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Original scientific paper

Allelopathic effects of actinobacterial isolates on seed germination and early seedling growth of velvetleaf (*Abutilon theophrasti* Medik.)

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SUMMARY

This study aimed to examine *in vitro* allelopathic effects of actinobacterial isolates on seed germination and early seedling growth of velvetleaf (*Abutilon theophrasti*). Thirty-five actinobacterial isolates were obtained from soil and compost in different phases of composting. Also, an experiment variant involving the herbicide mesotrione was set in the recommended amount of application, as a reference standard.

The experimental results indicate statistically significant differences ($p<0.05$) between controls and all treatments with actinobacterial isolates regarding seed germination, and radical and shoot length. The highest inhibition (100%) was observed in seed germination and radical length in treatments with the isolates A10 and NOV2, compared to uninoculated starch casein broth (SCB) as control. Shoot length was shown to be the most sensitive parameter, where 100% inhibition was observed in the following treatments with actinobacteria: A010, A017, NOV2, NOV3, NOV4 and NOV5. Actinobacterial isolates showed a higher inhibitory effect on seed germination than treatment with the reference herbicide mesotrione.

Keywords: actinobacteria, velvetleaf, seed germination, allelopathy

INTRODUCTION

Synthetic herbicides have had a central role in the history of weed management. Although they are efficient in controlling weeds, their continual use has resulted in weeds developing resistance to some herbicides (Santos, 2009). Furthermore, reacting to health and environmental concerns over their indiscriminate usage, numerous countries have banned many of those agrochemicals (Eisler, 2000). Although it is hard to leave off herbicides, it is possible to reduce their use by applying integrated weed management approaches in field crop production (Anderson, 2007). As a result, there is a rapidly growing trend at present towards discovering effective and safer alternatives to synthetic herbicides. Strategies that are complementary to herbicides are gaining increasing importance and aim to reduce dependence on chemical control, and to

alleviate negative impact that these compounds impose on the environment.

Allelopathy is a biological process representing the activity of various biochemical agents of one organism on the germination, growth or reproduction of another organism, and the process can be utilized as a method of weed control (Marcías et al., 2007). This method is considered to be an efficacious, cost-effective and environmentally friendly approach to weed control. Allelopathic control of weeds can be applied either as a unique strategy in certain systems, for example in organic production, or in combination with other methods of integrated plant protection (Saxena & Pandey, 2001; Jabran et al., 2015). Another aspect of allelopathy is the applicability of different groups of soil microorganisms and their metabolites for weed control, which offers an important alternative to the use of chemical products (Inderjit, 2005).

Actinobacteria (class *Actinobacteria*) represent one of the most widely distributed groups of microorganisms in nature. They have an outstanding role as agents of biodegradation in soil and formation of humus. They are Gram-positive bacteria whose DNA has a high content of GC pairs (55%) and they are the source of numerous secondary metabolites (Wang et al., 1999). Actinobacteria own a significant antagonistic potential against a variety of pathogens of cultivated plants and human pathogens (Ouhdouch et al., 2001; Šantrić et al., 2018). They are also important for the production of antibiotics (Agarwal & Mathur, 2016) and enzymes (Mukhtar et al., 2017). The most frequent genus of actinobacteria is *Streptomyces*. Many of their secondary metabolites show fungicidal, insecticidal and herbicidal properties (Shi et. al., 2020).

Weed interference in agricultural fields reduces the quality and quantity of agricultural products, causing huge economic losses to farmers. Velvetleaf (*Abutilon theophrasti* Medik.) is a major weed in maize and other summer row crops in many European countries, including Serbia (Sattin et al., 1992; Travlos et al., 2012; Vrbničanin et al., 2017). This species is generally highly competitive for nutrients, light and moisture, and it is able to drastically reduce yields and harvesting efficiency (Nurse & Di Tommaso, 2005). To our best knowledge, there are no available reports on the allelopathic potential of different actinobacterial isolates against this weed species. The present study therefore aimed to evaluate *in vitro* the allelopathic potential of actinobacterial isolates and their effects on seed germination and early seedling growth (radical and shoot length) of *A. theophrasti*.

MATERIALS AND METHODS

Isolation of actinobacteria

Isolation was performed from samples collected from arable fields and casing soils. Samples weighing 10 g were mixed with 90 ml of sterile distilled water and several serial dilutions were spread onto synthetic agar with sucrose, starch ammonia agar and starch casein agar (SCA) and incubated at 27°C for seven days. Thirty-five subcultured single colonies were transferred onto potato dextrose agar (PDA), and SCA was purified by streak plate technique (Jarak & Đurić, 2006).

Seed material

Seeds of the weed species *A. theophrasti* were collected in a field located in Belgrade's Jakovo suburb in October 2019. All seeds were cleaned and stored in paper bags in the laboratory at the temperature of 20-22°C.

Preliminary testing of allelopathic potential of actinobacterial isolates

In a preliminary test, primary inoculums of thirty-five isolates were prepared in Erlenmeyer flasks containing 25 ml of starch casein broth (SCB) and incubated for 7 days in an orbital shaker incubator at 120 rpm and 28°C. Culture filtrates were aseptically obtained by filtration through Whatman No 2 filter paper. Seed surface of *A. theophrasti* was sterilized with 5% sodium hypochlorite solution (NaOCl) for 2 minutes and then rinsed three times with sterilized distilled water to avoid possible inhibition of germination due to fungal or bacterial toxins. Ten disinfected seeds were placed into each sterilized Petri dish with filter papers and moistened with 2 ml of the primary filtrate of each actinobacterial isolate. Two controls were prepared in the same way in which uninoculated SCB broth and sterilized distilled water were used separately instead of culture filtrate. All dishes were sealed with parafilm to avoid evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 5 days, nine isolates with allelopathic potential were observed based on inhibition assessment of seed germination and early seedling growth (radical and shoot length).

Secondary test of allelopathic potential of actinobacterial isolates

In a secondary test, 60 ml of SCB was inoculated with 3 ml of primary inoculums of nine isolates and incubated for 7 days in an orbital shaker incubator at

120 rpm and 28°C. Culture filtrates were aseptically obtained by filtration through Whatman No 2 filter paper. Two controls were prepared in the same way as the uninoculated SCB broth and sterilized distilled water that were used separately instead of culture filtrate. All dishes were sealed with parafilm to avoid evaporation and placed in an incubator (VELP, Incubator FOC) at 28±1°C in the dark. After 5 days, germination percentage was calculated and seedling growth (radical and shoot length) was measured (Bataineh et al., 2008). Also, treatment with the herbicide mesotrione (commercial product CHIEF, mesotrione 100 g/l, Adama, Israel) was carried out at the recommended field rate of 120 g a.i./ha, i.e. 1.2 l/ha, as a reference standard. Inhibition percentage, based on the changed germination and seedling growth, was calculated using the formula:

$$\% \text{ inhibition} = [(Xc-Xt)/Xc]\times 100$$

where Xc = % of germination or seedling growth in control; and Xt = % of germination or seedling growth in treatments with actinobacterial isolates or the herbicide.

The experiment design was a randomized complete block with four replications (10 seeds per Petri dish), repeated twice, and data were combined for analysis.

Morphological and cultural characteristics of actinobacteria isolates

Nine actinobacterial isolates were inoculated on SCA and colony color was checked. Spores were examined under a microscope (CX41RF Olympus, Düsseldorf, Germany), while the Gram staining procedure for actinobacteria was performed using crystal violet and Lugol solution (Knežević-Vukčević & Simić, 2015).

The actinobacterial isolates were inoculated onto several different ISP (International Streptomycetes Project) media: yeast malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt starch agar (ISP4), glycerol asparagine agar (ISP5), peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7) (Himedia Laboratories, Mumbai, India). After 7 days, growth, pigmentation of aerial and substrate mycelium, and diffusible pigment production were observed (Shirling & Gottlieb, 1966; Wink, 2014).

Physiological and biochemical characteristics of actinobacterial isolates

Physiological characteristics were determined on SCA medium at different temperatures (5°C, 16°C, 26°C, 35°C) and pH levels (4, 7, 9), and the isolates were incubated at 27°C under different salt concentrations (3%, 5%, 7%). Their growth was measured after 7 days of incubation.

The starch hydrolysis test was performed on starch agar. Actinobacterial isolates were incubated for 5 days at 28°C. After incubation, the plates were flooded with Lugol's solution and inspected for the presence or absence of bright zones around colonies.

For utilization of carbon sources, the Hugh-Leifson medium was prepared and sterile carbon sources (1%) were added, including dextrose, sucrose and glucose. After incubation at 28°C for 7 days, a positive reaction was detected (green color around colony was turning yellow).

Gelatin hydrolysis was run on nutrient gelatin agar. Tubes were inoculated using the stub method and incubated at 27°C for 7 days along with uninoculated control tubes. After incubation, the tubes were kept at cold temperature. Hydrolyzed gelatin was detected in tubes with liquid media.

For catalase production, sterile yeast dextrose agar was inoculated with actinobacterial isolates and incubated at 28°C for 7 days. After incubation, a few drops of 3% hydrogen peroxide were added to each isolate, and the development of air bubbles indicated a positive reaction.

The use of citrate followed, and the SCA medium was inoculated with actinobacterial isolates and incubated at 30°C for 48 h. A change in color from green to blue indicated a positive reaction.

For melanin production, actinobacterial isolates were inoculated into the ISP6 and ISP7 media. The isolates were assumed to produce melanin when dark-brown or black diffusible pigments formed in the medium.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) using STATISTICA 8.0. software package. When F-values were statistically significant ($p<0.05$) treatments were compared using Fisher's Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Characteristics of actinobacterial isolates

Thirty-five isolates of actinobacteria from field soil and casing soil for button mushroom were collected. After a preliminary test with *A. theophrasti* seeds, 9 isolates (A01, A02, A07, A010, A017, NOV2, NOV3, NOV4 and NOV5) showed inhibitory effect (of 50% and more) on seed germination and early growth. Another test was set up with those 9 isolates to examine their allelopathic activity on *A. theophrasti* seeds. Morphological and biochemical characteristics of the selected isolates are described in Tables 1 and 2.

Table 1. Morphological and cultural characteristics of actinobacterial isolates

Characteristics	A01	A02	A07	A09	A010	A017	A018	NOV2	NOV3	NOV4	NOV5
Colony color	grey	pink	cream	grey	green	grey	grey	green	white	pink	cream
Gram reaction	+	+	+	+	+	+	+	+	+	+	+
Spore form	round	round	round	round	round	round	round	round	round	round	round
ISP2 gowth	++	++	++	++	++	++	++	++	++	++	++
a. mycelium	grey	pink	grey	grey	green	grey	white	white	white	white	grey
s.mycelium	green	purpl	brown	yellow	brown	brown	brown	green	orange	pink	brown
soluble pigment	brown	purpl	-	-	brown	brown	brown	-	orange	brown	-
ISP3 gowth	++	++	++	+	++	++	++	++	+	++	-
a. mycelium	grey	pink	cream	cream	grey	grey	white	cream	white	pink	-
s.mycelium	green	purpl	green	yellow	brown	brown	cream	brown	brown	pink	-
soluble pigment	-	purpl	-	-	yellow	brown	-	brown	brown	-	-
ISP4 gowth	++	+	++	++	+	++	++	++	+	++	++
a. mycelium	grey	pink	cream	cream	green	green	grey	cream	grey	grey	cream
s.mycelium	green	pink	green	green	yellow	brown	cream	green	cream	brown	cream
soluble pigment	-	-	green	green	-	green	-	-	yellow	grey	-
ISP5 gowth	++	++	++	++	++	++	+	++	++	+	++
a. mycelium	green	pink	cream	cream	cream	grey	white	cream	grey	pink	cream
s.mycelium	orange	pink	cream	cream	cream	brown	white	brown	brown	pink	cream
soluble pigment	yellow	pink	yellow	-	-	brown	-	brown	brown	-	-
ISP6 gowth	++	++	++	++	++	+	+	++	++	+	+
a. mycelium	grey	grey	grey	pink	grey	cream	white	pink	white	cream	cream
s.mycelium	green	black	brown	brown	brown	brown	black	cream	brown	cream	cream
soluble pigment	brown	brown	brown	-	brown	brown	black	-	brown	-	-
ISP7 gowth	++	++	++	++	++	++	++	++	+	+	+
a. mycelium	grey	grey	cream	cream	green	brown	white	cream	cream	brown	cream
s.mycelium	grey	black	green	brown	brown	brown	black	green	brown	brown	cream
soluble pigment	green	brown	-	-	brown	brown	black	brown	brown	brown	-

(-) no growth; (+) minimal growth; (++) optimal growth

Based on their morphological and biochemical properties, the isolates were identified as members of the genus *Streptomyces,* family *Streptomycetaceae* (Shirling & Gottlieb, 1966; Wink, 2014).

Bioassay with actinobacterial isolates and weed seeds

The germination results for *A. theophrasti* seeds obtained with different actinobacterial isolates are presented in Table 3 and Figure 1. Our data showed that the highest final germination of *A. theophrasti* seeds was in both control treatments (sterilized distilled water – 85.0% and uninoculated SCB broth – 68.0%). The data also showed that the highest inhibition (100%) of *A. theophrasti* seed germination was achieved by treatments A010 and NOV2, compared to the SCB broth control. Conversely, inhibition was the lowest when the herbicide treatment was used (50%), compared to the sterilized distilled water control. In other treatments, the inhibition of seed germination ranged from 59% (NOV5) to 72% (A07) (Figure 1a). The obtained data revealed a highly significant effect ($p<0.05$) between the controls and all treatments with actinobacterial isolates and the herbicide mesotrione (Table 3).

Table 2. Physiological and biochemical characteristics of actinobacterial isolates

Characteristics	A01	A02	A07	A09	A010	A017	A018	NOV2	NOV3	NOV4	NOV5
pH medium:											
4	-	+	-	-	-	-	-	-	-	-	-
7	++	++	++	+	++	++	++	++	++	++	++
9	++	++	++	+	++	++	+	++	+	+	++
N_aCl (%):											
3	++	++	++	++	++	++	++	++	+	++	+
5	++	-	++	++	+	++	++	++	+	+	++
7	++	-	++	++	-	+	+	++	+	+	++
Temperature: (C°)											
5	-	-	-	-	-	-	-	-	-	-	-
16	++	+	+	-	+	+	+	++	+	+	++
26	++	++	++	++	++	++	++	++	++	++	++
35	-	++	-	+	++	++	++	+	++	++	++
Hydrolysis of gelatin	+	-	+	+	-	+	+	-	+	-	-
Catalase	+	+	+	+	+	+	-	+	+	+	+
Starch hydrolysis	+	-	+	+	-	-	-	-	+	+	+
Citrate	+	-	-	-	-	+	-	+	-	-	-
Melanin production	-	+	-	-	+	+	+	-	-	-	-
Saccharose	-	+	-	-	+	+	-	-	-	-	-
Dextrose	-	+	-	-	+	+	+	+	+	+	+
Glucose	-	+	-	-	+	+	-	+	+	+	+

(-) no growth; (+) minimal growth; (++) optimal growth

Table 3. Effects of different actinobacterial isolates and a herbicide on *Abutilon theophrasti* seed germination and early seedling growth (radical and shoot length)

Treatments	Final germination (%)	Radical length (cm)	Shoot length (cm)
Control (SDW)	85.00±3.26 **a**	2.04±0.50 **a**	2.56±0.33 **a**
Mesotrione	42.50±1.31 **c**	0.24±0.00 **bcd**	0.00±0.00 **c**
Control (SCB)	68.00±2.79 **b**	1.87±0.26 **a**	1.98±0.41 **a**
A01	23.75±1.18 **d**	0.86±0.03 **bc**	0.28±0.01 **bc**
A02	22.50±1.18 **d**	0.92±0.10 **b**	0.80±0.02 **b**
A07	18.75±0.88 **d**	0.62±0.02 **bc**	0.25±0.01 **bc**
A010	0.00±0.00 **e**	0.00±0.00 **d**	0.00±0.00 **c**
A017	25.00±1.00 **d**	0.48±0.00 **bcd**	0.00±0.00 **c**
NOV2	0.00±0.00 **e**	0.00±0.00 **d**	0.00±0.00 **c**
NOV3	20.00±1.26 **d**	0.45±0.01 **bcd**	0.00±0.00 **c**
NOV4	20.00±1.09 **d**	0.37±0.00 **cd**	0.00±0.00 **c**
NOV5	27.50±1.09 **d**	0.45±0.00 **bcd**	0.00±0.00 **c**

SDW - Sterilized distilled water; SCB - Starch casein broth; Data are reported as the mean ± standard deviation. Differences in final germination, shoot and radical length of *Abutilon theophrasti* were evaluated by one-way analysis of variance (ANOVA), completed by Fisher's Least Significant Difference (LSD) test. Means in the same column referring to the same parameter are marked with different letters (a, b, c, d) only when they differ significantly ($p < 0.05$).

