Journal of Plant Physiology and Breeding
2022, 12(2): 105-116 ISSN: 2008-5168

Research paper

Micropropagation of Iranian native oregano (*Origanum vulgare* L.) using growth regulators

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Received: March 28, 2022 Accepted: June 17, 2022
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Abstract
Wild oregano (*Origanum vulgare* L.) belonging to the Labiate family is one of the most important medicinal plants in Iran and the world. It is widely used in the medicinal, food, and health industries. The present study aimed to investigate the *in vitro* culture of Iranian native oregano. Two experiments were designed to investigate the proliferation and rooting of this species. In the proliferation stage, benzyl adenine (BA) was used at four levels (0, 0.5, 1, and 1.5 mg/l) along with 0.25 mg/l indole butyric acid (IBA). For the rooting stage, IBA at four levels (0, 0.5, 1, and 1.5 mg/l) in combination with 0.5 mg/l naphthalene acetic acid (NAA) was used. The results showed that BA caused a significant effect on the leaf number, the number of shoots, the shoot length, chlorophyll index, and the leaf dry weight. The highest number of leaves and shoots and the highest value of chlorophyll index were seen in the medium containing 1.5 mg/l. The longest shoot and the highest leaf dry weight were related to 1 mg/l BA. In addition, the results related to the rooting stage showed that the root length, root diameter, and total dry weight of plantlets were significantly affected by IBA, while the number of roots was not influenced by IBA. The highest root diameter and total dry weight of plantlets were obtained in the medium containing 0.5 mg/l IBA, and the maximum root length was obtained in the MS medium without IBA.

Keywords: benzyl adenine; indole butyric acid; micropropagation, oregano; proliferation; rooting

How to cite: Ahmadi MR, Shahhoseini R, and Hakimi L, 2022. Micropropagation of Iranian native oregano (*Origanum vulgare* L.) using growth regulators. Journal of Plant Physiology and Breeding 12(2): 105-116.

Introduction

Origanum vulgare L. is one of the most important species of the genus *Origanum*, which is common not only in the Mediterranean region but also in other parts of the world, including Iran and Turan. The species includes six subspecies around the world (Spada and Perrino 1997), of which only three subspecies of Viride, Vulgare, and Gracile have been identified in Iran so far. These three subspecies are distributed in the northwest and west of Iran. Owing to the presence of phenolic compounds (mainly carvacrol and occasionally thymol) in the oil of thisplant, it's antimicrobial, antifungal, insecticidal, and antioxidative effects have received considerable attention in recent years (Moradi *et al.* 2021). At present, the rate of global extinction of this species has been about 1% of its total population over the last 100 years, and this amount is expected to increase by at least 10% by 2050. Factors affecting the extinction include land-use change, destruction and erosion of soil due to overgrazing and unprofessional agriculture, climate change, and illegal harvesting of plants for commercial purposes (Abrahamyan *et al.* 2014). It seems that one of the important strategies for satisfying the increasing demand of global and domestic markets and reducing the pressure on the habitats of wild plants is to cultivate this plant in agricultural systems and propagate it by micropropagation. Various factors such as mineral

nutrition, culture medium, and plant growth regulations are effective in plant micropropagation (Meena *et al.* 2010, Aghaye *et al.* 2013). Meftahizade *et al.* (2010) in their studies on lemongrass reported that BAP in combination with NAA had the highest regeneration in shoot tips explants. Sevindik *et al.* (2017) in an investigation on micropropagation of *Origanum sipyleum* reported that 85% of the nodes produced an average of 6 shoots per node on the MS medium supplemented with 0.5 mg/l BA and 0.2 mg/l GA_3. Beyrouthy *et al.* (2015) in a research on two *Origanum* species reported a mean rate of 2 to 3.7 new shootlets per explant from the nodal segments on MS medium supplemented with different concentrations of BAP (1, 1.5, and 2 mg/L). In another research on *Origanum vulgare*, Premi *et al.* (2021) showed that the presence of 2 and 4 mg L^{-1} KIN in the medium significantly enhanced shoot production. In the rooting phase, the induction of roots on the produced shoots can be an important part in micropropagation which is based on the type of culture, type of the plant, and age of the explant (Molassiotis *et al.* 2003; Thorpe *et al.* 2008). The capability of plant tissues to form adventitious roots is influenced by the interaction of many exogenous and endogenous factors including hormones and the chemical ingredients of the culture medium (Aghaye *et al.* 2013). In most reports, the exogenous auxins used for rooting were IBA, NAA, and IAA (Ainsley *et al.* 2001). Adventitious rooting in different *Origanum* species without using auxins has been reported in different studies (Kizil and Khawar 2017; Premi *et al.* 2021). However, successful rooting in *O. acutidens* was reported in the media containing 0.75 mg/l NAA (Kizil and Khawar 2017). In this regard, Abdallah *et al.* (2017) reported that te full strength MS medium supplemented with 1 mgl^{-1} IBA and the half strength MS containing 1 $mg^{l-}1$ IAA showed the highest number of main roots and root lengths.

