

PREVENTION OF BROWNING OF COCONUT (*COCOS NUCIFERA* L.) INFLORESCENCE AND LEAF TISSUES IN *IN VITRO* CULTURES

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Different antioxidants and their modes of application were investigated to reduce browning incidence in inflorescence and leaf tissues. Optimum size of inflorescence for tissue culture was also determined. Soaking of inflorescence tissues in antioxidants prior to slicing and culturing in media with activated charcoal (AC) gave best results. Inflorescences with outer spathe length of approximately 200 mm need not be soaked in antioxidants prior to slicing. Further slicing the inflorescence tissues on the first few subcultures when calloids are not yet produced is not recommended. Browning incidence was minimal when the number of tissues per vial was limited to five and when the subculture interval was three to four weeks. For coconut leaf tissues, the addition of AC to the nutrient medium will suffice as the antibrowning agent.

INTRODUCTION

Most coconut explants frequently turn brown shortly after inoculation. Such browning of tissues and medium occurs due to polyphenols which are released when tissues are wounded. Polyphenols inhibit growth and the tissues usually die. This can be prevented by several approaches, namely: a) culturing of explants initially in liquid medium in which phenols can readily diffuse; b) soaking of explants in water or pretreating them in an unsupplemented medium; c) adsorption of growth inhibiting substances by activated carbon (AC); d) adsorption by polyvinylpyrrolidone (PVP) which prevents the oxidation of polyphenols; and e) use of reducing agents by either submerging the explants in a solution of antioxidants such as ascorbic acid (AA) or citric acid (CA) or incorporating the reducing agents into the culture medium.

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This paper reports the methods employed to minimize browning of coconut tissues.

REVIEW OF LITERATURE

During the course of growth and development *in vitro*, plant tissues not only deplete the nutrients that are incorporated in the medium but also release toxic substances. These substances, such as phenols, may have profound physiological effects on the cultured tissues. Browning of the tissues and the adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinones which are highly reactive and toxic to the tissues (Maier and Metzler 1965).

Pelletier and Ilami (1972) working with tobacco demonstrated the existence of two major periods of intensive browning: one within the first 10 days and the other after 25 days of culture. Drastic browning in the early period of the culture may result from some wound or physiological changes stemming from the excision from the mother plant. The gradual browning is believed to be an expression of the process of senescence of the explants.

To minimize browning, Murashige

(1974) has suggested the presoaking of explants in AA and CA solutions and adding them to the culture medium to curtail the oxidation of the phenolics. Zaid and Tisserat (1983) soaked date palm explants in an antioxidant solution (150 mg/L CA and 100 mg/L AA) prior to the surface sterilization treatments. Apavatjrit and Blake (1977) suggested that browning could be eliminated by a nutritionally balanced medium. Excision of brown explant parts during culture was also advocated to prevent this problem (Smith and Thomas 1973).

Other means by which prominent browning problem can be minimized in coconut tissue culture was suggested by Sugimura and Salvaña (1989). One way was by reducing the thickness of the transverse slices of rachilla tissues; 1-mm thick slices gave 32% browning while 0.5-mm thick slices gave 11% only when inflorescences with a spathe length of 46 mm were taken. The use of Y3 formulation (vitamin source added at half strength) supplemented with 3% sucrose was found most effective as liquid slicing medium in preventing browning. Moreover, a relatively higher callus induction with minimal browning was obtained from explants taken from inflorescences with spathe lengths of 25 (stage 4) to 41 mm (stage 5). The lowest browning incidence was observed when the level of 2,4-D ranged from 20 -30 ppm and with 0.25% AC incorporated in the callus induction medium.

