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Full Length Article

Promotive Effects of Genistein on Photosynthesis, Flavonoid Biosynthesis and Antioxidant Enzyme Activities in *Ginkgo biloba*

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Abstract

The effects of exogenous genistein (GNT) on flavonoid content and antioxidase activity in *Ginkgo biloba* leaves were investigated. In particular, the flavonoids, chlorophyll, anthocyanins, and soluble sugar contents, as well as the enzymes included in flavonoid biosynthesis activities and antioxidant enzymes were measured. Leaves of one-year-old ginkgo seedlings were sprayed with 0 (control), 50, 100 and 150 μmol/L GNT. Results showed that 50, 100 and 150 μmol/L GNT significantly enhanced the intercellular CO_2 concentration, net photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll, soluble sugar and titratable acid contents in *G. biloba.* The contents of flavonoid and anthocyanins in the leaves of *G. biloba* treated with 50, 100 and 150 μmol/L GNT were significantly higher than of control. The results indicated that the key enzymes included in namely phenylalanine ammonia lyase, flavonoid biosynthesis, chalcone isomerase and chalcone synthase activities were markedly increased by GNT treatment. Likewise, application of GNT significantly enhanced the activities of ascorbate peroxidase, guaiacol peroxidase, catalase, and superoxide dismutase in *G. biloba*. These results suggest that foliar treatment with GNT may be effective measure of improving flavonoid content and beneficial antioxidant activity in the leaves of *G. biloba*. © 2016 Friends Science Publishers

Keywords: *Ginkgo biloba*; Genistein; Flavonoids; Chlorophyll; Antioxidant enzymes

Introduction

Ginkgo biloba L., commonly called maidenhair or sun tree, is a dioecious tree native to China. Often described as a "living fossil", it is the world's oldest relic plant. As early as 1970, studies showed that ginkgo extract exerts therapeutic effects on cardiovascular diseases. Since then, the medicinal value of ginkgo has increased and it has become one of the most widely researched medicinal plants (He and Wang, 2005). Aside from being the preferred natural medicine for treating Alzheimer's disease, ginkgo flavonoids also exhibit significant therapeutic effects against hypertension, atherosclerosis, diabetes and cardiovascular diseases (Zimmermann *et al*., 2002; Van and Montoro, 2009). Flavonoids are widely distributed in the plant kingdom and participate in the regulation of plant growth, protection of plants from UV rays, improvement of plant fertility and pollen germination. They also display antioxidant, anti-aging, and anti-pathogenic activities (Winkel-Shirley, 2002; Schijlen *et al*., 2004). In addition, the antioxidant property of ginkgo leaves is unlikely to be because of other antioxidant enzymes such as free-radical-scavenging enzymes. Goh *et al.* (2003) and Ellnain-Wojtaszek *et al.* (2002) obtained similar results with superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD) ; ascorbate peroxidase (APX) and glutathione reductase (GR) and mainly ascorbic acid (AsA), contents of antioxidant substances, and glutathione (GSH). Given their important pharmacological and physiological activities, flavonoids and antioxidant constituents have become a popular topic of research internationally. Here we sought to develop novel methods to increase flavonoid content and antioxidant level in ginkgo leaves.

Genistein (GNT), an isoflavone naturally present in soybean, is a tyrosine kinase inhibitor commonly used to investigate signal transduction of cellular G proteins (Zhu *et al*., 2007a, b and c). Several reports have showed that the activities of antioxidant enzyme, including ascorbic peroxidase, catalase, peroxidase, and superoxide dismutase are improved by GNT treatment (Liu *et al*., 2009). Recent studies have indicated that GNT can promote accumulation of secondary metabolites in plants. For example, exogenous GNT treatment promotes anthocyanin accumulation in apples (Wang *et al*., 2006), grapes (Zhu *et al*., 2010) and peaches (Zhu *et al*., 2007). These results indicate that GNT promotes flavonoid accumulation in plants. However, the effects of GNT on flavonoid accumulation in ginkgo remain unclear. The present study examined the effects of GNT on

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levels of photosynthetic products, flavonoids, and key flavonoid biosynthesis-related enzymes, and the activity of antioxidant enzyme in *G. biloba*. The study designed to research the physiological mechanism behind flavonoid production under GNT treatments and to explore potential application of GNT in promoting flavonoid production.

