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Is oxidative stress the cause of death when recalcitrant Spartina alterniflora seeds are dried?

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IS OXIDATIVE STRESS THE CAUSE OF DEATH WHEN RECALCITRANT *SPARTINA ALTERNIFLORA* SEEDS ARE DRIED?

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by
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ABSTRACT

Recalcitrant seeds, which die when desiccated, can be difficult to study because of their generally large size, high metabolism, and poor storage properties. However, recalcitrant seeds from the salt-marsh grass *Spartina alterniflora* are unique when compared to most other recalcitrant species because the seeds are dormant and small; *Spartina pectinata* and *S. spartinae*, which produce orthodox seeds, can be used as controls. Because of these somewhat rare characteristics, *S. alterniflora* is a good model system to study recalcitrance.

In the present study, the following physiological parameters were examined: Cardinal temperatures for germination, a viability test to determine if seeds are dormant or dead, stratification needed to alleviate dormancy, and the effects of dry down rates on viability.

For non-dormant seeds, the fastest germination rates occurred between 27-34°C. For dormant seeds, viability was established by cutting the coleoptile, which caused live seeds to germinate. Dormancy was alleviated with stratification, with average times to 50% germination of 2.1 and 2.6 months when seeds were stored at 2 and 10°C, respectively. Finally, *S. alterniflora* seeds lost viability when desiccated below 45% moisture content on a dry weight basis (DWB); however, drying rate did not influence death.

To investigate the causes of recalcitrant seed death, the putative role of oxidative stress was examined by assays for lipid peroxidation, leakage of cell components, total water-soluble antioxidant capacity (TAR), protein carbonylation and DNA fragmentation as *Spartina* seeds were dried. While lipid peroxidation was not associated with recalcitrant seed death, artifactual damage was observed when seeds were not freeze-clamped prior to extraction. TAR decreased during initial desiccation of orthodox and recalcitrant *Spartina* seeds. Protein carbonyl amounts (an indicator of protein oxidation) increased as *S. alterniflora* and orthodox *S. spartinae* seeds were desiccated. However, rehydration of dry, orthodox *S. pectinata*, and subsequent drying, did

not alter the TAR or protein carbonyls. DNA fragmentation was not evident during desiccation. These results suggest that lipid peroxidation, membrane damage and DNA fragmentation do not play a role in death due to drying. While protein oxidation and loss of antioxidant capacity changed, these are general responses to drying, rather than to recalcitrance.

CHAPTER 1
INTRODUCTION

A majority of seeds can be desiccated and stored dry for extended periods of time while remaining viable. However, some seeds are desiccation-intolerant (recalcitrant) and die when dried to low moisture contents (Bewley and Black 1994). Because recalcitrant seeds cannot be dried, long-term, gene bank storage of recalcitrant seed-germplasm is a major problem (Bewley and Black 1994). The majority of species with recalcitrant seeds are located in tropical forests and wetlands (Tweddle *et al.* 2003). Both of these habitats are disappearing at an alarming rate, and with no means of storing recalcitrant germplasm, there is a risk that these species could face extinction. To efficiently investigate how to store recalcitrant germplasm, the cause of desiccation-induced death needs to be determined. While the exact cause of death is unknown, a lack of sugars, protective proteins (e.g. late embryogenesis abundant proteins), or an increase in reactive oxygen species (ROS), which build up as recalcitrant seeds are desiccated, may contribute to death (Hoekstra *et al.* 2001).

Elucidating the cause of recalcitrance is a difficult task, in part, because of the physiological characteristics common to many recalcitrant seeds. Generally, recalcitrant seeds are shed non-dormant, are often chilling sensitive and are very large (Smith and Berjak 1995). Because most recalcitrant seeds are shed non-dormant and are chilling sensitive, they must be stored in conditions conducive to germination. This allows only a short time frame to work with most recalcitrant seeds. Furthermore, the large seed size makes it difficult to rapidly and uniformly dry the seeds. For example, the average seed weight of a recalcitrant *Aesculus hippocastanum* seed is ca. 13 g per seed (Flynn *et al*. 2006). Drying can take an extended period of time if the whole seed is dried, and there is a risk that aging effects could be misinterpreted as processes related to recalcitrance (Pammenter *et al.* 2002). If axes or seed parts are removed

from the seed to allow for faster drying, the dry down kinetics are altered, which changes the natural desiccation process. It is possible that phenomena related to physical damage to the seed may be confused with recalcitrance. Therefore, researchers in the recalcitrant field often face difficult alternatives: (1) if the whole seed is used, there is a risk of aging effects masking recalcitrance; (2) however, if the seeds are physically altered, some live tissue is damaged, and the dry down kinetics of the seeds are changed. In addition, much of the conflicting data in the recalcitrant seed field may stem from the use of many unrelated species to study recalcitrance. Even when the same species is used, it has often been stored and handled differently by various investigators. The use of a common model system to study recalcitrance may be beneficial and help to draw general conclusions about recalcitrance related phenomena.

The seeds of the lower salt-marsh grass *Spartina alterniflora* exhibit recalcitrant behavior (Mooring *et al*. 1971) and appear to be uniquely suited to study desiccation sensitivity in seeds because of their dormancy, chilling tolerance, small size, and the existence of desiccation-tolerant relatives in the same genus. Dormancy minimizes active germination metabolism (Bewley and Black 1994), assuring that results are due to recalcitrance and not germination. Also, because the seeds are dormant and chilling insensitive, the seeds can be stored for much longer time periods, compared to most other recalcitrant species. The small seed size allows for easier storage, larger sample sizes, and rapid more uniform drying.

In addition, *S. pectinata* and *S. spartinae*, which are in the same genus, produce desiccation-tolerant (orthodox) seeds. These orthodox seeds can be used as controls in each experiment to determine if the results are related to recalcitrance or are merely a general seed response to desiccation. These attributes are rare in recalcitrant seeds, and make *S. alterniflora* an attractive model system to study seed recalcitrance (Figure 1.1).

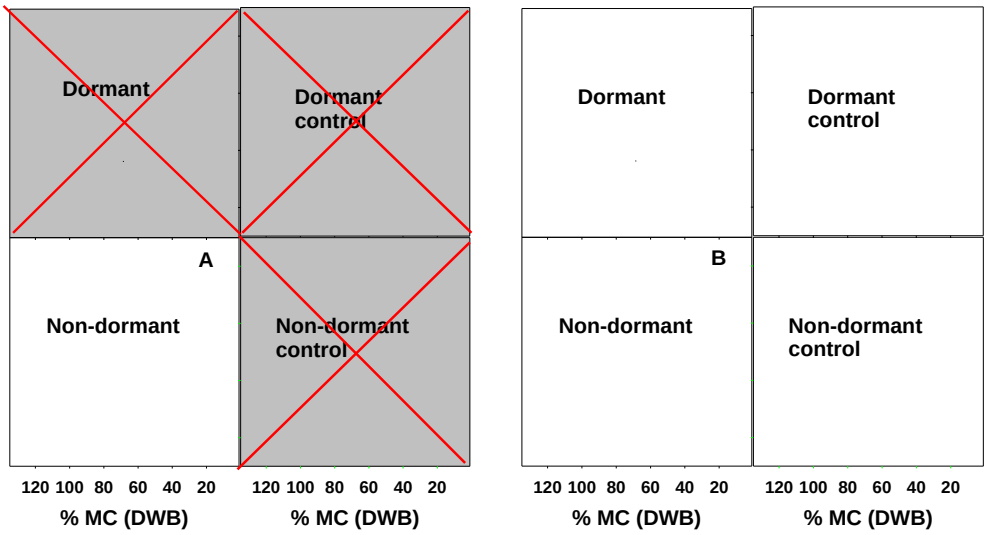

Figure 1.1. Comparison of the experimental design for a typical recalcitrant species (A), versus the *Spartina* system (B). A study with a typical recalcitrant seed only yields results for non-dormant seeds. With *Spartina*, each experiment has a four panel matrix, with dormant and desiccation-tolerant controls. MC, seed moisture content; DWB, dry weight basis.

In addition to serving as a model system to study recalcitrance, *S. alterniflora* is an important plant for coastal stabilization and erosion prevention in Louisiana and elsewhere (White *et al*. 1978). Coastal erosion is a global problem (Mitsch and Gosselink 2000). Restoration projects have begun replanting coastal species in marsh locations to help slow erosion and add biomass (Mitsch and Gosselink 2002). *Spartina alterniflora* is often used, as it is the dominant plant found in many salt-marshes along the East Coast of the United States (Bertness 1991). Transplanting *Spartina alterniflora* plants to the marsh can be very labor intensive and expensive (Oliver, 1925), so ideally large areas of coastline would be repopulated with *S. alterniflora* seeds. However, the use of *S. alterniflora* seeds has proved difficult because the seeds are shed dormant and are recalcitrant (desiccation-intolerant), making them difficult to handle and store. To assist conservationists with the use of *S. alterniflora* seeds, and to efficiently investigate the cause of their desiccation-induced death, it was necessary to first

investigate the physiological characteristics of *S. alterniflora* seeds. The physiological characteristics studied in this dissertation are: (1) determination of the maximum, minimum and ideal temperatures (cardinal temperatures) for germination of *S. alterniflora*; (2) development of a viability test to determine if the seeds are dormant or dead; (3) evaluate cold-stratification to break dormancy; (4) determine the effect of drying rates on desiccation tolerance of seeds.

The role of oxidative stress in death as *S. alterniflora* seeds are dried was also addressed. Oxidative stress is often cited as a possible reason for desiccation insensitivity in recalcitrant seeds (Leprince and Golovina 2002). Biomolecules such as lipids, proteins, DNA and RNA can be oxidized and damaged during drought- or desiccation-induced death of plant tissues (Leprince and Golovina 2002). To investigate the role of ROS in this study, I measured: (1) lipid peroxidation as dormant and non-dormant seeds were dried; (2) organic and inorganic leachates as indicators of physical membrane damage when seeds and embryos were dried; (3) the total water soluble antioxidant capacity of dormant and non-dormant seeds during desiccation; (4) the amount of protein carbonylation, as an indicator of protein damage, during desiccation; and (5) DNA fragmentation as an indicator of desiccation-induced damage.

CHAPTER 2
PHYISOLOGICAL BACKGROUND EXPERIMENTS WITH RECALCITRANT *SPARTINA ALTERNIFLORA* SEEDS

LITERATURE REVIEW

Spartina alterniflora Seed Biology

Spartina alterniflora Loisel. caryopses (called seeds from this point onward) consist of an embryo and endosperm encapsulated by a lemma, palea and two open glumes (Walsh 1990). The embryo is relatively long, and takes up one half of the seed, while the endosperm makes up the other half (Walsh 1990). The seeds mature along a spike, are shed dormant in the fall and germinate in the spring (Mooring *et al*. 1971, Walsh 1990).

Germination Temperatures for *S. alterniflora*

Non-dormant *S. alterniflora* seeds are typically germinated with an alternating temperature regime of 35°C (day) and 20°C (night) (Seneca 1974, Broome *et al.* 1974,Walsh 1990), or at a constant temperature of 26°C (Plyler and Carrick 1993); however, in these studies, the cardinal temperatures [minimum, optimal and maximum temperatures for germination (Copeland and McDonald 1995)], at which *S. alterniflora* germinates were not determined. Because of this, it is unclear if germination is adversely affected by temperature.

Storage and Dormancy of *S. alterniflora* Seeds

Spartina alterniflora seeds appear to require moist storage to remain viable. When the seeds were stored dry, their viability was much lower than moist stored seeds (Mooring *et al*. 1971, Broome *et al.* 1974). However, from these experiments, it was unclear if the low germination percentages were a result of dormancy or desiccation-intolerance. According to Broome *et al*. (1974), *S. alterniflora* requires several months of storage submerged in water at

2-3°C for germination to occur. In the experiments mentioned above, the seeds were stored dry from the time of harvest, so it is unclear if the seeds require moist conditions to remain viable or if they were viable in the dry state, but merely dormant.

Along with cold-stratification, dormancy was also alleviated in *S. alterniflora* seeds by cutting the scutellum, which resulted in 100% germination. Puncturing the scutellum also broke dormancy, but was not as effective as cutting, resulting in 40% germination. Removing the endosperm was not effective in breaking dormancy, suggesting that *S. alterniflora* dormancy is regulated in the embryo and not coat imposed, as coat imposed dormancy would most likely be alleviated by trauma to the either the scutellum or the endosperm (Plyler and Carrick 1993).

Recalcitrant Seeds and the Effect of Drying Rate on Their Viability

The causes of recalcitrant seed death when desiccated are controversial. The resolution of the problem is further complicated by common characteristics of recalcitrant seeds; they are often non-dormant, large, variable sized seeds that are chilling sensitive.

This is exemplified by the apparently simple effect of drying rate upon recalcitrant seed survival. It is often stated that recalcitrant seeds can survive at lower moisture contents if the seeds are rapidly dried (Berjak *et al.* 1993). However, conflicting reports exist in the literature regarding the speed of drying and its effect on the moisture content at which recalcitrant seeds lose their viability (Berjak and Pammenter 2008). For example, some recalcitrant species tolerate faster drying to lower moisture contents while still exhibiting signs of cellular viability [e.g. *Artocarpus heterphyllus* (Wesley-Smith *et al.* 2001) and *Telfaria occidentalis* (Ajayi *et al.* 2006)], while in other studies, faster drying causes the seeds to die at a higher moisture content [e.g.*Theobroma cacao* (Liang and Sun, 2000)]. Other investigators found no influence of drying rate on the moisture content at which recalcitrant seeds die [e.g. *Araucaria hunsteinii* (Tompsett 1982), *Quercus rubra* (Pritchard 1991) and *Quercus robur* (Finch-Savage *et al.* 1992)].

It has been proposed that rapid drying confers greater desiccation tolerance because the seeds spend little time at intermediate water contents, thus reducing harmful effects at this stage. The rate at which the seed moves through the intermediate water content is often deemed vital for increased tolerance (Daws *et al*. 2004, Pammenter and Berjak 2008); thus, it has been hypothesized that a lack of drying rate effect could simply be related to seed size. If the seed being studied is large, it may take too long for the tissue to move through the drying stages, allowing deleterious metabolites to accumulate. However, it must be noted that even when isolated axes are flash-dried in a matter of minutes, only a few viable cells remain, and healthy seedling establishment does not occur (Berjak and Pammenter 2008).

If drying rate does influence desiccation tolerance, it is possible that it is masked by the large size and germinative metabolism of many recalcitrant seeds. However, *S. alterniflora* seeds are dormant, thus reducing metabolism, and also relatively small (ca. 0.003g seed^{-1}) in comparison to other recalcitrant seeds. So, in the present study, different drying rates will be used to determine if there is an effect of drying rate upon whole seed viability.

In my studies, the physiological characteristics of *S. alterniflora* seeds were quantified, with the principle objectives to (1) develop a viability test for the seeds to determine if they are dead or dormant; (2) determine the cardinal temperatures at which *S. alterniflora* seeds germinate; (3) determine the treatments that will break dormancy and duration of cold-stratification (moist chilling) needed to break dormancy, and if this requirement varies as a function of accession and year of harvest; (4) to determine if rate of drying influences the critical moisture content of *S. alterniflora* seeds; and (5) determine the water potential at which desiccation damage occurs.

METHODS

Harvest and Storage of *Spartina alterniflora* Seeds

Spartina alterniflora seeds used in this study were collected in the years 2002-2006 from plants grown at Ben-Hur Experimental Station in Baton Rouge, Louisiana (2002 and 2004 harvests; accessions: Vermillion, 26-18, 27-2, 26-13, 7-14 and 7-8) or from wild plants in the marshes of Port Fourchon, Louisiana (2003, 2005, and 2006). The seeds were harvested by hand shattering at the time of natural dispersal. When collected, the seeds were immediately placed in sealed freezer bags. Upon arrival at the laboratory on the day of harvest, 10 gram aliquots of seeds were immediately placed in plastic GA-7 culture containers ('Magenta vessels') (Sigma Aldrich, St. Louis, USA) or high-walled glass Petri dishes containing 250 ml of distilled water, and placed at 2°C. The seeds could be stored for 8 months (breeding lines) or 11 months (wild type) before germination eventually occurred at 2°C storage. Prior to each experiment, the seeds were placed on a backlight to ensure only filled (containing a caryopsis) dispersal units were chosen, and the seeds were lightly patted with a Kim-Wipe™ disposable tissue to remove excess free water.

Cardinal Temperatures for Germination of *S. alterniflora* Seeds

To determine the cardinal temperatures at which *S. alterniflora* seeds germinate, the seeds (Vermillion, 2002) were placed at a range of temperatures (17, 22, 27, 34 and 37°C). Only seeds at 27°C had light in the incubator, but preliminary experiments indicated that light was not a requirement for *S. alterniflora* germination (Appendix Figure A-8). Three replications, containing 20 seeds each, were used for each of the temperatures tested, and each experiment was repeated at least twice, for a minimum of six total replications. Root and shoot emergence were scored every four hours (except for an eight hour gap each night) until maximum

germination was obtained. Root and shoot germination was scored separately, and seeds were deemed germinated when the axis (root or shoot) broke through the covering structures. To assist in scoring germination, the seeds were checked over a backlight so the root and shoot could be seen more easily.

Viability Test to Determine if Seeds Are Dormant or Dead

To determine if *S. alterniflora* seeds were viable, three replications of 20 filled seeds were selected, and each seed was cut ca. 1/3 of the way down from the apex of the seed, ensuring that the coleoptile was clipped. The seeds, in a 4 x 5 spacing pattern, were then placed in a 9 x 9 x 1.5 cm plastic Petri dish, which contained two pieces of brown germination paper (Anchor Paper Co., St. Paul, Minnesota, USA) and eight milliliters of distilled water. To evenly distribute the water, a folded Kim-Wipe™ was placed over the seeds. Each Petri dish was covered and positioned at ca. a 45° angle in a humidity box lined with moist paper towels. The humidity box was placed at 27°C in continuous light. Emergence of roots and shoots was recorded at 7 and 14 days. Shoot emergence was a more accurate indicator of viability in dormant seeds, as root emergence did not always occur when seeds were cut.

Cold-Stratification (2°C) Requirements to Alleviate Dormancy in *S. alterniflora* Seeds

The *S. alterniflora* seeds were subjected to a germination test each month after harvest to determine the effect of cold-stratification on dormancy. Three replications, with 20 intact seeds apiece, were placed in a standard germination test, which is the same as a viability test, with the exception of cutting the seeds. The seeds were checked for germination (root or shoot emergence) 14 days after the experiment began. After 14 days, any ungerminated seeds were subjected to the viability test detailed above. This experiment was repeated every four weeks for a total of 16 weeks, or until the seeds were completely non-dormant.

Dry Down Rates Versus Percent Viability of *S. alterniflora*

Whether *Spartina* seeds have a critical moisture content or are affected by drying rate was evaluated. Dormant (≤ 25% germination) and non-dormant (100% germination) *S. alterniflora* seeds were dried at different rates. To achieve different drying rates, seeds were air dried, desiccated at 33% RH, and flash dried at room temperature (22-24°C). The seeds for this experiment were harvested from Port Fourchon, Louisiana in 2006. Dormant seeds were stored for less than 2 months at 2°C, and non-dormant seeds were stored 4-5 months at 2°C. Healthy shoot emergence was used as the criterion for a viable seed. Shoots that emerged and then became necrotic in less than 24 hours were not deemed viable. A critical moisture content was determined from each dry down by fitting the data with a two segment linear regression (Liang and Sun 2000).

Slow Drying

Forty filled seeds were placed in a plastic 9 x 9 x 1.5 cm plastic Petri dish lined with two pieces of Anchor germination paper. Twenty seeds were designated for the germination/viability percentage calculation and placed on one side of the Petri dish, while the other 20 seeds, for percent moisture content determination, were placed on the opposite side of the dish. The Petri dish was covered and the seeds were placed on a bench-top at ca. 22°C for various drying times. After ambient air-drying, the seeds were immediately evaluated with a standard viability test and percent moisture content determination. Percent moisture content was calculated on a dry weight basis, and dry weights were obtained by placing the seeds at 105°C for 7 days, by which time seed weight was constant.

Intermediate Drying

Seeds were intermediately dried at 33% RH at 27°C. This atmosphere was created by placing 100 ml of a saturated magnesium chloride solution in a sealed GA-7 culture vessel at

27°C (Winston and Bates 1960). In order to place the seeds above the $MgCl_2$ solution, a seed stage was made from an unfolded butterfly clamp (Ideal brand, Office International Corporation, Edison, USA), which supported a 5 x 1.5 cm glass Petri dish half. The sealed culture vessel and stage were placed at 27°C for a minimum of 24 hours to allow for the relative humidity to equilibrate. Following equilibration, 40 *S. alterniflora* seeds were placed in the Petri dish, twenty for the moisture content determination and twenty for the viability test. The culture vessel was then covered with its plastic top and further sealed with Parafilm™ and placed at 27°C. Also, seeds were dried at 85% RH over potassium chloride (KCl) and 75% RH over sodium chloride (NaCl), both prepared in the same way as the $MgCl_2$ solution. The 85% RH and 75% RH dry downs are reported in a separate section because the seeds reached equilibrium at those RH values, whereas there was continuous dry down in the other dry down methods.

Rapid Drying

A flash dryer, as described in Wesley-Smith *et al.* (2001), was used to rapidly dry the *S. alterniflora* seeds. The dryer consisted of a 500 ml Nalgene jar (Thermo Fisher Scientific, Rochester, USA) with a layer of fresh 8 mesh, $CaSO_4$ desiccant (W.A. Hammond Drierite Company LTD, Xenia, OH, USA) on the bottom (ca. 75 g), a 12V computer fan (a 12V 1amp adapter was needed to run the fan from the wall current), and a Ball jar rim (Muncie, USA) lined with mesh that was used as a stage to hold the seeds. The computer fan, supported by four 200 µl pipette tips, was placed ca. 5 cm above the desiccant, and was oriented so it pulled dry air from the desiccant up, over the seeds (Figure 2.1). To obtain consistent dry down rates, it was necessary to use fresh desiccant for each dry down experiment (heat regenerated desiccant yielded inconsistent dry down patterns). The dryer was run for designated times, drying the 40 seeds to a wide range of moisture contents. After drying, the seeds were immediately placed in a

germination or moisture content test. Seeds designated for percent moisture content calculation and percent viability were both present in each dry down, so there is a matching moisture content value for each viability data point.

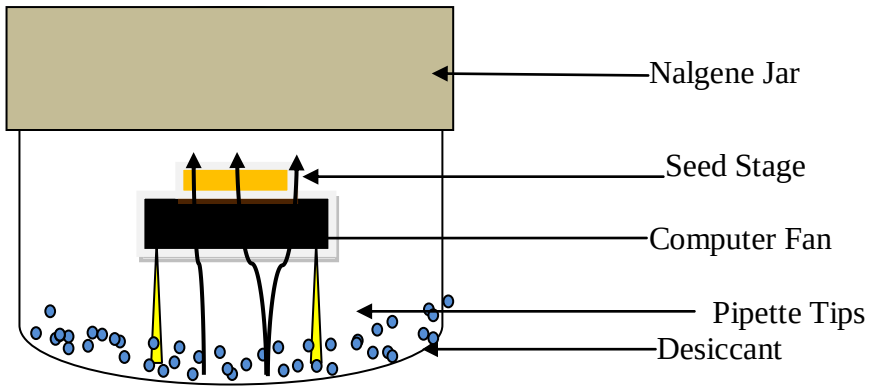

Figure 2-1. Seed flash dryer apparatus used in all experiments involving the rapid dry down of *Spartina sp.* seeds. A computer fan circulates dry air from the desiccant over the seeds.

Water Potential and Viability of *S. alterniflora*

Wild type *S. alterniflora* seeds, harvested in 2006 (Port Fourchon, Louisiana) and stored submerged at 2°C for 5 months were used to develop a moisture content/ relative humidity isotherm to determine seed water potential. Three replications of 20 seeds were equilibrated at 27°C for a minimum of 20 days over saturated salt solutions of KCl (85% RH), NaCl (75% RH), Mg $(NO_3)_2$ (55%) and $MgCl_2$ (33% RH) (Winston and Bates 1960). The experiment was repeated for a total of six replications. The water potential of the seeds was expressed in MPa and was calculated using the equation from Pritchard (1991):

$$\psi_w = (RT/V_w) \ln(\% \text{ RH}/ 100)$$

where $\psi_w =$ water potential (MPa), R = gas constant (8.314 J K^{-1} mol^{-1}), T = temperature in degrees Kelvin, V_w = partial molal volume of water (18 ml mol^{-1}), and % RH = relative humidity in the container.

Statistical Analysis

All of the results were expressed as mean $\pm$ standard error. Statistical significance was determined by using a one way analysis of variance (ANOVA) test along with Tukey's studentized range as a means separation test. A probability value of $\alpha < 0.05$ was considered significant. The critical moisture contents for all dry downs were determined by a two phase linear regression as described by Sun and Leopold (1993).

RESULTS

Cardinal Temperatures for Germination of *S. alterniflora* Seeds

In all temperature treatments, shoots emerged before the roots. Time to first shoot emergence averaged 11 $\pm$ 1 hours for all germination temperatures, and there was no significant difference for shoot emergence among any of the treatments (F statistic = 1.5, p = 0.261). Time of initial root emergence decreased between 17 and 34°C; however, root emergence for seeds germinated at 37°C was significantly delayed (F statistic = 13.77, p value = 0.0001) (Figure 2.2). Consistent with initial emergence, shoots also reached 50% emergence (G_{50}) faster than roots across all germination temperatures (Figure 2.3). The differences in the shoot and root G_{50} values were the most pronounced at the temperature extremes, with significant delays in shoot G_{50} occurring at 17°C (F statistic = 14.15, p value = 0.0001) and G_{50} values for root emergence taking > 60 hours at 17 and 37°C, which were significantly slower than the other treatments (F = 87.5, p <0.001). However, even with the delay, 100% germination was reached at all temperatures tested.

Viability Test to Determine If the Seeds Are Dormant or Dead

Seeds from each marsh harvest year, and all six accessions harvested in 2002 and 2004 had low initial germination percentages. However, cutting the seeds resulted in germination, revealing that the viabilities were > 80% across all harvest years and accessions (Figure 2.5A).

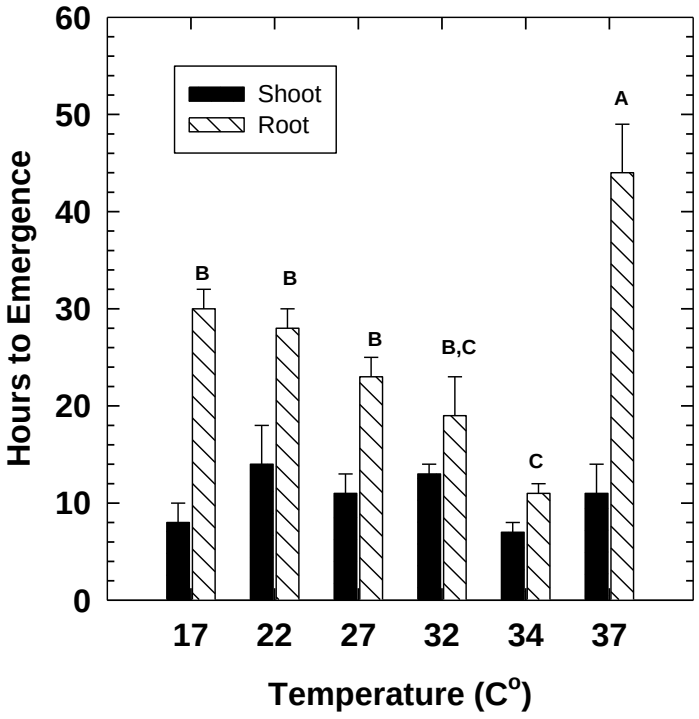

Figure 2.2. Effect of temperature on time to first visible protrusion for shoots (solid bars) and roots (hatched bars) of *S. alterniflora* (Vermillion 2001, 2002 and 2004). Seeds were stored at 2°C submerged in water for 6 to 11 months at the time of the experiments. All seeds were 100% non-dormant and viable at the time of the experiment. Seeds were germinated in darkness except for 27°C, where germination was evaluated in the light and dark. No differences were observed in seeds germinated in the light or dark (Appendix Figures A-7; A-8). Different letters indicate statistical difference at $\alpha = 0.05$ for root emergence (F statistic = 13.77, p value = 0.0001). There was no statistical difference for shoot emergence (F statistic = 1.50, p value = 0.261).

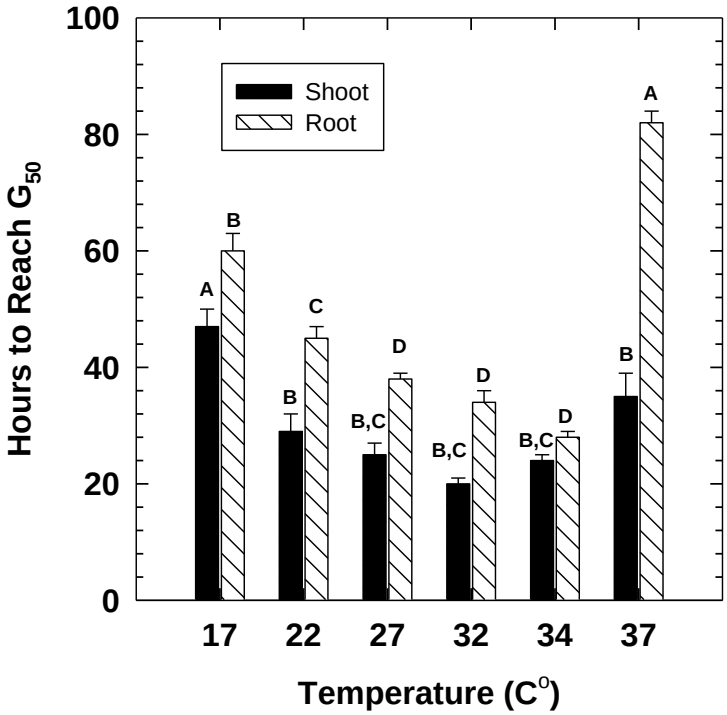

Figure 2.3. Effect of temperature on time to 50% germination (G_{50}) for shoots (solid bars) and roots (hatched bars) of *S. alterniflora* (Vermillion 2001, 2002 and 2004) seeds germinated at a range of temperatures. Seeds were stored for 6 to 11 months at 2°C, submerged in dH_20. All seeds were germinated in darkness, except for 27°C, where seeds were germinated in the light and dark. All seeds utilized were 100% non-dormant and viable. No differences were observed for seeds germinated in the light or dark (Appendix Figures A-7; A-8). There was a significant difference for time to G_{50} for shoots (F statistic = 14.15, p value = 0.0001) and roots (F statistic = 87.52, p value <0.001). Different letters indicate significance at $\alpha = 0.05$.

Cold-Stratification (2°C) Requirements to Alleviate Dormancy in *S. alterniflora* Seeds

After harvest, *Spartina alterniflora* seeds steadily lost dormancy when stratified at 2°C (Figure 2.4), and the time to 50 percent germination (G_{50}) ranged from 1.2 months to just greater than 3 months (Figure 2.5B). The average time to G_{50} across all accessions and harvest years was 2.1 months. The variation in stratification requirements among accessions and across harvest years was not statistically significant at $\alpha = 0.05$ (F statistics of 1.91 and 2.97 and p values of 0.1508 and 0.071, respectively). Seeds also lost dormancy when stratified at 10°C; however, the time to 50% germination was slower when compared to seeds harvested in the same year and stratified at 2°C, with an average time to G_{50} of 2.6 months.

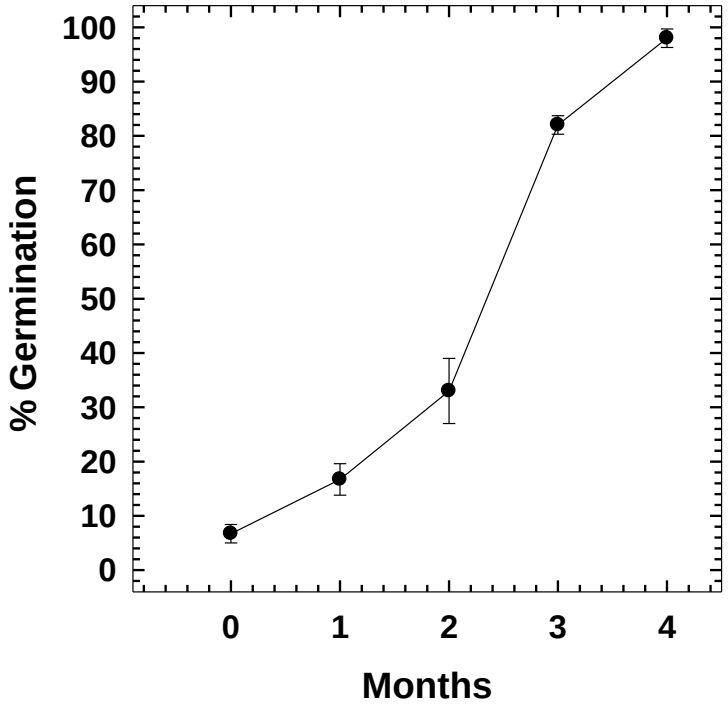

Figure 2.4. Relationship between time of cold-stratification and germination for *S. alterniflora* seeds (wild type, 2006). Immediately after harvest, seeds were stored submerged in GA-7 culture vessels at 2°C, which contained 250 ml of dH_20. To test for germination, every four weeks, three replications of 20 seeds each were germinated at 27°C with light. Germination was scored after 14 days when root or shoot emergence occurred. Error bars = standard error.

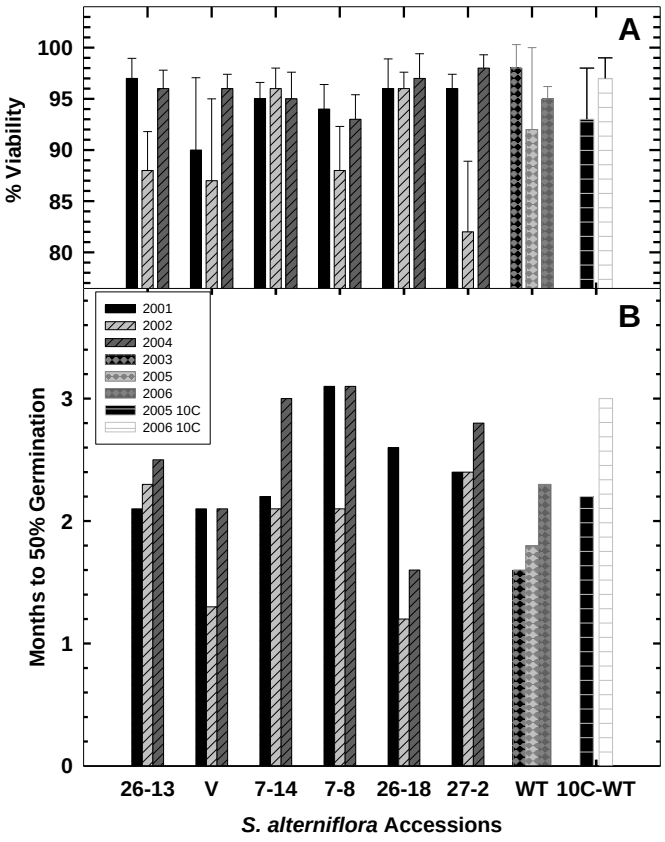

Figure 2.5. (A) Initial viability of *S. alterniflora* accessions after harvest. Viability was determined by cutting the coleoptile, which caused viable seeds to germinate. (B) Stratification time required for 50% germination for *S. alterniflora* accessions harvested in 2001 (closed bars), 2002 (light grey hatched bars), and 2004 (dark grey hatched bars) from Ben-Hur Experimental Station, Baton Rouge, Louisiana and wild type *S. alterniflora* seeds harvested in 2003 (black with grey checks), 2005 (light grey with grey checks), and 2006 (dark grey with grey checks) from Port Fourchon, Louisiana; and wild type seeds (Port Fourchon, LA) harvested in 2005 (black bar with horizontal stripes) and 2006 (white bar with horizontal stripes) stratified at 10°C. Seeds were stored in submergence conditions at 2°C or 10°C, and assayed at 4 week intervals for germination at 27°C with light for 14 days. Among accessions and harvest years, there were no significant differences at $\alpha = 0.05$.
Accessions: V, Vermillion; WT, wild type from Port Fourchon; 10C-WT, wild type from Port Fourchon stored at 10°C.

Determination of a Critical Moisture Content for *Spartina alterniflora* and the Effect of Drying Rates

The critical moisture content (defined in this study as the lowest moisture content achieved before visible damage occurred) of *S. alterniflora* was examined when desiccated at different rates. In three drying methods, the seeds attained < 20% MC (air dry, 33% RH, and rapid drying). Air drying yielded the slowest rate, and rapid drying in a flash dryer resulted in the fastest; however, drying at 33% RH and rapid drying produced a similar dry down rate (Figure 2.6).

