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Research Article

Effect of exogenous gibberellin on endogenous hormone and ginkgolide content in Ginkgo leaves

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Abstract

Ginkgolide is one of the important secondary metabolites of *Ginkgo biloba*. The synthesis of plant secondary metabolites is influenced by exogenous phytohormones. In this study, ten-year-old ginkgo grafted seedlings were used as the test material, and the effects of 0, 2, 4, 6, 8, and 10 mmol L^{-1} gibberellin (GA_3) on endogenous indole acetic acid (IAA), abscisic acid (ABA), GA_3, and ginkgolide contents in ginkgo leaves were investigated. Results showed that exogenous GA_3 treatment inhibited endogenous GA_3, changed the contents of endogenous IAA and ABA in ginkgo leaves, and affected the accumulation of secondary metabolite ginkgolides. Among the given concentrations, the 4 mmol L^{-1} GA_3 treatment could remarkably increase ginkgolide content. We found that the best harvesting period of ginkgo leaves were in late August and late September, in which the content of ginkgolides was the highest.

Keywords: abscisic acid; gibberellin; *Ginkgo biloba*; ginkgolide; indole acetic acid

Introduction

Ginkgo biloba (*G. biloba*), also known as Gongsun tree, is the only surviving species of Ginkgoaceae and the oldest relict plant of the gymnosperm left after the Quaternary glacier movement; hence, *G. biloba* is regarded as a "living fossil" and "panda of the plant kingdom" (Tian *et al*., 2017; Ye *et al*., 2020). The active constituents of *G. biloba* leaves are mainly flavonoids and ginkgolides compounds (Ye *et al*., 2019). Ginkgolide have a variety of pharmacological activities, such as antiplatelet aggregation factor, central nervous system protection, antibacterial and anti-inflammatory effects, antiviral and anticancer effects, anti-allergic effects, anti-shock effects, protective effects on ischemic injury, and antirejection in organ transplantation (Liao *et al*., 2006; Tian *et al*., 2017). Ginkgolide has a role in regulating nerves, and pure ginkgolide B (GB code BN52021) is clinically used in treating stroke, organ transplant rejection, hemodialysis, and shock (Hu *et al*., 2017). Since the 1980s, the unique pharmacological value of ginkgolide has caused an international upsurge in the study of *G. biloba*.

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Gibberellin (GA_3) is a broad-spectrum plant growth regulator that promotes plant growth and development, early maturity, yield, and quality improvement. GA_3 can quickly break the dormancy of organs, such as seeds, tubers, and bulbs; promote germination; reduce bud, flower, bell, and fruit shedding; increase fruit yield; or form seedless fruits (Niu *et al*., 2016; Vera-Sirera *et al*., 2016). In addition, Ülger *et al*. (2018) sprayed exogenous GA_3 on 'Memecik' olive cultivar and found that its abscisic acid (ABA), GA_3, indole acetic acid (IAA), and zeatin content increased considerably. Zhang *et al*. (2018) found that GA_3 regulated the synthesis and decomposition of cytokinin, which upregulated the expression levels of *A-IPT1* and tRNA-DMATase genes but downregulated the expression levels of *CYP735A*, *CKX1*, and *CKX2* genes. Guo *et al*. (2000) studied the effect of exogenous growth regulators on paclitaxel synthesis by applying exogenous GA_3 and found that the application of exogenous GA_3 could inhibit the synthesis of endogenous GA_3 through feedback inhibition and results in the accumulation of intracellular geranylgeranyl pyrophosphate (GGPP), which increases paclitaxel content. Ginkgolide and GA_3 are diterpenoids with the same precursor synthesis pathway (Ke *et al*., 2016). By applying exogenous GA_3 to inhibit the synthesis of endogenous GA_3, GGPP accumulation may be converted to the synthesis direction of ginkgolides to increase ginkgolide content.

Therefore, this paper studied the effects of different concentrations of exogenous GA_3 on ginkgolides and endogenous hormones to provide technical support and theoretical guidance for the screening and improving the regulation measures of ginkgolide content in production.

