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Induction of somatic embryogenesis from cotyledon explants of cashew (*Anancardium occidentale* L.)

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This experiment was aimed to induce somatic embryos from mature cotyledon explants of cashew (*Anacardium occidentale.* L). The sterilized cotyledon explants (10 x 5 mm^2) were cultured on MS basal medium with growth regulators. Morphological and embryogenic responses were examined at regular intervals. The results indicated that more than 92% of explants showed morphological response among tested treatments. Nodule induction was observed in all treatments and higher % (80%) was noted in MS medium supplemented with 2 mg/l BAP (benzylamino purine). The root formation was higher (47%) in MS medium contained 2 mg/l kinetin and 2 mg/l NAA (naphthaleneacetic acid) where longest roots (120 mm) were recorded. Further, it was noted that the medium contained 2 mg/l BAP showed higher % of somatic embryoid formation directly from cotyledon explant. Subsequently somatic embryos were noted 2-3 weeks after culture in MS basal medium without growth regulators. The developed protocol in the present study will be useful for the production of somatic embryos of cashew.

Introduction

Cashew (*Anacardium occidentale* L.), belonging to the family *Anacardiaceae,* is an evergreen tree and has been naturalized in many tropical and subtropical countries including Sri Lanka. The main product of this tree, the cashew nut, is globally popular as dessert. Cashew nut ranks third in the world market and it is considered as important cash crop because of its great social and economic significance for Asian countries.

In Sri Lanka, cashew is cultivated in almost all the districts, and is small-scale farmer crop. Major buyers of Sri Lanka's cashew nuts are the Middle East countries. Though cashew cultivation is expanding, it can be improved to take up future economic development by improving both production and productivity. For rapid expansion of cultivation, it requires an efficient method

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to produce uniform planting materials of improved varieties in a large scale. 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). The increasing demand for cashew nut in the international market has generated a strong potential for its export and this has resulted in the commencement of research and breeding programmes, leading to release of high yielding elite varieties for commercial exploitation (Gogte and Nadgauda, 2000).

Development and improvement of cashew cultivation in the Eastern region of Sri Lanka is very much realized after the recent Tsunami disaster, which destroy about 1441 acres of cashew plantation (Anon, 2007). However, the cashew production in Sri Lanka continues to be limited by low and variable nut yield, nut quality and susceptibility to pests and diseases. These drawbacks can be overcome by using genetically improved varieties *via* breeding programme. Since it is highly cross pollinated, woody and perennial in nature, the progress made in improvement of this crop is slow therefore there is a need to attempt circumventing these adverse factors through *in vitro* culture (Aliyu and Awopetu, 2005). The application of *in vitro* technologies has been employed for large varieties of trees, mostly temperate species (Kannan and Jasral, 1996). *In vitro* culture in cashew could offer an efficient, rapid and possibly a cost-effective system which could be used particularly for multiplying large numbers of plantlets of elite cashew genotypes for breeding and production purposes (Kenbo and Houng, 1999) and tissue culture technique of cashew through micropropagation and somatic embryogenesis has been attempted in the past. Das *et al*. (1996) however, reported that even though crops closely related to cashew have been propagated *in vitro*, satisfactory results have not been achieved for cashew. Mantel *et al*. (1997) also remarked that cashew is strongly recalcitrant to *in vitro* culture techniques and only limited success has been achieved. In efforts to develop a micropropagation protocol for cashew, a complete regeneration system has been obtained through organogenesis (Philip, 1984; Lievens *et al*., 1989; Falcone and Leva, 1990; Hedge *et al.*, 1991; D'Silva and D'Souza, 1992; Das *et al*., 1996; Thimmappaiah and Samuel, 1999).

Somatic embryogenesis as a tool for *in vitro* propagation and genetic improvement could play a more significant role in the production of cashew and the recent biotechnological advances like gene cloning and gene transfer offer great promise for rapid improvement of genotypes with desirable traits and integrate with the technique of somatic embryogenesis (Akula and Akula, 2005). Although considerable research focus and efforts have demonstrated the potential for Somatic embryogenesis propagation of a large variety of plant

species, success has been achieved only at the research scale (Aitken-Christie *et al*., 1995). Direct somatic embryogenesis from mature and immature cotyledon sections and from callus culture derived from nucellar tissue of cashew nut, has been reported (Hegde *et al*., 1991, 1994; Gogte and Nadgauda, 2000). *In vitro* culture techniques can provide adequate clonal planting materials however there are very few reports for the somatic embryogenesis from the cotyledon explants. Therefore, this experiment was done to select the most suitable medium for the induction of somatic embryos from cotyledon explants of cashew.

