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IN VITRO ORGANOGENESIS AND RAPID CLONAL PROPAGATION OF *WITHANIA SOMNIFERA* (L.) DUNAL

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ABSTRACT

Withania somnifera (L.) Dunal is an important medicinal species valued for its pharmacologically active with anolides; however, conventional propagation is limited by poor germination and non-uniform planting material. The present study aimed to standardize an efficient *in vitro* organogenesis protocol using shoot tip and nodal explants. Among different sterilization treatments evaluated, sequential treatment with 70% ethanol (30 s) + 0.1% $HgCl_2$ (30 s) + 10% NaOCl (10 min) produced maximum aseptic culture establishment (90%) with minimum contamination and browning. Shoot induction was successfully achieved on MS medium supplemented with cytokinins, with 2.0 mg L^{-1} BAP showing superior response in terms of shoot initiation and growth compared to kinetin treatments. Rooting of regenerated shoots was most effective on half-strength MS medium containing 0.4 mg L^{-1} IBA, producing higher rooting percentage, greater number of roots and longer roots than IAA and NAA treatments. The standardized regeneration system developed in this study provides a reliable method for rapid clonal multiplication and uniform propagation of *W. somnifera*.

***Key words*:** Ashwagandha, organogenesis, micropropagation, cytokinin, auxin

Introduction

Medicinal plants remain an important source of therapeutic compounds and continue to contribute significantly to traditional and modern healthcare systems. A large number of pharmaceutical and nutraceutical products are derived either directly or indirectly from plant metabolites, which necessitates reliable propagation and conservation of valuable medicinal species (Balandrin *et al*., 1985; Zenk, 1978). However, conventional propagation of many medicinal plants is often restricted by poor seed germination, slow multiplication rate and variability in planting material (Akerele *et al*., 1991).

Withania somnifera (L.) Dunal, commonly known as ashwagandha, is a well-known medicinal plant belonging to the family Solanaceae and widely used in traditional systems of medicine. The plant possesses diverse pharmacological properties such as adaptogenic, anti-inflammatory and immunomodulatory activities mainly attributed to with anolides. Increasing demand from pharmaceutical and herbal industries has created a need for large quantities of uniform and high-quality planting material.

Propagation through seeds is unreliable due to low germination percentage, genetic heterogeneity and inconsistent field establishment. *In vitro* culture techniques provide an effective alternative for rapid clonal multiplication and production of disease-free plants under controlled conditions. Among different approaches,

Table 1: Influence of various surface sterilants on aseptic culture establishment in *W. somnifera*.

Treatment	No. of explants established	Per cent aseptic culture (%)	Per cent contamination (%)	Days to initiate greening	Intensity of brwoning
T_1	0	0.00 (0.90)	100.00 (89.09)	0.00	0
T_2	18	60.00 (50.85)	40.00 (39.15)	7.67	+
T_3	22	73.33 (59.00)	26.67 (30.99)	7.00	+
T_4	19	63.33 (53.07)	36.67 (36.93)	10.67	+
T_5	15	50.00 (45.00)	50.00 (45.00)	9.67	++
T_6	11	36.67 (37.15)	63.33 (52.85)	11.00	+++
T_7	10	33.33 (34.92)	66.67 (55.08)	15.00	+++
T_8	15	50.00 (45.00)	50.00 (45.00)	11.67	+
T_9	22	73.33 (59.00)	26.67 (30.99)	8.67	+
T_{10}	18	60.00 (50.86)	40.00 (39.15)	11.34	++
T_{11}	27	90.00 (71.56)	10.00 (18.45)	7.67	+
T_{12}	25	83.33 (66.15)	16.67 (23.85)	5.67	++
Mean	16.83	56.67	43.33	9.56	
SE.m ±		3.24	3.24	1.17	
CD @ 1%		12.79	12.79	4.63	
CV (%)		**11.72**	**13.28**	**12.33**	

'0' – no browning, '+' – low browning, '++' – moderate browning, '+++' – high browning

Values represent mean ± SE of three replications with ten explants per replication.CD ($P \leq 0.01$) is indicated where applicable. Values in parentheses correspond to arc sine–transformed values [$\sin^{-1}(\sqrt{x}/100)$].

