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Micropropagation of *Annona squamosa* Linn. Using Explants (Shoot Tip and Node) of Field Grown Mature Plants

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Abstract: The investigation was based on axillary shoot proliferation, culture multiplication and rooting of the *in vitro* generated and proliferated shoots of *Annona.* Different strengths of MS and MMS media showed the best result. For axillary shoot proliferation nodal explant from field grown mature plants showed the best results on MMS medium containing 0.2 mg/l BA(6-benzyl adenine) + 0.1 mg/l NAA(α-naphthalene acetic acid) . The highest degree of axillary shoot proliferation from nodal explant was found 80% with 6.0± 0.42 shoots per culture. Root forming performance of IBA(Indole-3-butyric acid) was found to be the best among three auxins (NAA, IAA/Indole-3-acetic acid, IBA) tested. The highest percentage of root formation was obtained with 2.00± 0.21 roots per cutting on the medium containing 0.1 mg/l IBA. At the high concentration (0.1-0.2 mg/l) of all auxins callus formed from the cut margin of the micro cuttings. The regenerated shoots with well developed roots were gradually acclimatized and successfully transferred to the soil under natural conditions.

Key words: Micropropagation, mature plants, *Annona squamosa* Linn.

Introduction

Annona squamosa (Sharifa, Ata, Meoa, Sitaphal, Custard apple, Sugar apple) belongs to the family Annonaceae. *Annona* is indigenous to tropical America where from they spread to different parts of the world. A few species may be native of Africa. The plants of this species are shrubs or small trees with fairly short and smooth trunk reaching to a height of 5-6 meter. Due to hard nature and escape from animal damage, custard apples have become naturalized in many tropical and sub tropical parts.

The species produce aggregate fruits with edible pulp and are regarded as minor fruit crops. Custard apple is mostly consumed as table fruits. It is also used in ice-cream, puddings, and other milk products and preserved as jam, jelly or other products on limited scale. The nutritive value of the pulp of the *A. squamosa* fruits has been determined by Gopalan *et al.* (1987). The fruits contain carbohydrates, fat, vitamins (Thiamine, Riboflavin, Niacin, Ascorbic acid) and minerals (Calcium, Iron, Phosphorus etc.). The immature fruits, seeds, leaves and roots are of considerable medicinal value both in Ayurvedic and Yunani systems of medicine (Kirtikar and Basu, 1933). Its fruits are good tonic, enrich the blood, increase the muscular strength, lessen the burning sensation and relieves vomiting (Ayurveda). Ripe fruit is mild laxative and anthelmintic. The seeds are abortifacient and roots are drastic purgatives. Bark is used in diarrhoea. It has also some insecticidal properties.

A. squamosa is a very important plant both in horticulture and in ethnobotanical use. Although extensive studies have been made on tissue culture and biotechnology of a few tropical and subtropical fruit species, many important fruit plants and medicinal plants have just been studied only for establishing micro-propagation techniques. But still a large group of tropical and subtropical crops has yet not been exposed to plant tissue culture techniques. *A. squamosa* is one among the un-exploited fruit species which is commonly grown in Bangladesh. The plant produces profusion of berries during early rainy season. Selected genotypes hold promise for its large scale cultivation. In comparison with its importance, there are few reports on *in vitro* propagation of the plant. Therefore present investigation was undertaken to develop the tissue culture techniques for rapid micro propagation of *A. squamosa* using explants of mature plants. This investigation was performed in the Plant Tissue Culture Laboratory of Botany department in Rajshahi University during July, 1999 to September, 2000.

Materials and Methods

The explants used in the present study from healthy, disease free and actively growing shoots having tips and node were collected from mature plants. Then the plant material was washed thoroughly under running tap water to reduce the dust and surface contaminants. Surface sterilization includes treatment of the shoot segments using 1% savlon for 10 minutes with constant shaking. Then the materials were washed 3-4 times with distilled water. After rinsing in 95% alcohol for 30 seconds (explants) they were immersed in 0.1% $HgCl_2$ for different durations.

To remove every trace of the sterilant the material was then washed with distilled water. The segments containing nodes or shoot tips were prepared and were cultured singly in culture tube containing 15-20 ml of different growth regulators supplemented agar gelled media.

Different types of explants containing segments of node and shoot tips were collected from the field grown mature plant and cultured on proliferation medium containing cytokinin for axillary shoot multiplication. Following sequential sub-culturing, a stock of axillary shoot was raised from primary culture. Micro cuttings (1-3) cm were prepared from *in vitro* proliferated shoots for regenerating complete plantlets by rooting them *in vitro.*

MMS medium supplemented with different concentrations of auxins was used for rooting of micro shoots. Other substances like sucrose, agar, activated charcoal, PVP etc. were added directly to the media during preparation. Unless mentioned specially, all cultures were maintained in a growth room under 16h photo period with a light intensity of 50-70 μ Em^{-2} S^{-1}.

