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MICROPROPAGATION OF *POLIANTHUS TUBEROSA* (TUBEROSE) THROUGH CALLUS FORMATION

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Abstract

Callus was induced from bulb pieces and *in vitro* formed shoot buds in *Polianthus tuberosa* Willd., on Murashige & Skoog's (MS) medium supplemented with BAP and 2,4-D. Maximum callus induction from bulb pieces was 70% at 0.5 mg/l each of BAP and 2,4-D. Shoot buds showed 100% callus induction at 1.0 mg/l each of BAP and 2,4-D. Shoot regeneration response was observed only in buds derived callus. Highest number of shoots were regenerated from the callus at 3.0 mg/l of BAP. Best root development was achieved on MS medium supplemented with 0.5 mg/l NAA. Plantlets obtained were successfully transferred to the field with 100% survival rate.

Introduction

Polianthus tuberosa is one of the famous cut flowers belonging to the family Amaryllidaceae. It has exceptionally sweet smelling flowers and attractive foliage. Clusters of white, waxy flowers are produced on elongated inflorescence stalks. They are used as cut flowers, for making garlands and extracting essential oil. It is an economically important crop in tropical and subtropical regions and cultivated in Morocco, the Comores Island, France, Hawaii, South Africa, India, China and Pakistan. In Pakistan *Polianthus tuberosa* is mostly cultivated in Punjab province for commercial cut flowers production. The product is mostly consumed in big cities of Pakistan.

For commercial production the main aim is to produce attractive foliage and brilliant flowers at right time. To achieve this objective certain practices are common to all flowering bulbs and corms, like daughter bulb formation, scooping, twin scaling, forcing etc. These practices usually take ten or more years to build up commercial quantities from a single bulb. During this long time interval the material if indexed as virus-free could be re-infected (Alderson & Rice, 1986).

In recent years, there has been a considerable interest in the development of tissue culture techniques for the rapid propagation of healthy bulb stock and new varieties (Hussey, 1980). Many different types of tissues are used as explant for micro-propagation of bulbs, like bulb scales, very young leaf tissue and inflorescence stalk etc. Wang & Hu (1982) obtained virus-free plants of *Polianthus tuberosa* through tissue culture techniques. Zaidi *et al.*, (1994) obtained callus of *P. tuberosa* from nodal segments of floral shoots on MS medium containing BAP and NAA. Roots and shoots were regenerated with NAA, IAA kin & GA_3. Khan *et al.*, (2000) also obtained callus from leaf blades on MS medium supplemented with 1.0 mg/l of NAA and IAA, 2.0 mg/l kin and 7.0 mg/l BAP. The present study was conducted to initiate callus from two different explants of *P.tuberosa* (bulb pieces and shoot buds) and to find out their regeneration potential.

Materials and Methods

A young bulb of *P.tuberosa* with a number of lateral shoot buds was taken from a pot grown healthy plant in the Department of Botany, University of Peshawar. Leaves and roots were excised from the bulb and plant material was then washed thoroughly under tap water. A gentle washing with household detergent was also given. Upper few scales of the bulbs were removed and plant material was transferred to laminar air flow cabinet. It was surface sterilized with 70% ethyl alcohol for 2 minutes and 1% $HgCl_2$ for 3-4 minutes followed by several washings in sterile distilled water. Outer scales were removed from the bulb leaving the innermost two. It was then cut into 0.5cm small pieces by transverse and longitudinal cuts. Shoot buds were used as such after removing a single upper scale. Murashige & Skoog's (1962) basal medium was used at half strength supplemented with various growth regulators viz., Benzylaminopurine (BAP), Kinetin (Kin), 2,4-Dichlorophenoxyacetic acid (2,4-D) and Indoleacetic acid (IAA). The medium was fortified with 4% sucrose as carbon source and soldified with 0.9% agar. pH of the medium was adjusted to 5.6 with 0.1 N NaOH or 0.1 N HCl. The medium was autoclaved at 15 psi for 15 minutes. The cultures were incubated in biotrons with a 16 h light period in 24 h cycle at 25$\pm$ 1°C.

