Role of sterilants in establishment of aseptic culture using different explants in tuberose (*Polianthes tuberosa* Linn.)

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Abstract

The aim of the present study was to establish an efficient protocol for acquiring aseptic culture from Single and Double cultivars of tuberose (*Polianthes tuberosa* Linn.) namely, Prajwal and Vaibhav, respectively. Initially aseptic cultures for bulb scale, buds, petal segment and immature flower bud explants were established by pre-treatment using 0.1 per cent of carbendazim and mancozeb and 200 mg l^{-1} 8-HQC for 4h and 2h, respectively. The survival per cent observed was 89.20 per cent and 82.5 per cent in case of bulb scales and bud explants and 87.50 per cent and 90.83 per cent for flower based explants respectively. $HgCl_2$ and NaOCl applied at 1.0% for 8 min as surface sterilant has shown better results with respect to all the explants used in order to get the good amount of aseptic cultures in tuberose cultivars.

INTRODUCTION

Tuberose (*Polianthes tuberosa* Linn.) belonging to the family Amaryllidaceae is one of the important bulbous flower crops of tropical and subtropical regions. Among the commercially grown flowers in India, tuberose occupies a prime position owing to its popularity as a cut flower, loose flower and for perfumery. The serene beauty of the flower is because of its tall and straight spikes, bright snow-white flower and sweetness of blooms and delicacy of fragrance. In tuberose four main types *viz*., Single, Semi-double, Double and Variegated have been reported out of which only Single and Double types are under commercial cultivation.

Conventionally, tuberose (*Polianthes tuberosa* Linn.) is propagated from offsets and seeds, but commercially it is by bulbs and bulblets. However, *in vitro* propagation using emerging bud and scale section, petal segment, immature flower bud, leaf base segment and axillary bud is also possible. Very meager amount of work has been done for upgrading the conventional and non-conventional methods of propagation. Non availability of large quantity of disease-free genuine planting material is a major constraint for expansion of area under tuberose cultivation, for which *in vitro* propagation technique can be explored as an alternative tool to fulfill the demand of good quality disease-free planting material. Furthermore, with rapid area expansion and growing industrial uses like essential oil exraction, there is a dearth of growing planting material and here tissue culture technique can be alternative to meet the demand. Ornamental flowering bulbs (genotypes) are heterozygous and are primarily propagated vegetatively. Conventional propagation methods have some bottlenecks such as difficulty in producing disease free bulbs as well as spread of diseases from stock plants infected. *In vitro* propagation have several advantages like rapid mass multiplication of new cultivars, maintenance of disease-free stock, year round production of plantlets and storage of plantlets. In tuberose, *Tuberose mild mosaic virus* (TMMV) has been recognized as a major problem based on the serological and biological distinction (Lin *et al*. 2004). Recently more incidence of this viral disease was observed in farmers fields in the states of Karnataka, Tamil Nadu, Himachal Pradesh, Orissa, West Bengal, Uttar Pradesh and in countries like China, New Zealand, Taiwan etc. (Kirshna reddy *et al*. 2007). Viral disease decrease quality of flowers and bulbs which leads to decreased profit to growers and propagators. Tuberose is also affected by fungal disease like Sclerotium rot (basal rot) which is a devastating disease, which resulted in very low or no spike development, thus affecting the economy of this crop cultivation. Virus free Double-petalled tuberose cultivars were rescued by meristem tip tissue culture and as a result of which we can get a virus-free tuberose. In this context, the present study was undertaken to establish the aseptic culture from different explants in tuberose, which is a first and foremost step in any tissue culture method.

MATERIALS AND METHODS

The present investigation was carried out at the Division of Floriculture and Landscaping, Indian Agricultural Research Institute New Delhi and Central Tissue Culture Laboratory of National Research Centre on Plant Biotechnology, New Delhi, during 2006 to 2008.

The present investigation was conducted on two tuberose commercial cultivars *viz*., Prajwal (Single type), which is very floriferous and high concrete yielding and Vaibhav (Double type), which produces multi-whorled flower on the spikes having the exhibition value. The explants *viz*., bulb scales, buds, petal segments and immature flower buds of these two cultivars (Fig. 1) were taken from the Research Farm of Division of Floriculture and Landscaping, IARI, New Delhi. The stock plants maintained under insect-proof net in the previous season were selected for the study during October to December.

