INTERNATIONAL JOURNAL OF ENHANCED RESEARCH IN SCIENCE TECHNOLOGY & ENGINEERING
VOL. 2 ISSUE 3, MARCH-2013 ISSN NO: 2319-7463

In Vitro Regeneration and Phytochemical Profiling of *Withania somnifera* (L.): Enhancing Secondary Metabolite Production through Callus Culture and Elicitor Treatments

Jitendra Singh Rathore

Department of Botany, Government College Kherwara, Udaipur 313803, Rajasthan, India

Abstract

***Withania somnifera* (L.), commonly known as Ashwagandha, is an important medicinal plant in Ayurvedic and modern pharmacology, valued for its bioactive compounds called withanolides. The present study aimed to develop an optimized in vitro regeneration system and evaluate the influence of biotic and abiotic elicitors on secondary metabolite production in callus cultures. Leaf and nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations of auxins (2,4-D, NAA) and cytokinins (BAP, kinetin). Optimal callus induction (92%) was achieved with 2.0 mg/L 2,4-D and 0.5 mg/L BAP. Subsequent shoot regeneration was obtained using 1.5 mg/L BAP and 0.5 mg/L NAA, producing an average of 8.3 shoots per explant. Elicitation with 100 µM salicylic acid and 50 µM methyl jasmonate significantly enhanced withanolide content compared to the control. GC-MS analysis confirmed elevated levels of withaferin A and withanolide D. The study concludes that elicitor-based callus cultures provide an effective approach for sustainable production of valuable phytochemicals from *W. somnifera*.**

Keywords: *Withania somnifera*, callus culture, micropropagation, elicitors, secondary metabolites, GC-MS, withanolides.

1. Introduction

Withania somnifera (L.) Dunal, commonly known as Ashwagandha or Indian ginseng, is an important medicinal plant belonging to the family Solanaceae. It has been extensively used in Ayurvedic and traditional Indian medicine for more than 3000 years due to its wide range of pharmacological properties such as adaptogenic, anti-inflammatory, antioxidant, and immunomodulatory effects (Singh et al., 2010). The therapeutic efficacy of *W. somnifera* is primarily attributed to its diverse group of bioactive constituents known as withanolides, which are naturally occurring steroidal lactones (Mirjalili et al., 2009). These secondary metabolites play a vital role in the plant's defense mechanisms and are responsible for its medicinal significance. Among the known withanolides, withaferin A, withanolide A, and withanolide D are the most biologically active compounds exhibiting anticancer, antimicrobial, and anti-stress activities (Misra et al., 2008). Due to the growing demand for herbal medicines and pharmaceutical formulations derived from *W. somnifera*, the need for sustainable production of high-quality plant material has become critical. However, natural propagation is limited by low seed viability, poor germination rate, and genetic variability among plants (Sen and Sharma, 1991). Furthermore, uncontrolled harvesting and habitat degradation have led to a significant decline in natural populations of this valuable species. In recent years, plant tissue culture has emerged as a reliable and efficient tool for large-scale propagation and secondary metabolite production in medicinal plants. In vitro techniques such as callus induction, organogenesis, and somatic embryogenesis provide a controlled environment that allows manipulation of physiological and biochemical pathways to enhance the synthesis of desired compounds (Sivanesan and Murugesan, 2008). These systems enable the establishment of clonal propagation protocols, ensuring genetic uniformity and continuous biomass production independent of climatic conditions. An effective way to further enhance the accumulation of secondary metabolites in vitro is the use of elicitors — substances that mimic biotic or abiotic stress and activate plant defense responses, leading to increased biosynthesis of metabolites (Namdeo, 2007). Elicitors can be categorized into biotic, derived from biological sources such as yeast extract and chitosan, and abiotic, including compounds like salicylic acid, methyl jasmonate, silver nitrate, and heavy metal ions (Zhao et al., 2005). The elicitation process stimulates enzymes such as phenylalanine ammonia-lyase and squalene synthase, which are involved in the biosynthetic pathway of withanolides (Ghosh and Banerjee, 2003). In the case of *W. somnifera*, several studies have reported that in vitro cultures can be successfully established from various explants including leaf, root, and nodal segments, and that the production of withanolides can be enhanced through the application of specific growth regulators and elicitors (Rani et al., 2003; Ray and Jha, 2001). Auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) are known to promote callus formation by inducing cell dedifferentiation, while cytokinins such as 6-benzylaminopurine (BAP) and kinetin (KIN) are important for shoot regeneration and organogenesis (Murashige and Skoog, 1962; Thomas and Kumar, 2010). A precise balance between auxins and cytokinins is

