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In Vitro REGENERATION OF PLANTLETS FROM NODAL CULTURE OF *Verbena officinalis* L.

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Key words : *Verbena, In vitro* propagation, node, grwoth regulators, Callus, CMSR

Verbena officinalis L (Fam: Verbenaceae) is an ornamental plant known for its medicinal importance . A protocol for its *in vitro* propagation and genetic upgradation is highly desirable. Regeneration of shoots /plantlets was achieved using nodal segments of *V. officinalis* as explants. Sterilized explants were cultured on MS (Murashige and Skoog, 1962) medium containing 0.8 -1% agar, 3% sucrose and different combination and concentration of Kn/BAP and auxins (NAA, IBA, 2, 4-D).Dircet shoot multiplication was obtained on Kn (1-2 mgl^{-1}) supported medium and CMSR was obtained on medium supplemented with Kn and NAA. Highest % of shoot regeneration was noticed on 1.5 mgl^{-1} Kn. Callus in general was crystalline, hydrated, compact and white in appearance, callus grown on 1 mgl^{-1} each of NAA and Kn was slightly greenish-white in appearance. Excised shoots were rooted on MS (1/2 salt strength) medium containing IBA (2mgl^{1}). Kn and IBA supported medium was more economic as the complete plantlets (shoot and root) regenerated on this combination of hormones.

INTRODUCTION

Verbena officinalis L, belonging to family Verbenaceace is a beautiful, ornamental herb known for its medicinal importance. It is a decumbent and erect annual herb measuring about 1-2 ft in height with dissected tap root and beautiful liliac flowers disposed in corymbose spike. It shows luxuriant growth in the winter season and is abundantly found in the field till March-April. The beautiful verbenas consist of several species and hybrid varieties which are widely distributed frequently on waysides (Hains, 1961). This plant is commonly known as Pamukh or Faristarium (Urdu) and is also the source of herbal medicines.This plant contains glucosides, vernalin and verbenin (Chopra *et al.,* 1956), verbenin in mammals affects a strong and lengthy milk secretion (Chopra *et al.,*1956). Leaf juice of this plant is recommended for the treatment of rheumatism and joint pain (Chopra *et al.,* 1956; Watt, 1972) and roots are considered as a remedy for snake-bite and nerve complaints (Chopra *et al.,* 1956, Watt, 1972).

From the review of literature and data available from Natural Centre for Science Information, Indian Institute of Science, Banglore (NCSI / Bio Sci /BGS) 09 dated 22.09.2000), it appears that no tissue culture work on morphogenesis of this plant has been carried out. However, some works of seed physiology of this plant were conducted by Chojnowski *et al.* (1998) and Crezesik *et al.* (1998).Uniform and mass propagation through tissue culture would be beneficial for germplasm conservation, systematic cultivation and genetic upgradation as well as commercial exploitation of this ornamental plant for the production of vernenin and other active constituents at desirable level.

Keeping these into consideration, the present investigation was undertaken to develop a simple and efficient protocot for micropropagation using node as explant; the present study would probably form the basis for first report on this taxon.

MATERIAL AND METHODS

Young nodal explants collected from 1-2 months old *in vivo* grown plant(from garden of University Department of Botany, B.R.A.B.U., Muzaffarpur, Fig.,1) during December to March were washed thoroughly under running tap water for 15-20 minutes and then treated with 1% cetavelon (cetramide ip 20% w/v Isopropyl alcohol, BP 10% v/v solution) along with 4-5 drops of tween 80 for about 20 minutes with constant shaking followed by 3-4 times washing with double distilled water(DDW) to make the material free from detergent. Sterilized explants (8-10mm) were aseptically cut and inoculated singly in culture tube (25x150mm) containing MS (Murashige & Skoog, 1962) medium with 3% sucrose ,0.8% agar and different combination and concentration of auxin & cytokinin (Table 1&2). The pH of the medium was adjusted to 5.8 before autoclaving at 121°c for 20 minutes. Cultures were maintained at 25+ 2°C with light intensity of 2000 lux (cool, white) under continuous light .

Ten replicates were maintained for each experiment and were repeated twice. Shoot proliferation and elongation lasted for 5-6 weeks, callus induction lasted 4-5 weeks and rooting for 5 weeks, the time of each stage was fixed. Percentage of explants showing shoot regeneration, no. of shoots/ explants and length of shoots were taken as parameters for evaluating the morphogenetic potentialities of explants in the present experimental system. Calli were maintained for a long term (about 1½ years) by sub-culturing every 4-5 weeks on suitable medium.

