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RESEARCH ARTICLE

Protective enzyme activity and physiological properties of four mulberry varieties affected by drought stress in the Panxi Region of Sichuan Province, China

REN Ying-hong[*]

Xichang College, Xichang 615013, P. R. China

Abstract In order to identify the effects of drought stress on protective enzyme activity and physiological properties, four mulberry varieties, i.e.,‘Nanye-1’, ‘Yunsang-1’, ‘Xinyizhilai’ and ‘Husang-32’ in the Panxi Region of Sichuan Province, China, were selected. The activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in four mulberry varieties was determined. Soluble protein, soluble sugar, proline, net photosynthetic rate and transpiration rate of mulberry leaves were analyzed. The results show that during the early stages of drought stress, protective enzyme activities in four mulberry varieties continually increased. However, prolonged and intensified drought stress decreased their activities. After re-watering, they gradually returned to normal levels. Under drought stress and after re-watering, ‘Nanye-1’ and ‘Yunsang-1’ clearly showed smaller changes in soluble protein content than the ‘Xinyizhilai’ and ‘Husang-32’ varieties, whereas changes in their soluble sugar content were clearly greater than these last two varieties. When water deficit was protracted and intensified, ‘Nanye-1’ and ‘Yunsang-1’ still showed higher net photosynthetic, transpiration rates and water-use efficiency than ‘Xinyizhilai’ and ‘Husang-32’.

Key words drought stress, mulberry varieties, protective enzyme activity, leaf quality, physiology

1 Introduction

Mulberry has been listed as one of the important tree varieties in the “Grain for Green” project in China. With the implementation of a national strategy of “transferring mulberries in eastern regions to western regions”, western provinces are now attaching more importance to the development of silkworm-fed mulberries. Therefore, it is urgent to study the drought-resistant physiology of mulberry and to cultivate and select more drought-resistant mulberry varieties. Because of the need for artificial cultivation patterns and for easy harvesting, cultivated mulberry does not have as deep a root system as naturally growing mulberry; particularly, they have a lower SDR (stomatal diffuse resistance) and higher transpiration rates and are therefore weak in drought-resistance. Lack of water can slow down the growth of young leaves, hasten aging and falling of mature leaves, which reduces their light-absorbing space and curbs growth; meanwhile, drought stress also affects the physiological metabolism and leaf quality of mulberry (Ren, 2003). For all these reasons, the purpose of this study is to investigate the effect of drought stress on the physiology and leaf quality of different mulberry varieties.

2 Materials and methods

2.1 Materials and treatments

Samples were collected from four mulberry varieties, i.e., ‘Nanye-1’, ‘Yunsang-1’, ‘Xinyizhilai’ and ‘Husang-32’ in the Panxi Region of Sichuan Province, China.

‘Nanye-1’ and ‘Yunsang-1’ are high-quality mulberry varieties, extensively grown in the Panxi Region of Sichuan under conditions of drought stress. ‘Xinyizhilai’ is a high-quality variety grown in areas of Panxi, which specializes in the feeding of undeveloped silkworms. ‘Husang-32’ is a control species grown in Sichuan. ‘Nanye-1’ and ‘Yunsang-1’ are more tolerant to drought stress than ‘Xinyizhilai’ and ‘Husang-32’.

All treated mulberries are annually grafted with uniform grafts and individually planted outdoors in pots (0.35 m diameter × 0.40 m height). Two treatments were established: one under drought stress and the other under conditions of normal watering, with 15 pots of each variety for each treatment. With normal watering treatment as the control, they were put under drought stress after being provided with equal amounts of water. Determinations were undertaken

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[*] Author for correspondence. E-mail: renyinghong@163.com

when one plant among all treated mulberry plants showed temporary withering; it was undertaken once every other day and sampling was carried out at 10:00 am. After 7 days of drought stress treatment, watering was resumed and determinations were undertaken once every two days (Li, 2000; Shi et al., 2005).

