The Pharma Innovation Journal 2023; 12(1): 2691-2695

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(1): 2691-2695
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www.thepharmajournal.com
Received: 14-10-2022
Accepted: 24-11-2022

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Establishment and Intiation of axenic cultures using axillary buds in tuberose

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Abstract
Success of a commercially viable protocol for mass multiplication of plants using tissue culture begins with effective elimination of microbial contamination by pre-treatment and surface sterilization methods. In the present study, for development of axenic culture from axillary buds of tuberose. Among the various pre-treatment combinations tested, minimal microbial contamination and maximum survival percentage was found when the explants were treated with a combination of Carbendazim 0.1% + Mancozeb 0.1% + Streptomycin 100 ppm + Teepol 1% for overnight. Surface sterilization was achieved at the best by treating the pre-treated explants with NaOCl 4.0% (15 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (10 min.). Among the various treatments tested for culture establishment, the results were recorded when the explants were placed in MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA.

Keywords: Axillary buds, Tuberose, Pre-treatment, Surface sterilization, Culture intiation

Introduction
The tuberose (*Polianthes tuberosa*) is a perennial plant of the agave family Agavaceae, extracts of which are used as a middle note in perfumery (Edwards, 2006) [4]. The common name is derived from the Latin *tuberosa*, meaning swollen or tuberous in reference to its root system. It consists of about 12 species. Polianthes means "grey flower". The tuberose is a night-blooming plant thought to be native to Mexico along with every other species of *Polianthes*. It is a prominent plant in Indian culture and mythology (Sangavai and Chellapandi, 2008) [17]. The flowers are used in wedding ceremonies, garlands, decoration and various traditional rituals. The oil extracted from the flower is used as a perfume. Its scent is described as complex, exotic, sweet and floral. The tuberose grows in elongated spikes up to 45 cm (18 inch) long, and produces clusters of fragrant waxy white flowers that bloom from the bottom towards the top of the spike. It has long, bright green leaves clustered at the base of the plant and smaller, clasping leaves along the stem (Hutchinson *et al*., 2004) [7]. It thrives in sunny spots and bloom in late summer and its tall stems and rather sparse, grass-like foliage make them ideal for inter-planting (Nagar, 1995). In India, the commercial cultivation of tuberose is confined mainly to West Bengal, Tamil Nadu, Karnataka, Andhra Pradesh, Maharastra, Assam, Chattisgarh, Madhya Pradesh, Haryana and Uttarakhand. As the tuberose in particular in Andhra Pradesh it flourishes over an area of 1355 hectares. However there are several limitations like low multiplication rate, large quantity of propagation material is required, To meet the growing demand, of quality planting material of tuberose, massive *in vitro* propagation through tissue culture is the only option. *In vitro* plant regeneration of tuberose is an unique technique for production of new cultivars with commercial traits such as novel flower color, change plant architecture and fragrance (Datta, 2017) [3]. Even if, a few studies have been conducted in some laboratories in India and abroad to obtain prolific *in vitro* culture system of tuberose with limiting regeneration potential, no work has been done so far in Andhra Pradesh on these aspects. In view of this, an experiment was conducted on tuberose plant regeneration through *in vitro* culture of different explants.

Materials and Methods
Plant material collection
The explants used in the present study were axillary bud. Explants of the cultivar Prajwal were collected from the mother block at Horticulture Research Station, Kovvur, West Godavari district.

Explant selection and sterilization

Tuberose axillary bud explants were collected from healthy mother plants and were thoroughly washed under running tap water for 10-15 minutes. Axillary buds are collected from the bulb and bulblets removed from soil and washed under running tap water later top layer of scales, dry tunic and roots are removed by cutting with cutter which is sterilized by wiping with 70% ethanol. Later they were pre-treated and surface sterilized with different treatment combinations accordingly.

Sterilized different explants, namely, axillary bud were taken inside the laminar air flow chamber. Axillary bud as explant, outer scales were separated, removed and fresh cuts were made on top and bottom of bulb using forceps and scalpels on autoclaved papers and inoculated carefully in culture bottles.. During the inoculation, the cap of the culture vessel was removed and mouth of the culture vessel was flamed over the spirit lamp in the chamber. The sterilized and trimmed explants were quickly transferred to the culture medium by gently pressing with the sterilized forceps to secure their firm contact with the media and again the rim of the culture vessel were flamed and tightly capped and wrapped with polyethylene film.