T_1 – Control (no sterilant); T_2 – 0.1% mercuric chloride ($HgCl_2$) for 30 seconds; T_3 – 0.1% $HgCl_2$ for 1 minute; T_4 – 0.1% $HgCl_2$ for 1.5 minutes; T_5 – 70% alcohol for 30 seconds; T_6 – 70% alcohol for 1 minute; T_7 – 70% alcohol for 1.5 minutes; T_8 – 10% sodium hypochlorite (NaOCl) for 10 minutes; T_9 – 10% NaOCl for 15 minutes; T_{10} – 10% NaOCl for 20 minutes; T_{11} – 0.1% $HgCl_2$ (30 seconds) + 70% alcohol (30 seconds) + 10% NaOCl (10 minutes); T_{12} – 0.1% $HgCl_2$ (1 minute) + 70% alcohol (30 seconds) + 10% NaOCl (15 minutes)

organogenesis has been reported as a suitable method for regeneration in *W. somnifera*, although response varies depending on explant type and growth regulator composition (Rout *et al*., 2000; George *et al*., 2008).

Several regeneration protocols have been reported using different explants and hormonal combinations, yet reproducibility and culture establishment remain inconsistent under varying laboratory conditions (Govindaraju *et al*., 2003; Karuppusamy and Pullaiah, 2007; Autade *et al*., 2016). Therefore, optimization of sterilization procedure and growth regulator concentrations is essential for developing a reliable regeneration system.

The present investigation was undertaken to standardize an efficient *in vitro* organogenesis protocol for *W. somnifera* using shoot tip and nodal explants with the objective of improving culture establishment, shoot regeneration and rooting efficiency for rapid clonal propagation.

Materials and Methods

Healthy stock plants of *Withaniasomnifera* (L.) Dunal maintained under greenhouse conditions at the Department of Plantation, Spices, Medicinal and Aromatic Crops, College of Horticulture, Bagalkot, were used as the source of explants. Shoot tip and nodal segments were excised from actively growing shoots and used for culture initiation.

Excised explants were washed under running tap water followed by treatment with a mild detergent solution containing Tween-20 and rinsed thoroughly with distilled water. Surface sterilization was carried out inside a laminar airflow cabinet using different sterilant combinations. The optimized treatment consisted of 70% ethanol for 30 s followed by 0.1% $HgCl_2$ for 30 s and 10% sodium hypochlorite for 10 min, after which explants were rinsed four to five times with sterile distilled water prior to inoculation.

Murashige and Skoog (MS) basal medium supplemented with 3% sucrose and solidified with 0.8% agar was used for culture establishment. The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15–20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod provided by cool-white fluorescent light.

For shoot induction, explants were cultured on MS medium containing different concentrations of cytokinins, namely benzylaminopurine (BAP) and kinetin (KN),

Table 2: Effect of growth regulators on shoot induction from shoot tip explants of *W. somnifera*.

Treatment	No. of days for shooting	Shoot length (cm)	No. of leaves/ shoot	Main shoot length (cm)
T_1 – MS media (Control)	10.67 (3.40)	3.37 (2.09)	5.67 (2.52)	2.82 (1.95)
T_2 – MS + 1.0 mg/L BAP	13.00 (3.74)	3.25 (2.02)	3.34 (2.03)	2.18 (1.74)
T_3 – MS + 2.0 mg/L BAP	13.00 (3.74)	3.84 (2.19)	6.34 (2.69)	2.89 (1.96)
T_4 – MS + 3.0 mg/L BAP	10.33 (3.37)	3.46 (2.08)	5.67 (2.57)	2.27 (1.76)
T_5 – MS + 4.0 mg/L BAP	8.67 (3.07)	2.82 (1.95)	5.67 (2.58)	1.80 (1.67)
T_6 – MS + 1.0 mg/L KN	9.33 (3.19)	3.49 (2.11)	4.00 (2.22)	3.02 (1.99)
T_7 – MS + 2.0 mg/L KN	9.67 (3.26)	1.73 (1.62)	8.33 (3.05)	1.33 (1.49)
T_8 – MS + 3.0 mg/L KN	16.00 (4.12)	3.14 (2.03)	7.00 (2.80)	2.10 (1.76)
T_9 – MS + 4.0 mg/L KN	11.67 (3.56)	3.53 (2.12)	6.33 (2.67)	2.70 (1.92)
T_{10} – MS + 2.0 mg/L BAP + 2.0 mg/L KN	9.00 (3.16)	2.41 (1.85)	3.33 (2.06)	1.99 (1.73)
T_{11} – MS + 3.0 mg/L BAP + 1.0 mg/L KN	14.33 (3.91)	3.66 (2.13)	5.00 (2.43)	3.27 (2.03)
Mean	11.42	3.15	5.52	2.40
SE.m ±	0.17	0.16	0.24	0.18
CD @ 1%	0.50	0.67	0.98	0.73
CV (%)	**8.45**	**14.52**	**16.94**	**17.48**
Data presented in parentheses correspond to the square root of the mean values				

