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A rapid and effective method for dormancy breakage and germination of King of Bitters (*Andrographis paniculata* Nees.) seeds

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Abstract

Andrographis paniculata is a medicinal plant of the Acanthaceae family. The plant is important for its diterpenoids obtained from the mature plants grown normally through the seeds. However, the seed germination is slow and not efficient. Here, we report an effective method for breaking the seed dormancy to improve the germination percentage. Among the different physical and chemical scarification methods the fastest and the highest germination percentage were obtained from the seeds scarified with sandpaper. After three days of the scarification, 72.7% of the seeds germinated and this percentage was further increased to 90.4% after 15 days with a mean germination time (MGT_{a15}) of 4 days. Although, the other physical and chemical treatments showed moderate effects on germination (GP_{a15} ranged from 7% to 64%), but they failed to reduce the MGT_{a15}. Treating the sandpaper-scarified seeds with the seeds and the seeds coat protein extracts delayed the germination but did not affect the overall germination percentage, indicating the presence of unknown germinating factor(s) which may contribute to the dormancy of the seed. Together, the germination factor(s) and the hard seed coat caused the seed dormancy of *A. paniculata* which could be overcome by sandpaper scarification.

Keywords: *Andrographis paniculata*, dormancy breakage, sandpaper scarification, seed germination

Abbreviations: GP_{a3}, Germination percentage after three days; GP_{a15}, Germination Percentage after 15 days; GR_{a15}, Germination rate after 15 days; MGT_{a15}, Mean germination time after 15 days; SCPE, Seed coat protein extract; SPE, Seed protein extract.

Introduction

Andrographis paniculata from the family Acanthaceae, is one of the major medicinal plants of the tropics commonly known as Kalmegh or King of Bitters (Chauhan et al, 2009; Gomathinayagam et al, 2009; Valdiani et al, 2012). The plant grows abundantly in humid tropical climates particularly in the South East Asia region (Lattoo et al, 2008; Valdiani et al, 2012). Under this condition, the plant normally reaches a maturation stage after 90 to 120 days of germination (Mishra et al, 2007). The leaves of the mature plant contain abundant diterpenoids of medicinal benefit (Lattoo et al, 2008; Valdiani et al, 2012). Among the major beneficial diterpenoids are andrographolide, 14-deoxy-11, 12-didehydro-andrographolide, and neo-andrographolide, which were reported to possess immunostimulant, antipyretic, anti- inflammatory, and anti-diarrhea properties (Lattoo et al, 2008; Valdiani et al, 2012). In traditional medicine, the aerial parts of the plant particularly the mature leaves are used for the treatments of fever, sore throat, diarrhea, and other chronic and infectious diseases (Mishra et al, 2007; Valdiani et al, 2012). Since the plant's diterpenoids are extracted from the mature leaves, the medicinal and industrial values of the plant rely on the availability of the mature leaves harvested in the shortest possible time from the seeds.

One main problem of growing this plant from seeds is its poor seed germination performance. The germination percentage and rate are relatively low (Kumar et al, 2011; Saraswathy et al, 2004) which may indicate the presence of a combined dormancy of physical and innate nature (Saraswathy et al, 2004), a common survival strategy for a successful spread of plants in this world.

Dormancy is an intrinsic germination-preventing property of plant seeds which requires external stimulants to overcome (Thompson and Ooi, 2010). Dormancy and germination of seeds depend on seed structures and the factors that effect on the growth potential of the embryo (Koornneef et al, 2002). The basis of dormancy varies in different plant species but basically it is classified as physiological, morphologi-

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cal, morpho-physiological, physical and a combination of physical and physiological factors (Baskin and Baskin, 2001; Baskin and Baskin, 2004; Oliveira and Garcia, 2011). Depending on the dormancy, various scarifications based on the use of physical or chemical agents may be used to break seed dormancy. Seed dormancy of some plants are prone to chemical agents such as plant growth regulators (Basra, 2006), potassium nitrate (Hartmann et al, 1997; Kevsero Lu, 1993), hydrogen peroxide (Ghildiyal and Sharma, 2005) and sulphuric acid (Keshtkar et al, 2008). Other plants may require physical agents to break their seed dormancy such as hot water (Hermansen et al, 1999) and light and temperature (Finch Savage and Leubner Metzger, 2006; Merritt et al, 2007; Verma et al, 2010).