The use of charcoal is preferred over L-cysteine hydrochloride and other reducing agents, such as AA, CA, dithiothreitol, glutathione and mercaptoethanol (George and Sherrington 1984), because the latter are often toxic to the plant tissues at higher concentrations (Sharma et al. 1980). According to Reuveni and Lilien-Kipnis (1974), the addition of AC proved effective for a limited period only. After one or two transfers to the already familiar medium, browning occurred and tissues ceased growth. Zaid and Tisserat (1983) found that shoot tips and lateral buds cultured on nutrient medium without AC exhibited severe browning and growth inhibition.

Addition of 3 g/L AC caused substantial root and shoot growth of date palm embryos (Rebechault et al. 1976; Reynolds and Murashige 1979). Inclusion of AC reduced the availability of hormonal substances. It is, therefore, necessary to apply an abnormally high concentration of auxins (Tisserat 1979; Zaid and Tisserat 1983) up to a certain level not toxic to the tissues.

Age of media also affects browning (Ebert and Taylor 1990). While most explants on one-day-old medium had died or showed only minimal growth, a slightly better response was observed with 5-day-old medium. The best results, however, were obtained with 9-day-old medium. These results indicated that fresh medium contained toxic levels of a chemical compound(s), such as 2,4-D incorporated in the nutrient medium, which was subsequently adsorbed by AC thus preventing damage to the explants.

MATERIALS AND METHODS

Prevention of Browning of Inflorescence Tissues

Coconut inflorescences were collected without killing the bearing palms (Rillo 1989) and cultured in callus initiation medium with the following treatments:

- a. Incorporation of different antioxidants in the culture medium

Treatments used were:

1. +CA; -AC
2. +AA; -AC
3. +CA; +AA; -AC
4. +PVP; -AC
5. +AC (Control)

Legend:

- AC - activated carbon
CA - citric acid
AA - ascorbic acid
PVP - polyvinylpyrrolidone

- b. Soaking of inflorescence for 20 min in antioxidant solution, (CA, AA, CA + AA, PVP) before slicing. Sterile distilled water (SDW) was used as the control. Tissues were inoculated onto callus initiation medium supplemented with AC.

Prevention of Browning of Leaf Tissues

A suitable antioxidant for pretreatment to minimize browning of coconut leaf tissues was determined.

The spear leaf from healthy Catigan green dwarf palms was used as source of numerous leaf explants. The spear leaf was sterilized with 100% commercial bleach for 15 minutes, trimmed and then re-sterilized with 10% commercial bleach for 10 minutes. Sterilized leaflets were cut into approximately 0.5 to 1 cm sections while soaked in various antioxidant pretreatments, namely:

- | | |
|-----------------|-------------|
| no soaking (-S) | 1.0% PVP |
| 50 mg/L CA | 100 mg/L AA |

The pretreated leaf tissues were incubated for 3 days in liquid medium and then transferred onto semi-solid medium composed of Blake's nutrient formulation with AC incorporated.

Each treatment was replicated three times with 20 samples per replicate.

In the succeeding experiments, leaf tissues soaked in antioxidants were cultured onto semi-solid medium without AC incorporated. This was compared to nutrient medium with AC but without antioxidant soaking, and to the control (no AC, no antioxidant soaking).

Percent survival, tissue enlargement and extent of browning were noted 2 wks after inoculation. Extent of browning was rated as follows: B0 - without browning, B1 - browning up to one-third, B2 - browning up to two-thirds, and B3 - browning of the entire leaf tissue.

RESULTS AND DISCUSSION

Prevention of Browning of Inflorescence Tissues

The experiments showed that substituting AC with CA, combination of CA and AA, and PVP, resulted in severe browning of tissues (Table 1). The tissues did not survive until the next subculture. This confirms results of other studies that for coconut, incorporation of AC in the medium is essential (Ebert and Taylor 1990; Sugimura and Salvaña 1989).

