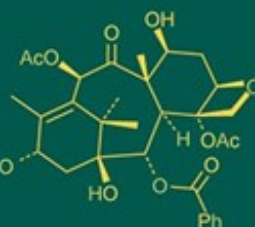
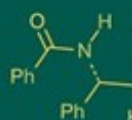


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Effect of pre-treatments on *in vitro* culture establishment of purple fleshed greater yam (*Dioscorea alata* var. *Purpurea*)

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ABSTRACT

Purple-fleshed greater yam (*Dioscorea alata* var. *purpurea*) is an important tuber crop valued for its nutritional, culinary and potential medicinal properties. Conventional propagation through tubers is relatively slow and may contribute to the accumulation and transmission of pathogens from the mother plant to the planting material. Micropropagation provides an efficient approach for rapid multiplication and production of healthy, uniform and disease-free planting material. However, the establishment of aseptic cultures in greater yam is considerably constrained by severe endogenous fungal and bacterial contamination associated with the explants. Therefore, the present investigation was undertaken to standardize an effective chemical pre-treatment protocol for reducing microbial contamination and improving the survival and establishment of nodal explants of greater yam. The experiment was conducted using nodal segments as explants and evaluated different combinations of fungicidal and antibacterial pre-treatments at different time durations. The pre-treatment consisted of Carbendazim + Mancozeb (0.1%), Streptocycline sulphate (200 ppm) and Tween-20 (2 drops/100 ml), applied for 30, 60, 90 and 120 minutes and a control. Among the treatments evaluated, Carbendazim + Mancozeb (0.1%) + Streptocycline sulphate (200 ppm) + Tween-20 (2 drops/100 ml) for 90 minutes recorded the highest explant survival of 75.00% and maximum culture establishment index of 83.33%, along with relatively lower fungal (14.64%) and bacterial contamination (10.36%). The same chemical combination applied for 120 minutes showed comparatively lower fungal (12.43%) and bacterial contamination (6.20%), but resulted in reduced explant survival (52.24%) and culture establishment index (69.86%), indicating that prolonged exposure although effective in suppressing microbial contamination, adversely affected explant viability. Among the different treatment durations, the 90-minute pre-treatment was identified as the most effective treatment for achieving a balance between microbial control, explant survival and successful culture establishment. No phenolic exudation was observed in any of the treatments. The results demonstrate that appropriate optimization of chemical pre-treatment duration is essential for successful *in vitro* establishment of *D. alata* var. *purpurea* nodal explants and provide a useful protocol for subsequent micropropagation and rapid multiplication of this valuable yam genotype.

Keywords: Micropropagation, Dioscoreaceae, Nodal segments, Axenic culture

Introduction

Dioscorea alata L. commonly known as greater yam, water yam or winged yam, is an important tropical and subtropical tuber crop belonging to the family Dioscoreaceae and is cultivated primarily for its large underground starchy tubers (Coursey, 1967) [1]. The species is one of the major cultivated members of the genus *Dioscorea* and has a broad pantropical distribution extending across Asia, Africa, the Pacific, the Caribbean and tropical America (Lebot *et al.*, 1998; Malapa *et al.*, 2005) [2, 3]. *Dioscorea alata* is a perennial, herbaceous and dioecious climbing plant characterized by vigorous twining stems and underground storage tubers that show extensive variation in size, shape, skin colour and flesh colour among cultivars (Lebot *et al.*, 1998; Abraham and Nair 1991) [2, 4]. Greater yam possesses considerable genetic and phenotypic variability, which is expressed through differences in tuber morphology, aerial tuber production, pigmentation, maturity, agronomic performance and biochemical composition (Girma *et al.*, 2017) [5]. The species consequently includes diploid, triploid and tetraploid cytotypes with chromosome numbers of $2n = 40, 60$ and 80 , respectively (Nemorin *et al.*, 2013; Arnau *et al.*, 2009) [7, 6].