essential to achieve optimum morphogenic responses and metabolite accumulation. Therefore, the present investigation aims to develop an optimized in vitro regeneration protocol for *W. somnifera* and to explore the potential of biotic and abiotic elicitors for enhancing secondary metabolite production. The specific objectives of this study are:

1. To establish an efficient callus induction and shoot regeneration system using different auxin–cytokinin combinations.
2. To evaluate the effect of selected elicitors, including salicylic acid, methyl jasmonate, and yeast extract, on withanolide accumulation in callus cultures.
3. To perform phytochemical profiling using GC-MS for identification and quantification of major withanolides produced under various culture conditions.

This study is expected to contribute to the development of a sustainable, reproducible, and high-yield system for producing valuable phytochemicals from *W. somnifera* through plant tissue culture technology, which may help in reducing pressure on wild populations and ensuring the consistent supply of bioactive compounds for the pharmaceutical industry.

2. Research Methodology

2.1 Plant Material and Explant Preparation

Healthy *W. somnifera* plants were collected from the forest. Young leaf and nodal segments (1–2 cm) were excised and surface sterilized using 0.1% (w/v) mercuric chloride for 2 minutes followed by three rinses with sterile distilled water.

2.2 Culture Medium and Growth Regulators

Murashige and Skoog (MS) basal medium supplemented with 3% sucrose and 0.8% agar was used. Growth regulator combinations tested included:

- 2,4-D (0.5–2.5 mg/L) + BAP (0.2–1.0 mg/L)
- NAA (0.5–2.0 mg/L) + Kinetin (0.5–1.0 mg/L)

The pH was adjusted to 5.8 before autoclaving at 121°C for 20 minutes.

2.3 Callus Induction and Shoot Regeneration

Explants were cultured in Petri dishes under controlled conditions (25 ± 2°C, 16 h photoperiod). Callus induction was recorded after 3 weeks. For shoot regeneration, green friable calli were transferred to MS medium supplemented with BAP (1.0–2.0 mg/L) and NAA (0.2–1.0 mg/L). Shoots were rooted on half-strength MS medium containing 0.5 mg/L IBA.

2.4 Elicitor Treatments

Well-established callus cultures were treated with:

- Salicylic acid (50, 100, 150 µM)
- Methyl jasmonate (25, 50, 75 µM)
- Yeast extract (100 mg/L)

Control cultures were maintained without elicitors. Samples were harvested after 7 and 14 days for phytochemical analysis.

2.5 Phytochemical Extraction and GC-MS Analysis

Dried callus samples (1 g) were extracted in methanol using Soxhlet extraction. The extracts were concentrated and analyzed using GC-MS (Agilent 7890B) under standard conditions. Peaks corresponding to withanolides were identified by comparing retention times and fragmentation patterns with authentic standards and NIST library data.

2.6 Statistical Analysis

All experiments were conducted in triplicates. Data were expressed as mean ± standard deviation (SD). Statistical significance was analyzed using one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

3. Analysis and Observations

The present study on *Withania somnifera* (L.) Dunal focused on the establishment of an efficient in vitro regeneration system and the evaluation of elicitor effects on secondary metabolite accumulation. Data were collected from multiple experimental trials, and all parameters were analyzed statistically to determine the most suitable hormonal and elicitor combinations for optimal growth and metabolite production. The results obtained from the observations are discussed below.

