



UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
TÂRGU MUREȘ

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TÂRGU-MUREȘ
ROMÂNIA

CATALOG DE SEMINTE
NOTE BOTANICE
Fasc. XLIII 2017



INDEX SEMINUM



2017



ISSN 1222-8982
ISSN 1222-6750

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PRELIMINARY STUDY OF *ARTEMISIA ANNUA* INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGI

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Introduction

Artemisia annua (Asteraceae) or sweet wormwood is an aromatic annual herb native to Asia, which has dispersed throughout the world covering a wide range of ecological regions: temperate environments, subtropical areas, arid or semiarid environments. Today it grows also in Argentina, Bulgaria, France, Hungary, Romania (where it is cultivated for its essential oil), Italy, Spain, USA and former Yugoslavia (Aftab et al. 2014).

A. annua is an important medicinal plant, which contains various chemical constituents including sesquiterpene lactones, flavonoids and essential oils (Ferreira 2010).

In the traditional Chinese medicine *A. annua* was used for over two millenia. Aqueous preparations of the dried herb were applied against fever, malaria, skin diseases, jaundice and hemorrhoids. It was known for its wound healing property and for the improvement of eye brightness. *A. annua* was also used to treat autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. In Iran has been used traditionally for infants as antispasmodic, carminative and sedative (Das 2012, Aftab et al. 2014).

The main natural constituent from the upper parts of the plant is artemisinin. This sesquiterpenelactone peroxide is an effective antimalarial agent, particularly for multi-drug resistant and cerebral malaria (Aftab et al. 2014). *A. annua* it is considered "the hope against malaria and cancer". Researchers reported the anticancer efficacy of artemisinin and its derivatives in cell cultures (rats and humans). It was shown that dihydroartemisinin is specifically toxic to breast cancer cells, but not to healthy cells (Singh et al. 2004, Firestone et al. 2009). Animal studies revealed important biological activities of the plant: antihypertensive (aqueous extract of the aerial parts), antimicrobial (the essential oils), anti-inflammatory (aqueous methanolic extract), antioxidant (flavonoids, the leaves and inflorescences), immunosuppressive (ethanolic extract), amelioration of collagen induced arthritis (water soluble artemisinin derivative SM905) and antiparasitic activity (artemisinin) (Das 2012). The essential oils of the plant are used not only in the pharmaceutical industry but also in cosmetic, flavoring and fragrance industries (Chaudhary et al. 2008). The high antioxidant activity of *A. annua* extract is due to its high phenolic content. Phenols from the plants also may synergize the effects of artemisinin against malaria and cancer (Ferreira 2010).

Organic synthesis of artemisinin is possible, but the low yields make it economically nonviable. Currently, *A. annua* is the source for the production of artemisinin and semi-synthetic artemisinin derivatives with reduced toxicity to humans (Awasthi et al. 2011, Jelodar et al. 2014). This plant still remains one of the major sources of drug throughout the world. To increase the artemisinin (and other active principle) content of the plant and reduce its costs of production became a global demand. Efforts are made on this line in cultivation and agronomical practices, breeding technologies, biotechnological and plant tissue culture studies (Jelodar et al. 2014).

An important area of investigation is the study of the effect of arbuscular mycorrhizal fungi (AMF) on active principles in *Artemisia annua*. It is known that AMF promote the accumulation of effective ingredients of medicinal plants, increase the yield, and also improve the quality of medicinal materials (Zeng et al. 2013). Only a few studies have approached the theme of *A. annua* mutualism with AMF (Kapoor et al. 2007, Chaudhary et al. 2008, Awasthi et al. 2011, Fortin et al. 2015). Most of these studies showed encouraging results. Further researches are necessary on the role of AMF in the cultivation of *A. annua*. Searching of efficient AMF strains adapted to different cultivars and production conditions must continue, given that the degree to which AMF affect different cultivars of the same species may vary greatly. In this sense, a project has been initiated with the main objective to develop a production procedure for *A. annua* seedlings with mycorrhiza (*Glomus intraradices*), in order to influence (quantitatively and qualitatively) the contents of active ingredients in the medicinal plant. In this article, a preliminary study will be presented, with the following objectives: (1) to make a seed germination test for the *Artemisia annua* cultivar A-3 Anamed (Winnenden, Germany); in the literature, the available information about the germination conditions for the A3 cultivar it refers to temperature (with a very large scale - from 20 to 30°C) (Anamed, Germany); (2) to perform the inoculation of the *Artemisia annua* A3 seedlings with the AMF spores of *Glomus intraradices*.

