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MICROPROPAGATION OF *AGERATUM CONYZOIDES* L.-AN IMPORTANT MEDICINAL PLANT

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A protocol was developed for rapid clonal propagation of an important medicinal plant, *Ageratum conyzoides* through *in vitro* culture of mature nodal explants. Multiple shoots were induced on Murashige and Skoog's medium supplemented with varying concentrations and combinations of auxins and cytokinins (indolyl-3 acetic acid , napthalene acetic acid , 2,4-dichlorophenoxy acetic acid, 6-furfuryl aminopurine and 6-benzyl aminopurine).Maximum number of multiple shoots were developed in plant medium fortified with 3.0 mg/l concentration of IAA-BAP combinations. The shoots were transferred for elongation to plant medium fortified with 3.0 mg/l of BAP & IAA with 600 mg/l activated charcoal. In the present study, out of all the auxin-cytokinin combinations tried, IAA-BAP combinations proved best effective for inducing multiple shooting and roots were formed at (2.0 and 3.0 mg/l) rather then IAA-KN combinations

Keywords : Micropropagation; Multiple shoots.

Introduction

Ageratum conyzoides L., (Asteraceae) is an annual herbaceous plant with a long history of traditional medicinal uses in several countries of the world and also has bioactivity with insecticidal and nematocidal activity[1]. This tropical species appears to be a valuable agricultural resources. There is high variability in the secondary metabolites of *A. conyzoides* which include flavonoids, alkaloids, cumarins, essential oils, and tannins[2]. Many of these are biologically active. In India, it is used to treat pneumonia, but the most common use is to cure wounds and burns[1].

Traditional communities in India use this species as a bacteriocide, antidysenteric, and antibiotic[2]. Roots are diuretic and are used in cardiac swellings, uterine disorders, piles, stones in the bladder and venereal diseases[3]. High variability in the secondary metabolites of *Ageratum conyzoides* which include flavonoids has great demand in pharmaceutical industries.

There are several reports in literature, where shoot bud formation has been reported in various explants in response to the combination of an auxin and cytokinin such as *Brassica compestris*[4-5]. Mathew and Hariharan[6] found that in *Syzygium aromaticum* BAP and NAA combination was most effective for promotion of shoot proliferation and multiple shoot formation for nodes. Similar findings for the same hormonal combination to induce and proliferate shoot buds in *Amaryllis* was found[7].

On the other hand, Kumar *et al.*[8] reported that 2,4-D incorporated singly in the MS medium, proved to be most effective for callus induction in the explants. 2-4-D has been found to induce callusing in all nodal explants of *A. conyzoides*,

Hence, it has become imperative to develop a rapid clonal propagation method to promote rapid production of secondary metabolites. Unfortunately no such protocol was available for the micropropagatin of *A. conyzoides*. The present paper describes a protocol for successful propagation of *A. conyzoides* using nodal explants from mature plant.

Materials and Method

The present study has been conducted on *Ageratum conyzoides* Linn. The plants were collected from the campus of University of Rajasthan, Jaipur. The museum specimen of the plant is deposited in the herbarium (RUBL No.-19877) at Department of Botany, University of Rajasthan, Jaipur.

Throughout the course of culture experiments, MS medium[9] was used. Stock solutions of this medium were prepared in double distilled water and stored in dark bottles under refrigeration. The stocks were mixed in required proportion, whenever needed.

Stock solution of auxins *viz.*, indole-3-acetic acid (IAA), napthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were prepared by dissolving in a few drops of absolute alcohol. Cytokinins

Table1. Response of nodal explants of *Ageratum conyzoides* to various concentrations of auxins added singly in MS medium (Culture age: 60 days).

S. No.	Auxins	Concentrations (mg/l)	Nature of Response	Percentage
1.	IAA	0.5	NR	100
		1.0	C+R*	80
		2.0	C+R**	80
		3.0	C+R**	100
2.	NAA	0.5	NR	100
		1.0	C+	80
		2.0	C+R***	60
		3.0	C+R*	80
3.	2,4-D	0.5	C+	100
		1.0	C++	100
		2.0	C++	80
		3.0	C+	80

NR = No response; R* = 0-5 Roots; C^{+} = Scanty callusing; R** = 6-10 Roots; C^{++} = Moderate callusing; R*** = 11-15 Root; C^{+++} = Profuse callusing; R**** = 16 Roots.

