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EFFICIENT *IN VITRO* MICROPROPAGATION OF PURPLE FLEABANE (*VERNONIA ANTHELMINTICA* WILLD.): A MULTIPURPOSE UNDER-EXPLOITED OIL-YIELDING HERB.

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ABSTRACT

Micropropagation of *Vernonia anthelmintica*, an important medicinal and under exploited oil-yielding herb of asteraceae has been achieved through seedling cotyledonary node explants. The explants if reared on Murashige and Skoog's (MS) medium supplemented with 0.1, 1, 5 and 10 µM levels of cytokinins, viz. N^6-benzyladenine (BA), kinetin (Kn), N^6-(2-isopentenyl) adenine (2iP) either alone or in combinations with auxins *i.e.* α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) or an adjuvant like adenine sulphate (Ad.S.), or coconut water (CW) induced direct multiple shoots in their axils. The best response was evoked on 5 µM BA in combination with 5% CW added to MS medium where cent percent cotyledonary nodes differentiated an average of 4.5±0.7 shoots per explant. The excised shoots rooted best in half strength MS basal medium with 2% sucrose. Nearly 75% shoots organized an average of 1.9±0.5 roots per shoot within 12 days. An early induction of i*n vitro* flowering has also been noticed in 33.3% cultures with 5% CW and 5 µM BA within six weeks. The tissue culture derived plantlets were successfully acclimatized to field with 60% survival. Thus, for the first time, a reliable method for efficient regeneration and achieving early flowering and fruiting, in an economically important crop has been developed.

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KEY WORDS

cotyledonary node, *in vitro* flowering, multiple shoots, plantlet, *Vernonia anthelmintica*.

ABBREVIATIONS

Ad.S. – adenine sulphate; B5 – Gamborg *et al*.'s medium; BA – N^6 –benzyladenine; CW-Coconut water; 2,4-D – 2,4-dichlorophenoxyacetic acid; GD – Gresshoff and Doy's medium; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; 2iP – N^6-(2-isopentenyl) adenine; Kn – kinetin; MS – Murashige & Skoog's medium; NAA – α-naphthalene acetic acid.

INTRODUCTION

Vernonia anthelmintica commonly known as Fleabane is multipurpose, under-exploited genus belonging to the family Asteraceae. The genus being a native of U.S has been widely scattered around the world[1]. This species contains 40% seed oil with 75% vernolic acid which is extensively used for epoxy coatings, manufacture of plastic formulations and other products[2,3]. Medicinally, it is effective against several diseases such as incontinence of urine, amoebiasis, eczema, ringworm, humed herpes, elephantiasis, diarrhoea, stomachache and rheumatism. Besides anthelmintic properties, the plant is also considered febrifuge and a valuable remedy for prolonging life, restoring youth and preventing the hair turning grey. The drug is also used in snake-bite and scorpion sting[4]. With the increased concern about rising health care costs, some governments are encouraging the use of indigenous form of medicines rather than expensive imported drugs and thus have proved as a strong driver for the resuscitation of herbal products. Therefore, realizing the current scenario, it is an exigency to evolve technique for mass propagation and conservation of the genetic resources of medicinal plants. Conventional propagation through seeds and cutting of stem and root is too slow to provide the answer to meet the demand for this valuable plant in time. Propagation through seeds is an inadequate solution due to low viability, a poor seed germination rate and scanty and delayed rooting of the seedlings on account of presence of germinating inhibitors in the genus[5,6]. Tissue culture technique has been developed using cotyledonary explants for its large scale propagation. Though some research papers have appeared on *in vitro* regeneration of other taxa of Asteracea *e.g. Chrysanthemum* spp[7,8], *Centaurea spachii* [9] and *Anthemis nobilis*[10] but no work has been conducted so far on this commercially important, under-exploited genus *Vernonia anthelmintica*.

MATERIALS AND METHODS

(i) Plant materials and surface sterilization:

Seeds of *Vernonia anthelmintica* were procured from M/s Pratap Nursery & Seed Stores, Panditwari, Dehradun, India. Healthy looking seeds were washed under running tap water for 15 min. They were then immersed in 1% citrimide (v/v) germicide-detergent solution (FCI India Ltd.) and stirred vigorously on magnetic stirrer at 30-40^{0}C for 10 min to provide better surface contact with the germicide. These were rewashed under running tap water to remove even the traces of citrimide. After

pouring out excess of water, the seeds were treated with freshly prepared 0.1% (w/v) $HgCl_2$ solution (Merck, India) for 10 min, keeping the vessel on stirrer. Finally, the seeds were given 4 or 5 times washings with sterilized distilled water under laminar flow cabinet to ensure complete surface sterilization of seeds.

