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A review on micro propagation of *Withania somnifera* – A medicinal plant

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The capacity for plant cell, tissue and organ cultures were produced and accumulated. Valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's market place for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is a commercial significance that drives the research initiatives. One of the most prolific producer plant, Withania (Ashwagandha in Hindi and Sanskrit, winter cherry in English) is the best source of withanolides particularly withaferin-A. It has been recognized as a plant with multiple forms of medicine in Ayurveda, the most descript and extensive which one of the major Indian systems of traditional medicines. Currently, there are several forms of pure herbs and herbal extracts of Withania sold all over the world as herbal health helping GRAS (generally regarded as safe) products, Ashwagandha forms active ingredient of more than 100 Ayurvedic and 30 Sidha medicines and also a good number of Unani medicines.

Key words: Secondary metabolites, Micropropagation, withanolides, *Withania somnifera*.

Introduction

Withania somnifera commonly referred as Indian ginseng. Modern herbalists classify *Ashwagandha* as an adaptogen, a substance said to increase the body's ability to withstand stress of all types. However, the evidence for an adaptogenic effect is limited to test tube and animal studies (Kupparanjan, 1976). Other proposed uses of *Ashwagandha* are based on even weaker evidence, including; preventing cancer (Devi and Sharada, 1992). Improving immunity (Ziauddin and Phansalkar, 1996) Reversal of paclitaxel induced neutropenia by *Withania somnifera* in mice, Enhancing mental function and combating anxiety and depression. Externally it has been applied as a poultice to boils, swellings and other painful pains. Root is harvested in the autumn and

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dried for some time for later use. The seed is diuretic and hypnotic. There are some research showed *Ashwagandha* is a promising alternative for cancer treatment and prevention. *Ashwagandha* seems to show positive effects on the endocrine, cardio, and central nervous systems. It is one herb that can help body produce its own thyroid hormones.

Active compounds

The major biochemical constituents of *Ashwaganda* root are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides (Atta and Dur, 1993). At present, 12 alkaloids, 35 withanolides and several sitoindosides from this plant have been isolated and studied. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. Much of *Ashwaganda's* pharmacological activity has been attributed to two main withanolides, withaniferin A and withanolide. The withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. *Ashwagandha* is thought to be amphoteric i.e, it can help regulate important physiologic processes. The theory is that when there is an excess of a certain hormone, the plant-based hormone precursor occupies cell membrane receptor sites so the actual hormone cannot attach and exert its effect. If the hormone level is low, the plant-based hormone exerts a small effect. They are the most important bioactive constituents of roots of *Ashwagandha*.

Withaferin A

Withaferin A is the most important of the withanolides isolated from *Withania somnifera* to which the curative properties of the leaves are attributed. It has been receiving a good deal of attention because of its antibiotic and antitumour activities. The patented unsaturated lactone-antibiotic, obtained from the Indian plant, is identical with withaferin A. For its separation, the leaves are extracted with cold alcohol; the extract is purified and dried, and finally crystallized from aqueous alcohol (yield, 0.18% air-dry basis). Withaferin A can also be obtained from the leaves by methanolic extraction, fractionation and chromatographic separation. It is insoluble in water and is administered in the form of suspension (Uma and Akagi, 1996). Roots of *Withania somnifera* contains four steroidal lactones, called withanolides, viz withaferin A 5, 20a (R)-dihydroxy- 6a, 7a-epoxy-1 -oxo-(5a)-witha-2,24 - dienolide (m.p. 282-84 °C) and two minor withanolides, of which one is probably 5a, 17a-dihydroxy-1-oxo-6a, 7a-epoxy-22R-witha-2,

24-dienolide (the so-called withanone). The first two withanolides also occur in the roots. Chlorogenic acid is present in the leaves (Subramanian, 1982).

