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Effect of the seed coat on dormancy and germination in *Stylosanthes humilis* H. B. K. seeds[1]

Izabel de Souza Chaves[2*], Nilo Cesar Queiroga Silva[2], Dimas Mendes Ribeiro[2]

ABSTRACT - Seed of Townsville stylo (*Stylosanthes humilis* H.B.K.) is known to exhibit a hard seed coat and when freshly harvested also show a physiological dormancy, however, the nature of the co-actions between seed coat and embryo growth that determine dormancy is poorly understood. In this study, physical dormancy of Townsville stylo seeds was not reduced during natural ageing at room temperature, in contrast to the physiological dormancy, which is gradually overcome during after-ripening. Furthermore, the permeability of seed coat was affected by scarification treatments as well as by low-pH solutions. Together, these data indicate that physical dormancy overcome of seed is prerequisite for radicle protrusion and physiological dormancy of Townsville stylo seeds contribute to its timing.

Index terms: physical dormancy, physiological dormancy, Townsville stylo.

Efeito do tegumento na dormência e na germinação de sementes de *Stylosanthes humilis* H. B. K.

RESUMO - Semente de estilosantes (*Stylosanthes humilis* H.B.K.) é conhecida por apresentar tegumento rijo e quando recém colhida também exibe dormência fisiológica, entretanto, a natureza da coação entre o tegumento e o crescimento do embrião que determina a sua dormência é pouco conhecida. Nesse estudo, a dormência física de sementes de estilosantes não foi reduzida durante o envelhecimento natural à temperatura ambiente, em contraste com a dormência fisiológica que foi superada gradualmente durante o envelhecimento pós-colheita. Além disso, a permeabilidade do tegumento foi afetada por tratamentos de escarificação bem como por soluções de baixo pH. Juntos, esses resultados indicam que a superação da dormência física da semente é um pré-requisito para a protrusão da radícula e a dormência fisiológica das sementes de estilosantes contribui para temporização da germinação.

Termo para indexação: dormência física, dormência fisiológica, estilosante.

Introduction

Townsville stylo (*Stylosanthes humilis* H.B.K.) is an annual forage legume common in natural pastures of tropical America (Williams et al., 1984; Stappen et al., 2000; Santos-Garcia et al., 2012). The species is utilizing for pasture improvement in tropical zones due to its high-quality forage for livestock, high seed production, and wide adaptability to low fertility soils (Edye, 1987). In addition, *Stylosanthes* are potentially useful species aiming at ecological purposes, such as rehabilitation of damaged ecosystems (Grigg et al., 2000; Starr et al., 2013). Seeds of Townsville stylo exhibit a physiological dormancy, which is gradually lost upon post-harvest ageing. Following harvesting physiological dormancy is lost very slowly; by six months germination increases substantially, and 12-15 after harvest seeds placed under a 30/25 °C day/night cycle with 60/70% relative humidity showed full germination (Vieira and Barros, 1994). Any stressing factor such as low pH solutions (Pelacani et al., 2005a,b), selenium compounds (Pinheiro et al., 2008) and ferric ions (Ribeiro et al., 2011), which induced ethylene production by seeds, promotes the overcome of physiological dormancy of *S. humilis* seeds. As with some other legumes such as *Senna multijuga* (Rodrigues-Junior et al., 2014) *Cassia*

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[2]Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 - Viçosa, MG, Brasil.
*Corresponding author <izabelchaves@yahoo.com.br>

leptophylla and *Senna macranthera* (Paula et al., 2012), seeds of Townsville stylo also exhibit physical dormancy, resulting from an impermeable seed coat (Smýkal et al., 2014).

The seed coat exerts its germination-restrictive action most of the time by being impermeable to water and/ or oxygen or by its mechanical resistance to radicle protrusion (Linkies et al., 2009; Smýkal et al., 2014). In legumes, a densely packed layer of palisade cells impregnated with water-repellent compounds causes mechanical resistance of seed coat (Baskin and Baskin, 2004; Smýkal et al., 2014, Baskin and Baskin, 2014). The seed becomes permeable to water only when the coat is disrupted in some way, particularly at the lens (strophiole) region, which is usually the physically weakest part of the seed coat (Moïse et al., 2005; Jaganathan et al., 2017). Thus, in the absence of physiological dormancy, overcoming of physical dormancy may lead to immediate germination of the seeds upon imbibition. Despite of the known association between seed coat permeability and embryonic growth potential, the nature of the co-actions between seed coat and embryo growth that determine dormancy is still unclear.

