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Studies on biotransformation of withanolides by bacteria isolated from infected plants of *Withania somnifera* (Dunal)

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A B S T R A C T

Endophytic microbes are commonly seen in all the plants including medicinal plants. The endophytes enhance the ability to synthesize bioactive compounds in the associated plants apart from the production of secondary metabolites. The present investigations were on analyzing these bacterial diversity associated with different medicinal plants that synthesises the secondary metabolite compounds under biotic stress in *Withania somnifera*. The bacterial strains were isolated and purified from the rhizosphere roots and leaves of *W. somnifera*. The isolated bacterial colonies were further sub-cultured and screened to species level using 16S rRNA sequencing. From the 16S rRNA sequences obtained, the strains were found to be in close match with *Bacillus velezensis*, *Bacillus amyloliquefaciens* and *Pseudomonas floridensis*. Further, the cell free extracts of the cultures with substrates of Withanolides and sterols were extracted and then analysed on HPLC and TLC. Of the strains obtained, B9 and B6 strains showed different bands when compared with control. Similarly, B1strain showed unique bands when compared with control. A significant rise in the production of secondary metabolites such as withaferin A, withanolide D was seen when treated with bacterial endophytes than the control. The present study could be used to trace the application of potent bacterial endophytes to nullify the pathogenicity.

Introduction

The quality and quantity of herbal drugs depend on the availability of soil nutrients (Ganie *et al.*, 2015) as well as the native geographical patterns (Liu *et al.*, 2015; Quan and Liang, 2017). So there has been a great deal of attention paid by researchers to study the native plants and their genetic diversity (Panda *et al.*, 2015). But studies conducted on the impact of bacterial endophytes on herbal drugs, which are crucial for pharmaceutical, is the need of the hour. By using endophytes, it is easy to understand the interactions between plants and microbes as well as the processes that take place during the production of herbal compounds with medicinal values. Microbes interact with host plants in a symbiotic

relationship, providing a valuable micro-niche for controlling plant diseases (Andreote *et al.,* 2014). Therefore, by using potent endophytes to enhance the production of crucial herbal drugs, it may be possible to enhance host-endophyte interactions.

Bacterial endophytes colonizing plants secrete antimicrobial compounds like saponins, essential oils, as a part of natural selection and contain genetic adaptation to overcome several biotic and abiotic threats. The bacterial endophytes produce detoxifying enzymes for colonization, including cellulase, protease, etc. Upon colonizing, these bacterial endophytes form a symbiotic relationship with the host. It is a necessity, through co-existence that they share genetic data to ensure that advantage of metabolic, genetic and physiological development will occur (Schmidt *et al.*, 2014). Plant hosts provide endophytes with nutrients, minerals, and favorable conditions to enable their growth and development, while microbes provide the host with disease resistance, growth, and bioactive compounds (Singh and Gaur, 2016). It may be a result of their genetic interaction that bacterial endophytes are able to produce plant bioactive compounds. Majority of recent studies demonstrate that endophytes are beneficial to plant growth and favour production of secondary metabolites (Singh and Gaur, 2016). Furthermore, endophytes are responsible for regulating plant health by soluble phosphorous, secreting antimicrobial agents, different phytohormones (IAA and GA3), as well as inhibiting pathogen growth by competing for nutrients

The erect, evergreen, perennial, and tomentose shrub *Withania somnifera* belonging to solanaceae (commonly known as Ashwagandha or Indian ginseng) has been used medicinally for over 3,000 years. Several of its therapeutically beneficial compounds have been cited in ancient texts of the Unani and Ayurvedic systems, as well as in modern WHO databases (Sen and Chakraborty, 2017). There are many secondary metabolites in leaves and roots of *W. somnifera* such as alkaloids, withanolides, glycowithanolides, flavonol glycosides, steroid lactones and polyphenolics that can be used as remedies to heal arthritis, cancer, inflammation and so on. They are also proved to be immunoregulatory and cardio protective aiding many untreated neuro degenerative disorders as well (Rai *et al.,* 2016).This plant is used extensively across the globe as an immuno-modulator, an anti-inflammatory, an anti-stress agent, anti-Parkinson's agent, an anti-Alzheimer agent, an immune system booster, a neuro-defensive agent, an anti-diabetic agent, an aphrodisiac, a memory enhancer, etc.

