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High Frequency *In vitro* Propagation of *Polianthes tuberosa*

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Abstract. Calli induced on MS medium supplemented with 10 µM α-naphthaleneacetic acid (NAA) grew extensively when cultured on MS medium modified with 4 µM 2,4-dichlorophenoxyacetic acid (2,4-D), producing on an average four shoots per callus culture. The addition of 1 mM L-arginine in the culture media enhanced the induction rate upto 10 shoots per callus culture in 12 weeks. When 2-3 cm long regenerated shoots were replanted on MS medium with 20 µM 6-benzylyadenine (BA) and 4 µM 2,4-D, shoots the proliferated at the cut ends. Floral axis buds produced 3-4 cm long multiple shoots on NAA and BA. New shoots regenerated from the calli produced at the base of shoots subcultured on 10 µM NAA. Repetition of shoot development, callus formation, and again shoot formation on 10 µM NAA and 2 µM BA greatly increased the number of plants from single shoots. Eighty five percent bulb explants produced 290 shoots in 12 weeks directly on 15 µM BA and 5 µM NAA. The somatic psuedoembryos formed in the calli were dormant.

Keywords: L-arginine, clonal propagation, *Polianthes tuberosa,* tuberose plant

Abbreviations: IAA = indol-3-acetic acid; IBA = indole-3-butyric acid; BA = N^6-benzylyadenine; CH = casein hydrolysate; CW = coconut water; 2,4-D = 2,4-dichlorophenoxyacetic acids; Kin = kinetin; NAA = naphthalene acetic acid; MS = Murashige and Skoog medium

Introduction

Polianthes tuberosa (family: Amaryllidaceae) is one of the important cut flower. Its fragrance particularly makes it second to none. It is widely used for the extraction of essential oils and aromatic compounds, used as raw material in the fast growing perfume industry. Attention has been, therefore, focused on developing new techniques for the genetic manipulation of this species, requiring the *in vitro* culture of tuberose tissues from which whole plant can be propagated.

Very few studies have been reported on the *in vitro* culture of this species. Amongst these the work of Narayanaswami and Prabhudesai (1979) was the foremost on the culture of the tuberose *in vitro*, which reported direct and indirect regenerations from its meristematic tissue explants. Gi and Tsay (1989) reported studies on anther culture, and induced somaclonal variations in *P. tuberosa.* Later on, Nisar *et al.* (1989) reported regeneration multiple shoots from callus cultures, which was followed by studies on multiple shoots from quiescent nodal buds of floral stalk, plantlets from nodal segments, and the evidence of formation of protocorm-like bodies from anther stalk calli (Zaidi *et al.*, 1994). Regenerative potential of bulb, leaf and scale of the tuberose were also investigated by Khan *et al.* (2000). Krishnamurthy *et al*. (2001) reported micropropagation of the 'single' and 'double' types of tuberose.

In an effort to explore further, *in vitro* cultural features about the enhancement in shoot multiplication rate, were investigated. The present study reports findings on shoot differentiation from bud explants and the calli, and somatically regenerated pseudoembryos.

Materials and Methods

Stock plant. *Polianthes tuberosa* plants, commonly called as tuberose, were obtained from local nurseries. Bulbs, inflorescence axis and floral buds of the plant were used as the source material. The floral stalks were separated from the bulbs at their bases. Leafless floral stalks and bulbs were scrubbed clean with detergent and treated individually for sterilization. Hot water and fungicide treatment of the bulbs preceded the surface sterilization process for bulb explants. The bulbs were treated with hot water at 58 °C for 30 min, and dried on filter paper at room temperature for one day. Roots were then removed, the tunica dried, and the bulbs submerged in 3 g/l solution of Diathane M-45 (AgrEvo Chemical Company, Berlin, Germany) for 30 min prior to explant preparation. Bulb explants (3-5 mm^3) were removed from the inner core of the peeled off bulbs. For axillary bud explants, 1 cm^3 lump of the bulb, with at least one bud near the basal plate, were obtained. Axillary bud and storage tissue explants of the bulb were surface sterilized in 0.1% (w/v) mercuric chloride solution with

