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Usijavanje ječma i kuruza u voćnjake oraha — procjena toksičnosti orahova lista na klijanje i rast klijanaca

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INTERCROPPING IN WALNUT ORCHARDS – ASSESSING THE TOXICITY OF WALNUT LEAF LITTER ON BARLEY AND MAIZE GERMINATION AND SEEDLINGS GROWTH

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SUMMARY

Intercropping arable crops between tree rows has proven to be a great alternative to conventional agriculture in terms of food production sustainability and climate change adaptation. However, close interactions between species in these systems sometimes yield adverse allelopathic effects. In this study, the possibility of intercropping barley and maize in the walnut orchard was investigated in terms of walnut leaf litter toxicity. Leaves from 15 and 30 years old walnut trees were used to prepare water extracts out of freshly fallen leaves and soil with leaves decomposed within. Barley and maize seeds were germinated in these extracts using between paper towels method. Fresh leaf litter extracts slightly reduced barley germination but significantly inhibited both barley and maize seedlings' growth. The extract from older walnut tree leaves had the most severe toxic effect, and seedlings shoot was more sensitive than root for both species. A significant correlation was observed between extracts pH and seedlings lengths, suggesting the hydrogen-induced injury to the root, which consequently influenced growth. Decomposed leaf litter extract had a promotional effect on barley and maize germination and growth, which shows that appropriate walnut litter management could improve the performance of intercropped walnut systems.

Key words: intercropping, allelopathy, walnut, barley, maize

INTRODUCTION

Intercropping arable crops between tree rows is a modern approach to a sustainable land use and food production. The addition of trees to the arable land offers a range of positive effects in improving soil health (Dollinger & Jose, 2018) and providing favorable microclimatic conditions (Quinkenstein et al., 2009). However, since several species cultivated in one area are in close interactions, it is essential that their below-ground and above-ground resource competition is reduced to a minimum, and that there are no negative allelopathic relationships between them. Allelochemicals from trees are natural compounds released into the atmosphere and rhizosphere by volatilization and rain leaching from tree canopies, decomposition of tree residues, and root exudation (Hadacek 2003). These compounds can affect the germination, growth, and development of many species grown nearby. Whether this effect will be inhibitory or beneficial depends on a lot of environmental factors and the susceptibility of the given crop to the toxicity of these compounds.

Therefore, prior to the establishment of an agroforestry system, it is important to evaluate the allelopathic relationships in tree-crop combinations to improve these systems' design and ultimately raise their productivity (John et al., 2006). Perhaps the oldest and the most researched example of negative allelopathic effects is walnut allelopathy due to the substance it secretes—juglone. Juglone (5-hydroxy-1,4-naphthoquinone) is an organic compound that enters the atmosphere and soil from all parts of trees from the Juglandaceae family. Walnuts secrete juglone in its reduced form, the so-called hydrojuglone, which is a colorless, non-toxic compound pre-