Three withanolides viz., withacoagin, coagulin, and withasomidienone have been isolated from the plant; along with other withanolides and withaferin A. 3ß-hydroxy-2, 3-dihydro withanolide F, isolated from the fruits, showed significant hepatoprotective activity, and anti-inflammatory activity equal to hydrocortisone **(**Rahman, 1993). In addition to the alkaloids, the roots are reported to contain starch, reducing sugars, hentriacontane, glycosides, dulcitol, withaniol (0.08%), an acid (m.p. 280-83°) and a neutral compound (m.p. 294-96°). Withaniol was later found to be a mixture of two withanolides, of which the major component was 5,20a-(R)-dihydroxy-6a, 7a-epoxy-1-oxo-(5a) - witha-2, 24- dienolide, and the minor component remained unidentified. The free amino acids identified in the roots include aspartic acid, glycine, tyrosine, alanine, proline, tryptophan, glutamic acid and cystine. This herb has been extensively evaluated for various pharmacological activities. It is found to be a potent anti-stress anxiolytic and anti-depressant (Kazmi and Noorali, 1991) action. It has been reported to have anti-convulsant activity too (Kulkarni and Joseph, 1998) Evidences have demonstrated anti-inflammatory activity. In addition *Withania somnifera* has the property of modulating the immune system (Begum and Sadique 1988). *Withania somnifera* has been found to have activity against the various cancer cell lines. *Withania somnifera* has been reported for its anti-microbial activity (Jaffer and Jawad, 1988) against Gram positive bacteria. It has also been reported for hypoglycemic, diuretic, hypocholesterolemi.

Withania somnifera contains flavonoids and many active ingredients of the withanolide class. Numerous studies over the past two decades indicate that it has anti-inflammatory, anti-tumor, anti-stress, antioxidant, mind-boosting, and rejuvenating properties (Kuroyanagi, 1999). Withanolides are the most important bio-active constituents present in roots of ashwagandha. Withanolides are believed to account for the multiple medicinal applications of *Withania somnifera*. These molecules are steroidal and bear a resemblance, both in their action and appearance, to the active constituents of Asian ginseng (Panax ginseng) known as ginsenosides. The Indian chemotypes-1 is designated having major steroidal lactone withanone (2.2 g/kg dry leaves) and withaferin (1.66 kg dry leaves). Four (1, 8-10) and six known (2-7) withanolides were isolated from the leaves of *Withania somnifera*. Among the new compounds, 10 possessed the rare 3-O-sulfate group with the saturation in A ring and 9 contained unusual 1,4-dien-3-one group. Compound 8 did not have usual 2, 3 unsaturation in A ring while 1 had the rare C-16 double bond. The structures of all the compounds were elucidated by spectroscopic methods and chemical

transformation (Dhar, *et al.,* 2006). Phytochemical and genetic analysis in selected chemotypes of *Withania somnifera* is reported by Shashi, *et al.* (2004) A reversed phase liquid chromatographic method for analysis and quantitative estimation of withaferin A in roots of *Withania somnifera* (L.) Dunal collected from different geographical zones, has been developed using a symmetry C18 column and a binary gradient profile. The various aspects of analysis such as extraction, efficiency, detection limits, reproducibility and peak purity were validated using photodiode array detector.

Significance of *in vitro* regeneration of *Withania somnifera*

Our study revealed that cultivation of medicinal plants especially high value medicinal plants is creating new dimension in the field of agriculture. Indian herbal industry is at blooming stage. However, cultivation of medicinal plant is not easy. It is a challenging task because very little knowledge of seed biology. Many effects have not been made to search elite specimen and their propagation. *Withania somnifera* generally grows on lower as well as higher altitude in rare so to conserve this medicinal plant. It should be propagated on lower altitudes to have its commercially significant products like secondary metabolites. Secondary metabolites of *Withania somnifera* have got potential application as therapeutics. As germination of seed in this plant is very poor, it is better to grow the plant, *in vitro* conditions from various explants. The technique of micropropagation is applied with the objective of enhancing the rate of multiplication. Through the culture over a million of plants can be grown from a small piece of plant tissue within 12 months. Such proliferative rate of multiplication can be expected by any *in vivo* methods. The natural habitats for medicinal and aromatic plants are disappearing fast and together with environmental and geographical disabilities. This is increasingly difficult to acquire plant derived component. This is promoted industries as well as scientists to consider the possibilities of investigation of *in vitro* culture as an alternative supply additional agriculture. The provision of alternative sources of *Withania somnifera* by encouraging its cultivation will go a long way in reducing their heavy dependence on the wild populations. Conventional propagation methods have proved to be inadequate to meet this challenge. Large scale production through plant *in vitro* regeneration will provide a means of putting the plant onto the market at lower prices. In addition, the technique is cost effective, relatively simple and can be performed by semi-skilled persons.

