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IMPROVED *in vitro* PROPAGATION OF *Hyacinthus orientalis* L. USING FRUITS CONTAINING IMMATURE ZYGOTIC EMBRYOS AND TENDER LEAF SHEATH AS EXPLANTS

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Abstract. Hyacinthus genus is an important group of ornamental plants that bear white, yellow, pink, red or purple coloured flowers. It has about 2000 species spread around the world that are grown commercially. Although, plant occurs naturally in Turkey yet efforts have not been made to adapt it for open field cultivation. There is need to transfer and establish these plants from wild to fields for commercial use through *in vitro* and *ex vitro* approaches, that will help local economy profitably. This study reports *in vitro* culture of *Hyacinthus orientalis* L. subsp. *orientalis*; using fruits containing immature zygotic embryos cultured on MS medium containing varying concentrations of Thidiazuron (TDZ) with and without 0.2 mg l^{-1} naphthaleneacetic acid (NAA) supplemented with 20 or 40 g l^{-1} sucrose. The study also reports induction of bulblets on tender leaf sheaths on MS medium containing different concentrations of benzylaminopurine (BAP) + 0.1 mg l^{-1} NAA supplemented with 30 g l^{-1} sucrose. The maximum bulblet regeneration (40%) with 31.33 bulblets/explant was noted on MS medium containing 0.15 mg l^{-1} TDZ supplemented with 40 g l^{-1} sucrose. Whereas, the best bulblet regeneration on tender leaf sheath explants was noted on 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA with 2.97 bulblets per explant of 0.55 cm bulb diameter and 1.20 leaves per bulblet. These bulblets were cultured singly on MS medium containing 20 mg l^{-1} GA_3 (Gibberellic acid) + 50 g l^{-1} sucrose and attained a diameter of 0.75–1.00 cm in 30 days time. The bulbs regenerated on both explants were successfully rooted and acclimatised in plant growth chamber using peat moss followed by their transfer to open field conditions.

Key words: *Hyacinthus orientalis* L. subsp. *orientalis*, micropropagation, fruits containing immature zygotic embryos, tender leaf sheath

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INTRODUCTION

Ornamental flower bulbs exhibit great diversity in their growth, developmental biology, morphology and physiological responses to environmental factors [Benschop et al. 2010].

Ornamental geophytes industry is dominated by 7 genera (Tulipa, Lilium, Narcissus, Gladiolus, Hyacinthus, Crocus, and Iris) out of more than 800 different genera found in the world. It is very interesting to note that most of the traditional flowering geophytes or bulbs are cultivated/grown in temperate regions. However, with increased global demand for new and old ornamental geophytes, development of innovative production and marketing techniques has become inevitable for several hundred taxa that exist in the wild [Bryan 1989]. Thus, there is need to focus on locally available flowering plants for their economic use as indoor or outdoor plants and save foreign exchange [Lee et al. 2007]. Two perennial taxons namely *Hyacinthus orientalis* subsp. *orientalis* and *H. orientalis* subsp. *chionophilus* are found in Turkey. They grow on calcareous rocky mountainous slopes at an altitude of 400–1600 m in Eastern Mediterranean, Southern Anatolia and Western Syria with blooming during March April [Tubives 2016]. They are mainly grown for aesthetic purpose in gardens and as cut flower. They are also used industries related to perfumery for obtaining essential oil extracts.

Seeds are not preferred for commercial multiplication; however, they are used for development of new cultivars. Their natural propagation rates are very slow and take 4–6 years to develop a bulb size capable of flowering and seed set; under ideal conditions.

Bulb chipping is most commonly used for *in vitro* micropropagation of geophytes with moderate success rates [Bach 1992]. Amano and Tsutsui [1980] suggests 43°C and 38°C temperature treatment for 4 and 30 d to induce offsets on 15 cm circumference bulblets. Commercial propagation of the plant is done by scoping or cross cutting by removing apical meristems on the cut surfaces or leaf cuttings and scales [Hartmann et al. 1990]. Plant tissue culture studies use leaves, scales, inflorescence stalk, ovaries and perianths [Hussey 1975, Pua and Chong 1984, Lu et al. 1988] to achieve the objectives.

The breeding and commercial method of propagation should be easy independent and efficient for propagation of plants year long. The study aimed to understand the best conditions for regeneration from fruits containing immature zygotic embryos for breeding purpose and tender leaf sheaths as explants to evaluate regeneration potential after treatment with different concentrations of plant growth regulators for commercial bulblet production.

MATERIALS AND METHODS

Plant material and experiments. Fruits containing immature zygotic embryos borne on racemes post 9–11 days post anthesis on first flowers of *H. orientalis* subsp. *orientalis* were randomly selected from the collection of "The Experimental and Ornamental Plants Garden" the Faculty of Agriculture, Dicle University, Diyarbakır Turkey. It may be mentioned that mating of closely related parent results in increased homozygosity due to inbreeding and produce uniform plants een if they are produced from seeds. Plant populations of *H. orientalis* subsp. *orientalis* grow here since last 11 years. Therefore although, the plant is cross pollinated, the fruits containing immature embryos were counted homozygous.

