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Allelopathic effects of leachates of *Juglans regia* L., *Populus tremula* L. and juglone on germination of temperate zone cultivated medicinal and aromatic plants

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Abstract The environmental benefits of agroforestry systems are well known. However, current knowledge of potential allelopathic interactions is inadequate. The decrease in soil fertility, the increasingly rhapsodic distribution of precipitation, and the special metabolism and cultivation of medicinal and aromatic plants are all harbingers of medicinal-agroforestry systems. The authors aimed to discover the allelopathic effects of *Juglans regia* L. and *Populus tremula* L. on germination of medicinal and aromatic plants cultivated in a temperate zone. Accordingly, an in vitro germination trial was conducted with leachates of these trees and two juglone concentrations. These allelopathic effects were evaluated for germination vigour, germination rate, and total fresh weight of seedlings of twelve different species. A pronounced species specificity was observed in tolerance of seeds and seedlings to the allelopathic effect of *Populus* and *Juglans*. In four of the species studied, the allelopathic effect may inhibit germination, but only initially. Poppy and angelica proved to be the most sensitive to the treatments. The following species had relative tolerance to the allelochemicals, so further research under natural conditions is suggested for: *Althea officinalis* L. (9.34 ± 5.04–68.66 ± 13.62 GR%), *Anethum graveolens* L. (12.00 ± 2.00–100.00 ± 6.12 GR%), *Cannabis sativa* L. (72.66 ± 9.02–91.34 ± 1.16 GR%), *Dracocephalum moldavica* L. (38.00 ± 2.00–80.00 ± 17.44 GR%), *Linum usitatissimum* L. (44.66 ± 2.00–58.00 ± 3.46 GR%), and *Satureja hortensis* L. (52.00 ± 28.22–82.00 ± 8.00 GR%). The aim would be to introduce them into agroforestry systems.

Keywords Tree–crop interactions · Screening · Bioassay · Maps · Medicinal-agroforestry

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Introduction

The Fourth Agricultural Revolution is currently thriving. It is characterized by two major trends: robotics and digitalisation, alongside immersion in traditions and patterns of nature (Gyuricza and Borovics 2018). There is a regenerative progression in agriculture, including the emergence of

Agroforestry Systems (AFS). The future of land use is facing many pressing challenges across the globe, challenges which can be alleviated by the widespread use of AFS (Garrity 2012).

The utilization of Medicinal and Aromatic Plants (MAPs) in the pharmaceutical, food and cosmetics industries is growing constantly, according to international surveys (Bernáth 2013). Meanwhile, eighty per cent of the world population uses herbal medicine for primary health care (Atmakuri and Dathi 2010). Silvoarable AFS, especially the alley-cropping type, is the most noteworthy for large-scale, eco-friendly production of MAP species in temperate zones.

Combining trees with crops raises new design considerations, because woody tree species influence the intercrops. Trees also modify their biochemical environment to ensure their own favourable growth. The key to the success of AFS is minimizing adverse interspecific interactions (competition, amensalism) and maximizing beneficial niche separations (Jose et al. 2004; Batish et al. 2008). Very limited scientific information is available on the effects of trees on the cultivation of MAPs. These effects include competition for water and nutrients, shade tolerance, and allelopathy (Jose and Gillespie 1998a, b; Gillespie et al. 2000).

Reisner et al. (2007) analysed the target regions for silvoarable agroforestry in Europe, based on the potential of those tree species from which high value products are made. Accordingly, there are hundred thousand square kilometres of arable land in Europe where the cultivation of *Populus* and *Juglans* species may be economically and ecologically beneficial. This represents more than forty per cent of the total arable land area of Europe (Reisner et al. 2007). MAPs cultivation in traditional walnut orchards in the AGFORWARD Project demonstrated a clear need for innovation and good techniques. On the other hand, the increase in poplar production through agroforestry systems is manifest in many countries worldwide. (Wani and Malik 2014; Burgess and Rosati 2019). In North America, it is thought that producing food and medicine in AFS is necessary to regenerate the environment and ensure food security (Osentowski 2017). Thus, both *Juglans* and *Populus* species are high priority trees in AFS. At the same time, there is an increasing need for MAP products from renewable resources, where an agroforestry system could serve.

Allelopathy is a natural phenomenon. It means one taxon is inhibited or destroyed whilst the other is unaffected through special metabolites triggered into the environment. This biochemical and ecophysiological interaction, executed by allelochemicals, is common both in both nature and in agroecosystems. Seen as a biological interaction it is a form of amensalism (Scavo et al. 2018). Allelochemicals are thoroughly soluble in water from the donor taxon tissues. This is how they are transported to the living tissue of the acceptor taxon. They typically have a developmental-physiological effect on the target organism (Szabó 2016). Allelochemicals are present in various concentrations in many plant parts, and they are released into the environment in several ways (decomposition of residues, volatilization, washout and root exudation). Seed size and permeability of the integument strongly influence the inhibitor effect in germination (Kruse et al. 2000).

