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In Vitro Seed Germination In *Withania Somnifera* L. (Dunal): A High-Value Medicinal Plant

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ABSTRACT

Withania somnifera L. (Dunal) is a valuable medicinal plant utilized in traditional medicine, Ayurveda, Unani, and traditional Chinese medicine to cure various disorders and diseases. This study aims to evaluate the percentage of *in vitro* seed germination in Murashige and Skoog (MS) media in the absence of plant growth regulators (PGRs) and supplemented with the α-naphthalene acetic acid (NAA) and benzylaminopurine (BAP). Seeds were cultured on quarter-, half-, and full-strength MS media (control treatment), in addition to quarter-, half-, and full-strength MS media supplemented with 0.5-1.5 mg/L NAA + 0.5-2.0 mg/L BAP (treatment with PGRs). The control treatment had a higher percentage of seed germination than the PGR treatment. In the case of control treatment, the maximum seed germination rate (80%) was found in quarter-strength (¼) of MS, followed by half- (1/2) and full-strength MS media. Similarly, in the case of treatment with PGRs, the maximum percentage (67%) of seed germination was found in quarter-strength (¼) of MS media supplemented with 0.5 mg/L NAA + 1.5 mg/L BAP as compared to other combinations. Seed germination occurred faster (after 3 weeks of culture) in the control treatment than in the PGR treatments (after 5 weeks). This research will be valuable for the production of sterile explants for the micropropagation of *W. somnifera* in a short time, and it might be useful for both *ex-situ* conservation and germplasm conservation of this species.

Keywords: Conservation, *In vitro*, MS medium, PGRs, Seed germination

INTRODUCTION

Withania somnifera L. (Dunal) (Solanaceae), often called Ashwagandha, Indian ginseng, poison gooseberry, or winter cherry, is an evergreen drought-resistant shrub, endemic plant to the Indian subcontinent that is utilized in traditional, homeopathic, and allopathic medicine to cure various diseases and ailments in the region of Southeast Asia (Gaurav *et al.*, 2023). *Withania* comprises 26 species that are scattered over South Asia and the Eastern Mediterranean region (Panwar & Tarafdar, 2006) in tropical and subtropical climates. *W. somnifera* is effectively cultivated in Madhya Pradesh, Gujarat, Uttar Pradesh, Haryana, Rajasthan, and Punjab in India (Bhatia *et al.,* 1987); however, it grows at an elevation of 1676 m in the Soon valley of Punjab and the Himalaya Range of Azad Jammu and Kashmir, Pakistan (Aslam *et al.*, 2017). In Nepal, it is cultivated in Rupandehi, Morang, Chitwan, Bara, Kailai, and Banke districts in the Terai region for its therapeutic uses. *Withania* roots are commonly utilized in herbal remedies, and the steroidal lactones (withanolides, & withaferin), alkaloids (isopelletierine and anaferine), and saponins found in them show a wide range of biological actions, including antimicrobial, anti-

phlogistic, tonic, against low blood pressure, sexual stimulant, psychostimulant, and diuretic properties (Jaffer *et al.,* 1988).

Seed germination can be described as the basic procedure through which diverse plant species develop from just one seed into a seedling or juvenile plant. Seed germination occurs through a series of processes that include water absorption, enzyme activation, and embryo conversion into seedlings, with seed germination favoring the presence of air, water, and temperature. The process of germinating seeds in a regulated, sterilized laboratory setting with nutrient-rich media instead of natural soil can be referred to as *in vitro* seed germination. This approach provides exact control over variables, including temperature, light, and nutrients, resulting in more rapid and consistent germination, decreased time required for germination, and may give a solution for completely inhibiting germination (Lopez-Encina & Gonzalez-Padilla, 1996). *In vitro* conditions promote germination as well as early growth of seedlings (Noleto & Silveira, 2004). This method promotes research, conservation, and sustainable plant production for a variety of uses.

Many medicinal plants are unable to germinate and grow from their seeds due to dormancy, nonviability, or long germination times (Singh & Singh, 2021). Similarly, plants like *Azadirachta indica* and *Rauvolfia serpentine* that are difficult to conserve in seed banks may have seed viability loss (Murthy and Saxena, 1998). In the case of *Panax ginseng*, germinating seeds is a complex process with multiple stratification processes, making seed banking challenging, and seed conservation might not be possible, especially in expensive field gene banks. Therefore, *in vitro* seed germination and *in vitro* culture techniques were used to conserve medicinal plants, including threatened, extremely uncommon, and vulnerable species (Sharma *et al.,* 2010), as well as producing plants with long seed dormancy periods, non-viable seeds, and epiphytic orchids.

