International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Genetic Fidelity in Micropropagated Plantlets of *Anacardium occidentale* L. (Cashew) an Important Fruit Tree

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Abstract: ***The meristem and axillary bud tissue culture were considered to be an ideal horticultural tool to generate morphologically and genetically true to type plants. In tissue culture derived plants of Anacardium occidentale L (cashew) variations in morphological traits like height and girth of the stem, the shape of the leaves and plagiotrophy are seen in the plants generated from the same cotyledonary nodes. In our experiments the multiple shoots were induced from cotyledonary nodes without the addition of growth regulators, without an intervening callus and within one generation; yet they show considerable amount of variation in morphology. Since no growth regulators were used or frequent subcultures were made it is suggested that the variation is due to stress induced by tissue culture procedures.***

Keywords: Multiple buds**,** Cotyledonary nodes, Morphology, Genetic variation, Plagiotrophy, Somaclonal variation.

Abbreviations: IBA- Indole -3- butyric acid, IAA- Indole Acetic Acid.

1. Introduction

Anacardium occidentale L (Cashew) is an important nut crop of economic and social importance for India. It is a source of the much needed foreign exchange and provides work for uneducated rural women. The production of raw nuts is not enough to meet the demand of the international market and to provide work for the women all through the year. This is due to the low yield of the trees grown at present as well as the loss of crop due to insect pests. Conventional methods for improvement of cashew can be supported by information on the process of germination, organogenesis and variation.

Variation among plants regenerated from tissue culture is termed 'somaclonal variation' (Larkin and Scowcroft, 1981). Some workers consider somaclonal variation to be caused by the *in vitro* culture system itself; others consider it to be the expression of pre-existing physiological or genetic variation in somatic cells (D`Amato, 1985; Swartz, 1991).

Several factors have been found to influence the level of somaclonal variation. For example, the regeneration system used, the cryopreservation protocol, the culture conditions and the number of subcultures which the cultures have passed through. (D`Amato, 1985; de Klerk, 1990; Harding, 1991; Harding, 1997; Jain et al. 1998; Aronen et al. 1999; Rani and Raina, 2000; Hao and Deng, 2002; Zhai et al. 2003). Other specific procedures which are reported to induce somaclonal variation are protoplast isolation (Li et al. 1994) or gene transfer (Bregitzer et al. 1998). Some species or clones are found to be more susceptible to somaclonal variation than others (Karp, 1989; Israeli et al. 1996).

According to Larkin and Scowcroft, 1981, somaclonal variation can be limited by selecting the correct explant source, developing *in vitro* medium supplement with minimal amounts of growth regulators, particularly BAP , avoiding exceeding the number of subculture cycles to beyond eight and avoiding frequent subculture.

Although somaclonal variation is undesirable in micropropagation, it can be a useful source of new variability in fruit crops where long generation time hinders conventional breeding (Hammerschlag, 1992). Variation in somaclones for karyotype, isoenzyme pattern, precocity for bearing, ploidy level, growth, yield, quality, pigmentation, disease resistance and resistance to adverse soil and climatic conditions have all been reported in different plants (Patil and Nevale, 2000). In general *in vitro* conditions can be extremely stressful on plant cell, and may set in motion highly mutagenic processes during explant establishment, callus induction, maintenance, embryo development and plant regeneration (Lorz et al. 1988).

2. Materials and Methods

Plant material

Mature seeds of cashew of the variety Ullal 3 obtained from Cashew Research Station of the University of Agricultural Sciences, Ullal were used for the experiment.

Sterilization of plant material

The nuts were kept under flowing water for 2 hours to remove the surface grouse, soil particles and also fungal spores prior to chemical sterilization so as to reduce contamination of cultures considerably. The nuts were then surface sterilized using 0.1% Bavistin (Carbendizim) for 45 minutes and in 0.1% mercuric chloride and 0.1% sodium lauryl sulphate for 20 minutes. After sterilizing they were rinsed thoroughly with sterile water till all the surface sterilants were washed away. They were then soaked in sterile distilled water for three days to soften the hard seed coat. The soaked nuts were opened under laminar air flow

and inoculated into 125 ml screw capped bottles and germinated aseptically in the culture room.