Materials and methods

Explant source

Mature cashew seeds of Trinidad variety were collected from Hardy estate, Kiran Plantation of Cashew Corporation of Sri Lanka, from open pollinated field grown trees of 39 years old and then cotyledons were separated carefully from seeds with sterile knife. Subsequently embryos were removed from cotyledons before being used in this experiment.

Sterilization of cotyledons

The collected cotyledons were washed under running tap water and then they were dipped in 70% of ethanol for 1 min and subsequent immersed in Clorox (30%) for 30 min with 2-3 drops of Tween 20. Thereafter, the cotyledons were rinsed thoroughly 5 times in sterilized distilled water until free from clorox residue under aseptic condition.

Induction media

Full strength of MS (Murashige and Skoog, 1962) salt media supplemented with vitamins, sucrose (3%), agar (0.8%) and various combinations of cytokinins and auxin (Table 1) were used for the induction of somatic embryos from cotyledon explants. The pH of each medium was adjusted to 5.8. Thereafter, 10 ml of each medium was poured into every culture bottle, and covered by plastic lid and labelled properly to avoid misuse and confusion of the media. Culture bottles containing media were autoclaved at 121^{o}C for 20 min at 15 psi, then, culture bottles were kept stationary and cooled. After four days, culture bottles were used for inoculation of cotyledon explants.

Table 1. Media used for induction of somatic embryos in this experiment

Treatments	Composition of media
*MS_1	MS + 2 mg/l kinetin + 2 mg/l NAA
MS_2	MS + 2 mg/l kinetin + 1 mg/l NAA
MS_3	MS + 2 mg/l BAP + 1 mg/l NAA
MS_4	MS + 2 mg/l BAP + 0 mg/l NAA
MS_5	MS + 2 mg/l kinetin+ 0 mg/l NAA

MS- Murashige and Skoog (1962).
Control treatment (Jha and Das, 2004).

Inoculation of explants

Sterilized cotyledon segments (10 mm long, 5 mm width) were excised aseptically under laminar hood and cultured on MS basal media containing 2 mg/l kinetin or 2 mg/l BAP in combination with 1 mg/l or 2 mg/l or without NAA. Five explants were cultured in each bottle having capacity of 125 ml. Each treatment had three replicates each having 20 explants. This experiment was repeated twice.

Culture incubation

After inoculation of explants, the culture bottles were incubated separately in photoperiod of 16 hrs light and 8 hrs dark, and temperature of $25\pm2^{o}C$ under aseptic conditions. Observation was done every week. After 4 weeks of culture, after removing the root organ, the cotyledon explants in different media were separately transferred to MS basal medium without growth regulators for the production of somatic embryos.

Statistical Analysis

Data were analysed using the SAS software. The percentage data were first subjected to Arcsine or Square root transformation and then analysed in analysis of variance. The significant difference between means was estimated using Duncan's Multiple Range Test at 5% significant level.

Results and discussion

In vitro response

Morphological responses such as swelling of explants, crack formations etc. were observed within one week of culture (Plate 1) and above 92% of

explants showed morphological response in all treatments. At first week of culture, explants turned to brownish green and root formations were noted in explants within two weeks. The size of the explant increased two third folds after three weeks of culture. It may be possibly due to active cell division and expansion of explant. After 3-4 weeks in culture, nodular structures and small globular structures were conspicuous on the excised cotyledons. The explants cultured in medium where NAA used as the auxin were dark in colour and hard in texture than explants cultured alone with BAP.

