African Journal of Biotechnology Vol. 8 (22), pp. 6185-6190, 16 November, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Sulfuric acid and hot water treatments enhance *ex vitro* and *in vitro* germination of *Hibiscus* seed

Hamidou F. Sakhanokho

USDA-ARS, Thad Cochran Southern Horticultural Laboratory, P. O. Box 287, 810 High Way 26 West, Poplarville, MS 39470. E-mail: Hamidou.Sakhanokho@ars.usda.gov.

Accepted 24 August, 2009

Seeds of *Hibiscus dasycalyx* S. F. Blake and Shiller, a federally listed candidate endangered species and native to North America and two variants of *Hibiscus acetosella* Welw. ex. Hiern were scarified using sulfuric acid and hot water. The effects of the scarification methods on i*n vitro* and *ex vitro* germination in both species were evaluated. Sulfuric acid scarification was very effective for *in vitro* and *ex vitro* germination of both forms of *H. acetosella* and *H. dasycalyx* seeds by dramatically increasing germination rate and decreasing germination time. Acid scarification of *H. acetosella* seeds for 10, 15, or 20 min resulted in close to 90% germination within a week. Germination rates of about 70% (*ex vitro*) and 80% (i*n vitro*) were obtained in *H. dasycalyx* seeds treated with sulfuric acid. Germination rates of 54% (*ex vitro*) and 95% (i*n vitro*) were achieved when *H. dasycalyx* seeds were treated with hot water for 5 min, but exposing the seeds for 10, 15, or 20 min produced poor results in *H. acetosella* and *H. dasycalyx* as hot water scarification appeared to result in severe injury or death of the embryos. The protocols described here constitute rapid, reliable and simple methods to germinate *H. acetosella* and *H. dasycalyx* seeds in *vitro* and *ex vitro*. These results can be valuable in commercial productions or research projects. In addition, the *in vitro* germination of *H. dasycalyx* can offer a valuable tool in conservation efforts for this threatened species.

Key word: *Hibiscus dasycaly*, *Hibiscus acetosella*, seed scarification, *in vitro* seed germination, *ex vitro* seed germination.

INTRODUCTION

The genus *Hibiscus* consists of over 300 species (Akpan, 2000) which are found in tropical, sub-tropical and temperate regions. The genus contains annuals, herbaceous perennials, shrubs and small trees. Some species are useful as sources of food and medicine (Wilson and Menzel, 1964); others are economically important as ornamentals or as bast fibers, particularly in tropical and sub-tropical regions. Among these species are *H. acetosella* Welw. ex. Hiern and *H. dasycalyx* S. F. Blake and Shiller. *H. acetosella* is native to Africa (USDA-ARS-GRIN, 2006) and has two variants, a green- and red-leaf form. The red-leaf form, often referred to as 'false roselle' or 'red leaf hibiscus', is an edible tropical shrub also grown as an annual ornamental for the attractiveness of its deep burgundy red, maple-like leaves. *H.* dasycalyx, also known as the Neches River rose mallow is a federally (USA) listed candidate endangered species that is native to North America (Creech et al., 1999). Currently, it can be found only in three wetlands in Eastern Texas as it is threatened by interspecific hybridization with *H. laevis* and *H. moscheutos* as well as loss of preferred wetland habitat along the Neches River and its tributaries (Klips, 1995; Smith and Creech, 1995; Creech et al., 1999).

Rapid and synchronized germination is necessary when there is a need to subject a uniform set of seedlings to a treatment. In addition, rapid germination is advantageous when seed is used in reclamation (Greipson, 2001). However, several factors, including variability in water absorption, seed age and vigor, can lead to poor and non-uniform germination of seeds. A seed propagation study including various collection dates and stratification periods prior to planting resulted in low germination percentages in *H. dasycalyx* (Smith et al., 1995). In conservation efforts, seed is preferred over vegetative material as a source of plant material since seed provides a wider genetic base (Pickens et al., 2003). Because i*n vitro* culture is conducted in a highly controlled environment, it constitutes a powerful tool that has been used to

increase seed germination percentages in hard to germinate seeds and in preservation of several rare or endangered species (Batty et al., 2001; Rasmussen et al., 1990; Willis et al., 2003; Buyun et al., 2004; Lopez et al., 2004). Both hot water and sulfuric acid scarification methods are routinely used to break dormancy and promote rapid and uniform seed germination. The objective of the present study was to establish protocols that promote rapid and uniform germination in *H. dasycalyx* and the two variants of *H. acetosella* under i*n vitro* and *ex vitro* conditions.

