension was diluted to achieve the final concentration of 10^6 conidia ml^{-1}. Inoculation of *T. aggressivum* f. *europaeum* T77 was performed two days after the spawned compost was placed into boxes, by pipetting 1 ml of spore suspension and 9 ml of tap water (10^6 conidia ml^{-1} per m^2) down the inner walls of each box. The plots were arranged in a completely random design with six replicates per treatment.

Efficacy in disease/pest control and impact on yield (biological efficiency) were evaluated by comparison with the uninoculated and inoculated control, respectively. The fruiting bodies were hand-picked in two successive production flushes. The harvested mushrooms were weighed and divided into two groups based on visual observation, i.e. with and without symptoms of green mould disease. The effect of fungicides on mushroom productivity was evaluated by biological efficacy (BE), calculated as the ratio of fresh weight of total fruiting body yield and weight of dry spawned substrate, expressed as %: BE = (fresh total fruiting body yield/dry spawned substrate mass) × 100 (Chrysayi-Tokousbalides et al., 2007). Fungicide effectiveness was calculated by Abbott's formula (Abbott, 1925): % effectiveness = [(Ic -It)/Ic] × 100, where Ic - disease incidence in inoculated control; It - disease incidence in treated samples. Disease incidence was recorded as a percentage of fruiting bodies with symptoms compared with those without symptoms.

All experimental boxes were placed inside the insect rearing cages (one box per cage). The density of *L. ingenua* was observed using yellow sticky traps inside each insect rearing cage to enable early observation of fly adults, and their abundance during the first mushroom flush. The yellow sticky traps were collected at five inspection periods (5, 8, 14, 18 and 22 days after treatment - DAT), and replaced with new ones. After each inspection period the collected traps were inspected under a binocular microscope to establish mushroom fly presence and density. The flies were identified to the species level based on the identification key given by Menzel & Mohrig (2000). The observed treatment parameter was the number of adult flies on yellow sticky traps per insect cage containing mushroom substrate. The trial was performed in six replicates.

Statistical analyses

Data were examined using the one-way analysis of variance (ANOVA), including comparison of means by Duncan's test. The test was used to compare the significance of differences in data based on the average efficacy in disease/pest control and biological efficiency (impact on yield) of different neem cake amendment concentrations in casing soil against *T. aggressivum* f. *europaeum* T77 and *L. ingenua* in experimental mushroom growing room. In all analyses, the level of significance was at least $p<0.05$ (Sokal & Rohlf, 2013). Statistical data analysis was performed using the software Statistica for Windows 6.0 (StatSoft Inc., 2004).

RESULTS

The highest statistically significant disease incidence was recorded in the control (2.97%), followed by the plot amended with 5% neem cake (2.94%) (Figure 1). Experimental plots amended with 1 or 2.5% neem cake showed the lowest number of green mould disease symptoms with respective disease incidence of 1.23 and 1.2%, without statistically significant differences. Those concentrations of neem cake reduced disease symptoms by 60% in comparison to control. Hence, the highest efficacy in control of *T. aggressivum* f. *europaeum* was found in these plots treated with neem cake 1 % (58.58%) or 2.5% (59.59%) (Figure 2). The lowest efficacy in reducing symptoms of green mould disease was noted in plots supplemented with 5% neem cake (1.01%).

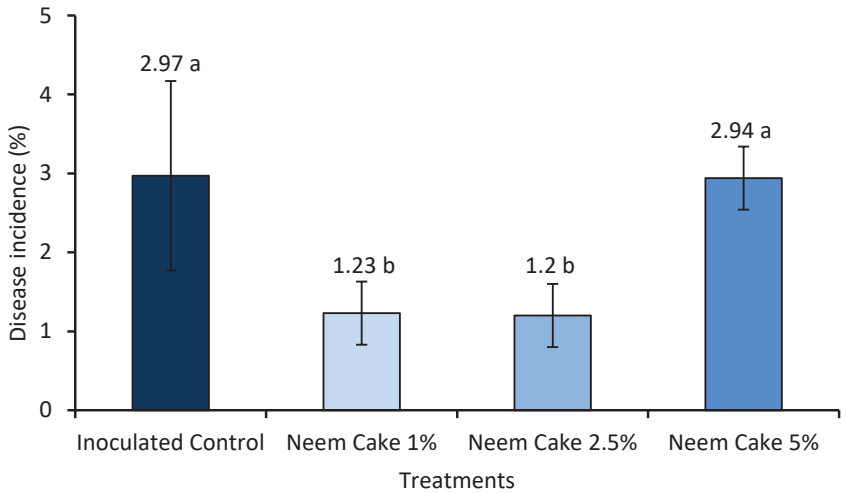

Figure 1. Green mould disease incidence on *Agaricus bisporus* after artificial inoculation of *Trichoderma aggressivum* f. *europaeum* T77; disease incidence average ± SE; SEDs, standard error of differences=11.61; SS=16.9; df, degrees of freedom=3; *F*=0.48; *P*-value=0.0001 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, p<0.05).

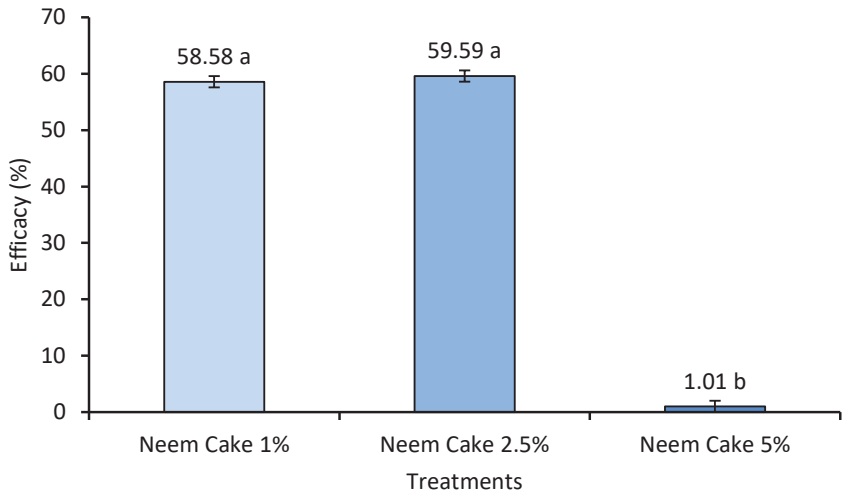

Figure 2. Efficacy of neem cake in control of *Trichoderma aggressivum* f. *europaeum* T77 after artificial inoculation on *Agaricus bisporus*; efficacy average SE; SEDs, standard error of differences=0.57; SS=16.9; df, degrees of freedom=3; *F*=0.48; *P*-value=0.0001 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, p<0.05).

Regarding the impact on yield, statistically significant differences were not found between the control, 1 or 2.5 % neem cake enhancement, with respective values of biological efficiency 82.1, 69.3 and 71.47% (Figure 3). Statistically significant negative impact on yield was noted in plots amended with higher concentrations of neem cake than previous (5, 10 and 15%). The lowest yield was recorded in plots supplemented with 5% neem cake (23.23%), while no fructification was noted when casing soil was supplemented with 10 or 15% neem cake. Neem cake at concentrations 1, 2.5 and 5% did not reduce the time taken for pinhead formation, compared to control.

Examination of the yellow sticky traps under a binocular microscope revealed only one species of mushroom fly pest, the mushroom sciarid fly *L. ingenua*, during the entire experimental period. The highest number of *L. ingenua* was found in plots covered with 10 and 15% neem cake (Figure 4). No statistically significant differences in the number of *L. ingenua* were found between the control plots and plots supplemented with 5 and 15% neem cake (Figure 4). The lowest number of mushroom fly adults was recorded in plots with 2.5% neem cake, followed by 1% neem cake at $p<0.05$ significance.

