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OPTIMIZATION OF GERMINATION PROTOCOL FOR RARE ENDEMIC *AJUGA VESTITA* BOISS. UNDER *IN VITRO* CONDITIONS

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Abstract

Since the seeds of endemic *Ajuga vestita* are difficult to germinate naturally they were subjected to *in vitro* culture. Highest rate of germination was obtained in 1/4 MS medium supplemented with 30 g sucrose in light. Microshoots subcultured in 1/1 MS supplemented with 0.125 mg/l Kn rooted well and transferred to soil with 80% success.

Due to geographical location Turkey is one of the world's richest countries in terms of its flora diversity and endemic plants. However, this rich diversity of plants is in danger of extinction in Turkey due to various reasons. Endemic plants are more sensitive to environmental changes compared to other species. Hence they often suffer from the danger of extinction and not only that they have less genetic diversity than the other common of species (Primarck 1993). *In vitro* propagation is considered as a powerful tool obtaining rapidly a large number of plants, and for *ex situ* conservation of endangered plants (Fay 1994). Plant tissue culture technique had been used previously with success for many endangered plants (Weilan *et al.* 2015, Shukla and Shamla 2017 and Mao *et al*. 2018).

The genus Ajuga (Lamiaceae) is represented by 13 species in Turkey; and 6 of them are known to be endemic (Güner *et al.* 2012). Most of the previous studies related to this genus reflect of their biological activities, ethnopharmacology (Cocoquyt *et al.* 2011), antimicrobial and antioxidant activities (Türkoğlu *et al*. 2010). There are a few studies on *in vitro* propagation of it species (Sivanesan *et al*. 2011 and Sivanesan and Park 2015). To our knowledge, only one report provided limited information related to the *in vitro* propagation of *Ajuga vestita* Boiss. However, no efficient germination method has been established for this plants as yet. Therefore, an attempt was made to investigate the optimization of germination conditions of *A. vestita* and its adaptation to soil. The developed protocol for germination may help propagation of this species in a short time and can adapt as natural populations.

Seeds of *Ajuga vestita* Boiss. were collected from their natural habitat in south-eastern Anatolia region of Turkey, in Savur district of Mardin (Fig. 1a). Prior to culturing, the seeds were washed under running tap water for 10 min, followed by rinsing with 70% (w/v) ethanol for 30 sec. subsequently they were kept in 5% (w/v) NaOCl solution for 10, 15 and 20 min. and finally, the seeds were rinsed with sterilized water 5 times for 5 min to remove the NaOCI. Following the sterilization, explants were cultured on MS (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose. The pH of all media was adjusted to 5.8 before adding agar (0.54%). The inoculated explants were kept at 25±2°C under 16 light/8 hrs dark photoperiod.

The effect of MS medium (1/1, 1/2, 1/4), light (dark, light-16/8 photoperiod) and different kinds of sugar (3% sucrose, glucose, lactose and fructose) on germination of seeds of *Ajuga* at broken/unbroken seeds were investigated. After four weeks, the germination percentage was

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expressed as a number of seeds germinated and for each experiment 20 seeds were used, with 3 repetitions. Twenty-day old micro shoots were cultured in 1/1 MS medium supplemented with 0.125 mg/l of Kn. *In vitro* raised seedlings (1.5 - 2 cm) were transferred to 1/2 MS medium supplemented with 0.5 mg/l NAA together with a control. Rooting experiments were conducted with 20 explants and each experiment was repeated at least three times. The rooting percentage, mean root number and length were recorded after four weeks of culture. Rooted plantlets were washed carefully under running tap water to remove the agar adhered to the roots. Rooted plantlets were transferred to pots containing 2 : 1 turf and perlite and were kept in growth room for 3 - 5 weeks until they were transferred to soil.

Simple completely randomized design was used to analyse the data. Experimental values are given as means (cm) ± standard deviation, the mean were compared by DMRT using SPSS 20.0 for Windows (SPSS Inc., USA) to evaluate if the means were significantly different.

