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Dormancy-breaking treatments for enhancing seed germination in plant *Kitaibela vitifolia* Willd.

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Abstract: Vine-leaved kitaibelia (*Kitaibela vitifolia* Willd.), also known as balkanmalva or chalice flower, is a critically endangered plant species with a high risk of extinction in the wild. A reason given for this is, among others, a low germination rate primarily caused by dormancy. The present study evaluated the seed germination and seedling growth parameters of vine-leaved kitaibelia in response to eight different pre-sowing treatments. The final germination percentage ranged from 0 % to 55 %, depending on the pre-sowing treatment. The most effective method for breaking dormancy and increasing vine-leaved kitaibelia seed germination was the treatment with seeds soaked in H_2SO_4 for 5 min. The mechanical scarification of vine-leaved kitaibelia seeds also improved germination as compared to control treatment, while treatments with nitric acid and gibberellic acid were not effective in enhancing seed germination. All evaluated seedling growth parameters were not affected by pre-sowing treatments. Considering that successful germination and seedling establishment are crucial for the regeneration of vine-leaved kitaibelia further studies are required in order to identify other pre-sowing treatments that could further enhance seed germination and, consequently, seedling development.

Key words: climate chamber; greenhouse; habitat restoration; seedling growth; plant protection

Postopki prekinitve dormance za pospeševanje kalitve kitajbelovke (*Kitaibela vitifolia* Willd.)

Izvleček: Kitajbelovka (*Kitaibela vitifolia* Willd.), poznana tudi kot balkanski slezenovec, je kritično ogrožena rastlinska vrsta z veliko nevarnostjo izumrtja v naravi. Med drugim je razlog za to majhna sposobnost kalitve, ki jo povzroča dormanca. V raziskavi so bili ovrednoteni kalitev semen in parametri rasti kitajbelovke kot odziv na osem različnih predsetvenih obravnavanj. Odstotek kalitve je bil med 0 % in 55 %, odvisno od predsetvenega obravnavanja. Najučinkovitejša metoda prekinitve dormance kalitve pri kitajbelovki je bilo namakanje semen pred kalitvijo v H_2SO_4 za 5 min. Mehanska skarifikacija semen je tudi izboljšala kalitev v primerjavi s kontrolo med tem, ko obravnavanji semen z dušikovo kislino in giberilini nista bili učinkoviti pri pospeševanju kalitve. Na vse ovrednotene rastne parametre sejank predkalitveno obravnavanje ni vplivalo. Upoštevaje, da sta uspešna kalitev in uspešen razvoj sejank odločilna za obnavljanje kitajbelovke, je iskanje primernih predsetvenih obravnavanj ključno za pospeševanje kalitve in razvoj sejank te rastline.

Ključne besede: klimatska komora; rastlinjak; obnova habitata; rast sejank; zaščita rastlin

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1 INTRODUCTION

Bosnia and Herzegovina (BIH) is a country in South-eastern Europe, situated in the western part of Balkan Peninsula (Gekić et al., 2022). As a result of unique orography, special geological past and pedo-climatic conditions, BIH is one of the richest European countries in terms of plant species diversity (Redžić, 2007).

Unfortunately, plant diversity in the BIH has been declining at an alarming rate in recent years, mainly because of habitat loss, climate change and all forms of pollution resulting from human activity. Accordingly, there is a pressing need to preserve plant diversity, especially endemic plants that are usually more vulnerable to environmental changes. One of such plant species is vine-leaved kitaibelia (*Kitaibela vitifolia* Willd.) also known as balkanmalva or chalice flower.

The given plant belongs to family Malvaceae and genus *Kitaibela* that was named in honour of Paul Kitaibel, a famous Hungarian Botanist. The natural distribution of vine-leaved kitaibelia is Balkan Peninsula and in its natural surroundings can grow up to 3 m. The stems are robust, simple or sparingly branched, with unusual vine-like leaves and large showy cup-shaped white flowers. The 5-petaled flowers are about 5 cm across. The petals are slightly longer than wide, with cuneate to oblate form and rounded or slightly notched apex. The fruit is a schizocarp, consisting of numerous one-seeded mericarps usually separating at maturity (Šilić, 1984). Vine-leaved kitaibelia is endemic to the western Balkan peninsula and the area of distribution of this plant species is constantly decreasing, especially in Bosnia and Herzegovina. According to the 'Red list of endangered wild species and subspecies of plants, animals and fungi for Federation of BIH', vine-leaved kitaibelia is a 'critically endangered' plant species with a high risk of extinction in the wild (OG F BIH, 2014).

