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Establishment of an *in vitro* method for micropropagation of ironwort, (*Sideritis raeseri* Boiss. & Heldr.)

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Establishment of an *in vitro* method for micropropagation of ironwort (*Sideritis raeseri* Boiss. & Heldr.)

Abstract: Ironwort / Mountain Tea (*Sideritis raeseri* Boiss & Heldr.) is an endangered (EN) plant species in Albania. This study aimed to develop a rapid clonal propagation protocol using *in vitro* methodologies. The ironwort seeds were pre-treated with three concentrations of GA_3 (250, 500, and 1000 mg l^{-1}). During the inoculation stage, two types of culture media, Murashige & Skoog (MS) and Woody Plant Medium (WPM), were tested, and the effects of both GA_3 concentration and culture media used were evaluated. For the subculture stage, three cytokinins (6-b*enzylaminopurine* / BAP, kinetin, zeatin) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}), were compared for the RGR index, while for the rooting stage, two different auxins (1-n*aphthaleneacetic acid* / NAA and i*ndole-3-butyric acid* / *I*BA) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}) were tested. GA_3 at 500 mg l^{-1} and MS medium resulted as more effective. The highest value of the RGR index during the subculture stage was obtained in the MS nutrient medium supplemented with BAP at 1.5 mg l^{-1}. For rhizogenesis response, IBA was more effective for roots and length number. Based on these results, *in vitro* methodologies can be a promising tool for the mass production of this endangered plant species and with possible applications for enhancing the production of valuable nutraceuticals.

Key words: mountain tea; micropropagation; seed germination; nutrient medium; GA_3 concentration

Vzpostavitev *in vitro* metode za mikropropagacijo albanskega sklepnjaka (*Sideritis raeseri* Boiss. & Heldr.)

Izvleček: Vrsta *Sideritis raeseri* Boiss & Heldr. je ogrožena (EN) rastlinska vrsta Albanije, sorodna vrsti *Sideritis scardica* Gris., poznani kot šarplaninski čaj. Namen raziskave je bil razviti protokol hitrega klonskega razmnoževanja te vrste z *in vitro* metodo. Semena so bila predhodno obdelana s tremi koncentracijami giberilinov (GA_3; 250, 500, in 1000 mg l^{-1}). Na stopnji inokulacije sta bili preiskuševani in ovrednoteni dve vrsti gojišč, Murashige & Skoog (MS) gojišče in gojišče za lesnate rastline (WPM) hkrati z učinki različnih koncentracij giberilinov. V prvi fazi gojenja so bili preiskušeni trije citokinini (6-benzilaminopurin *(*BAP), kinetin, zeatin) v štirih koncentracijah (0,5; 1,0; 1,5; 2,0 mg l^{-1}) in primerjani z indeksom relativne prirasti (RGR). V fazi ukoreninjanja sta bila preiskuševana dva auksina (1-naftalen ocetna kislina *(*NAA) in indol-*3*-maslena kislina (*I*BA) v štirih koncentracijah (0,5; 1,0; 1,5; 20 mg l^{-1}). Giberilini (GA_3) pri koncentraciji 500 mg l^{-1} in MS gojišče so bili najbolj učinkoviti. Največja vrednost indeksa relativne prirasti (RGR) je bila v prvi fazi gojenja dobljena v gojišču MS z dodatkom BAP 1.5 mg l^{-1}. Za nastanek korenin je bil IBA bolj učinkovit tako glede števila kot dolžine korenin. Na osnovi teh rezultatov lahko zaključimo, da je *in vitro* metoda obetajoče orodje za masovno razmnoževanje te ogrožene vrste z možnostjo uporabe pri pospešeni proizvodnji vrednih hranilnih snovi.

Ključne besede: albanski sklepnjak; mikropropagacija; kalitev semen; gojišča; GA_3 koncentracija

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1 INTRODUCTION

Mountain tea (*Sideritis raeseri* Boiss et Heldr.), also known as Ironwort, is an aromatic medicinal native plant of the western Balkans, including south Albania, southeastern parts of North Macedonia, and North Greece (Zekaj et al., 2008). It is considered a Balkan endemic species due to its restricted distribution range of only three countries. Due to its high quantity of bioactive compounds with a high percentage of antioxidants, mountain tea has been widely utilized in alternative medicine since ancient times (Romanucci et al., 2017; Tadić et al., 2021). According to Hodaj et al. (2017), it is also used as an herbal tea to treat digestive system disorders, coughs and as a dietary supplement for avoiding anemia. The European Medicinal Agency (EMA) has approved the use of mountain tea, due to its medicinal values and its marketing in the market or pharmacy, based on its traditional use for at least 30 years in Europe and particularly in the Balkans, where the population has the experience and accurate information on optimal daily dosing (EMA, 2016).

