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A novel *Wolbachia ftsZ* genotype in '*Candidatus* Phytoplasma solani' planthopper vector *Hyalesthes obsoletus* (Hemiptera: Fulgoromorpha: Cixiidae) associated with *Convolvulus arvensis*

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SUMMARY

Hyalesthes obsoletus (Hemiptera: Fulgoromorpha: Cixiidae) is a pan-European polyphagous planthopper known as a significant vector of the plant pathogenic bacterium '*Candidatus* Phytoplasma solani' (stolbur phytoplasma), which poses threats to various agricultural crops. A population of *H. obsoletus* associated with *Convolvulus arvensis* in Serbia was studied to investigate the presence and genetic diversity of *Wolbachia*, an endosymbiotic bacterium known for its promising biological control applications. Both insect-associated microorganisms, '*Ca*. P. solani' and *Wolbachia*, were found in the assessed *H. obsoletus* population. The analyzed vector population had a '*Ca*. P. solani' infection rate of 50%, while *Wolbachia* showed a high infection rate of 80%. *Wolbachia* presence displayed minimal variation across genders and was independent of individuals' phytoplasma-infection status. Genotyping of the identified '*Ca*. P. solani' strains revealed four previously described *stamp* genotypes (Rqg50/St1, Rqg31/St2, STOL/St4 and M5/St28). Notably, a single novel *Wolbachia ftsZ* genotype, designated WHo1, was found in the assessed *H. obsoletus* population, providing a valuable insight into the genetic diversity of *Wolbachia* endosymbionts within the Cixiidae family. Phylogenetic analysis demonstrated intricate relationships between WHo1 and other *Wolbachia* strains infecting hosts from diverse hemipteran suborders. Although *Wolbachia*-based strategies show promise for phytoplasma vector control, further research is needed to elucidate its potential interactions with '*Ca*. P. solani' and its effects on vector reproduction and fitness.

Keywords: *Hyalesthes obsoletus*, '*Candidatus* Phytoplasma solani', stolbur phytoplasma, *Wolbachia*, endosymbionts

INTRODUCTION

Hyalesthes obsoletus Signoret (Hemiptera: Fulgoromorpha: Cixiidae) is a pan-European polyphagous cixiid planthopper whose range extends eastward to the Middle East across the Mediterranean basin (Hoch & Remane, 1985). This insect is a prominent vector of the plant pathogenic bacterium '*Candidatus* Phytoplasma solani' (class Mollicutes), the stolbur phytoplasma, associated with plant diseases collectively referred to as "stolbur diseases" (Quaglino et al., 2013; Jović et al., 2019). *Hyalesthes obsoletus* occupies a broad ecological niche, primarily inhabiting xerothermic and ruderal environments, but its capacity for habitat exploitation allows it to extensively utilize agricultural landscapes whenever suitable host plants are available (Hoch & Remane, 1985; Holzinger et al., 2003; Nickel et al., 2003; Kosovac et al., 2018). Despite its polyphagous feeding strategy, *H. obsoletus* displays obligate host plant specialization for oviposition and nymphal development, being restricted in this context to a narrow range of hosts, specifically four plant species: *Urtica dioica* L., *Convolvulus arvensis* L., *Vitex agnus-castus* L., and *Crepis foetida* L. (Sforza et al., 1999; Sharon et al., 2005; Kaul et al., 2009; Kessler et al., 2011; Kosovac et al., 2018). Harboring '*Ca*. P. solani' as natural reservoirs, these plants serve as the starting points for diverse epidemiological cycles vectored by associated *H. obsoletus* populations that threaten crop yields across various agroecosystems (Langer & Maixner, 2004; Kosovac et al., 2016; 2019; 2023a; 2023b). Populations of *H. obsoletus* associated with *C. arvensis* have been linked to several '*Ca*. P. solani'-associated diseases of the tuf-b epidemiology in Serbia, including stolbur disease of pepper and eggplant, *Bois noir* disease of grapevine, potato stolbur disease and Rubbery Taproot Disease (RTD) of sugar beet (Aleksić et al., 1967; Mitrović et al., 2016; Kosovac et al., 2019; 2023a). Recent findings suggest that *H. obsoletus ex C. arvensis* populations may contribute to maize redness and tobacco stolbur, further expanding the range of the cultivated plants threatened by this vector and associated phytoplasma (Kosovac et al., 2023b). Molecular methods have significantly improved the research of stolbur disease epidemiology, enabling the tracing of '*Ca*. P. solani' strains through reservoir plants, vectors, and crops (Langer & Maixner, 2004; Aryan et al., 2014; Kosovac et al., 2023a; 2023b), and paving the way for more effective disease management strategies. Since traditional control measures, such as weed control and insecticide applications, have shown limited effectiveness (Mori et al., 2016; Riedle-Bauer & Brader, 2023), the challenge of stolbur disease control necessitates exploration of novel methods, including biological control that involves nematodes, fungi, and endosymbiotic bacteria (Gonella et al., 2011; Chuche et al., 2016; Iasur-Kruh et al., 2017; Moussa et al., 2021).

Wolbachia Hertig (α-Proteobacteria) are intracellular gram-negative bacteria with a host range that includes arthropods and nematodes (Werren, 1997). A specific classification scheme has been proposed for *Wolbachia,* classifying them within monophyletic supergroups (A-S) (Lefoulon et al., 2020). These well-known endosymbionts not only manipulate host reproduction (Hilgenboecker et al., 2008; Werren et al., 2008), but also significantly impact various host characteristics, such as fitness, metabolism, immunity, and the native microbiome (Stouthamer et al., 1999; Kambris et al., 2009; Hosokawa et al., 2010; Duan et al., 2020; Ju et al., 2020). *Wolbachia*'s unique manipulation of host biology has made it a powerful tool for control of mosquito-borne diseases, relying on mechanisms such as cytoplasmic incompatibility (CI) and pathogen blocking (PB) (Ross et al., 2019; Shropshire et al., 2020). The reported ability of *Wolbachia* to hinder viral replication in mosquitos (Sullivan, 2020) offers an impactful strategy potentially applicable for controlling insect-vectored plant pathogens.

Evidence of the *Wolbachia* CI effect has been documented in the planthopper virus vectors *Laodelphax striatellus* (Fallén) and *Sogatella furcifera* (Horváth). Despite being infected with the same *Wolbachia* strain, these vectors exhibited varying levels of CI, which appeared to correlate with different quantities of *Wolbachia* present in males (Noda et al., 2001). Research on the planthopper vector *Nilaparvata lugens* (Stål) revealed that the introduction of a specific *Wolbachia* strain isolated from *L. striatellus* can induce CI within its population (Gong et al., 2020). Moreover, this intervention has been shown to suppress the replication and transmission of rice ragged stunt virus (RRSV), highlighting the promising potential of *Wolbachia*-based strategies for managing plant diseases transmitted by hemipteran vectors (Gong et al., 2020).

Wolbachia infection has been documented in European cixiids, with the reported data for *Pentastiridius leporinus* (Linné), a vector of '*Ca*. P. solani' and '*Candidatus* Arsenophonus phytopathogenicus' to sugar beet

and potato (Bressan et al., 2008; 2009; Rinklef et al., 2024). Regarding the planthopper *H. obsoletus*, the presence of *Wolbachia* in its populations has been reported in Italy, and the detected *Wolbachia* strains were classified to supergroup B based on phylogenetic analysis of the 16S rRNA gene sequence (Gonella et al., 2011). Furthermore, *Wolbachia* was found in *H. obsoletus* populations associated with various plants across different regions, including *U. dioica*, *Salvia sclarea* L., and *Lavandula angustifolia* Mill. in France, *C. arvensis* in Germany, and *V. agnus-castus* in Israel (Chuche et al., 2016; Iasur-Kruh et al., 2017). These findings have allowed further research on the presence, diversity and potential applications of *Wolbachia* biocontrol strategies for managing this phytoplasma vector.

The presented study investigates the presence of the bacterial endosymbiont *Wolbachia* in *H. obsoletus* population associated with *C. arvensis* in Serbia, the primary vector of '*Ca*. P. solani' in this region. A two-step approach was employed: (1) analyzing '*Ca*. P. solani' infection and diversity in *H. obsoletus* using the stolbur phytoplasma-specific *stamp* gene (Fabre et al., 2011), and (2) examining *Wolbachia* presence, frequency, and genetic diversity employing the *ftsZ* gene, a well-established phylogenetic marker for *Wolbachia* (Schulenburg et al., 2000; Lo et al., 2002). Moreover, we have evaluated a potential correlation between '*Ca*. P. solani' infection and *Wolbachia* presence within the studied *H. obsoletus* population.

MATERIAL AND METHODS

Sampling insect material

Adult specimens of *H. obsoletus* were collected in July 2021 from the experimental fields of the Institute of Field and Vegetable Crops in Novi Sad, Serbia (GPS: 45°19'25.9"N 19°49'08.7"E). Entomological nets and mouth aspirators were used to sweep individual *C. arvensis* plants or patches within sugar beet plots and along their borders. The collected insects were transferred to 2 ml plastic tubes (Sarstedt, Germany) filled with 96% ethanol and transported to the laboratory. Morphological identification of the specimens was performed using a Leica S9E stereomicroscope. Following the taxonomic key of Hoch and Remane (1985), the specimens were identified as *H. obsoletus* based on the white pronotum and further examination of male genitalia. After confirming their identity, the insects were preserved in 96% ethanol at 4°C until further analysis.

DNA extraction

Total genomic DNA was extracted from individual *H. obsoletus* specimens using a non-destructive SDS-based protocol described by Rees et al. (2001) and Mahuku (2004), further modified by Kosovac et al. (2018). Initially, specimens were punctured and incubated overnight at 56°C in TES buffer (0.5% SDS, 20 mM Tris-HCl, 10 mM EDTA) supplemented with proteinase K (187.5 µg/ml). After homogenate separation and chloroform treatment (11,000 rpm, 10 min, repeated), the upper aqueous phase was precipitated with isopropanol and centrifuged (13,000 rpm, 15 min). The resulting DNA pellet was washed with 96% ethanol, air-dried, and resuspended in 50 µl TE buffer (50 mM of Tris, 1 mM of EDTA, pH 7.6). Extracted DNA was stored at -20°C.

Molecular detection and characterization of '*Ca*. P. solani' in *H. obsoletus*

Detection of '*Ca*. P. solani' was conducted using a nested PCR procedure targeting the *stamp* gene, which encodes the antigenic membrane protein of stolbur phytoplasma, in a total of 50 *H. obsoletus* specimens (25 males and 25 females). The PCR procedure employed primer pairs StampF/StampR0 and StampF1/StampR1 (Table 1). The final 25 µl PCR mixture contained 2 µl of isolated DNA, 1xDreamTaq PCR Master Mix (Thermo Scientific, Vilnius, Lithuania), and 0.4 µM of each primer. Thermal protocol followed the conditions described by Fabre et al. (2011). The '*Ca*. P. solani' positive control was the referent strain 429/19 (Ćurčić et al., 2021) from a collection of the Laboratory of Phytopathology of the Institute of Pesticides and Environmental Protection in Belgrade. PCR products (5 µl) were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. Nested *stamp* PCR amplicons from 10 '*Ca*. P. solani'-positive samples (five males and five females) were sequenced using the StampF1 primer by a commercial service (Macrogen Inc., South Korea). The obtained sequences were analyzed using the FinchTV v.1.4.0 software (http://www.geospiza.com) and aligned with ClustalW within the MEGA 11 software (Thompson et al., 1994; Tamura et al., 2021). The obtained sequences were compared with *stamp* genotypes available in NCBI GenBank database using BLAST (Basic Local Alignment Search Tool).

Table 1. Primers used for '***Ca***. P. solani' and *Wolbachia* detection and genotypisation, with their respective nucleotide sequences

Targeted gene	Primer name	Primer sequence 5'- 3'	Literature
stamp	StampF	GTAGGTTTTGGATGTTTTAAG	Fabre et al. (2011)
	StampR0	AAATAAAAGAACAAGTATAGACGA	
	StampF1	TTCTTTAAACACACCAAGAC	
	StampR1	AAGCCAGAATTTAATCTAGC	
ftsZ	ftsZunif	GG(CT)AA(AG)GGTGC(AG)GCAGAAGA	Lo et al. (2002)
	ftsZunir	GG(CT)AA(AG)GGTGC(AG)GCAGAAGA	

Molecular detection and characterization of *Wolbachia* in *H. obsoletus*

Following the detection of '*Ca*. P. solani' in *H. obsoletus* samples, a subset of 20 specimens (10 males and 10 females) was selected for *Wolbachia* analysis. This subset included an equal number of '*Ca*. P. solani'-positive and '*Ca*. P. solani'-negative individuals of both sexes. *Wolbachia* detection utilized the conventional PCR targeting the *ftsZ* gene (*Wolbachia* cell division gene) with primers ftsZunif and ftsZunir (Table 1). The final 25 µl PCR reaction mix contained 1 µl of isolated DNA, while the other components remained consistent with the protocol for the *stamp* gene. A thermal protocol according to Krstić (2017) was employed. Visualization of PCR products was performed as previously described. All *ftsZ* PCR amplicons were commercially sequenced (Macrogen Inc., South Korea) using the ftsZunir primer and the obtained sequences were compared with those available in the NCBI GenBank database using BLAST.