One of the potential limiting factors for the survival of wild plants in its natural environments is seed dormancy, which can be defined as the temporary blockade of the germination of a viable seed (Carrera-Castaño et al., 2020). Vine-leaved kitaibelia also produces seeds that are dormant upon maturity. Seed dormancy allows seeds to overcome periods that are unfavourable for seedling growth and is therefore important survival strategy of plants in their natural habitats (Li et al., 2022). On the other hand, insufficient level of seed germination or delayed germination may influence plant regeneration in its natural habitat (Chen, 2022).

There are two types of seed dormancy; exogenous and endogenous. Exogenous dormancy is caused by unfavourable conditions outside of the seed's embryo and is often broken down into three subgroups: (1) physical - caused by seed impermeability to oxygen or water, (2) chemical - caused by biologically inactive growth hormones that are present in the coverings around the embryo, and (3) mechanical - caused by hard seed that restrict growth. Endogenous dormancy occurs due to physiological or morphological changes within the seed's embryo. It includes: (1) physiological dormancy - caused by hormonal imbalance in the embryo, (2) morphological dormancy - caused by an undeveloped embryo, and (3) morphophysiological dormancy (combination of both) (Jursík et al., 2003).

Seed dormancy can be overcome by different pre-sowing dormancy-breaking treatments such us dry heat treatment, hot water treatment, mechanical scarification, chemical scarification, stratification and treatment with growth regulators (Phuyal et al., 2022). Physical, mechanical and chemical treatments reduce exogenous seed dormancy by removing or permeabilizing the seed coat, while stratification and treatment with growth regulators reduce endogenous seed dormancy by neutralizing seed germination inhibitors (Lavallee et al., 2021). However, inappropriate dormancy-breaking treatment can result in failure to break dormancy and at worst kill the seeds (Kildisheva et al., 2020). A better understanding of the mechanism and regulation of vine-leaved kitaibelia seed dormancy as well as dormancy breaking treatments ensures a greater chance for successful propagation of this plant, and thus for its preservation both in natural habitats and in ex-situ collections.

Therefore, the main goal of this study was to find out the appropriate dormancy-breaking treatments that maximize total germination and produce more vigour seedlings of vine-leaved kitaibelia. It was hypothesized that dormancy-breaking treatments applied in this study can facilitate the germination of the seeds in both the laboratory and greenhouse experiments.

2 MATERIALS AND METHODS

2.1 SEED COLLECTION

In this study, vine-leaved kitaibelia seeds were collected in September 2021 from the adult plant growing in the botanical garden which is part of the National Museum of Bosnia and Herzegovina (Latitude: 43.8563° N, Longitude: 18.4131° E). All seeds that were damaged or had irregular shapes, were removed. After collection, seeds were kept in paper bags in dark place at room temperature until further analysis.

2.2 DORMANCY-BREAKING TREATMENTS

Stratification (seeds exposed to low temperature), scarification (physically damage the seed coat to make it permeable to water and gases) and chemical methods (soaking the seeds in different chemicals) are the methods used for breaking seed dormancy (Kaur et al., 2020). In this study, the vine-leaved kitaibelia seeds were submitted to the following dormancy-breaking treatments: (T1) stratification at 4 ˚C for 7 days; (T2) immersion in 250 mg l^{-1} gibberellic acid solution (GA_3) for 24 h; (T3) immersion in 500 mg l^{-1} GA_3 solution for 24 h; (T4) immersion in 0.1 % HNO_3 for 24 h; (T5) immersion in 0.2 % HNO_3 for 24 h; (T6) mechanical scarification with sandpaper; (T7) immersion in sulfuric acid for 5 min; (T8) control treatment (without any seed manipulation). The volume of solution used in applied dormancy-breaking treatments was sufficient to completely submerge the seeds. Before any dormancy-breaking treatments, the seeds were soaked in distilled water for 24 hours.

