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Full Length Research Paper

Growth, physiological characteristics and total flavonoid content of *Glechoma longituba* in response to water stress

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The dried over-ground parts of the plant *Glechoma longituba* are one of the important ingredients used in traditional Chinese medicine (TCM). In this study, five different watering treatments, namely 95 to100% (T1), 80 to 85% (T2), 65 to 70% (T3), 50 to 55% (T4) and 35 to 40% (T5) of field capacity were used to investigate the effects of water deficiency on the growth, physiological characteristics and total flavonoid content of *G. longituba.* The study found that epidermis, palisade tissue, spongy tissue and vascular cylinder thickness peaked in the T2 treatment and then decreased in the other treatments in anatomy structure of leaf. And anatomical structure analysis of the stem revealed that the thickness of the cortex and the vascular cylinder were the highest in the T2 treatment, too. Water stress increased the thickness of the vessel walls in the xylem and the size of the parenchyma cells in the cortex, whereas it decreased the size of the parenchyma cells in the pith. As the water stress intensified, the resulting changes found in physiological indices suggested that water stress stimulated the level of redox activity. In addition, the yield of total flavonoids in the T2 treatment was higher than in the other treatments. Our results suggested that 80 to 85% of field capacity is the most suitable watering treatment for the growth of *G. longituba*. This procedure would be suitable for the domestication of *G. longituba.*

Key words: *Glechoma longituba*, water treatment, physiological characteristics, total flavonoids.

INTRODUCTION

Glechoma longituba (Nakai) Kupr. is a labiate perennial plant distributed mainly in the temperate zone of Asia (Flora of China Editorial Committee, 1977). Its dried aerial part is widely used in TCM in China. The medicines made from the plant exhibit important pharmacological effects, such as effective antipyretic, diuretic and choleretic functions for jaundice. They alleviate blood stasis and relieve swelling and are used to treat stranguria caused by urinary stones and heat, damp-heat jaundice, swollen sore furuncle carbuncle, and traumatic injury as well as other symptoms (China Pharmacopoeia Committee, 2010; Tao et al., 2004). Owing to the plant's pharmacological importance, at least two Chinese patent medicines, including Lithagogue Granules (Paishi-Keli in Chinese) and Cholagogue Capsule (Danle-Jiaonang in Chinese), contain components extracted from *G. longituba*. These medicines are used to treat urinary tract stones and cholelithiasis, respectively (Ni et al., 2010; Zheng, 1996). The major active ingredients of *G. longituba* are known to be include the total flavonoids of the plant. These flavonoids have been reported to exhibit

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Abbreviations: TCM, Traditional Chinese medicine; **MDA,** malondialdehyde; **TBA,** 2-thiobarbituric acid; **TCA,** trichloroacetic acid; **PAL,** phenylalanine ammonia-lyase.

a wide range of biological effects, including antiviral, anti-inflammatory, antibacterial, vasodilatory and antiallergic actions (Cook and Samman, 1996). Moreover, the content of total flavonoids is regarded as the important criterion for determining the quality of *G. longituba* (Cheng et al., 2005). Owing to the plant's high medicinal and economic value, the demand for *G. longituba* has increased steadily in recent years. However, the amount of wild *G. longituba* available is insufficient to meet the market demand. To increase the production of high-quality *G. longituba*, efforts should be made to develop optimal agrotechnologies for the plant's commercial cultivation. It is thus important to understand the mechanism by which the environment affects the plant's growth, physiological characteristics and total flavonoid content. Accumulation of secondary metabolites is known to be a defense mechanism of plants. This mechanism helps plants respond to and adapt to environmental stresses (Jwa et al., 2006; Kirakosyan et al., 2004). Indeed, biotic and abiotic stresses exert considerable influences on the levels of secondary metabolites in plants (Kirakosyan et al., 2004; Zhao et al., 2005). Water stress is one of the most important environmental factors that can regulate plant growth and development, limit plant production, and alter the physiological and biochemical properties of plants. Indeed, water stress is known to increase the amount of secondary metabolites in plants (Zobayed et al., 2007).

However, excessive water loss did not favor the accumulation of secondary metabolites in medicinal plants (Shang, 2003; Tan et al., 2008). In *Isatis indigotica*, extreme water stress has been found to reduce the production of indirubin. However, superior yield and quality could both be obtained at 45 to 70% of field capacity (Tan et al., 2008). A lot of investigations have showed that water stress increased secondary metabolite accumulation in medical plants, such as *Salvia miltiorrhiza* (Liu et al., 2011), *Bupleuri radix* (Zhu et al., 2009), *Catharanthus roseus* (Abdul et al., 2007), *Rehmannia glutinosa* (Gaertn.) (Chung et al., 2006). Appropriate water level in the environment is therefore vital for obtaining higher yield and quality in medicinal plants. In addition to the accumulation of certain secondary metabolites, plants have developed a wide diversity of mechanisms for morphological and physiological drought tolerance. However, though a number of recent studies have addressed the effects of abiotic stress treatments on the accumulation of secondary metabolites and on morphological and physiological variation, little information is available on the effects of environmental stress treatments on the production of total flavonoids and on morphological and physiological variation in *G. longituba*. Therefore, the current study therefore sought to investigate the effects of water deficiency on the plant growth, physiological characteristics and total flavonoid content of *G. longituba.* The objective of the current study was to increase the total flavonoid content of the experimental plants and to correlate the observed changes in this characteristic with the changes observed in the growth indices, malondialdehyde (MDA), proline, soluble sugars, and chlorophyll content of the plants, as well as in the anatomical structure of the stems and leaves of *G. longituba* under conditions of water deficiency.

