The Effect of Different Applications on In vitro Bulb Development of an Endemic Hyacinth Plant (Hyacinthus orientalis L. subsp. chionophyllus Wendelbo) Grown in Turkey

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A B S T R A C T

In this study the effects of different sucrose concentrations, and the combinations of jasmonic acid (JA) with auxins (IAA or NAA) or with cytokinin (2iP) on the bulb induction and rooting of in vitro plantlets of Hyacinthus orientalis subsp. chionophyllus Wendelbo, which is endemic in Turkey, were investigated. The effect of four different sucrose concentrations (30, 45, 60 and 90 g L⁻¹) on bulb formation in tissue culture was investigated. These plantlets were cultured on MS medium supplemented with several concentrations and combinations of JA (0.0, 1.0, 2.0 mg L⁻¹) and 2iP (0.0, 0.25 and 0.50 mg L⁻¹), IAA or NAA (0.5, 1.0 mg L⁻¹). In JA-2iP treatment, the highest number of bulblets (13.7 number/explant) was obtained by the combinations of JA 1.0 mg L⁻¹ + 2iP 0.25 mg L⁻¹. Also, the largest bulblets with the mean diameter of 7.9 mm were found on MS medium supplemented with JA 2.0 mg L⁻¹. In JA – Auxin treatment, the mean root number per bulblet was highest (17.9 number/explant) and root formation rate was maximum (81.14%) on MS medium supplemented with IAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹.

Introduction

The hyacinth plant (Hyacinthus orientalis L.) which was once thought to belong to the Liliaceae family is now classified in the Hyacinthaceae family. The hyacinth is a perennial herbaceous plant with 15-20 cm in height. A group of 5-15 flowers which has a strong scent makes a sparse bunch on the plant stem. Naturally grown hyacinth plants can be found in the Southwest Asia, Northwest of Syria and Lebanon. In Turkey, hyacinth plants are grown in the southern part of Anatolia and inner regions where Hyacinthus orientalis subsp. orientalis and Hyacinthus orientalis subsp. chionophyllus Wendelbo can be found in nature, but only the latter one is endemic to Turkey. Leaf diameters and flower tube lengths are the main criteria to distinguish the difference between these two taxons (Yüzbaşoğlu, 2003).

In Turkey, the B6, B7 and C6 sections, including the provinces of Kahramanmaraş, Sivas, Adana, Malatya, Tunceli, Erzincan and Kayseri, are the natural distribution areas of Hyacinthus orientalis subsp. chionophyllus Wendelbo. These hyacinth plants can be found mostly on the rocky slopes within these areas.

Like most geophyte plants, the hyacinth plant can be reproduced by the separation of its storage organs. However, the flowering of the multiplied plants is a long process which takes 4-5 years. In vitro culture of geophytes offers alternative methods instead of conventional methods, with many advantages such as increasing the multiplication coefficient, reducing the labour required, obtaining the virulent plant under controlled conditions in a short time.

There have been many studies on the growing techniques, propagation methods and chemical ingredients of the geophyte species which also might have economic importance as ornamentals. In these geophytes, in vitro studies are still being carried out with several objectives. These objectives include the use of plant growth regulators
on plant development (Pierik et al., 1975; Rice et al., 1983; Squires et al., 1991; Bach, 1992; Podwysynska, 2004; Mirici et al., 2005; Sun X et al., 2010), the treatments for breaking dormancy of tuberous plants (Li-Nagasuga et al., 2000; Jansky and Hamernik, 2015) and the use of different carbohydrate sources on tuber growth (Taeb et al., 1990, Sun et al., 2012). In Turkey, there are several works on the propagation and growing techniques for some of these endemic taxaons such as *Origanum sипileum* (Ołuk and Çakır, 2009; Sevindik et al., 2018); *Mascari azureum* (Urnabey, 2010); *Erodium somanum* (Çetin et al., 2016); *Silene bolanthoides* (Çördük et al., 2018), *Iris sari* (Doğan and Çağlar, 2020).

Hyacinth, as an ornamental plant, has also been explored by many ethnobotanists for their use in alternative medical treatments. The endemic *Hyacinthus orientalis* subsp. *chionophyllus* has been reported to benefit from antihemorrhoidal diseases, prostate diseases, hemostatis and wound healing leaves and bulb preparations (Kahraman and Kocabas, 2001; Çömelkeçioglu and Kahraman, 2008; Tuzlacı and Doğan, 2010; Altundag and Öztürk, 2011; Kayran and Özkan, 2017).

