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SCIENTIFIC ESTABLISHMENT OF THE CULTIVATION OF MEDICINAL AND AROMATIC PLANTS IN AGROFORESTRY SYSTEM

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1. INTRODUCTION AND OBJECTIVES

Over the past half-century, the startling symbiosis of both the empty promises at world climate summits and sustainability conferences and the unprecedented destruction of the natural living environment that surrounds and nurtures our societies has been reaching staggering heights. The resultant of the perceived destruction is the monoculture-based agriculture aimed solely at profit-maximization, the operation of which may be used as an indicator of the severity of the contrast between capital and environment. Phenomena (such as heavy rainfall in Southern Europe, severe drought and wildfire in Eastern Europe) resulting from the state of the degraded environment afflicted by negative externalities, along with the COVID-19 crisis cumulatively, seem to have consequences regarding the minor segment of European herbs & spice industry within the agricultural sector, such as the collapse of the supply chain or the case of raw material shortages (ANONYMUS 2021).

The time for sustainability is over within the era of climate change, as the condition is no longer to be maintained as a result of both our natural environments and agroecological systems needing regeneration instead. Agroforestry systems (AFS) are the reorganizations of an old-but-new concept where agriculture and forestry are combined on the same land (NERLICH et al., 2013; MOSQUERA-LOSADA et al., 2018). AFS are subsidized more and more, thus they may be one of the flagships of the regeneration process owing to their advantageous effects. For example: maintaining ecosystem-services and biodiversity, sequestering carbon, conservation of soil, preserving landscape and cultural heritage, supporting soil food webs, pollination and biological control, and improving competitiveness of farmers (SMITH et al., 2012; VAN ZANTEN et al., 2014; FAGERHOLM et al., 2016; TORRALBA et al., 2016; UDAWATTA et al., 2019; WILSON and LOVELL, 2016; MORENO et al., 2018). Plant production taking place within the AFS - due to numerous scientifically proven environmental benefits - represents the new paradigm, however, there is very little science-based information available on the potentially occurring interspecific interactions (allelopathy and shadow effect) within temperate medicinal agroforestry systems.

One fundamental task of medicinal agroforestry systems is to reveal the light intensity level requirement of different Medicinal and Aromatic Plants (MAPs), where the production of biomolecules can still be optimized according to pharmacopoeial expectations. Based on this knowledge, the most suitable species for production in medicinal-agroforestry systems could be determined. The shade effect is influenced by a wide variety of factors. They are: growth vigour, cutting cycle, vegetation length and cultivation technology of the tree species; the spacing and orientation of the AFS on one side, and the plant life-form and agrotechnical needs of the intercrop on the other side. In general, light may be a major limiting factor in AFS, however very limited scientific information is available on the relationship between shade tolerance and the accumulation of special metabolites of temperate zone MAPs.

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. 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 key to the utter success of medicinal and aromatic plant (MAP) production is the appropriate operation of plant production systems that supports both regeneration of agroecosystems and operational efficiency, as well as full compliance with quality requirements (drug quality, content and composition of active substances). The plant production of AFS seeks to achieve all these aspects collectively, however, the spread of AFS specialising in MAP's production is currently limited by the lack of advisory and modern decision support systems based on the theses proven by science under field conditions (SOLLEN-NORRLIN et al., 2020). The present PhD program intended to contribute to the professional establishment of medicinal agroforestry systems, was founded on the outright necessity of such an information base that is supportive of an agroforestry advisory & consulting system.

The scientific goal of the PhD program was to screen MAP species tolerant to allelochemicals of the targeted woody genuses (*Juglans/Populus*) frequently used within AFS, by performing *in vitro* pilot germination trials. Furthermore, to screen MAP species tolerant of reduced light conditions (30% and 50% artificial light reduction) occurring within AFS by performing small-scale experiments under open field conditions.

Based on the lack of professional knowledge presented in the introduction, we raised the following two main scientific questions:

- Are there any MAP species whose seeds are able to germinate properly - as recorded in the seed testing methods - in the presence of allelochemicals specific to the genus of *Juglans* and *Populus*?
- Are there any temperate zone cultivated MAP species the yield of which does not decrease even in low light conditions, and are able to produce drug and active ingredient that meet the pharmacopoeial quality?

Based on these scientific questions and in order to create a scientific background for medicinal agroforestry systems, we have set the following two research goals:

- Screening of the first group of MAP species tolerating the allelochemicals characteristic of the target woody genuses (*Juglans / Populus*) by performing *in vitro* pilot germination trials.
- Screening of the first group of MAP species tolerating reduced light conditions (30% and 50% artificial light reduction) occurs in AFS by performing three-year long small-scale field experiments.