A wide range of factors that may potentially disrupt seed-coat imposed dormancy under natural conditions have been identified, with differing implications for seed bank dynamics and seedling emergence patterns (Van Klinken et al., 2006; Gama-Arachchige et al., 2012). For example, high temperatures promoted dormancy overcome in impermeable seeds of *S. humilis* and *S. hamata* during the hot, dry season in northern Australia (McKeon and Mott, 1982). Mechanical abrasion by soil particle, decomposition of the seed coat by microbial action as well as smoke or heat shock from fire are the others possible environment factors affecting physical dormancy of seeds in nature (Briggs and Morris, 2008). A strong positive relationship between acidic solutions (low pH) and overcome of physiological dormancy has been found in scarified seeds of *S. humilis* (Pelacani et al., 2005a,b; Ribeiro et al., 2010). However, the importance of low pH for dormancy overcome in intact seeds of *S. humilis* has been poorly explored. Thus, the aim of the present study was to analyse the consequence of seed-coat-imposed dormancy on the physiological dormancy and germination of *S. humilis* seeds.

Material and Methods

Plant material and germination assays

Plants of *S. humilis* H.B.K. were grown in 3.0 L plastic pots in a greenhouse in Viçosa (20°45'S, 42°15'W), Minas Gerais, Brazil. Matures pods were harvested and stored in paper bags in laboratory at 30/25 °C day/night cycle with 60/70% relative humidity for 1, 6, 12, 24, 36 and 48 months prior to extraction from the pods. In this way, six seed lots (with different natural ageing time) were used for germination experiments. Following dehusking, intact seeds or mechanically (scarification with fine sandpaper number 150) and chemically (immersion in H_2SO_4 98% for 1 or 5 min and subsequent washing in water) treated seeds were sterilized with 0.5% NaOCl for 10 min and thoroughly washed with distilled water. Seeds (intact or scarified) were placed in glass Petri-dishes 90 mm diameter containing two layers of Whatman n° 1 filter paper moistened with 10 mL water (control), 1-aminocyclopropane-1-carboxylic acid (ACC), the biochemical precursor of ethylene, or 2-choroethylphosphonic acid (CEPA), an ethylene-releasing compound. To further evaluate the role of ethylene in seed germination, intact seeds were placed in 50 mL Erlenmeyer flasks containing two layers of Whatman n°1 filter paper moistened with 10 mL of water. Erlenmeyer flasks were immediately sealed with serum rubber caps and ethylene (to attain 10 μM) was injected in sealed flasks. The atmosphere of flasks was occasionally stirred with a syringe with needle inserted through the rubber seal. The effects of low pH solution on the mechanism of seed germination were also examined by treating seeds (intact or mechanically scarified with sand paper) with 10 mL MacIlvaine (10 mM) buffer solution at pH 4.0-7.0 in glass Petri-dishes 90 mm diameter containing two layers of Whatman n°1 filter paper. Petri-dishes and Erlenmeyer flasks containing fifty seeds were placed in the dark in a day/night growth chamber (Forma Scientific Inc., Ohio, USA) at 30 °C. The seed was considered as germinated upon protrusion of its radicle.

Seed coat permeability assay using tetrazolium staining

Entire seeds were incubated in a 1% (w/v) solution of 2, 3, 5-triphenyltetrazolium chloride (Sigma-Aldrich) at 30 °C in darkness for 5 d as described by Wharton (1955). The embryo and cotyledons of Townsville stylo seeds stain red upon entry of the tetrazolium salt solution in the viable seed, but stay whitish when the dye does not penetrate. Thus, enhanced staining intensity indicates enhanced seed coat permeability (Debeaujon et al., 2000).