Tender leaf sheath explants were obtained from juvenile bulblets induced on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose were abaxially cultured on MS medium containing 0.5, 1.0, 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (three combinations) plus MS medium used as control. The fruits were washed in slow-flowing tap water for 30 min to remove any visible contaminations followed by drying in laminar flow cabin and dark storing at 4 ±1°C in closed pair of Petri dishes. The fruits were surface-sterilised using 60% (v/v) hydrogen peroxide for 10 min followed by 3 × 3 min rinsing with double-distilled sterilised water. These were subsequently cultured on MS medium [Murashige and Skoog 1962] supplemented with 30 g sucrose solidified with 6.2 g l^{-1} agar (Duchefa, Haarlem, The Netherlands) for 7 d to screen for possible fungal and bacterial contaminations.

The screened fruits were regenerated on MS medium containing 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 mg l^{-1} TDZ (Thidiazuron) with and without 0.2 mg l^{-1} NAA (1-Naphthaleneacetic acid) using 20 and 40 g l^{-1} sucrose (6 + 6 combinations) + 2 g l^{-1} activated charcoal and solidified with 6.2 g l^{-1} agar to induce bulblets.

The tender leaf sheaths were also used as explant in another experiment. The explants were cultured on MS medium containing 0.0 (control) 0.5, 1.0, 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (three combinations excluding control) supplemented with 30 g l^{-1} sucrose + 2 g l^{-1} activated charcoal and solidified with 6.2 g l^{-1} agar.

Mean values for bulblet regeneration rate (%), number of bulblets per explant, and bulblet diameter (cm) were evaluated for fruits containing immature zygotic embryos based regeneration. Moreover, swelling percentage (%), number of bulblets per explant, bulb diameter (cm) and number of leaves per explant were also evaluated for tender leaf sheath explant based regeneration experiment.

Rooting. The mother (stalk) bulblets induced on immature embryos and tender leaf sheaths were cultured on MS medium containing 50 g l^{-1} sucrose + 2 g l^{-1} activated charcoal and solidified with 6.2 g l^{-1} agar to harden them, transform from juvenile vegetative phase to vegetative maturation phase and increase their bulblet diameter.

The developing bulblets were rooted on MS medium containing 1 mg l^{-1} IBA and 30 g l^{-1} sucrose for rooting. The rooting bulblets were transferred to 0.35 l pots containing 0.30 l of peat moss pH 6.5 ±0.5 in growth cabinet incubated for 7–9 weeks at depth of 1–1.5 cm. To prevent pushing of bulbs out, the pot surface was covered with approximately 0.5 to 0.60 cm sterilized grey sand. The pots were watered regularly to keep the rooting medium thoroughly moist avoiding wetting and flooding. The were subjected to 10º ±1ºC temperature and 80.0% relative humidity (TK 252, Nuve Turkey) with a 16 h light photoperiod at a photosynthetic photon flux density (PPFD) of 35 µmol m^{-2} s^{-1} for 30 d.

Statistical analysis. Each experimental treatment contained 60 explants divided into equally distributed 15 replications (15 replications × 4 explants = 60). The data were subjected to one-way ANOVA in "IBM® – SPSS® Statistics Version 20" software for Windows [http://www-01.ibm.com/support/ docview.wss?uid = swg24029274]. Data in percentages were subjected to arcsine square root transformation [Snedecor and Cochran 1967] before statistical analysis. The post hoc test was performed using Duncan's Multiple Range Test (DMRT) to compare differences among treatment means at $P < 0.05$ or $P < 0.01$.

RESULTS

Regeneration on immature fruits containing zygotic embryos. Effects of varying concentrations of TDZ with and without 0.1 mg l^{-1} NAA supplemented with 20 and 40 g l^{-1} sucrose on bulblet regeneration from fruits containing immature zygotic embryos were compared. Manual dissection of 400 randomly selected fruits on racemes showed average of 30–40% fruits with visible zygotic embryos. Rest of the fruits showed incompletely developed primary tissues at pre embryonic stage.

Rate (%) of bulblet regeneration. All fruits containing immature zygotic embryos at pre embryonic stage failed to induce bulblets. All regenerating fruits with immature embryos at post embryonic stage with visible embryonic structures induced callus before bulblet regeneration.

Significantly different and variable ($P < 0.05$) bulblet regeneration was noted on MS medium containing varying concentrations of TDZ with and without 0.2 mg l^{-1} NAA supplemented with 20 or 40 g l^{-1} sucrose (tab. 1).

No regeneration was noted on 0.05 mg l^{-1} TDZ and 0.15 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 20 g l^{-1} sucrose (tab. 1 column 3). Non regenerating immature fruits at pre embryonic stage were seen as black mass (of rounded fruit) in the culture medium.

Excluding non regenerating fruits containing immature zygotic embryos, regeneration percentage ranged 16.67 ±0.05–40.00 ±0.31% on varying concentrations of TDZ supplemented with 20 g l^{-1} sucrose. Whereas, it remained 20% (with different standard error) on varying concentrations of TDZ + 0.2 mg l^{-1} NAA supplemented with 20 g l^{-1} sucrose. Maximum bulblet regeneration was noted on 0.25 mg l^{-1} TDZ (fig. 1a). It seemed that the bulbs that contained post embryonic stage embryos regenerated and those containing pre embryonic stage embryos (described above) failed to regenerate.

