Micropropagation of *Withania somnifera* through forced axillary branching and synthetic seed production from vegetative propagules

Thesis Submitted in partial fulfillment for award of the degree of Master of Science in Biotechnology

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the thesis entitled " Micropropagation of *Withania somnifera* through forced axillary branching and synthetic seed production from vegetative propagules" in partial fulfillment for the award of degree of Master of Science in Biotechnology and Environmental Sciences,Thapar University, Patiala is an authentic record of my own work done during the period of six months from January 2013 to July 2013 under the guidance of Dr. Manju Anand, Assistant Professor, Thapar University Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled, "**Micropropagation of *Withania somnifera* through forced axillary branching and synthetic seed production from vegetative propagules**" submitted by Manveer kaur in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar University Patiala, is an authentic record of her own work carried out by her during the period of six months from January 2013 to July 2013, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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CONTENTS

ABSTRACT

The present investigation was carried out on an important medicinal plant *Withania somnifera* belonging to family solanaceae. The plant is commonly known as Ashwagandha or winter cherry and is documented in Ayurveda and indigenous medical system for the treatment of a variety of diseases and disorders. The nodal segments and shoot tips were excised from an elite field grown mature plant and thereafter planted on variously supplemented *Murashige and Skoog's medium* for the induction of multiple shoots. Shoot apices were encapsulated with sodium alginate to produce artificial seeds and their germination potential was evaluated after different periods of storage at 4^{o}C.

Withania somnifera exhibited good degree of multiple shoot proliferation from nodal explants and shoot tips. Multiple shoot proliferation from nodal segments was achieved on MS medium supplemented with BAP(4.4-6.6 μM) either alone or in combination with KN(4.65μM) or IAA(8.5μM) or TDZ(0.5μM) where 8-10 shoots were obtained after 8 weeks of culturing. Best results were, however, obtained on MS+ BAP(6.6μM) + IAA(8.5μM) where 12-15 shoots were formed per node after 8-10 weeks of culturing.

Shoot apices also exhibited multiple shoot proliferation when cultured on MS medium supplemented with BAP (6.6μM-8.8μM) either alone or in supplement with IAA (8.5μM) or KN (9.3 μM) or with NAA (2.6μM) . BAP alone was less effective in inducing multiple shoots while in combination with IAA or Kinetin significantly enhanced multiple shoot proliferation. Best medium for multiple shoot proliferation from shoot tip was BAP(6.6μM) +IAA(8.5μM) where a maximum of 20-25 shoots were regenerated after 10 weeks of culturing followed by BAP(8.8)+ KN(9.3μM) where nearly 10-12 shoots were formed.

Regenerated shoots were individually isolated and rooted on a separate root inducing medium which consisted of MS medium supplemented with varying concentrations of IBA (4.9μM, 9.8μM, 19.6μM). Best rooting was, howver, achieved on 9.8μM IBA. Rooting initiated at the base of the shoot after 30 days and well developed roots were formed after 40-45 days of inoculation . The plantlets with elongated root and shoot systems were subjected to hardening and attempts are underway to establish these plantlets in the soil.

Synthetic seeds were produced in *Withania* by encapsulating shoot apices using different concentrations (2%, 2.5% and 3%) of sodium alginate followed by their dropping in 75 mM calcium chloride. 2.5% sodium alginate turned out to be best composition for gel complexation and was prepared by dissolving either in distilled water or in MS medium devoid of agar. The seeds so formed were stored in refrigerator at 4^{o}C and their viability was checked after 10, 20, 30, 40 and 50 days of storage. There was gradual reduction in the conversion rates and plantlet regeneration upon storage and the seeds prepared using sodium alginate dissolved in water were viable for a longer period than those prepared using sodium alginate dissolved in MS medium.

INTRODUCTION

Medicinal plants-An overview

Healing with medicinal plants is as old as mankind itself. Awareness of medicinal plants usage is a result of many years of struggles against illnesses due to which man learned to pursue drugs in barks, seeds, fruit bodies and other parts of the plants. The knowledge of medicinal plants has accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Chinese medicine, Unani, Siddha and the Japanese Kampo. Herbal medicine is the oldest form of health care known to man and over 9000 herbs have known medicinal application in various cultures and countries (Farnsworth and Soejarto 1991). It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Of the 2,50,000 higher plant species on earth, more than 80,000 are medicinal.(Joy *et al*.,1998). Medicinal plants synthesize and accumulate variety of phytochemicals such as vitamins (A, C, E, and K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, enzymes and minerals etc. These phytochemicals possess antioxidant activities, which can be used in the treatment of multiple ailments. Medicinal plants form the resource base for rapidly growing pharmaceutical industry with 25% of drugs derived directly from the plants and many other compounds isolated as synthetic analogues. These herbal drugs are used in pharmaceuticals, neutraceuticals, cosmetics and as food supplements. Allopathic medicine too owes a tremendous debt to medicinal plants as one in four prescriptions filled in a country like United States contain one or more ingredients derived from higher plants. (Srivastava *et al*., 1995).

Over the past few years, the medicinal plants especially those used in Ayurveda and other systems of medicine have regained a wide recognition. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant material as a source of medicines for a wide variety of human ailments. Recognition and development of the medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations. Global estimates indicate that 80% of about 4 billion population can not afford the products of the Western Pharmaceutical Industry and have to rely

upon the use of traditional medicines which are mainly derived from plant material. However, a sustained supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labour cost, selection of the superior plant stock and over exploitation by pharmaceutical industry. Most often the medicinal plants are collected from the wild. This uncontrolled harvesting of these plants has resulted in receding the population of many species in their natural habitat. Vacuum is likely to occur in the supply of raw plant materials that are used extensively by the pharmaceutical industry as well as the traditional practitioners. Consequently, cultivation of these plants is urgently needed to ensure their availability to the industry as well as to people associated with traditional system of medicine. *In situ* conservation of these resources alone cannot meet the ever increasing demand of pharmaceutical industry. It is therefore, inevitable to develop cultural practices and propagate these plants in suitable agro climatic regions.(Farnsworth 1991)

Trade of medicinal plants

World Bank reports that trade in medicinal plants, botanical drug products and raw material is growing at an annual growth rate between 5 to 15%. The World Health Organization (W.H.O.) has estimated that the present demand for medicinal plants is approximately US $14 billion per year and the demand for medicinal plants is likely to increase more than US $5 trillion in 2050. In India, the medicinal plant related trade is estimated to be approximately US $1 billion per year. In China annual herbal drugs production is worth US$48 billion with export of US$3.6 billion(Sangita kumari *et al.*,2011).The highest account (63%) of world global herbal product market is mainly in Europe and North America. In North America itself the sale of medicinal plants has climbed to about $3 billion (Glaser, 1999) whereas in European market, herbal remedies stand at US$ 7.5 billion as of 1997. As far as the herbal import is concerned, China stands first with 45% herbal import for drug preparation, followed by USA which is 15.6 %, 10.5 % for Australia, 8.1 % for Indonesia and 3.7 % for India (Samy and Gopalakrishnakone, 2007). Presently the United States is the largest market for Indian botanical products accounting for about 50% of the total exports. Japan, Hong Kong, Korea and Singapore are the major importer of the herbal drugs making 66% share of China botanical drug export(Sangita kumara *et al.*,2011).

Status of medicinal plants in India

India is a gene rich country and although its total land area is only 2.4% of the world, it accounts for 8% of the total global diversity. Being the largest repository of medicinal herbs, India is called the "botanical garden" of the world. India is among twelve most biodiverse countries of world having 16 agro climatic zones, 10 vegetative zones and 15 biotic provinces (Samy and Gopalakrishnakone, 2007). There are about 45,000 plant species with continental hotspots in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar islands. India has a very widespread, safe and traditional usage record of herbal plants recognized through various Ayurveda, Unani, Siddha, Homeopathy and Naturopathy systems of health (Vaidya and Devasagayam, 2007 and Chaturvedi, 2007). More than 70% Indians or 1.1 billion population use these herbal-based formulations regularly as spices, home remedies and health foods, as these are non-narcotic and almost without any side effects. In India around 20,000 medicinal plant species have been recorded (Dev 1997), but more than 500 traditional communities use about 800 plant species for curing different diseases (Kamboj, 2000). Exploration for forest-based plant products for new pharmaceuticals and the demand for medicinal plants has increased tremendously but surprisingly, the bulk of the traded material is still from the wild and a very small number of species are cultivated. According to the data compiled by the International Trade Centre, Geneva, India is ranked second amongst the exporting countries, after China, with an annual export of 326 000 tonnes with a value of Rs 45.95 million (about US$ 1.4 million) during 1992-95. Recent trends have indicated further increase in this trade with the herbal cosmetic industry playing a major role in fuelling the demand for herbals worldwide. In addition to the international trade, there is a substantial volume of internal trade in medicinal plants in India. One estimate has projected the turnover of the herbal industry in India to be Rs 4000 million (about US$ 88 million) for the year 2000 (Ved 1997; Ved *et al*. 2001). The expanding trade in medicinal plants has serious implications on the survival of several plant species, many of which are under threat of becoming extinct. Though India has a rich biodiversity, about 90% of the medicinal plants used by industries are collected from the wild with 70% of the plant collections involving destructive harvesting because of the use of plant parts like roots, bark, wood, stem and the whole plant. Today this rich biodiversity of medicinal plants is facing a serious threat because of the rapid loss of natural habitats and

overexploitation of plants from the wild causing a number of plants to be either threatened or included in the endangered category. The assessments done so far for the prioritized native medicinal species have resulted in assignment of IUCN red list status to nearly 250 India's medicinal plant species (Ved and Kumar, 2000).