Materials and Methods

Plant Materials and Treatments

Ginkgo seedlings of one-year-old from Botanical Gardening of Yangtze University were used. The culture media comprised a mixed matrix of sand and humus at 1:2 ratio. The seedlings were planted in flowerpots (38 cm in height, 26 cm in diameter) with one seedling in each pot. The seedlings were placed in illumination incubator, light 14 h/day and relative humidity 60–70%. Each pot received 1L of liquid fertilizer containing1.4 g K, 3.6 g N and 1.2 g P weekly (Xu *et al*., 2011).

Seedlings with four blades were watered with 0 (control), 50, 100 and 150 μmol/L GNT solutions. Each treatment was divided into three sections, with each section watered three times. A total of 36 seedlings with similar growth rates were selected. The solutions were applied on 36 seedlings once a month, 20 mL each time, for 3 months. The front and back of the blades were sprayed every time. Sampling was started 4 weeks after the GNT treatments.

Measurement of Photosynthetic Rates and Soluble Sugar, Chlorophyll and Titratable Acid Contents

The photosynthetic parameters were determined on the fifth leaf by using a portable photosynthesis system (LiCor-6400, USA) from 8:00 am to 11:00 am when ginkgo leaves were under 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 60% humidity (Adamski *et al.*, 2011).

Soluble sugars of young leaf tissue were extracted with boiling ethanol and then analyzed enzymatically (Kunst *et al*., 1983) using a UV spectrophotometer. Soluble sugars content was showed with a percentage of leaf fresh weight (w/w, FW).

The content of chlorophyll was determined from 200 g samples of the fifth leaf of ginkgo. Total extinction coefficients and chlorophyll *a* and *b* contents were decided according to Graan and Ort (1984).

Tissue samples (15–20 g) were ground, filtered and centrifuged at 8, 000 × g for 15 min. To determine titratable acidity (Schmilovitch *et al.*, 2000), the supernatant was titrated with 0.1 mol/L NaOH. Results of titratable acid were expressed as percentage in the supernatant.

Measurement of Total Flavonoid and Total Anthocyanin Contents

Flavonoid was measured as described by Cheng (2004) with a little alters and expressed as mg rutin equivalent 100 g/FW and using rutin as the touchstone. The content of anthocyanin as described by Pirie and Mullins (1976) with minor modifications and determined as mg cyanidin equivalents 100 g/FW.

Determination of Enzyme Activity

Phenylalanine ammonia lyase (PAL) activity in ginkgo was determined by UV spectrophotometry (Carrera *et al*., 2012). The way was heated for 25 min in a water bath at 45°C. Spectrophotometric values were obtained at 290 nm. Enzyme activity unit (U) was set absorbing and changing 0.01 spectrophotometric values as the amount of enzymes required. The test was repeated three times, and the results were averaged.

Extraction and Determination of Antioxidant Enzymes

Activity of ascorbate peroxidase (APX) was calculated by measuring the reducing rate of ascorbate oxidation at 290 nm as described by Nakano and Asada (1981). The activity of superoxide dismutase (SOD) was estimated as described by Cakmak and Marschner (1992). Single unit of SOD activity was stipulated as the number of enzyme required to cause half inhibition of the proportion of nitro blue tetrazolium reduction at 560 nm. The activity of catalase (CAT) was immediately depended on disintegration of H_2O_2 at 240 nm (Yang *et al*., 2007). The activity of peroxidase (POD) was calculated with guaiacol as the substrate in a total volume of 3 mL according to Zhou and Leul (1999) with several modifications. Make the absorbance increase because of guaiacol oxidation was calculated at 470 nm.

Statistical Analysis

Data were analyzed by conducting one-way ANOVA in SAS 8.0 (SAS Institute Inc., North Carolina), and means were contrasted with Duncan's multiple scope test at $P < 0.05$.

Results

Effect of GNT on Leaf Photosynthetic Rates

GNT treatment influenced the photosynthetic activity of plants (Table 1). Compared with the control, GNT treatments at 50, 100 and 150 μmol/L increased the photosynthetic rate by 11.48%, 24.14% and 56.03%, respectively, and the intercellular CO_2 by 12.13%, 30.64% and 29.74%, respectively. Compared with the control, GNT treatments at 100 and 150 μmol/L GNT increased stomatal conductance by 9.09% and 31.81%, respectively and stomatal conductance by 30% and 40%, respectively.

