

MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF *CAMELLIA OLEIFERA* TO LOW-TEMPERATURE STRESS

JUANJUAN HU^{1*}, WEI WU^{2*}, ZHIHUA CAO³, JIA WEN¹, QINGLONG SHU^{1**} AND SONGLING FU^{1**}

¹School of Forest and Landscape Architecture, Anhui Agricultural University, Hefei, Anhui 230036, China

²Agricultural Engineering Research Institute, Anhui Academy of Agricultural Science, Hefei 230036, China

³Institute of Commercial Forest, Anhui Academy of Forestry, Hefei, Anhui 230031, China

**Corresponding authors contribute equally. The first corresponding author's email: juanzi5174@163.com;

Tel: 0086-551-65786561; Fax: 0086-551-65786512

*Authors contribute equally

Abstract

Camellia oleifera Abel originates from China and is high healthy effect food oil species. It is also a high additional plant in southern China and can help to keep some people of mountain area out of poverty. In recent years, climate change has been abnormal frequently. Abnormal low temperature in winter and late spring coldness may cause the hard hit to *C. oleifera* farmers. Freezing injury can be caused by sudden decreases in temperature in winter. However, *C. oleifera* varieties differ in their hardiness to low temperatures. The paper investigated cold-resistance mechanisms by determining and analyzing the morphological, physiological and biochemical characteristics of *C. oleifera* from eastern, western and southern Anhui, respectively. Sensitivity to low temperature was assessed via the number of leaves in spring shoots, leaf thickness, the activities of protective enzymes such as CAT, POD and SOD, and the inclusion contents of WSS, FPro, MDA, benzene-alcohol extracts and lignin. The results showed that *C. oleifera* varieties had different physiological and biochemical, and morphological responses to low winter temperatures. In different regions, the number of leaves, leaf thickness, WSS content, FPro content and MDA content varied from 5.2-7.8, 398.79 μ m-465.27 μ m, 23.41mg/g-24.74mg/g, 41.86 μ g/g-44.18 μ g/g and 10.08 μ mol/g-14.51 μ mol/g, respectively. The varieties from eastern Anhui, the leaf thickness were thicker. Meanwhile, the protective enzyme activities and inclusion contents were relatively higher. The protective enzyme activities and chemical components contents such as benzene-alcohol extract and lignin represented significantly difference ($p < 0.05$) among three regions. In the future, for the abnormal low temperature in winter, a serious of cultivation measures such as improving the contents of WSS, FPro, benzene-alcohol extract and lignin, were taken to enhance the cold resistance of *C. oleifera*. The result broadens the understanding of cold-resistance mechanisms in *C. oleifera*.

Key words: *Camellia oleifera*, Temperature stress, CAT, POD and SOD.

Introduction

Camellia oleifera Abel is one of the high economic tree species of Theaceae family. It is one of the world's four woody edible-oil trees. Compared to other old species such as palm, olive and coconut, *C. oleifera* has great significantly to the security of the grain and oil owing to it is mostly planted in mountainous regions. It originates from China (Shu & Zhang, 2009; Ma *et al.*, 2011; Zhang *et al.*, 2007a). It can not only reduce the high demand of edible oil supply, but also can replace the arable land for growing grain (Wu *et al.*, 2015). The planting area of *C. oleifera* in China is about 3.67×10^{10} m² (Wang *et al.*, 2008) and it has high economy of southern China. It is distributed in 18 provinces, cities or municipalities in southern China, including Guangxi, Yunnan, Fujian, Hunan and Jiangxi, but is rarer in the north of Southeast Asia. *C. oleifera* trees prefer warm and humid climatic conditions, with an average annual temperature of 14~22°C and annual rainfall of 800 mm or more. However, it is adaptable to different soil conditions. The tree species not only has high economic value, but also plays an important role in biological fireproofing, water and soil conservation, and ecological environment improvement.

C. oleifera oil, also known as tea oil, is the edible oil extracted from *C. oleifera* seeds (Ma *et al.*, 2011), which has also been called the "eastern olive oil" because it contains abundant unsaturated fatty acids, consisting of

oleic acid and linoleic acid (Long & Wang, 2008). Therefore, the planting of *C. oleifera* has already been expanded from the tropics to subtropical and northern subtropical regions. Unfortunately, damage due to cold temperatures has severely hindered *C. oleifera* production in northern regions of China. Therefore, research focused on the hardiness of *C. oleifera* to cold conditions has become of vital significance to improve its resistance to low temperatures. Previous studies have reported drought-induced stress including water control (Zhang *et al.*, 2013; Hu *et al.*, 2012), foliar fertilizer (Duan *et al.*, 2015), tea-oil composition (Ma *et al.*, 2011; Su *et al.*, 2014), disease resistance (Cao *et al.*, 2014; Zhang *et al.*, 2012), variety breeding and the chemical composition of shoots and leaves in *C. oleifera* (Li *et al.*, 2012; Wen *et al.*, 2013). However, we have not found researches about mature *C. oleifera* under natural low-temperature of different regions.