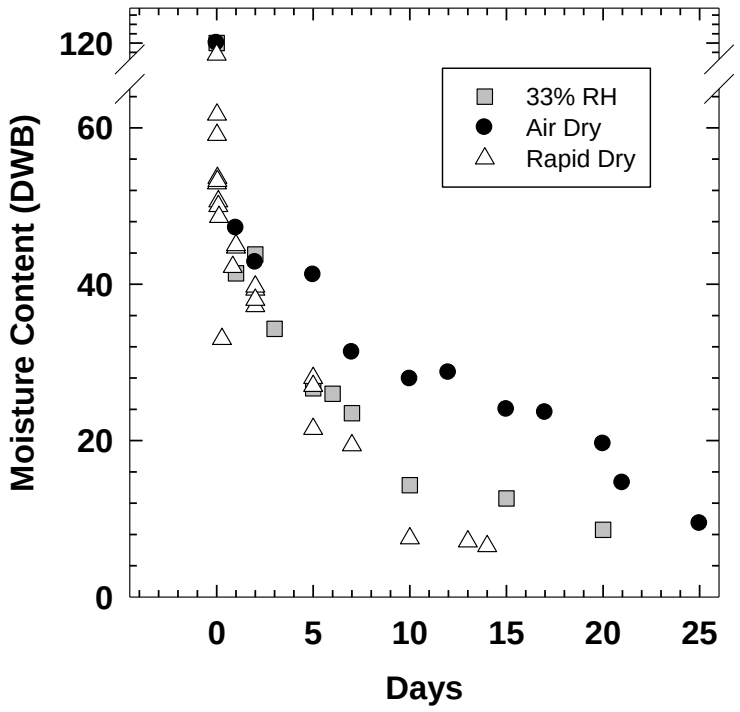

Figure 2.6. Three different methods of drying for recalcitrant *S. alterniflora* seeds (Port Fourchon, 2006). Air dried (●) and rapid dried (△) seeds were desiccated at room temperature (22-24°C) and seeds dried at 33% RH (■) were dried at 27°C. In all treatments, each dry down had matching points for moisture content and percent viability. Seeds were non-dormant and stored for 4-5 months submerged at 2°C. Each data point represents an independent drying experiment.

At all drying rates, *S. alterniflora* seeds began to lose their viability between 43 and 48% moisture contents (DWB) (Figure 2.7), and the dormancy status of the seeds did not affect the critical moisture content, as there were no discernable differences in the dry down curves between dormant and non-dormant *S. alterniflora* seeds (Figure 2.8). The average critical moisture content for all of the data presented in Figures 2.7 and 2.8 was 45% $\pm$ 1 (DWB), and the average critical moisture content for all the treatments presented in the study was 45% $\pm$ 1% (DWB).

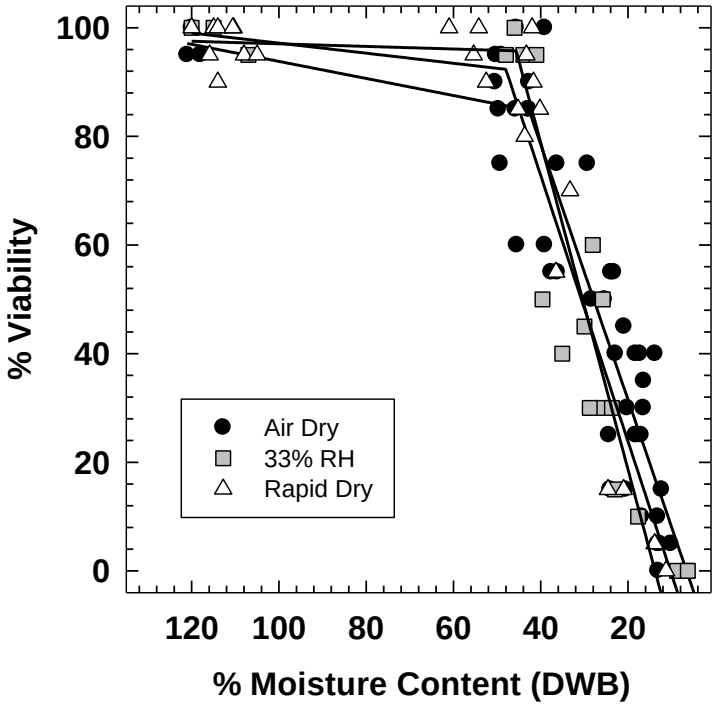

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Figure 2.7. Viability of non-dormant *S. alterniflora* seeds as a function of moisture content. Shoot viability was scored after three different drying regimes: rapid drying (Δ), 33 % relative humidity (RH) (■), and ambient air drying (●). Air dried and rapid dried seeds were dried at ambient temperatures and seeds at 33% RH dried at 27°C. Germination was scored after 14 days at 27°C, and viability was scored an after an additional 14 days at 27°C. Seeds were considered viable if shoot emergence was observed. Non-dormant, wild type seeds from the 2006 Port Fourchon harvest were used. Seeds were stored 4-5 months submerged at 2°C. Critical moisture contents were determined by fitting each dry down method with a two segment linear regression equation.

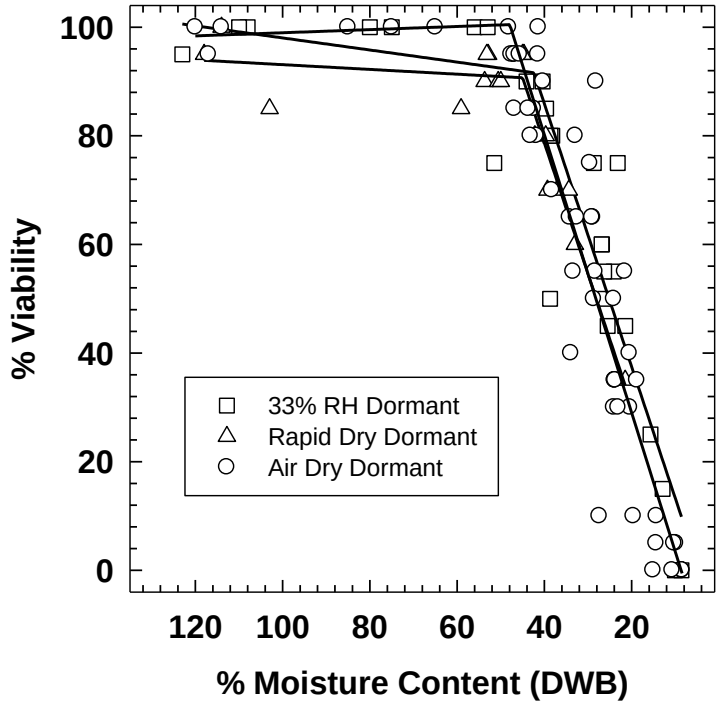

Figure 2.8. Viability of dormant *S. alterniflora* seeds as a function of moisture content. Shoot viability was scored after three different drying regimes: rapid drying (Δ), 33 % relative humidity (RH) (□), and ambient air drying (○). Ambient air dried and rapidly dried seeds were dried at ambient temperatures and seeds at 33% RH dried at 27°C. Dormant seeds had <25% germination before desiccation treatments. Germination was scored after 14 days at 27°C, and viability was scored after an additional 14 days at 27°C. Seeds were considered viable if shoot emergence was observed. Wild type seeds from the 2006 Port Fourchon harvest were used. Critical moisture contents were determined by fitting each dry down method with a two segment linear regression equation.

At the two other relative humidities (85% and 75% RH), the seeds did not dry completely and slowly dried to ca. 44 and 37% MC, respectively, after 10 days of drying (Figure 2.9). The seeds below 40% MC lost viability in the same manner as the other drying methods, and when the seeds equilibrated above 40%, they slowly began to lose their viability as storage time increase.

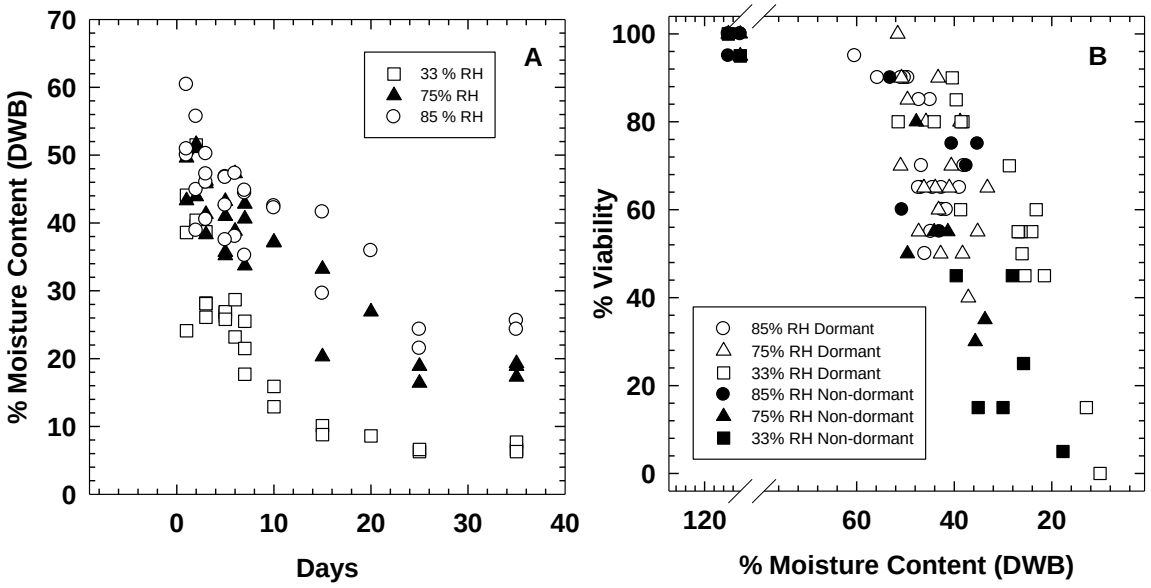

Figure 2.9. (A) Relationship between moisture content of dormant and non-dormant seeds and time of drying at elevated relative humidities. Seeds were dried at relative humidities of 85% RH (○), 75% RH (▲) and 33% RH (□). (B) Viability of non-dormant (closed symbols) and dormant (open symbols) *S. alterniflora* seeds as a function of moisture content when dried at 27°C at different relative humidities. Shoot viability was scored in dormant and non-dormant seeds after three different drying regimes: 85% RH (●, ○), 75% RH (▲, △) and 33% RH (■, □). Germination was scored after 14 days at 27°C, and viability was scored an after an additional 14 days at 27°C. Seeds were considered viable if shoot emergence was observed. Wild type seeds from the 2006 Port Fourchon harvest were used.

Water Potential and Viability of Non-Dormant *S. alterniflora* Seeds

Non-dormant *S. alterniflora* seeds equilibrated at 34% $\pm$1.3 moisture content (DWB) in a 93% RH atmosphere, which is equivalent to a water potential of -11MPa (Figure 2.10). As the relative humidity decreased, the moisture content at which the seeds equilibrated also decreased down to 7% at a relative humidity of 33% or a water potential of -161 MPa.

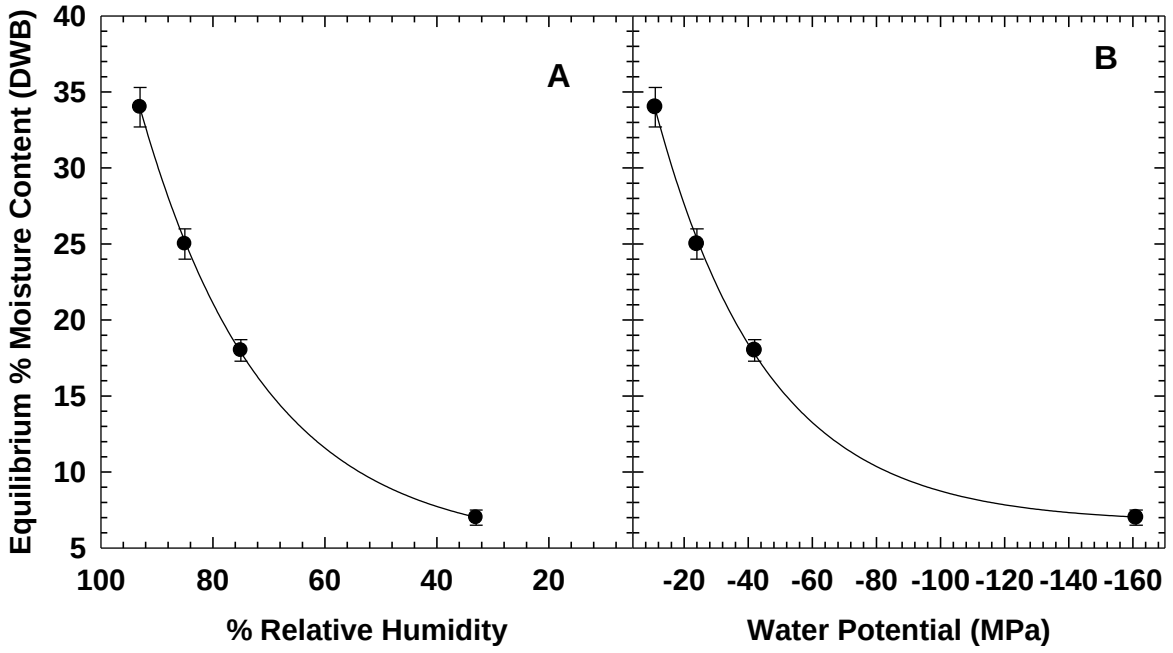

Figure 2.10. Desorption isotherm for non-dormant *S. alterniflora* (Port Fourchon, 2006) seeds stored at 27°C. (A) Equilibration moisture content of the seeds versus relative humidity and (B) equilibration moisture content of the seeds versus water potential (MPa). Seeds were equilibrated at different relative humidities at 27°C. Moisture content was determined after equilibrium was reached. Lines were fitted with the equation, $y = y_0 + ae^{bx}$ and the $r^2 = 0.99$.

Dry Down Rates of *Spartina alterniflora* and *Spartina pectinata* When Rapidly Dried

Spartina pectinata produces desiccation tolerant, orthodox seeds, that have a much faster drying rate when rapidly dried, reaching 40% MC in approximately 0.07 days, compared to 1 day for *S. alterniflora* (Figure 2.11).

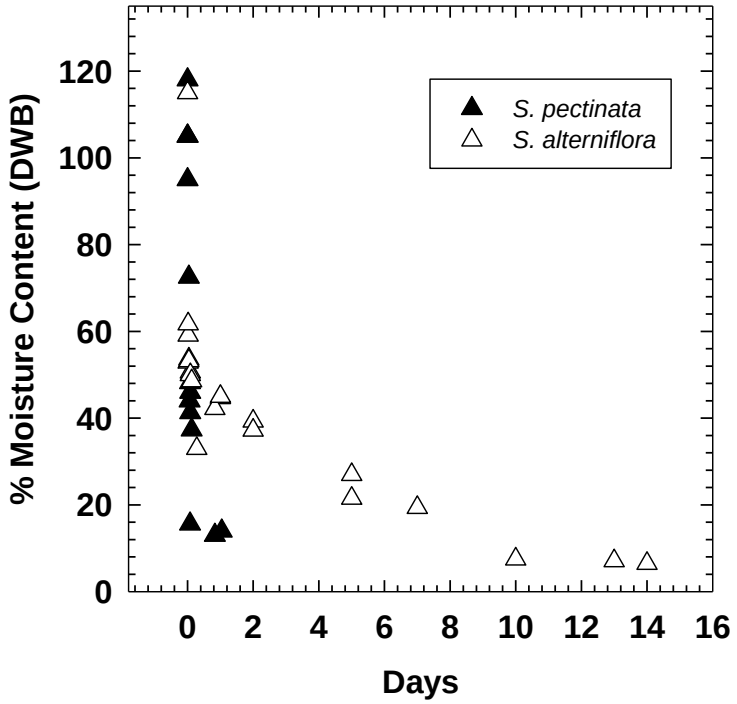

Figure 2.11. Representative dry down curve for the seeds of non-dormant orthodox *S. pectinata* (▲) and *S. alterniflora* (△) rapidly dried in a flash dryer.

DISCUSSION

Cardinal Temperatures for Germination of *Spartina alterniflora*

Spartina alterniflora seeds germinated at all the temperatures tested. However, root emergence was severely delayed at temperatures of 37°C and 17°C, but 100% germination was still attained. Eventually, after approximately 7-10 months, the seeds germinated in 2°C storage, and even at this low temperature, roots and shoots appeared healthy. From a germination standpoint, because marsh surface water temperatures along the coast of Louisiana range from

approximately 10°C to 30°C (White and Trapani 1982), any time of year should support *S. alterniflora* germination. However, because the seeds require cold-stratification, artificial seed establishment should probably take place in the winter months when the water temperatures are lower. If artificial seeding is done in the spring, germination may be delayed or not occur at all because the cold-stratification requirements are not met. If seeds germinate later than naturally, they may not establish as seedlings because environmental factors, such as air temperature and water levels, will differ from the time when germination and seedling establishment would occur naturally.

Viability Test to Determine if the Seeds Are Dormant or Dead

Cutting dormant *S. alterniflora* seeds caused high germination percentages (Figure 2.5 A), and is a simple, effective way to determine if a seed is dormant or dead. This test facilitates the use of dormant seeds to study desiccation-induced death. The use of dormant seeds in the study of recalcitrance is important, as results with dormant seeds reduce the risk of interpreting disruptions of germination-related metabolism as components of recalcitrant seed death.

Cold-Stratification (2°C) Requirements to Alleviate Dormancy in *S. alterniflora* Seeds

Results from the stratification experiment indicated that seeds from different harvest years and accessions of *S. alterniflora* do not lose dormancy at statistically different rates during stratification. This finding is somewhat expected, as all of the plants were grown in a similar climate. The seeds harvested in 2001, 2002 and 2004 were harvested from Baton Rouge, Louisiana (30°32' N, 91° 9' W), and the wild-type seeds were harvested from Golden Meadow, Louisiana (29° 37' N, 90° 26' W).

The cold-stratification requirement for *S. alterniflora* seeds in the laboratory resembled the response in nature. When *S. alterniflora* seeds are naturally shed in early November and late December along the coast of Louisiana, they are dispersed into standing water with an average

surface water temperature of 11°C. The average surface water temperature is then colder in January, with an average of 9.4°C (White and Trapani 1982). From results with seeds in the lab, 10°C is sufficiently cold to stratify the seeds, as an average G_{50} time of 2.6 months was observed for seeds stratified at 10°C (Figure 2.5B).

Knowledge of the stratification requirement for *S. alterniflora* is not only important for artificial establishment reasons, but also for the study of recalcitrance. Understanding the stratification requirements may allow manipulation of storage conditions to increase the length of the overall dormancy period and overall storage time. This is extremely valuable for the study of recalcitrant *Spartina* seeds because it allows more experiments to be performed with the same seed lot for almost a year (in contrast to a few weeks to months with other recalcitrant species), and lengthens the time interval for experimentation using dormant seeds.

Is There a Critical Moisture Content for *S. alterniflora* Seeds Dried at Different Rates?

Previous results have been interpreted to suggest that the degree of desiccation tolerance in recalcitrant seeds is dependent on the rate at which the seeds are dried (Sun 2002). However, results of the *S. alterniflora* dry down experiments suggest that viability is dependent on a narrow range of critical moisture contents (45%), and not the rate of drying; in all treatments, viability was lost as seeds were dried below 42 and 48% MC (DWB) (Figures 2.7, 2.8 and 2.9), with an average MC of 45% $\pm$ 1 (DWB). These results support the hypothesis that drying rate does not affect viability, as there is little variation between drying treatments. It is often proposed that seeds can tolerate greater desiccation when rapidly dried because seeds pass quickly through intermediate water stages before massive damage can occur (Berjak and Pammenter 2008). If this hypothesis is correct, it would be expected that *S. alterniflora* would lose viability at higher water contents when slowly dried, via the ambient air dry method or at elevated relative humidities, than when rapidly dried in a flash dryer. Even though whole *S. alterniflora* seeds

cannot be dried fast enough to constitute 'flash' drying (Berjak and Pammenter 2008), if death is truly caused by a build up of deleterious products, the slower dried seeds spend a much greater time at each drying stage and would therefore be expected die at a higher water content. Because there were no observed differences in *Spartina* death due to drying rate, it does not appear that a buildup of damaging products occurs at high water contents. Rather, an event at or near the critical moisture content appears to cause loss of viability. Another interesting result is that there were no differences in critical moisture contents between dormant and non-dormant *S. alterniflora* seeds (Figure 2.8). It is often proposed that an active metabolism plays a role in recalcitrant seed death (Berjak and Pammenter 2008) because most recalcitrant species produce only non-dormant seeds; however, if metabolism was the cause of death, it would be expected that non-dormant seeds would die at higher moisture contents than dormant seeds. When seeds were dried at a high relative humidity of 85%, the seeds remained just above the critical moisture content for approximately 10 days. During this time both dormant and non-dormant seeds began to lose their viability, but because of the extended time at a high relative humidity, this is most likely an affect of aging, rather than recalcitrance.

Why some studies find an effect of drying rate, and others do not, is uncertain. However, it is important to note that even when an effect of drying rate is reported, healthy seedlings are not produced at the lower moisture contents, but merely a few viable cells (Pammenter and Berjak 2008), which may be a symptom of dry-down kinetics and the non- uniformity of water removal, rather than recalcitrance.

Results from this study suggest that a critical moisture content, rather than drying rate, is the most significant feature of desiccation-induced death of *Spartina alterniflora* seeds. Also, the finding that dormant and non-dormant seeds have the same critical moisture content suggests that the active metabolism of a germinating seed is not the cause of death.

Water Potential and Viability of Non-Dormant *S. alterniflora* Seeds

Extrapolating from the results, it appears that *S. alterniflora* begins to lose viability at water potentials between -5 and -10 MPa. The onset of damage at this water potential appears to be common for many recalcitrant seeds (Sun and Liang 2001). For example, some common recalcitrant seeds, such as *Castanea sinensis*, *Acer pseudoplatanus*, and *Quercus rubra* have critical water potentials between -7.4 and -13 MPa (Sun and Liang 2001). Also, whole seeds of *Zizania palustris*, which is also a water grass with recalcitrant seeds, have a critical water potential of -3 MPa (Probert and Longley 1989), similar to the results observed with *S. alterniflora*. However, not all recalcitrant seeds were damaged in this range and could be desiccated much further. For example, *Artocarpus heterophyllus* axes and *Azadirachta indica* seeds had critical water potentials of -22.5 and -73.6 MPa, respectively (Liang and Sun 2001). This suggests that there are different levels of recalcitrance, and the onset of damage varies between species.

Water potentials were only calculated for the seeds of *S. alterniflora* for one harvest for this study. However, a multi-year study that examines the critical water potential, along with environmental parameters, such as temperature and length of maturation, may provide clues as to whether there are degrees of recalcitrance that can vary in a species from year to year.

Dry Down Rates of *Spartina alterniflora* and *S. pectinata* When Rapidly Dried

To compare the dry down rate of *S. alterniflora* with the seeds of orthodox *S. pectinata*, both were rapidly dried in the same manner. The seeds have a similar appearance and size, yet surprisingly, the dry down rate for *S. pectinata* is substantially faster than *S. alterniflora* (Figure 2.11). A similar trend was observed with seeds of *Acer* species, where the recalcitrant *Acer pseudoplatanus* took over 10 times longer to desiccate than orthodox *Acer platanoides*

(Greggains *et al*. 2000). It is unclear why *S. pectinata* dries at a faster rate, but it could be assumed that the prevention of water loss is a defense mechanism in *S. alterniflora*, aimed to prevent the seed from drying below the critical moisture content of 45% DWB. The molecular and/or structural basis for such differential desiccation rates merits further exploration.

CHAPTER 3
DOES LIPID PEROXIDATION OR MEMBRANE DAMAGE CAUSE DEATH AS RECALCITRANT *SPARTINA ALTERNIFLORA* SEEDS ARE DRIED?

LITERATURE REVIEW

Is Lipid Peroxidation a Cause or a Consequence of Recalcitrant Seed Death?

Increased lipid peroxidation has been proposed as a cause for viability loss in both desiccation-intolerant and aged orthodox seeds (Wilson and MacDonald 1986). The idea is still somewhat controversial, and the issue is unresolved because it is sometimes assumed that death by drying and aging occurs in the same way. This may be an unsubstantiated assumption, as it is possible that the role of lipid peroxidation differs between recalcitrant and aging seeds.

Most methods that determine lipid damage measure products of free radical attack upon polyunsaturated fatty acids; lipid peroxidation can generate hydroperoxides, endoperoxides, aldehydes and malondialdehyde (MDA) (Waterfall *et al.* 1997). The thiobarbituric acid reactive substances assay (TBARS), which measures malondialdehyde along with other aldehydes produced by lipid peroxidation, is a common method used to measure lipid peroxidation in both aged and recalcitrant seeds. However, no consistent results have emerged regarding a role for lipid peroxidation in the loss of viability as a consequence of seed drying (Table 3.1). For example, when the MDA content of tea, cocoa and jackfruit (all recalcitrant) was measured in freshly harvested seeds, and after 4 hours of desiccation, only cocoa had elevated MDA content following desiccation (Chandel *et al.* 1995). The tea and jackfruit seeds had a slight decline in MDA amounts following desiccation, but the baseline amounts in recalcitrant seeds were higher than amounts observed in orthodox french bean seeds, which were used as a control. However, this may not be a valid comparison because french bean is not in the same genus as the other seeds. In contrast, when Li and Sun (1999) measured lipid peroxidation in cocoa (*Theobroma*

cacao), they found a sharp increase in TBARS as seeds reached the critical moisture content, which coincided with a rapid decline in percent germination and a decline in reactive oxygen species (ROS) scavenging enzymes, peroxidase and superoxide dismutase. Results like these suggest that as recalcitrant seeds are dried, the ability to cope with oxidative stress is overwhelmed.

Products of lipid peroxidation also were observed as recalcitrant *Quercus robur* was dried, where a slight increase in TBARS products was observed as the cotyledons were dried and a much larger increase occurred as the axes were dried (Hendry *et al.* 1992, Finch-Savage *et al.* 1996), suggesting that lipid peroxidation may be concentrated in specific areas of the seed. While TBARS has been used with many different recalcitrant species, the results have been vastly different (Table 3.1), and it is still unclear what role, if any, lipid peroxidation plays in desiccation-induced death.

The thiobarbituric acid reactive substances assay has been the predominant method used to determine lipid peroxidation in recalcitrant seeds. However, the results must be viewed cautiously because carbohydrates and pigments, such as anthocyanins, can react with thiobarbituric acid (TBA) and cause lipid peroxidation values to be overestimated (Du and Bramlage 1992, Hodges *et al.* 1999). Also, Table 3.2 illustrates the numerous changes that have been made from the standard Heath and Packer method (1968) without documented justification when the TBARS method has been used to measure lipid peroxidation as recalcitrant seeds and seed parts are desiccated.

Table 3.1. Summary of TBARS amounts reported in the literature in many different recalcitrant seeds and recalcitrant seed parts

Species	TBA amounts in fully hydrated seeds	TBA amounts at critical moisture content	TBA amounts in 25 % viable seeds	Citation
Theobroma cacao/ axes	5 nmoles mg^{-1} protein	8.0 nmoles mg^{-1} protein	10 nmoles mg^{-1} protein	Li and Sun 1999
Shorea robusta/ axes	0.21 A 540/ g fw	0.8 A 540/ g fw	1.25 A 540/ g fw	Chaitanya and Naithani. 1998
Shorea robusta/ axes	0.1 g^{-1} fw O.D. 540 nm	0.26 g^{-1} fw O.D. 540 nm	0.33 g^{-1} fw O.D. 540 nm	Chaitanya and Naithani. 1994
Avicennia marina/ cotyledons	199 nmol g^{-1} dwt	210 nmol g^{-1} dwt	176 nmol g^{-1} dwt	Greggains *et al.* 2001
Avicennia marina/ root primordial	51 nmol g^{-1} dwt	78 nmol g^{-1} dwt	53 nmol g^{-1} dwt	Greggains *et al.* 2001
Avicennia marina/ hypocotyl	51 nmol g^{-1} dwt	40 nmol g^{-1} dwt	25 nmol g^{-1} dwt	Greggains *et al.* 2001
Acer pseudoplatanus/ radicles	215 nmol g^{-1} dwt	265 nmol g^{-1} dwt	250 nmol g^{-1} dwt	Greggains *et al.* 2000
Quercus robur/ axes	4.7 nmol g^{-1} dwt	4.7 nmol g^{-1} dwt	5.2 nmol g^{-1} dwt	Hendry *et al.* 1992
Quercus robur/ cotyledons	28 nmol g^{-1} dwt	48 nmol g^{-1} dwt	60 nmol g^{-1} dwt	Hendry *et al.* 1992
Camellia sinensis/ axes	0.079 TBA rp g^{-1} dwt	0.070 TBA-rp g^{-1} dwt[a]	**********	Chandel *et al.*1995
Theobroma cacao/ axes	0.202 TBA-rp g^{-1} dwt	**********	0.235 TBA-rp g^{-1} dwt[a]	Chandel *et al.* 1995

Continued, next page

Table 3.1. Continued.

Species	TBA amounts in fully hydrated seeds	TBA amounts at critical moisture content	TBA amounts in 25 % viable seeds	Reference
Azadirachta indica/ axes	0.72 nmol MDA g^{-1} fwt	1.8 nmol MDA g^{-1} fwt	3.6 nmol MDA g^{-1} fwt	Varghese and Naithani 2002
Azadirachta indica/ cotyledons	0.18 nmol MDA g^{-1} fwt	0.25 nmole MDA g^{-1} fwt	0.5 nmol MDA g^{-1} fwt	Varghese and Naithani 2002
Trichilia dregeana/ axes	275 nmol mg^{-1} protein	275 nmole mg^{-1} protein	330 nmol mg^{-1} protein	Song *et al.* 2004
Araucaria bidwilli/ embryo[b]	19.25 umol g^{-1} dwt	18.13 umol g^{-1} dwt	27.52 umol g^{-1} dwt	Francini *et al.* 2006
Araucaria bidwilli/ endosperm[b]	15.12 umol g^{-1} dwt	15.93 umol g^{-1} dwt	23.82 umol g^{-1} dwt	Francini *et al.* 2006

Notes: ***** indicates data were not available. rp is an abbreviation for reaction products. [a] indicates that only two TBARS points were given. For *Camellia sinensis* the critical moisture content TBARS point is actually just past the critical moisture content (germination = 75%). For *Theobroma cacao*, no 25% data point was given, seeds actually have 0% germination. The symbol [b] in the table indicates that TBARS points correlate with seedling establishment, not germination.

Table 3.2. For recalcitrant seeds, different thiobarbituric acid substances assay (TBARS) methods used in lipid peroxidation studies.

Tissue used	# of papers	Tissue amount	# of papers
axis	6	250 mg	1
		5 seeds, axes, or radicals	2
axis+cotyledons	3	Not given	4
(measured separately)		10 axes	1
whole seeds,		100 mg tissue	1
axes + endosperm	1	40 axes	1
(measured separately)			
Temperature of homogenization	# of papers	Homogenate used	# of papers
Ice	7	5% TCA	1
Room Temperature	1	Phosphate buffer	3
No temperature given	1	0.5% TBA in 20% TCA	2
26°C	1	Acetone	4
Centrifuge speed	# of papers	Methods to avoid artifactual oxidation	# of papers
5,367 g x 10 min	1	BHT	5
5,000 g x 10 min	5	None	5
15,000 g x 2 min	2		
13,000 g x 2 min	1		
5,000 g x 1 min	1		
Incubation time and temperature	# of papers		
100°C x 15 min.	1		
90°C x 30 min.	2		
95°C x 25 min	7		

TBARS Amounts As Orthodox Seeds Are Aged

Two studies reported that lipid peroxidation does not occur during natural seed aging (Priestley and Leopold 1979, Aiazzi *et al.* 1997). For example, when fresh *Atriplex cordobensis* seeds were compared with slightly detiorated seeds, MDA increased slightly, and decreased in highly deteriorated seeds (Aiazzi *et al.* 1997).

Other studies indicated that lipid peroxidation does increase as seeds are aged. When MDA content was measured in naturally aged soybeans, it increased significantly during aging (Sung and Chiu 1995, Sung 1996) and correlated with an increase in hydrogen peroxide (Sung 1996). The ROS increase suggests that either the antioxidants in the seeds are inactive, or the pool of ROS is too large to be completely quenched. Lipid peroxidation also occurred in naturally aged almond seeds (*Prunis dulchis*) (Zacheo *et al.* 2000), and both the MDA and hydroperoxide content [both TBARS and the ferrous-orange-xylenol (FOX) method were used] increased 60-70% during aging. Chang and Sung (1998) also reported an increase in lipid peroxidation in naturally aged corn kernels, and found that lipid peroxidation increased more in seeds stored at higher temperatures. In cotton seeds aged for twelve months at 25°C, the MDA content tripled, and the lipid peroxidation amounts appeared correlated with the decline in percent germination (Goel and Sheoran 2003).

It is important to note that all of the studies listed above worked with high oil seeds. Measuring lipid peroxidation via TBARS may not be appropriate in high oil seeds. The TBA product amounts may be dependent on the triacylglycerol content in the seed and mask processes occurring in the membranes (Leprince and Golovina 2002).

The literature for aged seeds reports a variety of results about whether lipid peroxidation causes a loss of viability. The inconsistencies regarding lipid peroxidation findings in seed aging are the result of many different species being used for the aging experiments, or the mixed results may derive from differences in the TBARS methods used. While every paper discussed above used TBARS to measure lipid peroxidation, Table 3.3 illustrates that, like recalcitrant studies, the methods for TBARS used for aged seeds were also not standardized, and could, therefore, lead to a cornucopia of results.

Furthermore, none of the cited protocols corrected for sugars or other interferences, as suggested by Hodges *et al.* (1999) and Du and Bramlage (1992). Such non-MDA interferences can drastically increase the values obtained by TBARS. By ignoring these corrections, the TBARS values can be vastly overestimated (Hodges *et al.* 1999).

Table 3.3. For aged orthodox seeds, different thiobarbituric acid substances assay (TBARS) methods used in lipid peroxidation studies.

Tissue used	# of papers	Tissue amount	# of papers
axis	2	3 repetitions of 5	1
whole seed	10	200mg	1
axis+cotyledons		20mg	2
(measured separately)	2	250mg	1
		1.0g	1
		0.5g	1
		5 seeds	1
		10 seeds	2
		100 seeds	1
		Not given	3
Temperature of homogenization	# of papers	Homogenate used	# of papers
4^0C	11	5% TCA	7
Room Temperature	1	Phosphate buffer	3
No temperature given	2	Potassium phosphate	1
		Water followed by TCA	1
		Ethanol	2
Centrifuge speed	# of papers	Methods to avoid oxidation during preparation	# of papers
8,000gX15 min .	1	Nitrogen gas to remove 0_2	1
14,000gX 20 min.	5	None	13
5,000gX5 min.	2		
45,000gX30min	1		
3,000gX15min	1		
10,000gX30min	2		
5,000gX1min	1		
Not given	1		

Incubation time and Temperature	# of papers
95^0C 30 min.	4
90^0C 25 min.	8
90^0C 30 min.	1
Not given	1

Measurement of Lipid Hydroperoxides With the Ferrous Oxidation-Xylenol Orange (FOX) Assay

To complement and confirm the findings of the TBARS test, it is necessary to use another method, such as the ferrous oxidation- xylenol orange (FOX) method, to measure lipid peroxidation. The FOX method measures hydroperoxides, which are early indicators of lipid peroxidation (DeLong *et al.* 2002), while TBARS measures malondialdehyde, a later product of lipid peroxidation. By using FOX along with TBARS, the results of both methods can be compared. If the results do not support one another, one of the techniques could be flawed, or something unique could be happening within the seeds. Hydroperoxide amounts increased in the cotyledons of recalcitrant *Telfaria occidentalis* seeds, from an absorbance of 0.02 in fully hydrated seeds, to 0.24 at the critical moisture content, and to 1.20 when the seeds were desiccated well past their critical moisture content (Nkang *et al.* 2000). With the exception of the Nkang *et al*. (2000) study, no other studies have measured lipid hydroperoxides in recalcitrant seeds.

Organic and Inorganic Leachates As Indicators of Physical Membrane Damage During Desiccation of Recalcitrant Seeds

Leachates are traditionally used as an indicator of membrane damage in seeds. However, there are conflicting findings in regards to leachate amounts as recalcitrant seeds are desiccated. For example, when leachates were measured as intact, recalcitrant *Quercus nigra* seeds were dried, there was no consistent correlation between viability and leachates (Bonner 1996). When *Q. nigra* seeds were dried slowly (95% RH, 27°C), a spike in leachates occurred after 10 days of drying (25% MC, DWB), but this is well before the seeds reached the critical moisture content of 18%, which did not occur until 15 days of drying. When *Q. nigra* seeds were dried quickly, or at a moderate rate (55% RH, 27°C,

with a stream of air from a fan, and air dried at 27°C, respectively), there was little change in leachates, and amounts never reached more than 2 uS g^{-1} dwt. At the critical moisture content, the seed leachates returned to baseline amounts. Leachates also increased well after the critical moisture content if the seeds were desiccated at 40°C. Becwar *et al.* (1982) found similar results with recalcitrant, intact *Acer saccharinum* seeds, as there was no leachate increase throughout the dry down. However, a significant increase in leachates was observed if the testa was removed before the leachate test. It has been proposed that the testa blocks leachate exchange, so it must be removed before leachates can be observed (Becwar *et al.* 1982); however, this contradicts the finding by Bonner (1996) mentioned earlier, where leachates were observed from intact seeds; they just did not draw a parallel with loss of viability.

When only the axes of recalcitrant seeds are dried, numerous studies have reported a significant increase in leachates during drying and suggest a direct correlation between membrane damage and viability (e.g. Wesley-Smith *et al.* 2001, Liang and Sun 2000, Pammenter *et al.* 1991). However, when jackfruit and tea axes were desiccated, leachates only increased 2 and 3%, respectively, less than the orthodox french bean control used, which had a 7% leachate increase during drying (Chandel *et al.* 1995).

From the various findings and different methods used to measure membrane damage and leakage (summarized in Table 3.4), it is unclear if physical membrane damage is a cause or the result of recalcitrant seed death. In the present study, the roles of lipid peroxidation and membrane damage were reevaluated using various recent improvements to the analytical methods, as well as the *S. pectinata* physiological controls.

Table 3.4. Summary of leachate data as recalcitrant seeds are desiccated.

