RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2005.31.409

ScienceAsia 31 (2005): 409-414

Different modes of plant regeneration and factors affecting *in vitro* bulblet production in *Ornithogalum virens*.

Pradeep Kumar Naik and Sanghamitra Nayak*

Department of Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan – 173215, Himachal Pradesh, India

* Corresponding author, email: sanghamitran@yahoo.com

Received 29 Sep 2004
Accepted 10 Jun 2005

ABSTRACT: Efficient procedures are outlined for plant regeneration through direct shoot bud formation and indirect organogenesis through callus formation in *Ornithogalum virens* Lindl using bulb scale as explant. Murashige and Skoog's (MS) medium containing 1 mg/L (5.4mM) NAA and 2 mg/L (4.4mM) BA was most effective in direct induction of shoot buds from explant. Callus cultures were raised from the bulb scale segment on MS medium supplemented with 2 mg/L (9.0mM) 2,4-D. Shoot regeneration from callus was optimum on the medium containing 2 mg/L (10.7mM) NAA and 0.5 mg/L (2.2mM) BA. Shoots developed roots on MS basal medium devoid of growth regulators. Regenerated plants grew profusely in MS liquid medium and were successfully transferred to pots. Bulblets were induced at the base of the regenerants upon transfer to MS basal medium supplemented with enhanced concentrations of sucrose (45 to 90 g/L). Direct induction of bulblets also occurred on the bulb scale grown on the MS media supplemented with 1 mg/L (5.4mM) NAA, 2 mg/L (8.9mM) BA and 60 g/L sucrose. Size of bulblets could be increased by decreasing the salt strength of MS basal medium to half. The effect of *in vitro* induced bulblet size on the *ex vitro* survival rate was also reported. Bulblets produced *in vitro* could be transplanted directly to potted soil. Chromosome analysis of direct explant-derived plants, callus-derived regenerates, and plants sprouted from *in vitro*-induced bulblets revealed only diploid cells with normal karyotypes comprising 2n = 6 chromosomes.

List of abbreviations: NAA, a-naphthalene acetic acid; BA, 6 benzyladenine; 2,4-D, 2,4 dichlorophenoxyacetic acid.

KEYWORDS: *Ornithogalum virens*, tissue culture, bulblet induction.

INTRODUCTION

Ornithogalum is a genus of bulbous plants native to the Mediterranean and South Africa. *Ornithogalum* species, commonly known as "Star of Bethelhem" have been cultivated commercially for a long time for their high ornamental value as cut-flowers and are becoming increasingly popular. *Ornithogalum virens* Lindl is economically important for its ornamental value. Besides, its low chromosome number (2n = 6) and simple karyotype makes this species an ideal material for *in vitro* studies involving chromosome behaviour during organ differentiation. It grows in India in temperate regions of the Himalyas at an altitude of 2000 m. Conventionally, the species is propagated vegetatively using mother bulbs, but the rate of propagation is very slow, producing 4-6 plants in a year. The horticultural interest in *Ornithogalum virens* necessitates the development of *in vitro* method to speed up the propagation rate. Tissue culture technique is powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of enhancing the rate of multiplication of desired genotypes[1,2]. The tissue culture technique has been successfully applied for rapid propagation of some *Ornithogalum* species[3,4]. The present paper deals with procedures for different modes of regeneration of plantlets directly from bulb scale explant and from callus, as well as *in vitro* production of bulblets. *In vitro* bulblet formation has been reported in *O. umbellatum*[3] and in other bulbous plant types such as tulips[5], Narcissus[6], Lachenalia[7], Lilium[8], Hyacinthus[9]. Bulblets have several advantages. They are hardier thereby increasing the survival rate after transplanting to soil. They are easy to handle and can be sown as seeds. They can be transported dry and no extra time or facilities are needed for hardening off. In this paper we have studied the factors affecting the formation of bulblets

in vitro, as well as transplantation success, which is one of the most important criteria in determing the success of cost-effective commercial micropropagation of *Ornithogalum virens.*

