

Leaf Functional Traits and Drought Resistance Evaluation of Five Mulberry Varieties in the Karst Region of Guangxi: Postprint

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Abstract

To screen for mulberry varieties with strong drought resistance suitable for cultivation in the karst regions of Guangxi, this study employed five mulberry varieties from the Huanjiang area, namely Guisang 5, Guisang 6, Guisangyou 12, Nongsang 14, and Yu 711, as experimental materials. Twenty-four leaf functional trait indicators were measured, including chlorophyll, osmotic adjustment substances, anthocyanin, malondialdehyde, antioxidant enzyme activity, vessel diameter, vessel reinforcement coefficient, leaf thickness, upper and lower epidermal thickness, palisade tissue thickness, spongy tissue thickness, stomatal size, and stomatal density. Principal component analysis, membership function, and cluster analysis were utilized to identify leaf functional trait indicators associated with drought resistance and to select varieties with superior drought resistance. The results demonstrated: (1) Principal component analysis revealed that the primary leaf functional trait indicators related to drought resistance were chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, malondialdehyde, superoxide dismutase, stomatal size, stomatal density, vessel reinforcement coefficient, and spongy tissue. (2) Based on membership function analysis, the drought resistance ranking of the five mulberry varieties was Nongsang 14 > Guisangyou 12 > Guisang 5 > Guisang 6 > Yu 711. Further cluster analysis indicated that Nongsang 14, Guisangyou 12, and Guisang 5 clustered into one group, while Guisang 6 and Yu 711 formed another group, with the two analytical approaches showing good consistency. (3) The superior drought resistance exhibited by Nongsang 14, Guisangyou 12, and Guisang 5 was associated with their more efficient physiological regulation mechanisms and optimized anatomical structures. These findings provide a scientific basis and theoretical reference for screening drought-resistant mulberry varieties in the karst regions of Guangxi.

Full Text

Preamble

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Functional traits and drought resistance evaluation of leaves from five mulberry varieties in the Guangxi karst region

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Abstract: In order to select drought-resistant mulberry varieties suitable for cultivation in the karst areas of Guangxi, this study investigated five mulberry varieties from Huanjiang county: Guisang 5, Guisang 6, Guisangyou 12, Nongsang 14, and Yu 711. A total of 24 leaf functional traits were measured, including chlorophyll content, osmotic regulatory substances, anthocyanins, malondialdehyde, antioxidant enzyme activities, as well as anatomical characteristics such as conduit diameter, conduit wall reinforcement, leaf thickness, upper and lower epidermis thickness, palisade mesophyll thickness, spongy mesophyll thickness, stomatal size, and stomatal density. Principal component analysis, membership function, and cluster analysis were employed to identify leaf functional traits associated with drought resistance and to select varieties with superior drought tolerance. The results showed: (1) Principal component analysis identified chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, malondialdehyde, superoxide dismutase, stomatal size, stomatal density, conduit wall reinforcement, and spongy tissue thickness as the key leaf functional traits related to drought resistance. (2) Membership function analysis ranked the drought resistance of the five varieties as: Nongsang 14 > Guisangyou 12 > Guisang 5 > Guisang 6 > Yu 711. Subsequent cluster analysis grouped Nongsang 14, Guisangyou 12, and Guisang 5 together, while Guisang 6 and Yu 711 formed a separate group, confirming the membership function results. (3) The superior drought resistance of Nongsang 14, Guisangyou 12, and Guisang 5 was attributed to their more efficient physiological regulation mechanisms and optimized anatomical structures. These findings provide a scientific basis and theoretical reference for screening drought-resistant mulberry varieties in Guangxi's karst regions.

Keywords: karst areas; mulberry; leaf functional traits; membership function; cluster analysis; drought resistance

Mulberry (*Morus alba*) has been cultivated in China for over 5,000 years and serves as the primary feed source for silkworm rearing (Jiao et al., 2020; Qin et al., 2010). Hechi City in Guangxi currently hosts the largest sericulture production base in China, leading the nation in mulberry plantation area and cocoon output for consecutive years, making the sericulture industry a distinctive local advantage (Chen et al., 2023; Tang et al., 2022). However, this region features typical karst topography, characterized by extensive limestone hills and depressions formed by karst development, with shallow soil layers and poor water retention capacity (Querejeta et al., 2007; Schwinning, 2008; Aritsara et al., 2023). As a high water-consumption species (Shen et al., 2019) with relatively shallow root systems (Shi et al., 2005), mulberry trees experiencing water deficit during growth exhibit leaf yellowing and reduced quality, ultimately affecting silkworm cultivation. Therefore, selecting drought-resistant mulberry varieties suitable for cultivation in Guangxi's karst regions is of paramount importance.

