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Article

Physiological Responses of *Dendrobium officinale* under Exposure to Cold Stress with Two Cultivars

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Abstract: This study aimed to explore the cold tolerance of two cultivars of *Dendrobium officinale* (MG1, MG2) grown in different regions of China. Under −2°C incubation, cultivar MG1 remained active after 3 d, and continued to grow after returning to room temperature. However, MG2 could only maintain its activity after 2 d treatment at −2°C, and the seedlings died with the low temperature treatment time. Investigation of the characteristics of the plants grown in the south (Hangzhou) or north (Zhengzhou) of China indicated that the leaves of MG1 also had reduced stomatal density, the highest thickness, and a compact microstructure. The contents of proline and soluble sugars were higher in MG1 than those in MG2. The cultivar MG1 had higher SOD enzyme activity than MG2, while CAT and POD activities in samples from Zhengzhou were higher than those from Hangzhou. The contents of polysaccharides and alkaloids in stems of in MG1 were higher than those in MG2, while the content of flavonoids in the Zhengzhou samples was higher than that in the Hangzhou samples. In addition, plant heights, stem diameters, and chlorophyll content were higher in MG1. Overall, MG1 had better cold resistance than MG2. MG1 is a cold tolerant cultivar with thick leaves and reduced stomatal density, higher contents of soluble sugars, proline, CAT, POD, polysaccharides, flavonoids and alkaloids, which together make it more adaptable to low temperatures. Thus, the cultivar MG1, with its demonstrated cold tolerance, can accordingly be grown on a large scale in cold regions, thereby expanding the available planting area for this important traditional medicinal plant to meet the increasing commercial demand for it.

Keywords: *Dendrobium officinale*; cold stress; microstructure characteristics; physiological characteristics; biochemical characteristics

1 Introduction

Dendrobium officinale Kimura et Migo, a member of the family *Orchidaceae*, is a traditional Chinese herbal medicine. *D. officinale* is used to alleviate diabetes, and enhance immunity with anti-oxidation and

antineoplastic functions [1,2]. *D. officinale* has many active components, including the major polysaccharides (20–40%) as well as alkaloids, flavonoids, amino acids, phenolic compounds and lignans, all of which may contribute to its broad medicinal efficacy. Wild *D. officinale* mainly grows on rocks at an altitude of 1,600 meters in the south of China, such as in southern Anhui, eastern Zhejiang, western Fujian, northwestern Guangxi, Sichuan, and southeastern Yunnan provinces.

As the demand for *D. officinale* increases, it has been necessary to enhance the yield by expanding the planting area of *D. officinale*. This has led to the northward shift of the planting range of *D. officinale*. However, low temperature is an important abiotic factor that limits the development and spread of plants [3]. Normal cultivars of *D. officinale* that have adapted to the warmer southern climate are vulnerable to injury by low temperature when they are grown in areas north of their native habitat regions. Low temperature can cause freezing of the cell membrane and oxidative stress and produces physiological and metabolic changes, resulting in irreversible damage and impact on plant growth and development [4]. It shows a significantly negative impact on the survival of the plant overall, having a detrimental effect on photosynthesis, water transport, nutrient absorption and growth; thus, the productivity and quality are greatly reduced [5]. Therefore, cold tolerant cultivars are accordingly required for *D. officinale* to adapt to the low temperature in the enlarged planting area of China.

Low temperature induces the expression of related stress-resistant genes, accumulates osmotic adjustment substances, activates the antioxidant system and changes the cell components, thereby alleviating the physiological and mechanical damage. Low temperature imposes dehydration, and plants accordingly accumulate high amounts of osmoprotectants to protect the cells from dehydration [6]. As common osmoprotectants, soluble sugars and proline, which are beneficial for increasing the osmotic potential of cells, usually accumulate significantly under cold stress, thereby maintaining the integrity of cell membranes, and reducing the damage to plants at low temperature [7]. The plants with higher accumulation of soluble sugars and proline accordingly display stronger tolerance against cold stress [8].

When subjected to low temperature stress, plants will be provoked into overproduction of reactive oxygen species (ROS) which disrupt the cellular homeostasis and the integrity of their overall metabolism [9]. A large amount of ROS can promote the degradation of unsaturated fatty acids in plant cell membranes to produce malondialdehyde (MDA), causing peroxidation of membrane lipids and damage to cell membranes [10]. In response to the damage from ROS, plants evolve enzymatic and non-enzymatic antioxidant defense mechanisms to protect themselves from oxidative stress, thus endowing plants with the ability to endure cold stress [11]. Enzymatic antioxidant systems include superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT), and their corresponding activities directly reflect the cold resistance of plants. Non-enzymatic compounds, such as ascorbate and glutathione, also contribute to the reduction of ROS damage [12]. Antioxidant enzymes play comparatively major roles in the protection of plants from cold stress.

In addition to the aforementioned antioxidants, important metabolites, such as flavonoids and polysaccharides, also play an important role against cold stress [13]. Increase in the content of flavonoids is often checked under cold stress for many plants. The removal or control of ROS produced by low temperature stress is one of the important mechanisms by which flavonoids protect plant organisms from damage [14]. Therefore, under low temperature environments, the accumulation of flavonoids in plants will be promoted [15]. Temperature is also one of the important factors regulating polysaccharides, and the content of polysaccharides significantly increases for *D. officinale* in winter. Compared with cold-sensitive varieties, the cold-tolerant varieties usually have higher polysaccharide content [16]. Polysaccharides are effective ROS scavengers and protect the plants from oxidative damage [17]. In addition, accumulation of the polysaccharides in plant cell walls results in increased plant resistance to cold [18].

In this study, two different cultivars of *D. officinale*, namely, MG1 (cold tolerant) and MG2 (cold sensitive) are investigated for their cold tolerance, and to determine their corresponding biological, physiological and biochemical properties. The ultimate purpose of this study is to find an alternative cultivar of *D. officinale* which is sufficiently cold tolerant so that it can be grown on a commercial scale in the north of China.

2 Materials and Methods

2.1 Cold Tolerance Experiment

The cold tolerant *D. officinale* cultivar "Mu Ge No.1" (MG1) and the cold sensitive cultivar "Mu Ge No. 2" (MG2) were obtained from Zhejiang Hangzhou Deqing Pastoral Ecological Agriculture Co., Ltd. Tissue culture seedlings (10 months) from these samples were kept at room temperature (20–25°C) for 2 weeks in tissue culture bottles. The tissue culture seedlings were then separated from the medium, cleaned, air dried for 2 d, and then transplanted into pots filled with growth matrix (pine bark). After maintaining at room temperature (20–25°C) for 7 d, the plantlets of MG1 and MG2 were placed in an incubator and subjected to continuous cold stress at −2°C. Three pots of each cultivar of plantlets were removed at intervals of 1 d, and 3 g of leaves were sampled from each pot to measure their relative electrical conductivity (REC) and malondialdehyde (MDA) content. The residue plantlets were kept at 25°C to test the survival growth.

2.1.1 Determination of Relative Electrical Conductivity

The REC was measured by ion conductivity meter as described in the literature [19]. For this, 1 g of the leaf blade was immersed in 8 mL deionized water for 5 h, and boiled for 30 min. The ion conductivity of the boiled water was measured using an ion conductivity meter (*DDS-11A, Aipli*, Shanghai, China). The REC value was then calculated based on the changing of the conductivity before and after boiling as follows:

$$E = E_0/E_1 \times 100\%$$

E: Relative electrical conductivity, %; E_0: Relative electrical conductivity before boiling, S/m; E_1: Relative electrical conductivity after boiling, S/m.