Materials and Methods

In vitro Regeneration

The bulbs of *O. virens* Lindl were taken as explants and outer scales were peeled off and discarded. The lower halves of bulbs containing the basal disc were cut into 4 sectors, washed thoroughly in 5% Teepal detergent for 15 min, surface sterilized with 0.1% mercuric chloride for 30 min, and rinsed 5 times in sterile distilled water. Each sector was further dissected to 2-3 explants, each explant with 1-2 scales joined at the base by a small part of the disc. The explants were aseptically inoculated onto Murashige and Skoog's basal medium[10] supplemented with different concentrations and combinations of NAA and BA, as mentioned in Table 1 and 2, for induction of shoots directly from explant and from callus. Regenerated shoots were rooted on MS medium devoid of any growth regulators and were maintained by regular subculture in liquid MS medium. *In vitro* grown plants were thoroughly washed in tap water, dipped in 0.2% "Bavistin" (a fungicide) solution for 10 min and transferred to potted soil in shade for acclimatization.

Induction of Bulblets

Scales of *in vitro* grown bulbs and culture derived regenerated shoots of about 3-4 cm long were inoculated onto MS medium supplemented with sucrose (30, 60 and 90 g/L) for *in vitro* formation of bulblets. For direct regeneration of bulblets, bulb scale explant were grown on media containing 1 mg/L NAA and 2 mg/L BA and different concentrations of sucrose (30, 60 and 90 g/L). The effect of different concentrations of sucrose on the percentage of shoot producing bulbs was studied. Different salt strength of media (full MS, ½ MS and ¼ MS) were used to determine the effect of salt strength of media on *in vitro* bulb formation. Bulblets thus produced could directly be transplanted to the pots containing mixtures of soil and sand (1:1).

Culture Conditions

For solidification of media 0.8% bacto-agar was used. Media were autoclaved at 121°C and 1.05 kg/cm^2 for 20 min and the pH was adjusted to 5.7. Cultures were inoculated at 25 ± 1°C and grown under white fluorescent light with 55 µmol m^{-2}s^{-1} light intensity. Each experimental set consisted of 15 replicates. Means were calculated by taking an average of 3 successive experiments.

Cytological Analysis

For analysis of chromosome number of regenerated plants, root tips were pretreated with a 2:1 mixture of saturated paradichlorobenzene solution: 0.002 M 8-hydroxyquinoline for 3 h, fixed overnight in 1:3 acetic acid: ethanol, and stained using the aceto orcein technique[11]. A detailed karyotypic study was made on the basis of the length of chromosome and position of the centromere as determined by F%, which denotes the ratio in percentage of the short arm to total length of each chromosome. Chromosome morphology was determined following the classification of Battaglia[12]. Chromosome counts were made on 8-10 countable metaphase plates per root tip. Fifty regenerated plants from each group *i.e.* direct explant derived plant, direct bulblet sprouted plants and callus regenerated plants, were analysed.

Statistical Analysis of Data

Data were subjected to analysis of variance[13] and statistical significance of different treatment means was determined.

Results and Discussion

In vitro Regeneration

Shoot buds were induced directly (Fig 1a) from the bulb scale when explanted on MS medium supplemented with NAA (0.5 - 1 mg/L) and BA (0.5 – 3 mg/L) (Table 1). Application of NAA alone had little effect on shoot bud induction, but the combination of NAA (1 mg/L) and BA (2 mg/L) was most effective in inducing an average of 10.4 ± 0.1 shoot buds in about 73% of explants within 30 days of culture (Table 1). This result showing requirement of NAA and BA in direct shoot bud formation is in agreement with that of Hussey[1]. The mean number of shoots formed per explant in this medium was significantly higher ($p = 0.001$) than those formed in media with other hormonal combinations (Table 1). On the other hand, the combinations of another auxin (IAA, 0.5 – 1 mg/L) and cytokinin (kinetin, 0.5 – 3 mg/L) were not effective in direct regeneration of shoot buds from the explant (data not shown). In *O. virens,* direct regeneration of shoot buds were conditioned to the attachment of the basal disc portion with the bulb scale, as reported earlier in *O. thyrsoides* and *O. umbellatum*[3,14].

Callus was induced from the bulb scale of *O. virens* at the basal region in proximity with the disc when cultured on MS basal medium supplemented with 1-4 mg/L 2,4-D. MS basal medium supplemented with 2 mg/L 2,4-D was most effective showing callus induction from 75% of explant in 2-3 weeks. Requirement of 2, 4-D for callus induction in *O. virens* is in contrast to the other reports in *Ornithogalum* species,

where callus initiation was favored by the presence of NAA in medium[3,4]. Faster proliferation of callus (Fig 1b) occurred by repeated sub-culturing on the MS basal medium with 2 mg/L 2, 4-D.