(ii) Culture medium:

Seeds were implanted on simple agar semisolid medium for germination. The root, hypocotyl and cotyledonary node explants excised from 20-d-old seedling were cultured on four different basal media *viz*. B5[11], GD[12], Knop's[13], MS[14]. The MS basal medium was used alone or in combination with cytokinins, *viz*. BA, Kn, 2iP, adjuvant *i.e*., Ad.S., CW and auxins such as. IAA, IBA, 2, 4-D and NAA. As a source of carbon, 3% sucrose (DCM, Daurala) was added to media for caulogenesis or 2% for rhizogenesis. The media were gelled with 0.8% agar (Qualigens, Mumbai). The pH of the media was adjusted to 5.8 using 0.1*N* NaOH or 0.1 *N* HCl prior autoclaving. Approximately, 20 ml medium was dispensed in each 2.5 x 15 cm test tube (Borosil) plugged with non-absorbent cotton wrapped in muslin cloth and autoclaved at 1.06 kg cm^{-2} at 121^0C for 15min.

(iii) Culture conditions and statistical analysis:

Cultures were maintained in the culture room at 25±2^0C temperature and relative humidity (55 ± 5%) under continuous white fluorescent light (450 - 640 μW.cm^{-2}) emitted by 40W Philips tubes. The explants were sub cultured at an interval of 28-30 days.

Results have been expressed as mean ± SE. The mean values calculated on the basis of a minimum of 18 replicates in each experiment, repeated twice or thrice depending on the experiments. Data (Table 1, 2 and 3.) were statistically analyzed using ANOVA (SAS). Between the treatments, the average figures followed by the same superscript were not significantly different at p≤0.05 level.

Table 1

Morphogenic responses of cotyledonary nodes excised from 20-d-old seedlings of Vernonia anthelmintica on different basal media. Experiment was repeated once. Data were recorded after 60 d of culture.

Basal Media	Explants developing shoots (%)		Average* No. of shoots per explants	Average* shoot length (cm)
	Single	Multiple		
B5	72.2^c	5.6^a	$0.8±0.1^a$	$3.6±0.7^a$
GD	83.3^b	5.6^a	$0.9±0.1^a$	$4.1±0.8^a$
Knop's	72.2^c	5.6^a	$0.8±0.1^a$	$1.2±0.3^b$
MS	94.4^a	5.6^a	$1.1±0.1^a$	$4.7±0.8^a$

** Mean of two replicates of 18 explants ± standard error. Values within the column followed by the same superscript are not significantly different as determined by SAS at p≤0.05.*

Table 2.

Morphogenic responses of cotyledonary nodes excised from 20-d-old seedlings of *Vernonia anthelmintica* on MS medium augmented with different growth regulators. Experiment was repeated twice. Data were recorded after 60 d of culture.

