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Studies on *in vitro* germination on endemic *Salvia* L. species

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Abstract
This study used seeds of two endemic sage plants (*Salvia siirtica* and *Salvia kronenburgii*) as a starting material. Mucilage causes dormancy in the seeds of these plants. Therefore, it is important to improve the germination performance of these plants' seeds, and in this study, some treatments were applied to the seeds before or during sowing. To this end, sodium hypochlorite, ethyl alcohol, gibberellic acid, seed cracking, removal of the seed coat, pre-cold treatment, and sulfuric acid treatments were applied to the seeds of the two species separately or in combination, and their germination performances were investigated in comparison with a control group. Considering the results higher germination rates were obtained for both plants compared to the control group in all treatments except sulfuric acid treatments. The best germination rate for both plants was obtained from the treatments where the seed coat was mechanically removed. In this treatment, the germination rate in *S. siirtica* increased 3.3 times, while it increased 2.4 times in *S. kronenburgii* compared to the control group. Additionally, GA treatments for *S. siirtica* and cold pre-treatments for *S. kronenburgii* significantly increased germination rates. In light of these results, the removal of the seed coat, gibberellic acid, and cold pre-treatment effectively broke dormancy in sage seeds and increased germination rates.

Keywords: Dormancy, Germination, *In vitro*, Endemic, *S.siirtica*, *S.kronenburgii*

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INTRODUCTION

Seed germination is one of the most critical stages of plant life and forms the basis of agricultural production (El-Keblawy and Al-Rawai, 2005). Detailed knowledge of germination patterns is important for successful cultivation (Pająk et al., 2019), and understanding the mechanism underlying species germination barriers (dormancy, abiotic factors, etc.). Seed germination, which significantly impacts the plant growth cycle, is temporarily delayed in some plants due to dormancy (Bu et al., 2008). The presence of germination-inhibitory substances in the seed coat, embryo, and seed is among the factors affecting seed dormancy (Latifi, 2001; Elamin et al., 2013). Seed germination studies, which are important for biodiversity protection, should know the germination conditions of seeds for the species studied.

The Lamiaceae, which has an important place among medicinal and aromatic plants, is the third largest family in Türkiye by the number of taxa and the fourth largest by the number of species. Among the members of the Lamiaceae family, which includes many economically and medicinally important species, *Salvia*, the largest genus of the family, contains approximately 950-1000 taxa. The genus *Salvia* is represented by 107 taxa in Türkiye, of which 58 are endemic and have an endemism rate of 54% (Celep and Dirmenci, 2017). *Salvia* is derived from the word "salvare," meaning healing (Amiri, 2007), and was used in ancient times as tea, for pain relief, sore throat, and influenza (Zhou et al., 2005; Mayekiso et al., 2008). Many studies have reported that the contents of *Salvia* species mainly include phenolic acids, flavonoids, terpenes, and terpenoids, and these secondary metabolites provide pharmaceutical and biological benefits due to their antimicrobial, antifungal, antiseptic, analgesic, anticancer, and insecticide properties (Lu and Yeap Foo, 2002; Fidan et al., 2021; Uysal et al., 2023). In Türkiye, which has rich biodiversity, the germination requirements of natural species are mostly unknown, and therefore,

studies on seed germination are insufficient. Various studies have recently investigated seed germination in the Lamiaceae, Asteraceae, and Brassicaceae families (Surgun-Acar et al., 2017).

With recent climatic changes, factors such as sudden rainfall, floods, droughts, desertification, and salinity, as well as excessive and unconscious collection of plants, overgrazing and human pressure have brought many plants to the point of extinction. Especially the future of endemic populations with few individuals is at a higher risk (Rakotonandrasana et al., 2023). Various *in vitro* tissue culture methods (micropropagation, germplasm preservation, etc.) are applied to conserve and produce endangered and difficult-to-propagate species. In these methods, seeds are an alternative explant source and enable the production of as many seedlings as desired indefinitely under aseptic conditions for various purposes, saving time and space (Babaoğlu et al., 2002; Gökdoğan et al., 2022). *Salvia* is propagated by seeds. However, since their seeds have a mucilaginous seed coat and dormancy, i.e., factors that inhibit germination (Tursun, 2019), tissue culture techniques can be considered an effective alternative method in the germination of these species.

