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Aggressiveness and trichothecene production of *Fusarium graminearum* isolates from cereals in Serbia

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SUMMARY

The aim of this study was to assess variations in aggressiveness and trichothecene production of *F. graminearum* isolates originating from maize, wheat and barley in Serbia. Analyzing *F. graminearum* isolates (98) obtained from various agroecological conditions of Serbia over the period from 1993 to 2010, using the HPLC method, the following two chemotypes were observed: 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON). A great diversity in the production of deoxinivalenol (DON) derivatives was observed. A majority of *F. graminearum* isolates, regardless of their origin (maize, wheat or barley) belonged to the 15ADON chemotype. The 3ADON chemotype was also detected, but in a significantly smaller number (13/98) samples, compared to the 15ADON chemotype (85/98). None of the tested isolates belonged to the NIV chemotype. The examined isolates showed different pathogenicity on barley leaf, wheat class and maize ears. The average pathogenicity of the tested isolates was the highest on barley leaf. It was observed that isolates originating from wheat had the highest average daily increase in mycelium growth rate (27.37 mm). Statistical analysis of the obtained results for mycotoxins synthesis showed that there was a highly significant statistical correlation between the production potentials of total DON, 3ADON and 15ADON in *F. graminearum* isolates belonging to various chemotypes. However, there was no statistically significant correlation between the aggressiveness of isolates and the production of total DON in isolates belonging to 3ADON and 15ADON chemotypes.

Keywords: cereals, *Fusarium graminearum*, mycotoxins, chemotypes, trichotecene, pathogenicity

INTRODUCTION

Small grains and maize predominate in total crop production in Serbia regarding both per unit area and total yield. However, cereal production is endangered by a large number of pathogens, particularly the species *Fusarium graminearum*. It is considered to be one of the most destructive and widespread pathogens of cereals and industrial crops world-wide. The importance of this species is that it does not only reduce yield, but grain quality also, due to its ability to synthesise mycotoxins in infected plants, and it has an adverse effect on human

and animal health. Damage that it causes is further increased by its ability to synthesise probably more than 17 mycotoxins, of which trichothecenes, such as deoxynivalenol (DON), are the most widespread and significant (Logrieco et al., 2002; Moretti et al., 2014).

F. graminearum is a species within the Fg complex (*Fusarium graminearum* Species Complex-FGSC), named *Fusarium graminearum sensu lato,* and it contains at least 15 different phylogenetic species (O'Donnell et al., 2000, 2004, 2008; Starkey et al., 2007; Sarver et al., 2011). Species of this complex synthesise various metabolites, including deoxynivalenol (DON) and its derivatives (3-acetyl deoxynivalenol [3ADON] and 15-acetyl deoxynivalenol [15ADON]) and nivalenol (NIV) (O'Donnell et al., 2000). Based on the mycotoxicological profile of synthesised trichothecenes, *F. graminearum* isolates can be grouped into one of three chemotypes (3ADON, 15ADON and NIV) (O'Donnell et al., 2004). As belonging to a particular trichothecene chemotype plays a significant role in the aetiology of cereal diseases, knowledge about the toxicological profile of *F. graminearum* is essential for agriculture, as well as the food industry of any country. Previous studies have shown that different mycotoxins had different toxicological properties: the toxicity of NIV to humans and animals is almost 10-fold higher than that of DON (Lee et al., 2015). Moreover, the phenotypic analyses of *F. Asiaticum,* which produces 3ADON, showed major advantages over *F. asiaticum* which produces NIV with regard to pathogenicity, growth rate, fecundity, conidial length, trichothecene accumulation and resistance to benzimidazole (Ward et al., 2008; Zhang et al., 2012). Variations in mycotoxin synthesis and affiliation to a certain chemotype (3ADON, 15ADON or NIV) may have important implications for toxicity (Luongo et al., 2010). Therefore, chemotype identification is an important information for establishing a risk assessment strategy in order to protect human and animal health.

Biogeographical studies of trichothecene chemotypes within the Fg complex have been carried out in many countries worldwide over the past two decades. According to literature data, all three chemotypes are widespread in Asia. It has been determined that *F. graminearum* isolates in wheat and barley predominantly produced 15ADON, in contrast to *F. asiaticum* isolates, which mainly produced 3ADON or NIV. Previous studies have shown that isolates of the 15ADON chemotype within the FGSC are dominant in the United States (Abramson et al., 2001; Miller et al., 1991), while the 3ADON chemotype has been observed in a much smaller percentage. However, an increase in the presence of 3ADON chemotype has been observed in the United States (Abramson et al., 2001; Gale et al., 2007) and Canada (Ward et al., 2008; Guo et al., 2008). *F. graminearum* is a cosmopolitan (O'Donnell et al., 2000; Backhouse, 2014) and a dominant member of the FGSC in Europe (O'Donnell et al., 2004; Toth et al., 2005; Yli-Mattila et al., 2009). In fact, *F. graminearum* was the only member of the FGSC in Europe, with the exception of a small number of isolates of *F. boothii* and *F. vorosii* in Hungary (Toth et al., 2005; Starkey et al., 2007), and *F. cortaderiae* in France and Italy (Boutigny et al., 2014; Somma et al., 2014). Data on the distribution of chemotypes in Europe show that the 15ADON chemotype is dominant in a majority of countries, in contrast to 3ADON and NIV, which have been detected at significantly lower frequency. In Europe, 15ADON has been observed in southern and central Europe (Toth et al., 2005; Boutigny et al., 2014; Jennings et al., 2004), while 3ADON is dominant in northwestern parts of Europe (Yli-Mattila et al., 2009; Fredlund et al., 2013; Nielsen et al., 2012). In Serbia, there is a lack of data on the distribution and aggressiveness, as well as the synthesis of 3ADON, 15ADON and NIV chemotypes by *F. graminearum*. The results of preliminary studies performed by Obradović et al. (2017) indicated that the 15ADON chemotype was dominant in Serbia.

Data found in literature so far indicate that isolates belonging to the 3ADON chemotype cause a more intense fusariosis and produce higher amounts of DON than isolates belonging to the 15ADON chemotype (Puri & Zhong, 2010). According to studies performed in Norway, 3ADON and 15ADON isolates have shown significant differences in the average growth rate of mycelia under *in vitro* conditions. A difference was also found regarding the aggressiveness of these two populations in wheat (Aamot et al., 2015). Studies conducted in Canada and China have shown that there were differences in phenotypic traits between 3ADON and 15ADON populations, which may explain the rapid change and spread of populations with the 3ADON chemotype. In Canada, Ward et al. (2008) noticed that 3ADON isolates produce more colonies and longer conidia, and have higher rate of mycelial growth, as well as greater synthesis of DON in comparison to the 15ADON population. In China, it has been observed that isolates of *F. asiaticum* belonging to the 3ADON chemotype rapidly replaced populations with the NIV chemotype because they were more aggressive in wheat and were more toxigenic (Zhang et al., 2010, 2012).

3ADON isolates are more aggressive than 15ADON populations regarding the rate of mycelial growth and DON synthesis *in vitro* (Ward et al., 2008). However, little data are available on the aggressiveness and potential of DON synthesis in 3ADON and 15ADON isolates of *F. graminearum*. The aim of this study was to assess variations in the aggressiveness and trichothecene production of *F. graminearum* isolates from maize, wheat and barley in Serbia.

MATERIALS AND METHODS

Isolate collection

This study encompasses 98 *F. graminearum* isolates from the collection of the Maize Research Institute at Zemun Polje, Belgrade, Serbia, which had been collected from grains with FHB symptoms in various locations in Serbia over the period from 1993 to 2010. The isolates were obtained from maize, wheat and barley grain (Table 1).

Table 1. List of tested *Fusarium graminearum* isolates

Isolates from wheat	Year	Chemotype	Isolates from maize	Year	Chemotype	Isolates from barley	Year	Chemotype
203	2003	15ADON	257	2004	15ADON	654	2005	15ADON
618	2005	15ADON	581	2005	3ADON	770/2	2005	15ADON
670	2005	15ADON	656	2005	3ADON	798	2005	15ADON
677	2005	15ADON	699	2005	15ADON	805	2005	15ADON
681	2005	15ADON	762	2005	15ADON	891/2	2006	15ADON
687/2	2005	15ADON	880	2006	15ADON	1217/2	2006	15ADON
744	2005	15ADON	914	1996	3ADON	1493	2007	15ADON
749	2005	15ADON	943/2	2006	3ADON	1517	2007	15ADON
763	2005	15ADON	971	2006	15ADON	1526	2007	15ADON
764	2005	15ADON	1010	2006	3ADON	1528	2007	3ADON
766	2005	15ADON	1030	1999	15ADON	1534	2007	15ADON
767	2005	15ADON	1133	2006	15ADON	1772	2008	15ADON
779/2	2005	15ADON	1165	2006	3ADON	1800	2008	15ADON
789	2005	15ADON	1195	2006	3ADON	1801	2008	15ADON
795	2005	15ADON	1199	2006	15ADON	1812	2008	15ADON
800	2005	3ADON	1211	2006	15ADON	1839	2009	15ADON
825	2005	15ADON	1249	2006	3ADON	2045	2009	15ADON
831	2005	15ADON	1255	2006	15ADON	2078	2009	15ADON
836	2005	15ADON	1268	2006	15ADON	2254	2009	3ADON
864	2005	15ADON	1282/2	2006	15ADON	2630	2010	15ADON
866	2005	15ADON	1368	2007	3ADON	2672	2010	15ADON
870	2006	15ADON	1408	2007	15ADON	2627	2010	15ADON
892	2006	15ADON	1419	2007	15ADON			
1012	2006	15ADON	1482/2	2007	15ADON			
1337	2006	15ADON	1495	2007	15ADON			
1343	2006	15ADON	1554/2	2007	15ADON			
1348	2006	15ADON	1649	2007	15ADON			
1351/2	2007	15ADON	1673	2008	15ADON			
1370	2007	3ADON	1696	2008	15ADON			
1443	2007	15ADON	1751	1994	15ADON			
1485	2007	15ADON	2533	2010	15ADON			
1486/2	2007	15ADON	2624	2010	15ADON			
1490/2	2007	15ADON	2811	1993	15ADON			
1746	2010	15ADON	2812	1998	15ADON			
2621	2010	15ADON	2813	1999	15ADON			
2625	2010	15ADON	2815	1999	15ADON			
2635	2010	15ADON						
2818	2002	15ADON						
2820	1997	15ADON						
2823	2002	15ADON						

In vitro fungal growth

Mycelial growth rate was evaluated by measuring the diameter of colonies after their subculturing on PDA at 25°C. The isolates were subculutred in 9 cm Petri dishes using 5 mm plugs of actively growing mycelium. Colony diameter was measured on the second and fourth day after subculturing. Within each of three replicate tests, the growth rate of each fungal isolate was estimated as a mean radial mycelial growth rate per day within the time period between two measurements of growth.

Aggressiveness evaluation

Variations in pathogenic properties of the 98 *F. graminearum* isolates examined (Table 1) were studied by applying artificial inoculation of plants in three pathogenicity tests: pathogenicity test on barley leaves, pathogenicity test on maize ears and pathogenicity test on wheat spikes.

Pathogenicity test on barley leaves. In order to test the pathogenicity of isolates, a method described by Imathiu et al. (2009) was used. According to this method, barley leaves were inoculated with spore suspensions of *F. graminearum* isolates. Eight leaves were placed in each Petri dish (Ø 150 mm) with filter paper soaked in distilled water. The central part of each leaf was inoculated with 5 µl of conidial suspension of the fungus *F. graminearum*. The inoculum was prepared from 14-days old isolates of cultures grown on synthetic nutrition agar (SNA) (Burgess et al., 1994) under a combination of fluorescent and ultraviolet light at 20°C. The concentration was adjusted using a hemocytometer to approximately $1x10^5$ conidia/ml. Leaves inoculated with sterile distilled water were used as a negative control. Incubation of inoculated leaves was performed at the temperature of 25°C, and the length of spots was measured after five days.

Pathogenicity test on maize ears. The pathogenicity test of *F. graminearum* isolates on maize was conducted under field conditions by artificial inoculation according to the method described by Reid et al. (1996). Artificial inoculation was completed three days after silking of plants by injecting 2 ml of conidial suspension with concentration of approximately $1x10^5$ conidia/ml into the maize silk channel. The inoculum was prepared in the same way as for the pathogenicity test performed on barley leaves. The same procedure was applied to control plants, and sterile water was used instead of the inoculum. The intensity of fusariosis on maize ears was evaluated on a 1-7 scale at the harvest maturity stage (Reid et al., 1996).

Pathogenicity test on wheat spikes. In order to test the pathogenicity of *F. graminearum* isolates in wheat under field conditions, artificial inoculation of wheat spikes was done according to the method presented by Mesterházy et al. (1999). Artificial inoculation of spikes was performed at the wheat flowering stage with a spore suspension of approximately $1x10^5$ conidia/ml. Thirty plants per isolate were inoculated in three replications. The amount of inoculum used per replicate was 30 ml, while control plants were treated with the same amount of distilled water. After inoculation, spikes were isolated with water-moistened PVC bags, which were removed 48 h later. The degree of infection was evaluated on the 1-7 scale two weeks after inoculation, based on the presences of fusariosis symptoms on spikes (Blandino et al., 2012).

Mycotoxin analyses

Samples for the trichothecene analysis were prepared on sterile maize kernels according to a method described by Logrieco et al. (1995). A total of 50 g of maize kernels (45% moisture) were poured into Erlenmeyer flasks, which were then inoculated with three sections of fungal colony (0.5x0.5 cm) developed on PDA. Following inoculation, Erlenmeyer flasks were sealed and covered with aluminium foil and then stored at 25°C. After incubation, inoculated maize kernels were transferred to aluminium dishes and dried at the temperature of 50°C, and then they were ground using the analytical mill (A11 IKA, Germany).

HPLC method (High Performance Liquid Chromatography)

This method was used to determine the affiliation of trichothecenes to a certain chemotype, as well as to qualitatively and quantitatively determine mycotoxin concentrations. To a 5 g sample, 25 ml of a mixture of acetonitrile and water (84:16, v/v) was added, and then the mixture was homogenised in a blender for 60 seconds. After filtration through Whatman filter paper no. 41, the filtrate was divided into two segments. The first segment of the filtrate was passed through MycoSep 113 Trich (Romer Labs, USA) and the second one through MycoSep 230 Niv (Romer Labs, USA). Once passed through the appropriate MycoSep column, extracts were filtered (17 mm, PTFE membrane 0.45 µm) and injected by the autosampler (WPS-300SL) into the Dionex Ultimate 3000 liquid chromatographic system with the DAD-3000 detector (Thermo Scientific, Germany). The chromatographic separation of 3ADON, 15ADON and NIV was performed on the analytical column Acclaim Polar Advantage II, C18 (150 × 4.6 mm, 3 µm) at 25°C.

A mixture of water and acetonitrile (90:10; v/v) at a linear flow rate of 1 ml/min for 15 minutes was used as a mobile phase for the separation of 3ADON and 15ADON. Chromatograms were generated at 221 nm. On the other hand, a mixture of water, acetonitrile and methanol (90:5:5; v/v) was used at a linear flow rate of 0.8 ml/min for 15 minutes as a mobile phase for NIV separation. Chromatograms were generated at 218 nm.

Data analysis

The Pearson correlation method was used to determine interdependence between the production potential of total DON of each chemotype and aggressiveness on maize, wheat and barley.

RESULTS

Growth rate

The average daily growth of *F. graminearum* colonies did not differ significantly among the tested isolates in relation to their origin. The lowest daily mycelial growth was observed in isolates obtained from barley. Variations in daily growth of mycelia were most pronounced in the isolates obtained from maize, with a range of variation from 13.16 mm to 32.00 mm. Daily mycelial growth of the isolates obtained from barley ranged from 14.83 mm to 30.16 mm. The lowest variation in daily mycelial growth was observed in isolates derived from wheat and it ranged from 19.5 mm to 30.83 mm (Tables 2-4).

Table 2. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (μg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from maize

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
581	3ADON	64.97	20.92	41.11	2.06	3.18	3.44	26.83
656	3ADON	29.68	10.78	14.78	2.67	2.92	19.19	30.83
914	3ADON	35.68	12.37	14.57	3.10	2.62	21.75	27.66
943/2	3ADON	3.18	0.63	1.03	3.38	3.68	15.75	30.33
1010	3ADON	6.38	0.71	3.01	2.07	3.33	4.94	26.66
1165	3ADON	16.18	1.77	10.65	2.67	2.72	17.94	24.33
1195	3ADON	97.41	24.72	59.60	2.88	2.87	21.56	27.66
1249	3ADON	159.25	72.47	72.85	2.58	2.67	12.94	31.33
1368	3ADON	10.55	ND	3.42	2.16	2.05	7.56	27.16
Average		47.03	18.05	24.56	2.62	2.89	13.89	28.08
257	15ADON	31.88	18.57	5.85	3.03	2.96	22.25	29.33
699	15ADON	54.75	41.32	6.68	4.47	4.21	29.30	29.66
762	15ADON	27.32	14.66	4.39	3.00	3.42	16.00	29.33
880	15ADON	64.96	36.73	22.64	4.95	5.02	31.44	28.00
971	15ADON	27.49	22.66	1.73	4.82	5.07	33.88	25.50
1030	15ADON	24.63	18.67	1.58	3.15	2.89	25.13	29.00
1133	15ADON	154.97	125.97	6.33	3.45	3.66	11.56	26.00
1199	15ADON	94.67	57.59	25.51	1.92	2.18	4.88	26.50
1211	15ADON	56.97	46.71	1.46	2.96	3.68	15.81	27.50
1255	15ADON	43.19	30.57	5.69	2.97	2.00	25.38	30.50
1268	15ADON	107.31	54.67	42.20	4.57	4.85	27.25	31.00
1282/2	15ADON	42.18	25.12	5.18	2.31	2.67	12.06	22.00
1408	15ADON	39.63	26.62	5.32	1.75	1.86	2.06	20.16
1419	15ADON	40.38	20.79	13.97	2.96	3.41	16.44	15.50
1482/2	15ADON	42.67	32.14	2.52	4.58	2.39	33.31	24.66
1495	15ADON	17.69	12.12	1.67	2.42	3.93	23.75	25.66
1554/2	15ADON	94.66	65.02	21.04	2.36	2.57	17.75	23.00
1649	15ADON	19.95	13.41	2.21	5.11	4.67	29.56	28.50
1673	15ADON	14.57	9.75	ND	3.47	5.42	14.50	26.50
1696	15ADON	31.62	25.36	0.19	2.34	2.82	5.88	27.16
1751	15ADON	22.23	15.36	1.96	4.43	3.83	26.13	27.50
2533	15ADON	19.22	11.40	0.59	2.26	4.68	12.44	27.00
2624	15ADON	78.47	55.61	20.10	2.42	2.95	24.31	29.50
2811	15ADON	135.67	114.68	7.74	2.92	2.12	13.19	27.33
2812	15ADON	8.97	5.47	0.89	2.95	3.50	21.00	30.16
2813	15ADON	45.58	33.58	6.40	2.90	2.26	21.63	13.16
2815	15ADON	100.97	93.07	0.82	2.57	2.20	4.63	32.00
Average		53.43	38.06	8.26	3.22	3.38	19.31	26.37
Total average		51.83	33.48	12.45	3.07	3.26	17.96	26.80

In this study, the 3ADON chemotype of *F. graminearum* isolates had generally higher daily growth rates of colonies compared to the isolates of the 15ADON chemotype. On average, mycelial growth rate was higher in 3ADON (28.08 mm) than in 15ADON (26.37 mm) isolates from maize. On the other hand, similar average values of mycelial growth rate were measured in 3ADON and 15ADON isolates obtained from wheat and barley (Tables 2-4).

Pathogenicity test on barley leaves

Pathogenicity tests showed that all isolates (98) caused necrotic spots with yellow halos on the fifth day after inoculation. In the majority of tested isolates, complete necrosis and leaf decay occurred seven days after inoculation. According to the obtained results, the tested isolates showed different pathogenicity on barley leaves.

Tabela 3. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (µg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from wheat

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
800	3ADON	12.31	2.73	3.73	3.6	4.07	20.31	27.00
1370	3ADON	64.36	18.99	36.51	2.43	2.65	14.88	27.83
Average		38.33	10.86	20.12	3.01	3.36	17.59	27.41
203	15ADON	38.15	29.11	2.50	2.56	2.27	5.31	28.33
618	15ADON	57.68	46.81	6.66	2.21	3.42	18.31	29.83
670	15ADON	32.19	26.94	ND	2.91	2.61	16.25	28.16
677	15ADON	103.59	69.97	26.82	2.65	2.65	13.38	27.00
681	15ADON	71.26	58.76	3.90	3.16	3.93	26.56	20.00
687/2	15ADON	39.63	31.88	2.36	2.38	2.35	17.81	30.33
744	15ADON	26.96	16.75	2.85	2.28	2.31	27.75	29.16
749	15ADON	95.95	78.14	9.77	2.46	4.07	12.75	26.00
763	15ADON	9.18	5.68	ND	2.29	2.17	4.30	26.33
764	15ADON	40.36	23.31	9.52	2.84	2.97	15.75	25.33
766	15ADON	6.09	3.18	0.93	3.02	4.63	22.10	30.16
767	15ADON	19.85	12.38	1.65	2.25	2.33	14.56	19.50
779/2	15ADON	72.22	46.58	17.81	2.7	4.05	17.56	30.33
789	15ADON	43.47	27.19	12.37	2.77	2.87	21.69	27.33
795	15ADON	18.56	11.69	1.61	2.11	2.16	4.44	29.33
825	15ADON	21.59	16.07	1.76	2.68	2.95	23.19	28.83
831	15ADON	9.13	6.67	0.43	2.01	2.07	6.31	30.00
836	15ADON	24.56	15.40	2.39	2.67	3.16	11.88	28.16
864	15ADON	28.95	22.07	2.04	2.86	2.97	29.38	30.00
866	15ADON	5.27	1.69	1.08	2.47	2.32	12.45	27.66
870	15ADON	37.86	24.77	5.29	2.82	2.26	11.56	26.83
892	15ADON	11.20	6.91	0.59	2.78	2.75	22.00	26.83
1012	15ADON	16.28	10.05	1.84	2.37	3.32	25.31	27.50
1337	15ADON	39.63	26.46	7.09	2.69	2.5	24.50	27.00
1343	15ADON	46.38	24.69	16.41	2.89	3.06	18.25	27.83
1348	15ADON	75.69	46.84	22.79	4.22	5.06	30.94	26.83
1351/2	15ADON	101.97	90.97	5.91	2.82	2.95	9.00	28.50
1443	15ADON	52.97	35.66	9.06	2.87	2.93	23.07	19.66
1485	15ADON	59.25	50.05	1.49	4.99	5.00	34.88	22.33
1486/2	15ADON	14.69	9.58	0.38	2.46	3.85	20.25	28.16
1490/2	15ADON	46.74	40.66	1.89	2.58	2.66	13.75	28.00
1746	15ADON	41.78	32.15	4.25	2.91	3.38	27.44	27.16
2621	15ADON	46.32	37.75	1.79	4.48	2.75	32.69	27.16
2625	15ADON	19.82	14.37	0.85	2.62	3.05	17.13	24.83
2635	15ADON	45.69	34.60	1.96	3.53	4.17	13.00	30.50
2818	15ADON	37.39	25.13	2.18	3.14	3.43	18.19	29.16
2820	15ADON	57.36	48.85	0.99	2.9	2.91	13.13	30.83
2823	15ADON	94.98	75.12	12.35	4.57	5.37	31.44	29.00
Average		42.38	31.18	5.65	2.86	3.15	18.64	27.36
Total average		42.18	30.19	6.39	2.87	3.16	18.58	27.37

No symptoms of disease appeared on leaves inoculated with sterile distilled water as a negative control.

The obtained results showed that 15ADON isolates expressed stronger pathogenicity under laboratory conditions than 3ADON isolates. The difference in pathogenicity between 3ADON and 15ADON isolates was more pronounced in isolates obtained from barley (3ADON-12.43 mm, 15ADON-18.28 mm) than in isolates from maize (3ADON-13.89 mm, 15ADON-19.32 mm). On the other hand, in isolates obtained from wheat the observed variations in isolate pathogenicity between 3ADON and 15ADON chemotypes were low (3ADON-17.59 mm, 15ADON-18.64 mm). Furthermore, 3ADON isolates originating from wheat (17.59 mm) were more aggressive than 3ADON isolates obtained from maize (13.89 mm) and barley (12.43 mm) (Table 2-4).

Pathogenicity test on maize ears

Pathogenicity of *F. graminearum* isolates in the field was confirmed after artificial inoculation of maize ears. The results indicated that all observed isolates were pathogenic as a characteristic symptom of red-pinkish rot appeared on all inoculated ears. Depending on isolate aggressiveness, ears were completely or partially affected by fungus mycelia. No disease symptoms occurred on ears in the negative control inoculated with sterile distilled water. The results indicated different degrees of aggressiveness that was evaluated based on the degree of infection. The 15ADON chemotypes were more aggressive than the 3ADON chemotypes isolated from maize and barley. The mean pathogenicity evaluation of 3ADON chemotype isolates ranged from 2.62 to 3.02 in isolates from maize and wheat, respectively, while the average evaluation of pathogenicity of the 15ADON chemotype ranged from 2.85 in isolates from wheat to 3.22 in isolates from maize. In contrast to isolates from maize and barley, those of the 3ADON chemotype from wheat showed greater aggressiveness (3.02) compared to the 15ADON isolates (2.85). Considering average values for the isolates obtained from maize, 15ADON isolates demonstrated higher aggressiveness (3.22) than the 3ADON isolates (2.62) (Table 2).

Table 4. Production of deoxynivalenol (DON), 3-acetyl-deoxinivalenol (3ADON) and 15-acetyl-deoxinivalenol (15ADON) (µg/g), aggressiveness and mycelial growth rate of *Fusarium graminearum* obtained from barley

Isolate	Chemotype	DON	15ADON	3ADON	Aggress. maize	Aggress. wheat	Aggress. barley	Mycelial growth (mm)
1528	3ADON	4.12	1.07	1.19	3.27	3.07	22.81	27.00
2254	3ADON	11.09	2.33	3.28	2.46	2.93	2.06	26.16
Average		7.60	1.70	2.23	2.86	3.00	12.43	26.58
654	15ADON	61.23	26.71	26.63	2.63	3.12	18.44	27.66
770/2	15ADON	14.21	6.21	1.35	3.03	2.80	12.50	28.66
798	15ADON	12.83	7.20	0.22	2.62	3.88	7.94	30.16
805	15ADON	46.41	32.81	4.85	4.33	4.06	24.69	18.16
891/2	15ADON	50.12	31.23	10.17	2.78	2.60	14.25	28.66
1217/2	15ADON	75.18	62.26	3.61	2.90	4.00	25.38	28.16
1493	15ADON	21.14	14.19	0.10	5.26	3.93	37.19	28.16
1517	15ADON	15.36	10.34	2.49	2.44	2.95	9.63	28.00
1526	15ADON	41.87	25.89	8.87	2.60	3.21	16.25	26.50
1534	15ADON	8.74	6.23	0.68	5.12	3.43	34.88	28.66
1772	15ADON	58.36	49.84	5.33	2.99	4.61	17.31	26.33
1800	15ADON	8.32	4.49	0.92	3.33	3.15	27.10	29.00
1801	15ADON	104.98	95.07	3.10	2.78	2.87	7.00	14.83
1812	15ADON	31.69	23.68	3.25	2.71	2.51	22.56	22.83
1839	15ADON	17.38	10.59	0.11	1.92	2.00	3.56	26.50
2045	15ADON	19.61	9.86	3.71	2.66	3.19	14.19	26.33
2078	15ADON	29.97	21.10	4.61	2.66	3.80	26.94	27.33
2627	15ADON	42.55	19.98	15.08	3.27	3.53	19.31	29.00
2630	15ADON	22.43	13.44	0.67	3.72	4.06	16.44	24.83
2672	15ADON	102.32	71.05	26.76	2.92	3.05	16.44	26.66
Average		39.23	27.11	6.12	3.13	3.34	18.60	26.32
Total average		36.36	24.79	5.77	3.11	3.31	18.04	26.34

Pathogenicity test on wheat spikes

The aggressiveness of *F. graminearum* isolates on wheat spikes was also tested under field conditions after artificial inoculation. Symptoms on wheat spikes occurred during the formation and maturation of grain. *F. graminearum* isolates differed in the expression of pathogenicity on wheat spikes in the field. No disease symptoms appeared on spikes inoculated with sterile distilled water that served as a negative control. The tested *F. graminearum* isolates expressed variations in aggressiveness on wheat spikes in the field. On average, the degree of aggressiveness ranged from 2.89 to 3.36 in 3ADON isolates, and from 3.15 to 3.38 in 15ADON isolates. The pathogenicity test of the isolates obtained from wheat spikes showed similar results to pathogenicity on maize ears, and isolates of the 15ADON chemotype were on average more aggressive than those of the 3ADON chemotype obtained from maize and barley, while 3ADON isolates obtained from wheat were more aggressive than isolates of the 15ADON chemotype (Tables 2-4).

HPLC method

Using the HPLC method to test *F. graminearum* isolates (98), the following two chemotypes were observed: 3ADON and 15ADON. According to the results, a great diversity was observed in the production of DON derivatives. The majority of *F. graminearum* isolates, regardless of their origin (maize, wheat or barley) belonged to the 15ADON chemotype. The 3ADON chemotype was also detected but a significantly smaller number of isolates (13/98) was found, compared to the 15ADON chemotype (85/98), while none of the tested isolates belonged to the NIV chemotype. The greatest number of isolates obtained from wheat belonged to the 15ADON chemotype (38/40), followed by isolates derived from barley (20/22), while the lowest number of isolates obtained from maize (27/36) belonged to the 15ADON chemotype (Tables 2-4).

The highest level of variation in 15ADON concentrations was observed in isolates derived from maize (5.47-125.97 μg/g) (Table 2), while the lowest variation in concentrations was observed in isolates obtained from barley (1.07-71.05 μg/g) (Table 4). Furthermore, isolates derived from maize also had the greatest range of variation in 3ADON concentrations (1.03-72.85 μg/g), while the lowest variation of this mycotoxin was observed in isolates derived from barley (0.10-26.76 μg/g) (Tables 2 and 4). There was no statistically significant correlation between the aggressiveness and production of total DON of 3ADON and 15ADON isolates (Table 5).

