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INFLUENCE OF *TRICHODERMA* INOCULATION ON FOUR CRUCIAL DEFENSE-RELATED ENZYMES AND LEAF SOLUBLE PROTEIN LEVEL OF POPLAR

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Abstract

Trichoderma spp. are beneficial endophytic plant symbionts known as plant growth improving factors and control agents for plant pathogens. We report in this study that the mechanisms for biological control of *T. asperellum* was the inducing of increases of crucial defense-related enzymes SOD, POD, PPO and PAL, and increasing the soluble protein level in leaf. Four *T. asperellum* strains each had slightly differential effects but all have positive effects. *T. asperellum* ACCC30536 had the best promoting effects. The activities of SOD and POD in poplar leaf responded to *T. asperellum* inducing as early as four hours after inoculation and both reached peak on the third day after inoculation. In contrast, the activities of both PPO and PAL in poplar leaf shortly showed suppression within one day after inoculation before the following drastic increase both peaking on the sixth day after inoculation. On the gene expression level, the regulation of SOD, POD and PAL in leaf showed consistent patterns with enzyme activities while PPO in leaf and the four enzymes in root indicated more complex regulatory mechanisms. Leaf soluble protein level were highly induced by each of the four *T. asperellum* strains and maintained almost stable from four hours after inoculation.

Key words: *Trichoderma asperellum*, Poplar, Antioxidant enzymes, Leaf soluble protein.

Abbreviations: (SOD) Superoxide dismutase, (POD) Peroxidase, (PPO) Polyphenol oxidase, (PAL) Phenylalanine ammonia-lyase, (qRT-PCR) Quantitative Real Time-Polymerase Chain Reaction.

Introduction

Plants are constantly exposed to the threats of pathogenic microorganisms in the environment. Thus, they have developed physiological defense mechanisms as well as adopted beneficial endophytes for protection. Species of the genus *Trichoderma* are beneficial endophytic plant symbionts and have been known as control agents for plant pathogens since the 1930s (Weindling, 1932). The mechanisms for biological control of *Trichoderma* spp. are multiple and varied, which include competition, antibiosis and mycoparasitism against the pathogens, and inducing the systemic resistance of the host plants (Harman *et al*., 2004). The increase of reactive oxygen species (ROS) to a damaging level is a common event in plants infected by pathogens or under abiotic stresses (Apel & Hirt, 2004; Bhar *et al*., 2017). Plants have enzymatic and nonenzymatic defense mechanisms against ROS damage. Superoxide dismutases (SOD, EC 1.15.1.1) and catalases (CAT, EC 1.11.1.6) are respectively the main enzymatic scavengers of superoxide and hydrogen peroxide which work in line (Noctor & Foyer, 1998).

Another well-known physiological event correlated with plant–pathogen interactions or plant resistance is the accumulation of phenolic compounds (Anesini *et al*., 2008; Lei *et al*., 2011; Yedidia *et al*., 2000). The enzymes peroxidase (POD, EC 1.11.1.7), polyphenol oxidase (PPO, EC 1.10.3.1) and phenylanlanine ammonialyase (PAL, EC 5.3.1.5) are the key enzymes for the biosynthesis of phenols and flavonoids in plants. PAL catalyzes the first reaction on the phenylpropanoid pathway, which in turn results in precursors of various secondary metabolites which are important for plant defense (Huang *et al*., 2010). POD and PPO are enzymes for the oxidization of phenols. PPOs catalyze the oxidation of polyphenols into antimicrobial compound quinones and are important for the further lignification of plant cell wall during pathogen infection. PODs participate in cell wall polysaccharides processes including phenol oxidation, thus is crucial for the suberization and lignification of plant cells in the defense reactions during pathogen invasion (Chittoor *et al*., 1999).

Many species of *Trichoderma* are also well known as plant growth promoter and have been applied widely in agriculture (Topolovec-Pintarić, 2019). However, the researches for use of *Trichoderma* on forestry and woody plants are far not sufficient. In our previous studies, we have reported that four *T. asperellum* strains each had growth-promoting effects and significantly promoted the photosynthesis efficacy of a hybrid poplar *Populus davidiana* × *P. alba* var. *pyramidalis*, among which *T. asperellum* ACCC30536 had the most pronounced promoting effects (Zhang *et al*., 2018; Jiang *et al*., 2016). In this study we report the activities and encoding-gene expression of the major defense-related enzymes SOD, POD, PPO and PAL in poplar inoculated with *T. asperellum* ACCC30536 under field conditions.

