

Effects of exogenous GA₃ and warm water on dormancy breaking germination characteristics of *Eucommia ulmoides*

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Abstract

Seeds of the plant *Eucommia ulmoides* Oliver, have prominent dormant characteristics. In the present study, seed dormancy and germination of *E. ulmoides* were investigated by treating them with exogenous GA₃ and different water temperatures. The results revealed that exogenous GA₃ and warm water soaking treatments were beneficial to the dormancy breaking and germination of seeds. The germination rate of seeds treated with 50°C water was 84.67%. In comparison, the seeds treated with 300 mg·L⁻¹ GA₃ had only a germination rate of 45%. This low germination rate resulted probably due to the low temperature of the GA₃ solution. Another important reason is the presence of eucommia gum in the seed coat of *E. ulmoides* which hinders the germination process. During germination, the SOD activity in the seeds treated with GA₃ (300 mg·L⁻¹) and warm water (50 °C) increased significantly. Increased SOD activity reduced the degree of oxidative damage of the plasma membrane and resulted a continuous decrease in TBARS content. Thereby, the seeds were prompted to develop in a direction conducive to germination. In addition, GA₃ (300 mg·L⁻¹) and warm water (50°C) treatments increased the content of endogenous GA₃, IAA, and ZR in the seeds, and decreased the content of endogenous ABA. This had resulted significantly higher ratios of GA₃/ABA, IAA/ABA and ZR/ABA than those ratios of distilled water (room temperature) treated plants. In short, 50 °C warm water treatment has a more marked germination promoting effect on *E. ulmoides* seeds.

Keywords: dormancy; endogenous hormones; enzyme activity; *Eucommia ulmoides*; germination; seed

Introduction

Eucommia ulmoides Oliver, is belonging to the Order Garryales and Family Eucommiaceae. It is a rare and valuable medicinal plant in the People's Republic of China. In China, the plant has been designated as a national second-class protected species, and is widely distributed in the subtropical to temperate regions. The provinces where the plant grows are Hunan, Guizhou, Sichuan, Henan and Shanxi (Wu *et al.*, 2019). China has a long history of knowledge and use of *E. ulmoides* for at least 2,000 years. The famous Chinese herbology 'Compendium of Materia Medica', published in the Ming Dynasty, has systematically preserved the documents on comprehensive knowledge and recorded the experiences on *E. ulmoides* from previous dynasties (Feng and Liang, 1996). Equipped with modern science and technologies, people now have re-discovered the medicinal

Received: 09 May 2023. Received in revised form: 25 May 2023. Accepted: 08 Jun 2023. Published online: 13 Jun 2023.

From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

value of *E. ulmoides*. In particular, the value of some secondary metabolites produced by *E. ulmoides* e.g., aucubin, pinoresinol diglycoside and chlorogenic acid, and these metabolites are the main components of antihypertensive drugs. Another secondary metabolite produced by this plant, syringin diglycoside, has the functions of enhancing memory, calming sedation, and lowering cholesterol (Li and Yan, 1986; Tang *et al.*, 1998). In addition to its exceptional medicinal value, *E. ulmoides* is also an important rubber-producing plant worldwide. Eucommia, as an isomer of natural rubber (Hamann *et al.*, 2002), has become the best natural polymer material alternative to natural rubber (Du *et al.*, 2003; Chen *et al.*, 2014). Furthermore, *E. ulmoides* is also a good timber plant species, and has been widely planted in urban gardening as an excellent ornamental plant as well.

