

The Development of Germination Tests and Breaking Dormancy Techniques of Bitter Seeds (*Andrographis paniculata* (Burm. f.) Wall. ex Nees)

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Abstract

Bitter seed (*Andrographis paniculata* (Burm. f.) Wall. ex Nees) is a medicinal species used in phyto-pharmacy and known as 'the king of bitterness'. Bitter seeds contain andrographolide, a diterpenoid lactone which is widely used in Indian medicine for snake bites and for the treatment of hepatitis. The research was conducted at the Seed Management Unit experimental field of the Indonesian Spice and Medicinal Plants Research Institute (IMACRI) and at the Seed Quality Testing Laboratory of IPB University, Indonesia, from January until May 2021. The objectives of the study is to determine the best germination test and effective breaking dormancy techniques to increase the germination percentage of bitter seeds. The study was arranged in a completely randomized design with two-factors. The first factor was seed treatments, i.e., control/untreated, chemical scarification with 5% H_2SO_4 for 10 minutes, and 5% H_2SO_4 followed by a 100 ppm GA_3 soaking treatment. The second factor conducted was germination test methods according to International Seed Testing Association, i.e., germinating the seeds on the top of paper and in pleated paper. Our study demonstrated that seed treatment with H_2SO_4 can break seed dormancy and speed up seed germination. Bitter seed germination on the pleated paper was higher than with the top-of-paper method. Bitter seed germination with the top-of-paper method was at 65 DAS and the final germination was at 80 days after sowing (DAS), and the final germination was 55.2%. With the pleated paper method, the seeds germinated at 30 DAS and the final germination was at 70 DAS, and the final the germination of 59.2%.

Keywords: chemical scarification, GA_3 , pleated paper and top of paper germination, seed viability, seed vigor.

Introduction

Bitter seed (*Andrographis paniculata* (Burm. f.) Wall. ex Nees) is originated from India (Valdiani et al., 2012) and it is one of the important medicinal plants in the tropics. Bitter seed in Indonesia has been long used as a traditional and medicinal ingredient because it contains andrographolide compounds which have various properties, such as an immune stimulator, anti-inflammatory, anti-cancer, hepatitis drug, and a COVID-19 inhibitor (Rajagopal et al., 2020). Bitter seed production in Indonesia has increased in the last five years (2017-2021), with an average annual production reaching 1,754705 kg per year (CAS, 2021).

One of the limitations in the cultivation of bitter seed is the limited availability of quality seeds. The seeds, including the freshly harvested seeds, generally have low germination rates. The bitter seeds have a dormancy period (Rusmin et al., 2007), and germination rate is highly affected by the environment. According to Rouhi et al., (2015), seed dormancy may also be influenced by pre-chilling after harvest and seed desiccation. Apart from the low germination rates, the seeds have rather low growth potentials, which can lead to misinterpretation in the evaluation of seed quality.

Bitter seed dormancy is classified as an 'exogenous,' or a physical dormancy due to the hard seed coat (Talei et al., 2012), resulting in a long period of germination that can reach 5-6 months (Rusmin et al., 2007). Physical dormancy was caused by one or more water-impermeable layers of palisade cells in the seed coat. Physiological dormancy is a complex, and can be classified as deep, intermediate, and non-deep dormancy (Baskin and Baskin, 2004). Chemical or mechanical scarification can promote germination of seeds with a non-deep physiological dormancy (Baskin and Baskin, 2004).

Information regarding the germination test methods of bitter seeds is still limited. Based on ISTA (2018), germination tests for small size seeds can use papers. A laboratory analysis is important to determine the inhibitory agent and the type of dormancy in selecting the best dormancy-breaking solution (Liu et al., 2011). Identification of the molecular mechanisms underlying the induction and release of dormancy after ripening in *Arabidopsis thaliana* seeds through cloning and characterization of the RDO2 mutant has been reported (Liu et al., 2011). Understanding of the molecular mechanisms are important information to further study other species with long seed dormancy.

Dormancy-breaking treatment of bitter seeds through the scarification of the seed coat and soaking in H_2SO_4 solution can potentially overcome the problem of seed dormancy. However, this technique still needs to be improved as the germination rate was still low (58%; Kataky and Handique, 2010). A study by Talei et al. (2012) reported that the soaking treatment of bitter seed in 5% (v/v) H_2SO_4 for 10 minutes resulted in 50% seed germination compared to 30% in the untreated seeds (control). Therefore, it is necessary to further study the dormancy mechanism of bitter seeds as a basis for determining an effective method of breaking dormancy and to obtain a higher germination percentage. This study aims to determine an optimum germination test method and effective breaking-dormancy techniques to increase bitter seed germination.