Results

Callus induction: Bulb pieces and shoot buds when transferred on MS medium supplemented with different combinations of BAP and 2,4-D showed that 70% of the bulb pieces produced callus on the basal medium supplemented with 0.5 mg/l each of BAP and 2,4-D after 2 weeks (Table 1). The callus was pale green in colour and soft in texture (Fig. 1). A slight callus formation was also observed in few cultures at 0.5 mg/l BAP and 0.1 mg/l 2,4-D. There was no reponse at 1.0 mg/l BAP and 0.5 mg/l 2,4-D. Callus got induced from all the shoot buds when inoculated on MS medium incorporated with 1.0mg/l of 2,4-D in combination with either 0.5 or 1.0mg/l of BAP (Table1). Callus formation started from the basal ends of the shoot buds and gradually it covered the whole buds within 4-6 weeks of culture. The callus obtained at 0.5 mg/l of BAP and 1.0mg/l of 2,4-D was yellowish white and compact in texture (Fig. 2). On the basal medium containing 1.0mg/l each of BAP and 2,4-D the callus formed was greenish white and soft.

Shoot buds when transferred on MS medium supplemented with 0.5mg/l of BAP alongwith 0.1mg/l of 2,4-D was found not effective for callus induction, however buds sprouted in few cultures (Table1).

Callus proliferation & morphogenesis: The calli obtained in the above experiments were transferred to basal MS medium supplemented with different concentrations of BAP (0.5-3.0mg/l) for their further proliferation and morphogenesis (Table 2). Shoot buds derived callus turned green after a little proliferation when subcultured on MS medium supplemented with either 1.0 or 2.0 mg/l of BAP. Small green buds appeared in the calli after few weeks that developed into shoots. However at 1.0mg/l of BAP shoot bud derived callus regenerated shoots after 8 weeks (Fig. 3) while at 2.0 mg/l of BAP shoot regeneration was observed after 6 weeks. At 3.0 mg/l of BAP, shoot bud derived callus, exhibited rapid proliferation during the first two weeks of culture. However in the third week callus growth stopped with the appearance of small green patches. In the next 2-3 weeks numerous shoot buds developed from these green patches (Fig. 4).

Table 1. Effect of different concentrations of BAP and 2,4-D on callogenesis of *P. tuberosa* when cultured on 1/2XMS medium. The cultures received around 1000 lux of 16h in a 24h cycle. The temperature was regulated at 25± 1°C.

Explant	Growth Hormones mg/l		No of cultures	Callogenesis	% of cultures produced callus	Remarks
	BAP	2,4-D				
Bulb Pieces	0.5	0.1	20	+	25%	Slight callus, greenish white, soft
Bulb Pieces	0.5	0.5	20	+	70%	Little callus, pale green, soft
Bulb Pieces	1.0	0.5	10	-	-	
Shoot Buds	0.5	0.1	10	-	-	Buds sprouted in two cultures
Shoot Buds	0.5	1.0	10	+	100%	Little callus, yellowish white, a bit hard
Shoot Buds	1.0	1.0	10	+	100%	Little callus, greenish white, compact but soft

- No Response
+ Slight Callus formation

Table 2. Effect of various cytokinins on callus proliferation and morphogenesis The culture conditions were identical with those of Table 1.

		Bulb Segment Derived Callus		Shoot Bud Derived Callus	
BAP mg/l	Kin mg/l	Callus Proliferation	Morphogenesis	Callus Proliferation	Morphogenesis
0.5		-	-	-	-
1.0		-	-	+	2-3 shoots arose after 8 weeks
2.0		+	-	+	2-3 shoots arose after 6 weeks
3.0		+	-	++	Numerous shoots after 3-4 weeks
1.0	0.5	-	-		
2.0	0.5	-			
3.0	1.0	-	Small embryoid like structures		

