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CONTROL OF EXCESSIVE BROWNING DURING IN-VITRO REGENERATION OF MUSA LATERITA.

DAYARANI M* AND DHANARAJAN M.S.[1]

**Research Scholar, Sathyabama University, Chennai,India.*
[1]Principal, Jaya College of Arts & Science, Chennai,India.

ABSTRACT

The effect of antioxidants in controlling the phenolic exudation was studied in in-vitro culture of *Musa laterita*. Citric and ascorbic acids were tried in two different concentrations viz., 50 and 100 mg l^{-1} and the explants were treated with antioxidants either prior to inoculation or incorporated into the culture medium. Observations on the phenolic content were recorded at regular intervals of six days up to a period of one month after inoculation. Soaking of explants for 30 minutes in an anti-oxidant solution prior to their culture caused the maximum reduction in phenol content on 24th DAI from 0.110 and 0.10 to 0.074 and 0.044 per cent at 50 and 100 mg concentrations respectively. In cases, where the antioxidants were incorporated into the culture medium, the reduction was too low. Results showed that the soaking of explants in an antioxidant mixture prior to their culture was effective at both the concentrations in controlling the phenolic exudation.

KEYWORDS: **Antioxidant, Ascorbic acid, Citric acid, Musa, Phenol**

DAYARANI M
Research Scholar, Sathyabama University, Chennai, India.

INTRODUCTION

Bananas and Plantains (*Musa* spp.), a staple food of developing countries, is commonly known as poor man's crop. It forms the subsistence crop of the farmers as it provides security for food and income. It belongs to the section Eumusa of the family Musaceae. There is one more section under the same family, called Rhodochlamys, which comprises species of ornamental view. They are important because of their attractive colour of bracts, short plant type and erect inflorescence. They can be used as cut flowers. Also, they are resistant gene sources for biotic and abiotic stresses,hence can be used in breeding programmes to improve the edible bananas. They are seen growing wild in regions of North Eastern parts of the country, Myanmar etc, [1] But with the threat of decline in forest area and zoom system of cultivation, these plants are under the verge of extinction. Therefore, these species need to be conserved under ex-situ conditions. In-vitro propagation is one of the efficient method of ex-situ multiplication and maintenance**.** However, *in-vitro* propagation of banana is quite often interrupted by the excess exudation of phenols as reported in banana cv. Williams[2]. These form a problem during in-vitro culture of banana explants, accompanied by the darkening of medium, an attribute of the phenolic compounds exuded from the plant tissue and accumulating in the culture medium resulting in high mortality.

Banana Explants are susceptible to tissue browning and elimination or minimization of this process is an essential prerequisite to successful culture establishment. Therefore identification of a suitable treatment to minimize tissue browning in the explants with particular emphasis on the use of antioxidants is the main objective of this study. Frequent subcultures prevented tissue browning caused by the action of phenols[3]. Similarly use of antioxidants along with aminoacids also prevented browning of tissues in *Musa* spp.[4,5]. But such frequent transfers and addition of aminoacids along with antioxidants is both time consuming and expensive procedure, which cannot be undertaken in a commercial situation. Therefore, in the present study, low cost antioxidants like ascorbic acid and citric acids were evaluated for their effect in controlling the phenolic exudation. The current study reveals the dynamics of phenolic exudates in shoot tips of *Musa laterita* as influenced by the exogenous antioxidants.