In plants with underground storage organs, carbohydrates that accumulate as a result of photosynthesis are transported to storage organs, which helps to develop onion, bulb or rhizome-like structures. The growth of these developing storage organs is also crucial to the flowering capacity of plants (Khodorova and Boitel-Conti, 2013). However, varying concentrations of sucrose and precooling applications can be very useful in the development of a plant or microbulblets from bulb explants of *hyacinthus*, tulips, lilium and glodiole (Takayama and Öztürk, 2011; Kayran and Özkan, 2017).

Materials and Methods

**Plant Material and Surface Sterilization**

The immature capsules of *Hyacinthus orientalis* subsp. *chionophyllus* Wendelbo were collected within the Kahramanmaras province (Turkey) in May. The capsules containing immature embryos were washed in detergent and surface-sterilized for 1 minute in 70% ethanol and then for 15 minute in 20% commercial bleach (Axion) adding 1-2 drop Tween-20 with continuous stirring. Then the seeds were rinsed three times with sterile distilled water. The sterilization of the media was done by autoclaving at 121°C for 15 min. after adjusting pH to 5.7 + 0.1 Plant material was cultured at 16 + 0.1°C in a 16-hour photoperiodic climate chamber and sub cultured at 6-week intervals to allow plants to develop.

**Culture Conditions**

Capsules were dissected longitudinally with a sterile lancet and the immature embryos were taken out and then transferred to Murashige and Skoog’s (MS) media consisted of 2 mg L⁻¹ BA+ 0.5 mg L⁻¹ IAA + 3.0% sucrose, 0.7% agar (Sigma) in petri dishes for germination. The medium pH was adjusted to 5.6 with 1N NaOH or 1N HCl before autoclaving at 121°C for 20 minutes. All cultures were kept at 24±1°C under cool-white fluorescent light (35 µ mol m⁻² s⁻¹) with 16h photoperiod. All growth regulators (N6-benzylamino-purine (BAP), 2-isopentenyladenenide (2iP), indole-acetic acid (IAA), naphthalene acetic acid (NAA) and jasmonic acid (JA) were filter-sterilized using a Millipore filter (0.22 µm pore size) and added to autoclaved medium before dispensing into petri dishes. The cultures were transferred to fresh media every six weeks. The average number of bulblets, root number, percent roots and size of bulblets recorded after two sub culturing were analysed statistically using factorial completely randomized design. Five bulbs were cultured per treatment and each experiment was repeated three or four times according to the experimental designs.

**Sucrose Treatment**

The effect of carbon source in MS (Murashige and Skoog, 1962) media formulation supplemented with 2 mg L⁻¹ BA+ 0.5 mg L⁻¹ IAA was evaluated by increasing the sucrose concentration (3.0, 4.5, 6.0 and 9.0%). For the regeneration of bulblets from embryos (approximately 0.5-1.0 cm length) were cultured in test tubes. Five test tubes containing one bulb were cultured per treatment and each experiment was repeated three times. The mean number of bulblets per explant and bulblets diameter was evaluated in two subcultures after culture initiation.

**JA and Cytokinin Treatment**

Bulblets (approx. 0.5-1.0 cm length) were cultured in MS solidified with 7.5 g L⁻¹ agar and containing 3.0% sucrose and three levels of JA (0.0, 1.0 and 2.0 mg L⁻¹) and 2iP (0.0, 0.25 and 0.50 mg L⁻¹). Bulblets from explants were cultured in vessels contained about 50 ml of medium for regeneration. Magentas containing five explants were cultured per treatment and each experiment was repeated three times. The mean number of bulblets per explant, number of roots and bulblets size was evaluated in two subcultures after culture initiation.
JA and Auxin Treatment

The medium with 3.0% sucrose was supplemented with three concentrations of JA (0.0, 1.0 and 2.0 mg L\(^{-1}\)) and IAA or NAA (0.0, 0.5 and 0.1 mg L\(^{-1}\)). Developed bulblets (approx. 3.0 mm in diameter) by MS medium supplemented with 2.0 mgI\(^{-1}\) BA + 0.5 mg L\(^{-1}\) IAA were determined for these treatments. Bulblets from explants were cultured in vessels containing 50 ml of medium for regeneration. Magentas containing five explants were cultured per treatment and each experiment was repeated three times. The mean number of roots, rooting percentage and bulb diameter were evaluated in two subcultures after culture initiation.

Statistical Methods

In this study, different sucrose concentration and several combinations of plant growth regulators such as IAA, NAA, 2iP, JA were tested. The data were analysed statistically using factorial completely randomized design consisting of each combination was three replicates consisted of five explants. Data was statistically analysed using the JMP 8.0. Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. The angle transformation values were calculated from the data in percentage (%). Variance analysis was done according the literature (Steel and Torrie, 1980; Yurtsever, 1984).