So far, research on *A. paniculata* seed dormancy breakage has limited to the effects of plant growth regulators and mechanical scarification on seed germination (Kumar et al, 2011). In the present study, the seed dormancy and germination of two *A. paniculata* accessions were investigated. We hypothesized that the dormancy is caused by a seed coat which requires physical scarification for germination. Our main purpose was to improve the germination percentage of *A. paniculata* seeds while at the same time establishing the basis of seed dormancy in this plant.

Materials and Methods

Seeds and chemicals

Seeds of two *A. paniculata* accessions namely, 11340 and 11347 were obtained from the Agro Gene Bank, University Putra Malaysia, Serdang, Selangor DE, Malaysia. The seeds were kept at 4°C until use. The chemicals used for the treatments were of analytical grade obtained from Fisher Scientific (Leicestershire, UK).

Sandpaper scarification method

The fundamental principle of the method is the removal of the seed coat using the rough surface of sand paper. The sand paper used in this technique was N° 120, sized 30 cm × 30 cm. The seeds were spread evenly on the rough surface of the paper on a flat surface, and then covered with another paper like a sandwich (Figure 1) so that the rough surface of the second paper was in contact with the seeds. Using the palm, the upper layer of the sandpaper was gently pressed against the bottom layer while at the same time the paper was moved slowly in a circular motion five times. The majority of the seeds are effectively scarified during this process without destroying the ability of the seed to germinate normally.

Preparation of seeds and seeds coat protein extracts

A. paniculata seeds were collected from the field and then the seeds coat were removed using sandpaper. With liquid nitrogen cooling, the seeds and seed coats were grounded to fine powder separately in a mortar and pestle. The fine powders were mixed with water in a ratio of 1: 2 and then were vigorously vortexed for 5 min to extract potential water-soluble substances. The homogenates were centrifuged at 15,000 g for 15 min at 4°C to secure the supernatant. Small aliquots of the supernatants were boiled to denature proteins.

Microscopic examination of seed embryo

The seeds were first soaked in a 5% (v/v) sulphuric acid solution for 10 min and then transferred into a petri dish covered with Whatman paper No.2. Using a fine knife and scalpel, the seeds were carefully cut from the edge to expose the embryo. The exposed embryo was then scanned using a dissecting microscope (Leica EZ4D).

Experimental design

We carried two experiments with experiment one to identify the best physical or chemical agents that improved the seed germination, and experiment two to determine whether or not the seed or the seed coat contain any substances that affect germination. Both of the experiments were conducted in a controlled growth chamber in the Department of Crop Science, University Putra Malaysia, between August and October 2011.

The first experiment was designed as a factorial randomized complete block (RCBD) with two factors and three replicates. The two factors evaluated in the experiment were: genetics of the plant based on the two different accessions (11340 and 11347); and scarification method based on 12 different chemical and physical agents comprising 2% and 5% (v/v) sulphuric acid for 10 min, 0.3% (w/v) potassium nitrate for 5 min, 1% and 2% (w/v) potassium chloride for 10 min, 1% and 2% (w/v) thiourea for 10 min, 25% (v/v) acetone for 5 min, normal water for 48 hours, boiling water 100°C for 5 min, scarification with two layers of

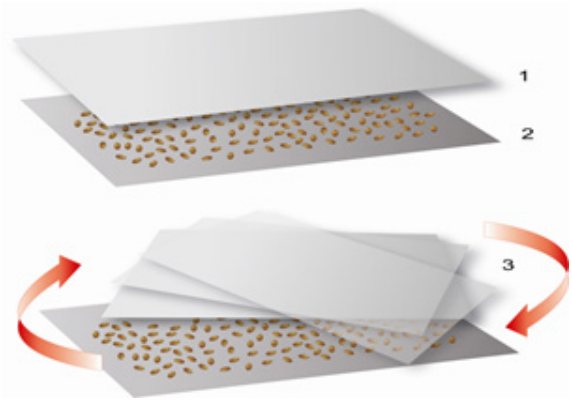

Figure 1 - Scarification of *A. paniculata* seeds using two layers of sandpaper. The seeds were spread evenly on the rough surface of the first sandpaper (1), the second sandpaper covered the seeds like a sandwich (2), using the palm the upper layer of the sandpaper was pressed gently against the seeds and then moved slowly in a circular motion five times (3).