S. siirtica Kahraman, Celep & Doğan, which is 'Critically Endangered' (CR) with very few individuals and confined to a very narrow range (Kahraman et al., 2011), and *S. kronenburgii* Rech.fil., which is categorized as 'Endangered' (EN), are endemic taxa growing in Türkiye (Kuşaksız, 2019). Seed germination studies on rare and endemic species are important in determining species conservation strategies for these plants. These species are in danger of extinction in many cases. Therefore, an accurate and precise understanding of the germination ability of these taxa is important for the continuity, conservation, and development of the species (Arslan et al., 2017). For all of the above-mentioned reasons, this study was conducted to determine the effect of different treatments on breaking the germination barrier and improving the germination potential of seeds of endangered endemic *S. siirtica* and *S. kronenburgii* plants using tissue culture methods.

MATERIALS AND METHODS

Plant material

The mature seeds of endemic *S. siirtica* (Siirt Tillo Çatılı Village - 37º 58' 19" N / 42º 01' 51" E, 1491 m) and *S. kronenburgii* (Van Tuşba Ayanis Village - 38º 41' 56" N / 43º 11' 44" E, 1741 m) used as starting material in this study were collected and identified by Hüseyin EROĞLU (PhD).

Seed Surface Sterilization

After the seeds were rinsed in tap water, they were kept in 70% ethanol (EtOH) for 30 s and then in 5% sodium hypochlorite (NaOCI) for 10 min for surface sterilization. After surface sterilization, the seeds were rinsed thoroughly with sterile distilled water to remove the chemicals.

Treatments

Different chemical substances and their combinations were used to break the seed coat's dormancy, increase germination rates, and reveal differences between the treatments in improving the germination potential. To this end, the mature seeds of each plant, whose surfaces were sterilized, were subjected to the following treatments for germination: 48% H_2SO_4 for 60 and 120 s; 96% H_2SO_4 for 60 and 120 s; -20ºC for 10 h with and without cracking; sanding; 250 and 500 ppm GA for 4 h; 250 and 500 ppm GA-supplemented ¼ Murashige&Skooge (Murashige and Skooge, 1962) medium cultivation and mechanical removal of seed coat (Table 1). The treatment group that was only pre-sterilized in 70% EtOH for 30 s and then in 5% NaOCI for 10 min was considered the “control” group.

Table 1. Different treatments for breaking the germination barrier

Treatments			
1	Control	8	Sanding
2	48% H_2SO_4	9	250 ppm GA
3	48% H_2SO_4	10	500 ppm GA
4	96% H_2SO_4	11	250 ppm GA + MS
5	96% H_2SO_4	12	500 ppm GA + MS
6	-20ºC (cracking)	13	Removal of the seed coat
7	-20ºC (non-cracking)		

To determine the effect of different treatments on dormancy breaking in seeds, a randomized experimental design was established with three replicates. A total of 100 seeds were sown for both plants, four seeds in each Magenta GA-7 culture vessel for each treatment.

Culture Conditions

A ¼ MS medium supplemented with 30 g L^{-1} sucrose and pH 5.8 was used for seed germination. Then, 5.465 g agar was added to the medium and sterilized in an autoclave at 1 atm and 121°C for 25 min. The prepared medium was equally divided into Magenta GA-7 culture vessels. The sterilized and pretreated seeds were cultured in these containers and left to germinate in the plant growth chamber where aseptic conditions were provided with 16 hours light/8 hours dark photoperiod and 24±1°C (during 24 hours) temperature. The emergence of a radicle on the seeds was considered a germination criterion, and germinated seeds were counted daily for about two weeks. Germination rates were calculated as % for each treatment.

