Progress in Nutrition 2017; Vol. 19, Supplement 1: 105-111 DOI: 10.23751/pn.v19i1-S.5768

Original article

Comparison of germination abilities of *Centaurea* sp. under *in vivo* and *in vitro* conditions

Sevda Demirel[1], Nalan Türkoğlu[1], Fethi Ahmet Özdemir[2]

[1]Department of Horticultural Science, Faculty of Agriculture, Van Yüzüncü Yıl University, Van, Turkey - E-mail: nalanturkoglu65@gmail.com; [2]Department of Molecular Biology and Genetics, Faculty of Science and Art, Bingol University, Bingol, Turkey.

Summary. In this study, *in vivo* and *in vitro* germination of endemic and under-threat *Centaurea fenzlii* and *Centaurea derderifolia* species were investigated. In *in vivo* germination, seeds were spread with forceps over the drying papers moisturized with distilled water in petri dishes. Then, petri dishes were closed and taken for germination in a climate chamber at 25°C and 16 hours light/8 hours dark photoperiods. In *in vitro* germination, following the sterilization period, seeds were cultured in MS, B5, SH and WH growth medium with 6 seeds in each in a climate chamber at 25°C under 16 hours light/8 hours dark photoperiod. Present findings revealed high germination rates for *C. fenzlii* and *C. derderifolia* seeds in WH growth medium.

Key words: Endemic, germination, *in vitro*, *in vivo*, *Centaurea*

Introduction

Turkey is the primary genetic differentiation center of *Centaurea* L. species (1). These species are represented with 189 taxa in Turkey and they are the third largest species of Turkey. The taxa of the species spread over Mediterranean and Iran-Turan phyto-geographical regions *Centaurea* species have significant number of endemic taxa and these endemic taxa can be endemic nationwide or be endemic in a limited area. These species have 112 endemic taxa in Turkey and endemism ratio is about 60%.

As the endemic species, *Centaurea fenzlii* Reich and *Centaurea derderiifolia* Wagenitz were used in this study. Of these species, *Centaurea derderiifolia* Wagenitz was categorized in Red Data Book of Turkish Plants under LR (cd) Lower Rısk (Conservatıon Dependent)-(Requiring preservation measures) category and *Centaurea fenzlii* Reich was categorized as LR (lc) Lower Rısk (Least Concern) category.

Although seed propagation methods are commonly preferred to maintain maximum genetic diversity, tissue culture techniques are preferred in some hard-to-germinate species with less seed production, or in *ex situ* preservation of some species not able to sustain growth after germination under greenhouse conditions (2). There are interesting outcomes available on germination characteristics of various species through *in vitro* germination experiments under controlled conditions.

Padilla and Encina (3) developed a germination procedure to identify the best *in vitro* environment for *Annona cherimola* seeds. Researchers achieved a quite high germination ratio (like 80%) in distilled water environment with vitamin-supplemented 8.67 μM gibberellic acid at 30°C in dark.

Pitcairn et al. (4) carried out an *in vivo* seed germination study with *C. calcitrapa* species and indicated that the greatest germination rates could be achieved with medium level seed bed temperatures (15-20°C).

In a study carried out by Özel (5) for *C. tchihatcheffii* propagation under *in vitro* conditions, seeds were subjected to +4˚C for 13 days and hold at 18˚C in a dark incubator. It was reported that only 1 seed germinated after 150 days and root development of the resultant plant was abnormal. The researcher indicated that *C. tchihatcheffii* was propagated with seeds in nature, but when the seeds were taken for germination with classical methods, outcomes would be negative even with the supply of basic requirements and the plant was hardly germinated from the seeds.

Tıpırdamaz et al. (6) germinated *C. tchihatcheffii* seeds under *in vitro* conditions and achieved 65% germination in Murashige and Skoog (MS) nutrient medium including 30 g/l sugar, 6 g/l agar, 1 mg/l GA_3 and 0.225 mg/l BAP.

Young et al. (7) carried out an *in vivo* germination study with *C. solstitalis* and observed the greatest germination in seeds alternatively held each other in cold and hot periods.