Reconstructing phylogenetic network of '*Ca*. P. solani'

To assess the genetic relatedness of '*Ca*. P. solani' genotypes infecting the studied *H. obsoletus* population, a phylogenetic median-joining network analysis was performed. This analysis compared the detected *stamp* genotypes with previously reported genotypes found in *H. obsoletus ex C. arvensis* in Serbia or plants infected by this vector (Mitrović et al., 2016; Kosovac et al., 2019; Kosovac et al., 2023a; 2023b). These included genotypes Rpm35/St3 (acc. no. KC703015), Vv24/St30 (KC703022), St81 (OP156885), St89 (OP156893), St94 (OP156898), St95 (OP156899), St97 (OR667032), St98 (OR667033), St99 (OR667034), and St102 (OR667037). The network analysis utilized a 474 bp fragment of the '*Ca*. P. solani' *stamp* gene sequence. The software NETWORK version 10.2 (Fluxus Engineering) (Bandelt et al., 1999) was employed with default settings. An ε parameter value of 0 was used, and maximum parsimony post-processing was implemented to create a network with the shortest possible trees.

Reconstructing phylogeny of *Wolbachia*

A *Wolbachia* genotype network was calculated to visualize the relationships between the *ftsZ* sequences from *H. obsoletus* and those of previously detected *Wolbachia* strains in Hemiptera insects (suborders Fulgoromorpha, Cicadomorpha, Heteroptera, and Sternorrhyncha). Median-joining analysis was performed based on a trimmed 435 bp *ftsZ* gene fragment using the previously defined values and settings. To infer the evolutionary relationships among the *Wolbachia* strains, a maximum likelihood phylogenetic tree was reconstructed using MEGA 11 (Tamura et al., 2021). The General Time Reversible (GTR) model was employed as a model of nucleotide substitution with 1000 replications. To root the tree, we included an *ftsZ* sequence from *Nasonia vitripennis* (Walker) (Hymenoptera), belonging to *Wolbachia* supergroup A, whereas all other strains employed in the median-joining and maximum likelihood analyses belong to supergroup B.

RESULTS

Genetic diversity of '*Ca*. P. solani'

Molecular detection of '*Ca*. P. solani' in the studied *H. obsoletus* population revealed a 50% infection rate (25/50 individuals). Sequencing of the '*Ca*. P. solani' *stamp* gene in 10 selected samples identified four previously described genotypes: Rqg50/St1 (acc. no. KC703019), Rqg31/St2 (KC703017), STOL/St4 (FN813261), and M5/St28 (KP337316). Rqg31/St2 was the most prevalent genotype, detected in 5/10 samples (Table 2). M5/St28 and STOL/St4 were each detected in two samples, while Rqg50/St1 was identified in only one isolate. All four '*Ca*. P. solani' genotypes were identified in females, while only Rqg50/St1 was absent in males, suggesting a minimal sex bias in genotype distribution (Table 2).

A median-joining network of the 14 *stamp* genotypes revealed the highest diversification from STOL/St4 genotype with six genotypes: St89, St95, St97, St98. St99, St102, clustering in a subnetwork derived from STOL (Figure 1). Forming a distinct cluster in the network, genotypes St81 and St94 were found to diverge from the centrally positioned Rqg31/St2, differing from it by only one nucleotide each. The Rqg50/St1 genotype, distant by 6 nucleotides from Rqg31/St2, clustered closely with Vv24/St30, while the genotype M5/St28 displayed genetic proximity to the Rpm35/St3 genotype (Figure 1).

Table 2. An overview of the '*Ca*. P. solani' and *Wolbachia* strains genotyped in the *H. obsoletus ex C. arvensis* subset. The table lists the '*Ca*. P. solani' original genotype name and its „St" variant (reviewed in Kosovac et al., 2023a); abbreviation HobsCa - *H. obsoletus ex C. arvensis*.

DNA isolate ID	HobsCa female/male (♀/♂)	'*Ca*. P. solani' presence (+/-)	'*Ca*. P. solani' *stamp* genotype	*Wolbachia* presence (+/-)	*Wolbachia ftsZ* genotype
113/22	♀	+	Rqg31/St2	+	WHo1
117/22	♀	+	Rqg31/St2	+	WHo1
118/22	♀	+	M5/St28	+	WHo1
230/21	♀	+	Rqg50/St1	+	WHo1
233/21	♀	+	STOL/St4	+	WHo1
246/21	♂	+	Rqg31/St2	+	WHo1
247/21	♂	+	STOL/St4	-	-
250/21	♂	+	Rqg31/St2	+	WHo1
251/21	♂	+	M5/St28	+	WHo1
92/22	♂	+	Rqg31/St2	+	WHo1
110/22	♀	-	/	+	WHo1
111/22	♀	-	/	+	WHo1
112/22	♀	-	/	+	WHo1
240/21	♀	-	/	+	WHo1
241/21	♀	-	/	+	WHo1
93/22	♂	-	/	-	-
95/22	♂	-	/	+	WHo1
96/22	♂	-	/	+	WHo1
242/21	♂	-	/	+	WHo1
244/21	♂	-	/	+	WHo1

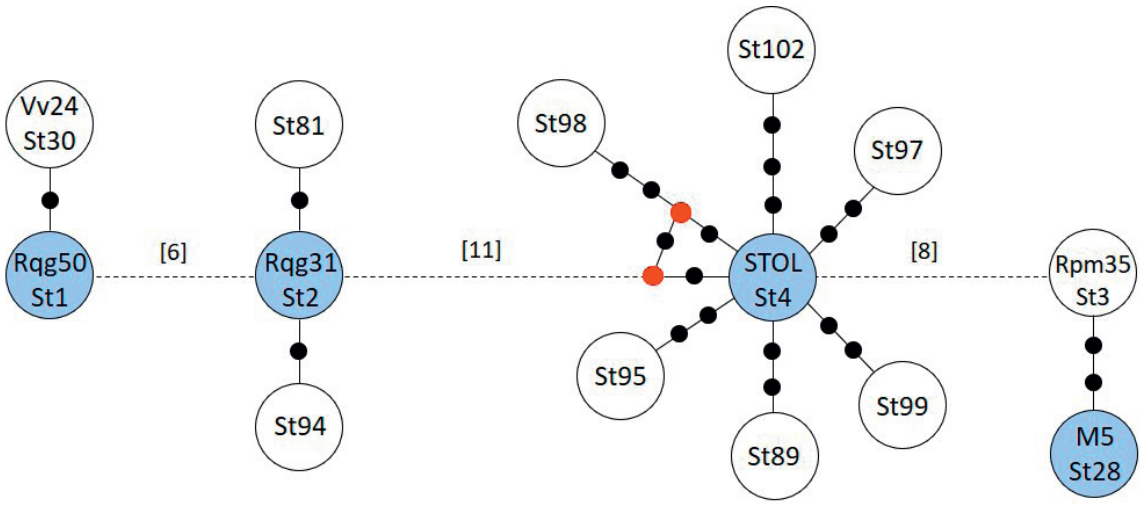

Figure 1. A median-joining network constructed using '*Ca*. P. solani' *stamp* genotypes found in the *H. obsoletus ex C. arvensis* population in this study (depicted with blue circles), and *stamp* genotypes previously found in populations of this vector associated with *C. arvensis* or transmitted by it on experimental plants (colourless circles). Within circles representing a specific *stamp* genotype, original genotype designations are provided alongside 'St' *stamp* sequence variant names (as reviewed in Kosovac et al., 2023a). The black dots on the connecting lines represent the number of nucleotide differences between linked genotypes. Dashed interconnecting lines represent disproportional distances between genotypes, with the number of mutations provided in square brackets above them. Red dots in the network are median vectors representing missing or unsampled intermediate genotypes.

Wolbachia infection patterns and *ftsZ* variability

Analysis of the *H. obsoletus* population revealed a high *Wolbachia*-infection rate, with 18/20 samples testing positive (Table 2). Notably, *Wolbachia* was detected in all ten female specimens (100% infection rate), while 8/10 males (80%) were positive. Sequence analysis of the *ftsZ* gene from these samples revealed the presence of a single *Wolbachia* genotype. Comparison with *ftsZ* sequences deposited in NCBI identified this genotype as a novel, so far unknown sequence variant, designated as WHo1 (acc. no. PP681126).

The analysis of *ftsZ* sequences using a median-joining network revealed a complex genetic relationship between *Wolbachia* and insect hosts (Figure 2A). The network encompassed *Wolbachia* strains from diverse hemipteran groups, including planthoppers (Fulgoromorpha), leafhoppers (Cicadomorpha), mirids and lace bugs (Heteroptera), and whiteflies and psyllids (Sternorrhyncha). The newly identified *Wolbachia* WHo1 *ftsZ* genotype from the planthopper *H. obsoletus* displayed intricate relationships with genotypes from all other insect groups. Within the network, a prominent double reticulation connected seven genotypes. WHo1 showed a genetic relatedness to *Wolbachia* strains found in the psyllid *Bactericera maculipenis* (abbreviated in Figure 2 as WBm) and the whitefly *Bemisia tabaci* (WBt). A *Wolbachia* strain from another planthopper, *Dictyophara europaea* (WDe), diverged from the whitefly *B. tabaci* (WBt) strain by three nucleotides. *Wolbachia* strains from the leafhopper *Philaenus spumarius* (WPhs1 and WPhs2) exhibited closer affinity to the mirid bug *Macrolophus pygmaeus* than to *Wolbachia* strains from the planthoppers (*H. obsoletus* and *D. europaea*). The network also revealed a second, distinct genetic cluster. This cluster comprised three *Wolbachia* strains from different suborders: a shared *ftsZ* genotype between the psyllid *Bactericera cockerelli* (WBc) and the lace bug *Pseudacysta perseae* (WPp), and a strain found in the leafhopper *Balclutha brevis* (WBb) (Figure 2A).

Maximum likelihood phylogenetic analysis corroborated the clustering pattern observed in the median-joining network (Figure 2A, 2B). All *ftsZ* sequences retrieved from the NCBI database for hemipteran hosts that belong to *Wolbachia* supergroup B, suggest the same affiliation for the newly described WHo1 genotype from *H. obsoletus*. Consistent with this, the maximum likelihood tree clearly separated the *N. vitripennis Wolbachia* strain (supergroup A) from the remaining sequences belonging to supergroup B

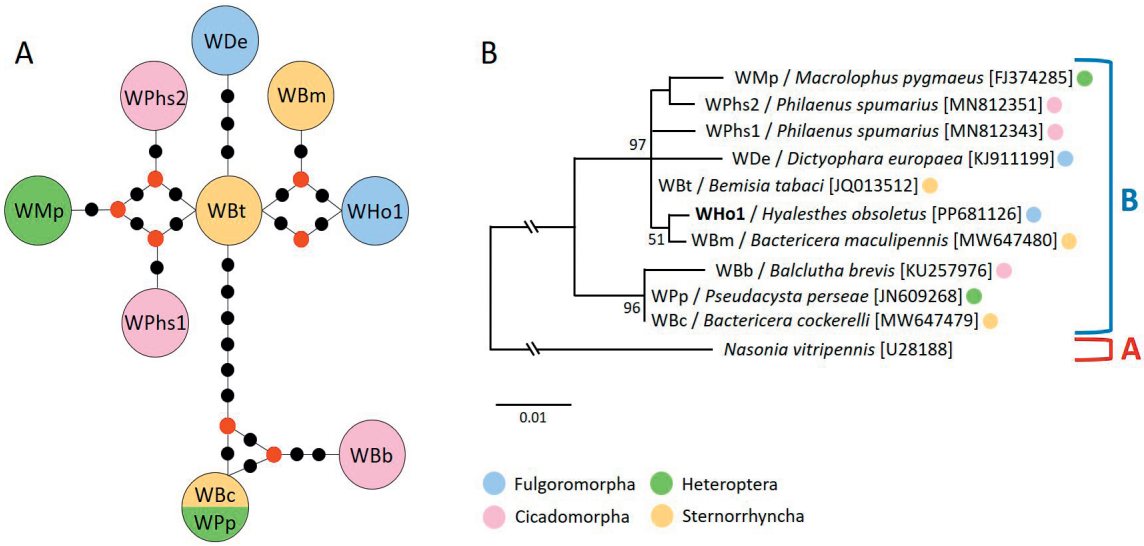

Figure 2. A – Median-joining network constructed using *Wolbachia ftsZ* genotypes found in *H. obsoletus ex C. arvensis* population in this study and genotypes from other hemipteran hosts. Each circle represents a unique *ftsZ* genotype, with given abbreviations provided for the purpose of this study and corresponding to the host. Black dots on connecting lines denote nucleotide differences between linked genotypes. Median vectors (red dots) indicate missing or unsampled intermediate genotypes. Colour coding (legend) corresponds to the specific suborder within the order Hemiptera. B – Maximum likelihood phylogenetic tree based on *Wolbachia ftsZ* genotypes including genotypes used in the median-joining network analysis (supergroup B) alongside a *Wolbachia* supergroup A strain isolated from *N. vitripennis*. Each branch is labelled with the *ftsZ* genotype abbreviation, insect host, and GenBank acc. number in square brackets. Circle colours next to the branches correspond to the specific Hemiptera suborder as defined in legend. Bootstrap support values above 50% are shown next to branches.

