In vitro Propagation of *Anacardium occidentale* L. through Embryo Culture

Sija.S.L, Potty.V.P, Santhoshlal.P.S

Abstract— An efficient and highly reproducible plant regeneration protocol has been developed from mature embryo of *Anacardium occidentale*. The recalcitrant nature of cashew has contributed to the limited success records in its *in vitro* culture. Browning of explant in cashew was found to be due to the oxidation of phenolic compound by polyphenolic oxidase enzyme present in the tissue when excised. In the current study, it has been reduced through the addition of activated charcoal, fast subculture passages and dark treatment. *In vitro* cultures of zygotic embryos were carried out on various media (Murashige and Skoog medium, Woody Plant medium) with different concentrations of plant growth regulators (PGRs). Maximum percentage of embryo germination and healthy plantlet formation was observed on WP medium containing 25mg/l 2,4-D and 10mg/l BAP compared to MS medium. *In vitro* developed plantlets were successfully acclimatized and transferred to soil. *In vitro* mature zygotic embryo culture thus allows for the production of cashew seedlings that can be transferred to natural orchards.

Index Terms— *Anacardium occidentale*, Activated charcoal (AC), *In vitro* zygotic embryo culture, Murashige and Skoog medium (MS), Plant growth regulators, Woody Plant medium (WP).

1 INTRODUCTION

CASHEW is the common name for a tropical and subtropical evergreen tree, *Anacardium occidentale* L., which belonging to the family Anacardiaceae [1]. It is one of the most important plantation crops earning huge foreign exchange through its kernel and cashew nut shell liquid (CNSL) [2]. The biological activities of CNSL components have attracted considerable attention in the areas of biological activities, such as anti-tumour activity [3], molluscicidal activity [4], antimicrobial activity [5], antioxidant activity [6] and xanthine oxidase inhibition [7] etc.

The application of tissue culture methods in cashew is limited by the difficulty of regenerating plants in a reproducible manner and one of the serious problems of micropropagation of cashew is attributed to the presence of secondary metabolites [8], which are oxidized after wounding and cause subsequent browning and necrosis of cashew explants [9]. 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 attempted using explants of both juvenile (seedling) and mature tree origin, successful regeneration has been achieved only in the explants of juvenile origin [10]. *In vitro* technologies have seen lot of applications in large variety of trees, mostly temperate species [11]. An attempt to develop a micropropagation protocol for cashew, an entire regeneration system has been accomplished through organogenesis [12, 13].

Embryo culture is the aseptic isolation and *in vitro* growth of an embryo under eventual culture conditions which helps in overcoming the post fertilization barriers of hybridization in woody plant species [14]. Efficient plant regeneration is a prerequisite for an entire genetic transformation protocol in plant species. Aiming this, the present study accomplished effective plant regeneration using mature embryos as a source material in *A.occidentale*. Even though immature inflorescence and immature embryos are best explant sources for *in vitro* culture in cashew [15, 16], neverthless they are available only for a limited period in a year. Mature embryos have always been optimal for *in vitro* studies for the fact that they can be handled easily over other explants and are accessible throughout the year [17].

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2 MATERIALS AND METHODS

2.1 Plant Material

Mature cashew (*A.occidentale*) seeds were acquired from the mother stock trees grown at the Kerala State cashew development corporation, Mundakkal, Kollam, Kerala, India.

2.2 Explants Disinfection and Culture Conditions

Seeds were thoroughly washed under continuous flashing of running tap water for 15 min, then washed with commercial detergent Tween-20 for 3 min and finally surface sterilized with $HgCl_2$ (0.1% w/v) for 5-10 min followed by rinsing with autoclaved distilled water for 3-5 times to remove any trace of mercuric chloride. After all, the seeds were soaked overnight. The sterilized and soaked seeds were displaced to the petriplate having the autoclaved filter papers. With the aid of a sterile blade, the seeds were broken. Mature nuts were cut longitudinally into two halves for to remove two sections of cotyledonary tissue with the embryonic axes seated in the upper portion of the nuts.

In the laboratory, these explants were washed thoroughly under running tap water for 20-30 minutes and then treated with 1% Labolene (Qualigens, India) detergent for 5 minutes to remove all debris and other contaminants. Again the mate-

rial was washed thoroughly with tap water to remove all detergent particles. More processing was done in sterile LAF cabinet. Inside the LAF, explants 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 and PVP alone as well as in combinations to reduce the discharge of phenolic substances.

