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BRIEF COMMUNICATION

In vitro shoot bud differentiation from leaf segments of *Achras sapota*

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Abstract

Direct shoot bud differentiation was achieved in leaf segments of *Achras sapota* cv. Cricket Ball inoculated on Schenk and Hildebrandt's medium supplemented with 5.0 μM thidiazuron and 8.88 μM benzylaminopurine. Leaves from middle part of the shoots and segments obtained from middle portion of leaf showed highest potential to regenerate shoot buds. Histological examination of developing shoot buds showed their *de novo* regeneration with clear vascular connection with the mother tissues.

Additional key words: adventitious shoot buds, benzylaminopurine, regeneration, thidiazuron, tissue culture.

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Achras sapota (*Sapotaceae*) a native of Mexico (Singh *et al.* 1970) is widely grown for its delicious and sugar rich fruits in many tropical countries including India. Lack of suitable methods for breeding and long breeding cycle make improvement of this plant very difficult. Recombinant DNA technology is emerging as an important alternative for genetic improvement of woody plants including fruit trees (Khurana and Khurana 1999). The ability to regenerate whole plants from somatic tissues is a pre-requisite either for *Agrobacterium* or biolistics mediated transformation. Development of a regenerative system for its integration in genetic manipulation program is an imperative need in *A. sapota*. In this species culture of mesocarp and endosperm (Bapat and Narayanaswamy 1977, Litz 1989), callus induction and early embryogenesis (Sachdeva and Mehra 1986) and micropropagation through enhanced axillary branching from cotyledonary nodes (Purohit and Singhvi 1998) have been reported. The present paper reports direct shoot bud differentiation in *A. sapota* from leaf segments.

Proliferating shoot cultures of *Achras sapota* L. cv. Cricket Ball (having an average 6 nodes) maintained on Schenk and Hildebrandt (1972; SH) medium containing 8.88 μM 6-benzylaminopurine (BAP) and which had passed through six sub-culture passages were used to obtain leaf explants (Purohit and Singhvi 1998). Leaves from apical, middle and basal part of the shoot were harvested and cut into 3 segments designated as proximal, middle and distal parts, perpendicular to the mid-rib. They were then placed on medium in the Petri dishes with abaxial side facing the medium.

Following medium compositions were tested: SH + BAP (2.22 - 22.2 μM); SH + kinetin (Kn) (2.32 - 23.23 μM); SH + thidiazuron (TDZ) (5.00 - 10.00 μM); SH + BAP (8.88 μM) + TDZ (1.00 - 10.00 μM), SH + α-naphthalene acetic acid (NAA) (2.69 - 26.85 μM); SH + 2,4-dichlorophenoxyacetic acid (2,4-D) (2.26 - 22.62 μM); SH + BAP (8.88 μM) + NAA (2.69 - 10.74 μM). All the cultures were kept under temperature of 28 ± 2 °C, a 16-h photoperiod with an irradiance of 45 μmol m^{-2} s^{-1} and relative humidity of 60 - 70 %. Each experiment was repeated thrice with 20 replicates.

Free hand sections of developing shoot buds on leaf segments were bleached in 2 % sodium hypochlorite solution (2 % active chlorine) for 10 - 20 s. After thorough washing with distilled water, they were kept in 1.0 % acetic acid solution (10 - 20 s) to get transparency. The sections were subsequently stained with safranine (4 - 5 min) and mounted in 30 - 40 % glycerine. Observations were made under phase contrast (microscope *Nikon*, Tokyo, Japan) and photographs taken.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; Kn - kinetin; NAA - α-naphthalene acetic acid; SH - Schenk and Hildebrandt; TDZ - thidiazuron.