3.1 Callus Induction and Morphogenic Response

Among the different combinations of auxins and cytokinins tested, the medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg/L 6-benzylaminopurine (BAP) was found to be the most effective for inducing callus formation. Leaf explants exhibited the highest callus induction frequency of 92%, while nodal explants showed a slightly lower induction rate of 86%. The callus obtained from leaf tissues appeared friable, cream to light green in color, and rapidly proliferated, indicating active cell division and dedifferentiation. In contrast, nodal explants produced a more compact and greenish callus.

The variation in callus texture and color between explant types suggests differential physiological responses to exogenous growth regulators, as reported previously by Rani et al. (2003) and Thomas and Kumar (2010). The presence of 2,4-D promoted cell dedifferentiation and auxin-induced callus proliferation, while BAP provided the cytokinin stimulus required for active cell division. This combination established a balanced auxin-to-cytokinin ratio favorable for initiating meristematic activity in the explants.

Callus formation was observed within 10–12 days of culture initiation, and maximum proliferation was recorded after four weeks. The mean fresh weight of the callus mass obtained on this medium was significantly higher compared to other hormonal combinations tested, confirming its suitability for large-scale biomass production.

3.2 Shoot Regeneration and Multiplication

Following successful callus induction, the green friable calli were subcultured on regeneration media containing different concentrations of cytokinins (BAP, kinetin) and auxins (NAA, IAA) to optimize shoot induction. The highest regeneration frequency was recorded on medium supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA. Under these conditions, multiple shoots developed directly from the callus surface within 18–20 days. The average number of shoots per explant was 8.3 ± 1.2, with an average shoot length of 6.5 cm after four weeks of culture.

The regeneration response was significantly lower in media containing kinetin in place of BAP, indicating that BAP is a more effective cytokinin for promoting adventitious shoot formation in W. somnifera. Similar observations have been reported by Sen and Sharma (1991) and Misra et al. (2008), who found that BAP in combination with a low concentration of NAA induces prolific shoot development and maintains tissue vigor. The synergistic effect of BAP and NAA stimulates cytokinin-mediated cell division and auxin-induced differentiation, thus facilitating organized shoot bud formation.

The regenerated shoots were healthy and morphologically normal, exhibiting well-developed apical meristems and expanded leaves. The frequency of regeneration declined at higher BAP concentrations (>2.0 mg/L), possibly due to hormonal imbalance leading to hyperhydricity and shoot vitrification.

3.3 Root Induction and Plantlet Formation

The elongated shoots were excised and transferred to half-strength MS medium supplemented with different concentrations of auxins (IBA, IAA, NAA) for root induction. The best rooting response, 88%, was achieved on half-strength MS medium containing 0.5 mg/L indole-3-butyric acid (IBA). Root initiation was observed after 10–12 days of culture, and the roots were long, white, and fibrous in nature. Shoots cultured on full-strength MS medium or media containing higher auxin concentrations exhibited lower rooting efficiency and callus formation at the basal ends. The superiority of IBA in inducing adventitious roots in W. somnifera has also been reported by Sivanesan and Murugesan (2008), who observed that IBA promotes early root differentiation and stronger root architecture compared to IAA and NAA. Rooted plantlets were successfully acclimatized in plastic pots containing a mixture of soil, sand, and vermicompost (1:1:1) under greenhouse conditions, with a survival rate of approximately 85%.

3.4 Effect of Elicitors on Biomass and Secondary Metabolite Accumulation

To evaluate the influence of elicitors on biomass production and withanolide accumulation, well-established callus cultures were treated with biotic and abiotic elicitors including yeast extract, salicylic acid, and methyl jasmonate at different concentrations. The untreated callus served as the control. Among all treatments, callus cultures exposed to 100 µM salicylic acid showed the maximum increase in both fresh weight and withanolide content. This treatment enhanced total biomass by approximately 35% over the