In the current study MS [18] and WP [19] were tested on mature embryos of *A.occidentale*. MS and WP media supplemented with 2,4-D and BAP alone and with its different combinations were used for plantlet development. pH was adjusted to 5.2 for WP medium and 5.8 for MS medium, and gelled with 0.8% agar. 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 ± 2^0C under dark.

3 Data 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 ($p < 0.05$).

4 Results and Discussion

4.1 Percentage Survival of Embryo Explants after Sterilizing With 0.1 % $HgCl_2$

Within the various surface sterilization methods tested, a primary 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 6 minutes followed by 4-5 times rinse in sterilized distilled water helped to reduce infection rate in mature embryo explants. The current study material following the above treatment, 66.66±1.47% 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 5 minutes. Each treatment had 30 explants and all the experiments were repeated thrice. The data on surface sterilization of explants are described in Fig.1.

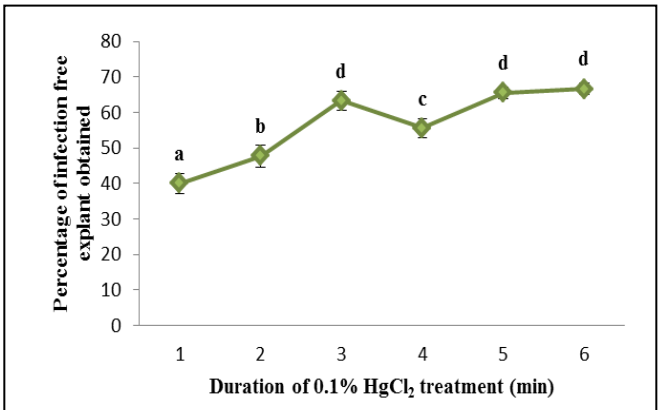

Fig.1. Surface sterilization of explants using $HgCl_2$ treatment; values with different letters are significantly different ($p < 0.05$), (n=3, error bars represent standard deviation).

4.2 *In vitro* Prevention of Browning in Cashew Cultures

Tissue browning and blackening are the major hindrance of *in vitro* culture of plants. It may be due to the presence of high secondary metabolites which are inhibitory to plant cellular growth. Accumulation of phenolic compounds to a level which is harmful for *in vitro* growth is common in some economically important plants such as coffee, mango, chickpea [20], sugarcane [21], guava, date palm [22], and cotton [23].

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. Frequent sub-culturing was performed to minimize the exudation of the phenolic compounds from the embryo explants. This observation was in agreement with previous reports [24, 25]. Though the browning was not very effectively reduced by the subculturing, however, the browning percentage was minimum with the addition of 4g/l activated charcoal and 3g/l PVP to the medium, which was supported by earlier workers [26, 24]. The present study materials following the above treatments, 82.78 % of the explants responded well in culture and it was significantly different ($p < 0.05$) with other antioxidant treatments (Fig.2).

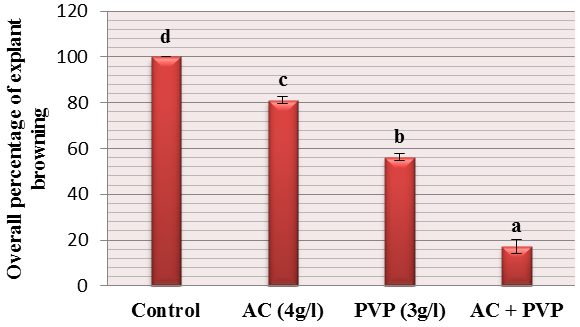

Fig. 2. Effect of various concentrations of antioxidants used for removing browning in embryo explants; values with different letters are significantly different ($p < 0.05$), (n = 3, error bars represent standard deviation).

4.3 Optimization of Culture Media and Growth Regulators on Plantlet Regeneration from Embryo Explants

Zygotic embryo culture is a method which comprises isolating and culturing of immature or mature zygotic embryos on a nutrient medium under aseptic conditions. This technique

also supports in understanding concepts associated to nutrient requirements of the growing zygotic embryo, shortening the breeding cycle and reducing seed dormancy [27]. *In vitro* propagation of *A.occidentale* using zygotic embryo explants was achieved by culturing the zygotic embryos on MS and WP media with various concentrations and combinations of 2,4-D and BAP. In the present study, zygotic embryos placed vertically showed 100% germination followed by the embryos with a small portion of cotyledon attached to it (Fig.3a). These observations highlighted the regeneration potential of the embryo with the associated tissue of the seed. There are also many reports on the rapid germination of embryo with a portion of cotyledon [28, 29]. Germination of the zygotic embryos started within 2-3 days of inoculation (Fig.3b). Noticeable colour changes were observed in zygotic embryo from white to light green, which is the first visible sign of embryo germination and was observed within 2 days of inoculation followed by elongation of radical. Shoot emergence was observed after one week of inoculation (Fig.3c) and well developed seedlings with healthy shoots and roots were formed by end of the fourth week of culture (Fig.3d).

