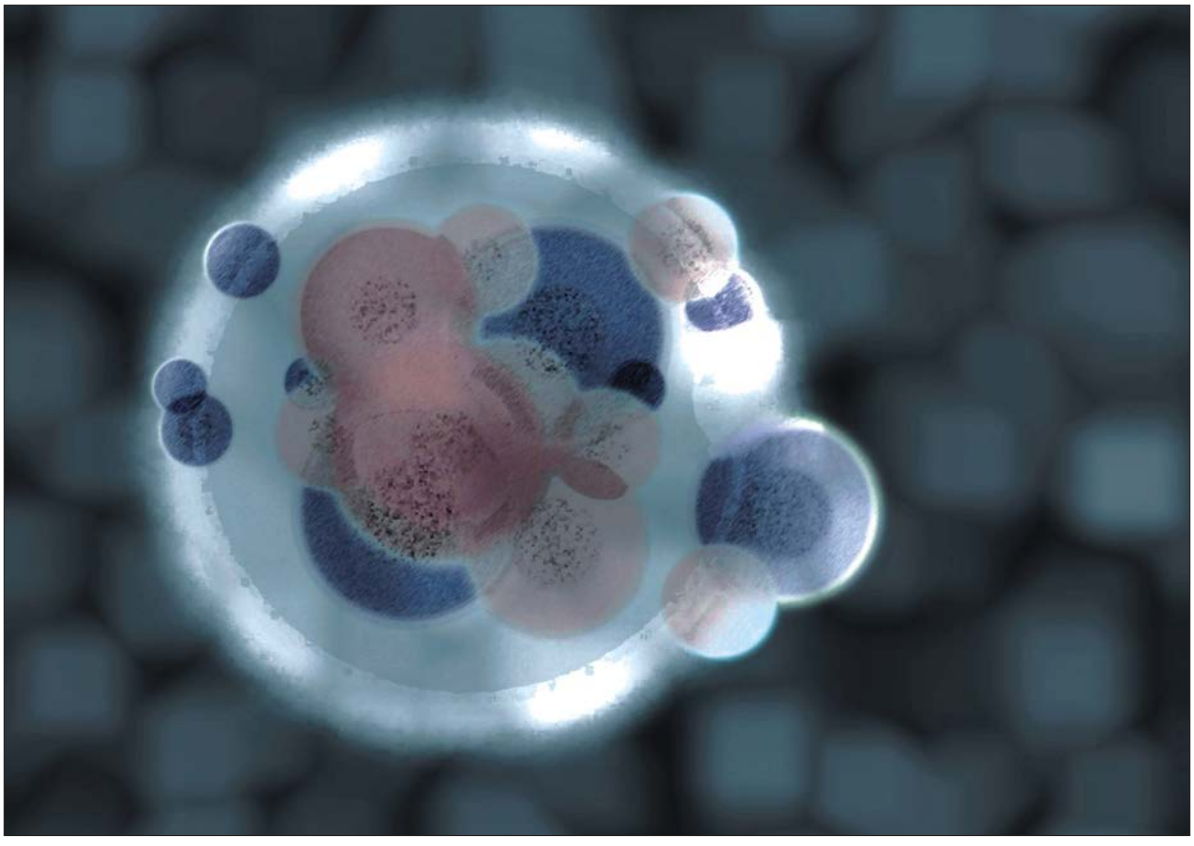

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Plant Regeneration of *Withania somnifera* (L.) Dunal (Ashwagandha) from Nodal Segments Derived Callus an Endangered Medicinal Plant in Bangladesh

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Abstract: Callus cultures were initiated from nodal segments on Murashige and Skoog[19] medium supplemented with 2,4-D, BAP and Kn. The highest frequency (85%) of organogenic callus induction was observed in MS medium containing 1 mg L^{-1} BAP and 2 mg L^{-1} Kn. Development of adventitious shoots occurred when the calli were subcultured in MS medium supplemented in the BAP and Kn. Shoots differentiated best (80%) from node derived callus on MS medium containing 1 mg L^{-1} BAP and 2.5 mg L^{-1} Kn. Regenerated shoots rooted best on MS medium containing IBA and Kn (1 mg L^{-1}). Plantlets were transferred to pots containing sand and soil mixture, acclimatized in a culture room and finally rooted plants were transferred to soil.