2.1.2 Determination of Malondialdehyde (MDA) Content

The MDA contents were measured spectrophotometrically as described in the literature [20]. This procedure involved taking 1 g of leaves, adding 2 mL of 10% (w/v) TCA and 2 mL of 0.6% (w/v) TBA solution, and mixing them completely. The mixture was then reacted in a boiling water bath for 15 min, and centrifuged at 4000 rpm for 10 min. The supernatant was taken and its absorbance (A) was measured at wavelengths of 532 nm, 600 nm and 450 nm, using a UV-visible spectrophotometer (*UV-2100, Shimadzu*, Kyoto, Japan). The content of MDA was calculated using the following formula:

$$C_1 = (6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}) \times 10$$

C_1: MDA content, μmol/L

2.2 Field Experiments

To test the cold tolerances of MG1 and MG2 in the field, both varieties were planted in representative regions in the south (Hangzhou) and the north (Zhengzhou) of China. For this, 10 month tissue culture seedlings of MG1 and MG2 in Hangzhou and Zhengzhou were transplanted in May, 2017. All the plants in both districts were planted in plastic greenhouses with pine bark/sawdust (9:1) as the substrate, watering daily and fertilizing once every half a month using complex fertilizer. For each planting district, 15 plants of each variety were sampled in May and September, 2018 and in January, 2019. The average temperatures (outdoor/greenhouse) in Hangzhou were: 17–26°C/18–22°C in May, 21–28°C/21–24°C in

September, and 2–8°C/8–14°C in January. The average temperatures (outdoor/greenhouse) in Zhengzhou were: 15–27°C/16–22°C in May, 16–27°C/16–22°C 21–28°C/21–24°C in September, and -4–6°C/0–4°C in January (outdoor weather information from the National Meteorological Centre, China). The sampled plants were used for the following experiments:

2.2.1 Growth Characteristics Determination

MG1HZ, MG2HZ, MG1ZZ, and MG2ZZ were sampled in May, September and January, as described above, and the corresponding budding rates, plant heights, stem diameters and leaf thicknesses of each branch of 3 × 15 clusters were randomly measured by the "Z" method (five points randomly selected according to the "Z" shape) using Vernier calipers [21].

2.2.2 Chlorophyll Determination

The determination of chlorophyll content was carried out by a spectrometric method [22]. A 0.5 g sample of frozen leaves was homogenized with 10 mL of acetone (80% v/v) using a pre-chilled pestle and mortar. The homogenate was then centrifuged (*5424R, Eppendorf*, Hamburg, Germany) at 8000 rpm at 4°C for 10 min, and the absorbance was measured at 663 nm and 645 nm with a UV-visible spectrophotometer (*UV-2100, Shimadzu*, Kyoto, Japan).

2.2.3 Structural Observations

1. Observation of leaf stomata

The observations of the stomata of the leaves were carried out by optical microscope (*E100, Nikon*, Kyoto, Japan) [23]. The selected fresh leaves of *D. officinale* were washed and air dried, and then placed in a sample bottle containing a fixing solution of anhydrous ethanol and acetic acid in a volume ratio of 3:1(v/v). The leaves were then placed in sterile water, soaked for about 5 min, removed, and photographed. The length and width of each pore and the number of pores in each image were measured using ImageJ (*Version: 1.52s*, USA) software.

2. Observation of the microstructure of leaves and roots

The combined microscopic observations were carried out by paraffin sectioning [24]. After selecting the leaves and roots from similar position in the sampled plants, which were then washed with water and dried, the middle part of the leaves was cut with scissors to dimensions of about 5 × 5 mm, and placed in FAA fixing solution (70% [w/v] ethanol 90 mL). Finally, observations of the microstructures were made via microscope.

2.2.4 Determination of Proline, Soluble Sugars and Antioxidant Enzymes

The determination of proline was carried out by a spectrophotometric method [25]. This method firstly involved weighing 0.2 g of *D. officinale* leaves, grinding them with 3% (w/v) sulfosalicylic acid, adding 5 mL sulfosalicylic acid dihydrate solution, and then boiling in a water bath for 10 min. After cooling, the solution was filtered, and made up to 5 mL using 3% (w/v) sulfosalicylic acid. After this, 2 mL of the extraction solution were placed into a test tube, to which 2 mL of glacial acetic acid and 2 mL of acid ninhydrin were added, and then extracted in a boiling water bath for 30 min. When the solution was cooled to room temperature, 4 mL of toluene were added and then shaken, and finally let stand for 2 h. The upper layer of the solution in the test tube was then analyzed, using toluene as a blank control, by determining the absorbance value at a wavelength of 520 nm which was then converted to proline content according to the cited method.

The method for measuring soluble sugars also followed a previously reported method [26]. Firstly, 0.5 g of leaves were placed into a mortar, distilled water and a small amount of quartz sand added, and then ground with a pestle into a homogenate. The homogenate, together with the residue, were then placed into a 100 mL volumetric flask, left at room temperature for 30–60 min, filtered, and the residue discarded. Next, 1 mL of

sample and 5 mL of anthrone reagent were added to a clean test tube, shaken in a boiling water bath while heating for 10 min, and, finally, the absorbance at 620 nm was measured after cooling. Glucose standard solutions of different concentrations were prepared, and their corresponding absorbance at 620 nm was measured to prepare a standard curve, from which the soluble sugar content was determined.

The antioxidant enzymes include SOD, CAT and POD. To determine these, 0.4 g of the leaves of *D. officinale* were placed in a pre-cooled mortar and pestle and ground, then added to 3.2 mL of phosphate buffer (pH 7.8, 123 mL of 0.2 mol/L Na_2HPO_4 and 877 mL of 0.2 mol/L NaH_2PO_4), and centrifuged at 8000 rpm at 4°C for 30 min. The resulting supernatant was the enzyme-containing solution which was used for the following measurements to determine enzyme activity.

SOD activity was estimated by monitoring 50% (w/v) inhibition of the photochemical reduction of NBT [27]. To a 10 mL centrifuge tube, 3 mL of the reaction mixture and 30 μL of the enzyme solution were added and shaken thoroughly, then 3 mL of the reaction mixture were taken and 30 μl of phosphate buffer (pH 7.8) added to this as a control. The corresponding absorbance was measured at 560 nm and the total SOD enzyme activity calculated from the absorbance. One unit of enzyme activity is defined as the amount of enzyme required to cause a 50% (w/v) reduction in NBT inhibition.

CAT activity was determined by measuring the disappearance of H_2O_2 [27]. To 3 mL of the reaction solution, 100 μL of the enzyme solution were added, with phosphate buffer solution (pH 7.0) as the control. The amount of H_2O_2 reduction was measured from the absorbance at 240 nm, and the extinction coefficient (40 $mM^{-1}cm^{-1}$) was used to calculate the activity of CAT. One unit of CAT activity is defined as the amount of CAT required to decompose 1 mole of H_2O_2 per min.

POD activity was assayed following the method of Liang and co-workers [27]. This method involved taking 30 μL of the enzyme solution, adding 200 mL phosphate buffer (pH 6.0), 114 μL of guaiacol, and 168 μL of 30% (w/v) H_2O_2, then shaking the mixture completely, and keeping it at room temperature for 3 min. The corresponding absorbance change of the brown guaiacol solution at 470 nm was recorded for calculating POD activity.