Material and Methods

Time and Location

The study was conducted from January to May 2021 at the Seed Management Unit Experimental field of the Indonesian Spice and Medicinal Plants Research Institute (IMACRI) in Bogor, and the Seed Quality Testing and Storage Laboratory, Faculty of Agriculture, IPB University.

Materials

Bitter seeds "Sambina 1" variety were harvested from the Experimental Station of the Seed Management Unit, IMACRI when the pods are uniformly in brown color. Chemical scarification treatment was implemented using H_2SO_4 , GA_3 , KNO_3 , and kinetin.

Development of Germination Test of Bitter Seed

The experiment was arranged using a two-factor completely randomized design. The first factor was seed treatment, which consisted of three levels:

no treatment (control), chemical scarification, and chemical scarification followed by GA_3 immersion. The second factor is the germination test methods: the top-of-paper (TP) and the pleated-paper (PP) methods. Each treatment was repeated four times, so there were 24 experimental units.

Chemical scarification treatment was conducted by soaking the seeds in 5% H_2SO_4 for 10 minutes (Talei et al., 2012). The seeds were rinsed thoroughly using running distilled water, and air-dried at room environment (26-32 °C, RH 61-79%) for one hour. The treatment with GA_3 (100 ppm) was conducted seeds 24 hours after chemical scarification (Cipcigan et al., 2020), followed by air drying for 12-18 hours (Powell et al., 2000).

Germination was carried out using two methods according to ISTA (2018): top-of-paper (TP), i.e., the seeds were placed on paper; and on pleated paper (PP), i.e., the seeds are placed between folds of a paper strip folded. For each method, one-hundred seeds were germinated on two-ply moist filter papers in a germination box set at 25 °C and relative humidity 80-90%.

Measurement and Data Analysis

Seed viability was measured based on seed viability and seed vigor. Data was analyzed using ANOVA at $\alpha = 5\%$ using Statistical Analysis System (SAS) 9.4. Significant differences between means were further analyzed using Duncan multiple range test at $\alpha=5\%$.

Observation and Measurement

Seed vigor, seed viability, speed of germination (SG), germination percentage (GP), and maximum growth potential (MGP) were measured. Seed germination is defined as the emergence of a radicle of >5 mm (Twala and Fisher, 2022). Seed vigor and seed viability was measured twice when the first-time seeds germinated, and on the final germination.

The highest percentage of the normal seedling was determined as the first count; the final count was determined by the highest percentage of cumulative normal seedlings. The data of the first and final count were presented in a curve analysis.

The speed of germination (SG) was measured every etmal, i.e., every 24 hours, from the first day the seeds were germinated to the last day (final count) of measurement. The speed of germination was calculated using the following formula:

$$SG (\%NS/etmal) = \sum_0^t \left(\frac{\%NS}{etmal} \right)$$

SG : speed of germination (%NS/etmal)
 %NS : percentage of normal germination at each observation time
 etmal : observation time every 24 hours
 t : observation time

The germination percentage (GP) was carried out after the first and the final count of bitter seeds were determined. Observations were made on seedlings that grew normally (ISTA, 2018). The normal seedling criteria of bitter seeds on paper were determined based on the presence of radicle, hypocotyl, and cotyledon. The criteria of healthy seedlings were the presence of a healthy root of a minimum 5 mm in length, a minimum hypocotyl length of 20 mm, and exhibiting no damage. The germination percentage was calculated using the following formula:

$$GP (\%) = \frac{\text{Number of NS I} + \text{Number of NS II}}{\text{Total seeds germinated}} \times 100\%$$

Where:

NS I: normal seedlings on the first count

NS II: normal seedlings on the final count

Maximum growth potential (MGP) is calculated based on the proportion of seeds germinating normally and abnormally until the last day of observation (final count). The MGP formula is as follows:

$$MGP (\%) = \frac{\text{Number of seeds germinated (normal and abnormal)}}{\text{Total seeds germinated}} \times 100$$

Result and Discussion

The bitter seed consists of testa (seed coat), embryo, and a radicle candidate (Figure 1). The testa is the maternal tissue, maternally inherited. In an angiosperm seed type, the embryo is surrounded by two layers: the testa (seed coat) and the endosperm (Savage and Matzger, 2006). The seed coat of bitter seed is separate from the cotyledons (Figure 2). Cotyledon, radicle and hypocotyl are important structures of the healthy seedlings (Figure 1C)

The first count of germination was determined based on the highest daily percentage of normal seedlings. The final count was determined on the day the highest cumulative normal seedlings were added or when the germination rate decreased until no more seeds germinated (Ningsih et al., 2021). The value when seedlings no longer grow was typically determined as the final count (Rusmin et al., 2016).

