UDC 632:615:89

eISSN 2406-1026

Pesticides & Phytomedicine

Pesticidi i fitomedicina

Scientific Journal of the Serbian Plant Protection Society

Vol. 40 * No. 1 * 2025

eISSN 2406-1026

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Vol. 40 * No. 1 * 2025

Pesticides & Phytomedicine

eISSN 2406-1026

Published triannually

PUBLISHER

Institute of Pesticides and Environmental Protection, Belgrade, Serbia

Phone: (011) 3076-133, 3076-136

Fax: (011) 3076-136

CO-PUBLISHER

Serbian Plant Protection Society, Belgrade, Serbia

PUBLISHER DIRECTOR

Emil Rekanović

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Cited in: ***Chemical Abstracts; CAB International; DOAJ; EBSCO; AGRIS; Scindeks***
Full text articles available at: www.pesting.org.rs; www.doaj.org;
http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949

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Pestic. Phytomed. (Belgrade), 40(1), 2025, 1–11

Botryosphaeriaceae fungi on apple fruit – identification and sensitivity to fungicides and essential oils *in vitro*

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SUMMARY

Apple production suffers significant economic losses and fruit quality reduction due to fungal pathogens, particularly ones that cause postharvest fruit rot, such as Botryosphaeriaceae fungi. Isolates used in this study were obtained from symptomatic apples and, based on morphological characteristics and sequence analysis of two genes (EF 1-α and β-tubulin), they were identified as *Diplodia seriata* and *Botryosphaeria dothidea*. Pathogenicity tests on healthy apple fruits revealed that *D. seriata* was more aggressive than *B. dothidea,* with significantly higher average values of lesion diameter and depth. Fungicide sensitivity tests showed that *D. seriata* was more sensitive to the combination fluopyram + tebuconazole (EC_{50}=0.00023 µg a.i. ml^{-1}), while *B. dothidea* exhibited higher sensitivity to pyraclostrobin (EC_{50}=0.025 µg a.i. ml^{-1}). With 98.44% and 97.56% percent growth inhibition (PGI) rate of *D. seriata* and *B. dothidea* (respectively) at 10 µg a.i. ml^{-1}, the tested combination of fungicides surpassed pyraclostrobin in inhibition potential. Four essential oils (thyme, rosemary, lavender and lemongrass) were also tested for antifungal activity using the fumigant macrodilution method. Thyme oil demonstrated the highest antifungal potential, completely inhibiting the mycelial growth of both species at 0.05 µl ml^{-1} of air. Strong inhibition potential was also shown by lemongrass oil with 100% inhibition of *D. seriata* and *B. dothidea* mycelial growth at 0.07 and 0.09 µl ml^{-1} of air, respectively. Rosemary oil showed a moderate inhibition potential, while lavender oil was the least effective. These findings highlight the inhibiting potential of fungicides against *D. seriata* and *B. dothidea*, but they also indicate that thyme and lemongrass essential oils could be used as viable alternatives. Further research is needed to determine their effectiveness in *in vivo* assays and potential impact on fruit quality and the environment.

Keywords: apple, fungal pathogens, postharvest fruit rot, fungicides, essential oils, antifungal activity

Article info

Original scientific paper

Received: 3 March 2025

Accepted: 25 March 2025

Publication is free of charge.

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DOI: https://doi.org/10.2298/PIF2501001M

INTRODUCTION

Fruit production accounts for 5.7% of Serbia's overall agricultural production, and apple was the leading fruit species in 2023. Serbia is a major apple producer in South East Europe with a total apple output of 379,690 tons, and 27,412 ha of harvested area (FAOSTAT, 2023; Statistical Office of the Republic of Serbia, 2024). Apple production is threatened by numerous postharvest phytopathogenic fungi, such as *Penicillium expansum*, *Botrytis cinerea*, *Fusarium avenaceum*, *Botryosphaeria* spp., *Diplodia* spp., *Alternaria* spp., *Monilinia* spp., *Neofabrea* spp., *Diaporthe*

spp. and some other, which cause severe fruit quality and yield losses (Konstantinou et al., 2011; Di Francesco et al., 2019; Vučković et al., 2022). The family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) includes a large and diverse group of fungi known for their global distribution in a wide variety of woody hosts (Batista et al., 2021). These fungi are described as aggressive pathogens, endophytes or opportunistic pathogens associated with host stress (Slippers et al., 2013). Many members of the family cause stem canker, die-back and fruit rot in apple-growing areas around the world (Tang et al., 2012). Several species of Botryosphaeriaceae have been reported as apple pathogens, but the most frequent ones are *Botryosphaeria dothidea* and *Diplodia seriata* (Delgado-Cerrone et al., 2016). The presence of these two species was previously confirmed on apple fruit in Serbia (Stojanović et al., 2003; Vasić et al., 2013; Vučković et al., 2022).

Traditional cultivation practices, such as pruning of infected branches, apple bagging technology or girdling (Dai et al., 2017), are labour intensive, so that preventive fungicide treatments are still an effective strategy for managing Botryosphaeriaceae pathogens. Their management is complicated due to their latent infection potential and wide host range. There are a number of fungicides from different chemical groups that have been registered against postharvest pathogens on apple, whose efficacy has already been evaluated and confirmed (Song et al., 2018; Fan et al., 2019; Thomidis & Prodromou, 2020; Fan et al., 2022). However, currently there are no fungicides registered in Serbia for the control of Botryosphaeriaceae in apple, although some active ingredients from the chemical groups of Quinone outside Inhibitors (QoI), DeMethylation Inhibitors (DMI) and Succinate-dehydrogenase Inhibitors (SDHI) have been registered against other apple postharvest pathogens (*Gleosporium* spp., *Monilinia* spp., *Penicillium* spp.) (Team of Editors, 2024). Regarding QoI fungicides, pyraclostrobin with its preventive, curative and eradicative effects has demonstrated exceptional degrees of efficacy against a broad spectrum of fungal pathogens (Yuan et al., 2015), including Botryosphaeriaceae species on apples, although it is not registered for its control (Fan et al., 2016; Fan et al., 2019). Fluopyram (SDHI fungicide) in combination with tebuconazole (DMI fungicide) is commercially available and registered for the management of rot-causing pathogens in apple orchards. Based on the FRAC classification, pyraclostrobin is categorized as high-risk, fluopyram as medium to high risk, and tebuconazole as medium risk active ingredient for fungicide resistance development (FRAC, 2024).

However, control measures against Botryosphaeriaceae pathogens need to be carefully addressed, considering the limitations inherent to fruit exportation, such as low fruit residue levels and environmental regulatory requirements restricting the selection of agrochemicals. The requirements for minimal pesticide residues in plant products, but also for a healthier environment, has led to the development of environmentally-friendly alternatives to synthetic fungicides, such as biological control agents or naturally derived compounds with antifungal potential (essential oils - EOs or plant extracts). EOs can have antifungal, antibacterial, antiviral, herbicidal or insecticidal effects (Sayed-Ahmad et al., 2017; Verdeguer et al., 2020). Many studies have explored the use of essential oils to control postharvest losses in fruit and vegetable crops (Abd-Alla et al., 2011; Grahovac et al., 2011; Lopez-Reyes et al., 2010, 2013; Antonioli et al., 2020; Kontaxakis et al., 2020; Di Francesco et al., 2022; Soppelsa et al., 2023). Therefore, EOs may be considered as an alternative resource for pest and disease control, since they constitute a rich source of bioactive compounds that are potentially suitable for use in integrated management programs (Merah et al., 2020; Verdeguer et al., 2020).

Hence, the aim of this study was to: 1) isolate and identify the pathogens that caused postharvest decay of apples; 2) evaluate *in vitro* effects of fungicides from three chemical groups (QoI, SDHI and DMI); and 3) evaluate the *in vitro* inhibitory activity of four EOs in the control of apple diseases caused by Botryosphaeriales fungi.

MATERIAL AND METHODS

Fungal isolation and morphology

Apple fruits with symptoms of rot were collected from a non-commercial orchard (with no history of fungicide application) in Rakari (Kolubara district, Serbia) in 2023. The fruits were surface-sterilized with 70% ethanol and isolation was performed by aseptically cutting small fragments from the margins of infected tissue and placing them on Potato Dextrose Agar (PDA). Fungal colonies showing characteristics of Botryosphaeriaceae were transferred to new PDA plates and incubated at 25°C for 14 days in darkness. Morphological characterization was performed based on macroscopic colony morphology (colour and mycelial type). Based on those characteristics, the isolates were divided into distinct groups.

DNA extraction and molecular identification of isolates

DNA was extracted from 7-day old mycelium grown on PDA following the 3% CTAB DNA isolation protocol described by Doyle and Doyle (1990) with

some modifications. The quality of DNA was tested by amplification of the ITS genomic region, while fungal identification to species level was performed by sequencing of two genes: translation elongation factor 1-α (EF1-α) and β-tubulin. These genomic regions were amplified employing ITS1/4 (White et al., 1990), EF1-728F/EF1-986R (Carbone & Kohn, 1999) and Bt2a/Bt2b (Glass & Donaldson, 1995) primer pairs, respectively. The PCR reactions were performed in Thermal Cycler (Biometra) at the final volume of 25 µl containing 1x PCR MasterMix (Thermo Fischer Scientific, Vilnius, Lithuania), 0.4 µM of each primer and 10x diluted sample DNA. Samples lacking the template DNA were considered as negative controls. The thermocycling pattern for amplification of the ITS region consisted of an initial denaturation at 94°C for 90 s; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with final elongation at 72°C for 9 min and 30 s. PCR conditions for the EF1-α and β-tubulin genes included initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 53°C for 1 min and 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were separated by 1% agarose gel electrophoresis and commercially sequenced with forward primers employed for their amplification (Macrogen, Netherland). Sequences were analyzed with the Gap4 program from Stadden Package (Staden et al., 2000), manually inspected and compared to sequences publicly available in NCBI's GenBank through BLAST search analysis.

Pathogenicity and aggressiveness tests

Apple fruits (cv. Golden delicious) without physical injuries were used in pathogenicity and aggressiveness tests. The fruits were surface disinfected with 2% sodium hypochlorite solution for 2 min, rinsed twice with sterile distilled water, and air dried in laminar flow. Three wounds (3x3 mm) were made aseptically on the equator of each apple prior to inoculation. PDA plugs of 3 mm in diameter containing mycelium were taken from a 5-day-old colony and used for artificial inoculation. For each isolate, two apple fruits with three inoculation sites were used. The fruits were placed in plastic boxes and incubated at room temperature for seven days. The lesion diameter was measured in two mutually perpendicular directions, and the average of two values was defined as the lesion diameter. Lesion depth was also recorded. Lesion diameters and depths in the pathogenicity test were subjected to an analysis of variance (ANOVA) and the mean values were separated by Tukey's test ($p = 0.05$).

In vitro fungicide sensitivity assay

A single isolate per each of the two identified species was chosen for a sensitivity assay. Isolates JR13/II, identified as *Diplodia seriata,* and JR57, identified as *Bothryosphaeria dothidea,* were tested for pyraclostrobin and fluopyram + tebuconazole sensitivity. The commercial fungicides Luna experience (200 g/L fluopyram + 200 g/L tebuconazole, Bayer CropScience LP, MO, USA) and Retengo (200 g/L pyraclostrobin, BASF SE, Ludwigshafen, Germany) were dissolved in sterile distilled water to prepare different fungicide concentrations. Adjusted fungicide concentrations were incorporated into autoclaved PDA medium cooled to 50°C to obtain final concentrations of active ingredients (a.i.) at: 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg a.i. ml^{-1} for Luna experience, and 0.01, 0.1, 1, 10 and 100 µg a.i. ml^{-1} for Retengo. The fungicide-amended medium (20 ml) was dispensed in 90 mm diameter Petri plates. Control Petri plates were not amended with fungicides. Both fungicide-amended and control Petri plates were inoculated with 6 mm mycelial plugs cut from the margin of actively growing cultures of both fungal isolates. All Petri plates were incubated in darkness at 25°C for 5 days. All assays, including controls, were performed in triplicates and the whole experiment was repeated twice. When mycelial mat of the controls reached ¾ of Petri plates, colony radial growth was measured in three directions. The percentage of inhibition was calculated for both fungicides using the formula: PGI = (a–b)/a × 100, where *a* was the colony diameter of control plates and *b* was the colony diameter of fungicide-amended plates. The concentration of each fungicide inhibiting mycelial growth by 50% (EC_{50}) was estimated using Probit analysis.

To determine antifungal properties (fungicidal or fungistatic) of the fungicides against the test pathogens, transfer experiments were performed. Mycelial plugs that failed to grow were transferred to fresh PDA medium (15 ml/plate) and incubated at 25°C. After 5 days, the activity of each fungicide was classified either as fungicidal if the pathogen failed to grow or as fungistatic if pathogen growth did occur.