Vegetable tuber crops represent an essential source of dietary starch, minerals, and bioactive compounds for global food security (Coursey, 1967) [1]. Among root vegetables, greater yam (*Dioscorea alata* L.) stands out due to its high yield potential and nutritional value, with specific purple-fleshed cultivars synthesizing deep purple pigments throughout their tuber tissues (Abdullah, 2020) [8]. The purple pigmentation of yam tubers

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is primarily associated with anthocyanins, which constitute an important class of water-soluble flavonoid pigments with recognized antioxidant properties (Champagne *et al.*, 2011) [9]. These purple pigments are primarily acylated cyanidin-based anthocyanins with high free-radical scavenging capacity that exert significant anti-inflammatory, anticancer, and cardioprotective health benefits (He *et al.*, 2010) [10].

Beyond phenolic pigments, *Dioscorea* tuber crops produce high levels of diosgenin, a major steroidal saponin (Bhandari *et al.*, 2004) [11] that serves as a critical natural precursor in the industrial synthesis of steroid hormones and oral contraceptives (Patel *et al.*, 2012) [12]. *Dioscorea alata* is predominantly propagated vegetatively through whole tubers, tuber pieces and other vegetative planting materials, because clonal propagation permits the maintenance of desirable cultivar characteristics (Lebot *et al.*, 1998; Vandenbroucke *et al.*, 2016) [2, 13]. However, traditional vegetative propagation using seed tubers yields low multiplication rates (Ezeh *et al.*, 2014) [14], and tuber-borne pathogens accumulate over successive field generations, leading to severe yield decline in yam crops (Ospina *et al.*, 2015; Acedo, 2003) [16, 15].

Plant tissue culture techniques provide an efficient platform for rapid clonal propagation and disease eradication in vegetable crops (Borges *et al.*, 2004) [17]. The restricted availability of healthy planting material and the relatively slow multiplication rate have stimulated interest in tissue culture and micropropagation as alternative approaches for the rapid multiplication and conservation of elite *D. alata* cultivars (Acedo, 2003; Rajitha *et al.*, 2011) [15, 18]. Nodal segments have been demonstrated to be suitable explants for *in vitro* multiplication of greater yam, and successful shoot regeneration and rooting have been obtained under appropriate combinations of basal medium, plant growth regulators and activated charcoal (Rajitha *et al.*, 2011) [18]. Nevertheless, initiating sterile *in vitro* cultures from field-grown subterranean yam explants remains a critical challenge (Nicoletti *et al.*, 2020) [19] because exogenous microbial epiphytes and deep-seated endophytes rapidly contaminate growth media during initial culture stages (Leifert *et al.*, 1994) [20]. Fungal pathogens frequently overgrow nodal explants and cause mortality (Webster *et al.*, 2003) [21], while vascular bacterial contaminants proliferate in nutrient media, causing tissue necrosis and culture degradation (Reed *et al.*, 2004) [22]. Furthermore, enzymatic oxidation of phenolic compounds exuded from cut tissues inhibits shoot bud initiation (Ahuja *et al.*, 2002) [23]. Standardizing chemical pre-treatments with systemic fungicides and bactericides is therefore mandatory to eliminate persistent pathogens (Cassells, 2012) [24].