Species	Method of Drying	Leachate Amounts Before Drying	Leachate Amounts at Critical Moisture Content	Leachate Amounts at 25% Viability	Reference
Landolpha kirkii	flash dryer, embryos	10 mamps/hr/g dwt	40 mamps/hr/g dwt	******	Pammenter *et al.* 1991
Ladolpha kirkii	buried in silica embryos	10 mamps/hr/g dwt	35 mamps/hr/g dwt	90 mamps/hr/g dwt	Pammenter *et al.* 1991
Acer pseudoplatanus	60-70% RH seeds (removed from samara)	10 % total seed leachates	15% total seed	42 % total	Pukacka *et al.* 2007
Trichilia dregeana	Silica axes	1 uS cm-1 mg-^{1} DW h^{-1}	2 uS cm-1 mg-1 DW h^{-1}	3.8 uS cm-^{1} mg^{-1} DW h^{-1}	Song *et al.* 2004
Camellia sinensis	Silica axes	988 uS cm^{-1} g-^{1} DW^{-1}	1017 uS cm-1 g-1 DW^{-1}	*******	Chandel *et al.* 1995
Theobroma cacao	Silica axes	572 uS cm^{-1} g-1 DW^{-1}	*******	1218 uS cm-1 g-1 DW^{-1}	Chandel *et al.* 1995
Theobroma cacao	95.5% RH axes	3 % of total	4 % of total	*******	Liang and Sun 2000
Theobroma cacao	79.5 % RH axes	3 % of total	8% of total	*******	Liang and Sun 2000
Theobroma cacao	6% RH axes	3 % of total	8 % of total	*******	Liang and Sun 2000
Theobroma cacao	60% RH axes	3 % of total	3 % of total	10% of total	Liang and Sun 2000
Theobroma cacao	60% RH cotyledons	3 % of total	12 % of total	38 % of total	Liang and Sun 2000

Continued, next page

Table 3.4. Continued.

Species	Method of Drying	Leachate Amounts Before Drying	Leachate Amounts at Critical Moisture Content	Leachate Amounts at 25% Viability	Reference
Quercus robur	intermediate dry axes	0% of total	62 % of total	100 % of total	Finch-Savage *et al.* 1996
Atrocarpus heterophyllus	slow dry axes	2360 uS cm^{-1} g^{-1} DW^{-1}	2407 uS cm^{-1} g^{-1} DW^{-1}	*******	Chandel *et al.* 1995
Quercus nigra	air flow seeds	0 uS g^{-1}	1.5 uS g^{-1}	1.8 uS g^{-1}	Bonner 1996
Quercus nigra	air dried seeds	0.8 uS g^{-1}	0.0 uS g^{-1}	1.2 uS g^{-1}	Bonner 1996
Quercus nigra	95% RH seeds	0.5 uS g^{-1}	4.8 uS g^{-1}	4.5 uS g^{-1}	Bonner 1996
Artocarpus heterophyllus	flash dryer axes	10 % of total	25% of total	*******	Wesley-Smith *et al.* 2001
Artocarpus heterophyllus	96 % RH axes	10% of total	22% of total	37% of total	Wesley-Smith *et al.* 2001
Shorea robusta	40-45% RH axes	0.2 m Mhos	1.3 m Mhos	1.4 m Mhos	Chandel *et al.* 1994
Shorea robusta	40-45% RH axes	0.1m Mhos	0.2 m Mhos	0.5 m Mhos	Chaitanya and Naithani 1998
Acer saccharinum	35-40% RH axes –post dry	150 uS g^{-1} dwt	600 uS g^{-1} dwt	2100 uS g^{-1} dwt	Pukacka and Ratajczak 2006

Continued, next page

Table 3.4. Continued

Species	Method of Drying	Leachate Amounts Before Drying	Leachate Amounts at Critical Moisture Content	Leachate Amounts at 25% Viability	Reference
*Acer saccharinum**	35◦C seed	7 % of total	8 % of total	8 % of total	Becwar *et al*. 1982
*Acer saccharinum**	35◦C testa removed	20 % of total	30 % of total	88 % of total	Becwar *et al*. 1982
Araucaria angustifolia	55 % RH axes	8 % of total	15 % of total	50 % of toal	Espindola *et al*. 1994
Araucaria angustifolia	55 % RH axes	9 % of total	30 % of total	63 % of total	Espindola *et al*. 1994

* It is unclear in this study if the testa was removed before or after desiccation. When a % of total value is given for leachates, that represents the % of leachates versus the total possible leachates from the tissue.

METHODS

Plant Material

Spartina alterniflora seeds used for the following experiments were harvested at the natural time of shattering from either the Ben-Hur Experimental Station in Baton Rouge, Louisiana (2004 harvest), or were harvested from wild-type plants from the marshes in Port Fourchon, Louisiana (2005, 2006, 2007 harvests). Subsequent to hand-shattering, the seeds were immediately placed in sealed freezer bags and transported to the laboratory for processing. Transport times from Ben-Hur Experimental Station and Port Fourchon were ca. 15 minutes and 3 hours, respectively. Once in the lab, the 10 gram seed lots were immediately placed in GA-7 culture vessels that contained 250 ml of deionized H_20 and stored at 2°C.

Spartina pectinata seeds were purchased from Western Native Seeds in Coaldale, Colorado (WNS-03A, Lot # 6042) and harvested in 2003. Upon arrival, the *S. pectinata* seeds had an average moisture content of 8.5 % $\pm$ 1. When received, the seeds were either placed in sealed Ball™ jars and stored at 23°C, or immediately submerged in H_20 and stored at 2°C. After several months of storage at 23°C, related studies indicated that *S. pectinata* seeds at low moisture contents could be safely stored at -20°C; to minimize the effects of aging, unimbibed seeds in the Ball jar were moved to -20°C.

Thiobarbituric Acid Reactive Substances Assay (TBARS) (Method from Hodges *et al.* 1999)

The TBARS method, from Heath and Packer (1968) and as modified by Hodges *et al.* (1999), was used to measure late products of lipid peroxidation as *Spartina* seeds were rapidly dried. Thiobarbituric acid reactive substances were quantified using 1, 1, 3, 3-tetraethoxypropane as a standard (Figure A-25).

A standard flash-dry with 90 seeds was used. After flash-drying, 40 seeds (20 each) were used for the viability test and percent moisture content calculation of the seeds after drying. The remaining 50 seeds were flash-frozen in liquid nitrogen and ground to a powder in a mortar and pestle that were pre-chilled with liquid nitrogen and buried in dry ice. To ensure reproducibility, the seeds were ground to the same degree, and liquid nitrogen was added liberally to prevent thawing. The seed powder was transferred to a 15 ml Tenbroek homogenizer embedded in ice, and the seeds were homogenized in 3 ml of ice-cold 80:20 ethanol:H_20 (v/v). Each sample was extracted with exactly 20 full strokes of the glass pestle to ensure complete and uniform homogenization. To maximize the transfer, two additional milliliters of ice-cold 80:20 ethanol: H_20 was used to rinse any tissue debris from the pestle and homogenizer (1 ml for each), bringing the total volume to 5 ml. The sample was then centrifuged (4°C) for 10 minutes at 12, 000 g to remove cellular debris. A 1 ml aliquot of the supernatant was added to a test tube that contained 2 ml of 20% (w/v) trichloroacetic acid (TCA), 0.01% butylated hydroxytoluene (BHT), and 1 ml of 0.67% TBA. A tissue blank also was used, where in place of TBA, distilled water was added. Samples were then vortexed (ca. 3 seconds), and heated in a 95°C water bath for 25 minutes (Figure A-26), followed by 10 minutes on ice to remove proteins and then centrifuged (4°C) at 10,000 g for 5 minutes. The absorbance of the supernatant was then measured at 440 (sugars), 532 (TBARS products) and 600 (turbidity) nm. To determine the overall MDA present the following calculations were used:

Heath and Packer (1968) uncorrected values:
1. $[(Abs_{532} + TBA) - (Abs_{600} + TBA) = A$
2. MDA total $(nmol \cdot ml^{-1}) = (A/157\,000\ M^{-1}\ cm^{-1})\ 10^6$

Du and Bramlage (1992) sugar corrected values
1. $[(Abs_{532} + TBA) - (Abs_{600} + TBA) = A$
2. $[(Abs_{440})\ 0.0571] = B$
3. MDA total $(nmol \cdot ml^{-1}) = (A-B/157\,000\ M^{-1}\ cm^{-1})\ 10^6$

Hodges *et al.* (1999) corrected values:
1. [(Abs $_{532}$ +TBA) - (Abs $_{600}$ + TBA) - (Abs $_{532}$-TBA-Abs $_{600}$-TBA) = A
2. [(Abs $_{440}$ +TBA-Abs $_{600}$) 0.0571] = B
3. MDA total (nmol $\cdot$ ml^{-1}) = (A-B/157 000 M $^{-1}$ cm^{-1}) 10^6

Conversion of nmol ml^{-1} to nmol$\cdot$seed^{-1}:
1. (nmol $\cdot$ ml^{-1}) (4 ml of reaction mix volume) = nmoles in reaction mix
2. Total nmoles in reaction mix = total nmoles in 1 ml of extract
3. [(Total nmoles/ml seed extract) (5 ml seed extract)] / 50 seeds = nmol $\cdot$ seed^{-1}

The TBARS assay was used with dormant and non-dormant seeds of *S. alterniflora* and *S. pectinata* seeds to determine if there was a difference in lipid peroxidation between recalcitrant and orthodox seeds, and between metabolically active (non-dormant) and inactive (dormant) seeds.

Finally, to have a comparable method to past TBARS experiments with recalcitrant seeds (e.g. Greggains *et al.* 2000), the same experiment as above was performed, with the exception that the seeds were initially ground in a mortar at 4°C, rather than freeze-clamping in liquid nitrogen.

Ferrous-Xylenol-Orange Method to Detect Early Products of Lipid Peroxidation (Method from Delong *et al.* 2002)

To measure hydroperoxides, the ferrous-xylenol orange method (FOX), as described by Delong *et al.* (2002), was used. A standard flash dry with 90 seeds was used to desiccate the seeds. After flash drying, 50 seeds were flash-frozen in liquid nitrogen and ground in mortar and pestle that were pre-chilled and buried in dry ice. To ensure reproducibility, the seeds were ground to the same degree, and liquid nitrogen was added liberally to prevent thawing. The seed powder was transferred to a 15 ml Tenbroek homogenizer embedded in ice, and the seeds were homogenized in 4 ml of ice-cold 80:20 ethanol:H_20 (v/v). Each sample was extracted with exactly 20 full strokes of the glass pestle to ensure complete and uniform homogenization. To

maximize the transfer, two additional milliliters of ice-cold 80:20 ethanol: H_20 was used to rinse any tissue debris from the pestle and homogenizer (1 ml for each), bringing the total volume to 6 ml. The sample was centrifuged (4°C) for 10 minutes at 12,000 g, and the supernatant was removed for assay. Interfering agents could be determined by adding 1 ml of supernatant to 1 ml of 10mM triphenylphosphine (TPP) in methanol, which reduces peroxides (Figure A-32). Seed extract samples containing methanol alone were compared with solutions containing TPP and methanol to determine the amount of peroxides present in the sample. After TPP was added, the samples were incubated at 23°C for 30 minutes to allow for the reduction of peroxides. Next, 2 ml of the FOX reagent was added. The FOX reagent consisted of 90% methanol (v/v), 10% 250 mM sulfuric acid (H_2S0_4) (v/v), 4 mM BHT, 250 uM of ferrous ammonium sulfate hexahydrate and 100 uM of xylenol-orange. The methanol, H_2S0_4, and BHT were mixed and stored at 4°C for up to one month; however, the iron and xylenol orange were prepared and added on the same day of the experiment.

Following the addition of the FOX reagent, the samples were left at 25°C for 90 minutes (Figure A-30), after which, the absorbance at 560 nm was recorded. Total peroxides were determined with the following equation (Delong *et al.* 2002):

A) [-TPP] – [+TPP] = [Abs_{560} LOOH + Abs_{560} interfering compounds] - [Abs_{560} interfering compounds] = Abs_{560} LOOH
B) [(Abs560) / (60,000 $M^{-1} cm^{-1}$)] (10^6) = nmoles ml^{-1}

Conversion of nmol ml^{-1} to nmol·$seed^{-1}$:

1. (nmol · ml^{-1}) (reaction mix volume) = nmoles in reaction mix
2. Total nmoles in reaction mix = total nmoles in 1 ml of extract
3. [(Total nmoles/ml seed extract) (6 ml seed extract)] / 50 seeds = nmol · $seed^{-1}$

Peroxide values were compared in dormant and non-dormant *S. alterniflora* and *S. pectinata* seeds as they were desiccated. A standard curve (Figure A-29) was constructed using hydrogen peroxide, using an extinction coefficient of 60,000 M^{-1} cm^{-1} (Delong *et al.* 2002).

Organic and Inorganic Leachates As Indicators for Physical Membrane Damage During Desiccation of *Spartina* Seeds

Organic and inorganic leachates are commonly used as indicators of physical membrane damage in seeds. To measure leachates, 90 seeds were flash dried, and after the seeds reached the desired moisture content, 40 seeds were used for viability and moisture content calculations, and the remaining 50 seeds were submerged in 10 ml of distilled H_20 for 24 hours at room temperature (ca. 23°C) (Figure A-33). After 24 hours, an aliquot of water was removed from the test tubes for leachate measurement.

Inorganic leachates were measured using a conductivity meter (model number, RC 16B2, Industrial Instruments, Cedar Grove, USA) and recorded as umhos ml^{-1}. Organic leachates were measured at 280 nm.

Also, in many recalcitrant studies, leachates were measured from isolated axes or embryos, rather than whole seeds. To have a valid comparison, two additional experiments were performed. In the first, seed embryos were isolated after desiccation and immediately placed in distilled H_20 to determine leachate amounts, and in the second, fresh embryos were isolated before desiccation and immediately placed in H_20 to measure leachate amounts. The embryos were isolated with a razor blade, and every effort was made to avoid embryo damage during cutting. After drying, a moisture content test and germination test were performed on the embryos in the same fashion as the whole seeds.

Finally, to determine if leachate amounts differed in aged seeds, *S. alterniflora* seeds were dried to 8% moisture content (DWB) and stored in sealed zip lock bags for 10 and 16 months at 2°C. The seeds stored for 16 months were Vermillion accession harvested in 2004 from Ben-Hur Experimental Station, Baton Rouge, Louisiana, and the seeds stored for 10 months were wild type seeds harvested in 2005 from Port Fourchon, Louisiana.

Statistical Analysis

Each data point represents an independent sample. Data were plotted on a scatter plot and fitted with best fit regression. A p value of ≤ 0.05 indicated that the overall test for the regression model was significant.

RESULTS

Thiobarbituric Acid Reactive Substances (TBARS) Amounts in *Spartina* Seeds During Desiccation

Lipid peroxidation is a common, yet controversial, explanation for desiccation-induced death in recalcitrant seeds (Leprince and Golovina 2002). To re-examine this hypothesis, the TBARS method was used to measure lipid peroxidation in recalcitrant *S. alterniflora* seeds and desiccation-tolerant (orthodox) *S. pectinata* seeds as they were desiccated. When the extraction for TBARS was carried out at 4°C (Heath and Packer 1968; standard published procedure), TBARS amounts increased in both *Spartina* species as the seeds were dried. There was a significant correlation in *S. pectinata* (p value = 0.001), yet the increase in *S. alterniflora* was minimal, and the regression was not significantly different than zero (p value = 0.712) (Figures 3.1 and 3.2). However, when the seeds were freeze-clamped in liquid nitrogen before extraction, TBA-reactive substances were detected at constant, low amounts in both species (Figure 3.3 and 3.4). Using the Heath and Packer (1968) calculations, average TBARS amounts were higher in *S. alterniflora* than in *S. pectinata*. However, after application of the Hodges *et al.* (1999) correction factor, TBARS amounts were not different between the *Spartina* species. Dormant *S.alterniflora* (Figure 3.4) and *S. pectinata* seeds (Appendix Table A-28) that were freeze-clamped prior to extraction had very similar values to the non-dormant seeds.

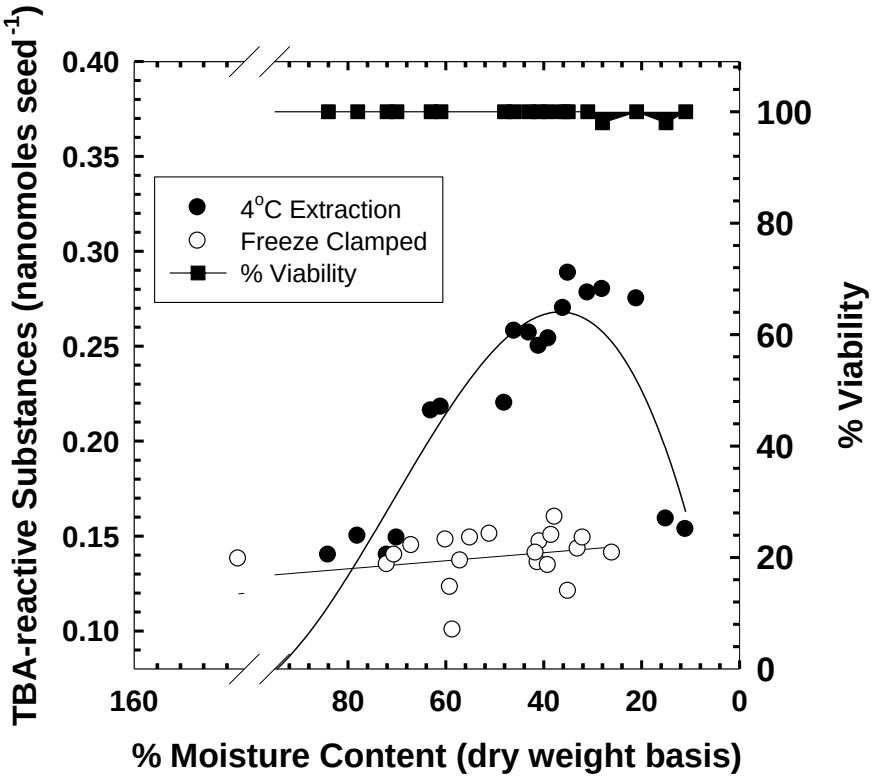

Figure 3.1. TBA-reactive substances in non-dormant *S. pectinata* seeds (WNS-03A, lot # 6042, Coaldale, CO) when the seeds were freeze- clamped in liquid N_2 before extraction (○) and when extraction was carried out at 4^0C (●). Percent viability of the seeds during drying is represented by the closed squares. TBARS calculations are uncorrected Heath and Packer (1968) values, and do not account for possible interferences. Absorbances were taken at 532 and 600 nm. Formula used to fit the unclamped data is $y = y_0+ax+bx^2+cx^3$. The r^2 for unclamped *S. pectinata* = 0.86 and the overall fit of the model was significant ($F = 34.1$ and $p = 0.001$).

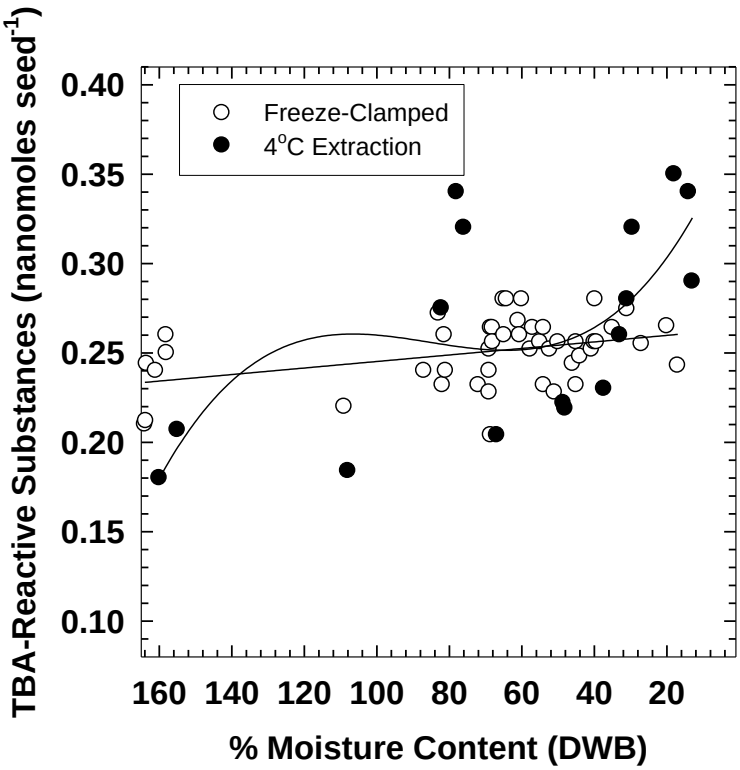

Figure 3.2. TBA-reactive substances (TBARS) in non-dormant *S. alterniflora* (Vermillion, 2004) when the seeds were freeze-clamped in liquid nitrogen before extraction (○), and when extraction was carried out at 4°C (●). TBARS calculations are uncorrected Heath and Packer (1968) values and do not account for possible interferences. Absorbances were taken at 532 and 600 nm. Regression equation used was, $y = y_0 + ax + bx^2 + cx^3$. The r^2 for unclamped TBARS was 0.42, and the curve was not significantly different from a slope of 0 ($F = 2.97$ and $p = 0.0712$).

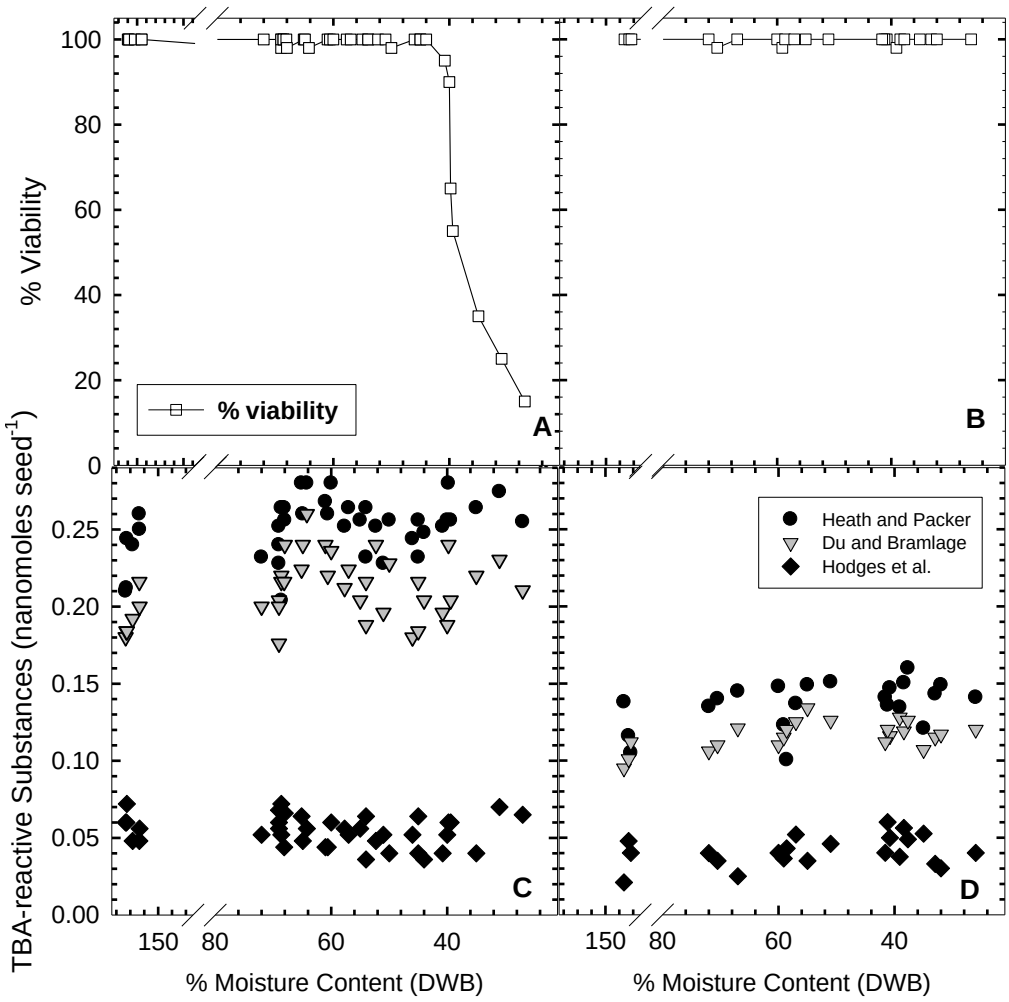

Figure 3.3. The effect of drying upon viability and TBARS values, calculated according to Heath and Packer (1968), Du and Bramlage (1992) and Hodges *et al.* (1999). (A) Viability of non-dormant *S. alterniflora* (Vermillion, 2004 harvest) shoots as the seeds were flash-dried; (B) viability of non-dormant *S. pectinata* seeds (WNS 03A, lot # 6042, Coaldale, CO) as they were dried. (C) TBARS amounts of non-dormant *S. alterniflora* seeds during flash-drying. Values calculated according to Heath and Packer (●), Du and Bramlage (▼), which corrects for sugars, and Hodges (♦), which corrects for sugars and other interferences. (D) TBARS amounts in non-dormant *S. pectinata* as the seeds are dried. The symbols are the same as panel C. All seeds were freeze-clamped with liquid nitrogen prior to extraction.

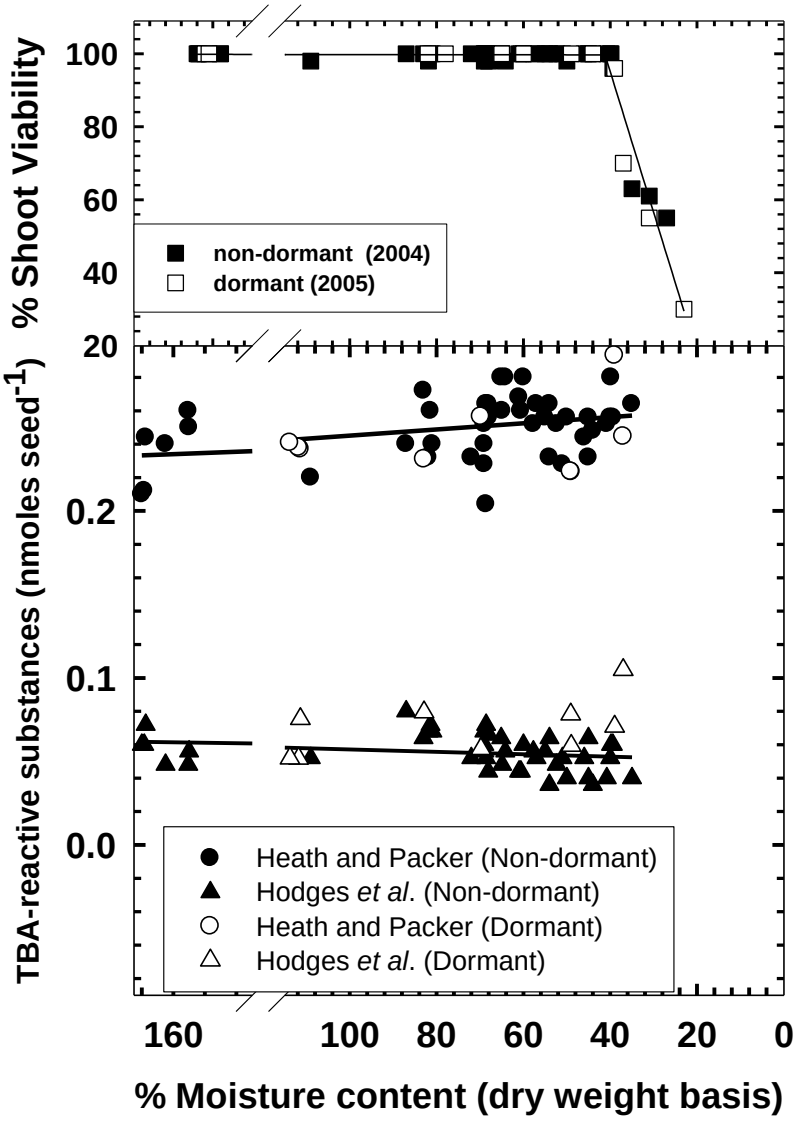

Figure 3.4. Comparison of TBARS values between dormant (Port Fourchon, 2005) and non-dormant *S. alterniflora* seeds (Vermillion, 2004). The upper panel represents the viability of dormant (□) and non-dormant (■) *S. alterniflora* seeds as they are flash-dried. The lower panel is TBARS amounts as dormant seeds are dried (open symbols) and TBARS amounts as non-dormant seeds are dried (closed symbols). Seeds were freeze-clamped in liquid nitrogen before extraction. TBARS amounts were calculated by the Heath and Packer (○, ●) method or using the Hodges correction (▲, △).

Ferrous Xylenol-Orange Assay (FOX) Products During the Desiccation of *Spartina* Seeds

The TBARS assay has known deficiencies, and while some of these deficiencies were accounted for in the present work, it was prudent to employ another lipid peroxidation assay to confirm the trends observed with the TBARS assay. The FOX method measures both hydrogen peroxide and lipid hydroperoxides, which are early indicators of lipid peroxidation and oxidative stress (Delong *et al.* 2002). A spike in FOX positive material occurred in both non-dormant *S. alterniflora* and *S. pectinata* seeds between the moisture contents of 85 and 60% (DWB); however, FOX positive substances remained at a low and constant value when dormant *S. alterniflora* and *S. pectinata* seeds were dried (Figure 3.5). The FOX spike in non-dormant *S. alterniflora* was observed in seeds from two different harvest years, with either Vermillion (Ben-Hur) or wild-type seeds (Port Fourchon), and seeds from two different locations (Figure 3.5A).

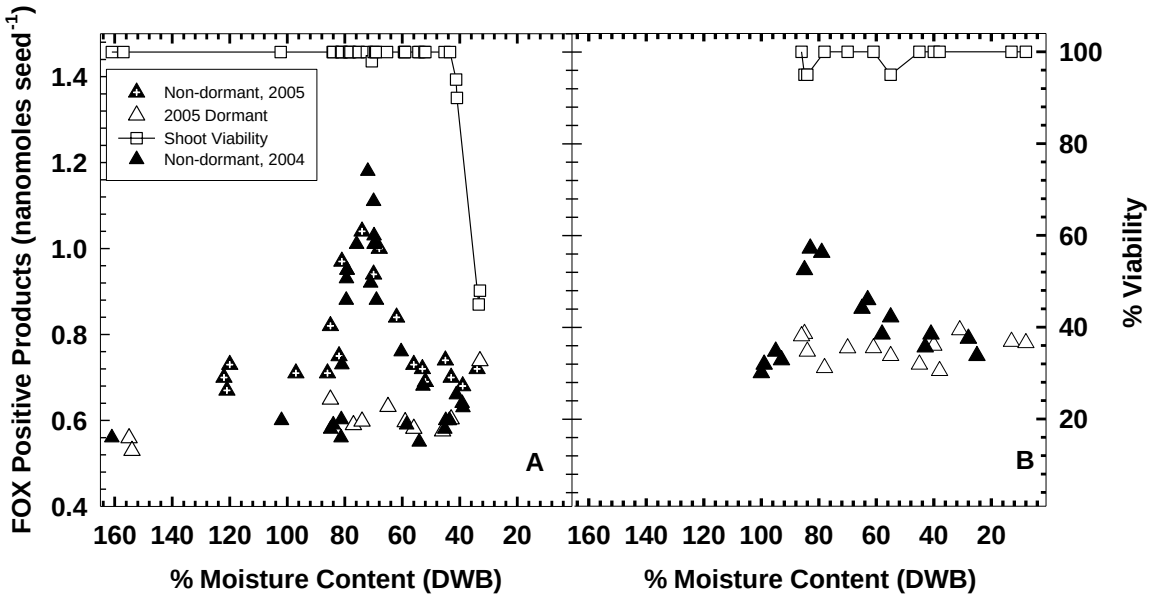

Figure 3.5. Ferrous xylenol orange (FOX) assay to measure hydrogen peroxide and lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. alterniflora* harvested in 2004 and 2005 from Ben-Hur Experimental Station, Baton Rouge, Louisiana and Port Fourchon, Louisiana, and (B) dormant and non-dormant *S. pectinata* seeds harvested in 2003 (purchased from Western Native Seed, Coaldale, USA, lot # 6042), and stored dry at -20°C until needed for an experiment, at which time they were brought to room temperature in sealed Ball jars and then submerged in H_20 and placed at 2°C. Dormant seeds for both species had ≤ 25% germination. Non-dormant seeds germinated 100%.

Organic and Inorganic Leachates As Indicators for Physical Membrane Damage During Desiccation of *Spartina* Seeds

Inorganic and organic leachates, which are used as indicators of membrane damage, did not increase in whole *Spartina alterniflora* (Figure 3.6) or *S. pectinata* (Figure 3.7) seeds during desiccation, and both species had similar leachate amounts in both dormant and non-dormant seeds. However, when *S. alterniflora* seeds were stored dry at 2°C, there was a substantial increase in both inorganic and organic leachates (Figure 3.8). Also, if embryos were isolated either before or after desiccation, there was a significant increase in both inorganic and organic leachates in both *S. alterniflora* and *S. pectinata* (Figures 3.9, 3.10).

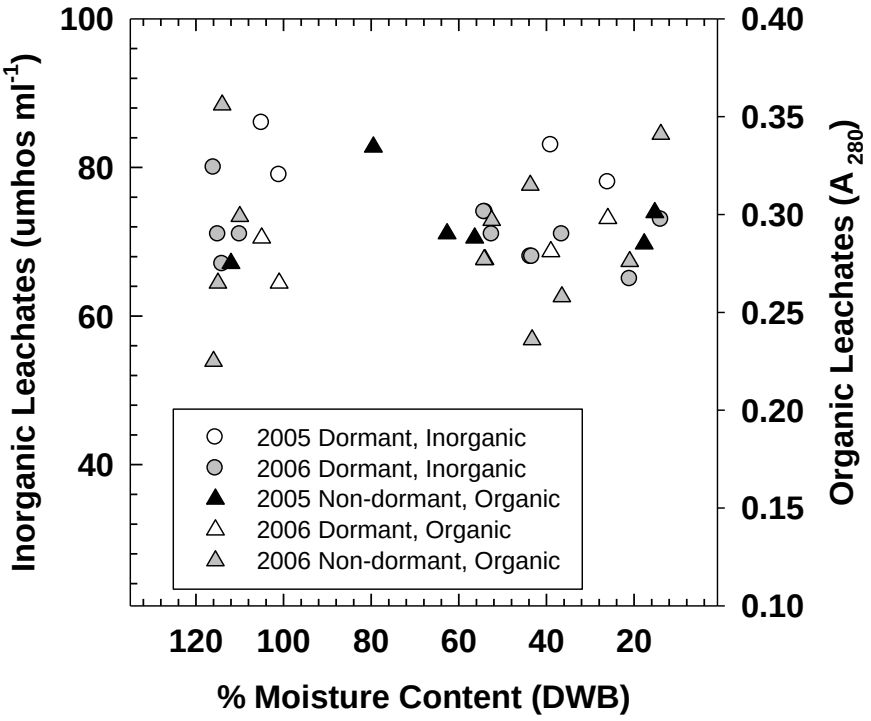

Figure 3.6. Organic and inorganic leachates amounts as *S. alterniflora* (Port Fourchon) seeds were desiccated. Leachates amounts were measured in both dormant (2006, △, ○) and non-dormant (2005, ▲, ●; 2006 ▲, ●) seeds. Measurements were taken after the seeds were imbibed in 10 ml of H_20 for 24 hours. Each value represents an independent sample.

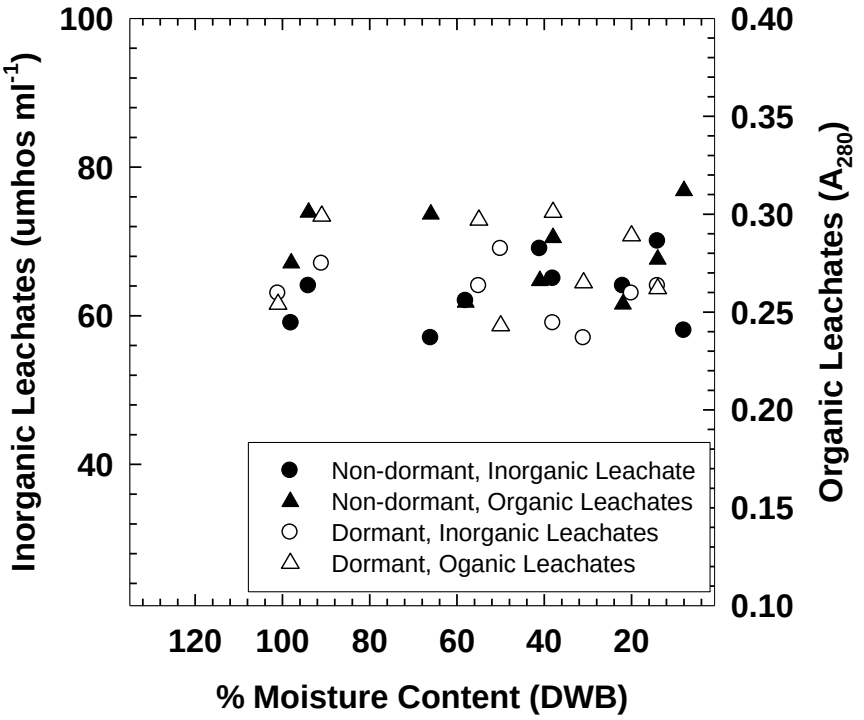

Figure 3.7. Organic and inorganic leachates amounts as *S. pectinata* seeds were desiccated. Leachate amounts from intact dormant (△, ○) and non-dormant (▲, ●) *S. pectinata* seeds during desiccation. Seeds were harvested in 2003 and purchased from Western Native Seeds (WNS, lot # 6042) (Coaldale, CO, USA.) Leachate amounts were recorded after 24 hours in 10 ml of H_20. Seeds were stored in sealed Ball jar at -20°C for 3 years prior to use. Each value represents an independent sample.