Shoot buds (Fig 1c) were induced from 2 months old callus grown on MS media when 2,4-D was replaced by a combination of NAA (1, 2 or 3 mg/L) and BA (0.1, 0.5 or 1 mg/L) (Table 2). Callus grown on the media containing 2 mg/L NAA and 0.5 mg/L BA showed the best response, inducing 7.4 ± 0.2 shoots per culture (Table 2) from about 82% of the calli within 3-4 weeks. The requirement of auxin and cytokinin in combination for shoot bud formation from calli of *O. virens* is like that of *O. umbelaatum*[4], but unlike *O. thyrsoides*[3], where shoot induction occurred on the basal medium devoid of any growth regulators.

Shoot buds obtained both through direct and indirect organogenesis developed into shoots which attained the length of 3-4 cm within 15 days. Elongated shoots about 4 cm long were excised from the base and placed in MS basal medium for rooting. Roots were formed in 3-4 days. Completely regenerated plants were maintained in MS liquid medium, where they multiplied (Fig 1d) rapidly through formation of lateral shoots, resulting in production of 8-10 plants from a

Table 1. Effect of different combinations of NAA and BA on direct shoot formation from explants of *Ornithogalum virens*.

MS+Growth regulator (mgL^{-1}) NAA	BA	Percent of regeneration of explant (Mean ± SEM)*	Number of shoots per explant (Mean ± SEM)*
0.5	-	22.9 ± 0.3	2.5 ± 0.1
1	-	28.3 ± 0.5	2.6 ± 0.2
0.5	0.5	30.1 ± 0.3	2.6 ± 0.2
0.5	1.0	30.2 ± 0.4	5.4 ± 0.2
0.5	2.0	47.9 ± 0.5	7.2 ± 0.2
0.5	3.0	36.4 ± 0.5	3.3 ± 0.1
1.0	0.5	58.4 ± 0.5	5.2 ± 0.2
1.0	1.0	62.3 ± 0.5	8.5 ± 0.3
1.0	2.0	73.1 ± 0.6	10.4 ± 0.1
1.0	3.0	55.4 ± 0.5	5.2 ± 0.2
2.0	0.5	19.4 ± 0.5	2.4 ± 0.2
2.0	1.0	20.4 ± 0.4	3.5 ± 0.2
2.0	2.0	26.6 ± 0.3	3.5 ± 0.1
2.0	3.0	10.6 ± 0.5	3.0 ± 0.1
F value		926.28	132.829
P value		< 0.001	< 0.001

The difference in the mean values amongst the treatment group is statistically significant ($p < 0.001$). * Data represents 15 replicates per treatment in three repeated experiments.

