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STANDARDIZED PROTOCOL FOR IN VITRO SEED GERMINATION OF *WITHANIA SOMNIFERA*

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ABSTRACT An efficient and improved protocol for rapid *in vitro* seed germination and seedling development technique of *Withania somnifera* (L.) Dunal has been developed. The treatment in which the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS media with 0.3% GA_3 was found to be the best for seed germination (100%) with an average shoot length of 5 to 6 cm in *W. somnifera*. The germination was found on 6th day of inoculation.

KEYWORDS : In vitro, seed germination, Withania, seed development

INTRODUCTION

Withania somnifera (L.) Dunal of the Solanaceae family is a highly reputed plant of the Indian traditional system of medicines (Kaileh *et al.,* 2007). It is an important herb in the ayurvedic and indigenous medical systems for over 3000 years (Sharma *et al.,* 2010). Medicinal properties of this plant has been attributed to the presence of a group of steroidal lactones, known as withanolides present in leaves and roots (Sangwan *et al.,* 2004). Their roots are prescribed as medicines for hiccups, several female disorders, bronchitis, rheumatism, dropsy, stomach and lung inflammation, and skin diseases. The active pharmacological components of *W. somnifera* are steroidal lactones of the withanolide type. Several chemotypes have been found differing in their withanolide content. The principal withanolide in Indian *W. somnifera* are withaferin A and withanolide D (Ganzera *et al.,* 2003). This medicinally important plant species has been largely depleted from its natural habitat and is now included in the list of threatened species by the International Union for Conservation of Nature and Natural Resources (Kavidra *et al.,* 2000; Siddique *et al.,* 2005; Rahman, 2001).Withania is propagated commercially using seeds because of the lack of its natural ability for vegetative propagation. Also the seed viability is limited to one year only making the long duration seed storage futile (Farooqi and Sreeramu, 2004). Seed propagation, however, is not always satisfactory, as the percentage of germination is low, due to the presence of certain inhibitory compounds in the fruits and the high risk of getting various diseases (De Silva and Senarath, 2009). However, the conventional propagation methods cannot meet the increasing demand of this plant which is used as raw material for the preparation of various pharmaceutical products. Due to the increasing demand, poor viability of stored seeds and little information available regarding seed germination of *W. somnifera* an alternative procedure of propagation through *in vitro* seed germination and seedling development is essential. The immature seeds obtained from green pods of *W. somnifera* can be germinated asymbiotically *in vitro* for rapid micro propagation (Sen *et al.,* 1991) and this method can be exploited for the rapid propagation and conservation of *W. somnifera*. GA_3 induces the germinating seed cells to generate molecules of mRNA that code for hydrolytic enzymes. Gibberellic acid is a very potent hormone whose natural occurrence in plants controls their development. In two ways, GA_3 exerts its effect, first by growing embryo growth potential and secondly by inducing hydrolytic enzymes (Govindaraju *et al.,* 2003). Therefore, the present study was carried out to optimize the concentration of gibberellic acid (GA_3) in MS media for *in vitro* seed germination and seedling development of *W. somnifera* by using *in vitro* techniques.

MATERIALS AND METHODS

The seeds of variety Arka Aswagandha were collected from IIHR Bangalore. The seeds were kept in air tight containers and stored in refrigerator at 20ºC. For germination, the seeds were inoculated in jam bottles with standard Murashige and Skoog medium containing 3% sucrose and 0.5% agar along with various treatments. The pH of the medium was adjusted to 5.8 before the addition of 0.5% (w/v) agar. The treatments used were S1 in which the seeds were soaked in water overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes, 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated to half strength MS media without hormones. In treatment S2 the seeds were soaked in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media without hormones. The seeds were soaked in tender coconut water for 1hour and then in 250ppm GA_3 overnight in treatment S3 and the next day surface sterilized with 5% Tween 20 for 5 minutes, 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3. Another treatment was S4, in which the seeds were soaked in water overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes, 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3. In treatment S5, time of exposure of the seeds to $HgCl_2$ treatments was reduced to 5 minutes. In S6, the concentration of $HgCl_2$ was reduced to 0.05% but the time of exposure was 10 minutes. In S7, both the concentration of $HgCl_2$ and time of exposure of the seeds to $HgCl_2$ treatments were reduced to 0.05% and for 5 minutes. The *in vitro* cultures were incubated under controlled condition at 25 ± 2°C temperature, 60 ± 10% relative humidity and 16 h photoperiod. The cultures were kept in dark condition till the germination was observed and then kept under light. They were observed daily to collect the data on seed germination, mainly the effect of these treatments on the percentage of seed germination, average shoot length and time taken for the induction of seed germination.