According to Tomasini & Theilade (2019), mountain tea is a culturally and commercially significant important species locally consumed as a tea and used for the treatment of flu symptoms and respiratory problems, as well as harvested for trade. However, due to its widespread use, it has been subjected to uncontrolled and destructive harvest practices in its distribution area of occurrence in Albanian territory, resulting in a decrease in the wild populations of the mountain tea (Bojadzi et al., 2012; Tomasini & Theilade, 2019).

Mountain tea populations have been reduced by 50 % in the Prespa area from 1990 until today, and since its population in Albania has been reduced by 30 % and destructive harvest practices of the natural population continue, it has been assessed as endangered (EN) species by the Albanian government (Shuka & Malo, 2010; MoE, 2013; Shuka et al., 2021). Furthermore, *S. raeseri*, with its closest relative, *S. scardica*, is listed as species of high conservation interest for the western Balkan countries (Aneva & Zhelev, 2018).

In light of the preceding, it is critical to improving the situation through *in situ* and *ex situ* cultivation and conservation techniques. Furthermore, *in vitro* technologies are effectively applied with the goal of rapid mass production via clonal propagation and the establishment of a genetic collection for the conservation of endangered plant species of economic importance. The medicinal and aromatic plants (MAPs), among others, are the main focus of these techniques' application because of their importance and widespread use in pharmacy and medicine (Neergheen-Bhujun et al., 2017; Moraes et al., 2021).

Seasonal variations, growing practices, the expense of production, as well as other factors all impede large-scale phytochemical production from field-grown plants. The application of biotechnological techniques would be of significant interest in this area, not only for biomass production but also for optimizing the production of secondary metabolites (Georgiev et al., 2009; Cardoso & Silva, 2013; Kapoor et al., 2018). Efforts are being made to modify the organogenesis of the secretory structures of MAPs in terms of density or glandular diameter (Vantu & Gales, 2009; Sota et al., 2019; Sota et al., 2020), as well as the possibility of increased synthesis of essential secondary metabolites, using specific physic-chemical parameters under in vitro conditions (Avato et al., 2005; Tousi et al., 2010; Sharma et al., 2015; Radić et al., 2016; Jamwal et al., 2018).

The success of *in vitro* stabilization and multiplication of plant germplasm is determined by several parameters, including the explant chosen, the physical or chemical treatments applied, and any pretreatment used. Some studies (Shtereva et al., 2015; Papafotiou & Kalantzis, 2009; Danova et al., 2013) employed seeds as primary explants and isolated nodal explants for further multiplication via subcultures for *Sideritis* sp. In many cases, when seeds are used as initial explants, pretreatment with GA_3 is seen as effective in order to enhance their germination and faster proliferation under *in vitro* conditions (Khuat et al., 2022; Cornea-Cipcigan et al., 2020; Rout et al., 2017; Arabaci et al., 2014; Gashi et al., 2012). This effect is related to the synthesis of α-amylases, essential enzymes that help and promote breaking seed dormancy (Finch-Savage & Leubner-Metzger, 2006). For *Sideritis leucantha* Cav., a Spanish endemic species, Juan-Vicedo et al. (2021) refined a micropropagation and cryopreservation strategy using shoot explants. Sarropoulou & Maloupa1 (2015) studied the effects of various dikegulac sodium concentrations, a PGR that enhance lateral growth, on *in vitro* regeneration of *S. raeseri* using shoot tips as primary explants. In some of these studies, efforts have been made to find a suitable plant growth regulators (PGRs) ratio in different stages of micropropagation that enhanced *in vitro* regeneration with potential uses for other purposes such as conservation or secondary metabolites production.

Because many *Sideritis* species are indigenous to certain places, they are adapted to the native growing conditions in their natural habitats. Hence, adaptation abilities are likely to vary significantly among species in the genus. This study aimed to stabilize an effective micropropagation protocol by using various concentrations of GA_3 for enhancing seed germination under *in vitro* conditions and confronting some PGRs ratios for *in*

vitro regeneration and rooting induction on the derived plantlets.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL COLLECTION AND DISINFECTION

As primary explants were used mature seeds of *Sideritis raeseri* Boiss. & Heldr. collected in the National Park of Prespa, Albania. The seeds were left for 30 min in tap water and, after that, were sterilized with 5.20 % sodium hypochlorite solution for 15 minutes.

2.2 GA_3 PRETREATMENT FOR SEED GERMINATION ENHANCEMENT

Before inoculation in culture vessels, the seeds were treated for 24 h in GA_3 solution. Three concentrations of gibberellic acid (GA_3), specifically I: 250 mg l^{-1}; II: 500 mg l^{-1}; III: 1000 mg l^{-1}, were tested and compared. After this treatment, the explants were inoculated in the nutrient medium, and their *in vitro* cultivation was initiated.