Antifungal effect of essential oils *in vitro*

Antifungal activity of four commercially available EOs (Herba, Belgrade): thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), lavender (*Lavandula* spp.) and lemongrass (*Cymbopogon citratus*), was tested using the fumigant macrodilution method against the same two isolates used in fungicide sensitivity assays (JR13/II

and JR57). The experiment was conducted in glass Petri plates (diameter: 90 mm) on PDA medium with mycelial fragments (diameter: 6 mm) placed at the center of each plate. Five different volumes of selected EOs were pipetted onto filter paper cuttings attached to the inner side of plate covers to achieve the final air phase concentrations: 0.02, 0.05, 0.07, 0.09 and 0.12 µl ml^{-1} of air. The plates were inverted, sealed with parafilm to prevent oil evaporation and incubated at 25°C for 5 days. Distilled water was used as negative control. The percentage growth inhibition (PGI) was calculated as previously described. The experiment was repeated twice.

RESULTS

Fungal identification

After the isolation protocol, 17 isolates were obtained from apple fruits and, based on their colony morphological traits, they were divided into two groups. The cultures of both groups were initially white, turning pale olive-brown after three days, and becoming dark olive brown to grey brown after seven days. Finally, the cultures became dark grey on the surface and black on the reverse after 14 days. Isolates in the first group (7 isolates) formed moderately dense to dense mycelium, with woolly, cottony and fluffy aerial appearance, while isolates in the second group (10 isolates) formed abundant fluffy aerial mycelium occasionally grouped in tufts reaching the lid of Petri plates. Based on colony morphological characteristics, the isolates from the first and second group were identified as *Diplodia seriata* and *Bothryosphaeria dothidea*, respectively.

One representative isolate from each group (JR 13/II and JR 57) was subjected to molecular identification. For both isolates ITS, EF1-α and β-tubulin genomic regions were successfully amplified. The obtained sequences of EF1-α and β-tubulin genes were 212 and 396 nt long for isolate JR13/II, and 229 and 377 nt long for isolate JR 57, respectively. BLAST analysis of both genes of isolate JR 13/II showed that they shared 100% identity with all 100 strains of *D. seriata* listed (such as strain Bot-2018-S7 from an apple tree from Chile - MH745087/MH908101, culture strain CBS:121110 from *Prunus armeniaca* from South Africa - MT592554, and CBS:134700 from *Prunus laurocerasus* from Italy - KC869998) while isolate JR 57 was identified as *B. dothidea,* also with 100% identity with all isolates listed. The listed strains included strains SHP6H3I9_2020 from walnut from France (PQ303808), as well as voucher MFLU 22-0097 strain from *Prunus serrulata* from Taiwan (ON677459), and voucher CGMCC 3.19257 from *Vaccinium uliginosum* L. from China (MK085916).

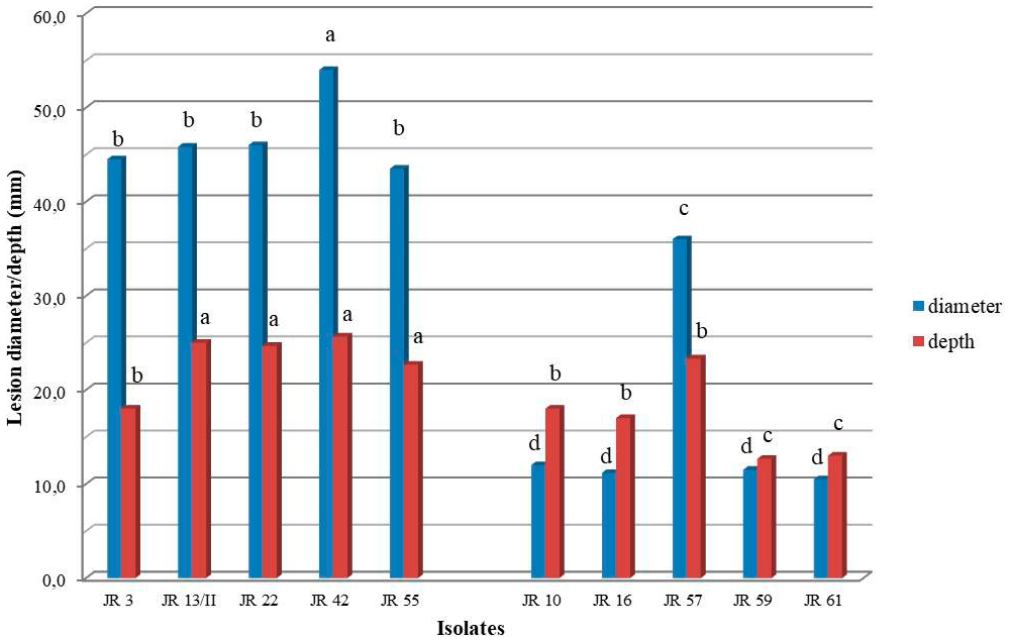

Figure 1. Lesion diameter and depth on apples inoculated with *D. seriata* (JR 3, JR 13/II, JR 22, JR 42, and JR 55) and *B. dothidea* (JR 10, JR 16, JR 57, JR 59, and JR 61)

Pathogenicity and aggressiveness tests

Five isolates from each of the two morphological groups were selected for pathogenicity and aggressiveness tests. The *D. seriata* isolates (JR 3, JR 13/II, JR 22, JR 42 and JR 55) showed significantly higher aggressiveness than *B. dothidea* (JR 10, JR 16, JR 57, JR 59 and JR 61) based on the average values of lesion diameter and depth (Figure 1), which were 46.8±4.17 mm and 23.2±3.11 mm, respectively. The average lesion diameter was 16.2±11.06 mm and depth 16.8±4.35 mm on apple fruits inoculated with B. *dothidea.*

In vitro fungicide sensitivity assay

The radial growth of *D. seriata* and *B. dothidea* isolates was significantly inhibited by both tested fungicides. The combination of fluopyram and tebuconazole showed higher efficacy against the isolates of both tested species, compared to pyraclostrobin. However, some differences in sensitivity were noted between the species. Calculated EC_{50} values showed that *D. seriata* was more sensitive to fluopyram + tebuconazole (EC_{50}=0.00023 µg a.i. ml^{-1}) in comparison with *B. dothidea* isolate (EC_{50}=0.00108 µg a.i. ml^{-1}). The concentration of 10 µg a.i. ml^{-1} of the fungicide combination highly inhibited mycelial growth of both isolates tested, with PGI values of 98.44% and 97.56%, respectively. The maximal concentration (100 µg a.i. ml^{-1}) totally inhibited mycelial growth of both isolates, and showed fungicidal effect on the *D. seriata* isolate, while the same concentration demonstrated fungistatic activity against the *B. dothidea* isolate.

The EC_{50} values obtained for radial mycelial growth suggested that pyraclostrobin was less effective than the combination fluopyram + tebuconazole. The calculated EC_{50} values for pyraclostrobin showed that *D. seriata* was less sensitive to that fungicide (EC_{50}=3.895 µg a.i. ml^{-1}) than the *B. dothidea* isolate (EC_{50}=0.025 µg a.i. ml^{-1}). The mycelial growth of *D. seriata* and *B. dothidea* at the maximum tested concentration (100 µg a.i. ml^{-1}) was inhibited by 81.88% and 88.60%, respectively.

Antifungal effect of essential oils *in vitro*

Thyme essential oil demonstrated the highest antifungal activity against both fungi, achieving complete inhibition of mycelial growth at oil concentration of 0.05 µl ml^{-1} of air phase. Lemongrass essential oil also revealed a strong inhibition potential with PGI ranging from 85.1% at 0.02 µl ml^{-1} of air phase to 100% at 0.07 µl ml^{-1} of air phase against *D. seriata*, while *B. dothidea* was inhibited from 73.6% at 0.02 µl ml^{-1} of air phase to 100% at 0.09 µl ml^{-1} of air phase.

Moderate antifungal activity was demonstrated by rosemary EO with PGI values ranging from 34.2% against *D. seriata* and from 20.6% against *B. dothidea* at the lowest concentration, up to 53.8% against *D. seriata* and 59.7% against *B. dothidea* at the highest concentration tested.

Lavender EO showed the lowest inhibition potential against both isolates with PGI values ranging from 0% to 13.8% and 5.4% to 13.2% for *D. seriata* and *B. dothidea*, respectively (Figures 2 and 3).

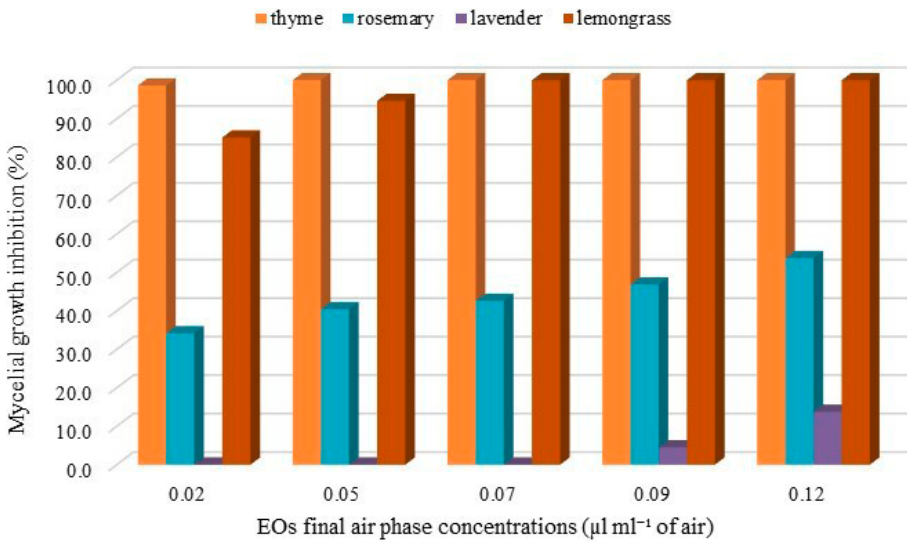

Figure 2. Effects of different EOs to mycelial growth of *D. seriata*

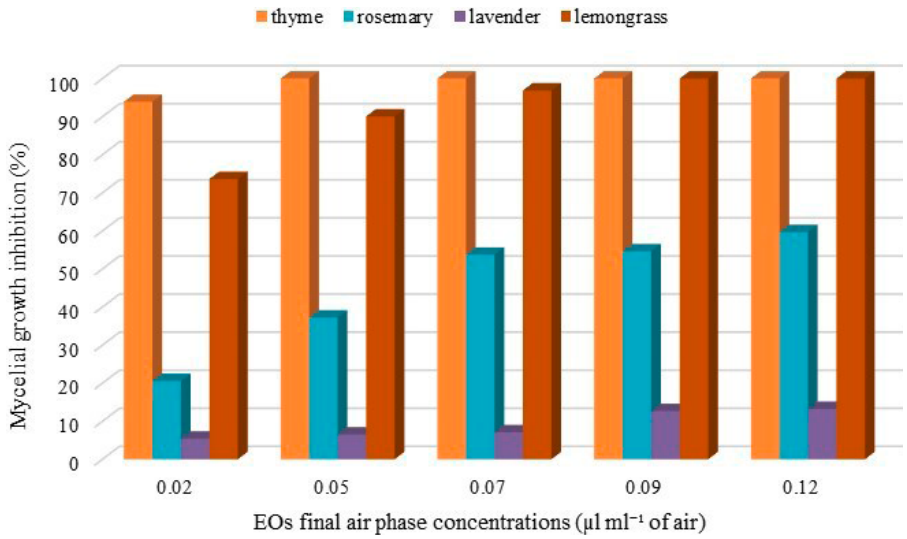

Figure 3. Effects of different EOs to mycelial growth of *B. dothidea*

DISCUSSION

Botryosphaeriaceae is a fungal taxon within Ascomycota representing a serious threat to several perennial species, including fruit crops (Billones-Baaijens & Savocchia, 2019; Batista et al., 2021; Bezerra et al., 2021; Martino et al., 2024) that can be colonized and damaged by species in this family. Several species of the family Botryosphaeriaceae can cause postharvest fruit rot that can adversely affect apple yield in major growing regions around the world, and the most frequent are *Diplodia seriata* and *Botryosphaeria dothidea* (Delgado-Cerrone et al., 2016).

Identification of Botryosphaeriaceae species is mainly based on colony morphology and molecular analyses of genomic regions (Phillips et al., 2013; Zhou et al., 2015; Delgado-Cerrone et al., 2016). Based on morphological characteristics and comparisons of DNA sequences of the translation elongation factor 1-α (EF1-α) and β-tubulin genomic regions, 17 isolates of Botryosphaeriaceae in this study were identified either as *D. seriata* (7 isolates) or *B. dothidea* (10 isolates). The presence of both species as dominant pathogens causing fruit rot had been previously reported on apple fruit in Serbia (Stojanović et al., 2003; Vasić et al., 2013; Vučković et al., 2022). Both species were found to cause postharvest apple fruit rot. Pathogenicity tests showed statistically significant higher aggressiveness of *D. seriata* in comparison with *B. dothidea* isolates.