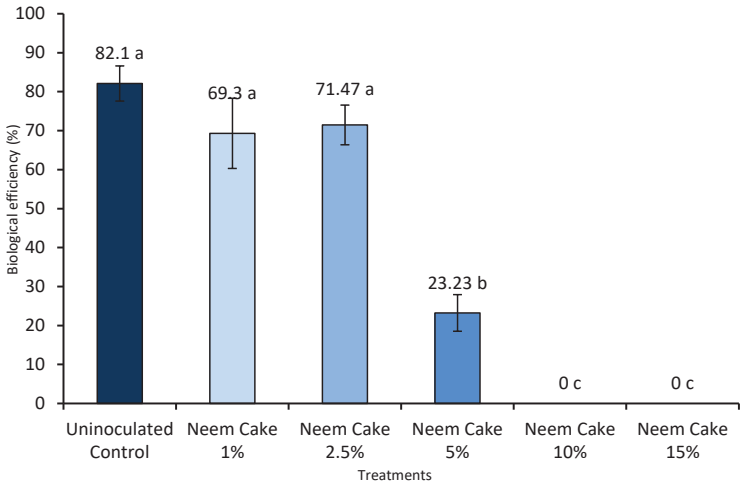

Figure 3. Impact of treatment on yield of *Agaricus bisporus* shown through biological efficiency of neem cake; biological efficiency average ± SE; SEDs, standard error of differences=4.99; SS=277980; df, degrees of freedom=3; *F*=6.16; *P*-value< 0.004 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, $p<0.05$).

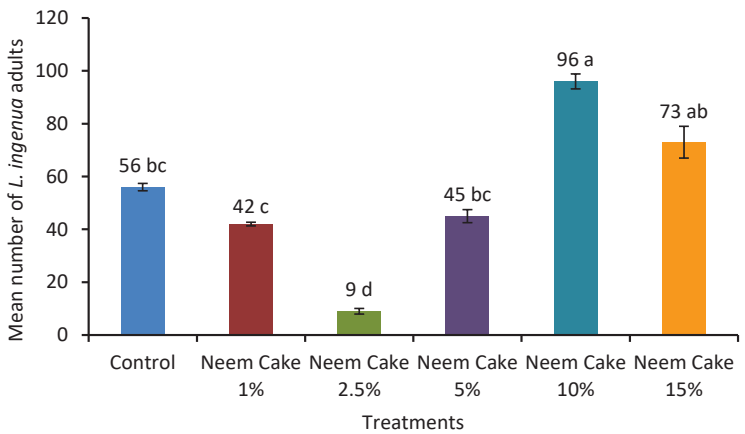

Figure 4. Abundance of *Lycoriella ingenua* adults per insect cage depending of concentrations of neem cake throughout the experimental period (average±SE). SEDs, standard error of differences=11.61; SS=2188.75; df, degrees of freedom=5; *F*=12.19; *P*-value<0.004 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, $p<0.05$).

During the experimental period, the highest statistically significant number of mushroom fly adults was recorded on day 14 after treatment (DAT) (Figures 5 and 6). Lower numbers of *L. ingenua* were noted in all other inspection periods (5, 8, 18 and 22 DAT) with no significant differences among them at $p<0.05$ level of significance.

DISCUSSION

The neem cake biopesticide was successfully applied at the concentration of 2.5% in casing soil, both as an insecticide against mushroom fly *L. ingenua* adults and as a fungicide reducing *T. aggressivum* incidence. The abundance of *L. ingenua* adults decreased by 83.93%

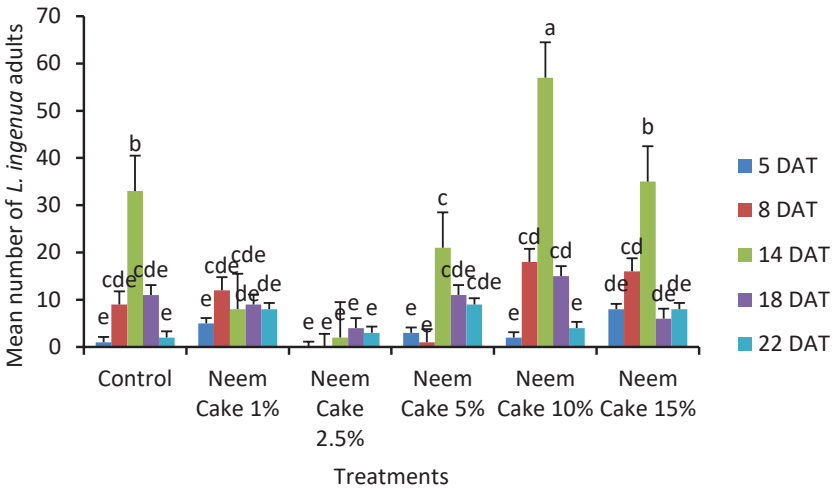

Figure 5. Abundance of *Lycoriella ingenua* adults depending of concentration of neem cake 5, 8, 14, 18 and 22 days after treatment (DAT) (average±SE). SEDs, standard error of differences=9.05; SS=1593.33; df, degrees of freedom=29; *F*=7.93; *P*-value<0.0001 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, $p<0.05$).

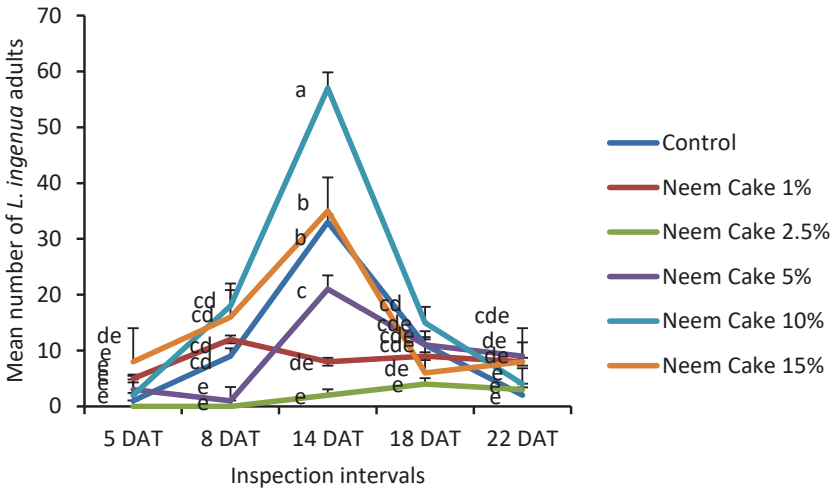

Figure 6. Abundance of *Lycoriella ingenua* adults depending of neem cake concentration during the experimental period (average±SE). SEDs, standard error of differences=9.05; SS=1593.33; df, degrees of freedom=29; *F*=7.93; *P*-value<0.0001 (One-way ANOVA; *Means marked by different letters are significantly different; Duncan's test, $p<0.05$).