MATERIALS AND METHODS

Plant material

The species used were H. dasycalyx and two variants of *H. acetosella*. Mature seeds were collected from greenhouse grown plants and air dried before storage in paper bags kept at room temperature (about 22°C).

In vitro germination experiment

The *in vitro* experiment consisted of sulfuric acid and hot water scarification treatments. Seeds of the two forms (red and green forms) of *H. acetosell*a and *H. dasycalyx* were immersed in 98% sulfuric acid concentration in 125 ml beakers each containing about 40 ml H_2SO_4. A batch of 50 seeds was placed in each 125 ml beaker. To assure uniform coverage, the solution was stirred continuously during the acid treatment under a fume hood. The H_2SO_4 treatment durations were 5, 10, 15 and 20 min. The experiment was repeated twice. Because of consistent bacterial and fungal contamination and poor germination rate with seeds of *H. dasycalyx in vitro* during the study, seeds of this species were subjected to an additional and longer treatment period of 25 min, so the H_2SO_4 treatment durations for *H. dasycalyx in vitro* were 5, 10, 15, 20 and 25 min. This additional duration (25 min) was also included in the *ex vitro* germination test for *H. dasycalyx* even though contamination was not a major problem in that case. Following acid treatment, the seeds were rinsed promptly and thoroughly for about 5 min with running tap water to remove acid residues. The controls were immersed in water with no H_2SO_4. The seeds were surface sterilized under a laminar flow hood by dipping in 100% ethanol for 3 min with gentle shaking, except for *H. dasycalyx* seeds, which were dipped in 100% ethanol for 5 min. They were then transferred into sterilized 250 ml beakers containing 40% (v/v) bleach (NaOCl) solution and one drop of Tween^{TM} 20 (Sigma Aldrich Corporation, St. Louis, MO) and shaken for 20 min at 110 rpm. The seeds were then rinsed with sterile distilled water and stored overnight in distilled sterile water on a shaker at 110 rpm. The next day, the seeds were rinsed again three times with distilled sterile water and transferred into 100 mm x 15 mm Petri dishes (3 - 4 seeds per dish maximum) containing MS (Murashige and Skoog, 1962) basal medium with 20 g l^{-1} sucrose, 0.75 mg l^{-1} $MgCl_2$ and 2 g l^{-1} Gelrite (Sigma Aldrich Corporation, St. Louis, MO). The pH of the MS medium was adjusted to 5.8 before addition of the gelling agent and autoclaving for 15 min at 121°C. The Petri dishes were then placed into an incubator where the temperature was maintained constant at 28°C with a light intensity of 100 µmol.$m^{-2}s^{-1}$ and a photoperiod regime of 16 h light and 8 h dark. Petri dishes were arranged in a completely randomized design (CRD) in the incubator. Hot water scarification was accomplished by immersing seeds in 99°C tap water and removing them after 0, 5, 15, 20, or 25 min. For the control treatments, seeds were immersed in non heated tap water and removed as described with the hot water treatments. Seeds were then sterilized and transferred onto MS medium. Seeds were checked weekly for germination for a total period of three weeks and the number of germinated seeds was counted and the result expressed in percentages.

Ex vitro germination experiment

For the *ex vitro* experiment, both the acid and hot water scarifications were performed as described above, except that no surface sterilization was performed after scarification. Instead, seeds were placed in moist Whatman NO 1 (Fisher Scientific, Atlanta, GA) filter paper and transferred directly into 15 x 95 mm Petri dishes (10 seeds per dish) which were wrapped with aluminum foil and kept in laboratory drawers at room temperature (about 22°C). Filter paper was moistened as needed and seeds were checked for germination weekly for a total period of 21 days. For both *in vitro* and *ex vitro* experiments, the mean weekly percentage germination and the total percentage germination after three weeks were determined. Data was subjected to the analysis of variance (ANOVA) and mean separation was done using SAS software (SAS, Inc., 2003).

RESULTS AND DISCUSSION

Effect of H_2SO_4 on *ex vitro* seed germination

H_2SO_4 scarification was very effective for both forms of *H. acetosella* and *H. dasycalyx* by substantially enhancing the germination rate and also reducing the germination period (Tables 1, 2 and 3). For the green form of *H. acetosella*, there were significant (P = 0.05) germination differences among scarification durations in week 1, 2, 3 or in total percentage germination (Table 1). For this genotype in week 1, significantly germination perecentages (up to 92% compared to only 6%) were obtained when seeds were scarified with 98% H_2SO_4 for 10, 15, or 20 min. Not only did the acid scarification improved seed germination but it also shortened the germination time as germinated seeds could be observed after only two days. The highest total germination rate for the green form of *H. acetosella ex vtiro* was obtained when seeds were acid scarified for 20 min even though this result was not significantly different (P = 0.05) from those obtained with the 5, 10, or 15 min treatments (Table 1). For the red-leaf hibiscus, the results obtained *ex vitro* with H_2SO_4 scarification were similar to those obtained with the green *H. acetosella* genotype (Tables 1 and 2). As in the case of the green genotype, most of the red-leaf hibiscus seeds germinated within one week when they were acid scarified for 5, 10, 15, or 20 min (Table 2). The highest total germination rate (98%) after three weeks was obtained when seeds were acid treated for 10 or 20 min even though these results were not significantly different (P = 0.05) from those obtained with the acid treated seeds for 5 or 15 min.