Juglone (5-hydroxy-1,4-naphthoquinone) is a unique allelopathic metabolite of the *Juglandaceae* family. It occurs together with many other metabolites with potential allelopathic effect (phenolic acids, flavonoids and terpenes), (Willis 2000; Frohne and Pfander 2005; Nour et al. 2012). The concentration of juglone is influenced by the season, the plant part and its location in the tree. All the leaves, roots and husks of walnut contain juglone in different concentrations (Jose 2002). Experiments on this subject have given contradictory results with regard to the juglone content during the vegetation period. No clear trend can be established (Coder 1983; Cosmolescu et al. 2011, 2014; Strugstad and Despotovsky 2012). Juglone may occur in the soil of a walnut plantation in concentrations ranging from 10^{-4} to 10^{-6} M, depending on walnut species and season, distance from the tree, and many other biotic and abiotic factors (Jose and Gillespie 1998a, b; Thevathasan et al. 1998; Terzi 2008). The bacterium *Pseudomonas putida* J1 utilizes the carbon content of juglone to generate energy. Accordingly juglone decomposes into 3-hydroxy-juglone, but further research is needed to understand the bacterium's detoxifying role (Rettenmaier et al. 1983; Schmidt 1988; Williamson and Weidenhamer 1990).

Populus trees synthesize compounds typically derived from the shikimate-phenyl-propanoid pathway (phenol glycosides, hydroxy-cinnamates, flavonoids and condensed tannins). However, terpenoids and fatty acids are also present in considerable concentrations (Chen et al. 2009). The most likely responsible compounds are the phenolic acids. They decompose from decaying leaves in the following concentrations: (60.60–132.54 mg/100 g) to soil (6.19–29.33 mg/100 g). This may be significant (Singh et al. 2001). Previous studies highlight the allelopathic potential of *Populus* species on different MAP species (Melkania 1984; Raj et al. 2010; Chauhan et al. 2013).

There is hardly any scientific information regarding the inhibitory effect of *Juglans/Populus* species' allelochemicals on the germination of temperate zone MAPs. Much of the evidence on the implementation of MAPs to AF systems relates to studies in tropical and Far Eastern countries and their environmental conditions (Rao et al. 2004). It is rather challenging to apply all this knowledge to temperate zone circumstances.

The objective of this work is to take the first step in revealing the species-specific inhibitory effect of *Populus/Juglans* allelochemicals on the germination of MAPs cultivated in temperate zones. This study was done by means of a screening type laboratory bioassay.

Materials and methods

Collection of plant material and preparation of aqueous extracts

The study was conducted at the laboratory of the Department of Medicinal and Aromatic Plants, Institute of Sustainable Horticulture, Szent István University (SZIU), Budapest, Hungary. All plant materials of *Juglans* and *Populus* were collected from the Experimental and Research Farm of the Faculty. Leaves of 10 different thirty-year-old walnut trees (*Juglans regia* L.) were collected. Also, leaves of 10 poplar trees (*Populus tremula* L.) were collected from eight to ten-year-old trees. The leaves of both species were picked in August, 2018 as juglone content of walnut leaves is highest in August (Strugstad and Despotovski 2012). Mature leaves were collected from the lower branches of the trees. Leaves were dried in the shade and crushed into a fine powder with a hammer mill. Ground leaf powder was refrigerated in an airtight plastic bag before use.

The treatments were as follows: *Populus tremula* leaf aqueous extract, *Juglans regia* leaf aqueous extract, juglone (Sigma-Aldrich) solution 10^{-3} M, juglone solution 10^{-4} M and distilled water as control.

The 10^{-3} M and 10^{-4} M juglone solutions were prepared with 300 ml distilled water. The mixture was placed in an Erlenmeyer flask and submitted to ultrasonic bath for 30 min. Subsequently it was stirred continuously for 24 h.

Both leaf extracts were prepared by adding 30 g of ground leaf powder to 350 ml of distilled water. Then the ingredients were mixed and placed in an ultrasonic bath for two hours, and filtered with ten to twelve µ of filter paper. Thereafter the solution was topped up with 300 ml with distilled water and mixed. (Kocacaliskan and Terzi 2001; Ercisli et al. 2005).

Tested seeds

We examined the germination and seedling growth of 10 temperate zone cultivated MAP species (Table 1). All accessions originated from certified seeds except for flax and poppy, where gene bank material of the Department of Medicinal and Aromatic Plants (SZIU) was used.

Quantification of allelochemicals in the leaves

To confirm presence of juglone in *Juglandis folium* samples, we applied the TLC method stated in the VII. Hungarian Pharmacopoeia (Pharmacopoeia Hungarica 1986). The test showed a positive result, so the leaves contained juglone. Approximate juglone concentration value for leaves of *Juglans regia* based on literature data is 5.42 to 22.82 mg/100 g (Cosmolescu et al. 2011). To determine total phenolic content in poplar leaf samples, we used the Singleton and Rossi (1965) modified method (Szabó et al. 2016). Mean total polyphenol content of the plant material was 356.22 ± 6.65 mg GAE/g d.w.