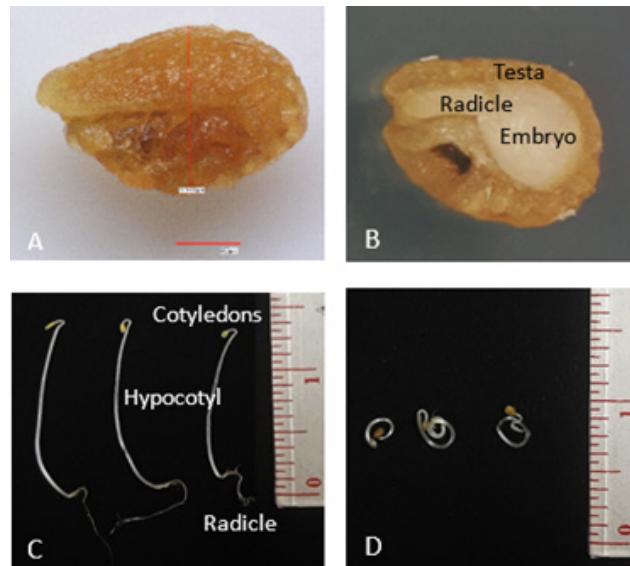


Figure 1. Morphology of bitter seed and seedlings; an entire seed (A), a cross section of bitter seed (B), normal seedlings (C), and abnormal seedlings (D).

Based on the curve analysis results of the TP and PP methods (Figures 2 and 3), the first bitter seedlings appeared in 5-10 days after sowing (DAS). The number of germinated seeds with the TP method and the first count of untreated seeds was determined based on the highest daily normal seedling at 65 DAS. The final count was determined based on the highest cumulative normal seedling count at 80 DAS (Figure 2A). The first count of seeds treated with chemical scarification using 5% H_2SO_4 for 10 minutes was set at 60 DAS, and the final count was 75 DAS (Figure 2B). Observations of the first and final count of seeds treated with chemical scarification followed by soaking in 100 ppm GA_3 were determined at 55 and 75 DAS, respectively (Figure 2C).

Contrary to the PP method, the first and final count of the untreated seeds were set at 35 and 70 DAS, respectively (Figure 3A), while the first and final count of seeds treated with chemical scarification were conducted at 35 and 65 DAS (Figure 3B). The chemical scarification was followed by a 100 ppm GA_3 soaking treatment and resulted in the first and final count observations at 30 and 70 DAS (Figure 3C). Chemical scarification with 5% H_2SO_4 for 10 minutes resulted in a shorter first and final count observation period than the untreated seeds in each TP and PP method (Figures 2 and 3).

There are two main categories of seed dormancy, exogenous and endogenous, i.e., the presence of inhibitors in the seed embryo, which inhibit the emergence of radicles in fully imbibed seeds

