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Direct regeneration of *Withania somnifera* (L.) Dunal – A medicinal plant

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ABSTRACT

Plants have been an important source of medicine for thousands of years. Medicinal plants are the most important source of life saving drugs. Even today, the WHO estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines. Medicinal plants are resources of new drugs. Approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant substances. Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. In-vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine. An efficient protocol was developed for high frequency and rapid regeneration was achieved in *Withania somnifera* (L.) Dunal. *Invitro* cultures were initiated by meristematic explants on M.S–B5 medium with various growth hormones individually as well as combinations. Highest shoot multiplication rates were observed when explants grown in the medium supplemented with 2.0 mg/l of BAP. M.S–B5 medium fortified with 1.0 mg/l BAP was found suitable for optimum growth and elongation of shoots. The best rooting response were observed when the medium containing 1.0 mg/l of IBA. The rooted plantlets were successfully established in soil.

***Keywords*:** *Withania somnifera*; meristematic explants; M.S–B5 medium; BAP and IBA

1. INTRODUCTION

Nature has provided a rich source of herbal medicines to cure various ailments of mankind. Plants are one of the most important sources of drugs, since every species in nature is of potential value to human beings. Medicinal plants are of great interest as pharmaceutical industries depend in part on plants for the production of secondary compounds. A large number of medicinal plants are exploited from the natural flora for the commercial production of drugs. During the last few decades as a result of technology advancement and increasing demand of phytomedicines, medicinal plants used in various traditions were investigated chemically and phmacologically for the active chemical constituents and to elaborate new stand recipes for more effective treatments for specific ailments. In view of growing world populations, increasing anthropogenic activities, rapidly eroding natural ecosystems etc., the natural habitat for a great number of herbs and trees are dwindling and many of them are facing extinction. To cope up with this alarming situation, the recent exciting developments in biotechnology have come as a boon, now plant tissue culture become an integral part of biotechnology, which can be of immense help to overcome the problem of depletion of natural herbal wealth through rapid micropropagation methods. There are number of constraints for the propagation and conservation of many taxa through conventional methods like vegetative and seed propagation, which includes slow rate of multiplication, edaphic and climatic factors, dormancy, low percentage of seed set and germination. Sometimes, in spite of a high rate of germination, clonal uniformity cannot be maintained through seeds. Under these circumstances, in vitro techniques have been successfully applied to solve many constraints related to conventional clonal propagation in a large number of medicinal plants (Molnar et al., 2010, Sridhar, 2011 and Kumar et al., 2009).

India is richly endowed with a wide variety of plants having medicinal value. These plants are widely used by all sections of the society whether directly as folk medicines or indirectly as pharmaceutical preparation of modern medicine .The use of different parts of several medicinal parts to cure specific diseases has been in vogue from ancient times. The use of different parts of several medicinal parts to cure specific diseases has been in vogue from ancient times (Pandey et al., 2013). Plants has been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80 per cent of people still rely mainly on traditional remedies such as herbs for their medicines. Plants are also the source of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances (Mohammed and Ali, 2010).

Tissue culture is the in vitro aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions (Thorpe., 2007) often to produce the clones of plants. The resultant clones are true-to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH medium, adequate temperature and proper gaseous and liquid environment. Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites.

Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu et al., 2009). Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space. In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants.

The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities.

Withania somnifera (L.) Dunal. of Solanaceae is a viable plant used in traditional ayurvedic medicine and is often taken for its nervous sedative, hypnotic, tonic, astringent and aphrodisiac properties (Matsuda, 2000). Ashwgandha has long been considered as an excellent rejuvenator, a general health tonic and a cure for a number of health complaints. It is a sedative, diuretic, anti-inflammatory and generally respected for increasing energy, endurance, and acts as an-adaptogen that exerts a strong immunostimulatory and ananti-stress agent. Ashwagandha is taken for treating cold and coughs, ulcers, emaciation, diabetes, conjunctivitis, epilepsy, insomnia, senile dementia, leprosy, Parkinson's disease, nervous disorders, rheumatism, arthritis, intestinal infections, bronchitis, asthma, impotence and a suppressant in HIV/AIDS patients.