and *T. aggressivum* incidence by 59.6% in comparison with control plots. After neem cake application, adverse effects on mushroom formation, yield and quality of fruiting bodies were not observed. The 5% concentration of neem cake drastically decreased mushroom yield (71.7%), while 10 or 15% neem cake amendement completely prevented fructification, which is in accordance with numerous other studies. In agreement with the results of the present study, Inam-Ul-Haq et al. (2010) found that concentrations of neem cake higher than 4% decreased mushroom yield. Previous studies have also shown that all neem tree derivatives, such as essential oils, neem cake and plant extracts, were efficient in pest/disease control in various mushroom crops. Shah et al. (2011) revealed that neem oil inhibited *Trichoderma harzianum* Rifai isolated from the oyster mushroom [*Pleurotus sajor-caju* (Fr.) Singh] by 34.1% in *in vitro* experiments. Furthermore, the authors reported that 1, 2, and 3% neem cake amendments increased oyster mushroom yields by 29, 34.3 and 35.3% (32.8% mean value) and reduced disease incidence by 33.3, 27.7 and 22.2 (mean value 27.7%), respectively, in comparison with control in *in vivo* tests. Besides, Shah et al. (2011) found that neem cake slightly reduced the time taken for pinhead formation as compared to control (7.6 days). Regarding the use of neem cake against disease agents, Sharma and Jarial (2000) reported that this neem three derivative inhibited the growth of *Diehliomyces microsporus* (Diehl & E.B. Lamb.) Gilkey, the false truffle disease agent of *Agaricus* spp. *in vitro.* The authors also discovered that neem cake incorporated in compost, drastically reduced disease incidence and increased the yield of button mushroom in *in vivo* experiments. Additionally, Sharma and Rajesh (2005) noted that 10% neem leaf extract inhibited the growth of *Sepedonium chrysospermum* (Bull.: Fr.) Link, causing yellow mould in button mushroom. Mishra (2009) reported that several neem three derivatives, i.e. neem leaf extract, neem cake solution and neem sawdust, inhibited *Trichoderma viride* Pers. on *Agaricus* spp. Grewal and Grewal (1988) noticed that incorporation of dried leaves of neem tree into mushroom compost eliminated pathogenic fungi belonging to *Fusarium* and *Sependonium* species. Moreover, Sharma and Jandiak (1994) reported that neem tree leaves incorporated in compost inoculated with various weed fungi, increased mushroom yield. Concerning the impact of neem cake on mushroom yield, Khade et al. (2019) reported that 2% neem cake per 3 kg substrate resulted in a significantly higher number of fruiting bodies of elm oyster mushroom [*Hypsizygus ulmarius* (Bull. Ex Fr.) Redhead] (89.56%). The authors also found that neem cake supplement achieved maximum mushroom yield (841.11 g per kg substrate), compared with different other organic and inorganic supplements. Also, the impovement of yield of oyster mushrooms *Pleurotus florida* Cetto and *P. sajor-caju* was detected after 2 and 4% neem cake supplementation (Sharma & Kumar, 2009). Furthermore, Inam-Ul-Haq et al. (2010) discovered that 2% neem cake amendment was the most promising in improving the yield of oyster mushroom [*Pleurotus ostreatus* (Jacq. Ex Fr.) Kumer] as it increased it up to 4%. Besides, Kumar et al. (2012) found that neem cake as a casing supplement of milky mushroom (*Calocybe indica* Purkay & A. Chandra) significantly inhanced its yield and pinhead initiation in comparison with control mushrooms, by 40-45%. It is noteworthy to highlight that neem tree based products have extremely low mammalian toxicity (Kleeberg, 1992), and they are relatively safe to non-target organisms (Schmutterer, 1995).

As in the case with *T. aggressivum*, we are not aware of any earlier research conducted to determine the impact of a neem cake on regulation of *L. ingenua* populations. Drobnjaković et al. (2019) found that a bioinsecticide based on azadirachtin (Ozoneem trishul 1%), the component of neem oil, succesfully controlled *L. ingenua*, compared to the malathion-based chemical insecticide Etiol tečni. Ozoneem trishul was applied at 2 ml m^{-2}, split in four applications, during casing time and later at seven-day intervals. The azadirachtin-based bioinsecticide supressed populations of the mushroom fly significantly better than malathion applied in the control chambers. The highest average number of mushroom flies was recorded 30 days after treatment (25 days after casing). There have been only a few reports with some other agricultural pests demonstrating that neem-based products may suppress pest populations and provide good alternative to conventional insecticide control. Depressed development and mortality were also recorded for neem-treated phorid larvae (Erler et al., 2009) and some dipteran pests (Stark et al., 1990; Okumu et al., 2007).

Pest and disease control in mushroom crops in Serbia is mainly based on using chemical pesticides, toxic to non-target organisms, humans and the environment. The European Commission Regulation 1107/2009 stimulates the application of low-risk active ingredients and use of sustainable alternatives

to chemical pesticides (Villaverde et al., 2014). Beside the European and national policies, the United Nations (UN) Sustainable Development Goals also urgently promote environmentally-friendly disease/pest control in mushroom production, which will improve the productivity and income of smallholder farming families (as part of a vulnerable population) (UNDP, 2023). Neem cake 2.5% amendment in casing soil could be recommended for application in mushroom production to control the mushroom sciarid fly and the causal agent of green mould disease without any negative impact on mushroom yield. The use of neem cake will reduce the use of chemical pesticides in mushroom industry and allow the processing and export of mushroom substrate and fresh mushrooms in accordance with the required standards for product safety and quality. It will further promote competitiveness among local farmers in regional markets. Furthermore, sustainable mushroom production will improve human health and safekeep the environment, strengthen the ecosystem's capacity to adapt to climate change, and improve land and soil quality.

CONCLUSION

Neem cake added as a supplement to casing soil for mushroom growing, applied at the concentration of 2.5%, reduced the number of *L. ingenua* adults by 83.93% and *T. aggressivum* green mould disease incidence by 59.6%, compared to control. After the application of that neem cake concentration, no adverse effects on mushroom formation, yield or quality of fruiting bodies was observed. Neem cake applied as a 2.5% amendment in casing soil could be recommended for application in white button mushroom production to control the mushroom sciarid fly and symptoms of green mould disease without negative impact on mushroom yield. The use of neem cake at the recommended application rate is in accordance with the basic postulates of the European Commission Regulation 1107/2009 and the UN Sustainable Development Goals.

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Uticaj dodavanja neem cake-a pokrivci za gajenje šampinjona na suzbijanje *Trichoderma aggressivum* Samuels & W. Gams i *Lycoriella ingenua* (Dufour) i prinos šampinjona

REZIME

Cilj rada je unapređenje integralne zaštite šampinjona *Agaricus bisporus* (Lange) Imbach od prouzrokovača bolesti zelene plesni *Trichoderma aggressivum* Samuels & W. Gams i šampinjonske mušice *Lycoriella ingenua* (Dufour). Ispitivan je uticaj *neem cake*-a dodatog pokrivci u smanjenju broja odraslih jedinki šampinjonske mušice, pojave simptoma zelene plesni i uticaj na prinos šampinjona. Pokrivka za gajenje šampinjona je obogaćena različitim koncentracijama *neem cake*-a: 1; 2,5; 5; 10 i 15%. Dodavanje *neem cake*-a pokrivci u udelu od 2,5% smanjilo je broj šampinjonskih mušica 83,93% i pojavu simptoma bolesti zelene plesni 59,6% u poređenju sa kontrolom. Nisu uočeni nepovoljni uticaji na obrazovanje, prinos i kvalitet plodnosnih tela šampinjona pri primeni navedene koncentracije. Dodatak 2,5% *neem cake*-a pokrivci se može preporučiti za primenu u proizvodnji šampinjona za suzbijanje šampinjonske mušice i simptoma bolesti zelene plesni, bez negativnog uticaja na prinos.