- No Response
+ Slight callus proliferation
++ Moderate callus proliferation

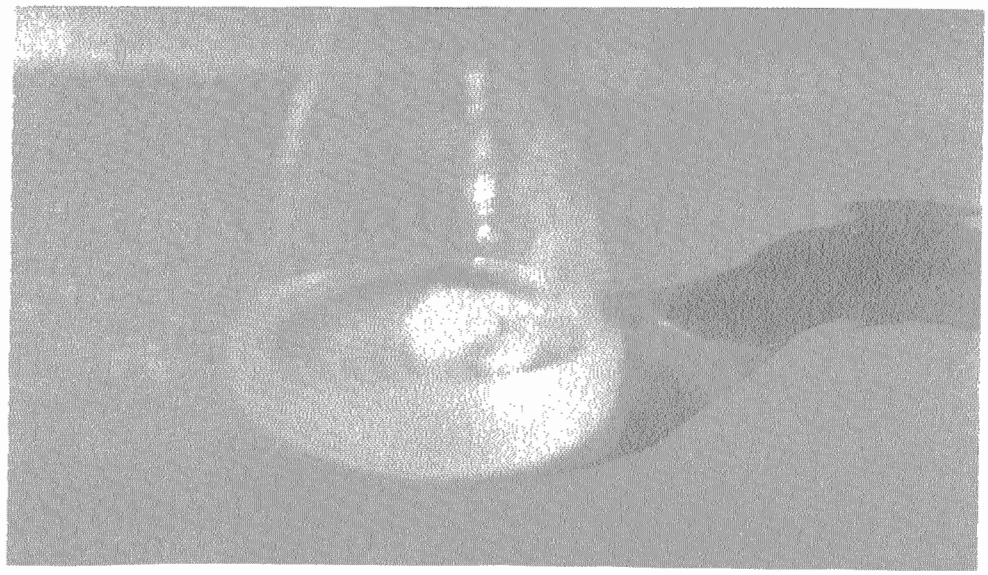

Fig. 1. Callus formation in bulb segment explant of *P. tuberosa* at 0.5 mg/l each of BAP and 2, 4 D after 4 weeks of cultures at 25 ± 1° C. The cultures received 16 h light in 24 h cycle.

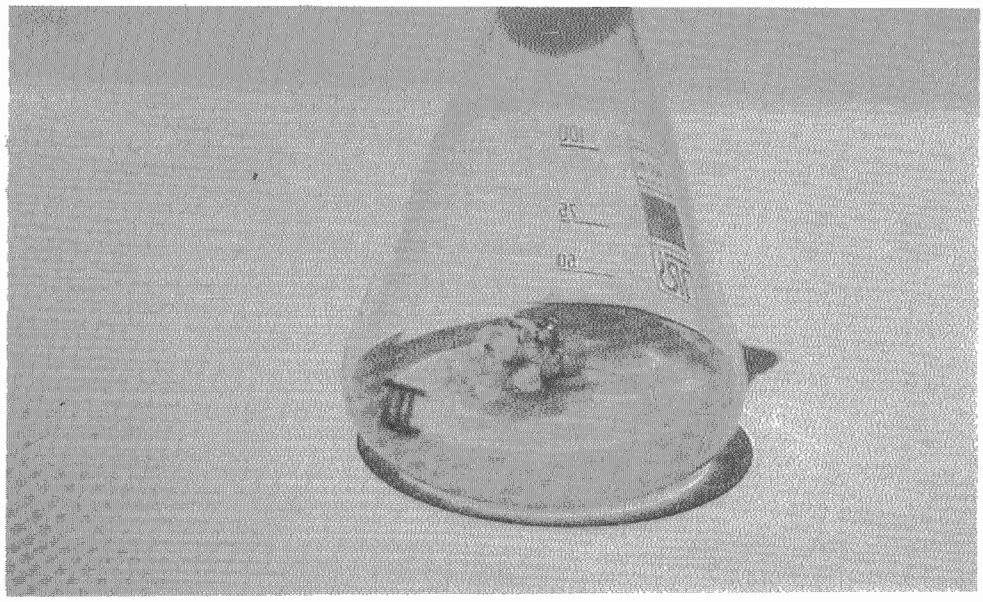

Fig. 2. Callus formation on ½ XMS medium supplemented with 0.5 mg/l of BAP and 1.0 mg/l 2,4-D from shoot bud explant of *P. tuberosa.* Small amount of yellowish white callus was found after 4 weeks of culture period.

Fig. 3. Shoot regeneration from shoot bud derived callus of *P. tuberosa* at 1.0 mg/l of BAP after 8 weeks of culture.

Shoots thus obtained from different calli were further subcultured for multiple shoot formation on MS medium supplemented with 3.0 mg/l of BAP. Large number of multiple shoots were obtained within 4 weeks of subculture. Bulb segments derived callus did not respond to 0.5 and 1.0 mg/l level of BAP. However, a little callus proliferation was observed on MS medium supplemented with either 2.0 or 3.0 mg/l of BAP after 4 weeks of subculture. Moreover callus turned green at 3.0 mg/l level of BAP without any morphogenetic response. This green callus was then again subcultured on MS medium supplemented with different combinations of BAP and Kin to test for morphogenesis (Table 2). There was no significant effect of this combination except the appearance of small embryoid like structures. These structures did not show any further growth or development on transfer to different levels of BAP (0.5-2 mg/l).