According to Indian Herbal System (Ayurveda), Ashwagandha is considered one of the most important herbs and the best adaptogenic. It contains constituents like cuseohygrine, anahygrine, tropine, and anaferine, glycosides, withenolide with starches and amino acid. Withanolide consists of steroidal molecules which is said to fight inflammation. Ashwagandha stimulates the immune system, combats inflammation, increases memory, and helps maintain general health and wellness. Ashwagandha is known to increase the production of bone marrow, semen, and acts anti-aging. Ashwagandha anti-tumor and antiinflammatory agents are approved in several studies. Its steroidal is much higher than that of hydrocortisone which is a common treatment in cancer cases. Diseases like TB, chronic upper respiratory diseases and HIV have been added to the list of Ashwagandha due to its strong immunostimulatory activity, and it is recognized as a blood tonic, especially in gynecological disorders including anemia and irregular menstruation. Patients with anxiety can also benefit from Ashwagandha.

The roots of ashwagandha are a constituent of over 200 formulations in Ayurveda, Siddha and Unani medicine. It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as Withanolides.

This drug is known to have anti-inflammatory, antitumor, antioxidant, anticonvulsive and immunosuppressive properties (Baldi *et. al*., 2008). Commonly it's propagated commercially by the means of seeds, but the seed viability is limited to one year (Rani and Grover, 1999), making the long duration seed storage futile (Farooqi and Sreeramu, 2004). Due to poor viability of seed, alternative procedure of propagation is essential for constant supply for industrial level.

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as in-vitro regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants

as bioreactors. This paper reviews the achievements and advances in the application of tissue culture and genetic engineering for the in-vitro regeneration of medicinal plants from various explants and enhanced production of secondary metabolites. *In vitro* technology can be used as an alternative because the advantage of tissue culture technology lies in the production of high quality planting material on a year–round under disease–free condition any where irrespective of the season and eather. A micropropagation technique for rapid and large scale propagation of medicinal plants has significantly increased (George and Sherington, 1984; Arora and Bhojwani, 1989). Thus the present paper reports an efficient protocol for rapid propagation of *Withania somnifera* (L.) Dunal.

2. MATERIALS AND METHODS

Shoot tip and nodal segments collected from widely growing healthy plants of *Withania somnifera* were used as explants. The explants of shoot apices and nodal segment were 3-5 mm approximately in length. Explants were surface sterilized by washing initially under tap water, then with liquid soap (Teepol) for few minutes and with 0.1% mercuric chloride for 2-3 minutes. They were rinsed with sterile distilled water for many times to remove any traces of mercury chloride and inoculate on M.S. (Murashige and Skoog, 1962) medium with B5 vitamins (Gamborg et, al., 1968) supplemented with various concentrations of growth regulators such as BAP, Kn, 2,4-D, NAA and IBA. The pH of the medium was adjusted to 5.8 before sterilization and cultures were maintained at 25 ±2 °C with 16 hours photoperiod.

For each treatment three replicates each of 25 culture tubes were used. Multiple shoots obtained directly and where elongated then they were separated from each other and rooted successfully on full strength and half strength M.S.–B5 medium supplemented with various concentrations of IBA.

3. RESULTS AND DISCUSSION

Nature is an infinite resource for drug development and created almost an inexhaustible array of molecular entities. It is not possible to obtain a precise figure for the total number of species existing on earth (Pimm et al. 1995) made an effort to express biodiversity in number, estimating the total number of species to be 10-100 million and plants existing range from 2,50,000 to 7,50,000. Only 5-10% of these species however have been acknowledged through scientific evaluation to have therapeutic value (Verpoorte., 1998). Plant derived drugs are being widely used not only in developing countries but also in the most advanced countries. It has been reported by WHO that about 80% of the world's population rely on medicinal plants for their primary health care. Until recently plants are being the important source of novel pharmacologically active compounds, with many blockbuster drugs derived directly or indirectly from plants. Despite the current occupation with synthetic chemistry as a vehicle to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous (Raskin et al., 2012 and Newman et al., 2000). According to recent report utility of natural products as sources of novel structures is still alive and well. Up to 50% the approved drugs during the last 30years are from either directly or indirectly from natural products.