Plant Tissue Culture And Medicinal Plants

In the search for new drugs and natural remedies, medicinal plants or herbs used in folk and traditional medicines are promising candidates and consumption of herbal medicines is on continues increase. The rising demand of plant based drugs is creating heavy pressure on some selected high valued medicinal plants in wild due to overharvesting. Several of these medicinal plant species have slow growth rates, low population densities and narrow geographical range, therefore they are more prone to extinction Most of the medicinal plants either do not produce seeds or have low germination rates thus making their cultivation difficult. Moreover the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise majority of the medicinal plants are not amenable to conventional vegetative propagation methods as they have often proved cumbersome and are more liable to be infected with systemic infections which deteriorates their quality and genetic vigor, thus limiting the multiplication of desired cultivars. Due to the shortage of high quality planting material, cultivation and domestication of medicinal plants is facing great problem. It is therefore imperative to adopt alternative methods of propagation having high multiplication rates to produce large number of plants of improved quality and shortened rotation. In this regard, *in vitro* propagation or micropropagation can be used as an effective supplementation to conventional methods of vegetative propagation with the objective of enhancing the multiplication rates. Moreover the plant multiplication can continue throughout the year irrespective of season and the stocks of germ plasm can be maintained for many years.

MICROPROPAGATION:

Micropropagation is the science of multiplying plants under controlled physical and chemical conditions. Micropropagation has number of advantages like:-

1. Higher rate of multiplication
2. Environment can be controlled or altered to meet specific needs of the plant
3. Plant available all year round (independent of regional or seasonal variation)
4. Identification and production of clones with desired characteristics
5. Production of secondary metabolites
6. New and improved genetically engineered plant can be produced
7. Conservation of threatened plant species
8. Preservation of genetic material by cryopreservation

METHODS OF MICROPROPAGATION

Micropropagation of plants can be achieved through three main techniques:-

1. **Enhanced axillary shoot proliferation:**

Micropropagation through apical and axillary shoot proliferation is the most common, reliable and applicable method for *in vitro* mass multiplication. Axillary and apical shoots contain quiescent or active meristems depending on the physiological state of the plant. When grown on culture media containing cytokinins , axillary shoots develop precociously which proliferate to form clusters of secondary and tertiary shoots. Cells of the meristems are uniformly diploid and least susceptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of the clones.

2. ***De novo*** **formation of adventitious shoots**: New adventitious shoots can develop either:

Directly from the explants like root, stem, petiole, leaf lamina and flower parts etc.

Or

Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

3. **Somatic embryogenesis:**

It involves the formation of bipolar embryos from the somatic cells that parallel the developmental path of zygotic embryos and can develop into fully functional plants under appropriate conditions.

Stages in micropropagation

Micropropagation involves 4 definite stages. These are as follows:

Stage 0: Selection of healthy disease-free elite mother plant for culture initiation.

Stage I: Initiation and establishment of aseptic cultures. (Main steps: explants isolation, surface sterilization and establishment of explants on appropriate culture medium).

Stage II: Shoot multiplication or rapid somatic embryo formation using a defined culture medium.

Stage III: Rooting of regenerated shoots

In this stage, shoots or shoot clusters from stage II are prepared to transfer to soil.Shoots are separated manually from clusters and transferred on a rooting medium containing an auxin or on a medium having low salt concentration Elongation of shoots prior to rooting, rooting of shoots (individual or clumps), and prehardening cultures to improve survival are

some of the activities carried under this stage.

Stage IV: Transfer of plantlets to natural environment through acclimatization or hardening. Hardening of plantlets imparts some tolerance to moisture stress and a shift from heterotrophic to autotrophic nutrition. During hardening, plantlets develop cuticle and their stomata start functioning. Hardened plantlets are then transferred to glass or polyhouse and finally shifted to normal environmental conditions.

SYNTHETIC SEEDS

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage. Earlier synthetic seeds were referred only to somatic embryos that were of economic use in crop production and plant delivery to the field or green house. Few years past, however, other propagules like shoot buds, shoot tips, organogenic or embryogenic calli, etc. have also been employed for the production of synthetic seeds. Artificial seeds have the potential for providing an inexpensive plant delivery system.

In some of the horticultural crops seeds propagation is not successful due to

- Heterozygosity of seeds particularly in cross pollinated crops
- Minute seed size eg; orchids
- Presence of reduced endosperm
- Some seeds require mycorrhizal fungi association for germination eg: orchids
- No seeds are formed

Application of synthetic seeds

By combining the benefits of a vegetative propagation system with the capability of long-term storage and with the clonal multiplication, synthetic seeds have many diverse applications in the field.

1) Multiplication of non-seed producing plants, ornamental hybrids or polyploids plants

2) Propagation of male or female sterile plants for hybrid seed production

3) Germplasm conservation of recalcitrant species

4) Multiplication of transgenic

RATIONALE AND OBJECTIVES

The present investigation was carried out an important medicinal plant-*Withania somnifera*. The lack of proper cultivation practices, loss of habitats and the illegal and indiscriminate collection of this plant from its natural habitat pose a serious threat to its existence in the wild. Due to overexploitation, this plant had entered in the endangered plants list. *Withania* is commonly propagated by means of seeds because of lack of natural ability for vegetative propagation but seed viability is limited to a period of one year making long duration seed storage fugile. In view of the difficulties in the propagation of this medicinal plant, the present study was conducted with the following objectives:

- To develop a reliable protocol for rapid and mass propagation of *Withania somnifera* through forced axillary branching.

- To obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions.

- Encapsulation of shoot tip propagule for constructing artificial seeds for short term conservation of germ plasm.

REVIEW OF LITERATURE

The most successful and widely used discipline of plant tissue culture technique is micrpopropagation which has contributed significantly towards the enhanced production of high quality planting material. By using this technique, better quality and disease resistant plants can be obtained without seasonal restriction. This technique is based upon the concept of totipotency as proposed by Harberlandt which means that single cell can give rise to whole plant. Micropropagation is now a well established technique commercialized globally for rapid production of a number of commercially important plants.

In order to meet the growing demands of medicinal plants, micropropagation can be effectively used for large-scale multiplication and conservation of endangered, rare and threatened plant species including *Saussaurea lappa, Picorrhiza kurroa, Ginkgo biloba, Swertia chirata, Gymnema sylvestre, Tinospora cardifolia, Salaca oblonga, Celastrus paniculata, Oroxylum indicum, Glycyrrhiza glabra, Tylophora indica, Bacopa mooniera, Rauwolfia serpentina, Withania somnifera, Aloe vera, Allium sativum, Zingiber officinale, Dioscorea deltoidea, Costus speciosus, Solanum khasianum* etc. (Chaturvedi *et al*., 2007 and Sharma *et al*., 2010).

For micropropagation of medicinal plants, all the three techniques of *in vitro* propagation viz. forced axillary branching, *de novo* adventitious shoot formation and somatic embryogenesis have been exploited.

Micropropagation through enhanced axillary branching:

Micropropagation through apical and axillary shoot proliferation is the most common, reliable and applicable method for *in vitro* mass multiplication. It is the stimulation of axillary buds,which are usually present in the axil of each leaf to develop into a shoot. A shoot tip and an axillary bud when grown under high cytokinin concentration, usually develop axillary shoots which can be subdivided into smaller clumps of shoots which in turn can develop similar clusters after subculturing on fresh medium. This process can go on indefinitely and can be maintained throughout the year and a large number of plants can be raised starting from a single shoot tip or an axillary bud. This method ensures genetic stability of the clones as cells of shoot meristem are uniformly diploid and are least susceptible to genotypic changes.

The role of cytokinins in inducing bud break is well documented and supported by many researchers. Among different cytokinins, BA and K when used either alone or in combination with lower concentration of auxins have been very effective in inducing sprouting of axillary buds in several medicinal plant species.

Amin *et al.,* (2003) reported multiple shoots from nodal segments in *Paederia foetida* on MS medium supplemented with 4.4µM BA . Likewise Chandra *et al.,* (2006) reported successful propagation of *Picrorrhiza kurroa* Royle from nodes on the same medium. Nath and Buragohin (2004) developed a protocol for multiple shoot generation from shoot tips of *Adhatoda vasica* using 8.8µM BAP likewise Lubaina *et al*., (2011) induced multiple shoot formation in *Plumbago zeylanica* an important medicinal plant using nodes as explants on the same medium. Parabia *et al.,* (2007) successfully achieved multiple shoot proliferation (6 shoots per node) from nodal explants on MS medium supplemented with BAP (17.6µM) and NAA (2.6µM) in *Leptadenia reticulate*. Karnawat *et al*., (2011) induced multiple shoots from nodal explants of *Verbesina encelioides* on MS medium supplemented with 13.2µM BAP likewise Ananthi *et al.,* (2011) reported successful multiple shoot formation from nodes (42 shoots) and shoot tip explants (37 shoots) in *Rorippa indica* on the same medium. Sahzad *et al.*,(2011) found 0.5 µM 6-benzyladenine (BA) to be most effective for shoot development of an important medicinal plant *Veronica anagallis-aquatica* L. through mature nodal segments.

