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Effect of Antioxidants in Controlling Phenol Exudation in Micropropagation of Litchi cv. Purbi

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Conventionally litchi is propagated by vegetative means mainly by air layering or marcottage, grafting and budding. Although for large-scale production of elite litchi clones, micropropagation can be used as a potential alternative to conventional means of propagation. The presence of phenolic compounds causing the death of explants has been one of the significant bottlenecks of litchi micropropagation which causes death of explants after turning brown and these exudates appear as a reaction to injury and/or infection. Litchi cultivar Purbi grown at Horticulture Garden of Bihar Agricultural College, Sabour was selected for the investigation and the required planting material used was Leaf and Nodal segment as a source of the explant. Different antioxidants were used as treatments to control the phenol exudation. The results of the study revealed that all the antioxidants supplemented into the media significantly reduced phenolic exudation for both the explants. However, minimum phenolic exudation (+) was observed for both the explants, when media was supplemented with ascorbic acid 300 mg per litre which also results in maximum per cent shoot regeneration (54.0±0.47) for nodal segments and maximum per cent callus induction (60.9±0.61) for leaf explants after (29.0±0.81 days) and (46.4±0.55 days) of culturing, respectively. Phenolic exudation was recorded highest (++++) under control when no antioxidants were supplemented into the media. Though all other antioxidants used in our study including citric acid, PVP and activated charcoal significantly reduced percent oxidative browning, ascorbic acid was found to be the most effective antioxidant in controlling lethal browning during micropropagation of litchi followed by the combination of ascorbic acid with citric acid (150 mg/l each) in the media for both the explants.

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Keywords: Litchi; micropropagation; phenol; browning; antioxidants.

1. INTRODUCTION

Litchi (*Litchi chinensis* Sonn.), is one of the most popular subtropical fruits belonging to the Sapindaceae or soapberry family and subfamily Nephelae which comprises not less than 150 genera and 2000 species. The fruits of the litchi tree are highly prized for its luscious white arils. Litchi originated between latitudes 23° & 27°N in the regions circumventing Southern China, Northern Vietnam and Malaysia and has now been widely distributed across the tropical and subtropical regions of the world. It was introduced in India at the end of the 17th century. India enjoys a prominent position in the litchi map of the world both regarding production and productivity.

The increasing demand of litchi fruits across the globe has led to an increase in the area under production consequently leading to an increase in the demand for propagation materials. Of the total production of litchi in India, 45% is contributed by Bihar itself. Conventionally litchi is propagated by vegetative means mainly air layering or marcottage, grafting & budding [1]. However, the success rate with air layering is less in many litchi growing areas while grafting technique has yet not been standardized. Although various means to increase the efficiency of these methods have been tried, for example, use of younger branches, small earth balls and rooting hormones like 1,4-indole-3-butyric acid (IBA), the process is still slow and inefficient. Such being the circumstances, micropropagation has emerged as a potential alternative for the large-scale, round the year production of elite litchi clones. Hitherto, litchi, like other woody perennials, is considered to be recalcitrant to *in vitro* propagation methods owing mainly to the secretion of polyphenols by the explants which lead to contamination of media.

Phenolic acids are intermediates of phenylpropanoid metabolism and precursors of lignin and phenylpropanoid phytoalexins. Their deposition in cell walls is an important defence mechanism against pathogen infection. Being an important group of secondary metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid (IAA) catabolism. They are effective in plant growth regulation, cell differentiation and organogenesis [2]. In tissue culture studies, phenolic substances, especially oxidised phenolics generally have an adverse effect on *in vitro* proliferation [3].

Tissue browning and blackening also hinder *in vitro* culture of many economically important plants. When cells are damaged, the contents of cytoplasm and vacuoles are mixed, and phenolic compounds can readily become oxidised by air. Oxidised phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants. The phenolic browning takes place due to the action of copper-containing oxidase enzyme: polyphenoloxidases like tyrosinases, which are released or synthesised in oxidative conditions after tissue wounding and oxidise o-diphenols released due to cellular wounding to o-quinones [4,5].