There was no statistically significant correlation between aggressiveness and the production of total DON by 3ADON and 15ADON isolates. The tested isolates originating from maize synthesised the highest average concentration of total DON (51.83 μg/g) (Table 2), followed by isolates from wheat (42.18 μg/g) (Table 3) and barley (36.36 μg/g) (Table 4). Moreover, a comparison of average concentrations of total DON between isolates of the 3ADON and 15ADON chemotypes indicated that 15ADON isolates

Table 5. Correlation coefficient (r) between the production potential of total DON of each chemotype and aggressiveness on maize, wheat and barley

Evaluation of aggressiveness	Total DON (μg/g)	
	Chemotype 15ADON	Chemotype 3 ADON
maize	0.07^{ns}	-0.19^{ns}
wheat	0.06^{ns}	-0.28^{ns}
barley	-0.08^{ns}	0.04^{ns}

ns - not statistically significant

Table 6. Corellation coefficient (r) between two chemotypes in production potential of total DON, 3ADON and 15ADON

	Chemotype 15ADON		Chemotype 3ADON	
	Total DON	15ADON	Total DON	15ADON
15ADON	0.968**		0.966**	
3ADON	0.583**	0.373**	0.979**	0.899**

** Statistically highly significant difference (P ≤ 0,01)

synthesised higher concentrations of total DON. The highest differences in the synthesis of total DON between isolates of the 3ADON chemotype (7.6 μg/g) and 15ADON chemotype (39.2 μg/g) were found in isolates obtained from barely, while these differences were smaller in isolates derived from wheat and maize (Table 2-4). There was a highly significant statistical correlation between the production potentials of total DON, 3ADON and 15ADON in *F. graminearum* isolates belonging to different chemotypes (Table 6).

DISCUSSION

Previous data reported from around the world had shown that the 15ADON chemotype was dominant, while 3ADON chemotype was observed at a much smaller percentage (Gale et al., 2007; Ward et al., 2008; Prodi et al., 2011; Boutigny et al., 2014; Somma et al., 2014: Bozac et al., 2016). Recently, the presence of 3ADON chemotype has increased in the USA and Canada (Gale et al., 2007; Kuhnem et al., 2015), while in Europe it has been detected in Norway (Aamot et al., 2015), Sweden and Finland (Fredlund et al., 2013; Lindblad et al., 2013; Kuhnem et al., 2015), France (Boutigny et al., 2014) and Italy (Somma et al., 2014). The present study shows that the 15ADON chemotype of *F. graminearum* isolates is dominant in Serbia, while the NIV chemotype was not detected. The 3ADON chemotype was observed in a significantly lower percentage than the 15ADON chemotype (13.28% vs. 86.73%, respectively). The highest percentage (25%) of 3ADON chemotype was detected in isolates obtained from maize, followed by barley (9.09%) and wheat (5%). Previous results had shown that 15ADON chemotype produced both 3ADON and NIV but in much lower concentrations (Ward et al., 2008; Puri & Zhong, 2010).

Furthermore, this study shows that the examined *F. graminearum* isolates differed regarding their average concentrations of total DON. The highest average concentrations of DON were determined in isolates collected from maize grain (51.83 μg/g), then from wheat grain (42.18 μg/g) and finally from barley grain (36.36 μg/g). Contrary to our results, previous studies had shown that *F. graminearum* isolates obtained from wheat grain had synthesised higher average concentrations of DON than those derived from maize grain (Tančić et al., 2015). Moreover, Kuhnem et al. (2015) observed significant differences in DON production between isolates from different sources. Isolates derived from wheat produced 73.96 μg/g of DON, which was 30 μg/g higher on average than DON from isolates obtained from maize (44.57 μg/g).

The results indicate that 15ADON isolates of *F. graminearum* synthesised higher total amounts of DON than 3ADON isolates. However, in Canada, Ward et al. (2008) observed that 3ADON isolates of *F. graminearum* had significantly higher production of trichothecenes compared to 15ADON isolates. Similar to these results, in North America, Puri and Zhong (2010) found that 3ADON isolates of *F. graminearum* synthesised 1.5 and 86 times higher total amounts of DON and 3ADON, respectively, than 15ADON isolates. Likewise, 3ADON isolates synthesised six times less 15ADON than 15ADON isolates. In that study, higher concentrations of 15ADON (up to 125.97 μg/g) were observed in 15ADON isolates than in 3ADON isolates (up to 72,85 μg/g). In contrast to the findings in the United States reported by Ward et al. (2008) and Puri and Zhong (2010), the largest differences in the synthesis of total DON in our studies were between 3ADON and 15ADON isolates from barley (five times higher concentrations of total DON in 15ADON isolates). However, Kuhnem et al. (2015) observed that total DON production in wheat did not differ between 3ADON (53.1 μg/g) and 15ADON (47.6 μg/g) isolates.

In the present study, a connection between the pathogenicity of isolates and their origin was not established. These results are in accordance with data reported by Lee et al. (2016), who studied the pathogenicity of *F. graminearum* isolates originating from different cereals and found that it did not depend on the origin of isolates. Namely, isolates obtained from maize were less aggressive than those derived from barley and rice. Similarly, Kuhnem et al. (2015) studied the pathogenicity of *F. graminearum* isolates originating from maize and wheat, and concluded that there was no connection between the pathogenicity and origin of isolates. Nevertheless, when observing variations in pathogenic properties of *Fusarium* spp. obtained from maize and wheat grain, Tančić et al. (2015) detected that intraspecies variation in aggressiveness was the most pronounced in *F. graminearum* isolates. Also, these authors found that *F. graminearum* isolates originating from maize grain were more pathogenic than isolates originating from wheat grain, when pathogenicity was tested on maize.

In previous studies, researchers paid attention to differences in colony growth rate of different chemotypes within the Fg complex (Aamot et al., 2015; Ward et al., 2008; Puri & Zhong, 2010). Conducting the current study, we observed that the average daily growth of mycelium was higher in 3ADON isolates. Similarly, Ward et al. (2008), studying the spread of Fg populations in North America, observed that *F. graminearum* isolates

with the 3ADON chemotype had a significantly higher colony growth on PDA than isolates with the 15ADON chemotype. The present study shows that the greatest variation in daily growth rate between isolates with the 3ADON and 15ADON chemotypes was observed in isolates obtained from maize, compared to those derived from wheat and barley. The results showed that a large variation in mycelial growth was observed among the studied *F. graminearum* isolates, but the difference between isolates of various trichothecene chemotypes (3ADON and 15ADON) was not significant. Furthermore, certain authors had determined that belonging to a chemotype did not affect the mycelial growth rate of isolates. Puri and Zhong (2010), studying *F. graminearum* isolates, observed that there were no significant differences in the growth rate of colonies between 3ADON and 15ADON isolates. Moreover, no significant correlation was observed between the degree of aggressiveness in wheat and the mycelial growth rate of 20 studied *F. graminearum* isolates on PDA (Aamot et al., 2015). There is a large number of different literature data on the association between pathogenicity of isolates and affiliation to trichothecene chemotypes (Ward et al., 2008; Zhang et al., 2012; Puri & Zhong 2010; Aamot et al., 2015; Kuhnem et al., 2015; Carter et al., 2002; Von der Ohe et al., 2010; Li et al., 2010). Trichothecene production can increase the pathogenicity of some species of the genus *Fusarium* depending on their host species (Villafana et al., 2019). Studying the pathogenicity of *F. graminearum* isolates, Carter et al. (2002) proved that different chemotypes were equally pathogenic to wheat, while the NIV chemotype was more pathogenic to maize. Zhang et al. (2012) studied *F. graminearum* isolates obtained from wheat and observed that the pathogenicity of 3ADON and 15ADON chemotypes was significantly higher than the pathogenicity of NIV chemotypes in wheat. Similar results were reported by Li et al. (2010), who also observed that isolates with the 3ADON chemotype were more virulent than NIV populations in China. Furthermore, Gale et al. (2011) established that isolates that synthesised DON caused disease symptoms that spread significantly faster on wheat spikes than those that synthesised NIV. However, Goswami and Kistler (2005) analysed isolates of the Fg complex derived from various hosts, and observed a great variation in the pathogenicity of isolates that did not depend on the type of produced mycotoxin. A statistically significant correlation was observed between the concentration of trichothecene produced by each species and the degree of aggressiveness in wheat.

The obtained results show that isolates with the 15ADON chemotype were more aggressive than those with 3ADON in the pathogenicity test on barley leaves. Moreover, pathogenicity tests on maize and wheat showed that 15ADON isolates derived from maize and barley were more aggressive than isolates with the 3ADON chemotype. The same conclusion was drawn by Aamot et al. (2015), who observed differences in aggressiveness of certain isolates of *F. graminearum* in Norway, and it was found that the aggressiveness of isolates in wheat was associated with trichothecene chemotypes, i.e. the authors detected a significantly higher average aggressiveness in 15ADON isolates than in 3ADON isolates. According to the obtained results on the pathogenicity of isolates derived from maize and wheat, 3ADON isolates obtained from wheat were more aggressive than 15ADON isolates. Kuhnem et al. (2015) established a statistically significant positive correlation between total accumulation of trichothecene (deoxynivalenol [DON] and its acetyl derivatives) and pathogenicity in wheat and maize. One tested isolate produced neither DON nor ADON in wheat and maize grain, but was aggressive to both hosts. Similar results were reported by Puri and Zhong (2010), who observed that 3ADON isolates were more aggressive and caused significantly higher disease intensity in wheat than 15ADON isolates. However, Ward et al. (2008) and Von der Ohe et al. (2010) compared the aggressiveness and production of deoxynivalenol between 3ADON and 15ADON chemotypes and established that there was no significant difference in pathogenicity between them. Similar to our present results, zero or negative correlation between the aggressiveness and production of DON in grain were also revealed by Atanassov et al. (1994) and McCormick (2003).

Global studies on trihotecene synthesis within the Fg complex in cereals are important so as to establish a genotype database which will enable that each shift in populations can be traced in the future (Starkey et al., 2007; Ward et al., 2008; Gale et al., 2007, 2011; Guo et al., 2008; Schmale et al., 2011). In recent years, global climate change has caused variations in agro-climatic conditions, which may stimulate the synthesis of higher concentrations of mycotoxins in cereal grain during the growing season and cause economic losses in the production, as well as increased risk to human and animal health (Moretti et al., 2018). The mentioned reasons indicate a need for permanent monitoring of these toxigenic species in the production of cereals. Knowledge about all factors that directly or indirectly affect disease development is a necessary prerequisite for successful prevention of damage caused by toxigenic and pathogenic fungal species.

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REFERENCES

Aamot, H.U., Ward, T.J., Brodal, G., Vrålstad, T., Larsen, G.B., Klemsdal, S.S. ... Hofgaard, I.S. (2015). Genetic and phenotypic diversity within the *Fusarium graminearum* species complex in Norway. *European Journal of Plant Pathology*, 142, 501-519.

Abramson, D., Clear, R.M., Gaba, D., Smith, D.M., Patrick, S.K., & Saydak, D. (2001). Trichothecene and moniliformin production by *Fusarium* species from western Canadian wheat. *Journal of Food Protection, 64*, 1220-1225.

Atanassov, Z., Nakamura, C., Mori, N., Kaneda, C., Kato, H., Jin, Y-Z. ... Murai, K. (1994). Mycotoxin production and pathogenicity of Fusarium species and wheat resistance to Fusarium head blight. *Canadian Journal of Botany, 72*, 161-167.

Backhouse, D. (2014). Global distribution of *Fusarium graminearum*, *F. asiaticum* and *F. boothii* from wheat in relation to climate. *European Journal of Plant Pathology, 139*, 161-173.

Blandino, M., Haidukowski, M., Pascale, M., Plizzari, L., Scudellari, D., & Reyneri, A. (2012). Integrated strategies for the control of Fusarium head blight and deoxynivalenol contamination in winter wheat. *Field Crops Research, 133*, 139-149.

Boutigny, A.L., Ward, T.J., Ballois, N., Iancu, G., & Ioos, R. (2014). Diversity of the *Fusarium graminearum* species complex on French cereals. *European Journal of Plant Pathology, 138*, 133-148.

Bozac, P., Popescu, S., Botau, D., Boldura, O.M., & Pirvulescu, P. (2016). Molecular characterization for some new *Fusarium* isolates collected from the West Part of Romania. *Romanian Biotechnological Letters, 21*(3), 11560-11568.

Burgess, L.W., Summerell, B.A., Bullock, S., Gott, K.P., & Backhouse, D. (1994). *Laboratory manual for Fusarium research* (3rd ed.). Sydney, Australia: University of Sydney & Royal Botanic Gardens.

Carter, J.P., Rezanoor, H.N., Holden, D., Desjardins, A.E., Plattner, R.D., & Nicholson, P. (2002). Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum*. *European Journal of Plant Pathology, 108*, 573-583.

Fredlund, E., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., & Lidlblad, M. (2013). Deoxynivalenol and other selected *Fusarium* toxins in Swedish oats–occurrence and correlation to specific *Fusarium* species. *International Journal of Food Microbiology, 167*, 276-283.

Gale, L.R., Harrison, S.A., Ward, T.J., O'Donnell, K., Milus, E.A., Gale, S.W., & Kistler, H.C. (2011). Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. *Phytopathology, 101*, 124-134.

Gale, L.R., Ward, T.J., Balmas, V., & Kistler, H.C. (2007). Population subdivision of *Fusarium graminearum sensu stricto* in the upper Midwestern United States. *Phytopathology, 97*, 1434-1439.

Goswami, R.S., & Kistler, H.C. (2005). Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice. *Phytopathology, 95*, 1397-1404.

Guo, X.W., Fernando, W.G.D., & Seow-Brock. H.Y. (2008). Population structure, chemotype diversity, and potential chemotype shifting of *Fusarium graminearum* in wheat fields of Manitoba. *Plant Disease, 92*, 756-762.

Imathiu, M.S., Ray. V.R., Back, M., Hare, C.M., & Edwards, G.S. (2009). *Fusarium langsethiae* pathogenicity and aggressiveness towards oats and wheat in wounded and unwounded *in vitro* detached leaf assays. *European Journal of Plant Pathology, 124*, 117-126.

Jennings, P., Coates, M.E., Walsh, K., Turner, J.A., & Nicholson, P. (2004). Determination of deoxynivalenol- and nivalenol-producing chemotypes of *Fusarium graminearum* isolated from wheat crops in England and Wales. *Plant Pathology, 53*, 643-652.

Kuhnem, P.R., Del Ponte, E.M., Dong, Y., & Bergstrom, G.C. (2015). *Fusarium graminearum* isolates from wheat and maize in New York show similar range of aggressiveness and toxigenicity in cross-species pathogenicity tests. *Phytopathology, 105*, 441-448.

Lee, T., Paek, J.S., Lee, K.A., Lee, S., Choi, J.H., Ham, H. ... Ryu, J.G. (2016). Occurrence of Toxigenic *Fusarium vorosii* among Small Grain Cereals in Korea. *The Plant Pathology Journal, 32*, 407-413.

Lee, T., Zhang, H., Diepeningen, A., & Waalwijk, C. (2015). Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. *Food Additives and Contaminants, Part A, 32*, 453-460.

Li, W., Hu, Y.C., Chen. Y., Zhang, A.X., & Chen, H.G. (2010). Phylogenetic analysis, chemotype diversity, and pathogenicity of the *Fusarium graminearum* clade in the Yangtze basin. *Mycosystema, 29*, 51-58.

Lindblad, M., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., & Fredlund, E. (2013). Deoxynivalenol and other selected *Fusarium* toxins in Swedish wheat–Occurrence and correlation to specific *Fusarium* species. *International Journal of Food Microbiology, 167*, 284-291.

Logrieco, A., Moretti A., Ritieni, A., Bottalico, A., & Corda, P. (1995). Occurrence and toxigenicity of *F. proliferatum* from preharvest maize ear rot and associated mycotoxins in Italy. *Plant Disease, 79*, 727-723.

Logrieco, A., Mule, G., Moretti, A., & Bottalico, A. (2002). Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. *European Journal of Plant Pathology, 108*, 597-609.

Luongo, D., Severino, L., Bergamo, P., D'Arienzo, R., & Rossi, M. (2010). Trichothecenes NIV and DON modulate the maturation of murine dendritic cells. *Toxicon, 55*, 73-80.

McCormick, S. (2003). The role of DON in pathogenicity. In Leonard. K.J. Bushnell. W.R. (Eds.), *Fusarium head blight of wheat and barley* (pp 165-183). St. Paul, MN, USA: American Phytopathological Society.

Mesterházy, A., Bartók, T., Mirocha, C.G., & Komoróczy, R. (1999). Nature of wheat resistance to *Fusarium* head blight and the role of deoxynivalenol for breeding. *Plant Breeding, 118*, 97-110.

Miller, J. D., Greenhalgh, R., Wang, Y. Z., & Lu, M. (1991). Trichothecene chemotypes of three *Fusarium* species. *Mycologia, 83*, 121-130.

Moretti, A., Panzarini, G., Somma, S., Campagna, C., Ravaglia, S., Logrieco, A.F., & Solfrizzo, M. (2014). Systemic growth of *F. graminearum* in wheat plants and related accumulation of deoxynivalenol. *Toxins, 6*, 1308-1324.

Moretti, A., Pascale, M., & Logrieco, A.F. (2018). Mycotoxin risks under a climate change scenario in Europe. *Trends in Food Science and Technology, 84*, 38-40.

Nielsen, L.K., Jensen, J.D., Rodríguez, A., Jørgensen, L.N., & Justesen, A.F. (2012). TRI12 based quantitative real-time PCR assays reveal the distribution of trichothecene genotypes of *F. graminearum* and *F. culmorum* isolates in Danish small grain cereals. *International Journal of Food Microbiology, 157*, 384-392.

Obradović, A., Stanković, S., Krnjaja, V., Nikolić, A., Ignjatović-Micić, D., Stepanović, J., & Duduk, B. (2017). Trichothecene chemotype diversity of *Fusarium graminearum* isolated from wheat, maize and barley in Serbia. *Genetika, 49*(1), 355-364.

O'Donnell, K., Kistler, H.C., Tacke, B.K., & Casper, H.H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum,* the fungus causing wheat scab. In: *Proceedings of the National Academy of Sciences of the USA, 97*(14), 7905-7910.

O'Donnell, K., Ward, T.J., Aberra, D., Kistler, H.C., Aoki, T., Orwig, N. ... Klemsdal, S.S. (2008). Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia. *Fungal Genetics and Biology, 45*(11), 1514-1522.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kiestler, H.C, & Aoki, T. (2004). Genealogical concordance between mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology, 41*, 600-623.

Prodi, A., Purahong, W., Tonti, S., Salomoni, D., Nipoti, P., Covarelli, L., & Pisi, A. (2011). Difference in chemotype composition of *Fusarium graminearum* populations isolated from durum wheat in adjacent areas separated by the Apennines in NorthernCentral Italy. *Plant Pathology Journal, 27*, 354-359.

Puri, D.K., & Zhong, S. (2010). The 3ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15ADON population in spring wheat. *Phytopathology*, *100*, 1007-1014.

Reid, L.M., Hamilton, R.I., & Mather, D.E. (1996). Screening maize for resistance to gibberella ear rot (Technical Bulleten 5E). Canada: Research Branch Agriculture and Agri-Food Canada.

Sarver, B.A.J., Ward, T.J., Gale, L.R., Broz, K., Kistler, H.C., Aoki, T. ... O'Donnell, K. (2011). Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. *Fungal Genetics and Biology, 48*, 1096-1107.

Schmale, D.G., Wood-Jones, A.K., Cowger, C., Bergstrom, G.C., & Arellano, C. (2011). Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA. *Plant Pathology, 60*, 909-917.

Somma, S., Petruzzella, A.L., Logrieco, A.F., Meca, G., Cacciola, O.S., & Moretti, A. (2014). Phylogenetic analyses of *Fusarium graminearum* strains from cereals in Italy and characterisation of their molecular and chemical chemotypes. *Crop and Pasture Science, 65*, 52-60.

Starkey, D.E., Ward, T.J., Aoki, T., Gale, L.R., Kistler, H.C., Geiser, D.M. O'Donnell, K. (2007). Global molecular surveilliance reveals novel Fusarium head blight species and trichotecene toxin diversity. *Fungal Genetics and Biology, 44*, 1191-1204.

Tančić, S., Stanković, S., Lević, J., & Krnjaja, V. (2015). Correlation of deoxynivalenol and zearalenone production by *Fusarium* species originating from wheat and maize grain. *Pesticides and Phytomedicine, 30*, 99-105.

Toth, B., Mesterhazy, A., Horvath, Z., Bartok, T., Varga, M., & Varga, J. (2005). Genetic variability of central European isolates of the *Fusarium graminearum* species complex. *European Journal of Plant Pathology, 113*, 35-45.

Villafana, R.T., Ramdass, A.C., & Rampersad, S.N. (2019). Selection of *Fusarium* trichothecene toxin genes for molecular detection depends on TRI gene cluster organization and fene function. *Toxins, 11*,1, 36.

Von der Ohe, C., Gauthier, V., Tamburic-Ilincic, L., Brule-Babel, A., Fernando, W.G.D., Clear, R. ... Miedaner, T. (2010). A comparison of aggressiveness and deoxynivalenol production between Canadian *Fusarium graminearum* isolates with 3-acetyl and 15-acetyldeoxynivalenol chemotypes in field-grown spring wheat. *European Journal of Plant Pathology, 127*, 407-417.

Ward, T.J., Clear, R.M., Rooney, A.P., O'Donnell, K., Gaba, D., Patrick, S. ... Nowicki, T.W. (2008). An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. *Fungal Genetics and Biology, 45*, 473-484.

Yli-Mattila, T., Gagkaeva, T., Ward, T.J., Aoki, T., Kistler, H.C., & O'Donnell, K. (2009). A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. *Mycologia, 101*, 841-852.

Zhang, H., van der Lee, T., Waalwijk, C., Chen, W., Xu, J., Xu, J. ... Feng, J. (2012). Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive isolates. *Plos One, 7*(2). e31722.

Zhang, H., Zhang, Z., van der Lee, T., Chen, W.Q., Xu, J., Xu, J.S.... Feng, J. (2010). Population genetic analyses of *Fusarium asiaticum* populations from barley suggest a recent shift favoring 3ADON producers in Southern China. *Phytopathology, 100*, 328-336.

Agresivnost i sinteza trihotecena izolata *Fusarium graminearum* poreklom sa zrna žitarica u Srbiji

REZIME

Cilj ovog rada bio je da se utvrde razlike u agresivnosti i sintezi trihotecena kod izolata *F. graminearum* poreklom sa zrna kukuruza, pšenice i ječma u Srbiji. Proučavanjem izolata *F. graminearum* (98), izolovanih iz različitih agroekoloških uslova Srbije u periodu od 1993. do 2010. godine, primenom HPLC metode, identifikovana su dva trihotecen hemotipa: 3-acetil-deoksinivalenol (3ADON) i 15-acetil-deoksinivalenol (15ADON). Uočen je veliki diverzitet u sintezi deoksinivalenol (DON) derivata. Bez obzira na poreklo (kukuruz, pšenica ili ječam), većina ispitivanih izolata *F. graminearum*, pripadala je 15ADON hemotipu. Identifikovan je i 3ADON hemotip, ali u znatno manjem broju (13/98), u poređenju sa 15ADON hemotipom (85/98). Nijedan od ispitivanih izolata nije pripadao NIV hemotipu. Ispitivani izolati su pokazali različitu patogenost na listu ječma, klasu pšenice i klipu kukuruza. Prosečna patogenost ispitivanih izolata bila je najveća na listu ječma. Uočeno je da su izolati poreklom sa pšenice imali najveći prosečan dnevni porast micelije (27.37 mm). Utvrđena je statistički visoko značajna pozitivna korelacija između 3ADON i 15ADON izolata *F. graminearum* i sinteze ukupnog DON, 3ADON i 15ADON. Međutim, između stepena agresivnosti ispitivanih izolata i sinteze ukupnog DON nisu utvrđene statistički značajne korelacije.

Ključne reči: žitarice, *Fusarium graminearum*, mikotoksini, hemotipovi, trihoteceni

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Original scientific paper

Impact of natural products on *Acyrthosiphon pisum* density on *Pisum sativum* L. and forage quality

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SUMMARY

A field trial was conducted at the Institute of Forage Crops (Pleven, Bulgaria) from 2015 to 2017. It studied the effects of natural products on *Acyrthosiphon pisum* density, as well as changes in the chemical composition, content of plant fibre components and enzyme degradability in forage pea. Treatments with the natural insecticides Madex and Agricolle, applied alone or in combination with the organic fertilizers Lithovit and Nagro were performed twice - at the beginning of the flowering stage and one week later. The fertilizers used in the trial are environmentally safe and approved for use in organic production. The synthetic products Kristalon, a foliar fertilizer, and Proteus 110 OD, an insecticide, were used for comparison. The application of natural products, either alone and in combination, resulted in a reduction in pea aphid density. Applying Agricolle with Nagro, followed by Lithovit with Agricolle, led to the highest aphid number decrease (70.0 and 51.1%, respectively). An optimal combination of decrease in the content of plant cell wall fibre components, cellulose and lignification degree with a significant increase in forage *in vitro* enzyme digestibility was established after applying Agricolle with Lithovit and Agricole with Nagro. Digestibility reached 71.8 and 69.8%, respectively, an increase of 8.2-5.2%, while ADF, cellulose and lignification degree decreased from 7.1 and 7.7%, 8.0 and 23.4%, amd 10.5 and 6.8% after applying Agricolle with Lithovit and Agricole with Nagro, respectively. In comparison, the synthetic products Kristalon and Kristalon with Proteus increased forage quality, but to a relatively lesser extent. A stronger linear relationship was found between aphid density and dry matter digestibility, compared to the content of neutral detergent fibres. Pea forage with low content of plant cell wall fibre components, cellulose and lignification degree, high protein content, and digestibility after treatment with the natural product Agricolle, and its combinations with Lithovit and Nagro, make it a very good complement to other forages in dairy cow rations.

Keywords: pea aphid, natural products, pest control, forage pea, chemical composition, forage quality

INTRODUCTION

Legumes usually result in higher intake and animal production than grass silages of comparable digestibility. This is true both for silage (Dewhurst et al., 2003) and pasture herbaceous plants (Fraser et al., 2004). An additional benefit of legumes is that the rate of decrease in digestibility is lower than in grasses as maturity is progressing (Dewhurst et al., 2009).

Peas are very tasty and digested completely, which leads to greater intake and profit. In any case, pea provides equal or improved animal productivity. The advantage of using it for feeding is that it can be purchased for less than the comparative value of other forages based on nutrient content. Virtually all producers who have used pea in animal diets appreciate the positive results and nutrition, safety and palatability of this legume (Anderson et al., 2006). An increase in pea growing area under organic production would help to increase the pea supply on the feed market.

Organic production is known as a specific way to safeguard the production environment, in particular biodiversity, and provide healthy and quality nutrition (Grigorova & Arabska, 2013). Pea plants provide a valuable source of nitrogen in organic farming (Gerdgikova et al., 2012). Organic cultivation of *Pisum sativum* and the use of organic products in Bulgaria is at an early stage and needs further research. It is necessary not only to evaluate the productive potential of this crop in organic agriculture but analyse additionally its chemical composition, nutritive value, energy yield and feed units.

Pea aphids have become a serious pest of feed and grain peas in recent years in Bulgaria. Research has focused mainly on understanding the potential for aphid damage and how to manage them in relation to pea productivity. Feed infestation with these species has been extremely severe, especially in organic farming, but there are no available data on actual damage to yield and feed quality from these infestations.

The aim of the current study was to determine the effect of natural products on *Acyrthosiphon pisum* density, as well as changes in the chemical composition, content of plant fibre components and enzyme degradability of forage spring pea.

Table 1. Trial variants and product characteristics

Variants	Application rates, per ha	Active ingredients	Producer
1. Control	-	treated with distilled water	
2. Lithovit natural leaf nano-fertilizer	2000 g ha^{-1}	contains calcium carbonate from natural reserves with micronutrients: 79.19 % $CaCO_3$; 4.62 % $MgCO_3$; 1.31 % Fe	Ctheo Vita Ltd., Germany
3. Nagro bio-organic nano-fertilizer	500 ml ha^{-1}	contains micro- and macro-elements (molybdenum, magnesium, cobalt, manganese, zinc, iron, copper, boron, nitrogen and phosphorus), meso elements, microhumates, vitamins, fulvo acid, amino acids, phytohormones, organic solvents, silicon compounds, organic calcium, antioxidants, adaptogens, metabolites, nitrogen fixators	Scientific Production Association "Bioplant", Russian Federation
4. Madex	100 ml ha^{-1}	Cydia pomonella Granulovirus - CpGV-V15 3 x 10^{13} granules/liter	Switzerland
5. Agricolle	300 ml / 100 l water	natural polysaccharides for sticking small insects	Cal-Agri products LLC, USA
6. Lithovit+ Madex	2000 g ha^{1}+100 ml ha-1		
7. Lithovit+ Agricolle	2000 ml ha^{-1} + 300 100 l water		
8. Nagro+ Madex	500 ml ha^{-1}+100 ml ha-1		
9. Nagro+Agricolle	500 ml ha^{-1}+ 300 100 l water		
10. Kristalon	2000 g ha^{-1}	nitrogen 17.0%, nitrate nitrogen 8.0%, ammonium nitrogen 9.0%, phosphorus (P_2O_5) 6.0%, potassium 18.0%, magnesium 2.0%	Nu 3 BV, Netherlands
11. Proteus 110 OD	600 ml ha^{-1}	Thiacloprid 100 g/l; Deltamethrin 10 g/l	Bayer CropScience
12. Kristalon+Proteus	2000 g ha^{-1} + 600 ml ha^{-1}		

MATERIAL AND METHOD

A study was conducted from 2015 to 2017 to examine the effects of natural products, applied either alone or in combination, on pea aphid control (*Acyrthosiphon pisum*, Hemiptera, Sternorrhyncha Aphididae) in spring forage pea (*Pisum sativum* L.), variety Pleven 4, in the experimental field of the Institute of Forage Crops, Pleven, Bulgaria. Synthetic products, a foliar fertilizer and an insecticide, were used for comparison. Trial variants and product characteristics are shown in Table 1. The bio-organic universal nano fertilizer Nagro is an environmentally safe fertilizer, allowed for use in organic production according to producers (Chekmarev et al., 2018). Lithovit is a suitable fertilizer for use in organic farming under Council Regulation (EEC) No. 2092/91 – European Community. Treatments were performed twice: at the beginning of the flowering stage and one week later. Aphid density was calculated by counting them per individual plant every two to three days over a period beginning one day before treatment until the harvest of green fodder mass.

Products with different levels of efficacy against pea aphids were used either alone or in combination, as well as the control, to create different degrees of infection with aphids. The experiments were set out using the long plot method with three replications of each variant, and the plot size was 6.50 m^2. Each product used and the untreated check were applied to the randomized replicated plots.