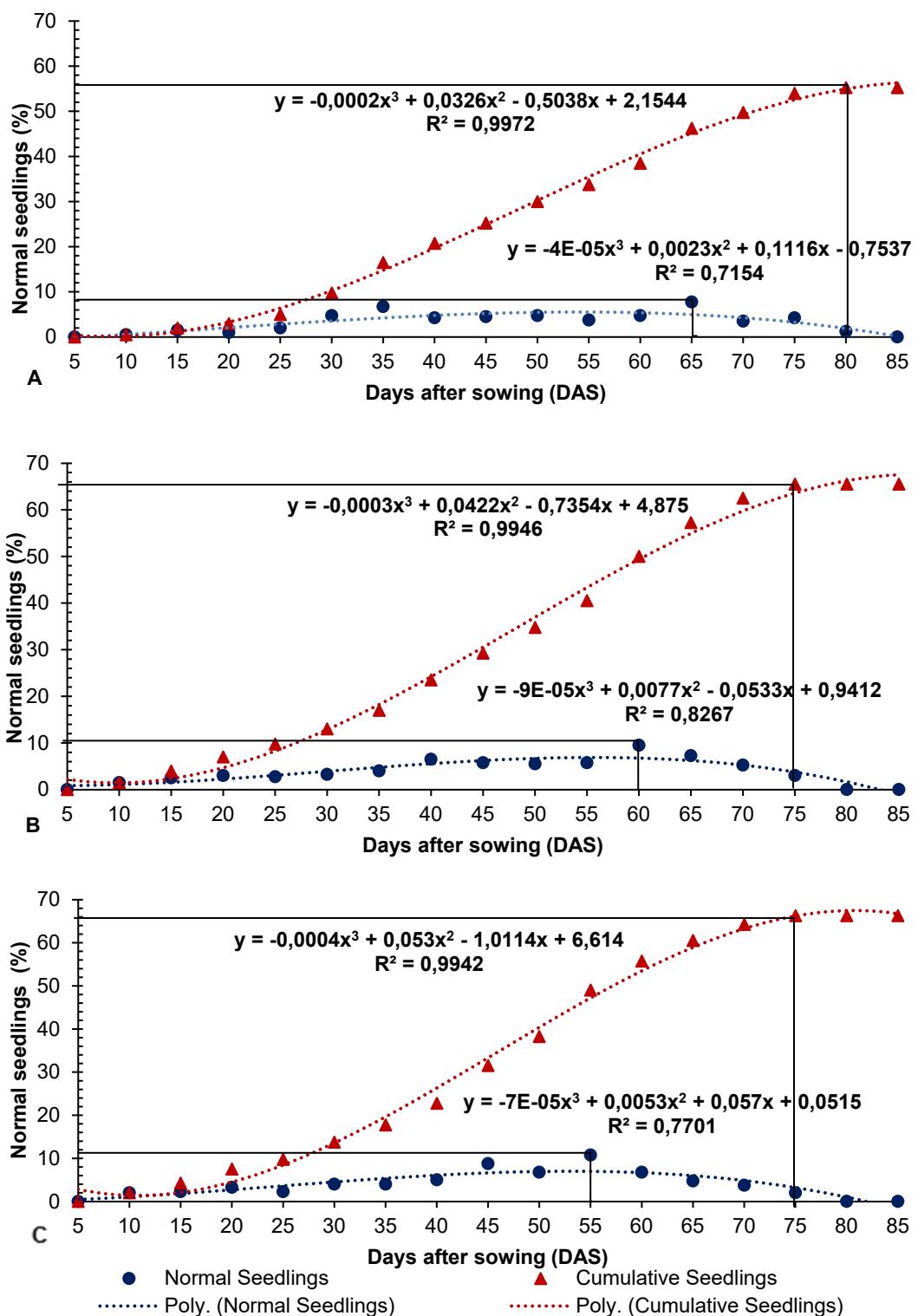


Figure 2. The curves for determining bitter seed germination percentage using the top-of-paper (TP) method: (A) control, (B) chemical scarification, and (C) chemical scarification followed by GA₃ treatment.