Bioassay for seed germination

The investigation was carried out in a completely randomized arrangement, with four treatments and one control group in three replicates. Each replicate contained 50 seedlings. Seeds were placed in nine-

Table 1 The MAP species which were investigated, and their origins

Scientific name	Common name	Origin
Althaea officinalis L.	Marsh mallow	Jelitto Staudensamen GmbH
Anethum graveolens L.	Dill	Hermes ÁFÉSZ
Angelica archangelica L.	Angelica	Jelitto Staudensamen GmbH
Cannabis sativa L.	Hemp	Tetragenom Ltd
Carum carvi L.	Caraway	Hermes ÁFÉSZ
Centaurium erythraea Rafn.	Centaury	Jelitto Staudensamen GmbH
Dracocephalum moldavica L.	Moldavian dragonhead	Jelitto Staudensamen GmbH
Levisticum officinale Koch.	Lovage	Jelitto Staudensamen GmbH
Linum usitatissimum L.	Flax	Genebank SZIU Department of Medicinal and Aromatic Plants
Papaver somniferum L.	Poppy	Genebank SZIU Department of Medicinal and Aromatic Plants
Satureja hortensis L.	Savory	Hermes ÁFÉSZ
Sinapis alba L.	Mustard	Kertimag Romania S.R.L

centimetre Petri dishes containing two layers of filter paper. Ten ml of extract, juglone solution, or distilled water was put into each Petri dish. Due to the small seed size, *Centaurium erythraea* was only administered in four ml of each treatment type. The Petri dishes were placed in a climate chamber (Sanyo MLR 351 H) set to 10 h/23 °C for the light period and 14 h/20 °C for the dark period. Seeds were considered as germinated when they developed a radicle and shoot extension longer than 2 mm. Germinated and healthy seeds were counted two times (Table 2), one time during and one time at the end of the experiment. The fresh weight of ten average, randomly selected seedlings of each replication/treatment was measured on laboratory scales (Kern, ALJ 220-4NM) on the second inspection day of the experiment. Where less than ten seeds germinated, we took the mean of all the germinated seeds, and hereinafter this value was used. Every adjustment of the germination experiments was set according to Hungarian standards for seed testing (MSZ 6354–3:2008). The germination rate—GR (%)—was calculated using the following formula:

$$GR(\%) = \left(\frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds}\right) x100$$

The determination of the GR (%) was calculated based on the data of the final inspection day. The germination vigour—GV (%)—is the ratio of intact, normally developed seedlings on the first inspection day, determined by the standard.

Table 2 Inspection days for counting of germinated seeds from the start of the experiment

Species	Day of the 1st inspection	Day of the final inspection
Althea officinalis L.	6	14
Anethum graveolens L.	7	21
Angelica archangelica L.	10	21
Cannabis sativa L.	3	14
Carum carvi L.	7	13
Centaurium erythraea L.	6	21
Dracocephalum moldavica L.	10	14
Levisticum officinale Koch.	10	16
Linum usitatissimum L.	3	7
Papaver somniferum L.	5	10
Satureja hortensis L.	6	13
Sinapis alba L.	3	14

Statistical analyses

Repeated measurements with ANOVA models were used to evaluate statistical differences between germination data of different treatments. A treatment was excluded from the model of a species if no germination was observed in a minimum of two out of three replicates, or if no germination was observed in one replicate and only one seed germinated in each of the other two. In species where interaction between treatment and time was significant, data were displayed graphically. This research is exploratory in nature. That is why the univariate one-way ANOVA models were evaluated for all species on both sampling dates. Post hoc analysis was by Tukey's HSD or Games-Howell's test, depending—as in many other studies—on the homogeneity of variances, which was tested by Levene's method. We also evaluated differences in mean weight of 1 seedling per treatment. For this, we used univariate one-way ANOVA models for 10 species, based on the average weight data of the seedlings per replicates. Poppy and centaury seedlings did not have measurable weight. In these cases as well, Tukey's HSD or Games-Howell's method were used as post hoc tests. Results were considered significant if $p < 0.05$. All the statistical analyses were performed with IBM SPSS Statistics 25.

Results and discussion

Althea officinalis L.

The juglone 10^{-4} M solution treatment was not effective, whilst the juglone 10^{-3} M solution treatment had a drastic allelopathic effect on both GV and GR (Table 3). *Populus* and *Juglans* leaf extract treatments showed significant depression on GV; ultimately the effect of leaf extract treatments was not significant on GR (Table 3). Seedling weight was significantly reduced by both of the extract treatments and none of the juglone treatments (Table 4). We suggest that *Althea officinalis* be further studied for allelopathy and agroforestry research.

Anethum graveolens L.

The GV of dill was significantly reduced by both leaf extracts but finally there was no significant reduction of GR. While there was no significant difference for the juglone 10^{-4} M solution either on GV or GR, the juglone 10^{-3} M treatment drastically reduced both GV and GR (Table 3). The leaf extract treatments did not influence seedling weight. Based on the results, *Anethum graveolens* L. is recommended for further experiments, especially for poplar-based AFS research. The leaf extracts neither had negative effect on GR nor on seedling weights. Furthermore, the puffer effect of soil through changes of soil microbial communities may further mitigate initial allelopathic effects. Thus, dill might prove viability in combination with the candidate tree species (Yang-Ping et al. 2017).

Angelica archangelica L.

