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MICRO PROPAGATION AND TISSUE CULTURE OF THE ENDANGERED MEDICINAL PLANT *WITHANIA SOMNIFERA* BY THE DIRECT SHOOT AND ROOT INITIATION METHOD

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ABSTRACT: Leaf and Cotyledon explants of *Withania somnifera* (L). Dunal were used to evaluate the effect of different growth regulators on the in vitro direct shoot and root initiation methods. Four different explants were used to establish callus shoot and root direct regeneration. In the first experiment leaf segments were cultured on MS basal supplemented with 2,4 – Dichlorophenoxyacetic acid (2,4 – D, 0.1-20.0 mg/L), with combination of Naphthalene acetic acid (NAA 0.1-20 mg/L) and Benzylaminopurin (0.1-20 mg/L). This new protocol was standardized for easy mass propagation of *W. somnifera* medicinal plant. Callus initiation was observed best in MS media with (2,4- D 1.0-5.0 mg/L) after 16-20 days (93%). Highest maximum number of multiple shoots was obtained on MS medium (BAP 3.0 – 5.0 mg/L). The shoots were seaperated from the multiple-shoots, transferred to MS medium supplemented with 1.5 – 20 mg/L NAA favored roots formation occurred in most of the shoot let 88% were successfully achieved in the MS media. The rooted plantlets were transferred to polythene bags which was containing vermi compost, sand and red soil in the ratio of 1:2:2 and kept in a mist house. After acclimatization in the mist house for 2-months, it transferred to greenhouse. The plantlets were successfully planted in the field.

Key Words: *Withania somnifera*, **2,4**-Dichlorophenoxy acetic acid, α- Naphthalene acetic acid, Benzylaminopurine, **MS** -Murashige and Skoog Medium.

INTRODUCTION

Withania somnifera (L.) Dunal, is an erect, evergreen, perennial shrub and member of Solanaceae family is a widely used medicinal plant useful in the treatment of inflammatory, anti-tumour agent (Naidu et al., 2003). it is well known for years as an important drug in Ayurvedic literature. Root of the plant W*ithania somnifera* (Ashwagandha) reportedly exhibit antioxidant, immunomodulatory and haematopoietic properties (Mishra et al., 2000).

Ashwagandha roots used in Ayurveda and Unani medicines. Roots are prescribed as medicines for hiccups, several female disorders, bronchitis, rheumatism, dropsy, stomach and lung inflammation, and skin diseases. The ingredient in medicines prescribed for curing disability and sexual weakness in males (Joshi et al., 2010). According to red list of threatened species, 44 plant species are critically endangered, 113 endangered and 87 vulnerable. *W. Somnifera* proved to be 99.75% of the endangered medicinal plant (Siddique et al., 2005; Rahman, 2001). As over harvesting of *W. somnifera* that plant root is going to be endangered condition in the Southern India (Manickam et al., 2000). The active pharmacological components of *Withania somnifera* constituents are withanolides (Steroidal lactones with ergostane skeleton) and alkaloids (Elsakka et al., 1990). The active cintent of Indian *Withania somnifera* are withaterin-A and withanolide-D, both are present leaves and roots of the plant are used as a source of drugs. Total alkaloid content in the root of the Indian type has been reported to be between 0.13 to 0.31% of this plant showed antitumor and radio sensitizing effects in animal models (Sharma et al., 2009). It also possesses anti-stress, immunomodulatory, anti-oxidant and anti-bacterial activity (Kupchan et al., 1965 ; Devi et al., 1992; Devi et al., 1993).

Due to the medicinal value, these plants are collected and used as raw material for large-scale medicinal industry, leading to over exploitation and it becomes an endangered plant species. One of the problems for commercial cultivation, it takes long periods for seed germination strains productivity. Micro propagation of *Withania somnifera* employs different explants such as shoot tips (Sen and Sharma, 1991), auxiliary meristems (Roja and Heble, 1991) auxiliary leaves, auxiliary shoot and hypocotyl and root segments (Rani and Grover,1999) has been demonstrated. Propagation use by seed, but seed viability is limited to more than one year. (Roja and Heble, 1991) reported, callus formation from explant. Due to poor viability of stored seed and lack of protocol for *in vitro* Multiplication, the present study was carryout to the potential of different explants leaf, cotyledon, hypocotyls and epicotyls. The direct root and shoot regeneration under *in vitro* mass clonal multiplication through the tissue culture method with possibility of developing a new protocol was standardized.