Growth regulators (µM)							Explants developing shoots (%)		Average* No.of shoots per explant	Average* shoot length (cm)	Culture Inducing flowers (%)
BA	Kn	2iP	2,4-D	NAA	Ad.S	CW(%)	Single	Multiple			
0							94.4[a]	5.6[i]	1.1± 0.1[d]	3.2±0.4[a]	0[d]
0.1							77.8[c]	11.1[i]	1.1± 0.1[d]	0.8±0.5[c]	0[d]
1							44.4[f]	50[e]	1.8±0.3[d]	0.9±0.2[c]	0[d]
5							5.6[i]	94.4[a]	3.3±0.4[b]	0.4±0.1[d]	5.6[c]
10							16.7[h]	83.3[b]	2.7±0.3[c]	0.4±0.1[d]	0[d]
	0.1						77.8[c]	11.1[i]	1.1±0.1[d]	0.6±0.2[c]	0[d]
	1						77.8[c]	11.1[i]	1.1±0.1[d]	1.0±0.2[c]	0[d]
	5						61.1[d]	33.3[g]	1.4±0.2[d]	0.7±0.1[c]	0[d]
	10						11.1[i]	77.8[c]	2.1±0.2[c]	0.6±0.1[c]	5.6[c]
		0.1					83.3[b]	16.7[h]	1.2±0.1[d]	1.9±0.7[b]	0[d]
		1					72.2[c]	27.8[g]	1.3±0.2[d]	1.2±0.2[c]	0[d]
		5					55.6[e]	44.4[f]	1.4±0.2[d]	1.0±0.2[c]	0[d]
		10					11.1[i]	88.9[b]	1.7±.0.3[d]	0.9±0.2[c]	0[d]
5			0.1				88.9[b]	11.1[i]	1.1±0.2[d]	1.1±0.2[c]	11.1[c]
5			1				22.2[h]	27.8[g]	1.3±0.3[d]	0.7±0.2[c]	0[d]
5			5				22.2[h]	16.7[h]	0.6+0.3[d]	0.3±0.1[d]	0[d]
5			10				5.6[i]	0[j]	0.1±0.1[d]	0.1±0.1[d]	0[d]
5				0.1			88.9[b]	5.6[i]	1.0±0.2[d]	0.6±0.14[c]	0[d]
5				1			88.9[b]	11.1[i]	1.1±0.4[d]	0.6±0.20[c]	0[d]
5				5			77.8[c]	22.2[h]	2.03+0.4[c]	0.7±0.07[c]	0[d]
5				10			88.9[b]	11.1[i]	1.1±0.2[d]	0.4±0.06[d]	0[d]
					0.1		94.4[a]	0[j]	0.9±0.1[d]	0.8±0.1[c]	0[d]
					1		88.9[b]	5.6[i]	1.0±0.2[d]	1.5±0.4[bc]	22.2[b]
					5		88.9[b]	5.6[i]	1.0±0.2[d]	2.2± 0.7[b]	0[d]
					10		77.8[c]	11.1[i]	1.1±0.1[d]	1.0± 0.3[c]	0[d]
5					0.1		22.2[h]	72.2[e]	2.1±0.3[c]	0.3± 0.0[d]	0[d]
5					1		33.3[g]	61.1[d]	2±0.3[c]	0.2± 0.0[d]	0[d]
5					5		44.4[f]	55.6[e]	1.8±0.1[d]	0.3± 0.0[d]	0[d]
5					10		50[e]	44.4[f]	1.6±0.3[d]	0.3± 0.1[d]	0[d]
5						5	5.6[i]	94.4[a]	4.5±0.7[a]	1.6± 0.3[bc]	33.33[a]
5						10	5.6[i]	94.4[a]	3.6±0.2[b]	1.2± 0.4[c]	0[d]
5						15	5.6[i]	94.4[a]	3.4±0.2[b]	1.1± 0.3[c]	0[d]
5						20	5.6[i]	94.4[a]	3.2±0.3[b]	0.9± 0.3[c]	0[d]

**Mean of two replicates of 18 explants ± standard error. Values within the column followed by the same superscript are not significantly different as determined by SAS at p□0.05.*

Table 3

Rhizogenesis of excised shoots of *Vernonia anthelmintica* cultured on full and half strength MS and B5 medium alone and half strength MS with different auxins. Experiment was repeated twice. Data were recorded after 30 d of culture.