Different methods have been employed to reduce phenolic oxidation and improve regeneration of explants including use of liquid media, frequent sub-culturing, use of antioxidants (eg. citric acid and ascorbic acid), PVP (polyvinyl pyrrolidone) and activated carbon [6,7]. *In vitro* propagation or micropropagation holds great potential as an efficient propagation technique in litchi to meet the increasing demand of quality planting material free from diseases and pest and needs extensive research to develop protocols that can help in reducing or controlling phenolic oxidation. Of the different methods mentioned above, our study focused on the use of different antioxidants in controlling the *in vitro* phenolic exudation in litchi.

2. MATERIALS AND METHODS

Litchi cultivar Purbi maintained litchi orchard of Horticulture Garden, Bihar Agricultural College, Sabour was selected for this study as a source of the explant. Leaf and Nodal segment obtained from the newly emerged panicles were used a planting material (explant). The culture media included Murashige and Skoog (MS) medium [8] consisting of 0.8% Agar, 3% sucrose and pH 5.8 as the basal medium to which 2.5 mg/l of BAP was added for the nodal segment related study and 2.5 mg/l 2, 4-D was added for leaf related study. The inoculated cultures were incubated at 25±2 °C in an air-conditioned culture room with a light intensity of 2000-3000 lux from cool white fluorescent tubes. The light/dark cycles of photoperiod were maintained at 16/8 hours daily. Young shoot branches were cut from the healthy

plants of selected genotype, and the nodal segments of 1-2 cm length containing axillary bud were cut out using scalpel and forceps. Apart from this the young leaves of 1 cm × 1cm were also collected to be used as explant.

The explants of suitable sizes were washed in running tap water 4-5 times followed by washing in a solution containing 2- 3 drops detergent (tween -20) and 1-2 ml Dettol for about 10 minutes and subsequent washing with sterilised water for 2-3 times. Furthermore, the washed nodal segment and leaf explant were dipped in 0.2% bavistin solution for 50 minutes and 30 minutes respectively to control the fungal contamination.

All the aseptic manipulations such as surface sterilisation of explants, preparation and inoculation of explants and subsequent subculturing were carried out under aseptic conditions in the hood of clean laminar airflow chamber. Sterilisation with 0.2 % $HgCl_2$ for 3 min and 0.1 % $HgCl_2$ for 1 min was given to the nodal segment and leaf explant, respectively under the laminar air flow before inoculation. The main study was carried out to see the effect of anti-oxidants in the media at different concentrations and time on phenolic exudations. The observations recorded included per cent oxidative browning, days taken for shoot proliferation (nodal segment), days are taken for callus induction (leaf), per cent shoot proliferation, per cent callus induction and degree of phenolic exudation. The degree of phenolic exudation and medium staining was recorded as per visual observation on colour intensity (browning) of media and scoring was given as (++++) high browning, (+++) medium browning, (++) low browning, (+) almost no browning.

3. RESULTS AND DISCUSSION

In this study, the explants were put into the media containing varying concentration of antioxidants and the results demonstrated that minimum phenolic exudation (+) was observed in all explants, when media was supplemented with ascorbic acid 300 mg per litre resulting in maximum per cent shoot regeneration (54.0±0.47) (Table 1) and per cent callus induction (60.9±0.61) (Table 2) after (29.0±0.81) and (46.4±0.55) days of culturing, respectively. This was because ascorbic acid (300 mg per litre), when used in media, controlled the per cent oxidative browning in both shoot and leaf cultures to the maximum extent followed by ascorbic acid (450 mg per litre) in the media. The degree of phenolic exudation was recorded highest in control (++++) where no antioxidants were used. Though all the other antioxidants including citric acid, PVP and activated charcoal significantly reduced per cent oxidative browning, ascorbic acid was found to be the most effective antioxidant followed by the treatment combination of ascorbic acid with citric acid (150 mg/l each) in the media for all the explants. These findings are also in consistence with the findings of Pankaj et al. [7]; Das et al. [9]; Amin and Razzaque, [6] and Kantharajah et al. [10]. Moreover, when the concentration of ascorbic acid was increased to 450 mg per litre, the per cent oxidative browning was low but per cent shoots regeneration (Table 1), and per cent callus induction (Table 2) reduced considerably. The per cent shoot regeneration as well as per cent callus induction showed an increment with the decreasing per cent oxidative browning (Graph 1&2). Martinez and Whitaker [11] have reported that the mechanism of ascorbic acid inhibition involves the reduction of quinones generated by PPO. It was also observed that the explants under controlled condition regenerated earlier though, at a very lower frequency than the explants treated with antioxidants, this indicated the inhibitory and deleterious effect of antioxidants on the morphogenesis of the explants when used at higher concentrations.