Diagnosing the pathogens of apple fruit rot is crucial for successful control of this disease. The availability of control strategies against diverse Botryosphaeriales pathogens is essential for integrated management of fruit rot of apple. Although some cultivation practices have been used to protect apple orchards from species in Botryosphaeriaceae family, preventive applications of fungicides from different chemical groups are still a practical and effective strategy for managing many pathogens, including those responsible for apple fruit rot. Even though some active ingredients from QoI, DMI and SDHI chemical groups are registered against apple postharvest pathogens (*Gleosporium* spp., *Monilinia* spp., *Penicillium* spp.), no fungicides are currently approved in Serbia for the control of Botryosphaeriaceae in apple (Team of Editors, 2024). Among the commercial chemical formulations registered for use against other fungal diseases of apple, two products with three active ingredients and different modes of action were selected in this study to examine their inhibition activity against two causal agents of apple fruit rot: *D. seriata* and *B. dothidea*. The study considered their inhibitory effects on mycelium growth of pathogens. The tested products (fluopyram + tebuconazole and pyraclostrobin) strongly inhibited mycelial growth of both fungi and some differences in sensitivity between the species were noted. Variation in fungicide inhibitory effects had been previously confirmed between Botryosphaeriaceae species, even within the same region and crop (Bester et al., 2007; Amponsah et al., 2012; Pitt et al., 2012). The calculated fluopyram + tebuconazole EC_{50} values for *D. seriata* (EC_{50}=0.00023 µg a.i. ml^{-1}) and *B. dothidea* (EC_{50}=0.00108 µg a.i. ml^{-1}), as well as

pyraclostrobin EC_{50} values for *D. seriata* (EC_{50}=3.895 μg a.i. ml^{-1}) and *B. dothidea* (EC_{50}=0.025 μg a.i. ml^{-1}) demonstrated high inhibition potential of these active ingredients. Previously, Fan et al. (2019) tested 97 isolates of *B. dothidea* from apple for their sensitivity to pyraclostrobin and the EC_{50} values ranged from 0.7010 to 7.1378 μg ml^{-1}, with the mean EC_{50} of 3.0870±0.1560 μg ml^{-1}, confirming strong inhibition effects of pyraclostrobin. Also, EC_{50}s between 0.004 and 2.15 mg a.i. l^{-1} showed that pyraclostrobin, fluazinam, tebuconazole, fludioxonil and prochloraz were highly effective and similar in their inhibitory effects on mycelium growth and conidium germination of *D. seriata* (Antony et al., 2024). *In vitro* results obtained in a study by Torres et al. (2013) demonstrate strong effectiveness of tebuconazole in the control of conidial germination and radial growth of *D. seriata* and *D. mutila*. Fluopyram, tebuconazole and pyraclostrobin were also among the tested fungicides and endorsed for their efficacy against species of the Botryosphaeriaceae family in apple (Song et al., 2018), almond (Olmo et al., 2017), and grapevine (Bester et al., 2007; Amponsah et al., 2012; Pitt et al., 2012).

Despite their high efficacy, synthetic chemical fungicide treatments pose many risks, including mounting health concerns voiced by consumers and health authorities, which has led to demands to reduce human and environmental exposure to chemicals, increased restrictions imposed by regulatory agencies on specific agro-chemicals and/or their allowable residues, especially after harvest (Romanazzi & Droby, 2016). The need for reduced pesticide residues in plant products has led to the development of alternatives to synthetic fungicides, such as naturally derived compounds with antifungal potential. In recent years, as a strategy to control apple fruit fungal pathogens, the application of plant essential oils has been considered a natural alternative to synthetic fungicides (Lopez-Reyes et al., 2010; Di Francesco et al., 2022; Soppelsa et al., 2023). Each of the four essential oils (thyme, rosemary, lavender, and lemongrass) tested in this study had some reducing effect on the mycelial growth of *D. seriata* and *B. dothidea*. The results of the present study indicated that the volatile phases of thyme and lemongrass essential oils possess high antifungal activity against both fungi, achieving a complete inhibition of mycelial growth at oil concentrations of 0.05 and 0.07 μl ml^{-1} of air phase, respectively. Moreover, the phenolic monoterpenoid compound thymol (major constituent of thyme EO) has an important activity against fungal plant pathogens, attributing to its antioxidant activity (Elshafie et al., 2015) and stimulating plant defences (Lopez-Reyes et al., 2013; Sivakumar & Bautista-Baños, 2014). Moderate antifungal activity was demonstrated by rosemary EO, while lavender EO showed the lowest inhibition potential against both tested isolates. A large variety of volatile compounds have been shown to have strong antifungal effect when tested under laboratory and small-scale *in vivo* conditions. Strong effects of EOs against *Botryosphaeria* species was confirmed in a study by Sarkhosh et al. (2018). The results of their *in vitro* testing of essential oils extracted from eight plant species showed a 100% reduction in the mycelium growth of *Botryosphaeria*, *Colletotrichum*, *Fusarium* and *Phytophthora* species after applying thyme EO at a concentration of 100 μg l^{-1}. Also, in a study by Wang et al. (2023), cinnamon and clove EOs exhibited high inhibitory activity against the mycelial growth of *B. dothidea*, both in vapor and contact phases under *in vitro* conditions. *In vivo* testing showed that the EO vapor treatments also alleviated the severity of fruit rot in artificially infected apples. Several studies have also reported efficient activities of thyme, lemongrass and rosemary essential oils (Lopez-Reyes et al., 2010; 2013; Grahovac et al., 2011; Ali et al., 2015; Císarová et al., 2016; Servili et al., 2017; Di Francesco et al., 2022).

In the current study, the results demonstrated that the main Botryosphaeriaceae pathogens causing postharvest decay of apple fruits were *D. seriata* and *B. dothidea*. *In vitro* fungicide sensitivity assays indicated that both pathogens were sensitive to the active ingredients pyraclostrobin, fluopyram and tebuconazole, which are registered in Serbia against some other apple postharvest pathogens, including *Gleosporium* spp., *Monilinia* spp., and *Penicilium* spp. The vapour phase of thyme and lemongrass essential oils effectively limited mycelial growth of both tested fungi, showing that these oils can be potential biocontrol agent candidates for preventing and controlling apple fruit rot. Despite their proven effectiveness under laboratory conditions, their efficacy still needs to be confirmed under large scale and commercial conditions, and potentially undesirable effects on postharvest fruit, human health and the environment require further detailed investigation.

ACKNOWLEDGEMENT

This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia: Grant No 451-03-66/2024-03/200214.

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Botryosphaeriaceae kao patogeni jabuke – identifikacija i osetljivost na fungicide i etarska ulja *in vitro*

REZIME

Proizvodnja jabuke suočava se sa značajnim ekonomskim gubicima usled smanjenog kvaliteta plodova kao posledice prisustva velikog broja fitopatogenih gljiva, naročito prouzrokovača truleži plodova nakon berbe kao što su gljive iz familije Botryosphaeriaceae. Izolati korišćeni u ovom istraživanju prikupljeni su sa zaraženih plodova jabuka i na osnovu morfoloških karakteristika i analize sekvenci dva genska regiona (EF 1-α and β-tubulin) identifikovani kao pripadnici vrsta *Diplodia seriata* i *Botryosphaeria dothidea*. Na zdravim plodovima jabuka sproveden je test patogenosti kojim je na osnovu merenja prečnika i dubine truleži utvrđen značajno viši stepen agresivnosti izolata *D. seriata*. Veća osetljivost na kombinaciju fluopiram + tebukonazol uočena je kod izolata *D. seriata* (EC_{50}=0,00023 µg a.s./ml), dok je piraklostrobin jače inhibitorno delovanje ispoljio na izolat *B. dothidea* ($EC_{50=}$0,025 µg a. s./ml). Ispitivana kombinacija fluopiram + tebukonazol pokazala je veći inhibitorni potencijal u poređenju sa piraklostrobinom, sa inhibicijom porasta micelije od 98,44% za *D. seriata* i 97,56% za *B. dothidea* pri koncentraciji od 10 µg a.s./ml. Antifungalni efekat četiri etarska ulja (majčine dušice, ruzmarina, lavande i limunske trave) ispitan je primenom fumigantne makrodilucione metode. Ulje majčine dušice ispoljilo je najizraženije delovanje, potpuno inhibirajući porast micelije izolata obe vrste pri koncentraciji 0,05 µl/ml vazdušne faze. Jako inhibitorno delovanje pokazalo je i ulje limunske trave sa kompletnom inhibicijom porasta micelije pri koncentraciji 0,07 µl/ml vazdušne faze (*D. seriata*), odnosno 0,09 µl/ml vazdušne faze (*B. dothidea*). Umeren inhibitorni potencijal zabeležen je kod ulja ruzmarina, dok je ulje lavande ispoljilo najniži antifungalni efekat. Rezultati prikazani u ovom radu ukazuju na visok stepen osetljivosti izolata *D. seriata* i *B. dothidea* na ispitivane fungicide, ali i ulja majčine dušice i limunske trave kao njihove ekološki prihvatljivije alternative. Dalja istraživanja su potrebna kako bi se ispitala efikasnost ovih ulja u uslovima *in vivo*, kao i potencijalni uticaj na kvalitet plodova i životnu sredinu.

Ključne reči: jabuka, fitopatogene gljive, trulež plodova, fungicidi, etarska ulja, antifungalno delovanje

Pestic. Phytomed. (Belgrade), 40(1), 2025, 13–25

Natural and semi-synthetic insecticides protect onion from wireworms

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SUMMARY

Wireworms, the larval stage of click beetles (Elateridae: *Agriotes* spp.), pose a significant threat to global agriculture, particularly to root vegetables, such as onion. Their subterranean lifestyle, as well as the withdrawal of some traditional synthetic insecticides, make them challenging to control. Therefore, this research aimed to compare the effects of natural, semi-synthetic and synthetic insecticides in controlling wireworm damage in an onion field.

A field trial for testing the effects of different insecticidal treatments on plant density, wireworm damage (%) and total onion yield, was conducted at the Institute for Vegetable Crops (Smederevska Palanka, Serbia) in 2024. The experiment consisted of six treatments: an untreated control, three natural insecticides (two formulations of spinosad a.i. - granular and liquid, and *Beauveria bassiana* ATCC 74040 2.3×107 conidiospores/ml), a semi-synthetic insecticide (a.i. spinetoram) and a synthetic insecticide (a.i. tefluthrin). The insecticides were applied during planting, following their label application rates per hectare. Assessments were conducted 20 and 42 days after treatment (DAT) to determine plant density. Wireworm damage was specifically evaluated 42 DAT, and yield was calculated by weighing the harvested onion bulbs. The results showed that the granular spinosad formulation, applied in furrows at planting, significantly increased plant density 20 DAT, while its liquid formulation, applied as a soil treatment during planting, resulted in the lowest density. Spinetoram showed the highest plant density 42 DAT and the highest percentage of wireworm damage (15%) of all insecticides tested. The control had the highest percentage of damaged plants and the lowest yield. Onion yield was at the maximum after treatment with spinetoram, whereas the lowest yield was achieved after treatment with the granular spinosad formulation.

Field trials show that natural and semi-synthetic insecticides can effectively control wireworms, ensuring adequate crop protection and viable yields. This study supports developing and adopting environmentally conscious soil pest management.

Keywords: Elateridae, biopesticides, insecticides, pest control, yield, onion

Article info

Original scientific paper

Received: 4 April 2025

Accepted: 16 April 2025

Publication is free of charge.

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DOI: https://doi.org/10.2298/PIF2501013P

INTRODUCTION

Onion (Amaryllidaceae: *Allium cepa* L) is recognized globally as one of the oldest cultivated vegetable crops, which has been grown for 4000 years. In the Republic of Serbia, onion is cultivated on approximately 4,114 ha, constituting 0.76% of the global onion cultivation area. (Bošnjak et al. 2007; Takač & Vuković 2023). Climate change, with its associated warmer temperatures, droughts, and altered precipitation, has amplified the impact of soil pests, particularly wireworms, on onion yield. A three-year field study investigating wireworm activity in sunflower under these changing conditions underscored this trend (Gvozdenac et al., 2022).