Table 1 - Variance analysis of the different physical and chemical scarification effects on the measured traits of two accessions of *A. paniculata*.

Source	df	Mean Square					
		GP_{a3}	GP_{a5}	GP_{a10}	GP_{a15}	MGT_{a15}	GR_{a15}
Treatment (T)	11	3458.8**	6581.2**	5977.4**	4642.7**	29.7**	2275.7**
Accession (A)	1	10.89ns	12.5ns	12.5ns	14.22ns	0.01ns	5.82ns
T×A	11	5.80ns	5.59ns	3.89ns	3.19ns	0.12ns	1.61ns
Error	46	4.45	11.01	12.89	11.01	0.24	2.26

** and ns, refer to 1% and not significant, respectively. GP_{a3}: Germination percentage after three days, GP_{a5}: Germination percentage after five days, GP_{a10}: Germination percentage after ten days, GP_{a15}: Germination Percentage after 15 days, MGT_{a15}: Mean germination time after 15 days, and GR_{a15}: Germination rate after 15 days.

sandpaper (N°120), and distilled water.

One hundred seeds from each accession were soaked in separate Petri dishes in each level of treatment.The seeds were surface sterilized by soaking them in 10% (v/v) sodium hypochlorite (NaOCl) solution for 10 minutes (Talei et al, 2011) and thoroughly rinsed with distilled water. The seeds were subjected to the treatments, and were distributed evenly on a Whatman paper (N° 2) placed in a sterile petri dish. The petri dish was sealed with parafilm, and incubated in a growth chamber. The average temperature of the growth chamber was set between 26-32°C with relative humidity between 60-75%. The seeds were watered every week by saturating the Whatman paper with sterilised water. After three days, the germinated seeds were counted every day. The germination percentages after three days (GP_{a3}) and 15 days (GP_{a15}) of the experiment were then calculated. The mean germination time (MGT_{a15}) was calculated using the described formula of Ellis and Roberts (1981). The germination rate (GR_{a15}) was calculated by dividing the germination percentage obtained at each counting to the certain number of the counting day. The effects of the treatments were evaluated based on the germination percentage and time.

The second experiment was designed as a randomized complete block design (RCBD) with five treatments and three replicates. The treatments were: seed protein extract (SPE), seed coat protein extract (SCPE), boiled SPE, boiled SCPE, and distilled water as a control. For this experiment, fifty seeds were used for each of the treatments. The seeds were first scarified using two layers of sand paper (N°120) as described earlier and then treated with the extracts prior to germination. After three days, the germination percentage (GP_{a3}) of the treated seeds was calculated and the germinated seeds were counted daily thereafter. At the end of the experimental period of 15 days, the GP_{a15}, MGT_{a15} and GR_{a15} were calculated.

Statistical analysis

Initially, the raw data were tested for normality using the SAS software 9 and then the main data were then analyzed for the studied traits using analysis of variance and Duncan's multiple range test ($P < 0.01$).

Results

Effects of chemical and physical scarification on seed dormancy and germination

Applying different physical and chemical treatments prior to seed germination significantly accelerated the germination of *A. paniculata* seeds. Variance analysis of the treatment effects on the measured characteristics (GP_{a3}, GP_{a5}, GP_{a10}, GP_{a15}, MGT_{a15} and GR_{a15}) showed that the treatments significantly improved the germination of the seeds while the genetic factor (two accessions) played no role in the germination (Table 1). The seeds were successfully germinated as early as three days after the treatments giving germination percentages between 7 to 72.7%. The GP_{a3} values showed that the scarification with two layers of sandpaper was the most effective method to break seed dormancy in which 72.7% of the seeds germinated after three days (Figure 2A). None of the control seeds germinated during this period. The second most effective method was soaking the seeds in 2% (w/v) thiourea for 10 min, giving a GP_{a3} value of 64%. The other chemical and physical agents demonstrated varying effectiveness as reflected by GP_{a3} values ranging from 7 to 58%. Within three days, all of the physical and chemical agents improved the germination percentage of the seeds with scarification with sandpaper giving the best performance.