MATERIALS AND METHODS

Collection of plants: *Withania somnifera* explants and seeds were collected from the Irula Tribe Women's Welfare Society **(**ITWWS) in 2011, Kanchipuram, Tamil Nadu.

Surface sterilization: The explants of auxiliary leaves 1 - 2 cm, segments were taken from 4-5 month old plants while hypocotyl, Cotyledon and epicotyls segment of 0.5 – 1 cm size explants were removed from 10-15 days old seedling and all the explants were washed thoroughly with running tap water for 20 min, after that immersed in Teepol (1% v/v) for 2 – 3 min and washed thoroughly with distilled water. Subsequently they were sterilized on the surface with 0.1% $HgCl_2$ solution for 1-2 minutes and again washed well in distilled water for 3-4 times to remove the traces of $HgCl_2$ (Mercuric chloride).

Culture media: The all explants (1-2cm) were implanted on sterile medium consisting of salts and vitamins of MS medium (Murashige and Skoog, 1962), supplemented with 0.2% (w/v) Agar and 3% (w/v) sucrose.

Culture conditions: All cultures were maintained at 25±2°C under 16 hrs photoperiod at a photosynthetic flux of 12.6μmol m^{-2} s^{-1}, provided by cool daylight fluorescent lamps.

Shoot and root initiation:

All the explant were produced for shoots and roots initation with the MS media supplemented in the range of BAP 0.1-20 mg/L and NAA 0.1-20 mg/L were used to produce maximum number of multiple shoots and roots repectively.

Hardening: For hardening-off, 7 to 8 week old rooted shoots were removed from the culture flacks. After washing away the Agar with water they were transferred in to small pots containing sterile vermi compost, sand, and red soil in the ratio of 1:2:2 and were kept in a mist house (Figure: E & F). After acclimatization in the mist house for 2-months, they were transferred to greenhouse.

Histological analysis: During the culture period, developing regenerated shoot buds samples were collected and fixed with in formalin-aceto-alcohol (FAA) solution (70% ethanol, 5% glacial acetic acid and 5% formaldehyde) kept it to 24-h at room temperature. After fixation, the samples were dehydrated through a graded series of butanol solution and then embedded in paraffin. For histological analysis, the tissue was sectioned to 750 μm with a microtome, stained with hematoxylin solution for 3 minutes for examined under a light microscope and take out the photographs.

RESULTS AND DISCUSSION

Effect of various explants used to different contraction of 2, 4-Dichlorophenoxyactic acid with MS media on callus induction (%): Callus initiation was observed within 3 to 4 weeks on MS media supplemented with 2, 4-D (0.1-20 mg/L). The response shown by different explants varied widely depending on the concentration of 2-4-D (Table-1). The overall callus induction frequency on MS medium varied from 42-93 percent with leaf explants, while in hypocotyl, epicotyl and cotyledon the frequency range was observed in the order of 36-84, 25-89 and 32-80 percent respectively.

The optimum frequency of callus induction was elicited with the supplementation of 1.5-3.0 mg/L of 2, 4-D more or less uniformly for all the explants (Figure: A & B). Among the 4 explants maximum callus induction was observed with leaf explants followed by the L > E > H > C.

Table 1. Effect of various explants used to different contraction 2, 4-Dichlorophenoxyactic acid with MS media on callus induction (%)

MS + 2,4-D (mg/L)	Explants' of Callus Induction %			
	L	C	H	E
0.1	42	42	62	25
0.5	58	48	48	39
1.0	72	45	62	45
1.5	80	60	65	72
2.0	92	62	72	82
3.0	93	80	84	89
4.0	80	65	62	87
5.0	69	48	43	78
10.0	65	32	36	46
20.0	-	-	-	-
SD±SE	16.28 ± 5.85	14.47 ± 5.16	14.84 ± 5.32	23.84 ± 7.94

L – Leaf, C – Cotyledon, H – Hypocotyl, E – Epicotyl, **-** No response.
SD - Standard Deviation, SE - Standard Error.