No regeneration was noted on 0.05 mg l^{-1} TDZ, 0.2 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA and 0.25 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA. It ranged 20 ±0.03–33.33 ±0.19% on varying concentrations of TDZ and 20 ±0.03–40 ±0.17% on different concentrations of TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose (tab. 1 column 4). The highest regeneration percentage was obtained on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose. Variable anthocyanin pigmentation was also noted on developing bulblets (fig. 1b).

Number of bulblets per explant. Irrespective of the concentration of sucrose, varying concentrations of TDZ (with and without 0.2 mg l^{-1} NAA) failed to induce any significant statistical difference for number of bulblets induced on each fruit containing immature zygotic embryo (tab. 1 column 5 and 6). Except non regenerating explants, number of bulblets per immature zygotic embryo on MS medium containing varying concentrations of TDZ supplemented with 20 or 40 g l^{-1} sucrose showed mean number of 13.33 ±0.13–23.33 ±0.18 and 21.33 ±0.05–31.33 ±0.09 bulblets per explant respectively.

The maximum numbers of 23.33 ±0.18 bulblets were regenerated on MS medium containing 0.10 mg l^{-1} TDZ supplemented with 20 g l^{-1} sucrose. Whereas, the maximum numbers of 31.33 ±0.09 bulblets were regenerated on MS medium containing 0.15 mg l^{-1} TDZ supplemented with 40 g l^{-1} sucrose.

Table 1. Effects of carious concentrations of TDZ + NAA on bulblet regeneration and bulblet diameter of *H. orientalis* cultured on 20 and 40 g l^{-1} sucrose from fruits containing immature zygotic embryos

Medium		Rate (%) of bulblet regeneration **		Number of bulblets per explant		Bulblet diameter (cm)**	
TDZ (mg l^{-1})	NAA (mg l^{-1})	20 g l^{-1} sucrose*	40 g l^{-1} sucrose*	20 g l^{-1} sucrose*	40 g l^{-1} sucrose*	20 g l^{-1} sucrose**	40 g l^{-1} sucrose*
0.05	0.20	20.00 ±0.01 ab	20.00 ±0.03ab	20.00 ±0.07	25.67 ±0.03	0.19 ±0.01a	0.28 ±0.11b
0.10	0.20	20.00 ±0.06ab	40.00 ±0.17a	20.00 ±0.07	28.83 ±0.02	0.18 ±0.03a	0.37 ±0.11a
0.15	0.20	0.00 ±0.00b	26.67 ±0.42 a	0.00 ±0.00	26.67 ±0.06	0.00 ±0.00c	0.21 ±0.01c
0.20	0.20	20.00 ±0.04 ab	0.00 ±0.00b	19.10 ±0.03	0.00 ±0.00	0.11 ±0.05b	0.00 ±0.00d
0.25	0.20	20.00 ±0.03ab	0.00 ±0.00b	20.00 ±0.22	0.00 ±0.00	0.11 ±0.02b	0.00 ±0.00d
0.30	0.20	20.00 ±0.08ab	26.67 ±0.04a	21.00 ±0.47	24.23 ±0.09	0.12 ±0.01b	0.18 ±0.01c
0.05	0.00	0.00 ±0.00b	0.00 ±0.00b	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00d	0.00 ±0.00d
0.10	0.00	16.67 ±0.05ab	26.67 ±0.07ab	23.33 ±0.18	22.32 ±0.06	0.21 ±0.03a	0.26 ±0.09a
0.15	0.00	20.00 ±0.16ab	20.00 ±0.03ab	20.00 ±0.01	31.33 ±0.09	0.14 ±0.03b	0.25 ±0.02a
0.20	0.00	20.00 ±2.32ab	20.00 ±0.02ab	20.00 ±0.02	21.33 ±0.05	0.13 ±0.05b	0.29 ±0.031a
0.25	0.00	40.00 ±0.31a	30.00 ±0.12a	18.83 ±0.19	22.52 ±0.03	0.16 ±0.04b	0.16 ±0.05c
0.30	0.00	26.67 ±0.43ab	33.33 ±0.19a	13.33 ±0.13	23.11 ±0.06	0.10 ±0.01c	0.20 ±0.02b

* – all values given in a column followed by different letters are significantly different at 0.05 level using Tukeys test
** – all values given in a column followed by different letters are significantly different at 0.01 level using Tukeys test
** – mean values shown by different letters in a single column are statistically different using Duncan's Multiple Range Test at $p < 0.01$ level of significance
ns – non significant