Statistical analysis

All experiments were performed in triplicate. The results of the activity assays are shown as means ± standard error (SE). The data analysis was performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, USA). The significance of differences was tested using a one-way analysis of variances (ANOVA) and Tukey's test at a 0.05 level of significance.

RESULTS

Table 2 and Figure 1 show the effects of different treatments and their combinations on the germination of *S. siirtica* and *S. kronenburgii* seeds.

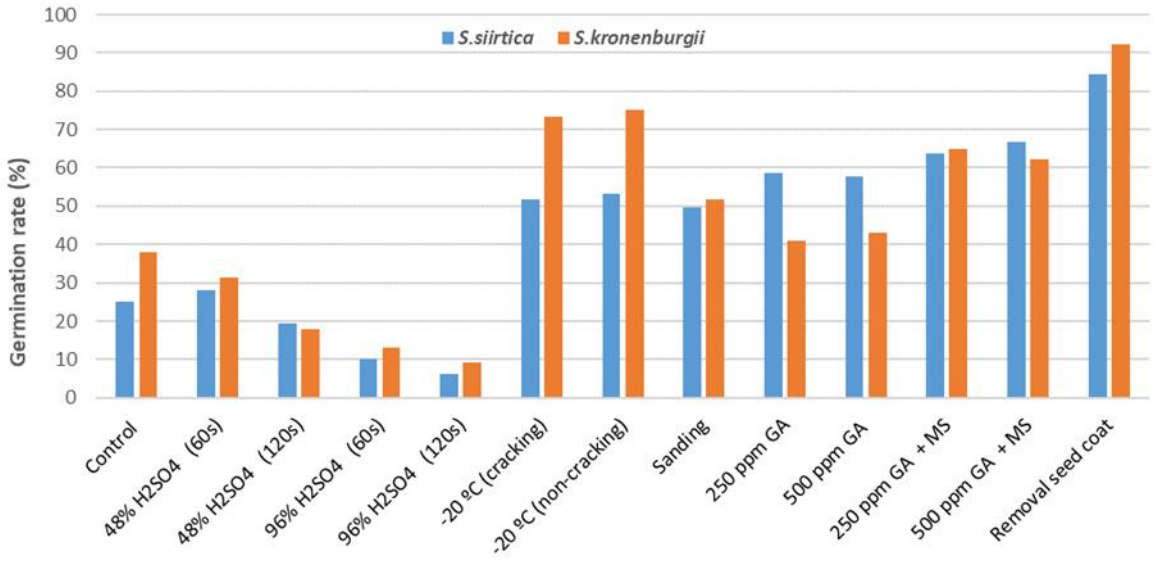

Figure 1. Effects of different treatments on the germination percentage of *S. siirtica* and *S. kronenburgii* seeds under *in vitro* conditions (%)

Considering the results for *S. siirtica*, the highest germination rate was obtained from the treatment where the seed coat was mechanically removed at 84.4%, followed by the treatment where seeds were cultured in a GA-supplemented MS medium with 66.7% and 63.6% germination rates and these treatments were found to be statistically different. Approximately half of the seeds germinated in cold pretreatment and sanding treatments. However, cracking/non-cracking treatment did not contribute significantly to the germination rate. Sulfuric acid treatments reduced the germination rate of seeds compared to the control group (except 48% 60 s). Furthermore, the germination rate decreased significantly in these treatments with the increased concentration and duration. These decreases were found to be statistically significant. For *S. kronenburgii*, all treatments, except sulfuric acid treatments, resulted in higher germination rates in comparison with the control group. The highest germination rate (92.2%) was obtained from the treatment where the seed coat was mechanically removed, followed by -20ºC cold pretreatment with 75.0% and 73.3% germination rates, while cracking had no significant effect. The effects were not statistically different. Seeds cultured on the GA-supplemented MS medium had higher germination rates than those pretreated with GA (Figures 2 and 3).

