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"Enhancement of Secondary Metabolites of *Withania somnifera* using Biological Approaches"

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SRIVIPRA PROJECT 2025

Title : **Enhancement of Secondary Metabolites of *Withania somnifera* using Biological Approaches**

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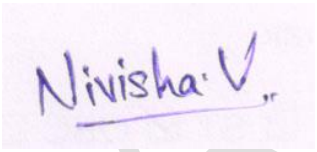
1		Nivisha V.	**1423038**	B.Sc. (H) Botany Sem V	
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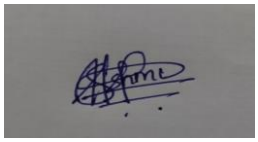
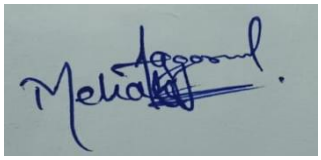
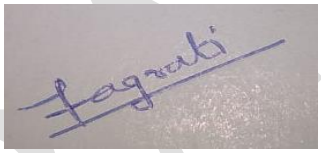
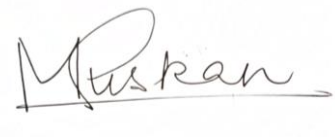
4		Sreelakshmi S	**1423054**	B.Sc. (H) Botany Sem V	

5		Mehak Aggarwal	**1123187**	B.Sc. Life Science Sem V	
6		Jagrati Bhardwaj	**1423048**	B.Sc. (H) Botany Sem V	
7		Muskan Modi	**1424045**	B.Sc. (H) Botany Sem III	
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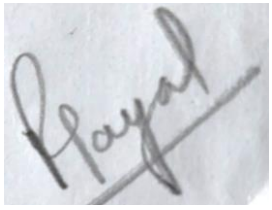

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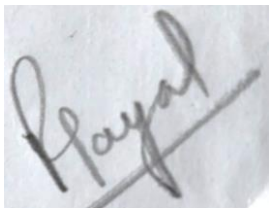
Certificate of Originality

This is to certify that the aforementioned students from Sri Venkateswara College have participated in the summer project SVP 2517- titled "**Enhancement of Secondary Metabolites of *Withania somnifera* using Biological Approaches**". The participants have carried out the research project work under our guidance and supervision from 1st July, 2025 to 30th September 2025. The work carried out is original and carried out in an online/offline/hybrid mode.



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Introduction

Withania somnifera (L.) Dunal, commonly known as Ashwagandha, is a revered medicinal plant in Ayurveda with over 3,000 years of traditional use for promoting vitality and managing stress-related ailments [Poojari et al., 2019]. It is native to arid regions of India, the Middle East, and parts of Africa. It is primarily cultivated for its roots and leaves that contain pharmacologically active compounds [Verma and Shukla, 2015]. These therapeutic properties are largely attributed to its diverse group of secondary metabolites, especially withanolides and withanosides, which are steroidal lactones exhibiting anti-inflammatory, antioxidant, antitumor, and immunomodulatory effects [Mohammad et al., 2022].

Modern studies have confirmed the efficacy of Ashwagandha in reducing oxidative stress by elevating antioxidant enzyme levels and in regulating inflammatory pathways through cytokine modulation [Sridharan and Sakthivel, 2015; Anastasia, 2021]. Withanolide A and Withaferin A, among the most potent constituents, are responsible for enhancing neuroprotection, reducing tumor progression, and supporting endocrine function [Semenzato and Fani, 2024]. Traditional extraction methods such as Soxhlet and maceration are still widely used. However, recent advances include microwave-assisted and ultrasonic techniques that yield higher metabolite concentrations while preserving their bioactivity [Mohammad et al., 2022].

However, reliance on chemical fertilizers and pesticides in cultivation raises environmental and health concerns by potentially altering the plant's phytochemical profile and contributing to soil degradation [Rana et al., 2021]. This has driven the exploration of sustainable biological alternatives such as Plant Growth-Promoting Rhizobacteria (PGPR), Arbuscular Mycorrhizal Fungi (AMF), and microbial elicitors, which can enhance metabolite production while maintaining soil health [Bogani et al., 2021; BALDI et al., 2020]. These bio-based interventions not only improve plant biomass and nutrient uptake but also trigger stress-related metabolic pathways that stimulate secondary metabolite biosynthesis [Kavitha et al., 2022; Verma et al., 2022].

The current review synthesizes findings on biological approaches for enhancing secondary metabolites in W. somnifera, emphasizing their mechanisms, efficacy, and practical applications for sustainable medicinal plant production.

Plant Growth Promoting Rhizobacteria (PGPR)

Traditional methods of cultivation often yield inconsistent results regarding the production of these metabolites, prompting exploration into more sustainable and efficacious approaches. One such method is the use of plant growth-promoting rhizobacteria (PGPR), which have been shown to influence the metabolic processes of plants, thereby enhancing the synthesis of secondary metabolites (Bogani et al., 2021). The incorporation of PGPR can lead to increased biomass and improved phytochemical profiles, making them invaluable in agricultural practices aimed at optimising the yield of medicinal plants (Mohammad A et al., 2022). This exploration holds promise for both ecological sustainability and the pharmaceutical industry (BALDI et al., 2020). Plant Growth Promoting Rhizobacteria (PGPR) represent a diverse group of beneficial bacteria residing in the rhizosphere, the soil region surrounding plant roots. These microorganisms establish symbiotic relationships with plants, thriving in the nutrient-rich environment near the roots (Bhattacharyya & Jha, 2011). PGPR facilitates plant growth and health through both direct and indirect mechanisms. They enhance nutrient acquisition by fixing atmospheric nitrogen, increasing the solubility of minerals such as phosphorus, and synthesizing phytohormones like

auxins and cytokinins, which regulate plant growth (Lugtenberg & Kamilova, 2009). Additionally, PGPR can function as biocontrol agents by competing for nutrients or producing antimicrobial compounds that inhibit the proliferation of plant pathogens. This symbiotic interaction enhances soil productivity and nutrient availability, thereby improving plant growth, root development, and crop yield. Consequently, this reduces the dependency on costly chemical fertilizers, promoting environmental sustainability (Vejan et al., 2016).

Uses and role of PGPR

The interaction between PGPR and plant roots fosters enhanced growth through mechanisms such as nutrient solubilisation, production of growth hormones, and suppression of plant diseases (Anastasia et al., 2021). For instance, these bacteria can enhance the bioavailability of essential nutrients such as phosphorus and nitrogen, thereby contributing to improved plant health and productivity (Bogani et al., 2021). Specifically, studies have shown that endophytic bacteria enhance the synthesis of secondary metabolites in medicinal plants, leading to increased concentrations of bioactive compounds (Semenzato et al., 2024). This phenomenon is particularly relevant for *Withania somnifera*, where PGPR can potentially elevate the levels of valuable secondary metabolites that confer medicinal benefits (Kavitha et al., 2022). Therefore, harnessing PGPR not only optimises growth conditions but also enriches the phytochemical profile of important medicinal flora. Specifically, the consortium works at the cellular level in the target plant's rhizosphere directly or indirectly. It makes the plant produce a higher level of secondary metabolites through the given possible mechanisms.