Results and Discussion

Sucrose Treatment

After two weeks in culture, most immature hyacinth (*Hyacinthus orientalis* subsp. *chionophyllus* Wendelbo) embryos formed shoots in test tubes. Increased sucrose concentrations in the growing media improved the bulblet formations. The rate of explants producing shoots and the number of bulblets per explant was determined after 12 weeks of culture initiation. The largest bulblets with a mean diameter of 7.4 mm and 7.2 mm were found on MS medium with BAP 2.0 mg L\(^{-1}\) + IAA 0.5 mg L\(^{-1}\) containing 6.0 % sucrose and 9.0% sucrose respectively (Table 1). The highest number of bulblets (18.2 and 16.9) per explant was on MS medium containing 9.0% sucrose and 3.0% sucrose respectively.

*In vitro* shoot initiation and bulblet regeneration in hyacinth with different sucrose treatments were shown in Figure 1.

Table 1. The effects of different sucrose concentrations in the MS medium (2.0 BAP mg L\(^{-1}\) + 0.5 IAA mg L\(^{-1}\)) on *in vitro* bulblet formation in hyacinth.

| Sucrose concentration (%) | Mean bulb diameter (mm) | Mean number of bulblets (number/explant) |
|---------------------------|-------------------------|----------------------------------------|
| 3.0                       | 5.8\(^{a}\)              | 16.9\(^{a}\)                           |
| 4.5                       | 5.2\(^{b}\)              | 11\(^{b}\)                             |
| 6.0                       | 7.4\(^{a}\)              | 14.5\(^{ab}\)                         |
| 9.0                       | 7.2\(^{a}\)              | 18.2\(^{a}\)                          |

Different letters within a column are significantly different (LSD test; P≤0.05)

Figure 1. a) Initiation of shoots with sucrose in MS medium supplemented with 2.0 BAP mg L\(^{-1}\)+ 0.5 IAA mg L\(^{-1}\). b) Bulb growth of shoots with 3.0% sucrose. c) Diameter measurement of a developing bulblet. d) Bulblet formation on the medium containing different sucrose concentration. e) Bulblet formation with 9.0% sucrose.
There are many studies indicating the positive effects of increased sucrose concentrations in the growing media on the bulb formations in several plants. Sun et al. (2012) reported that *Lilium davidii* var. *unicolor* had 100% percent success in bulblet formation at the sucrose concentration of 100 g L⁻¹, while the diameter of bulblets occurs to the maximum. The increase in sucrose concentration, except standard sucrose concentration (3.0%), induced a high occurrence of bulblets per explant and a well-developed bulb diameter (approx. 1-36 shoots and 3.46-12.6 mm diameter, respectively). Sucrose at high concentrations promoted *in vitro* bulbing of shallot (*Allium cepa*), where a concentration of 50 g L⁻¹ sucrose induced 10% of the plants to form bulbs, whereas bulbing in shallot was not promoted both above and below this concentration (Saos et al., 2002). An increasing sucrose concentration, from 3 to 60 mM, stimulated *in vitro* bulb formation in Narcissus (Staikidou et al., 1994, 2005). Our study with hyacinth plants also confirmed the positive effect of increased sucrose concentration in the media on *in vitro* bulb formation.

**JA - 2iP Treatment**

In this treatment different concentrations of JA and 2iP (as a cytokinin source) alone or with their several combinations were used for bulblet formation, shoot and root regeneration from the microbulb explants of hyacinth. Many newly formed shoots started to grow after 10-12 days of culture in MS medium (3.0% sucrose) with JA-2iP combinations.

In this experiment, the mean number of bulblets per explant with combinations was often considerably higher than with JA treatments alone (Table 2). The highest number of bulblets (13.7 and 12.4 bulblets per explant) was obtained by using JA 1.0 mg L⁻¹ + 2iP 0.25 mg L⁻¹ and 2iP 0.50 mg L⁻¹, respectively. This was followed with 10.2 bulblets by using JA 2.0 mg L⁻¹ + 2iP 0.25 mg L⁻¹ and with 8.4 bulblets by 2iP 0.25 mg L⁻¹, which were in the same statistical group.