Micropropagation is particularly relevant for purple yam because it permits rapid multiplication of selected genotypes from limited starting material and can contribute to the production of uniform planting material and conservation of valuable germplasm (Acedo, 2003; Belarmino *et al.*, 2008) [15, 25]. However, establishment of aseptic cultures remains a critical step because nodal explants derived from field-grown or greenhouse-grown plants may harbour surface-associated and endogenous microbial contaminants (Acedo, 2003; Rajitha *et al.*, 2011) [15, 18]. Thus, *Dioscorea alata* var. *purpurea* represents a nutritionally and economically valuable purple-fleshed yam genotype with considerable genetic, cytological and biochemical diversity, while its conventional propagation limitations emphasize the need for efficient *in vitro* multiplication systems (Arnaud *et al.*, 2009; Lebot *et al.*, 1998) [6, 2]. Development of a reliable aseptic culture-establishment protocol is therefore an essential prerequisite for subsequent shoot multiplication, conservation and rapid production of healthy planting material of purple-fleshed greater yam (Acedo, 2003; Rajitha *et al.*, 2011) [15, 18]. In this context, the present investigation was undertaken to standardize an effective chemical pre-treatment protocol for reducing microbial contamination and improving the survival and establishment of nodal explants of greater yam.

Materials and Methods

The present experiment was carried out at Tissue culture laboratory, Regional Horticultural Research Station, Dr. YSR Horticultural University, Kovvur, Andhra Pradesh to standardize aseptic culture establishment in *Dioscorea alata* var. *Purpurea*. The experimental site is geographically located at 17.00 N latitude, 81.43 E longitude and altitude of 20 m above MSL.

The experiment was conducted in Complete Random Design (CRD) with four replications. Tubers were raised in pots under aseptic conditions to grow healthy and disease to collect nodal segments of 2-3 cm length as explants for the study. Immediately after collection of the nodal segments (explants), they were washed with distilled water to remove dust. Explants showing no visible mechanical injury were selected for inoculation.

The selected explants were treated with Carbendazim + Mancozeb (0.1%), Streptocycline sulphate 200 ppm and Tween 20 (2 drops/100 ml) for different time durations 30, 60, 90, 120 minutes and they were compared with control distilled water. Following pre-treatment, the explants were rinsed three times with autoclaved distilled water inside a laminar airflow chamber to remove the chemical residues. The pre-treated explants were subsequently subjected to surface sterilization using 0.1% mercuric chloride (HgCl₂) for 5 minutes. After chemical surface sterilization, the explants were washed three times with sterile distilled water to minimize the residual toxic effects of the sterilizing agents.

The thoroughly rinsed explants were then considered ready for establishment under *in vitro* conditions. After completion of sterilization, the explants were placed on sterile tissue paper to remove excess moisture. The dried leaf sheaths and petioles were carefully removed, and a fresh cut was made at the basal end of each explant to expose the healthy internal tissue. The sterilized explants were then inoculated onto the prepared culture medium using sterile forceps.

Each explant was gently pressed against the medium to ensure proper and firm contact with the surface. The inoculated test tubes were closed using polypropylene caps and further sealed with cling film to maintain aseptic conditions. Following evaluation of the most suitable pre-treatment procedure, the explants were cultured on Murashige and Skoog (MS) medium (Murashige *et al.*, 1962) [26] supplemented with sucrose 30g, activated charcoal 1g, BAP (0.75 mg L⁻¹) and NAA (0.5 mg L⁻¹) solidified with agar-agar 8g L⁻¹. Before inoculation of explants in MS media, the pH of the media adjusted to 5.8 before adding the gelling agent and then autoclaved 121°C for 15 minutes. After inoculation, the cultures were maintained in a growth chamber at 26 ± 1 °C under a photoperiod of 16 h light and 8 h dark. Illumination was provided using LED lighting with a light intensity of approximately 3000 lux.

Observations were recorded for the type and incidence of contamination, percentage of fungal and bacterial contamination, percentage of chemical toxicity, percentage of phenolic exudation, percentage of explant survival and culture establishment index. The observations recorded for the different parameters were statistically analysed using OPSTAT according to the experimental design. Analysis of variance (ANOVA) was carried out following the procedures outlined by Panse and Sukhatme (1985) [27].

Results and Discussion

Pre-treatment of nodal segments of purple-fleshed greater yam (*Dioscorea alata* var. *purpurea*) prior to surface sterilization had a significant effect on microbial contamination, chemical toxicity, and subsequent culture establishment (Table 1).

