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Alkanna tinctoria: An Approach Toward Ex situ Cultivation

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Abstract. *Alkanna tinctoria* (L.) Tausch (Boraginaceae) is a perennial herbaceous medicinal plant species, with limited distribution and small populations. It is included in the Red Data Book of Bulgaria as endangered species, and protected by the Biodiversity Act. *Ex situ* cultivation is recommended as conservation measure for *A. tinctoria*. Seed germination rate under laboratory conditions: *in vivo*, *in vitro*, and in soil, was very low, up to 1%. The present study deals with stimulation of seed germination, acceleration of plants' growth using hydroponic technologies, and assessment of the photosynthetic apparatus of the adapted plants. Seeds gathered from 4 natural populations were treated by gibberellic acid (GA_3), and irradiation with red, blue, or infrared light emitting diodes, in order to stimulate their germination. Seedlings were grown on Cutting board hydroponic system for 6 weeks, than potted in soil substrate. Photochemical activity of adapted plants was characterized by Pulse-Amplitude-Modulated chlorophyll *a* fluorescence. Germination rate was enhanced mainly by GA_3 ($p < 0.001$) although seed origin and light quality influenced germination as well; furthermore, interaction of the 3 factors was also observed. In the best variant, 20% of the seeds germinated. No significant differences were noticed in the maximum quantum yield of primary photochemistry in dark-adapted state Fv/Fm between plants obtained from seeds germinated under different light quality. A pilot agriculture was established on the experimental field plot of IBER. Owing to the application of hydroponic technologies, plants' growth was accelerated and time from seed germination to plant flowering was twice shortened.

Key words: dyer's alkanet, seed germination, hydroponics, fluorescence of PS II, ABC analysis.

Introduction

Alkanna tinctoria (L.) Tausch is a perennial herbaceous medicinal plant species from Boraginaceae family. Its general distribution includes the Mediterranean area of Europe and Africa, from Spain and Morocco to Asia Minor and Jordan, the southern part of Central Europe (Hungary and Slovakia), and Caucasus (Pre-Caucasus and Transcaucasia) (Kozhuharov, 1989; Hoskovec, 2014). The species has a limited distribution with small populations in Bulgaria (Fig. 1). It is included in the Red Data Book of Bulgaria (Evstatieva, 2015) and the Red List of Bulgarian vascular plants (Petrova & Vladimirov, 2009) as endangered

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species, and protected by the Biological Diversity Act (2002).

A. tinctoria plants are harvested from its natural habitats for use as a red dye to give colour to wines, alcoholic tinctures, vegetable oils, and varnishes since antiquity in the Mediterranean region (Grieve, 1995-2020). In folk medicine it is applied as an excellent remedy for treating abscesses and inflammations (Duke, 2002). Plant extracts have expressed antiviral, antibacterial, anticancer, anti-inflammatory, anti-skin aging, and antiradical activities (Assimopoulou & Papageorgiou, 2005; Genova et al., 1995; Khan et al., 2015; Ozer et al., 2010; Tung et al., 2013).

In nature, reproduction of *A. tinctoria* is realized through seeds enclosed in monospermic achenes (Gerardi et al., 1998), however, the germination rate of its seeds is very low (Qi et al., 1993). The data on cultivation of *Alkanna tinctoria* are scantly. Attempt to its introduction in culture was done by Pluhar et al. (2001) who studied the germination rate and the propagation possibilities of the species. It was reported that alkanet could be propagated by raising seedlings, sowing the seeds in cold beds in autumn, or in heated equipment in early spring (Nemeth, 2009). *A. tinctoria* plants are maintained in the Botanical Garden of the Slovak Agricultural University in Nitra, grown by seeds sowed into prepared seed-bed on the experimental field (Becarova et al., 2012). The species is cultivated as a dye plant in Central and Southern Europe (Grieve, 1995-2020). Experimental work for *ex situ* cultivation is among the recommended conservation measures for *A. tinctoria* in the Red Data Book of Bulgaria.

The present study deals with stimulation of seed germination, acceleration of plants' growth using hydroponic technologies, and assessment of the photosynthetic apparatus of the adapted plants of *A. tinctoria*. The study was carried out within a research project supported by the Bulgarian National Science Fund. It is related to the development of effective protocols for cultivation of the species, as a part of a larger investigation on the possibilities for initiation of agricultures of medicinal plants with resource deficit from the Bulgarian flora.

Fig. 1. *Alkanna tinctoria* (L.) Tausch *in situ*.

Material and Methods

Plant material. Seeds were gathered from 4 Bulgarian natural populations in early June 2019, close to the villages Ilindentsi (N 41.65349, E 23.23062), Mikrevo (N 41.63887, E 23.17440), General Todorov (N 41.45800, E 23.28071), and Struma (N 41.55849, E 23.23130), all along the valley of the Struma River.