Materials and Methods

***Trichoderma* strains and inoculum preparation:** The fungal strains used in this study were *T. asperellum* ACCC32492 (T1), *T. asperellum* ACCC31650 (T2) and *T. asperellum* ACCC30536 (T3), purchased from Agricultural Culture Collection of China, ACCC) and *T. asperellum* T4 (T4) isolation, all preserved in Northeast Forestry University (Fig. 1). Fungal strains were each cultured on potato dextrose agar (PDA) medium at 28°C in dark for seven days. Then conidia were collected by washing with tap water and the concentration of conidia suspension was calculated using a hemocytometer under microscope. The conidia suspension was used as inoculum.

mock-inoculated with the equal volume of tap water. Each group contained ten individual poplar plants.

Sample collection: the comparatively youngest mature leaf approximately 1cm beneath the shoot apex of each poplar plant was collected for enzyme activity assays and soluble protein concentration assays. The same leaves were also used for fluorescent quantitative real-time PCR (qRT-PCR) assays. Root of three individual poplar plants as three biological replicates were excavated from pot soil to be used for the qRT-PCR assays. Plant material collected in the field were preserved in liquid nitrogen for the sequential lab experiments.

Total RNA isolation and quantitative real-time PCR (qRT-PCR): Total RNA was isolated a using plant RNA isolation kit (USA omega Bio-Tek, Norcross, GA Extracted). Total RNA was digested with DNase I (Promega) to remove DNA residues. 1 µg of total RNA was reverse transcribed to synthesize cDNA in a 10µL volume using PrimeScript RT reagent kit (TaKaRa). A light Cycler 96 real-time PCR detection system (Roche) and FastSart essential DNA Green Master Mix (Roche) were used to perform qRT-PCR experiments according to manufacturers' instructions. Three genes, *PdPapActin1, PdPapEF1-α* and *PdPapUBQ* were used as reference genes and their mean expression was used to calculated the relative expression of target genes. All primers are shown in Table 1.

Plant material and growth conditions: Seedlings of subcultured *Populus davidiana* × *P. alba* var. *pyramidalis* clones preserved by Northeast Forestry University were transplanted into pot soil containing 6 L soil and placed under field conditions in a tree nursery of Northeast Forestry University, Harbin, China (45° 45' 0.0000" N and 126° 37' 59.9916" E) from June 10th to July 11th 2013 and well-watered. On July 11th the poplar plants of each *Trichoderma* treatment group were inoculated by 1×10^3 cfu/m^3 soil. One Control group was mock-inoculated with the equal volume of tap water. Each group contained ten individual poplar plants.

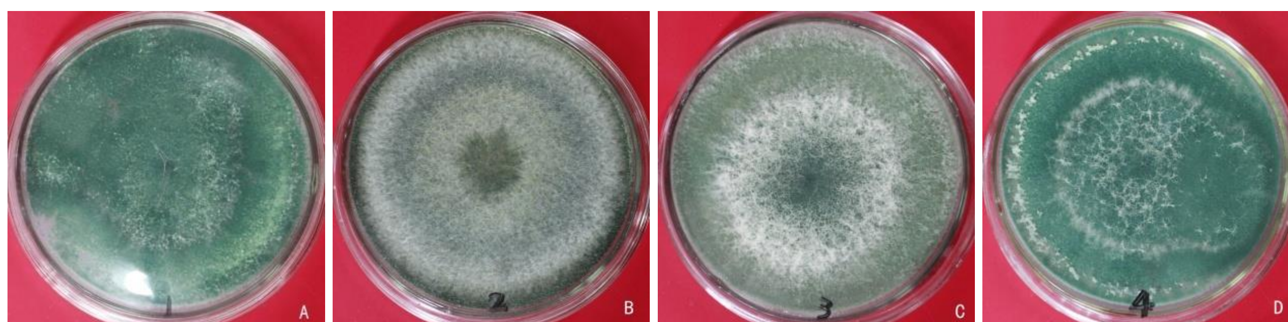

Fig. 1. *T. asperellum* cultured on PDA medium, T_1 (A), T_2 (B), T_3 (C) and D (T_4).

Table 1. Primers used in qRT-PCR.