applied individually and in combination. Observations were recorded on days to shoot initiation, shoot length, number of leaves per shoot and regeneration response. Regenerated shoots were excised and transferred to half-strength MS medium supplemented with different auxins including indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA) for root induction. Rooting response was evaluated based on days to rooting, rooting percentage, number of roots per shoot and root length.

Experiments were conducted in a completely randomized design with three replications, each consisting of ten explants. Data were analysed using standard error of mean (SEm) and critical difference (CD) at 1% probability level.

Results

Aseptic culture establishment

Surface sterilization treatments significantly influenced culture establishment and contamination. Among the treatments evaluated, T_{11} [70% ethanol (30 s) + 0.1% $HgCl_2$ (30 s) + 10% NaOCl (10 min)] recorded the highest aseptic culture establishment (90.00%) with minimum contamination (10.00%) and low browning. The next best response was observed in T_{12} (83.33% aseptic culture), whereas untreated control (T_1) showed no culture establishment. Treatments involving single sterilants resulted in comparatively lower aseptic response and higher contamination levels. Differences among treatments were significant at 1% level.

Fig. 1: Explant source used for *in vitro* regeneration of *Withania somnifera* (a) Field-grown mother plant, (b) shoot tip explant, (c) nodal segment and (d) cotyledon explant.

Shoot induction from shoot tip explants

Shoot induction was obtained in all cytokinin-supplemented media. MS medium containing 2.0 mg L^{-1} BAP produced improved shoot growth with greater shoot length (3.84 cm) and higher number of leaves per shoot (6.34) compared to other treatments. Higher concentrations of BAP reduced shoot growth, while kinetin treatments showed comparatively lower shoot response. The control medium recorded moderate growth but lower regeneration efficiency. Variations among

Fig. 2: Effect of cytokinin treatments on shoot induction from shoot tip explants of *withania somnifera* (a) MS medium without growth regulators (control), (b) MS medium supplemented with BAP, (c) MS medium supplemented with KN and (d) MS medium supplemented with combined BAP and KN.

Table 3: Effect of growth regulators on shoot induction from nodal explants of *W.somnifera*.

Treatment	No. of days for shooting	Shoot length (cm)	No. of leaves/ shoot	Main shoot length (cm)
T_1 – MS media (Control)	13.00 (3.71)	2.87 (1.96)	3.21 (1.79)	1.85 (1.67)
T_2 – MS + 1.0 mg/L BAP	12.00 (3.59)	3.35 (2.08)	3.83 (1.96)	2.50 (1.87)
T_3 – MS + 2.0 mg/L BAP	11.33 (3.50)	3.21 (2.05)	3.49 (1.87)	2.01 (1.72)
T_4 – MS + 3.0 mg/L BAP	11.67 (3.53)	3.33 (2.08)	5.62 (2.37)	2.53 (1.88)
T_5 – MS + 4.0 mg/L BAP	9.33 (3.18)	2.34 (1.82)	4.20 (2.05)	1.75 (1.65)
T_6 – MS + 1.0 mg/L KN	11.33 (3.51)	3.22 (2.05)	2.98 (1.73)	2.77 (1.94)
T_7 – MS + 2.0 mg/L KN	11.00 (3.43)	2.20 (1.77)	3.51 (1.87)	1.52 (1.56)
T_8 – MS + 3.0 mg/L KN	11.33 (3.50)	3.26 (2.06)	6.48 (2.55)	2.37 (1.83)
T_9 – MS + 4.0 mg/L KN	13.67 (3.82)	2.38 (1.84)	5.18 (2.28)	2.01 (1.73)
T_{10} – MS + 2.0 mg/L BAP + 2.0 mg/L KN	12.33 (3.65)	3.30 (2.07)	4.08 (2.02)	2.98 (1.99)
T_{11} – MS + 3.0 mg/L BAP + 1.0 mg/L KN	14.67 (3.95)	1.93 (1.70)	3.77 (1.94)	1.45 (1.66)
Mean	11.97	2.85	4.21	2.16
SE.m ±	0.24	0.12	0.15	0.13
CD @ 1%	0.96	0.49	0.59	0.52
CV (%)	**11.59**	**11.01**	**12.75**	**12.93**
Data presented in parentheses correspond to the square root of the mean values				

treatments were significant at 1% level.