Plant leaf functional traits encompass a series of measurable indicators including leaf anatomical structure and physiological characteristics (Sun et al., 2017), which are closely associated with drought resistance (Cheng et al., 2017; Li et al., 2020; Li et al., 2021). Under drought stress, plants generate large quantities of reactive oxygen species within cells (Cruz de Carvalho, 2008), leading to lipid peroxidation of plasma membranes and production of malondialdehyde (MDA), causing oxidative damage. To counteract this damage, plants activate radical scavenging antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) to eliminate reactive oxygen species (Ye et al., 2014; Wang et al., 2019; Huang et al., 2024). As drought stress intensifies beyond a critical threshold, chloroplasts become deformed and their lamellar structures are damaged, resulting in decreased chlorophyll content (Zhang et al., 2014). Additionally, plants enhance drought resistance by increasing the content of osmotic regulatory substances such as proline, soluble sugars, and soluble proteins to lower osmotic potential, with these substance contents directly reflecting plant adaptability to adverse conditions (Wang et al., 2019; Huang et al., 2024). Research indicates that drought-resistant plants exhibit lower MDA content, stronger antioxidant enzyme activity, and higher levels of osmotic regulatory substances compared to drought-sensitive plants (Li et al., 2024). Under drought conditions, leaf functional traits in anatomical structure also display distinctive characteristics, with drought-resistant plants typically possessing thicker cuticles, well-developed palisade tissue, higher ratios of palisade to spongy tissue, greater stomatal density, and tightly arranged, elongated cells (Qiu et al., 2014; Oliveira et al., 2017). For instance, studies on the relationship between anatomical structure and drought resistance in autopolyploid mulberry leaves found that drought-resistant tetraploids exhibited increased spongy tissue thickness, palisade tissue thickness, lower epidermis thickness, leaf thickness, and leaf compactness compared to their less drought-resistant diploid parents (Han et al., 2021).

Current research on mulberry drought resistance has primarily focused on molecular aspects (Zeng et al., 2022), anatomical structure (Han et al., 2021; Shen et al., 2019), or physiological responses to drought stress (Mo et al., 2017; Liu et al., 2022) from a single perspective, with few studies integrating both anatomical and physiological leaf functional trait indicators. However, plant drought resistance results from the combined effects of multiple indicators, making it difficult to accurately assess using a single metric. Sun et al. (2024) and Sun et al. (2024) emphasized that plant drought resistance evaluation requires multi-indicator comprehensive analysis methods such as principal component analysis, membership function analysis, and cluster analysis to avoid the limitations of single indicators. Therefore, this study selected five common mulberry varieties from the Huanjiang region as experimental subjects, measuring 12 physiological indicators including chlorophyll, osmotic regulatory substances, MDA content, and antioxidant enzyme activity, along with 12 leaf anatomical structure indicators including leaf thickness, upper and lower epidermis thickness, palisade tissue, spongy tissue, stomatal size, and stomatal density, totaling 24 leaf functional trait indicators. By combining principal component analysis, membership function analysis, and cluster analysis, this research aimed to: (1) identify leaf functional trait indicators associated with drought resistance in the five mulberry varieties; and (2) evaluate the drought resistance of these five varieties. The results provide a scientific basis and theoretical reference for screening drought-resistant mulberry varieties in Guangxi's karst regions.

1.1 Study Site and Materials

All mulberry materials were collected from Guangxi Nongtou Shiyi Smart Mulberry Plantation (Huanjiang Park, 107°51'–108°43' E, 24°44'–25°33' N, elevation 220 m). Five varieties were selected as experimental materials: Guisang 5, Guisang 6, Guisangyou 12, Nongsang 14, and Yu 711. Selected experimental individuals were three-year-old plants growing in the same plantation area, with leaves sampled from new shoots that emerged in spring (March, during the dry season) after winter pruning. Following the methodology of Wang (2018), leaves from the 6th to 7th leaf positions were collected from 10 plants with similar growth status in each variety for physiological indicator measurement. Additionally, healthy mature leaves from the 6th to 7th leaf positions were collected from five different individuals of each variety for anatomical structure measurement. All samples were placed in ziplock bags, transported to the laboratory, and stored at 4°C for subsequent analysis.

