

*Full Length Research Paper*

# Physico-protein based dormancy in medicinal plant of *Andrographis paniculata*

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***Andrographis paniculata* is an important medicinal herb in the family of the Acanthaceae. Low seed germination is one of the main problems in *A. paniculata*. The current study focused on the seed dormancy and effective methods to enhance the seed germination. For this purpose, two experiments were carried out: (1) Testing different mechanical and chemical scarification methods; (2) studying the impact of seed and seed coat proteins on the scarified seeds. The results showed that all scarification methods increased the germination percentage, while seed and seed coat protein extracts decreased it. The fastest and highest germination was obtained using scarification with sandpaper (71.33% at the third day after the scarification). The germination was further increased up to 94% after 20 days. Finally, to identify the role of seed and seed coat proteins on the seed dormancy and germination, the proteins were analyzed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Three polymorphic bands were detected in total. However, the hard seed coat layer may physically affect the seed dormancy, but the presence of a specific protein sized 75 kDa in the seed coat and two proteins sized 31 and 69 kDa in endosperm may cause the physiological dormancy. Our findings suggested that a combination of physical and physiological mechanisms control the dormancy of *A. paniculata* seed.**

**Key words:** *Andrographis paniculata*, seed protein, scarification, seed dormancy, seed germination.

## INTRODUCTION

*Andrographis paniculata* is one of the major medicinal plants that belong to the plant family Acanthaceae, commonly known as Kalmegh or King of Bitters (Chauhan et al., 2009; Gomathinayagam et al., 2009; Kumar et al., 2011a; Rajpar et al., 2007). The economic importance of the herb has been considered recently (Valdiani et al., 2012). The plant grows well in tropical climates, particularly in the South-East Asia region (Lattoo et al., 2008). Importance of the species lies in

presence of Deterpenoids and Flavonoids and especially companionship of Andrographolide as the major constituents in it which let the plant to enjoy several curative effects such as immunostimulant, antipyretic, anti-inflammatory and anti-diarrhea (Saralamp et al., 1996).

Other diterpenoids including: 14-deoxy-11, 12-didehydro-andrographolide, neoandrographolide, andrographiside, carry a lower degree of medicinal eminence (Lattoo et al., 2008; Valdiani et al., 2012). The main problem of *A. paniculata* propagation is the seed germination which is considered relatively low (Kumar et al., 2011b; Saraswathy et al., 2004). In general, the low germination indicates the presence of seed dormancy, which is a common survival strategy for an effective

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dispersal of plants (Saraswathy et al., 2004). However, seed dormancy is an inborn seed characteristic that determines the environmental conditions in which the seed can germinate (Finch and Leubner, 2006), but it troubles the seed germination in laboratorial conditions and dormant seeds of *A. paniculata* get seriously stagnated after imbibitions (Talei et al., 2011).

The basis of dormancy differs in various plant species, but principally it is classified as physiological, morphological, morpho-physiological, physical or a combination of them (Baskin and Baskin, 2001, 2004). Understanding the ways for breaking the seed dormancy depend on the basis of the dormancy and it can be overcome by using physical or chemical treatments such as plant growth regulators (Basra, 2006; Jaskani et al., 2006), potassium nitrate (Hartmann et al., 1997; Kevsero Lu, 1993), hydrogen peroxide (Ghildiyal and Sharma, 2005), sulphuric acid (Keshtkar et al., 2008), hot water (Hermansen et al., 1999), light, temperature (Finch and Leubner, 2006), and microbial (Finch and Leubner, 2006; Koornneef et al., 2002). On the other hand, it is necessary to determine the inhibitor agents and the type of dormancy to choose the best solution. Here, we demonstrate the basis of the seed dormancy in *A. paniculata* and discuss the methods to break it. In addition, we hypothesized that the dormancy is mainly caused by two different mechanism consist of: (1) a hard seed-coating layer which requires a physical scarification to be removed and (2) existence of unknown inhibitor proteins in the seed and seed coat. Attempts to utilize the different methods to break the seed dormancy and improve the seed germination are mentioned in the present article.

## MATERIALS AND METHODS

### Plant materials and chemicals

Seeds of *A. paniculata* were obtained from Agro Gene Bank, University Putra Malaysia. The chemicals used for the treatments were of analytical grade obtained from Fisher Chemicals.