RESULTS AND DISCUSSION

The seeds were subjected to different treatments as indicated in Table (1) and the *in vitro* seed cultures were maintained in culture room (air conditioned) in dark condition till germination was noticed. After germination they were transferred to light condition in the culture room for proper growth of the seedlings. The effect of these treatments on seed germination was observed based on different parameters *viz.* days of germination, percentage of seed germination and shoot length of seedlings.

Out of these seven treatments, only the treatments S3 and S5 have shown germination. In S3 (seeds soaked in tender coconut water for 1hour and then in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3), the induction of germination (40%) was found on 14th day of inoculation. The average shoot length of the seedlings was about 3-4 cm. But in the case of S5 (seeds soaked in water for overnight and next day treated with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3), seed germination (100%) was noticed on 6th day of inoculation and then the seed cultures were transferred to light conditions. The average shoot length of the seedlings was about 5-6 cm. The treatments S1, S2, S4, S6 and S7 did not show any induction of germination. Thus S5 treatment was identified as the fastest method for seed germination.

Thus the treatment S5 is found as the best one for *in vitro* seed germination of *W. somnifera.* S5 has shown maximum germination rate (100%) with an average shoot length of 5-6 cm. Germination was found within 6 days of inoculation. Compared to the rest of the treatments, S5 has shown not only maximum germination rate but also faster development of the seedlings.

Gibberellins are a family of 136 tetracyclic diterpenes, a small subset of which are active as plant hormones and known to stimulate seed germination in a wide range of plant species and the predominant active GA_3 depends on the species (Thomas et al., 2005). Gibberellic acid is a very potent hormone whose natural occurrence in plants controls their development. In two ways, GA_3 exerts its effect, first by growing embryo growth potential and secondly by inducing hydrolytic enzymes. Murashige and Skoog (MS) medium containing 3.0 mg l^{-1} gibberellic acid (GA_3) and 3.0 mg l^{-1} Kinetin (Kn) was reported to be effective for maximum germination percentage (92.67) in a study conducted by Sharma *et al.,* 2016. Thus in the present study, a rapid and effective procedure for *in vitro* seed germination of *Withania somnifera* has been found out.

CONCLUSION

From the present investigation, it is concluded that the growth hormones gave significantly better response than control both in seed germination and seedling development in *W. somnifera* (L.) Dunal. The treatment in which the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS media with 0.3% GA_3 was found to be the best for seed germination. It showed a germination rate of 100% with an average shoot length of 5-6 cm and the germination was noticed on 6th day of inoculation with a rapid development of the seedlings and so this treatment is adjudged as the best one for *in vitro* seedling development. Since the germination percentage of *Withania* seeds in the natural environment is very poor, the present protocol will be helpful to produce quality seedlings in large quantities and to conserve this rare species from the dangers of natural selection.

Table1: Different treatments and their effect on *in vitro* seed germination of *W. somnifera*

Sl No.	Pretreatment of seeds	Seeds were treated with 5% Tween 20 for 5 minutes	$HgCl_2$ treatment	Media treatment	Days of germination	Percentage of seed germination (%)	Shoot length of seedlings (cm)
S1	Overnight soaking in water		0.1% for 10 minutes	Half MS without hormones	No germination found	0	-
S2	Overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS without hormones	No germination found	0	-
S3	Tender coconut water treatment for 1hour then overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	14th day	40	3-4
S4	Overnight soaking in water		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	No germination found	0	-
S5	Overnight soaking in water		0.1% for 5 minutes	Half MS with 0.3ppm GA_3	6th day	100	5-6
S6	Overnight soaking in water		0.05% for 10 minutes	Half MS with 0.3ppm GA_3	No germination found	0	0
S7	Overnight soaking in water		0.05% for 5 minutes	Half MS with 0.3ppm GA_3	No germination found	0	0

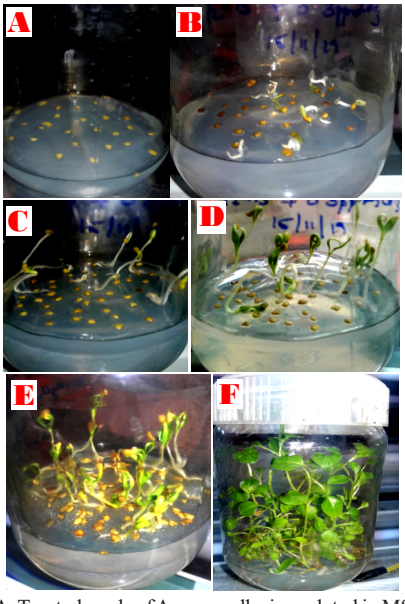

Figure 1: A. Treated seeds of Aswagandha inoculated in MS media, B. Induction of germination on 6th day of inoculation, C. Stage of seedlings after 12th day of inoculation, D. Stage of seedlings after 18th day of inoculation, E. Stage of seedlings after 24th days of inoculation, F. Stage of 2 months old seedlings.

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