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sent in all tree parts . However, it oxidizes to juglone in the presence of air, which may have toxic effects on the nearby plants and other organisms (Islam & Widhalm, 2020). These toxic effects include an inhibition of germination and growth by creating harmful free radicals such as superoxide and hydroxide, which disrupt the mitochondrian, chloroplastic (Willis, 2000), and cell-cycle activities, may modify the DNA, inhibit the mRNA synthesis, induce an alkylation of thiol or amine groups of essential proteins, etc. (Strugstad & Despotovski 2012). A variability in the juglone effects on the nearby grown plants is due to the differences among various walnut species and cultivars, seasonal differences in the occurrence of juglone in the walnut tree and soil, differences in the plant species' susceptibility to allelopathy, and geographical differences due to light conditions, soil conditions, and soil microbiology (Willis, 2000). Although it is to be expected that most of the juglone in the soil is present in autumn due to leaf litter and fruits on the soil surface, in their study Jose and Gillespie (1998a) did not detect significant variations in the juglone concentration in the soil through the year and concluded that the amount of juglone from leaves and fruits is probably negligible compared to the constant excretion of juglone through the walnut root. The research, however, showed that juglone could occur in high concentrations in the leaves, with a peak in June/July for *Juglans nigra* and May and July for *Juglans regia* (Willis, 2000). In a previous research of walnut allelopathy's impact on agricultural crops' germination and growth, a positive correlation was observed between the effects of pure juglone and walnut green leaf extracts, which was mainly inhibitory (Ercisli et al., 2005; Kocacë Aliskan & Terzi, 2001), indicating a significant presence of juglone in the green leaves. However, Wang et al. (2014) identified twenty-eight compounds in the walnut leaf litter, with many of them reported as phytotoxic, suggesting that juglone is not the only compound responsible for walnut allelopathy. Furthermore, Matok et al. (2009) demonstrated that the bioactive compounds, such as phenolic acids, flavonoids, and terpenes present in walnut leaves, might be responsible for their toxicity. Considering the tree age, leaves' vegetative stage, and the crops selected for intercropping, the allelopathic effect can vary significantly. In their review paper, Scott and Sullivan (2007) discussed various estimations on the number of years subsequent to the black walnut planting whereafter toxicity may appear. However, the majority of literature suggests it is not sooner than 12 years. Regarding leaf maturity, it seems that the fresh, green leaves may exert a stronger inhibitory effect than the dried fallen ones or a decomposed leaf litter, but that also depends on the observed crop (Bahuguna et al., 2014a, 2014b; Ercisli et al., 2005). Given a research results variability, this study aimed to explore the germination and seedling growth of barley (*Hordeum vulgare* L.) as a winter crop and maize (*Zea mays* L.) as a summer crop under the treatments by fresh and decomposed walnut (*Juglans regia* L.) leaf litter extracts.

MATERIALS AND METHODS

Leaf litter collection and extracts preparation

Since winter barley is a crop that is sown in October/November and grain maize is usually sown in April/May, the effects of two walnut leaf extracts were examined: a freshly fallen leaf litter, which would affect barley sown in November, and a soil-decomposed walnut leaf litter, which would affect maize sown in May. The decomposed leaf extract was prepared according to the methodology proposed by Zhang et al. (2015), which was also modified to prepare the freshly fallen leaf extract. In late October, walnut leaf litter was collected from two sites in Eastern Croatia: Đakovo, where walnut orchard is 15 years old, and Šag, where the walnut trees are over 30 years old. Subsequent to the removal of damaged or diseased leaves, the litter was washed with the distilled water and dried in an oven at 60 °C for 24 hours, then ground to <1mm. For the first fresh litter extract, the prepared powder was mixed with the distilled water in a ratio of 1:25 (w/v) and left for 48 hours. The mixture was then centrifuged, filtered, and supernatant kept in the fridge at 4 °C. For the decomposed leaf litter extract, the leaf powder previously prepared was mixed with soil from the local arable plot in ratio 1:8 (w/w) (according to an approximation of annual walnut leaf litter production and the weight of surface soil). The distilled water was added to the mixture until it was saturated to 60% of field saturated water capacity. The pots were covered with plastic foil with three to four holes to allow for a ventilation and to reduce a high evaporation and were left at room temperature for 150 days to decompose. Every few days, the pots were weighted, and the distilled water was added according to a mass difference. After 150 days, the soil-leaf mixture was mixed with the distilled water in a ratio of 9:25 (w/v), left for 48 hours, and then centrifuged and filtered.

Germination and seedling growth experiment

Germination rate, shoot and root length, and the total length of the seedlings were determined after growing barley and maize between the paper towels, according to the applicable ISTA rules (ISTA, 2005). For both species, the paper towels were soaked with 100 mL of the distilled water or walnut leaf extract in four repetitions. In each repetition, 50 seeds were placed on the paper towels, which were then folded and rolled into tubes, put into plastic bags to retain moisture, and deposited into a growing chamber for seven days. Barley was grown at 20 ºC with changing light conditions of 12 hours of light and 12 hours of dark, and maize at 20ºC for 16 hours in dark and at 30ºC for 8 hours in light.

Measurements

The germination rate was determined by counting the properly developed seedlings after seven days. The root and shoot were manually measured using a ruler, and those values were added up to determine the total seedling length. The extracts' pH was measured by the

Mettler Toledo pH-meter while applying the electrometric method.

Data analysis

Statistical analysis was conducted in the *R* software (R Core team, 2021). The univariate one-way ANOVA was applied to assess the differences between the treatments for each dependent variable separately (germination rate, seedling length, shoot, and root length), and the post hoc Tukey HSD was performed for multiple pairwise comparisons ($p < 0.05$) for each species. Pearson's product-moment correlation was computed to determine a relationship between the extracts' pH and the seedlings' length.

