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RESEARCH ARTICLE

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The effects of phlorizin stress on the protective enzyme and metabolic regulation substances in the root of *M. micromalus*

Abstract The effects of phlorizin stress on the protective enzyme (PE) and metabolic regulation substances (MRS) in the *Malus micromalus* root were studied by tissue culture method. Under four concentration gradients of phlorizin stress, the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), the content of malondialdehyde (MDA), proline (Pro) and soluble proteins were tested. The results showed that phlorizin stress could lead to different changing tendencies for different detection indicators. The changes of SOD activity, POD activity and Pro content appeared more evident. The difference between the treatments under phlorizin stress and the control without phlorizin stress was not significant in the initial period. With the extension of processing time the difference became more and more evident and the protective enzyme activities and metabolic regulation substances were influenced greatly under different concentrations of phlorizin stress. When the concentration of phlorizin got to 1 $g \cdot L^{-1}$, the effect was the most obvious. The activities of protective enzymes were significantly improved, with the MDA production reduced, and Pro and soluble protein (SP) accumulation accelerated.

Keywords phlorizin stress, *Malus micromalus*, protective enzyme, metabolic regulation

1 Introduction

Allelopathy refers to a chemical ecology phenomenon where plants release certain chemical substances to affect their own growth and development or others. The allelopathy of plants is put to effect by the secondary metabolism and allelochemicals (Evans, 1997; Zhang et al., 2007). This phenomenon has been widely reported in agroecosystems. Lettuce (*Lactuca sativa* L.), cucumber (*Cucumis sativus* L.), soybean (*Glycine max* (L.) Merr) and watermelon (*Citrullus lauatus* (Thumb.)) cause autotoxic growth inhibition (Bai et al., 2009). Allelopathy is one of the main reasons that result in continuous cropping obstacles, therefore, the research on the effect of root allelopathy to soil surroundings has gradually become a hot topic, revealing the theory of continuous cropping obstacles (Sanderson et al., 2004; Wu et al., 2001). The apple tree is one of the fruit trees planted on a large scale in China as a mainstay industry. However, due to the difficulties in farmland limitation and crop rotation, China is facing a severe problem of continuous cropping obstacles. Phlorizin belongs to glycosides and it is distributed in apple tree barks and roots, and released to the soil by the root system (Schieber et al., 2001). It is the main factor (Yang et al., 1994; Wu et al., 2001) that causes apple replantation diseases. It was reported that 1 $mmol \cdot L^{-1}$ phlorizin can produce an inhibiting effect on the growth of seedlings, and with the increase of its concentration, the effect can be strengthened (Zhang et al., 2007).

A key way to ease the problem of replantation is to select the rootstocks resistant to phlorizin. In our study, under the conditions of *in vitro* combination of plant tissue culture technology from the toxic chemicals at cellular levels, the selection of disease-resistant mutant plants is an attempt to breeding higher plants (Shu, 1993; Liu et al., 2002; Wang et al., 2002; Zhang, 2002; Lu et al., 2006; Bai et al., 2009). *Malus micromalus* was used as the experiment material to be stressed under different concentrations of phlorizin, and then the stressed plants were inoculated with 3 $g \cdot L^{-1}$ phlorizin to survey the protective enzymes and the content of malondialdehyde (MDA), praline (Pro) and soluble proteins. The aim of this research was to make sure of the effect of phlorizin stress on the protective enzymes and metabolic regulation substances in the root of *M. micromalus*, which will provide references for selecting resistant plants against apple replantation diseases.