### Protein extraction for seed treatment

The seed coats of the collected seeds were first removed using sand paper and then the seeds and seed coats were frozen and ground in liquid nitrogen separately using mortar and pestle to obtain a fine powder. The seeds and seed coats powders were mixed with sterilized water separately and vortexed vigorously for 10 min followed by centrifugation at 15000 rpm for 20 min at 4°C to extract the potential water-soluble inhibitors. Supernatants were collected and total protein content determined using Bradford's method (Bradford, 1976).

### Protein extraction for biochemical profiling

1 g of the above-mentioned powdered samples was subjected to extraction with buffer (2 ml) containing: 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) KO/H (Merck, Darmstadt,

Germany) pH 7.5, 40 mM KCl, 1mM ethylene diamine tetraacetic acid (EDTA), 10% (v/v) Glycerol and 1mM phenylmethylsulfonyl fluoride (PMSF). Samples were vortexed for 10 min and centrifuged in Allegra 25 R model centrifuge (Beckman Coulter Inc, Germany) at 15000 rpm for 20 min at 4°C.

The supernatants were collected into a sterile tube and the total protein concentration was determined by Bradford's method employing bovine serum albumin (Sigma) as the standard (Bradford, 1976). The measurements were done in triplicate, at 595 nm, using a Perkin Elmer Lambda25 ultraviolet/ ultraviolet visible (UV/VIS) spectrophotometer.

### Separation of protein by SDS-PAGE

In order to establish the extraction efficiencies, the protein samples were run on the SDS-PAGE separation with following Laemmli method (Laemmli, 1970). 20 µg of proteins were solubilised with sample buffer (62.5 mM Tris(hydroxymethyl) Tris-HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2- mercaptoethanol and 0.01% (w/v) bromophenol blue) and loaded in each lane of the 12% concentrated separating gel.

Electrophoresis was accomplished at 100 V for 90 min using Bio-Rad, Mini Protein electrophoresis system. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 40% (v/v) methanol and 7% (v/v) acetic acid for 1 h and destained with 40% (v/v) methanol and 7% (v/v) acetic acid until the background was clear. The gel scanning and visualization was done by a densitometer (GS-800, Bio-Rad, USA). Further analysis was performed by GelPro Analyzer software (version 3.1).

### Experimental design

As it was mentioned the first experiment was to determine a rapid and effective method to enhance the germination percentage and the second experiment was to identify the impact of the seed and seed coat proteins on the seed dormancy and germination of *A. paniculata* during May to August 2011. Both experiments were carried out based on Randomized Complete Block Design (RCBD) with three replicates.

Five different evaluated treatments in the first experiment were consisting scarification with two layers of sandpaper (SP), 0.3 % (w/v) potassium nitrate (PN) for 10 min, 25% (v/v) acetone (AC) for 10 min, boiling water (BW) 100°C for 10 min and distilled water (control). Three of the different treatments were applied in the second experiment including seed protein extract (SPE), seed coat protein extract (SCPE) and scarification with two layers of sand paper (control). Fifty seeds were used for each treatment. The seeds were treated with five ml of the different treatment on the filter paper in sterilized Petri dishes, separately. Petri dishes were sealed with parafilm to prevent any water loss during incubation. The average temperature in the growth chamber was set between 26 to 32°C and the relative humidity (RH) varied between 60 and 75%.

