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Research Article

Organogenesis from Leaf-derived Calli of *S. potatorum* L. f.: A Vulnerable Medicinal Tree Species

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Abstract

An efficient protocol for *in vitro* regeneration of *Strychnos potatorum* L. f. through indirect organogenesis was developed using mature leaf disc explants. The explants were selected from twenty years old tree. Cultured on Murashige and Skoog's medium supplemented with 2,4-dichloro phenoxy acetic acid (2,4-D) was used for the induction of organogenic calli. A pale yellow coloured friable callus developed at 2 mg/L^{-1} 2,4-D where showed higher morphogenic potential. Further *de-novo* shoots formation and multiplication in optimal concentrations of cytokinins with different combinations of auxins. An average mean shoots number (14.2±0.42) and shoots height (3.5±0.27cm) was obtained from piece of calli in 6-benzylaminopurine (BAP) 1 mg/L^{-1} with 0.5 mg/L^{-1} α-naphthalene acetic acid (NAA). BAP in combinations with NAA was found to be superior to shoot development than other combination used. The well developed shoots were transferred to root induction medium for effective rooting. The rooting medium consisted of in half-strength MS medium augmented with 0.5 mg/L^{-1} NAA was used. The maximal mean number of roots per shoots 6.5±0.53 was recorded in 4 weeks. The rooted plantlets were removed from the culture tubes, gently washed with sterile double distilled water to remove agar adhering on the plant surface. The regenerated plants were transferred to plastic cups filled with a potting mixture containing sterile sand and soilrite (1:1 ratio) and allowed to grow in the greenhouse. The slowly acclimatized plants were further transferred to substrate containing red soil and farmyard manure (1:1) for effective hardening. 75% survival rate was recorded. This protocol can be useful for the *ex-situ* conservation and rehabilitation of this vulnerable medicinal tree *S. potatorum*.

Keywords: Indirect organogenesis, friable callus, shoot development, 6-benzylaminopurine, α-naphthalene acetic acid

INTRODUCTION

Strychnos potatorum L. f. (Loganiaceae) is a medium sized deciduous tree found in India and other parts of the world [1]. It is naturally growing in forests up to 1200 m altitude in the forest [2]. Seeds are popularly known as 'clarifying nuts' and are utilized by rural household for generations to clarify turbid water [3]. This property was attributed to the presence of anionic polyelectrolytes having -COOH and free -OH surface groups that are present in the seed proteins [4]. It is a tropical tree species used in traditional medicinal systems. The roots are used to cure leucoderma. The ripe fruit is emetic, diaphoretic, alexiteric, cures inflammation, anemia, jaundice [5]. Its bark and root is used to control microbial infections. In Ayurveda, the plant is used for treating eye and urinary tract infections [6]. Due to indiscriminate collection and over-exploitation for medicinal and commercial uses leads to decline its numbers in the natural habitat. Further, the plant fruits severely damaged by the invasion of fungal mycelium as a result seeds showed a hollow without embryos. Moreover, fewer seed germination rate and poor vegetative cuttings due to erratic rooting behavior are also encompasses major bottlenecks of this tree species. It is one of the fast disappearing vulnerable medicinally important forest tree species [7-8]. To the best of our knowledge, only a single report describing *in-vitro* regeneration of *S. potatorum* was documented [8]. Till date, no reports are available on *in-vitro* organogenesis from leaf disc explants. Hence, the present study was under taken to demonstrate efficient protocol for organogenesis of *S. potatorum*.

MATERIAL AND METHODS

Explants source and explant preparation

The tender leaves were collected from natural habitat (latitude 10°16'01.4"N and longitude 77°59'57.6"E 750 feet elevation) in Eastern Ghats, Tamil Nadu, India during autumn season. The leaves were thoroughly washed with running tap water for 1 hr. to remove debrices. The leaves were surface disinfected with 0.1% bavistin and 2-3 drops of Tween 20 followed by 0.03% (w/v) mercuric chloride $HgCl_2$ (Himedia Mumbai, India) for 5 minutes. The explants were rinsed for 5 or 6 times with sterile double distilled water, in order to remove chemical residues. Aseptically, the leaves were made into leaf-discs 1cm using cork borer and used further for *in-vitro* studies.

