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Callus induction and *in-vitro* Micropropagation of *Vitex negundo*: A multipurpose dynamic medicinal plant of India

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ABSTRACT

Vitex negundo is a commonly used medicinal plant in Indian subcontinent. It has broad range of uses including antimicrobial, analgesic, antifertility, antioxidants, antihyperglycemic to anti-inflammatory and antitumor effects. Considering its medicinal importance, an attempt was made with an aim to protect, conserved the germplasm and to improve genotype of V.negundo by in-vitro technique of tissue culture through callus induction. Explants collected from field grown plants were cultured on MS medium with different concentration and combinations of Auxin (2, 4D, NAA) and cytokinin (BAP, Kin) with or without additive like coconut water for callogenesis. Among 12 different hormonal combinations of MS media, media supplemented with 2, 4D, NAA BAP alone or in combination with C12 showed better response for callussing and C18 for multiple shooting and R14 for rooting showed high efficacy to induce callus and organogenesis. Callus induction was observed within 6 days and the callus was subcultured once in 2 week in subculturing media. The multiple shoot regeneration was observed. Maximum number of shoot regeneration was observed (8-12) in S18 media, supplemented with BAP-10-28 ml and coconut water 5 ml/l. The regenerated shoots having size of 10-28 mm were excised and transferred to liquid medium supplemented with low concentration of IBA, NAA for rooting. Among the different concentration of Auxin tested was more effective for inducing roots within 2 weeks of culture with maximum number of 8-16/explants measuring size of 16-20 mm. 85-90% of the microshoots produced long healthy root system.

Key words: Germplasm conservation, *V. negundo*, Micropropagation, Callus Induction.

INTRODUCTION

Vitex negundo commonly called Sindvar or Nishinda, it is a deciduous, woody aromatic and multipurpose medicinal shrub belonging to the Verbanaceae family. It is usually found in South-Asian countries [1]. It has also many pharmacological properties including analgesic [2] anticontraceptive [3] anti-inflammatory [4] anti-fertility [5] anti-feedant [6] anti-histamine antioxidant [7] cytotoxic for human cancer cell lines [8]. The plant parts are also reported to have anti-microfilarial [9] anti-viral [10] anti-bacterial [11] anti-fungal [12] insecticidal [13] larvicidal [14] as well as significant effect on antagonizing the *Vipera russellii* and *Naja kaouthia* venom induced lethal activity in both *in vitro* and *in vivo* studies [15]. The plant is reported to contain potent and novel therapeutic agents for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product, peroxynitrite. Administration of *V. negundo* extracts also potentiated the effect of commonly used anti-inflammatory drugs [16] analgesics sedative-hypnotic drugs [17] and anti-convulsive agents [18].

In recent years, due to its dynamic medicinal properties, unrestricted removal and over exploitation of this medicinal plant led to its drastic reduction [19]. Presently, the wild genetic stock of *V. negundo* has been depleted heavily due to various biotic and abiotic stresses. Conventional method of propagation of *V. negundo* and several other medicinal plants through vegetative cuttings is unfortunately slow and needs large number of stem segments from the mother plants, leading to more destruction of available genetic stocks. The efficacy of rooting is strictly age dependent besides several other limiting factors. Alternatively, seed propagation of *V. negundo* is possible only with very poor rate of germination. Owing to these reasons, it is very important to protect and restore *V. negundo* by developing a viable protocol for its large-scale micropropagation method and also to supply adequate quantity of elite clones for establishment in the field.

Callus is a de-differentiated state of tissue through the exogenous application of plant growth hormones *in vitro*. This callus can solve the problem of unavailable plant materials for *in vitro* studies. Different physiological and morphogenic responses can also be observed through callus culture like somaclonal variations, somatic embryogenesis, organogenesis; it can also pave the path for isolating economically valuable phytochemicals, which can avoid the collection of plant materials from natural sources [20]. In the areas of plant biotechnology, callus and cell culture carries a special role for producing medicinal and bioactive compounds in large-scale from plants [21, 22]. Phytochemicals are serving as a major source of pharmaceuticals, flavors, agrochemicals, colors, biopesticides, and food additives [23]. In this perspective, callus culture already has been studied for different medicinal plants like *Artemisia annua* for artemisinin, *Aspidosperma ramiflorum* for ramiflorin [24], *Camellia chinensis* for flavones [25], *Capsicum annum* for capsaicin [26], and *Centella asiatica* for asiaticoside [27] but there is still limited information on these medicinal plant resources.