Seedling cultivation by sowing, is one of the main means for producing *E. ulmoides* seedlings. But so far, the researchers have mainly focused on the dormancy and germination characteristics of seeds. However, there is no research on the physiological characteristics during the germination of seed. Shen and He (2005) revealed two reasons regarding the germination of *E. ulmoides* seeds, and these are the sensitivity to germination temperature and the mechanical restraint from the peel gum which restrict and hinder the germination of seeds and cause dormancy. Wu and Huang (1989) reported that the most suitable conditions for the germination of seeds are: mixed sand, wet storage and a temperature of 20-25 °C. The research of Zhou (2016) showed that soaking seeds with warm water or solutions of plant growth regulators (i.e., gibberellin) can significantly increase the germination rate. The seed germination process is accompanied by a series of complex and orderly physiological changes such as respiratory metabolism, metabolism and transformation of storage substance. Exploring the changes in enzymes and endogenous hormones involved in these physiological activities can provide insights into the germination and emergence mechanisms of *E. ulmoides* seeds.

Materials and Methods

In October 2018, seeds were collected from the *E. ulmoides* Planting Centre in Yichuan County, Henan Province. Healthy seeds were screened via soaking with tap water in a pot. The seeds were stirred thoroughly in the pot for 8-10 min. The floating seeds were discharged as abortive. The seeds remained in the bottom of the pot were rinsed with water, dried and used as the experimental material. Seeds having full shape and uniform size were selected and sterilised by soaking with 5% sodium hypochlorite solution for 10 min. The sterilised seeds were then rinsed with distilled water for three times and dried.

Seed germination assay

The dried seeds were soaked separately in two groups. Group 1: at room temperature (18 °C ± 2 °C), the seeds were separately soaked in solutions containing 0 (CK), 100, 150, 200, 250, 300 mg L⁻¹ GA₃ for 24 h. In this treatment CK is a distilled water control. Group 2: the seeds were put into beakers in a water bath setting temperatures at 0 (CK), 40, 50, 60 and 70 °C. In this treatment seeds of CK were incubated at room temperature and considered as control of the experiment. The germination assay was carried out by using the 'Petri dish/filter' paper method. The treated seeds were uniformly arranged in a 9 cm Petri dish (10 seeds · dish⁻¹) covered with double-layer filter paper. For each replicate, 5 Petri dishes were used. Each treatment had three replicates and placed in the growth chambers with a setting of 12/12 h and 25/20°C day/night cycle. The germination rate assay was performed for a total of 15 days. Germination of the experimental seeds were considered when the radicle length reached 1/2 of the seed length. The number of seed germinations was counted every day and distilled water was added regularly to keep the filter paper moist. The seed germination rate was calculated at the end of the experiment.

Determination of physical and biochemical indicators of sprouting

Different physiological indicators were determined at 0 (CK), 5, 10, and 15 d after the seeds treated with distilled water (room temperature; CK), 300 mg L⁻¹ GA₃ solution, and 50 °C warm water. Using the nitrogen blue tetrazole photoreduction and the thiobarbituric acid method to measure the superoxide dismutase (SOD) activity and the content of thiobarbituric acid (TBARS) (Zou, 2000; Li, 2000). Using enzyme-linked immunosorbent assay (EMSA) to measure the endogenous hormones gibberellin (GA₃), auxin (IAA), abscisic acid (ABA) and zeatin nucleoside (ZR) contents (Li, 2000), and calculate the ratios of GA₃/ABA, IAA/ABA and ZR/ABA.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences in the same index after with exogenous GA₃ or different water temperatures treatments. Statistical values are expressed as mean (\pm SE). All statistical analysis were carried out using SPSS 18.0.

Results

With the increase of GA₃ concentration, the germination rate of seeds increased rapidly (Figure 1). For the 300 mg·L⁻¹ treatment, the germination rate reached 45%, and it was significantly different from the CK ($P < 0.05$) (Figure 1). In addition, soaking seeds in warm water also significantly promoted the germination. But the germination rate gradually decreased with the increase of water temperature (Figure 1 A). Among them, the germination rate of the 50 °C warm water treatment reached its maximum value (84.67%) and was significantly higher than the CK ($P < 0.05$) (Figure 1 B).