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RESULTS AND DISCUSSION

Shoot regeneration

Direct regeneration of shoots was obtained from nodal explants on MS medium supplemented with cytokinin (Kn & BAP) in various concentrations within a range of 0.5 -5mgl^{-1} (Table 1), the best response (3-4 shoots /culture) in terms of shoot regeneration (SR) as well as shoot elongation (SE) was achieved on medium supported with Kn(1.5mgl^{-1}). SE on this combination was more promising (a height of about 5.6 cm in 25 day old culture, Fig. 2), bulging and hypertrophy in nodal explants were prominent before initiation of shoot buds (SBS). Regeneration of SBS on suitable medium was encountered after 10 days of inoculation, callus also started to from at the basal end of the explants after 15 -17 day of implantation. Table-1 shows that promising number of shoots was also obtained on MS medium containing Kn + NAA (2 + 1)mgl^{-1}. Direct regeneration of shoots in the persent system depends on specific combination of Kn as well as quantitative interaction of auxin and cytokinin, this was also repoted in *Aloe vera* (Khanarn *et al.,* 2014) and *Rauwolfia serpentina*, (Kumari *et al.,*2015). Tissue culture studies on a number of medicinal plants including *V. officinalis* (Naseem & Jha, 1997 and Kumar *et al.,* 2010) suggest that a fine balance of exogenous cytokinin and auxin is necessary before successful regeneration can occur; hormones above 2mgl^{-1} had adverse effect on shoot multiplication. *In vitro* regeneration in *Verbena* from axillary buds has proved to be the most acceptable and reliable method (Mallick *et al.,* 2012 and Sushila *et al.,* 2013); however, the technique needs to be further investigated for better promising results. Nodal explants as a means of micropropagation have been reported in a number of taxa including *V. officinalis* as an ideal explant for direct shoot multiplication (Salma et. al., 2008; Kumar *et al.,* 2010; Mallick *et al.,* 2012 and Kumari and Naseem, 2015), direct regeneration of shoots from nodal explants is highly desirable since the regenerants are genetically identical to the mother plant (Sen & Sharma, 1991 and Mallick *et al.,* 2012).

Callus induction

Callus started to form at the basal end of nodal explants after 15 day of enoculation on MS medium having different combination and concentration of hormones (Table 1), the best callus biomass was obtained on NAA + Kn (1 + 1) mgr^{-1} and Kn + 2, 4D (1 +1l)mgl^{-1} and % response of the explants was also promising on the above combination of hormones (Table 1, Fig. 3).

Callus formation in explants was encountered in all the suitable combination of hormones tested (Table 1); NAA / 2, 4-D and Kn above 2 mgl^{-1} were inhibitory and explants finally turn brown . These findings are congruent with the observations of Singh et. al., (2009) and Kumari et. al., (2015). Callus in general was crystalline, hydrated ,compact and white in appearance; callus grown on 1 mgl^{-1} each of Kn and NAA was slightly greenish-white in appearance (Fig. 5)

2, 4-D is usually the choice auxin for callus induction in the present experimental system but 2,4 -D alone or in combination with cytokinin (Kn /BAP) was not suitable for long term culture and callus mediated organogenesis as also reported by Naseem and Jha (1994), Choudhary & Qu(2000) and Kumari *et al.* (2015).The callus was conserved for a period of 1½ years by regular sub culturing at an interval of 30-35 day on MS medium supported with 1mgl^{-1} each of Kn & NAA. This combination of hormones could not induce any regeneration in culture and was an ideal combination for callus preservation. Callus induction was limited by several factors, when these requirements where adequate (temperature 25 ± 2°C, pH.5.8 and 2000 lux light), the cultre response was optimum.

Callus mediated shoot regeneration (CMSR)

CMSR was encountered on sub culture medium (MS) containing NAA and Kn within a concentration range of 0.5 - 3mgl^{-1} , the best shoot regeneration (SR) from callus was obtained on Kn + NAA (2 : 1 mgl^{-1} , Fig. 7) after 20 day of sub culture and % response on this combination was also promising (about 80%), non green calli cloud not respond to the medium and showed complete loss of differentiation and regeneration even on different hormonal combination; this was also reported by Narayana Swami(1997) and Kumari and Naseem (2015). 3-4 roots were also found to regenerate from callus lump on sub culture after 25 day of culture in presence of NAA + Kn (2 : 1 mgl^{-1}), *in vitro* grown roots were hairy and white (Fig. 6) and no SR was recorded on this combination of hormones . From these findings, it is evident that cytokinin alone as well as high cytokinin and low auxin promotes SR and SE(shoot elongation), whereas low cytokinin and high auxin favours root formation (Singh *et al.,* 2009; Kumar *et al.,* 2010 and Sushila *et al.,* 2013).

