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Control of Contamination and Browning in Micropropagation of Cashew (*Anacardium occidentale* L.) using Axillary Buds

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ABSTRACT

Cashew (*Anacardium occidentale* L.) is highly cross pollinated in nature, therefore, superior characteristics of the improved varieties are difficult to maintain by seed propagation. Multiplication through microporpagation is the most efficient way for mass propagating the improved plants. This research was carried out to establish a protocol for surface sterilization of axillary buds of the field grown mature cashew trees without losing the tissue viability. Investigations were also undertaken to eliminate the browning in the cultured explant. Among the five protocols tested, the protocol consisting the steps of dipping the axillary buds in 70% ethanol for 30 seconds, 10% sodium hypochlorite for 20 minutes and thermotherapy at 60 °C for 5 minutes in water bath was effective in controlling the fungal contamination. Tissue viability test with Evans Blue indicated that the tissues were remained viable after applying the above sterilization protocol. Browning of the tissues and media, one of the serious constrains in cashew tissue culture, could be reduced by optimizing certain conditions. Axillary buds extracted from the basal position of the immature branches showed lower browning over the top and the middle positions. Maintenance of the cultures in the dark was effective in reducing the browning of the culture.

KEYWORDS: *Anacardium occidentale* L., Axillary buds, Browning, Cashew, Surface sterilization

INTRODUCTION

Cashew is an evergreen perennial tropical tree belonging to family Anacardiaceae, with the natural order Sapindales. It is a native of tropical central and South America (Paul, 1936), however, now it can be found in many tropical and subtropical countries including Sri Lanka. Cashew is one of the most important plantation crops and it has been ranked second in the international trade amongst the major edible nuts (Bhalekar and Patil, 2015). By expanding the cashew cultivation, it can be taken for future economic development by improving both production and productivity. Total production of cashew in Sri Lanka is 10,000 metric tons and total export is 145.7 metric tons in 2012 (Anon, 2013). Cashew is cultivated in almost all districts of Sri Lanka as a small scale plantation crop. However, few large scale plantations have been established in the past with identified varieties.

The total extent of land area under cashew cultivation in 2013 is 48,200 hectares (Anon, 2013). Reported average national cashew yield is approximately 4-5 kg/tree/year, but it has a potential yield of about 10-15 kg/tree/year (Jayasekara and Kodikara, 2003). Use of poor planting materials is one of the major reasons for low average yield. As a result of continuous collaborative research and development work by Sri Lanka Cashew Corporation and Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, new high yielding varieties that are suitable for planting in all cashew growing areas in Sri Lanka have been introduced (Aththanayake and Wijethunga, 2012). The first three varieties developed in Sri Lanka are WUCC-9, WUCC-19, WUCC-21 introduced in 2005 (Jayasekara and Jayasekara, 2005). Varieties WUCC-1 and WUCC-23, are the newly released high yielding varieties (Aththanayake and Wijethunga, 2012). In addition to these varieties, existing improved varieties would help to overcome the present scarcity of genetically improved planting materials for cashew growers.

An efficient method is required for mass production of uniform planting materials from improved varieties for rapid expansion of the plantation. Cashew is highly cross pollinated, woody and perennial in nature (Aliyu and Awopetu, 2005). Therefore, superior characteristics of the improved varieties are difficult to be maintained by seed propagation. Vegetative propagation is being increasingly used for clonal multiplication and conventional vegetative propagation methods are not powerful enough to produce planting materials in a large scale (Wickramasinghe, 2002).

Cashew *in vitro* micropropagation could offer an efficient, rapid and possibly a cost-effective method which could be used particularly for multiplying large numbers of plantlets of elite cashew genotypes for breeding and production purposes.

However, there is no reliable method of developing true to type plants from field grown mature trees due to several constraints. Strong levels of surface sterilization is required to decontaminate the explant materials collected

from the field grown plants, that cause loosing of the viability of the tissue (Das *et al.*, 1996). Presence of secondary metabolites which are oxidized after wounding that cause subsequent browning and necrosis of cashew explants (Aliyu, 2005) is another constraint.

This research was carried out to optimize the sterilization protocol while maintaining the tissue viability. Furthermore, attempts were taken to control browning in the explant materials used for cashew micropropagation.

MATERIALS AND METHODS

Experimental Location

This experiment was carried out at the tissue culture laboratory of the Department of Horticulture and Landscape Gardening, Faculty of Agriculture and Plantation Management, Wayamba university of Sri Lanka from January to May 2016.

Plant Materials

Axillary buds were used as the explant material. Explants were extracted from the immature branches of the field established mature cashew trees cultivated in Makandura premises of Wayamba University of Sri Lanka.