W. somnifera seeds can germinate in the soil, and conventionally it is a seed-propagated plant (Sen & Sharma, 1991). However, the viability of seeds is poor and limited only for a single year, rendering long-term storage of seeds useless (Rani & Grover, 1999; Siddhique *et al.*, 2004). Another concern is low seed germination in *W. somnifera* (Vakeswaran & Krishnasamy, 2003), which is most likely caused by the existence of chemical inhibitors in the seeds and fruit wall (De Silva *et al.*, 2009). Germination of seeds in *in vitro* conditions in suitable nutrient media is an essential stage in the regeneration of disease-free plants in any season via micropropagation. Various parts of the plant, such as nodes, leaves, and meristem tips, that were germinated from *W. somnifera* seeds, can be used as sterile explants for its micropropagation. It helps minimize time and money on the explant sterilizing process during micropropagation while also preventing contamination in culture. Several researches were carried out regarding *W. somnifera* seed germination in a controlled lab condition (Khanna *et al.*, 2013; Niyaz & Siddiqui, 2014; Kumar *et al.*, 2016; Himangini & Thakur, 2018); however, a few studies on *in vitro* seed germination in *W. somnifera* (Arun *et al.*, 2011; Pandey *et al.*, 2013; Sharma *et al.*, 2016) were carried out. Similarly, there is a lack of literature regarding comparative studies on seed germination of *W. somnifera* in MS media with and without using plant growth regulators (PGRs). Therefore, this study aims to examine the percentage of seed germination of *W. somnifera* in MS media without plant growth regulators (PGRs) in comparison to those supplemented with PGRs in MS media under controlled conditions.

MATERIALS AND METHODS

Seeds collection

Seeds of *W. somnifera* (Figure 1) were obtained in September 2022 from the National Botanical Garden in Godavari (1515 m), Lalitpur, Nepal. It was validated by comparing it to herbarium specimens from the National Herbarium and Plant Laboratories (KATH), Godavari, Lalitpur, Nepal.

Figure 1. (A): *Withania somnifera* plant, (B): Fruits of *W. somnifera*, and (C): Seeds of *W. somnifera*

Surface sterilization of seeds

Seeds were thoroughly washed with tap water and then soaked in water for 24 hours. They were surface sterilized with mercuric chloride ($HgCl_2$) and ethanol before being inoculated in nutrient media. The seeds were first dipped in 1% $HgCl_2$ for 10 min, washed with sterilized distilled water, and then immersed in 70% ethyl alcohol for 2 minutes. The seeds were washed three times with sterilized distilled water to remove disinfectants.

Nutrient media preparation

MS medium (Murashige & Skoog, 1967) was made with stock solutions A, B, C, and D. As a control treatment, several strengths of MS media were made from the stock solution, including full, half, and quarter strength. Similarly, plant growth regulators (PGRs) such as NAA (0.5-1.5 mg/L) and BAP (0.5-2.0 mg/L) were combined with various strengths of MS media to form a treatment group with PGRs. MS media was combined with 3% sucrose, 0.8% agar, and adjusted to 5.6 pH before being autoclaved for 15 minutes at 121°C and 15 pounds of pressure.

Inoculation and maintenance of culture for seed germination

Seeds (30) were inoculated in culture jars with various strengths of MS media (control treatment) and various combinations of NAA and BAP with MS media (treatment group with PGRs) in a laminar airflow cabinet. The culture jars were sealed with aluminium foil and transferred to the growth room for incubation. The culture was maintained at 26±2°C, a 16-hour light period, and light intensity of 3000-4000 lux.

Data analysis

Data on seed germination were collected from triplicate observations of each control treatment and PGR treatment. Average seed germination, standard deviation, and percentage germination of seeds were calculated using Microsoft Excel 2010. One-way ANOVA test with Duncan's Multiple Range Test (DMRT) for the analysis of percentages of seed germination with various strengths of MS media and PGRs at a significance level of 0.05 was carried out using the IBM Statistical Package for the Social Sciences (SPSS) version 2020.