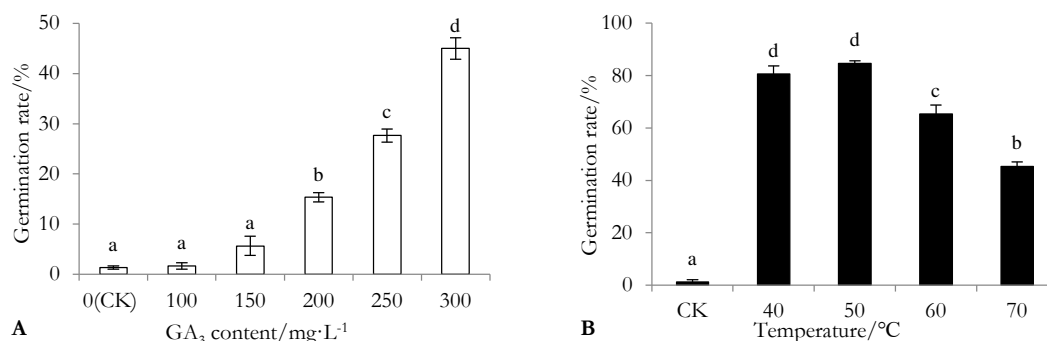


Figure 1. Effect of GA₃ (A) and warm water (B) soaking treatment on germination rate of *E. ulmoides* seeds

The values are shown as mean \pm SE. Different letters above the bars denote significant differences ($P < 0.05$) in germination rate with Duncan's test with different concentration of GA₃ content or different temperature of warm water treatment.

The SOD activity of seeds with different soaking treatments continued increased during the germination process (Figure 2). Among them, the SOD activity of GA₃ (300 mg L⁻¹) and warm water (50 °C) treatment showed significant differences compared to the CK on the 10th and 5th day respectively ($P < 0.05$) (Figure 2A). In addition, the SOD activity values of GA₃ (300 mg L⁻¹) and warm water (50 °C) treatments were significantly higher than that of distilled water (room temperature) treatment from the 5th day. The TBARS content of seeds with different treatments continued to decreased during the germination process (Figure 2B). Among them, the TBARS content of GA₃ (300 mg L⁻¹) and warm water (50 °C) treatment showed significant differences compared to the CK on the 10th and 5th day respectively ($P < 0.05$). However,

the difference between TBARS content in seeds treated by distilled water (room temperature) compared to CK did not reach a significant level on the 15th day.

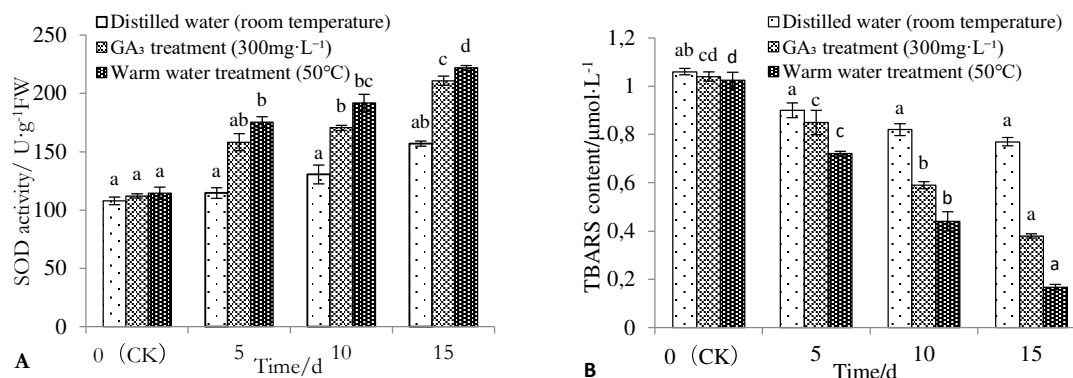


Figure 2. Changes of the SOD activity (A) and TBARS content (B) in *E. ulmoides* seed during germination stages with different soaking treatment

The values are shown as mean±SE. Different letters above the bars denote significant differences ($P < 0.05$) in SOD activity of TBARS content with Duncan's test with different treat time.