Results and Discussion

Different types of explants e.g. shoot tip and node were used for the development and proliferation of axillary shoots.

Axillary shoot proliferation: MMS produced the best shoot proliferation (Table 1). Axillary shoot proliferation with two kinds of cytokinin, BA and Kn with or without auxin was investigated. Of them node showed the best result (100%) in BA (concentration 2.0 mg/ml) without auxin. Number of shoots per culture and average shoot length were 6.1 ± 0.39, 5 ± 0.42 cm for BA and 5 ± 0.55, 4.75 ± 0.42 cm for Kn respectively (Table 2) (Fig. 1a-b). The nodal explants were taken from field grown mature plant and cultured on MMS medium supplemented with 36 different combinations and concentrations of BA with NAA, IBA and IAA for investigating axillary shoot proliferation. Among these 0.2 mg/l BA and 0.1 mg/l NAA combination showed the best results.

The highest number of shoots per culture was 6.0 ± 0.42 and the longest length of shoots was 6.2 ± 0.59 cm (Table 3), (Fig. 1c).

Table 1: Effect of different strengths of MS medium on axillary shoot proliferation from the explants (shoot tip and node) of field grown plants.

Growth regulator mg/l	Different nutrient media	Explants showing proliferation	No. of shoots per culture	Average length of the longest shoots (cm)
BA 2.0	MS	75	3.80 ± 0.43	3.50± 0.46
	MMS	100	6.30 ± 0.42	5.50± 0.36
	M_3	65	3.50 ± 0.45	3.20± 0.40

Table 2: Effect of different concentrations of cytokinin in MMS medium in direct regeneration of shoot from nodal segments of *A. squamosa*.

Concentrations of growth regulators (mg/l)		% of explant showing proliferation	No. of shoots per culture	Average length of shoot per culture	Days to sprout the axillary buds
BA	1.0	90	5.06± 0.45	4.05± 0.39	18-20
	1.5	92	5.12± 0.54	4.18± 0.43	15-18
	2.0	100	6.10± 0.39	5.00± 0.42	15-20
Kn	1.0	70	3.87± 0.53	2.80± 0.34	20-22
	1.5	75.5	4.12± 0.41	3.37± 0.43	22-25
	2.0	83	5.00± 0.55	4.75± 0.42	15-20

MS = Murashige and Skoog (1962) medium MMS = Modified Murashige and Skoog's medium
M_3 = Fruit Plant Medium
There were 15-20 explants for each treatment and data ($\bar{x}$± SE) were recorded after 6 weeks of culture.

Table 3: Effect of different concentrations and combinations of BA with NAA, IAA and IBA on axillary shoot proliferation from nodal explants of field grown mature plant.

Growth regulators (mg/l)	% of explant showing proliferation	No. of total shoots per culture	No. of usable shoots per culture	Average length of longest shoots per culture(cm)	Callus formation
BA + NAA					
0.2 + 0.1	80	6.00± 0.42	5.70± 0.73	6.20± 0.59	-
+ 0.2	60	5.60± 0.38	5.00± 0.48	5.70± 0.45	+
+ 0.5	40	3.66± 0.46	3.50± 0.39	3.90± 0.49	-
0.5 + 0.1	50	4.86± 0.55	4.60± 0.56	5.00± 0.49	-
+ 0.2	65	5.83± 0.53	5.44± 0.52	5.89± 0.52	-
+ 0.5	30	2.89± 0.38	2.80± 0.44	2.94± 0.43	+
BA + IAA					
0.2 + 0.1	33	3.34± 0.41	3.16± 0.44	3.31± 0.44	-
+ 0.2	46	4.06± 0.52	3.93± 0.50	4.30± 0.37	-
+ 0.5	23	2.27± 0.47	2.15± 0.19	2.45± 0.46	+ + +
0.5 + 0.1	48	4.61± 0.57	4.45± 0.55	4.63± 0.54	+
+ 0.2	63	5.70± 0.50	5.23± 0.41	5.84± 0.53	+
+ 0.5	30	2.90± 0.33	2.88± 0.46	3.06± 0.33	+
BA + IBA					
0.2 + 0.1	70	5.89± 0.57	5.66± 0.63	5.90± 0.62	-
+ 0.2	60	5.50± 0.63	5.18± 0.51	5.75± 0.61	-
+ 0.5	45	5.00± 0.49	5.12± 0.50	5.53± 0.45	-
0.5 + 0.1	20	2.18± 0.23	2.20± 0.41	2.75± 0.38	+
+ 0.2	65	5.70± 0.60	5.44± 0.52	5.63± 0.53	-
+ 0.5	35	3.46± 0.45	3.35± 0.44	3.56± 0.43	-

(-) Indicates no response (+) Slight callusing (+ +) Considerable callusing (+ + +) Profuse callusing
There were 15-20 explants for each treatment and data ($\bar{x}$± SE) were recorded after 6 weeks of culture.