The chemical composition of aboveground biomass harvested at the flowering stage was determined by standard methods and included: crude protein (CP) by Keldahl (N x 6.25) and crude fibre (CF) by Weende system (AOAC, 2007). The content of plant cell wall fiber components was analysed as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid-detergent lignin (ADL), according to Goering and Van Soest (1970), and degree of lignification coefficient=ADL/NDFx100. *In vitro* enzyme digestibility of dry (IVDMD) and organic (IVOMD) matter was determined using a two-stage pepsin-cellulase method proposed by Aufrere (1982, cited by Todorov et al., 2010).

The data were summarized and presented as average for 2015-2017. The data were exposed to one-way ANOVA, the mean being compared by Tukey's test at 5% probability ($p \leq 0.05$). Simple correlation and regression analyses were used to establish the relationship between pea density and some forage quality components (NDF, ADF and IVDMD).

RESULTS AND DISCUSSION

Forage pea plots were checked weekly during May for initial infestation of *A. pisum*. At the beginning of May, pea aphids were found in the test plots, and one week later treatments were performed.

Impact of natural products on *Acyrthosiphon pisum* density

Aphid density in treated plots and untreated check were similar during the three-year study period, and the differences were statistically insignificant when plants were treated with the foliar fertilizer Lithovit ($F_{11,59}$=13.593; p=0.021) (Table 2). In contrast, aphid numbers after treatment with the natural fertilizer Nagro decreased by significant 52.2% and approached the density noted after using the bioinsecticide Agricolle. Nagro is a highly concentrated complex liquid bio-organic fertilizer that has the properties of a bioinsecticide with antiferromonic action. In addition to increasing resistance to unfavourable climatic factors (drought, low temperatures) and increasing leaf area, and subsequently the intensity of plant photosynthesis (according to the manufacturer), Nagro also had a repellent effect against aphids, which resulted in a reduction in their numbers.

Table 2. Average number of *Acyrthosiphon pisum* Harr. specimens per plant during the growing season affected by treatment with products of different biological action in spring forage pea

Products	2015-2017	Decrease %
1. Control	70.8 d*	–
2. Lithovit	60.3 cd	–14.8
3. Nagro	33.8 ab	–52.2
4. Madex	57.2 c	–19.2
5. Agricolle	31.8 ab	–55.0
6. Lithovit +Madex	55.4 c	–21.8
7. Lithovit +Agricolle	34.6 ab	–51.1
8. Nagro+Madex	37.6 b	–46.8
9. Nagro+Agricolle	21.3 a	–70.0
10. Kristalon	72.2 d	+1.9
11. Proteus 110 OD	28.6 ab	–59.6
12. Proteus+Kristalon	23.6 a	–66.6

*Means in each column marked by the same letter are not significantly different (p<0.05)

The biological insecticides Madex and Agricolle decreased aphid numbers to different degrees, based on their modes of action. Madex applied alone reduced their numbers by 19.2%, and the difference was significant compared to the untreated check. A significantly lower density and considerable reduction of 55.0 % was found after Agricolle treatment. The product has a natural impact, which is based on the properties of some natural polysaccharides for sticking small insects (such as aphids, whiteflies, fleas, etc.) on plants, soil or other surfaces.

The interaction of bioinsecticides with the natural fertilizers Lithovit and Nagro had different effects on *A. pisum* density. The use of Lithovit with Madex decreased aphid density by 21.8%, while the difference between Madex and Lithovit+Madex combination was insignificant. The application of Lihtovit with Agricolle was associated with a considerably stronger reduction - an average of 51.1% due to the bioinsecticide action. Compared to Agricolle used alone, the difference was insignificant.

Applying Agricolle with Nagro led to the best interaction of active substances, compared to the other combinations, and the largest reduction in pea aphid density. The reduction in numbers by an average of 70.0% was the highest decrease among all other products and combinations, and a statistically significant decrease was observed compared to all treatments with Madex. The most successful combination was Nagro with Agricolle, followed by Lithovit with Agricolle.

The combination of the synthetic product Proteus 110 OD with Kristalon provided a marked reduction in the population density of *A. pisum* by an average of 66.6%, and differences in treatment with Agricolle and Nagro, and the combined use of Agricolle were insignificant.

Product applications revealed statistical differences between treatments and the untreated check. Those differences in aphid density created different degrees of damage over time until forage plots were harvested, and affected the forage quality components.

Impact of natural products on forage quality

Forage quality reflects the ability of forage to meet nutritional needs of consuming animals. Plants differ regarding quantitative contents of different nutrients, depending on aphid damage. Nutrients vary in the amounts of fats, proteins, carbohydrates, fibre and other micro-nutrients that are present in plant tissues.

Dry matter contains the essential nutrients in a feed. Nutrient contents of feeds are determined on the basis of ash (moisture included) or dry matter (no moisture) contents. Nutrient content will always be higher in DM, compared to ash in each feed. The determination of DM can be used to assess whether the moisture content in a feed is within expected limits. Moisture content should not exceed 15% of dry forage, as this amount of moisture is necessary for promoting mould growth.

In the present study, moisture did not exceed the defined limit, and dry matter after treatment with natural products ranged from 93.24 -94.6% (Table 3).

Table 3. Principal composition, content of fiber components, and digestibility of dry forage pea biomass after treatment with products of different biological action

V	DM	Ash	CP	CF	NDF	ADF	HEMI	CELLU	LIGNIF	IVDMD	IVOMD
1	93.81 abc*	6.08 ab	171.2 a	242.3 e	395.1 c	329.1 c	66.0 bcd	263.1 c	13.3 abc	66.33 cd	66.75 cd
2	94.31 bc	7.11 c	184.0 d	240.9 e	431.2 ef	363.6 e	67.6 cde	296.0 e	15.6 cd	58.97 def	59.73 def
3	94.04 abc	6.78 bc	185.6 d	223.5 c	376.7 ab	319.7 b	57.0 b	262.7 b	13.5 abc	66.91 cde	67.28 de
4	94.60 c	6.82 bc	190.4 e	220.4 b	434.5 f	360.9 e	73.7 de	287.2 e	17.8 d	61.12 b	60.99 ab
5	93.41 bc	5.71 a	170.3 a	212.3 a	380.8 b	304.4 a	76.4 e	228.0 a	13.3 abc	67.9 a	68.12 a
6	93.39 bc	7.07 c	197.4 f	241.4 e	408.2 d	343.1 d	65.1 bcd	278.0 d	13.3 abc	66.33 c	66.75 c
7	93.71 abc	5.48 a	170.5 a	223.8 c	369.3 a	305.7 a	63.6 bc	242.1 a	11.9 ab	71.79 g	72.28 h
8	93.90 abc	6.12 ab	178.5 c	262.7 f	409.2 d	340.4 d	68.8 cde	271.6 c	15.2 cd	61.46 b	61.96 b
9	93.24 ab	6.11 ab	176.8 c	224.8 c	405.6 d	303.6 a	102.0 f	201.6 a	12.4 abc	69.76 f	69.86 g
10	92.94 a	6.10 ab	173.8 b	218.8 b	424.6 e	303.5 a	121.1 j	182.4 a	11.1 a	68.42 ef	69.32 fg
11	93.46 bc	5.69 a	170.4 a	237.5 d	389.8 c	317.5 b	72.3 cde	245.2 a	15.1 bcd	65.59 c	65.70 c
12	93.67 c	6.39 abc	177.4 c	237.7 d	369.2 a	325.9 c	43.3 a	282.6 b	15.4 cd	68.55 ef	68.74 efg

V – Variant; DM - dry matter, %; CP - Crude protein, g kg^{-1}; CF - Crude fibre; NDF - Neutral-detergent fibre; ADF - Acid-detergent fibre; HEMI - Hemicellulose; CELLU - Cellulose; LIGNIF - Degree of lignification; IVDMD - *In vitro* dry matter digestibility, %; IVOMD - *In vitro* total matter digestability, %; 1 - Control; 2 - Lithovit; 3 - Nagro; 4 - Madex; 5 - Agricolle; 6 - Lithovit+Madex; 7 - Lithovit+Agricolle; 8 - Nagro+Madex; 9- Nagro+ Agricolle; 10- Kristalon; 11- Proteus; 12- Kristalon+ Proteus;

*Means in each column marked by the same letter are not significantly different ($p<0.05$)

Although different hypotheses have been proposed about the use of crude protein as a feed quality measure, it continues to be a frequently used parameter. The crude protein content is very different in feeds, but higher protein concentration is usually associated with higher quality in various feeds. As feed plants mature, their crude protein is diluted by increasing fibre content.

The results demonstrate that the applied natural products provided significantly higher contents of crude protein ($F_{11,2}$=2.506; p=0.001) (from 1.5 to 15.3%) and lower crude fibre contents ($F_{11,2}$=2.446; p=0.017) (0.6 – 12.4%), compared to the untreated control. Exceptions were observed for Agricolle and Lithovit + Agricolle, where crude protein and fibre had lower contents, and insignificant differences compared to the control. The optimal combination for aphid control, associated with the most pronounced increase in crude protein and decrease in crude fibre was found after the use of Nagro, Madex and Nagro + Agricolle.

The application of synthetic products impacted slightly the values of crude protein and crude fibre, compared to the natural products. Only Proteus led to a greater decrease in crude fibre (by 9.7%).

Sulc et al. (2015) reported comparable results. According to them, crude protein content in untreated alfalfa was lower ($p < 0.05$) than it was in plants subjected to an early insecticide treatment against *Empoasca fabae* Harris. According to other studies, the use of biologically active substances had positive effects on protein content and increased crude protein productivity (Stakhova et al., 2000; Zhelyazkova, 2007).

The detergent feed analysis system is used to characterize fibre or total cell wall content of forage or feed. That portion of forage is termed neutral detergent fibre (NDF), which contains primary components of plant cell walls, namely hemicellulose, cellulose, and lignin. Another parameter of fibre is acid detergent fibre (ADF), a subset of NDF. Acid detergent fibre contains poorly digestible cell wall components, namely cellulose, lignin, and other very resistant substances. NDF and ADF are good indicators of feed quality, and their lower values in feed suggest higher-quality feed and *in vitro* dry matter digestibility. According to Fahey and Hussein (1999), structural polyosides in forage plants account for 300 to 800 g kg^{-1} (30-80 %) of forage dry matter and they are the main source of nutritional energy for ruminants, although less than 50% of them are digestible and utilized.

In the present study, differences in pea aphid density had a different impact on forage quality and the content of structural fibre fraction (polyosides) in plant cell walls. The impact of the products was diverse, depending on their origin.

Treatments with Lithovit, Madex and its combinations with natural fertilizers increased the NDF and ADF components due to weak protective effects against *A. pisum* and pronouncedly stronger aphid infection compared to other treatments (Table 2). A similar trend was observed regarding cellulose (Table 3).

The use of Nagro, as well as the natural insecticide Agricolle, alone or in combination with natural fertilizers, had entirely positive effects on feed quality, associated with statistically significant reductions in NDF ($F_{11,2}$=8.193; p=0.001), ADF ($F_{11,2}$=5.994; p=0.034) and cellulose ($F_{11,2}$=6.918; p=0.018) values. That was due to the protective effect of Agricolle and Nagro, which led to a strong reduction in *A. pisum* density. The most favourable effect, expressed as a significant reduction in fibre content, was found after treatment with Lithovit and Agricolle (decrease by 6.5, 7.1 and 8.0% for NDF, ADF and cellulose, respectively), Agricolle (by 3.6, 7.5 and 13.3% for NDF, ADF and cellulose) and Nagro (by 4.7, 2.9 and 0.2% for NDF, ADF and cellulose). Compared to them, the synthetic products reduced fibre components and cellulose to a lesser extent, and NDV and cellulose values for Kristalon and its combination with Proteus significantly exceeded the control value, respectively.

The ADF values were found to be highly correlated ($P < 0.05$) with CP (r=0.700), and ADF with cellulose (r=0.845). Similar results were reported by Jančík et al. (2008).

Total digestible animal polyoside hemicellulose in pea dry mass was several times lower - from 43.3 to 121.1 g kg^{-1}, compared to that of non-digestible cellulose - from 182.4 to 296.0 g kg^{-1}, a trend confirmed in this study as well. There was an increasing trend in hemicellulose contents, while a significant decrease was noted for Kristalon + Proteus 110 OD only, and insignificant for Nagro, and the combinations Lithovit+Madex and Lithovit+Agricolle ($F_{11,2}$=10.060; p=0.033).

Lignification extent was not impacted by the activity of products, and not found to be significantly different in comparison to the control, except for Madex ($F_{11,2}$=3.274; p=0.001).

Decreasing the content of fibre components, determined as NDF, ADF and cellulose, and increasing *in vitro* digestibility of dry matter, are considered as important effects in forage quality evaluation.

The variation range of *in vitro* dry matter digestibility (IVDMD) was from 66.33 to 71.79%. The treatment process for lower aphid density did not lead to increased *in vitro* digestibility in all variants. The digestibility of forage pea investigated was higher after application of the bioinsecticide Agricolle and its combinations, as well as Nagro.

Forage quality in terms of IVDMD was the highest when the combination Lithovit with Agricolle was used (an increase of 8.2%), followed by Agricolle with Nagro (an increase of 5.2%), and the difference in comparison to control data was significant ($F_{11,2}$=3.581; p=0.011). Increase in IVDMD values in other treatments mentioned above was insignificant and varied from 0.9 to 2.4%.

According to Vilela et al. (2020), higher crude protein content and digestibility resulted in their experiment in a higher predicted intake and high-quality feed because of lower contents of cellulose, hemicellulose and lignin. That trend was confirmed in the present study.

Identical trends were noted regarding *in vitro* total matter digestibility, where the combined use of Lithovit with Agricolle, and Agricolle with Nagro led to significant increase of 8.3 and 4.7%, respectively ($F_{11,2}$=3.677; p=0.027). Compared to organic products, treatments with Kristalon, and Kristalon + Proteus increased the IVDMD and IVOMD values, but to a lesser extent.

Figure 1 shows the relationship between pea aphid density and some forage quality components. *Acyrthosiphon pisum* infestation had a negative effect on forage quality associated with an increase in the content of neutral detergent fibres and decrease in dry matter digestibility. A stronger linear relationship (R^2 = 0.59) was found between aphid density and dry matter digestibility. Linear regression indicated there is a 0.18% loss in IVDMD for each number increase in density. The relationship between pea aphid density and other feed quality components was weak.

Therefore, the presented data indicate that the level of aphid infestation caused during the flowering development stages impacted the quality of forage pea at harvest. The use of the bioinsecticide Agricolle, alone or in combination with biofertilizers, protected plants and improved forage quality.

Various authors have reported that crude protein, lignin, ADF, and IVDMD were affected by insecticide treatments, as well as by damage from sucking insect pests in untreated control. All parameters were negatively affected by insect damage in the untreated variant. For example, Singh et al. (2007) assessed qualitative losses in sorghum following *Pyrilla perpusilla* Walker infestation and revealed a significant decrease in *in vitro* dry matter digestibility of the whole plant. The increasing fibre components (NDF and ADF) were acting as an additional factor in reducing dry matter digestibility.

Banyal et al. (2015) reported data on cowpea quality parameters which clearly revealed that the incidence of insects-pests was directly correlated with forage quality.

In the present study, there was a significant increase in dry matter, crude protein, ash and *in vitro* dry matter digestibility in the protected crop, as compared to unprotected crop. The NDF, ADF, hemicellulose and phenolics were lower in the protected crop than in unprotected crop.

In a later study, Bynum and Bell (2019) confirmed a positive impact of insecticides on forage quality by controlling aphid density - a trend also found in the present study after treatment with natural insecticide products. The authors reported that sugarcane aphid damage impacted negatively sorghum silage quality. The studied components in terms of forage quality, such as ADF, lignin, IVTDMD, milk per ton and relative feed quality, had statistically significant levels of difference in the untreated variants when compared to insecticide treatments. According to these authors, heavy aphid pressure had a negative effect, resulting in increase in ADF and lignin values, and decrease in IVTDMD, milk per ton and relative feed quality. Additionally, they found that ADF and lignin were positively correlated to sugarcane damage, indicating that plants became lignified under increased aphid pressure, decreasing forage quality and its digestibility.

High-quality forage pea is palatable and often maximizes intake and productivity by dairy cows. Low

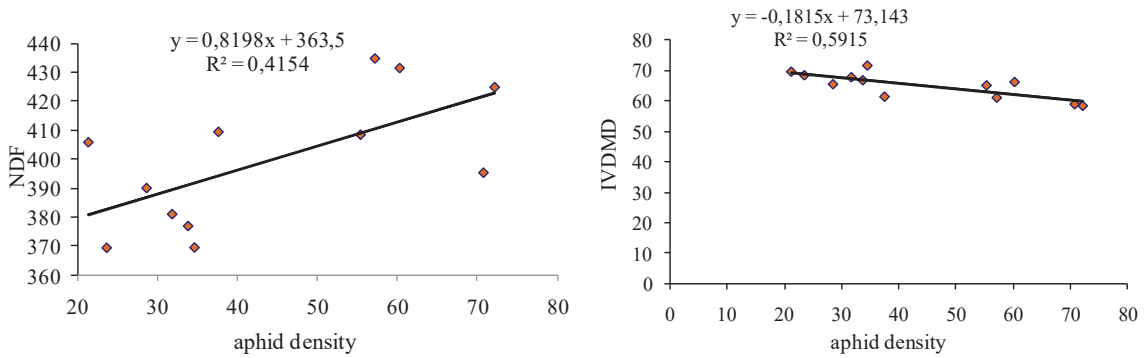

Figure 1. Relationship between pea forage quality components and pea aphid density

fibre, high protein content and digestibility of that forage after treatment with the natural product Agricolle, and its combinations with Lithovit and Nagro, made it a very good complement to grains and other forages in dairy rations.

CONCLUSIONS

- The application of natural products, either alone or in combinations, resulted in a reduction in pea aphid density. Applying Agricolle with Nagro, followed by Lithovit with Agricolle, led to the highest aphid number decrease (70.0 and 51.1%, respectively).

- An optimal combination of decrease in the content of plant cell wall fibre components, cellulose and lignification degree with a significant increase in forage enzyme *in vitro* digestibility was established after applying Agricolle with Lithovit, and Agricolle with Nagro. Digestibility reached 71.8 and 69.8%, respectively, increasing 8.2 and 5.2%, while ADF, cellulose and lignification degree decreased 7.1 and 7.7%, 8.0 and 23.4%, and 10.5 and 6.8% after applying Agricolle with Lithovit and Agricole with Nagro, respectively.

- A stronger linear relationship was found between aphid density and dry matter digestibility compared to the content of neutral detergent fibres.

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REFERENCES

Anderson, V.L., White, L., & Ilse, B. (2006). The feeding value of field peas. *Carrington Research Extension Center Feedlot Research Report*, *29*, 42-45.

AOAC (2007). *Official methods of analysis* (18-th ed.) Association of Analytical Chemists, Gaithersburg, MD, USA. http://files.b00kpedia.com/download/download+official+methods+of+analysis+of+aoac+international+18th+edition.pdf

Banyal, D., Chaudhary, J., & Katoch, R. (2015). Effect of diseases and insect-pest on forage quality of cowpea. In M.M. Roy, D.R. Malaviya, V.K. Yadav, Tejveer Singh, R.P. Sah, D. Vijay, & A. Radhakrishna (Eds.), *The 23rd International Grassland Congress "Sustainable Use of Grassland Resources for Forage Production, Biodiversity and Environmental Protection", Theme 2: Grassland production and utilization*, (pp 1-3). New Delhi, India: Range Management Society of India.

Bynum, E. & Bell, J. (2019). Sugarcane aphid damage to forage sorghum silage yield and quality induced by different infestation levels for the Texas High Plains. Texas Grain Sorghum Board Final 2019 Report. Texas A&M AgriLife Extension, 1-32. Retrieved from https://www.sorghumcheckoff.com/assets/media/2017-2019%20TGSB%20Final%20Report%20SCA%20Forage%20Sorghum%20Damage_distribution.pdf

Chekmarev, P.A., Glinushkin, A.P., & Startsev, V.I. (2018). Production of organic products - competitive position of the Russian Federation APRK. *Achievements of Science and Techniques APK*, *32*(3), 5-6.

Dewhurst, R.J., Delaby, L., Moloney, A., Boland, T., & Lewis, E. (2009). Nutritive value of forage legumes used for grazing and silage. *Irish Journal of Agricultural and Food Research, 48*(2), 167-187.

Dewhurst, R.J.., Fisher, W.J., Tweed, J.K.S., & Wilkins, R.J. (2003). Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. *Journal of Dairy Science, 86*, 2598-2611. doi: https://doi.org/10.3168/jds.S0022-0302(03)73855-7

Fahey, G.C., & Hussein, H.S. (1999). Forty years of forage quality research: Accomplishments and impact from an animal nutrition perspective. *Crop Science, 39*, 4-12. doi: https://doi.org/10.2135/cropsci1999.0011183X003900010002x

Fraser, M.D., Speijers, M.H.M., Theobald, V.J., Fychan, R., & Jones, R. (2004). Production performance and meat quality of grazing lambs finished on red clover, lucerne or perennial ryegrass swards. *Grass and Forage Science, 59*, 345-356. doi: https://doi.org/10.1111/j.1365-2494.2004.00436.x

Gerdgikova, M., Videva, M., Pavlov, D., & Dobreva, A. (2012). Chemical composition, nutritive value, energy yield and feed units of the winter pea grain grown after different predecessors using conventional and organic production. *Agricultural Science and Technology, 4*(3), 271-276.

Goering, H.K., & Van Soest, P.J. (1970). *Forage fiber analyses (apparatus, reagents, procedures, and some applications)*, *Agriculture Handbook No. 379*. Washington, WA: Agricultural Research Service, US Department of Agriculture.

Grigorova, Z., & Arabska, E. (2013). Opportunities of organic farming for biodiversity preservation and sustainable development. *New Knowledge Journal of Science*, *2*(1), 136-145.

Jančík, F., Homolka, P., Čermák, B., & Lád, F. (2008). Determination of indigestible neutral detergent fibre contents of grasses and its prediction from chemical composition. *Czech Journal of Animal Science, 53*(3), 128-135.

Singh, S., Luthra, Y. & Joshi, U. (2007). Biochemical differences in some forage sorghum varieties in relation to *Pyrilla perpusilla* Walker infestation. A*cta Phytopathologica et Entomologica Hungarica, 42*(1), 17-23. doi: https://doi.org/10.1556/aphyt.42.2007.1.3

Stakhova, L.N., Stakhov, L.F., & Ladygin, V.G. (2000). Effect of exogenic folic acid on the yield and amino acid composition of the seeds of *Pisum sativum* L. and *Hordeum vulgare* L. *Applied Biochemistry and Microbiology*, *36*(1), 85-89. doi: https://doi.org/10.1007/BF02738142

Steg, A., van Straalen, W.M., Hindle, V.A., Wensink, W.A., Dooper, F.M., & Schils, R.L.M. (1994). Rumen degradation and intestinal digestion of grass and clover at two maturity levels during the season in dairy cows. *Grass and Forage Science, 49*, 378-390. doi: https://doi.org/10.1111/j.1365-2494.1994.tb02014.x

Sulc, R.M., McCormick, J.S., Hammond, R.B., & Miller, D.J. (2015). Forage yield and nutritive value responses to insecticide and host resistance in alfalfa. *Crop Science, 55*, 1346-1355. doi: 10.2135/cropsci2014.09.0658

Todorov, N., Atanassov, A., Ilchev, A., Gantchev, G., Mihailova, G., Girginov, D. ... Tchobanova, S. (2010). *Practices in animal nutrition*. Sofia, Bulgaria: East-West Publishing House.

Vilela, A., González-Paleo, L., Ravetta, D., Murrell, E. & Van Tassel, D. (2020). Balancing forage production, seed yield, and pest management in the perennial sunflower *Silphium integrifolium* (Asteraceae). *Agronomy, 10(*1471), 1-14. doi: 10.3390/agronomy10101471

Zhelyazkova, C. (2007). Study the influence of some growth regulators on productivity, the chemical composition and nutritional value of spring peas (*Pisum sativum* L.) and spring vetch (*Vicia sativa* L.). Ph.D. thesis. Faculty of Plant Technology, Agrarian University, Plovdiv, Bulgaria.

Uticaj prirodnih preparata na brojnost *Acyrthosiphon pisum* na *Pisum sativum* L. i kvalitet stočne hrane

REZIME

U Institutu za krmno bilje (Pleven, Bugarska) u periodu 2015-2017 izveden je poljski ogled kako bi se ispitao uticaj prirodnih proizvoda na brojnost *Acyrthosiphon pisum*, kao i promene u hemiskom sastavu, sadržaju komponenti biljnih vlakana i degradaciju enzima u krmnom grašku. Tretmani prirodnim insekticidima Madex i Agricolle primenjeni su samostalno i u kombinaciji sa organskim đubrivima Lithovit i Nagro dva puta, na početku cvetanja i nedelju dana kasnije. Primenjena đubriva su ekološki bezbedna i odobrena za primenu u organskoj proizvodnji. Sintetičko folijarno đubrivo Kristalon i insekticid Proteus 110 OD, bili su primenjeni uporedno. Primena prirodnih preparata samostalno ili u kombinaciji dovelo je do redukcije brojnosti zelene graškove vaši. Primena Agricolle sa Nagro, a zatim Lithovit sa Agricolle, dovela je do najvećeg smanjenja brojnosti vaši (70.0 i 51.1%, respektivno). Optimalna kombinacija smanjenja sadržaja biljnih vlakana ćelijskog zida, celuloze i stepena lignifikacije sa značajnim povećanjem enzimske svarljivosti *in vitro* dobijena je nakon primene preparata Agricolle sa Lithovit, kao i Agricolle sa Nagro. Svarljivost je dostigla 71.8 i 69.8%, respektivno, što je porast od 8.2-5.2%, dok su ADF, celuloza i stepen lignifikacije opali 7.1 i 7.7%, 8.0 i 23.4%, 10.5 i 6.8%, respektivno. U poređenju sa tim, sintetički proizvod Kristalon, kao i Kristalon + Proteus, povećali su kvalitet stočnog graška, mada u relativno manjem stepenu. Dobijena je veća linearna zavisnost između brojnosti vaši i svarljivosti suve mase nego sadržaja neutralnih vlakana deterdženta. Stočni grašak sa niskim sadržajem komponenti vlakana ćelijskog zida, celuloze i stepenom lignifikacije, kao i visokim sadržajem proteina i svarljivošću nakon tretmana prirodnim proizvodom Agricolle, kao i njegovim kombinacijama sa preparatima Lithovit i Nagro, čine ga dobrim dodatkom drugim krmivima za ishranu krava muzara.

Ključne reči: zelena graškova vaš, prirodni proizvodi, suzbijanje štetočina, krmni grašak, hemijski sastav, kvalitet stočne hrane

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Original scientific paper

In vitro and *in vivo* toxicity of fungicides and biofungicides for the control of Verticillium and Fusarium wilt of pepper

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SUMMARY

A survey of *in vitro* and *in vivo* sensitivity of *Verticillium dahliae* and *Fusarium oxysporum* to several commercial fungicides and biofungicides was undertaken. In *in vitro* assays, the tested isolate of *V. dahliae* proved to be very sensitive to difenoconazole (EC_{50} = 0.02 mg/l). However, under greenhouse conditions, the highest efficacy in *V. dahliae* control on inoculated pepper plants was recorded for a product based on thiophanate-methyl (83.10% compared to control). Among the tested fungicides, the lowest efficacy was recorded for a product based on azoxystrobin (23.10 %) with no significant difference compared to control ($p > 0.05$). In *in vitro* assays, the tested *F. oxysporum* isolate was the most sensitive to prochloraz (EC_{50} = 0.07 mg/l) and the least sensitive to fluopyram (EC_{50} = 1075.01 mg/l). In *in vivo* assay, the highest efficacy was achieved by products based on captan (95.60%), and the lowest by a product based on thiophanate-methyl (54.40%). Antagonistic activity of the bacterium *B. subtilis* under laboratory conditions was not satisfying. Also, the antifungal activity and spectrum of a tested product based on tee tree oil was not efficient in suppressing pepper wilting caused by *V. dahliae* and *F. oxysporum*.

Keywords: soil-borne plant pathogens, wilt disease, pepper, fungicides, tea tree oil, *Bacillus subtilis*

INTRODUCTION

Pepper production is severely affected by soil-borne plant pathogens worldwide. *Verticillium* spp. and *Fusarium* spp., the causal agents of wilt disease, are among the most important pathogens in economic terms (Mijatovic et al., 2005; Fradin & Thomma, 2006). They can infect pepper plants at any stage of development, causing yellowing, wilting and shriveling of leaves, followed by stunting, bark cracking and twig or branch dieback. Eventually, diseased plants may die, leading to significant yield losses. Due to the endophytic

growth of these pathogens, as well as long persistence of their resting survival structures (microsclerotia, chlamydospores) in soil, wilt disease is very difficult to control (Alström, 2001).

Over the past several decades, soil fumigation with methyl bromide has been the primary method of controlling soil-borne diseases. However, in 1992 methyl bromide was listed as a Class I ozone-depleting substance that should be removed from the market by 2015 (Bell, 2000; UNEP, 2006). Without proper control, soil-borne diseases could increase crop losses to unpredictable levels. Although crop rotation slowly reduces inoculum density, it is not always profitable to practice it in intensive cropping. In addition, resistant pepper cultivars are not commercially available, while grafting of plants on resistant rootstock would not be cost-effective under practical conditions (Gilreath et al., 2004).

It is believed that the application of naturally-occurring and widespread substances as crop protectants could give a convenient solution. Thus, essential oils from many plants, as well as secondary metabolites of many microorganisms are well-known for their strong antimicrobial activity (Daferera et al., 2003). Tea tree essential oil has a long history of use as a topical microbicide in human pharmacology (Carson et al., 2006). Its suppressive activity against many phytopathogenic fungi, including *Aspergillus fumigatus*, *Fusarium solani*, *Penicillium expansum*, *Botrytis cinerea* and *Rhizopus oryzae*, has also been documented (Bishop & Reagan, 1998; Inouye et al., 1998; Bowers & Locke, 2000; Inouye et al., 2000; Angelini et al., 2006). In addition, the tea tree oil-based formulated product Timorex Gold has already been registered in more than 25 countries for the control of foliar and fruit diseases in both conventional and organic farming. However, its possible use against soil-borne plant pathogens has not been tested before. On the other side, *Bacillus* species, including *Bacillus subtilis,* are successfully used for the control of numerous plant and animal diseases (Fravel, 1988; Weller, 1988; Pandey et al., 1997; Mihajlović, 2014). Not only that they produce toxins (Mukry et al., 2010) and other metabolites that reduce pathogenic organisms, but they also increase plant growth (Awais et al., 2007) and significantly improve the uptake of fertilizing elements by plants. Due to its very high plant stimulating activity, a strain known as Ch-13 of *B. subtilis* has been registered in several countries as a biofertiliser under the trade name Extrasol. Further studies, however, have shown that it is also effective against some important plant pathogens (*Fusarium*, *Helminthosporium, Alternaria, Puccinia, Phythophtora,* etc.). Nevertheless, its effects on soil-borne pathogens and particularly wilt disease causal agents remains unknown (Pertot et al., 2015).