2.3 GERMINATION EXPERIMENTS

Two experiments, i.e. lab experiment and greenhouse experiment, were conducted to evaluate the impact of different dormancy-breaking treatments on germination and seedling growth of vine-leaved kitaibelia. Germination test was emphasised in the lab (petri dish) experiment, while seedling emergence and vigour were emphasised in the greenhouse (pot) experiment. Both experiments were carried out as a randomized complete block design. In each experiment, a total of 8 treatments were tested, and 100 seeds per treatment were evaluated. Each treatment was replicated five times.

Germination tests were conducted by placing 20 seeds in a sterile plastic Petri dishes (9 cm diameter) lined within two sheets of filter paper. The filter papers in Petri dishes were moistened daily with distilled water during the experiment. To minimize the effect of environmental factors, germination test was carried out under controlled conditions in a growth chamber at 25 ˚C ± 3 ˚C and 80 % relative humidity under a 12 h photoperiod. Germination was considered complete once the radicle had protruded 2 mm in length (Tobe et al., 2005).

The following germination parameters were evaluated: seed germination time, germination pattern, percent germination, germination energy and shoot and seminal root length. Germination was recorded daily, from the day of sowing through to the end of the experiment. Shoot and seminal root length was measured manually with a graduated ruler at the end of the experiment (10th days of the experiment).

Seed germination time (SGT) represents the number of days from first observed germination to where there was no more germination. Germination pattern was determined by counting the number of seeds that germinated at the different days after sowing (Viswanath et al., 2002).

The percent germination (GP) illustrates the number of germinated seeds on the final day of a germination test and it was calculated according to Eq. 1:

$$GP = \frac{germinated\ seeds\ at\ the\ end\ of\ experiment}{total\ seeds} \times 100 \quad (1)$$

Germination energy (GE) is a measure of the speed of germination and hence, a measure of the vigour of seedlings. It represents the number of seeds that germinated within a definite period under optimum or stated condition. GE was determined on Day 5 after setting up the germination test (Elezz and Ahmed, 2021) and it was calculated according to Eq. 2:

$$GE = \frac{germinated\ seeds\ after\ 5\ days}{total\ seeds} \times 100 \quad (2)$$

In order to see if the positive response to dormancy-breaking treatments would occur outside the germination chamber, pot experiment with treated seeds was also performed. The pot experiment was carried out under controlled conditions, in the greenhouse located in Kakanj (Latitude: 44.1280° N, Longitude: 18.1178° E) from the mid-June 2022 to mid-September 2022. Seeds obtained through breaking-dormancy treatments were sown with 3 cm depth in each pot (6 cm diameter x 5 cm high) containing a compost and sand mixture (one seed per pot). All pots (30 pots per treatment) were placed in a greenhouse under the same conditions (temperature ranging from 15 to 28 °C with 75 % of relative humidity). The pots were irrigated as needed to keep the soil moist during the experiment. Number of emerged seedlings was counted at 30 days after planting, while the seedling height and number of leaves and flowers per seedlings were evaluated at the end of the experiment (90 days after the start of the experiment). Plant height was measured manually with a graduated ruler.

2.4 STATISTICAL ANALYSIS

All data collected was subjected to Analysis of Variance using Microsoft Excel software program. When Fisher's F values were significant, the analysis was continued by comparing the means using the least significance difference (LSD) test at the threshold of $p < 0.05$.