2. MATERIALS AND METHODS

The test species of the allelopathy research were *Althaea officinalis* L.; *Anethum graveolens* L.; *Angelica archangelica* L.; *Cannabis sativa* L.; *Carum carvi* L.; *Centaurium erythraea* Rafn.; *Dracocephalum moldavica* L.; *Levisticum officinale* Koch.; *Linum usitatissimum* L.; *Papaver somniferum* L.; *Satureja hortensis* L. and *Sinapis alba* L. In the *in vitro* pilot germination trails, an aqueous extract of the leaves of *Populus tremula* L. and *Juglans regia* L. and a solution of 10^{-3} M and 10^{-4} M juglone were used as treatments and distilled water as a control.

10 ml of 10^{-3} M and 10^{-4} M juglon solutions containing 1.76 mg and 176 μg of juglon were administered per treatment. The concentrations of the juglone treatments were chosen based on that juglone may occur in the soil of a walnut plantation in concentrations ranging from 10^{-4} to 10^{-6} M, and from this it is worth performing treatment with an order of magnitude higher concentration according to the lessons of previous allelopathic experiments (JOSE and GILLESPIE 1998; THEVATHASAN et al. 1998; TERZI 2008). To validate the presence of juglon in *Juglandis folium* samples, TLC method defined in the Hungarian Pharmacopoeia (Ph. Hg. VII. 1986) was used. A modified version (SZABÓ et al. 2016) of the method of SINGLETON and ROSSI (1965) was used to determine the total polyphenol content of poplar leaves. The monitored germination parameters were the germination vigor, the germination rate and the fresh weight of the seedlings according to the regulations of MSZ 6354–3: 2008. Statistical analysis was performed using ANOVA model as well as Tukey HSD or Games-Howell post hoc tests.

The following taxons were investigated in the *in vivo* shade effect research: *Achillea collina* Becker 'Azulenka'; *Calendula officinalis* L.; *Cannabis sativa* L. 'KC Dóra'; *Carum carvi* L.; *Dracocephalum moldavica* L.; *Linum usitatissimum* L.; *Melissa officinalis* L. 'Lemona'; *Ocimum basilicum* L. and *Satureja hirtensis* L. In the case of the small-scale open field study on shade-tolerance, shade treatments (30% and 50%) were established using one and two layer of commercially available agro green shade nets on frames. Raschel mesh with a material thickness of 35 g/m^2 provides a light reduction of 30% according to the manufacturer's product specification. The microclimatic data of the treated and control plots, including the light reduction provided by Raschel nets, were measured based on 3472 measurements from sunrise to sunset in June and July of 2020. NBIoT sensor set as the measuring instrument was put into experimental operation in cooperation with KIBU Innovation Nonprofit Ltd. Based on the results provided by the sensor, the experimental treatments were named A30 (30% light reduction all day) and A50 (50% light reduction all day). The changes of morphological (horizontal and vertical extension), yield (fresh and drug weight) and phytochemical (essential oil content; total flavonoid content; rosmarinic acid content; proazuelen content; carotenoid content; essential oil composition; polyphenol composition; carotenoid composition; fatty acid composition) characteristics was measured as a result of the shade-effect.

The essential oil content was determined by Clevenger-type apparatus, according to the method recommended in the Hungarian Pharmacopoeia VIII (Ph. Hg. VII. 1986). The assay of total flavonoid content determination was done according to the method given in the Hungarian Pharmacopoeia VIII (Ph. Hg. VIII., 2004) for *Calendulae flos*. The determination of rosmarinic acid content was based on the method published by Szabó et al. (2016). The proazulene content in the EO samples of Millefolii herba was determined by the spectrophotometric method at 608 nm, as described in the European Pharmacopoeia VIII. (Ph. Hg. VIII. 2004). The fatty oil content of the dried flax seeds (*Lini semen*) and hemp seeds was determined by soxhlet extraction. The fatty acid composition was determined by gas chromatography (GC) flame ionization (FID). GC-MS was used to determine the composition of the essential oil and the proportion of components by area %. HPLC analysis was performed to determine the carotenoid composition. The polyphenol composition of the marigold samples were quantified by the UHPLC-ESI-MS/MS multicomponent method. Student's t test was used to compare the different parameters of the treated and control plants. In the case of the analysis of the change in the composition of the essential oils carotenoids, composition analysis was performed, which shows how the change in the *relative ratios* of the different components of a matrix affects the overall composition of the matrix and thus its quality.

