

# Amelioration of phlorizin-induced autotoxicity in *Malus hupehensis* by rhizobium

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## Research article

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# Abstract

**Background:** Phlorizin can cause autotoxic reaction in apple plant, resulting in severe yield losses. It is noted that rhizobium can both degrade phenolic compounds and form mutually beneficial relationships with non-legumes. However, phenolic-degrading rhizobia have rarely been studied for its potential to alleviate the autotoxicity in apple plant.

**Results:** The aim of this study is to determine the capability of phenolic-degrading rhizobia for eliminate the toxic effects caused by phlorizin in *Malus hupehensis*. Results showed bioaugmentation with rhizobial strain y5077 not only efficiently degrades phlorizin, but also eliminates growth inhibition caused by phlorizin. Rhizobia inoculation enhanced melatonin accumulations and reduced H<sub>2</sub>O<sub>2</sub> and malondialdehyde production. Meantime, activities of antioxidant enzymes and the expression of genes in the ascorbate-glutathione cycle were maintained at a lower level under phlorizin stress. In addition, exogenous melatonin treatment abolished the induction of antioxidant enzymes by phlorizin. The results showed that rhizobia inoculation could alleviate phlorizin-induced oxidative stress by inducing melatonin production.

**Conclusions:** The facts suggested phenolic-degrading rhizobia might be exploited for soil bioremediation in autotoxic apple orchard.

## Background

Apple tree is one of China's large-scale cultivation of fruit trees, is a pillar of fruit industry in China. But, the problem of apple replant disease (ARD) is very prominent in China, due to arable land restrictions and crop rotation difficulties. ARD is resulting from continuous cropping of apple trees in the same field, leading to reduced yield and growth retardation. The symptoms is caused by both microbial factors, increased pathogens and reduced beneficial bacteria, and chemical factors, accumulations of autotoxic phenolic-compounds (Mazzola and Manici, 2012).

Phlorizin is a major autotoxic substance in apple cropping. It mainly released from decomposing apple debris, can accumulate up to 1% in the soil (Gosch et al., 2010; Huang et al., 2013; Hofmann et al., 2009). There were reports that phlorizin can specifically inhibit the respiratory process and the tricarboxylic acid cycle pathway in apple roots (Wang et al., 2012; Yin et al., 2016). Numerous studies have demonstrated that autotoxicity induced by phenolic compounds can cause the burst of reactive oxygen species (ROS); hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide radical (O<sub>2</sub><sup>•-</sup>), and hydroxyl radical (OH<sup>•</sup>), causing severe oxidative damage in plants (Yu et al., 2003; Bais et al., 2003). Oxidative stress not only results in disrupting the structures of nucleic acids and proteins, but it also destroy cell membranes by generating a by-product malondialdehyde which can cause secondary oxidative damage (Lara-Nunez et al., 2006; Ye et al., 2006).

Nevertheless, it has been well documented that rhizobia is available for degrade aromatic pollutants including phenolic compounds (Yessica et al., 2013; Wei et al., 2008; Sallabhan et al., 2013). Rhizobia is a plant growth promoting rhizobacteria, often used as a biological fertilizer. It is also noted that rhizobia

can be associated with non-legumes in mutually beneficial manner without forming root nodules. Many non-legumes are reported to be able to use rhizobia as a growth-promoting bacteria, i.e. rice (Nadarajah et al., 2017), maize (Nyoki et al., 2018) and spinach (Alejandro et al., 2018). Rhizobia can promote the growth of non-legumes directly via inducing phytohormones production (Zahir et al., 2010), and increased availability of insoluble phosphorus (Pandey and Maheshwari, 2007). Also, rhizobia could alleviate the toxic effects of ROS caused by biotic and abiotic stresses by enhanced the activities of antioxidant enzymes in plant, i.e. superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Moran et al., 2003; Saadani et al., 2016; Abdelkrim et al., 2018; Duan et al., 2018).

Regarding to the capability of rhizobia in degrading phenolic compounds, and also in promoting growth and oxide stress defense of non-legume plants, bioaugmentation with rhizobia might be a promising practice for amelioration of soil in autotoxic orchard. Therefore, the two aims of the present study were: (1) to screen rhizobia strains that can efficiently degrade phlorizin, and (2) to determine the ability of selected strains to promote growth and resistance to toxicity of phlorizin in apple plants.

## Results

### Strain selection and colonization

In this experiment, four rhizobia strains (y4071, y5077, y4091 and y4103) with the capacity to degrade 10 mM phlorizin within 96 h (degradation rate >80%), were subjected to higher concentrations of phlorizin. The results showed the ability of y4091 and y4103 to degrade phlorizin were limited at higher concentrations. Meantime, y4071 had a degradation rate over 50%, and *Ensifer meliloti* y5077 was the most efficient phlorizin-degrading isolate which can reach 100% degradation rate (**Fig. 1**).

To evaluate the vitality of rhizobia in the presence of phlorizin, root colonization rates were recorded. As shown in **Table 3**, the root colonization rates of four rhizobia were close without phlorizin treatment. But, it was higher for *E. meliloti* y5077 compared to the other strains under phlorizin treatment. The data implied that high concentration of phlorizin might also have toxic effects on rhizobia, which limited the bacterial colonization on *M.hupehensis* root. And y5077 had the highest tolerance to phlorizin toxicity in this study. Taken together, the foregoing results suggested the ability of *Rhizobium* to withstand the toxicity of phenolic acids and to utilize phenolic acids were varies widely.

### The protective effect of rhizobia on *M.hupehensis* under phlorizin stress

Plant biomass was measured to evaluate the tolerance of *M.hupehensis* growing in phlorizin treated soil. As shown in **Table 4**, the growth of shoot and root were both significantly hindered, and the effect was enhanced with increasing phlorizin concentration. The results of root shoot ratio suggested that the damage of phlorizin to roots was more direct and stronger than that of shoots.