Impact of PGPR on Secondary Metabolite Production

The intricate relationship between *Withania somnifera* and plant growth-promoting rhizobacteria (PGPR) significantly impacts the production of secondary metabolites, which are vital for the plant's therapeutic efficacy (Pandey et al., 2018). Studies have demonstrated that the presence of PGPR can markedly elevate the levels of key bioactive compounds, such as withanolides and alkaloids, which are notable for their medicinal properties (Mohammad A et al., 2022). Additionally, the modulation of phytohormonal pathways by these beneficial microbes enhances the plants' stress responses, thereby fostering a conducive environment for metabolic processes that produce these valuable metabolites (Semenzato et al., 2024).

Mechanisms of action of PGPR in enhancing plant growth and health

PGPR acts in various pathways, which ultimately can result in a gradual increase in the gross yield of the medically important contents of the plant. The enhancement of secondary metabolite production in *Withania somnifera*, facilitated by plant growth-promoting rhizobacteria (PGPR), involves a complex interplay of physiological and biochemical mechanisms. These microorganisms influence biomass accumulation, alter gene expression associated with metabolite biosynthesis, and modulate phytohormone levels, which collectively drive increased secondary metabolite yields (Tsukanova et al., 2017). Studies have shown that specific PGPR strains, such as Pseudomonas and Bacillus, can enhance the expression of genes involved in secondary metabolite pathways, thereby resulting in higher concentrations of bioactive compounds (Vacheron et al., 2013). Additionally, the role of phytohormones, such as auxins and cytokinins, released by PGPR is critical in stimulating plant growth and enhancing metabolite production

(Cassán et al., 2013; Bogani et al., 2021; Semenzato et al., 2024). These interactions facilitate the secretion of growth-promoting substances, including phytohormones such as indole-3-acetic acid (IAA), which directly influence plant growth and resilience to stressors (Bogani et al., 2021). Furthermore, endophytic bacteria within medicinal plants contribute to stress mitigation by eliciting defensive responses, subsequently leading to an increase in the biosynthesis of bioactive compounds, fundamental for the therapeutic properties of these plants (Semenzato et al., 2024). As a result, harnessing PGPR not only optimises growth but also significantly enhances the pharmacologically relevant secondary metabolites in *Withania somnifera*, underlining their potential in sustainable agriculture (Mohammad A et al., 2022). By employing various methodologies, including flow cytometry and transcriptomic analyses, researchers are able to systematically compare the efficacy of different microbial strains in augmenting biomass and secondary metabolite levels, providing a deeper understanding of the mechanisms at play (Del Rosario Cappellari et al., 2015; Bogani et al., 2021). Furthermore, Table 1.1 illustrates the differential outcomes of these interactions across various PGPR strains, underlining their potential in agricultural and pharmaceutical applications (Mohammad A et al., 2022).

Specific secondary metabolites enhanced by various PGPR types in Withania somnifera

The intricate relationship between *Withania somnifera* and plant growth-promoting rhizobacteria (PGPR) significantly impacts the production of secondary metabolites, which are vital for the plant's therapeutic efficacy. Studies have demonstrated that the presence of PGPR can markedly elevate the levels of key bioactive compounds, such as withanolides and alkaloids, which are notable for their medicinal properties (Mohammad A et al., 2022). Additionally, the modulation of phytohormonal pathways by these beneficial microbes enhances the plants' stress responses, thereby fostering a conducive environment for metabolic processes that produce these valuable metabolites (Semenzato et al., 2024). (Table 1.1)

Table 1.1 Different PGPR bacterial types and their effects on the *W. somnifera* seedlings treated in standard growth conditions, and their effect on the increase of different types of secondary metabolites, like withaferin, withanolides

PGPR Type/Strain	Description & Methodology	Pros	Cons	Effect on Secondary Metabolites	Reference
Azospirillum sp.	Free-living N2-fixer; isolated from rhizosphere. Used as a seed coat/root dip or in consortium; applied before or at transplantation.	Enhances N uptake, shoot/root growth, and increases yield	The effect varies with soil type/strain; alone, it may be less effective	Increases root withanolides and withaferin A content	Rizvi, A., Ahmed, B., Khan, M. S., El-Beltagi, H. S., Umar, S., & Lee, J. (2022). Bioprospecting Plant Growth Promoting Rhizobacteria for Enhancing the Biological Properties and Phytochemical Composition of Medicinally Important Crops. Molecules (Basel, Switzerland), 27(4), 1407. https://doi.org/10.3390/molecules27041407

Azotobacter chroococcum	Nitrogen-fixing bacterium; typically applied as a soil inoculant or root dip with saplings before planting; often part of a consortium.	Fixes atmospheric N, improves plant vigor	May show limited vigor effects in nutrient-rich soils	Boosts total alkaloid (withanolide) levels in roots	R. Elango, S. R., and.. (2011). Effect of microbial consortium on plant growth and improvement of alkaloid content in *Withania somnifera* (Ashwagandha). Current Botany, 2(8). Retrieved from https://updatepublishing.com/journal/index.php/cb/article/view/1371
Pseudomonas fluorescens	Siderophore producer; often introduced as a root drench or soil inoculant with or without other PGPR; survives UV-B and abiotic stress.	Disease suppression, phosphate solubilization, and abiotic stress tolerance	Needs viable living cells; less persistence under harsh conditions	Enhanced with aferin A, overall withanolides; improved resilience	Rathaur, P., *, Raja, W., *, Ramteke, P. W., & John, S. A. (2012). Effect of UV-B Tolerant Plant Growth Promoting Rhizobacteria (PGPR) on Seed Germination and Growth of *Withania somnifera*. Advances in Applied Science Research, 3, 1399–1404.
Bacillus subtilis/megaterium	Spore-forming PGPB; inoculum prepared in broth/carrier (liquid or solid); used alone or with others; survives diverse soil/weather conditions.	Increases shoot and root weight, stress resistance	Some strains may not colonize all soils equally	Promotes biosynthesis of withaferin A and other alkaloids	
PGPR Consortium	Mix of Azospirillum, Azotobacter, Pseudomonas, Bacillus; roots dipped or soil inoculated before planting; can be used alone	Synergy boosts growth/alkaloids more than individual strains	Interaction effects are sometimes unpredictable, and technical complexity	45–189% increase in alkaloid (withanolide) content, max. vigour	

	or repeated.				

Arbuscular mycorrhizal fungi [AMF]

What are Arbuscular mycorrhizal fungi?

Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota, are essential for plant health and ecological sustainability due to their widespread symbiotic associations with vascular plants (van der Heijden et al., 1998; Smith & Smith, 2011; van der Heijden et al., 2015). These fungi improve a plant's resistance to environmental stressors, particularly under conditions of drought and nutrient scarcity, while also facilitating efficient uptake of vital minerals, especially phosphorus (Chen M et al., 2018). Beyond nutrient acquisition, their role in maintaining soil fertility, reducing negative impacts of intensive farming practices, and enhancing biodiversity has made AMF a cornerstone of sustainable agriculture (Browne et al., 2010; Riedo J et al., 2021; Eduardo K. Mitter et al., 2021). Recent studies suggest that AMF colonization in Ashwagandha roots improves biomass, enhances nutrient dynamics, and significantly modulates the synthesis of withanolides, thereby directly increasing its medicinal quality and value (Alfiky A et al., 2021; Avio L et al., 2020). Studies report that AMF inoculation helps Ashwagandha plants tolerate abiotic stresses such as drought and salinity, conditions under which medicinal plants often show decreased productivity and metabolite accumulation (Kaur et al., 2019).

By improving soil structure and microbial diversity in the rhizosphere of Ashwagandha, AMF supports a healthier growing environment, which reduces the need for chemical fertilizers and pesticides, aligning with organic and sustainable cultivation practices (Singh et al., 2020). AMF can influence the expression of genes associated with secondary metabolite pathways in Ashwagandha, suggesting a molecular-level enhancement of its medicinal properties through fungal symbiosis (Sharma et al., 2021). Incorporating AMF in Ashwagandha cultivation can be a strategic biofertilizer approach that complements traditional farming, especially in nutrient-poor soils where Ashwagandha is often grown for herbal medicine production (Gupta et al., 2022). Given the increasing global demand for high-quality Ashwagandha, utilizing AMF not only boosts plant productivity but also promotes higher quality and consistency in bioactive compound content, which is essential for pharmaceutical uses (Mishra et al., 2023).

Role of Arbuscular Mycorrhizal Fungi (AMF)

Research reveals that AMF inoculation promotes the accumulation of primary metabolites such as proteins and carbohydrates, coupled with secondary metabolites such as phenolics, resulting in increased biomass yields for *W. somnifera.* (Reddy M et al.). Furthermore, Arbuscular mycorrhizal fungi may enhance the accumulation of active substances in medicinal plants such as Withania somnifera, making mycorrhizal technology a potential and sustainable tool for improving growth and secondary metabolite production" (Johny et al., 2021). Additionally, these fungi enhance the therapeutic properties of plants like ashwagandha by promoting nutrient absorption and increasing resilience to environmental stress (ABD-ALLAH et al.). According to (Bora et al.), the investigation of AMFs' effects on the biochemical profile of *W. somnifera* thus identifies encouraging directions for further study. Arbuscular mycorrhizal

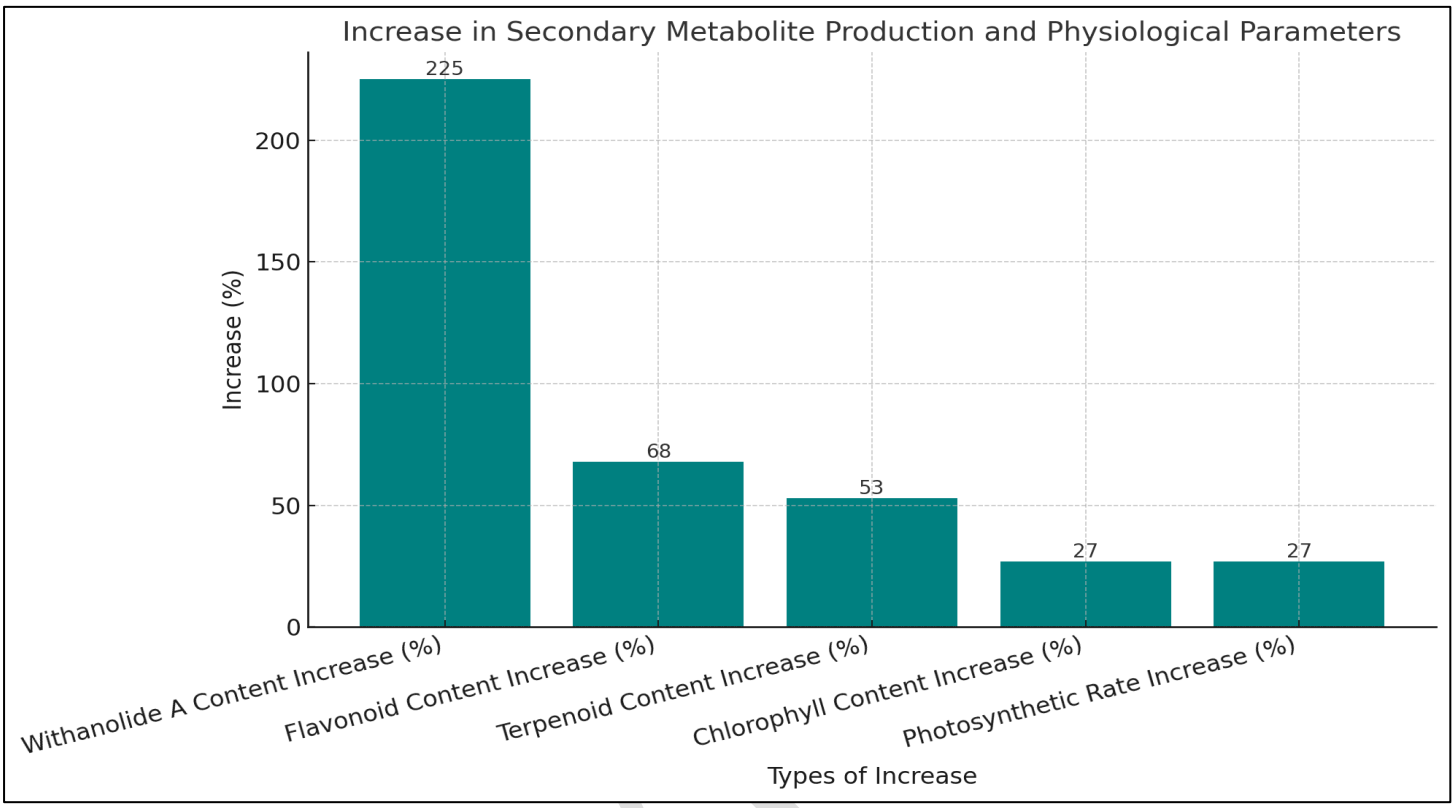
fungi (AMF) and plant roots have a symbiotic interaction that is essential for promoting plant development, especially in situations with low nutrient levels.