2.2.5 Determination of Total Polysaccharides, Flavonoids and Alkaloids

Total polysaccharides were determined according to a method described in the literature [28]. Dried stems of *D. officinale* were ground into powder and extracted with 85% (w/v) ethanol for 24 h at room temperature. The solution was then filtered to remove fat-soluble ingredients. The residue obtained after the ethanol extraction was dried and then extracted 3 times with hot water (70°C–75°C), combined and filtered for 1.5 h each time. The filtrate was then evaporated under reducing conditions and absolute ethanol was added during precipitation until the ethanol concentration reached 85% (w/v). The mixture was stored in the refrigerator at 4°C for 24 h. The resulting precipitate was collected and centrifuged, then dissolved in distilled water and lyophilized to produce crude polysaccharides. The crude polysaccharides were hydrolyzed with trifluoroacetic acid (6 mol L^{-1}). The absorbance of the hydrolyzed solution was measured at 488 nm using a spectrophotometer. Glucose standard solutions of different concentrations were used to prepare a standard curve, and their corresponding absorbances were measured at 488 nm. The polysaccharide content of the samples was then determined according to the standard curve.

To determine the flavonoid content, the method of Zhou et al. [29] was utilized. *D. officinale* samples were ground into powder after drying; 1.0 g of this powder was added into a 100 mL Erlenmeyer flask and subjected twice to ultrasound extraction for 45 min each time with 50 mL of methanol. After this, the total extract was evaporated to dryness and the residue dissolved again in a solution with an amount of methanol: water (80:20, v/v). The solution was then transferred to a 2 mL volume and diluted to constant volume (10 mL). The absorbance of the treated samples was measured at 510 nm using a spectrophotometer. Rutin standard solutions of different concentrations were prepared, and their

absorbances at 510 nm were measured to prepare a standard curve. The flavonoid content of the samples was determined according to the standard curve.

The alkaloid content was determined from a method previously published in the literature [30]. This method involved taking 0.5 g of *D. officinale* stems or leaves which were placed in a round bottom flask, with 2 mL of 10% (w/v) ammonia water injected into the flask, which was then sealed for 30 min. After this, 10 mL of chloroform were added, and then the solution was condensed and refluxed in a 65°C water bath for 2 h, cooled and filtered. Finally, 2 mL of the filtrate were placed in a 50 mL centrifuge tube, with 8 mL of chloroform added, and shaken well. The absorbance of the processed sample was measured at 620 nm using a spectrophotometer. Alkaloid standard solutions of different concentrations were prepared, and a standard curve produced, based on the absorbances of the standard solutions at 620 nm. The alkaloid content was then determined according to the standard curve.

2.3 Statistical Analysis

For each treatment, 5 plants were analyzed, and all the experiments were repeated three times. The data were analyzed using SPSS (version 22.0, IBM Corp., Armonk, NY, USA) software for statistical analysis. ANOVA followed by Duncan's LSD tests were performed, and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Cold Tolerance of D. officinale

The tolerance of *D. officinale* to cold was investigated under a stress of −2°C. As shown in Fig. 1A, the cultivar MG1 had intact, green leaves after 2–3 days treatment, and the plants maintained good vigor, as evident by their consistent green leaves, strong stems and general healthy appearance after 10 days of growth at room temperature. However, the plants were completely destroyed after treatment of 4 or more days without recovery during 10 days of growth, as evident from their blighted, withered, discolored and shriveled appearance, which did not improve over time. Therefore, the number of cold tolerant days for cultivar MG1 was 3. For the cultivar MG2, the plants were completely destroyed after 3 or more days of treatment, without recovery during 10 days of growth. Therefore, the number of cold tolerant days for cultivar MG2 was 2 d.

For REC and MDA, both values increased with the number of days of cold stress. For comparison of the two cultivars, MG2 showed significantly higher REC than MG1 (Fig. 2B). The same results were obtained for MDA, except for the 5d treatment, in which MG2 had higher MDA content than did MG1 (Fig. 2A). In addition, the MDA content for MG1 of 3 d stress was close to that for MG2 of 2 d stress, which was consistent with the results of recovery growth for MG1 of 3 d stress compared to MG2 of 2 d stress.

3.2 Characteristics of Cold Tolerance

Characteristics of cold tolerance for *D. officinale* were determined for samples from the planting fields in Hangzhou and Zhengzhou. These characteristics included the microstructures of the leaves, impermeable components, antioxidant enzymes, and important chemical constituents (alkaloids, total polysaccharides and flavonoids).

3.2.1 Microstructure

The structures of leaves are normally closely related to cold tolerance. Thus the thickness and microstructure of leaves as well as roots were accordingly analyzed. As shown in Fig. 3A, some differences were observed for the leaf thickness between the two cultivars during September. Cultivar MG1 had greater leaf thickness than MG2; when comparing the planting location for the same cultivar, no difference was found between the plants from Hangzhou and those from Zhengzhou.

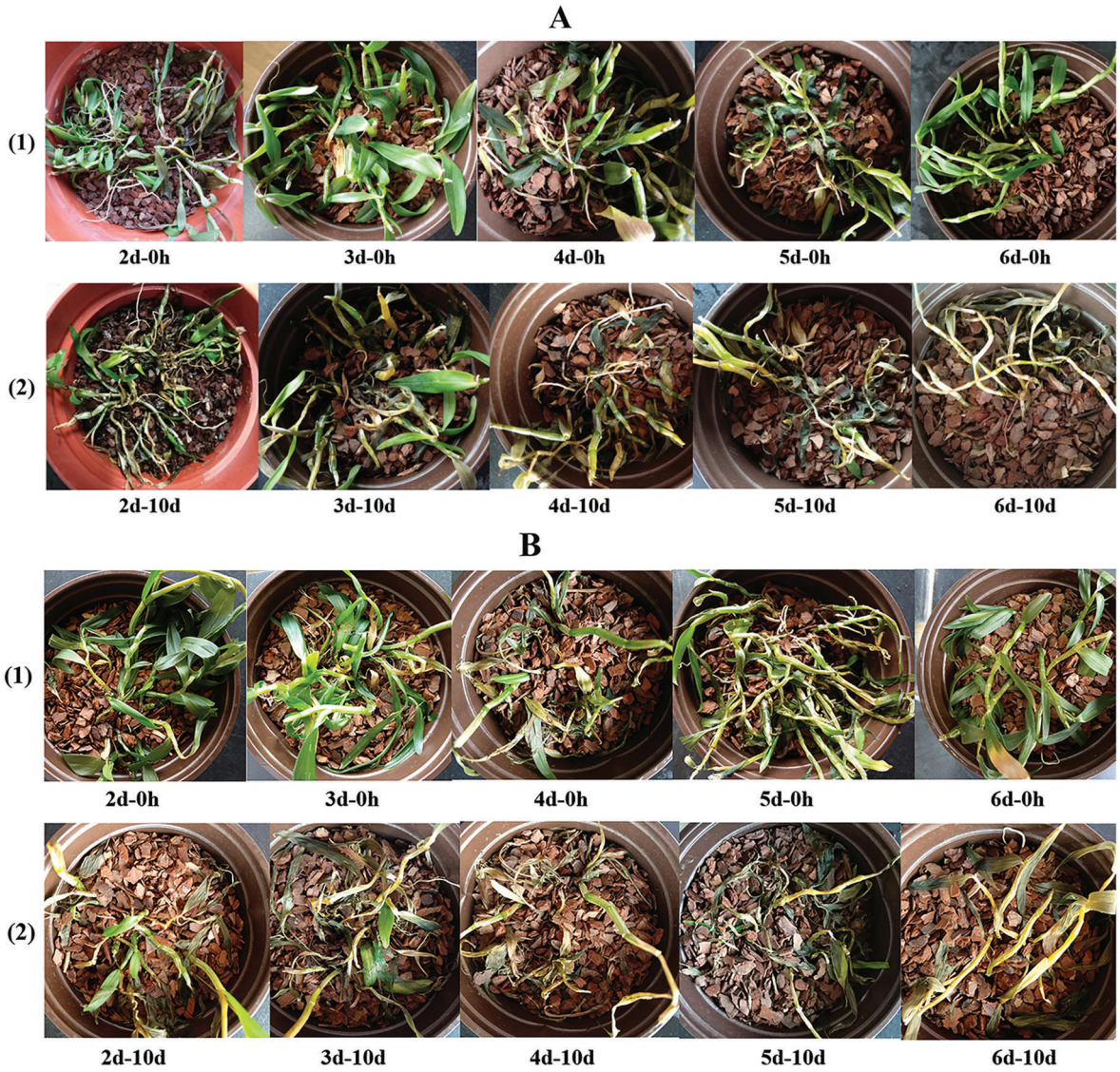

Figure 1: Recovery of *D. officinale* seedlings 10 d post stress under −2°C. A: MG1; B: MG2; (1) Seedlings under cold stress for 2 d–6 d; 0h means no recovery; (2) Seedlings recovered 10 d post cold stress