(Figure 2B). Within the B genetic group, the tree revealed two high-supported (96-97% bootstrap value) clades. Notably, the novel genotype WHo1 clustered closely with *Wolbachia* from *B. maculipennis* (WBm) within a secluded branch, overall mirroring the grouping observed in the median-joining network (Figure 2A). Likewise, genotypes WBb, WPp, and WBc formed a group on a separate branch, consistent with the network's second genetic cluster (Figure 2A).

One-sided co-occurrence of '*Ca*. P. solani' and *Wolbachia* in *H. obsoletus* population

Potential correlation between the occurrence of '*Ca*. P. solani' and *Wolbachia* in the *H. obsoletus* population was assessed through molecular detection and genotyping of both insect-associated microorganisms (Table 2). Nearly all samples positive for '*Ca*. P. solani' were found to be infected with *Wolbachia* (9/10), showing a 90% co-occurrence rate. However, the presence of *Wolbachia* in nearly all *H. obsoletus* individuals (18/20) was irrespective of sex and '*Ca*. P. solani' infection. Moreover, a single *Wolbachia* genotype, WHo1, was detected across all '*Ca*. P. solani'-infected samples, irrespective of the present *stamp* genotype. The two *H. obsoletus* individuals negative for *Wolbachia* were males, one infected with the '*Ca*. P. solani' STOL/St4 genotype and the other uninfected.

DISCUSSION

This study provides the first insight into *Wolbachia* presence and its genetic diversity within a population of the planthopper vector *H. obsoletus* associated with *C. arvensis* in Serbia. Additionally, the potential patterns of co-occurrence between *Wolbachia* and '*Ca*. P. solani' in assessed vector population were explored. Detection of four '*Ca*. P. solani' *stamp* genotypes (Rqg50/St1, Rqg31/St2, STOL/St4, and M5/St28) aligns with previous studies characterizing '*Ca*. P. solani' associated with *H. obsoletus ex C. arvensis* in Serbia (Mitrović et al., 2016; Kosovac et al., 2019; 2023a; 2023b). These genotypes belong to the tuf-b '*Ca*. P. solani' epidemiology, with STOL/St4 bridging both the tuf-b1 and newly described tuf-d cycles (Ćurčić et al., 2021; Kosovac et al., 2023a).

While the performed phytoplasma characterization relied solely on the *stamp* gene, it provided sufficient information to confirm the existing knowledge about '*Ca*. P. solani' epidemiology within this vector-host association.

The performed analysis of *Wolbachia* in the *H. obsoletus* population from Serbia, based on the *ftsZ* gene, provides some foundational data that pave the way for further investigation of this endosymbiont's role within this vector host. A novel *ftsZ* genotype was unveiled, WHo1, which lacks any matches with *Wolbachia* strains identified in other organisms. Identifying a novel *Wolbachia ftsZ* genotype in *H. obsoletus* expands the understanding of this endosymbiont diversity within the Cixiidae family. *Wolbachia* was previously characterized in populations of the planthopper *D. europaea* in Serbia, a vector of the *Flavescence dorée* phytoplasma (16SrV group) (Krstić et al., 2018). Our findings, demonstrating that the investigated *H. obsoletus* population harbours a single *Wolbachia* genotype (WHo1) alongside four '*Ca*. P. solani' genotypes, align with those of Krstić et al. (2018), revealing a higher level of diversity in the phytoplasma population (five genotypes) compared to the single *Wolbachia* strain observed within several assessed *D. europaea* populations. This consistency suggests a potential lack of direct correlation between the diversities of these co-occurring microorganisms within their insect hosts. However, Krstić et al. (2018) reported a higher *Wolbachia* infection rate in the vector's populations with low phytoplasma infection, suggesting a potential interaction between the two microorganisms. To gain a more reliable understanding of co-occurrence patterns between *Wolbachia* and '*Ca*. P. solani' in *H. obsoletus*, investigation across multiple vector's populations with varying phytoplasma infection rates would be valuable.

Further research, especially genotyping of the *Wolbachia* surface protein *wsp* gene and MLST analysis of the genes *gatB*, *coxA*, *hcpA*, and *fbpA*, should be conducted to thoroughly characterize the detected *Wolbachia* strains in *H. obsoletus* and precisely determine their sequence type (ST) and supergroup affiliation (Stouthamer et al., 1999; Baldo et al., 2006). Based on the performed phylogenetic analysis, it can be concluded that WHo1 genotype belongs to supergroup B, which is consistent with previously published data on this planthopper (Gonella et al., 2011). *Wolbachia*-infection levels detected in our study also follow a previously documented pattern with more females infected than males (Gonella et al., 2011). Full genotypisation of the *Wolbachia* strains infecting *H. obsoletus* is especially important since the presence of this endosymbiont was confirmed in its three out of four host plant associations (Chuche et al., 2016). It would be especially interesting to perform a comparative analysis of *Wolbachia* strains found in *H. obsoletus* and *P. leporinus* as both cixiids are vectors of '*Ca*. P. solani' (Bressan et al., 2008; 2009; Jović et al., 2019; Rinklef et al., 2024).

While *Wolbachia*-based strategies have been proven effective in targeting arbovirus transmission by female mosquitoes (reviewed in Gong et al., 2023), their applicability for controlling phytoplasma transmission still poses a challenge, given that both sexes are effective vectors (Alma et al., 2019). Although this endosymbiont holds promise for control of the vectors of plant pathogens (Gong et al., 2023), several key challenges remain. The most critical is identification of specific *Wolbachia* strains that induce CI, a necessary effect for population suppression, while the PB would be desirable to further enhance control. Unfortunately, many *Wolbachia* strains lack these effects. Once a suitable *Wolbachia* strain is identified and established within a vector's population, interventions like population suppression or replacement could be implemented in insect control programs.

The presented study, based on a limited sample size, cannot establish a correlation between *Wolbachia* and '*Ca*. P. solani' infection in *H. obsoletus*, nor any potential pathogen interference by *Wolbachia*. Future studies utilizing larger *H. obsoletus* sample size would elucidate the complex relations between *Wolbachia* and its insect host, as well as *Wolbachia*'s influence on vector-borne pathogen transmission dynamics (Zug & Hammerstein, 2015). Additionally, investigating the impact of the *Wolbachia* strain genotyped as WHo1 on the *ftsZ* gene on host fitness, as well as its potential for vector biocontrol within a multidisciplinary framework, holds potential for sustainable phytoplasma disease management. Therefore, further research on *Wolbachia* in *H. obsoletus* is a promising study field, as this vector plays a central role in disseminating '*Ca*. P. solani' across agroecosystems in Central and Southeastern Europe, especially considering the current limitations in stolbur disease control.

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Novi *ftsZ* genotip *Wolbachia* endosimbionta cikade *Hyalesthes obsoletus* (Hemiptera: Fulgoromorpha: Cixiidae) vektora '*Candidatus* Phytoplasma solani' asocirane sa *Convolvulus arvensis*

REZIME

Hyalesthes obsoletus (Hemiptera: Fulgoromorpha: Cixiidae) je polifagna cikada poznata kao značajan vektor fitopatogene bakterije '*Candidatus* Phytoplasma solani' (stolbur fitoplazma) koja nanosi štetu u proizvodnji brojnih poljoprivrednih kultura. U okviru odabrane populacije *H. obsoletus* asocirane sa biljkom *Convolvulus arvensis* u Srbiji, istraženi su prisustvo i genetička raznovrsnost endosimbiontske bakterije *Wolbachia*, poznate kao perspektivnog agensa u biološkoj kontroli. U ispitivanoj populaciji *H. obsoletus* pronađena su oba mikroorganizma, '*Ca*. P. solani' i *Wolbachia*. Stopa '*Ca*. P. solani' infekcije *H. obsoletus* populacije je bila 50%, dok je u slučaju *Wolbachia* iznosila 80%. Prisustvo *Wolbachia* nije bilo uslovljeno infekcijom fitoplazmom i pokazalo je minimalne varijacije između polova insekta. Genotipizacijom identifikovanih sojeva '*Ca*. P. solani' otkrivena su četiri prethodno opisana *stamp* genotipa (Rqg50/St1, Rqg31/St2, STOL/St4 i M5/St28). Novi *Wolbachia ftsZ* genotip, označen kao WHo1, je identifikovan u populaciji *H. obsoletus*, što pruža novi uvid u genetičku raznovrsnost *Wolbachia* endosimbionta insekata iz familije Cixiidae. Filogenetska analiza je pokazala složene odnose između WHo1 i *Wolbachia* sojeva iz domaćina različitih Hemiptera podredova. Iako strategije biološke kontrole zasnovane na *Wolbachia* imaju potencijal u kontroli vektora fitoplazmi, potrebna su dalja istraživanja kako bi se objasnile interakcije ovog endosimbionta sa '*Ca*. P. solani' kao i uticaj *Wolbachia* na reprodukciju i fitnes vektora.

Ključne reči: *Hyalesthes obsoletus*, '*Candidatus* Phytoplasma solani', stolbur fitoplazma, *Wolbachia,* endosimbionti

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Original scientific paper

Evaluation of the viability of old seeds of several important agricultural weeds

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SUMMARY

Persistent seed banks are equally important in agriculture and invasion biology. While seed vitality persistence exemplifies an eternal uphill battle for weed control in agriculture, it signals a potential invasiveness of species in invasion biology. Considering yield losses caused by *Amaranthus retroflexus*, *Abutilon theophrasti*, *Chenopodium album* and *Datura stramonium* in agriculture, and the importance of *Ambrosia trifida* as an emerging invader in Europe, the objective of this study was to test the viability and longevity of the aged seeds of these economically important weeds. Three seed viability/longevity tests were conducted: the crush test, germination test in Petri dishes, and 3,5-triphenyltetrazolium chloride (TTC) test. The results revealed a significant variation in germination potential among the tested species. The highest vitality was observed in 7-year-old seeds of *A. retroflexus* (41.67 %), followed by 16-year-old *A. theophrasti* seeds (17.78 %), 13-year-old *C. album* seeds (15.00 %) and 17-year-old *D. stramonium* seeds (7.5 %). Furthermore, a remarkable seed longevity was documented in the tested species (with the exception of *A. trifida*), preserving their germination potential for over half a century. Seed germination was recorded in 49-year-old seeds of *D. stramonium*, 53-year-old seeds of *A. retroflexus*, 58-year-old seeds of *A. theophrasti* and 59-year-old seeds of *C. album*, in strong evidence of the persistence of these weed species' seeds in the environment.

Keywords: germination, seed longevity, seed persistence, viable seeds, weed seeds

INTRODUCTION

Amaranthus retroflexus, Abutilon theophrasti, Chenopodium album and *Datura stramonium* are considered to be among the world's worst weed species (Costea et al., 2004; McDonald et al., 2004; Ziska, 2013; Sarabi et al., 2013; Bajwa et al., 2019), while *Ambrosia trifida* has recently been detected in Serbia, where it currently invades agricultural areas (Vrbničanin, 2015) and has the potential to cause health problems (Ghosh et al., 1991; Savić et al., 2019). In general, all of these weed species cause significant losses in agriculture. In maize

and soybean crops, *A. trifida* can cause yield losses of over 50% at densities of only 1 plant m^{-2} (Baysinger & Sims, 1991; Webster et al., 1994; Harrison et al., 2001). Studies on sugar beet yield losses caused by *A. retroflexus* have shown 12% yield reduction at densities of 4000 plants per ha, and 31% at 15000 plants per ha (Stebbing et al., 2000). In general, weeds compete with crops for light, nutrients and humidity. A well-planned management of commercial seed production and storage, and successful weed control, require that weed seed viability/longevity be determined. Additionally, data on seed longevity and seed bank are important indicators of the invasiveness potential of studied species (Daehler et al., 2004).

Viability is the percentage of viable seeds in the seed bank which have the potential to germinate. Genetic factors are the key determinants of seed persistence (Bekker et al., 2003), enabling weed seeds to remain viable for long periods of time in the soil, where seed dormancy is also an important trait. However, Conn and Farris (1987) showed that there is no significant relationship between initial seed dormancy and seed longevity. Seed dormancy is cycling, ensuring the survival of a weed, making it one of the most important adaptive traits in plants (Gonzáles-Alday et al., 2009). Viable seeds are often remarkably similar to nonviable seeds visually, which often leads to erroneous conclusions, and therefore makes seed viability/longevity testing a requirement in estimating the number of viable seeds in seed banks.

