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EFFECT OF THIDIAZURON AND 2,4-DICHLORO PHENOXY ACETIC ACID ON CALLUS DEVELOPMENT IN TEA CLONE UPASI 9 TDZ AND 2,4-D ON TEA CALLUS INITIATION

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Abstract– A commonly cultivated tea clone, UPASI 9, is currently propagated through cuttings, an endeavor that has been proved to be environmentally hazardous (due to the use of large quantities of excavated soil) and unprofitable. As an alternative, this study highlights tissue culture as a viable option for the robust and easy regeneration strategy for the tea clone UPASI 9. A study was initiated to establish an *in vitro* culture protocol for *Nilgiris tea (Camellia sinensis*) by standardizing explant sources, disinfestation methods, and the constitution of culture media. Segments were dissected from greenwood stem (current year growth) internodes of field-growntea plants. Disinfestation was achieved by successive treatments of fungicide (bavistin), 0.1% mercuric chloride, and 7.5% $CaCl_2$. MS medium supplemented with sucrose (30 g/l), citric acid (100 mg/l), and ascorbic acid (100 mg/l) was used with combinations of the auxins (2,4-dichlorophenoxyacetic acid; 2,4-D) and cytokinin (Thidiazuron; TDZ). It was found that 1.1 mg of 2,4-D and 1.1 mg of TDZ induced the most callus proliferation. Though equal proportions of TDZ (1.1, 2.2, 3.3 mg) and 2,4-D (1.1, 2.2, 3.3 mg) were effective in inducing callus formation, it was noticed that 1.1 mg each of TDZ and 2,4-D made a major impact by reducing the time required for the callus initiation and proliferation.

INTRODUCTION

Tea (*Camellia sinensis* [L.] O. Kuntze) is an evergreen, perennial, cross-pollinated tree species, and young leaves are processed to prepare a non-alcoholic beverage globally known as 'tea'. Apart from being a stimulant and an antioxidant, tea is valued for its several other health benefits including anti-cancer properties (Mondal *et al*., 2004). Especially green tea has been shown to reduce the frequency of cancers of the mouth, stomach, small intestine, pancreas, lung, breast, skin, urinary bladder, prostate, and esophagus (Vasisht, 2003). Tea polyphenols can inhibit tumor-cell proliferation and induce apoptosis (Lambert and Yang, 2003), while tea catechins are capable of inhibiting angiogenesis and tumor cell invasiveness (Zaveri, 2006). Tea leaves are rich in polysaccharides, essential oils, vitamins, minerals, purines, flavonoids, alkaloids like caffeine, and polyphenols like catechins. Current tea production is not sufficient to meet the demands of the world market as the yield of tea is greatly reduced by several biotic and abiotic stresses. Further, the scope for extending tea cultivation at the expense of important food crops is also largely limited. Thus, tea genetic improvement combining both yield and quality is of utmost importance. At present, the main method

Abbreviations: 2,4-D = 2,4-dichloro phenoxy acetic acid; MS =Murashige and Skoog Medium (1962); TDZ = Thidiazuron (N-phenyl- N'-1,2,3-thiadiazol-5 yl urea); PVPP = polyvinyl polypyrrolidone; $HgCl_2$ = Mercuric chloride; $Ca(OCl)_2$ = Calcium hypo chloride; mg = milligram; mins = Minutes; mg/l= milligram per liter; RH= Relative humidity; PGR= Plant Growth Regulators; g/l= gram per liter.

of breeding tea is through conventional breeding. Though it takes decades, considerable progress has been made in conventional breeding to release desirable tea clones for the Indian production system. However, limitations such as the long growth cycle, self-incompatibility as well as highly selfing recession, make slow progress in the genetic improvement of tea. To accelerate the tea breeding efficiency, it is proposed to employ biotechnology that enables desired changes in Tea in terms of yield and quality. Advances in biotechnology speed up the genetic improvement program by offering several tools such as tissue culture-mediated crop improvement (such as protoplast fusion and embryo rescue of immature hybrids), genetic engineering, genome editing, and micropropagation. Especially, micropropagation ensures high-throughput true-to-type seedling production.