MATERIALS AND METHODS

Plant materials and treatments

G. longituba was obtained from the Chinese Traditional Medicine Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. In May 2008, *G. longituba* clonal fragments with two oppositifolious leaves were transplanted into plastic pots (20.5 cm in upper diameter, 4.7 cm in lower diameter and 7.8 cm in height) filled with fertile sandy loam soil and were maintained for approximately one month in a growth chamber. After their recovery from transplantation, uniform and healthy seedlings were chosen, cultivated and watered daily in the greenhouse (25±5ºC day/night, maximum illumination 1000 to 1200 μmol $m^{-2}s^{-1}$, relative humidity of approximately 70%). The field capacity of the soil used in this experiment was 23.65%. The experiment used five watering treatments, namely 95 to 100% (T1), 80 to 85% (T2), 65 to 70% (T3), 50 to 55% (T4) and 35 to 40% (T5) of field capacity. The physiological parameters of the plants were determined in July 2008, and the experiment was conducted over a period of approximately five months. All treatments were arranged in a completely randomized block design with five replicates.

Growth

Five plants per treatment were harvested, weighed and dried in an oven at 60℃ for 48 h to determine the initial aerial biomass at the beginning of the experiment. The final aerial biomass was also determined at the end of the experiment (December 2008). The length of the main stems and the leaves in each treatment were both determined subsequent to treatment.

Tissue anatomy

At the end of each treatment, samples were taken from the stem (the mature stem) and leaf (the fully expanded leaf), cut transversely into approximately 0.5 cm segments, and fixed in 70% alcohol-formalin-glacial acetic acid (V/V/V = 90/5/5) for at least 24 h. The material was then dehydrated by passing through an ethanol series, cleared in xylene and embedded in paraffin wax (melting point: 52 to 56℃). Transverse 8 μm thick sections were obtained using a rotary microtome (RM2016, China), double stained with safranin solution (safranin: 70% ethanol, W/V: 1 g/100 ml) and Fast Green solution (Fast Green: 100% ethanol, W/V: 0.5 g/100 ml), mounted in Canada balsam and photographed with an Olympus Bx40 microscope (Olympus Optical, Tokyo, Japan).

Measurements of MDA (malondialdehyde) content

Malondialdehyde (MDA) was measured as an indicator of lipid peroxidation according to the method of Cakmak and Horst (1991). The levels of lipid peroxidation in leaves were determined as 2-thiobarbituric acid (TBA) reactive metabolites, chiefly MDA. Fresh leaf tissue (approximately 0.3 g) was homogenized and extracted in

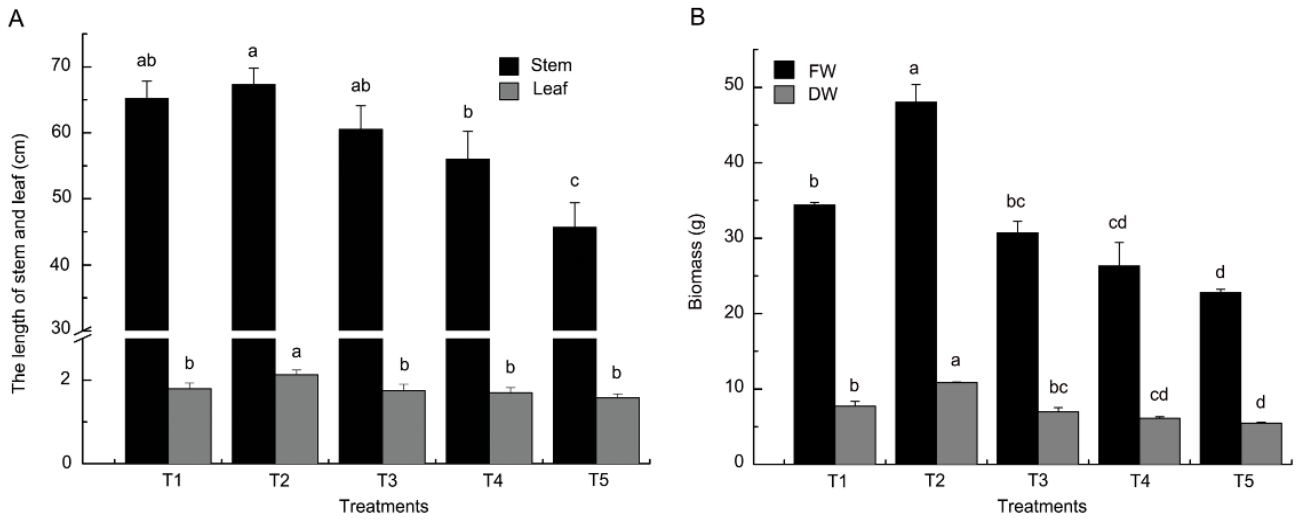

Figure 1. The effects of different water treatments on the length of stem and leaf (A), biomass (B) in *Glechoma longituba.* FW: Fresh weight, DW: Dry weight. T1, T2, T3, T4 and T5 meant 95 to100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

10 ml 0.25% (w/v) TBA dissolved in 10% (w/v) trichloroacetic acid (TCA). The extract was heated at 95°C for 30 min and then cooled quickly on ice. After centrifugation at 10,000×g for 10 min, the absorbance of the supernatant was measured at 532 nm. A correction for non-specific turbidity was obtained by subtracting the absorbance value taken at 600 nm. All spectrophotometric measurements were conducted on a 756 CRT type ultraviolet-visible spectrophotometer (Shanghai Precision Scientific Instrument Co., Limited).

Determination of chlorophyll, proline, soluble sugars and total flavonoid content

Chlorophyll content was determined according to Arnon (1949). A 0.5 g sample of fresh leaf was incubated in 10 ml of 80% acetone in the dark for 48 h at room temperature (approximately 25°C). The absorbance of the extract was measured at 470 nm, 645 nm and 663 nm. Proline concentration was determined according to the method of Bates et al. (1973). Proline was extracted from approximately 0.5 g of leaf sample and homogenized in 10 ml of 3% sulfosalicylic acid. A total of 2 ml of extract was allowed to react with 2 ml acidninhydrin and 2 ml of glacial acetic acid for 75 min at 100°C. The reaction was then terminated by using an ice bath. The reaction mixture was extracted with 4 ml of toluene and vortexed. Absorbance of the toluene layer was read in a spectrophotometer at 520 nm. Soluble sugars were determined by the anthrone method according to Spiro (1966). A 100 ml leaf extract was added to 3 ml extraction buffer containing 1.08 M H_2SO_4, 1.09 mM thiourea and 2.1 mM anthrone. The mixture was heated at 100ºC for 10 min. Its absorbance was then read at 620 nm. Total flavonoids were extracted from dried and powdered material using the aluminum chloride colorimetric method (Jia et al., 1999) with a slight modification. Samples of approximately 1.0 g of powder were subjected to extraction for 1 h at 90ºC using 50 ml of ethanol/water (40:100, v/v) as an extraction solvent.