Wireworms, the subterranean larvae of click beetles (Coleoptera: Elateridae, genus *Agriotes* spp. Eschscholtz, 1829), are a significant and persistent challenge to global agricultural productivity. These polyphagous pests are characterized by their extended larval stage, which may last for several years while they reside in soil, which makes them very difficult to detect and control (Parker & Howard, 2001; Sufian, 2013; Stolpe Nordin, 2017; Toscano et al., 2017). *Agriotes* larvae are polyphagous herbivores that specialize in feeding on underground plant structures of a large number of plant species, including field crops, vegetables and ornamentals. Damage to root and tuber vegetables, especially during planting and preharvest stages, is economically significant. The initial signs include small holes and tunnels, which later develop into skin scarring, impacting product marketability (Barsics et al., 2013; Furlan et al., 2017; Antwi et al., 2018; Poggi et al., 2021; Gvozdenac et al., 2022). This feeding behavior leads to significant economic losses for farmers worldwide as damaged crops become unusable or show reduced yields. The impact of wireworms is particularly severe in regions with temperate climate, where their long life cycle and adaptability to different soil types contribute to their prevalence. The economic impact of wireworm infestation on potato crops is substantial, given the high susceptibility of potato tubers to damage, while even minor injuries diminish their market value (Parker & Howard, 2001; Staudacher et al., 2013). Furthermore, sunflower, as well as some other field and vegetable crops, are vulnerable to these pests (Gvozdenac et al., 2019; Gvozdenac et al., 2022). Onion seedlings are also extremely vulnerable to wireworm damage, making early stage protection critical. Therefore, chemical pesticides are often considered essential, especially during planting (Bošnjak et al., 2007; Barsics et al., 2013).

There are several challenges in controlling these pests, including difficulties in diagnosing their presence in the soil, prolonged development and presence of larval stages, and traditional management strategies that rely heavily on treatments with conventional insecticides, including organophosphates, pyrethroids, and neonicotinoids (Van Herk et al., 2008; Furlan & Kreutzweiser, 2015; Traugott et al., 2015). However, there has been in recent years a significant decrease in the use of compounds from the groups of synthetic pesticides, due to strict bans and/or restrictions as a result of concerns over their impact and persistence in the environment, their non-selective action, especially on beneficial organisms, and the development of resistance in wireworm populations to these insecticides. Experimental evidence suggests that sublethal doses of conventional insecticides such as neonicotinoids, pyrethroids, and fipronil result in extended morbidity in wireworm populations. Crucially, the documented recovery of some individuals underscores a significant risk of evolving insecticide resistance (Antwi et al., 2018). As mentioned, many of these traditional insecticides have been consequently withdrawn from the market or subjected to strict regulatory restrictions, necessitating the development of alternative and more sustainable control measures (Wilde et al., 2007; Van Herk et al., 2008; Antwi et al., 2018; de Oliveira Cantao & Mian, 2023).

The lack of effective chemical controls has prompted increased research into integrated pest management (IPM) strategies, including biological control agents, agro-technical measures, and innovative technologies. Biological control approaches, such as the use of entomopathogenic fungi and nematodes, are being explored as environmentally friendly alternatives (Kabaluk et al., 2007; Milosavljevic, 2015; Poggi et al., 2021; Nikoukar & Rashed, 2022). This study evaluated the impact of natural, semi-synthetic and synthetic insecticides on wireworm control in onion crop, comparing the effects of three natural insecticides - two spinosad formulations (granular and liquid), one *Beauveria bassiana* Balsamo-Crivelli (1936) (Ascomycota: Hypocreales) biological preparation, the semi-synthetic spinetoram, and the synthetic tefluthrin insecticide.

A study by Ladurner et al. (2009) evaluated the efficacy of *B. bassiana* in biological control of wireworm populations in organic production systems in Germany and Italy. Naturalis-L, a microbial insecticide containing *B. bassiana* strain ATCC 74040, targets a wide range of arthropod pests, including whiteflies, tetranychid mites, thrips, and wireworms (Ladurner et al., 2009;

Prijović et al., 2011; Paluch, 2022). Its primary mode of action is contact-based, fungal spores adhere to the insect cuticle, germinate, and produce hyphae that penetrate the host. This process is facilitated by the release of cuticle-hydrolyzing enzymes (Vernon & Van Herk, 2022; CBC Europe, 2025). The biological product containing *B. bassiana* shows a promising effectiveness in reducing wireworm damage in potatoes. Application at planting is recommended, especially in non-irrigated fields. While effective against some wireworm species, its efficacy varies, and further research is needed to optimize fungal biocontrol strategies (Ester & Huiting, 2007; Ladurner et al., 2009; Wraight et al., 2009).

Spinosyns (spinosad and spinetoram) have been used for over 25 years against various pests, and despite the IPM guidelines, pests have developed resistance. Spinosad, a naturally derived biopesticide from *Saccharopolyspora spinosa* Mertz and Yao (1990), offers a valuable combination of high selectivity against target insect pests and low toxicity to beneficial arthropods. Registered globally for seed treatment and planting use, it provides effective control both by contact and ingestion of a broad spectrum of pests, including Coleoptera. To date, the unique mechanism of action of nicotinic acetylcholine and gamma-aminobutyric acid (GABA) receptors in the insect nervous system has resulted in no observed cross-resistance (Salgado et al., 1997, 2010; Salgado, 1998; Racke, 2006; Bacci et al., 2016; de Oliveira Cantao & Mian, 2023; Sparks et al., 1995, 2021, 2025). The use of spinosad to protect crops from wireworms and other soil-borne pests has been studied extensively. Studies have consistently shown that spinosad significantly reduces wireworm damage, and seed treatment is a particularly effective method of application (Ericsson et al., 2007; Van Herk et al., 2015; Arrine et al., 2017). In addition, in-furrow applications at planting have shown good results in crop protection (Vernon et al., 2013; de Oliveira Cantao & Mian, 2023). Spinetoram, a ''reduced-risk," semi-synthetic analog of spinosad, shares a similar mode of action (Yee, 2018). While spinetoram is effective against a broad spectrum of pests, its ecotoxicological impact, particularly on beneficial insects, remains understudied (Chloridis et al., 2007; Drobnjaković et al., 2023, 2025). Although spinosad has shown some efficacy against wireworms, the effectiveness of spinetoram against this pest varies, as evidenced by mixed results reported in studies using the insecticide Radiant 120 SC (Zhang et al., 2018; Poggi et al., 2021).

Pyrethroids, typically applied as foliar sprays, have also been explored for in-furrow wireworm control with varying results (Traugott et al., 2015). In wheat, tefluthrin seed treatments have provided good stand and yield protection under heavy *Agriotes obscurus* L pressure, although, like neonicotinoids, it did not reduce wireworm populations (Vernon et al., 2009). Laboratory studies suggest that tefluthrin acts as a repellent rather than a lethal agent to wireworms when applied to wheat seeds, potentially explaining the observed stand protection in both wheat and maize crops (Van Herk and Vernon, 2014). In Serbia, tefluthrin-based soil insecticides are registered for protecting various field and vegetable crops, including maize, sugar beet, potato, carrot, celery, parsley, and onion (Plant Protection Directorate, 2025). Specifically, Saturn Terra New, a granular formulation (GR) containing tefluthrin, is used to control wireworms in maize, potato, onion and carrot (Agrosava, 2025).

This research aimed to evaluate the efficacy of environmentally friendly insecticides against synthetic alternatives, establishing precise application rates, so as to optimize onion yield and quality, while minimizing ecological impact. Ultimately, this study sought to equip onion growers with effective and sustainable tools for long-term wireworm management, thereby enhancing the stability of onion production.

MATERIAL AND METHODS

The 'Stuttgarter Yellow' Dutch yellow onion variety, commercially known as 'Stuttgarter Riesen', was selected for this experiment. This variety is characterized by round yellow bulbs and excellent storage capacity, and is suitable for sowing over the period from March to April.

Wireworms, the larval forms of various click beetles *Agriotes* spp., were used as test organisms to assess the effects of insecticides. The risk of wireworm infestation was assessed by soil sampling. Wireworm density was estimated per m² based on the number of specimens found in spring soil samples collected before the experiment was established. Samples were collected using the standard square method (50 × 50 cm, approximately 40 cm deep) with 10 replicates distributed across the experimental field (Čamprag, 1983; Štrbac, 2005).

Experimental field and treatments

The trial was conducted in an experimental field specifically designed for controlled pest management studies of the Institute for Vegetable Crops (Smederevska

Palanka, Serbia; 44^0 22' N, 20^0 57' E) in 2024. This field allowed for precise treatments and monitoring of wireworm populations, as well as onion crop responses (Photo 1). The experimental field soil was a vertisol (alluvial loam), which was significantly influenced by the nearby Kubršnica and Jasenica rivers. A multiannual crop rotation with moderate fertilization ensured an adequate macroelement supply. Winter wheat was used as a preceding crop. Prior to sowing, the soil received a mixed mineral fertilizer of 46 kg/ha N, P_2O_3 and K_2O. Following winter plowing and seedbed preparation, sowing was conducted with crop-specific interrow spacing and plant density.

The Dutch yellow onion variety 'Stuttgarter Yellow' was sown directly in the field on March 29, 2024.

The experimental design, including treatment arrangement and plot size, was based on EPPO (2005) guidelines and was implemented in four replicates.

Six treatments were tested as follows: untreated control, three insecticides of natural origin, a semi-synthetic and a synthetic insecticide (Table 1). The insecticides were applied directly to furrows at planting following the label application rates per hectare. A pre-emergence herbicide application significantly reduced weed density. To eliminate the risk of phytotoxic effects on onion plants, residual weeds were manually removed. Manual weed control and crop hoeing were consistently implemented throughout the experimental period with no other pesticides used, ensuring that the wireworm response was solely attributable to the tested insecticides.

a b

Photo 1. Experimental onion field during planting (a) and second assessment (b)

Plant density and wireworm damage (%)

Owing to a lack of specific method for assessing the impact of wireworms on onion crops, assessment methods for maize and cereals were adapted for use in this study. The efficacy of the applied insecticides was estimated based on plant density and wireworm damage (%) on onion plants, observed 20 and 42 days after treatment (20 DAT and 42 DAT). For both evaluations, plant density was assessed by randomly selecting ten 1-meter sections within a row per plot and counting the number of emerged plants within each section. The first assessment was conducted at the time of emergence after approximately 75% of onion plants have emerged, and the second assessment was conducted at the stage of 5-6 leaves. The number of plants showing symptoms of wireworm infestation was recorded during the second assessment and transformed into %.

Onion yield

Bulb mass per replicate was measured on an analytical balance upon harvest (July 25, 2024) and converted to average onion yield (t/ha) for each treatment. The percentage yield change, indicating insecticide effects, was calculated relative to the untreated control and synthetic insecticide.

Statistical analysis

Wireworm density was expressed as a total number of individuals per square meter (m^2).

Plant density, expressed as the mean number of plants per linear meter, was used to estimate the effects of insecticides. The difference in mean plant density between the insecticide-treated plots and untreated control plots was used to calculate the percentage effect of each insecticide.

The mean onion bulb yield was determined by measuring the total bulb yield per experimental plot for each treatment. Insecticide effects were expressed as a percentage relative to both the untreated control and the synthetic insecticide treatment.

The results were statistically analyzed using STATISTICA 10. Fisher's least significant difference test (Fisher's LSD) was employed to determine significant differences in mean values at a 95% confidence level ($p < 0.05$).

RESULTS

The results of the present study demonstrated significant differences in all measured variables (the efficacy of different insecticide treatments expressed as plant density, wireworm damage, and total onion yield) across experimental treatments.

Table 1. List of insecticides tested in the experimental onion field

Product	A.i.	Concentration (F)	Company name	Rate (ha)	Rate (fr)
Natural origin insecticides					
Gestikal 001GR	Spinosad	1 g/kg (GR)	Agrosava, Serbia	48 kg	16 g
Laser 240 SC	Spinosad	240 g/l (SC)	Corteva Agriscience, Switzerland	0.3 l	0.1 ml
Naturalis-L	*Beauveria bassiana* ATCC74040	2.3×10^7 conidiospores/ml(l)	CBC (Europe) S.r.l. Biogard division, Italy	3 l	1 ml
Semi-synthetic insecticide					
Radiant 120 SC	Spinetoram	120 g/l (SC)	Corteva Agriscience, Switzerland	0.4 l	0.13 ml
Synthetic insecticide					
Saturn Terra New	Tefluthrin	5 g/kg (GR)	Agrosava, Serbia	15 kg	5 g
Untreated Control	–	–	–	–	–

A.i. - active ingredient; F – formulation of prodact; GR – granules; SC - concentrated suspension; L – liquid; ha – per hectare; fr – per furrow

Baseline soil sampling confirmed wireworm population density of 3 larvae/m^2 per experimental plot (Photo 2), a level exceeding the economic threshold requiring insecticidal treatment for effective crop protection (Čamprag, 1983; Furlan, 2014; Bažok et al., 2018).