Çakırlar et al. (8) pointed out that *C. tchihatcheffii* seeds were not able germinate at high temperatures and pH levels might be effective on seed germination. It was also reported that the seeds with pre-cooling treatments and kept at -79±2˚C temperature did not lose their germination ability and they exhibited a germination ratio of 10%. Researchers indicated that the seeds passed through a certain dormancy period yielded higher germination ratios, the seed left about 9 months following the collection from the field completed their germination in 7-10 days in soil + perlite mixture and germination rates reached to 90% levels. It was also reported that flowering stage was reached in 50-55 days, dormancy in seeds kept under laboratory conditions (20˚C, 50-60% humidity) lasted about 9 months and dormancy was probably originated from embryo rather than seed testa.

Materials and Methods

Centaurea fenzlii Reich

It is a biennial plant with thickened taproots. Stem is upright, 40-120 cm tall and upper sections are branched with couple large capitula (Figure 1). Leaves are stiff with short firm hairs and without a petiole. Flowers are yellow, achenes are 6 mm and pappuses are 6-10 mm. Blooming time is 6-7th months. They exist over steppes, forests, field sides and 1150-1820 m altitudes (9).

Centaurea derderiifolia Wagenitz

The stem is slanted, 25-103 cm tall, branched in upper part with a couple capitula (Figure 2). Leaves seem to have parallel lobes externally, second and third order lobes are not distinct, narrow, parallel sided, firm and lanceolate. Leaf surface is covered with thin smooth hairs. The leaf is attached to the stem without a petiole, the top is wide and sharp tipped and the bottom is butt based. Flowers are yellow and blooming is generally observed in July-August. The number of immature seeds is between 10-20. Achene length including pappus is around 2.6-3.3 cm (10).

In vivo works

Centaurea seeds were kept in 0.5-1% KNO_3 and 50-100 ppm GA_3 for 24 hours. They were rinsed through distilled water once before sowing. Then, autoclaved drying papers were placed in petri dishes and moisturized with distilled water. The seeds were spread over the drying paper with forceps. Petri dishes were closed and placed into a climate chamber. Since *Centaurea* sp. is a long-day plant, petri dishes were exposed to 16 hours light/8 hours dark photoperiod at 25˚C. A distinctive separation of radicle from testa was considered as germinated. Germinated seeds were counted at

Table 1. Descriptive statistics and comparison results for germination rate (%) of *C. fenzlii*

	Treatment Dose	GA_3		KNO_3	
		Avr.	St.Err.	Avr.	St.Err.
Germination rate (%)	50 ppm	0.17	0.10	0.28	0.11
	100 ppm	0.11	0.06	0.17	0.10
Average germination time (day)	%0.5	10.83	5.51	16.44	1.56
	% 1	10.00	5.29	11.00	5.57

the same hour of every day for 3 weeks. Experiments were conducted in 3 replications each of with 24 seeds.

In vitro works

Since high rate *Armillaria* fungus contamination was observed during *in vivo* germination of *Centaurea* sp. seeds, the seeds were treated with fungicide against this disease factor before *in vitro* germination processes. About 5 g Antracol combi fungicide was added to 250 ml distilled water. The seeds were kept in this solution for 20 minutes and kept in 20% sodium hypochlorite solution for 30 minutes. Then they were washed through distilled water 3 times. MS (11) solid growth medium (Duchefa Biochemie M0222.0010) was added 4.2 g per liter, WH solid growth medium (Duchefa Biochemie W0227.0010) was added 0.96 g per liter, B5 (12) solid growth medium (Duchefa Biochemie G0209.0010) was added 3.05 g per liter and SH (13) solid growth medium (Duchefa Biochemie M0225.0010) was added 3.18 g per liter. The pH of ready growth mediums was adjusted to 5.7. Except for WH medium, 30 g sucrose was added to MS and SH mediums and 20 g sucrose was added to B5 medium. All growth mediums were supplemented with agar (0.8%) and then they were subjected to sterilization process in an autoclave at 121˚C for 20 minutes (14). These treatments were implemented to determine germination rates under *in vitro* conditions. Thus, the seeds which were kept in 0.5 % KNO_3 and 50 ppm GA_3 dose for 24 hours yielding well outcomes under *in vivo* conditions were sown. Seeds of both species were also kept in a fridge at +4°C for 3 months to improve germination rates. Following the sterilization process, in each species, chilled (control group 4 replications), non-chilled (control group 3 replications), chilled + 0.5% KNO_3 (4 replications), non-chilled + 0.5% KNO_3 (3 replications), chilled + GA_3 50 ppm (4 replications), non-chilled + GA_3 50 ppm (3 replications) seeds were cultured in MS, B5, SH and WH mediums with 6 seeds in each for 3 weeks under 16 hours light/8 hours dark photoperiod at 25˚C in a climate chamber. Emergence of radicle from seed testa was accepted as germinated and germinated seed counts were taken at the same hour of every day throughout the culture period. The plantlets with emerged and parallel cotyledon leaves were accepted as completed the emergence. They were then counted and emergence rates were calculated.