Six day old seedlings obtained from the sterilized and aseptically germinated seeds were used as source of explants. The shoot was cut off 0.25 cm above the cotyledonary node. The radicle was cut off leaving 1 cm of the hypocotyl. Cotyledonary nodes with the cotyledons intact were inoculated into 125 ml capacity screw capped bottles, containing MS medium. The explants were inoculated with the hypocotyl stump inserted in the MS medium without any growth hormones.

The multiple micro-shoots induced at the axil of the cotyledonary nodes were harvested and rooted on MS medium supplemented with 2.85 µM IAA + 4.92 µM IBA + 116.86 mM sucrose according to the protocol of D'Silva, (1991). The rooted plantlets were hardened by transferring them to a mixture of sand: soil: powdered coconut husk (1:1:0.25), in containers covered with plastic covers to avoid desiccation and kept for 4 weeks in the greenhouse. The hardened plants were transferred to soil in the green house.

Three different sets each of five one- year-old plants were selected randomly from among these plants. The plants of each set were generated from the same cotyledonary node. The plants were assessed for their morphological characters. The height was measured using a meter scale and the diameter of the stem one cm above the surface of the soil was calculated using electronic calipers (Mitutoyo PC-15JN). Occurrence of plagiotropic branching and the shape of the leaves were noted visually.

3. Results

Morphology: Variations in the morphology of the plants with respect to all the four parameters studied were seen. Variations in height and diameter of the stem were observed both between the plants of the three sets as also within the plants of the same set derived from one cotyledonary node (Table 1). Some plants derived from the same cotyledonary node differ very much from others in height (Fig.1. A). Plagiotrophy was noted in 100% of the plants of one set, 80% of the plants of another set and 40% of the plants of the third set (Table 2, Fig.1.B). There is considerable variation in the shape of leaves. Whereas the majority of plants of two of the three sets had typical obovate leaves, some of them had oblong lanceolate and a few had elliptic leaves (Table 3, Fig.1.C).

4. Discussion

Variations were reported in tissue culture derived plants as early as 1964 by Hollings and Stone (1964) although plants derived through tissue culture were in early years assumed and accepted to be true clones of the mother plant. The meristem and axillary bud culture were considered to be an ideal horticultural tool to generate morphologically and genetically true to type plants. However variations in tissue culture derived plants have now been reported in most plants including the major crop plants rice, wheat, maize, barley, sugarcane, potato and forage grasses (Buitatti et al. 1986; Vasil, 1986). The variants include chlorophyll-deficient plants, changed morphology, single-gene mutation, polyploidy, aneuploidy, chromosomal rearrangements, modified yield, quality and disease resistance and occasionally novel variants not present in the natural gene pools (Ahloowalia, 1986).

In cashew, variation is seen in all the four parameters studied even among plants derived from the same cotyledonary node. Variation is seen in height and diameter of the stem as well as in the shape of the leaves and in the plagiotrophic habit. It is interesting to note that many leaves have the typical oblong lanceolate shape of leaves of *Magnifera indica* which belongs to same family. Altered growth habit and leaf shape have also been reported in other tissue cultured woody plants by Antonetti and Pinon (1993), Son et al. (1993) and Saieed et al. (1994).

Plagiotropic branching is a common phenomenon met with in cashew trees raised from cuttings. Though the micro shoots got from the cotyledonary nodes correspond to cuttings, plagiotropic branching was not seen in all the plants. It has been noted in a study of the long term effects of cashew trees generated by tissue culture and planted on the field that plagiotropy was not observed in the *in vitro* grown trees (Hegde et al. 1996; Nivas and D`Souza 2004). The tissue cultured trees showed rapid growth during the first year upon transferring to the field. There were no lateral branches; as a result, the trees were straight and tall. According to Nivas et al. (2004) this observation suggests the possible long- term effects of the growth substances added *in vitro* resulting in strong apical dominance during the period of the first year in the field. In the present study however, no growth regulators were added to the medium and hence there is considerable amount plagiotropic branching similar to that observed in trees raised from cuttings.