The treatments were as follows:

- control: the distilled H_2O
- fresh_15: fresh leaf litter extract, 15-year-old walnut
- fresh_30: fresh leaf litter extract, 30-year-old walnut
- decomp_15: decomposed leaf litter extract, 15-year-old walnut
- decomp_30: decomposed leaf litter extract, 30-year-old walnut

RESULTS AND DISCUSSION

The impact of different walnut leaf litter extracts on barley and maize was observed and recorded in terms of germination, total seedlings length, root, and shoot length. Walnut extracts did not affect maize germination, that is, there were no statistically significant differences between a control and treatments (Table 1). This result is consistent with those observed by Kocacë Aliskan and Terzi (2001), who found that maize, in terms of germination, was resistant even to fresh, green leaf extract, which hypothetically could have a greater juglone content and thereby have a stronger inhibitory effect. As suggested by Terzi (2008), it could be that the resistant species have a mechanism of tolerance to juglone or other toxic compounds in their seed coats. Both fresh walnut leaf litter extracts, however, reduced barley germination. This result was unexpected, since barley was somewhat tolerable to juglone and walnut leaf extract treatments in previous research (Bahuguna et al., 2014b; Kocacë Aliskan & Terzi, 2001). Although the reduction amounted to only 1.75% for both treatments in regards to control, it was statistically significant ($p < 0.05$; Table 1).

Table 1. Barley and maize germination, root and shoot length, and total seedlings' length under different treatments
Tablica 1. Klijavost, duljina korijena i hipokotila i ukupna duljina klijanaca ječma i kukuruza pod različitim tretmanima

Treatment	Germination (%)		Shoot length (cm)		Root length (cm)		Seedling length (cm)	
	Barley	Maize	Barley	Maize	Barley	Maize	Barley	Maize
control	99.50 ^{a}	99.75 ^{a}	10.09 ^{a}	9.95 ^{a}	13.53 ^{ab}	11.69 ^{a}	23.62 ^{a}	21.64 ^{a}
	(0.58)	(0.50)	(1.00)	(1.00)	(1.05)	(1.07)	(1.69)	(1.47)
fresh_15	97.75 ^{b}	99.00 ^{a}	8.26 ^{b}	8.72 ^{b}	13.16 ^{bc}	10.36 ^{b}	21.41 ^{b}	19.08 ^{b}
	(0.50)	(0.82)	(1.04)	(1.17)	(1.21)	(1.28)	(1.89)	(1.98)
fresh_30	97.75 ^{b}	98.75 ^{a}	8.12 ^{b}	7.95 ^{b}	12.62 ^{c}	10.26 ^{b}	20.74 ^{b}	18.21 ^{b}
	(0.96)	(0.50)	(0.95)	(0.84)	(1.23)	(1.11)	(1.74)	(1.79)
decomp_15	99.75 ^{a}	99.50 ^{a}	10.20 ^{a}	10.19 ^{a}	14.01 ^{a}	11.77 ^{a}	24.21 ^{a}	21.96 ^{a}
	(0.50)	(0.58)	(1.10)	(1.00)	(1.03)	(1.21)	(1.92)	(1.68)
decomp_30	99.50 ^{a}	99.25 ^{a}	9.82 ^{a}	9.69 ^{a}	13.79 ^{ab}	11.83 ^{a}	23.61 ^{a}	21.52 ^{a}
	(0.58)	(0.50)	(1.10)	(1.39)	(0.85)	(1.21)	(1.62)	(2.07)

*Data are represented as average (standard deviation).

**Different letters indicate significant differences between treatments ($p < 0.05$), based on Tukey HSD tests.

Regarding seedlings' length, both barley and maize were not significantly affected by the decomposed leaf litter extracts but were inhibited by the fresh ones. Contrary to their effect on germination, fresh leaf litter extracts had a stronger inhibitory effect on the maize growth than the barley growth, with the lowest maize seedling length observed with the fresh_30 treatment. Barley seedling length was equally inhibited by the fresh_15 and the fresh_30 extract, respectively (Figure 1).