3 RESULTS

3.1 LAB EXPERIMENT

Generally, germination started early; in most pre-sowing treatments where germination occurred, the time period from sowing to the first emergence of seedlings ranged between four and six days. The only exception was in the sulphuric acid treatment where germination started on the third day after sowing. In all treatments where germination occurred, seed germination was completed within eight days from the beginning of the experiment, indicating that the vine-leaved kitaibelia seed germination time lasted from 3 to 4 days, depending on the pre-sowing treatment. Most pre-sowing treatments reached their peak germination 4th or 5th day after sowing. In this study, pre-sowing treatment with sulphuric acid for five min (T7) resulted in a higher germination compared with other seed-dormancy-breaking treatments. The mechanical scarification of vine-leaved kitaibelia seeds (T6) also improved germination as compared to control treatment. Unfortunately, in this study pre-sowing treatments with GA_3 and HNO_3 (T2 - T5) did not significantly improve the seed germination. Moreover, germination was completely inhibited when the seeds treated with 250 mg l^{-1} GA_3 (T2) and 0.2 % HNO_3 (T4) (Table 1).

The highest percentage germination of vine-leaved kitaibelia seeds (55 %) was observed in treatment with seeds soaked in H_2SO_4 for 5 min (T7). The next best results were found in mechanical scarification treatment (T6) and cold stratification treatment (T1). Germination was 13.3 % and 6.7 %, respectively for these treatments, not differing among them. The lowest germination percentages were obtained by treatments T2, T4, T8, T3 and T5, which presented germination varying from 0 to 3.3 %, respectively, with no statistical difference.

Germination energy was determined on Day 5 after setting up the germination test and it varied from 0 % to 43.3 % among the pre-sowing treatments. The highest germination energy (43.3 %) was calculated for T7, followed by T6 (13.3 %) and T1 (5 %) (Table 1).

Table 1: Germination percentage and germination energy in response to different pre-sowing treatments

Pre-sowing treatment	Percent Germination (%)	Germination Energy (%)
T1 (stratification at 4 ˚C for 7 days)	$6.7 \pm 15.0^{bc*}$	5 ± 2.0^{c}
T2 (GA_3 250 mg l^{-1} for 24 h)	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
T3 (GA_3 500 mg l^{-1} for 24 h)	3.3 ± 5.0^{c}	0.0 ± 0.0^{c}
T4 (0.1 % HNO_3 for 24 h)	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
T5 (0.2 % HNO_3 for 24 h)	3.3 ± 5.0^{c}	3.3 ± 2.0^{c}
T6 (mechanical scarification)	13.3 ± 5.0^{b}	8.3 ± 3.5^{b}
T7 (sulfuric acid treatment for 5 min)	55.0 ± 15.0^{a}	43.3 ± 5.0^{a}
T8 (control treatment)	1.7 ± 5.0^{c}	1.7 ± 2.5^{c}
$LSD_{0.05}$	7.9	1.8

Averages denoted by the same letter indicate no significant difference ($p \leq 0.05$)

Table 2: Effects of different pre-sowing treatments on shoot and seminal root length

Pre-sowing treatment	Shoot length (mm)	Seminal root length (mm)
T1 (stratification at 4 ˚C for 7 days)	9.0 ± 6.0^{a}	15.0 ± 6.0^{ab}
T2 (GA_3 250 mg l^{-1} for 24 h)	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}
T3 (GA_3 500 mg l^{-1} for 24 h)	9.1 ± 7.4^{a}	15.3 ± 9.6^{ab}
T4 (0.1 % HNO_3 for 24 h)	0.0 ± 0.0^{b}	0.0 ± 0.0^{c}
T5 (0.2 % HNO_3 for 24 h)	8.4 ± 6.1^{a}	14.3 ± 6.1^{b}
T6 (mechanical scarification)	10.2 ± 5.3^{a}	20.0 ± 8.9^{a}
T7 (sulfuric acid treatment for 5 min)	10.6 ± 6.0^{a}	17.1 ± 7.3^{ab}
T8 (control treatment)	10.3 ± 5.8^{a}	18.3 ± 6.6^{ab}
$LSD_{0.05}$	4.7	5.3

Averages denoted by the same letter indicate no significant difference ($p \leq 0.05$)

At the end of the experiment (10 days after the start of the germination test), plumule and radicle length were determined for sprouted seeds of each petri dish (Table 2).

The longest mean shoot was noted in T7 (treatment with H_2SO_4 for 5 min), followed by T8 (control treatment) and T6 (mechanical scarification); however, no significant difference was observed between the treatment where germination occurred in regards to shoot length. The longest mean seminal root was also noted in T6, T7 and T8 treatments, not differing among them. Statistically significant difference in seminal root length between all pre-sowing treatments where germination occurred was only observed between T7 (seeds soak in H_2SO_4 for 5 min) and T5 treatment (seeds soak in 0.2 % HNO_3 for 24 h).

