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In Vitro Shoot Proliferation from Excised Shoot Tip and Nodal Segment of *Anacardium occidentale* L.

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A B S T R A C T

An efficient *in vitro* shoot proliferation protocol was established through excised shoot tip and nodal segment of *Anacardium occidentale*. The effect of different growth regulators alone or in combination on multiple shoot production from different explants of *A.occcidentale* was studied. Shoot proliferation were obtained from excised shoot tips and nodal segments from *in vitro* grown seedlings of cashew were cultured on MS medium supplemented with NAA alone or in combination with BAP and Kinetin. Maximum number of shoots (3-5 per explants) was obtained from excised nodal segment.

Introduction

Cashew (*A.occidentale* L.) is medicinally and economically important tropical horticultural crop, a member of the family *Anacardiaceae*. It is indigenous to north eastern Brazil (Azam-Ali and Judge, 2000), which is now widely grown in other tropical countries like India and is a multi-purpose plant (Togan 1977). Cashew nut and liquid from nut shell (CNSL) are the two most important parts have lot of applications in medical and industrial field (Rajesh *et al*., 2009).

According to Ammirato *et al*., (1984) *in vitro* micropropagation has been successful for several horticultural fruit species. In *in vitro* studies, cashew has been seen to be more recalcitrant (Mantell *et al*., 1998). In *in vitro* clonal propagation, herbaceous plants can be propagated by using mature or immature tissue whereas it is too difficult in the case of perennial plants (Sumita and Sudripta 2005). Cashew nut was found difficult to propagate *in vitro* from mature plant tissues (shoot apices or nodal explants)

due to recalcitrant nature, microbial contaminations and high phenolic exudation. Though micropropagation in cashew has been experimented using explants of both juvenile (seedling) and mature tree origin, favorable regeneration has been accomplished only in the explants of juvenile origin (Thimmappaiah and Shirly 1999). Philip (1984) reported the direct plantlet regeneration from mature nut cultures. Attempts to develop a micropropagation protocol for cashew, a complete regeneration system has obtained through organogenesis (Philip 1984; Thimmappaiah *et al*., 2002).The present study was aimed at raising both regeneration efficiency and the multiple shoot production for mass propagation of *A. occidentale.*

Materials and Methods

Plant Materials

Juvenile twigs were collected from mature trees grown at Kerala State Cashew Development Corporation, Mundakkal, Kollam, Kerala, India. Shoot tips and nodal explants were treated with a fungicide (Bavistin 1 g/l) for 1 h followed by sterilization with 0.1% $HgCl_2$ for 10 min, and 5 washes with sterile distilled water. The explants were then cultured on MS (Murashige and skoog 1962) medium enhanced with different concentrations of the growth regulators NAA, BAP and Kinetin independently or in combinations. The pH of the medium was adjusted to 5.8 before autoclaving. The media were sterilized by autoclaving at 121^0C for 15 min. The cultures were incubated at 25± 2^0 C under dark.

Mature cashew seeds were collected from Kerala State Cashew Development Corporation, Mundakkal, Kollam, Kerala, India. Seeds were efficiently washed in sterile distilled water consist of a pinch of detergent. Sterilization was carried out in 70% ethanol (10 min) followed by agitation in 0.1% $HgCl_2$ (20 min). Later they were rinse thrice with sterile distilled water and germinated under dark condition in culture bottles.

In vitro and *Ex vitro* Germination of Seeds

The MS basal media were tested for their effectiveness in promoting the *in vitro* germination of cashew seeds. The basal media tested for seed germination were fortified with 2% sucrose without plant growth regulators (PGRs). In order to achieve a high germination rate, MS medium was then supplemented with various PGRs at fixed concentrations (2-5 mg/l) individually. In all cases, MS medium was altered by adding 2 g/l activated charcoal. All media were solidified with 0.8% agar and adjusted to p^H 5.8 before autoclaving.

Sterile media were allocated into culture bottles. Surface-sterilized seeds were inoculated to culture bottles with media under dark for germination and maintained. At the end of 2 weeks, seed germination was observed and germination percentages were determined by dividing the number of germinated seeds by the total number of viable seeds in the same media.

Seeds were planted on plastic trays, containing washed and sterilized sand as substrate in laboratory conditions to obtain *ex vitro* growing plantlets for further experiments.

In vitro Shoot Proliferation from Shoot Tip and Nodal Segment

Detopped shoots from *ex vitro* germinated seedlings were defoliated and segmented into nodal and shoot tip cuttings for

culturing. The explants were then cultured on MS medium supplemented with various concentrations of the growth regulators NAA, BAP and Kinetin separately or in combinations. The pH of the medium was modified to 5.8 before autoclaving. The media were sterilized by autoclaving at 121^0C for 15 min. The cultures were incubated at 25± 2°C under dark.