After each treatment, observations on nature of microbial contamination, percent fungal contamination, percent bacterial contamination, percent survival, mortality of explants due to chemical toxicity were recorded replication wise after 20 days of culture initiation.

Statistical analysis

The data collected for all characteristics involved in the study were subjected to statistical analysis through OPSTAT package as per the design adopted in corresponding experiments. The analysis of variance (ANOVA) was performed as per the outlines described by Panse and Sukhatme (1985) [15]. Statistical significance was tested by employing 'F' table value at 5 per cent level of significance. The critical difference value at 5 per cent level of significance was calculated wherever the differences among the treatment means were found significant. The appropriate standard errors of mean (SE m±) were calculated in each case.

Results and Discussions

Standardization of different pre-treatment methods by using axillary buds as explants in tuberose

The data from table 1 showed that among different chemical pre-treatments the minimum fungal contamination (9.50%), bacterial contamination (11.85%), total contamination (21.35%) percentage was obtained in explants overnight treated with carbendazim 0.1% + mancozeb 0.1% + streptomycin 100 ppm + teepol 1% (P_4). While, maximum fungal contamination (63.28%), bacterial contamination (35.71%), total contamination (98.19%) percentage was recorded under control (distilled water wash) (P_0). Thereby, the maximum (78.65%) explant survival percentage, with hihest (74.47) culture establishment index was recorded with carbendazim 0.1% + mancozeb 0.1% + streptomycin 100 ppm + teepol 1% (P_4) for overnight followed by carbendazim 0.1% + mancozeb 0.1% + streptomycin 100 ppm + teepol 1% (P_3) for 5 h (64.32%) and minimum (1.81%) explant survival percentage was recorded in control (distilled water wash) (P_0) with minimum (1.22)culture establishment index.

In the present experiment (table-1), it was revealed that pretreatment of axillary bud explants of tuberose cv. Prajwal with carbendazim 0.1% + mancozeb 0.1% + streptomycin 100 ppm + teepol 1% for overnight effectively controlled the total contamination. It was reported that decontamination of explants of underground parts is a difficult task. While following different procedures including hot water treatment of bulbs, high concentration of decontaminates or by using fungicide containing media for longer exposure period may reduce the contamination problem. Similar findings were observed with combination of bavistin 1000 ppm + kavach 500 ppm + cetrimide 500 ppm for overnight was found to effective in controlling the contamination was reported in tuberose (Krishnamurthy *et al.* 2001) [13]. While, the axillary bud explants treated with 0.3% bavistin along with 0.4% 8-HQC for three hours reported the minimum microbial contamination in tuberose (Surendranath *et al.* 2015) [18]. Similar findings were also recorded by Raghuvanshi *et al.* (2013) [16] in tuberose cv. Mexican single and Kanchana *et al.* (2019) [11] in tuberose cultivars. Long-term chemical treatments can be effective in reducing microbial contamination, but they can also be harmful to explants and have a negative impact on *in vitro* survival rates (Verma *et al.*, 2012) [9]. Dosage of chemical and duration of exposure of explant depend on the nature, type and tenderness of explant. All these factors might be leading to different results in observations. Axillary bud explants in tuberose, which may have had more endogenous microbial contamination than other explants due to their collection from the underneath the soil, were used in the current experiment. For the purpose of creating *in vitro* axenic cultures and reducing microbial contamination, treating these explants for an extended period of time without causing tissue damage is quite beneficial.

Table 1: Effect of different pre-treatment methods on development of axenic culture in axillary bud cultures of tuberose cv. Prajwal.