Germination rate (%), average germination time (day) and average emergence time (day) were investigated in this study. Following equation was used to determine average germination time (day);

$(Ç_{50}) = \sum(g*n) / Sn$ (15).

g: day of count

n: Number of germinated seeds at the day of count

Sn: Total number of germinated seeds at the end of test

The following equation was used to calculate the average emergence time (day)

$$OÇS=\frac{N_1 \times D_1 + N_2 \times D_2 \ldots\ldots N_n \times D_n}{N_1 + N_2 + N_n} \quad (16).$$

Results

Results obtained under ın vivo conditions

Before to set up the experiments, tetrazolium test was conducted to determine seed vigor and the value was calculated as 20%. Descriptive statistics and comparison results for germination rate (%) of *C. fenzlii* are provided in Table 2. When the pre-treatments and their doses were compared (Table 3 and Table 4), it was observed in both species that the differences in germination rates and average germination times at different doses were not significant. Thus, it was con-

Table 2. Descriptive statistics and comparison results for germination rate (%) of *C. derderiifolia*

	Treatment Dose	GA_3		KNO_3	
		Avr.	St. Err.	Avr.	St. Err.
Germination rate (%)	50 ppm	0.55	0.15	0.11	0.11
	100 ppm	0.17	0.10	0.33	0.19
Average germination time (day)	%0.5	13.86	1.12	4.00	4.00
	% 1	8.50	4.37	8.58	4.29

cluded that both pre-treatments and the doses did not have significant effects on investigated traits.

Results obtained under ın vitro conditions

Since the germination rates (%) and average germination times (day) of *C. fenzlii* and *C. derderiifolia* species were different, species were not compared, only pre-treatments, chilling treatments and growth mediums were compared in both species separately.

It was observed in Table 4 providing germination rates (%) of C. *fenzlii* that the differences between germination rates of chilled and non-chilled seeds were not significant, but the differences between pre-treatments and growth mediums were significant. Among the growth mediums, WH was not different in control group from the pre-treatments and yielded the greatest germination rate (50%) in both chilled and non-chilled seeds. The germination rates in the other growth mediums varied between 0-11% and the differences among treatment means were not found to be significant. Similarly, the differences among pre-treatments of the other growth mediums were not also found to be significant. Therefore, it can be stated that pre-treatments and other growth mediums apart from WH medium did not have significant effects on germination rates (%) of both chilled and non-chilled seeds. In GA_3 treatment on the other hand, the lowest average germination time (12 days) in both chilled and non-chilled seeds was observed in WH medium. In chilled seeds, germination was not observed in MS medium, therefore average germination time was not able to be calculated for this medium and the average germination time was calculated as 16.33 days for SH medium and 15.33 days for B5 medium.

In Table 5 that the differences in germination rates of growth mediums, chilled and non-chilled seeds of treatment groups except for control group were not significant. In control group, the greatest germination (17%) was observed in SH medium of chilled seeds, it was followed by MS and WH mediums (4%)

Table 3. Descriptive statistics and comparison results for germination rate (%) of *C. fenzlii*

Growth medium	Chilling	Control		GA_3		KNO_3	
		Avr.	St.Err.	Avr.	St.Err.	Avr.	St.Err.
WH	Chilled	0.50 a A	0.10	0.25 a A	0.05	0.25 a A	0.11
	Non-chilled	0.50 a A	0.00	0.22 a B	0.05	0.33 a AB	0.10
SH	Chilled	0.04 b A	0.04	0.04 b A	0.04	0.04 b A	0.04
	Non-chilled	0.11 b A	0.06	0.06 b A	0.06	0.11 ab A	0.06
MS	Chilled	0.00 b A	0.00	0.00 b A	0.00	0.00 b A	0.00
	Non-chilled	0.06 b A	0.06	0.00 b A	0.00	0.06 b A	0.06
B5	Chilled	0.09 b A	0.05	0.04 b A	0.04	0.04 b A	0.04
	Non-chilled	0.11 b A	0.06	0.11 b A	0.11	0.06 b A	0.06