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2 Materials and methods

2.1 Materials

M. micromalus seedlings used in this study were kept in our laboratory. Phlorizin was bought from Sigma Company.

2.2 Methods

Flasks were filled with 40 mL MS medium. 0, 0.5, 1, 2 and 3 $g \cdot L^{-1}$, as CK, T1, T2, T3 and T4 of phlorizin respectively, were added into each flask with pH 5.8–6.0 and sterilized under 121°C for 17 min. The crops of the same growth (0.1–0.3 mm) were selected for the application of the medium with different concentrations of phlorizin. One plant was set in each bottle per concentration, with 10 replications per treatment, which were cultured in 25±2°C, 70%–90% contrast moisture, light/dark (14h/10h) for 50 d. Then, the crops that survived were selected for the next cultivation for 40 d with three replicates. Then the same growing plants were transplanted to the root-producing medium. After 60 d, the plants were transplanted to hexagonal nutrition bowls with 9.5 cm height and 3.3 cm width. 3 $g \cdot L^{-1}$ phlorizin was added at the same time. When three new leaves appeared, the indexes were measured every two days, including SOD (Zou, 1995), POD (Zou, 1995), (CAT), the content of MDA(Zou, 1995), Pro (Yu et al., 2003), SG; (Zhang, 1992) and SP (Zhang, 1992).

2.3 Data statistics and analysis

All the data were analyzed by DPS (Data processing system) software version 7.05. Differences among treatments were identified with Duncan multiple comparison test.

3 Results and analysis

3.1 Effects of phlorizin stress on protective enzyme system in *M. micromalus* root

M. micromalus was stressed under different concentrations of phlorizin, and then the stressed plants were inoculated with 3 $g \cdot L^{-1}$ phlorizin. The activity of protective enzymes in *M. micromalus* root was tested and shown in Table 1. It showed that phlorizin stress acclimation can slow down the decline of the protective enzyme activity caused by phlorizin significantly. The protective enzyme activity of the treatments under phlorizin stress acclimation, especially that of T3 and T4, was significantly higher than that of the treatments without stress acclimation. It indicated that phlorizin stress acclimation increased the activity of the three enzymes in *M. micromalus* root. After two days the protective enzyme activity in T1 and T2 had no change or no obvious change compared to CK while the protective enzyme activity in T3 and T4 treatments showed a significant difference compared with CK. With the extension of processing time, all of the protective enzyme activity increased significantly. However, there was still no difference or no significant difference between T1, T2 treatments and CK. All of the highest activity of SOD, CAT and POD appeared in T4 treatment, which was 176.48, 286.67 and 282.25 $U \cdot g^{-1}s^{-1}$ FW respectively. We concluded that *M. micromalus* under different concentrations of phlorizin acclimation showed different resistances against the phlorizin, which was reflected by the activity changes of protective enzyme. The effect of T4 treatment was the most obvious in all of the treatments.

3.2 Effects of different concentrations of phlorizin stress mutagenesis on MDA and osmotic content in *M. micromalus* root

M. micromalus was stressed under different concentrations of phlorizin, and then the stressed plants were inoculated with 3 $g \cdot L^{-1}$ phlorizin. The contents of MDA, Pro, SG and SP in *M. micromalus* root were tested and shown in Table 2 and Table 3.

On the whole, the result in Table 2 showed that the MDA content appeared to have an increasing tendency with the increase of concentrations of phlorizin stress in each treated-time, the MDA content also appeared to have an increasing tendency with the extension of treated-time in each treatment. On the second day there was no significant difference in the MDA content between T1, T2 and CK while the MDA content in T3 and T4 was significantly lower than that of CK. After 4 d and 8 d, the MDA content in T1 and T2 still had no significant difference from that of CK, but there was significant difference between T3, T4 and CK. There was a significant difference in T4 from that of all other treatments after being treated for 4 d and 8 d.