We intended to develop a suitable protocol for studying the callus culture of *V. negundo*. In the present study we opted to investigate the effects of phytohormones for callus induction and to observe genetic improvement in traits of the plant materials after the exogenous application of plant growth regulators in the culture medium on this material.

MATERIALS AND METHODS

Culture Media

The most important factor governing the growth & morphogenesis of plant tissues in culture is the composition of the culture medium. The basal media is Murashige and Skoog's (MS) media [27]. In the experiment 12 different hormonal combinations of media was made for callogenesis, 15 different combinations for subculturing and shoot regeneration and 23 different combinations for rooting was prepared.

Actively growing young shoots of *V.negundo* with 4 to 5 nodes were collected from the motive field, grown plants were maintained in the Nakshatra Van (A botanical & medicinal garden of Ranchi, Developed by Forest Department of Jharkhand Government). For callus initiation, internode segments and shoot tips of young vegetative stem with a size of 1.5-2.0 cm were excised from the *ex-vivo* plant and washed under running tap water for 30 min and then with liquid detergent (1% SDS), later rinsed with sterile double distilled water. The explants were then disinfected using 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) for 2 min, there after the segments were washed 5-7 times with sterile distilled water. Nodal explants were trimmed using a sterile surgical blade under a mixture of ascorbic acid solution (0.1% each) and blotted on sterile filter paper before implanting on the culture media. The processes were aseptically done under laminar air flow (LAF).

Culture media preparation and explants implantation

MS medium was supplemented with 3% sucrose. The media was further augmented with different hormonal concentration as shown in Table 1. The pH of media was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, Mumbai, India) later autoclaved at 121 °C for 20 min. After 24 h explants were prepared and implanted on to the media. The media was poured in borosillicates test tubes (12 x 10) and incubated at 25 ± 1 °C for 16 h photoperiod. Cultures were maintained at 23 ± 2 °C with a photoperiod of 16/8 h under illumination of 4000 lux.

Subculturing of Callus for organogenesis

Shooting Response

The *in vitro* developed calli subcultured on 25 different combinations of auxin and cytokinin for shoot bud differentiation and multiplication. Primary cultures with single and multiple shoots regenerated from the original

nodal explants were carefully taken out from the culture vessels and sectioned into nodal segments. Nodal segments were once again cultured on MS medium fortified with hormones as given in Table 1 for further multiplication. Multiplication of shoots was carried out by repeated harvest of microshoots followed by subculturing of nodal explants on medium supplemented with various hormonal regimes. The data was recorded in different parameter viz percentage response, response day, number of shoot, length of shoot (Table 2).

Rooting Response

For *in vitro* rooting, healthy microshoots regenerated from the above experiments were subjected to rooting on half-strength MS medium fortified with different concentrations of indole butyric acid IBA, IAA, NAA alone or in combination with additives tyrosine and PVP (0.10%). Microshoots measuring the height of 12 - 20 mm with 2 - 3 pairs of leaves were cultured on R14 media and maintained under the cultural conditions as described earlier.

For *ex vitro* rooting, long healthy microshoots were trimmed at the basal end to expose the fresh tissues for facilitating the absorption and treated with 4000 ppm IBA solution and planted in the plastic pots containing soil, sand and vermicompost (6:2:1). Plantlets with well developed root system were recovered from the culture vessels and gently rinsed to remove the adhering media before transfer into plastic pots containing a mixture of soil, sand and vermicompost for acclimatization. Plantlets were initially grown under diffused natural light in a small polythene tunnel with frequent misting to maintain the humidity. Regenerated plants were nourished with diluted macro and micro nutrient of MS medium (1/8) for better growth and development. After 45 days, 75% of the acclimatized plants with well developed root and shoot system were transferred to natural conditions under a shade. These plants were subsequently established in the field by providing frequent watering with organic compost.

RESULTS AND DISCUSSION

Callogenesis

Within two weeks of culture period, most 100% callogenic responses were activated in medium C_{12}, C_5, C_{10}, C_8 and C_7. Best callogenic response was found with gremin tenture for both types of explants. The I.N. explants produced better callogenic response than the S.T. explant in all of the 12 different hormonal combinations.