1.2.1 Measurement of Physiological Indicators

Chlorophyll and carotenoid contents were determined using the acetone-ethanol extraction method (Shi and Wei, 2021). Soluble sugar content was measured by anthrone colorimetry (Wang, 2018). Soluble protein content was determined using Coomassie brilliant blue G-250 staining (Shi and Wei, 2021). Free proline content was measured by acidic ninhydrin colorimetry (Wang, 2018). Antho-

cyanin content was determined using the hydrochloric acid-methanol method (Shi et al., 2024). Superoxide dismutase activity was measured by the nitroblue tetrazolium (NBT) photoreduction method (Wang, 2018). Peroxidase activity was determined by the guaiacol method (Liu and Liu, 2018). Catalase activity was measured by the hydrogen peroxide method (Wang, 2018). Malondialdehyde content was determined by the thiobarbituric acid (TBA) method (Wang, 2018).

1.2.2 Measurement of Anatomical Structure

For anatomical analysis, leaves were first cut into approximately 1 cm² pieces, avoiding main and secondary veins. The samples were placed in centrifuge tubes containing Franklin's maceration fluid (H₂O₂:CH₂COOH = 1:1) and heated in a 70°C oven for 4 hours. After cooling and rinsing with clean water, the samples were stained with 1% alcian blue for 15 minutes to prepare temporary mounts. Stomata were observed under an optical microscope, and five random field-of-view images were captured and saved. Stomatal size was measured as stomatal area using ImageJ 1.53c software, while stomatal density was calculated as the number of stomata divided by the field area.

For each variety, five leaves were hand-sectioned along the main vein and leaf blade. The sections were bleached for 2 minutes, then sequentially stained with 1% safranin solution for 15 minutes and 1% alcian blue solution for 5 minutes to prepare temporary mounts. Observations and photography were performed under an optical microscope. ImageJ 1.53c software was used to measure upper and lower epidermis thickness, palisade mesophyll thickness, spongy mesophyll thickness, leaf thickness, conduit major and minor axes, and conduit wall thickness. Conduit wall reinforcement was calculated as the ratio of conduit wall thickness to conduit major axis. Individual conduit diameter was calculated based on elliptical area. Conduit diameter was calculated using the following formula (Scholz et al., 2013):

$$d = \sqrt{\frac{4N}{\pi}}$$

where d represents individual conduit diameter and N represents the conduit coefficient.

1.3 Data Processing and Analysis

Bar charts were generated using the ggplot2 package in R version 4.1.0. One-way ANOVA, principal component analysis, and cluster analysis were performed using SPSS 27.0 statistical software. One-way ANOVA was used to analyze differences in various indicators among different varieties. Principal component analysis was employed to select main drought resistance indicators representing

the five mulberry varieties for comprehensive evaluation using membership function and cluster analysis. The membership function was calculated as follows (Wang et al., 2014):

$$U(X_j) = \frac{X_j - X_{min}}{X_{max} - X_{min}}$$

where X represents the measured value of a given indicator, $X_{\{min\}}$ represents the minimum value of that indicator, and $X_{\{max\}}$ represents the maximum value. An inverse membership function was used for indicators negatively correlated with drought resistance:

$$U(X_j) = 1 - \frac{X_j - X_{min}}{X_{max} - X_{min}}$$

The membership function values for each indicator were summed and averaged to obtain a comprehensive evaluation index, with higher values indicating stronger drought resistance.

2 Results and Analysis

2.1 Leaf Functional Traits of Five Mulberry Varieties

Nongsang 14 exhibited the lowest soluble sugar content and also the lowest carotenoid content (Figure 1 [Figure 1: see original paper]A; Table 1), but had the highest chlorophyll a, chlorophyll b, and total chlorophyll contents (Table 1). Guisang 6 showed the highest contents of soluble protein, anthocyanin, and malondialdehyde, as well as the highest POD activity (Figure 1B, D, E, F), but had the lowest proline content (Figure 1C). Guisang 5 had the highest proline content and the highest carotenoid content (Figure 1C; Table 1), but the lowest malondialdehyde, chlorophyll a, chlorophyll b, and total chlorophyll contents (Figure 1E; Table 1). Guisangyou 12 exhibited the highest SOD activity (Figure 1G), while Yu 711 had the lowest SOD activity and also the lowest POD activity (Figure 1F, G), but showed the highest carotenoid content, soluble sugar content, and CAT activity (Table 1; Figure 1A, H).