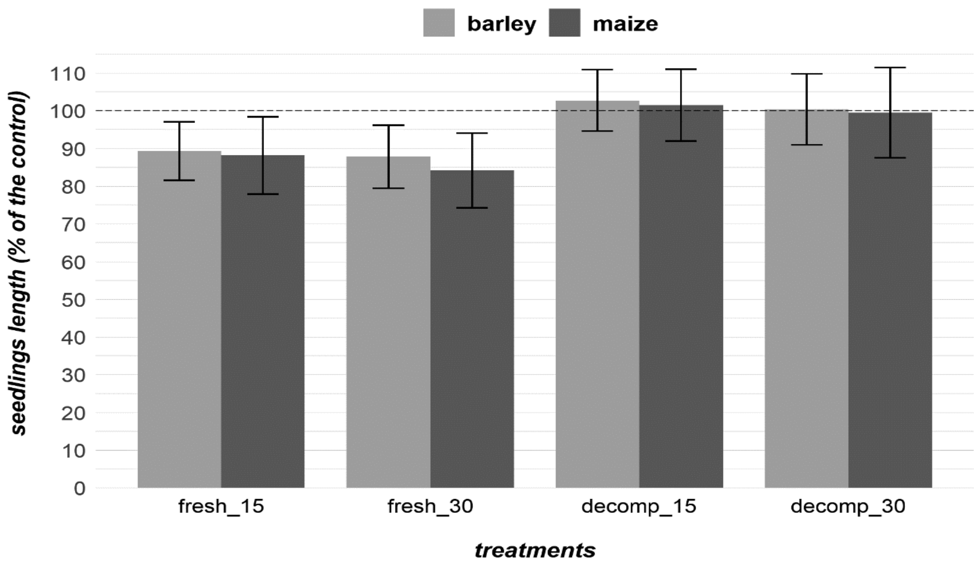

Figure 1. Barley and maize seedlings' length under different treatments, expressed as a control percentage

Grafikon 1. Duljina klijanaca ječma i kukuruza pod različitim tretmanima izražena u postotku od kontrole

Bahuguna et al. (2014b), who used a leaf extract prepared out of the fallen walnut leaves in October, observed greater sensitivity of barley root than that of the shoot growth. Contrary, in this bioassay, the shoot was more sensitive to the fresh leaf litter extracts for both species (Figures 2 and 3). These results are consistent with those obtained by Kocacë Aliskan and Terzi (2001), who found that the barley shoot growth was most sensitive to the fresh, green walnut leaf extract. A shoot growth inhibition can happen due to a disruption in photosynthesis or due to the metabolic changes caused by an oxidative stress (Wang et al., 2014). Jose and Gillespie (1998b)4-napthoquinone observed a significant reduction of net photosynthetic rate in the leaves of hydroponically grown maize and soybean in the juglone treatments with 10 and 100 M concentrations, as well as a reduced transpiration and stomatal conductance. However, Hejl and Koster (2004) found no effect of the juglone treatments on chlorophyll fluorescence and chloroplasts, but they detected a significantly reduced maize and soybean water uptake and H+-ATPase activity. This lead to the conclusion that photosynthesis and, consequently, the seedlings' growth was probably indirectly inhibited by a disrupted root plasma membrane function and the resulting stomatal closure.