The current study was aimed to optimize the proliferation and rooting stages of the Iranian native oregano to provide conditions for large-scale micropropagation.

Materials and Methods

Seeds of oregano were prepared by the Research Institute of Forests and Rangelands (RIFR), Iran. The seeds were planted in pots containing a mixture of pneumatic sand, perlite, and peat moss, and maintained in a greenhouse at a temperature of 25 ºC and 16 hours of light. Growing plants were carefully taken care of for 1 month to prepare suitable explants for the whole period of the research.

The culture medium and explants

The current experiment was conducted to evaluate the establishment and proliferation phases of oregano. For the micropropagation of oregano, 10-cm stem samples with 3-5 fresh and strong buds were taken from the mother plant. MS medium (Murashige and Skoog 1962) containing sucrose (30 g/l) and agar (7 g/l) was used. The pH of the culture medium was adjusted to 5.8 using the HCl 0.1 N and NaOH 0.1 N solutions. The studied growth regulators were added to the media before autoclaving. The prepared culture medium (20 ml) was poured into the glass jars (200 ml) and autoclaved for 20 minutes. After removing the bud explants from the stem under a laminar hood (JTLVC2), they were washed with 70% alcohol for

30 seconds and 5 times with sterile distilled water for 1 minute. Next, the explants were immersed in 5% sodium hypochlorite solution for 10 minutes, then washed with the sterile distilled water 5 times and finally transferred to the sterile filter paper for drying. To assay the effect of BA on proliferation rate, the sterilized explants were cultured on media with 0.25 mg/l IBA and different concentrations of BA (0, 0.5, 1, and 1.5 mg/l). Cultures were maintained at the temperature of 25±1 °C under a 16/8 h light regime. Assayed traits were recorded after 12 weeks.

The proliferated shoots were transferred to the rooting media after six weeks. In this phase, four levels of IBA (0, 0.5, 1, and 1.5 mg/l) were combined with 0.5 mg/l NAA. The basal culture medium used for rooting also was MS. All cultured media were maintained in a growth chamber at 25 ± 1 °C and 45% moisture under a 16/8 h light regime. After eight weeks of culture, the assayed traits were measured.

Traits measured

Assessed traits in the present study included the number of leaves, number of shoots and roots, chlorophyll index (SPAD), leaf dry weight, shoot length, root length and root diameter, and dry weight of whole plantlets.

Statistical analysis

Both proliferation and rooting experiments were conducted as a completely randomized design with four replications and three explants per replication. The data were subjected to analysis of variance using SAS software (version 9.1). Duncan's multiple range test was used for the comparison of the means at $p \leq 0.05$. Charts were drawn by Excel software.

Results

The results obtained from the analysis of variance (Table 1) showed that BA had a significant effect on the number of leaves and shoots, shoot length, chlorophyll index (SPAD), and leaf dry weight ($p \leq 0.01$). Also, in the rooting phase, IBA showed a significant effect on the root length and diameter, and dry weight of the whole plant ($p \leq 0.01$) but did not have a significant effect on the leaf number.

Effect of BA on the leaf number

As the results in Figure 1 exhibit, plantlets cultured in the treatment of 1 mg/l BA with 19 leaves showed the highest number of leaves, and plantlets of the control with 11 leaves showed the lowest number of leaves. The results reported on *in vitro* culture of Selva strawberry cultivar by Jalili Marandi *et al*. (2011) showed that the highest

Table 1. Analysis of variance of the effect of benzyl adenine (BA) on the evaluated traits of oregano.