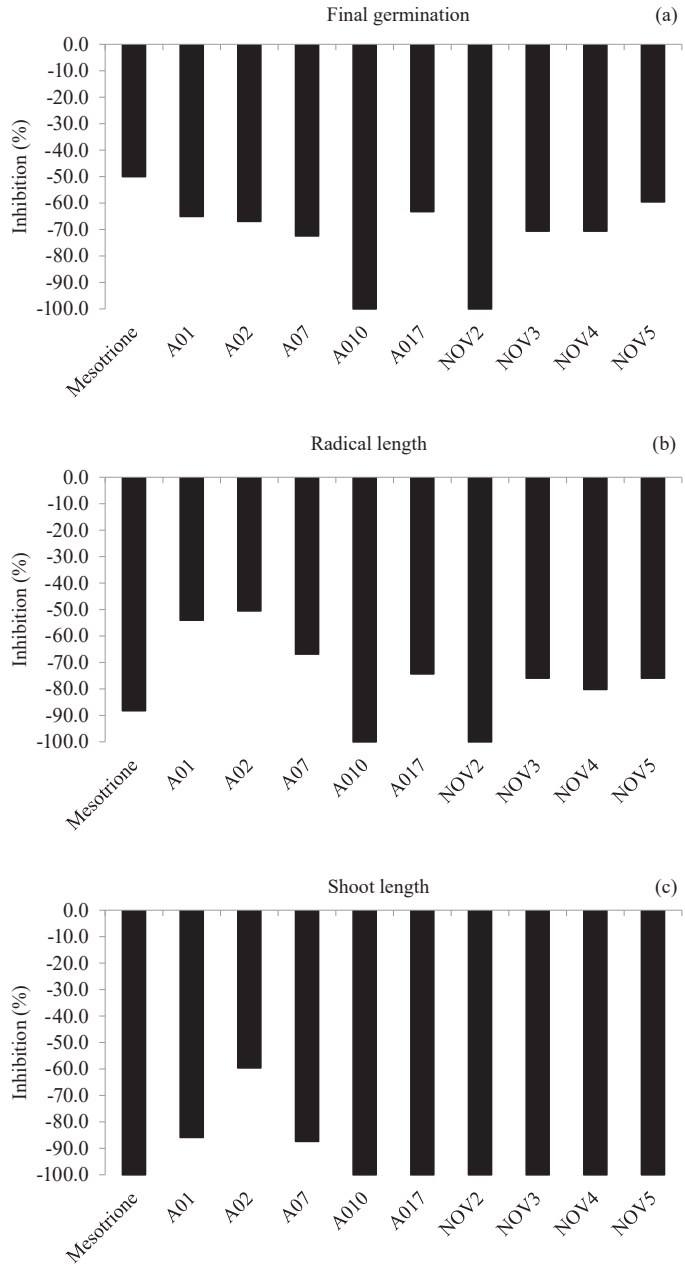

Figure 1. Inhibition (%) of final seed germination (a), radical length (b) and shoot length (c) under impact of different actinobacterial isolates and mesotrione herbicide

Literature data indicate a great allelopathic potential of actinobacteria, i.e. their secondary metabolites, on weed species. Priyadharsini et al. (2013) tested *in vitro* the herbicidal activity of a *Streptomyces* sp. isolate (KA1-3) on the weed species *Cassia occidentalis* L. and *Cyperus rotundus* L. The research showed that the bioactive compound N-phenylpropanamide had an inhibitory effect of up to 80% on seed germination of those weed species. Working with a *Streptomyces* sp. 8E-12 strain, Lee et al. (2003) isolated a herbicidal metabolite marked MHM, which stimulated leaf bleaching of the monocotyledonous weed species *Digitaria sanguinalis* and *Echinochloa crus-galli* under *in vivo* conditions.

The present study suggests that the tested actinobacterial isolates had adverse effects on the radical and shoot length of *A. theophrasti* (Table 3). The results of the seed germination test showed that two isolates, A010 and NOV2, achieved the highest inhibition (100%) of *A. theophrasti* radical length. Inhibition by other treatments with actinobacteria ranged from 50% to 80%, while mesotrione treatment caused 88% inhibition. On the other hand, shoot length was shown to be more sensitive as a parameter because 100% inhibition was observed in several treatments with actinobacteria (A010, A017, NOV2, NOV3, NOV4, and NOV5) and mesotrione (Figure 1b and 1c). Statistical analysis revealed signi ficant differences ($p<0.05$) between the controls and all treatments regarding both parameters (Table 3). Similarly, Singh et al. (2018) found several isolates with herbicidal action during a screening test that included over 300 endophytic actinobacteria. Applying secondary metabolites of those isolates on the weed species *Parthenium hysterophorus, Ageratum conyzoides,* and *Bidens biternata* before and after emergence, they observed a significant reduction in the length of their shoots and roots.

CONCLUSION

Of the 35 isolates of actinobacteria isolated from field soil and mushroom casing soil, 9 isolates (A01, A02, A07, A010, A017, NOV2, NOV3, NOV4 and NOV5) demonstrated allelopathic effects on seed germination and early shoot growth of the weed species *Abutilon theophrasti*. Based on morphological and biochemical characteristics, the isolates were identified as belonging to the genus *Streptomyces* of the family *Streptomycetaceae*. The isolates A010 and NOV2 demonstrated the best inhibitory potential (100%) for all measured parameters. In other treatments, inhibition of seed germination ranged from 59% to 72%, while shoot growth (i.e. radical and shoot length) decreased from 50% to 87%. The potential of actinobacteria for producing bioherbicide metabolites makes them a good candidate for biological weed control.

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Alelopatski efekti aktinobakterija na klijanje semena i rani porast klijanaca Teofrastove lipice (*Abutilon theophrasti* Medik.)

REZIME

Cilj ovog rada je bio da se ispitaju alelopatski efekti aktinobakterijskih izolata u *in vitro* uslovima na klijavost semena i rani porast klijanaca semena *Abutilon theophrasti*. Iz zemlje i komposta u različitim fazama kompostiranja dobijeno je 35 izolata aktinobakterija. Takođe, u ogled je uključen i tretman herbicidom (mezotrion) u preporučenoj količini primene, kao referentni standard. Na osnovu dobijenih rezultata može se utvrditi da postoji statistički značajna razlika ($p<0,05$) između kontrole (neinokulisana tečna skrobno-kazeinska podloga) i svih tretmana sa aktinobakterijskim izolatima na klijavost semena, dužinu korenka i stabaoceta. Najveća inhibicija (100%) klijanja semena i dužine korenka zabeležena je u tretmanima sa izolatima A10 i NOV2. Pokazalo se da je dužina stabaoceta osetljiviji parametar, jer je zabeležena inhibicija od 100% u većini tretmana A010, NOV2, NOV3, NOV4 i NOV5. Izolati aktinobakterija su pokazali veći inhibitorni efekat na klijanje semena u odnosu na tretman herbicidom mezotrionom.

Ključne reči: aktinobakterije, Teofrastova lipica, klijanje semena, alelopatija

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Fusarium species associated with wheat head blight disease in Algeria: characterization and effects of triazole fungicides

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SUMMARY

Fusarium head blight is an important disease of durum wheat which requires several fungicide treatments of seeds to achieve satisfactory control. The current study was carried out to evaluate commercially available fungicides *in vitro* for their efficacy against eighteen *Fusarium* spp. isolates collected from different fields in the north-eastern part of Algeria. The morphological and molecular characterization reveals the presence in wheat seeds of the main species complexes *F. acuminatum, F. equiseti, F. avenaceum, F. solani, F. culomorum, F. incarnatum-equiseti,* as well as *F. tricinctum* species complex and *F. chlamydosporum* species complex. Antifungal activity of fungicides shows that all triazoles tested have proven their effectiveness in inhibiting the mycelial growth of various strains of *Fusarium* tested. However, their sensitivity varies between them significantly ($p<0.05$) depending on the dose applied and period of exposure to each fungicide. The results showed that tebuconazole (Raxil and Tébuzole) and the combination fludioxonil + difenoconazole greatly reduced the mycelial growth of *Fusarium* isolates by 84.31%, 82.94%, 81.33%, respectively, as compared to difenoconazole alone (73.16%) at the recommended dose after five days of exposure. Regarding their effect on conidia germination, tebuconazole was more effective than fludioxonil + difenoconazole, which leads to deformation of cell wall structure and fragmentation of conidia. These results will provide useful information to select suitable fungicides for seed treatment and management of wheat head blight disease.

Keywords: wheat, *Fusarium*, fungicides, toxicity

INTRODUCTION

Wheat is one of the major cereal crops produced worldwide with an output of 776.7 million tons over 2021-2022 (FAO 2022). Durum wheat (*Triticum durum*) takes a strategic place in the food system and national economy of Algeria with a production of 2.5 million tons in 2021 (FAO, 2022). Several abiotic and biotic stressors may reduce this production. Among them, Fusarium head blight (FHB) is one of the most economically destructive diseases affecting cereal production worldwide (Goswami & Kistler, 2004; Wegulo et al., 2015). Infected grains become shrivelled and discoloured (white and/or pink), and premature bleaching and death of spikelets or entire heads may occur (Petronaitis et al., 2021).

Generally, up to 19 species in the genus *Fusarium* have been reported as causing FHB disease of wheat (Liddell, 2003), constituting a complex of toxigenic pathogens belonging to the genus *Fusarium* (teleomorph = *Gibberella*) and the non-toxigenic genus *Microdochium* (Nielsen et al., 2011). Among different species causing FHB, *F. graminearum* is regarded as the most common causal agent worldwide because of its extensive occurrence and aggressiveness (Goswami & Kistler, 2004; Kazan et al., 2012). However, other causal agents are less commonly reported, such as *F. poae, F. cerealis* and *F. equiseti*, and to a lesser degree *F. oxysporum, F. verticillioides* and *F. solani* (Bottalico & Perrone, 2002). Additionally, different regions may have different dominant FHB-causing species. For example, in Canada, *F. avenaceum* was the main causal agent of FHB in durum wheat (Tittlemeier et al., 2013), while *F. asiaticum* is the main FHB pathogen present in Asia (Zhang et al., 2012; Ueda et al., 2007). In Algeria, the FHB species *F. culmorum* was the most frequent and aggressive species on wheat seedlings (Abdallah-Nekache et al., 2019). The various FHB causal agents affect grain quality by accumulation of various mycotoxins, which cause health risks to both humans and animals. Aside from the health risk posed by mycotoxins, FHB has a double negative effect on returns to the producer through yield loss and reduced price for diseased commodity, reaching 52% of durum wheat yield losses in Australia, 50% in USA, 46% in Iran and 44% in Tunisia (Petronaitis et al., 2021). In recent decades, market discounts in the USA extend from USD 1.84 to 3.67 per tonne per 0.5 ppm of DON in grain (Dahl & Wilson, 2018).

According to new strategies of integrated pest management (IPM), many agronomic, genetic, biological tools, as well as agricultural practices, are now available to protect or restrict fungal diseases and related mycotoxin accumulation. The most effective control methods to minimize FHB impact are fungicide treatments (Malbrán et al., 2020), while anthesis applications can also be efficient (Rojas et al., 2020), and the use of resistant cultivars (Willyerd et al., 2012). Currently, chemical control of fungal pathogens can be achieved by several fungicides with different target sites, which are distinguished by their mode of action. The most recent target site fungicides are succinate dehydrogenase inhibitors (SDHIs), as well as the well-known phenylpyrroles (PP fungicides) that affect the fungal osmotic signal transduction cascade. There are also pathogen osmoregulators (fludioxonil is the best known compound), benzimidazole carbamates and demethylation inhibitors (DMI) which affect sterol biosynthesis in membranes (Masiello et al., 2019).

Nowadays, triazoles are the most important fungicides applied in FHB control in the main wheat producing countries (Becher et al., 2011), likewise in Algeria. FHB is best monitored with triazole fungicides (Nakajima, 2010; Paul et al., 2010; Paul et al., 2008) which inhibit the cytochrome P450 sterol 14α-demethylase (CYP51), an enzyme required for ergosterol biosynthesis, causing fungal membrane structure to be disrupted (Ma & Michailides, 2005). Among triazoles, metconazole and tebuconazole are widely employed active substances to suppress FHB symptoms (Kotowicz et al., 2014), while difenoconazole, as well as other DMI fungicides, have strong activity in controlling plant pathogenic fungi, including *Fusarium* species (Suty-Heinze & Dutzmann, 2004). The increasing use of triazole fungicides for FHB control has led to an emergence of resistant fungal pathogens, which have been recorded in populations of many major phytopathogenic fungi, including *Botrytis cinerea* (Stehmann & De Waard, 1996), *Venturia inaequalis* (Köller et al. 1997), *Blumeria graminis f. sp. tritici* (Godet & Limpert, 1998), *Mycosphaerella graminicola* (Mavroeidi & Shaw, 2005), *Colletotrichum cereale* (Wong & Midland, 2007), and *F. graminearum* (Yin et al., 2009). Studies associate decrease in DMI sensitivity to mutations in and over expression of the cyp51 gene (Leroux et al., 2007; Yin et al., 2009). Hence, determining the pathogenic population sensitivity to the most commonly used fungicides in disease control is an initial phase in developing an anti-resistant strategy (Lu et al., 2012). For this reason, the present study aimed to evaluate *in vitro* the sensitivity of *Fusarium* species occurring on durum wheat to four commercial products containing difenoconazole, fludioxonil and tebuconazole, currently used for wheat seed treatment in Algeria. The efficacy of fungicides at different doses and over different exposure periods on *Fusarium* species was tested *in vitro* in solid medium to evaluate the inhibition of mycelial growth, and in liquid medium to examine their effects on spore germination.

MATERIAL AND METHODS

Sample collection and fungal isolation

After the harvest season 2017-2018, the CNCC (National Center for Seeds and Plants Certification and Control) of Setif state supplied sixty durum wheat samples (diseased seeds and ears) from six varieties, namely Bousselam, Mohamed Ben Bachir, GTAdur, Cirta, Waha and Vitron, collected from districts in the north-eastern parts of Algeria, including: Setif, Bordj Bou Arreridj (BBA), M'sila, Batna, Khenchela, Biskra and Mila. The samples were stored in paper bags at a temperature of 4°C until further use.

The pathogens were isolated from durum wheat seeds using a method developed by the National Plant Protection Laboratory, France (LSV, 2008). The seeds were superficially disinfected by soaking in 1.5% sodium hypochlorite solution for 10 min and then thoroughly rinsed with sterile distilled water. After that, they were dried with sterile filter paper for 20 to 30 minutes under aseptic conditions. Surface disinfected seeds were plated on Potato Sucrose Agar (PSA) medium, seven to eight seeds per plate, and incubated at 25°C for 5-7 days. Different types of fungal colonies were observed on the PSA medium, but only typical colonies and conidia with *Fusarium* traits were selected, purified (using the single-spore technique) and then submitted to morphological features examination.

