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Original Research Article

Impact of culture media and growth hormones on callus induction and root organogenesis in cotyledon segment of *Anacardium occidentale* L.

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Abstract: The present study aimed to determine the optimal cultural conditions for the initiation of callus and root organogenesis in cotyledon segment of cashew (*Anacardium occidentale* L). One of the major problems in *in vitro* propagation of cashew is the blackening/browning of tissue, which is mainly due to the exudation of phenolic compound from the cut end of explants. In the current study, inhibitory effects of different treatments such as adding activated charcoal, polyvinylpyrrolidone (PVP), ascorbic acid to the medium, continuous subculture passages or dark incubation were investigated against browning problem of *A.occidentale*. Adding a combination of 3g/L activated charcoal, 75 mg/L ascorbic acid and 2.5g/L polyvinylpyrrolidone (PVP) to the Murashige and Skoog (MS) basal medium and Woody Plant (WP) medium was found as the most effective treatment. The cotyledonary explants, excised from mature seeds were screened at various concentration and combinations of BAP and 2,4-D in the range 0-75mg/L for callus production. The developed callus in this medium was pale yellow, light to dark brown, semi friable, compact and slow growing. The result also revealed that WP medium with high concentration of 2,4-D (50 mg/L) and BAP (50 mg/L) exhibited profuse root organogenesis than MS medium.

Key words: *Anacardium occidentale*, activated charcoal (AC), polyvinylpyrrolidone (PVP), growth regulators, callus induction, root organogenesis.

Introduction

Cashew (*A.occidentale* L.) is an important tropical horticultural cash crop. Nutritive kernals and liquid from nut shell (CNSL) are considered as the most economic parts of this crop. CNSL mainly consists of anacardic acid, cardol and cardanol. The biological activities of CNSL components includes molluscicidal activity (Kubo *et al.,* 1986), anti-tumour activity (Kubo *et al.,* 1993), anti-microbial activity (Muroi and Kubo 1993), anti-oxidant activity (Amorati *et al.,* 2001) and xanthine oxidase inhibition (Masuoka and Kubo 2004) etc. *In vitro* technologies have applications in large variety of trees, chiefly temperate species (Kannan and Jasari 1996). Persely (1992) also highlighted the need for the applications of bio techniques to many tropical crops with a view to rectifying constraints to their productivity. *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 (Kembo and Hornung 1999). Limited success has been achieved in cashew culture because of its recalcitrant nature (Mantell *et al*., 1998; Boggetti *et al*., 1999). Cashew was found difficult to induce callus from mature plant tissues (nodal segments or shoot apices) due to excess phenolic exudation, microbial contaminations and resistive nature (D'Souza *et al.,* 1996). However, callus induction has been reported from seedlings explants (Lakshmi 1989; Leva and Falcone 1990), cotyledonary tissues excised from mature seeds (Kamshananthi and Thayamini 2012) and immature zygotic embryos (Ananthakrishnan *et al.,* 1999).

The problem of phenolic exudation during tissue culture was reported in many plant species including fruit trees like cashew (Aliyu 2005), litchi (Chandra and Padaria 1999), avocado (Castro *et al.,* 1995) and guava (Meghwal *et al.,* 2000). Success or failure of tissue culture largely depends on browning of the culture medium especially in fruit trees like cashew. The objectives of the present study were to investigate the possible means for successful initiation and establishment of callus cultures from cotyledon explants via preventive approaches

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to overcome failures caused by phenolic browning.

Materials and Methods

Plant material

Mature cashew (*A.occidentale*) seeds were procured from the mother stock trees growing at the Kerala State cashew development corporation, Mundakkal, Kollam, Kerala, India and cotyledons were carefully excised.