No seed germinated after the first inspection day, therefore GV can not be evaluated. On GR, juglone treatments were not significantly different from the control; however, juglone 10^{-3} M solution caused relevant reduction (19.32%). *Populus* extract, as well as *Juglans* extract, had a drastic effect on GR (Table 3). Both of them significantly reduced the weight of the seedlings (Table 4). Based on these results, angelica might not be suitable to combine with poplar or walnut.

Cannabis sativa L.

None of the treatments had any significant effect on either germination or seedling growth. However, we point out a slight stimulatory effect of juglone treatments on both GV and GR (Tables 3 and 4). There was no negative impact of the studied allelochemichals on germination. Seeds germinated intensively in spite of the presence of allelochemicals. *Cannabis sativa* is reported to be suitable for intercropping with poplar. Furthermore, shaded conditions may promote fiber production (Jha and Gupta 1991; Zubay et al. 2020). For all these reasons, hemp is highly recommended for further AFS research and application.

Carum carvi L.

Initially, both the juglone 10^{-3} M solution and *Populus* extract inhibited GV of caraway seeds more effectively than, the *Juglans* extract. Considering GR,

Table 3 Means and standard deviations of germination vigour (GV, %) and germination rate (GR, %) of the investigated MAP species influenced by different allelochemicals

MAP species	Parameters (%)	Treatment				
		Populus extract	*Juglans* extract	Juglone 10^{-4} solution	Juglone 10^{-3} solution	Control
Althea officinalis	GV	$16.00^{ab} \pm 5.30$	$17.34^{b} \pm 3.06$	$46.66^{c} \pm 7.02$	$4.00^{a} \pm 2.00$	$52.66^{c} \pm 3.06$
	GR	$47.34^{b} \pm 15.14$	$47.34^{b} \pm 1.16$	$52.00^{b} \pm 11.14$	$9.34^{a} \pm 5.04$	$68.66^{b} \pm 13.62$
Anetheum graveolens	GV	$30.66^{b} \pm 12.06$	$26.66^{b} \pm 4.16$	$78.66^{c} \pm 1.16$	$6.66^{a} \pm 3.06$	$91.34^{c} \pm 7.58$
	GR*	$86.66^{ab} \pm 19.74$	$76.00^{ab} \pm 15.1$	$99.34^{b} \pm 1.16$	$12.00^{a} \pm 2.00$	$100.00^{b} \pm 6.12$
Angelica archangelica	GV	ng	ng	ng	ng	ng
	GR	$17.34^{a} \pm 5.78$	$22.66^{a} \pm 11.54$	$52.66^{b} \pm 11.72$	$41.34^{ab} \pm 10.06$	$60.66^{b} \pm 10.26$
Cannabis sativa	GV	$50.00^{ns} \pm 21.64$	$63.34^{ns} \pm 14.04$	$84.00^{ns} \pm 8.72$	$86.00^{ns} \pm 6.92$	$69.34^{ns} \pm 23.00$
	GR	$72.66^{ns} \pm 9.02$	$85.34^{ns} \pm 4.16$	$90.00^{ns} \pm 6.92$	$91.34^{ns} \pm 1.16$	$82.66^{ns} \pm 12.06$
Carum carvi	GV	$12.66^{ab} \pm 3.06$	$25.34^{b} \pm 5.04$	$70.66^{c} \pm 11.02$	$9.34^{a} \pm 3.06$	$81.34^{c} \pm 2.30$
	GR*	$32.00^{a} \pm 0.00$	$60.66^{b} \pm 2.30$	$83.34^{bc} \pm 9.86$	$24.00^{a} \pm 4.00$	$87.34^{c} \pm 4.16$
Centaurium erythraea	GV*	$12.66^{abc} \pm 15.14$	$4.00^{a} \pm 3.46$	$56.66^{bc} \pm 12.86$	$6.66^{ab} \pm 2.30$	$54.66^{c} \pm 9.46$
	GR	$22.00^{a} \pm 16.38$	$16.66^{a} \pm 2.30$	$82.00^{b} \pm 10.58$	$37.34^{a} \pm 8.32$	$82.00^{b} \pm 9.16$
Dracocephalum moldavica	GV*	$46.00^{a} \pm 3.46$	$34.66^{a} \pm 1.16$	$72.00^{ab} \pm 14.00$	$69.34^{ab} \pm 9.86$	$71.34^{b} \pm 4.62$
	GR*	$48.66^{ns} \pm 5.06$	$38.00^{ns} \pm 2.00$	$80.00^{ns} \pm 17.44$	$56.66^{ns} \pm 20.42$	$66.66^{ns} \pm 11.38$
Levisticum officinale	GV	$17.34^{a} \pm 2.30$	$11.34^{a} \pm 2.30$	$48.00^{b} \pm 8.72$	$41.34^{b} \pm 8.32$	$58.00^{b} \pm 9.16$
	GR	$32.66^{ab} \pm 12.86$	$27.34^{a} \pm 6.42$	$59.34^{bc} \pm 8.08$	$52.00^{abc} \pm 13.12$	$61.34^{c} \pm 8.32$
Linum usitatissimum	GV	$19.34^{a} \pm 3.06$	$16.66^{a} \pm 7.02$	$48.66^{b} \pm 4.62$	$44.66^{b} \pm 14.46$	$47.34^{b} \pm 8.08$
	GR*	$46.66^{a} \pm 1.16$	$48.00^{a} \pm 0.00$	$58.00^{b} \pm 2.00$	$44.66^{ab} \pm 2.00$	$58.00^{ab} \pm 3.46$
Papaver somniferum	GV*	ng^{b}	ng^{b}	$67.34^{a} \pm 20.52$	ng^{b}	$97.34^{a} \pm 1.16$
	GR*	ng^{b}	ng^{b}	$86.66^{a} \pm 1,.16$	ng^{b}	$92.66^{a} \pm 11.02$
Satureja hortensis	GV	$37.34^{a} \pm 13.32$	$60.00^{ab} \pm 5.30$	$74.66^{b} \pm 5.04$	$62.00^{ab} \pm 14.42$	$57.34^{ab} \pm 6.42$
	GR	$52.00^{ns} \pm 28.22$	$73.34^{ns} \pm 9.02$	$81.34^{ns} \pm 5.04$	$68.00^{ns} \pm 8.00$	$82.00^{ns} \pm 8.00$
Sinapis alba	GV*	ng^{b}	ng^{b}	$85.34^{a} \pm 4.62$	$73.34^{a} \pm 7.02$	$80.66^{a} \pm 1.16$
	GR	$31.34^{a} \pm 5.78$	$17.34^{a} \pm 6.12$	$85.34^{b} \pm 4.62$	$73.34^{b} \pm 16.16$	$72.00^{b} \pm 19.08$