SOV	df	Mean squares				
		Number of leaves	Number of shoots	Shoot length	Chlorophyll index (SPAD)	Leaf dry weight
Treatment (BA)	3	47.33**	3.41**	0.29**	31.38**	0.00058**
Error	12	6	0.95	0.065	3.003	0.00001
Total	15	53.33	4.36	0.355	34.383	0.00059
C.V (%)		14.84	34.05	13.01	8.33	9.39

**Significant at 1% level of probability.

Table 2. Analysis of variance of the effect of indole butyric acid (IBA) on the evaluated traits of oregano

SOV	df	Mean squares			
		Number of roots	Root length	Root diameter	Weight of whole seedlings
Treatment (IBA)	3	8.99^{ns}	292.139**	0.781**	0.027**
Error	12	21.35	1.004	0.004	0.0005
Total	15	30.34	293.143	0.785	0.0275
C.V (%)		38.89	9.83	13.23	15.35

. ^{ns} Not statistically significant; **Significant at 1% level of probability.

number of leaves was obtained in media supplemented with 1 or 2 mg/l benzyl adenine (BA). Although there was no statistically significant difference among the concentrations of 0.5, 1, and 1.5 mg/l BA in the number of oregano leaves, the best concentration regarding the number of leaves was 1 mg/l BA, which is consistent with the results of Jalili Marandi's *et al.* (2011). On the other hand, Premi *et al.* (2021) investigated the *in vitro* culture of *Origanum vulgare* and reported that the use of BAP in the medium resulted in the lowest number of leaves per shoot when compared to chitosan and kinetin.

Effects of BA on the number of shoots

As shown in Figure 2, there was a significant difference among different levels of BA regarding the number of shoots. Shoot number increased as the concentration of BA increased to 1.5 mg/l, however, no significant difference was found between the levels of 1 and 1.5 mg/l. The highest number of shoots (3.75) was observed in the medium containing 1.5 mg/l BA, and the lowest (1.75) was seen in the hormone-free medium. Asghari *et al.* (2013) investigated the interaction of genotype with benzyl adenine in the *in vitro* culture of basil and reported that the highest number of shoot regeneration (7.1) was obtained at 2 mg/l benzyl adenine in the Hungarian genotype, and the lowest number of regeneration (zero) was obtained in the BA-free medium. Darroudi *et al.* (2015) studied the effect of different treatments of BA (0, 0.5, 1, 3, and 5 mg/l) on the micropropagation of Redcurrant and reported that the highest number of shoots was obtained at a concentration of 3 mg/l BAP. Also, Kizil and Khawar. (2017) showed that the maximum number of 10.28 shoots per explant was obtained in the MS medium supplemented with 0.8 mg/l BAP. BA is an important cytokinin that is mostly used in the proliferation phase, and its superiority over other types of cytokinin in inducing axillary branching and multiple shoots has been reported by different researchers (Acemi *et al*. 2013; Türker and Hatipoğlu 2018; Premi *et al*. 2021).

Effects of BA on the shoot length

Based on Figure 3, the shoot length was affected by different concentrations of BA. However, there was no significant difference among the concentrations of 0, 0.5, and 1.5 mg/l BA, as well as between the concentrations of 1 and 1.5 mg/l BA. The longest shoot length was observed in medium supplemented with 1 mg/l BA (3.43 cm)

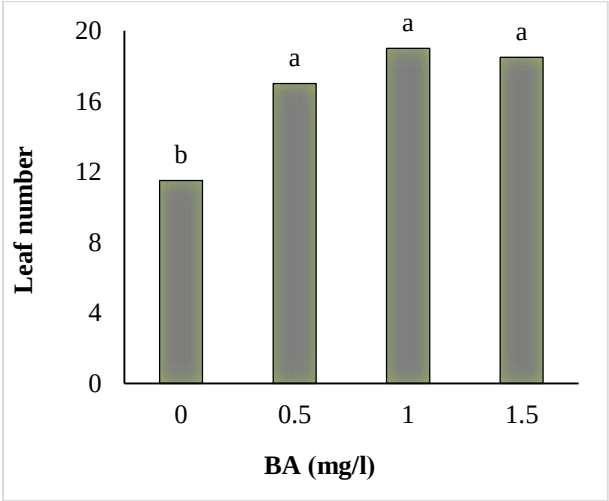

Figure 1. Effect of different concentrations of benzyl adenine (BA) on the number of oregano leaves under the *in vitro* culture.