Table 4: Effect of different auxins in MMS medium on development and growth of roots from *in vitro* grown micro shoots.

Hormonal supplement		% of root formation	No. of roots per rooted cutting	Average length of roots (cm)	Days of root formation	Callus formation at the cutting base
IBA	0.1	100	2.00± 0.21	4.50± 0.20	15-20	-
	0.2	70	1.70± 0.10	3.00± 0.25	25-30	+ +
	0.5	30	1.00± 0.02	3.00± 0.35	25-30	+ + +
	1.0	-	-	-	-	+ + +
NAA	0.1	60	2.50± 0.30	3.00± 0.15	15-20	-
	0.2	40	1.98± 0.21	2.30± 0.25	20-25	+
	0.5	30	1.20± 0.10	1.00± 0.30	25-30	+ +
	1.0	-	-	-	-	+ + +
IAA	0.1	80	2.00± 0.15	3.00± 0.35	18-20	-
	0.2	65	2.20± 0.20	2.50± 0.39	20-22	+
	0.5	35	1.70± 0.15	2.00± 0.30	25-30	+ +
	1.0	-	-	-	-	+ + +
IBA+ NAA	0.2+ 0.1	15	1.5± 0.40	1.10± 0.10	20-25	+
	0.2+ 0.2	25	2.5± 0.20	2.00± 0.10	25-30	+ +
	0.2+ 0.5	20	2.0± 0.40	1.20± 0.10	20-22	+ + +

(-) Indicates no response (+) Slight callusing (+ +) Considerable callusing (+ + +) Profuse callusing
There were 15-20 explants for each treatment and data ($\bar{x}$± SE) were recorded after 7 weeks of culture.

Fig. 1: (a-d): *In vitro* plantlet regeneration of *Annona squamosa* from mature explants. Effects of cytokinin and auxin on axillary shoot proliferation from nodal explants on MMS + 0.2 mg/l BA + 0.2 mg/l NAA after 3 weeks (a), after 5 weeks (b) and after 7 weeks (c). Rooting of *in vitro* proliferated shoots (d)

Rooting of shoots: Two cm long micro cuttings were prepared for *in vitro* rooting. Various concentrations (0.1,0.2,0.5 and 1.0 mg/l) of auxins were tested and 0.1mg/l of all three auxins produced the highest frequency of root proliferation. The frequency of root formation was 100% with IBA, 60% with NAA and 80% with IAA. When micro cuttings were cultured on MMS medium supplemented with 0.5 mg/l either of IBA, IAA or NAA they produced callus at the cut bases of the shoots and very few number of roots. Maximum length of the longest root was 4.5± 0.2 cm in IBA supplemented medium and 3.0± 0.15 cm & 3.0 ± 0.35 cm in NAA & IAA supplemented medium respectively. Different combinations and concentrations of NAA+ IBA did not produce satisfactory rooting than single auxin treatments. Combination, 0.5 mg/l NAA+ 0.2 mg/l IBA produced only 20% root proliferation (Table 4), (Fig. 1d).

The present research work was undertaken with a view to develop a reproducible protocol for large scale propagation of custard apple (*Annona squamosa*). For the primary establishment of *in vitro* culture, surface sterilization of the explants (node and shoot tip) was a necessary step. Shoot tip and node segments of mature plant of *Annona squamosa* were used for shoot regeneration. Among the three strengths of MS medium (viz. MS, MMS and M_3) used, MMS medium produced significantly best results than those produced by other two media (MS and M_3) irrespective of growth regulator supplements. Similar results were obtained by Nair *et al.* (1983) working on *A. squamosa*. Many authors reported that several fruit plants highly responded on MMS medium e.g. *Malus prunifolia* L. (Ranjit *et al.*, 1987) *Citrus aurantifoliata* (Radhamani *et al.*, 1992; Sharma *et al.*, 1984), *Syzygium cumini* (Yadav *et al.*, 1990) etc.

It has been mentioned that BA is the cytokinin of choice at a concentration range of 1.0-2.0 mg/l for producing axillary shoots from explants of most of the fruit plants (Hutchinson, 1981; Litz *et al.*, 1985; Litz and Jaiswal, 1990).

Among the three types of auxins (IBA, NAA, IAA) used IBA supplemented medium was found to be more satisfactory for root induction. In this experiment, rooting response of micro cuttings cultured on IBA containing medium was found to be better than that cultured on medium containing IAA and NAA. The superiority of IBA over other auxin has also been reported for other tropical fruit trees like Guava (Jaiswal and Amin, 1987), Jackfruit (Amin *et al.*, 1992) and Pummelo (Amin and Akhter, 1993).

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