Morphological identification of *Fusarium* isolates

Preliminary identification of *Fusarium* spp. was carried out according to Leslie and Summerell (2006). The critical characteristics that were assessed included macroscopic traits on the PSA (growth rates, presence of aerial mycelium, colony appearance and texture, pigmentation on both top and reverse plates) and microscopic traits on Carnation Leaf Agar (CLA). The evaluation of microscopic criteria was done using a method suggested by the National Plant Protection Laboratory, France (LSV, 2008). Plates with *Fusarium* spp. cultures on CLA were first placed under a stereo microscope to observe sporodochia (disposition, color, abundance), and then two samplings were systematically carried out for observation in a drop of dye (lactophenol cotton blue). The first sampling consisted of collecting aerial mycelium, and noting the characteristics of microconidia (shapes size, abundance, conidiophore appearance, conidiogenesis). The second sampling consisted of collecting sporodochia for observation of macroconidia produced in sporodochium (shapes size, abundance). The presence and appearance of chlamydospores was studied from samples taken from the aerial mycelium of cultures on PSA.

Molecular identification of *Fusarium* isolates

Genomic DNA was extracted using E.Z.N.A. Fungal DNA Mini Kit (OMEGA, Bio-tek) following the manufacturer's instructions. For molecular identification at the genus level, amplification of the Internal Transcribed Spacers of ribosomal DNA (rDNA-ITS) region was done using the primers ITS1/ITS4 (5'-TCCGTAGGTGAACCTGCGG-3'/5'TCCTCCGCTTATTGATATGC-3') (White et al., 1990), while identification at the specie level was done by amplification of the Transcription Elongation Factor 1 alpha (TEF-1α) gene using the primers EF1/EF2 (5'-ATGGGTAAG GAG GACAAG AC-3'/5'-GGAAGTACCAGT GAT CAT GTT-3') (O'Donnell et al., 2000; O'Donnell et al., 2004; Proctor et al., 2009). PCR amplification was performed using the KAPA3G Plant PCR Kit (Kapa Biosystems, Boston, USA). PCR assay was carried out in the thermal cycler (T100™ Thermal Cycler; Bio-Rad, Irvine, CA) and PCRs were conducted in 25µl volume reactions containing 1x buffer, 2.0 mM$MgCl_2$, 0.2 mM each dNTPs, 0.3µM of each primer, 1U of HOT FIREPol® DNA Polymerase (Solis Biodyne) and 1µl of fungal suspension from 5 to 7-day-old subcultures in PDA as a template. A non-template negative control was included in each amplification reaction. The thermal cycling parameters for ITS and TEF-1α locus were as follows: initial denaturation (95°C, 15 min), denaturation (95°C, 20 sec), annealing (for ITS: 50°C, 15 sec and for TEF-1α: 53°C, 15 sec), extension (72°C, 1 min) and final extension (72°C, 1 min) for 40 cycles. The PCR products were separated by electrophoresis on 1.5% agarose gels and were visualized by ethidium bromide staining and UV light. Using the MoBio UltraClean® PCR cleanup kit (Carlsbad, CA, USA), positive PCR products were purified according to the manufacturer's instructions and sent to the BIOfidal laboratory (CEDEX-France) for forward sequencing, following the company protocol. Subsequently, sequences were compared with those in the public databases (GenBank for rDNA-ITS for non-*Fusarium* species and FUSARIUM MLST for rDNA-ITS and TEF-1α for *Fusarium* species) and only similarity levels ≥ 99% were retained for identification of *Fusarium* isolates.

Fungicides used in *in vitro* assay

Four fungicides, registered for seed coating of cereals and belonging to DMIs: difenoconazole (Dividend 30g/l) and tebuconazole (Raxil 060 FS, Tébuzole 60 g/l FS)

grouped as triazoles, and a mixture of fludioxonil (belongs to PPs) + difenoconazole (Celest Extra 25g/l + 25g/l), were tested in this study. Based on the label dose recommended by the manufacturers, we tested, for each fungicide, the manufacturers' recommended dose (D) and two lower dilutions, half (0.5D) and decimal (0.1D) as reported in Table 1. Stock solutions were prepared to obtain specific concentrations of the active ingredient.

Effect of fungicides on mycelial growth of *Fusarium* isolates

The purpose of this experiment was to determine the efficacy of four fungicides and the behaviour of eighteen *Fusarium* strains based on mycelial growth *in vitro*, using the poisoned food technique (Nene & Thapliyal, 1993) and Potato Sucrose Agar (PSA) as a basic culture media. Based on the active ingredient, appropriate amounts of each fungicide were determined and aseptically added to the sterilized and cooled (50°C) PSA medium to obtain required concentrations in conical flasks separately, which were thoroughly shaken before being poured into 8.5 cm sterile Petri dishes. Three plates per treatment and per replication were maintained for each fungicide and its target concentration, and PSA Petri dishes without fungicide were used as controls.

The prepared dishes were aseptically inoculated with 5 mm diameter fungal plugs taken from the border of one week old culture and incubated at 25°C for 15 days. The results were recorded on the 5th, 10th and 15th day of incubation by measuring the average diameter (in mm) of fungal colonies from two perpendicular diameters. The mycelial growth inhibition (MGI, %) was determined using the following formula (Askarne et al., 2012):

$$MGI\ (\%) = \frac{Dc - Dt}{Dc} \times 100$$

where Dc is the diameter of colony in control, and Dt is the diameter of colony in treatment.

Effects of fungicides on conidia germination of *Fusarium* isolates

In order to achieve a concentration of spores equal to 1×10^5 conidia ml^{-1}, necessary for testing the effects of fungicides on the germination of spores, several culture media were used. For strongly sporulating strains, we used PSA and Spezieller Nährstoffärmer Agar (SNA), and CLA for less sporulating ones (Leslie & Summerell, 2006). However, for weakly sporulating strains, we used Pine Needle Medium (Su et al., 2012) for 10 days at 25°C.

Conidia were then obtained by scrubbing each colony surface with 10 ml of sterile distilled water containing 0.1% (v/v) tween 20 (for better conidia separation) and then filtering the suspension through two layers of sterile muslin to remove hyphal fragments. The resulting conidia in suspension were counted in Malassez cells and adjusted to 1×10^5 conidia ml^{-1}. In order to evaluate the effect of the fungicides on conidia germination, a modified method of Li et al. (2022) was applied, where solutions of three fungicides (fludioxonil + difenoconazole, and tebuconazole: Raxil and Tébuzole) at their recommended and half doses were prepared in Potato Dextrose Broth (PDB). For each concentration, a fungicide aliquot (75 µl) was mixed with 75 µl of conidia suspension (~1×10^5 conidia ml^{-1}) in a 96-well plate, in triplicate. Controls were performed with 75 µl of sterile PDB and 75µl of the conidia suspension. The prepared plates were incubated at 25°C for 18 h and then observed with an optical microscope at ×10 magnification (B-290 Series, Optika). Germination and conidia anomalies (especially in macroconidia) were evaluated in nine replicates (three wells per treatment and three microscopic fields per well). Conidia were counted as germinated when the germ tube length was equal to or longer than the spore diameter (Klosowski et al., 2018). Conidia germination inhibition (CGI, %) was calculated using the following formula :

$$CGI\ (\%) = \frac{Nt - Ng}{Nt} \times 100$$

where Nt and Ng are the total number of conidia examined and total number of germinated conidia, respectively.

Table 1. Fungicides tested in colony growth and conidial germination assays with *Fusarium* spp.

Fungicides		Doses of a.i. tested (mg/l^{-1})		
Active ingredients	Trade names	D	0.5D	0.1D
Difenoconazole	Dividend 30 FS	60	30	6
Fludioxonil + Difenoconazole	Celest Extra 25 + 25g/l	50+50	25+25	5+5
Tebuconazole	Raxil 60 g/l FS	30	15	3
Tebuconazole	Tébuzole 60 g/l FS	30	15	3

a.i.: active ingredient

Statistical analysis

In order to further compare the effectiveness of fungicides included in the study, mycelial growth inhibition and conidia germination inhibition of *Fusarium* species were analysed for each fungicide and concentration using the analysis of variance (ANOVA). Means were separated using Tukey's New Multiple Range Test B (P=0.05). The SPSS 25 software (IBM, 2017) was used for all data analysis.

RESULTS

Pathogen isolation and identification

Based on morphological characteristics of the fungal isolates (Leslie & Summerell, 2006) obtained from diseased seeds and ears, eighteen fungal isolates belonging to the *Fusarium* genus, disseminated in various proportions throughout the study areas, were revealed. Most of the fungal isolates were present in Mila District, 33.33%, followed by BBA, Batna and M'sila Districts, 22.22%, 16.66%, 11.11%, respectively. The lowest percentage, 5.55%, was in Setif, Khenchla and Biskra Districts.

Molecular identification was based on their rDNA-ITS region and the TEF-1α gene of all fungal strains isolated in this study, and they were successfully amplified with primers ITS1-ITS4/ EF1-EF2, which resulted in amplicons of 500 bp (Figure 1a) and 700 bp (Figure 1b), respectively.

This analysis showed that the isolated strains belonged to five species, namely *F. avenaceum* (FusBi7, FusBi21), *F. acuminatum* (FusBi15, FusBi23, FusBo11.5, FusBo6.12, FusBo33), *F. culmorum* (FusBo50, FusBo59), *F. equiseti* (FusBo25, FusBo28, FusBo49) and *F. solani* (FusBo35), and three complexes, including *F. incarnatum-equiseti* (FusBi8, FusBi1, FusBi2), *F. tricinctum* (FusBi6) and *F. chlamydosporum* (FusBo26) based on the sequences of rDNA-ITS region and TEF-1α gene for each of them, which were ≥99% similar reference sequences.

Effect of fungicides on mycelial growth of *Fusarium* isolates

The analysis of variance shows a very highly significant fungicidal effect at 5% threshold on the mycelial growth of *Fusarium* strains studied as a function of doses applied and periods of fungicide exposure (Table 2). This shows a highly variable behaviour between the *Fusarium* isolates included in this study with respect to the fungicides tested.

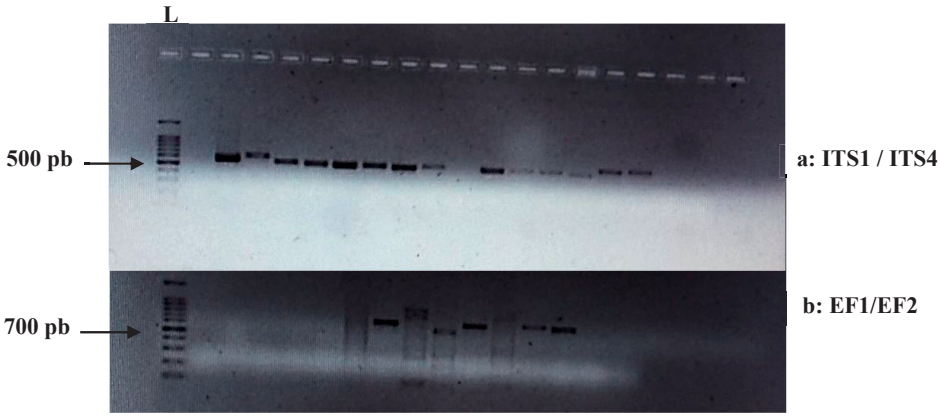

Figure 1. Electrophoresis products of amplified DNA of isolates of *Fusarium* spp. **a:** Amplification with universal primers ITS1/ ITS4, **b:** Amplification with universal primers EF1/EF2, **L:** Ladder.

Table 2. Variance analysis of fungicide effects depending on *Fusarium* isolates, doses and exposure periods

Source of variation	Sum of squares	Df	Medium square	F	Signification
Fusarium isolates	296716.79	17	17453.93	251.88	0.000
Fungicides	88184.37	3	29394.79	424.20	0.000
Dose of fungicides	232544.28	2	116272.14	1677.95	0.000
Periods of exposure	5226.68	2	2613.34	37.71	0.000
Total	10762039.80	1944			
a. R-square = 0.919 (Adjusted R-square = 0.878)					

Efficiency of fungicides against *Fusarium* isolates

The effects of different concentrations on mycelial growth of *Fusarium* isolates were studied, and the results of three doses used: namely the recommended dose, half the recommended dose and one tenth of the recommended dose, on inhibition of mycelial growth revealed a significant difference at 5% threshold and correlated positively with the dose and exposure period to fungicides.

From the results obtained, we noted that the recommended dose of all fungicides was the most effective and reached its maximum after only 5 days of exposure of *Fusarium* strains to the fungicides, while a slight difference was observed between 5 days and 10 days of exposure (Table 3).

By reducing the recommended dose by half, a slight difference in efficacy was observed. On the other hand, when the doses were divided by ten, the differences were quite noticeable. Thus, with difenoconazole (Dividend), we had a reduction in efficiency of around 48.18%, followed by tebuconazole, Raxil and Tebuzole, with 26.80% and 26.06%, respectively, and it was only equal to 19.21% with fludioxonil + difenoconazole (Celest Extra).

It was also noted that the active ingredient tebuconazole, represented by the generic tebuconazole product Tébuzole and the innovative tebuconazole product Raxil, achieved effectiveness which was very close; we recorded inhibition rates of 82.94% and 84.31% by the recommended dose, respectively. Tukey's test B confirmed that they belong to the same group, proving that the generic product can have the same level of effectiveness as the innovative product.

The lowest average inhibition of mycelial growth was recorded with the FusBi11.5 strain, 65.03%, which seems moderately resistant to the action of the fungicides tested. In contrast, the highest effectiveness of 93.28% was obtained with the FusBo28 strain after only 5 days of exposure to fungicides (Table 3). This isolate (FusBo28) was the most sensitive to all fungicides used, tebuconazole (Raxil and Tébuzole), difenoconazole, and fludioxonil + difenoconazole. On the other hand, FusBi6 was the most resistant strain to difenoconazole and to fludioxonil + difenoconazole with inhibition rates of 29.66% and 50.68%, respectively. FusBi7 was the most resistant strain to tebuconazole (Tébuzole) with an inhibition rate of 62.00%, while the most resistant strain to tebuconazole (Raxil) was FusBo11.5 with only 59.66%. Thus, a great variability was observed between *Fusarium* strains and it materialized by the formation of 15 groups through the statistical Tukey's B post hoc test.