Nodule formation

Among tested combinations, nodules induced in all treatments but all explants did not exhibited embryogenic response within 4 weeks of culture. Higher % (80%) of nodule formation was observed in MS medium contained 2 mg/l BAP (Table 2) and MS_4 was not significantly differed from other treatments except MS_1. More nodules were formed at the cut end of the cotyledon explant. Not all the nodules become embryogenic. Seran *et al*. (2006) described that, nodules obtained surface of leaves did not show embryogenic potential when they were maintained in ½ MS medium supplemented with 0.2 mg/l NAA and 2 mg/l BAP. In this study, nodules in cultures were smaller in size and were of various sizes. Formation of such nodular structures is reported in somatic embryogenesis of *Cocus sativus*, *Oryza sativa* and in *Musa* cv Bluggoe (ABB) (Sannasgala, 1989). Seabrook and Douglass (2001) demonstrated, that pale yellow, nodular callus formed at the cut surface of leaf, root and tuber slice explants in potato when cultured in 0.15 µm/l BAP. In this experiment also greenish yellow friable nodular callus was noted in cotyledon explants cultured in media containing NAA.

Table 2. Nodule formation in cotyledon explants cultured in various combinations of NAA, kinetin and BAP at 4th week

Treatments	**Growth Regulators (mg/l)**			**% explants shown**
	BAP	Kinetin	NAA	**nodule formation**
MS_1	0	2	2	53.3 ± 6.7 ab
MS_2	0	2	1	36.7 ± 6.7 b
MS_3	2	0	1	46.7 ± 8.8 b
MS_4	2	0	0	80.0 ± 10.0 a
MS_5	0	2	0	46.7 ± 14.5 b
F test				*

Value represents mean ± stand error of two independent experiments, each with 60 explants per treatment. F test: - *: $p < 0.05$.

Mean followed by the same letters in each column are not significantly different in each column according to Duncan's multiple Range Test at 5 % significant level.