Effect of GNT on Chlorophyll Contents

As shown in Table 2, GNT treatments at both 100 and 150 μmol/L improved chlorophyll contents. The contents of chlorophyll *a* and *b* after treatment with 100 μmol/L GNT

Table 1: Effect of GNT on the photosynthetic parameters in *G. biloba* leaves

GNT treatment concentrations (μmol /L)	P_n (CO_2 μmol m^{-2} s^{-1})	G_s (H_2O mol m^{-2} s^{-1})	C_i (CO_2 μl/L)	T_r (H_2O mmol m^{-2} s^{-1})
0 (Control)	10.19 ± 0.71 d	0.22 ± 0.03 c	259.85 ± 28.69 c	1.18 ± 0.04 d
50	11.36 ± 0.17 c	0.21 ± 0.01 c	291.36 ± 19.81 b	1.25 ± 0.05 c
100	12.65 ± 0.59 b	0.24 ± 0.02 b	339.48 ± 11.57 a	1.57 ± 0.10 b
150	15.90 ± 1.13 a	0.29 ± 0.01 a	337.15 ± 21.24 a	1.64 ± 0.06 a

P_n, G_s, C_i, and T_r represent net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate, respectively. Values are mean ± standard error (SE) of six replications. Different letters within the same column indicate significant differences between treatments according to Duncan's multiple range test at $P<0.05$ level

Table 2: Effect of GNT on chlorophyll contents in *Ginkgo biloba*

GNT treatment concentrations (μmol/L)	chlorophyll a (mg/g FW)	chlorophyll b (mg/g FW)	chlorophyll a+b (mg/g FW)	chlorophyll a/b
0	0.952 ± 0.021 b	0.481 ± 0.028 b	1.433 ± 0.083 b	1.979 a
50	1.074 ± 0.045 b	0.489 ± 0.015 b	1.563 ± 0.051 b	2.196 a
100	1.386 ± 0.020 a	0.647 ± 0.036 a	2.033 ± 0.104 a	2.142 a
150	1.325 ± 0.062 a	0.623 ± 0.010 a	1.948 ± 0.129 a	2.127 a

Values are mean ± standard error (SE) of six replications. Different letters within the same column indicate significant differences between treatments according to Duncan's multiple range test at $p<0.05$ level

were 1.386 and 0.647 mg/g FW, respectively with marked increases of 45.58% and 34.51%, respectively, compared to treatment with the control ($P < 0.05$). The range ability of chlorophyll *a*+*b* fits the change in chlorophyll *a* and *b* contents. Non-significant difference was observed in chlorophyll *a*/*b* under different GNT concentration treatments.

Effects of GNT on Soluble Sugar and Titratable Acid Contents

Exogenous GNT treatments increased the contents of titratable acid (61.99%, 28.05% and 63.80%) and soluble sugar (20.04%, 10.11% and 10.11%) compared with control treatment. This showed that spraying GNT promotes carbohydrate accumulation in ginkgo leaves (Table 3).

Effects of GNT on Chalcone Synthase (CHS), Chalcone Isomerase (CHI) and Phenylalanine Ammonia Lyase (PAL) Activities

Exogenous GNT treatment significantly ($P < 0.05$) induced the CHS, CHI, and PAL activities. Among them, 100 μmol/L GNT had the highest PAL activity of 344.592 U/mg, which was 28.99% higher than after control (267.15 U/mg). Treatment with 150 μmol/L GNT increased the activities of CHS and CHI by 97.98% and 127.57%, respectively compared with the control ($P < 0.05$) (Fig. 1).

Effects of GNT on Flavonoids and Anthocyanins

100 and 150 μmol/L GNT treatments significantly improved the flavonoid (11.41% and 20.88%, respectively) and anthocyanin (27.01% and 12.54%, respectively) contents compared with control treatment. This indicated that GNT can increase the contents of flavonoid and anthocyanin in the leaves of *G. biloba* within a certain concentration range (Table 4).

Table 3: Effects of GNT on soluble sugar and titratable acid contents

GNT treatment concentrations (μmol/L)	soluble sugar (mg/g FW)	titratable acid (mg/g FW)
0	10.482 ± 1.005 c	0.221 ± 0.024 c
50	12.586 ± 0.942 a	0.358 ± 0.018 a
100	11.543 ± 0.897 b	0.283 ± 0.014 b
150	12.060 ± 1.141 ab	0.362 ± 0.020 a

Table 4: Effects of GNT on flavonoids and anthocyanins

GNT treatment concentrations (μmol/L)	flavonoids (mg/100 g FW)	anthocyanins (mg/100 g FW)
0	132.152 ± 10.242c	0.311 ± 0.018 c
50	132.171 ± 12.351 c	0.314 ± 0.023 c
100	147.238 ± 17.260 b	0.395 ± 0.015 a
150	159.745 ± 14.642 a	0.350 ± 0.030 b

Values are mean ± standard error (SE) of six replications. Different letters within the same column indicate significant differences between treatments according to Duncan's multiple range test at $p<0.05$ level

Effects of GNT on Antioxidant Enzyme Activity

As shown in Fig. 2, 50, 100 and 150 μmol/L GNT treatments significantly improved the activity of antioxidant enzymes, including SOD (34.27%, 40.45% and 89.07%, respectively), CAT (14.14%, 26.24% and 33.77%, respectively), and POD (46.96%, 74.24% and 59.63%, respectively), compared with control treatment. Treatment with 50 μmol/L GNT exerted no significant effect on APX activity, but 100 and 150 μmol/L GNT significantly improved APX activity by 54.18% and 49.53%, respectively, compared with the control.