Then, 3 ml of extract (20 mg/ml) was added to a 10 ml volumetric flask, brought to volume. A total of 4 ml was then added to the 10 ml volumetric flask. This addition was followed by the addition of 0.3 ml of a 5% aqueous solution of sodium nitrite. The contents of the bottle were mixed thoroughly. After 6 min, 0.3 ml of a 10% aqueous solution of aluminum chloride was added, and the mixture was shaken and allowed to stand for 6 min. Next, 4 ml of a 4% aqueous solution of sodium hydroxide was added to the bottle, and the mixture was shaken up and allowed to stand for 15 min at room temperature (25ºC). Following the reaction period, absorbance of the samples were measured at 510 nm using the ultraviolet-visible spectrometer with solvent as a blank for the extracts. The same procedure was repeated for all standard lutin solutions at different concentrations (0, 0.206, 0.412, 0.618, 0.824 and 1.030 mg/ml). The data were expressed as the average of three measurements.

Statistical analyses

To assess the statistical significance of the treatment differences, a one-way analysis of variance (ANOVA) was conducted using SPSS 16.0 software. The least significant difference test ($p < 0.05$) was performed. The data were expressed as means ± standard errors.

RESULTS

The growth variables of *G. longituba*

In order to investigate the effect of soil water content on the growth of *G. longituba,* we examined the growth of the plants in five different water treatments, as stated above (Materials and Methods). The results showed that different water treatments had definite effects on the stem and leaf lengths of *G. longituba* (Figure 1A). The leaf length in the T2 treatment was significantly higher than in the other treatments, whereas the T1, T3, T4 and T5 treatments exhibited no significant differences in leaf

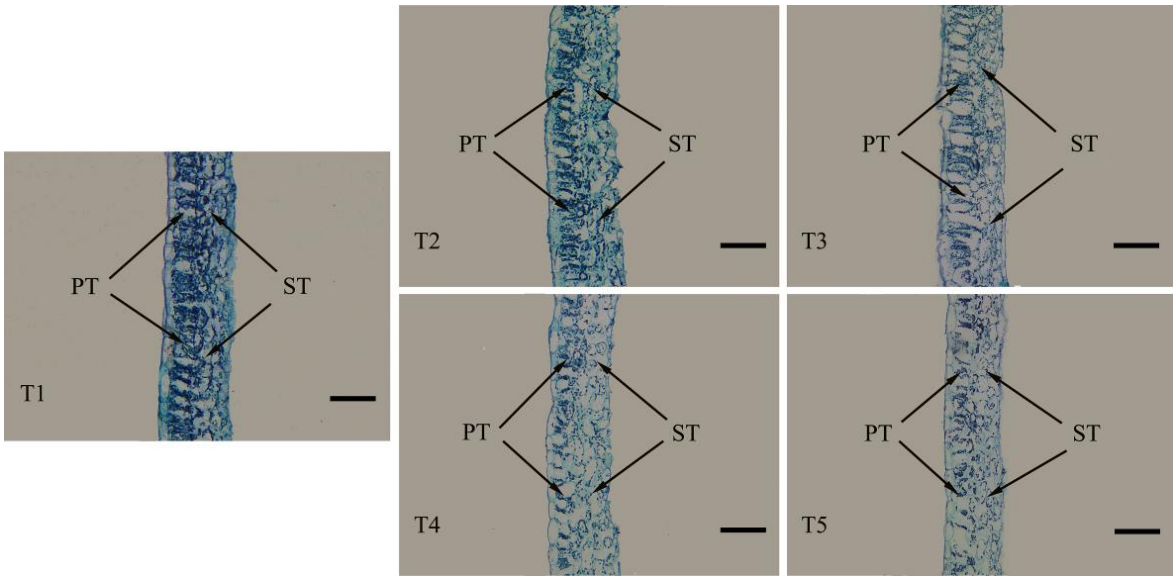

Figure 2. Transverse leaf sections of *Glechoma longituba* under different water treatments. PT: palisade tissue; ST: spongy tissue; Scale bars=100 μm. T1, T2, T3, T4 and T5 meant 95 to100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

Table 1. Effects of different water treatments on anatomy structure of *Glechoma longituba* leaf.

Treatments	Leaf thickness (μm)	Upper epidermis thickness (μm)	Palisade tissue thickness (μm)	Spongy tissues thickness (μm)	Lower epidermis thickness (μm)
T1	185.70±1.50^{b}	20.39±0.25^{b}	85.32±1.20^{b}	64.96 ±1.29^{b}	17.98±0.29^{b}
T2	196.33±2.86^{a}	22.34±0.73^{a}	90.64±1.73^{a}	72.61±2.66^{a}	19.03±0.50^{a}
T3	163.20±1.23^{c}	17.45±0.24^{c}	70.56±0.98^{c}	55.98 ±0.88^{c}	16.51±0.24^{c}
T4	151.55±1.08^{d}	16.73±0.25^{c}	62.09±0.85^{d}	50.08±0.78^{d}	15.14±0.36^{d}
T5	130.89±1.34^{e}	14.38±0.34^{d}	55.81±0.84^{e}	42.69±1.09^{e}	13.71±0.29^{e}

Different letters indicated significant differences at $P < 0.05$ by the least significant difference (LSD) (n=15). T1, T2, T3, T4 and T5 meant 95-100%, 80-85%, 65 to70%, 50 to 55% and 35 to 40% of field capacity, respectively.

length. The greatest stem length was observed in the T2 treatment and was significantly higher than the corresponding values for the T4 and T5 treatments. The highest accumulation of biomass was found in the T2 treatment, and the next highest value was observed in the T1 treatment (Figure 1B). The T5 treatment exhibited the lowest value of biomass. Similar results were obtained from the data of fresh weight.