Rooting of micoroshoot and Plantlet formation

In vitro grown shoots (4-6 cm) either through direct or callus mediated caulogenesis (3,1 & 3.3) were isolated and singly implanted on MS as well as rooting media i.e., 1/2 MS+PGR (plant growth regulator) for rhizogenesis; rooting was obtained on ½ MS medium in presence of IBA either used singhly or in combination with Kn within concentration range of 0.5- 5mgl^{-1} and optimum rooting of microshoots (Fig. 4) was achieved on 1/2 MS medium supplemented with 2mgl^{-1} IBA within 15 days, IBA above 3ml^{-1} was inhibitory for rhizogenesis. Low salt medium with proper auxin has been found to have stimulatory effect on root induction in many plant species including *Verbena* (Laxmi-Sita *et al.,* 1986; Naseem and Jha; 1994 and Kumari and Naseem; 2015); no rhizogenesis was recorded on MS basal medium.

Induction of roots and shoots (complete plantlets) was encountered on MS medium fortified with Kn(1 – 3 mgl^{-1}) and IBA (1-3mg^{-1}, Table-1), optimum results were obtained on 1mg$^{-/}$ each of Kn and IBA (Fig. 8) this combination of hormones is more economic and needs to be further standardized for better growth as the growth rate of regenerants was much slow. Physical growth conditions described above were also optimal for rooting. Regenerated plantlets were transferred in plastic pots having sterilized soil, vermiculite and sand(1: 1 : 1) and little fungicide; plantlets were acclimatized for a week in culture room and finally transferred to shade house.

In vitro raised plantlets were healthy, green and morphologically identical to mother plant and the survival rate of plantlets was also promising (>78%)

CONCLUSION

In the present experiments, shoots developed directly and indirectly via callus formation from nodal explants can be used as an ideal system for shoot regeneration and *in vitro* cloning of *V. offidnalis*; however, techniques need to be further standardized for mass propagation of superior clones.

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Table-1 Direct shoot regeneration & Callus induction from nodal explants of *V. officinalis* under effective combination and concentration of growth hormones (25 d old culture)*

Hormonal concentration (mgl^{-1})				% culture showing response	Total no. of shoots/culture	Callus growth (mg)	Other response
Kn/BAP	NAAA	IBA	2, 4-D				
0.5	–	–		–	–	–	–
1.0				80.2	1 ± 0.5	Callusing (basal part, bp)	–
1.5				94.4	4 ± 0.38	Callusing (bp, 550 ± 30.0)	–
5.0				58.6		Callusing	Browning
1	1			95		Excellent callus (1090 ± 40.20)	(Greenish-White)
2	1			80.4		Callus (895 ± 33.74)	CMSR (callus sub culture)
1	2			92.3		Callus (895 ± 3.574)	CMRR (Callus sub culture)
5	5			56.2		callusing	browing
1		1		94.8	Complete plantlets		Slow growth rate
1		2		65.6	”		
3		3					Browning
1		.1		94.8		Excellent callus (1085 ± 35.84)	
2		.1		85.4		Callus (886 ± 30.78)	
5		5		55.8		Callus	(Browning)

***Data scored (Mean ± SE) from 10 replicates of nodal explants which were repeated twice.**

Table-2 Rooting of microshoots of *V. offcinalis* on media having effective concentration and combination of phytohormones (25d old culture)*

Medium	Hormonal concentration (mgl^{-1}) NAA	IBA	Rooting days	% response	Total no. of roots/culture	Other response
		0.53	–	–	–	Shoots survived
MS		5	–	–	–	Shoots deformed
	0.5-3	–	–	–	–	–
		05	11	72.4	2.4 ± 0.6	
RM						
(½ MS		1	10-11	86.8	5.2 ± 0.4	Good root growth
Salts)		2	8-10	9.4	6 ± 0.4	Excellent root growth
		5	9-10	62.2	2.0 ± 0.7	Browning
	1	–	12-13	70.2	2.4 ± 0.6	–
	2	–	11	82.4	3.4 ± 0.7	Good root growth
	5	–	11-12	58.2	1.9 ± 0.2	Browning

* Data scored (Mean ± SE) from 10 replicates of micro shoots which were repeated twice.