AMF's mechanisms for boosting secondary metabolite synthesis and plant growth

Arbuscular mycorrhizal fungi colonize plant root cortical cells, forming arbuscules that facilitate nutrient exchange (Smith and Read, 2008). AMF improves the absorption of phosphorus, nitrogen, and micronutrients, which are crucial for plant metabolism (Jiang et al., 2021). AMF enhances nutrient uptake, especially phosphorus, which is essential for the synthesis of several metabolites, including those present in *Withania somnifera*, by developing symbiotic relationships with plant roots. Because AMF-treated plants have substantially higher fresh and dry weights than their uninoculated counterparts, this improved nutrient absorption helps to increase biomass production (Reddy et al.). Additionally, it has been demonstrated that the connection with AMF increases the content of secondary metabolites, including phenolics, which are essential for the plant's defensive mechanisms and possible medicinal advantages (Johny et al.). Further research highlights AMF's capacity to enhance phytochemical synthesis in stressful environments, boosting *Withania somnifera* effectiveness as a therapeutic agent (Amasha et al.). Therefore, a viable path for agricultural biotechnology is represented by the processes through which AMF promote the formation of secondary metabolites as well as plant growth (Anastasia et al.). Similarly, Verma et al. (2022) found that AMF symbiosis stimulates secondary metabolite biosynthesis both directly, by increasing nutrient uptake and biomass, and indirectly, by inducing stress-related metabolic pathways. These findings highlight the dual role of AMF in metabolic enhancement (Kumar et al., 2023).

AMF's Interaction Mechanisms with *Withania somnifera*

Arbuscular mycorrhizal fungus (AMF) and host plants develop complex connections that are essential for improving the uptake of nutrients and the synthesis of secondary metabolites. Through a vast network of fungal hyphae, mycorrhizal connections allow plants such as Withania somnifera to access limited soil nutrients, hence enhancing the plant's potential for absorption. These interactions have been demonstrated in studies that show that inoculating plants with specific AMF species—specifically, *Glomus mosseae, G. fasciculatum, and G. macrocarpum*—significantly enhances both primary metabolites and phenolic compounds. These microbes have demonstrated marked increases in plant biomass and metabolite production when compared to uninoculated controls (Reddy M et al., 2014). Additionally, studies highlight mycorrhizas as strong elicitors of antioxidant chemicals in root systems, which may promote increased phytochemical variety and plant resistance (Srivastava et al., 2015). Together, these results highlight the complex function of AMF in supporting plant development as well as aiding in the manufacture of medicinal chemicals necessary for Withania somnifera's therapeutic qualities (Johny et al., 2015; Mansotra et al., 2015).



The bar chart illustrates the enhancement in secondary metabolite production in *Withania somnifera* when inoculated with arbuscular mycorrhizal fungi (AMF) compared to those without inoculation. The plants with AMF inoculation show a concentration of 35 mg/g dry weight, while the control group without AMF has a concentration of 20 mg/g dry weight. This highlights the crucial role of AMF in boosting the production of beneficial therapeutic compounds.

Effect of AMF on Secondary Metabolites

AMF is necessary for the production of numerous secondary metabolites, such as alkaloids and steroids, which are vital for the defence mechanisms and therapeutic properties of plants (Debi & Parkash, 2020). Research has shown that AMF increases metabolite profiles by causing physiological and biochemical alterations in host plants in addition to increasing nutrient availability (Pandey et al., 2023). Furthermore, the application of ecological farming techniques, such as organic mulching and no-tillage, has shown promising results in sustaining AMF populations, which has contributed to the synthesis of bioactive compounds (Da-Hao C et al., 2023). Arbuscular mycorrhizal fungi (AMF) engage in a complex, highly regulated symbiosis with plants that profoundly affects gene expression related to secondary metabolite production and root growth (Chauhan et al., 2023) The symbiotic process begins with molecular signaling between the plant and fungi, leading to root colonization and arbuscule formation in root cortex cells, which serve as the nutrient exchange interface (Yang, 2019). This interaction triggers transcriptional reprogramming, activating specific transcription factors such as GRAS family proteins that regulate downstream gene cascades essential for arbuscule development and function (Rich et al., 2017). AMF upregulate genes involved in key biosynthetic pathways for secondary metabolites—including alkaloids, flavonoids, terpenoids, and phenolics—as well as genes responsible for lipid biosynthesis and transport,

which are critical for maintaining the symbiotic interface (Xu, 2023). Concurrently, AMF modulate phytohormone signaling pathways, especially involving auxin and ethylene, which orchestrate changes in root architecture like increased lateral root formation and branching. This root remodeling enhances nutrient uptake capacity and metabolic activity. ("Erratum," 2018). Genes encoding phosphate and ammonium transporters are also upregulated, increasing the plant's ability to acquire essential minerals, thereby supporting greater metabolic energy for secondary metabolite synthesis (Tarnabi et al., 2019; Pimprikar & Gutjahr, 2018). Together, the coordinated regulation of these diverse gene sets by AMF results in enhanced secondary metabolite accumulation and optimized root growth, facilitating improved plant health, nutrient acquisition, and overall adaptability in various soil conditions (Tarnabi et al., 2019b). This transcriptional orchestration highlights AMF's critical role as biotic modulators of plant metabolism and development through multi-layered genetic and biochemical pathways, underscoring their importance in sustainable agriculture and plant productivity (Tarnabi et al., 2019; Pimprikar & Gutjahr, 2018).

AMF Species	**Withaferin-A Increase (%)**
Rhizophagus irregularis	11.5 to 43.5
Claroideoglomus etunicatum	11.5 to 43.5
Claroideoglomus claroideum	11.5 to 43.5
Acaulospora delicata	11.5 to 43.5
Glomus hoi	11.5 to 43.5

S.No	**Experimental Approach**	**Description**	**Reference**
1	Pot Experiment with AMF	Growing *W. somnifera* seedlings in sterilized soil with AMF (e.g., *Glomus mosseae*) to observe growth and metabolite changes	Rana and Singh, 2020
2	In Vitro Tissue Culture with AMF	Using micropropagated plantlets with AMF in sterile media to monitor metabolite production under controlled conditions	Verma et al., 2022
3	Field Trials	Applying AMF in open field conditions to evaluate plant growth and secondary metabolite yield under natural stress	Kapoor et al., 2021
4	Biochemical Analysis	Measuring levels of withanolides, phenolics, etc., using HPLC after AMF treatment	Kumar et al., 2022
5	Molecular Analysis	Analyzing the expression of genes involved in metabolite biosynthesis through qRT-PCR post-AMF inoculation	Jiang et al., 2021
6	Stress Simulation Studies	Combining AMF with stress factors (drought, salinity) to evaluate the impact on secondary metabolite accumulation	Verma et al., 2022

Microbial elicitors

Introduction

Among the various biotechnological strategies used to enhance secondary metabolite production in *Withania somnifera*, elicitation using microbial agents has emerged as one of the most efficient and sustainable approaches. Microbial elicitation involves the use of biologically derived molecules or cells, such as bacterial lipopolysaccharides, fungal chitin, or whole endophytic microbes, that simulate pathogen attacks and trigger complex plant defense signaling cascades (Bogani et al., 2021; Kavitha et al., 2022).