Large areas north of the Yangtze River in Anhui Province have been planted with *C. oleifera* in recent years. However, eastern Anhui is located on the most northern margin of where *C. oleifera* is naturally distributed in China. Considering the preferred annual temperature of *C. oleifera*, the northern margin meets only basic needs for the growth of *C. oleifera*. Furthermore, although trees may exist in extremely low temperatures, such conditions lead to cold-induced stress in plants. In addition, the growth and maturing characteristics of *C. oleifera* vary with climatic changes

(Zhuang, 2008). However, we have not found *C. oleifera* under natural low-temperature with regards to its hardiness as previous reports have been based on cold-related stress induced within an indoor environment. Consequently, as a part of this study *C. oleifera* trees from eastern, western and southern Anhui were selected as experimental materials to investigate the effect of natural low-temperature variation on the morphological, physiological and biochemical characteristics of *C. oleifera*. The study will help explain the differences between the physiological characteristics and the inclusion contents of *C. oleifera* in different regions of China and provide information on their response to low-temperature stress with the aim of facilitating information for cold-resistant *C. oleifera* breeding, variety extension and cultivation in commercial production.

Materials and Methods

Site description: Experiments were conducted in Fengyang County in eastern Anhui, Shucheng County in western Anhui and She County in southern Anhui, which represented typical ecotypes of the distribution of *C. oleifera* in this region. Fengyang County faces Huaihe River and *C. oleifera* was found on hills, where it is known to grow best with regards to the northern margin of the *C. oleifera* distribution in China. Shucheng County is in the Dabieshan mountain range and is on the northern fringe of where *C. oleifera* can typically grow in China. She County is in the mountains area of southern Anhui, which has proved to be a suitable area for the growth of *C. oleifera*. The ecological characteristics of the three regions are shown in Table 1.

Table 1. The environmental conditions of the three ecotype regions for *C. oleifera* in Anhui Province.

Site	Eastern Anhui	Western Anhui	Southern Anhui
Longitude (N)	32°37'~33°03'	31°01'~31°34'	29°30'~30°07'
Latitude (E)	117°19'~117°57'	116°26'~117°15'	118°15'~118°53'
Average elevation/m	35	220.3	100
Landform	Hill	Mountain	Mountain
Average annual temperature/°C	14.9	15.6	16.4
Average temperature of the coldest month	0.9	2.4	3.8
Annual average frost-free duration/d	212	224	228

Experimental materials: A strong cold period came at the end of December in 2012 and resulted in a large temperature drop at the beginning of January, 2013, in Anhui. In mid-January, 2013, the Fengyang varieties (Fengyang No. 1–5 of eastern Anhui), the Shucheng varieties (Dabieshan No. 1–4 of western Anhui) and the Shexian varieties (Huangshan No. 1–5 of southern Anhui) were investigated in 50-year-old trees sampled from the three ecotypes. And the temperature of collecting samples among Fengyang County, Shucheng County and She County were -4°C~6°C, -1°C~7°C and 0°C~9°C, respectively.

Determination of spring leaves and morphology from different ecotype cultivars of *C. oleifera*: Three samples were used per cultivar and for each region. The numbers of spring shoot leaves were counted, the lengths and widths of the leaves were measured with a graduated scale, and the leaf thickness and the thicknesses of the spongy mesophyll and palisade mesophyll were determined by hand sectioning with an electron microscope (Olympus BX53, Japan) at a magnification of 100 times.

Determination of catalase, peroxidase and superoxide dismutase activity: Five grams of leaves were ground into a homogenate with quartz sand at a low temperature. Then, 5 mL phosphate buffer (pH 7.5) was added and the mixture was centrifuged at 1200g, 4°C for 30 min to obtain the crude catalase (CAT) enzyme extract in the supernatant (Zhang *et al.*, 2012).