Therefore, the aim of this study was to determine whether tea tree oil and *B. subtilis*-based products could be effectively used for suppressing wilt diseases in pepper. As no standardized method for such evaluation is available, a reference fungicide, as well as the best plant quantitative parameter(s) for disease assessment, were applied based on *in vitro* and *in vivo* experiments. Fungicides with different modes of action were chosen based on literuture data and professional experience.

MATERIALS AND METHODS

Isolates

V. dahliae and *F. oxysporum* were isolated from infected pepper plants sampled from two locations in Serbia: Padinska Skela and Smederevska Palanka, respectively, using a method described by Dhingra and Sinclair (1995). Small fragments of diseased xylem tissue were washed under running tap water for 30 minutes, surface disinfected by 2% NaClO, placed aseptically on potato dextrose agar (PDA) and incubated at 25±1°C for 7-10 days. The developed mycelia were transferred to fresh PDA medium to obtain pure cultures. The isolates were maintained on PDA slants at 5°C in the culture collection of the Institute of Pesticides and Environmental Protection, Belgrade, Serbia. Preliminary identification of the isolates was based on morphological and pathogenic characteristics, according to Waterhouse and Waterston (1964). The identity of the isolates was confirmed by amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using the primers ITS1 and ITS4 (White et al., 1990).

Crop protection products

Crop protection products for *in vitro* and *in vivo* studies were selected based on available literature data on their modes of action and spectra of activity, on pathogens' biology, as well as the authors' practical experience. Tables 1 and 2 summarize the products used against *V. dahliae* and *F. oxysporum*, respectively.

Table 1. Active ingredients, trade names, producers and *in vivo* application rates of products tested in this study against *Verticillium dahliae*

Active ingredient	Product	Producer	Concentration (%)
B. subtilis	Ekstrasol	Bisolbi Inter	1×10^7 cfu/ml
Tea tree oil	Timorex Gold	Stockton	1
Thiophanate-methyl	Funomil	Agromarket	0.1
Difenoconazole	Score 250-EC	Syngenta	0.05
Fluopyram	Luna Privilege	Bayer CropScience	0.1
Azoxystrobin	Quadris	Syngenta	0.075
Prochloraz	Spartak 450-EC	Sinochem Ningbo	0.08

Table 2. Active ingredients, trade names, producers and *in vivo* application rates of products tested in this study against *Fusarium oxysporum*

Active ingredient	Product	Producer	Concentration (%)
B. subtilis	Ekstrasol	Bisolbi Inter	1×10^7 cfu/ml
Tea tree oil	Timorex Gold	Stockton	1
Fluopyram	Luna Privilege	Bayer CropScience	0.1
Captan	Agrokaptan	Agromarket	0.3
Prochloraz	Spartak 450-EC	Sinochem Ningbo	0.08
Propiconazole	Bumper 25-EC	Magan Agrochemicals	0.03
Thiophanate-methyl	Funomil	Agromarket	0.1

In vitro sensitivity of the isolates studied

Sensitivity of *V. dahliae* and *F. oxysporum* isolates to fungicides and tea-tree-oil *in vitro* was determined in a radial growth assay described by Leroux and Gredt (1972). Mycelial plugs (10 mm in diameter), cut from the edge of actively growing colonies of *V. dahliae* and *F. oxysporum* isolates on PDA, were used for inoculation of fungicide-amended and fungicide-free media. Preliminary concentrations of all investigated fungicides ranged from 0.0001 to 100 mg a.i./l, while tea tree oil was applied at 100 to 5000 mg a.i./l (Löcher & Lorenz, 1991).

V. dahlia – Based on preliminary studies, the following concentrations of fungicides were used in the medium: thiophanate-methyl 1.25, 2.5, 3.5, 5, and 7.5 mg/l; difenoconazole - 0.09, 0.19, 0.37, 0.75, and 1.5 mg/l; fluopyram 5000 mg/l; azoxystrobine 25, 50, 75, and 100 mg/l; prochloraz 0.01, 0.1, and 1 mg/l. The tea tree oil concentration range was 1500, 2500, 3000, and 5000 mg/l.

F. oxysporum – The selected concentrations of thiophanate-methyl were 25, 50, 75, and 100 mg/l; captan 50, 75, 100, and 250 mg/l; fluopyram 250, 500, 1000, 1500, and 2000 mg/l; propiconazole 1.56, 3.12, 6.25, 12.5, and 25 mg/l; prochloraz 0.06, 0.125, 0.25, 0.5, 1 mg/l, and tea tree oil 125. 250, 500, and 1000 mg/l.

Instead of fungicide dispersion, control plates were amended with the same amount of sterile distilled water. Three replicates per each fungicide concentration and each isolate were used. After 7-15 days of incubation at 25°C in the dark, the mean colony diameter of each isolate was measured and growth inhibition (PI [%]) was calculated using the formula below:

$$PI\ (\%) = [(a - b)/a] \times 100$$

where a = the mean colony diameter of control plates, and b = the mean colony diameter of fungicide-amended plates. The median effective concentration value (EC_{50}, fungicide concentration which inhibited mycelial growth by 50%) was determined for each isolate by probit analysis (Finney, 1971).

Azoxystrobin sensitivity of the *V. dahlia* isolate was determined as described above with a slight modification. In order to inhibit an alternative respiratory pathway, salicylhydroxamic acid (SHAM) (Sigma-Aldrich, Saint Louis, MO) was added at the concentration of 0.1 mg/l into both fungicide-amended and fungicide-free media (Ziogas et al., 1997).

In vitro antagonistic activity assay

Fungal pathogens, *V. dahliae* and *F. oxysporum* isolates grown in potato-dextrose broth (PDB) at 24°C for 48 h and homogenized on magnetic stirrer, were used as an inoculum source.

The antagonistic microorganism *B. subtilis* strain Ch-13 was isolated from the commercial product Ekstrasol (Bisolbi Inter, Russia) by plating and grown in submersed culture in Erlenmeyer flasks on the shaker (200 rpm) at 28°C for four days on Meynell media containing: molasses – 20.0, K_2HPO_4 – 7.0, KH_2PO_4 – 3.0; $MgSO_4$ – 0.1; sodium citrate – 0.5; $(NH_4)_2SO4$ –1.0; H_2O – adjusted to 1 l; pH 7.0 (Meynell et al, 1967). After cultivation, a sample of the cultivation medium was centrifuged at 10 000 g for 10 min and the supernatant was used for *in vitro* antagonistic activity assay.

Two-layer-PDA medium in 90-mm petri dishes was used in the antagonistic assay. The first layer was 2% PDA, while the second layer consisted of 1.2% PDA containing previously prepared suspension of each fungal pathogen. One 10-mm well per plate was made and 100 µl of prepared antagonistic supernatant was added to each well. Antagonistic activity was tested in four replicates against each isolate. As reference treatments, 100 µl of conventional fungicide dispersions (thiophanate-methyl for *V. dahliae* and prochloraz for *F. oxysporum*) were used at the rate of 1% a.i., while 100 µl of sterile, distilled water was added to the wells in control plates. The whole experiment was repeated twice.

The assessment of antagonistic activity was performed after 48 h incubation at 25°C by visual observation of the presence of clear inhibition zones around the wells, as well as by measuring of the diameter of the whole activity zone that consisted of clear inhibition zone + a partial mycelial growth inhibition zone (mm). Since experimental conditions were identical in all replications, the obtained data were pulled together and the average values were presented.

In vivo studies

Inoculum preparation: A pure culture of *V. dahliae* grown on PDA at 25°C for 2 weeks was used as a source of inoculum. The medium from 10 petri dishes, each containing 15-day-old fungal cultures, was blended with 1000 ml of distilled water until complete homogenization. Pepper plants were previously dipped in the inoculum suspension for two to three minutes. The prepared amount of inoculum was used for inoculation of 20 pepper plants (D'Ercole et al., 2000). After transplanting, the plants were first watered with the remaining inoculum suspension (5 ml per plant) and then with water.

Inoculum of *F. oxysporum* was prepared by growing the isolate in glass bottles containing 150 g double sterilized barley grains at 25°C for 21 days. Then, the inoculum was mixed thoroughly with sterilized clay soil at the rate of 3% and added into pots (Hashem et al., 2010).

Greenhouse experiment: Five-week-old pepper plants (cv. Novosadska babura), grown in 60-celled polystyrol trays, were transplanted into 10 cm × 5 cm pots filled with 400 ml sterile growth substrate (Floragard®, Germany). Sixty ml of each fungicide, at the rates given in Table 1 and Table 2, were added to each pot prior to inoculation, while inoculated plants (methods described previously in the chapter *Inoculum preparation*), watered with 60 ml of sterile distilled water, served as a positive control (C). Uninoculated pepper plants, watered with 60 ml sterile distilled water, served as a negative control (AC). The pots were kept in a greenhouse (24±2°C). The degree of wilting was recorded daily, while final evaluation was performed 25 (*F. oxysporum*) and 45 days (*V. dahliae*) after inoculation. Disease severity was estimated by visual observation based on a scale 0-5, where 0 = no symptoms, 1 = chlorosis of lower leaves, 2 = slight wilting with pronounced chlorosis, 3 = slight wilting and necrosis, 4 = pronounced wilting and necrosis, and 5 = death of plant. The experimental design was a complete randomized block with five replicates per treatment and five plants per replicate. The experiment was conducted twice. Infection degree (ID) was evaluated using Townsend-Heuberger's formula (Puntner, 1981):

$$ID = \Sigma(nv)100/NV$$

where: n = degree of infection rated on a scale of 1-5, v = number of plants in a category, N = highest degree of infection rate, and V = total number of plants screened. The efficacy was determined using Abbott's formula. The data were analyzed separately for each trial using ANOVA and the means were separated by Duncan's multiple range test.

In addition to visual assessment of both Verticillium and Fusarium wilt incidence, measurements of height and fresh weight of plants from the soil level to the uppermost leaf tip was performed. In case of Verticillium wilt, the length of the vascular necrotic zone on longitudinal section was also recorded. The data were processed by ANOVA and the means separated by Duncan's multiple range test.

RESULTS

Morphological characteristics of isolates

V. dahliae: The isolates grown on PDA formed white mycelium, which later became black when microsclerotia formed (50-100 µm). Conidiophores were abundant, hyaline, verticilliately branched. Conidia were hyaline, ellipsoidal to irregularly sub-cylindrical, with an average size of 4.9 µm (2.7-7.5 µm) × 2.6 µm (2.0-3.2 µm). Based on macroscopic and microscopic characters of the isolate, it was established that they apparently belong to the species *V. dahlia*.

F. oxysporum: The isolate produced delicate, white to pink mycelium on PDA media. After seven days of incubation at 25°C in the absence of light, the fungus formed a colony of 7 cm in diameter. The presence of conidia on the PDA substrate was not observed. However, in the 5-day-old culture on synthetic nutrient-poor agar (SNA) media, a large number of unicellular, elyptical, oval-shaped microconidia and straight to slightly curved macroconidia with 3 septates formed. Based on the studied characteristics, it was determined that the test isolate belongs to *F. oxysporum*.

Molecular identification of isolates

Using ITS1/ITS4 primers, PCR products of approximately 450 bp and 600 bp were noted. No amplicons occurred in negative control. BLAST analysis of the nucleotide sequence of the amplified product of 450 bp in size showed identity (was identical) with the KC156634.1 sequence of *V. dahliae*, while an analysis of nucleotide sequence of the amplified product 600 bp in size showed it was identical with the sequence of *F. oxysporum* EF495230.1.

In vitro sensitivity of the studied *V. dahliae* isolate

Sensitivity of the studied isolate to test fungicides was determined based on EC_{50} values (Table 3). Of all tested products, difenoconazole exhibited the greatest toxicity, severely inhibiting hyphal growth at the concentration of 0.09 mg/l. The calculated EC_{50} value for mycelial growth inhibition was 0.02 mg/l. The isolate also showed high susceptibility to difenoconazole (0.002 mg/l) and prochloraz (EC_{50}=0.03 mg/l). On the other hand, azoxystrobin exhibited a significantly lower toxicity than the mentioned fungicides (EC_{50}=71.95 mg/l), whereas fluopyram was completely ineffective even at the concentration of 5000 mg/l. It was also found that all conventional fungicides were more toxic than the studied formulated tea tree oil product. The tested *V. dahlia* isolate demonstrated an ability to tolerate tee tree oil at concentrations higher than 1500 mg/l. The calculated EC_{50} value for mycelial growth inhibition was 1507.65 mg/l.

In vitro sensitivity of the studied *F. oxysporum* isolate

Sensitivity of the *F. oxysporum* isolate to the tested fungicides and tea tree oil is presented in Table 4. The isolate expressed very high sensitivity to prochloraz with an EC_{50} value of 0.07 mg/l. The isolate was capable to grow well on the medium containing 0.06 mg/l of prochloraz, while its growth was severely inhibited at 0.25 mg/l and higher concentrations. Propiconazole was also highly toxic (EC_{50}=4.69 mg/l), while the toxicity of captan was moderate (EC_{50}= 19.14 mg/l). Tea tree oil showed the lowest toxicity to *F. oxysporum* with an EC_{50} value of 1205.77 mg/l.

Table 3. *In vitro* sensitivity of *Verticillium dahliae* to fungicides and tea tree oil

Fungicide	EC_{50} (mg/l)		b	
	Value	Range	Value	Range
Tee tree oil	1507.65	1351.73-1681.93	3.46	3.10-3.82
Prochloraz	0.03	0.02-0.06	0.66 ±0.98	
Fluopyram	>5000	*NS	*NS	
Difenoconazole	0.02	0.0004-0.05	0.51	0.37-0.65
Azoxystrobine	71.95	61.05-89.56	2.19	1.86-2.52
Thiophanate-methyl	3.48	3.03-3.87	2.72	2.42-3.02

EC_{50} – fungicide concentration which inhibits mycelial growth by 50%; *95% confidence interval (p=0.05)
*NS – not specified

Table 4. *In vitro* sensitivity of *Fusarium oxysporum* to fungicides and tea tree oil

Fungicide	EC_{50} (mg/l)		b	
	Value	Range	Value	Range
Tea tree oil	1205.77	711.74-3470.31	0.71	0.57-0.85
Prochloraz	0.07	0.04-0.10	0.87	0.68-1.06
Fluopyram	1075.01	670.50-2321.21	0.56	0.39-0.73
Propiconazole	4.69	3.27-6.35	0.86	0.72-1.00
Captan	19.14	2.79-35.75	1.10	0.80-1.40
Thiophanate-methyl	71.95	61.05-89.56	2.19	1.86-2.52

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; *95% confidence interval (p=0.05)

Table 5. Fungal growth inhibition zones in treatments (%) with *Bacillus subtilis* and conventional fungicide, compared with control

Pathogen	Inhibition zone (%)	
	Bacillus subtilis strain Ch-13	Conventional fungicide
Verticillium dahliae	+	+++
Fusarium oxysporum	–	+++

– no inhibion zone;
+ partia l inhibition of mycelial growth only;
++ clear inhibition zone > 25 mm;
+++ clear inhibition zone > 50 mm;

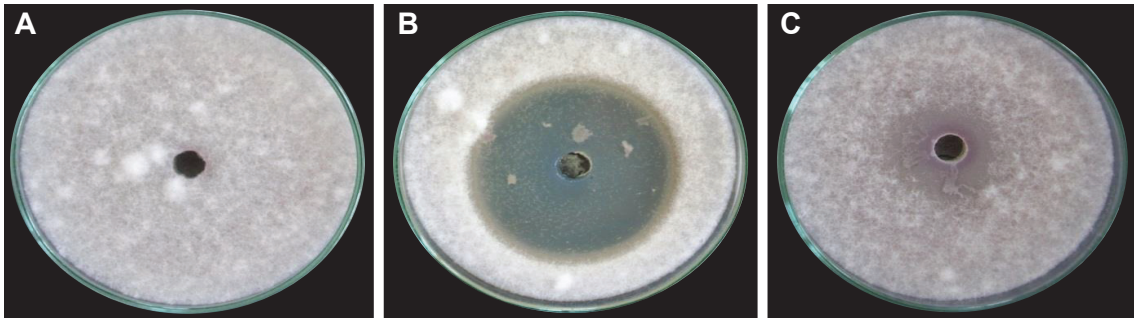

Figure 1. *In vitro* antagonistic activity assay of *B. subtilis* against *Fusarium oxysporum* A) Total activity zone; B) Clear inhibition zone C) Partial mycelial growth inhibition zone

Assessment of antagonistic activity of *B. subtilis* strain Ch13 *in vitro*

Assessment of th e antagonistic activity of the bacterium *B. subtilis* strain Ch-13 was conducted after 48 h incubation at 25°C by measuring inhibition zone diameter (mm) (Table 5).

The *B. subtilis* strain Ch-13 showed higher antagonistic activity against *V. dahliae* than against *F. oxysporum* isolate. Mycelial growth of *V. dahliae* in the activity zone of *B. subtilis* was not completely inhibited, yet a zone of partial inhibition of mycelial growth with a diameter of 16 mm was observed. Low level of antagonistic activity of the *B. subtilis* strain Ch-13, again with a zone of partial inhibition of mycelial growth, was recorded for the isolate of *F. oxysporum* (70 mm). Inhibition zone in the prochloraz treatment of *F. oxysporum* was 61.7 mm, while the radius of inhibition zone in the treatment with thiophanate-methyl was 52.75 mm (Figure 1).

Table 6. Verticillim wilt severity and treatment efficacy on pepper plants 45 days after fungicide and biocontrol agent application

Fungicide	Rate (%)	Disease index (%)		Efficacy (%)
		Ms[1]	Sd[2]	E[3]
B. subtilis	1.00	42.00 c*	25.10	35.40
Tee tree oil	1.00	35.00 bc	13.70	46.20
Thiophanate-methyl	0.10	11.00 a	8.90	83.10
Difenoconazole	0.05	19.00 ab	8.20	70.80
Fluopyram	0.10	16.00 ab	8.20	75.40
Azoxystrobin	0.075	50.00 cd	25.00	23.10
Prochloraz	0.08	17.00 ab	21.10	73.80
Control	–	65.00 d	13.70	0.00
AC[3]	–	0.00 a	0.00	100.00

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly ($p<0.05$) different according to Duncan's test; [2]Standard deviation; [3]Efficacy (%). [3]AC - non-inoculated control plants

Table 7. Height (in cm), fresh weight (in g) and necrosis zone (in cm) of pepper plants inoculated with *Verticillium dahliae* 45 days after fungicide and biocontrol agent treatments

Fungicide	Rate (%)	Height (cm)		Fresh weight (g)		Necrosis (cm)	
		Ms[1]	Sd[2]	Ms[1]	Sd[2]	Ms[1]	Sd[2]
B. subtilis	1.00	10.40 ab*	7.52	2.63 a	1.33	1.30 c	0.84
Tee tree oil	1.00	9.00 a	2.24	1.72 a	0.68	2.25 d	0.75
Thiophanate-methyl	0.10	24.60 de	2.07	7.37 cd	2.76	0.45 ab	0.32
Difenoconazole	0.05	26.60 ef	2.97	7.97 d	1.46	0.06 ab	0.13
Fluopyram	0.10	17.30 bc	7.51	3.94 ab	1.51	0.75 bc	0.43
Azoxystrobin	0.075	11.50 b	5.09	1.98 a	0.58	1.25 c	0.56
Prochloraz	0.08	19.00 cd	6.16	5.32 bc	3.46	0.45 ab	0.32
Control	–	12.20 abc	6.73	1.72 a	1.06	2.30 d	0.57
AC[3]	–	31.80 f	1.30	11.34 e	1.65	0.00 a	0.00

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly ($p<0.05$) different according to Duncan's test; [2]Standard deviation; [3]AC - non-inoculated control plants

Greenhouse experiment

***V. dahliae*:** Table 6 summarizes the results of Verticillium wilt severity and the efficacy of substances applied prior to inoculation. Under greenhouse conditions, the lowest disease index was found in pepper plants treated with thiophanate-methyl (11%), corresponding to the efficacy of 83.10%, compared to the inoculated untreated control. However, the observed differences in disease index among treatments with thiophanate-methyl (11%), fluopyram (16%), prochloraz (17%), and difenoconazole (19%) was not statistically significant. In this experiment, tea tree oil- and *B. subtilis*-based products were moderately effective (46.20 and 35.40%, respectively). Among the tested products, the highest disease incidence was recorded in plants treated with azoxystrobin (23.10%). Moreover, the difference between azoxystrobin treatment and untreated control was not significant ($p > 0.05$).

Plant height, fresh weight and vascular necrotic zone length of plants treated with the investigated products are presented in Table 7. Maximum plant height was recorded in treatments with difenoconazole (26.60 cm) and thiophanate-methyl (24.60 cm), while the height of plants treated with tea tree oil and *B. subtilis* was not significantly different from the untreated control. Similarly, the highest fresh weight was recorded in treatments with difenoconazole (7.97 g) and thiophanate-methyl (7.37 g), whereas tee tree oil and azoxystrobin treatments resulted in the lowest fresh weight (1.72 g and 1.98 g, respectively).

The average length of vascular necrotic zone in inoculated untreated plants was 2.30 cm. The lowest

values of this parameter were found in treatments with difenoconazole (0.06 cm), thiophanate-methyl (0.45 cm) and prochloraz (0.45 cm), respectively. In the tea tree oil treatment, the length of necrosis was not significantly different from that in inoculated untreated plants, while a significantly lower value was found in the treatment with *B. subtilis*.

High negative correlations between disease severity and plant height (r = 0.81) and between disease severity and fresh weight of pepper plants (r = 0.84) were found. On the other hand, the length of vascular necrotic zone was in positive correlation with disease severity (r = - 0.84).

F. oxysporum: Table 8 summarizes the results of Fusarium wilt severity and efficacy of the products applied prior to inoculation. Based on the disease severity observation, captan (95.60%) and prochloraz (92.20%) showed the highest efficacy. High efficacy of *B. subtilis* was also noted.

The height and fresh weight of pepper plants inoculated with *F. oxysporum* and treated with the investigated substances are presented in Table 9. As compared to control pepper plants (0.50 cm), all inoculated treatments other than propiconazole (0.84 cm) and prochloraz (1.10 cm) exhibited significantly higher values. Maximum plant height was recorded in treatments with captan (4.84 cm) and *B. subtilis* (4.46 cm). Treatments with thiophanate-methyl (0.64 g) and fluopyram (0.91 g) showed the lowest plant fresh weight, while maximum values were noted in treatments with prochloraz (2.04 g) and captan (2.03 g). Statistically significant differences were not observed in the weight of plants in all inoculated treatments, when compared with untreated uninoculated control pepper plants (14.24 g).

Correlation between disease incidence and plant height was moderate (r = 0.56), while correlation between efficacy and fresh weight of plants was weak (r = 0.43).

Table 8. Fusarium wilt severity and treatment efficacy on pepper plants 25 days after fungicide and biocontrol agent application

Fungicide	Rate (%)	Disease index (%)		Efficacy (%)
		Ms [1]	Sd[2]	E[3]
B. subtilis	1.00	20.00 ab*	32.60	77.80
Tea tree oil	1.00	85.00 c	22.40	5.60
Fluopyram	0.10	30.00 ab	27.40	66.70
Captan	0.30	4.00 ab	5.50	95.60
Prochloraz	0.08	7.00 ab	11.00	92.20
Propiconazole	0.03	22.00 ab	43.80	75.60
Thiophanate-methyl	0.10	41.00 b	43.40	54.40
Control	–	90.00 c	22.40	0.00
AC[4]	–	0.00 a	0.00	100.00

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly (p<0.05) different according to Duncan's test; [2]Standard deviation; [3]Efficacy (%); [4]AC - non-inoculated control plants

Table 9. Height (in cm) and fresh weight (in g) of pepper plants inoculated with *Fusarium oxysporum* 25 days after fungicide and biocontrol agent application

Fungicide	Rate (%)	Height (cm)		Fresh weight (g)	
		Ms[1]	Sd[2]	Ms[1]	Sd[2]
B. subtilis	1.00	4.46 cd*	1.70	1.98 a	1.18
Tea tree oil	1.00	1.90 ab	1.60	1.06 a	0.89
Fluopyram	0.10	3.74 cd	1.30	0.91 a	0.58
Captan	0.30	4.84 d	1.40	2.03 a	1.09
Prochloraz	0.08	1.10 a	0.70	2.04 a	0.76
Propiconazole	0.03	0.84 a	0.70	1.82 a	1.27
Thiophanate-methyl	0.10	2.94 bc	1.50	0.64 a	0.39
Control	–	0.50 a	0.30	1.50 a	0.91
AC[3]	–	10.50 e	1.90	14.24 b	1.28

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly (p<0.05) different according to Duncan's test; [2]Standard deviation; [3]AC - non-inoculated control plants

DISCUSSION

Data in the present study showed that the tested *V. dahliae* and *F. oxysporum* isolates were sensitive to the tested fungicides. The fungicides exhibited different levels of toxicity, and different EC_{50} values indicate heterogeneity in responses to fungicides with different modes of action.

Our results provide novel information on the efficacy of the succinate dehydrogenase inhibitor (SDHI) fungicide fluopyram in controlling Verticillium and Fusarium wilt of pepper and efficacy of difenoconazole in suppressing *V. dahliae*.

Fluopyram provided moderate control against these two diseases under greenhouse conditions, causing 75.40% and 66.70% reduction, respectively. However, in the laboratory test, the isolate of *V. dahliae* did not show sensitivity to fluopyram, and it was ineffective even at the concentration of 5000 mg/l. Of all *in vitro* tested fungicides, fluopyram also exhibited the lowest toxicity to the studied *F. oxysporum* isolate with its relatively high EC_{50} value of 1075.01 mg/l. Fluopyram ineffectiveness in *in vitro* studies could be attributed to its mode of action. Fluopyram is an SDHI fungicide that specifically inhibits fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II and plays an important role in integrated management programs for many plant diseases (Avenot & Michailides, 2010).

Difenoconazole was effective against *V. dahliae* in our experiments. This demethylation inhibitor (DMI) fungicide caused a 70.80% reduction in Verticillium wilt severity. Again, another DMI fungicide, prochloraz, showed a high toxicity to propiconazole-tested isolates of *V. dahliae* (EC_{50}=0.03 mg/l) and *F. oxysporum* (EC_{50}=0.07 mg/l) under laboratory conditions. These results appear to be in partial agreement with results published by Kurt et al. (2003) for *V. dahliae*. They showed that the mean effective concentration (EC_{50}) for *V. dahliae* isolates from Turkey ranged from 0.52 to 0.84 mg/l. The EC_{50} values recorded in our experiments with *F. oxysporum* were similar to those reported by Amini and Sidovich (2010). They found that isolates of *F. oxysporum* f. sp. *lycopersici* were most sensitive to prochloraz in a group of fungicides used in that test (benomyl, carbendazim, fludioxonil, bromuconazole and azoxystrobine) (EC_{50} = 0.005 mg/l).

In our experiment, antagonistic activity of the bacterium *B. subtilis* was generally not satisfying under laboratory conditions. The strain Ch-13 showed no inhibitory effects *in vitro* against the tested isolates. However, the efficacy of *B. subtilis* strain Ch-13 (77.80%) against *F. oxysporum* was relatively high. It could be related to the mechanisms of biological control. Besides direct antagonism to plant pathogen growth, *B. subtilis,* as a plant growth promoting rhizobacterium, can promote plant growth. It could be indirectly by reducing mobilizing nutrients in soils, producing numerous plant growth regulators that protect plants from phytopathogens by controlling or inhibiting them. Also, *B. subtilis* affects them by improving soil structure and bioremediating polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (like pesticides) (Rajkumar et al., 2010; Braud et al., 2009). Therefore, our results show that the local strain Ch-13 of *B. subtilis* is an efficient tool to control Fusarium wilt but it was not as efficacious as the conventional chemical pesticides (captan and prochloraz >90%).

Numerous studies have demonstrated that vascular pathogens are able to activate all factors that affect plant growth rate (Yadeta & Thomma, 2013; Rekanovic et al., 2007; Tanovic et al., 2004). The present study also indicates high negative correlations between Verticillium wilt disease severity and plant height, and between disease severity and fresh weight of pepper plants. However, our experiments showed that plant height and fresh weight are not reliable parameters for evaluating fungicide efficacy in controlling *F. oxysporum*. Correlation between disease incidence and plant height was moderate, while it was weak between the efficacy and fresh weight of plants.

Also, the antifungal activity and spectrum of the tested product based on tee tree oil were not efficient in suppressing pepper wilting caused by *V. dahliae* and *F. oxysporum*. Under laboratory conditions, this biofungicide exhibited low toxicity to the tested isolates with EC_{50} values of 1507 mg/l and 1205.77, respectively. In the present study, the results obtained in *in vitro* tests partially confirmed those obtained under greenhouse conditions. Considering all products tested against the *F. oxysporum* isolate, the lowest control efficacy was recorded in the treatment with tee tree oil (5.60%), while a slightly higher value was found for *V. dahliae*. The same observation was reported by Tanovic et al. (2004). They confirmed a very low antimicrobial activity of this oil against *V. dahliae* isolated from pepper and *Fusarium oxysporum* f. sp. *lycopersici*.

CONCLUSION

A product based on thiophanate-methyl showed the best result in controlling Verticillium wilt, while azoxystrobine was the most effective against *F. oxysporum* on pepper. The activity spectrum of the

tested biofungicides was not satisfying. The results therefore reveal a risk associated with the application of these products in contemporary conventional model of pepper production. However, the high efficacy of *B. subtilis* strain Ch-13 found against *F. oxysporum* (77.80%) could provide a basis for using these substances after pathogens have been detected and identified in soil on fields intended for pepper production.

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REFERENCES

Alström, S. (2001). Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. *Journal of Phytopathology*, *149*(2), 57-64.

Amini, J., & Sidovich, D.F. (2010). The effects of fungicides on *Fusarium oxysporum* f. sp. *lycopersici* associated with Fusarium wilt of tomato. *Journal of Plant Protection Research, 50*(2), 172-178.

Angelini, P., Pagiotti, R.P., Menghini, A., & Vianello, B. (2006). Antimicrobial activities of various essential oils against foodborne pathogenic or spoilage moulds. *Annals of Microbiology*, *56*(1), 65-69.

Avenot, H.F., & Michailides, T.J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection*, *29*(7), 643-651.

Awais, M., Shah, A.A., Hameed, A., Hasan, F. (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pakistan Journal of Botany, 39*(4), 1303-1312.

Bell, C.H. (2000). Fumigation in the 21st century. *Crop Protection*, *19*(8-10), 563-569.