Culture media and culture conditions

The nutrient medium consisted of Murashige and Skoog's media [9], with 3% (w/v) sucrose (Nice, Chemicals, India) and 0.8% (w/v) agar (Himedia, Mumbai, India). The pH of the medium was adjusted to 5.8 by 0.1 N NaOH or 0.1 N HCl after

adding the plant growth regulators (PGRs) (Himedia, Mumbai, India). The medium was autoclaved at 1.05 kg/cm^2 pressure and 121 °C temperature for 15 minutes. The culture was maintained at 25 ± 2 °C for 16 h under a light intensity supplied by cool white fluorescent lamps (Philips, India) and > 80% relative humidity.

Callus induction, *de-novo* shoot formation and further proliferation

The effect of various concentrations of 2,4-dichloro phenoxy acetic acid (2,4-D) was tested for callus induction. The calli were subcultured after the interval of 30 days in the same medium. After 30 days, the colour and texture, were observed and recorded. Percentage of callus formation was also calculated. The suitable ideal calli were selected for the regeneration through organogenic pathways of this species. Further, shoot development and multiplication on MS medium was supplemented with various concentrations (0.5 to 3.0 mg/L^{-1}) of 6 benzylaminopurine (BAP), thidiazuron (TDZ), and kinetin (Kn) with Naphthalene acetic acid (NAA) was studied. Subcultures were made at four weeks intervals. The frequencies of shoots numbers and shoot heights was recorded after 8-weeks incubation.

Rooting and acclimatization

The healthy regenerated shoots from multiple shoot clusters were separated and transferred to a rooting medium. The rooting medium contains half-strength MS medium supplemented with different concentration (0.2 to 2.0 mg/L^{-1}) of α-naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA), and Indole-3-acetic acid (IAA) individually. Well-developed rooted plantlets were removed from the culture tube, gently washed with sterile distilled water to removed agar adhering on plant surface. The plantlets were transferred to plastic cups filed with soilrite. The cups were covered with polythene bags to maintain relative humidity in the controlled environmental condition. The slowly acclimatized plants were transferred to polythene cups containing potting mixture consisted of sterile sand and soilrite (1:1 ratio) and allowed to grow in the greenhouse. The survival rate was recorded 4 weeks after transplantation.

Data analysis

Experiments have been set up indefinitely in a randomized design. Data had been collected at the end of the 8-weeks for callusing and formation of multiple shoots. The rooting was evaluated 4-weeks from culture initiation. Each experiment were repeated at least twice consisted of 30 explants. Significance was once determined by using calculating the Mean ± Standard Deviation in One-way ANOVA using SPSS statistical software package (Trial version: 16) was used.

RESULTS AND DISCUSSION

The present investigation describes high-frequency shoot induction from the callus-derived from tender leaf disc were taken as an explants. Leaves selected from mature trees were amenable to obtain calli and effective for organogenesis. Moreover, initially plant regeneration using young leaves may reduce the chances of genetic variation [10]. A reddish- pale brown, friable callus formation was seen at the edges of the leaf lamina. No callus formation was noticed in PGRs-free control medium. Explants were turned necrotic after few days of inoculation. In this study the microbial contaminants are found on both surface as well as inside the leaflets. Similar observation was made in [11]. At the early stages of the culture period, the proliferation frequency rate of calli was very low. At the cut end of the explants became blackened due to phenolic exudation. Following successive for two or three subcultures to control browning of explants and calli rapidly proliferation frequency increased. These calluses were distinguished into three types based on their color and surface texture (Fig. 1 a & b). Callus formation decreased with increasing concentrations of the plant hormones.

A white friable callus was induced on MS medium supplemented with 2,4-D. Interestingly, no callus formation was observed on MS medium supplemented with low 2,4-D. MS medium containing 2,4-D at 1.0 mg/L^{-1} produces compact callus whereas soft friable callus was produced in MS medium containing 2,4-D (2.0 mg/L^{-1}). Among the different concentrations of 2,4-D tested, the best callus production (71%) was achieved at 2.0 mg/L^{-1} 2,4-D (Table 1). Similarly callus induction from leaf-discs were reported from earlier studies on *de-novo* organogenesis in *Gymnema sylvestre* [12]. Furthermore, 2,4-D influenced the molecular and physiological processes of calli by activating proteins, regulating endogenous IAA metabolism and regulating DNA methylation [13-14]. In contrast, in other trees the combination of auxin with cytokinins obtained regenerable green callus was also reported [15].