Means with the same letter are not significantly different from each other. (Tukey's and Games-Howell's $p < 0.05$)

ng not germinated, *ns* not significant

*Games-Howell's post-hoc test was used

although the juglone 10^{-3} M solution and *Populus* extract treatments had strongest inhibitory effect, the *Juglans* extract treatment also differed significantly from the control (Table 3). On the other hand, inhibitive effect on seedling development was strongest in the leaf extract treatments, followed by the two juglone solutions (Table 4). According to additional data published on *Carum carvi,* using it in AFS could influence the quality of the *Carvi fructus* drug. The essential oil content can be reduced by strong wind during ripening of the fruits. In AFS, however, wind erosion can be mitigated by alleys and shelterbelts (Böhm et al. 2014; Brandle et al. 2004). The growth inhibitory effect of the tested allelochemicals is definite, however further research on cultivation possibilities with other multipurpose tree species is approved.

Centaurium erythraea Rafn

Compared to the other treatments, both *Juglans* extract and the juglone 10^{-3} M solution treatments showed the largest decreases in GV. *Populus* extract treatment caused a less pronounced reduction of GV, whilst the

Table 4 Means and standard deviations of avarage weight of the germinated seedlings (mg/seedling) of the investigated MAP species influenced by different allelochemicals

MAP species	Treatment				
	Populus extract	*Juglans* extract	Juglone 10^{-4}	Juglone 10^{-3}	Control
*Althea officinalis**	$0.0097^{a} \pm 0.0009$	$0.0104^{ab} \pm 0.0007$	$0.0144^{bc} \pm 0.0013$	$0.0173^{abc} \pm 0.0031$	$0.0158^{c} \pm 0.0015$
Anetheum graveolens	$0.0070^{ab} \pm 0.0015$	$0.0063^{ab} \pm 0.0010$	$0.0094^{b} \pm 0.0010$	$0.0043^{a} \pm 0.0035$	$0.0085^{ab} \pm 0.0005$
Angelica archangelica	$0.0189^{a} \pm 0.0019$	$0.0194^{a} \pm 0.0042$	$0.0236^{ab} \pm 0.0016$	$0.0239^{ab} \pm 0.0018$	$0.0261^{b} \pm 0.0010$
Cannabis sativa	$0.0461^{ns} \pm 0.0079$	$0.0568^{ns} \pm 0.0083$	$0.0522^{ns} \pm 0.0044$	$0.0495^{ns} \pm 0.0124$	$0.0572^{ns} \pm 0.0154$
*Carum carvi**	$0.0073^{a} \pm 0.0007$	$0.0098^{a} \pm 0.0004$	$0.0111^{b} \pm 0.0006$	$0.0120^{ab} \pm 0.0023$	$0.0130^{b} \pm 0.0004$
*Dracocephalum moldavica**	$0.0080^{ns} \pm 0.0006$	$0.0108^{ns} \pm 0.0020$	$0.0140^{ns} \pm 0.0039$	$0.0096^{ns} \pm 0.0008$	$0.0168^{ns} \pm 0.0031$
Levisticum officinale	$0.0141^{ab} \pm 0.0028$	$0.0119^{a} \pm 0.0040$	$0.0154^{ab} \pm 0.0024$	$0.0104^{a} \pm 0.0009$	$0.0199^{b} \pm 0.0016$
Linum usitatissimum	$0.0195^{ns} \pm 0.0019$	$0.0219^{ns} \pm 0.0066$	$0.0296^{ns} \pm 0.0036$	$0.0196^{ns} \pm 0.0025$	$0.0266^{ns} \pm 0.0080$
Satureja hortensis	$0.0042^{ns} \pm 0.0024$	$0.0052^{ns} \pm 0.0003$	$0.0071^{ns} \pm 0.0001$	$0.0072^{ns} \pm 0.0013$	$0.0070^{ns} \pm 0.0005$
*Sinapis alba**	$0.0213^{a} \pm 0.0016$	$0.0181^{a} \pm 0.0045$	$0.0358^{abc} \pm 0.0117$	$0.0350^{b} \pm 0.0034$	$0.0487^{c} \pm 0.0010$