Explants disinfection and culture conditions

Cotyledonary explants were washed thoroughly under running tap water for 20-30 minutes and then soaked in 1% Labolene (Qualigens, India) detergent for 5 minutes to remove all debris and other contaminants. Again the material was washed thoroughly with tap water to remove all detergent particles. Further processing was done in sterile LAF cabinet. Inside the LAF, explants were cut into small pieces of 5 mm width and 10 mm length and its surface sterilization was done by using 70% (v/v) ethanol for 30 seconds, followed by 0.1% aqueous $HgCl_2$ solution for 3-6 minutes. After rinsing with sterile water for 4-5 times, these explants were inoculated to different medium with the various concentrations of activated charcoal, ascorbic acid and PVP alone as well as in combinations to reduce the exudation of phenolic substances.

In the present study, two types of media viz., MS (Murashige and Skoog 1962) and WP (Lloyd and Mc Cown 1980) media were evaluated for their ability to support the morphological responses of cotyledon cultures. WP media supplemented with 2,4-D and BAP alone and with its different combinations were used for callus induction in cotyledon. p^H was adjusted to 5.2 for WP medium and 5.8 for MS medium, and gelled with 0.8% agar (Sigma, INDIA). The media were sterilized by autoclaving at 121^0C for 15 min. For initial *in vitro* response, 10-15mL media were dispensed in pre - sterilized culture tubes and plugged with non-absorbent fresh cotton. The cultures were incubated at 25 ± 2^0 C under dark.

Data analysis

Percentage of callus induction from cotyledonary explants, the morphology and colour of callus were recorded after 2 weeks of culture. Data were expressed as means and standard deviation (SD) of three replicate determinations. All statistical analyses were carried out using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008). To determine whether there were any differences among the means, one way analysis (ANOVA) and the Duncans New Multiple range test were applied to the result at 0.05 level of significance ($p < 0.05$).

Results and Discussion

Effect of different time duration in the control of infection with 0.1% $HgCl_2$ in in vitro cultured A. occidentale

Among the various surface sterilization procedures tried, an initial thorough wash with running tap water for 15 minutes followed by a wash with an aqueous solution of Labolene and thus treatment with 0.1% $HgCl_2$ for 5 minutes followed by 4-5 times rinse in sterilized distilled water helped to reduce infection rate in cotyledonary explants. The present study material following the above treatment, 77.77±1.57% of the explants were recovered in culture without any infection and it was not significantly different ($p < 0.05$) as compared with the time duration of 3 and 6 minutes. Each treatment had 30 explants and all the experiments were repeated thrice. The data on surface sterilization of explants are depicted in Fig.1.

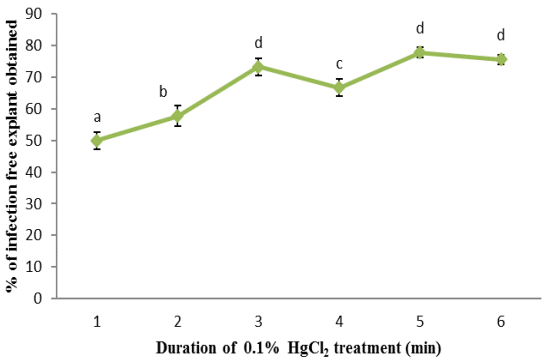

Figure 1: Surface sterilization of explants using $HgCl_2$ treatment; values with different letters are significantly different ($p < 0.05$), (n=3, error bars represent standard deviation).

In vitro prevention of browning in cashew culture

Tissue browning is a constant detriment that renders tissue culture work difficult in this species. The presence of phenolic compounds and high ployphenol

oxidase activity cause explants browning which limits morphogenic response. Result of the present study revealed that explants maintained on a medium without antioxidants leached out phenolic compound into the medium, which leads to necrosis of the explants. While the addition of 3g/L activated charcoal, 75mg/L ascorbic acid and 2.5g/L PVP to the medium showed better results to control browning in various explants of cashew, which was supported by earlier workers (D'Souza *et al.,* 1996; Das *et al.,* 1996). Results of the present study also showed that, continuous subculture and dark incubation were more effective in controlling oxidative browning of cashew explants. This observation was in agreement with previous reports (Das *et al.,* 1996; Mantell *et al.,* 1998). The present study materials following the above treatments, 86.78 % of the explants were responded well in culture and it was significantly different ($p < 0.05$) with other antioxidant treatments (Fig.2).