The results of the present study showed that almost all explants on the medium without antioxidants and those with lower concentration levels of antioxidants exhibited a high level of browning. This may be attributed to the fact that exudation of phenolics is a natural phenomenon in plants after wounding. The accumulation of these compounds leads to browning and possibly death of the explants. Hence, we conclude that phenolic browning, a major bottleneck in litchi micropropagation, can be controlled to a greater extent using antioxidant, ascorbic acid at the rate of 300 mg per litre or a combination of ascorbic acid and citric acid (150 mg/l each) in the culture media.

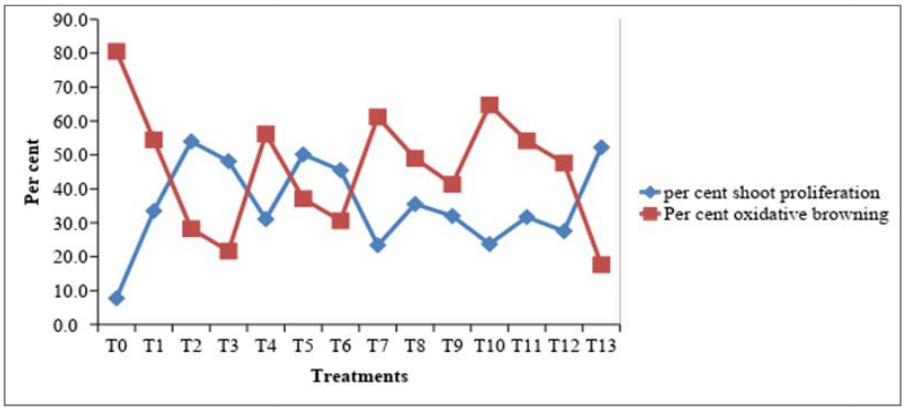
Our findings can be helpful in leading other research works related to the *in vitro* establishment of other woody perennial crops.

Table 1. Effect of antioxidants and its concentration in media to control phenolic exudation (Nodal segment)

