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In vitro cloning of an-antidiabetic drug plant *Vernonia divergens* benth through nodal and shoot-tip cultures

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Abstract
Medicinal plants are in demand since the beginning of human civilization and the plant products feature prominently in traditional therapeutics. Diabetes is one of the most common diseases in human population and more than 30% Indian population are suffering from this disease. There are large number of plants which are recommended for its treatment in herbal system of medicine. *Vernonia divergens* Benth commonly known as insulin plant is a potent sugar killer and is used as an excellent medicine for diabetes mellitus. This plant has restricted distribution in our locality and some people suffering from diabetes grow this plant in their courtyards. Due to ever increasing demand of this plant for officinal uses and to analyse biochemical constituents, it is desirable to develop a simple and efficient protocol for mass propagation and conservation to meet the growing need. Keeping in view its medicinal use, the *in vitro* studies of this plant were being undertaken to develop a protocol for mass propagation as well as to analyse its biochemical constituents.
Regeneration of plantlets and callus differentiation were obtained using stem (node, internode) and shoot-tip of *Vernonia divergens* as explants. Sterilized explants taken from *in vivo* grown plant (about 4 years old) were cultured on MS (Murashige & Skoog, 1962) medium containing 0.8% agar, 3% sucrose and different combination and concentration of NAA/2, 4-D and Kinetin (Kn). Techniques were standardized for shoot regeneration directly from node and shoot-tip explants as well as shoots from callus. The development of shoots/multiple shoots was more frequent in culture, the best response was obtained on 3 mg/l Kn in nodal and shoot tip culture. Multiple shoots were obtained in nodal culture on 2 mgl-1 NAA + 3 mgl-1 Kn. Callus mediated shoot regeneration was frequent in culture and was achieved on 5 mgl-1 Kn and 2 mg/l-1 NAA on subculture. 2, 4-D alone or 2, 4-D + Kn resulted in callus differentiation from explants. Callus in general was greenish-white/white compact, hydrated and crystalline in appearance. Rooting of shoots (3-4 cm) was obtained on rooting medium (½ strength of MS salts) fortified with 1 mgl-1 NAA & 2 mgl-1 IBA. *Ex-vitro* rooting (dipping of shoots in IBA) technique was more promising in woody shoots and plantlets were successfully transferred to soil and survived well in nature. The success rates of transferred plantlets were satisfactory. Higher concentration (>5 mgl-1) of auxin and Kn was inhibitory for culture growth, nodal explants was superior to all other explants (internode, shoot-tip) with respect to shoot regeneration. Protocol was also developed for conservation of callus which survived till 2 years. *In vitro* developed plantlets were morphologically identical to parent plants. Works are in progress to develop protocol for isolation of active constituents and to determine the active compound which has ant diabetic properties.

Keywords: *Crystalline, diabetes, mellitus, restricted, potent, vernonia*

Introduction
Diabetes is one of the most common diseases in human population and more than 30% Indian population are suffering from this disease. There are large number of plants which are recommended for its treatment in herbal system of medicine (Ansari 2007, Naseem and Ansari 2008) [3, 18]. *Vernonia divergens* Benth (Fam: Asteraceae) commonly known as insulin plant is a potent sugar killer and is used as an excellent medicine for diabetes mellitus. This plant has restricted distribution in our locality and some people suffering from diabetes grow this plant in their courtyard (Singh 2011 & Kumar *et al*. 2010, 2014) [23, 12, 10].
Medicinal plants are in demand since the beginning of human civilization and the various plant products feature prominently in traditional therapeutics (Ahuja, 1994, Khalafalla *et al*. 2009, Nag & Johri 1970 & Naseem 1990) [2, 13, 15, 16]. Over 25% of all drugs dispensed world over include plant constituents. Diabetes is a global problem. The prevelance of diabeties in India is showing a sharp upswing, it is reporting by United Nations that India would be the diabetic capital of the world in coming 20 years. Diabetes is a pathologiical condition in which there is uncontrolled increase in blood sugar level.

The two primary pathogenic factors leading to diabetes are decreased insulin secretion and insulin resistance which arise from abnormalities within liver, skeletal muscle and pancreatic cells. Insulin resistance increases the risk of heart disease and also decreases high density lipoprotein cholestrol level. Now a days, diabetes is a globel problem, the treatment of which is recommended by various homeopathic and herbal drugs. There are large number of plants and their mother tinctures which are recommended for its treatment in herbal system of medicine and homeopathy (Ansari, 2011, Kumar *et al*, 2012, Naseem & Jha 1994, Dhawan 1909) [4, 17, 11]. *Vernonia* plant directly taken by the diabetic patients from nature. This plant has no systematic cultivation and biochemical constituents and mass propagation. *Vernonia* antidiabetic drug plant need domestication and cultivating drug plants on large scale for commercial demand.