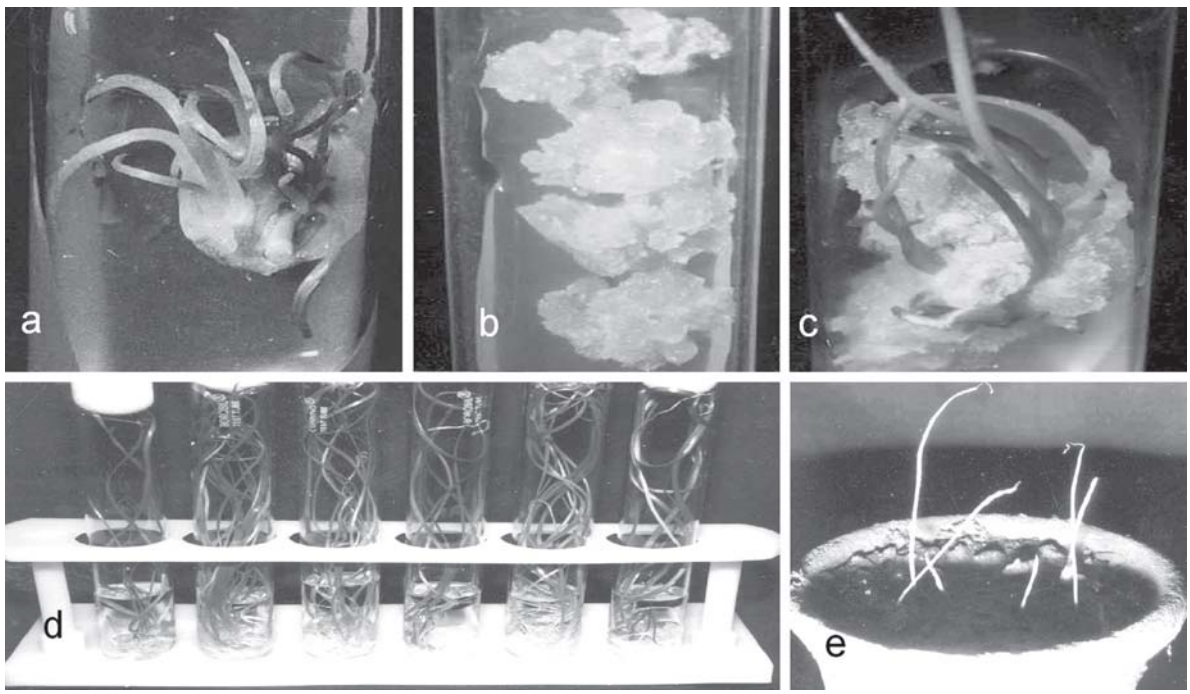

Fig 1. a. Direct regeneration of shoots from bulb scale of *O.virens* grown on MS + 1 mgL^{-1} NAA + 2mgL^{-1} BA.
b. Growth of callus of *O. virens* on MS + 2 mgL^{-1} 2,4-D.
c. Shoot regeneration from callus of *O. virens* grown on MS + 2 mgL^{-1} NAA + 0.5 mgL^{-1} BA.
d. Multiplication of *O. virens* plantlets in MS liquid medium.
e. Regenerated plants of *O. virens* in pot.

Table 2. Effect of different combinations of NAA and BA on multiple shoot formation from callus.

MS+Growth regulator (mgL^{-1}) NAA	BA	Percent of regenerating callus (mean ± SEM)*	Number of shoot per callus (mean ± SEM)*
1.0	-	-	-
2.0	-	-	-
3.0	-	-	-
1.0	0.1	30.5 ± 0.5	2.2 ± 0.1
2.0	0.1	61.3 ± 0.5	3.0 ± 0.1
3.0	0.1	40.2 ± 0.3	3.2 ± 0.1
1.0	0.5	66.2 ± 0.5	6.4 ± 0.2
2.0	0.5	82.2 ± 0.6	7.4 ± 0.2
3.0	0.5	47.2 ± 0.5	6.3 ± 0.2
1.0	1.0	70.5 ± 0.2	2.4 ± 0.2
2.0	1.0	80.5 ± 0.2	6.3 ± 0.1
3.0	1.0	50.1 ± 0.4	2.7 ± 0.1
F value		490.90	255.70
P value		< 0.001	< 0.001

The differences in the mean values among the treatment groups are statistically significant ($p < 0.001$). *Data represents 15 replicates for treatment in three repeated experiments.

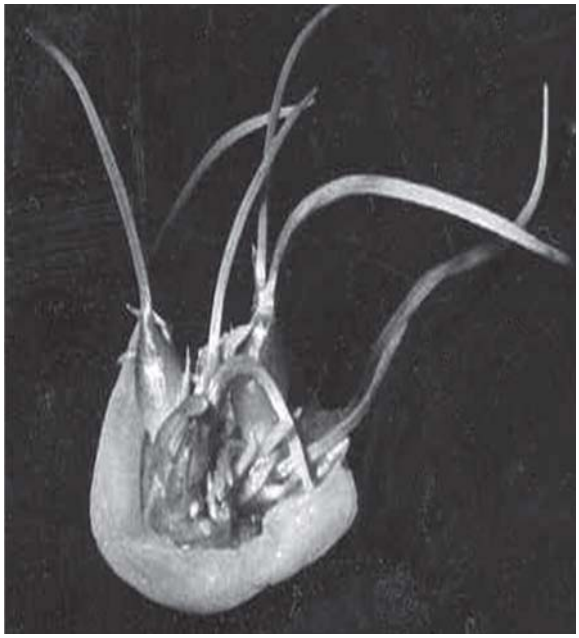

Fig 2. Bulb formation at the base of *in vitro* grown plants of *O. virens* after 60 days of culture.