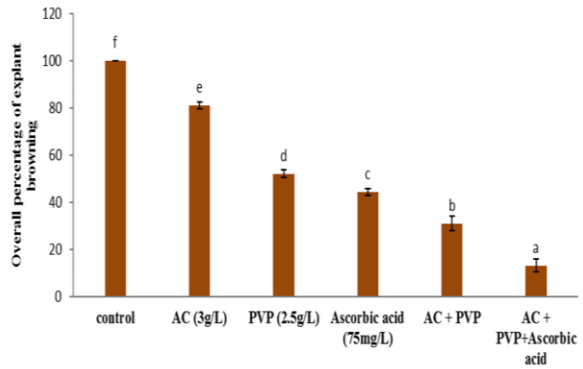

Figure 2: Effect of various concentrations of antioxidants used for removing browning in *A.occidentale*; values with different letters are significantly different ($p < 0.05$), ($n = 3$, error bars represent standard deviation).

Cotyledon culture

Morphological Response: Noticeable colour changes were observed in cotyledon explants cultured in MS and WP media at the first week of culture, and they turned light brown in colour. Most of the explants enlarged around 3/4 fold; which may be due to active cell division and expansion. Crack formation was started at the first week on the edges of explants; cracks were formed due to the swelling of explants (Fig.3B). Browning of media was also occurred around the explants at the second week of culture. Exudation of secondary metabolites from the explants may be the reason for browning of media. The number of cracks found to be high in those explants cultured on WP medium supplemented with combination of 2,4-D and BAP compared to MS medium. Greenish or yellowish white nodular like structures observed on the edge of the surface, concentrated towards the concave side of the explants cultured on WP medium at 50mg/L (2,4-D) (Fig.3C,D). After four weeks of culture they became light brown in colour. This concentration and combination for nodular formation in cotyledonary explants was not reported earlier and it was in contrast with the finding of earlier studies (Bavathrine and Thayamini 2010; Kamashananthi and Thayamini 2012).

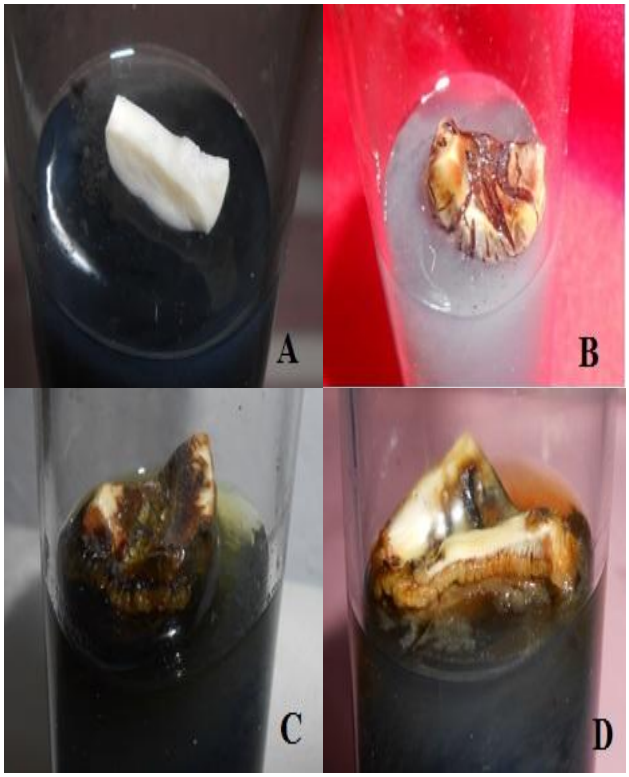

Figure 3: *In vitro* morphogenic response of cotyledon segment cultured on WP medium at 50 mg/L 2,4-D. (A) Inoculated cotyledon explant, (B) Crack formation on the cotyledon explant at the first week of culture, (C) Greenish white nodular like structures on cotyledon explants after one week of culture, (D) Yellowish white nodular like structures on cotyledon explants after two weeks of culture.