MATERIALS AND METHODS

The present study on the effect of antioxidants in controlling the browning by phenolic exudation under in-vitro conditions was conducted in *Musa laterita*, which belongs to the Rhodochlamys section of Musaceae. Citric acid (CA) and Ascorbic acid (AA) were used as antioxidants. Shoot tips (5 cm^3) of this cultivar were extracted from 2-3 months old suckers and brought to the laboratory in 0.1 per cent cetrimide solution. Equal proportion of Citric acid and Ascorbic acid at two different concentrations viz., 50 and 100 ppm were used. One set of explants were soaked for 30 minutes in an anti-oxidant solution prior to its culture (T_2 and T_4) while in the other set, the anti-oxidant was incorporated into the culture medium (T_1 and T_3). The shoot tips were then trimmed to a size of 2 cm^3 and washed with 0.5% detergent solution of Tween-20 and then washed well with distilled water so as to remove the detergent. Later the explants were taken to the laminar air flow chamber where they were surface sterilized for 10 min in 5 per cent sodium hypochlorite and for 5 minutes in 0.1 per cent mercuric chloride with sterile water rinsing in between. After the final rinse with the sterile water, outer layers were removed to get a cube of 1.5 cm.

The explants were then initiated on MS medium[6] containing 3.0 mg BAP, 30g sucrose and 7g of Agar per liter. The cultures were incubated at 25 $\pm$ 2^0C with 70 per cent relative humidity. Artificial illumination was provided through cool white fluorescent lamps and the light intensity was maintained at 1600 lux with a photoperiod of 14/10 hrs. light/dark cycle. Care

was taken so that explants were trimmed approximately to a constant size of 2.0g before initiation and similarly the medium was poured at a constant volume of 15ml per culture tube. Phenol content of the explants along with the medium was estimated at regular intervals of six days i.e., 0^{th}, 6^{th}, 12^{th}, 18^{th}, 24^{th} and 30^{th} day after inoculation (DAI). The standard colorimetric method of Malik and Singh[7] was adopted for phenol estimation. The experiment was laid out in a Completely Randomized Design (CRD) with five treatments and four replications. The studies were concentrated on the initial period of one month, which is very crucial for the cessation of apical dominance leading to the production of adventitious buds.

RESULTS AND DISCUSSION

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds. Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups. Phenolic substances tend to be water soluble since they most frequently occur combined with sugar as glycosides and are usually located in the cell vacuoles. Phenols are collectively called polyphenols. One of the most common problems associated with the *in vitro* establishment of Banana tissue culture is deleterious effects of oxidized phenols. The browning of the surface of the explants is due to the oxidation of phenolic compounds resulting in the formation of quinines which is highly reactive to the plant tissue[8].

The interference of the phenols with morphogenesis is a well established fact and they develop browning around the explants due to their accumulation. Phenol induced suppression of tissue growth has been well documented in Tobacco[9] and toxic effects on cashew explants[10]. The effect of exogenous antioxidants in controlling the levels of phenolic exudates on *in-vitro* shoot tip culture of *Musa laterita* is presented in Fig. 1. Initial levels of phenolics produced were high. This is because when cells are damaged, the contents of cytoplasm and vacuoles are mixed and phenolic compounds can readily become oxidized by air. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants. But the amount of phenolics produced declined over time. The phenol content on various Days After Inoculation (DAI) was statistically significant among the treatments except for 18 DAI. The mean values of the phenolic contents are shown in Table 1.After 30 DAI, soaking the explants in antioxidant stock has shown better control over blackening compared to addition of antioxidant in the medium. In control, the phenol content increased gradually and reached the maximum of 0.141% on 30^{th} DAI, which could be attributed to the absence of antioxidant treatments. In T_1 and T_3 where the antioxidants were incorporated into the culture medium, the phenol content increased gradually and later decreased indicating that inclusion of antioxidants in the culture medium is quite effective in controlling the phenolic exudation as reported earlier in banana [5,11] and coffee cultures[12]. In *Cinnamomum tamala*, when the foliar explants and immature seeds were soaked in different antioxidant solution immediately after harvest, the phenolic compounds and other secondary compounds were released in the antioxidant solution. Explants collected and processed without soaking in the antioxidant solution turned necrotic and degenerated subsequently[13]. There is one more report about addition of ascorbic acid to the media which inhibited the exudation of phenols in banana tissue culture[14].