Plant density and wireworm damage (%)

An analysis of plant density data indicated significant differences in the efficacy of applied insecticides (Table 2). Treatment with Gestikal 001 GR at 48 kg/ha resulted in a plant density increase by 202.5%, 20 DAT, compared to the control, thereby demonstrating a substantial positive impact on plant emergence and plant density. Conversely, treatment with Laser 240 SC (0.3 l/ha) achieved the lowest plant density increase (165.0%, 20 DAT) compared to the control, considering all insecticides tested. Over the longer period of 42 DAT, Radiant 120 SC (applied at a rate of 0.4 l/ha) resulted in a statistically significant increase in plant density of 121.1% relative to the control, whereas Gestical 001 GR showed the smallest increase (110.2 %), compared to the other insecticides.

Photo 2. Visual confirmation of wireworms (*Agriotes* spp.) in a soil sample from an experimental plot (original photo)

Table 2. The number of emerged onion plants in different insecticidal treatments and plant density (%) in comparison to untreated control

Insecticides (Rates liters/ha or kg/ha)	Mean number (± SE)	Plant density (%)	
		Ec	Es
20 DAT			
Gestikal 001 GR 48 kg/ha	4.05 (0.83) **a**	202.5	108.6
Laser 240 SC 0.3 l/ha	3.30 (0.61) **ab**	165.0	88.5
Radiant 120 SC 0.4 l/ha	3.60 (0.79) **ab**	180.0	96.5
Naturalis-L 3 l/ha	3.40 (0.58) **ab**	170.0	91.2
Saturn Terra New 15 kg/ha	3.73 (0.69) **ab**	186.5	100.0
Untreated Control	2.00 (0.68) **b**	100.0	–
	F=1.16; p<0.37		
42 DAT			
Gestikal 001 GR 48 kg/ha	7.33 (0.61) **ab**	110.2	94.6
Laser 240 SC 0.3 l/ha	7.68 (0.39) **ab**	115.5	99.1
Radiant 120 SC 0.4 l/ha	8.05 (0.42) **a**	121.1	103.9
Naturalis-L 3 l/ha	7.90 (0.52) **ab**	118.8	102.0
Saturn Terra New 15 kg/ha	7.75 (0.09) **ab**	116.5	100.0
Untreated Control	6.65 (0.32) **b**	100.0	–
	F=1.48; p<0.25		

DAT = days after treatment; Mean values marked by different letters are significantly different (Fisher's LSD test, $p < 0.05$); Ec – efficacy compared to untreated control; Es – efficacy compared to synthetic insecticide

The frequency of wireworm damage, quantified as the percentage of plants with wireworm damage 42 DAT showed significant treatment effects. Laser 240 SC showed the lowest incidence of damage (10%), indicating superior protection. Radiant 120 SC, which enabled the highest plant density, recorded the highest incidence of damage (15%) of all insecticide treatments. The untreated control showed a statistically significant increase in the frequency of damage (26%) compared to all treatments, indicating a significant impact of wireworm infestation. Figure 1 provides a graphical representation of these data.

Photo 3 shows a comparison of healthy onion plants and plants with symptoms. Plants on the left, originating from the Laser 240 SC-treated field, exhibited healthy growth. In contrast, plants on the right from the untreated control displayed characteristic wireworm damage symptoms, including stunting, underdeveloped root systems, chlorosis, and dried outer leaves.

Photo 3. Symptoms of wireworm damage in onion plants: left - healthy plants, and right - plants with symptoms (original photo)

Onion yield

The maximum average onion yield (21.80 t/ha) was obtained from plots treated with Radiant 120 SC. As anticipated, the untreated control exhibited the lowest yield (15.23 t/ha). All insecticide treatments significantly enhanced yield, relative to the control.

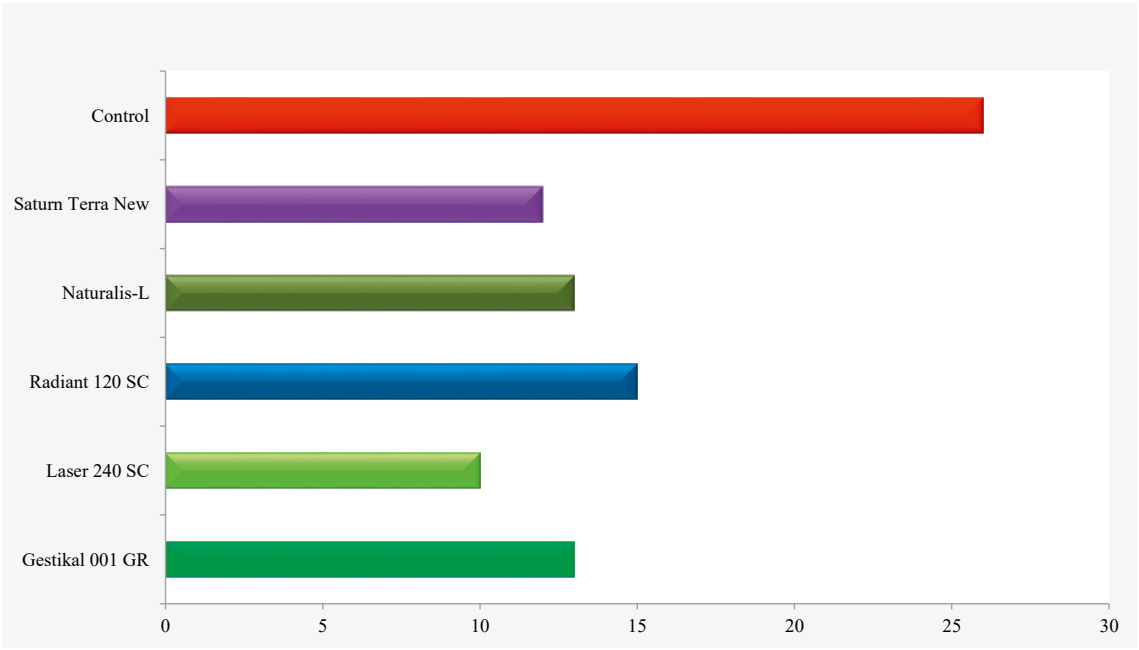

Figure 1. Percentage of wireworm-damaged onion plants

Table 3. Onion yield (t/ha) after different insecticidal treatments and effects of insecticide treatments on onion yield (%) in comparison to untreated control

Insecticides (Rates liters/ha or kg/ha)	Repeats				Mean yield (SE)	Onion yield (%)	
	I	II	III	IV		Ec	Es
Gestikal 001 GR 48 kg/ha	18.1	18.8	20.5	19.5	19.23 (0.51) **c**	126.3	89.4
Laser 240 SC 0.3 l/ha	19.9	18.3	20.6	19.0	19.45 (0.51) **bc**	127.7	90.4
Radiant 120 SC 0.4 l/ha	24.3	21.1	21.5	20.3	21.80 (0.87) **d**	143.1	101.3
Naturalis-L 3 l/ha	22.0	20.7	21.7	20.6	21.25 (0.35) **bd**	139.5	98.8
Saturn Terra New 15 kg/ha	23.3	22.5	19.1	21.1	21.50 (0.92) **d**	141.2	100.0
Control	14.5	14.7	15.9	15.8	15.23 (0.37) **a**	100.0	–
					F=17.17; p<0.01		

Mean values marked by different letters are significantly different (Fisher's LSD test, $p < 0.05$); Ec – efficacy compared to untreated control; Es – efficacy compared to synthetic insecticide

The 6.57 t/ha yield increase observed with Radiant 120 SC correlated with its peak plant density result 42 DAT. In contrast, Gestical 001 GR resulted in the lowest yield of all insecticides (19.23 t/ha) and also demonstrated the lowest plant density in the second assessment (Table 3).

DISCUSSION

Wireworms, well-known for their polyphagous nature, are globally recognized as major agricultural pests that inflict substantial economic damage across a wide range of crops (cereals, vegetables, including onion, maize, potato, sugar beet and ornamentals), weeds and non-crop plants (Poggi et al., 2021). Their complex biology and ecology, coupled with resistance to numerous insecticide classes, pose significant challenges for effective control (Hays, 1933; Parker & Howard, 2001; Gvozdenac et al., 2022). Implementation of Directive 128/2009/EC (European Commission, 2009), which has resulted either in a ban or restricted use of a substantial number of effective synthetic insecticides, presents significant obstacles for the protection of major field and vegetable crops from soil pests, including wireworms. Consequently, the limitations imposed on traditional chemical control have spurred a surge in research focused on the development and refinement of IPM strategies. These comprehensive approaches encompass a diverse range of tactics, including the implementation of biological control agents, optimization of agronomic practices, and integration of innovative technologies, aimed at achieving sustainable and effective pest control, while minimizing environmental impact (Kabaluk et al., 2007; Ladurner et al., 2009; Poggi et al., 2021; Nikoukar & Rashed, 2022).

Pre-treatment population density assessments are crucial for accurate evaluation of insecticide effects on wireworm populations. Wireworm samples can be collected by soil sampling, bait traps or sex pheromone traps (Traugott et al., 2015). Conversely, bait traps offer a more rapid assessment, and sex pheromone traps are preferred for long-term population monitoring (Burgio et al., 2012; Traugott et al., 2015). Therefore, the current study employed soil sampling, which is labor-intensive but provides reliable estimates of wireworm abundance per unit area (Parker, 1994; Parker & Howard, 2001; Sufian, 2013; Stolpe Nordin, 2017; Toscano et al., 2017). Based on the economic risk assessment of wireworm infestation (Čamprag, 1983; Štrbac, 2005), which identified the harmful abundance threshold for onion crops, the application of tested insecticides in these studies was justified.

The current findings confirm a significant efficacy of the entomopathogenic fungus *B. bassiana*-based bioinsecticide Naturalis-L in controlling wireworms in onion plants. The other tested bioinsecticides also demonstrated satisfactory results in terms of enabling good plant density, reducing wireworm damage, and enhancing yield relative to untreated controls. Numerous studies have explored the impact of natural insecticides on soil pests, including wireworms. Specifically, the entomopathogenic fungi *B. bassiana* and *Metarhizium anisopliae* Metschnikoff (1879) (Ascomycota: Clavicipitaceae) are known to infect larval and adult

Agriotes spp, and have been employed in biocontrol experiments against these pests (Parker & Howard 2001; Wraight et al. 2009; Poggi et al., 2021; Paluch, 2022). Zacharuk and Tinline (1968) conducted initial laboratory studies on the effects of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* on larvae and adults of *Agriotes* spp. They observed increased wireworm mortality, reduced crop damage, and higher yields when the spores were applied directly to adult cuticles, feeding larvae with inoculated crop seeds or spreading into soil furrows at planting. In a later study, Kleespies et al. (2013) investigated the effects of microbial antagonists on wireworm larvae in soil and concluded that *B. bassiana* caused the highest fungal infestation and increased wireworm mortality in Germany. In addition, laboratory and field tests were conducted using various strains of *B. bassiana* and *M. anisopliae* at different application rates, and with different wireworm species. In potato field trials in northern Italy, Naturalis-L achieved 54%-94% efficacy against *Agriotes* spp. (Ladurner et al., 2009). The results of Kölliker et al. (2011), who evaluated the efficacy of the Swiss *M. anisopliae* strain ART-2825 and Naturalis against three wireworm species in laboratory, greenhouse and field plots, indicated a higher efficacy under laboratory conditions than in field settings, suggesting a need for further research on wireworm control in potato crops grown in Northern Switzerland. A research in spring wheat against wireworms in Montana, where the efficacy of 10 biopesticides, applied alone, in mixtures or in combination with imidacloprid was tested, showed that the *B. bassiana* ANT-03 biopesticide, its combination with imidacloprid and *B. bassiana* GHA, and combinations of *Metarhizium brunneum* F52 with spinosad or imidacloprid provided significant wireworm control (Antwi et al., 2018). Plots treated with biopesticide-imidacloprid combinations showed significantly higher yields, which highlights the potential of combined biopesticide and insecticide treatments and identifies promising candidates for further research.

In the present study, both spinosad formulations applied (granular, spread in furrows during planting, and liquid, applied as a soil treatment at planting) significantly improved plant density and yield, and reduced plant damage. Similarly, de Oliveira Cantao & Mian (2023) monitored wireworm impact on maize yield in northern Italy and found that spinosad application at planting significantly increased yield, compared to untreated plots, leading them to conclude that spinosad can be incorporated into IPM strategies. Additionally, Arrine et al. (2017) evaluated a microgranular spinosad-based product for the control of *Agriotes* spp. pests in maize and potatoes, and the product showed good efficacy against wireworms in both crops, with favorable environmental behavior. In a study assessing wheat seed treatments, Van Herk et al. (2015) evaluated the efficacy of 11 treatments against two economically significant wireworm species, and high-dose spinosad treatments resulted in high wireworm mortality within 24 h, whereas tefluthrin did not induce significant mortality.