Statistical Analysis

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

Juvenile twigs procured from mature trees were inadequate as explant source. Shoot tip and nodes had only a 2% and 15% survival, respectively, after the decontamination procedures necessary for field grown twigs. The few explants that survived sterilization turned brown and died within a week. Hence seedlings germinated *ex vitro* were the source for explant material in the following experiments.

In vitro and *Ex vitro* Germination of Seeds

Results of the present study indicated that existence of growth regulators significantly affected *in vitro* seed germination. It was observed that the auxin 2,4-D alone was poor in inducing seed germination. Combination of 2,4-D, BAP and NAA was found to be most effective that showed 72.2 ± 0.83% germination in 8days (Table 1 and Figure 1) and it was found to be significantly different ($p < 0.05$) as correlated with the other hormonal concentrations. Whereas the seed germination percentage was only 27.83 ± 1.16% in MS media without growth regulators. Earlier researchers have worked on *in vitro* seed germination of *A.occidentale* on MS medium (Thimmappaiah and Shirly 1999; Sudripta *et al*., 1996). The *ex vitro* germination of seeds started after 10 days and it showed 90% of germination. Microshoots and leaves sprouted from decapitated seedling were used as the explants for the present study (Figure 2).

In vitro Shoot Proliferation through Shoot Tip and Nodal Segment

In the present study cashew explants were tested for *in vitro* multiplication.Excised shoot tips inoculated on basal MS medium failed to show shoot proliferation. Whereas excised shoot tips inoculated on MS medium supplemented with either NAA (2-5 mg/l) or BAP (2-5 mg/l) alone also produced shoot proliferation. But the highest shoot proliferation efficiency (57.2 ± 0.14%) was in the presence of NAA (5 mg/l), BAP (3 mg/l) and Kinetin (1 mg/l) (Table 2 and Figure.3). This concentration and combination for shoot proliferation from excised shoot tip were not seems to be reported earlier and it was in contrast with the findings of earlier study where maximum percentage of shoot proliferation (2 buds/ explant) from shoot tip were observed on MS medium (Sudripta *et al.*, 1996).

Nodal segments inoculated on MS basal medium showed 3-5 shoots per culture. There was no induction of multiple shoot on MS medium without growth regulators.

Table.1 *In Vitro* Germinating Cashew Seeds on MS Medium

SI.No	Growth hormones (mg/l)			Germination period (days)	Seed germination (%)
	2,4-D	BAP	NAA		
1	0	0	0	9	27.83±1.16a
2	2	-	-	8	39.2±1.58bc
3	2.5	-	-	10	46.4±1.14e
4	3	-	-	9	35.6±1.75b
5	3.5	-	-	7	43.5±1.22d
6	5	-	-	6	54.3±1.03f
7	2.5	2	1	9	55.6±1.14f
8	3	2	1	12	63.8±0.83h
9	3	2.5	1	10	47.6±1.14e
10	3	3	1	12	68.2±0.83i
11	5	2	1	7	56.4±1.81g
12	5	2.5	1	8	72.2±0.83j
13	5	3	1	11	58.2±0.83g
14	5	3.5	1	12	55.1±0.89f

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.2 Effect of Various Concentrations of Plant Growth Regulators on *In Vitro* Shoot Proliferation from Shoot Tip Explants of *A.occidentale* After Four Weeks of Culture

SI.No	Growth hormones (mg/l)			% of shoot formation of excised shoot tip
	NAA	BAP	KINETIN	
1	0	0	0	0
2	2	-	-	16.4±1.14a
3	2.5	-	-	36.8±1.30d
4	3	-	-	44.2±1.30e
5	3.5	-	-	16.4±.89a
6	5	-	-	25.2±1.30b
7	-	2	-	44.8±1.14e
8	-	2.5	-	16.4±1.30a
9	-	3	-	25.2±1.30b
10	-	3.5	-	53.2±.70g
11	-	5	-	24.0±.83b
12	2	2	1	53.2±1.30g
13	2.5	3	1	33.2±1.14c
14	5	2.5	1	36.4±.83d
15	5	3	1	57.2±0.14h
16	5	3.5	1	47.2±.83f

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 Effect of Various Concentrations of Plant Growth Regulators on *In Vitro* Shoot Proliferation from Nodal Explants of *A.occidentale* after Four Weeks of Culture

SI.No	Growth hormones (mg/l)			% of shoot formation of excised nodal segment
	NAA	BAP	KINETIN	
1	0	0	0	0
2	2	-	-	17.5±1.04b
3	2.5	-	-	34.33±1.21e
4	3	-	-	42.28±1.60g
5	3.5	-	-	24.83±0.75c
6	5	-	-	46.66±1.36h
7	-	2	-	43.71±1.11g
8	-	2.5	-	47.71±1.11h
9	-	3	-	51.33±1.21i
10	-	3.5	-	53.33±0.81j
11	-	5	-	24.85±0.89c
12	2	2	1	47.66±0.81h
13	2.5	3	1	15.28±1.11a
14	5	2.5	1	37.8±0.83f
15	5	3	1	64.2±1.30k
16	5	3.5	1	28.28±1.11d