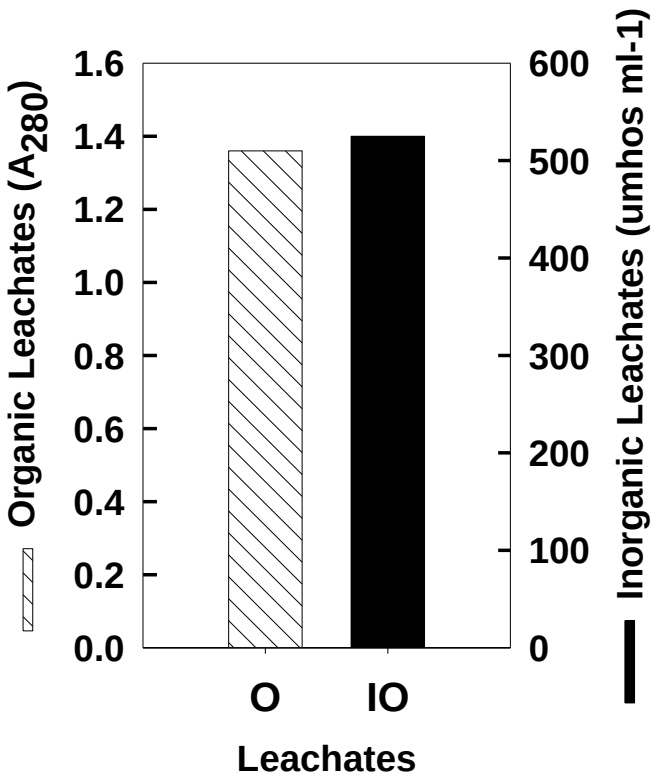

Figure 3.8. Inorganic (solid bar) and organic (hatched bar) leachates from the seeds of dried, aged *S. alterniflora* (Vermillion, 2004). Seeds were dried (8% MC, DWB) and stored at 2°C for an average of 10 months. This graph represents two independent leaching assays. Leachate measurements were recorded after seeds were incubated in 10 ml of distilled H_2O for 24 hours at room temperature (22-24°C).

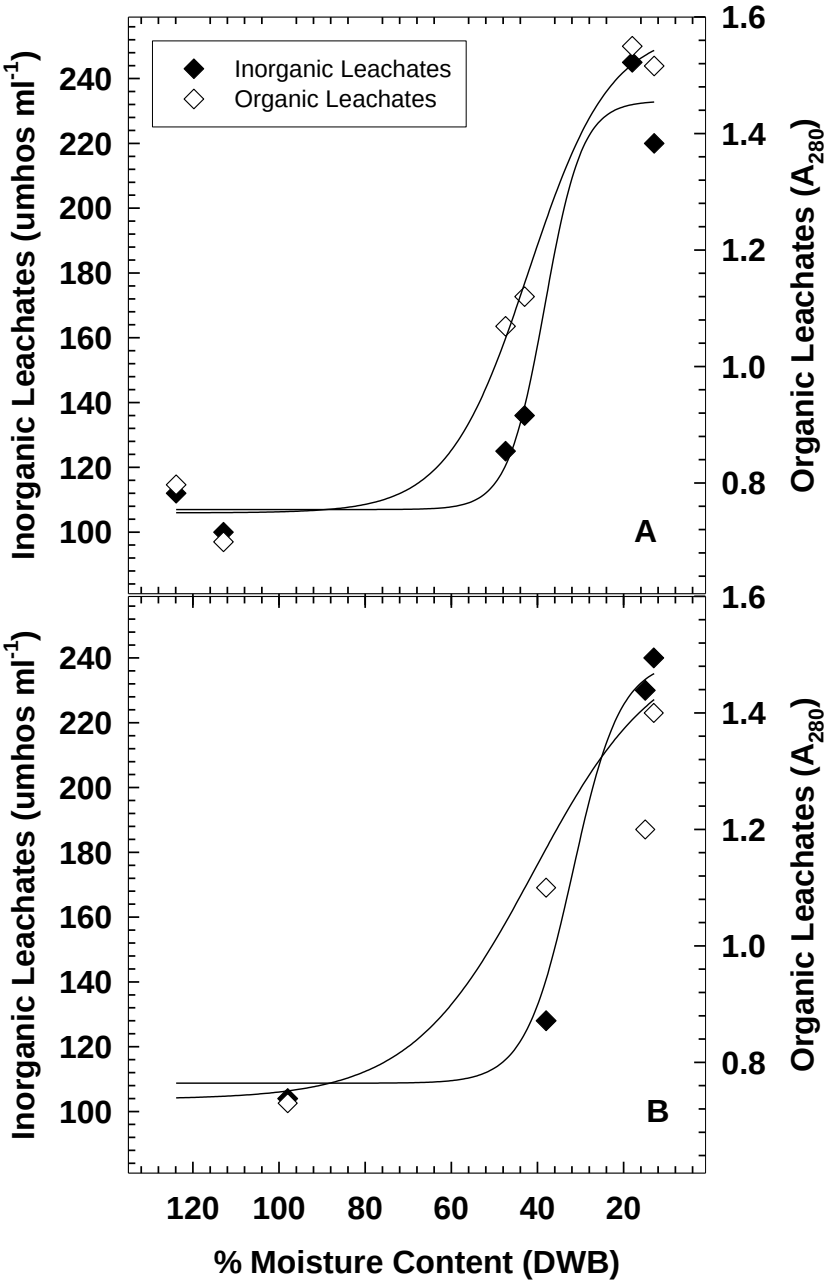

Figure 3.9. Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated after drying. (A) Inorganic (♦) and organic (◊) leachates from non-dormant *S. alterniflora* (Port Fourchon, 2006) and (B) *S. pectinata* (WNS, lot # 6042) embryos isolated after desiccation. Fifty seeds were placed in 10 ml of H_20. Leachate amounts were recorded after 24 hours at room temperature. Each value represents an independent sample. Embryos were isolated on a backlight with and razor blade. Every attempt was made to avoid embryo damage during the cutting. Regression equation used to fit data was, $y = y_0 + a/ 1+ e^{-(x - x0/b)}$. The p value was <0.01 for each curve.

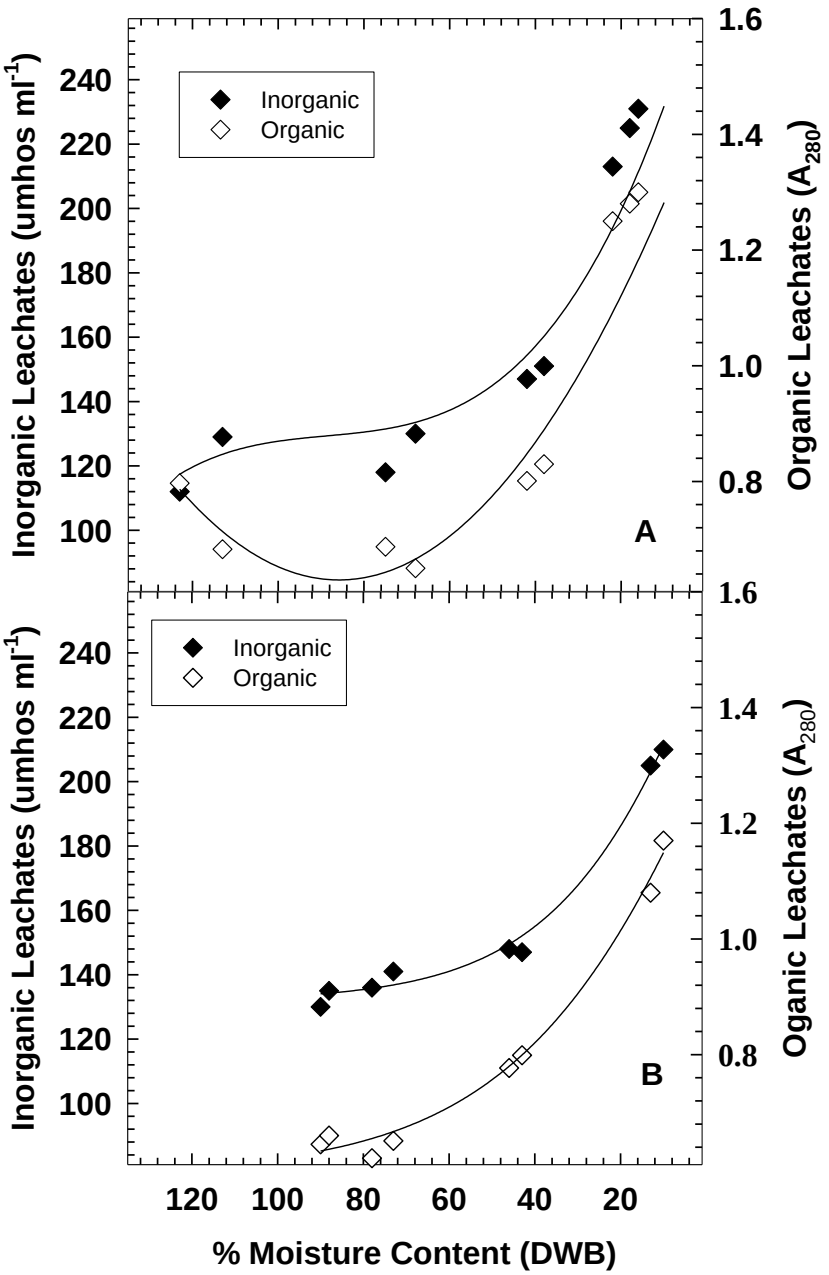

Figure 3.10. Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated before drying. (A) Inorganic (♦) and organic (◊) leachates from non-dormant *S. alterniflora* (Port Fourchon, 2006) and (B) *S. pectinata* (WNS, lot # 6042) embryos isolated before desiccation. Fifty seeds were placed in 10 ml of H_20 at room temperature. Leachate amounts were recorded after 24 hours. Each data point represents an independent sample. Embryos were isolated on a backlight with and razor blade. Every attempt was made to avoid embryo damage during the cutting. The data in the top panel was fitted with the equation, $y = y_0 + ax + bx^2 + cx^3$ and the bottom panel with, $y = y_0 + a/ 1+ e^{-(x - x0/b)}$. The p values for each curve were < 0.001.

DISCUSSION

Thiobarbituric Acid Reactive Substances (TBARS) Amounts in *Spartina* Seeds During Desiccation

The hypothesis that lipid peroxidation plays a role in desiccation-induced death is common, yet controversial. While some studies (Table 3.1) found increased TBARS amounts, it is still unclear if lipid peroxidation is associated with desiccation-induced death of recalcitrant seeds. Very few studies measured lipid peroxidation in closely related orthodox seeds (Greggains *et al*. 2001), so it is unclear if results found are common to all seeds, or are exclusive to recalcitrant seeds. Also, in many past studies (Table 3.1) seed extraction for TBARS has been carried out at 4°C. In the present study, extraction at 4°C caused increased TBARS in both *Spartina* species (Figures 3.1 and 3.2) with a substantial increase during drying of orthodox *S. pectinata*; however, when the seeds of either species were freeze-clamped before extraction, there was no increase in TBARS (Figures 3.3 and 3.4). This suggests that lipid peroxidation products were artifacts of extraction on ice, and not produced during seed drying, and therefore, are not an indicator of oxidative stress in recalcitrant seeds, nor a cause of their death. Also, it is known that several interfering substances can yield positive TBARS and inflate the values (Du and Bramlage 1992, Hodges *et al*. 1999). Corrections to the method have been devised to eliminate these artifacts, but very few seed studies employ them. In both *Spartina* species, TBARS was overestimated when the corrections were not employed (Figures 3.3 and 3.4). This suggests that if the corrections are not used, some TBA-reactive substances are not products of lipid peroxidation, but rather sugars and phenolics.

Also, from the TBARS data, the absorbance at 440 nm (Du and Bramlage 1992) suggests that *S. alterniflora* seeds have more sugars present than orthodox *S. pectinata* seeds. While only an indirect indicator of overall sugar concentration, this result is intriguing, as it is often

hypothesized that sugars may play a role in desiccation tolerance (e.g. Berjak and Pammenter, 2001). Sucrose and fructose reportedly have the highest absorbance in the presence of TBA (Du and Bramlage 1992), and sucrose is often associated with tolerance and fructose, which does not have the protective attributes of sucrose, may be linked to intolerance (Kermode and Finch-Savage 2002). It would be surprising to find that sucrose amounts are higher in *S. alterniflora*; however, high fructose amounts could indicate that *S. alterniflora* is lacking key sugar protectants (e.g. sucrose).

Ferrous Xylenol -Orange Assay (FOX) Products During the Desiccation of *Spartina* Seeds

No increase in FOX positive substances occurred during drying of dormant *S. alterniflora* and *S. pectinata* seeds. However, FOX products did increase when non-dormant *S. alterniflora* and *S. pectinata* seeds were moderately dried to 85% MC (DWB) (Figure 3.5). As the seeds were dried further, the FOX products disappeared. The FOX spike occurred in two different harvest lots of *S. alterniflora* from different years and locations, minimizing the chance that the spike was a one-time artifact of seed manipulation. Because the FOX spike occurs in both *S. pectinata* seeds and *S. alterniflora* seeds, and only in the non-dormant state, it does not appear to be related to seed death because both dormant and non-dormant *S. alterniflora* seeds are desiccation sensitive. The spike may be related to cellular signaling, rather than seed death. Lipid hydroperoxides are involved in many signaling pathways, such as stress responses, seed maturation and possibly germination (Loiseau *et al.* 2001, Trawatha *et al.* 1993). Because the spike was only present in non-dormant seeds, it may be a signal related to germination processes that began during storage at 2°C, rather than the drying process, which took 60 minutes in *S. alterniflora* and 5-10 minutes in *S. pectinata* for the moisture content to reach 85% MC (DWB). The less pronounced spike observed in *S. pectinata* may be related to the shorter time interval required for *S. pectinata* to traverse the 85 to 50% moisture content range, thus reducing the time

for hydroperoxides to accumulate and quenching them more rapidly. The transient FOX spike could be a lipid hydroperoxide by-product of the synthesis of jasmonic acid, which is a stress response hormone (Creelman and Mullet 1997). A jasmonic acid spike has been observed in both non-dormant, recalcitrant *Quercus robur* seeds just before they reached their critical moisture content (Finch-Savage *et al.* 1996), and also drought stressed *Carica papaya* seedlings (Mahouachi *et al*. 2007)

Finally, the FOX spike may be a seedling response, rather than a seed response. The FOX assays of non-dormant seeds were done after *Spartina* seeds had been stored for several months at 2°C, which is necessary to break dormancy. During this time, the seeds may have started the germination process and the caryopses may have started to elongate within the covering structures. If the germination process had started, the FOX spike may represent a pre-emergent seedling response to drying, rather than a seed response.

Organic and Inorganic Leachates as Indicators for Physical Membrane Damage During Desiccation of *Spartina* Seeds

Numerous studies with other recalcitrant species have observed dramatic increases in leachates as seeds were desiccated (Table 3.4). However, when whole *Spartina alterniflora* seeds were desiccated and assayed immediately after drying, there were no changes in leachates. However, if the embryo of either *Spartina* species was isolated before or after drying, there was a significant increase in leachate amounts. There are multiple explanations as to why an increase in leachates was observed in isolated embryos and not whole seeds. One explanation is that the intact covering structures (glumes and pericarp) prevent leakage to the outside media. Becwar *et al.* (1982) proposed this explanation when they observed no leakage with intact *A. saccharinum* seeds, and large increases once the testa was removed. This is a valid argument; however, the covering structures do not appear to be blocking leachate exchange, because massive leachate

amounts are observed in dried, dead and aged *S. alterniflora* seeds (Figure 3.8). Alternatively, by removing part of the seed (i.e. the pericarp), the dry down kinetics of the seed are changed. Possibly, when part of the seed is removed, and the embryo is exposed, deleterious events occur that would normally be buffered if all seeds parts were present. Also, regardless of the care taken, it is inevitable that live tissue will be cut as embryos are isolated. In dicots, cotyledon tissue must be cut to isolate the axes, and even in monocots, such as *S. alterniflora*, the aleurone layer must be cut to remove the endosperm. It is documented that wounding can create ROS (Salin and Bridges 1981). If even small amounts of ROS are produced during the removal of covering structures, a chain reaction might occur, causing significant damage. This damage may then be exacerbated by drying, causing an increase in leachates. Finally, if the embryo is isolated after drying, the seeds are much more brittle and more prone to crack when the embryo is dissected, thus causing increased leachates. Since increased embryo leachates were observed during drying of both recalcitrant and orthodox *Spartina* seeds, it is difficult to conclude that such leachate release is associated with recalcitrant seed death.

SUMMARY

From the results of the two lipid peroxidation assays and the leachate assay, it does not appear that lipid peroxidation and membrane damage are the causes of death when recalcitrant *S. alterniflora* seeds are desiccated. When increased damage products were observed, they appeared to be artifacts of the method (TBARS) or associated with the developmental status (non-dormant vs. dormant) (FOX) of the seed, rather than related to recalcitrant death. The results suggest the importance of (1) employing an orthodox species control; (2) evaluating seeds in the dormant and non-dormant state; and (3) careful comparisons when the physical

characteristics of seeds are altered (i.e. embryo isolation). The choice of model seed systems to study recalcitrant seed death will be limited by these constraints, especially since most recalcitrant species exist only in the non-dormant state (Farnsworth 2000).

CHAPTER 4
CHANGES IN PROTEIN CARBONYL CONTENT AND TOTAL ANTIOXIDANT CAPACITY IN *SPARTINA ALTERNIFLORA* AND *SPARTINA PECTINATA* SEEDS DURING DRYING

LITERATURE REVIEW

Does a Decrease in Total Antioxidant Capacity Cause Recalcitrance?

Antioxidant amounts measured in recalcitrant seed experiments have yielded inconsistent results (Table 4.1); it is unclear whether a reduction in antioxidants is a cause or effect of recalcitrant seed death, or if it is just a general desiccation response of all seeds. Measuring individual antioxidants can be very laborious and time consuming, and when only a few antioxidants are measured, only a narrow snapshot of the antioxidant activities within the seed can be obtained. In contrast, a total antioxidant response (TAR) experiment estimates the total antioxidant capacity (Erel 2004) of the seeds. Several methods exist to measure total antioxidant response (e.g. Miller and Rice-Evans 1996, Erel 2004), but all are based on a similar principle. The methods require the addition of the free radical $^{\cdot}OH$, which will oxidize another chemical added to the reaction. When this chemical is oxidized, it forms a color that can be measured spectrophotometrically. As tissue antioxidants quench $^{\cdot}OH$, less of the chemical will be oxidized; hence, less color will develop (Erel 2004). If a difference in absorbance occurs among the treatments, it suggests different total antioxidant capacities. The only published study of total antioxidants in seeds evaluated various genotypes of tomato, where higher lycopene amounts contributed to greater antioxidant capacity (Ramirez-Rosales *et al.* 2005). To date, no study has measured total antioxidants in recalcitrant seeds during desiccation. This chapter summarizes the total antioxidant response data for recalcitrant and orthodox *Spartina* species during seed desiccation.

Table 4.1 Antioxidant amounts as recalcitrant seeds and seed parts are desiccated

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25% viability	Reference
Tocopherol	*Avicennia marina*/ Plumule	10 ug g^{-1} dwt	16 ug g^{-1} dwt	30 ug g^{-1} dwt	Greggains *et al.* 2001
Tocopherol	*Avicennia marina*/ Cotyledons	4 ug^{-1} dwt	4 ug g^{-1} dwt	7 ug g^{-1} dwt	Greggains *et al.* 2001
Tocopherol	*Avicennia marina*/ Root primordia	16 ug^{-1} dwt	13 ug g^{-1} dwt	12 ug g^{-1} dwt	Greggains *et al.* 2001
Tocopherol	*Avicennia marina*/ Hypocotyls	12 ug^{-1} dwt	11 ug^{-1} dwt	10 ug^{-1} dwt	Greggains et al 2001
Tocopherol	*Araucaria bidwillii*/ Embryo	100 ug g^{-1} dwt	100 ug g^{-1} dwt	*******	Francini *et al.* 2006
Tocopherol	*Araucaria bidwillii*/ Endosperm	130 ug g^{-1} dwt	125 ug g^{-1} dwt	*******	Francini *et al.* 2006
Tocopherol	*Acer pseudoplatanus*/ Whole seeds	3 ug g^{-1} dwt	10 ug g^{-1} dwt	12 ug g^{-1} dwt	Greggains *et al.* 2000
Tocopherol	*Quercus robur*/ Axes	80 ug g^{-1} dwt	60 ug g^{-1} dwt	35 ug g^{-1} dwt	Hendry *et al.* 1992
Tocopherol	*Quercus robur*/ Cotyledons	15 ug g^{-1} dwt	18 ug g^{-1} dwt	20 ug g^{-1} dwt	Hendry *et al.* 1992
SOD	*Avicennia marina*/ Plumue	23 umol min^{-1} g^{-1} dwt	19 umol min^{-1} g^{-1} dwt	30 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001
SOD	*Avicennia marina*/ Cotyledons	6 umol min^{-1} g^{-1} dwt	4 umol min^{-1} g^{-1} dwt	4 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001

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Table 4.1. Continued

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25% viability	Reference
SOD	*Avicennia marina*/ Root primordia	12 umol min^{-1} g^{-1} dwt	13 umol min^{-1} g^{-1} dwt	15 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001
SOD	*Araucaria bidwillii*/ Embryo	310 U mg^{-1} protein	350 U mg^{-1} protein	*******	Francini *et al.* 2006
SOD	*Araucaria bidwillii*/ Endosperm	275 U mg^{-1} protein	175 U mg^{-1} protein	*******	Francini *et al.* 2006
SOD	*Shorea roubsta*/ Axes	0.0 U g^{-1} g fwt	0.0 U g^{-1} fwt	0.0 U g^{-1} fwt*	Chaitanya and Naithani 1994
SOD	*Theobroma cacao*/ Axes	260 U mg^{-1} protein	230 U mg^{-1} protein	220 U mg^{-1} protein	Li and Sun 1999
SOD	*Theobroma cacao*/ Cotyledons	300 U mg^{-1} protein	250 U mg^{-1} protein	210 U mg^{-1} protein	Li and Sun.1999
SOD	*Acer pseudoplatanus*/ Whole seeds	33 umol min^{-1} g^{-1} dwt	38 umol min^{-1} g^{-1} dwt	31 umol min01 g^{-1} dwt	Greggains *et al.* 2000
SOD	*Trichilia dregeana*/ Axes	13 U mg^{-1} protein	10 U mg^{-1} protein	9 U mg^{-1} protein	Song *et al.* 2004
SOD	*Quercus robur*/ Axes	80 U mg^{-1} protein	60 U mg^{-1} protein	67 U mg^{-1} protein	Hendry *et al.* 1992
SOD	*Quercus robur*/ Cotyledons	190 U mg^{-1} protein	150 U mg^{-1} protein	300 U mg^{-1} protein	Hendry *et al.* 1992
SOD	*Azadirachta indica*/ Axes	15 U g^{-1} protein	18 U g^{-1} protein	20 U g^{-1} protein	Varghese and Naithani 2002
SOD	*Azadirachta indica*/ Axes	1.9 U g^{-1} protein	2.5 U g^{-1} protein	6 U g^{-1} protein	Varghese and Naithani 2002

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Table 4.1. Continued.

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25% viability	Reference
Catalase	*Azadirachta indica*/ Axes	0.08 nmol H202 min^{-1} g^{-1} protein	0.33 nmol H202 min^{-1} g^{-1} protein	0.18 nmol H202 min^{-1} g^{-1} protein	Varghese and Naithani2002
Catalase	*Trichilia dregeana*/ Axes	15 umol H202 mg^{-1} protein min^{-1}	32 umol H202 mg^{-1} protein min^{-1}	35 umol H202 mg^{-1} protein min^{-1}	Song *et al.* 2004
GR	*Quercus robur*/ Axes	12 nmol min^{-1} mg^{-1} protein	7 nmol min^{-1} mg^{-1} protein	11 nmol min^{-1} mg protein	Hendry *et al.* 1992
GR	*Quercus robur*/ Cotyledons	17 nmol min^{-1} mg^{-1} protein	19 nmol min^{-1} mg^{-1} protein	35 nmol min^{-1} mg^{-1} protein	Hendry *et al.* 1992
GR	*Trichilia dregeana*/ Axes	30 nmol NADH mg^{-1} protein min^{-1}	37 nmol NADH mg^{-1} protein min^{-1}	48 nmol NADH mg^{-1} protein min^{-1}	Song *et al.* 2004
GR	*Araucaria marina*/ Embryo	0.26 U mg^{-1} protein	0.1 U mg^{-1} protein	*******	Francini *et al.* 2006
GR	*Araucaria marina*/ Endosperm	0.2 U mg^{-1} protein	0.2 U mg^{-1} protein	*******	Francini *et al.* 2006
GR	*Acer pseudoplatanus*/ Axes	1000 nmol NADPH min^{-1} mg^{-1} protein	1900 nmol NADPH min^{-1} mg^{-1} protein	2000 nmol NADPH min^{-1} mg^{-1} protein	Pucacka and Ratajczak 2007
GR	*Acer pseudoplatanus*/ Cotyledons	510 nmol NADPH min^{-1} mg^{-1} protein	700 nmol NADPH min^{-1} mg^{-1} protein	775 nmol NADPH min^{-1} mg^{-1} protein	Pucacka and Ratajczak 2007
GPOD	*Avicennia marina*/ Plumule	1 umol min^{-1} g^{-1} dwt	5 umol min^{-1} g^{-1} dwt	5 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001
GPOD	*Avicennia marina*/ Cotyledons	2 umol min^{-1} g^{-1} dwt	5 umol min^{-1} g^{-1} dwt	5 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001

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Table 4.1. Continued.

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25 viability	Reference
GPOD	*Avicennia marina*/ Root primorida	210 umol min^{-1} g^{-1} dwt	220 umol min^{-1} g^{-1} dwt	130 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001
GPOD	*Avicennia marina*/ Hypocotyls	0 umol $min^{-1}g^{-1}$ dwt	0 umol min^{-1} g^{-1} dwt	0 umol min^{-1} g^{-1} dwt	Greggains *et al.* 2001
Glutathione	*Acer saccharinum*/ Axes	2400 umol g^{-1} dwt	2000 umol g^{-1} dwt	1500 umol g^{-1} dwt	Pukacka and Ratajczak 2006
Glutathione	*Acer saccharinum*/ Cotyledons	0 umol g^{-1} dwt	1000 umol g^{-1} dwt	950 umol g^{-1} dwt	Pukacka and Ratajczak 2006
Glutathione	*Acer pseudoplatanus*/ Whole seed	6 umol g^{-1} dwt	5 umol g^{-1} dwt	6 umol g^{-1} dwt	Greggains *et al.*2000
Glutathione[%]	*Azadirachta indica*/ Embryo-Harvested in Ouaga	1.5 umol g^{-1} dwt	0.9 umol g^{-1} dwt	0.4 umol g^{-1} dwt	Sacande *et al.* 2000
Glutathione[%]	*Azadirachta indica*/ Embryo-Harvested in Bobo	0.25 umol g^{-1} dwt	0.65 umol g^{-1} dwt	0.25 umol g^{-1} dwt	Sacande *et al.* 2000
Glutathione	*Acer pseudoplatanus*/ Axes	1.0 nmole $axis^{-1}$	2.0 nmole $axis^{-1}$	1.0 nmole $axis^{-1}$	Pukacka and Ratajczak 2007
Glutathione	*Acer pseudoplatanus*/ cotyledons	13 nmole $cotyledon^{-1}$	30 nmole $cotyledon^{-1}$	9 nmole $cotyledone^{-1}$	Pukackaand Ratajczak 2007
Ascorbate	*Acer saccharinum*/ Axes	23 umoles g^{-1} dwt	37 umol min^{-1} dwt	19 umoles g^{-1} dwt	Pukacka and Ratajczak 2006
Ascorbate	*Acer saccharinum*/ Cotyledons	6 umoles g^{-1} dwt	5.5 umol g^{-1} dwt	2.8 umol g^{-1} dwt	Pukacka and Ratajczak 2006

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Table 4.1. Continued

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25 viability	Reference
Ascorbate	*Araucaria bidwillii*/ Embryo	90 umol g^{-1} dwt	145 umol g^{-1} dwt	******	Francini *et al.* 2006
Ascorbate	*Araucaria bidwillii*/ Endosperm	14 umol g^{-1} dwt	12 umol g^{-1} dwt	******	Francini *et al.* 2006
Ascorbate	*Quercus robur*/ Axes	210 nmol g^{-1} dwt	450 umol g^{-1} dwt	410 umol g^{-1} dwt	Hendry *et al.* 1992
Ascorbate	*Quercus robur*/ Cotyledons	80 nmol g^{-1} dwt	23 umol g^{-1} dwt	13 umol g^{-1} dwt	Hendry *et al.* 1992
Ascorbate	*Acer pseudoplatanus*/ Axes	20.0 nmole $axes^{-1}$	17.0 nmole $axes^{-1}$	20.0 nmole $axes^{-1}$	Pucacka and Ratajczak 2007
Ascorbate	*Acer pseudoplatanus*/ Cotyledons	125 nmole $cotyledon^{-1}$	110 nmole $cotyledon^{-1}$	120 nmole $cotyledon^{-1}$	Pucacka and Ratajczak 2007
APX	*Araucaria bidwillii*/ Embryo	2.7 U mg^{-1} protein	5.7 U mg^{-1} protein	*******	Francini *et al.*2006
APX	*Araucaria bidwillii*/ Endosperm	1.0 U mg^{-1} protein	1.0 U mg^{-1} protein	*******	Francini *et al.* 2006
APX	*Theobroma cacao*/ Axes	110 nmol mg^{-1} protein min^{-1}	90 nmol mg^{-1} protein min^{1}	80 nmol mg^{-1} protein min^{-1}	Li and Sun 1999
APX	*Theobroma cacao*/ Cotyledons	0 nmol mg^{-1} protein min^{-1}	0 nmol mg^{-1} protein min^{-1}	0 nmol mg^{-1} protein min^{-1}	Li and Sun 1999
APX	*Acer pseudoplatanus*/ Whole seeds	190 umol min^{-1} g^{-1} dwt	135 umol min^{-1} g^{-1} dwt	145 umol min^{-1} g^{-1} dwt g^{-1}	Greggains *et al.* 2000

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Table 4.1. Continued

Antioxidant	Species	Amount before drying	Amount at critical moisture content	Amount at 25 viability	Reference
APX	*Quercus robur*/ Axes	7.0 nmol min^{-1} mg^{-1} protein	2.5 nmol min^{-1} mg^{-1} protein	1.5 nmol min^{-1} mg^{-1} protein	Hendry *et al.* 1992
APX	*Quercus robur*/ Cotyledons	0.0 nmol min^{-1} mg^{-1} protein	0.0 nmol min^{-1} mg^{-1} protein	0.0 nmol min^{-1} mg^{-1} protein	Hendry *et al.* 1992
APX	*Trichilia dregeana*/ Axes	255 nmol mg^{-1} protein min^{-1}	225 nmol mg^{-1} ^{1} protein min^{-}	180 nmol mg^{-1} protein $^{-1}$	Song *et al*. 2004
APX	*Acer pseudoplatanus*/ Axes	75 nmol min^{-1} mg^{-1} $protein^{-1}$	190 nmol min^{-1} mg $protein^{-1}$	200 nmol min^{-1} $protein^{-1}$	Puckackaand Ratajczak 2007
APX	*Acer pseudoplatanus*/ Cotyledons	50 nmol min^{-1} mg^{-1} $protein^{-1}$	90 nmol min^{-1} mg^{-1} $protein^{-1}$	100 nmol min^{-1} mg^{-1} $protein^{-1}$	Puckackaand Ratajczak 2007
Peroxidase	*Theobroma cacao*/ Axes	455 nmol mg^{-1} protein min^{-1}	430 nmol mg^{-1} $protein^{-1}$	330 nmol mg^{-1} $protein^{-1}$	Li and Sun 1999
Peroxidase	*Theobroma cacao*/ Cotyledons	3800 nmol mg^{-1} protein min^{-1}	1200 nmol mg^{-1} $protein^{-1}$	600 nmol mg^{-1} protein	Li and Sun 1999
Peroxidase	*Telfairia occidentalis*/ Cotyledons	328.7 umol product 1^{-1} min^{-1}	1205 umol product 1^{-1} min^{-1}	876.4 umol product 1^{-1} min	Nkang *et al.* 2000

% -This study was more of an aging study, rather than desiccation tolerance. Abbreviation codes, SOD, superoxide dismutase; GR, glutathione reductase; GPOD, glutathione peroxidase; APX, ascorbate peroxidase

Does Protein Oxidation (Carbonyl Content) Increase During Desiccation of Recalcitrant Seeds?

Oxidation of proteins can lead to loss of protein function, increased susceptibility to proteolytic degradation, unfolding and protein aggregation (Grune *et al.* 1997, Butterfield *et al.* 2006). Oxidized proteins can form several products, such as methionine sulfoxides, glutamic semialdehydes, and carbonyls (Requena *et al.* 2003). Increased protein oxidation has been observed in degenerative diseases and as humans age (Berlett and Stadtman 1997). Oxidized proteins also are correlated with age and death in other species, such as yeast, insects and mammals (Levine and Stadtman 2001, Dukan and Nystrom 1998 and Nystrom 2002). If protein oxidation is prevented, life-span is increased in *Musca domestica* (houseflies) (Sohal 2002). Protein carbonyls are irreversible products of protein oxidation, and are commonly used as an overall indicator of protein oxidation (Job *et al.* 2005) When orthodox soybeans were desiccated, there was a slight decrease in protein carbonyls; however, when rapidly aged, there was no increase in protein carbonyls in the axes or cotyledons of soybeans (Sun and Leopold 1995). Also, Job *et al.* (2005) observed increased protein carbonyls as non-dormant *Arabidopsis thaliana* seeds were imbibed at the start of the germination process. It was proposed that the proteins oxidized were storage proteins, which were being marked for degradation during germination. In *Arabidopsis thaliana* plants, carbonylated proteins increased initially as the plants matured; however, the carbonylated proteins then decreased prior to bolting and flowering (Johansson *et al.* 2004). The protein carbonyl amounts were also not correlated with leaf senescence, which suggests that protein oxidation is not correlated with death in plants, unlike other organisms, such as yeast and bacteria. No studies have measured protein oxidation amounts as recalcitrant seeds are dried.

METHODS

Storage of *Spartina spartinae* and Maturing *Spartina pectinata*

Spartina spartinae seeds were harvested from Port Fourchon, Louisiana in 2007 by clipping spikes at different visually assessed maturation stages. Upon collection, the spikes were immediately placed in sealed Hefty™ freezer bags (Pactiv, Lake Forest, USA) for transportation to the laboratory. After the seed spikes were transported approximately 3 hours in the freezer bags, the bags were freeze-clamped in liquid nitrogen, placed in a larger freezer bag and stored at -80^{o}C. Upon use, a 50 ml plastic centrifuge tube was filled with liquid nitrogen and buried in dry ice. The spikes were removed from -80°C and plunged into the tube, which contained liquid nitrogen. The seeds were then stripped by hand from the spikes and quickly evaluated using a backlight. Filled seeds were selected and placed in a mortar filled with liquid nitrogen to avoid thawing during the counting process.

S. pectinata spikes were harvested at Brookings, South Dakota in 2007 by Dr. Jose Gonzalez, from plants that were at different maturation stages. Upon collection, the spikes were immediately freeze-clamped and stored at -80^{o}C for less than one week. The spikes were then placed on dry ice and shipped over night to Louisiana State University. Upon arrival, the spikes were separated visually by maturation stage, freeze-clamped in liquid nitrogen, placed in freezer bags and stored at -80^{o}C. Upon use, a 50 ml centrifuge tube was filled with liquid nitrogen and buried in dry ice. The spikes were removed from -80°C and plunged into the tube, which contained liquid nitrogen. The seeds were then stripped from the spikes and placed on a backlight. Filled seeds were selected and placed in a mortar filled with liquid nitrogen to avoid thawing during the counting process.

Total Water Soluble Antioxidant Capacity (TAR) of *S. alterniflora* and *S. pectinata* Seeds As They Are Dried (Method from Erel 2004)

After flash-drying, 50 seeds were freeze-clamped in liquid nitrogen and ground to a powder in a mortar and pestle buried in dry ice. The seed powder was then transferred to a Tenbroek tube embedded in ice, which contained 5 ml of ice-cold ethanol: water (80:20 v/v). Each sample was extracted with exactly 20 full strokes of the glass pestle to ensure complete and uniform homogenization. The extract was then centrifuged at 4°C for 10 minutes at 12,000g. After centrifugation, the supernatant was collected and added to 250 ul of reagent 1, followed by the addition of 50 ul of reagent 2, which starts the Fenton reaction. The reagents for the experiment were prepared as follows:

Reagent one was made up of 5.6 g of KCl dissolved in 1000 ml of distilled water. Reagent grade HCl (6.4 ml, 36.5%) was diluted with 1000 ml of dH_20. KCl (800 ml) solution was mixed with 200 ml of the HCl solution, and the final pH was 1.8. Reagent grade ortho-dianisidine dihydrochloride (3.17g) was dissolved in the KCl-HCl solution. Finally, 0.0176 g of Fe $(NH_4)_2(SO_4)_2 \cdot 6H_20$ was dissolved in the KCl-HCl solution. Reagent 1 can be stored at 4°C for several months (Erel 2004). Reagent 2 consisted of 0.641 ml of 35% H_20_2 solution diluted to 1000 ml with reagent 1. This reagent was made up fresh daily. As mentioned in Erel (2004), it is important to use only high grade orthodianisidine dihydrochloride (product # D3252, Sigma-Aldrich, St. Louis, USA) characterized by a lack of color, as lesser grades will distort results.