Microscopy

We performed microscopic analysis of mature seed coat to correlate the germination behaviour of Townsville stylo seeds with precise seed coat characteristics. Two treatments were compared for their effectiveness in overcoming hard seed coat dormancy (i) manual scarification - seeds were subject to manual scarification by gently rubbing between fine sand paper (n° 150) for 1 min, (ii) chemical scarification

- seeds were immersed in 20 mL of 98% H_2SO_4 for 1 min or 5 min at ambient temperature (~ 25 °C). The seeds were then washed thoroughly under running water for 5 min. Seeds were mounted directly on double-sided adhesive tape affixed to scanning electron microscopy stubs. Then, the samples were coated with gold-palladium (15 nm) in a sputter coater and examined using a scanning electron microscope (LEO; EVO 50 XVP, Cambridge, UK).

Statistical analysis

The statistical design of the assays was based on a completely randomized distribution with five replicates with 50 seeds each for germination test in Petri-dishes or Erlenmeyer flasks. Germination percentage was transformed to arcsin $(\% G/100)^{1/2}$, prior to analysis and all data were checked for normality. Analysis of variance (ANOVA; $p < 0.05$) was carried out to determine effects of treatments. If ANOVA showed significant effects, Tukey test was used to determine differences among treatments. All mean comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 for Windows Statistical Software Package.

Results and Discussion

The degree of dormancy of Townsville stylo seeds was assessed by determining the germination percentage of the seed lots with different post-harvest age (Figure 1a). After mechanical scarification of the seeds, freshly harvested seeds of Townsville stylo were dormant, but this physiological dormancy has disappeared after 12 months after-ripening (Figure 1a). This agreed with the suggestion of Vieira and Barros (1994) that, for Townsville stylo seeds, a dry storage for up to 1 year would be required for the enhancement of germination. However, compared with freshly harvested seeds, the non-dormant seeds displayed a similar permeability of seed coat, demonstrating that natural ageing time did not increase permeability of Townsville stylo seeds to tetrazolium solution (Figure 1b). Cell expansion growth required for radicle protrusion and seed coat rupture depends on environmentally and hormonally regulated cell wall-loosening mechanisms (Van Sandt et al., 2007; Linkies et al., 2009; Muller et al., 2009; Morris et al., 2011). In Townsville stylo embryo hypocotyl-radicle axis growth are associated with ethylene production