Microbial elicitors fall under the category of biotic elicitors, originating from bacteria, fungi, or viruses, and can include molecules such as microbial cell wall fragments, exopolysaccharides, glycoproteins, and other microbial metabolites (Namdeo, 2007; Sivanandhan et al., 2014). Their application leads to systemic resistance and metabolic reprogramming, channeling plant energy and resources into the enhanced biosynthesis of bioactive compounds like withanolide A, withaferin A, and other therapeutically important phytochemicals in *W. somnifera* (Mohammad et al., 2022; Singh et al., 2020). This technique not only increases target compound levels but also contributes to sustainable and eco-friendly cultivation strategies by reducing the dependence on synthetic inducers or chemical stressors.

In contrast, abiotic elicitors consist of physical or chemical factors such as UV light, salinity, drought, temperature, heavy metals, and compounds like methyl jasmonate (MeJA), salicylic acid (SA), or hydrogen peroxide (H_2O_2) (Kavitha et al., 2022; Saini et al., 2023). These elicitors also induce plant defense responses and secondary metabolism through signal perception and transduction pathways, often overlapping with those triggered by microbial elicitors. While abiotic elicitation is commonly used in cell suspension and tissue cultures for controlled enhancement of metabolite biosynthesis, biotic elicitors offer an added advantage of long-term plant-microbe symbiosis and sustainability in field conditions (Verma et al., 2018; Abdel Latef et al., 2021).

Mechanism of microbial elicitors in enhancing secondary metabolites

Microbial elicitors interact with plant cells through surface molecules such as lipopolysaccharides, glycoproteins, or chitin fragments that are recognized by pattern recognition receptors (PRRs) on the plant plasma membrane (Zhao et al., 2021). The interaction between plant cells and microbial elicitors often initiates a cascade of signaling events, including the generation of reactive oxygen species (ROS), ion fluxes, activation of MAPKs (mitogen-activated protein kinases), and upregulation of defense-related genes (Anastasia et al., 2021). These events collectively enhance the expression of genes involved in the biosynthesis of key metabolites, such as withanolides in W. somnifera (Semenzato & Fani, 2024). Microbial elicitors also modulate plant hormone levels, such as jasmonic acid and salicylic acid, which further regulate metabolite synthesis pathways (Kavitha et al., 2022; Abdel Latef et al., 2021).

Microbial elicitation methods for *Withania somnifera*

1. Foliar Spray Method

In this method, microbial elicitors or their metabolites are applied directly to the leaf surface through a fine mist sprayer, enabling uptake through stomata or cuticular pores. This technique is advantageous because it is scalable, cost-effective, and induces systemic resistance in a non-invasive manner (Abdel Latef et al., 2021). However, it has limitations such as dependence on environmental conditions like rainfall and the necessity of multiple treatments for consistent results (Egamberdieva et al., 2017). A case

study by Egamberdieva et al. (2017) and Rana et al. (2021) demonstrated that application of *Bacillus subtilis* and *Pseudomonas fluorescens* as foliar sprays significantly enhanced biomass and withanolide content in greenhouse conditions.

2. Root Drench Method

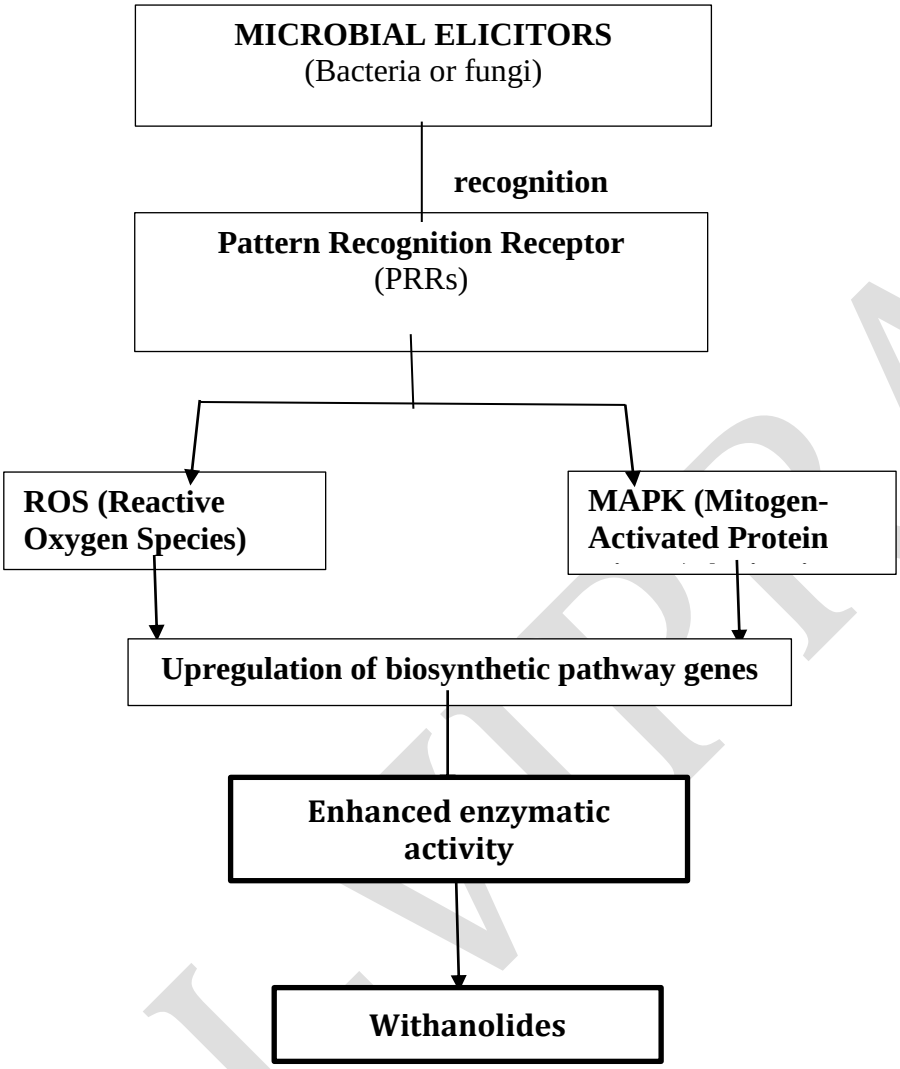
This technique involves applying microbial suspensions directly to the rhizosphere. The method was successfully applied in a study where root inoculation with PGPR (Plant Growth-Promoting Rhizobacteria) significantly enhanced withaferin A levels through rhizospheric colonization (Egamberdieva et al., 2017; Rana et al., 2021). Another case study includes endophyte-associated enhancement of isoprenoid pathway genes (DXS/DXR) and photosynthetic performance in *W. somnifera* (Pandey et al., 2018). This method facilitates sustained microbial interaction, promotes root development, and improves nutrient uptake, often synergizing with mycorrhizae (Egamberdieva et al., 2017). However, the variable composition of soil microbiota can reduce reproducibility, making it less predictable compared to in vitro systems (Rana et al., 2021).