Materials and methods

Seed germination test

Seeds of *Artemisia annua* A3 were germinated in March (2017), in Linhart germination trays (Fig. 1), under the following laboratory conditions: air humidity of 40%, 16 h light and 8 h dark photoperiod, and two different temperatures (20 and 22°C). In the Linhart tray, four seed lots (100 seeds / lot) were sown. One seed lot consisted of ten columns of seeds, each with ten rows. A total of 800 seeds were germinated under the two established laboratory conditions.

For the determination of the final germination percentage (FGP) and the germination index (GI), the numbers of germinated seeds were daily counted until no further germination occurred.

The FGP was calculated according to Anjum et al. (2005), with the following equation:

$$FGP = \frac{N_T \cdot 100}{N} \quad (1)$$

where N_T is the proportion of germinated seeds in each treatment, and N is the number of seeds used in the bioassay.

The GI was calculated as described by the Association of Official Seed Analysts (Dezfuli et al. 2008), with the following equation:

$$GI = \frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \dots + \frac{N_n}{n} \quad (2)$$

where $N_1, N_2, N_3, \dots$ and N_n represents the number of seed which germinated in day 1, 2, 3... and n .

Mycorrhizal inoculation

The *Artemisia annua* seeds were germinated at the end of March (2017), under the pre-tested laboratory conditions (air humidity of 40%, 16 h light and 8 h dark, and temperature of 22°C), on peat discs with coconut fibre (Anamed, Germany). The fourteen day-old seedlings (in the 2 leaf stage) were transplanted in pots (with 50 cm length, 19.5 height and 16.3 cm width) containing sterile commercial peat (Blondy Romania SRL). The 5-6 leaf stage (about thirty-five day-old) seedlings were used for inoculation with AMF spores. The seedlings were placed separately in 4 l pots with sterile peat and were watered with 400 ml water/day. The inoculation was made with the arbuscular mycorrhizal fungi (AMF) spores of *Glomus intraradices* purchased commercially (Italpollina SPA, Italy). Because the product of AMF spores was supplied as a high concentrated powder (1400 spores/g), 0.5 g mycorrhizal inoculum was added to each pot after dissolving in 400 ml water. The pots were not watered in the first 24 h after the AMF treatment. During this period the mutualistic relationship between the plant and AMF could be established. In the subsequent three weeks the plants inoculated with AMF (a total of 100 plants) were kept separate to those without AMF treatment (a total of 100 plants) in a greenhouse.

The AMF colonisation was evaluated on 12 randomly selected plants / treatment (12 plants inoculated with AMF, and 12 control plants) at early vegetation period, one week after inoculation. A random sub-sample of approximately 150 root fragments (of 1 cm length) per plant was collected. The roots were cleared in 2.5% KOH (90°C) for 45 min, acidified with 1% HCl for 15 min, stained with 0.05% Trypan Blue in acid glycerol (90°C) for 45 min,

and then stored in acid glycerol (Koske and Gemma, 1989). From each sub-sample, 10 randomly selected root fragments were mounted in glycerine on slides. The fragments were aligned parallel to the long axis of the slide. Pictures from the root fragments were taken at 200x magnification by a photographic camera (Canon EOS 1100D, Taiwan) mounted on a microscope (Ceti Topic-T, Belgium). One picture, at 200x magnification, covered a field of view with a length of 0.882 mm, and a width of 0.588 mm. For the root fragment, five pictures were obtained. The distance between the pictures was constant for each sub-sample. In the few cases, when the root fragments were too wide to fit into the field of view of the camera, pictures were taken in two width portions.

Data analyses

Count data obtained from seeds germination were first tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Because the data did not fulfil the assumption of normality, therefore the two-tailed Wilcoxon test was used to compare germinated seed numbers on different temperatures.

The percentage of root colonisation by AMF was determined using the program ImageJ Image Processing and Analysis in Java version 1.51j8 (National Institute of Mental Health, Bethesda, Maryland, USA). On the image a grid of lines was created with an area per point of 0.1 mm² (Fig. 2). The points were counted in the following categories: with arbuscules, with vesicles, with hyphae only, with fungal material (one or more types of fungal material), with no fungal material, all examined points. The arbuscular colonisation (AC) and the vesicular colonisation (VC) were calculated by dividing the number of categories by the total number of examined points. The hyphal colonisation (HC) was calculated as the proportion of points with fungal material.