Seed germination. First tests of seed germination were carried out in different ways in parallel, under laboratory conditions: *in vivo*, *in vitro* as well as in soil substrate, and on a hydroponic system, 30 seeds per population. *In vivo* test was done on moistened filter paper in Petri dishes, at 25°C, while *in vitro* test was performed after standard seed disinfection, in plastic containers with basal MS medium, in a culture room at 23 ± 2 °C and 16/8 h photoperiod. For the soil test, seeds were put in a terrine with light soil and sand in proportion 1:1, in a room phytotron under natural daily light and temperature between 20 and 26 °C. The Cutting board hydorponic system (GHE), with 27 meshy pots with peat cubes on keramzit (5 seeds per meshy pot), was in the same phytotron; the nutrient solution, agitated by an aquarium pump and airstones, consisted of water and equal quantities of Flora Micro, Flora Grow, and Flora Bloom (GHE), its pH was maintained between 5.5 and 6.5, and the electrical conductivity (EC) – between 0.40 and 0.98 mS.cm^{-1}.

In order to stimulate seed germination, 2 factors were tested: soaking in gibberellic acid (GA_3) and irradiation with monochromatic light emitting diodes (LED). Seed origin (population) was considered as third factor with potential influence on seed germination. A total of 180 seeds per population were used, distributed in 2 consecutive trials (first one beginning in December 2019); half of the seeds were treated with 0.35% GA_3 for 4 h prior to germination, and the others were soaked in water for the same time, noticed as 'control'. Both GA_3-treated and control seeds were placed in Petri dishes, in 3 cameras at constant temperature of 21±1 °C, under 12 h daily illumination with red (660 nm), blue (469 nm), or infrared (730 nm) light of the same quantum fluence rate of 110 mol/(m^{-2} s^{-1}). As a result, 24 variants were compared, 30 seeds per variant. Seeds were light-stimulated as long as one week after the end of seed germination.

Statistical analyses. The germination rate was assessed 4 weeks after the beginning of the tests, and expressed as percentage of germinated seeds. Seeds with hypocotyl were considered as germinated. The effect of GA_3 on seed germination was evaluated using Excel t-test paired. The output variable assumes values in the standard unit interval (0; 1). The values of seed germination were classified in 3 groups according to ABC analysis (Ultsch & Lötsch, 2015), and presented graphically using ggplot2 package functions (R Core Team, 2020; Wickham, 2016).

Soilless cultivation and plants' adaptation to soil. Seedlings were grown on the same Cutting board hydroponic system (GHE), described above, in mesh pots with peat cubes on keramzit, one per pot, for 6 weeks. Rosettes with diameter about 10 cm were potted in soil substrate consisting of light mix and sand, in proportion 2:1, and grown in the room phytotron.

Analysis of plant photochemical activity. After developing large rosettes, diameter of 15-20 cm, plants were analysed for their photochemical activity. They were dark-adapted for 5 min, than the induction of Chlorophyll *a* fluorescence was measured by Pulse-Amplitude-Modulated (PAM) fluorimeter (model 101/103, H. Walz, Effeltrich, Germany) as described by Fedina et al. (2003). The initial fluorescence level (F_0) was measured at frequency of 1.6 kHz. To evaluate the maximal fluorescence level (Fm), saturating flashes of 3000 μmol m^{-2} s^{-1} PFD with duration of 1s were provided by Schott lamp KL 1500 (Schott Glaswerke, Mainz, Germany). The maximum quantum yields of PSII photochemistry (Fv/Fm) were calculated according to Kitajama and Butler (1975). Five measurements were done for each plant.

Results and Discussion

Seed germination rate without stimulation, under *in vivo* and *in vitro* laboratory conditions, as well as in soil, was very low, from 0% in the soil terrine and *in vitro* conditions, up to 1% for the *in vivo* test in the Petri dishes. On the Cutting board hydroponic system, only one seed from Mikrevo population germinated i.e. 0.74% of all 135 seeds (Fig. 2).

Similar results were reported by Becarova et al. (2012) for *A. tinctoria* seed germination, where 51 seedlings were obtained from 925 seeds, diasporas being sowed in special seed-bed in the Botanical garden of the SAU in Nitra, Slovakia. Extremely low seed germination was reported not only for *A. tinctoria* but also for the whole Boreginaceae family (Gerardi et al., 1998). Presence of seed dormancy was proved for *Alkanna orientalis* during a testing of the seed germination behaviour of this species (Moustafa, 2002). Very likely, the dormancy is one of the reasons for the low seed germination in *A. tinctoria*, as well.