Though conventionally tea is successfully propagated through cuttings over several decades, it is limited by long periods of production, and the prevalence of soil/host-bornpests and diseases, etc., Micropropagation produces rapid, healthy, and authenticated seedlings. Although micro propagation in tea has already been reported (Kato, 1985, Mondal *et al*., 1998), little effort has been done in Nilgiris tea in this direction. There have been other clones created for the Nilgiris region, but UPASI 9 is the most popular among planters due to its adaptability to a variety of environmental conditions, better yield, and higher-quality tea products. UPASI 9 is currently propagated using cuttings, an endeavor that has been proved to be environmentally hazardous (due to the use of large quantities of excavated soil) and unprofitable. For this reason, in this research, we chose tissue culture as a viable option for the robust and easy regeneration strategy for the tea clone UPASI 9.This paper reports the impact ofdifferent concentrations of TDZ and 2,4-D on callus formation in C*amellia sinensis* when nodal and leaf segments are used as explants.

MATERIALS AND METHODS

This experiment was conducted at the Department of Plant Biotechnology, Centre for Plant Biotechnology and Molecular Biology, Tamil Nadu Agriculture University, Coimbatore (Tamil Nadu) India (11.017208078453807, 76.9325308662057). The experimental material was tea genotype UPASI 9 explants which were collected from Horticultural Research Station, Doddabetta, Udhagamandalam (Wood House Farm) (11.4189664952854, 76.72909021819251). Explants with three leaves and two internodes were collected and disinfested the internodes and leaves with a series of baths with agitation on a C1 platform shaker (New Brunswick Scientific classic series, USA). The explant treatment series included: an initial fungicide wash (30 min), 2 times distilled water wash, an Antioxidant wash (Citric acid 100 mg/l + ascorbic acid B˜ 100 mg/l,15 mins), 2 times distilled water wash, Tween 20 wash (5 drops of tween –20, 4 times, 20 washes; each wash was done for 2 mins), 0. 1 N HCl (1 to 2 mins), distilled water wash (2 mins), 0.1% $HgCl_2$ wash (10 mins), 7.5% $CaCl_2$ (10 min), and three final rinses in autoclaved distilled water (2 min each). The surface sterilized explants were then inoculated to MS media fortified with TDZ and 2,4-D (modified from Mondal *et al.*,1998), and the pH of the media was maintained between 5.6-5.8 before autoclaving. Cultures were then stored at 25±2 °C under a photoperiod (light intensity: B˜3000 lux, RH:53%) of 16 hours. Arc sine transformed Data analysis for mean and standard deviation was performed using the General R-shiny based Analysis Platform Empowered by Statistics (GRAPES KAU) and MS Excel.

Fig. 1. UPASI 9 - Mother plant collecting field and explant collected

RESULTS AND DISCUSSION

Effect of different sterilizing Agents on nodal explants

Different sterilizing agents help to understand how they affect the explants. Exudation of phenols from explants was an issue when using $Ca(OCl)_2$ for 15 mins whereas $HgCl_2$ didn't show any browning of explants or phenolic exudation after sterilization. The effect of different sterilization on different concentrations is given in Table 1.

For the sterilization of nodal and leaf explants mercuric chloride ($HgCl_2$) and calcium hypochlorite

Table 1. Effect of different sterilizing Agents on nodal explants

Treatment	Sterilization		Total number of inoculations	Surviving explants	Percentage of surviving nodal explants Mean + SE*
	Agent	Timing			
S1	$HgCl_2$	10mins	80	60	61.76 (75) + 9.05[a]
S2	$Ca(OCl)_2$	15 mins	80	0	0.00 (0) + 0.00[b]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parenthesis

($Ca(OCl)_2$) were used to treat the explants for a period of 10 mins with 0.1% of $HgCl_2$ and 15 mins with 1% $Ca(OCl)_2$, respectively. Treatment S1 performed well in sterilization when compared with S2 (Table 1). After sterilization of the explants, which were inoculated to MS media supplemented with different combinations of PGRs. Fungal contamination was easily seen in the media after one week of inoculation, as the explants were not treated with fungicides (bavistin). This leads to treating the explants with different concentrations of fungicide (bavistin) (Table 2). The sterilization process from the fungicide treatment: bavistin wash to hydrochloric acid wash takes one hour and the addition of sterilizing agents and anhydrous calcium chloride wash was given in an aseptic condition in laminar air flow which takes 30 mins. So, the whole sterilization technique lasts for 1 hour and 30 mins.

