In Vitro Response of Cotyledon Explants of Cashew (*Anacardium occidentale* L.) Cultured in Different Concentrations of BAP

G. Bavatharine and Thayamini H. Seran

ABSTRACT

This experiment was carried out to study the embryogenic response of cashew (*Anacardium occidentale* L.) cotyledon explants cultured in different concentrations of BAP under *in vitro* conditions. Sterilized cotyledon segments (10 mm long, 5 mm width) were cultured on full strength MS basal medium containing various concentrations (0-10 mg/L) of BAP. The result revealed the medium without BAP exhibited profuse root organogenesis which decreased with the increase in the concentration of BAP. Greenish or yellowish white nodular structures appeared on the edge of the surface concentrated towards the convex side of the explants. Consequently, these structures gave rise to somatic embryoids and the degree of embryoid formation increased with the increasing concentration of BAP. MS medium with 8 mg/L BAP was the most optimal concentration for the induction of somatic embryos among the tested. Cytological study confirmed the development of somatic embryoids directly from cultured cotyledon explants.

KEYWORDS: Cashew, cotyledon, explants, BAP, somatic embyroids

INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an important cash crop and grown in tropical countries. It is valued for its delicious and nutritive kernels that are the most economic part of crop and also for its oil from the shell (CNSL). More than half of the cashew extent is confined to the dry zone of Sri Lanka (Wickramasinghe, 2002) and breeding programs have been implemented leading to commercial release of high yielding elite varieties (Rao et al., 1998). However there is inadequate supply of planting materials produced through grafted method to meet the demand (Nambiar et al, 1990) and for this method of propagation, adequate amount of seeds are required as the getting root stock. Further, low percentage of fruit set (3%-4%) has been reported in cashew nuts and conventional methods of propagations are not efficient enough to provide high yielding planting materials (Sivantham et al., 1990). To overcome these problems, tissue culture technique is being studied for obtaining large number of plant material within a short period. *In vitro* plant regeneration technique through organogenesis or embryogenesis provides rapid multiplication of superior varieties.

Even though *in vitro* clonal propagation of perennial plants is generally difficult than that in herbaceous plants they can be propagated by using mature or immature tissues (Jha and Das, 2005). Members of the family Anacardiaceae are in general very recalcitrant but promising results have been reported in mango (Litz et al., 1984). The application of tissue culture methods in cashew is limited by the difficulty of regenerating of plants in a reproducible manner and one of the serious constrains of micropropagation of cashew is attributed to the presence of secondary metabolites (Mantell et al., 1998) which are oxidized after wounding and cause subsequent browning and necrosis of cashew explants (Jha, 1988)). Das et al. (1996) reported that the strong surface sterilization requires for decontaminating the shoot explants obtained from field grown mature plants.

Somatic embryogenesis offers an efficient system for mass clonal propagation within a short period but success has been limited with woody species (Ammirato, 1989) and leads to the formation of bipolar structures possessing both a shoot and a root meristem and embryos regenerate from somatic cells of

Department of Crop Science,
Faculty of Agriculture,
Eastern University, Chenkalady, Sri Lanka.

Psidum guajava L. cv. Banarasi local (Rai et al., 2007) and Jha (1988) reported morphogenesis in callus cultures derived from zygotic embryos of cashew and occurrence of globular protuberances. Cotyledonary segments (Hegde et al., 1992) and nucellus tissues from developing seeds (Cardoza and D'Souza, 2000) from cashew have also been used for the induction of somatic embryogenesis. Ananthakrishnan et al. (1999) reported induction of calli from nuclear explants excised from one-month-old developing fruits of cashew. Kebebew et al. (1998) stated that the use of mature seeds as explants for plant regeneration is the simplest and is potentially more efficient method to be applied breeding programmes. Therefore, the objective of the present study is to determine the embryogenesis potential of cotyledon explants of cashew (*Anacardium occidentale* L) cultured *in vitro* with different concentrations of BAP.

MATERIALS AND METHODS

Cotyledon Explants

Cashew seeds were collected from Kiran Plantation of Cashew Cooperation in the Eastern region of Sri Lanka and cotyledons were carefully excised. They were thoroughly washed with distilled water and then dipped in 70% ethanol for one min subsequently immersed in 30% Clorox™ (sodium hypochlorite, 5.25% active ingredient) with few drops of tween 20 for 20 min with agitation. Thereafter they were rinsed in sterile distilled water in order to remove the traces of Clorox and placed in a sterile beaker with sterilized distilled water until further use.