The studies reported by Neetu Singh (2021) that it can also be used for treating various types of cancer and other conditions of the colon, the mammary, the lung, the prostate, the skin, the blood, the liver, and the kidney. In wild and cultivated conditions, this plant is highly susceptible to fungal phytopathogens, such as *Alternaria alternata*, *Fusarium oxysporum*, *Myrothecium roridum*, among others. *Alternaria alternata* is the foremost cause of leaf spot disease, which has substantial toxic effects on plant bioactives. In humans, the association of *A. alternata* with *W. somnifera* may be responsible for serious lung infections (Chowdhary *et al.,* 2016). Therefore, it is forbidden to use infected tissues for therapeutic purposes based on guidelines from the World Health Organization (WHO, 2016). Moreover, leaf spot disease results in plant cell death by affecting the photosynthesis and C: N ratio via deteriorating plant tissues at a cellular level. The withanolide content was drastically reduced by 76.3% in diseased plant (Shivanna *et al.,* 2014). Application of chemicals to manage *A. alternata* infections in *Withania somnifera* is restricted as they are harmful to humans (Nicolopoulou-Stamati *et al.*, 2016).

Ashwagandha, or *Withania somnifera,* synthesizes tri-terpenoid steroidal lactones called withanolides. Through oxidation at C-22 and C-26 to form a steroidal lactonering, withanolides are chemically nomenclatured as 22-hydroxyergostane-22-oicacid26-δlactones. Within Solanaceae, withanolides are produced by only a few genera, with the highest abundance and most structurally

diverse forms found in *Withania somnifera* (Misra *et al.*, 2008). Considering the ever-increasing demand for secondary metabolites from *W. somnifera*, efforts must be made to find an ecologically-friendly potent antagonist to prevent *W. somnifera* from being infected by several fungal groups that inhibit secondary metabolites synthesis. Researchers have demonstrated that major withanolides, like Withaferin A and Withanolide A, are effective in the treatment of cancer, Parkinson's disease, and Alzheimer's disease (Bisht and Rawat, 2014). In addition to being potential sources of drugs, plant secondary metabolites also offer structural scaffolds for the synthesis of new drug candidates, due to their chemical diversity and complexity (Belbei *et al.*, 2008; Harvey, 2008). In contrast, the chemical complexity is a significant barrier in chemically synthesizing pharmaceuticals. Typically, natural sources also yield low yields when attempting to isolate pharmaceutically active components (Balbei *et al.*, 2008). Developing novel pharmaceuticals using biotransformation technology may be possible by using a variety of microbial metabolic processes. The bio-transformation process has been successfully used to produce many pharmaceutical candidates (Shanu-Wilson *et al.*, 2020). Moreover, microbial transformation can produce larger quantities of metabolites than experimental animals, cell cultures, or mammalian enzymes (Pham *et al.*, 2019).

Therefore, the current biotransformation study was aimed to determine whether the isolated bacteria from infected plants are capable of metabolizing withanolides to compounds beneficial for their survival and also have pharmaceutical advantage.

Materials and Methods

Isolation of bacteria from rhizosphere and rhizoplane of *Withania somnifera*

Bacteria associated with plants ample were isolated from both rhizoplane and rhizospheric soil of *W. somnifera*. Both roots and soil samples were collected from a root depth of about 5cm and 15cm, respectively. Roots were submerged in 50ml of sterile phosphate buffer (PBS) for about 2hr and shaken on rotary shaker. The supernatants thereafter were serially diluted from 10^{-1} to 10^{-8}. 10^{-4} and 10^{-6} dilutions were plated onto plates with Luria Beratani medium for isolating bacteria and incubated at 37°C for 24-48 hours. The colonies obtained were further sub-cultured on LB agar and screened at species level using 16S rRNA sequencing.

Isolation of endophytic bacteriafrom infected plants of *W. somnifera*

The excised leaves and roots from infected plants of *W.somnifera* were subjected to three step surface sterilization procedure. The samples were washed with 99% ethanol followed by 6min wash with 3% sodium hypochlorite. Then again subjected to a 30sec wash with 99% ethanol followed by a final wash with sterile distilled water. The surface sterilized leaves and roots were then aseptically seized into 1cm fragments and inoculated onto nutrient agar for bacterial isolation. The plates were then incubated at 37°C for 24-48 hours. The colonies obtained were further sub-cultured on their respective medium and screened at species level using 16S rRNA sequencing (Asal *et al.*, 2016).

