In vitro Plant Regeneration from nodal and Shoot-tip explants of *Vernonia divergens*

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ABSTRACT

Diabetes is a global problem, the treatment of which is recommended by various homeopathic and herbal drugs. There are large number of plants which are recommended for its treatment in herbal system of medicine, *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 a restricted distribution in Muzaffarpur and some diabetic people grow this plant in their courtyards. Keeping in view its officinal use, the *in vitro* studies of this plant were being under taken to develop a protocol for mass propagation as well as to analyse its biochemical constituents.

Regeneration of shoots and callus differentiation was obtained using shoot-tip and stem segment of *Vernonia divergens* as explants. Sterilized explants were cultured on MS (Murashige & Skoog, 1962) medium containing 0.8% agar, 3% sucrose and different combinations and concentrations of auxin and cytokinin. Technique have been developed for shoot regeneration directly from stem & shoot-tip explants as well as shoot regeneration from callus. 2 mgl^{-1} each of 2,4-D & Kn was most effective and induced the formation of direct shoots in culture of shoot-tip. 2,4-D (1-5 mgl^{-1}) alone or 2,4-D + Kn resulted in callus differentiation from both the explants. Highest % of result was obtained on 2 mgl^{-1} each of 2,4-D & Kn. Callus was creamy white, hydrated and crystalline in appearance. Callus turned brown on higher concentration (5 mgl^{-1}) of 2,4-D on subculture. Rooting of shoots was obtained on rooting medium (RM, ½ MS salt + full strength vitamins & amino acids) supplemented with 1mgl^{-1} NAA & 2mgl^{-1} IBA technique was more promising in woody shoots and plantlets were successfully transfer to soil and survive well in nature. Work are in progress to develop protocol for isolation of active constituents of pharmaceutical importance known for antidiabetic property.

Keywords: ***Callus, Herbal, mellitus, protocol, restricted, technique, Vernonia.***

INTRODUCTION

Diabetes is one of the most common disease in human population. The prevelance of diabeties in India is showing a sharp up swing, it is a reporting by United Nations that India would be the diabetest capital of the world in 20 years. Diabetes is a pathological condition in which there is uncontrolled increase in blood sugar level. In this disease, sugars are not properly abserbed by the body. This disease is more common in persons having sedentary habit, rich diet, anxities and stress, it is also hereditary (Naseem & Ansari 2008, Khalafalla *et al*. 2009, Kumar *et al*. 2010,2014, Ansari 2011). The disease is caused due to malfunctioning and disorder of pancreas which fails 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. 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. The disease increases many folds during any sort of stress. Pathological features attributed to

diabetes mellitus are flow of glucose in urine (glucosuria), Polyurea, Polydipsia, polyphagia, asthenia and acidosis (sembulingam and sembulingam, 2005).

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Medicinal plants are in demand since the beginning of human civilization and the various plant products feature prominently in traditional therapeuties (Ahuja 1994, Naseem & Jha 1994, Ansari 2011). In Unani Ayurvedic, Allopathic and homoeopathic system (Naseem 1990, Dhawan 2009). *In vitro* protocol for mass propagation of *Vernonia divergens*^{Benth} commonly known as insulin plant in nursery is a stout perennial shrub of 4-8 feet. This plant has restricted distribution in Muzaffarpur and some diabetic people grow this plant in their courtyards (Naseem *et al*. 2009 , Ansari 2011). Leaves of this plant is a potent sugar killer i.e. Hypoglyceric and is used as excellent medicine for diabetic mellitus (Singh 2011, Kumar *et al*. 2012, 2014). No studies on *in vitro* propagation & Biochemical constituents of this plant are known and reported in Books and Journals. Tissue culture studies on this plant is highly desirable for pharmaceutical point of view. However some reports or biochemical constituents of other species of *Vernonia* are available in literature (Chopra *et al*. 1956; The Wealth of India 2005, Ethnomedicinal plant 2006).

MATERIALS AND METHODS

The study has given a method for rapid *in vitro* propagation by multiple shoot induction of *Vernonia divergens,* an important antidiabetic drug plant belonging to Asteraceae family. A mature plant about (4 year's old) is a rare perennial evergreen shrub about 4-8 ft. in height (Haines 1961). Tissue culture studies on vegetative parts (nodal & shoot–tip) explant of this plant were collected during September to May are highly responsive. The explants were cut into small pieces of 5-7 to 10-20 mm long thoroughly washed in running tap water for surface sterilization the explants were treated with 0.2% $HgCl^2$ and washed with distilled water and finally washed with sterile distilled water for 4-5 times. After proper sterilization of (nodal & shoot–tip) segments were cut into pieces (5-7 mm long) and explants were aseptically cultured on MS (Murashige & Skoog's, 1962) medium. Supplemented with 3% sucrose, 0.8 % Agar and different combinations and concentration of growth regulators (Table-1). The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 min. at 120º C. Culture (Borosilicate glass tube) were exposed to continuous light from cool white fluorescent tube (2000 lux) at a temperature 25±2º C (Skoog and Miller 1957, Nag & Johri 1970,Chatterjee & Prakashi 1997). 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 their 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.

Technologies have been standardize for shoot regeneration directly from node and shoot-tip explants. Callus 2 mgl^{-1} each of 2,4- dichlorophenoxy acetic acid and cytokinins was most effective and induced the formation of direct shoots in culture of shoot-tip and node callus mediated shoot were obtained on Kn & NAA supported media on subculture, 2, 4-D (1 mgl^{-1}) alone or 2, 4-D+Kn. Resulted in callus differentiation from both the explants callus was creamy white hydrated and crystalline in appearance. In the present system, a high cytokinin to auxin ratio promotes shoot regeneration. Ten replicates were maintained for each experiment and was reported twice callus turn. Explant taken during September to may were highly responsive.