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1-2 drops of Tween-20 for 15 min and rinsed with sterile distilled water, 5 times prior to inoculation. Nodal and internodal explants (1 cm segments) were obtained from the non-flower bearing portion of the floral axis. The explants were sterilized in 0.1% (w/v) mercuric chloride solution with 1-2 drops of Tween-20 for 10 min. The explants were finally rinsed 5 times with sterile distilled water in aseptic environment. Prior to inoculation, floral shoot explants were trimmed at both ends.

Culture medium preparation. A number of modifications to the MS medium (Murashige and Skoog, 1962) were tried during the studies. The culture medium was supplemented with the following organic addenda: 100 mg/l myoinositol, 10 mg/l thiamin HCl, 50 mg/l glycine, 3% sucrose, 0.8% agar or 0.3% phytagel, auxins, cytokinins and reduced nitrogen sources, such as casein hydrolysate (CH) and 15% (v/v) coconut water (CW) in combination and concentrations as necessary. The plant growth regulators used were N^6-benzyladinine (BA), kinetin (Kin), naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxy acetic acid (2,4-D) and picloram. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH HCl prior to the addition of agar and autoclaved at 121 °C for 30 min at 138 kpa.

Cultural conditions. The explants were cultured in 100 × 15 mm petri plates (25 ml culture medium per plate), 150 × 18 mm test tubes (15 ml culture medium per tube), and magentas (50 ml culture medium per magenta), whose covers were wrapped with parafilm to avoid contamination. Cultures were kept in a growth room at 25 ± 2 °C and relative humidity of 50-60%. The cultures were exposed to illumination of 2000 lux light intensity, 16 h photoperiod. Callus cultures were kept in the dark. Each treatment consisted of 10 replicates.

Transplantation. As the plantlets developed to 10-15 cm in height and 4-5 number of roots, the plantlets were transferred to pots. The roots were washed with luke warm water to remove the medium and dipped in the fungicide solution (3 g/l Dithane M-45) to protect the plantlets from soil born diseases. The potting compost consisted of sterilized soil, sand and peat moss in 1 : 1 : 1, ratio supplemented with Hogland's solution to meet the nutrient requirements of the plants. Pots were covered with transparent polythene bags, placed in the growth room at 25 ± 2 °C for 4-6 weeks, until the plants were established in the pots and gained appropriate size for transplanting in field.

Results and Discussion

Multiple shoots from callus. Callus induction was tried with bulb tissue explants. Calli were induced on MS medium supplemented with 5, 10, 15 and 20 µM NAA. Greenish-white friable calli formed in 3-4 weeks. Preliminary studies with the freshly induced callus indicated that hormonal requirement for the growth of tuberose was specific. Extensive callus growth occurred with 4 µM 2,4-D, which 20 µM was primarily obtained with 10 µM NAA. Several attempts were made to induce redifferentiation in the bulb calli, using MS medium supplemented with different combinations and concentrations of 2,4-D and BA (Table 1). Calli were cultured on regeneration media and subcultured after every 4-week period. After second subculture on the same medium, visible morphogenetic changes were observed in the calli. Most of the calli turned green and the signs of shoot proliferation were evident in eight weeks. Within 10-14 weeks after first subculture from each callus, on an average four shoots were produced. The number of shoots per callus increased with the passage of time, without subculturing and inspite of necrosis of the parent calli. However, the addition of 1 mM L-arginine to the culture medium reduced the time for shoot production. The number of shoots per callus increased up to 10 shoots per callus within 12 weeks (Fig. 1). Sabapathi and Nair (1992) also reported improvement in the rate of shoot proliferation in the case of *Colocasia esculenta* on modified Linsmaier and Skoog medium (Linsmaier and Skoog,1965) supplemented with L-arginine and ornithine in addition to NAA and Kin. Todd and Gifford (2003) observed that application of exogenous L-arginine to cotyledons of the seedlings of *Pinus taeda* germinated in the absence of megagametophyte resulted in an increase in the total shoot pole argenase activity. These findings are suggestive of the positive physiological role of L-arginine in enhancing shoot multiplication *in vitro.* The 2-3 cm long regenerated shoots were removed from the calli and replanted on the same MS medium supplemented with 20 µM BA and 4 µM 2,4-D. Shoots were observed to resume growth. In addition, shoot proliferation also occurred from the basal part of the detached shoots. The successive shoot proliferations, one after the other, resulted in the formation of small clumps of rootless shoots (Fig. 2). For callus induction, NAA

