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Rapid multiplication of Withania somnifera using axillary leaf segments

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Abstract: *An efficient micropropagation protocol has been developed for rapid micropropagation of Withania somnifera (L.) Dunal. Multiple shoots were induced by culturing axillary leaf explants excised from mature plants on Murashige and Skoog's basal medium supplemented with various combinations of BAP (N6 benzyle-aminopurine), Kn (Kinetin) & IAA (indole-3-acetic acid) formed direct shoot regeneration and indirect organogenesis through callus. The optimal level of BAP and IAA supplementation to the culture medium was 4.4 µM and 2.8 µM for 7 days to 10 days induction period followed by subculturing on Modified MS medium devoid of IAA produced maximum number of multiplication frequency (86%), mean number of shoots (20.5±0.4) and shoot length (1.6±0.3 cm) per explants. The regenerated shoots rooted best on half-strength MS medium supplemented with IBA 6.8 µM or NAA (α- naphthalene acetic acid) 7.5 µM. The micropropagated shoots with well developed roots were successfully established in pots containing a mixture of garden soil, sand and vermicompost (1:1:1 v/v) and grown in green house with 100% survival rate. The regenerated plants were morphologically uniform and exhibited similar growth characteristics and vegetative morphology to the donor plants.*

Keywords*: Withania somnifera (L.) Dunal., leaf explants, micropropagation, medicinal plants, secondary metabolites.*

Abbreviations: IBA - indole-3-butyric acid; MS – Murashige and Skoog medium; Kn – Kinetin; BAP - N6 benzyl aminopurine; IAA - indole-3-acetic acid; NAA – α- naphthalene acetic acid, PGRs – plant growth regulators.

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I. Introduction

Withania somnifera (L.) Dunal (Solanaceae) commonly known as Indian Ginseng or Ashwagandha, is a native to East Asia and Africa (Kulkarni *et al*. 1996), a middle sized under shrub, branching and perennial, about 30 cm to 1.5 m in height and also a commercially cultivated plant. This plant is well known as an important drug in Ayurvedic and Unani medicines (Roja *et al*. 1991). The plant is beneficial, having medicinal properties for anti-tumor and anti-inflammatory activity (Bolleddula *et al*. 2003), immunomodulatory activity (Ziauddin *et al*. 1996), cardioprotective agent (Mohanty *et al*. 2004), central nervous system (Jing *et al*. 2002, Kulkarni and Ninan 1997), growth promoter activity (Budhiraja and Sudhir 1987), cytokine production and stem cell proliferation (Diwanay *et al*. 2004), enhancer of white blood cells and platelet counts (Agarwal *et al*., 1999) and anticancer activities (Menon *et al*. 1997, Khanna *et al*. 2006). These properties are due to the presence of a number of use alkaloids like withanolides and withaferins etc. in the roots as well as in the leaves (Mohan *et al*. 2004, Balashashi *et al*. 2004).

There are a number of reports on *in vitro* studies on shoot tips and seedlings (Sen and Sharma 1991, Supe *et al*. 2006), axillary buds (Rani and Grover 1999, Vadawale *et al*. 2004, Sivanesan 2007), node (Sivanesan and Murugesan 2008, Biswal *et al*. 2008), internode, hypocotyls and embryo explants (Kulkarni *et al*. 2000), meristem culture (Teli *et al*. 1999) and also from leaf explants (Ghimire *et al*. 2010, Joshi and Padhya 2010, Abhayankar and Chinchanikar 1996, Kulkarni *et al*. 1996). The applications of *in vitro* studies on leaf explants are not only useful for developmental studies but also for investigating biochemical properties for secondary metabolites through *in vitro* genetic manipulation. However, there are limited reports on leaf explants being used for direct or indirect regeneration studies. The present work is being focused on an efficient protocol

for direct as well as indirect plantlet regeneration which is a prerequisite for manipulation of secondary metabolites production.