Research on the efficacy of spinetoram against wireworms and soil pests is scarce. Our findings demonstrated that spinetoram-based insecticides positively influenced onion yield and plant density. Nevertheless, the impact of spinetoram on wireworm-induced damage to onions showed inconsistent results. According to Kuhar et al. (2013), spinetoram shares activity against similar pest groups as spinosad and may offer comparable benefits in controlling various pests, including those residing in soil.

The current experiments demonstrated that the tested tefluthrin-based insecticide provided high efficacy in protecting onion from wireworm damage, leading to increased yields and reduced plant damage. This aligns with the findings of a three-year study by Vernon et al. (2009), in which tefluthrin and neonicotinoid seed treatments (imidacloprid, clothianidin and thiamethoxam) effectively protected wheat, probably by wireworm intoxication or repulsion/morbidity, without significantly decreasing wireworm populations in subsequent spring sampling. In addition, a significant wireworm repellency of tefluthrin was recorded (Van Herk et al., 2015). Furthermore, Gvozdenac et al. (2022) compared a *Metarhizium brunneum* Cb15-III-based bioinsecticide to some conventional insecticides, including a tefluthrin-based one, for wireworm control in sunflower, employing an 'Attract and Kill' strategy. Three-year trials demonstrated that tefluthrin treatments consistently yielded the highest plant density and lowest damage, while bioinsecticides exhibited comparable efficacy to conventional insecticides in low wireworm populations.

The onion yields obtained in this study notably surpassed the established two-year average of 18.83 t/ha for the Dutch Yellow onion variety under comparable cultivation conditions, as previously reported (Brdar-Jokanović et al., 2011). This marginal yield increase suggests that the experimental conditions and tested treatments did not detrimentally impact the overall productivity of onion crop relative to previous data for this variety.

Our research demonstrated that natural and semi-synthetic insecticides significantly enhanced onion

plant density, vigor, and overall yield. Furthermore, these compounds exhibited comparable levels of efficacy to synthetic insecticides, while offering notable environmental advantages. Consequently, we recommend further investigation of the potentials of biopesticides for wireworm control to improve integrated pest management strategies.

ACKNOWLEDGEMENT

This study was funded by the Ministry of Science, Technological Development and Innovation, Republic of Serbia (451-03-66/2024-03/200214 and 451-03-66/2024-03/200216).

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Prirodni i polusintetički insekticidi u zaštiti luka od šteta koje izazivaju žičnjaci

REZIME

Larve žičnjaka (Elateridae: *Agriotes* spp.), predstavljaju značajnu pretnju globalnoj poljoprivredi, posebno korenasto-krtolastom povrću kao što je luk. Njihov podzemni način života čini ih izazovnim za suzbijanje koji je dodatno komplikovan povlačenjem tradicionalnih sintetičkih insekticida iz upotrebe. Stoga, ova studija upoređuje efekte prirodnih, polusintetičkih i sintetičkih insekticida u kontroli oštećenja od žičnjaka na usevu luka.

Terenski ogledi efikasnosti različitih insekticidnih tretmana na gustinu biljaka, oštećenje od žičnjaka (%) i ukupan prinos luka sprovedeni su 2024. godine u Institutu za povrtarstvo (Smederevska Palanka, Srbija). Eksperiment je podrazumevao šest tretmana: netretirana kontrola, tri prirodna insekticida (dve formulacije a.s. spinosada: granule (GR) i koncentrovana suspenzija (SC), i *B. bassiana* ATCC 74040 2,3×107 konidiospora/ml), polusintetički insekticid (a.s. spinetoram) i sintetički insekticid (a.s. teflutir). Tretiranje je rađeno u vreme sadnje crnog luka prema preporučenoj količini primene po hektaru. Ocene su urađene 20 i 42 dana posle tretmana (DPT) da bi se odredila gustina biljaka. Oštećenja biljaka luka od žičnjaka zabeležena su 42 DPT, a prinos je izračunat vaganjem ubranih lukovica luka nakod žetve. Rezultati su pokazali da je spinosad formulacija GR, razbacana u brazde pri sadnji, značajno povećala gustinu biljaka nakon 20 DPT, dok je formulacija SC, primenjena kao tretman zemljišta pri sadnji, rezultirala najnižom gustinom. Spinetoram je pokazao najveću gustinu biljaka 42 DPT i najveći procenat oštećenja od žičnjaka (15%) od svih testiranih insekticida. Kontrola je imala najveći procenat oštećenih biljaka i najmanji prinos. Spinetoram je dao maksimalan prinos luka, dok je spinosad formulacija GR dala najmanji prinos među insekticidima.

Poljski ogledi su pokazali da prirodni i polusintetički insekticidi efikasno suzbijaju žičnjake, obezbeđujući adekvatnu zaštitu useva i održive prinose. Ova studija podržava razvoj i usvajanje ekološki svesnog upravljanja štetočinama u zemljištu.

Ključne reči: Elateridae, biopesticidi, insecticidi, suzbijanje štetočina, prinos, crni luk

Pestic. Phytomed. (Belgrade), 40(1), 2025, 27–35

Analytical approach for simultaneous determination of azoxystrobin, prothioconazole and trifloxystrobin in plant protection products

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SUMMARY

In this study, an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method with diode array detection (DAD) was developed and validated for simultaneous determination of azoxystrobin, trifloxystrobin and prothioconazole in plant-protection products. Chromatographic separation of the active substances was achieved using 0.1% acetic acid and acetonitrile (30:70 v/v) at a flow rate of 0.52 ml/min on a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm × 1.8 µm) and UV detection at 210 nm. Validation was done by evaluating the linearity and precision of the method, repeatability of injections, accuracy, and limits of detection and quantification (LOD and LOQ). Under the conditions, correlation coefficients of linearity were 0.996-0.997, the precision of method, expressed as relative standard deviation, was lower than the modified Horwitz values, the accuracy of all individual substances was within the range of 94.61-107.35%, while repeatability of the injectons was satisfied with RSD of 0.94-1.35%. LOD and LOQ were 0.0063 mg/ml and 0.019 mg/ml for azoxystrobin, 0.0051 mg/ml and 0.015 mg/ml for prothioconazole and 0.0051 mg/ml and 0.015 mg/ml for trifloxystrobin, respectively. A simple, precise, accurate, and fast analytical method for simultaneous determination of the fungicides azoxystrobin, prothioconazole and trifloxystrobin can be proficiently used for their detection and quantification in formulated products. The developed and validated method was applied to real samples, confirming the method's applicability.

Keywords: pesticide formulations, active ingredients, analytical methods, reversed phase high-performance liquid chromatography (RP-HPLC/DAD), fungicides

Article info

Original scientific paper

Received: 23 December 2024

Accepted: 19 March 2025

Publication is free of charge.

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DOI: https://doi.org/10.2298/PIF2501027L

INTRODUCTION

The main challenges to achieving high yields include a wide range of pests, weeds and diseases that cause damage and great losses, which can be prevented or controlled by plant protection products (PPPs). Without effective and quality PPPs, and their conscientious application, agricultural production would be severely compromised, resulting in seriously affected global food availability and agricultural economy.

Thus, for such plant protection to be implemented, it is necessary before application of PPPs to determine their quality, and this is achieved by analyzing the physicochemical properties and impurities of toxicological significance, and identification and determination of amounts of active substances in formulations (FAO & WHO, 2022). These characteristics need to be evaluated according to CIPAC (Collaborative International Pesticides Analytical Council) or AOAC (Association of Official Agricultural Chemists) standard methods. If official methods are not available, it is necessary to develop and validate a suitable method.

Diseases caused by phytopathogenic fungi represent a serious challenge for agricultural production worldwide. Controlling such diseases often requires the application of PPPs or some other agricultural practices, and their effective control is crucial for preserving agricultural productivity. Their timely identification and adequate control play a key role in ensuring a stable and sustainable food supply for the growing world population. However, the problem arises when several diseases occur simultaneously, and resistance is developed to frequently applied active substances. For this reason, mixing two or more active substances with different spectrums and modes of action is becoming increasingly common.

Hence, to expand the spectrum of action, reduce instances of pest resistance, replace multiple applications with a single one, and reduce the number of treatments, multi-pesticide PPPs are being introduced. A good example are PPPs based on two or three active substances, such as a multipesticide formulation based on azoxystrobin, prothioconazole and trifloxystrobin.

Azoxystrobin and trifloxystrobin are strobilurin fungicides, synthetic analogs of natural fungal metabolites (Clough, 1993). According to FRAC, these active substances belong to the fungicide group C, whose mode of action is described as inhibition of cellular respiration (Quinone Outside Inhibitors) (FRAC, 2023). It is reflected in binding to the external site of the cytochrome b-c1 complex (quinone), where coenzyme Q10 (ubiquinone), which is responsible for transferring electrons to proteins, would otherwise be bound. These systemic fungicides are transported by the xylem, and in some cases also act as contact fungicides with a wide range of action (Team of editors, 2020). They are registered for the control of fungi from the divisions Basidiomycota, Ascomycota, Deuteromycota and Oomycetes (Bartlett et al., 2002; Anonymous, 2023) (Table 1 and Table 2).

Table 1. Phytopathogenic fungi controlled by azoxystrobin

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Erisiphe gramminis, Puccinia striiformis, Puccinia recondite, Rhynchosporium secalis, Fusarium spp., Septoria nodorum*
Sugar beet	*Cercospora beticola, Rhizoctonia solani*
Sunflower	*Sclerotinia sclerotiorum, Diaporthe helianthi, Phomopsis helianthi*
Oilseed rape	*Sclerotinia sclerotiorum*
Tomato/ Potato	*Phytophthora infestans, Aternaria solani*
Paprika	*Leveillula taurica*
Cucumber/ Watermelon/Melon/ Zucchini	*Pseudoperonospora cubensis, Alternaria cucumerina, Erysiphe cichoracearum, Cladosporium cucumerinum*
Cabbage	*Peronospora parasitica, Alternaria brassicae, Albugo candida*
Carrot	*Erysiphe heraclei, Alternaria dauci*
Strawberry	*Mycosphaerella fragariae, Podosphaera aphanis*
Raspberry	*Dydimella applanata*
Blackberry	*Kuehneola uredinis*
Grapevine	*Plasmopara viticola, Uncinula necator*

Prothioconazole is a fungicide in the group of triazolinthiones, which belongs to the G group according to FRAC, and affects sterol biosynthesis in membranes (FRAC, 2023). It is used as a systemic fungicide with protective, curative and eradicative effects (Team of editors, 2020), affecting ergosterol in the cell membrane, which plays a significant role in its structure and permeability, and is essential for cell growth (Parker et al., 2013). Plant species and phytopathogenic causing agents for which prothioconazole is registered are given in Table 3 (Anonymous, 2023).

For individual determination of azoxystrobin, the available method implies the use of gas chromatography (Dobrat, W. & Martijn, 2009), while the available methods for determination of trifloxystrobin and prothioconazole suggest the use of high-performance liquid chromatography (HPLC) (De Oliveira & Garvey, 2017; Partian & Garvey, 2021). However, methods for simultaneous determination of these fungicides are still lacking. The current study therefore aimed to develop and validate a method for simultaneous analysis of azoxystrobin, prothioconazole and trifloxystrobin in formulated products.

MATERIALS AND METHODS

Analytical standards of azoxystrobin (97%), prothioconazole (99.5%) and trifloxystrobin (98%) were obtained from Dr Ehrenstorfer (Augsburg, Germany). Acetonitrile (HPLC grade), ultrapure water and acetic acid were purchased from J.T. Baker (Netherlands). The plant protection product (SC formulation) used in this study contained azoxystrobin (111.9 g/l), prothioconazole (143 g/l) and trifloxystrobin (111.2 g/l).

Individual stock solutions of analytical standards of azoxystrobin, prothioconazole, and trifloxystrobin were prepared by dilution in acetonitrile and ultrasonic homogenization. Stock solutions were then used to obtain a series of mixture solutions in concentrations of 0.0218-0.5135 mg/ml for azoxystrobin, 0.0162-0.3815 mg/ml for prothioconazole and 0.0213-0.5016 mg/ml for trifloxystrobin (Table 4). All standard solutions were stored at 4 °C in the dark.

