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Effect of dry heat on seed germination of *Desmodium* and *Stylosanthes* species

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Abstract

Mechanical scarification with a scalpel is the best treatment to break physical dormancy and reach high germination percentages in many legumes. However, it is highly time-consuming. Given the ecological relationship between the presence of physical dormancy and high temperatures in tropical grasslands, dry heat treatment could also promote breaking of physical dormancy in *Desmodium* and *Stylosanthes* species. This study assessed seed germination of several accessions of nine species of *Desmodium* and *Stylosanthes*. Seeds were treated with dry heat (80°C for 30 minutes) and scarified with a scalpel to determine whether dry heat is a reliable alternative treatment to overcome physical dormancy. Mechanical scarification with a scalpel was effective and resulted in high germination for all species. In *S. guianensis*, both treatments had an equivalent effect, making dry heat a feasible alternative. Dry heat could also be a reliable alternative in *D. heterocarpon*, *D. velutinum*, *S. hamata*, and *S. scabra*, but tetrazolium tests may be necessary to confirm viability. For *D. barbatum* and *D. scorpiurus*, dry heat could be an alternative but further research is needed to confirm this, while in *S. capitata* and *S. viscosa* dry heat is not a reliable alternative.

Keywords: *Desmodium*, dry heat, genebank standards, physical dormancy, seed germination, *Stylosanthes*, viability test

Introduction

Desmodium spp. and *Stylosanthes* spp. are key tropical forage crops with a wide distribution from the humid to semi-arid tropics (Williams *et al.*, 1984; Chandra, 2009). Several species of both genera are herbaceous plants adapted to tropical dry woodlands, natural savannas and grasslands (Stace and Edye, 1984; Baskin and Baskin, 2014). They are extensively used by the livestock sector due to their adaptability to infertile, acid soils and stressful climatic conditions. In addition, *Stylosanthes* spp. have high nutritional value (Burt *et al.*, 1983; Lascano, 1991; Santos-Garcia *et al.*, 2012), while *Desmodium*

spp. are beneficial as intercrops for soil recovery (Khan *et al.*, 2008). Seeds of several *Desmodium* and *Stylosanthes* species exhibit physical dormancy (PY), which is a major difficulty when assessing viability in genebanks and is also responsible for inefficient crop establishment (Mott and McKeon, 1982; Anand *et al.*, 2011). High environmental temperatures are closely related to the breakdown of PY and seed germination of these tropical legumes (Mott *et al.*, 1981; McKeon and Mott, 1982; McKeon and Brook, 1983; Veasey and Martins, 1991). A study of the response to high temperatures is not only important for a better understanding of *Stylosanthes* and *Desmodium* germination ecology, but also to improve the use of seeds and to manage seeds more efficiently in genebanks.

The seed coat of *Desmodium* and *Stylosanthes* species has a palisade layer of compressed macrosclereid cells that have high concentrations of hydrophobic compounds, (Serrato-Valenti *et al.*, 1993; Castillo and Guenni, 2001) which cause impermeability (Baskin *et al.*, 2000). This is expected since both genera belong to the Fabaceae (sub-family Papilionoideae), one of the 18 angiosperm families with PY (Baskin *et al.*, 2000; Gama-Arachchige *et al.*, 2013). As in all families with PY, there are specialised structures (water gaps) in the seed coat of legumes that are sensitive to physical environmental signals such as high temperatures or highly fluctuating temperatures (Baskin *et al.*, 2000; Baskin, 2003). In Papilionoideae, the lens is the “signal detector” by which seeds become permeable and able to germinate during the most favourable conditions after the dry season (Lersten *et al.*, 1992; Serrato-Valenti *et al.*, 1993; Morrison *et al.*, 1998; Baskin and Baskin, 2000, 2014; Baskin *et al.*, 2000; Baskin, 2003; Burrows *et al.*, 2009; Jaganathan and Liu, 2014; Jaganathan, 2015, 2016).

Field and laboratory experiments have demonstrated the importance of high temperatures for the germination of seeds of *Stylosanthes* spp. and *Desmodium* spp. Field research with *S. hamata*, *S. scabra*, *S. humilis* and *S. viscosa* have found that temperatures above 50°C are required to achieve a high proportion of seedling establishment (Mott *et al.*, 1981; McKeon and Mott, 1982; McKeon and Brook, 1983; Nicolaeva *et al.*, 1985). In *Desmodium* spp., temperatures close to 40°C reduced the number of hard seeds (Veasey and Martins, 1991). In addition, there are a range of artificial treatments for breaking PY, including acid scarification, microwaves, percussion with hot plates, hot water, dry heat and mechanical scarification with sandpaper or a scalpel (Mott, 1979; Erasmus and Pieterse, 2001; Bhatt *et al.*, 2008; Burrows *et al.*, 2009; Anand *et al.*, 2011; Chaves *et al.*, 2017). Mechanical and acid scarification are the most effective treatments in a wide range of legume species (Silveira and Fernandes, 2006; Clifton-Cardoso *et al.*, 2008; Perez-Garcia, 2009; Pereira and Ferreira, 2010; Wang *et al.*, 2011; Baskin and Baskin, 2014). However, these procedures are risky and/or time-consuming. The CIAT (International Center for Tropical Agriculture) genebank has a viability standard of 70%, i.e., seed lots in storage should have at least 70% viability and seeds are sampled for viability testing every 5 - 10 years. The average rate per trained person for conducting mechanical scarification with a scalpel in the Seed Viability Laboratory at CIAT is 30 accessions (with 100 seeds each) per person in one working day. Heat treatments that mimic natural habitat effects and disrupt the lens structure could offer an alternative, practical method for overcoming PY in seeds of *Stylosanthes* and *Desmodium* species. There is some evidence that dry oven heat (75-85°C) increases the germination percentage in some *Stylosanthes* species

(Gilbert and Shaw, 1979; Mott and McKeon, 1979; Nicolaeva *et al*., 1985); there is less literature about the effect of dry heat on the germination of *Desmodium* species, but a similar response is expected since some are adapted to grasslands.

In the present study, we aimed to establish if dry heat treatment (80°C for 30 minutes) is a reliable alternative to mechanical scarification with a scalpel in viability tests of key *Desmodium* and *Stylosanthes* species, and whether sowing between paper (BP) or on top of paper (TP) has any influence on the effect of these treatments. To address these objectives, three experiments were carried out and the proportions of normal and abnormal seedlings, and hard (not able to imbibe water), fresh (able to imbibe water but without germination) and dead (rotten/mouldy) seeds were analysed. Seeds were treated with dry heat or by scarification with a scalpel and sown with BP and TP techniques.