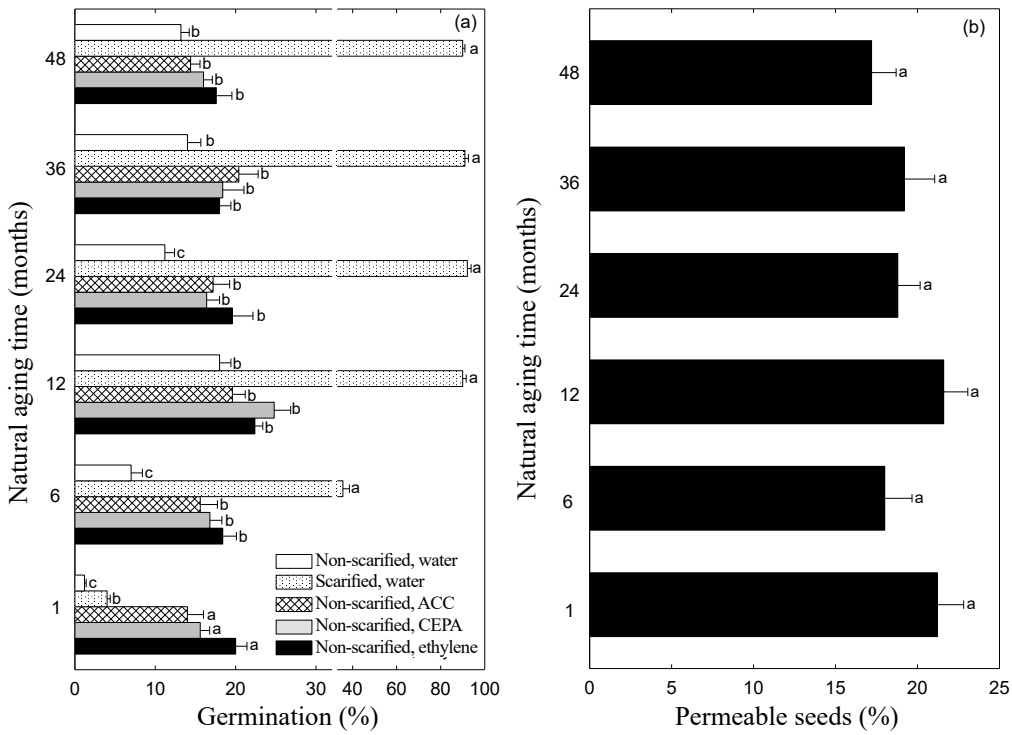

Figure 1. Effect of dry storage on dormancy release of Townsville stylo seeds. (a) Germination was determined in mechanically scarified seeds imbibed just in water or in intact seeds treated with ACC (1 mM), CEPA (1 mM) and ethylene gas (10 µM). (b) Permeability of intact seeds to tetrazolium solution. Remaining fraction of seed to 100% corresponds to impermeable seeds. Germination and permeability of seeds to tetrazolium salt were carried out on the 5th day. Values with the same letter within (a) each natural aging time and (b) among natural aging time are not statistically different at the 5% level by Tukey test. Data points are means of five replicates ± standard error.

as a mechanism for cell expansion growth (Vieira and Barros, 1994; Ribeiro and Barros, 2006). Freshly harvested seed and non-dormant seeds germinated only about 20% when treated with ACC (immediate precursor of ethylene), CEPA (an ethylene-releasing compound) or ethylene gas (Figure 1a). The observation that intact seeds were permeable to tetrazolium salt solution in a manner related to germination behaviour of seeds treated with ACC, CEPA or ethylene gas demonstrates that seed coat play a major role on the imposition of germination of Townsville stylo seeds and that seed coat weakening is not the consequence of ethylene action. Therefore, ethylene requirement for physiological dormancy alleviation as well as for germination of Townsville stylo seed appears to be under control of physical dormancy imposed by the hard seed coat. The hardseededness and water impermeability of many legume seeds is due to a palisade epidermal layer of thick-walled Malphighian cells in the outer testa (Moïse et al., 2005). Thus, the germination behaviour of intact seeds of Townville stylo can be related with structure of the seed coat: a cuticle and a palisade layer composing the outer integument followed by an endothelium layer and a crushed parenchymatic layer forming the inner integument (Figures 2a-d).

The important restrictive role of the seed coat in the germination of Townsville stylo seeds was supported further by scarification experiments. It is known that physical and chemical scarification overcome physical dormancy of *Stylosanthes* seeds (Mott and Mckeon, 1979; Anand et al., 2011). In this context, seed coat permeability to tetrazolium salt solution was increased in dormant and non-dormant seeds scarified with sand paper or treated with H_2SO_4 (Table 1). However, scarification treatments increased germination of non-dormant seeds *i.e.*, seeds without physiological dormancy, but not in seeds with physiological

Table 1. Permeability of Townsville stylo seed coat to tetrazolium solution and germination of dormant (15 post-harvest days old) and non-dormant (765 post-harvest days old) seeds after treatment with different physical dormancy breaking methods. Permeability of seeds and germination were carried out on the 5th day.

Treatment	Permeable seed fraction (%)	Germination (%)
Dormant seed		
Non-scarified (Control)	20.4 ± 1.3 c	2.9 ± 0.7 a
Mechanical scarification	98.8 ± 1.2 a	3.4 ± 0.9 a
Chemical scarification – 1 min H_2SO_4	74.8 ± 3.0 b	3.1 ± 0.5 a
5 min H_2SO_4	97.6 ± 1.0 a	4.0 ± 1.0 a
Non-dormant seed		
Non-scarified (Control)	18.8 ± 2.1 c	16.4 ± 1.5 c
Mechanical scarification	98.8 ± 0.8 a	94.0 ± 2.1 a
Chemical scarification – 1 min H_2SO_4	77.2 ± 3.1 b	74.4 ± 4.7 b
5 min H_2SO_4	99.2 ± 0.8 a	94.4 ± 2.6 a

In each column, means do not differ significantly at the 5 % level by Tukey test, when followed by the same letter. Data are means ± standard error of five replicates.

