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The endophytic fungi as natural elicitors of secondary metabolites, antioxidant enzymes, physiological traits, and associated genetic indicators of *Polygonatum sibiricum*

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Abstract

This study explores the impact of four fungal endophytes, *Fusarium oxysporum*, *Aspergillus terreus*, *Alternaria alternata*, and *Penicillium chrysogenum*, on the metabolites, antioxidants, physiological, and genetic traits of *Polygonatum sibiricum*. The experiment performed in randomized complete block design (RCBD) and fungal strains were applied to plants through root dipping. The results indicated significant ($p \leq 0.05$) variations in secondary metabolites, physiological traits and antioxidant enzyme activities. *F. oxysporum* illustrated the significant rise alkaloids, saponins, phenolics, and antioxidant enzyme activities (POD, SOD, CAT). Likewise, *P. chrysogenum* significantly enhanced flavonoid content, and *A. terreus* showed a marked increase in terpenoids, while *A. alternata* depicted mild impact on saponins and phenolics. Additionally, fungal treatments significantly influenced Pn and chl content, with *F. oxysporum* resulting in the highest net Pn and chl. Gene expression analysis showed that *F. oxysporum* and *P. chrysogenum* significantly ($p \leq 0.05$) upregulated the defense-related genes, such as *PAL*, *CYP450* and *BAS*, correlating with enhanced secondary metabolite production. Furthermore, the correlation analysis and principal component analysis (PCA) confirmed strong relationship between fungal treatments and plants metabolic, physiological and genetic responses. Besides, these results further illustrated the potential of fungal endophytes as natural modulators for enhancing plant physiological, biochemical and molecular ability that reshape overall response of *P. sibiricum*.

Keywords: Metabolites; Correlation; PCA; Hetamap; Antimicrobial Potential

1. INTRODUCTION

Plants establish intricate relationships with endophytic microbes that lives within plant tissues or rhizosphere asymptotically. Among these endophytic fungi impart dynamic roles in enhancing plant physiological, biochemical and molecular health that purposely increase plants resilience under stress conditions. Besides, endophytic fungi effect the bioactive properties of their host plants, often producing bioactive compounds (BACs) having antimicrobial, physiological and biochemical properties [1, 2]. Currently, this feature has acquired significant importance owe to its applications in agriculture, biotechnology and drug discovery [3]. *Polygonatum* species are medicinal plants, also known as Solomon's seal, are globally important for their rich profile of BACs (flavonoids, saponins, alkaloids, phenolic), which depict potent antimicrobial, anti-inflammatory and antioxidant activities [4]. The biosynthesis of these BACs is regulated by a group of genes encoding key enzymes involved in secondary metabolic pathways. For example, saponin biosynthesis in *Polygonatum* involves genes encoding β -amyrin synthase (*BAS*), a key enzyme in the synthesis of triterpenoid saponins [5] and cytochrome P450 monooxygenases (*CYP450*), which catalyze various oxidation reactions integral for saponin synthesis [6]. In the same way, the formation of flavonoids in *Polygonatum* is controlled by genes such as chalcone synthase (*CHS*) and chalcone isomerase (*CHI*), which are involved in the phenylpropanoid pathway that leads to the production of flavonoids and other associated compounds [4].

Besides, the transcriptomic and genomic analyses have found some other genes involved in the biosynthesis of BACs in *Polygonatum*, including those involved in the phenylalanine ammonia-lyase (*PAL*) pathway, which is vital for the synthesis of phenolic compounds [7]. Moreover, *PAL* is an important enzyme in the production of precursors for flavonoids, lignins, and other secondary metabolites. Moreover, genes regulating the synthesis of glutathione S-transferase (*GST*) and UDP-glucose:flavonoid 3-O-glucosyltransferase (*UGT*) are significant for the stabilization and modulation of flavonoids and other BACs [8]. Moreover, Tropinone reductase (*TR*) and norbelladine synthase (*NBS*) are key enzymes in the alkaloid biosynthesis that have been identified in the plant's gene expression profiles [9]. These alkaloids depict significant pharmacological activity and contribute to the medicinal properties of plants. The symbiotic association between endophytic fungi and *Polygonatum* gives an interesting opportunity to elucidate the microbial production of these BACs. Recent researches have illustrated that endophytic fungi symbiotically associated with medicinal plants not only produce BACs similar to their host, but also exhibit enhanced antimicrobial properties, indicating their functional significance in plant defense

mechanisms [10-13]. Besides, endophytic fungi can regulate the expression of plant genes involved in secondary metabolism, potentially enhancing the biosynthesis of BACs [14].

The capability of endophytic fungi to rectify the metabolic, antioxidant, genetic and physiological properties of *Polygonatum* species, highlights their potential as alternative sources for BACs. In this context the present study aimed to elucidate the role of different endophytic fungi isolated from *P. sibiricum* on biochemical, antioxidant and physiological properties of *Polygonatum*, and their ability to regulate the bioactive profiles of the host plant. We hypothesized that endophytic fungi function as natural biological elicitors that differentially regulate secondary metabolite biosynthesis in *Polygonatum sibiricum* through coordinated modulation of antioxidant enzymes and key biosynthetic genes. Therefore, the objectives of this study were to investigate the effects of selected endophytic fungi on secondary metabolite accumulation, antioxidant activities, physiological performance, and gene expression, and to identify the most effective fungal species for enhancing bioactive compound production in *P. Sibiricum*. Besides, physiological measurements were used to assess changes in plant performance, the metabolite quantifications were used to evaluate variations in secondary-metabolite accumulation, antioxidant enzyme activities were used to characterize the biochemical defense response, and expression analysis of selected biosynthetic genes was used to examine molecular responses associated with these physiological and biochemical changes. Furthermore, the findings are expected to shed the light on the biochemical and genetic and basis of the plant-fungal symbiosis and will open new horizons for bioprospecting endophytes as sustainable and integral sources of therapeutic agents.

2. MATERIALS AND METHODS

The experiment was conducted at the experimental site of King Abdulaziz University, Jeddah, Saudi Arabia. The experiment was conducted using an RCBD with three biological replicates (blocks) within glass house. Each treatment consisted of ten plants per block. Data from five randomly selected plants within each block were averaged before statistical analysis. Therefore, the experimental unit was the block-level treatment mean rather than individual plants, eliminating pseudoreplication. The experiment was optimized at 25⁰C/15⁰C day and night temperature, 60% humidity, 12h photoperiod and 20000lux light intensity. Four endophytic fungal isolates, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Aspergillus terreus*, and *Alternaria alternata*, were selected for comparative evaluation of their effects on *P. sibiricum*. The isolates

were included to determine whether taxonomically distinct endophytes induce differential biochemical, physiological, and molecular responses in the host

2.1 Extraction and Preparation of Fungal Inoculum

The fungal strains were obtained from first fungal culture bank of Pakistan (FCBP), University of the Punjab, Lahore, Pakistan. These fungi were cultured on nutrient-rich potato dextrose agar (PDA). To prepare the inoculum, a liquid form was created by obtaining the fungal culture, and suspending it in sterile water, for ensuring a spore concentration between 10^6 to 10^8 spores/mL. Besides, a solid inoculum was prepared by combining fungal biomass with carriers like vermicompost, peat, or rice bran [15].

2.2 Inoculum Application and Post-Application Management of Plants

Before the treatment of endophytic fungi, the roots of four leafed stage of *Polygonatum* seedlings were sterilized following the methods opted by [16]. To confirm the surface sterility, the pieces from roots were kept on PDA and incubated at 25°C to 28°C for 5 days. The absence of fungal growth ensured the effectiveness of sterilization. Besides, Quantitative colonization levels of the inoculated endophytes within plant tissues were not determined; hence, the present results are interpreted as responses to fungal inoculation. Moreover, quantitatively equivalent colonization was not established among the four fungal treatments. Afterwards, the sterile roots of *Polygonatum* seedlings were immersed in the fungal solution for 30 to 60 minutes before planting in pots (30 cm diameter and 35 cm depth) containing potting mix. One plant was planted in each pot, and for each treatment ten plants were used. The pots were filled with autoclaved potting mix (40% peat moss, 30% loamy soil, 20% perlite, 10% organic compost) having pH 6 to 7, as used by [17]. On the other hand, during experiment, the temperature and humidity were well maintained and fungicide were avoided to ensure the colonization of endophytic fungi. Moreover, the control set of plant was sown in pots containing potting mix, without treating their root with endophytic fungi. The data were collected after 6 to 8 weeks of fungal inoculation from five randomly selected plants of each treatment in each replicate.