Scarifying the seeds with sandpaper, thiourea and KCl not only accelerated the germination as shown by the MGT_{a15} value but also improved the overall germination percentage as demonstrated by the GR_{a15} and GP_{a15} values. The sandpaper-scarified seeds rapidly germinated with MGT_{a15} value of four days (Figure 2C), eight to ten times faster than the control (Figure 2D). The GP_{a15} (Figure 2B) and GR_{a15} (Figure 2D) values of the treatment were far higher than the values obtained from the other agents. More than 90% of the sandpaper-scarified seeds and 95% of 2% (w/v) thiourea-scarified seeds were successfully germinated at the end of the experiment. Based on these values, scarifying the seeds with 2% (w/v) thiourea and sandpaper outperformed the other methods in terms of speed and percentage of germination.

The other physical and chemical agents showed moderate effects on germination except for the

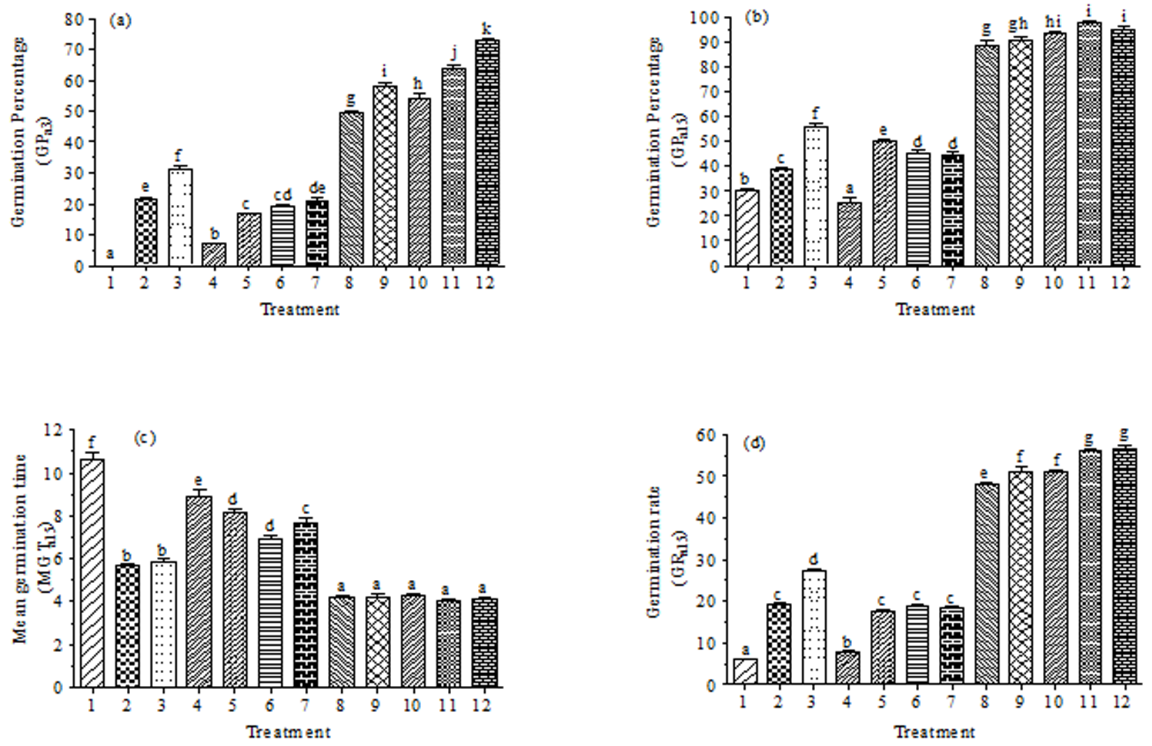

Figure 2 - Seed germination of *A. paniculata* under various physical and chemical scarification treatments. (A) Germination percentage after three days of treatment, (B) Germination percentage after 15 days, (C) Mean germination time after 15 days and (D) Germination rate after 15 days. Mean values ± S.E are from three independent replicates and values superscripted by different letters are significantly different by Duncan's multiple range test ($p \leq 0.01$).The treatments were: [1] Control, [2] Boiling water for 5 min, [3] Normal water for 48 h, [4] 5% (v/v) H_2SO_4 for 10 min, [5] 2% (v/v) H_2SO_4 for 10 min, [6] 25% (v/v) acetone for 5 min, [7] 0.3% (w/v) KNO_3 for 5 min, [8] 1% (w/v) KCl for 10 min, [9] 2% (w/v) KCl for 10 min, [10] 1% (w/v) Thiourea for 10 min, [11] 2% (w/v) Thiourea for 10 min, and [12] Scarifying with sand paper.