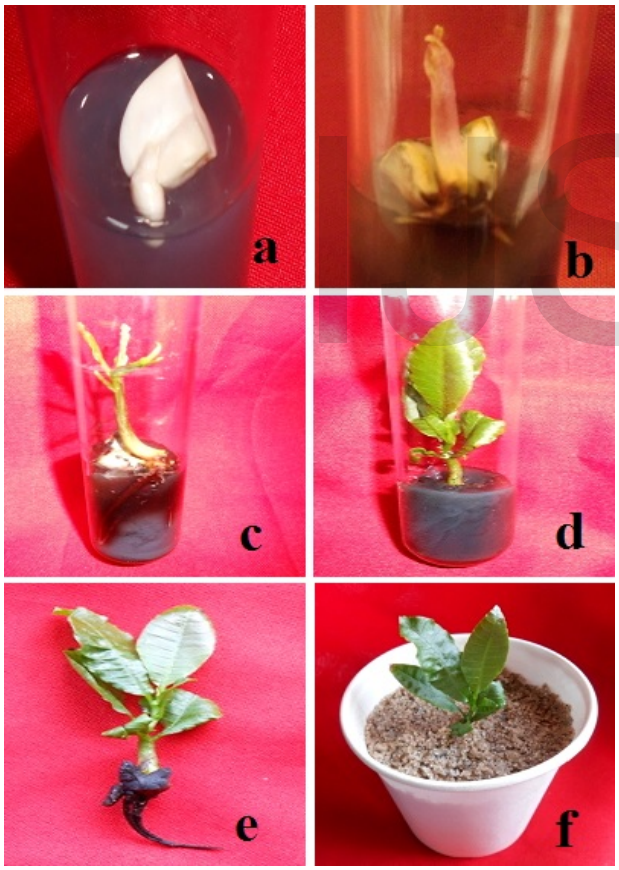

Fig. 3. Plantlet regeneration from mature zygotic embryos in *A.occidentale*

a) Inoculation of embryo on basal WP medium b) Germination of embryo on basal WP medium c) Direct shoot and root organogenesis in WP medium d) Two week old aseptically germinated seedlings e) Rooted plantlets ready for transplantation f) Acclimatized plantlets after 7 days of transplantation onto soil mix.

In the present investigation, it was observed that 2,4-D alone was poor in inducing germination from mature zygotic embryo explants. It only produced 35.3 ± 1.43 % of germination response in WP medium (Table 1). Besides that, 2,4-D when used in combination with BAP produced increased percentage of embryo germination than when it used alone. Maximum percentage of germination (63.5 ± 1.37 %) was noticed on the zygotic embryo

TABLE 1
THE EFFECT OF PLANT GROWTH REGULATORS ON GERMINATION OF ZYGOTIC EMBRYOS OF *A.OCCIDENTALE* AFTER 4 WEEKS OF CULTURE ON WP MEDIA

SI.No	Growth hormones (mg/l)		Germination of embryos (%)
	2,4-D	BAP	
1	0	0	0
2	5	-	22.2±1.09a
3	10	-	35.3±1.43c
4	25	-	26.9 ± 1.69b
5	-	5	NC
6	-	10	NC
7	-	25	26.7 ± 1.40b
8	10	5	59.6 ± 1.36f
9	10	10	38.9±0.72d
10	25	5	60.1±2.74f
11	25	10	63.5±1.37g
12	25	25	55.3±1.15e

The values represent the mean (±SD) of three independent experiments. Mean values within a column followed by different letters are significantly different by Duncan's multiple range test (P<0.05).

TABLE 2
THE EFFECT OF PLANT GROWTH REGULATORS ON GERMINATION OF ZYGOTIC EMBRYOS OF *A.OCCIDENTALE* AFTER 4 WEEKS OF CULTURE ON MS MEDIA

SI.No	Growth hormones (mg/l)		Germination of embryos (%)
	2,4-D	BAP	
1	0	0	0
2	5	-	15.2±1.09b
3	10	-	10.6±1.05a
4	25	-	21.3±0.74c
5	-	5	NC
6	-	10	NC
7	-	25	11.3±0.94a
8	10	5	22.1±1.27c
9	10	10	39.9±0.72f
10	25	5	41.3±1.15f
11	25	10	33.1±0.40e
12	25	25	29.1±1.25d

The values represent the mean (±SD) of three independent experiments. Mean values within a column followed by different letters are significantly different by Duncan's multiple range test (P<0.05).

explants grown on WP basal medium supplemented with 25mg/l 2,4-D and 10mg/l BAP (Table1) and it was significantly different ($p < 0.05$) with other hormonal concentrations. This concentration and combination for healthy plantlet regeneration from mature zygotic embryo were not reported earlier and it was in contrast with the findings of earlier studies where maximum percentage of plantlet regeneration from immature zygotic embryos were observed on MS medium [15,16].