Extraction of DNA from bacteria

Total genomic DNA from the selected strains was extracted using N-Cetyl-N, N, N-trimethyl-ammonium bromide (CTAB) method as described by Khamael *et al.*, (2018). About 1ml bacterial broth culture was centrifuged for 2min at 10000 rpm at 4°C and the pellet obtained was resuspended in 675µl of extraction buffer (100mM Tris HCl, 100mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K - 0.03µg/µl). The contents were mixed thoroughly and incubated for 30min at room temperature. To the contents 75µl of SDS (20%) was added and incubated at 65°C for about 2hours and later centrifuged at maximum speed. To the supernatant Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at maximum speed. The upper aqueous layer was separated to a fresh tube

and DNA was precipitated with 0.6 volumes of isopropyl alcohol and centrifuged at10000 rpm. The pellet obtained was then washed with 70% ethanol and stored at -20^{0}C.

16S rRNA Sequencing

Bacterial strains isolated were identified using 16S rRNA gene based analysis as described by Abdul Rasool *et al.,* (2015). Extracted DNA was used as the template for amplifying 16S rRNA using PCR. The PCR was performed with forward and reverse primers with an initial denaturation for 4min at 94° C, followed by 35 cycles of 94°C denaturation for 30s, 51°C for annealing for 40s, and extension at 72°C for 2min.

Finally the reaction was held at 72°C for 5min. Amplification was done with the universal primers (16S rRNA gene universal primers (Forward 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; Reverse 1392 R 5′-ACGGGCGGTGTGTAC-3′) as mentioned by Dastager *et al.,* (2009). The amplicon size was expected to be 1700bp. The amplified PCR products were purified (1.7kb) from low melting agarose gel stained with ethidium bromide as per the standard protocol (Sambrook *et al.,* 2001) for further sequencing.

The PCR product was then sequenced using ABI PRISM Big Dye Terminator sv1.1cycle sequencing kit (Applied Biosystems Foster City, CA, USA) as prescribed in the instructions using both the forward and reverse primers.

Maintenance of bacterial cultures

Pure cultures of the bacterial isolates were maintained by streak plate on nutrient agar (pH 7.2). Single colony from the bacterial culture plate were inoculated into 15ml sterile nutrient broth in a screw caped glass tube, incubated in shaker (24hr, 180 rev/min, 28°C) and used as stock cultures for biotransformation studies of withanolides and sterols. Pure cultures were stored at 4°C for subsequent use.

Incorporation of the substrates

The substrates selected for the bacterial biotransformation studies were withanolides and sterols (cholesterol, ergosterol and stigmasterol). In brief, about 50ml of the nutrient broth was dispensed into a series of 10 conical flasks and autoclaved at 15lbs for 30min. 50µL of pure bacterial broth cultures were inoculated into each flask and incubated in a shaker incubator (30hr, 180rev/min, 28°C). The withanolide substrates were dissolved in methanol at concentration of 5mg/0.6 ml and the final volume was made up to 3 ml using sterile MQ water. 0.4 ml of the stock withanolide substrate solution (5mg/0.6 ml) was added to the actively growing 28-30hr bacterial cultures. 500µL of the stock sterol mixture (4mg of each sterol was dissolved in equal volumes of ethanol and ethyl acetate) was added to the actively growing 28-30hr bacterial cultures and incubated in an orbital shaker (28hr, 180 rev/min 28°C) (Erin, 2012).

Extraction and estimation of Withanolide and sterols

Cell free extracts of the bacterial cultures with substrates along with their respective control cultures were extracted three times with equal volumes of chloroform in a separating funnel. The pooled chloroform fractions were air dried and dissolved in HPLC grade methanol, filtered and subjected to TLC and HPLC as described by Sangwan *et al.,* (2008).