Conservation and mass propagation of antidiabetic drug plants through organ culture are highly recommended and enormous efforts in the area of tissue culture (Guha & Maheshwari 1966, Razdan 1993 & Tyagi & Kothari 2005) [7, 20, 24]. Screening of secondary metabolites of pharmaceutical importance.

Diabetes is more common in persons having sedentary habit, rich diet, anxities and stress, it is also hereditary. (Naseem & Ansari 2008) [18]. This disease is caused due to malfunctioning and disorder of pancreas which fail to secrete adequate amount of insulin or sometime insulin secretion is stopped as a result proper assimilation of sugar in body does not take place and excess sugars flow out in blood and urine (Skoog & Miller 1957, Steward *et al.*, 1958) [23, 24]. This disease has serious implications and the main symptoms in the diseased persons are general weakness, nervousness, loss of appetite, increased thirst, loss of vigour and loss of memory. It has serious side effects on eye, heart, kidney and wound healing. Pathological features attributed to diabetes mellitus are flow of glucose in urine (glucosuria), Polyurea, Polydipsia, polyphagia, asthenia and acidosis (Sembulingam and Sembulingam, 2005) [21].

Our investigation is based using explants collected from mature *in vivo* grown plant (about 4 years old) and the cultures were maintained under continuous cool white fluorescent light (2000 lux) during the whole experiment (White 1949, Haberlandt 1902) [8]. In my opinion, the present investigation would probably form the basis for first report on this taxa. *In vitro* cloning of *Vernonia divergens* through direct and callus mediated shoot regeneration using explants taken from *in vivo* grown plant under different hormonal regimes.

Material and Methods

Micropropagation of plantlets was obtained using stem (node, internode) and shoot- tip of *Vernonia divergens* as explants. Node (8-10mm), internode (10mm) and shoot tip (10- 15 mm) collected from young shoots of *in vivo* grown plant (about 4 years old) were cultured on MS (Murashige & Skoog, 1962) [14] medium containing 0.8% agar, 3% sucrose and different combination and concentration of NAA/2, 4-D and Kinetin (Kn). Rooting of shoots (3-4cm) was obtained on rooting medium RM (½ MS Salts) fortified with 1mgl-1 NAA & 2 mgl-1 IBA (Cooking 1960, Nitsch 1969 & Skoog 1994) [5, 19]. In the present investigation, a preliminary studies were made on antidiabetic drug plants *Vernonia divergens* Benth. (Fam: Asteraceae) commonly known as insulin plant in nursery (Hains 1961) [9]. Leaves of this plant is a potent sugar killer and is used as an excellent medicine for diabetes mellitus.

The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC for 20 minute. Various growth regulators and adjuvants used as supplement of the basal medium were IBA, NAA, 2,4-D & Kn. Stem segments (nodes and internodes) and leaf segments from youngest shoots and shoot-tip segments collected from *in vivo* grown mature plant (about 4 years old) of *Vernonia divergens* during December to January were used as explants and were surface sterilized. These adjuvants were used in a wide range of concentration (1-10 mgl-1) either alone or in various combinations. The stocks of various growth regulators were prepared.

The cultures were incubated in culture room mainted at 25 + 20 C with a relative humidity of about 60% under continuous fluorescent light (2000 lux, cool and white). Calli obtained from different explants were taken out of the culture tubes aseptically and kept in a presterilized. The callus was cut into several pieces of almost equal sizes with the help of a sterilized blade. Pieces of calli from the growing portions were inoculated into the culture tubes containing MS medium with different combinations and concentrations of growth regulators.

Microshoots (3-4 cm) obtained from shoot-tip, nodal segment and regenerative callus in *Vernonia divergens* were cultured on MS and rooting media (RM 1/2 MS salts + full strength vitamins & amino acid) supplemented with IBA and NAA singly and in combination for rhizogenesis. Culture conditions were kept constant as in shoot regeneration. The plantlets growing under very humid culture conditions on highly nutritious media require careful conditioning to enable them to grow in soil, otherwise, on transfer to soil usually they die off. Special case was taken for the proper growth of tender roots and shoots.