II. Materials and Methods

Healthy young leaves of size 1 cm to 2 cm diameter were taken from the third axil (from tip) of a two months old *Withania somnifera* plant maintained in Medicinal Plant Garden, Trident School of Biotech Sciences, Bhubaneswar. The discs were washed under running tap water and then with teepol (5%, v/v) for 10 minutes followed by running water and 70% alcohol. They were surface sterilized with 0.1% $HgCl_2$ (w/v) and washed three times with sterile double distilled water. The leaf discs were cut into four pieces and aseptically cultured with either their abaxial or adaxial surface touching the Murashige and Skoog's medium (1962). The medium was supplemented with different concentrations and combinations of IAA (1.14 µM – 2.85 µM), BAP (2.66 µM – 4.4 µM) and Kn (0.93 µM – 1.86 µM). The pH of the medium was adjusted to 5.8 prior to autoclaving at 1.1 kg/cm^2 (121^{0}C) for 20 minutes and it was solidified with 0.8% agar. Culture vessels, containing 25 ml solidified medium were used. All the cultures were incubated under white fluorescent light (2000 lux) for 16/8 hours light/dark cycle at 25±2^{0}C.

After induction of shoot buds, these were subcultured on basal medium supplemented with only cytokinins (BAP/Kn). Shoot buds of size 1-2 cm long with distinct apical meristems excised from the explants and transferred to basal medium with IBA (4.9 µM) alone or a combination of IBA (2.95-4.9 µM)) and NAA (2.1-4.2 µM) formed roots. Regenerated plantlets with healthy and profuse roots were transferred to pots containing autoclaved mixture of garden soil, sand and vermicompost (1:1:1 v/v) and acclimatized for 2 weeks under natural diffuse sunlight and 70 % humidity in a greenhouse before transferring to the field. All the experiments were repeated thrice with ten replicates each time.

III. Results and Discussion

When very young leaves and very old or senescent leaves were used as explants, white callusing was observed and failed to regenerate in any of the treatments (data not shown). Hence young leaves of an intermediate size of 1-2 cm diameter from the apex were chosen.

There was no significant result when the leaf pieces were inoculated on basal medium devoid of PGRs or with PGRs used separately (data not shown). However, in the present study, a combination of BAP (3.11 µM - 6.2 µM), Kn (0.93 µM – 1.86 µM) and IAA (1.14 µM – 2.85 µM) induced more shoot buds as compared to any other combinations reported earlier (Abhayankar and Chinchanikar 1996, Kulkarni *et al*. 1996). The formation of shoot buds occurred both on the abaxial or adaxial surface touching the medium. This is comparable with the earlier studies of Kulkarni *et al*. 1996. The induction of shoot buds (70-90 %) was the same irrespective of the orientation of explants on the medium. The percentage of response of explants forming shoot buds increased with increase in concentration of PGRs up to a certain concentration i.e. BAP (5.3 µM), Kn (2.32 µM) and IAA (2.95 µM), above that resulted into a decrease in number of shoot buds and formation of non-organogenetic callus or callus with rhizogenesis occurred, suggesting the preferential nature of auxin for caulo-rhizogenesis (Fig. 1*A*). It was also noticed that, if any explants failed to induce any shoot buds, produced shoot buds after first transfer on the same basal medium and PGRs.

The optimum response of shoot buds formed was observed on the basal medium supplemented with BAP (4.4 µM), Kn (0.93 µM) and IAA (2.28 µM) (Table 1, Fig. 1*B*) after one to two weeks. The number of shoots formed per explant was 4.4 and an average height up to 2.3 cm. The frequency of explants forming shoots decreased at an increase in concentrations of both auxin (IAA 3.94 µM) and cytokinin (BA 9.8 µM and Kn 2.32 µM). This may be due to the presence of high endogenous auxins, as observed by Moore (1989) young leaves fail to support adventitious shoot bud formation as these are the site for actively synthesizing the auxins. Both the number of shoot buds and size of the buds greatly varied according to the concentration and combination of PGRs on the basal medium.