Sen and Sharma(1991) achieved shoot multiplication from shoot tips of *Withania somnifera* on MS medium supplemented with BA. An efficient protocol for *in vitro* propagation of *Withania somnifera* was reported by Sivanesan and Murugesan (2008) from nodal segments using IAA(8.5µM)+ BAP(6.6µM) .The maximum no. of shoots formed were 22. Shukla *et al*.,(2010) obtained multiple shoots from nodal and shoot tip explants in *Withania* on MS medium supplemented with 1.1µM, 2.3µM and 4.65µM Kinetin. Nodal segments formed maximum number of shoots(16 shoots per explant) on MS medium supplemented with 4.65µM Kinetin. Fatima and Anis (2011) found that TDZ at a concentration of 0.5µM to be most effective in inducing multiple shoots from nodal segments forming a maximum of 23 shoots.
Kumar *et al.*,(2011) reported that KN (9.3µM) +BAP (8.8µM) was effective for shoot regeneration from shoot tips(max. no. of shoots 54) and BAP(6.6µM) and Kinetin (6.9µM) for shoot regeneration from nodal segments(Max. no. of shoots-38) in *Withania*. Tuhin and Biswajit

(2012) obtained a maximum of 21 shoots in presence of BAP(4.4µM)+KN(4.65µM) in *Withania somnifera* using nodal segments as explant.

Kanungo and Sahoo (2011) initiated shoot development from apical buds of *Withania* on Revised Tobacco medium(RT) supplemented with 4.5µM 2,4-D. Soni *et al*., (2012) reported BAP 4.4µM to be most effective for multiple shoot proliferation from axillary buds in *Withania somnifera*.The nodal segments showed 100 % bud break while shoot tips showed no response. Baba *et al*., (2013) initiated shoot proliferation from shoot tips of *Withania* on MS medium containing BA (2.2 – 8.8µM) in conjunction with (NAA 2.2-5.6 µM) while Nayak *et al*.,*(2013)* achieved multiple shoot proliferation from nodal segments of *Withania* on MS medium supplemented with 4.4µM BA.

De novo formation of adventitious shoots

Many medicinal plants have been successfully propagated in vitro by adventitious shoot initiation. Formation of shoots can be directly or indirectly through the formation of callus.

Directly from explants:. *De novo* formation of adventitious shoots through direct regeneration is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. The direct regeneration method has the advantage of omitting the callus and embryoids phases and significantly reducing the total number of stages in culture. New adventitious shoots can develop directly from the explants like leaf, stem, petiole and flower parts.

The direct method of shoot formation was reported from shoot tips of *Catharanthus roseus* (Seth and Marthur,2005), *Embelia ribes* (Raghu *et al*.,2006),, *Aegle marmelos* (Das *et al*.,2008). Efficient plant regeneration system has been established by Chuan *et al*., (2000) from stem internode explants of *Adenophora triphylla* on MS medium supplemented with 2.22-35.51 µM of BA in combination with 0.54 µM NAA.

Manjkhola *et al*.,(2005) induced organogenesis (12.2 shoots per culture) in *Arnebia euchroma*, a highly

valued, critically endangered medicinal plant of the Himalaya on MS medium supplemented with 1 µ*M* indole-3-butyric acid combined with 2.5 µ*M* 6-benzyladenine. Multiple shoots were induced from leaf and petiole explants through adventitious shoot bud regeneration in P*yrethrum* by Hedayat *et al*.,(2009). Logesh *et al.,* (2010) developed shoots from the leaf explant cultures in *Withania* on basal MS medium supplemented with IAA (3.15µM) and BAP (8.8µM) likewise Joshi and Padhya (2010) reported induction of shoot buds(max. no. of shoots-12) from the midrib on the abaxial side of leaves in *Withania* in presence of Kn and BAP (4 µM each). Udayakumar *et al*.,(2012) initiated shoots directly from epicotyl explant on 6-benzyl amino purine (BAP: 8.8µM) along with indole-3-acetic acid (IAA:12.6µM), and the maximum of 15.5 ± 0.90 shoots/explant were achieved by subsequent subcultures at 4 weeks interval in the same medium in *Withania*.

2)Indirectly through callus:Plant regeneration in this method involves the initiation of basal callus and then shoot bud differentiation. Callus is produced on explants *in vitro* as a result of wounding and in response to hormones supplied in the medium. Auxin at a moderate to high concentration is the primary hormone used to produce callus.

Callus mediated shoot organogenesis has been reported in several medicinal plants including *Gymnema sylvestre* (Gopi, 2002), *Eurycoma longifolia* (Siregar *et al*., 2003), *Saussurea obvallata* (Dhar and Joshi, 2005), *Euphorbia nivulia* (Sunandakumari *et al*., 2005) *Cassia angustifolia* (Agarwal and Sardar, 2006), *Arctium lappa* (He *et al*., 2006), *Mucuna pruriens* (Faisal *et al*., 2006), *Sarcostemma brevistigma* (Thomas and Shankar, 2009), *Cassia angustifolia* (Siddique *et al*., 2010), *Curcuma kwangsiensis* (Zhang *et al*., 2011) and *Rauwolfia serpentina* (Panwar *et al*., 2011).

Sharma and Wakhlu, (2003) achieved shoot differentiation from petiole callus of *Heracleum candicans* on MS medium containing 4.4µM BAP and 1µM NAA.

Sahai *et al*., 2010 reported high frequency plant production by shoot organogenesis from leaf callus of *T. indica* on BA (5 µM) supplemented MS medium . Kaur *et al*., 2011 reported induction of green callus from leaf and stem explants of *T. indica* on MS supplemented with NAA and kinetin which exhibited prolific shoot differentiation when transferred to (8.8 µM) BA.

Siddiqui *et al.,* (2004) induced organogenic callus from nodal segments in *Withania somnifera* on MS medium containing 4.4µM BAP and 9.3µM Kinetin. Shrivastav *et al*.,(2007) initiated callus cultures and

shoot differentiation from shoot tips of *Withania* on medium supplemented with 24D, BA and Kin alone or in combination. Madhavilatha and Singh (2008) reported callus formation from axillary leaves on MS + 2,4-D (9µM) and KN (4.65µM) in *Withania*. Udayakumar *et al*.,(2012) developed a protocol for initiation of shoots through the formation of callus from epicotyl explant on 2,4-dichlorophenoxy acetic acid (2,4-D: 9µM) along with kinetin (Kn 2.8µM), and shoots were initiated from calli on BAP (4.4µM) along with adenine sulphate in *Withania*.

Plants raised through calli may not be true elites because of various morphological, physiological and genetic variations found in callus cells, resulting in high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it. Moreover, shoot multiplication through callus phase is not applicable to many important crop species and wherever applicable, the initial plant regeneration capacity of the tissues may decline with the passage of time and is eventually lost. Still callus constitutes one of the unique materials for rapid multiplication of plants, since large number of plants can be obtained from a small tissue.

Somatic embryogenesis : Somatic embryogenesis is a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue. The somatic embryos are bipolar structures having a radical and plumule and are similar to zygotic embryos in development and can develop into fully functional plants under aseptic conditions. Since somatic embryos carry a pre-formed radical, there is no need of rooting as is required in organogenesis.
Induction of somatic embryogenesis requires a single hormonal signal while in the organogenesis two different hormonal signals are needed to induce first a shoot organ, then a root organ. Embryoid formation has been reported in tissue and organ cultures of a number of plant species derived from leaf, petiole, root, floral parts and nucellar tissue of
Chrysanthemum (Mani and Senthil, 2011), *Withania somnifera* (Sharma *et al*., 2010), *Datura stramonium* (Sundar and Jawahar, 2010), *Rauvolfia serpentina* (Singh *et al*., 2009), *Podophyllum peltatum* (Kim *et al*.,2007) *Tylophora indica* (Chandrasekhar *et al*, 2006), *Syngonium podophyllum* (Zhang *et al.*, 2005), *Ceropegia candelabrum* (Beena and Martin, 2003), *Podophyllum hexandrum* (Pandey *et al*., 2002) and in many other medicinal plants.

Pandey *et al*.,(2002) reported somatic embryogenesis in *Podophyllum hexandrum* from cotyledonary

leaves of germinated embryos on MS medium containing various concentrations and combinations of NAA (2.6-26µM), BAP (2.2-5.5µM) and GA_3(2.8µM). Embryogenic calli were obtained by Jha *et al.,(*2007) from leaf explants on MS medium supplemented with 9.3µM KN likewise Saxena *et al*., (2011) obtained embryogenic callus with 0.9µM IAA from cotyledonary leaves in *Jatropha curcus*. Sathyanarayana *et al*., (2007) reported somatic embryogenesis from stem explants of *Leptadenia reticulata* on NAA+BAP. Lee *et al*., (2009) also developed a protocol for induction somatic embryogenesis in *Cnidium officinale* from immature flower cultures on 2,4-D and BAP.

There are only a few reports regarding somatic embryogenesis in *Withania somnifera*.

Gita *et al*.,(2004) induced somatic embryos from calli obtained from axillary shoots , internodal segments (from in vitro-raised plants), and root and cotyledonary leaf segments (from in vitro-raised seedlings) of *Withania somnifera*. .Sharma *et al.,* (2010) obtained embryogenic calli from leaf explants of *Withania somnifera* on MS medium supplemented with 2, 4-D and BAP . High frequency of somatic embryo mass induction was noticed on 2, 4-D and BAP along with casein hydrolysate.

Synthesis of artificial seeds

In recent years, encapsulation technology has drawn much attention for the production of artificial seeds as it helps in minimizing the cost of micropropagated plants, ensures high volume of propagation and can be useful for the conservation of germplasm of elite and endangered plants and also facilitates exchange and distribution of germplasm across different laboratories (Rai *et al*., 2009 and Verma *et al*., 2010).