Leaves (0.5 g each) were also ground into a homogenate with 3 mL distilled water and 1 mL of the crude enzyme extract, and centrifuged at 14,000g, 4°C for 15 min to obtain the supernatant, which contained crude

peroxidase (POD) enzyme extracts. Then, 3 mL phosphate-buffered saline (PBS), 0.05 mL methyl catechol reagent and 0.1 mL hydrogen peroxide were added to 0.1 mL of the supernatant (Mbouobda *et al.*, 2010).

0.5 g leaves were ground into a homogenate with quartz sand and ice. Then, 5 mL phosphate buffer (pH 7.8) was added and the mixture was centrifuged at 10,000g, 4°C for 30 min to obtain the supernatant, which contained the crude superoxide dismutase (SOD) enzyme extracts (Zhang *et al.*, 2012). Enzyme activity was expressed in U (min·g fresh weight). The determination of CAT, POD and SOD activity was repeated three times.

Determination of water-soluble sugar, free proline and malonyldialdehyde content: Fresh leaves (0.5 g) were ground into a homogenate with quartz sand, placed in a 50-mL triangular flask containing 25 mL distilled water and heated at 60°C for 30 min. The extract was then filtered and rinsed with hot water. The water-soluble sugar (WSS) contents were assayed following the anthrone method (Plummer, 1987).

Weighed leaves (0.5 g) were ground into a homogenate. Then, 5 mL sulfosalicylic acid (5%) was added and the mixture was heated at 100°C for 10 min, followed by centrifugation at 3000g for 10 min to obtain the supernatant. Free proline (FPro) contents were assayed following the ninhydrin method (Plummer, 1987).

Leaves (0.5 g each) were ground into a homogenate with 10 mL 10% trichloroacetic acid (TCA) and quartz sand. The resulting mixture was centrifuged at 4000g, 4°C for 10 min to obtain the supernatant, which contained the crude enzyme extracts. Then, 2 mL extraction supernatant and 2 mL 0.6% *tert*-butyl alcohol (TBA) were mixed and

heated at 100°C for 15 min prior to rapid cooling. The mixture was centrifuged at 4000g for 15 min to obtain the supernatant. Malondialdehyde (MDA) contents were assayed following the TBA method (Plummer, 1987). The determination of WSS, FPro and MDA content was performed with three replications.

Determination of chemical component content: Leave samples were pre-dried to a constant weight at 85°C (the mass was determined once per hour; if the differences of two sequential results were within 2 mg, the latter was taken as the constant mass) and the weights recorded. Samples were then smashed and completely mixed. The determinations of all the chemical components were carried out in accordance with national standards (Qu, 1992), of these, the benzene-alcohol extracts was determined by GB/T 2677.6-1994. The contents of all chemical components refer to the mass fraction; lignin was determined by GB/T 2677.8-1994 with a volume fraction of 72% in H₂SO₄.

Statistical analysis: Data from this study were presented as the means ± SE for at least three independent experiments. Analysis of variance (ANOVA) and Duncan's test were used to compare the variations of physiological and biochemical parameters following different treatments using SPSS 17.0 software for Windows. *P* values less than 0.05 were considered significant.

Results

Comparison of the morphological structure of *C. oleifera* from different regions: As shown in Table 2, the numbers of leaves in the spring shoots from samples taken from eastern, western and southern Anhui were 5.2, 6.3 and 7.8, respectively. The average leaf size from the three regions was 5.71 cm × 2.80 cm, 5.25 cm × 2.60 cm and 5.78 cm × 2.86 cm, respectively. There was a significant difference with regards to the number of leaves for the different regions, where samples taken from eastern and western Anhui had fewer leaves. The leaf thickness decreased gradually from eastern Anhui to western Anhui to southern Anhui. The total leaf thickness for samples taken from eastern Anhui was 11.40% higher than that of western Anhui, and was 16.67% higher than that of southern Anhui. The leaf thickness and the thicknesses of the palisade and spongy tissue all differed significantly between the different regions (*p*<0.01). This suggested that as the annual average temperature was lower, the number of leaves decreased and the leaf thickness increased in the eastern region. This may be an adaptive mechanism of *C. oleifera* to cope with low-temperature conditions.

Comparison of protective enzyme activity among the three regions: CAT, POD and SOD activity in the leaves of samples taken from eastern, western and southern Anhui were determined and the results were shown in Fig. 1.

CAT activity in eastern Anhui *C. oleifera* plants was the highest of the three regions and was 54.74% higher than in western Anhui samples and 60.13% higher than southern Anhui samples. However, the difference in CAT activity between western and southern Anhui was not significant (*p*>0.05).