2.2 Determination of enzyme activity

Preparation of enzyme solution: a 0.5 g of leaf material from the same position of each mulberry variety was weighed accurately, put it into a mortar and 4 mL (pH 7.8, 0.05 mol·L^{-1}) of an orthophosphoric acid buffer was added, ground sufficiently under ice-bath conditions, centrifuged for 20 min (12000 r·min^{-1}) under 5°C and then the supernatant, used in this experiment, was extracted.

Determination of enzyme activity: a nitroblue tetrazolium photoreduction oxidation-reduction method was used to determine the activity of superoxide dismutase (SOD), a guaiacol method was used to determine the activity of peroxidase (POD) and a potassium permanganate titration method to determine the activity of catalase (CAT) (Li, 2000; Hao et al., 2004).

2.3 Measurement of physiological and biochemical indices

A portable photosynthetic system (T1X5-1) was used to determine net photosynthetic and transpiration rates from leaves selected at the 8th position of each variety. Parameters were measured from 9:00 to 11:00 am on sunny, windless days and the experiment was replicated six times (Ren and Ren, 2001).

Soluble protein content was estimated by a coomassie blue dye-binding method, the soluble sugar content by the anthrone method, the proline content by a ninhydrin test and the content of glyceryl dialdehyde by a bi-component spectrophotometric method (Ren and Ren, 2001).

3 Results and analysis

3.1 Effect of drought stress on protective enzyme activity

Under drought conditions, accumulated free radicals from the dehydration of plant tissues, induction-generated perhydrols and other toxic substances directly or indirectly trigger a peroxidation process of membrane lipid, bringing about many changes in the activity of SOD, POD and CAT that are closely related to the generation of free radicals and active oxygen in plants (Jiang and Song, 2005; Lei et al., 2008).

3.1.1 Effect of drought stress on SOD activity

SOD activity was assayed over a period of nine days (Fig. 1). Under conditions of aggravated drought stress, SOD activity of the more drought-resistant variety ‘Nanye-1’ clearly increased and had increased by 100% by the 7th day, while the SOD activity of ‘Yunsang-1’ increased gradually during the first five days, then declined to normal levels. After drought stress was relieved, the SOD activity of ‘Nanye-1’ decreased dramatically to normal levels. As water deficit was prolonged and intensified, the SOD activity of the less drought-resistant variety ‘Xinyizhilai’ had increased by 54% on day 7. After the relief of drought stress, SOD activity of ‘Xinyizhilai’ revived more slowly. During the entire drought stress and re-watering period, the SOD activity of ‘Husang-32’ fluctuated slightly.

3.1.2 Effect of drought stress on POD activity

During the entire drought period, the POD activity of different mulberry varieties continually increased with prolonged and intensified drought stress (Fig. 2). By the 5th day, the POD activity of ‘Yunsang-1’ had increased by 57%, but then began to decrease gradually after re-watering. The POD activity of ‘Nanye-1’ had increased by 59% by the 7th day of drought stress and returned to normal levels soon after re-watering. During the first day of the treatment, the POD activity of the less drought-resistant variety ‘Xinyizhilai’ increased by 77%, reached a peak on day 7 and then sharply declined after re-watering. Under prolonged drought stress, the POD activity of ‘Husang-32’ showed a progressive increase. It began to decrease after re-watering.