In our pilot agroforestry study, the behavior of three MAP species (*Origanum vulgare* L. subs. *vulgare; Melissa officinalis* L.; *Thymus pannonicus* All.) was investigated in a pilot-scale, close-space designed poplar plantation featuring all interspecific interactions (shade, allelopathy, competition for water and nutrients) occur in AFS. The effect of the presence of tree rows was assessed in terms of yield (fresh and drug weight) and essential oil content results. The treatment effect was evaluated by MANOVA for the yield data and by ANOVA for the essential oil content.

3. RESULTS AND DISCUSSION

In the case of the *in vitro* allelopathy research the behaviour of ten MAP species was undertaken, the small-scale open field study covered nine MAP species, while three MAP species were assessed during the pilot agroforestry research, all under different experimental conditions. The results of our allelopathy research has demonstrated that there are MAP species that are able to germinate properly in the presence of allelochemicals specific to the Juglans and Populus genuses as recorded in the seed testing methods. 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. We have shown 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. We have shown that there is a significant interaction between the duration and intensity of the allopathic effect in the germination of seeds of some MAP species - and this potency decreased in each case as the treatment progressed.

The results of our *in vivo* study demonstrated that there are temperate grown species of MAPs, for which it is possible to produce the quality drug specified in the professional standards and with adequate yields even under shade (30%) conditions, and that different species respond specifically to the shade effect. Our data proved, that semi shade (30 %) exerted favourable effects on several examined MAP species of a variety of experimental parameters; however, the 50 % shade effect, conversely, had a favourable effect on none of these examined MAPs of any of the examined parameters. Consequently, in the case of medicinal agroforestry land use in temperate zones, for the model species we studied, the system of the trees and MAPs should be designed in such a way that the shade created by the trees does not cause 50% reduction in light in the growing area of MAPs, or not even a light reduction of 30%.

In the pilot agroforestry study, we demonstrated that the cultivation of *Melissa officinalis* L. is not negatively affected by the presence of the trees and that the cultivation of *Thymus pannonicus* All. can be effective in AFS providing adequate lighting. The cultivation of *Origanum vulgare* L subsp. *vulgare* does not appear to be economical under agroforestry conditions at present, and certainly not in the case of closed tree plantation.

4. CONCLUSION

Bioassays are kick-off methods in allelopathic research to measure the response of germinating seeds and seedlings to the activity of allelochamicals. 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. According to the results of our *in vitro* germination trails, the species of *Althaea officinalis* L., *Anethum graveolens* L., *Cannabis sativa* L., *Carum carvi* L., *Dracocephalum moldavica* L., *Linum usitatissimum* L. and *Satureja hortensis* L. are recommended for further pot culture and small-scale medicinal agroforestry studies. We do not recommend *Angelica archangelica* L., *Centaurium erythraea* Rafn., *Levisticum officinale* Koch., *Papaver somniferum* L., *Sinapis alba* L. species for further experiments focusing on the introduction of MAPs to poplar and walnut based medicinal agroforestry systems.

According to the results of our *in vivo* small-scale open field shade-tolerance studies, the screened MAP species was divided into two groups. *Achillea collina* Becker., *Cannabis sativa* L., *Carum carvi* L., *Linum usitatissimum* L. and *Ocimum basilicum* L. species were recommended with suggested research topic orientations for further small-scale experiments. *Calendula officinalis* L., *Dracocephalum moldavica* L., *Melissa officinalis* L. and *Satureja hortensis* L. are recommended for large-scale experiments for the introduction of them into medicinal agroforestry systems. MAPs respond species-specifically to 30 % light reduction. Based on the specificity of these species, in the case of fields with poor soil conditions, as well as setting up scale-up cultivation experiments, we recommend the species mentioned below for medicinal agroforestry cultivation for the following reasons. *Calendula officinalis* L.: drug yield was not decreased by mild shadow (30 %); however, it increased the carotenoid content. *Dracocephalum moldavica* L.: mild shade (30 %) increased *Dracocephali herba* yield while not decreasing its EO content. *Melissa officinalis* L.: drug yield can be increased by mild shade (30 %). *Satureja hortensis* L.: mild shade (30 %) did not decrease drug yield but increased its EO content. Species less tolerant of the 30 % shade are recommended for further agroforestry research with the following suggestions considering research topic orientation: *Achillea collina* Becker: screening of genotypes most tolerant of reduced light conditions (maintaining organ proportions, homogeneous floral horizon). *Cannabis sativa* L.: investigation of the fiber and multi-purpose cultivation in agroforestry conditions. *Carum carvi* L.: setting up experiments for both variety and nutrient replenishment targeting the improvement of EO accumulation; determining the optimal economic centre of gravity for increasing yield and decreasing EO accumulation due to shade. *Linum usitatissimum* L.: assessment of mild (<30%) light reduction for the optimum of production and quality parameters. *Ocimum basilicum* L.: exploring the optimization of cultivation for light conditions typical of the local agroforestry systems (very mild shade testing).