Mostly somatic embryos have been used in the encapsulation technology for the production of synthetic seeds. In recent years, the possibility of encapsulating vegetative propagules such as axillary buds, shoot tips and nodal segments as an alternative for somatic embryos has also been explored (Machii, 1992; Mandal *et al*., 2000; Chand and Singh, 2004; Rai *et al*., 2008 a, b; Singh *et al*., 2009; Verma *et al*., 2010; Singh and Chand, 2010; Singh *et al*., 2010; Kumar *et al*., 2010).

Pandey *et al*.(2002) developed synthetic seeds from somatic embryos of *Podophyllum hexandrum* using 3% (w/v) sodium alginate emulsion and 1% calcium chloride solution. , Makowezynska and Andrzejewska(2006) encapsulated shoot tips of *Plantago asiatica* to form artificial seeds and studied their regeneration potential to form complete plantlets.

Faisal *et al*., 2007 used nodal segments of *Tylophora indica* to synthesize artificial seeds. The best gel

complexing was achieved with 3% sodium alginate and 100 mM $CaCl_2.2H_2O$ and maximum frequency (91%) of conversion of these encapsulated beads into plantlets was achieved on Murashige and Skoog's (MS) medium containing 2.5 µM 6- benzyladenine (BA) and 0.5 µM α-naphthalene acetic acid (NAA) after 6 weeks of culture.

Kant *et al*., (2008) encapsulated shoot tips of *Spilanthes acmella*(L.),a medicinally important plant species using 3% sodium alginate and 100mM calcium chloride while Verma *et al*., (2010) encapsulated shoot tips of *Solanum nigrum* L. Kumar *et al*., (2010) encapsulated shoot tips excised from *in vitro* proliferated shoots derived from nodal explants of *Simmondsia chinensis* to produce artificial seeds. A gelling matrix of 3% sodium alginate and 100 mM calcium chloride was found to be most suitable for formation of ideal beads. Thiruvengadam and Praveen (2012**)** developed an efficient protocol for regeneration of encapsulated shoot tip explants of spine gourd (*Momordica dioica*).

There is only one report regarding construction of artificial seeds using vegetative propagules in *Withania somnifera*. Singh *et al*.,(2006) encapsulated shoot tips using 3% sodium alginate and 75 mM $CaCl_2.2H_2O$ and reported the maximum conversion response(87%) on MS medium supplemented with 2.4µM IBA after 5 weeks of culture.

MATERIAL AND METHODS

Choice of material

Withania somnifera (Family: Solanaceae) a popular Indian medicinal plant commonly known as ashwagandha, ginseng, and winter cherry was selected as an experimental material**.** It has been an important herb in the ayurvedic and indigenous medical system for over 3000 years. This plant is reputed to have adaptogenic, tonic, analgesic, antipyretic, anti-inflammatory and abortifacient properties.

Distribution

It is a xerophytic plant which grows wildly in all drier parts of subtropicai India i.e. in Madhya Pradesh, Uttar Pradesh, Punjab plains and North-western parts of India like Gujarat and Rajasthan. It is also found in Congo, South Africa, Egypt, Morocco, Jordan, Pakistan and Afganistan. It grows wildly in dry parts

in waste areas and on road sides.

Morphology

Withania somnifera is a small, erect, evergreen woody under shrub belongs to Solanaceae family that grows or reaches about 30-150cm in height The roots are stout, long tuberous, fleshy, whitish brown and aromatic. The roots are the main part of the plant that are widely used as therapeutic agents.The leaves are simple, alternate or sub-opposite, round-oval shaped. Flowers are inconspicuous, greenish or lubrid-yellow, in axillary, umbellate cymes. The fruit is a round orange-red berry, enclosed in green enlarged calyx. The fruit resembles that of red cherries. The seeds are many, yellow kidney shaped and discoid.

Historical Uses of *Withania Somnifera*

The leaves, berries and tubers of Ashwagandha have been in use for centuries in India as a home remedy and the extract is an important part of Indian Ayurvedic medicine. It has been used in the following ways:

- General tonic and "adaptogen", helping the body adapt to stress, especially for geriatrics; to promote strength and vigor;
- used as sedative or calming agent and for insomnia: the species Latin name "somnifera" means "soporific" "tending to cause sleep";
- Sexual vitality;
- Liver tonic;
- Anti-inflammatory agent; it is used to treat rheumatic pain and arthritis;

Coagulant: the berries are used as a substitute for rennet, to coagulate milk in cheese making.

Medicinal properties

Ahwagandha possesses antioxidant antitumor, antistress, anti-inflammatory, immunomodulatory, hematopoetic, anti-ageing, anxiolytic, antdepressive rejuvenating properties and also influences various neurotransmitter receptors in the central nervous system(Pattipati *et.al.*, 2003). Withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. Withaferin A is the most important withanolide and has good antibiotic and antitumor properties. It also has specific immunosuppressive effects on human B and T lymphocytes viz. antigen recognition and proliferative capacity of B and T lymphocytes. Antifungal and antibacterial properties have been demonstrated in the withanolides isolated from the ethanolic extract of the whole plant and leaves. In recent studies done on human breast, lung and colon cancer cell lines, plant extracts inhibited the growth of these cell lines. The researchers revealed that a specific extract from the plant, Withaferin A, was more effective in the inhibition than the common cancer chemotherapy drug, doxorubicin. The roots of the plant are categorised as rasayanas, which are reputed to promote health and longevity by augmenting defence against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and creating a sense of mental wellbeing (Prakash *et.al.*, 2002).

Chemical Constituents

The plant is chemically very complex and more than 80 compounds are known from it (Van Wyk B,Oudtshoorn BV and Gericke N(2000) . The biologically active chemical constituents are alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc), steroidal compounds, including ergostane type steroidallactones, withaferin A, withanolides Ay, withasomniferin-A, withasomidienone, withasomniferolsA-C, withsomniferin A, withasomnidienone, withasomniferols A-C, withanone etc. The constituents of Withania roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called the withanolides (Elsakka M,GrigoreseuE,Stanescu U 1990; Mishra L,Singh B and Dagenais S. 2000) ,with the main active chemical constituent Withaferin A, a phytosteroid (Lavi D,Glotter E,Shro Y. 1965). Other constituents include saponins containing an additional acyl group (sitoindoside VII and VIII), and withanoloides with a glucose at carbon 27 (sitoindoside ix and x) (Elsakka M,Grigoreseu E,Stanescu U 1990 ; Ganzera MI,Chodhary IA and Khan 2003). Apart from

these contents plant also contain chemical constituents like withaniol, acylsteryl glucosides, starch, reducing sgar, hantreacotane, ducitol, a variety of amino acid including aspartic acid, proline,tyrosine,alanine, glycine, glutamic acid, cystine, tryptophan, and high mount of iron. Much of *Withania*'s pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D.

Toxic Effects

Ashwagandha can irritate the gastrointestinal (GI) tract,so its use should be prohibited during conditions of stomach ulcer. It also might cause the immune system to become more active, and this could increase the symptoms of auto-immune diseases.The use of Withania should be avoided during pregnancy as it may cause miscarriage.

Glassware:

The glassware used for culture work comprised of 6"x1" Borosil test tubes, 100 ml, 250 ml, 500 ml, and 1000 ml corning and Borosil flasks, pipettes, and measuring cylinders (100 ml, 500 ml). Before use, glassware were brushed with alkaline detergent teepol and then washed in running water. These were then treated with hot chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4+ H_2O) followed by thorough washing with tap water. The glasswares were then inverted in a clean tray and left to dry in the oven. Plugs for the tubes and flasks were made out of absorbent surgical cotton wrapped in muslin. 5 - 10 ml water was then poured into every culture vessel which was tightly plugged. The glassware was then steam sterilized by autoclaving at 121^0 C and 1.1 kg/cm^2 for 20 minutes.

Culture Medium:

The medium formulation described as Murashige and Skoog (1962) referred as MS medium was selected as the optimal culture medium. Stock solutions of generally 8-10 times major elements, 1000 times minor elements, 100 times organic constituents were prepared. These stock solutions were stored in a freeze chest at – 4^oC and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days.

Table 1:

Composition of Murashige and Skoog's medium(1962):

INGREDIENT	AMOUNT(mg/l)
MAJOR ELEMENTS	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	490
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Na_2EDTA	37.3
$FeSO_4.7H_2O$	27.8
MINOR ELEMENTS	
H_3BO_3	6.2
$MnSO_44H_2O$	2.3
$ZnSO_47H_2O$	8.6
KI	8.3
$Na_2MoO_4.2H_2O$	0.025
$CuSO_45H_2O$	
$CoCl_2.6H_2O$	0.025
ORGANIC SUPPLEMENTS	
Glycine	2.0
Myo-inositol	100
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	20000

Agar-0.7% was used.

All the constituents except agar were mixed and then the pH of the solution was adjusted to 5.5 - 5.8. Later, agar was added and the medium was heated to boil so as to homogenize agar.

Following are some of the supplements which were used either singly or in combination for the induction of multiple shoot formation.