2.3 Quantification of Secondary Metabolites

The total saponin content in the leaves of *Polygonatum* were determined using the vanillin-sulfuric acid method [18]. The air-dried leaves were ground in 70% ethanol and extracted via

maceration. Afterward the extracted mixture was filtered and concentrated. Furthermore, the standard curve was prepared using 20–100 µg/mL diosgenin solution. Afterward this solution was mixed with 0.5 mL of 5% vanillin in acetic acid, and 4 mL of 72% sulfuric acid. Subsequently, the mixture was heated for 10 minutes at 60°C, then cooled, and its absorbance was recorded at 560 nm. Correspondingly the plant extract was treated and the absorbance was compared with the standard curve for estimating the total saponins. Besides, the total phenolic content was calculated by Folin-Ciocalteu method [19]. For this purpose, 0.5 mL methanolic extract was combined with 2.5 mL Folin-Ciocalteu reagent (diluted 1:10) and 2 mL of 7.5% Na₂CO₃. The whole mixture was placed in dark for 30 minutes. Afterward the absorbance was recorded at 765 nm against gallic acid as the standard. The total flavonoid content was recorded by mixing 0.5 mL ethanolic extract to 2 mL of water and 0.15 mL of 5% NaNO₂ [20]. Furthermore 0.15 mL of 10% AlCl₃ was added in mixture after 5 minutes. After another 6 minutes, 1 mL of 1 M NaOH was added and the absorbance was then measured at 510 nm, with quercetin serving as the standard. Besides, for estimating the terpenoids, 1 mL of the methanolic extract was combined with 2 mL chloroform and 3 mL concentrated sulfuric acid. The mixture was kept for 2 hours, and its absorbance was at 538 nm against linalool as the standard [21].

2.4 Estimation of the Activities of Antioxidant Enzymes

For the measurement of enzymatic activity, the detached leaf samples from *P. sibiricum* were stored at -20°C and washed repeatedly using double distilled water. The 10 g of leaf were thoroughly homogenized in 100 mM sodium-phosphate buffer. The mixture was left in buffer for 5 mins at 4°C. The filtered mixture was centrifuged for 5 mins at 5000 x g to obtain the supernatant for enzymatic activity assessment [22]. For The activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) in *Polygonatum* leaves were estimated using standard spectrophotometric methods. For POD activity, the guaiacol was oxidized in the presence of H₂O₂ and the absorbance was recorded against the standard absorption curve at 470 nm. Moreover, SOD activity was recorded spectrophotometrically based on its ability to impede the photoreduction of nitro blue tetrazolium (NBT) at 560 nm, in a reaction mixture having riboflavin, methionine, and EDTA. Besides, the CAT activity was measured by recording the decomposition of H₂O₂ at 240 nm.

2.5 Quantification of Chlorophyll and photosynthesis

The physiological indices such as photosynthesis-rate (Pn) was measured by a portable device IRGA (ADC Bioscientific, UK) from attached flag leaves while the chlorophyll (Chl) content was estimated with the help of SPAD-502plus apparatus (ADC Bioscientific, UK).

2.6 Gene Expression Analysis

For gene expression analysis, the plants were selected from both treated and untreated groups of one year old *Polygonatum* plants. Leaf samples from the selected plants were immediately frozen in liquid nitrogen and stored at -80°C until further processing. RNA was extracted using the RNeasy Kit (Qiagen, Germany) according to the manufacturer's instructions. Besides, agarose gel electrophoresis was used to check the integrity of the extracted total RNA. The samples depicted clear rRNA bands of 28S and 18S rRNA (Figure S1). Subsequently it showed limited degradation or smearing, indicating that the extracted RNA was sufficiently intact. A total of 2 µg of RNA was utilized to construct a cDNA library using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). The transcript levels of metabolites (BACs) related genes, (as referenced in Table 1), from leaf tissues, were assessed through qRT-PCR using the SYBR Green Kit (BioFACT, Korea). Gene expression was normalized using the Actin1 reference gene (MIQE data provided in Table S1 and Figure S2). Actin (ACT) was used as the endogenous reference gene for normalization of transcript abundance. Each reaction was performed with appropriate technical replicates together with no-template controls to monitor contamination and nonspecific amplification. Each qRT-PCR analysis was conducted with three technical replicates and three biological replicates, as described by [23]. Relative gene expression for each sample was calculated using the $2^{-\Delta\Delta C_t}$ method [23]. Details of the primers used for qRT-PCR are provided in Table 1.

2.7 Statistical Analysis:

The statistical model has been explicitly included as: $Y_{ij} = \mu + T_i + B_j + \epsilon_{ij}$, where T_i represents treatment effects and B_j represents block effects. Treatments were considered fixed effects, while blocks were treated as random effects. The Analysis of Variance (ANOVA) was conducted using the Statistix software version 8.1 (McGraw-Hill, 2008) with a significance level of 5%. Before final ANOVA (Table S2), residual normality was assessed using the Shapiro–Wilk test and homogeneity of variance was assessed using Levene's test. The assumptions were considered satisfactory, and no data transformation was required. Following significant ANOVA

results, treatment means were separated using Tukey's Honest Significant Difference (HSD) test at $p \leq 0.05$. Figure lettering is based on Tukey HSD groupings. Additionally, RStudio version 1.1.456 (RStudio Team, 2020) was utilized to generate the correlation chart, principal component analysis (PCA) biplot, and heatmap clusters. The PCA biplot was created using the R packages "factoextra" and "FactoMineR," while Pearson's correlation was analyzed with the help of the "GGally" and "ggplot2" packages. The heatmap cluster dendrogram was developed using the R packages "pheatmap" and "ComplexHeatmap." Furthermore, before PCA, variables were mean-centered and scaled to unit variance., while principal components with eigenvalues >1 were retained for interpretation. Heatmap clustering was performed using distance and linkage. For qRT-PCR data, the mean value obtained from the technical replicates was used for each biological replicate, and treatment effects were evaluated using the same RCBD.

3. RESULTS

3.1. Secondary Metabolite

3.1.1 Total Saponins

The total saponin content differed significantly among treatments. The *F. oxysporum* treatment had the highest saponin content, which was significantly ($p \leq 0.05$) greater than the control (Figure 1). *A. terreus* and *P. chrysogenum* showed statistically similar saponin levels, both of which were significantly ($p \leq 0.05$) higher than the control but lower than *F. oxysporum* (Figure 1). *A. alternata* exhibited a moderate increase in saponin levels, which was different from the control but lower than *F. oxysporum* (Figure 1).

3.1.2 Total Flavonoids

Flavonoid levels showed significant ($p \leq 0.05$) variation among treatments. The *P. chrysogenum* treatment had the highest flavonoid content, which was significantly ($p \leq 0.05$) higher than all other treatments and the control (Figure 1). *F. oxysporum*, *A. terreus*, and *A. alternata* had similar flavonoid levels, all of which were significantly higher than the control (Figure 1).

3.1.3 Total Phenols

The total phenolic content was significantly ($p \leq 0.05$) affected by the treatments. *F. oxysporum* had the highest phenolic content, which was significantly ($p \leq 0.05$) greater than all other treatments and the control (Figure 1). *A. terreus*, *A. alternata*, and *P. chrysogenum* had

comparable phenolic levels, which were significantly ($p \leq 0.05$) higher than the control but lower than *F. oxysporum* (Figure 1). The control group exhibited the lowest phenolic content, which was significantly ($p \leq 0.05$) different from all treated groups (Figure 1).

3.1.4 Alkaloids

The alkaloid content varied significantly among the treatments. *F. oxysporum* had the highest alkaloid content, which was significantly ($p \leq 0.05$) greater than all other treatments and the control (Figure 1). *P. chrysogenum* exhibited the second-highest alkaloid level, which was significantly ($p \leq 0.05$) lower than *F. oxysporum* but higher than the other treatments (Figure 1). *A. terreus* and *A. alternata* had statistically similar alkaloid levels, which were significantly ($p \leq 0.05$) greater than the control (Figure 1). The control group had the lowest alkaloid content, which was statistically significantly different from all other treatments.