2.3 MEDIA COMPOSITION IN EACH STAGE OF MICROPROPAGATION

Inoculation and seeds germination stage: after GA_3 treatment, the explants were inoculated in a nutrient medium for their germination. Two different basal media, specifically Murashige-Skoog (MS) medium (Murashige & Skoog, 1962) and Woody Plant Medium (WPM) (Lloyd & McCown, 1980) were compared, each of them supplemented with 1-naphthaleneacetic acid (NAA) at 0.1 mg l^{-1} and 6-benzylaminopurine (BAP) at 1 mg l^{-1}. Seeds' germination started 6 – 7 days of culture, but germination percentage and morphometric parameters (shoot length and leaves number) were evaluated after 30 days of culture.

Subculture stage: For shoots regeneration, MS basal medium was used, and the effect of three different cytokinins (BAP, kinetin, zeatin) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}) were tested and compared. The plant material was weighed before inoculation in each treatment (initial mass - M1), while after 30 days, the biomass obtained in each treatment was weighed (final mass - M2). After that, the relative growth rate (RGR) following the formula: $RGR = (lnM2 - lnM1) / (no.\ of\ days) \times 100$ was evaluated, where *ln* is the natural logarithm, and FM is the fresh mass (Gatti et al., 2017).

Rooting stage: For rhizogenesis induction, MS basal medium was used, and the effect of two different auxins, specifically -naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) at four concentrations (0.5; 1.0; 1.5; 2.0 mg l^{-1}), were tested and compared. In this stage, roots number and lengths were evaluated.

In all cases, all media were enriched with sucrose at 3 % and agar at 0.57 %. The pH was adjusted to 5.7 prior to autoclaving.

2.4 INCUBATION CONDITIONS

The cultures were maintained in the growth chamber at a temperature 25 °C ± 2 °C with a 16 h light / 24 h photoperiod with cool, white fluorescent light of intensity 43.4 mmol m^{-2} s^{-1}.

2.5 STATISTICAL ANALYSES

For each treatment, 30 explants were used, and all experiments were repeated at least three times. Experimental data was elaborated by the Student's Test and the analysis of variance (ANOVA) with JMP 7.0 statistical software. Seeds germination, morphometric parameters, and RGR index in each cultivation stage were measured after 30 days of culture.

3 RESULTS

3.1 EFFECT OF GA_3 AND MEDIA TYPE ON *IN VITRO* SEEDS GERMINATION

In this experiment, seed germination and shoots development of *S. raeseri* affected by GA_3 and the type of culture media were investigated. Seeds started germination after 6 7 days of culture, and shoot / root organogenesis was observed due to the proliferation of zygotic embryos (Fig. 1 a). Within a week, these organs are differentiated (Fig. 1 b, c). The obtained results showed that seeds pre-treatment with GA_3 solution gave high germination rates in all concentrations used.

From the variability chart (Fig. 2), it can be observed that the differences in this parameter were not influenced by the type of basal media used but only by the GA_3 concentration. The results clearly show no statistical differences between MS and WPM media for the same concentration of GA_3. Therefore, treatment with GA_3 at 500 mg l^{-1} was evaluated as the most effective concentration, resulting specifically in an 88.4 % of germination rate for MS medium and 86.4 % for WPM medium.