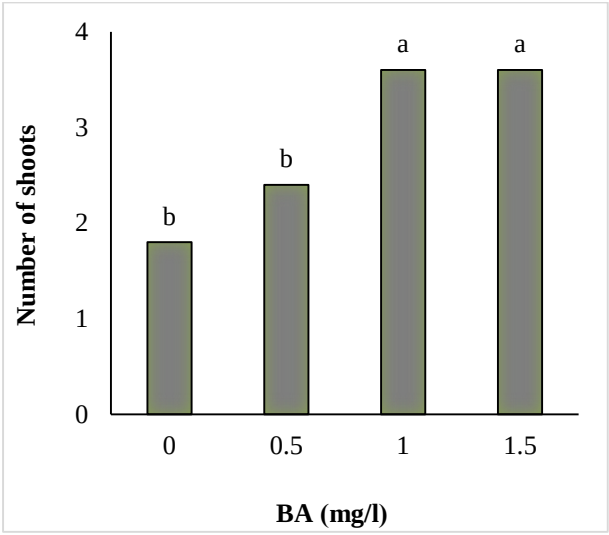

Figure 2. Effect of benzyl adenine (BA) concentrations on the number of oregano shoots under the *in vitro* culture.

and the shortest shoot length was seen in the control (1.7 cm). Consistent with our results, Jalili Marandi *et al.* (2011) in their study on the micropropagation of strawberry cv. Selva. reported that the longest shoot length was observed in the MS medium containing 30 mg chitosan and 1 or 2 mg/l BA. Similarly, Kizil and Khawar (2017) showed that a maximum shoot length of 3.25 cm was recorded on the MS medium supplemented with 1.6 mg/l BAP.

Effects of BA on the chlorophyll index (SPAD)

Increasing the concentration of BA hormone significantly increased the chlorophyll index (SPAD) in oregano (Figure 4). The highest value of the index was related to the concentration of 1.5 mg/l BA (23.4) and the lowest amount was related to the BA-free medium (17). Jalili Marandi *et al.* (2011) reported the highest amount of chlorophyll in the media containing a combination of 1 or 2 mg/l BA and 30 mg/l chitosan.

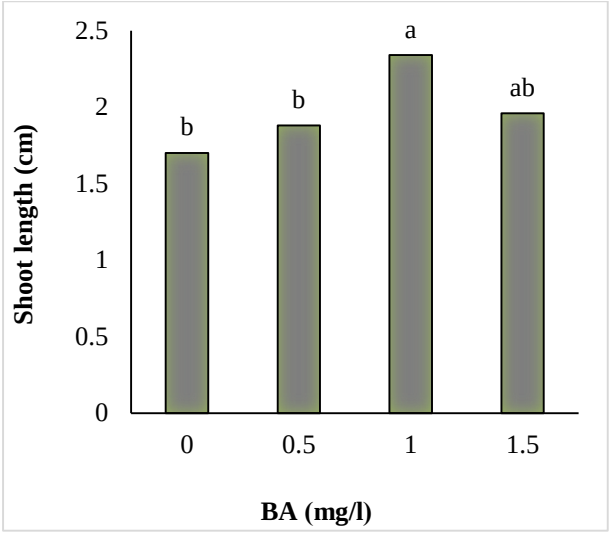

Figure 3. Effect of benzyl adenine (BA) concentrations on the shoot length of oregano under the *in vitro* culture.

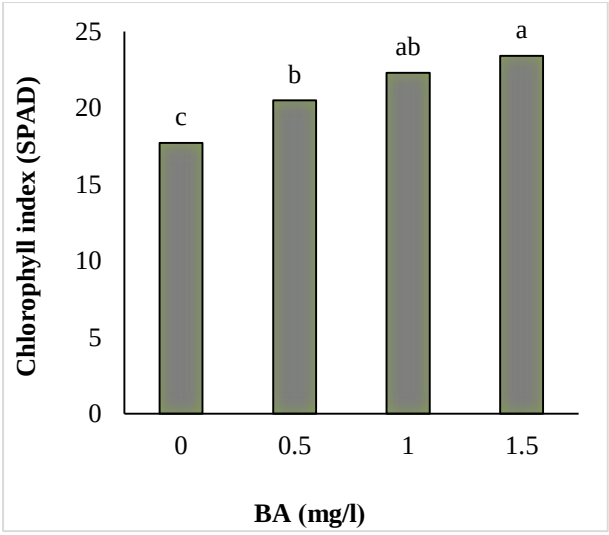

Figure 4. Effect of benzyl adenine (BA) concentrations on the chlorophyll index (SPAD) of oregano under the *in vitro* culture.