Materials and Methods

Plant materials and GA_3 treatments

Ten-year-old grafted seedlings of *G. biloba* cv. 'Jiafoshou' were as experiment planted in Ginkgo Science and Technology Park of Yangtze University. The tree is moderate, and the growth is basically the same. All seedlings were treated by foliar sprayed with aqueous solutions of GA_3 (containing 0.01% Tween 20, pH 5.8) at six concentrations of 0 (the control), 2, 4, 6, 8, and 10 mmol L^{-1} from June 25. The leaves were sampled from July 10 to October 25 by random stratification sampling method, and one hundred leaves with uniform size, uniform leaf colour, and no injury were randomly picked from similar branches with the same growth potential every fifteen days. Harvested ginkgo leaves were divided into two parts. One part was frozen in liquid nitrogen and stored in a refrigerator at -80 °C for the determination of endogenous hormone content in the leaves. The other part was washed and dried immediately, dried at 105 °C for 20 min or 85 °C for constant weight and grinding, and stored in the dryer for the measurement of ginkgolide content.

Detection of endogenous hormones and ginkgolide content

Endogenous hormone testing was performed following the method of Zhu *et al*. (2010). Endogenous hormones IAA, ABA, and GA_3 in *G. biloba* leaves were extracted with 80% precooled methanol aqueous solution. The target hormone was further purified by SEP-C18 small-column solid-phase extraction technique and detected by high-performance liquid chromatography with diode-array detection. A 10% methanol solution was finally selected as the washing solution by a preferred method, and 65% methanol solution was used as the elution solution. Column: Waters ODS-C18 column (4.6 mm × 250 mm, 5 μm); mobile phase: methanol-water-acetic acid (volume ratio = 54 : 45.2 : 0.8) mixture (pH 3.5), flow rate 1.0 mL min^{-1}; Column temperature: 40 °C; water 2,996 PDA detector; injection volume: 20 μL. Quantification was done through an external standard method. Hormone concentrations were expressed in microgram per gram of fresh sample (FW). Ginkgolide content was determined via large-caliber capillary gas chromatography separation and determination (Liao *et al*., 2008). Standard gingkolide A, ginkolide B, ginkolide C, and bilobalide were provided by the China National Institute for the Control of Pharmaceutical and Biological Products. Ginkgolide concentrations are expressed in microgram per gram of dry sample (DW).

Statistical analysis

Design of the experiments was completely randomized with three replications. All data were processed using Excel, SPSS 22 and OriginPro 9.0 software. Duncan method ($P < 0.05$) was used in comparing differences.

Results

Effect of GA_3 on endogenous IAA content in ginkgo leaves

Seasonal variation in the content of endogenous hormone IAA in ginkgo leaves treated with different concentrations of GA_3 was analysed. As shown in Figure 1, the IAA content of ginkgo leaves treated with different concentrations of GA_3 showed a similar trend over time. In the annual harvest period, the IAA values detected in each treatment level were significantly higher than those in the control group, and the annual peak appeared on July 25, when all treatment levels were at their peak. The IAA content of the 8 mmol L^{-1} treatment group was 45.59 μg g^{-1} FW, which was the highest content during the peak period of July 25. This hormone concentration was remarkably higher than the hormone concentrations in other treatment levels and then began to decrease. After IAA content dropped to the bottom of the valley on August 25, IAA began to rise again, rose to a secondary peak on September 10, and then gradually decreased until the end of sampling. IAA content in the 6 mmol L^{-1} treatment group showed no significant difference from that in the 8 mmol L^{-1} treatment group on July 25 and August 10 but was markedly lower than that in the 8 mmol L^{-1} treatment group in other sampling times. The longitudinal comparison of the whole harvest period showed that the annual average content of IAA at the 8 mmol L^{-1} treatment level was the highest, whereas IAA content in the 2 mmol L^{-1} treatment level was remarkably lower than the IAA contents in other treatment levels.