Gene name	GenBank Accession number	Primer sequence
PdPapCu/ZnSOD	KP893248	(F) GCAGTGAAGGTGTGAGTGGCA (R) CCCAGATCACCAGCATGACGAT
PdPapPOD21	KP893251	(F) CGGATCCCGATCCACAGGAAGT (R) ACAACAACACCACAGCCCTTGA
PdPapPAL3	KP893250	(F) GGCAAGCTCCTATTCGCTCAGT (R) GATTGTGCTGCTCGGCACTTTG
PdPapPPO1	KP893252	(F) ACCTGGAGCTGGTTCACTCGA (R) AATGCTGCGTCAAGCCAATCAG
PdPapActin1	KP973950	(F) GCTGAGAGATTCCGTTGCCCTG (R) GGCGGTGATCTCCTTGCTCATT
PdPapEF1-α	KP973951	(F) TGGGTCGTGTTGAAACTGGTGT (R) GGCAGGATCGTCCTTGGAGTTC
PdPapUBQ	KP973952	(F) TGTTGTGATCAACGCGAACTCG (R) GAGGATGCCTAGTGCTACGCAT

Defense enzymatic activity and extract of protein of poplar seedlings: About 0.50 g (-80°C) frozen leaves were used for analysis of superoxide dismutase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase enzyme activity and isolation soluble proteins in poplar seedling leaves according to the methods of Jian-Hui *et al*., (2017). All were assayed using spectrophotometer (PG Instruments Limited, Beijing, China). Three biological replicates were performed with three or four individual plants pooled as one replicate.

Data analysis: SPSS 22.0 (SPSS Inc., IBM company, the USA) was used for statistical analyses. Data are presented as mean ± standard deviation (SD).

Results

***T. aspereullm* inoculation regulated defense-related enzyme activities in poplar leaves:** We investigated the activities of four crucial defense-related enzymes, SOD, POD, PPO and PAL in poplar leaves after the inoculation of four *T. aspereullm* stains. The enzyme activities in the poplar leaves before inoculation (0HAI), four hours after inoculation (4HAI), and 1, 3, 6, 9, 12, 15 days after inoculation (DAI) were determined. Our results showed that each of the four *T. aspereullm* strains induced both SOD and POD activities after inoculation and that SOD and POD activities showed significant ($p<0.05$) increases as early as 4HAI. The activities of SOD and POD maintained higher levels compared to the uninoculated plants while demonstrating continuously increasing patterns from 4HAI to 1DAI (SOD) or 3DAI (POD) before finally remaining at a higher level ($p<0.05$) than the control (Fig. 2A-B). The T3 treatment with *T. asperellum* ACCC30536 inoculation resulted in the highest increase of both SOD and POD activities, suggesting the highest potential of scavenging ROS (Noctor & Foyer, 1998) thus the best effects for poplar.

The activity of PPO and PAL were also largely upregulated eventually and both showed a peak on 6DAI (Fig. 2C-D). However, steady up-regulated patterns were not formed till 3DAI (for PPO) or 1DAI (for PAL). The activities of PPO and PAL were once regulated differentially by different *T. aspereullm* stains, even showed temporal down regulation before 1DAI. The differential early responses of poplar towards the inoculation of different *T. aspereullm* strains reflect differential mechanisms of interactions between poplar and *T. aspereullm* strains. However, after 3DAI all the four strains induced much higher activities of PPO and PAL in poplar than the control, indicating stronger tolerance against biotic stress (Huang *et al*., 2010). Again *T. asperellum* ACCC30536 demonstrated the best inducing effects in poplar.

Gene expression analysis of defense-related enzymes in poplar leaf: We have found out in the enzyme activity assays that T3 was the optimal inoculation for poplar grown under field conditions. Thus, we further investigated the early response of SOD, POD, PPO and PAL enzymes to T3 inoculation on gene expression level. The relative expression of the genes *PdPapCu/ZnSOD*, *PdPapPOD21*, *PdPapPPO1* and *PdPapPAL3* at 0, 0.5, 1, 2, 4, 24, 72HAI in poplar leaf were determined by qRT-PCR (Fig. 3). The expression of both *PdPapCu/ZnSOD* and *PdPapPOD21* responded to T3 inoculation at a very early stage (2HAI), suggesting the efficient inducing of ROS-scavenging enzymes by T3 (Fig. 3A-B). The expression of *PdPapCu/ZnSOD* peaked at 24HAI then dropped, the pattern of *PdPapCu/ZnSOD* expression coincided well with SOD activity. The expression peak of *PdPapPOD21* was at 72DAI, which also coincided well with its enzyme activity. The expression of *PdPapPPO1* showed down-regulation before 4HAI but continuously increased during 4HAI to 72 HAI; the expression of *PdPapPAL3* was not induced by T3 till 4HAI then kept increasing till 72 HAI. Both were consistent with the regulation pattern of their enzyme activities (Fig. 3C-D, Fig. 2C-D8).