In the area of cancer, over the time frame from around the 1940s to date, of the 175 small molecules 85 actually being either natural products or directly derived there from (Newman and Cragg 2012). Although many of the drugs are made by synthetic chemistry, most of the core structures or scaffolds for synthetic chemicals are based upon natural products. Not all natural products can be fully synthesized and many natural products have very complex structures that are too difficult and expensive to synthesize on an industrial scale. Because of their complex structures and in spite of extensive efforts to develop partial or total chemical synthesis, isolation of such plant derived compounds from their natural source remains the only viable option, with very few exceptions. And most of the medicinal plants are not cultivated; rather they are collected from wild. In the past, quantities needed to meet demand were relatively low; however, increasing commercial demand is fast outpacing supply. Production and isolation of the chemicals by conventional techniques face several problems leading the plants to become endangered and resulting in loss of biodiversity (Kolewe et al., 2008).

India has 2.4% of world's area with 8% of global bio-diversity. It is one of the 12 mega-diversity hot-spot regions of the world. Across the country, the forests are estimated to harbor 90% of India's total medicinal plants diversity. Only about 10% of the known medicinal plants of India are restricted to no forest habitats (Wakdikar, 2004). According to Schippmann et al. (1990), one fifth of all the plants found in India are used for medicinal purpose. The world average stands at 12.5% while India has 20% plant species of medicinal value and which are in use.

Nodal and shoot tip explants cultured on M.S – B5 medium without growth regulators thrived only few days and shrivelled off. When BAP was added to the culture medium (0.5 – 5.0 mg/l) the explants showed growth range from single to multiple shoots. Similar observation was already noted in *Dolichos biflorus* (Soundar Raj et.al., 1989; Handique and Bora, 1999). Nodal explants showed solitary shoot development at 0.5 – 1.0 mg/l of BAP. Multiple shoot was observed from 1.5 – 3.5 mg/l of BAP. Maximum percentage of response and number of shoots were noticed on the medium containing 2.0 mg/l BAP (Table – 1). Medium containing 0.5 mg/l of BAP the shoot tip explants produce single shoots. Multiple shoot were observed for 1.0 – 3.5 mg/l of BAP. Culture medium containing 2.5 mg/l BAP noticed maximum percentage of response. Maximum shoot length was observed in shoot tip and nodal explants on the medium containing 1.5 mg/l of BAP and minimum at 4.5 mg/l of BAP (Table – 1).

Nodal and shoot tip explants occurred on M.S–B5 medium supplemented with different concentration of Kn showed initiation of single shoot to multiple shoots. The medium containing Kn 0.5 – 1.5 mg/l formed the growth of solitary shoot, whereas 2.0 – 3.5 mg/l showed maximum percentage response and 4.0 and 4.5 mg/l of Kn supported lower response and callus formation. Both NAA and 2,4–D in various concentration (0.5 – 5.0 mg/l) the explants response callus formation after two week of inoculation. Between two explants node produced maximum amount of callus. Shoot elongation was noticed on lower concentration of BAP 1.5 mg/l containing medium (Table – 1). These results confirm earlier reports made by Kawata et. al., 1995.

The well developed shoots were excised and sub cultured in medium supplemented with half strength and full strength concentration of M.S – B5 medium with IBA (0.5 – 5.0 mg/l). Half strength M.S – B5 medium with 1.5 mg/l of IBA induced maximum number of roots (Table – 2).

Table 1. Effect of BAP and Kn on nodal and shoot tip explants of *Withania somnifera.*

Growth Regulator (mg/l)	Culture showing response (%)		Average shoot length (cm)		Mean number of shoots / explants	
	Node	Shoot tip	Node	Shoot tip	Node	Shoot t ip
BAP						
0.5	58	52	4.8	4.2	16.8	12.4
1.0	63	61	5.2	4.6	17.7	14.5
1.5	86	77	5.7	4.9	19.5	17.2
2.0	91	84	6.2	5.8	22.4	18.9
2.5	94	92	6.6	6.2	25.6	19.3
3.0	82	78	5.9	5.4	23.2	17.6
3.5	76	65	4.8	4.7	21.4	15.7
4.0	68	53	4.3	3.9	17.6	14.8
4.5	52	42	3.9	3.3	16.4	13.1
5.0	41	36	3.2	2.8	14.2	11.2
Kn						
0.5	62	60	4.2	3.1	14.3	12.8
1.0	71	67	4.9	3.8	15.9	14.2
1.5	88	73	5.6	4.7	18.2	15.8
2.0	91	88	6.0	5.2	20.6	16.2
2.5	82	79	5.3	4.8	17.4	15.7
3.0	78	75	4.7	4.3	15.3	14.6
3.5	64	63	4.1	3.9	14.1	13.7
4.0	59	57	3.4	3.2	13.3	11.5
4.5	41	38	2.8	2.4	11.5	9.4
5.0	38	32	2.3	1.8	8.5	7.8