Effect of GA_3 on endogenous ABA content in ginkgo leaves

Changes in endogenous hormone ABA in ginkgo leaves were detected by treating ginkgo leaves with different concentrations of GA_3. It was shown in Figure 2 that after treatment with different concentrations of GA_3, the change trend of ABA content in ginkgo leaves was similar in different periods, and the difference between groups was small. The ABA values of each treatment level were considerably higher than ABA value in the control group during the annual harvest period. After a peak appeared on August 25, the ABA content in the later sampling period increased until the end of sampling. On August 25, September 10, and September 25, the ABA content of the 2 mmol L^{-1} treatment group was substantially higher than that of the control group, whereas no significant difference was found between the rest of the samples and the control group. ABA content in the 8 mmol L^{-1} treatment group was high during the whole sampling period and was significantly different from the control group. The highest ABA content was 6.39 μg g^{-1} FW in the 8 mmol L^{-1} treatment group during the peak period on August 25, when no significant difference was observed with other treatment groups. ABA content of the 4 mmol L^{-1} treatment group was five periods of that of the control group, whereas ABA contents of the 6 and 10 mmol L^{-1} treatment groups were six periods of that of the control group, and no significant difference was found between the remaining sampling tests and the control group. The differences between the 4, 6 and 10 mmol L^{-1} treatment groups were not significant, except that the ABA content in the 10 mmol L^{-1} treatment group was remarkably lower than that in the 6 mmol L^{-1} treatment group on July 25 and that in the 4 mmol L^{-1} on September 10; ABA content in the 4 mmol L^{-1} treatment group was significantly higher than that in the 6 and 10 mmol L^{-1} treatment groups on September 25; ABA content in the 10 mmol L^{-1} treatment group was significantly higher than ABA contents in the 4 and 6 mmol L^{-1} treatment groups on October 10. Longitudinal comparison of the whole harvest period showed the annual ABA content of each treatment level had no significant difference with the control group.