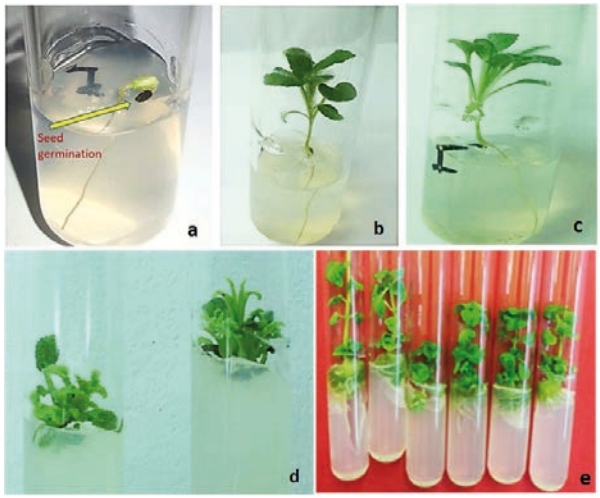


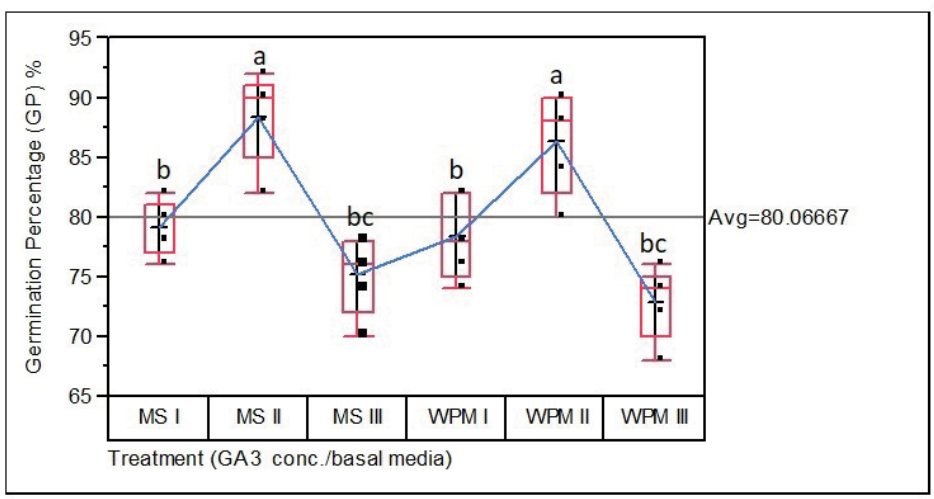
Figure 1: *Sideritis raeseri* Boiss. & Heldr. micropropagation **a)** Seed germination under *in vitro* conditions **b, c)** Shoot and root differentiation **d)** Shoots regeneration during subculture **e)** Rhizogenesis induction

After seeds germination, morphological characteristics such as shoots length (cm) and the number of leaves were monitored to detect if the pre-treatment of seeds or the basal media used caused any significant difference in these monitored parameters. For the above, the obtained results were interesting (Fig. 3). Regarding shoots length (cm), no differences were observed caused by the basal medium type. Even for this parameter, the highest results were obtained in GA_3 solution at 500 mg l^{-1}, precisely 2.31 cm for MS medium and 2.16 cm for WPM medium. The lowest results were obtained in GA_3 solution at 1000 mg l^{-1} for both basal media used (Fig. 2 a). The same trend was observed even for the number of leaves, where the best results were obtained in GA_3 solution at 500 mg l^{-1}. However, significant differences were observed between MS and WPM medium for this concentration (precisely, 17.01 in MS medium and 14.22 in WPM medium). For the other GA_3 concentrations, no differences were observed between MS and WPM media for the leaves number parameter (Fig. 2 b). In an overall analysis, we conclude that pretreatment with GA_3 at 500 mg l^{-1} is the most effective concentration, and cultivation in MS basal medium is more advantageous than WPM.

GA_3 is the plant hormone that is crucial in breaking seed dormancy. From our data, it is clear that GA_3 positively affects *in vitro* organogenesis by stimulating the proliferation of embryos within the seed to give shoots and roots. In all treatments with GA_3, is observed not only the germination rate at high values but also the increase of biomass of the monitored biometric parameters. So, it is evidenced that the GA_3 beneficial role in promoting seed development of *S. raeseri*.