Table 3: Mean effects of fungicides on mycelial growth of *Fusarium* isolates depending on doses tested

Fusarium isolates	Recommended dose (D)				Half recommended dose (O,5D)				Tenth of recommended dose (0.1D)			
	Celest Extra	Dividend	Raxil	Tébuzole	Celest Extra	Dividend	Raxil	Tébuzole	Celest Extra	Dividend	Raxil	Tébuzole
FusBi1	86.25±1.77	85.33±1.02	59.80±8.20	87.24±1.16	73.70±1.77	83.23±1.53	58.42±7.94	82.23±1.64	81.95±3.46	81.11±2.05	75.18±5.95	78.96±4.07
FusBi11.5	58.60±2.63	76.94±0.83	65.20±7.99	64.91±2.02	81.00±2.47	72.87±3.71	65.50±7.86	63.99±2.79	75.85±5.28	28.84±6.21	60.39±9.18	52.82±4.27
FusBi15	68.86±1.07	34.94±1.53	73.61±4.95	82.47±1.52	76.99±1.37	36.58±1.98	70.45±5.21	50.44±3.20	22.24±2.64	61.88±3.69	68.78±7.94	38.70±4.73
FusBi2	86.24±2.98	70.70±3.19	72.40±4.74	83.01±1.09	84.41±3.82	43.36±5.95	69.91±7.02	74.40±2.14	76.47±6.62	26.86±9.24	68.05±11.14	85.16±2.89
FusBi21	84.00±4.48	71.04±7.24	64.01±9.32	94.12±0.00	63.37±5.30	39.54±7.31	62.47±9.69	73.11±2.67	76.08±6.80	16.01±8.05	69.22±12.72	52.29±7.23
FusBi23	90.37±0.40	86.66±1.24	84.58±2.22	90.31±0.47	93.29±0.41	82.10±2.49	82.92±2.33	73.55±5.66	85.44±1.74	38.20±4.12	59.19±7.79	76.36±1.53
FusBi25	93.29±0.41	88.37±0.81	83.41±2.21	73.92±2.83	93.26±0.43	93.26±0.43	78.86±4.69	85.29±5.46	75.14±1.82	60.94±4.15	57.79±7.57	59.23±2.44
FusBi28	93.26±0.43	93.26±0.43	84.06±2.13	93.26±0.43	89.84±0.53	78.63±1.90	78.60±4.76	83.84±1.35	74.15±1.99	57.26±3.89	49.23±8.11	49.34±4.28
FusBi35	77.85±2.58	83.34±1.43	63.75±7.72	82.57±2.18	85.97±2.06	76.62±3.36	56.14±9.14	64.69±4.19	82.71±4.26	10.78±4.05	65.04±7.61	52.04±4.05
FusBi49	91.83±1.00	89.16±1.75	75.54±4.47	67.75±2.85	81.78±1.32	66.59±2.53	73.31±5.26	91.38±1.30	44.69±2.27	80.02±2.43	62.68±8.02	66.95±2.15
FusBi6	50.68±2.14	29.66±3.06	65.56±8.01	83.80±1.73	85.59±1.95	75.51±3.71	56.70±7.62	85.22±1.45	80.55±3.49	29.20±3.89	74.29±6.26	85.00±1.26
FusBi6.12	90.57±0.37	91.59±0.62	86.24±1.80	90.71±0.71	89.34±0.30	91.12±0.58	86.52±1.81	89.31±1.40	93.26±0.43	93.26±0.43	79.15±4.80	81.48±2.22
FusBi7	78.79±1.64	55.29±1.97	65.59±5.52	62.00±2.19	50.21±1.77	72.98±1.70	59.38±7.84	56.80±4.39	78.58±3.20	36.84±4.64	78.22±4.74	69.28±3.26
FusBi8	86.78±1.51	70.16±6.24	67.74±5.64	81.19±1.82	91.44±1.50	76.08±4.22	74.66±4.20	94.12±0.00	35.00±2.99	7.53±1.01	73.48±5.17	43.90±1.35
FusBo26	88.65±0.77	75.27±1.84	76.71±4.60	91.38±1.30	55.84±1.22	22.05±3.72	72.10±4.98	69.55±1.00	75.98±2.08	2.89±1.85	57.88±9.38	23.67±3.77
FusBo33	65.91±4.37	50.68±6.31	61.04±8.81	76.02±2.63	86.12±4.09	79.61±2.70	71.23±7.42	94.12±0.00	51.99±6.78	3.98±2.65	61.53±11.58	57.80±5.58
FusBo50	88.11±2.82	75.43±3.79	77.55±4.74	94.12±0.00	63.60±2.95	20.83±1.24	73.90±4.55	73.17±1.26	32.06±5.96	10.26±3.51	63.90±10.53	36.72±3.12
FusBo59	83.86±4.83	89.08±0.73	64.07±9.35	94.12±0.00	75.82±8.08	71.89±4.34	91.18±1.39	81.64±2.36	69.61±9.26	36.54±9.26	94.12±0.00	94.12±0.00
Mean	81.33±2.01	73.16±2.45	84.31±1.25	82.94±1.39	78.98±2.30	65.71±2.97	81.14±1.69	77.05±2.35	67.32±3.95	37.91±4.17	61.71±3.04	61.32±3.23

Results given in Mean±SE

Regarding the effect of exposure duration of *Fusarium* isolates to the fungicides tested, it appears that its extension did not in general increase their effectiveness through greater inhibition of mycelial growth. Overall, the rates of inhibition of mycelial growth induced by the four fungicides after 5, 10 and 15 days of exposure varied in a non-significant manner. By way of example, we obtained the average inhibition rates of 84.82±2.33% and 84.50±2.54% after 5 days, 82.97±2.67% and 82.72±3.06% after 10 days, and 82.98±2.95%, 81.86±3.26% after 15 days of exposure to Raxil and Tébuzole, respectively (Table 4).

The differences observed in Table 4 are due much more to the dose effect and to the behaviour of *Fusarium* isolates, and also to the depletion of nutrients in culture medium.

Effects of fungicides on spore germination of *Fusarium* isolates

The fungicides that showed greater efficiency in the mycelial growth test (fludioxonil + difenoconazole, tebuconazole: Tébuzole and Raxil) were also tested *in vitro* for their effect on conidial germination. The results showed a highly variable impact at 5% threshold between *Fusarium* isolates, fungicides and doses (Table 5).

The results of inhibition of conidia germination following treatment with fungicides revealed that tebuconazole (Tébuzole) was the most effective fungicide with 73.461±1.18%, followed by tebuconazole (Raxil) with 69.753±0.892%, even better than fludioxonil + difenoconazole, which only inhibited spore germination by 62.16±0.789% at the recommended dose (Table 5). In addition, we noticed that the half dose proved to be much less effective than the recommended dose, so that inhibition rate was reduced by more than half, particularly with fludioxonil + difenoconazole and tebuconazole (Raxil), giving only 27.558% and 33.582%, respectively. It is clear that the impact of fungicides on spore germination differs remarkably from their effect on mycelial growth in terms of efficacy and also in terms of ranking of the fungicides tested.

The results revealed that the fungicidal effect of Raxil is very limited on the FusBi1 strain by inhibiting only 3.060±0.197% of spore germination, while FusBo33 was particularly resistant to fludioxonil + difenoconazole and tebuconazole (Raxil), germinating only up to 14.443± 3.408% (Table 5). However, the effect was very pronounced on other strains, such as FusBo26 and FusBi15 with 96.863±0.256% and 96.010±0.173% inhibition rates recorded with fludioxonil + difenoconazole and tebuconazole (Tébuzole), respectively. But it is even more pronounced with the F usBi15 strain, which achieved

Table 4: Mean effects of fungicides on mycelial growth of *Fusarium* isolates depending on exposure period

Fusarium isolates	5 days exposure (P1)				10 days exposure (P2)				15 days exposure (P3)			
	Celest Extra	Dividend	Raxil	Tébuzole	Celest Extra	Dividend	Raxil	Tébuzole	Celest Extra	Dividend	Raxil	Tébuzole
FusBi1	87.10±1.17	76.53±8.11	89.63±1.12	87.29±2.39	83.01±2.91	68.19±8.98	84.12±2.26	87.21±1.89	80.00±4.17	77.67±6.14	76.91±4.13	84.03±2.55
FusBi11.5	53.24±4.20	71.79±5.33	84.26±3.30	82.33±4.94	56.66±3.53	68.75±5.40	85.02±2.94	83.31±3.77	57.57±3.42	66.79±5.20	83.17±2.11	75.19±4.25
FusBi15	57.32±3.09	62.50±4.70	83.28±2.11	75.83±2.90	58.15±3.62	67.10±4.95	84.20±2.96	80.51±4.37	57.77±4.04	71.15±5.96	84.22±3.31	83.51±4.35
FusBi2	88.60±1.30	85.05±2.28	90.21±1.71	90.59±0.88	87.11±1.18	76.48±8.10	89.18±1.25	87.80±2.50	72.73±5.63	63.45±7.67	77.66±4.32	75.24±4.38
FusBi21	85.20±2.37	70.06±9.77	89.18±1.25	86.94±2.48	62.77±4.67	60.45±6.10	77.61±2.86	76.35±3.48	61.47±4.58	82.98±2.05	91.17±1.02	89.84±1.23
FusBi23	88.25±1.27	81.03±6.42	88.75±1.85	89.06±2.20	81.97±2.97	73.69±8.20	83.50±2.17	86.88±2.10	84.32±2.66	78.81±5.45	82.19±1.73	86.09±2.37
FusBi25	84.24±2.64	83.25±1.69	80.32±0.95	85.56±2.41	81.95±4.27	76.60±6.61	77.34±4.23	79.45±4.07	63.40±6.11	63.38±6.34	78.95±3.40	75.56±3.85
FusBi28	88.71±1.33	86.10±2.05	89.55±1.75	90.82±0.95	91.78±0.70	87.39±2.22	90.52±1.88	92.17±1.02	90.87±0.89	86.21±2.08	90.52±1.88	91.89±1.00
FusBi35	58.62±3.67	60.02±6.07	79.24±3.14	75.19±2.89	57.52±3.39	63.19±4.57	83.29±2.76	78.68±4.03	56.44±4.35	74.92±5.41	82.57±3.26	84.30±4.70
FusBi49	81.95±4.27	74.84±6.53	78.13±4.40	78.47±3.74	84.21±2.76	78.14±6.22	79.35±3.23	84.66±2.70	74.21±5.93	70.14±6.44	81.50±3.77	77.47±4.79
FusBi6	60.23±4.80	61.30±6.41	78.50±3.26	74.71±3.68	57.96±3.30	65.75±5.63	83.41±2.03	77.51±3.54	56.34±3.82	63.91±4.86	82.25±2.48	75.55±4.34
FusBi6.12	88.45±1.26	79.33±6.22	89.84±1.65	89.06±2.20	85.66±2.29	64.16±10.02	86.95±2.08	85.02±2.39	71.74±5.83	62.23±6.51	79.31±3.48	73.45±3.96
FusBi7	84.47±2.60	84.51±1.81	79.35±3.23	85.57±2.90	85.09±2.35	64.05±10.01	89.64±1.37	86.61±2.45	82.45±3.16	73.04±8.04	83.18±2.17	86.88±2.10
FusBi8	83.62±2.62	70.24±9.49	85.83±2.12	87.44±1.97	78.26±5.35	72.67±6.77	79.03±4.67	76.46±4.34	67.53±5.60	64.62±6.90	79.34±3.48	76.01±3.64
FusBo26	84.15±2.76	69.80±9.36	85.43±2.25	87.21±1.89	86.42±2.33	63.94±9.98	88.65±1.69	85.77±2.51	74.24±5.94	73.10±6.82	79.41±4.67	75.16±4.32
FusBo33	72.01±5.72	66.89±6.50	76.81±4.33	74.47±4.21	62.20±4.80	62.81±6.49	77.05±2.71	75.12±3.47	55.02±4.94	76.50±4.63	86.17±3.44	88.81±1.22
FusBo50	90.34±0.80	86.13±2.06	90.06±1.83	91.43±0.97	83.81±2.53	83.68±1.88	77.63±2.80	84.70±2.74	71.18±0.00	86.54±0.03	90.29±1.25	90.88±1.53
FusBo59	87.82±1.29	80.94±6.39	88.36±1.74	89.06±2.20	81.75±4.29	75.75±6.39	76.95±4.14	80.73±3.63	56.66±3.81	69.92±5.64	84.93±3.14	83.54±4.03
Mean	79.13±2.62	75.02±5.62	84.82±2.33	84.50±2.54	75.91±3.18	70.71±6.58	82.97±2.67	82.72±3.06	68.55±4.16	72.52±5.34	82.98±2.95	81.86±3.26

Results given in Mean±SE

100% germination in the presence of tebuconazole (Raxil). In the case of the FusBo33 strain, the results of fungicide effects on spore germination, unlike the mycelial growth test, should be taken with great caution because of its low sporulation; despite testing several culture media that promote sporulation, we were unable to achieve the required concentration of 10^5 sp/ml.

The microscopic examination of samples taken from spore suspensions of different *Fusarium* strains amended with fungicides revealed changes at the structural level compared to those that were not treated with fungicides (Figure 2a). Thus, tebuconazole (Raxil) caused deformation (Figure 2b) and fragmentation (Figure 2c) of conidia, while fludioxonil + difenoconazole only altered conidia through fragmentation (Figure 2c). It is also important to note that the effect of fungicides was notable in inhibiting germ tube elongation in all strains.

Table 5: Average results of fungicide effects on spore germination of *Fusarium* isolates

Fusarium isolates	Fludioxonil + Difenoconazole		Tebuconazole (Tébuzole)		Tebuconazole (Raxil)	
	D	0.1D	D	0.1D	D	0.1D
FusBi1	20.030±0.296	13.423±0.377	40.883±6.135	6.907±0.395	3.060±0.197	0.000±0.000
FusBi11.5	25.707±0.083	15.663±0.055	93.437±0.353	86.140±0.598	71.357±0.930	25.460±0.501
FusBi15	100.00±0.000	79.767±0.319	100.00±0.000	96.863±0.388	100.00±0.000	85.023±0.481
FusBi2	27.830±0.514	0.000±0.000	86.937±1.104	88.677±0.333	85.627±0.468	0.000±0.000
FusBi21	54.383±2.420	1.233±0.291	100.00±0.000	98.147±0.437	84.287±0.563	25.993±1.442
FusBi23	96.723±0.435	67.750±1.432	100.00±0.000	98.167±0.218	100.00±0.000	86.907±0.619
FusBi25	29.647±0.229	22.510±0.797	32.410±1.170	41.817±2.160	48.863±3.924	21.430±0.785
FusBi28	84.820±0.765	0.950±0.137	74.917±1.468	67.637±1.050	80.960±0.626	8.800±0.478
FusBi35	81.033±0.112	10.787±0.166	54.850±0.430	29.380±0.993	42.193±0.578	8.967±0.357
FusBi49	89.153±1.460	23.027±1.320	53.787±2.670	11.573±0.109	75.460±2.162	7.563±0.114
FusBi6	96.977±0.535	31.020±0.806	100.00±0.000	100.00±0.000	98.890±0.262	75.427±0.500
FusBi6.12	59.160±0.374	45.157±0.215	76.343±1.197	73.400±0.759	76.663±0.425	44.560±0.246
FusBi7	97.187±0.366	14.617±0.432	93.027±0.111	99.470±0.125	97.090±0.172	62.900±0.294
FusBi8	24.247±1.311	12.917±0.528	89.303±0.840	89.303±0.840	58.153±1.529	18.220±1.188
FusBo26	96.863±0.265	97.870±0.248	89.073±0.489	91.703±0.245	97.907±0.143	94.550±0.431
FusBo33	14.443±3.408	13.130±3.098	14.443±3.408	13.130±3.098	14.443±3.408	13.130±3.098
FusBo50	90.620±0.330	18.660±0.292	96.010±0.173	91.667±0.447	95.843±0.324	7.807±0.431
FusBo59	30.060±1.314	27.570±0.276	26.880±1.694	24.583±1.997	24.773±0.362	17.753±0.192
Mean	62.16±0.789	27.558±0.599	73.461±1.18	67.142±0.788	69.753±0.892	33.582±0.619

Results given in Mean±SE

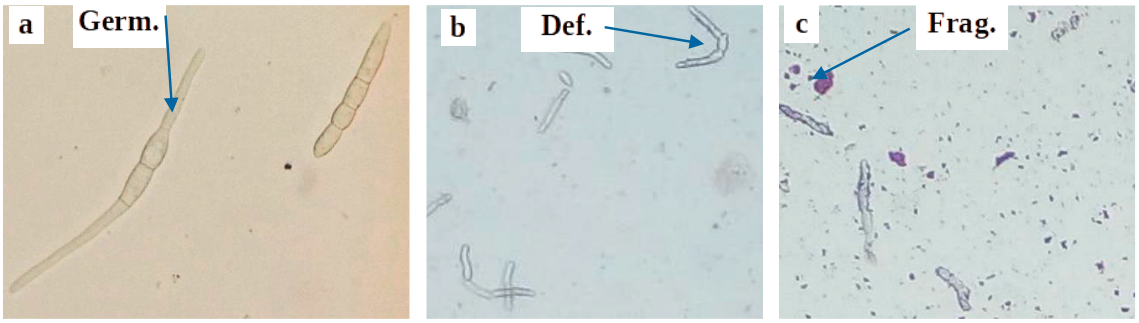

Figure 2. Effects of fungicides on the morphology of *F. avenaceum* conidia (FusBi7). Fungicides were mixed with conidia suspension at 25°C for 18 h and morphological differences were observed under optical microscope at ×10 magnification. (**a**) Conidia free of fungicide treatment germinated normally (Germ.). (**b**) Deformation (Def.) and distortion (**c**) of conidia caused by tebuconazole (Raxil); fragmentation (Frag.) of conidia caused by tebuconazole (Raxil) and fludioxonil + difenoconazole

DISCUSSION

The aims of this study were to identify the fungal species causing FHB of wheat and assess *in vitro* their sensitivity to the main fungicides currently used in several crops in Algeria. This provides critical information for disease control strategies. Identification of *Fusarium* isolates was performed with morphological and molecular techniques using PCR with primer sets. Some variation was found in the overall prevalence among species of the 18 *Fusarium* isolates collected in the study area. *F. acuminatum* was the most prevalent with a frequency of 27.7% of *Fusarium* isolates, followed by *F. incarnatum-equiseti* species complex and *F. equiseti* with a frequency of 16.6% each. The least commonly isolated species were *F. solani, F. tricinctum* species complex and *F. chlamydosporum* species complex with 5.5% frequency. *F. acuminatum* was the dominant species isolated from the head of wheat in north-eastern districts of Algeria which is quite equivalent to the results reported by Shikur et al. (2018) where it was the second most frequent species isolated from the crown of wheat in Turkey. However, *F. culmorum* was reported as the species most frequently isolated in other districts of northern Algeria (Abdallah-Nekache et al., 2019; Hadjout et al., 2022). But on the other hand, *F. graminearum* species complex was the predominant species isolated from heads of wheat in several other countries, including Iran (Sharifi et al., 2016) and Brazil (Pereira et al., 2021).

