



Compatibility of Inherent Fungal Endophytes of *Withania somnifera* with *Trichoderma viride* and its Impact on Plant Growth and Withanolide Content

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Abstract

Withania somnifera (Ashwagandha), also known as Indian ginseng, is an important ancient medicinal plant, used in the Indian traditional systems of medicine. In view of increasing demand for roots of Ashwagandha, the present study was undertaken to investigate the compatibility of inherent fungal endophytes along with the biocontrol agent, *Trichoderma viride*, for enhancing *W. somnifera* plant growth and root secondary metabolites (withaferin A). It has frequently been emphasized by the World Health Organization the use of healthy roots of Ashwagandha for therapeutic applications. To maintain quality of *W. somnifera* roots, an option could be eco-friendly management of root-knot diseases and co-inoculation of native endophytes along with *T. viride*. The in vitro antagonistic activity of *T. viride* (TV) against the *W. somnifera* pathogens, *Alternaria alternata* and *Sclerotium rolfsii*, showed 64.3% and 69.5% growth inhibition, respectively. Here, we investigated the compatibility of TV along with the native endophytic fungi *Aspergillus terreus* strain 2aWF (2aWF), *Penicillium oxalicum* strain 5aWF (5aWF), and *Sarocladium kiliense* strain 10aWF (10aWF) for the cultivation of *W. somnifera*. The co-inoculation of TV and native endophytic fungi resulted in increased shoot, root weight, and plant height to 65–150%, 35–74.5%, and 15–35%, respectively, compared to untreated plants. Withanolide A content in leaves of TV-treated plants increased significantly by 260%, whereas in co-inoculation treatments, it was enhanced up to 109–242%. However, no considerable change was noticed with withaferin A content in leaves, except the 2aWF + TV treatment significantly increased by 27%. In contrast, withanolide A content in roots was not affected by TV alone but co-inoculation with endophyte treatments significantly increased its content (19–73%). TV alone had increased chlorophyll a by 23%; however, in combination treatments, it increased up to 115–164% compared to control. Besides secondary metabolites in roots and leaves, co-inoculation of TV and native endophytes modulated the expression of the withanolide biosynthetic pathway genes *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, *CAS*, *SMT1*, *STE1*, and *CYP710A1* compared to control treatments. Apart from withanolide biosynthetic pathway genes, co-inoculation of TV also ameliorated the host-resistant-related gene *NPR1* which was upregulated by ninefold in the TV treatment and 3- to 7-fold in the combination treatment. Overall, our results show that co-inoculation of TV along with inherent endophytes of *W. somnifera* enhanced plant growth and withanolides accumulation.

Keywords Fungal endophytes · Withanolides · *Withania somnifera* · *Trichoderma viride*

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Introduction

Trichoderma spp are effective biological means for plant disease management in sustainable agriculture practices to manage various kinds of abiotic and biotic stresses (Waghunde et al. 2016; Hermosa et al. 2011). *Trichoderma* spp recruited for cultivation of the medicinal plants *Coleus forskohlii*, *Artemisia pallens*, and *Withania somnifera* showed better management of root-knot disease and plant biomass (Saikia et al. 2013; Singh et al. 2011). Application of *Piriformospora indica* for cultivation of *W. somnifera* and *Spilanthes scalva* increased net primary productivity (NPP), growth, and biomass of plants (Rai et al. 2001). Of late, *Trichoderma* spp are reported as endophytes from lentil roots, pods of *Theobroma gileri*, and roots of *Coffea arabica* (Mulaw et al. 2013; Bae et al. 2009; Rinu et al. 2013). The deteriorative effects of fertilizers and pesticides on ecosystems have forced a shift in agriculture practices towards organic farming through use of plant growth promoting or biological controlling microorganisms.

Endophytes are bacteria or fungi that inhabit ubiquitously in all parts of tissue without causing any harm to plants, and have been reported from different crop plants as well as from medicinal plants (Jia et al. 2016; Compant et al. 2010; Rodriguez et al. 2009). They induce accumulation of secondary metabolites, plant growth, biomass, and resistance against biotic and abiotic stresses in hosts (Waller et al. 2005; Rodriguez et al. 2008; Sherameti et al. 2008). Native endophytes from opium poppy, *Catharanthus roseus* and *Atractylodes lancea*, have been reported to increase secondary metabolites, net photosynthetic efficiency and the biomass of plants (Pandey et al. 2016a, b; Zhou et al. 2016).

Withania somnifera (L.) belongs to the Solanaceae family, popularly known as Indian ginseng and has been adopted in conventional use in various health conditions in Indian society from ancient times. Withanolides, a type of steroidal lactones that account for the medicinal value, are abundantly present in the root and leaf tissue of *W. somnifera*. To date, all steroidal lactones along with their glycosylated derivatives have been reported such as withanolide A, withaferin A, withanolide D, withanone, withanoside IV, withanoside V, and so on (Trivedi et al. 2016). Due to increasing global demand for withanolides, various attempts have been made to increase the production of withanolides in in vivo or in vitro conditions. Development of hairy root and suspension cultures, precursor feeding, and elicitation by biotic or abiotic elicitors could be alternative options for withanolide production (Ahlawat et al. 2017; Sivanandhan et al. 2014a, b). An approach using transgenic technology for overexpression of withanolide biosynthetic pathway genes for withanolide

accumulation in plants is also gaining prominence. Few withanolide pathway genes are well characterized and the putative withanolide biosynthetic pathway as depicted in Fig. 1 (Gupta et al. 2013). Precursor feeding with phytosterols is a key component for various kinds of withanolide biosynthesis, silencing or overexpression of any methylerythritol 4-phosphate (MEP)/mevalonate (MVA)/any downstream pathway genes, affect directly on phytosterol content and also on withanolide content in leaves and roots (Singh et al. 2016; Saema et al. 2015). The overexpression of the *WsCAS* or *GrDXS* gene in *W. somnifera* leads to higher withanolides content in leaves as compared with control plants (Jadaun et al. 2016; Mishra et al. 2016). In another transgenic plant, the overexpression of squalene synthase (*SQS*) leads to enhanced withanolides biosynthesis in roots, stem, and leaf tissues (Patel et al. 2015). Apart from genetic manipulation or tissue culture methods, the application of the phytohormone salicylic acid (SA) or methyl jasmonate (MeJA) as an elicitor transiently enhanced the significant level of withanolide content in the leaves (Jadaun et al. 2016; Mishra et al. 2016; Rana et al. 2014). Recently, an endophytic fungus, *Talaromyces pinophilus*, was isolated from leaves of *W. somnifera* which were independently producing withanolide in the culture media (360 mg/L) which was reported by Sathiyabama and Parthasarathy (2017) and in fact it could be further explored for an alternative source for withanolide production under in vitro conditions at an industrial scale to meet the market demand.

Trichoderma spp are the most successful biofungicides used in today's agriculture. They grow in the rhizosphere and are capable of penetrating internally plant roots (Harman et al. 2004; Harmosa et al. 2011). The presence of *Trichoderma* in the rhizosphere evokes a beneficial effect on plant growth, yield, and resistance to pathogens (Bae et al. 2010). *T. viride* and *T. harzianum* are aggressive parasites of phytopathogenic fungi and these species are effective in the stimulation of plant defense responses (Viti et al. 2016). However, the use of *Trichoderma* spp along with crop-specific endophytes for cultivation of the medicinal plant, *W. somnifera*, has not been explored. In the present study, we report for the first time, the compatibility of the biofungicide, *T. viride*, with three inherent fungal endophytes *Aspergillus terreus* strain 2aWF (2aWF), *Penicillium oxalicum* strain 5aWF (5aWF), and *Sarocladium kiliense* strain 10aWF (10aWF) and their interaction on plant growth, enhanced withanolide accumulation, and plausible modulation of the withanolide pathway genes in *W. somnifera* host plant.

