Propagation of Ornamental Plants
Vol. 00, № 0, 2000: 00-00

EFFECT OF PHOTOPERIOD, AUXIN, AND CYTOKININ ON THE MULTIPLICATION RATE AND GROWTH OF AMARY LLIS (*HIPPEASTRUM JOHNSONII*) BULBS *IN VITRO*

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Abstract

Amaryllis (*Hippeastrum johnsonii*) is a bulbous plant and has a high potential for breeding programs. First step to reach this aim is to obtain high rate of multiplication. Since conventional propagation of Amaryllis by bulb offsets is slow, *in vitro* culture might be developed to overcome this deficiency. The present study describes the effects of photoperiod (16/8, 14/10, and 12/12 h light/dark), different concentrations of NAA (0, 1, 2, and 4 mg l^{-1}) and 2iP (0, 14, 16 and 18 mg l^{-1}) on micropropagation of *Hippeastrum johnsonii* using bulblets cultured on MS basal medium. For optimal micropropagation by bulblets explants MS medium supplemented with 16 mg l^{-1} 2iP + 4 mg l^{-1} NAA was most effective. Maximum bulblets diameter (6.70 mm), leaf length (14.90 cm), root length (3.30 cm), longest root (4.80 cm), root number (4.00), fresh weight (8.00 g), and dry weight (4.30 g) were obtained on this medium. In most cases, minimum of these traits were observed on medium without NAA and 2iP (control). The least root length (0.23 cm) was recorded in medium containing the highest concentration of 2iP (18 mg l^{-1}). Also, in most cases, 16/8 h light/dark was more effective on Amaryllis micropropagation.

Key words: Amaryllidaceae, bulbous plants, ornamental plants, organogenesis, plant growth regulator

INTRODUCTION

Amaryllis (*Hippeastrum johnsonii*) is an ornamental bulbous flowering plant belonging to the family Amaryllidaceae. The species of genus *Hippeastrum* are native to Central and South America, but are currently cultivated all over the world, and are especially easily grown in the tropical and subtropical regions (Okubo 1993, Jana 1995). Propagation of Amaryllis can be done by seed, offset bulblets, and twin scaling (Vijverberg 1981). These conventional propagations of *Hippeastrum* are slow, seasonal, and variable with some hybrids not producing offsets (Smith et al. 1999). In addition, normally a plant produces two to three bulblets in a year of growth (Dohare 1989, Bose and Yadav 1989). Propagation of plant from seed results in a high variation in flower color, plant shape, time of flowering etc. (Siddique et al. 2007). Thus, a suitable alternative method for the rapid propagation of the bulbous plants with stable morphological features is micropropagation. The successful micropropagation of Amaryllidaceae plants has been reported (Fountain and Rourke 1980, Saker et al. 1998, El-Shamy 2005, Siddique et al. 2007, Zayed et al. 2011). However, studies on micropropagation of *H. johnsonii* and develop a protocol for *in vitro* establishment of *in vitro* produced plantlets and bulblets are relatively scarce. El-Shamy (2005) studied the effect of N^6-(2-Isopentenyl) adenine (2iP) and a-naphthaleneacetic acid (NAA) on micropropagation of *Hippeastrum vittatum* under continuous darkness. For rooting of *H. hybridum* plantlets, Siddique et al. (2007) used different concentrations of NAA only. Sultana et al. (2010) observed the maximum bulb formation on medium supplemented with 6.0 mg l^{-1} 6-benzylaminopurine (BAP) and 500 mg l^{-1} cycocel fortified with 90 g l^{-1} sucrose.

Studies on *in vitro* propagation of *H. johnsonii* are few. Thus, the objective of this study was to improve the micropropagation of Amaryllis (*Hippeastrum johnsonii*) by using different concentrations of 2iP, NAA, and different photoperiods.

MATERIALS AND METHODS

Bulbs of Amaryllis about 7.00 cm in diameter were collected from a greenhouse in Abas Abad city, located in northern part of Iran (Fig. 1A). These bulbs were excised from 4-year old stock plants. The mother plants

Received: January 14, 2013 *Accepted: March 30, 2013*

had been grown in the greenhouse at 23-25°C, a 14-h photoperiod of natural light, and a relative humidity of 85%. Bulbs were washed under running tap water with a few drops of liquid soap for 20 min, and then immersed in fungicide solution of Carboxytyrame (2 g l^{-1}) for 2 min. Bulbs were thoroughly rinsed with sterile distilled water three times, followed by soaking in sodium hypochlorite solution at 10% for 20 min along with some drops of Tween-20, and rinsed again with sterile distilled water for 15 min. Then they were transferred in aseptic conditions under a laminar air flow cabinet and immersed into ethanol 70% for 10 s, followed by soaking in 1% mercuric chloride solution for 12 min, then transferred to 20% sodium hypochlorite solution for 10 min. Finally, bulbs were washed 3-4 times by double distilled water and then separated into so-called twin scales, consisting of a basal plate and two to four scales. The twin scales of size 15 mm were used as explants (Fig. 1B). The explants were cultured on Murashige and Skoog (1962) basal medium (MS) supplemented with different levels of α-naphtaleneacetic acid (NAA) (0, 1, 2, and 4 mg l^{-1}) and N^6-(2-Isopentenyl) adenin (2iP) (0, 14, 16, and 18 mg l^{-1}). The medium pH was adjusted to 5.7 before autoclaving at 121°C, 118 kPa for 20 min. Experiments were carried out in three replications and three explants were inoculated in each glass dish. Cultures were incubated in a growth chamber at 25 ± 2°C, 70-80% relatively humidity, under a photosynthetic photon density flux of 50 µmol m^{-2} s^{-1} with different photoperiods (16/8, 14/10, and 12/12 h light/dark). Samples were subcultured each 14 days. Data (bulblets diameter, leaf length, root length, longest root, root number, fresh weight and dry weight) were recorded after 6 weeks from the first inoculation. The statistical analysis was completely randomized block design. The recorded data were statistically analyzed using SPSS software, and the means were compared using Duncan's test at 5% probability level.