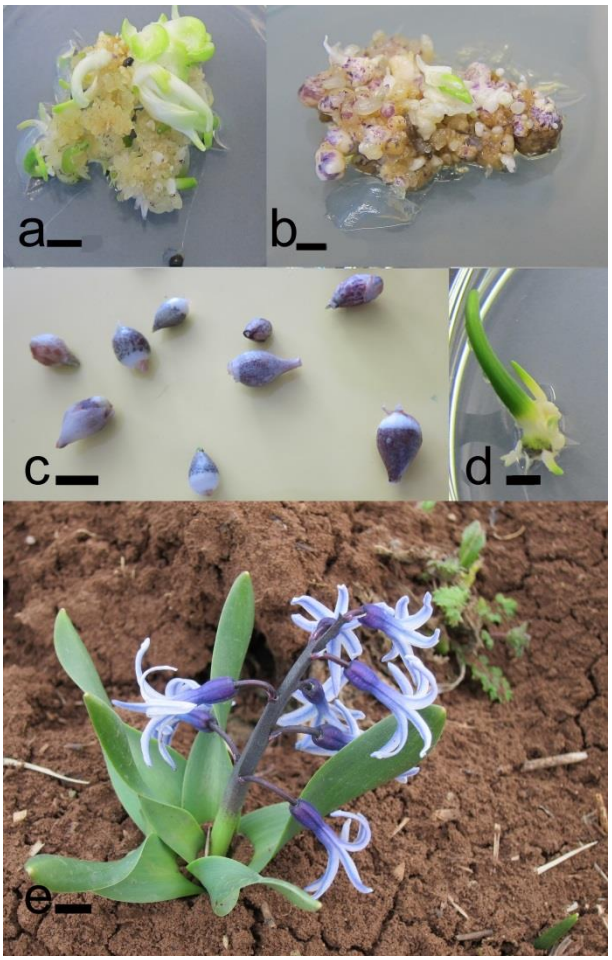

Fig. 1. Regeneration on immature fruits containing zygotic embryos (a) regeneration on MS medium containing 0.25 mg l^{-1} TDZ supplemented with 20 g l^{-1} sucrose (b) 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose (c) increase in bulblet diameter on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA (d) the developing bulblets on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA with induction of roots and shoots after two weeks (e) hardening in the field. Bar frame a = 0.5 cm, frame b = 0.4 cm, frame c = 1 cm, frame d = 1 cm, frame e = 1.40 cm

Bulblet diameter. Bulblet diameter ranged 0.10 ±0.01–0.21 ±0.03 cm and 0.11 ±0.05–0.19 ±0.01 cm on MS medium containing varying concentrations of TDZ or TDZ + 0.2 mg l^{-1} NAA supplemented with 20 g l^{-1} sucrose respectively (tab. 1 column 7).

Bulblet diameter ranged 0.16 ±0.05–0.29 ±0.031 cm and 0.18 ±0.01–0.37 ±0.11 cm on MS medium containing varying concentrations of TDZ and different concentrations of TDZ + 0.2 mg l^{-1} NAA supplemented 40 g l^{-1} sucrose respectively (tab. 1 column 8). A general comparison of bulblet diameter on two sucrose concentrations showed an

increased bulblet diameter on varying concentrations of TDZ with and without 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose. Maximum bulblet diameter (0.37 ±0.11 cm) was noted on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA (fig. 1c). All bulblets induced 1–2 leaves per bulblet (data not shown in Table).

Rooting and acclimatisation. They attained average mass of 0.75–1.00 cm on MS medium post GA3 (Gibberellic acid) treatments followed by culture on MS medium containing 50 g^{-1} sucrose. The developing bulblets on MS medium containing 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA were rooted on MS medium containing 1 mg l^{-1} IBA and 30 g l^{-1} sucrose. These bulblets induced 1–2 cm long healthy and sturdy roots and 4–5 cm long shoots in 2 weeks' time (fig. 1 d).

All of the rooted bulblets were not difficult to acclimatize when transferred to 0.35 l pots containing 0.30 litre peat moss and subjected to 10 ±1°C temperature and 80.0% relative humidity in a growth chamber under 16 h light photoperiod at 35 µmol m^{-2} s^{-1} for 30 d. They were transferred to fields after hardening in the growth chamber at the end of 12 months (fig. 1e). They induced flowering after 3–4 months depending on tissue maturity and transformation from vegetative to generative phase. They induced 5–6 leaves per bulb with inflorescence and did not show any problem of stress during field performance.

Bulblet induction on tender leaf sheath explant under the influence of different concentrations of BAP and NAA. All regenerating tender leaf sheath explants swelled and elongated before regeneration. Regeneration was noted with formation of bulb initials at the leaf sheath bases very close to bulb tunics (fig. 2a). The swellings on explants had range of 66.67 ±1.04–93.33 ±4.65% with 1.70 ±0.65–2.97 ±0.94 bulblets per explant. Tender leaf sheath grew to variable length on all cultures including control. All of them had curly leaf tips. No swelling and regeneration was noted on control. Maximum swelling and bulblets per explant was noted on MS medium containing 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA. Bulblet diameter had range of 0.55 ±0.06–0.65 ±0.04 cm. Maximum bulblet diameter was noted on 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (fig. 2b).

These bulblets induced 1.20 ±0.07 to 2.67 ±0.04 leaves per explant. Maximum number of leaves was noted on 0.5 mg l^{-1} BAP + 0.10 mg l^{-1} NAA. Significant reduction in number of leaves per explant was noted on 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (1.60 ±0.06 leaves per explant) and 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (1.20 ±0.07 leaves per explant).