Shoot organogenesis from callus and plant regeneration

Organogenic green calli were transferred to regeneration medium for shoot development. The medium for calli-derived shoot differentiation was achieved with different combinations of plant growth regulators. Organogenic calli was considered to be one of the most efficient regeneration and transformation system for plants [16-17]. The shoot differentiation started 4-weeks after transferring the calli to regeneration medium (Fig.1 c). The plant regeneration was characterized by the development of different concentrations of cytokinins in combinations with NAA. In the above medium improved the shoot induction both in terms of height and number of shoots per calli piece. The TDZ with NAA combination showed low frequency of shoot formation was observed (Fig 1 d & Table 2). However, BAP 1.0 mg/L^{-1} with NAA at 0.5 mg/L^{-1} produced maximum mean shoots (14.2 ± 0.42) with average mean shoot height (3.5 ± 0.27 cm) was recorded (Fig. e & f). The induction frequency and number of shoots per calli was low in other cytokinins used in this study. The synergistic effect of BAP & NAA combinations on shoot organogenesis reported for several woody species [18-20]. Different concentrations of TDZ in combination with NAA and Kn (Table 2) were also tested in this study aforesaid. The combination showed, the maximum average mean value for callus induction frequency (57% and 64%) was recorded.

***In-vitro* rooting, hardening, and acclimatization**

The *in vitro* regenerated healthy shoots were excised from the base and transferred to half-strength MS medium supplemented with different concentration of IBA,NAA and IAA (0.2 and 3.0 mg/L^{-1}) individually. Rooting was observed within 4 weeks of culture as shown in Fig. 1 g. A significant rooting with maximum mean shoot and root length were observed with the increasing in concentration NAA. The highest rooting frequency (70%) were achieved on ½ MS medium supplemented with 0.5 mg/L^{-1} NAA the maximum number of root per shoots (6.5 ± 0.53) and highest root length (3.0 ± 0.24cm) was observed (Table 3). Auxins are known to promote adventitious root formation and further development. NAA is the best auxins showed effective rhizogenesis. The similar findings of high root induction were reported in *Neolamarkia cadamba* [21], *Coccoloba uvifera* [22]. This may be due to an appropriate concentration of auxin acting on cells, which is beneficial for binding with ATP enzyme on the plasma membrane. This acidifies the cell wall environment, and some unstable hydrogen bonds are easy to break, so that the molecular structure of cell wall polysaccharides is intertwined. The cell wall tends to relax, making the cells that

form the adventitious roots easily broken through. Well-rooted plantlets were transplanted into sterile plastic cups containing sterile sand and soilrite (1:1) mixture, acclimatized in the controlled condition then the plants were transferred to green house after 30 days. The survival percentage of such plants was 75%.

Figure 1: In-direct organogenesis from leaf-derived calli of *S. potatorum*. a - Callus induction from mature leaf disc, **b** – Callus formation after 10 days, **c** – Initiation of shoots from callus, **d** - Multiple shoot formation in TDZ at 1.0 mg/L^{-1} 0.5 mg/L^{-1} NAA, **e** - Multiple shoot formation in BAP 1.0 mg/L^{-1} 0.5 mg/L^{-1} NAA, **f** – Shoots elongation, **g** – Root formation in 0.5 mg/L^{-1} NAA, **h** – Plant hardening in paper cups containing sand and soilrite.

Table 1: Effect of 2,4-D on organogenic calli induction from leaf disc.

2,4-D (mg/L^{-1})	% callusing response	Degree of callus formation	Colour and texture of callus
0.0	00	0.0	0.0
0.5	12	-	-
1.0	29	+	White, friable
1.5	42	++	Yellowish brown and friable
2.0	71	+++	Pale yellow and friable
2.5	59	++	Yellow and compact
3.0	40	+	Yellow and compact

Induction of callus given in different grades viz. (-) Nil, (+) Minute, (++) Moderate, (+++) High

Table 2: Effect of adventitious shoots formation and regeneration of *S. potatorum* leaf cultured on MS medium.

PGRs (mg/L^{-1})				Shooting response (%)	Mean number of shoots	Height of the shoot (cm)
TDZ	BAP	Kn	NAA	0.0	0.0	0.0
0.5			0.5	23	4.8±0.42^{d}	2.5±0.46^{d}
1.0			0.5	64	10.6±0.52^{g}	2.4±0.43^{c}
1.5			0.5	52	8.3±0.48^{ef}	2.3±0.35^{c}
2.0			0.5	47	6.6±0.52^{e}	2.3±0.22^{c}
2.5			0.5	41	4.3±0.48^{d}	1.9±0.41^{ab}
3.0			0.5	36	2.5±0.53^{b}	1.7±0.32^{a}
	0.5		0.5	35	8.5±0.53^{f}	2.4±0.36^{b}
	1.0		**0.5**	**76**	**14.2±0.42^{h}**	**3.5±0.27^{c}**
	1.5		0.5	67	10.4±0.52^{gh}	2.8±0.31^{b}
	2.0		0.5	54	9.5±0.53^{g}	2.2±0.48^{b}
	2.5		0.5	45	8.3±0.48^{ef}	1.8±0.08^{a}
	3.0		0.5	30	7.5±0.53^{ef}	1.6±0.41^{a}
		0.5	0.5	18	2.7±0.48^{b}	2.1±0.09^{b}
		1.0	0.5	57	6.6±0.52^{ef}	2.5±0.29^{b}
		1.5	0.5	50	5.8±0.42^{e}	2.1±0.16^{b}
		2.0	0.5	44	3.7±0.48^{c}	1.9±0.45^{ab}
		2.5	0.5	39	2.9±0.32^{b}	1.8±0.41^{ab}
		3.0	0.5	28	1.7±0.48^{a}	1.7±0.50^{a}

