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METHODS FOR RAPID SEED GERMINATION OF *BROUSSONETIA PAPYRIFERA*

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Abstract

Seed germination is one of the main methods of reproduction and expansion of the distribution area of *Broussonetia papyrifera* in the wild. There are many factors affecting seed germination, such as sterilization, temperature, light and exogenous hormones. The sterilization treatment with 70% EtOH for 30 seconds and 0.1% $HgCl_2$ for six minutes show the best disinfection effect and the least toxicity. The optimum temperature and light for seed germination were 30 °C and 16 h/d light respectively. 6-Benzylaminopurine (6-BA) and gibberellins (GA_3) improved seed germination, and GA_3 had a better effect, especially at the concentration of 1600 mg/L. NAA had inhibitory effect on seed germination. Our study may help develop a seed germination system for *B. papyrifera* and be a valuable method for the conservation and regeneration of seedbanks.

Key words: *Broussonetia papyrifera*, Seed germination, Sterilization, Temperature, Light, Exogenous hormones.

Introduction

Broussonetia papyrifera is a member of Moraceae family and it is a common economic tree mainly found in the Asia-Pacific countries (Chang *et al*., 2015). *B. papyrifera* is a kind of raw material for paper production; hence, it is also known as "paper mulberry" (González-Lorca *et al*., 2015). *B. papyrifera* displays strong vitality and resistance ability (such as under the stress of heavy metals, dehydration and salt and alkali), and is widely used in medicine, as animal feed and for ecological remediation (Si *et al*., 2018; Ryu *et al*., 2019; Zhang *et al*., 2020). *B. papyrifera* reproduction includes sexual reproduction and asexual reproduction (Maan *et al*., 2020). Sexual reproduction mainly refers to the germination of seeds. Seed germination is one of the main reproduction methods of *B. papyrifera* in the wild, and also an important way to expand the distribution area of *B. papyrifera* (Maan *et al*., 2021). Researchers have studied the effects of sowing season, growth regulator and different maturity of fruit on seed germination of *B. papyrifera* (Yan *et al*., 2018; Xu *et al*., 2019). However, the poor germination issues of *B. papyrifera* seeds still persist.

As the first step in the growth of seed plants as well as a vulnerable and risky step (Kadereit *et al*., 2017), seed germination includes multiple steps, starting from the absorption of water by the dry seeds and ending with the embryonic axis emergence (Li *et al*., 2021). Wild seeds are often contaminated with several fungi and bacteria and do not germinate properly (Munkager *et al*., 2020). Further, many factors, such as sterilization, temperature and light, as well as exogenous hormones, affect seed germination (Cochavi *et al*., 2018; Sun *et al*., 2019). Gibberellins and cytokinins, which are two kinds of phytohormones, regulate the physiological and biochemical process of seed germination along with various changes in seed cells (Simlat *et al*., 2019; Li *et al*., 2020). Seeds treated with phytohormones before germination initiates biochemical changes in their cells (Cabello-Conejo *et al*., 2014). 1-naphthylacetic acid (NAA)can enhance the activity of peroxidase and catalase in seeds, improve the integrity of the cell membranes and promote germination (Li *et al*., 2018). The successful stimulation on the rate of *Calanthe hybrids* seed germination by NAA has previously been reported (Shin *et al*., 2011). However, the type of hormone, the concentration used, and the treatment duration will produce various responses.

Although the propagation of *B. papyrifera* in the wild is not affected to a considerable extent, the germination rate of the seeds is relatively low. In order to explore the influence of temperature, light and exogenous hormones (6-BA, GA_3 and NAA) on seed germination, the research conducted a controlled experiment close to reality. Our study will help build a seed germination system of *B. papyrifera* with an improved success rate and be a valuable method for conservation and regeneration of seedbanks.

Materials and Methods

Seed collection: *Broussonetia papyrifera* has a heterothallic and collective fruits (Zhang & Wu 1998). The fruit on every female plant in the wild is rich. Seed collection was conducted at the campus of Central South University of Forestry and Technology (CSUFT), Changsha (E:112°59′40.2″, N:28°8′4.2″), P.R. China, in September 2017 and August 2018. The collected fresh fruit of *B. papyrifera* were washed with tap water. After the pulp and impurities were removed, the seeds were dried at 25°C and then stored and sealed in glass bottles for later use (Fig. 1).