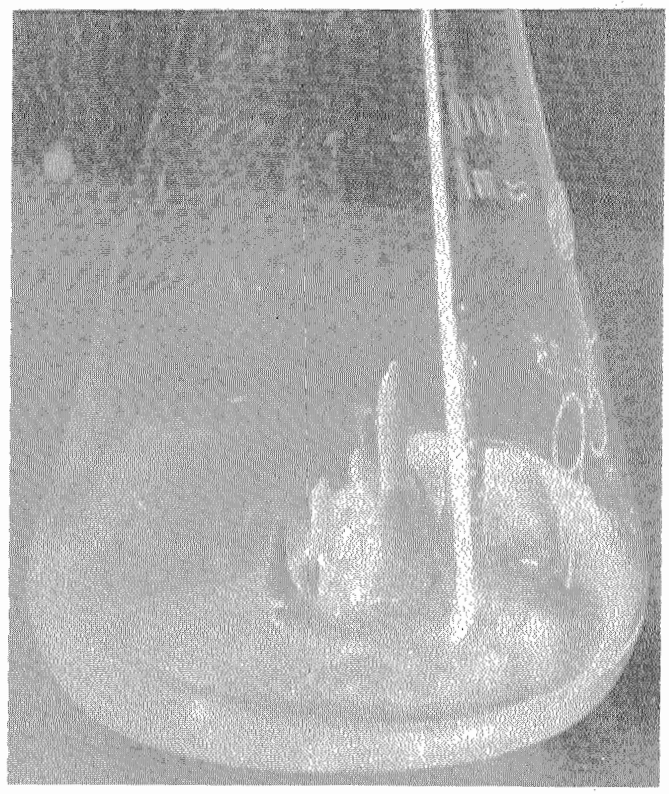

Fig. 4. Shoot regeneration from shoot bud derived callus at 3.0 mg/l of BAP after 3 weeks.

Rooting of the microshoots: With the objective of obtaining whole plantlets, shoots obtained from the above experiments were isolated and further transferred to root induction medium. Microshoots attaining the size of 3-4 cm were subcultured singly on 1/2XMS medium supplemented with either 0.1 or 0.5 mg/l of IAA. Best results were achieved when 0.5mg/l of IAA was used. All the shoots rooted within two weeks of culture period that were thin, long and large in number (Fig. 5). A few new leaves also produced from the shoots. The plantlets attained a height of 7-8cm in the next 2-3 weeks. At 0.1 mg/l of IAA, 90% of the shoots gave rooting after 4 weeks. These roots were however, short, thin and few in number as compared with those induced at 0.5mg/l of IAA.

Transfer to the soil: Plantlets attaining a height of 7-8 cm were subjected to further hardening before transfer to the field. Plantlets were transferred aseptically to the test tubes containing about 5 ml of ¼ strength MS solution with 1% sugar and covered with cotton plugs. They were placed under the laboratory conditions for about two weeks after which tubes were uncovered and kept as such for 2-3 days. Plantlets were then transplanted into the pots containing sandy loam soil (1:2) and covered with transparent polythene bags. After about two weeks polythene bags were removed when plantlets had established themselves in the soil (Fig. 6). Transplantation was done in the months of September and October. After establishment plantlets showed normal vegetative growth upto December with the formation of new leaves and shoots. At the start of December,

portion of the plants above the soil wilted with the formation of underground bulbs like mother plant. However size of the bulbs was smaller in *in vitro* raised plants as compared to mother plant. After a period of perennation in the soil bulbs sprouted again in the months of March and April with healthy shoots. Survival rate of the transplants was 100%. About 3 ½ to 4 months are required to obtain healthy rooted plantlets (7-8cm) for field transfer starting from the callus induction on shoot buds.

Fig. 5. Root induction on ½ XMS medium supplemented with 0.5 mg/l IAA after 2 weeks of culture

Discussion

Polianthus tuberosa is an economically important ornamental crop. It is a slow growing bulb taking at least 3-4 years to mature. A mature bulb produces only one floral stalk and then dies off (Khan *et al.*, 2000). In recent years plant tissue culture is widely used for large scale production of ornamental crops. It allows the rapid propagation of a

large number of clonal propagules within a short time span. Methods for *in vitro* production of many kinds of bulbs and corms have been developed (George & Sherrington, 1984). In the present study rapid clonal propagation of *Polianthus tuberosa* was achieved through callus formation.