Bishop, C.D., & Reagan, J. (1998). Control of the storage pathogen *Botrytis cinerea* on Dutch white cabbage (*Brassica oleracea* var. *capitata*) by the essential oil of *Melaleuca alternifolia*. *Journal of Essential Oil Research, 10*(1), 57-60.

Bowers, J.H., & Locke, J.C. (2000). Effect of botanical extracts on the population density of *Fusarium oxysporum* in soil and control of Fusarium wilt in the greenhouse. *Plant Disease*, *84*(3), 300-305.

Braud, A., Jézéquel, K., Bazot, S., & Lebeau, T. (2009). Enhanced phytoextraction of an agricultural Cr-and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. *Chemosphere*, *74*(2), 280-286.

Carson, C.F., Hammer, K.A., & Riley, T.V. (2006). *Melaleuca alternifolia* (tea tree) oil: a review of antimicrobial and other medicinal properties. *Clinical Microbiology Reviews*, *19*(1), 50-62.

Daferera, D.J., Ziogas, B.N., & Polissiou, M.G. (2003). The effectiveness of plant essential oils on the growth of *Botrytis cinerea, Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. *Crop Protection*, *22*(1), 39-44.

D'Ercole, D.N., Nipoti, P., di Pillo, L., & Gavina, F. (2000). *In vitro* and *in vivo* tests of *Trichoderma* spp. as a biocontrol agent of *Verticillium dahliae* kleb. in eggplants. Advances in Verticillium research and disease management. In *Proceedings of the International Verticillium Symposium* (pp. 260-263). St. Paul, MN: American Phytopathological Society.

Dhingra, O.D., & Sinclair, J.B. (1995). *Basic plant pathology methods* (2nd edn). Boca Raton, FL: CRC Press.

Finney, D.J. (1971). *Probit analysis* (3rd edn.). Cambridge, UK: Cambridge University Press.

Fradin, E.F., & Thomma, B.P. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology*, *7*(2), 71-86.

Fravel, D.R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology*, *26*, 75-91.

Gilreath, J.P., Santos, B.M., Gilreath, P.R., Jones, J.P., & Noling, J.W. (2004). Efficacy of 1,3-dichloropropene plus chloropicrin application methods in combination with pebulate and napropamide in tomato. *Crop Protection*, *23*(12), 1187-1191.

Hashem, M., Moharam, A.M., Zaied, A.A., & Saleh, F.E.M. (2010). Efficacy of essential oils in the control of cumin root rot disease caused by *Fusarium* spp. *Crop Protection*, *29*(10), 1111-1117.

Inouye, S., Tsuruoka, T., Watanabe, M., Takeo, K., Akao, M., Nishiyama, Y., & Yamaguchi, H. (2000). Inhibitory effect of essential oils on apical growth of *Aspergillus fumigatus* by vapour contact. *Mycoses*, *43*(1-2), 17-23.

Inouye, S., Watanabe, M., Nishiyama, Y., Takeo, K., Akao, M., & Yamaguchi, H. (1998). Antisporulating and respiration-inhibitory effects of essential oils on filamentous fungi: Hemmeffekte ätherischer Öle auf Sporulation und Atmung von Fadenpilzen. *Mycoses*, *41*(9-10), 403-410.

Kurt, S., Dervis, S., & Sahinler, S. (2003). Sensitivity of *Verticillium dahliae* to prochloraz and prochloraz–manganese complex and control of Verticillium wilt of cotton in the field. *Crop Protection*, *22*(1), 51-55.

Leroux, P., Gredt, M. (1972) Etude de l'action in vitro des fongicides, méthode de l'incorporation ou milieu. Versailles, France: Laboratoire de Phytopharmacie - INRA, 1-10.

Löcher, F.J., & Lorenz, G. (1991). Methods for monitoring the sensitivity of *Botrytis cinerea* to dicarboximide fungicides. *EPPO Bulletin, 21*, 341-34.

Meynell, G.G., Meynell, E., Mekler, L.B., Kriviskij, A.S., & Urbah, V.J. (1967). *Experimental microbiology*. Moscow, USSR: Mir.

Mihajlović, M. (2014). *Soil-borne pathogens of pepper and possibilities of fungicide control* (Doctoral dissertation). University of Belgrade, Agriculture faculty, Belgrade, Serbia.

Mijatovic, M., Zecevic, B., Ivanovic, M., & Obradovic, A. (2005). Diseases of pepper in Serbia and results of breeding for resistance. *Folia Horticulturae*, *17*, 53-60.

Mukry, S.N., Ahmad, A., & Khan, S.A. (2010). Screening and partial characterization of hemolysins from Bacillus sp.: Strain S128 & S144 are hemolysin B (HBL) producers. *Pakistan Journal of Botany*, *42*(1), 463-472.

Pandey, A., Palni, L.M.S., & Coulomb, N. (1997). Antifungal activity of bacteria isolated from the rhizosphere of established tea bushes. *Microbiological Research*, *152*(1), 105-112.

Pertot, I., Alabouvette, C., Esteve, E.H., & Franca, S. (2015). The use of microbial biocontrol agents against soil-borne diseases. *Retrieved from European Commission EIP-AGRI Focus Group: : https://ec. europa. eu/eip/agriculture/sites/agrieip/files/8_eip_sbd_mp_biocontrol_final. pdf.*

Rajkumar, M., Ae, N., Prasad, M.N.V., & Freitas, H. (2010). Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in Biotechnology*, *28*(3), 142-149.

Rekanovic, E., Milijasevic, S., Todorovic, B., & Potocnik, I. (2007). Possibilities of biological and chemical control of Verticillium wilt in pepper. *Phytoparasitica, 35*(5), 436-441.

Tanović, B., Obradović, A., & Potočnik, I. (2004). Effects of thyme essential oil on Pythium sp., In: Proceedings of ESNA XXXIV Annual Meeting (pp 502-505). Novi Sad, Serbia: Faculty of Agriculture, University of Novi Sad.

United Nations Environment Programme (UNEP), Ozone Secretariat. (2007). *Handbook for the Montreal protocol on substances that deplete the ozone layer*. UNEP/Earthprint.

Waterhouse, G.M., & Waterston, J.M. (1964). *Phytophthora syringae*. *CMI Descriptions of pathogenic fungi and bacteria*, *32*, 1-2.

Weller, D.M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology*, *26*(1), 379-407.

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., Eds., *PCR Protocols. A guide to methods and applications* (pp 315-322). New York, USA: Academic Press.

Yadeta, K., & Thomma, B. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science*, *4*, 97.

Ziogas, B.N., Baldwin, B.C., & Young, J.E. (1997). Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in *Septoria tritici*. *Pesticide Science*, *50*(1), 28-34.

Efekti fungicida i biofungicida u uslovima *in vitro* i *in vivo* u suzbijanju verticilioznog i fuzarioznog uvenuća

REZIME

U radu je ispitivana *in vitro* i *in vivo* osetljivost izolata *Verticillium dahliae* i *Fusarium oxysporum* na nekoliko komercijalnih fungicida i biofungicida. U *in vitro* testovima izolat *V. dahliae* je ispoljio visoku osetljivost na difenokonazol (EC_{50} = 0,02 mg/l). Međutim, u uslovima staklenika, najveća efikasnost na inokulisanim biljkama paprike utvrđena je kod preparata na bazi tiofanat-metila (83,10%). Među testiranim fungicidima, najniža efikasnost koja se nije statistički značajno razlikovala u poređenju sa inokulisanom i netretiranom kontrolom ($p > 0,05$), utvrđena je u tretmanu azoksistrobinom (23,10%). Prohloraz je bio najtoksičniji fungicid u laboratorijskim uslovima ispitivanja za izolat vrste *F. oxysporum*, sa vrednošću EC_{50} = 0,07 mg/l. U *in vivo* ispitivanjima najveća efikasnost utvrđena je kod preparata na bazi kaptana (95,60%), a najmanja u tretmanu tiofanat-metilom (54,40%). Antagonistička aktivnost biološkog preparata na bazi bakterije *Bacillus subtilis* u laboratorijskim uslovima nije bila zadovoljavajuća. Takođe je utvrđeno da biopreparat na bazi ulja čajnog drveta nije bio efikasan u suzbijanju uvenuća paprike čiji su prouzrokovači vrste *V. dahliae* i *F. oxysporum*.

Ključne reči: zemljišni patogeni biljaka, uvenuće, paprika, fungicidi, ulje čajnog drveta, *Bacillus subtilis*

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Impact of water quality on pesticides and fertilizer compatibility

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SUMMARY

Mixtures of two or more pesticides are very common in contemporary agriculture. However, changes in their efficacy or biological activity, such as synergism and antagonism, phytotoxicity, persistence, toxicity to non-target organisms, may occur as a consequence. This study was conducted in order to evaluate the compatibility of insecticides (cyantraniliprole – Exirel, chlorantraniliprole – Coragen 20 SC), a fungicide (captan – Merpan 50 WP) and a foliar fertilizer (Folia Stim Mix TE), as well their mixtures, in spray liquids, depending on water quality (well water from two locations in Serbia – Mala Remeta and Čerević). These products are used to control the most significant peach pests, and as an additional source of nutrients. Water analysis (pH, hardness, electroconductivity, chloride, nitrate, nitrite, ammonia, calcium and iron content) and tests of physico-chemical properties of the spray liquids (pH, suspensibility, dispersibility, surface tension, and electroconductivity) were performed in a laboratory experiment according to standard methods. The physico-chemical properties of the liquids changed depending on water quality and components incorporated in the mixture. However, all tested spray liquids showed consistency and compatibility over a period of 24 hours.

Keywords: pesticides, fertilizer, mixture, water quality, physico-chemical properties, compatibility

INTRODUCTION

Chemical control is the most important tool in integrated pest management (IPM) because it is time-saving, accessible, and often low-cost when appropriately applied (Castro, 2009). Mixtures of plant protection products (PPPs) are commonly and intensively used for broadening the spectrum of activity, slowing down or delaying the appearance of resistant populations, etc., all for the ultimate goal of increasing yield and ensuring high quality of agricultural products. Therefore, they are also essential for resistance management programs. However, inappropriate chemical control can cause irreversible environmental and economic impact (Moraes et al., 2019), e.g. change the efficacy or biological activity (synergism and antagonism, phytotoxicity or persistence). The commonest negative effect on treated plants is phytotoxicity (Vuković et al., 2014). It occurs as temporary or permanent damage of vegetative/generative organs of crops and non-target plants, slows down or completely stops germination, and causes physiological and morphological changes. Phytotoxicity can occur as a

result of simultaneous application of two or more PPPs, which are normally used for different target purposes, or due to double amounts of non-pesticidal components (solvents, wetters, emulsifiers, etc.). The main factors that influence phytotoxicity are water characteristics (pH, temperature, hardness), plant species, the sensitivity of varieties and the growth stage of plants (Vuković, et al., 2014). It is also important to note that pesticide mixtures can be highly toxic to non-target species, such as aquatic organisms and mammals (Mihajlović et al., 2019).

This study is based on previous knowledge that the quality of water used in plant protection affects the physico-chemical and biological properties of fungicides, insecticides and other components (fertilizers) in mixtures (Vuković et al., 2013). However, information regarding this subject is still lacking, especially for new active substances.

In this study, the compatibility of liquid sprays of a novel group of anthranilic diamide insecticides (cyantraniliprole, chlorantraniliprole), a fungicide (captan), a foliar fertilizer and their mixtures were analyzed as depending on water quality. These mixtures are intensively used currently for the protection of peach from its most important pest (*Grapholita molesta* Busck) and the causative agent of shot hole disease (*Stigmina carpophila* Lev).

MATERIALS AND METHODS

Well water used in this study was collected from fields at two locations in Vojvodina Province, Serbia (Mala Remeta – location 1 and Čerević – location 2) just prior to treatment. Water analysis (pH, hardness, electroconductivity, and chloride, nitrate, nitrite, ammonia, calcium and iron contents), as well as physico-chemical properties of spray liquids (pH, suspensibility, dispersibility, surface tension, and electroconductivity), were performed according to standard methods in the laboratory. The physico-chemical properties were chosen according to the Manual on the Development and Use of FAO and WHO Specifications for Pesticides (2016), based on their influence on the biological efficacy of pesticides.

The pH value of the spray liquids prepared in well water was determined according to the CIPAC MT 75 method (Dobrat & Martijin, 2007a). For determination of suspensibility, the CIPAC MT 15 method (Dobrat & Martijin, 2007b) was used, while dispersion stability was determined according to CIPAC MT 180 (Dobrat & Martijin, 2007c), both expressed as percentage (%). Surface tension of spray liquids (mJ/m^2 with an accuracy of ±0.1 mJ/m^2) was determined using the tensiometer (Lecomte du Nouy) (Šovljanski et al., 2002). Electroconductivity was determined using the conductometer (WTW pH/cond 740), and the CIPAC MT 32 method (Dobrat & Martijin, 2007c), with pre-calibration in standard hard water at 25 °C, expressed in μS/cm. Analysis of the physico-chemical properties was performed in triplicates, immediately after preparation and after 24 h.

For the analysis, insecticides based on cyantraniliprole (Exirel, 100 g a.i./l, SE [suspo-emulsion]) and chlorantraniliprole (Coragen 20, 200 g a.i./l, SC [suspension concentrate]), a fungicide based on captan (Merpan 50, 500 g a.i./kg, WP [wettable powder]) and a plant nutrition agent (Folia Stim Mix TE, a concentrated liquid formulation with 100% EDTA chelate micronutrients) were applied at application rates shown in Table 1.

The results of the analyzed parameters are expressed as average values. The significance of differences of the test parameters was evaluated using ANOVA for a threshold of 0.05 (statistic software Statistica 12).

Tabela 1. PPPs, foliar fertilizer, their mixtures and application rate

PPPs and fertilizer	Application rate (kg, l/ha)
Exirel	0.6 l/ha
Coragen 20 SC	0.2 l/ha
Merpan 50 WP	2.5 kg/ha
FoliaStim Mix TE	1.5 l/ha
Exirel SE + Merpan 50 WP	0.6 l/ha+2.5 kg/ha
Coragen 20 SC + Merpan 50 WP	0.2 l/ha+2.5 kg/ha
Exirel SE + FoliaStim Mix TE	0.6 l/ha+1.5 l/ha
Coragen 20 SC + FoliaStim Mix TE	0.2 l/ha+1.5 l/ha
Merpan 50 WP + FoliaStim Mix TE	2.5 kg/ha+1.5 l/ha
Exirel SE + Merpan 50 WP + FoliaStim Mix TE	0.6 l/ha+2.5 kg/ha+1.5 l/ha
Coragen 20 SC + Merpan 50 WP + FoliaStim Mix TE	0.2 l/ha+2.5 kg/ha +1.5 l/ha

RESULTS AND DISCUSSION

Water analysis

Water properties, such as pH, electroconductivity and hardness, can affect the quality and efficacy of pesticides and their mixtures in the process of application. It may also happen during mixing pesticides with non-pesticide substances (complex fertilizers, adjuvants, protectants), increasing the risk of undesired effects (Vuković et al., 2013). These water properties can cause accelerated degradation of active substances, changes in suspensibility or biological effect (antagonism, additive effect, synergism), and finally toxicity to plants. The results of chemical analyses of water are shown in Table 2.

Based on the analysis, water samples were classified as slightly alkaline and hard water. The results of the analysis of water samples from both locations showed high concentrations of ammonium (Federal Minister of Labor, 1998). The other tested parameters had values below the prescribed maximum allowable concentration (MAC). Based on a scale for determination of water hardness, both samples (locations 1 and 2) were classified as hard water, with $CaCO_3$ contents of 355 to 361 mg/l, respectively.

pH of spray liquids

The values of pH of well water from location 1 ranged from 7.9 to 8.2 over a period of 24 h (Figures 1 and 2). The spray liquids of insecticides, fungicide and foliar

Tabela 2. The quality of water used in the experiment

Location	Analyzed parameters								
	pH	E* (mS/cm) on 20°C	NO_3^- mgN/l	NO_2^- mgN/l	NH_4+ mgN/l	Chloride mgCl/l	$CaCO_3$ mg/l	Ca mg/l	Fe mg/l
1	8.1	654.0	3.1	0.01	**0.3**	7.1	**355.0**	80.4	<0.1
2	8.2	757.0	1.2	0.01	**0.4**	31.4	**361.0**	115.0	<0.1
MAC**	**6.8-8.5**	**≤1000**	**50**	**0.03**	**0.1**	**200**	***	**200**	**0.3**

* Electroconductivity
** MAC – maximum allowable concentration for II class water quality (Official Gazette SRJ, 1998)
*** water hardness scale (0-4 very soft; 4-8 slightly soft; 8-16 slightly hard; 16-30 hard; over 30 very hard)

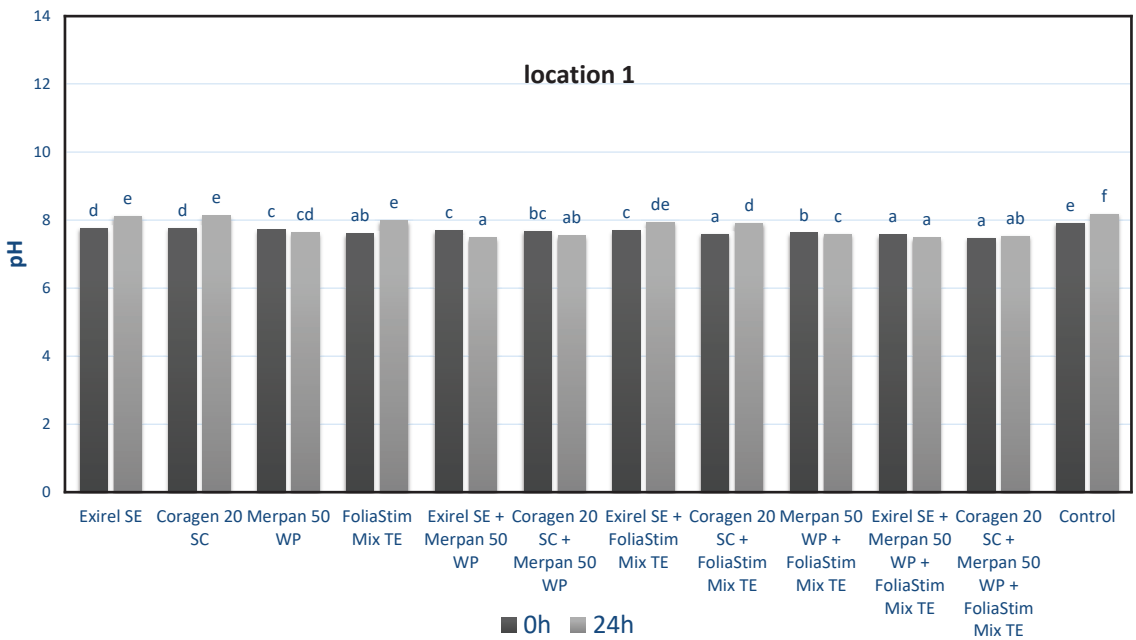

Figure 1. pH values of spray liquids of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after mixing and after 24 h (location 1)

fertilizer showed slightly alkaline reaction (7.6-7.7) at the moment of mixing. After 24 h, there was a slight increase in pH values (7.5-8.2), while the mixtures Exirel + Merpan 50 WP, Coragen 20 SC + Merpan 50 WP and Exirel + Merpan 50 WP + Folia Stim Mix TE showed decreasing pH. Most spray liquids of the tested insecticides, fungicide, foliar fertilizer, and their mixtures showed increasing pH from the moment of preparation until the expiry of 24 hours. Based on the pH of spray liquids made with water from location 1, it is evident that the medium reaction changed depending on the components and age of spray liquids.

The pH values of well water from location 2 ranged between 7.7 and 7.8 over 24 h (Figures 1 and 2).

Spray liquids of Exirel, Coragen 20 SC, Merpan 50 WP and FoliaStim Mix TE showed slightly alkaline reaction (7.5-7.9), while only with the spray liquid of Merpan 50 WP fungicide had decreasing pH after 24 h (7.5). Spray liquids of Exirel + Merpan 50 WP and Coragen 20 SC + Merpan 50 WP showed decreasing pH, while it increased in the other mixtures after 24 h. All insecticide and fungicide mixtures with foliar fertilizer displayed neutral to slightly alkaline reaction (7.3-7.7) right after mixing, and all mixtures showed

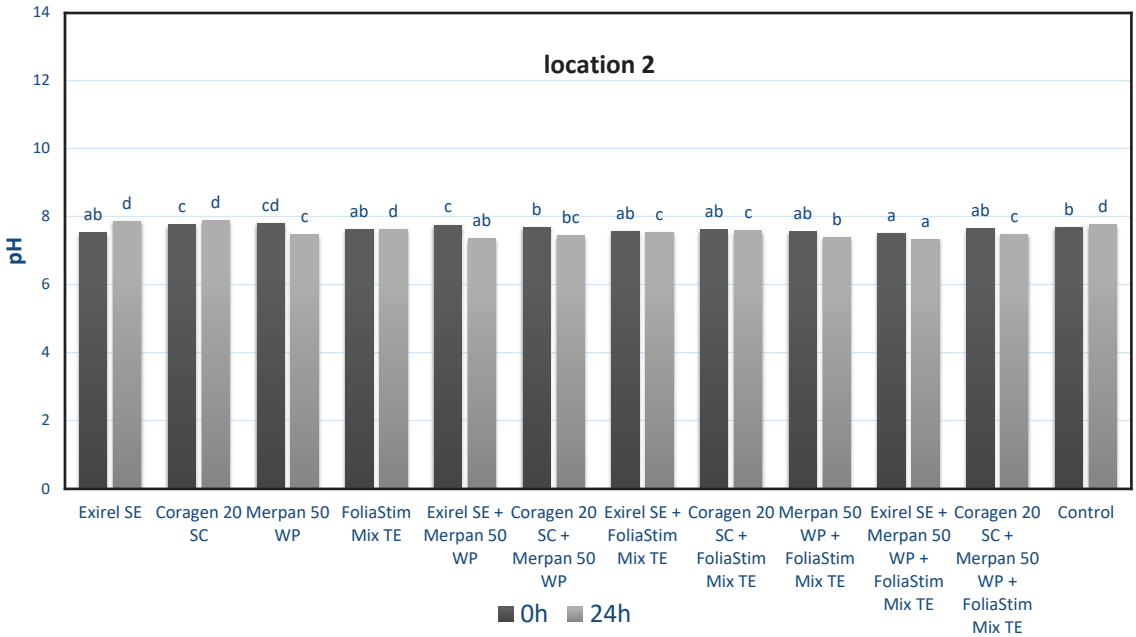

Figure 2. pH values of spray liquids of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after mixing and after 24 h (location 2)

Table 3. Suspensibility of spray liquids of insecticides (SC), fungicide (WP) and foliar fertilizer

Insecticides, fungicide, foliar fertilizer and their mixtures	Suspensibility (%)	
	Location 1	Location 2
Coragen 20 SC	99.9	99.9
Merpan 50 WP	54.4	56.3
Exirel + Merpan 50 WP	54.1	60.0
Coragen 20 SC + Merpan 50 WP	90.5	88.2
Coragen 20 SC + FoliaStim Mix TE	99.9	99.9
Merpan 50 WP + FoliaStim Mix TE	88.8	88.4
Exirel + Merpan 50 WP + FoliaStim Mix TE	85.8	88.4
Coragen 20 SC + Merpan 50 WP + FoliaStim Mix TE	96.4	95.1

decrease in pH after 24 h. In general, spray liquids of fungicides (azoxystrobin, mancozeb) and insecticides (thiamethoxam, cypermethrin) insignificantly changed pH, compared to control water, during the 24 h test period. However, the presence of the complex fertilizer, regardless of other components and water pH, changed the medium reaction in all variants from slightly alkaline to neutral and slightly acid (Vuković et al., 2013).

Suspensibility depending on water quality

Suspensibility of the SC, WP and WG formulations of PPPs dissolved in water shows that the persistence of active substances and other components in spray liquids over a specific time has an acceptable value of 60% (Federal Minister of Economy, 2001). In this study, the suspensibility of the pesticides, fertilizer and their mixtures ranged from 54.1 to 99.9%. The suspensibility of the insecticide Coragen 20 SC was very high in both tested waters (99.9%), as well as its mixture with Folia Stim Mix TE. However, the fungicide Merpan 50 WP, formulated as a wettable powder, showed reduced suspensibility with values below the limit (54.4-56.3%), and its mixture with the insecticide Exirel had the same result (54.1-60%) (Table 3).

The rate of coagulation is the most important property for evaluation of components in mixtures. Mixtures with rapid precipitation trend involve a risk in their use as precipitates contain high concentrations of non-pesticide ingredients and active substances that are phytotoxic. Over 2/3 of unstable products coagulate and precipitate within 5-15 minutes (Hrlec, 1999). The stability of most insecticides (pyrimifos-methyl and imidacloprid) and fungicides (propineb and mancozeb) in spray liquids prepared with well water is reduced, compared to the same suspensions in tap water, which indicates the dependence of pesticide stability on water quality and on the choice of tank-mix. This further indicates changes in suspensibility caused by the quality of water for treatment and the choice of ingredients in a mixture (Vuković et al., 2013).

Dispersion stability

Dispersion stability of the test PPPs and foliar fertilizer, as well as their mixtures, was observed immediately after mixing and after 0.5 h, 1 h, 2 h and 24 h (Table 4). After 24 h, redispersion was also evaluated (0.5 h). During observation, all spray liquids in the tested waters exhibited stability without any separation.

Surface tension of spray liquids

Surface tension depends on the treated surface, temperature of spray liquids, intermolecular forces of fluid whereby polar liquids (water) have higher surface tension than non-polar ones (Šovljanski et al., 2002).

Surface tension of well water (location 1) was 48 mJ/m^2 and 46.3 mJ/m^2 immediately after sampling and after 24 h, respectively. Surface tension of all spray liquids ranged from 34-45 mJ/m^2 after mixing, while 24 h later it was 35-41 mJ/m^2 (Figures 3 and 4). The obtained results on surface tension of Exirel and Coragen 20 SC spray liquids indicate a decrease in analyzed values after 24 h. The foliar fertilizer had a high surface tension (47 mJ/m^2) after mixing, while its value after 24 h matched the control.

Table 4. Dispersion stability of insecticides, fungicide, and their mixtures with foliar fertilizer

Insecticides, fungicide, foliar fertilizer and their mixtures	Dispersion stability				
	0 h*	0.5 h	1 h	2 h	24 h
Location 1					
Exirel	s	s	s	s	s
Exirel + Merpan 50 WP	s	s	s	s	s
Exirel + FoliaStim Mix TE	s	s	s	s	s
Exirel + Merpan 50 WP + FoliaStim Mix TE	s	s	s	s	s
Location 2					
Exirel	s	s	s	s	s
Exirel + Merpan 50 WP	s	s	s	s	s
Exirel + FoliaStim Mix TE	s	s	s	s	s
Exirel + Merpan 50 WP + FoliaStim Mix TE	s	s	s	s	s

*- immediately after preparation; s-stable

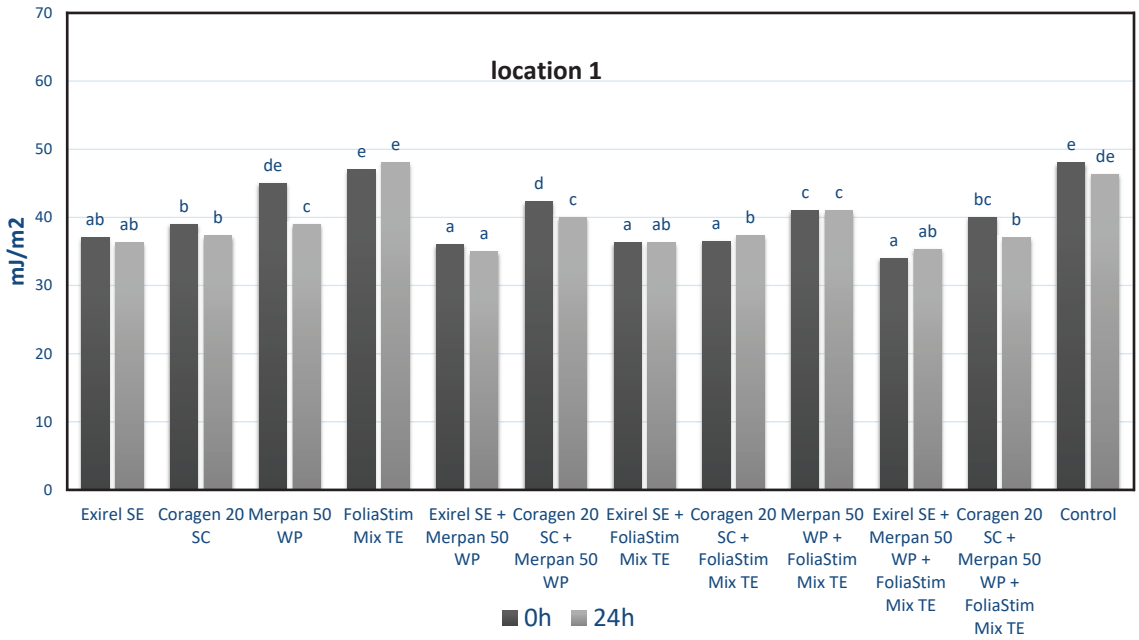

Figure 3. Surface tension of spray liquids of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after mixing and after 24 h (location 1)

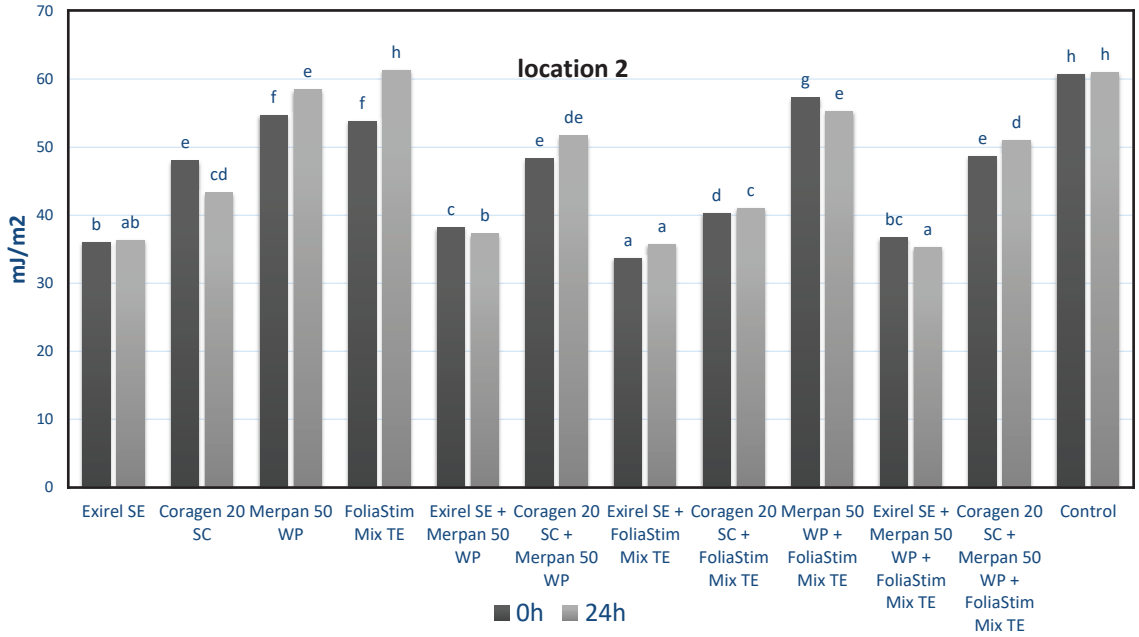

Figure 4. Surface tension of spray liquids of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after mixing and after 24 h (location 2)

The surface tension of mixtures of insecticides and fungicide slightly decreased after 24 h. The surface tension of insecticides, fungicide, and foliar fertilizer mixtures was almost the same 24 h after preparation. Finally, surface tension of spray liquids decreased in comparison with control liquid, and the changes depended on components and the expiration time of 24 h.