seeds treated with sulphuric acid for 10 min. All of the agents significantly improved the germination percentage and rate (GP_{a15} and GR_{a15}) but overall failed to reduce the germination time to about half of the time required for germination without any treatment (Figure 2C). The GR_{a15} values of the treatments ranged from 8 to 56.6 reflecting the effectiveness of the agents in breaking the seed dormancy. Among the better scarification methods judging by the GR_{a15} and MGT_{a15} values were treating the seeds with 1 and 2% (w/v) KCl for 10 min.

Although soaking the seeds in 2% (v/v) of H_2SO_4 for 5 min significantly improved the germination performance, a prolonged 10 min treatment with 5% (v/v) of H_2SO_4 failed to improve the germination. Microscopic examination of these seeds showed that the embryos were damaged as indicated by the darken embryo (Figure 3A) compared to a more whitish embryo of the untreated seed (Figure 3B). Therefore, H_2SO_4 could break the seed dormancy with an improved germination percentage when use at 2% (v/v) for 5 min.

Effect of the seed and the seed coat protein extracts on germination

We attempted to confirm that the dormancy of the seed was purely physical in nature and no other factors were involved. To prove this, we treated the sand-scarified seeds with the seed and the seed coat protein extracts so that the effect of water-soluble substances if present could be seen in the reduction of germination. Variance analysis of the treatment effects on the measured characteristics (GP_{a3}, GP_{a15}, MGT_{a15} and GR_{a15}) showed that the treatments significantly affected the GP_{a3}, GP_{a5}, GP_{a10}, MGT_{a15} and GR_{a15} but not GP_{a15} (Table 2). The protein extracts delayed the germination of the seeds as shown by the GP_{a3} values (Figure 4) indicating the presence of germinating factor(s) in the seeds and the seed coats. Judging by the presence of this germinating factor(s) which delayed the germination of the seeds, the dormancy of the seed was likely to be a combination of physical and physiological factors.

Discussion

The ability to reduce the time required to establish *A. paniculata* seedlings and to increase the germination percentage of the seeds is important from an industrial perspective. As a matter of fact, the seed

Table 2 - Variance analysis of the seed and seed coat protein effects on the measured traits of *A. Paniculata*.

Source	df	Mean Square					
		GP_{a3}	GP_{a5}	GP_{a10}	GP_{a15}	MGT_{a15}	GR_{a15}
R	2	6.67ns	33.07ns	46.67ns	63.2ns	0.42ns	6.06ns
Treatment	4	1638**	438.93**	184.67*	96.93ns	3.93**	358.62**
Error	8	5	14.73	40.67	49.53	0.13	3.12

**, * and ns, refer to 1%, 5% and not significant, respectively. GP_{a3}: Germination percentage after three days, GP_{a5}: Germination percentage after five days, GP_{a10}: Germination percentage after ten days, GP_{a15}: Germination Percentage after 15 days, MGT_{a15}: Mean germination time after 15 days, and GR_{a15}: Germination rate after 15 days.

germination of the plant is relatively poor. According to a recent report, the dormancy of of *A. paniculata* is controlled by a combination of physical (hard seed coat) and physiological (unknown proteins) mechanisms (Talei et al, 2012). Therefore, the mechanical or chemical methods, which can remove the hard seed coat layer or dissolve the inhibitor proteins could be effective methods to break the seed dormancy and improve the tendency of germination in *A. Paniculata* seeds.

Among the studied scarification methods the sandpaper successfully solved this problem with the germination percentage record more than 72% after just three days of treatment. The method is not only accelerating the germination, but it also improves the overall germination percentage to more than 94% after 15 days on the treatment.