This study offers new data on the sensitivity of most important *Fusarium* species associated with FHB of wheat to *Fusarium*-controlling fungicides that are necessary to limit crop losses. Triazoles are the most frequently applied fungicides for managing FHB because they are more effective than other active ingredients (Mateo et al., 2011, 2013; Haidukowski et al., 2012; Hellin et al., 2018). However, little is known about the impact of sublethal doses of these fungicides on the emergence of fungal resistances (Hellin et al., 2018). In fact, declining tebuconazole sensitivity has been reported in Germany (Klix et al., 2007) and China (Yin et al., 2009) because of the extensive use of fungicidal DMIs over the last 30 years.

With regard to the obtained results of *in vitro* effects of fungicides, a significant effect of the tested commercial fungicides was recorded on radial mycelial growth of all strains of *Fusarium* along the concentration gradient. Compared to the untreated control, all fungicides reduced the growth rates of all *Fusarium* strains, and the growth rates decreased as fungicide concentrations increased. Three fungicides (fludioxonil + difenoconazole, tebuconazole: Tébuzole and Raxil) were highly effective against all head blight isolates at all concentrations. However, difenoconazole was a moderately effective fungicide. Generally, a positive correlation was observed between fungicide concentrations and inhibition of mycelial growth of *Fusarium* isolates. The inhibition rate reached its maximum after only five days of exposure, and stagnated at this level, while increase in exposure periods of *Fusarium* isolates to the fungicides tested did not influence mycelial growth inhibition.

In agreement with our results, the efficacy of fludioxonil in a mixture with difenoconazole against *F. solani* and *F. oxysporum* causing potato dry rot was demonstrated by Vatankhah et al. (2019). Fludioxonil action may be related to modification of the signal transduction pathways of *F. oxysporum*, which affects mycelial growth (Kim et al., 2007; Yang et al., 2011). A study conducted by Ochiai et al. (2002) also found that fludioxonil can disturb the CANIKI/COSI signal transduction pathway, which results in dysfunction of glycerol synthesis and inhibition of hyphae formation in *Candida albicans*. In contrast, difenoconazole alone was the least effective among the fungicides tested with only 58.93% inhibition rate. These results concur with those reported by Gxasheka et al. (2021), who found a slight decrease in mycelial growth of *F. graminearum* under the activity of higher concentrations of difenoconazole.

Decrease in mycelial growth due to tebuconazole, represented by the generic Tébuzole or the innovative product Raxil, was similar in our study to the results obtained by Bhimani et al. (2018), who found an 87% reduction in mycelial growth of *F. oxysporum* by tebuconazole at low concentrations. Gxasheka et al. (2021) studied the effects of fungicides on *Fusarium* species causing maize ear rot disease in China, and also found that tebuconazole reduced mycelial growth of *F. oxysporum* by 67% with its lowest concentration. This could be explained by inhibition of the cytochrome P450 sterol 14α-demethylase (CYP51), an enzyme required for ergosterol biosynthesis, causing fungal membrane structure to be disrupted, which inhibits fungal growth (Ma & Michailides, 2005).

As the fungicides used in this test had the same concentration of active molecules, the isolates and different species showed different sensitivities to the same fungicides, which is in agreement with other studies. For example, fludioxonil + difenoconazole had different efficacy against *F. solani* and *F. oxysporum* isolates (Vatankhah et al. 2019). Gxasheka et al. (2021) also found that the same concentration of tebuconazole and difenoconazole had different efficacy results against *F. graminearum* and *F. oxysporum* isolates. Differences in the effectiveness of the same fungicide in inhibiting

mycelial growth of different *Fusarium* species and strains could be due to genetic polymorphism (higher or lower sensitivity of a strain) (Falcão et al., 2011). According to Hellin et al. (2018), *F. culmorum* could adapt to triazole pressure by major transcriptome modifications in response to triazole fungicides, including overly expression of drug resistance transporter, and the same mechanism is expected to occur in other species. Fungicide efficacy is influenced by fungal species, strains, ecological factors, and interactions among these factors (Mateo et al., 2011).

In vitro efficiency of fungicides regarding conidial germination indicated a significant effect between the fungicides selected and *Fusarium* strains studied. Triazoles inhibit 14-α-demethylase from taking part in the synthesis of ergosterol, the most common sterol in fungal cell membranes (Ma & Michailides, 2005). According to Shcherbakova et al. (2020), triazole fungicides effectively prevent the growth of a wide range of plant pathogenic fungi. It is often assumed that they are unable to inhibit the germination of their spores with the same efficacy because fungal spores already contain ergosterol, which is consistent with the results we obtained for tebuconazole. However, fludioxonil in a mixture with difenoconazole showed a germination inhibition rate of 62.16%, contrary to the results obtained by Rosslenbroich and Stuebler (2000), who reported that fludioxonil inhibited spore germination, germ tube elongation, and mycelium growth of *Botrytis cinerea* by affecting the osmoregulatory signal transmission pathway of that fungus. Moreover, our data also showed that the active ingredient tebuconazole represented by the innovative product namely Raxil caused more fragmentation and conidial malformations of strains, such as FusBi7, FusBo59 and FusBo26, than fludioxonil + difenoconazole, which caused conidial fragmentation in the FusBi7 strain. Malformation of conidia can be explained by findings that ergosterol biosynthesis-inhibiting fungicides frequently cause hitting morphological malformations, and irregular thickening of the cell wall (Ramirez et al., 2004), which can sometimes progress to fragmentation of conidia. Another possible explanation for conidia fragmentation could be related to the additive chemical products that differ in innovated and generic products, which are added to fungicides to improve their activity. The results indicate that these fungicides also inhibited the germination of conidia through degradation of cell structures, and not only by inhibiting germ tube elongation. To our knowledge, this is the first time that conidial fragmentation caused by the tested fungicides has been reported. This new finding has major implications on the management of *Fusarium* head blight.

CONCLUSION

It was concluded that *in vitro* effects of fungicides have revealed a range of inhibitory activities against *Fusarium* isolates responsible for durum wheat head blight disease, including inhibition of mycelial growth, germination of spores, elongation of the germ tube and breakdown of cellular structures. Furthermore, none of the tested *Fusarium* strains showed resistance to triazoles applied under *in vitro* conditions. Given the importance and the need to control *Fusarium* wilt of durum wheat, *in vivo* experiments are necessary to validate these results. The information provided by this study may be useful for selecting the best active molecules against FHB and contribute to the evolution of an effective management strategy for this disease.

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Vrste roda *Fusarium* prouzrokovači fuzarioze klasa pšenice u Alžiru: karakterizacija i delovanje triazolnih fungicida

REZIME

Fuzarioza klasa pšenice je važna bolest durum pšenice koja zahteva primenu nekoliko tretmana semena fungicidom kako bi se na zadovoljavajući način suzbila bolest. Istraživanje je sprovedeno kako bi se u uslovima *in vitro* procenila efikasnost komercijalno dostupnih fungicida protiv 18 izolata *Fusarium* spp. koji su sakupljeni na različitim poljima u severo-istočnom delu Alžira. Morfološkom i molekularnom karakterizacijom, na semenu pšenice otkriveno je prisustvo glavnih kompleksa vrsta *F. acuminatum, F. equiseti, F. avenaceum, F. solani, F. culomorum, F. incarnatum-equiseti*, kao i kompleksi vrsta *F. tricinctum* i *F. chlamydosporum*. Antifungalno delovanje fungicida pokazuje da su svi testirani triazoli dokazali efektivnost u inhibiciji porasta micelija različitih testiranih sojeva roda *Fusarium*. Ipak, njihova osetljivost je značajno ($p<0.05$) varirala u zavisnosti od doze i dužine izlaganja pojedinačnim fungicidima. Rezultati su pokazali da su tebukonazol (Raxil i Tébuzole) i kombinacija fludioksonil + difenokonazol u velikoj meri smanjili porast micelija izolata iz roda *Fusarium,* i to respektivno 84.31%, 82.94% i 81.33%, u poredjenju sa samostalnom primenom difenokonazola (73.16%) u preporučenoj dozi nakon pet dana izlaganja. Što se tiče delovanja na klijanje konidija, tebukonazol je bio efikasniji od kombinacije fludioksonil + difenokonazol, koja je uzrokovala deformacije strukture ćelijskog zida i fragmentaciju konidija. Rezultati su dali korisne informacije kao osnovu za odabir pogodnih fungicida za semenski tretman i suzbijanje fuzarioze klasa kao bolesti pšenice.

Keywords: pšenica, *Fusarium*, fungicidi, toksičnost

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Original scientific paper

Oxidative stress response as biomarker of exposure of a freshwater invertebrate model organism (*Unio mancus* Lamarck, 1819) to antifouling copper pyrithione

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SUMMARY

Sublethal effects of copper pyrithione (CuPT) on an invertebrate model organism, freshwater mussel (*Unio mancus*), were assessed using oxidative stress parameters and histopathology. The environmentally relevant concentration of 5 µg/l copper pyrithione was applied as a 96 h semi-static exposure. MDA levels in digestive gland (DG) significantly increased, compared to a control group ($p < 0.001$). Gill MDA levels also increased, but not significantly ($p > 0.05$). GSH level in DG increased significantly ($p < 0.05$), and gill tissue levels also increased but not significantly ($p > 0.05$). Levels of SOD, GPx activities, and AOPP levels did not change significantly ($p > 0.05$). The most prominent histopathological alterations in the gills were haemocyte infiltration, lipofuscin aggregates and lamellar deformations with tubule degeneration and accumulated lipofuscin aggregates in DG tubules. CuPT impacts antioxidant defense systems even during sublethal exposure of *Unio mancus* as the freshwater model organism.

Keywords: biocides, antifouling copper pyrithione, biomarkers, oxidative stress, freshwater mussel

INTRODUCTION

Freshwater ecosystems are under pressure of continuous water quality and biodiversity decline, impacting global human and animal populations with health risks, augmented by agriculture, forestry and modern technical developments. Comprehensive biomonitoring programs using biomarkers for changes in biological responses enable early warning of human, animal and ecosystem health risks. They extend to risk assessment and ecotoxicology (Guidi et al., 2017).

Aquatic organisms accumulate on submerged external surfaces of marine structures, such as boat hulls, and cause biofouling. Extra cleaning and maintenance expenditure, higher fuel consumption, reduced manoeuvrability and increased frictional drag on boats are other detriments enhancing the impact. Furthermore, the affected surface may pose food safety risks (Muller-Karanassos et al., 2019; Nogueira et al., 2018). Marine biofouling not only damages boat surfaces, but also increases carbon dioxide emission while reducing boat performance. Antifouling paints (coatings) containing biocides are used to prevent this significant marine concern. (Martins et al., 2018). Tributyltin (TBT) and its derivatives were effective antifouling paints during the 1970s, with annual global production of around 4000 tons, providing protection for up to five years. TBTs have been considered advantageous due to their long-term protection effect (Arai et al., 2009). However, their long half-life caused accumulation in sediments and aquatic organisms, including non-target aquatic organisms even at low concentrations. Furthermore, biomagnification in the food web at increasing levels raise concern. Representative marine products at different trophic levels in Danish coastal waters were shown to accumulate in the food web. Levels were determined in flounder (60-259 ng g^{-1}, w/w), eider duck (12-202 ng g^{-1}, w/w), and harbour porpoise (134-2283 ng g^{-1}, w/w) (Strand & Jacobsen, 2005). Shell malformations were reported in larvae of *Crassostrea gigas* found in the Gulf of Arcachon, France (Arai et al., 2009). In 1990, tin-free antifouling paints were developed as alternatives. The IMO (International Maritime Organization) recommended that countries ban the use of TBT-based antifouling paints for vessels shorter than 25 m due to serious environmental impact. Some organisms exposed to TBT showed imposed imposex and the USA, Canada, Sweden and The Netherlands have imposed restrictions on TBT release from ship surface (Okay, 2004; Gittens et al., 2013).

Alternative antifouling compounds, such as diuron, irgarol, zinc pyrithione (ZnPT, presently used also in antidandruff shampoos as antimicrobial) and copper pyrithione (CuPT) are proposed chemicals developed for use as "booster" biocides in Cu-based antifouling paints. Pyrithione salts, ZnPT and CuPT, were commercialised together during the 1990s. Environmental pollution by direct leaching from hull and paint particle discharges during maintenance and cleaning has raised concern since both biocides are usually present as mixtures in the marine environment. Some had the potential to cause harmful effects on many non-target organisms, algal growth and photosynthesis (Dupraz et al., 2018), energy production, biological response to genomic activities, such as stress responses, genotoxic damage, immune-supressed protein expression, oxidation and nerve conduction (Qian et al., 2013). Their apolar properties and high degradability have made them choice biocides as replacements for TBT (Martins et al., 2018). Copper pyrithione, registered for antifouling use, was approved by the European Union in 2015 as an active ingredient in anti-degradation products, as part of its program for evaluation of active substances in biocidal products.

Copper pyrithione, a Cu(I) used as the main biocide in antifouling paints, is a highly effective broad-spectrum biocide, but it also has adverse effects on several aquatic organisms. CuPT degrades rapidly by photolysis in seawater/sediment systems. Significant metabolites (pyrithione sulfonic acid [OMSA] and 2-pyridine sulfonic acid [PSA]) are produced via aerobic degradation (Mochida et al., 2008). CuPT is very toxic to aquatic invertebrates at the µg/l level. CuPT 96 h EC_{50} was 11 µg/l and 96 h NOEC growth was 6.9 µg/l to the marine eastern oyster (*C. virginica*). Tresnakova et al. (2020) reported sublethal toxicities of CuPT and ZnPT alone (10 µg/l) and in combination (5 µg/l) to the freshwater mussel *Unio crassus*. CuPT toxicity to the copepod *Tigriopus japonicus* (24 h LC_{50} = 41 µg/l) was higher than ZnPT (24 h LC_{50} > 500 µg/l) (Shipbuilding Research Association of Japan, 2002, cited in Yamada, 2006). Another early life stage embryotoxicity and embryo mortality report was made for sea urchins (Wang et al., 2011; Xu et al., 2011). An EC_{50} value > 100 mg/l was determined for *Paracentrotus lividus* embryotoxicity, where this exposure concentration was not very toxic to early embryos (2-cell stage) but inhibited the pluteus stage of larval development (EC_{50} = 0.011 mg/l) (Gutner-Hoch et al., 2019).