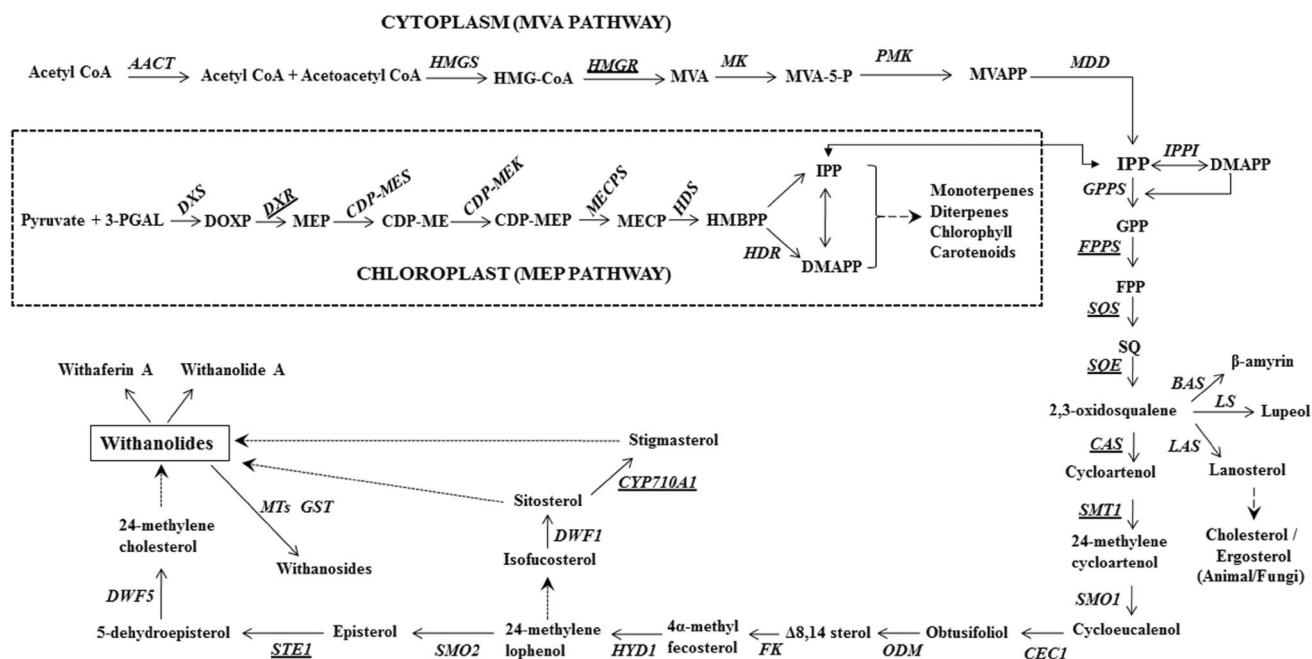


Fig. 1 Schematic diagram of withanolides biosynthetic pathway in *W. somnifera*. Solid arrows indicate single-step reactions, dashed arrows indicate several steps, dotted arrows represent unidentified steps, and the double arrow shows the exchange between cytosolic and plastid compartments AACT (acetyl-CoA thiolase), BAS (β -Amyrin synthase), CAS (Cycloartenol Synthase), CDP-ME (4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol), CDP-MEK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), CDP-MEP (2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol), CDP-MES (2-C-methyl-D-erythritol 4-phosphate cytidyl transferase), CECI (Cycloecalenol cycloisomerase), CYP710A1 (C-22 Sterol desaturase), DMAPP (dimethylallyl diphosphate), DOXP (1-deoxy-D-xylulose 5-phosphate), DWF1 (Delta-24 sterol reductase), DWF5 (Sterol delta-7 reductase), DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), DXS (1-deoxy-D-xylulose-5-phosphate synthase), FK (Delta 14-sterol reductase), FPP (Farnesyl diphosphate), FPPS (FPP synthase), GPP (Geranylgeranyl diphosphate), GPPS (Geranyl diphosphate Synthase), HDR (4-hydroxy-3-methylbut-2-enyldiphosphate reductase), HDS (4-hydroxy-3-methylbut-2-enyldiphosphate synthase), HMBPP (4-hydroxy-3-methylbut-2-enyl diphosphate reductase), HMG-CoA (3-hydroxy-3-methylglutaryl-CoA), HMGR (HMG-CoA reductase), HMGS (HMG-CoA synthase), HYDI (C-7,8 sterol isomerase), IPP (isopentenyl diphosphate), IPPI (isopentenyl diphosphate isomerase), LS (Lupeol synthase), LAS (Lanosterol synthase), SMT1 (Sterol methyltransferase 1), SQ (Squalene), SQE (Squalene monooxygenase/epoxidase), SQS (Squalene Synthase), and STE1 (C-5 sterol desaturase)

Materials and Methods

Plant material and Growth Conditions

Withania somnifera Dunal (var. Poshita) seeds were collected from the National Gene Bank for Medicinal and Aromatic Plants at CSIR-CIMAP, Bangalore. Seeds were sown in soil-filled polythene bags (7.5 cm x 13 cm), grown in a polyhouse under natural photoperiod conditions. Light irrigation was provided with a hand sprinkler on alternate days up to 4 weeks. After 4 weeks, healthy and equal height plantlets were selected for transplantation to the research field at CSIR-CIMAP, Research Centre, Bangalore. The polyethylene bag cover was removed before transplantation and the plants were transplanted in 13-cm-deep soil up to the lower stem level. The bed dimension was 3 m x 3 m

(length x width) comprising 16 plants per plot; 4 plants in each rows and 4 plants per column, with a distance of 50 cm and plant to edge distance was fixed to 50 cm.

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Treatment of *W. somnifera* with *Trichoderma viride* and Combination with Fungal Endophytes

Three native endophytic fungi *Aspergillus terreus* strain 2aWF (MH196570), *Penicillium oxalicum* strain 5aWF (MH196571), and *Sarocladium kiliense* strain 2aWF (MF800957) isolated from *W. somnifera* were used in the present study. *Trichoderma viride*, a biofungicide, was obtained from a local market (NIPROT 0.5% W.P, Pest Control Pvt. Ltd. India). After 4 weeks of transplantation in the experimental plot, all 16 plants of *W. somnifera* plants were treated with *Trichoderma viride* (TV) alone and in

combination with the above-mentioned *W. somnifera* fungal endophytes (Suppl. Table 1). Spore suspension culture (1×10^8 spore/conidia mL^{-1}) of individual endophyte and TV were prepared in phosphate buffered saline (PBS) (NaCl 8 g L^{-1} , KCl 0.2 g L^{-1} , Na_2HPO_4 1.44 g L^{-1} , K_2HPO_4 0.24 g L^{-1} ; pH 7.40). Each plant in the individual bed was treated with 30 mL of spore suspension of TV culture. However, in the combination treatment, each plant received 15 mL of TV spore suspension and 15 mL of individual 2aWF/5aWF/10aWF spore suspension.

Measurement of Plant Height, Biomass, and Analysis of Photosynthetic Pigments

Plants were harvested at 150 days post inoculation (dpi). Before harvesting, average plant height was randomly measured from each treatment and control bed. After harvesting, leaf and root samples from the center of each treatment bed were subjected for analysis. Thereafter, selected plants were kept in shade at room temperature for dry shoot and root weight measurement. At 150 days post culture inoculation, the third leaf from the top of the plant in each treatment was considered for estimation of plant pigments. A uniform circular leaf area (6 mm diameter) with the help of cork borer was taken, followed by incubation in chilled 100% methanol overnight at 4 °C. The methanolic extract was measured at wavelengths 666 nm, 653 nm, and 470 nm for three biological and three technical repeats (Eppendorf biospectrometer). Chlorophyll and carotenoids were calculated as described by Lichtenthaler and Wellburn (1971). Chlorophyll a = $(15.65 A_{666} - 7.34 A_{653})$, Chlorophyll b = $(27.05 A_{653} - 11.21 A_{666})$, and Carotenoids = $[1000 A_{470} - (2.86 \times \text{Chl b} - 129.2 \times \text{Chl a})]$.

Withanolides Estimation from *W. somnifera* Root and Leaves

At 150 dpi, *W. somnifera* leaf and root samples were made into fine powder for withanolide extraction and analysis using high-performance liquid chromatography (HPLC). One hundred mg of dry leaf powder or root powder were extracted in 2 mL of ethyl acetate (100%) and kept overnight at room temperature. For complete extraction of withanolides from the tissue (root or leaf) sample, the above procedure was repeated thrice. The combined extract of ethyl acetate was filtered through a 0.45 μm syringe filter and concentrated by drying at room temperature and finally dissolved in 100% methanol for HPLC analysis. Twenty millilitres of samples were manually injected into a HPLC (Shimadzu 10 AVP; Shimadzu, Kyoto, Japan) containing a Zodiac C-18 (250 mm \times 4.6 mm, 5 μ) column. The mobile phase for HPLC analysis consisted of two solvents, 0.1 M of ammonium acetate ($\text{CH}_3\text{COONH}_4$) dissolved in 18 M Ω MQ water (solvent A) and acetonitrile

(solvent B). Withanolides were eluted under gradient programming of the mobile phase initially at 95% A, then changed to 55% A and 20% A at 18.0 and 25.0 min, respectively. Solvent A was maintained at 20% A from 25 to 30 min then increased to 55% A at 35 min and finally reached to 95% at 40 min. The flow rate was fixed at 0.5 mL/min with the UV detector set at 227 nm wave length throughout the analysis. Standard withaferin A and withanolide A prepared in methanol were used as reference compounds. The specificity of the withaferin A and withanolide A peaks in samples was identified and quantified by their elution at specific retention times (Rt).