The surface tension of well water from location 2 slightly increased 24 h after sampling. The value of surface tension of the spray liquid containing the fungicide Merpan 50 WP and FoliaStim Mix TE increased, in contrast to the other spray liquids. An analysis of surface tension data shows that the Exirel insecticide data were 36 mJ/m^2 and 36.3 mJ/m^2 after mixing and after 24 h, respectively. Coragen 20 SC produced surface tension of 48 mJ/m^2, which decreased down to 43.3 mJ/m^2 after 24 h. In the case of foliar fertilizer, initial surface tension (53.8 mJ/m^2) increased after 24 h (61.3 mJ/m^2). All insecticide and fungicide spray liquids manifested a slight decrease in surface tension 24 h after preparation, compared to the values measured immediately after mixing, except the Coragen 20 SC + Merpan 50 WP mixture, which showed a slight increase in surface tension after the resting period of 24 hours.

Surface tension of the tested spray liquids increased in value after 24 h, except for the Merpan 50 WP + Folia Stim Mix TE mixture. Spray liquids of Coragen 20 SC + Merpan 50 WP + Folia Stim Mix TE mixtures showed an increase in surface tension after 24 h, when it was 51 mJ/m^2. Based on the obtained results, it is evident that the surface tension of spray liquids depends on the quality of water, components and its expiration after 24 h.

Electroconductivity of spray liquids

Electroconductivity of the slightly alkaline well water from location 1 remained the same after 24 h (624.3-624 µS/cm). Electroconductivity values of the PPPs Exirel, Coragen 20 SC and Merpan 50 WP increased after 24 h (Figures 5 and 6), while electroconductivity of the fertilizer ranged 1004.3-1029 µS/cm. Electroconductivity values in mixtures of the tested insecticides and fungicide ranged from 700-709 µS/cm after mixing, and increased (726.3-737 µS/cm) after 24 h. Electroconductivity in mixtures of the foliar fertilizer with fungicide, and fertilizer with insecticides increased as well. It can be inferred that electroconductivity depends primarily on the liquid components and partially on the standstill time.

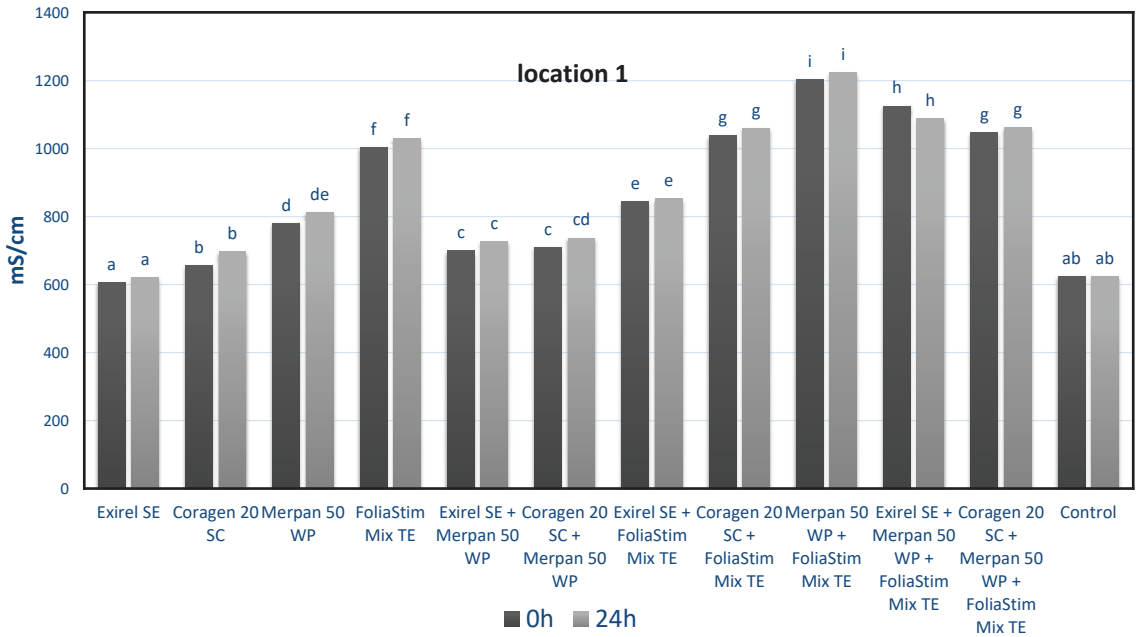

Figure 5. Electroconductivity of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after preparation and after 24 h (location 1)

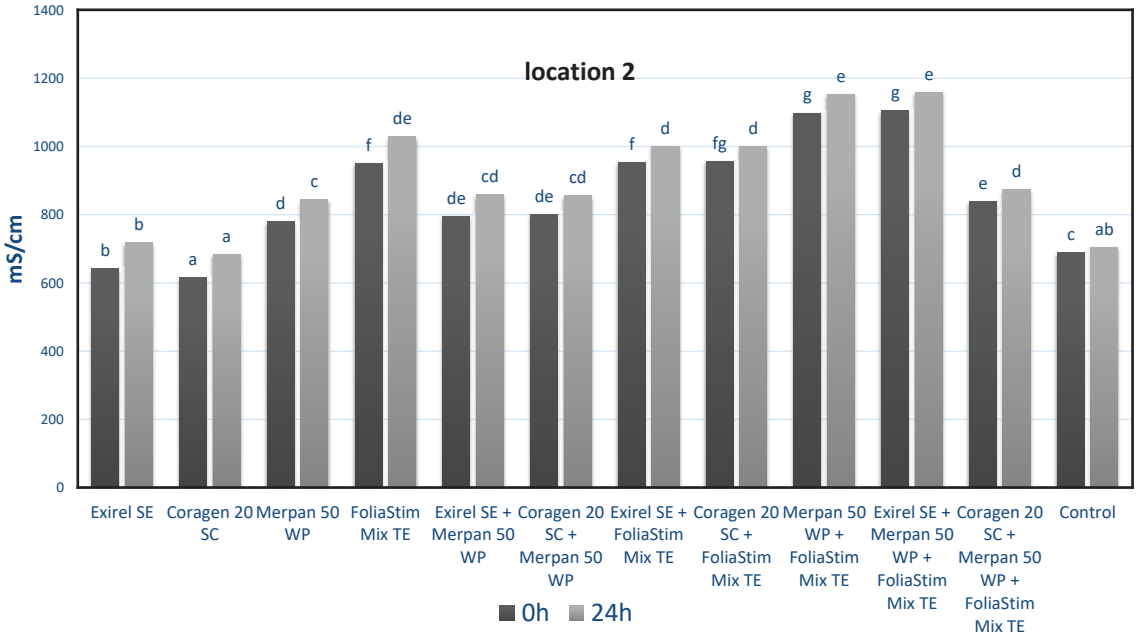

Figure 6. Electroconductivity of insecticides, fungicide, foliar fertilizer and their mixtures in well water immediately after preparation and after 24 h (location 2)

Electroconductivity of the alkaline well water from location 2 was 689.3 µS/cm, and it slightly increased over 24 h (704.3 µS/cm). Electroconductivity of the tested insecticides, fungicide, foliar fertilizer and their mixtures in water from location 2 increased after 24 h (Figure 6).

The lowest electroconductivity was measured in the spray liquid of Coragen 20 SC (614.6-684 µS/cm), while the highest was in the triple mixture of Exirel + Merpan 50 WP + FoliaStim Mix TE, which ranged from 1100-1172 µS/cm, similar to the results obtained in the water test at location 1.

CONCLUSION

Changes in physicochemical properties of pesticide mixtures can cause different consequences for crops and other plants, as well as the environment. However, most researchers focus on the effects of individual substances.

This study analyzed the influence of water quality on spray liquids of individual substances (cyantraniliprole, chlorantraniliprole, captan and foliar fertilizer), and their mixtures, testing the most important properties. Based on the obtained results, it could be concluded that the physico-chemical properties of spray liquids of insecticides, fungicide, fertilizer, their dual and triple mixtures, vary depending on the quality of water and the components included in their composition. However, all tested spray liquids exhibited consistency, and compatibility, over 24 h.

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REFERENCES

De Castro, V.L.S.S. (2009). Uso de misturas de agrotóxicos na agricultura e suas implicações toxicológicas na saúde. *Journal of the Brazilian Society of Ecotoxicology*, *4* (1-3), 87-94.

Dobrat, W., & Martijn, A. (Eds.) (2007a). *Determination of pH values, General method*. CIPAC Handbook F, MT 75. Harpenden, UK: Collaborative International Pesticides Analytical Council Limited, England, 205-206.

Dobrat, W., & Martijn, A. (Eds.) (2007b). *Suspensibility of wettable powders in water*. CIPAC Handbook F, MT 15. Harpenden, UK: Collaborative International Pesticides Analytical Council Limited, England, 45-52.

Dobrat, W., & Martijn, A. (Eds.) (2007c). *Determination of conductivity*. CIPAC Handbook F, MT 32. Harpenden, UK: Collaborative International Pesticides Analytical Council Limited, England, 103-107.

Federal Minister of Economy and Internal Trade, Republic of Serbia (2001). Pravilnik o metodama za ispitivanje pesticida (Guidance on methods for pesticides analysis). *Službeni list SRJ (Official Gazette SRJ), 63/2001-1; 65/2001-14; RS 93/2005-7, RS 21/2012-75***.** Retrieved from: https://www.pravno-informacioni-sistem.rs/SlGlasnikPortal/eli/rep/slsrj/ministarstva/pravilnik/2001/63/1/reg

Federal Minister of Labor, Health and Social Policy, Republic of Serbia (1998). Pravilnik o higijenskoj ispravnosti vode za piće (Water quality regulation). *Službeni list SRJ (Official Gazette SRJ), 42/1998-4, 44/1999-19, 28/2019-114.* Retrieved from: http://www.pravno-informacioni-sistem.rs/SlGlasnikPortal/eli/rep/slsrj/ministarstva/ pravilnik/1998/42/2/reg

Hrlec, G. (1999). Kompatibilnost formulacija. *Glasnik zaštite bilja*, *4*, 187-196.

Manual on development and use of FAO and WHO specifications for pesticides (2016). Rome, Italy: FAO/WHO Joint Meeting on Pesticde Specifications (JMPS). Retrieved from WHO/FAO: http://www.fao.org/3/i5713e/i5713e.pdf

Mihajlović, V., Tomić, T., Tubić, A., Molnar Jazić, J., Ivančev Tumbas, I., Šunjka, D., ... Teodorović, I. (2019). The impact of humic acid on toxicity of individual herbicides and their mixtures to aquatic macrophytes. *Environmental Science and Pollution Research, 26*(23), 23571-23582.

Moraes, H.M.F., Costa, J.O., Pereira, G.A.M., Souza, W.M., Silva, A.A., & Paixão, G.P. (2019). Physical compatibility and stability of pesticide mixtures at different spray volumes. *Planta Daninha., 37*, e019214004; doi: https://doi.org/10.1590/s0100-83582019370100125

Šovljanski, R., Klokočar Šmit, Z., & Lazić, S. (2002). Praktikum iz opšte fitofarmacije – za studente Poljoprivrednog fakulteta. Novi Sad, Serbia: Faculty of Agriculture.

Vuković, S., Inđić, D., & Gvozdenac, S. (2014). Phytotoxic effects of fungicides, insecticides and nonpesticidal components on pepper depending on water quality. *Pesticides and Phytomedicine*, *29*(2), 145-153.

Vuković, S., Inđić, D., Lazić, S., Grahovac, M., Bursić, V., Šunjka, D., & Gvozdenac, S. (2013). Water in pesticide application. *Journal of Environmental Protection and Ecology*, *14*(1), 132-141.

Uticaj kvaliteta vode na kompatibilnost pesticida i đubriva

REZIME

Smeše dva ili više pesticida vrlo su česte u savremenoj poljoprivredi. Međutim, promene u efikasnosti ili biološkoj aktivnosti preparata, poput sinergizma i antagonizma, fitotoksičnosti, postojanosti, toksičnosti za neciljane organizme, mogu se javiti kao posledica primene takvih smeša. Ovo istraživanje je sprovedeno u cilju procene kompatibilnosti radnih tečnosti insekticida (cijantraniliprol - Exirel, hlorantraniliprol - Coragen 20 SC), fungicida (kaptan - Merpan 50 WP) i folijarnog đubriva (Folia Stim Mix TE), kao i njihovih smeša, u zavisnosti od kvaliteta vode (bunarska voda sa dva lokaliteta u Srbiji - Mala Remeta i Čerević). Navedeni preparati se koriste za suzbijanje najznačajnijih štetnih organizama breskve i kao izvor hranljivih sastojaka za biljku. Analize vode (pH, tvrdoća, elektroprovodljivost, hloridi, nitrati, nitriti, amonijak, sadržaj kalcijuma i gvožđa) i fizičko-hemijskih svojstava radnih tečnosti (pH, suspenzibilnost, disperzibilnost, površinski napon i elektroprovodljivost) izvedene su u laboratorijskom uslovima prema standardnim metodama. U zavisnosti od kvaliteta vode i komponenti koje su uključene u smešu, došlo je do promene fizičko-hemijskih svojstava radnih tečnosti. Međutim, sve testirane radne tečnosti su pokazale konzistentnost i kompatibilnost tokom 24 sata.

Ključne reči: pesticidi, đubrivo, smeše, kvalitet vode, fizičko-hemijska svojstva, kompatibilnost

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Original scientific paper

Nephroprotective effect of aqueous acetonic extract of *Morus alba* and its underlying mechanisms against glyphosate-induced toxicity - *in vivo* model

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SUMMARY

Glyphosate, the active substance in Roundup®, is the most widely used pesticide in the world and may be present as a residue in derived foods and drinking water. Previous reports have confirmed that extracts from leaves of *Morus alba* exert many pharmacological activities. However, renoprotective effects of *M. alba* extract and its underling molecular mechanism is still unknown.

Wistar rats (180-200 g) were used in this study (n=5-6). A control group received 0.2 ml normal saline intraperitoneally (i.p) once daily for two weeks. Control animals received standard diet. Treated groups received either polyphenolic extract (100 mg/kg,i.p) or glyphosate (100 mg/kg, i.p), or co-administration (extract µg ml^{-1} kg b.w. and glyphosate 100 mg kg^{-1} b.w, i.p), daily until the 15thday of treatment. Lactate deshydrogenase LDH, serum concentrations of blood urea, creatinine and nitric oxide were measured using standard coloromertic methods.

Renal oxidative stress, evidenced by increased malondialdehyde (MDA) and protein carbonyl levels and decline in superoxide dismutase (SOD) activity, was significantly alleviated by mulberry leaves extract (MLE) administration. MLE also appears to be able to modulate altered biochemical parametres by maintaining free iron and Ca_2^+ homeostasis, and regulate the endogenous antioxidant enzymes system. It seems that concurrent use of the aqueous acetonic fraction of *M. alba,* rich in chlorogenic acid and its isomeres, can protect kidneys from glyphosate-induced nephrotoxicity. Overall, MLE may possess protective activity against glyphosate-induced toxicity, which may be attributed to chlorogenic acid and its isomers, the most abundant phenolic acids present in its extracts.

Mulberry leaves are a source of phenolic compounds and can be a good start towards discovering a new chemical compound which may lead to a new drug. A mulberry extract supplement could serve as a candidate for developing a safe, and promising nutraceutical product for the management of nephrotoxicity.

Keywords: glyphosate, oxidative stress, nephrotoxicity, mulberry, nephroprotective effect

INTRODUCTION

Glyphosate is one of the most commonly used herbicides in the world, including Tunisia. Residual amounts of glyphosate have been detected in soil, vegetables, grains and other food products. A recent study revealed that glyphosate residues can be accumulated in chicken organs and muscles after consuming glyphosate in their feed (Shehata et al., 2014). Furthermore, detection of glyphosate residues in human urine has indicated that many diseases currently on the rise, such as hepatotoxicity, gastrointestinal, cardiovascular and respiratory disorders, are associated with glyphosate use as the cause of multi-organ toxicity. However, the toxic effect of glyphosate or its surfactant in renal function has not been well established. Kidney damage is also frequent and usually reflects reduced organ perfusion. Renal failure requiring haemodialysis, metabolic acidosis and hyperkalaemia may supervene in severe cases (Bradberry et al., 2004).

Since kidney may also be an organ for the excretion of glyphosate components (Sribanditmongkol al., 2012), there is an early evidence of kidney damage that could be used to predict the risk of fatal outcome in glyphosate toxicity. The plant protection product glyphosate was used in the current study, and the surfactant used in Roundup® may contribute to herbicide nephrotoxicity (Seok et al., 2011). Renal dysfunctions have been reported in cases of acute intoxication. Recent studies have reported that the mechanism of pesticide nephrotoxicity might be related to oxidative stress, apoptosis or an inflammatory response, and therefore several chemical and natural compounds with antioxidant and/or anti-inflammatory activity have been examined for their protective effects against pesticide-induced nephrotoxicity. Intoxication with glyphosate has been found to induce early kidney damage, i.e. acute tubular, glomerular necrosis and apoptosis (Naqvi, 2017). These modes of cell death occurred in tubules and glomeruli during the acute stages of Roundup® toxicity. Wunnapuk et al. (2014) investigated a panel of kidney injury biomarkers in terms of suitability to detect acute kidney injury (AKI) as a major renal disease associated with high mortality rate and increasing prevalence (Wunnapuk et al., 2014). After oral administration of glyphosate to rats, suitable biomarkers are able to detect the early stages of kidney injury, and glyphosate has been shown to be a causative agent of vasculitic neuropathy, while exposure to a large quantity of glyphosate-based herbicide over a short time through transcutaneous or inhalation pathways without protection may cause vasculitis, not only in the peripheral nervous system but also in other organs (Kawagashira et al., 2017).

Recently, much attention has been focused on the protective effects of antioxidants and naturally occurring substances against oxidative stress damage. *Morus alba* L. or white mulberry (family Moraceae) is widely distributed and cultivated in Iran, India, China, southern Europe and North America (Yang et al., 2010). Several pharmacological, biological and clinical properties, including antibacterial, antiviral (Wang et al., 2008), antitussive, hypoglycemic (Naowaboot et al., 2004), hypotensive (Emami et al., 2004), antiatherogenic, antihyperlipidemic (Nickavar & Mosazadeh, 2009), diuretic, astringent and antioxidant (Yang et al., 2010; Kobayashi et al., 2011), have been reported for *M. alba* leaves.

In folk medicine *M. alba* has been used for treatments of urinary incontinence due to its strong diuretic property (Yeung , 1985). Its leaves in the forms of infusion and decoction are well-known in different parts of the world and used for preventing or treating urinary disorders (Yang et al., 2010). They are also reported to have diuretic, antiviral, and bacteriostatic properties (Chu et al., 2006). Mulberry fruits also have a tonic effect on kidneys (Duke & Ayensu, 1985). Various phenolic compounds have been identified from mulberry leaves, such as flavonoids and other derivatives; these compounds are responsible for most of the potential health benefits of mulberry leaves and help maintain the body against cellular injuries.

Numerous studies have focused on the nephroprotective activity of *M. alba*. A study has investigated the nephroprotective effect of hydroalcoholic extract and flavonoid fraction of *M. alba* leaves on cisplatin-induced nephrotoxicity in rats. Flavonoids from *M. alba* could also prevent cisplatin-induced pathological damage of the kidney. Cisplatin as an important anti-tumor drug causes nephrotoxicity mainly by oxidative stress and renin-angiotensin system (RAS). *M. alba* leaf extracts have been reported to have protective effects on cisplatin-induced nephrotoxicity in rats (Nematbakhsh et al., 2013) and prevent renal functioning alterations expected with the use of gentamicin, the most effective bactericidal drug against a wide range of Gram negative micro-organisms, and its nephrotoxic side effects (Ullah et al., 2016). Moreover, nephroprotective effects of hydroalcoholic extracts of *M. alba L*. against isoniazid (INH) have been studied in albino rabbits. Isoniazid is the first line

drug for the treatment of tuberculosis and can cause nephrotoxicity in humans and animals (Faqir et al., 2014). The effect of mulberry tea supplement on the cellular damage of kidney induced by supratherapeutic acetaminophen administration was also investigated (Salih et al., 2015). Acetaminophen, also known as paracetemol, is widely used as an analgesic and antipyretic, prescribed as pain reliever and fever reducer. Mulberry tea extract supplement did help to maintain kidneys closer to normal and served to protect them from severe damage due to nephrotoxicity, compared to animals that received no such supplement (Salih et al., 2015).

Positive correlation between glyphosate exposure and health deterioration has been increasingly recognized, and chronic exposure to glyphosate may be the cause of widespread nephrotoxicity. Previous studies have demonstrated that extracts from *M. alba* had renoprotective properties but the effects of MLE on glyphosate-induced kidney injury remained unclear. The aim of this study was to examine the effects of MLE on glyphosate-induced kidney injury and elucidate its molecular mechanisms.

MATERIALS AND METHODS

Glyphosate [N-(phosphonomethyl) glycine] (GLP), Roundup® plus 450g/L(H.029-11), is a commercial formulation, purchased from ATLAS AGRICOLE, which has been registered with the Tunisian Ministry of Agriculture. Thiobarbituric acid (TBA), 2,6,-di-tert-butyl-4-hydroxytoluene (BHT), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), 2-methoxyphenol (gaiacol), bovine catalase 4-(1-hydroxy- 2-methylamino-ethyl)-benzene-1,2-diol (epinephrine), and 2,4-dinitrophenyl hydrazine (DNPH) were obtained from Sigma-Aldrich (Germany). Buffer salts (KCl, NaHCO3, Na_2HPO_4, NaH_2PO4, $K2HPO_4$, andKH_2PO_4) were purchased from Baker Inc. (Phillipsburg, USA).

M. alba leaf extract preparation

Mulberry (*Morus alba* L.) leaves were collected in May-June in the north of Tunisia where the species grows wildly. The leaves were then washed with distilled water and extracted with 70% cold acetone (–20 °C). The supernatant was collected and pooled, then concentrated to dryness under vacuum, using a rotary evaporator (60 °C), to obtain a final volume of 3 ml (Rebai et al., 2017). Then, the aqueous extract was lyophilized to obtain MLE, which was then stored at –20 °C before use. Phenolic compounds from the mulberry leaves were assayed by the Folin-Ciocalteau method, and MLE (100 μg ml^{-1} kg^{-1} b.w.) was used at the concentration of 100 μg/ml.

HPLC-DAD analysis and LC-electrospray ionization (ESI)-MS

Phenolic compounds were separated by reverse phase HPLC analysis under conditions previously described (Fattouch et al., 2008) with UV or DAD detection. Analytical RP-HPLC analysis was performed with a C18 column. The mobile phase consisted of 1% aqueous formic acid (solvent A) and methanol (solvent B). The elution was allowed to run with 95% A and 5% B, 75% A for 10 min, 65% A for 3 min, 55% A for 35 min, 40% A for 40 min, 50% A for 45 min, 45% A for 50 min, 30% A for 53 min, 25% A for 56 min, 20% A for 60 min and 95% A for 95 min for 10 min. The flow rate was 1 ml/min. Polyphenols in the eluted fractions were detected at 280 nm and 350 nm with a diode array detector. The LC-ESI-MS system consisted of an Agilent LC 1100 series (Agilent Technologies, Inc., CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000þ (Bruker Daltonics, GmbH, Germany) mass spectrometer, equipped with an ESI source and ion trap mass analyzer. The ESI was operated in the positive mode with ESI source probe at 250°C; CDL at 250°C, block at 240°C, flow gas (N2) at 4.5 l/min, probe voltage 4.5 kV, fragmentor voltage 20 V, and a nominal mass range up to m/z 800. Compounds were identified by comparing their retention times and spectra to those of standards, when available. Quantification was then confirmed by comparison with the calibration curves obtained with standards, i.e. reference solution of phenolic compounds. Unknown chromatographic peaks were tentatively identified via their spectral features in comparison with literature data.

Animals

Wistar rats (180–240 g) were purchased from the Society of Pharmaceutical Industries Tunisia (SIPHAT), allowed to acclimatize in the laboratory environment for 1 week at room temperature 22 ± 1 °C, and supplied with standard diet and tap water *ad libitum*. Procedures with the laboratory animals and their care were in accordance with the NIH guidelines.

Experimental procedure and treatment

The animals were randomly divided into four groups of six animals each: group 1 received standard diet (control), group 2 was injected (i.p.) with glyphosate (100 mg kg^{-1} b.w.), group 3 was injected with MLE (100 µg ml^{-1} kg^{-1} b.w.) (i.p.), and finally group 4 was injected with both glyphosate and MLE according to Rebai et al. (2017). The rats were observed daily for mortality and signs of toxicity. At the end of the experimental period (15 days), the animals were anesthetized with urethane (40 mg ml^{-1}) and then sacrificed. Blood samples were collected and allowed to clot at room temperature before centrifuging at approximately 3000 rpm for 15 minutes. The serum was stored at -20° C until assaying for biochemical parameters. Kidneys were removed and dissected free from the surrounding fat and connective tissue, then homogenized in PBS buffer of pH 7.4 with an Ultrathurax T25 homogenizer, and centrifuged (10 at 10,000 g, 4 °C) and the supernatant was used for determination of markers for oxidative stress and biochemical parameters.

Measurement of lactate dehydrogenase (LDH) released

The amount of LDH released is measured with an enzymatic reaction which converts iodonitrotetrazolium or INT (a tetrazolium salt) into a red color formazan. When LDH is present in the cell culture, it reduces NAD^+ to NADH and H^+ through the oxidation of lactate to pyruvate. The catalyst (diaphorase) then transfers H/H^+ from NADH + H^+ to the tetrazolium salt INT to form a colored formazan salt. The amount of color produced is measured at 490 nm by standard spectroscopy and is proportional to the amount of damaged cells in the culture.

Kidney protein quantification

Total soluble proteins were determined according to Ohnishi and Barr (1978) using the Biuret method. At acidic pH, a blue-colored complex of soluble proteins with copper was spectrophotometrically measured at 546 nm using NanoSpec Double UV (Germany) (Ohnishi & Barr, 1978).

Urea and creatinine assessment in plasma

Urea and creatinine in plasma were measured using a commercially available spectrophotometric enzymatic kit (commercial kit from Biomaghreb, Tunisia) according to Larsen (1972).

Antioxidant enzyme activity

Catalase (CAT) assay

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm. The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and 5 µl of sample. CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}*cm^{-1}$ for H_2O_2. One unit of catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 mmol of H_2O_2 per minute at 37 °C and specific activity corresponding to transformation of substrate (in mmol) (H_2O_2) per minute per milligram protein (Aebi, 1984).

Peroxidase (POD) assay

Peroxidase activity was measured at 25 °C using guaiacol as hydrogen donor. The reaction mixture contained 9 mM guaiacol, 19 mM H_2O_2 in 50 mM phosphate buffer pH 7, and 10 µl samples in 1 ml final volume. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase in absorbance at 470 nm each 30 s for 3 min. Peroxidase activity was expressed as nanomoles of guaiacol oxidized per minute with a molecular extinction coefficient of 26.2 mM^{-1} for calculation.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by using the modified epinephrine assay. At alkaline pH, superoxide anion (O_2^-) causes the auto-oxidation of epinephrine to adrenochrome. One unit of SOD is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%. Samples were added to 2 ml reaction mixture containing 10 µl bovine catalase (0.4 U $µl^{-1}$), 20 µl epinephrine (5 mg ml^{-1}), and 62.5 mM sodium carbonate/sodium bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm each 30 s for 3 min (Misra & Fridovich, 1972).

Calcium determination

Extracellular ionizable calcium was determined using a commercially available kit from Biomaghreb, Tunisia. At basic pH, calcium was constituted with

cresolphtalein, a purple-colored complex measurable at 570 nm. Briefly, 50 µl of sample was added to the reaction mixture containing 2-amino-2-methyl 1-propanol buffer (500 mmol L^{-1}), cresolphtalein (0.62 mmol l^{-1}), and hydroxy-8 quinoleine (69 mmol l^{-1}). Incubation was carried out at room temperature for 5 min assuming the complex was stable during 1 h (Stern & Lewis , 1957).

Free iron determination

Free iron was determined according to Leardi et al. (1998) using a commercially available kit from Biomaghreb (Ariana, Tunisia). Briefly, at acidic pH 4.8, all Fe^{3+} released from transferrin were reduced by ascorbic acid into Fe^{2+}, which constitutes with ferrozine a colourful purple complex measurable at 560 nm. Heart extract was added to 250 µl of reaction mixture containing ascorbic acid (5 g/L) and ferrozin (40 mM), and incubation was performed at 37°C for 10 min (Leardi et al., 1998).

Lipid peroxidation determination

Lipid peroxidation was carried out using the method of MDA measurement according to Draper and Hadley (1990). An aliquot of brain homogenate was mixed with BHT-TCA solution containing 1% BHT and 20% TCA. After centrifugation, the supernatant was mixed with a second solution containing 0.5 N HCL and TBA (120 mmol l^{-1}) and then heated at 80 °C for 10 min. After cooling, the absorbance of the resulted chromophore was measured at 532 nm using a Nanolytik® NanoSpec Double UV-visible spectrophotometer (Nanolytik, Quality from Germany). Malondialdehyde (MDA) contents were expressed as millimoles MD per milligram protein with an extinction coefficient of 1.56105 mol l^{-1} cm^{-1}(Draper & Hadley, 1990).