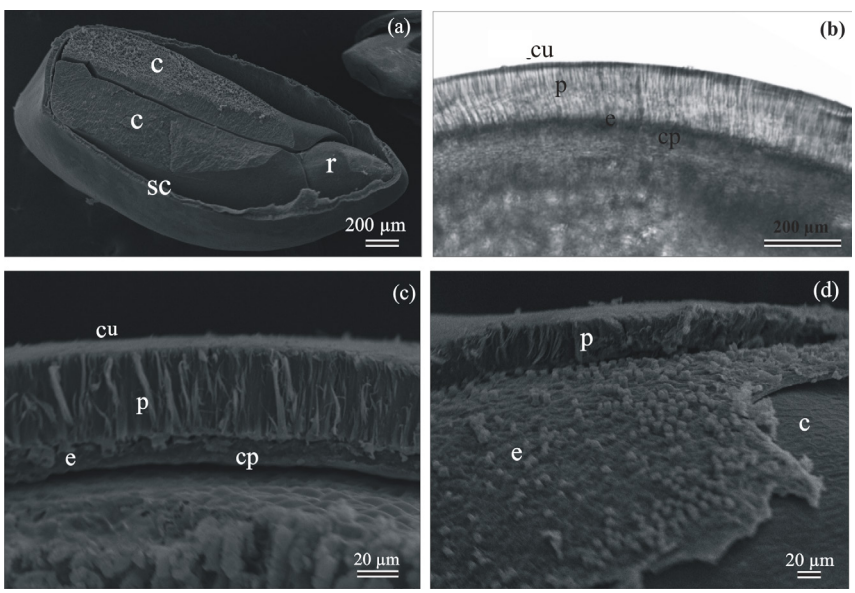

Figure 2. Scanning electron micrographs of Townsville stylo seed. (a) Entire mature seeds (15 post-harvest days old) showing radicle tip (r), cotyledons (c) and seed coat (sc). (b,c and d) Structure of the seed coat showing cuticle (cu), palisade layer (p), endothelium layer (e), crushed parenchymatic layer (cp) and cotyledons (c).

dormancy (Table 1). Scanning electron micrographs revealed that the surface of mechanically scarified seeds peeled at various places, resulting in a weakening of the seeds coat (Figure 3b). In contrast, scarification with H_2SO_4 overcame physical dormancy of seeds by causing many randomly located cracks in the seed coat, which than act as sites of water entry (Figures 3c-g). However, germination of freshly harvested seed was not increased after scarification treatments (Figures 4a, e), indicating that Townsville stylo seeds depend on a stepwise physiological dormancy-overcoming behaviour in timing their germination. In agreement with this, scarified dormant-seeds were able to germinate quite rapidly when imbibed in CEPA (Figure 4c), an ethylene-releasing compound. On the other hand, mechanical or chemical scarification of aged Townville stylo seeds can be substitute for the exogenous CEPA requirement for germination (Figures 4d, f). Together, these data indicate that physical dormancy overcome is prerequisite for radicle protrusion and physiological dormancy of Townsville stylo seeds contribute to its timing.

In many ecosystems, soil moisture and temperature are the two most important factors that determine seasonal germination pattern and modulate persistence and dormancy of soil seed banks (Benech-Arnold et al., 2000; Walck et al., 2005; Batlla and Benech-Arnold, 2010). For example, McKeon and Mott (1982) showed that seeds of *S. humilis* and *S. hamata* were softened by temperature fluctuation during the