[NAA-naphthalene acetic acid, K_n– kinetin, IBA-Indole butyric acid, 2, 4-D, 2, 4 dichlorophenoxy acetic acid, BAP-6, benzyl amino purine, CMSR-callus mediated shoot regeneration, CMRR-callus mediated root regenration.]

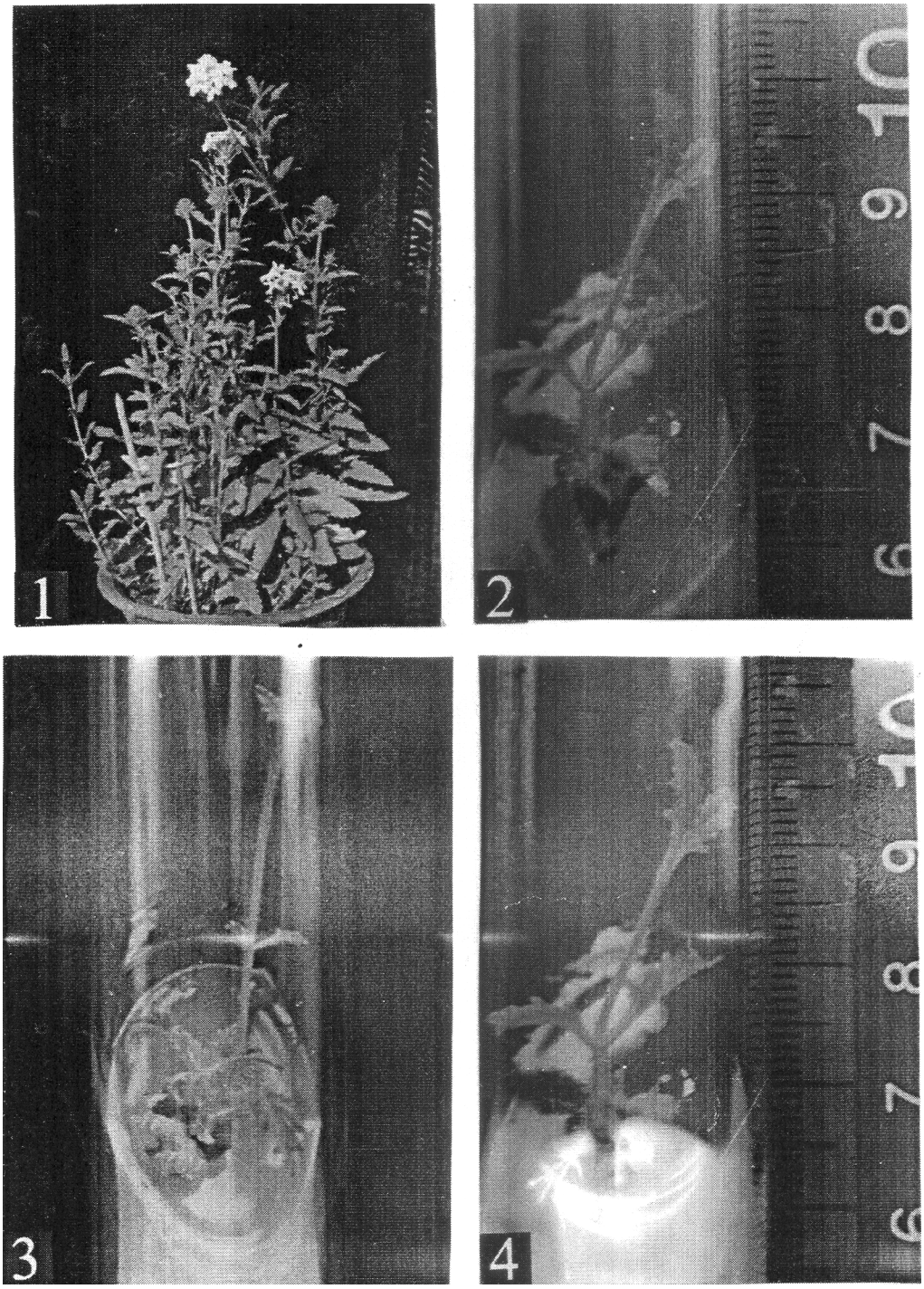

Fig.1 : 2 Months old *in vivo V. officinalis* plant

Fig. 2 : Shoots emerging from nodal segment (NS) on MS + 1.5 mgl^{-1} Kn (21d old).

Fig. 3 : Callus induction from base of NS on MS 1.5 mgl^{-1} Kn (25 d old).