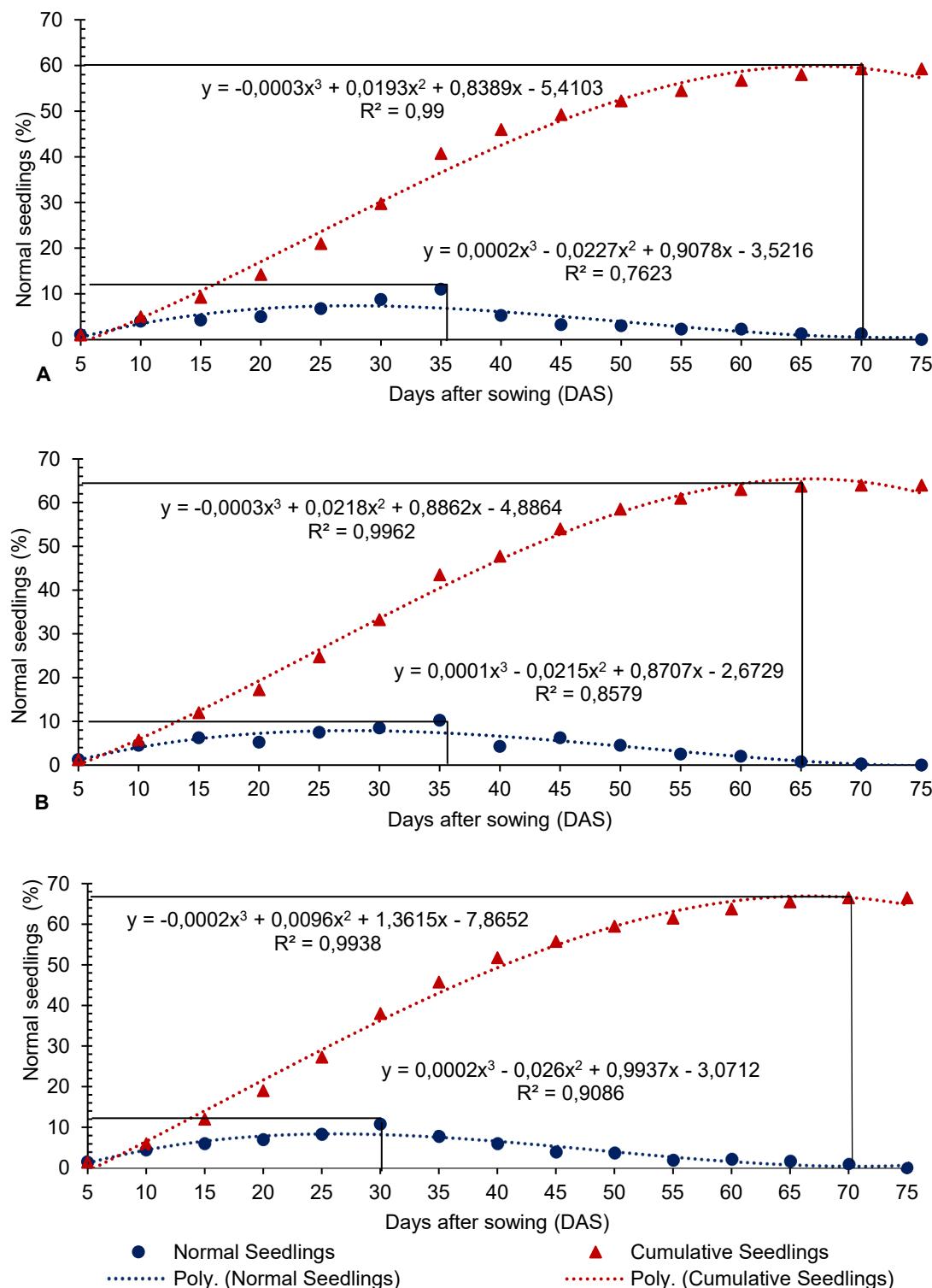


Figure 3. The curves for determining bitter seed germination count using the pleated-paper (PP) method: (A) control, (B) chemical scarification, and (C) chemical scarification followed by GA₃ treatment.

(Baskin and Baskin, 2004). Bitter seed dormancy is exogenous or physical, due to the thick and hard seed coat; the seed coat is the primary modulator of the interaction between the internal structure of the seed and the surrounding environment, as well as keeping the embryo from germinating (Baskin and Baskin, 2004). Tieu et al. (2001) stated that the specific metabolic pathways in seeds can be blocked by several inhibitory compounds, such as cutin and suberin, found in seeds with a hard-shell structure. Breaking seed dormancy that is caused by hard seed coat can be done mechanically and chemically (Ertekin, 2011).

Table 1 shows that seed treatment and test methods highly significantly affected speed of germination. The speed of germination in the control group was significantly lower than the chemical scarification with 5% H_2SO_4 for 10 minutes and the chemical scarification followed by 100 ppm GA_3 soaking treatment (Table 1). However, the speed of germination between 5% H_2SO_4 with and without GA_3 were not significantly different.

Table 1 also shows that the germination using the PP method were twice as fast as the TP method. This is likely due to differences in the humidity in the germination media which affect the acceleration of the germination of bitter seeds. Seeds that were germinated with TP method were laid flat on the filter paper, so the evaporation occurred quickly. In contrast, the seeds with the PP method were laid between damp papers; the moist environment with this method accelerated germination as hard and thick seed testa require high humidity to germinate.

Regardless of the chemical scarification treatment, the germination percentage (GP) and maximum growth potential (MGP) in the PP method were not significantly different from the TP method (Table 2). This might be related to the optimal light condition that allow seeds to germinate in the TP method. In line with the Baskin and Baskin (2004) report, in addition to moisture, light is one of the important factors to break dormancy and to allow morphogenesis (Muham et al., 2015). Phytochromes and other light receptors regulate various morphogenesis, from seed germination, seedling development, the formation of flowers, fruits, and seeds (Muham et al., 2015).