For *H. dasycalyx*, there were significant differences (P = 0.05) among the H_2SO_4 treatment durations in week 1, 2, 3, as well as for the total percentage germination (Table 3). In week 1, the highest percentage germination

Table 1. Effect of sulfuric acid and hot water scarification on *in vitro* and *ex vitro* seed germination of *Hibiscus acetosella* (green variant).

Scarification type	Duration (min)	Germination (%)			
		Week 1	Week 2	Week 3	Total
H_2SO_4 *ex vitro*					
	0	6c	0b	0b	6b
	5	72b	12a	2a	86a
	10	86a	2b	0b	88a
	15	92a	0b	0b	92a
	20	92a	2b	2a	96a
H_2SO_4 *in vitro*					
	0	18c	2b	5a	25b
	5	72b	16a	4a	92a
	10	88a	2b	0b	90a
	15	86a	0b	6a	92a
	20	88a	0b	0b	88a
Hot water *ex vitro*					
	0	15a	0b	0a	15a
	5	0b	6a	0a	6b
	15	0b	0b	0a	0c
	20	0b	0b	0a	0c
Hot water *in vitro*					
	0	18a	5a	4a	27a
	5	2b	0b	0b	2b
	15	0b	0b	0b	0b
	20	0b	0b	0b	0b

Means with the same letter belonging to the same scarification type and within the same week column are not significantly different at P = 0.05 according to Tukey's test.

was achieved when *H. dasycalyx* seeds were acid scarified for 20 min even though this germination percentage was not significantly different (P = 0.05) than those obtained with 15 or 25 min (Table 3). However, this result (58% germination) was a significant improvement over the control that produced only 6% germination.

Effect of hot water on *ex vitro* seed germination

Hot water treatment was ineffective for both variants of *H. acetosella* as the highest germination rates were only 6 and 5% for the green and red genotypes, respectively (Tables 1 and 2). No seed germination was obtained for any of the two forms of *H. acetosella* treated with hot water for a duration that lasted over 5 min, probably because the embryos were severely injured or killed. Thus, hot water treatment is not a suitable scarification method for *H. acetosella*. On the other hand, 54% of *H. dasycalyx* seeds treated with hot water for 5 min germinated within after three weeks (Table 3), but as this was the case with the two *H. acetosella* genotypes, no *H. dasycalyx* seeds treated with hot water for more than 5 min germinated, even after three weeks (Table 3).

Effect of H_2SO_4 on *in vitro* germination

There were significant differences (P = 0.05) among acid treated seeds for the various scarification durations for all three hibiscus genotypes (Tables 1, 2 and 3). For the green variant of *H. acetosella*, the best results were obtained when seeds were acid treated for 10, 15, or 20 min (Table 1). A total of 25 percent of the non scarified *H. acetosella* (green form) seeds germinated over a period of 21 days compared to 92% germination when seeds were treated with H_2SO_4 for 5 or 15 min (Table 1). The effect of acid scarification on seed germination was similar in the red form of *H. acetosella* (Table 2). Only 27% of the control seeds germinated during the 21 day period compared to the 98% germination achieved with H_2SO_4 treated seeds for 15 or 20 min and high percentages of seeds germinated in one week after acid treatment for 10, 15, or 20 min (Table 2).

For *H. dasycalyx*, only 4% of the control seeds germinated *in vitro* after three weeks (Table 3). Germination rate was also low when seeds were acid treated for 5, 10, or 15 min. In addition, seeds that germinated after acid treatment for these initial durations were often lost due to bacterial and or fungal contamination. Consequently, seeds

Table 2. Effect of sulfuric acid and hot water scarification on *in vitro* and *ex vitro* seed germination of *Hibiscus acetosella* (red variant).