Culture Media

MS (Murashige and Skoog, 1962) basal media supplemented with vitamins, sucrose (30 g/L) and various concentrations (0-10 mg/L) of BAP were prepared and the pH was adjusted within the range of 5.7 – 5.8 with drop by drop addition of 1 N HCl or 1 N NaOH by using a pH meter. Thereafter it was placed on a hot plate with mechanical stirrer and heated. When the solution was boiling 8 g of agar was added. After dissolving the agar, approximately 10 ml of full strength MS medium were poured into each sterilized bottles and covered immediately by the lid. Subsequently bottles were autoclaved at 121 ˚C under 15 psi for 20 min and then the culture bottles were kept stationary and cooled. The culture bottles were kept for four days to check for microbial contaminants before being inoculated.

Culture Inoculation

This was done to select the most suitable concentration of BAP for the induction of somatic embryos in excised cotyledon explants. Sterilized cotyledon segments (5 mm width and 10 mm length) were excised aseptically under a sterile laminar flow with the flame sterilized scalpel and forceps. Consequently they were cultured on MS media with different concentrations (0-10 mg/L) of BAP. Each treatment had 20 explants and this experiment was repeated twice.

Culture Conditions

Each culture bottles (125 ml capacity) containing three cotyledon explants were incubated at 25±2^{o}C under white fluorescent light in photoperiod of 16 hrs and 8 hrs dark. Morphological changes were observed daily and data were recorded.

Samples for Histological Study

Explant samples incubated in media were fixed in fixative [70% Ethanol, 40% formaldehyde, 5 ml glacial acetic acid] overnight. The fixed samples were dehydrated in a graded ethanol series, infiltrated and embedded in paraffin wax and then embedded samples were cut into 7 µm thick sections using manually operated microtome (Leica™, Germany). Thereafter the sections were dewaxed and rehydrated through ethanol series as described by

Harris *et al*. (1994). The longitudinal sections obtained were stained with Toluidene blue for two min prior to microscopic observation to confirm the embryogenic response in explants incubated in different concentrations of BAP.

Statistical Analysis

Data were first subjected to square root and log transformation and then they were analyzed using the SAS statistical computer software. The mean comparisons between treatments were done by using tukey's studentized range test (HSD) at 5% significant level.

RESULTS AND DISCUSSION

Morphological Response

Colour change of explants was observed in the explants cultured in 10 mg/L BAP at the first week of culture. From second week onwards, distinct change was noted in all cultures thus they turned light brown in colour. The extent of browning was more in the explants cultured in media containing 0, 2, 4 and 6 mg/L BAP and less in explants in 8 mg/L and 10 mg/l BAP at the fourth week of culture. Greenish or yellowish white nodular like structures appeared on the edge of the surface, concentrated towards the convex side of the explants (Figure 1A). There was no distinct changes in colour occurred after four weeks of culture. Leva and Falcone (1990) reported that pale-green calli exhibited morphogenic activity while the brown ones grew in an unorganized form.

The colour of media around explants remained unchanged during the first week of inoculation except the media supplemented with 10 mg/L BAP, which turned slight brown in colour. All media around the explants turned light brown at the second week. At the third week, media supplemented without or with 2 mg/L BAP turned moderate brown. The reason for browning of media may be possibly due to the exudation of secondary metabolites to the media. Mantell et al. (1998) reported the release of secondary metabolites from the duct of primary phloem elements of all organs of cashew which has resulted into serious browning.

Gradual increase in the size of explants occurred from first week to fourth week of culture, thereafter no remarkable changes occurred with the size of explants. Most of the explants increased around 3/4 fold; it may be possibly due to active cell division and expansion. Explants cultured in 8 and 10 mg/L BAP was comparatively larger than the other explants. Baldan et al. (2003) reported that the growth regulators closely regulate the events of cell proliferation, elongation and differentiation that ultimately build up the embryo body. Crack formation was started at the first week; cracks were produced as the result of swelling of explants. The number of cracks found to be high in those explants cultured in 8 and 10 mg/L BAP. According to Kiong et al. (2008), the cotyledon explants incubated at the higher concentration of hormones were swollen, turned green in colour and ruptured.

Root Organogenesis

The average number of root organs per cultured explants had significant difference ($P<0.05$) among the treatments (Table 1). At the third week, the highest number of root organs (3.33) was observed in the explant cultured without growth regulators whereas there was no any formation of root organ exhibited in explants cultured in the concentrations of 6, 8 and 10 mg/L BAP. At the sixth week, maximum number of root organs was observed on the medium without growth regulators (Figure 1B) and also root occurrence was started in explants supplemented with 6 mg/L BAP thereafter root organs remained unchanged in number. Formation of root organs was not occurred with the increasing in the concentration of BAP (8-10 mg/L).