On the other hand, higher phlorizin concentration also affected photosynthetic capacity of *M.hupehensis* plants (**Fig. 2**). The inhibitory effect begun to show at 100 mmol Kg<sup>-1</sup> concentration in soil in this study,

which might be better reflect the toxic concentration of rhizosphere soil in the orchard. However, the rhizobia inoculation noticeably alleviated this negative impact. *E. meliloti* y5077 significantly increased root and shoot growth of *M. hupehensis* plant under phlorizin stress. It is noted that, the root shoot ratio of rhizobia treated plant also remained almost unchanged under different concentrations of phlorizin (**Table 4**).

To further assess the protective effect of *E. meliloti* y5077 on the *M. hupehensis* roots, the root architecture was closely examined. Compared with the control, respective reductions for non-rhizobia and rhizobia inoculated plants were 37% vs. 3% in root lengths, 31% vs. 6% in average diameter, 52% vs. 12% in root volume, 35% vs. 18% in tips number and 63% vs. 9% forks number (**Table 5**).

### Induction of melatonin

The H<sub>2</sub>O<sub>2</sub> concentration and malondialdehyde (MDA) content were detected to evaluate oxidative stress level of *M. hupehensis* plant under phlorizin treatment (**Fig. 3**). In comparison with the control, H<sub>2</sub>O<sub>2</sub> production was stimulated in phlorizin treated plant since the fifth day and elevated dramatically thereafter. The trend of MDA increasing was consistent with H<sub>2</sub>O<sub>2</sub> production. However, the H<sub>2</sub>O<sub>2</sub> production as well as MDA content was significantly suppressed in *E. meliloti* y5077 inoculated plants. Studies have shown that the protective response of rhizobia on plants against oxidative stresses is primarily regulated by various phytohormones (Zahir et al., 2010).

Melatonin, an important phytohormone which functioned as an oxidation state regulator in plant, may participate in the elimination of oxidative stress in rhizobia inoculated plants. The data shown that the melatonin accumulation was significantly enhanced in y5077 inoculated plants. On the other hand, rhizobia inoculation could alleviate the inhibition of melatonin accumulation by phlorizin (**Fig. 3**). Melatonin is synthesized from tryptophan by a series of identified enzymes in plants (Back et al., 2016; Hardeland et al., 2008). In order to confirm the involvement of melatonin in alleviating phlorizin-induced oxidative stress, the expression of genes (*MdTDC1*, *MdAANAT2* and *MdASMT1*) involved in its production was analyzed. As shown in **Figure 4**, the expression of all three genes was slightly suppressed by phlorizin treatment while it was significantly induced in y5077 inoculated plants. Moreover, the expression of all genes were enhanced by y5077 inoculation under phlorizin stress. The results indicated that melatonin might involve in the protective effect of rhizobia on *M. hupehensis* plants.

### Effect on antioxidant system in *M. hupehensis*

The antioxidant enzymes activities were measured to evaluate the protective effects of rhizobia on *M. hupehensis*. The results showed that SOD activity was elevated in 2 week old greenhouse seedlings in response to 100 mmol Kg<sup>-1</sup> phlorizin, but not in rhizobia inoculated group. The SOD activity decreased gradually after 2 hours of exogenous melatonin treatment, and continue to decrease over time. After 12 hours, the SOD activity of melatonin treated plant was reduced to a level close to that in rhizobia inoculated plant (**Fig. 5a**). The effect of exogenous melatonin on CAT and POX activities were similar as

the change of SOD activity (**Fig. 5b, c**). These data suggested that rhizobium might improve *M. hupehensis*'s resistance to phlorizin by enhancing endogenous melatonin production.

In plants, antioxidant enzymes were not the only anti-oxidative systems. The non-enzymatic antioxidants mechanisms such as ascorbate-glutathione cycle was also recruit to eliminate ROS to prevent oxidative damage in cellular level (Wei et al., 2018). The expression of genes involved in ascorbate-glutathione cycle *MdcAPX*, *MdDHAR1*, *MdDHAR2* and *MdcGR* was detected. Compared with control group, all genes detected were markedly up-regulated after the seedling was transplanted into phlorizin treated soil. However, the rhizobia inoculation could significantly abolished the induction of genes expression by phlorizin (**Fig. 6**).

## Discussion

It is believed that plant-derived phenolic acid can destruct the cell membranes and inhibit the activity of critical enzymes of bacteria (Bahman et al., 2019). However, rhizobia have evolved mechanisms to counteract, nullify and even utilize these allelopathic compounds in root exudates as nutrients along with their ability to establish plant-bacteria interactions (Santi et al., 2010). In addition to the symbiosis between rhizobia and legumes – formation of root nodules, it is known that rhizobia can also colonize roots surface to using root exudates as nutrient sources and promote the growth of many non-legumes (Bhattacharjee, 2009). Our results showed the population of viable epiphytic bacterial cells on the root surface was affect by phlorizin in *M. hupehensis* seedlings, which indicated the toxic effects of plant allelochemicals on rhizobia.

Since the past decade, rhizobium has been found to have the ability to degrade various organic pollutants in the environment, including aromatic hydrocarbons, and highly resilient and toxic heterocyclic and chlorinated compounds (Yessica et al., 2013; Shen et al., 2014; Jabeen et al., 2014). Hence, rhizobia might be exploited for soil bioremediation in autotoxic apple orchard. But, there has been very little research reported on the protective effect of rhizobia against plant autotoxicity. In the present work, *E. meliloti* y5077 not only shown efficient degradation of phlorizin, but also alleviated the oxidative stress caused by phlorizin. Numerous studies have shown phenolic acid compounds including phlorizin served as allelochemicals, can cause imbalance of oxidation-reduction equilibrium in recipient plant (Bais et al., 2003; Weir et al., 2006; Ding et al., 2007; Zhang et al., 2007). As the results, excessive production of ROS leads to local tissue necrosis or whole plant death. Plant scavenging mechanisms are needed to prevent the toxic effects of ROS to protect cells from deleterious effects of allelochemicals (Ding et al., 2007; Cheng et al., 2015). Antioxidant enzymes (SOD, CAT, and POX) and non-enzymatic antioxidants are both play important roles in scavenging ROS (Andréia et al., 2016). The present study showed that rhizobia inoculation could alleviated the oxidative stress not by inducing the antioxidant system of plants, but melatonin production (Fig. 3, Fig. 5).