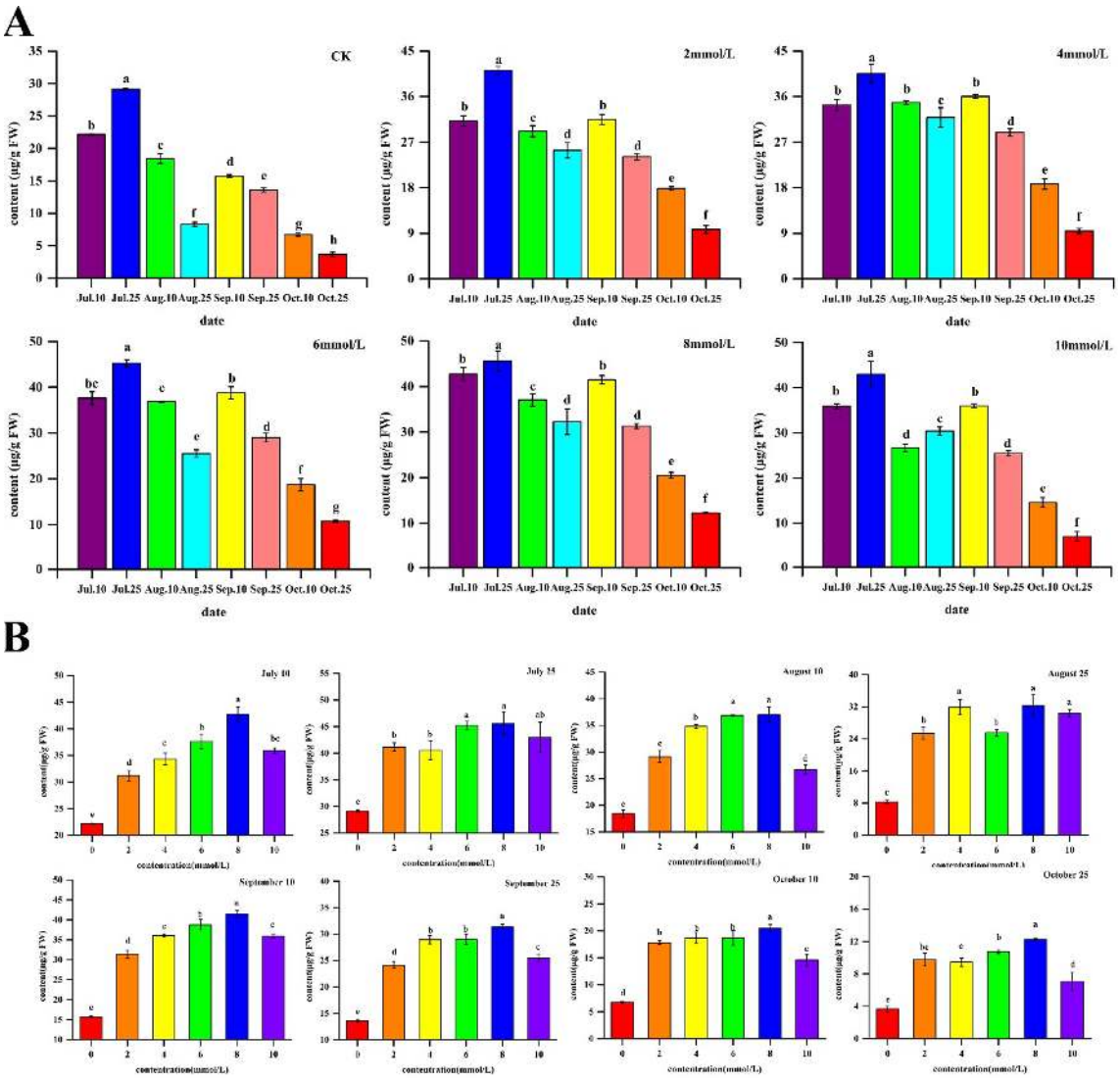

Figure 1. Effect of GA_3 on the content of endogenous IAA in ginkgo leaves. Endogenous IAA content in ginkgo leaves at different peroids (A) and endogenous IAA content in ginkgo leaves treated with different concentrations of GA_3 (B)

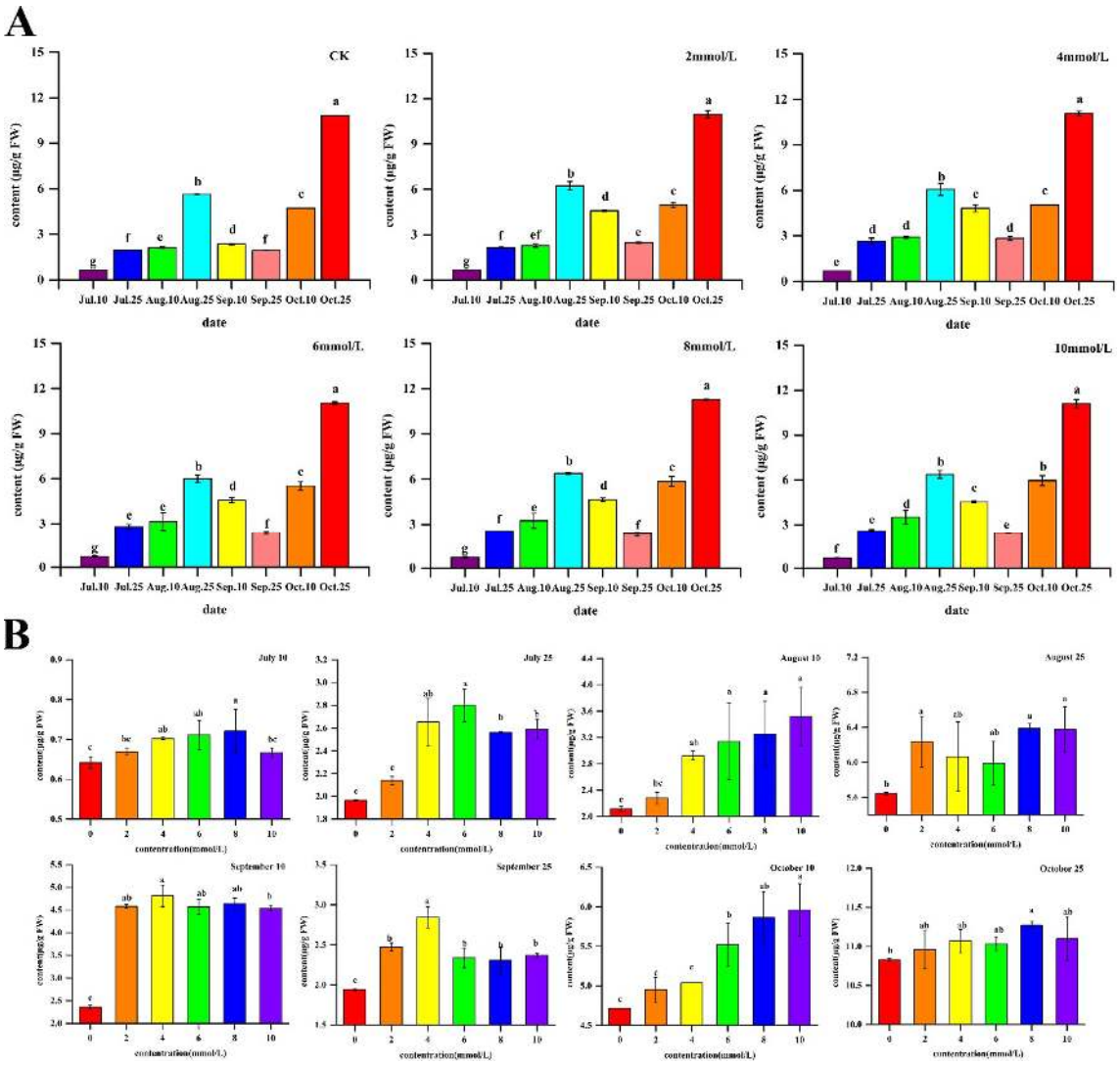

Figure 2. Effect of GA_3 on the content of endogenous ABA in ginkgo leaves. Endogenous ABA content in ginkgo leaves at different peroids (A) and endogenous ABA content in ginkgo leaves treated with different concentrations of GA_3 (B)