Variation in *in vitro* generated plants has been attributed to various causes. One of these is the source of the explants. Variation is said to be due to the explants which are collected from several plants of the same variety or from several shoots of the same plant (Chandra and Sreenath, 1982). Variations in the explants would lead to variation in the plants derived from these explants. However in case of shoots derived from cotyledonary nodes of cashew, variation is seen even in plants derived from the axil of one cotyledon. The addition of growth regulators, particularly BAP is said to be another cause of variations (Oono, 1982). In our experiments the shoots from cotyledonary nodes were induced without the addition of growth regulators and yet they show considerable amount of variation. It is also said that exceeding the number of subculture cycles to beyond eight, as well as frequent subculture results in variation of the microshoots (Rodrigues et al. 1998). The shoots induced from cotyledonary nodes of cashew and showing variation were derived in the primary culture without the need for any subculture. It is also suggested that shoots derived through an intermediary callus could be morphologically and genetically variable, whereas shoots derived directly without callus are uniform (Son et al. 1993; Saieed et al. 1994). But the shoots derived from cotyledonary nodes of cashew are formed directly without an intervening callus. It is therefore suggested that stress induced by touch or by wounding,

causes release of biomolecules which interfere in the functioning and development of the organism (Jaffe et al. 1984). Brown and Lorz (1986) have also attributed variations *in vitro* produced plants to response to tissue culture stress.

Examining variation in isozymes, which results from changes in protein coding sequences, has been a common technique for different kinds of genetic analysis attributed to somaclonal variation (Eastman et al. 1991). The strength of this approach is its emphasis on DNA sequences that are expressed phenotypically. However, protein studies investigate only a part of the variation in the most conserved class of DNA sequences. Other techniques detect polymorphism directly at the DNA level (genetic molecular markers) and thus give access to more variable segments within structural genes (for example introns and flanking sequences) as well as to non-coding regions of the genome.

In the present work , it has been shown that in cashew there is considerable variation even though the shoots were derived from one and the same cotyledonary node and were produced without intermediary callus. Some authors failed to observe somaclonal variation using RAPD analysis of various woody species including *Picea abies* (Fourre et al. 1997), *Eucalyptus camaldulensis* (Rani and Raina, 1998) and *Azadirachta indica* (Singh et al. 2002); *hybrid aspen* (Jokipii, et al. 2004); *Prunus* (Helliot et al. 2002); *Vitis sp. and Actinidia sp.* (Zhai et al. 2003); *Betula pendula* (Ryynänen and Aronen, 2005). However intraclonal RAPD polymorphisms have been observed during or after *in vitro* culture in *Picea glauca* (Isabel et al.1996); *Populus nigra* (Wang et al. 1996); *Musa species* (Damasco et al. 1996); *Prunus species* (Hashmi et al. 1997); peach (Rani and Raina, 2000), Almond (Channuntapipat et al. (2003) and *Camellia sinensis* (L.) O Kuntze (Jibu et al. 2006).

According to Siwaporn et al. (2008) morphological variations were observed in the field grown papaya plants maybe due to the somaclonal variation derived from indirect somatic embryogenesis in cotyledon explants of papaya. In tissue cultured date palms somaclonal variation has been observed and the plants have been analyzed as off types due to tissue culture procedures which are exhibiting morphological changes in various aspects including flowering patterns Al Kaabi et al. (2008). Morphological variations have been observed by Paul Schellenbaum et al. (2008), in Vitis. However there is no single molecular technique that will unequivocally demonstrate the presence or absence of somaclonal variation in regenerated plants (Ryynänen and Aronen, 2005). It would be necessary to have long term field trials for assessing the real quality of *in vitro* produced woody plants. Changes produced after several years of planting have been observed by us in the *vitro* trees of cashew (Nivas and D'Souza, 2004). Besides, Linhart et al.(1989) caution that the relationship between protein variation and morphological and physiological variation is poorly understood. It has been thought that relatively rapid analyses of protein variation can be used as predictors of variation at other levels. However it is not clear how well protein variation predicts variation at other levels because comparative studies have not been carried out with trees and very few have been done with other plants.

