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Original Article

In vitro Plant Regeneration from Mature Seed Explants of *Withania somnifera* (L.) Dunal, an Important, Rare and Endangered Medicinal Plant

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Abstract

Withania somnifera (L.) Dunal a member of the Solanaceae family, is a traditional medicinal plant commonly known in India as Ashwagandha. It is used for different diseases such as hiccup, cough, rheumatism, tuberculosis, and exhibits excellent antitumor and anti-bacterial activities as well. Direct organogenesis of plants using mature seeds provides faster response and is also a time saving approach, thus the present study was conducted to investigate the optimal concentrations and combinations of plant growth regulators with MS medium for the establishment of an efficient regeneration system in *W. somnifera* using mature seed as an explant. Therefore, an efficient *in vitro* protocol for high frequency regeneration has been developed using mature seeds as explant. In the present study, the multiple shoots along with embryogenic callus induction was best seen in MS medium supplemented with BAP (1.5 mg/L) and IAA (0.5 mg/L). Furthermore, MS medium fortified with GA_3 (0.3 mg/L) and IBA (3.0 mg/L) alone was suited for shoot elongation and rhizogenesis respectively. The rooted plantlets were hardened and successfully established in the soil. The establishment of a highly reproducible regeneration system would greatly influence the efforts of improvement of the hereby studied medicinal plant species through useful gene transfer technology.

Keywords: BAP; GA_3; IAA; IBA; mature seed; Medharasayana

Introduction

Withania somnifera (L.) Dunal commonly known as Indian ginseng is a member of the family Solanaceae. It plays an important role in the indigenous medicine of India and is also used as an energy-enhancing tonic known as Medharasayana (Nadkarni, 1976; Williamson, 2002). The medicinal value of this plant is high, especially to treat hiccup, cough, rheumatism, dropsy, inflammations, tuberculosis and exhibits excellent antitumor and anti-bacterial activities (Kiritikar and Basu, 1975; Devi and Sharada, 1992; Devi, 1996). The conventional method of propagation of this plant is through seeds, but the seed viability is very poor and low germination limits its multiplication.

Raising the demand for wild source, herbal drugs has abetted over the exploitation of medicinal plants, leading to cumulative and sustainable use of forest wealth.

Clonal propagation through tissue cultures offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication of uniform genotypes, resulting in short term gains (Gupta *et al*., 1993), while selection of explant is one of the important steps for successful *in vitro* regeneration studies especially for recalcitrant plants. Direct organogenesis of plants using mature seed provides faster response and is also a time saving approach (Aasim *et al*., 2011).

However, there are few reports in the past that demonstrated the *in vitro* plant regeneration with mature seeds as an explant in the plant species like spinach (Al-Khayri *et al*., 1992), chickpea (Polisetty *et al*., 1997), peanut (Pacheco *et al*., 2007) and *Phelloderndron amarense* (Yang *et al*., 2011). Very few reports are available in regard with *in vitro* plant regeneration system of *Withania somnifera* using mature seeds and have been published (Sen and Sharma, 1991; Supe *et al*., 2006; Sharma *et al*., 2015).

Though some micropropagation studies have been conducted so far, the present paper deals with the development of an efficient plant regeneration using mature seeds of this rare and endangered medicinal plant, *Withania somnifera*. Further, the establishment of a highly reproducible regeneration system would greatly influence the efforts of improvement of the medicinal plant species through useful gene transfer technology.

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Materials and Methods

The mature seeds of *Withania somnifera* (L.) Dunal were collected during sunny days and were used as explants. The collected mature seeds were initially washed with tap water, followed by Tween 20 solution. After that, the seeds were surface sterilized by dipping them in 70% alcohol for about 40 seconds, followed by treatment with 0.3% $HgCl_2$ for 3 minutes and then washed several times with sterile double distilled water and inoculated onto the culture medium. All the operations were done aseptically under a laminar air flow cabinet.

MS (Murashige and Skoog, 1962) media was used for the current study. The sucrose content of the media was 3% (W/V) and the pH of the media was adjusted at 5.8 before autoclaving at 121 °C temperature and 15 pound pressure for 15 minutes. Gelling of the media was done with 0.8% agar agar.

The cultures were maintained under cool fluorescent light intensity of 200-300 lux for 16-8 h light-dark period and the temperature of 22 ± 2 °C. The effect of cytokinin (6-benzylaminopurine (BAP)) in combination with auxin (indole-3-acetic acid (IAA)), GA_3 (gibberellic acid) and IBA (indole 3 butyric acid) alone with MS medium were observed on propagation, shoot elongation and rooting responses after 2 and 4 weeks respectively.

After root development, the seedlings were transferred into a beaker containing sterilized soil and covered with polyethylene bag, where it was kept in the mist chamber for 15 to 20 days for hardening. Then the seedlings were transferred into pots containing sterilized soil and watered regularly.

Results and Discussion

The present study was conducted to investigate the optimal concentrations and combinations of plant growth regulators with MS medium for the establishment of an efficient regeneration system in *Withania somnifera* using mature seeds as an explant.