Discussion

The total anthocyanins and flavonoids contents are important indices for measuring the quality of ginkgo

leaves. This study is the first to report that exogenous GNT treatment can significantly improve ginkgo flavonoid and anthocyanin contents. Wang *et al*. (2006) and Zhu *et al*. (2007) reported similar results in apples and grapes, respectively. However, whether GNT can regulate the synthesis of flavonoids and anthocyanins remains unclear. The present data showed that GNT treatment significantly increased the contents of chlorophylls *a* and *b* and thus improved quantum efficiency. Our results are consistent with Zhu *et al.* (2007) regarding the fluorescent characteristics of chlorophyll in peaches. However, chlorophyll content of peach leaves was found to be reduced in their study. This discrepancy may be due to exogenous GNT treatments of different plant organs. The observed increase in chlorophyll *a*+*b* content may be associated with increase in the contents of flavonoid and anthocyanin caused by a positive relationship among chlorophyll, flavonoid contents and anthocyanin (Xu *et al.,* 2011).

Many hypotheses have been proposed to explain how the phenotypes and evolutionary patterns of carbon compounds influence the distribution of secondary plant metabolites (Heyworth *et al*., 1998; Mosaleeyanon *et al*., 2005). For example, the metabolic overflow theory states that excess carbohydrates are used to produce secondary metabolites in plants when the amount of carbon compounds exceeds the demand for plant growth (Matsuki, 1996). Accordingly, the increase in flavonoid content in ginkgo may be due to increased production of carbohydrates such as soluble sugar and titratable acid after GNT treatment (Table 3). Sugar is the primary ingredient of flavonoids and anthocyanin (Saure, 1990), and may also regulate gene expression of enzymes participating in anthocyanin biosynthesis (Gollop *et al*., 2001). Our previous studies also confirmed that sugar is a signal molecule that induces gene expression of key enzymes including chalcone isomerase (CHI), flavonols synthetase (FLS) and anthocyanidin synthase (ANS) involved in flavonoid biosynthesis in ginkgo. However, further studies are needed to establish if a relationship exists between GNT and gene expression of key enzymes mediating biosynthesis of soluble sugar and flavonoids in ginkgo.

SOD clears superoxide anions, while POD, APX, and CAT clear the SOD decomposition product H_2O_2 from cells. These antioxidant enzymes collectively work to maintain the balance of intracellular reactive oxygen metabolism (Xu *et al.,* 2009). Considering the strong antioxidant activities of flavonoids and anthocyanins, researchers have suggested that plant flavonoids and anthocyanin accumulation are related to antioxidant activity. GNT is an effective antioxidant that can inhibit the production of free radicals. Cai and Wei (1996) initially demonstrated that GNT treatment can enhance the activities of GST, SOD, GSH-PX and CAT, in rats. Recent studies on horticultural plants have confirmed that exogenous GNT can improve the activity of antioxidant enzymes in peach (Zhu *et al*., 2007) and hot pepper (Liu *et al*., 2009) leaves.

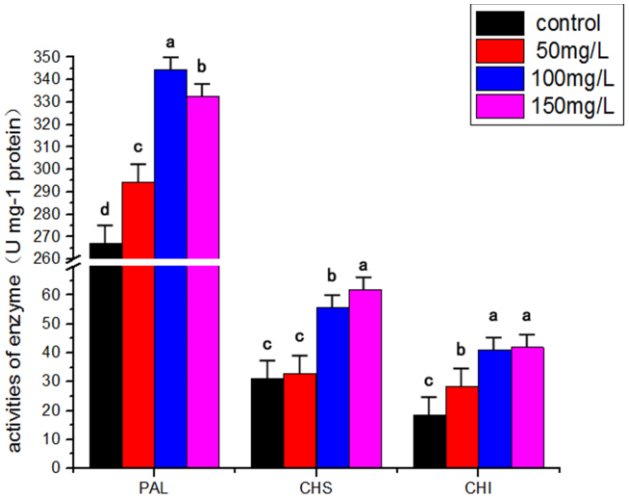

Fig. 1: Effects of GNT on phenylalanine ammonia enzyme, chalcone synthetase and chalcone isomerase chalcone activity in ginkgo leave. Values are mean ± standard error (SE) of six replications. Different letters within the same column indicate significant differences between treatments according to Duncan's multiple range test at $p<0.05$ level