Real-time Quantitative PCR (RT-qPCR) Analysis

Leaf samples (third leaf from top) were collected randomly from each treatment and kept at -80 °C for RNA extraction. One hundred mg of leaf tissue was homogenized in liquid nitrogen and RNA extraction was carried out according to the protocol of the RNA isolation kit (Sigma–Aldrich). Genomic DNA trace in isolated RNA was removed by treating with endonuclease DNase I (RNase-free DNase I, Ambion). The quality and quantity of total RNA were estimated with a spectrophotometer (Eppendorf biospectrometer) at OD 260/280. First-strand cDNA synthesis preceded with 2 μg of total RNA using the ReadyScript® cDNA Synthesis Kit (Sigma–Aldrich) according to the steps mentioned in the manufacturer's protocol. All primers used in real-time-quantitative PCR (RT-qPCR) for withanolide and host-resistant transcript quantification were adopted as mentioned by Singh et al. 2015 (Suppl. Table 2). RT-qPCR was accomplished using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma–Aldrich), the reaction proceeded with three technical and three biological replications from each treatment. RT-qPCR reaction conditions were started with 10 min at 95 °C, subsequently followed by 40 cycles of program as denaturation at 95 °C for 15 s each and annealing/extension at 60 °C for 1 min each. SYBR® Green fluorescent signals were detected and the threshold cycle (Ct) of each gene displayed by the StepOnePlus™ Real-Time PCR System (Applied Biosystems). After completion of RT-qPCR, the specificity of the amplicon was evaluated by a melting-curve analysis using the dissociation method (Applied Biosystems). Average Ct values were calculated from three technical repeats. The Ct value for each gene was normalized with the 18S gene, which was referred to as the constitutive reference transcript. The relative levels of each transcript of withanolide pathway genes and defense-related genes in endophyte-treated plants were compared with untreated *W. somnifera* plants (Livak and Schmittgen 2001; Schmittgen and Livak 2008).

Screening of TV Against *W. somnifera* Damping-off and Leaf Spot Pathogen

Damping-off and leaf spot infected *W. somnifera* plant samples were collected from an agriculture field of CSIR-CIMAP, Research Centre, Bangalore. Damping-off diseased stem samples were collected with the help of sterilized forceps, inoculated on a PDA plate and kept at 28 °C for 5 days. Leaf spot diseased samples were collected from infected plants and the sample with the help of a sterilized needle was inoculated on a PDA plate for 5 days at 28 °C. Pure cultures of both pathogenic fungi were maintained on fresh PDA plates. Isolated pathogens were confirmed by the morphology and structure of conidiophores and spores through a bright-field microscope. *Trichoderma viride* (TV) was used for antagonistic activity against the damping-off and leaf spot pathogen by the dual culture method (Campanile et al. 2007). The plates were incubated at 28 °C for 3–5 days and the treatments were performed in triplicate. The percent inhibition of the phytopathogenic fungi was calculated according to Ghildial and Pandey (2008) by using the formula: $(R1-R2/R1)100$. Where R1 is the radial growth of the pathogen without TV, and R2 represents the radial growth of the pathogen in the presence of V.

Statistical Analysis

For statistical analysis, one-way ANOVA with a Dunnett multiple test was calculated by using Graph Pad prism, version 5.01 (GraphPad Software, San Diego, CA). The statistical significance of differences between control and treated samples was at different *p* levels shown in legends.

Results

Effect of *T. viride* and Fungal Endophytes on *W. somnifera* Plant Growth

Withania somnifera plant height and biomass were measured at 150dpi of TV alone and the combination with native endophytic fungi. TV alone treatments did not significantly increase the fresh shoot and root weights, moreover plant height was less compared to control. Co-inoculation of TV with 2aWF, 5aWF, and 10aWF endophytes significantly increased the fresh shoot weight up to 150%, 110%, and 65%, respectively, as compared to control (Fig. 2a). Similarly, the same set of treatments increased the fresh root weight significantly up to 74.5%, 65%, and 35% in 2aWF + TV, 5aWF + TV, and 10aWF + TV, respectively, compared to untreated plants (Fig. 2a). Dry shoot weight was significantly higher in both TV alone as well as in combination treatments. TV alone increased only

20% the dry shoot weight, whereas in the combination with endophytes 2aWF, 5aWF, and 10aWF it increased up to 121%, 85%, and 132%, respectively (Fig. 2b). Dry root weight was significantly higher with co-inoculation treatments (16–56%), whereas in TV-treated *W. somnifera* plants, dry root weight was similar to control (Fig. 2b). The treatment of TV alone did not significantly increase plant height, however, combination treatments increased it up to 18–42% compared to control (Fig. 2c). Chlorophyll and carotenoid pigments per unit leaf area at 150 dpi with TV alone showed enhanced content of chlorophyll a up to 23%, whereas co-inoculation treatments 2aWF + TV, 5aWF + TV, and 10aWF + TV were noticeably higher 152, 115, and 164% than untreated plants, respectively (Fig. 3a). Analogously, chlorophyll b content remained unaffected in TV and 5aWF + TV treatments, whereas in combination treatments of TV along with 2aWF and 10 aFW it increased by 22 to 33%, respectively, compared to control (Fig. 3b). Interestingly, the carotenoid content was drastically enhanced in all the treatments and it increased up to fivefold as compared to control plants (Fig. 3c).

Co-inoculation of *T. viride* (TV) and Fungal Endophytes Induce withanolides

The effect of TV and combination treatments with native fungal endophytes (2aWF + TV, 5aWF + TV and 10aWF + TV) on the withanolides content in leaves through HPLC at 150 dpi is shown in Fig. 4. Withaferin A content in leaves was not significantly affected by the treatment of TV alone or the combination treatments, except in the TV + 2aWF treatment, which was enhanced 29% compared to control (Fig. 4g). Interestingly, withanolide A, which is present in less content in leaves, increased greatly with TV treatment alone as well as in combination treatments of fungal endophytes. The relative enhancements of withanolide A in leaves after treatment with TV, 2aWF + TV, 5aWF + TV and 10aWF + TV were 260%, 205%, 109%, and 242%, respectively, as compared to control plants (Fig. 4h).

Withanolide A is a prominent bioactive compound in the roots of *W. somnifera* that accounts for its pharmaceutical value. In the present investigation, powdered dry roots of all treatments were subjected to withanolide extraction and quantification through HPLC (Fig. 5). Withanolide A content was not significantly changed in the TV alone treatment, whereas in combination treatments, withanolide A content in roots was increased significantly as compared to control plants. Withanolide A content in 2aWF + TV, 5aWF + TV, and 10aWF + TV treatments were 27.0, 19.0, and 73.0% higher than the untreated plants (Fig. 5g).

