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Research Article

In Vitro Seed Germination and Seedling Development of An Endemic Medicinal Plant *Hypericum adenotrichum* Spach

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Abstract

In this study, it has been aimed to describe an appropriate procedure for *in vitro* seed germination and seedling development of *Hypericum adenotrichum* Spach which is an endemic plant under *in vitro* conditions. At he end of experiments, It has been found that seed germination of *Hypericum adenotrichum* is effected by stratification, photoperiod, dark, different temperature and Murashige Skoog media (MS) with macro salt ingradient in different levels (MS, ½ MS and ¼ MS) and vitamin additions (MS vitamins, B5 vitamins and Galzy vitamins) to these media. The highest germination value has been held when sterilized seeds were incubated in ¼ MS/Galzy nutrient medium under 16/8 photoperiod and 18 ºC conditions after stratification at 4ºC for 2 months. All seeds were observed during 8 weeks (22 ºC and 16/8 photoperiod) and radicula emergence was mostly in 7th days of incubation. At the end of these processes, parameters such as the highest number of shoots per seedling, mean number of lateral roots, mean length of shoots and number of leaves per shoot were evaluated by one-way analysis of variance and it has been concluded that ¼ MS/Galzy medium is the most proper media for seedling development.

Keywords: *Hypericum adenotrichum*, endemic plant, germination, *in vitro*, MS, seedling.

Endemik ve Tıbbi Bir Bitki Olan *Hypericum adenotrichum* Spach'un *In Vitro* Tohum Çimlenmesi ve Fide Gelişimi

Öz

Bu çalışmada, endemik ve tıbbı bir bitki olan *Hypericum adenotrichum* Spach'ın *in vitro* tohum çimlenmesi ve fide gelişimi için uygun bir yöntem geliştirilmesi amaçlanmıştır. Denemelerin sonunda; stratifikasyon, fotoperiyod, karanlık, farklı sıcaklık ve farklı seviyelerde makro tuzlara sahip Murashige ve Skoog besi ortamlarının (MS, ½ MS ve ¼ MS) ve bu ortamlara ilave edilen vitamin bileşenlerinin (MS vitaminleri, B5 vitaminleri, Galzy vitaminleri) *Hypericum adenotrichum*'un tohum çimlenmesini etkilediği belirlenmiştir. En yüksek çimlenme değeri; 2 ay süre ile + 4ºC'de stratifikasyon uygulanmış tohumların, 18 º C'de 16/8 fotoperiyot koşulları altında ve Galzy vitaminleri ile desteklenmiş ¼ MS ortamında inkübe edilmesi ile elde edildiği görülmüştür. Çoğunlukla inkübasyonun 7. gününde radikula çıkışı gösteren tohumlar, fide gelişimi için 8 hafta boyuna izlenmiştir (22 º C ve 16/8 fotoperiyot koşullarında). Bu sürenin sonunda fide başına ortalama en yüksek sürgün sayısı, ortalama yan kök sayısı, ortalama sürgün uzunluğu ve sürgün başına ortalama yaprak sayısı parametrelerinin tek yönlü varyans analizi ile değerlendirilmesi sonucunda ¼ MS/Galzy ortamının fidelerin gelişimi için de en uygun ortam olduğu tespit edilmiştir.

Anahtar Kelimeler: *Hypericum adenotrichum*, endemik bitki, çimlenme, *in vitro*, MS, fide.

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1. Introduction

For the protection and management of biodiversity, first of all, information about the reproductive biology and seeds of the endangered species should be investigated and seed germination needs should be determined [1]. Seed is the most widely used part of the plant to protect the plant germplasm and transport it to the place where it will be used [2]. The most important stage of seed multiplication is to obtain an efficient germination procedure. Low germination rates in plants can be caused by unsuitable environmental conditions during seed germination, low seed strength or seed dormancy [3]. Seed germination behavior is an inseparable part of *ex situ* protection to provide efficient populations especially for germplasm and to improve the standard vigor monitoring protocol [4]. Plant tissue culture techniques are a powerful alternative technique for the production of plants that are difficult to reproduce by conventional methods [5]. Many plants that have problems in their production have been germinated and reproduced by using this method, [6-8].

