Available online at www.elixirpublishers.com (Elixir International Journal)

Applied Botany

Elixir Appl. Botany 45 (2012) 7850-7852

ISSN: 2229-712X

In vitro Regeneration of Plantlets via Shoot tip Culture in *Withania somnifera* L

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ARTICLE INFO

Article history:
Received: 6 February 2012;
Received in revised form:
25 March 2012;
Accepted: 10 April 2012;

Keywords
Micropropagation,
Shoot tip,
Withania somnifera ,
BAP,
NAA.

ABSTRACT
A protocol was developed for high frequency plantlet regeneration from shoot tip explants of *Withania somnifera* on MS media supplemented with BAP and Kn at different concentrations. These *in vitro* developed shoots were separated and rooted on half strength of MS medium supplemented with IBA. Complete plantlets were hardened and acclilimatized in field condition. The regeneration protocol developed in this study provides a basis for germplasm conservation and for further investigation of bioactive constituents of this medicinal plant.

Introduction

Techniques of micropropagation or *in vitro* cultivation have emerged as alternatives for species that do not have the property of producing viable seeds, that is, species that cannot germinate and develop adequately in their natural environment (González et al. 2004). Micropropagation still remains the most pragmatic and useful aspect of plant tissue culture but there are some other facts too, which are of immense practical value. This area has tremendous potential in solving global problems of medicine, food, fibre, and fuel in developing countries, it has emerged as an invaluable aid for production of clones and development of high quality plantation, economical morphological and medicinally important crop (Krishna and Singh, 2007). *In vitro* production which also concerns prominent plant species is widespread in developing countries. For orchids, *in vitro* clonal propagation is the only commercially viable method of micropropagation. Clonal multiplication of the cultivar is very important in horticulture and silviculture.

Withania somnifera L. (Dunal) belongs to family Solanaceae and is classically known for its rejuvenate benefits. It has recently been referred to as Indian ginseng for its reputed restorative benefits. The wild plant is generally an erect branching shrub, grows approximately up to a height of one meter. The plant is used for the treatment of tuberculosis, rheumatism, inflammatory conditions, and a potential antitumor agent (Suffness and Dourous 1982; Chopra 2006). The plant contains tropane alkaloids such as tropine hygrine anferine and a number of steroidal lactones known as Withanolides. The various withanolides, withaferin A and its 5-hydroxy-6-chloro derivatives have been reported to exhibit marked cytostatic activity against cell derived from human carcinoma, experimental mouse tumours and Hela 229 cells *in vitro* (Uma Devi et al. 1992). Recently *W. somnifera* L. was also used to inhibit the development of tolerance and dependence on chronic use of various phytotropic drugs (Gupta and Rana 2007). The tribal especially *Bheel* and *Garasodia* give root powder orally to the male patients of asthma and bronchitis (Singh and Pandey 1998). Recently *W. somnifera* L. was also used to inhibit the development of tolerance and dependence on chronic use of various phytotropic drugs. There are some reports of *in vitro* regeneration using shoot tip culture (Anand *et al*, 2011; Ananthi *et al*, 2011)

Materials and Methods

Shoot tips were sterilized and inoculated on MS medium strength medium using various concentrations of phytohormones (auxins, cytokinin). In (*Withania somnifera* L. cotyledons, epicotyls, hypocotyls were excised from *in vivo* grown seedling. Soot tips were used as explants.

Two days prior to the inoculation of explants, the mother explants were sprayed with 1% Bavistin solution (a fungicide). Small young branches having about 8-10 nodes were cut and immersed in 1% Bavistin solution for 20 minutes followed by three rinses with tap water. Further sterilization was carried out under a laminar air flow cabinet. Explants were treated with 0.1% mercuric chloride ($HgCl_2$) solution for 3-5 minutes followed by 3 rinses with autoclaved distilled water.

Hardening and acclimatization

The well developed plantlets was transferred to pots containing vermiculite and sterilized soil (1:3) under fluorescent light (3000-4000 lux, 16/8 hour photoperiod) condition in growth chamber, where temperature was maintained at 25±2ºC for about 3-4 weeks. Initially they were covered with inverted glass beakers for retention of humidity (50 ± 5ºC) and watered every day with half strength of MS medium for 3 weeks [Fig.A-F]

A-D

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D-F

Fig. 1

A- Initial stages of shoot development
B- Elongation and maturation of shoots
C- Induction of rooting
D- Root induction from shoots
E- Hardening
F- Acclimization