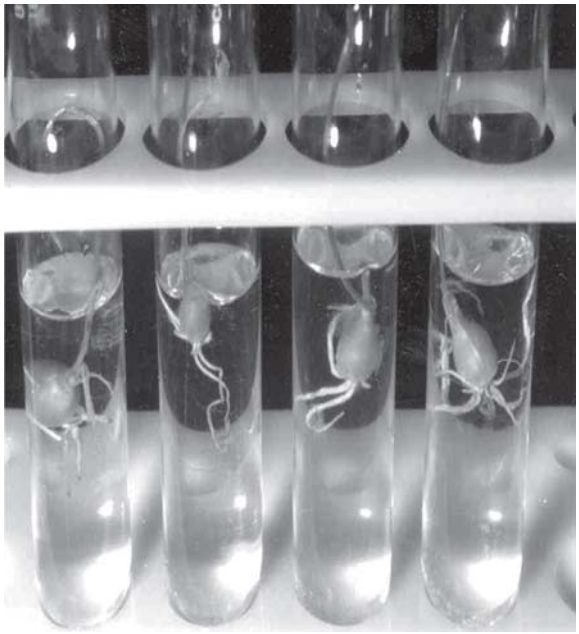

Fig 3. Induction of bulblets on bulb scale of *O. virens* grown on MS + 1 mgL^{-1} + 2 mgL^{-1} BA + 60 gL^{-1} sucrose.

single plant. Thus, approximately 80-100 small plantlets from a single explant could be produced within 90 days and 60-70 plantlets per callus within 150 days of culture. The regenerated plants were transferred to pots containing a 1:1 sand and soil mixture (Fig 1e) and maintained in a shade house.

Induction of Bulblets

Regeneration of plants of *O. virens* was accompanied by bulb formation (Fig 2) at the base of shoots grown *in vitro* on MS basal medium supplemented with enhanced levels of sucrose (45-90 g/L) (Fig 6). Of all the concentrations of sucrose used, MS medium supplemented with 60 g/L sucrose was found to be optimum for formation of bulbs *in vitro*. The percentage of *in vitro* grown shoots (explants) forming bulbs was approximately 70%. Tiny bulbs were visible at the base of shoots after 30 days of explantation on MS medium. No bulbs could be induced *in vitro* on MS medium containing a normal amount of sucrose (30 g/L). Sucrose is considered to be stored in the form of starch in the storage tissue *i.e* bulb scales of most bulbous plants[15]. Sucrose has been found to promote formation of various storage organs (bulbs, corms, tubers and rhizomes) in most cases studied so far[5,16,17].

Direct Bulblet Formation on Bulb Scale

Bulblets were produced directly on the bulb scale (Fig 3) when the later was grown on MS medium supplemented with NAA (0.5, 1, 2 mg/L), BA (0.5, 1, 2 mg/L) and elevated levels of sucrose (45-90 g/L). MS basal medium supplemented with NAA (1 mg/L), BA (2 mg/L) and sucrose 60 g/L was found to be most effective in inducing 12-15 bulblets of different sizes (2-10mm) within 4-5 weeks. Bulblet production directly on bulb scale explants has also been reported in other species, including *O. umbellatum*[5,8,14].

In vitro Bulblet Size

Bulblets formed *in vitro* at the base of regenerated plants or directly on bulb scale were of varying sizes,

i.e small (2-3 mm), medium (4-6 mm) and large (7-10 mm). Sizes of bulblets could be increased by decreasing

Table3. Strength of medium on size of bulb formation *in vitro*.

Strength of medium	Sucrose	Percentage of bulbs formed in each size class. Small (2-3 mm)	Medium (4-6 mm)	Large (7-10 mm)
Full MS	30	97	3	0
	60	95	5	0
	90	92	8	0
Half MS	30	32	64	4
	60	36	56	8
	90	41	54	5
1/4th MS	30	68	31	1
	60	77	22	1
	90	70	30	0

the salt strength of MS medium (Table 3). It was observed that the percentage of medium size bulblet formation on medium with 45% sucrose increased from 3-64%, and that with 60% sucrose, it increased from 5-56% when the salt strength of the medium was reduced to half (Table 3). An effect of mineral salts on *in vitro* bulblet formation has also been reported in *Lilium speciosum*[15].