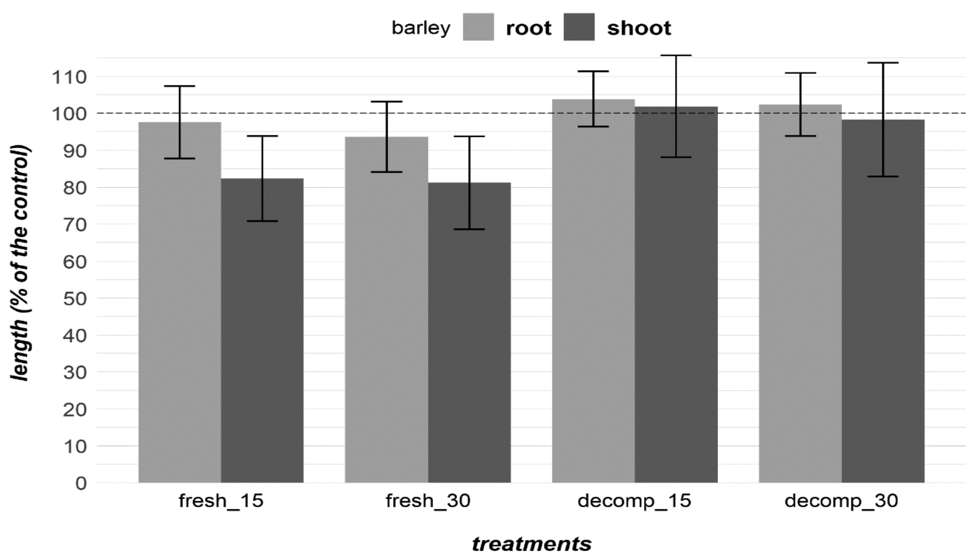

Figure 2. Barley root and shoot length under different treatments, expressed as a control percentage

Grafikon 2. Duljina korijena i hipokotila ječma pod različitim tretmanima u postotku kontrole

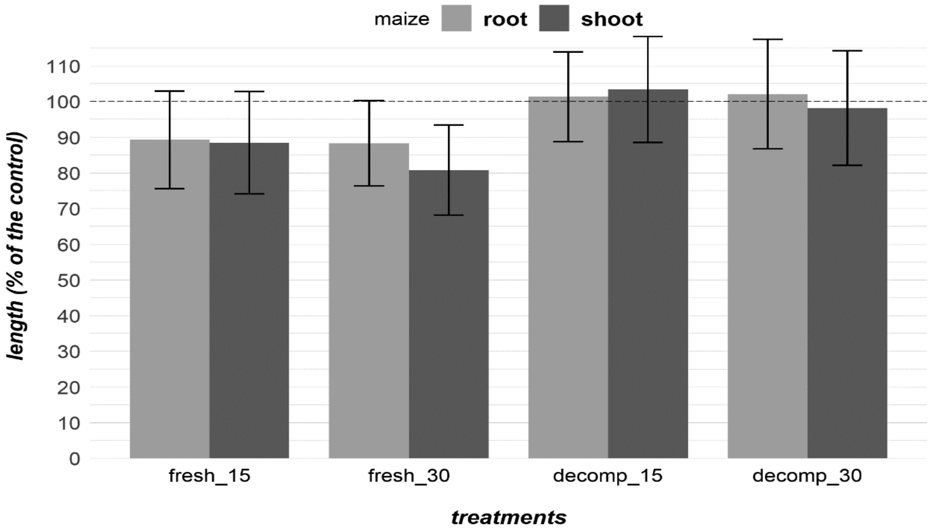

Figure 3. Maize root and shoot length under different treatments, expressed as a control percentage

Grafikon 3. Duljina korijena i hipokotila kukuruza pod različitim tretmanima u postotku kontrole

Furthermore, in this study, an interesting observation during the treatments of fresh litter extracts was that of the brownish barley roots with little or no lateral roots. Murata et al. (2003) suggest that this can be due to a low solution pH, which can cause a hydrogen-induced root injury, make its membrane leaky, and thereby interfere in the absorption of nutrients, thus causing a loss of sugars and amino acids. These changes contribute to the growth inhibition of the whole plant. Indeed, both fresh litter extracts had a lower pH than that of their corresponding decomposed extracts and control (i.e., the distilled H_2O; Table 2), and significant correlations ($p < 0.05$) were observed between the extracts' pH and the seedlings' length; $r = 0.98$ and $r = 0.97$ for barley and maize, respectively.

Table 2. The extracts' pH values

Tablica 2. pH vrijednosti ekstrakata

Treatment	pH
control	7.01
fresh_15	5.3
fresh_30	5.03
decomp_15	7.61
decomp_30	7.57