Morpho-anatomical response to different water treatments

Cross-sections of leaves of *G. longituba* are of the dicotyledonous type (Figure 2) are consisted of epidermis and mesophyll tissue. The palisade mesophyll is composed of exactly one layer. The spongy tissue mesophyll cells were characterized by many intercellular spaces. As the water deficit increased, the resulting water stress induced marked changes in the characteristics of the leaf anatomy. Compared to the T1 treatment, the T2 treatment produced a distinct increase in the thickness of epidermis, palisade tissue and spongy tissue as well as in leaf thickness, but these characteristics decreased in the T3, T4 and T5 treatments (Table 1). The shape of the palisade tissue in the T4 and T5 treatments resembled a short pillar rather than a long pillar. The chloroplasts of the palisade tissue and spongy tissue gradually decreased in volume and number in the leaves across the T1 to T5 treatments. The cells, especially the green cells of the palisade tissue, were relatively less densely arranged compared to the corresponding characteristics of the leaves in the T1 treatment. The amount and the magnitude of intercellular spaces of the palisade tissue in water-stressed leaves were relatively larger than the corresponding values in the leaves of the T1 treatment. In

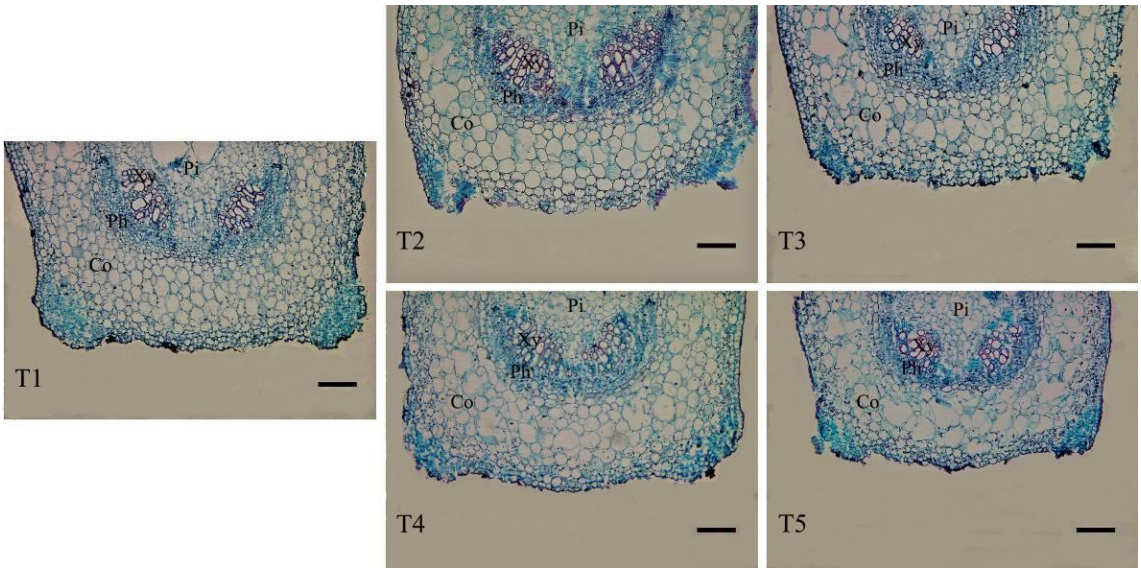

Figure 3. Transverse stem sections of *Glechoma longituba* under different water treatments. Co: cortex; Ph: phloem; Xy: xylem; Pi: pith. Scale bars=100 µm for stems. T1, T2, T3, T4 and T5 meant 95 to100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

addition, larger gas chambers formed in the spongy tissue. The stem of *G. longituba* is composed of epidermis, a ring of sclerenchyma cells, exodermis, stem cortex and pith, and all characteristics of the dicotyledonous type (Figure 3). As shown in Figure 3, the size of the parenchyma cells of the cortex in the T2, T3, T4 and T5 treatments increased gradually and was larger than that observed in the T1 treatment. The vessels of the xylem were well developed in the T2 treatment, but they were poorly developed and the number of vessel in the xylem was decreased in the T1, T3, T4 and T5 treatments. Moreover, the parenchyma cells of the pith in T2, T3, T4 and T5 showed greater compactness and decreased vacuolation compared to T1.

The thickness of the cortex, vascular cylinder, phloem, and xylem and the diameter of the pith and the vessel increased in the T2 treatment, but they decreased in the T3, T4 and T5 treatments (Table 2). The thickness of the vessel increased from T2 to T5 in response to the increase of water stress (Table 2).

MDA content of the leaves

MDA is one of the main products of membrane lipid peroxidation. Its content can be used as an index of the degree of membrane lipid peroxidation. Table 3 shows that as water stress increased, the MDA content of the leaves of *G. longituba* increased gradually and the rate of increase became greater. This finding indicates that membrane lipid peroxidation increased gradually as water stress increased. The highest MDA content was observed in the T5 treatment. This value differed significantly from those found in the other treatments. It was approximately 100% greater than the value observed in the T1 treatment.

Proline content in the leaves

Table 3 shows that the accumulation of proline varied with water stress. The proline content in the leaves of *G. longituba* gradually increased as the drought stress intensified. The proline content of the leaves in the T2, T3, T4 and T5 treatments was significantly higher than that observed in the T1 treatment. This variable increased by 17.43, 48.35, 89.86 and 133.60%, respectively, compared to that observed in the T1 treatment.

Soluble sugar content in the leaves

The change in the soluble sugar content in the leaves under the different treatments is shown in Table 3. The soluble sugar content ranged from 30.83 to 58.25% as the water stress gradually intensified. These results show that the soluble sugar concentrations in the leaves increased significantly as the soil water content declined. Compared to T1, the increased amount of soluble sugars in T5 was higher than that found in the other treatments. These results indicate that *G. longituba* has the ability to adapt to water stress through osmoregulation.

Chlorophyll content of the leaves

As the degree of drought stress increased, the content of

Table 2. Effects of different water treatments on anatomy structure of *Glechoma longituba* stem.