Protein carbonylation

The most commonly used marker of protein oxidation was used to quantify protein carbonyls (PC) in the brain homogenate by the reaction between 2,4-dinitrophenylhydrazine and DNPH, and protein carbonyls. After the precipitation of proteins in the sample with 20% TCA, and centrifugation at 10,000 g for 10 min at 4 °C, DNPH-containing buffer reacted with protein carbonyls and dissolved in 20 mmol l^{-1} potassium phosphate (pH 2.3) containing guanidine HCL 6 mol l^{-1}. The amount of protein carbonyls produced is quantified spectrophotometrically at an absorbance of 366 nm using the molar extinction coefficient of 22,000 mol l^{-1} cm^{-1}. Carbonyl content can be standardized to protein concentration and expressed as nanomoles of carbonyl residues per milligram of protein.

Statistical analysis

Data are presented as the mean ± SEM from three independent experiments performed in quadruplicate. Statistical analysis of the data was performed by using Student's t-test and ANOVA, followed by Bonferroni's test. One asterisk $P < 0.05$ vs control two asterisks $P < 0.05$ vs control, one number sign $P < 0.05$, two number signs $P < 0.01$ vs pesticide-treated rats.

RESULTS

Evaluation of body and kideney weights

The animals were checked for both body and kidney weight gain or loss. The results show that weight variations were time-dependent effects of glyphosate in the exposed rat group during glyphosate treatement period at a dose of 100 ppm. The final body weight of rats moderately decreased in the glyphosate-exposed groups, compared to controls, as shown in Figure 1. Weight loss was observed when rats were administered daily glyphosate dose (100 µg kg^{-1} b.w.), while co-treatement with both MLE and glyphosate induced a significant gain in body weight, compared with the glyphosate-treated group. On the other hand, no statistically significant difference in absolute kidney weight was observed between the control and MLE-treated group ($P>0.05$). However, absolute kidney weight showed a significant increase in glyphosate-treated groups, compared to control group. Moreover, the absolute kidney weight of rats submitted to glyphosate co-treatement and to MLE-treatment was compared to the control group, and no statistically significant changes were observed ($P>0.05$).

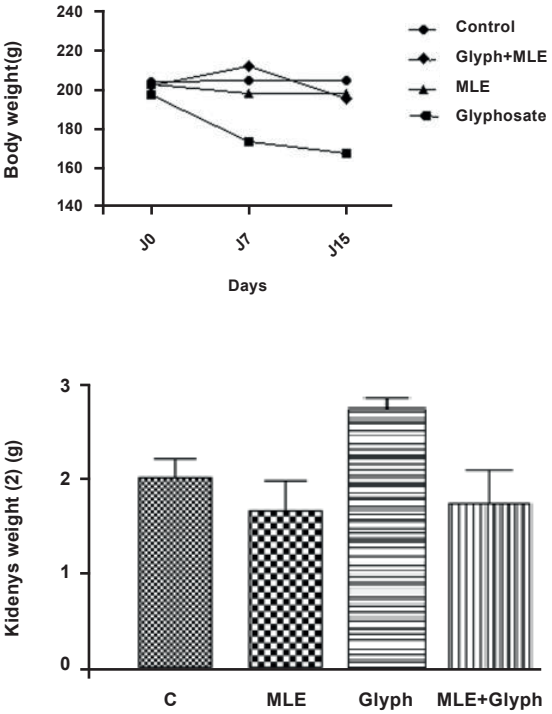

Figure 1. Body weight evolution and kidney weight variations during treatment. Glyphosate and MLE were administered in a single dose i.p. to rats daily for 15 days. Data are expressed as mean ± SEM

MLE protects kidneys from toxicity induced by glyphosate exposure

Lactate dehydrogenase (LDH), an important intracellular enzyme used as a sensitive parameter that reflects oxidative stress and altering of the enzymatic system in tissues, was used to evaluate the nephroprotective effect of MLE against toxity induced by glyphosate. As shown in Figure 2A, administration of a single dose of glyphosate at 100 ppm 100 g^{-1} b.w for two weeks induced cell death by increased LDH release from kidney tissue, about +50% compared to the non-treated control group. Moreover, the co-administration of glyphosate and the phenolic extract MLE attenuated the toxic effect of the pesticide and decreased LDH levels in kidney homogenate in rats.

Then, focusing on renal specific biomarkers toxicity using creatinine, and urea levels, our results show that both creatinine and urea levels decreased in the glyphosate and MLE co-treated group (Figure 2B,C) to correct the deleterious effet of glyphosate in the renal function of rtas exposed only to glyphosate.

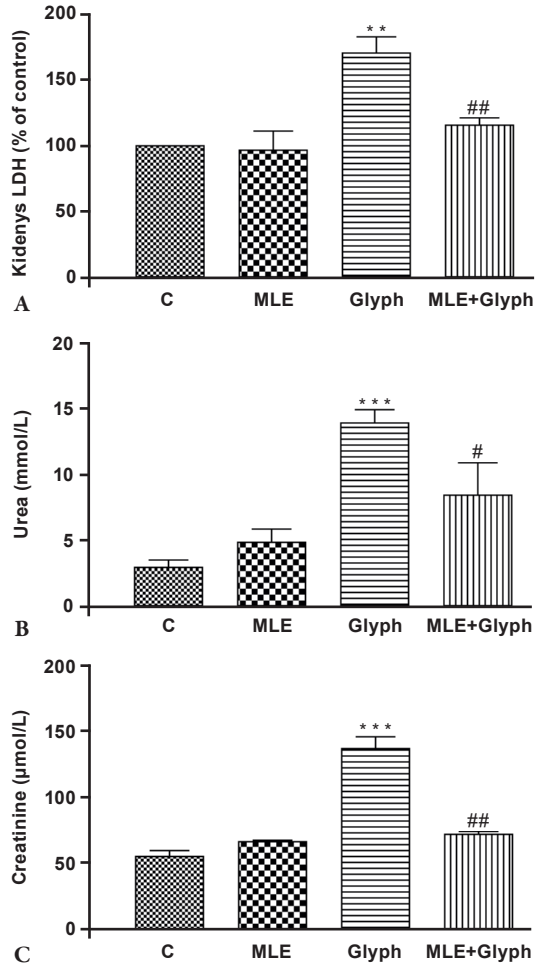

Figure 2. Nephroprotective Effect of MLE agianst on glyphosate-induced renal toxictity.

(A) Glyphosate-induced release LDH from kidenys. The results are expressed as percentages of lactate dehydrogenase (LDH) released, quantified by using an LDH activity kit assay.

(B, C) MLE restores renal toxicity biomarkers (urea and creatinine) induced by glyphosate. Results are expressed as mean ± SD (n = 5). Asterisk $P < 0.05$ vs control, number signs $P < 0.01$ vs pesticide treated rats

Enzymatic and antioxidant status in kidney

The results showed that oxidative stress induced by glyphosate altering the enzymatic system affects antioxidant enzyme activities. Catalase (CAT) and peroxidise (POD) activities increased significantly in the exposed groups, compared with control groups, but the levels of superoxide dismutase (SOD) activity decreased markedly in kidneys by -87.5% in the exposed groups, compared to controls. MLE administered in co-treatment

with glyphosate decreased both CAT and POD activities near to the level in non-treated group. Total SOD activity was corrected by MLE administration, bringing it to near control levels (Figure 3).

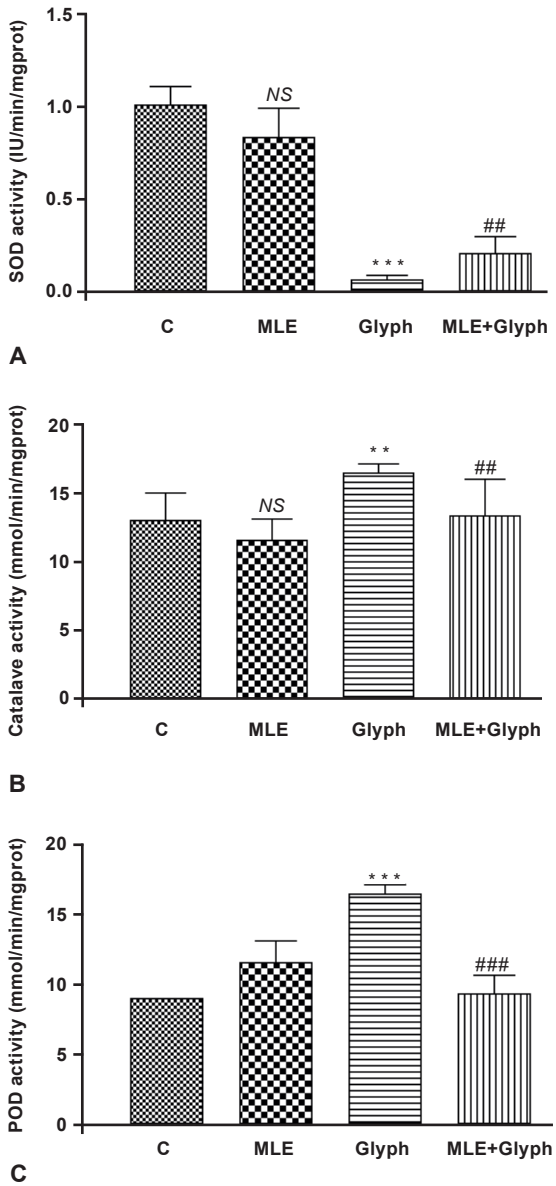

Figure 3. Effect of MLE extract and glyphosate on kidneys antioxidant enzymatic system. Wistar rats were administered i.p. with *Morus alba* leaf extract (MLE), glyphosate 100 mg kg^{-1} b.w. (Glyph), or glyphosate plus MLE (MLE + Glyph). (A) superoxide dismutase (SOD), (B) kidney catalase, and (C) peroxidase (POD) activities were determined. Results were expressed as means ± SD (n = 5). One asterisk $P < 0.05$ vs control, two asterisks $P < 0.05$ vs control, one number sign $P < 0.05$, two number signs $P < 0.01$ vs pesticide-treated rats.

Estimation of MDA and protein carbonyl levels in kidneys

Glyphosate exposure provoked lipid peroxidation, inferred from an almost double increase in MDA levels, and three-fold in protein carbonyls in the groups treated with 100 ppm glyphosate for two weeks, compared with those in control groups (Figure 4). Co-treatment with mulberry leaf extract abrogated the toxic effect of glyphosate, bringing it near to control group levels.

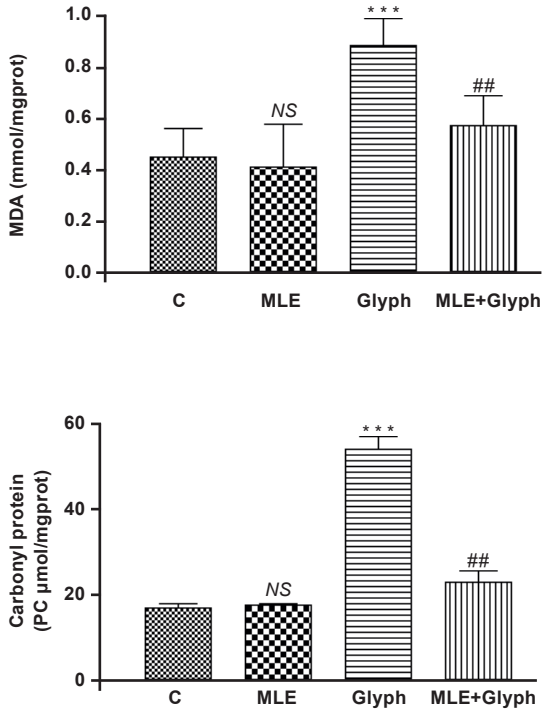

Figure 4. Renoprotective effect of MLE on glyphosate-induced lipoperoxidation and protein carbonylation. Rats were treated with glyphosate (100 mg kg^{-1} b.w.). MLE was administered i.p. with the dose of 100 µg ml^{-1} daily for 15 days. Results are expressed as mean ± SD (n = 5). Asterisk $P < 0.05$ vs control, number signs $P < 0.01$ vs pesticide-treated rats

Glyphosate and intracellular mediators in kidneys

Calcium pathway and free iron accumultion, the intracellular mediators involved in the molecular target of glyphosate-induced oxidative stress against which MLE could exert its neuroprotective effects, were investigated. Glyphosate provoked an increase in renal free iron (Figure 5A), and ionizable calcium (Figure 5B). Co-treatment of rats with glyphosate and MLE significantly decreased these mediators when compared to the control group.

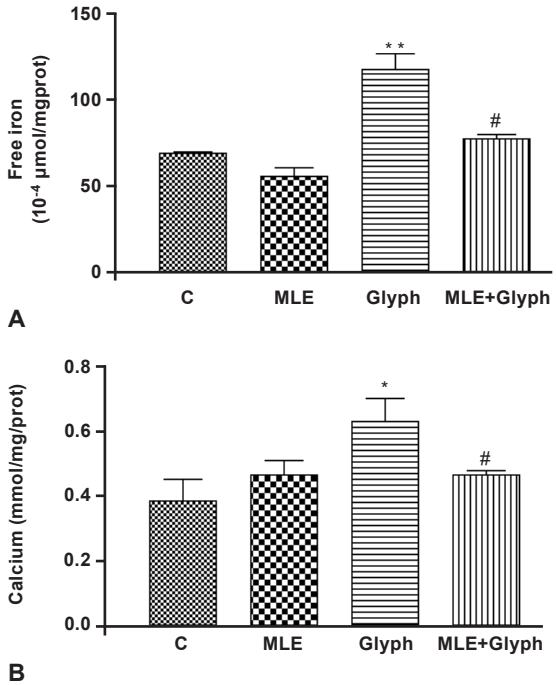

Figure 5. Protective effects of MLE on glyphosate-induced free iron (A) and Ca_2 + (B), in kidneys. Rats were administered i.p. with *Morus alba* leaf extract (MLE), glyphosate 100 mg kg^{-1} b.w. (Glyph), or glyphosate plus MLE (MLE + Glyph). Results are shown as means ± SD (n = 5). Asterisk $P < 0.05$ vs control, number signs $P < 0.01$ vs pesticide-treated rats

DISCUSSION

Glyphosate [N-(phosphonomethyl) glycine] is an organophosphate and one of the most popular herbicides in the world and the active ingredient of the plant protection product Roundup®. As previous investigations performed on various organs have revealed, glyphosate causes hepatotoxicity (El-Shenawy, 2009), neurologic disorders (Negga et al., 2012; Rebai et al.,2017), and nephrotoxicity (Astiz et al., 2009). Importantly, detection of glyphosate residues in human urine has proposed that early evidence of kidney injury could be used to predict the risk of glyphosate toxicity (Valcke et al., 2017).

Many studies have reported that accidental or intentional ingestion of glyphosate or other surfactant-based herbicide, such as Roundup(®), leads to nephrotoxicity and severe renal function disorders. More recently, it has been reported that exposure to glyphosate-based herbicides in transcutaneous or inhalation pathways without protection may cause vasculitis neuropathy, not only in the peripheral nervous system but also in other organs (Kawagashira et al., 2017). Moreover, other studies have reported that glyphosate is able to trigger dysregulation of numerous metabolism pathways, which may contribute to oxidative damage and generation of reactive oxygen and nitrogen species (ROS, RNS) (Astiz et al., 2009), and to cause mitochondrial oxidative phosphorylation by damaging the mitochondrial membrane (Bradberry et al., 2004), to mediate toxicity by lipid peroxidation and protein carbonyl formation (Mensah et al., 2012; Rebai et al., 2017), to enhance DNA damage (Navarro & Martinez, 2014) and to inhibit RNA transcription (Marc et al., 2005). Glyphosate may also induce apoptosis by inducing caspase 3 in *in vitro* studies (Culbreth et al., 2012).

Since kidneys are the main site of xenobiotic biotransformation and elimination, and are considered a major homeostatic organ, while it may also be an organ for the excretion of glyphosate components (Sribanditmongkol et al. 2012), several *in vitro* and *in vivo* research studies have been performed and a relatively large number of chemicals and natural compounds tested for their potential protection against pesticides-induced nephrotoxicity. Çavuşoğlu et al. (2011) showed that treatment of Swiss albino mice with *Gingko biloba* L. leaf extract (150 mg/kg b.w) produced an improvement in indices of nephrotoxicity, lipid peroxidation, and genotoxicity induced by glyphosate (50 mg/kg body weight). These *in vivo* results showed that *G. biloba* may present a significant protective effect against toxicity induced by glyphosate (Çavuşoğlu et al., 2011).

Based on these observartions, changes in renal biochemical parameters in the kidney in our experimental study were examined in rats after applying sub-lethal doses of glyphosate (100 mg/kg b.w.) to identify suitable biomarkers able to detect the early stages of kidney injury and molecular mechanism involved in the nephroprotective effect of polyphenolic extract of *M. alba* leaves. The *M. alba* plant is reported to have an anti-inflammatory potential (Chen et al., 2013) and possess strong antioxidant properties (Sadighara & Barin. 2010). Several studies have also demonstrated that *M. alba* extract has significant beneficial kidney protection effect in drug-induced nephropathy. It has been reported to have a protective effect on cisplatin-induced nephrotoxicity in rats, and to prevent renal functioning alterations and nephrotoxicity caused by therapeutic treatement drugs, e.g gentamicin as a bactericide against Gram negative micro-organisms (Nematbakhsh et al., 2013), isoniazid (INH) as a tuberculosis drug (Faqir et al., 2014), supratherapeutic acetaminophen as a paracetamol analgesic and anti-pyretic (Salih et al., 2015).

To produce nephrotoxicity in experimental animals, different doses of glyphosate have been employed in different studies, and administration of large doses of glyphosate were reported to produce alteration in kidney functioning. A dose of 50 mg/kg causes renal tubular damage and glomerular filtration impairment and significant rise in serum creatinine (Çavuşoğlu et al., 2011). A dose of 126 mg/kg causes peritubular inflammatory reaction and nephrose characterized by cellular vacuolation and limited tubular necrosis. El-Shenawy. (2009) used a sub-lethal concentration of glyphosate alone (134.95 mg/kg) to induce nephrotoxicity, and thus to produce nephrotoxicity in experimental animals, while approximately 100 mg/kg has been employed in different other research experiments. Therefore, a daily dose of 100 mg of glyphosate/kg was selected in the current study to produce significant nephrotoxic effects in experimental animals (El-Shenawy, 2009).

Therefore, a reduction in kidney weight was observed in the group of rats treated with glyphosate and this loss of weight was ameliorated when rats received a daily dose of *M. alba* extract during the experimental period (Figure 1). This reduction in kidney weights can be attributed to oxidative and cellular damage caused by glyphosate. This renal atrophy may be the consequence of renal fibrosis and renal healing due to kidney inflammation, cellular phenotype transformation and renal parenchyma deficiency. In accoardance with these observations, morphological changes in kidney following pesticide exposure were also reported by Hamdaoui et al. (2016), demonstrating that albino rats intraperitoneally treated with a sub-lethal concentration of glyphosate alone (126 mg/kg) led to significant reduction in kidney weight. However, it was not the case with El-Shenawy (2009), who demonstrated an increase in kidney weight between control and Roundup-treated groups when rats were treated with a sub-lethal concentration of glyphosate (134.95 mg/kg) at 2-day intervals during two weeks.

Generally, intracellular enzymes were used as important biomarkers for detection of hepatotoxic and nephrototoxic effects of pesticides. Benedetti et al. (2004) showed that glyphosate causes liver damage in rats by intracellular enzymes leakage in liver. Herein, kidney excretion of lactate dehydrogenase was studied to investigate the nephroprotective properties of *M. alba* extract. Lactate dehydrogenase (LDH), an intracellular enzyme, is recognized as a potential marker for assessing the toxicity of chemical products (Agrahari et al., 2007). An increase in LDH can reflect damage to a number of different tissues (skeletal or cardiac muscles, kidney or liver). LDH levels may rise whenever there is cell necrosis or when neoplastic proliferation of cells causes an increased LDH production (Dzoyem et al., 2014). In this our study, LDH increased in animals treated with glyphosate. This can be explained to be the result of oxidative stress induced by LDH leaking from kidneys in the experimental model, which showed early detection of renal damage. However, it decreased to the normal range with MLE administration (Figure 2).

Therefore serum creatinine and blood urea have usually been used as an early and sensitive indicators, or biomarkers, for kidney injury in human pesticide intoxication. Serum Cr is the most commonly studied biomarker in glyphosate-induced clinical nephrotoxicity (Mohamed et al., 2016). In our study, a significant increase in serum urea and creatinine levels was observed after exposure to glyposate. Renal tubular damage and glomerular filtration impairment were observed in the kidneys of mice exposed to glyphosate and these forms of damage may account for the increase in serum urea and creatinine levels of the animals receiving glyphosate (Çavuşoğlu et al., 2011; Mohamed et al., 2016). Our results clearly showed that the polyphenolic fraction of *M. alba* inhibited the glyphosate-induced increase in kidney damage biomarkers (urea and creatinine) as an earlier stage of nephrotoxicity (Figure 2). Also, Mansour and Mossa (2010) reported that pesticides can alter plasma urea, uric acid and creatinine levels as a result of impairment of the glomerular function and tubular damage in kidneys (Mansour & Mossa, 2010). Since creatinine is eliminated throughout glomerular filtration and tubular secretion in the proximal tube, the creatinine level is considered as a good risk marker for chronic renal insufficiency. Urea is the ultimate end product of protein catabolism in the body. The elevation of blood urea is also an indicator for renal failure and kidney dysfunction (Baudin et al., 2013). These results were in accordance with the results of Zhang et al. (2017) and Mesnage et al. (2015) who reported significant changes in kidney hemato-biochemical indices, including statistically increased levels of creatinine and urea in rats treated with glyphosate (Mesnage et al., 2015).

Furthermore, controlling the antioxidant enzyme system, MLE can modulate the increased levels of CAT and POD activities. Our results are in accordance with the findings of Peluso et al. (1998), who detected the induced formation of DNA and enhanced hepatic CAT activity in rats treated with glyphosate. Among the antioxidant enzymes, SOD is the primary step of the defense mechanism against oxidative stress by catalyzing dismutation of superoxide radicals (O_2^-) into molecular oxygen (O_2) and H_2O_2 (McCord et al., 1971). H_2O_2 is neutralized by the combined action of CAT in all vertebrates. SOD enzymes play critical

roles in the regulation of cellular oxidative stress. The important SOD decline in glyphosate-induced toxicity was significantly restored by MLE (Figure 3). However, the interaction between glyphosate and the activities of SOD enzymes is not yet understood. An hypothesis is that glyphosate exposure leads to a SOD decline in kidneys, which results from the loss of copper and zinc, which directly binds to sulfhydryl groups on cysteine. Further studies should specify whether the cytoplasmic Cu/Zn SOD or the mitochondrial Mn-SOD were most affected. Another hypothesis is that modulation of antioxidant enzymes could correspond to post-translational modification as oxidative phosphorylation. Furthermore, glyoxylate, a breakdown product of glyphosate, is a potent glycating agent and would cause DNA damage by attacking Cu and Zn-SOD (Kaneta et al.,1994).

Generally, it is well established that elevated intracellular MDA and the protein carbonyl (PC) affect membrane integrity which is correlated to oxidative stress and pathological conditions (Rizvi & Maurya, 2007). Especially renal cells are highly suscepetible to oxidative damage because of the high polyonsaturated fatty acid content of their membrane. It has already been repotred that glyphosate induces lipid peroxidation (El-Shenawy, 2009) and its administration generates overproduction of free radicals, causing oxidative damage, and increases lipid peroxidation levels in kidney tissues even at lower doses (10 mg kg^{-1}). Our results show that MLE reduces glyphosate-induced intracellular lipid peroxidation by decreasing levels of MDA as a final product of peroxidation. (Figure 4). This protective effect can be due to scavenging MDA molecules by the active ingredient content of MLE or by inhibiting mitochondrial chain reactions. Furthermore, a significant increase in protein carbonyl levels suggests that the oxidative protein damage might be one of the explanations of glyphosate toxicity that is restored by the administration of daily dose of MLE.

In fact, the kidney is the major organ involved in the regulation of calcium and phosphate homeostasis (Wei et al., 2016). We found that glyphosate alters intracellular mediators in kidneys as well as in brain, as demonstrated by Rebai et al. (2017) under the same experimental conditions. MLE is able also to cancel increases in the levels of both calcium and iron triggered by the toxic effect of glyphosate (Figure 5).

Indeed, as glyphosate increased free iron levels, it could also increase hydroxyl radicals, toxic radicals that may in turn modify calcium homeostasis. Several studies have demonstrated that glyphosate promotes calcium uptake by L-type voltage-activated channels leading to calcium-overload cell death (De Liz Oliveira et al., 2013). Moreover, Rebai et al (2017) demonstrated that *in vivo* exposure to the same dose of glyphosate induced Ca_2^+ uptake in brain homogenates. Further experimentation should investigate the putative involvement of calcium channels in the mechanism of action of glyphosate in kidney tissue using calcium channel blockers.

Another possible target that can be involved in the protective effect of MLE against glyphosate-induced toxicity in the kidneys is its ability to reduce the levels of free iron. It is already known that free iron acts like a catalyst of auto-oxidation and there is an evidence that increase in free iron is correlated with oxidative stress status. Its role in disease processes seems to be a common theme of cellular injury. Thus, it is of interest that experimental evidence exists for the role of antioxidant molecules in contrast-induced injury kidneys, in particular AKI (Bakris et al., 1990).

Importantly, MLE counteracted glyphosate-induced renal damage and this nephroprotective activity could result from synergism between various *M. alba* extract-containing phenolic compounds. The phenolic constituents of the investigated MLE were analyzed by RP-HPLC, and monitored by UV or diode-array detector and mass spectrometry (ESI-MS) analysis (Figure 6 and Table 1). Considering the elution profile of chlorogenic acid isomers from plant foods reported in the literature on C18 HPLC columns (Fang et al., 2002), chlorogenic acid (5-CQA) and its isomers, as members of cinnamic acid, are the most abundant constituent in acetonic-aqueous mulberry leaves extract. This finding is in agreement with previous reports suggesting that 5-CQA is the major constituent of mulberry leaves (Thabti et al., 2012; Sánchez-Salcedo et al., 2015). The findings of many researches have confirmed that these compounds are responsible for useful effects in oxidative stress. Thus, antioxidant compounds from mulberry (*M. alba*) leaf extract are absorbed in the small intestine and then pass into blood circulation and retain their antioxidant activity in the animal (Lee et al., 2007). Chlorogenic acid (5-CQA) is a class of cinnamic acid, which possesses different isomeric forms, and it is the predominant phenolic compound in coffee and berries. Pharmacological effects of chlorogenic acid in kidneys have been demonstrated and it appears to exhibit renoprotective activity. A dose of CGA (100 mg/kg body weight) was given to mice for 8 days and it was reported to reverse lipid peroxidation, cause inactivation of cytochrome P450 and increase cellular defense (Kapil et al., 1995).

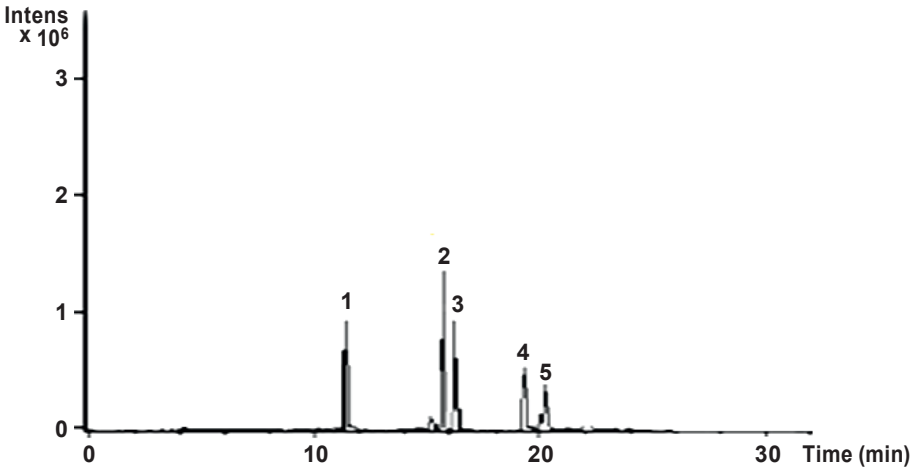

Figure 6. Typical liquid chromatography profile of *Morus alba* leaves extract detected by absorbance at 280 nm. Peaks were identified by LC-MS as shown in Table 1.

Table 1. RP-HPLC-DAD and LC-MS analyses of main phenolics in *Morus alba* leaf aqueous-acetone extacts

Pic N°	tR (min)	m/z $(M-H)^+$	Identity	Concentration (mg/100g fw)
1	11.5	353	Neochlorogenic acid (3-CQA)	5.12
2	15.5	353	Chlorogénique acid (5-CQA)	6.95
3	16.3	353	3,4 –Dicaffeoly quinic (isochlorogenic acid)	3.71
4	16.8	353	Crypto chlorogenic acid (4-CQA)	1.28

The renoprotective activity of caffeic acid (3, 4-dihydroxycinnamic acid), a major metabolite of chlorogenic acid, has been investigated through *in vitro* studies. Caffeic acid (CA) is already considered a potent antioxidant, and its function dependents on its chemical structure. Coffee acid can improve chronic renal failure and reduce protein urea, blood urine nitrogen and blood creatinine, as well as oxidation stress in the kidney. CA administration alleviated glomerular sclerosis scores and tubulointerstitial injuries and this effect may be due to its anti-oxidation and inhibiting accumulation of extracellular matrix (Jingqiu et al., 2016). Several others studies have also demonstrated that CA has a significant beneficial kidney protection effect in drug-induced nephropathy (Domitrović et al., 2014) and diabetic nephropathy (Jin et al., 2015). The anti-hypertension effect of CA is well-established (Zhao et al., 2012), a property that may be a good marker to control progress of chronic kidney dysfunction (Suzuki et al ., 2006). Also, it has been suggested that CA attenuates CP-induced kidney injury through suppression of oxidative stress, inflammation, apoptosis and autophagy, along with improvement in kidney regeneration which protects the kidneys from nephrotoxicity by reducing the burden of tubular cells (Domitrovic et al., 2014).

CONCLUSION

In this study we reported that glyphosate may induce oxidative stress leading to alterations in enzymatic redox, antioxidant endogenous system and scavengers, and other biochemical parameters which cause damage in the functional integrity of kidneys in rats. Our findings have shown that the use of mulberry leaves extract provides a protective effect to kidney tissue against nephrotoxicity caused by glyphosate, which indicates a significant improvement and reduction in tissue damage in the treated group. These findings suggested that mulberry's high phenolic levels, in particular chlorogenic acid and its isomers, have a potential to reduce or maintain renal toxicity. Finally, it suggests that mulberry leaves extract supplement may serve as a candidate for developing a safe and promising nutraceutical product for the management of renal toxicity induced by glyphosate residues in food of plant origin.