The medium fortified with BAP (2.5 mg/d.) + WAA (0.5 mg/l) C12 indicated better (100%) response within 6 days that that of the medium fortified with single combination of 2, 4D, NAA and combination of 2, 4D + NAA (Fig 1). Synergism of 2, 4D and NAA found in the study was correlated with the results obtained by Nikolaeve et al. [25] with *Camellia chinerusis*. However, use of single auxin (WAA) for induction of calli was also confirmed in our experiment. Such finding has also been reported by Choudhary et al. [28], when MS medium was fortified with different conclusions of NAA for *Cananga Oberata* culture. Results described by Choudhary et al. [28] were also in agreement with our result for using fera, *Cardiospermum halicacabum* Linn, *Abrul precatorious*, *Vitex negundo* respectively.

Our finding in the study regarding synergistic effect of hormone (A + C) differed from the finding of Choudhary et al. [28] on same plant and also from the Findira of Oliveira et al. [24], We have noted that the effect of cbett callogenis (IN) hormone was explants dependent for *V. negundo*, the similar finding was reported by Hanan et al. [30], which reported that the best responses was only from young leaves with same texfures of calluses but this finding of the present study differed from the findia of Choudhary et al. [28] and Sahoo and Rout [29] on sea plant.

Shoot regeneration and multiplication

After callus induction, further morphogenic response were observed i.e. shooting was observed in SC and SN when the callus was submitted on 25 different combinations of Auxin (NAA) and cytokinin (BAP and Kin) alone on in various combination along with cow (Fig 2). 5 ml/l callus developed both the shoot tip and nodal explants calli were structured morphogeneris that S.T. callus and within 8 to 14 days 90% of shoot induction was observed in (1.5 BAP 0.5 NAA) 18 media top both the explants followed by S_{10}, S_{17}, S_{13}, media also given better response of about 83-80% respectively. These combination an average of 8-12 shoot/regenerated from nodal callus while calli of S.T. produces having length of 24-28 mm shoots having length of 10-19mm. The medium cibtaububg jub akibe did not from multiple shoots. Similar finding was reported by Roy et al. [31]. On the medium containing BAP + NAA, both the explants calli responded well and produced more shoots than the medium containing only cytokinin. Roy et at. [32] observed similar response in case of *B. diffusa*. Noman et al. [33] reported cytokinin induced shoots at a lesser frequency compared to the media supplemented with combination treatment of cytokinin and Auxin.

Between the 2 cytokinin tested (BAP and Kin) BAP was found to be more effective than Kin in the induction of multiple shoots from the nodal explants. Similar observations were reported by Noman et al. [33] and Roy et al. [31]. In the present study BAP was reported with shoat (number and length). Similar reporting was observed in study conducted by Sahoo and Chand [34]. Results described by Noman et al. [33], is also in agreement without result for multiplier. In the study it was observed that in each explants C-9 auxiliary buds were formed within 10 - 15 days after imbruting. Later 25 - 30 days after inoculation, new shoots were developed adjacent to these auxiliary buds.

Root induction and acclimatization

For root induction well developed callus were directly cultured on rooting 23 different combinations of MS media for rooting. Different concentration of IBA, IAA and NAA were used in ½ strength of MS for root induction in addition to additives like PVP and tyrosine. The best response were observed in R14 media supplemented with 1.0 mg/l BA + 1.0 mg/l IAA + 0.5 mg/l BAA followed by R18, R15 and R19 (Table 3). In R14 media it was observed 90% calli showed direct root induction with maximum no of roots 8-16 calli having the size of 12-20 mm Fig 3).

Various studies on root induction from *in vitro* raised microshoots were reported but no reporting was found on direct root induction from callus on this plant. A combination of two auxins for root induction was reported in *Sysgium cumini* by Yadav et al. [35] and by Stephen et al. [19] in *V. nrgundo* but no reporting was found for direct root induction from calli in combination of two auxins and cytokinin (BAP) in addition to PVP and tyrosine. The calli having 6 - 8 roots with 16 - 20 mm in size were further subcultured on R14 medium (supplemented with PVP and tyrosine after 20 days of root induction. It was found that 85% of subcultured material produces microshoots with healthy roots with increase in number and size within 2 weeks of culture and no callus formation was observed from the proximal end of the shoots.