Table 4. Descriptive statistics and comparison results for germination times (day) of *C. fenzlii*

Growth medium	Chilling	Control		GA_3		KNO_3	
		Avr.	St.Err.	Avr.			Avr.
WH	Chilled	12.38 a A	0.33	11.63 a A	0.38	11.83	2.68
	Non-chilled	13.55 a A	0.91	11.33 a B	1.76	12.06	0.92
SH	Chilled	19.00 b A	-	12.00 b A	-	19.00	-
	Non-chilled	17.50 b A	1.50	19.00 b A	-	21.50	0.50
MS	Chilled	NG b A	-	NG b A	-	NG	-
	Non-chilled	19.00 b A	-	NG b A	-	21.00	-
B5	Chilled	20.50 b A	1.50	12.00 b A	-	21.00	-
	Non-chilled	23.00 b A	1.0	16.00 b A	-	13.00	-

and germination was not observed in B5 medium. In non-chilled seeds on the other hand, except for WH growth medium and KNO_3 pre-treatment (6%), germination was not observed in other mediums and pre-treatments. While the differences in germination rates of SH medium and chilled seeds of control group and the other GA_3 and KNO_3 groups were found to be significant, the differences in other mediums, chilled and non-chilled seeds of pre-treatment groups were not found to be significant.

Average germination times (day) for *C. derderiifolia* are provided in Table 6. While germination time was observed as 14.67 days in SH medium and chilled seeds, the value was observed as 22 days in non-chilled seeds and germination was not observed in other growth mediums. Therefore, average germination times were not able to be calculated for these mediums.

Discussion

Gibberellins with high levels in developing seeds have significant functions in seed germination and dormancy control. Effects of Gibberellic Acid (GA_3) treatments on germination have long been known and concentration and duration of GA_3 have significant impacts on germination.

Dormant seeds cannot germinate because of insufficient conditions. Non-dormant seeds can germinate when the required conditions were met. Water is alone sufficient for seeds of some species. In some other species, light, soil conditions and temperature fluctuations are also required for germination. Absence of these factors may hinder germination and seed may get into pseudo-dormancy. Dormancy is mostly related to internal gibberellic acid (GA_3) and abscisic acid (ABA).

In a previous study, *C. depressa* seeds which were kept at room temperature for 1, 6 and 12 months fol-

Table 5. Descriptive statistics and comparison results for germination rates (%) of *C. derderiifolia*

Growth medium	Chilling	Control		GA_3		KNO_3	
		Avr.	St.Err.	Avr.			Avr.
WH	Chilled	0.04 ab A	0.04	0.00 a A	0.00	0.00 a A	0.00
	Non-chilled	0.00 a A	0.00	0.00 a A	0.00	0.06 a A	0.06
SH	Chilled	0.17 a A	0.07	0.00 a B	0.00	0.00 a B	0.00
	Non-chilled	0.00 a A	0.00	0.00 a A	0.00	0.00 a A	0.00
MS	Chilled	0.04 ab A	0.04	0.00 a A	0.00	0.00 a A	0.00
	Non-chilled	0.00 a A	0.00	0.00 a A	0.00	0.00 a A	0.00
B5	Chilled	0.00 b A	0.00	0.00 a A	0.00	0.00 a A	0.00
	Non-chilled	0.00 a A	0.00	0.00 a A	0.00	0.00 a A	0.00

Table 6. Descriptive statistics and comparison results for germination times (day) of *C. derderiifolia*

Growth medium	Chilling	Control		GA_3		KNO_3	
		Avr.	St.Err.	Avr.			Avr.
WH	Chilled	16.00	-	NG	-	NG	-
	Non-chilled	NG	-	NG	-	8.00	-
SH	Chilled	14.67	4.81	NG	-	NG	-
	Non-chilled	22.00	-	NG	-	NG	-
MS	Chilled	NG	-	NG	-	NG	-
	Non-chilled	NG	-	NG	-	NG	-
B5	Chilled	NG	-	NG	-	NG	-
	Non-chilled	NG	-	NG	-	NG	-

lowing seed harvest were subjected to germination experiments at 5, 10, 15, 25 and 30°C temperatures. The greatest germination was observed as 42% in 1-month waited seed at 5°C, as 44% in 6-month waited seeds at 5 and 10°C and as 56% in 12-month waited seeds at 10°C. Effects of light were also investigated in that study and the germination rate was observed as 28% under 12 hours light + 12 hours dark conditions and as 21% under 24 hours dark conditions (17). Although the differences between KNO_3 doses of the present study were not significant, germination time was 5 days earlier in 1% treatment than in 0.5% KNO_3 treatment.