The supplementation of the MS media with different concentrations of cytokinin (BAP) and auxin (IAA) showed effective multiplication of shoots from the mature seeds. MS medium combination with various concentrations of BAP (05-3.0 mg/L) with IAA (0.5 mg/L and 1.0 mg/L) tested for multiple shoot induction in mature seed explants (Table 1). Embryogenic callus with multiple shoots were initiated directly from the seeds after four weeks of culture on all combinations of MS media with BAP and IAA. While, the mature seeds on MS medium without any phytohormones were shrivelled and produced a single shoot, without forming the callus and multiple shoots even after four weeks of culture. The obtained findings suggest that the endogenous levels of phytohormones present in the seeds are not sufficient to sustain their growth and proliferation in the basal MS medium.

In the present study, it was reported that the simultaneous maximum induction of embryogenic callus and multiple shoots took place at high concentration of BAP with low concentrations of IAA. Similar findings were also observed by a few researchers (Joshi and Kothari, 2007; Rahman *et al*., 2008; Ashrafuzzaman *et al*., 2009; Otroshy *et al*., 2011). The synergistic effect of cytokinin (BAP) and an auxin (IAA) has been demonstrated in many medicinal plants, for example *Santolina canescens* (Casado *et al*., 2002), *Bupleurum fruticosum* (Fraternale *et al*., 2002) and turmeric (Salvi *et al*., 2002). One observation of these researchers was that low concentrations of an auxin in combination with a cytokinin, positively modified the frequency of shoot induction and growth. An increased concentration of auxins facilitated more callus formation. BAP at higher concentrations not only reduced the number of shoots, but also resulted in stunted growth of the shoots.

Maximum response resulted when the seeds were cultured on MS medium supplemented with BAP (1.5 mg/L) and IAA (0.5 mg/L), with the highest mean number of shoots (52.0 ± 0.22 per seed) along with extensive yield of callus (Figs. 1a and 1b). Hence, this was opted as the most suitable medium for mass propagation of *Withania somnifera* through seeds when compared to the previous reports of Sen and Sharma (1991), Supe *et al*. (2006) and Sharma *et al*. (2015).

The shoots of 1.0-1.5 cm in length were excised from the multiple shoot cluster and transferred individually to culture tubes containing MS media with various levels of 0.1-0.5 mg/L GA_3 for shoot elongation (Table 2). Elongation of shoots was higher, i.e., (15.6 ± 0.23 cm per shoot) on MS medium containing GA_3 (0.3 mg/L) (Fig. 1c). This may be due to the action of GA_3 on cell elongation (Qin *et al*., 2005). The elongated shoots were then implanted on ½ MS medium containing 3% (W/V) sucrose with various concentrations of IBA (1.0-4.0 mg/L) for rhizogenesis (Table 3). The medium without IBA did not show any root induction. In contrast, root induction was observed when regenerated shoots were cultured on medium with low concentrations of auxins, whereas at higher concentrations shoots formed callus at the cut end of the stem. These results are in agreement with earlier investigations published (Rout *et al*., 1999). The optimum rooting frequency (100%), root number (22.2 ± 0.08 per shoot) and root length (15.0 ± 0.12 cm per shoot) was obtained on MS medium supplemented with IBA (0.3 mg/L) (Fig. 1d). Similar findings had also been reported by Chandran *et al*. (2007) and Rani *et al*. (2014).

Acclimatized plantlets were transferred to the field with a 95% survival rate, which was an improvement over 64.9% survival rate obtained by Sharma *et al*. (2015). There were no observable differences between the parent plant and *in vitro* raised plants.

Conclusions

The protocol developed in the present study appears to be a promising method of propagation of the valuable, endangered medicinal plant *Withania somnifera* through seeds. It will also be of use in conservation and genetic transformation studies aimed at improving the species.

Table 1. Evaluation of media for callus formation and multiple shoots proliferation from mature seed explants of *Withania somnifera* (L.) Dunal

S. No.	Media	*In vitro* response	
		Intensity of callus forming	Shoot no./Seed
1.	MS basal	-	1.0 ± 0.00
2.	MS + BAP (0.5 mg/L) + IAA (0.5 mg/L)	+++	12.0 ± 0.10
3.	MS + BAP (1.0 mg/L) + IAA (0.5 mg/L)	+++	29.7 ± 0.18
4.	MS + BAP (1.5 mg/L) + IAA (0.5 mg/L)	++++	52.0 ± 0.22
5.	MS + BAP (2.0 mg/L) + IAA (0.5 mg/L)	++	26.4 ± 0.25
6.	MS + BAP (2.5 mg/L) + IAA (0.5 mg/L)	+	18.6 ± 0.18
7.	MS + BAP (3.0 mg/L) + IAA (0.5 mg/L)	+	14.5 ± 0.15
8.	MS + BAP (0.5 mg/L) + IAA (1.0 mg/L)	+++	8.0 ± 0.20
9.	MS + BAP (1.0 mg/L) + IAA (1.0 mg/L)	+++	10.1 ± 0.28
10.	MS + BAP (1.5 mg/L) + IAA (1.0 mg/L)	+++	20.3 ± 0.23
11.	MS + BAP (2.0 mg/L) + IAA (1.0 mg/L)	++	28.1 ± 0.20
12.	MS + BAP (2.5 mg/L) + IAA (1.0 mg/L)	+	15.2 ± 0.17
13.	MS + BAP (3.0 mg/L) + IAA (1.0 mg/L)	+	11.2 ± 0.08