Effects of BA on leaf dry weight

The results shown in Figure 5 indicated that the leaf dry weight in the *in vitro* culture of oregano was affected by different concentrations of BA. The highest amount was seen at the concentration of 1 mg/l (0.046 g) and the lowest amount was obtained at the concentration of zero BA (0.018 g). Jalili Marandi *et al.* (2011) indicated that cytokinin increases dry matter by stimulating cell proliferation and division in organs and tissues, including leaves.

Effects of IBA on the root number

As the results in Figure 6 show, the highest number of roots was obtained at the concentration of 1 mg/l IBA (13.7) and the lowest in the control treatment (10.2). However, no significant difference was found between the levels of IBA hormone, which

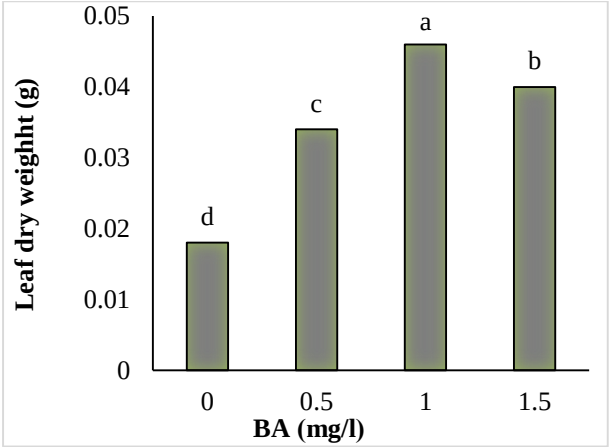

Figure 5. Effect of benzyl adenine (BA) concentrations on the leaf dry weight of oregano under the *in vitro* culture.

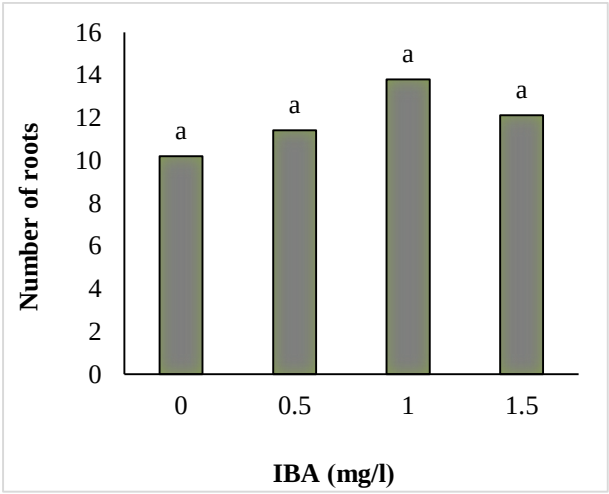

Figure 6. Effect of indole butyric acid (IBA) concentrations on the number of oregano roots under the *in vitro* culture.

which could be due to the presence of internal auxin in the oregano plant. Internal auxin is resistant to different concentrations of IBA.

Effects of IBA on the length of the roots

Based on the results demonstrated in Figure 7, IBA had a significant effect on the root length of oregano. The longest root length was observed in the control treatment (22.87 cm), followed by concentrations of 1.5, 0.5, and 1 mg/l IBA (7.74, 5.32, and 4.83 cm, respectively). Therefore, it seems that the addition of IBA to the medium reduces the root length of oregano. Saber Hamishgi *et al.* (2011) studied the micropropagation of stevia and showed that among the concentrations of 0.25, 0.5, and 1 mg/l IBA the maximum root length (2.91 cm) was obtained in the MS culture medium containing 1 mg/l IBA. The difference between these results may be due to the different plant species tested.

Effects of IBA on the root diameter

Figure 8 exhibits that among the different concentrations of IBA, 0.5 mg/l level had the greatest effect on the root diameter of oregano, and increasing the concentration of this hormone from this level upwards reduced the root diameter. In line with our results, Shakouri *et al*. (2012) studied the effect of different concentrations of NAA hormone (0, 100, 200, 300, and 400 ppm) on the cuttings of lucky bamboo, and reported that the largest root diameter was obtained in the medium supplemented with 100 ppm NAA.