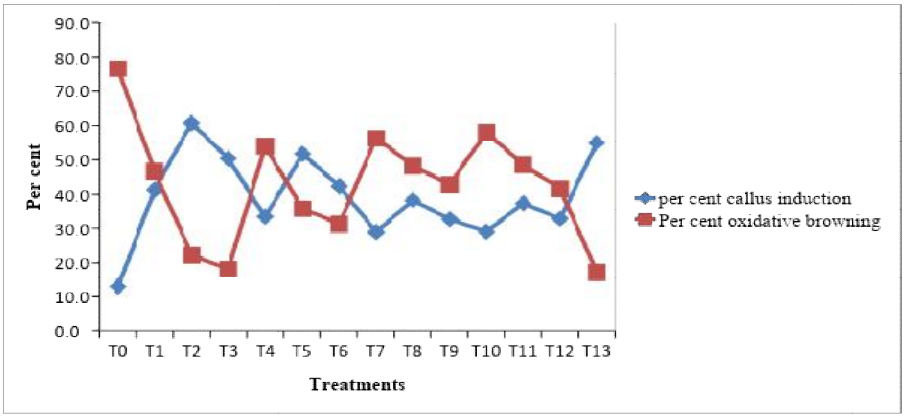
Treatment	Antioxidants	Concentration	Degree of phenolic exudation	Per cent oxidative browning	Days taken for shoot proliferation	Per cent shoot regeneration
T_0	Control	-	++++	97.3 (80.5±0.94)	22.0±0.23	1.8 (7.8±0.36)
T_1	**Ascorbic acid**	150 mg/l	+++	66.2 (54.4±1.21)	26.5±0.72	30.5 (33.5±0.83)
T_2		**300 mg/l**	**+**	**22.5 (28.2±1.54)**	**29.0±0.81**	**65.5 (54.0±0.47)**
T_3		450 mg/l	+	13.7 (21.7±0.70)	31.1±0.87	55.5 (48.1±0.38)
T_4	Citric acid	150 mg/l	+++	69.1 (56.2±0.56)	30.1±0.84	26.7 (31.1±0.25)
T_5		300 mg/l	++	36.4 (37.1±0.25)	33.1±0.92	59.0 (50.2±0.53)
T_6		450 mg/l	+	25.9 (30.6±0.63)	34.4±0.95	50.9 (45.5±0.44)
T_7	PVP	150 mg/l	+++	76.7 (61.2±2.21)	30.1±0.84	15.8 (23.3±1.00)
T_8		300 mg/l	++	57.0 (49.0±1.01)	32.9±0.89	33.8 (35.5±1.64)
T_9		450 mg/l	+	43.6 (41.3±0.54)	33.9±0.92	28.1 (32.0±1.43)
T_{10}	Activated	150 mg/l	+++	81.8 (64.7±0.77)	23.5±0.63	16.2 (23.7±0.41)
T_{11}	charcoal	300 mg/l	+++	65.7 (54.2±1.27)	25.0±0.69	27.6 (31.7±0.57)
T_{12}		450 mg/l	++	54.6 (47.6±0.66)	26.1±0.72	21.4 (27.5±0.49)
T_{13}	Ascorbic acid + Citric acid	150 mg/l + 150 mg/l	+	9.2 (17.6±0.65)	32.3±0.89	62.5 (52.2±1.21)
CD (P=0.05)				3.04	2.33	2.42
SEm (±)				1.04	0.80	0.83

Table 2. Effect of antioxidants and its concentration in media to control phenolic exudation (Leaf explant)

Treatment	Antioxidants	Concentration	Degree of phenolic exudation	Per cent oxidative browning	Days taken for callus induction	Per cent callus induction
T_0	Control	-	++++	94.7 (76.6±0.75)	38.7±0.17	5.0 (12.9±0.38)
T_1	**Ascorbic acid**	150 mg/l	+++	53.0 (46.7±0.98)	46.0±1.97	43.7 (41.3±1.07)
T_2		**300 mg/l**	**+**	**14.5 (22.3±0.87)**	**46.4±0.55**	**76.3 (60.9±0.61)**
T_3		450 mg/l	+	9.9 (18.2±1.80)	49.4±0.60	59.4 (50.4±0.42)
T_4	Citric acid	150 mg/l	+++	65.4 (53.9±1.17)	43.5±0.68	30.4 (33.4±0.29)
T_5		300 mg/l	++	34.3 (35.8±1.06)	44.5±0.68	61.7 (51.7±0.54)
T_6		450 mg/l	+	27.1 (31.3±0.89)	46.6±0.70	45.6 (42.4±0.40)
T_7	PVP	150 mg/l	+++	69.4 (56.4±1.67)	43.4±3.51	23.5 (28.9±1.27)
T_8		300 mg/l	++	55.8 (48.3±0.91)	44.3±3.58	38.6 (38.3±1.83)
T_9		450 mg/l	+	46.4 (42.9±1.71)	45.4±3.67	29.3 (32.7±1.47)
T_{10}	Activated charcoal	150 mg/l	+++	72.1 (58.1±0.78)	42.2±1.37	23.6 (29.0±0.51)
T_{11}		300 mg/l	+++	56.3 (48.6±1.05)	43.2±1.40	37.0 (37.4±0.71)
T_{12}		450 mg/l	++	44.2 (41.6±0.90)	43.9±1.43	29.5 (32.8±0.60)
T_{13}	Ascorbic acid + Citric acid	150 mg/l + 150 mg/l	+	8.9 (17.3±1.14)	52.4±1.67	67.2 (55.0±1.33)
CD (P=0.05)				3.41	5.68	2.74
SEm (±)				1.17	1.95	0.94



Graph 1. Effect of different treatment of antioxidants in media on per cent shoot proliferation & oxidative browning (nodal segment).