The contents of endogenous GA₃, IAA and ZR in the seeds of different soaking treatments increased continuously, while the content of ABA decreased continuously during the germination process (Figure 3). Among them, the ABA content decreased to a significant level ($P < 0.05$) compared to CK on the 15th day (Figure 3C), but the endogenous GA₃, IAA and ZR contents were not different from CK in distilled water treatment (room temperature). In addition, the endogenous GA₃ and IAA contents were significantly different from the control group on the 10th day ($P < 0.05$) (Figure 3A, B), but the ABA content was significantly different from the control group on the 5th day ($P < 0.05$) in GA₃ (300 mg·L⁻¹) and warm water (50 °C) treatments. Finally, the ZR content increased significantly compared to CK on the 15th day in the GA₃ (300 mg·L⁻¹) treatment ($P < 0.05$), while the warm water (50 °C) treatment showed a significant difference from the CK on the 5th day ($P < 0.05$) (Figure 3D).

The ratios of endogenous GA₃/ABA, IAA/ABA and ZR/ABA of the seeds under different soaking treatments continued to increase during the germination process (Figure 4). The endogenous GA₃/ABA and IAA/ABA of the distilled water (room temperature) treatment increased significantly compared to CK on the 15th day ($P < 0.05$) (Figure 4A, B), while the ZR/ABA values was not significant difference from the CK on 15th day (Figure 4C). In the treatment of GA₃ solution (300 mg·L⁻¹), endogenous GA₃/ABA and IAA/ABA increased significantly compared to CK on the 5th day ($P < 0.05$), while ZR/ABA values increased significantly compared to CK on the 15th day ($P < 0.05$). However, in warm water (50 °C) treatment, endogenous GA₃/ABA, IAA/ABA, and ZR/ABA values all increased significantly compared to CK on the 5th day ($P < 0.05$).