Effects of IBA on the total dry weight

The results displayed in Figure 9 showed that the dry weight of the oregano plantlet was affected by different levels of IBA. The greatest dry weight was obtained at the concentration of 0.5 mg/l (0.24 g) and the lowest was obtained in the hormone-free treatment (0.07 g). However, the differences

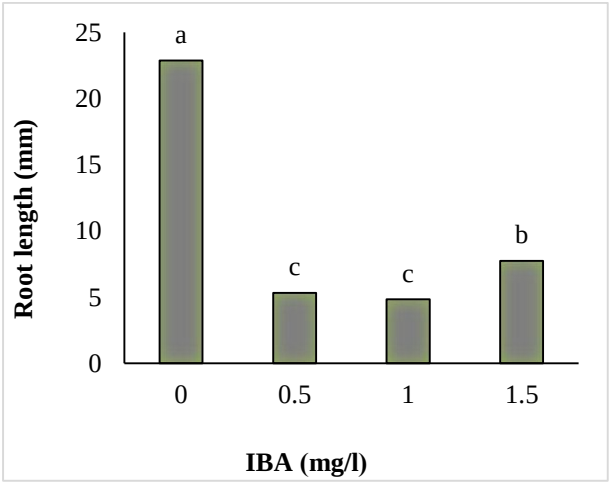

Figure 7. Effect of indole butyric acid (IBA) concentrations on the root length of oregano under the *in vitro* culture.

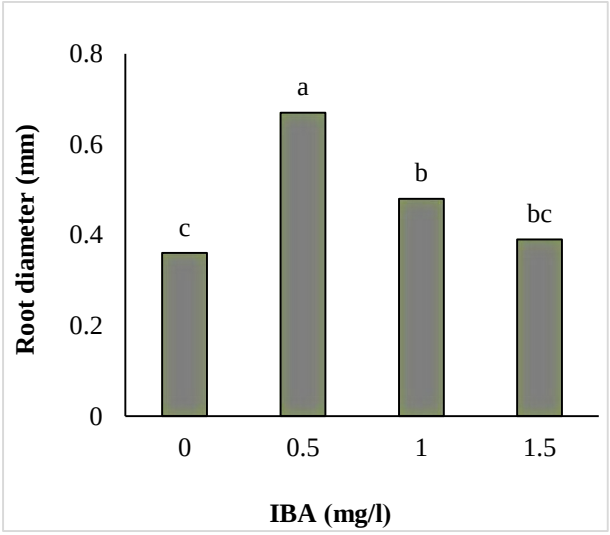

Figure 8. Effect of indole butyric acid (IBA) concentrations on the root diameter of oregano under *in vitro* culture.

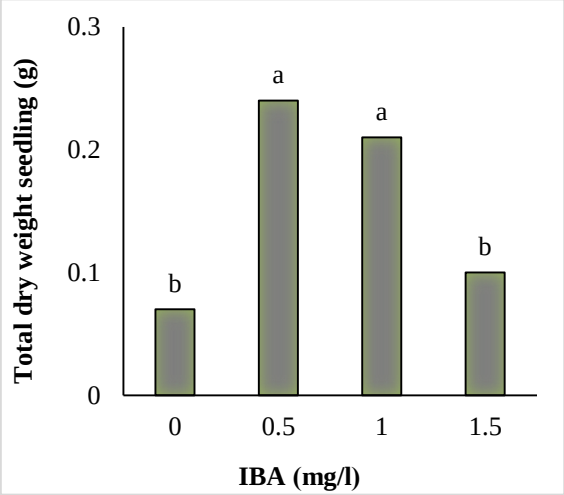

Figure 9. Effect of indole butyric acid (IBA) concentrations on the dry weight of oregano seedlings under the *in vitro* culture.

between the zero and 1.5 mg levels, as well as between 0.5 and 1 mg concentrations were not significant. Karfarma and Shirzadi (2015) stated that the interactions of different levels of IBA hormone (1000, 2000, 3000, and 4000 mg/l), treatment methods and different tomato cultivars had a significant effect on the seedling dry weight. So that the greatest seedling dry weight with an average of 1.53 g was related to the combination of 3000 mg/l IBA, Calj N3 cultivar, and the priming method, and the lowest seedling dry weight with an average of 0.4 g was related to the treatment containing 2000 mg/l IBA hormone, Calj N3 cultivar, and the foliar application method. Our results were consistent with those of Karfarma and Shirzadi (2015) and Islam *et al.* (2020).

