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RAPID CALLUS INDUCTION AND REGENERATIVE STUDIES IN SHOOT TIP EXPLANTS OF *WITHANIA SOMNIFERA* (L.) DUNAL-INDIAN GINSENG

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ABSTRACT

The efficient large scale in vitro regeneration protocol was developed by indirect organogenesis of *Withania somnifera* (L.) Dunal. *In vivo* Shoot tip explants of 2-3 months old seedlings were grown on Murashige and Skoogs (MS) medium supplemented with various concentrations of different auxins (1-4 mgl^{-1}) and cytokinins (0.5-1 mgl^{-1}). The shoot initiation was observed when the callus were sub cultured on MS medium containing different concentration of cytokinins (1-4 mgl^{-1}) and low concentration of Auxins (0.5-1 mgl^{-1}). Shoots derived from callus in primary cultures were transferred to proliferation medium (multiple shoot regeneration) of different cytokinins. Rapid shoot multiplication was observed on MS medium supplemented with KN (2 mgl^{-1}) and BA (0.5 mgl^{-1}). Shoots were rooted on MS medium supplemented with 1mgl^{-1} of IBA. Plantlets with well developed roots were transferred to sterile soil mixture. The pots were maintained in green house condition and transferred to field.

Key words: **Shoot tip, *Withania somnifera*, Auxins, Cytokinins, Proliferation medium.**

INTRODUCTION

Withania somnifera (L.) is an important medicinal plant belongs to the family Solanaceae and popularly known as Ashwagandha / winter cherry. It is a shrub found in drier parts of India, Srilanka, South Africa, Egypt and Pakistan. The plant extract used as an herbal tonic in Vedas in the form of Medharasayana (promoter of learning and

memory) and considered as an Indian Ginseng in traditional system of medicines **[1]**. The root and leaves extract were used as nerve tonic, anti ageing and for the treatment of neurodegenerative disorders **[2]**. The extract also exhibits antimicrobial, anti-inflammatory, antitumor and antioxidant properties **[3]**. In recent years, much attention has been received due to pharmacological activity attributed to two main withanolides viz. Withaferin - A and Withanolide - D.

Traditionally *W. somnifera* is propagated from seeds, but the mature and healthy seeds are not always available for germination. The viability period of seeds is very short and their germination is also poor **[4]**. To meet the growing demand for pharmaceutical based industry, it is found necessary to multiply these species by adopting tissue culture techniques.

Regarding regeneration of *W. somnifera*, there are several reports describing the induction of shoots from germinating seeds and shoot tips **[5, 6]**, nodal explants with axillary buds **[7]**, node and leaf explants **[8]**, nodal segments **[9]**, internode and leaf callus **[10]**, apical bud **[11]**, epicotyls **[12]**.. Therefore, the present study was aimed at developing an efficient and rapid indirect regeneration protocols from shoot tip explants of *W. somnifera.*

MATERIALS AND METHODS

Collection and sterilization of seeds

W. somnifera seeds were collected from wild plants grown in Kolar fields, Karnataka, India. The seeds were washed thoroughly under running tap water for 20 minutes and rinsed with Teepol solution 5% (v/v), 0.3% (w/v) sodium hypochlorite, 0.2% mercuric chloride (2 minutes) and washing with sterile double distilled water 3-4 times after each treatment with sterilants. The sterilized seeds were transferred to pots containing a mixture of sterile soil, sand and manure (2:1:1). These pots were incubated in green house condition for 45- 60 days.

Tissue culture medium and plant growth regulators

The axillary and terminal shoot tip explants were transferred into culture tubes containing MS medium with different concentrations (1-4 mgl^{-1}) of auxins (2, 4-D, NAA, IAA and IBA) and in combination with cytokinins (BA and KN). The cultures were incubated at 25 ± 2^0C under 16 hour photoperiod with light intensity (3000-4000 Lux) provided by white florescent bulbs.

Shoot initiation and elongation of multiple shoots

Callus initiated from MS medium supplemented with auxins and in combination with cytokinins were sub cultured with different concentration of cytokinins and in

combination with auxins. Individual shoots derived from the callus in primary cultures were excised and transferred to proliferation medium of different cytokinins regimes of BA and Kn. Shoot differentiation and rapid multiplication was initiated under application of constant KN (0.5-1 mgl^{-1}) to varying BA (0.5- 4 mgl^{-1}) and vice versa. Individual shoots are separated and inoculated on rooting media.

Statistical tools

The data are expressed as Mean ± S.E. All the experiments were repeated thrice and 15 replicates for each experiment. The data were analyzed statistically by one way analysis of variance followed by Duncan's multiple range tests using SPSS software. Probability values $P < 0.05$ were considered Significant.