TLC and HPLC analysis of the substrates

Withanolides and sterol substrate extracts from the bacterial cultures were initially analyzed on TLC (Farzana *et al.,* 2011). Substrate extracts along with their respective controls and standards were spotted onto 20 x 20cm TLC plates procured from Sigma Ltd. Sterols were dissolved in chloroform, methanol and water (70:30:5) and withanolides were dissolved in chloroform, toluene, methanol and ethyl acetate (70:24:8:4). The spraying solvent was common which included ansialdehyde, water, acetone and

perchloric acid (250µL, 40ml, 10ml and 5ml respectively). The spotted TLC plates were kept in the TLC chamber containing respective solvents for 1hr, sprayed with the spraying solvent, and dried for 15min. The plates were kept in the hot air oven maintained at 120-150°C for a few minutes until the spot development.

HPLC analysis was performed on Waterpad (Model 996: Milford, MA, USA) and separations were achieved using reverse phase column (150 X 39mm i.d: 4µm) subjected to gradient elution for sample 6 alone. The solvent system (0.6 ml per/min) comprised methanol and water (each containing 0.1% acetic acid in the gradient mode 45:55-65:35, 45 min). Detection was done at 227nm using an online UV detector (SPD-10A) and the chromatogram reports were generated through integrated software (Empower). Amounts of 10µL withanolide sample (methanolic solution) or 5µLwas injected for each run.

Results and Discussion

Isolation and identification of bacteria

Fifteen isolated bacterial colonies from rhizosphere, rhizoplane, roots and leaves on nutrient agar plates were sub cultured and maintained as pure cultures (B1 to B15) (Plate.1). A total of 97endophytic bacterial isolates (about 35% of total counted) were isolated from different parts of the infected plants of *W. somnifera.*

The average population density ranged from 3.124 to 1.683 log CFU g^{-1} of endophytic bacterial communities among various plant parts (Fig. 1). The maximum colony count was seen in roots 3.124 log CFU g^{-1} whereas the least was observed in leaves (1.683 log CFU g^{-1}). Similar findings were reported wherein the endophytes were predominant in roots rather than leaves (Aradhana *et al.,* 2018). In the present study, surface sterilized roots also showed colony count of about 2.054 log CFU g^{-1}.

Identification of the bacterial cultures was done by 16S rRNA gene based assay. Genomic DNA isolated from the fourteen pure bacterial cultures was used as template for PCR amplification of 16S rRNA gene. The presence of genomic DNA of the bacterial isolates was confirmed on 0.8% agarose gel stained with ethidium bromide. An intense single band was seen in all the wells along with DNA marker.16S rRNA gene was amplified using PCR, the primers selected were specific. An intense single band of size 1.7kb was visible on 1% agarose gel stained with ethidium bromide. Amplification was seen in bacterial isolates B1, B6, B8, B9, B10, B11, B14 and B15 (Fig. 2), non amplification in other bacterial isolates might be due to less concentration of DNA.16S rRNA gene was purified andbacterial species were identified by sequencing.

Phylogenetic tree

The phylogenetic tree was built using MEGA7 by Neighbor-Joining method (Jones *et al.,* 1992). The distances were calculated using the Maximum Composite Likelihood method and showed close relation with their respective strains. *Bacillus velezensis* and *Pseudomonas floridensis* sequences from NCBI database were used as reference sequences with their respective counter parts.

All the sequences showed similarity without many substitutions stating the minimal variations within the species. From the 16S rRNA sequences obtained, the strains showed close match with *Bacillus velezensis* and *Pseudomonas floridensis.* The strains 1 and 14 are 96.79 and 98.58% close match to *Bacillus velezensis* and *Pseudomonas floridensis* respectively (Fig. 3).

Plate.1 LB agar plates of the bacterial isolates (B1, B6, B8, B9, B10, B11, B14 and 15) from leaf, roots, rhizosphere and rhizoplane regions from infected plants of *Withania somnifera*