Results

Effect of Basal media

For multiplication and shoot proliferation different types of nutrient media. viz. MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al*.,1968), were tried to explore their regeneration potential . MS media gave the best result for multiple shoot proliferation through nodal stem segment (Table 1)

Effect of sucrose concentration

Sucrose satisfies the carbohydrate requirement of most tissue cultures. Optimal level of sucrose required for maximum shoot production was determined. However, various carbohydrates viz., sucrose, glucose and maltose were tried in different concentrations. Sucrose (3%) was found to be most favourable (Table 2)

Effect of cytokinins

During the present research work experiments were conducted to study the effect of various concentrations of cytokinin alone or/in combination (BAP/Kn) on the preconditioned explant to induce multiple shoot production in *Withania somnifera* L. with nodal segment as a explant (Table 3)

Effect of 6-benzyl amino purine (BAP)

During the experiment when 0.5-8 mg/l concentrations of BAP were tried on *Withania somnifera* L the results proved that BAP (3.0 mg/l) elicited 3-4 shoots per explant within 30-35 days of inoculation. The data pertaining to response of the explant following a one month inoculation period are presented. However, the number of cultures showing regeneration of explant into shoots varied with the concentration of BAP. The best results were obtained with 3.0 mg/l of BAP in the medium with production of higher number of shoots per explant

Effects of Kn

In order to study the effect of kinetin on multiple shoot formation MS-medium containing Kn (0.5 to 8.0 mg/l) alone was used.Lower concentrations of Kn proved to be effective in both medicinal plant species for shoot proliferation. However no further growth of the shoot was obtained (1-2 shoots per explant) in this concentration of Kn (1 mg/l) in case of *Withania sonmifera* L.

Effect of BAP in combination with Kn

The optimal concentration of BAP (1.0 mg/l) + Kn (1.0 mg/l) and activated charcoal in combination were incorporated in the MS media to study the response for multiple shoot formation in *W. somnifera* L. The results presented in the table showed that the maximum number of shoot produced was 13. This indicated the higher concentration of BAP and Kn reduced the number of multiple shoot.

Different auxins viz. IAA/IBA/NAA and 2,4-D in combination with BAP (1.0 mg/l) were tried to multiple shoot proliferation in *W. somnifera* L. In the present study maximum multiple shoot (13) was observed on MS medium supplemented BAP (1.0 mg/l), IBA (0.25 mg/l) and activated charcoal (Table 4)

Root induction in vitro condition

During the work *in vitro*, shoot regeneration of *Withania somnifera* L. was observed through axillary and apical meristem. The shoot was transferred on rooting media with auxins like IAA/IBA/NAA/2,4-D at concentrations of 5 - 8.0 mg/l. In *Withania somnifera* L. best root induction was achieved in ½ strength MS-media supplemented with IBA (1.0 mg/l) (Table 5)

Discussion

Micropropagation is a combination of the arts and science of plant multiplication *in vitro* and plant acclimatization. It is propagation of a genotype that comprises many steps with stock plant care, explants selection and sterilization, media manipulation to obtain proliferation, rooting, acclimatization and usually associated with commercial production. The purpose of micropropagation is to produce carbon copies of original unique parts or more simply put, to grow clones in quantity. The micropropagation process is the culmination of all the inventions, theories and discoveries, man has made regarding the Anatomy and physiology of the living world. About 150 plants have been commercially micropropagated many of these have reached the limits of their improvement by traditional methods. The emphasis on sustainable agriculture, increasing world population and the loss of prime land to housing and industry make this method of propagation indispensable (Sen *et al.*, 2004)

During present experiments, multiple shoots were obtained from axillary buds in *W. somnifera* Axillary bud proliferation proved to be simple and reliable method for rapid production of desired clones of medicinal important plants

Multiple shoot formation through auxillary buds

BAP and Kn in combination gave the positive results in shoot multiplication with 1.0 mg/l Kn in combination with BAP (1.0 mg/l) and adenine sulphate (10 mg/l).

W. Somnifera gave 12-20 shoots from nodal explant when cultured on MS medium with BAP, Kn 1.0 mg/l each. This combination gave the best result on multiplication of auxillary bud.

Constituents of culture media plays important role for successful plant regeneration (Murashige & Skoog, 1962).Optimum results were obtained in the plant species with full strength MS medium for shoot multiplication and half strength of MS salts for root induction in *W. somnifera* L.).

Carbohydrate like glucose, sucrose and fructose were used on energy source. 3% of sucrose was found to be most suitable for shoot multiplication during the study. Results were in accordance with (Shekhawat *et al.,* 2005, Gentry and Emmons, 2004).

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