POD activity in southern Anhui *C. oleifera* plants was decreased compared to western and eastern Anhui, with POD activity in southern Anhui samples 25.95% lower than in eastern Anhui samples and 29.01% lower than in western Anhui samples. POD activity differed significantly between western and southern Anhui (*p*<0.05).

SOD activity in leaf samples from southern Anhui was slightly lower than in leaf samples from eastern and western Anhui (14.71% and 11.26% lower, respectively). SOD activity was significantly different between eastern and southern Anhui, and between western and southern Anhui (*p*<0.05).

Comparison of WSS, FPro and MDA contents among the three regions: The natural environments of the ecological regions of eastern, western and southern Anhui differed considerably and the average temperatures in winter were distinct. The inclusion contents of the leaves differed significantly among the three regions and generally decreased from eastern Anhui to western Anhui to southern Anhui. The results are presented in Table 3.

The WSS content was highest in leaves collected from eastern Anhui, while it was lowest in leaves from trees grown in southern Anhui. The changes in WSS contents between the regions were significantly different (*p*<0.05).

C. oleifera leaf samples from eastern and western Anhui showed obviously higher FPro contents than leaves from southern Anhui. Furthermore, the FPro content of leaves from trees grown in southern Anhui was 5.25% less than leaf samples from eastern Anhui and 2.06% less than leaf samples from western Anhui. The FPro contents differed significantly among the different regions (*p*<0.05) (Table 3).

The MDA contents from eastern Anhui were 23.70% and 43.95% higher, respectively, than the corresponding contents from western Anhui and southern Anhui (Table 3). There were extremely significant differences in the MDA contents among three regions (*p*<0.01). These results suggested that the cytomembranes of the leaves were seriously damaged in eastern Anhui.

Table 2. Average numbers^a of spring leaves and leaf morphology for *C. oleifera* from different ecotypes.

Ecotype	Number of leaves	Leaf size/cm		Thickness/μm		
		Length	Width	Leaf	Palisade tissue	Spongy tissue
Eastern Anhui	5.2 aA	5.71 aA	2.80 aA	465.27 aA	175.31 aA	189.26 aA
Western Anhui	6.3 bAB	5.25 bB	2.60 aA	417.64 bB	142.97 bB	167.84 bB
Southern Anhui	7.8 cC	5.78 aA	2.86 aA	398.79 cC	133.38 bB	159.72 cC

^aValues given are the means of three replicates. Mean values with the same small letter (in the row) and capital letter (in the column) letters were not significantly different by Duncan's test at *p*<0.05

Table 3. Average WSS and FPro contents^a of *C. oleifera* leaves from different ecotypes.

Collection area	WSS (mg/g)	FPro ($\mu\text{g/g}$)	MDA($\mu\text{mol/g}$)
Eastern Anhui	24.74 \pm 0.36 aA	44.18 \pm 0.67 aA	14.51 \pm 0.38 aA
Western Anhui	23.54 \pm 0.23bAB	42.74 \pm 0.79 abA	11.73 \pm 0.96 bB
Southern Anhui	23.41 \pm 0.23 bB	41.86 \pm 0.71 bA	10.08 \pm 0.79 cC

^aValues given are the means of three replicates. Mean values with the same lowercase (in the row) and uppercase (in the column) letters were not significantly different by Duncan's test at $p < 0.05$

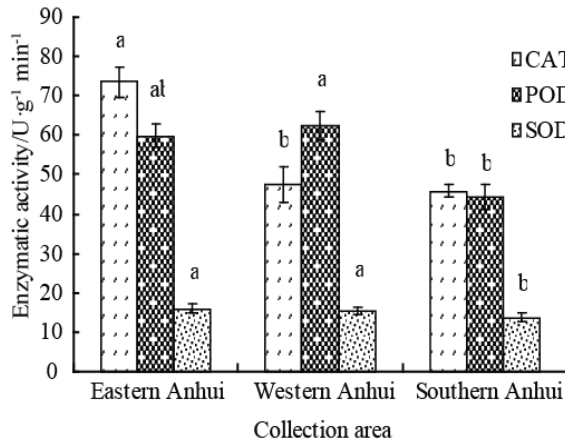


Fig. 1. Average activities of CAT, POD and SOD of *C. oleifera* leaves from different ecotypes. Values with different letters are significantly different at $p < 0.05$.