The explants *viz*., bulb scales 1 cm and buds 0.5 cm were excised from fresh bulbs harvested from the field. These were then washed thoroughly under the running tap water for 30 minutes. The explants were then subjected to pre-treatment, using fungicides namely, carbendazim (0.1%), mancozeb (0.1%) and bactericide 8-HQC (200 mg l^{-1}) in different combinations for 2 - 4h for bulb scale and bud explants, followed by several washings with distilled water. The petal segment and immature flower bud explants of the size 10-12mm and 10-15mm, respectively were excised from freshly harvested spike of 30cm length from both the cultivars. These flower based explants were subjected to pre-treatment with the same treatment combination which was used for bulb scales and bud explants but with different time durations *viz*, 1-2h. The pre-treated explants were taken to the laminar air-flow chamber, where they were surface sterilized using two agents namely; mercuric chloride (0.1%) and sodium hypochlorite (1.0%), for 6 – 8 min, followed by 3-4 washings in sterile distilled water. The explants were then cultured on medium (Murashige and Skoog, 1962) supplemented with 3% sucrose. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 21 min. The best pre-treatment was further used for culturing the surface sterilized explants on basal medium. Twenty explants were cultured per replication per treatment and the experiment was repeated three times. The observations *viz*., per cent contamination and per cent survival were recorded after 21 days of culture period.

RESULTS AND DISCUSSION

For the present investigation, explants were excised from underground part of the plants. Usually explants collected from field grown plants harbor microbes. Decontamination of explants obtained from underground parts has been reported to be a very difficult task by several workers (Seabrook, 1990 and Hol *et al*., 1992). Results obtained in the present study reveals that per-treatment of explants with 0.1 per cent of carbendazim, mancozeb and 200 mg l^{-1} of 8-HQC for 4h gave the maximum culture survival, 89.20 per cent and 82.5 per cent in case of bulb scale and bud, respectively (Table1). In petal segment and immature flower bud explants with same pre-treatment combination for 2h, gave the maximum culture survival, 87.50 per cent and 90.83 per cent for these flower based explants, respectively (Table 2). With all four explants we also observed varied cultivar performance i.e. cultivar Prajwal responded in