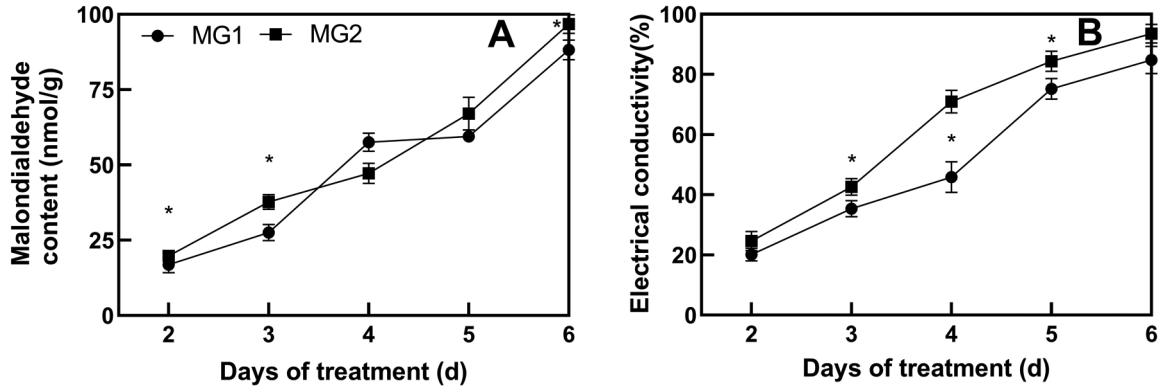

Figure 2: Effects of low temperature on MDA content (A) and REC (B) of *D. officinale* seedlings. (* indicates significant difference, $p < 0.05$)

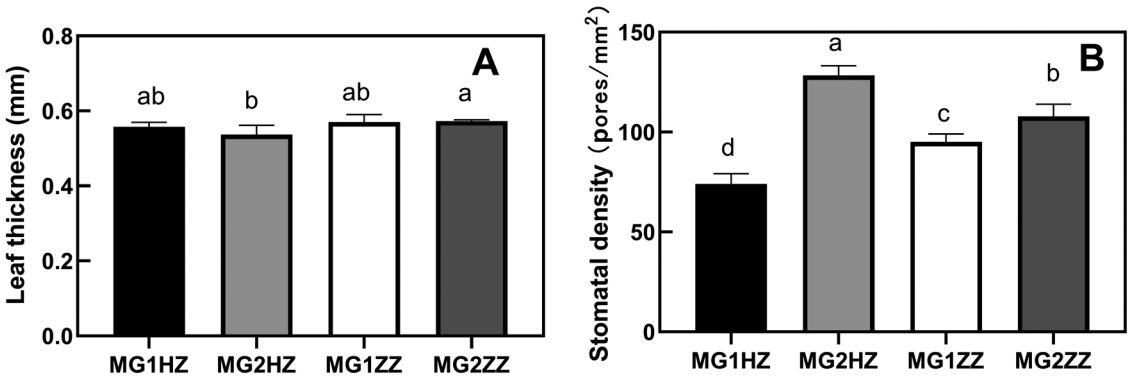

Figure 3: The thickness (A) and stomatal density (B) of the leaves of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). HZ: plants from Hangzhou; ZZ: plants from Zhengzhou

The stomas on the back surface of the leaves were observed. A significant ($p < 0.05$) difference in stomatal density was observed between the two cultivars. As shown in Figs. 3B and 4, cultivar MG1 from Hangzhou had the lowest stomatal density, followed by MG1 from Zhengzhou. Cultivar MG2 from Hangzhou had the highest stomatal density, followed by MG1 from Zhengzhou. These results further indicated that cultivar MG1 had lower ($p < 0.05$) stomatal density than MG2.

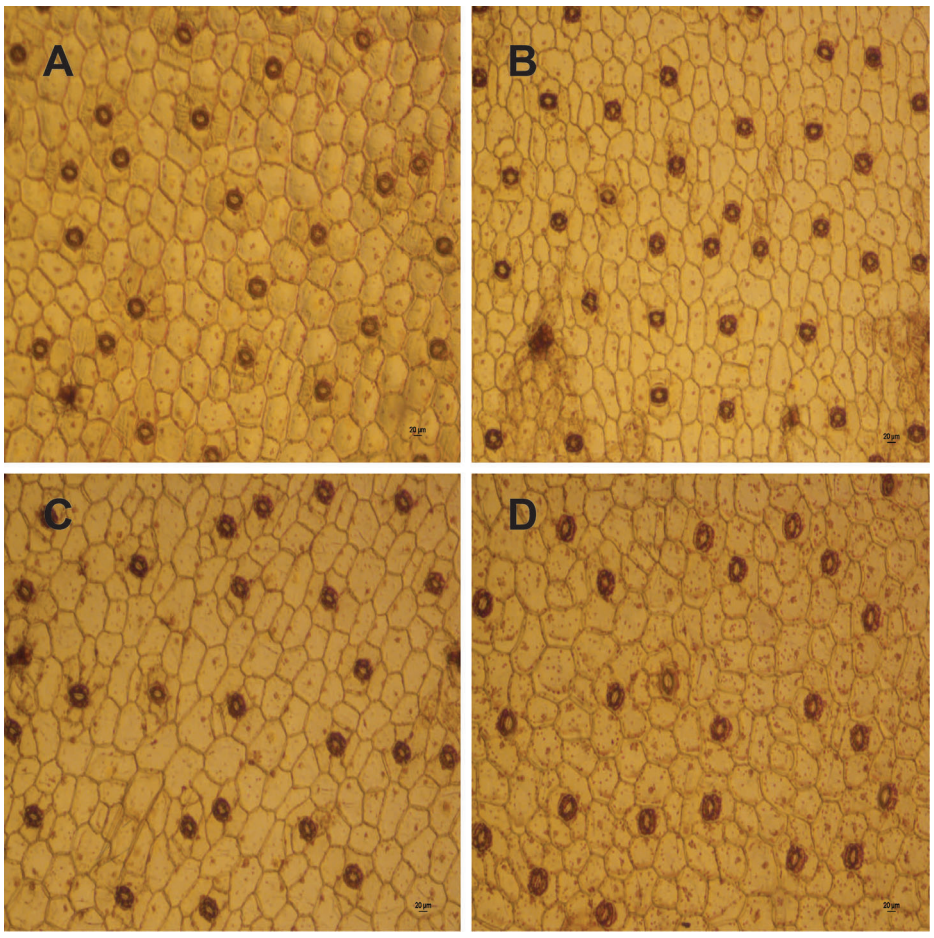

Figure 4: Profiles of stomata on the back surface of the leaves for MG1 and MG2 planted in Hangzhou (HZ) and Zhengzhou (ZZ) (10 × 40). A: MG1HZ; B: MG2HZ; C: MG1ZZ; D: MG2ZZ

For the microstructures of the leaves, a significant difference existed between the two cultivars from the two different fields (Fig. 5). A more compact structure was found in the leaves of MG1ZZ, which had the highest thickness of palisades cells and spongy mesophyll, and the cells were closely arranged. However, for MG2HZ, the palisades cells and spongy mesophyll were not so well defined; the palisades cells in particular were loose. It is apparent that cultivar MG1 had greater thickness, and the structure of the same cultivar form Hangzhou was more compact than its counterpart from Zhengzhou.