Materials and methods

Experiment 1

Seeds of *Desmodium barbatum* (CIAT accession number: 23527), *D. heterocarpon* (23303), *D. scorpiurus* (43266), *D. velutinum* (23272), *Stylosanthes capitata* (2536) and *S. guianensis* (165) were used in this experiment. These accessions had been vacuum-packed in aluminium bags and stored at 5°C at the Genetic Resources Program (PRG) in CIAT, and originated from Mexico, Indonesia, Australia, Brazil and Colombia (table 1).

A sample of 1,200 seeds was taken for each accession. Each sample was divided among six paper bags corresponding to the combination of two sowing methods, between paper (BP) and on top of paper (TP), and three treatments (control, mechanical scarification, and dry heat). Paper bags were placed at room temperature (25°C) for one week. During this time, seeds from two of the paper bags were mechanically scarified with a scalpel, by making a small cut on the testa on the opposite side to the hilum. Two other bags with seeds inside were placed in a preheated oven at 80°C for 30 minutes and then returned to room temperature. The temperature and duration of the treatment were determined from preliminary experiments with small samples of *D. heterocarpon*, *D. velutinum* and *S. capitata* seeds using temperatures from 70 to 90°C and periods of 10 to 60 minutes: 80°C for 30 minutes was the treatment that consistently generated high percentages of normal seedlings in all species (unpublished data). No treatment was given to the seeds from the two remaining bags (control). For each treatment, four replicates of 50 seeds were either rolled in saturated germination paper (BP) or sown on the top of saturated filter paper in Petri dishes (TP), according to ISTA BP and TP methods (ISTA, 2020). The Petri dishes were randomly placed inside a germination chamber with a photoperiod of eight hours with white light at 35°C and 16 hours of darkness at 20°C. The BP rolls were packed in pairs inside plastic bags with holes and randomly placed inside a basket, which was then put inside the same germination chamber where the Petri dishes were placed. BP rolls were watered once per week, while the Petri dishes were watered every two or three days as they lost moisture faster.

The numbers of normal and abnormal seedlings, and hard (impermeable), fresh (permeable but not germinated) and dead (rotten) seeds, were recorded 7, 14 and 21 days

after sowing. Seedlings with an intact axis, cotyledons, and stem, without any irregularity in shape or colour were considered normal (ISTA, 2020). Counts were summed and the total numbers were used in the analysis described below.

Experiment 2
In this experiment, 10 accessions from each of *D. heterocarpon*, *D. velutinum*, *S. capitata* and *S. guianensis*, with varying storage periods and originally collected from 11 different countries (table 2), were assessed to compare the germination of seeds treated with 30 minutes of dry heat at 80°C against seeds treated with mechanical scarification with a scalpel. Seed lots of these accessions had been vacuum-packed in aluminium bags with between 4 and 8% moisture content and conserved in the long-term storage room (-18°C) at the Genetic Resources Program in CIAT. For each accession, 320 seeds were sampled and divided into two groups, then placed at room temperature (25°C) for one week. Seeds from one group were scarified with a scalpel (mechanical scarification) and seeds from the other group were given a dry heat treatment as described for experiment 1. Treatments were represented by five replicates, three of them had 20 seeds and the other two 50 seeds. Each replicate was sown in a 90 mm-diameter Petri dish (TP procedure) as explained in experiment 1. Petri dishes were randomly placed within a germination chamber with photoperiod of eight hours with white light at 35°C and 16 hours of darkness at 20°C. The same data collection protocol described in experiment 1 was followed in experiment 2.

Experiment 3
Based on the results of experiments 1 and 2, it was decided to more broadly evaluate whether the current CIAT protocol could be replaced by a simpler, less labour-intensive method. The current CIAT viability-testing protocol consists of scarifying with a scalpel the seeds and then sowing them between paper sheets, while the alternative protocol involved applying dry heat (80°C for 30 minutes) to seeds and then sowing on top of paper. Current and alternative protocols will be referred to as ‘scalpel’ and ‘dry heat’ treatments. Germination of 10 accessions of *S. capitata*, 25 accessions of *S. hamata*, 43 accessions of *S. scabra* and 12 accessions of *S. viscosa* (table 3) were evaluated by using the current CIAT viability protocol and the alternative protocol. Seed lots, after drying, had been conserved at -18°C since 2006. For each accession, 200 seeds were sampled and placed in two paper bags (100 seeds in current CIAT protocol group and 100 seeds in alternative protocol group). Bags were placed at room temperature (25°C) for two weeks. Then dry heat and mechanical scarification treatments were applied, and the seeds were sown on top of paper (TP) and between paper (BP), following the procedure explained in experiment 1 (ISTA, 2020). Dry heat treatment was represented by four replicates of 25 seeds sown in Petri dishes, while scalpel treatment was represented by two replicates of 50 seeds sown in two paper rolls.

Statistical analysis
Data obtained in this study did not match normal-distribution and homogeneity-of-variance assumptions, which is quite common in germination data (Hay *et al.*, 2014; Gianinetti, 2020). Inaccurate use of statistical methodologies like ANOVA (Sileshi,

2012; Gianinetti, 2020) was thus avoided and instead a Generalized Linear Model for binomial family data with logit link function was fitted using *R* (Dunn *et al*., 2018). This modelling approach considers the variable number of seeds that were used in the different experiments (necessary for logistical reasons). The occurrence of over-dispersion was determined by comparing the values of both residual degrees of freedom and the Pearson statistic against the GLM model deviance. In cases where over-dispersion was detected, the GLM model was adjusted to a quasibinomial GLM. Wald tests were implemented to calculate the statistical differences among treatments when the GLM model did not require adjustment, while an F-test was used in those cases where the quasibinomial GLM model was required (Dunn *et al*., 2018).

Results

Experiment 1

A significant ($P < 0.001$) effect of pre-sowing treatment on the proportion of normal seedlings was observed for each species when the three treatments (control, dry heat and mechanical scarification with a scalpel) were considered together (table 1). The percentage of normal seedlings from non-treated (control) seeds was always less than that of dry heat- and scalpel-treated seeds (figure 1). Control seeds had high percentages of non-germinated seeds, most of which were hard seeds. When only dry heat and mechanical scarification treatments were considered, the differences became less pronounced, and in *D. velutinum* and *S. guianensis*, the treatment effect was not significant (table 1). In *D. barbatum*, *D. heterocarpon*, *D. scorpiurus* and *S. capitata*, there was a significant effect of treatment, with normal percentages of mechanically scarified seeds always greater than that of dry heat-treated seeds. Meanwhile, only *S. guianensis* had a significant effect of sowing method (BP or TP) on the percentage of normal seedlings. However, this significance was lost when only dry heat and scalpel treatments were considered, which means that the difference between sowing methods only occurred in the control group, with a higher normal percentage in the TP method (figure 1). Seeds of *D. heterocarpon* and *S, guianensis* treated at 80°C and sown using the BP method showed wide variation in contrast with those sown on TP.