Sterilization of explants is very important *in vitro* studies because the ratio and duration of the solution used for sterilization can vary depending on the plant species or explant type. In our study, three different durations (10, 15 and 20 min.) at 5% NaOCl were tested with a control group (non-sterile seeds). Each one of the duration tested showed 100% effectiveness in sterilization, but there were no germination. For this reason, a part of the seeds slightly have broken under sterile conditions and transferred to the same medium. The differences between in the germination percentage of broken and unbroken seeds were observed. After about one week, root formation was seen on broken seeds, but this was not observed on unbroken seeds. The germination percentages were significantly found different between the treatments. Treatment for different durations in the 5% NaOCI solution was examined, it was found that the treatment for 15 min was better for both germination and morphological development than other treatments (10 and 20 min). As a result, 15 minute treatment was taken as the optimum time for decontamination and germination of *A. vestita* seeds. Also, it was found that breaking of seed shell was necessary for germination.

Table 1. Effect of the strength of MS and sugar kinds on germination and development of *Ajuga vestita* seeds.

Applications		Germination percentage		Morphological development	
		Broken seed	Unbroken seed	Av. root length (cm)	Av. plants length (cm)
Carbon source	3% (w/v) sucrose	80	0	0.00 ± 0.00 ^{c}	1.84 ± 0.33 ^{a}
	3% (w/v) lactose	100	10	0.81 ± 0.14 ^{a}	1.30 ± 0.09 ^{ba}
	3% (w/v) fructose	90	50	0.00 ± 0.00 ^{c}	0.48 ± 0.18 ^{c}
	3% (w/v) glucose	100	10	0.31 ± 0.09 ^{b}	0.88 ± 0.11 ^{cb}
Strength of MS salts	Control	100	0	0.66 ± 0.17 ^{b}	0.86 ± 0.15 ^{b}
	¼ MS	100	0	2.04 ± 0.49 ^{a}	2.88 ± 0.64 ^{a}
	½ MS	100	0	1.77 ± 0.26 ^{a}	2.34 ± 0.37 ^{a}
	1/1 MS	80	0	0.00 ± 0.00 ^{b}	1.84 ± 0.33 ^{ba}

Each value represents the mean of three replicates per treatment (n = 20). Values of means within a column followed by the same letter(s) are not significantly different according to DMRT ($p \leq 0.05$).

It is important to determine the germination strength and nutrient media conditions for seeds so that *in vitro* studies can be carried out without harming the endemic plants in their natural

habitats (Erdoğan 2010). It was also investigated the effect of MS strength on germination in this study. It was observed that the development of germinated seeds was slow in the control group after a while, but the seeds in the ¼ MS continued to develop at the same rate (Fig. 1b). The morphological development of microshoots on 1/4 MS medium was found to be more significant than the other applications (Table 1). It has been reported that the mineral requirement in the germination process can vary from species to species, and this is probably due to reserves in the seed (Padilla and Encina 2003). Similarly in our study, it was determined that only water and agar were sufficient in the first days for germination to occur but the necessity of MS nutrients for the development of micro shoots in the following days. Comparatively sucrose and lactose were more effective than fructose and glycose for seed germination. Lactose promoted development of root (Table 1, Fig. 1c). In addition, it was also found that the seeds germinated in both dark and light but these seeds germinated better in light. It was observed that seeds germinated well ¼ MS medium with 30 g sucrose. In seedlings obtained from mature seeds were transferred to 1/1 MS medium with 0.125 mg/l Kn to stimulate seedlings development.

