Influence of growth regulators and nitrogenous compounds on *in vitro* bulblet formation and growth in oriental lily

S. Kumar, V. Awasthi, J. K. Kanwar

Department of Biotechnology, University of Horticulture & Forestry, Solan, India

ABSTRACT: The influence of growth regulators and nitrogenous compounds on *in vitro* bulblet formation and growth was studied in two hybrids of *Lilium*. Bulbscales isolated from pre-cooled bulbs of hybrids Rosato and Marco Polo were used. The basal portion with plate (5 × 6 mm) of inner bulbscales was cultured on Murashige and Skoog (MS) medium containing 0.5 or 1 mg/dm^3 naphthaleneacetic acid (NAA) and/or benzyladenine (BA). The presence of NAA (0.5 mg per dm^3) showed higher explant regeneration, producing about three bulblets per explant as compared to control. About four bulblets per explant were produced at both concentrations of BA. The bulblets with significantly higher fresh weight were obtained on medium containing NAA. Approximately a three-fold increase of bulblet fresh weight was observed with all the concentrations of TDZ in both cultivars. The bulblets cultured with nitrogenous compounds after attaining the size of 14–16 cm flowered during the second year of the growing period without any phenotypic variations.

Keywords: lily hybrids; Rosato; Marco Polo; NAA; BA; TDZ; thiourea; phenylthiourea; urea; bulblet regeneration and production

Lilium is one of the leading cut flowers all over the world. Among various types of lilies, hybrids of asiatic and oriental lilies and *L. longiflorum* seem the most promising in florist trade. Oriental lilies are the most expensive among various lily forms, as their bulbs are highly valuable and require a special technology for bulb production program; they have a wide acceptability in floral industry, mainly as cut flowers and potted plants. Several attempts have been made to multiply lily through tissue culture. However, although the bulblets raised through tissue culture showed a high multiplication rate, for commercial use they remained small in size (Niimi, Onozawa 1979; Tanaka et al. 1991; Maesato et al. 1994; Niimi 1995; Kumar et al. 2001). In bulbous crops, vigour and growth of the plants are directly correlated with size of the underground organs. A certain minimum size of the bulb is essential for the plant to flower. Urea and thiourea derivatives, the most important group of non-purine cytokinins (Mok et al. 1982; Yonova et al. 1989) are widely distributed in plants and have an important regulatory role in plant growth and development (Galston, Kaur-Sawhney 1980; Altman, Bachrach 1981; Yonova, Guleva 1997). Cytokinin-like compounds, e.g. thidiazuron, are receiving much attention (Fellman et al. 1987). Some analogs of N, N'-phenylurea and thiourea were found to be more active than kinetin in lateral bud development in pea seedling bioassay (Bruce et al. 1965; Bruce, Zwar 1966). Different urea derivatives are applied *in vitro* and *ex vitro* to enhance the growth and mass production in *Lilium* and other bulbous crops (Dhua et al. 1987; Woo et al. 2000; Nhut et al. 2002). In the present research, keeping in view the growth regulating activity of urea and thiourea derivatives, an attempt was made to study the effect of NAA, BA and some urea compounds on productivity and *in vitro* bulblet growth in oriental lily hybrids.

MATERIALS AND METHODS

Preparation of material

Pre-cooled bulbs (2°C for 6 weeks) of oriental lily hybrids Rosato and Marco Polo were procured from the Department of Floriculture and Landscaping, Solan, India. The bulbscales were excised from bulbs and a lower portion with basal plate (5–6 mm) of the inner scales was used as explant. The explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 3–4 min and rinsed three times under aseptic conditions with sterile distilled water to remove the sterilization solution.

Cultural conditions

The sterilized explants were cultured on the Murashige and Skoog (MS; 1962) regeneration medium supplemented with 8 g/dm^3 (w/v) agar, 30 g/dm^3 (w/v) sucrose and 0.5 and 1 mg/dm^3 of naphthaleneacetic acid (NAA) and benzyladenine (BA), respectively. After four weeks of *in vitro* cultivation formed bulblets were transferred to MS medium supplemented with 0.05, 0.1, 0.5 and 1 mg/dm^3 of urea, thiourea, thidiazuron (TDZ) and phenylthiourea, respectively. The cultures without growth regulators served as control. The explants were cultured in 100 ml Erlenmeyer flasks (Borosil) containing 30 ml of medium. The flasks were plugged with non-absorbent cotton. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C, at the pressure of 1.1 kg/cm^2 for 15 min. All the cultures were incubated in a room with controlled conditions of 24 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 to 60 µmol/m^2/s.

Data on explants forming bulblets were recorded after 4 weeks, and on number of bulblets and average fresh weight after 13 weeks of culture.

