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Development of triploid plantlets from immature endosperm of *Garcinia gummi-gutta* var. gummigutta (L) Rob.

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The inoculated immature endosperm bits of 1cm size onto ½ MS supplemented with 3.0 mgl^{-1} each of BA and Kinetin induced adventitious buds and subsequent proliferation of multiple shoots. These triploid plantlets were rooted, hardened and planted out. Development of regeneration protocol from triploid parenchymatous tissue of endosperm not only overcome the dearth of demands for true-to-type propagules but also surpassed the gender dimorphism. Evolving seedlessness through *in vitro* endosperm culture may also be helpful to avoid the processing difficulty and to support the establishment of domestic HCA extraction unit for Women empowerment.

Key words : *Garcinia gummi-gutta* var. gummigutta, Polygamodioecious, Kodampuli, Immature seeds, Endosperm culture, Triploid, Seedlessness, Life style diseases, Shoot bud differentiation, Regeneration, Direct organogenesis.

INTRODUCTION

Garcinia gummi-gutta var. gummigutta (L) Rob. known in vernacular as Kodampuli or Malabar Tamarind belongs to the family Clusiaceae (Lewis and Neelakantan, 1965). This under-exploited perennial backyard spice tree has excited the scientific world because of the presence of anti-obesity plant metabolite (-) hydroxy citric acid (HCA) in its fleshy fruit rind. Though its cultivation has confined to coastal Kerala and Sri Lanka, the vast stretches of coastal saline belts of Indian sub-continent and the entire south east Asian countries may be used for extensive cultivation. Piling up of the obesity related life style diseases, diabetes and heart ailments, especially in the third world countries has warranted the extensive cultivation of this miscellaneous backyard spice tree for supporting the indigenous pharmaceutical industry.

During processing removal of seeds is highly laborious, time consuming and expensive. Where seed is not commercially important, development of seedless types will be a great boon (Wang and Chang, 1978). *In vitro* technique is an effective tool for the production of seedless types through triploid endosperm culture. The seeds of Kodampuli possess endosperm throughout its developmental stages (Richard, 1990). Triploid nature of endosperm is the characteristic feature of angiosperms and is formed as a result of triple fusion around the time of fertilization (Thomas and Chathurvedi, 2008). When one sperm nuclei inside a pollen grain fuses with the two polar nuclei at the centre/interior of the embryo sac of female gametophyte forming a primary endosperm cells. This process of triple fusion develops into endosperm is called double fertilization. The endosperm tissue being triploid, the plantlets developed from it will also be triploids. Furthermore, the seedless triploids in general are observed to have heterotic vigour as compared to other ploidy levels. The conventional approach to develop triploids is to cross tetraploids with diploids (Straub, 1973). But the lengthy pre-bearing age of perennial crop like Kodampuli nullify the importance of tetraploid development and subsequent production of triploids through conventional crossing techniques (Rajendran *et al.*, 2002). This approach is not only laborious in the perennial trees but also requires more than 100 years to release seedless variety with stable characteristics (Esen and Soost, 1973; Gupta, 1982; Rajendran *et al.*, 2004). Hence, the totipotency of triploid cells of endosperm may be profitably exploited as an alternative method for evolving seedless types in crop improvement programme of perennial tree species like Kodampuli.

Endosperm of some species is responsible for seed dormancy (Basra, 1994). Seed dormancy of 7-8 months, dioecious nature and the lack of large scale multiplication protocol for productive female trees stand in the way of extensive cultivation of this therapeutically important crop. However, it is noteworthy to emphasize the regeneration of plantlets from triploid endosperm tissue of Kodampuli or Malabar Tamarind to compensate incompetent

vegetative propagation, prolonged seed dormancy, difficulty in identifying productive females and the seed removal for processing. The cells of the endosperm of the Kodampuli at the time of excision (30-45 days after pollination) are meristamatic particularly the outermost layer of cells. An attempt has been made in this direction to produce plantlets by culturing immature endosperm tissues of Kodampuli at CPBMB, College of Horticulture, Vellanikkara of Kerala Agricultural University.

MATERIALS AND METHODS

Origin and preparation of explants:

Explant source for *in vitro* endosperm culture was procured from the field of Department of Olericulture, College of Horticulture, Vellanikkara during the period of 2006-09. The immature fruits after 30-45 days of fertilization were harvested and pre-treated with systemic fungicide Indofil at 0.1 per cent concentration and a few drops of detergent extran for 30 minutes. Then rinsed thoroughly thrice with distilled water for removing the fungicidal residue. These fruits were brought to the aseptic condition of laminar hood, wiped with absolute alcohol and scooped out the seeds. These seeds were surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) for 3 minutes (Rajendran *et al.*, 2002). Both ends of the surface sterilized seeds were excised and discarded for avoiding the embryo germination. Thereafter, the seed coat removed and the remaining endosperm portion were excised into equal halves of approximately 1.0 cm size.