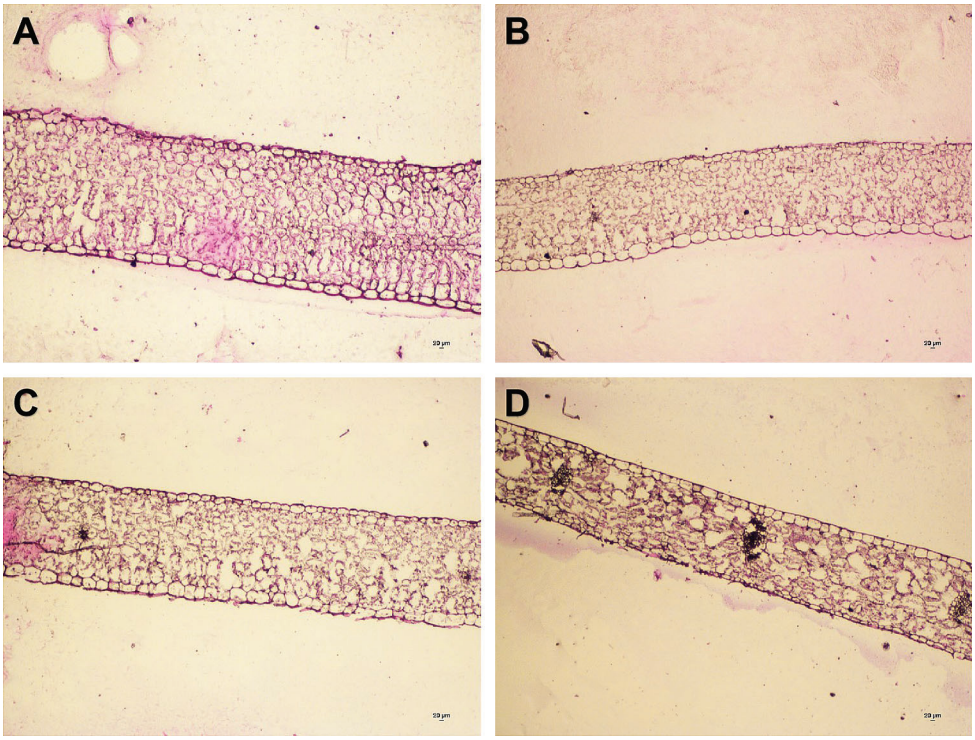

Figure 5: The leaf paraffin section maps of *D. officinale* (10 × 10). A: MG1HZ; B: MG2HZ; C: MG1ZZ; D: MG2ZZ. MG1HZ had the largest cross-sectional area. The leaf tissue of MG1 was dense, the thicknesses of palisade cells and spongy mesophyll cells are the largest, and the cells are densely arranged. The leaf thickness and tissue of MG2 are generally small, and the cells are sparsely arranged

For the microstructure of the roots, no significant differences existed between the two cultivars from the two different fields (Fig. 6). It was difficult to determine any difference in epidermis, cortex, or vascular bundle of the plants between MG1 and MG2 or between samples from Hangzhou and Zhengzhou.

3.2.2 Physiological and Biochemical Characteristics

Proline and soluble sugars are important cold tolerance osmolytes. Soluble sugars are also important cold tolerance and protective substances for plants. The results (Fig. 7A) showed that the soluble sugar content of *D. officinale* was similar between cultivars MG1 and MG2 from Hangzhou from the same time. However, a significant ($p < 0.05$) difference was observed between the two cultivars from Zhengzhou. Higher sugar content was found in MG1 than in MG2 during the same period. For January, the soluble sugar content of MG1ZZ was 2.3 times that of MG1HZ, and MG2ZZ was 1.6 times that of MG2HZ, indicating that the low temperatures in Zhengzhou in January significantly ($p < 0.05$) promoted the accumulation of soluble sugars in MG1ZZ and MG2ZZ.

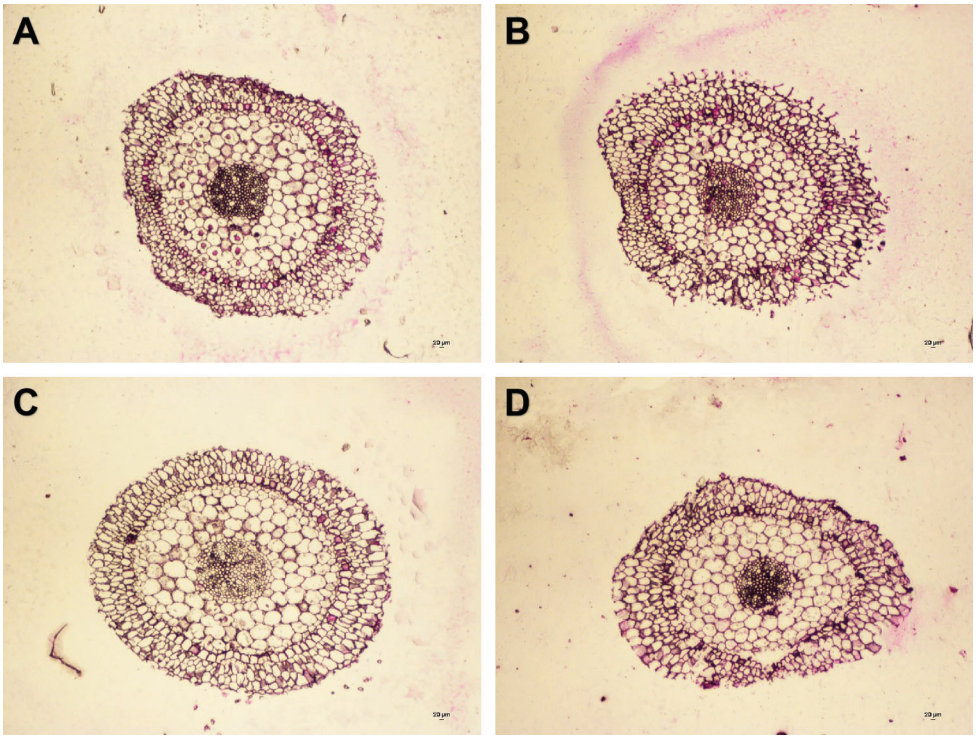

Figure 6: The root paraffin section maps of *D. officinale* (10 × 10). A: MG1HZ; B: MG2HZ; C: MG1ZZ; D: MG2ZZ. MG1HZ has 5–6 layers of root coat cells. The cells of MG1 in the phloem are larger, and the cells are neatly arranged. MG1 has more cell layers than MG2, and the cells are densely arranged