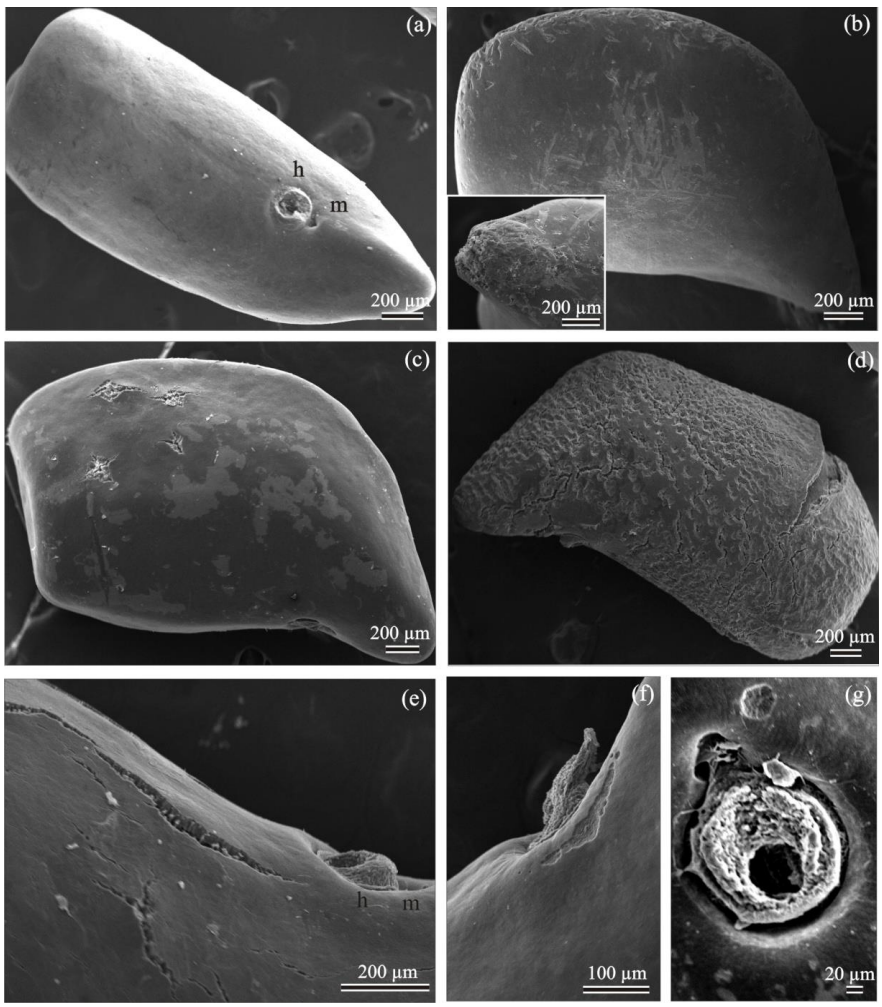

Figure 3. Scanning electron micrographs of Townsville stylo seed coat after pre-treatment with different dormancy breaking methods. (a) Intact seeds (15 post-harvest days old) showing hilum (h) and micropyle (m); (b) mechanical scarification with fine sand paper. Detail shown the damage caused by sandpaper occurs preferentially in the curved region and at the extremity where the protrusion of the radicle occurs; (c and d) acid scarification with H_2SO_4 for 1 and 5 min, respectively. (e) Cracks are see on the surface of seeds treated with H_2SO_4 for 1 min. (f and g) Morphology of the hilum area after treatment with H_2SO_4 for 5 min.