Out of the fresh extracts, the one obtained from the older walnut trees was more inhibitory. It was previously reported that the walnut toxicity does not appear in the first 12-25 years (Scott & Sullivan, 2007), which may be related to a physiological difference between the young and older walnut trees. It is not yet confirmed, however, that the older walnut trees produce more juglone and other toxic compounds than the younger ones. Also, the observed effects of a more severe allelopathy in the older walnut plantations may result from a juglone buildup in soil (Willis, 2000). The decomposed walnut leaf litter extracts had a slightly promotional effect on the seedlings' growth (Figure 1). Zhang et al. (2015) also observed a positive effect of the decomposed walnut leaf litter extracts on the rape germination and growth. Allelochemicals in the walnut leaves are under the influence of different factors during their decomposition in the soil, such as the temperature, nutrient content, and microbial activity, which can weaken their toxicity, or in some cases, even transform them into the more active substances (Zhang et al., 2015). Nonetheless, the nutrients released from the decaying leaves can weaken the toxic effects of such compounds, as well as a decomposition-produced humus, which can absorb them (Loffredo et al., 2005). Furthermore, humus can promote the overall plant growth and thereby indirectly decrease a plant's sensitivity to the the toxic compounds in the walnut leaves. Even though there was no statistically significant difference between the control and the decomposed walnut leaf extracts (Table 1) either in the total seedling lengths or in the shoot or root lengths, a slight inhibitory effect for shoot lengths was observed in a treatment with the decomp_30 for both species. Trogisch et al. (2016) pointed out that the physicochemical litter traits (e.g., the leaf toughness, lignin:N ratio, C:N ratio and phenolic content), can significantly alter the leaf litter decomposition rate, in addition to the environmental factors and soil properties. The litter produced by the young trees has a lower C:N ratio and lower lignin concentrations than the one produced by the older trees, so the leaf litter from the older trees has a slower decomposition rate (Trap et al., 2013). A slower decomposition rate could result in the presence of inhibitory allelochemicals in the leaf litter extract from a 30-year-old walnut, even five months subsequent to the decomposition. However, this should yet be confirmed by a chemical analysis of litter and the prepared extracts.

CONCLUSION

This study suggests that the allelochemical properties of fresh walnut leaf litter could have an inhibitory effect on the barley and maize growth. A decrease in both the shoot and root length was observed, with the shoot being more sensitive with regard to the both species. Although similar results obtained by other studies also suggest that these species would not be a good choice for intercropping with walnut, this study proved that the walnut leaf toxicity could be weakened by decomposition, and the decomposed walnut leaf litter could even have a promotional effect on the barley and maize growth. Therefore, an appropriate walnut litter management could improve the performance of the intercropped walnut systems. For comparability reasons, this study was solely focused on a laboratory bioassay and early development of tested species, which is why the leaf litter extracts were used. In nature, the freshly fallen leaves could exert different effects under variated field conditions, which are usually hard to be separated from the actual allelopathic effect. This study, however, can be a good basis for further walnut leaf toxicity research under different growing conditions.

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USIJAVANJE JEČMA I KURUZA U VOĆNJAKE ORAHA — PROCJENA TOKSIČNOSTI ORAHOVA LISTA NA KLIJANJE I RAST KLIJANACA

SAŽETAK

Usijavanje poljoprivrednih kultura između redova drveća (konsocijacija) već se pokazalo kao izvrsna alternativa konvencionalnoj poljoprivredi u smislu održivosti proizvodnje hrane i adaptacije na klimatske promjene. Međutim, bliske interakcije između biljnih vrsta u takvim sustavima ponekad rezultiraju štetnim alelopatskim učincima. Ovim radom istražena je mogućnost usijavanja ječma i kukuruza u voćnjake oraha iz aspekta toksičnosti orahova lista. Lišće oraha starih 15 i 30 godina korišteno je za pripremu vodenih ekstrakata iz svježe otpalog lišća te smjese tla i razgrađenoga orahova lišća. Sjeme ječma i kukuruza naklijavano je metodom između filter-papira natopljenih priprmljenim ekstraktima. Vodeni ekstrakti svježeg lišća smanjili su klijavost ječma, a značajno su inhibirali rast klijanaca i ječma i kukuruza. Ekstrakt sježeg lišća starijega oraha imao je najznačajnije toksično djelovanje, a hipokotili klijanaca obiju vrsta bili su osjetljiviji nego njihovo korijenje. Uočena je značajna korelacija između pH ekstrakta i duljine klijanaca, što ukazuje na oštećenje korijena vodikom, a posljedično je utjecalo na ukupni rast klijanaca. Vodeni ekstrakti smjese tla i razgrađenog orahova lišća imali su promotivni učinak na klijanje i rast klijanaca ječma i kukuruza, što pokazuje kako bi pravilno gospodarenje otpalim lišćem oraha moglo poboljšati uspješnost konsocijacijskih sustava oraha i ratarskih kultura.

Ključne riječi: konsocijacija, alelopatija, orah, ječam, kukuruz

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