Although in MS medium, maximum percentage of zygotic embryo germination (41.3± 1.15 %) was obtained when 2,4-D at 25 mg/l was used in combination with BAP at the concentration of 5 mg/l (Table 2) and it was not significantly different ($p < 0.05$) with other hormonal concentration such as 2,4-D (10 mg/l)+ BAP (10 mg/l). These results recommended that WP medium was better than MS medium for maximum percentage of zygotic embryo germination and plantlet formation. Healthy and mature seedlings formed from zygotic embryos cultured on WP and MS media were taken out from the culture tubes and washed with distilled water to remove traces of agar (Fig.3e). The plantlets thus obtained were acclimatized by transferring them to plastic cups containing sterile vermiculite: garden soil (1:1) (Fig.3f), shifted to green house and transferred to the research field.

5 Conclusion

Cashew is treated to be one of the most recalcitrant species *in vitro*. It is necessary to find out the optimal culture conditions to organize it in a culture medium and proliferate. The present study accomplished efficient plant regeneration using mature embryos as a source material in *A.occidentale*. Mature embryos have always been ideal for *in vitro* studies for the reason that they can be handled easily and are usable throughout the year. While immature inflorescence and immature embryos are the best explant sources for *in vitro* culture in cashew, they are available only for a limited period in a year. Results of the present investigation highlighted that *in vitro* seedling development from mature embryo was optimal in full-strength WP medium augmented with 2,4-D (25mg/l) with combination of BAP (10mg/l).

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References

[1] Johnson, D., 1973, "The botany, origin and spread of cashew (*Anacardium occidentale* L.) ", J. Plant. Crop., 1, pp.1-7.

[2] Pillai, C.K.S., Prasad, V.S., Sudha, J.S., Bora, S.C., and Menon, A.A.R., 1990, "Polymeric resins from renewable resources", J.Applied Sci., 4, pp.2487-2501.

[3] Schmourlo, G., Mendonça, F.R.R., Alviano, C.S., and Costa, S.S., 2005, "Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants", J. Ethnopharm., 96, pp.563-568.

[4] Goncalves, J.L.S., Lopes, R.C., Oliveira, D.B., Costa, S.S., Miranda, M.M.F.S., Romanos, M.T.V., Santos, N.S.O., and Wigg, M.D., 2005, "*In vitro* anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea", J. Ethnopharmacol., 99(3), pp.403-407.

[5] Abas,F., Lajis,N., Israf,D., hozirah,S.,and Umi,K.Y.,2006,"Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables", Food Chemistry., 95(4), pp.566–573.

[6] Amorati, R., Pedulli, G.F., Valgimgli, L., Attanasi, O.A., and Filippone, P., 2001, "Absolute rate constants for the reaction of peroxyl radicals with cardanol derivatives", Journal of the Chemistry Society Perkin Transactions., 2, pp.2142-2146.

[7] Masuoka, N., and Kubo, I., 2004, "Characterization of xanthine oxidase inhibition by anacardic acids", Biochim. Biophys. Acta., 1688, pp. 245-249.

[8] Mantell, S.H., Boggetti B., Bessa, A.M.S., Lemos, E.E., Abdelhadi, A and Mneney, E.E., 1998, "Micro propagation and Micro grafting methods suitable for the safe international transfer of cashew". In Tropper, C. P., Caligari, P. D. S., Kullaya, A. K., Shomari, S. H., Kasuga, L. J., Masawe, P. A. L. and Mpuinami, A. A. (eds.). Proceedings of International Cashew and Coconut conference, Dar es Salam. Bio-Hybrid International Ltd, Reading, pp.128-133.

[9] Jha, T.B., 1988, "*In vitro* morphogenesis in cashew nut (*Anacardium occidentale* L.) ", Indian J. Exp. Biol., 26, pp.505-507.

[10] Thimmappaiah, and Shirly, R.S., 1999, "*In vitro* regeneration of cashew (*Anacardium occidentale* L.) ", Indian J. Exp. Biol., 37, pp.384-390.