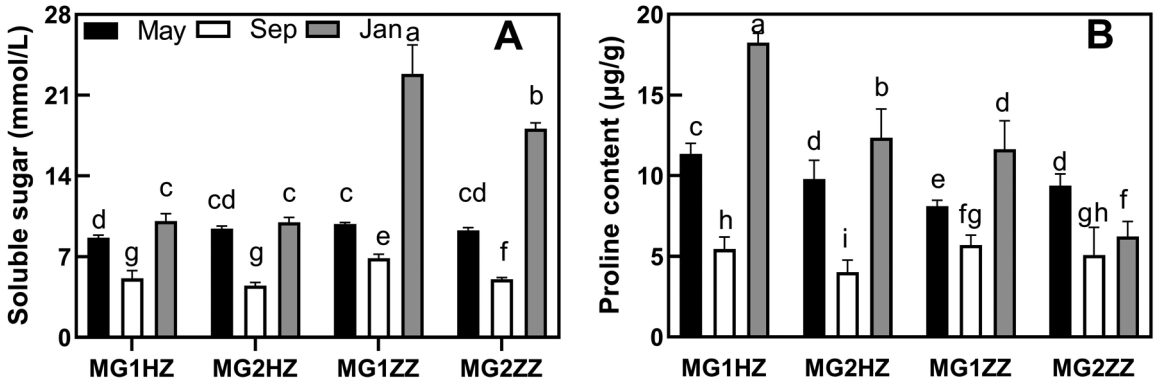

Figure 7: Content of soluble sugars and proline in the leaves of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). HZ: plants from Hangzhou; ZZ: plants from Zhengzhou

The highest proline content was obtained in January, followed by May and July (Fig. 7B). Comparing the two cultivars, MG1 showed higher proline content than MG2. Concerning the growth location, the proline content in Hangzhou was higher than that in Zhengzhou. The proline content of MG1HZ was 3 times that of MG1ZZ, and MG2HZ was 1.8 times that of MG2ZZ, indicating that the low temperature in Hangzhou in January significantly ($p < 0.05$) promoted the accumulation of proline in both MG1HZ and MG2HZ.

The activities of SOD, CAT and POD enzymes in the leaves of *D. officinale* were measured (Fig. 8) at various times. For SOD (Fig. 8A), the highest activity was found in September, followed by May, and much lower SOD activity was obtained in January. Comparing the two cultivars, no significant ($p > 0.05$) difference was found between MG1 and MG2, except that MG1 showed higher ($p < 0.05$) SOD activity than MG2 in May and September. The highest SOD content in September reached 362 U g^{-1} for MG1. Comparing the two districts, slightly higher ($p < 0.05$) SOD activity was found in samples from Hangzhou than in those from Zhengzhou.

For CAT (Fig. 8B), the highest activity was found in January, followed by September, while much lower CAT activity was obtained in May. Comparison of the two cultivars indicated that cultivar MG1 had higher ($p < 0.05$) activity than MG2. The highest CAT value was 324 U g^{-1} for MG1 from Zhengzhou. Comparing the two districts, higher ($p < 0.05$) CAT activity was found in the Zhengzhou samples compared to those from Hangzhou.

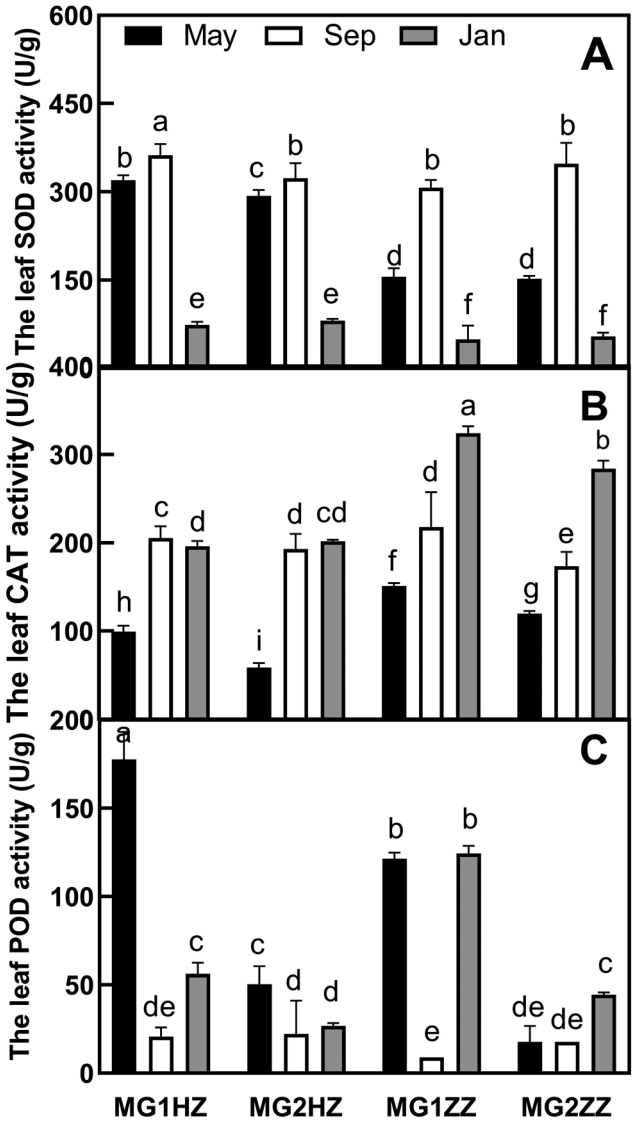

Figure 8: Activities of antioxidase enzymes in the leaves of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). HZ: plants from Hangzhou; ZZ: plants from Zhengzhou. A: SOD; B: CAT; C: POD

For POD (Fig. 8C), the highest activity was found in May, followed by January, while much lower POD activity was obtained in September. Comparing the two cultivars, cultivar MG1 had higher ($p < 0.05$) activity than MG2. The highest observed POD activity was 324 U g^{-1} for MG1 in Zhengzhou. Comparison of the two districts showed that higher ($p < 0.05$) POD activity was found in the samples from Zhengzhou compared to those from Hangzhou.

Polysaccharides, flavonoids and alkaloids are all important constituents of *D. officinale*. The contents of polysaccharides, alkaloids and flavonoids in the stems and leaves of *D. officinale* were measured in May, September and January (Fig. 9). The content of total polysaccharides in the stems was greater than that in the leaves. It can be seen from the figure (Figs. 9A, 9B) that the content of polysaccharides in the stems was the highest in January while the content of polysaccharides in the leaves was the highest in September. In January, the polysaccharides content of *D. officinale* increased significantly ($p < 0.05$), with MG1 increased by 18.85% and MG2 increased by 22.5% in the Hangzhou samples. As far as the cultivars are concerned, MG1 showed significantly ($p < 0.05$) higher content in both leaves and in stems. For the districts, much higher ($p < 0.05$) content was present in the Hangzhou samples than in those from Zhengzhou.