Key words: Solanaceae, aromatic and medicinal plant, organogenesis, callus, node

INTRODUCTION

Withania somnifera (L.) Dunal, (Ashwagandha) a member of the Solanaceae, is one of the most widespread tranquillizers used in Bangladesh. It is also rare and endangered. It is a widely used medicinal species useful in the treatment of inflammatory conditions, tuberculosis, rheumatism, as a tonic, or as an anti-tumor agent, the reproductive and nervous systems, having a rejuvenative effect on the body and is used to improve vitality and aid recovery after chronic illness[1,2]. This species also contains tropane alkaloids and withanolides[3]. Propagation is mainly by seed, but seed viability is limited to one year. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils. It cannot grow in the shade. It requires moist soil. The whole plant, but especially the leaves and the root bark, are abortifacient, adaptogen, antibiotic, aphrodisiac, deobstruent, diuretic, narcotic, strongly sedative and tonic. Internally, it is used to tone the uterus after a miscarriage and also in treating post-partum difficulties. It is also used to treat nervous exhaustion, debility, insomnia, wasting diseases, failure to thrive in children, impotence, infertility, multiple sclerosis etc. Externally it has been applied as a poultice to boils, swellings and other painful parts. The root is harvested in the autumn and dried for later use. Some caution is advised in the use of this plant since it is toxic. Roja *et al.*[4] reported callus formation from axillary meristem explants in MS medium with 2,4-D (2 mg L^{-1}). Baburaj and Gunasekaran[5] have also reported callus induction from leaf explants of *W. somnifera* using MS medium supplemented with NAA (2 mg L^{-1}) and Kn (0.5 mg L^{-1}). Due to poor viability of stored seed and a lack of a protocol for *in vitro* (mass) multiplication, the present study was undertaken to examine the potential, of different explants, to respond under *in vitro* conditions with the possibility of developing a protocol for the *in vitro* multiplication of *W. somnifera*. Huge quantities of plant materials and extracts are imported for the manufacture of Ayurvedic, Unani and Homeopathic Medicines[6]. Making health care and medical facilities available to the people is now a major concern of a large number of countries[7]. Due to the toxic and adverse reactions of synthetic and chemical medicines being observed round the globe herbal medicine has made come back to improving the fulfillment of our present and future health needs. Religious-cultural faith, weak economy in accessibility and consequently lack of modern medicinal facilities in these villages seems to be the cause of dependence on these medicinal plant species in addition to their proven ameliorative effects[8]. For further research into the biochemical compositions and potential medicinal values of this plant, an efficient *in vitro* regeneration system for the production of plants is required because field-grown plants may be subject to seasonal and somatic variations, infestations of bacteria, fungi and insects as well as environmental pollution that can affect the medicinal value of the harvested tissues[9]. In addition, *in vitro* propagation

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methods offer powerful tools for germplasm conservation and the mass-multiplication of threatened plant species[10].

MATERIALS AND METHODS

Explants were collected from *in vivo* grown medicinal plants *Withania somnifera* (L.) Dunal, (Ashwagandha) and its nodal segments were used for establishing callus. Nodal segments (1-2 cm) were washed thoroughly under running tap water, then treated with a few drops of Tween-80 and 1% Savlon for 10 min with constant shaking. This followed by successive three washing with distilled water to make the material free from savlon. Surface sterilization was carried out with 0.1% $HgCl_2$ for seven min followed by gentle shaking. After surface sterilization the segmented parts were thoroughly washed for several times with sterile distilled water. Then explants were transferred in 25×150 mm culture tubes with 15 ml basal media (MS) supplemented with different hormone (2,4-D, Kn and BAP) concentrations for callus induction. Cultures were incubated at 25+2°C under the warm fluorescent light with intensity varied from 2000-3000 lux. The pH was adjusted to 5.7 prior to autoclaving. Cultures were incubated at 25±1°C with 16 h photoperiod. Callus from these primary cultures was transferred to MS medium containing different concentration and combinations of BAP, Kn, NAA and IAA for shoot differentiation and incubated in light. Data on shoot proliferation efficiency were recorded after 8 weeks of culture. Proliferated shoots were transferred to MS with different concentrations of Kn, IBA, NAA and IAA for adventitious root formation.