Table 2. Response of nodal explants of *Ageratum conyzoides* to various concentrations of cytokinins added singly in MS medium (Culture age: 60 days).

S.No.	Cytokinins	Concentrations (mg/l)	Nature of Response	Percentage
1.	KN	0.5	NR	100
		1.0	NR	100
		2.0	Enl.	80
		3.0	Enl.	100
2.	BAP	0.5	NR	100
		1.0	C+	100
		2.0	C+++	100
		3.0	C++	100

NR = No response; Enl. = Enlargement; C^{+} = Scanty callusing; C^{++} = Moderate callusing; C^{+++} = Profuse callusing.

viz., 6-furfuryl aminopurine (Kinetin) and 6-benzylaminopurine (BAP) were dissolved in a few drops of 1N HCl and final volume of all the growth regulators was made by adding distilled water.

Nodal part of the field grown young plants were collected and thoroughly washed with tap water initially containing a few drops of detergent and then by plain water. It was followed by surface sterilization with 0.1 percent mercuric chloride for 3-4 minutes. Explants were given 3-4 successive washings with sterilized distilled water to remove traces of mercuric chloride. Callus cultures were maintained through regular subculturing at an interval of 3 weeks. For evaluation of growth, fresh weight was determined after removing the adhering agar and moisture

Table 3. Response of nodal explants of *Ageratum conyzoides* to various concentration and combinations of kinetin and auxins added in MS medium (Culture age: 60 days).

Auxins	Concentration (mg/l)	Kinetin (mg/l)			
		0.5	1.0	2.0	3.0
IAA	0.5	NR	C+R**	C+, R**	C+, R*
	1.0	C+, R*	C+, R*	C+, R*	C+, R**
	2.0	C+, R**, MS	C+, R**, MS	C+, R**, MS,	C+, R*, MS
	3.0	C+, R*, MS	C+, R*, MS	C+, R**, MS	C++,MS, R***
NAA	0.5	NR	C+	C+	C+, R*
	1.0	C+R*	C+, R*	C+, R**	C++, SS
	2.0	C+, R*,SS	C+, R*, SS	C+, SS	C+, R*
	3.0	C+, SS	C+, SS	C+, SS	C++, SS
2,4-D	0.5	NR	NR	C+	C++
	1.0	C+	C+	C++	C++
	2.0	C++	C+++	C++	C+
	3.0	C++	C++	C+	C+

NR = No response; R* = Roots initiation; C+ = Scanty callusing; R** = 0-5 Roots; C++ = Moderate callusing; R*** = 6-10 Roots; C+++ = Profuse callusing; MS = Multiple shoots; SS = Single shoots.

Table 4. Response of nodal explants of *Ageratum conyzoides* to various concentration and combinations of BAP and auxins added in MS medium (Culture age: 60 days).

Auxins	Concentration (mg/l)	BAP (mg/l)			
		0.5	1.0	2.0	3.0
IAA	0.5	NR	C+, MS,	C+, MS	C+, MS
	1.0	C+, MS	C+, R*, MS	C++,MS,R*	C++, R*MS
	2.0	C+, MS,R*	C+, MS,R**	C+, MS,R**	C++, MS, R***
	3.0	C+, MS, R*	C+, MS,R**	C+, MS,R***	C+, R***, MS,
NAA	0.5	NR	C+,SS	C+, SS	C+, SS
	1.0	C++, SS	C+, SS	C+, SS, R**	C+++, SS
	2.0	C++, R*,SS	C++,SS,R*	C++, SS	C+,SS,R**
	3.0	C++, R*,SS	C++, SS, R**	C++,R**,SS	C++,SS,R**
2,4-D	0.5	NR	NR	C+	C++
	1.0	C+	C+	C+,SS	C+,SS
	2.0	C+,SS	C++,SS	C++,SS	C++,SS
	3.0	C++, SS	C+++, SS	C+, SS	C++,SS

NR = No response; R* = Roots initiation; C+ = Scanty callusing; R** = 0-5 Roots; C++ = Moderate callusing; R*** = 6-10 Roots; C+++ = Profuse callusing; MS = Multiple shoots; SS = Single shoots.