*Mean ± SD; mean for each experiment marked with the same letter does not differ significantly (P< 0.05)

Table 3: *In vitro* root formation of *S. potatorum* in different auxins on half-strength MS medium.

Auxin (mg/L^{-1})			Rooting response (%)	Mean number of roots per explant	Length of the roots (cm)
NAA	IBA	IAA	0.0	0.0	0.0
0.2			42	4.0±0.47^{e}	1.9±0.29^{ab}
0.5			**70**	**6.5±0.53^{f}**	**3.0±0.24^{c}**
0.7			59	4.9±0.57^{cd}	2.2±0.15^{b}
1.0			43	3.3±0.48^{c}	2.1±0.41^{b}
1.5			36	2.0±0.47^{b}	1.8±0.08^{a}
2.0			28	1.3±0.48^{a}	1.6±0. 50^{a}
	0.2		39	3.4±0.52^{c}	2.1±0.18^{ab}
	0.5		63	4.5±0.53^{cd}	2.6±0.10^{b}
	0.7		51	3.8±0.42^{d}	2.4±0.29^{b}
	1.0		39	2.9±0.32^{b}	2.1±0.18^{b}
	1.5		32	1.8±0.42^{a}	1.9±0.41^{a}
	2.0		29	1.3±0.48^{a}	1.7±0.25^{a}
		0.2	35	2.2±0.42^{bc}	2.0±0.12^{b}
		0.5	58	3.3±0.48^{bc}	2.5±0.29^{bc}
		0.7	49	2.5±0.53^{b}	2.1±0.46^{b}
		1.0	36	2.2±0.42^{b}	1.9±0.66^{ab}
		1.5	27	1.3±0.48^{a}	1.8±0.41^{a}
		2.0	25	1.1±0.32^{a}	1.7±0.50^{a}

*Mean ± SD; mean for each experiment marked with the same letter does not differ significantly (P< 0.05)

CONCLUSION

In conclusion, this study demonstrated a complete feasible protocol for indirect-organogenesis of *S. potatorum* under *in vitro* using leaf-derived callus. BAP at 1.0 mg/L^{-1} along with 0.5 mg/L^{-1} NAA was found best combination was optimized for effective *de-novo* shoot formation. Effective root formation was achieved using NAA. This protocol can be useful for large scale propagation in a short span of time and also helpful in *ex-situ* conservation of this vulnerable medicinal tree species.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest.

REFERENCES

1. Sanmugapriya E, Venkataraman S. Anti-ulcerogenic potential of Strychnos potatorum Linn. seeds on aspirin plus pyloric ligation induced ulcers in experimental rats. Phytomedicine. 2007; 14:360-5. https://doi.org/10.1016/j.phymed.2006.12.025

2. Mishra SB, Verma A, Vijayakumar M. Preclinical evaluation of anti-hyperglycemic and antioxidant action of Nirmali (Strychnos potatorum) seeds in streptozotocin-nicotinamide- induced diabetic wistar rats: a histopathological investigation. Biomark. Genom. Med. 2013; 5:157-163. https://doi.org/10.1016/j.bgm.2013.07.010

3. Tripathi PN, Chaudhuri N, Bokil SD. Nirmali seed a naturally occurring coagulant. Indian J Environ Health. 1976; 18:72-81.

4. Sarawgi G, Kamra A, Suri N, Kaur A, Sarethy IP. Effect of Strychnos potatorum Linn. Seed extracts on water samples from different sources and with diverse properties. Asian J Water Environ. Pollut. 2009; 6:13-17.

5. Kirtikar KR, Basu BD. Illustrated Indian Medicinal Plants.Edited by: Mhaskar K S, Blatter E, Cains J F. Sir Satguru's Publications. 2000. pp. 2271.