Number of roots under all applied concentration were higher than in the control group of *Hyacinthus orientalis* subsp. *chionophyllus* Wendelbo (Table 2). The addition of only high JA concentration significantly increased the mean number of roots per explant. Similarly, Silva Maia and Pedroso-de-Moraes, (2017) reported a positive increase in the number of roots when the addition of JA to the culture medium showed the best result for rooting TDZ was used with NAA. The highest number of roots (19.9 per explant) was obtained by using 2.0 JA alone. Also the other combinations had a higher number of roots (ranged from 10.3 to 15.7) per explant than that of the control treatment which had only 6.3 roots. But these differences were not statistically significant (P≤0.05).

**Table 2. Effects of JA, 2iP and their combinations in the MS medium (containing 3% sucrose) on the number of bulblets and roots, and bulb diameter of the hyacinth plants.**

| Plant growth regulator concentrations and combinations (mg L⁻¹) | Mean number of bulblets (number/explant) | Mean number of roots (number/explant) | Mean bulb diameter (mm) |
|---------------------------------------------------------------|-----------------------------------------|--------------------------------------|------------------------|
| JA 1.0                                                        | 5.1                                     | 11.6<sup>c</sup>                    | 7.0<sup>b</sup>        |
| JA 2.0                                                        | 5.2<sup>b</sup>                         | 19.9<sup>a</sup>                    | 7.9<sup>a</sup>        |
| 2iP 0.25                                                      | 8.4<sup>ab</sup>                        | 14.5<sup>b</sup>                    | 6.9<sup>ab</sup>       |
| 2iP 0.50                                                      | 12.4<sup>a</sup>                        | 10.3<sup>b</sup>                    | 6.4<sup>bcd</sup>      |
| JA 1.0 + 2iP 0.25                                            | 13.7<sup>a</sup>                        | 15.7<sup>b</sup>                    | 5.7<sup>bcd</sup>      |
| JA 1.0 + 2iP 0.50                                            | 6.8<sup>b</sup>                         | 12.5<sup>bc</sup>                   | 5.2<sup>bcd</sup>      |
| JA 2.0 + 2iP 0.25                                            | 10.2<sup>b</sup>                        | 13.0<sup>bc</sup>                   | 6.3<sup>bcd</sup>      |
| JA 2.0 + 2iP 0.50                                            | 6.6<sup>b</sup>                         | 13.3<sup>bc</sup>                   | 6.4<sup>bcd</sup>      |
| Control                                                       | 6.5<sup>b</sup>                         | 6.3<sup>c</sup>                     | 4.4<sup>a</sup>        |

Different letters within a column are significantly different (LSD test; P≤0.05)

**Table 3. Effect of Auxins and JA and their combination of the root number, percentage of the roots and the bulb diameter of *in vitro* grown hyacinth plants.**

| Plant growth regulator concentration and combinations (mg L⁻¹) | Mean number of roots (number/explant) | Rooting percentage(%) | Mean bulb diameter (mm) |
|---------------------------------------------------------------|--------------------------------------|-----------------------|------------------------|
| IAA 0.5                                                       | 1.0<sup>b</sup>                      | (46.92)<sup>abc</sup> | 6.92<sup>bcd</sup>     |
| IAA 1.0                                                       | 1.5<sup>b</sup>                      | (46.92)<sup>abc</sup> | 7.62<sup>abcd</sup>    |
| IAA 0.5 + JA 1.0                                              | 13.1<sup>a</sup>                     | (72.29)<sup>ab</sup>  | 8.5<sup>a</sup>        |
| IAA 0.5 + JA 2.0                                              | 3.2<sup>b</sup>                      | (76.92)<sup>a</sup>   | 6.9<sup>bcd</sup>      |
| IAA 1.0 + JA 1.0                                              | 2.7<sup>b</sup>                      | (63.84)<sup>ab</sup>  | 5.1<sup>bcd</sup>      |
| IAA 1.0 + JA 2.0                                              | 17.9<sup>a</sup>                     | (81.14)<sup>a</sup>   | 9.1<sup>a</sup>        |
| NAA 0.5                                                       | 3.8<sup>b</sup>                      | (76.92)<sup>a</sup>   | 5.8<sup>abcd</sup>     |
| NAA 1.0                                                       | 1.6<sup>b</sup>                      | (47.71)<sup>ab</sup>  | 5.8<sup>abcd</sup>     |
| NAA 0.5 + JA 1.0                                              | 4.1<sup>b</sup>                      | (72.29)<sup>ab</sup>  | 5.9<sup>abcd</sup>     |
| NAA 0.5 + JA 2.0                                              | 0.9<sup>b</sup>                      | (42.29)<sup>abc</sup> | 4.3<sup>cd</sup>       |
| NAA 1.0 + JA 1.0                                              | 0.4<sup>b</sup>                      | (21.93)<sup>c</sup>   | 3.6<sup>d</sup>        |
| NAA 1.0 + JA 2.0                                              | 0.2<sup>b</sup>                      | (30.78)<sup>bc</sup>  | 4.2<sup>cd</sup>       |
| Control                                                       | 4.4<sup>b</sup>                      | (64.22)<sup>ab</sup>  | 5.6<sup>abcd</sup>     |

<sup>a</sup>Values in parentheses indicate arcsine transformed % values.
As seen in Figure 2, the best bulb and root development of the hyacinth plant was obtained on MS medium supplemented with JA 2.0 mg L⁻¹.