Effect of GA_3 on endogenous GA_3 content in ginkgo leaves

We analysed the effect of exogenous GA_3 on the content of endogenous hormone GA_3 in ginkgo leaves. As shown in Figure 3, the general variation trend of endogenous GA_3 content in ginkgo leaves treated with different concentrations of GA_3 was similar, but the endogenous GA_3 in the control group was significantly higher than that in the test treatment group. Results showed that the higher exogenous GA_3 concentration, the lower endogenous GA_3 detected in the treatment groups. Endogenous GA_3 content in the experimental and control groups began to decrease starting from the initial sampling but began to rise at the end of July until a peak appeared on August 10. Then, the endogenous GA_3 content of each treatment level began to decrease slowly until around September 10, when endogenous GA_3 content was the lowest. Afterwards, endogenous GA_3 rose again and reached a second peak on September 25. At the beginning of sampling (the whole month of July), the endogenous GA_3 content of the control group was significantly higher than that of the treatment groups, and the endogenous GA_3 content in the 2 and 4 mmol L^{-1} treatment groups was greatly higher than

that in the 6, 8, and 10 mmol L^{-1} treatment groups. Subsequently, the endogenous GA_3 content of each group began to rise; however, the content increase rate of the 8 mmol L^{-1} treatment group was higher than that of the 6 and 10 mmol L^{-1} treatment group. In August, the endogenous GA_3 content of the 8 mmol L^{-1} treatment group was substantially higher than contents in the 6 and 10 mmol L^{-1} treatment group. On September 10, the endogenous GA_3 content of the control group was lower than contents of the 2 and 4 mmol L^{-1} treatment group for the first time. Subsequently, the variation trend of GA_3 content in each group was similar to that in the control group. Longitudinal comparison of the mean value of the whole harvest period showed that the annual average endogenous GA_3 content was the lowest at the treatment level of 10 mmol L^{-1}.