Shoot induction from nodal explants

Nodal explants also responded to cytokinin supplementation, though the response was lower than shoot tip explants. MS medium containing 3.0 mg L^{-1} BAP produced higher leaf number (5.62) and improved shoot growth. Kinetin treatments showed moderate regeneration response, while higher cytokinin concentrations reduced shoot growth. The control treatment showed minimal regeneration. Treatment differences were significant at 1% level.

Root induction from regenerated shoots

Auxin treatments significantly affected rooting response. Half-strength MS medium supplemented with 0.4 mg L^{-1} IBA recorded the highest rooting percentage (85.66%), greater number of roots per shoot (7.34) and longer roots (15.84 cm). IAA and NAA treatments produced moderate rooting, while higher auxin concentrations reduced root formation. Combined auxin treatments also produced good rooting but were not superior to IBA alone. The control treatment showed poor rooting response. Differences among treatments were significant at 1% level.

Fig. 3: Effect of cytokinin treatments on shoot induction from nodal explants of *withania somnifera* (a) MS medium without growth regulators (control), (b,c) shoot induction and proliferation on MS medium supplemented with BAP and (d) shoot induction on MS medium supplemented with KN.

Discussion

Successful establishment of aseptic cultures is a prerequisite for any *in vitro* regeneration system. In the present study, sequential treatment with ethanol, mercuric chloride and sodium hypochlorite resulted in maximum aseptic culture establishment with minimal contamination and browning. The improved response obtained with combined sterilants may be attributed to the complementary action of disinfectants, where ethanol removes surface debris and enhances penetration, $HgCl_2$ eliminates persistent microorganisms and sodium hypochlorite removes remaining spores. Similar sterilization strategies have been reported effective in

Fig. 4: Effect of auxin treatments on root induction from *in vitro* regenerated shoots of *Withania somnifera* (a) Control medium without auxins, (b) rooting on IBA-supplemented medium and (c,d) enhanced root induction on combined auxin treatments (IBA+IAA+NAA)

Table 4: Effect of growth regulators on root induction from regenerated shoots of *W. somnifera*.

Treatment	No. of days for rooting	Rooting percent (%)*	No. of roots per explants	Root length (cm)
T_1 – ½ MS (Control)	19.67 (4.54)	12.34 (20.51)	1.00 (1.41)	4.78 (2.39)
T_2 – ½ MS + 0.2 mg/L IBA	12.00 (3.59)	55.34 (48.07)	4.34 (2.31)	12.67 (3.69)
T_3 – ½ MS + 0.4 mg/L IBA	10.67 (3.42)	85.66 (68.18)	7.34 (2.89)	15.84 (4.10)
T_4 – ½ MS + 0.6 mg/L IBA	12.34 (3.65)	76.67 (61.12)	5.67 (2.58)	14.13 (3.89)
T_5 – ½ MS + 0.8 mg/L IBA	13.34 (3.79)	47.43 (43.45)	2.60 (1.91)	10.90 (3.44)
T_6 – ½ MS + 1.0 mg/L IBA	15.67 (4.08)	21.34 (27.40)	1.67 (1.66)	9.94 (3.23)
T_7 – ½ MS + 0.2 mg/L IAA	16.34 (4.16)	47.67 (43.66)	3.00 (2.00)	11.30 (3.49)
T_8 – ½ MS + 0.4 mg/L IAA	13.67 (3.83)	67.34 (55.17)	4.67 (2.38)	13.60 (3.82)
T_9 – ½ MS + 0.6 mg/L IAA	12.67 (3.69)	78.00 (62.14)	6.00 (2.65)	14.30 (3.91)
T_{10} – ½ MS + 0.8 mg/L IAA	14.34 (3.91)	59.00 (50.19)	3.67 (2.16)	12.24 (3.63)
T_{11} – ½ MS + 1.0 mg/L IAA	15.34 (4.04)	59.34 (50.38)	2.67 (1.91)	10.67 (3.39)
T_{12} – ½ MS + 0.2 mg/L NAA	16.34 (4.16)	57.34 (49.26)	3.67 (2.16)	12.50 (3.66)
T_{13} – ½ MS + 0.4 mg/L NAA	13.67 (3.83)	66.34 (54.57)	5.00 (2.45)	13.34 (3.78)
T_{14} – ½ MS + 0.6 mg/L NAA	13.00 (3.74)	75.67 (60.52)	5.67 (2.58)	14.10 (3.89)
T_{15} – ½ MS + 0.8 mg/L NAA	14.34 (3.91)	56.34 (48.66)	3.34 (2.07)	12.20 (3.63)
T_{16} – ½ MS + 1.0 mg/L NAA	15.34 (4.04)	36.67 (37.26)	2.00 (1.73)	10.78 (3.40)
T_{17} – ½ MS + IBA (0.2 mg/L) + IAA (0.2 mg/L) + NAA (0.2 mg/L)	11.34 (3.51)	82.62 (65.44)	7.34 (2.89)	15.57 (4.07)
T_{18} – ½ MS + IBA (0.4 mg/L) + IAA (0.3 mg/L) + NAA (0.2 mg/L)	10.34 (3.37)	89.67 (71.39)	8.67 (3.10)	16.67 (4.20)
T_{19} – ½ MS + IBA (0.6 mg/L) + IAA (0.4 mg/L) + NAA (0.3 mg/L)	11.34 (3.51)	84.34 (66.75)	7.34 (2.89)	15.34 (4.04)
Mean	13.67	62.45	4.58	12.72
SE.m ±	0.06	1.87	0.06	0.18
CD @ 1%	0.17	7.18	0.18	0.51
CV (%)	**2.77**	**6.26**	**4.8**	**8.46**