Ključne reči: *Agaricus bisporus*, *Azadirachta indica*, bolest zelene plesni, šampinjonska mušica

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Species of the genus *Parthenolecanium* (Hemiptera: Coccidae) in urban environments in Serbia

Marina Dervišević[1]* and Draga Graora[2]

[1]Institute of Pesticides and Environmental Protection, Banatska 31b, 11080 Belgrade, Serbia
[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Belgrade, Serbia
**Corresponding author: marina.dervisevic@pesting.org.rs*

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SUMMARY

Four species of soft scales from the genus *Parthenolecanium* have been registered in urban areas in Serbia: *Parthenolecanium corni*, *P. fletcheri*, *P. pomeranicum*, and *P. rufulum*. They all develop one generation annually and overwinter as second-instar nymphs on host twigs. *P. corni* reproduces by gamogenesis, while the other three species reproduce by parthenogenesis. The species were recorded on the territory of Serbia in 22 locations on 20 host plants, whereby *P. corni* was identified on 8 new host plants, and *P. fletheri* on one new host. The intensity of scale attacks and damage symptoms on the infested plants were variable. *P. corni*, *P. fletcheri*, and *P. rufulum* formed numerous colonies on some woody and bushy plants, causing branches of individual plants to dry and decay.

Keywords: scale insects, *Parthenolecanium*, urban trees, ornamental plants, Serbia

INTRODUCTION

The family Coccidae (Hemiptera: Coccoidea) includes many, primarily polyphagous, species that inhabit mainly perennial plants, including fruit trees, vines, forest trees, shrubs, and ornamentals worldwide (García-Morales et al., 2016). In tropical and subtropical regions, they are economically significant pests as they limit the production of citrus, olives, tea, and vines. However, in recent years, an increasing economic importance of these insects has been recorded in countries with temperate climate, which is probably a consequence of global warming and milder winters, as well as intensified transportation of plants and plant material (Goliszek et al., 2011).

Species of this family suck the sap from aboveground plant parts, causing premature drying and falling of leaves, drying of twigs, and the whole plant may decay due to long-term and continuous feeding (Japoshvili et al., 2008). In addition, Coccidae secrete large amounts of honeydew, which is a suitable substrate for development of sooty mold. As a result, photosynthesis and transpiration in plants are reduced, and plants look unsightly and dirty, spoiling their aesthetic value as ornamentals, and accelerating their decay (Basheer et al., 2011).

According to literature data, the family Coccidae includes over 1,230 species, 16 of which belong to the genus *Parthenolecanium* (García-Morales et al., 2016). In Serbia, the family Coccidae is represented with 17 species, and the genus *Parthenolecanium* with five species

(Kozarževskaja & Vlainić, 1982; Dervišević, 2019). Certain species of this genus occasionally start overpopulating when they cause significant damage to plant production. Thus, the European fruit lecanium, *P. corni*, is an important pest of grapevines in Croatia and Portugal (Masten-Milek et al., 2007; Silva et al., 2016), and it has also been recorded on many fruit species in Poland and Turkey, where it can cause minor or major damage (Tartanus et al., 2023; Gülmez et al., 2023). *P. corni* is one of the most common scales inhabiting ornamental plants in urban environments (Johnson & Lyon, 1991). Significant damage has been recorded in Georgia and the USA on plants in the genera *Quercus* and *Fraxinus* (Hodges & Braman, 2004; Japoshvili et al., 2008; Robayo-Comacho, 2015). In Turkey, *P. rufulum* has been registered as a significant pest of hazel (Saruhan & Tuncer, 2001) and oak in urban areas (Ülgentürk & Çanakçıoğlu, 2004).

In Serbia, *P. corni* was the most harmful species in fruit orchards and forest stands until the mid-20th century (Mitić-Mužina, 1960; Mitić-Mužina, 1964), while the peach scale, *P. persicae*, was registered as a grapevine pest (Graora et al., 2012). Studies of this group of insects in urban areas are few and date from about 40 years ago when data on host plants and the biology of scales were reported for *P. corni*, *P. pomeranicum*, *P. rufulum*, and *P. fletcheri* (Kozarževskaja & Vlainić, 1982).

Considering that there is no detailed literature on *Parthenolecanium* species on forest and ornamental plants in Serbia, the need for a comprehensive study of these insects has arisen. Therefore, this work aimed to determine the distribution of these species in Serbia, to study their life cycle, host plants, infestation intensity, and symptoms of damage in urban areas.

MATERIALS AND METHODS

The study of soft scales from the genus *Parthenolecanium* were executed from 2014 to 2017 in the field and in the Laboratory of Entomology and Agricultural Zoology of the Faculty of Agriculture in Belgrade, Serbia.

To determine the presence and distribution of scales, the material was sampled from 22 locations in Serbia: Ada Ciganlija (44°47′30″N, 20°24′45″E), Aranđelovac (44°18’28”N, 20°33’07”E), Banjica (44°46′18″N, 20°28′16″E), Bežanijska kosa (44°49′50″N, 20°22′31″E), Blok 45 (44°47′48″N, 20°22′45″E), Galenika (44°51’27”N, 20°21’54”E), Čukarica (44°41′30″N, 20°23′50″E), Konjarnik (44°46′54″N, 20°30′31″E), Kosmaj (44°28′17″N, 20°34′32″E), Košutnjak (44°45′53″N, 20°26′11″E), Novi Beograd (44°49′11″N, 20°23′56″E), Pančevo (44°51′53″N, 20°39′23″E), Radmilovac (44°45′15″N, 20°34′59″E), Svilajnac (44°13′54″N, 21°11′32″E), Topola (44°14’25”N, 20°40’47”E), Ušće (44°49′36″N, 20°26′13″E), Voždovac (44°46’25.8”N, 20°28’17.6”E), Vračar (44°47′51″N, 20°28′35″E), Zemun (44°50′26″N, 20°24′31″E), Zemun Polje (44°52′19″N, 20°19′27″E), Zvezdara (44°46′36″N, 20°31′56″E), Žagubica (44°11′51″N, 21°47′18″E).

Methods of visual examination of plants were determined, infested plant material was sampled from parks and tree rows, and infestation intensity and damage symptoms evaluated. The intensity of scale attacks on plants was assessed according to the Borchsenius (1963) scale. The life cycle of scales was monitored in different locations and on different host plants. Thus, the development of *P. corni* on *Ulmus minor* was monitored (location Radmilovac), *P. fletcheri* on *Thuja occidentalis* var. Tiny-Tim (location Pančevo), *P. pomeranicum* on *Taxus baccata* (location Svilajnac), and *P. rufulum* on *Quercus robur* (location Ada Ciganlija). Throughout the year, plant material was sampled at intervals of 7 to 10 days during the vegetation season and once a month during vegetative rest. Five two-year or one-year old twigs of 20 cm length were collected from each infested plant. The twigs were packed in labeled plastic bags with detailed information on the sampling location, date, and host plant, and then stored in a fridge until further examination in the laboratory.

The sampled plant material was examined in the laboratory, permanent microscopic slides were made, and the soft scales were reared and identified. To analyze their morphological characters, permanent microscopic slides of females were made according to the relevant method of Kosztarab and Kozár (1988), and species were identified based on the keys of Gill (1988), Kosztarab & Kozár (1988), and Stepaniuk & Łagowska (2006).

To rear the scales, the sampled twigs with scale colonies were placed in glass cylinders covered with dense synthetic mesh. The time and number of eggs laid, and duration of embryonic and post-embryonic development of scales on twigs were examined daily. The average number of eggs laid by females per species was determined based on counting the eggs of 10 females.