RESULTS

Effects of NAA, 2iP, and photoperiod on bulblets diameter

Bulblets diameter was varied with 2iP and NAA concentrations and photoperiod (Tables 1 to 7). Minimum bulblets diameter was recorded in the absence of exogenous 2iP. Variant 2iP at 16 mg l^{-1} in the MS medium performed the best for increasing bulblet diameter, resulting in the highest number of bulblets for more explants and in the greatest bulblets diameter (6.70 cm). NAA at 4 mg l^{-1} has been shown to have highest influence on bulblets diameter. All the combined treatments of 2iP and NAA showed a synergistic effect, with the increasing bulblets diameter greater than that of the singular treatments. The combination of 2iP at 16 mg l^{-1} and NAA at 4 mg l^{-1} was the most efficient for increasing of bulblets diameter (6.70 and 6.34 cm). After 6 weeks of culture, fully developed bulblets of *H. johnsonii* were produced from the explants (Fig. 1). The lowest bulblet diameter (0.96 cm) was obtained on the medium without 2iP and NAA (Table 7). NAA and 2iP had significant effect on bulblet diameter when combined with each other. Interaction effects of photoperiod with each one of two plant growth regulators and with both of them were not significant. The bulblet diameter was significantly affected by photoperiod (Table 8). However, differences of bulblets diameter in explants grown under photoperiods of 16/8 h (6.70 cm), 14/10 h (6.23 cm), and 12/12 h (6.10 cm) were not significant (Table 1).

Effects of NAA, 2iP, and photoperiod on leaf length

Light had a significant effect on increasing leaf length. Data presented in Table 8 show that photoperiods of 16/8 and 14/10 h increased leaf length more than 12/12 h (Table 8). Evaluation of the role of photoperiod on leaf length revealed that the maximum (9.80 cm) and minimum (7.95 cm) leaf length were obtained in explants incubated in photoperiods of 16/8 and 12/12 h, respectively (Table 1). There was no significant difference between photoperiods of 16/8 and 14/10 h. The positive influence of 2iP and NAA was clear in enhancing the length of leaves those treated with 16 mg l^{-1} 2iP along with 4 mg l^{-1} NAA being the longest ones (14.90 and 14.22 cm) (Table 7, Fig. 1). To the contrary, the number of leaves per bulb was least (1.97 and 2.13 cm) in medium without 2iP and NAA (control) (Table 7).

Effects of NAA, 2iP, and photoperiod on root number

Of the different concentrations of NAA, 4.0 mg l^{-1} showed the best (2.78) effect on root number. Root number increased as concentrations of NAA increased (Table 3). The treatment without NAA resulted in minimum root number (1.25). Among different concentrations of 2iP, maximum root number (2.33) was recorded in explants grown on medium containing 16 mg l^{-1}. The number of root per twin scales did not increase with increasing the concentration of 2iP (Table 2). The maximum number of roots (4.00) was recorded at combination of 16 mg l^{-1} 2iP and 4 mg l^{-1} NAA (Table 7, Fig. 1C). Average number of roots was minimum (0.70) in absence of 2iP and NAA. It appeared that higher average number of roots was found at plant growth regulators concentrations than the control. Data in Tables 1 to 7 show that in most cases, the number of root was lowest at the base of bulblets grown in medium without NAA. Differences in root number in explants grown under photoperiods of 16/8, 14/10 and 12/12 h were not significant (Table 1). NAA and 2iP had significant effect on root number, but the effect of different photoperiods was not significant (Table 8).

Fig. 1. Micropropagation process of *Hippeastrum johnsonii*. A) Bulbs, B) the twin scales cultured on MS medium, C) explants containing root, D) explants containing leaf, E) plantlets formed on medium containing 16 mg l^{-1} 2iP + 4 mg l^{-1} NAA, and F) hardening of plantlets. Plantlets were transferred to plastic pots containing a mixture of cocopeat, compost and sand (1 : 1 : 1).

Table 1. The mean comparison for the main effect of photoperiod on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of Hippeastrum johnsonii.

Treatment	Levels	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
Photoperiod	P1	3.15 ± 0.19 a	9.80 ± 0.55 a	1.97 ± 0.11 a	2.18 ± 0.11 a	2.69 ± 0.16 a	5.25 ± 0.29 a	2.25 ± 0.13 a
	P2	2.87 ± 0.17 ab	8.83 ± 0.55 b	1.97 ± 0.11 a	1.81 ± 0.11 b	2.05 ± 0.15 b	4.84 ± 0.28 b	2.10 ± 0.13 a
	P3	2.63 ± 0.18 b	7.95 ± 0.55 c	1.93 ± 0.22 a	1.56 ± 0.10 c	1.88 ± 0.14 b	4.66 ± 0.28 b	2.01 ± 0.13 a

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$. P1, P2, and P3: photoperiods of 16/8, 14/10 and 12/12 h, respectively.

Table 2. The mean comparison for the main effect of different concentrations of 2iP (0, 14, 16, and 18 mg l^{-1}) on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of *Hippeastrum johnsonii*.

Treatment	Levels	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
2iP	I1	1.48 ± 0.15 c	3.29 ± 0.56 c	1.61 ± 0.43 c	1.75 ± 0.11 a	1.97 ± 0.15 b	1.87 ± 0.19 c	0.85 ± 0.09 c
	I2	2.76 ± 0.16 b	10.40 ± 0.11 b	1.94 ± 0.32 b	1.83 ± 0.11 a	2.25 ± 0.17 ab	5.60 ± 0.29 b	2.32 ± 0.13 b
	I3	4.66 ± 0.19 a	12.27 ± 0.57 a	2.33 ± 0.10 a	2.03 ± 0.15 a	2.60 ± 0.19 a	6.97 ± 0.30 a	3.15 ± 0.15 a
	I4	2.64 ± 0.19 b	9.49 ± 0.67 b	1.97 ± 0.10 b	1.78 ± 0.11 a	2.00 ± 0.15 b	5.22 ± 0.29 b	2.16 ± 0.13 b

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$. I1, I2, I3, and I4: concentrations of 0, 14, 16, and 18 mg l^{-1} 2iP, respectively.