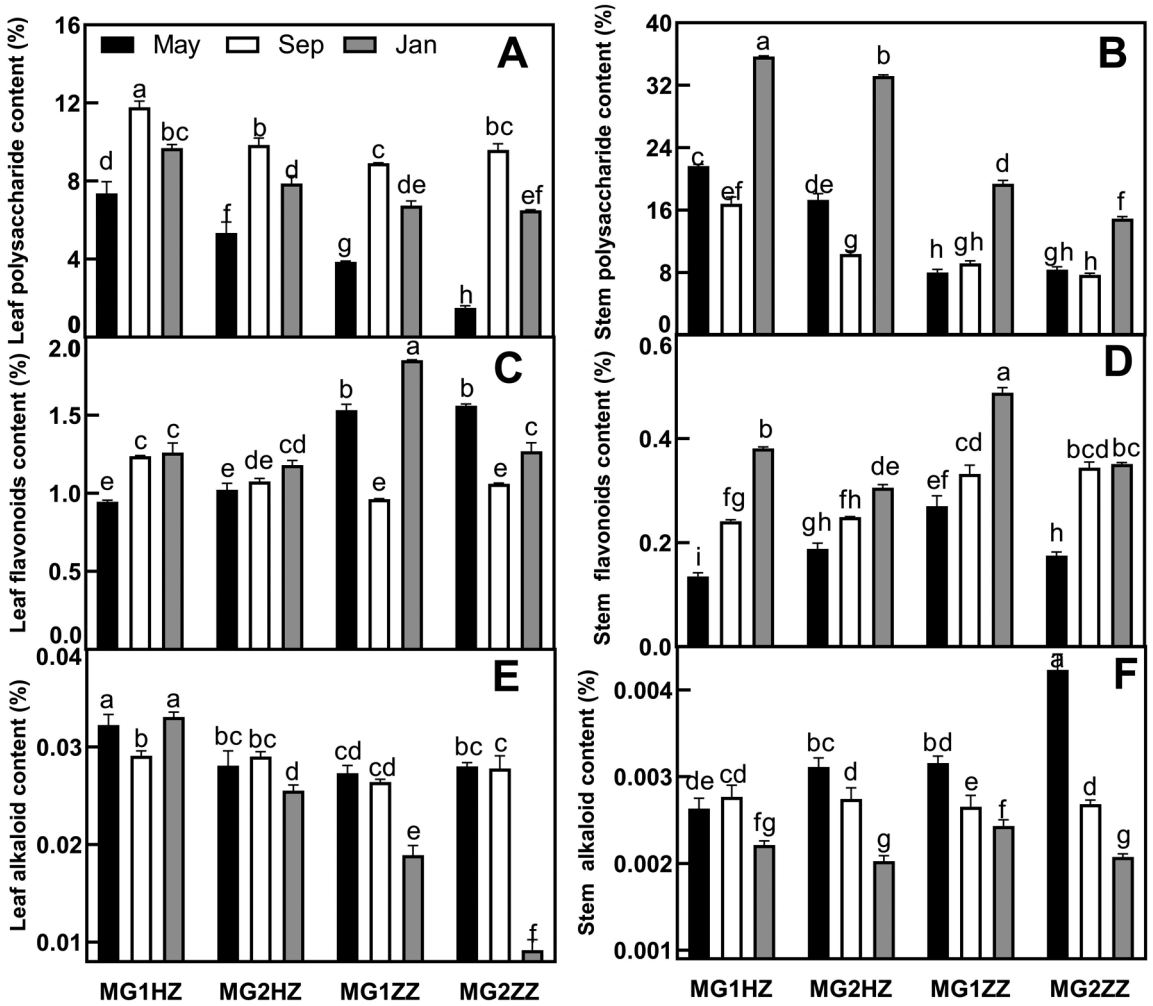

Figure 9: Contents of important constituents in the leaves and stems of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). A: Total Polysaccharides; B: Flavonoids; C: Alkaloids

As shown in Figs. 9C, 9D, flavonoids mainly existed in the leaves of *D. officinale*. The flavonoids level increased significantly ($p < 0.05$) from May to January in the leaves of the two cultivars. For comparison of the cultivars, MG1 had lower ($p < 0.05$) content of flavonoids than MG2. For the districts, the flavonoids content was higher ($p < 0.05$) in the Zhengzhou cultivar than in the one from Hangzhou. The highest content was found in MG1 from Zhengzhou, with 1.8% in leaves and 0.5% in stems.

As shown in Figs. 9E, 9F, the alkaloids of *D. officinale* also existed mainly in the leaves of *D. officinale*. The alkaloids level decreased continuously from May to January in the leaves of the two cultivars. In comparing the cultivars, MG1 had higher ($p < 0.05$) content of alkaloids in the stems than did MG2. For the districts, the alkaloids content was higher ($p < 0.05$) in the Zhengzhou samples than in the Hangzhou samples. The highest content was found in MG1 from Hangzhou, with 0.035% in the leaves.

3.2.3 Growth Indices of D. officinale Under Artificial Planting Conditions

The budding rate, plant height, stem diameter, and chlorophyll content of the different species of *D. officinale* in May, September and January were measured as growth indices. It was found that the budding rate, plant height, and stem diameter all increased significantly with the growth time, and that the cold tolerant strain was larger than the non-cold tolerant strain.

The budding rate significantly affected the yield of *D. officinale*. Fig. 10A shows that the budding rates of *D. officinale* planted in different areas had significant differences ($p < 0.05$). The budding rate of MG1ZZ was the highest (105%), followed by MG1HZ. Notably, the budding rate of MG1 was significantly ($p < 0.05$) higher than that of MG2. The budding rate of *D. officinale* in Zhengzhou was higher than that in Hangzhou.

May to July was the main vigorous growth period of *D. officinale*, and from September to November was also a vigorous growth period. As can be seen from Fig. 10B, the plant height of *D. officinale* significantly ($p < 0.05$) increased in each growth period. The growth of the two cultivars in May was relatively close in the two regions, while the MG1HZ growth was significantly ($p < 0.05$) higher than that from Zhengzhou. In September, the highest plant height was found in MG1HZ, followed by MG2HZ, MG1ZZ and MG2ZZ. Similar results were obtained for the plants in January. The plant height of *D. officinale* was noticeably higher in MG1 than in MG2 in Hangzhou but not in Zhengzhou.

In Fig. 10C, the stem diameter of *D. officinale* was significantly ($p < 0.05$) increased in each growth stage. Overall, both MG2ZZ and MG1HZ had approximately the largest stem diameters in different months, followed by MG1ZZ and MG2HZ.

In Fig. 11, the chlorophyll content of the cold tolerant strains in Hangzhou was the highest, reaching 42.41 mg g^{-1}, and the chlorophyll content did not change significantly ($p > 0.05$) with the extension of the growing season. In May, MG1ZZ had the lowest chlorophyll content, which was 26.93 mg g^{-1}. From May to September, the chlorophyll content of *D. officinale* in general reached the highest level. After the temperature decreased in September, the chlorophyll content in all the lines except MG1HZ decreased significantly ($p < 0.05$), and the decrease rates were similar.

4 Discussion

Low temperature, one of the important abiotic stresses, not only affects the growth, development, yield and quality of plants, but also limits the geographical distribution of many wild plants [31,32]. *Orchidaceae* plants live mainly in tropical and subtropical regions with high temperature and humidity, and their cold tolerance is weak [33]. Therefore, low temperature is the main factor restricting the growth of orchid plants. Low temperature usually promotes overproduction of ROS, which induce oxidation of lipids in membranes to produce MDA. Therefore, the integrity of cell membranes is damaged, thus REC increases [34,35]. In this study, *D. officinale* cultivar MG1 showed higher cold tolerance than MG2 based on corresponding MDA and REC values. Cultivar MG1 showed higher cold tolerance, with greater leaf