Callus induction: In this investigation, it was observed that the auxin 2,4-D alone was poor in inducing callus from cotyledon explants. In WP medium, maximum callusing (62.21 ± 5.09 %) was obtained when 2,4-D at 50 mg/L was used in combination with BAP at the same concentration (Table 1) and it was found to

be significantly different ($p < 0.05$) as correlated with the other hormonal concentrations. This concentration and combination for callus induction in cotyledon segments were not seems to be reported earlier. The callus so formed was compact in texture and light to dark brown in colour and slow growing (Fig.4). Callus induction was occurred from cut end of cotyledon segments after 2 weeks of culture; furthermore, entire surface of explants were covered with callus within 4 weeks of culture (Fig.4D). All callus cultures obtained from cotyledon explants were transferred to fresh WP medium supplemented 2,4-D and BAP once in every weeks for proliferation. Callus induction in cotyledon segment was also reported on MS medium with high concentration of 2,4-D and BAP (Kembo and Hornung 1999).

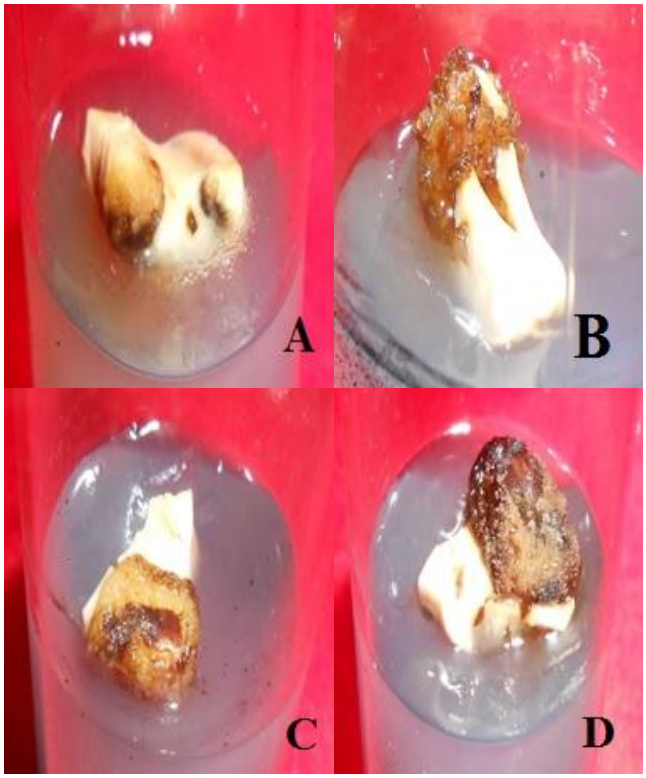

Figure 4: Induction and proliferation of callus from cotyledon explant of *A.occidentale* on WP medium (A) Direct callus initiation after one week of culture, (B) Callus after 2 weeks of culture, (C) Callus after 3 weeks of culture, (D) Callus after 4 weeks of culture.

Table 1: The callus induction from the cotyledon explants of *A.occcidentale* inoculated on the WP media supplemented with 2, 4-D and BAP alone and with different combinations after 4 weeks of culture.

S.No	Growth hormones (mg/L)		Days to callus initiation	Callus score	Explants forming callus (%)	Callus texture	Callus colour
	2,4-D	BAP					
1	0	0	NC	NC	NC	NC	NC
2	10	-	NC	NC	NC	NC	NC
3	25	-	NC	NC	NC	NC	NC
4	50	-	15	++	19.99±3.33a	semi friable	light brown
5	-	10	NC	NC	NC	NC	NC
6	-	25	NC	NC	NC	NC	NC
7	-	50	NC	NC	NC	NC	NC
8	25	10	NC	NC	NC	NC	NC
9	25	25	NC	NC	NC	NC	NC
10	50	10	21	++	47.66±5.13c	compact	dark brown
11	50	25	19	++	27.77±5.09ab	compact	dark brown
12	50	50	9	+++	62.21±5.09d	compact	dark brown
13	75	75	17	++	29.99±3.33b	compact	light brown

NC = no callus formed; + = callus only formed at the edge of the explant; ++ = callus formed more at the edge; +++ = callus covered the surface; ++++ = callus covered more on the surface. For each treatment the means within the column by different letters are significantly different at $P < 0.05$. Each value is expressed as the means ±SD.