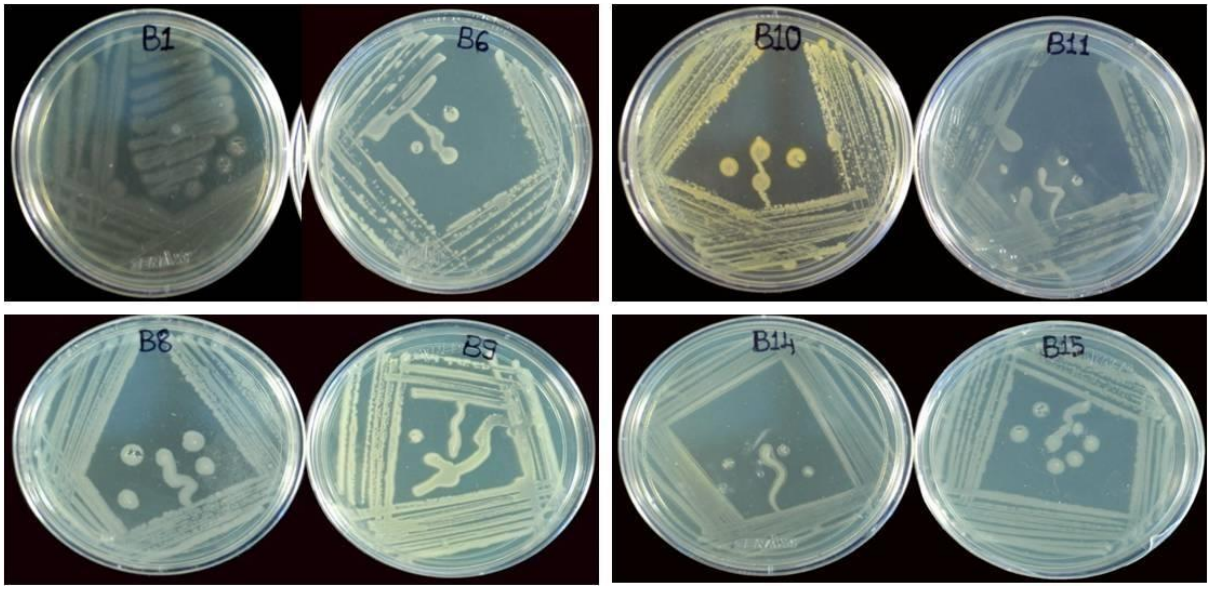


Fig.1 Graph showing the population density of endophytes isolated from parts of plant samples. Values are expressed as CFU/gm and value ±SD.

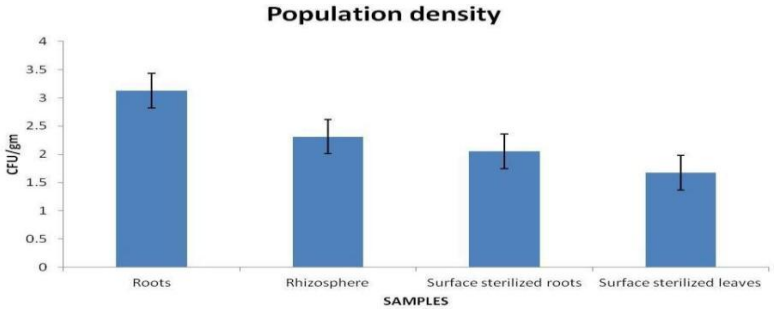
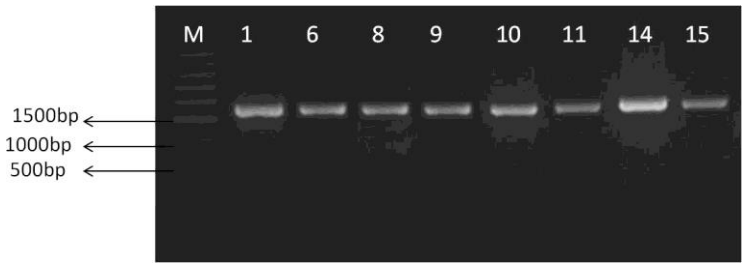


Fig.2 Agarose gel electrophoresis of 16S rRNA gene amplicon of size 1.5 kb of nine bacterial isolates.



Lane M – 500 bp DNA ladder, Lane 1, 6, 8, 9, 10, 11, 14, 15- 16S rRNA gene amplicon of bacterial isolates of their respective numbers.

Fig.3 Image showing the evolutionary tree of the DNA sequences using the Neighbor-Joining method.