Numerous tests enable seed longevity/viability verification: germination test directly from soil samples (Bekker et al., 1998); controlled ageing test (CAT) (Newton et al., 2009); standard germination test (International Seed Testing Association, 1985); 3,5-triphenyltetrazolium chloride test (TTC, International Seed Testing Association, 1985), and seed crush test (Sawma & Mohler, 2002). In general, each test has its specific advantages and disadvantages. Germination seed testing directly from soil is optimal as it does not require seed extraction from soil, unlike Petri dish tests. However, its downside is that it can take up to two years to complete, until all seeds have germinated. Meanwhile, Petri dish tests offer a more rapid and time-efficient procedure due to the flexibility of using variable temperature conditions. Of course, it is important to note that a portion of seeds can degrade or show high level of dormancy, in both germination tests (Roberts & Dawkins, 1967; Roberts & Feast, 1973). The crush test requires seeds to be removed from soil, germinated and then crushed. Its advantage is that the entire seed sample can be used directly, with those seeds that are brittle or discolored instantly being classified as nonviable (Warnes & Anderson, 1984; Wilson, 1985; Wilson & Lawson, 1992). The TTC test is considered a very quick and reliable method, superior to other methods. However, depending on the analyst's training and application to smaller seeds, the TTC test requires a certain level of skill (Borza et al., 2007).

Different species have characteristic seed longevities and their relative longevity may vary depending on storage conditions and seed dormancy (as a genetic trait of a species; Naylor, 1983). Temperature and moisture content are considered crucial factors that primarily affect seed longevity (Priestley, 1986). Seed longevity and germination capacity have been studied in many plant species (Lueschen & Anderson, 1980; Sawma & Mohler, 2002; Uremis & Uygur, 2005; Conn et al., 2006; Csontos et al., 2016; Mercado & Delgado, 2018; Mercado et al., 2020; Wiebach et al., 2020). The oldest dry-stored seeds known to have germinated were those of *Canna compacta,* about 620 years old (Lerman & Cigliano, 1971). Due to the significant economic influence of the selected weed species (*A. retroflexus, A. theophrasti, C. album* and *D. stramonium*) on crop yields and human health (*A. trifida*) this study aimed to test the hypothesis that seed physiology and tendency to survive/propagate will preserve its germination capacity, i.e. preserve seed viability and secure weed seed longevity. Consequently, this paper reports on the viability and longevity of old seeds of some economically important agricultural weeds.

MATERIAL AND METHODS

Plant material

Seeds of *A. theophrasti, A. trifida, A. retroflexus, C. album* and *D. stramonium* were collected in the field many years ago (20/30/40 years ago), while the only exception was *A. trifida,* which has been recorded in Serbia only recently. The seeds were kept at room temperature, in dry glass containers in the laboratory, as part of a seed collection. The seeds of newer date were collected in the field and kept in paper bags at room temperature in the laboratory until further analysis.

Viability/longevity testing

Viability/longevity testing of seeds was done using three methods: crush test (Sawma & Mohler,

2002), germination test in Petri dishes (International Seed Testing Association, 1985) and TTC (3,5-triphenyltetrazolium chloride) test (International Seed Testing Association, 1985).

Crush test

The method was described by Sawma and Mohler (2002). Seeds were observed under the microscope (Stereo trinocular microscope, MICRO-SC2). Damaged seeds (incomplete or with ruptures in seed coat) were removed from the analysis. A total of 30 seeds in 3 replicates were analyzed for each tested weed species. A single seed was wrapped in a small sheet of paper and forced to break, without pulverizing it.

Germination test in Petri dishes

The test in Petri dishes (International Seed Testing Association, 1985): seeds were placed on filter paper in 9 cm Petri dishes with 5 ml of distilled water (every population containing 30 seeds/Petri dish, in 3 replicates). Petri dishes were set down to incubate at different temperatures: 15, 20 and 25 °C. The seeds were kept in the dark for 15 days and their germination was evaluated daily (with daily removal of germinated seeds). A seed was considered to have germinated when its radicle was visible (about 0.5 mm in length).

TTC test (3,5-triphenyltetrazolium chloride)

The TTC test (International Seed Testing Association, 1985): seeds were kept in distilled water for 18 hours. Subsequently, 30 seeds/test tube in 3 replicates, representing each population, were kept in 1% TTC at 30 °C for 24 h. The seeds were then dissected under the microscope (Stereo trinocular microscope, MICRO-SC2), and seeds with pink to red embryos were considered as viable (Grabe, 1970, Egley & Chandler, 1978; Leist et al., 2003).

Data Analysis

The number of viable seeds was calculated following the PS=Z1+Z2 formula (Uremis & Uygur, 2005) and viability percentage for each population according to formula PV= PS/UB×100 (out of a total of 360), with Z1 being the number of viable seeds in the germination test, Z2 the number of viable seeds in the 1% TTC test and UB the initial number of seeds. Data was processed using the analysis of variance, and Tukey's post hoc test (α=0,05) in Statistica 7.

RESULTS AND DISCUSSION

Longevity and seed germination in agriculture are pertinent because of the maximum period certain seeds can remain in the seedbank and still retain a high germination percentage. On the other hand, in invasion biology, these data can be used as criteria for assessing species invasiveness potential (Daehler et al., 2004; Hiebert, 1997). Conn and Deck (1995) determined that only 2-5 % of weed seeds (*C. album, Stellaria media, Capsela bursa-pastoris, Descurania sophia*) were viable 9,7 years after their burial. Seed viability varies among weed species (Holm et al., 1977), depending on their age, with individual differences in seed viability often being more pronounced between individual seeds than between seeds of different ages (Milberg, 1994). Additionally, their germination potential varies greatly between seed batches and, in limited data sets, no clear effect of seed age can be evident between older batches (Milberg, 1994). In preliminary testing, the crush test was shown to be the most difficult to execute and the least reliable. The seeds were wrapped in paper and crushed but the obtained products and their consistency did not adhere to the criteria described by Sawma and Mohler (2002). According to them, *A. theophrasti, A. retroflexus* and *C. album* seeds can be classified as viable if the crushed product is creamy and the flesh oily, while seeds are considered nonviable if they have deteriorated (or appear powdery) or their color is brown or black. The crush test results were not analyzed because the majority of seeds from all tested populations had deteriorated or become powdery (except *D. stramonium* seeds that could not be crushed without strong pressure, which consequently caused the seeds to become powdery). This method was only proved to be useful for the majority of tested *A. trifida* seeds. Due to the generally low germinability of all seed samples, when the results of the other seed viability methods were considered, the crush test results were not taken into consideration. The results gained by the TTC and germination tests in Petri dishes were further elaborated.

Starting from the initial hypothesis, germination was expected in the oldest seed samples of all tested weed species. The hypothesis regarding seed longevity was confirmed: 58-year-old seeds of *A. theophrasti* (4.72 % vitality), 49-year-old seeds of *D. stramonium* (1.67 %), 59-year-old seeds of *C. album* (3.89 %) and 53-year-old seeds of *A. retroflexus* (0.28 %) (Table 1).

Table 1. Viability percentage (PV) and number of viable seeds (PS) of the total number of 360 seeds per tested species

A. theophrasti			*D. stramonium*			*C. album*			*A. retroflexus*			*A. trifida*		
age	PV	PS	age	PV	PS	age	PV	PS	age	PV	PS	age	PV	PS
58	4.72	17	54	0	0	59	3.89	14	53	0.28	1	8	0.28	1
20	6.67	24	49	1.67	6	27	7.22	26	30	1.94	7	7	1.94	7
17	7.22	26	29	0	0	26	14.1	51	20	0.28	1	6	5.83	21
16	17.78	64	20	0.28	1	17	5.28	19	18	2.22	8	2	6.11	22
14	11.67	42	18	1.94	7	16	10.3	37	17	11.67	42	1	5.56	20
13	6.94	25	17	7.5	27	13	15.0	54	16	1.39	5			
11	10.56	38				4	5.83	21	15	7.5	27			
10	5.28	19				1	2.50	9	13	12.78	46			
9	8.06	29							7	41.67	150			
7	6.11	22												
6	4.44	16												

PV - viability percentage, PS- number of viable seeds

The longevity of these seeds can be attributed to genetic factors as a key influence in seed persistence. The survival/viability of seeds under conditions of stress (inadequate moisture and temperature) in the laboratory is thought to be a good predictor of their potential persistence in the environment (Bekker et al., 2003). Although it is clear that seedling emergence is related to seed size (Benvenuti et al., 2001) and seed energy storage (Mennan & Ngouajio, 2006), the duration of delayed germination has varied among weed species, germination rate varied from year to year, but variation was also common within the same species (Toole & Brown, 1946; Darlington & Steinbauer, 1961). Confirmed germination/seed longevity (Table 1) is of significance for agriculture, as it implies an eternal battle against weeds, while at the same time gives a clear signal of the persistence of such species in the environment. By analyzing the results, it can be concluded that each of the tested species (with the exception of *A. trifida*, whose seeds were of more recent dates) has retained its germination potential for over 50 years (Tables 1, 2, 3, 4, 5, 6). The highest vitality percentage, observed within a single species, was detected in 16-year-old *A. theophrasti* seeds (17.78 %), 17-year-old *D. stramonium* seeds (7.5 %), 13-year-old *C. album* seeds (15.00 %) and 7-year-old seeds of *A. retroflexus* (41.67 %). In contrast, small viability percentage in relatively young seeds of *A. trifida* is surprising (Table 1). Low germination rates of *A. trifida* seeds and the fact that seeds of that species are relatively short-lived (Davis et al., 2005; Harrison et al., 2007) is of equal importance for both agronomy and invasion biology (as it is an emerging invasive species in Serbia; Vrbničanin, 2015).

Germination percentage and significance of differences in the longevity of different age seeds of the tested species *A. theophrasti, A. retroflexus, A. trifida, C. album* and *D. stramonium* are shown in Tables 2, 3, 4, 5 and 6.

Abutilon theophrasti

Sixteen-year-old seeds of *A. theophrasti* germinated at all tested temperatures, while the TTC test showed 0 % vitality (Table 2). Inconsistencies in vitality percentage can be explained by the species metabolism. Vivrette and Meyr (2002) explained the absence of red/pink color by the slow metabolic rate of embryos (which can lead to erraneous, negative conclusion regarding longevity/viability). In the 9-year-old seeds, the results differed. Seeds did not germinate at the tested temperatures, while the TTC test showed 32.22 % viability/longevity (Table 2).

Seiler (2010) points to a possibility of coming to questionable conclusions as a result of using different vitality/longevity testing methods. After testing the germination of wild *Helianthus annus* and *H. petiolaris seeds* (20-year-old, kept at room temperature, 20-22 °C, 22% RH), Seiler (2010) observed low viability/germination in both species, 13 and 1.5%, respectively. Contrary to these results, the TTC test showed a high vitality percentage in the populations of 90 and 80.20 %, respectively.

After analyzing germination at the tested temperatures (15, 20 and 25 °C), it was concluded that *A. theophrasti* seeds have the highest germination rate at 25 °C, regardless of seed age, which was confirmed by the analysis of variance (with the exception of 17-, 14- and 6-year-old seeds, Figure 1, Table 2). However, the t-test showed that there were no significant differences between the tested temperatures in 58-, 20-, 17-, 14-, 11-,

Table 2. Germination rates and statistical significance of differences in seed vitality of *A. theophrasti*

	age	58	20	17	16	14	13	11	10	9	7	6
	15 °C	0	0	0	21.11	0	0	6.67	2.22	0	2.22	0
GT	20 °C	6.67	3.33	2.22	20	2.22	7.78	10	0	0	0	0
	25 °C	7.78	4.44	6.67	30	20	20	2.22	6.67	0	10	2.22
TTC	30 °C	7.78	18.89	20	0	24.44	0	23.33	12.22	32.22	12.22	15.56
	PS	17	24	26	64	42	25	38	19	29	22	16
						t-test						
	15-20 °C	ns	ns	ns	ns	ns	*	ns	ns	-	ns	ns
GT	15-25 °C	ns	ns	ns	*	ns	0	ns	ns	-	ns	ns
	20-25 °C	ns	ns	ns	ns	ns	**	ns	ns	-	*	ns
						ANOVA-LSD test						
	15-20 °C	ns	*	ns	ns	ns	**	ns	ns	-	ns	ns
GT	15-25 °C	*	**	ns	ns	ns	**	ns	**	-	**	ns
	20-25 °C	ns	ns	ns	*	ns	**	**	**	-	**	ns

Number of live seeds PS; ns-differences are not statistically significant; p<0.05 *, p<0.01**; ANOVA-analysis of variance; TTC test; GT–germination test

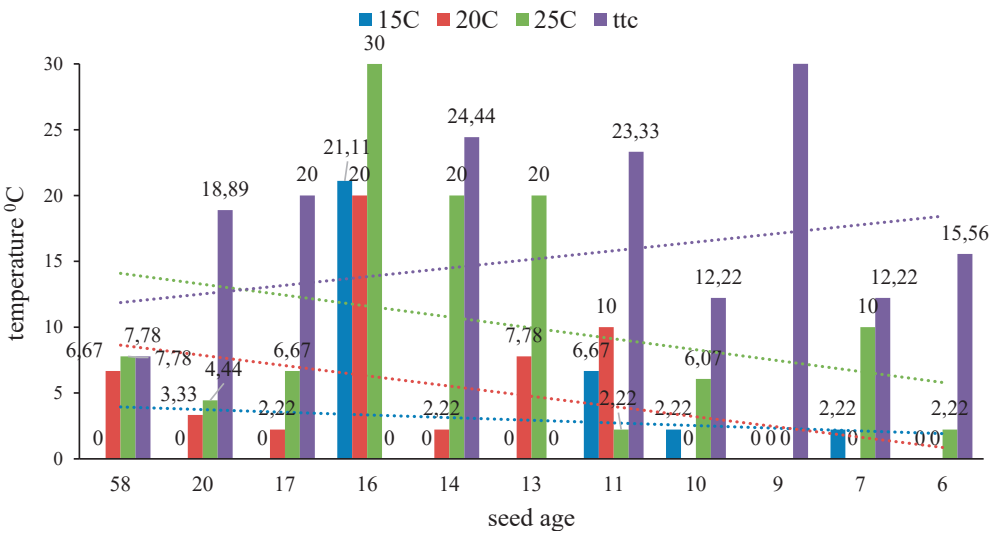

Figure 1. *Abutilon theophrasti* seed longevity

10- and 6-year-old seeds. It is interesting that 58- and 6-year-old seeds showed similar vitality (4.72% and 4.44%, respectively, Tables 1 and 2). A study done by Horowitz and Taylorson (1984) showed that the optimal germination temperature in this species is within 24-30 °C range, while germination declines above 35 °C. They highlighted that the permeability of seeds varied depending on a set of factors and their combinations, which in turn reflects on the germination process. Seed permeability changes after drying at 34 °C. In 3-year-old seeds, the seed coat remained hard, but in 15-year-old seeds, this did not affect (i.e. improve) their permeability.