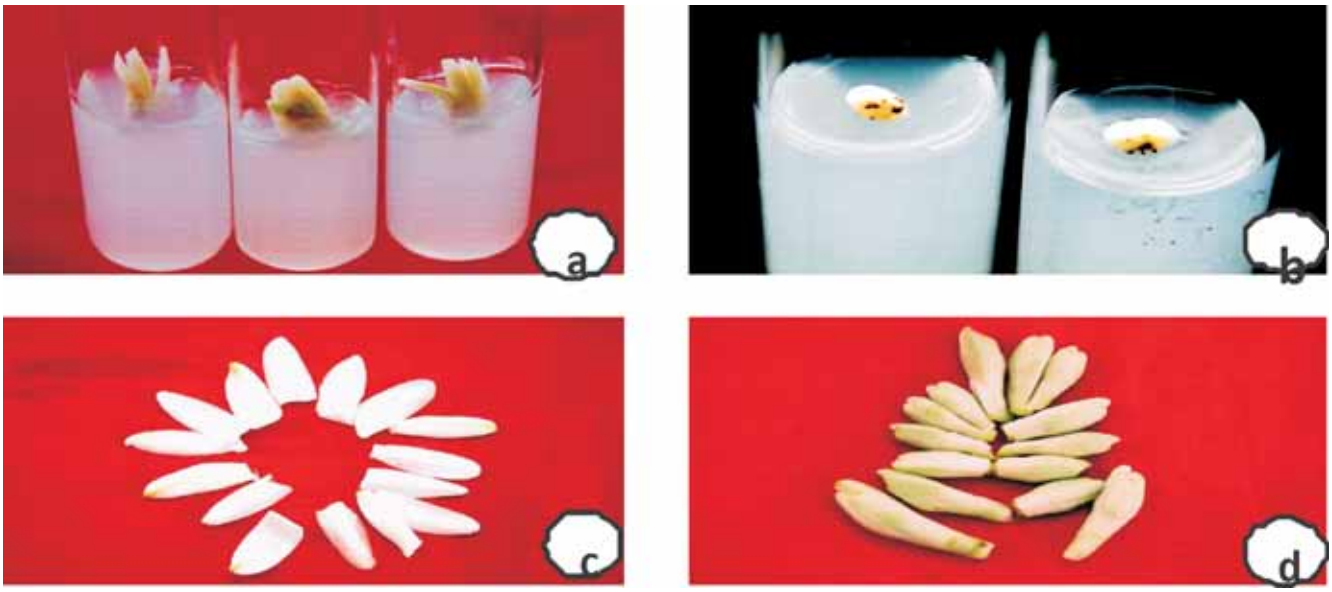

Figure. 1. a. Bulb scales; **b.** buds; **c.** Petal segments; **d.** immature flower buds

Table 1. Effect of pre-treatments on culture establishment in tuberose cultivars using bulb scale and bud explants

Treatment	Per cent survival (bulb scale)			Per cent survival (bud)		
	Prajwal	Vaibhav	Mean	Prajwal	Vaibhav	Mean
P_1	3.30	1.60	2.45	6.70	11.70	9.20
P_2	76.60	83.30	79.95	66.70	65.00	65.85
P_3	66.60	65.00	65.80	57.70	53.30	55.50
P_4	91.70	86.70	89.20	83.30	81.70	82.50
Mean	59.50	59.15	59.33	53.60	52.92	53.25
CD at 5%	Treatment = 2.76 Cultivar = N.S. Treatment x Cultivar = 3.91		Treatment = 4.3 Cultivar = N.S. Treatment x Cultivar = 6.8			

P_1= Control (Distilled water) for 2 h; P_2= Carbendazim (0.1%) + 8-HQC (200 mg l^{-1}) for 2 h; P_3= Mancozeb (0.1%) + 8-HQC (200 mg l^{-1}) for 2 h; P_4= Carbendazim (0.1%) + Mancozeb (0.1%) + 8-HQC (200 mg l^{-1}) for 4 h. N.S. = Non Significant

Table 2. Effect of pre-treatments on culture establishment in tuberose cultivars using petal segment and immature flower bud explants

Treatment	Per cent survival (Petal segment)			Per cent survival (Immature flower bud)		
	Prajwal	Vaibhav	Mean	Prajwal	Vaibhav	Mean
P_1	8.33	6.67	7.5	6.67	5.00	5.83
P_2	63.33	61.67	62.50	70.00	63.33	66.66
P_3	70.00	55.00	62.50	66.67	61.67	64.17
P_4	88.33	86.67	87.50	91.67	90.00	90.83
Mean	57.50	52.50	55.00	58.75	55.00	56.89
CD at 5%	Treatment = 3.66 Cultivar= 2.57 Treatment x Cultivar = 5.13		Treatment = 3.74 Cultivar = 2.67 Treatment x Cultivar = 5.28			

P_1= Control (Distilled water) for 1 h; P_2= Carbendazim (0.1%) + 8-HQC (200 mg l^{-1}) for 1 h; P_3= Mancozeb (0.1%) + 8-HQC (200 mg l^{-1}) for 1 h; P_4= Carbendazim (0.1%) + Mancozeb (0.1%) + 8-HQC (200 mg l^{-1}) for 2 h.

better way to get aseptic culture than the cultivar Vaibhav.

As the explants were obtained from above and underground parts from field grown plants which harbour microbes, decontamination was a difficult task and therefore had to resort to harsh treatment. The conventional disinfection method (Bapat and Narayanaswamy, 1976; Hassey, 1976) had to be adopted to reduce the level of contamination present on the tissue. Pre-treatment efficiency depends upon the nature of fungi or bacteria infecting the stock plant part which is excised.

Pre-treatments are known to improve *in vitro* culture establishment in different plant species (George, 1993). Earlier, Shield *et al*., (1984) suggested the use of different chemicals in combination in controlling microbial infections in tobacco cultures. It is well established fact that bactericide along with a fungicide have great efficiency in controlling systematic infections. Furthermore, it has been proposed that these compounds should be used at non-phytotoxic levels to obtain the desired results.