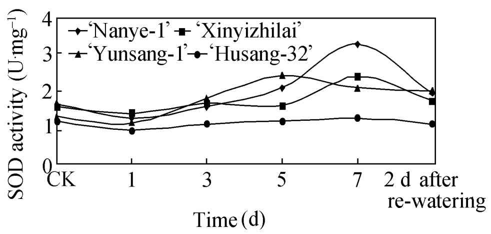

Fig. 1 Effect of drought stress on SOD activity

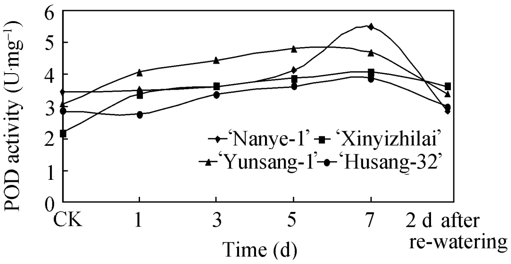

Fig. 2 Effect of drought stress on POD activity

3.1.3 Effect of drought stress on CAT activity

As shown in Fig. 3, during the entire drought period, CAT activity showed significant differences among the different mulberry varieties. Under intensified drought stress, CAT activity of the more drought-resistant varieties 'Nanye-1' and 'Yunsang-1' fluctuated significantly and reached 5.905 and 3.647 U·mg^{-1} respectively on day 7. The CAT activity of 'Yunsang-1' on day 7 was lower than that on the 5th day (4.104 U·mg^{-1}). After the relief of drought stress, CAT activity of 'Nanye-1' declined drastically and returned to normal levels. With the aggravation of drought stress, the CAT activity of the less drought-resistant 'Xinyizhilai' was clearly lower than that of the more drought-resistant varieties. During the first two days of the treatment, the CAT activity of 'Husang-32' increased dramatically. Furthermore, as water deficit was prolonged and intensified, the CAT activity of 'Husang-32' decreased gradually. After the relief of drought stress, CAT activities of 'Xinyizhilai' and 'Husang-32' recovered slowly.

3.2 Effects of drought stress on leaf quality

3.2.1 Effect of drought stress on soluble protein of leaves

The effect of drought stress on the soluble protein of leaves of all four varieties is shown in Fig. 4. With a prolonged drought stress, the soluble protein of leaves of all mulberry varieties decreased continuously and recovered gradually after the relief of drought stress. Under normal conditions, 'Nanye-1', 'Yunsang-1' and 'Xinyizhilai' have lower soluble protein content than 'Husang-32'. With the aggravation of drought stress, the soluble protein content of 'Nanye-1' and 'Yunsang-1' showed obvious decreases with rates of 29.6% and 49% on day 5 and 32% and 61% on day 7. Under progressive drought stress, the soluble protein content of 'Husang-32' dropped to 2.4 mg·g^{-1}, a rate of decrease of 95.6% at day 7. After the relief of drought stress, the more drought-resistant varieties recovered faster than the less drought-resistant varieties.

3.2.2 Effect of drought stress on soluble sugar of leaves

As shown in Fig. 5, during the entire drought period, the soluble sugar content in leaves of all varieties fluctuated significantly. Under normal conditions, the more drought-resistant 'Yunsang-1' and 'Nanye-1' varieties have low soluble sugar content, but under drought stress they showed a considerable increase. During the first three days of drought stress, 'Yunsang-1' had a dramatic increase in soluble sugar content, which reached a maximum of 3.104 μg·g^{-1}, an increase of 293% by day 3 and was clearly higher than that of 'Nanye-1'. The less drought-resistant 'Husang-32' and 'Xinyizhilai' showed smaller increase in soluble sugar content than the more drought-resistant varieties. When drought stress was prolonged and intensified, the soluble sugar content in the leaves of all varieties clearly declined and only returned to normal levels after the drought stress was relieved.