RESULTS AND DISCUSSION

Callus induction was observed in MS media containing different concentrations and combinations of 2, 4-D, Kn and BAP. Within 10-12 days of incubation the nodal explants depending upon the concentration and combination of hormones were induced calli. There was a wide range of variation in percentage of callus formation and average fresh weight of callus. The highest percentage of callus induction (85) was observed in MS medium containing 1 mg L^{-1} BAP and 2 mg L^{-1} Kn and followed by 81% in MS medium containing 2 mg L^{-1} BAP (Table 1).

Highest callus growth in terms of fresh weight (995±9.90 mg) was observed in MS medium fortified with 2 mg L^{-1} BAP and highest dry weight of calli (145±0.34 mg) was observed in MS+1 mg L^{-1} Kn. Color of calli was mostly light green to dark green. It was observed that only light green calli produced shoot buds. Proliferation of shoot buds was observed in MS+1 mg L^{-1} BAP; MS+2 mg L^{-1} BAP+0.5 mg L^{-1} IAA and MS+1 mg L^{-1} BAP+2.5 mg L^{-1} Kn. The shoot buds first appeared as nodular growth within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordial. Maximum number of shoot buds was obtained in MS+1 mg L^{-1} BAP+2.5 mg L^{-1} Kn.

In the present investigation it was observed that BAP alone produced callus but BAP in combination with Kn was more suitable than BAP alone. Gita and Grover [11] used Kn and with BAP for callus induction with 84% frequency.

For shoot differentiation light green compact calli were subcultured in MS medium supplemented with different concentrations of BAP or Kn alone or BAP incombination with Kn. Such a combined effect has also been reported in *Petasites hybridus* of family Asteraceae[12]. Significant improvement in shoot formation over control has previously been achieved with the addition of cytokinins like BAP and Kn in many composites for example Conchou *et al.*[13], Le[14], Nin *et al.*[15], Fauconnier *et al.*[16], Wildi *et al.*[12], Cuenca *et al.*[18]. and BAP and Kn in combination with different concentrations of NAA and IAA. The highest 80% of shoot regeneration was observed in 1 mg L^{-1} BAP with 2.5 mg L^{-1} Kn and number of shoots per callus was 4.35±0.95 and this was followed by 50.00% in 1 mg L^{-1} BAP and number of shoots per callus was 3.75±0.80 (Table 2).

In the present investigation it was observed that when calli were subcultured on media with different concentrations of Kn alone or BAP and Kn in combination with NAA and IAA failed to produce any shoots. Calli produced shoots only when BAP alone or was combined with Kn. It was also observed that calli subcultured on media with lower concentrations of BAP and Kn alone produced roots.

In the present investigation it was recorded that BAP in combination with Kn produced shoots from calli better than that of BAP alone but Gita and Grover[11] was also showed that BAP alone produced shoots from axillary shoot derived calli.

For adventitious root formation, axillary shoot callus excised and cultured on MS medium with different concentration of auxins and cytokinins. It was observed that IBA and Kn (1, 2 mg L^{-1}) alone in MS medium was the most effective for rooting of shoots in *Withania somnifera* (L.) Dunl (Table 3). After 12-15 days post transfer to rooting medium, roots appeared and by day 30, many were found to be 4.3-5.5 cm. long. The plantlets were transferred to pots containing a sand/ soil mixture (1:1) initially covered with beakers.