The schematics of the reaction are as follows (Erel 2004): Reagent 1 (Fe_2+-o-dianisidine complex) + reagent 2 (H_20_2) → Fe_3+-o-diansidine + $^{\bullet}OH$ → dianisidyl radical (causes bright yellow-brown color development) dianisidyl radical → further oxidation reactions of dianisidyl radical (darkening of color).

To determine the total antioxidant amounts in the sample, 250 ul of the sample extract (Figure A-36) were added to 1 ml of reagent 1, followed by 50 ul of reagent 2. The samples were briefly vortexed and incubated in a 23°C water bath for 15 minutes (Figure A-35). Dianisidyl formation was measured with a spectrophotometer at 444 nm. The assay was calibrated against an aqueous Trolox standard (soluble form of vitamin E) (Figure A-34). The results are expressed as percent suppression of dianisidyl radical formation.

Protein Carbonyl Assay (Method from Reznick and Packer 1994)

Fifty seeds were freeze-clamped in liquid nitrogen and ground to a fine powder in a mortar and pestle buried in dry ice. The powder was then homogenized in a 15 ml Tenbroek tissue homogenizer, which contained 5 ml of ice-cold homogenizing buffer (50 mM KH_2P0_4, pH 7.4, plus 1mM EDTA, 0.1% digitonin and 5 ul of a protease inhibitor cocktail) (Sigma, St Louis, USA, product # P9599). Each sample was extracted with exactly 20 full strokes of the glass pestle to ensure complete and uniform homogenization. The extract was then transferred to a 15 ml Corex test tube and centrifuged at 4°C for 10 minutes at 12,000 g. The supernatant was transferred to a clean Corex test tube, and 10% streptomycin sulfate (w/v) solution was added to give a final concentration of 1%. The solution was vortexed (ca. 3 seconds) and left at room temperature (ca. 23°C) for 15 minutes to precipitate nucleic acids. To fully remove nucleic acids, the extract was centrifuged at 4°C for 10 minutes at 12,000 g. One milliliter of the supernatant was then added to either 4 ml of 10 mM dinitrophenyhydrazine (DNPH) in 2.5 M hydrochloride (HCl), or 4 ml of 2.5 M HCl, which was used as control. The tubes were vortexed and placed at room temperature in the dark for 1 hour, and were vortexed every 15 minutes. The protein was precipitated from the solution by adding 5 ml of ice-cold 20% TCA. The solutions were incubated on ice for 10 minutes and centrifuged at 4°C for 10 minutes at 10,000 g. The supernatant was discarded, 10% TCA was added, and the protein pellet was dispersed with a

glass rod to remove any possible contaminants. The solution was centrifuged (4°C) for 5 minutes at 10,000 g, and the supernatant was discarded. On ice, the pellet was then washed 3 times with 4 ml of ice-cold 1:1 ethyl acetate: ethanol mixture to remove excess free DNPH and non-protein carbonyls. To re-solubolize the protein pellet, 2 ml 6 M guanidine hydrochloride was added, and the solution was incubated at 37°C for 10 minutes. The absorbance of both the DNPH treated sample and HCl control was taken at 370 nm to measure DNPH absorbance and 280 nm to determine total protein amounts. The assay was calibrated against a DNPH standard, which has a molar absortivity of 22,000 M^{-1} cm^{-1} (Figure A-40) and a bovine serum albumin (BSA) standard to determine protein extract amounts (Figure A-39). The total protein carbonyl value was calculated with the following equation:

$(A_{370}) / 22{,}000\ M^{-1}\ cm^{-1}\ (10^6) = \text{nmoles ml}^{-1}$

RESULTS

Total Water Soluble Antioxidant Response

To determine if the antioxidant capacity changed as *S. alterniflora* seeds were dried, a total antioxidant capacity assay was performed (Erel 2004). When both dormant and non-dormant *S. alterniflora* seeds were flash-dried, their antioxidant capacity decreased from 30% suppression to <15% in desiccated seeds. This trend was consistent with two different harvest years (2005 and 2006) (Figure 4.1A). Antioxidant capacity also decreased in desiccation tolerant *S. pectinata* and *S. spartinae* seeds during the initial desiccation of the seeds during maturation in the field (Figures 4.2A and 4.2B). However, when mature *S. pectinata* seeds were rehydrated and dried again, the decrease in antioxidant capacity was irreversible, and the TAR values remained at the low values (Figure 4.1B).

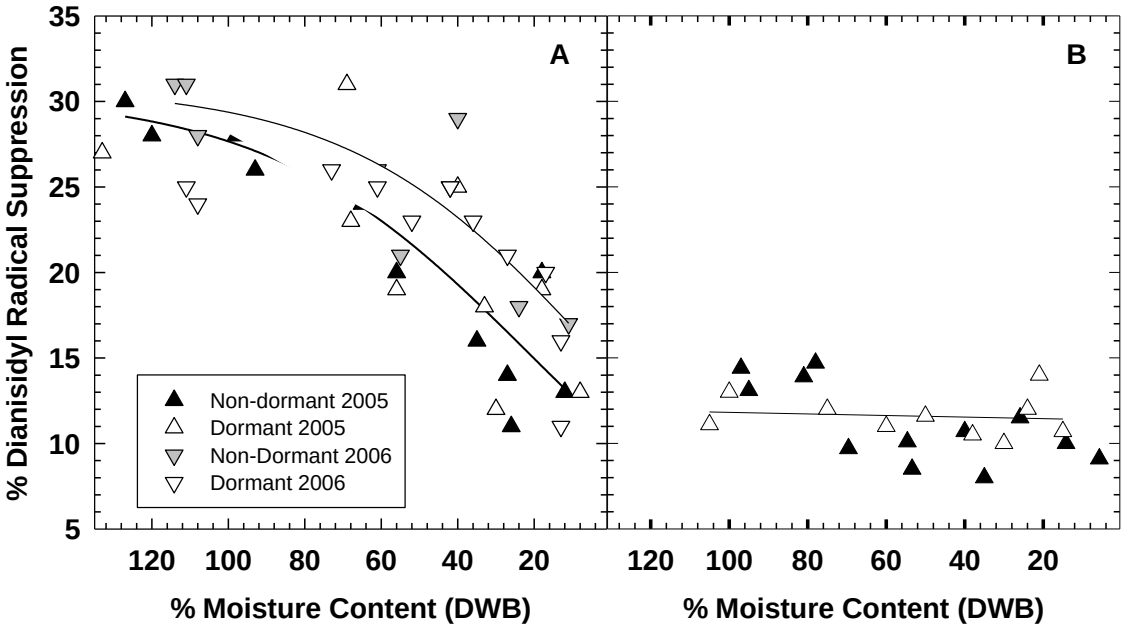

Figure 4.1. Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. (A) dormant and non-dormant *S. alterniflora* seeds harvested from Port Fourchon, Louisiana in 2005 (non-dormant, ▲; and dormant,△) and 2006 (non-dormant, ▼; dormant, ▽) and (B) dormant (△) and non-dormant(▲) mature *S. pectinata* seeds purchased from Western Native Seed Company, Coaldale Colorado (lot # 6042). The *S. pectinata* seeds are orthodox and underwent their first desiccation on the mother plant. The seeds were received dry (8% MC, DWB) and were rehydrated by submerged storage. Dormant seeds used had <25% germination and non-dormant seeds were 100% non-dormant. Absorbance for the antioxidant assay was taken at 444 nm after 15 minutes of reaction time at 23°C. For clarity, non-linear regression for one year's harvest (2005 harvest) is shown. Both years for *S. alterniflora* had similar trends and the p value was < 0.04 for all regression lines.

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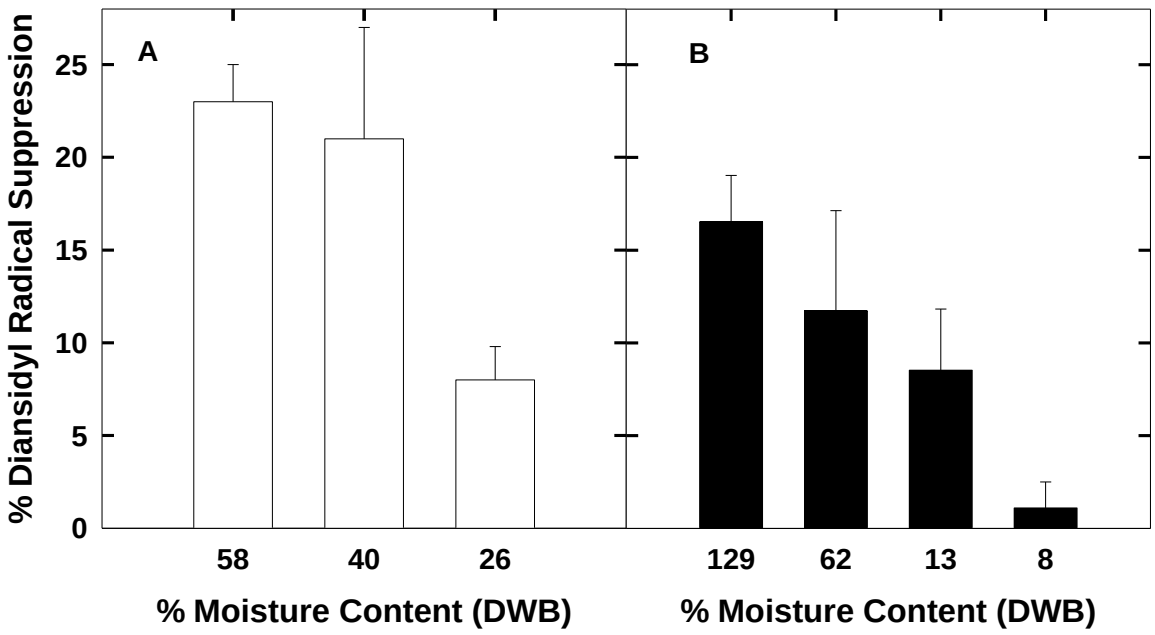

Figure 4.2. Total water-soluble antioxidant amounts in maturing (A) *S. pectinata* and (B) *S. spartinae*. *Spartina pectinata* seeds were harvested at different stages of maturation drying in the field. *Spartina pectinata* seeds were harvested from Brookings, South Dakota (2007) and *S. spartinae* seeds were harvested from Port Fourchon, Louisiana (2007). The *S. pectinata* and *S. spartinae* seeds were flash-frozen in liquid N_2 after collection, separated into maturation stages while on dry ice, flash frozen again in liquid N_2 and stored at -80^{o}C. Each mean represents 3 independent determinations. Absorbance was taken at 444 nm after a 15 minute reaction at 23°C. Error bars ± SE.

Protein Oxidation (Carbonylation) During Desiccation

During drying of *S. alterniflora*, carbonyl content increased significantly, and began before the seeds reached the critical moisture content of 45% (DWB). The carbonyl content increased in both dormant and non-dormant seeds from around 1.5 nanomoles mg^{-1} protein in hydrated seeds, to >5 nanomoles of carbonyls mg^{-1} protein in desiccated seeds. This trend was consistent with two different seed harvests from 2005 and 2006 (Figure 4.3A). When protein carbonyls were measured during the first dry down of *S. pectinata* seeds maturing in the field (maturation dry), there was no change in protein oxidation amounts, with an average carbonyl amount of 4.5 nanomoles of protein carbonyls mg^{-1} protein (Figure 4.4A). Similar results were obtained for *S. pectinata* seeds that had been rehydrated and desiccated again (Figure 4.3B).

When the orthodox *S. spartinae* seeds that were maturing in the field to various water contents were assayed, protein carbonyls increased from 7 to 12 nanomoles of carbonyls mg^{-1} protein (Figure 4.4B) as the seeds matured and water content decreased. When protein carbonyl data were corrected for the changing amounts of total protein, which increased during maturation (Figure A-41), both maturing *S. pectinata* and *S. spartinae* had increased protein carbonyls (per 50 seeds) as the seeds dried on the plant (Figure 4.4C and D).

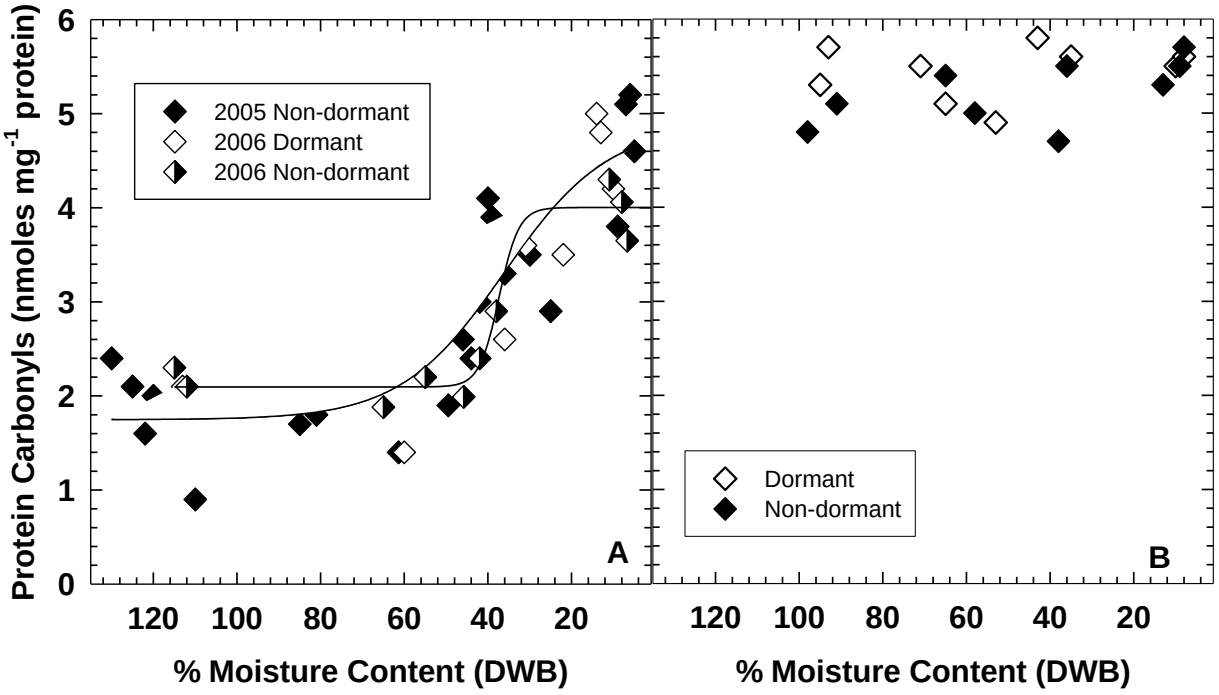

Figure 4.3 Protein carbonyl amounts in (A) *S. alterniflora* and (B) *S. pectinata* seeds during desiccation. Protein carbonyl amounts were determined for dormant *Spartina alterniflora* seeds (open symbols) harvested from Port Fourchon, Louisiana in 2006 (open diamonds), and non-dormant *S. alterniflora* seeds (closed symbols), harvested from Port Fourchon in 2005 (closed diamonds) and 2006 (half-shaded diamonds). Both dormant and non-dormant seeds were stored submerged at 2°C; dormant seeds for less than 2 months and non-dormant seeds for 5-8 months. Carbonyl amounts were also determined in dormant (open diamonds) and non-dormant (closed diamonds) mature *S. pectinata* (Lot 6042) seeds that were rehydrated at 2°C and subsequently rapidly dried. Regression equation = $y = y_0 + a/ 1+ e^{-(x - x_0/b)}$. For clarity, only 2006 dormant and non-dormant regression lines are shown. 2005 non-dormant seeds have an almost identical regression line as 2006 dormant seeds. All p values for non-linear regression of *S. alterniflora* are ≤ 0.002, indicating the overall fit of the model is significant.

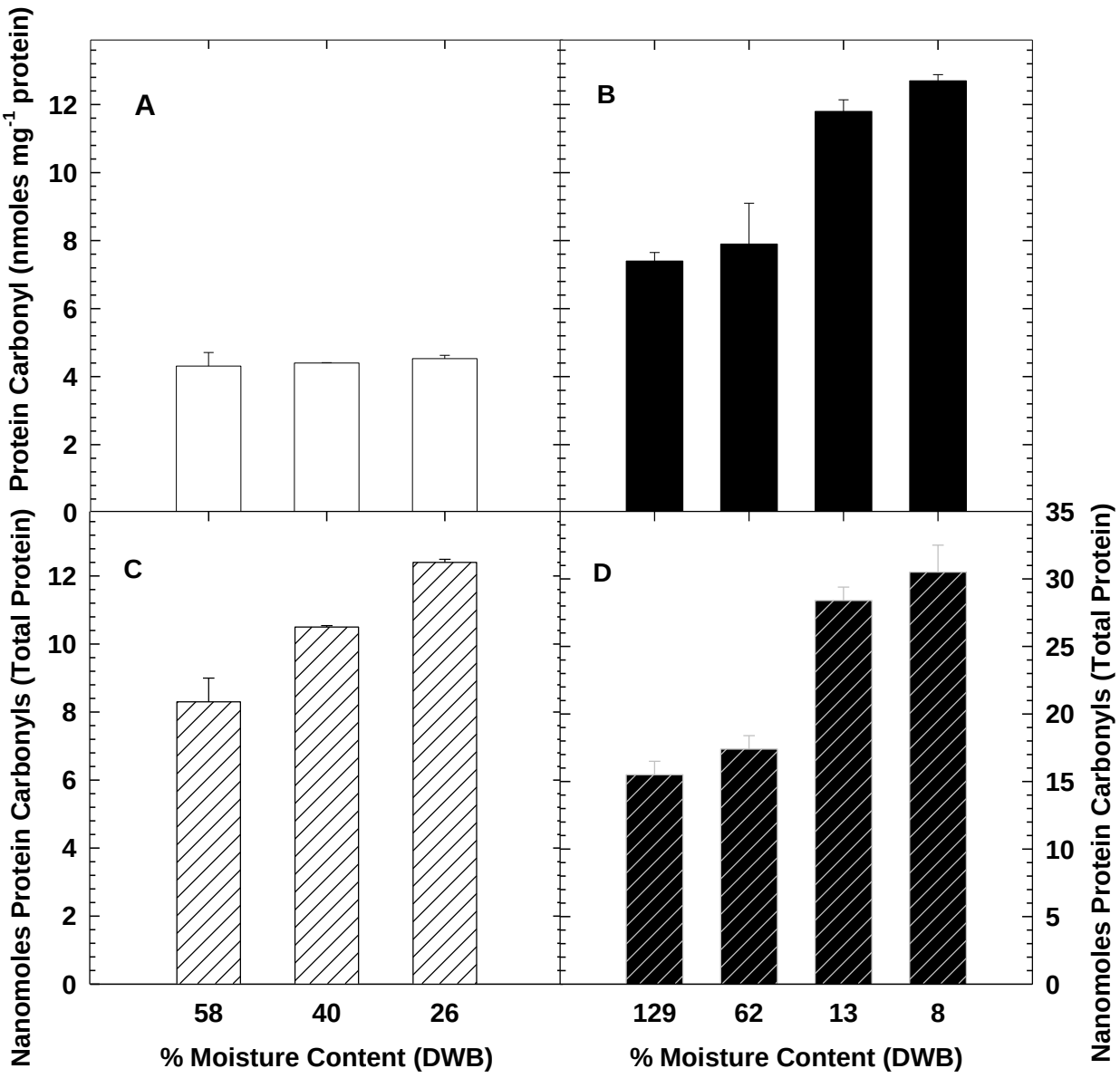

Figure 4.4. Protein carbonyl amounts during the first dry down (maturation drying) of (A) *S. pectinata* (South Dakota, 2007 harvest) (B) *Spartinae spartinae* (Port Fourchon, 2007). (C) The data from panel A were expressed as nanomoles of carbonyls in the total amount of protein extracted from *S. pectinata* (South Dakota, 2007); (D) data from panel B, expressed as nanomoles of carbonyls from the total protein extracted from the 50 seeds. N**ote that the scale of the y axis in panel D is different than panels A, B and C.** The *S. pectinata* and *S. spartinae* seeds were flash-frozen in liquid N_2 after collection, separated into maturation stages while on dry ice, flash frozen again in liquid N_2 and stored at -80^{o}C. Error bars = SE.

DISCUSSION

Total Water Soluble Antioxidant Response

A lack of antioxidants in recalcitrant seeds has been cited as a possible cause for desiccation-induced death (Hoekstra *et al.* 2001). Many antioxidants have been measured as recalcitrant seeds are dried (Table 4.1); however, because of inconsistent results, it is still unclear if antioxidant deficiencies contribute to death when recalcitrant seeds are desiccated. In the present study, the antioxidant capacity decreased as both dormant and non-dormant *S. alterniflora* seeds were desiccated (Figure 4.1A), and the trends were consistent in dormant and non-dormant seeds across harvest years (Figure A-37). This suggests that water-soluble, non-enzymatic antioxidants were exhausted as the seeds were desiccated. When mature *S. pectinata* seeds purchased from Western Native Seeds were assayed, the baseline TAR was low, similar to the values observed in desiccated *S. alterniflora*. Because the purchased *S. pectinata* seeds had already dried down on the mother plant prior to harvest, and were rehydrated in the lab, it is possible that the antioxidants were exhausted during normal maturation drying in the field, and the antioxidant capacity is irreversible. To address the question, *S. pectinata* seeds were obtained at different stages of drying on the mother plant, and these seeds had a decrease in antioxidant capacity similar to what was observed in recalcitrant *S. alterniflora* seeds. This suggests that the decline in antioxidants is merely a response to the desiccation during seed maturation and is not related to recalcitrance. This is consistent with the finding by Puckacka and Ratajczak (2007), who observed a decrease in several antioxidants (e.g. ascorbate peroxidase, glutathione reductase) in both recalcitrant *Acer platanoides* and orthodox *Acer pseudoplatanus* seeds during their initial desiccation. This finding demonstrates the importance of not only having an orthodox control, but also having the control in the same physiological state as the recalcitrant seeds.

Protein Oxidation (Carbonylation) During Desiccation

A significant increase in protein carbonylation occurred as *S. alterniflora* was dried, and the increase began before the seeds reached the critical moisture content (Figure 4.4A). As the antioxidant capacity decreased (Figure 4.1), protein carbonyls increased. Protein carbonyls increased in both *S. alterniflora* and *S. pectinata* if the results are expressed as total protein carbonyls from 50 seeds (Figure 4.4C). However, there was no significant change in protein carbonyls in the initial or second dry down of *S. pectinata* if data were expressed on a carbonyl mg^{-1} protein basis (Figures 4.4B and 4.5A). The lack of difference in *S. pectinata* if the results are expressed on a mg^{-1} protein basis could signal a difference between orthodox and recalcitrant seeds; however, there are caveats that temper a comparison between the initial dry down of *S. pectinata* and *S. alterniflora*. First, there are only 3 moisture contents stages of *S. pectinata* to measure, ranging from a moisture content (DWB) of 56 % to 26%; because of this narrow range, the protein carbonyl increase may have already occurred while the plants were still on the mother plant. Secondly, the *S. pectinata* seeds harvested at different dry down stages had to be frozen in liquid nitrogen and shipped on dry ice to stop biochemical reactions. The moisture contents of the *S. pectinata* seeds were taken after shipping and arrival in Baton Rouge; therefore, it may not be valid to compare the moisture content of those seeds to the moisture contents of *S. alterniflora*, which were not freeze-clamped prior to determination of moisture contents. However, the similar carbonyl amounts between the South Dakota seeds, which were stored at -80°C, and the purchased seeds from Western Native Seeds suggest that storage at -80°C did not affect carbonyl amounts. Thirdly, while *S. pectinata* and *S. alterniflora* are closely related (Baumel *et al*. 2002), they grow in drastically different environments. The natural habitat of *Spartina alterniflora* is coastal salt-marshes, whereas *S. pectinata* is most commonly in prairies. This difference in climates may cause differences unrelated to recalcitrance. Finally, *Spartina*

pectinata seeds are undergoing numerous other physiological processes during normal maturation drying, e.g. reserve deposition and induction of dormancy that are already completed at the stage when *S. alterniflora* was harvested, and therefore strict comparisons with *S. alterniflora* may not be possible. Nevertheless, it is curious that TAR comparisons between the two species showed consistent trends, in contrast to the protein carbonyl data. Since this issue cannot be resolved directly, seed protein carbonyls were assayed in another orthodox species, *Spartina spartinae*, which grows in the same environment as *S. alterniflora.* In *S. spartinae*, the protein carbonyls increased significantly during maturation drying on the mother plant. This suggests that when plants are grown in the same environment, protein carbonylation increases regardless of desiccation tolerance.

Another possibility for the differences could be the proteins oxidized in *S. alterniflora* are different from those carbonylated in *S. pectinata*. It is possible that *S. alterniflora* is missing chaperone proteins (e.g. heat shock proteins), which often protect other proteins, membranes, and DNA (Buitink *et al.* 2002). If chaperone proteins are missing in *S. alterniflora*, vital proteins needed for function may be damaged during desiccation, eventually leading to death. However, if protection proteins are present in the orthodox seeds, they may act as a shield for the vital proteins.

CHAPTER 5
DNA FRAGMENTATION DURING DRYING OF *SPARTINA ALTERNIFLORA* AND *SPARTINA PECTINATA* SEEDS

LITERATURE REVIEW

Does DNA Fragmentation Occur As *S. alterniflora* Seeds Are Dried?

When cells are damaged, DNA can be fragmented into smaller pieces. This fragmentation is usually caused by extensive base oxidation or apoptosis. DNA fragmentation has been observed extensively in humans and other mammals that are aged or under a physiological stress (Friedberg *et al*. 1995). DNA fragmentation has also been observed in seeds and seedlings. For example, in *Medicago truncatula* seedlings, DNA fragmentation was observed if the seedlings were dried at a desiccation-intolerant stage of development (Faria *et al.* 2005). Conversely, if the seedlings were treated with polyethylene glycol before drying, which induces desiccation tolerance in the seedlings, no DNA fragmentation was observed. This result suggests that DNA fragmentation may play a role in desiccation intolerance of *Medicago truncatula* seedlings. However, the DNA pattern was only measured in viable and dead seedlings, so it is unclear if the fragmentation is a cause of death or merely a result. Also, as pea seeds lost viability during aging, there was continuous increase in DNA fragmentation (Kranner *et al.* 2006). These previous studies measured DNA damage, but did not examine whether the DNA damage was prevented in healthy stages, or if the DNA was more efficiently repaired at those stages. Osborne and Boubriak (1994) examined DNA damage induced by UV light in barley seeds, and found that DNA was repaired in a matter of hours during the desiccation tolerant phase of germination. However, in intolerant stages, the barley was unable to repair DNA fragmented by UV light, suggesting that DNA was damaged in all stages, but in tolerant stages, efficient repair was able to occur. Also, Boubriak *et al.* (2000) found that fully hydrated *Avicennia marina* hypocotyl tips could efficiently repair irradiated DNA. When hypocotyl tips were desiccated 22% or more, the

DNA was unable to be repaired back to high molecular weight DNA. No survival data were given, so it is unclear if the seeds desiccated past 22% are alive or dead. It would be expected that if the tissue is dead, DNA repair would not take place.

DNA fragmentation has never been measured as recalcitrant seeds are dried, and it is unclear if it occurs, or plays a role in recalcitrant seed death.

METHODS

Genomic DNA Fragmentation (Method from Faria *et al.* 2005)

To determine if DNA fragmentation occurred as dormant and non-dormant *Spartina alterniflora* (Port Fourchon, 2006) and non-dormant *S. pectinata* (lot # 6042), seeds were flash-dried, 50 seeds were freeze-clamped in liquid nitrogen and ground to a fine powder in a mortar embedded in ice. The powder was weighed quickly, and 50 mg was transferred to a 1.5 ml sterile plastic Eppendorf tube. The genomic DNA was then extracted using the Invitrogen Chargeswitch® DNA kit (Invitrogen, Carlsbad, CA, USA) for plants, according to the manufacturer's instructions (www.invitrogen.com/content/sfs/manuals/chargeswitch_plant_man.pdf). After extraction, the DNA concentration was measured with a spectrophotometer at 260 nm, and to ensure minimal protein contamination, a 260/280 nm ratio was calculated. The DNA was then either used immediately or stored at -80°C. Two micrograms of the DNA, plus 6X blue/orange loading dye (Promega, Madison, USA) were loaded onto a 0.8% agarose gel made from 0.5X TBE (0.89 M Tris, 0.89 M boric acid, 0.02 M sodium EDTA) buffer, and electrophoresed at 40 volts for 6 hours (Faria *et al.* 2005) at room temperature A 1kb marker from Invitrogen was used as a molecular marker for all gels. Following electrophoresis, the gel was stained with 0.5 mg/ml of ethidium bromide for 30 minutes, followed by de-staining in ddH_20 for 20 minutes. The gel was

then visualized and photographed in a Biorad Gel Doc 1000 UV box and imaged with Molecular Analyst software (Biorad Laboratories, Hercules, USA).

In addition to flash-dried seeds, DNA was extracted from aged wild type *S. alterniflora* seeds (Port Fourchon, 2005) stored in a sealed freezer bag at 2°C for 10 months. DNA was extracted from the aged seeds and treated in the same manner as detailed above.

RESULTS

DNA Fragmentation

When both dormant (Figure 5.1) and non-dormant (Figure 5.2) *Spartina alterniflora* seeds were desiccated, there was no visible fragmentation, even in seeds desiccated to <15% MC (DWB).

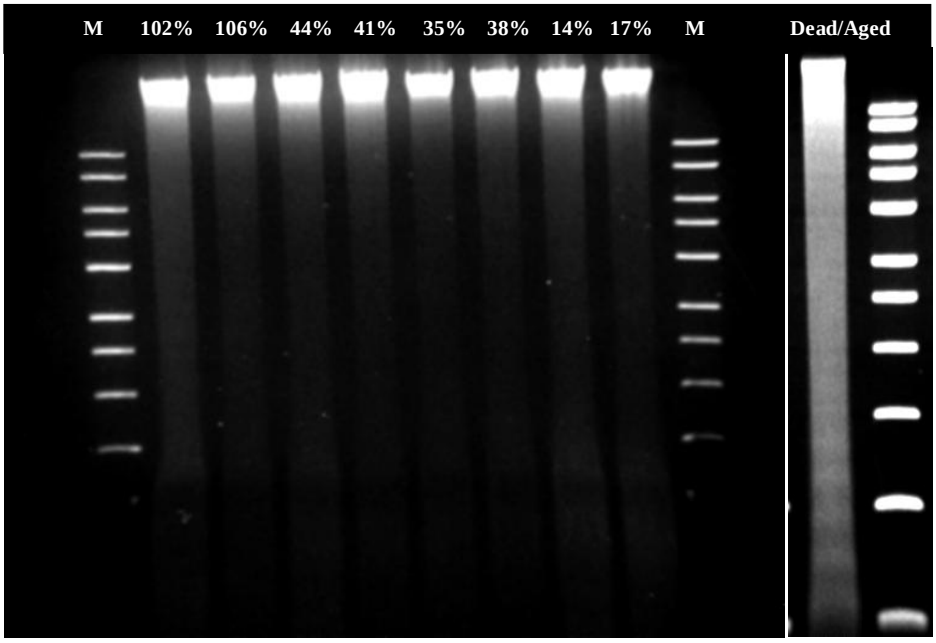

Figure 5.1. DNA fragmentation during desiccation of dormant *S. alterniflora* seeds. Genomic DNA was extracted from dormant *S. alterniflora* seeds (Port Fourchon, 2006) and desiccated to various moisture contents. DNA (1.8 ug per lane) was loaded onto a 0.8% agarose gel. The gel was run at 40 V for approximately 6 hours. DNA was stained with ethidium bromide. Lane 1- 1kb marker; 2. Fully hydrated; 3. Fully hydrated; 4. 44% MC; 5. 41% MC; 6. 35% MC; 7. 38% MC; 8. 14% MC; 9. 17% MC; 10. 1kb marker; Lane 11 – DNA from dried seeds aged and stored at 2°C for 10 months; Lane 12 – 1 kb marker. Two independent experiments were performed with identical results.

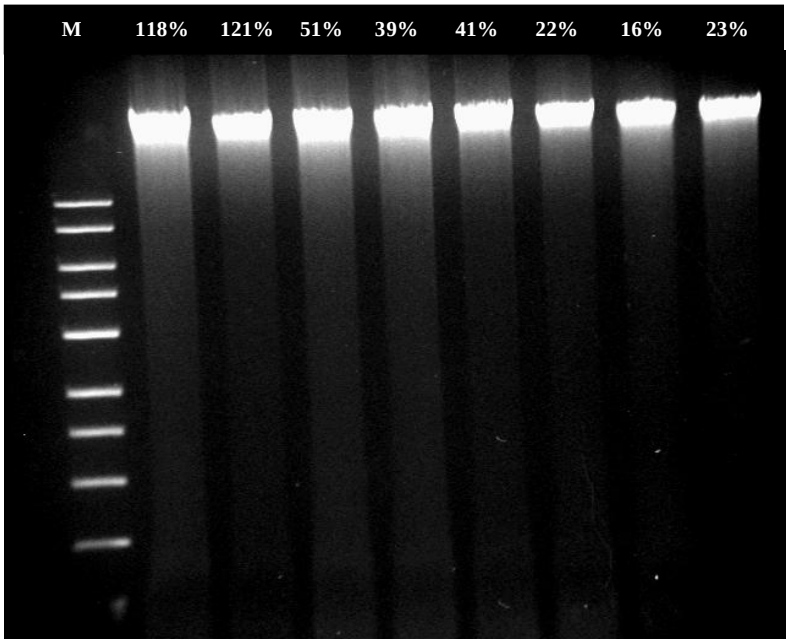

Figure 5.2. DNA fragmentation during desiccation of non-dormant *S. alterniflora* seeds. Genomic DNA was extracted from non-dormant *S. alterniflora* seeds (Port Fourchon, 2006) desiccated to various moisture contents. DNA (1.8 ug per lane) was loaded onto a 0.8% agarose gel. The gel was run at 40 V for approximately 6 hours. DNA was stained with ethidium bromide. Lane 1-1kb marker; 2. Fully hydrated; 3. Fully hydrated; 4. 51% MC; 5. 39% MC; 6. 41% MC; 7. 22% MC; 8. 16% MC; 9. 23% MC. Two independent experiments were performed with identical results.

Additionally, there was no fragmentation in desiccation-tolerant *S. pectinata* seeds (Figure 5.3). However, when *S. alterniflora* seeds were flash-dried and stored dry at 2°C for 10 months, there was massive DNA fragmentation (Figure 5.1), which appeared as a smudge of DNA on the gel, rather than the distinct bands observed in the unaged seeds.

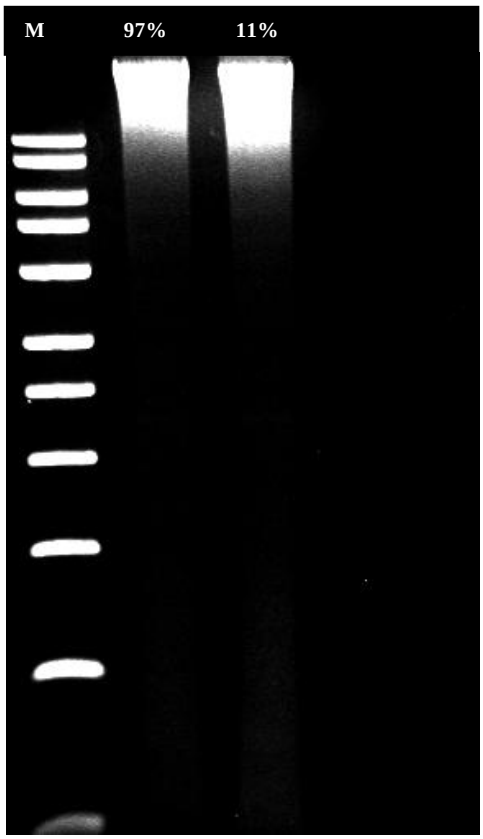

Figure 5.3. DNA fragmentation in non-dormant, fully hydrated and dry *S. pectinata* (WNS-03A, Coaldale, USA, lot 6042) seeds. DNA (1.8 ug per lane) was loaded onto a 0.8% agarose gel. Lane 1 is a 1 kb molecular marker; 2. fully hydrated; 3. 11% MC. The gel was run at 40 volts for 6 hours and stained with 0.5 ug/ml of ethidium bromide. Two independent experiments were performed with identical results.

DISCUSSION

DNA Fragmentation

No visible DNA fragmentation was observed as dormant and non-dormant *S. alterniflora* seeds were desiccated (Figures 5.1 and 5.2). This finding, along with the reports of DNA

fragmentation in aged seeds and desiccated seedlings, suggests that death by drying in recalcitrant seeds is different from death in dried seedlings and aged seeds. This is significant because this means that desiccated seedlings should not be used as an analogy for death in recalcitrant seeds. The fragmentation observed in the *Medicago* seedlings (Faria *et al.* 2005) may be a result of desiccation damage, but also could be from DNA fragmentation that can occur as seeds begin to germinate normally (Wang *et al.* 1998). The *Spartina* data also serve as a caution against slow drying (days to weeks) of seeds, where effects of desiccation can be confounded by seed aging (Figure 5.2). DNA fragmentation has been observed in artificially aged seeds of *Pisum sativum* (Kranner *et al.* 2006). With slowly dried seeds, results that appear related to recalcitrant death may actually be an aging process or a post-mortem event.