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REFERENCES

Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology, 105*, 121-126. doi.org/10.1016/S0076-6879(84)05016-3

Agrahari, S., Pandey, K., & Gopal, K. (2017). Biochemical alteration induced by monocrotophos in the blood plasma of fish, *Channa punctatus* (Bloch). *Pesticide Biochemistry and Physiology, 88*(3), 268-272. https://doi.org/10.1016/j.pestbp.2007.01.001

Astiz, M., De Alaniz, M.J.T., & Marra, C.A. (2009). Antioxidant defense system in rats simultaneously intoxicated with agrochemicals, *Environmental Toxicology and Pharmacology, 28*, 465-473. doi: 10.1016/j.etap.2009.07.009.

Bakris, G.L., Lass, N., Gaber, A.O., Jones, J.D., & Burnett, J.C. (1990). Radiocontrast medium-induced declines in renal function: A role for oxygen free radicals. *Americal Journal of Physiology, 258*, 115-120. doi: 10.1152/ajprenal.1990.258.1.F115.

Baudin, E., Caron, P., Lombard-Bohas, C., Tabarin, A., Mitry, E., Reznick, Y. ... Do Cao, C. (2013). Malignant insulinoma: recommendations for characterisation and treatment,. *Annales d'Endocrinolgie* 74, 523-533. doi: 10.1016/j.ando.2013.07.001

Benedetti, A.L., De Lourdes Vituri, C., Trentin, A.G., Domingues, M.A.C., & Alvarez-Silva, M. (2004). The effects of sub-chronic exposure of Wistar rats to the herbicide glyphosate-biocarb. *Toxicology Letters*, *153*, 227-232. doi: 10.1016/j.toxlet.2004.04.008.

Bradberry, S.M., Proudfoot, A.T., & Vale, J.A. (2004). Glyphosate poisoning. *Toxicological Reviews, 23*, 159-167.

Çavuşoğlu, K., Yapar, K., Oruç, E., & Yalçın, .E. (2011). Protective effect of *Ginkgo biloba* L. leaf extract against glyphosate toxicity in Swiss albino mice. *Journal of Medicinal Food, 14*, 1263-1272. doi: 10.1089/jmf.2010.0202

Chen, Y.C., Tien, Y.J., Chen, C.H., Beltan F.N., Amor, E.C., Wang, R.J. ... Yang, R.C. (2013). *Morus alba* and active compound oxyresveratrol exert anti-inflammatory activity via inhibition of leukocyte migration involving MEK/ERK signalling. *BMC Complementary and Alternative Medicine*, *13*, Art. No. 45. doi: 10.1186/1472-6882-13-45

Chu, Q., Lin, M., Tian, X., &Ye, J. (2006). Study on capillary electrophoresis-amperometric detection profiles of different parts of *M. alba* L. *Journal of Chromatography, A, 1116*, 286–290.

Culbreth, M.E., Harrill, J.A., Freudenrich, T.M., Mundy, W.R., & Shafer, T.J. (2012). Comparison of chemical-induced changes in proliferation and apoptosis in human and mouse neuroprogenitor cells. *Neurotoxicology, 33,* 1499-1510.

De Liz Oliveira Cavalli, L.V., Cattani, D., Heinz Rieg, C.E., Pierozan, P., Zanatta, L., Benedetti Parisotto, E... Zamoner, A. (2013). Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free Radical Biological Medecine*, *65*, 335–346. doi: doi.org/10.1016/j.freeradbiomed.2013.06.043

Domitrović, R., Cvijanović, O., Šušnić, V., & Katalinić, N. (2014). Renoprotective mechanisms of chlorogenic acid in cisplatin-induced kidney injury. *Toxicology*, *324,* 98-107.

Draper, H.H., & Hadley, M. (1990). Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology, 186*, 421-431.

Duke, J. A. & Ayensu, E. S. (1985). Medicinal plants of China (2 Vols). *Journal of Botanical Taxonomy and Geobotany, Feddes Repertorium*, *98*, 398. doi.org/10.1002/fedr.19870980707

Dzoyem, J.P., & Eloff, J.N. (2014). Biochemical parameters in toxicological studies in Africa: Significance, principle of methods, data interpretation, and use in plant screenings. In Victor Kuete (ed.), T*oxicological Survey of African Medicinal Plants* (pp 659-715). London, UK: Elsevier. doi.org/10.1016/B978-0-12-800018-2.00023-6

El-Shenawy, N.S. (2009). Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. *Environmental Toxicology and Pharmacology, 28*, 379-385. doi: 10.1016/j.etap.2009.06.001.

Emami, A., Shams Ardekani, M.R., Mehregan, I. (2004). *Color atlas of medicinal plants.* Tehran, Iran: ITMRC Publications, 240.

Fang, N., Yu, S., & Prior, R.L. (2002). LC/MS/MS characterization of phenolic constituents in dried plums. *Journal of Agricultural and Food Chemistry, 50*, 3579-85.

Faqir, M., Zafar, M.V.S., Tanweer, K., Javed, I., & Saleemi, M.K. (2014). Nephroprotective effects of *Morus Alba* Linn against isoniazid-induced toxicity in albino rabbits. *Pakistan Veterinary Journal, 34*, 499-503.

Fattouch, S., Caboni, P., Tuberoso, C., Angioni, A., Dessi, S., Marzouki, M.N., Cabras, P. (2008). Comparative

analysis of polyphenolic profiles and antioxidant and antimicrobial activities of Tunisian pome fruit pulp and peel aqueous acetone extracts. *Journal of Agricultural and Food Chemistry, 56*(3), 1084-1090. doi.org/10.1021/jf072409e

Hamdaoui, L., Naifar, M., Mzid, M., Ben Salem, M., Chtourou, A., Makni-Ayadi, F. ... Rebai, T. (2016). Nephrotoxicity of Kalach 360 SL: biochemical and histopathological findings. *Toxicology Mechanisms and Methods, 26*(9), 685-691.

Jin, S., Chang, C., Zhang, L., Liu, Y., Huang, X., & Chen, Z. (2015). Chlorogenic acid improves late diabetes through adiponectin receptor signaling pathways in *db/db* mice. *PLoS ONE, 10*(4), 120-842.

Jingqiu, L., Xiaoxia, G., Yinghua, X., Jing, C., Wei, L., & Yan, Z. (2016). Chlorogenic acid slows down proteinuria and renal fibrosis in 5/6-nephrectomized rats by anti-oxidation and inhibiting accumulation of extracellular matrix. *International Journal of Clinical and Experimental Medicine, 9*(8), 15719-15727.

Kaneto, H., Fujii, J., Suzuki, K.,. Kasai, H., Kawamori, R., Kamada, T., & Taniguchi, N. (1994). DNA cleavage induced by glycation of Cu, Zn-superoxide dismutase. *Biochemical Journal*, *304*, 219-225.

Kapil, A., Koul, I.B., & Suri, O.P. (1995). Antihepatotoxic effects of chlorogenic acid from *Anthocephalus cadamba*. *Phytotherapy Research*, *9*, 189. doi: doi.org/10.1002/ptr.2650090307

Kawagashira, Y., Koike, H., Kawabata, K., Takahashi, M., Ohyama, K., Hashimoto, R. ... Sobue, G. (2017). Vasculitic neuropathy following exposure to a glyphosate-based herbicide. *Internal Medicine, 56*, 1431-1434.

Kobayashi, Y., Miyazawa, M., & Kojima, T. (2011). The use of *Morus alba* L.(mulberry) and *Eucommia ulmoides* (Tochu) leaves as functional foods: a promising approach in the management of hyperlipidemia. *Journal of Traditional Medicine, 27*, 225-230.

Larsen, K. (1972). Creatinine assay by a reaction-kinetic principle. *Clinica Chimica Acta, 41*, 209-17. doi: 10.1016/0009-8981(72)90513-x.

Leardi, A., Caraglia, M., Selleri, C., Pepe, S., Pizzi, C., Notaro, R. ... Tagliaferri, P. (1998). Desferioxamine increases iron depletion and apoptosis induced by ara-C of human myeloid leukaemic cells. *British Journal of Haematology, 102*, 746-752.

Lee, C.Y., Sim, S.M., & Cheng, H.M. (2007). Systemic absorption of antioxidants from mulberry (*Morus alba* L) leaf extracts using an in situ rat intestinal preparation. *Nutrition Research, 27*, 492-497. doi: 10.1016/J.NUTRES.2007.06.004

Mansour, S.A., & Mossa, A.H. (2010). Oxidative damage, biochemical and histopathological alterations in rats exposed to chlorpyrifos and the antioxidant role of zinc. *Pesticide Biochemistry and Physiology, 96*, 14-23.

Marc, J., Breton, M.L., Cormier, P., Morales, J., Belle, R., & Mulner Lorillon, O.A. (2005). A glyphosate-based pesticide impinges on transcription. *Toxicology and Applied Pharmacology, 203,* 1-8. doi: 10.1016/j.taap.2004.07.014

McCord, J.M., Keele, B.B., & Fridovic, I. (1971). An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. *Proceedings of the National Academy of Sciences of the USA, 68*, 1024-1027.

Mensah, P.K., Palmer, C.G., Muller, W.J. (2012). Lipid peroxidation in the freshwater shrimp *Caridina nilotica* as a biomarker of Roundup herbicide pollution of freshwater systems in South Africa. *Water Science and Technology, 65*, 1660-1666.

Mesnage, R., Arno, M., Costanzo, M., Malatesta, M., Séralini, G.E., Antoniou, M.N. (2015). Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. *Environmental Health*, *14,* 70.

Misra, H.P., & Fridovich, I. (1972). The role of superoxide anion in autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, *247*, 3170- 3175.

Mohamed,F., Endre, Z.H., Pickering, J.W., Jayamanne, S., Palangasinghe, C., Shahmy, S. ... Buckley, N.A. (2016). Mechanism-specific injury biomarkers predict nephrotoxicity early following glyphosate surfactant herbicide (GPSH) poisoning. *Toxicology Letters*, *7*, 258, 1-10.

Naowaboot, J., Pannangpetch, P., Kukongviriyapan, V., Kongyingyoes, B., & Kukongviriyapan, U. (2004). Antihyperglycemic, antioxidant and antiglycation activities of mulberry leaf extract in streptozotocin-induced chronic diabetic rats. *Plant Foods for Human Rationion, 64*, 116-121.

Naqvi, R. (2017). Acute kidney injury from different poisonous substances. *World Journal of Nephrology, 6(*3), 162–167.

Navarro, C.D., & Martinez, C.B. (2014). Effects of the surfactant polyoxyethylene amine (POEA) on genotoxic, biochemical and physiological parameters of the freshwater teleost *Prochilodus lineatus*. *Comparative Biochemistry and Physiology, C - Toxicology and Pharmacology, 165*, 83-90.

Negga, R., Stuart, J.A., Machen, M.L., Salva, J., Lizek, A.J., Richardson, S.J. ... Fitsanakis V.A. (2012). Exposure to glyphosate- and/or Mn/Zn-ethylenebisdithiocarbamate-containing pesticides leads to degeneration of

γ-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. *Neurotoxicity Research, 21*, 281–290

Nematbakhsh, M., Hajhashemi, V., Ghannadi, A., Talebi, A., & Nikahd, M. (2013). Protective effects of the *Morus alba* L. leaf extracts on cisplatininduced nephrotoxicity in rats. *Research in Pharmaceutical Sciences, 8*(2), 71-77.

Nickavar, B., & Mosazadeh, G. (2009). Influence of three *Morus* species extracts on α-amylase activity. *Iranian Journal of Pharmaceutical Research,* 8(2),115-119.

Ohnishi, S.T., & Barr, J.K. (1978). A simple method of quantitating protein using the biuret and phenol reagent. *Analitical Biochemistry, 86*, 193-200.

Peluso, M., Munnia, A., Bolognesi, C., & Parodi, S. (1998). 32P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. *Environmental and Molecular Mutagenesis 31*, 55-59.

Rebai, O., Belkhir, M., Boujelben, A., Fattouch, S., & Amri, M. (2017). *Morus alba* leaf extract mediates neuroprotection against glyphosate-induced toxicity and biochemical alterations in the brain. *Environmental Science and Pollution Research International, 24*(10), 9605-9613.

Rizvi, S.I., & Maurya, P.K. (2007). Markers of oxidative stress in erythrocytes during aging in humans. *Annals of the New York Academy of Sciences, 1100*, 373-382.

Sadighara, P., & Barin, A. (2010). The study of antioxidant potential of *Morus alba* L. leaves extract. *Journal of Herbal Drugs, 1*(3),43-46.

Salih, N.D., Hazir, M.N.S., & Abo Hamid, M.H. (2015). The effect of mulberry (*Morus* sp.) tea supplementation on acetaminophen induced renal failure in rats. *World Journal of Pharmacy and Pharmaceutical Sciences, 4*(4), 111-125.

Sánchez-Salcedo, E.M., Mena, P., Garcia-Viguera, C.,& Hernandez, F. (2015). Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties. *Journal of Functional Foods, 12*, 399-408.

Seok, S.J., Park, J.S., Hong, J.R., Gil, H.W., Yang, J.O., Lee, E.Y. ... Hong, S.Y. (2011). Surfactant volume is an essential element in human toxicity in acute glyphosate herbicide intoxication. *Clinical Toxicology (Phila), 49*(10), 892-899.

Shehata, A.A., Schrödl, W., Schledorn, P., & Krüger, M. (2014). Distribution of glyphosate in chicken organs and its reduction by humic acid supplementation. *Journal of Poultry Science, 51*(3), 333.

Sribanditmongkol, P., Jutavijittum, P., Pongraveevongsa, P., Wunnapuk, K., & Durongkadech, P. (2012). Pathological and toxicological findings in glyphosate-surfactant herbicide fatality: A case report. *The American Journal of Forensic Medicine and Pathology, 33*(3), 234-237.

Stern, J., & Lewis, W.H. (1957). The colorimetric estimation of calcium in serum with ocresolphthalein complexone. *Clinica Chimica Acta,* 2, 576-580.

Suzuki, A., Yamamoto, N., Jokura, H., Yamamoto, M., Fujii, A., Tokimitsu, I. & Saito, I. (2006). Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. *Journal of Hypertension, 24*, 1065-1073.

Thabti, I., Elfalleh, W., Hannachi, H., Ferchichi, A., & Campos, M.D.G. (2012). Identification and quantification of phenolic acids and flavonol glycosides in Tunisian *Morus* species by HPLC-DAD and HPLC-MS. *Journal of Functional Foods, 4*, 367-374.

Ullah, N., Khan, M.A., Khan, S., Ahman, H., Asif, A.H., & Khan, T. (2016), Nephro-protective potential of *Morus alba*, a prospective experimental study on animal models, *Pharmaceutical Biology, 54*, 530-535.

Valcke, M., Levasseur**,** M.E., Da Silva**,** A.S., & Wesseling**,** C. (2017). Pesticide exposures and chronic kidney disease of unknown etiology: an epidemiologic review. *Environmental Health***,** *16*, 49.

Wang, J., Wu, F.A, Zhao, H. Liu, L., & Wu, Q.S. (2008). Isolation of flavonoids from mulberry (*Morus alba* L.) leaves with macroporous resins. *African Journal of Biotechnology, 7*(13), 2147-2155.

Wei, K., Yin, Z., & Xie, Y. (2016). Roles of the kidney in the formation, remodeling and repair of bone. *Journal of Nephrology, 29*, 349-357.

Wunnapuk, K., Gobe, G., Endre, Z., Peake, P., Grice, J., Roberts, M.S. ... Liu, X. (2014). Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers. *Toxicology Letters, 225*, 192–200.

Yang Y, Gong T, Liu C, Chen RY (2010). Four new 2-arylbenzofuran derivatives from leaves of *Morus alba* L. *Chemical and Pharmacelutical Bulletin, 58*(2), 257-60.

Yeung, H.C. (1985). Handbook of Chinese herbs and formulas, Vol 1. Taos, NM, USA: Redwing Book

Zhang, F., Pan, L.P., Ding, E.M., Ge, Q.J., Zhang, Z.H., Xu, J.N., Zhang, L., & Zhu, B.L. (2017). Study of the effect of occupational exposure to glyphosate on hepatorenal function. *Zhonghua Yu Fang Yi Xue Za Zhi*, *51*(7), 615-620.

Zhao, Y., Wang, J., Ballevre, O., Luo, H. & Zhang, W. (2012). Antihypertensive effects and mechanisms of chlorogenic acids. *Hypertension Research, 35*, 370-374.

Nefroprotektivno delovanje vodenog acetonskog ekstrakta *Morus alba* i mehanizam toksičnosti indukovane glifosatom - *in vivo* model

REZIME

Glifosat, aktivna materija preparata Roundup®, je pesticid koji je u najširoj upotrebi u svetu i njegovi ostaci se mogu naći u prerađenoj hrani i pijaćoj vodi. Ranija istraživanja su potvrdila da ekstrakti lista *Morus alba* poseduju farmakološko dejstvo. Međutim, renoprotektivno delovanje ekstrakta *M. alba* i njihovog molekularnog mehanizma još uvek je nepoznato.

U ogledu su korišćeni laboratorijski beli pacovi (180-200 g) (n=5-6). Kontrolna grupa je intraperitonealno (i.p) dobijala 0.2 ml normalnog fiziološkog rastvora jednom u toku dana tokom dve nedelje. Kontrolnim životinjama je davana standardna hrana. Tretirane grupe su dobijale ili polifenolni ekstrakt (100 mg/kg, i.p) ili glifosat (100 mg/kg, i.p), ili pak njihovu smešu (ekstrakt μg ml^{-1} kg t.m. i glifosat 100 mg kg^{-1} t.m, i.p), jednom dnevno do petnaestog dana tretmana. Merene su vrednosti laktat dehidrogenaze LDH, koncentracije uree, kreatitina i azot-monoksida u serumu, a korišćene su standardne kolorometrijske metode.

Renalni oksidativni stress, konstatovan preko povišenih vrednosti malondialdehida (MDA) i nivoa proteinskih karbonila, kao i snižene aktivnosti superoksida dismutase (SOD), bio je značajno povišen delovanjem primenjenog ekstrakta lista duda (MLE). MLE je pokazao da može da modulira izmenjene biohemijske parameter održavanjem nivoa slobodnog gvožđa i homeostaze Ca_2^+, kao i da reguliše endogeni sistem antioksidativnih enzima. Izgleda da istovremena primena vodenog acetonskog rastvora frakcije *M. alba* koji je bogat hlorogenom kiselinom i njenim izomerima može imati zaštitno delovanje na bubrege nakon nefrotoksične aktivnosti glifosata. Ukupno gledano, MLE može delovati zaštitno protiv toksičnosti indukovane glifosatom, što se može pripisati hlorogenoj kiselini i njenim izomerima, koji predstavljaju najprisutnije fenolne kiseline u ekstraktima.

List belog duda je izvor fenolnih jedinjenja i stoga može predstavljati osnovu za potragu za novim hemijskim jedinjenjem koje će omogućiti dobijanje novog lekovitog sredstva. Suplement od ekstrakta belog duda može biti kandidat za razvoj bezbednog i perspektivnog preparata kao dodatka ishrani namenjenog zaštiti od nefrotoksičnosti.

Keywords: glifosat, oksidativni stres, nefrotoksičnost, beli dud, nefroprotektivno delovanje

Homage to Dr Neško K. Nešković (1943-2021)

Our dear colleague and friend Dr Neško Nešković passed away on February 19 after a long illness. He was a principle research fellow and one of the early founders of toxicological and ecotoxicological research of pesticides in the former Yugoslavia, an expert who focused his professional efforts on elucidating the problem of "bound" pesticide residues and toxic effects on users. He was also an editor-in-chief of the scientific journal "Pesticides and Phytomedicine" for many years.

Dr Nešković was born in the village Donja Ljubovića near Ljubovija on 24 October 1943. He completed elementary education in his home village and in Ljubovija, and secondary education in the Šabac Agricultural School. He graduated from the University of Belgrade, Faculty of Agriculture (Plant Protection Department) in Zemun-Belgrade in 1966, and later earned a MSc title at the Faculty of Natural Sciences in Belgrade, and received a PhD degree from the Faculty of Agriculture, Plant Protection Department (Pesticide Toxicology) in Zemun-Belgrade in 1976.

After completing education, he was employed at the Institute for Implementation of Nuclear Energy in Agriculture, Veterinary Medicine and Forestry (INEP) in Zemun-Belgrade. His exceptional expertise resulted in his quick promotion to the position of head of the Laboratory for Toxicology and Ecotoxicology, and then to the position of director of the Institute for Pesticides and Environmental Protection, which was founded as a constituent department of INEP. Over the period 1991-2003, he was employed at the Institute of Plant Protection and the Environment in Belgrade, where he set up a Laboratory for Toxicology and headed it. He demonstrated his leadership skills again as president of the Managing Board and Science Committee of the Institute, then as deputy director, and finally as director of the Institute. In 2003, he returned to the Institute of Pesticides and Environmental Protection and headed its Laboratory for Toxicology until retirement in 2008. Beside his regular duties, Dr Nešković was also an active member of various commissions, boards

and other professional bodies in relevant ministries of the Republic of Serbia and the Federal Republic of Yugoslavia responsible for research and other activities relating to agriculture and environmental protection.

Dr Nešković participated in many national and international research projects, and headed some of them. He led the international project „*The Study of Bioavailability and Possible Toxicological Effects of Bound Pesticide Residues to Non-Target Organisms*", a grant of the Vienna-based International Atomic Energy Agency, and the national project „*Pesticides and the Environment*", funded by the Ministry of Science and Technology of the Republic of Serbia. He cooperated with the National Institute of Environmental Health Sciences (NIEHS), North Carolina, USA, as a visiting researcher. During his second study visit in the USA, he visited the North Carolina State University (Department of Toxicology and Pesticide Residue Research Laboratory) in Raleigh, as well as several other research institutes and laboratories. In the course of his long research career,

Dr Nešković participated in a great number of national and international research conferences and published research paper in the most prominent national and international journals. His research contribution included over 150 articles and lectures.

The activities of Dr Nešković went beyond research work as he was engaged in tutelage within undergraduate and postgraduate studies at the Faculty of Agriculture, University of Belgrade, in General and Specializing Plant Protection Study Programmes, as well as Agricultural Toxicology and Ecotoxicology. Besides, Dr Nešković was either a consultant or mentor to several undergraduate and MSc theses, as well as PhD dissertations at the Belgrade University Faculty of Agriculture and Faculty of Natural Sciences, and served as member of a number of commissions for evaluation of MSc theses and PhD dissertations. He was a lecturer in two rounds of the international study programme in pesticide toxicology (*International Training Course on Toxicology - Toxicology of Pesticides*) for experts in developing countries, organized by UN specializing agencies (WHO, FAO, ILO) and several domestic institutions.

Dr Nešković was one of the founders (in 1986) and early members of the Editorial Board of the scientific journal "*Pesticides*", which later changed the title to "*Pesticides and Phytomedicine*", and was its editor-in-chief from 1987 until 2007. Owing to his efforts, the journal managed to overcome a difficult period of economic and political sanctions and international isolation of our country, and important technical and organizational changes were made at that time. An international editorial board was set up, editorial procedures were improved, a new publisher was found, the journal concept was altered to enable that articles in a broader range of subjects relating to plant protection be covered, and the quality of print and other technical aspects were improved. The result of all those changes was an improved rating of the journal. After retirement, Dr Nešković continued to support the journal as editor emeritus. Dr Nešković was also a member of editorial boards of several other international research journals ("*Archives of Toxicology, Kinetics and Xenobiotic Metabolism", "Journal of Environmental Science and Health - Part B: Pesticides, Food Contaminants and Agricultural Wastes*") and member of the Editorial Board of the journal „*Plant Doctor*". He was also active as a reviewer of numerous articles in prominent international research journals focusing on toxicology.

Dr Nešković was a member of various national and international research associations: Serbian Medical Society (Toxicology Section), Yugoslav Association of Toxicologists, Serbian Association of Toxicologists, Serbian Plant Protection Society, Serbian Biological Society, Serbian Ecological Society, Society for Environmental Toxicology and Chemistry, Association of European Toxicologists and Toxicological Societies and International Society of Ecotoxicology and Environmental Safety. As an active member of the Serbian Plant Protection Society, he presided the organization committees of two research conferences and was one of the editors of the monograph "Plant Protection – Today and Tommorow". Dr Nešković was also a member of the editorial board of the publishing programme „Man and the Environment" of the Serbian Academy of Sciences and Arts (SANU).

The results of Dr Nešković's work and his significant contribution to the profession focusing primarily on plant protection and plant protection products have received public recognition and he was awarded several prizes: Badge of Honour with Silver Wreath for Work Results, INEP Plaque, Gold Plaque of the Institute of Pesticides and Environmental Protection, Certificate of Appreciation of the Institute of Plant Protection of the Belgrade Faculty of Agriculture, Memorial Plaque of the Serbian Plant Protection Society, Charter of the Assembly of Agricultural Engineers and Technical Staff of Loznica, Krupanj, Mali Zvornik and Ljubovija.

We have parted from a man of broad interests, a true patriot, a brisk and humorous personality who was always faithful to his principles, and rigorous to himself and others. His biography remains to tell of the times he lived in and profession he devoted his professional life to with so much energy and passion from the first to the last day of his rich career. All his colleagues will remember his exceptional contribution to the science and profession of plant protection, and his close associates and friends will keep in warm memory his good-hearted support as a fellow professional, and his human kindness and generosity.

Milka Budimir
Dragica Brkić

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.

Manuscript submission

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.

Manuscript preparation

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).

Title should be concise and refer to the subject. Full names and surnames of all authors, details of their respective affiliations and emails should be indicated below the title. If discrepancy in such data occurs between the textual document and submission metadata in Assistant, the former will be given precedence.

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.

Discussion should emphasize the importance of the results, as well as their place within the context of previous research. Wherever possible, Results and Discussion should be separate sections.

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

References cited in the text need to include the author's/ authors' surname(s) and year of publication:

- author, year;
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References mentioned in the manuscript must be listed in the References section at its end, in alphabetic order and using the **APA citation style** (see description at e.g. https://owl.english.purdue.edu/owl/resource/560/01/).

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.

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

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

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

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

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

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.

Diagrams should be processed in Corel Draw (version 9 or later) or in Adobe Illustrator (version 9 or later) and all data written in Times New Roman font. Diagrams should be submitted as supplementary files and their approximate locations in the text marked.

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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The editorial staff practice a policy of plagiarism prevention.

Uputstvo autorima

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

Časopis *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* predstavlja nastavak publikacije *Pesticidi*, koja je pod tim imenom izlazila u periodu 1986-2003.

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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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Publikovanje u časopisu *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* podrazumeva da rad sadrži rezultate originalnih istraživanja koji nisu objavljeni, odnosno nisu dostavljeni nekom drugom časopisu za objavljivanje. Pregledni radovi treba da sadrže sveobuhvatan prikaz određene teme zasnovan na referentnoj literaturi i publikovanim rezultatima sopstvenih istraživanja. Svi radovi se recenziraju, a recenzija je obostrano anonimna.

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Autori u radu NE NAVODE ključne reči. Njih će glavni urednik ekstrahovati iz *KWASS* tezaurusa (rečnika), što će značajno poboljšati vidljivost rada. Autori imaju pravo da dodeljene ključne reči prihvate ili da neke od njih zamene.

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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.

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

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

Zahvalnica se navodi na kraju teksta rada, pre literature.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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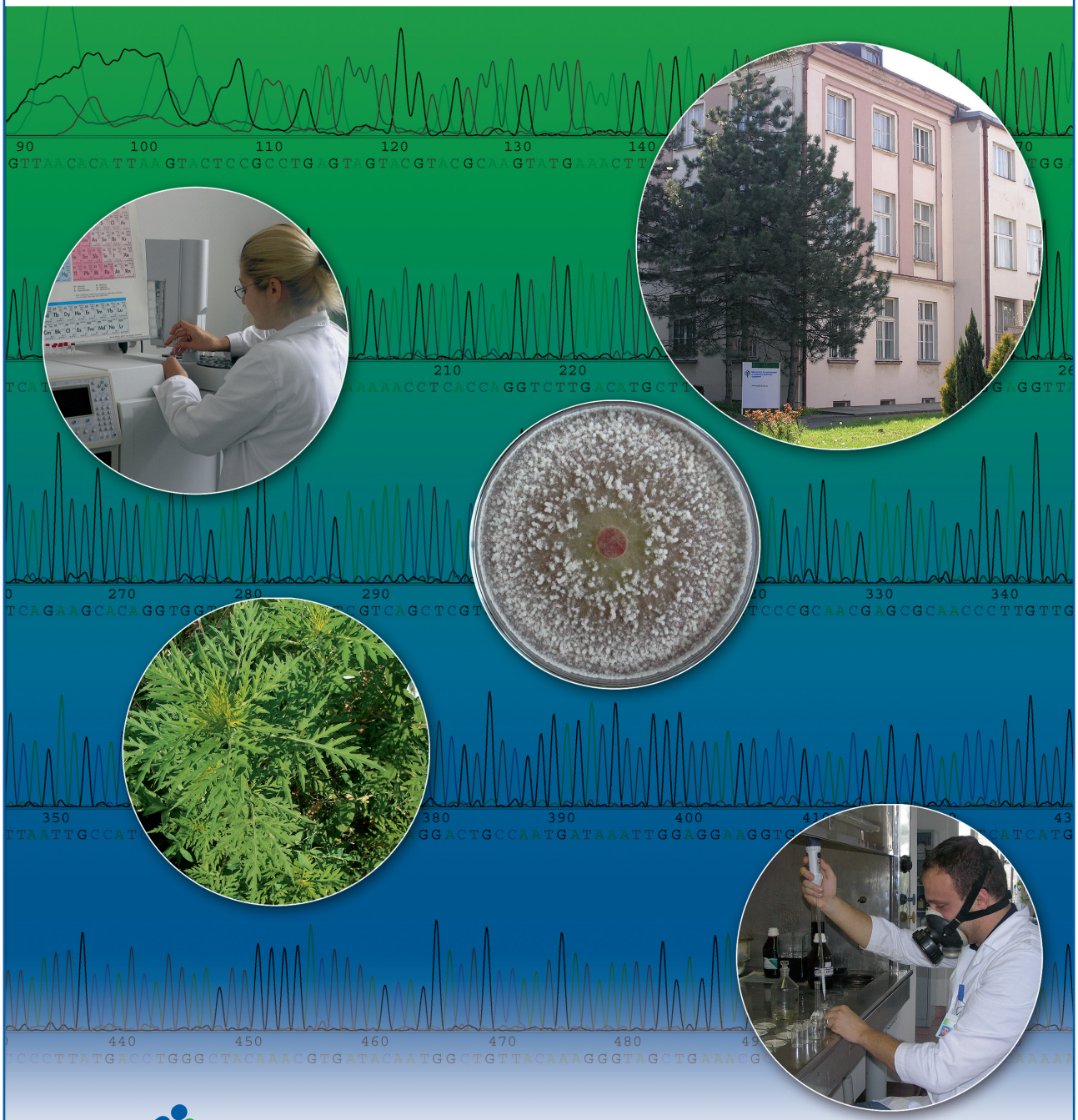

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