Experiment 2

Dry-heat and mechanical-scarification treatments showed different effects among the species. A significant effect ($P < 0.001$) of pre-sowing treatment on the proportion of normal seedlings was observed in all accessions of *D. heterocarpon.* Scalpel treatment resulted in > 90% normal seedlings for all ten accessions (figure 2). Meanwhile, dry heat had differential effects between accessions. For seven accessions, dry heat gave normal seedling percentages above 70% (23902, 23628, 13105, 13137, 3667 and 23618; table 2). Accessions 13515, 13651 and 13129 showed hard-seededness in several seeds; very few seeds were dead or abnormal with dry heat and scalpel treatments. Accessions 23902, 23628 and 23618 are notable cases, presenting high percentages of normal seedlings in both treatments despite 29, 32 and 32 years of storage, respectively. These three accessions are the oldest seed lots used in the entire study.

Table 1. Information on the accessions of *Desmodium* and *Stylosanthes* species used in experiment 1, with the statistical significance (*P*) of the effect of sowing method and pre-sowing treatment on the proportion of normal seedlings. Accessions were stored at 5°C after drying. *P-values* are given for the statistical significance considering all three treatments (control, scarification with a scalpel and dry heat) and considering only two treatments (without control).

Species	Accession number (CIAT-)	Country of origin	State	Post-harvest storage (months)	Moisture content (% f.wt.)	Factor	*P*	
							3 treatments	2 treatments
Desmodium barbatum (L.) Benth.	23527	Mexico	Chipas	15	5.21	Sowing method	0.990	0.370
						Pre-sowing treatment	**< 0.001***	**0.02***
Desmodium heterocarpon (L.) WC.	23303	Indonesia	E. Nusa-tenggara	17	4.96	Sowing method	0.526†	0.133†
						Pre-sowing treatment	**< 0.001*†**	**< 0.001*†**
Desmodium scorpiurus (Sw.) Poir.	43266	Australia	Unknown	17	4.86	Sowing method	0.411†	0.732†
						Pre-sowing treatment	**< 0.001*†**	**0.010*†**
Desmodium velutinum (Willd.) DC.	23272	Australia	Unknown	13	7.17	Sowing method	0.933 †	0.392 †
						Pre-sowing treatment	< **0.001*†**	0.392 †
Stylosanthes capitata Vogel	2536	Brazil	Ceara	13	6.68	Sowing method	0.635 †	0.288 †
						Pre-sowing treatment	< **0.001*†**	**0.015*†**
Stylosanthes guianensis (Aubl.) Sw.	165	Colombia	Cauca	16	6.55	Sowing method	**0.004*†**	0.140 †
						Pre-sowing treatment	< **0.001*†**	0.341 †

* Values that were significant at $P < 0.05$ when considering the effect of all three treatments (control, dry heat, and mechanical scarification) or when considering just two treatments (dry heat and mechanical scarification).
† F-test (quasibinomial GLM) instead of Wald-test (Binomial GLM) for cases with overdispersion or with all replicates having 100% as response in any treatment.

For *D. velutinum*, accessions 23995 and 23272 had > 95% normal seedlings regardless of whether the seeds had been subjected to the dry heat or scalpel treatment (i.e. no significant difference; figure 2, table 2). The remaining eight accessions showed significant differences, with scalpel-treated seeds having higher germination than dry heat-treated seeds. For accessions 33464, 13212, 33003 and 23991 the scalpel treatment had the highest percentages of normal seedlings, although the dry heat treatment always exceeded 70% of normal seedlings. In accessions 23320, 13697, 23319 and 33353, the oven treatment did not result in 70% of normal seedlings while scalpel treatment always resulted in > 80% normal seedlings. These accessions showed high proportions of non-germinated seeds, which were mainly hard seeds, when treated with dry heat.

Four accessions of *S. guianensis* (1605, 10482, 1553 and 2100) showed significant differences in normal seedlings between treatments, but both treatments gave percentages of normal seedlings above 90% (figure 2, table 2). None of the other *S. guianensis*

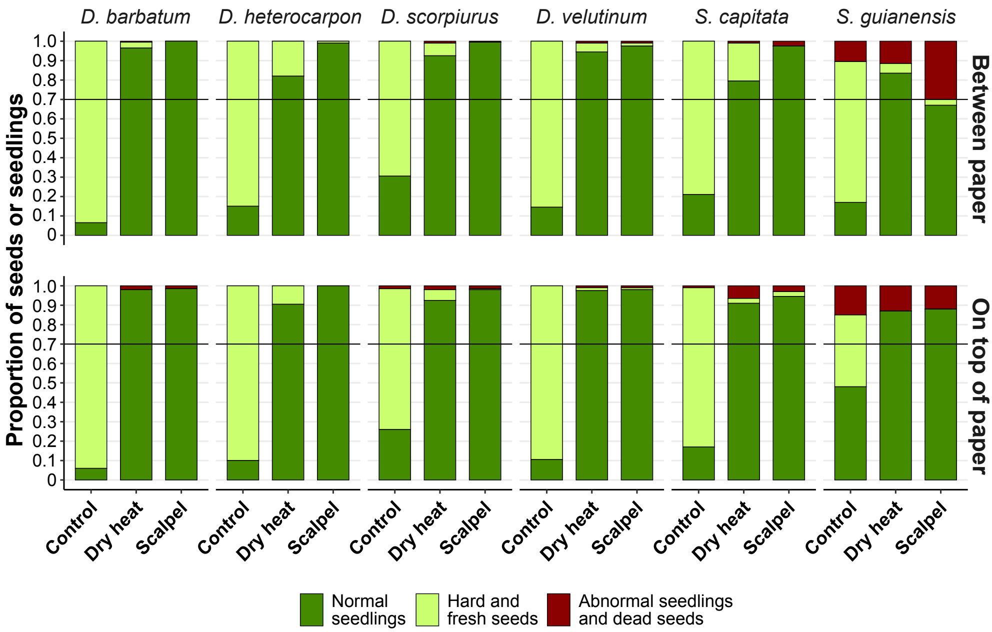

Figure 1. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal and dead seeds (red) in germination tests applied to seeds from six *Desmodium* and *Stylosanthes* species treated with dry heat (in an oven at 80°C for 30 minutes), with mechanical scarification with a scalpel, and without treatment (control), and sown in either between paper or on top of paper). Seeds were incubated with alternating 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