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Plant growth regulators are necessary for shoot bud induction on leaf segments. Although BAP alone (8.88 μM) induced shoot buds in *ca.* 18 % explants, the best response however, was obtained when 8.88 μM BAP was combined with 5.0 μM TDZ (Fig. 1*A*, *B*, Table 1). Both TDZ and Kn on the other hand evoked comparatively poor response when used individually. Occasional rooting was observed at 1.0 μM concentration of TDZ. Incorporation of auxins (NAA and 2,4-D) individually either caused callusing or induced rooting in some cases (Fig. 1*C*). A combination of different concentrations of NAA with 8.88 μM BAP induced shoot buds in all the treatments but the response percentage and the number of shoots per regenerating leaf segments were very low as compared to BAP used alone. It was therefore concluded that 8.88 μM BAP combined with 5.0 μM TDZ was the best treatment for induction of maximum number of shoot buds (*ca*. 25 per explant) in a larger percentage (35 %) of leaf explants. The elongation of shoot buds and their further multiplication are being attempted to recover complete plantlets through *de novo* adventitious shoot bud regenerative pathway of micropropagation.

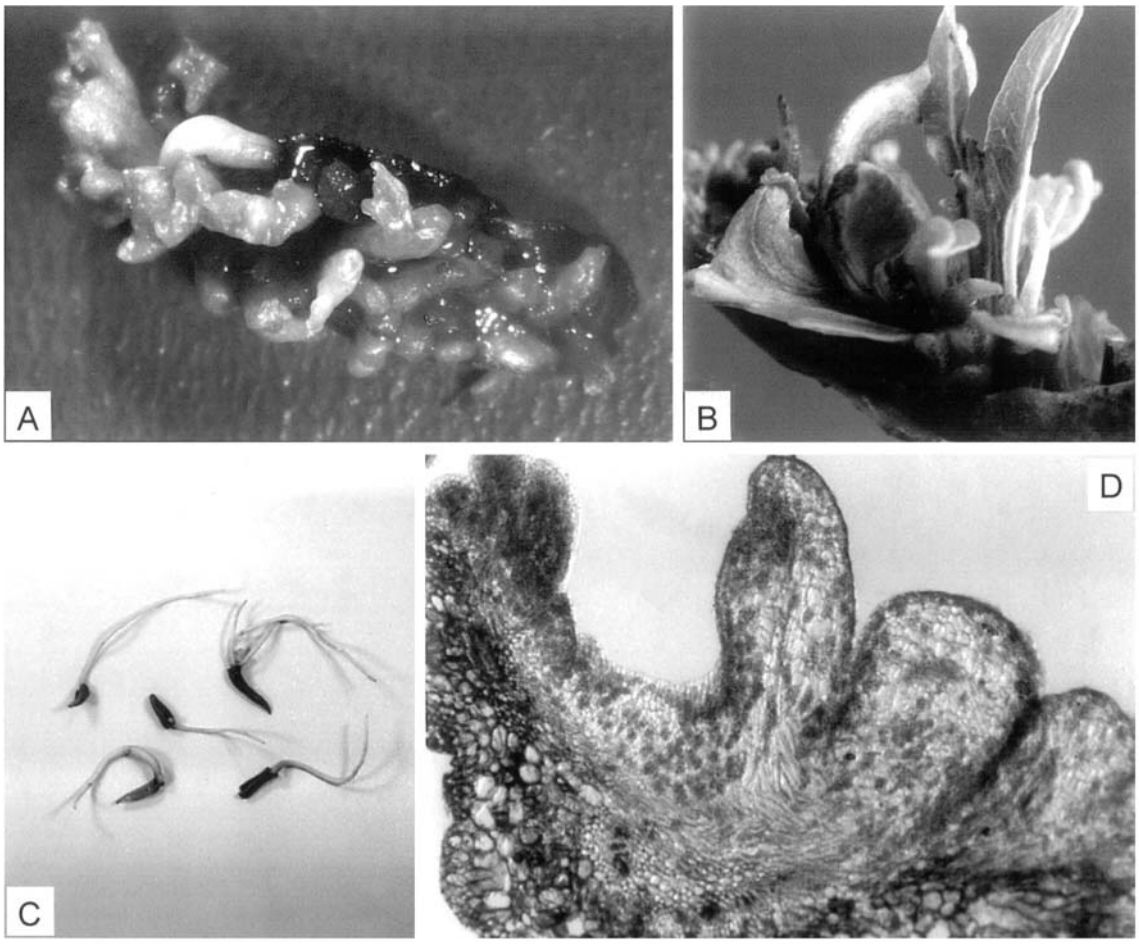

Fig. 1. Effect of PGRs on shoot bud differentiation in *A. sapota* leaf explants cultured on SH medium. Shoot bud differentiation and proliferation with 8.88 μM BAP and 5.0 μM TDZ (*A*, *B*), induction of roots with 26.85 μM NAA (*C*), and transverse section of cultured leaf explant showing adventitious shoot buds and their vascular connection with mother tissue (*D*).