CHAPTER 6
GENERAL CONCLUSIONS AND PROSPECTS FOR FURTHER RESEARCH

The cause of desiccation-induced recalcitrant seed death is still unknown. It has been proposed that death may be caused by a build up of reactive oxygen species during desiccation (reviewed in Pammenter and Berjak 2008). However, the data in the present study suggest that oxidative stress is not the cause of desiccation induced death in recalcitrant *S. alterniflora* seeds. There is no visible membrane damage as evidenced by the TBARS assay and leachate measurements when whole seeds are dried (Figures 3.3 and 3.6). Also, there is no change in FOX products when dormant seeds are measured; however, the spike in FOX products occurs in both non-dormant *S. alterniflora* and *S. pectinata* early in the drying process (Figure 3.5). There is a decrease in water-soluble antioxidant capacity, and an increase in protein carbonylation as *S. alterniflora* seeds are dried; however, because the same changes can be observed in the initial dry down of orthodox *S. pectinata* and *S. spartinae* (Figure 4.4), these phenomena appear to be a response to initial desiccation *per se*, rather than related to recalcitrant seed death. Also, these processes do not seem to be reversible, even in orthodox seeds, which makes it imperative to not only have an orthodox control, but also use a control in the proper physiological state (i.e. never experienced desiccation) as the recalcitrant seed. As illustrated by Figure 5.4, the absence of an appropriate physiological context would lead an investigator to a totally misleading conclusion. Without the appropriate orthodox control seeds in a comparable physiological state, and without the ability to compare dormant versus non-dormant seeds of a recalcitrant species, an excellent, but incorrect interpretation of the results would be produced.

Without the beneficial controls provided by the *Spartina* system, it would have been easy to wrongly conclude that oxidative stress played a significant role in recalcitrant seed death. Unfortunately, the experimental design issues raised by the present study substantially compromise a large body of existing data concerning the possible causes of recalcitrant seed death.

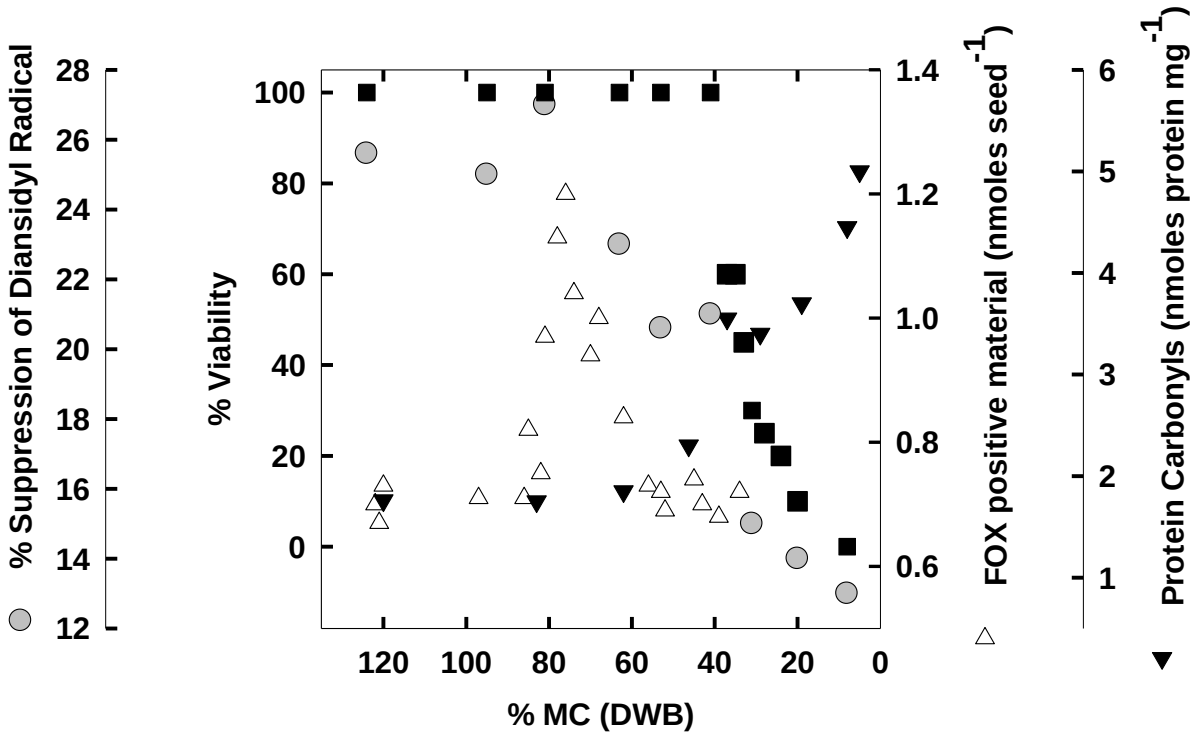

Figure. 6.1. Illustration of how the results of this study may have been misinterpreted without the proper physiological controls. Data are from non-dormant *S. alterniflora* only. Shown on the graph are: FOX products (△), protein carbonyls (▼), water-soluble antioxidant capacity (●), and viability (■).

The physiological controls of the *Spartina* system make *S. alterniflora* an attractive model system to study recalcitrance, and help differentiate between processes related to recalcitrance and those that are part of the general maturation drying and desiccation of all seeds. The results observed in this study point to the fact that an orthodox control, in the same physiological state as the recalcitrant seed, must be used in order to truly understand the

mechanisms of recalcitrance. It is hoped that the concepts illustrated in this study of *Spartina* will improve subsequent investigations of recalcitrant seed death in this and other species.

FUTURE WORK

The future work with *S. alterniflora* should focus on both the physiological characteristics of the seeds, which will help with the use of *S. alterniflora* seeds for applied problems, and mechanistic studies to try and determine the causes of recalcitrant seed death.

Physiological Experiments

Storage of *S. alterniflora* Seeds in Salt-Solutions

Storage of *S. alterniflora* seeds is a complex problem because the seeds must remain at high moisture contents to remain viable. However, at these moisture contents the seeds will germinate. Even when stored at 2°C, the seeds will germinate after approximately 10 months of storage. It would advantageous if the storage time of *S. alterniflora* could be dramatically increased, which would allow for germplasm storage and the ability to perform a greater quantity of experiments with the same seed lot. While dry storage does not appear to a viable option at the present time, it may be possible to store the seeds in salt solutions at temperatures below 0°C. The addition of salt will lower the freezing point of water, and if a sufficient amount of salt enters the seed and the water freezing point is lowered internally, it may enable long-term storage of *S. alterniflora* seeds at low temperatures.

Drying and Rehydration of *S. alterniflora* Seeds

Spartina alterniflora seeds appear to have a critical moisture content of 45%, but it is still unclear what causes the loss of viability. One experiment that may lend clues as to why the seeds die would be to repeatedly flash dry the seeds to just above the critical moisture content and then rehydrate them. If death is a result of a physical phenomenon that occurs at the critical moisture content, the seeds should tolerate drying and rehydration indefinitely. However, if death is

caused by a gradual build-up of deleterious products that cannot be repaired, the seeds should eventually lose their viability at a moisture content above the critical moisture as the damaging products gradually increase during the dry downs. If the cause of recalcitrant death can be narrowed down, it may allow a more focused effort in elucidating the cause of death.

Q_{10} Dry Down Experiments to Determine Desiccation-Induced Death

Another method to determine if death is a physical or chemical event is to dry the seeds at different temperatures. The temperature dependence of a biochemical reaction is referred to as $Q_{10,}$ because the biochemical rate of a reaction should roughly double when the temperature is raised by 10°C (Mohr and Schopfer 1995). Drying *S. alterniflora* at temperatures that differ by 10°C should indicate if death is caused by a chemical/ physical or biochemical reaction. If the rate of death is roughly doubled when the temperature is increased by 10°C, it suggests a biochemical cause of death. However, if the rate of death is increased, but less than double, it suggests a chemical cause, and finally, if the rate of death is unaffected by temperature, death is most likely a physical phenomenon. Like the repeated desiccation experiment, this straightforward experiment can lend valuable clues to the cause of desiccation-induced death, and help dictate the possible causes of desiccation-induced death that should be examined more closely.

Seed Imbibition in Abscisic Acid (ABA) and Tetcyclacis to Delay Recalcitrant Death

Storage of recalcitrant seeds is a major issue because the seeds must be stored at high moisture contents to remain viable. A simple method to quickly confer desiccation-tolerance to a recalcitrant seed would be beneficial because the seeds could be stored dry, which would prevent germination, and is more economical than moist storage. One method that appears to increase desiccation-tolerance, and merits further investigation is the imbibition of recalcitrant seeds in tetcyclacis and abscisic acid (ABA). Desiccation-tolerance was dramatically increased in the

seeds of recalcitrant *Acer saccharinum* when they were imbibed in either ABA or tetcyclacis, which induces endogenous ABA production (Beardmore and Whittle 2005). This experiment needs to be repeated with recalcitrant *S. alterniflora* to determine the effect of ABA on the critical moisture content. This experiment is particularly intriguing with *S. alterniflora* because the seeds are shed dormant, so it assumed that ABA is already present in the seeds. If the addition of ABA increases the desiccation-tolerance in *S. alterniflora*, it will not only raise questions about the induction of desiccation-tolerance, but also the induction of dormancy in *S. alterniflora* seeds. If the ABA treatment does not alter the desiccation-tolerance of *S. alterniflora*, it may suggest different causes of recalcitrance between *S. alterniflora* and *A. saccharinum* seeds. The successful induction of desiccation-tolerance via ABA would not only help determine the causes of recalcitrance, but it would also provide a dry storage method for *S. alterniflora* seeds, which would make it easier to use *S. alterniflora* in coastal restoration projects.

Mechanistic Studies with *S. alterniflora*

Enzyme Activity and Structure During Desiccation

To determine if damage to enzymes is a cause of recalcitrant seed death, enzyme activity and structure can be measured during desiccation. Certain enzymes are damaged and aggregate when desiccated (e.g. citrate synthase) (Chakrabortee *et al*. 2007). Orthodox seeds, such as *S. pectinata*, must avoid or prevent this enzyme damage when desiccated, thus allowing the seed to remain viable at low moisture contents. It is unclear if orthodox seeds survive desiccation because their enzymes and other proteins are able to withstand desiccation without aggregating, or if there is a system in place to repair the proteins damaged by desiccation. To determine if damage and repair differ in orthodox *S. pectinata* and recalcitrant *S. alterniflora*, protein structure and activity can be measured as the seeds are desiccated. To start, the most efficient

method to compare the enzymes of *S. alterniflora* and *S. pectinata* is to choose a suite of known desiccation-sensitive enzymes to study. If during desiccation, protein damage is observed in *S. alterniflora* and not *S. pectinata*, it suggests that damage to key enzymes may be a cause of recalcitrant seed death. If damage is observed in both species, it may be necessary to desiccate the seeds to just above the critical moisture content for *S. alterniflora* and rehydrate them to determine if proteins are repaired during rehydration. If a difference in protein damage or repair is observed between the two *Spartina* species, it is still necessary to explore the cause of the difference.

Protective Proteins

Certain classes of proteins, such as late embryogenesis abundant (LEAs) and heat shock proteins (HSPs) are thought to prevent and repair damage during the desiccation of an organism (Goyal *et al.* 2005, Torok *et al.* 2001). If enzyme damage or lack of repair is observed in the study detailed above, it may indicate a deficiency in one of the HSPs or LEA proteins. Also, the type of damage observed in the above study may lend a clue as to which class of proteins is missing. If protein damage is observed in *S. alterniflora*, but not *S. pectinata*, it may suggest that *S. alterniflora* lacks certain LEAs, which appear to prevent protein aggregation (Chakrabortee *et al*. 2007). When human cells were desiccated, the presence of LEA proteins significantly reduced protein aggregation (Chakrabortee *et al.* 2007). However, not only must the LEA proteins be present, but they have to be at a fairly high ratio to prevent aggregation. For example, when the human cells were desiccated, even when the LEA proteins were present in a 2:1 molar ratio compared to the other proteins, aggregation was not significantly prevented. The LEA molar ratio had to be 5:1 before a significant decrease in protein aggregation was observed (Chakrabortee *et al.* 2007). Therefore, it is important in future experiments to not only identify the LEA proteins that are present, but it also imperative to quantify their amounts if any

conclusion regarding protection is to be made. Along with LEAs, HSPs are also thought to protect against protein damage. However, unlike LEAs, which prevent damage, HSPs are most likely to repair the damage by refolding denatured proteins (Torok *et al*. 2001). If in the experiment above, protein denaturation is observed in orthodox *S. pectinata,* yet can be repaired, it suggest a role for HSPs. Heat shock proteins have been observed in recalcitrant *Castanea sativa* cotyledons (Collada *et al.* 1997); however, it was not determined which HSPs were present. It is possible that recalcitrant seeds have many HSPs, but they lack the ones needed for desiccation-tolerance. Similar to the LEA proteins, it is important in the future to identify all of the HSPs present, and not just one or two, as the mere presence of any HSP does not sufficiently address their role in recalcitrance.

The successful identification and quantification of protective proteins, such as LEAs and HSPs, in both recalcitrant seeds and a closely related orthodox relative may elucidate the mechanism by which orthodox seeds can successfully tolerate desiccation.

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APPENDIX A
SUPPORTING FIGURES AND TABLES

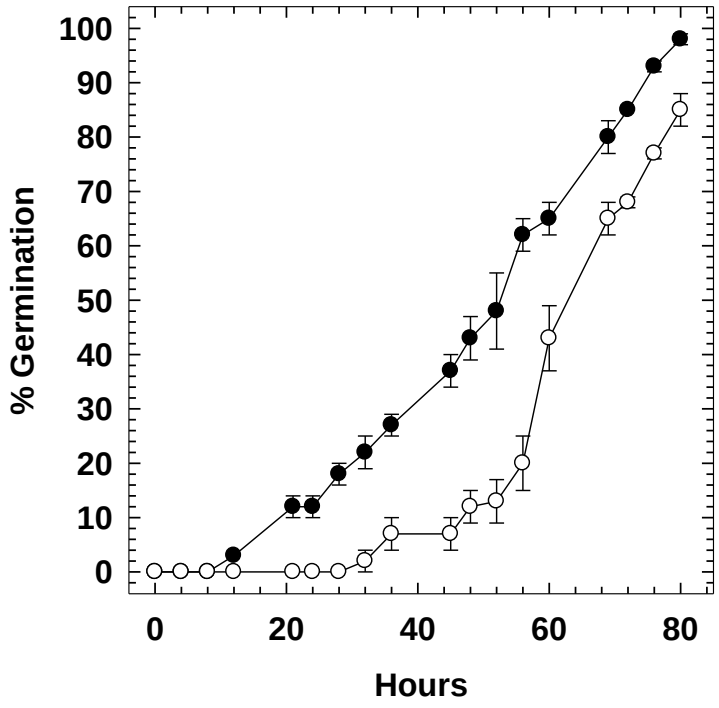

Figure A-1. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 17°C in darkness. Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. First experiment, Databook 4: 16-17

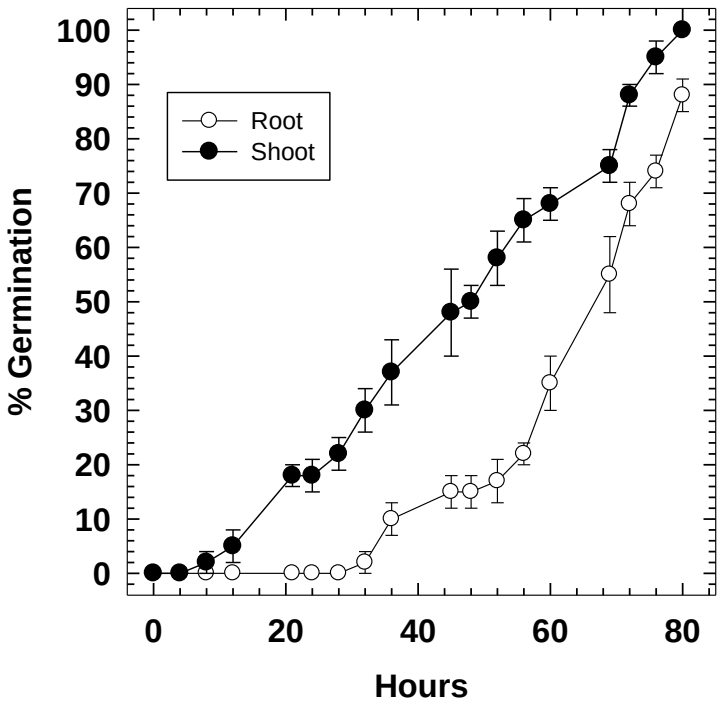

Figure A-2. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 17°C in darkness. Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Second experiment, Databook 4: 30-31.

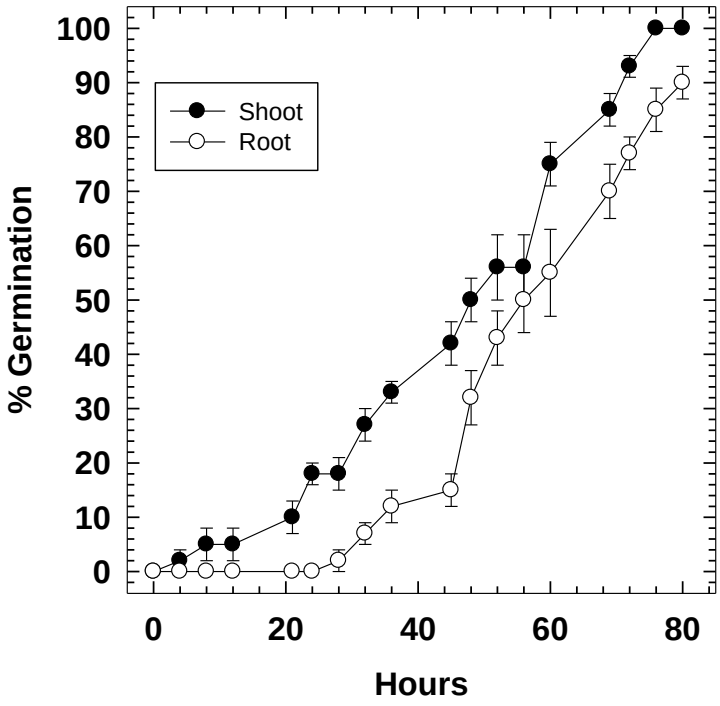

Figure A-3. Germination versus time for *S. alterniflora* (Vermillion accession, 2004) seeds subjected to 17°C in darkness. Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering. 3rd experiment, Databook 8: 95.

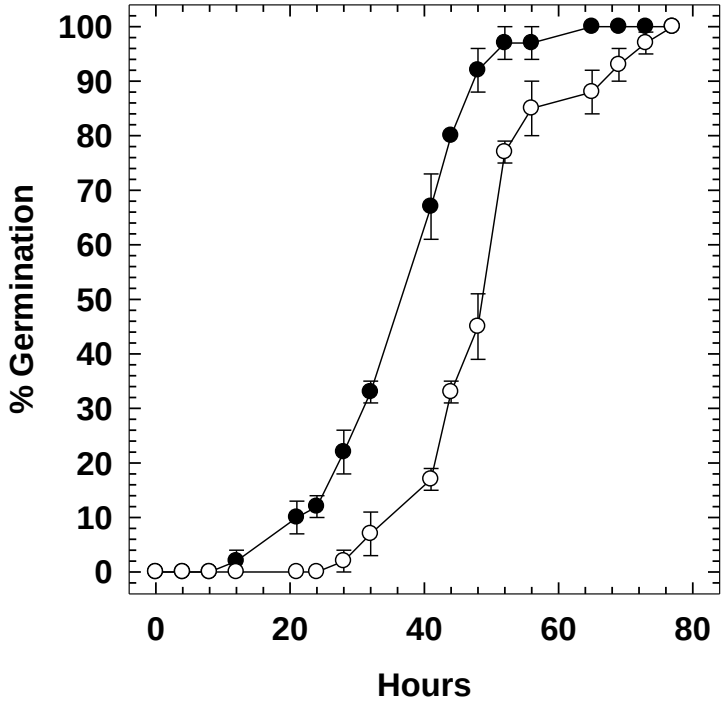

Figure A-4. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 22°C in darkness. Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. 1st experiment, Databook 4: 29

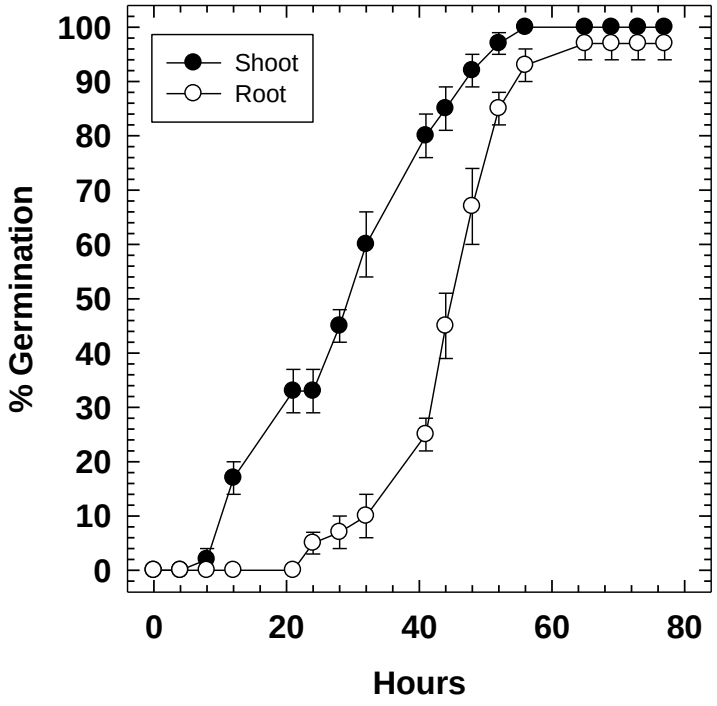

Figure A-5. Germination versus time for *S. alterniflora* (Vermillion accession, 2004) seeds subjected to 22°C in darkness (Experiment 2). Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Databook 8: 94.

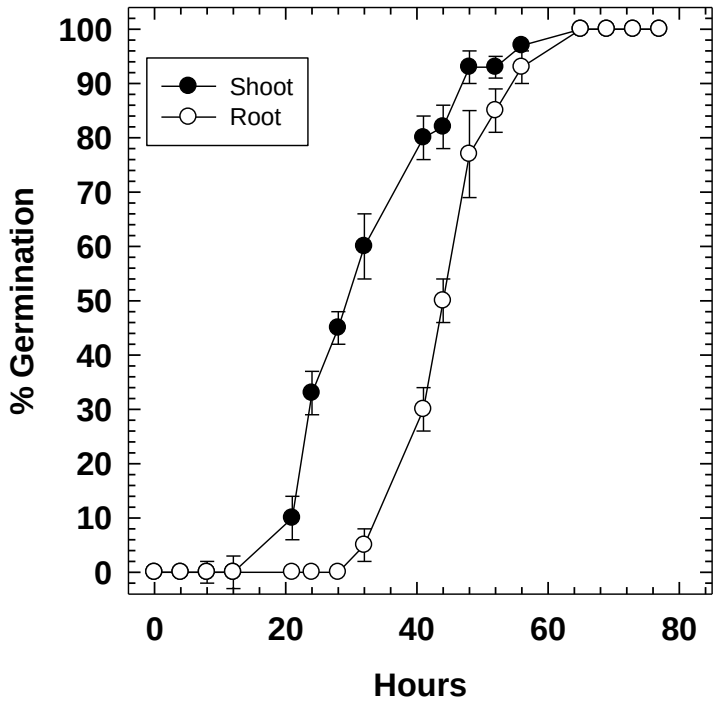

Figure A-6. Germination versus time for *S. alterniflora* (Vermillion accession, 2004) seeds subjected to 22°C in darkness (Experiment 3). Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Databook 8: 96.

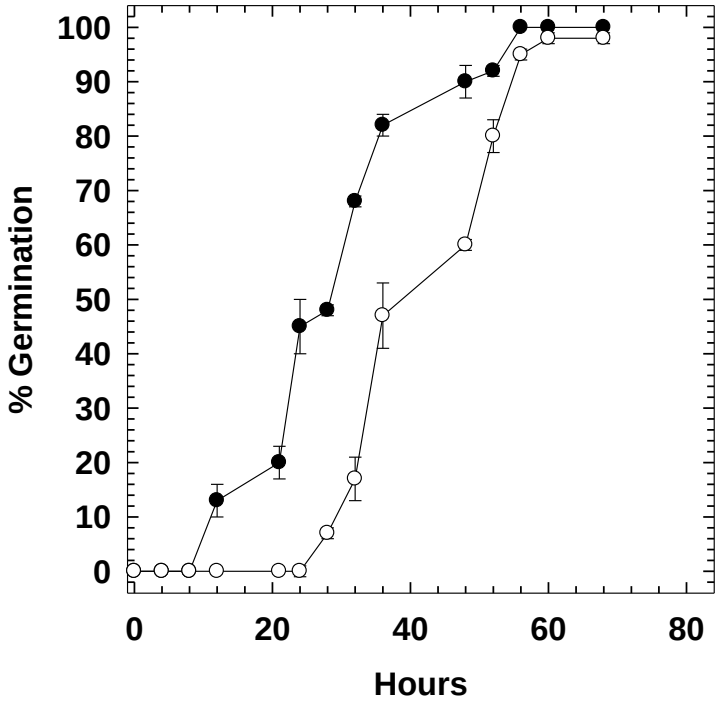

Figure A-7. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 27°C with light (Experiment 1). Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Databook 3: 69-71

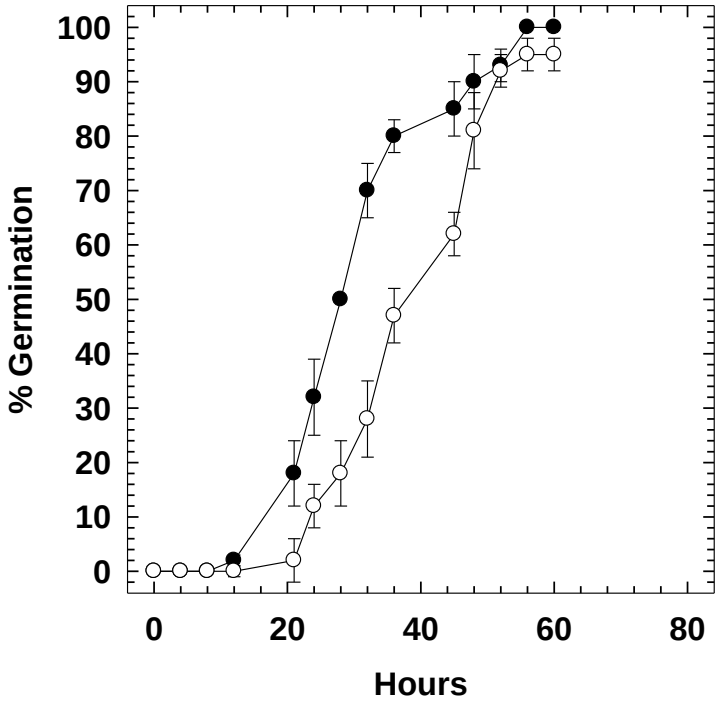

Figure A-8. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 27°C in darkness (Experiment 2). Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Databook 3: 74-76.

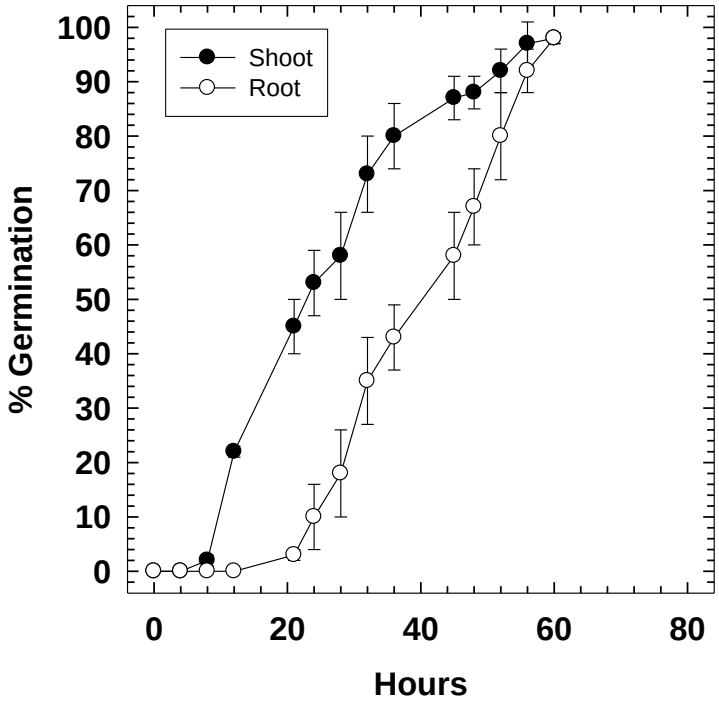

Figure A-9. Germination versus time for *S. alterniflora* (Vermillion accession, 2002) seeds subjected to 27°C in darkness (Experiment 3). Seeds were stratified at 2°C for four months submerged in dH_20. Root (○) and shoot (●) germination was scored separately, and germination was scored when the axis broke through the covering structure. Databook 4: 22-31.

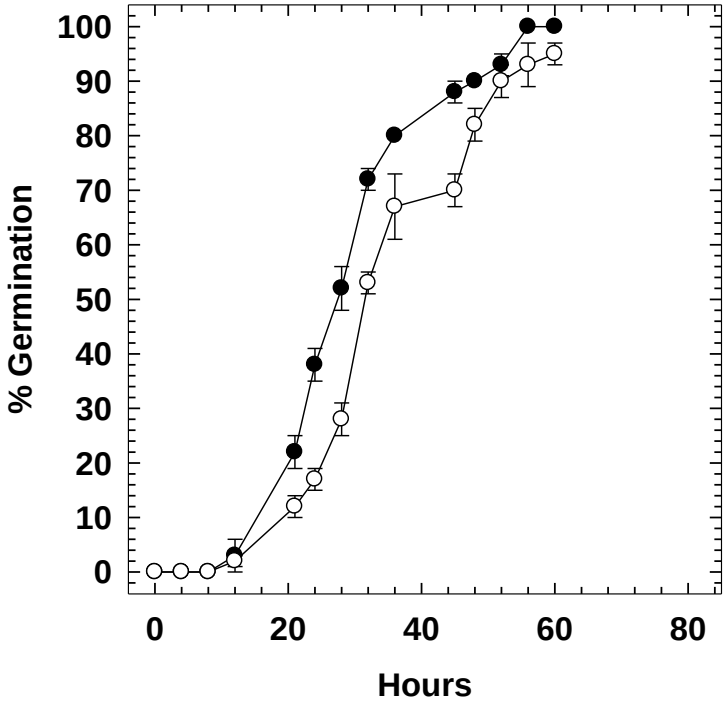

Figure A-10. Germination versus time of *S. alterniflora* seeds (Vermillion, 2002) germinated at 32°C in darkness (experiment 1). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 3: 27-29.

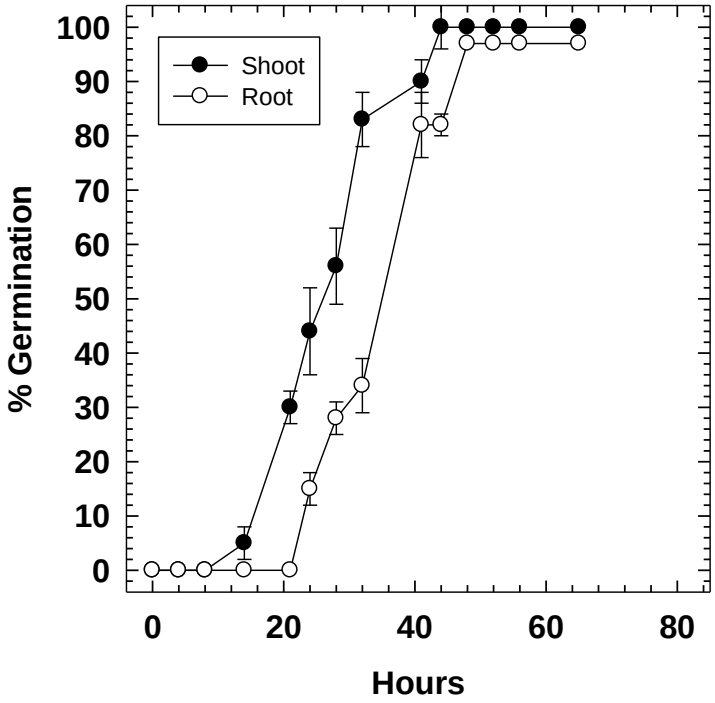

Figure A-11. Germination versus time of *S. alterniflora* seeds (Vermillion, 2004) germinated at 32°C in darkness (Experiment 2). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 8: 98.

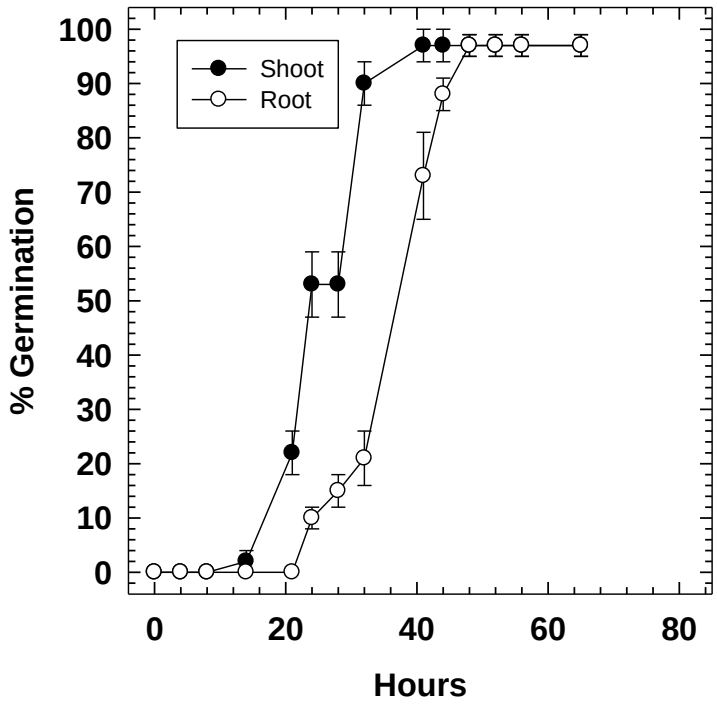

Figure A-12. Germination versus time of *S. alterniflora* seeds (Vermillion, 2004) germinated at 32°C in darkness (Experiment 3). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 8: 99.

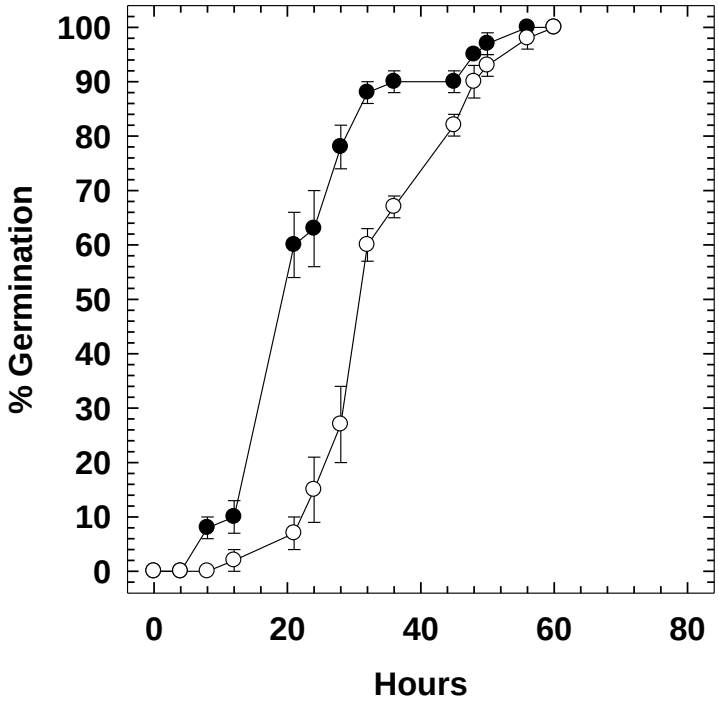

Figure A-13. Germination versus time of *S. alterniflora* seeds (Vermillion, 2002) germinated at 34°C in darkness (Experiment 1). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 4: 22.

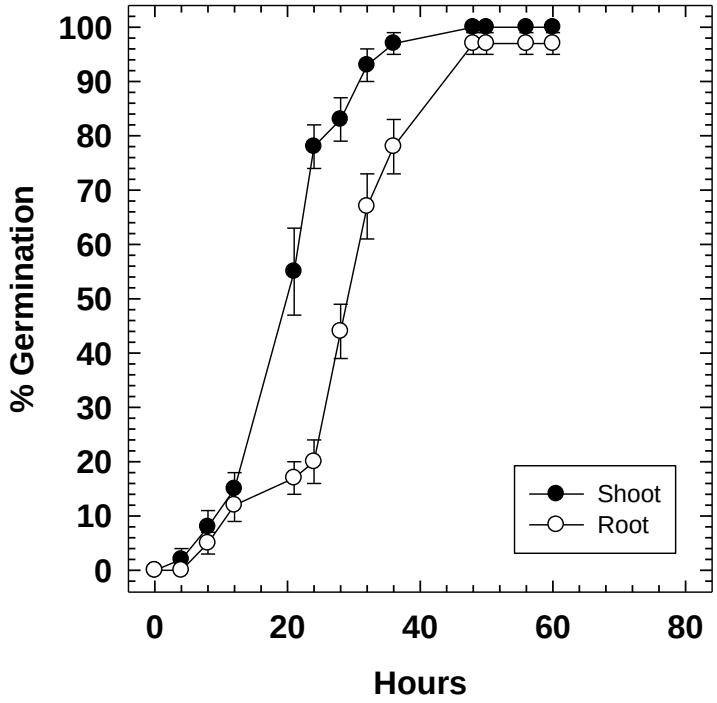

Figure A-14. Germination versus time of *S. alterniflora* seeds (Vermillion, 2004) germinated at 34°C in darkness (Experiment 2). Seeds were stored at 2°C in dH_20 for 6 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 4: 31.