Sandpaper scratched the seed coat and this prompt the germination rate by allowing water to get through the embryo. The germination of a number of plant species with hard seed coat such as *Tephrosia purpurea* (Sundararaj et al, 1971), *Melilotus alba*, and *Trigonella Arabica* (Baskin, 2003; Baskin and Baskin, 2001) was improved when the seeds were scarified with sandpaper. Scarifying the seed coat simply breaks the dormancy of the seed by exposing the embryo to water. Water uptake is one of the critical factors that active the initial biochemical changes responsible for protein synthesis during germination (Siddiqui and Khan, 2010). The importance of water in seed germination was demonstrated previously in many plant species including the family Fabaceae (Baskin et al, 2000), *Tamarindu sindicus* (Muhammad and Amusa, 2003), *Leucaenia leucocephala* and *Acacia nilotica* (Duguma et al, 1988), and *Atropabella donna* (Genova et al, 1997). Together, these findings support the concept that the water impermeable seed coat layer is primarily responsible for the seed dormancy of *A. paniculata* and by breaking this layer, germination can be initiated.

While scarifying with sandpaper was the most effective method, some other chemical treatments also were evaluated as effective approaches. For instance, soaking the seeds in 1 and 2% thiourea and KCl for 10 min was also quite effective in promoting seed germination. The results revealed that thiourea as an organosulfur compound, improved the seed germination. That might be due to the ability of

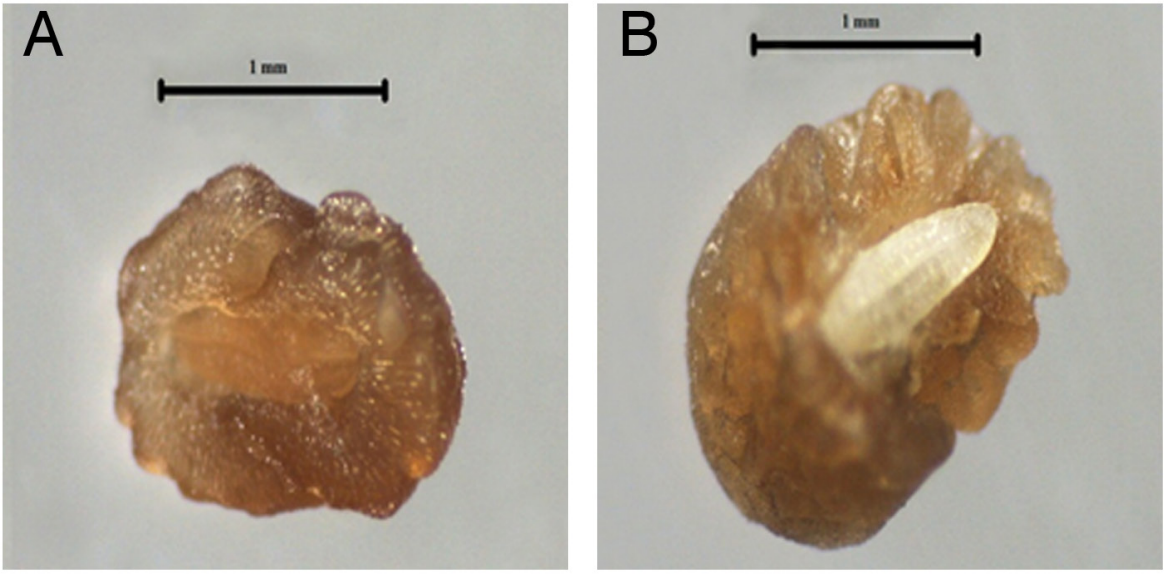

Figure 3 - The destructive effect of sulphuric acid on the embryo of the seed. (a) A damaged embryo of the treated seed looked darker. (b) A healthy embryo of the untreated seed was white. The seeds were soaked in 5% (v/v) H_2SO_4 for 10 min.