accessions had a significant difference in the percentage of normal seedlings between the two treatments, and it was always above 90%. For three *S. capitata* accessions (1019, 2054 and 2246), there was no significant difference between treatments (table 2). Accessions 1019 and 2054 showed normal seedling percentages above 70%, but 2246 had normal percentages below 50% in both dry heat and scalpel treatments, with considerable proportions of dead/abnormal seeds and non-germinated seeds (figure 2), which were mostly fresh. Accessions 12291, 11568 and 1924 showed a statistical difference among dry heat and scalpel treatments (table 2), with higher percentages of normal seedlings after the scalpel than the heat treatment. Nonetheless, dry heat treatment resulted in percentages of normal seedlings > 75%. In accessions 2246 and 2666, both dry heat and scalpel treatments resulted in low proportions of normal seedlings (< 40%) with high percentages of fresh seeds (figure 2). There were significant differences between treatments, with dry heat presenting the lowest percentages of normal seedlings (table 2). There were again differences among dry heat and scalpel treatments for accessions 2220, 1914 and 12750, with normal seedling percentages following scalpel treatment always reaching the > 70% while dry heat treatment resulted in < 70% of normal seedlings. In these three accessions, there were high percentages of fresh seeds in these three accessions for both treatments. Accessions 22020 and 1914 also had dead seeds with dry-heat and scalpel treatments.

Table 2. Information on the 40 accessions of *Desmodium* and *Stylosanthes* species used in experiment 2, with the statistical significance (*P*) of the effect of pre-sowing treatment on the proportion of normal seedlings. Accessions were stored at -18°C after drying.

Species	Accession number (CIAT-)	Country of origin	State	Storage period (years)	*P*
Desmodium heterocarpon	3667	Unknown	Unknown	2	< **0.001***
	13105	Malaysia	Perak	20	< **0.001***†
	13129	Thailand	Phangnga	2	< **0.001***
	13137	Thailand	Ranong	14	< **0.001***†
	13515	China	Hainan	18	< **0.001***†
	13651	Thailand	Trat	24	< **0.001***†
	23618	Laos	Tourakhom	32	**0.003***†
	23628	Australia	Unknown	32	**0.006***
	23646	Indonesia	West Sumatra	9	< **0.001***†
	23902	China	Hainan	29	**0.004***†
Desmodium velutinum	13212	Thailand	Khon Kaen	13	**0.011***†
	13697	Thailand	Chachoengsao	10	**0.001***†
	23272	Indonesia	W. Nusatenggara	2	0.245†
	23319	Indonesia	E. Nusatenggara	3	< **0.001***†
	23320	Indonesia	E. Nusatenggara	2	< **0.001***†
	23991	Thailand	Phrae	9	**0.009***
	23995	Thailand	Nan	5	0.056†
	33003	Thailand	Tak	3	**0.022***†
	33353	Vietnam	Quang Ngai	11	< **0.001***†
	33464	Thailand	Kalasin	7	**0.001***†
Stylosanthes capitata	1019	Brazil	Minas Gerais	8	0.570
	1914	Venezuela	Monagas	1	< **0.001***†
	1924	Venezuela	Monagas	11	**0.003***
	2054	Brazil	Bahia	5	0.714†
	2220	Brazil	Bahia	5	**0.003***
	2246	Brazil	Piaui	2	0.398†
	2666	Brazil	Tocantis	1	**0.001***
	11568	Brazil	Bahia	9	**0.015***
	12291	Australia	Unknown	13	**0.001***†
	12750	Australia	Unknown	6	< **0.001***†
Stylosanthes guianensis	1147	Colombia	Antioquia	1	0.055†
	1553	Colombia	Meta	5	**0.003***†
	1605	Unknown	Unknown	5	**0.039***†
	2100	Brazil	Bahia	9	**0.001***†
	10142	Australia	Unknown	1	0.999
	10482	Brazil	Piaui	20	**0.006***†
	11027	Colombia	Huila	16	0.864†
	11148	Panama	Cocle	16	0.999†
	11269	Mexico	Guerrero	21	0.320
	12392	Australia	Unknown	9	0.250

* *P*-values that were significant at $P < 0.05$ when considering the effect of the two treatments (dry heat and mechanical scarification). † F-test instead of Wald-test.