3.1.5 Terpenoid Content

The terpenoid content was significantly affected by the treatments. *A. terreus* exhibited the highest terpenoid content, which was statistically significantly ($p \leq 0.05$) greater than all other treatments and the control (Figure 1). *P. chrysogenum* showed the second highest terpenoid content, which was significantly ($p \leq 0.05$) higher than *A. alternata*, *F. oxysporum*, and the control (Figure 1). *A. alternata* and *F. oxysporum* had moderate terpenoid levels, which were significantly higher ($p \leq 0.05$) than the control but lower than *A. terreus* and *P. chrysogenum* (Figure 1). The control group had the lowest terpenoid content, which was significantly different from all treated groups.

3.2. Antioxidant Enzymes Activity

The treatments significantly ($p \leq 0.05$) affected the activity of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) enzymes (Figure 1). *F. oxysporum* exhibited the highest activity for all three enzymes, significantly ($p \leq 0.05$) greater than all other treatments and the control (Figure 1). *P. chrysogenum* and *A. terreus* showed similar intermediate levels of POD, SOD, and CAT activity, all significantly ($p \leq 0.05$) higher than *A. alternata* and the control (Figure 1). *A. alternata* had moderately increased enzyme activity compared to the control, but it was significantly lower than the other treatments (Figure 1). The control group consistently had the lowest enzyme activity, which was statistically distinct from all treatments for each enzyme (Figure 1).

3.3. Physiological Parameters

3.3.1 Net Photosynthetic Rate (Pn)

The net Pn was significantly ($p \leq 0.05$) affected by the treatments. *F. oxysporum* resulted in the highest Pn, which was statistically significantly greater than all other treatments and the control (Figure 1). *A. terreus* showed the second highest Pn, which was significantly ($p \leq 0.05$) higher than *A. alternata*, *P. chrysogenum*, and the control (Figure 1). *A. alternata* and *P. chrysogenum* had similar Pn levels, both significantly ($p \leq 0.05$) higher than the control but lower than *F. oxysporum* and *A. terreus* (Figure 1). The control group exhibited the lowest Pn, which was statistically significantly different from all treated groups.

3.3.2 Chlorophyll Content (Chl)

All fungal treatments (*F. oxysporum*, *A. terreus*, *A. alternata*, and *P. chrysogenum*) significant ($p \leq 0.05$) increase in chl content compared to the control (Figure 1). Conversely, there were no statistically significant ($p \leq 0.05$) differences in chl content among the fungal treatments, indicating that all treatments had a comparable effect (Figure 1). The control showed the lowest chl, which was significantly different from all treated plants (Figure 1).

3.4 PCA, Correlation and Heatmap Analysis

The PCA biplot highlighted the clustering of treatments based on physiological and biochemical profiles, showing that *P. chrysogenum* and *A. terreus* are distinct from the control and other treatments (Figure 2). Correspondingly, PCA with confidence ellipses indicated the variability and distribution of treatments (Figure 3). In this context, *P. chrysogenum* showed greater variation and a more dispersed cluster, whereas *A. alternata*, *F. oxysporum* and *A. terreus* formed tighter and more closely overlapping clusters as indicated in figure 3. Besides, the correlation matrix revealed strong positive associations among antioxidant enzymes, secondary metabolites, and several physiological traits (Figure 4). Subsequently phenolics, flavonoids, terpenoids, Pn, chl, SOD, POD, and CAT exhibited positive correlations as indicated in figure 4. In parallel, the heatmap with hierarchical clustering showed the distinct patterns in the physiological and biochemical profiles of the treatments. In this context *P. chrysogenum* was characterized alongs comparatively higher flavonoids and phenolics but lower SOD and terpenoid levels (Figure 5). On the other hand, the control shows consistently lower values across the measured parameters (Figure 5). The

clustering pattern further confirmed that the treatments varied in their effects on biochemical and antioxidant responses.

3.5. Gene Expression Analysis

The amplification of the target genes *BAS*, *CYP450*, *CHS*, *CHI*, *PAL*, *GST*, *UFGT*, *TR*, and *NBS* was confirmed using agarose gel electrophoresis across the control and fungal-treated samples of *P. sibiricum* as indicated in figure 6. Distinct amplification bands were observed in the respective sample lanes, where clearly defined bands at the expected positions indicated specific amplification of the target genes (Figure 6). Furthermore, the differences in band intensity among treatments were consistent with variation in transcript abundance observed in the corresponding gene-expression analysis as shown in figure 7.

Each gene's expression pattern demonstrated a highly significant ($p \leq 0.05$) variation across the treatments. For instance, *BAS* gene showed its highest expression under *F. oxysporum*, with expression levels significantly ($p \leq 0.05$) higher than other treatments, including *P. chrysogenum* as illustrated in figure 7. On the other hand, the expression under *A. terreus* and *A. alternata* was significantly ($p \leq 0.05$) lower, reflecting their comparatively weaker elicitation of basal defense.

For *CYP450*, the expression was significantly ($p \leq 0.05$) upregulated under *F. oxysporum* and *P. chrysogenum* treatments compared to *A. terreus* and *A. alternata*, as mentioned in Figure 7. This rise of expression was consistent with the rise of BACs as depicted in figure 1. Besides, the *CHS* and *CHI* genes, essential for flavonoid biosynthesis, showed significantly high expression with *P. chrysogenum* followed by *F. oxysporum*, *A. terreus* and *A. alternata* (Figure 7). Besides, complementary to levels of phenolics the expression of the *PAL* gene was most significantly ($p \leq 0.05$) induced by *F. oxysporum*, followed by *P. chrysogenum*, *A. alternata* and *A. terreus* as mentioned in figure 7. Similarly, *GST* gene exhibited significantly ($p \leq 0.05$) higher expression under *P. chrysogenum*, that was followed by *A. terreus* and *F. oxysporum*. (Figure 6). Correspondingly, *UFGT*, critical for glycosylation of flavonoids, was most significantly ($p \leq 0.05$) upregulated by *P. chrysogenum*, followed by *A. alternata*, further validating the specificity of this gene's response to these endophytes (Figure 7).

For *TR*, *F. oxysporum* and *P. chrysogenum* induced the most significant ($p \leq 0.05$) expression levels, reflecting their strong role in maintaining redox homeostasis (Figure 7). Lastly, the *NBS* gene exhibited its highest expression under *F. oxysporum*, with *P. chrysogenum* also inducing statistically significant ($p \leq 0.05$) expression (Figure 7). This suggests that *F. oxysporum* strongly

activates immune-related signaling pathways compared to the moderate effects of *A. terreus* and *A. alternata*.

In conclusion, the differential gene expression patterns observed in response to fungal endophytes were statistically significant, indicating a highly specific modulation of metabolic and defense pathways. *F. oxysporum* and *P. chrysogenum* emerged as the most effective endophytes, eliciting robust upregulation of key defense-related genes. These findings rectify the ability of fungal endophytes as natural stimulators for increasing the *P. sibiricum* resilience and BACs production, as supported by statistically significant data that validate the reliability of these results.