In Turkey, *Hypericum* species is represented in 19 sections by about 100 taxa and 45 of these taxa are endemic [9]. Turkey is a crucial gene center for *Hypericum* L. species. *Hypericum adenotrichum* Spach is a perennial herbaceous and endemic plant, which grows in Turkey. Species-related studies are focused on the identification of the chemical and pharmacological properties and the determination of the secondary metabolite content [10-14]. Within the literature research, it is not found any information about the *in vitro* germination and seedling development of *H. adenotrichum*. This work aims to identify the necessary conditions for *in vitro* germination desires and seedling development of *H. adenotrichum*. The results of the study will be the starting step for the *ex situ* protection programs of the species.

2. Material and Method

Our study material, the seeds of *H. adenotrichum* Spach were collected from the Karıncalı Mountain (Karacasu, AYDIN, TURKEY), about 1400 meters, where the plant has a natural distribution. As a result of the morphological observations of the seeds in the laboratory conditions, most of the seeds were found to be empty and full seeds were determined by using flotation technique [15]. According to this technique, the seeds were first taken in a beaker containing distilled water and were periodically mixed with a glass baguette. After a period of 30 minutes, the seeds remaining on the surface of the water were removed by means of a strainer. Thus, the seeds (empty) left on the surface of the water are separated from the seeds (full) in the bottom of the beaker. The results were evaluated as %.

As a result of the morphological observations, the seeds which were thought to be full took the Tetrazolium test [16] and the number of live seeds was determined as percentage.

For *in vitro* germination experiments, full seeds were used. In this way, it was tried to eliminate the possibility of empty seeds to reduce the germination rate randomly. The seeds were washed out under the tap water for about half an hour to remove the rough dirt. After this, the seeds were sterilized in 70% ethyl alcohol (1 minute) and in 1-2 drops of Tween 80 added NaOCl (2.25%) at 2.5 minutes. The seeds were then washed 3 times with sterile distilled water.

In the preliminary trials, the germination cannot be achieved, which suggested that the seed of *H. adenotrichum* have special requirements for germination or may have dormancy. As known, a number of basic factors such as temperature, humidity, oxygen, light play a role in the realization of germination. Considering these key factors, to promote *in vitro* germination and also growth and development; cold application (stratification), different temperature applications, dark-light conditions and different culture media were tried.

During the stratification process, which is one of the ways to break the dormancy, the seeds were exposed to +4 ºC for 2 months. To investigate the effect of temperature on seed germination and development, incubation temperatures at 15, 18, 22 and 25 ºC were tried and 16/8 photoperiod and completely dark conditions were created to study the effects of light.

In the experiments, full Murashige and Skoog medium (MS) [17], ½ and ¼/ MS medium and MS medium supplemented with B5 [18] and Galzy vitamins [19] were used as incubation media.

½ MS and ¼ MS media were prepared by reducing MS macro salts respectively at ½, ¼ rate. ½ MS/B5, ¼ MS/B5 media were prepared by reducing MS macro salts respectively at ½, ¼ rate and adding vitamin concentrations belonging to the B5 medium instead of MS vitamins. ¼ MS/Galzy medium was prepared by reducing MS macro salts at ¼ rate and adding Galzy vitamin concentrations instead of MS vitamins.

In trials, radicle emergence was taken as germination criterion. Approximately 2 weeks after the beginning of the tests, three-way variance analysis was used to evaluate the data of seed germination. The data of germination was used in the variance analysis by transforming with $x' = \arcsin \sqrt{(x/100)}$. The differences that were considered to be significant among averages were compared by the Tukey multiple comparison test. One-way variance analysis was used in the assessment of the data of seedling development parameters. The differences that were considered to be significant among averages were compared by the Duncan multiple comparison test. For statistical analysis, SPSS 15.0 program was used.