Table 1. The effect of 2,4-D and BA on callus growth from the bulbs of *Polianthes tuberosa* and shoot development after 14 weeks

Concentration of 2,4-D (µM)	Concentration of BA (µM)					
	1	5	10	15	20	25
2	-	-	++	sh	sh	sh
4	++	++	sh	sh	sh	sh

– = no effect; ++ = callus growth; sh = shoot formation

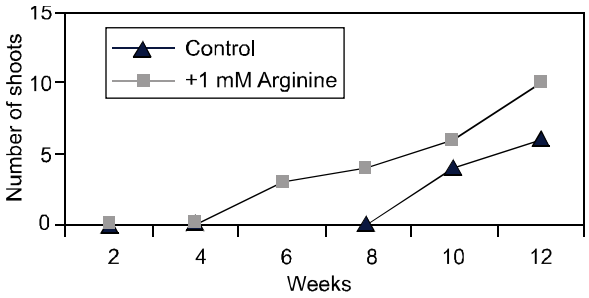

Fig. 1. The effect of addition of L-arginine to MS medium on the calli of *Polianthes tuberose* to form shoots, as compared to the control calli cultured on MS medium alone after 12 weeks of culture.

appears to be a key factor for bulb tissue. In previous studies, Narayanaswami and Prabhudesai (1979) found that explanted tissue of the bulb required a high concentration of 2,4-D in conjunction with 15% CW (v/v) and a longer incubation period to elicit callus induction. Both NAA and 2,4-D are very strong auxins. According to studies reported earlier, NAA should be used occasionally and the use of 2,4-D should be avoided for callus induction as they may cause rapid increase in poloidy in cultures from a wide range of plant tissues (Wilmer and Hellendoorn, 1968; Sunderland, 1973). However, these results indicated that tuberose requires a higher exogenous level of auxins for callus induction. Extensive callus growth occurred on MS medium when 4 µM 2,4-D was used. Though 2,4-D is also regarded as a strong auxin, which causes cell elongation and proliferation, yet its behaviour is modified in combination with BA favouring shoot growth and proliferation. Marin and Rubluo (1995) also experienced unexpected morphogenetic responses induced by auxins alone in *Mammillaria sanangelensis,* a severely endangered cactus, suggesting that it is the genetic constitution of the plant that modifies the expected morphogenetic behaviours of phytohormones in *in vitro* conditions.

Multiple shoots from floral axis buds. The nodal explants of floral axis carrying quiescent buds cultured on modified MS media with different combinations of 5 µM NAA, 15 µM

Fig. 2. The axillary shoot proliferation of *Polianthes tuberose* resulted in the formation of a small clump of rootless shoots after 12 weeks.