3. DISCUSSION

The significant variations in metabolic, physiological and antioxidant contents among the treatments confirmed the diverse metabolic responses of *P. sibiricum* to fungal endophytes, indicating the potential of fungal interactions to increase plant secondary metabolites and stress-related pathways. For instance, *F. oxysporum* was observed to induce the highest levels of saponins, alkaloids, phenols, and antioxidant enzyme (POD, SOD, and CAT) activities compared to all other treatments and the control, highlighting a robust induction of plant defense mechanisms (Figure 1). Correspondingly this was consistent with conclusion of Selwal et al. [24] who reviewed the role of *F. oxysporum* in regulating plant metabolic pathways, including its effect on secondary metabolites like alkaloids and phenolics, which are important for plant defense and stress tolerance. Moreover, *F. oxysporum* triggered higher gene expression of defense-associated genes such as *BAS*, *CYP450*, *PAL*, and *NBS* (Figure 6), further supporting its strong role as a defense activator as reported by Yang et al. [25] and Kumar et al. [26]. Likewise previous studies, *F. oxysporum* has been reported to enhance BACs production through upregulation of various secondary metabolic pathways as explicated by Chakraborty et al. [27], thereby increasing plant resilience. Besides, *P. chrysogenum* illustrated the highest flavonoid content, significantly ($p \leq 0.05$) enhancing flavonoid biosynthesis (Figure 1). This result aligned with Thuerig et al. [28] that had shown *P. chrysogenum* to elicits flavonoid production through the upregulation of genes like *CHS* and *CHI* (Figure 1, 6 and 7). In line with the findings of Sikandar et al. [29] and Dargiri, et al. [30], the observed increase in flavonoids under *P. chrysogenum* treatment was accompanied by moderate but notable increases in antioxidant enzyme activity, which corroborates findings that flavonoids play an important role in mitigating oxidative stress. Interestingly, while *P.*

chrysogenum made significant ($p \leq 0.05$) changes in secondary metabolites and antioxidant activities, its terpenoid content was lower than other treatments, which might indicate a specific regulation of flavonoid biosynthesis over other metabolic pathways in this species (Figures 1). These results are consistent with Nikalje et al. [13] emphasizing the ability of fungal endophytes to modulate specific secondary metabolite biosynthesis, which ultimately contributes to improved plant stress tolerance.

A. terreus also enhanced the synthesis of secondary metabolites, especially terpenoids, with levels surpassing those of other fungal treatments and the control (Figure 1). This result was consistent with the findings of Chen et al. [31] that reported *A. terreus*' ability to induce terpenoid production in plants, hence enhancing both stress tolerance and metabolism. However, its impact on phenolic and alkaloid content was relatively moderate compared to *F. oxysporum* and *P. chrysogenum*, suggesting a more specialized role in terpenoid biosynthesis rather than a broad activation of secondary metabolite pathways. The role of *A. terreus* in regulating plant metabolic networks and enhancing specific BACs production is increasingly recognized in plant-microbe interactions [32].

The impact of *A. alternata* on secondary metabolites was more subdued, although it led to moderate increase in saponins and phenols, its overall effect was less pronounced compared to the other treatments (Figures 1). This probably reflect the comparatively weaker triggering effects of *A. alternata*, as observed in similar study by Zhao et al. [33]. However, it is noteworthy that *A. alternata* induced higher levels of certain stress-related genes, such as *UFGT*, than the control (Figure 7), illustrating its potential tendency in regulating specific defense pathways [5]. As suggested by Chung [34], *A. alternata* may have a more specialized impact on plant defense pathways, as it can trigger oxidative stress responses and promote specific phytohormonal changes.

Figure 1 indicates the clear difference in metabolite content, enzyme activity, chl and Pn across treatments, reinforcing the role of fungal endophytes in modulating plant biochemical and physiological profiles. Besides, the PCA analysis further supports these findings by showing clustering of fungal treatments based on secondary metabolite profiles, Pn, chl and enzyme activity, with *P. chrysogenum* and *A. terreus* forming distinct clusters due to their strong association with specific metabolites like terpenoids and flavonoids as indicated in Figures 2 and 3. The PCA analysis was consistent with the results obtained from individual trait analyses and

provided an integrated visualization of treatment-associated response patterns. Treatments such as *F. oxysporum* and *P. chrysogenum* were located closer to metabolite and antioxidant-related variables, suggesting coordinated regulation of these traits under fungal inoculation. The correlation matrix from figure 5, illustrated positive association between antioxidants (SOD, POD, and CAT) and secondary metabolites (phenolics, alkaloids, flavonoids), consistent with the understanding that antioxidant systems often work in association with secondary metabolite biosynthesis to protect plants from oxidative damage as reported by Saini et al. [35] and Llauradó-Maury et al. [36]. Furthermore, the heatmap rectified these patterns, showing how different treatments modulate multiple biochemical pathways simultaneously, with *P. chrysogenum* notably enhancing flavonoid and phenolic production while *F. oxysporum* boosts alkaloid and antioxidant activities (Figure 5).

In terms of gene expression, the different fungal treatments led to significant differential regulation of metabolite-related genes, further validating the biochemical data (Figures 6 and 7). The high expression of *BAS*, *CYP450*, *PAL*, and *NBS* genes under *F. oxysporum* treatment confirmed its strong role in activating secondary metabolite biosynthesis that was in line with the findings of Yang et al. (2022). In contrast, the upregulation of *CHS* and *CHI* genes under *P. chrysogenum* emphasizes its specificity in driving flavonoid biosynthesis as reported by Ye et al. [23] (2025). Similarly, corresponding to the findings of Chen et al. [23] (2021) present study reported the highest *GST* expression under *P. chrysogenum* that confirms the role of endophyte in imparting the resilience in *P. sibiricum*.

The findings from present study has provided strong evidence for the potential role of fungal endophytes, particularly *F. oxysporum* and *P. chrysogenum*, in actively provoking the secondary metabolites and antioxidant systems, making them promising candidates for natural elicitors in the production of BACs. Overall, the interaction of endophytic fungi enhances the expression of genes associated with the production of secondary metabolites (BACs) that triggers the antioxidant system of *P. sibiricum* as explicated in proposed model shown in figure 8. An important feature of the present dataset was the differential response breadth among the four endophytes. Rather than producing a uniform host response, the fungal treatments showed distinct patterns across secondary metabolites, antioxidant enzymes, physiological traits, and gene expression. *F. oxysporum* showed comparatively broad changes across several response variables, whereas *P.*

chrysogenum, *A. terreus*, and *A. alternata* showed more selective associations with particular metabolite or biosynthetic traits. These findings indicate that the response of *P. sibiricum* was dependent on the identity of the fungal endophyte. The integrated response pattern indicates that fungal inoculation was associated with coordinated changes across biochemical, physiological and molecular levels. In treatments showing increased secondary-metabolite accumulation, these changes were considered together with antioxidant enzyme activity and expression of genes associated with the corresponding biosynthetic pathways. The results also highlighted the complexity of fungal-plant interactions, where the specific fungal species and their ability to modulate distinct metabolic pathways play a crucial role in determining the overall plant response. This work further supports the growing body of literature demonstrating the potential of fungal endophytes as biotechnological tools to improve the yield and quality of BACs in medicinal plants.

Declarations:

Abbreviation: Not Applicable

Ethics approval and consent to participate: Not applicable. Experimental research, field studies on plants, and collection of plant material is complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication: Not Applicable

Availability of data and materials: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests: The authors declare no competing interests

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Authors' contributions: YA: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing—original draft. HA: Validation, Data curation, Writing—review and editing.

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Gene	Primers
β-amyrin synthase (BAS)	ATGGCCTGGAGGAGGAAGAA (F) TTAGGAGGCTTGGGAGAGT (R)
cytochrome P450 monooxygenases (CYP450)	TGGACTGGACAGACTGGGACCATAA (F) TCGGAAGAAGCCGATATTGTGGAA (R)
chalcone synthase (CHS)	ATGGCTAGCTTCAGTTGGTGGT (F) TCAGTAGTAGCACCACGTTCTT (R)
chalcone isomerase (CHI)	ATGGAGTTCTGGGAAGCAAATG (F) TCAGTAGAGTCCACCGCATTAC (R)
phenylalanine ammonia-lyase (PAL)	ATGGAAGCCGGTGACCTCAAGA (F) TCAGCCTTGGAGAGCTTGGTGA (R)
glutathione S-transferase (GST)	ATGGCAAGATCTTCCGTTGG (F) TCAAGGCGGTTTCTGCCGTT (R)
UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT)	ATGGCAAGACAAAGGAGGAG (F) TCAAGGCGGTTTCTGCCGTT (R)
Tropinone reductase (TR)	ATGGAGCTTCAGGGAGAGTG (F) TCAAGGAGCGTTGAGGTGTC (R)
norbelladine synthase (NBS)	ATGGAGGTGAGGAGGAGGAG (F) TCAAGGAGAGGTGAGGAGTGG (R)
β-actin (ACT)	ATGGCTGGGGTGTTGAAGGT (F) ATGGCTGGGGTGTTGAAGGT (R)

Antioxidants in plants: a valorization potential emphasizing the need for the conservation of plant biodiversity in Cuba. Antioxidants. 2020;9048.