Bulblet maturation medium. All those bulblets that were not treated with 20 mg l^{-1} GA_3 tended to induce necrosis on leaves without any increase in diameter of developing bulblets.

BAP + NAA (three combinations) regenerated bulblets preconditioned for 15 days on MS medium containing 20 mg l^{-1} GA_3 tended to accelerate transition from a juvenile vegetative phase to vegetative maturation and stimulated fast induction of new leaves. Except few non-significant changes, conditioning of bulblets did not show any significant differences on bulblet diameter. The bulblets showed statistically non-significant increase or decrease in bulblet diameter. The bulblets induced diameter of 0.65 ±0.03 cm or an increase of 0.3 mm on bulblets that were induced on 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (fig. 2 c). However, a diameter of 0.62 ±0.06 and 0.53 ±0.04 cm or 0.3 and 0.2 mm diameter decrease was noted on bulblets regenerated on 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA and 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA, respectively.

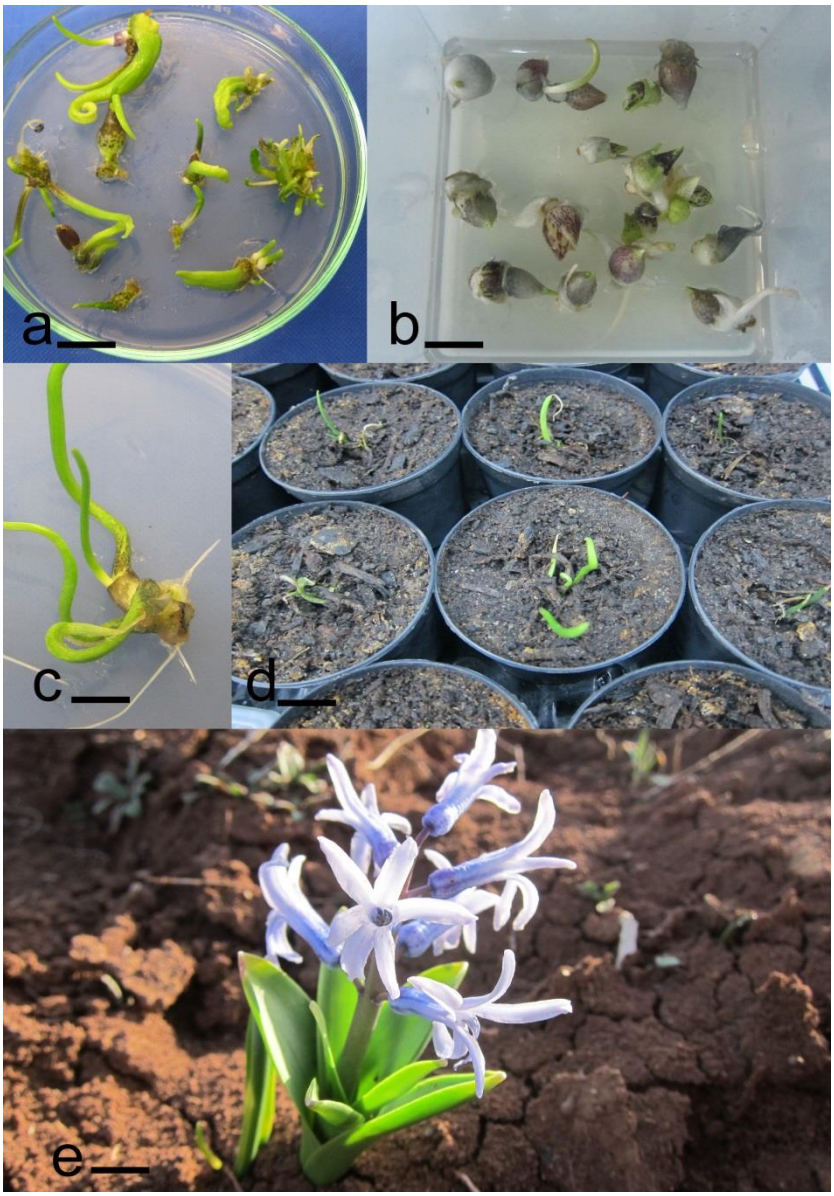

Fig. 2. Bulblet induction on tender leaf sheath explant under the influence of different concentrations of BAP and NAA (a) regeneration on swelled and elongated leaf sheath bases very close to bulb tunics (b) maximum bulblet diameter on 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (c) induction of improved diameter on 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA (d) hardened bulblets on MS medium containing 50 g l^{-1} sucrose transferred to peat moss at 10 ±1°C and 80.0% relative humidity under 16 h light photoperiod (e) the bulblets transferred to fields after acclimatisation with induction of racemes on flowering stalks. Bar frame a, b = 1 cm, frame c = 1.4 cm, frame d = 0.5 cm, frame e = 1.20 cm

Irrespective of the effects on bulblet diameter, all bulblets induced increased number of leaves in range of 1.20 ±0.07–2.67 ±0.04 before and 2.73 ±0.05 to 4.67 ±0.09 after GA_3 treatment of bulblets. Maximum number of leaves was induced on bulblets regenerated on MS medium containing 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA. It was followed by significantly reduced 2.73 ±0.05 and 2.93 ±0.02 leaves per bulb on bulbs regenerated on 1.0 mg l^{-1} BAP–0.1 mg l^{-1} NAA and 1.5 mg l^{-1} BAP–0.1 mg l^{-1} NAA induced bulblets respectively.