[11] Kannan, V.R., and Jasrai, Y.T., 1996, "Micropropagation of *Gmelina arborea*", Plant Cell Tissue and Organ Cult., 46, pp.269-271.

[12] Das, S., Jha, T.B. and Jha, S., 1996, "*In vitro* propagation of cashew nut", Plant Cell Reports., 15,pp. 615-619.

[13] Thimmappaiah, R.A., and Samuel, S.R., 1999, "*In vitro* regeneration of cashew (*Anacardium occidentale* L.) - shoot tip culture and node culture and propagation", Indian Journal of Experimental Biology., 37(4), pp.384-390.

[14] Bonga, J.M., and Aderkas, P., 1992, "*In vitro* culture of trees, Kluwer Acadeemic Publ. Dordrechet, The Netherlands 2(4), pp.236.

[15] Aliyu., and Olawale, M.,2005, "Application of tissue culture to cashew (*A.occidentale* L.) breeding: An appraisal", African Journal of Biotechnology., 4(13), pp.1485-1489.

[16] Sumita, Jha., and Sudripta, Das., 2005, "Tissue culture of cashew nut", Plant biotechnology and molecular markers, Edited by Srivastava.P.S, Alka Narula, Sheela S, Anamaya publishers,New Delhi, India, pp.244-260.

[17] Kishore, S.N., Visarada, K.B.R.S., Lakshmi, A.Y., Pashupatinath, E., Rao, S.V., and Seetharama, N., 2006, "*In vitro* culture methods in Sorghum with shoot tip as the explant Material", Plant Cell Rep., 25, pp. 174-182.

[18] Murashige, T., and Skoog, F., 1962, "A revised medium for rapid growth and bioassays with tobacco tissue cultures", Physiol. Plant., 15, pp. 473- 497.

[19] Lloyd, G., and Mc Cown, B., 1980, "Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*", Intl Plant Prop Soc Proc., 30, pp.421-427.

[20] Iqbal, J., Naz, S., Nazir, S., Aftab, F., and Ahmed, M.S., 1991, "Total phenolics, phenylalanine ammonia lyase and polyphenol oxidases in in vitro calli of chick pea", Pak. J. Bot., 23, pp. 227-235.

[21] Chen, H.K., M. Mic., and D.W.S. Mok., 1990. "Somatic embryogenesis and shoot regeneration from interspecific hybrid embryos of Vigna glabrescens and V. radiate", Plant Cell Report., 9, pp.77-79.

[22] Daayf, F., E. Bellajm, E. Hassni, F. Jaiti and E. Hadrami., 2003, "Elicitation of soluble Phenolics in date palm callus by *Fusarium oxysporum*.sp. albedinis culture medium", Environmental and Experi-

mental Botany., 49, pp.41-47.

[23] Ozyigit, I.I., M.V. Kahraman and O. Ercan., 2007, "Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.) ", African J. of Biotechnology., 6(1), pp.3-8.

[24] Das, S., Jha, T.B., and Jha, S., 1996, "*In vitro* propagation of cashewnut", Plant Cell Re., 15, pp.615-619.

[25] Mantell, S.H., Boggett, B., Bessa, A.M.S., Lemos, E.E.P., and Abdelhad, A.R., 1998, "Micropropagation and micrografting methods suitable for safe international transfer of cashew", in Proceedings of International Cashew and Coconut Conference, (Dar Es Salaam), pp.95-107.

[26] D'Souza, L., D'Silva, I., Mallaya, S., Augustine, A.C., and Rajendra, K., 1996, "*Anacardium occidentale* L. (Cashew nut) ", in Biotechnology in agriculture and forestry, edited by Bajaj YPS, 35 (New York: Springer-Verlag), pp. 31-49.

[27] Hu, C., and Wang, P., 1986, "Embryo culture technique and applications", In: D.A.Evans, W.R.Sharp and P.V.Ammirato(ed). Handbook of plant cell culture.4 (Macmillan, New York), pp.43-96.

[28] Archana, P., Mahitha, B., and Rama, S.N., 2014, "Optimization of culture media for invitro zygotic embryo culture of *Senna alata* L. Roxb- An ethno medicinal plant", IOSR Journal of Pharmacy and Biological Sciences., 9, 3(6), pp.1-6.

[29] Ram, B. M., Ujjwala, D., Ugandhar, T., Praveen, M., and Upender, M., 2006, "*In vitro* zygotic embryo culture of an endangerd forest tree. *Givotia rottleriformis* and factors affecting its germination and seedling growth", *In vitro* cellular and developmental biology-Plant., 42(5), pp.418-421.