Table 3 shows that the changes of the osmotic substance content in *M. micromalus* root caused by different concentrations of phlorizin stress acclimation were different. As for SP content, after 2 d, there was a significant difference in all treatments compared with that of CK except for T1, and after 4 d the SP content of T2, T3 and T4 showed slight decline, with no significant difference in T3 and T4 with CK. The SP content in T3 and T4 reached 37.01 and 37.23 after 6 d. The inducing capacity of SP in T1 and T2 was very poor and that in T3 and T4 performed well. As for SG content, it did not change regularly. Only the SG content in T4 was always significantly higher than that in CK treatment from 2 d to 8 d, while that in T1 and T4 performed better. After 2 d, there was no difference between all treatments with CK. With the extension of processing time, the Pro content of T2, T3 and T4 was significantly increased, and the Pro content in T3 and T4 increased the most obviously after 6 d, which reached

Table 1 The effect of *M. micromalus* on the protective enzyme activity in root under phlorizin stress

treatments	SOD activity ($U \cdot g^{-1}s^{-1}$ FW)				CAT activity ($U \cdot g^{-1}s^{-1}$ FW)				POD activity ($U \cdot g^{-1}s^{-1}$ FW)			
	2 d	4 d	6 d	8 d	2 d	4 d	6 d	8 d	2 d	4 d	6 d	8 d
CK	75.67±0.36cd	97.82±0.36d	132.81±0.71c	123.91±0.62d	166.67±6.67b	206.67±6.67b	233.33±6.67b	206.67±11.54b	45.44±0.79d	138.35±0.60d	91.89±1.80c	67.29±0.80c
T1	77.34±0.24 c	110.28±0.40c	135.21±0.53b	142.65±3.96c	193.33±6.67b	226.67±6.67b	226.67±6.67b	220.00±11.54ab	53.88±0.18c	149.30±0.95c	95.86±0.45c	63.55±1.71c
T2	77.46±0.33 c	110.57±0.90 c	137.1±0.12b	147.68±0.33c	226.67±6.67a	220.00±0.00b	246.67±6.67b	233.33±6.67ab	48.24±0.96cd	133.04±3.48d	80.27±0.51d	67.47±0.39c
T3	91.89±0.26 b	117.60±1.42 b	126.35±0.25d	157.7±2.88b	193.33±6.67b	233.33±6.67b	246.67±6.67b	226.67±6.67ab	74.63±0.81a	167.73±0.37b	120.79±0.17b	101.19±0.45b
T4	100.58±0.42a	150.34±0.34a	160.85±0.25a	176.48±0.50a	233.33±6.67a	266.67±6.67a	286.67±6.67a	246.67±6.67a	66.63±3.04b	282.25±1.25a	167.68±0.49a	147.41±0.32a

Note: Data followed by same letters are not significantly different at $P = 0.05$ level.

Table 2 Effects of *M. micromalus* on the MDA and osmotica content in root under phlorizin stress

treatments	MDA content ($umoL \cdot L^{-1}FW$)			
	2 d	4 d	6 d	8 d
CK	0.53±0.01ab	0.81±0.01a	0.92±0.00a	1.13±0.01b
T1	0.56±0.01a	0.76±0.02ab	0.93±0.01a	1.21±0.01a
T2	0.54±0.00ab	0.74±0.01b	0.96±0.01a	1.17±0.01b
T3	0.46±0.03c	0.62±0.01c	0.73±0.01b	1.01±0.01c
T4	0.48±0.01c	0.52±0.01d	0.63±0.01c	0.69±0.01d

Note: Data followed by same letters are not significantly different at $P = 0.05$ level.

Table 3 Effects of *M. micromalus* on the SP, SG and Pro content in root under phlorizin stress