Effect of different bavistin concentrations on fungal and phenolic contaminations

For controlling the fungal contamination, the fungicide bavistin was used, the chemical presents in the bavistin is carbendazim which effectively controls the fungal contamination after inoculation. To reduce the fungal contamination, explants were

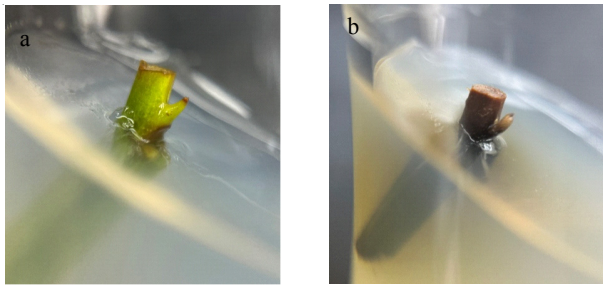


Fig. 2. Effect of sterilizing agent on nodal explants: a) $HgCl_2$, b) $Ca(OCl)_2$

treated with different concentrations of fungicide (Bavistin) for a period of 30 mins (Table 2).

This experiment was conducted with different concentrations of fungicides (0%, 0.2%, 0.25%, 0.3%, 0.4%, 0.6%, 1%). Treatments B4, B5, B6, and B7 performed well in eliminating the fungal contamination from the nodal explants (Table 2). But when the concentration of bavistin increased a sudden increase in phenolic exudation resulted from the explants, which lead to the browning of explants and simultaneously the drying up of explants (Table 3). So, for better controlling and maintaining the live explants, bavistin treatments with 0.2% (B2) and 0.25% (B3) were found to be useful. After a bavistin wash of 30 mins, the plants

Table 2. Effect of bavistin concentrations on fungal contamination of Nodal explants

Treatment	Bavistin		Total number of inoculations	Explants without fungal contamination	Percentage of culture without fungal contamination Mean + SE
	Concentration	Timing			
B1	0%	30 mins	80	54	55.54 (67.5) + 8.1[c]
B2	0.20%		80	75	66.12 (76) + 27.94[bc]
B3	0.25%		80	77	82.16 (96.25) + 9.33[ab]
B4	0.30%		80	80	90.00 (100) + 0.00[a]
B5	0.40%		80	80	90.00 (100) + 0.00[a]
B6	0.60%		80	80	90.00 (100) + 0.00[a]
B7	1%		80	80	90.00 (100) + 0.00[a]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parentheses.

were washed in distilled water two times.

Bavistin-treated nodal explants produced phenolic contamination when compared with the

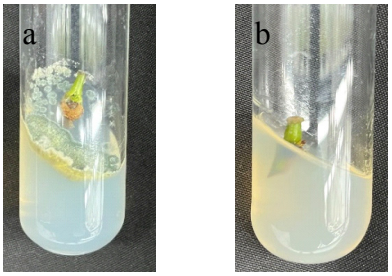


Fig. 3. Fungal contamination: a) explants not treated with fungicide (0%), b) explants treated with fungicide (0.2%)

explants not treated with bavistin (Table 3). The non-treated explants produced fungal contamination as mentioned in Table 2. So, the treatments that are apt for sterilization or to reduce the fungal contamination and phenolic contamination were 0.25% (B3) of bavistin concentration and then 0.2% (B2). Comparing Table 2 and 3 the better treatment to reduce fungal contamination is 0.25% (B3) bavistin. The phenolic contamination can be due to the deposition of catechins in the xylem tissue (Liu *et al*., 2009).

Effect of different antioxidants on phenolic contaminations of nodal explants

Tea is rich in phenolics, the major problem faced during inoculation of nodal explants is the phenolic contamination. Phenolic exudation was seen in explants after the series of sterilization techniques, to eliminate the phenol exudation from the explants after inoculation antioxidants such as citric acid, ascorbic acid, PVPP, and glutamine were used to fortify the MS media (Rana *et al*., 2016) with