Table 3. The mean comparison for the main effect of different concentrations of NAA (0, 1, 2, and 4 mg l^{-1}) on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of *Hippeastrum johnsonii*.

Treatment	Levels	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
NAA	N1	2.48 ± 0.17 c	7.73 ± 0.55 c	1.25 ± 0.11 d	1.07 ± 0.08 d	1.07 ± 0.11 d	4.34 ± 0.25 c	1.76 ± 0.10 c
	N2	2.59 ± 0.18 c	8.41 ± 0.55 bc	1.58 ± 0.23 c	1.46 ± 0.11 c	1.57 ± 0.09 c	4.74 ± 0.25 bc	2.11 ± 0.10 b
	N3	2.97 ± 0.19 b	9.09 ± 0.56 b	2.25 ± 0.11 b	2.14 ± 0.14 b	2.64 ± 0.13 b	4.97 ± 0.25 b	2.13 ± 0.10 b
	N4	3.49 ± 0.19 a	10.21 ± 0.66 a	2.77 ± 0.54 a	2.71 ± 0.15 a	3.53 ± 0.18 a	5.62 ± 0.25 a	2.49 ± 0.15 a

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$. N1, N2, N3, and N4: concentrations of 0, 1, 2, and 4 mg l^{-1} NAA, respectively.

Table 4. The mean comparisons for the effects of two-fold interactions of photoperiod and different concentrations of 2iP on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of *Hippeastrum johnsonii*.

Treatments	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
P1I1	1.40 ± 0.17 a	3.47 ± 0.67 a	1.75 ± 0.16 a	2.18 ± 0.13 a	2.74 ± 0.14 a	1.92 ± 0.11 a	0.78 ± 0.07 a
P1I2	2.88 ± 0.18 a	11.20 ± 0.44 a	2.16 ± 0.17 a	2.23 ± 0.14 a	2.83 ± 0.15 a	5.84 ± 0.29 a	2.39 ± 0.11 a
P1I3	5.15 ± 0.19 a	13.70 ± 0.34 a	2.25 ± 0.09 a	2.36 ± 0.21 a	3.01 ± 0.17 a	7.33 ± 0.33 a	3.26 ± 0.12 a
P1I4	3.15 ± 0.19 a	10.84 ± 0.56 a	1.75 ± 0.09 a	1.94 ± 0.11 a	2.15 ± 0.16 a	5.93 ± 0.29 a	2.57 ± 0.12 a
P2I1	1.70 ± 0.17 a	3.46 ± 0.77 a	1.50 ± 0.08 a	1.79 ± 0.11 a	1.66 ± 0.12 a	1.94 ± 0.19 a	1.01 ± 0.10 a
P2I2	2.71 ± 0.18 a	10.20 ± 0.34 a	1.91 ± 0.09 a	1.69 ± 0.11 a	2.05 ± 0.17 a	5.54 ± 0.29 a	2.34 ± 0.15 a
P2I3	4.72 ± 0.15 a	12.18 ± 0.65 a	2.41 ± 0.08 a	2.00 ± 0.14 a	2.35 ± 0.18 a	6.79 ± 0.34 a	3.07 ± 0.15 a
P2I4	2.36 ± 0.18 a	9.46 ± 0.81 a	2.08 ± 0.10 a	1.75 ± 0.12 a	2.11 ± 0.16 a	5.08 ± 0.28 a	2.00 ± 0.15 a
P3I1	1.34 ± 0.18 a	2.94 ± 0.71 a	1.58 ± 0.18 a	1.27 ± 0.10 a	1.50 ± 0.13 a	1.75 ± 0.12 a	0.76 ± 0.03 a
P3I2	2.68 ± 0.18 a	9.78 ± 0.53 a	1.75 ± 0.11 a	1.57 ± 0.11 a	1.85 ± 0.14 a	5.42 ± 0.29 a	2.25 ± 0.20 a
P3I3	4.11 ± 0.19 a	10.91 ± 0.47 a	2.33 ± 0.09 a	1.73 ± 0.13 a	2.44 ± 0.17 a	6.81 ± 0.29 a	3.13 ± 0.10 a
P3I4	2.41 ± 0.16 a	8.17 ± 0.57 a	2.08 ± 0.12 a	1.66 ± 0.12 a	1.73 ± 0.14 a	4.65 ± 0.27 a	1.91 ± 0.10 a

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$.

Table 5. The mean comparisons for the effects of two-fold interactions of photoperiod and different concentrations of NAA on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of *Hippeastrum johnsonii.**