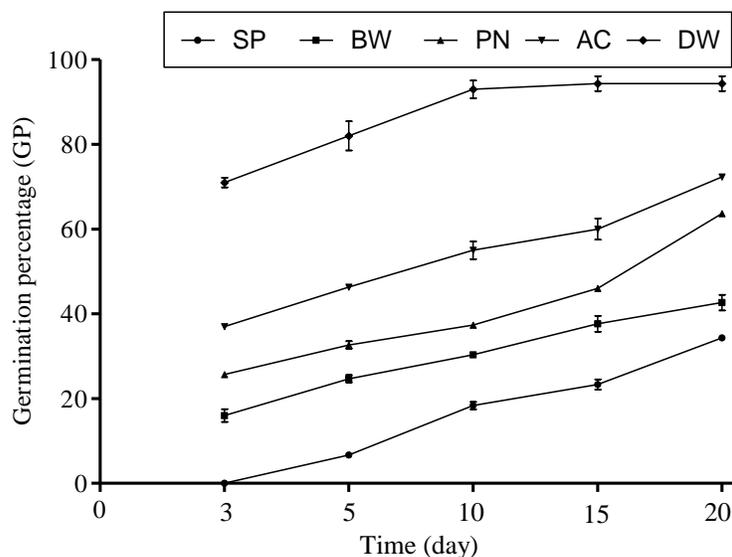
### Studied traits and used equations

The seed counting was started from the third day and was continued until the 20<sup>th</sup> day. The mean germination time after 20 days ( $MGT_{a20}$ ) was calculated using the described formula of Ellis and Roberts (1981). The average germination percentage ( $AGP_{a20}$ ) was calculated using the following equation:  $AGP = Dt / \sum (1/Gt)$ , where Gt is the percentage of the seeds germinated for a particular day, and Dt is the number of days after the seed treatment ( $1 \leq D \leq 20$ ). The germination rate ( $GR_{a20}$ ) was calculated by dividing the germination percentage obtained at each counting to the certain

**Table 1.** Variance analysis of different mechanical and chemical scarification treatments (EXP 1) and seed and seed coat protein effects (EXP 2) on measured characteristics of AP.

Source	df	Mean square								
		GP <sub>a3</sub>	GP <sub>a5</sub>	GP <sub>a10</sub>	GP <sub>a15</sub>	GP <sub>a20</sub>	MGT <sub>a20</sub>	AGP <sub>a20</sub>	GR <sub>a20</sub>	
R	2	4.07 <sup>ns</sup>	11.27 <sup>ns</sup>	0.2 <sup>ns</sup>	1.87 <sup>ns</sup>	1.07 <sup>ns</sup>	0.49 <sup>ns</sup>	1.43 <sup>ns</sup>	1.62 <sup>ns</sup>	
EXP (1)										
Treatment	4	2133.57**	2394.27**	2529.60**	2189.23**	1719.60**	47.02**	2472.31**	1247.63**	
Error	8	1.82	7.767	7.7	10.533	5.4	0.129	5.735	1.729	
EXP (2)										
R	2	1.33 <sup>ns</sup>	2.11 <sup>ns</sup>	18.11 <sup>ns</sup>	27.44 <sup>ns</sup>	25.44 <sup>ns</sup>	0.17 <sup>ns</sup>	5.74 <sup>ns</sup>	1.42 <sup>ns</sup>	
Treatment	2	2275**	735.44**	320.44*	192.11 <sup>ns</sup>	183.11 <sup>ns</sup>	14.29**	365.06**	573.85**	
Error	4	5.333	28.611	31.111	32.944	30.944	0.179	24.042	6.32	

\*\* , \* and ns, refer to 1%, 5% and not significant, respectively. GP<sub>a3</sub>, GP<sub>a5</sub>, GP<sub>a10</sub>, GP<sub>a15</sub> and GP<sub>a20</sub>: Germination percentage after 3, 5, 10, 15 and 20 days, respectively, MGT<sub>a20</sub>: Mean germination time after 20 days, AGP<sub>a20</sub>: Average germination percentage after 20 days and GR<sub>a20</sub>: Germination rate after 20 days.



**Figure 1.** The trend of GP during the experimental period under various scarification methods. SP: sand paper, BW: boiling water, PN: potassium nitrate, AC: acetone and DW: distilled water (control).

number of the counting day.