control. Similarly, 50 µM methyl jasmonate significantly improved secondary metabolite production without adversely affecting callus growth. The combined elicitation effects were time-dependent, with peak metabolite accumulation recorded after 14 days of exposure. Quantitative analysis revealed that salicylic acid-treated callus accumulated higher levels of withaferin A and withanolide D, as confirmed by GC-MS profiling. These results suggest that elicitors act by inducing stress-responsive signal transduction pathways, which activate key enzymes involved in withanolide biosynthesis. Similar findings have been reported by Ray and Jha (2001) and Namdeo (2007), who demonstrated enhanced withanolide accumulation in elicitor-treated hairy root cultures of *W. somnifera*. The use of elicitors such as methyl jasmonate and salicylic acid mimics plant defense responses, leading to increased synthesis of secondary metabolites through upregulation of genes involved in terpenoid and steroidal pathways. The observed improvement in metabolite yield indicates that elicitor-mediated manipulation of cell cultures offers a viable biotechnological strategy for producing high-value phytochemicals in a controlled environment.

3.5 Overall Observations

From the overall analysis, it can be concluded that:

- Leaf explants respond better than nodal explants for callus induction and proliferation.
- The combination of 2.0 mg/L 2,4-D and 0.5 mg/L BAP is ideal for callus formation, while 1.5 mg/L BAP and 0.5 mg/L NAA yield optimal shoot regeneration.
- Rooting is most effective on half-strength MS medium supplemented with 0.5 mg/L IBA.
- Among elicitors, 100 µM salicylic acid and 50 µM methyl jasmonate significantly enhance biomass and withanolide production.

The findings are consistent with previous reports (Rani et al., 2003; Misra et al., 2008; Thomas and Kumar, 2010) and confirm that careful optimization of plant growth regulators and elicitor concentrations can substantially improve both morphogenic response and secondary metabolite synthesis in *W. somnifera* cultures.

4. Results

The experimental data obtained from the elicitor treatments demonstrated significant variations in secondary metabolite production among the different culture conditions. Quantitative estimation of withanolide content was performed using GC-MS analysis, and the results are summarized in Table 1. Statistical analysis indicated that the responses were significantly different ($p < 0.05$) between control and treated samples, confirming the positive influence of elicitor application on withanolide accumulation.

4.1 Effect of Elicitors on Withanolide Content

Treatment	Withanolide A (mg/g DW)	Withanolide D (mg/g DW)	Total Withanolides (mg/g DW)
Control	3.25 ± 0.18	2.10 ± 0.12	5.35 ± 0.22
Salicylic acid (100 µM)	6.80 ± 0.25	4.32 ± 0.19	11.12 ± 0.31
Methyl jasmonate (50 µM)	6.45 ± 0.27	4.05 ± 0.17	10.50 ± 0.33
Yeast extract (100 mg/L)	5.12 ± 0.22	3.45 ± 0.15	8.57 ± 0.28

The quantitative results clearly indicate that all elicitor treatments enhanced withanolide accumulation compared to the control cultures. The highest total withanolide content (11.12 ± 0.31 mg/g DW) was recorded in callus cultures treated with 100 µM salicylic acid, followed closely by those treated with 50 µM methyl jasmonate (10.50 ± 0.33 mg/g DW). Yeast extract also produced a considerable increase (8.57 ± 0.28 mg/g DW) compared to the untreated control (5.35 ± 0.22 mg/g DW), although the effect was less pronounced than that of abiotic elicitors. The increase in withanolide concentration under elicitor treatment suggests that both salicylic acid and methyl jasmonate acted as potent stress-inducing agents that triggered the plant's defense metabolism. These compounds likely activated signal transduction pathways involving reactive oxygen species and jasmonate signaling cascades, which subsequently upregulated key enzymes in the terpenoid biosynthetic pathway responsible for withanolide formation. Similar trends have been observed in earlier studies on *W. somnifera* by Ray and Jha (2001) and Rani et al. (2003), where abiotic elicitors led to enhanced accumulation of withaferin A and withanolide D.