Table 1. List of primers used for gene expression analysis

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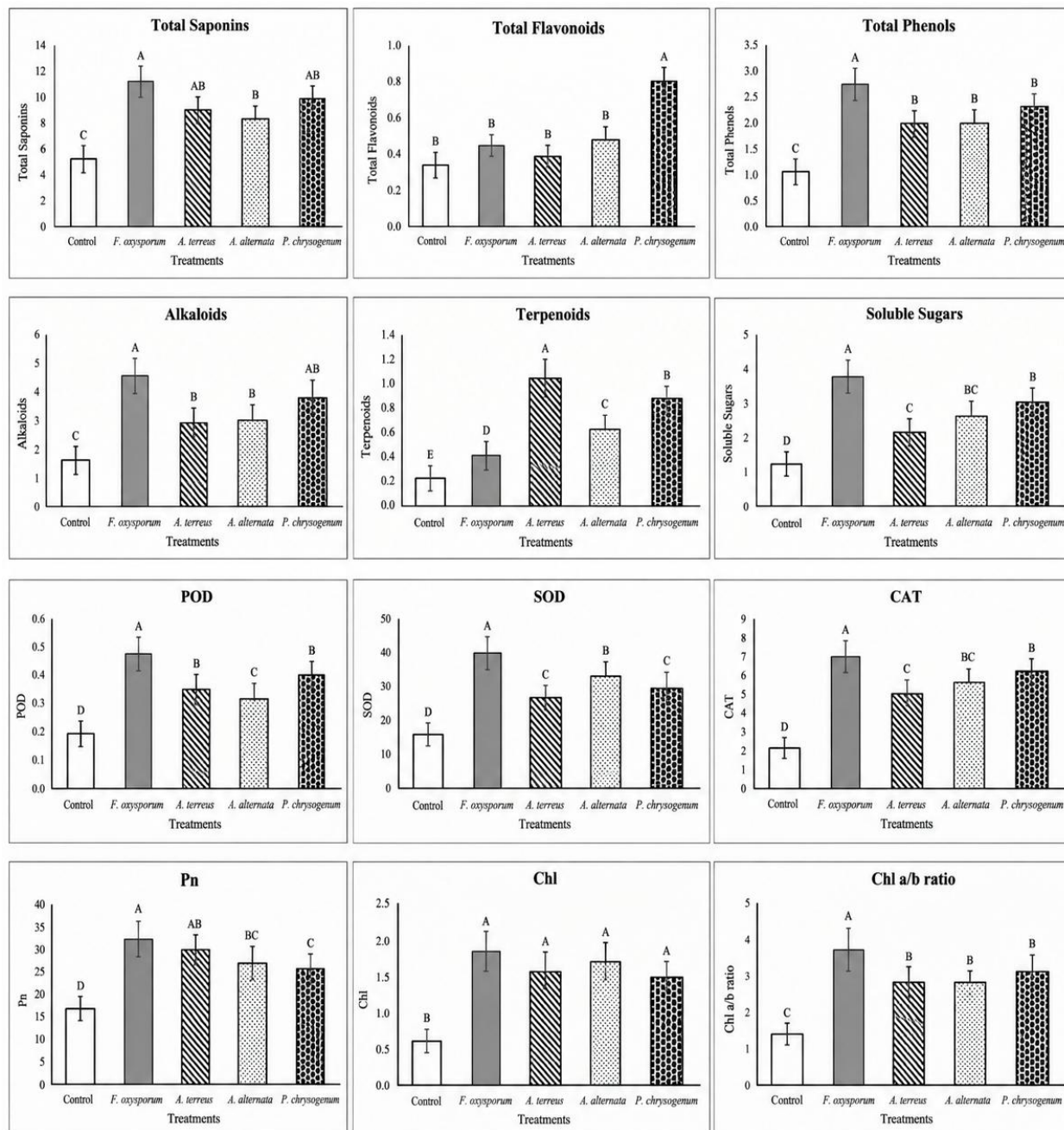


Figure 1. Effect of different endophytic fungi on secondary metabolites percentage, antioxidant enzymes activity ($U\ mg^{-1}\ of\ protein$) and photosynthesis ($\mu mm^{-1}\ S^{-2}$) and chlorophyll ($g\ kg^{-1}$) of *Polygonatum sibiricum*. Values shown in graph are mean values analysed at $p \leq 0.05$ during tri-replicate experiment. Besides, the bar values having different letters vary significant at $p \leq 0.05$.

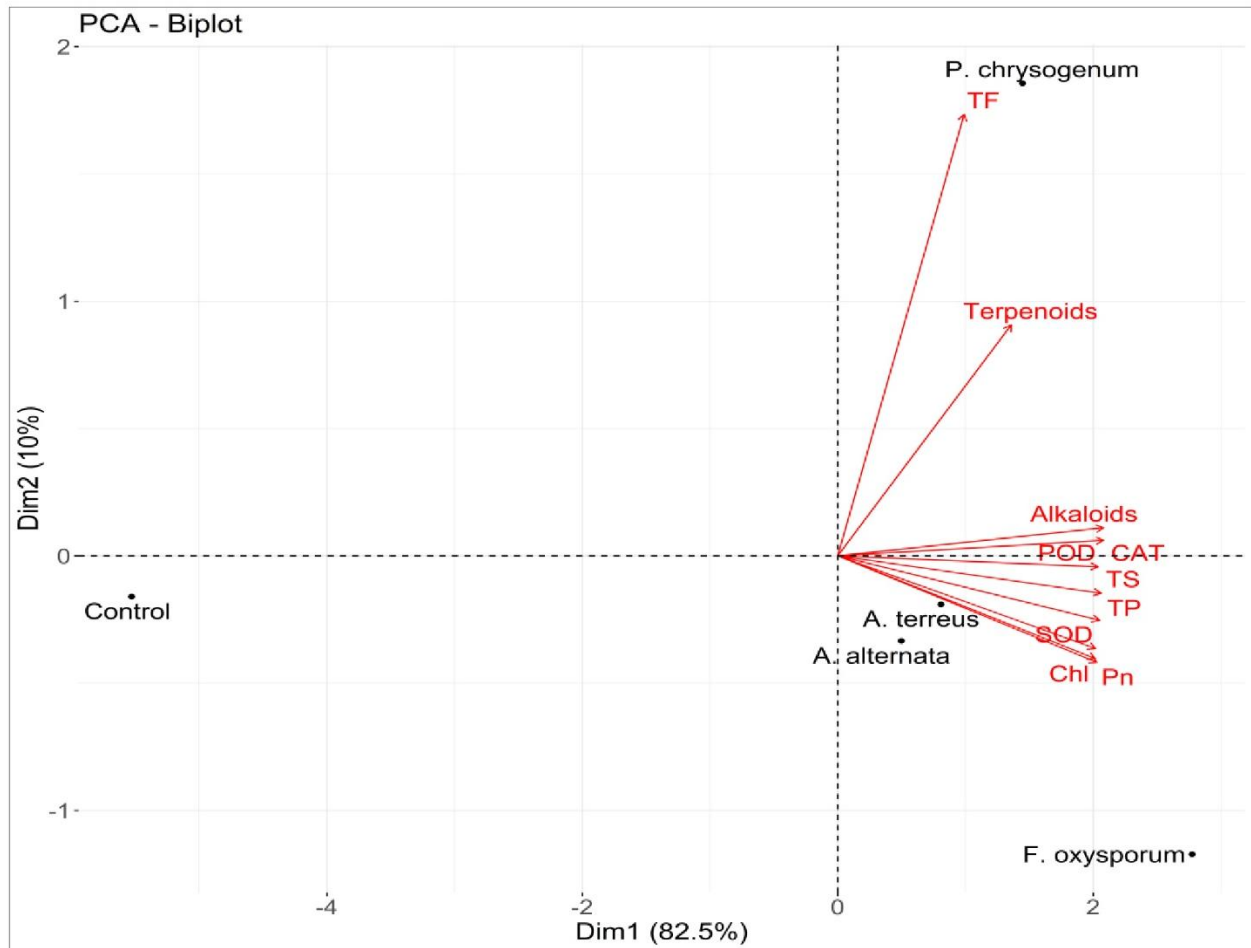


Figure 2. The PCA biplot illustrate the clustering of treatments based on metabolic, antioxidant and physiological profiles. The biplot is showing that *F. oxysporum*, *P. chrysogenum* and *A. terreus* are distinct from the control and other treatments due to their strong association with enzymes (SOD, POD, CAT) and secondary metabolites like terpenoids, alkaloids, and phenolics.