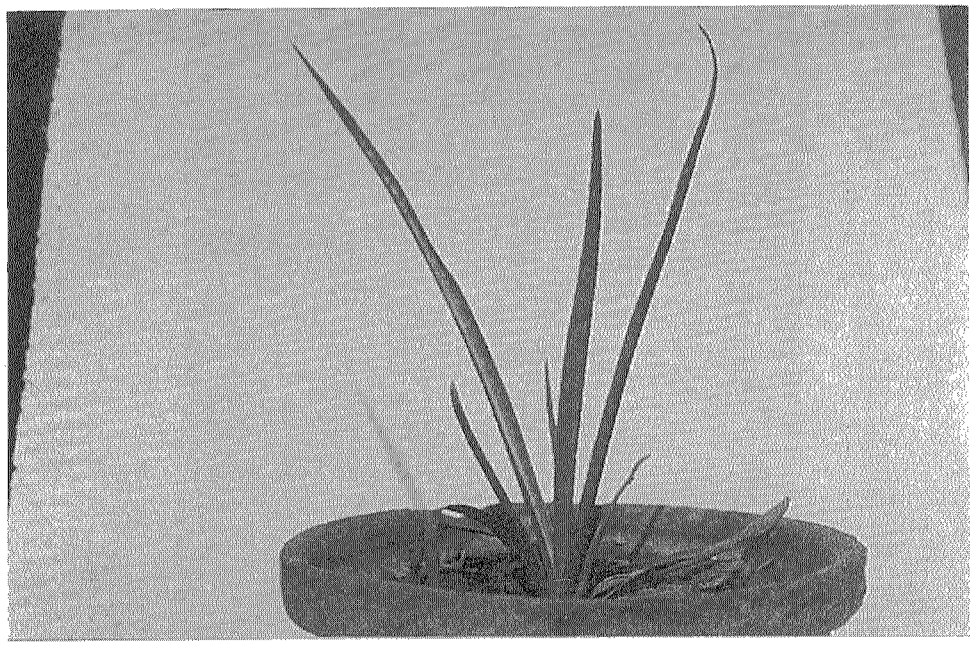

Fig. 6. *In vitro* raised plant established in the soil after 4 weeks.

Callus of *P.tuberosa* was obtained from two different explant sources i.e bulb pieces and shoot buds on ½XMS medium supplemented with different combinations of BAP and 2,4-D. It was observed that higher level of BAP as compared to 2,4-D inhibited callus induction. However when the two hormones were used in equal concentrations or 2,4-D level was increased, the explants exhibited callus induction. Slight callus was however formed from bulb pieces in few cultures (25%) at 0.5 mg/l BAP and 0.1 mg/l 2,4-D. Moreover callus induction response was greater in shoot buds as compared to bulb pieces. Callus formed was small in amount with slow multiplication rate whether explants used were bulb pieces or shoot buds. Afridi & Ghori (1993) also induced callus from shoot explants of *Colchicum luteum* at 0.5 mg/l 2,4-D and 0.1 mg/l BAP. However Khan *et al.*, (2000) used various combinations of NAA, IAA, Kin & BA for callus induction in leaf blades of *Polianthes tuberosa*. They obtained a good amount of nodular and friable callus at 1.0mg/l IAA and NAA, 2.0mg/l Kin., and 7.0mg/l BAP. This callus developed protocorm like bodies that could not show further growth.

Shoot bud derived callus regenerated shoots when cultured on various concentrations of BAP (0.5-3.0mg/l). At low concentration (0.5) there was no response. However increase in BAP level showed considerable effect on shoot regeneration. Maximum

number of shoots developed at 3.0mg/l level of BAP. Zaidi *et al.*, (1994) regenerated shoots on different combinations of NAA, IAA, GA_3, BAP and Kin., in the callus of *P. tuberosa*

Bulb piece derived callus did not show any response when cultured at either 0.5 or 1.0mg/l of BAP. However a little increase in callus mass was observed at higher BAP level (2.0 - 3.0 mg/l). The callus turned green at 3.0mg/l level of BAP without any morphogenic response.This callus was differentiated into small embryoid-like bodies when subcultured on 3.0mg/l of BAP and 1.0mg/l Kin.These embyoid-like structures could not develop further. These results were similar to those of Zaidi *et al.*, (1994) and Narayanaswami & Prabhudesai (1979). They also obtained embryoid-like bodies from calli of *P.tuberosa* that did not show further differentiation and development.

Shoots successfully rooted with 0.5 mg/l of IAA and transferred to the field after hardening. Plants survived transplanting shock with 100% survival rate. They showed vegetative growth for few months (September-December) and then perennated with the formation of underground bulbs. The bulbs sprouted again in the months of March and April and showed vegetative growth. Thus as a result of these studies an efficient method has been developed for rapid clonal propagation of tuberose.

Acknowledgment

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