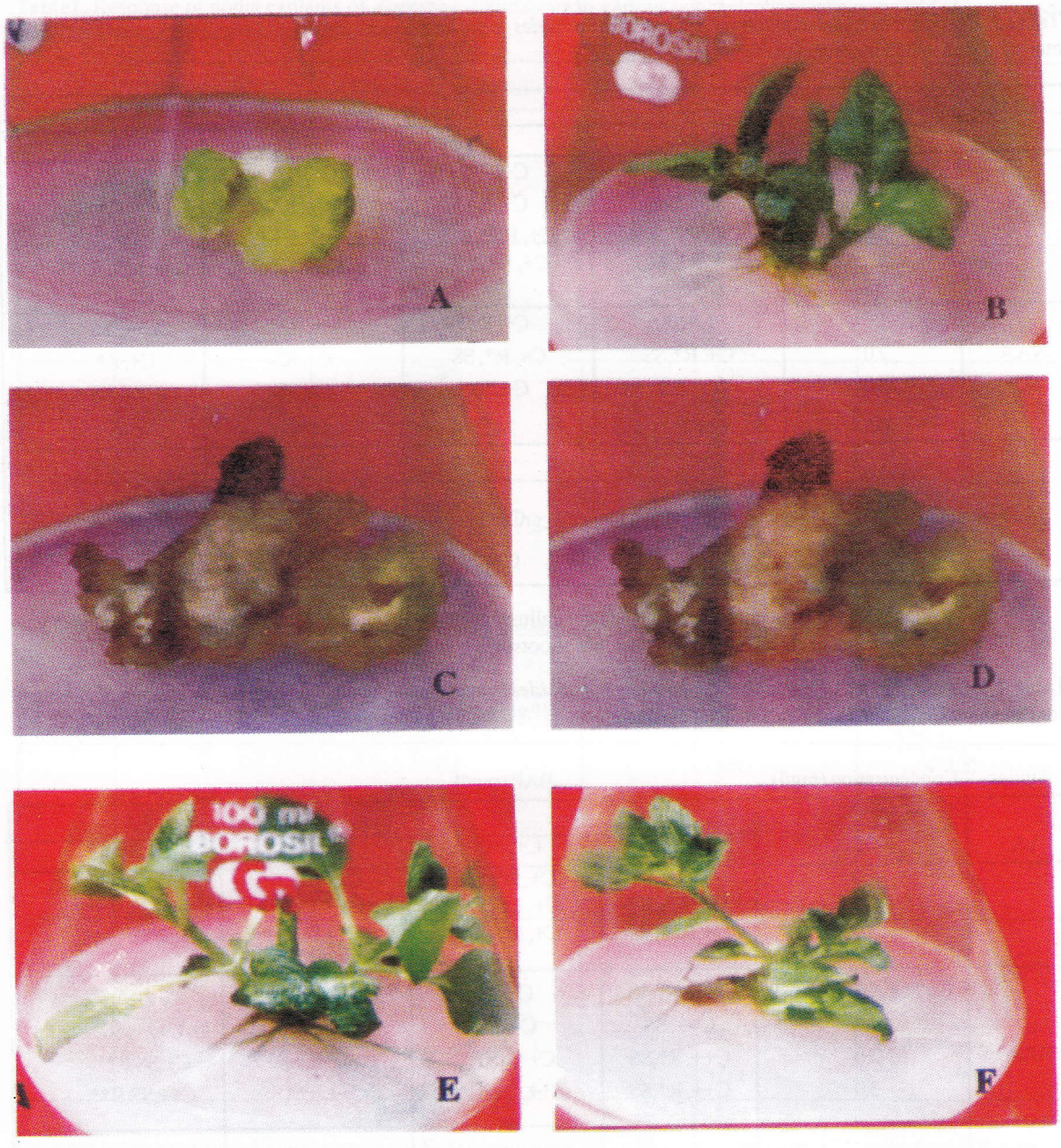

Fig.1. (A-F). Micropropagation of *Ageratum conyzoides.*
(A) Profuse callusing with 2.0 mg/l of BAP
(B) Multiple shooting, rooting and callusing with KN (3.0 mg/l) + IAA (3.0 mg/l)
(C) Single shooting & scanty callusing with KN (3.0 mg/l) + NAA (2.0 mg/l)
(D) Moderate callusing with KN (1.0 mg/l) + 2,4-D (2.0 mg/l)
(E) Multiple shooting, rooting and callusing with BAP (3.0 mg/l) + IAA (3.0mg/l)