Table 2. Mean values of callus formation, number of leaf per explant and number of bulblets per explant obtained from different concentration of BAP and 0.1 mg l^{-1} NAA supplemented with basal MS medium from *H. orientalis*

Medium		Callus formation rate (%)*	Number of bulblets per explant*	Bulb diameter (cm)*	Number of leaves per ulblet**
BAP (mg l^{-1})	NAA (mg l^{-1})				
0.5	0.1	66.67 ±1.04c	2.53 ±0.91 ab	0.62 ±0.01a	2.67 ±0.04a
1.0	0.1	80.00 ±2.76b	1.70 ±0.65b	0.65 ±0.04a	1.60 ±0.06b
1.5	0.1	93.33 ±4.65a	2.97 ±1.94a	0.55 ±0.06b	1.20 ±0.07b
Control		0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

* – values in each column followed by a different lower-case letter are statistically different at $P \leq 0.05$ by Duncan's multiple range test
** – values in each column followed by a different lower-case letter are statistically different at $P \leq 0.01$ by Duncan's multiple range test

Table 3. Stimulation of leaves induction on bulbs and roots using MS medium containing 20 mg l^{-1} GA_3

Medium (mg l^{-1})		Bulb diameter (cm)*		Number of leaves per explant**		Number of daughter bulblets per mother bulb*	
BAP	NAA	before	after	before	after	before	after
0.5	0.1	0.62 ±0.01a	0.65 ±0.03 a	2.67 ±0.04a	4.67 ±0.09a	0.00 ±0	1.73 ±0.12b
1.0	0.1	0.65 ±0.04a	0.62 ±0.06 a	1.60 ±0.06b	2.73 ±0.05 b	0.00 ±0	2.20 ±0.11 ab
1.5	0.1	0.55 ±0.06b	0.53 ±0.04 b	1.20 ±0.07b	2.93 ±0.02b	0.00 ±0	2.40 ±0.23 a

* – values in each column followed by a different lower-case letter are statistically different at $P \leq 0.05$ by Duncan's multiple range test
** – values in each column followed by a different lower-case letter are statistically different at $P \leq 0.01$ by Duncan's multiple range test

During this phase initial vegetative phase of bulblets had changed to late adult maturing phase. Accelerated transition from a juvenile vegetative to an adult maturation phase due to GA_3 treatment also caused positive effects on induction of new axillary bulblets. GA_3 conditioning induced 1.73 ±0.12 to 2.40 ±0.23 daughter bulblets

per mother (stalk) bulb. Maximum number of daughter bulblets were regenerated on 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA regenerated bulblets.

Bulblet hardening medium. Thereafter, these bulblets were transferred to MS medium containing 50 g l^{-1} sucrose to harden them; where the bulbs developed anthocyanin pigmentation. Non-significant changes were noted on bulblets diameter. The bulblets induced on 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA showed a non-significant reduction in bulblet diameter compared to those regenerated on 1 mg l^{-1} BAP + 0.1 mg l^{-1} NAA showed no change in diameter and those on 1.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA showed an increase in bulblet diameter in the hardening medium (tab. 4).

Table 4. Means of bulb diameter, number of per leaf and number of bulblets per explant measured before and fifteen days later bulbs incubated MS medium contain 50 g l^{-1} sucrose to obtain more bulb diameter

Medium (mg l^{-1})		Bulb diameter (cm)*		Number of leaves per explant**		Number of daughter bulblets per explant*	
BAP	NAA	before	after	before	after	before	after
0.5	0.1	0.65 ±0.03 a	0.61 ±0.01	4.67 ±0.01a	5.33 ±1.11	1.73 ±0.12b	4.27 ±0.05 a
1.0	0.1	0.62 ±0.06 a	0.62 ±0.07	2.73 ±0.21 b	3.40 ±0.12	2.20 ±0.11 ab	2.43 ±0.02 b
1.5	0.1	0.53 ±0.04 b	0.62 ±0.02	2.93 ±0.61b	4.90 ±0.51	2.40 ±0.23 a	2.47 ±0.06 b

Mean values shown by different letters in a single column are statistically different using Duncan's Multiple Range Test at $P < 0.05$ level of significance

The bulbs induced on all concentrations of BAP + NAA after hardening showed significantly increased number of leaves per bulb when compared to leaves induced on maturation medium. They had range of 3.40 ±0.12 to 5.33 ±1.11 leaves per bulb after the hardening treatment; which induced maximum number of 5.33 ±1.11 leaves on 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA induced bulbs. New daughter bulblets were induced that ranged 2.43 ±0.02 to 4.27 ±0.05. Maximum number of daughter bulblets were regenerated on MS medium containing 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA after hardening. No necrosis was noted on the developing bulblets. The explants that were not hardened on sucrose failed to regenerate new leaves and had stunted growth and necrosis on developing leaves.