RESULTS AND DISCUSSION

In vitro seed germination and growth in *W. somnifera* were observed in various strengths of MS media (control treatment) as well as in various strengths of MS media combined with PGRs (treatment with PGRs). Seed germination occurred earlier and at higher percentages in the control treatment compared to the PGR treatment.

Seed germination in MS media without PGRs (control treatment)

Seed germination and growth occurred in full, half, and quarter-strengths of MS medium, however, the highest percentage (80%) of seed

germination was found in quarter-strength (¼) of MS media, followed by half-strength (½) (51%), and full-strength (27%) of MS media after the 3 weeks of culture (Figures 2 and 3). This result was supported by the findings of Mahmod *et al.* (2021) on seed germination of *Hylocereus undatus* in quarter-strength (¼) MS media. According to them, reduced nutrient concentrations in quarter-strength MS may help seeds absorb water more effectively, increasing germination rates. Trajkovic *et al.* (2019) found that *Viola cornuta* seed germination rates were higher in half-strength (½) MS media compared to full-strength after 4, 8, and 12 weeks of *in vitro* culture at 4, 10, and 22˚C. Similarly, Kulkarni *et al.* (2000), Arun *et al.* (2011), and Pandey *et al.* (2013) found that ½ MS media resulted in greater seed germination rates in *W. somnifera*. It may be due to decreased osmotic stress, a lower risk of nutrient toxic effects, and an adjusted nutrient balance appropriate for early growth stages in quarter- and half-strength MS medium (Murashige & Skoog, 1962). Full-strength MS medium is nutrient-rich, which can result in a high osmotic level in the medium, and some seeds are sensitive to high concentrations of specific nutrients (e.g., nitrogen, potassium, or phosphorus) that exist in full-strength MS media. The excess nutrients in full-strength MS media may limit water uptake and induce nutritional imbalances, preventing germination and early root and shoot growth.

Moreover, the one-way ANOVA test found a significant difference ($p<0.001$) between different strengths of MS media and percentages of seed germination at the 0.05 significance level. This shows that seed germination is dependent on the concentration of nutrients present in various strengths of MS media.

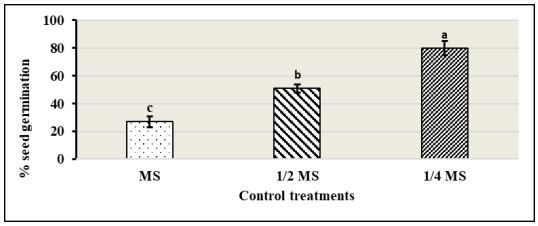

Figure 2. Seed germination in various strengths of MS media in *W. somnifera* after 3 weeks of culture. [DMRT (α=0.05) indicated no significant difference between means in bars with the same letter, (n=9)].

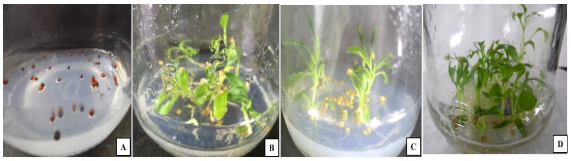

Figure 3. Seed germination in control treatment: (A) seeds in a nutrient medium, (B) Germination in MS medium, (C) Germination in ½ MS, and (D) Germination in ¼ MS

Seed germination in MS media with PGRs (treatment with PGRs)

Seed germination in various strengths of MS media with the combination of NAA and BAP (0.5-1.5 mg/L) (treatment with PGRs) was found after 5 weeks of culture and was less effective than in various strengths of MS medium in the absence of PGRs (control treatment). However, seed germination percentages were higher in ¼ MS + NAA + BAP compared to ½ MS + NAA + BAP, and full-strength MS + NAA + BAP (Figures 4, 5, 6, & 7).

In the case of seed germination in 1/2 MS + NAA + BAP, the highest percentage of seed germination (38%) was found in 1/2 MS media containing 0.5 mg/L NAA and 1.5 mg/L BAP, which was lesser than the control treatment of ½ strength of MS media (51%) (Figure 4). The one-way ANOVA test found a significant difference ($p<0.001$) between half-strength of MS medium combined with various strength of NAA and BAP and percentages of seed germination at the 0.05 significance level.