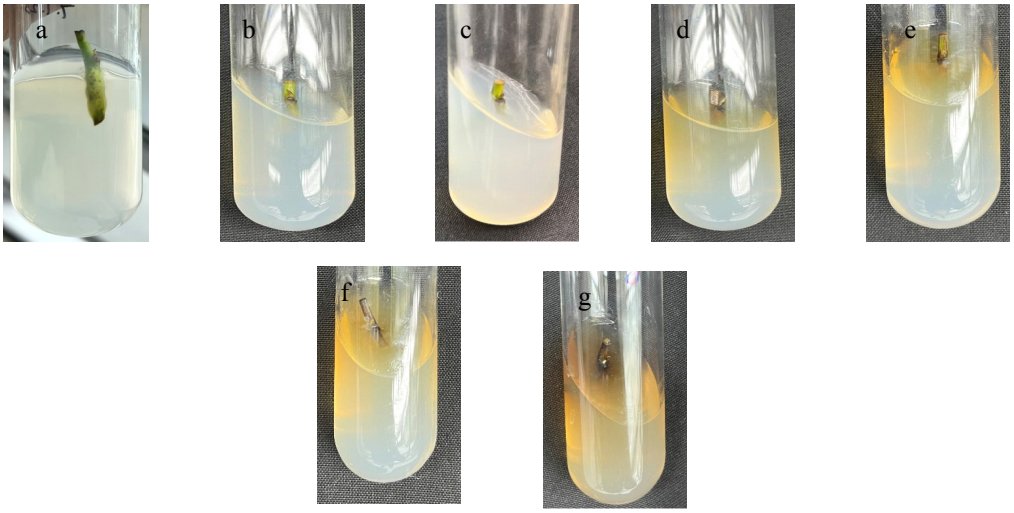


Fig. 4. Effect of different concentrations of bavistin in phenolic exudation from plants: a) 0% b) 0.2% c) 0.25% d) 0.3% e) 0.4% f) 0.6% g) 1% respectively

Table 3. Effect of bavistin concentrations on phenolic contamination of Nodal explants

Treatment	Bavistin		Total number of inoculations	Explants without phenolic contaminations	Percentage of culture without phenolic contamination Mean + SE
	Concentrations	Timing			
B1	0%	30 mins	80	80	90.00 (100) + 0.00[a]
B2	0.20%		80	75	77.84 (93.75) + 9.34[a]
B3	0.25%		80	77	82.16 (96.25) + 9.33[a]
B4	0.30%		80	25	33.22 (31.25) + 11.06[b]
B5	0.40%		80	27	35.05 (33.75) + 9.49[b]
B6	0.60%		80	3	7.83 (3.75) + 9.33[b]
B7	1%		80	13	23.06 (16.25) + 7.38[c]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parentheses.

Table 4. Effect of Antioxidants on phenolic contamination of Nodal Explants

Treatment	Antioxidants	Total number of inoculations	Total number of explants without phenolic contamination	Percentage of cultures without phenolic contamination Mean + SE
P1	Without antioxidants	80	30	37.49 (37.5) + 8.14[d]
P2	100 mg/l of citric acid + 100 mg/l ascorbic acid	80	58	68.02 (85) + 7.75[b]
P3	5g/l of PVPP	80	64	55.75 (67.5) + 9.83[c]
P4	100 mg/l of citric acid + 100 mg/l glutamine	80	80	90.00 (100) + 0.00[a]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parentheses.

different concentrations, and to know which antioxidant favors the initiation of callus.

100 mg/l of citric acid + 100 mg/l glutamine (P4) performed well in reducing the phenolic contamination when compared with the rest of the treatments (Table 4), but the formation of calli was delayed. 100 mg/l of citric acid + 100 mg/l ascorbic acid (P2) controlled the phenolic exudation and the rate of callus formation in this treatment was much higher when compared with the P2 treatment. So, P2 treatment was used to control phenolic contamination.