5. Acknowledgement

Facilities provided by the Mangalore Jesuit Educational Society as well as the technical help given by Vinod Robin D`Souza and Rahul are gratefully acknowledged.

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Table 1 : Height and diameter of the stem of three sets of five plants generated from cotyledonary nodes of cashew

Plantlet Number	Height (cms)			Diameter (mm)		
	Set I	Set II	Set III	Set I	Set II	Set III
1	17.0	33.0	20.0	10.5	21.1	9.2
2	12.5	26.0	21.0	8.1	16.5	12.2
3	10.0	18.5	36.0	6.8	12.8	17.9
4	15.0	21.5	14.0	6.7	14.1	8.6
5	11.0	15.5	16.5	7.8	12.0	8.1

Table 2 : Occurrence of plagiotropism in three sets of five plantlets generated from the cotyledonary nodes of cashew.

Plantlet number	Set I	Set II	Set III
1	+	+	+
2	+	+	+
3	_	+	+
4	_	+	_
5	_	+	+

Table 3: Shape of leaves in three sets of five plantlets generated from the cotyledonary nodes of cashew.

Plantlet number	*Set I*	*Set II*	*Set III*
1	Lanceolate	Obovate	Obovate
2	Lanceolate	Obovate	Lanceolate
3	Lanceolate	Obovate	Obovate/ eliptic
4	Lanceolate	Lanceolate	Obovate
5	Lanceolate	Obovate	Obovate

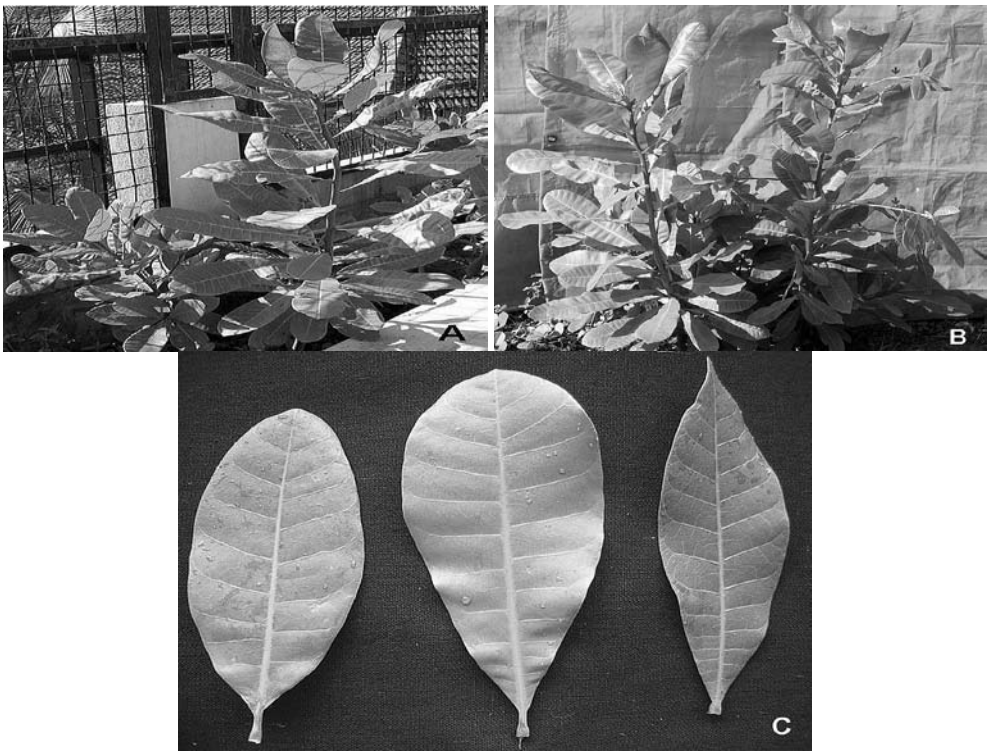

FIG.1. Plants originating from the same cotyledonary node.
A. Plants showing remarkable difference in height.
B. Plants with (right) and without (left) plagiotropic branching(→).
C. Leaves showing elliptic (left), obovate (center) and oblong lanceolate (right) shapes.