Table 2. Phytopathogenic fungi controlled by trifloxystrobin

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Puccinia striiformis, Puccinia recondite, Erisiphe gramminis, Fusarium spp.*
Sugar beet	*Cercospora beticola*
Sunflower	*Phoma spp.*
Tomato	*Botrytis cinerea, Leveillula taurica*
Apple	*Venturia inaequalis, Podosphaera leucotricha, Colletotrichum spp.*
Cherry/Sour cherry	*Monilia laxa*
Blueberry	*Botrytis cinerea*

Table 3. Phytopathogenic fungi controlled by prothioconazole

Plant species	Phytopathogenic fungi
Wheat/ Barley	*Erisiphe gramminis, Puccinia striiformis, Puccinia recondite, Rhynchosporium secalis, Fusarium spp., Septoria nodorum, Pyrenophora teres*
Sugar beet	*Cercospora beticola*
Sunflower	*Sclerotinia sclerotiorum, Botrytis cinerea, Phoma spp.*
Oilseed rape	*Sclerotinia sclerotiorum, Alternaria brassicae*

Table 4. Concentrations of azoxystrobin, prothioconazole and trifloxystrobin in a series of solutions

	azoxystrobin (mg/ml)	prothioconazole (mg/ml)	trifloxystrobin (mg/ml)
mix 1	0.51348	0.38148	0.50160
mix 2	0.36677	0.27249	0.35829
mix 3	0.18339	0.13624	0.17914
mix 4	0.13099	0.09732	0.12796
mix 5	0.06549	0.04866	0.06398
mix 6	0.02183	0.01622	0.02133

The Agilent 1100 Series with DAD detector and a reversed-phase Zorbax Eclipse XDB-C18 (50 mm × 4.6mm × 1.8μm) (Agilent Technologies, USA) were used for an HPLC analysis.

Applying the IAEA guidelines (IAEA, *2009*), validation was performed based on several analytical performance parameters: linearity, method precision, repeatability of injections, accuracy, and limits of detection and quantification (LOD and LOQ) (CIPAC, 1999).

Evaluation of the linear relationship between analyte concentration and the corresponding detector response is essential. The linearity should be tested at several concentration levels, and this assessment should confirm a linear growth of the detector's response with increasing analyte concentrations.

Precision and repeatability are parameters that are similar but refer to different aspects of the performance of the method being validated. Precision reflects the congruity of results obtained under modified and predicted conditions. It clarifies the ability of a method to give consistent results under different conditions (apparatus, analyst, environment, time) and it employs statistical parameters, such as the relative standard deviation of repeated measurements, as well as the Horwitz limit (European Commission, 2019).

On the other hand, repeatability indicates the method's precision in repeated measurements of the same sample under identical conditions (apparatus, analyst, environment, time). Also, it indicates the convenience of the method for repeated consecutive applications on the same sample, which ensures its applicability in routine analytical practice.

Accuracy is determined by comparing the obtained results with the results obtained using certified reference material. When certified reference material is not available, standard samples to which a known amount of analyte is added can be used. Accuracy is most often expressed as a percentage of analytical procedure „recovery" for a known additional amount of analyte in the sample, so it is essential for estimating in what percentage the method reflects the actual concentration of the analyte in a tested sample (ICH, 2022).

Finally, LOD and LOQ need to be determined. LOD is the lowest possible value that can be identified and not expressed as an exact value, while LOQ is the lowest value of an analyte that can be quantified and precisely determined (Zuas et al., 2016).

RESULTS AND DISCUSSION

HPLC analysis

A simultaneous analysis of azoxystrobin, prothioconazole and trifloxystrobin was performed using HPLC/DAD. During the process of method validation, different wavelengths, combinations of mobile phases, their flow rates and column temperatures were tested. The most effective for the analysis were the conditions shown in Table 5.

The appropriate separation and appearance of analytical signals of azoxystrobin, prothioconazole and trifloxystrobin were achieved under these conditions at all tested wavelengths (Figure 1). However, the best response was achieved at the wavelength of 210 nm (Figure 2), and it was therefore chosen for further analysis. The UV spectra of all three active substances were recorded at the same wavelength (Figure 3).

The applicability of the method in terms of specificity was demonstrated by example chromatograms (standard and formulation with active substance) (European Commission, 2019).

Table 5. Conditions for HPLC-DAD determination of azoxystrobin, prothioconazole and trifloxystrobin

Mobile phase	0,1% acetic acid: acetonitrile
Mobile phase ratio	30:70
Column temperature	20 °C
Flow	0.520 ml/min
Wavelength	254 nm, 210 nm, and 230 nm
Injected volume	1 μl
Retention time of azoxystrobin	1.793min
Retention time of prothioconazole	2.403min
Retention time of trifloxystrobin	4.191min

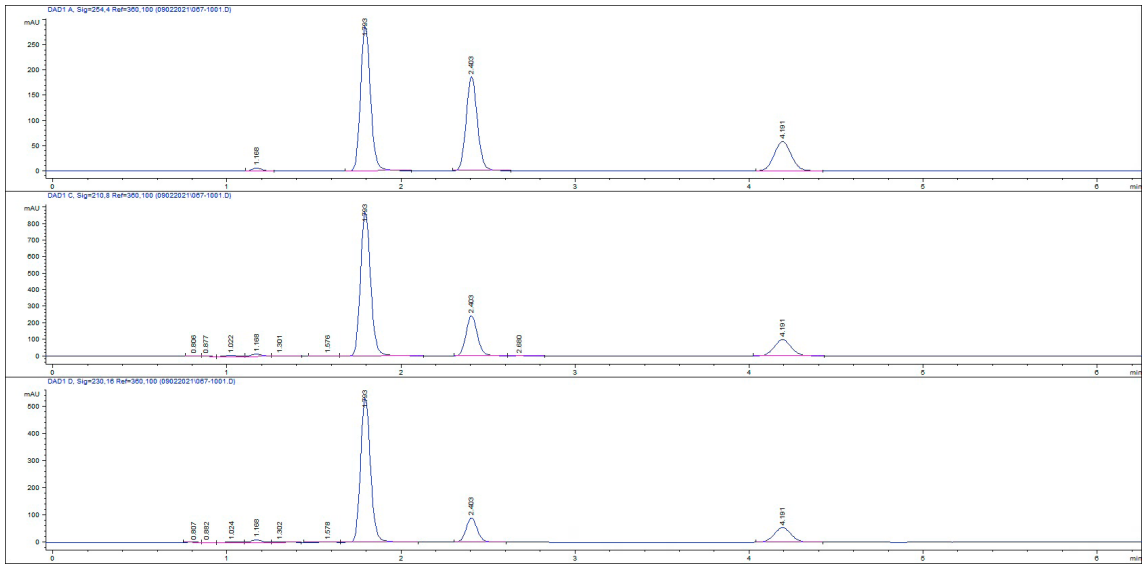

Figure 1. Chromatogram of the mixture of analytical standards of azoxystrobin (0.183385 mg/ml), prothioconazole (0.136243 mg/ml) and trifloxystrobin (0.179143 mg/ml) in acetonitrile at different wavelengths under conditions specified in Table 5 - azoxystrobin (1.793 min), prothioconazole (2.403 min) and trifloxystrobin (4.191 min)

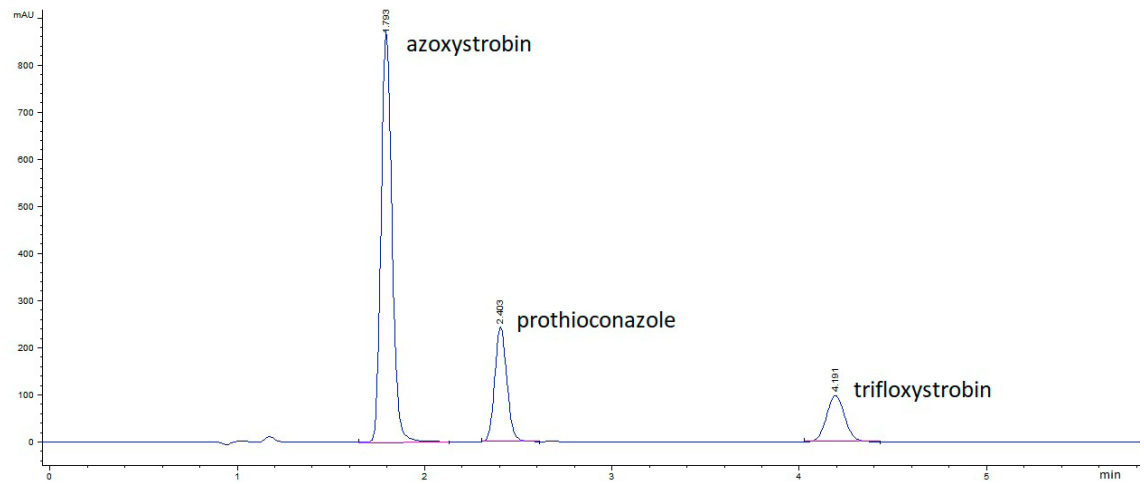

Figure 2. Chromatogram of the mixture of standards of azoxystrobin (0.183385 mg/ml), prothioconazole (0.136243 mg/ml) and trifloxystrobin (0.179143 mg/ml) in acetonitrile at the wavelength of 210 nm

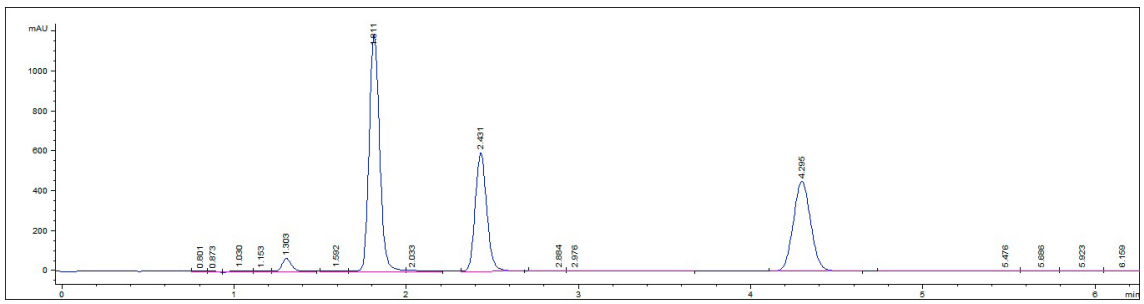

Figure 3. Chromatogram of PPP in acetonitrile - azoxystrobin (1.611 min), prothioconazole (2.431 min) and trifloxystrobin (4.295 min)

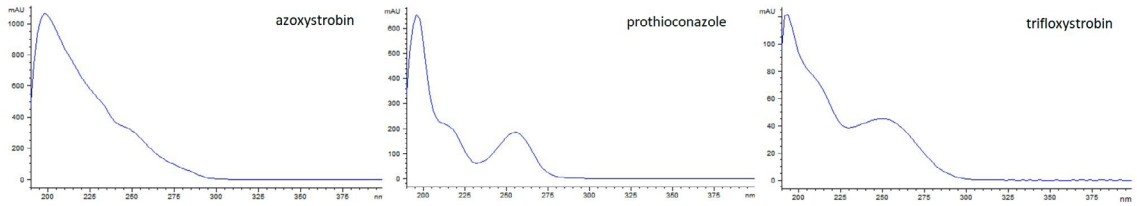

Figure 4. UV spectra of analytical standards of azoxystrobin, prothioconazole and trifloxystrobin in acetonitrile at the detector wavelength of 210 nm

Validation of method

The chromatographic conditions were checked by examining the linearity of the detector response, precision, repeatability and accuracy of the method, and by determining the LOD and LOQ, and the dependence of the peak area on the injected volume.

The linearity of detector response was determined at six concentration levels. Analytical and statistical data obtained by the linear regression method are shown in Table 6.

A good linearity of detector response was achieved within the range of tested concentrations of azoxystrobin, prothioconazole and trifloxystrobin (Table 6). The obtained values indicate that the increase in contents of the introduced compounds linearly follows the increase in area of analytical signal. In the regression equation, the correlation coefficient of linear dependence for the analyzed compounds was 0.996-0.997, which indicates a high sensitivity of this method for determination of azoxystrobin, prothioconazole and trifloxystrobin since the acceptable coefficient of determination is ≥0.98 (European Commission /3030/99).

The repeatability of injections of azoxystrobin, prothioconazole and trifloxystrobin was established by injecting 1 µl of the mixture of standard solutions of these compounds seven times (European Commission /3030/99). The values of the obtained peak areas for the tested compounds are shown in Table 7.

The relative standard deviation (RSD%) values of 0.94, 1.35 and 1.07 for azoxystrobin, prothioconazole and trifloxystrobin, respectively, indicate that good repeatability of determination of these compounds was achieved by the applied method (RSD≤2).

The precision of the method was determined by injecting 1 µl of the PPP solution in acetonitrile five times, and the relative standard deviation (RSD%) was 0.79, 0.98, and 1.27 for azoxystrobin, prothioconazole and trifloxystrobin, respectively (Table 8). The results are acceptable when the experimental RSDr values are below those obtained by the modified Horwitz equation (CIPAC, 1999).

The Horwitz limit value was calculated based on the equation %RSDr=2(1-0.5 log C), which was modified to %RSDr=%RSDr×0.67. The relative standard deviation (RSD) of reproducibility of azoxystrobin, prothioconazole and trifloxystrobin determination using the peak area is well below the modified Horwitz limit values of 2.00, 1.93 and 2.01% (Table 9).