Treatments	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
P1N1	2.85 ± 0.18 a	9.34 ± 0.65 a	1.16 ± 0.17 a	1.51 ± 0.09 a	1.55 ± 0.09 a	4.99 ± 0.27 a	1.99 ± 0.10 a
P1N2	2.86 ± 0.18 a	9.06 ± 0.47 a	1.33 ± 0.09 a	1.77 ± 0.09 a	1.94 ± 0.11 a	4.99 ± 0.27 a	2.19 ± 0.14 a
P1N3	3.29 ± 0.19 a	10.06 ± 0.64 a	2.33 ± 0.14 a	2.59 ± 0.15 a	3.30 ± 0.18 a	5.36 ± 0.23 a	2.31 ± 0.14 a
P1N4	3.59 ± 0.19 a	10.75 ± 0.48 a	3.08 ± 0.15 a	2.85 ± 0.24 a	3.97 ± 0.23 a	5.67 ± 0.23 a	2.51 ± 0.13 a
P2N1	2.50 ± 0.20 a	8.08 ± 0.76 a	1.50 ± 0.08 a	1.04 ± 0.08 a	0.85 ± 0.05 a	4.37 ± 0.20 a	1.75 ± 0.08 a
P2N2	2.72 ± 0.21 a	8.02 ± 0.34 a	1.75 ± 0.08 a	1.49 ± 0.12 a	1.55 ± 0.10 a	4.50 ± 0.20 a	2.10 ± 0.19 a
P2N3	2.85 ± 0.19 a	9.28 ± 0.63 a	2.16 ± 0.07 a	1.81 ± 0.13 a	2.35 ± 0.16 a	4.95 ± 0.29 a	2.08 ± 0.13 a
P2N4	3.41 ± 0.20 a	9.92 ± 0.63 a	2.50 ± 0.10 a	2.88 ± 0.15 a	3.43 ± 0.16 a	5.53 ± 0.29 a	2.49 ± 0.10 a
P3N1	2.10 ± 0.23 a	8.78 ± 0.56 a	1.08 ± 0.05 a	0.67 ± 0.06 a	0.82 ± 0.05 a	3.67 ± 0.18 a	1.54 ± 0.10 a
P3N2	2.19 ± 0.23 a	8.14 ± 0.45 a	1.66 ± 0.06 a	1.12 ± 0.11 a	1.21 ± 0.11 a	4.72 ± 0.19 a	2.04 ± 0.10 a
P3N3	2.76 ± 0.21 a	7.92 ± 0.37 a	2.25 ± 0.08 a	2.03 ± 0.12 a	2.29 ± 0.16 a	4.59 ± 0.19 a	2.00 ± 0.10 a
P3N4	3.48 ± 0.22 a	9.95 ± 0.75 a	2.75 ± 0.11 a	2.41 ± 0.13 a	3.20 ± 0.18 a	5.65 ± 0.20 a	2.46 ± 0.15 a

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$.

Table 6. The mean comparisons for the effects of two-fold interactions of different concentrations of 2iP and NAA on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight and bulblets dry weight of *Hippeastrum johnsonii.**

Treatments	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
I1N1	1.33 ± 0.24 c	2.42 ± 0.55 c	1.00 ± 0.04 a	0.96 ± 0.07e	0.93 ± 0.06 e	1.31 ± 0.09 g	0.56 ± 0.05 c
I1N2	1.23 ± 0.19 c	2.90 ± 0.55 c	1.22 ± 0.11 a	1.49 ± 0.13 cde	1.33 ± 0.11 de	1.70 ± 0.09 g	0.77 ± 0.07 c
I1N3	1.63 ± 0.19 c	3.55 ± 0.55 c	2.00 ± 0.11 a	2.02 ± 0.14 bcd	2.44 ± 0.15 bcd	2.04 ± 0.12 g	1.01 ± 0.10 c
I1N4	1.72 ± 0.19 c	4.30 ± 0.55 c	2.22 ± 0.11 a	2.52 ± 0.16 ab	3.17 ± 0.16 b	2.43 ± 0.14 g	1.05 ± 0.10 c
I2N1	2.33 ± 0.23 c	7.81 ± 0.56 c	1.22 ± 0.12 a	1.01 ± 0.09 e	1.05 ± 0.12 e	4.18 ± 0.17 f	1.79 ± 0.15 c
I2N2	3.00 ± 0.18 b	10.17 ± 0.67 b	1.66 ± 0.13 a	1.55 ± 0.14 cde	1.72 ± 0.13 cde	5.79 ± 0.17 b-e	2.43 ± 0.15 b
I2N3	2.67 ± 0.19 c	11.09 ± 0.67 b	2.33 ± 0.14 a	2.16 ± 0.16 bcd	2.78 ± 0.16 bc	5.86 ± 0.17 b-e	2.57 ± 0.11 ab
I2N4	3.02 ± 0.19 b	12.51 ± 0.49 b	2.55 ± 0.11 a	2.61 ± 0.18 ab	3.43 ± 0.16 b	6.56 ± 0.23 abc	2.51 ± 0.13 ab
I3N1	3.50 ± 0.17 b	10.39 ± 0.49 b	1.44 ± 0.12 a	1.27 ± 0.11 de	1.40 ± 0.10 de	6.39 ± 0.23 bcd	2.53 ± 0.13 ab
I3N2	4.11 ± 0.18 b	11.69 ± 0.68 b	2.00 ± 0.11 a	1.54 ± 0.11 cde	1.81 ± 0.11 cde	6.46 ± 0.23 bcd	2.92 ± 0.13 ab
I3N3	4.71 ± 0.14 b	12.96 ± 0.78 b	2.00 ± 0.14 a	2.05 ± 0.15 bcd	2.66 ± 0.12 bc	7.15 ± 0.29 ab	3.08 ± 0.13 ab
I3N4	6.34 ± 0.15 a	14.01 ± 1.00 a	3.88 ± 0.14 a	3.26 ± 0.19 a	4.54 ± 0.19 a	7.91 ± 0.30 a	4.08 ± 0.15 a
I4N1	2.79 ± 0.15 c	10.31 ± 1.01 b	1.33 ± 0.11 a	1.05 ± 0.11 e	0.92 ± 0.08 e	5.49 ± 0.22 c-f	2.16 ± 0.10 ab
I4N2	2.03 ± 0.20 c	8.87 ± 0.89 c	1.44 ± 0.11 a	1.26 ± 0.11 de	1.42 ± 0.10 de	5.00 ± 0.20 def	2.32 ± 0.10 ab
I4N3	2.86 ± 0.22 c	8.75 ± 0.69 c	2.66 ± 0.11 a	2.35 ± 0.17 bc	2.68 ± 0.16 bc	4.83 ± 0.20 ef	1.86 ± 0.10 c
I4N4	2.90 ± 0.21 c	10.02 ± 0.55 b	2.44 ± 0.12 a	2.47 ± 0.19 ab	3.00 ± 0.16 b	5.57 ± 0.20 c-f	2.30 ± 0.13 ab

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$.