Results and Discussion

Shoot were obtained directly from node, internode and shoot-tip explants after 10 days of inoculation on MS medium supplemented with 2, 4-D/NAA and Kn. While nodal, intermodal and shoot-tip segments produced callus on MS medium fortified with 3 mgl-1Kn in nodal and shoot-tip culture. Shoot tip and nodal segment were most responsive explants for callus induction and regeneration 2,4-D (Table-1) was the beat hormone for callus growth and optimum response for callus growth was obtained on 5 mg-1 2,4-D in nodal and 2 mg-1 2,4-D in shoot tip culture (Fig. 2). Callus in general was greenish white/white compact, hydrated and crystalline in appearance in nodal and shoot-tip explant. The nodal segment on basal medium supplemented with 2,4-D (1-5 mgl-1) and Kn (1-5 mgl-1) showed hypertrophy, in enlargement of explant. Shoot regeneration and callus growth after 8 days of culture (Table-1). Callus growth was comparatively faster than Kn and NAA supplemented media. Callus obtain on higher concentration 5 mgl-1 of 2,4-D and Kn finnaly turn brown. On suitable combination of hormones (2 mgl-1 2,4-D + 2 mgl-1 Kn). Shoot regeneration and callus growth was excellent (Fig. - 4). 3-4 shoots/culture was obtain on this combination. Callus biomass was taken on 21 days of culture (Fig.-5) and the best callus biomass and percentage response were achieved on 2 mgl-1 each of 2,4-D and Kn. Callus obtain on 2,4-D supported media was not suitable for long term culture. Calli were maintained in culture till one year for regeneration on 1 mgl-1 each of NAA and Kn.

***In-vitro* Shoot Regeneration**

Direct formation of shoots/multiple shoot was observed in nodal and shoot-tip cultures on MS medium supplemented with different concentrations of Kn and NAA + Kn. The best shoot regeneration in nodal explant was obtained on 3mgl-1 Kn and 2mgl-1 NAA + 3mgl-1 Kn and in shoot tip explant on 3mgl-1 Kn, 2mgl-1 Kn+2mgl-1 NAA and 2mgl-1 2,4-D+2mgl-1 Kn above 5mgl-1 was inhibitory for shoot regeneration. Callus mediated shoot formation on sub-culture (node, internode & shoot-tip derived calli) was obtained on medium supplemented with 1-3 mgl-1 NAA abd 2-5 mgl-1 Kn. Caulogenesis through callus subculture was frequent in the present experimental system on NAA and Kn fortified media. The *in vitro* developed shoots either through direct regeneration or via callus phase were isolated or aspetically cultured on basal medium without any phytohormone for better growth and seasoning (Table-2). Shoot elongation was promising on basal medium and attained a length of about 4-5 cm in culture. Rooting was not achieved on hormone free medium.

Rooting of Micro Shoots

In vitro grown shoots (3-4 cm) obtained from nodal segment, shoot tip segment and regenerative callus was subculture on MS and rooting medium (½ MS salts + full strength of vitamins & amino acids) supplemented with or without IBA (1- 5mgl-1) and NAA (1-5 mgl-1). Regeneration of roots was difficult in culture, rooting was however, obtained in some cultures on Rooting medium (RM) supported with 2mgl-1 NAA and 1mgl-1 IBA after 15 days of implantation. Besides rooting, hypertrophy of shoot and callusing were also noticed in culture, browning of callus started after 25 days of culture on the above noted media (Table-2, Fig. -6). Better rooting of micro shoots (about 70%) was obtained on 1mgl-1 NAA and 2mgl-1 IBA. Rooting was not obtained on hormone free and NAA (1-5mgl-1)/IBA (1-5mgl-1) supplemented media (both MS and RM). Hormone free medium only favoured shoot elongation. Stout root emerged from micro shoot (60.2%) on RM containing 2mgl-1 NAA and 1mgl-1 IBA after 20 days of culture but shoots subsequent deformed with marked hypertrophy.

Effect of Seasonal Variation on Regeneration

Influence of explant age and season of explant collection from *in vivo* grown *Vernonia* plant were studied on suitable combinations of hormones [MS+3mgl-1 Kn, MS+1-2mgl-1 NAA+ 2-3 mgl-1 Kn] in case of nodal and shoot tip culture. Age of the explant and seasonal variations (Mach-May, June Aug, Sept.-Nov., Dec.-Feb.) greatly influence shoot regeneration in culture, the frequency of shoot regeneration was highly promising in explants collected during September to May, however, Juvenile explants collected during December to May were most regenerative (Table - 3).

Table 1: Effect of different combinations of growth regulators on callus formation in shoot-tip cultures of Vernonia divergens.

Hormones (mg1-1) (MS medium)	% of cultures Showing response		No. of shoots Per culture		Other Response
	S	ST	S	ST	
2,4D					
(1,0)	72.5	78	-	-	callus
(2,0)	74.2	84.5	-	-	callus & shoot regeneration
(3,0)	-	58	-	-	callus
(5,0)	45	57.5	-	-	callus (show growth)
2,4-D+Kn					
(1.0+1.0)	65.5	77.5	-	-	callus
(2.0+2.0)	78.5	90.2	-	-	white callus
(3.0+3.0)	60.5	68	-	-	callus
(5.0+5.0)	52.5	66.5	-	-	callus (browning)
Kn					
(1,0)	45	55.5	-	-	callus (slow growth)
(2,0)	64.5	72.5	-	-	white callus & shoots)
NAA + KN					
(2.0+2.0)	-	-	-	-	callus (show growth)
Sub culture					
(5.0+5.0)	-	-	-	-	callus (browning)