Table 1
Effect of antioxidants in controlling the levels of phenolic exudates encountered during the in-vitro shoot tip culture of Musa laterita.

Treatments	0th day	6th day	12th day	18th day	24th day	30th day
Control (without antioxidants)	0.021	0.043	0.054	0.109	0.119	0.141
T1 - medium (50 mg each of CA + AA)	0.020	0.040	0.054	0.101	0.100	0.099
T2 - soaking (50 mg each of CA + AA)	0.018	0.051	0.060	0.110	0.074	0.054
T3 - medium (100 mg each of CA + AA)	0.046	0.062	0.079	0.093	0.101	0.089
T4 - soaking (100 mg each of CA + AA)	0.045	0.070	0.091	0.100	0.044	0.042
CD (p=0.05)	0.004	0.010	0.009	NS	0.012	0.016
CV%	9.180	12.276	8.516	10.141	8.946	12.001

However, the reduction in phenol content was more pronounced (i.e., from 0.101 to 0.089%) at higher concentrations (100 mg l^{-1}) as against the lower concentrations of antioxidants (50 mg l^{-1}). This gives an indication that the effect could further be improved by increasing the concentration of antioxidants to an optimum level. While in T_2 and T_4 where the explants were soaked for 30 minutes in an anti-oxidant solution prior to its culture, the phenol content increased gradually up to 18th DAI and then declined drastically on 24th DAI i.e., from 0.110 to 0.074% in T_2 and from 0.100 to 0.044% in T_4(Fig.1). The present results are in agreement with the findings in Guava[15].

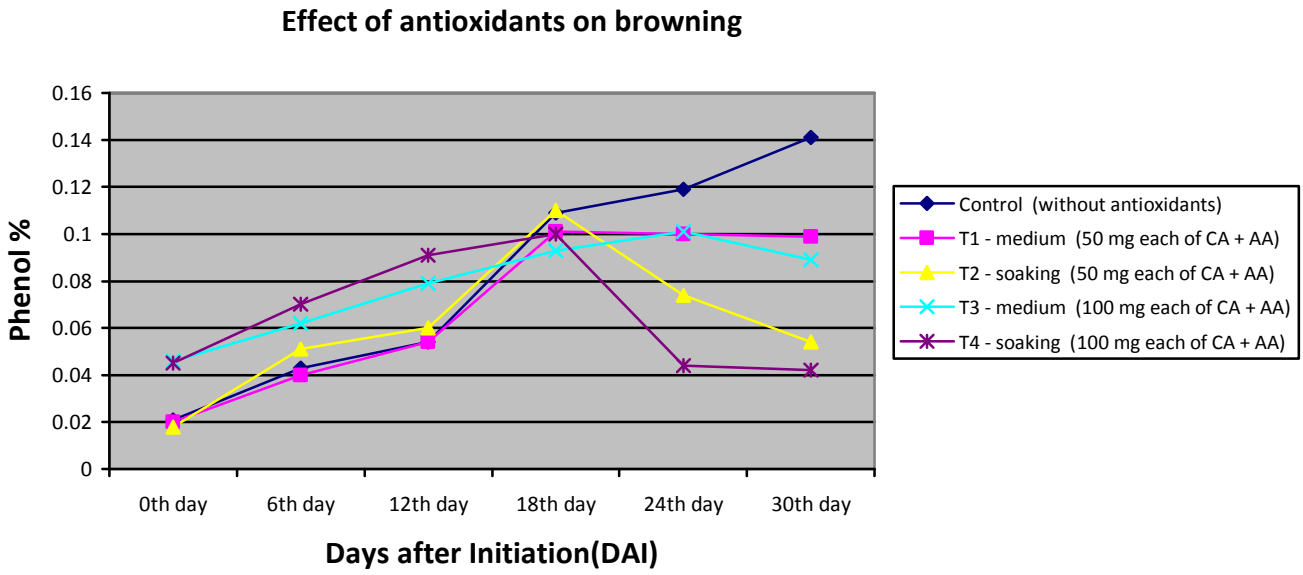

Figure 1
Effect of antioxidants in controlling the levels of phenolic exudates encountered during the in-vitro shoot tip culture of Musa laterita