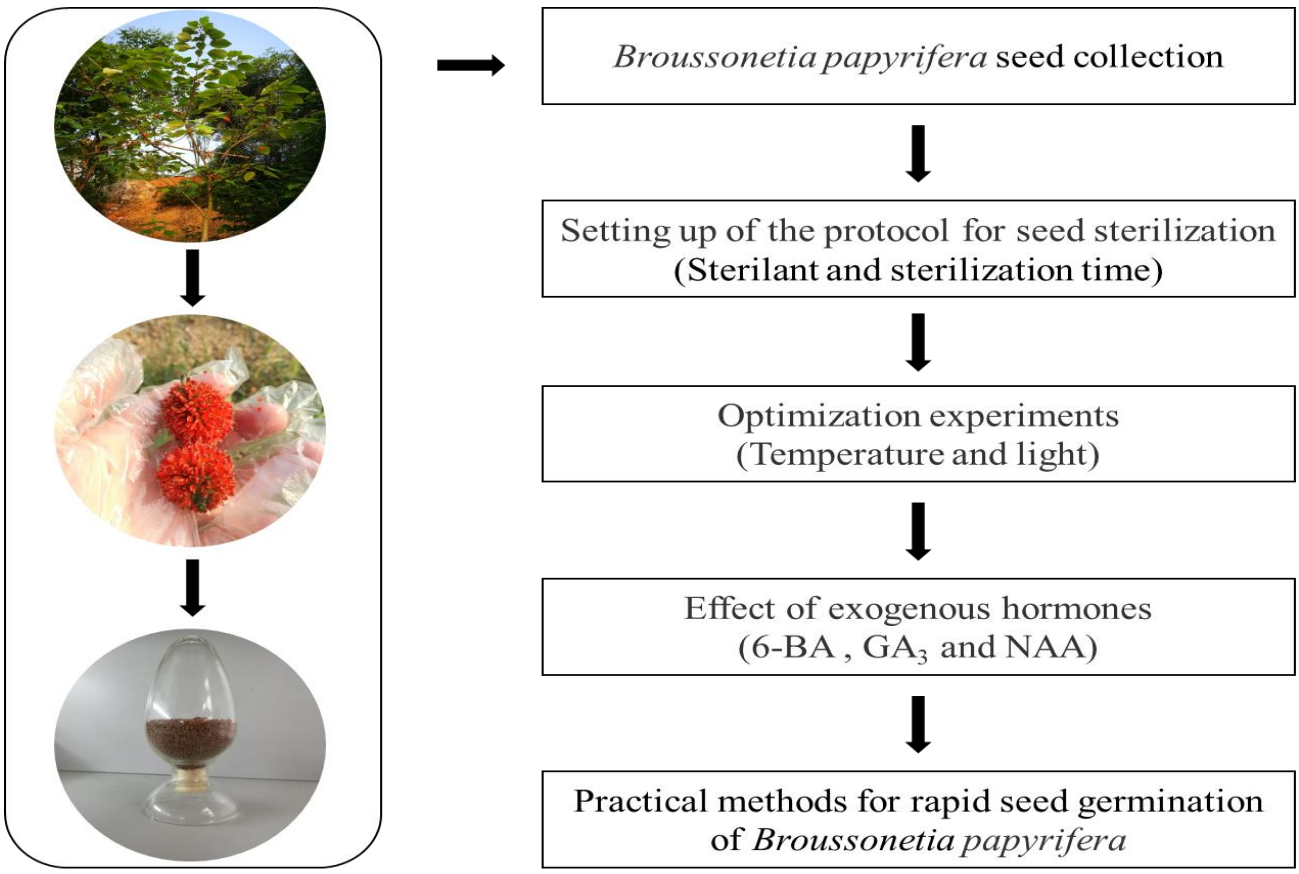


Fig. 1 The scheme for germination experiment of *B. papyrifera* seeds.

