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EFFICIENT *IN VITRO* BULBLET PRODUCTION OF ENDANGERED *HYACINTHELLA Micrantha* (BOISS.) CHOUARD

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ABSTRACT

Hyacinthella micrantha (BOISS.) Chouard with their attractive flowers is one of the important endemic and endangered species of Turkey. We aimed to improve a standard *in vitro* multiplication protocol using basal scales for bulblet production and conservation of the species. Bulb scales of the species collected from natural habitat were cultured in the different basal Murashige and Skoog (MS), Orchimax and Lindeman Orchid Medium embryonic callus induction medium. Then, they were transferred to MS, Orchimax and Lindeman Orchid Medium basal bulblet induction medium supplemented with cytokinins (1.0, 2.0, 4.0 and 8.0 mg/l N6-benzylamino-purine (BAP), Zeatin and 2-isopentyladenine (2-iP) at 16 °C. After ten months of culture initiation, high frequency bulblet regeneration was developed using bulb scales on different basal media containing different concentrations of cytokinintypes. The highest ratio of bulb scales forming shoot/bulblets per explant (87%) and bulblets per explants (5.60) were induced on a Orchimax media supplemented with 8 mg/L Zeatin. MS and Orchimax medium gave the best result in terms of embryonic calli formation and *in vitro* bulblet production compared to Lindeman Orchid medium. 2,4-D, BAP and Zeatin were more effective than2-iPfor *in vitro* bulblet production. Also, regenerated bulblets subjected to pre-cold treatments for 3 months at 4-6 ºC resulted in increasing of new mini bulblets (approximately 15%). Regenerated bulblets were acclimatized with high survival ratio (82 %).

Keywords: bulblets, *Hyacinthella micrantha*, *in vitro,* micropropagation.

INTRODUCTION

The genus *Hyacinthella* Schur is a member of the *Liliaceae* family and there are 17 species spread out largely Mediterranean regions (Anonymous, 2010). *Hyacinthella* genus is quite similar to *Muscari* and *Bellevalia* genus and there are *12 Hyacinthella* species which 10 of them are endemic (endemism rate is 83.3 %) and also one hybrid, *H. micrantha* (Baker) Chouard × *H. heldreichii* (Boiss.) Chouard distributed naturally in Turkey(Güner *et al.,* 2000; Arslan, 2004; Yetişen *et al.,* 2012). *H. micrantha* is endemic and also involved in the endangered category (Öztürk and Bilgili, 2015) in Turkey. The species is bulbous, annual and herbaceous species the species is mainly distributed in rocky areas of Central and Transitional zones (Bolu, Kastamonu, Amasya, Ankara, Çorum and Samsun provinces) of Turkey. The species with their attractive sky blue and white flowers is under threat because of having slow propagation in nature, destruction of pasture areas, irregular grazing and collection. It was reported that the species should be protected and cultivated (*in-situ* or *ex-situ*) for designing works (Öztürk and Bilgili, 2015). The natural low germination and reproductive rate of the species by bulbs obstruct cultivation and marketing of this ornamental plant. We have observed that each bulb produces average only 1–2 mature big sized (2 cm diameter) bulblets in a 3 years periods in nature or greenhouse.

Plant tissue cultures applications are commonly used for multiplication of pathogen-free plants and conservation of germplasm. Plant regeneration through adventitious shoots and somatic embryos was achieved using different explant types, basal medium or growth regulators on similar and close relative *Muscari* and *Bellevalia* species *Bellevalia romana* (Lupi *et al.,* 1985),*Muscari armeniacum* Leichtlin ex Baker (Suzuki and Nakano, 2001),*Muscari comosum* var. *plumosum* (Ko *et al.,* 2006), M*uscari comosum* var. *plumosum* (He *et al.,* 2006), *Muscari macrocarpum* Sweet (Ozel *et al.,* 2007),*Muscari aucheri* (Uranbey *et al.,* 2010), *Muscari neglectum* (Karamian and Ranjbar, 2010), *Muscari mirum* (Nasırcılar *et al.,* 2011), *Muscari armeniacum* (Lee *et al.,* 2012; Yücesan *et al*. 2014), *Bellevalia* dubia (Canhoto, 2018). However, there is no reports about callus induction and shoot or bulblet regeneration of *H.micrantha* or other *Hyacinthella* species. Therefore, the first objective of this study was to improve efficient micropropagation system for *H. micrantha* and the second goal was to investigate the influences of different

commercial medium and concentrations of cytokinin on bulblet multiplication of proliferated calli.