RESULTS AND DISCUSSION

The MS medium supplemented with various concentrations of different auxins and cytokinins either singly or combinations to analyze the effects of the hormones on morphogenetic potential of the explants. Shoot tip explants were responded positively for the induction of callus and multiple shoots. However, Vadawale *et al.* **[7]** have reported the rapid *in vitro* propagation through the auxiliary bud or shoot derived callus were found to be initiation of callus and multiple shoot induction in the same taxon.

Effects of auxins on callus induction

Table 1, Figure-A, indicates the induction of callus by different Auxins. The maximum response was evident in IBA and 2, 4-D at $2mgl^{-1}$. The white to brown callus induction was observed in different concentrations of auxins ranging from 1.5 to $3mgl^{-1}$. Studies by **[13]** also reported the callus initiation occurred in leaf, cotyledon, hypocotyls and epicotyls explants on MS medium supplemented with different concentration of 2, 4-D and NAA.

Effect of auxins and cytokinins on callus induction

The callus initiation was observed in presence of BA and KN (0.5 mgl^{-1}) along with auxins (1-4 mgl^{-1}) as recorded in **Table 2**, **Figure B**. The significance rate of callus induction was evident at IBA (2 mgl^{-1}) and KN (0.5 mgl^{-1}). Similar response was recorded at 2 mgl^{-1} of auxins and 0.5 mgl^{-1} of cytokinins. The results obtained were similar to the studies of **[14]**. Where in the maximum callus induction was observed in 2, 4-D (2 mgl^{-1}) and KN (0.5 mgl^{-1}). Rout *et al*. **[10]** was also reported the highest initiation of callus from leaf explants cultured on 2, 4-D (1 mgl^{-1}) and BA (1 mgl^{-1}).

Effect of auxins with cytokinins on shoot regeneration

The shoot regeneration was maximum in 2 mgl^{-1} of KN and 0.5 mgl^{-1} of IAA as indicated

in Table 3, Fig-D. However, no response was observed at 4 mgl^{-1} of different cytokinins. Studies of **[9]** reported similar response in MS medium supplemented with 2 mgl^{-1} of BA and 0.5 mgl^{-1} of IBA. Udayakumar *et al.* [12] stated that epicotyl explants induced multiple shoots on MS medium containing BAP (2 mgl^{-1}) with IAA (0.2 mgl^{-1}).

Effect of combination of cytokinins on multiple shoot regeneration

Rapid multiple shoots were obtained from *in vitro* regenerated shoot tips were transferred to medium containing BA (1-4 mgl^{-1}) and KN (0.5 mgl^{-1}) and vice versa. **Table 4, Figure E** shows the maximum number of multiple shoots in combination of cytokinins at (2 mgl^{-1}) and (0.5 mgl^{-1}). Vadawale *et al.* **[7]** reported the effect of combination of cytokinins on multiplication of shoots from nodal explants.

Root initiation and acclimatization of tissue cultured plants

Root formation was induced in the *in vitro* proliferated shoots by culturing them on MS medium containing IBA (0.5-1mgl^{-1}) and was found to be best and produced maximum roots (20-25) per plant. Maximum roots (90-95%) obtained from shoots, when cultured on media containing 1mgl^{-1} of IBA. However frequency of root formation was comparatively low in higher concentrations of IBA **[15]**. The results are concurrent with studies of **[16]** reported the different concentration of IBA on rooting of *in vitro* regenerated shoots. About 95% of the excised shoots developed roots within 30-40 days. Well rooted plantlets were taken out from the culture vessels and washed with running water to remove the medium and transferred to nursery trays containing coconut peat mass. When the plants were twenty days old, they were successfully transferred to pots containing mixture of sterile soil, manure and sand (1:2:1) and were maintained in the green house condition **(Figure F)**. Plantlets with well developed roots were successfully transferred to soil.

CONCLUSION

The present study developed the protocol for indirect organogenesis of *Withania somnifera* from shoot tip explants. The *in vitro* regeneration technique minimizes indiscriminate collection, overexploitation of natural plants for commercial purposes. Therefore, large scale multiplication of the plants through tissue culture might be an alternative source to fulfill the global demands for its high medicinal value.

REFERENCES

[1] Gupta, GL and Rana, AC, PHCOG MAG: Plant review. *Withania*

somnifera (Aswagandha): A Review, Pharmacognosy, 1, 2007, 129–136.

[2] Bhattacharya, SK, Bhattacharya, D, Sairam, K and Ghosal, S, Effect of *Withania somnifera* glycowithanolides on rat model of tardie dyskinesia, Phytomedicine, 9, 2002, 167-170.