Gene expression analysis of defense-related enzymes in poplar root: the root is in direct contact with *T. aspereullm.* Hence, we subsequently investigated the relative expression of the genes *PdPapCu/ZnSOD*, *PdPapPOD21*, *PdPapPPO1* and *PdPapPAL3* at 0, 0.5, 1, 2, 4, 24, 72HAI in poplar root in response to T3. In the root, the expression of all four enzyme genes responded at 0.5 HAI or 1HAI, even earlier than in poplar leaf. These responses are consistent with the fact that poplar root was firstly in direct contact with T3 and a series of signal transduction events might have been involved between root responses and leaf responses, causing a 0- to 3-hour time lag between the regulation of enzyme gene expression (Fig. 4). By 72 HAI, the expression of all four enzyme genes was maintained at a significantly higher level than control, suggesting induced systemic resistance in root as in leaf.

Inoculation with four *T. aspereullm* strains increased soluble protein level in poplar leaf: compatible solutes such as nitrogen-containing compounds or carbohydrates have a wide range of regulatory functions while plants are under abiotic stress, especially osmotic stress (Azevedo Neto *et al*., 2009). In this study we reported that the four *T. aspereullm* strains each induced increased systemic resistance in poplar against biotic stress. Subsequently, we investigated the concentration of soluble protein in poplar leaf after inoculation with the four *T. aspereullm* strains to estimate the induced resistance against osmotic stress. Our results showed that each of the four *T. aspereullm* strains induced high levels of soluble protein in poplar leaf from an early stage after inoculation (Fig. 5). The high levels of soluble protein were maintained throughout our sampling period, from as early as 4HAI to 15DAI. Consistent with the defense-related enzyme activities, T3 also showed the best effects in inducing soluble protein level (Fig. 5).