RESULT AND DISCUSSION

Experiments were carried out with the explants (nodal & shoot–tip) collected from *in vivo* grown plant (about 4 years old) of *Vernonia divergens*. Explants collected during early phase of growth were more responsive than explants collected during late phase of growth.

To select the most suitable basal medium, different explants were cultured on different basal medium (MS & Nitsch) with or without phytohormones and results have been presented in Table – 1 & 2). MS medium was found most suitable for differentiation and regeneration (Skoog & Miller 1957, Razdan 1993).

In vitro plant regeneration

Shoot-tip and nodal segments were most responsive explants for callus induction and regeneration 2, 4-D 2mgl^{-1} was the best hormone for callus growth and optimum response for callus growth was obtained on 5mgl^{-1} 2, 4-D in nodal and 2mgl^{-1} 2, 4-D in shoot-tip culture. Callus mediated shoot regeneration was promising in culture and was obtained on 5mgl^{-}1 Kn and 2mgl^{-1} NAA on sub culture. Callus in general was greenish white/white, compact, hydrated and crystalline in appearance in nodal and shoot-tip explants. Callus turn brown on higher concentration (5mgl^{-}1) of auxin and cytokinin on sub culture. Rooting was not achieved on hormone free medium. Calli were maintained in culture till one year for regeneration on 1 mgl^{-1} each of NAA & Kn (Table-2). Shoot regeneration was best achieved on 3 mgl^{-l} Kn in nodal and shoot-tip culture. Multiple shoots were obtained from nodal culture on 2 mgl^{-1} NAA+ 3 mgl^{-1} Kn (Skoog 1944, Steward *et al*. 1958). In the present system, a high cytokinin to auxin ratio promotes shoot regeneration.

Rooting of micro shoot

Regeneration of roots was difficult in culture. Rooting of microshoots was obtained on rooting medium (RM½ MS salt full strength vitamins & amino acids) supplemented with 1 mgl^{-1} NAA and 2 mgl^{-1} IBA in culture. Better rooting of micro shoots (70 %) was obtained on 1 mgl^{-1} NAA and 2 mgl^{-1} IBA. Rooting was not obtain on hormone free medium. Basal medium only favour shoot elongation (Table-2). RM containing 2 mgl^{-1} NAA and 1 mgl^{-1} IBA after 20 days of culture but shoot subsequent deformed with marked hypertrophy. Better rooting of micro shoots was obtained on RM supplemented with 1 mgl^{-1} NAA and 2 mgl^{-1} IBA.

Table 1: Effect of different combinations of growth hormones on callus formation in nodal and shoot-tip cultures of *Vernonia divergens*

Hormones (mgl^{-1}) (MS medium)	% of cultures showing response		No. of shoots per culture		Other response
	S	ST	S	ST	
2, 4-D					
(1,0)	72.5	78	-	-	hypertrophy
(2,0)	74.2	84.5	-	-	callusing vigorous
(3,0)	-	58	-	-	callus
(5,0)	45	57.5	-	-	callusing (browning)
2, 4-D+Kn					
(1.0+1.0)	65.5	77.5	-	2±0.3	callus, hypertrophy
(2.0+2.0)	78.5	90.2	-	3±0.6	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 (Sub culture**)**					
(2.0+2.0)	-	-	-	-	callus , greening of explants
(5.0+5.0)	-	-	-	-	callus (browning)

10 replicates per treatment
Growth period : 28 days.

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

Medium	Auxin (mgl-1)		% 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 (½ MS Salts+ full strength vits & amino acids	1-5	-	-	-	-
	-	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	-	-	(Browning)

*Culture period : 21 days

Culture replicate : 20

RM : ½ MS salts + full strength vitamins & amino acids

Effect of seasonal variation on Regeneration

In vitro plant regeneration of *Vernonia divergens* were standarized using different combination and concentration of auxin and cytokinin. Age of the explant and seasonal variations (March – May, June – Aug., Sept. – Nov.,Dec. – Feb.) greatly influence multiple shoot regeneration in cultures, the frequency of shoot regeneration was highly promising in explants collected during September to May, However, Juvenile explant collected during Dec. to May were most regenerative (Table -3) (Conger 1987 & Ansari 2011)

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

Age of explants (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})

Conclusion

The result of this studies shown that tissue culture techniques can play an important role of clonal propagation of *Vernonia divergens*. The protocol for conservation of callus which survived till 2 years in *in vitro* developed plantlets were morphological identical to parent plants. Work are in progress to solve the problem regarding browning in callus on long term culture and to develop a simple and efficient protocol for rooting and to determine the active compound which has antidiabetic properties.

Fig.1 - *Vernonia divergens* plant growing in nature (4 years old)

Fig. 2 - 28 days old culture showing excellent growth of crystalline callus on MS + 2mgl^{-1} 2,4-D + 3 mgl^{-1} Kn

Fig.3- Culture showing regeneration of green shoots on degenerated callus (sub culture) on MS+5mgl^{-1}NAA + 5mgl^{-1} Kn

Fig.4- 18 days old callus sub culture showing development of single green & stout leafy shoot on MS+2mgl^{-1}NAA +5mgl^{-1} Kn

Fig. 5 - Development of numerous green Shoots on MS+2mgl^{-1}NAA+5mgl^{-1}Kn

Fig. 6- 45 days old *ex vitro* established plantlet of *Vernonia divergens*

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