Means with the same letter are not significantly different from each other (Tukey's and Games-Howell's $p < 0.05$)

ns not significant

*Games-Howell's post-hoc test was used

juglone 10^{-4} M solution treatment did not differ from the control. After fifteen days of administering the potential allelopathic agents, the GR of centaury was significantly reduced by all of the treatments except the juglone 10^{-4} M solution. The leaf extracts, especially *Juglans* extract, caused the most conspicuous inhibitor effect on GR (Table 3). The seedlings were too small to get reliable data on their weight. *Centaurium* is a species whose cultivation technology remains under development, but its natural habitat—ranging from mostly sparse, open, sunny fields to semi-shaded forests and meadows—indicates the potential for agroforestry cultivation. Present results reveal that germination is drastically inhibited by allelopathy. Therefor the species is not recommended for direct sowing into poplar and walnut alleys, although seedling technology can modify the manifestation of allelopathy in future tests.

Dracocephalum moldavica L.

At the beginning, leaf extract treatments caused a relevant depression of GV. But at the end, no significant difference was found either on GR or on the weight of the seedlings (Tables 3 and 4). Although no significant effect was statistically justified, the differences are considerable. Therefore, further allelopathic and AF research is definitely needed for this species.

Levisticum officinale Koch

Populus and *Juglans* extract treatments had severe negative effects on both GV and GR of lovage. The inhibitory effect of leaf extracts was significant compared to control, however that of the juglone treatments was not. (Table 3). All treatments caused some degree of reduction regarding weight development of the seedlings, yet only *Juglans* extract and juglone 10^{-3} M treatments proved to be significant (Table 4). Nevertheless, lovage is a semi shade-tolerant, root-drug providing medicinal plant. That is the main reason to tag it for agroforestry utilization (Chauhan et al. 2013). We suggest setting up a field experiment to investigate whether the inhibitory effect of allelochemicals of the candidate tree species would appear under field conditions, especially in the case of propagation via seedlings.

Linum usitatissimum L.

Populus and *Juglans* extract treatments significantly reduced GV, but did not have serious inhibitory effect on GR because of the good recovery of the seedlings.

None of the juglone treatments had any effect on germination (Table 3). There was no significant difference in seedling weight (Table 4). Overall, flaxseed tolerates allelopathic effects well, a fact which may be related to the larger seed size and possibly less permeable integument compared to the other species studied. Two things cause significant yield losses and lower oil content of flax: the phenomenon of stresses at flowering time and during seed filling (Cloutier 2016). This indicates the relevance of further research and small-scale experimental cultivation of *Linum usitatissimum* in alley-cropping systems.

Papaver somniferum L.

The seeds of poppy did not germinate for juglone 10^{-3} M, *Populus* and *Juglans* extract treatments. There was no significant difference for juglone 10^{-4} M compared to the control, either on GV or on GR. The poppy seedlings were too small for reliable data on their weight. *Papaver somniferum* is not recommended for further research on poplar- or walnut-based AFS cultivation.

Satureja hortensis L.

On GV, the *Populus* extract treatment differed from the juglone 10^{-4} solution treatment, whilst none of the treatments differed from the control. No significant effect was registered on GR or on weight development (Tables 3 and 4). The germination bioassay did not show any negative influence on early development and germination of savory. Thus, this species is highly recommended for further AFS research.

Sinapis alba L.

Both GV and GR of mustard seed was reduced significantly by extract treatments of *Juglans* and *Populus*, whilst none of the juglone treatments had a significant impact (Table 3). Seedling weight was lowered considerably by the juglone 10^{-3} M solution treatment, while the effect of the dilutes was less pronounced. The *Populus* and *Juglans* extracts had a reduction impact one order of magnitude stronger than the juglone treatments (Table 4). Based on the present results, mustard is not recommended for poplar- and walnut-based agroforestry systems.

Bioassays are kick-off methods in allelopathic research to measure the response of germinating seeds and seedlings to the activity of allelochamicals. Along with this, germination bioassays are fraught with some criticisms of absence of soil testing and ignoring the role of microorganisms in the process (John et al. 2006). On the other hand, bioassays are not affected by the complex network of soil food web nor by other biotic and abiotic factors playing part simultaneously in open field conditions, since the purpose of them is the isolation of only one factor to be examined. During the discussion, we sought to ensure that the results were broadly interpretable to the scientific agroforestry knowledge base, while not ignoring the fact that the results of future's field experiments may specify and differentiate the present results. The present in vitro investigations are the first step for screening the most promising species in order to set up field experiments and finally establish medicinal-agroforestry systems.