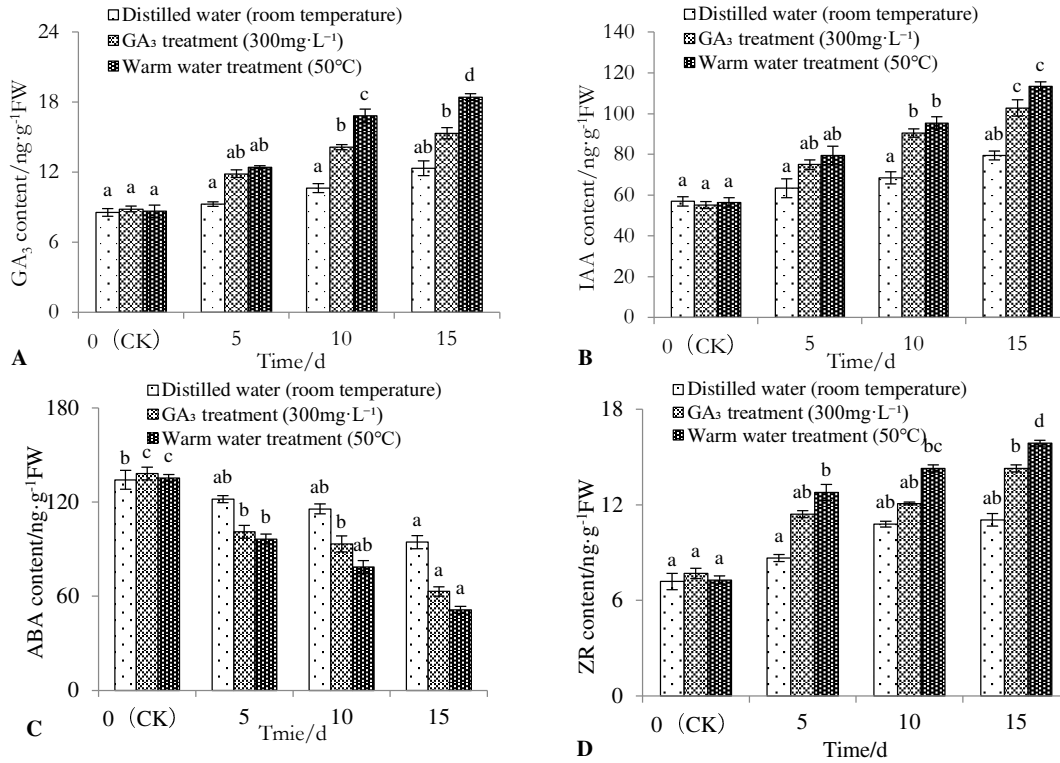


Figure 3. Changes of endogenous hormone GA₃ (A), IAA (B), ABA (C) and ZR (D) contents in *E. ulmoides* seed during germination stages with different soaking treatment

The values are shown as mean±SE. Different letters above the bars denote significant differences ($P<0.05$) in endogenous hormones content with Duncan's test with different treat time.

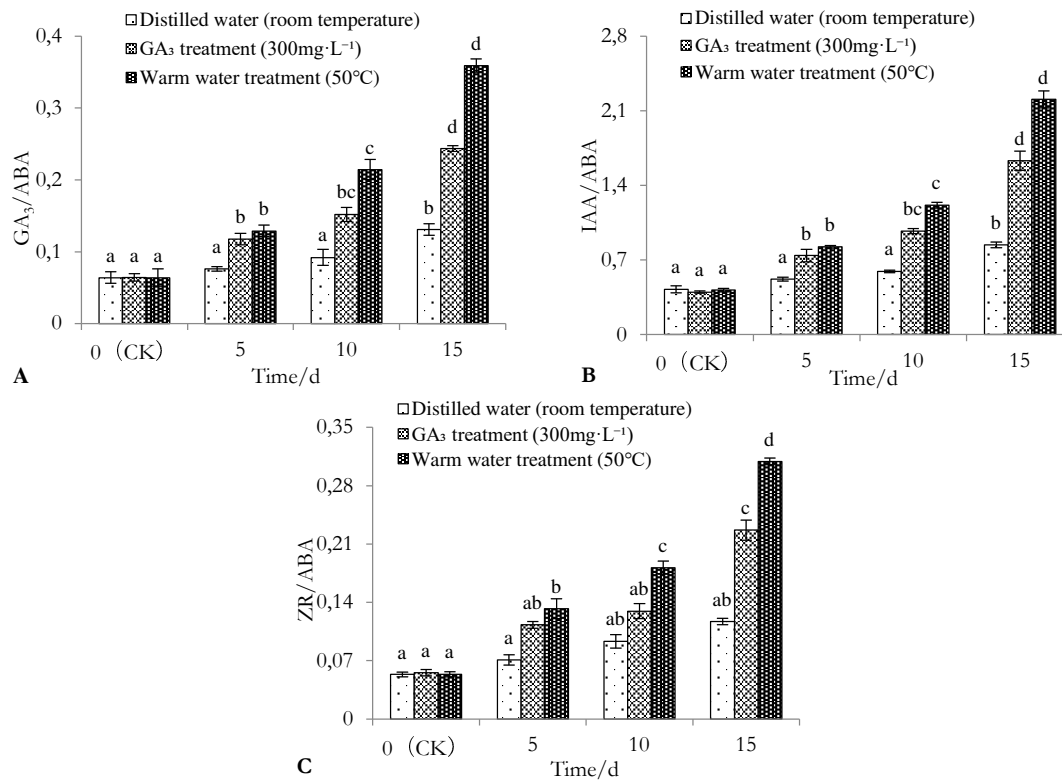


Figure 4. The ratios of endogenous GA₃/ABA (A), IAA/ABA (B) and ZR/ABA (C) in *E. ulmoides* seed during germination stages with different soaking treatment. The values are shown as mean±SE. Different letters above the bars denote significant differences ($P < 0.05$) in endogenous hormone ratio with Duncan's test with different treat time.