In all tested populations seed germination began at the end of the first week, first in variants with GA_3-treated seeds, and prolonged until the end of the third week. Germination rate was enhanced mainly by GA_3 ($p < 0.001$, t-test paired). Best results were noticed for seeds from Mikrevo population, treated with GA_3 and irradiated with blue light: 20% of 30 seeds germinated (Fig. 3). The stimulating effect of GA_3 on the germination of *A. tinctoria* seeds was demonstrated by Pluhar et al. (2001) as well, who reported up to 50% germination rate after seeds treatment with 400 ppm GA_3 for overnight before sowing.

Fig. 2. Germination of non-stimulated seeds on the Cutting board hydroponic system.

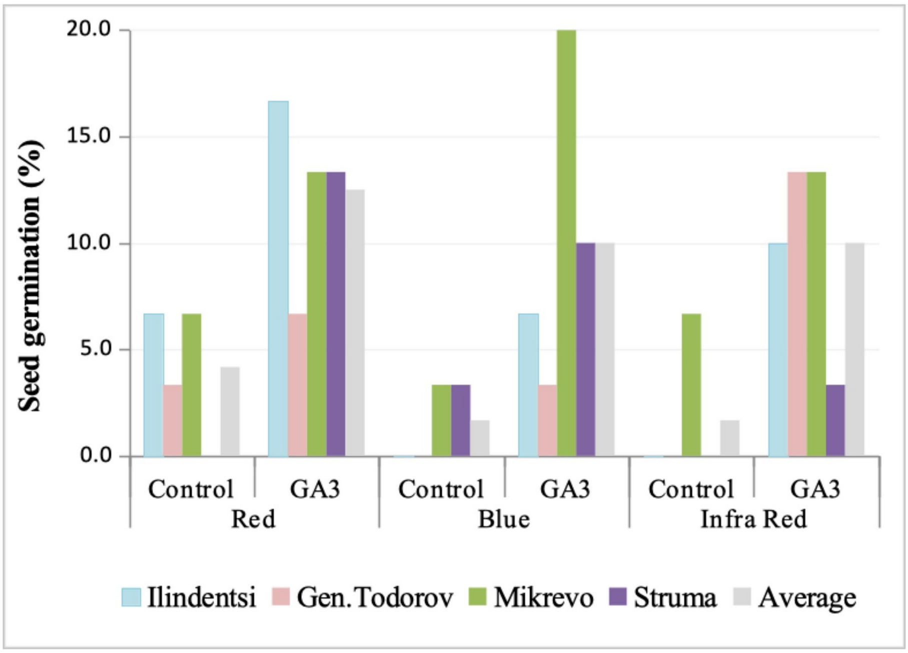

Fig. 3. Seed germination in all tested variants.

According to the germination rate, variants were grouped into 3 groups, comprising 6, 7, and 11 variants, respectively (Fig. 4) group A (count = 6, median = 13.4); group B (count = 7, median = 6.6); and group C (count = 11, median = 0). In 6 variants of the control seeds, belonging to 3 of the populations, no germination was observed at all (Fig. 3). Detailed analysis revealed relation between the seed origin and the germination rate (Table 1). Population near Mikrevo village was the only one with seeds germinating in all tested variants, and those close to villages of General Todorov and Struma manifested the worst germination potential. This might be genetically determined and additionally influenced by some differences in the environmental conditions such as soil characteristics, habitat exposure, and microclimatic features.

All of the 3 tested factors: GA_3, light quality, and seed origin, influenced seed germination; furthermore, interaction of the factors was also observed. Thus, red light was the best one for the seeds originating from Ilindentsi and Struma populations, blue light – for seeds from Mikrevo, and infrared light – for seeds from Gen. Todorov. The effect of different monochromatic lights on seed germination was reported in other species too (Costa et al., 2016; Nasrullah, 2001; Strydom et al., 2017). It could be specific and stimulating or inhibiting, as for ex. in chilli, where the red light was proved to increase seed germination in highest degree, while the violet light slacked it (Nasrullah, 2001). The red light enhanced also seed germination and survival in seagrass *Halophila ovalis* (Strydom et al., 2017).

The growth of 82% of the plants was accelerated on the Cutting board hydroponic system owing to the well-developed axial root and numerous root branches (Fig. 5). Plants were successfully adapted to soil substrate in pots, and after 2 months in the room phytotron (Fig. 6-A) they were transferred for a month to the greenhouse where they strengthened, formed ramified stems, and the biggest ones began to flower (Fig. 6-B). In mid-May, 27 plants were transferred outdoors, in the *ex situ* collection of IBER (Fig. 6-C), where the first flowering individuals were observed at the end of May 2020 (Fig. 6-D).