After two weeks, the cultures were transferred to basal medium with BAP (2.6 µM – 4.4 µM) and Kn (0.93 µM) and maintained for four weeks, which these produced an average of 20-23 shoots and many small proliferating shoot buds from a single explant (Table 2, Fig. 1*CD*). The observation revealed that the explant in presence of individual cytokinin does not regenerate shoot buds. When two cytokinins are incorporated in the medium, their synergistic effect induces shoot bud regeneration. This is inconsistent with the earlier studies (Joshi and Padhya 2010, Abhayankar and Chinchanikar 1996). Vincent *et al*. 1992 have also reported in *Kaempferia galanga* that MS medium supplemented with Kn and BAP together proved synergistic for induction of shoot buds from leaf explants.

Micro-shoots of 1-2 cm in height were separated from the explant and transferred to half-strength basal medium with IBA or NAA alone or in combinations. Root induction was successfully achieved when half-strength basal medium was supplemented with IBA (4.9 µM), produced healthy roots with many laterals (Fig. 1*E*). This is a similar report to the findings of Rani and Grover 1999, where root induction was effective alone or in combination with NAA (10.7 µM) and IBA (9.8 µM). The beneficial effects of reduced salt and sucrose concentrations during the rooting phase has been described by several authors (Ajithkumar and Senni 1998; Joshi and Thengane 1996) and also in several species like *Solanum surattense* (Rout *et al*. 2006), *W. somnifera* (Biswal *et al*. 2008) and *C. roseus* (Biswal *et al*. 2007). The effectiveness of IBA in rooting has been reported in many species by different authors. According to Ludvig-Muller (2000), transport velocity of IBA was marked slower as compared to that of NAA and IBA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus it functions better in root induction by Nickell (1982). An increase in IBA or NAA to 6.8 µM showed decrease in response of cultures i.e. slightly thick and short or callus with rhizogenesis (Fig. 1*F*). This finding suggests that higher concentration of auxin is responsible for callusing at base and a decline in root induction (Biswal *et al*. 2007). Root induction was also observed from shoots of *Tylophora indica* from leaf callus (Faisal and Anis 2003).

Plantlets from culture medium when transferred directly to small pots followed by green house conditions (Fig. 1*G*) and acclimatization showed 100 % survival. Growth revived after two weeks of transplantation, about 70 plantlets were transferred. All plantlets survived and the plantlets exhibited similar characters to those of the *in vivo* mother plants.

The present study describes an effective protocol for clonal propagation of *Withania somnifera* using combinations of PGRs with MS basal medium in appropriate concentration and the culture system produced masses of plant material that would be suitable for commercial as well as developmental applications. The protocol could be helpful for the improvement of secondary metabolite content by genetic manipulation of this multi-medicinal and pharmaceutically active plant species.

Table1. Effects of different concentrations of PGRs on regeneration of shoot buds from leaf explants of *Withania somnifera* after 2 weeks of culture. Data (Mean ± SE) of three independent experiments each with 10 replicates. Means followed by the same letter within the columns are not significantly different. ($P < 0.05$) as tested by the multiple range test of Duncan (1955).

MS (1962) basal medium with plant growth regulators [µM]			Regeneration [%]	Number of shoots [explants^{-1}]	Shoot length [cm]
BA	Kn	IAA			
0.6	0.2	0.2	92	3.5±0.3^{d}	1.9±0.3^{cd}
0.6	0.2	0.5	95	4.9±0.3^{bc}	1.9±0.2^{cd}
0.8	0.2	0.4	96	6.1±0.2^{a}	2.2±0.2^{b}
1.0	0.2	0.5	100	5.5±0.2^{ab}	2.3±0.2^{a}
1.0	0.4	0.4	95	4.5±0.3^{bc}	2.0±0.3^{bc}
1.2	0.2	0.4	99	5.0±0.3^{b}	2.1±0.3^{ab}
1.4	0.2	0.5	100	6.1±0.3^{a}	2.0±0.3^{bc}

Table 2. Effects of different concentrations of PGRs on multiplication shoot buds from leaf explants of *Withania somnifera* after 4 weeks of subculture. Data (Mean ± SE) of three independent experiments each with 10 replicates. Means followed by the same letter within the columns are not significantly different. ($P < 0.05$) as tested by the multiple range test of Duncan (1955).