Effects of NAA, 2iP, and photoperiod on root length

The highest average of the root length (3.31 cm) and longest root (4.87 cm) were measured with treatment 16 mg l^{-1} 2iP and 4 mg l^{-1} NAA, respectively (Table 7, Fig. 1). Concerning the root length per plantlets, NAA at 4.0 mg l^{-1} induced the maximum length (2.72 cm) with the longest roots (3.54 cm) that was followed by the treatment NAA at 2.0 mg l^{-1} (2.15 and 2.65 cm). 2-iP at 16 mg l^{-1} induced the maximum length (2.03 cm) with the longest roots (2.60 cm) that was followed by the treatment 2iP at 14 mg l^{-1} (1.84 and 2.25 cm). Lowest root length (0.23 cm) was measured in control plantlets (Table 7). In most cases the length of root was shortest at the base of bulblets grown in medium without NAA. Differences of root length in explants grown under photoperiods of 16/8, 14/10 and 12/12 h were significant (Table 8). Light had a significant effect on increasing root length (Table 1). Data presented in Table 1 show that photoperiods of 16/8 and 14/10 h increased leaf length more than 12/12 h. Evaluation of the effect of photoperiod on root length revealed that the maximum and longest root (2.18 and 2.70 cm) and minimum and shortest root (1.56 and 1.90 cm) were obtained in explants incubated in photoperiods of 16/8 and 12/12 h, respectively (Table 1). There was no significant difference between photoperiods of 16/8 and 14/10 h. NAA and photoperiod had significant effect on root length, but interaction effect of them was not significant. 2iP had no significant effect on root length. In case of average root length, NAA and photoperiod had significant effect, but 2iP and interaction effect of them was not significant (Table 8).

Effects of NAA, 2iP, and photoperiod on fresh and dry weight of bulblets

The effects of different concentrations of NAA and 2iP were significant on the fresh and dry weight of bulblets (Table 8). The highest average fresh (8.00 g) and dry (4.20 g) weight of bulbs was found with 16 mg l^{-1} 2iP and 4 mg l^{-1} NAA (Table 7). Average fresh (1.03 g) and dry (0.42 g) weight of bulbs was minimum in absence of 2iP and NAA (control). It appeared that higher average weight of bulbs had found at hormone concentration of 16 mg l^{-1} 2iP in combination with 4 mg l^{-1} NAA (Table 7). Differences of bulblets fresh and dry weight in explants grown under different photoperiods showed that of 14/10 h was better than 16/8 h and 12/12 h (Table 1).

About 50% of the rooted plantlets were transplanted to plastic pots (cocopeat: compost: sand 1 : 1 : 1) when they were approximately 17 cm in length, and 90% of plantlets were successfully grown at 25°C, 75% shade and 80% relative humidity greenhouse. Acclimatized plants were morphologically and genetically identical with mother plants and no visible morphological alteration was observed in these plants.

DISCUSSION

The combined effects of different concentrations of 2iP and NAA were significant on most growth traits in *H. johnsonii*. The best growth was achieved at 16 mg l^{-1} 2iP in combination with 4 mg l^{-1} NAA. It might be due to the combined beneficial effect and positive response to both 2iP and NAA. The efficacy of 2iP and NAA for

Table 7. The mean comparisons for effects of two-fold interactions of photoperiod and different concentrations of 2iP and NAA on bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight, and bulblets dry weight of *Hippeastrum johnsonii.**