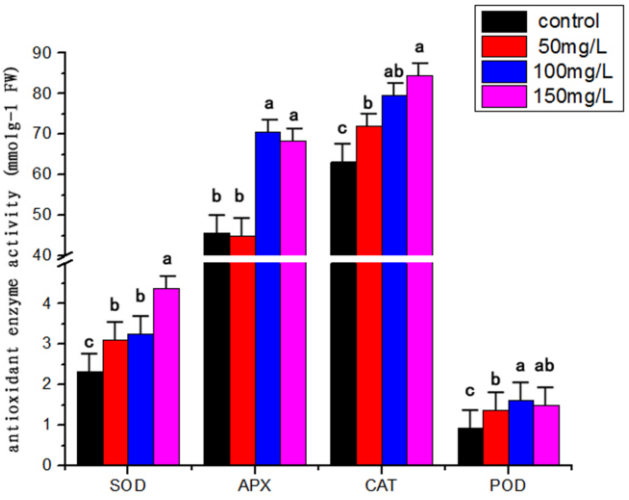

Fig. 2: Effect of genistein on salinity-induced changes in ascorbate peroxidase (APX), guaiacol peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) activities of ginkgo leave.

Values are mean ± standard error (SE) of six replications. Different letters within the same column indicate significant differences between treatm ents according to Duncan's multiple range test at $p<0.05$ level

In the same way, the present study found that exogenous GNT treatments significantly increased the POD, SOD, CAT and APX activities in the leaves of *G. biloba*. This finding suggests that the GNT-induced accumulation of flavonoids and anthocyanins is related to improved activity of antioxidant enzymes.

Intracellular GNT tyrosine kinase inhibitors are related to G protein activation and cGMP signaling systems. Previous researches suggested that GNT promoted anthocyanin synthesis in apples (Wang *et al*., 2006) and hypothesized that tyrosine kinase negatively regulates phytochrome-mediated production of anthocyanin. Thus, GNT may also be effective in regulating the phytochrome-mediated synthesis of flavonoids and anthocyanin. Meanwhile, almost all enzymes related to flavonoid and anthocyanin synthesis are light regulatory enzymes whose expression levels are mediated by phytochromes (Kim *et al*., 2002). This observation is similar to the results obtained by Wang (2006) and Zhu (2007) in apples and peaches. The present study showed that GNT treatment significantly improved the accumulation of flavonoids and anthocyanin, and induced the CHS, CHI and PAL activities in the leaves of *G. biloba* in a concentration-dependent manner. Our study further supports the hypothesis of Wang (2006). A positive relationship was observed between the activities of CHS, CHI, and PAL (Mato *et al.*, 2000) and contents of flavonoid, anthocyanin and total polyphenol in a lot of plants (Ju *et al.*, 1995; Obinata *et al.*, 2003). Jaakola *et al*. (2002) research have also indicated that the transcript levels of some genes encoding enzymes (i.e., CHS, CHI and PAL) participated in anthocyanin and flavonoid biosyntheses correlate positively with accumulation of anthocyanins and flavonoids. CHS, CHI and PAL played a key role in biosynthesizing flavonoid regulatory enzymes in the leaves of *G. biloba* (Xu *et al.*, 2008) and 5-aminolevulinic acid induced the transcription of a *CHI* gene that regulates total flavonoid accumulation in the leaves of *G. biloba* (Cheng *et al.*, 2011). Taken together, these data indicate that GNT treatment can increase the contents of anthocyanin and flavonoid by enhancing the CHS, CHI and PAL activities.

Conclusion

The study indicated that GNT at 50, 100 and 150 μmol/L greatly promoted the photosynthetic and respiratory capacities, chlorophyll content, soluble sugar content, titratable acid content, three crux flavonoid biosynthetic enzymes activities, including PAL, CHS and CHI and the contents of flavonoids and anthocyanins, which are often considered major constituents of quality of the leaves of *G. biloba*. In addition, GNT application also significantly enhanced the expression of antioxidant enzymes (APX, POD, CAT and SOD). Thus, 150 μmol/L GNT application may be effective measure for improving the medicinal value of the leaves of *G. biloba.*

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