Discussion

Effects of different soaking treatments on the germination of seeds

Gibberellin, as a signaling substance, plays an important role in promoting seed germination (Hamdaoui *et al.*, 2021). A large number of studies have shown that exogenous GA₃ treatment is helpful for seed dormancy and germination (Lai *et al.*, 2017; Ma *et al.*, 2018; Shang *et al.*, 2019; Agostini *et al.*, 2022). In addition, soaking seeds in warm water has the effect of softening the seed coat and increasing the permeability of the seed coat. Therefore, warm water soaking at a suitable temperature is beneficial for seed germination (Chen *et al.*, 2017; Seng and Cheong, 2020; Yousif *et al.*, 2020). This study confirmed that exogenous GA₃ and warm water soaking treatments are beneficial to the dormancy breaking and germination of *E. ulmoides* seeds. However, when the concentration of GA₃ solution reached 300 mg·L⁻¹, the seed germination rate was only 45%, which was far from the result obtained by Zhou (2016). According to Zhou (2016), the germination rate of seed over 80% occurred at 150 mg·L⁻¹ GA₃ solution. The main reason for the low germination rate in this study was the selection of experimental time period (November - December) when a relatively low temperature (experimental room temperature 18 °C ± 2 °C) and short time prevailed. So, the soaking treatment with GA₃ solution, though, can soften the seed coat with the effect of replacing its physiological post-ripening to some extent, the optimum was not fulfilled in the present study. In this experiment, the seed soaking with warm water promoted the germination of seeds to a great extent. Among them, the germination rate of warm water treatment at 40 °C and 50 °C both reached more than 80%. Afterwards, as the water temperature continues to

rise, the germination rate of the seeds gradually decreases. This may be related to the fact that seeds have already entered the stage of water swelling are still in a high-temperature water environment, which damages the embryo of the seeds and causes some seeds to die. This result is consistent with the observations obtained from *Astragalus membranaceus* Bge. var. (Chen *et al.*, 2017) and *Swainsonia salsula* (Pall.) DC (Wang *et al.*, 2011).

Changes in physiological and biochemical characteristics of seed germination

During the germination process, various metabolic activities are initiated inside the seeds. These active metabolic processes will produce a large number of reactive oxygen species, which will cause certain oxidative damage to cells and tissues (Yang *et al.*, 2015; Farooq *et al.*, 2021; Zhang *et al.*, 2022). Plant antioxidant enzyme systems play an important role in removing and mitigating the damage caused by active oxygen to seed germination (Manjavachi *et al.*, 2022). SOD, as the primary substance for scavenging free radicals in plants, converts the more toxic $O^{\cdot 2}$ into less toxic H_2O_2 through a disproportionation reaction. In this study, during the germination process, the SOD activities in seeds treated with GA_3 ($300\text{ mg}\cdot\text{L}^{-1}$) and warm water (50°C) were significantly higher than that of the distilled water (room temperature) treatment. This indicates that different soaking treatments can significantly increase SOD activity, thereby reducing the damage of reactive oxygen species to the cell membrane system.

TBARS is an important indicator of the damage of plant plasma membranes. The changes in the membrane system will have a certain impact on seed viability (Xu *et al.*, 2022). In the present study, during the germination process, the TBARS content in the seeds of different treatments continued to decrease. The TBARS contents in seeds treated with GA_3 ($300\text{ mg}\cdot\text{L}^{-1}$) and warm water (50°C) were significantly lower than that of the distilled water (room temperature) treatment. These results indicate that different soaking treatments can reduce the degree of oxidative damage to the plasma membrane, which is beneficial for breaking seed dormancy.