comparison of wss and fpro contents among the three regions: the benzene-alcohol extracts content in *C. oleifera* leaves from eastern and western anhui were higher than that of leaves from southern anhui by 44.90% and 32.20%, respectively ($p = 0.05$). the changes in benzene-alcohol extracts contents among the regions were significantly different ($p < 0.05$).

the lignin content of leaves from eastern, western and southern anhui were also determined. the results showed that the lignin content of leaves from eastern anhui were highest at 15.40%. the average annual temperature of eastern anhui is lowest. thus, we can confer that there was a negative correlation between the lignin content and the temperature. the lignin content was significantly different between eastern and western anhui, and between eastern and southern anhui ($p < 0.05$) (fig. 2).

the above results suggested that the accumulation of wss, fpro, benzene-alcohol extracts and lignin in *C. oleifera* leaves and effective osmoregulatory substances may play important roles in the cold-resistance mechanism under natural low temperatures.

discussion

temperature is a very important ecological factor during the plant growth process. the average annual and winter temperatures in eastern anhui are both lower in comparison to both western anhui and southern anhui. thus, *C. oleifera* varieties from different regions likely have different degrees of resistance to cold. compared with western anhui and southern anhui, *C. oleifera* varieties sampled from eastern anhui were strongly

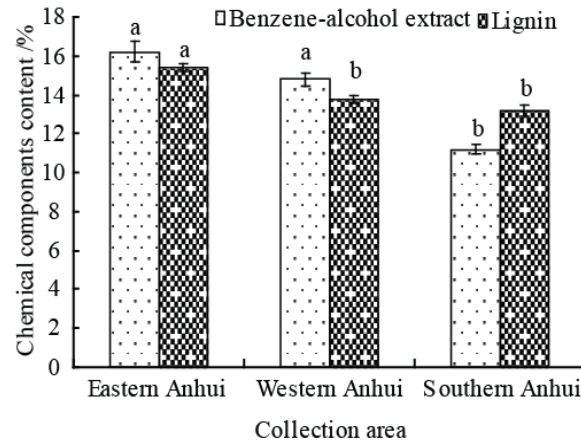


Fig. 2. Average chemical components contents of *C. oleifera* leaves from different ecotypes. Values with different letters are significantly different at $p < 0.05$.

resistant to cold. during the natural cooling process, *C. oleifera* plants changed with regards to their growth and morphology due to low-temperature stress. the number of leaves on spring shoots and the leaf size can be considered to directly reflect a plant's growth state. furthermore, total leaf thickness, and the thicknesses of palisade tissue and the spongy mesophyll are indirect indices that reflect a plant's ability to synthesize chemicals and thus reflect its chemical composition. strongly cold-resistant *C. oleifera* plants from eastern anhui had smaller leaves than samples from southern anhui and the leaf thickness, and the thicknesses of the palisade tissue and spongy mesophyll were relatively thicker than the samples collected from western and southern anhui. these results are consistent with the findings of zhuang *et al.* (1992) and li & bao (2014).

sod, pod and cat are considered protective enzymes and form part of the plant cell defense system, which can eliminate peroxide and harmful substances produced by plants under low-temperature stress. the activities of these enzymes may also protect the plant cell membrane and biomacromolecules, allowing the plant to survive or adapt during periods of low temperature (feng *et al.*, 2011; gao *et al.*, 2011). in this study, the sampling temperature of eastern anhui was the lowest and the activity of cat and sod in leaves sampled from this region was highest. in general, lower temperatures enhanced the activities of the protective enzymes, allowing more free radicals to be eliminated through a series of physiological regulatory mechanisms. these mechanisms may protect *C. oleifera* plant cells, preventing harm and serious damage due to the low temperatures, which demonstrates that maturing

c. oleifera trees have the ability to withstand cold conditions. this also suggested the presence of a reaction oxygen species (ros) system, which may be promoted through enzymatic and non-enzymatic pathways when plants are exposed to low-temperature conditions. under normal ambient conditions, toxic peroxidating substances are eliminated to maintain normal physiological metabolism in plants (feng *et al.*, 2005; he *et al.*, 2007). however, as abnormal low temperature is prolonged, the activity of normal physiological metabolism may be damaged due to the presence of excessive free radicals and ros, which must be eliminated to prevent damage. ros lead to peroxidation of the lipid membrane and decrease the activities of protective enzymes, resulting in mda-membrane lipid peroxidation products (zhang *et al.*, 2007; he *et al.*, 2012).