Medium + auxins (μM)	shoot developing roots(%) with callus	without callus	Average* No. of roots per shoot	Average* root length(cm)
MS(F) 0	0^{g}	0^{c}	0.0 ± 0.0^{c}	$0.0\pm0.^{d}$
MS(1/2) 0	16.7^{e}	75^{a}	1.9 ± 0.5^{a}	3.6 ± 0.8^{a}
B5(F) 0	0^{g}	0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}
B5(½) 0	8.3^{f}	33.3^{b}	1.4 ± 0.6^{a}	1.7 ± 0.6^{b}
MS (½) + IBA				
0.1	25^{d}	0^{c}	0.4 ± 0.1^{b}	0.7 ± 0.3^{c}
1	12.5^{e}	0^{c}	0.1 ± 0.1^{b}	0.5 ± 0.3^{c}
5	79.2^{b}	0^{c}	1.7 ± 0.8^{a}	0.6 ± 0.2^{c}
10	87.5^{a}	0^{c}	1.2 ± 0.6^{ab}	0.4 ± 0.2^{c}
MS (½) + IAA				
0.1	12.5^{e}	0^{c}	0.3 ± 0.1^{b}	0.4 ± 0.2^{c}
1	16.7^{e}	0^{c}	0.2 ± 0.1^{b}	0.4 ± 0.2^{c}
5	25^{d}	0^{c}	0.5 ± 0.1^{b}	0.5 ± 0.3^{c}
10	33.3^{c}	0^{c}	0.5 ± 0.2^{b}	0.4 ± 0.2^{c}
MS (½) + NAA				
0.1	12.5^{e}	0^{c}	0.2 ± 0.1^{b}	0.5 ± 0.2^{c}
1	33.3^{c}	0^{c}	0.2 ± 0.1^{b}	0.4 ± 0.2^{c}
5	79.2^{b}	0^{c}	0.6 ± 0.2^{b}	0.3 ± 0.1^{c}
10	87.5^{a}	0^{c}	0.8 ± 0.3^{b}	0.2 ± 0.0^{c}

** Mean of two replicates of 24 explants ± standard error. Values within the column followed by the same superscript are not significantly different as determined by SAS p□0.05.*

RESULTS

(i) Differentiation of shoots:

Of the several explants, *i.e.* root, hypocotyl and cotyledonary node tried, only cotyledonary node induced multiple shoots. If reared on four different basal media, *viz*. B5, GD, Knop's and MS, MS proved best for eliciting optimum shoot differentiation in cent percent (94.4% single + 5.6% multiple) cultures with an average of 1.1±0.1 shoots per explant having an average shoot length of 4.7±0.8 cm (Table 1). Though addition of cytokinins (BA, 2iP, Kn) to the basal medium promoted average shoot number on all their levels (0.1, 1, 5 and 10 μM), yet 5 μM BA yielded the best response in cent percent (94.4% multiple + 5.6% single) cultures with an average of 3.3±0.4 shoots per explant. (Table 2, Figure 1B). However, percentage of multiple shoots induction as well as average shoot number was significantly less on 0.1, 1 and 10 μM BA (Fig. 1A). Compared to BA other cytokinins (2iP or Kn) proved less effective in promoting organogenesis (Table 2).

BA in combination with Ad.S. did not facilitate an increase in the number of shoots. Nevertheless, 5 µM BA in combination with CW was most effective for shoot bud multiplication, which developed an average of 4.5±0.7 shoots in cent percent explants (Table 2; Fig.1B). Influence of auxins (2, 4-D or NAA) in combination with optimum level (5 µM) of BA did not promote shoot proliferation (Table 2). However, on such media, induction of greenish-brown, nodular and compact calli were initiated after 4 to 5 days of culture covering the entire explants within 15 days. The percentage of calli increased with increase in levels of auxins. Besides, root and hypocotyl explants induced only callus when cultured at different concentrations (0.1, 1,5 and 10) of cytokinins (BA, 2iP, Kn) alone or in combinations with adjuvant (Ad.S., CW) and auxins (2,4-D, NAA).

A Culture showing induction of single shoots on MS + 1µM BA 1.68x

B Culture showing induction of multiple shoots on MS + 5µM BA + 5% CW 1.84 x.

(ii) In vitro flowering:

The explants cultured on MS medium enriched with adjuvant (Ad.S. or CW) or cytokinins (BA or Kn) alone or in combination with 2,4-D induced *in vitro* flowering within six weeks of initial inoculation (Fig. 1C). Percentage of flowering varied from an optimum of 33.3% on 5%CW+ 5 µM BA to 22.2% on 1 µM Ad.S. and 11.1% on 5 µM BA + 0.1 µM 2,4-D medium. However, the percentage of *in vitro* flowering was significantly less (5.6 %) on cultures reared on cytokinins alone (5µM BA or 10 µM Kn). The other combinations tried, did not favour early *in vitro* flowering (Table 2).

C Well developed in vitro flower on MS + 1µM Ad.S. 1x.