Setting up of the protocol for seed sterilization: Trials were conducted wherein the seeds were subjected to several sterilization treatments before germination. 100 seeds were placed on three layers of sterilized filter paper and in a Petri dish, steeped in clear water for 48 h, and then soaked in a combined sterilizer of 70% ethanol (ETOH) and 0.1% mercury ($HgCl_2$) within different time. The treatments were the following: (1) 70% ETOH for 30 seconds; (2) 0.1% $HgCl_2$ for three minutes; (3) 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for three minutes; (4) 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for six minutes; (5) 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for nine minutes; (6) 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for 12 minutes. In each trial, the cultured seeds were monitored each day in observation period of 14 days. On the half of observation period, the amount of moldy seeds during seed germination was counted to estimate the contamination rate (CR). On the final day, the amount of germinated seeds was counted to estimate germination rate (GR).

$$CR = \frac{n_0}{N} \times 100\% \qquad (1)$$

where n_0 and N are the number of contaminated seeds and the total number of tested seeds, respectively.

$$GR = \frac{n}{N} \times 100\% \qquad (2)$$

where n is the number of germinated seeds.

General germination test: The seeds were steeped with sterile water for 48 h and then soaked in sterilizing agent (70% ETOH for 30 seconds and 0.1% $HgCl_2$ for six minutes). Later, 50 seeds were placed in a tissue culture bottle containing five layers of sterilized filter paper for seed germination test. All culture bottles were placed in an artificial climate incubator at 30°C with a 16 h/d light. The light intensity was 2000 lux and the humidity was 75%. Upon the start of the first seed germination, the number of germinated seeds was recorded each in the observation period of 14 days. Six replicates were set in each treatment. Seeds were considered to have germinated when the radicle protruded prominently. Germination rate (GR), germination energy (GE) and germination index (GI) were calculated as follows.

$$GE = \frac{n_7}{N} \times 100\% \qquad (3)$$

where n_7 is the number of germinated seeds in the first 7 days.

$$GI = \sum \frac{G_t}{D_t} \qquad (4)$$

where G_t is the number of germinated seeds observed in a certain day and D_t is the corresponding germination day.

Optimization experiments: Seeds were incubated at constant temperatures of 25, 30 and 35°C for 14 days under 12 hours of light irradiation to determine the

optimum temperature for germination. The duration of light irradiation was optimized by probing the seed germination under 0/24, 8/16, 16/8 and 24/0 h light/dark treatments per 24-h cycle. Prior to these optimizing experiments, the seeds were steeped with sterile water for 48 hours and sterilized.

Effect of exogenous hormones: To determine the types and concentrations of exogenous hormones for germination, seeds were steeped with 6-BA (0, 5, 10, 50, 100, 200 mg/L), GA_3 (0, 200, 400, 800, 1200, 1600, 2000 mg/L) and NAA (0, 1, 5, 10, 25, 50, 100 mg/L) for 24 hours after sterilization. All the culture bottles were placed in an artificial climate incubator at 30°C with a 16 h/d light.

Statistical Analysis

Data were recorded and counted using the Excel 2010 software. The final germination percentage was expressed as the mean ± standard error (SE) of the six replicates. SPSS software (version 20.0) was used to perform a one-way ANOVA of variance on the data to evaluate all major effects. The significance of differences between treatment groups was tested by Duncan's test. Figures were drawn using Origin Pro 9.0.

Results and analysis

Seed sterilization: Different treatment duration combinations (70% EtOH and 0.1% $HgCl_2$) were used to sterilize the seeds. The results of CR and GR are shown in (Table 1). The CR calculated from treatment with a single sterilizing agent (EtOH or $HgCl_2$) was significantly higher than the treatment using dual sterilization agents. In the treatment of dual sterilization agents, the CR gradually decreased when the sterilization time using $HgCl_2$ was prolonged, and the CR and GR decreased to zero after 12 minutes. This indicates that sterilizing for too long poisoned seeds instead, which impeded the normal germination process. The best sterilization effect and the highest GR were seen with 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for six minutes. This sterilization method was used in subsequent experiments.

Effect of temperature: Fig. 2 shows the effect of temperature on germination potential of *B. papyrifera* seeds. GR, the most critical index for characterizing seed germination, was achieved highest in the *B. papyrifera* seeds at 30°C, reaching 31.6 ± 1.67%, which was 1.61 times and 2.87 times higher than that observed at 35°C and 25°C, respectively. GE, the statistical indicator of germination regularity of seeds, observed in the treatment of 30°C was 17.6 ± 2.61%, which was 1.76 times and 2.51 times higher than that at 35°C and 25°C, respectively. This also indicates that the GE and GR of *B. papyrifera* seeds showed the same trend under different temperature conditions. The early germination was neat, the seedling emerged quickly, and the later germination period was prolonged. GI, an indicator of seed vigor, at 30°C of *B. papyrifera* seeds was 5.41 ± 1.25%, which was 3.89 times and 2 times higher than that at 35 ° C and 25 ° C, respectively. This shows that temperature improves the vigor of *B. papyrifera* seeds and extends their germination period. Considering all the germination indices, 30°C was postulated to be the best temperature for the germination of *B. papyrifera* seeds.