Melatonin is an amphipathic molecule which can functioning as antioxidant signaling molecule under stress conditions (Back et al., 2016). Oxidative stress arises from an imbalance between ROS production

and the detoxification of their reactive intermediates, which is of vital importance if plants are to maintain intracellular ROS pools at low levels. Various mechanisms control temporal and spatial coordination between ROS and other signals that are activated in separate parts of the plant at different times (Bais et al., 2003). Among the ROS, H<sub>2</sub>O<sub>2</sub> participates in a series of processes for plant development, stress responses, and programmed cell death (Veselin et al., 2015). We noted here that H<sub>2</sub>O<sub>2</sub> content were significantly increased along with the decreasing in melatonin production in phlorizin treated seedlings. On the other hand, the melatonin content was increased significantly along with the decreasing in H<sub>2</sub>O<sub>2</sub> content in rhizobia inoculated plants (Fig. 3). Meanwhile, the exogenous application of melatonin was also associated with reduction of oxidative stress in phlorizin treated seedlings (Fig. 5). Taken together, the results implied that melatonin might serve as a direct scavenger of free radicals and through a scavenging cascade (Galano et al., 2013).

## Conclusions

In conclusion, phlorizin stress lead to a significant losses in biomass and photosynthetic efficiency in *M.hupehensis*. Inoculation with *E. meliloti* y5077 resulted in increasing of all the growth indices by reducing the oxidative stress. This protective effect was more noticeable along with other resistance promoting characteristics such as melatonin production induced by rhizobia inoculation. Not to mention that, rhizobia is an excellent source of phenolic compound degrading bacteria and its inherent probiotic properties. The results from this study suggest that rhizobia with the capacity to degrade phlorizin and to ameliorate phytotoxic effects of phlorizin could be an environmentally friendly method to improve the productivity of the apple farming.

## Materials And Methods

### Soil preparation

Soil samples were collected from campus of Yantai Institute of Coastal Zone Research, Shandong China, on March 20, 2018. It was air-dried for 2 weeks, removed larger debris use a 10-mesh sieve, then autoclaved at 121°C for 15 min before use. Then the physical and chemical properties of soil was analyzed (**Table 1**). Phlorizin was sterilized by 0.22 mm filter before it was added into soil to achieve different soil concentrations, 50, 100, 150 and 200 mmol/kg.

### Rhizobia isolation and plant cultivation

Rhizobia strains were originally isolated from legume plants in saline soil area (salt content 0.3-0.7% ) of Yellow River Delta, Shandong China (37.77°N, 118.97°E) . A total of 119 isolates were obtained. All isolates were subjected to molecular identification and phylogenetic analysis using housekeeping gene *recA* and 16S rDNA as conserved sequences, respectively (Ren et al., 2016). In order to screen for strains capable of using phlorizin as the sole carbon source, isolates were propagated on basal mineral agar plates (NH<sub>4</sub>Cl, K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, pH 6.5) with 10 mM phlorizin (Sigma; St Louis, MO, USA) at

room temperature. Four isolates (y4071, y5077, y4091, y4103) that can grow on screening medium were selected for follow-up experiments. Phlorizin degradation by the isolates was performed three times in liquid basal mineral medium with 10, 20, 40 and 60 mM phlorizin respectively. Isolate y5077 was identified as *Ensifer meliloti* strain in our previous work (Ren et al., 2016).

Seeds of *M.hupehensis* were collected from Yantai, Shandong China, with mean annual precipitation 650 mm, mean annual temperature 12.7°C and elevation 100-920 m. *M. hupehensis* plant was reproduced by apomixes. After stratification at 4°C for 1 month, seeds were surface-sterilized before use. After 2 weeks growth, every three uniform seedlings were transferred into a single 5-L pot with prepared soil, cultivated in a climate chamber, day/night temperatures 28/20 °C, 50 ± 2 % relative humidity, and 12/12 h light/dark cycle. Rhizobia broth ( $0.8 \times 10^9$  CFU mL<sup>-1</sup>) used as inoculant, 1 mL inoculant was sprayed into 1 kg soil.

After 4 weeks cultivation, the *M.hupehensis* root were harvested to evaluate bacterial colonization. Root systems were rinsed gently to remove all attached debris. Then it was placed in a 100 ml tube contain 0.1% peptone and 0.2% Na-hexametaphosphate in deionized water, shaken vigorously for 10 min at 220 rpm. The suspensions was serially diluted and spread on basal mineral medium agar plates to determine the number of viable cells (cfu g<sup>-1</sup> fresh weight).

### **Biomass and root architecture**

After 10 weeks of cultivation, the *M.hupehensis* seedlings were measured for photosystem II efficiency (FPSII) and non-photochemical quenching of chlorophyll fluorescence (NPQ) as previously described (Kong et al., 2017). The plants were oven-dried and weighed to record biomass. The roots were rinsed to remove all attached debris. After thorough cleaning, roots system were arranged and scanned. The images of root system was analysis using the WinRHIZOR image system V4.1c (Regent Instruments, Quebec, Canada) for total lengths, surface area, volume and the number of root tips and forks.

### **HPLC analysis of phlorizin and melatonin**

For phlorizin assay, 10 g soil was extracted using 90% (v/v) ethanol at room temperature. After centrifugation, the supernatant was filtered with 0.45 mm filter, lyophilized, then dissolved in acidified methanol (0.2M acetic acid) for analysis. HPLC system(1200, Agilent, Santa Clara, USA) was equipped with a Hypersil-ODS C18 column (4.6mm×250 mm, 5 mm; Thermo Fisher Scientific Inc., Waltham, MA). The mobile phase was 30% methanol and 70% 0.15M acetic acid, flow rate was at 0.8 mL min<sup>-1</sup>.The detector wavelength was at 286 nm, temperature was 28°C. Each sample was replicated three times. Phlorizin was identified by comparison with the standards. The retention times was 7.14 min (**Table 2**).