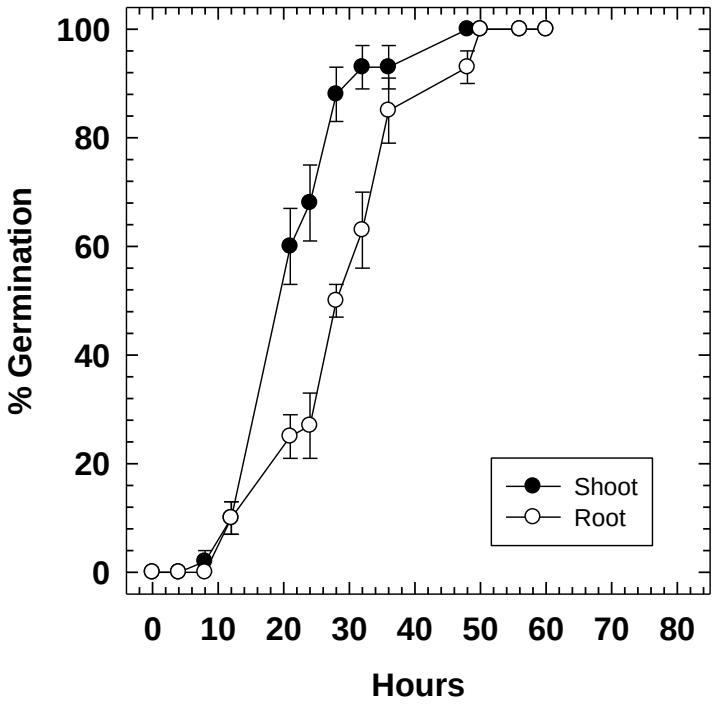

Figure A-15. Germination versus time of *S. alterniflora* seeds (Vermillion, 2004) germinated at 34°C in darkness (Experiment 3). Seeds were stored at 2°C in dH_20 for 6 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 8: 97.

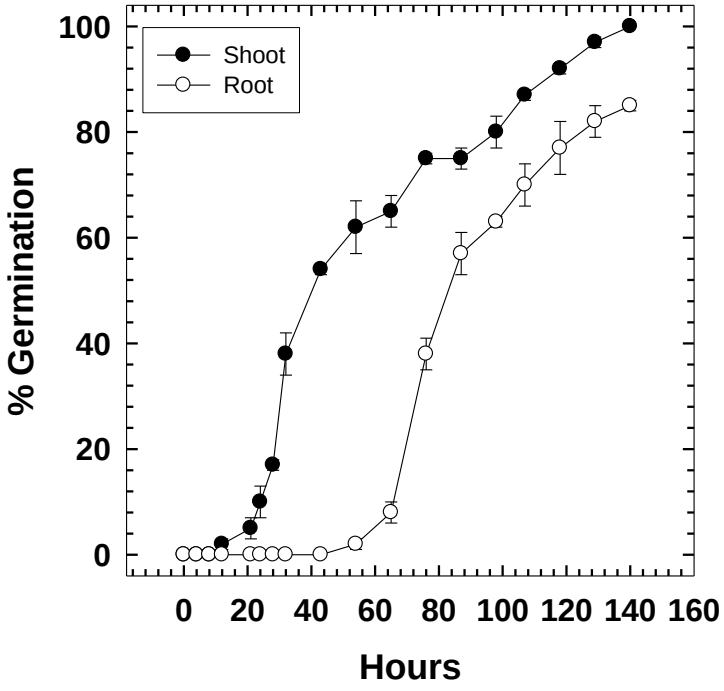

Figure A-16. Germination versus time of *S. alterniflora* seeds (Vermillion, 2002) germinated at 37°C in darkness. Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 4: 34-36.

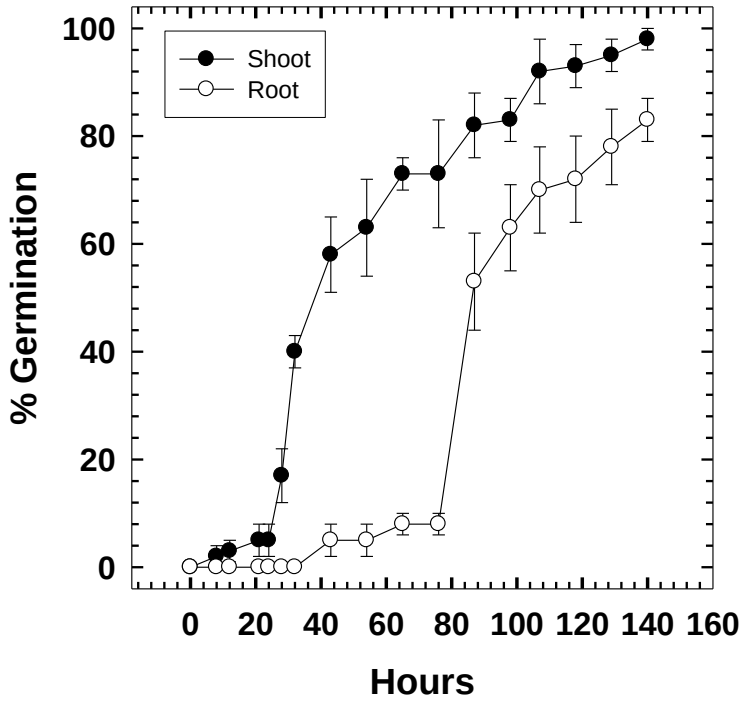

Figure A-17. Germination versus time of *S. alterniflora* seeds (Vermillion, 2002) germinated at 37°C in darkness (Experiment 2). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 4: 37-38.

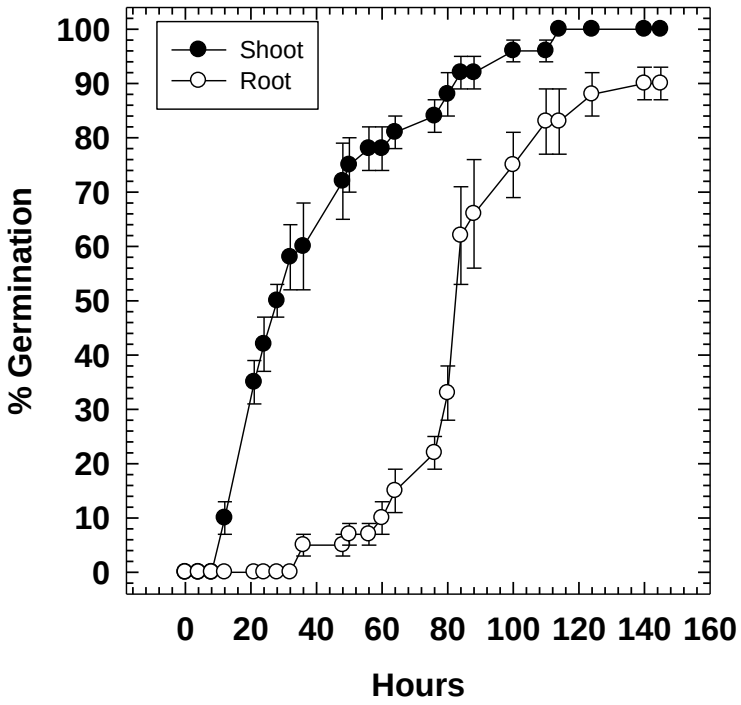

Figure A-18. Germination versus time of *S. alterniflora* seeds (Vermillion, 2004) germinated at 37°C in darkness (Experiment 3). Seeds were stored at 2°C in dH_20 for 4 months. Root (○) and shoot (●) germination were scored separately, and germination was recorded when the axis broke through the covering structure. Databook 8: 100.

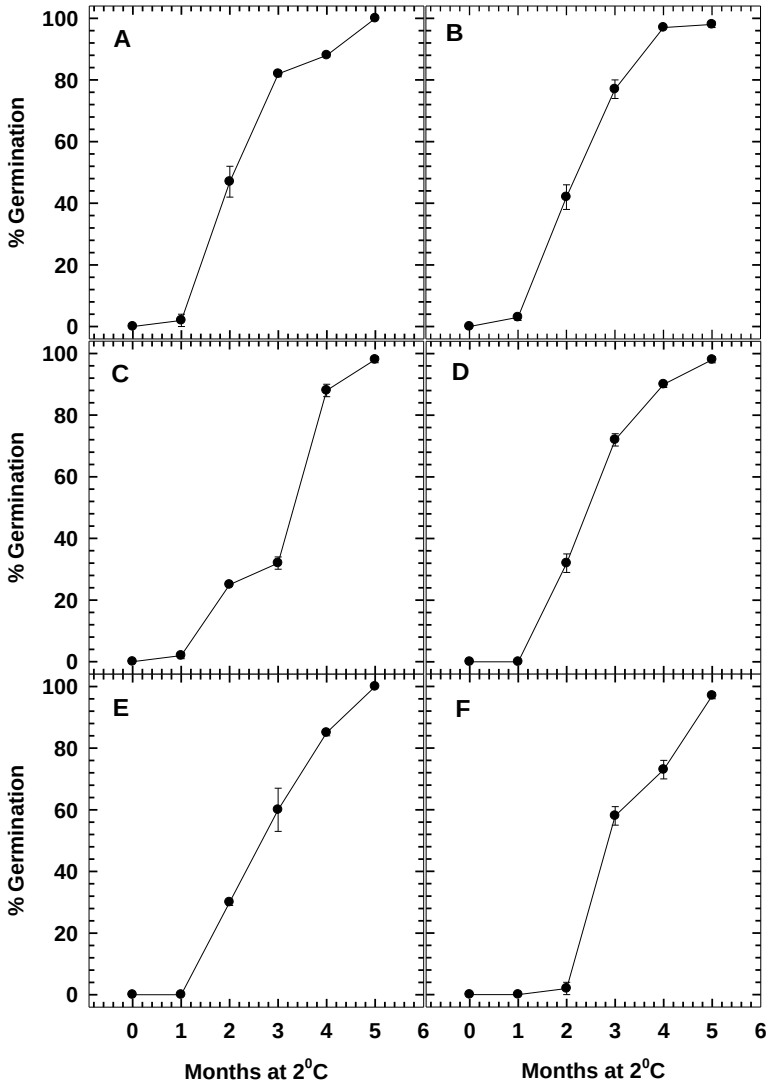

Figure A-19. Comparison of stratification (2°C) time versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2001 (Ben-Hur Experimental Station in Baton Rouge, Louisiana). Upon harvest, seeds were stored submerged in dH_20 at 2°C. Germination tests were performed at 27°C every four weeks on three replications of 20 seeds each. Germination was recorded after 14 days. (A) Vermillion, (B) 26-18, (C) 26-13, (D) 27-2, (E) 7-8, (F) 7-14. Databook 1: 59-71.

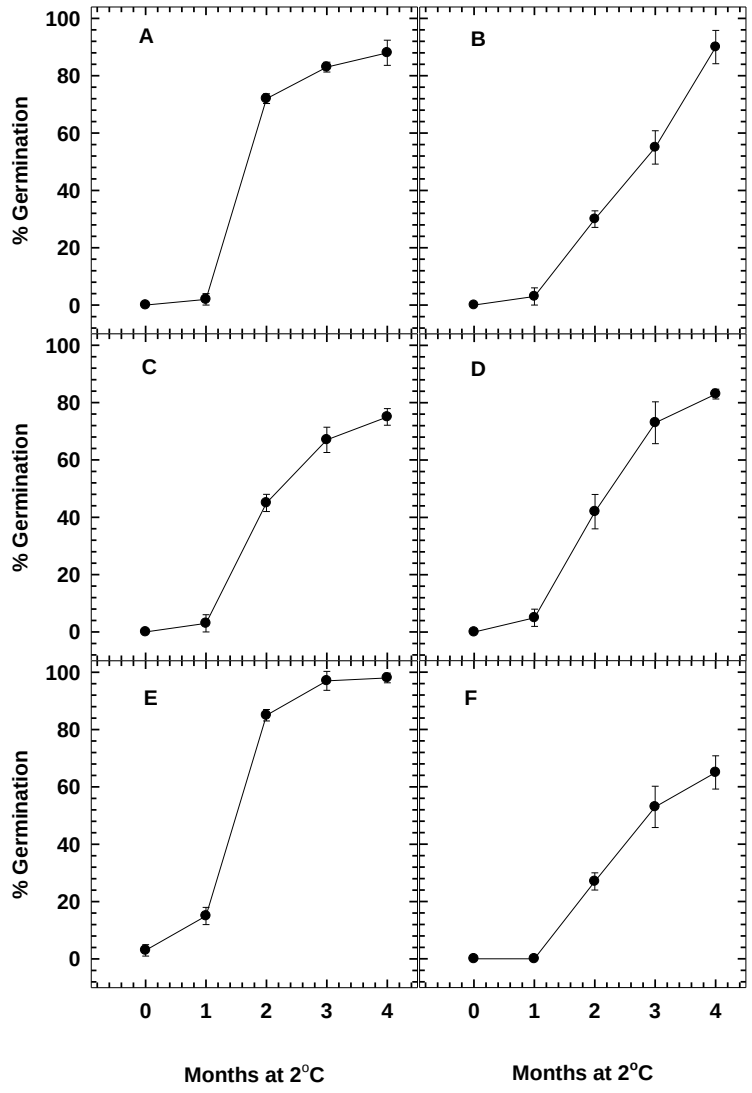

Figure A-20. Comparison of stratification (2°C) time versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2002 (Ben-Hur Experimental Station in Baton Rouge, Louisiana). Upon harvest, seeds were stored submerged in dH_20 at 2°C. Germination tests were performed at 27°C every four weeks on three replications of 20 seeds each. Germination was recorded after 14 days. (A) Vermillion, (B) 26-18, (C) 26-13, (D) 27-2, (E) 7-8, (F) 7-14. Databook 3: 6-8, 23-24, 51-52, 61-62, 67-68.

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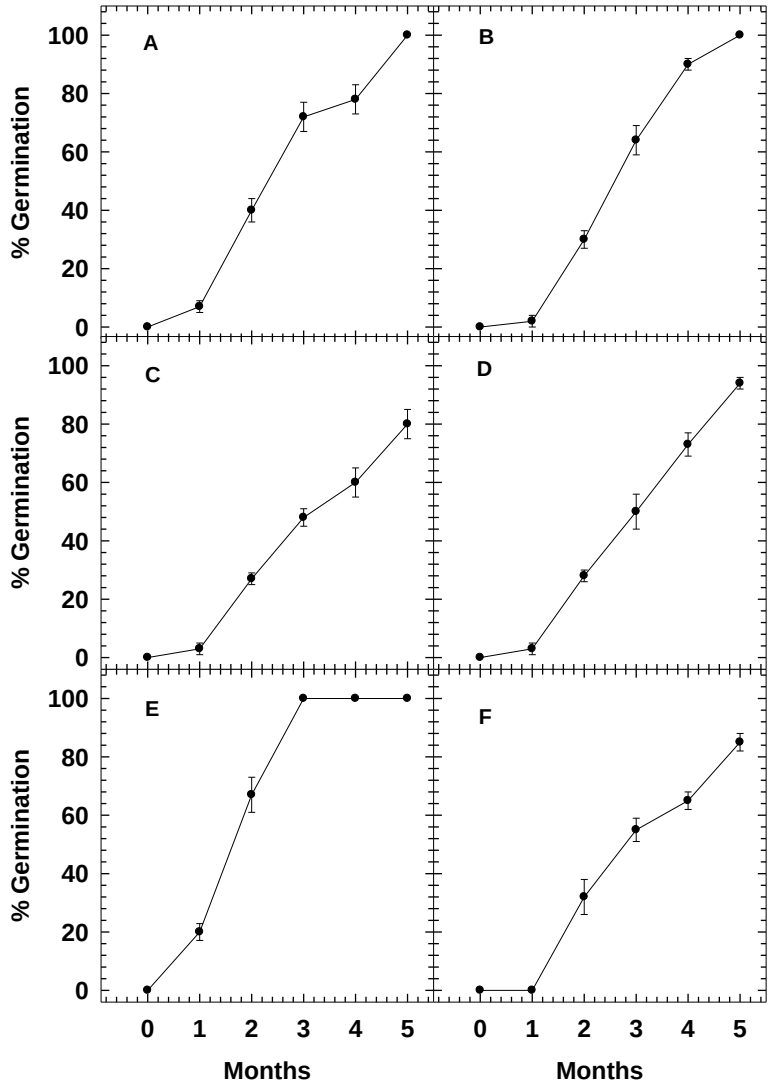

Figure A-21. Comparison of stratification (2°C) time versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2004 (Ben-Hur Experimental Station in Baton Rouge, Louisiana). Upon harvest, seeds were stored submerged in dH_20 at 2°C. Germination tests were performed at 27°C every four weeks on three replications of 20 seeds each. Germination was recorded after 14 days. (A) Vermillion, (B) 26-18, (C) 26-13, (D) 27-2, (E) 7-8, (F) 7-14. Databook 7: 44-47, 72, 92-93; Databook 8: 40, 56, and 73.

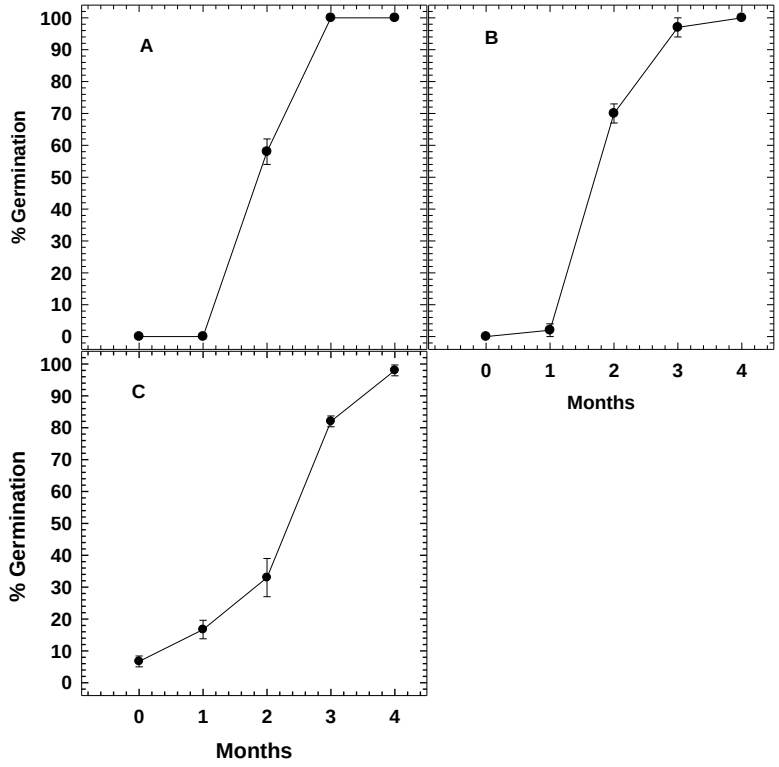

Figure A-22. Comparison of stratification (2°C) time versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2003, 2005, 2006 (wild-type seeds, harvested from Port Fourchon, Louisiana). Upon harvest, seeds were stored submerged in dH_20 at 2°C. Germination tests were performed at 27°C every four weeks on three replications of 20 seeds each. Germination was recorded after 14 days. (A) 2003, (B) 2005, (C) 2006. Databook 4: 55, 67; 5: 25, 44; databook 10: 30-31, 58, 78; databook 12: 20-21, 35, 85, 97, 100.

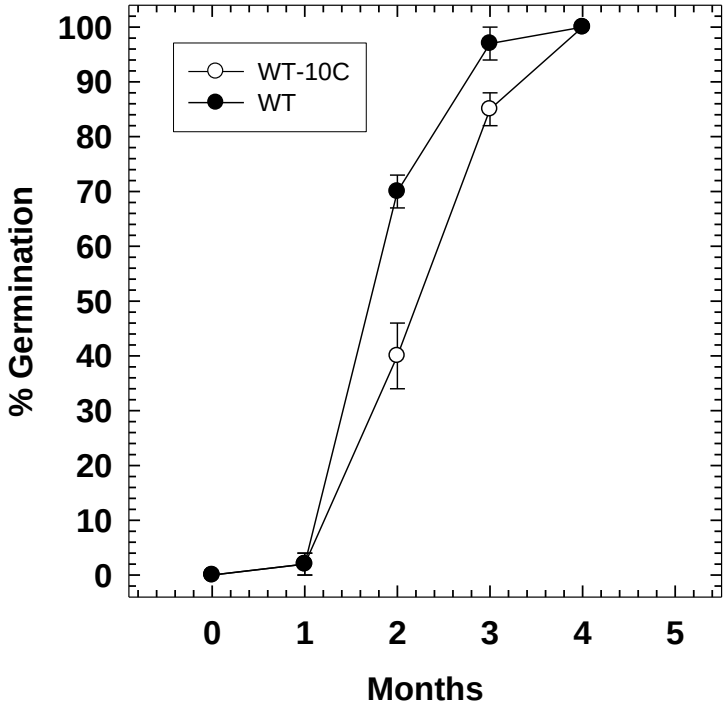

Figure A-23. Comparison of stratification at two temperatures versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2005 (wild type seeds harvested from Port Fourchon, Louisiana). Stratification was carried out at 2°C (●) and 10°C (○). To determine degree of dormancy, germination tests were carried out every four weeks at 27°C on three replications of 20 seeds each. Germination was recorded after 14 days. Databook 10: 30-31, 58, 78.

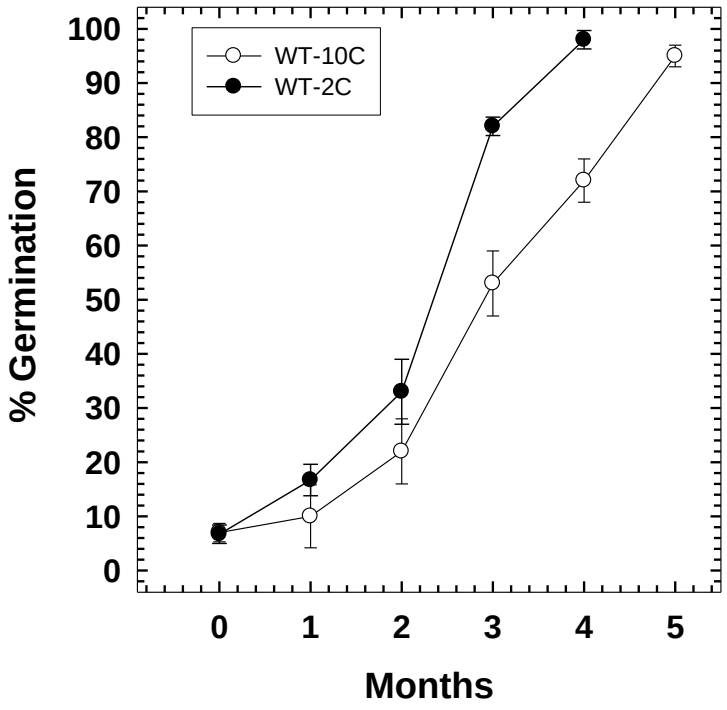

Figure A-24. Comparison of stratification at two temperatures versus loss of dormancy for the seeds of *S. alterniflora* harvested in 2006. Seeds were harvested from Port Fourchon, Louisiana and stored at either 2°C (●) or 10°C (○). To determine degree of dormancy, germination tests were carried out every four weeks at 27°C on three replications of 20 seeds each. Germination was recorded after 14 days. Databook 12: 20-21, 35, 85, 97, 100.

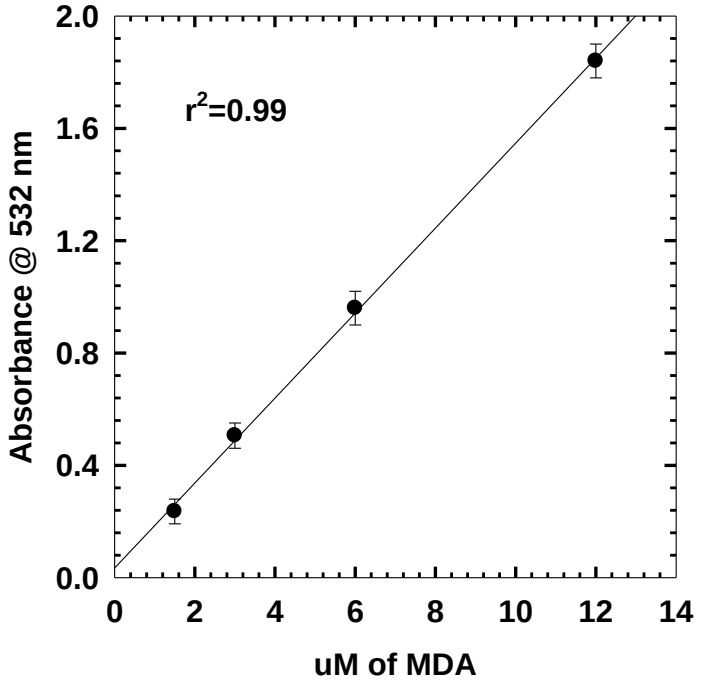

Figure A-25. Representative standard curve for the TBARS assay using 1, 1, 3, 3-tetraethoxypropane (malondialdehyde). Reaction was carried out at 95°C for 25 minutes, and absorbance was read at 532 nm. Error bars = standard error. Databook 7: 9-15, 21-22, 73

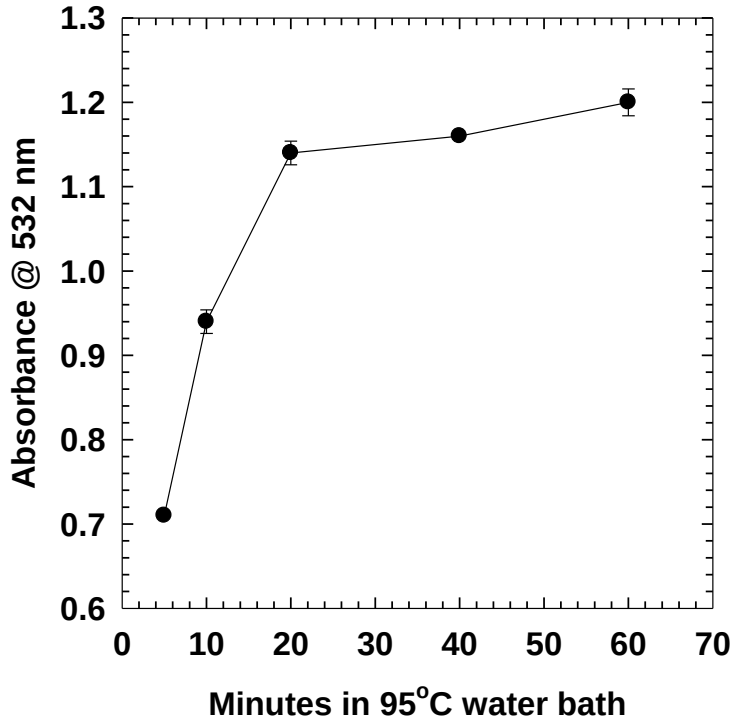

Figure A-26. Determination of the time needed in a 95°C H_20 bath for the TBARS reaction to complete. Seed extract, plus 6.25 uM MDA, was added to the TBARS assay. Reaction was run at 95°C and absorbance (532 nm) was taken at intervals to determine when the absorbance reached a plateau. Databook 7: 16-19.

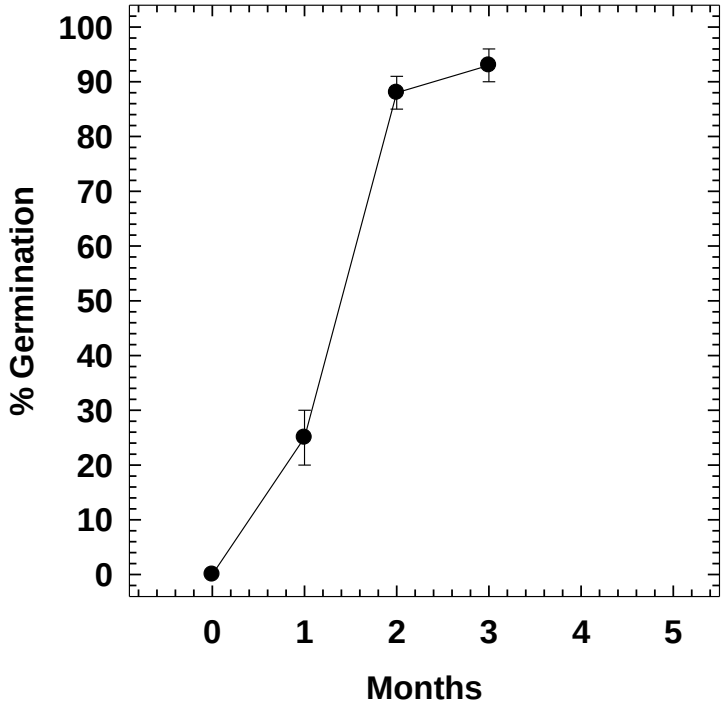

Figure A-27. Time of stratification at 2°C versus loss of dormancy in the seeds of orthodox *Spartina pectinata.* Seeds were purchased from Western Native Seed Company (lot # 6042), and the seeds in this experiment were immediately submerged in H_20 and placed at 2°C. To test for germination, three replications of 20 seeds each were placed at 27°C with light for 14 days. Error bars = standard error. Databook 7: 24-33.

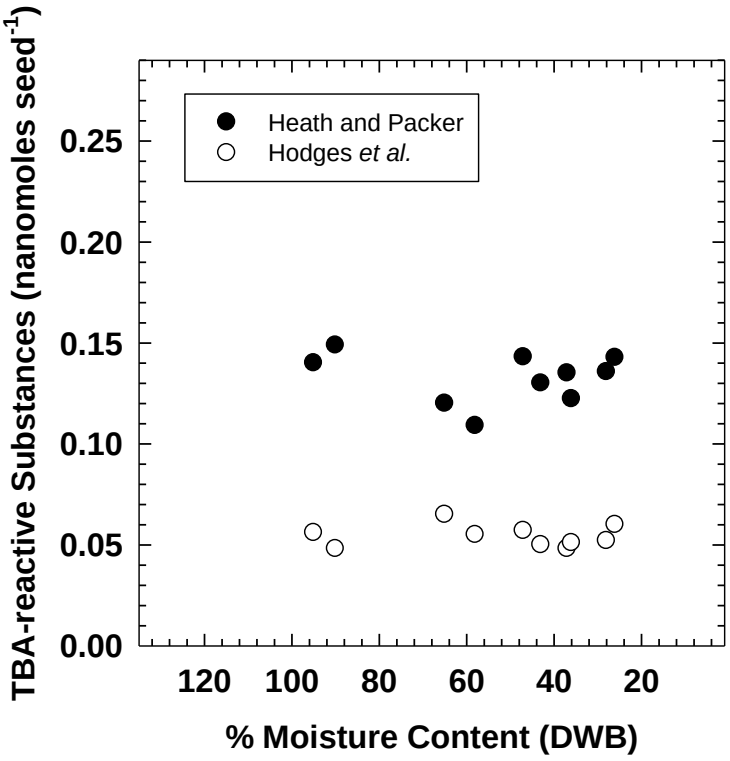

Figure A-28. TBARS values in dormant *S. pectinata* seeds during desiccation. The seeds used were purchased from Western Native Seeds and harvested in 2003 (WNS 03A, lot # 6042, Coaldale, USA). Seeds were freeze-clamped before extraction. TBARS amounts were calculated by the Heath and Packer (●) method or using the Hodges correction (○). Absorbance was measured at 440, 532 and 600 nm after 25 minutes in a 95°C water bath. Databook 8: 33-34, 65-67.

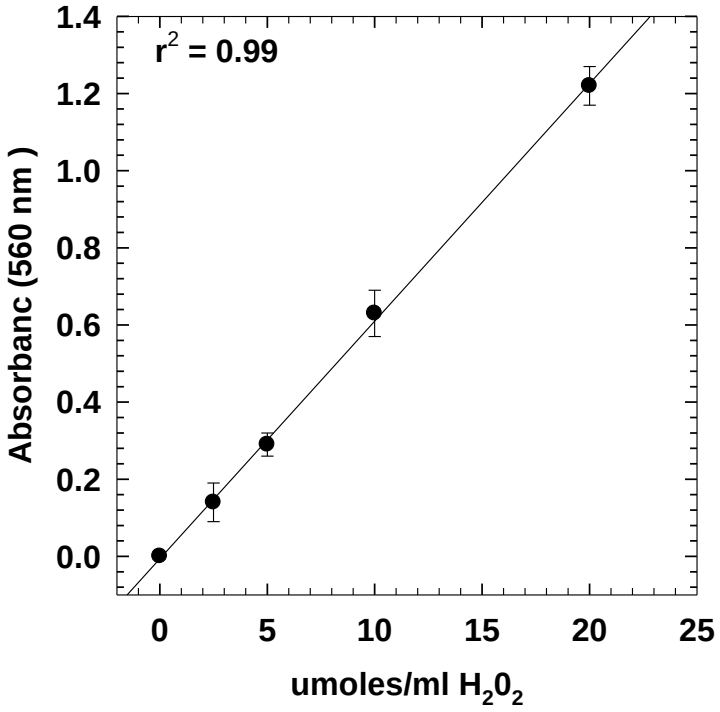

Figure A-29. Representative standard curve for the ferrous xylenol orange assay (FOX) using H_20_2 as the standard. Reaction was carried out for 90 minutes in a 25°C water bath and absorbance was read at 560 nm. Error bars = standard error. Databook 9: 13-14, 22, 33, 44, 77, 88; databook 10: 50

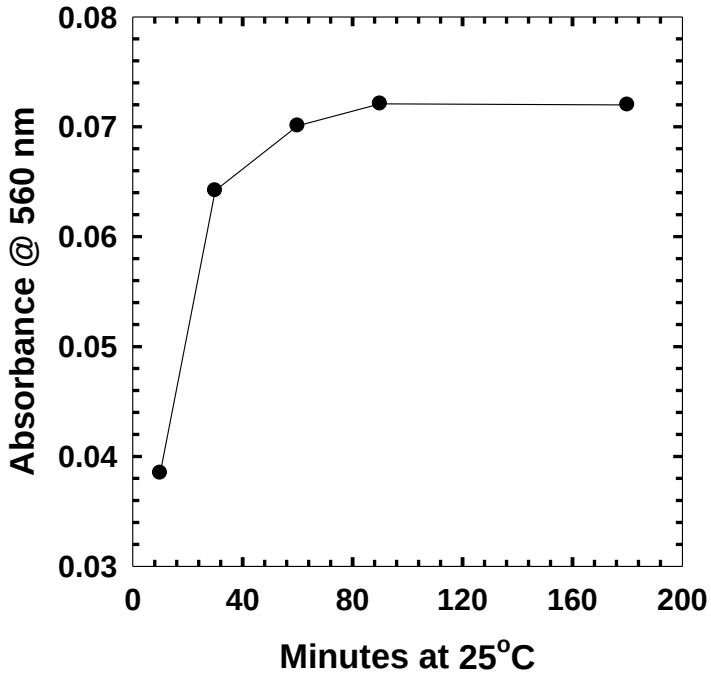

Figure A-30. Determination of time needed in a 25°C H_20 bath for the FOX reaction to plateau. Seed extract (1 ml) was added to FOX reagents and incubated in water bath at 25°C. Absorbance was measured at intervals to determine when the reaction reached a plateau. This determined how long samples should be left in the water-bath to allow the reaction to complete. Databook 8: 76, 81-82

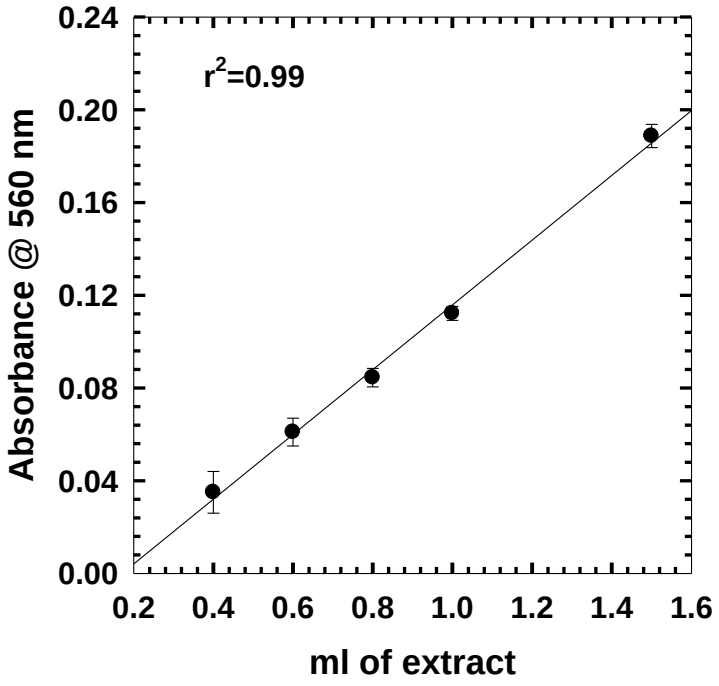

Figure A-31. Increasing amounts of *S. alterniflora* extract added to the FOX assay to determine linearity. Sample volumes were adjusted with ethanol/water (80:20 v/v). Reaction was carried out in a 25°C water bath for 90 minutes. The absorbance was read at 560 nm. These results indicate that the reaction is linear with respect to the FOX positive material in the extract. Databook 8: 75.