treatments	soluble protein ($ug \cdot g^{-1}FW$)				soluble sugar ($ug \cdot g^{-1}FW$)				proline ($ug \cdot g^{-1}FW$)			
	2d	4 d	6 d	8 d	2d	4 d	6 d	8 d	2d	4 d	6 d	8 d
CK	32.25±0.03c	32.79±0.03ab	32.61±0.04c	33.14±0.08b	30.45±0.03d	31.12±0.02c	32.35±0.03b	33.07±0.03a	10.54±0.31a	11.99±0.04c	14.17±0.58c	10.42±0.02d
T1	32.90±0.34bc	33.42±0.07ab	35.21±0.03b	34.78±0.04b	31.04±0.04a	31.45±0.02c	32.64±0.01a	33.10±0.02a	9.94±0.40a	12.59±0.02c	15.23±0.26c	12.96±0.03c
T2	33.69±0.34ab	32.49±0.36b	35.03±0.13b	34.81±0.04b	30.72±0.02b	31.03±0.03b	31.98±0.02d	32.36±0.03b	9.75±0.34a	14.77±0.07b	17.39±0.36b	12.47±0.15c
T3	34.07±0.50ab	33.99±0.36a	37.01±0.09a	36.20±0.36a	30.59±0.03c	31.56±0.02b	32.08±0.02c	32.10±0.01c	10.06±0.30a	14.97±0.25b	19.20±0.38ab	16.17±0.17b
T4	35.08±0.04a	33.78±0.34a	37.23±0.04a	35.96±0.04a	31.12±0.02a	31.96±0.03a	32.28±0.01b	33.12±0.02a	10.26±0.57a	16.10±0.33a	19.37±0.29a	18.71±0.20a

Note: Data followed by same letters are not significantly different at $P = 0.05$ level.

19.20 and 19.37, respectively. The Pro content in all treatments showed a downward tendency after 8 d; however, the Pro content of T4, was significantly higher than that of the other treatments.

Overall, after phlorizin stress acclimation in different concentrations and forms, T4 can significantly decrease the content of MDA and accelerate accumulation of Pro and SP.

4 Discussion

Plant autotoxicity substance affects the metabolic activity of plants, inhibits the electron transport in the mitochondrion and respiration, and then affects photosynthesis leading to both plant growth retardation and resistance reduction (Marci et al., 1986; Wu et al., 2002; Santos et al., 2004). Zhang considered that high concentration of phlorizin affected the protective enzyme activities in seedling cell and with the increase of its concentration the inhibition effect becomes stronger, including the following aspects: decreasing dry material accumulation, affecting the activity of protective enzyme and photosynthesis speed. The changes of protective enzymes in apple seedlings can make MDA content and active oxygen increase in the organ residual. The MDA content is an important sign of membrane lipid peroxidation and also a final product of membrane lipid peroxidation, which is related to the damage of plant cell membrane. More and more studies have shown that the stress can decrease the activity of anti-oxidation enzymes of plants and the rapid accumulation of MDA can lead to an increase of membrane permeability (Liang et al., 2003). As for Proline, its content in plant materials will increase obviously in the face of adversity, therefore, it can be used as an indicator of plant stress resistance (Zhang et al., 2005). This study showed that after being induced by different concentrations of phlorizin stress the autotoxicity of *M. micromalus* rootstocks could be reduced and with the increase of its concentrations the activities of the antioxidant enzyme, SOD, POD and CAT increased significantly, with the content of MDA reduced simultaneously. This is an important response of plants to the resistance against diseases in the early time as well as one of the responses of plants to the early recognition of pathogen, then starting the defense mechanism (Xu et al., 1995).

Lu (2000) reported that the stronger the SOD and POD activities were in continuous-cropping cucumber, the more adversity-resistance there would be, which showed that seedlings can have a certain resistance by means of stress inducement. In our study it indicated that the phlorizin stress in different concentrations had a great impact on the protective enzyme activities and the osmoregulation substance content. The effect of phlorizin in the concentration of T4 on plant physiologic changes was most obvious, which can significantly improve the activities of SOD, POD and CAT, reducing the generation of MDA and increasing the accumulation of Pro and SP. The changes induced by phlorizin in the concentrations of other treatments were not regular and obvious. Overall, each index in T4 showed the best. However, how the phlorizin stress in the concentration of T4 took effect, which may be at the gene level or merely physiologic adaptation to stress, needs further studies by molecular biology method. If it is just physiologic adaptation, the concentration of phlorizin will be increased in further studies to screen for resistant plants.

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