The leaf anatomical structure was similar across all five mulberry varieties, consisting of upper and lower epidermis, palisade mesophyll, spongy mesophyll, and vascular bundles. Both upper and lower epidermis were single cell layers. Palisade mesophyll, located beneath the upper epidermis, consisted of elongated columnar cells arranged densely but with varying lengths. Notably, Nongsang 14 contained palisade tissue not only beneath the upper epidermis but also near the lower epidermis, with spongy tissue situated between these two palisade layers comprising mostly short columnar cells arranged compactly (Figure 2 [Figure 2: see original paper]). Vascular bundles formed the core of the leaf

main vein, composed of xylem and phloem, with xylem located innermost and primarily consisting of conduits (Figure 3 [Figure 3: see original paper]).

As shown in Table 2, conduit diameter ranged from 13.67 to 17.62 μm , with the maximum value observed in Guisang 5, which also had the minimum conduit wall reinforcement value of 0.08 μm . Guisang 5 additionally exhibited the thinnest upper epidermis and leaf thickness at 17.82 μm and 86.55 μm , respectively, the smallest stomatal size at 121.47 μm^2 , and the highest stomatal density at 47.6 $\text{ind.} \cdot \text{mm}^{-2}$. The highest conduit wall reinforcement was found in Guisang 6 (0.09 μm), followed by Nongsang 14 and Guisangyou 12. Guisang 6 also showed the thickest upper epidermis and leaf thickness at 22.78 μm and 107.47 μm , respectively, and the lowest stomatal density at 30.6 $\text{ind.} \cdot \text{mm}^{-2}$. Guisangyou 12 had the thinnest lower epidermis (7.38 μm) but the thickest spongy mesophyll (42.09 μm), the lowest leaf tissue tightness (21.98%), the highest leaf tissue looseness (43.95%), and the lowest palisade-to-spongy ratio (0.54). Nongsang 14 exhibited the thickest lower epidermis (8.43 μm) and the thickest palisade mesophyll (26.66 μm), with the highest leaf tissue tightness (27.95%). Yu 711 showed the highest palisade-to-spongy ratio (0.91) and the largest stomatal size (201.59 μm^2).

In summary, the five mulberry varieties displayed varied performance across different indicators with inconsistent rankings, making it difficult to evaluate drought resistance based on a single metric.

2.2 Principal Component Analysis of Leaf Functional Trait Indicators

Principal component analysis of the 24 leaf functional trait indicators revealed that three principal components accounted for 90.219% of the cumulative variance, retaining most of the information from the original indicators (Table 3). Based on the principle that larger loadings indicate greater contribution and stronger typicality to the principal component, the three components were analyzed. The first principal component contributed the most variance (40.749%), with high loadings for malondialdehyde, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, stomatal size, and stomatal density. The second principal component contributed 25.132% of variance, with superoxide dismutase showing high loading. The third principal component contributed 24.338% of variance, with conduit wall reinforcement and spongy tissue thickness showing high loadings. Ten indicators with high loadings across the three principal components contained substantial information and were therefore selected as key indicators for evaluating drought resistance among the five mulberry varieties: malondialdehyde, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, superoxide dismutase, stomatal size, stomatal density, conduit wall reinforcement, and spongy tissue thickness.

2.3 Membership Function Analysis of Drought Resistance-Related Leaf Functional Traits

Based on principal component analysis, ten indicators were selected from the original 24 for membership function analysis: malondialdehyde, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, superoxide dismutase, stomatal size, stomatal density, conduit wall reinforcement, and spongy tissue thickness. Higher average membership function values indicate stronger drought resistance. Three indicators negatively correlated with drought resistance (malondialdehyde, stomatal size, and spongy tissue thickness) were calculated using the inverse membership function, while seven positively correlated indicators used the standard membership function. As shown in Table 4, the average membership values for the five mulberry varieties were 0.574, 0.559, 0.538, 0.404, and 0.367, respectively, indicating the following drought resistance ranking from strongest to weakest: Nongsang 14 > Guisangyou 12 > Guisang 5 > Guisang 6 > Yu 711.

2.4 Cluster Analysis of Drought Resistance-Related Leaf Functional Traits

Cluster analysis was performed using the ten indicators selected by principal component analysis. The dendrogram (Figure 4 [Figure 4: see original paper]) shows that at a standardized scale of 25, the varieties could be divided into two groups: Group I comprised Yu 711 and Guisang 6, while Group II included Guisangyou 12, Nongsang 14, and Guisang 5. Varieties within the same group exhibited greater structural similarity and evolutionary resemblance, suggesting similar drought resistance. This grouping aligns with the membership function analysis results, with Group I showing lower drought resistance compared to Group II.