RESULTS

During the three-year research in urban areas in Serbia, four *Parthenolecanium* species were registered: *Parthenolecanium corni* (Bouché) (Figure 1), *Parthenolecanium fletcheri* (Cockerell) (Figure 2), *Parthenolecanium pomeranicum* (Kawecki) (Figure 3), and *Parthenolecanium rufulum* (Cockerell) (Figure 4).

Figure 1. Females of *P. corni* (orig.)

Figure 2. Female of *P. fletcheri* (orig.)

Figure 3. Females of *P. pomeranicum* (orig.)

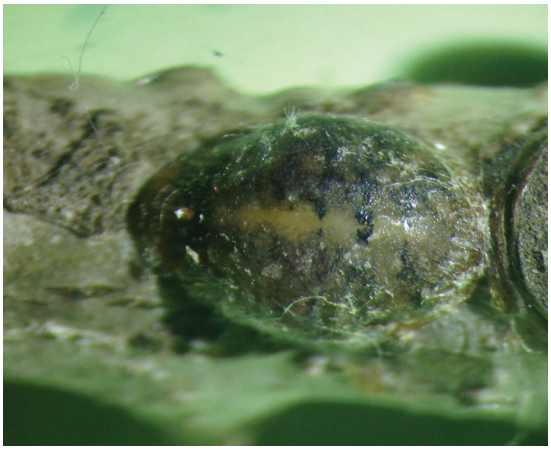

Figure 4. Female of *P. rufulum* (orig.)

Life cycle of *Parthenolecanium* species

All four identified species develop one generation per year and overwinter as second-instar nymphs on host twigs. They reproduce by gamogenesis or parthenogenesis. Since the life cycle of the four species was very similar during the three-year research, while the timing of emergence of all development stages differed by no more than a few days per year, the obtained results are presented only for the year 2017.

P. corni reproduces by gamogenesis. During development, females pass through two nymphal stages, while males go through prepupal and pupal stages in addition to two nymphal stages. In the spring, the overwintering second-instar nymphs (Figure 5) become active and resume feeding. They usually concentrate around leaf buds on younger twigs. The nymphs of future males form a wax covering to undergo prepupal and pupal transformation underneath. The appearance of prepupae (Figure 6) was recorded in mid-March, and pupae at the end of March. Males (Figure 7) ecloded in the first decade of April. After intensive feeding, the nymphs of future females molted to become females (Figure 8). Females were found to appear in the first decade of April, 3-4 days after the eclosion of males (Table 1). Females laid an average of 924.3±14.1 eggs (Figure 9). After embryonic development, which lasted about 20 days, first-instar nymph hatching (Figure 10) was recorded in mid-May. The hatched nymphs remained under the scales of females for a variable period of time before leaving and moving to young twigs and leaves to feed during the summer months. Second-instar nymphs formed at the end of August, and fed until October, when they usually retreat to tree forks or cracks on thicker branches to overwinter.

P. fletcheri, *P. pomeranicum*, and *P. rufulum* reproduce by parthenogenesis. Overwintering second-instar nymphs feed intensively in the spring, especially on young twigs whose bark is thin and juicy, thus significantly increasing their body size, and after molting, they form females. The appearance of *P. rufulum* and *P. pomeranicum* females was noted in April and *P. fletcheri* in early May (Table 1). The females started oviposition in May, and the number of eggs laid depended on scale species. On the average, the highest number of eggs was recorded in *P. pomeranicum* (1001.1±3.7), followed by *P. rufulum* (694.6±19.8), and *P. fletcheri* (350.3±4.1). First-instar nymphs hatched after 3-4 weeks, which is the duration of embryonic development. During the summer months, from June to August, they sucked plant sap, usually on the apical, young parts of the host plants. In September, they formed second-instar nymphs which continued feeding until the beginning of October, when they migrated to thicker branches to overwinter.

Table 1. The life cycle of *Parthenolecanium* species in Serbia in 2017

Development stage	Species				
	P. corni		*P. fletcheri*	*P. pomeranicum*	*P. rufulum*
	Female development	Male development			
prepupa	-	15.03.	-	-	-
pupa	-	27.03.	-	-	-
male	-	07.04.	-	-	-
female	10.04.	-	03.05.	29.04.	10.04.
eggs	25.04.	25.04.	18.05.	16.05.	03.05.
N_1	16.05.	16.05.	14.06.	12.06.	02.06.
N_2	27.08.	27.08.	12.09.	14.09.	03.09.

N_1 – first-instar („crawler")
N_2 – second-instar

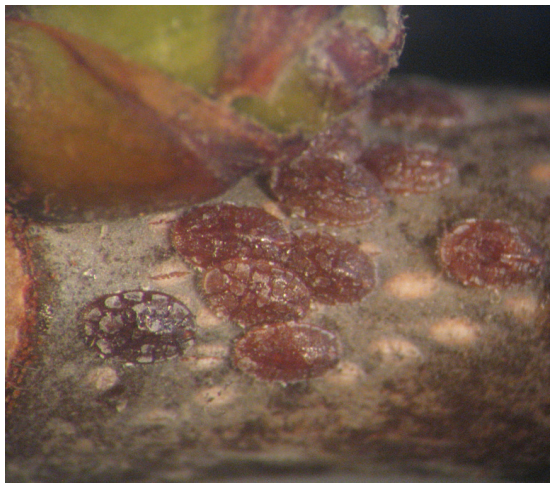

Figure 5. Overwintering second-instar nymphs of *P. corni* (orig.)

Figure 6. Prepupa of *P. corni* (orig.)

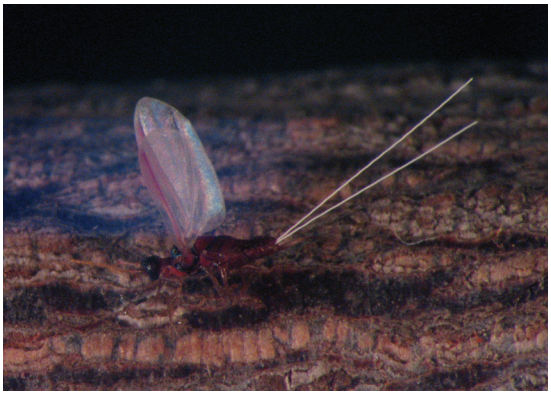

Figure 7. Male of *P. corni* (orig.)

Figure 8. Females of *P. corni* (orig.)

Figure 9. Eggs of *P. corni* (orig.)

Figure 10. First-instar nymph of *P. corni* (orig.)

Distribution, host plants, infestation intensity, and damage symptoms of *Parthenolecanium* species in Serbia

Soft scales of the genus *Parthenolecanium* were recorded in Serbia on 22 locations and 20 host plants (Table 2).

P. corni was the most widespread species, infesting the largest number of host plants. It was detected on 14 locations and 18 plant species, of which 8 species, *Buxus sempervirens*, *Celtis australis*, *C. occidentalis*, *Rosmarinus officinalis*, *Styphnolobium japonicum*, *Syringa vulgaris*, *Tilia cordata* and *Tilia tomentosa*, were new hosts of this scale insect in Serbia.

P. fletcheri was registered on *Thuja occidentalis* on three locations. Also, this species was found on *Taxus baccata* L. (Kosmaj location), which is a new host in Serbia.

P. pomeranicum was identified only on *Taxus baccata* on the location Svilajnac.

P. rufulum was found on nine locations on *Quercus robur*. This species was also found on *Ulmus minor* (Ada Ciganlija location).

The listed species of soft scales most often inhabit woody and shrubby plants and occur much less on herbaceous plants. The intensity of attack on infested plants ranged from 1 to 4, i.e. from individual specimens being found on a plant to an entire plant being covered with colonies.