MS (1962) basal medium with plant growth regulators [µM]		Regeneration [%]	Number of shoots [explants^{-1}]	Shoot length [cm]
BA	Kn			
0.6		92	8.6±0.3^{h}	2.0±0.3^{cd}
0.7		97	10.5±0.2^{g}	2.0±0.3^{cd}
0.8		98	14.5±0.2^{ef}	2.1±0.2^{c}
0.9		99	16.3±0.3^{d}	2.2±0.3^{b}
1.0		100	20.0±0.2^{bc}	2.2±0.2^{b}
1.0	0.2	100	23.8±0.3^{a}	2.3±0.3^{a}
	0.6	82	5.2±0.4^{jk}	1.8 ±0.3^{fg}
	0.7	84	6.1±0.3^{i}	1.8 ±0.2^{fg}

0.8	85	7.1 ± 0.3^{jh}	1.9 ± 0.3^{e}
0.9	87	7.2 ± 0.2^{jh}	2.0 ± 0.5^{cd}
1.0	88	8.7 ± 0.3^{h}	2.0 ± 0.3^{cd}

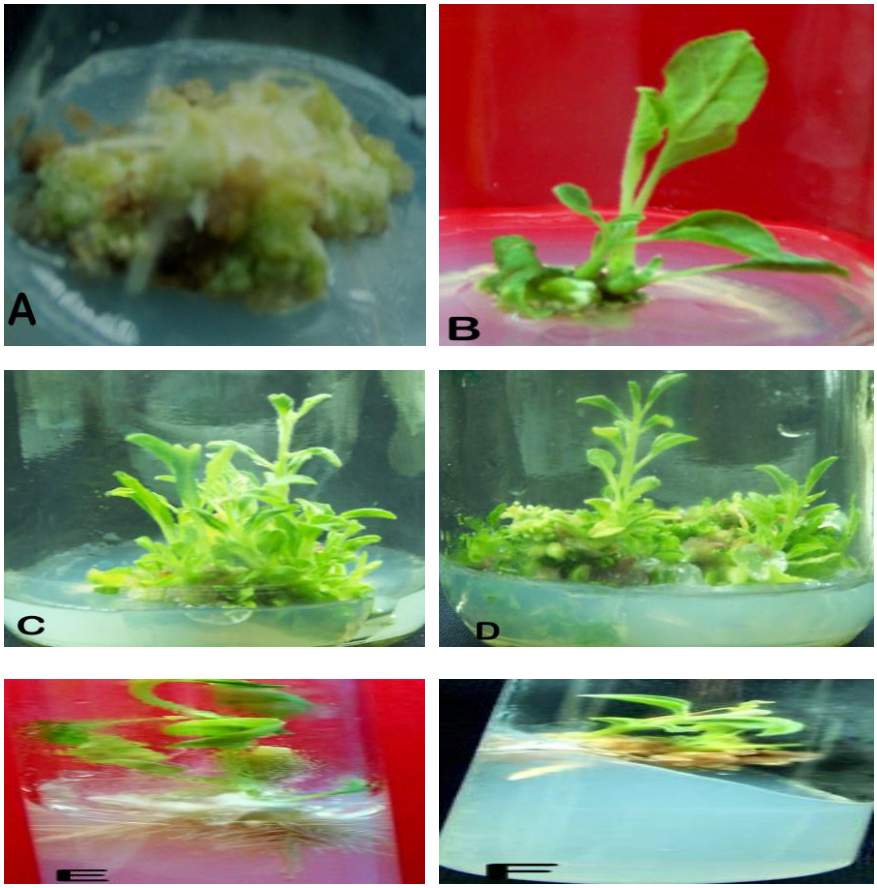

Fig. 1. Effect of PGRs on shoot bud induction from axillary leaf explants of *W. somnifera*. *A* – callus with rhizogenesis in 4 weeks of culture; *B* – induction of shoot buds in 2 weeks of culture; *CD*- proliferation and multiplication of shoots after 4 weeks; *E* – root induction on half strength MS medium supplemented with IBA 4.9 µM after 2 weeks of culture; *F* – single root induction after 2 weeks of culture; *G* – an acclimatized plant.

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