TISSUE CULTURE STUDIES OF CERTAIN PROMISING CASHEW GERMPLASMS.

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Abstract: Cashew (*Anacardium occidentale* L.) is a costly horticultural crop. It is a woody perennial tree of tropical origin and is being cultivated in India, Brazil and African countries. The existing plantations in India, however, are showing a trend of gradual decline in productivity. Moreover, the yield is not satisfactory and difficult to forecast each and every year. The serious limitation in Cashew production is that like many other tree plantation crop, cashew is also an extremely heterozygous and cross pollinated crop. The present study aims to standardize a reliable protocol for cashew micro propagation using cotyledonary node, terminal and nodal explants of germinated seedlings. Standardization of a reliable protocol for establishment and efficient multiplication is presented in the present chapter after a series of experiments on manipulations of culture media.

Key words: In vitro clonal production, horticultural, micro propagation, MS basal medium, cotyledonary node, terminal/auxiliary shoot.

Introduction: Cashew (*Anacardium occidentale* L.) is a costly horticultural crop. It is a woody perennial tree of tropical origin and is being cultivated in India, Brazil and African countries. In India it is grown in an area of 10 lakh ha with the production of 5.8 lakh MT with an average productivity of 840 kg/ha and has earned a foreign exchange worth Rs. 1623 corers during the year 2016-17 (Thimmappaiah and Samuel 2018). The existing plantations in India, however, are showing a trend of gradual decline in productivity. Moreover, the yield is not satisfactory and difficult to forecast each and every year. The serious limitation in Cashew production is that like many other tree plantation crop, cashew is also an extremely heterozygous and cross pollinated crop. Moreover, the plantations are of mostly non descripting seedling origin. Hence, existing area with cashew plantation needs to be replanted with clonal material of elite lines to boost up the production. The production of clonal stocks in large scale is limited, even through vegetative production techniques such as air-layering, grafting, budding and epicotyls grafting are available.

In vitro clonal production has recently been used successfully in a number of tree crops to produce large number of identical clones from selected ‘elite’ plants (Bajaj, 1986; Fiorinol G lorcti 1987; Mescarenhas G Muralidharan, 1989). In Vitro studies, however, are very limited in cashew, particularly due to the interference to phenols and tannins during the establishment of cultures (Hegde et al., 1993). It makes successful in vitro propagation of cashew from mature elite plant of proven high yield quite impossible. The target, naturally has shifted towards using juvenile explants, embryos etc. and reports of in vitro culture of Cashew seedlings and regeneration of multiple plantlets from mature cotyledons (Hegde et al., 1993); morphogenesis from immature embryo – derived callus (Jha, 1988); multiplication from cotyledonary nodes (D’ Silva and D’ Souza. 1992); and regeneration through axillary bud proliferation from shoot tip/nodal explants from in vitro grown seedlings (Thimmappaiah and Samuel. 1999) are available. The present study aims to standardize a reliable protocol for cashew micro propagation using cotyledonary node, terminal and nodal explants of germinated seedlings. Standardization of a reliable protocol for establishment and efficient multiplication is presented in the present chapter after a series of experiments on manipulations of culture media.

Materials and Methods:

a) Plant materials: Seeds were collected from selected elite donor plants of four varieties viz. Madras Mixed Aroct (MMA), Uulal-1, Vengurla-3 & WBDC –IV.

b) Seed Germination: Seeds were germinated both in vivo (in sand) and in vitro and the explants, viz. cotyledonary node; terminal and nodal shoot buds were excised from the germinated seedlings for further culture in medium. In vitro seed germination, seed coats were removed aseptically after surface sterilization and the two intact cotyledons containing embryos were incubated in germination medium; the medium being either MS basal or MS basal supplemented with different concentrations of gibberelic acid (GA_3).

c) Surface sterilizations and culture of explants: Surface sterilization of the explants and their subsequent culture in media containing different combination of growth regulators were done. It was observed that there was browning in the medium around the cut and of the explants within 2-3 days of inoculation. To overcome this problem, explants trial for rooting were inoculated to fresh medium supplemented with PVP (Polyvinyl Pyrolidone) or activated charcoal (0.3%). For root induction attempts were made by transferring the micropropagated shoots in MS/B_5/DKW basal medium supplemented with either IBA alone or combinations of IBA and NAA. Attempts were also made by infecting the lower cut ends of the explants with *Agro bacterium rhizogenes* strain LBA 9407.

Results:

A) Seed germination: About 90-95% Cashew seeds germinated in vivo (in sand) irrespective of the four varieties under present study. Rate in vivo germination was comparatively lower and of the eight treatments. MS basal medium supplemented with 6uM/1 GA_3 resulted in highest (in vitro) rate of germination; maximum in case of variety MMA (72%) followed by varieties like Ullal-1(69%), Vengurla-3(64%) and WBDC-IV (61%).