Rooting and acclimatisation. The bulbs rooted on MS medium containing 1 mg l^{-1} IBA were hardened and transferred to peat moss at 10 ±1°C and 80.0% relative humidity under 16 h light photoperiod (35 µmol m^{-2} s^{-1} for 30 d (fig. 2d). The bulbs induced more number of leaves and conceived more compactness after transfer to MS medium containing 50 g l^{-1} sucrose for 15 days of hardening. These hardened bulbs induced healthier roots compared to non hardened bulbs and grew well when transferred to pots containing peat moss. These bulblets were transferred to fields after acclimatisation. All bulbs induced stalks with racemes (fig. 2e) and set

seeds under field conditions after a period of 13 months when they met appropriate photoperiod during March–April.

Comparing success of 120 bulbs each regenerated on two explants, both showed easy acclimatisation under field conditions. No difference was noted on plants regenerated from two explants and induced racemosus flowering after 12–13 months under field conditions.

DISCUSSION

Plant biotechnology provides an excellent opportunity for micropropagation of bulbous plants. Newly growing *H. orientalis* subsp. *orientalis* plants face a continuous threat due to biotic and abiotic stresses throughout growth under natural conditions. Despite many bulblet regeneration protocols developed for *H. orientalis*, the regeneration frequency is low and varies highly among different reports [Hussey 1975, Pierik and Post 1975, Lu et al. 1988, Ault 1995]. This study reports micropropagation of *H. orientalis* using fruits containing immature zygotic embryos and tender leaf sheath as explant.

It is well known that *H. orientalis* has a raceme containing unbranched, indeterminate type of inflorescence. It was found that indeterminate type of flowering habit in *H. orientalis* after flowering on raceme type inflorescence affected seed set quantitatively throughout the seed set period [pers observations, Beckett 1993] by delay in reproductive development in some flowers with their variable performance. This affects their rate of multiplication under natural conditions [Anonymous 2016]. This could have resulted in variable growth, poor development or non development of embryos on immature fruits resulting in low regeneration from them. Immature fruits with developed embryos showed regeneration. Fruits with partial development of embryos or no embryos failed to regenerate and divide their cells. No regeneration or poor regeneration (20–40%) of immature fruits/seeds collected from fields could be attributed to this. The results of the study are in agreement with Mukhtar et al [2012]. The results of the study are also in agreement with Steeves and Sussex [1989] and Lyndon [1994], with similar observations on other plants.

Although, TDZ is necessary for regeneration, NAA played an important role in controlling cell differentiation [Bassuner et al. 2007, Petrasek and Friml 2009, Rademacher et al. 2012]. It was found that TDZ with and without NAA affected regenerating explants for bulblet differentiation variably in agreement with previous studies [Khawar and Ozcan 2002, Khawar et al. 2004, Vanneste and Friml 2009, Cheng et al. 2010, Zhao et al. 2010]. Sucrose is used as exogenous source of carbohydrate and as osmotic agent in plant cell and tissue culture studies. The results confirmed that irrespective of use of varying concentrations of TDZ with and without NAA; differential osmotic stress induced by 20 and 40 g l^{-1} sucrose in the regeneration media had significantly different ($P < 0.05$) effects on rate of bulblet regeneration percentage and increase in bulb diameter. Low sucrose did not need supplementary auxins to induce maximum bulblet regeneration, whereas, high sucrose needed supplements with NAA to induce maximum bulblet regeneration.

Similar number of bulblets per explant were induced on 0.25 mg^{-1} TDZ supplemented with 20 g l^{-1} sucrose and 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose. However, the bulblets induced on 0.10 mg l^{-1} TDZ + 0.2 mg l^{-1} NAA supplemented with 40 g l^{-1} sucrose were significantly large in diameter. It is assumed that they up took more exogenous sucrose and hydrolysed it into glucose and fructose subsequently in agreement with Roitsch [1999], Hirose et al. [2002], Cho et al. [2005], Ji et al. [2005] and Wang et al. [2008, 2010]. This intake of sucrose promoted cells growth and physiological metabolism by its accumulation.

Furthermore, variable supply of osmotic stress (due to 20 or 40 g l^{-1} sucrose) in the culture media had differential effects on the regenerating bulblets in agreement with Geng et al. [2008], Huang and Liu [2002], Pan et al. [2010] and Huang et al. [2012]. Earlier report on bulblet regeneration by Pierik and Post [1975], made use of basal bulb scale segments for micropropagation. Hussey [1975] reported that bulb scale and basal plate, leaf, stem and ovary tissue are excellent explants to tissue culture on low concentrations of the auxins IAA and NAA. They report that at higher concentrations, NAA induce calli. Ault [1995] also confirmed use of bulb scales for micropropagation on BA and NAA from a single mother bulb scale within 6 months of culture initiation.

However, these reports do not suggest use of fruits containing immature zygotic embryos and role sucrose in gaining diameter. The results of this study are not compatible with any of the previous studies on *H. orientalis*, as all studies followed different pathways to achieve regeneration. The results are also not in agreement with Chung et al. [2006], who reported scarification of scale segments at 4°C for 4 months for regeneration of bulblets.