Table 2. Effects of different treatments on the germination percentage of *S. siirtica* and *S. kronenburgii* seeds under *in vitro* conditions (%)*

No.	Treatments	Time	*S.siirtica*	*S.kronenburgii*
1	Control	10 minutes	25.1±0.37^{l}	38.0±0.54^{f}
2	48% H_2SO_4	60 seconds	28.0±0.23^{k}	31.5±0.69^{g}
3	48% H_2SO_4	120 seconds	19.3±0.15^{m}	17.8±0.81^{h}
4	96% H_2SO_4	60 seconds	10.0±0.73^{n}	13.1±0.32^{k}
5	96% H_2SO_4	120 seconds	6.1±0.18^{o}	9.2±0.15^{l}
6	-20ºC (cracking)	10 hours	51.9±0.42^{g}	73.3±1.29^{b}
7	-20ºC (non-cracking)	10 hours	53.4±0.95^{f}	75.0±1.09^{b}
8	Sanding	-	49.6±1.03^{h}	51.7±0.87^{d}
9	250 ppm GA	4 hours	58.8±0.45^{d}	41.0±0.73^{e}
10	500 ppm GA	4 hours	57.9±1.27^{e}	43.0±0.35^{e}
11	250 ppm GA + MS	-	63.6±0.75^{c}	64.8±0.29^{c}
12	500 ppm GA + MS	-	66.7±1.02^{b}	62.1±0.99^{c}
13	Removal of the seed coat	-	84.4±0.87^{a}	92.2±1.09^{a}

*Vertical bars indicate ± SE. Letters (a-k) indicate significant differences ($p \leq 0.05$) compared to the control group.

Figure 2. 28-day *in vitro* development of *S. siirtica* with the seed coat removal treatment

Figure 3. 28-day *in vitro* development of *S. kronenburgii* with the seed coat removal treatment

DISCUSSION

Seeds of most medicinal plants adapt to environmental conditions differently. Therefore, it is necessary to create optimal conditions for the germination of seeds of medicinal plants and determine the ecological/physiological factors affecting dormancy in their production (Khakpoor et al., 2015). Studies on the seed pretreatment of *Salvia* plants have shown that they exhibit dormancy caused by mucilage in the hard seed coat (Khakpoor et al., 2015; Yaman, 2020). Therefore, to break this dormancy and improve the germination potential, some pretreatments (cold pretreatment, soaking in gibberellic acid, immersion in sulfuric acid, mechanical removal of the seed coat, etc.) were applied to the seeds in the present study. In our study, germination by the mechanical removal of the seed coat gave the highest germination rate (92.2% and 84.4%, respectively) compared to the other treatments for both species (*S. kronenburgii* and *S. siirtica*). Likewise, in a study conducted to improve the germination of *Vitex doniana*, whose population was declining due to low yield, long germination time and overharvesting, removing the seed coat followed by germination on filter paper resulted in the highest germination percentage (87%) (Haruna et al., 2024). In another study, to increase the *in vitro* seed germination success of *Jatropha curcas*, some pretreatments (imbibition, stratification, scarification, and removal of the seed coat) were performed before sowing. The dormancy of the seeds was broken only by removing the seed coat and the seeds were kept at 25 ± 2˚C in the dark and cultured in a 1/1 MS medium and 100% germination was achieved.