Figure 1 shows vitality tendencies (in %) in *A. theophrasti* seeds of different ages. A trend analysis showed that seed vitality increases in newer seeds (TTC trend line), which was expected. However, an increase in germination rate is not evident (in all ages) with the increase in temperature (Figure 1). In older seeds (58-, 20-, 17-, 16-, 14- and 13-years-old) better germination rate was evident at 25 °C than on 20 °C, which was not the case with 11-, 10-, 9-, 7- and 6-year-old seeds. Contrary to that, Mennan (2003) recorded a significant decrease in seed vitality/longevity over time (after 1 year of storage of *Galium aparine* and 22 months of *Bifora radians*).

In general, an increase in seed germination rate was observed in younger seeds (TTC test). Nevertheless, no consistency was observed in the number of viable seeds (PS, Tables 1 and 2), depending on their age. The highest number of viable seeds was documented in 16-year-old seeds and the lowest in 6-year-old. It is interesting that the number of viable seeds among 58-year-old seeds was 17, while this number in 6-year-old seeds was 16 (Tables 1 and 2). It is difficult to discern the initial germination rate in test samples, but it was presumably lower on average. This is consistent with the results of a study by Uremis and Uygur (2005), which tested the seed germination rate of several weed species following their storage in soil (over seven years). A significant reduction in germination rate (from 95% to 6.71%) was observed in *A. theophrasti*. The fact that very old seeds kept their ability to germinate can be useful in weed seed bank analyses and suggest an adequate weed control method in agricultural fields. This result is especially meaningful for sugar beet production, where herbicide-resistant *A. theophrasti* populations are found with increasing frequency (Heap, 2023). Earlier studies had also shown that seeds of this weed species are able to remain viable for up to 50 years in soil (Dorado et al., 2009).

Datura stramonium

Datura stramonium is an economically important species, causing yield losses of 26-71% at densities of 3-11 pm^{-2} in tomato fields (Monaco et al., 1981), and 15-45% losses in soybean crops (Hagood et al., 1981). It has also been shown to exhibit a very high competitive index in soybean production in Serbia (Meseldžija et al., 2020). Additionally, this species is poisonous for people and animals as it contains tropane alkaloids, principally scopolamine, hyoscyamine and atropine (Miraldi et al., 2001; Ogunmoyole et al., 2019). Data analysis showed that *D. stramonium* seeds had the lowest vitality/longevity and germination rate of all tested weed species (Tables 1 and 3). In the tested seeds of varying ages (54, 49, 29, 20, 18 and 17 years old), the first recorded germination was in 20-year-old seeds (1.1%), only at 25 °C, while the highest germination rate was characteristic for 17-year-old seeds (16.67%, Table 3 and Figure 2).

Nevertheless, the TTC test confirmed that 49-year-old seeds have preserved their vitality/longevity (6.67%, i.e. there were 6 viable seeds among 360 tested). Due to their low germination rate, data could not be analyzed further, except for 17- and 18-year-old seeds (Table 3). According to Conklin (1976), low germination rate or complete lack of germination in *D. stramonium* seeds can be attributed to: (1) unfavorable environmental conditions, (2) impermeable seed coat, (3) the presence of endogenous inhibitors, and (4) slow metabolic rate of seeds (no pink/red coloration after soaking in 1% tetrazolium solution; Vivrette & Meyr, 2002). Low germination rate of this weed species, with only 3% of

Table 3. Germination rate and statistical significance of differences in seed vitality of *D. stramonium*

	age	54	49	29	20	18	17
	15 °C	0	0	0	0	0	0
GT	20 °C	0	0	0	0	1.11	10
	25 °C	0	0	0	1.11	3.33	16.67
TTC	30 °C	0	6.67	0	0	3.33	3.33
	PS	0	6	0	1	7	27
				T-test			
	15-20 °C	-	-	-	-	ns	*
GT	15-25 °C	-	-	-	-	ns	*
	20-25 °C	-	-	-	-	ns	ns
				ANOVA-LSD test			
	15-20 °C	-	-	-	-	ns	**
GT	15-25 °C	-	-	-	-	ns	**
	20-25 °C	-	-	-	-	ns	*

Number of live seeds PS; ns-differences are not statistically significant; $p<0.05$ *, $p<0.01$**; ANOVA-analysis of variance; TTC test; GT-germination test

germinated seeds in 19.7-year-old seeds was also observed by Conn et al. (2006). In contrast, a study by Toole and Brown (1946) showed that *D. stramonium* seeds germinated after 39 years of burial 34 cm below soil surface.

Literature data shows that the optimal temperature for germination of *D. stramonium* seeds is between 20 and 35 °C (Andersen, 1968; Conklin, 1976). Therefore, the observed lack of germination at 20 and 25 °C could be explained as a complete loss of seed vitality, i.e. by the fact that the tested seeds were not viable. The occurrence of seed mortality can be explained as physiological or chemical damage (Priestley, 1986).

Chenopodium album

The tests conducted and analysis of the obtained results have shown that all *C. album* seeds germinated regardless of their age (apart from 17-year-old seeds at 20 °C, Table 4). It is especially important to highlight that 59-year-old seeds germinated in all tested temperature treatments, with a total of 14 viable seeds and viability percentage of 3.89 % (Tables 1 and 4).

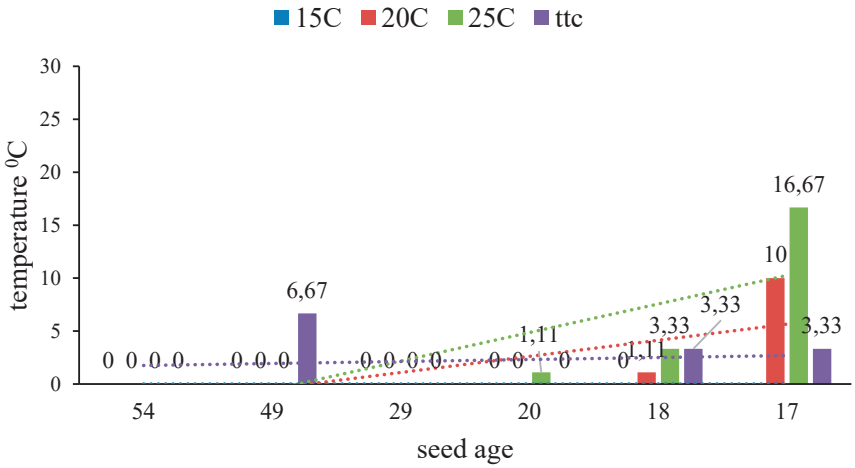

Figure 2. *Datura stramonium* seed longevity

Table 4. Germination rate and statistical significance of differences in seed vitality of *C. album*

	age	59	27	26	17	16	13	4	1
	15 °C	1.11	16.67	27.78	4.44	6.67	23.33	6.67	1.11
GT	20 °C	5.55	7.78	14.44	0	17.78	13.33	4.44	4.44
	25 °C	8.89	3.33	11.11	16.67	16.67	23.33	10	4.44
TTC	30 °C	0	1.11	3.33	0	0	0	2.22	0
	PS	14	26	51	19	37	54	21	9
					T-test				
	15-20 °C	ns	ns	ns	ns	**	ns	ns	ns
GT	15-25 °C	*	ns	*	ns	*	ns	ns	ns
	20-25 °C	ns	ns	ns	*	ns	ns	ns	ns
					ANOVA-LSD test				
	15-20 °C	ns	*	*	ns	**	ns	ns	ns
GT	15-25 °C	*	**	**	**	**	ns	ns	ns
	20-25 °C	ns	ns	ns	**	ns	ns	ns	ns

Number of live seeds PS; ns-differences are not statistically significant; p<0.05 *, p<0.01**; ANOVA-analysis of variance; TTC test; GT-germination test

The most vital seeds of *C. album* were 26 and 13 years old (14.17% and 15%, respectively). T-test and analysis of variance showed that differences in germination rates at different temperatures were not statistically significant and germination rate was higher at 25 °C (Table 4, Figure 3). In general, differences in vitality can be associated with the length of primary dormancy in this weed species. It differs between various populations and can also vary between individual plants of the same population (Holm et al., 1977).

The TTC test determined a low vitality of the tested seeds, even though the number of viable seeds (in all groups) was between 9 and 54 (Table 4). Low vitality (3%), i.e. short seed longevity in this species, was also confirmed by Conn et al (2006). As with all other tested species, no pink/red coloration after exposing seeds to 1% TTC could be linked to slow metabolic rate (Vivrette & Meyr, 2002). In general, the TTC method is not efficient enough with small seeds, as they are difficult to manipulate (cut) and the color is difficult to determine due to their small size. Nevertheless, the fact that 59-year-old seeds have germinated points to the fact that this can be a serious problem in agriculture. This is also in line with the results of Gioria et al. (2020), which showed that low-mass seeds are characterized by highly persistent seed banks. *Chenopodium album* is widely distributed, has a high seed production, its seeds are persistent and can maintain germination capacity in soil for many years (Bajwa et al., 2019). Due to the long-term application of herbicides, this species has also evolved herbicide resistance. The species is especially a problem in sugar beet production (as both species belong to the family *Amaranthaceae*), considering that the number of herbicides which are efficient in its control is decreasing.

Jursík et al. (2003) showed that *C. album* has low germination energy at temperatures between 15 and 24 °C. They found the highest germination rate (75%) at 18°C, while every subsequent temperature increase beyond 24 °C caused a decrease in its germination rate (51% at 24°C). This result supports our findings to some degree. It was concluded that germination rate was higher at 20 °C, but there were no statistically significant differences between germination rates at 20 and 25 °C (Table 4, Figure 3). The analysis of each seed age group showed that seeds germinated better at 20 °C than 25 °C (27-, 26- and 16-year-old seeds), while the opposite was true for 59-, 13- and 4-year-old seeds.

Amaranthus retroflexus

It can be inferred from the results shown in Table 5 that *A. retroflexus* seeds had the best germination rate at 25 °C (showing high viability, i.e. keeping its longevity). Analysis of the obtained results has shown that seed vitality/longevity increases in younger seeds (17-, 15-, 13- and 7-years old). Furthermore, differences were detected in the seed vitality/longevity of different age groups, depending on temperature (t-test and ANOVA, Table 5 and Figure 4). It was also found that 53-year-old seeds did not germinate, even though the TTC test showed 1% longevity (1 viable seed, PV = 0.28%, Table 1) in the tested sample. Only 20-year-old seeds germinated at 25 °C (1 viable seed, PV = 0.28%, Table 1), although the TTC test showed 0% longevity (Table 5).