Differences in the time to germinate can be caused by the interaction of various environmental factors including water, light, and temperature (Gupta and Chakrabarty, 2013). Light and temperature can affect GA biosynthesis and signaling, hence, affecting seed germination (Skubacz and Golec, 2017). A suitable germination substrate is characterized by adequate

porosity that can supply oxygen and retain water which is required for seed germination (Santos et al., 2022).

The low bitter seed germination is also caused by the hard seed coat, which makes it impermeable to gas and water (Rusmin et al., 2007). A study by Sun et al. (2018) demonstrated that *Glycyrrhiza uralensis* hard seed coat contains hydrophobic chemicals such as cutin, suberin, cellulose, hemicellulose, and a waxy coating, which prevented the seeds from imbibing water. Chemical scarification treatment can effectively accelerates the germination by creating cracks and cavities in the seed coat tissue, hence improved water imbibition and increased seed metabolism (Yuniarti and Djaman, 2015).

The chemical scarification followed by 100 ppm GA_3 soaking for 24 hours resulted in a shorter first count observation period (± 5 days) than the control and chemical scarification treatments (Figure 3). Gibberellic acid is a natural regulator of the seed germination; it stimulates the production of the hydrolytic enzyme α -amylase in the aleurone layer of germinating seeds (Gupta and Chakrabakti, 2013). Gibberellic acid can exert its influence in two ways, by increasing the growth potential of the embryo, and by stimulating the production of hydrolytic enzymes (Gupta and Chakrabakti, 2013).

Table 2 showed that the germination of the untreated seeds were significantly higher in the pleated paper method (59.2%) than those in the top of paper method (55.2%). Seed germination with 5% H_2SO_4 with or without GA_3 were similar (63.5% – 64%) in both test method (PP and TP). Seeds treated with chemical scarification followed by 100 ppm GA_3 soaking showed that the PP method produced significantly higher mean growth potential (MGP) than those in the TP method. According to the Indonesian Ministry of Agriculture (2019), bitter seeds fulfill the criteria as an extension seed class if the GP reached 60%, therefore, the seed treatment developed in this study has met the germination percentage of bitter seeds met this criteria.

Table 1. Effect of seed treatment and test methods on germination speed

Seed treatment	Germination speed (%NS per day)
Control	2.06 b
5% H ₂ SO ₄	2.40 a
5% H ₂ SO ₄ + 100 ppm GA ₃	2.51 a
Test methods	
Top-of-paper	1.69 b
Pleated paper	2.96 a

Note: Values in the same column followed by the same letters are not significantly different based on the DMRT at $\alpha=0.05$. NS = normal seedlings.

Table 2. The interaction effects of seed treatments and test methods on germination percentage (GP) and maximum growth potential (MGP)

Seed treatment	GP (%)		MGP (%)	
	TP	PP	TP	PP
Control	55.2 Bb	59.2 Ab	74.0 Ab	73.5 Ab
5% H ₂ SO ₄	65.5 Aa	64.0 Aa	77.2 Aa	79.0 Aa
5% H ₂ SO ₄ + 100 ppm GA ₃	63.7 Aa	66.5 Aa	76.7 Ba	81.0 Aa
CV (%)	3.34		2.30	

Note: At the same variable, values followed by the same lowercase letter in a column or capital letter in a row show not significant difference based on the DMRT at $\alpha=0.05$. * = significant at $P < 0.05$, ** = significant at $P < 0.01$, ns = not significant. TP = top-of-paper, PP = pleated-paper, CV = coefficient of variation.

Conclusion

Our study demonstrated that treatment with H₂SO₄ can break seed dormancy and speed up germination. Bitter seed germination on the pleated paper was higher than with the top-of-paper method. The first and final count of germination with 5% H₂SO₄ with the top-of-paper method was at 60 and 75 DAS with germination percentage of 65.5%, whereas with the pleated paper method it was at 35 and 65 DAS with germination percentage of 64%. The first and final germination with the 5% H₂SO₄ + 100 ppm GA₃ with the top-of-paper method was at 55 and 75 DAS with germination percentage of 63.7%, whereas with the pleated paper method at 35 and 70 DAS with germination percentage of 66.5%.

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