Furthermore, many methods, such as chemical etching, physical etching, and hormone treatments, are used to break dormancy in various plant seeds (Uludağ and Özer, 1999; Serim and Sözeri, 2011; Bozdoğan et al., 2019). Among the dormancy-breaking procedures performed on *Adonis aestivalis* seeds, the most effective method was GA treatment at 150 ppm, and the germination rate was 20.0% (Taşkesen, 2007). Another study reported that 250 and 300 ppm GA solutions applied to *Hippomarathrum microcarpum* seeds increased the germination rate above 50.0% (Ertuş et al., 2011). In support of the researchers, Tursun (2019) obtained the highest seed germination rate (74%) for breaking dormancy in *Salvia verticillata* seeds with 2000 ppm GA and complete darkness treatment. Subaşı and Güvensen (2010) detected very low germination in the pre-chilling and non-pre-chilling groups for *Salvia smyrnaea* seeds and revealed that pre-chilling was insufficient for breaking dormancy and gibberellin was also necessary. Our results regarding the significant increase in the germination percentage of the seeds cultivated in the GA-supplemented ¼ MS medium agree with the data in the literature. In their study on breaking the high dormancy in *Sinapis arvensis* seeds, Ateş and Üremiş (2021) provided the best germination rates with 100% in 2000 ppm GA_3 and 91.9% in H_2SO_4 (60 s), respectively. Tursun (2019) reported that when the exposure time to sulfuric acid was increased from 60 seconds to 15 minutes in *Salvia verticillata* seeds, the germination rate also increased but then decreased significantly after 15 minutes. Likewise, Tuncer and Ummuhan (2017) found high germination rates for Molehiya (*Corchorus olitorius* L.) seeds subjected to sulfuric acid treatment for 5-10 minutes and reported statistically significant decreases with the increased duration. Bhardwaj et al. (2016) examined the effects of stratification, scarification, acids (H_2SO_4, HNO_3, and HCl), gibberellic acid, and alcohol on germination to break dormancy in various medicinal plants. The best germination rate for all plants was obtained in seeds treated with H_2SO_4 alone. In the present study, H_2SO_4 treatments displayed the lowest germination percentage for both species, generally lower than the control group.

Ismaili et al. (2023) examined the effects of different temperature treatments to determine the optimum germination conditions for *Stachys mouretii* and obtained the highest germination percentage (66.5%) at 25/10°C. Young and Young (1992) reported that most *Salvia* taxa require cold pretreatment for germination. Yücel and Yılmaz (2009) determined high germination rates (78%) in *Salvia cyanescens* after pre-chilling at constant temperature of -5ºC for 5 minutes. In our study, cold pretreatment significantly increased the germination rate for both plants, while this rate was higher (75%) for *S. kronenburgii*.

Özcan et al. (2014) determined that gibberellin (95.1% and 50.2%, respectively) for *Salvia officinalis* and *Salvia fruticosa* and pre-drying (42.0%) and ethylene (47.6%) for *Salvia pomifera* species were the best media for germination. The researchers reported significant differences between the species' germination rate and germination strength values. Another study by Arslan et al. (2017) measured the highest germination and rooting rates (21.25% and 17.97%, respectively) for *S. siirtica* seeds in a 28-day stratification treatment. In the study, GA, citric acid, and +4ºC retention treatments applied to *S. siirtica* seeds did not achieve success in germination. In another study in which seeds of *Salvia verticillata* species were treated with sanding and hot water, the effect of sanding on germination was higher, at 53% (Khapor et al., 2011). In a study on the effects of different dormancy-breaking treatments on the germination of *Zaleya pentandra* seeds, the treatment with sandpaper was highly effective in breaking seed dormancy (Munawar et al., 2015). The effects of different concentrations of gibberellic acid, potassium nitrate, and mechanical scarification were investigated to break dormancy and improve germination in yarrow seeds. It was stated that sanding by mechanical scarification increased germination (Nejad et al., 2022). In our study, sanding before sowing germinated about half of the seeds in both plant species.

CONCLUSION

S. siirtica and *S. kronenburgii*, which are the subject of our study, are endangered plant species whose seed coat contains mucilage inhibiting germination. In our research conducted to improve germination in these plant species, different pretreatments were applied to the seeds before germination under *in vitro* conditions and mechanical removal of the seed coat promoted germination at the highest rate. However, the other treatments (except the H_2SO_4 treatment) generally increased the germination by 50% or more compared to the control group. In conclusion, the removal of the seed coat used to break mucilage-induced dormancy may improve the germination potential of other species and enable mass multiplication. Furthermore, these results provide basic information on possible treatments to restore endemic and endangered species in their natural habitat.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

P.O., İ.S.K.- Analysis, interpretation, literature review and writing. All the authors read and approved the final manuscript. All the authors verify that the text, figures, and tables are original and that they have not been published before.

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