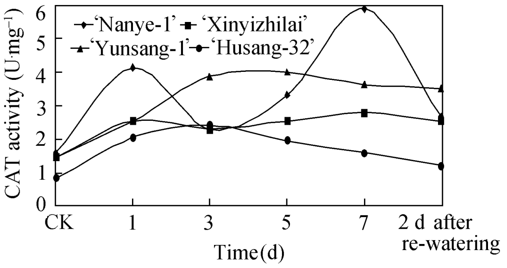

Fig. 3 Effect of drought stress on CAT activity

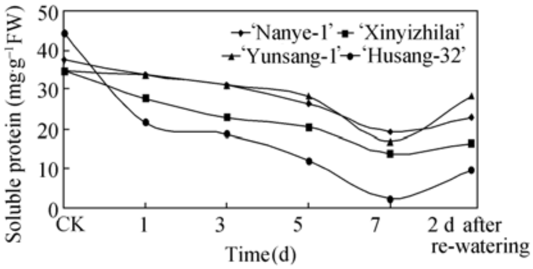

Fig. 4 Effect of drought stress on leaf soluble protein

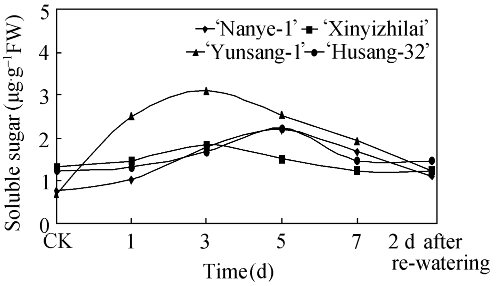

Fig. 5 Effect of drought stress on soluble sugar content

3.3 Effect of drought stress on physiological properties

3.3.1 Effect of drought stress on proline content of leaves

The effect of drought stress on the proline content of leaves of all mulberry varieties is shown in Fig. 6. During the first five days of the treatment, the more drought-resistant 'Nanye-1' and 'Yunsang-1' showed a marked increase in the proline content of leaves, with an increase of 4.744 and 3.654 mg·g^{-1} respectively at day 5, whereas the leaf proline content of the less drought-resistant ‘Husang-32’ and ‘Xinyizhilai’ had an increase of 0.267 and 1.495 mg·g^{-1} respectively on day 3. After the relief of drought stress, the proline content of leaves from all varieties steadily returned to normal levels, but that in ‘Nanye-1’ recovered more slowly.

3.3.2 Effect of drought stress on net photosynthetic rate of leaves

As shown in Fig. 7, during the early stages of drought stress, the net photosynthetic rate of all four varieties declined sharply, after which the drop slowed down. Under the intensified drought stress, the net photosynthetic rates became even negative. During the process of drought stress treatment, net photosynthetic rates of all mulberry varieties showed a similar trend. This change in drought response may provide some useful clues that the net photosynthetic rate is correlated with drought-resistance.

3.3.3 Effect of drought stress on transpiration rate of leaves

The transpiration rate of leaves of all four mulberry varieties sharply declined during the first five days of the treatment. After that however, the decline slowed down (Fig. 8). After the relief of drought stress, all transpiration rates returned to normal levels.

As shown in Fig. 9, during the first five days of the treatment, the water-use rate fluctuated slightly. With the progressive drought stress, water-use rates fell significantly and became negative values on day 7. After the relief of drought stress, water-use rates increased significantly.

4 Discussion

1) Drought stress changes the structure, permeability and function of plant membranes and increases active oxygen in cells, leading to physiological function disorders and even death of plant cells (Wang, 1985; Zhu and Bao, 1995; Zhao et al., 2005; Lei et al., 2008). Plants have two methods to fend off damage caused by active oxygen-enzyme systems such as SOD, CAT, POD, and non-enzyme systems such as ascorbic acid, carotenoid, glutathione. Mulberry procures an enzyme system to protect it against active harm caused by oxygen under drought stress; this system can remove free active oxygen radicals and, to a certain extent, reduce the damage (Wu, 2006). In this study, during the early stage of drought stress, protective enzyme activities of mulberry continually increased; however, protracted and intensified drought stress decreased their activities because the protective enzymes were proteins containing a metal prosthetic group and were affected by the oxidation of active oxygen. Simultaneously, the enzymes of the four different mulberry varieties may have different structures and stabilities and perform differently under drought stress, owing to various active substrates for different protective enzymes and their various strategies adapted to the environment. When drought stress does not exceed their tolerance levels, they can induce and synthesize protective enzymes and increase enzyme activities; when these exceed the tolerance level, drought stress will lead to decreases of enzyme activities (Shi et al., 2005). Pan and Luo (2007) cloned part of the oxidase gene sequence of 1-Aminocyclopropanecarboxylic acid (ACC) of mulberries, which showed an increased expression under drought stress. This expression var-