MATERIALS AND METHODS

Plant material and explant preparation: *H. micrantha* were determined in Turkey, square C5 botanical region and bulbs of the species collected on March during the period of flowering, in 2010–2011 (Fig 1. a-b). They were planted in pots containing compost in a greenhouse for 3-4 days. The bulbs were removed from the pots, outer leaves of the bulbs were discarded and washed with sterile water and detergent. Then, the bulbs were treated with fungicide (N-(trichloromethyl thio) cyclochox-4 ene-1, 2-dicarboximide) and kept in a dry paper for 24 hours at 20 °C. The bulbs were kept for 3 min in 95% C_2H_6O. The bulbs were sterilized in 40 min in 40% (v/v) commercial bleach and cleaned 4 times with water. The bulbs were divided into four parts and explants (with 3-4 scales in 2-3 mm width 6-7 mm lengths) containing basal tissue and were isolated.

Embryonic callus and bulblet induction medium: Bulb scale explants were transferred to MS (Murashige and Skoog, 1962), Orchimax (Duchefa Biochemie B.V. Netherlands, catalog number: O0257) and Lindeman Orchid (Duchefa Biochemie B.V. Netherlands, catalog number: L0216**)** *embryonic callus induction medium* containing activated charcoal, casein, 2,4-D and gelrite. After 2-4 weeks of culture, all explant covered with embryonic calli were moved to *bulblet induction medium* containing the same basal medium containing various concentrations of cytokinin as follow.

Embryonic callus induction medium I: MS minerals and vitamins, 400 mg / l casein, 2.5 mg / l 2,4-D, 1 g /L activated charcoal and 2 g / l gelrite.

Bulblet induction mediumI: MS minerals and vitamins,40 g / l sucrose, 1.0, 2.0, 4.0 and 8.0 mg / l BAP, Zeatin and 2-iP, 0.1 mg / l NAA, 7 g / l agar

Embryonic callus induction medium II: Orchimax minerals and vitamins, 400 mg / l casein, 2.5 mg / l 2,4-D, 1 g/l activated charcoal and 2 g / l gelrite.

Bulblet induction medium II: Orchymax minerals and vitamins, , 40 g / l sucrose, 1.0, 2.0, 4.0 and 8.0 mg / l BAP, Zeatin, 2-iP, 0.1 mg / l NAA and 7 g / l agar

Embryonic callus induction medium III: Lindeman Orchid Medium and minerals, 400 mg / l casein, 1 g / l activated charcoal, 2.5 mg / l 2,4-D and 2 g / l gelrite.

Bulblet induction medium III: Lindeman Orchid Medium and minerals + 0.1 mg / l NAA, 40 g / l sucrose, 1.0, 2.0, 4.0 and 8.0 mg / l BAP, Zeatin and 2-iP, 7 g / l agar

Maturation and acclimatization of the bulblets: The bulblets are separated from each other and transferred to following maturation mediums and basal medium remained same for 4-6 weeks.

Bulblet maturation medium I : MS minerals and vitamins, 40 g / l mannitol, 1000 mg / l casein, 50 g / l sucrose, 7 g / l agar;

Bulblet maturation medium II : Orchimax minerals and vitamins, , 40 g / l mannitol, 1000 mg/l casein , 50 g / l sucrose and 7 g / l agar;

Bulblet maturation medium III : Lindeman Orchid minerals and vitamins + 50 g / l sucrose, + 40 g / l mannitol, 1000 mg / l casein, 7 g / l agar;

Bulblets were subjected to pre-cold treatments at 4-6 ºC. for 4-8- and 12 weeks. The bulblets reached to the diameter of 1-1.5 cm were transferred to pots containing peat and acclimatized in growth chamber with % 70-90 humidity at 20-22°C.

Statistical analysis: Three replicates were applied for each treatment and all experiments were repeated at least once. Results were processed and analysed by analyses of variance (ANOVA) the differences between the means were compared by Duncan's multiple range (Düzgünes *et al*. 1987)

RESULTS AND DISCUSSION

Contaminations were notreported on bulb scale explants of *H.micrantha*. The bulb scales swelled and produced yellow coloured and embryogenic calli structure after 15-20 days on all embryonic callus induction medium containing different basal media(Fig 1.c). Embryogenic calli and somatic embryos were observed after 4-6 weeks culture initiation (Fig1. d-e). Embryonic calli clusters were detached from the basal and transferred to bulblet maturation medium forshoot proliferation. Shoot initials and bulblet development occurred after 13-16 weeks and of culture. Within 16 weeks, each bulblet turned into shoots. These bulbs turned into semi-mature bulbs after 24-28 weeks of culture initiation and in *vitro* bulbing was induced almost in all media tested. Bulblet induction was recorded after 20 weeks of culture initiation for each basic medium. The frequency of bulblet regeneration were statistically influenced ($p<0.01$) for each basal media containing various of concentrations of BAP, 2-Ip and Zeatin (Table 1, Table 2 and Table 3.).