Fig. 4 : Successful rooting of microshoot on medium fortified with ½ MS + 2 ml^{-1} IBA (25d old).

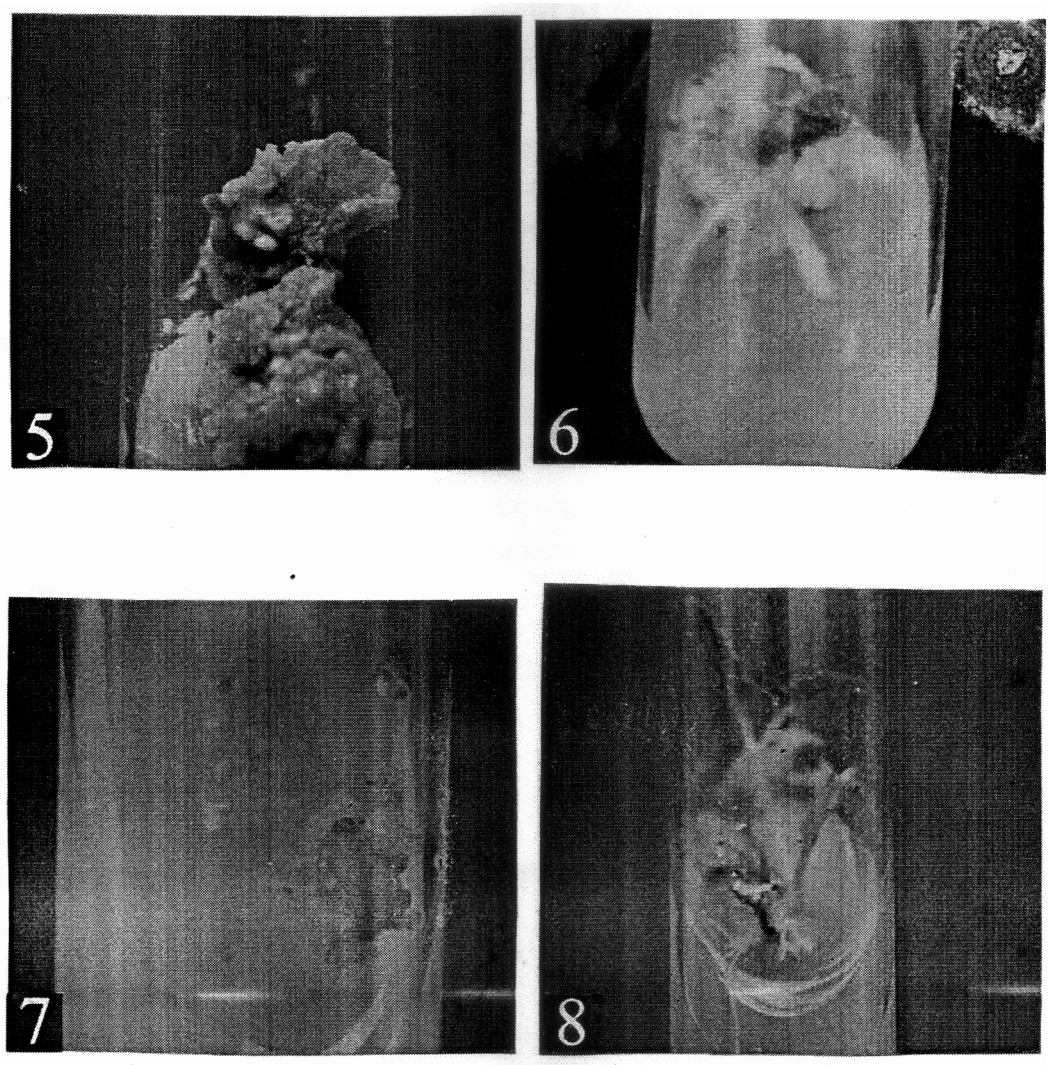

Fig. 5 : Callus induction from NS on MS + 1 mgl^{-1} NAA + 1mgl^{-1} KN (25 d old), mark greenish–white Callus.

Fig. 6 : CMRR on MS + 2mgl^{-1} NAA + 1mgl^{-1} Kn (Subculture medium, 30 d old).

Fig. 7 : CMSR on MS + 2mgl^{-1} Kn + 1mgl^{-1} NAA on subculture medium (25 d old)

Fig. 8 : Development of complete plantlets (Shoot and roots) from NS on same medium (MS + 1 mgl^{-1} KN + 1 mgl^{-1} IBA, 25 d old).