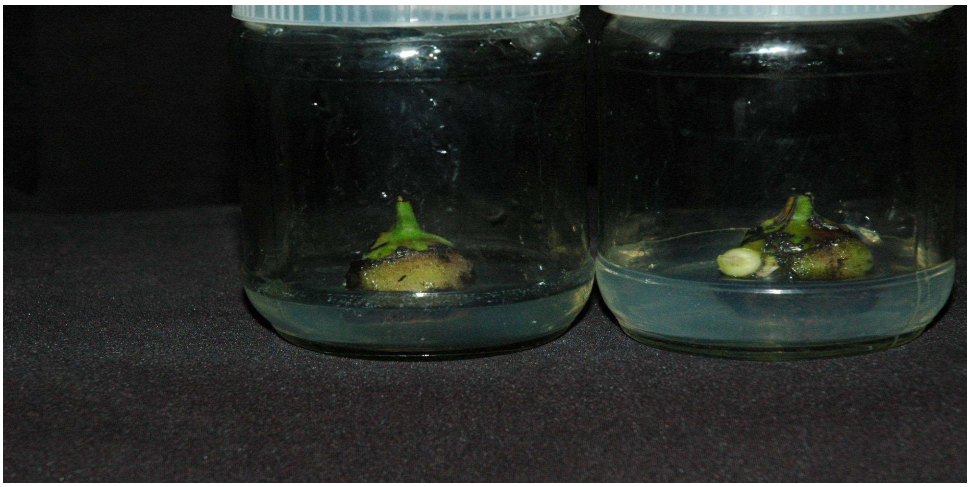

Figure 2
Proliferating suckers with reduced blackening after soaking in 50 mg/l(left) 100mg/l (right) antioxidants on 24 DAI.

This drastic reduction in phenol content beyond 18 DAI could be attributed to the following reasons.

1. Exudation of phenols generally ceases once after shoot multiplication begins.
2. Rapid oxidation of ascorbic acid in the medium to dihydro-ascorbic acid, which would have been absorbed by the tissues leading to the control of phenolic exudation in the later days of culture.
3. Soaking of explants in a mixture of ascorbic acid and citric acid exposes them to reducing agents that prevent blackening through the oxidation of phenols and also lowers the pH as the PPO activity is greatest only at a pH of 6.5 and above[16].
4. The conjugational properties of the phenolic compounds with sugars, amino acids and proteins.

From the above results, it is concluded that i) the activity of ascorbic acid and citric acid in preventing the phenolic exudation was reduced when they were incorporated in the culture medium. This might be attributed to the thermo-labile nature of ascorbic acid, which would have lost its effect on autoclaving of the medium and ii) accumulation of phenolics during *in-vitro* culture may not only depend on the strength of the medium employed, but also on the key enzymes involved in the biosynthesis of phenolics, substrate availability, exposure to oxygen and other physiological conditions.

CONCLUSION

Equal proportion of citric and ascorbic acids were tried in two different concentrations viz., 50 and 100 mg l^{-1} to control browning in in-vitro culture of *Musa laterita*. The explants were treated with antioxidants either prior to inoculation or incorporated into the culture medium. Soaking of explants for 30 minutes in an anti-oxidant solution prior to their culture caused the maximum reduction in phenol content on 24th DAI from 0.110 per cent and 0.100 per cent to 0.074 per cent and 0.044 per cent at 50 mg and 100 mg/l concentrations respectively. In cases, where the antioxidants were incorporated into the culture medium, the reduction on 24th DAI was too low. Results showed that the soaking of explants of *Musa laterita* in an antioxidant mixture of Citric acid and Ascorbic acid for 30 min. prior to their culture was effective in controlling the phenolic exudation.

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