Table 2: Response of auxins on rooting of microshoots in Vernonia divergens*

Medium	Auxin (mgl')		% of shoots that rooted	No of roots/shoot	Other Response
	IBA	NAA			
	In vitro rooting[1]				
BM	0.0	0.0	-	-	Shoot elongation
	1-5	-	-	-	-
	-	1-5	-	-	-
	1.	2	-	-	-
	2	2	-	-	-
	5	5	-	-	(Browning)
RM	1-5	-	-	-	-
(1/2 MS Salts+full strength vits & amino acids	-	1-5	-	-	-
	1	1	-	-	Poor rooting
	1	2	60.2	1.0±0.2	Stout root
	2	1	70.2	5.0±0.6	Root in bunch
	5	5	-	-	(Browing)
	ex-vitro rooting[2]				
	1	-	-	-	-
	2	41.5	3±0.2	-	-
	3	89.6	3±0.2	-	Roots in bunch

1. Culture period : 21 days
   Culture replicate : 20
   RM : ½ MS salts + full strength vitamins & amino Acids
2. Shoot cutting dipped in IBA solution for 8-10 min. Mean was calculated for 20 shoots.

Table 3: Effect of Seasonal variations on regeneration potential of nodal (N) and shoot tip (ST) segment vernonia divergens*

Age of explant (Duration in month)	Explant	Total no. of treated explants	Number Regenerating	% Regeneration
March-May	N	30	28	93. Fast growth
	ST	30	27	91. Fast growth
June-August	N	30	15	50. Slow growth
	ST	30	14	42. Slow growth
Sept-Nov	N	30	23	76. Moderate growth
	ST	30	22	73. Moderate growth
Dec-Feb	N	30	26	87. Fast growth
	ST	30	25	84.Fast growth

*Explants were taken from 4 years old *in vivo* grown plant (evergreen, period was taken from March).
Growth period : 21 days
Media : N: MS+Kn (3mgl.1), MS+NAA (2mgl.1) + Kn(3mgl.1)
ST : MS+KN(3mgl.1), MS+NAA (1mgl.1) + Kn (2mgl.1)

Fig 1: A flowering twig of *Vernonia divergens* [Beth]

Fig 2: 25days old culture showing excellent Growth of cellus on MS 5mgl-1 2, 4-D

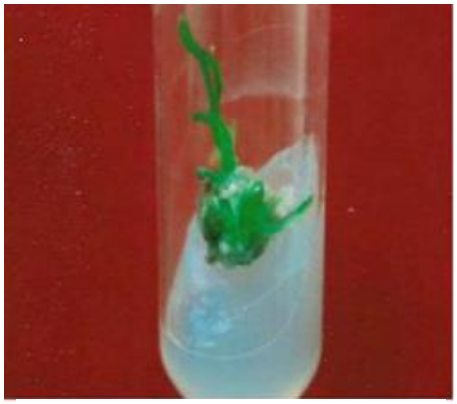

Fig 3: 25days old culture showing callus with D Multiple shoots formation on MS+2mgl + 2, 4-D+2mgl +kn.

Fig 4: 28 days old culture on MS+5mgl-1 2, 4- and 5mgl-1 Kn: mark browing of Culture

Fig 5: 25 Days old culture showing shoot formation from nodal segment on MS 3mgl-1Kn.

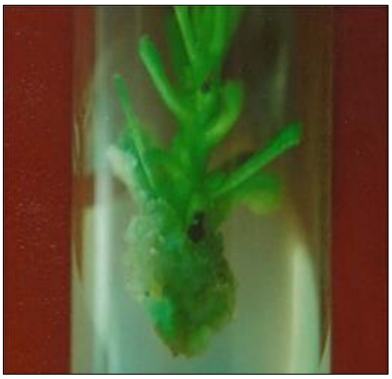

Fig 6: Multiple shoot formation from nodal segment on MS 2mgl-1NAA+3mgl-1Kn

Fig 7: Development of numerous green Shoots on MS+2mgl-1NAA+5mgl-1Kn

Fig 8: *Ex-vitro* established plantlets of *Vernonia divergens*

Conclusion

The present investigation indicates that growth and proliferation of organ cultures from shoot tip and nodal segments in *Vernonia divergens* can be stimulated easily by using *in vitro* techniques. The result of this study shown that tissue culture techniques can play an important role of clonal propagation of *Vernonia divergens* rich in secondary metabolites with antidiabetic properties. Identification and enhancement of active constituents in culture would be a boon for millions of persons suffering from diabeties.

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