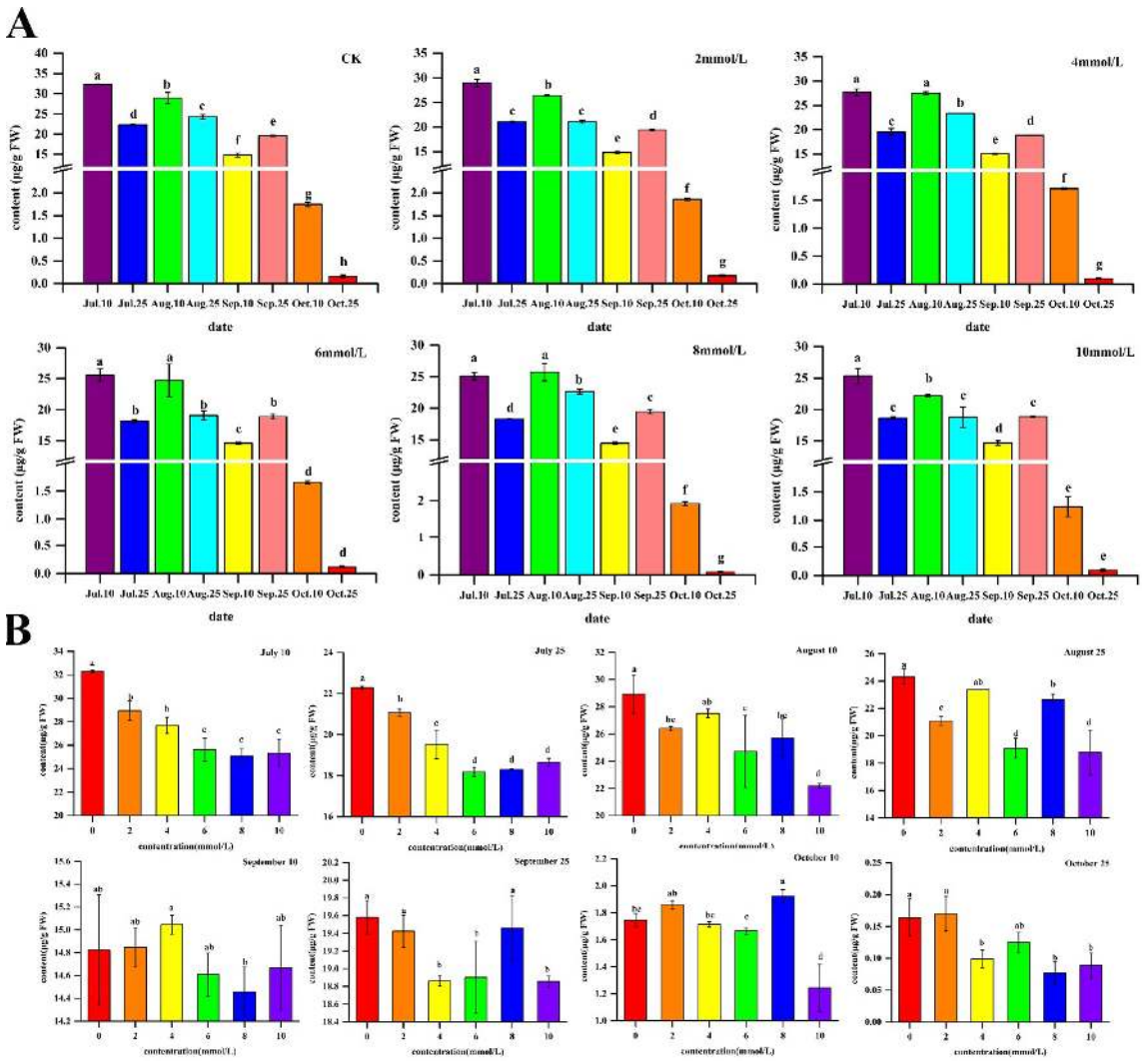

Figure 3. Effect of GA_3 on the content of endogenous GA_3 in ginkgo leaves. Endogenous GA_3 content in ginkgo leaves at different peroids (A) and endogenous GA_3 content in ginkgo leaves treated with different concentrations of GA_3 (B)

Effect of GA_3 on ginkgolide content in ginkgo leaves

We detected the ginkgolide content in *G. biloba* leaves treated with different concentrations of GA_3 to analyse the effect of GA_3 treatment on ginkgolide content. As shown in Figure 4, ginkgolide content under different concentrations of GA_3 showed similar trends in different periods. In the whole year of harvesting, the ginkgolide content of the 4 mmol L^{-1} treatment group was significantly higher than that of the control group, especially when the ginkgolide content reached the peak of the entire harvest season on August 25. At this time, the ginkgolide content in the 4 mmol L^{-1} treatment group was the highest (4,770 μg g^{-1} DW). Subsequently, the ginkgolide content of the treatment and control groups began to decrease. After reaching the "valley bottom" on September 10, the ginkgolide content of the 4 mmol L^{-1} treatment group was 4,010 μg g^{-1} DW, which was still higher than that of other treatment groups. By the time of sampling on September 25, the ginkgolide content reached the second highest value of the whole year, and the level of 4mmol L^{-1} (4,660 μg g^{-1} DW) was significantly higher than that of the other groups. This ginkgolide content was greatly higher than that of the other groups. Subsequently, the ginkgolide content of each treatment group began to decrease until the end of sampling. The ginkgolide content remained at a high level during the entire picking period in August and then began to decline. However, the ginkgolide content rose again in late September, reached the second peak of the whole year, and then began to decline. During the two peak periods on August 25 and September 25, the total terpene content of the 10 mmol L^{-1} treatment group was significantly lower than that of the control group. This result indicated that the 10 mmol L^{-1} treatment level might have exceeded the optimal treatment concentration. Compared with the whole harvest period, the annual average ginkgolide content of the 4 mmol L^{-1} treatment group was the highest.