For nodal segments

MS+ KINETIN (4.65µM, 9.3µM, 18.6µM)
MS+ BAP (4.4µM, 8.8µM, 17.6µM)
MS+ BAP(6.6µM) +IAA (8.5µM)
MS+ TDZ(0.65µM, 0.5uM)
MS+ BAP (4.4µM) + KINETIN(4.65µM)

For shoot tips

MS+ KINETIN (4.65µM, 9.3µM, 18.6µM)
MS+ BAP (4.4µM, 8.8µM, 17.6µM)
MS+ BAP (6.6µM) + IAA (8.5µM)
MS+ BAP(8.8µM) + KINETIN (9.3µM)
MS + NAA(2.6µM) + BAP(8.8µM)

Sterilization of inoculum

The explants were washed under running tap water for 30 minutes.They were then treated with detergent Teepol for 10 minutes.The nodal segments were treated with bavistin for 5 minutes and shoot tips were immersed for 4 minutes. The explants were then treated with 0.1% mercuric chloride.(4 minutes for nodal segments and 2 min. for shoot tips) in the laminar hood.The explants were then thoroughly washed (4 - 5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. Fresh cuts were given to the explants after sterilization to remove undesirable or dead portions. The explants were then planted on variously augmented MS medium.

Inoculation

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a bactericidal U. V. tube (15 W, peak emission 2637 A^o). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, scalpels, blade etc.), gas burner, lighter, tube containing absolute alcohol etc were also cleaned with alcohol. The fresh material to be inoculated was kept in a Petri dish covered with a piece of black paper in order to protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with U.V. rays continuously on for one hour.

Cultural conditions:

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4^{o}C. All the inoculated cultures were incubated in growth room in controlled conditions at a temperature of 25.0 ± 2^{o}C with a photoperiod of 12 hours per day. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) at 50 μmol/m^2/s^1.

PRODUCTION OF ARTIFICIAL SEEDS

For the production of artificial seeds, shoot tips of *Withania somnifera* after proper sterilization were subjected to encapsulation with sodium alginate by dropping method (Redenbaugh *et al*.,1987)**.** In this method the Na alginate is released drop-by-drop (each drop containing a single explant) into a sterile solution of the complexing agent (a di or tri-valent metal salt, such as calcium chloride or calcium nitrate.

Alginate is by far the most used gelling agent for synthetic seed preparation. The main advantages of this compound are: the excellent water solubility and moderate viscosity of Na-alginate at room temperature, its easy availability at low cost, the long-term storability of the Na-alginate solution, the easy use of calcium salts for quick gellation and bead hardening at room temperature, the possibility to prepare synthetic seeds of different hardness by changing the concentration of Na-alginate and or the duration of the ion-exchange reaction, the absence of any kind of toxicity of the Ca alginate matrix for explants, the possibility to mix the alginate with a nutritive medium to obtain an artificial

endosperm(Lambardi *et al.*,2006*)* .

Procedure

1) Sodium alginate (in the range of 1 to 3% w/v) was prepared by two different methods.In the first method it was prepared by dissolving in sterile distilled water and in the second case it was prepared by dissolving in full strength MS medium devoid of sucrose.
2) Explants mixed with sodium alginate were dropped into pre-chilled calcium chloride(75 mM) solution and were kept as such for 20-30 minutes for polymerization.
3) Shoot tips were completely gelled by calcium alginate and after hardening, these beads were recovered by decanting the calcium chloride solution and washing them in sterile water 3 to 4 times.
4) The alginate beads about (5mm in diameter) were collected on a sterile filter paper in a Petri dish sealed with parafilm. These were then stored in refrigerator at 4^{o}C.
5) In order to retrieve plantlets from stored encapsulated shoot tips, they were cultured on growth regulator free agar solidified full strength MS medium after an interval of 10 days.

RESULTS AND OBSERVATIONS

Nodal explant culture

Fresh nodal segments were collected from healthy , field grown plant of *Withania somnifera*. Atleast 1 cm node having one dormant lateral bud was excised and then surface sterilized with 0.1% mercuric chloride. Damaged internodal tissue on both sides of the sterilized segment was cut off. The nodal segment 3-4 mm in size was then cultured on MS medium supplemented with various growth regulators.

The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used. Multiple shoot formation was induced from nodal explants on MS medium supplemented with BAP either alone or in conjunction with other cytokinins i.e. KN or TDZ or in combination with the auxin IAA. However, maximum shoot proliferation occured on BAP(6.6µM)) and IAA(8.5µM) supplemented medium. On this medium, initial bud break was noticed after 7 days (Fig.2) and multiple shoot initiation occurred leading to the formation of 4-5 shoots after 2-3 weeks of culture(Fig. 3). Shoots multiplied further leading to the formation of 12-15 shoots from a single nodal explant after 6-8 weeks of culturing (Figs.4&5). Subculturing was done after every 8 weeks and the number of shoots increased manifold thereafter (Fig. 6).

Axillary bud break was also observed when 4.4 µM BAP was used in conjunction with 4.65 µM KN(Fig.7) leading to the formation of a few shoot initials after 10-12 days(Fig.8). These shoot initials sprouted into green leafy shoots later on(Fig.9). On subsequent subculturing on the fresh medium, the shoot proliferation continued leading to the formation of many shoots (Fig 10). BAP (6.6 µM) when used in conjunction with TDZ (0.5µM) also promoted the formation of 4-5 shoots from the axillary position. Response of various growth regulators on shoot proliferation from nodal segment is depicted in table 2. and Fig. 11.

Table 2:

Serial number	PLANT GROWTH REGULATOR	NO. OF SHOOTS PER EXPLANT
1	MS+ BAP(4.4µM)	4
2	MS+ BAP(8.8µM)	2
3	MS+ BAP(17.6µM)	2
4	MS+ KN(4.65µM)	2
5	MS+ KN(9.3µM)	1
6	MS+KN(18.6µM)	1
7	MS+ BAP (4.4µM) + KN(4.65µM)	8-10
8	MS+ BAP(6.6µM) + IAA(8.5µM)	12- 15
9	MS+ BAP(6.6µM) + TDZ(0.5µM)	4-5

Shoot tip culture

Young healthy shoot apices were collected from field grown mature plant and cultured on MS medium supplemented with different concentrations of BAP (4.4 – 17.6 µM) used either alone or in combination with KN(4.65-18.6µM) Or IAA (µM) or NAA (2.6µM). Shoot apices when cultured on MS medium supplemented with BAP (8.8µM) initiated multiple shoots (Fig.12) forming 8-9 shoots after 8 weeks of culture (Fig.13) . After subculturing on the same medium, clusters of shoot initials were formed (Fig.14) which developed into green leafy shoots subsequently (Fig.15). A change in the concentration level of BAP above and below the optimum value (8.8 µM) resulted in decline in the number of shoots formed.

Synergistic effect of BAP (6.6µM) with IAA(8.5µM) resulted in highest shoot proliferation forming 20-25 shoots after subculturing in 75% of cultures. Shoot bud break occurred after 7-8 days (Fig.16) leading to the formation of many shoots after 5 weeks (Fig.17). Figures 18 and19 depict numerous well developed shoots and clusters of shoot initials formed on further subculturing after 5 and 10 weeks respectively.

MS medium supplemented with 8.8 µM BAP in conjunction with KN (9.3 µM) was also effective in inducing bud break and forming multiple shoots at the rate of 10-12 shoots per explant in 60% of cultures (Fig.21). The shoots multiplied prolifically when transferred to the fresh medium (Fig. 22). Multiple shoot formation was also observed on MS medium supplemented with BAP (8.8 µM) and NAA (2.6µM) where 4-5 shoots were regenerated from single shoot apex after 5 weeks of culturing. Fig. 23 and Table 3 shows effects of different plant growth regulators on multiple shoot proliferation from shoot tip explants.

TABLE 3:

SERIAL NO.	PLANT GROWTH REGULATOR	NO. OF SHOOTS
1	MS + BAP (4.4µM)	2
2	MS + BAP (8.8µM)	8- 9
3	MS + BAP (17.6µM)	2
4	MS + KN (4.65µM)	2
5	MS + KN (9.3µM)	1
6	MS + KN (18.6µM)	1
7	MS+ BAP(6.6µM) + IAA(8.5µM)	20-25
8	MS + BAP(8.8)+KN(9.3µM)	10- 12
9	MS + NAA(2.6µM)+BAP(8.8µM)	4

Rooting of regenerated shoots

Regenerated shoots were carefully rescued from the culture bottles and test tubes in laminar airflow on a sterile plate. Each of the shoots was carefully inoculated upright in the MS medium supplemented with different combinations of IBA and NAA for root initiation (Fig. 24). Induction of roots occurred after 4 weeks on MS medium containing 9.8µM IBA (Fig. 25).The roots elongated further (Fig. 26) leading to the formation of well developed roots measuring 6-7 cm in length after 40-45 days (Fig. 27). The roots were long, white and devoid of root hairs. A complete plantlet with shoot and root systems is depicted in Fig. 28.

Acclimatization of plantlets

The rooted plantlets were successfully transferred to the field conditions through successive hardening stages. The rooted plantlets were gently removed from the cultures tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical injury to the plantlets. Roots were thoroughly washed with tap water to remove any remaining agar sticking to them. Plantlets were then transferred to plastic pots containing sterile vermiculite (A mixture of soil and vermicompost 1:1), then covered with plastic bags having holes and kept under the culture room condition for 15 days (Figs. 29 & 30). The plants were thoroughly watered. The plantlets were then shifted to polybags containing same potting mixture and was kept in the growth room for another 15 days (Fig. 31).The plants with newly formed leaves were shifted to green house and attempts are underway to establish these plantlets in the natural environment.