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

Figure.1(a-e) Different Stages of *In Vitro* Germination of Cashew Seeds on MS Medium

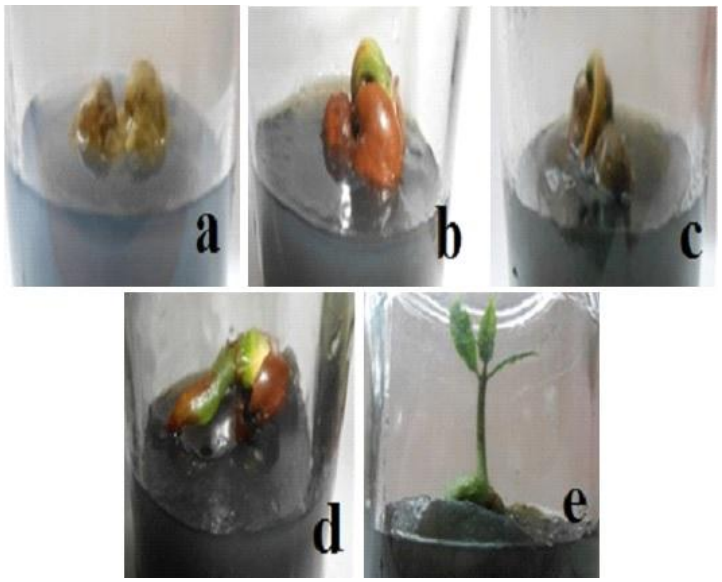

Figure.2 *Ex Vitro* Germination of Cashew Seeds

Figure.3 (a-d) *In Vitro* Shoot Proliferation from Shoot Tip Explants of *A.occidentale*

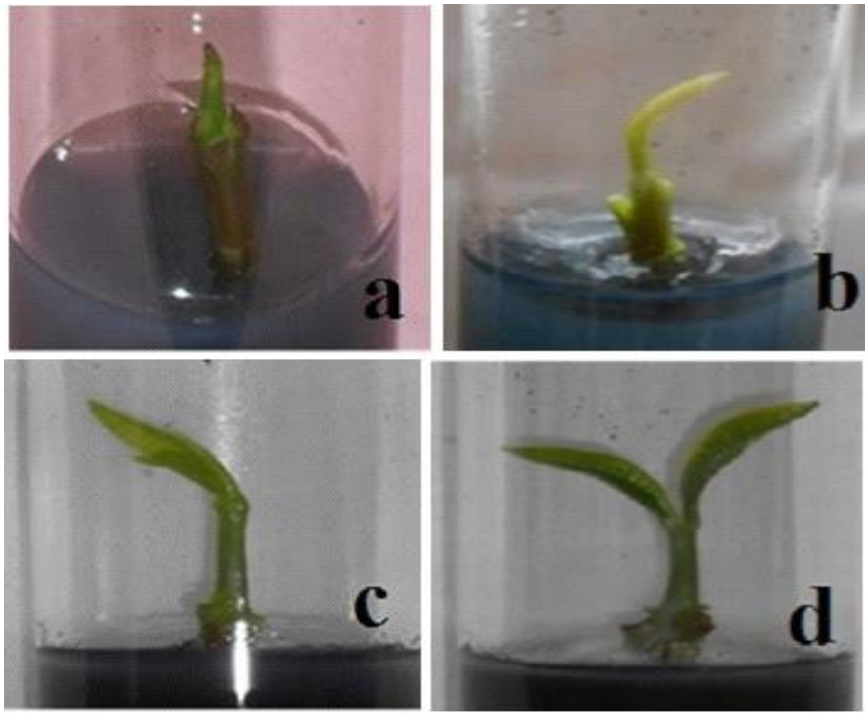

Figure.4(a-e) *In Vitro* Shoot Proliferation from Nodal Explants of *A.occidentale*

MS medium supplemented with either NAA (2-5 mg/l) or BAP (2-5 mg/l), alone showed alteration in the induction of multiple shoots which ranged between 17.5 to 46.66% in the case of NAA and 24.85 to 53.33% in the case of BAP alone. The highest percent of shoot proliferation (64.2 ± 1.30%) was obtained on MS medium supplemented with NAA (5 mg/l) and BAP (3 mg/l) and Kinetin (1mg/l) (Table 3 and Figure.4). It was significantly different ($p < 0.05$) as compared with other hormonal concentrations. This concentration and combination for multiple shoot induction from nodal segment were not seems to be reported earlier. Sudripta *et al*., (1996) also reported the role of cytokinins (BAP, Kinetin and Zeatin) in efficient regeneration of cotyledonary nodal explants of *A.occidentale* when cultured on MS medium.

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