Data presented in parentheses correspond to the square root of the mean values
* For rooting per cent (%), data presented in parentheses correspond to the arcsine transformation of the mean values.

Withaniasomnifera by Rout *et al.*, (2000), Karuppusamy and Pullaiah (2007) and Shah *et al*., (2013), who emphasized that sequential chemical sterilization is necessary to control endogenous contamination in medicinal plant explants. The higher contamination observed in single-sterilant treatments indicates incomplete surface disinfection and supports the need for combined sterilization procedures.

Shoot regeneration varied with explant type and cytokinin composition. Shoot tip explants exhibited comparatively better regeneration response than nodal segments, which may be due to the presence of actively dividing meristematic cells and higher physiological competence. Similar observations were reported in ashwagandha and other medicinal plants where apical meristems showed superior organogenic potential (Rout *et al*., 2000; George *et al*., 2008). Among cytokinins, BAP produced better shoot growth than kinetin. The effectiveness of BAP may be related to its ability to promote cell division and release apical dominance, thereby enhancing shoot initiation. Earlier studies in *W. somnifera* also reported improved shoot proliferation with BAP-supplemented medium compared to kinetin (Govindaraju *et al*., 2003; Autade *et al*., 2016). Higher cytokinin concentrations reduced shoot growth, possibly due to hormonal imbalance leading to suppression of elongation, a phenomenon commonly reported in tissue culture systems.

Root induction was significantly influenced by auxin type and concentration. Half-strength MS medium supplemented with IBA produced superior rooting compared to IAA and NAA. IBA-induced roots were longer and more numerous, suggesting its stability and slow degradation in plant tissues, which allows sustained root differentiation. Similar results have been documented in ashwagandha where IBA promoted strong root formation and better plantlet establishment (Govindaraju *et al*., 2003; Nandagopal *et al*., 2011; Autade*et al*., 2016). Reduced rooting at higher auxin concentrations may be due to inhibitory effects of excess auxin causing callusing or suppression of root elongation.

Overall, the regeneration response obtained in the present investigation confirms that successful micropropagation in *Withaniasomnifera* depends on optimization of sterilization procedure, cytokinin-mediated shoot induction and auxin-mediated rooting. The protocol developed provides a reproducible regeneration system comparable with earlier reports but with improved culture establishment and rooting efficiency, indicating its suitability for rapid clonal multiplication.

Conclusion

An efficient *in vitro* regeneration protocol was standardized for *Withaniasomnifera* using shoot tip and nodal explants. Sequential surface sterilization using ethanol, $HgCl_2$ and NaOCl ensured maximum aseptic culture establishment. MS medium supplemented with 2.0 mg L^{-1} BAP proved optimal for shoot induction, while half-strength MS medium containing 0.4 mg L^{-1} IBA produced superior rooting. The developed protocol is reliable and suitable for rapid clonal multiplication and production of uniform planting material in ashwagandha.

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