Treatments	Fungal contamination (%)	Bacterial contamination (%)	Total Contamination (%)	Explant survival (%)	Culture establishment index
P_0-Control (distilled water wash)	63.28 (52.68)	34.91 (36.19)	98.19 (83.42)	1.81 (7.72)	1.22
P_1-Carbandazim 0.2% + Mancozeb 0.2% + Streptomycin 200 ppm + Teepol 1% – 3 h	38.06 (38.06)	30.95 (33.78)	69.01 (56.19)	30.98 (33.80)	20.19
P_2-Carbandazim 0.2% + Mancozeb 0.2% + Streptomycin 200 ppm + Teepol 1% – 4 h	26.14 (30.71)	23.78 (29.16)	49.92 (44.93)	50.08 (45.02)	37.31
P_3-Carbandazim 0.2% + Mancozeb 0.2% + Streptomycin 200 ppm + Teepol 1% – 5 h	19.13 (25.92)	16.64 (24.04)	35.77 (36.71)	64.32 (53.30)	52.35
P_4- Carbandazim 0.1% + Mancozeb 0.1% + Streptomycin 100 ppm + Teepol 1% – over night	9.50 (17.88)	11.85 (20.08)	21.35 (27.47)	78.65 (62.48)	74.47
Mean	31.22 (33.05)	23.62 (28.65)	54.84 (49.74)	45.16 (40.46)	**37.10**
SE m±	1.20	1.02	1.43	0.68	0.56
CD at 5%	3.67	3.10	4.36	2.07	1.72

Standardization of different surface sterilization methods in tuberose cv. Prajwal using axillary bud as explants

Explants collected from field grown beneath the soil contain variety of microbes that need to be eliminated by following suitable sterilization protocols before inoculation in culture media. present data from table 2 showed that, among different surface sterilant combinations and durations used in treatments (S_6) NaOCl 4.0% (15min) + 75% ethanol (1min) + $HgCl_2$ 0.1% (10 min) recorded the effective performance with minimum fungal (4.50%), bacterial (7.03%), total contamination (11.53%), highest explant survival (79.02%) and maximum culture establishment index (118.04). The explants mortality other than microbial contamination was noticed high under axillary bud explant treated with $HgCl_2$ 0.1% (10 min) + 75% ethanol (1min) + $HgCl_2$ 0.1% (6 min) (S_9) than in control treatment. This might be due to longer exposure of axillary bud to $HgCl_2$ may have negative effects on the explants, as the cut portion ends are the entry points for active compounds, so the long period of exposure to $HgCl_2$ had led to browning and death of the tissue in explants (Emoghene *et al.* 2020) [5]. Other reason may be due to more fungal, bacterial contamination may lead to high mortality and low survival rate of cultures.

The use of $HgCl_2$ and NaOCl combinations are found effective as sterilants as demonstrated by Bisen and Tiwari (2006) [2] in case of guava axillary buds. The utilization of $HgCl_2$as an ideal surface sterilant for underground plant parts was strongly justified by various workers such as Hussain (1975) [6] in gladiolus, Krishnamurthy (2000) [12] in tuberose, Babu *et al.* (2004) [1] in ginger, Kadam *et al.* (2010) [8], Surendranath *et al.* (2015) [18] and Kanchana *et al.* (2019) [11] in tuberos.

Table 2: Effect of different surface sterilization methods on development of axenic culture in axillary bud cultures of tuberose cv. Prajwal.

Treatments	Fungal contamination (%)	Bacterial contamination (%)	Total Contamination (%)	Mortality other than microbial contamination (%)	Explant survival (%)	Culture establishment index
S_0-Control (Distilled water wash)	50.33 (45.17)	47.77 (43.70)	98.10 (83.95)	0 (0.00)	1.90 (7.91)	4.56
S_1-$HgCl_2$ 0.1% (6 min.) + 75% ethanol (1min.)	40.00 (39.21)	42.15 (40.46)	82.15 (64.98)	0 (0.00)	17.85 (24.85)	35.70
S_2 -$HgCl_2$ 0.1% (8 min.) + 75% ethanol (1min.)	31.45 (34.09)	38.50 (38.33)	69.95 (56.74)	5.33 (13.34)	24.72 (29.80)	49.44
S_3-$HgCl_2$ 0.1% (10 min.) + 75% ethanol (1min.)	26.72 (31.11)	29.11 (32.64)	55.83 (48.32)	12.45 (20.65)	31.72 (34.26)	63.49
S_4-NaOCl 4.0% (15 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (6 min.)	15.54 (23.16)	20.00 (26.55)	35.54 (36.57)	0 (0.00)	64.46 (53.38)	88.92
S_5-NaOCl 4.0% (15 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (8 min.)	5.00 (12.92)	9.11 (17.55)	14.11 (22.05)	6.87 (15.18)	79.02 (62.71)	118.04
S_6-NaOCl 4.0% (15 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (10 min.)	4.50 (12.24)	7.03 (15.36)	11.53 (19.84)	16.73 (24.13)	71.74 (57.67)	104.35
S_7-HgCl20.1% (6 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (6 min.)	24.66 (29.76)	26.54 (30.99)	51.20 (45.66)	16.50 (23.95)	32.30 (34.62)	64.60
S_8-$HgCl_2$ 0.1% (8 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (6 min.)	20.11 (26.63)	18.64 (25.57)	38.75 (38.48)	23.56 (29.02)	37.69 (37.85)	75.38
S_9-$HgCl_2$ 0.1% (10 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (6 min.)	12.37 (20.58)	14.81 (22.62)	27.18 (31.41)	35.62 (36.63)	37.20 (37.56)	74.42
Mean	23.06 (27.49)	25.36 (29.37)	48.43 (44.80)	11.70 (16.29)	39.86 (38.06)	67.89
SE m±	0.48	0.49	0.75	0.21	0.46	1.03
CD at 5%	1.43	1.47	2.23	0.64	1.38	3.06