The reason for root formation may be due to the endogenous auxin presented in cotyledons of cashew. Pareek and Shashi (1998) reported that presence of

endogenous auxin in explants and induction of roots in culture is a common feature of *in vitro* system of woody plant. Further, it was noted that with the increasing concentration of BAP, the induction of root organs was reduced. Moreover, it was observed that root organs were formed on the abaxial side of explants in the present study. The finding is supported by Choi *et al.* (1999) who reported that cotyledon explants cultured on basal medium in the absence of any exogenous growth regulators never differentiated to produce somatic embryos but occasionally formed roots.

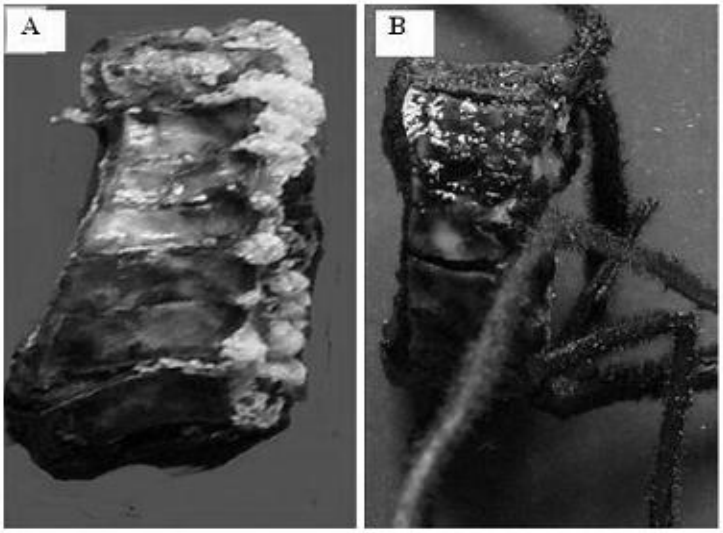

Figure 1A: Morphological response of cotyledon explants cultured on MS medium with 8 mg/L BAP at the fifth week (x 3)
Figure 1B: Root organogenesis from cultured cotyledon explants on MS medium without BAP at the fifth week (x 3)

Table 1: The average number and length of root organs per cultured explants

BAP (mg/L)	Number of root organs per explants		Length (cm) of root organs per explants	
	3 week	6 week	3 week	6 week
0	3.33 ± 0.33 a	9.00 ± 0.58 a	8.00 ± 0.58 a	12.00 ± 1.15 a
2	2.33 ± 0.33 b	4.33 ± 0.33 b	4.00 ± 0.88 b	4.67 ± 0.33 b
4	1.00 ± 0.00 c	1.33 ± 0.33 c	3.00 ± 0.58 b	3.33 ± 0.66 b
6	0.00 ± 0.00 d	1.00 ± 0.00 c	0.00 ± 0.00 c	0.83 ± 0.17 c
8	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d
10	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d
F test	*	*	*	*

Data based on the availability of survived explants. Value represents mean ± standard error
F test: *- P<0.05. Means with the same letter are not significantly different according to Tukey's Studentized test at 5% level.

The highest length (12 cm) of root organ was observed at the sixth week in the medium devoid of growth regulators while the shortest root organ length (0.83 cm) was noticed in the medium supplemented with 6 mg/L BAP (Table 1). Formation of root organs was not occurred with the increasing in the concentration of BAP (8-10 mg/L). The growth of main root organs ceased after fifth week of culture and no further

growth was observed. There was significant variation ($P<0.05$) in the average length of root organs among the treatments. The average number of lateral root organs per explant showed a similar pattern to that of main root organs.

Embryoid Formation

At the first week, no nodular formation occurred in cultured explants. Small greenish white structures were started to initiate between the concave and convex epidermal portion of explants supplemented with 8 mg/L BAP and slight swelling having yellowish white protuberance was observed on the edge of the surface of explants cultured in 10 mg/L BAP at the fourth week of culture. Formation of such nodular structures is reported in somatic embryogenesis of *Cocus sativus*, *Oryza sativa* and *Musa* cv Bluggoe (ABB) (Sannasgala, 1989). Explants cultured in media with 4 mg/L and 6 mg/L BAP showed slight embryoid formation at the fifth week week, afterwards most of them became brown in colour. Kiong et al. (2008) reported citrus explant swollen and turned to dark green in colour after two weeks of culture and subsequently turned to yellow in colour and died after 12 weeks of culture. The degree of embryoid formation increased with the increasing concentration of BAP.