Statistical analysis

Three replications with 10 explants in each replication (30 explants) were maintained for each treatment and the data were analyzed statistically using factorial completely randomized design (Gomez, Gomez 1984). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD multiple range test (P = 0.05) was used to determine differences between treatments.

Bulblet storage

The individual bulblets were separated and transferred to the MS medium without growth regulators. When the leaves were dried, the bulblets were removed, washed thoroughly and dried at room temperature. The bulblets were treated with Bavis-

Table 1. Effect of NAA and BA on regeneration of explant after 4 weeks of culture

Treatment (mg/dm^3)	Explant regeneration (%)		
	Rosato	Marco Polo	Mean
Control	22.0 (17.32)	18.0 (12.95)	20.0 (15.14)
NAA, 0.5	90.0 (84.07)	88.0 (78.33)	89.0 (80.01)
1	80.0 (73.89)	74.0 (68.13)	77.0 (69.22)
BA, 0.5	54.0 (48.13)	51.0 (43.91)	52.5 (47.13)
1	60.0 (53.93)	59.0 (50.13)	59.5 (51.14)
NAA, 0.5 + BA, 1	70.0 (64.18)	68.0 (61.13)	69.0 (62.41)
1 + BA, 0.5	65.0 (58.11)	66.0 (55.11)	65.5 (54.14)
Mean	63.0 (56.13)	60.5 (53.99)	61.7 (54.00)

LSD (P = 0.05); treatment (A) = 1.90; cultivar (B) = 1.18; A × B = 1.53
Figures within parenthesis are arc sine transformed values

Table 2. Effect of NAA and BA on average number of bulblets after 13 weeks of culture

Treatment (mg/dm^3)	Number of bulblets per explant		
	Rosato	Marco Polo	Mean
Control	2.3	2.0	2.2
NAA, 0.5	3.0	2.7	2.8
1	3.3	2.7	3.0
BA, 0.5	3.7	3.7	3.7
1	4.0	4.0	4.0
NAA, 1 + BA, 0.5	3.5	3.2	3.3
0.5 + BA, 1	4.3	4.0	4.2
Mean	3.6	3.2	3.4

LSD (P = 0.05); treatment (A) = 0.84; cultivar (B) = 0.45; A × B = 1.16

Table 3. Effect of NAA and BA on average fresh weight per bulblet after 13 weeks of culture

Treatment (mg/dm^3)	Average fresh weight per bulblet (mg)		
	Rosato	Marco Polo	Mean
Control	108.3	97.3	102.8
NAA, 0.5	204.7	186.0	195.3
1	194.0	177.0	185.5
BA, 0.5	100.7	99.3	100.0
1	126.0	104.0	115.0
NAA, 1 + BA, 0.5	161.0	151.3	156.2
0.5 + BA, 1	193.0	114.0	153.5
Mean	155.4	132.7	144.1

LSD (P = 0.05); treatment (A) = 1.89; cultivar (B) = 1.01; A × B = 2.68

tin (0.1%, w/v) and stored at 2°C for six months (till next growing season) before transferring to soil in earthenware pots (25 cm in diameter). The process was repeated until the size of bulblets required for flowering was achieved.

RESULTS AND DISCUSSION

Effect of NAA and BA on regeneration and bulblet growth

Table 1 summarizes the range of responses after four weeks of culture. In the absence of NAA and BA, only a few cultures (20%) were established from the basal portion of the explant, which produced 2.2 bulblets per explant indicating that the endogenous hormones required for such morphogenetic responses were already present in the explant. Similar results were also reported for other *Lilium* species (Novak, Petru 1981; Niimi 1985; Maesato et al. 1994). Without BA, the rate of explants with bulblets increased significantly with both concentrations of NAA. A four-fold increase (89%) in the regeneration response was observed when the medium was supplemented with 0.5 mg/dm^3 NAA. About three bulblets per explant regenerated in the presence of