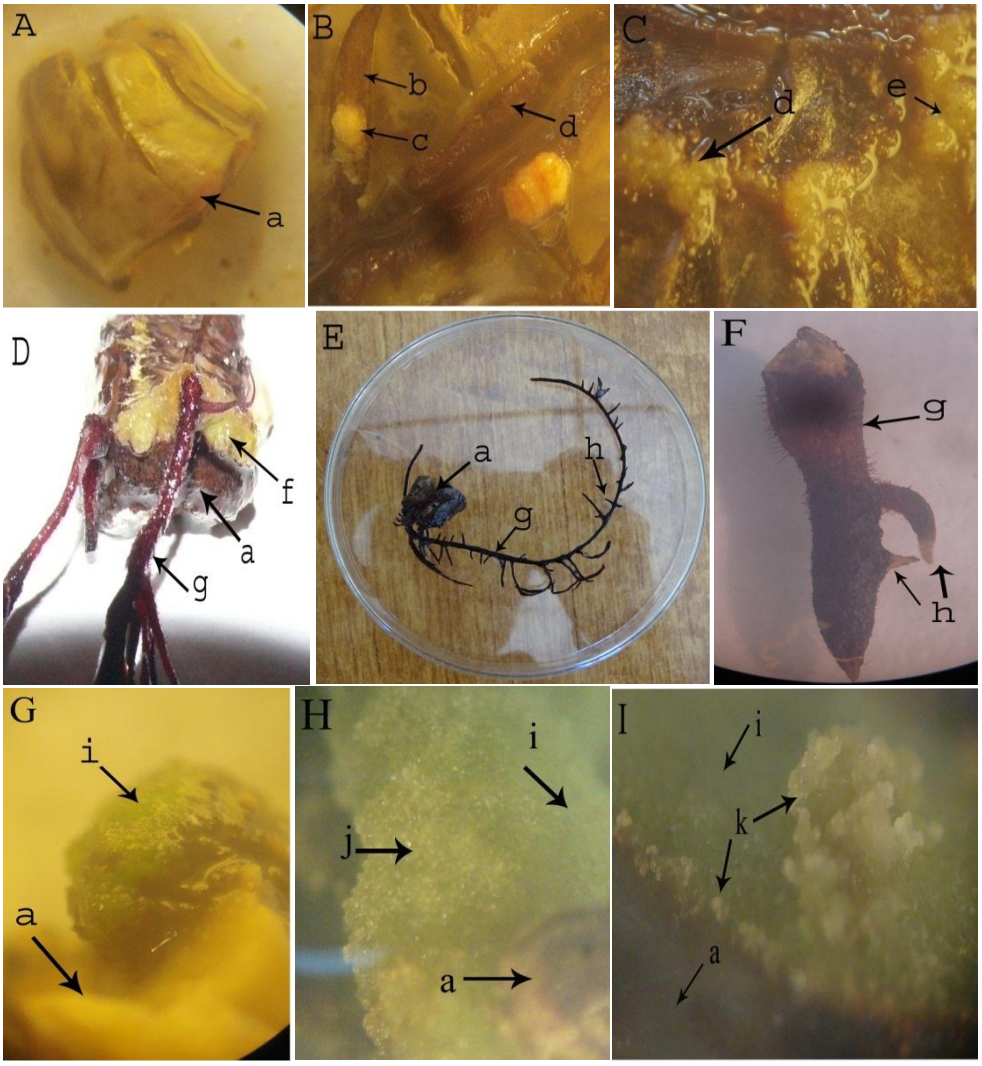

Plate 1: Induction of somatic embryos from cotyledon explants.

A-Crack formation on cotyledon explant at 1st week of culture (x200), B-Formation of protuberant tissue on explant cultured in MS1 at 2nd week of culture (x200), C- Nodular formation on explant cultured in MS + 2 mg/l kinetin + 1 mg/l NAA at 3rd week of culture (x200). D-Morphogenetic response of explant cultured in MS + 2mg/l kinetin + 2 mg/l NAA (x 3), E-F. Longest and shortest roots formed from explants cultured in MS + 2 mg/l kinetin + 2 mg/l NAA. (E: x0.6; F: x200), G- Green callus formation in MS + 2 mg/l kinetin + 2 mg/l NAA at 4th week (x 200), H- Yellowish green friable callus formation from explant at 4th week after culture in MS + 2mg/l BAP + 1mg/l NAA(x200), I- Formation of embryos from top of the callus and cut end surface of explant cultured in MS + 2 mg/l BAP (8th week after initial culture) (x200).

Note: a) explant; b) crack; c) swelling of tissue; d) reddish nodule; e) yellowish nodule; f) yellowish callus; g) main root; h) lateral root; i) greenish callus; j) globular structure; k) embryo formation.

Callogenesis

Callus induction was observed at the cut edges and on the abaxial surface of the explants within 4 weeks. They were green in colour and friable. No callus was induced when explants were cultured on MS media supplemented with cytokinins (kinetin / BAP) alone. Higher percentage of callus was induced in MS media supplemented with 2 mg/l NAA + 2mg / l BAP. Molina (2004) described that 80-100 percentage of callus induction from petiole explants of *Salvia canariensis* L. at high concentrations of BAP (8.88 µM) and NAA (21.48 µM). And also highest callusing rate of 50 % was observed in the medium containing 1.0 mg/l NAA + 1.0 mg /l BA in intermodal explants of potato cultures (Shirin *et al*, 2007). In *Acacia sinuata (Lour.) Merr* culture calluses were induced from cotyledon explants on Murashige and Skoog (MS) medium containing, 8.1 µM NAA and 2.2 µM BA (Vengadesan et al, 2003). The combinations 5.4 µmol / l NAA and 4.44 µmol / l BAP or 2.7 µmol / l NAA and 13.32 µmol / l BAP induced callogenesis in 90% of the explants of cotyledonary leaves of *E. Camaldulensis.*(Dibax *et al*., 2005).

Root organogenesis

A high rate (46.7%) of root formation was observed in MS_1 at 4^{th} week of culture. Hamidou *et al*. (2004) described the cotyledon tissues of cotton have the propensity for excessive root formation, resulting in less callus formation in MS medium with 1 mg/l kinetin and 2 mg/l NAA. In the present study, MS_1 significantly differed from other treatments on root organogenesis % at 4^{th} week (Table 3). It is lower in MS_4 which contained BAP only when compared to other treatments. Reason for the root formation among tested treatments in the present study may be presence of endogenous and exogenous auxin in explants. Pareek and shash (1998) indicated that induction of roots in culture is the common feature of *in vitro* system of woody plant. More number of roots per explant (6%) obtained in MS_1 and root length ranged from 5 mm to 120 mm (Plate 1). Dutta Gupta *et al*. (1997) described that high concentration of NAA (1-4 mg/l) with low concentration of BA (0.1-0.5 mg/l) yields roots with little callus formation. More lateral roots were induced from induced main roots and increases in root length were also noted until subculturing.