Root organogenesis: The results of the present study revealed that the root induction was occurred in cotyledon segment cultured on WP and MS medium augmented with and without growth regulators. At the fourth week, maximum number of root organs (9.66 ± 1.03) was observed on the WP medium at the concentration of 50 mg/L 2,4-D and 50 mg/L BAP and it was significantly different ($p < 0.05$) with other hormonal concentrations (Table 2). Though in MS medium it was (8.00 ± 0.81) at the concentration of 10 mg/L BAP and it was also significantly different ($p < 0.05$) with other

hormonal concentration (Table 3). These results are in contrast to the findings of earlier study (Bavatharine and Thayamini 2010) where the formation of root organs was not seen with increase in the concentration of BAP (8-10 mg/L) in MS medium.

Table 2: The average number and length of roots per cultured explants in WP medium.

S.No	Growth hormones (mg/L)		No: of roots per explants		Length (cm) of roots per explants	
	2,4-D	BAP	2 weeks	4 weeks	2 weeks	4 weeks
1	0	0	1.52± 1.2	3.40 ± 1.14ab	1.50 ± 0.53	2.14 ± 0.69a
2	10	-	2.50 ± 1.2	5.60 ± 1.15c	2.12 ± 0.75	4.00 ± 0.70bc
3	25	-	4.11 ± 0.69	6.00 ± 0.66c	1.50 ± 0.54	3.00 ± 0.44ab
4	50	-	4.66 ± 1.52	8.00 ± 0.81d	3.85 ± 0.67	4.12 ± 0.83bc
5	-	10	1.50 ± 1.3	3.00 ± 0.70a	2.00 ± 0.53	5.10 ± 0.78cde
6	-	25	1.52 ± 1.2	3.40 ± 1.14ab	2.80 ± 0.69	5.66 ± 0.70de
7	-	50	4.33 ± 1.55	6.23 ± 0.83c	1.25 ± 0.46	3.70 ± 0.82bc
8	25	10	3.33 ± 1.52	5.00 ± 0.70bc	1.63 ± 0.80	2.75±0.88ab
9	25	25	3.50 ± 0.57	5.87 ± 0.83c	5.60 ± 0.95	6.23±0.83e
10	50	10	3.66 ± 0.44	4.00 ± 0.81ab	3.33 ± 1.52	5.83±0.93de
11	50	25	4.50 ± 1.3	5.83 ± 0.83c	4.00 ± 0.81	6.00±0.66de
12	50	50	7.52 ± 1.31	9.66 ± 1.03e	2.00±0.89	4.62±0.90cd

Each treatment had 30 explants and all the experiments were repeated thrice. For each treatment the means within the column by different letters are significantly different at $P < 0.05$. Each value is expressed as the means ±SD.

Table 3: The average number and length of roots per cultured explants in MS medium