Mochida et al. (2006) reported that CuPT was more toxic than ZnPT to the fish *Pagrus major* and toy shrimp *Heptacarpus futilirostris* (LC_{50} values of CuPT and ZnPT: 9.3 and 98.2 µg/l for *P. major*, and 2.5 and 2.5 µg/l for *H. futilirostris*, 96 h, respectively). Skeletal muscle atrophy

and peripheral nerve damage are the result of repeated exposures to high concentrations (Arch Chemicals, 2008). In addition, potential endocrine disrupting effects have raised concerns over mutagenicity and impairment of endocrine signalling pathways (Cui et al. 2014).

Globally the aquaculture industry is a good example of sustainable ecosystem services, providing a healthy protein source in seafood, where bivalves are of economic importance. Bivalve molluscs, both marine (*Mytilus*) and freshwater (*Unio*, *Anodonta*), respond to rapid changes in their environment, such as pollutant load, habitat salinity, temperature (e.g. climate change stressors), pH or pollutant mixtures, and therefore they serve as bioindicator species for biomonitoring (Kholodkevich et al., 2019).

Today bivalves also have another unprecedented role as invertebrate model organisms for research in chronobiology, neuroendocrinology, bacterial endosymbiosis, innate immunity, biomineralization, aging and various biotechnological applications, as well as for environmental health monitoring (Robledo et al., 2019). Mussels are abundant in seas; they accumulate metals in high concentrations and keep them in their bodies for a long time and are one of the biological indicators that reflect pollution in water. *Unio mancus* is an indicator species physiologically suitable for ecotoxicological studies. It is the first species reported to disappear after exposure to pollution stress. The *Unio* genus has the advantage of being sensitive to even low levels of environmental pollutants, have a long life, are widely distributed, and large enough for providing tissues for analyses (Van Hassel & Farris, 2007).

Biochemical, molecular and physiological biomarkers are reliable integral responses of model/indicator organisms both for whole organism level changes in the environment and state of the habitat (ecological status) of aquatic organisms and lower organisational levels under the impact of environmental stressors (Newton & Cope, 2006). Metabolic and physiological monitoring provides invaluable information about prolonged thermal stress depleting energy sources (Ganser et al., 2015) and adjustments in filtration rates (Ferreira-Rodriguez, 2019).

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are enzymatic defense line antioxidants. When oxidative stress persists, enzyme inactivation, lipid peroxidation, DNA damage and even cell death are unavoidable. Reactive oxygen species (ROS) production is also caused by the loss of mitochondrial integrity, damage to critical cellular macromolecules and activation of programmed cell death (Wang et al., 2018). Aerobic life forms are under a threat of ROS targeting lipids where cellular level membrane lipid peroxidation damage is measured by malondialdehyde (MDA, a secondary product) (De Las Heras et al. 2018). Parameters used as biomarkers of ROS and defense systems are widely employed in life sciences and ecotoxicological research.

There is limited toxicity data on antifoulants, and more specifically on CuPT effects on non-target freshwater organisms. Pollution biomonitoring is mostly limited to crustaceans, which are the most affected benthic organisms. Marine coastal ecosystems depend on benthic organisms for energy transfer between pelagic and benthic ecosystems. Biocidal metals, Cu and Zn, were shown to accumulate in experiments with non-target organisms after exposure to antifouling biocides (Muller-Karanassos et al., 2019). No studies have been conducted to investigate ecotoxicological effects of the antifouling compound copper pyrithione on freshwater mussels. This study aimed to test the hypothesis that CuPT toxicity to the non-target model organism *Unio mancus* acts through multiple mechanisms with diverse biomarkers involved, such as those of oxidative stress (MDA, GSH, SOD, GPx, AOPP) in gill and digestive gland (DG) tissues. In addition, total hemocyte counts and histopathological effects were assessed.

MATERIALS AND METHODS

Freshwater mussels and exposure experiments

Freshwater mussels (Mollusca: Bivalvia: Unionidae), *Unio mancus*, Lamarck 1819 (Bourguignat, 1860) were collected manually from shallow locations of Karasu Irmağı (Karasu River), Sinop, NE Turkey. Mussels were brought to the Ecotoxicology Laboratory of the Biology Education Department, Gazi Faculty of Education, in containers providing cool and humid environment where direct contact with daylight was avoided. The specimens were acclimatized for at least two weeks in aerated glass aquaria. In this process, the mussels were fed on freshwater algae. Fifteen mussels were distributed to each 20 l glass aerated aquarium with 15 l of water. The mean total weight and length of the control (24.83 ± 7.50 g; 59.37 ± 5.37 cm) and experimental groups (25.90 ± 8.91 g; 59.74 ± 0.06 cm) of freshwater mussels were measured. Water quality parameters were: temperature 19.2 ± 0.14°C, conductivity 249.1 µS/cm, pH 8.12 ± 0.2, dissolved oxygen (DO) 6.30 mg/l.

In preliminary dose-range finding experiments (1, 2, 4, 8, 10, 12 and 50 µg/l, 100% mortality was recorded for the highest concentration) copper pyrithione (CuPT) (Arch Chemicals, UK, purity: 95-100%; CAS: 14915-37-8;

molecular weight: 315.86 g/mol) was weighed and a stock solution was prepared by dissolving the amount in a specified volume of DMSO, and dosing solutions were diluted from this stock using DMSO. Exposure concentrations were based on environmentally relevant concentrations in available literature (Almond & Trombetta, 2016). All chemicals, solutions and aquaria were covered with aluminium foil to avoid photodegradation. In a sublethal experiment, 15 specimens were exposed to 5 µg/l CuPT (in DMSO) in 20 l volume aquaria. Two control 20 l aquaria (DMSO and negative control) contained 15 specimens each. Sublethal exposure duration was 96 h. The bioassay system was constructed according to the OECD (1993) and national legislation (Turkish Official Gazette, 1991).

Total haemocyte counting

Hemolymph was obtained from each mussel from the anterior adductor muscle using disposable syringe with 1:1 dilution with 4% formaldehyde. Haemolymph total hemocyte counts (THCs/ml) were estimated by a modified hemocytometer method of Sepici-Dinçel et al. (2013).

Tissue analysis

After hemolymph collection, mussels were dissected and tissues (gill and DG) of 10 specimens were reserved for biochemistry. The tissues were wrapped in aluminium foil before immediate freezing in liquid nitrogen and storage at -80°C.

Oxidative stress biomarker analyses (MDA, GSH, AOPP, SOD, GPX)

Dissected, frozen tissues were thawed on ice and about 100 mg was weighed and homogenized using 900 µl trichloroacetic acid (TCA, ice-cold 10%, 1:10, w:v). Homogenate supernatants were kept on ice or stored at -20°C until analysis.

Lipid peroxidation levels (MDA analysis) were measured as TBARS equivalents by slightly modified methods of Casini et al. (1986), Kurtel et al. (1992) and Yildirim et al. (2011). To 250 µl supernatant, 10 µl 1% BHT (butylated hydroxytoluene) was added, and 250 µl 0.67% TBA (thiobarbituric acid). The mixture was boiled at 100°C for 15 min, and absorbance measured at 535 nm. Molar extinction coefficient of $\varepsilon_{535} = 1.56 \times 10^5\ M^{-1}cm^{-1}$ was used to calculate TBARS equivalents (nmole/g tissue).

Reduced glutathione levels (GSH). A slightly modified method of Parihar et al. (1997)

Kurtel et al. (1992), Yildirim et al. (2011) and Flohe & Günzler (1984) was used. Supernatant in the amount of 100 µl was mixed into 400 µl 0.3 M Na_2HPO_4 (disodium hydrogen phosphate) and 50 µl DTNB (Ellman's reagent [1959]; 5,5′-dithiobis[2-nitrobenzoic acid]), incubated at room temperature for 10 min and absorbance was measured at 412 nm. Reduced GSH values were calculated as µmole/g tissue, assuming a molar extinction coefficient of $\varepsilon_{412} = 14.150 \times 10^5\ M^{-1}cm^{-1}$.

AOPP levels: Ice-cold Tris-HCl buffer, 20 mM, pH=7.4, was used as dilution medium (100 mg gill and DG tissue sample +400 µl buffer, 1:5, w:v) for tissue homogenization and the homogenate centrifuged at 5.000*xg* for 10 min. Supernatant was analyzed at 340 nm for AOPP levels using the spectrophotometric method of Witko-Sarsat et al. (1996) (Biochrom Libra S22® UV-VIS spectrophotometer) in the presence of potassium iodide. When necessary the supernatant was diluted 1:3 with PBS and 200 µl of sample was analyzed, calibrated with chloramine-T solution. AOPP levels were expressed in micromoles chloramine-T equivalents per liter. The values are expressed as nmole/mg protein.

SOD enzyme assay: The principle of the assay is based on tissue superoxide degradation by SOD enzyme and the production of O_2^- with xanthine oxidase. After the reaction with nitroblue tetrazolium (NBT), color change due to the end product was determined spectrophotometrically (Sun et al., 1988). Tissue samples, 50 mg of DG and gill each, were weighed and diluted 1:5 (w:v) with 20 mM HEPES (pH=7.2, containing 1 mM EDTA, 210 mM mannitol, 70 mM sucrose) before homogenization. Homogenates were centrifuged at 1.500*xg* for 5 min at +4°C. Tissue SOD activity (U/mg protein) was calculated using the Cayman SOD assay kit at 440-460 nm following manufacturer's instructions.

GPx enzyme assay: Cumene hydroperoxide is formed by GPx activity after oxidation of GSH; glutathione reductase (GR) and NADPH reduce the oxidized glutathione to reduced GSH. $NADP^+$ is the oxidized product of the reaction (Paglia & Valentine, 1967). Tissue samples, 50 mg of DG and gill each, were weighed and diluted 1:5 (w:v) with 50 mM Tris-HCl (pH=7.5, containing 5 mM EDTA and 1 mM DTT) before homogenization. Homogenates were centrifuged at 10.000*xg* for 15 min at +4°C. Tissue GPx activities (nmole/min/mg protein) were calculated using the Cayman GPx assay kit at 340 nm following manufacturer's instructions. Bradford's micromethod version was used for tissue protein level measurements

using bovine serum albumin (BSA) as the standard (Bradford, 1976). Absorbance measurements were made using the Molecular Devices VERSAmax tunable microplate reader®.

Histopathological analysis

Following ice anesthesia, a complete necropsy of the Unionid mussels was performed and tissues (digestive gland, mantle, intestine, nephridium, gill, gonads) transferred to prelabelled histology cassettes and stabilized into invertebrate Davidson's fixative (composed of 115 ml of acetic acid (glacial), 220 ml of formalin, 330 ml of 95% ethyl alcohol and 335 ml distilled water) for 48 hours. Afterwards, histological cassettes were embedded in 70% ethanol. Routine histological protocols were processed (dehydration in ethanol series, clearing with xylene, embedded in paraffin, sectioned with microtome, stained via H & E). Preparations were visualized under light microscope.

Statistical analysis

The exposure group mean values of MDA, GSH, AOPP, SOD and GPx were tested for homogeneity of variances and normal distribution. If these conditions were met, then parametric t-test and one-way ANOVA were used for comparison between groups. Otherwise, the non-parametric counterparts Mann-Whitney U and Kruskal Wallis-H tests were used for statistical analyses. The significance level was taken as 0.05.

RESULTS

Total hemocyte counts (THCs)

The mean total hemocyte counts of freshwater mussel *Unio mancus* exposed for 96 h to sublethal CuPT were 179667 ± 20552 THCs/ml and 139667 ± 10165 in the controls. The slightly higher cell numbers in the experimental group were not significantly different from the control group ($p > 0.05$).

Lipid peroxidation levels (malondialdehyde levels, MDA, as TBARS equivalents) of DG and gill tissues

Lipid peroxidation and oxidative stress biomarker malondialdehyde (MDA) levels were measured in freshwater mussel DG and gill tissues exposed to sublethal copper pyrithione for 96 h. The mean experimental group DG level increased almost five-fold that of the control group, and the increase was strongly significant ($p < 0.001$). Gill MDA levels did not differ significantly between groups ($p < 0.05$) (Figure 1).

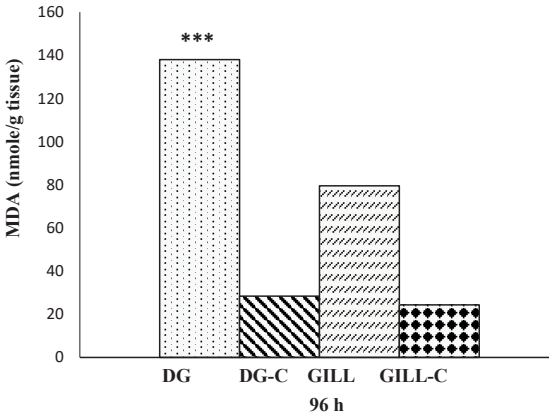

Figure 1. MDA activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 µg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: GILL tissue, GILL-C: Gill-Control tissue, 96 h. *** $p < 0.001$

Tissue reduced glutathione levels (GSH)

Mean reduced glutathione (GSH) levels showed a similar trend with MDA levels in gill and DG tissues of mussels after 96 h exposure to sublethal CuPT. Experimental groups mean GSH levels were almost 2.5-fold that of the control group, and the increase was significant ($p < 0.05$). In the gill tissue experimental group, GSH level increased slightly but not significant ($p > 0.05$) (Figure 2).

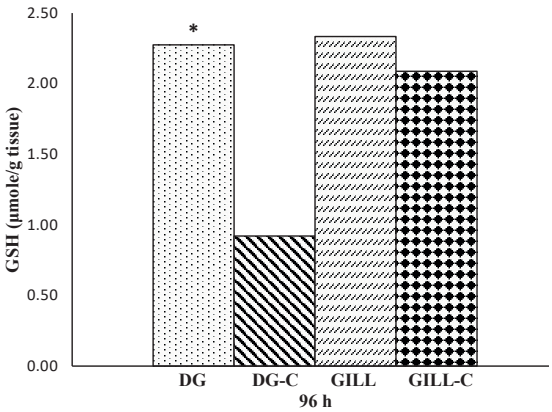

Figure 2. GSH activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 µg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h. * $p < 0.05$

Tissue AOPP levels

Mean AOPP levels were determined in DG and gill tissues of organisms exposed to sublethal CuPT. AOPP levels decreased in both tissues compared to their respective controls, however this effect was not significant ($p > 0.05$) (Figure 3).

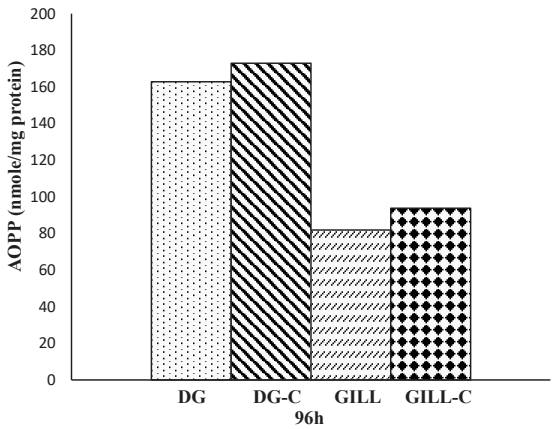

Figure 3. AOPP activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Tissue superoxide dismutase (SOD) activity

Mean SOD activities in both tissues increased in the experimental groups but the increase was not significant ($p > 0.05$) (Figure 4).