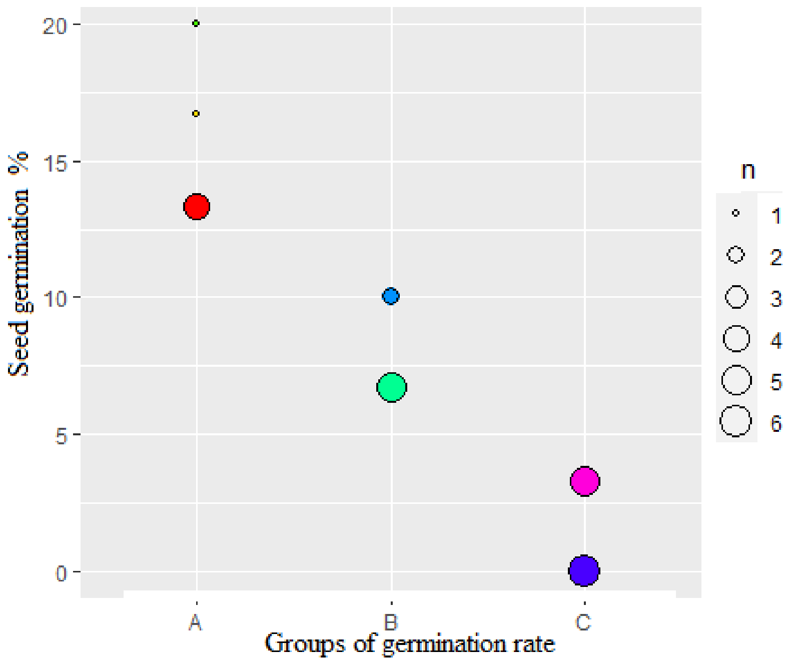

Fig. 4. Groups of variants with relatively high (A), medium (B) and low (C) germination rate. (*Note*: **n** indicates the number of variants with equal values, circle size corresponding to the number of variants with the same values, concerning seeds from all tested populations).

Table 1. Relation between the seed population origin and the germination rate of the seeds: groups with relatively high (A), medium (B) and low (C) germination rate.

Population	Number of participation in the ABC-groups		
	A	B	C
Mikrevo	3	2	1
Ilindentsi	1	3	2
Gen.Todorov	1	1	4
Struma	1	1	4

Fig. 5. Plants with vigour root system, on the Cutting board hydroponic system: **A**) top view; **B**) bottom view; **C**) entire plant before the transfer from the hydroponic system to pots.

Fig. 6. Plant acclimation: **A**) Well developed potted plant rosettes in the room phytotron; **B**) Plant with ramified stems in the greenhouse; **C**) Plants on the *ex situ* collection of IBER; **D**) Beginning of flowering in late May 2020.

Our results showed the advantages from the application of the hydroponic techniques in the propagation of *A. tinctoria*: in the present study, the whole cycle, from the sowing seeds to the plants flowering in open air, took 6 months, while in the experiment conducted by Becarova et al. (2012) flowering plants were obtained after one year from its onset.

Usually, seeds and cuttings are used as initial material for acceleration of plants' growth by hydroponic techniques (Texier, 2013). Concerning *A. tinctoria*, seedlings were proved to be the only appropriate starting material. To our knowledge, in the literature there are reports on few medicinal plant species cultivated hydroponically, among them *Mentha, Stevia, Arnica, Ocimum* (Giurgiu et al., 2014). The present experiment was one more successful application of the soilless cultivation in the propagation of medicinal plants with resource deficiency.

Photochemical activity of adapted plants was characterized by PAM chlorophyll *a* fluorescence. The use of monochromatic light can be perceived by the plant as a stress factor; therefore, we compared the photochemical activity of the potted plants grown from seeds, germinated under red, blue, and infrared light. To avoid the possible influence of population based features, only plants from one and the same population (Mikrevo) were tested. No significant differences were noticed in the maximum quantum yield of primary photochemistry in dark-adapted state Fv/Fm (Table 2). In general, the maximum quantum yield of PSII (Fv/Fm) is about 0,80-0,83 (Drozdova et al., 2001) which means that treatment of seeds with monochromatic light did not affect the subsequent development of the plants.

Table 2. Maximum quantum yield of PSII (Fv/Fm) of plants grown from seeds of Mikrevo population.

Variant		Fv/Fm ± SD
Control	InfraRed	0.820 ± 0.002
GA_3	InfraRed	0.815 ± 0.001
	Red	0.826 ± 0.004
	Blue	0.835 ± 0.004

Conclusion

First trials of *ex situ* cultivation of *Alkanna tinctoria* by seeds, using hydroponic technologies, were successful and promising. The application of this method, together with the stimulation of the seed germination by gibberellic acid, shortened twice the time necessary for the vegetative period from seed germination to plant flowering. A pilot agriculture was established on the experimental field plot of IBER. It will be used for development of effective protocols for cultivation of the species under control conditions. This experiment could be also used as a model for initiation of agricultures of other medicinal plants from the Bulgarian flora, with resource deficit and conservation status.

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