For melatonin assay, 1 g fresh leaf tissues was collected from seedlings after dark adaption for 2 hours, homogenized in pre-cooled 80% (v/v) methanol. After centrifugation at 5000g for 5 min, the supernatant was filtered with 0.45 mm membrane. Melatonin content was analyzed using the same HPLC system as above with an isocratic elution of 0.15 M acetic acid and methanol (60:40, v:v) , flow rate was at 0.9 mL

min<sup>-1</sup>. The detector wavelength was at 220 nm, temperature was 30°C. Each sample was replicated three times. Melatonin was identified by comparison with the standards (Sigma; St Louis, MO, USA). The retention times was 3.53 min (**Table 2**).

### **Measurements of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation**

Fresh leaf tissue was homogenized with 5% (w/v) trichloroacetic acid on ice. The H<sub>2</sub>O<sub>2</sub> was measured as described by the method of Brennan and Frenkel (1977). One unit of H<sub>2</sub>O<sub>2</sub> was defined as the chemluminescence caused by the internal standard of 1mM H<sub>2</sub>O<sub>2</sub> g<sup>-1</sup> fresh weight. Lipid peroxidation was determined by measuring malondialdehyde (MDA), using the thiobarbituric acid reaction as our previously described (Ren et al., 2016). Leaf tissue was homogenized in 0.1 % trichloroacetic acid on ice. After ultra-speed centrifugation, the supernatant was reacted with thiobarbituric acid. The MDA concentration was calculated base on the extinction coefficient,  $\epsilon=155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### **Measurement of antioxidant enzymes activity**

Fresh leaf tissues were homogenized with buffer solution containing 1% (w/v) polyvinylpolypyrrolidone, 50 mM potassium phosphate, pH 7.8, 1 mM EDTA-Na<sub>2</sub> on ice. Superoxide dismutase (SOD; E.C.1.15.1.1), catalase (CAT; E.C. 1.11.1.6) and peroxidase (POX; E.C. 1.11.1.7) activities were measured separately according to our previous work (Ren et al., 2016).

### **Quantitative real time PCR**

Genes expression of enzymes involved in ascorbate-glutathione cycle or melatonin synthesis was analyzed with quantitative real time PCR (qRT-PCR). All primers used in this work are listed (**Supplementary information**). Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Three bio-replicates were prepared from each treatment. The cDNA was synthesized using GoScript™ kit (Promega, Madison, WI, USA). The qRT-PCR was performed using iCycler iQ Real-time PCR Detection System (Bio- Rad). The reaction system comprising 0.2 mM primers, 1ml template cDNA, and 10 ml SYBR Green MasterMix (TaKaRa Bio Inc., Dalian, China). The program was 95 °C for 20 s; 32 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72°C for 28 s. *Malus* EF-1 *a* gene (GenBank: DQ341381) were used as the internal controls, to standardize expression levels using 2<sup>-ΔΔCt</sup> method.

### **Statistical analysis**

All data were analyzed using Microsoft Excel (Redmond, WA, USA) and the values for each treatment are presented as the mean ± standard deviation of three replicates. A one-way analysis of variance was completed with the SPSS Statistics 17.0 software (SPSS, Inc., Chicago, IL, USA). Duncan's multiple range test was used to compare mean values at the  $p < 0.05$  significance level.



# Declarations

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## *Availability of data and materials*

All data generated or analysed during this study are included in this published article [and its supplementary information files].

## *Authors' contributions*

RCG designed and performed the experiment, analysed the data and wrote the manuscript. KCC and AM perform extraction and identification the phytohormone using HPLC. RCG and KCC participated in revisions of the manuscript. All authors have read the manuscript, given comments and approved the final version of the manuscript.

## *Ethics approval and consent to participate*

Not applicable

## *Consent for publication*

Not applicable

## *Competing interests*

The authors declare that they have no competing interests.

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## Tables

Table 1. Physical and chemical characteristics of the soil.

Soil properties	
sand (%)	49
silt (%)	26
clay (%)	25
soil pH (1:1 soil/water ratio)	7.2
NO <sub>3</sub> -N (mg/kg)	11.3
NH <sub>4</sub> -N (mg/kg)	14.7
P (mg/kg)	8.2
organic mater content (%)	1.13
phlorizin	—

‘—’ indicated the phlorizin was not been detected in the soil.

Table 2. Measurement on main parameters of phlorizin and melatonin by HPLC

	Retention time min	Linear equation	R2	Detection limit mg L <sup>-1</sup>	Linear range mg L <sup>-1</sup>
phlorizin	7.14	Y=1.66×10 <sup>-6</sup> X+0.214	0.9981	2.4	0-500
melatonin	3.53	Y=4.23×10 <sup>-6</sup> X+0.638	0.9994	1.7	0-1000

‘y’ indicated the concentration of phlorizin or melatonin mg L<sup>-1</sup>; ‘x’ indicated the peak area μAU\*s.

Table 3. Colonization of *M.hupehensis* roots (cfu g<sup>-1</sup> fresh weight) by phlorizin degrading bacterial isolates.

Iolates	- phlorizin	+ phlorizin
Control	0	0
y4071	2.7±0.6 × 10 <sup>5</sup>	1.4±0.2 × 10 <sup>6</sup>
y5077	1.9±0.5 × 10 <sup>5</sup>	3.2±1.1 × 10 <sup>7</sup>
y4091	3.3±1.3 × 10 <sup>5</sup>	3.9±0.5 × 10 <sup>5</sup>
y4103	2.3±0.7 × 10 <sup>5</sup>	7.1±0.5 × 10 <sup>5</sup>

Table 4. Biomass (g dry weight pot<sup>-1</sup>) and root shoot ratio (Wroot/Wshoot) of different concentrations phlorizin soils.