Plant drought resistance is influenced by multiple factors involving various leaf functional trait characteristics, necessitating comprehensive evaluation using multiple indicators (Li et al., 2017; Sun et al., 2017). For example, studies on *Camellia oleifera* in alpine regions identified palisade-to-spongy ratio, leaf area, vein thickness, and palisade tissue thickness as primary drought resistance indicators (Sun et al., 2024), while palisade tissue thickness, leaf tissue tightness and looseness, and palisade-to-spongy ratio were typical indicators for five hickory varieties (Ji et al., 2023). Zhang et al. (2018) found that CAT, POD, MDA content, and two other physiological indicators were important for evaluating drought resistance in different strawberry varieties. In this study, although Guisang 6 exhibited the highest stomatal density and conduit wall reinforcement in anatomical structure, its high MDA content and low SOD activity resulted in relatively low drought resistance. In contrast, the most drought-resistant variety, Nongsang 14, not only possessed high conduit wall reinforcement but also showed the highest chlorophyll a, chlorophyll b, and total chlorophyll contents and strong SOD activity, demonstrating that mulberry drought resistance must be evaluated based on multiple indicators of leaf functional traits rather than a

single metric.

Principal component analysis of the 24 indicators identified ten key traits for evaluating mulberry drought resistance: chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, SOD activity, MDA content, stomatal size, stomatal density, conduit wall reinforcement, and spongy tissue thickness. Previous studies on mulberry drought resistance utilized five anatomical indicators (palisade tissue thickness, leaf thickness, spongy tissue thickness, lower epidermis thickness, and upper cuticle thickness) (Han et al., 2021) and eight physiological indicators (MDA, CAT, POD, SOD, chlorophyll, proline, soluble protein, and soluble sugar) to evaluate drought tolerance in nine mulberry varieties (Liu et al., 2022). The current results differ partially from these studies, as palisade tissue thickness, leaf thickness, lower epidermis thickness, soluble protein, soluble sugar, POD, and CAT showed loadings below 80% in our principal component analysis and were not selected as relevant drought resistance indicators. These discrepancies may be attributed to genetic differences among mulberry varieties or different growth environments. On one hand, different mulberry varieties possess distinct drought resistance genes that may result in varying leaf functional trait responses to drought stress (Zeng et al., 2022), making certain indicators more reflective of drought resistance in specific varieties. On the other hand, plant drought resistance is closely related to the severity of drought stress in the growth environment. Liu et al. (2022) applied different gradients of drought stress treatment, which inevitably caused corresponding changes in physiological indicators. In contrast, this study focused on mulberry trees growing under natural karst environmental conditions, where drought stress may differ from laboratory conditions and may not elicit equivalent responses across all physiological indicators.

Drought resistance evaluation requires mutual verification through membership function analysis and cluster analysis to confirm result reliability. This study analyzed the ten indicators selected by principal component analysis using both methods. Membership function analysis ranked the five varieties as Nongsang 14, Guisangyou 12, Guisang 5, Guisang 6, and Yu 711 from most to least drought-resistant. Cluster analysis grouped Nongsang 14, Guisangyou 12, and Guisang 5 together, with Guisang 6 and Yu 711 forming a separate group. The consistent results from both analyses enhance reliability, aligning with previous studies that employed similar analytical frameworks to comprehensively evaluate stress resistance in eight Moraceae *Ficus* species based on anatomical structure and to assess drought resistance in different strawberry varieties under drought stress (Zhang et al., 2018; Zhu et al., 2012). The study found that the three drought-resistant varieties (Nongsang 14, Guisangyou 12, and Guisang 5) all had average membership function values of 0.5 or higher for the representative indicators, indicating more efficient physiological regulation mechanisms and optimized anatomical structures compared to the other two varieties. Under drought stress, drought-resistant plants can typically maintain higher chlorophyll content to sustain normal photosynthesis, and their stronger SOD activity can scavenge reactive oxygen species and reduce MDA formation,

thereby mitigating oxidative damage (Zhang et al., 2018; Huang et al., 2024). Furthermore, in terms of anatomical structure, conduit wall reinforcement is closely related to plant anti-embolism capacity (representing drought resistance, generally indicated by the xylem water potential at 50% hydraulic conductivity loss, P_{50}) and serves as a good predictor of P_{50} , with higher values indicating stronger drought resistance (Scholz et al., 2013).

The selected drought-resistant varieties provide a scientific basis for future mulberry breeding and cultivation in karst regions. Future research should explore gene expression related to these drought resistance indicators at the molecular level.

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