Explant Preparation and Surface Sterilization

The stem cuttings containing the axillary buds were washed thoroughly with Teepol followed by running tap water for ten minutes. Axillary buds were excised carefully under the laminar flow hood from the washed cuttings in 400 mg/L ascorbic acid solution to minimize the browning.

Five surface sterilization techniques were tested to select the most appropriate method. Steps included the sterilization with 10% commercial bleach (sodium hypochlorite) with 2-3 drop of polysorbate-20 (Tween 20) for 20 minutes by continuous shaking either with or without 70% ethanol for 30 seconds and thermotherapy at 45, 50 or 60 °C (Table 1).

Table 1. Tested protocols for sterilization of cashew explants

Treatment	Sterilization protocol
1	10% commercial bleach (20 min.)
2	70% EtOH (30 s) + 10% commercial bleach (20 min.)
3	70% EtOH[1]+ 10% commercial bleach (20 min.) + Thermotherapy at 45 °C (5 min.)
4	70% EtOH[1]+ 10% commercial bleach (20 min.) + Thermotherapy at 50 °C (5 min.)
5	70% EtOH[1]+ 10% commercial bleach (20 min.) + Thermotherapy at 60 °C (5 min.)

EtOH-Ethanol

After disinfection, the axillary buds were thoroughly washed three times with autoclaved distilled water until free from Clorox residues. In the protocols 3-5, the sterilized explants were applied with thermotherapy at 45, 50 or 60 °C for 5 min in water bath with autoclaved distilled water.

Sterilized explants were cultured on to full strength MS medium (Murashige and Skoog, 1962). Each treatment was consisted with three replicates. Records were taken on the number of contaminated cultures and source of contaminant after seven days of culture initiation. The experiment was repeated three time.

Testing the Tissue Viability after Sterilization

A viability assay was done on the sterilized explants by staining with Evans Blue. A positive control of fresh axillary buds and a negative control of a dead axillary bud containing non-viable cells were used as the controls. The tissues were separately put in to 0.25% Evans Blue solution. After 25 hours these buds were separately washed with phosphate buffer and hand sections were laid onto the microscopic slides and observed under light microscope (40×10).

Culture Conditions for Controlling Browning and Shoot Development

Vegetative axillary buds extracted from three different positions of immature branches, top, middle and bottom, were tested for browning intensity after inoculation. Explants were cultured on MS medium supplemented with 2% sucrose, 2 mg/L Benzylaminopurine (BAP), 0.1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). The same medium supplemented with different concentrations of Gibberellic acid (GA_3; 0, 0.1, and 0.5 mg/L) were also tested for controlling browning and shoot development. Cultured explants were kept under room temperature and normal light condition. Browning intensity was recorded by measuring the diameter of browning circles and any effect on shoot elongation was recorded after seven days of culture initiation.

Vegetative axillary buds excised from immature cashew branches were cultured on both full and half strength of MS media supplemented with 2% sucrose, 0.5 mg/L Naphthaleneacetic acid (NAA) and 5 mg/L BAP. Three replicates were exposed to 18/6 photoperiod for seven days. Other three replicates were maintained in the dark continuously. Browning intensity of the cultured explants were recorded after seven days.

Data Analysis

Data were statistically analyzed using Statistical Analysis System (SAS version 9.2).

RESULT AND DISCUSSION

Effect of Surface Sterilization Techniques

The best method for sterilization of cashew axillary buds was identified after seven days of culture establishment. All the cultures except the culture which received fifth sterilization protocol were contaminated by fungi. The detailed optimized sterilization protocol identified in the experiment is given in the Figure 1.

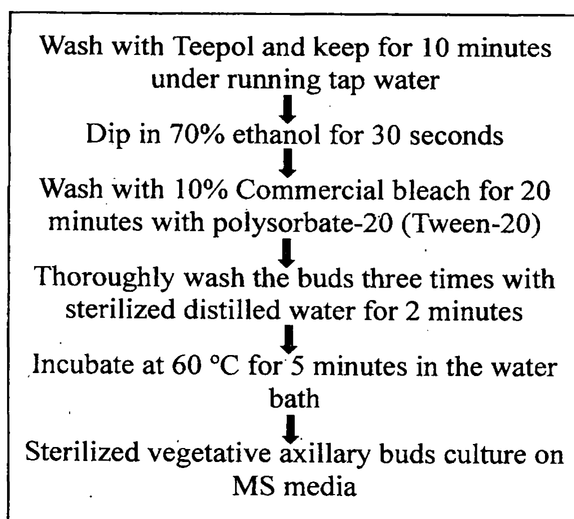

Figure 1. Optimized sterilization protocol

Results revealed that the thermotherapy is effective in controlling the contamination of fungi; and, the temperature is shown to be critical. Among the tested temperatures, 60 C was highly effective over the other two temperatures, 45 and 50 °C. Thermotherapy has been used for controlling fungi in different planting materials such as Horseradish (Eranthodi and Babadoost, 2010).