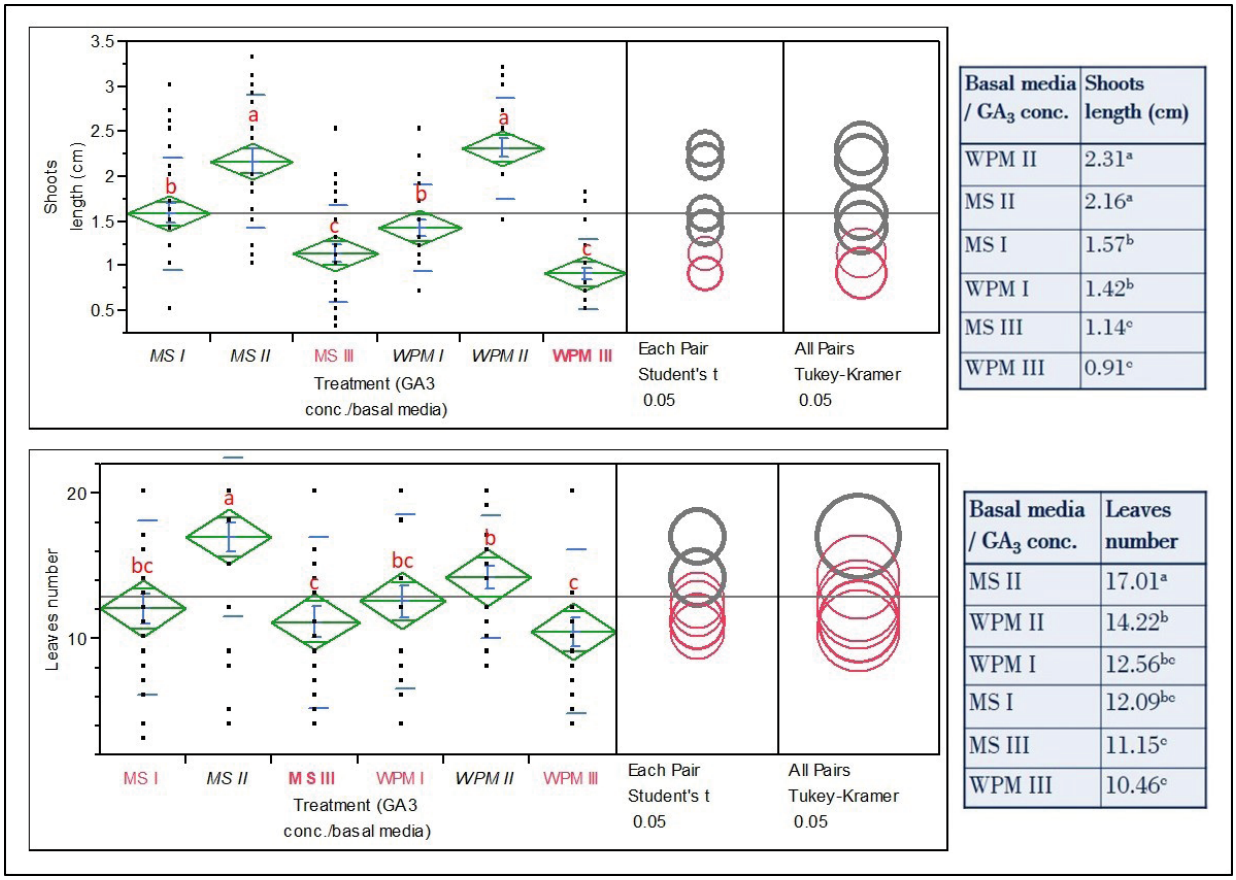
3.2 BIOMASS PRODUCTION UNDER DIFFERENT CONCENTRATIONS AND TYPES OF CYTOKININS

The proliferated plantlets were subcultured for further multiplication, whereas before inoculation in the nutrient medium, the roots were removed, and small shoots were used for this purpose. A few days after cultivation in the subculture stage, new shoots and leaves



Basal media / GA_3 conc.	Mean
MS II	88.40[a]
WPM II	86.40[a]
MS I	79.20[b]
WPM I	78.40[b]
MS III	75.20[bc]
WPM III	72.80[bc]

Figure 2: Variability chart for germination rate depending on the GA_3 concentration and basal media used



Basal media / GA_3 conc.	Shoots length (cm)
WPM II	2.31[a]
MS II	2.16[a]
MS I	1.57[b]
WPM I	1.42[b]
MS III	1.14[c]
WPM III	0.91[c]

Basal media / GA_3 conc.	Leaves number
MS II	17.01[a]
WPM II	14.22[b]
WPM I	12.56[bc]
MS I	12.09[bc]
MS III	11.15[c]
WPM III	10.46[c]

Figure 3: Oneway Analysis of **a)** Shoots length (cm); **b)** Leaves number; depending on the GA_3 concentration and basal media used

were formed in all concentrations or types of cytokinins supplemented in the nutrient media (Fig. 1 d). The initial and final mass results indicated that both type and cytokinin concentration affected *in vitro* regeneration of plantlets during this stage (Tab. 1).

Regarding the variability for the RGR index between different cytokinins for the same concentration (Fig. 4a), it can be said that except for the lowest concentration used of 0.5 mg l^{-1} where kinetin showed the highest effectiveness of the three cytokinins used, in all other concentrations the highest value of RGR was obtained from the use in the nutrient medium of BAP. In most cases, kinetin and zeatin are very close to each other in their effectiveness concerning the value of RGR according to the measurements performed, except for the RGR value at 1.5 mg l^{-1} of cytokinins concentration, where zeatin gave the lowest value. Regarding comparing different concentrations within the same type of cytokinin (Fig. 4b) for kinetin and BAP, the best results for RGR value were obtained when using 1.5 mg l^{-1} of each cytokinin, precisely 5.72 for kinetin and 6.11 for BAP. While for zeatin, the most optimal concentration resulted the one at 2.0 mg l^{-1}. An overall analysis of the obtained data showed that the most effective treatment, depending on the cytokinin type and concentration, was the use of BAP at 1.5 mg l^{-1}, where the RGR value obtained is equal to 6.11.