Contamination: Fungal contamination declined progressively with each successive pre-treatment, from 58.33% in the untreated control (P₁, distilled-water wash) to 12.43% in explants treated with Carbendazim + Mancozeb (0.1%), Streptocycline sulphate (200 ppm) and Tween-20 for 120 minutes (P₅). Bacterial contamination followed the same declining trend, from 24.63% (P₁) to 6.20% (P₅). No phenolic exudation was recorded in any treatment (0% throughout).

The progressive fall in fungal and bacterial contamination with increasing pre-treatment duration is consistent with the established antimicrobial efficacy of carbendazim + mancozeb + streptocycline regimens in explant establishment protocols. Carbendazim is a broad-spectrum systemic benzimidazole fungicide that has long been used as a pre-treatment or medium additive to suppress fungal contaminants in tissue culture, and combined carbendazim–mancozeb antifungal formulations have been shown to sharply reduce fungal infection rates in other recalcitrant, contamination-prone in vitro systems (Xing *et al.*, 2025) [28]. Similarly, carbendazim has been reported to control fungal contamination effectively when incorporated into pre-treatment or culture media at working concentrations broadly comparable to those used here (Permadi *et al.*, 2023) [29].

The parallel decline in bacterial contamination can be attributed to the streptocycline sulphate component of the pre-treatment. Antibiotic pre-treatments and antibiotic supplemented media, including streptocycline based combinations, have been used successfully to eliminate persistent surface and endophytic bacterial contaminants in nodal explants based micropropagation of other vegetatively propagated species, where combining streptocycline with a second antibiotic achieved fully contamination-free cultures after initial surface sterilization alone had proved insufficient (Ray *et al.*, 2017) [30]. The Tween-20 included in all pre-treatments in the present study would have further aided contaminant removal by lowering the surface tension of the treatment solution and improving its penetration into the nodal crevices and axillary bud scales where epiphytic and endophytic microflora often persist.

The complete absence of phenolic exudation across all treatments in this study suggests that nodal tissue of *D. alata* var. *purpurea* is comparatively low in oxidisable phenolics relative to other tuber and woody species, where phenolic browning caused by polyphenol oxi-

dase activity is a major constraint on explant survival and typically requires supplementation with antioxidants or adsorbents to control (Permadi *et al.*, 2023) [29].

Chemical toxicity: Chemical toxicity remained at 0% for pre-treatment durations up to 90 minutes (P₁–P₄) but rose sharply to 29.13% at 120 minutes (P₅).

Survival and culture establishment: Survival percentage and the culture establishment index improved progressively with pre-treatment duration, peaking at 90 minutes (P₄) with 75.00% survival and an establishment index of 83.33. Beyond this, at 120 minutes (P₅), both parameters declined to 52.24% survival and a 69.86 establishment index, despite this treatment showing the lowest contamination overall.

The sharp increase in chemical toxicity (29.13%) and the accompanying decline in survival and establishment index at the longest exposure duration (P₅, 120 minutes) reflect a well-documented dose/duration trade-off in chemical pre-treatment of explants. While carbendazim-based fungicides are generally regarded as safe for plant tissue at working concentrations and have even been used as cytokinin-like growth-promoting supplements in some tissue culture systems (Debergh *et al.*, 1993) [31], prolonged or excessive exposure can produce measurable phytotoxic effects on plant tissue, as demonstrated in whole-plant studies where carbendazim application caused weak but detectable phytotoxicity in treated plants (Garcia *et al.*, 2002) [32]. This concentration-and-duration-dependent toxicity explains why the 90-minute pre-treatment (P₄) rather than the 120-minute pre-treatment (P₅) produced the best overall outcome in the present study: it achieved a substantial reduction in contamination (14.64% fungal, 10.36% bacterial) without crossing the phytotoxicity threshold, thereby yielding the highest survival (75.00%) and culture establishment index (83.33) among all treatments.