### Statistical analysis

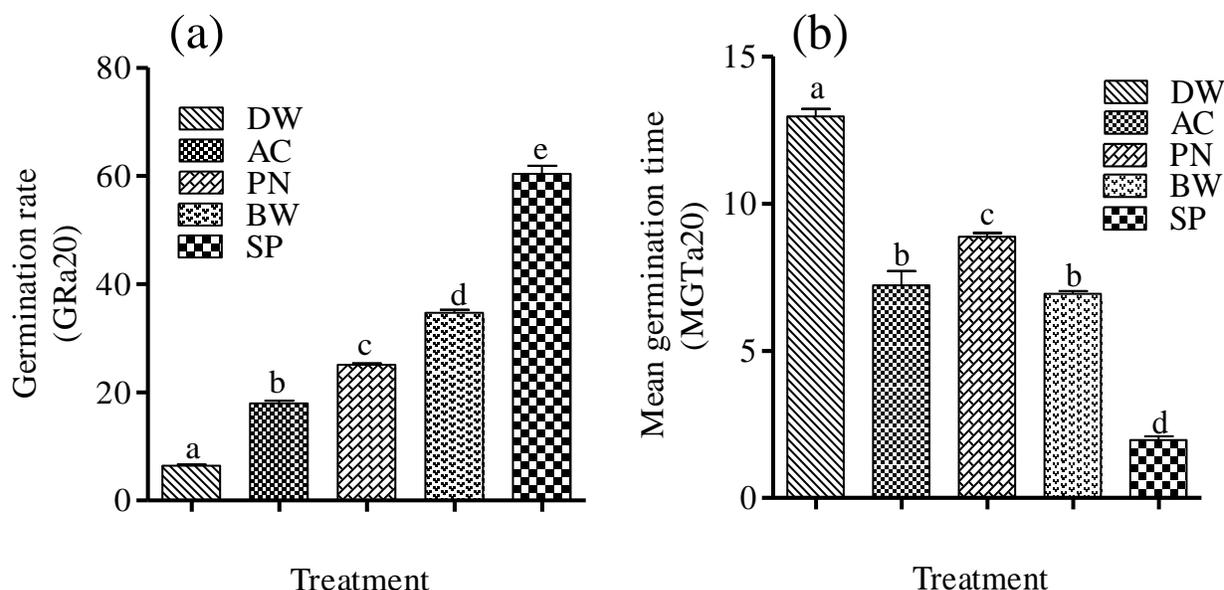
The raw data was subjected initially to normality test using SPSS software (version 19) and the main data were then used for analysis of variance (ANOVA). Duncan's multiple range tests at 1% level was employed to compare the means of different test parameters.

## RESULTS

### Effects of mechanical and chemical scarification on the germination of *A. paniculata* seeds

The results showed that all scarification methods were

efficient on the studied parameters significantly (Table 1). All the scarification methods increased the GP<sub>a20</sub> and decreased the MGT<sub>a20</sub>. The first emergence of the seedlings was observed three days after being treated with the studied agents. The treated seeds with two layers of sand paper were germinated faster than others with 71% and the minimum was obtained in control treatment with 0%. The germination was increased up to 94.33 and 34.33%, respectively in sand paper method and control treatment after 20 days (Figure 1). Among the treatments, scarification with sand paper produced the highest GP<sub>a20</sub> (94.33%), AGP<sub>a20</sub> (90.58%), GR<sub>a20</sub> (60.37) and the lowest MGT<sub>a20</sub> (1.96), while distilled water (the control treatment) showed the lowest GP<sub>a20</sub> (34.3%), AGP<sub>a20</sub> (14.44%) and GR<sub>a20</sub> (6.44) (Figure 2a) and the highest MGT<sub>a20</sub> (12.96) (Figure 2b). The correlation



**Figure 2.** Comparison of the germination rate; **a** and the mean germination time; **b** of AP seeds under various treatments using Duncan's multiple range test ( $P < 0.01$ ). Different letters indicate the significant difference between the values of pair of treatments. SP: sand paper, BW: boiling water, PN: potassium nitrate, AC: acetone and DW: distilled water (control).

between studied traits was significant at  $p < 0.01$ . Interestingly, the correlation between  $AGP_{a20}$  and  $GR_{a20}$  exhibit the highest significance ( $r = 0.999^*$ ), while the lowest correlation was established between  $GP_{a20}$  and  $MGT_{a20}$  ( $r = -0.85^{**}$ ). Besides, the correlation between  $GP_{a20}$ ,  $AGP_{a20}$  and  $GR_{a20}$  were significantly positive, while a negative correlation was observed between  $MGT_{a20}$  and  $GP_{a20}$ ,  $AGP_{a3}$  and  $GR_{a20}$ .

#### Effects of the seed and seed coat proteins on the germination of *A. paniculata* seeds

The seed and seed coat protein extracts affected the measured traits significantly (Table 1). Moreover, the results indicated that the  $GP_{a3}$ ,  $AGP_{a20}$  and  $GR_{a20}$  were significantly decreased when the seeds were exposed to the seed and seed coat proteins, while at the same situation  $MGT_{a20}$  got increased. The first emergence of seedlings was happened three days after treating with the protein extracts. The lowest germination percentage at the third day was found when the seeds were treated with seed protein extract (16.33%) and the highest was obtained in scarified seeds with sand paper (control) (71.33%). These percentages were increased up to 82 and 94%, respectively in seed protein extract and control 20 days (Figure 3). The effect of the seed and seed coat protein extracts were significant on  $GP_{a3}$ . During the experimental period, the germination percentage increased but no significant differences were observed among the treatments in term of  $GP_{a15}$  and  $Gp_{a20}$