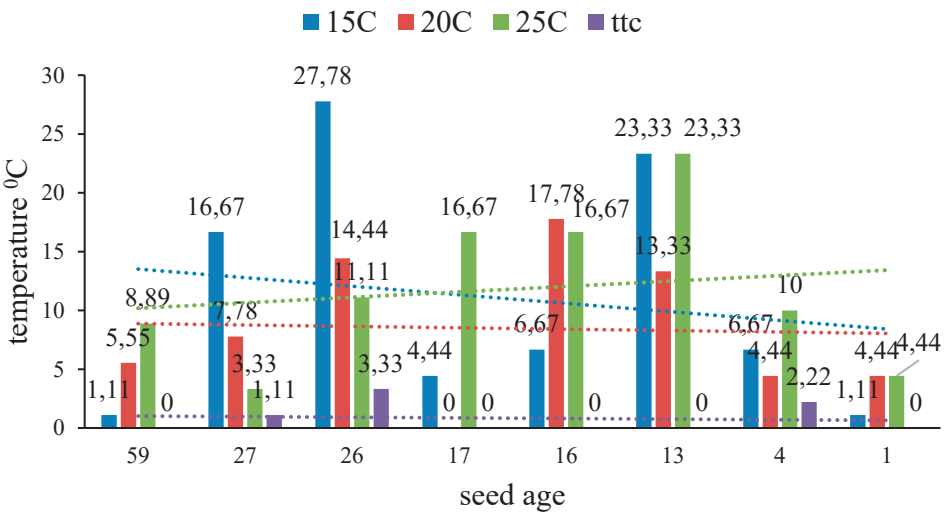

Figure 3. *Chenopodium album* seed longevity

Variations in the germination rate of *A. retroflexus* seeds depending on temperature treatments have been explained by Weaver and Thomas (1986), claiming that *Amaranthus* species seeds are relatively temperature insensitive. Given that the seeds were kept under stable conditions, at room temperature, the observed low degree of vitality/longevity was not surprising. Nevertheless, it is unclear whether, had the seeds been kept in soil, the germination rate/capacity would have been higher and enabled this weed species' persistence over longer periods of time. In general, it is a well-known fact that smaller seeds, such as those of *A. retroflexus,* have a limited food reserve to support germination (Webb et al., 1987). Of all tested weed species, *A. retroflexus* seeds showed the highest viability, with 13-year-old seeds having 46 viable seeds, and twice younger seeds (7 years old) having 3.3 times higher viability (150) (Table 5). No initial germination rate data were available for any of the tested seeds. However, according to Uremis and Uygur (2005), *A. retroflexus* seeds lose their initial germination capacity after seven years following their retrieval from soil (it reduces from 95 to 0.26%).

Table 5. Germination rate and statistical significance of differences in seed vitality of *A. retroflexus*

	samples	53	30	20	18	17	16	15	13	7
	15 °C	0	1.11	0	1.11	0	0	3.33	3.33	0
GT	20 °C	0	1.11	0	6.67	10	2.22	0	0	67.78
	25 °C	0	4.44	1.11	0	36.67	3.33	26.67	44.44	98.89
TTC	30 °C	1	1.11	0	1.11	0	0	0	3.33	0
	PS	1	7	1	8	42	5	27	46	150
					T-test					
	15-20 °C	ns	ns	ns	**	ns	ns	ns	ns	**
GT	15-25 °C	ns	ns	ns	ns	*	ns	**	**	**
	20-25 °C	ns	ns	ns	ns	ns	ns	**	**	**
					ANOVA-LSD test					
	15-20 °C	ns	ns	ns	**	ns	ns	ns	ns	**
GT	15-25 °C	ns	ns	ns	ns	**	*	**	**	**
	20-25 °C	ns	ns	ns	**	**	ns	**	**	**

Number of live seeds PS; ns-differences are not statistically significant; p<0.05 *, p<0.01**; ANOVA-analysis of variance; TTC test; GT-germination test

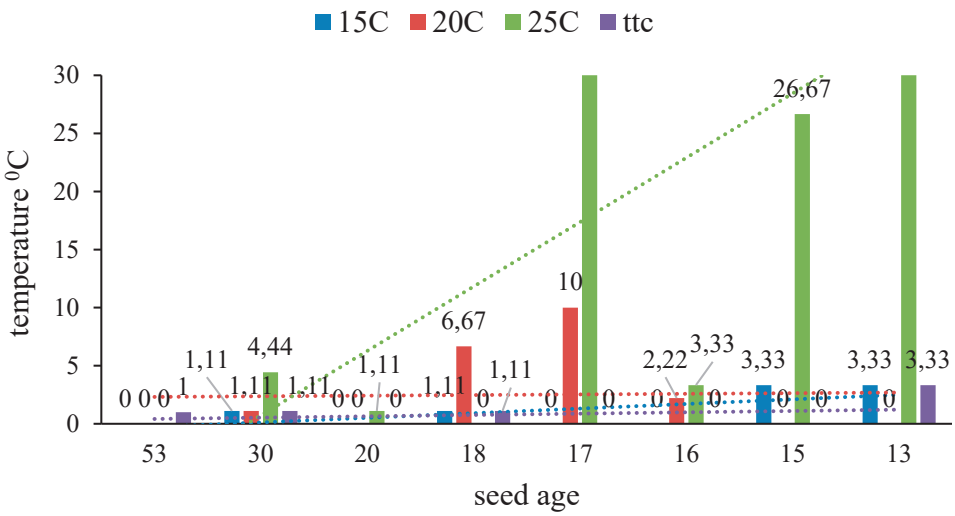

Figure 4. *Amaranthus retroflexus* seed longevity

Ambrosia trifida

Ambrosia trifida, as one of the most competitive weeds in maize and soybean production in the USA, is also a threat to human health (alergies), crop production and natural plant communities (Harrison et al., 2001; Kil et al., 2004; Schutte, 2007). The species is currently spreading in Serbia (Vrbničanin, 2015; Savić et al., 2019). Due to its habitus, the presence of one *A. trifida* plant per m^{-2} can lead to maize and soybean yield losses of over 50 % (Baysinger & Sims, 1991; Harrison et al., 2001). All new scientific data pertaining to this invasive weed species are equally important for agriculture, invasion biology and healthcare system (Follak et al., 2013). Even though this species is known for its relatively low fecundity (Harrison et al., 2001) and transient seed bank characteristics (Abul-Fatih & Bazzaz, 1979; Leck, 1989), its vitality has not been studied sufficiently, which is therefore very important to do. An analysis of results showed that the number of viable seeds (PS) increases with decreasing seed age (Table 1). Harrison et al. (2007) concluded that some *A. trifida* seeds recovered their viability after 9 years of burial (20 cm burial depth). The optimal temperature for seed germination was shown to be 10-24 °C (Abul-Fatih & Bazzaz, 1979), which is in line with the results obtained in the present study. *A. trifida* seeds had the highest germination rate at 15 °C temperature (Tables 1 and 6; Figure 5). In general, both tests (TTC and germination test) have confirmed low germination rates of the seeds of this species.

Table 6. Germination rate and statistical significance of differences in seed vitality of *A. trifida*

	samples	8	7	6	2	1
	15 °C	0	0	11.11	14.44	14.44
GT	20 °C	0	6.67	6.67	5.55	4.44
	25 °C	1.1	1.1	0	1.11	0
TTC	30 °C	0	0	5.56	3.33	3.33
	PS	1	7	21	22	20
			t-test			
	15-20 °C	ns	ns	ns	*	*
GT	15-25 °C	ns	ns	*	*	**
	20-25 °C	ns	ns	ns	ns	ns
			ANOVA-LSD test			
	15-20 °C	ns	*	**	**	**
GT	15-25 °C	ns	ns	**	**	**
	20-25 °C	ns	*	**	*	ns

Number of live seeds PS; ns-differences are not statistically significant; p<0.05 *, p<0.01**; ANOVA-analysis of variance; TTC test; GT–germination test

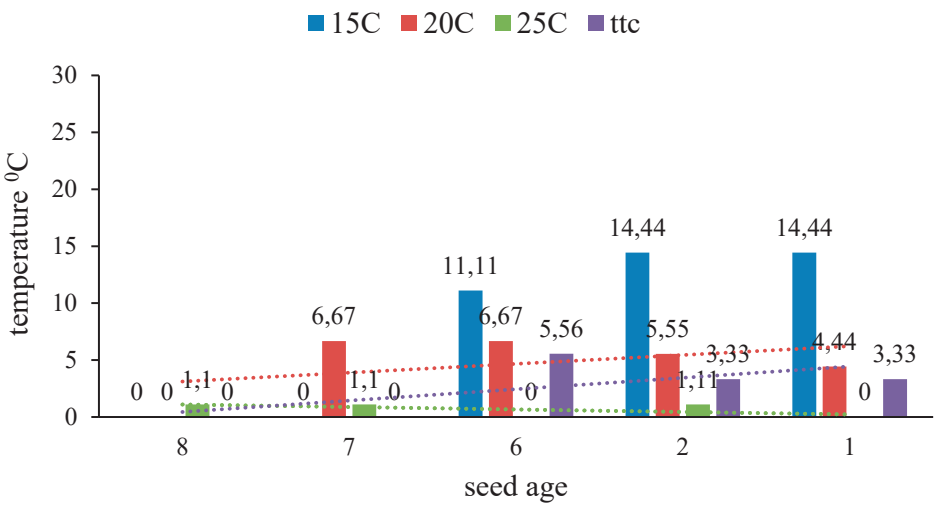

Figure 5. *Ambrosia trifida* seed longevity

The oldest *A. trifida* seeds had the lowest number of viable seeds (8-year-old seeds had 1 viable seed out of 360 in total, viability percentage PV=0,28 %), while the 6-, 2- and 1-year-old seeds had a similar number of viable seeds: 21, 22 and 20, respectively (PV=5.83%; 6.11%; 5.56%, respectively; Table 1). The analysis of variance confirmed statistically significant differences in germination rates, depending on temperature, in 7-, 6-, 2- and 1-year-old seeds (Table 6). Figure 5 shows that the seeds of this weed species have the best germination rate at 15 °C, regardless of seed age, while 25 °C temperature treatment leads to a reduction in germination rate. The results of the germination and TTC test have aligned only for *A. trifida* of all analyzed weed species. The trend line of seed germination rates at 25 °C shows a tendency of reduction, unlike the trend lines in the TTC test treatments at 15 °C temperature. Similar is also true for the trend line at the temperature treatment of 20 °C (Figure 5). Such results can be explained by the fact that *A. trifida* seeds germinate at lower temperatures, and in a wide range of soil depths and soil moistures (Abul-Fatih & Bazzaz, 1979). Due to all of the above, the fact that seeds of this weed species germinate early (before those of the other tested species), and its strong competitiveness, *A. trifida* becomes a dominant species in weed communities that it invades. The fact that this species can also show a delayed emergence by adapting to crop field conditions (Clements et al., 2004) is of particular importance, especially bearing in mind the fact that it also possesses mechanisms to develop herbicide resistance (to glyphosate and acetolactate synthase inhibitors; Heap, 2020).

CONCLUSION

Overall, this study has shown that the seed germination potential of different weed species varies greatly. The highest vitality values were observed in 7-year-old seeds of *A. retroflexus* (41.67 %), 16-year-old *A. theophrasti* seeds (17.78 %), 13-year-old *C. album* seeds (15%) and 17-year-old *D. stramonium* seeds (7.5 %). Furthermore, the most important result of this research was the finding that each of the tested weed species (excepting *A. trifida*) has preserved its germination potential for half a century. Remarkable seed longevity was found in the tested weed species: 49 years in *D. stramonium,* 53 years in *A. retroflexus*, 58 years in *A. theophrasti,* and 59 years in *C. album*, despite the relatively unfavorable conditions of their storage. Such results on weed seed viability/longevity are of critical importance for agriculture, especially organic agriculture, essentially demonstrating that such exceptional longevity and strong seed vitality imply an eternal uphill battle in weed control, giving us irrefutable proof of these weed species' persistence in the environment.

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Vijabilnost starih semena poljoprivredno značajnih korovskih vrsta

REZIME

Prisustvo banke semena je podjednako važno za poljoprivrednu praksu i oblast bioloških invazija. U poljoprivredi vitalnost semena predstavlja borbu protiv korova, a u oblasti invazivne biologije to je signal invazivnog potencijala vrste. Uzimajući u obzir smanjenje prinosa zbog prisustva vrsta *Amaranthus retroflexus, Abutilon teophrasti, Chenopodium album* i *Datura stramonium* u poljoprivrednoj praksi i značaj invazije *Ambrosia trifida* u Evropi, cilj rada je bio da se ispita vitalnost i dugovečnost starih semena navedenih ekonomski značajnih vrsta. Urađena su tri testa za proveru vitalnosti/dugovečnosti semena: test gnječenja, test klijanja u Petri posudama i tetrazolijum test (3,5 trifeniltetrazolijum hlorid-TTC). Dobijeni rezultati su pokazali velike varijacije potencijala klijavosti. Najveća vitalnost je utvrđena za seme *A.retroflexus* starog 7 godina (41,67%), zatim semena *A. teophrasti* starog 16 godina (17,78%), trinaestogodišnjeg semena *C. album* (15%) i sedamnaestogodišnjeg semena *D. stramonium* (7,5%). Takođe, utvrđena je izuzetna vitalnost semena testiranih vrsta (osim *A. trifida*) starog više od pola veka. Zabeležena je klijavost 49 godina starog semena *D. stramonium*, 53 godina starog semena *A. retroflexus*, 58 godina starog semena *A. teophrasti* i 59 godina starog semena *C. album,* čime se potvrđuje njihova perzistentnot u životnoj sredini.