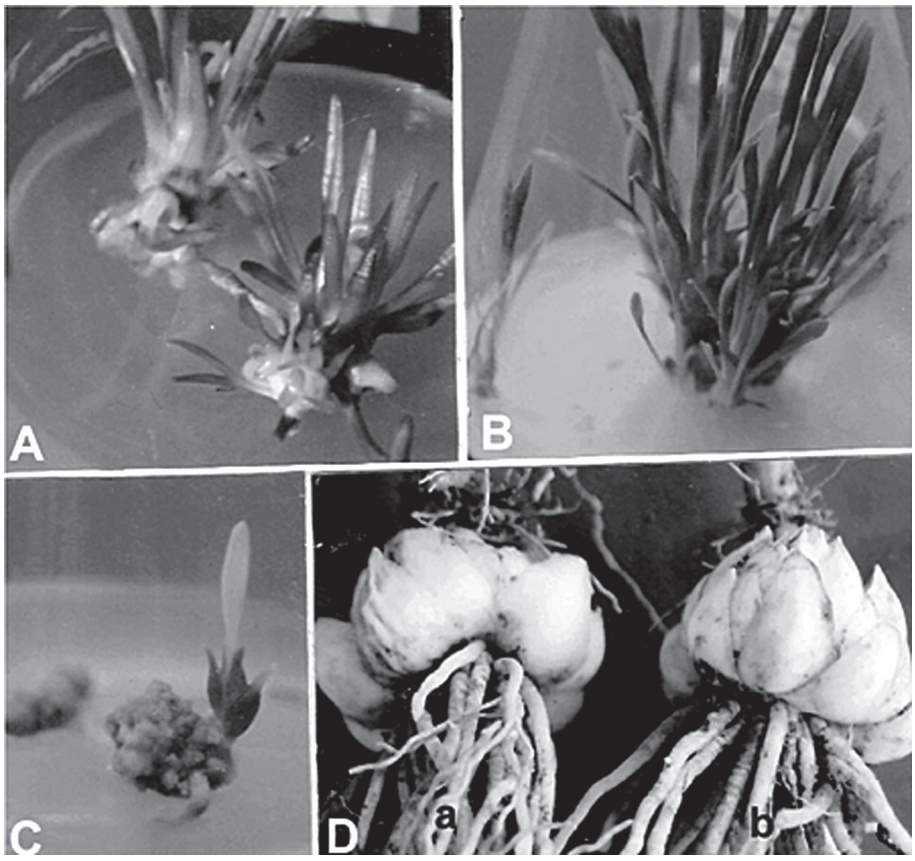

Fig. l. (A) Bulblet formation on MS medium supplemented with 1 mg/dm^3 BA in Marco Polo after 13 weeks. (B) Bulblet production on MS medium supplemented with 0.05 mg/dm^3 urea in Rosato after 13 weeks. (C) Formation of bulblets without leaves with phenylthiourea (1 mg/dm^3). (D) TDZ derived bulbs of Rosato (a) and Marco Polo (b) after the second year of the growing period

Table 4. Effect of urea, thiourea, phenylthiourea and TDZ on average number of bulblets after 13 weeks of culture

Treatment (mg/dm^3)		Number of bulblets per explant		
		Rosato	Marco Polo	Mean
Control		2.3	2.0	2.2
Urea,	0.05	5.3	4.7	5.0
	0.1	4.7	4.3	4.5
	0.5	4.3	3.7	4.0
	1	3.3	3.0	3.2
Thiourea,	0.05	3.7	3.7	3.7
	0.1	4.3	4.0	4.2
	0.5	3.3	3.0	3.2
	1	2.7	2.7	2.7
Phenylthiourea,	0.05	3.7	2.7	3.2
	0.1	2.7	3.0	2.8
	0.5	2.0	2.0	2.0
	1	1.0	1.0	1.0
TDZ,	0.05	3.3	2.7	3.0
	0.1	4.0	3.3	3.7
	0.5	4.7	3.7	4.2
	1	3.0	4.3	3.7
Mean		3.4	3.2	3.3

LSD (P = 0.05); treatment (A) = 0.58; cultivar (B) = 0.19; A × B = 0.82

NAA at both concentrations (Table 2) without any callus formation and the results were statistically significant as compared with control. Addition of only BA into medium increased regeneration of bulblets to moderate level (52.5–59.5%) as compared with control and produced approximately four bulblets per explant (Fig. 1A).

Both concentrations of NAA in combination with BA did not affect the regeneration response and number of bulblets per explant (Tables 1 and 2). Niimi (1985) also reported that NAA at 0.05 and 0.1 mg/dm^3 stimulated bulblet formation in *L. rubellum* and the addition of BA had a little effect. A different effect of growth regulators, under similar environmental conditions,

Fig. 2. Flowering plant of Rosato in earthenware pots containing soil:sand:FYM mixed in the ratio 1:1:1

Table 5. Effect of urea, thiourea, phenylthiourea and TDZ on fresh weight per bulblet after 13 weeks of culture

Treatment (mg/dm^3)		Average fresh weight per bulblet (mg)		
		Rosato	Marco Polo	Mean
Control		108.3	97.3	102.8
Urea,	0.05	273.0	267.0	270.3
	0.1	222.7	231.0	226.8
	0.5	211.0	205.0	208.0
	1	187.3	179.3	183.3
Thiourea,	0.05	219.0	206.0	212.5
	0.1	271.0	193.7	205.3
	0.5	217.7	210.7	214.2
	1	198.7	192.0	195.3
Phenylthiourea,	0.05	273.3	227.3	250.3
	0.1	240.7	271.0	255.8
	0.5	220.0	216.7	218.3
	1	305.0	318.7	311.8
TDZ,	0.05	365.0	345.7	355.3
	0.1	343.3	316.3	329.8
	0.5	263.7	352.3	358.0
	1	318.7	373.0	345.8
Mean		252.1	247.2	249.7

LSD (P = 0.05); treatment (A) = 5.82; cultivar (B) = 1.99, A × B = 8.22

was observed on fresh weight of regenerated bulblets. Auxin NAA induced higher bulblet fresh weight than control and/or BA (Table 3). Similar observation was also made in the case of *L. japonicum* (Maesato et al. 1994). No differences were observed between cultivars in bulblet regeneration of explants or bulblet number, although more weighty bulblets were found in Rosato as compared with Marco Polo (Table 3).