**JA – Auxin treatment**

The results of root formation from plantlets obtained on MS media containing various concentrations of IAA and NAA and with or without JA after 12 weeks in culture were given in Table 3.

The mean root number per bulblet was highest (17.9 number/explant) in MS medium supplemented with IAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹ combination, followed by IAA 0.5 mg L⁻¹ + JA 1.0 mg L⁻¹, combinations with 13.1 roots (Table 3). The root number of certain combinations was as low as 0.4 (NAA 1.0 mg L⁻¹, NAA 1.0 mg L⁻¹ + JA 1.0 mg L⁻¹ or NAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹). The root formation rate was highest in IAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹ with 81.14%. This was closely followed by IAA 0.5 mg L⁻¹ + JA 2.0 mg L⁻¹ and IAA 0.5 mg L⁻¹ + JA 2.0 mg L⁻¹ combinations with the same percentage (76.92%), being in the same statistical group (Table 3).

The bulb diameters were the largest of the MS medium with the combination of IAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹ with 9.1 mm or by IAA 0.5 mg L⁻¹ + JA 1.0 mg L⁻¹ with 8.5 mm. The bulb and root development of the plants in MS medium with several combinations of NAA-JA did not differ significantly compared to that of the control plants. The lowest bulb diameter with 3.6 mm was recorded on MS medium supplemented with NAA 1.0 mg L⁻¹ and JA 1.0 mg L⁻¹. It was also observed that the root development was directly proportional to the bulb diameter. In this experiment the best root regeneration and bulb development were obtained with the combination of IAA 1.0 mg L⁻¹ + JA 2.0 mg L⁻¹ (Figure 3).

The results revealed that bulb formation was better with the application of IAA-JA combination than with the combination of NAA-JA. Saniewski (1974) reported that the best concentration for the induction of bulb formation in *Hyacinthus orientalis* was the medium containing 10 ppm BA + 1 ppm NAA, and indicated that the concentration of NAA was necessary for root differentiation and callus formation. On the contrast, in our study NAA-JA combination did not effectively promote root production. Xiao Mei (2010) reported that the best concentration for the induction of root formation in *Hyacinthus orientalis* was MS medium supplemented with ½ MS + 0.2 mg L⁻¹ NAA. However, in this present study another form of auxin (IAA) supplemented with JA was more effective in promoting the rooting percentage of *in vitro* plantlets of endemic *Hyacinthus orientalis* subsp. *chionophyllus* Wendelbo grown in Turkey.

**Conclusion**

Turkey has a rich endemic plant pool, however, most of the endemic plants are at the risk of extinction (Ekim et al., 2000). Unfortunately, a comprehensive conservation program, including the proper propagation methods of certain endemic plants have not been established yet (Bulut and Yılmaz, 2010). Therefore more studies are needed to develop suitable *in vitro* propagation protocols. Since the interaction of the plant growth regulators in the process of *in vitro* propagation of the ornamental geophyte plants
might differ according to the species or even with the explant types used. Efficient bulblet regeneration has been obtained from immature embryo explants of different geophytes such as *Stenbergia fischeriana* (Mirici et al., 2005), *Tulipa karamanica* and *Tulipa sintenensis* (Kalyoncu et al., 2006), *Muscaria mirum* (Nasircilar et al., 2009), *Hyacinthus orientalis* (Kizil et al., 2016), *Iris kirkwoodii* (Doğan and Çağlar, 2018). The most important factors affecting *in vitro* plant regeneration are plant growth regulators, plant genotype, plant age, plant collect time, habitat and explants type (Nasircilar et al., 2009; Özcan, 2002, Doğan and Çağlar, 2020). In this study, the addition of JA or in combination with IAA or 2iP in the growing media containing sucrose improved the micropropagation success and bulb formation of the hyacinth explants. Moreover, this protocol, in which immature embryos are used as explant source can be applied for rapid propagation of other geophytes species with potential for ornamental The methods and findings of the present study might also be used in further studies for obtaining a better proliferation rate and improving the bulb development in other Hyacinth species as well as in other geophyte species.

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