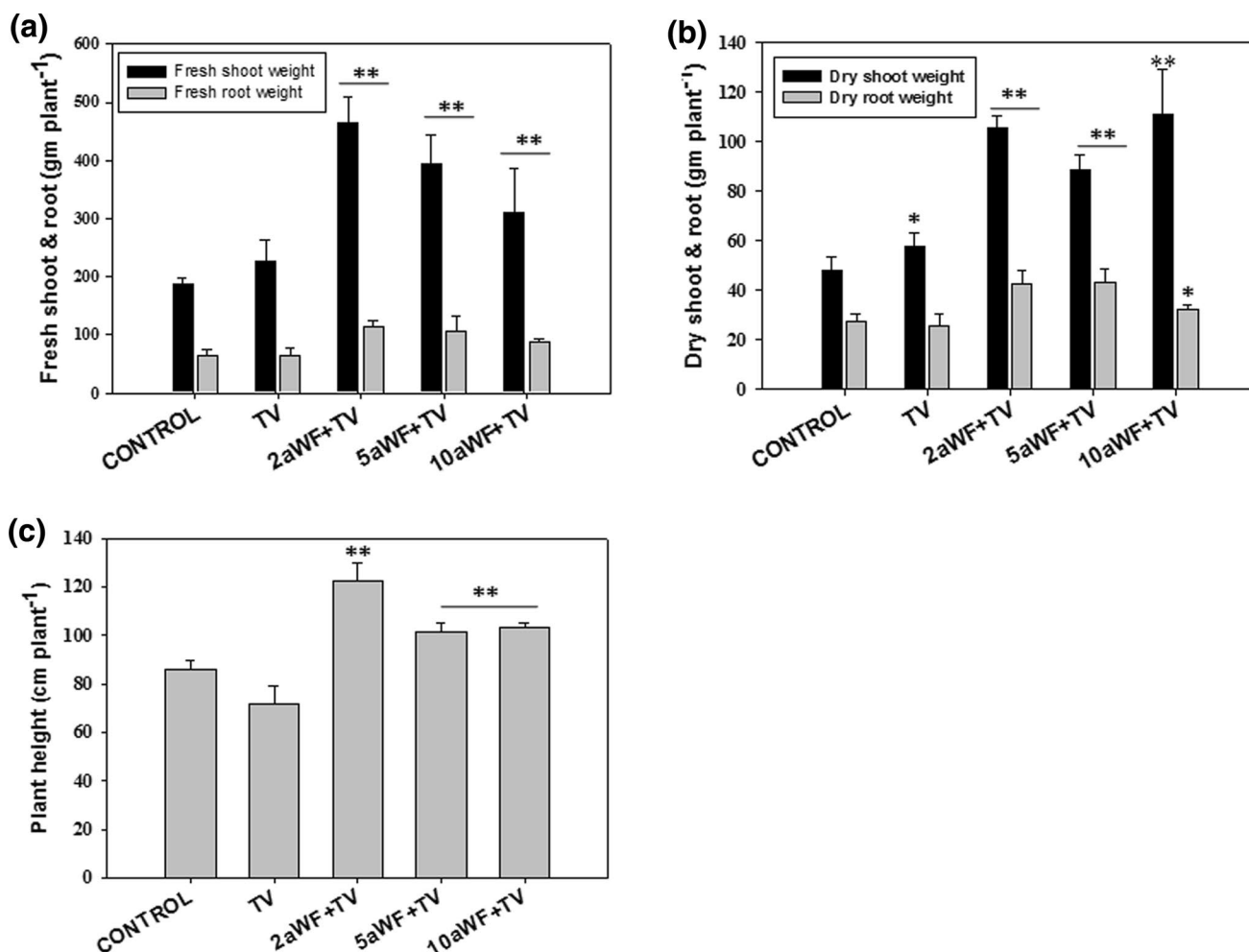


Fig. 2 Combined effect of *T. viride* (TV) and fungal endophytes on height and biomass of plants at 150 dpi. **a** Fresh shoot and root weight. **b** Dry shoot and root weight. **c** Height of plants. Each treat-

ment was followed with four biological replicates. Error bars represent standard deviation of four replicates. Asterisks indicate a significant difference from the control at p value * < 0.1 , ** < 0.05

Real-time Quantitative PCR (RT-qPCR) Analysis of Withanolide Biosynthetic Genes

Expression analysis of withanolide biosynthetic pathway genes through real-time quantitative PCR at 150 dpi was carried out to understand the impact of TV and fungal endophyte treatments. Generally, *HMGR* (3-hydroxy-3-methylglutaryl-CoA reductase), *DXR* (1-deoxy-Dxy-lulose 5-phosphate reductoisomerase), *FPPS* (farnesyl diphosphate synthase), *CAS* (cycloartenol synthase), *SQS* (squalene synthase), *SQE* (squalene epoxidase), *SMT1* (sterol methyltransferase), *STE1*(C-5 sterol desaturase), and *CYP710A1*(C-22 sterol desaturase) genes are known to play a key role in withanolide biosynthesis (Singh et al. 2015, 2016). *HMGR* and *DXR* are key genes of the MVA and MEP pathways, respectively, and contribute to the formation of the common precursor isopentenyl diphosphate (IPP). In the present investigation, the *HMGR* expression

level in the treatment of TV, 2aWF + TV, and 10aWF + TV was significantly increased 6-, 3-, and 3.5-fold, respectively, as compared with control, whereas the 5aWF + TV treatment remained unaffected (Fig. 6a). *DXR* expression was drastically increased in all treatments of TV, 2aWF + TV, 5aWF + TV, and 10aWF + TV by 16-, 25-, 9-, and 46-fold, respectively, compared to untreated plants (Fig. 6b). *FPPS* expression was enhanced by 9.8-, 33.6-, 3.2-, and 31.3-fold in TV, 2aWF + TV, 5aWF + TV, and 10aWF + TV treatments, respectively (Fig. 6c). Downstream pathway genes of withanolide biosynthesis, such as *SQS* gene expression was increased by 11- to 111-fold in TV-, 2aWF + TV-, 5aWF + TV-, and 10aWF + TV-treated plants compared to controls (Fig. 6d). 2,3-oxidosqualene, a precursor of β -amyrin, lupeol, lanosterol, and cycloartenol (Fig. 1), mediates through the *SQE* gene. The treatments of TV, 2aWF + TV, 5aWF + TV, and 10aWF + TV profoundly upregulated *SQE* expression by 2.1- to 6.9-fold compared

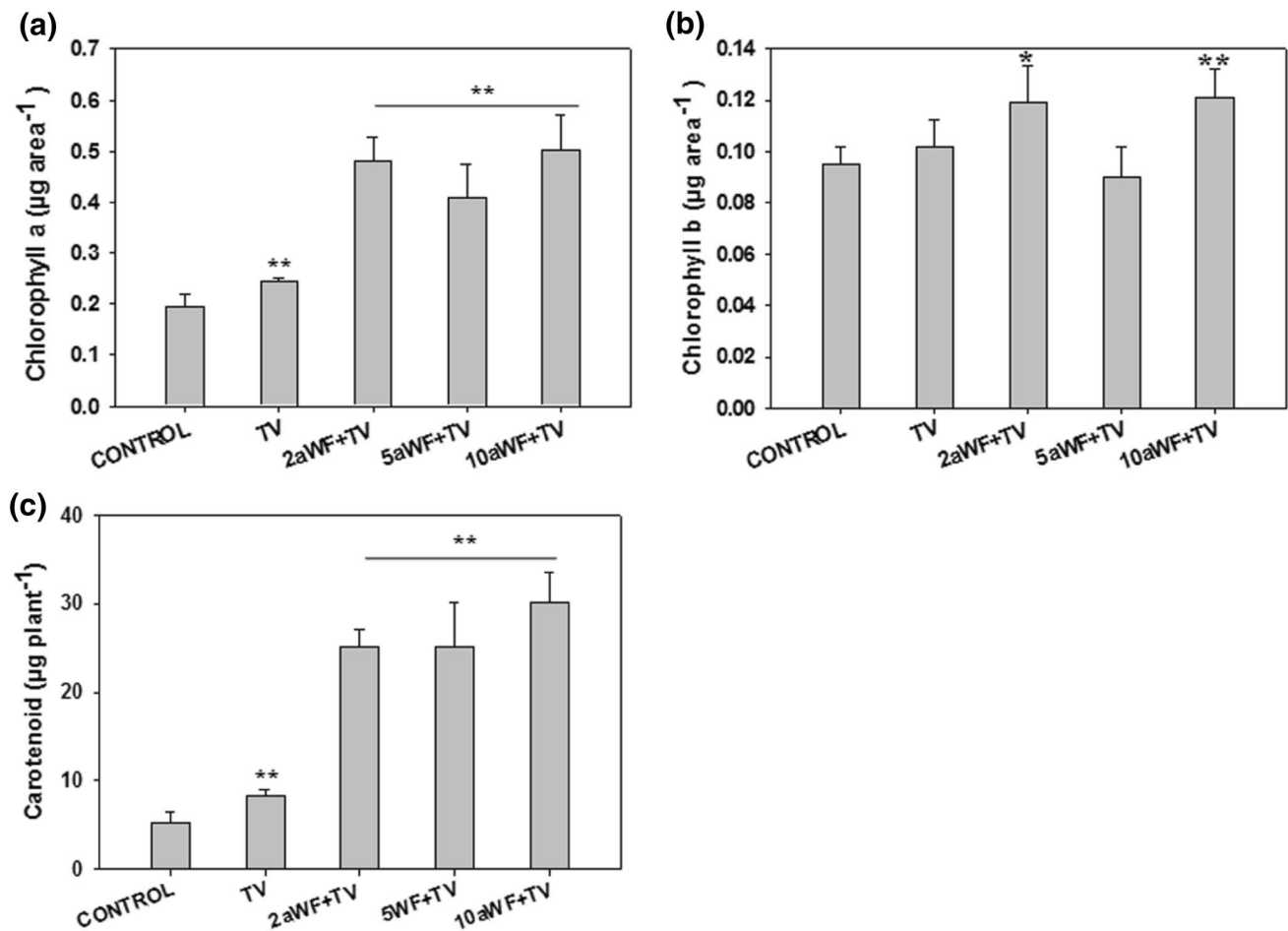


Fig. 3 Combined effect of *T. viride* (TV) and fungal endophytes on photosynthetic pigments in control and treated plant leaves at 150 dpi. **a** chlorophyll a, **b** chlorophyll b, **c** carotenoid. Each treatment was fol-