Scarification type	Duration (min)	Germination (%)			
		Week 1	Week 2	Week 3	Total
H_2SO_4 *ex vitro*					
	0	6c	8c	0ab	14b
	5	72b	15a	2a	89a
	10	86a	10ab	2a	98a
	15	94a	2d	1ab	97a
	20	93a	3d	2a	98a
H_2SO_4 *in vitro*					
	0	19c	4b	4b	27b
	5	70b	20a	3b	93a
	10	89a	5b	0c	94a
	15	87a	3bc	8a	98a
	20	93a	2bc	3b	98a
Hot water *ex vitro*					
	0	17a	3a	0a	21a
	5	0b	5a	0a	5b
	15	0b	0b	0a	0c
	20	0b	0b	0a	0c
Hot water *in vitro*					
	0	21a	3a	2a	26a
	5	2b	0b	0b	2b
	15	0b	0b	0b	0b
	20	0b	0b	0b	0b

Means with the same letter belonging to the same scarification type and within the same week column are not significantly different at P = 0.05 according to Tukey's test.

were scarified for a longer time period (25 min). Surface sterilization protocol was also modified as follows. Seeds were placed in 100% ethanol for 5 min (instead of 3) and the bleach concentration was increased from 40 to 50%. The rest of the procedure remained unchanged. *H. dasycalyx* has calyx, bracteoles and mature seeds that are densely pubescent, creating a favorable environment for bacteria and fungi. Bacterial or fungal contamination could be reduced by removing the seed coats and plating fewer seeds in each Petri dish. Seed coat removal was found to improve germination rate and reduce contamination problem in cotton (Sakhanokho et al., 2001). However, the drawback of this procedure is that it is more time and supply consuming. The total germination percentage significantly (P = 0.05) improved when *H. dasycalyx* seeds were acid treated for 20 or 25 min, which produced 80 and 84% germination, respectively compared to only 4% germination for the control (Table 3).

Effect of hot water on *in vitro* seed germination

As this was the case in the *ex vitro* test, hot water treatment was ineffective for both variants of *H. acetosella* as the highest germination rate after hot water treatment was only 2% after a 5 min treatment for both the green and red genotypes (Tables 1 and 2). Similar to the *ex vitro* seed germination test, no seed germination was obtained for any of the two forms of *H. acetosella* treated with hot water for a duration that lasted over 5 min. On the other hand, there was a marked improved germination rate in *H. dasycalyx* as 95% of the seeds treated with hot water for 5 min germinated after three weeks and noticeably, 74% of the seeds germinated in the first week (Table 3). However, as this was the case with the *ex vitro* test, no *H. dasycalyx* seeds treated with hot water for more than 5 min germinated *in vitro*, even after three weeks (Table 3).

Conclusion

In summary, sulfuric acid scarification was effective for *H. dasycalyx* and *H. acetosella* germination under both *in vitro* and *ex vitro* conditions. However, hot water scarification was only effective for *H. dasycalyx* seed germination *in vitro* and *ex vitro* when seeds were treated for no longer than 5 min. It was ineffective for *H. acetosella*

Table 3. Effect of sulfuric acid and hot water scarification on *in vitro* and *ex vitro* seed germination of *Hibiscus dasycalyx*.

Scarification type	Duration (min)	Germination (%)			
		Week 1	Week 2	Week 3	Total
H_2SO_4 *ex vitro*					
	0	6c	3c	6c	15b
	5	8c	4c	7c	19b
	10	14b	48a	4a	66a
	15	54a	10b	0c	64a
	20	58a	9b	3b	70a
	25	57a	11b	2b	70a
H_2SO_4 *in vitro*					
	0	0c	2c	2b	4c
	5	0c	0c	0c	0c
	10	0c	0c	0c	0c
	15	16b	14b	4a	34b
	20	54a	26a	0c	80a
	25	56a	28a	0c	84a
Hot water *ex vitro*					
	0	9a	8b	6b	23b
	5	0b	36a	18a	54a
	15	0b	0c	0c	0c
	20	0b	0c	0c	0c
Hot water *in vitro*					
	0	11b	5b	4a	20b
	5	74a	18a	3a	95a
	15	0c	0c	0b	0c
	20	0c	0c	0b	0c

Means with the same letter belonging to the same scarification type and within the same week column are not significantly different at P = 0.05 according to Tukey's test.

seed germination *in vitro* and *ex vitro*. The protocols described here constitute rapid, reliable and simple methods to germinate *H. acetosella* and *H. dasycalyx* seeds in *vitro* and *ex vitro*. They can be very valuable in commercial productions or research projects involving these species since poor and erratic seed germination can be a hindrance in commercial nursery systems as well as in research settings where there is a need to subject a uniform group of seedlings to a treatment. In addition, the *in vitro* germination of *H. dasycalyx* can offer a valuable tool in conservation efforts for this threatened species.

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