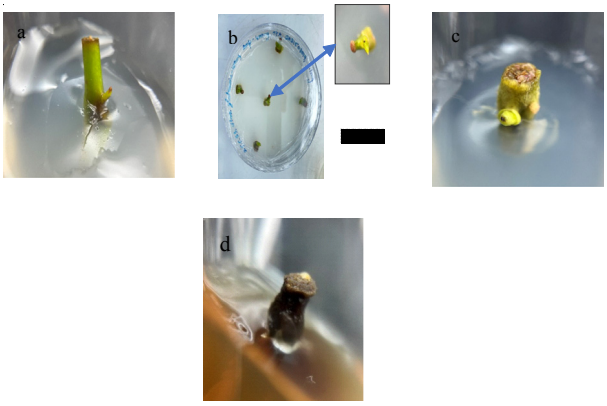


Fig. 5. Explants in MS media fortified with different anti-oxidants a) citric acid and ascorbic acid b) citric acid and glutamine c) PVPP d) Phenolic exudation in MS media without anti-oxidants

Effect of TDZ and 2,4-D combination on callus induction

Among the different combinations of TDZ and 2,4-D used, MS media supplemented with 1.1 mg/l TDZ and 1.1 mg/l 2,4-D (T1) gave a maximum callusing frequency of 81.25%, whereas MS media fortified with 1.1 mg/l TDZ and 2.2 mg/l 2,4-D resulted in poor callus formation with 22.5% (Table 1). T1 treatment was chosen for the regeneration of tea genotype UPASI 9 as the rate of formation of calli is comparatively higher than the rest of the treatment (Data not shown). The inoculated explants were kept in the dark for 3 days and then under illumination (16 hours of light and 8 hours of dark) for the initiation of callus. Inoculated explants were stored at 26 °C and 9 °C. Callus induction was noticed only in the nodal explants kept at 26 °C after 6 days of inoculation.

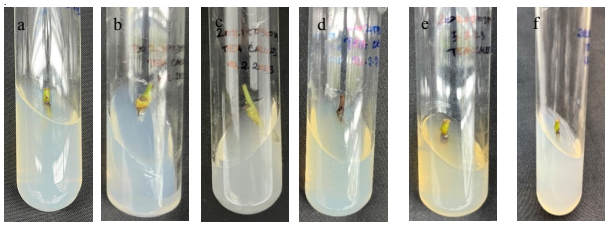


Fig. 6. Nodal explants in MS media supplemented with different PGR concentrations: a) T1 b) T2 c) T3 d) T4 e) T5 f) T6 respectively

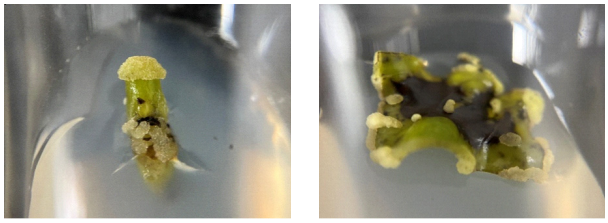


Fig. 7. Callus formation on different explants after 24 days of inoculation

Effect of 1.1mgTDZ and 1.1mg 2,4-D combination on callus induction of differentex plants

Leaf segments and nodal explants were inoculated to 1.1 mg TDZ and 1.1 mg 2,4-D supplemented MS media to know which explant performs better (Table 6).

Table 5. Effect of TDZ combination with 2,4-D on callus induction on Nodal explants

Treatment	MS basal media supplemented with concentrations of PGR's (mg/l)		Total number of inoculations	Total number of primary calli	Callusing frequency (%) (Mean+ SE)
	TDZ	2,4-D			
T1	1.1	1.1	80	65	64.75 (81.25) + 6.26[a]
T2	2.2	2.2	80	43	53.75 (47.16)+ 2.76[a]
T3	3.3	3.3	80	60	60.292 (75) + 6.14[a]
T4	1.1	2.2	80	18	27.613 (22.5) + 9.09[a]
T5	1.1	3.3	80	57	57.928 (71.25) + 7.28[b]
T6	2.2	1.1	80	62	61.953 (77.5) + 5.76[c]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parentheses.

Table 6. Effect of 1.1mg TDZ and 1.1mg 2,4-D on different explants

Explant	Total number of inoculations	Number of regenerations				Total percentage
		6 days after inoculation	12 days after inoculation	18 days after inoculation	24 days after inoculation	
Leaf	80	4	10	14	14	17.5[b]
Stem	80	20	60	65	65	81.25[a]

*Mean and standard deviation are arc sine transformed values of 4 replication with 20 explants per replication. Non-transformed data are given in parentheses.