lowed with four biological replicates. Error bars represent standard deviation of four replicates. Asterisks indicate a significant difference from the control at p value * <math>p < 0.1</math>, ** <math>p < 0.05</math>

to untreated plants (Fig. 6e). Expression of the *CAS* gene was upregulated by 1.6- to 3.9-fold by TV treatment alone and in combination with endophytes for formation of cycloartenol, a key component of withanolide biosynthesis (Fig. 6f). The *SMT1* expression level in TV, 2aWF + TV, and 10aWF + TV treatments was enhanced by 3.8-, 7.5- and 8.6-fold, respectively, whereas 5aWF + TV-treated plants showed no noticeable change as compared with control (Fig. 6g). Expression of *STE1* was increased by 1.2- to 4.7-fold with the treatment of TV, 2aWF + TV, 5aWF + TV, and 10aWF + TV (Fig. 6h). *CYP710A1* expression, which is responsible for stigmaterol biosynthesis, was upregulated by 2.5- to fivefold in the TV treatment alone and also in the combination with 2aWF, and 10aWF, however, in the 5aWF + TV treatment, its expression was equivalent to control plants (Fig. 6i). Overall, the expression analysis of withanolide biosynthetic pathway genes was prominently modulated in all treatments of TV and in combination with 2aWF/5aWF/10aWF endophytes as compared to untreated *W. somnifera* plants. Apart from the analysis of withanolide biosynthetic pathway genes, *NPRI*

(nonexpressor of pathogenesis-related genes 1) expression analysis was carried out to understand plant immunity and regulation of plant disease resistance. The *NPRI* gene was also differentially upregulated by TV treatment and also in combination with native endophyte treatments (Fig. 6j). In TV alone treated plants, *NPRI* expression increased up to ninefold, whereas in combination with 2aWF increased threefold (Fig. 6j). However, in 5aWF + TV- and 10aWF + TV-treated plants, *NPRI* was upregulated up to 7-fold as compared with control plants (Fig. 6j).

Antagonistic Effect of TV on *W. somnifera* Pathogen

Pathogenic fungi isolated from diseased *W. somnifera* plants were identified as *Sclerotium rolsii* and *Alternaria alternata* on the basis of colony characters and spore structure. *A. alternata* and *S. rolsii* are potent pathogens that cause serious diseases in many crops and severely affect the chlorophyll pigment, plant health, secondary metabolites, and overall crop yields (Singh et al. 2017). The antagonistic activity

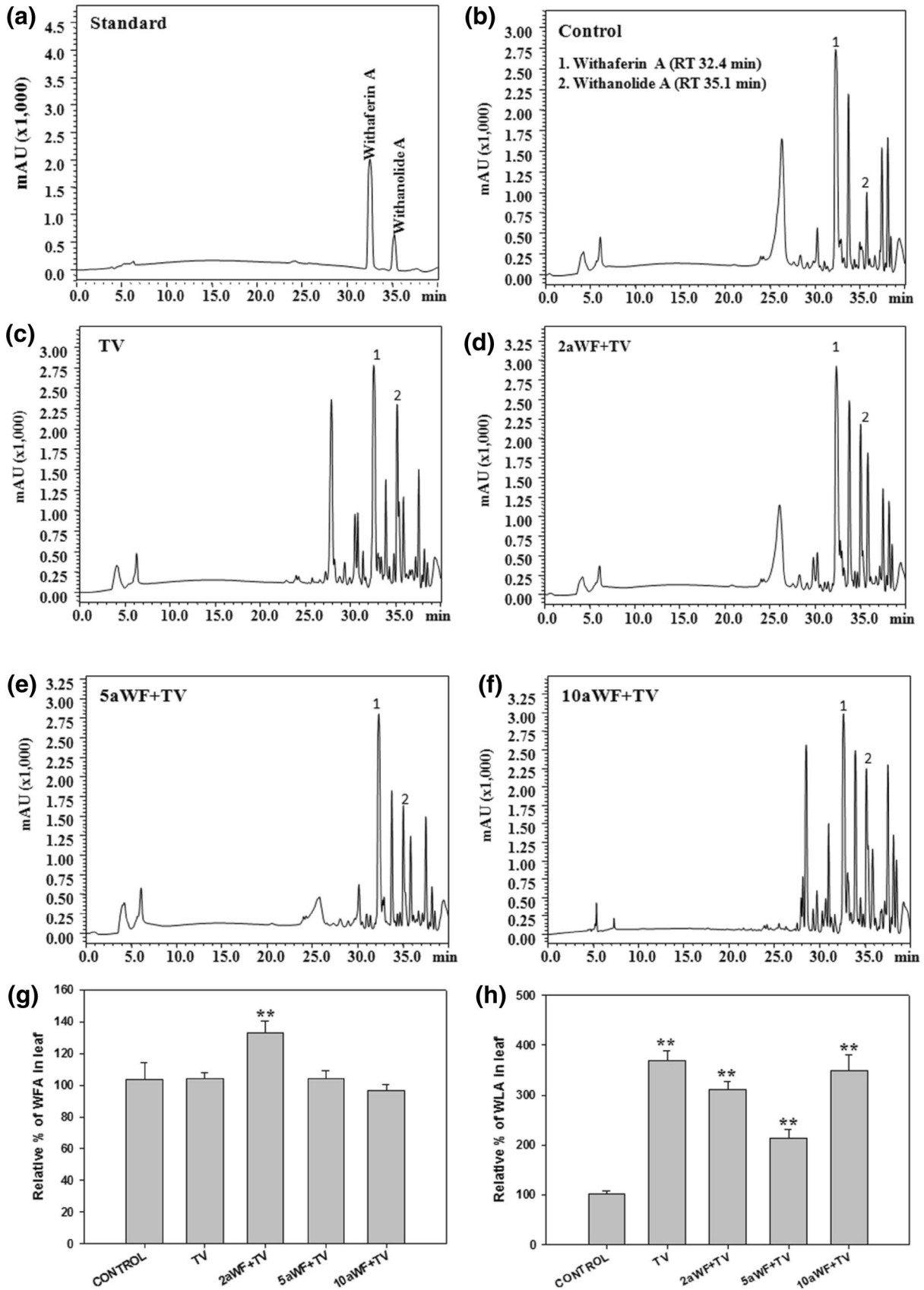


Fig. 4 HPLC chromatogram and withanolides content in leaves of treated and untreated plants at 150 dpi. **a** Chromatogram of standard withaferin A and withanolide A. **b** Chromatogram of untreated plant leaves. **c** Chromatogram of TV-treated plant leaves. **d** Chromatogram of 2aWF and TV-treated plant leaves. **e** Chromatogram of 5aWF and TV-treated plant leaves. **f** Chromatogram of 10aWF and TV-treated plant leaves. **g** Withaferin A content (%) in leaf. **h** Withanolide A content (%) in leaf. HPLC analysis for each sample was carried out in triplicate. Error bars represent standard deviation. Asterisks indicate a significant difference from the control at p value $** < 0.05$. (1) Withaferin A, (2) Withanolide A., WFA-withaferin A (RT 32.4 min), WLA-withanolide A (RT 35.1 min)

of TV was observed against the leaf spot and damping-off pathogens in the dual culture plate technique. *In vitro*, TV inoculation significantly inhibited the growth of both pathogens, *S. rolfii* and *A. alternata*, and the percentage of growth inhibition was 69.5 and 63.5%, respectively (Table 1).