Table 1: Response of callusing in different hormonal composition

Media code & Hormonal composition	Explants source	Percentage of Response	Callogenesis	Time required for response(days)	Color & texture
C1 (1.0mg/l)	ST	30	-	20	White & friable
	NT	80	++	15	White & friable
C2(2.0mg/ l, 2, 4D)	ST	60	++	15	White & friable
	NT	100	+++	8	White & friable
C3(3.0 mg/l, 2, 4D)	ST	25	+	20	White & friable
	NT	80	++	12	White & friable
C4(1.0mg/l NAA)	ST	60	++	16	Greenish white & friable
	NT	100	+++	11	Greenish white & friable
C5(1.5 mg/l NAA)	ST	100	+++	12	Greenish white & friable
	NT	100	+++	8	Greenish white & friable
C6(2.0 mg/l NAA)	ST	40	+	18	Greenish white & friable
	NT	80	++	12	Greenish white & friable
C7(1.0 mg/ l, 2, 4D + 1.0 mg/l NAA)	ST	80	+++	10	White & loose
	NT	100	+++	10	White & loose
C8(1.0 mg/ l, 2, 4D + 1.5 mg/l NAA)	ST	80	+++	10	White loose & wet
	NT	100	+++	8	White loose & wet
C9(1.5 mg/ l, 2, 4D + 1.0 mg/l NAA)	ST	60	++	12	White loose & wet
	NT	80	+++	8	White loose & wet
C10(1.5 mg/l BAP + 1.5 mg/l NAA)	ST	80	+++	8	Whitish Green & wet
	NT	100	+++	8	Whitish Green & wet
C11(2.0 mg/l BAP + 1.0 mg/l NAA)	ST	60	++	15	Whitish Green & loose
	NT	80	+++	12	Whitish Green & loose
C12(2.5 mg/l BAP + 0.5 mg/l NAA)	ST	100	+++	10	Green & wet
	NT	100	+++	6	Green & wet

Following acclimatization about 75% of shoots produced healthy root system and exhibited good shoot growth. Rooted microshoots could be hardened within 45 days and subsequently established in the field. Sahoo and Chand [34] evaluated different combination of substrate (vermiculite, vermicompost, soilrite mix, garden soil) for acclimatization of micropropagated *V.negundo.* This report was found in accordance with the present study that vermicompost supported maximum survival of micropropagated plants with enhanced shoot and root development.

In the present study use of PVP and tyrosine increase the rate and response of root induction was significantly observed. This happened due to extra cellular signaling for differentiation of tyrosine. Observation on use of AgNO3

such as promoting root induction in apple, chicory plants and *Iecalepsis hamiltonir* was reported in earlier studies Stephen et al. [19].

Fig 1a: Callusing in C12 media, green and wet

Fig 1b: Callusing in C8 media, greenish brown and loose

Table 2: Effect of different hormonal composition on organogenesis (shoot length and number)

Media code & Growth Regulators(mg/l) + Coconut water	Average length of shoot explants		Explants showing shoot regeneration (%)		Average no. of shoot/explants	
	ST	IN	ST	IN	ST	IN
S1(0.5 BAP)	12.12±0.26	14.25±2.60	40	50	2±0.2	4±0.2
S2(1.0BAP)	15.26±0.62	20.23±1.32	50	50	4±0.3	5±0.3
S3(1.5 BAP)	12.12±0.33	12.00±2.70	60	70	4±0.2	6±0.2
S4(2.0 BAP)	12.00±1.70	14.00±1.72	50	60	2±0.2	4±0.3
S5(2.5 BAP)	10.42±2.10	10.62±1.70	45	50	3±0.2	3±0.4
S6(0.5 Kn)	-	10.42±0.62	10	10	-	3±0.2
S7(1.0Kn)	-	12.12±0.33	20	25	-	3±0.4
S8(1.5 Kn)	-	10.42±1.72	30	30	-	3±0.2
S9(2.0Kn)	04.04±0.22	14.25±2.60	25	30	2±0.2	4±0.4
S10(2.5Kn)	04.02±0.22	10.16±1.12	20	25	2±0.2	3±0.2
S11(0.5 BAP + 0.25 NAA)	05.01±0.33	18.50±1.70	50	50	3±0.4	5±0.3
S12(1.0 BAP + 0.25 NAA)	06.04±0.24	12.25±2.20	60	75	4±0.2	6±0.2
S13(1.5 BAP + 0.25 NAA)	10.42±0.62	25.23±1.32	70	80	4±0.4	8±0.3
S14(2.0 BAP + 0.25 NAA)	10.62±1.72	24.42±0.33	70	80	5±0.2	8±0.2
S15(2.5 BAP + 0.25NAA)	11.12±1.60	18.50±1.70	60	70	4±0.2	6±0.3
S16(0.5BAP + 0.5NAA)	08.02±0.22	14.16±1.12	45	50	2±0.3	4±0.3
S17(1.0 NAA + 0.5NAA)	13.36±0.33	25.50±2.60	70	80	4±0.2	10±0.2
S18(1.5 BAP + 0.5NAA)	19.25±1.62	28.10±1.70	85	90	5±0.2	10±0.3
S19(2.0 BAP + 0.5NAA)	16.22±2.10	26.00±2.20	70	85	5±0.2	12±0.2
S20(2.5 BAP + 0.5 NAA)	12.10±0.32	16.22±2.10	60	65	4±0.3	5±0.2
S21(0.5 BAP + 0.5 Kn)	08.04±1.70	15.12±1.12	50	60	3±0.2	5±0.3
S22(1.0 BAP + 0.5 Kn)	08.10±1.72	10.42±0.33	50	50	3±0.2	3±0.2
S23(1.5 BAP + 0.5 Kn)	06.05±0.33	14.25±2.60	45	50	2±0.3	4±0.3
S24(2.0 BAP + 0.5 Kn)	06.04±0.22	12.25±2.20	40	50	2±0.4	4±0.8
S25(2.5 BAP + 0.5 Kn)	04.04±0.24	12.12±0.33	40	50	2±0.4	4±0.3