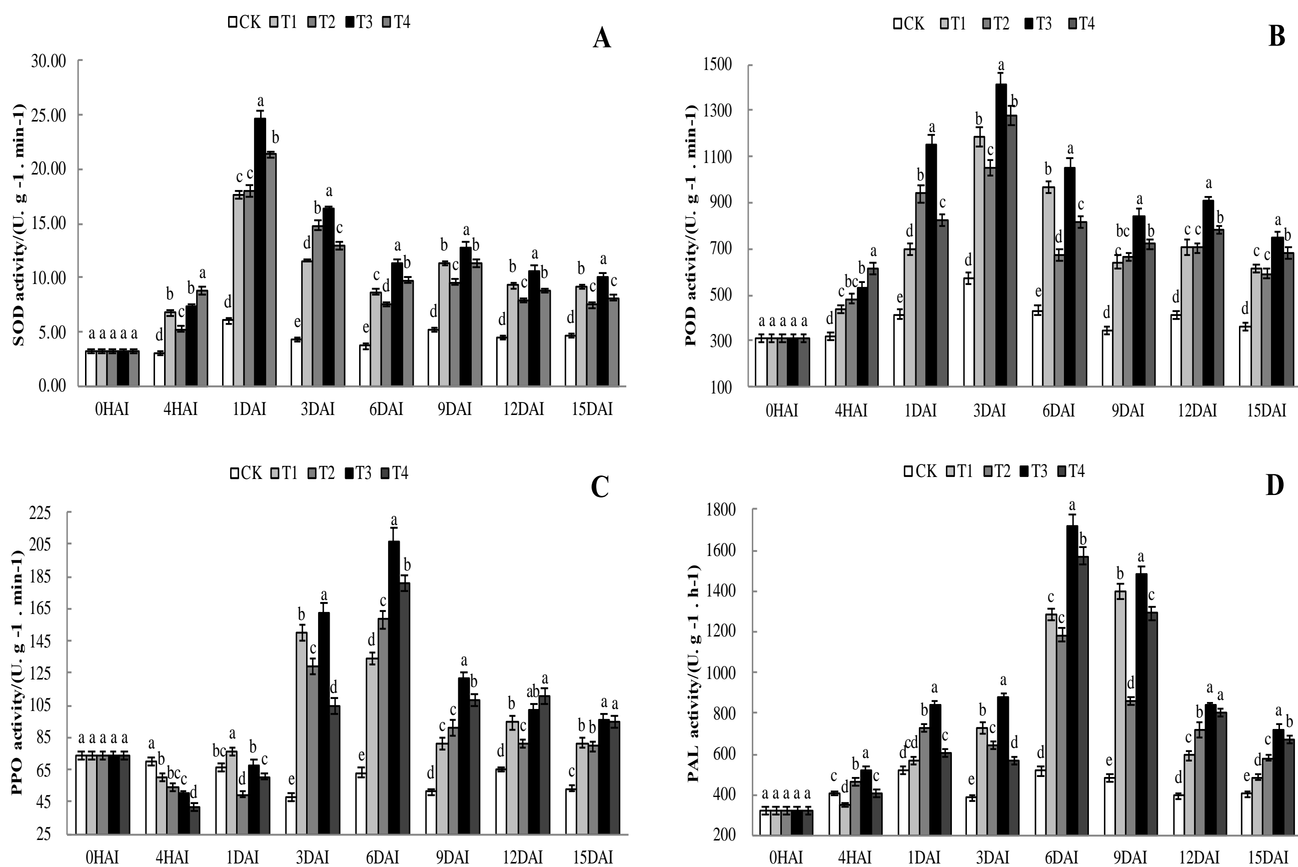

Fig. 2. Temporal changes in the activities of SOD (A), POD (B), PPO (C) and PAL (D) in poplar leaf after inoculation ($p<0.05$).