Discussion

Effect of GA_3 on endogenous hormones in ginkgo leaves

In this study, the IAA contents of all experimental groups were significantly higher than that of the control group after exogenous GA_3 was sprayed. Giuliano *et al*. (1993) found that GA_3 can promote the synthesis and increase the content of IAA by inhibiting the production of IAA oxidase and preventing the decomposition of IAA. In this study, it was found that the difference of IAA content between the treatment group and the control group increased with the delay of time in the early sampling period. This difference may be due to the accumulation of synthesized IAA content after the inhibition of IAA oxidase activity under the influence of exogenous GA_3 at the early stage of treatment (Wu *et al*., 2001). At the same time, ABA gradually increased and inhibited the outward transport of IAA. Hence, the content of IAA in the treatment group was more different from the control group. However, the influence of exogenous GA_3 was gradually weakened, IAA oxidase was increased, and IAA metabolism was strengthened with the continuation of sampling. Therefore, the difference in content gradually decreased in the later sampling period. As shown in Figure 2, the ABA content of each treatment was approximately similar to the change trend in the control group. At the end of sampling, the trend of ABA content was similar between groups, and no significant difference was observed possibly due to the disappearance of exogenous GA_3 and the decrease of ambient temperature in the later stage. The ABA anabolic pathway with GGPP as the synthesis precursor returned to normal; therefore, the difference in ABA content gradually decreased, and this difference gradually disappeared by the end of sampling. Exogenous GA3 can inhibit the synthesis of GA3-oxidase and 3β-hydroxylase via feedback to inhibit the endogenous GA3 anabolic process, resulting in a decrease in its content (Zhu *et al*., 2007; Yamaguchi, 2008; Gil and García-Martinez, 2010). In our experiment, the endogenous GA_3 detected was significantly lower than the GA_3 content of the control group, which was consistent with the results of Guo *et al*. (2000).