(Figure 3). Despite an increase in  $GP_{a20}$ , decrease in  $MGT_{a20}$ ,  $AGP_{a3}$  and  $GR_{a20}$  were obviously seen. The highest  $GR_{a20}$  (60.44) and lowest  $MGT_{a20}$  (1.90) were found in the control treatment, while the lowest  $GR_{a20}$  (32.89) (Figure 4a) and highest  $MGT_{a20}$  (6.21) (Figure 4b) were obtained in the seed protein treatment.

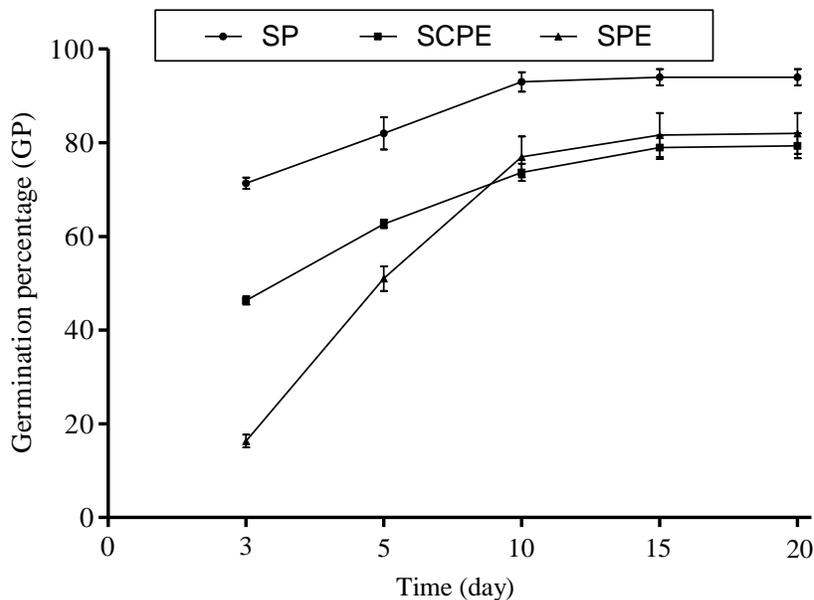
#### Analysis of protein profiles

Proteins' analysis manifested that 15 different types of protein are detectable in the seed and seed coat of *A. paniculata*. Theoretically, the total seed protein must contain an accumulation of both the endosperm and the seed coat proteins. This point was completely confirmed by SDS-PAGE running (Figure 5). The seed's compartments generated a total of 42 protein bands in each triple group where the proportion of the seed coat, the endosperm and the whole seed were 13, 14 and 15 bands, respectively. The molecular weight of the proteins was ranged from 17 to 127 kDa approximately (Figure 5).