Effect of nitrogenous compounds on bulblet growth

A single bulblet regenerated two bulblets on the medium without urea or its derivatives (Table 4). An addition of urea, thiourea and TDZ (at all concentrations) and phenylthiourea (0.05 mg/dm^3) into the medium resulted in formation of 3–5 bulblets per explant. Urea was the most effective in production of bulblets (Fig. 1B). Probably, nitrogenous compounds might increase the protein level in the explant during differentiation resulting in higher number of bulblets. Ishioka and Tanimoto (1993) observed three major bands of denatured proteins from bulbscale explants with differentiated bulblets in *Lilium longiflorum*.

In *Lilium,* the role of TDZ on shoot multiplication was well documented (Park et al. 1996; Woo et al. 2000). Urea and its derivatives significantly stimulated fresh weight of bulblet over control after 13 weeks of cultivation. About a three-fold increase in average fresh weight was observed with TDZ as compared with control (Table 5). The results are contrary to those of Park et al. (1996), who reported that TDZ at 0.01 mg/dm^3 inhibited bulblet development although it produced multiple bulblets from bulbscales in *Lilium*. All levels of urea, thiourea and TDZ produced leafy bulblets whereas phenylthiourea suppressed leaf formation in bulblets (Fig. 1C). Yonova and Guleva (1997) also reported that non-purine cytokinin derivatives of urea and thiourea play important regulatory role in plant growth and development.

Among the cultivars, the heaviest bulblets were observed in Rosato as compared with Marco Polo (Table 5).

The bulblets attaining the size of 14 to 16 cm (Fig. 1D) after repeated storage at 2°C and transfer to soil flowered during the second year of the growing period in pots (25 cm in diameter) containing soil: sand: FYM mixed in the ratio 1:1:1, without any phenotypic variations (Fig. 2). Bacchetta et al. (2003) successfully rooted and acclimatized regenerated shoots of *Lilium* under glasshouse conditions where they flowered after the second

year of the growing period with true-to-type shape and colour.

From our results, it is concluded that a certain minimum size of bulbs is essential for the plant to get into its reproductive phase. A bigger size of the bulb resulted in a larger size of the plant. Hence, in the present investigation some nitrogenous compounds were used to increase the size of *in vitro* bulblets to have early flowering in lilies. These nitrogen-containing compounds increase the solubility and recovery of proteins, and thus lead to a better bulblet growth.

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Vliv růstových regulátorů a dusíkatých látek na tvorbu cibulek a růst orientální lilie

ABSTRAKT: Byl studován vliv růstových regulátorů a dusíkatých látek na *in vitro* tvorbu cibulek u dvou hybridů lilie. Byly použity šupiny izolované z předchlazených cibulí hybridů Rosato a Marco Polo. Bazální část s plátkem (5 × 6 mm) vnitřních šupin byla kultivována na Murashige a Skoog (MS) médiu, obsahujícím 0,5 nebo 1 mg/dm^3 naftyloctové kyseliny (NAA), benzyladenin (BA) a thidiazuron (TDZ). V přítomnosti 0,5 mg/dm^3 NAA došlo k vyšší regeneraci explantátů, které tvořily ve srovnání s kontrolou kolem tří cibulek na explantát. Kolem čtyř cibulek na explantát vznikalo při obou koncentracích BA. Na médiu s NAA byly zjištěny cibulky s průkazně vyšší čerstvou hmotností. Asi třikrát vyšší nárůst čerstvé hmotnosti cibulek byl pozorován u obou kultivarů při všech koncentracích TDZ. Cibulky kultivované s nitrátovými sloučeninami vykvetly po dosažení velikosti 14–16 cm během druhého roku růstové periody bez fenotypických změn.

Klíčová slova: hybridy lilie; Rosato; Marco Polo; NAA; BA; TDZ; thiourea; phenylthiourea; urea; regenerace a produkce cibulek

Corresponding author:

Prof. Surinder Kumar, Ph.D., University of Horticulture & Forestry, Department of Biotechnology, Solan – 173 230 (H.P.), India
tel.: + 91 01792 252310, fax: + 91 01792 252242, e-mail: skuhf@yahoo.com