In cases where individual specimens and small colonies of scales were found (infestation intensity 1 and 2), the plants had no visible damage symptoms. On the other hand, when numerous scale colonies were found (infestation intensity 3 and 4) (Figures 11-12), symptoms of yellowing and falling of leaves, weak growth of shoots, and drying of individual twigs were observed. The presence of honeydew and sooty mold further impaired the aesthetic appearance of plants.

The greatest damage was recorded in Radmilovac, where the attack of *P. corni* caused branches of *Ulmus minor* to dry up, and in Ada Ciganlija, where an attack of *P. rufulum* caused the drying of individual branches of *Q. robur*. Similarly, *Thuja occidentalis* plants in Pančevo were found to have dirty appearance due to a strong infestation of *P. fletcheri* and the presence of sooty mold, which reduced their aesthetic value.

Table 2. Host plants, distribution, and infestation intensity of *Parthenolecanium* species in Serbia

Scale insects	Host Plant	Location	Infestation intensity
P. corni	*Acer negundo*	Bežanijska kosa	2
		Zemun	3-4
	Acer pseudoplatanus	Ada Ciganlija	3-4
		Aranđelovac	1-2
		Banjica	2-3
		Novi Beograd	3
		Topola	1-2
		Ušće	3-4
		Zemun Polje	3-4
	Aesculus hippocastanm	Blok 45	1-2
		Novi Beograd	1-2
	*Buxus sempervirens**	Žagubica	1
	Carpinus betulus	Zemun	4
	*Celtis australis**	Novi Beograd	1
	*Celtis occidentalis**	Novi Beograd	1
	Cercis siliquastrum	Novi Beograd	2
	Cornus sanquinea	Radmilovac	1-2
	Fraxinus excelsior	Zemun	3-4
	Prunus cerasus	Žagubica	1
	*Rosmarinus officinalis**	Bežanijska kosa	2
		Voždovac	1
	*Styphnolobium japonicum**	Blok 45	3-4
	*Syringa vulgaris**	Blok 45	1
		Galenika	1
	*Tilia cordata**	Novi Beograd	3-4
	*Tilia tomentosa**	Zemun	3-4
	Ulmus minor	Radmilovac	3-4
	Quercus robur	Ušće	1
P. fletcheri	*Taxus baccata**	Kosmaj	3-4
	Thuja occidentalis	Novi Beograd	1-2
		Zvezdara	1-2
		Pančevo	3-4
P. pomeranicum	*Taxus baccata*	Svilajnac	1-2
P. rufulum	*Quercus robur*	Ada Ciganlija	3-4
		Čukarica	1
		Konjarnik	1
		Kosmaj	1
		Košutnjak	3-4
		Novi Beograd	1
		Voždovac	1
		Vračar	3-4
		Zemun	3-4
	Ulmus minor	Ada Ciganlija	1

*plants first identified as hosts of species in the genus *Parthenolecanium* in Serbia

Figure 11. Colony of *P. corni* on *T. tomentosa* (orig.)

Figure 12. Colony of *P. rufulum* on *Q. robur* (orig.)

DISCUSSION

Four species of the genus *Parthenolecanium* were identified in urban areas in Serbia: *P. corni*, *P. fletcheri*, *P. pomeranicum*, and *P. rufulum*. All of them develop one generation annually and overwinter as second-instar nymphs on twigs of their host plant. *P. corni* reproduces by gamogenesis, while *P. fletcheri*, *P. pomeranicum*, and *P. rufulum* reproduce by parthenogenesis. Data about the number of generations and the way of overwintering of soft scales are similar in most European countries (Kosztarab & Kozár, 1988; Ülgentürk et al., 2008). An exception is *P. corni*, which has been found to develop two generations on vines in Croatia and on peaches in Pennsylvania (Kosztarab, 1996; Masten-Milek et al., 2007). In Serbia, *Parthenolecanium* species are univoltine (Kozarževskaja & Vlainić, 1982; Graora et al., 2012), although *P. corni* can also develop a second generation on acacia (Mitić-Mužina, 1960).

The genus *Parthenolecanium* includes mainly polyphagous and less often monophagous species, which inhabit primarily perennial woody and shrubby plants. In the course of this study, four species of scales were found on 20 plants. *P. corni* was found on 8 new hosts, and *P. fletcheri* on one new host. In a previous research study in Serbia, *P. corni* was detected on plants from at least 13 plant genera, *P. rufulum* on 9 plant genera, and *P. fletcheri* and *P. pomeranicum* on one plant species each (Kozarževskaja & Vlainić, 1982).

P. corni, *P. fletcheri,* and *P. rufulum* were found in the present research to cause infestation intensity 3 and 4, resulting in the drying of branches and decay of individual ornamental plants.

There are similar data on the harmfulness of these species in urban areas in other European countries. Thus, *P. rufulum* is a significant pest of *Quercus* sp. in Georgia (Japoshvili, 2001), *P. corni* is the most abundant and economically important scale species on ornamentals in Poland (Goliszek et al., 2011), and *P. pomeranicum* is the most abundant species in Turkey (Ülgentürk et al., 2008). In England, *P. pomeranicum* was a serious pest of *Taxus baccata* in the 1930s. After that period, the species lost its importance, probably due to the actions of natural enemies (Malumphy et al., 2011). *P. fletcheri* has been recorded in the US as a pest of ornamental plants in the genera *Thuja* and *Taxus*, while in Europe, it does not cause significant damage. In some countries, such as Great Britain, this species occasionally forms numerous colonies on ornamental plants in urban areas without causing visible symptoms of damage (Malumphy, 2011).

CONCLUSION

In urban areas, plants grow under challenging conditions outside their natural habitats and are therefore more susceptible to harmful effects of many abiotic and biotic factors. Many of their pests are soft scales, which occasionally form dense colonies on plants. Considerable harmfulness of these species is due to their polyphagy, high fecundity, and ecological plasticity. As chemical control measures are rarely applied against these pests in urban areas, further research should focus on studying the complex of natural enemies and their role in regulating the abundance of *Parthenolecanium* soft scale species.

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Vrste roda *Parthenolecanium* (Hemiptera: Coccidae) u urbanim sredinama u Srbiji

REZIME

U urbanim sredinama u Srbiji registrovane su četiri vrste štitastih vaši iz roda *Parthenolecanium*, i to: *Parthenolecanium corni*, *P. fletcheri*, *P. pomeranicum* i *P. rufulum*. Sve utvrđene vrste razvijaju jednu generaciju godišnje i prezimljavaju u stadijumu larve drugog stupnja na grančicama domaćina. *P. corni* se razmnožava gamogenezom, dok se ostale vrste razmnožavaju partenogenezom. Utvrđene vrste zabeležene su na području Srbije u 22 lokaliteta na 20 biljaka domaćina, pri čemu je *P. corni* utvrđena na 8 novih biljaka domaćina, a *P. fletheri* na jednom novom domaćinu. Intenzitet napada vaši kao i simptomi oštećenja na infestiranim biljkama su se razlikovali. Na pojedinim drvenastim i žbunastim biljkama, vrste *P. corni*, *P. fletcheri* i *P. rufulum*, su obrazovale brojne kolonije prouzrokujući sušenje grana i propadanje pojedinačnih biljaka.