Table 3. Effect of different media on root organogenesis in cultured cotyledon explants

Treatments	Root organogenesis %		Number of roots per explant At 4th week
	At 2nd week	At 4th week	
MS_1	40.0 ± 9.0 a	46.7 ± 8.8 a	6.0 ± 0.6 a
MS_2	20.0 ± 0.0 b	23.3 ± 3.3 b	5.0 ± 0.6 a
MS_3	16.7 ± 3.3 b	23.3 ± 3.3 b	2.7 ± 0.3 b
MS_4	13.3 ± 3.3 b	20.0 ± 0.0 b	2.7 ± 0.3 b
MS_5	26.7 ± 3.3 ab	30.0 ± 0.0 b	5.3 ± 0.7 a
F test	*	*	**

Value represents mean ± stand error of two independent experiments, each with 60 explants per treatment. F test: * $p < 0.05$.
Mean followed by the same letters in each column are not significantly different in each column according to Duncan's multiple Range Test at 5 % significant level.

Embryogenic response

Higher % of somatic embryoid formation was observed in medium containing 2 mg/ l BAP alone (Table 4) after removing the root organ from cultured explants at 4th week. Subsequently the cotyledon explants were subcultured into MS basal media without hormones. Somatic embryos were noted 2-3 weeks after transfer of explants. BAP was marginally more effective than kinetin (Park and Facchini, 1999) and higher rate of embrygenic response in MS medium containing BAP at 5 mg/l observed in tea cotyledons (Jha *et al*., 1992) and also cotyledonary type of somatic embryos were formed on cotyledon segments in the presence of 0.2 mg/l NAA with 2mg/l BAP (Seran *et al*., 2007). Direct somatic embryogenesis was also obtained from hypocotyl explants of *Eucalyptus camaldulensis* without an intervening callus phase on MS basal medium containing 0.5 mg/l BA (Prakash and Gurumurthi, 2009). Somatic embryos are produced in clumps and were translucent formed directly from cotyledons and indirectly from friable yellowish green callus (Plate 1).

Most of the somatic embryos induced at the cut end. This is supported by Akula and Dodd (1998) on tea nodal explants cultures on MS with 0.5-1.0 mg/l BAP. Embryogenic areas were clearly visible from the rest of the callus by their globular appearance through light microscope. Ravishankar Rai and McComb (2002) demonstrated that the globular structures observed in embryo explants of sandalwood cultured on MS medium with BAP at 1.6-6.4 µm/l. BAP was found to be essential for obtaining morphogenetic differentiation of the callus. Absence of BAP in the medium resulted poor induction (Gallo-Meagher *et al*., 2005). Mandal *et al*. (1995) described that, concentration of

BAP higher than 0.5 mg/l favoured the callusing of cotyledonary leaves, obtained in safflower.

Table 4. The embryogenic response of cotyledon explants cultured in different media

Treatments	Callogenesis at 4th week	Somatic embryogenesis (%) at 8th week of culture	
		Indirect method	Direct method
MS_1	20.0	8.3	0.0
MS_2	28.3	6.7	3.3
MS_3	33.3	5.0	6.7
MS_4	0	0	16.7
MS_5	0	0	10.0
F Test			ns

*8weeks after initial culture

Value represents mean ± stand error of two independent experiments, each with 60 explants per treatment. F test: * $p < 0.05$; F test: ns – not-significant

Mean followed by different letters are significantly different in each column according to Duncan's Multiple Range Test at 5 % significant level.

Conclusion

The present study describes a protocol for the induction of somatic embryos from mature cotyledon explants of cashew. Higher nodule formation and high rate of somatic embryogenic response exhibited in MS medium supplemented with 2 mg/l BAP. Direct and indirect somatic embryos were formed at the end of explant and from friable yellowish green callus respectively, when cotyledon explants cultured in MS medium without or with NAA. The embryogenic systems can be used as a method of multiplication of the selected superior variety of cashew plant. This protocol would help in rapid propagation of cashew (*Anacardium occidentale* L.) and other tropical woody plants. Further, investigations are necessary on large scale production of somatic embryos and their subsequent germination of somatic embryos *in vitro.*

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