In the four species we investigated, we found a significant interaction between treatment and duration. The emergence of seedlings during the experiment showed very distinct trends, depending on the treatment types. For the following three species, both *Populus* and *Juglans* extracts showed a significantly lower allelopathic effect as the treatment progressed: *Althea officinalis* ($p = 0.005$), *Anetheum graveolens* ($p = 0.002$) and *Linum usitatissimum* ($p = 0.006$) (Figs. 1, 2 and 3). In the case of *Carum carvi,* the same tendency was very considerable ($p = 0.000$). It means that the seeds germinated more intensively over time (Fig. 4). Consequently, the allelopathic effect is likely to cause only initial inhibition of germination for many species. Further inhibitory effect may be reduced with upgraded soil conditions, increased soil organic carbon, soil nutrient concentration and the high soil microbial activity typical of AF systems (De Stefano and Jacobson 2018; Udawatta et al. 2008; Pardon et al. 2019; Kaur et al. 2009).

Low concentrations of juglone could promote plant growth (Chou 1995), whilst juglone 10^{-3} M—as the negative control in this experiment—is not considered to be a concentration that occurs in field conditions and reportedly causes developmental disorders in seedlings of many species (Rietveld 1983). The seeds of five species: *Althea officinalis*, *Anetheum graveolens*, *Carum carvi*, *Centaurium erythraea,* and *Papaver somniferum;* reacted quite differently to the lower and

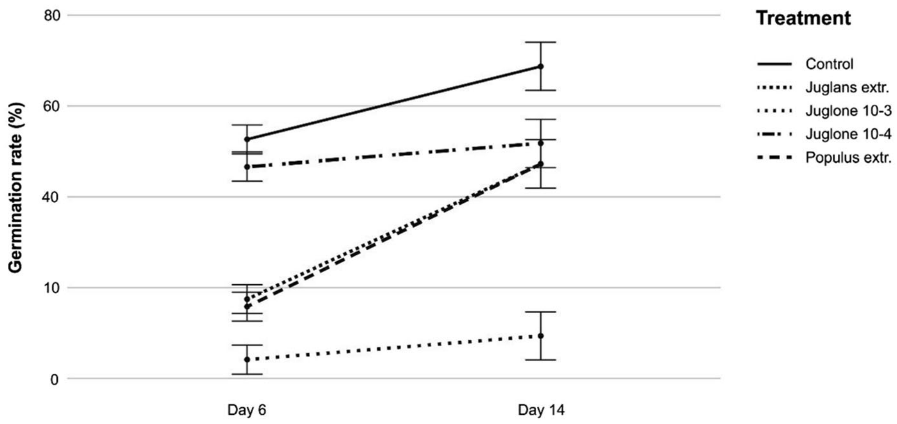

Fig. 1 Interaction between treatment and time on germination of *Althea officinalis* L. (p = 0.005)

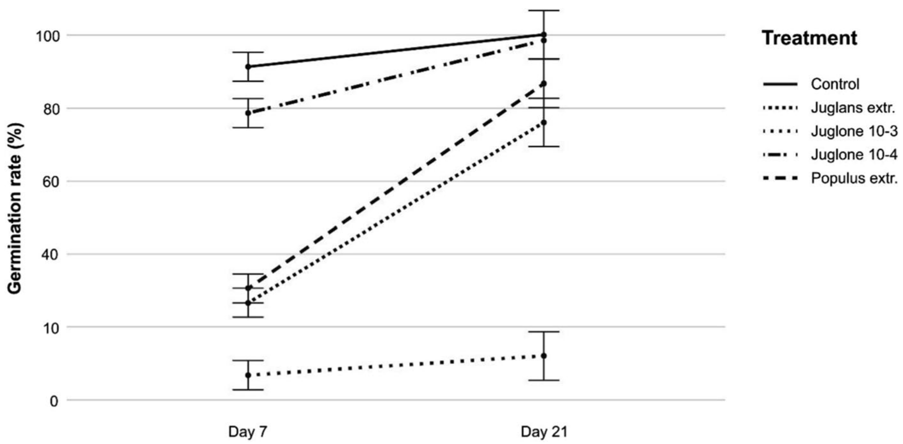

Fig. 2 Interaction between treatment and germination time of *Anetheum graveolens* L. (p = 0.002)

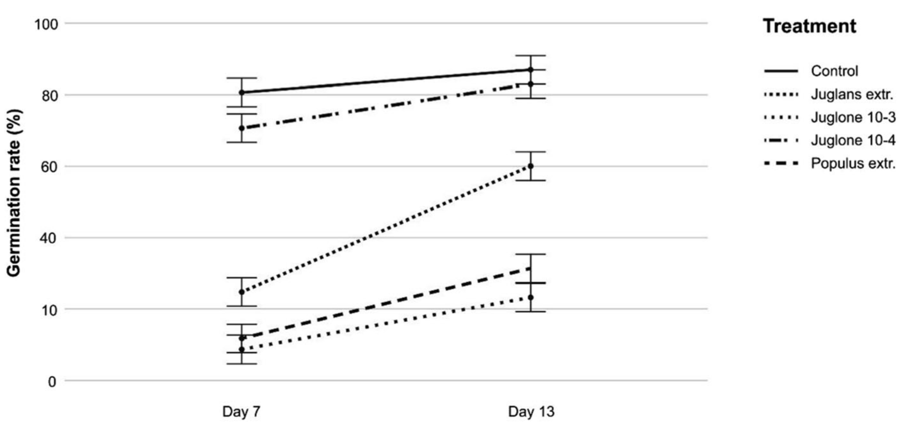

Fig. 3 Interaction between treatment and germination time of *Linum usitatissimum L.* (p = 0.006)

higher concentrations of juglone solutions. In all of these cases, the stronger concentration resulted in poorer germination vigour and rate. For *Cannabis sativa*, *Dracocephalum moldavica* and *Sinapis alba,*