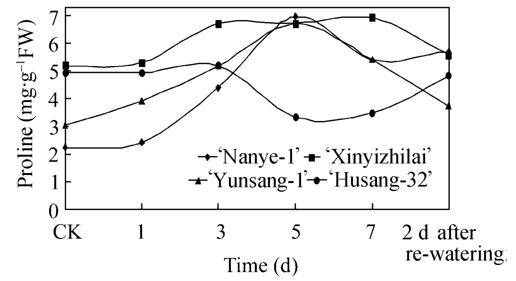

Fig. 6 Effect of drought stress on proline content

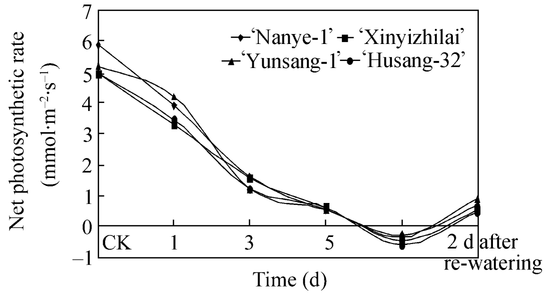

Fig. 7 Effect of drought stress on net photosynthetic rate

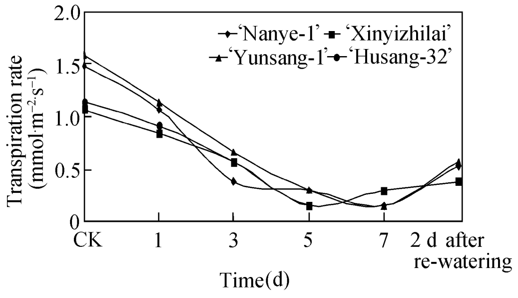

Fig. 8 Effect of drought stress on transpiration rate

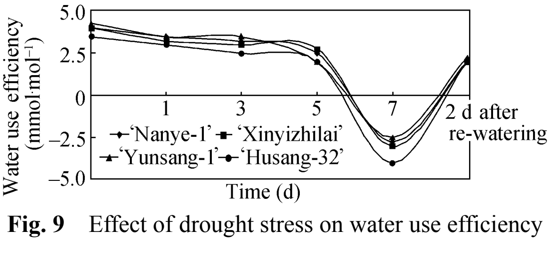

Fig. 9 Effect of drought stress on water use efficiency

ied among different individuals, which is assumed to be correlated with their differences in drought-resistance. Shi et al. (2005) suggested that, under drought stress, membranes of mulberry cells were damaged, membrane permeability and electro-conductivity increased, the malondialchehyche (MDA) as hyperoxide of membrane lipid increased in amount and damage to the cell membranes showed up differently in the various mulberry varieties. In this study, the activity of protective enzymes (SOD, POD and CAT) all tended to increase at first and decreased later, but the patterns varied, indicating that drought resistance of these four varieties may differ to a greater extent than expected.

2) Soluble protein content and soluble sugar content are critical indices for the quality of mulberry leaves, which directly affects the development of silkworms and the yield and quality of cocoons. Under drought stress and after re-watering, the two more drought-resistant varieties clearly showed smaller changes in the soluble protein content than the less drought-resistant varieties, whereas their soluble sugar content changes were obviously greater than those of the less drought-resistant varieties. So, drought resistance of mulberry may not be much correlated with the absolute amount of its soluble protein and sugar content, but is closely correlated with the changes of their content under drought stress (Zhang and Tan, 2002; Ji et al., 2004; Cheng et al., 2004).