Need of tissue culture for in vitro production of secondary metabolites

Since the secondary metabolites have complex stero structure with many chiral centers, which may be essential for biological activity, many of these cannot be synthesized economically on a commercial basis. They are therefore extracted from plants cultivated in fields or growing in wild stands. It has been reported that nearly 95% of the plants used traditionally as ingredients of crude drugs are collected from forest and other natural resources. The continuous and non organized exploitation has resulted in many plants becoming rare and some even come extinct. Besides these plants collected as minor forest produce, show a wide disparity in their values, due to lack of information on their life cycles, maturity and regeneration times, all of which change the quality and quantity of active chemical ingredients present. There are fluctuation in the concentration and quantities of secondary metabolites in field grown plants as biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental influence. To overcome these limitation biotechnologists suggested the use of plant cell and tissue culture rather than to use whole plants for the multiplication of pharmaceutical important plant and extraction for secondary metabolites. Schielder (1985) reported that plant cell and tissue culture, as well as genetic engineering may be an alternative to the conventional method for the improvement of medicinal plants.

Ayurveda is an ancient Indian system of medicine. Ayervedic medicinal plants are rich in secondary metabolites, which are potential source of drugs. It has been estimated that the about 1000 Ayurvedic remedies, prepared from around 750 plants are being used at present. Plant metabolites are source of Pharmaceuticals, food additives, fragrance and pesticides. It is estimated that the market potential for herbal drugs in the western would alone could be from US $ 4.9 million to 47 billion by the year 2000 if the AIDS epidemic continues unchecked. Nearly 75-80 % of the world population depends upon crude plant preparation to tackle their health problems since plant cell are totipotent, all the necessary genetic and physiological potential for the natural product formation should be present in an isolate cell (Chen, *et al.,* 1969). The root, stem and leaves of regenerated plants or the induced callus may be used fresh or dried, as raw drugs or different secondary metabolite are extracted from them. The plant tissue part selected for culture may be critical for regeneration and production. Assessing the amount of the products, either the regeneration or callus culture of the desired plants may be maintained in tissue culture. Till now very few reports of cell culture which can accumulate alkaloids at level significantly higher than the parent plants. Plants tissue culture may very well contain metabolic pathways that have been modified from that of the plant (Gita *et al.,* 2003). Cell culture technology has been applied to number of medicinal plants

to obtain pharmaceutically important drugs. But the results have not been encouraging, as the yield is too low to be commercially feasible. Only a few products such as shikonin and ginseng biomass are manufactured at a larger scale.

Micropropagation of *Withania somnifera*

Shoot multiplication was achieved *in vitro* from shoot tips of aseptically germinated seedlings of *Withania somnifera* L. using low concentrations of 6-benzyladenine (BA), viz. 2.2, 4.4 and 8.9 µM . Maximum number of shoots were obtained when 2.3 µM 2,4-dichlorophenoxyacetic acid (2,4-D) or 2.5 µM indolebutyric acid (IBA) was added to medium containing 4.4 µM BA during initiation of shoot multiplication, but not when added later. Direct multiple shoot initiation was also obtained from germinating seeds in the presence of BA alone. Rooting was successful in excised shoots grown on growth regulator-free MS medium. Rooted shoots were successfully established in soil in a greenhouse (Sen and Sharma, 1991). Gita rani *et al.* (2003) obtained direct rhizogenesis from *in vitro* leaves of *Withania somnifera* (L.), dunal by using an IBA dip treatment. The segments dipped in IBA formed roots along the midrib region of the abaxial surface when placed on Murashige and Skoog's (MS) basal medium containing no plant growth regulators. The length of the dip treatments (10, 20 and 30 min) and strength of the MS media (1/4, 1/2, and full-strength) treatments had no apparent effect on rooting, although maximum rooting (85.3 percent of the cultures) occurred when the leaf segments were placed on 1/2strength MS medium after a dip treatment with 100 mg/liter IBA solution for 20 min. The average number and length of roots were 32.3 per culture and 5.6 cm, respectively. Only 20 percent of the cultures produced roots if explants were grown on full-strength MS medium supplemented with IBA. An efficient protocol was developed for large scale propagation using seed as explant with MS medium supplemented with BAP 0.6 mg/l and IAA 0.4 mg/l (Fig 1. A, B and Fig C). About 90% rooting was achieved with 0.4 mg/l IBA and 0.4 mg/l IAA (Fig 1. D) (Supe *et al.,* 2006).