Shoot branching is dependent on the activity of axillary meristems, which are chiefly regulated by cytokinin (Dobranszki and Silva 2010). The cytokinin BA has a key role in promoting cell division, shoot multiplication, and axillary bud formation (Sutter 1996). Shoot proliferation in the culture medium is mostly influenced by a combination of high levels of cytokinin and low concentrations of auxin (Roberson *et al.* 2005). The effect of cytokinin on tissue or organ cultures can be varied depending on the type of culture, the variety of plant, and the age of the explant (Thorpe *et al.* 2008). so that shoot length and leaf dry weight reached their peaks at the concentration of 1 mg/l BA. It was shown that when the concentration of BAP was in excessive amounts, it resulted in the reduction of shoot number. One of the possible reasons can be the reductive effect of higher concentrations of BAP (Aghaye *et al*. 2013). Thus, a certain amount of BA is required to have the best effect on the proliferation rate of explants.

Root induction in tissue culture is an important step in plant micropropagation (George 1996). Rooting is controlled by various factors such as growth regulators, basal salt composition, genotype, and culture conditions. For most species, exogenous auxins such as IBA, NAA, and IAA and their interaction with the endogenous auxins lead

to the induction of root formation in tissue culture (Thorpe *et al.* 2008). Also the ratio of auxin to cytokine is important for induction and root growth, so that higher auxin and lower cytokinin lead to the formation of roots (Torabi *et al.* 2001).

Conclusion

The results of the current investigation indicated that BA at the concentration of 1.5 mg/l brought about the highest number of leaves and shoots and the highest value of chlorophyll index in the proliferation phase. BA at the 1 mg/l concentration, resulted in the longest shoot and the greatest leaf dry weight. In the rooting stage, IBA at 1 mg/l concentration resulted in the highest number of roots, and at 0.5 mg/l led to the maximum root diameter and the highest total dry weight. It can be concluded that BA at 1-1.5 mg/l and IBA at 0.5-1 mg/l lead to the best results in the proliferation and rooting phases, respectively.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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ریزازدیادی مرزنجوش (*Origanum vulgare* L.) بومی ایران با استفاده از تنظیم کننده‌های رشد

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چکیده

مرزنجوش وحشی (*Origanum vulgare* L.)، متعلق به خانواده Labiate یکی از مهمترین گیاهان دارویی ایران و جهان است که به طور گسترده در صنایع دارویی، غذایی و بهداشتی استفاده می‌شود. پژوهش حاضر با هدف بررسی کشت درون شیشه‌ای مرزنجوش بومی ایران انجام شد. دو آزمایش برای بررسی تکثیر و ریشه زایی این گونه گیاهی طراحی شد. در مرحله تکثیر، بنزیل آدنین (BA) در چهار سطح (۰، ۰/۵، ۱ و ۱/۵ میلی گرم در لیتر) همراه با ۰/۲۵ میلی گرم در لیتر ایندول بوتیریک اسید (IBA) استفاده شد. برای مرحله ریشه زایی از IBA در چهار سطح (۰، ۰/۵، ۱ و ۱/۵ میلی گرم در لیتر) همراه با ۰/۵ میلی گرم در لیتر نفتالین استیک اسید (NAA) استفاده به عمل آمد. نتایج نشان داد که BA بر تعداد برگ، تعداد شاخساره، طول شاخساره، شاخص کلروفیل و وزن خشک برگ تأثیر معنی‌داری دارد. بیشترین تعداد برگ و شاخساره و بیشترین مقدار شاخص کلروفیل در محیط کشت حاوی ۱/۵ میلی گرم در لیتر مشاهده شد. بیشترین طول شاخساره و وزن خشک برگ در مقایسه با سایر غلظت‌ها مربوط به ۱ میلی گرم در لیتر BA بود. علاوه بر این، نتایج مربوط به مرحله ریشه‌زایی نشان داد که طول ریشه، قطر ریشه و وزن خشک کل گیاهچه‌ها به‌طور معنی‌داری تحت تأثیر IBA قرار گرفتند، در حالی که تعداد ریشه تحت تأثیر غلظت IBA قرار نگرفت. بیشترین قطر ریشه و وزن خشک کل گیاهچه‌ها در محیط کشت حاوی ۰/۵ میلی گرم در لیتر IBA و حداکثر طول ریشه در محیط MS بدون IBA به دست آمد.

واژه‌های کلیدی: ایندول بوتیریک اسید؛ بنزیل آدنین؛ تکثیر؛ ریزازدیادی؛ ریشه زایی؛ مرزنجوش