Table 1: Effect of different concentrations and combination of 2,4-D, Kn and BAP on induction of callus from nodal segment of *Withania somnifera* after four weeks of culture

Treatments (mg L^{-1})	Days to callus initiation	% of callus formation	Color	Texture of callus	Fresh weight of callus (mg) $\bar{x}$ ± SD	Dry weight of callus (mg) $\bar{x}$ ± SD
MS+2,4-D 0.5	12	-	-	-	-	-
MS+2,4-D 1.0	12	11	DG	F	385±106.0	78±0.22
MS+2,4-D 2.0	12	21	DG	F	445±10.0	87±0.25
MS+ BAP 0.5	12	41	DG	F	567±10.7	85±0.32
MS+ BAP 1.0	12	70	LG	F	701±5.3	87±0.32
MS+ BAP 2.0	12	81	LG	F	995±9.9	98±0.31
MS+ Kn 0.5	12	56	LG	C	888±8.9	133±0.42
MS+ Kn 1.0	12	75	LG	C	907±7.9	145±0.34
MS+ Kn 2.0	12	67	LG	C	808±5.7	110±0.28
MS+BAP 0.5 + Kn 0.5	12	46	DG	C	705±10.9	123±0.30
MS+BAP 0.5+ Kn 1	12	54	DG	C	987±10.5	97±0.30
MS+BAP 0.5+ Kn 2	12	67	LG	F	875±8.9	89±0.31
MS+BAP 1.0+ Kn 0.5	12	71	LG	F	789±8.7	125±0.28
MS+BAP 1.0+ Kn 1.0	12	76	LG	C	764±8.9	103±0.42
MS+BAP 1.0+ Kn 2.0	12	85	LG	F	982±9.8	98±0.37
MS+BAP 2.0+ Kn 0.5	12	67	LG	C	897±7.9	89±0.20
MS+BAP 2.0+ Kn 1.0	12	78	LG	C	835±9.7	95±0.31
MS+BAP 2.0+ K n 2.0	12	49	DG	F	791±10.8	126±0.32

LG = Light green, DG = Dark green, C = Compact, F = Friable

Table 2: Effect of BAP and Kn alone or in combination with NAA or IAA and BAP in combination with Kn in MS medium on organogenesis of node derived callus after 8 weeks of culture

	% of organogenic calli			
Growth regulars	Root	Shoot	Number of shoot/callus $\bar{x}$ ± SD	Length of shoot (cm) $\bar{x}$ ± SD
BAP 0.5	+	-	-	-
BAP 1.0	-	50	3.75± 0.80	2.60± 1.17
BAP 2.5	-	20	2.25± 0.72	2.45± 1.15
BAP 3.5	-	-	-	-
BAP 2.5 + NAA 0.5	-	-	-	-
BAP2.5+ NAA 1.0	-	-	-	-
BAP2.5+ IAA 0.5	-	30	2.00 0.53	3.50± 1.10
BAP2.5 + IAA 1.0	-	-	-	-
Kn 0.5	+	-	-	-
Kn 1.0	+	-	-	-
Kn 2.5	-	-	-	-
Kn 3.5	-	-	-	-
Kn 2.5 + NAA 0.5	-	-	-	-
Kn 2.5 + NAA 1.0	-	-	-	-
Kn 2.5 + IAA 0.5	-	-	-	-
Kn 2.5 + IAA 1.0	-	-	-	-
BAP1.0+Kn 1.0	+	45	2.00±0.52	2.50±0.9
BAP1.0+Kn2.5	+	80	4.35±0.95	3.00±1.00
BAP1.0+Kn 3.5	-	35	2.25±0.54	2.25±1.09

Table 3: Effect of Kn, IBA, IAA and NAA alone or in combination in MS medium on rooting after 35 days of culture