Graph 2. Effect of different treatments of antioxidants in media on per cent callus induction & oxidative browning (Leaf explant).

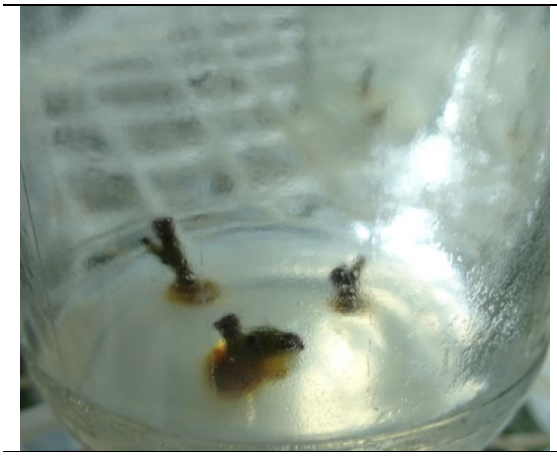
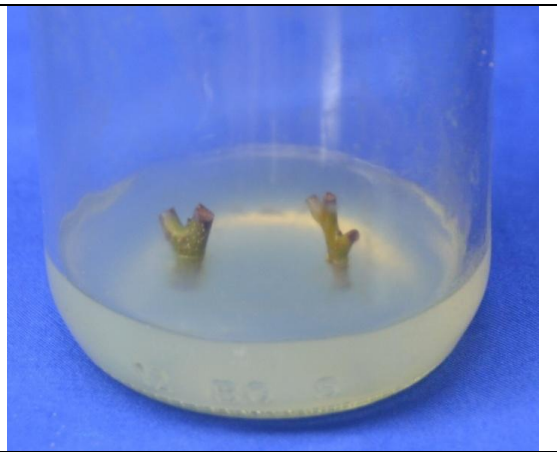
Culture showing phenolic browning	Phenolic browning controlled
	
Control	Ascorbic acid- 300 mg/l

Plate 1. Control of phenolic exudation of nodal explants with antioxidant treatment.

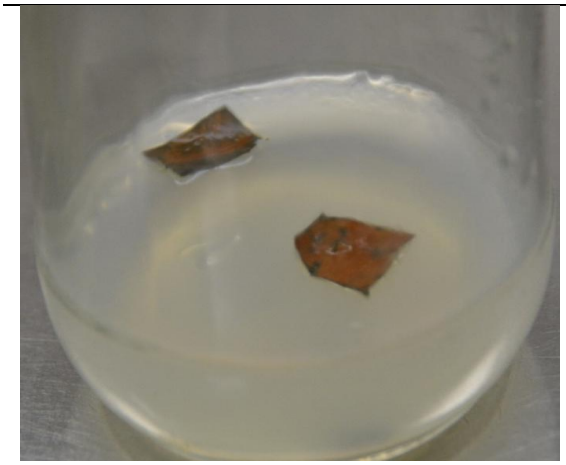
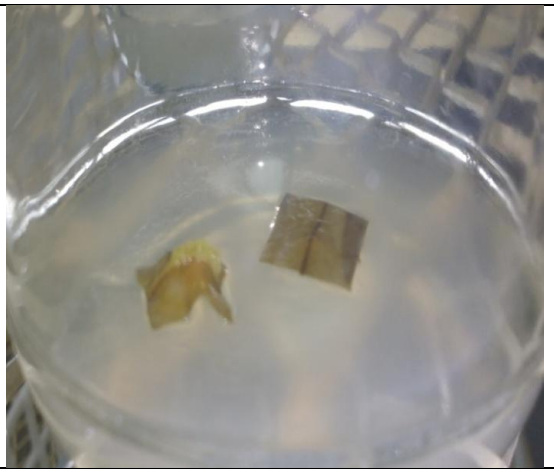
Culture showing phenolic browning	Phenolic browning controlled
	
Control	Ascorbic acid- 300 mg/l

Plate 2. Control of phenolic exudation of leaf explants of Purbi cultivar of litchi with antioxidant treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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