Artificial seeds

In the present investigation, seeds were constructed by encapsulating shoot tips in different concentrations of sodium alginate (2%, 2.5% and 3%.) and calcium chloride (75 mM) as the gel matrix. Sodium alginate at 2.5% concentration was found to be the most appropriate for encapsulation resulting in the formation of clear, transparent isodiametric beads approximate 5 mm in diameter (Fig. 32). Figure 33 shows the magnified view of shoot- tip encapsulated beads. The seeds prepared by using 2% sodium alginate were soft and easily diffusible while the seeds prepared using 3% sodium alginate were hard and took longer time to dissolve and germinate. Sodium alginate was prepared by two methods. One by dissolving in distilled water and the other by dissolving in MS medium devoid of sucrose. Encapsulation was done by dropping method. The alginate beads of

5 mm in diameter were then collected on a sterile filter paper in a petridish and sealed with parafilm. The seeds were then stored in refrigerator at 4^{o}C.

For retrieving plantlets, the encapsulated shoot tips were cultured on basal MS medium with 2% sucrose. The alginate coat started dissolving after 9-10 days followed by sprouting of shoot apex (Fig. 34), which elongated further (Fig. 35) and formation of shoots was observed within 18 days (Fig. 36).

The frequency of conversion of these synthetic seeds into plantlets was evaluated after different storage periods of 0, 20, 30, 40 and 50 days respectively. It was observed that there was a gradual reduction in the conversion rates and shoot regeneration thereafter. Shoot tips encapsulated seeds showed 100 % germination after 10 days . The percentage germination of seeds prepared by dissolving sodium alginate in water decreased gradually with 33% germination(table 4) while those prepared by dissolving sodium alginate in MS medium devoid of sucrose showed 25% germination after 50 days(table 5). The percentage response for conversion of these synthetic seeds stored for different periods is shown in Figure 37 .

NODAL EXPLANT CULTURE

Fig. 1 Inoculation of nodal segment on MS+IAA(8.5µM)+BAP(6.6µM)

Fig. 2 Shoot bud break after 7 days on the same medium

Fig. 3 Shoot initiation on MS+ IAA(8.5µM)+BAP(6.6µM) after 2-3 weeks of culture

Fig. 4 Numerous multiple shoots formed after 6 weeks on MS+BAP+IAA


1


2


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Fig. 5 Formation of multiple shoot cluster after 8-weeks of culture on the same medium.

Fig. 6 Multiple shoots formed on MS+ IAA(8.5μM)+BAP(6.6μM) after subculturing.

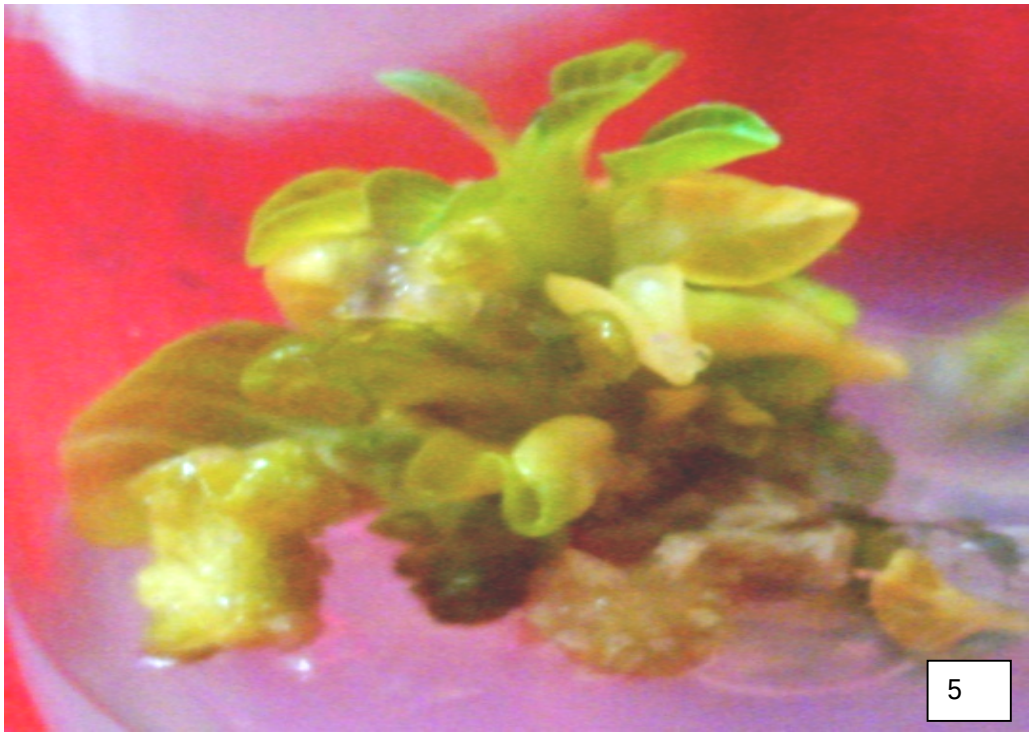
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Fig. 7 Shoot bud break on MS+ KN (4.4μM) +BAP (4.65μM) after 7 days.

Fig. 8 Shoot initiation on MS+ KIN (4.4μM) +BAP (4.65μM) after 10 days.

Fig. 9 Group of shoot initials formed on same medium after 3 weeks.

Fig. 10 Formation of mutliple shoots after fresh culturing on the same medium.


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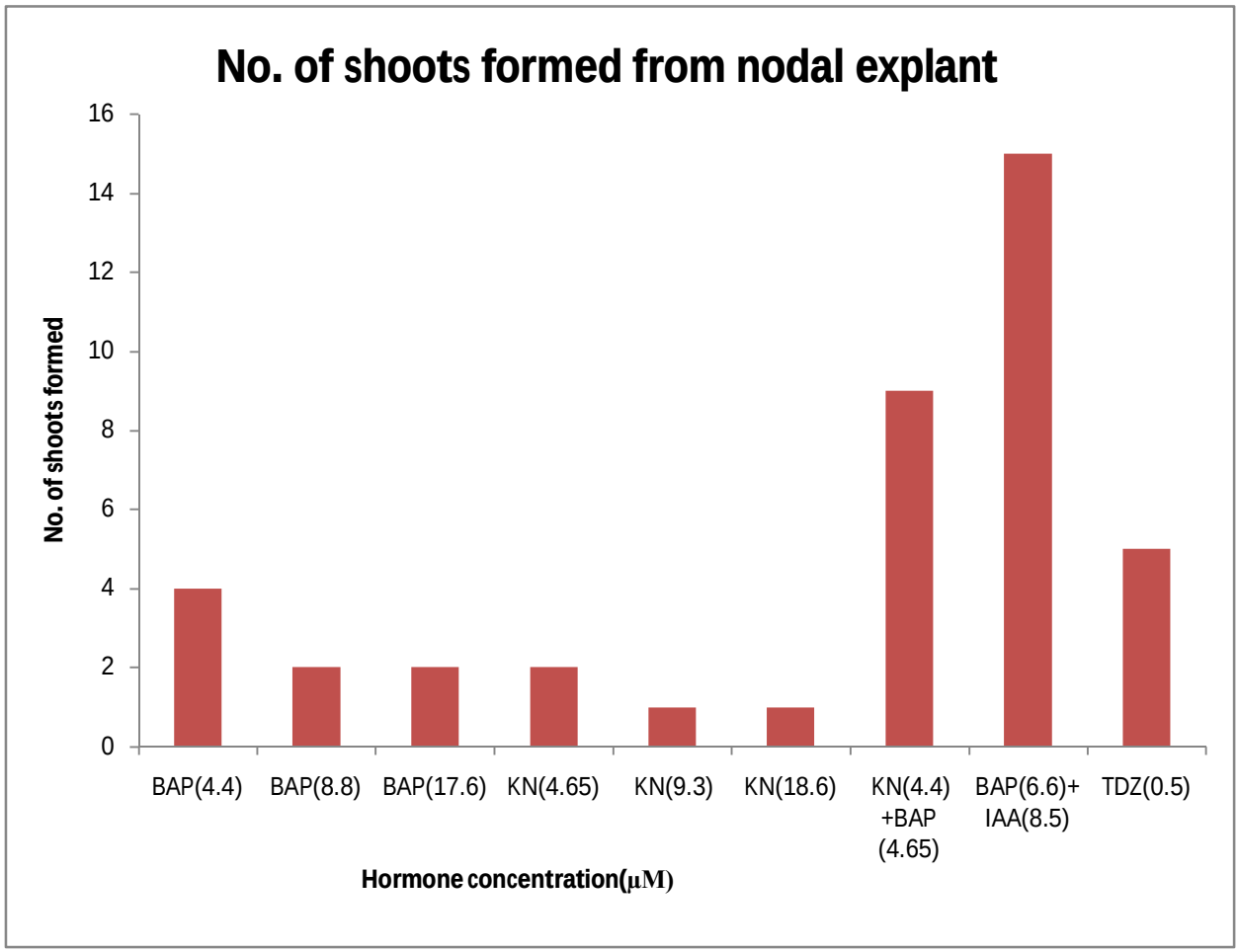

Fig. 11

Histogram depicting the response of various growth regulators on shoot proliferation from nodal segment.

Shoot tip culture

Fig. 12 Formation of multiple shoots after 4 weeks of inoculation on MS+ BAP (8.8µM).

Fig. 13 Cluster of few shoots initiating on same medium after 8 weeks of culturing.

Fig. 14 Initiation of multiple shoots after subculturing on MS+ BAP (8.8µM).

Fig. 15 Multiple shoots proliferated from shoot tips on MS+ BAP (8.8µM).