Bulblet regeneration on tender leaf sheaths. Varying concentrations of BAP + NAA had variable effects on the regeneration of bulblets on tender leaf sheath explants that promoted growth and development of bulblets in different way. The results showed that more number of bulblets were induced on MS medium containing 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA compared to other BAP + NAA concentrations. Preconditioning of regenerating bulblets with GA_3 induced precocious and faster maturing from juvenile bulblets induction phase to tissue maturation phase resulting in regeneration of number of axillary daughter bulblets in agreement with the studies of Poethig [1990] and Reid, [1986]. They reported regeneration of number of daughter bulblets on mother bulblets using cytokinin + auxins. Sun et al. [2010], reported adventitious shoots from scale segments and tender leafs of *H. orientalis*. Unlike report in this study, Pierik and Woets [1971] reported that continuous light inhibited regeneration compared to darkness and IAA promoted bulblet regeneration, whereas kinetin slightly decreased regeneration.

Daughter bulblet regeneration needs a long period of 3–4 years to regenerate under natural conditions from a juvenile vegetative phase to vegetative maturation. The leaf induction phase that started after preconditioning with GA_3, and transformed a juvenile vegetative phase, continued even when the bulbs were transferred to hardening medium containing sucrose. The explants that were not preconditioned with GA_3 showed variable oxidative stress related necrosis and inhibition on leaf growth. It was observed that GA_3 treatment inhibited oxidative stress related necrosis in agreement with Adkins et al [1993] and Ptak et al. [2009]. Induction of increased number of leaves after sucrose based hardening suggested that sucrose accelerated rate of photosynthesis helped in

induction of more dry matter with healthy bulbs without any type of dormancy in agreement with Barpete et al. [2014].

CONCLUSION

Profuse regeneration was noted on immature fruits containing immature zygotic embryos compared to regeneration on tender leaf sheath explants with different regeneration potential. The results of the study are novel, very detailed, interesting and have never been reported in any of the previous regeneration experiment on *H. orientalis.* The results provide a meaningful insight to accelerate micropropagation of *H. orientalis* subsp. *orientalis* using immature fruits and leaf sheath as explants for breeding purpose. Both could be employed for regeneration depending on breeding or propagation based objectives.

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ULEPSZONE ROZMNAŻANIE *in vitro Hyacinthus orientalis* L. PRZY UŻYCIU OWOCÓW ZAWIERAJĄCYCH NIEDOJRZAŁE ZARODKI ZYGOTYCZNE ORAZ POCHWY LIŚCIOWEJ JAKO EKSPLANTÓW

Streszczenie. Rodzaj Hyacinthus jest ważną grupą roślin ozdobnych, które mają biało, żółto, różowo, czerwono lub fioletowo zabarwione kwiaty. Na świecie istnieje około 2000 gatunków uprawianych w celach handlowych. Chociaż roślina ta występuje w warunkach naturalnych w Turcji, to konieczne są wysiłki, aby zaadaptować ją do uprawy na otwartym polu. Istnieje potrzeba, aby przenieść i zasiedlić te rośliny z warunków dzikich do polowych za pomocą metod *in vitro* i *ex vitro,* co wspomoże lokalną gospodarkę. Badanie dotyczy hodowli *Hyacinthus orientalis* L. subsp. *orientalis* w warunkach *in vitro*, przy użyciu owoców zawierających niedojrzałe zarodki zygotyczne wyhodowane na podłożu MS zwierającym różne stężenia Thidiazuronu (TDZ) oraz z i bez 0,2 mg l^{-1} kwasu naftylooctowego (NAA) z dodatkiem 20 lub 40 g l^{-1} sacharozy. Badanie dotyczy również indukcji cebulek na pochwie liściowej na podłożu MS zawierającym różne stęże-

nia benzyloaminopuryny (BAP) + 0.1 mg l^{-1} NAA z dodatkiem 30 g l^{-1} sacharozy. Maksymalna regeneracja cebulek (40%) przy 31,33 cebulkach/ eksplantach została zaobserwowana na podłożu MS zawierającym 0,15 mg l^{-1} TDZ z dodatkiem 40 g l^{-1} sacharozy. Natomiast najlepszą regenerację cebulek na eksplantach z pochewek liściowych zaobserwowano na 1,5 mg l^{-1} BAP + 0,1 mg l^{-1} NAA przy 2.97 cebulkach na eksplant o średnicy cebulki 0,55 cm i 1,20 liściach na cebulkę. Cebulki te były hodowane pojedynczo na podłożu MS zawierającym 20 mg l^{-1} GA_3 (kwas giberelinowy) + 50 g l^{-1} sacharozy i osiągały średnicę 0,75–1,00 cm w ciągu 30 dni. Cebulki zregenerowane na obydwu eksplantach były dobrze zakorzenione i zaaklimatyzowały się w komorze hodowlanej z mchem torfowym, po czym następowało przeniesienie do warunków otwartego pola.

Słowa kluczowe: *Hyacinthus orientalis* L. subsp. *orientalis*, mikrorozmnażanie, owoce zawierające niedojrzałe zarodki zygotyczne, pochewka liściowa

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