Effect of light: *Broussonetia papyrifera* seeds can germinate under light and dark conditions, which indicates that light is not a limiting factor for the germination of *B. papyrifera* seeds. Under dark conditions, *B. papyrifera* seeds germinated normally, but the stems grew excessively, the cotyledon growth time was delayed, and the leaves were prone to yellowing because of the inability to photosynthesis (Fig. 3).

Figure 3 shows the effects of light on GR, GE and GI of *B. papyrifera* seeds. The GR of seeds was the highest at 26 ± 2.45% under the 16 h/d light treatment. Also, there were no significant differences in the germination rates between 8 h/d and 0 h/d light treatments. The GR observed in the 24 h/d light treatment was the lowest, at only 10%.

Different irradiation times had varied effects on the GE of *B. papyrifera* seeds. The GE value was the highest (19.2 ± 3.63%) after 16 h/d light treatment and the lowest after 24 h/d light treatments. This may be because the suitable temperature and continuous light exposure speed up the enzymic reactions inside the seeds, consume the stored nutrients, and enhance the germination of the seeds.

By comparing the effects of different light treatments on the GI of *B. papyrifera* seeds, it was found that although the GI of *B. papyrifera* seeds treated with 16 h/d light was higher than that of 0 h/d and 8 h/d, the difference was not significant (p>0.05). This shows that light has limited effect on improving the vigor of *B. papyrifera* seeds and prolonging their germination time. The GI of the 24 h/d light treatment was the lowest at 1.17%. Based on these results, 16 h/d light is proposed as the best light exposure for the germination of *B. papyrifera* seeds.

Table 1. Effects of different sterilizing agents and different sterilization time on germination of seeds of *B. papyrifera*.

Treatment	Sterilizing agents	Time	CR (%)	GR (%)
1	70% EtOH	30 s	100	0
2	0.1% $HgCl_2$	3 min	85	4
3	70% EtOH+0.1% $HgCl_2$	30 s+3 min	62.5	9
4	70% EtOH+0.1% $HgCl_2$	30 s+6 min	5.5	15
5	70% EtOH+0.1% $HgCl_2$	30 s+9 min	6.5	10
6	70% EtOH+0.1% $HgCl_2$	30 s+12 min	0	0

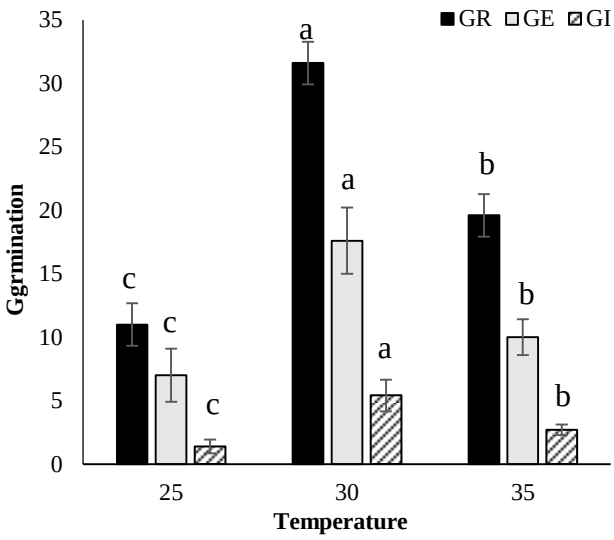
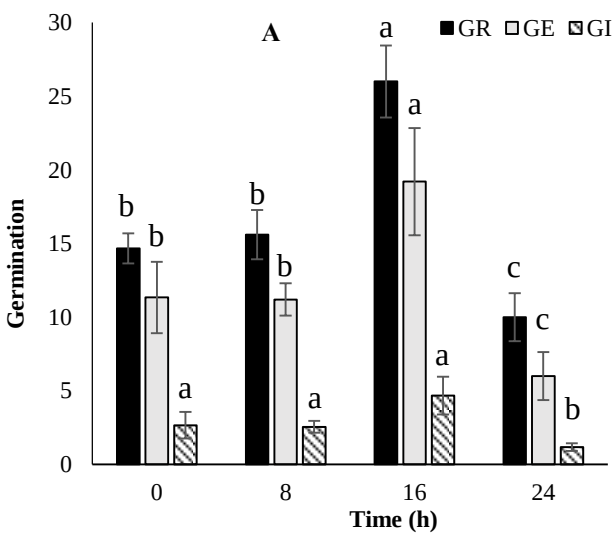


Fig. 2. Effects of temperatures on germination rate (GR), germination energy (GE) and germination index (GI) of *B. papyrifera* seeds.



