IN-VITRO STUDIES IN NEEM & CASSIA

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Abstract: Almost 26-32% of the drugs currently used are still of plant origin. Most of the plants producing these constituents are wild in nature, only a few are cultivated. As the plants have often been collected from the natural flora, there is an increasing depletion of the natural resources. The present study highlights the application of in-vitro culture techniques for rapid multiplication of elite plants through micro propagation of neem. Meristem cultures leading to multiple shoot formation have been exploited in neem (*Azadirachta indica)*. Explants from shoot apices, intermodal meristem cultured on MS supplemented with BA and NAA gave good responses in shoot multiplication. *Cassia fistula* is another important species of Cassia for anthraquinones. Callus tissue obtained from leaf explants were allowed to grow on MS media supplemented with hormones 2,4-D and kinetin. After successful growth of callus, the tissues were harvested after 60 days. Anthraquinones were extracted by ether and ethyl acetate and subjected to TLC with reference to standard commercial anthraquinone.

Key words: Explants, meristem cultures, anthraquinones, in-vitro culture, micropropagation, azadirachtin, pharmaceutic, TLC,

Introduction: With the progress in the technique of plant tissue and cell culture and its studies of biosynthesis and biotransformation, industrial production of plant medicinal substances by in vitro techniques as received more attention in current years (Carew et al, 1965 Puhan, 1971; Ahuja, 1965; Stockigt et al 1695, Ni, 1997).The system possesses a number of advantages for example, it is not subjected to the limitation a soil and seasonal condition and also provides high rate to growth it has been now possible to industrially produce some plant drugs by cell culture system. It has been demonstrated also that this technique is much superior to the traditional cultivation of plant drug and thus opens up a new way for the production of plant medicinal substances in general. In the present paper, to techniques such as micropropagation of A. indica and callus culture of C. fistula with particular reference anthraquinone production has been discussed.

Azadirachta indica A Zuss (Meliaceae) is a multipurpose tree of its medicinal and insecticidal properties. It grows in different agro climatic zones of our country with a variation in azadirachtin content (1.5 to 4.5%) from seed kernel. (Ermel et; 1986). Moreover, traditional methods to genetic improvement through sexual propagation have several limitations (Tomar. 1989). From the literature survey it appears that in vitro regeneration of *A. indica* have been made from callus at somatic embryoids and also direct shoot formation from seedling explants (Gautam, al 1968, Narayan & Jayswal, 1985). To the best of my knowledge there is no report on the in vitro cloning methods of adult neem tree. This paper reports methodology of in vitro clonal propagation of *A. indica* tree.

Cassia fistula L. (Fabaceae) is one of the important species of Cassia which a rich source of anthraquinones and is widely known for pharmaceutic properties. From perusal of literature (Ahuja and Prasad, 1987) it appears that many useful anthraquinones are present in non - differentiating callus culture of *C. fistula*. The objective of the present programme is to estimate the anthraquinone in vitro callus culture qualitatively as well as quantitatively.

Materials & methods:

A) Young nodal segments of *A. indica* were collected from the mature trees. Explants were surface sterilized with 10% teepol for 15 minutes followed by washing with distilled water. Explants were then treated with 0.15% $HgCl_2$ for 5-6, minutes and washed with distilled water for 3 times. Sterilized explants were then inoculated in MS medium supplemented with different combination and concentration of BA, Kin, BAP, NAA and 2.4-D (Table-1). The cultures were then maintained at 26+1^o C with 16 hrs photoperiod and light intensity of 2000 lux (approx).

B) Leaflets were taken as explants from young branches of *C. fistula* tree. Surface sterilization was made with 0.2% $HgCl_2$ solution for 5 minutes followed by washing with sterile distilled water. Explants were then punched from the leaflet lamina with sterile cork borer (5 mm diameter) and single explants was inoculated on solidified MS medium supplemented with 2.4-D, kinetin, BA, NAA, BAP in different combinations and concentration (Table 2)

C) The powdered Leaf cells (1gm) from callus were extracted with 30 ml of water at 80^o C for 1 hour with frequent agitation. The aqueous extract was decanted and filtered the mare was re-extracted with another portions of 30 ml water for 30 minutes. The extract was decanted and filtered through same filter paper.

A Portion of the aqueous was transferred to a separating funnel. Then acidified with 1 drop of HCl and extracted with two successive portion of 40 and 20 ml of either. The combined ether extract was washed with 2 x 5 ml fraction of water. The ether phase was discarded (free aglycone), in aqueous solution 8 ml of 25% HCl was added and refluxed for 20 minutes. The mixture with new precipitation was extracted with 3x 30 ml fraction of ethyl acetate. The brown deposits (at the interphase) were taken with the filter, washed with small volume of ethyl acetate.