Ključne reči: klijanje semena, dugovečnost semena, perzistentnost semena, vitalnost semena, seme korova

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Assessing sister chromatid exchanges in human peripheral lymphocytes exposed to tetrachlorvinphos during G_0 phase

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SUMMARY

While pesticides undeniably contribute to enhancing agricultural productivity, the escalating trend in their usage has given rise to a myriad of environmental and public health challenges over time. Tetrachlorvinphos, an organophosphate pesticide deemed potentially carcinogenic by the International Agency for Research on Cancer, is commonly employed to combat flies, mites, and larvae in animals, safeguarding public health in open spaces, and managing pest issues in domestic animals. We aimed to investigate the genotoxic and cytostatic effects of tetrachlorvinphos on human lymphocytes in the G_0 phase of the cell cycle using the sister chromatid exchange (SCE) assay. We found that tetrachlorvinphos increased SCE values at 3 concentrations (5, 25, 50 μM). On the other hand, the increase in SCE values was found to be statistically significant only at the highest concentration (50 μM, $p<0.05$). We also found that the SCE value showed a linear dose-dependent increase ($p=0.005$). We concluded that exposure to tetrachlorvinphos had genotoxic potential on human lymphocytes in the G_0 phase of the cell cycle. Additionally, exposure of cells in the G_0 phase of cell cycle to tetrachlorvinphos was found to have no discernible impact on cell cycle kinetics.

Keywords: pesticides, organophosphates, genotoxic effects, cytostatic effects, sister chromatid exchange assay, G_0 phase

INTRODUCTION

Organophosphates are one of the pesticide groups that are widely used today for many applications, primarily to protect plants and agricultural products against various organisms in order to increase crop yield, and safeguard public and animal health (Jaga & Dharmani, 2003; Stoytcheva, 2011). Although these pesticides play a significant role in increasing agricultural productivity, gradual rise in their usage rate over time has resulted in various environmental and health problems (Ragnarsdottir, 2000). They pose a significant risk of an emergence of long-term effects because organophosphates are absorbed and accumulated in fatty tissues due to their lipophilic nature (Bolognesi, 2003; Katzung et al., 2012; Kwong, 2002). Numerous

studies have consistently indicated an elevated risk of various diseases, such as Parkinson's and cancer, associated with exposure to pesticides such as tetrachlorvinphos (TCVP), while at the molecular level, these substances induce a range of genotoxic effects, including DNA damage and chromosomal abnormalities (Hung et al., 2015; Jamil et al., 2005; Lerro et al., 2015; Li et al., 2015; Narayan et al., 2013; Timoroğlu et al., 2014; Yang et al., 2020). This situation results in general consideration of organophosphates as a threatening factor to human health in the case of chronic exposure through water, air and food contamination. Therefore, determining the potential effects of commonly used chemicals, such as pesticides, on genetic material is of great importance to protect public health and minimize global risks.

TCVP (Figure 1), an organophosphate pesticide classified into Group 2B (possibly carcinogenic) by the International Agency for Research on Cancer (IARC), is generally used for fly, mite, and larva control in animals, protecting public health in open areas, and pest control in domestic animals (Guyton et al., 2015). It is incorporated into powders and flea and tick collars for domestic animals, and is additionally supplemented to feeds for goats, pigs and horses as a larvicide. Occupational exposure to TCVP typically occurs on farmlands during crop application. Additionally, the general population may be exposed to TCVP by various pathways, including contact with domestic pets, ingestion of residues on vegetables and fruits or direct inhalation during routine applications in residential settings (Davis et al., 2008; Cobanoglu & Cayir, 2021). Due to its potential carcinogenicity, the use of TCVP has been prohibited for any purpose in European Union countries. In contrast, its usage is permitted in the United States, but solely for field crops (Guyton et al., 2015; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017). Although there are some *in vivo* and *in vitro* studies related to TCVP toxicity, data on its toxicity to humans is still limited (Parker, 1985; National Toxicology Program, 1978; Ergun & Cayir, 2021; Cobanoglu & Cayir, 2021).

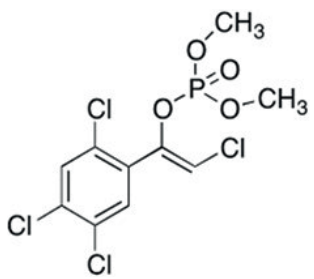

Figure 1. The structure of tetrachlorvinphos

The sister chromatid exchange (SCE) assay is a rapid and sensitive method commonly used in genetic toxicology to assess cytotoxicity and genotoxicity by determining qualitative and quantitative DNA damage. This method allows for a measure of exchange of genetic material between sister chromatids caused by physical, chemical or biological factors. SCE is known as an early indicator of genetic instability (Lialiaris, 2013; Wolff, 1977). That is why the SCE assay has been used in many studies investigating the genotoxic and cytotoxic potentials of various chemicals (Celik et al., 2010; Khabour et al., 2011; National Toxicology Program, 1978). The genotoxic potential of TCVP on circulating human peripheral lymphocytes (HPL) was reported in a previous study (Cobanoglu & Cayir, 2021). On the other hand, no data is available regarding the genotoxic potential of TCVP on human lymphocytes in the G_0 phase of the cell cycle. However, Fenech recommends testing each chemical at different stages of the cell cycle when investigating its genotoxic potential (Fenech, 2000). Therefore, the current study was planned to investigate the genotoxic and cytostatic effects of TCVP on G_0 phase cells using the SCE assay.

MATERIALS AND METHODS

TCVP (99.5 % purity) was obtained from Sigma (USA) and dissolved in dimethylsulfoxide (Merck, Germany, DMSO). Blood samples were taken from 2 voluntary donors (22 and 23 years old) into sterilized heparin tubes. The ethical approval of the study was granted by the Canakkale Onsekiz Mart University Clinical Research Ethics Committee (Decision number: 2021-10).

Controls and concentration ranges

Mitomycin-C (MMC, Sigma, 0.05 μg/ml) was used as a positive control. DMSO was used as a solvent control (<%1, v/v). Kirsch-Volders et al. (2003) suggest that the concentration leading to approximately 50-60% cytotoxicity should be considered as the highest concentration in experimental setups. Therefore, 4 different concentrations (1, 5, 25, 50 μM) were selected as recommended by Kirsch-Volders et al. (2003).

SCE assay

The SCE assay was conducted according to Moorhead's method with minor modifications

(Moorhead et al., 1960). For the preparation of HPL cell cultures, heparinized whole blood should be added to culture medium containing a mitogen, such as phytohemagglutinin (PHA), and incubated at 37 °C for 72 h. SCE formation can be visualized when cells are cultured in the presence of a synthetic nucleoside, an analogue of thymidine, such as 5-bromo-2 deoxyuridine (BrdU), for at least two or more cell cycles. BrdU's incorporation in newly synthesized DNA of replicating cells (S phase) enables visualization and evaluation of SCE. In the present study, each culture was duplicated and was incubated at 37°C for 72 hours. For G_0 exposure, the blood was treated with TCVP (1, 5, 25, 50 μM) for 24 h without phytohaemagglutinin (PHA, Biological Industries, Israel). At the conclusion of the 24-hour period, the cultures were washed three times with medium. Subsequently, the cultures were reestablished using a medium mixture containing PHA. The amounts of 10μg/ml of BrdU (Sigma, USA), and 0.3 μg/ml of colcemid (Biological Industries, Israel) were added to each culture 24 and 70 h after culture initiation, respectively. A KCl amount of 0.075 M was heated at 37°C, and methanol/acetic acid (M/A,3:1) was prepared for harvest (Figure 2). The cells were treated once with KCl and then rinsed 3 times with M/A. Finally, according to Perry and Wolff, the slides were stained with fluorescence plus Giemsa (Perry & Wolff, 1974).

Microscopic evaluation for determining SCE/cell

In the case of each donor and replicate, 25-second metaphases were assessed, with chromosomes stained to distinguish one arm as light and the other arm as dark (Figure 3. b). For each concentration, a total of 100-second metaphases were evaluated (2 donors, 2 replicates) on a light microscope (Olympus, CX31) at 1000 X magnification to determine the mean SCE/cell value.

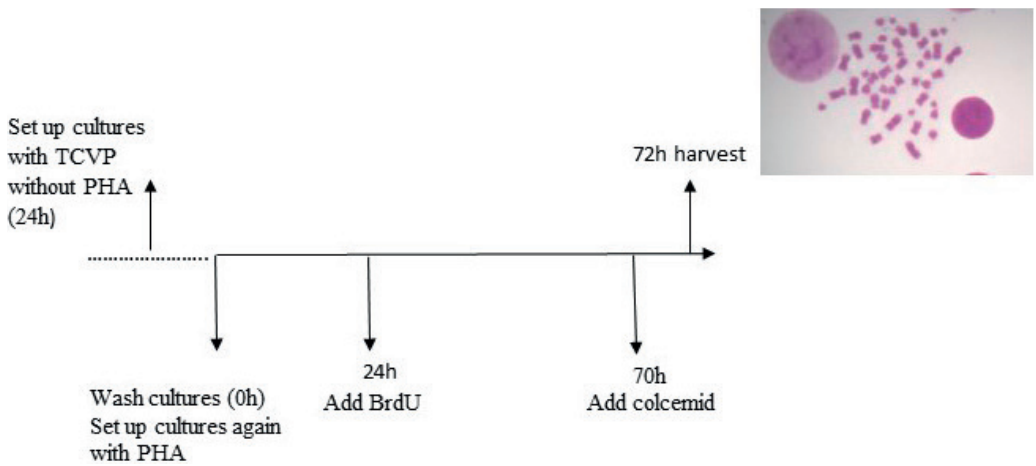

Figure 2. Abstract of experimental design

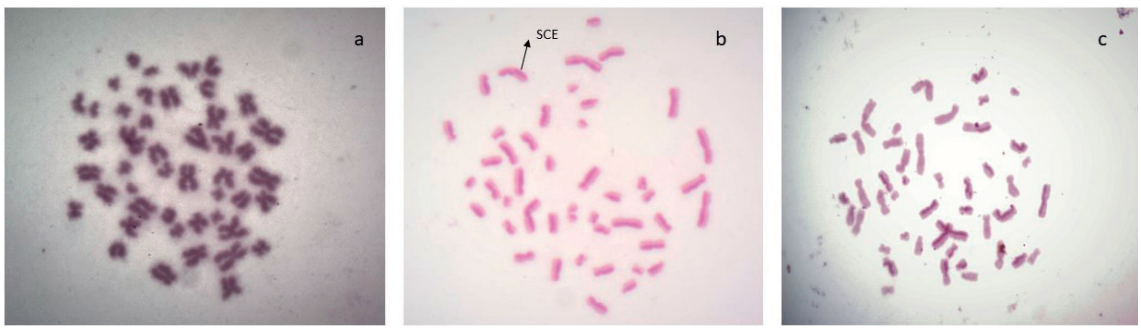

Figure 3. a: first (M1), b: second (M2), c: third (M3) division metaphases

Cytotoxic and cytostatic effects of tetrachlorvinphos

Cytotoxic evaluation for the selected concentrations was performed by mitotic index (MI). While calculating the MI value for each concentration, 1000 cells were scored and the following formula was used:

$$MI = \frac{100 \times \text{cell in metaphase}}{1000}$$

For each concentration, 100 metaphases were evaluated to determine the proliferation index (PI) value representing the cytostatic effect. While calculating the PI values, the following formula was used:

$$PI = \frac{(M1 \times 1) + (M2 \times 2) + (M3 \times 3)}{N}$$

In this formula, M1 (both arms uniformly dark chromosomes), M2 (one arm stained dark, the other arm stained light chromosomes), and M3 (uniformly light and one arm stained dark, the other arm stained light chromosomes) represent the first, second and third division metaphases, respectively (Figure 3).

Statistical analysis

In the study, the statistical analysis of the SCE and PI values was conducted using Kruskal–Wallis and Dunn's multiple comparison tests. A linear regression analysis was performed to show the dose dependence. Data analyses were performed in the Prism software (GraphPad Software Inc) and Excel (Microsoft).

RESULTS

All results are presented as the means (±SE) for two donors and two parallel experiments. Table 1 shows the effect of TCVP on SCE values at four different concentrations. It was found that TCVP increased SCE values at 3 concentrations (5, 25, 50 μM). On the other hand, the increase in SCE value was found to be statistically significant only at the highest concentration (50 μM, $p<0.05$). It was also found that the SCE value showed a linear dose-dependent increase ($p=0.005$, $R2=0.95$). The data obtained for the PI are shown in Table 2. It was determined that TCVP did not change PI values statistically significantly at any tested concentration ($p>0.05$).