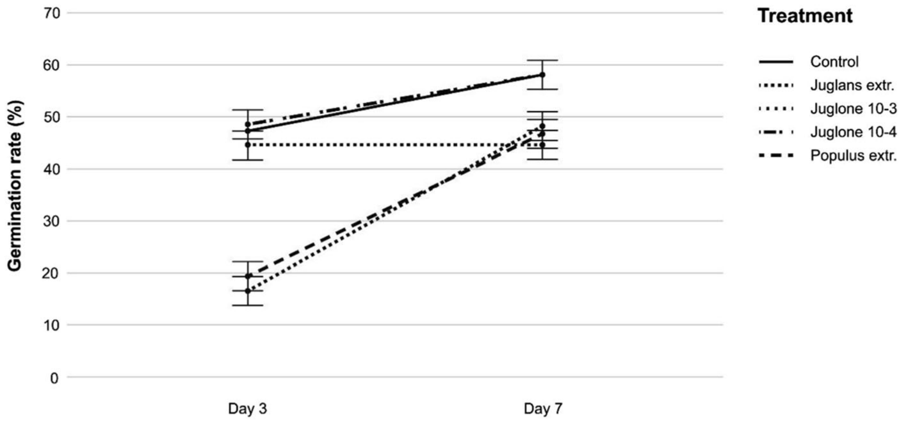

Fig. 4 Interaction between treatment and germination time of *Carum carvi* L. (p = 0.000)

the juglone 10^{-4} concentration treatment seemed to have a slight stimulatory effect on both GV and GR. In the cases of *Angelica archangelica*, *Centaurium erythraea*, *Levisticum officinale,* and *Sinapis alba,* we detected a more drastic effect on the germination with the *Juglans* extract treatment than with the juglone 10^{-3} solution alone. This incident may confirm the preconception that there are other biologically active compounds with allelopathic activity in *Juglandis folium,* and they may have a synergistic impact on each other beyond juglone. Additionally, for *Cannabis sativa,* higher germination parameters were triggered by all juglone solution treatments rather than by the *Juglans* extract. The results showed that in some cases, both leaf extracts had a more depressive effect on germination than the juglone solution in any concentration. The depressive effect was fairly noticeable for *Angelica archangelica*, *Centaurium erythraea*, *Levisticum officinale* and significant for *Sinapis alba.*

We established that a pronounced species specificity can be observed in terms of tolerance of seeds and seedlings to the allelopathic effect of *Populus* and *Juglans* species. Previous researchers on other horticultural and crop species have also reported species specificity for tolerance to allelopathic effects. The results of germination bioassays show juglone tolerance for *Cucumis melo cv. Galia* and contradictory results for *Triticum aestivum.* Meanwhile, germination and growth inhibition were observed in *Cucumis sativus cv. Beith Alpha, Solanum lycopersicum, Fragaria* × *ananassa, Citrullus lanatus, Raphanus sativus* and *Medicago sativa.* (Terzi 2008; Ercisli et al. 2005; Kocacaliskan and Terzi 2001). Germination and field experiments led to the conclusion that the allelopathic effect of *Populus* species was tolerated by *Melissa officinalis. Cicer arietinum* was inhibited, whilst experiments with *Triticum aestivum* gave mixed results (Melkania 1984; Raj et al. 2010; Majeed et al. 2017).

The data resulting from this experiment may provide an important basis for further pot culture studies and field experiments. The goal is to base the growth of MAPs in temperate-zone agroforestry systems on the most favorable interactions.

Conclusions

The present in vitro investigations are the first step for screening the most promising species in order to establish a sustainable MAP AF system. Open field and pot experiments should further clarify the response of these species and their interactions with the trees. The in vitro germination trial of twelve MAP species proved that their reaction to the allelopathic effect of *Juglans regia* and *Populus tremula* is definitely species-specific. Germination vigour, germination rate and seedling phytomass production data showed highly significant differences among different allelochemicals in certain species. Likewise the germination and development of other species was not influenced at all by any of the allelopathic effects. Based on our results, the following species are recommended for allelopathy and agroforestry research in combination with the target tree species:

Althea officinalis, *Anethum graveolens*, *Cannabis sativa*, *Dracocephalum moldavica*, *Linum usitatissimum*, and *Satureja hortensis*. We recommend them especially for further pot culture studies and field experiments. Germination results of four species add strength to the idea that there might be several other biomolecules in *Juglandis folium* besides juglone that have an active and synergistic allelopathic effect. The study showed that despite the allelopathic effects inhibiting the germination of certain temperate zone cultivated MAP species, germination of many other MAPs in a walnut/poplar-based AFS could be steady.

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