12


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15

Fig.16 Initial bud break after 7 days on MS+ BAP(6.6µM) + IAA(8.5µM).

Fig. 17 Initiation of multiple shoot buds on MS+ BAP(6.6µM) + IAA(8.5µM).

Fig. 18 Group of shoot initials formed after 5 weeks of subculturing.

Fig. 19 A 10 weeks old culture showing formation of multiple shoots from the shoot initials.


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Fig. 20 Axillary bud break on KN (9.3μM) +BAP (8.8μM).

Fig. 21 Formation of multiple microshoots (10-12) after on the same medium.

Fig. 22 Formation of numerous shoots on the same medium after subculturing on same medium.


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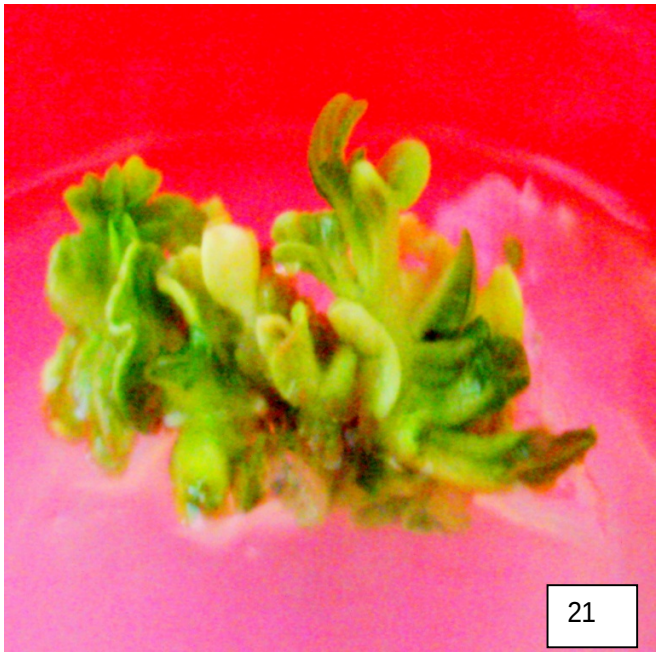
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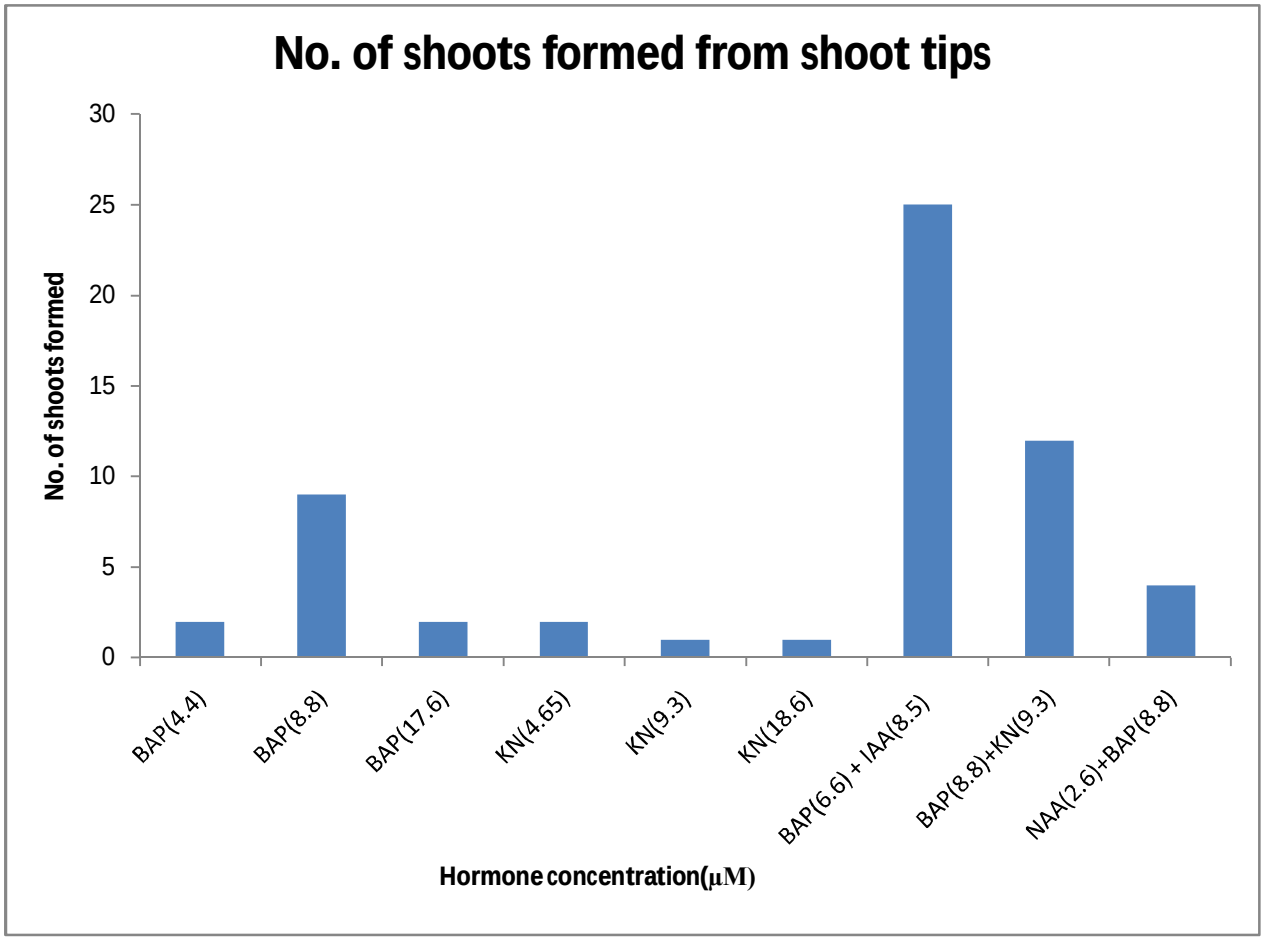

Fig. 23

Histogram depicting the effect of various plant growth regulators on shoot proliferation from shoot tips.

Fig. 24 Inoculation of shoot on rooting medium(MS + 9.8 μM IBA).

Fig .25 Initiation of roots from basal end of shoot after 4 weeks.

Fig .26 Further elongation of roots on the same medium.

Fig .27 Fully developed roots formed after 40-45 days.

Fig.28 A complete plantlet with well developed root and shoot system.


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Fig. 29 Plants transferred to plastic cups containing soil and vermicompost(1:1)under controlled environmental conditions.

Fig. 30 Plants covered with polyethylene bag.

Fig. 31 Plant transferred to plastic bag and kept under the culture room conditions.

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Fig. 32 Synthetic seed formation by encapsulating shoot tips with 2.5% sodium alginate and 75mM $CaCl_{2..}$

Fig. 33 Magnified image of synthetic seeds.

Fig. 34 Germination of synthetic seeds on basal MS after 8 days.

Fig. 35 Sprouting of shoots after dissolution of alginate coat.

Fig. 36 Further elongation and growth of shoot.


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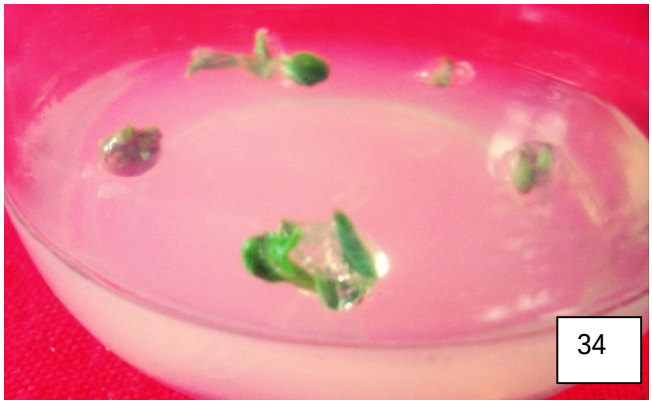
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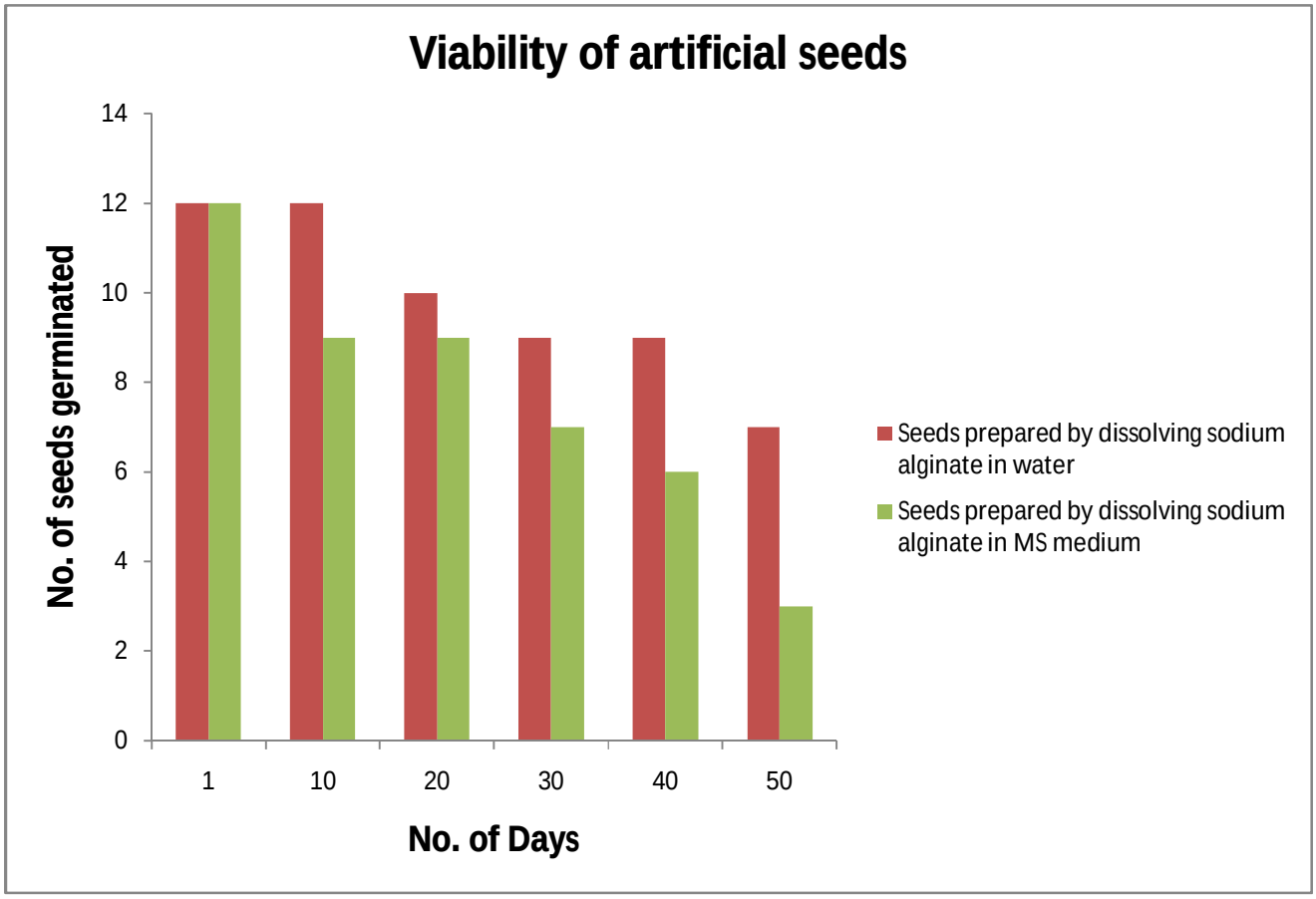