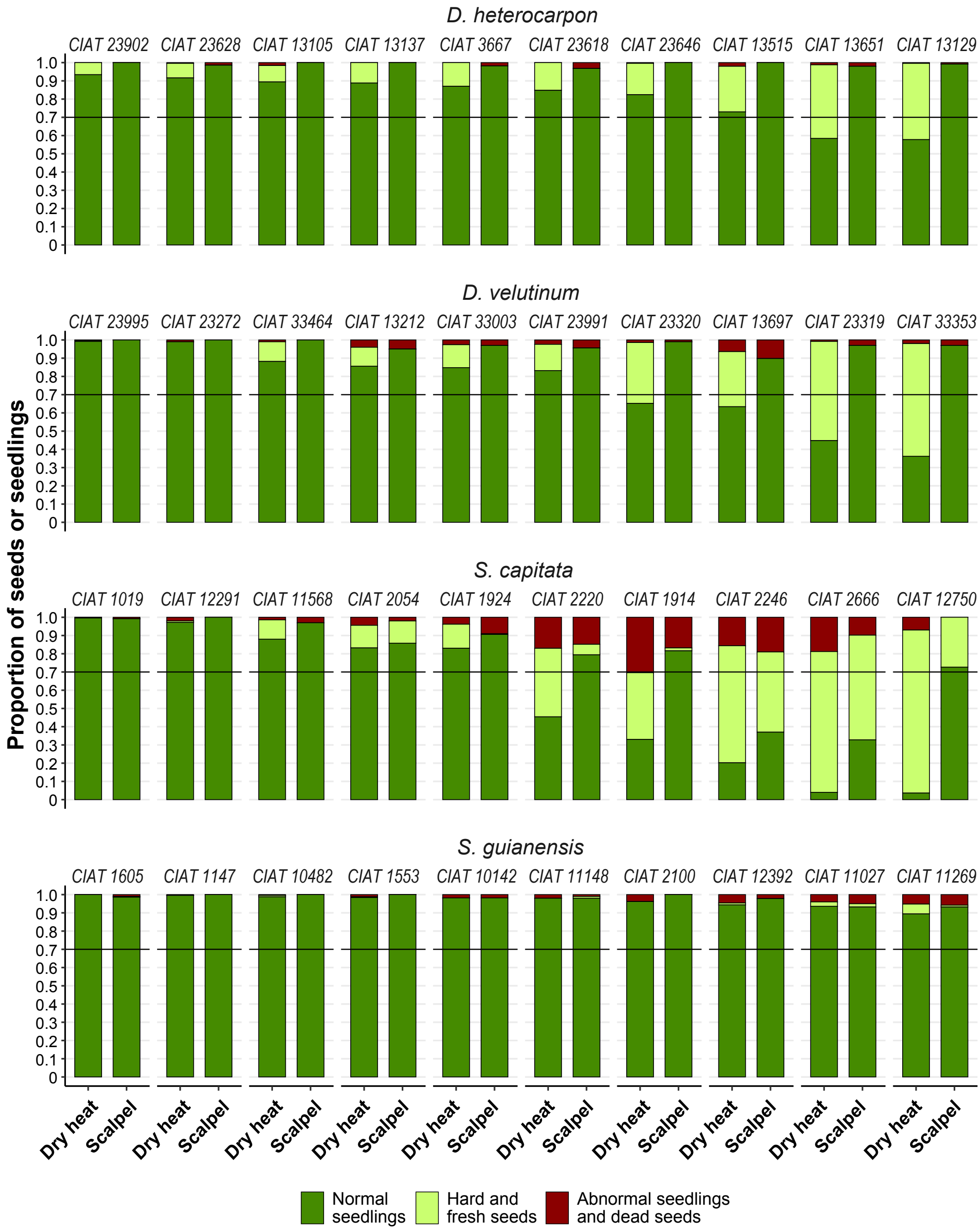

Figure 2. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal or dead seeds (red) in germination tests applied to seeds from ten accessions of four *Desmodium* and *Stylosanthes* species treated with dry heat (in an oven at 80°C for 30 minutes) and mechanically scarified with a scalpel. Seeds were sown on top of paper and incubated with alternating 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

Table 3. Information on the 90 accessions of *Stylosanthes* species used in experiment 2, with the statistical significance (*P*) of the effect of breaking PY treatment on the proportion of normal seedlings. Accessions were stored at -18°C after drying.

Species	Accession number (CIAT-)	Country of origin	State	Storage period (months)	*P*
Stylosanthes capitata	12126	Australia	Unknown	13	**0.041***†
	12173	Australia	Unknown	13	**0.009***†
	12181	Australia	Unknown	13	0.570
	12746	Australia	Unknown	13	0.120
	12734	Australia	Unknown	13	0.430
	12735	Australia	Unknown	13	**0.009***
	12737	Australia	Unknown	13	< **0.001***
	12747	Australia	Unknown	13	**0.010***
	12748	Australia	Unknown	13	0.32
	12751	Australia	Unknown	13	< **0.001***
Stylosanthes hamata (L.) Taub.	121	Venezuela	Zulia	13	**0.001***
	141	Colombia	Atlantico	13	< **0.001***
	142	Colombia	Atlantico	13	< **0.014***
	167	Colombia	Guajira	13	**0.042***†
	1010	Australia	Unknown	13	< **0.001***
	1453	Ant&Bar	Antigua	13	0.552†
	1936	Venezuela	Guarico	13	0.999
	2770	Venezuela	Yaracuy	13	**0.001***
	2858	Colombia	Magdalena	13	0.770
	10583	Venezuela	Falcon	13	0.097
	11206	Colombia	Magdalena	13	0.510
	11208	Colombia	Magdalena	13	< **0.012***†
	11583	Venezuela	Falcon	13	< **0.001***
	11776	Venezuela	Falcon	13	0.190
	11797	Venezuela	Trujillo	13	0.784†
	11781	Venezuela	Nueva España	13	0.580
	11794	Venezuela	Trujillo	13	0.180
	12023	Australia	Unknown	13	**0.009***
	12440	Australia	Unknown	13	0.135†
	12455	Australia	Unknown	13	0.351†
	12510	Australia	Unknown	13	0.350
	11786	Venezuela	Zulia	13	**0.024***
	12442	Australia	Unknown	13	0.250
	12447	Australia	Unknown	13	0.535
	12513	Australia	Unknown	13	< **0.001***
Stylosanthes scabra	66	Colombia	V. del Cauca	13	0.680
	1047	Brazil	Bahia	13	0.090†
	1055	Brazil	Bahia	13	< **0.001***
	1068	Brazil	Bahia	13	0.170
	1077	Brazil	Bahia	13	0.250
	1083	Brazil	Bahia	13	**0.025***
	1086	Brazil	Bahia	13	0.180
	1092	Brazil	Espiritu Santo	13	**0.001***
	1096	Brazil	Bahia	13	**0.037***

Table 3. *cont'd*

Table 3. *Continued.*

Species	Accession number (CIAT-)	Country of origin	State	Storage period (months)	*P*
Stylosanthes scabra Vogel	1271	Brazil	Mato Grosso-Sul	13	< **0.001***
	1293	Brazil	Goias	13	**0.047***
	1381	Unknown	Unknown	13	**0.039***†
	1434	Brazil	Sao Paulo	13	**0.037***
	1928	Venezuela	Monagas	13	0.200
	1946	Brazil	Goias	13	**0.017***
	1995	Brazil	Distrito Federal	13	0.130
	2003	Brazil	Goias	13	0.790
	2075	Brazil	Bahia	13	**0.016***†
	2086	Brazil	Bahia	13	0.870
	2089	Brazil	Bahia	13	0.585†
	2099	Brazil	Bahia	13	**0.032***
	2108	Brazil	Bahia	13	< **0.001***†
	2111	Brazil	Bahia	13	0.343†
	2121	Brazil	Bahia	13	**0.020***†
	2143	Brazil	Bahia	13	0.066†
	2164	Brazil	Bahia	13	0.540
	2308	Brazil	Unknown	13	0.986†
	2339	Colombia	Casanare	13	0.775†
	2377	Brazil	Bahia	13	< **0.001***†
	2478	Brazil	Paraiba	13	0.510
	2507	Brazil	Sergipe	13	0.340
	2574	Brazil	Goias	13	0.390
	2637	Brazil	Tocantins	13	**0.007***
	2681	Brazil	Tocantins	13	0.220
	2796	Venezuela	Monagas	13	0.146†
	10037	Brazil	Bahia	13	0.104†
	10088	Brazil	Bahia	13	**0.028***
	10156	Colombia	Cauca	13	< 0.001†
	10581	Venezuela	Tachira	13	0.067
	10614	Colombia	Tolima	13	0.380
	10623	Brazil	Espiritu Santo	13	0.630†
	10659	Brazil	Minas Gerais	13	0.166†
	10669	Brazil	Minas Gerais	13	0.409†
Stylosanthes viscosa (L.) Sw.	9	Brazil	Bahia	13	0.200
	2001	Brazil	Goias	13	0.800
	2060	Brazil	Bahia	13	0.160
	2110	Brazil	Bahia	13	**0.002***†
	2118	Brazil	Bahia	13	0.377†
	2123	Brazil	Bahia	13	0.790†
	2255	Brazil	Bahia	13	0.093†
	2341	Colombia	Casanare	13	**0.002***
	2371	Brazil	Bahia	13	< **0.001***
	2372	Brazil	Bahia	13	< **0.001***
	2374	Brazil	Bahia	13	< **0.012***†
	2380	Brazil	Bahia	13	< **0.019***†

* *P*-values that were significant at $P < 0.05$ when considering the effect of the two treatments (dry heat and mechanical scarification). † F-test instead of Wald-test.