Surface sterilants, their levels and durations of exposure is known to influence the *in vitro* culture establishment. Further, combinations of sterilants are also known to influence the culture establishment. Results obtained in the present study advocate that exposure of explants to $HgCl_2$ (0.1%) and NaOCl (1.0%) for 8 min was best for surface sterilization, which gave maximum culture survival i.e. 88.30% and 87.45% with bulb scales and bud explants, respectively (Table 3). The flower based explants were also have showed better response to the same surface sterilant treatment combination and for same time duration. From our study, we have obtained 92.40 per cent of aseptic culture in case of petal segment explant and 90.83 per cent with respect to immature flower bud explant after surface sterilization treatment (Table 4). We have also observed the cultivar variation with respect to treatment combinations with all the explants used.

Table 3. Effect of surface sterilization treatments on culture establishment in tuberose cultivars using bulb scale and bud explants

Treatment	Per cent survival (bulb scale)			Per cent survival (bud)		
	Prajwal	Vaibhav	Mean	Prajwal	Vaibhav	Mean
S_1	11.70	6.70	9.16	8.33	6.70	7.51
S_2	66.60	63.30	64.95	65.00	40.00	52.50
S_3	86.60	83.30	84.95	80.00	66.60	73.30
S_4	91.60	85.00	88.30	88.30	86.60	87.45
Mean	64.12	59.57	61.81	60.40	49.97	55.2
CD at 5%	Treatment = 1.43		Treatment = 5.61			
	Cultivar = 0.98		Cultivar = 4.11			
	Treatment x Cultivar = 2.61		Treatment x Cultivar = 9.83			

S_1= Control (Distilled water) 15 min; S_2= $HgCl_2$ (0.1%) 4 min + NaOCl (1.0%) 6 min; S_3= $HgCl_2$ (0.1%) 6 min + NaOCl (1.0%) 8 min; S_4= $HgCl_2$ (0.1%) 8 min + NaOCl (1.0%) 8 min.

Table 4. Effect of surface sterilization treatments on culture establishment in tuberose cultivars using petal segment and immature flower bud explants

Treatment	Per cent survival (Petal segment)			Per cent survival (Immature flower bud)		
	Prajwal	Vaibhav	Mean	Prajwal	Vaibhav	Mean
S_1	8.33	10.00	9.17	18.33	8.3	13.33
S_2	73.33	70.00	71.67	65.0	63.33	64.12
S_3	81.67	76.67	79.17	88.33	86.67	87.50
S_4	93.33	91.67	92.50	91.67	90.00	90.83
Mean	64.17	62.09	63.13	65.83	49.97	63.95
CD at 5%	Treatment = 4.11		Treatment = 3.55			
	Cultivar = 2.19		Cultivar = 2.53			
	Treatment x Cultivar = 5.82		Treatment x Cultivar = 5.05			

S_1= Control (Distilled water) 15 min; S_2= $HgCl_2$ (0.1%) 4 min + NaOCl (1.0%) 6 min; S_3= $HgCl_2$ (0.1%) 6 min + NaOCl (1.0%) 8 min; S_4= $HgCl_2$ (0.1%) 8 min + NaOCl (1.0%) 8 min.

Uses of mercuric chloride and sodium hypochlorite have been reported for effective surface sterilization of explants in tuberose and other bulbous crops. However, duration of application varied with cultivars, (Mishra *et al*., 2005; Bora and Paswan, 2003; Dilta *et al*., 2000). In the present study, combination application of sterilants proved to be more effective. Mercuric chloride has been one of the most commonly used sterilent by George and Shrrington (1984), while sodium hypochlorite is also efficient for different plant species. The phytotoxicity of sterilant usually depends upon the nature of the tissue, age and type of plant part. Mercuric chlorite has been one of the mostly used sterilant (George, 1993) while sodium hypochloride is also efficient for different plant species. The bactericidal action of NaOCl is due to HOCl and OCl^- ions, which are greatly effective being less phyto-toxic. Earlier, efficiency of dual sterilant has been suggested by Mishra *et al*., (2005) in tuberose, while Gupta and Durzon (1985) and Hennerlg *et al*., (1988) found its effectiveness in woody plant tissue culture. Effective decontamination of geophytic storage organ as explant through sterilization with sodium hypochlorite and mercuric chloride was one of the best combination in bulbous plants (Debergh and Maene, 1981).

With this study, it was concluded that the under ground excised explants could harbour more number of microbes than the above ground excised explants. With respect to two cultivars used Single type cultivar Prajwal was more responsive than the Double type cultivar Vaibhav which may be attributed to the effect of genotypes.

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