TS, total saponins; TP, total phenols.

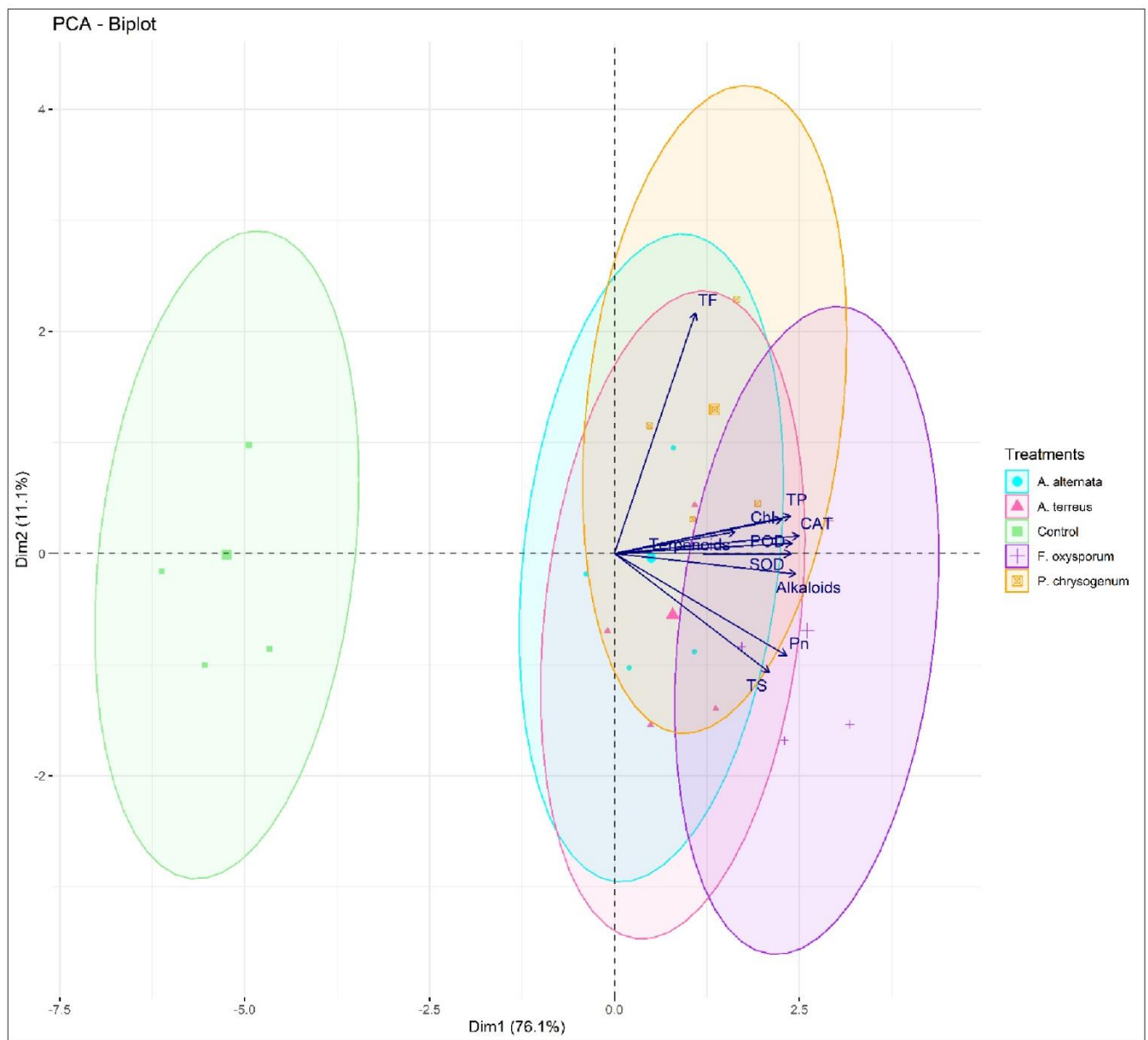


Figure 3. The varying orientations of PCA eclipses indicates significantly varying impacts of endophytic fungi on metabolic, antioxidant and physiological traits of *Polygonatum sibiricum* as compared to control. Besides, *P. chrysogenum* display significant variation, while others, like *A. alternata* and *A. terreus*, form tighter, more overlapping groups.

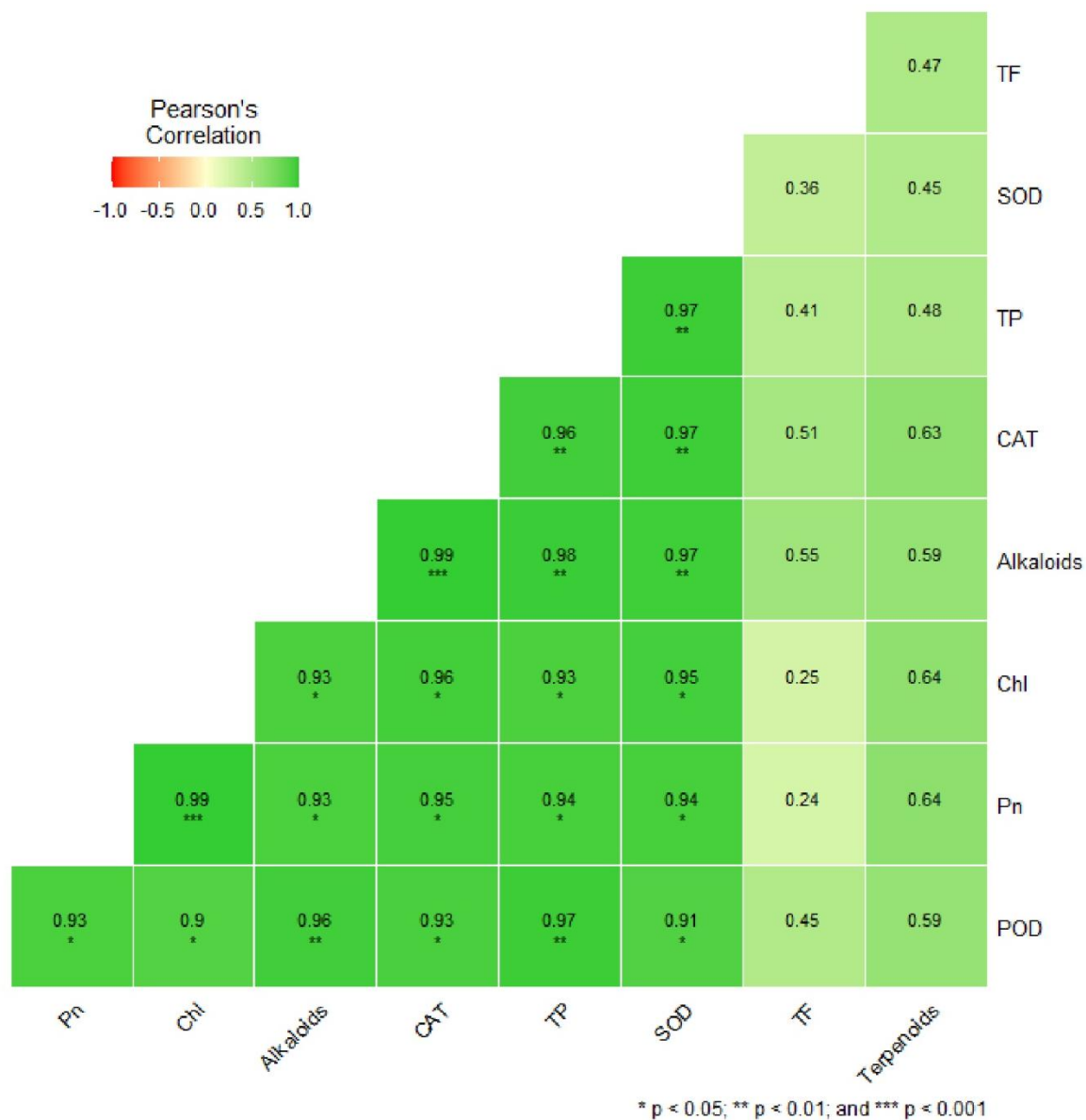


Figure 4. The correlation matrix illustrating the extent of paired association of secondary metabolites, antioxidant enzymes and physiological traits.