Experiment 3

Dry-heat and scalpel treatments did not show significant differences in the percentages of normal seedlings in four out of 10 *S. capitata* accessions (12181, 12746, 12734 and 12748; table 3). For these accessions, normal percentages were above 70% in both treatments. The six remaining accessions showed a significant effect of treatment ($P < 0.005$; table 3). Seeds of accession 12126 treated with dry heat produced normal seedling percentages greater than 80%, while scalpel-treated seeds did not reach the 70% threshold (figure 3). Scalpel treatment showed higher percentages of normal seedlings than dry heat in accession 12747, but dry heat-treated seeds still achieved percentages > 75%. In four accessions (12735, 12737, 12751 and 12173), dry heat-treated seeds did not reach the 70% normal seedling threshold, while scalpel-treated seeds did. Accessions 12737 and 12751 presented non-germinated seeds (mainly fresh), while accessions 12735 and 12173 had many dead and abnormal seeds with dry heat.

Out of 25 accessions of *S. hamata*, 14 showed no effect of treatment on the percentage of normal seedlings (table 3), with high normal percentages following both treatments (figure 4). The remaining accessions showed significant differences, two of them having higher normal seedling percentages with dry heat than with scalpel treatment (12023 and 11208). Accessions 11786, 167 and 2770 had higher percentages of normal seedlings with scalpel treatment, but dry heat treatment always exceeded 70% normal seedlings (figure 4). Accession 12513 had a high percentage of normal seedlings following scalpel treatment, but not after dry heat treatment, where there were many non-germinated seeds (mainly fresh). High percentages of fresh seeds were also found in accession 11583, but this accession had higher percentages of normal seedlings than 12513. In four accessions (121, 141, 1010 and 142), dry heat treatment resulted in dead seeds and abnormal seedlings.

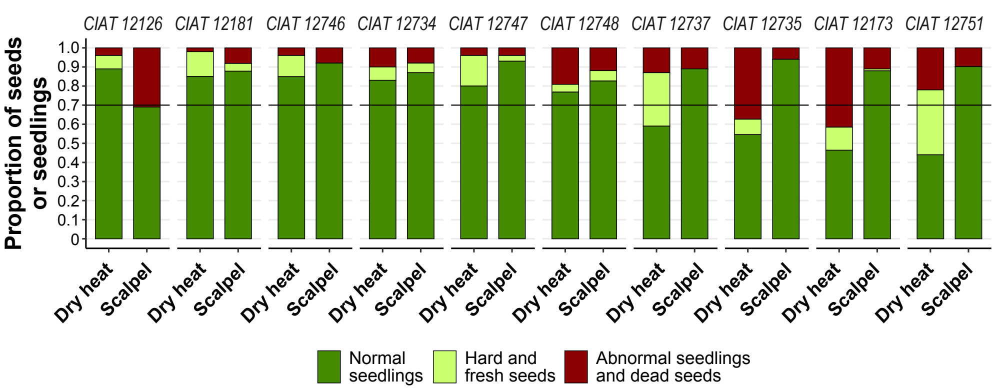

Figure 3. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal and dead seeds (red) in germination tests applied to seeds from ten accessions of *S. capitata* following two treatments: 1) dry heat at 80°C during 30 minutes and sown on top of paper (Dry heat); and 2) mechanical scarification with a scalpel and sown between paper (Scalpel). Seeds were incubated with alternating temperatures of 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

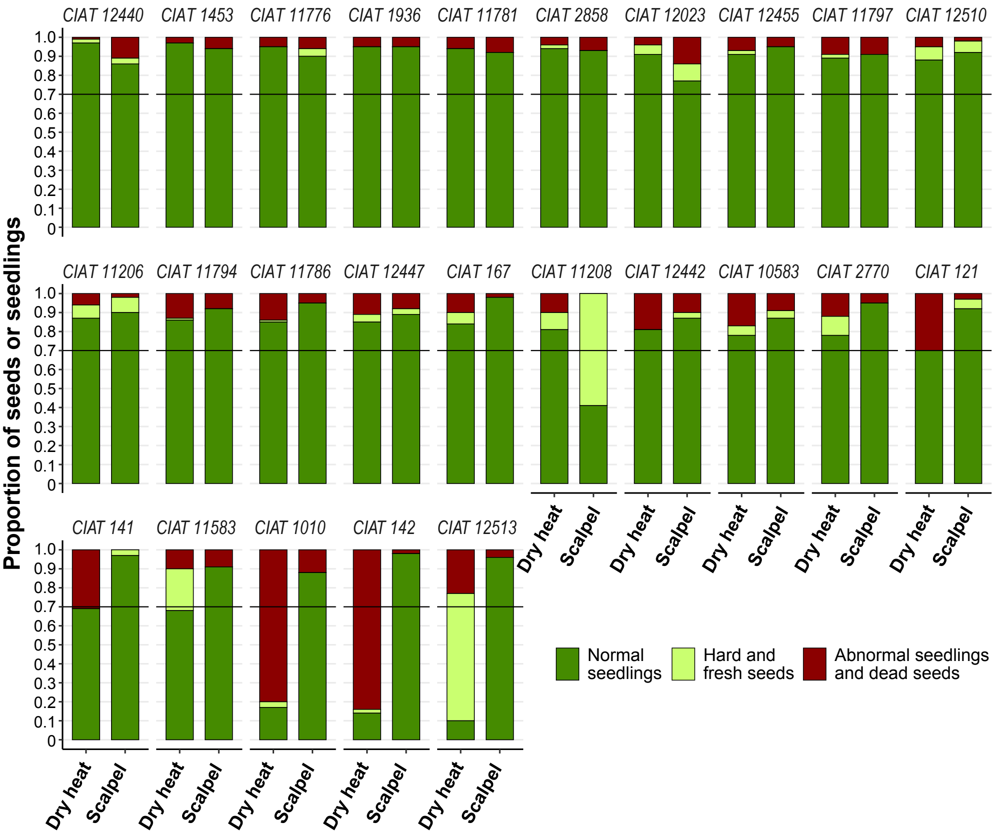

Figure 4. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal or dead seeds (red) in germination tests applied to seeds from 25 accessions of *S. hamata* following two treatments: 1) dry heat at 80°C for 30 minutes and sown on top of paper (Dry heat); and 2) mechanical scarification with a scalpel and sown between paper (Scalpel). Seeds were incubated with alternating temperatures of 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