3.2 GREENHOUSE EXPERIMENT

Effect of pre-sowing treatments on vine-leaved kitaibelia seed germination, evaluated in the greenhouse, is presented in Figure 1. Among the eight applied pre-sowing treatments, only three of them (T1, T6 and T7) stimulated seed germination.

The highest percentage germination (65 %) was observed in T7 (sulfuric acid treatment for 5 min), followed by T6 (mechanical scarification) and T1 (stratification at 4 °C for 7 days). Interestingly, the mechanical scarification and seed treatment with sulfuric acid for 5 min resulted in greater germination in potted soils than in Petri dishes, while the seed stratification at 4 °C showed an opposite trend; i.e., germination was higher in Petri dishes.

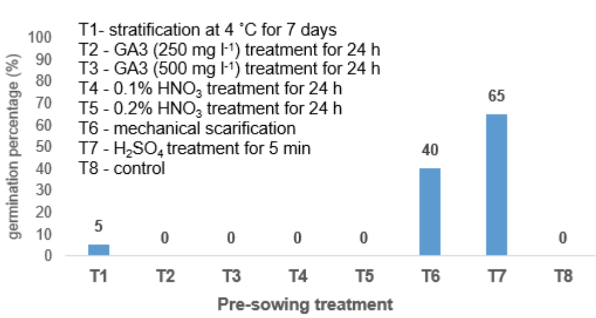

Figure 1: Vine-leaved kitaibelia seed germination in response to different pre-sowing treatments

Effect of pre-sowing treatments on growth parameters of vine-leaved kitaibelia seedlings in the pot experiment is presented in Table 3. Seedling height as well as the number of leaves and flowers per seedlings were evaluated at the end of the experiment (90 days after the start of the greenhouse experiment).

The highest mean seedling height was noted in T6 (mechanical scarification), followed by T1 (stratification at 4 °C for 7 days) and T7 (sulfuric acid treatment for 5 min); however, no significant difference was observed between them. Furthermore, these pre-sowing treatments (T6, T1, T7) had no significant influence on the number of leaves and flowers of vine-leaved kitaibelia seedlings.

4 DISCUSSION

Many wild plants, including vine-leaved kitaibelia, produce seeds that require a period of dormancy before they will germinate (Wang et al., 2021). Since the seed dormancy can reduce the vine-leaved kitaibelia restoration success, it is very important to identify the most efficient method for overcoming seed dormancy. Among the eight pre-sowing treatments applied in this study, only few of them significantly stimulated vine-leaved kitaibelia seed germination, and that is, T7 (sulfuric acid treatment for 5 min), T6 (mechanical scarification) and T1 treatment (stratification at 4 °C for 7 days). The most efficient treatment to break vine-leaved kitaibelia seed dormancy was treatment with sulfuric acid in both laboratory and greenhouse experiment. It seems that immersion seeds in concentrated sulfuric acid remove or permeabilize the seed coat, leading to a significant increase in seed germination as compared to control and other dormancy-breaking treatments. It could lead to a conclusion that dormancy in vine-leaved kitaibelia seeds is imposed by seed coat impermeability which prevents oxygen and/or water permeating into the seed or by hard seed coat which mechanically restricts embryo expansion, thus preventing shoot and seminal root emergence. Seed dormancy caused by an impermeable or hard seed

Table 3: Seedling growth parameters in response to different pre-sowing treatments

Pre-sowing treatment	Seedling height (cm)	Number of leaves per seedlings	Number of flowers per seedlings
T1 (stratification at 4 °C for 7 days)	81.9 ± 13.1	43.3 ± 20.0	1.1 ± 4.0
T6 (mechanical scarification)	82.4 ± 18.0	40.6 ± 19.0	1.4 ± 5.0
T7 (sulfuric acid treatment for 5 min)	80.9 ± 14.4	38.1 ± 18.0	1.8 ± 4.0

coat is known as physical dormancy, and that is, the major type of exogenous dormancy (Baskin et al., 2006).