The effect of 1.1 mg TDZ and 1.1 mg 2,4-D on leaf and stem were noticed with an interval of 6 days. Nodal segments performed well in the MS media fortified with 1.1 mg TDZ and 1.1 mg 2,4-D when compared with leaf explant (Table 6). Leaf cupping was visible in 65% of the leave that was inoculated in the same media but these leaves didn't form callus even after 20 days of inoculation. With this, we concluded that the nodal explants are a good source for the formation of calli in MS media supplemented with 1.1mg of TDZ and 2,4-D. Within a short period, nodal segments could initiate the formation of the callus.

Different stages of callus formation in MS media supplemented with 1.1 mg of TDZ and 1.1 mg of 2,4-D

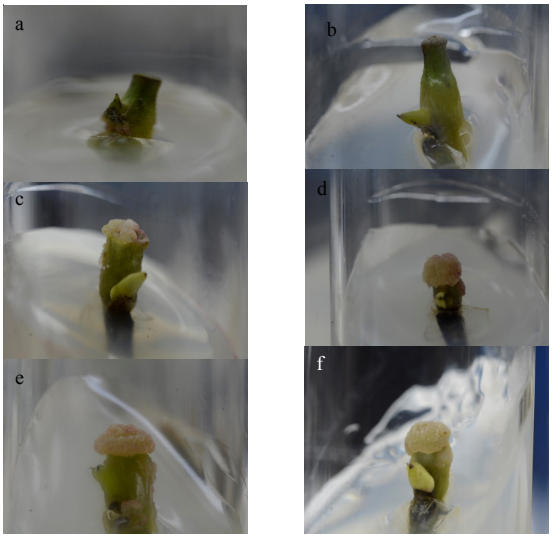


Fig. 8. a) Explant after inoculation to MS media, b) Explant after 6 days of inoculation, c) Explant after 12 days of inoculation, d) Explant after 18 days of inoculation, e) Explant after 24 days of inoculation, f) Explant after 30 days of inoculation

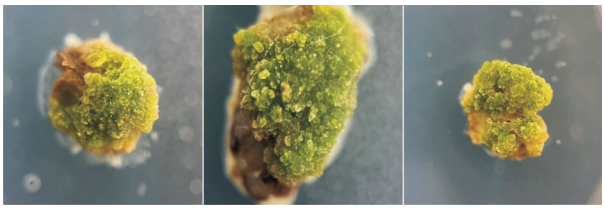
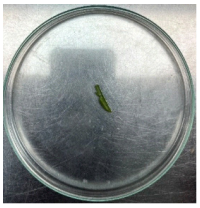


Fig. 9. Callus growth in MS media fortified with TDZ and 2,4-D (1.1mg)

CONCLUSION

This study establishes a protocol for the callus regeneration of the tea clone UPASI 9 from the nodal and leaf segments usingMS media supplemented with TDZ and 2,4-D. From this study, we could understand that the chemical used for sterilization canaffect the explant's callus-inducing ability and this can lead to the deposition of catechins in the



Trimmed explant (1.5-2 cm)

Explants inoculated in MS media fortified with 1.1mg TDZ and 2,4-D each

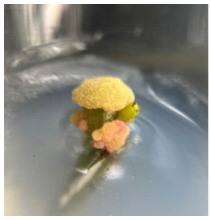
Explant after sterilization (sterilization time:1hour and 30 mins)

Explants kept in the dark for 2 days

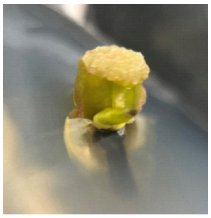
Callus Initiation

Callus grew on MS media fortified with 1.1mg TDZ & 2,4-D each

Explants kept under illumination after keeping it in dark



Explant after 30 days of inoculation



Explant after 18 days of inoculation

Explant after 12 days of inoculation

xylem tissue. Increased concentration of bavistin for the sterilization of the plant resulted in less callus initiation and antioxidant PVPP delayed the rate of callus initiation when compared to other antioxidants used in the experiment these might be due to the deposition of the catechins in the xylem vessels (Liu *et al*., 2009). It was also noticed that for high rates of callus formation, a 1:1 ratio of auxin and cytokinin is necessary.

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Authors' contribution

Conceptualization and designing of the research work (NMB); Execution of lab experiments and data collection (RM); Analysis of data and interpretation (RM, NMB); Preparation of manuscript (RM, NMB, PMS, DKL, MK).

Declaration

The authors declare that they have no potential conflict of interest

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