Of the 43 accessions of *S. scabra* evaluated, 27 showed no significant difference in normal percentage between the dry heat and scalpel treatments and this percentage always exceeded the 70% viability threshold (table 3). Six accessions (2108, 2099, 1083, 1434, 1092 and 1096) with a significant effect ($P < 0.005$) of treatment on normal seedling percentage showed higher percentages of normal seedlings with dry heat treatment than with scalpel (figure 5). Meanwhile, six accessions (2075, 10156, 1293, 1946, 2637 and 1088) showed a significant effect of treatment and higher percentage of normal seedlings following scalpel treatment than with dry heat; nevertheless, dry heat treatment always exceeded the 70% threshold. Accession 1381 also showed a significant effect of treatment, but with both treatments the threshold was reached; in dry heat some fresh seeds remained. In three accessions, dry heat treatment resulted in low normal percentages compared with scalpel treatment (always > 70%). Accessions 1055 and 2121 showed persistence of hard-

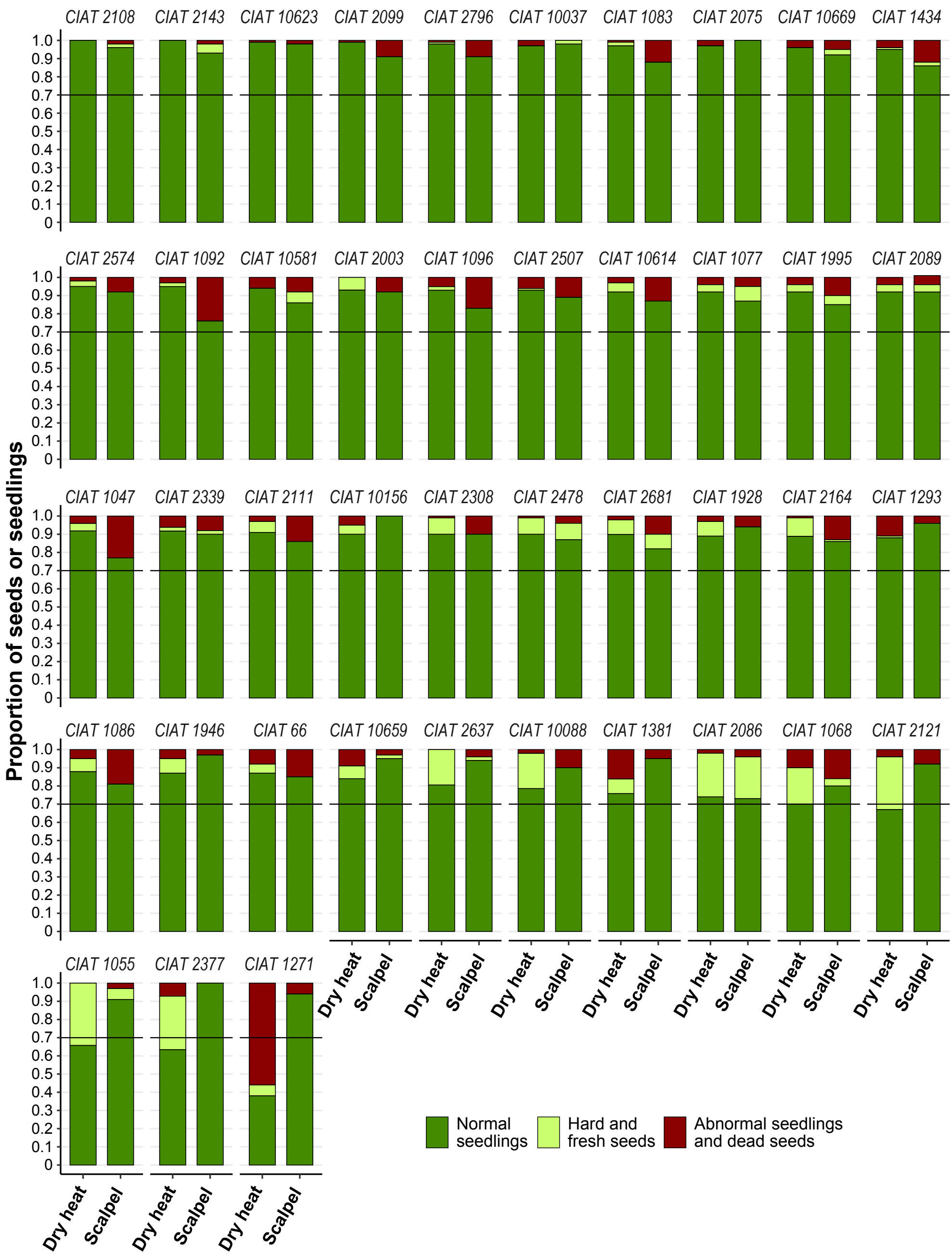

Figure 5. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal or dead seeds (red) in germination tests applied to seeds from 43 accessions of *S. scabra* following two treatments: 1) dry heat at 80°C for 30 minutes and sown on top of paper (Dry heat); and 2) mechanical scarification with a scalpel and sown between paper (Scalpel). Seeds were incubated with alternating temperatures of 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

seededness while accession 2377 had high proportion of fresh seeds. Accession 1271 was the only one that showed many dead seeds or abnormal seedlings with dry heat. Six accessions (2255, 2001, 2060, 2118, 2123, and 9) did not show a statistical difference between treatments in the percentage of normal seedlings, and both were always being above 70% (table 3, figure 6). However, the remaining six accessions did have significant differences between treatments, with dry heat-treated seeds never reaching the 70% threshold. These showed high percentages of hard and fresh seeds.