4.2 Comparative Analysis of Elicitors

Among the tested elicitors, salicylic acid showed the most pronounced effect on total metabolite content, producing approximately a twofold increase over the control. The strong response to salicylic acid may be attributed to its role as a systemic acquired resistance (SAR) inducer, which stimulates the phenylpropanoid pathway and promotes the accumulation of secondary metabolites such as withanolides (Namdeo, 2007). Methyl jasmonate, a key signaling molecule in plant stress responses, also enhanced withanolide biosynthesis significantly, consistent with its ability to upregulate genes involved in steroidal lactone formation (Zhao et al., 2005). The yeast extract, representing a biotic elicitor, also improved metabolite content, though to a lesser extent. The response of biotic

elicitors is often variable and depends on the composition and concentration of the extract, as well as the developmental stage of the culture. The increase in withanolide yield under yeast extract treatment indicates that components such as polysaccharides and peptides within the extract may have activated plant defense mechanisms and induced secondary metabolite synthesis (Ghosh and Banerjee, 2003).

4.3 GC-MS Profiling and Peak Identification

The GC-MS chromatograms of methanolic extracts from the treated callus cultures revealed distinct peaks corresponding to major withanolides. The retention times (RT) for withaferin A and withanolide D were recorded at 21.4 and 25.7 minutes, respectively. The comparison of chromatograms between control and treated samples showed a marked increase in the intensity of these peaks under elicitor treatments, confirming the quantitative enhancement in metabolite concentration. Salicylic acid-treated samples exhibited the highest peak area for withaferin A, whereas methyl jasmonate treatment resulted in a relatively higher peak for withanolide D. This suggests that different elicitors may differentially regulate specific branches of the withanolide biosynthetic pathway. The overall GC-MS profile confirmed that the elicited cultures not only produced greater quantities of metabolites but also maintained the chemical identity of the naturally occurring compounds found in field-grown plants.

4.4 Biomass Correlation with Metabolite Content

A positive correlation was observed between biomass accumulation and total withanolide content in all treatments. Salicylic acid-treated cultures showed approximately 35% higher fresh weight compared to the control, which corresponded to the highest total withanolide yield. This observation supports the hypothesis that elicitor treatments not only stimulate secondary metabolism but may also improve overall cellular growth and metabolic activity (Sivanesan and Murugesan, 2008). The results demonstrate that biomass productivity and metabolite accumulation can be simultaneously enhanced under optimized elicitation conditions.

4.5 Statistical Significance and Comparative Evaluation

Statistical analysis using one-way ANOVA revealed that differences in withanolide content between control and elicitor-treated cultures were highly significant ($p < 0.05$). The standard deviations obtained from triplicate experiments were within acceptable limits, confirming the reproducibility of the results. Among the treatments, salicylic acid produced the highest fold increase (2.08 times the control) in total withanolide content, followed by methyl jasmonate (1.96 times) and yeast extract (1.60 times). The comparative evaluation highlights that abiotic elicitors are generally more effective than biotic ones in stimulating withanolide biosynthesis in *W. somnifera* cultures, possibly due to their more direct role in signal transduction pathways associated with stress metabolism (Namdeo, 2007).

4.6 Summary of Results

From the present investigation, it can be summarized that:

1. All elicitor treatments significantly enhanced the accumulation of withanolides in comparison to untreated controls.
2. Salicylic acid at 100 µM concentration was the most effective elicitor for total withanolide production, yielding 11.12 ± 0.31 mg/g DW.
3. Methyl jasmonate at 50 µM also exhibited strong stimulatory effects on metabolite accumulation.
4. Yeast extract, though less effective, still contributed to increased metabolite content and biomass growth.
5. GC-MS analysis confirmed the presence and enhancement of characteristic withanolide peaks in elicited cultures.

The findings clearly establish that elicitor-mediated manipulation of callus cultures can serve as a sustainable and reproducible biotechnological approach for the large-scale production of pharmacologically valuable withanolides from *W. somnifera*. The results also align with earlier studies by Ray and Jha (2001), Rani et al. (2003), and Misra et al. (2008), confirming the reliability of elicitation strategies in enhancing secondary metabolite yields in medicinal plants.