Table 1. The effect of tetrachlorinphos on SCE in G_0 lymphocytes

Concentration of tetrachlorvinphos	Metaphase	Mean SCE/cell ± SE
Solvent control	100	4.75 ± 0.14
MMC (0.05 μg/ml)	100	22.50 ± 0.71
1 μM	100	4.75 ± 0.21
5 μM	100	5.03 ± 0.04
25 μM	100	5.27 ± 0.32
50 μM	100	*6.52 ± 0.18

Abbreviations: MMC: mitomycin-C, SCE: sister chromatid exchange, SCE/cell: total number of SCE in a cell, SE: standard error. *$p< 0.05$

Table 2. The effect of tetrachlorvinphos on mitotic index and proliferation index in G_0 lymphocytes

Concentration of tetrachlorvinphos	Cell	Mean MI	Cell	Mean PI
Solvent control	1000	1.67	100	1.69
MMC (0.05 μg/ml)	-	-		-
1 μM	1000	1.68	100	1.70
5 μM	1000	1.62	100	1.69
25 μM	1000	1.61	100	1.65
50 μM	1000	1.60	100	1.65

Abbreviations: MMC: mitomycin-C, PI: proliferation index, MI: mitotic index

DISCUSSION

The current study aimed to investigate whether TCVP has a genotoxic potential in G_0 phase cells. For this purpose, HPLs with more than 95% in the G_0 phase were selected for experiments (Banasik et al., 2005). In the present study, when the SCE values induced by TCVP were compared to the solvent control, it was found that TCVP increased SCE values in a dose-dependent manner. However, a statistically significant increase in the SCE value was observed only at the highest concentration. It was also determined that there was no cytostatic effect of TCVP.

It is well-known that organophosphate pesticides (OPs) can cause DNA damage by interacting with nitrogenous bases or producing reactive oxygen species (Prathiksha et al., 2023). Until now, there have been numerous studies focusing on the genotoxic potential of OP exposure. For example, Garry et al. (1990) demonstrated that malathion induced chromosomal aberration and elevation in SCE on human lymphocytes. In another study, it was revealed that two different OPs, phorate and trichlorfon, caused an increase in SCE and had mutagenic potential on human lymphocytes (Timoroğlu et al., 2014). It was determined that the commercial form of TCVP, named Gardona®, induced a significant increase in chromosome aberrations (Kurinnyĭ & Pilinskaia, 1977). On the other hand, toxicological evaluation of TCVP, a member of OPs, was conducted in only a few human studies (Cobanoglu & Cayir, 2021; Ergun & Cayir, 2021). Ergun & Cayir (2021) investigated whether TCVP caused DNA methylation and cytotoxicity The authors reported that TCVP did not cause DNA methylation but had a cytotoxic effect in A549 lung epithelial cells. Another study reported that TCVP increased micronucleus (MN) frequency on HPL progressing through the cell cycle in all studied concentrations, but that none of these increases were statistically significant (Cobanoglu & Cayir, 2021). In the same study, it was also found that TCVP had no cytostatic effect on HPL progressing through the cell cycle. This finding regarding the cytostatic effect of TCVP is consistent with the findings obtained in the current study. In this context, it may suggest that TCVP does not affect the cell cycle kinetics.

DNA, which is highly sensitive to changes caused by various chemical agents, is the basic unit of heredity. Damaged DNA can play a crucial role in a variety of outcomes, such as genomic instability (Prathiksha et al., 2023). Little is known about the molecular basis of SCE, which represents the exchange of homologous loci during the S phase as a biomarker for genomic instability. It was reported that the target molecules of xenobiotics in the formation of SCE might be the DNA topoisomerase II complex, DNA replication enzymes, and DNA repair enzymes (Wilson & Thompson, 2007; Pommier et al., 1985). A previous study reported that TCVP induced significant changes in SCE values at the three studied concentrations (5, 25, and 50 μg/ml) in cells progressing through the cell cycle (Cobanoglu & Cayir, 2021). On the other hand, in the present study, the SCE value was found to be statistically significant only at the highest concentration. When comparing the results of the two studies, we can speculate two scenarios. Firstly, TCVP might be more genotoxic in HPL cells progressing through the cell cycle than in G_0 phase HPL cells. This possibility would not be surprising because it is expected for cells to be much more sensitive against chemicals when they enter the S, G2, and M phases of the cell cycle (Fenech, 2000). Secondly, for DNA damage to result in SCE, cells must pass through the S phase. Furthermore, the induced lesions can be repaired before the cells enter the S phase (Kopjar & Garaj-Vrhovac, 2000). Therefore, a lesser increase in the frequency of SCE may have been observed in cells damaged in the G_0 phase by TCVP. According to both scenarios, TCVP has genotoxic potential. In addition, we also determined that the SCE value showed a linear dose-dependent increase. The result tells us that longer exposure times to TCVP may be genotoxic in G_0 phase HPLs not only at the 50 μM concentration but also at lower concentrations.

In conclusion, the obtained results showed that exposure to TCVP in the G_0 phase of the cell cycle did not affect cell cycle kinetics. In addition, the results of the current study revealed that TCVP had the potential to induce DNA damage in G_0 lymphocytes, possibly resulting in the formation of SCE. The detection of TCVP's genotoxic potential in the G_0 phase of the cell cycle strengthens the hypothesis presented in a previous study, indicating that TCVP may indeed possess genotoxic properties (Cobanoglu & Cayir, 2021). On the other hand, for a more comprehensive understanding of the molecular mechanisms underlying the potential genotoxic effects of TCVP, future studies could benefit from focusing on specific aspects. First and foremost, exploring whether TCVP triggers oxidative stress, similar to certain other OP pesticides, could

provide valuable insights. Additionally, investigating the capacity of TCVP to bind to DNA warrants attention as it constitutes another pertinent avenue for further exploration.

ACKNOWLEDGEMENT

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Test razmene sestrinskih hromatida u humanim perifernim limfocitima izloženim delovanju tetrahlorvinfosa tokom faze G_0

REZIME

Iako pesticidi nesporno doprinose povećanju poljoprivredne proizvodnje, rastući trend njihovog korišćenja je tokom vremena doveo do pojave raznovrsnih izazova vezanih za životnu sredinu i javno zdravlje. Tetrahlorvinfos, organofosfatni pesticid koji Međunarodna agencija za istraživanje raka smatra potencijalno kancerogenim, koristi se za borbu protiv muva, grinja i životinjskih larvi, u svrhu zaštite javnog zdravlja na otvorenim površinama i suzbijanja štetnih organizama kod domaćih životinja. Cilj istraživanja je bio da se ispita genotoksično i citostatično delovanje tetrahlorvinfosa na humane limfocite u fazi G_0 životnog ciklusa ćelije, koristeći test razmene sestrinskih hromatida (SCE). Našli smo da tri koncentracije tetrahlorvinfosa (5, 25, 50 μM) povećavaju vrednosti SCE. Ipak, to povećanje SCE vrednosti bilo je statistički značajno samo kada je primenjena najviša koncentracija (50 μM, $p<0.05$). Takođe, SCE vrednosti su pokazale linearno povećanje zavisno od doze ($p=0.005$). Zaključili smo da izlaganje humanih limfocita u fazi G_0 životnog ciklusa ćelije delovanju tetrahlorvinfosu ima genotoksičan potencijal. Takođe, izlaganje ćelija u fazi G_0 tetrahlorvinfosu nije imalo primetan uticaj na kinetiku životnog ciklusa ćelije.

Ključne reči: pesticidi, organofosfati, genotoksično delovanje, citostatičko delovanje, test razmene sestrinskih hromatida, faza G_0

Instructions for Authors

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

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

Authors need NOT specify keywords in their articles. The Editor-in-Chief will propose a choice of keywords, and the authors are entitled to accept or change some of them.

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. All authors will be required to confirm their co-authorship in writing during the copyediting stage of manuscript preparation.

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

Original scientific 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:

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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**.

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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

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 black 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.

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

Photos need to be submitted in JPG or TIFF formate and the resolution should be at least 300 dpi. 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.

Any use of artificial inteligence tools (e.g. ChatGpT) should be explained (e.g. for improving English text, which is acceptable).

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.

Publishing in *Pesticidi i fitomedicina (Pesticides and Phytomedicine)* is free of charge.

The editorial staff practice a policy of plagiarism prevention.

Uputstvo autorima

O časopisu

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 zvanična veb stranica izdavača (http://www.pesting.org.rs/).

Časopis se indeksira u sledećim bazama: Chemical Abstracts, CAB International; DOAJ, EBSCO, AGRIS, Scindeks.

Tokom 2011. godine, časopis je prešao na sistem onlajn uređivanja (Elektronsko uređivanje – e-Ur) na portalu SCIndeks Asistent **http://scindeks.ceon.rs/journaldetails.aspx?issn=1820-3949**. Ovaj sistem uređivanja omogućava funkcionalniju prijavu radova i komunikaciju uredništva sa recenzentima i autorima, a obuhvata i servise za kontrolu kvaliteta radova: *CrossRef* za dodelu DOI i iThenticate za prevenciju plagijarizma. Elektronsko uređivanje je usaglašeno sa Aktom o uređivanju časopisa Ministarstva prosvete, nauke i tehnološkog razvoja RS i obezbeđuje vođenje evidencije koje ovaj akt nalaže.

Prijavljivanje radova

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 predložiti urednik, a autori imaju pravo da dodeljene ključne reči prihvate ili da neke od njih zamene.

Priprema rada

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

Naslov treba da bude kratak i da upućuje na temu. Puna imena i prezimena svih autora, puni nazivi i adrese institucija svih autora i njihove email adrese treba navesti ispod naslova rada. U slučaju neslaganja ovih podataka u samom tekstu rada i u prijavi na platformi za uređivanje, prioritet će se dati podacima u samom tekstu rada. U toku faze lekture, tražiće se da svi autori u pisanom obliku potvrde svoje autorstvo.

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

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

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

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

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

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

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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

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

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

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

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

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.

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

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

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

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

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

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

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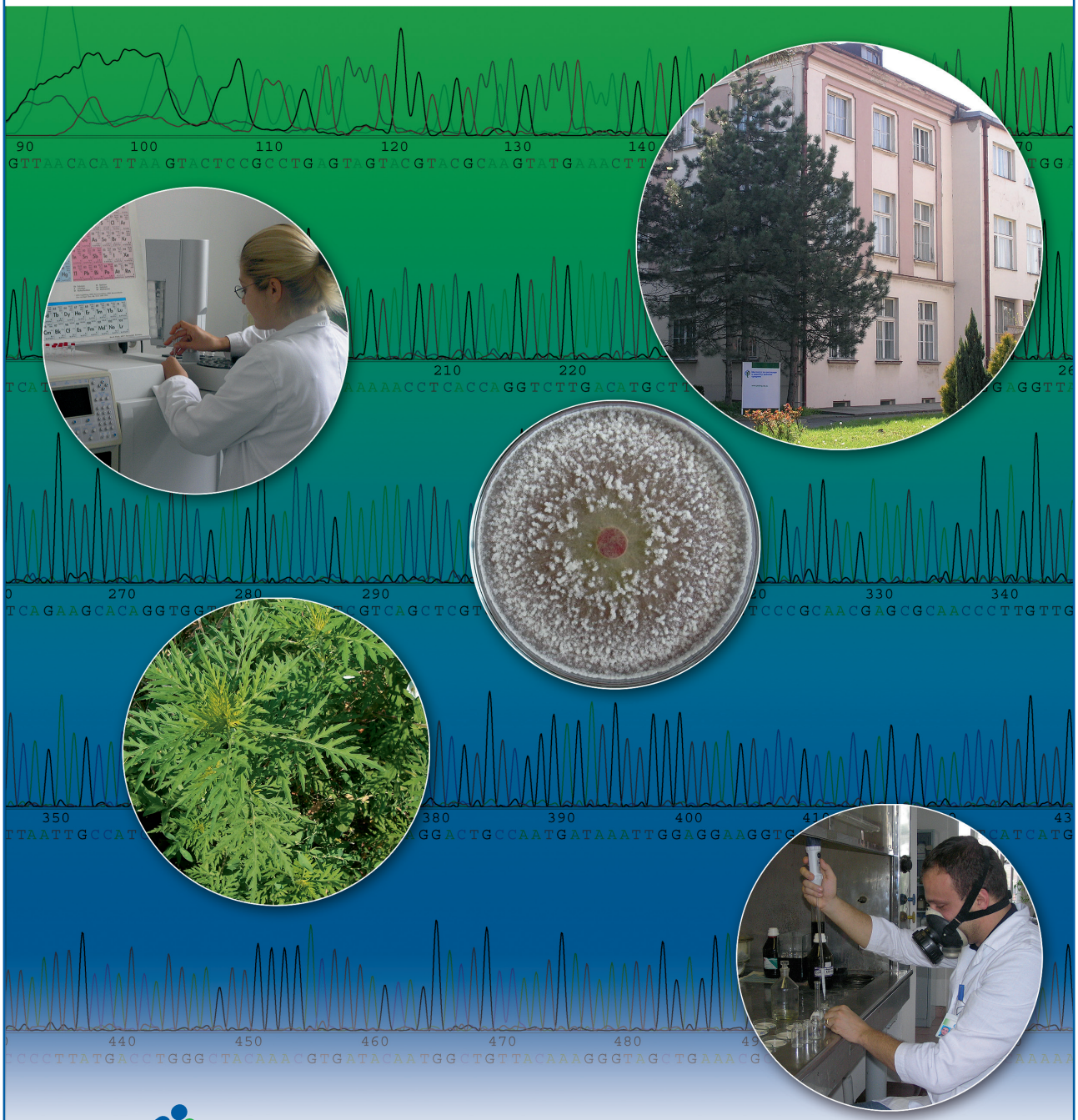

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