3. Results and Discussion

Morphological observations were revealed that approximately 70% of the seeds were empty. According to the flotation technique, the seeds sinking into the bottom of the bowl were “full” and the floating seeds were considered in empty. As a result of the flotation technique, approximately 67% of the 100 seeds were found to be empty and 33% were full. These values are almost the same as the results obtained from the morphological observations made by observing the seeds individually. In addition, the tetrazolium test applied to the seeds determined to be “full” gave the result that the seeds are alive 100%. The flotation method helps in separating many empty, broken, diseased or insect-damaged seeds. This method is a method especially applied in heavy seed species and gives very good results for large seeds with high moisture content [20]. Although the method was generally applied to large seeds, it yielded successful results in distinguishing relatively small *H. adenotrichum* seeds. It was concluded that buoyancy method could be effective in preventing seed loss especially in experiments with endemic plants.

Table 1. Germination percentages obtained in dark condition

Germination percentages in dark (%)								
Germination media	Cold application (+4ºC)				Without cold application			
	Temperatures (ºC)				Temperatures (ºC)			
	15	18	22	25	15	18	22	25
MS medium	25	30	20	10	10	10	5	0
½ MS medium	25	40	25	10	15	15	10	0
¼ MS medium	30	40	35	20	15	20	10	5
MS / B5 vitamins	25	30	20	10	10	10	5	0
½ MS /B5 vitamins	25	35	25	10	10	15	5	0
¼ MS/B5 vitamins	30	40	30	20	15	20	10	0
¼ MS / Galzy vitamins	25	40	30	20	15	20	10	5

Full seeds were included in sterilization trials. Sterilization method of the seeds yielded 100% sterile cultures. In the experiments performed in dark conditions, germination percentages varied depending on germination media, temperature and cold application (Table 1).

Three-way variance analysis was conducted to determine whether the interactions of the obtained values had a statistically significant effect. As a result of variance analysis, the importance level (p) was less than 0.05 depending on germination media, germination temperatures and cold application in terms of seed germination in the dark (Table 2). According to this result, it is possible to say that germination percentages differed with the confidence level of $p \leq 0.05$ of germination media, germination temperatures and cold application.

Table 2. Results of the three- way Anova Test under dark condition

Source of variance	Sum of Squares	Degrees of freedom	Mean Square	F-Ratio	Significant (p)
Media (M)	0.372	6	0.062	38.907	0.000
Temperature (T)	2.143	3	0.714	448.030	0.000
Stratification (ST)	2.552	1	2.552	1.601.001	0.000
M x T	0.116	18	0.006	4.049	0.000
M x ST	0.017	6	0.003	1.750	0.116
T x ST	0.115	3	0.038	24.090	0.000
M x T x ST	0.055	18	0.003	1.916	0.021

From the values in the medium * stratification line of the table, it is seen that the common effect of the medium and stratification on germination percentage ($p = 0.116$, $p > 0.05$) is not statistically significant. In other words, germination percentage of seeds germinated in various media does not differ depending on stratification and it can be said that there is no interaction between medium and stratification.

From the values in the medium*temperature line of the table, it is seen that the common effect of the medium and stratification on germination percentage ($p=0.000$, $p< 0.05$) is statistically significant.

From the values in the medium*temperature*stratification line of the table, the common effect of medium, temperature and stratification on germination percentage ($p = 0.021$, $p < 0.05$) appears to be statistically significant and these 3 factors have a common interaction on germination. After these differences, the Tukey test was performed to determine what a grouping occurred in terms of all the procedures applied (Table 3).

Table 3. Three-way analysis of variance and Tukey multiple comparison test results in dark.

Factors		Groups
Media	MS	c
	½ MS	b
	¼ MS	a
	MS / B5 vitamins	c
	½ MS / B5 vitamins	bc
	¼ MS/B5 vitamins	a
	¼ MS/Galzy vitamins	a
Temperatures (ºC)	15	b
	18	a
	22	c
	25	d
Stratification	With stratification	a
	Without stratification	b

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According to the Tukey test results, 4 different groups were formed depending on seed germination media. The germination percentage was highest in the seeds germinated in ¼ MS, ¼ MS/B5, ¼ MS/Galzy media and no statistically significant difference could be found between these media with $p<0.05$ confidence level.

4 different groups were formed in terms of germination temperatures and the highest germination percentage was determined at 18 ºC. A statistically significant difference was found between the seeds with stratification and seeds without stratification in terms of the germination percentage and the percentage and the highest germination percentage was determined in the seeds with stratification.