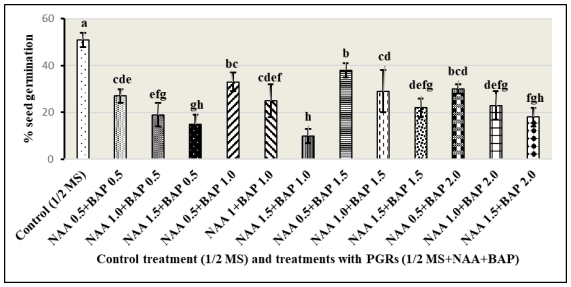

Figure 4. Seed germination in 1/2 MS media in combination with NAA and BAP in *W. somnifera* after 5 weeks of culture. [DMRT (α=0.05) indicated no significant difference between means in bars with the same letter, (n=39)]

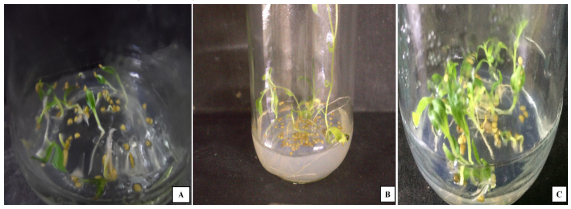

Figure 5. Seed germination in PGRs treatment: (A) Seed germination in MS + 0.5 mg/L NAA + 1.5 mg/L BAP, (B) Seed germination in ½ MS + 0.5 mg/L NAA + 1.5 mg/L BAP, and (C) Seed germination in ¼ MS + 0.5 mg/L NAA + 1.5 mg/L BAP

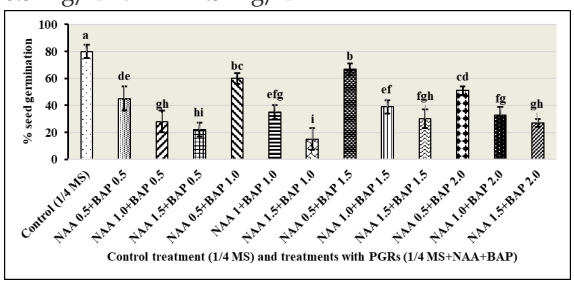

Figure 6. Seed germination in 1/4 MS media in combination with NAA and BAP in *W. somnifera* after 5 weeks of culture. [DMRT (α=0.05) indicated no significant difference between means in bars with the same letter, (n=39)].

In the case of seed germination in 1/4 MS + NAA + BAP, the highest percentage of seed germination (67%) was obtained in 1/4 MS medium containing 0.5 mg/L NAA and 1.5 mg/L BAP, which was lesser than the control treatment of ¼ strength of MS media (80%) (Figure 6). The one-way ANOVA test found a significant difference ($p<0.001$) between quarter-strength of MS medium combined with various strength of NAA and BAP and percentages of seed germination at the 0.05 significance level.

Similarly, in the case of seed germination in MS + NAA + BAP, the highest percentage of seed germination (19%) was obtained in full-strength MS medium containing 0.5 mg/L NAA and 1.5 mg/L BAP, which was lesser than the control treatment of full-strength of MS media (27%) (Figure 7). The one-way ANOVA test found a significant difference ($p<0.001$) between full-strength of MS medium combined with various strength of NAA and BAP and percentages of seed germination at the 0.05 significance level. This shows that seed germination is dependent on the concentration of nutrients present in different strengths of MS medium (1/4 MS, ½ MS, & FMS) in conjunction with different concentrations of NAA and BAP.

This study showed that the percentage of seed germination was higher in control treatments (1/4 strength MS, ½ strength MS, & full-strength MS) as compared to the treatment with the combination of different concentrations of NAA and BAP in ¼, ½, & full-strength MS media (treatment with PGRs). It could be because MS medium alone delivers the necessary nutrients while maintaining the internal hormonal balance required for seed germination. The absence of additional PGRs such as NAA and BAP reduces the danger of hormonal disorders, resulting in greater germination rates. MS media, particularly at varying strengths (e.g., full, 1/2, or 1/4), include necessary macronutrients (such as N, K, and P) and micronutrients (such as Ca, Fe, and Mg), providing an ideal environment for germination. These nutrients actively promote basic cellular activities, stimulate root and shoot growth, and aid seeds in moving from dormancy to vigorous growth. Seeds are extremely

sensitive to hormonal factors in the early phases of growth, and large amounts of exogenous hormones might disrupt the hormonal balance necessary for germination. Auxins, particularly NAA, can cause a buildup of ethylene, a chemical known to hinder germination when generated in excess. Cytokinin, such as BAP, can also delay germination by changing the attention away from early root and shoot emergence and toward later-stage development activities (Basra *et al.*, 2005).