		Phlorizin contents in soil mmol Kg <sup>-1</sup>				
		0	50	100	150	200
Control	Shoot	9.41±1.02d	9.66±0.23d	8.16±0.23c	6.25±0.36b	4.63±0.4a
	Root	12.35±0.58c	12.42±0.33c	11.02±0.33b	10.38±0.25b	7.56±0.73a
	W <sub>root</sub> /W <sub>shoot</sub>	0.76	0.67	0.67	0.60	0.53
y5077	Shoot	9.67±0.52b	9.31±0.35b	9.7±0.81b	8.94±1.2b	7.41±1.02a
	Root	14.34±0.7b	12.72±0.26b	13.54±0.92b	12.36±1.58b	10.35±0.48a
	W <sub>root</sub> /W <sub>shoot</sub>	0.67	0.73	0.71	0.72	0.71

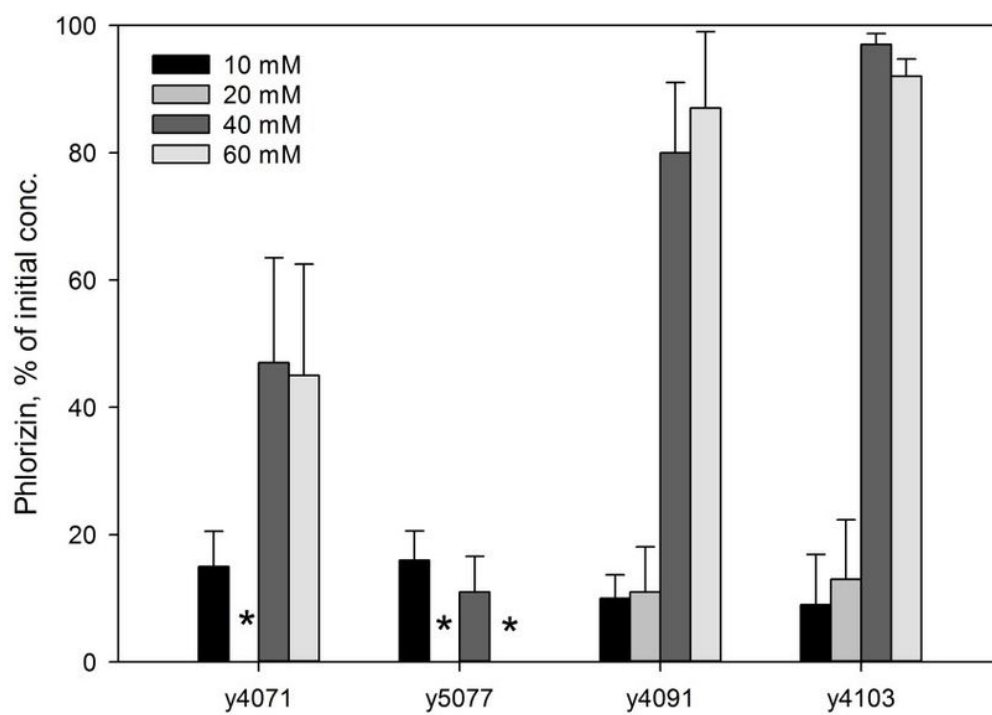
Means with different letters within each row are significantly different (one-way ANOVA, Duncan's multiple range test,  $p < 0.05$ ).

Table 5 .Effects of rhizobia on root system architecture of *Malus hupehensis* under 200 mmol Kg<sup>-1</sup>phlorizin induced stress.

	Control	phlorizin	rhizobia	phlorizin+rhizobia
Length (cm)	511±20.2a	322±14.6b	550±32.5a	497±34a
Diam (mm)	0.48±0.02b	0.33±0.02c	0.57±0.014a	0.45±0.03b
Volume (cm <sup>3</sup> )	0.77±0.05a	0.37±0.03b	0.83±0.06a	0.68±0.05a
Number of root tips	1522±134a	982±101c	1332±74b	1244±82b
Number of root forks	3522±251a	1291±147c	3381±236b	3192±127b

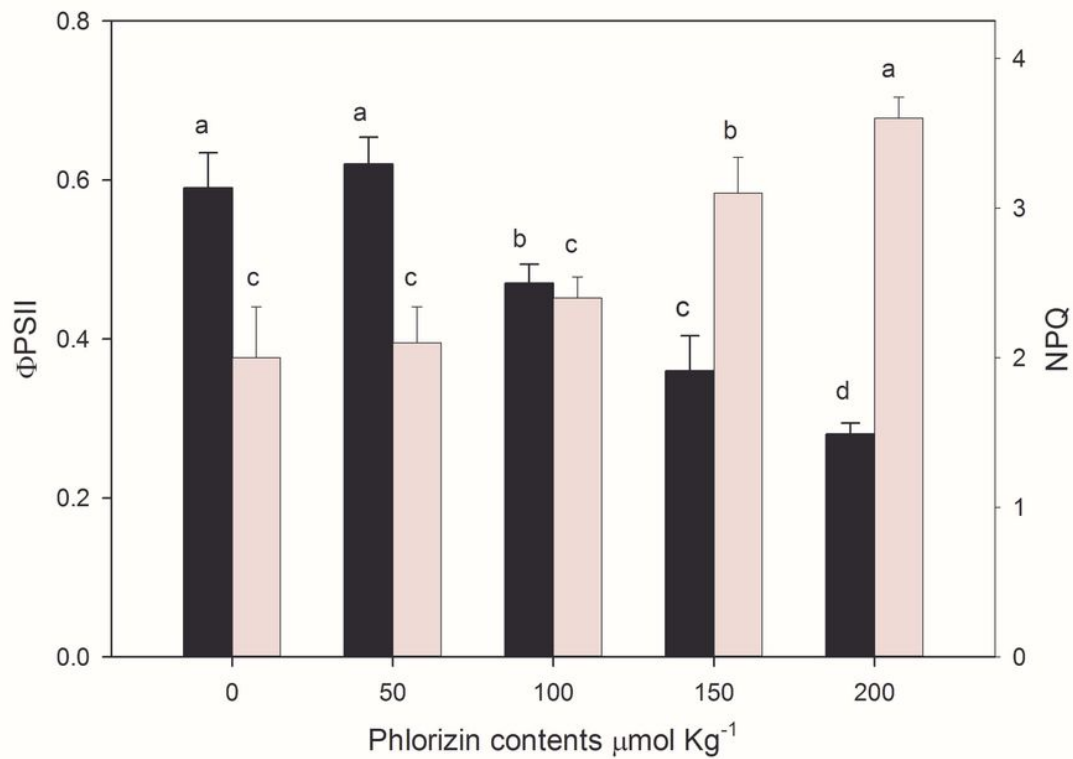
Means with different letters within each row are significantly different (one-way ANOVA, Duncan's multiple range test,  $p < 0.05$ ).