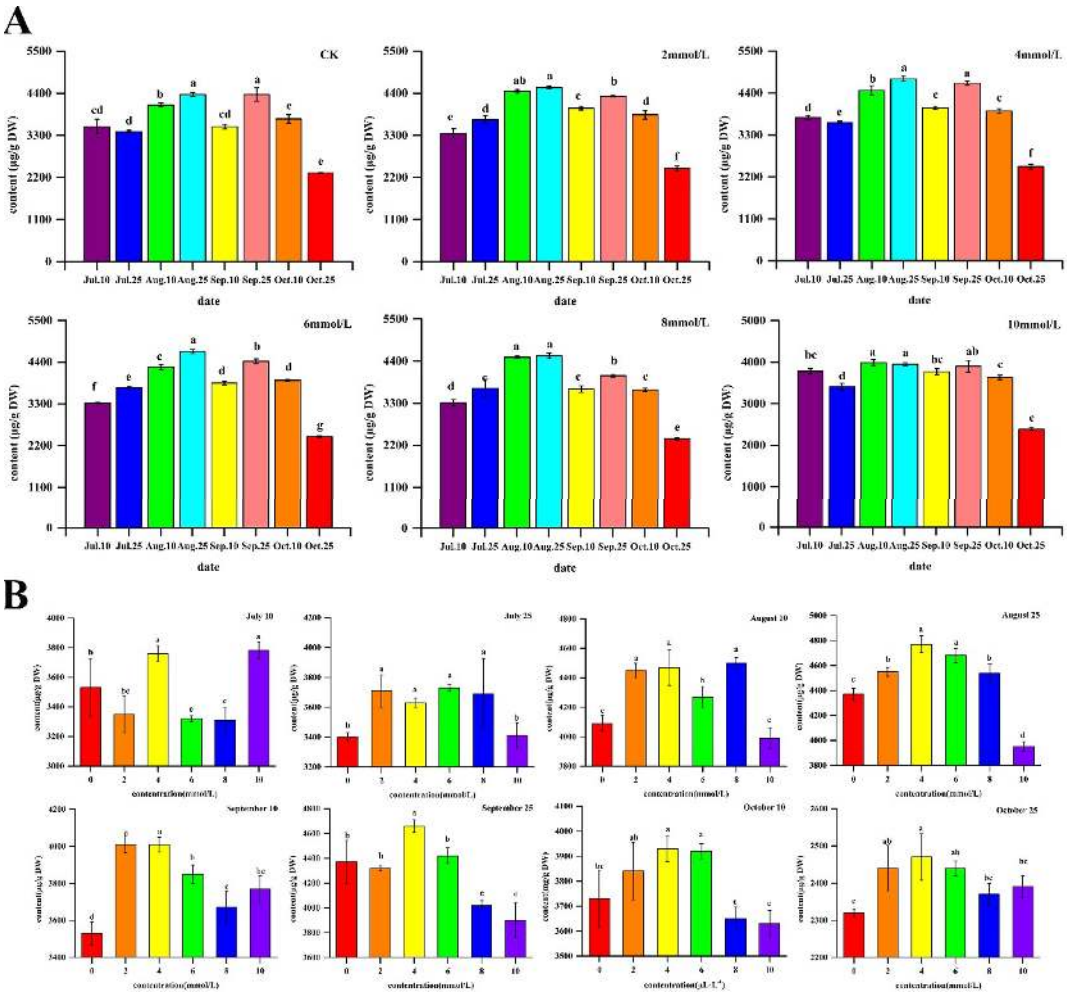

Figure 4. Effect of GA_3 on the content of endogenous ginkgolide in ginkgo leaves. Endogenous ginkgolide content in ginkgo leaves at different periods (A) and endogenous ginkgolide content in ginkgo leaves treated with different concentrations of GA_3 (B)

Effect of GA_3 on ginkgolide content in ginkgo leaves

GA_3 is a kind of functional growth-regulating hormone that can significantly promote plant growth, delay plant senescence, and stimulate plant cell division, including cell division and cell enlargement. GA_3 can promote DNA synthesis and reduce cell wall stretch ability; hence, current research focuses on the effects of GA_3 on watermelon growth cycle and fruit tree bearing (Ju and Wang, 2002). GA_3 can inhibit the accumulation of GA20-oxidase and 3-beta hydroxylase mRNA via feedback. The study of Xu *et al*. (2011) and Xie *et al*. (2002) showed that the spray of growth regulator on ginkgo had an effect on the content of ginkgolides.

The effect of exogenous GA_3 on ginkgolide content was multifold. First, exogenous GA_3 enhanced the photosynthesis of *G. biloba*. The study of Li *et al*. (2010) showed that applying exogenous GA_3 can improve the net photosynthetic rate and stomatal conductance of sugarcane. Therefore, exogenous GA_3 can affect the content of primary products and secondary metabolites of *G. biloba* and increase the carbon source of the plant. GA_3 provides more precursors for the synthesis of ginkgolides and finally improves the content of ginkgolides. Second, exogenous GA_3 can cause the feedback inhibition of the synthesis of GA3-oxidase and 3β-hydroxylase

to inhibit the anabolic processes of endogenous GA_3. In this experiment, endogenous GA_3 detected in treatment groups were significantly lower than the GA_3 content of the control group probably because the exogenous GA_3 feedback inhibited the synthesis of endogenous GA_3 and thus decreased its content. The inhibition of endogenous GA_3 synthesis increased GGPP, a substrate for the synthesis of ginkgolides. Therefore, ginkgolide content was increased. Third, IAA regulates the synthesis of GGPP in the DXP pathway, and although the concentration of precursors for GA_3 and ginkgolides was increased, the ginkgolide content synthesized from precursors was increased due to the inhibition of endogenous hormone synthesis.

From the analysis of each treatment concentration, ginkgolide content in the 10 mmol L^{-1} treatment group was remarkably lower than the control group during the two peak periods, August 25 and September 25. Therefore, this concentration may be higher than the optimal treatment concentration. The 4 mmol L^{-1} treatment group had substantially higher ginkgolide content than the other treatment groups at the two peak periods. The three concentrations (2, 6, and 8 mmol L^{-1}) had a lesser tendency to influence the level of ginkgolide content than the 4 mmol L^{-1} treatment, especially in the peak period of September 25. These three treatment groups (2, 6, and 8mmol L^{-1}) were not significantly different from that of the control group or lower than that of the control group Only the 4 mmol L^{-1} treatment group had significantly higher ginkgolide than the control and other treatment groups. Therefore, the 4 mmol L^{-1} treatment level may be the optimal treatment concentration.

In addition, exogenous GA_3 also has a certain impact on the optimal harvesting period of ginkgo leaves. Exogenous GA_3 had no obvious effect on the peak time of ginkgolides but remarkably increased the ginkgolide content, extended the harvest time of ginkgo leaves, and the ginkgolide content of more than 0.4% was changed from 40 days in the control group to 60 days in treatment groups, greatly increasing the harvesting time.

Conclusions

Although the effects of different concentrations of GA_3 on the ginkgolide content are different, it can be effectively used to extend the harvest time of ginkgo leaves. Further research is needed on the possible mechanisms of GA_3 regulation.

Acknowledgements

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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