Treatments	Stem thickness (mm)	Cortex thickness (μm)	Vascular cylinder thickness (μm)	Phloem thickness (μm)	Xylem thickness (μm)	Pith diameter (μm)	Vessel diameter (μm)	Thickness of vessel wall (μm)
T1	1.35±0.04^{b}	290.87±5.17^{b}	750.30±11.37^{b}	90.45± 4.31^{b}	149.30±4.25^{b}	381.73±5.85^{b}	36.15±1.03^{b}	2.93± 0.28^{e}
T2	1.44±0.03^{a}	300.66±5.13^{a}	819.05±16.05^{a}	97.96± 3.30^{a}	162.27±4.82^{a}	427.80±5.46^{a}	40.65±2.70^{a}	3.23±0.33^{d}
T3	1.28±0.03^{c}	282.58±4.99^{c}	689.49±14.21^{c}	85.78± 3.71^{c}	138.35±3.00^{c}	341.49±5.13^{c}	33.50±1.68^{c}	3.60±0.39^{c}
T4	1.25±0.02^{c}	279.31±4.72^{c}	670.86±10.49^{d}	83.12±2.75^{c}	135.50±4.51^{c}	330.02±3.09^{d}	31.61±1.79^{d}	4.09±0.26^{b}
T5	1.17±0.02^{d}	260.62±5.29^{d}	630.68±5.43^{e}	76.22±3.49^{d}	122.82±3.08^{d}	307.56±4.35^{e}	27.60±2.69^{e}	4.61±0.39^{a}

Different letters indicated significant differences at *P < 0.05* by the least significant difference (LSD) (n=15). T1, T2, T3, T4 and T5 meant 95 to 100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

Table 3. Effects of different water treatments on MDA, proline and soluble sugar content.

Treatments	MDA umol·g^{-1} (FW)	Proline ug·g^{-1} (FW)	Soluble sugar % (FW)
T1	4.00±0.09^{d}	18.84±0.57^{d}	30.83±0.34^{e}
T2	4.70±0.07^{c}	22.05±1.25^{c}	37.36±0.29^{d}
T3	5.42±0.03^{b}	27.95±1.14^{b}	43.53±0.29^{c}
T4	5.77±0.04^{b}	35.77±1.42^{b}	46.03±1.25^{b}
T5	8.03±0.04^{a}	44.01±7.37^{a}	58.25±1.06^{a}

Different letters indicated significant differences at P < 0.05 by the least significant difference (LSD) (n=3), T1, T2, T3, T4 and T5 meant 95 to 100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

Table 4. Chlorophyll content in the leaves of *Glechoma longituba* under different water conditions in mg. g^{-1}.Fw.

Treatments	Chlorophyll a	Chlorophyll b	Chlorophyll a/ Chlorophyll b	Total Chlorophyll	Carotenoid	Carotenoid/Chlorophyl
T1	1.04±0.00^{a}	0.32±0.00^{a}	3.22±0.03^{ab}	1.36±0.00^{a}	0.32±0.00^{a}	0.23±0.00^{a}
T2	0.91±0.00^{b}	0.27±0.00^{b}	3.36±0.03^{a}	1.18±0.00^{b}	0.22±0.01^{b}	0.19±0.01^{c}
T3	0.86±0.01^{c}	0.29±0.01^{b}	2.98±0.09^{bc}	1.15±0.00^{bc}	0.24±0.00^{b}	0.21±0.00^{b}
T4	0.83±0.01^{d}	0.30±0.01^{ab}	2.77±0.13^{cd}	1.13±0.02^{c}	0.23±0.01^{b}	0.20±0.01^{bc}
T5	0.60±0.00^{e}	0.23±0.02^{c}	2.64±0.17^{d}	0.83±0.01^{d}	0.15±0.01^{c}	0.18±0.01^{c}

Different letters indicated significant differences at *P < 0.05* by the least significant difference (LSD) (n=3). T1, T2, T3, T4 and T5 meant 95 to 100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