Table 2. Effect of IBA on root induction of *Withania somnifera.*

Medium	IBA (mg/l)	Culture showing response in %	Average number of root / explants
M.S – B5	0.5	81	13
M.S – B5	1.0	87	16
M.S – B5	1.5	78	14
M.S – B5	2.0	75	11
M.S – B5	2.5	72	9
M.S – B5	3.0	70	6
M.S½ - B5	0.5	84	15
M.S½ - B5	1.5	90	19
M.S½ - B5	2.0	82	16
M.S½ - B5	2.5	76	13
M.S½ - B5	3.0	73	09

Rooted plant let were hardened in mist chamber with survival rate of 70 percent after that it was transferred to soil. Efficient plant regeneration is the primary objective of many studies in plant tissue culture. Micro propagation system provides a method for rapid regeneration of various medicinal crops of high economic value.

The improved in vitro plant culture system has the potential for commercial production of medicinal crops on large scale. During the past decade remarkable progress resulted in plant biotechnology has been witnessed with a constant flow of improved transformation regeneration protocols for many medicinal crops.

A good regeneration protocol is always needed for genetic transformation studies for up-regulation of secondary compounds. Supplementation of natural organic extracts as additives for standardizing regeneration protocols of commercially important medicinal crops has been increased significantly.

These promissory organic extracts described in this review would certainly be of increasing importance in near future in the field of medicinal plants research, such as genetic transformation studies and scale-up of secondary compounds through cell suspension cultures in bioreactors.

4. CONCLUSIONS

Withania somnifera, best known as ashwagandha has been used for centuries for the treatment of vivid health disorders. Multiple health benefits featured in this herbal supplement makes it as a perfect rejuvenator of physical and psychological health. As per research, this medicinal herb is mainly found in the regions of North America and India. Powerful antioxidant compounds enriched in this herb scavenges free radicals and reduces aging impact on person. Apart from consuming this extract, diet taken by person plays an important role in increasing the level of antioxidants in body. In order to obtain good level of antioxidants, it is advised to include surplus amount of fruits and vegetables in diet. Direct regeneration was achieved from the nodal and shoots tip explants. The protocol standardized in present study is reproducible and can be used n future medicinal plants improvements programme.

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Plate - 1. Morphology of *Withania somnifera*.

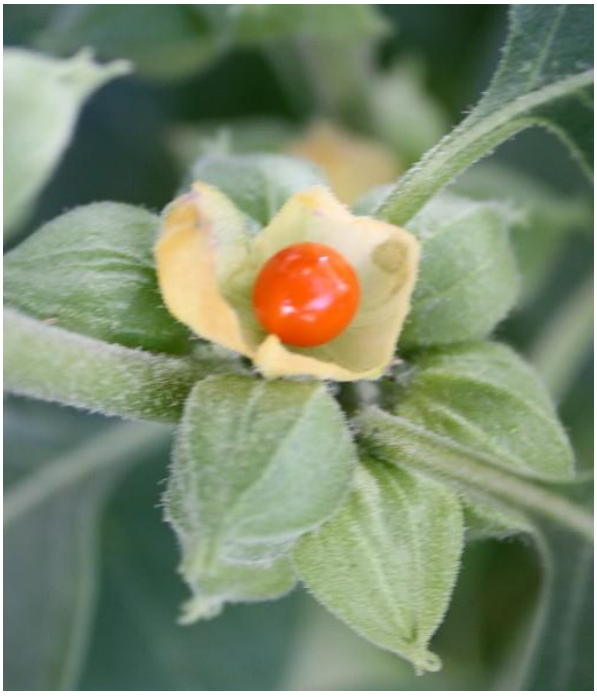

Plate - 2. Regeneration of *Withania somnifera in vitro* condition.

Fig. 1. Shoot elongation of nodal explants on M.S–B5 + BAP

Fig. 2. Rooting response of shoot tip explants on M.S½ – B5 + IBA

Fig. 3. Hardening of *invitro* obtained plantlet