In the present study, there were no nodules or white protuberance observed in explants cultured without growth regulators. Similar observation was recorded in cashew (Gogate and Nadguda, 2003) whereby explants did not show any embryogenic response or callus formation in medium that was lacked of plant growth regulators. In this study, embryoids like structures were observed and concentrated towards the convex side on the surface and the degree of embryoid formation decreased towards the concave side at the sixth week. Among the different concentrations (0-10 mg/L) of BAP, 8 mg/L was the most responsive concentration of BAP for the induction of somatic embryoids from cotyledon explants of cashew. Seran et al. (2007) reported that somatic embryos were first induced and developed on cotyledon segments but this phenomenon was not observed when callus was induced in the cotyledon segments.

Histological Study

Histological studies ravealed that somatic embryoids developed directly from cotyledon explants without intervening callus stage. Similar finding is reported by Seran et al. (2007). The protuberances were mainly found on epidermal tissues at the edge margin located between convex and concave surface of cotyledon segments. The outgrowths were formed by the proliferation of subepidermal cells and the initial growth was often followed by the rupture of epidermis (Puigderrajols et al., 2000).

The epidermal cells near the edges of cultured cotyledon explants divided and formed undifferentiated parenchyma cells (Figure 2A) which have thin wall and largely vacuolated. Parenchyma cells are capable of cell division and divided into meristematic cells (Figure 2B) which are small and had nucleus in centre. These meristematic cells further differentiated into embryogenic cells. Typical embryogenic cells are small with a large nucleus with a very densely stainable nucleous, dense cytoplasm and small vacuoles (Sharp et al., 1980). The continuous cell division led to form somatic embryoids where the cells were arranged in organized manner. The two distinct poles of root and shoot were clearly observed in the longitudinal section of somatic embryos (Figure 2C). In the present study, vascular connection was clearly observed between the vascular stands of the mother plant and developing embryos (Figure 2D).

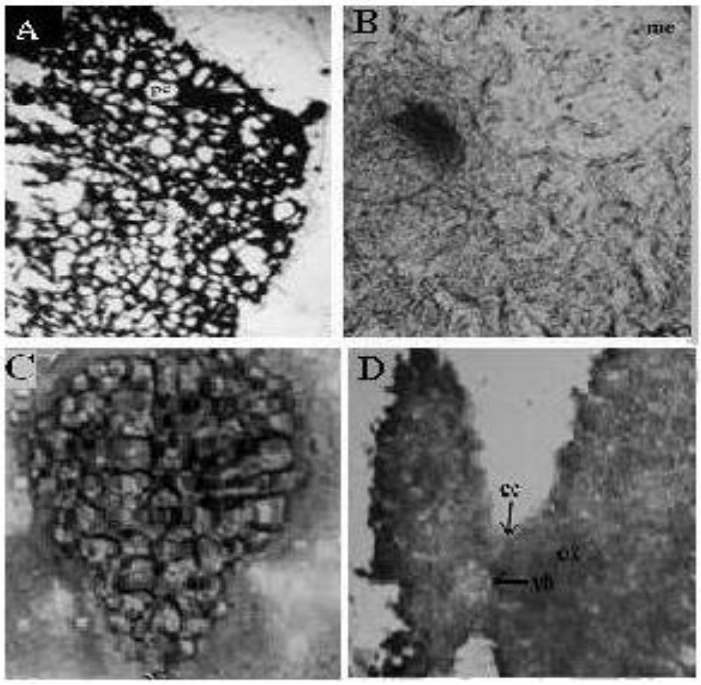

Figure 2: Histology of cotyledon explants cultured in MS medium

A: Transverse section of parenchyma cells from cultured explant in 8 mg/L at the second week (×160)
B: Cross section of meristematic cells from cultured explant in 8 mg/L BAP at the third week (×100).
C: Transverse section of somatic embryo from cultured explant in 8 mg/L BAP at the fourth week (×160)
D: Transverse section of somatic embryo directly originated from cultured explant in 10 mg/L BAP at the fourth week (×100)
Note: pc- parenchyma cell; mc- meristematic cell; cc- vascular connection.

CONCLUSION

In the present study, the results revealed that the incidence of root organogenesis increased with the decrease of concentration of BAP. The medium without exogenous plant growth regulators exhibited more root organ formation in cultured cotyledon explants. Among the different concentrations (0-10 mg/L) of BAP, 8 mg/L was the most responsive concentration of BAP for the induction of somatic embryoids in cultured cotyledon explants of cashew under *in vitro* conditions.

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