Growth regulators	Number of shoots per treatment	Shoots rooted (%)	Root length (cm) $\bar{x}$ ± SD	Root morphology
Kn 1.0	25	90	4.60±0.5	Fragile, long
Kn 2.0	25	85	4.30±0.4	Fragile, long
Kn 4.0	25	70	3.25±0.9	Fragile, long
IBA 0.5	25	75	3.50±0.9	Thin, long
IBA 1.0	25	90	4.40±0.6	Thin, long
IBA 2.0	25	85	4.80±0.4	Thin, long
IBA 2.0 + IAA2.0	25	80	5.50±0.4	Thin, long
IBA 4.0 + IAA 2.0	25	80	5.20±0.5	Thin, long
IBA 6.0+ IAA 2.0	25	80	2.25±0.2	Thin, long
IBA 2.0 + NAA1.0	25	70	4.20±0.7	Thick, long
IBA 4.0+ NAA2.0	25	70	5.20±0.6	Thick, long
IBA 6.0+ NAA4.0	25	25	5.10±0.4	Thick, long

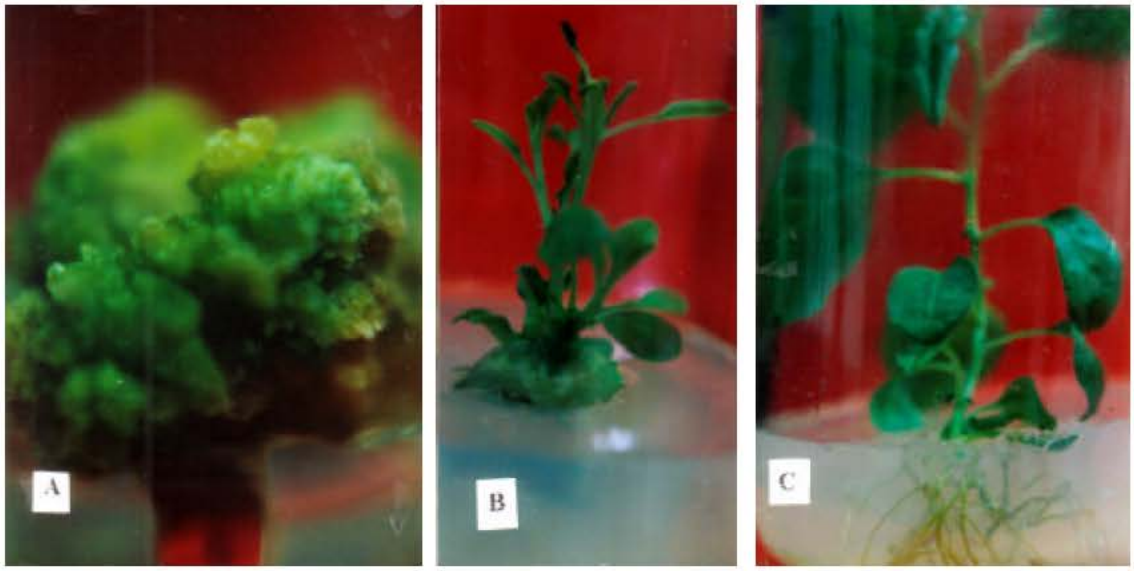

Fig. 1: Callus induction and shoot proliferation from nodal segment explants of *Withania somnifera* L.
A: Induction of light callus from nodal segment on MS+2 mgl^{-1} BAP
B: Induction of multiple shoot buds from node derived callus in MS+2 mgl^{-1} BAP
C: Rooting of regenerated shoot

Withania somnifera is normally propagated through seeds and being an open pollinated plant, the inherent variability attributable to recombination is expected. It is, therefore, not unusual that chemotypes on the basis of withanolides have been recognized[20,17]. This species is of economic interest for its wide ranging pharmacological activity and one of the major constraints in utilizing natural populations is the existence of plant to plant chemovariability. It is hoped that a standard protocol to induce multiple shoots in culture may provide a more homogeneous source of plants.

In conclusion, we report an efficient and easy to handle protocol for micropropagation of the endangered medicinal plant (*Withania somnifera*). This protocol provides a successful and rapid technique that can be used for ex-situ conservation. As a part of domestication strategy, these plants can be grown and further cultivated in fields. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of the valuable flora of the Bangladesh.

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