Discussion

Trichoderma and its biological control activities along with plant growth promotion were reported ever since 1930 (Weindling 1932). Present day agriculture demands new avenues for management of emerging phytopathogens to reduce disease incidence and also for soil health (Waghunde et al. 2016). The application of *T. harzianum* and *T. viride* for cultivation of *W. somnifera* and management of root-knot disease along with improved root biomass was reported by Pandey et al. (2011) and Rawal et al. (2014). Deployment of endophytic fungi for cultivation of *Spilanthes calva* and *W. somnifera* improved net primary plant productivity (Rai et al. 2001; Pandey et al. 2018). Application of the *W. somnifera* native bacterial endophytes, *Bacillus amyloliquefaciens* (MPE20) and *Pseudomonas fluorescens* (MPE115), significantly enhanced withaferin A, withanolide A and withanolide B content in leaves (Mishra et al. 2018a). However, an integrated approach for the use of *Trichoderma* sps along with crop-specific endophytes had not been reported so far. Hence, the present investigation was focused on compatibility of *T. viride* along with three fungal endophytes (2aWF, 5aWF, and 10aWF) for cultivation of the medicinal plant, *W. somnifera* and the impacts on *in planta* modulation of the key secondary metabolites, withaferin A and withanolide A. Previous reports of associations between endophytic fungi and medicinal plants (Jia et al. 2016), the use of native endophytes of *Atractylodes lancea* (Zhou et al. 2016), *Papaver somnifera* (Pandey et al. 2016a), and *Catharanthus roseus* (Pandey et al. 2016b) showed no adverse effects on primary plant productivity. Recently, Pandey et al. (2018) reported that endophytic fungi and bacteria inhabiting roots, leaves, and seeds of *W. somnifera* could improve photosynthetic efficiency, plant growth, and biomass of *W. somnifera* in pot experiments.

In the present study, the co-inoculation of *T. viride* along with native, endophytic fungi for cultivation of *W. somnifera* increased the fresh shoot weight, fresh root weight, and plant height by 65–150%, 35–74.5%, and 15–35%, respectively. Furthermore, the combined effect of TV along with native, endophytic fungi showed no disadvantage on primary plant productivity moreover, it induced secondary metabolite content in both leaves and roots. Withaferin A content in 2aWF + TV treatment leaves increased (29.0%), whereas in other treatments, there were no noticeable effects. Moreover, leaf withanolide A was drastically increased by 260% in TV and up to 109–242% in combination treatments. A similar observation was noticed with application of the native bacterial endophytes of *W. somnifera*, *Bacillus amyloliquefaciens* (MPE20), and *Pseudomonas fluorescens* (MPE115) significantly increased withaferin A, withanolide A and withanolide B contents in leaves by 1.52–1.96, 3.32–5.96, and 12.49–21.47-fold, respectively (Mishra et al. 2018b).

Owing to the limited knowledge of the terminal pathway of withanolide biosynthesis, it is very contrary to understand the conversion of withanolides into their functional derivatives (Singh et al. 2016). Interestingly, withanolide A was the only prominent source from roots and its contents in roots of 2aWF + TV, 5aWF + TV, and 10aWF + TV treatments were 27.0, 19, and 73% higher than the untreated plant roots. Transport of withanolide A from the underground part is not supported because the root itself has *de novo* withanolide A biosynthesis capacity (Sangwan et al. 2008) and this result inferred that only TV and its combination treatments with fungal endophytes had induced withanolide A biosynthesis in the roots. The native endophytes of other medicinal plants such as *Atractylodes lancea*, *Catharanthus roseus*, and poppy plant (Zhou et al. 2016; Pandey et al. 2016a, b) have been reported as prominent inducers of secondary metabolites. Apart from endophyte treatment, foliar spraying of salicylic acid (SA) and methyl jasmonic acid (MeJA) has induced withanolides accumulation in the leaves of *W. somnifera* (Jadaun et al. 2016; Mishra et al. 2016; Rana et al. 2014). The enhancement of secondary metabolites in the leaf or root might be possibly due to fungal endophytes or *Trichoderma* itself acting as biotic elicitors or some type of abiotic component was secreted inside the plant that could modulate withanolides biosynthesis. Apart from induction of secondary metabolites *in planta*, use of the root endophytic fungus *Serendipita vermifera* to poplar plants attenuates the effects of toxic metal stress and maintains plant growth (Lacercat-Didier et al. 2016). The co-inoculation of endophytic fungi and AMF (arbuscular mycorrhizal fungi) on *Verbascum lychnitis* plants is responsible for increased survival and growth in post-mining waste (Węzowicz et al. 2017). Nonetheless, the majority of research is concerned with AMF and *Trichoderma* sps use as fertilizer for organic

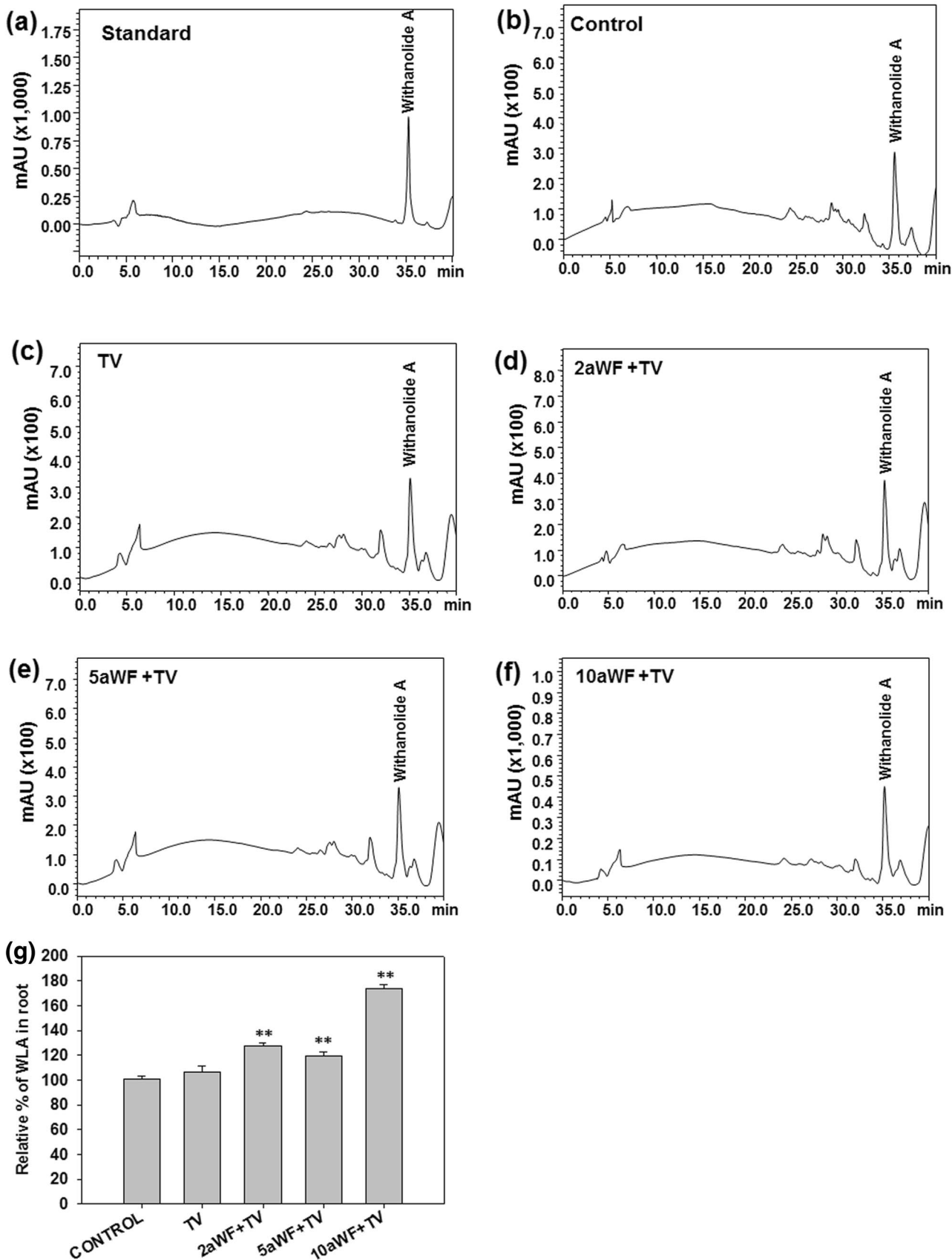


Fig. 5 Chromatogram and withanolides content in roots of treated and untreated plants at 150 dpi. **a** Chromatogram of standard withanolide A. **b** Chromatogram of untreated plant roots. **c** Chromatogram of TV-treated plant roots. **d** Chromatogram of 2aWF and TV-treated plant roots. **e** Chromatogram of 5aWF and TV-treated plant roots. **f** Chromatogram of 10aWF and TV-treated plant roots. **g** Withanolide A content (%) in root. HPLC analysis for each sample was carried out in triplicate. Error bars represent standard deviation. Asterisks indicate a significant difference from the control at p value $** < 0.05$. Withaferin A (RT 32.4 min) and Withanolide A (RT 35.1 min)

farming. Endophytic fungi should also receive appropriate attention for use with AMF or *Trichoderma* sps in organic farming.