Results and discussions

The numbers of germinated seeds, together with the final germination percentages (FGP) are presented in the Tables 1 and 2. The counts per day of germinated seeds, together with the germination indices (GI) are presented in Tables 3 and 4.

Table 1. Final germination percentage (FGP) of *Artemisia annua* seeds (germination temperature of 20°C)

Germinated seeds (N)				
Columns	Seed lot 1	Seed lot 2	Seed lot 3	Seed lot 4
1	7	5	5	6
2	7	10	7	8
3	7	8	6	6
4	6	6	10	8
5	7	9	8	8
6	8	7	8	9
7	8	7	9	6
8	8	8	5	5
9	8	9	7	9
10	8	9	7	6
Total	74	78	72	71
Mean ± SD	7.4 ± 0.699	7.8 ± 1.54	7.2 ± 1.619	7.1 ± 1.449
N_T	295			
FGP (%)	73.75			

Note: N_T - the proportion of germinated seeds in each treatment; FGP (%) - final germination percentage.

Table 2. Final germination percentage (FGP) of *Artemisia annua* seeds (germination temperature of 22°C)

Germinated seeds				
Column	Seed lot 1	Seed lot 2	Seed lot 3	Seed lot 4
1	8	7	7	7
2	9	10	9	8
3	9	9	8	10
4	8	10	10	10
5	8	9	10	9
6	10	8	9	10
7	9	10	10	10
8	10	10	8	9

9	8	10	9	9
10	10	9	8	8
Total	89	92	88	90
Mean $\pm$ SD	8.9 $\pm$ 0.875	9.2 $\pm$ 1.032	8.8 $\pm$ 1.032	9 $\pm$ 1.054
N_T	359			
FGP (%)	89.75			

Note: see Table 1.

Table 3. Germination index (GI) of *Artemisia annua* seeds (germination temperature of 20°C)

Germinated seeds					
Day	Seed lot 1	Seed lot 2	Seed lot 3	Seed lot 4	Total
1	0	0	0	0	0
2	4	4	3	3	14
3	22	21	21	19	83
4	23	24	20	19	86
5	10	14	14	14	52
6	10	10	10	9	39
7	5	5	4	7	21
GI					76.07

Table 4. Germination index (GI) of *Artemisia annua* seeds (germination temperature of 22°C)

Germinated seeds					
Days	Seed lot 1	Seed lot 2	Seed lot 3	Seed lot 4	Total
1	0	0	0	0	0
2	3	3	4	5	15
3	21	21	20	19	81
4	25	27	26	27	105
5	25	27	29	28	109
6	15	12	9	11	47
7	0	0	0	0	0
GI					90.38

Germination started in the second day. At temperature of 20°C, the seeds of *Artemisia annua* germinated within 7 days, while on temperature of 22°C within 6 days. Germination of seeds on 22°C was significantly higher than on 20°C (N=40, z=-5.034, p<0.0001, two-tailed Wilcoxon test) (Fig. 3). Also the values calculated for FGP and GI were higher when temperature was of 22°C.

No AMF colonization was observed in plant roots without AMF spores treatments. For the plants inoculated with AMF the AC was of 34.75% (SD: 10.50), the VC of 2.65% (SD: 1.29), and the HC of 50.37% (SD: 11.10) (Table 5). These results are in good agreement with Awasthi et al. (2011), where the HC of *Artemisia* plants (sixty day-old) roots with *Glomus intraradices* was of 53.66%.

Table 5. Arbuscular colonization (AC), vesicular colonization (VC) and hyphal colonization of the roots of about two month-old *Artemisia annua* seedlings

AMF plants	AC (%)		VC (%)		HC (%)	
	Mean	SD	Mean	SD	Mean	SD
1	18.657	8.433	3.308	3.324	30.837	9.201
2	31.717	13.006	5.569	4.103	50.248	5.759
3	42.448	12.978	3.656	3.521	58.073	7.063
4	16.257	5.353	2.133	2.267	29.361	8.143
5	29.201	13.900	1.685	1.228	45.363	6.067
6	50.417	10.370	2.712	3.946	63.833	9.915
7	43.303	8.291	1.251	1.493	55.310	6.946
8	45.023	11.085	1.607	2.066	63.781	7.082
9	42.613	8.065	2.186	2.247	56.898	6.972
10	29.923	4.729	1.336	1.778	46.685	5.135
11	32.727	9.778	4.074	4.556	51.018	7.513
12	34.808	8.152	2.286	2.894	53.117	8.278