5. Conclusion

The present investigation successfully established an efficient and reproducible in vitro regeneration system for *Withania somnifera* (L.) Dunal, along with a validated elicitation strategy to enhance secondary metabolite production. The study demonstrated that the careful optimization of plant growth regulators and elicitor concentrations plays a decisive role in influencing morphogenic responses and metabolite biosynthesis. Among the tested hormonal combinations, the medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BAP proved to be the most effective for inducing callus from leaf explants, achieving a high induction rate of 92%. The callus obtained under these conditions was friable, fast-growing, and morphogenetically competent, indicating a high degree of cellular totipotency suitable for subsequent regeneration experiments. Shoot organogenesis was found to be most successful when the cultures were transferred to a medium containing 1.5 mg/L BAP and 0.5 mg/L NAA. Under these optimized conditions, the callus readily differentiated into multiple shoots, with an average of 8.3 shoots per explant. The regenerated shoots were vigorous

and morphologically normal, and their rooting response was best achieved on half-strength MS medium supplemented with 0.5 mg/L IBA, yielding a rooting efficiency of 88%. These results indicate that the balanced interaction of auxins and cytokinins determines both callus morphogenesis and shoot regeneration potential, consistent with earlier reports on *W. somnifera* by Sen and Sharma (1991) and Thomas and Kumar (2010). In addition to the regeneration protocol, the study explored the impact of different elicitors—salicylic acid, methyl jasmonate, and yeast extract—on the enhancement of secondary metabolite accumulation in callus cultures. Among these, abiotic elicitors demonstrated a stronger stimulatory effect on withanolide production. The treatment with 100 µM salicylic acid resulted in the highest total withanolide content (11.12 ± 0.31 mg/g DW), followed by 50 µM methyl jasmonate (10.50 ± 0.33 mg/g DW), whereas yeast extract (100 mg/L) also produced a noticeable improvement (8.57 ± 0.28 mg/g DW) compared to the control (5.35 ± 0.22 mg/g DW). These findings confirm that elicitor-mediated induction effectively activates stress-responsive metabolic pathways that regulate the biosynthesis of steroidal lactones such as withaferin A and withanolide D. GC-MS profiling further validated the biochemical responses induced by the elicitors. Distinct chromatographic peaks corresponding to withaferin A and withanolide D were observed at retention times of 21.4 and 25.7 minutes, respectively. The intensity of these peaks was markedly higher in elicited samples compared to the control, confirming a substantial increase in metabolite concentration. These results align with earlier observations made by Ray and Jha (2001) and Rani et al. (2003), who reported enhanced withanolide accumulation in elicitor-treated in vitro cultures of W. somnifera. The established protocol thus provides a reliable and sustainable alternative to conventional field cultivation for producing high-quality, withanolide-rich biomass. This approach offers several advantages, including year-round production, genetic uniformity, disease-free plant material, and controlled metabolite synthesis under defined environmental conditions. From a commercial standpoint, such an in vitro system can be used to supply standardized plant extracts for the pharmaceutical, nutraceutical, and herbal industries, reducing the dependency on natural populations and supporting biodiversity conservation.

Furthermore, the results of this study provide a foundation for future advancements in metabolic engineering and large-scale cultivation of *W. somnifera* under bioreactor-based systems. Integration of cell suspension cultures with optimized elicitor combinations could further increase metabolite yield and process efficiency. Additionally, studies employing molecular and transcriptomic approaches may help identify key regulatory genes involved in the withanolide biosynthetic pathway, facilitating targeted enhancement through genetic manipulation. In conclusion, the current research contributes to the growing field of plant biotechnology by demonstrating that the integration of in vitro regeneration techniques with elicitor-mediated secondary metabolite enhancement offers a sustainable, scalable, and environmentally friendly method for the commercial production of valuable phytochemicals. The findings not only support the conservation of *W. somnifera* but also strengthen its industrial and therapeutic potential as a consistent source of bioactive compounds for modern medicine.

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