Transverse section of cultured leaf explant collected on day-one showed normal histology of leaf showing epidermis, mesophyll and vascular bundles. Each of these tissue layers consisted of differentiated cells and possessed characteristic cellular organization of a normal dicot leaf. After 18 d, proliferation of new cells in the midrib region was observed. These cells continued to divide forming a group of small cells. Within 22 d in culture this group of cells became pronounced and could easily be identified as a meristemoid, the precursor of an adventitious shoot bud. Continued proliferation of small meristemoid cells resulted in rapid expansion of the surface area and the total volume. A consequence of this expansion of cell mass was protrusion of a bud

primordium above the leaf surface which became visible after 32 - 34 d in culture. After 40 d the bud primordia progressed into the early stage of adventitious shoot bud development. Such shoot buds showed clear vascular connection with mother tissues (Fig. 1*D*).

Table 1. Effect of PGRs on adventitious shoot bud differentiation from leaves of *A. sapota* (middle portion of the leaves from middle part of shoots) on SH medium (explant response and average number of adventitious buds were recorded after 63 d of inoculation). Means followed by different letters differ significantly at 5 %.

PGR	Concentration [μM]	Response [%]	Number of buds [explant^{-1}]
BAP	4.44	5.00	1.33^{d}
	8.88	18.33	6.50^{a}
Kn	9.28	3.33	2.50^{c}
	22.22	6.16	3.33^{b}
TDZ	5.00	15.12	4.52^{e}
	10.00	18.00	5.56^{d}
BAP+TDZ	8.88+ 1.00	8.66	7.58^{c}
	8.88+ 5.00	36.44	25.32^{a}
	8.88+10.00	25.56	10.82^{b}
BAP+NAA	8.88+ 2.69	11.00	1.83^{c}
	8.88+ 5.38	13.30	4.33^{a}
	8.88+10.74	0.90	2.50^{b}

The data presented above demonstrate clearly that adventitious shoot bud regeneration is possible in leaf segments of *A. sapota* cv. Cricket Ball. Hormones controlling organogenetic process in plant tissues and organs cultured *in vitro* have already been examined by many investigators and role of BAP in adventitious bud differentiation has been demonstrated (*e.g.* Tanimoto and Harada 1982). In present studies, BAP appeared to be more effective than Kn for inducing adventitious shoot buds in leaf segments of *A. sapota*. BAP in combination with IAA alone (Arockiasamy *et al.* 2002) or along with adenine sulphate (Mishra 2002) was found useful in shoot bud differentiation from leaf explants of *Eryngium* and *Cajanus*, respectively. TDZ, a cytokinin like substance, has been effectively used to induce shoot regeneration on leaf explants of many dicots (Huettman and Preece 1993). Fiola *et al.* (1990) have shown that TDZ somehow interact with cytokinins to increase their activity. In *A. sapota* leaf explants, a combination of TDZ with BAP proved stimulatory in induction of adventitious shoot buds. Similar results were also reported for *Picea glauca* (Ellis *et al*. 1991). Both the position of the leaf on shoot and the leaf part influence the capacity to regenerate adventitious shoots *in vitro*. New leaves near the tip of the shoot and basal part of the leaf had greater regeneration capacity (Caboni and Tonelli 1999) while a gradient of response from tip to the base of shoot with the distal leaves being more responsive and tending to form the more shoots per regenerating leaf has been reported in *Malus domestica* (Fasolo *et al*. 1989). In *A. sapota* leaf segments from the middle part of the leaves obtained from second or third nodes of *in vitro* developed shoots regenerated more shoot buds. Shoot buds can either originate endogenously from perivascular cambium or exogenously from single epidermal cells but they show definite procambial strands in the maternal tissues establishing a conducting connection between the young shoots and the vascular system of the mother plant (Haccius 1978). Histological studies in present investigation have clearly established endogenous origin of shoot buds showing continuity of vascularization.

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