- : not observed; + : low; ++ : medium; +++ : moderate; ++++ : high;
values are mean ± SE of fifty replicates

Table 2. Effect of plant growth regulator GA_3 on *in vitro* shoot elongation of *Withania somnifera* (L.) Dunal

S. No.	Media	Shoot length (cm)
1.	MS basal	5.0 ± 0.00
2.	MS + GA_3 (0.10 mg/L)	9.2 ± 0.08
3.	MS + GA_3 (0.20 mg/L)	12.0 ± 0.18
4.	MS + GA_3 (0.30 mg/L)	15.6 ± 0.23
5.	MS + GA_3 (0.40 mg/L)	12.4 ± 0.19
6.	MS + GA_3 (0.50 mg/L)	8.3 ± 0.15

values are mean ± SE of ten replicates

Table 3. Effect of growth regulator IBA on *in vitro* rooting of *Withania somnifera* (L.) Dunal

S. No.	Media	Rhizogenesis		
		Root induction (%)	Root no. /shoot	Root length (cm)
01.	MS basal	00.0 ± 0.00	00.0 ± 0.00	0.0 ± 0.00
02.	MS + IBA (1.0 mg/L)	70.0 ± 0.18	12.8 ± 0.12	6.8 ± 0.04
03.	MS + IBA (1.0 mg/L)	82.0 ± 0.21	14.4 ± 0.10	11.8 ± 0.11
04.	MS + IBA (2.0 mg/L)	94.0 ± 0.11	18.0 ± 0.22	10.4 ± 0.08
05.	MS + IBA (3.0 mg/L)	100.0 ± 0.00	22.2 ± 0.08	15.0 ± 0.12
06.	MS + IBA (4.0 mg/L)	72.0 ± 0.21	16.0 ± 0.22	10.4 ± 0.08

values are mean ± SE of ten replicates

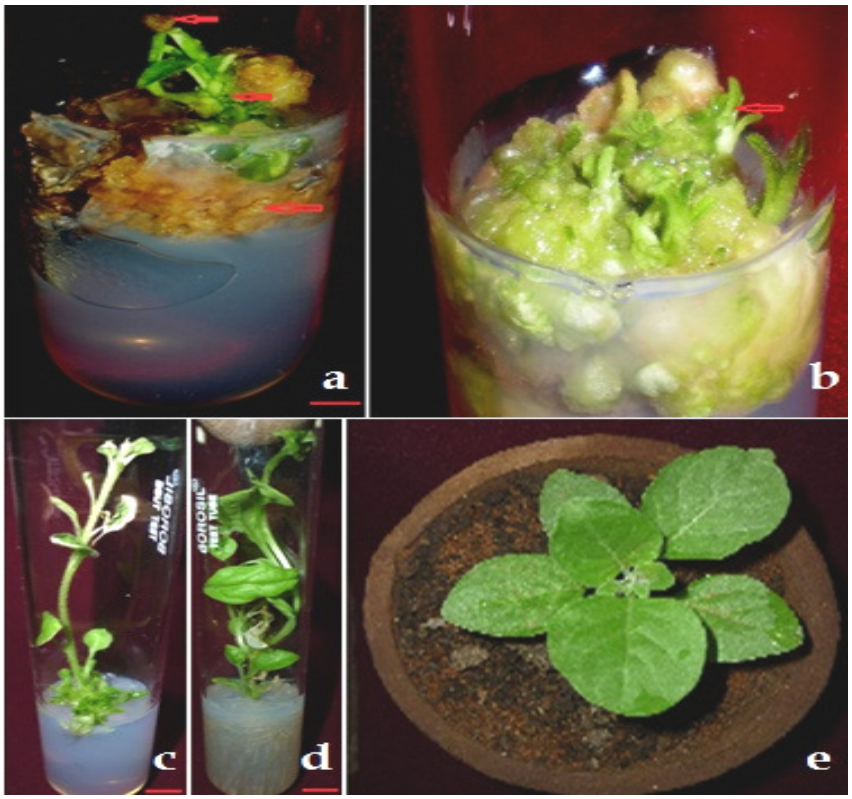

Fig. 1. *In vitro* plant regeneration from mature seed explants of *Withania somnifera* (L.) Dunal; a) multiple shoots and embryogenic callus developed from seed explant (arrows show multiple shoots, embryogenic callus and seed coat); b) Emergence of multiple shoots (arrow) from the embryogenic callus; c) Elongated shoot; d) well developed roots consisting plant; e) acclimatized plant. Scale bar length is 0.5 cm

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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