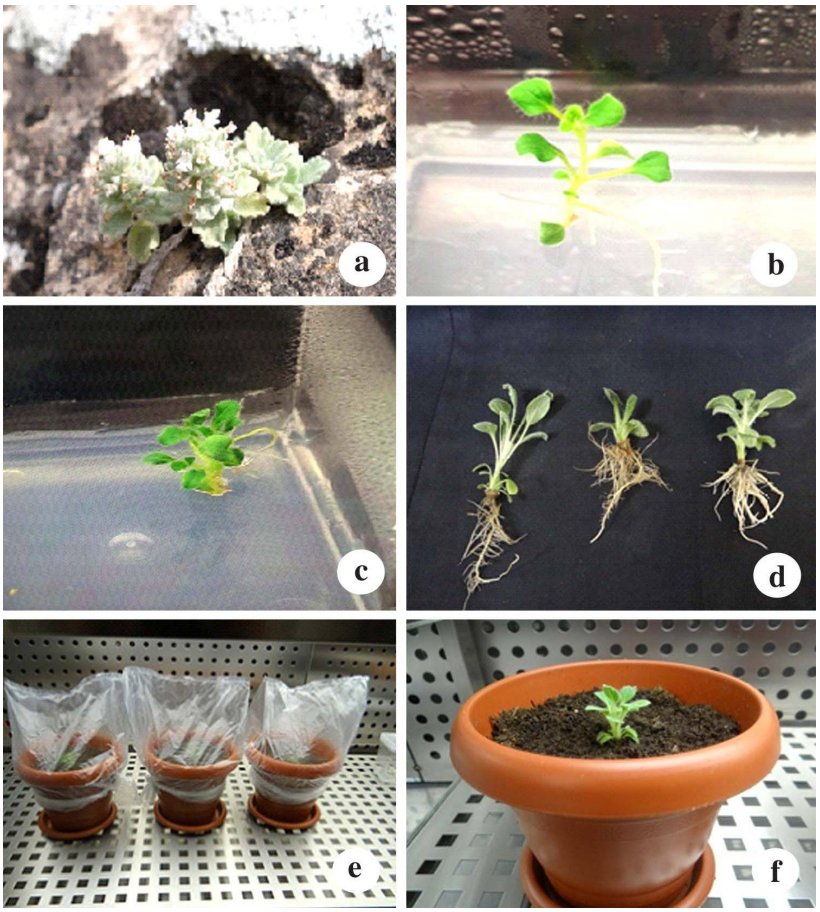

Fig. 1. Under *in vitro* conditions, germination of *Ajuga vestita* seeds. a. Endemic *Ajuga vestita* plants in its natural habitat. b. Germinated seeds in ¼ MS medium. c. Germinated seeds in the MS medium supplemented with 3% (w/v) sucrose. d. Rooted plantlets *in vitro* conditions. e,f. Adaptation of plantlets to soil.

MS medium supplemented with 0.5 mg/l NAA and with a hormone-free was used for rooting of seedlings of *A. vestita*. For this purpose, the elongated seedlings (2 - 3 cm) were clipped and transferred to the rooting medium. About two weeks after culture, rooting occurred in hormone-free medium but the culture medium supplemented with 0.5 mg/l NAA was found not to be effective enough for the rooting of shoots. Whereas, a large number of primary and secondary root formation were observed in both media after four weeks. While there was no difference between two media in terms of the number of roots, it was found that hormone-free medium gave better results for root length. Nevertheless, it was seen that the formation of new shoots were produced in the medium containing 0.5 mg/l NAA was healthy than the control. Gao *et al.* (2003) stated that *in vitro* rooting in different plant species depends on the internal hormone levels of plants. Similarly, Piatczak *et al.* (2005) suggested that MS medium without hormones can be successfully used for rooting. On the contrary, Kaul *et al.* (2013) reported that in the rooting of *in vitro* shoots of *Ajuga bracteosa*, obtained 100% success with 20 roots per shoot in medium containing 0.5 mg/l IBA. The results of this study showed that use of NAA was not necessary to promote rooting of *A. vestita*.

Rooted seedlings were planted in pots containing a mixture of peat and perlite (Fig. 1d). These pots were covered with a transparent pouch and kept in growth chamber. The transparent pouches were opened at regular intervals for 10 days from day 2, and the humidity of the environment was kept under control. The pots were completely opened at the end of the 10 th day, and after 2 weeks, the said plantlets were transfered to greenhouse (Fig. 1e,f). During acclimatization, the shoots were elongated and the leaves were widen. The plants continued to grow for one month, and finally were transferred to the soil with 80% success.

In the present study, the suitable procedure was established for *in vitro* germination of *A. vestita* and its acclimatization. The developed protocol for germination may help propagation of this species in a short time and can be gain back to their natural populations. Consequently the development of a reliable *in vitro* germination protocol is of great importance for production or conservation of rare plant species.

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