SI.No	Growth hormones (mg/L)		No. of root per explants		Length (cm) of root per explants	
	2,4-D	BAP	2 weeks	4 weeks	2 weeks	4 weeks
1	0	0	1.52 ± 1.2	3.40 ± 1.14bc	1.50 ± 0.53	2.14 ± 0.69a
2	10	-	3.50 ±0.57	5.87 ± 0.83de	1.63 ± 0.80	2.75 ± 0.88ab
3	25	-	2.50 ± 1.2	5.60 ± 1.15de	2.00 ± 0.89	3.00 ± 0.44ab
4	50	-	4.11 ±0.69	6.00 ± 0.66de	2.12 ± 0.75	3.70 ± 0.82ab
5	-	10	4.66 ±1.52	8.00± 0.81f	3.33 ± 1.52	4.12 ± 0.83b
6	-	25	0.56 ±0.56	1.50± 1.3a	1.63 ± 0.80	2.75 ± 0.88ab
7	-	50	1.68 ± 1.3	3.00 ± 0.70ab	1.96 ±0.59	2.89±1.14ab
8	25	10	3.66 ± 0.44	5.00 ±0.70cde	2.08 ±0.49	3.10±0.55ab
9	25	25	2.50 ± 1.2	3.40 ± 1.14bc	1.50 ± 0.53	2.75 ± 0.88ab
10	50	10	3.00 ± 0.70	4.66 ±1.52bcde	1.25 ± 0.46	3.60 ± 0.82ab
11	50	25	4.33 ± 1.55	6.23± 0.83e	2.02 ± 0.71	2.98 ± 0.88ab
12	50	50	2.80 ± 0.69	4.12 ± 0.83bcd	2.13 ± 0.89	3.72 ± 0.66ab

Each treatment had 30 explants and all the experiments were repeated thrice. For each treatment the means within the column by different letters are significantly different at $P < 0.05$. Each value is expressed as the means ±SD.

In the current study it was observed that root organs were formed on the concave side of explants (Fig.5 & 6). It may be due to the accumulation of endogenous auxin in this region of cotyledons (Pareek and Shashi 1998). The highest length (6.23 ± 0.83 cm) of root organ was observed at the fourth week in WP medium which augmented with 25 mg/L 2,4-D and 25mg/L BAP (Table 2) while the highest root length (4.12 ± 0.83 cm) was noticed in MS medium supplemented with 10 mg/L BAP (Table 3) which was in confirmatory with earlier report (Bavatharine and Thayamini 2010). The results also revealed that WP medium showed profuse root organogenesis while MS medium produced thin and short roots (Fig.5 & 6).

Figure 5: A & B - Root organogenesis from cultured cotyledon explants on WP medium after four weeks of culture.

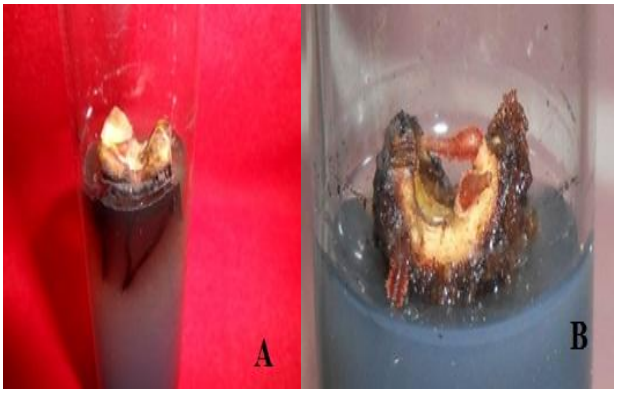

Figure 6: A & B - Root organogenesis from cultured cotyledon explants on MS medium after four weeks of culture.

Conclusion

The present investigation showed that amendment of MS and WP medium with 3g/L activated charcoal especially when mixed with 2.5g/L PVP and 75 mg/L ascorbic acid, continues subculture or dark incubation were exhibited positive effect on control of browning in *in vitro* culture of cashew explant. Thus combinations of MS and WP medium were considered as standard one for further experiments for callus induction in *A.occidentale*. The cotyledon tissue part of *A.occidentale* was optimum explant of callus induction. The result highlighted that there was ample of variations in days to callus initiation, percentage of explants developed callus, callus texture, callus colour and degree of callus formation depending on culture media formulations. Callus initiation on cut ends of *in vitro* cultured explants could be observed in all hormone combinations after 7 - 20 days. The best callus growth was obtained under the culture on WP medium containing 50mg/L 2,4-D and 50mg/L BAP. The callus was semi friable and compact in texture and yellow, light to dark brown in colour and covered more on the surface after 4 weeks of culture. The result also revealed that WP medium with high concentration of 2,4-D (50mg/L) exhibited profuse root organogenesis than MS medium.

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