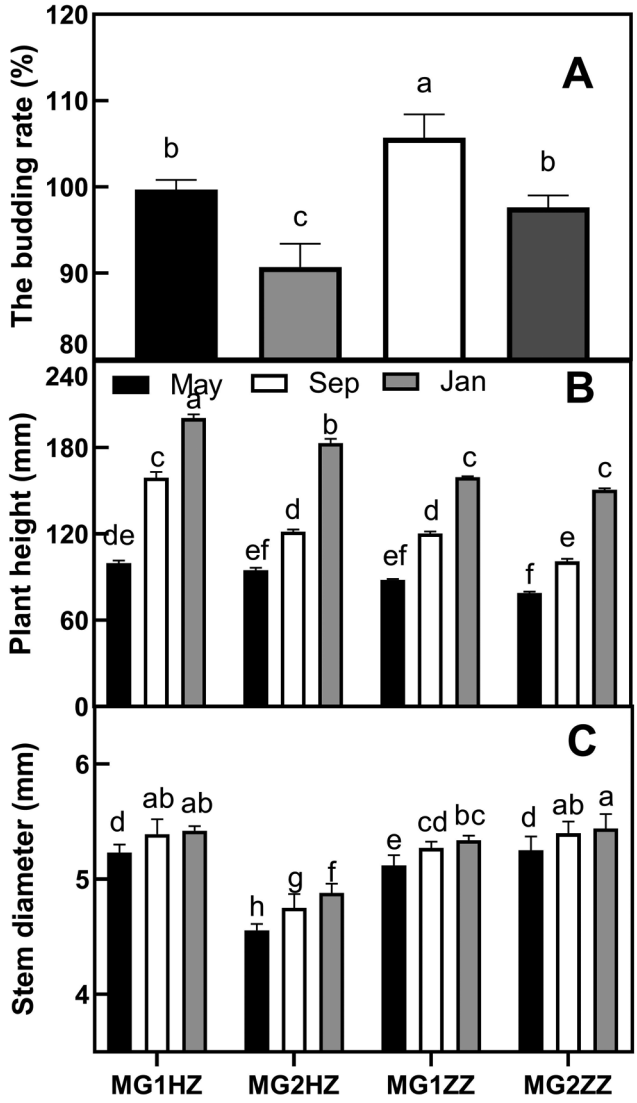

Figure 10: The growth indices of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). A (budding ratio); B (plant height); C (stem diameter). HZ: plants in Hangzhou; ZZ: plants in Zhengzhou

thickness and lower stoma density, higher contents of proline, POD, total polysaccharides and flavonoids and greater budding rates. In Zhengzhou, MG1 had higher soluble sugars and CAT than MG2, while in Hangzhou, MG1 showed higher plant height, stem diameter, and chlorophyll content than MG2. Therefore, MG1 is, overall, a cold tolerant cultivar with better cold tolerance and growth properties than MG2, which allows it to grow in cold districts, even in some places in northern China.

As a cold-tolerant cultivar, MG1 has some unique characteristics or properties, such as microstructure, content of osmolytes, antioxidant enzymes, and other important constituents. Regulation of structure is an important way for plants to adapt to low temperature. Low stoma density, thick palisade tissues and tightly packed cells [36] are shared by cold-resistant walnut varieties and cold-tolerant sweet potato genotypes, which help them reduce transpiration to resist cold and thus achieve self-protection [37]. Increased leaf thickness and mesophyll cell volume were observed for *Arabidopsis* after being moved

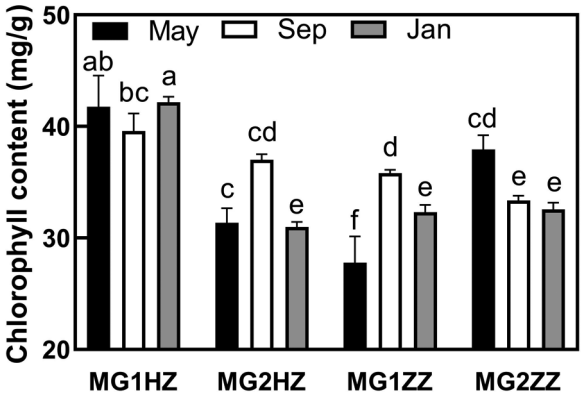

Figure 11: The content of chlorophyll of *D. officinale* planted in different districts. Different letters represent significant differences ($p < 0.05$). HZ: plants from Hangzhou; ZZ: plants from Zhengzhou

from 20°C to 5°C for 2 months [38], indicating that low temperature induced the plant to regulate its structure to adapt to the changed environment. It is apparent that cold tolerant *D. officinale* MG1 holds the same inherent structural strategy to resist cold.

Proline and soluble sugars are important osmolytes. Genes related to osmotic regulation in plants participate in the expression and increasing content of various osmolytes imparts certain plants with a variety of osmotic regulation capabilities, thereby improving their cold tolerance [39]. Under low temperature stress, the degradation of starch and other macromolecular substances in plants accelerates, which promotes the accumulation of soluble sugar content and the increase of cytosol concentration. Therefore, water potential and freezing point decrease, which is important for protecting sensitive proteins and thus maintaining membrane integrity [40]. Proline (Pro) is widely present in plants, and mainly functions as an osmolyte, a protective substance for protein molecules, and a scavenger for active oxygen, thereby playing a cold-resistant role [41]. Mu found that under low temperature stress, *Phalaenopsis*, a family of orchids increased cold tolerance by increasing their contents of proline and soluble sugars [42]. Jan found that after incubating *C. offcinalis* in a room at 25°C and then moving it to low temperature stress at 4°C, the proline and soluble sugar content gradually increased with time [43]. For the cold tolerant cultivar MG1 of *D. officinale*, proline and soluble sugars are the major osmolytes. The significantly increased content of proline in the samples from Zhengzhou indicates that proline plays a great role in cold tolerance under low temperature in the north of China.

Low temperature promotes overproduction of ROS through the breaking down of normal metabolic and photosynthetic processes [44]. SOD, POD and CAT are very important antioxidant enzymes [45]. SOD, the first enzyme involved in antioxidant activity, functions as a scavenger of superoxide anion O_2^- to convert it into H_2O_2 [46]. POD and CAT degrade excess H_2O_2 by enzymatic action to prevent plants from being damaged by peroxidation, and POD is also related to lignin synthesis and cell resistance [1]. Under low temperature stress, SOD, CAT and POD all increase rapidly and maintain high, stabilized activity [47]. For *D. officinale* in the present study, higher CAT and POD activities were found in the plants from Zhengzhou than in those from Hangzhou, which indicates that these two antioxidases play significant roles in cold tolerance.

Polysaccharides, flavonoids and alkaloids are all important components of *D. officinale*. As important active substances, polysaccharides, flavonoids and alkaloids achieve anti-oxidation by eliminating free radicals, and thus play roles in cold tolerance [48–50]. They are usually induced under stresses, such as low temperature, desiccation, and UV radiation. Low temperature was found to induce accumulation of polysaccharides of leaf and stem in rapeseed [15] and *Cyclocarya paliurus* [51]. Accumulation of

flavonoids and alkaloids was also observed in spinach [52] and in hairy roots [53]. In this study, cultivar MG1 presented higher levels of polysaccharides, flavonoids and alkaloids, which might endow MG1 with cold tolerance. When comparing plants from the two districts, the contents of polysaccharides and alkaloids were higher in Hangzhou samples than in those from Zhengzhou. However, the content of flavonoids was higher in plants from Zhengzhou than in those from Hangzhou, indicating that flavonoids might be a major antioxidant helping *D. officinale* adapt to low temperature.

In conclusion, MG1 exhibits better cold tolerance than MG2, with better survival characteristics, and lower REC and MDA production under low temperature. The cultivar MG1 of *D. officinale* is a cold tolerant cultivar with thicker leaves and reduced stomatal density, and higher contents of soluble sugars, proline, CAT, POD, polysaccharides, flavonoids and alkaloids, which allow it adapt to low temperature, including some places in northern China. Therefore, it is anticipated that the cultivar MG1 can be suitably planted on a large scale in relatively cold regions like Zhengzhou, having a certain natural degree of protection from low temperature damage, thereby expanding its cultivation area to more northerly regions.

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