A preliminary trial showed that soaking of inflorescence rachillae in the above-mentioned antioxidants for 20 minutes prior to slicing and culturing in media with AC gave best results. For inflorescence with 240 mm outer spathe length, the addition of PVP and the combination of CA and AA resulted in the least browning of tissues (41.3%) followed by AA (42.7). Tissues not soaked in any antioxidant had 50.7% browning incidence (Table 2). In a follow-up experiment using inflorescence with 270 mm outer spathe length, tissues soaked in AA showed a browning incidence of 8.5%. The percentage of browning increased slightly when tissues were soaked either in CA + AA (11.5%) or in sterile distilled water (control) (14.5%). The pretreatment of tissues with either CA or PVP further enhanced browning incidence to 30%, thus these chemicals were not considered as effective antioxidant agents.

Inflorescences of different ages were also cultured using the inflorescence number as the age determinant. The inflorescence number, however, was later observed to be not precise enough for determining the right physiological age of the explant for tissue culture. As can be seen in Table 2 where inflorescence no. 6 was used in all three experiments, the size of the inflorescence based on the outer spathe length varied widely among various palm sources within the same cultivar. This factor partly contributed to an inconsistent response observed from experiment to experiment in terms of

the degree of browning, with the smallest inflorescence giving the lowest browning incidence. For tissues with outer spathe length of 240 mm, the percent browning in the control did not differ greatly from tissues soaked in antioxidants. Due to minimal browning incidence observed from inflorescences with outer spathe length of approximately 200 mm, these need not be soaked in any antioxidant prior to slicing. Sterile distilled water would suffice as the soaking medium.

It was also observed that during subculture of enlarged inflorescences, browning was severe on tissues which had been further sliced even when soaked in different antioxidants. The combination of CA and AA showed a browning incidence of 72.2%, SDW - 84%, AA - 84.8%, CA-89.2% and PVP - 95.5% (Table 3). Wounding increases the wound surface area causing an increment in the uptake of nutrients and growth regulators (Pierick 1989). Further, it stimulates ethylene production. Ethylene accumulation in the culture medium is known to inhibit growth of tissues. Avoiding further slicing of the original cultures when calloids are not yet formed gave a browning incidence of 29.2% (Table 3).

It was also observed that with 10 mL medium per vial, the number of tissues should be limited to five per vial with subculture interval of 3 - 4 wk. It was noted that browning incidence was high when the subculture interval was 6 - 8 wk or more (unpublished results).

Prevention of Browning of Leaf Tissues

Since all the treatments contained AC, this additive was not incorporated into the medium in the succeeding experiments to avoid masking of the effect of the antioxidants used. Based on Duncan's Multiple Range Test no significant difference was observed among all antioxidant pretreatments after 2 wks from initial culture (Table 4).

The effect of the following treatments, namely: a) no antioxidant

TABLE 1

Percent Browning of Explants from Inflorescence No. 6 with Outer Spathe Length of 240 mm Using Different Antioxidants Incorporated in the Callus Initiation Medium

TREATMENT	% BROWNING	
	2 Wk	4 Wk
+CA; -AC	32.0	64.0
+AA; -AC ¹	-	-
+CA; +AA; -AC	52.0	64.0
+PVP; -AC	36.0	74.7
+AC (Control)	20.0	53.4

¹ No data were collected because all the cultures got contaminated.

TABLE 2

Percent Browning of Different Sizes of Inflorescence No. 6 Soaked for 20 min. in Different Antioxidants Taken After 4 wk from Initial Culture.

EXPERIMENT NO.: LENGTH OF SPATHE:	% BROWNING		
	I 200 mm	II 240 mm	III 270 mm
TREATMENT:			
SDW (Control)	0.0	50.7	14.5
CA	0.0	46.7	30.0
AA	2.8	42.7	8.5
CA + AA	0.0	41.3	11.5
PVP	0.0	41.3	30.0

TABLE 3

Percent Browning of Inflorescence Tissue Soaked in Different Antioxidants Prior to Slicing and First Subculture