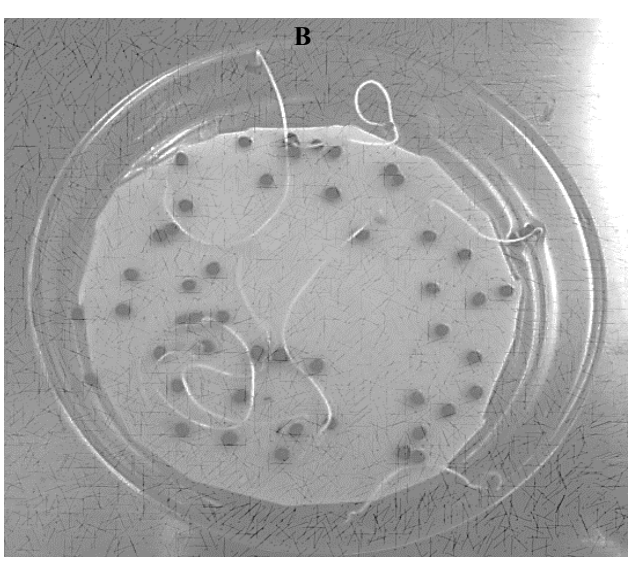


Fig. 3. Effects of light on GR, GE and GI of *B. papyrifera* seeds (A) and seed germination of *B. papyrifera* under dark conditions (B).

Effect of exogenous hormones: Exogenous hormones treatment is the most effective way to regulate seed germination efficiency and dormancy (Afroze and O'Reilly 2016). Different exogenous hormones have different effects on *B. papyrifera* seeds.

6-BA enhanced the germination of *B. papyrifera* seeds and the enhancement became higher until 6-BA concentration increased to 50 mg/L (Fig. 4). GR was the highest (21.5%) at a concentration of 50 mg/L. It was significantly different from all other treatment groups of 6-BA (p<0.01). The GR increased by 2.24 times compared with the control group. The GE and GI had the same trend as the GR and reached the maximum when the 6-BA concentration was 50 mg/L.

GA_3 at low concentrations (<1600 mg L^{-1}) enhanced the germination of *B. papyrifera* seeds, however, this enhancement decreased until the GA_3 concentration increased to 2000 mg L^{-1} (Fig. 5). When adding 1600 mg/L of GA_3, the GR of seeds was the highest as 72.4±1.67%, which was7.54 times higher than the control (p<0.01). The GR reached 59.6% and 61% at 2000 and 1200 mg/L, respectively. However, the growth time of cotyledons of the germinated *B. papyrifera* seeds treated with 2000 mg/L was prolonged, and the sprout growth was weak. This may be because the concentration of GA_3 was very high, which had a toxic effect on the germination and growth of the seedlings. Similarly, the addition of GA_3 increased the GE of *B. papyrifera* seeds. The GE of the 1600 mg/L treatment group was 52.4%, which was significantly higher than that of the other experimental groups (p<0.01). In addition, when the concentration of GA_3 was 1600 mg/L, the GI reached its the maximum value. The dormancy of *B. papyrifera* seeds was broken, and the germination time of *B. papyrifera* seeds was significantly shortened. Therefore, the germination cycle was reduced, and *B. papyrifera* seeds germinated more neatly.

Fig. 6 shows the effect of NAA on the germination of seeds of *B. papyrifera*. The GR of NAA treatment groups were significantly lower than that of the control group (p<0.05). As the increase of NAA concentration, the GR gradually decreased. The highest germination rate was obtained in the absence of NAA, and more pronounced inhibitory effects were seen as the concentration of NAA increased. GE and GI also showed a similar trend, indicating that NAA reduced germination vigor, delayed germination, and seedling emergence. In summary, NAA has a strong inhibitory effect on the germination of *B. papyrifera* seeds.