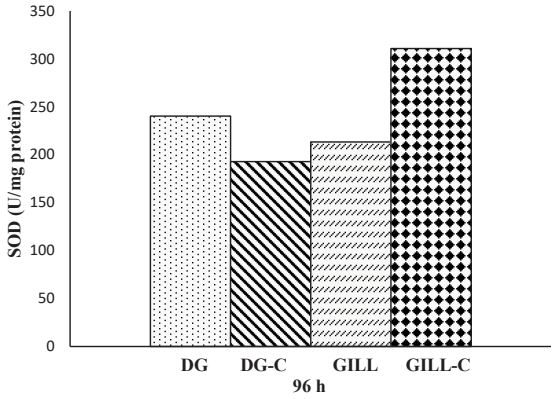

Figure 4. SOD activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Tissue glutathione peroxidase (GPx) activity

Mean GPx activity in DG decreased in the experimental group, whereas gill level increased when compared with the control group ($p > 0.05$) (Figure 5).

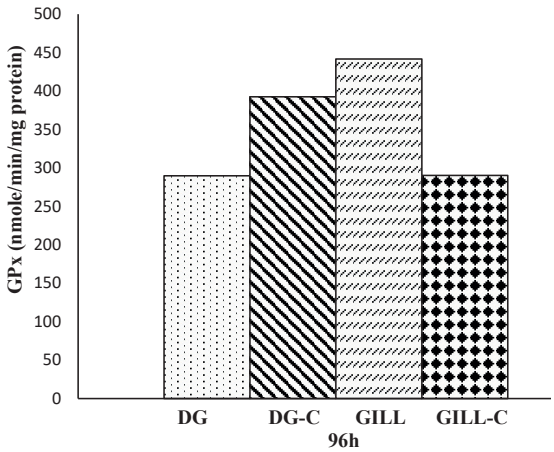

Figure 5. GPx activity in digestive gland and gill tissues of *Unio mancus* exposed the copper pyrithione (5 μg/l), DG: Digestive gland tissue, DG-C: Digestive gland-Control tissue, GILL: Gill tissue, GILL-C: Gill-Control tissue, 96 h.

Histological findings

Unionid mussel tissues of the mantle, intestine, nephridium and gonads did not reveal significant adverse effects when exposed to sublethal antifouling copper pyrithione for 96 h. Histopathology is generally the gold standard for evaluating toxic effects at tissue level in toxicology, as well as in ecotoxicology. The gill filaments of control mussels exhibited no pathological changes (Figure 6a). In the control group, gills showed well-preserved lamellae composed of a single layer of epithelial cells and tight hemolymphatic sinus. Compared to control, exposure to copper pyrithione led to mild histopathological alterations in the gills, such as haemocyte infiltration, lipofuscin aggregates and lamellar deformations (shorthening) (Figure 6b). The DG of control Unionids showed a normal structure with digestive tubules composed of epithelial cells (Figure 7a-b). Sublethal copper pyrithione exposed mussels exhibited mild histological change in the structure of DG and accumulated lipofuscin aggregates (Figure 7c-d).

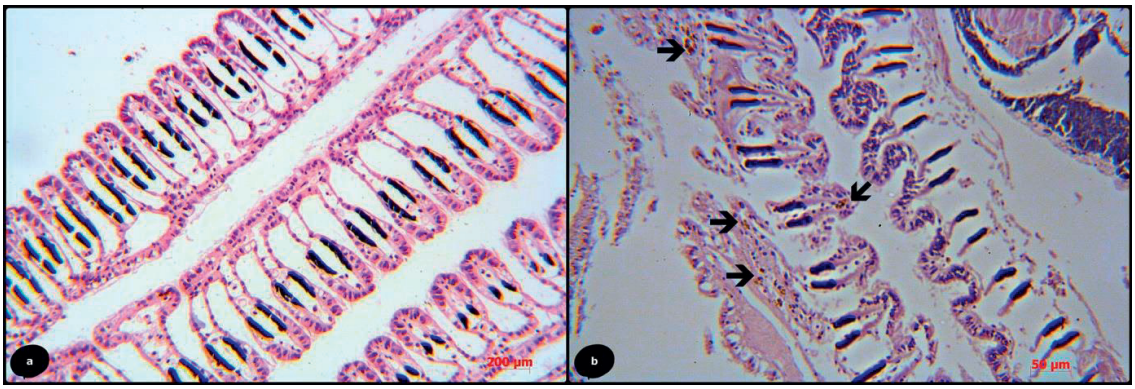

Figure 6. Histologic illustration of the gills of *Unio mancus* exposed to copper pyrithione (H&E). (a) Control mussel gills with normal structure; (b) Lamellar deformation of gill epithelium (shorthening) and lipofuscin aggregates (arrows).

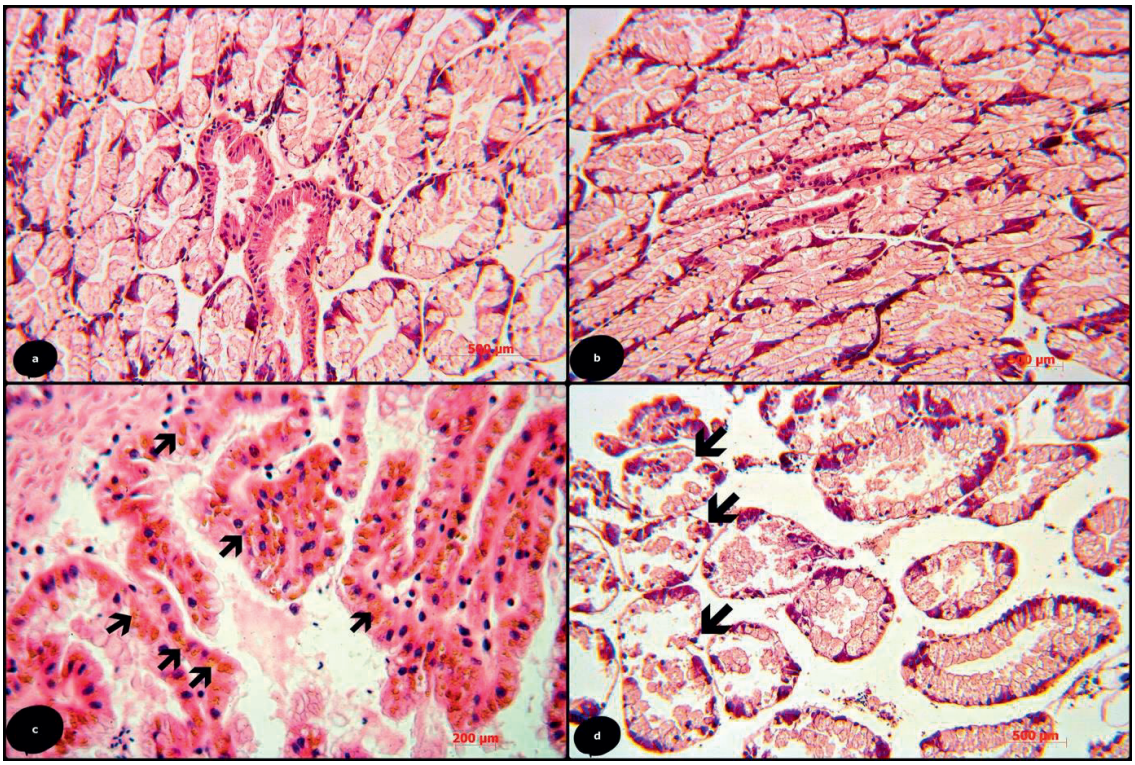

Figure 7. Histologic illustration of the DG of *Unio mancus* exposed to copper pyrithione (H&E). (a-b) Control mussel digestive gland with typical structure; (c) Lipofuscin aggregates; (d) Digestive tubule degeneration (arrows).

DISCUSSION

Contamination of freshwater and marine ecosystems with heavy metals, organic pollutants, pollutants of emerging concern, persistent pollutants and biocidal substances is a major concern worldwide. Macroinvertebrates are widely accepted indicator species in ecotoxicology studies, representing impacts due to biotic and abiotic stressors using a series of parameters for biochemical, physiological, survival, feeding and reproduction activities. Over 95% of all animal species are invertebrates and they have invaluable ecological roles in the lower levels of the food web. Bioindicator mussels have been used for pollution biomonitoring since the early 1950s and their use has increased since then (Van Hassel & Farris, 2007).

Unio mancus mussels were exposed to sublethal CuPT for 96 hours and morphometric, hematological and biochemical parameters were studied. CuPT did not affect weight, length, height or thickness of mussels ($p > 0.05$). Similarly, total hemocyte counts increased after exposure but the change was not significant ($p > 0.05$). Tresnakova et al. (2020) reported significant increase in THCs after 48 h exposure of *Unio crassus* to CuPT and its combination with ZnPT but a significant decrease followed after 7 days.

The micronutrient Cu is essential both for aquatic plants and animals, but may pose toxicity risk at high concentrations, caused by heavy boat traffic, maritime activity and limited water exchange rate regions. Risk is mainly due to intensive use of Cu-based (Cu alone or Cu in combination with booster biocide) antifouling paints (Schiff et al., 2007). Cu as a cofactor and redox component of cellular metabolism may be toxic above optimum levels to aquatic life, including microalgae. In photosynthesis, the mechanism of toxic action (MoA) of Cu is thought to inhibit electron transport of the PSII (Dupraz et al., 2018), decreasing its efficiency. ZnPT and CuPT may both impact cell membrane integrity and membrane transport, and inhibit ATP synthesis. A further impact of CuPT could be through mitochondrial oxidative metabolism and even mediating the apoptotic process through ROS formation and oxidative stress (Gutner-Hoch et al. 2019).

The bioavailable form of copper is the dissolved ionic form Cu^{2+}. Elevated concentrations of Cu in sediments and surface waters of California, USA (Schiff et al. 2007) and Mexico (Jonathan et al. 2011) were attributed to Cu-containing antifouling paints. Cu exhibits higher toxicity in mixtures, compared with pure solutions, due to synergistic interactions.

Antifouling paint particles (APPs) of between 500 mm and > 2 mm in diameter have been identified in silty, intertidal estuarine sediments, in guts of sediment-dwelling ragworm, *Hediste diversicolor*. The ragworm tissues showed high Cu residues (Muller-Karanassos et al. 2019). These sites have high APPs loads due to abandoned boats or boating activities. Epibenthic harpaticoid) *Nitokra* sp. was also studied for APPs residues in sediments and decreased fecundity was reported as potential risk (Soroldoni et al., 2017).

Another study examined the effects of TBT and benzo[a]pyrene (BaP) on the gastropod *Haliotis diversicolor* (abalone) immune system, and long-term chronic exposure to sublethal BaP concentration was observed to modulate immunocompetance as a change in the activity of immune-related parameters. The immunotoxicity biomarkers THC, phagocytosis, membrane stability and lysosome activity were significantly reduced after 21 d of sublethal TBT exposure. After 21 d, 14 d BaP-exposed gastropod parameters recovered. Fourteen days of improvement in TBT-exposed organisms showed no variation. In this case, TBT is thought to be more harmful than BaP (Gopalakrishnan et al., 2011). CuPT is an alternative to the banned TBT, but it also shows non-target species toxicity. In our study, an increase in hemocyte count was observed as a result of exposure to copper pyrithione. This difference can be explained by the different functioning of the immune system. Hemocyanin is the blood pigment in mussels, and the hemoglobin of higher organisms in the hierarchy of species' has iron. In our study, iron binding as the element can be assumed as effective in increasing hemocyte counts. In addition, the use of different model organisms in experiments with CuPT, which is considered as toxic as TBT, suggests that the duration of exposure is significant and that some chemicals may have different effects on each animal species. In fact, the improvement of immune parameters in BaP treatments supports this view.

Ecotoxicity of CuPT and HPT (2-mercaptopyridine N oxide, degradation product) to the brine shrimp, *Artemia salina* was studied together with Cu^{2+} using natural seawater and organic matter-free artificial seawater as exposure media (Lavtizar et al. 2018). Artificial seawater media, with the highest salinity and no organic matter content, caused the highest CuPT toxicity (48 h EC_{50} = 250 µg/l). The corresponding natural seawater EC_{50} value was 556 µg/l, showing less toxicity; organic matter content and salinity decrased toxicity. The marine crustacean *Tigriopus japonicus* was more sensitive than *Artemia salina* to CuPT exposure, and the 24 h EC_{50} = 23 µg/l showed CuPT to be highly toxic (Onduka et al. 2010). Koutsaftis and Aoyama (2007) reported 24 h EC_{50} of 830 µg/l for *Artemia salina* exposed to CuPT in standard artificial seawater, which is highly toxic. Also in agreement were the results reported by Mochida et al. (2006), 96 h LC_{50} = 2.5 µg/l for *Heptocarpus futilirostris* (toy shrimp). CuPT 24 h LC_{50} values for *Tigriopus japonicus* (copepod) (and for the rotifer *Brachionus koreanus*) were in µg/l order of magnitude and highly temperature-dependent (Li et al., 2014). Although nauplii are tolerant to marine toxins to a small extent, 24 h LC_{50} for *Balanus amphitrite* barnacle nauplii exposed to CuPT were in the 4.0-6.1 µg/l range and imposex was recorded in sites heavily polluted by PAH and other endocrine disruptors (Romano et al. 2010). Our results showing high toxicity of CuPT to non-target freshwater

mussels are in agreement with other studies and confirm them (Koutsaftis & Ayoma, 2007; Koutsaftis & Ayoma, 2008; Onduka et al., 2010; Romano et al., 2010; Bao et al., 2011; Li et al., 2014; Lavtizar et al., 2018; Tresnakova et al., 2020).

Biomarker-based results showed prooxidant activity, where catalase and cholinesterase activities increased significantly. Overload of ROS together with weakened antioxidant capacity predispose aquatic species to oxidative stress. Malondialdehyde (MDA) levels of DG increased dramatically with respect to controls, showing lipid peroxidation and tissue unable to cope with oxidative stress caused by CuPT after 96 h exposure, leading us to consider that oxidative stress may contribute to CuPT toxicity in *Unio*. In agreement with results of other researchers, our MDA levels increased in DG and gills of *Mytilus edulis* (mussel) and *Crassostrea gigas* (oyster) when exposed to heavy metals, silver, cadmium, copper and zinc for 21 days (Geret et al. 2002). However, no differences were reported in MDA levels of *Unio crassus* neither with CuPT alone nor in combination with ZnPT.

Flakes of CuPT and ZnPT, which are directly released and reach bottom sediments in a way similar to TBT accumulation in the sediment, have a relatively short half-life of just several months in water bodies but can last for years bound in anaerobic sediment at ppm levels (Warford et al. 2022). Risk due to long-term effects of TBTs is higher and raises concern specifically in estuarine areas, necessitating comprehensive ecotoxicity assessment of sediment organisms such as Polychaete species for pyrithiones that are marketed as alternatives to TBTs. ZnPT has been reported to be toxic to the polychaete *Hediste diversicolor* (Nunes & Costa, 2019. Parameters of oxidative stress (catalase, glutathione S-transferases (GSTs), and lipid peroxidation (GPx), total proteins and neurotoxicity (acetylcholinesterase) were studied after 96 h acute exposure to a 10-160 µg/l dose range. TBARS levels did not change, while AChE decreased and catalase increased, leading the authors to conclude that no clear toxicity mechanisms have been elucidated for ZnPT so far. Evidently, toxicological data are sparse, and more than one MoA may be effective (Nunes & Costa, 2019). As a cofactor for a number of enzymes, Cu^{2+} participates in oxidative stress and mitochondrial morphology with a narrow optimal range between essential and toxic concentrations. Numerous studies in a variety of species have shown that Cu^{2+} exposure resulted in apoptotic and autophagic cell death because of elevated ROS levels (Wang et al. 2018).