Different concentration of 2, 4-Dichlorophenoxyactic acid with MS media - Leaf induction of callus fresh and dry weight: The explants of leaf maximum callus yield was obtained on MS medium with different concentration of 2,4-D after 4 week (Table-2). The fresh and dry weight data indicated that, the optimum levels of 2, 4-D required for maximum yield of callus ranged from 1.5-3.0 mg/L. The similar result got in the range of 2, 4-D (2.0-3.5 mg/L) (Baumert et al., 1992).

Table 2. Different concentration 2, 4-Dichlorophenoxyactic acid with MS media leaf explants induction of callus fresh and dry weight

MS +2,-4-D (mg/L)	Leaf explants of Callus induction %	
	F.W	D.W
0.1	169.5	25.03
0.5	325.2	31.2
1.0	336.2	50.2
1.5	354.2	54.3
2.0	337.8	58.2
3.0	375.2	48.3
4.0	325.2	34.8
5.0	332.0	35.4
10.0	323.2	25.1
20.0	-	-
SD±SE	58.79 ± 19.43	12.64 ± 4.49

F.W - Fresh weight, D.W - Dry weight, **-** No response.
SD - Standard Deviation, SE - Standard Error.

Effect of different explants and Benzyl Aminopurine (BAP) with MS media on Shoots Induction (%): Benzyl amino purine (BAP) is a synthetically produced N6-adenine compound. BAP is a synthetic cytokinin with a capacity to promote cell division and influences steps in the cell cycle for a long time has been considered. The addition of BAP is necessary to obtain callus and shoot growth. The level of BAP required for optimum shoot formation may be (3.0-5.0mg/L) but this depends upon the type of explants (Figure: C) Anatomical study of callus cross section developed on the shoot (Figure: G & H), The leaf explants callus produced highest shoot formation 6-85% (Table-3). Compare to order of 23-80, 8-52 and 6-19 respectively **L > C> H >E**. The same plant reported in another study that IBA was efficient in inducing roots (100%) in regenerated shoots obtained from nodal explants (Sivanesan and Murugesan, 2008).

Table 3. Effect of different explants and Benzyl Aminopurine (BAP) with MS media on Shoots Induction (%)

MS +BAP (mg/L)	Explants' of Shoot Induction %			
	L	C	H	E
0.1	-	23	25	-
0.5	22	65	26	-
1.0	25	25	32	6
1.5	28	58	35	8
2.0	34	65	52	12
3.0	52	49	48	13
4.0	85	80	39	19
5.0	82	72	20	18
10.0	16	50	12	14
20.0	6	52	8	6
SD±SE	28.24 ± 9.11	18.57 ± 6.03	14.39 ± 4.65	5.04 ± 2.06

L – Leaf, C – Cotyledon, H – Hypocotyl, E – Epicotyl, **-** No response.
SD - Standard Deviation, SE - Standard Error.

Effect of different explants and Naphthalene acetic acid (NAA) with Ms media on Roots induction (%): Callus initiation occurred in leaf, cotyledon, hypocotyl and epicotyl explants on MS media supplemented with different concentrations of NAA (0.1-10.0mg/L) (Table-4). After 4 weeks of induction, different explants were varied in callus roots induction response depending on the levels of NAA. In lower concentration of NAA (0.1-0.5mg/L) callus did not produced root induction. A highest root formation was obtained by leaf explants up to 88% at 5-10mg/L NAA concentration (Figure: D). Compare to hypocotyl, epicotyl and cotyledon the frequency range was observed in the order of 0-85, 0-87 and 0-50 respectively **L > E > H > C**. The same plant reported in their multiple roots on MS medium supplemented with NAA at optimum concentration (0.5 mg/L) (Sharma et al., 2009).

Table 4. Effect of different explants and Naphthalene acetic acid (NAA) with MS media on Roots induction (%)

MS +NAA (mg/L)	Explants' of Root Induction %			
	L	C	H	E
0.1	-	-	-	-
0.5	-	-	22	-
1.0	-	10	54	50
1.5	25	12	54	54
2.0	52	18	65	70
3.0	54	22	70	72
4.0	65	47	82	75
5.0	88	45	85	87
10.0	88	50	70	85
20.0	26	24	26	22
SD±SE	25.83 ± 9.47	16.31 ± 5.71	22.33 ± 7.46	21.54 ± 7.66

L – Leaf, C – Cotyledon, H – Hypocotyl, E – Epicotyl, **-** No response.
SD - Standard Deviation, SE - Standard Error.