## Figures



**Figure 1**

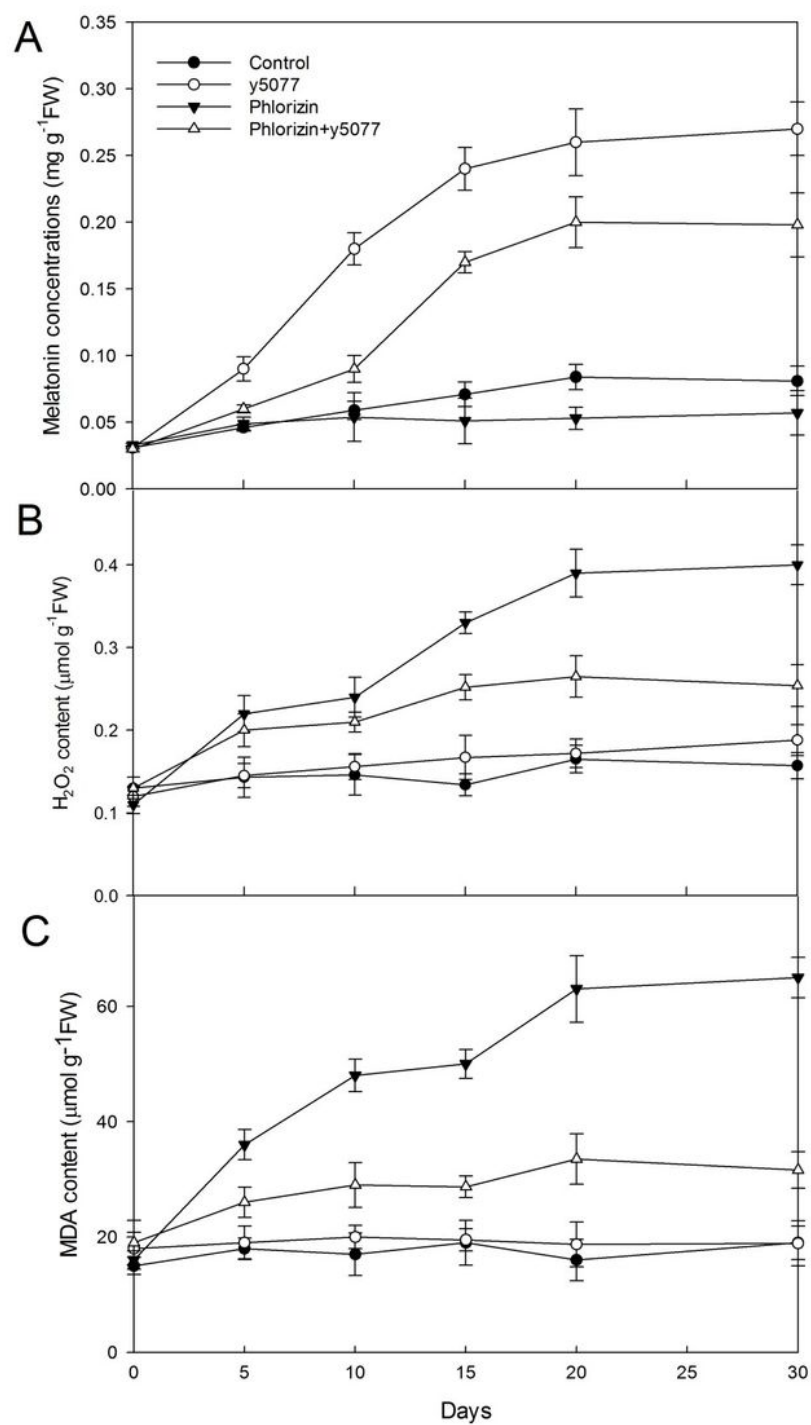
Phlorizin degradation rate by the rhizobia isolates over 96 h in basal mineral medium. \* = not detected. Means  $\pm$  SEM of three replicates.