BA and/or 15 µM Kin produced multiple shoots (Table 2). Visible morphogenetic changes were observed just after four weeks. Shoots were formed on M1 and M2 media, while on M3 and M4 media explants turned brown (Table 2). The shoot proliferation depended on the presence of NAA with BA, irrespective whether Kin was present or not. Twenty five percent explants produced 20 shoots in 8 weeks, while the remaining 75% produced less than that. The reason for this may be that the bud explants derived from the nodes close to the subterrestrial zone of the floral axis would have lost their meristematic activity due to ageing. Following induction, shoot growth continued without subculturing and attained 3-4 cm height in 11-12 weeks. At that stage, the shoots were excised and subcultured on fresh media. Subsequent development of the excised shoots was determined by the hormonal content of the medium. As compared to induction medium, callus pro-

Table 2. The effect of combination of auxins and cytokinins on the floral axis shoot buds of *Polianthes tuberosa*

Media code	Growth regulators (µM)			Observations
	NAA	BA	Kin	
M1	5	15	15	Shoot proliferation
M2	5	15	-	Shoot proliferation
M3	5	-	15	Senescence of explants
M4	-	15	15	Senescence of explants

M1-M4 = basal MS medium + mentioned growth regulators

liferation occurred around the base of excised shoots on the media containing 10 µM NAA. The calli were highly caulogenic, giving rise to new shoots within 4 weeks. In turn, when recultured on media supplemented with 10 µM NAA + 2 µM BA, these produced more callus leading to further shoot production. The alternate concurrence of shoot development, callus formation, and adventive shoot formation were repeated several times, which greatly increased the number of plants from single shoots. The multiple shoots rooted well on MS medium containing 10 µM IBA in the medium veried in expression in contrast to tuberose cultures, depending on the concentration and genetic constitution of the explants as in *Lilium japonicum.* BA and NAA.

The effect of combination of BA and NAA in the medium veried in expression in contrast to tuberose cultures, depending on the concentration and genetic constitution of the explants as in *Lilium japonicum.* BA and NAA also caused bulbet regeneration from mother scale callus (Mizuguchi and Ohkawa, 1994) and active biomass production in *Cattleya aurantiaca* shoot explants reported by Mauro *et al* (1994).

Multiple shoot formation from bulb explants. Bulb explants carrying dormant buds cultured on MS medium supplemented with 15 µM BA and 5 µM NAA produced multiple shoots directly. Efficiency of shoot production obtained from 100 such explants is shown in Fig. 3, on an average 30 shoots appeared in 10% of the explants in 6 weeks. After 12 weeks, 85% of the explants produced a total number of 290 shoots, while average shoot count was 4 per explant. The shoots normally gained a height of 8-10 cm in 6 weeks. Similar high frequency direct shoot regeneration from corm axillary buds and the rapid clonal propagation of *Colocasia esculenta* is reported by Chng and Goh (1994), indicating that axillary buds on the underground modified stems, whether a corm or bulb, actively responded to hormonal stimulus in *in vitro* condition, giving rise to multiple shoots.

Psuedosomatic embryogenses. Extensive callus growth occurred on MS medium supplemented with 4 µM 2,4-D. The shifting of 2-week-old calli on basal MS medium induced characteristic changes in its appearance. Nodular overgrowths resembling embryoid-like structures were produced in quick successions (Fig. 4). Such overgrowths retained their independent identity, not resembling with meristematic nodules. The tadpolelike embryoids showed root like appendages at one end and presumptive shoot apex at the other end, apparently resembled proembryos of zygotic origin. The somatic redifferentiation was obtained only in those calli, which were incubated on media supplemented with 4µM 2,4-D. On prolonged incubation in nutrient medium upto 8 weeks, the nodular bodies were somewhat elongated. In order to promote their further growth and differentiation, isolated embryos were transferred to MS liquid medium containing twice the concentration of ammonium nitrate and 15% CW (v/v), and shakern at 50-60 rpm at 25 °C. Within 3 weeks of culture, elongation of ovoid structures was observed, which resembled typical embryoids of somatic origin. To regenerate the embryoids, or to succeed in breaking down their dormancy, they were cultured on different hormonal compositions, with complex addenda which, however did not yield the desired result, showing browning and senescence of the cultures.