Table 1: Plantlets' weight before and after subculture stage

	Kinetin				Zeatin				BAP			
Concentration (mg l^{-1})	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Initial mass (g) (M1)	1.04	1.12	1.08	1.20	1.10	1.08	1.28	1.15	1.12	1.04	1.10	1.13
Final mass (g) (M2)	3.38	4.21	6.01	5.77	2.81	3.92	4.49	5.57	3.07	4.31	6.88	5.98
RGR index	3.93	4.41	5.72	5.23	3.13	4.30	4.18	5.26	3.36	4.74	6.11	5.55

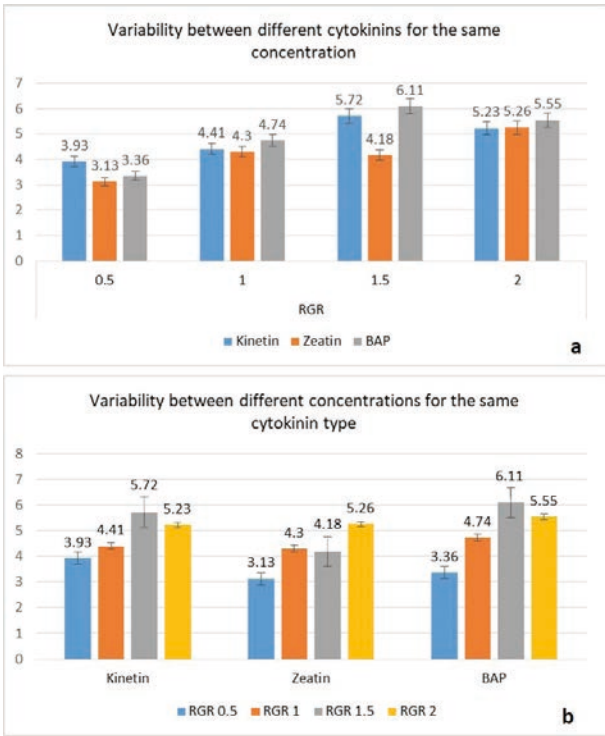


Figure 4: Variability for RGR index between **a)** different cytokinins for the same concentration **b)** different concentrations for the same cytokinin