**Figure 2**

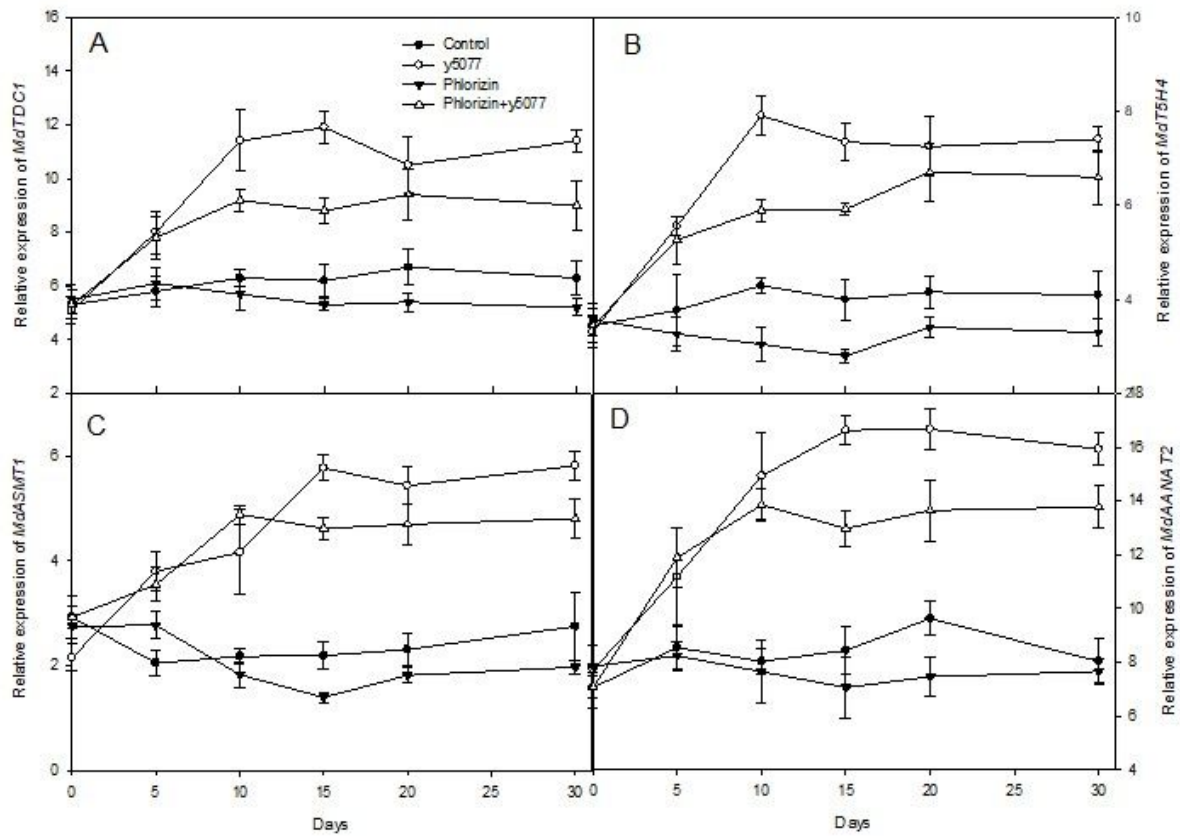
Effect of different phlorizin concentrations on photosynthetic parameters of *M. hupehensis*. Left, photosystem II efficiency ( $\Phi\text{PSII}$ ); Right, non-photochemical quenching of chlorophyll fluorescence (NPQ). Values are means of three independent experiments. The error bar represents the standard error. Data were separated using Duncan's multiple range test; different letters above the error bars indicate statistical significance at  $p < 0.05$ .





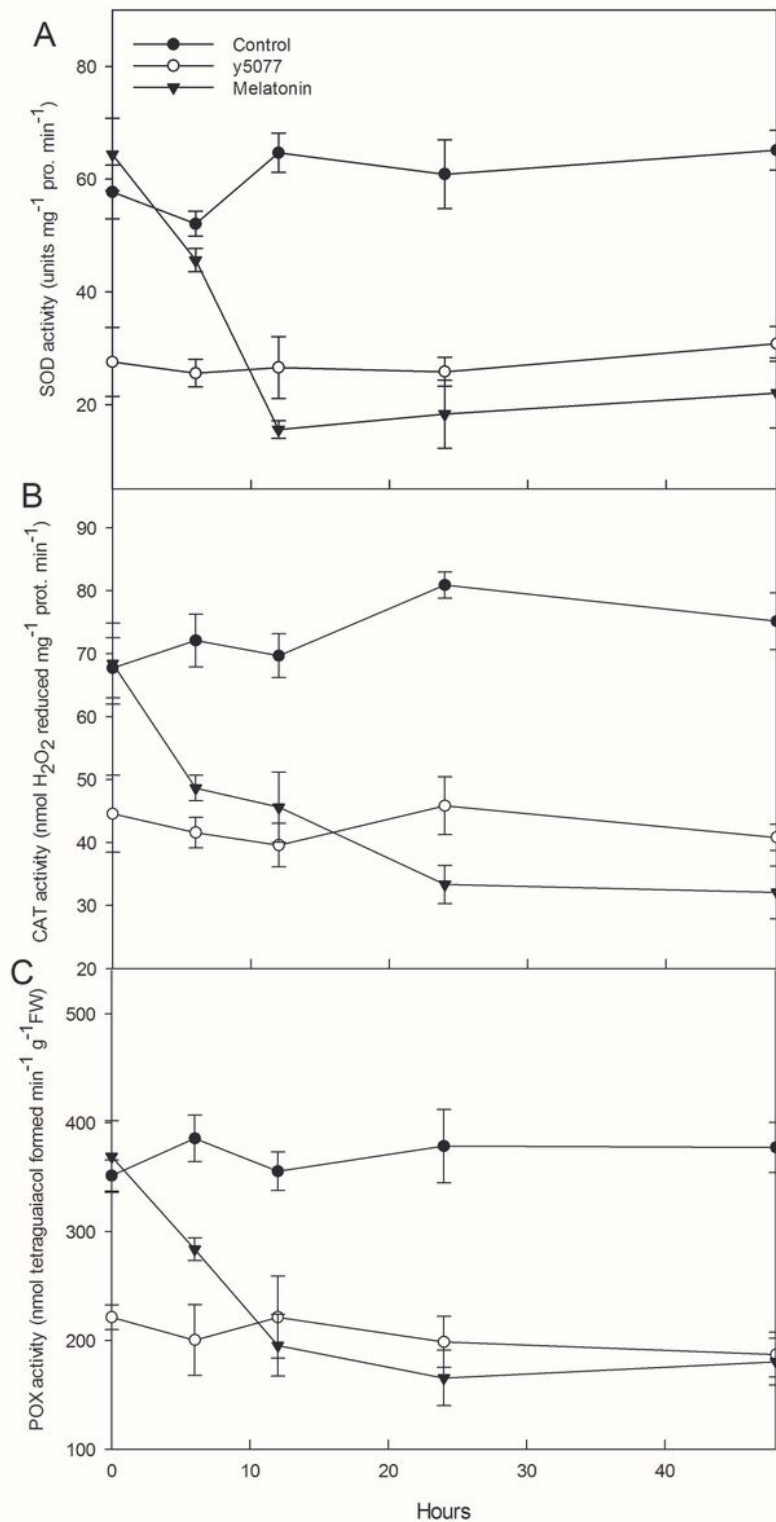
**Figure 3**

Effect of rihzobia inoculation on melatonin,  $\text{H}_2\text{O}_2$  and MDA content in *M. hupehensis* under phlorizin stress. A, contents of melatonin; B, contents of  $\text{H}_2\text{O}_2$ ; C, contents of MDA. Phlorizin concentration applied in the experiment was  $100 \mu\text{mol Kg}^{-1}$ . Different letters above the error bars indicate statistical significance at  $p < 0.05$  (one-way ANOVA, Duncan's multiple range test,  $P < 0.05$ )