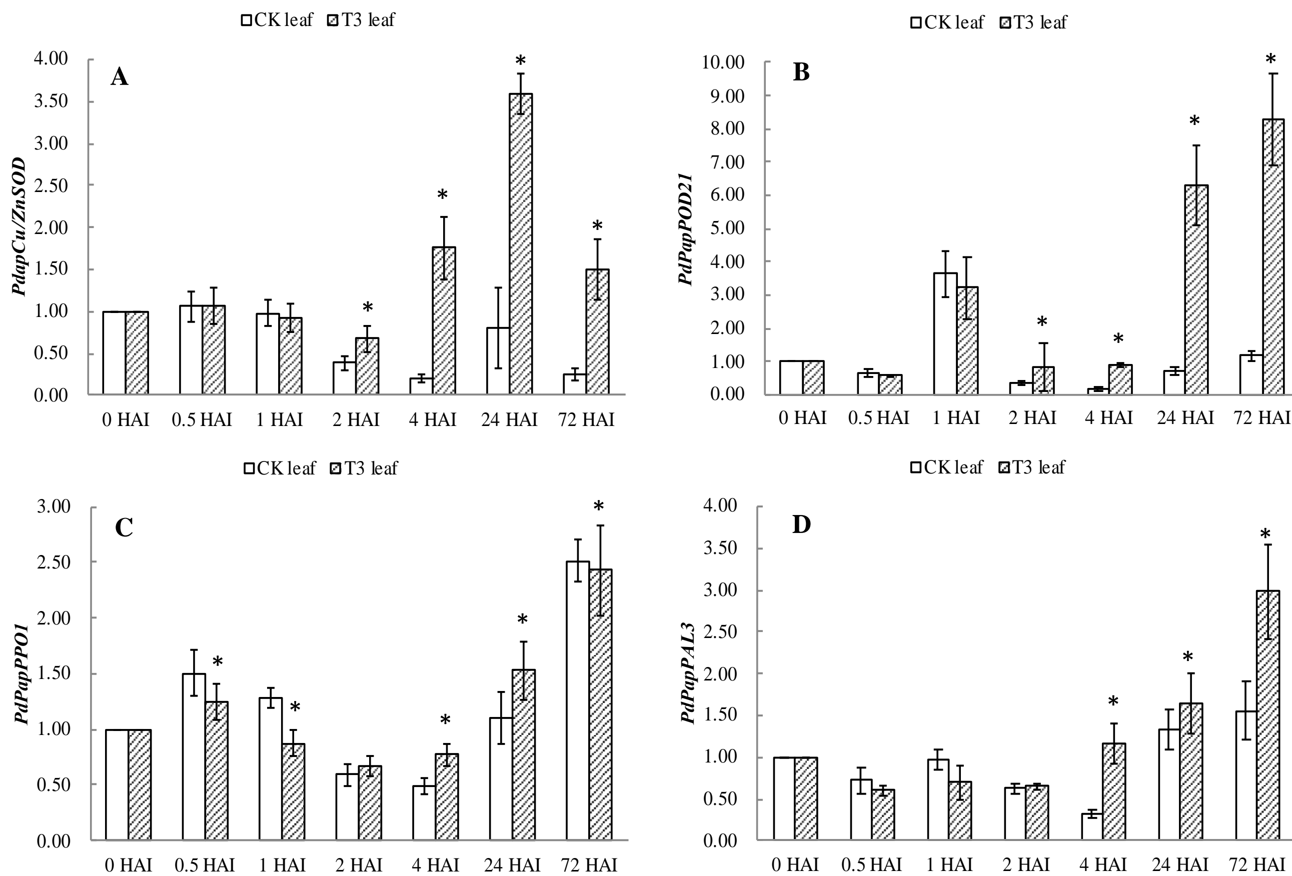

Fig. 3. Relative expression of *PdPapCu/ZnSOD* (A), *PdPap/POD21* (B), *PdPapPPO1* (C) and (D) *PdPapPAL3* in poplar leaf (*significant difference, $p<0.05$).