[3] Mayura, R and Akanksha, J, Chemistry and pharmacology of *Withania coagulants*: an Ayurvedic remedy, Journal of Pharmacognosy and Pharmacology, 62, 2010, 153-160.

[4] Vakeswaran, V, and Krishnasamy, Improvement in storability of Ashwagandha (*W. somnifera* Dunal) Seeds through pre-storage treatments by triggering their physiological and biochemical properties, Seed Technology, 25, 2003, 203.

[5] Sen, J, and Sharma, AK, Micropropagation of *Withania somnifera* from germinating seeds and shoot tips, Plant Cell Tissue Organ Culture, 26, 1991, 71-73.

[6] Furmanowa, M, Gajdzis-Kuls, D, Ruszkowska, J, Czarnocki, Z, Obidoska, G, Sadowska, A, Rani, R and Upadhyay, SN, *In vitro* propagation of *Withania somnifera* and isolation of withanolides with immunosuppressive activity, Planta Medica, 67, 2001, 146-149.

[7] Vadawale, AV, Bhatt, PM, and Dave, M, Rapid *in vitro* propagation of Ashwagandha (*W. somnifera*) through axillary bud multiplication and indirect organogenesis, Phytomorphology, 54, 2004, 59-64.

[8] Sinha, A and Kothari, SL, High frequency shoot bud regeneration from node and leaf explants of *W. somnifera* (L.) Dunal, Proceedings of National Symposium on Plant Biotechnology: New Frontiers, 2007, 167- 171.

[9] Valizadeh, J and Valizadeh, M, Development of efficient micropropagation protocol for *W. coagulans* (stocks) Dunal, African Journal of Biotechnology, 10(39), 2011, 7611-7616.

[10] Rout, JR, Sahoo, S and Das, RR, An attempt to conserve *W. somnifera* (L.) Dunal- A highly essential medicinal plant through *in vitro* callus culture, Pakisthan Journal of Botany, 43 (4), 2011, 1837-1842.

[11] Kanungo, S and Sahoo, SL, Direct organogenesis of *Withania somnifera* (L.) from apical bud, International

Research Journal of Biotechnology, 2(3), 2011, 58-61.

[12] Udayakumar, R, Choi, CW, Kim, KT, Kim, SC, Kasthurirangan, S, Mariashibu, TS, Sahaya Rayan, JJ and Ganapathi, A, *In vitro* plant regeneration from epicotyls explants of *Withania somnifera* (L.) Dunal, Journal of Medicinal plant research, 7(1), 2013, 43-52.

[13] Armugam, A and Gopinath, K, Micropropagation and tissue culture of the endangered medicinal plant *Withania somnifera* by the direct shoot and root initiation method, International Journal of Applied Biology and Pharmaceutical Technology, 2(3), 2011, 315-321.

[14] Rani, G and Grower, IS, *In vitro* callus induction and regeneration studies in *Withania somnifera*, Plant Cell Tissue Organ Culture, 57, 1999, 23-27.

[15] Sivaneson, I, Direct regeneration from apical bud explants of *W. somnifera* Dunal, Indian Journal of Biotechnology, 6, 2007, 125-127.

[16] Tuhin, C and Biswajith, G, Mass propagation and *in vitro* conservation of Indian Ginseng- *Withania somnifera* (L.) Dunal, Global Journal of Research Medicinal Plants and Indigenous Medicine, 1(10), 2012, 529-538.

Table 1: Effect of different concentrations of Auxins for callus induction

Sl. No.	Conc. (mgl^{-1})	2,4-D	NAA	IAA	IBA
1	0.5	1 ± 1^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	2 ± 0.577^{ab}
2	1	4 ± 2^{abc}	2 ± 0.00^{a}	4 ± 0.577^{bc}	4 ± 1.15^{bc}
3	1.5	6 ± 1.52^{bc}	2 ± 0.577^{a}	4 ± 0.577^{bc}	6 ± 1.73^{c}
4	2	13.66 ± 0.88^{d}	12 ± 1.52^{c}	12.33 ± 0.88^{d}	13.66 ± 0.33^{e}
5	2.5	7 ± 0.577^{c}	6 ± 0.577^{b}	6 ± 1.15^{c}	10 ± 1.15^{d}
6	3	4 ± 0.577^{abc}	5 ± 0.577^{b}	3 ± 1.15^{b}	7.33 ± 0.88^{cd}
7	3.5	3 ± 1.15^{ab}	2 ± 0.577^{a}	2 ± 1.15^{ab}	$5\pm1.15b^{c}$
8	4	2 ± 0.577^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0.66 ± 0.66^{a}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level