Standardization of culture initiation in tuberose cv. Prajwal using axillary bud

In the present study (table-3), the culture initiation was effectively found when axillary bud explants of tuberose cv. Prajwal were kept under MS media supplemented with 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA (T_3) recorded the maximum culture establishment (80.34%), lesser number of days to shoot emergence (3.20), more number of shoots per explant (3.52) and highest culture establishment index (257.08), while the least culture establishment (9.56%), maximum number of days to shoot emergence (12.67), minimum number of shoots per explant (1.20) and lowest culture establishment index (11.47) was noticed in control (MS media devoid of growth regulators) (T_1)

From these observations it was found that BAP at lower concentrations can be effective in inducing shoots and culture establishment which showed the positive correlation between the observations but further increase in BAP concentration lead to decrease in culture establishment in tuberose. This might be due to the antagonistic effect of higher levels of BAP on the explants or by the presence of endogenous cytokinins in axillary bud cultures or sensitivity of axillary bud cultures to higher levels of cytokinin in tuberose were reported by Mishra *et al.* (2006) [14], Kadam *et al.* (2009) [9] and Kanchana *et al.* (2019) [11].

Table 3: Effect of cytokinin concentration on per cent culture establishment and number of days for shoot emergency, number of shoots and culture establishment index in axillary bud cultures of tuberose cv. Prajwal

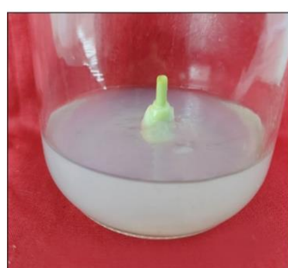
Treatments	Percent culture establishment (%)	Number of days for shoot emergence	Number of shoots per explant	Culture Establishment Index
T_1-Control (MS Media devoid of growth regulators)	9.56 (17.95)	12.67	1.20	11.47
T_2-MS + 1.0 mg L^{-1} BAP + 0.1 mg L^{-1}NAA	55.13 (47.93)	6.03	2.21	164.74
T_3-MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	80.34 (63.67)	3.20	3.52	257.08
T_4-MS + 3.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	75.52 (60.34)	4.00	2.60	196.35
Mean	55.13 (47.47)	6.475	2.38	157.41
SE m±	1.15	0.60	0.51	0.95
CD at 5%	3.61	1.88	1.58	2.96

Culture Inoculation

Fugal Contamination

Bacterial Contamination

Microbial Contamination



Culture Establishment

Conclusion

The present study advocated that amon various pretreatment suface sterilents used for establishment of axenic culture, combination of Carbendazim 0.1% + Mancozeb 0.1% + Streptomycin 100 ppm + Teepol 1% for overnight followed by surface sterilization with NaOCl 4.0% (15 min.) + 75% ethanol (1min.) + $HgCl_2$ 0.1% (10 min.) have performed better. Among the various treatments tested for culture establishment, the best results were recorded when the explants were placed in MS + 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA.

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