Seed germination is closely related to changes in the content of endogenous hormones. Various hormones in seed can induce the decomposition and synthesis of storage substances and eventually cause seed germination (Ge *et al.*, 2023). Endogenous GA_3 accelerates the decomposition and synthesis of internal materials by increasing various enzyme activities, thereby promoting seed germination (Wang *et al.*, 2020). ABA has the effect of antagonizing GA_3 . By inhibiting the normal metabolism of nucleic acids and interfering with the synthesis of ribonucleic acids, the seeds cannot conduct normal metabolic activities, which eventually leads to dormancy of the seeds (Wang *et al.*, 2020; Gao *et al.*, 2021). In this study, the endogenous GA_3 contents of seeds with GA_3 ($300\text{ mg}\cdot\text{L}^{-1}$) and warm water (50°C) treatments were significantly higher than that in the distilled water (room temperature) treatment. It may be due to the continuous infiltration of external GA_3 or because soaking seeds in warm water caused more bound GA_3 in the seeds to gradually become free, and eventually led to an increase in endogenous GA_3 content. In addition, the endogenous ABA contents of seeds in different treatments were significantly lower than that in distilled water (room temperature) treatment, and they continued to decline during the germination process. This shows that exogenous GA_3 and warm water soaking treatment promote the increase of the endogenous GA_3 content of the seeds. The treatment also reduces the endogenous ABA content, thereby promoting the seed to develop in a direction favourable to germination.

There are different research conclusions about the relation between IAA and seed dormancy. Some studies have suggested that IAA can promote the termination of seed dormancy (Zhao *et al.*, 2020; Wu *et al.*, 2022). While others suggested that the content of IAA has little relation with the termination of seed dormancy (Lu *et al.*, 2014). ZR has an antagonistic effect on seed germination inhibitors, and to a certain extent it is negatively correlated with seed dormancy (Wang *et al.*, 2020). In this study, the contents of endogenous IAA and ZR in seeds treated with GA_3 ($300\text{ mg}\cdot\text{L}^{-1}$) and warm water (50°C) treatment were significantly higher than those treated with distilled water (room temperature). It again showed that exogenous GA_3 and

warm water-soaked seeds enhanced the content of endogenous hormones that promote germination and eventually led to the germination of *E. ulmoides* seeds.

In contrast to a single endogenous hormone, changes in the balance between different hormones are more important for seed dormancy and germination. Especially, the ratio between promoting and inhibiting growth hormones (Su *et al.*, 2018; Wang *et al.*, 2020). In this study, the ratio of GA₃/ABA, IAA/ABA, and ZR/ABA in seeds treated with GA₃ (300 mg·L⁻¹) and warm water (50 °C) were significantly higher than those treated with distilled water (room temperature). GA₃ and warm water soaking treatments not only increased the growth hormone content in the seeds, but also reduced the content of endogenous hormones that inhibit growth. Thereby creating internal conditions that were beneficial to the germination of seeds. In addition, starting from the 5th day, the endogenous GA₃, IAA and ZR contents, and the values of GA₃/ABA, IAA/ABA, and ZR/ABA were significantly higher than the GA₃ (300 mg·L⁻¹) treatment. On the other hand, while the ABA content, which is not conducive to seed germination, was lower than that of GA₃ (300 mg·L⁻¹) treatment.

Conclusions

Exogenous GA₃ and warm water soaking treatments were beneficial to the dormancy breaking and germination of seeds. But compared to 300 mg·L⁻¹ GA₃, 50°C warm water treatment has a more marked germination promoting effect on *E. ulmoides* seeds. This result is also consistent with the results of seed germination rate.

Authors' Contributions

NW wrote the manuscript and designed experiments; SSX performed out experiments; NW and SSX analysed the experimental results. Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research was supported by the Natural Science Foundation of Henan Province (182300410092) and Public Welfare Industry Special Research Projects of Luoyang (2022087).

Conflict of Interests

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

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