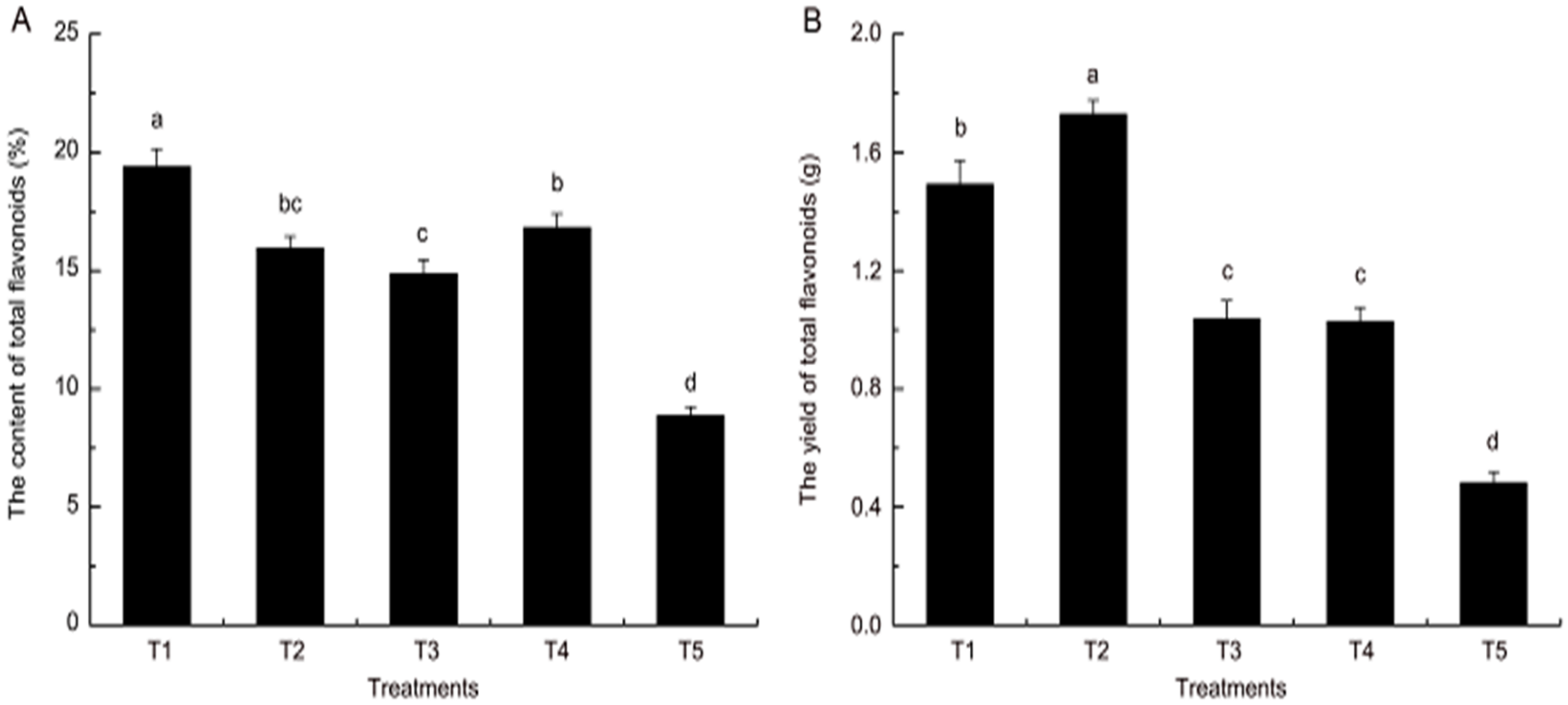

Figure 4. The content of total flavonoids (A) and the yield of total flavonoids (B) in *Glechoma longituba* under different water treatments (n=3). T1, T2, T3, T4 and T5 meant 95 to 100%, 80 to 85%, 65 to 70%, 50 to 55% and 35 to 40% of field capacity, respectively.

chlorophyll a significantly decreased (Table 4). The contents of chlorophyll b and carotenoids showed an initial decline across treatments, then rose, and finally followed a downward trend, reaching a very low level in the T5 treatment compared to the T1 treatment. Under the conditions of water deficit, the chlorophyll a/b ratio first increased, and then decreased. This result showed that the decomposition rate of chlorophyll a was quicker than that of chlorophyll b. Water stress led to the decrease of the carotenoid: chlorophyll ratio.

The content and the yield of total flavonoids in *G. longituba*

As shown in Figure 4, the content of total flavonoids in *G. longituba* reached its highest value at 95 to 100% (T1) of field capacity. No great differences were observed among the treatments in which soil water content ranged from 50 to 85% of field capacity (T2, T3 and T4). If the soil water content was lower than 50% (T5) of field capacity, the content of total flavonoids decreased drastically. The yield of total flavonoids in *G. longituba* was calculated based on the biomass (DW) and the content of total flavonoids. The results of this calculation showed that the yield of total flavonoids in T2 was the highest, the

yield in T1 was the next highest, and the yield in T5 was the lowest.

DISCUSSION

Growth responses to different water treatments

Soil water potential is a critical factor in the growth and development of land plants, and the water content of the soil has a great impact on plant growth. Under different levels of water treatments, *G. longituba* showed different growth patterns: the stem and leaf lengths in T2 was the greatest, and their stems were the strongest. In T5, the plants had short and fine stems and exhibited the worst growth, with the smallest leaves and some yellowing. Water deficiency and excess water both caused the growth of *G. longituba* to decrease. This result is consistent with previous findings for other water-stressed plants (Dai et al., 2007). These results indicate that excessive drought may be a serious obstacle to the normal growth of this plant and that 80 to 85% (T2) of field capacity represents a suitable condition for the growth of *G. longituba*.

The physiological response to water stress

The long-term evolution of plants has produced mechanisms for adaptation to adversity. These mechanisms are subject to hereditary constraints. Reactive oxygen metabolism plays an important role in the primary response to environmental stress. Free radicals will accumulate in plant cells if the balance between the production and the elimination of these free radicals is destroyed by stressful conditions that trigger or exacerbate the peroxidation of the lipids of the cell membrane (Asada, 2006). MDA is one of the main products involved in membrane lipid peroxidation. Its accumulation responds to the degree of membrane lipid peroxidation.

As shown in Table 3, a pronounced lipid peroxidation was detected in the T2, T3, T4 and T5 treatments, and the MDA content in the T5 treatment doubled compared to the T1 treatment. This result demonstrates the inadequacy of antioxidant defenses for combating AOS-mediated damage. Proline is an important organic osmolyte that accumulates in a variety of plant species in response to environmental stress, such as drought (Ashraf and Foolad, 2007). Plants will actively accumulate proline to decrease the intracellular osmotic potential, to improve the hydrophily of protoplasm, to help cell tissue hold water and resist dehydration, to maintain the intracellular pressure, and to enhance the ability to delay the intensification of water stress (Slama et al., 2007; Xu and Zhan, 2009). With a decrease in water content, *G. longituba* could accumulate proline to maintain the balance of osmotic potential and thereby protecting the membrane system from harm. The content of proline observed in T5 was much higher than that observed in the other treatments. These results showed that the alteration of proline content resulted from the severity of water stress and further suggested that this alteration was a symptom of cell damage in the leaves of *G. longituba* (Lutts et al., 1999). Soluble sugars are important osmoregulatory substances in plants grown under different water treatments. These sugars can accumulate and lower the osmotic potential in plant tissues, thereby maintaining the driving force for extracting soil water under conditions of water stress (Nakayama et al., 2007). In *G. longituba*, the soluble sugar content in the leaves increased in response to increased water stress at an increasing rate. These results show that soluble sugars play a vital role in *G. longituba* and make a major contribution to osmotic adjustment under severe water stress (Hessini et al., 2008; Sánchez et al., 2004).

The accumulation of proline, soluble sugars and other materials plays an important role in osmotic adjustment, protection of tissue, and the removal of reactive oxygen species, thereby promoting growth and other functions (Mi et al., 2004). The gradual upward trend observed in the content of proline and soluble sugars in this experiment shows that the accumulation of proline and soluble sugars could activate the antioxidant defense mechanisms and improve the drought tolerance of *G. longituba*.