Fig 2a: Multiple shooting in S18 media

Fig 2b: Shoot regeneration in S13 media

Table 3: Effect of different hormonal composition on rooting

Media Code and Growth regulators (mg/l) + PVP(0.1%) + Tyrosine(0.1mg/l)	Rooted shoot (%)	No of roots/culture	Average root length (mm)
R1(0.5 IBA)			2.15±0.18
R2(0.5 IBA)			4.57±0.17
R3(1.5 IBA)	50	8±0.3	3.63±0.12
R4(2.0 IBA)	70	10±0.2	3.89±0.14
R5(0.5 IAA)	-	-	-
R6(1.0 IAA)	30	2±0.2	1.67±0.12
R7(1.5 IAA)	10	2±0.2	1.53±0.14
R8(2.0 IAA)	-	-	-
R9(0.5 IBA + 0.5 IAA)	60	5±0.3	6.83±0.17
R10(1.0 IBA + 0.5 IAA)	75	9±0.2	7.00±0.20
R11(1.5 IBA + 0.5 IAA)	70	10±0.4	8.00±0.04
R12(2.0 IBA + 0.5 IAA)	50	04±0.2	3.20±0.02
R13(0.5 IBA + 1.0 IAA)	65	04±0.2	4.55±0.12
R14(1.0 IBA + 1.0 IAA)	90	16±0.4	20.00±0.20
R15(1.5 IBA + 1.0 IAA)	80	14±0.2	16.00±0.40
R16(2.0 IBA + 1.0 IAA)	70	10±0.2	12.39±0.12
R17(0.5 IBA + 0.5 NAA)	70	10±0.2	12.35±0.20
R18(1.0 IBA + 0.5 NAA)	80	15±0.2	18.00±0.40
R19(1.5 IBA + 0.5 NAA)	80	12±0.3	15.00±0.20
R20(2.0 IBA + 0.5 NAA)	70	10±0.4	8.05±0.04
R21(1.0 IBA + 0.5 IAA + 0.5 NAA)	50	6±0.2	3.05±0.17
R22(1.0 IBA + 1.0 IAA + 0.5 NAA)	60	6±0.4	4.55±0.12
R23(1.5 IBA + 1.0 IAA + 1.0 NAA)	70	8±0.2	5.30±0.11

Fig 3a: Rooting in R14 media

Fig 3b: Rooting in R19 media

CONCLUSION

Considering the importance of medicinal and other properties of *V. negundo* callus induction and direct callogenesis micropropagation protocol can be effectively used in their species for various applications such as genetic improvement, in vitro conservation, large scale genetic multiplication and genetic transformation. Isolation of bioactive compounds from the callus can also be an additional application. The present study revealed the usefulness of PVP and tyrosine for enhancing root induction and shoots regeneration in the target species and may also be applied on other species.

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