Ključne reči: štitaste vaši, *Parthenolecanium*, gradsko zelenilo, ukrasne biljke, Srbija

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Corrigendum to "Sensitivity of *Cuscuta* species and their hosts to *Anethum graveolens* essential oil"

Marija Sarić-Krsmanović*, Jelena Gajić Umiljendić, Ljiljana Radivojević, Ljiljana Šantrić, Tijana Đorđević and Rada Đurović-Pejčev

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**Corresponding author: marijasaric.msaric@gmail.com*

The original published version of the article contained an error:

(1) Figure 2 contains research data repeated from Figure 1.

Corrigendum presents the corrected version of Figure 2.

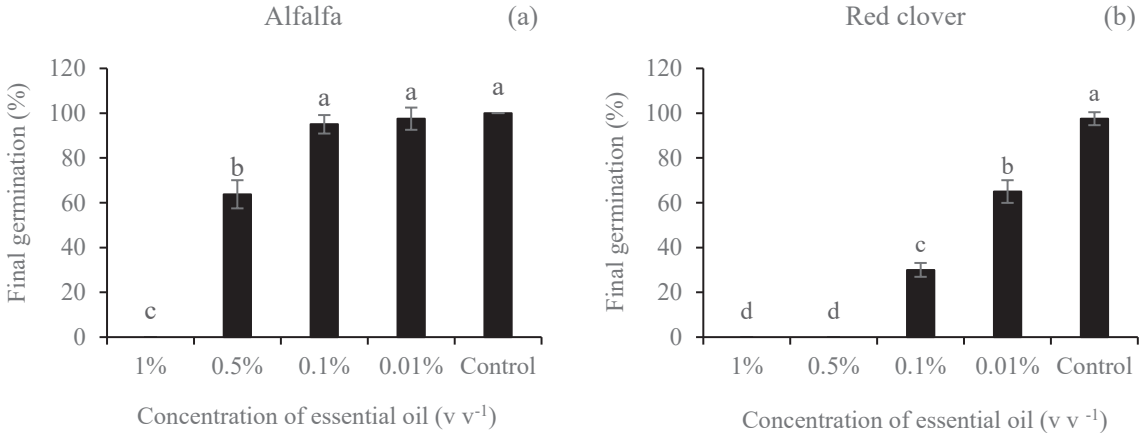

Figure 2. Effects of different concentrations of *A. graveolens* essential oil on seed germination of alfalfa (a) and red clover (b). Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p < 0.05$. Means marked by different letters (a, b, c, d) differ significantly ($p < 0.05$) for final germination at different concentrations.

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Photos need to be taken by digital camera (resolution at least 150 dpi, photo dimension A4, file format JPG or TIFF). If authors are unable to submit original photos, those should be scanned in RGB mode (colour) or as Grayscale (black and white), with 300 dpi resolution in original size. Photos need to be marked with Arabic numerals in consecutive order. Provide each photo with a caption, mark its approximate location in the text and submit it as a supplementary file.

Authors are expected to use the accepted International System of Units (SI). Abbreviations should be defined in brackets at their first in-text mention. Provide full Latin names along with common names of organisms, and italicize only Latin names of genera and species, e.g. Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). After first mention, the Latin name can be abbreviated (e.g. *L. decemlineata*).

Review articles need to contain an introduction, appropriate subtitles and a reference list.

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Uputstvo autorima

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Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* objavljuje naučne radove iz oblasti: toksikologije i ekotoksikologije pesticida; fitopatologije; primenjene entomologije i zoologije; herbologije; zaštite bilja i prehrambenih proizvoda; primene pesticida u poljoprivredi, komunalnoj higijeni i javnom zdravstvu.

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Od 2020. godine, časopis izlazi četvoromesečno (tri broja godišnje).

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Rad treba pripremiti u programu za obradu teksta Word (format A4, margine 25 mm, font Times New Roman 12 pt). Radovi treba da budu isključivo na engleskom jeziku sa naslovom i rezimeom na oba jezika (engleskom i srpskom).

Naslov treba da bude kratak i da upućuje na temu. Puna imena i prezimena svih autora, puni nazivi i adrese institucija svih autora i njihove email adrese treba navesti ispod naslova rada. U slučaju neslaganja ovih podataka u samom tekstu rada i u prijavi na platformi za uređivanje, prioritet će se dati podacima u samom tekstu rada.

Rezime (obima do 300 reči) treba da predstavi ono što je za rad najznačajnije.

Rad treba, po pravilu, da sadrži sledeća poglavlja: Uvod, Materijal i metode, Rezultati, Diskusija, Zahvalnica i Literatura.

Uvod treba da sadrži najnužniji pregled istraživanja u datoj oblasti i ciljeve istraživanja.

Materijal i metode treba opisati dovoljno detaljno da omogućе ponavljanje ispitivanja. Poznate metode i tehnike označiti samo odrednicom iz literature.

Rezultate predstaviti logičnim redosledom, jasno i precizno, koristeći prigodne tabele i grafičke prikaze. Izbegavati ponavljanje rezultata u tabelama i grafikonima, ali i u tekstu rada.

Diskusija treba da istakne značaj dobijenih rezultata, kao i njihovo mesto u kontekstu prethodnih istraživanja. Kad god je to moguće, diskusiju treba odvojiti od rezultata.

Zahvalnica se navodi na kraju teksta rada, pre literature.

Literatura se u tekstu rada citira navođenjem prezimena autora i godine:

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Dedić, B. (2012). Testing sunflower inbred lines for tolerance to phoma black stem. *Pesticides & Phytomedicine*, *27*(4), 299-303. doi:10.2298/PIF1204299D

Abbaspoor, M., & Streibig, J.C. (2005). Clodinafop changes the chlorophyll fluorescence induction curve. *Weed Science, 53*(1), 1-9. doi:10.1614/WS-04-131R

Abbaspoor, M., Teicher, H.B., & Streibig, J.C. (2006). The effect of root-absorbed PSII inhibitors on Kautsky curve parameters in sugar beet. *Weed Research, 46*(3), 226-235. doi:10.1111/j.1365-3180.2006.00498.x

Knjige: autor(i) ili editor(i), godina publikovanja, naslov, mesto publikovanja i naziv izdavača.

Timbrell, J. (2000). *Principles of biochemical toxicology* (3rd ed). London, UK: Taylor and Francis Ltd.

Frank, R. H., & Bernanke, B. (2007). *Principles of macroeconomics* (3rd ed.). Boston, MA: McGraw-Hill/Irwin.

Saari L.L., & Thill, D.C. (Eds.). (1994). *Resistance to acetolactate synthase inhibiting herbicides: Herbicide resistance in plants*. Boca Raton, FL, USA: CRC Press.

Disertacije: autor, godina odbrane, naslov, i puni naziv institucije u kojoj je disertacija odbranjena.

Stepanović, M. (2012). *Osetljivost izolata Alternaria solani (Sorauer) iz različitih krajeva Srbije na fungicide i rizik rezistentnosti*. (Doktorska disertacija). Univerzitet u Beogradu, Poljoprivredni fakultet, Beograd.

Poglavlja u knjigama i radovi u zbornicima: autor(i), godina publikovanja, naslov poglavlja/rada/apstrakta, naslov izvornika sa imenom (imenima) urednika, strane priloga, mesto publikovanja i naziv izdavača.

Hammond, K. R., & Adelman, L. (1986). Science, values, and human judgment. In H. R. Arkes & K. R. Hammond (Eds.), *Judgement and decision making: An interdisciplinary reader* (pp 127-143). Cambridge, UK: Cambridge University Press.

Edwards, J.P., Fitches, E.C., Audsley, N. & Gatehouse, J.A. (2002). Insect neuropeptide fusion proteins – A new generation of orally active insect control agents. In T. Margini (Ed.), *Proceedings of the BCPC – Pests and diseases* (pp 237-242). Brighton, UK: University of Brighton Press.