Fig. 37

Histogram showing the effect of storage on the viability of seeds.

DISCUSSION

The present investigation was carried out on an important medicinal plant *Withania somnifera* with the aim to develop an efficient, reliable and reproducible protocol for its clonal propagation through forced axillary branching under *in vitro* conditions.

Withania is conventionally propagated by seeds but due to low percentage germination, conventional method cannot meet the increasing demand of this plant used as raw material for preparation of pharmaceutical products. Over exploitation of this plant from wild creates a need to develop an efficient protocol for micropropagation of *Withania somnifera* to meet the demand of its commercial cultivation.

Micropropagation through enhanced axillary branching is the most common and reliable technique for mass propagation since it ensures genetic stability of clones. For shoot proliferation, cytokinins are one of the most important factors affecting the response (Lane 1979 and Bhojwani 1980). Cytokininins used in plant cell culture regulate cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation and stimulate protein and enzyme activity (Gross and Parthier, 1994). A wide range of cytokinins like kinetin, BAP, 2-iP, and zeatin have been employed for shoot proliferation (Bhojwani and Razdan 1983). However a wider survey of literature suggested that BAP is the most reliable and effective cytokinin for shoot proliferation. A number of plants such as *Stevia rebaudiana* (Debnath 2008*), Gynura procumbens* (Keng *et al.*,2009) and *Rorippa indica* (Ananthi *et al.*, 2011) have been successfully multiplied using BAP.

In the present investigation, axillary shoots were induced from the nodal segments and shoot apices on MS medium supplemented with different concentrations of BAP (4.4-8.8µM) alone or in combination with KIN (9.3µM) or IAA(8.5µM). For nodal explants best results were obtained on MS+ BAP(6.6µM) + IAA(8.5µM) where 15 shoots were formed after 8 weeks of culturing while on MS+ KIN(4.65µM) +BAP (4.4µM) the maximum number of shoots formed were 10. Similar results were also reported by other workers in *Phyllanthus caroliniensis* (Catapan *et al.*, 2000), *Phyllanthus amarus* (Bhattacharya and bhattacharya, 2001), *Coleus forskohlii* (Dube *et al.*, 2011).

Multiple shoot proliferation from shoot tips was achieved on MS+BAP (6.6µM) +IAA (8.5µM) where a maximum of 25 shoots were regenerated after 10 weeks of culturing whereas 12 shoots were formed on media supplemented with BAP(8.8)+KN(9.3µM). Our results are in agreement with other workers who

have reported the synergistic effect of BAP with IAA or BAP with KN for multiple shoot proliferation from shoot tips and nodal segments in *Withania somnifera*(Sivanesan 2006;Sivanesan and Murugesan., 2008; Aniel *et al.*, 2011; Kumar *et al.*,2011; Tuhin and Biswajit.,2012;) Aniel *et al.*,(2011) reported a maximum of 38 shoots per nodal segment when cultured on MS medium fortified with BAP(6.6µM) and IAA(8.5µM) in *Withania somnifera*. Similarly Govindaaraju *et al.*,(2003) Kulkarni *et al.*,*(*2000) and Rani and Grover(1999) found that BAP and IAA are the best growth regulators for multiple shoot induction in *Withania* tissue culture.

Initiation and development of roots from the basal end of regenerated shoots is an important and indispensable step to establish tissue culture derived plantlets to the soil. Auxins play a provital role in inducing roots at the base of microshoots, the importance of auxins such as IBA, IAA or NAA for rooting has already been documented. In the present investigation , well developed roots were formed on full strength MS medium supplemented with 9.8µM IBA after 40-45 days of inoculation. Role of IBA in root induction has been demonstrated in *Withania somnifera* by a number of other workers such as Sivanesan(2007); Sivanesan and Murugesan(2008); Tuhin and Biswajit.,(2012); Kumar *et al.*,(2011); Fatima and Anis(2011) Joshi and Padhya(2010). Nayak *et al*,.(2013) found that half strength MS medium containing 4.9µM IBA induced rooting in 95% of the shoots within 10-15 days in *Withania* while Sivanesan (2007) reported half strength MS media supplemented with 9.8µM IBA to be the best medium for rooting in *Withania*.

Encapsulation of vegetative propagules offers an efficient and cost effective system for clonal propagation of plant species (Singh *et al.*, 2006).In addition these encapsulated propagules can also be used for conservation of germplasm and exchange of axenic plant material.(Hasan and Takagi 1995).

Artificial seeds have been produced by encapsulating different cell types in a number of plants (Redenbaugh *et al*., 1986). Verma *et al*.,(2010) found that in *Solanum nigrum* synthetic seed developed from shoot tips yield good response than other non-embryogenic vegetative propagules. The response may be related to mitotic activity being greater in the meristem of the shoot tips than in lateral buds, which are subjected to apical dominance. Encapsulation of shoot tips in sodium alginate and calcium chloride has also been reported in a wide range of plant species such as *Camellia japonica* (Ballester *et al*.,1997) Apple rootstock(Sicurani *et al.*, 2001; *Withania somnifera* (Singh *et al.*,2006) and *Psidium guajava* (Rai *et al*.,2008).

During the present investigation synthetic seeds were prepared from shoot tips in *Withania somnifera* using 75 mM calcium chloride and 2.5% sodium alginate which was the best gel complex. Lower concentrations (1%, 2%) of sodium alginate resulted in the formation of fragile beads and higher concentration (3%) favored the formation of hard beads which had a marked effect on the germination or conversion process later on. Singh *et al.,(*2009) reported that 3% sodium alginate and 100 mM calcium chloride was best composition for shoot tip encapsulation in *Spilanthes acmella*. Likewise Chung *et al*., (2012) reported that lower concentrations of sodium alginate 1-2% (w/v) and $CaCl_2$.$2H_2O$ (50 mM) in *Momordica dioica* resulted in beads without a defined shape and were too soft to handle, whereas at higher concentrations of sodium alginate (4-5%) or $CaCl_2.2H_2O$ (150 mM), the beads were isodiametric but were hard enough to cause considerable delay in shoot emergence.

Verma *et al*.,(2010) prepared synthetic seeds using shoot tips in *Solanum nigrum* by encapsulating them with 3% sodium alginate prepared in MS medium devoid of sucrose or in double distilled water. Sodium alginate combined with full strength MS medium demonstrated significant superiority over the double distilled water with respect to shoot growth. Synthetic seeds with endosperm (alginate gel containing MS medium) showed promoting response on shoot development and conversion in *Phyllanthus amarus* (Singh *et al.,* 2006). In our investigation, however, synthetic seeds prepared by dissolving sodium alginate in water gave better results giving 33% conversion efficiency after 50 days whereas seeds constructed in sodium alginate dissolved in MS medium gave lower conversion efficiency of 25% after 50 days.

There is only a single report regarding preparation of synthetic seeds from shoot tips in *Withania* by Singh *et al*.,(2006). Synthetic seeds were prepared by encapsulating shoot tips of *Withania somnifera* using 3% sodium alginate and 75mM calcium chloride .The maximum percentage response (87%) for conversion of encapsulated shoot tips into plantlets was achieved on MS medium supplemented with 2.4µM IBA after 5 weeks of culture.

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