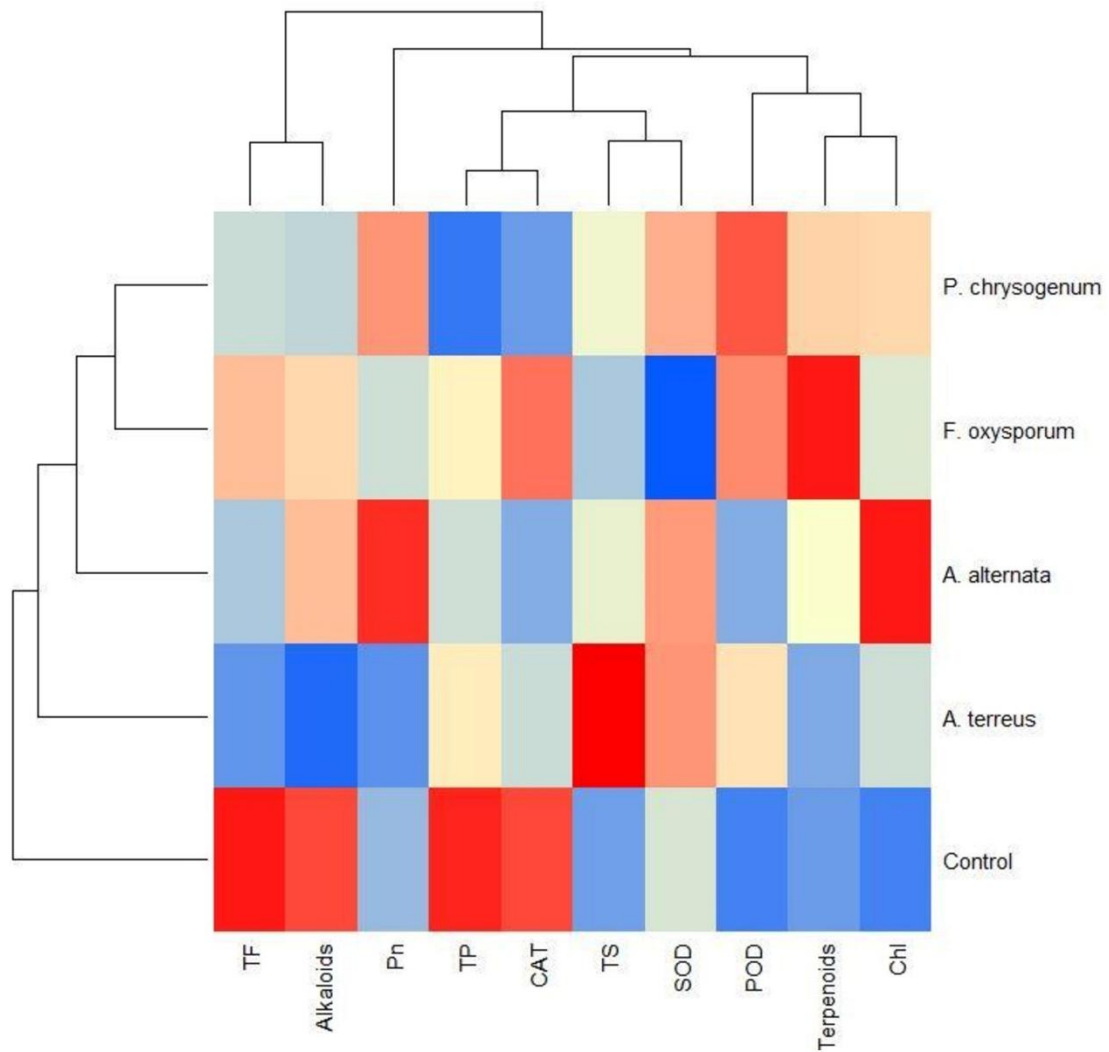


Figure 5. The heatmap cluster reveals varying expression of metabolic, antioxidant and physiological traits due to endophytic fungi.

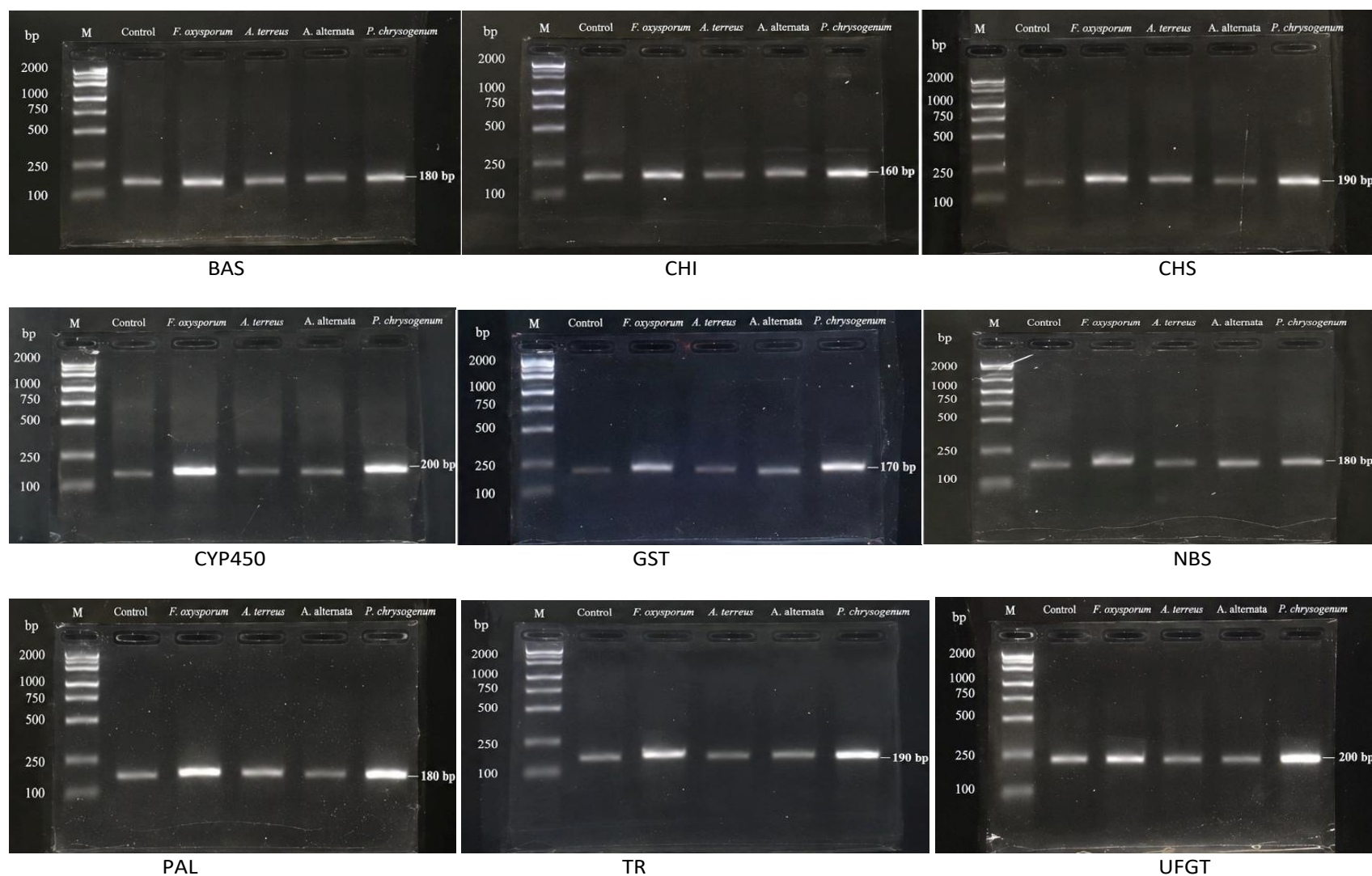


Figure 6. Gel illustrating amplification profile of target genes (BAS, CYP450, CHS, CHI, PAL, GST, UFGT, TR, and NBS) in control, *F. oxysporum*, *A. terreus*, *A. alternata*, and *P. chrysogenum* treatments. **M** represents molecular-weight marker, and distinct bands corresponding to the respective target genes observed across the samples.