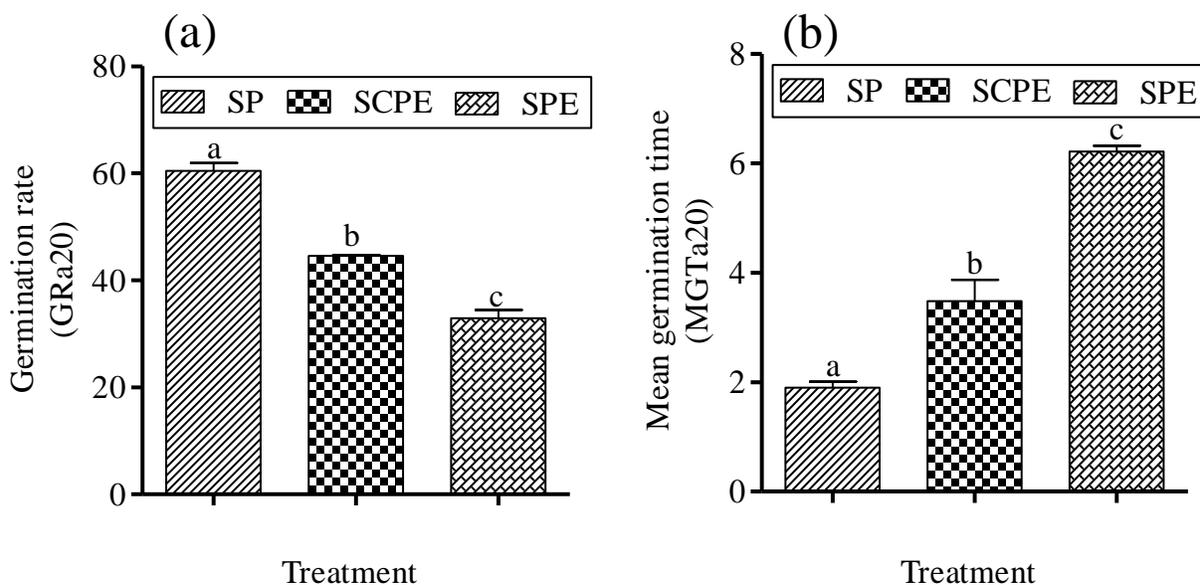
## DISCUSSION

#### Physical dormancy of AP was due to the hard seed coat

*A. paniculata* seeds have a hard seed coat layer, and the GP is relatively poor. Breaking the seed dormancy using sand paper is not a new technique and it has been used in other plant species as well. The fundamental principle



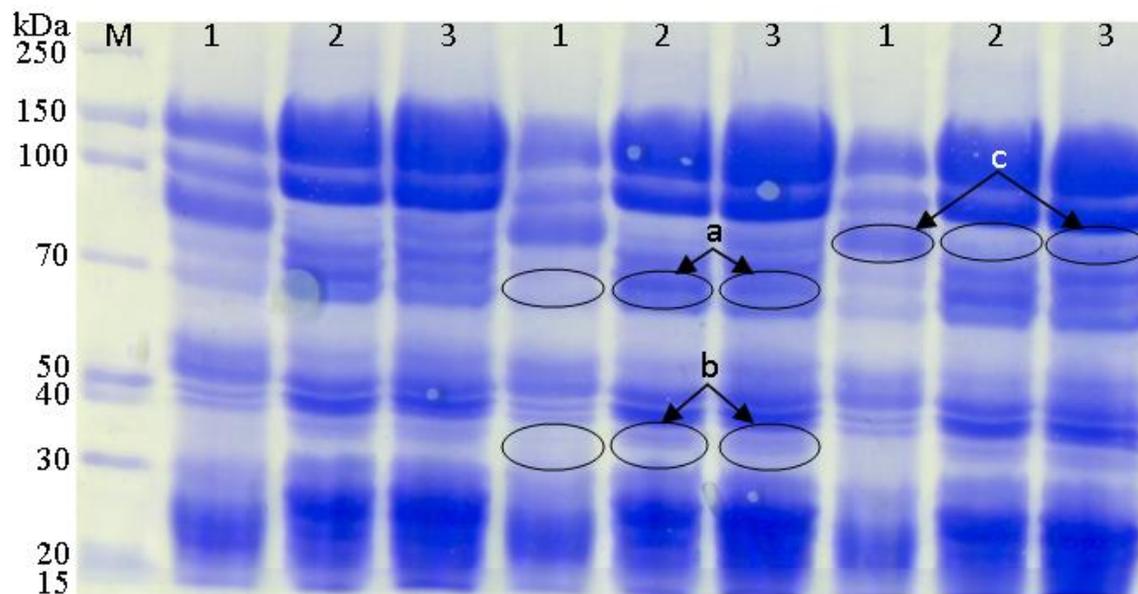
**Figure 3.** The trend of GP during the experimental period under the seed and seed coat protein extract treatments. SP: Sand paper (control), SPE: seed protein extract, SCPE: seed coat protein extract.



**Figure 4.** Comparison of the germination rate; **a** and the mean germination time; **b** of AP seeds under the seed and seed coat protein extract treatments using Duncan's multiple range test ( $P < 0.01$ ). Different letters indicate significant difference between the values of pair of treatments. SP: sand paper (control), SPE: seed protein extract, SCPE: seed coat protein extract.

of the method using the sand paper is removing the hard seed's coat to allow the water to penetrate inside the seed tissues. The majority of the seeds are effectively scratched during this process without destroying the

ability of the seed to germinate normally. Removing this hard seed-coating simply breaks the dormancy. Improvement of the germination performance has been reported when the seeds were scarified with sand paper



**Figure 5.** SDS-PAGE polypeptide profile of the seed, seed coat and endosperm of *A. paniculata*. The lane M represents the protein marker, lane 1 represent the seed coat, lane 2 represent endosperm and lane 3 represent the seed protein isolated from *A. paniculata* seeds. Protein samples were loaded in equal amount of 20  $\mu$ g.

in different plant species with the hard seed-coating layer such as *Tephrosia purpurea* (Sundararaj et al., 1971), *Melilotus alba* and *Trigonella Arabica* (Baskin, 2003; Baskin and Baskin, 2001). The hard seed-coating layer in *A. paniculata* seeds provides a combination of physical and physiological dormancy that prevents water uptake by the embryo.