Interestingly in this study, the seed germination tests with 0.1 % and 0.25 % nitric acid did not enhance vine-leaved kitaibelia seed germination as compared with control treatment. The assumption is that the low concentration of nitric acid caused inadequate removal of seed coat, resulting with poor seed germination. The results of the study also showed that the mechanical scarification with sandpaper had a significant effect on germination as compared to control treatment. These findings support the hypothesis that mechanical scarification can cause cracks in the seed coat, thus allowing for water movement to the embryo to trigger germination (Salazar & Ramírez, 2018). Similar findings were reported by Orsenigo et al. (2019) and Gao et al. (2021).

In this study, there were no significant differences in germination percentage and germination energy among treatments with GA_3 (T2 and T3) and control treatments (without any seed manipulation) in both laboratory and greenhouse experiment. The data suggested that the GA_3 in concentration of 250 mg l^{-1} and 500 mg l^{-1} failed to overcome dormancy of vine-leaved kitaibelia seeds. Tang et al. (2019) also found that treatments with gibberellic acid did not improve the seed germination of *Sorbus alnifolia* (Siebold & Zucc.) K. Koch. Similar results were obtained by Stejskalová et al. (2015) in sycamore seeds. Contrastingly, there are many studies that have demonstrated improved seed germination by treatment with GA_3 (Gashi et al., 2019; Cornea-Cipcigan et al., 2020; Uçarli, 2021; Guariz et al., 2022).

Many researchers agree that the hormonal signals, especially those of abscisic acid (ABA) and gibberellic acid (GA), play a dominant role in the regulation of seed dormancy and germination. ABA and GA are widely recognized as essential endogenous factors that regulate seed germination; ABA represses seed germination, while GA release seed dormancy and promote germination (Tuan et al., 2018; Ali et al., 2022). It is also well known that changes in the balance of a seed's ABA and GA levels can exert a significant influence in a seed germination (Finkelstein et al., 2008). However, in the present study, increased GA content did not improve the germination of vine-leaved kitaibelia seeds. On the basis of the above, it can be assumed that the dormancy in vine-leaved kitaibelia seeds is primarily controlled by factors outside the embryo. However, to our knowledge, there is no study that has examined the influence of GA treatments or any other pre-sowing treatments on vine-leaved kitaibelia seed germination. Further investigation is therefore needed to investigate the impact of the present findings.

In this study, cold stratification i.e., seeds stored at 4 °C for 7 days, enhanced the vine-leaved kitaibelia seed germination as compared to control in both laboratory and greenhouse experiment; however, the germination percentage and germination energy of cold stratified seeds was significantly lower than that of seeds treated by sulfuric acid or mechanically scarified seeds.

The results obtained in this study also showed that there were no significant differences among pre-sowing treatments in regards to shoot length in vine-leaved kitaibelia seeds. A similar pattern was found for seminal root length. Statistically significant difference in seminal root length between all pre-sowing treatments where germination occurred was only observed between T7 (sulfuric acid treatment) and T5 (0.2 % nitric acid treatment).

In this study, no significant differences were observed for all evaluated vine-leaved kitaibelia seedling growth parameters (seedling height and number of leaves and flowers) among pre-sowing treatments. This implies that the pre-sowing treatments may improve seed germination; however, this does not necessarily lead to improved seedling growth and development. The results of the present study are consistent with this hypothesis.

5 CONCLUSIONS

In general, the results revealed that the seed treatment with sulfuric acid was the most effective pre-sowing treatment from the view of seed germination both in the laboratory and greenhouse experiment. The mechanical scarification of kitaibelia seeds also improved germination as compared to control treatment, while treatments with nitric acid and GA_3 were not effective in enhancing seed germination. All evaluated vine-leaved kitaibelia seedling growth parameters were not affected by pre-sowing treatments. Future experiments should focus on identifying other pre-sowing treatments that could further enhance vine-leaved kitaibelia seed germination. The mechanism of seed breaking dormancy and germination of vine-leaved kitaibelia also needs further study.

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