Attempts to induce the embryoids to develop into normal plantlets on a basal medium under the influence of natural adjuvant such as water extract of bulb, malt extract, casein

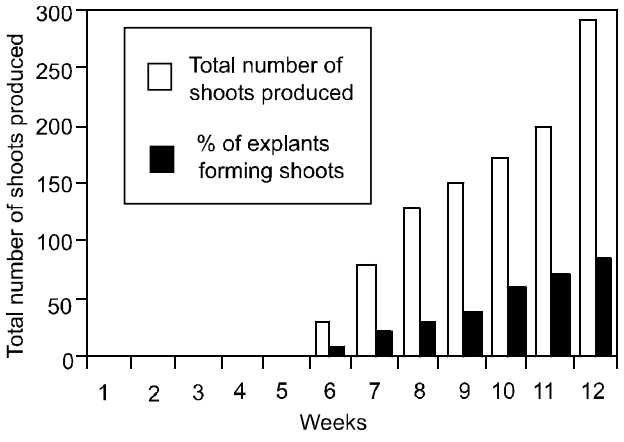

Fig. 3. Efficiency and rate of axillary shoot formation from bulb explants of *Polianthes tuberose* after 12 weeks of culture.

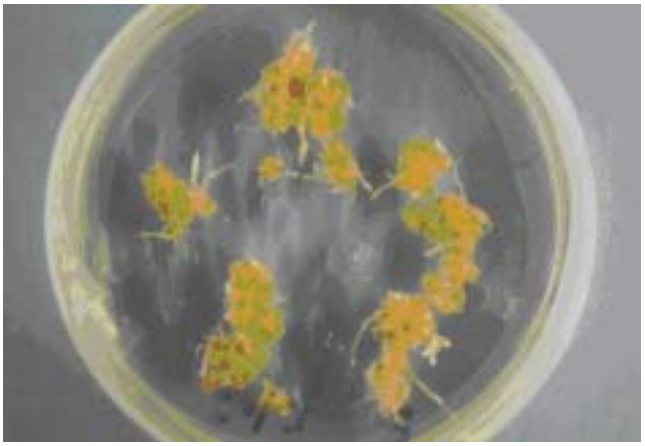

Fig. 4. Psuedoembryogensic clumps developed in calli on growth regulator free medium showing out growth of root like structure.

hydrolysate or change of carbon source, and increasing the concentration of sucrose (upto15%) were of no avail. Physical factors, such as exposure to high intensity light or continuous chilling at 4 °C prior to incubation also proved ineffective in inducing the embryoids to undergo normal differentiation except rhizogenesis. On prolonged incubation, the embryoids, which were imperfect, accumulated starch grains in their cells and remained quiescent indefinitely. Eventually, all attempts to regenerate plantlets from somatic embryos were not successful. The embryo-like structures induced in the tuberose calli were of the nature of shootless embryoids. This is comparable to the occurrence of rootless shoots, prevalent in some cultures of Pergularia (Prabhudesai and Nayranaswamy, 1974) and many other plants in the *in vitro* condition. This has its parallel in the species *Escholtia calfornica*, where embryoids, although produced in abundance, failed to develop into normal plantlets. On differentiating medium, the primary root was well developed representing growth of the axial structures in continuity with the shoot region and was never adventitious in origin from a callus mass. However, owing to lack of typical organization of shoot apex it could not initiate leaf primordia and thereby was arrested in development. Nayranaswamy and Prabhudesai (1979) and Zaidi *et al.* (1994) also obtained elongated embryoids of *Polianthes tuberosa.* However, none of the innumerable embryoid-like structures grew into plantlets cultured even under aforsaid variety of nutrients and hormonal conditions, such structure were not embryoids but psuedoembryoids. Interpretations of Tian and Yang (1984) are fully applicable to these abortive regenerants. They observed similar situation in gynogenetic embryoids and callus in ovary culture of *Oryza sativa*. Chang *et al*. (1986) have reasoned that abnormality occurred due to the absence of nurse tissue-like endosperm and some inadequacy in the culture medium.

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