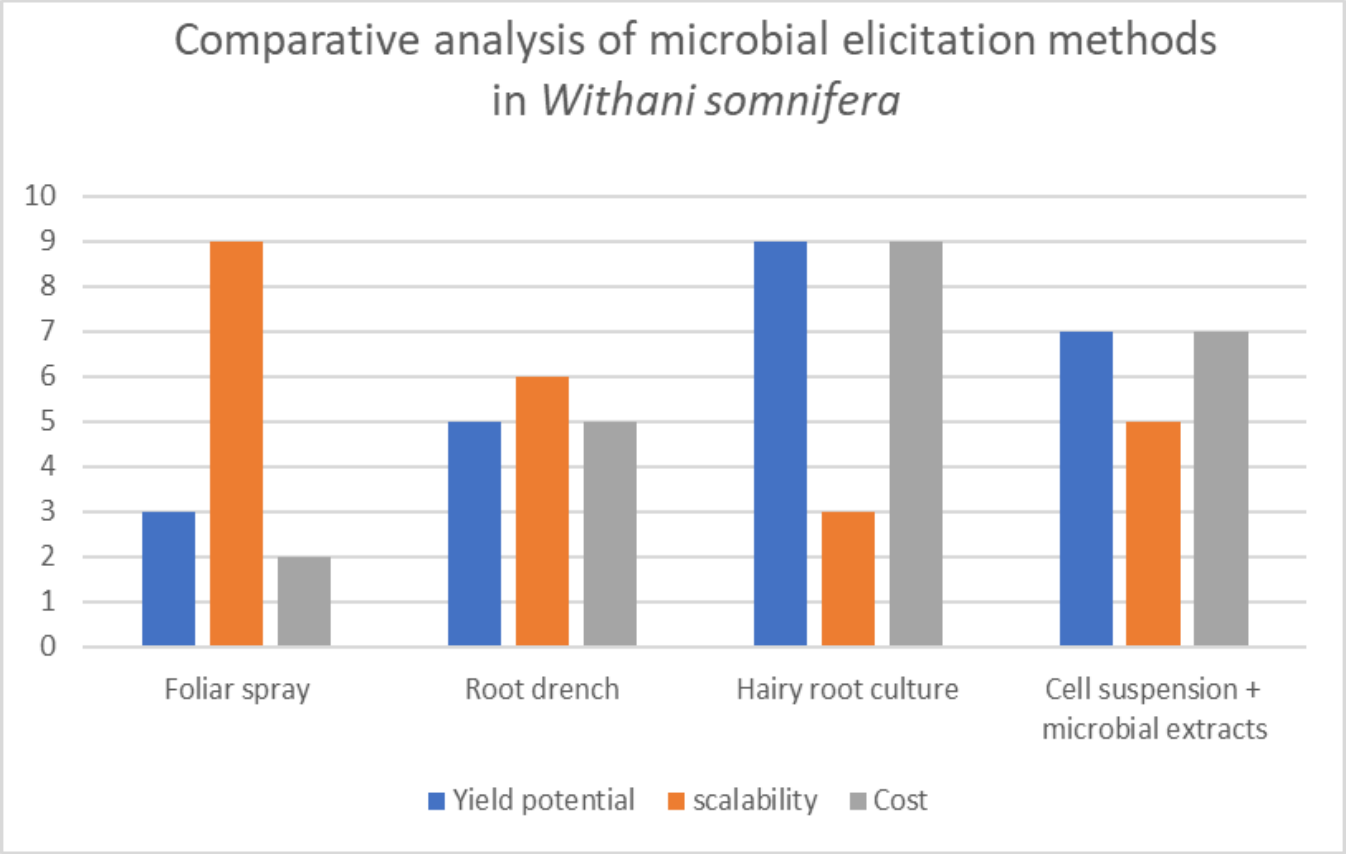
3. Hairy Root Culture

Hairy roots are induced via infection with Agrobacterium rhizogenes, which integrates root-inducing (Ri) genes into plant genomes, producing stable, fast-growing root tissues. Studies have shown that hairy root cultures produce 3-5 times more withanolides than intact plants (Mohammad et al., 2022; Saravanakumar et al., 2021). While this method allows for large-scale metabolite production and is genetically stable, it requires strict sterile conditions, technical expertise, and may encounter regulatory concerns due to its transgenic nature (Giri & Narasu, 2000; Saravanakumar et al., 2021).

4. Cell Suspension Culture with Microbial Extracts

This method involves culturing plant callus in liquid MS medium and treating it with microbial elicitors like lipopolysaccharides (LPS), fungal chitin, or crude. This method is highly controllable and precise, making it ideal for pathway analysis, transcriptomics, and optimization studies (Vinod et al., 2022). However, the requirement for sterile culture conditions and equipment makes it less viable for field-scale or resource-limited settings (Namdeo, 2007). Vinod et al. (2022) demonstrated that treatment with *Kappaphycus alvarezii* (seaweed) extract in bioreactor cultures led to a 3.05-fold increase in withaferin A content. Similar enhancements were observed using LPS and fungal chitin in a dose-dependent manner in callus cultures (Namdeo, 2007; Sivanandhan et al., 2014).


MICROBIAL ELICITORS
(Bacteria or fungi)
recognition
Pattern Recognition Receptor
(PRRs)
ROS (Reactive Oxygen Species)
MAPK (Mitogen-Activated Protein
Upregulation of biosynthetic pathway genes
Enhanced enzymatic activity
Withanolides



Co-cultivators

Researchers have been exploring ways to boost the production of secondary metabolites in medicinal and agriculturally important plants. These valuable compounds can help us make better use of the natural resources these plants offer. By using various microbial approaches, we can increase the secondary metabolites in Ashwagandha.

What are Co-Cultivators?

Co-Cultivators (Usually bacteria or Fungi) are the organisms that are purposely added to a microbial culture to observe the combined effect caused due to their interactions with them. These interactions can be mutualistic, competitive, antagonistic, or synergistic, and they often lead to enhanced metabolic activities, activation of silent biosynthetic gene clusters, or production of novel bioactive compounds that are not expressed in monocultures. Co-cultivation is widely used in biotechnology, ecology, medicine, and bioprocessing to harness the collective potential of microbial communities.