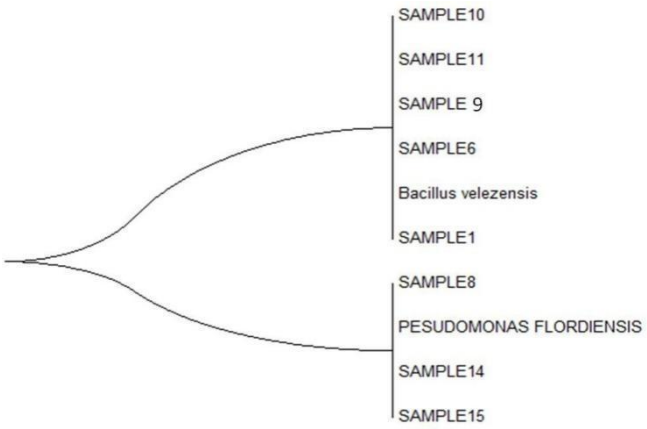


Fig.4 TLC analysis for withanolide substrate for the selected bacterial isolates. Unknown band can be seen in the image. 1 and 2 are B6 and B9 samples respectively. C: Control; T: treatment. S: Standard.
Middle: TLC analysis for withanolide substrate for B11 and B12 bacterial isolates.
Right: TLC analysis for sterol substrate for selected bacterial isolates

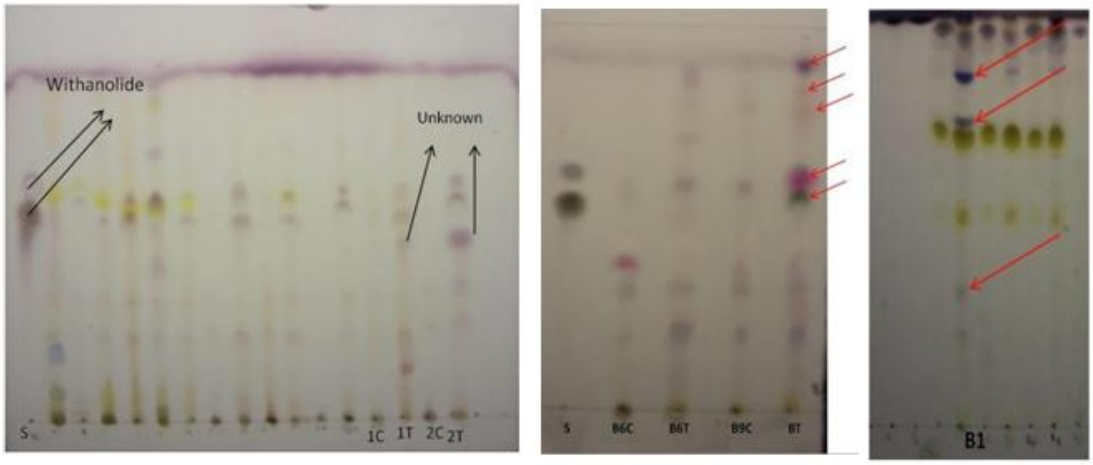


Fig.5 HPLC chromatogram of sample 6 obtained with isocratic elution. A: withaferinA; D: withanolideD.

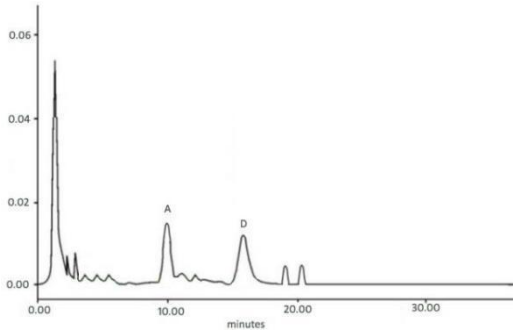
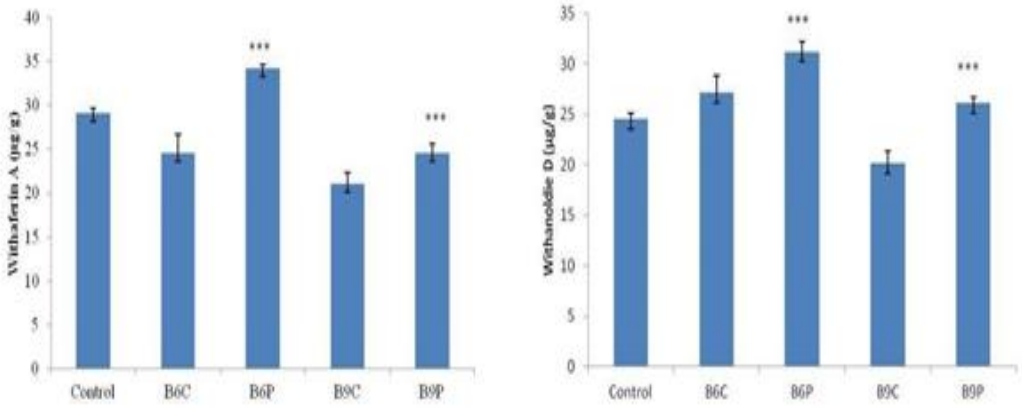