3) Under normal conditions, proline accumulation is one of the key features of protein metabolism under drought stress. However, under aggravating drought stress, the proline content of all four mulberry varieties decreased. Perhaps the biological synthesis of proline originates from glutamic acid, the constant regeneration of which needs a supply of carbohydrates, whereas drought stress obstructs the supply of carbohydrates and generation of glutamic acid which, in turn, affects the synthesis of proline and results in a decrease of proline content (Ma and Mu, 2006). More recently, most investigators have come to the conclusion that proline has two roles to play in drought resistance: to maintain a permeability balance between protoplasm and the environment as a permeability adjusting substance and to maintain the structural integrity of membranes. As a result, when leaves lack water, the proline content is used as a physiological indicator for drought resistance (Buchanan et al., 2002). Ji et al. (2004) studied another adverse stress related non-protein amino acid, i.e., γ-aminobutyric acid (GABA), which showed the differences in the GABA content of various varieties; the GABA content in leaves at the early stages was higher than that in leaves at later stages at the same leaf positions. The GABA content gradually decreased when leaves aged. It was therefore thought that the GABA content may be correlated with drought resistance of the mulberry (Doi et al., 2000; Ji et al., 2007). Zhan (2007) proposed that drought also affects the activity of glycolisome and photorespiration in mulberry.

4) Under moderate drought stress, the photosynthesis of most plants begins to decrease (Luo, 1984). Under serious drought stress, it clearly decreases and may even stop altogether. With a protracted drought stress, transpiration rates further drop and with it, the net photosynthetic rate as well. But due to the closure of stomata, the fixed-rate of CO_2 decreases and at the same time rising photorespiration leads to a rapid decrease in the net photosynthetic rate which may even become negative (Ren et al., 2001). Although the closure of stomata may also result in a drop in the rate of transpiration, an amount of water can still physically evaporate, even when the stomata close completely. So drought stress affects the net photosynthetic rate more than it affects the rate of transpiration, and the rate of water-use by mulberry would drop and become negative (Ma and Mu, 2006). Yu et al. (1993) undertook analyses on leaf water potential, stoma conductance, transpiration rates and other physiological water indices of various mulberry varieties. Their results show that 'Nongsang-12' and 'Select-792' had high rates of water-use, whereas 'Husang-32' and 'Xinyizhilai' had low rates. The rate of water-use of a single leaf was most directly relevant in fundamental biological research into water-saving agriculture, reflecting a numerical relationship among the respiration metabolism of leaves, plant growth and water use (Luo, 1984; Li and Zhao, 1997; Li et al., 2003). There is great potential in a study about the rate of water-use of plants. Transpiration-resistant agents can be used to reduce the transpiration of plants and thus improve their drought-resistance. Transpiration-resistant agents applicable to mulberry plants should meet the following requirements: non-toxic, inexpensive and enduring effect, no harmful effect on mulberry leaves and quality. Chen and Shu (1999) showed that spraying of acetylsalicylic acid on mulberry leaves could reduce stomatal aperture, lower their water transpiration and slightly promote photosynthesis of plants. The effect could last for about 12 days.

5) So far, there has been little fundamental research into the genetic stability of indices of drought-resistant plants. More research on the reliability of indices on drought-resistant plants has been carried out which focuses on two aspects: physiological indices (net photosynthetic rate, transpiration rate and water-use rate) and biochemical indices (activities of protective enzymes, proline, malonaldehyde, soluble sugar and soluble protein). Although various physiological and bio-chemical indices can indicate differences at various levels in the drought-tolerance capacity of mulberry, these various indices differ in their reliability to mark drought-tolerance. The relevance of the various indices to drought resistance and their genetic stabilities also deserves further investigation. Because drought stress exerts a number of different effects on

the growth and development of plants, as well as on their physiological and bio-chemical processes, a comprehensive set of indices rather than a single index should be used in the select-breeding processes of plant varieties, root systems, leaves and stem growth. Physiological and bio-chemical indices should be integrated into a single comprehensive selection index, which can then be used to improve the reliability of selections (Chen and Shu, 1999).

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