In experiments carried out by culturing seeds in the photoperiodic medium, germination percentages varied depending on germination media, temperature and cold application (Table 4).

Three-way variance analysis was conducted to determine whether the interactions of the obtained values had a statistically significant effect. As a result of variance analysis, the importance level (p) was less than 0.05 depending on germination media, germination temperatures and cold application in terms of seed germination in photoperiod condition (Table 5). According to this result, it is possible to say that germination percentages differed with the confidence level of $p\leq0.05$ of germination media, germination temperatures and cold application.

Table 4. Germination percentages obtained in 16/8 photoperiod condition

Germination percentages in photoperiod 16/8 (%)								
Germination media	With stratification (+4ºC)				Without stratification			
	Temperatures (ºC)				Temperatures (ºC)			
	15	18	22	25	15	18	22	25
MS medium	40	60	35	20	20	25	15	0
½ MS medium	50	65	40	20	20	30	15	5
¼ MS medium	55	75	50	30	25	35	20	5
MS/B5 vitamins	40	55	35	20	20	25	15	0
½ MS/B5 vitamins	50	65	35	20	20	30	15	5
¼ MS/B5 vitamins	55	70	40	30	25	35	15	5
¼ MS/Galzy vitamins	55	70	40	30	20	35	20	5

Table 5. Results of the three- way Anova Testing of the effects on germination under 16/8 photoperiod condition

Source of variance	Sum of Squares	Degrees of freedom	Mean Square	F-ratio	Significant (p)
Media (M)	0.390	6	0.065	75.933	0.000
Temperature (T)	4.133	3	1.378	1.610.606	0.000
Stratification (ST)	4.300	1	4.300	5.026.659	0.000
M x T	0.067	18	0.004	4.373	0.000
M x ST	0.020	6	0.003	3.911	0.001
T x ST	0.071	3	0.024	27.684	0.000
M x Tx ST	0.107	18	0.006	6.943	0.000

It is seen from the values in the line of medium * stratification, medium*temperature, temperature*stratification and medium*temperature*stratification that the common effect of these factors on the germination percentage is statistically significant at $p<0.05$, that is, these factors interact with germination. After these differences, the Tukey test was performed to determine what a grouping occurred in terms of all the procedures applied (Table 6).

According to the Tukey test results, 3 different groups were formed depending on the seed germination medium. The germination percentage was highest in the seeds germinated in ¼ MS, ¼ MS/B5, ¼ MS/Galzy media and no statistically significant difference could be found between these media with $p<0.05$ confidence level.

4 different groups were formed in terms of germination temperatures and the highest germination percentage was determined at 18 ºC. A statistically significant difference was found between the seeds with stratification and seeds without stratification in terms of the germination percentage and the highest germination percentage was determined in the seeds with stratification.

High germination percentages in dark media were obtained with the germination of the stratified seeds at 18 °C in ¼ MS, ¼ MS/B5, ¼ MS/Galzy media. High germination percentages in photoperiodic media were obtained by the germination of stratified seeds at 18 °C in ¼ MS, ¼ MS/B5, ¼ MS/Galzy media.

When we compare the germination percentages in dark media and the germination percentages in photoperiodic media, it is seen that the highest germination percentage (75%) is reached in photoperiodic media.

Table 6. Three-way analysis of variance and Tukey multiple comparison test results under 16/8 photoperiod condition

Factors		Groups
Media	MS	c
	½ MS	b
	¼ MS	a
	MS/B5 vitamins	c
	½ MS/B5 vitamins	b
	¼ MS/B5 vitamins	a
	¼ MS/Galzy vitamins	a
Temperatures (ºC)	15	b
	18	a
	22	c
	25	d
Stratification	With stratification	a
	Without stratification	b

Light is seen to have an important effect on the germination of *H. adenotrichum* seeds. The highest germination percentage was reached under 16/8 photoperiod. In seed germination, light is as important as temperature. Light has a regulatory effect on germination and endogenous seed dormancy is often associated with the absence of light in some plant species [21]. While some seeds germinate similarly in the dark and under light [22], others can germinate more easily either under light [23] or in dark conditions [24].