Fig.6 Graph showing the effect of the endophytes on secondary metabolites withanolide A & D content of *W. somnifera*. Values are expressed as value ±SE. Asterisks (***) indicate the treatments are significantly higher than the control without pathogen stress ($p < 0.05$). B6 and B9 are samples. C: control.



TLC analysis of withanolides and sterol substrates

Bacterial isolates B1, B6, B8, B9,B10, B11, B14 and B15 were selected for bio-transformation studies of withanolides andsterol substrates. Culture extracts after extracting thrice with chloroform were dried and the dried residue was dissolved in minimum amount of methanol and analyzed on TLC separately for withanolides and sterols with respective standards and controls. Spots detected on TLC for withanolide and sterols are shown Fig. 4. B9 and B6 strains showed different bands (indicated by arrows) when compared with controls, which was not seen with other bacterial extracts in case of withanolides. Similarly B1 bacterial strain showed different bands (indicated by arrows) when compared with control and these bands are not detected in the extracts of other bacterial cultures in case of sterol TLC analysis. The detection of different bands in TLC analysis suggests that the bacterial species might be performing biotransformation changes with respect to withanolides and sterol substrates. B9and B6 bacterial isolates were further incorporated with high concentration of withanolide substrates and analyzed by TLC (Fig. 4). Different bands were again detected (indicated by arrows).

In the present study, about 8 bacterial isolates were screened for biotransformation studies for withanolides and sterols. TLC analysis of the cell free extracts of bacterial cultures along with their respective controls showed some observable difference in the bands detected. In case of TLC analysis of cultures incorporated with withanolide substrates, isolates B6 and B9 showed different detectable bands which were not seen in their control and other culture extracts.

In TLC analysis of cultures incorporated with sterol substrate isolates, B1 showed different detectable bright bands which were not seen in their control and other culture extracts.

Different bands detected in extracts of isolates B1, B6 and B9 could be due to bioconversion changes with respect to their sterol and withanolide substrates. Biotransformation of withanolides to compounds of pharmaceutical importance by cell suspension cultures have been previously studied and reported (Farzana *et al.*, 2011).

Depletion of withanolide content has been reported in *W. somnifera* due to the infection of *A.alternata*. HPLC profiling was done to evaluate the impact of pathogenic stress on the production of valuable

secondary metabolites. A significant elicitation was seen in the secondary metabolites like withaferin A and withanolide D, when treated with bacterial endophytes than the control. The production of withaferin A and withanolide D seemed to be over expressed by 1.4 fold and 2.4 fold respectively under pathogenic stress. Withaferin A content was observed to be 34.23µg/g and 24.56µg/g under pathogenic stress for B6 and B9 respectively when compared to their respective controls. Withanolide D was significantly increased under pathogenic stress with 31.21µg/g and 26.11µg/g for B6 and B9 respectively when compared to their respective controls (Fig. 5 & 6).

By using a wide variety of microbial metabolic activity, biotransformation technology could be used to develop novel pharmaceuticals. Many pharmaceutical candidates were successfully produced by biotransformation processes (Belbei *et al.*, 2008 and Harvey, 2008). In this context the results of present study suggest that microbes from their native plants can be exploited for biotransformation of plant secondary metabolites into other important molecules. There is no report in the literature regarding the biotransformation of withanolides and sterols by microbes. But Kim *et al.*, (2010) have reported that a *Mycobacterium sp*. is able to biotransformdecursin to a compound of pharmaceutical importance.

Microorganisms play an important role in producing and transforming natural secondary metabolites which might have great pharmaceutical importance. Biotransformation technology uses a wide variety of microbial metabolic activity that could be potentially used to develop novel pharmaceuticals. In the present study, detection of new compounds of withanolide and sterols formed by the isolated bacterial were detected through TLC and HPLC analysis. These new substrates have diversified advantage in pharmaceutical industries.

Conflicts of Interest

The authors declare no conflicts of interest.

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