The highest bulblet regeneration frequency (72 %) was recorded for MS basal medium containing 2.0 mg/l Zeatin. Whereas, the maximum number of bulblets per explant (5.33) was found on a MS medium supplemented with 2.0 mg/l BAP (Fig. 1. f). Presence of BAP and Zeatin in MS medium affected bulblet multiplication when compared to MS basal medium that do not contain cytokinine, however, different concentrations of 2-Ip did not positively afftect bulblet regeneration.

The best regeneration frequency (87 %) and number of bulblets per explants (5.60) were recorded for

Orchimax basal medium containing 8.0 mg/l Zeatin (Fig. 1. g). Comparing cytokinine types included in Orchimax media, the species responded better to Zeatin and high concentrations of Zeatin comparatively induced higher bulblet regeneration frequency.

The maximum bulblet regeneration (41.4% and 39.9 %) occurred on Lindeman Orchid media containing 8 mg/l BAP and 4.0 mg/l Zeatin respectively (Fig. 1. h). Similarly, the highest mean number of bulblets per explant (3.13) was found on Lindeman Orchid medium containing 8 mg/l BAP.

Table 1. *In Vitro* bulblet multiplication on*H. micrantha* on embryonic callus and bulblet induction medium I containing MS, BAP, 2-iP and Zeatin.

Cytokinine types (mg/l)			Rgeneration ratio of producing shoots or bulblets [%]	Number of bulblets per bulb scale
BAP	**2-iP**	**Zeatin**		
-	-	-	22.2 cd*	0.60 b*
1.0			23.3 cd	1.43 b
2.0			54.7 abc	5.33 a
4.0			68.5 ab	1.93 ab
8.0			40.0 a-d	1.40 b
	1.0		10.0 d	1.10 b
	2.0		28.5 a-d	1.87 ab
	4.0		33.0 a-d	3.93 ab
	8.0		19.4 cd	0.70 b
		1.0	26.3 bcd	0.63 b
		2.0	72.0 a	3.70 ab
		4.0	20.0 cd	0.50 b
		8.0	29.7 a-d	0.63 b
			LSD %1: 23.75	LSD %1: 3.185

*) Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test

Table 2. *In Vitro* bulblet multiplication on*H. micrantha* on embryonic callus and bulblet induction medium II containing Orchimax, BAP, 2-iP and Zeatin.

Cytokinine types (mg/l)			Rgeneration ratio of producing shoots or bulblets [%]	Number of bulblets per bulb scale
BAP	**2-iP**	**Zeatin**		
-	-	-	13.0 cd*	1.07 cd*
1.0			0.0 e	0.0 d
2.0			22.5 cd	2.47 bcd
4.0			32.9 bc	1.37 cd
8.0			23.3 cd	4.80 ab
	1.0		13.0 cd	1.37 cd
	2.0		15.7 cd	0.70 cd
	4.0		3.4 de	0.17 cd
	8.0		20.0 cd	2.20 bcd
		1.0	26.3 c	2.57 bcd
		2.0	21.0 cd	3.03 abc
		4.0	60.7 b	4.47 ab
		8.0	87.0 a	5.60 a
			LSD %1: 16.13	LSD %1: 2.585

*) Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test

Table 3. *In Vitro* bulblet multiplication on *H. micrantha* on embryonic callus and bulblet induction medium III containing Lindeman Orchid, BAP, 2-iP and Zeatin.

Cytokinine types (mg/l)			Rgeneration ratio of producing shoots or bulblets [%]	Number of bulblets per bulb scale
BAP	**2-iP**	**Zeatin**		
-	-	-	19.4 ab	0.90 cd
1.0			6.6 b	1.30 bcd
2.0			26.3 ab	2.17 abc
4.0			28.5 ab	1.30 bcd
8.0			41.4 a	3.13 a
	1.0		23.3 ab	1.33bcd
	2.0		13.0 ab	0.90cd
	4.0		39.9 a	1.67bcd
	8.0		30.0 ab	0.77cd
		1.0	16.5 ab	1.50bcd
		2.0	23.3 ab	2.47ab
		4.0	6.6 b	0.53d
		8.0	16.5 ab	1.30bcd
			LSD %1 17.44	LSD %1 1.251

*) Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test

When compared to basal media, MS and Orchimax media responded better than Lindeman Orchid media to higher bulblet formation irrespective of concentration. Existence or lack of 2-ipin the culture medium did not changed to bulblet production in Lindeman Orchid media. Content of the basal nutrient medium in plant tissue cultures studies is one of the most chief factors inducing callus induction and regeneration of plant species (Gamborg *et al.,* 1976). *In vitro* bulblet formation of *Muscari* and *Bellevalia* species has been observed on wide range of media MS medium, N_6 (Chu *et al.,* 1975), Orchimax (Duchefa Biochemie B.V. Netherlands product code O0257) and culture conditions were frequently used for organogenesis of these plants. MS basal medium generally gave the best response for bulblet induction of *Muscari* and *Bellevalia* species (Lupi *et al.,* 1985; Ozel *et al.,* 2007; Ozel *et al.,* 2009; Karamian and Ranjbar, 2010; Azad and Amin, 2012; Ozel *et al.,* 2015) using mature bulb scales. Orchimax and MS medium gave better results compared to Orchid medium in our study. Uranbey *et al,* (2010) similarly found that considerable increases bulblet regeneration were accomplished on Orchimax mineral salts and vitamins when compared to MS and N_6 basal media for close relative *Hyacinthus azureus*, *Hyacinthella azurea* or *Muscari azureum* species.

Previous studies about bulblet proliferation for bulbous species revealed that addition of cytokines to basal media drastically stimulated bulblet formation (Ulrich *et al.,* 1999; Wawrosch *et al.,* 2001; Paek and Murthy, 2002; Mirici *et al.,* 2005; Uranbey *et al.,* 2010,Yücesan *et al*. 2014). Efficiency of BAP for high-frequency bulblet production was reported in mature bulb scale explants of *Muscari* and *Bellevina* and other geophytes species (Saniewski, 1979; Suzuki and Nakano, 2001; Malabadi and Van Staden, 2004; Nakano *et al.,* 2005; Mirici *et al.,* 2005; Ko *et al.,* 2006; Nasircilar *et al.,* 2011, Suh *et al.,* 2015). Our results also indicated that BAP and Zeatin were very effective for callus formation and bulblet induction. Moreover, healthy bulblets with larger half-diameters were observed in all basal media containing BAP in our study.

Bulblets with 0.5-1.5 cm diameter were visible on *bulblet maturation medium* after 7-8 months culture initiation (Fig 1. i-j). The bulblets are separated from each other and transferred the fresh maturation mediums. Each bulblet developed average 3-5 roots for each bulblet during culture. Rooting occurred in all media including MS, Orchimax and Lindeman Orchid and Rooting quality in MS medium was high with number of roots bulblet. Bulblets started rooting in the maturation medium were subjected to pre-cold treatments 1,2 and 3months at 4-6 ºC before being transferred to the soil. Increasing the duration of the cold treatment to three months resulted in proliferation of leafy structures producing new mini bulblets. Proliferation of new mini bulblets kept 4-6 ºC was occurred 4.5% for the first month, 9.6% for the second month and 15.2% for the third month.The bulblets reached to the diameter of 1-1.5 cm were transferred to containers filled up peat, sterile field soil (1:1) and acclimatized in climate room with 70-90% moisture. 82 % of regenerated bulblets formed healthy leafy structures after acclimatization period.

Figure 1. *In vitro* bulblet production of *H. micrantha* using bulb scales on different basal media (a-b) endangered species *H. micrantha* with blue and white flowers in its natural habitat (c) Embryogenic callus clusters after 2 weeks in culture (d) somatic embryogenic callus clusters after 4 weeks in culture (e) developed somatic embryo callus clusters after 6 weeks in culture (f) bulblet formation on bulblet maturation MS basal medium containing 2 mg/l BAP (g) bulblet formation on bulblet maturation Orchimax basal medium containing 8 mg/l Zeatin (h) bulblet formation on bulblet maturation Lindeman Orchid basal medium containing 8 mg/l BAP (i-j) Bulblets with 0.5-1.5 cm diameter on bulblet maturation medium after 7-8 months in culture.

Conclusion: Orchimax medium and MS medium supplemented with BAP and Zeatin resulted in robust shoot proliferation and bulblet formation using mature bulb scales of *H. micrantha.* Pre-**c**hilling was effective for bulb induction before being transferred to the soil and cold treatment should not be performed in a short period

of less than one month. This protocol developed can be used for conserving this endangered species. This study also efficient for the micropropagation of other valuable *Hyacinthella* species.

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