In addition, the light requirements for germination may differ with temperature. According to the studies, some species need a constant temperature and light for germination, while others germinate either in the dark or under light, but require a temperature fluctuation [25]. In other species, stratification [26] or high temperatures [27] replace the need for light for germination. The absence of light was reported to have a negative effect on *Hypericum* species such as *H. gramineum* [28], *H. brasiliense* [29], *H. perforatum* [30, 31] and *H. avicullariifolium* [32].

After the seeds were stored at + 4 ºC for 2 months, on the basis of optimum conditions by beginning *in vitro* germination experiments, germination percentage was found to increase approximately twice. This shows us that there are seed dormancy and this dormancy has been broken with cold application. Seeds of many species require pre-planting applications to overcome dormancy. Cold application (stratification) is one of the pre-planting seed applications that can be used to promote germination in species with dormant seeds [33] and helps to eliminate internal dormancy [34].

Temperature is seen to have an effect on seed germination. The highest germination percentage was reached at 18 ºC. Germination percentage decreased as temperature increased. In a study conducted on *Hypericum* species, it was reported that germination rates of 4 *Hypericum* species (*H. hirsutum*, *H. polphyllum*, *H. xylosteifolium* and *H. erectum*) decreased substantially when temperature was 25 ºC [35]. Temperature plays a role in germination in 3 different ways; it determines the capacity and percentage of germination, eliminates primary and/or secondary dormancy and induces secondary dormancy [4]. Temperatures of 25 ºC and above are thought to induce secondary dormancy on *H. adenotrichum* seeds.

In experiments performed for the effect of different media types on the germination of *H. adenotricum*, high germination percentages were obtained at optimum conditions (photoperiod-stratification -18°C) from ¼ MS¼ MS/B5, ¼ MS/Galzy media. This shows that vitamin contents did not have a significant effect on germination. Achieving the best germination percentage at ¼ MS salt concentrations may be related to the decrease in germination rate due to the increase in salt concentration in the medium and the difficulty in absorption (water uptake) caused by the high osmotic pressure in the germination medium. In some plants, salinity affects seed germination negatively, reduces nodule formation, delays plant growth and decreases crop formation. Salinity can affect seed germination either by creating an osmotic potential that prevents water uptake or by the toxic effect of ions on embryo viability [36]. Although there was a difference between germination media according to the three-way variance analysis and Tukey test, germination percentages were 65% for ½ MS and 60% for MS under optimum conditions, indicating that salt concentration did not affect the germination as much as light, temperature and stratification.

One week after the germination, the seeds were placed under 16/8 photoperiod conditions at 22 ± 1 °C and seedling growth parameters in different media were evaluated after approximately 8 weeks (Table 7).

The highest number of shoots per seedling was obtained from ¼ MS/Galzy medium. The number of shoots in ¼ MS and ¼ MS/B5 media is lower than ¼ MS/Galzy medium. When these media, whose macro and micro salt concentrations were same but vitamin contents were different, were compared, no significant effect was observed on the number of shoots of MS and B5 vitamins, and Galzy vitamins increased the number of the shoots substantially. The number of shoots in ½ MS and ½ MS/B5 media is higher than the number in MS and MS/B5 media. There was a decrease in the number of shoots as the salt concentration increased and the vitamins MS and B5 had no significant effect on the number of shoots.

¼ MS/Galzy medium was determined as the most suitable medium for seedling growth in terms of average number of lateral roots, average shoot length and the average number of leaves per shoot. The difference between the media in respect of average root length is not statistically significant.