Morpho-anatomical response to different water treatments

Some studies have demonstrated that increased water stress reduces leaf weight and leaf thickness (Gregoriou et al., 2007; Huang et al., 2004; Zavala and Ravetta, 2001). Under aggravated water stress, the thickness of palisade and spongy tissue of water-stressed leaves were lower than in T1. Similar results were observed for the entire thickness of the lamina. The thickness of the mesophyll cells decreased in the stressed plants. This result indicated a reduction in cell size. Furthermore, a decreased density of spongy parenchyma cells and an increase of intercellular spaces were also evident. Similar results have also been found in *Persea americana* (Chartzoulakis et al., 2002). In the stems, the larger parenchyma cells in the exodermis and the compact parenchyma cells in the pith indicated that *G. longituba* exhibited anatomical adaptations to the water stress in the experiment.

The change of total flavonoids of *G. longituba* under different water treatments

The acclimation of a plant to its growing environment is

reflected in such physiological responses as accumulation of reactive oxygen and secondary metabolism (Singh and Singh, 2006; Yin et al., 2009; Zhu et al., 2009). Total flavonoids in many medicinal plants play important roles in pharmacology, and their content is an important index for estimation of medicinal quality (Ebrahimzadeh et al., 2008; Zhao et al., 2003). In our study, the excessive water loss greatly decreased the yield of total flavonoids in *G. longituba* (Figure 4B). These results showed that water is an important factor restricting the production of total flavonoids. They further indicated that an appropriate water level is important for the synthesis of total flavonoids in *G. longituba*. These results were consistent with previous findings that the content of total flavonoids in *Tribulus terrestris* under high levels of water treatments is higher than that occurring in low-water treatments (Yang et al., 2010). Although *G. longituba* was found to require more moisture for its growth process, a finding consistent with the fact that *G. longituba* usually occurs in humid wastelands, hillsides, understory and roadside environments, excess water or water deficiency did not favor higher yields and a higher content of total flavonoids simultaneously. In *Ginkgo biloba*, drought stress has been found to promote the growth of quercetin content and to inhibit the increase of rutin in the leaves (He and Zhong, 2003). In *Scutellaria Baicalensis* Georgi, baicalin increased steadily in the stems and leaves under lower water stress, and it decreased sharply under heavy water stress (Liu et al., 2010). In the tea tree, soil drought could not cause an increase in phenylalanine ammonia lyase (PAL) activity (Jeyaramraja et al., 2003). The variation in the content of total flavonoids in *G. longituba* was similar to that found in *S. baicalensis* Georgi and *T. terrestris* (Liu et al., 2010; Yang et al., 2010). This finding may indicate that the mechanism of flavonoid synthesis in *G. longituba* differs from those found in these other two species.

Conclusions

This study has shown that stem and leaf length, biomass, leaf thickness, chlorophyll content, and the content of total flavonoids tend to decrease under water stress, whereas the content of MDA, proline and soluble sugars simultaneously tend to increase. This finding implies that the increase of osmotic adjustment in *G. longituba* plants under water stress, the formation of the well-developed aerenchyma of the exodermis and the compact parenchyma cells of the pith in the stem, and an increase in intercellular spaces in the leaf could indicate an adjustment and acclimation to water stress. These changes would prevent or alleviate the injury caused by water stress. Moreover, a value of 80 to 85% of field capacity was found to be optimal for growth and for maximizing the yield of total flavonoids.

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REFERENCES

Abdul JA, Manivannan P, Kishorekumar A, Sankar B, Gopi R, Somasundaram R, Panneerselvam R (2007). Alterations in osmoregulation, antioxidant enzymes and indole alkaloid levels in *Catharanthus roseus* exposed to water deficit. Colloid Surface B., 59: 150-157.

Arnon DI (1949). Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol., 24: 1-15.

Asada K (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol., 141: 391-396.

Ashraf M, Foolad MR (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot., 59: 206-216.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-207.

Cakmak I, Horst J (1991). Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). Physiol. Plantarum., 83(3): 463-468.

Chartzoulakis K, Patakas A, Kofidis G, Bosabalidis A, Nastou A (2002). Water stress affects leaf anatomy, gas exchange, water relations and growth of two avocado cultivars. Sci. Hortic., 95: 39-50.

Cheng MJ, Feng GY, Huang QH (2005) Study on the extraction process for total flavones from *Herba Glechomae* by orthogonal design. Chin. Hosp. Pharm. J., 25: 147-149.

China Pharmacopoeia Committee (2010). Chinese Pharmacopoeia of People's Republic of China. China Medical Science and Technology Press, Beijing, pp. 158-159..

Chung IM, Kim JJ, Lim JD, Yu CY, Kim SH, Hahn SJ (2006). Comparison of resveratrol SOD activity, phenolic compounds and free amino acids in *Rehmannia glutinosa* under temperature and water stress. Environ. Exp. Bot., 56: 44-53.

Cook NC, Samman S (1996). Flavonoids--Chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem., 7: 66-76.

Dai ZW, Wang LJ, Zhao JY, Fan PG, Li SH (2007). Effect and after-effect of water stress on the distribution of newly-fixed ^{14}C-photoassimilate in micropropagated apple plants. Environ. Exp. Bot., 60: 484-494.

Ebrahimzadeh MA, Pourmorad F, Bekhradnia AR (2008). Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. Afr. J. Biotechnol., 7(18): 3188-3192.

Li HW (1977). Flora of China. Science Press, Beijing, 65(2): 316-318..

Gregoriou K, Pontikis K, Vemmos S (2007). Effects of reduced irradiance on leaf morphology, photosynthetic capacity, and fruit yield in olive (*Olea europaea* L.). Photosynthetica, 45: 172-181.

He BH, Zhong ZC (2003). Study on variation dynamics of modular population of *Ginkgo Biloba* under different conditions of environmental stress. J. Southwest Agric. Univ., 25: 7-10.

Hessini K, Ghandour M, Albouchi A, Soltani A, Werner KH, Abdelly C (2008). Biomass production, photosynthesis, and leaf water relations of *Spartina alterniflora* under moderate water stress. J. Plant Res., 121: 311-318.