Culture media and techniques:

The basal medium consisted of MS supplemented with 3.0 per cent sucrose, 0.01 per cent inositol and 0.25 per cent neutralized activated charcoal. It was solidified with 7.5 gl^{-1} agar and the pH was adjusted to 5.7 ± 0.2 before adding agar (Rajendran *et al.*, 2002). Sterilization of media was done by subjecting them to temperature of 121^0C at a pressure of 15 psi for 20 min. After sterilization, the media were allowed to cool to room temperature and stored in cool dry place. The inoculated endosperm bits onto various hormonal combinations of MS media (Murashige and Skoog, 1962) were incubated under dark condition of culture room for a fortnight to reduce the phenolic interference (Rajendran *et al.*, 2002). Thereafter the cultures were incubated 26 ± 2^0C for 16 h photoperiod with 1000-1500 Lux supplied by fluorescence tubes.

Sub-culturing was carried out at monthly intervals. The sub-cultured immature endosperms were transferred to half strength MS media containing growth hormones to initiate shoot and root growth in explants. (Table 1 and 2). The rooted plantlets were hardened, planted out in the plastic pots and kept under the green house of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara.

Optimization of growth regulators for shoot bud differentiation:

A series of treatments were employed to define the optimum concentration of plant growth regulators and additives in the MS basal media for shoot bud differentiation. The studies were tried at different concentration of cytokinin like N^6 Benzyl Adenine (BA) and Kinetin (K) ranging from 1-3 mgl-1 (Table 1).

Optimization of growth regulators for rooting and hardening:

Different combinations of plant growth regulators like IBA, NAA, K, 2,4-D were tried for standardizing optimum level for rooting and hardening at concentration ranging from 1-2 mgl^{-1} (Table 2). The half strength MS liquid medium containing 3.0 $mg1^{-1}$ BA plus 3.0 mgl^{-1} K was tested for hardening the rooted plantlets.

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below:

Shoot bud differentiation and organogenesis

Basra (1994) reported that the endosperm of some species is responsible for seed dormancy. In the case of Kodampuli the seed dormancy of 7-8 months was also reported by Mathew and Sarah (1995) and Rajendran et al (2004). Hence, the occurrence of prolonged dormancy might be due to the presence of endosperm in Kodampuli. The regeneration from immature endosperm exploiting its meristamatic activity may be an ideal research strategy to overcome the inherent lacuna.

Around 300 excised endosperm pieces from immature seeds surface sterilized by 0.1 per cent $HgCl_2$ for 3.0 minutes were registered survival per cent in the range of 23.3-98 without phenolic exudation to the culture media (Table 1). The initial fortnight incubation in dark followed by light incubation of the *in vitro* endosperm cultures were observed 98 per cent survival onto ½ MS medium fortified with 3.0 mgl^{-1} BA plus 3.0 mgl^{-1} Kinetin (Table 1) (Fig. 1).

The shoot bud differentiation from inoculated endosperm was observed 21 days after inoculation onto MS medium supplemented with 3 mgl^{-1} each of BA and K (Table 1) (Fig. 1d and 1e). The nodules or adventitious

Table 1 : Effect of media combinations for culture establishment and multiple shoot induction

Pretreatment + surface sterilization	Treatment no.	Basal media + hormones (mgl^{-1})	No. of explants inoculated	No. of explants survived	Survival (%)	Nature of response	Days taken to initiate regeneration	Nature of loss	
								Reason	No.
90% Alcohol wiping + 0.1% Bavistin +0.1% $HgCl_2$ (3 min^{-1})	A1	MS + 3 Kinetin	300	70	23.3	No response	22	FC	230
90% Alcohol wiping + 0.1% Bavistin + 0.1% $HgCl_2$ (3 min^{-1})	A2	MS + 3 BA	300	192	64	Callusing	15	BC	108
90% Alcohol wiping + 0.1% Bavistin +0.1% $HgCl_2$ (3 min^{-1})	A3	MS + 1 BA + 1 Kinetin	300	210	70	White friable callusing	20	FC	90
90% Alcohol wiping + 0.1% Bavistin +0.1% $HgCl_2$ (3 min^{-1})	A4	MS + 2 BA + 2 Kinetin	300	227	75.6	Callusing	20	FC and BC	73
90% Alcohol wiping + 0.1% Bavistin +0.1% $HgCl_2$ (3 min^{-1})	A5	MS + 3 BA + 3 Kinetin	300	294	98	Multiple shoot formation	14	FC	6