Discussion

Seed germination is one of the most complex and active periods of the plant life cycle (Wojtyla *et al*., 2016). The characteristics of germination varies from plant type (Anniwaer *et al*., 2020).Therefore, different measures should be taken to enhance seed germination. This study indicated that temperature, light, exogenous hormones, and several other factors significantly affected the germination of *B. papyrifera* seeds. The results obtained are of practical significance for establishing a scientific sowing system to break the dormancy of *B. papyrifera* seeds and promote germination.

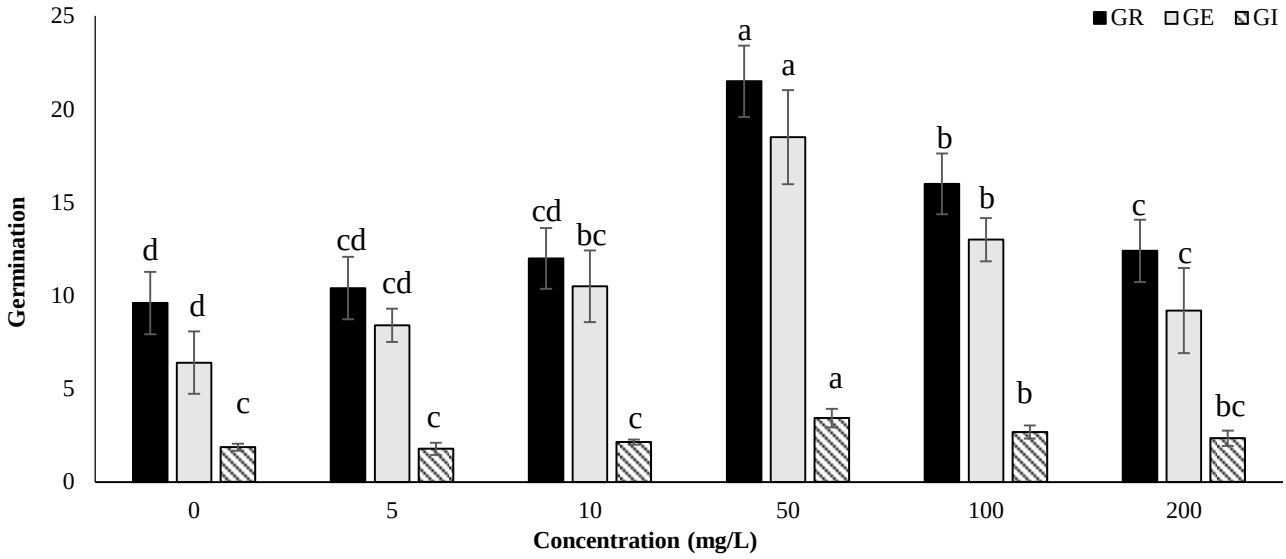


Fig. 4. Effects of 6-BA on GR, GE and GI of *B. papyrifera* seeds.