Table 7. The effect of different media on in vitro growth of Hypericum adenotrichum seedlings

		Seedling growth parameters			
Media	Average number of shoots per seedling	Average number of lateral roots	Average shoot lengths (cm)	Average root lengths (cm)	Average number of leaves per shoot
MS	1.8d*	1.6^{c}	2.1^{b}	2.5^{a}	9.5^{b}
½ MS	2.5^{bc}	3.6^{bc}	2.2^{b}	2.7^{a}	9.8^{b}
¼ MS	3.6^{b}	6.0^{ab}	3.0^{ab}	3.0^{a}	11.8^{ab}
MS/B5 vitamins	2.0^{d}	1.6^{c}	2.2^{b}	2.3^{a}	9.6^{b}
½ MS/B5 vitamins	2.6^{bc}	3.8^{bc}	2.2^{b}	2.6^{a}	10.0^{b}
¼ MS/B5 vitamins	3.6^{b}	5.8^{ab}	2.9^{ab}	2.9^{a}	11.5^{ab}
¼ MS/Galzy vitamins	5.8^{a}	6.3^{a}	3.3^{a}	3.0^{a}	12.3^{a}

* In each column, the means followed by different letter(s) show significant differences at the $p \leq 0.05$

In general, seedling growth was negatively affected due to increasing salt concentrations other than root lengths. Therefore, the development of seedlings in ¼ MS medium gave much better results than MS medium.

Minerals are the important components of the culture media and there are many options for the combinations of the macro and micro salt mixtures of the media [37]. MS medium has a high salt content (especially in terms of potassium and nitrate salts) compared to other media types and is widely used as culture medium in tissue culture studies [38] Due to the high salt content, this growth medium may not always be the most suitable medium for the development and growth of *in vitro* small plants or explants. Salt stress affects plant growth in physiological aspects. Shoot growth and dry matter decrease with salinity and the root: shoot ratio may increase [39]. Shoot growth is reduced by salinity due to inhibitory effects of salt on cell division and growth at growth points [40]. Inhibition of plant growth by salinity may depend on the inhibitory effect of ions, or high salinity may inhibit the elongation of roots and shoots by causing a slow uptake of water by the plant [41] germinated the seeds of three types of *Coffea arabica* L. in MS,

½ MS, ¼ MS and agar media and compared germination percentages and seedling growths [42]. At the end of the experiments, it was found that the germination percentage, hypocotyl and root length increased proportionally from MS medium towards the agar medium. In our study, there was no statistically significant difference in root length in terms of macro salt concentrations. However, there was a significant difference in the number of roots, shoot lengths, number of shoots and number of leaves. Macro salt concentration was especially more effective on the number of shoots and roots.

In our experiments, no significant difference was observed between MS and B5 vitamins in terms of seedling growth, and Galzy vitamins had a significant effect on seedling growth. Galzy vitamins stand out especially with their biotin and Ca–pantothenate, content, differently from MS and B5 vitamins. Plants normally synthesize vitamins for growth and development [43].However, *in vitro* plant cells can synthesize the necessary vitamins only below the optimal amount; therefore, the culture medium is often supplemented with vitamins to enhance growth [44].

4. Conclusions and Recommendations

A final protocol was determined for seedling development and seed germination of *H. adenotrichum* at the end of experiments. Seeds were kept under 4 º C during 2 months and then they rinsed 30 min. under tap water. Rinsed seeds were kept in 70% ethanol for 1 min and following this they transferred into 2.25 % NaOCl (containing 1-2 drop tween 80). Following all these treatments, seeds were rinsed again by using distilled water.

Low seed germination rates widely commented as it dependent upon to exogenic and endogenic dormancy has been reported in some studies. Despite our results supports dormancy, one should keep in mind that selection of healty/empty seeds by floating them on water increases germination rates. There is no record about healty/empty *Hypericum* seeds selection by floating them on water in literature. Our study presents the first evaluation of interactive effects of light, dark, temperature, stratification and nutrient medium interactive effects on seed germination. The highest germination value has been held when sterilized seeds were incubated in ¼ MS/Galzy vitamins containing medium under 16/8 photoperiod and 18 ºC conditions after stratification at 4 ºC for 2 months.

The seeds having emerged radicles were transferred *in vitro* conditions (22 ºC and 16/8 photoperiod) mainly 7th days of incubation. By observing development of seedlings, ¼ MS/Galzy medium was found to be the most suitable media for development. Concordantly, number of shoots per seedling, mean number of lateral roots, mean length of shoots and number of leaves per shoots were the highest in the medium.

Present study aiming determination of conditions of *in vitro* seed germination and seedling development of *H. adenotrichum* may serve as a first step for future conservation studies of the species.

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