Treatments	Bulblet diameter (cm)	Leaf length (cm)	Root number	Root length (cm)	Longest root length (cm)	Bulblets fresh weight (g)	Bulblets dry weight (g)
P1I1N1	1.00 ± 0.19 k	2.13 ± 0.55 qr	0.66 ± 0.14 d	1.43 ± 0.16 b-i	1.43 ± 0.13 f-k	1.19 ± 0.10 jk	0.48 ± 0.08 no
P1I1N2	1.03 ± 0.22 k	2.56 ± 0.55 pqr	1.00 ± 0.07 cd	1.81 ± 0.18 a-i	1.90 ± 0.15 d-k	1.42 ± 0.10 ijk	0.59 ± 0.08 mno
P1I1N3	2.00 ± 0.16 h-k	4.83 ± 0.79 n	2.33 ± 0.09 a-d	2.68 ± 0.21 a-d	3.56 ± 0.18 a-g	2.65 ± 0.12 f-k	1.06 ± 0.09 k-o
P1I1N4	1.56 ± 0.17 ijk	4.36 ± 0.89 no	3.00 ± 0.19 abc	2.80 ± 0.20 a-d	4.06 ± 0.19 a-d	2.42 ± 0.12 g-k	1.00 ± 0.08 k-o
P1I2N1	2.80 ± 0.24 e-k	9.00 ± 0.43 l	1.33 ± 0.09 cd	1.38 ± 0.11 c-i	1.50 ± 0.11 f-k	4.84 ± 0.20 b-h	2.04 ± 0.13 d-l
P1I2N2	2.96 ± 0.24 e-k	10.03 ± 0.42 jkl	1.33 ± 0.12 cd	1.96 ± 0.13 a-i	2.06 ± 0.12 c-k	5.52 ± 0.20 a-e	2.35 ± 0.13 d-j
P1I2N3	2.40 ± 0.24 g-k	11.91 ± 0.33 d-h	3.00 ± 0.29 abc	2.73 ± 0.14 a-d	3.56 ± 0.13 a-g	6.01 ± 0.20 a-e	2.64 ± 0.13 d-h
P1I2N4	3.36 ± 0.30 e-j	13.86 ± 0.23 ab	3.00 ± 0.23 abc	2.87 ± 0.15 a-d	4.20 ± 0.16 abc	6.98 ± 0.23 ab	2.52 ± 0.13 d-h
P1I3N1	3.76 ± 0.25 d-h	13.53 ± 0.32 abc	1.33 ± 0.10 cd	1.80 ± 0.11 a-i	1.90 ± 0.10 d-k	7.40 ± 0.29 ab	2.74 ± 0.15 d-g
P1I3N2	4.83 ± 0.20 b-e	13.10 ± 0.23 bcd	2.00 ± 0.16 a-d	1.78 ± 0.11 a-i	2.26 ± 0.12 c-k	6.86 ± 0.28 abc	3.06 ± 0.15 b-e
P1I3N3	5.33 ± 0.19 a-d	13.30 ± 0.43 bcd	1.66 ± 0.23 bcd	2.56 ± 0.14 a-e	3.10 ± 0.12 a-i	7.22 ± 0.30 ab	3.20 ± 0.15 a-d
P1I3N4	6.70 ± 0.12 a	14.90 ± 0.55 a	4.00 ± 0.23 a	3.31 ± 0.19 a	4.87 ± 0.19 a	8.00 ± 0.29 a	4.30 ± 0.15 a
P1I4N1	3.80 ± 0.23 d-h	12.70 ± 0.57 b-f	1.33 ± 0.10 cd	1.43 ± 0.11 b-i	1.36 ± 0.10 g-k	6.54 ± 0.25 a-d	2.69 ± 0.14 d-h
P1I4N2	2.66 ± 0.16 f-k	10.56 ± 0.65 h-l	1.00 ± 0.09 cd	1.51 ± 0.10 b-i	1.53 ± 0.10 f-k	6.17 ± 0.25 a-e	2.78 ± 0.14 d-g
P1I4N3	3.43 ± 0.31 e-j	10.21 ± 0.55 e-j	2.33 ± 0.15 a-d	2.40 ± 0.19 a-g	2.96 ± 0.16 a-i	5.58 ± 0.25 a-e	2.33 ± 0.14 d-j
P1I4N4	2.73 ± 0.19 f-k	9.89 ± 0.67 jkl	2.33 ± 0.11 a-d	2.42 ± 0.18 a-g	2.83 ± 0.16 a-j	5.43 ± 0.25 a-e	2.49 ± 0.15 d-j
P2I1N1	1.63 ± 0.17 ijk	3.16 ± 0.67 o-r	1.33 ± 0.13 cd	0.63 ± 0.05 hi	0.63 ± 0.06 j-k	1.72 ± 0.12 ijk	0.77 ± 0.10 mno
P2I1N2	1.73 ± 0.22 h-k	3.66 ± 0.88 n-q	1.33 ± 0.13 cd	2.05 ± 0.11 a-h	1.43 ± 0.11 f-k	2.21 ± 0.14 h-k	1.10 ± 0.10 j-o
P2I1N3	1.60 ± 0.17 ijk	3.13 ± 0.55 o-r	1.66 ± 0.13 bcd	1.66 ± 0.11 a-i	1.86 ± 0.11 e-k	1.66 ± 0.10 ijk	1.17 ± 0.10 i-o
P2I1N4	1.83 ± 0.44 h-k	3.90 ± 0.55 nop	1.66 ± 0.11 bcd	2.81 ± 0.15 a-d	2.73 ± 0.21 b-j	2.19 ± 0.13 h-k	1.00 ± 0.10 k-o
P2I2N1	2.30 ± 0.19 g-k	7.23 ± 0.55 m	1.33 ± 0.11 cd	0.73 ± 0.07 f-i	0.73 ± 0.07 jk	3.71 ± 0.17 e-k	1.62 ± 0.12 g-o
P2I2N2	3.00 ± 0.19 e-k	10.33 ± 0.55 h-l	2.00 ± 0.12 a-d	1.47 ± 0.11 b-i	1.66 ± 0.12 f-k	5.94 ± 0.23 a-e	2.46 ± 0.13 d-h
P2I2N3	2.70 ± 0.19 f-k	11.82 ± 0.67 d-i	2.00 ± 0.12 a-d	1.55 ± 0.11 b-i	2.43 ± 0.12 c-k	6.31 ± 0.28 a-e	2.74 ± 0.13 d-g