The effect of TV and endophyte combination treatments on plant health can be associated with photosynthetic pigments, chlorophyll a content. In the present study, TV-treated *W. somnifera* plants had 23% higher and in the combination treatment had 115–164% higher chlorophyll a contents, albeit carotenoid content was up to five times higher than control plants. Previous reports also support the application of endophytes to increase plant growth and photosynthetic parameters as well (Marks and Clay 1996; Spiering et al. 2006). Alexandru et al. (2013) reported that application of *Trichoderma* sps influenced the rate of photosynthesis and pigment in tomato plants.

Induction of withanolide accumulation can be possible through the upregulation of MVA or MEP and downstream pathway genes such as *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, *CAS*, *SMT1*, *STE1*, and *CYP710A1*. The phytosterol pathway is common in all plants and its accumulation within cells has been influenced by suppression or overexpression of particular genes such as *CAS* (Babiychuk et al. 2008; Mishra et al. 2016), *SQE* (Han et al. 2010), *SQS* (Singh et al. 2015), *DXS* (Jadaun et al. 2016), *HMGR* and *STE1* (van Deenen et al. 2012), *FPPS* (Closa et al. 2010), *HMGR*, and *DXR* (Singh et al. 2014) that modulate phytosterol accumulation. In the present study, we noted modulation of transcripts of withanolide pathway genes with discrete expression levels in TV and endophyte co-inoculation treatments (Fig. 7). Independently, endophytes 2aWF, 5aWF, and 10aWF appreciably induced the expression of *HMGR*, *DXR*, *FPPS*, *SQE*, *CAS*, *STE1*, and *CYP710A1* transcript levels, whereas *SQS* was induced by 2aWF and 5aWF only (data not shown). *Trichoderma* has been reported to manage root knot (Pandey et al. 2011) and to induce systemic resistance only (Hoitink et al. 2006) but no one claimed on withanolide pathway genes. In this result, TV alone and in combination with 2aWF or 10aWF treatments differentially regulated and significantly enhanced the transcript levels of *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, *CAS*, *STE1*, and *CYP710A1* genes. However, in 5aWF + TV treatment except *STE1* and *CYP710A1*, all

other genes *HMGR*, *DXR*, *FPPS*, *SQS*, *SQE*, and *CAS* were substantially higher. A similar observation was noticed with treatment of native endophytic bacteria in enhancing withanolides by modulating the expression of *SQS*, *SE*, *CAS*, *FPPS*, *SMT*, *ODM*, *HMGR*, *SGT*, *DXS*, *DXR*, *CPR1*, and *CPR2* genes in root and leaf tissue (Pandey et al. 2018). The treatment of native bacteria *B. amyloliquefaciens* and *P. fluorescens* leads to the overexpression of *WsHMGR*, *WsDXS*, *WsFPPS*, *WsSQS*, *WsCYP710A1*, *WsSTE1*, *WsDWF5*, and *WsSGT10* genes under pathogen *A. alternata* stress (Mishra et al. 2018b). Hence, these research findings support our present investigation. Pandey and his group reported that native fungal endophytes could enhance vindoline alkaloid production in *C. roseus* and a few bacterial endophytes could stimulate benzene isoquinoline alkaloid accumulation in *Papaver somniferum* by modulating the expression of key regulatory genes (Pandey et al. 2016a, b). The application of seaweed (*Gracilaria edulis* and *Sargassum wightii*) extract and MeJA hormone enhanced the expression levels of *SQE*, *SQS*, *HMGR*, and *FPPS* genes in in vitro hairy root cultures of *W. somnifera* (Sivanandhan et al. 2015; Saxena et al. 2016). The treatment of MeJA and SA to Ashwagandha plants resulted in significant expression of *WsCYP98A* and *WsCYP76A* genes in leaves (Rana et al. 2014), remarking the application of abiotic and biotic factors ameliorates withanolide accumulation and its biosynthetic pathway genes.

Beside inhibition of soil pathogens, *Trichoderma* sps are also known for induction of systemic resistance (ISR) in plants (Hoitink et al. 2006). NPR1 a common regulator for ISR and SAR (systemic acquired resistance) genes. It regulates different kinds of pathogen-related (PR) genes and provides tools for resistant against various kinds of pathogens (Pieterse et al. 2014; Hoitink et al. 2006; Dutt et al. 2015). In the present investigation, TV alone treatment in *W. somnifera* induced a ninefold expression level of NPR1, whereas in the fungal endophyte combination treatments, it increased up to 3- to sevenfold higher than control plants. Endophytic fungi played an associative role with the host and complemented increases in secondary metabolites in leaves and roots and induced the NPR1 gene to increase immunity (Jia et al. 2016). Few endophytes, targeted to inhibit the pathogen by producing bioactive secondary metabolites or also the tomato root endophyte *Fusarium solani*, elicited induced systemic resistance against the tomato foliar pathogen (Kavroulakis et al. 2007; Gao et al. 2010). In the present study, in vitro antagonistic activity of *T. viride* could bring 64–69% growth inhibition of the fungal pathogens *A. alternata* and *S. roffsii*, respectively. Increased overall plant growth or biomass might be due to nutrient availability or phytohormones are responsible for the increase in plant height and biomass (Khan et al. 2015; Fouda et al. 2015).