Germination performance of the seeds can also be enhanced by soaking the seeds in boiling water for 10 min. This process could soften and destroy the seed-coating layer to allow the water uptake by the embryo. Improvement in seed germination using boiling water has been delineated for this plant by Kumar et al. (2011b). There are statements from other researchers that highlight the role of boiling water as a germination enhancer in hard coated seeds in some plant families like Fabaceae, Malvaceae, Cannaceae, etc. (Baskin et al., 2000), testa (Aydin and Uzun, 2001; Msanga and Maghembe, 1986; Ren and Tao, 2004), *Tamarindu indicus* (Muhammad and Amusa, 2003), *Leucaenia leucocephala* and *Acacia nilotica* (Duguma et al. 1988) and *Atropabella donna* (Genova et al., 1997).

#### **Physiological dormancy of *A. paniculata* was due to unknown protein**

The upshots of the current exploration confirmed that the dormancy of *A. paniculata* seeds is a combination of physical and physiological factors. To prove this, the scarified seeds were exposed to the seed and seed coat proteins separately to differentiate their prohibitive effects

on germination. The results indicated that the seed and seed coat proteins affected  $GP_{a3}$  significantly. During the experimental period GP increased but no significant differences were observed among the treatments in terms of  $GP_{a15}$  and  $GP_{a20}$ . This could be due to the denaturation (or deactivation) of the proteins during the experimental period. Low  $GP_{a3}$  and high  $GP_{a20}$  indicated that proteins have no certain inhibition effects on the seed germination, but they can postpone the germination procedure and this delay increases the mean germination time, subsequently. The significance of the obtained results from each compartment led us to subject the case to a bio-molecular investigation with looking at the protein profiling of the seed, seed coat and endosperm as reliable evidence.

In fact, various protein resources caused different prohibition effects on  $GP_{a3}$  and this was a logic clue to predict a variant protein profiling for the mentioned seed compartments. However, three polymorphic protein bands were detected in the gel analysis in which two of them sized approximately 31 and 69 kDa (bands A and B) were absent typically in the seed coat (present in endosperm and whole seed), and the band C sized 75 kDa was absent in endosperm (present in the seed and seed coat) (Figure 5). Hence, the proteins A and B could be considered as the reason of the germination delay in first three days ( $GP_{a3}$ ). In contrast, the protein C can be introduced as a non functional or so-called structural protein, which has no inhibitor effect on the seed germination in *A. paniculata*. This justification for protein C is in agreement with its physical effects on the seed coat hardness, which can simultaneously cause a physical

physical dormancy in this species. The results of the present study matched well with the previous findings that showed some plant seeds contain protein-based compounds that inhibit the seed germination process such as rice seeds (Horiguchi and Kitagishi, 1971), barley seeds (Papageorgakopoulou and Georgatsos, 1978), lettuce seed (Black and Richardson, 1967; Schultz and Small, 1991), flax (Rosu et al., 2010), *Candida albicans* (Cho et al., 2000) and *Pinus bungeana* (Hao et al., 2005).

## Conclusions

Our results revealed the important role of SP and BW in breaking the physical dormancy (caused by hard seed coat layer) and physiological dormancy (caused by the seed and seed coat proteins). However, the mechanisms of the above-mentioned factors are totally different (removing the hard seed coat layer and removing or dissolving the inhibitor proteins), but they will meet at the same point which results from the improvement in *A. paniculata* germination. According to decelerating effect of the seed and seed coat proteins on GP<sub>a3</sub> even in scarified seeds it can be concluded that the presence of proteins in both functional and structural forms can facilitate the occurrence of physiological and physical dormancy. Both the proteins caused delay but no absolute inhibition in germination of the scarified seeds, suggesting that the dormancy in *A. paniculata* is controlled by a combination of physical (presence of a hard seed coat) and physiological (presence of unknown proteins) mechanisms.

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