In summary, we contribute to the prosperity of the collective knowledge base of Hungarian agricultural research and the professional establishment of temperate medicinal agroforestry systems by the synthesys and publication of four new universal and seven new special scientific findings. We have done all this, with the aspiration of expediting the blooming of agricultural practices as a force for revitalizing the diversity and beauty of the living natural environment.

5. NEW SCIENTIFIC FINDINGS

New universal scientific findings

1. We have proven a species-specific allelopathic effect on allelochemicals of *Juglans* and *Populus* tree species for other MAP taxons under *in vitro* conditions, ones that have already been cited in literature for other agricultural crops. Allelochemicals of *Juglans* and *Populus* are being tolerated based on the germination parameters of *Althea officinalis* L., *Anethum graveolens* L., *Cannabis sativa* L., *Dracocephalum moldavica* L., *Linum usitatissimum* L. and *Satureja hortensis* L. On the basis of the germination parameters of *Carum carvi* L. it does seem to partially tolerate allelochemicals of *Juglans*. Furthermore, according to the germination parameters of those of *Angelica archangelica* L., *Centaurium erythraea* Rafn., *Levisticum officinale* Koch., *Papaver somniferum* L. and *Sinapis alba* L., none of these species tolerate the allelochemicals of either *Juglans* or *Populus* species.
2. There tends to be a statistically significant interaction between the duration of exposure to the allelochemicals and the degree of allelopathic effect for

certain MAP taxons - according to which, allelopathic tolerance may change in a species-specific manner as the allelopathic effect gradually progresses, whereas in the case of other taxons the intensity of allelopathic effect significantly decreases instead when *in vitro* circumstances.

3. Different MAP taxons tolerate a 30% decrease of light according to the specificity of their species. My results have demonstrated that in *in vitro* circumstances, all *Calendula officinalis* L., *Dracocephalum moldavica* L., *Melissa officinalis* L. and *Satureja hortensis* can indeed be cultivated under conditions of 30% light reduction according to both production (fresh and drug weight) as well as quality (content and composition of active substance) criteria.
4. In the case of MAP taxons already examined 50% decrease in light has not improved once any of the properties of any of the taxons yet in many cases even caused deterioration considering *in vivo* circumstances.

New special scientific findings

5. *In vivo* shade conditions (30%), did not show any detrimental impact to the development of fresh nor the drug weight of *Achillea collina* Becker 'Azulenka'. Moreover, the proazulene active ingredient does indeed accumulate within the taxon even under 30% light reduced cultivation conditions, in accordance with regulations (*Millefolii herba*) set by Ph. Hg. VIII.
6. It has also been proven by three-year *in vivo* experiment, that neither 30 % nor 50% light reduction affected the drug production of *Calendula officinalis* L. Therefore it can be said that, a pharmacopoeial-grade calendula drug can be successfully produced even under semi-shad growing conditions.
7. I initiated, contrary to the previous scientific consensus, considering the plant materials under my examination, that ß-carotene, lutein and the cis-isomer of lutein ((9'Z) -Lutein) are indeed the main carotenoid components of *Calendula flos sine calycibus*, followed only by the epimer of luteoxanthine I. and the cis-isomer of γ-carotene ((5'Z) - γ-carotene) in quantitative order.
8. Being the first within scientific literature, I identified the presence of gondoic acid - as the cis-isomer of eicosanoic acid - concerning the fatty oil of *Cannabis sativa* L. 'KC Dora' variety.

9. Based on two years of data, I demonstrated that, in *in vivo* circumstances, the production of *Dracocephali aetheroleum* under 30% and 50% reduced light conditions significantly increased the amount of the isomeric constituents (neral and geranial) forming the citral molecule compared to the control exposed to full sunlight.
10. The essential oil content of *Saturejae herba* can be significantly increased by a 30% reduction in light, whilst it does not affect either the fresh or the drug weight, nor the quality of the essential oil composition as such.
11. *Melissa officinalis* L. can be cultivated efficiently under agroforestry conditions in accordance with fresh and drug weight criteria.

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