P2I2N4	2.86 ± 0.19 e-k	11.44 ± 0.77 e-j	2.33 ± 0.16 a-d	3.02 ± 0.30 abc	3.40 ± 0.16 a-h	6.21 ± 0.29 a-e	2.53 ± 0.13 d-h
P2I3N1	3.53 ± 0.19 d-h	10.59 ± 1.03 h-l	1.66 ± 0.11 bcd	1.31 ± 0.11 c-i	0.90 ± 0.09 ijk	6.07 ± 0.29 a-e	2.45 ± 0.13 d-h
P2I3N2	4.60 ± 0.19 b-f	11.20 ± 0.19 f-j	2.00 ± 0.17 a-d	1.57 ± 0.12 b-i	1.96 ± 0.14 d-k	6.06 ± 0.29 a-e	2.68 ± 0.15 d-h
P2I3N3	4.53 ± 0.26 b-f	12.73 ± 0.55 b-f	2.33 ± 0.18 a-d	1.94 ± 0.11 a-i	2.60 ± 0.14 c-j	7.02 ± 0.28 ab	2.95 ± 0.15 c-f
P2I3N4	6.23 ± 0.26 ab	14.21 ± 0.51 ab	3.66 ± 0.20 ab	3.17 ± 0.18 ab	3.96 ± 0.16 a-e	8.00 ± 0.35 a	4.19 ± 0.20 ab
P2I4N1	2.57 ± 0.26 f-k	11.35 ± 0.52 e-j	1.66 ± 0.19 bcd	1.50 ± 0.11 b-i	1.16 ± 0.09 ijk	6.00 ± 0.20 a-e	2.15 ± 0.20 d-k
P2I4N2	1.56 ± 0.27 ijk	6.90 ± 0.53 m	1.66 ± 0.11 bcd	0.88 ± 0.08 e-i	1.16 ± 0.09 ijk	3.80 ± 0.07 d-j	2.15 ± 0.20 d-k
P2I4N3	2.60 ± 0.27 f-k	9.45 ± 0.54 kl	2.66 ± 0.11 a-d	2.09 ± 0.11 a-h	2.50 ± 0.13 c-j	4.83 ± 0.14 b-h	1.45 ± 0.10 h-o
P2I4N4	2.73 ± 0.21 f-k	10.15 ± 0.55 jkl	2.33 ± 0.12 a-d	2.54 ± 0.16 a-e	3.63 ± 0.19 a-f	5.71 ± 0.23 a-e	2.24 ± 0.10 d-k
P3I1N1	1.33 ± 0.22 jk	1.96 ± 0.55 r	1.00 ± 0.07 cd	0.83 ± 0.03 e-i	0.73 ± 0.08 jk	1.03 ± 0.08 k	0.43 ± 0.04 o
P3I1N2	0.96 ± 0.22 k	2.46 ± 0.55 pqr	1.33 ± 0.08 cd	0.60 ± 0.03 hi	0.66 ± 0.08 jk	1.46 ± 0.10 ijk	0.63 ± 0.05 mno
P3I1N3	1.30 ± 0.33 kj	2.70 ± 0.34 pqr	2.00 ± 0.09 a-d	1.72 ± 0.06 a-i	1.90 ± 0.09 d-k	1.80 ± 0.10 ijk	0.81 ± 0.05 l-o
P3I1N4	1.76 ± 0.50 h-k	4.63 ± 0.35 no	2.00 ± 0.09 a-d	1.95 ± 0.06 a-i	2.73 ± 0.13 b-j	2.69 ± 0.10 f-k	1.16 ± 0.70 i-o
P3I2N1	1.90 ± 0.15 h-k	7.20 ± 0.34 m	1.00 ± 0.05 cd	0.93 ± 0.02 e-i	0.93 ± 0.07 ijk	4.01 ± 0.16 d-i	1.71 ± 0.70 f-n
P3I2N2	3.05 ± 0.15 e-k	10.16 ± 0.56 jkl	1.66 ± 0.07 bcd	1.21 ± 0.11 d-i	1.43 ± 0.10 f-k	5.91 ± 0.15 a-e	2.49 ± 0.80 d-h
P3I2N3	2.93 ± 0.25 e-k	9.55 ± 0.78 kl	2.00 ± 0.11 a-d	2.21 ± 0.15 a-h	2.36 ± 0.13 c-k	5.26 ± 0.15 a-f	2.32 ± 0.10 d-j
P3I2N4	2.83 ± 0.26 e-k	12.23 ± 0.75 c-g	2.33 ± 0.16 a-d	1.95 ± 0.11 a-i	2.70 ± 0.13 b-j	6.49 ± 0.15 ab	2.48 ± 0.10 d-h
P3I3N1	3.20 ± 0.25 e-j	7.07 ± 0.55 m	1.33 ± 0.11 cd	0.69 ± 0.03 ghi	1.40 ± 0.10 g-k	5.70 ± 0.15 a-e	2.40 ± 0.10 d-i
P3I3N2	2.90 ± 0.25 e-k	10.79 ± 0.44 g-k	2.00 ± 0.13 a-d	1.28 ± 0.11 c-i	1.20 ± 0.08 h-k	6.48 ± 0.15 a-e	3.04 ± 0.13 b-e
P3I3N3	4.26 ± 0.34c-g	12.86 ± 0.89 b-e	2.00 ± 0.13 a-d	1.66 ± 0.11 a-i	2.30 ± 0.16 c-k	7.20 ± 0.29 ab	3.08 ± 0.13 b-e
P3I3N4	6.10 ± 0.35abc	12.93 ± 0.34 b-e	4.00 ± 0.20 a	3.30 ± 0.18 a	4.86 ± 0.19 a	7.87 ± 0.29 a	4.02 ± 0.13 abc
P3I4N1	2.00 ± 0.17h-k	6.90 ± 0.78 m	1.00 ± 0.08 cd	0.23 ± 0.01 i	0.23 ± 0.02 k	3.93 ± 0.14 d-i	1.64 ± 0.10 g-o
P3I4N2	1.86 ± 0.15h-k	9.16 ± 0.89 kl	1.66 ± 0.11 bcd	1.40 ± 0.09 c-i	1.56 ± 0.12 f-k	5.03 ± 0.16 b-g	2.02 ± 0.10 d-l
P3I4N3	2.56 ± 0.19e-k	6.60 ± 0.45 m	3.00 ± 0.15 abc	2.55 ± 0.13 a- e	2.60 ± 0.13 c-j	4.09 ± 0.14 c-i	1.81 ± 0.10 e-m
P3I4N4	3.23 ±0.19e-j	10.03 ± 0.55 jkl	2.66 ± 0.14 a-d	2.46 ± 0.13 a-f	2.53 ± 0.13 c-j	5.56 ± 016 a-e	2.18 ± 0.13 d-k