6. Bisset NG, The Asian species of Strychnos part III: the ethnobotany. Lloydia. 1974; 37:62-107.

7. Kagithoju S, Godishala V, Kairamkonda M, Kurra H, Nanna RS. Recent advances in elucidating the biological and chemical properties of Strychnos potatorum Linn. f.-a review. Int. J Pharm. Bio. Sci. 2012; 3:291-303.

8. Kagithoju S, Godishala V, Kairamkonda M, Nanna RS. Embryo culture is an efficient way to conserve a medicinally important endangered forest tree species Strychnos potatorum. J For Res. 2013; 24:279-283. https://doi.org/10.1007/s11676-013-0350-0

9. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 1962;

15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

10. Singh CK, Raj SR, Patil VR, Jaiswal PS, Subhash N. Plant regeneration from leaf explants of mature sandalwood (Santalum album L.) trees under in vitro conditions. In Vitro Cell. Dev. Biol. 2013; 49:216-222. https://doi.org/10.1007/s11627-013-9495-y

11. Omamor IB, Asemota AO, Eke CR, Ezia EI. Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). Afr. J. Agric. Res. 2007; 2:534-537.

12. Isah T. De-novo in vitro shoot morphogenesis from shoot tip-induced callus cultures of Gymnema sylvestre (Retz.) R.Br. ex. Sm. Isah Biol Res. 2019; 52:3. https://doi.org/10.1186/s40659-019-0211-1

13. Pan Z, Zhu S, Guan R, Deng X. Identification of 2, 4-D-responsive proteins in embryogenic callus of valencia sweet orange (Citrus sinensis Osbeck) following osmotic stress. Plant Cell Tiss Organ Cult. 2010; 103:145-153. https://doi.org/10.1007/s11240-010-9762-0

14. Hesami M, Daneshvar MH, Najafabadi YM, Alizadeh M. Effect of plant growth regulators on indirect shoot organogenesis of Ficus religiosa through seedling derived petiole segments. J. Genet. Eng. Biotechnol. 2018; 16:175-180. https://doi.org/10.1016/j.jgeb.2017.11.001

15. Qing ZY, Jie ZM, Deng Z, Jie ZJ, Jian LJ, Yang CX. In vitro plant regeneration of Zenia insignis Chun. Open Life Sci. 2018; 13:34-41. https://doi.org/10.1515/biol-2018-0005

16. Koetle MJ, Finnie JF, Balázs E, Staden JV. A review on factors affecting the Agrobacterium-mediated genetic transformation in ornamental monocotyledonous geophytes. S. Afr. J. Bot. 2015; 98:37-44. https://doi.org/10.1016/j.sajb.2015.02.001

17. Li J, Zhang D, Ouyang K, Chen X. High frequency plant regeneration from leaf culture of Neolamarckia cadamba. Plant Biotechnology. 2019; 36:13-19. https://doi.org/10.5511/plantbiotechnology.18.1119a

18. Venkatachalam P, Kalaiarasi K, Sreeramanan S. Influence of plant growth regulators (PGRs) and various additives on in vitro plant propagation of Bambusa arundinacea (Retz.) Wild: A recalcitrant bamboo species. J. Genet. Eng. Biotechnol. 2015; 13:193-200. https://doi.org/10.1016/j.jgeb.2015.09.006

19. Das P, Tanti B, Borthakur SK, In vitro callus induction and indirect organogenesis of Brucea mollis Wall. ex Kurz - A potential medicinal plant of Northeast India. J. Genet. Eng. Biotechnol. 2018; 119:203-211. https://doi.org/10.1016/j.sajb.2018.09.012

20. Patricia D, Stephen B, John A. Shoot organogenesis from leaf discs of the African ginger (Mondia whitei (Hook.f.) Skeels), an endangered medicinal plant. In Vitro Cell.Dev.Biol. 2021; 57:1-6. https://doi.org/10.1007/s11627-020-10146-0

21. Huang H, Wei Y, Zhai Y, Ouyang K, Chen X, Bai L. High frequency regeneration of plants via callus-mediated organogenesis from cotyledon and hypocotyl cultures in a multipurpose tropical tree (Neolamarkia cadamba). Scientific Reports. 2020; 10:4558. https://doi.org/10.1038/s41598-020-61612-z

22. Manokari M, Priyadharshini S, Shekhawat M S, Micropropagation of sea grape (Coccoloba uvifera (L.) L.). S. Afr. J. Bot. 2021; 250-258. https://doi.org/10.1016/j.sajb.2020.04.028