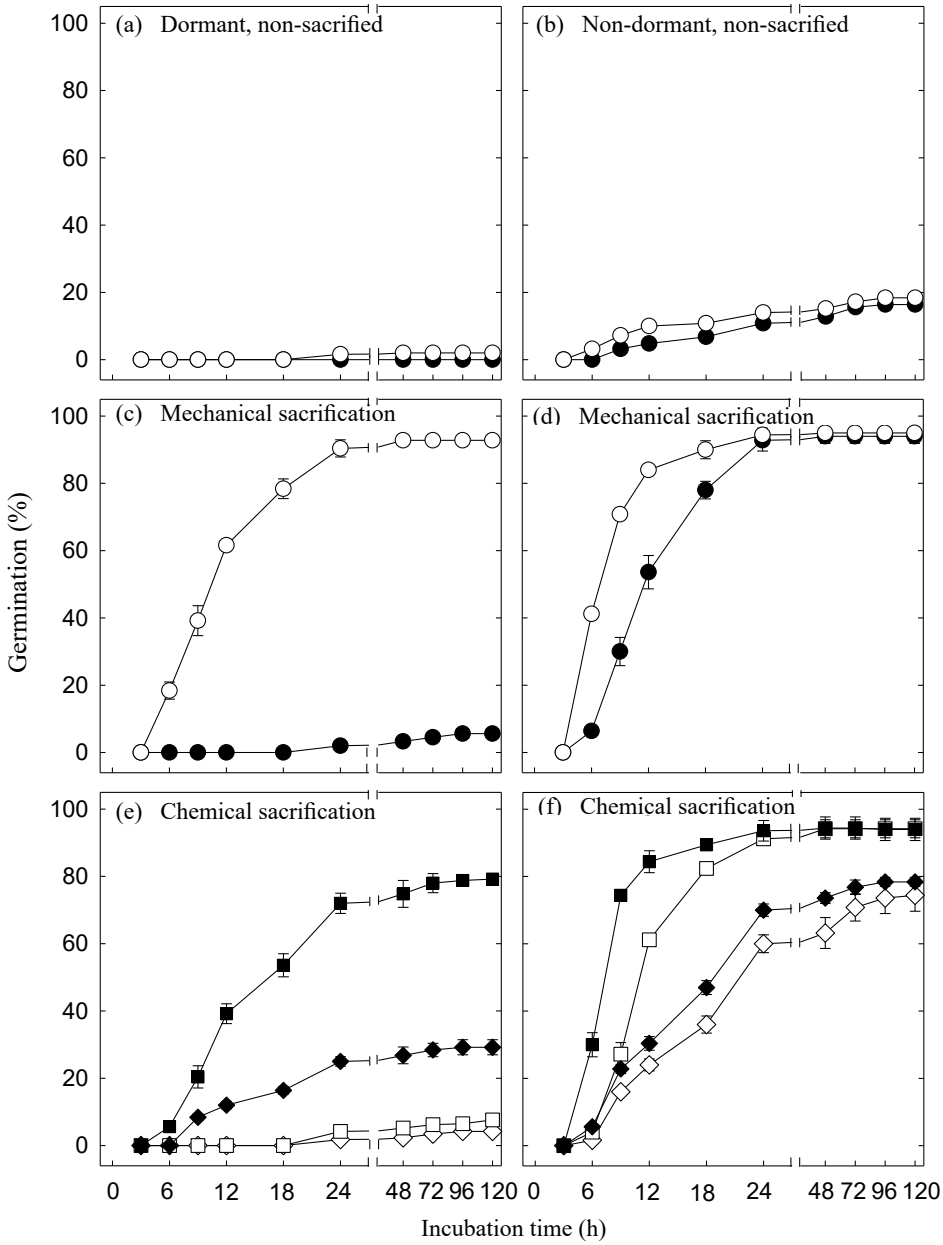

Figure 4. Time course of germination for dormant seed (15 post-harvest days old) and non-dormant seed (765 post-harvest days old) after pre-treatment with different physical dormancy breaking methods. (a, b, c and d) Seeds were imbibed in water (filled circle) or 1 mM CEPA (open circle). (e and f) Seeds were pre-treated with H_2SO_4 for 1 and 5 min, and imbibed in water (open diamond, open square) or 1 mM CEPA (filled diamond, filled square), respectively. Data points are means of five replicates ± standard error.

hot, dry season in northern Australia. Soils in which species of *Stylosanthes* genus is distributed naturally are generally acidic (pH 4.0-5.5) (Williams et al., 1984), as the Cerrado (the Brazilian savannah) soils (Ruggiero et al., 2002). Low-pH solutions were effective in overcoming physiological dormancy of scarified seeds of Townsville stylo (Pelacani et al., 2005a,b; Ribeiro et al., 2010). In the present work, those data were expanded to include the effects of low-pH solutions on physical dormancy overcome of Townsville stylo seeds (Figures 5a-d). Low pH of the incubation medium-mediated increase in the seed coat tetrazolium salt permeability during dormant and non-dormant seed imbibition (Figures 5a, b). The maximum significant effect induced by acidic solution occurred at pH 4.0 when permeability of the dormant and

non-dormant seeds to tetrazolium was increased by 2.1- and 2.3-fold, respectively, as compared with the control (pH 7.0) (Figures 5a, b). In line with this finding, acidic solutions increased germination of non-escarified seeds (Figures 5c, d, black bars). The maximum effect promoted by applied of low pH solutions occurred at pH 4.0 when seed germination of dormant and non-dormant seeds was increased by 22.0- and 2.8-fold, respectively, as compared with the control (pH 7.0) (Figures 5c, d, black bars). The occurrence of germination indicates that the permeability to tetrazolium salt can be used to monitor the permeability of Townsville stylo seeds to water entry. As expected, physiological dormancy of scarified seeds was partially broken by acidic solution at pH 4.0 and 5.0 (Figure 5c, white bars). In addition, low pH solutions had no toxic effect on the germination response of scarified non-dormant seeds (Figure 5 d, white bars). In other words, freshly harvested seeds exhibited a reduced germination compared with non-dormant seeds, indicating that the germination behaviour of freshly harvested seeds treated with acid solutions is related to the degree of the physiological dormancy in the seeds. Together, these data indicate that low pH solutions causes seed coat weakening, which in turn may require a lower embryo force for its rupture.