Types of Co-cultivators

Co-cultivators are broadly classified on 2 bases :

1. Based on the Microbial species involved.
2. Based on the type of interactions among them.

Based on the Microbial species involved :

- **Based on bacterial-bacterial co-cultures**: Two different strains of bacteria are cultured together in shared environments, to study their interaction behavior and combined effects. For ex, the Co-cultivation of Escherichia coli and Bacillus paratyphosus to study biochemical interactions.
- **Based on Fungi-fungi co-culture**: Fungal-fungal co-culture involves growing two or more fungal strains together in a controlled environment to stimulate the production of novel secondary metabolites (SMs) or enhance enzyme activity, often mimicking their natural competitive interactions. For example, the Co-cultivation of *Trichoderma harzianum* and *Talaromyces pinophilus* produces siderophores like ferrirubin and harzianic acid, which aid in iron solubilization and plant growth promotion.
- **Based on bacteria-fungus co-culture:** Bacteria-fungi co-culture involves growing bacteria and fungi together to study their interactions, often to enhance the production of secondary metabolites, improve bioprocessing, or explore ecological relationships. This approach mimics natural microbial communities where the interactions, like competition, symbiosis, or antagonism, can activate silent biosynthetic gene clusters, leading to novel compounds or improved yields. Ex: Co-culture of *Saccharomyces cerevisiae* (yeast) and lactic acid bacteria (LAB) to induce biofilm formation in yeast, enhancing microbial survival in harsh conditions.
- **Based on Microbe plant and microbe host culture**: Co- Co-cultivation of plants and microbes, or microbes with a host culture, involves growing these organisms together in a controlled setting to study their interactions, enhance plant growth, or produce valuable compounds. For example, the Co-cultivation of nitrogen-fixing bacteria (Rhizobium) with legume plants to form nodules for nitrogen fixation.

Based on interactions between the microbes

- **Mutualistic**: Both species benefit, e.g., cyanobacteria providing organic carbon to heterotrophic bacteria for product synthesis.
- **Commensalistic**: One species benefits, while the other is unaffected, e.g., one microbe producing a metabolite that another uses without reciprocation.
- **Competitive**: Microbes compete for resources like nutrients or space, often triggering the production of secondary metabolites like antibiotics.
- **Antagonistic**: One microbe inhibits another, e.g., through the production of siderophores that limit iron availability to competitors.
- **Synergistic**: Combined metabolic activities produce outcomes not achievable by individual species, e.g., co-cultures degrading complex substrates like lignocellulose.

How do co-cultivators work?

Co-cultivator microbes interact through physical, chemical, or metabolic mechanisms, which can be harnessed for specific purposes.

Physical Contact

When microbes (like bacteria or fungi) physically touch the plant's roots, root hairs, or cells, they can trigger changes in the plant's behavior, like turning on specific genes or forming protective layers. Direct contact between *Withania somnifera* roots and microbes like arbuscular mycorrhizal fungi (*Glomus intraradices*) or endophytic fungi (*Trichoderma harzianum*) can activate defense-related genes in the

plant. For example, fungal hyphae penetrating root cells signal the plant to produce more withanolides as a defense response.
Example: In co-cultures, *Trichoderma* physically interacting with *Withania somnifera* roots has been shown to increase withanone production by 1.5-fold, likely due to direct contact triggering the plant's stress response pathways.

Chemical signaling
Microbes release chemical "messages" (like quorum-sensing molecules) that act like signals to tell the plant or other microbes to activate certain genes, often leading to the production of new or more secondary metabolites. Microbes like Pseudomonas fluorescens or *Aspergillus* species release signaling molecules (e.g., lipopolysaccharides or volatile organic compounds) that "talk" to *Withania somnifera* cells. These signals can switch on genes in the mevalonate (MVA) or responsible for making withanolides.
Example: Co-culturing with *Streptomyces* species, which produce quorum-sensing molecules like N-acyl homoserine lactones, can activate silent biosynthetic genes in *Withania somnifera*, leading to increased diversity of withanolides.

Nutrient Cross-feeding
One organism produces a nutrient that another uses to grow or make compounds. It's like one microbe "feeding" the plant or another microbe to help it thrive. Arbuscular mycorrhizal fungi (AMF) like *Rhizophagus* irregularis provide *Withania somnifera* with nutrients like phosphorus or nitrogen from the soil. In return, the plant supplies the fungi with sugars. This nutrient boost helps the plant grow stronger and produce more secondary metabolites, such as withanolide D (increased by 15% in AMF-colonized plants).
Example: In soil-based systems, *Azotobacter chroococcu*m fixes nitrogen for *Withania somnifera*, improving plant vigor and increasing total withanolide content by 20–25% compared to non-inoculated plants.

Competition for resources
Microbes and plants compete for limited resources like nutrients (e.g., iron), which can push them to produce specialized compounds to outcompete others. When co-cultured with *Trichoderma harzianum*, *Withania somnifera* faces iron competition, prompting the fungus to produce siderophores (iron-binding molecules). These siderophores not only help *Trichoderma* grab iron but also signal the plant to ramp up its defense compounds, like withaferin A, to protect itself. In a study, *Trichoderma* co-cultivation with *Withania somnifera* hairy roots increased siderophore production, which indirectly boosted withanone levels by 1.5-fold due to competitive stress responses.

Applications of Co cultivators

Enhanced Production of Medicinal Compounds
Co-cultivation boosts the yield of bioactive secondary metabolites like withanolides, which are used in pharmaceuticals for their anti-inflammatory, anticancer, adaptogenic, and neuroprotective properties.
Agricultural improvement:
Co-cultivators improve *Withania somnifera* growth, stress resistance, and yield in agricultural settings, making cultivation more sustainable and productive.
Biotechnological production of novel compounds

Co-cultivation activates silent or cryptic biosynthetic genes in *Withania somnifera*, leading to the production of novel or rare secondary metabolites for biotechnology applications.

Bioremediation and Environmental Applications

Co-cultivators enhance *Withania somnifera's* ability to thrive in contaminated or nutrient-poor soils, aiding bioremediation and sustainable agriculture.

Research and Development:

Co-cultivation systems are used to study plant-microbe interactions, metabolic pathways, and gene regulation in *Withania somnifera*, advancing scientific knowledge and biotechnology

Stress Induction

Secondary metabolites (SMs) are bioactive compounds that help plants survive environmental stresses while offering valuable medicinal properties, including anti-cancer compounds like taxol (Cragg & Newman, 2005) and anti-malarial artemisinin (Weathers et al., 2011). These compounds have become crucial for pharmaceutical and nutraceutical industries, yet their natural production is often limited (Ncube et al., 2012).

Stress induction has emerged as an effective strategy to boost SM yields by triggering plant defense mechanisms (Baenas et al., 2014). This eco-friendly approach enhances metabolite production through controlled abiotic (e.g., drought, UV light) or biotic (e.g., microbial elicitors) stresses (Goyal et al., 2020). Compared to chemical synthesis, stress induction is cost-effective, scalable, and maintains natural biosynthetic pathways (Isah et al., 2018).