Transplantation of Bulblets into Soil

All *in vitro* plantlets with bulbs induced in culture showed a survival rate of about 80% when transferred to the potted soil. But, survival rates of bulblets produced directly on explants varied for different size bulblets. Small bulblets (2-3 mm diam) showed a survival rate of 40-50%, whereas, the larger bulblet (4-10 mm diam) had a 70-80% survival rate. The effect of *in vitro* induced bulblet size on *ex vitro* survival rate has also been reported in Lachenalia[7]. Transplanting of *in vitro* plantlets is more labor intensive then planting bulblets directly on soil. However, in order to successfully implement the use of *in vitro* generated bulblets rather then *in vitro* rooted plantlets of *O. virens* for commercial production, all stages of *in vitro* bulblet production must be optimized.

Cytological Analysis

Regenerants obtained directly from bulb scale from callus and those sprouted from *in vitro* induced bulblets were subjected to cytological analysis. A total of 50 plants from each group was analysed. It was found that all plants were diploid (2n = 6) (Fig 4) with normal karyotypes (Fig 5a–c). Detail karyotypic analysis revealed no gross structural changes in any chromosomes of regenerated plants of *O. virens* when compared with the karyotype of the source plant. This suggests the relative stability of all types of regenerants

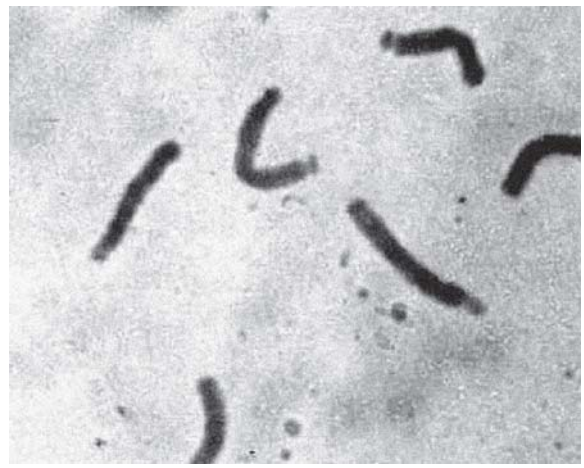

Fig 4. Diploid cell showing six chromosomes in root tip of a regenerated plant.

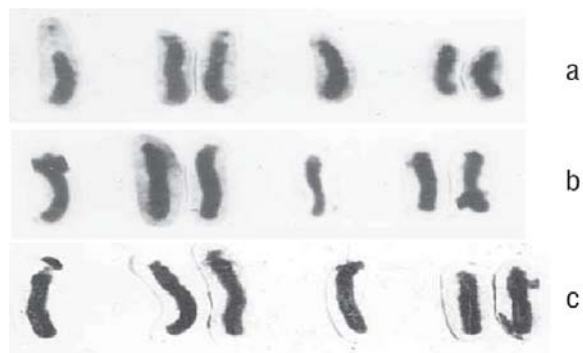

Fig 5. Normal karyotypes in root tips of regenerated plants.
a. Direct explant derived plant
b. Callus derived plant
c. Plant developed from *in vitro* induced bulblets.

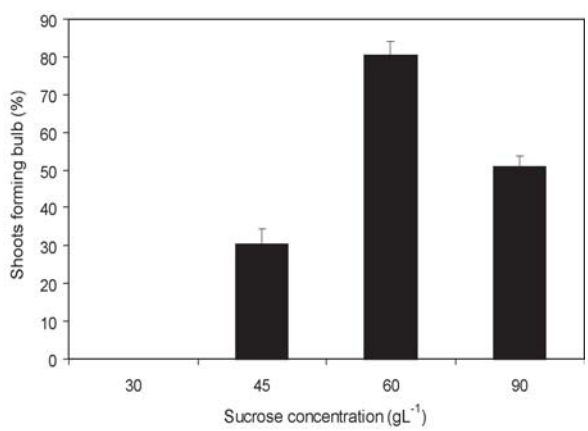

Fig 6. Effect of sucrose concentration in the medium on *in vitro* bulb formation in *O. virens*.

obtained through different modes of regeneration. Among other Liliaceous ornamentals, genetic stability of explant and callus derived regenerants has also been reported in *Ruscus hyphophyllu*[18], *O thyrsoides*[19] and *O. umbellatum*[4].