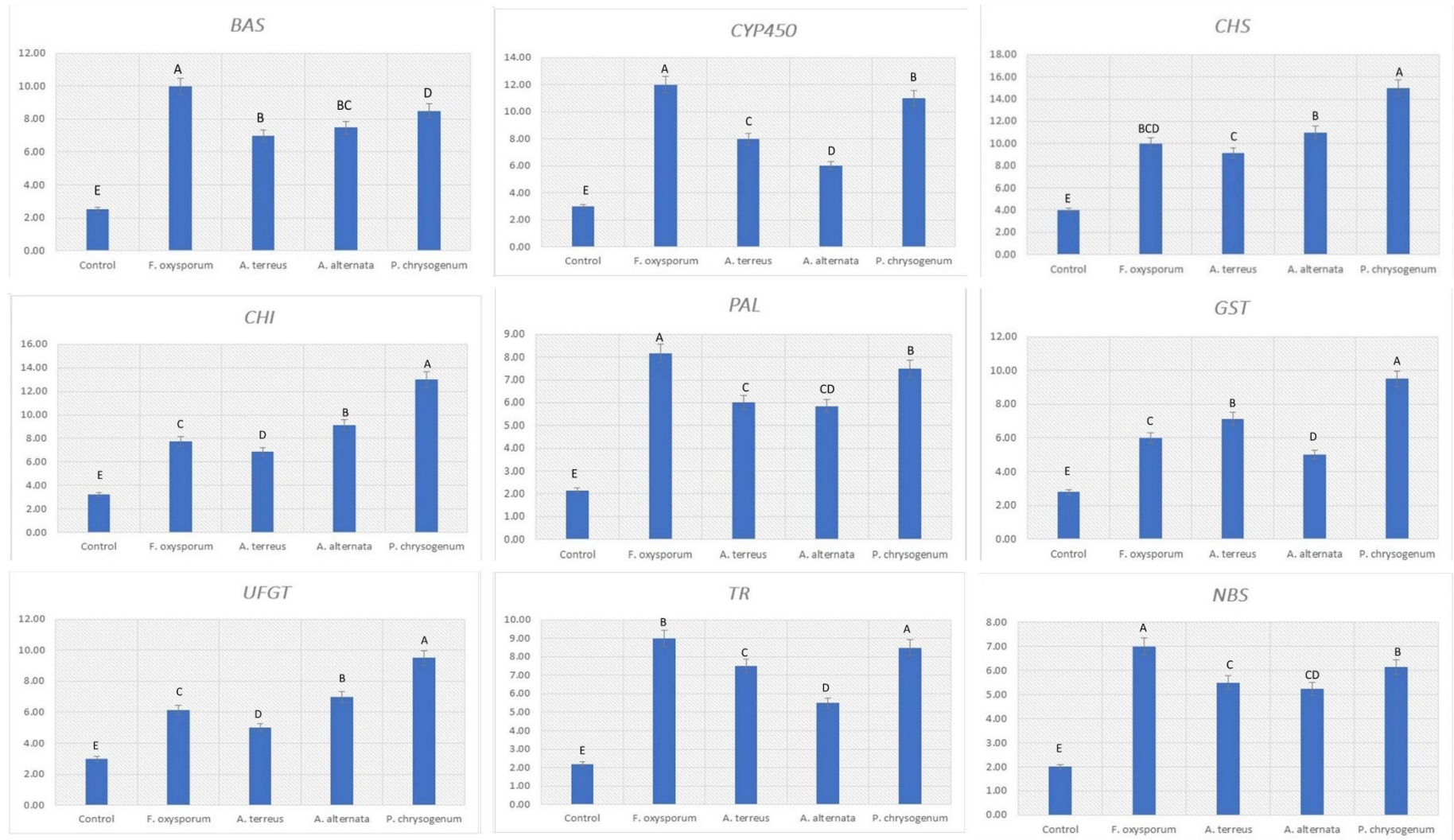
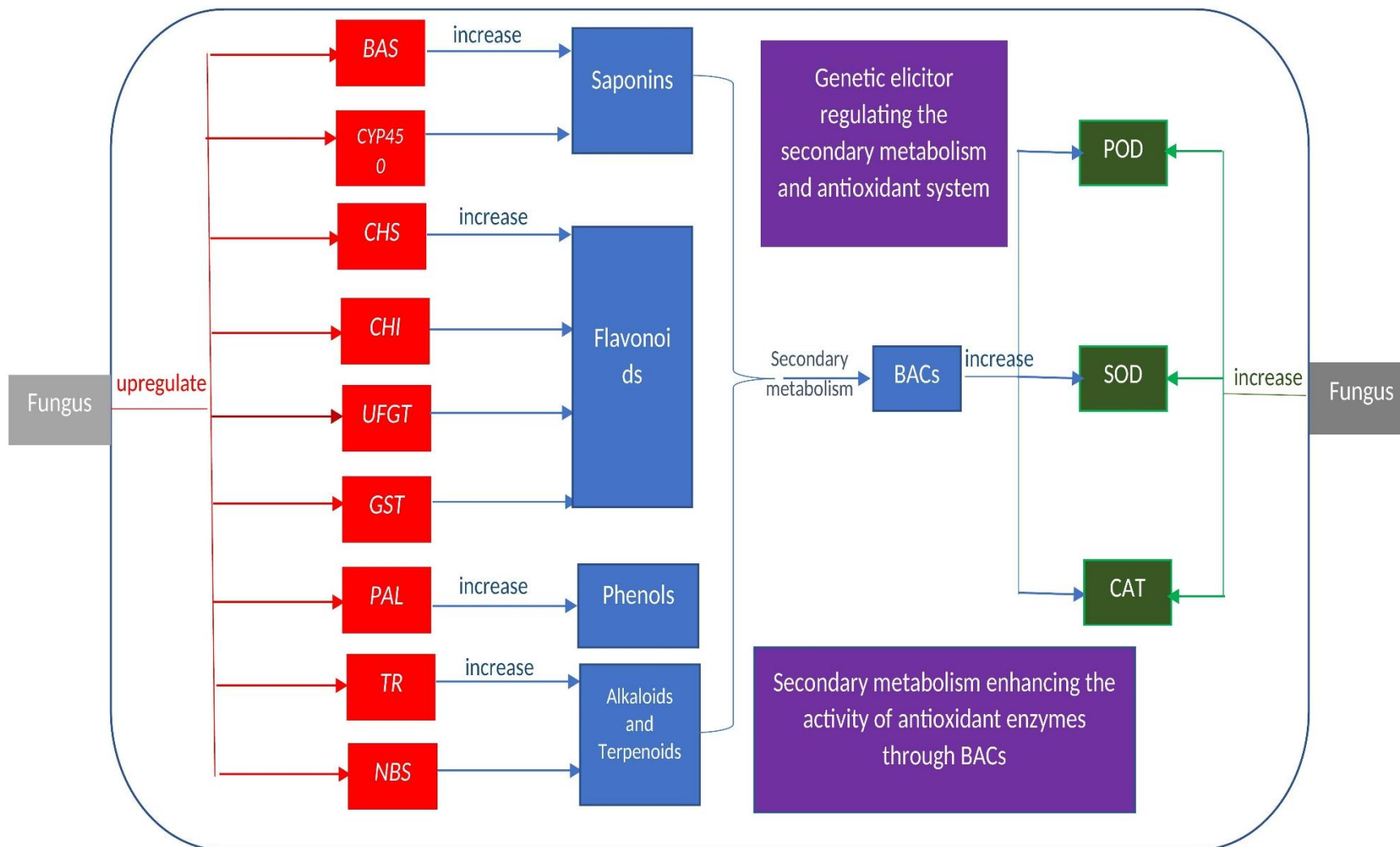


Figure 7. Effect of different endophytes on the expression of genes regulating the production of metabolites enhancing the antimicrobial activity of *Polygonatum sibiricum*.



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582 **Figure 8.** Proposed model showing the regulation of secondary metabolism and antioxidant system of *P. sibricum* due to modulation of
 583 genetic elicitors by endophytic fungi.

Supplementary Files

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