3.3 *IN VITRO* ROOT FORMATION USING DIFFERENT CONCENTRATIONS OF IBA AND NAA

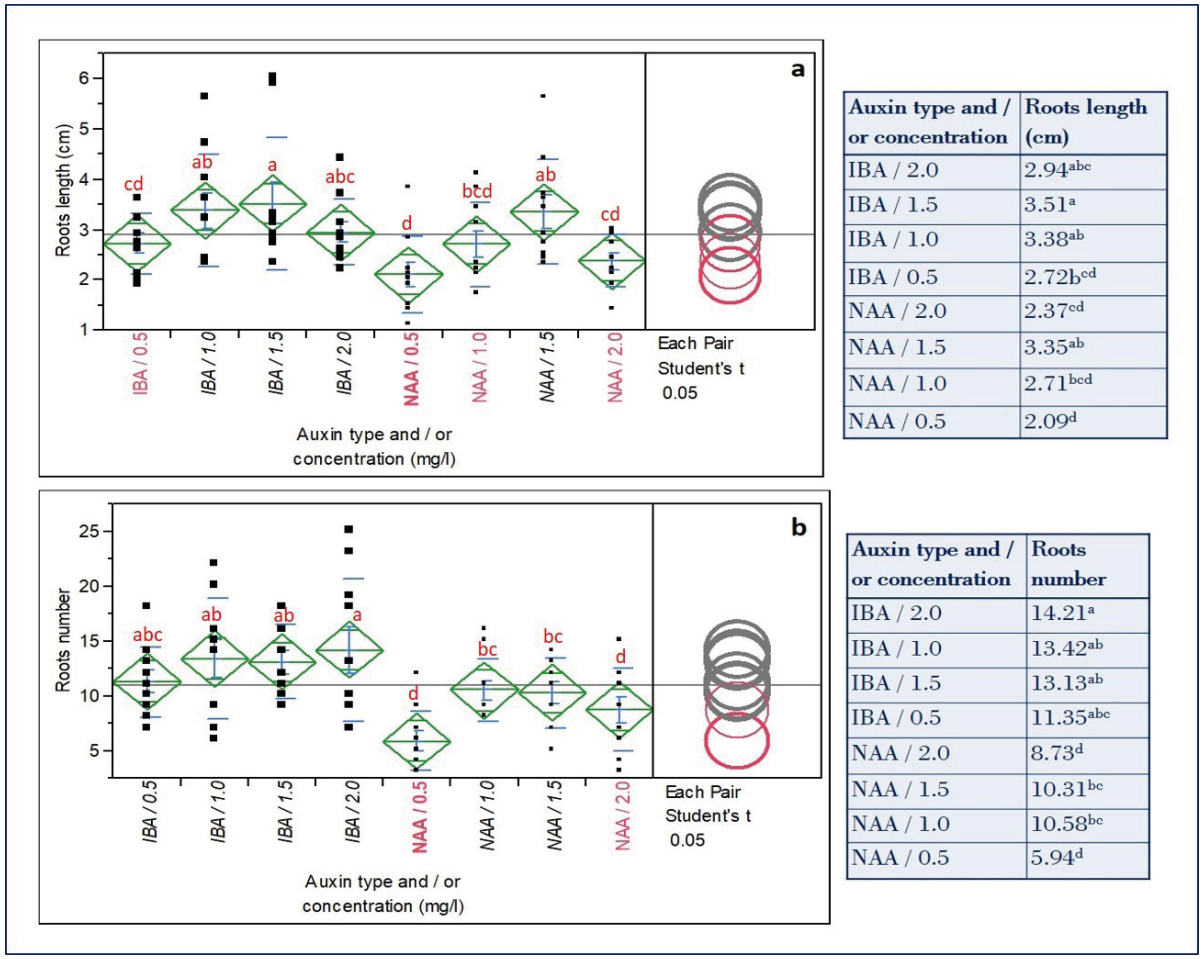
Root formation is a crucial stage for the micropropagation of plants reproduced *in vitro*. In this part of the study, the effect of IBA and NAA added separately in four concentrations on *in vitro* rooting of *S. raeseri* was investigated. The rooting response was observed at a high rate in all treatments (Fig. 1 e), but even in this stage was observed that the monitored morphometric parameters (number of roots and their length) are highly affected by the type and auxin concentration (Fig. 5 a; b). From an overall evaluation, IBA resulted more effective than NAA for both parameters under evaluation. Regarding roots length, the most effective treatment resulted in the use of IBA at 1.5 mg l^{-1}, a value (3.51 cm) statistically different from all the other values obtained, followed by the use of IBA at 1 mg l^{-1} and NAA at 1.5 mg l^{-1}, with mean values respectively 3.38 and 3.35. As for roots number, all the treatments with IBA showed higher values of this parameter, and the best result was obtained at 2.0 mg l^{-1} of IBA, with a respective value of 14.21 roots/plantlet.

4 DISCUSSION

Seed germination is a complex process, and GA_3 plays a crucial role in controlling and encouraging germination in many plant species. In this respect, exogenous applications of GA_3 are primarily used during *in vivo* or *in vitro* plant cultivation for enhancing seeds germination. Our study showed that adding GA_3 to the culture medium, regardless of concentration, increased the percentage of in vitro *Sideritis raeseri* germination, indicating the role of GA in breaking dormancy. Furthermore, the results revealed that the seed treatments significantly affected the germination and seedling growth parameters. Maximum germination and other seedling growth parameters were observed with 500 mg l^{-1} of GA_3. Otherwise, seedlings derived from GA_3-treated seeds showed normal morphology. In this regard, in their report, Cornea-Cipcigan et al. (2020) concluded that exogenous applications of GA_3 stimulated not only the germination of *Cyclamen* sp. but also higher rates of biometric parameters in the obtained plantlets.

Similarly, Gashi et al. (2012) found that using 1000 mg l^{-1} GA_3 + 0.3 % KNO_3 highly stimulated the germination of *Ramonda serbica* Pančić seeds grown in *Petri* dishes under controlled physical conditions. Also, Arabaci et al. (2014) mentioned that the pre-treatment of *Sideritis perfoliata* L. seeds with GA_3 at 100 mg l^{-1} for two hours resulted in a 100 % of germination rate. Furthermore, in their study on seed germination of *Vasconcellea stipulate* V.M. Badillo, Vélez-Mora et al. (2015) found effective the use of GA_3 at 1.44 µM in Nitch & Nitch basal medium, which significantly stimulated seeds germination. Similarly, Ake et al. (2007) obtained positive results on *in vitro* germination of coconut embryos by supplementing the semi-solid medium with GA_3 at 4,6 µM. Meanwhile, Nikam & Barmukh (2009) found effective the soaking of *Santalum album* L. seeds at 4 mM of GA_3 solution and obtained an 80.67 % of germination rate after seeds *in vitro* inoculation in MS medium.