when varieties are exposed to natural low temperatures stress during growth and maturation, a resistance mechanism is triggered to help the plant withstand the cold by increasing the wss content. thus, the wss content is an important osmotic adjustment that not only lowers the cyto-solution freezing point and strengthens the infiltration potential of cells, but also buffers excessive dehydration in the cytoplasm to prevent solidification. this could be helpful to reduce the freezing point and buffer excessive cytoplasmic dehydration to protect the cytoplasm in the flow state through effective osmoregulation under low temperature (wang *et al.*, 2014). the wss content can promote the accumulation of abscisic acid and indirectly induce protein synthesis, which can strongly enhance cold resistance in plants (wang *et al.*, 1996). therefore, reducing adverse reactions in response to low-temperature stress and damage to the cytoplasm may assist plants to adapt to changes in the external environment (li & wang, 2002). the accumulation of wss under low-temperature stress could be considered a cold hardiness index and has been used in various research focused on plant resistance. it was reported that the content of wss showed a positive correlation with cold resistance (luo *et al.*, 2002). the results of the present study demonstrated that *c. oleifera* from eastern anhui (the coldest region of those sampled) had the highest cold tolerance and the accumulation of wss in eastern anhui *c. oleifera* leaves was the highest of the sampling ecotypes.

wss and fpro are considered protective substances and it has been reported that the fpro content is significantly and positively correlated with the cold hardiness of many plants (jiang *et al.*, 2014). fpro plays an important role in maintaining the cytoplasm structure, as well as transportation and osmotic adjustment in cells; therefore, it helps to enhance the water-holding capacity of plants and prevents the plant tissue from being injured under low-temperature stress (liu *et al.*, 2003; bai *et al.*, 2003). increased fpro accumulation has been found in plants resisting various environmental stressors (jiang *et al.*, 1997). in the present study, accumulation of fpro in samples collected from eastern anhui was the highest, and was the lowest in samples collected from southern anhui. the fpro content differed significantly between the different regions ($p < 0.05$). this suggested that the higher contents of fpro in samples from eastern anhui, where the temperature was lowest, may be the result of a physiological reaction to cold adaptation. this is consistent

with reports from artus *et al.* (1996) and burbulis *et al.* (2012). on the contrary, the results are not consistent with a report by wang *et al.* (2014), proline content decreased as temperature declined.

mda is a cytoplasm membrane peroxidation product and its content reflects the level of cytoplasm membrane peroxidation and can indicate cell injury (he *et al.*, 2015). under low temperature, the accumulation of reactive oxygen free radicals beyond a certain limit in *c. oleifera* tissue may cause membrane lipid peroxidation, resulting in the accumulation of mda (robert & bewlery, 1980; jiang *et al.*, 2008). in the present study, as the varieties due to exposure to different low temperatures, the mda contents sampled in eastern anhui were relatively higher. this suggested that the varieties in eastern anhui suffered more injury than that of the other regions. when the ambient conditions continued to decline to a critical level, membrane lipid peroxidation was aggravated, which had a detrimental effect on membrane lipid function and led to increasing mda contents. this is consistent with the reports of huang *et al.* (2002) and huang *et al.* (2015).

lignin is responsible for rigid plant cell walls and protects plant cells and tissue from adverse effects (chen *et al.*, 2005; han *et al.*, 2000; xu *et al.*, 2007). benzene-alcohol extracts from *c. oleifera* leaves contain resin, wax, fat, tannins and phenolic compounds. variation of the total phenols and lignin is an important evaluation index of low-temperature stress. the lignin content positively correlates with stress resistance in plants (zhang *et al.*, 2009). the results of the present study showed that the lignin content and the content of benzene-alcohol extracts from plants sampled from eastern anhui were higher than those of western and southern anhui. it was also found that increased lignin and benzene-alcohol extract contents were helpful to improve the stress resistance of *c. oleifera*, which is consistent with reports from zhang & xiao (1997) and zhu *et al.* (1990).

Conclusions

To investigate changes of *C. oleifera* under low-temperature stress, we performed a comparative morphology, physiological and biochemical characteristics analysis of *C. oleifera* from eastern, western and Southern Anhui under natural low temperature. in this study, the leaf morphological characteristics of *C. oleifera* obtained from eastern anhui was increased compared with those from western and southern Anhui. protective enzyme activities and inclusion contents were relatively higher in eastern Anhui. Such the response mechanisms will provide insights into cold-resistance research and for further dissection of cold tolerance mechanisms in *C. oleifera*.

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