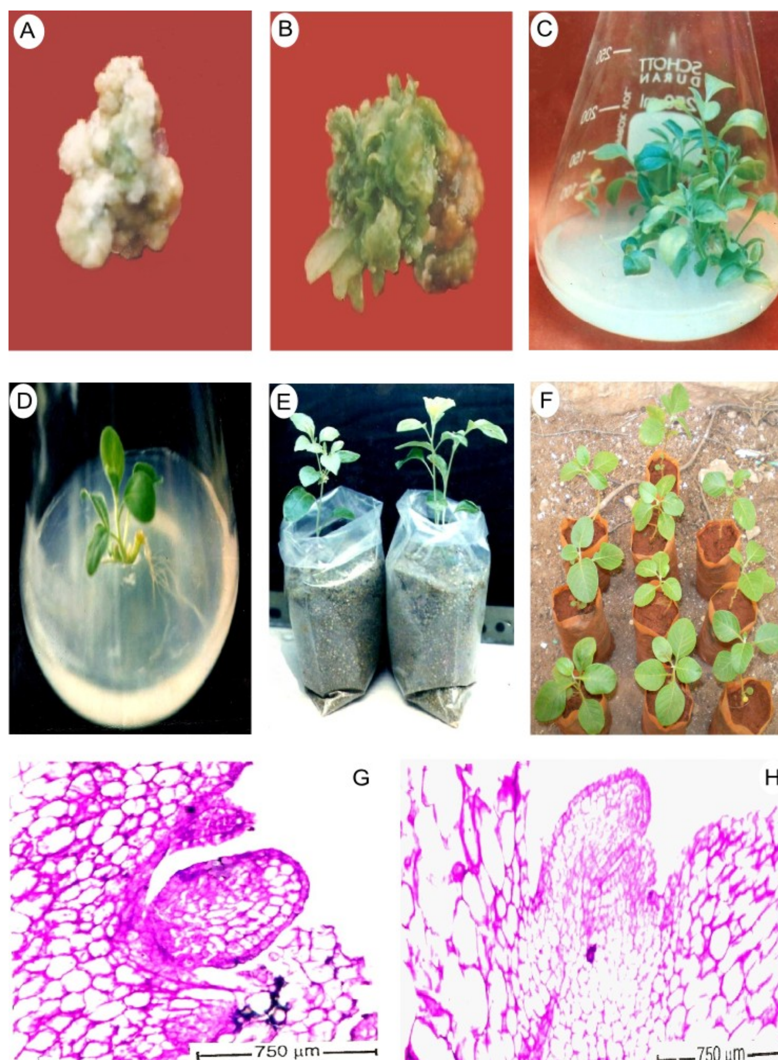

Figure: **A** - Development of Callus (Calli), **B** - Callus regeneration, **C** - Multiple shoots induction, **D** - Roots induction, **E** - Plantlets were transplanted with polythene bags to acclimatization for 2 month in mist house, **F** - Plant Hardening for *in vivo* condition, **G** – Cross section showing meristematic region after two weeks culture, **H** - Cross section of shoot bud connected to leaf explant showing leaf primodia and the shoot eapical meristem,

Histological analysis of shoot development:

Histologial analysis of the regenerating shoots was performed on explants at different stages of development and showed that the shoot buds had emerged from epidermal parenchymal cells, with no intermediate callus formation (Figure -G). After 14 days of culture, the shoot buds showed well developed leaf primordia and apical meristem (Figure -H). The meristematic cell were much samllar than the surrounding cells, which consisted of closely arranged and highly cytoplasmic cells.

CONCLUSION

As suggested in the published literature, very less significant micropropagation work has been done on this plant species. With regard to the tissue culture aspect, already some of work has been done. This experiments were carried out to propagate the selected explants of leaves, cotyledon, hypocotyls and epicotyls segment were used for different growth regulators on the in vitro direct shoot and root initiation using MS medium.

In our study the result shows *W. somnifera* micropropagation were enabled the mass propagation of *W. somnifera* plant and an efficient protocol was developed for direct regeneration through tissue culture method by using the leaves explants.

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