**Figure 4**

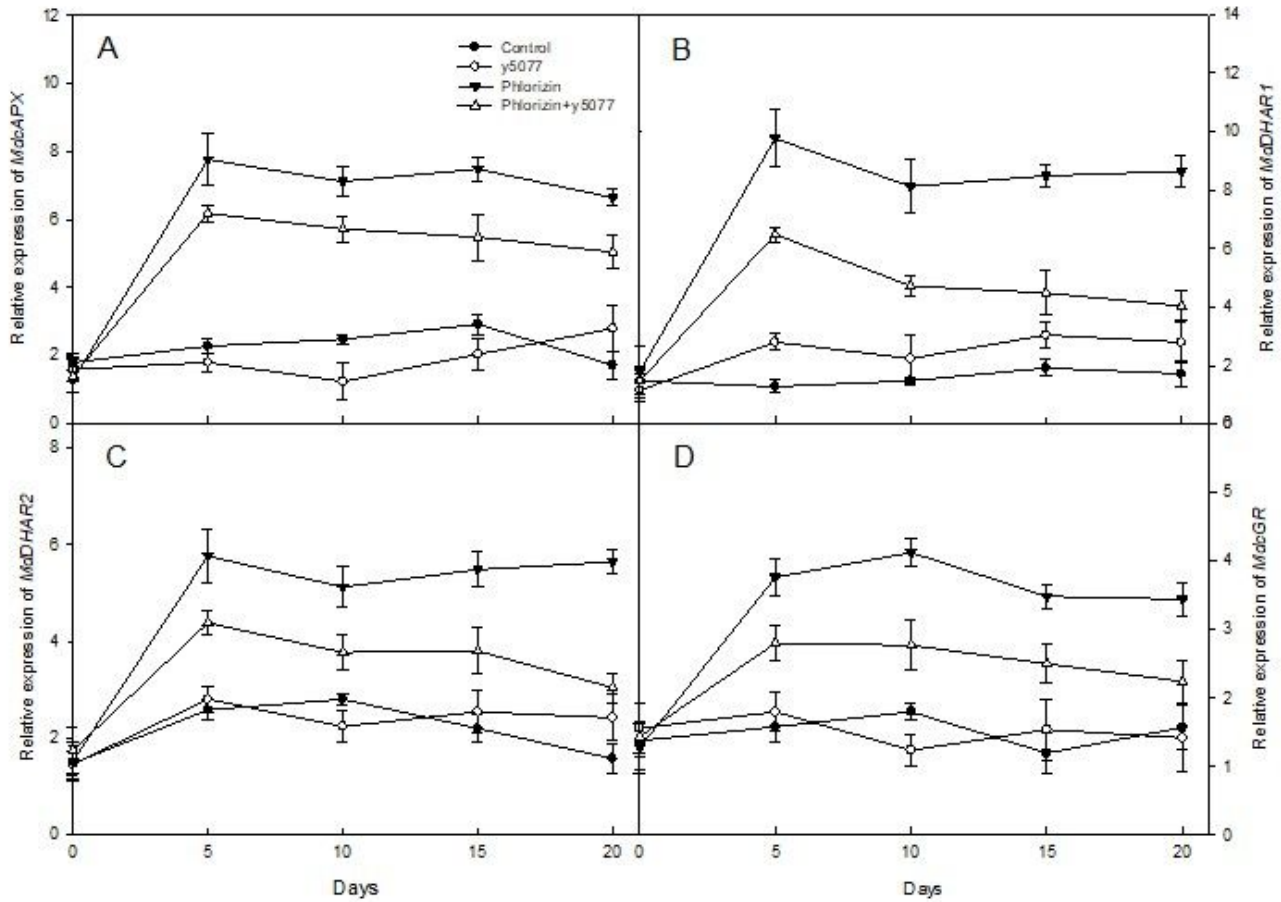
Effect of rihzobia inoculation on expression patterns of melatonin synthesis genes in *M. hupehensis* under phlorizin stress. A, expression of MdTDC1; B expression of MdT5H4; C, expression of MdAANAT2; D, expression of MdASMT1. The total RNA was extracted from leaf after 2 hours dark adaption. Different letters above the error bars indicate statistical significance at  $p < 0.05$  (one-way ANOVA, Duncan's multiple range test,  $P < 0.05$ )



**Figure 5**

Effect of rihzobia inoculation and exogenous melatonin on the antioxidant enzymes activity in *M.hupehensis* plants under phlorizin stress. A, SOD activity; B CAT activity; C, POX activity. Two weeks old greenhouse seedling, with or without rihzobia inoculation, grown in 100  $\mu$ mol Kg<sup>-1</sup> phlorizin were used in this experiment. Melatonin (1 mM melatonin in distilled water; Sigma) was sprayed on front and back of all leaves. Sampling started after 0, 6, 12, 24, and 48 of melatonin treatment. Different letters above the

error bars indicate statistical significance at  $p < 0.05$  (one-way ANOVA, Duncan's multiple range test,  $P < 0.05$ )



**Figure 6**

Effect of rihzobia inoculation on expression patterns of ascorbate-glutathione cycle genes in *M. hupehensis* under phlorizin stress. A, expression of MdcAPX; B, expression of MdDhAR1; C, expression of MdDhAR2; D, expression of MdcGR. Different letters above the error bars indicate statistical significance at  $p < 0.05$  (one-way ANOVA, Duncan's multiple range test,  $P < 0.05$ )