Why Use Stress Induction in Ashwagandha?

Stress induction has emerged as a particularly effective strategy for enhancing secondary metabolite production in *Withania somnifera* (Ashwagandha) due to its ability to stimulate the plant's natural defense mechanisms. This medicinal herb produces valuable withanolides (e.g., withaferin A and withanolide D) that exhibit potent anti-inflammatory, anti-cancer, and neuroprotective properties, but their natural concentrations are often suboptimal for commercial extraction (Mirjalili et al., 2009). Controlled application of abiotic stresses (e.g., drought, salinity) or biotic elicitors (e.g., chitosan, yeast extract) has been shown to significantly upregulate key biosynthetic pathways, increasing withanolide yields by 2-5-fold compared to unstressed plants (Sabir et al., 2012; Sivanandhan et al., 2013). This approach offers distinct advantages over conventional cultivation methods, including reduced production costs, avoidance of genetic modification concerns, and maintenance of the natural phytochemical profile (Giri & Zaheer, 2016). Moreover, stress induction techniques are scalable from laboratory tissue cultures to field cultivation, making them particularly attractive for commercial applications (Nagella & Murthy, 2010). The method's effectiveness is attributed to its activation of stress-responsive transcription factors and enzymatic pathways that naturally enhance withanolide biosynthesis as part of the plant's survival strategy (Dhar et al., 2014).

Types of Stress and Their Effects on Secondary Metabolites

ABIOTIC STRESS

Stress Type	Example	Effect on SMs	Reference
UV Radiation	UV-B exposure	Resveratrol(grapes),	Zhang&Björn(2009)

		flavonoids	
Drought	Water deficit	Artemisinin(Artemisia annua)	Arsenault et al.(2010)
Temperature	Heat shock	Ginsenosides(Panax ginseng)	Ali et al.(2006)
Heavy Metals	Cadmium (Cd)	Phenolic compounds	Mithöfer et al.(2004)
Salt Stress	NaCl	Betalains(beetroot)	Savitha et al.(2011)

BIOTIC STRESS

Elicitor Type	**Example**	**Effect on SMs**	**Reference**
Fungal Elicitors	Chitosan	Taxol(*Taxusspp*.)	Sonja et al.(2002
Bacterial Elicitors	Yeast extract	Alkaloids(*Catharanthus roseus*)	Namdeo(2007)
Herbivore Attack	Wounding	Nicotine(*tobacco*)	Baldwin(1999)

Mechanism of Stress-Induced Secondary Metabolite Production

Plants enhance secondary metabolite (SM) production under stress through coordinated molecular responses. When exposed to stressors (drought, pathogens, UV), plants first perceive signals through membrane receptors, triggering reactive oxygen species (ROS) bursts that act as secondary messengers (Gill & Tuteja, 2010). This activates:

1. Phytohormone Signaling:

- Jasmonic acid (JA) and salicylic acid (SA) pathways upregulate key enzymes (PAL, CHS) in SM biosynthesis (Pieterse et al., 2012)

2. Transcriptional Regulation:

- Stress-responsive transcription factors (MYB, WRKY) bind to promoter regions of SM genes (Vom Endt et al., 2002)

3. Metabolic Reprogramming:

- Carbon flux shifts from primary to secondary metabolism (e.g., phenylpropanoid pathway under UV stress) (Zhao et al., 2005)

4. Defense Compound Accumulation:

- Final SM products (e.g., withanolides, artemisinin) accumulate in specialized tissues (Dhar et al., 2014)

Stress Signal → ROS Burst → JA/SA Signaling → TF Activation → SM Gene Expression → Enzyme Induction → Metabolic Shift → SM Accumulation

Case Studies and Applications of Stress-Induced Secondary Metabolite Production

Plant & Stress Type	Secondary Metabolite Increased	Study & Year	Mechanism	Application
Artemisia annua (Drought stress)	Artemisinin (anti-malarial)	Arsenault et al., 2010	Drought stress upregulates ADS (amorpha-4,11-diene synthase)	Increases yield for ACT (Artemisinin-based Combination Therapy)
Vitis vinifera (UV-C Resveratrol exposure)	Resveratrol (antioxidant)	Tassoni et al., 2012	UV-C activates stilbene synthase (STS) and phenylpropanoid pathway	Used in nutraceuticals anti-aging supplements
Taxus brevifolia (Chitosan elicitor)	Taxol (anti-cancer)	Sonja et al., 2002	Fungal elicitor triggers jasmonate signaling and taxane biosynthesis genes	Enhances paclitaxel production for cancer treatment
Withania somnifera (Salt stress - 150 mM NaCl)	Withaferin A (anti-cancer)	Sabir et al., 2012	Salt-induced oxidative stress boosts withanolide biosynthesis	Applied in Ayurvedic formulations
Catharanthus roseus Vincristine (Cadmium stress)	Vincristine (anti-leukemia)	Gupta et al., 2013	Cd stress activates defense genes in TIA (terpenoid indole alkaloid) pathway	Supports anticancer alkaloid production.

Conclusion

This review details the various biotechnological and ecological approaches employed to significantly enhance secondary metabolite yield in Withania somnifera, a high-value medicinal crop renowned for its significant pharmacological value. Applying microbial elicitors, which include components from bacteria and fungi, has been found to effectively trigger plant defence mechanisms, leading to increased yields of valuable secondary metabolites, specifically withanolides and alkaloids. Complementing this, PGPR helps in enhanced nutrient assimilation and overall plant growth as well as having a significant effect on the activation of pathways associated with secondary metabolite accumulation, thereby enhancing the therapeutic potential of ashwagandha. The symbiotic relationships formed by AMF improve the uptake of nutrients, increase stress resistance, and hence increase the production of secondary metabolites, thereby furthering the medicinal value of the plant and biomass yield. Stress-induced approaches, which include various abiotic stresses such as drought, salinity, and UV exposure, effectively trigger stress response

transcription factors and cause metabolic reprogramming, leading to elevated levels of key withanolides like withaferin A. In addition, co-cultivation techniques, wherein W. somnifera is in contact with microbial consortia or other plant species, have shown promising results by promoting the production of secondary metabolites through interspecies signaling and synergistic interactions. Despite these advances, challenges persist in the establishment of standardized protocols, fully understanding strain-specific responses, and the effective translation of these methods into industrial applications. Future research must prioritize integrative approaches that combine microbial elicitation, varied stress induction methods, and advanced omics-based tools. An integrated strategy will be instrumental in uncovering the intricate molecular mechanisms behind these advances and in sustainably achieving maximal metabolite yields. These holistic strategies will increase the pharmaceutical value of Withania somnifera and encourage sustainable agricultural practices, effectively meeting the increased global demand for natural therapeutics while conserving ecological balance.

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