TREATMENT	% BROWNING
Control (no slicing)*	29.2 ^c
CA + AA	72.2 ^b
SDW	84.0 ^{ab}
AA	84.8 ^{ab}
CA	89.2 ^a
PVP	95.5 ^a

* Tissues were directly transferred onto the callus initiation medium. Means with the same letter are not significantly different at 5% level by DMRT.

soaking (-S), incorporation of AC in medium (-S; +AC), b) no antioxidant soaking, no AC (-S;-AC), c) soaking in CA, no AC (+CA; -AC), d) soaking in PVP, no AC (+PVP; -AC), or e) soaking in AA, no AC (+AA; -AC) were evaluated. After 2 wk from antioxidant pretreatment, percentage

survival (86.7%) and tissue enlargement (90%) were lowest in the control. The rest of the treatments did not show any significant difference. In terms of the extent of browning, the incorporation of AC alone in the medium gave the least percentage of browning (6.7%) that extended up to 1/3 of the leaf tissue (B1) (Table 5).

CONCLUSION AND RECOMMENDATION

Incorporation of activated charcoal in the medium is the optimum antibrowning agent for coconut inflorescence and leaf tissue cultures.

Soaking of inflorescence tissues in AA, CA + AA, or PVP for 20 min may lessen browning incidence, but inflorescences with outer spathe lengths of approximately 200 mm need not be soaked in any antioxidant prior to slicing. Sterile distilled water would suffice as the soaking medium.

TABLE 4
Duncan's Multiple Range Test of Percent Survival, Tissue Enlargement and Extent of Browning of Leaf Tissues Pretreated with Various Antioxidants Prior to Culture onto Medium with AC

ANTIOXIDANT PRE- TREATMENT	% DATA ¹					
	SURVIVAL	TISSUE ENLARGEMENT	EXTENT OF BROWNING			
			B0	B1	B2	B3
-S; +AC	90.0 ^a	90.0 ^a	45.0 ^a	38.3 ^a	6.7 ^a	10.0 ^a
+CA; +AC	90.0 ^a	90.0 ^a	60.0 ^a	20.0 ^a	10.0 ^a	10.0 ^a
+PVP; +AC	91.7 ^a	91.7 ^a	63.3 ^a	20.0 ^a	8.3 ^a	8.3 ^a
+AA; +AC	93.3 ^a	93.3 ^a	60.0 ^a	26.7 ^a	6.7 ^a	6.7 ^a

¹ Means having a common letter are not significantly different at 5% level DMRT; data taken after 2 wk from pretreatment; values represent the mean of 3 replications with 20 samples per replicate.

TABLE 5
Duncan's Multiple Range Test of Percent Survival, Tissue Enlargement and Extent of Browning of Leaf Tissues After 2 Wk from Antioxidant Pretreatment

TREATMENT	% DATA ¹					
	SURVIVAL	TISSUE ENLARGEMENT	EXTENT OF BROWNING			
			B0	B1	B2	B3
-S; +AC	100.0 ^a	100.0 ^a	93.3 ^a	6.7 ^c	0.0 ^c	0.0 ^b
-S; -AC (Control)	86.7 ^b	90.0 ^b	0.0 ^b	46.7 ^b	40.0 ^a	13.3 ^a
+CA; -AC	100.0 ^a	100.0 ^a	6.7 ^b	81.7 ^a	11.7 ^b	0.0 ^b
+PVP; -AC	100.0 ^a	100.0 ^a	1.7 ^b	78.3 ^a	20.0 ^b	0.0 ^b
+AA; -AC	96.7 ^a	96.7 ^a	3.3 ^b	50.0 ^b	43.3 ^a	3.3 ^b

¹ Means having a common letter are not significantly different at 5% level; values represent the mean of 3 replications with 20 samples per replicate.

Further slicing of tissues during the first few subcultures when calloids are not yet produced is not recommended. It is also suggested to limit explants to 5 tissues per vial and maintain subculture intervals of 3 - 4 wk.

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