by rolling the tissues softly between two folds of blotting paper.

All aseptic manipulations were done in a walk-in-type transfer chamber or laminar flow air bench (HM-104, Horizontal type), pre sterilized with ultraviolet light for half an hour. The chamber was also fumigated with potassium permanganate and formic acid, each time before use. All the cultures, after inoculation, were kept in a growth chamber under a continuous diffused illumination from fluorescent tubes and incandescent bulbs. The temperature was maintained at 26 ± 2°C and 50 to 55 percent relative humidity.

Results and Discussion

The effect of various concentrations of different auxins (IAA, NAA, and 2,4-D) as well as cytokinins (kinetin and BAP) were studied individually as well as in various combinations. For each concentration tried, ten replicates were taken and experiments were repeated thrice. Corresponding controls were set on hormone free MS basal nutrient medium. Observations were recorded after 60 days.

Effect of auxins, incorporated singly in the basal medium (Table 1 and Fig.1.)

(A) Response to IAA concentrations - Concentrations of IAA (1.0, 2.0 and 3.0 mg/l) to be effective as small number of roots except the lowest concentration of IAA (0.5 mg/l) where there was no response. Higher concentration of IAA (3.0 mg/l) induced scanty callus with thin and white hairy roots in 100 percent of the cultures.

(B) Response to NAA concentrations - Lower concentration of NAA (1.0 mg/l) induced scanty callus without any rooting. 0.5 mg/l concentration of NAA showed no response. However, higher concentration of NAA (2.0 mg/l) showed scanty callusing with profuse rooting in 60 percent of the cultures.

(C) Response to 2,4-D concentrations - 2,4-D (0.5 and 3.0 mg/l) induced scanty callusing, whereas at (1.0 and 2.0 mg/l) moderate callusing was obtained with no rhizogenesis. Callus was slightly green in color and friable in morphology.

Effect of cytokinins incorporated singly in the basal medium (Table 2 and Fig.1.)

(A) Response to kinetin concentrations - Kinetin at 0.5 and 1.0 mg/l showed no response but concentrations 2.0 and 3.0 mg/l of Kn incorporated in the medium brought about an increase in size of the explants, the increase being directly proportional to the level of kinetin in the medium.

(B) Response to BAP concentrations - Explants showed no response with 0.5 mg/l BAP concentration. Presence of 1.0 mg/l concentration, scanty callusing was observed.

The best result was observed with concentration of BAP (2.0 mg/l), where profuse callusing induced in 100 percent cultures. BAP concentration (3.0 mg/l) induced moderate callusing on hormone-free MS basal medium, explants turned black within a week.

Effect of various combinations of kinetin and auxins (Table 3 and Fig.1.)

(A) Effect of kinetin and IAA - Lower concentration of kinetin (0.5 and 1.0 mg/l) with lower concentration of IAA (0.5 and 1.0 mg/l) showed scanty callusing with small amount of rooting. The higher concentration of IAA (2.0 mg/l and 3.0 mg/l) with Kn (2.0 and 3.0 mg/l) induced multiple shooting with scanty callusing and good rooting. The overall best result for profuse rooting, multiple shooting and moderate callusing were observed with IAA (3.0 mg/l) in combination of kinetin (3.0 mg/l) in 100 percent cultures.

(B) Effect of kinetin and NAA - Low levels of NAA (0.5 and 1.0 mg/l) concentration resulted in scanty callusing with low levels of kinetin (0.5 and 1.0 mg/l). Higher concentration of NAA (2.0 and 3.0) with higher concentration of kinetin (2.0 and 3.0 mg/l) induced moderate callusing with single shooting observed where no rooting were observed.