Huang WD, Wu LK, Zhan JC (2004). Growth and photosynthesis adaptation of dwarf-type chinese cherry (*Prunus pseudocerasus* L. cv. Laiyang) leaves to weak light stress. Sci. Agric. Sinica., 37: 1981-1985.

Jeyaramraja PR, Pius PK, Raj KR, Jayakumar D (2003). Soil moisture stress induced alterations in bioconstituents determining tea quality. J. Sci. Food Agric., 83: 1187-1191.

Jia ZS, Tang MC, Wu JM (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64(4): 555-559.

Jwa NS, Agrawal GK, Tamogami S, Yonekura M, Han O, Iwahashi H, Rakwal R (2006). Role of defense/stress-related marker genes, proteins and secondary metabolites in defining rice self-defense mechanisms. Plant Physiol. Biochem., 44: 261-273.

Kirakosyan A, Kaufman P, Warber S, Zick S, Aaronson K, Bolling S, Chul Chang S (2004). Applied environmental stresses to enhance the levels of polyphenolics in leaves of hawthorn plants. Physiol. Plantarum., 121: 182-186.

Liu HY, Wang XD, Wang DH, Zou ZR, Liang ZS (2011). Effect of drought stress on growth and accumulation of active constituents in *Salvia miltiorrhiza* Bunge. Ind. Crop Prod., 33: 84-88.

Liu JH, Zhang YQ, Li J, Hu JH, Li ZH (2010). Influence of water stress on the physiological and biochemical characteristics of *Scutellaria Baicalensis* Georgi. Plant Physiol. Biochem., 11: 22-25.

Lutts S, Majerus V, Kinet JM (1999). NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. Physiol. Plantarum., 105: 450-458.

Mi HL, Xu X, Li SH, He J, Zhang YP, Zhao TC, Ma YM (2004). Effects of soil water stress on contents of chlorophyll, soluble sugar, starch, C/N of two desert plants (*Cynanchum komarovii* and *Glycyrrhiza uralensis*). Acta Bot. Boreal-Occident Sin., 24: 1816-1821.

Nakayama N, Saneoka H, Moghaieb Reda EA, Premachandra GS, Fujita K (2007). Response of growth, photosynthetic gas exchange, translocation of ^{13}C-labelled photosynthate and N accumulation in two soybean (*Glycine max* L. Merrill) cultivars to drought stress. Int. J. Agri. Biol., 9: 669-674.

Ni SM, Qian DW, Duan JA, Guo JM, Shang EX, Shu Y, Xue CF (2010). UPLC-QTOF/MS-based screening and identification of the constituents and their metabolites in rat plasma and urine after oral administration of *Glechoma longituba* extract. J. Chromatogr. B., 878(28): 2741-2750.

Sánchez FJ, De Andres EF, Tenorio JL, Ayerbe L (2004). Growth of epicotyls, turgor maintenance and osmotic adjustment in pea plants (*Pisum sativum* L.) subjected to water stress. Field Crops Res., 86: 81-90.

Shang XH, Wang Y, Yan XF (2003). Effect of soil moisture on growth and root-salidroside content in *Rhodiola sachalinensi*. Plant Physiol. Commun., 39: 335-336.

Singh B, Singh G (2006). Effects of controlled irrigation on water potential, nitrogen uptake and biomass production in *Dalbergia sissoo* seedlings. Environ. Exp. Bot., 55: 209-219.

Slama I, Ghnaya T, Messedi D, Hessini K, Labidi N, Savoure A, Abdelly C (2007). Effect of sodium chloride on the response of the halophyte species *Sesuvium Portulacastrum* grown in mannitol-induced water stress. J. Plant Res., 120: 291-299.

Spiro RG (1966). Analysis of sugars found in glycoprotein. In: Neufeld, E.S., Ginsburg, V.(Eds.), Methods in Enzymology, Complex Carbohydrates. Academic Press, pp.3-26.

Tan Y, Liang ZS, Dong JE, Hao HY, Ye Q (2008). Effect of water stress on growth and accumulation of active components of *Isatis indigotica*. China J. Chin. Mater. Med., 33: 18-22.

Tao Y, Xiao YX, Shi MY, Yi JP (2004). Effect of the ethanol extracts of *Glechoma longtiba* on isolated ileum smooth muscle of guinea pig and intestinal movement function of mice. Chin. J. Hosp. Pharm., 24: 65-67.

Xu YG, Zhan YG (2009). Progress of the research on plant drought-resistant mechanism and related genes. Biotechnol. Bull., 2: 11-17.

Yang L, Han ZM, Yang LM, Han M (2010). Effects of water stress on photosynthesis, biomass, and medicinal material quality of *Tribulus terrestri*. Chin. J. Appl. Ecol., 21: 2523-2528.

Yin DM, Chen SM, Chen FD, Guan ZY, Fang WM (2009). Morphological and physiological responses of two chrysanthemum cultivars differing in their tolerance to waterlogging. Environ. Exp. Bot., 67: 87-93.

Zavala JA, Ravetta DA (2001). Allocation of photoassimilates to biomass, resin and carbohydrates in *Grindelia Chiloensis* as affected by light intensity. Field Crop Res., 69: 143-149.

Zhao FP, Strack D, Baumert A, Subramaniam R, Goh NK, Chia TF, Tan SN, Chia LS (2003). Antioxidant flavonoids from leaves of *Polygonum hydropiper* L. Phytochemistry. 62: 219-228.

Zhao J, Davis LC, Verpoorte R (2005). Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv., 23: 283-333.

Zheng XL (1996). Danle capsules-example of combine biochemical. Chin. J. Biochem. Pharmaceuti., 17: 35-36.

Zhu ZB, Liang ZS, Han RL (2009). Saikosaponin accumulation and antioxidative protection in drought-stressed *Bupleurum chinense* DC. Environ. Exp. Bot., 66: 326-333.

Zobayed SMA, Afreen F, Kozai T (2007). Phytochemical and physiological changes in the leaves of St. John's wort plants under a water stress condition. Environ. Exp. Bot., 59: 109-116.