Table 6. Linearity parameters for azoxystrobin, prothioconazole and trifloxystrobin determination by HPLC/DAD

	Peak area		
	azoxystrobin	prothioconazole	trifloxystrobin
mix 6	476.85	146.1	86.35
mix 5	1058.25	319.35	189.1
mix 4	2234.7	899.25	501.3
mix 3	3322.25	1114.7	663.55
mix 2	6021.5	2210.35	1340.85
mix 1	7965.2	3217.1	1987.35
Regression equation	y = 15444 + 215.7	y = 8391.x – 12.19	y = 3953.x – 30.18
Section	215.7	12.19	30.18
Slope	15444	8391.x	3953.x
Correlation coefficient	**0.996**	**0.997**	**0.997**

The accuracy of the method was assessed using the standard addition method, i.e. by adding a known amount of the analyte to the sample (Table 10). The appropriate known amounts of analytical standards of azoxystrobin, prothioconazole and trifloxystrobin were added to PPP samples in which the content of active substances was previously determined. After the analysis, the expected concentrations were compared with the concentrations obtained using the described method. The high agreement between values obtained in the procedure (94.61-107.35%) and actual values confirms the accuracy of the applied method for determination of azoxystrobin, prothioconazole and trifloxystrobin, given that the acceptable value is between 90 and 110 % of target concentration (European Commission /3030/99).

Table 7. Repeatability of determination of azoxystrobin, prothioconazole and trifloxystrobin

Concentration	0.51348	0.38148	0.5016
	azoxystrobin	prothioconazole	trifloxystrobin
Peak area	3149.0	954.3	591.1
	3100.9	948.8	578.1
	3167.6	979.0	572.5
	3170.1	978.4	573.9
	3106.1	955.4	581.8
	3114.5	952.4	577.1
	3115.0	951.3	577.2
Mean value	3131.9	959.9	578.8
SD	29.6	12.9	6.1
RSD	**0.94**	**1.35**	**1.07**

Table 8. Precision of determination of azoxystrobin, prothioconazole and trifloxystrobin

	azoxystrobin	prothioconazole	trifloxystrobin
Peak area	5031.2	2815.2	3137.4
	5052.4	2869.1	3220.3
	5137.7	2852.5	3193.2
	5073.9	2826.7	3157.6
	5084.6	2800.8	3122.6
Mean value	5075.9	2832.9	3166.2
SD	40.2	27.7	40.19
RSD	**0.79**	**0.98**	**1.27**

Table 9. Horwitz limit values for the active substances azoxystrobin, prothioconazole and trifloxystrobin

		γ=1.0996 g/cm^3			*Horwitz* value RSDr	modified *Horwitz* value
	g/l	am/γ/10	C	logC	2(1-0.5logC)	RSDr × 0.67
azoxystrobin	111.9	10.18	0.10	-0.99	2.99	2.00
prothioconazole	143.0	13.00	0.13	-0.89	2.89	1.93
trifloxystrobin	111.2	10.11	0.10	-1.00	3.00	2.01

Table 10. Accuracy of determination of azoxystrobin, prothioconazole and trifloxystrobin in PPPs

Active substances	I mg/ml	I Rec.%	II mg/ml	II Rec.%	Mean Rec.%	SD
azoxystrobin	0.10269	94.61	0.03668	95.91	95.26	0.92
prothioconazole	0.07629	101.23	0.02725	99.32	100.28	1.35
trifloxystrobin	0.10032	107.35	0.03583	100.96	104.16	3.19

Table 11. Analyses of azoxystrobin, prothioconazole and trifloxystrobin in PPPs

Fungicide and type of formulation	Amount in PPP declared by manufacturer	Measured concentrations
Prothioconazole, EC	250 g/l	252.7±0.3 g/l
Azoxystrbin, SC	250 g/l	250.4±0.1 g/l
Trifloxystrobin, WG	500 g/l	501.6±0.5 g/l
Prothioconazole + azoxystrbin, SC	150 g/l +250 g/l	152.4±0.4 g/l +251.1±0.5 g/l
Prothioconazole + trifloxystrobin, SC	175 g/l +150 g/l	175.2±0.3 g/l +150.4±0.2 g/l

Based on the described method validation, this HPLC protocol can be successfully used for determining the exact contents of all three active substances in PPPs.

Analyses of PPPs

The developed analytical method was applied to determine the contents of active substances in formulated products. Five commercially available plant protection products were analyzed using the method and compared with reference values (Table 11). The calibration curve method and an external standard were applied. Based on the regression equations of calibration curves, the amounts of active substances in the measured mass of samples were calculated. The obtained results confirmed an agreement between the measurement concentrations and reference values given by the manufacturer.

In this study, an analytical method for simultaneous determination of azoxystrobin, prothioconazole and trifloxystrobin in pesticide formulations by HPLC–DAD was developed and validated. Parameters such as linearity, precision and accuracy confirmed its reliability. Thus, the method can be used to analyze products containing the three active substances, and equally successfully those that contain a combination of two or a single one of them. Conditions were set to a lower flow rate of the mobile phase, as the applied short column requires, and uniform separation of analytical signals was obtained at the wavelength of 210 nm. Using the short column, it was managed to develop a method which is fast, simple, accurate and economical in solvents consumption.

ACKNOWLEDGEMENT

This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (grants No. 451-03-65/2024-03/ 200117 and 451-03-66/2024-03/ 200117) and Joint Research Project Serbia-Slovakia (Grant No. 337-00-3/2024-05/06).

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Analitička metoda za istovremeno određivanje azoksistrobina, protiokonazola i trifloksistrobina u sredstvima za zaštitu bilja

REZIME

U ovoj studiji, razvijena je i validovana izokratska metoda za istovremeno određivanje azoksistrobina, trifloksistrobina i protiokonazola u sredstvima za zaštitu bilja primenom reverzno-fazne visokoperformantne tečne hromatografije (RP-HPLC/DAD). Hromatografsko razdvajanje aktivnih supstanci postignuto je upotrebom 0,1% rastvora sirćetne kiseline i acetonitrila (30:70 v/v) kao mobilne faze, pri protoku od 0,52 ml/min. Korišćena je Zorbax Eclipse XDB-C18 (50 mm x 4.6 mm x 1.8 µm) kolona, a detekcija je vršena na talasnoj dužini od 210 nm. Validacija metode obuhvatala je procenu linearnosti i preciznosti metode, ponovljivosti injektovanja, tačnosti metode, kao i limita detekcije i kvantifikacije (LOD i LOQ). Pod navedenim uslovima postignuti koeficijent korelacije linearnosti kretao se 0.996-0.997. Preciznost metode, izražena kao relativna standardna devijacija (RSD), bila je niža od modifikovanih Horwitz-ovih vrednosti. Tačnost određivanja svake pojedinačne aktivne supstance kretala se 94,61-107,35%, dok je ponovljivost injektovanja bila zadovoljavajuća sa RSD vrednostima 0,94-1,35%. Limiti detekcije i kvaktifikacije iznosili su 0,0063 mg/ml i 0,019 mg/ml za azoksistrobin, 0,0051 mg/ml i 0,015 mg/ml za protiokonazol, 0,0051 mg/ml i 0,015 mg/ml za trifloksistrobin. Stoga, razvijena i validovana jednostavna, precizna, tačna i brza analitička metoda za istovremeno određivanje fungicida azoksistrobina, protiokonazola i trifloksistrobina se može uspešno primenjivati za njihovo pojedinačno, ali i istovremeno određivanje u formulisanim proizvodima. Daljom primenom opisane metode na realne uzorke, potvrđena je njena namena.

Ključne reči: formulacije pesticide, aktivne supstance, analitičke metode, reverzno-fazna visokoperformantna tečna hromatografija (RP-HPLC/DAD), fungicidi

Instructions for Authors

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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. U toku faze lekture, tražiće se da svi autori u pisanom obliku potvrde svoje autorstvo.

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

Originalni naučni 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:

- autor, godina;
- prvi & drugi autor, godina;
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Reference u časopisima treba da sadrže sledeće podatke: autor(i), godina publikovanja, naslov rada, naslov časopisa, volumen, broj (ako se paginacija ponavlja), brojeve stranica (od – do) i doi broj (ukoliko postoji).

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, u crnoj boji. 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 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 u JPG ili TIFF formatu, sa rezolucijom najmanje 300 dpi. 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.

Svako korišćenje alata zasnovanog na veštačkoj inteligenciji (npr. ChatGpT) treba da bude opisano (npr. za popravak kvaliteta engleskog jezika, što je dopušteno).

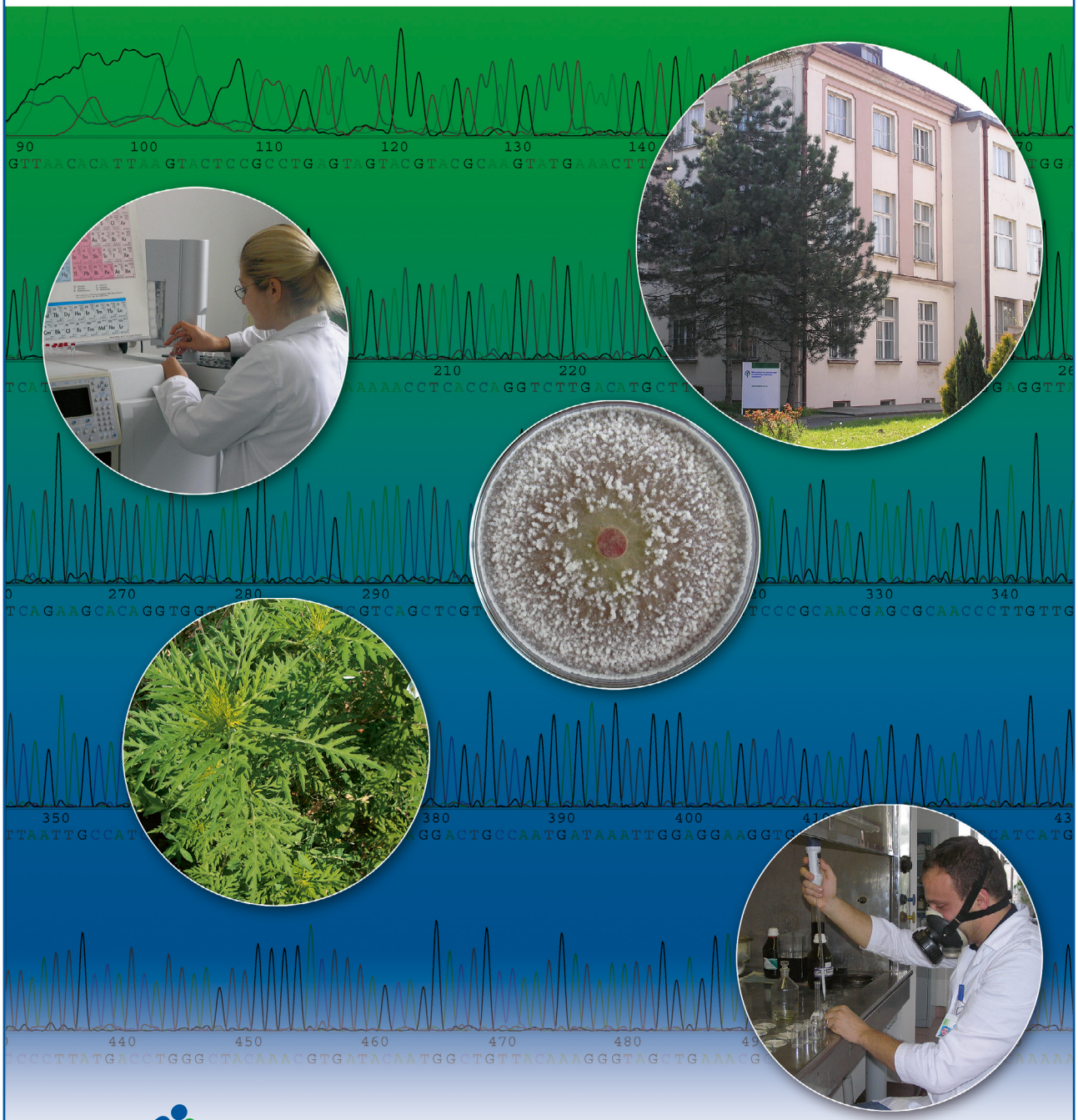
Od autora se očekuje da koriste preporučene jedinice međunarodnog sistema (SI). Skraćenice je potrebno definisati u zagradama nakon prvog pominjanja u tekstu. Narodni nazivi organizama se daju uz pun latinski naziv, a kurzivom se obeležavaju samo latinski nazivi rodova i vrsta, npr. krompirova zlatica, *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.

Objavljivanje radova u časopisu *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* je besplatno.

Uredništvo preduzima mere protiv grubog kršenja etičkih normi u vezi sa plagijarizmom.

Nauka u službi zdravlja bilja, čoveka i prirode



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