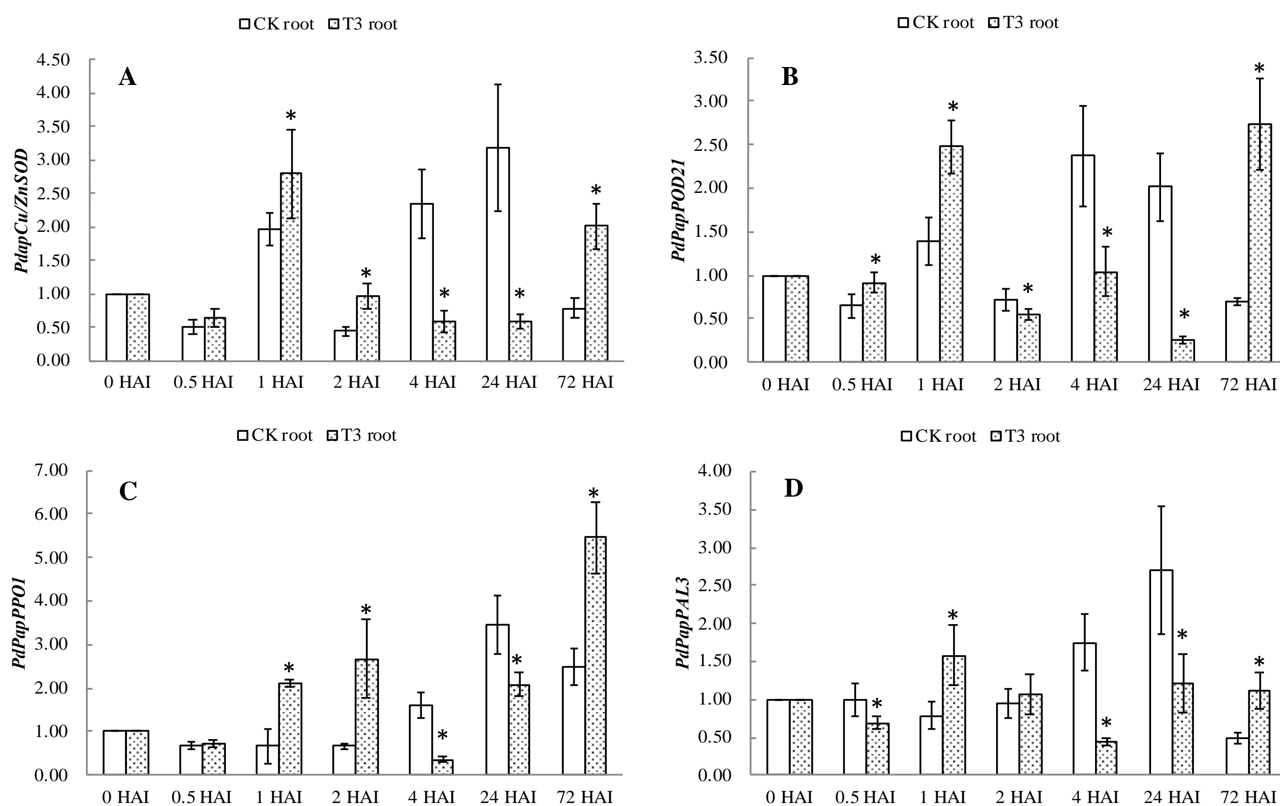

Fig. 4. Relative expression of *PdPapCu/ZnSOD* (A), *PdPap/POD21* (B), *PdPapPPO1* (C) and (D) *PdPapPAL3* in poplar leaf (*significant difference, $p<0.05$).

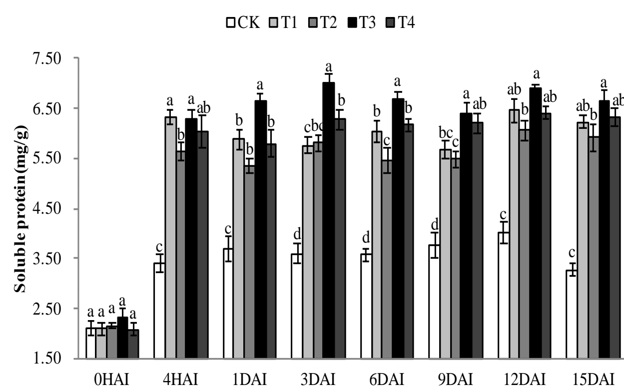

Fig. 5. Concentration of soluble protein in poplar leaves inoculated with four *T. aspereullm* strains.

Discussion

In this study we have reported that four *T. aspereullm* stains each induced the activities of ROS scavenging enzymes and enhanced phenols/flavonoids production in plants. The ROS scavenging mechanism were activated at a very early stage after inoculation (4HAI). In contrast, the PPO and PAL activities were maintained almost stable or even suppressed, differentially by different stains, for a time (4HAI to 1DAI) before drastic increase, which was consistent with a period for *T. aspereullm* to colonize and settle on poplar root. This time-course pattern we reported indicated that *T. aspereullm* strains might suppress the host plant's systematic immune mechanisms shortly while it colonized plant root and rhizosphere but eventually raise a peak of immune responses then maintain it at a high level. Meanwhile, the ROS scavenging enzymes were induced almost instantly and maintained at high levels after inoculation, possibly to minimize the damage on plants during the colonization of *T. aspereullm.*

The results of our qRT-PCR assays showed upregulations of *PdPapCu/ZnSOD*, *PdPapPOD21* and *PdPapPAL3* at 2HAI (for *PdPapCu/ZnSOD* and *PdPapPOD21*) or 4HAI (for *PdPapPAL3*), prior to or no later than our first sampling time point (4HAI) when significant increases in enzyme activities were detected. This pattern suggested that the increase of enzyme activities might have resulted directly from enzyme transcript and protein levels. However, the up-regulation of *PdPapPPO1* expression occurred prior to the increase of PPO enzyme activity, suggesting other complex regulatory mechanisms. The regulation of gene expression in poplar root showed fluctuating patterns within the first day after inoculation, which were quite different from the leaves, suggesting differential regulation mechanisms what required further investigation. Soluble protein level were reported to increase in maize or tomato under salt stress (Amini & Ehsanpour, 2005; Azevedo Neto *et al*., 2009). Inducing of soluble protein level by four *T. aspereullm* strains may indicate a stronger tolerance against osmotic stress at the early stage. To conclude, this study reports the induced effects and time-course regulation patterns of poplar systematic resistance represented by four major defense-related enzymes, SOD, POD, PPO and PAL under field conditions after *T. aspereullm* inoculation. We report that all four *T. aspereullm* strains have positive influence on poplar systematic resistance within 1DAI and could be effective as early as 0.5HAI. Comprehensively, *T. asperellum*

ACCC30536 was optimal for inoculating the hybrid poplar *Populus davidiana* × *P. alba* var. *pyramidalis*.

Author contributions

RZ designed the experiments. YW wrote the manuscript. JD performed treatments of plants, culturing of plant and fungal material, sample collection and preparation of qRT-PCR. AMB and AWB performed data analyses. XH and RM performed defense enzymatic activity and extract of protein of poplar seedlings.

Acknowledgments

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