Tissue Viability after Sterilization

The basis of Evans Blue used for viability assay is the penetration of the dye into non-viable cells giving the blue colour in the cells of the dead tissue. Live cells are not stained due to the inability of the dye to penetrate through the cell membrane resulting the colorless tissue after staining. Complete blue colour development in the negative control of dead tissue and the absence of colour development in the positive control indicated accuracy of the staining method. Observations revealed that the sections of the stained axillary buds after sterilization were viable indicating the absence of any stained cell within the tissue, except the cells in the cutting surface. The non-viable part of the sterilized tissue can be a barrier for absorbing the culture medium components. This can be overcome by removing two or three cell layers of the cut surface after surface sterilization. The results revealed that the optimized sterilization technique did not cause any damage to the tissue and thus, is well suited for culture initiation.

Control of Browning

Browning of the tissue and media is a serious constrain in the tree tissue culture due to exudation of phenolic compounds. It is extremely severe in cashew because it contains high concentrations of polyphenols that leach out and cause browning of medium and necrosis of the explant (Bajaj, 1996). It also releases secondary metabolites from the duct of primary phloem elements of all organs, which has resulted into serious browning in cashew (Mantell *et al.*, 1998). There are several methods of elimination of phenolic compounds in *in vitro* culture of tree species; however, complete success was achieved very rarely (Rajaguru *et al.*, 2008). Even though the tissues were excised in 400 mg/L ascorbic acid, it was not sufficient to reduce the browning completely; thus, further attempts were made in controlling the browning. Results revealed that there is an impact of the position of the axillary bud on the immature branches on releasing the polyphenolic compounds (Table 2).

Table 2. Browning intensity of the media from the cultured buds collected from different positions of the branches

Positions of axillary bud	Average browning intensity
Top	1.17 ± 0.04^{a}
Middle	0.88 ± 0.05^{b}
Bottom	0.29 ± 0.04^{c}

Means with different letters are significantly different at p<0.05 at 95% confident level. n= 36

Highest browning was observed in the axillary buds extracted from the top part of the branch whereas the lowest was in the most mature part. A gradual decreasing of browning intensity was observed with increasing the maturity of the branch from where the explants are extracted. It has been reported that the young immature tissues contained the higher total phenolic contents (Padda and Picha, 2007).

Gibberellic acid is the plant growth regulator which is normally used in plant tissue culture for promoting shoot elongation, however, effect of GA_3 in reducing the browning in different crops has also been reported (Li *et al.*, 2006). Any shoot development or reduction in browning intensity was not observed in the media of the three GA_3 concentrations tested (Table 3). Further experiments should be carried out to optimize

the effective GA_3 concentration.

Table 3. Browning intensity in the media with different concentrations of gibberellins (mg/L)

Gibberellins (mg/L)	Average browning intensity
0.0	0.79 ± 0.09^{a}
0.1	0.78 ± 0.09^{a}
0.5	0.77 ± 0.09^{a}

Means with different letters are significantly different at $p<0.05$ at 95% confidence level. n=36

Results revealed that browning could be reduced by maintaining the cultures in the dark (Table 4).

Table 4. Effect of light on browning intensity after seven days of culture establishment

Condition	Average browning intensity
Light	1.85 ± 0.03^{a}
Dark	0.15 ± 0.05^{b}

Means with different letters are significantly different at $p<0.05$ at 95% confident level. n=24

Concentration of the culture medium did not affect on browning (Table 5) or initiation of shoot development.

Table 5. Effect of media composition on browning intensity

Media	Average browning intensity
MS media	1.10 ± 0.07^{a}
Half MS media	1.09 ± 0.09^{a}

Means with different letters are significantly different at $p<0.05$ at 95% confident level. n=24

However, according to observations obtained from the cultures in the dark condition, browning was not observed for two days after culture initiation. After two days to a small extent browning in the explant was observed and a gradual increase occurred. Thus, explants were sub cultured into fresh media to maintain the tissue viability. However, any bud sprouting was not observed in dark condition.

CONCLUSIONS

The results from the present study clearly demonstrated that the identified surface sterilization method for axillary buds collected from field established mature plant of cashew was successful in maintaining tissue viability and controlling contamination. Thermotherapy at 60 °C was effective on controlling the fungi. Axillary buds which were excised from bottom portion of the immature cashew shoots were the best to reduce browning, over the buds from other positions. By maintaining the cultures in the dark, browning could be eliminated. Browning of the cultures cannot completely eliminated using a single method, thus, a combination of several methods is best to be practiced. Further investigations should be carried out to develop the axillary buds of cashew plants.

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