Internet reference: autor(i), godina publikovanja, naslov, naziv izvornika, link.

Graora, D., & Spasić, R. (2008). Prirodni neprijatelji *Pseudaulacaspis pentagona* Targioni-Tozzetti u Srbiji. *Pesticidi i fitomedicina*, *23*(1) 11-16. Retrieved from http://www.pesting.org.rs/media/casopis/2008/no.1/23_1_11-16.pdf

Radunović, D., Gavrilović, V., Gašić, K., Krstić, M. (2015). Monitoring of *Erwinia amylovora* in Montenegro. *Pesticides and Phytomedicine*, 30(3), 179-185. doi 10.2298/PIF1503179R or http://www.pesting.org.rs/media/casopis/2015/no.3/30-3_179-185.pdf

Kerruish, R.M. & Unger, P.W. (2010). *Plant protection 1 – Pests, diseases and weeds*. Retrieved from APPS at http://www.appsnet.org/Publications/Kerruish/PP1.pdf

Tabele se obeležavaju arapskim brojevima prema predviđenom redosledu. Tabele se izrađuju isključivo u programu Word for Windows, kroz meni Table-Insert-Table, koristeći font Times New Roman, 12 pt i osnovni prored. Fusnotama neposredno ispod tabela treba dati prednost nad drugim objašnjenima u zaglavlju tabela ili u samim tabelama, a tekst se daje u fontu Times New Roman, 10 pt. Svaka tabela mora imati zaglavlje. Tabele se prilažu kao dopunske (zasebne) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

Grafikoni treba da budu urađeni i dostavljeni u programu Excel, sa podacima u fontu Times New Roman. Potrebna objašnjenja daju se u legendama obeleženim arapskim brojevima prema redosledu. Grafikoni se prilažu kao zasebne (dopunske) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

Dijagrami treba da budu urađeni i dostavljeni u programu Corel Draw (verzija 9 ili novija), ili u programu Adobe Illustrator (verzija 9 ili novija). Za unos podataka treba koristiti font Times New Roman. Grafikoni se prilažu kao zasebne (dopunske) datoteke, a u samom tekstu se obeležava njihovo približno mesto.

Fotografije treba da budu snimljene digitalnim fotoaparatom (rezolucija najmanje 150 dpi, dimenzija fotografije A4, a format zapisa JPG ili TIFF). Ukoliko autori nisu u mogućnosti da dostave originalne fotografije, treba ih skenirati u RGB modelu (ukoliko su u boji), odnosno kao Grayscale (ukoliko su crno-bele), sa rezolucijom 300 dpi u originalnoj veličini. Fotografije je potrebno obeležiti arapskim brojevima prema predviđenom redosledu. Za svaku fotografiju se daje legenda i obeležava njeno približno mesto pojavljivanja u tekstu. Svaka fotografija se prilaže kao zasebna (dopunska) datoteka.

Od autora se očekuje da koriste preporučene jedinice međunarodnog sistema (SI). Skraćenice je potrebno definisati u zagradama nakon prvog pominjanja u tekstu. Narodni nazivi organizama se daju uz pun latinski naziv, a kurzivom se obeležavaju samo latinski nazivi rodova i vrsta, npr. krompirova zlatica, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). Nakon prvog pojavljivanja, latinsko ime dalje treba pisati skraćeno (npr. *L. decemlineata*).

Pregledni radovi treba da sadrže uvod, odgovarajuće podnaslove i spisak literature.

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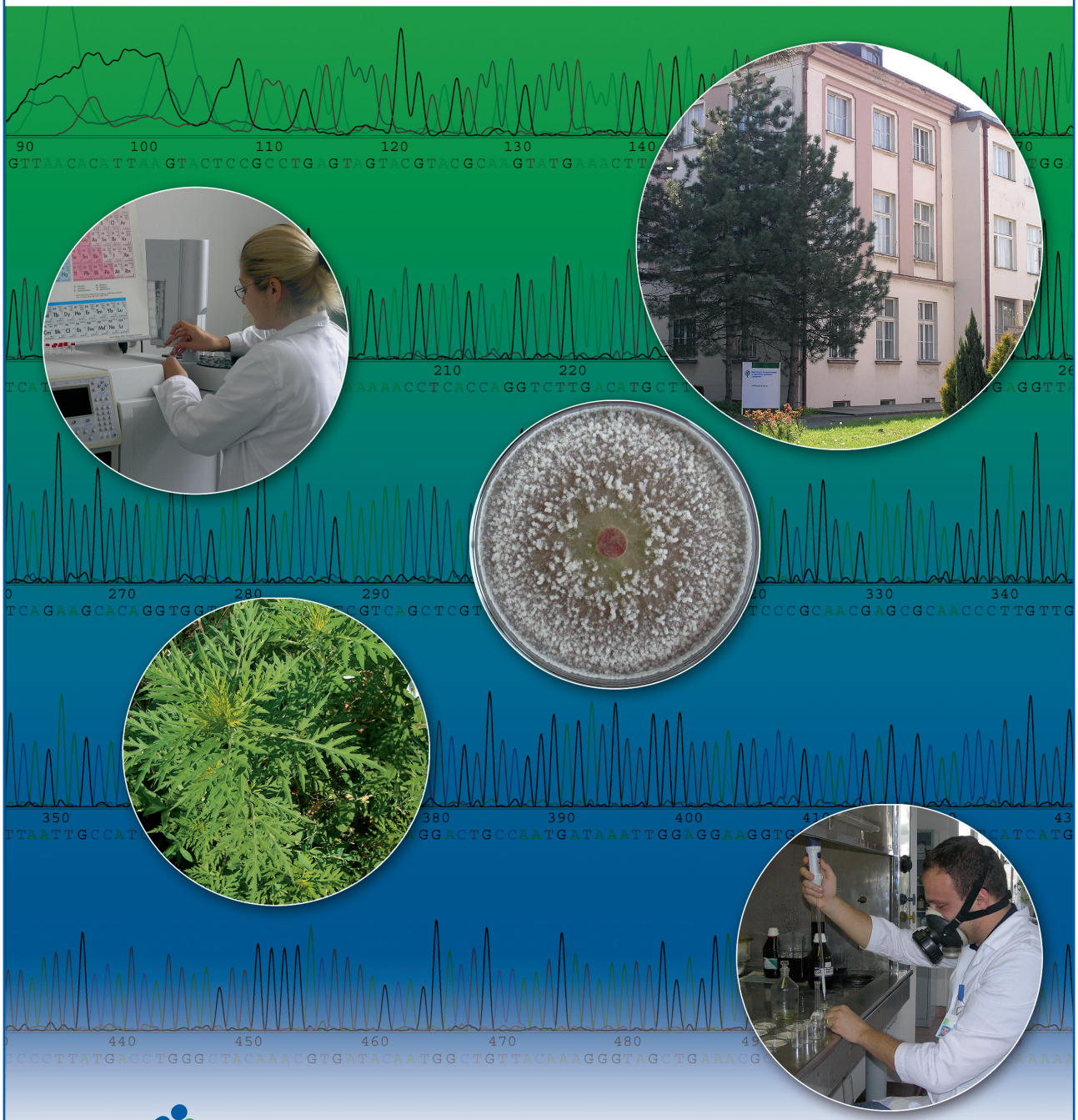

Institut za pesticide i zaštitu životne sredine

11080 Beograd - Zemun, Banatska 31b
Tel/fax: (011) 3076-133, 3076-136
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