In our study, there are no observed significant differences between MS and WPM basal media used for most of the results. Regarding the efficiency of MS media for *in vitro* regeneration of *S. raeseri* plantlets under *in vitro* conditions, our results are similar to those reported by other authors for micropropagation of different *Sideritis* species (Juan-Vicedo et al., 2021; Sevindik et al., 2019; Shtereva et al., 2015; Papafotiou & Kalantzis, 2009). On the other hand, Yavuz (2016) found compelling the use of B5 medium for *in vitro* regeneration of *Sideritis stricta* Benth. plantlets.



Auxin type and / or concentration	Roots length (cm)
IBA / 2.0	2.94^{abc}
IBA / 1.5	3.51^{a}
IBA / 1.0	3.38^{ab}
IBA / 0.5	2.72b^{cd}
NAA / 2.0	2.37^{cd}
NAA / 1.5	3.35^{ab}
NAA / 1.0	2.71^{bcd}
NAA / 0.5	2.09^{d}

Auxin type and / or concentration	Roots number
IBA / 2.0	14.21^{a}
IBA / 1.0	13.42^{ab}
IBA / 1.5	13.13^{ab}
IBA / 0.5	11.35^{abc}
NAA / 2.0	8.73^{d}
NAA / 1.5	10.31^{bc}
NAA / 1.0	10.58^{bc}
NAA / 0.5	5.94^{d}

Figure 5: Oneway Analysis of a) Roots length (cm); b) Roots number, depending on the auxin type and/or concentration

Cytokinins have been used to stimulate plant growth and development as they favor cell division and cytokinesis, thus stimulating lateral shoots growth. In tissue culture, the types and concentrations of cytokinin added to culture media are the most important factors affecting the *in vitro* multiplication of plant propagules. In the present work, variations in the response of the multiplication parameters of *Sideritis raeseri* were observed depending on the type and concentration of cytokinin. Among three types of cytokinins, i.e., BAP, kinetin, and zeatin, used for in vitro shoot multiplication, BAP at 1.5 mg l^{-1} was the most effective treatment. Shoot multiplication in the present study was obtained by enhancing shootlets' fresh mass, which is crucial in employing tissue culture techniques for *Sideritis raeseri* micropropagation. Similar to our results, other authors, when confronting different types or concentrations of cytokinins, also have reported the effective use of BAP for *in vitro* multiplication of *Sideritis* sp. (Yavuz, 2016; Papafotiou & Kalantzis, 2009). Meanwhile, Juan-Vicedo et al. (2021) evidenced that for *S. leucantha*, the best results for shoot morphogenesis were obtained on a nutrient medium supplemented with 0.44 μM 2-isopentenyladenine. On the other hand, Shtereva et al. (2015) stated that for micropropagation of *S. scardica*, the use of zeatin at 2 mg l^{-1} combined with 0.2 mg l^{-1} indole-3-acetic acid (IAA) was the best combination for shoot proliferation.

For *in vitro* root formation, most plant species require a medium supplemented with essentially auxin-specific PGRs. Usually, IBA, IAA, or NAA are used for the rhizogenesis of plant microshoots. In the present study, IBA was found to be superior over the NAA for *in vitro* root formation of *S. raeseri* since the highest values of root numbers and lengths were observed with IBA. Furthermore, the obtained roots' appearance was healthy and suitable for successful acclimatization. Our findings on the effect of IBA in rhizogenesis induction of *S. raeseri* are in line with the ones reported by Yavuz (2016), who achieved the best rooting rate of *S. stricta* on B5 medium supplemented with 4.5 mg l^{-1} IBA. On the other hand,

for *Sideritis leucantha* the best auxin for rooting response resulted 1-naphthaleneacetic acid (Juan-Vicedo et al., 2021). In this respect, Ragavendran et al. (2012) rooted *in vitro* raised shoots of *Passiflora foetida L.* by use of IBA.

5 CONCLUSIONS

An effective micropropagation method of *Sideritis raeseri* Boiss. & Heldr. was recognized using seeds as the initial explant. The *in vitro* germination rate and shoots length were strongly affected by the GA_3 concentration, where the treatment with GA_3 at 500 mg l^{-1} gave the best results. Many plants were obtained in the subculture stage, where the most effective cytokinin was found to be the cytokinin BAP. For the monitored parameters (RGR), it can be concluded that the concentration of 1.5 mg l^{-1} of BAP and kinetin was the most optimal concentration that strongly influences the *in vitro* regeneration of plantlets. At the same time, zeatin was found effective at the concentration of 2 mg l^{-1}. For rhizogenezis induction, IBA was more effective than NAA for roots length and number. Effective clonal growth enables the creation of a plant collection with the potential for utilization in conservation programs, which are very important to apply to this endangered plant species. Also, this may further extend studies toward optimizing protocols for *in vitro* production of secondary metabolites from this important medicinal species.

6 ACKNOWLEDGEMENTS

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