Thus, the process of regeneration of plantlets from bulb scale explant of *O. virens* through different modes of regeneration ensures rapid multiplication of true-to-type traits for commercial use.

Acknowledgements

The authors are grateful to Dr. Y. Medury, Vice Chancellor and Brig. Balbir Singh, Registrar of Jaypee University of Information Technology for providing facilities and encouraging throughout.

References

1. Hussey G (1986) Problems and prospects in the *in vitro* propagation of herbaceous plants. In: Withers LA and Alderson PG (Eds) Plant Tissue Culture and its Agricultural application. London: Butterworths, pp 113-22.
2. Murashige T (1990) Plant propagation by tissue culture. A practice with unrealized potential- In: Ammirato PV; Evan DR, Sharp WR, Bajaj Y.P.S (ed): Hand book of Plant Cell Culture, Mc Graw Hill Publishing Company, New York, Vol 5, pp3-9.
3. Hussey G (1976) Plant regeneration from callus and parent tissue in *Ornithogalum thyrsides*. J. Expt. Bot. **27**, 375-382.
4. Nayak S and Sen S (1995a) Rapid and stable propagation of *Ornithogalum umbellatum* L. in long term culture. Plant Cell Rep **15**, 150-3.
5. Rice RD, Alderson PG, Wright NA (1983) Induction of bulbing of tulip shoots *in vitro*. Sci Hortic **20**, 377-396.
6. Squires WM, Langton FA, Fenlon JS (1991) Factors influencing the transplantation success of micropropagated narcissus bulbils. J Hortic Sci **66**, 661-71.
7. Slabbert MM and Niederwieser JG (1999) *In vitro* bulblet production of Lachenalia. Plant Cell Rep **18**, 620-4.
8. Kim K W, Buyn MS, Choi JD, Park KI, Kim EY, Kim JS (1996) Morphological differences related to developmental pattern of lily plantlets regenerated from scaler *in vitro* (Proc Int Symp Lilium) Acta Hortic 414, 263-268.
9. Bach A (1992) Micropropagation of hyacinth (*Hyacinthus orientalis* L.) in : Bajaj . YPS (ed) Biotechnology in agriculture and forestry, vol 4. Springer. Berlin Heidelberg, New York. pp 144-59
10. Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant **15**, 473-97.
11. Sharma AK and Sharma A (1980) Chromosome techniques: Theory and practice, 3rd edition. Butterworths, London.
12. Battaglia E (1955) Chromosome morphology and terminology. Caryologia **8**, 178-87.
13. Sokal PR and Rohlf FJ (1973) Introduction to biostatistics, W. H. Freeman and Co., San Francisco.
14. Nayak S and Sen S (1995b) *In vitro* propagation of *Ornithogalum umbellatum* L. through direct organogenesis. Indian J. Exp. Biol **33**, 144-6.
15. Van Aartrijk J and Blom-Barnhoorn GJ (1980) Effects of sucrose, mineral salts and some organic substances on the adventitious regeneration *in vitro* of plantlets from bulb-scale tissue of *Lilium speciosum* 'rubum'. Acta Hortic **109**, 297-302.
16. Arora JS, Singh K, Grewal HS, Gosal SS and Chanana YR (1996). *In vitro* cormel production from nodal buds and cormel tips in Gladiolus. In: Islam AS(ed) Plant Tissue Culture. New Delhi: Oxford and IBH Publishing Co Pvt. Ltd, pp 50-3.
17. Nayak S (2000) *In vitro* multiplication and microrhizome induction in *Curcuma aromatica* Salisb. Plant Growth Regul **32**, 41-7.
18. Jha S and Sen S (1985) *In vitro* regeneration of *Rucas hypophyllum* L. plants. Plant Cell Tiss Org Cult **5**, 79-87.
19. Nayak S and Sen S (1991) Cytological and cytophometric analysis of direct explant and callus derived plants of *Ornithogalum thyrsides* Jacq Cytologia **56**, 297-302.