Values are mean ± standard error. Means in a column followed by the same letter are not significantly different at $p \leq 0.01$.

Table 8. Analysis of variance of the studied factors and their combinations on the bulblets diameter, leaf length, root number, root length, longest root length, bulblets fresh weight and bulblets dry weight of *Hippeastrum johnsonii*.

		Mean square						
Source of variations	df	Bulblets diameter	Leaf length	Root number	Root length	Longest root length	Bulblets fresh weight	Bulblets dry weight
P (Photoperiod)	2	0.57 *	4.66 **	0.06 ns	4.66 **	8.71 **	6.61 *	0.49 ns
I (2iP)	3	3.13 **	0.57 ns	3.22 **	0.57 ns	3.06 **	4.49 **	19.68 **
N (NAA)	3	62.62**	19.09 **	3.13 **	19.09 **	43.74 **	169.00 **	32.67 **
P × I	6	0.06 ns	0.24 ns	0.78 ns	0.24 ns	0.70 ns	0.25 ns	3.17 ns
P × N	6	0.02 ns	0.49 ns	0.40 ns	0.49 ns	0.19 ns	0.79 ns	0.42 ns
I × N	9	2.34 **	0.38 ns	0.75 ns	0.38 ns	0.79 ns	1.12 ns	20.09 **
P × I × N	18	0.09 ns	0.42 ns	0.4 ns	0.42 ns	0.45 ns	2.19 ns	0.93 ns
Error	94	0.62	0.43	0.75	0.43	0.70	1.14	0.24
C.V. (%)	-	27.41	35.45	44.32	35.45	38.08	21.79	23.07

**: Significant at α = 1%, *: Significant at α = 5%, ns = Not significant.

micropropagation has been demonstrated in other species of *Hippeastrum*. In *H. vittatum* the highest number of bulbs per explants with moderate leaf length and bulb fresh weight was observed on medium supplemented with 16 mg l^{-1} 2iP and 4 mg l^{-1} NAA (Zayed et al. 2011). Results of the present study showed that the heaviest bulb was achieved at 16 mg l^{-1} 2iP and 4 mg l^{-1} NAA levels. Cytokinins and auxins are known to stimulate cell division and cell expansion. These phenomena are required for bulb formation and development. In *Lilium longiflorum* (a bulbous plant), the highest bulb diameter was observed in MS medium supplemented with 0.03 mg l^{-1} BAP and 0.3 NAA mg l^{-1} (Mojtahedi and Azadi 2008). In *H. hybridum* maximum bulb formation has been reported on MS medium containing 6.0 mg l^{-1} BAP, while the minimum in absence of BAP (Sultana et al. 2010). The number of *in vitro* bulbs per twin scales increased with increasing the concentration of BAP up to 6 mg l^{-1} and then it gradually decreased with further increase of BAP concentration. Cytokinins have been considered to be important for *in vitro* bulb formation.

Results of the present study showed that NAA was effective on increasing the number and length of roots, but NAA in combination with 2iP was more effective. In current work, NAA and 2iP had synergism on root number and root length. There are some findings in agreement with our results. In *Eustoma grandiflorum* the highest root number was seen in medium supplemented with both auxins and cytokinins (Ahmadi Hesar et al. 2011). The rooting experiment in *H. hybridum* plantlets showed that well developed root system was achieved in the MS medium supplemented by 0.2 mg l^{-1} NAA (Siddique et al. 2007). Stimulation of root induction and growth by NAA and NAA + 6-Furfurilaminopurine (kinetin) treatments has been reported by Kaviani et al. (2011) in *Matthiola incana*, an ornamental plant. The effectiveness of NAA for rooting of plants has already been demonstrated (Hammaudeh et al. 1998, Lee-Epinosa et al. 2008, Jain and Ochatt 2010). Rooting is a crucial step to the success of micropropagation. Without effective root system plant acclimatization will be difficult and the rate of plant propagation may be severely affected (Gonçalves et al. 1998). Results of the present study showed the positive effect of cytokinin on rooting. The same results were obtained in some other plants (Jain and Ochatt 2010, Gomes et al. 2010). On the other hand, there are several studies indicating that cytokinins do not have any effect on rooting (Han et al. 2004). Fuller and Fuller (1995) demonstrated that the highest percentage of explant regeneration with root percent (65.0%) in *Brassica* spp. was obtained in culture medium supplemented with 2 mg l^{-1} indole-3-butyric acid (IBA) without cytokinins. The lowest rooting in *Bambusa arundinacea* was observed on medium without cytokinins (Nayak et al. 2010). In *Arbutus unedo* L., shoots produced on higher cytokinin-containing medium were more amenable to root induction than shoots obtained with the lowest concentrations of cytokinin (Gomes et al. 2010). Evaluation of some researches clearly points out a negative effect of cytokinins on shoot rooting (Van Staden et al. 2008), although a positive role has been occasionally referred (Jain and Ochatt 2010). Gautam et al. (1983) findings on *in vitro* regeneration of plantlets from somatic explants of *Matthiola incana* showed that 1 and 4 mg l^{-1} of NAA induced profuse rooting in explants. Studies of Isutsa (2004) on micropropagation of *Passiflora edulis* varieties showed that the shoots did not initiate roots on all IBA-augmented medium but they initiated roots only on NAA-augmented medium. In tissue culture of orchid, NAA stimulated root growth (Kalimuthu et al. 2007).

Results of the present study showed that rooting and bulb diameter is affected by photoperiod. There are

only a few reports on the role of light on rooting and bulb formation. Pati et al. (2006) showed that usually 16 h photoperiod is suitable for tissue culture experiments. Light plays an important role for satisfactory micropropagation (Burger et al. 1990, Pati et al. 2006, Ebrahimzadeh et al. 2006). Ebrahimzadeh et al. (2006) observed that *Anthurium* explants grown under darkness did not produce any roots. These explants produced roots following exposure to light. Burger et al. (1990) found that longer light period proved to be better than shorter one for rooting. Longer photoperiod induced larger bulb diameter in *Polianathes tuberosa* (a bulbous plant) (Khan et al. 2000). These findings confirm our results about the effect of photoperiod on bulb diameter.

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