It was reported that *C. repens* seeds germinated between 0.5-35°C temperatures and optimum 20-30°C temperatures were sufficient, light was not required and varying temperature, light and dark periods improved germination (18). Despite insignificant differences between heat and cold treatments, the success ratios were higher in chilled seeds of the present study.

The effects of different GA_3 doses on *in vitro* and *in vivo* germination of clove seeds were investigated in a previous study. Plant seeds were exposed to 0, 10, 50, 100 and 250 ppm GA_3 concentrations for 24 hours to remove the inhibiting materials and to promote germination. Then the treated seeds were planted into MS medium under *in vitro* conditions and into 3 different mediums (peat, peat + perlite (1:1) and perlite) under *in vivo* conditions. With regard to germination rates both under *in vitro* and *in vivo* conditions, the best results were obtained from the control group and GA_3 doses had a decreasing effect on germination rates (19). These findings comply with the present findings.

Holding seeds in osmotic solutions has been a method of pre-sowing treatment since 1970s. The principle objective in these treatments is to create a difference in pressures between the water in seed and the surrounding solution and to allow the sufficient entrance of water to initiate germination. Theoretically, germination-stimulated seeds may exhibit faster and high rate emergence. As osmotic solutions, potassium nitrate, potassium dihydrogen phosphate and similar solutions are used at certain concentrations (20).

Türkoğlu et al. (21) carried out a study to determine germination rates and germination percentages of *Centaurea balsamita, Centaurea iberica* and *C. virgata* seeds at different temperatures and reported the best germination percentage as 90.22% at 15°C and germination rate as 36.71% at 15°C in *C. balsamita* species. Among the present species, *C. fenzlii* exhibited the best germination rate in WH medium under *in vitro* conditions and *C. derderifolia* exhibited better germination power and rate under *in vivo* conditions. The *C. derderifolia* and *C. fenzlii* species of the present study both had lower germination rates and percentages than the species studied by Türkoğlu et al. (21).

Plant biotechnology has been used to eliminate extinction threats exerted on plants by either humans or the other factors since 1970s and such technologies bring about significant alternatives to *in situ* preservation methods. In this study, single-point endemic (in Tunceli province) *Centaurea derderifolia* and *Centaurea fenzlii* seeds were successfully germinated under *in vivo* and *in vitro* conditions without any problems. Also, several number of plants were obtained in a short time and supports provided in this way for the propagation of these single-point endemic species. Exposure to various biotic (disease factors, wild plants, insects, microorganisms, animals), abiotic (physical impacts (drought, salinity, high or low temperature, radiation, plant nutrients, leaf roll and folding (heat stress), floods), mechanic (wind, snow and ice cover), chemical (air pollution, plant nutrients, pesticides, toxins, salts, soil pH) stressors and dormancy restricts seed-propagation of these species in their natural habitats. Single or combined treatments and some chemicals can be used to improve germination rate and speed of dormant *Centaurea* species. In present study, seeds were treated with KNO_3 and kept at 5°C and germinated in sugar-free WH medium. In further studies, seeds of these significant medicinal and ornamental plant species can be germinated in WH medium, then they can be used to get new plantlets through sub-cultures and ultimately a support can be provided for the preservation of these endemic species. These species with rapid propagation can also be used in landscape design as a dried flower garden plant or rock garden plant and they can also be marketed both in domestic or foreign markets. Since there aren't any previous studies about *in vivo* and *in vitro* germination of these species, current findings may lead further studies to be carried out with these species.

Acknowledgements

Authors thank to Van Yüzüncü Yıl University, Scientific Research Projects Department for the financial support provided to this project entitled as "Germination possibilities *in vivo* and *in vitro* conditions of some *Centaurea* sp." (Project No: 2015-FBE-YL055). (The paper is a part of the M.Sc. thesis of this project work).

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Correspondence:
Nalan Türkoğlu
Department of Horticultural Science, Faculty of Agriculture, Van Yüzüncü Yıl University, 65100, Van, Turkey.
E-mail: nalanturkoglu65@gmail.com