Table 1. Effects of pre-treatment on establishing axenic culture of purple fleshed greater yam

Treatments	Fungal contamination (%)	Bacterial contamination (%)	Phenol exudation (%)	Chemical toxicity (%)	Survival (%)	Culture establishment index (%)
P1	58.33 (49.80)	24.63 (29.67)	0	0	17.04 (24.16)	25.00 (29.93)
P2	43.64 (41.32)	31.36 (34.03)	0	0	25.00 (29.83)	34.25 (35.78)
P3	32.33 (34.53)	26.00 (30.57)	0	0	41.67 (40.13)	57.92 (49.54)
P4	14.64 (22.46)	10.36 (18.66)	0	0	75.00 (60.52)	83.33 (66.67)
P5	12.43 (20.56)	6.20 (14.40)	0	29.13 (31.20)	52.24 (46.27)	69.86 (56.72)
SE _m ±	2.24 (1.43)	1.51 (1.08)		0.59 (0.51)	4.04 (2.58)	2.69 (2.04)
C.D at 5%	6.80 (4.34)	4.59 (3.27)		1.56 (1.54)	12.30 (8.02)	8.19 (6.22)
CV	13.86 (8.46)	15.30 (8.43)		19.25 (16.20)	19.17 (12.82)	9.96 (8.57)

*Values mentioned in parentheses are angular transformed values

P₁: Control (Distilled water wash)

P₂: Carbendazim + Mancozeb (0.1%), Streptocycline sulphate 200 ppm and Tween 20 (2 drops/100 ml for For 30 minutes

P₃: Carbendazim + Mancozeb (0.1%), Streptocycline sulphate 200 ppm and Tween 20 (2 drops/100 ml for 60 minutes

P₄: Carbendazim + Mancozeb (0.1%), Streptocycline sulphate 200 ppm and Tween 20 (2 drops/100 ml) for 90 minutes

P₅: Carbendazim + Mancozeb (0.1%), Streptocycline sulphate 200 ppm and Tween 20 (2 drops/100 ml) for 120 minutes

This pattern is consistent with reports on other *Dioscorea* species, where nodal segments taken from actively growing vines and subjected to appropriately timed, graded pre-treatment and sterilization

sequences achieved a favourable balance between contamination control and explant viability, whereas overly aggressive sterilant regimens reduced survival despite superior decontamination (Fotso *et al.*, 2013; Das *et al.*, 2013; Mosisa *et al.*, 2014) [34, 33, 35]. Comparable

graded pre-treatment protocols combining fungicides, antibiotics and surfactants have also been optimised in other *Dioscorea* species and related tuber crops specifically to maximise axenic culture establishment while minimising phytotoxic explant loss (Hussein *et al.*, 2019; Bhat *et al.*, 2022) [37, 36].

Conclusion

This study clearly demonstrates that pre-treatment of nodal explants is a critical, controllable variable in establishing axenic *in vitro* cultures of purple-fleshed greater yam (*Dioscorea alata* var. *purpurea*), and that its effectiveness cannot be judged by contamination control alone, explant viability must be weighed equally. Progressive exposure to Carbendazim + Mancozeb (0.1%), Streptocycline sulphate (200 ppm) and Tween-20 steadily reduced both fungal and bacterial contamination with increasing duration, confirming that this fungicide–antibiotic–surfactant combination is well suited to eliminating the surface and endophytic microflora typically associated with field-collected yam nodal tissue. However, the data reveal an important threshold effect: decontamination efficiency and explant safety do not improve in tandem indefinitely. Beyond 90 minutes of exposure, the incremental gain in contamination control was outweighed by a sharp rise in chemical phytotoxicity, which in turn depressed survival and culture establishment. This identifies 90 minutes (P₄) as the practical optimum not simply the treatment with the least contamination, but the one that best balances microbial control against tissue health.

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