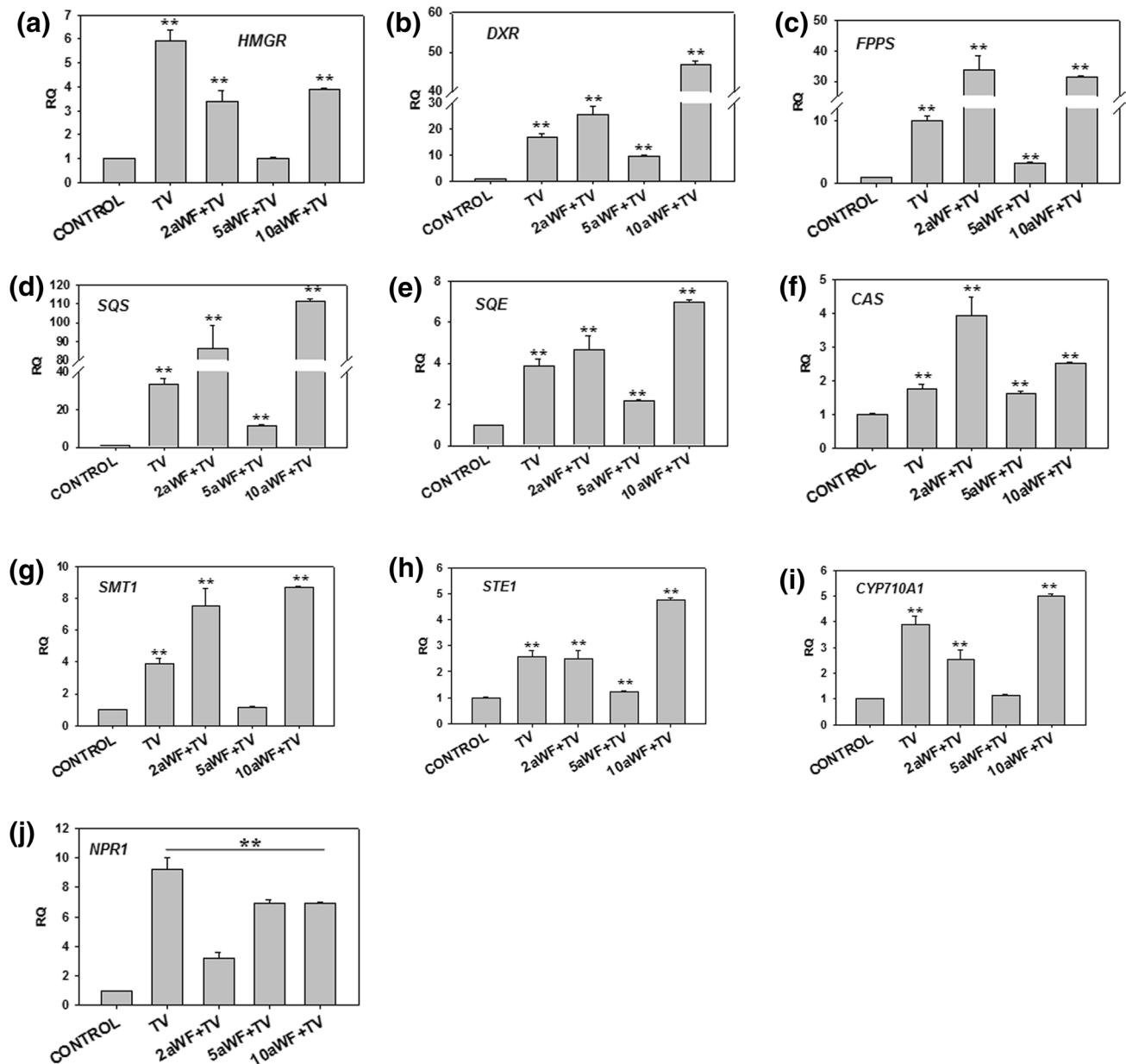


Fig. 6 Real-time qPCR analysis showing relative expression levels of upstream and downstream withanolide pathway genes in leaves at 150dpi. **a** *HMGR*, **b** *DXR*, **c** *FPPS*, **d** *SQS*, **e** *SQE*, **f** *CAS*, **g** *SMT1*, **h** *STE1*, **i** *CYP710A1*, and **j** *NPR1*. Expression levels of these genes were normalized to 18S rRNA and are represented in comparison

with control. Data are mean \pm SD ($n=3$ biological replicates) and y axis represents relative quantity (RQ). RQ was calculated using the equation; $RQ=2^{-\Delta\Delta C_t}$. Error bars represent standard deviation. Asterisks indicate a significant difference from the control at p value $**<0.05$

Table 1 Percent growth inhibition of *Sclerotium rolfii* and *Alternaria alternata* by TV after 3 days of incubation

Pathogen	% of inhibition
<i>Sclerotium rolfii</i>	69.5 \pm 0.86 ^{ab}
<i>Alternaria alternata</i>	64.3 \pm 1.40 ^{ab}

Numbers followed by the \pm is standard deviation (SD)

^aAverage value of three replicates

^bSignificantly different at p value <0.05

In conclusion, there is no deleterious effect after treatment with TV or the combination with native endophytes on *W. somnifera* plant growth and withanolides content. Inoculation of fungal endophytes along with TV increased overall plant biomass and yield. Apart from primary plant productivity, co-inoculation treatments of fungal endophytes with TV, induced secondary metabolite contents, mainly withanolide A in both leaf and root tissues. Furthermore, the key secondary metabolites (withanolides)

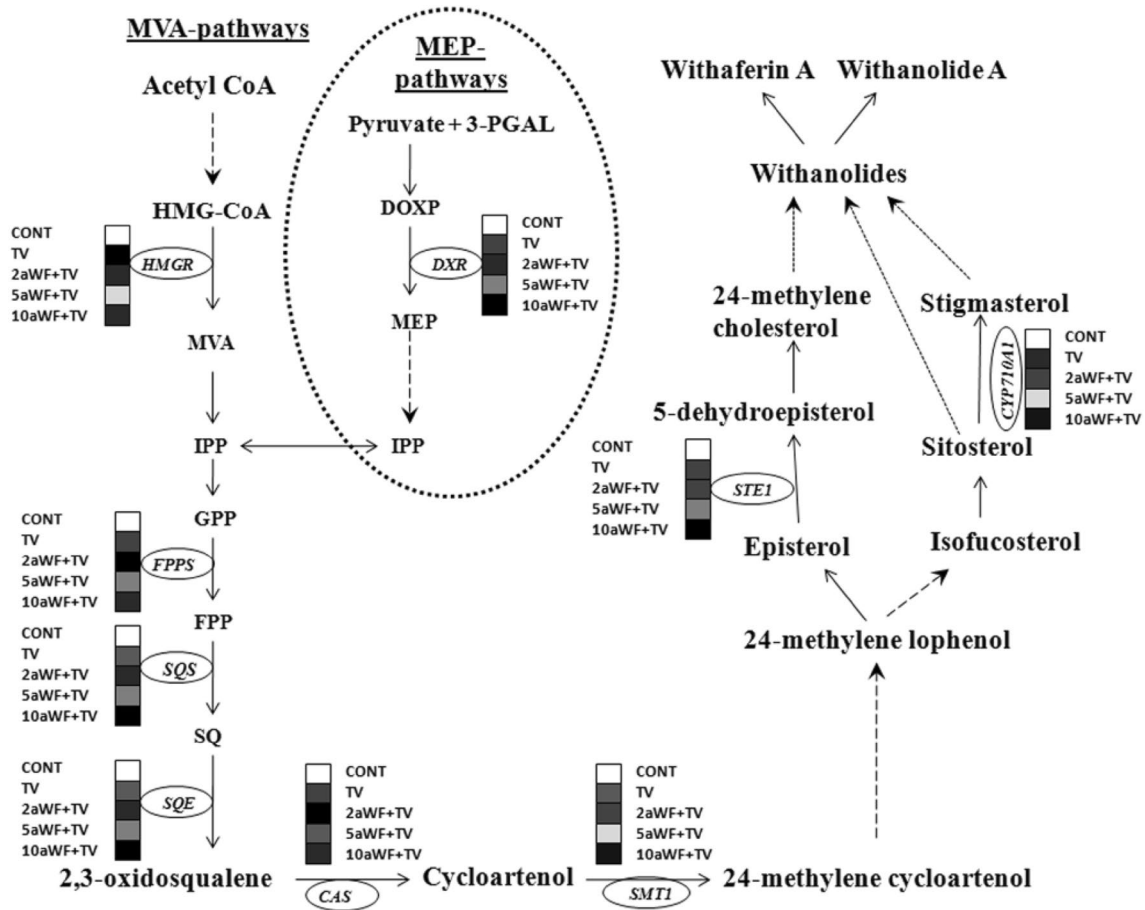


Fig. 7 Schematic representation of differential regulation of withanolide biosynthesis in *W. somnifera* by TV alone or in combination with endophytic fungi. Color intensity represents expression level of respective genes with different treatments of TV (*Trichoderma viride*), 2aWF+TV (*Aspergillus terreus*+TV), 5aWF+TV (*Penicillium oxalicum*+TV), and 10aWF+TV (*Sarocladium kiliense*+TV). Solid arrows indicate single-step reactions, dashed arrows indicate

several steps, and dotted arrows represent unidentified steps. Enzyme acronyms: HMGR (3-hydroxy-3-methylglutaryl-CoA reductase), DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), FPPS (Farnesyl diphosphate synthase), CAS (Cycloartenol synthase), SQS (squalene synthase), SQE (squalene epoxidase), SMT1 (sterol methyltransferase), STE1 (C-5 sterol desaturase), and CYP710A1 (C-22 Sterol desaturase)

ameliorated from their respective genes were differentially regulated in TV or in combination with 2aWF, 5aWF, and 10aWF endophyte treatments that lead to substantial withanolide A enhancement in leaves and roots as well. The treatment of TV or with 2aWF, 5aWF, and 10aWF endophytes, significantly increased the content of chlorophyll a, chlorophyll b, and carotenoid pigments. However, an overall mechanism for the interaction or signal communication between the host and endophytes is still illusive. Prospective avenues could be explored through use of three inherent fungal endophytes *Aspergillus terreus* 2aWF, *Penicillium oxalicum* 5aWF, and *Sarocladium kiliense* 10aWF along with the prevalent biofungicide, *T. viride* for its potential integration towards disease management and *in*

planta enhancement of secondary metabolites during *W. somnifera* cultivation.

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Compliance with Ethical Standards

Conflict of interest Authors declare that they have no conflict of interest.

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