The series of separators was prepared by 25 ml of ethyl acetate solution to the first and 20 ml of pure ethyl acetate to each of the other two. The three separators were shaken in a consecutive manner with the same two portions of 25 and 20 ml of freshly prepared sodium bicarbonate (saturated) solution. Ethyl acetate portions were discarded and combined bicarbonate solutions were acidified with 10 ml of HCl, the mixture was shaken very gently. Then the mixture was extracted successively with 25 ml and 20 ml of ethyl acetate. The combined ethyl acetate solution was washed with 10 ml of water transferred into 50ml of volumetric flask and completed the volume.

D) Identification and Determination of Anthraquinone: For TLC, extracts (ether & ethyl acetate) were concentrated and subjected to TLC on Cellulose (Chromedia CC41) along with standard anthraquinone and allowed to reach the solvent front 10 cm from base line. Developing mixtures were on butane: Pyridine: water (6:4:3) and detected by 15% aniline phthalate in n-butane and leaf at 105^o C for 5 minutes. RF value was determined with formula. (Table-3)

Solute	Ditance travel by	Distance travel by	Rf	hRf (Rf x 100)	Anthraquinone content mg/gm
Ether extract	2.3	10	0.23	23	0.04
Standard Sample	7.9	10	0.79	79	
Ethyl acetate extract	8.4	10	0.84	84	0.06

Table – 3: Determination & estimation of anthraquinone.

Results and Discussion:

A) Neem: Plantlets regeneration of neem explants were initiated within 2-3 weeks of culture on MS basal nutrient media containing BA and NAA. These plantlets grown fully with micro shoots of 3-4 in each with 4-6 weeks of culture. From the experimental results in becomes clear that MS medium supplemented with NAA 1.5 mg/l and BA 2.5 mg/l yielded best result. The results of other treatments have been presented in table 1. In the initiation of culture there were the formations of compact, greenish callus from the base of the shoot explants. For shoot bud proliferation and shoot growth. MS medium containing NAA (1.5mg/l) and BA (2.5 mg/l) was found to be the best. NAA in combination with other cytokinin were found inhibitory on proliferation and growth of shoots (table.1). Hutchinson (1981) and Litz and Jaiswal (1990) were of opinion and BA is an effective cytokinin for producing axillary shoot. The superiority of BA over other cytokinin for multiple shoot formation in mulberry has been reported by Ora and Ohyama (1986).

B) Cassia: Callus induced within 3-4 weeks of culture with 2, 4-D and kinetin was initially dark grown in color. The callus was globular and friable. These calli were subsequently subculture on MS fresh media with 2, 4-D and kinetin in high concentration shows color variation from greenish to blackish. The growth was very good with the auxin 2, 4-D (3.5 mg/l) and kinetin (0.5 to 1.5 mg/l) (Table.2). It was observed that the extraction of glycoside by following ethyl acetate (0.06 mg/gm) is quantitatively more than ether extraction (0.04 mg/gm) (Table.3). This may be due to ether extraction is in free aglycon where as ethyl acetate is rich in carboxylic anthraquinone.

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SL NO	MEDIA	HORMONE CONCENTRATION mg/l					RESPONSE			NATURE OF CALLUS
		2,4D	NAA	KIN	BAP	BA	A	B	C	
01	MS	2	0	0	1	0	-	-	-	
02		0	2	0	1	0	-	-	-	
03		2	2	0	2	0	-	-	-	
04		0	2	1	0	0	-	-	-	
05		0	1	0	0	1	+	30	-	Compact, hard, green.
06		0	0	1	0	2	-	-	-	
07		0	1.5	0	0	2.5	++	60	+	Compact & green
08		0	0	1	0	3	*	30	-	Compact, white
09		0	2	0	0	3	*	65	-	
10		3	0	1	0	0	-	-	-	
11		0	0	0	2	0	-	40	++	2-3 branches
12		0	0	0	3	0	-	20	+	branchless
13		5	0	2	0	0	-	-	-	

+= Poor response. ++= good response,-= no response.

A= Intensity of callus initiation. B= explants showing responses %.C= Intensity of plantlets.

TABLE – 1. EFFECT OF HORMONES ON MORPHOGENETIC RESPONSE IN NEEM.

SL NO	MEDIA	HORMONE CONCENTRATION mg/l					RESPONSE			NATURE OF CALLUS
		2,4D	NAA	KIN	BAP	BA	A	B	C	
01	MS	0	0	0	2.5	1	-	-	-	
02		0	0	0	3	1.5	-	-	-	
03		0	0	0	4	2	-	-	-	
04		0	0	1.5	3.5	0	+++	80	-	Globular,brown,compact
05		0	1	0	4	3	++	60	-	Globular, green, friable.
06		0	0	1.5	4	0	+	30	-	
07		1	1	0	0	0	-	-	-	
08		1.5	0	0	3	0	-	-	-	
09		0	1	1	0	0	-	-	-	
10		1	1	0	2	0	-	-	-	

+= Poor response. ++= good response,-= no response. +++ = excellent response.

A= Intensity of callus initiation. B= explants showing responses %.C= Intensity of plantlets.

TABLE – 2. EFFECT OF HORMONES ON MORPHOGENETIC RESPONSE IN CASSIA.