B) Establishment:

1. Effect of Basal media and growth regulators on percentage of establishment.

a. Explants: Cotyledonary node

i) MS- Percentage of establishment was low (3-9) in MS basal medium alone, i.e. without supplementation of any growth regulator, in all four varieties. Rate of establishment increased with increasing concentration of BAP and it was 53%, 50%, 48% and 41% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively when media was supplemented with 2 mg /1 BAP. A combination of BAP with either of the auxins (NAA or 2, 4-D) did not result in better response. The combination TDZ, the cytokinin, with NAA (each at 0.1 mg/1), however, resulted in 51% , 47%, 42% and 39% establishment in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively. The combinations of two cytokinins along with one auxin (either NAA or IBA) also did not result in better or comparable rate of establishment except in one case (BAP, 0.5 mg/1+TDZ, 0.1 mg/1 I+IBA, 0.05 mg/I) where the rate of establishment was 50%, 47%, 43% and 39% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively

ii) B_5 – Percentage of establishment was low (5-11) in general when explants were cultured in B_5 basal medium without supplementation of any growth regulator. Rate of establishment increased with increase in concentration of BAP in B_5 medium and it was 67%, 64%, 60% and case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively when medium was supplemented with 2 mg/1 BAP. A combination of BAP with either of the two auxins (NAA or 2,4-D) did not promote better rate of establishment. The combination of TDZ, the other cytokinin with NAA (each at 0.1 mg/1), however, resulted in 66%, 63%, 58% and 56% establishment in varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV

respectively. The combinations of two cytokinine (BAP and TDZ) along with one auxin (NAA or IBA) also did not result in better or comparatively rate of establishment except in one case (BAP 0.5 mg/I+TDZ, 0.1 mg/I+IBA, 0.05 mg/I) where the rate of establishment was 65%, 67%, 59% and 55% in varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively.

iii) DKW- the response of DKW basal medium was very poor in comparison to the other basal media (MS and B_5) and the maximum rate of establishment was 34%, 33%, 30% and 32% in verities MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively when basal medium was supplemented with BAP (0.5mg/I), TDZ, (0.1mg/I) and IBA (0.05 mg /I).

b. Explants: Terminal and nodal shoot buds

I) MS- Percentage of establishment was low (2-6) when explants were cultured on basal medium alone. Significant rate of establishment was attained in only two cases when growth regulators were used : when medium was supplemented with BAP (2 mg/I) 4(6%, 47%, 44% and 40% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively); and BAP (0.5 mg/I) in combination with TDZ (0.1 mg/I) and IBA (0.05 mg /I) (44%, 46%, 42% and 44% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) .

ii) B_5- Percentage of establishment was low (2-8) when explants were cultured on basal medium alone. Considerable rate of establishment was attempted only after addition of growth regulator (s) basal medium : when medium was supplemented with BAP (2 mg/I) (52%, 51%, 49% and 45% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively); and BAP (0.5 mg/I) in combination with TDZ (0.1 mg /I) and IBA (0.05 mf/I) (51%, 49%, 47% and 45% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively.

iii) DKW- The response of DKW basal medium was very poor in comparison to the other basal media (MS and B_5) and more or less uniform rate of establishment was attained in all four varieties when DKW basal medium was supplemented with TDZ and NAA (each at 0.1 mg/I) and the rate was 30%, 23%, 25% and 20% in case of varieties like MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively.

Multiplication:

1. Effect of basal media and growth regulators on percentage of multiplication.

a. Explants: Cotyledonary node.

i) MS – The established explants were subculture in media supplemented with respective combinations of growth regulators as initially used during the experiments on establishment. Media with combinations of growth regulators (s) which induced maximum percentage of establishment also showed maximum percentage of multiplication. Of all the combination of growth regulators tested, maximum rate of multiplication (41%, 38%, 34% and 35% in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively was attained when MS basal medium was supplemented with BAP (2mg/I).

ii) B_5 – Of all the combination of growth regulators tested, maximum rate multiplication (53%, 58%, 54% and 50% in case of MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) was achieved when B_5 basal medium was supplemented with BAP (2 mg/I).

iii) DKW- Very few established explants showed further proliferation in DKW medium. Maximum rate of multiplication was 19%, 27%, 25% and 22% (in case of MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) when this basal medium was supplemented with BAP (2 mg/I).

b. Explants: Terminal and nodal shoot buds.

i) MS – of all the combination of growth regulators tested, maximum rate of multiplication (31%, 39%, 41% and 38% in case of MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) was attained when MS basal medium was supplemented with BAP (2 mg/I).

ii) B_5 – Of all the combinations of growth regulators tested maximum rate of multiplication (37%, 45%, 43% and 40% in case of MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) was attained when B_5 medium was supplemented with BAP (0.5 mg/I), TDZ (0.1 mg/I) and IBA (0.05 mg/I).

iii) DKW – Few established explants showed proliferation and multiplication in DKW medium. Maximum rate of multiplication was 22%, 15%, 11% and 10% (in case of varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) when this basal medium was supplemented was BAP (1 mg/I) and 2.4 OD (3 mg/I) .