Fig. 1 : Direct organogenesis from immature endosperm (Triploids) : (a) Bearing fruit : (b) Immature fruit : (c) Endosperm bits : (d) Shoot bud differentiating : (e) Shoot regeneration : (f) and (g) Multiple shoot proliferation : (h) Root initiation :(i) Rooted plantlets : (j) Hardening : (k) and (l) Plant out of triploid plantlets

buds were formed either from the peripheral cells or the cells beneath. So, the meristamatic activity of the cells was intensified and uniform throughout the excised endosperm explant bits. The similar results were reported

Table 2 : Response for rooting, hardening and plant out

Basal media + hormones (mgl^{-1})	No. of shoot kept for rooting	Days taken for rooting	Survival (%)	No. of plants kept for hardening	No. of plantlet planted out	Remarks
½ MS + 1 2,4 – D	60	-	-	-	-	
½ MS + 1 2,4 – D + 1 NAA	65	-	-	-	-	
½ MS + 1 2,4 – D + 1 IBA	50	-	-	-	-	
½ MS + 1 2,4 – D + 1 Kinetin	65	-	-	-	-	
½ MS + 2 2,4 – D + 1 Kinetin	75	16	21.3	14	11	Remaining cultures were subcultured for adventitious shoot multiplication and rooting

Table 3 : Response of endosperm onto MS basal medium supplemented with 3.0 mgl^{-1} each of BA and K

No. of endosperm taken	No. of endosperm bits inoculated	No. of shoot buds differentiated per culture	Total no. of differentiated shoot buds	No. of adventitious shoots per subculture	Total no. of adventitious shoots per subculture
12	24	16	384	7	168
12	24	19	456	6	144
10	20	20	400	7	140
17	34	15	510	6	204
8	16	15	240	6	96
11	22	16	352	7	154
12	24	20	480	7	168
12	24	20	480	6	144
8	16	17	272	6	96
9	18	15	270	6	108
10	20	15	300	6	120
11	22	15	330	7	154

by Bhojwani and Razdan (1996) in many species of angiosperms. These adventitious buds differentiated or originated from the peripheral cell of the endosperm and possessed distinct shoot apices and well differentiated vasculature (Bhojwani and Razdan, 1996; Bhojwani and Bhatnagar, 1999). Their findings were also in line with our results (Fig. 1d).

The direct organogenesis from endosperm culture has favoured the exogenous cytokinin application of 3.0 mgl^{-1} each of BA and K. These cultures exhibited direct organogenesis at the rate of 15-20 shoots per explant within 28-30 days after inoculation. This finding was in corroboration with the results of Bhojwani and Johri (1970), Johri and Nag (1970, 1974) in Loranthaceae family (*Scurrula pulverulenta, Taxillus vestitus*). The addition of cytokinin to the MS basal medium (half strength) was indispensable for shoot regeneration has been well-documented by Gill (1992), Raman *et al.* (1992) and Srivastava and Sandhu (2002) in citrus cultivars Mosambi, Baramasi lemon, Kinnow and Kagzi lime.

Rooting, hardening and planting out:

The regenerated adventitious shoots were excised from above cultures when they were 8 cm in height and subcultured to ½ strength MS with 2:1 auxin-cytokinin ratio. Half strength MS medium supplemented with hormonal concentration ranging from 1-2 mgl^{-1} promoted roots during subculturing (Fig. 1h). Roots were induced directly from the regenerated shoots. Roots were initiated from the bases of shoots on half strength MS + 2mgl^{-1} 2, 4-D + 1.0 mgl^{-1} K after 21 days when the cultures were grown in light. Similar results were reported by Tejavathi and Sunita (2002) in *Linium usitatissimum*. They further stated that the root initiation was delayed by 15 days when the cultures are exposed to light.

After 16 days, the well rooted plantlets were transferred to half strength MS basal liquid medium containing 2 per cent sucrose and 3 mgl^{-1} each of BA and K for hardening. The plantlets thus hardened were first transferred to sterilized vermicompost: sand (2:1) mixture and finally to normal soil in small pots.

Fleshy fruit rind of Kodampuli has cherished the Asian culinary art as well as the integral part of indigenous system of medicine for the treatment of rheumatism, bowel complaints, rickets and uterine contraction after delivery. Long stretches of coastal saline belts of the humid tropical regions of the third world are the potential areas for its extensive cultivation. At present, high economic value of this therapeutically important perennial spice has hindered the extensive cultivation due to the lack of regeneration protocol for large scale multiplication and sex identification at early stage. Development of regeneration protocol from triploid parenchymatous tissue of endosperm not only overcome the dearth of demands for true-to-type propagules but also surpasses the gender dimorphism. Evolving seedlessness through *in vitro* endosperm culture may also be helpful to avoid the processing difficulty and to support the establishment of domestic HCA extraction unit for Women empowerment.

Abbreviations:

MS – Murashige and Skoog (1962), $HgCl_2$ – Mercuric Chloride, BA – N^6 Benzyl adenine, K-Kinetin, HCA- Hydroxy Citric Acid.

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