In all the cases of treatment with PGRs, it was found that the higher concentration of cytokinin (1.5 mg/L BAP) and lower concentration of auxin (0.5 mg/L NAA) enhanced seed germination in *W. somnifera* over the other combinations. Seed germination rates increased from 0.5 mg/L BAP to 1.5 mg/L at a constant NAA concentration of 0.5 mg/L. If cytokinin and auxins are mixed in MS media, seed germination is determined by the ratio of the two. Greater cytokinin and lesser auxin levels encourage germination by prioritizing shoot growth over root elongation. High auxin concentration, on the other hand, can limit germination by suppressing shoots and promoting root-centric development (Wareing and Phillips, 1981). As a result, the combination of cytokinins and low auxin levels in MS media promotes seed germination. Similarly, while auxins normally stimulate root initiation and lengthening, they can hinder germination by reducing the growth of shoots at greater concentrations (Steffens & Rasmussen, 2016). Furthermore, higher auxin levels may increase the production of abscisic acid (ABA), a hormone recognized to promote dormancy and so reduce the rate of germination (Kucera *et al.*, 2005).

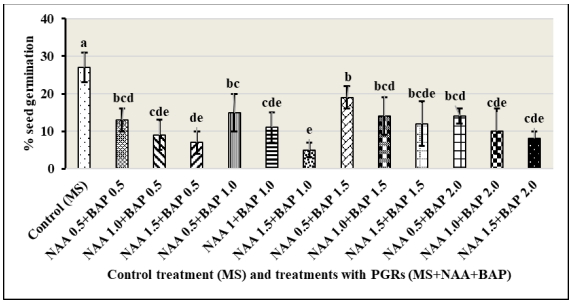

Figure 7. Seed germination in full-strength MS medium in combination with NAA and BAP in *W. somnifera* after 5 weeks of culture. [DMRT (α=0.05) indicated no significant difference between means in bars with the same letter, (n=39)].

Previous studies on *W. somnifera* seed germination in MS media using some PGRs showed different results than this study, such as higher percentage of germination in ½ MS medium containing 750 mg/L GA_3 than in potassium nitrate and sodium nitrate (Arun *et al.*, 2011), and higher percentage of seed germination (92.67%) in full-strength of MS medium containing 3.0 mg/L GA_3 and 3.0 mg/L KN (Sharma *et al.*, 2016).

In vitro seed germination on MS media enriched with NAA and BAP was reported successfully in numerous orchid species. For example, studies on *Cymbidium aloifolium* found that 1/2 MS media enriched with 0.5 mg/L NAA and BAP significantly increased seed germination rates and resulted in vigorous growth of seedlings (Bhowmik and Rahman, 2017). Similarly, research in *Spathoglottis plicata* showed that full-strength MS medium with an additional 0.5 mg/L NAA and BAP significantly increased protocorm growth and differentiation into seedlings in comparison to 1/4 MS and other media (Bhowmik and Rahman, 2020).

CONCLUSION

The protocol for *in vitro* seed germination in *W. somnifera* was developed using different strengths of MS medium (control treatment) and a combination of various strengths of MS media and PGRs (PGRs treatment). In the case of control treatment, the ¼ MS media produced earlier and higher seed germination rates than other treatments. Full-strength MS media contains higher concentrations of nutrients that may be hazardous to seed germination in *W. somnifera*. Likewise, in the case of treatment with PGRs, ¼ MS media combined with 0.5 mg/L NAA and 1.5 mg/L BAP was the most effective media for seed germination than the other treatments. Seed germination percentage is dependent on various strengths of MS media and PGR concentrations. This research work will aid in the development of sterile seedlings that can be utilized as explants for *in vitro* propagation of *W. somnifera*.

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