Conclusions

Artemisia annua seeds showed a good percentage and rate of germination (FGP of about 90%, GI = 90.38) on the following laboratory conditions: air humidity of 40%, 16 h light and 8 h dark photoperiod, and temperature of 22°C. The test resulted in an efficient germination procedure which can be used in further experiments regarding the cultivation of the *Artemisia annua* A3 cultivar. Root colonisation with AMF of *Artemisia* plants

was also successful. The next step in the project will be to investigate plant property variations regarding plant biomass, glandular trichome numbers and artemisinin production of *Artemisia annua* plants when these plants are in mutualism with AMF.

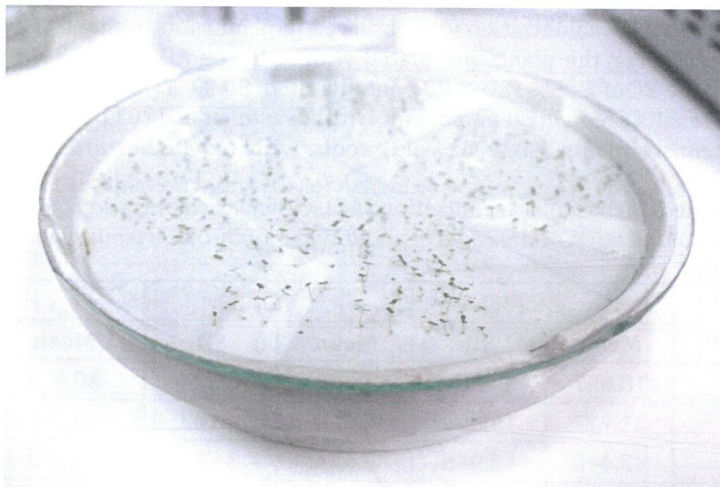


Figure 1. The Linhart germination tray with *Artemisia annua* A3 seeds

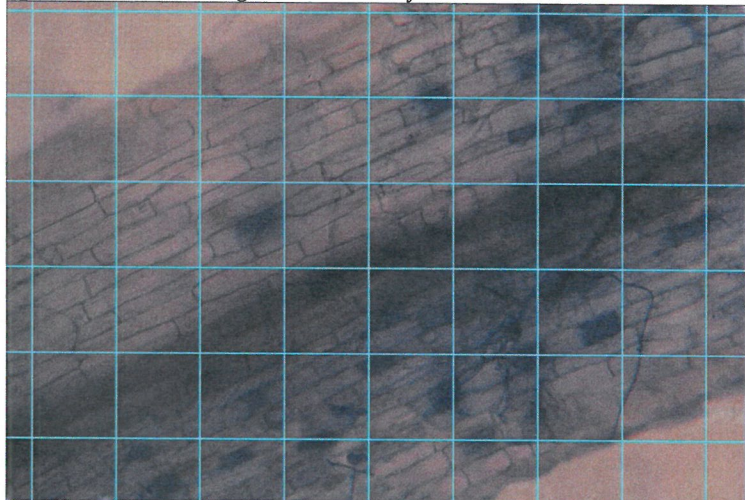


Figure 2. Image greed of root colonization of *Artemisia annua* A3 with AMF [Image] Image Processing and Analysis in Java version 1.51j8) (Photo by Erzsébet Domokos)

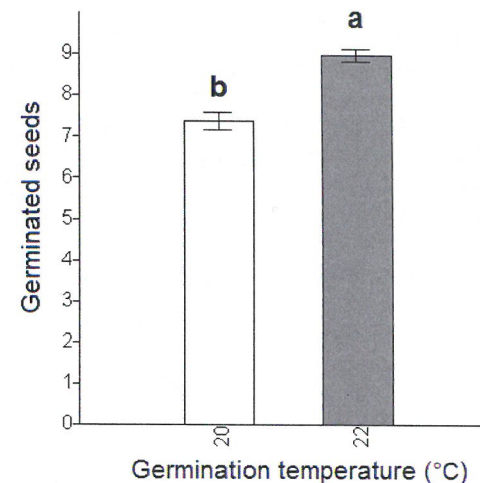


Figure 3. Effect of temperature on *Artemisia annua* seeds germination. The two-tailed Wilcoxon test was used to compare variables. Different letters means statistical significant differences at $p < 0.05$ level. Error bars = ± 1 standard error.

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