Table 2: Effect of different concentrations of Auxins (1 - 4 mgl^{-1}) with Cytokinins (0.5 mgl^{-1}) on callus initiation

Sl. No.	Conc. (mgl^{-1})	2,4-D + BA	2,4-D+ KN	NAA+BA	NAA+KN	IAA+BA	IAA+KN	IBA+BA	IBA+KN
1	1+0.5	2 ± 1.15^{a}	3 ± 0.57^{b}	2.33 ± 0.33^{b}	2.33 ± 0.33^{a}	3 ± 1.15^{a}	3 ± 0.57^{a}	5 ± 1.73^{ab}	4 ± 0.57^{a}
2	2+0.5	12 ± 1.52^{c}	14 ± 0.57^{d}	12.66 ± 0.33^{d}	12 ± 0.57^{c}	12.66 ± 1.85^{b}	13.33 ± 1.20^{c}	13.33 ± 1.66^{c}	14.66 ± 0.33^{c}
3	3+0.5	7 ± 1.15^{b}	9 ± 0.57^{c}	6 ± 1.15^{c}	6±1.15b	4 ± 1.73^{a}	7 ± 1.15^{b}	8 ± 2.30^{bc}	9 ± 1.15^{b}
4	4+0.5	2 ± 1.15^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	1 ± 0.57^{a}	1 ± 0.57^{a}	0.33 ± 0.57^{a}	2 ± 0.57^{a}	2 ± 0.00^{a}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level

Table 3: Effect of different concentrations of Auxins (0.5 mgl^{-1}) with Cytokinins (1 - 4 mgl^{-1}) on shoot initiation/explant

Sl. No.	Conc. (mgl^{-1})	BA+2,4-D	KN+2,4-D	BA+NAA	KN+NAA	BA+IAA	KN+IAA	BA+IBA	KN+IBA
1	1+0.5	2.33 ± 0.33^{b}	2.33 ± 0.33^{b}	2.33 ± 0.57^{b}	2.33 ± 0.33^{b}	2 ± 0.00^{b}	2.33 ± 0.33^{b}	2.33 ± 0.33^{b}	2.66 ± 0.33^{b}
2	2+0.5	3 ± 0.57^{b}	3.33 ± 0.33^{c}	2.66 ± 0.57^{b}	2.66 ± 0.33^{b}	2.66 ± 0.33^{b}	3.33 ± 0.33^{c}	3.33 ± 0.33^{c}	4.33 ± 0.33^{c}
3	3+0.5	2 ± 0.00^{b}	2.33 ± 0.33^{b}	2.33 ± 0.57^{b}	2 ± 0.00^{b}	2.33 ± 0.33^{b}	2 ± 0.00^{b}	2.33 ± 0.33^{b}	2.66 ± 0.33^{b}
4	4+0.5	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}	0 ± 0.00^{a}

Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level

Table 4: Effect of different concentrations and combination of cytokinins on rapid multiple shoots initiation/explants

Sl.No.	Conc. (mgl^{-1})	BA+KN		KN+BA	
		1	2	1	2
1	1+0.5	7.66 ± 0.88^{b}	3.33 ± 0.33^{a}	8 ± 0.577^{b}	4.33 ± 0.33^{b}
2	2+0.5	12 ± 0.57^{c}	5.33 ± 0.66^{b}	13.66 ± 0.33^{c}	7 ± 0.57^{c}
3	3+0.5	5.66 ± 1.20^{bc}	3.66 ± 0.33^{ab}	9 ± 2.30^{b}	3.66 ± 0.33^{ab}
4	4+0.5	3.66 ± 0.33^{a}	2.33 ± 0.33^{a}	3.33 ± 0.33^{a}	2.66 ± 0.33^{a}

1-Number of Explants responded; 2. Number of Shoots initiated/explants; Values represent the Mean ± SE. Means with the different letters in columns indicate significant differences at 5% level

Induction of callus on MS + IBA (2 mgl^{-1})

Callus induction on MS+ IBA (2 mgl^{-1}) with Cytokinin (0.5 mgl^{-1})

Initiation of shoots from dark green callus MS+IBA (2 mgl^{-1}) + KN (0.5 mgl^{-1})

Proliferation of shoot induction on MS + KN (2 mgl^{-1}) + IBA (0.5 mgl^{-1})

Multiple shoot induction on MS + KN (2 mgl^{-1}) + BA (0.5 mgl^{-1})

Hardened regenerated plants maintained in green house

Figures A-F: Callus induction and regeneration of plantlets of *Withania somnifera* from shoot tip