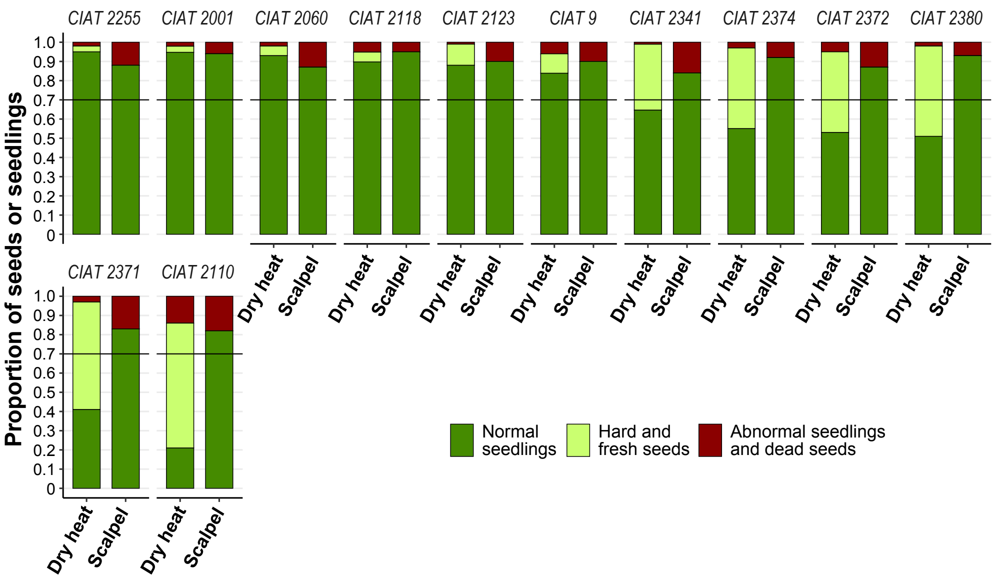

Figure 6. Final proportion of normal seedlings (dark green), hard and fresh seeds (light green), and abnormal or dead seeds (red) of germination tests applied to seeds from 12 accessions of *S. viscosa* following two treatments: 1) dry heat at 80 °C for 30 minutes and sown on top of paper (Dry heat); and 2) mechanical scarification with a scalpel and sown between paper (Scalpel). Seeds were incubated with alternating temperatures of 35/20°C with a photoperiod of eight hours light during the warm phase/16 hours dark during the cool phase for 21 days.

Discussion

The results of this study indicated high percentages of hard-seededness in seed lots of the evaluated species. Mechanical scarification with a scalpel was found to consistently be the best treatment for overcoming the physical dormancy in all nine species, as previously demonstrated for other legume species (Silveira and Fernandes, 2006; Clifton-Cardoso *et al.*, 2008; Perez-Garcia, 2009; Pereira and Ferreira, 2010; Wang *et al.*, 2011; Baskin and Baskin, 2014). However, this type of scarification, especially for tiny seeds, demands a lot of staff time for viability testing, causing a bottleneck when working with thousands of accessions in a genebank. This study has confirmed the observations of previous studies that a dry heat treatment also has a positive effect on seed germination of *Stylosanthes* spp. (Mott *et al.*, 1981; McKeon and Mott, 1982; McKeon and Brook, 1983) and *Desmodium* spp.,

and highlighted the potential for the use of dry heat treatment in routine viability testing as an alternative to mechanical scarification. This would increase the throughput of viability testing as at the CIAT genebank, one person can simultaneously apply dry heat treatment to 120 accessions (with 100 seeds each) by using two ovens, with no labour required, other than sampling the seeds and putting them in labelled paper bags.

The cost reduction from using dry heat, does however depend on the species as the dry heat effect varied among species and, in several cases, differed from the effect of scarifying seeds with a scalpel. Dry heat treatment generated similar percentages to the maximum viability obtained in seeds mechanically scarified with a scalpel in all evaluated accessions of *S. guianensis*. This is a particularly important species in the forages collection of the CIAT genebank, represented by 1,486 accessions. Dry heat at 80°C for 30 minutes was also a reliable alternative for *S. scabra*, which is represented by 777 accessions in the CIAT genebank. However, fresh seeds are expected in some accessions of *S. scabra*, and therefore dry heat must be complemented with a tetrazolium test to confirm maximum viability percentages in those cases where there are still fresh seeds at the end of the germination test. The tetrazolium test is also a costly procedure, but according to results in *S. scabra* it would be expected that a small proportion of seeds of a few accessions may require it. Therefore, in overall terms, it is probable that even when tetrazolium tests are required, a saving in time and resources will be achieved by using dry heat instead of mechanical scarification with a scalpel in the monitoring of viability of *S. scabra* accessions. The great majority of *S. hamata* accessions also showed satisfactory viability percentages with dry heat, but for this species, more accessions are expected to be susceptible to high temperatures than for *S. guianensis* or *S. scabra*. There are 361 accessions of *S. hamata* in the CIAT genebank. Meanwhile, mechanical scarification with a scalpel gave high percentages of viable seedlings in most of the evaluated accessions of *S. capitata* and *S. viscosa,* but dry heat treatment was efficient only in approximately half of their accessions. Thus, dry heat treatment is not a suitable alternative for viability monitoring purposes in these two species. The use of tetrazolium in these cases is expensive since it would be needed for many accessions, and for high proportions of the tested seeds.

For *D. heterocarpon* and *D. velutinum*, the results also suggested that dry heat is an alternative to mechanical scarification with a scalpel. However, some proportion of hard seeds remained in a few accessions. Therefore, for confirming the maximum viability tetrazolium tests could be an option to complement dry heat treatment. Both species are represented by 601 accessions at CIAT genebank. For *D. barbatum* and *D. scorpiurus*, where only one accession was assessed, dry heat resulted in high percentages of normal seedlings as did mechanical scarification with a scalpel. Thus, dry heat is a potential alternative to mechanical scarification in these two species, but more experimentation is necessary with larger representation at the accession level.

In this study, no significant difference was found between sowing methods (BP or TP) when dry heat and mechanical scarification with a scalpel were applied to *Desmodium* and *Stylosanthes* spp., except for control seeds of *S. guianensis*. Thus, since sowing seeds on top of paper has many operational benefits (e.g. to constantly monitor the progress of germination, reusability of dishes and the availability of light for seedlings to grow

more vigorously (Baskin and Baskin, 2014), it is proposed to use this methodology instead of between paper as ISTA suggests for seeds of *D. intortum* (Mill.) Urb. and *D. uncinatum* (Jacq.) DC. (with sulphuric acid scarification), *S. guianensis*, *S. hamata*, *S. humilis* Kunth and *S. scabra* (ISTA, 2020).

These findings have important implications for laboratories working with the evaluated species, given that dry heat reduces the time spent in pre-sowing treatments that break PY. It is suggested that for accessions of *D. heterocarpon*, *D. velutinum*, *S. guianensis*, *S. scabra* and *S. hamata*, dry heat treatment should be used for the initial test soon after harvest and first viability monitoring test, or for every monitoring test until the germination of a certain proportion of accessions from the same harvest fails to reach 70% normal seedlings which is the regeneration standard for wild varieties of the CIAT´s forages collection (when a new seed lot is produced to represent the accession in the genebank). Additionally, these results have relevance for the tropical livestock sector. Physical dormancy has already been identified as a major factor limiting crop establishment (Mott and McKeon, 1982; Anand *et al.*, 2011). Mass mechanical scarification methods have been employed, but they tend to generate large quantities of damaged seeds (Baskin and Baskin, 2014).

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