Glutathione (GSH), as a major cellular antioxidant defense agent, decreases in the process of oxidative stress due to utilization as antioxidant. GSH levels in exposed mussel DG increased 2.5-fold compared to controls ($p < 0.05$), but increase in gills was not significant ($p > 0.05$) after 96 h exposure. *Unio tumidus* mussels were introduced to four different contaminated areas and exposed for 15 days. In the digestive gland, GSH levels decreased by 70%. It shows that the defense mechanism against oxidative stress was working and reduced glutathione depots (Cossu et al., 2000). The GSH levels in *Unio crassus* tissues were significantly raised after 48 h exposure to 10 µg/l CuPT, while they declined significantly after 7 days exposure. In another experimental study, the effects of 1-4 h high temperature exposure to SOD, the activities of GSH-Px and GSH were investigated in *Heteropneural fossilis*. After exposure at elevated temperatures ranging from 25 (control) to 37°C, SOD activity increased, while GSH-Px activity and GSH content decreased significantly ($p < 0.05$) at 32 and 37°C after 14 h in comparison to control. Nevertheless a transient increase in GSH-Px activity was observed after 1 and 2 h at 32°C. The results at 27°C temperature were non-significant ($p < 0.05$) in comparison to control. During the extended hours (1 to 4 h) of each elevated temperature, a general trend of increase in SOD activity was observed at 32 and 37°C. However, GSH-Px activity and GSH content did not change significantly for most of the extended period of elevated temperature.

Oxidative stress and histopathological parameters were determined in a study examining the effect of heavy metals on *Leuciscus cephalus* species. Fish samples were collected from two sites in the Tur River, NW Romania, upstream and downstream of a pollution source. Histopathological changes were associated with metal bioaccumulation, being more severe in kidneys than in liver. Malondialdehyde (MDA) and advanced oxidation protein products (AOPP) increased significantly in the liver and kidney of fish at the downstream site, compared to the upstream one, whereas GSH decreased. The activities of SOD, catalase (CAT) and glutathione-S-transferase (GST) increased significantly in livers, whereas SOD increased in kidneys (Hermenean et al. 2015). ROS formed during xenobiotic metabolism causes direct inactivation of GST isoenzymes. Furthermore, the use of engineered micro/nanomaterials (EMNMs) as carriers of antifouling booster biocides to control their release and reduce harmful effects on living biota are considered as a potential mitigation strategy, and they are under investigation (Gutner-Hoch et al. 2019).

Although mussels are important bioindicator model organisms in ecotoxicology of aquatic systems, the published research has been carried out mostly on marine bivalves. Histopathological investigations of Unionid mussels related to ecotoxicology are scarce. As sessile filter-feeder organisms, they can easily accumulate water-borne toxicants from the environment and reflect changes in water quality. The studies indicated that DG and gill tissues lead as the most sensitive tissues exposed to chemical toxicants. Since gills have a large surface in direct contact with water-borne toxicants, they are considered a sensitive organ affected by water quality (Aarab et al., 2011; Pinto et al. 2019). Exposure of 96 h to sublethal copper pyrithione revealed haemocyte infiltration, lipofuscin aggregates and lamellar deformations. Similar to the findings in this study, Tresnakova et al. (2020) noticed hemocytic infiltration and lipofuscin-like structure in gill tissues of *Unio crassus* following exposure to copper and zinc pyrithione and their combination for 48 h and 7 days. Lopez-Galindo et al. (2010) observed lipofuscin aggregates in gill tissues with haemocytic infiltrations on gill filaments following exposure to the antifoulants NaClO and Mexels432. Previous research with *Ruditapes phillippiranum,* exposed to contaminated sediments, reported results similar to our study (Martin Diaz et al. 2008). Haemocyte infiltration was noticed in gill tissues of *Mytilus edulis* exposed to treated and untreated sewage (Akaishi et al., 2007). *Mytilus galloprovincialis* and *Ruditapes phillipinarum* field samples were reported to show lipofuscin in cells due to environmental stressors of heavy metals and anoxic conditions (Sarasquete et al. 1992).

Overall mussel health is mainly evaluated looking at DG tissue and the impact of xenobiotics is compared with other tissues (Faggio et al. 2018). Digestive tubule degeneration has heretofore been associated with toxicity of xenobiotic exposure (Bignell et al. 2008). Exposure to antifouling copper pyrithione showed accumulated lipofuscin aggregates and caused mild degeneration of digestive tubules. In agreement with our present research, Tresnakova et al. (2020) observed deformations and loss of digestive tubules of *U. crassus* following exposure to copper-zinc pyrithione combination for 48 h and 7 days. Changes in the structure of DG epithelium in response to toxicants have been investigated for some marine bivalves (Sarasquete et al. 1992). Previous studies have demonstrated degeneration of DG following exposure to different toxicants and an increase in the frequency of DG degeneration may lead to digestive gland dysfunctions. (Lowe & Clarke, 1989). Results of the present study also support that oxidative stress in bivalves can be the source of lipofuscin accumulation in association with xenobiotic exposures.

In conclusion**,** there have been limited data so far on the environmental occurrence, fate, toxicity and persistence of these booster biocides, as well as their potential risks for aquatic ecosystems due to their increased use (Bao et al., 2011). The use of these antifouling biocides is expected to further increase after the complete ban on TBT, yet the results of this study (and others) suggest that some of them could be even as toxic as TBT to aquatic systems. Further studies should be carried out to understand how they affect aquatic life.

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Oksidativni stres kao biomarker kod izlaganja slatkovodne školjke (*Unio mancus* Lamarck 1819) kao model organizma sredstvu protiv obrastanja bakar piritionu

REZIME

Ocenjivani su efekti bakar piritiona (CuPT) na jednu vrstu beskičmenjaka, slatkovodnu školjku (*Unio mancus*) kao model organizma, korišćenjem parametara oksidativnog stresa i histopatoloških nalaza. Koncentracija bakar piritiona od 5 µg/l, koja je značajna za životnu sredinu, primenjena je u polustatičnom izlaganju u trajanju od 96 h. Nivoi MDA u digestivnoj žlezdi (DG) bili su značajno povećani, u poređenju sa kontrolnom grupom ($p < 0.001$). MDA u škrgama su takođe bili povećani, ali ne značajno ($p > 0.05$). Nivo GSH u DG se značajno povećala ($p < 0.05$), dok je u tkivu škrga povećanje bilo bez značaja ($p > 0.05$). Nivoi SOD, GP i AOPP nisu se značajno izmenili ($p > 0.05$). Najznačajnije histopatološke promene u škrgama predstavljali su infiltracija hemocita, akumulacija lipofuscina i lamelarna deformacija sa degeneracijom tubule i akumulacijom lipofuscina u tubulama DG. CuPT utiče na antioksidativni odbrambeni sistem čak i tokom subletalnog izlaganja *Unio mancus* kao slatkovodnog model organizma.

Ključne reči: biocidi, sredstvo protiv obrastanja bakar pirition, biomarkeri, oksidativni stres, slatkovodna školjka

Instructions for Authors

About Journal

Pesticidi i fitomedicina (Pesticides and Phytomedicine) is dedicated to the following research fields: toxicology and ecotoxicology of pesticides; phytopathology; applied entomology and zoology; weed science; plant and food products protection; use of pesticides in agriculture, sanitation and public health.

The journal continues the title *Pesticidi*, which was published over the period 1986-2003.

Pesticidi i fitomedicina (Pesticides and Phytomedicine) publishes original scientific papers and review papers that have not been published previously.

Pesticidi i fitomedicina (Pesticides and Phytomedicine) is an Open Access journal.

Contributions to the journal must be submitted in English, with summaries in English and Serbian (Serbian-speaking authors only).

As of 2020, *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* is issued triannually (three issues annually).

As of 2021, Pesticides and Phytomedicine (Pesticidi i fitomedicina) will be published **online only,** and paper copies of future issues will no longer be available. The primary platforms for journal publication will continue to be: Scindeks (http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949) and the publisher's official web site (http://www.pesting.org.rs/).

The journal is indexed in: Chemical Abstracts, CAB International; DOAJ, EBSCO, AGRIS, Scindeks.

In 2011, the journal converted to an electronic online journal management system on the SCIndeks Assistant portal at **http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949.** The system enables easy article submission and communication among the editorial staff, reviewers and authors. It also includes several quality control services: *CrossRef* for DOI assignment, *CrossChek* for plagiarism prevention and *KWASS* for equipping articles with keywords extracted from a dictionary/thesaurus. Electronic editing is in compliance with the Journal Editing Act of the Ministry of Education, Science and Technological Development of the Republic of Serbia, and provides record-keeping stipulated in the Act.

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To be published in *Pesticidi i fitomedicina (Pesticides and Phytomedicine),* an article must be based on original scientific results that have not been previously published and are not under consideration for publication elsewhere. Review articles should contain a comprehensive survey of a particular subject based on referenced literature and published results of the author(s) own research. All contributions are peer reviewed in a double blind process.

A click on "submit a manuscript" on the left-hand side of the journal home page in SCIndeks Assistant will lead users to a registration page and further on into a guided process of electronic manuscript submission. Serbian authors are requested to fill out the application form in both English and Serbian. Each visual or graphic item (table, chart, diagram or photo) should be submitted as a separate (supplementary) file.

Authors need NOT specify keywords in their articles. They will be extracted and selected by the Editor-in-Chief from the *KWASS* thesaurus (dictionary), which will significantly improve article visibility. Authors are entitled to accept or change some of the keywords.

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The manuscript should be prepared in Microsoft Word (A4 format, all margins 25 mm, font Times New Roman 12 pt). Articles have to be written in the English language, and only the title and abstract in both English and Serbian (Serbian summary will be furnished by the copyeditor for foreign authors'manuscripts).

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Abstract (not exceeding 300 words) should briefly state the main results and conclusions.

Articles should contain the following sections: Introduction, Material and Methods, Results, Discussion, Acknowledgement and References.

Introduction should present the state-of-the-art in a particular research field, as well as research intent.

Material and Methods should provide sufficient detail to allow the work to be reproduced. Conventional methods should only be referenced.

Results should be presented in a logical order, clearly and concisely, using adequate tables and graphics. Avoid repetition of the results in tables and graphics, or in the text.

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Acknowledgement should be collated at the end of the manuscript before References.

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Journal references are required to contain the following information: name(s) of author(s), year of publication, title of article, title of journal, volume, issue number (unless pagination is continuous), pages (from-to) and DOI if available.

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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

Books: name(s) of author(s) or editor(s), year of publication, title, place of publication and name of publisher.

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.

Dissertations: author's name, year of presentation, title, full name of the institution at which dissertation was defended.

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.

Book chapters and articles in conference proceedings: author(s), year of publication, title of chapter/article/abstract, source title (with editors names), pages, place of publication and publisher.

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, England: 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 references: author(s), year of publication, title, source title, 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

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Tables need to be numbered in Arabic numerals consecutively as they appear in text. Tables should be made exclusively in Word for Windows using the toolbar menu Table-Insert-Table, Times New Roman font, 12 pt, and single line spacing. Footnotes immediately below the table body should be given priority over other explanation in table header or in table cells, and text should be in Times New Roman font, 10 pt. Each table must have a header. Tables should be submitted as supplementary (separate) files, and their approximate location in the text marked.

Graphs should be processed in Microsoft Excel and all data in Times New Roman font. Explanations should be provided in captions, consecutively and marked with Arabic numerals. Graphs should be submitted as supplementary files, and their approximate location in the text marked.

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

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

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

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

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Radovi koji se prilažu moraju biti napisani na engleskom jeziku, sa rezimeom na engleskom i srpskom jeziku.

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

Od 2021. godine, časopis Pesticidi i fitomedicina (Pesticides and Phytomedicine) objavljuje sveske samo u elektronskom obliku, bez štampane verzije. Osnovne platforme na kojima se postavljaju sadržaji časopisa su: Scindeks (http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949) i zvanični veb sajt izdavača (http://www.pesting.org.rs/).

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

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

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

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

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

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

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

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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.

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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.

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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.

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

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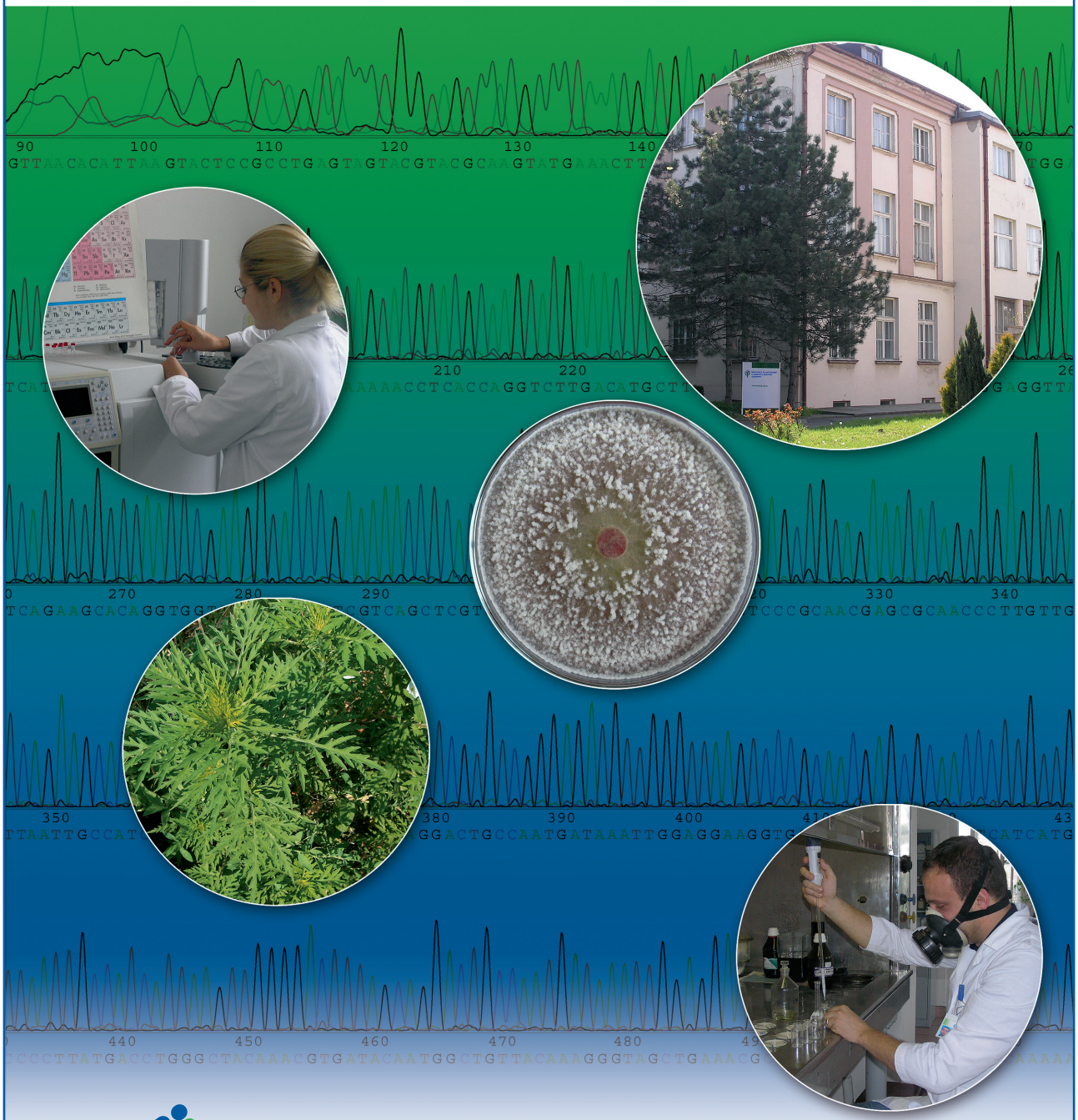

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