Conclusions

Physical dormancy of Townsville stylo seed was not reduced during natural ageing at room temperature, in contrast to the physiological dormancy, which is gradually overcome during post-harvest ageing. Furthermore, the permeability of seed coat was affected by scarification treatments as well as by low-pH solutions, which leads to effects on germination. Given

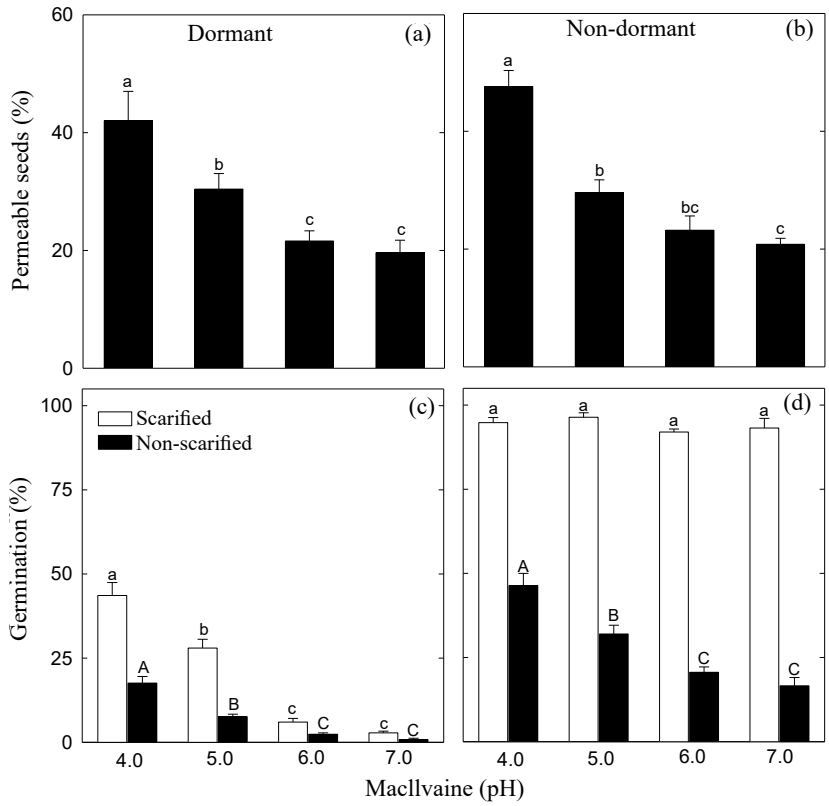

Figure 5. Response of Townsville stylo seeds to MacIlvaine buffer solutions at several pH(s). (a and b) Permeability of intact seed to tetrazolium salt supplied in MacIlvaine buffer at several pH(s). Remaining fraction of seed to 100% corresponds to impermeable seeds. (c and d) Germination of seeds incubated in serial pH MacIlvaine buffer solutions. Test-solutions were renewed 24 hours after start of imbibition. Permeability of seeds and germination were registered on the 5th day. After mechanical scarification, permeability of seed coat to tetrazolium salt supplied in MacIlvaive pH 7.0 was 95 ± 0.9% and 93 ± 0.7% in dormant (15 post-harvest days old) and non-dormant seeds (765 post-harvest days old), respectively. Bars followed by the same small or capital letter across the range of MacIlvaine solutions do not differ statistically at the 5% level by Tukey test.

that under treatment with acidic solutions a tight relationships linking seed coat permeability and germination are observed, it is possible that low pH soils would play an important ecological role in the successful establishment of *Stylosanthes* species population. In summary, seed coat weakening was a prerequisite for radicle protrusion and physiological dormancy of Townsville stylo seeds contribute to its timing.

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