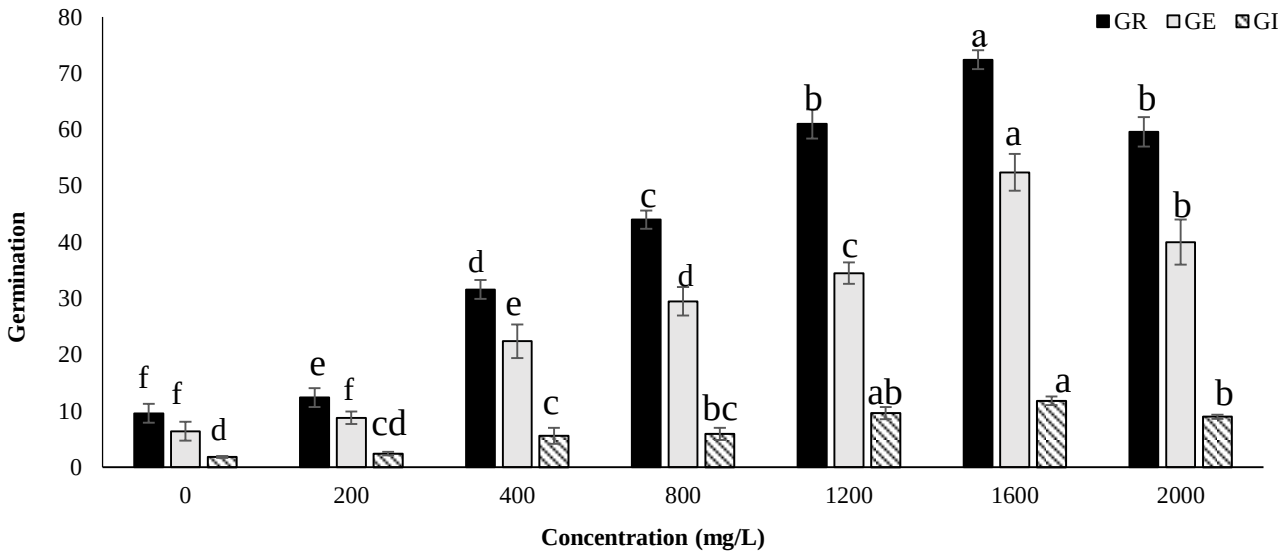


Fig. 5. Effects of GA_3 on GR, GE and GI of *B. papyrifera* seeds.

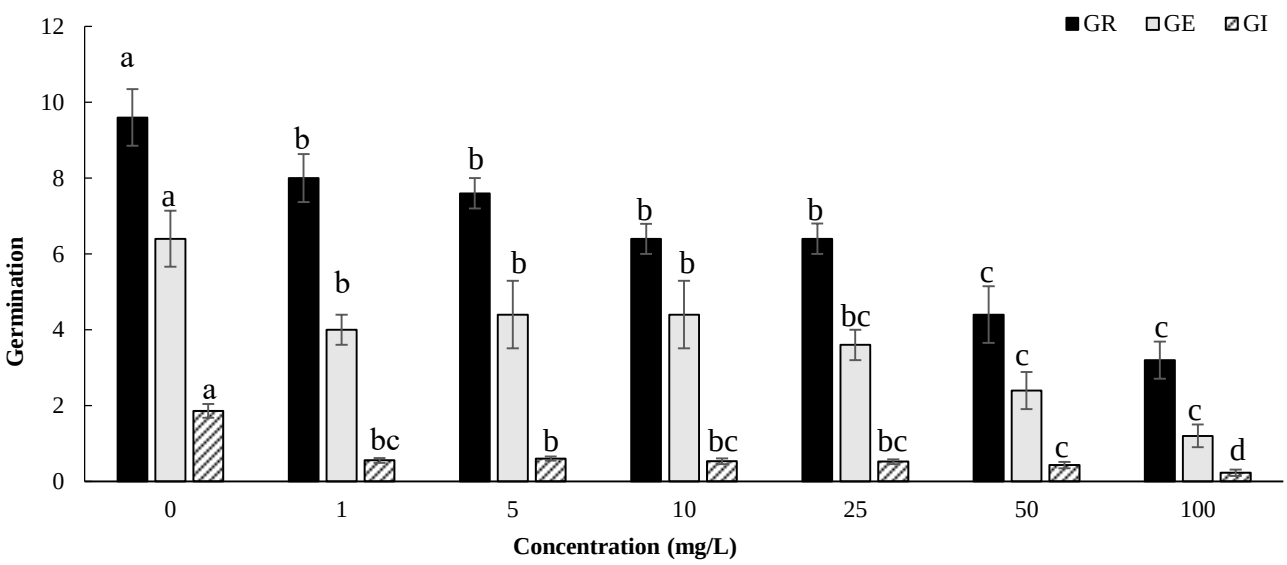


Fig. 6. Effects of NAA on GR, GE and GI of *B. papyrifera* seeds.

Seeds of *B. papyrifera* collected from the open field or germinated in the natural state are often loaded with microbes and cannot germinate. The GR of *B. papyrifera* seeds in the natural state was found to be approximately 5%, while that of seeds sown in the field was less than 4% (Ji *et al*., 2017). *B. papyrifera* seeds are easily contaminated and moldy during natural germination, resulting in extremely low germination rates. Ethanol, mercuric chloride, calcium hypochlorite, sodium hypochlorite and chlorine gas are commonly used for surface sterilization of plants and seeds (Barampuram *et al*., 2014). Using 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for six minutes, a significant reduction in the microbial load carried on the seeds was observed, thereby reducing the contamination rate during the seed germination process; and minimizing the toxicity to the internal structures of *B. papyrifera* seeds. The combination of EtOH and $HgCl_2$ sterilant has been used in the sterilization of tissue culture and seed germination of many plant such as *Narcissus pallidulus* and *Dendrobium aqueum*, however, the sterilization time varies (Parthibhan *et al*., 2017, Berkov *et al*., 2021).