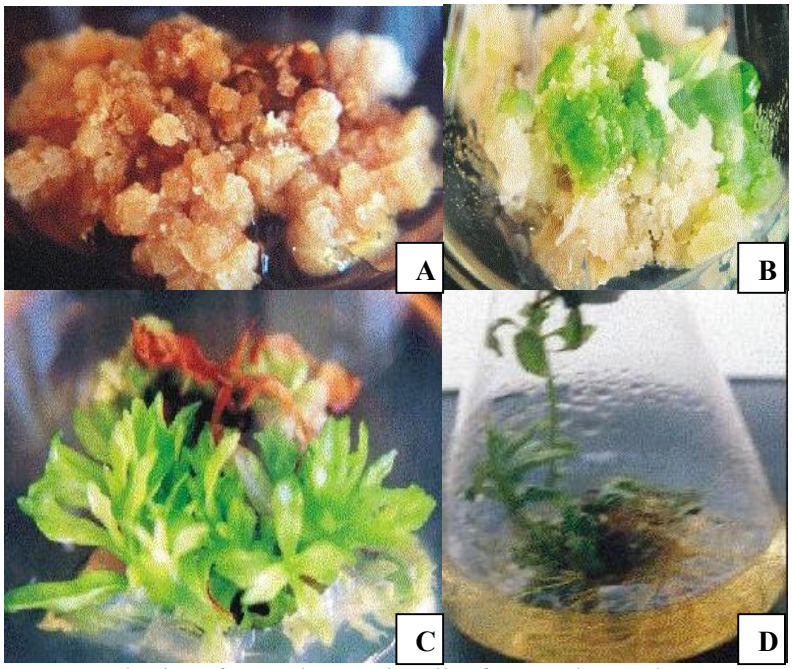

Fig 1. A. Induction of nonembryogenic callus from seed as explant.
B. matured embryogenic friable callus of *Withania somnifera*
C. Multiple shoots induction from embryogenic callus.
D. Adventitious root induction from separated shoot of regenerated plant

Kurkarni *et al*. (2006) reported that induced direct shoot regeneration from node, internode, hypocotyl and embryo explants of *Withania somnifera*). Direct regeneration of shoot buds was observed in MS basal medium supplemented with various concentrations of either benzyladenine (BA) or thidiazouron (TDZ) depending on the explant. Nodal explants formed multiple shoots both from pre-existing and de novo buds on Murashige and Skoog's medium (MS) containing 0.1–5.0 mg l−1 BA and a ring of de novo shoot buds on MS medium containing 0.2 and 0.3 mg l−1 TDZ. Internodal explants formed shoot buds on MS with 1.0 and 5.0 mg l−1 BA while the hypocotyl explants gave rise to multiple shoots only on MS with 0.5 mg l−1 BA. Isolated embryos gave rise to many shoot buds on MS with 0.2 and 0.3 mg l−1 TDZ. The shoot buds elongated and rooted either on MS medium with 0.01 mg l−1 BA or on half strength MS medium lacking growth regulators, which depended upon the growth regulator used in the shoot bud induction medium. Except for the embryo-derived plantlets, all other plantlets could be acclimatized with 100% success. Callus cultures were initiated from nodal segments on Murashige and Skoog 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 ha-1 BAP and 2 mg L ha-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 ha-1 BAP and 2.5 mg L ha-1 Kn. Regenerated shoots rooted best on MS medium containing IBA and Kn (1 mg L ha-1). Plantlets were transferred to pots containing sand and soil mixture, acclimatized in a culture room and finally rooted plants were transferred to soil (Siddique *et al.,* 2004). *In vitro* Culture of Italian *Withania somnifera* and composition of Withanolide was determined using HPLC by Vitali *et al.*(1996).

Direct rooting from leaf explants of *Withania somnifera* was achieved on half strength Murashige and Skoog's medium supplemented with 15 g l^{-1} sucrose, and different concentrations of growth regulators. Basal medium supplemented with 2.85 µM indole acetic acid and 9.85 µM indole butyric acid achieved maximum number of roots with 100% response. The roots were cultured on MS liquid medium for the establishment of root-organ culture with the same plant growth regulators and incubated on an orbital shaker at 80 rpm at (250 ± 02)°C. A root biomass of 6.150 ± 00.17 g was obtained after 5 weeks. When 1 g roots were inoculated to 2.5 l bubble column reactor, 47 g roots were obtained after 6 weeks. The concentration of alkaloids was increased as compared to field grown roots. The maximum concentration of withanolides (10 mg g^{-1} dry weight) was obtained in the bioreactor (Wadegaonkar, *et al.,* 2005). Abhyankar and Chinchanikar(1996), Furmanowa, *et al*. (2001), Rani *et al,* (2003) and Teli and Patil (1999) also done the great work on *Withania somnifera*.

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