The overall best result was with observed NAA (2.0 mg/l) and kinetin (3.0 mg/l) combination in which moderate callus and single shoot was developed in 100 percent cultures

(C) Effect of kinetin and 2, 4-D - Lower concentration of kinetin (0.5 and 1.0 mg/l) in combination of 2, 4-D (0.5 and 1.0 mg/l) induced no response. Kinetin (2.0 and 3.0 mg/l) concentration with 2,4-D (0.5 and 1.0 mg/l) showed scanty and moderate callusing, where no rooting and shooting was observed. The increasing concentration of 2,4-D (2.0 mg/l) with 1.0 mg/l of kinetin showed profuse callusing but the increasing concentration of 2,4-D (3.0 mg/l) and kinetin (2.0 and 3.0 mg/l) induced scanty callusing.

4. Effect of various combinations of BAP and auxins (Table 4 and Fig.1.)

(A) Effect of BAP and IAA - Minimum concentration of BAP (0.5 mg/l) with minimum concentration of IAA (0.5 mg/l) showed no response while increasing concentration of BAP (1.0 and 3.0 mg/l) with lower concentration of IAA (0.5 and 1.0 mg/l) showed moderate callusing with multiple shooting and moderate amount of rooting. Increasing concentration of IAA (2.0 and 3.0 mg/l) in combination of BAP (2.0 and 3.0 mg/l) induced good callusing with profuse amount of multiple shoots and profuse amount of rooting also. At 3.0 mg/l concentration of both IAA and BAP best

results for rooting and shooting were observed.

(B) Effect of BAP and NAA - Combination of BAP and NAA in nodal explants induced single shooting and moderate amount of rooting. Lower concentration of NAA (0.5 and 1.0 mg/l) with lower concentration of BAP (0.5 and 1.0 mg/l) showed scanty callusing and single shooting without rhizogenesis. But higher concentration of both BAP and NAA (2.0 and 3.0 mg/l) induced moderate callusing with profuse rooting and single shooting.

(C) Effect of BAP and 2,4-D - No response observed with 0.5 mg/l concentration of BAP and 2,4-D. Increasing concentration of BAP (1.0, 2.0 and3.0 mg/l) with lower concentration of 2,4-D (0.5 and 1.0 mg/l) induced scanty callusing with single shooting. Increased concentration of 2,4-D (2.0 and 3.0 mg/l) with BAP (2.0 and 3.0 mg/l) showed profuse callusing with single shooting

In the present experiments, among the various auxins tested, NAA incorporated singly in MS medium proved to be more effective for callusing in the explants. Similarly, the role of 2,4-D was better than NAA for the induction of callus from the cultured explants of *Ageratum conyzoides*. Kumar *et al.*[8], reported that 2,4-D incorporated singly in the MS medium, proved to be most effective for callus induction in the explants. 2-4-D has been found to induce callusing in all nodal explants of A. *conyzoides*, when supplemented in varied concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) to the MS basal medium during the present study. Similarly, significance of 2-4-D induced callusing have been reorted in *Brassica* species[9,10]. In the present study, no callusing was evoked in the explants on the concentration of kinetin tried, only enlargement of the explants was observed. Cytokinins, incorporated singly in basal medium are known to induce shoot buds in various explants of different plants. Higher concentration of kinetin/BAP (1.0 to 3.0 mg/l) showed no differentiation of shoot buds in all the explants tried in the present study. Whereas at high concentration of kinetin (1.0 to 5.0 mg/l), differentiation of shoot buds were observed in hypocotyls segments[11]. In the present study, though auxin, incorporated singly, were able to induce a rhizogenic response, cytokinins could not bring about any morphogenetic effect when they were incorporated singly in the MS medium (Fig.1.). Auxin-cytokinin interactions are also not always helpful in obtaining morphogenesis in spite of their extensive effects. De Fossard and Lee[12] tested 175 different cultures of *Eucalyptus boncroftii* with no distinctive morphogenetic response.

In nodal explants, BAP-Auxin combinations proved better than kinetin-auxin combinations for callus induction or morphogenetic response.

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