Temperature is one of the dominant factors affecting seed germination (Wang *et al*., 2020, Xue *et al*., 2021), because it affects seed deterioration and can shorten dormancy period and other stages of germination (Teimori *et al*., 2021). Amylase and protease enzymes, along with specific metabolic processes in seeds, affect the germination process with temperature changes (Ozden *et al*., 2021). In this study, we found that 30–35 ° C is the most suitable for germination of *B. papyrifera* seeds, and the temperature of 30°C show the best result. Zhang *et al*.'s study show the same findings and the author also proved that the germination rate is higher when the seeds are germinated under the variable temperature condition of 30°C /20°C, in relation to the constant temperature condition at 20 °C (Zhang *et al*., 2012). Duan *et al*. found that the suitable germination temperature of *B. papyrifera* seeds in greenhouse is 24.5-27.5°C, and seeds cannot germinate normally under low temperature (Duan *et al*., 2013). Previous studies have shown that *B. papyrifera* seeds need high temperature conditions for germination. These results indicate that sowing seedlings in summer would be the most beneficial for the germination of *B. papyrifera* seeds due to the relatively higher temperatures in the season.

Seeds which need light during seed germination process are known as photoblastic seeds (Savaedi *et al*., 2019) Seeds that do not require light during germination are called shady seeds. Obviously, the *B. papyrifera* seeds are light-neutral seeds. They germinate successfully under both light and dark conditions. However, the germination of *B. papyrifera* seeds is blocked under dark conditions. The stems grow excessively, the cotyledons grow late, and photosynthesis cannot be carried out normally. This is consistent with the conclusion of Sun *et al*. who also found impeded germination of *B. papyrifera* seeds under dark conditions (Sun *et al*., 2007). Our study indicated that the 16 h/d light treatment significantly increased the GR of *B. papyrifera* seeds, which is also due to the characteristics of strong positive species of *B. papyrifera* (Miyazawa *et al*., 2014).

Exogenous phytohormones are essential in regulating plant seed germination and seedling growth, and development. The main source of energy during seed germination is the hydrolysis release of starch stored in endosperm (Zaynab *et al*., 2021). GA_3 is one of the phytohormones proposed to control primary dormancy by inducing germination (Darrudi *et al*., 2015). Our study showed that 6-BA and GA_3 improved the seed germination, and GA_3 had better effect, especially at the concentration of 1600 mg/L. As reported previously, GA_3 effectively relieved seed dormancy and increased germination rate of mulberry which belongs to Moraceae as same as *B. papyrifera* (Ye *et al*., 2015). In addition, GA_3 has different effects on different plant seeds. GA_3 inhibited the germination of *Nicotiana benthamiana* seeds (Wünschová *et al*., 2009). This finding corroborate those of H.R. KESHTKAR (Keshtkar *Et Al*., 2009) who found that higher concentrations (1000-2000 mg/kg) were more effective than lower concentrations (0-500 mg/kg). By contrast, lower GA_3 concentrations significantly improved germination of *Platycodon grandiflorum* (Zhao *et al*., 2006) and *Avena sativa* (Ge 2019). In this study, we confirmed that NAA delayed or inhibited the germination, reduced the germination rate, and prolonged the germination time of *B. papyrifera* seeds. However, it was confirmed that NAA had a positive regulatory effect on seed dormancy, and the inhibitory effects on germination increased with increasing NAA concentration.

Conclusion

This study establishes a system for the seed germination of *B. papyrifera* that includes sterilization, germination conditions and hormone treatment. The sterilization treatment with 70% EtOH for 30 seconds and 0.1% $HgCl_2$ for six minutes showed the best disinfection effect with the least toxicity on *B. papyrifera* seeds. Temperature, light and exogenous hormones had a different impact on the germination of seed of *B. papyrifer*a. The conditions under which seeds of *B. papyrifer*a could germinate efficiently were: (1) soaking with 70% ETOH for 30 seconds and 0.1% $HgCl_2$ for six minutes; (2) steeping with 1600 mg/L of GA_3 for 24 hours; (3) at 30°C; (4) under 16/8 h light/dark regimes per 24-h cycle.

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