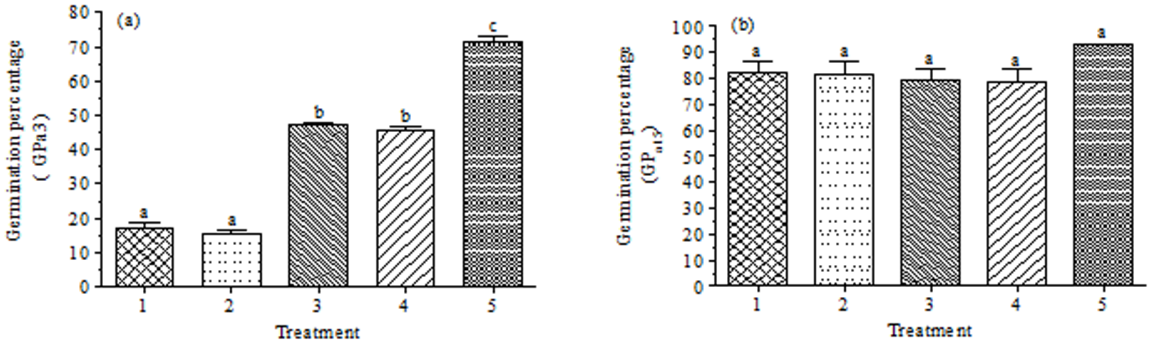

Figure 4 - Effect of seed and seat coat protein extracts on the seed germination of *A. paniculata*. (A) Germination percentage after three days (GP_{a3}), (B) Germination percentage after 15 days (GP_{a15}). Mean values ± S.E are from three independent replicates. Values superscripted by different letters are significantly different by Duncan's multiple range test ($p \leq 0.01$). The treatments were: [1] seed protein extract (SPE), [2] boiled SPE, [3] seed coat protein extract (SCPE), and [4] boiled SCPE [5] Scarified seeds with sand paper in distilled water as a control.

thiourea in solubilizing proteins especially the seed coat protein (Rabilloud et al, 1997). Besides, scarification of the seeds with KCl, significantly increased the seed germination. This can be due to dissolving the proteins by salting effects of KCl (some proteins mainly seed proteins and cell wall proteins are only solubilized by high salt (KCl) concentrations) (Carpentier et al, 2009). The 2% (w/v) thiourea method for 10 min also improved the overall germination percentage up to 97% after 15 days on the treatment.

Soaking the seeds in normal water for 48 h and boiled water for 5 min could also improve the seed germination percentage compared with control treatment. The result matched up with the finding of Kumar et al (2011), who showed that by soaking the seeds in hot water for 5 min increased the germination percentage. Hot water would soften the seed coat allows the water to be absorbed by the embryo. In this case, the delay in germination was associated with either lack of an effective method to break the dormancy or it could also be due to the presence of germination prohibitor factor(s) that were released during the seed coat softening.

Since the negative effect of some proteins on seed germination have been highlighted in the previous studies (Talei et al, 2012), we further tested this hypothesis by using the seed and seed coat protein extracts, and interestingly both of the extracts significantly delayed the germination as demonstrated by the germination percentage after three days. The results of the present study matched up well with the previous findings that showed some plant seeds contain inhibitors of seed germination (Horiguchi and Kitagishi, 1971; Rosu et al, 2010; Schultz and Small, 1991). However, the germination percentage after 15 days was not affected by both of the protein extracts, suggesting that the protein extracts might contain chemicals that delay the dormancy breakage.

Apart from the physical agents, potassium nitrate as a common chemical agents for promoting the seed germination, affected the germination of A paniculata seeds positively. Although the efficacy of this agent, was not as good as the other treatments such as scarifying with sandpaper. Additionally, KNO_3 improved the average germination percentage in comparison to the control. The positive effect of KNO_3 on the germination had been reported previously (Hilton, 1984; Nadjafi et al, 2006). For example, nitrogenous compounds (KNO_3 and NH_4NO_3) improved the seed germinations of some plants including *Medicago Arabica*, *Foeniculum vulgare*, *Thapsia villosa*, *Epilobium hirsutum* and *Rumex crispus* (Perez-Fernandez et al, 2006). In tune with our outcomes, the International Seed Testing Association (ISTA) recommended the use of potassium nitrate at 0.1 and 0.2 % for seed germination.

Conclusions

The dormancy of *A. paniculata* seeds is primarily attributed to the hard seed coat. The seed coat protects the embryo and its environment from water and any external threats. In the other word, it acts as a physical barrier inducing the seed dormancy. Scarifying or softening this layer by physical or chemical agents especially using sandpaper or thiourea is an efficient way to break the dormancy and to accelerate the seed germination. The seed or the seed coat may contain germination inhibitor factor(s). These agents delay the breakage of the dormancy as they might provide a physiological basis for dormancy of the seed. Further investigations on the structure of these delaying substances and their influence mechanism, provide a better understanding about the seed dormancy and germination processes in this species.

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