2. Effect of basal media growth regulator on growth of multiplied shoots

a. Explant: Colytedonary node.

i) MS - Of all the combinations of growth regulators tested, maximum length of multiplied shoots (after 30 days) was attained (3.75 cm, 3.00 cm, 2.75 cm and 3.00 cm in varieties MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) when MS basal medium was supplemented with BAP (2 mg/I) .

ii) B_5 – Of all the of all the combinations of growth regulators tested, maximum length of multiplied shoots (after 30 days) was attained (3.90 cm, 3.50cm, 3.25 cm and 3.00 cm in verities MMA, Ullal-1, Vengurla-3, and WBDC-IV respectively) when B_5 basal medium was supplemented with BAP (2 mg/I).

iii) DKW – Even the best growth promoting growth regulator, i.e. BAP at concentration 2 mg/I could not in DKW basal medium promote the length of multiplied shoots beyond 2.00 cm., in any of the varieties under present study.

3. Rate of multiplication: Rate of multiplication in Cashew was low in general and it was true for all the four varieties under present study as well as two types of explants. The highest rate of multiplication (2-3 auxiliary proliferated shoots / explants) was attained in the variety MMA when the cotyledonary node – explants were cultured on B_5 basal medium supplemented with BAP (2 mg/I).

Discussion: As mentioned in the introduction clonal propagation from explants of mature ‘elite’ plants of proven qualities virtually impossible in case of cashew due to the interference of phenols and tannins, the present study aims at micro propagation of cashew from juvenile explants. Reports of similar types of endeavor are available (D’ Silva and D’ Souza, 1992; Thimmappaiah and Samuel 1999).

Successful seed germination of this type of recalcitrant material is the first obstacle before manipulating with juvenile explants such as cotyledonary node or terminal/auxiliary shoot buds. Result of the present study indicate that an in vitro approach rather than an in vitro one yields better percentage of seed germination in all the varieties of cashew under present study. An in vitro approach, nevertheless, was to some explants satisfactory as about 70% seeds germinated in varieties like MMA, Ullal – I only when the germination medium (MS basal) was supplemented with gibberelic acid (6um/1). A similar treatment resulted in 64% and 61% seed germination in varieties Vengurla – 3 WBDC – IV respectively. The gibberelic acid (GA_3) most probably erased the inherent dorunancy of the recalcitrant cashew seeds resulting in successful seed germination.

Though both was types of juvenile explants, i.e. cotyledonary node and terminal nodal explants, showed considerable percentage of culture establishment but the former (i.e. cotyledonary node) was found to be marginally superior. That supplementation of growth regulator was a must to induce culture establishment in

case of cashew was evident from the result that nether of the basal medium without the supplementation of growth regulator of any sort could promote consider able percentage of establishment of any of the four varieties under present study. Of the three types of basal medium MS, B_5 and DKW the last mentioned one was found to be unsuitable for establishment in all the cases. Furthermore, of MS and B_5, cashew showed a slight preference for B_5, the Gamborg's basal medium (1963) as the highest percentage of establishment was attained when the explants were grown on this basal medium after on this basal medium after supplementation of different growth regulators various combinations and concentrations. Probably high inorganic, especially the nitrate salts as provided by the B_5 were reassured for this plant material. Regarding response among four genotypes, the variety MMA seemed to be most amenable for culture as the rate of establishment was noticed in this variety. The variety Ullal – 1 comes as a close second. Although thirty combinations of growth regulators were tried but medium supplemented with BAP alone (at 2mg/I) provided rate of established irrespective of explants, variety of basal medium the ruling out the unnecessary of a combination of two or more growth regulators for establishment. The highest rate of establishment in B_5 basal medium supplemented BAP alone (at 2mg/I) as observed in the present study this differed from the available literature on cashew micro propagation as most of the reports at claimed success using MS basal medium and a wide array of combinations of growth regulations (Thimmappaih and Samuel,1999) one significant finding of the present study was that the best medium for establishment was equally good for multiplication to as B_5 basal medium supplemented with 2 mg/I BAP promoted highest percentage of multiplication of the established shoots. Moreover maximum proliferation of as auxiliary shoots was also attained in this medium as these (the auxiliary shoots) grew up to 3.90cm on an average. Rate of multiplication, however, was far from satisfactory as the highest rate of multiplication 2-3 auxiliary shoots per explants in case of variety MAA

Rooting of the micro propagated shoots nevertheless could not be induced in spite of a number of trails. Even use of the rooting inducible bacterial strain *Agrobacterium rhizogemes* (LBA 9407) could not induce rooting leaving further scope of work of a viable micro propagation protocol for cashew.

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