(iii)In vitro rooting and field transfer:

To induce rhizogenesis, nearly 2 cm long, axenically grown shoots were excised and cultured on half or full strength MS or B5 basal alone or half strength MS with varying levels (0.1,1,5 & 10 µM) of different auxins *viz.* IAA, IBA and NAA along with 2% sucrose (Table 3). The best response was observed on half strength MS medium where 75% cultures organized roots without intervening callus with an average of 1.9±0.5 roots per culture having an average root length of 3.6±0.8 cm (Fig. 1 D). Though, the percentage of rooting was maximum (87.5%) on IBA/ NAA supplemented medium but that preceded intervening callus formation (Table 3).

D Induction of rhizogenesis in excised in vitro raised shoots and development of plantlets on half strength MS medium after 12 days of transfer 1.68 x.

The *in vitro* raised plantlets were taken out of the test tubes, washed thoroughly with tap water and dipped in bavistin for half an hour and rewashed. They were transferred to autoclaved soilrite kept in jars and watered with ¼ strength MS salts twice a week up to 15 days for acclimatization. These were transplanted in earthen, pots containing pre-autoclaved garden soil and maintained under high humidity conditions for acclimatization. The percentage of survival in the field was about 60% after three months (Fig. 1 E).

E In vitro raised plantlet transferred to garden soil after 3 months 0.65x.

Fig.1

A-E In vitro plantlet development through cotyledonary node explants of Vernonia anthelmintica Willd.

DISCUSSION

In vitro regeneration has been found to be extremely difficult in the member of family Asteraceae on account of several constraints such as (i) presence of inhibitors during seed germination (ii) non availability of the elite germplasm and, (iii) poor seed germination due to short term viability[5,6,7]. However, some research papers have appeared on *in vitro* regeneration of other taxa using various explants of asteraceae[7,8,9,10]. But no work has been conducted so far on this commercially important genus *V. anthelmintica*. During present investigation *in vitro* regeneration has been achieved successfully employing cotyledon explants.

While comparing the responses of various growth regulators only BA elicited the optimum number of shoots in maximum percentage of cultures. Besides, addition of CW (5%) further improved the response. Similar to this, promotary effect of CW in organogenesis has been also reported earlier[15,16,17]. Promotary effect of BA has already been well established in several other herbaceous taxa e.g., nodal segments from micro propagated plants of *Lavandula vera*[18], rhizome buds of turmeric and ginger[19], leaf culture of *Arachis pintoi*[20], node, internode and leaf explants of *Bacopa monniera*[21], root explants of *Swertia chirata*[22]. The probable reason for the induction of multiple shoots through cytokinins may be due to the fact that the latter activate and regulate the expression of genes associated with cell division and differentiation. Cytokinin is most likely to set in motion a cascade of developmental events which promote shoot regeneration[23,24]. BA being an artificial cytokinin has been proved effective in shoot regeneration in many systems, because it not only mimics the activity of endogenously produced hormones but is also metabolically biostable and resistant to enzymatic degradation[25, 26]. Besides, the plant tissues metabolize the natural hormones more rapidly than the artificial growth regulators or could also be due to their ability to induce production of natural hormones such as zeatin within the tissues[27].

The requirement of low salt concentration without auxin for rooting further makes this method more suitable for mass propagation. Reduced effect of MS salts either alone or in combination with auxins for induction of rhizogenesis in herbaceous taxa has already been reported earlier [22,28,29,30,31]. Thus this plant can be easily grown even in waste lands.

Early induction of *in vitro* flowering in the cotyledonary cultures of this genus has been seen as a novel feature which offers an attractive alternative to obtain flower and seeds throughout the year. Though flowering has been seen on BA or Kn supplemented medium yet the optimum response has been seen on medium containing 1 µM Ad.S. Earlier, Bernier[32], Joshi and Nadgauda[33], too, have reported that cytokinins have promotary as well as inhibitory effects on induction of flowering in a number of plant species, and promotary effect is much more than inhibitory ones. *In vitro* flowering was also reported earlier in hypocotyl explants of cucumber[34] and in nodal explants of Gentian[35] under the influence of 0.5-1 µM BA+ 1.5-5 µM 2,4-D or 2 µM Kn alone.

Since seeds of this taxon are the economically valuable part, it is worth doing extensive work on this taxon using this protocol. Further study for enhancing percentage of flowering and quality of seeds is in progress so that this could be effectively employed for extraction of oil at commercial

level. The protocol can be used even for raising transgenic plants with improved seeds having elevated level of epoxy oil.

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