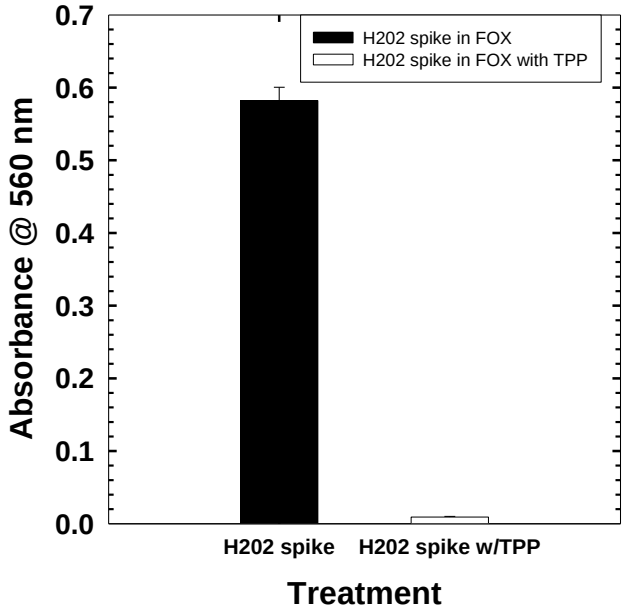

Figure A-32. Determination if triphenylphosphine (TPP) reduces hydrogen peroxide (H_20_2) in the ferrous xylenol orange assay (FOX). A H_20_2 spike was added to a FOX standard, with and without TPP. Samples were incubated for 30 minutes with the TPP and then placed in a 25^0C water bath for 30 minutes. Absorbance was taken at 560 nm. Error bars =SD. Delong *et al.* (2002) imply that TPP reduced LOOH, but not H_20_2. This experiment was done to verify that statement. Based upon these data, TPP reduces H_20_2. Therefore, to determine the amounts of LOOH alone, catalase must be included to destroy the H_20_2 prior to addition of the FOX reagent. Catalase was not added in the data reported in the body of the text. Databook 9: 55.

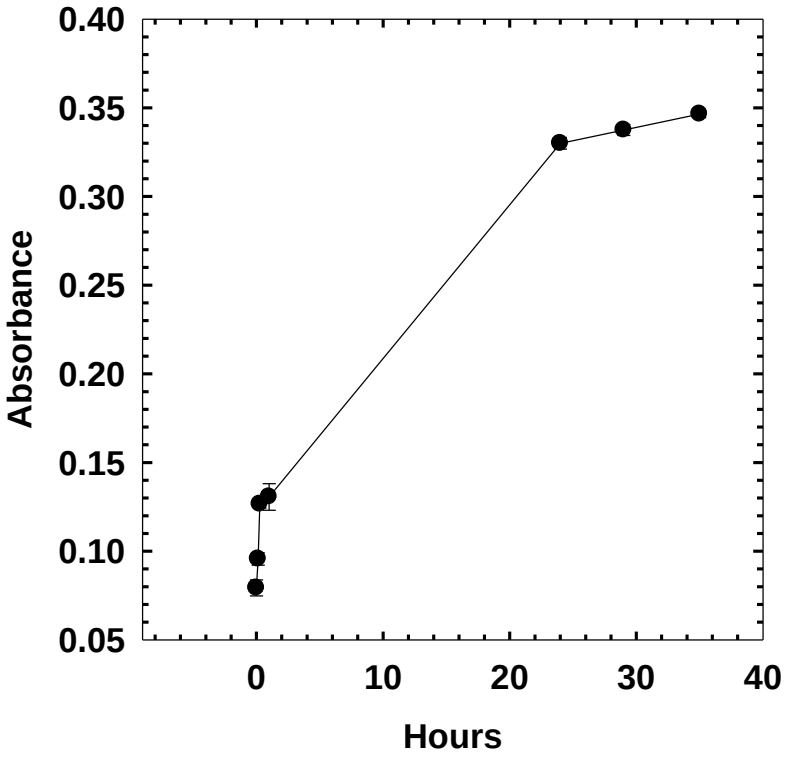

Figure A-33. Organic leachate amounts from fully hydrated *S. alterniflora* (Port Fourchon, 2005) versus time at room temperature (22-24°C). To determine leachate amounts, the seeds were placed in 10 ml of dH_20, and leachate amounts were recorded at different time intervals until the leachate amount reached a plateau. Organic leachates were measured with a spectrophotometer at 280 nm. Databook 10: 83-84.

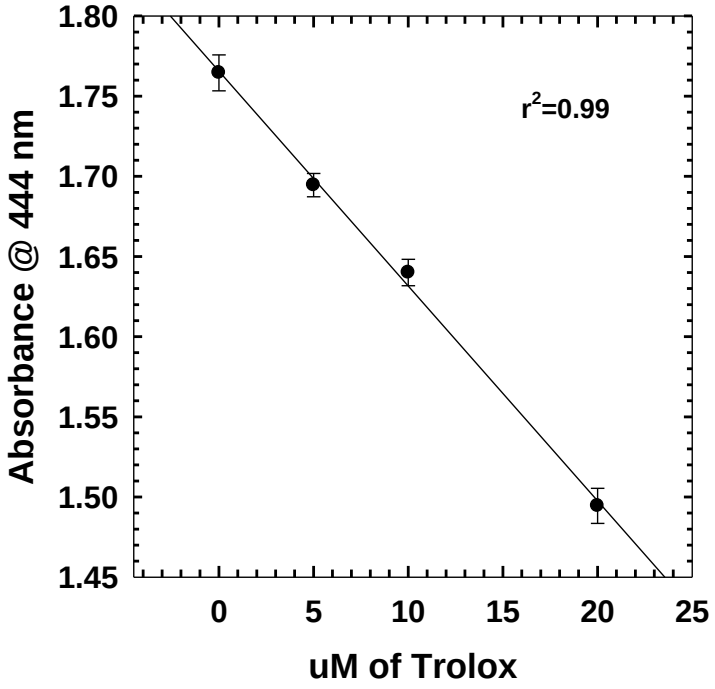

Figure A-34. Standard curve for the total antioxidant response assay using the vitamin E analog Trolox as the standard. Samples were incubated at 25°C for 15 minutes and absorbance was taken at 444 nm Databook 10: 20, 60, 66; Databook 11: 13, 20, 49, 53, 58, 73, 77; Databook 12: 29.

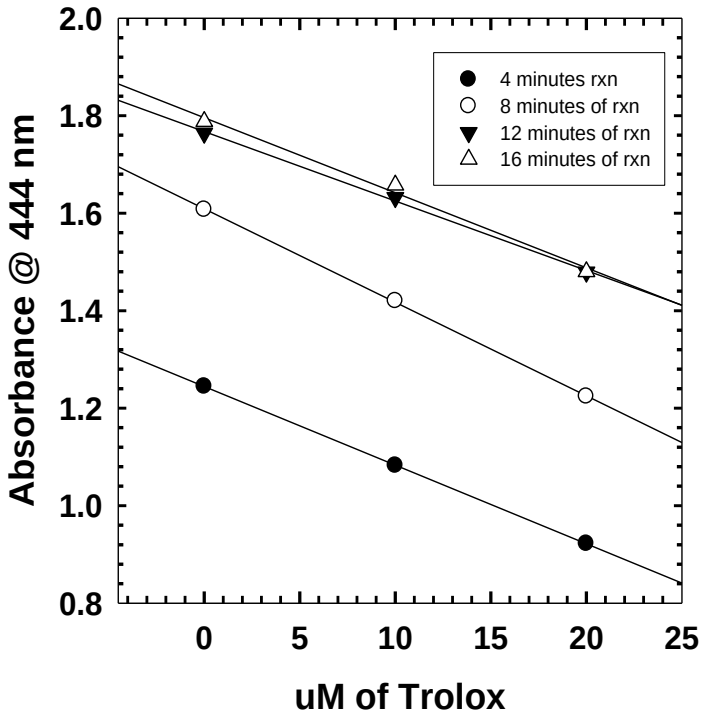

Figure A-35. Trolox standard for the TAR assay, measured at different time points to determine when the reaction would plateau, and if reaction was linear throughout the time course. Absorbance was measured at 444 nm after 4(●), 8 (○), 12 (▼) and 16 (▽) minutes of reaction time at 25°C. Databook 10: 21-22.

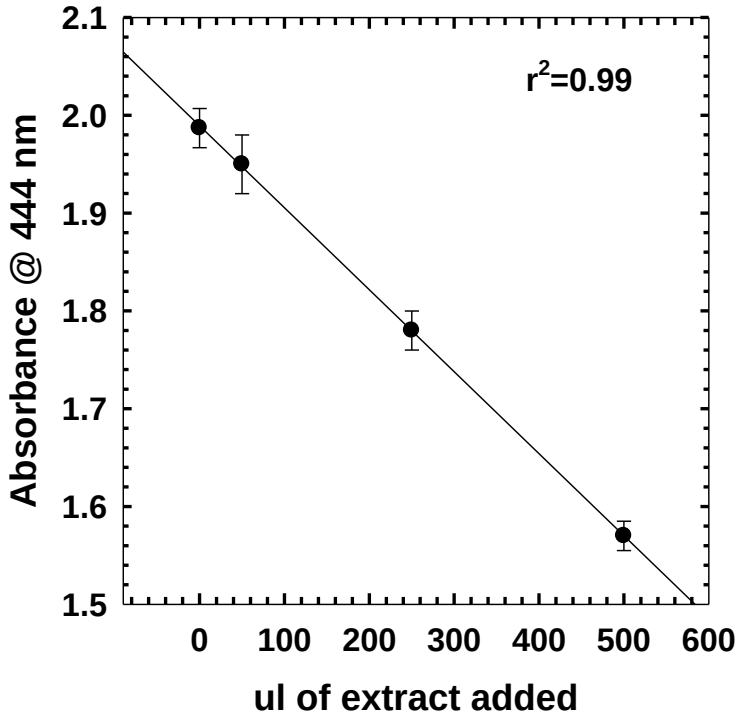

Figure A-36. Percent suppression of dianisidyl radical formation in the TAR assay, when different amounts of seed extract were added to ensure linearity. TAR reaction was recorded at 444 nm after 15 minutes of reaction at 25°C. Each data point represents 3 independent determinations. Error bars = standard error. Databook 10: 23-24.

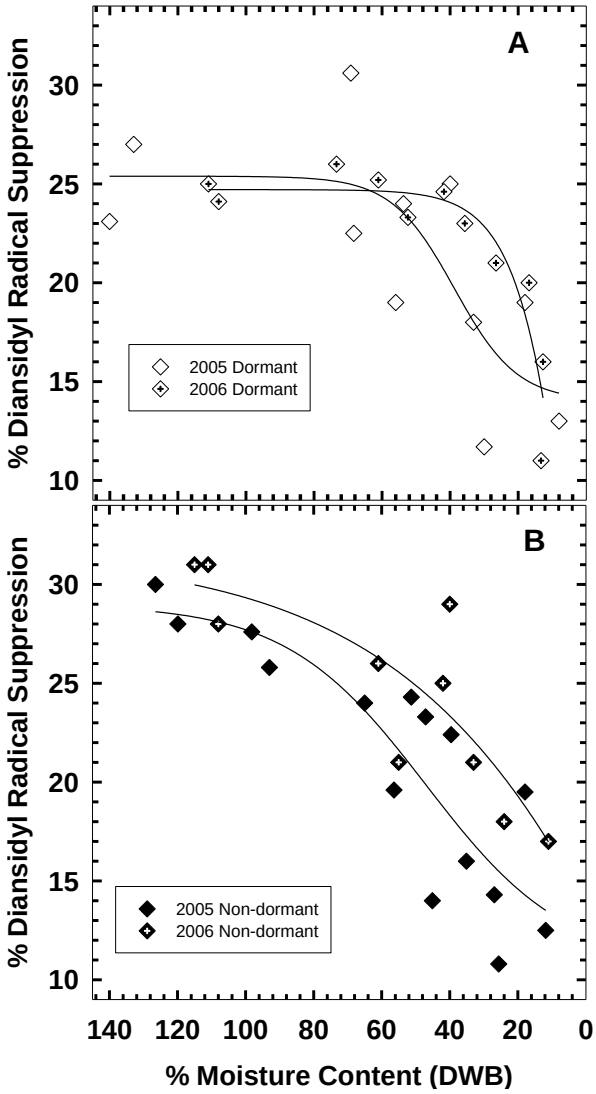

Figure A-37. Total water-soluble antioxidant capacity versus desiccation of *S. alterniflora* seeds harvested in two different years. Both (A) dormant and (B) non-dormant *S. alterniflora* seeds harvested from Port Fourchon, Louisiana in 2005 and 2006. Dormant seeds were stored submerged at 2°Cfor less than 2 months, and non-dormant seeds were stored in the same conditions for 5-8 months. The TAR reaction was carried out at room temperature and the absorbance was measured after 15 minutes at 444 nm. Databook 10: 60-67; Databook 11: 12, 21; Databook 12: 28-34; Databook 13: 12-19.

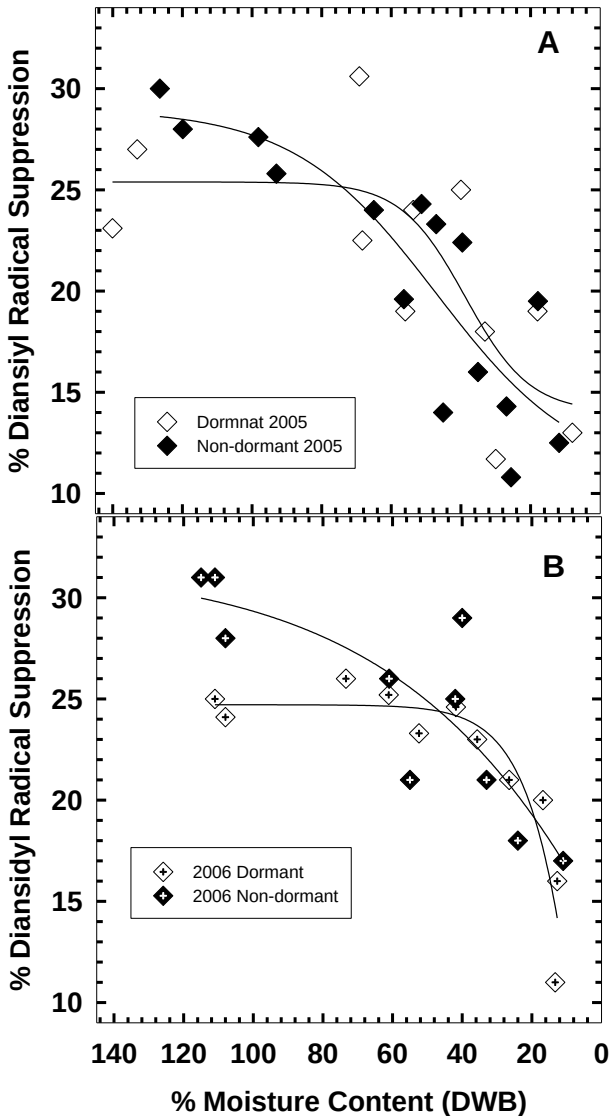

Figure A-38. Comparison of the total water soluble antioxidant response of dormant and non-dormant *S. alterniflora* seeds harvested in two different years. The seeds were harvested from Port Fourchon, Louisiana in (A) 2005 or (B) 2006. Dormant seeds are represented by the open symbols and non-dormant with closed symbols. Dormant seeds were stored submerged at 2°C for less than 2 months and non-dormant seeds were stored in the same manner for 5-8 months. The TAR reaction was carried out at room temperature for 15 minutes and the absorbance was taken at 444 nm. Databook 10: 60-67; Databook 11: 12, 21; Databook 12: 28-34; Databook 13: 12-19.

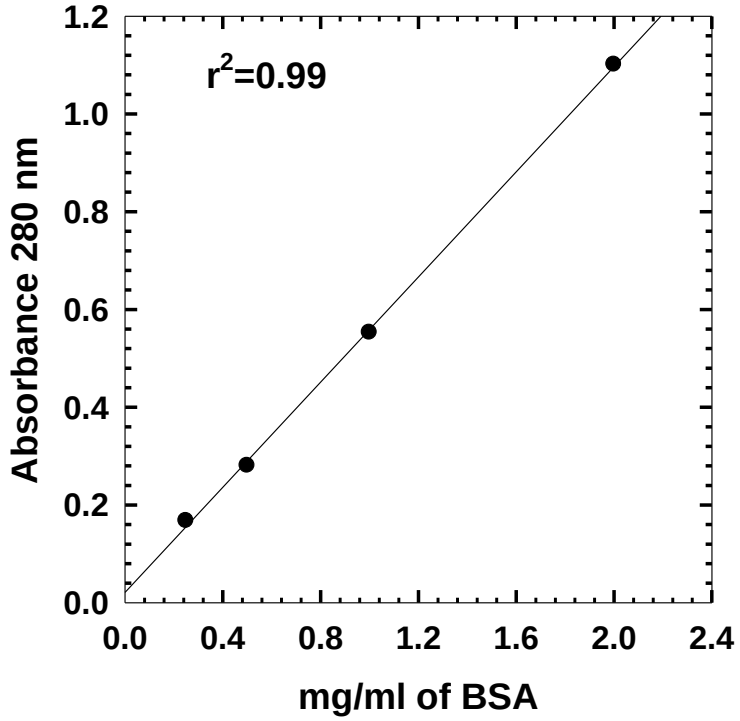

Figure A-39. Standard curve constructed from bovine serum albumin for the protein carbonyl assay. The curve was constructed to determine the absorbance of proteins at 280 nm. This was measured for the protein carbonyl assay, so nanomoles of carbonyl per mg of protein could be accurately calculated. Databook 10: 94; 12: 8.

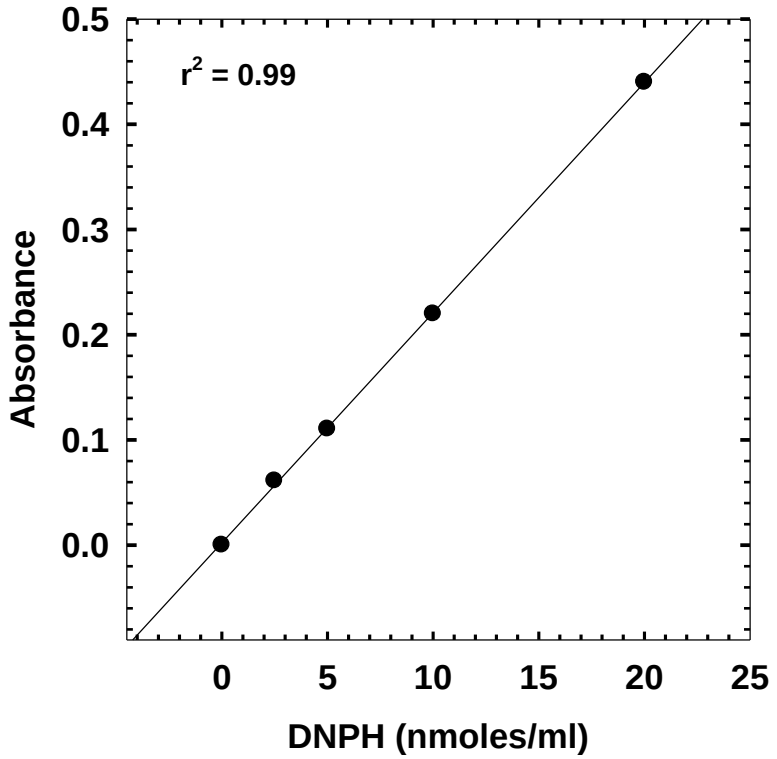

Figure A-40. Standard curve for protein carbonyl assay using 2,4- dinitrophenylhydrazine (DNPH). The DNPH reacts with the carbonyls and has a maximum absorbance as 395 nm. A standard curve was constructed to accurately calculate the total protein carbonyls present. The DNPH was dissolved in 2.5M HCl, and absorbance was measured at 395 nm. Databook 10: 96; databook 11: 6.

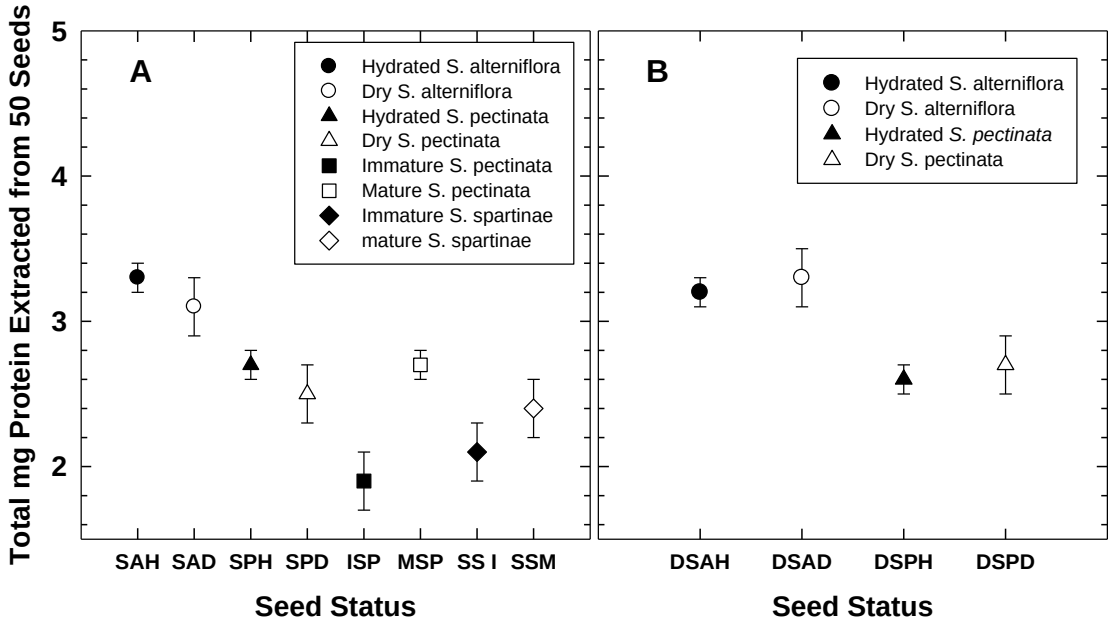

Figure A-41. Total protein amount extracted from 50 *Spartina sp*. seeds used for the protein carbonyl assay. Protein amounts were determined for (A) non-dormant and (B) dormant *Spartina sp.* seeds. Protein amount was determined after the proteins were precipitated with TCA and re-dissolved in 2 ml of 6 M guanidine- HCl. Concentration was determined by total absorbance at 280 nm. Graph coding is as follows: SAH, fully hydrated *S. alterniflora*; SAD, dry *S. alterniflora*; SPH, fully hydrated *S. pectinata*; SPD, dry *S. pectinata*; ISP, immature *S. pectinata*; MSP; mature *S. pectinata* (South Dakota seeds just completing maturation dry); SSI, immature *S. spartinae*; SSM, mature *S. spartinae* (mature seeds just completing maturation dry); (B) DSAH, dormant, fully hydrated *S. alterniflora*; DSAD, dry, dormant *S. alterniflora*; DSPH, dormant, fully hydrated *S. pectinata*; DSPD, dormant, dry *S. pectinata.* Databook 10: 88-90, 93-98; Databook 11: 7-11, 22-25, databook 12: 7-15, 25-27, databook 13: 55-62; databook 14: 25-31, 33-37.

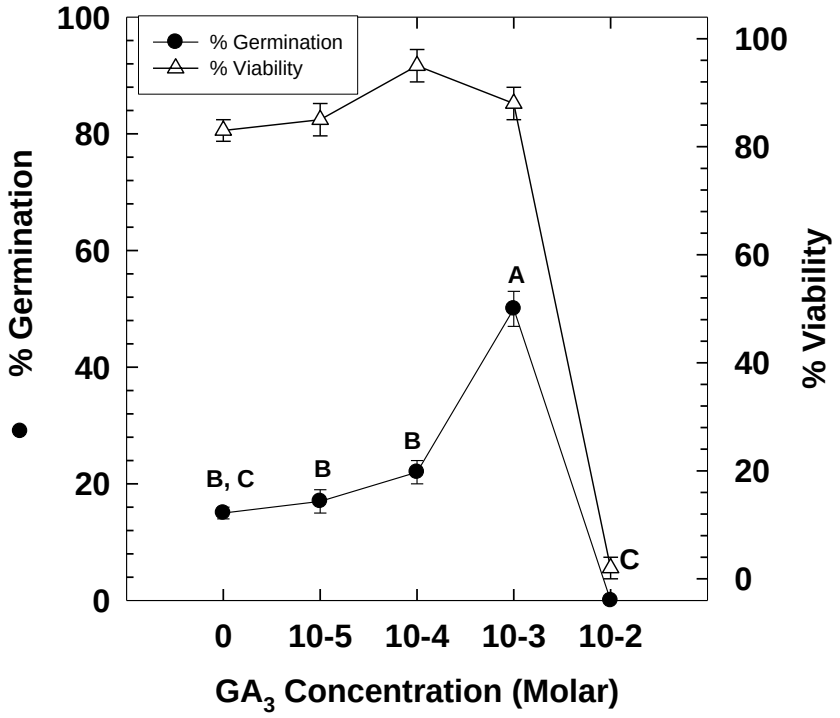

Figure A-42. Germination of *S. alterniflora* (26-18, 2002 harvest) seeds treated with gibberellic acid (GA_3) after 1 month of stratification at 2°C. Seeds were left 7 days with continuous contact on GA_3. After 7 days contact, seeds were rinsed and moved to a standard germination test at 27°C for 14 days. Different letters indicate a significant difference at $\alpha = 0.05$. These data are contrary to the findings of Plyler and Carrick (1993), where no effect of GA_3 on *S. alterniflora* was found. Databook 3: 26-29.

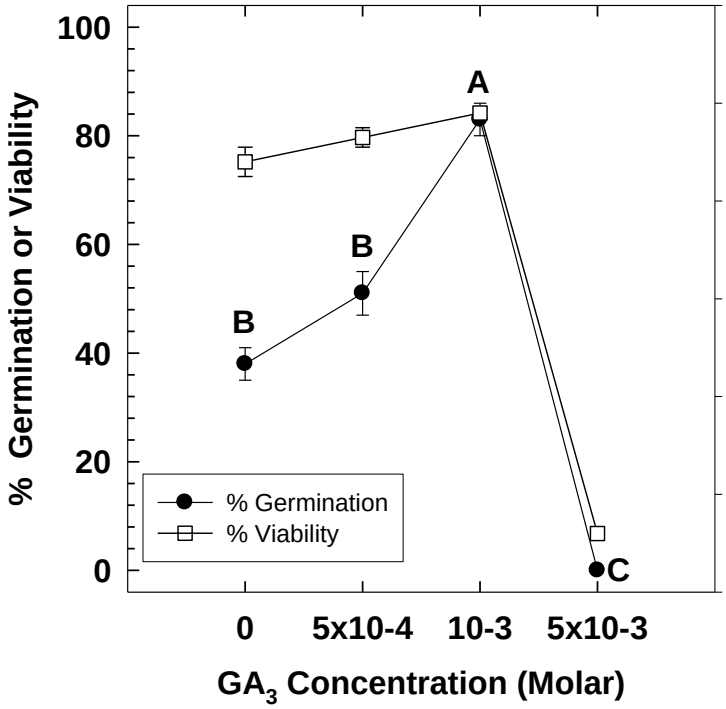

Figure A-43. Germination of *S. alterniflora* (26-18, 2002 harvest) seeds treated with gibberellic acid (GA_3) after 55 days of stratification at 2°C. Seeds were left 7 days with continuous contact on GA_3. After 7 days contact, seeds were rinsed and moved to a standard germination test at 27°C for 14 days. Different letters indicate a significant difference at $\alpha = 0.05$. This finding is similar to that of Figure A-42, except, the background germination increased as a result of stratification; however, the optimum GA_3 concentration remained the same. Databook 3: 43-46.

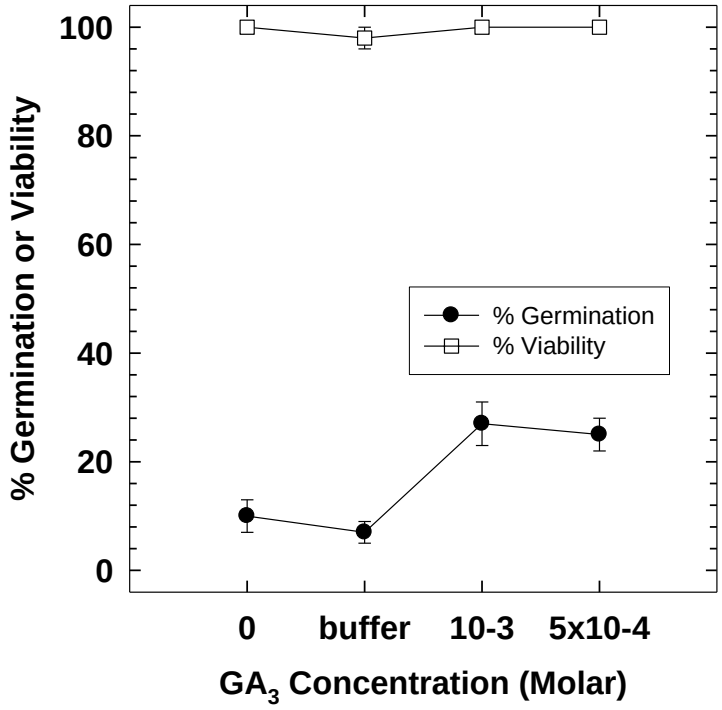

Figure A-44. Germination of wild-type *S. alterniflora* (Port Fourchon, 2005 harvest) seeds treated with gibberellic acid (GA_3) after 2 days of stratification at 2°C. Seeds were left 7 days with continuous contact on GA_3 + citrate phosphate buffer (pH 4.70). After 7 days contact, seeds were rinsed and moved to a standard germination test at 27°C for 14 days. There were no significant differences in germination for this treatment at $\alpha = 0.05$. Databook 10: 34-35.

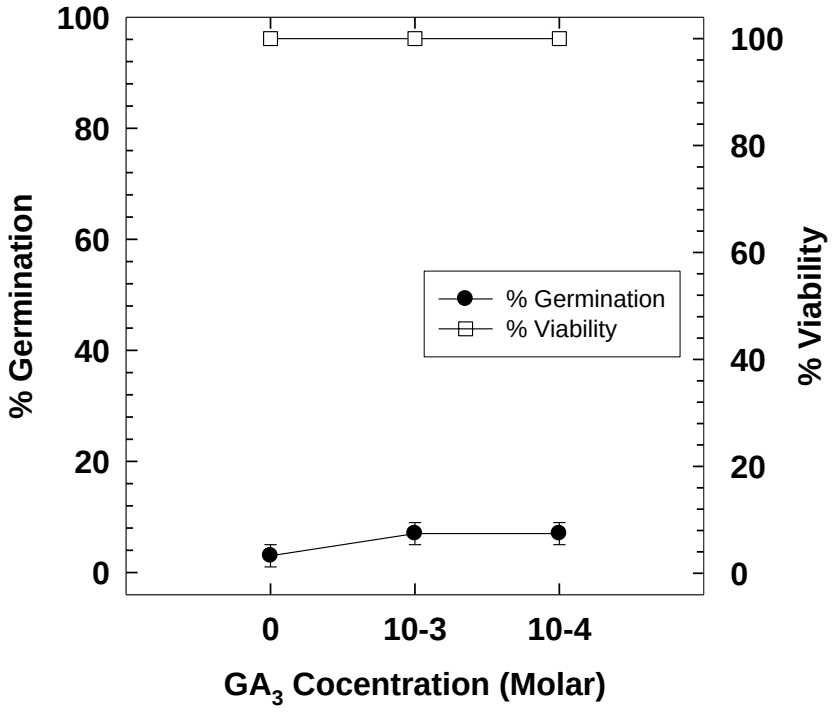

Figure A-45. Germination of wild-type *S. alterniflora* (Port Fourchon, 2005 harvest) seeds treated with gibberellic acid (GA_3) with no buffer used after 2 days of stratification at 2°C. Seeds were left 7 days with continuous contact on GA_3 (no buffer, initial pH 4.1). After 7 days contact, seeds were rinsed and moved to a standard germination test at 27°C for 14 days. There were no significant differences in germination for this treatment at $\alpha = 0.05$. These data, along with Figures A-42- A-44 suggest that GA_3 sensitivity increases as *S. alterniflora* seeds are stratified. Databook 10: 36-37.

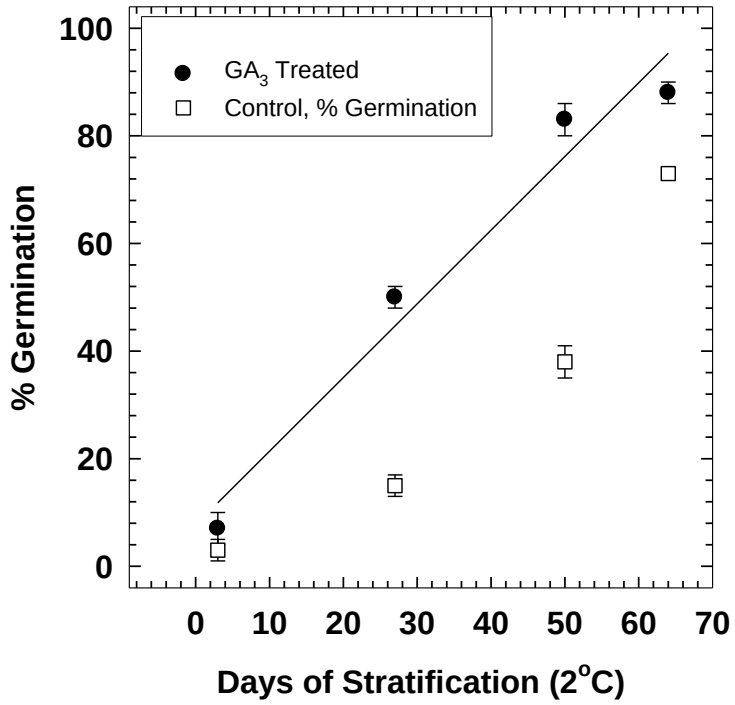

Figure A-46. Germination versus time of stratification for *S. alterniflora* (26-18 and Port Fourchon) seeds treated with 10^{-3} M GA_3. Seeds were left 7 days with continuous contact on GA_3 (no buffer, initial pH 4.1). After 7 days contact, seeds were rinsed and moved to a standard germination test at 27°C for 14 days. First two points are 26-18 accession seeds, and the experiment was performed in 2002; the upper two points are wild-type seeds harvested in Port Fourchon, Louisiana and the experiments were performed in 2005. These data suggest that stratification of *S. alterniflora* increases sensitivity to GA_3. Databook 3: 26-29, 43-46; Databook 10: 34-37.

Table A-1. Different contact times of propanol treatment to break dormancy of *S. alterniflora* (26-18, 2002 harvest). After prescribed contact time, a standard germination test at 27°C was performed. Databook 3:10-12

Concentration	Contact Time	Germination ± SE	Viability ± SE
0 mM	1 day	2 ± 2	80 ± 3
0 mM	2 day	2 ± 2	83 ± 3
0 mM	3 day	2 ± 2	82 ± 3
40 mM	1 day	10 ± 5	78 ± 4
40 mM	2 day	0 ± 0	77 ± 3
40 mM	3 day	0 ± 0	75 ± 5
80 mM	1 day	8 ± 4	83 ± 4
80 mM	2 day	3 ± 3	82 ± 3
80 mM	3 day	5 ± 3	75 ± 3

Table A-2. Treatment of dormant *S. alterniflora* seeds with propanol. *S. alterniflora* (26-13, 2002 harvest) seeds were stored 60 days submerged at 2°C. After 1 day of contact time with propanol, a standard 14 day germination test at 27°C was performed. Databook 3: 48-49.

Concentration	Contact Time	Germination ± SE	Viability ± SE
0 mM	1 day	22 ± 1	83 ± 3
100 mM	1 day	22 ± 3	83 ± 3
120 mM	1 day	38 ± 3	75 ± 2
140 mM	1 day	30 ± 2	70 ± 2

Table A-3. Kinetin treatment to break dormancy in *S. alterniflora*. At the time of the experiment the *S. alterniflora* (26-18)was stratified for 18 days at 2°C and treated with kinetin (initial pH 3.5, no buffer used) with 7 days continuous contact. Following kinetin treatment, seeds were placed in a standard germination test for an additional 14 days. Databook 3: 15-18.

Concentration	Contact Time	Germination ± SE	Viability + SE
0 M	7 days	13 ± 4	95 ± 2
10^{-5} M	7 days	12 ± 2	98 ± 2
10^{-4} M	7 days	13 ± 4	100 ± 0
5 x 10^{-3} M	7 days	7 ± 4	15 ± 4

Table A-4. Benzylaminopurine (cytokinin) treatment to break dormancy in *S. alterniflora* seeds (26-18, 2002 harvest).Seeds were stratified 33 days at 2°C and treated with benzylaminopurine (cytokinin) (pH 3.5, except for 5 x 10^{-3}M, which was 2.6; higher concentrations were insoluble) for 7 days of continuous contact. Following cytokinin treatment, seeds were rinsed with H_20 and placed in a standard germination test for an additional 14 days. Databook 3: 31-32.

Concentration	Contact Time	Germination ± SE	Viability + SE
0 M	7 days	8 ± 1	87 ± 2
10^{-5} M	7 days	18 ± 2	88 ± 3
10^{-4} M	7 days	13 ± 2	87 ± 1
10-3 M	7 days	8 ± 1	83 ± 2
5 x 10^{-3} M	7 days	8 ± 1	12 ± 3

APPENDIX B
EFFECTS OF FUNGICIDES ON ROOT AND SHOOTS OF *SPARTINA ALTERNIFLORA*

Does Cutting the Seed and Captan Application Inhibit Root Growth in Germinating *S. alterniflora* Seedlings?

RATIONALE

Previous experiments in this lab have shown that when the fungicide Captan is added during germination of *S. alterniflora* seeds, the root lengths are much shorter than in seeds with no Captan added. Also, when seeds are cut, which promotes germination in dormant and viable seeds, the roots are also reduced. This experiment tested that observation to determine the effects cutting and Captan application to *S. alterniflora* seeds.

MATERIALS AND METHODS

To determine if cutting, or the fungicide Captan, affected root length in seedlings, a germination test was performed with and without Captan on cut and intact seeds. This experiment was performed with non-dormant seeds (Vermillion) that were stored submerged for three months at 2°C.

The treatments used were intact seeds without Captan (Voluntary Purchasing Group, Bonham, USA), intact seeds with ca. 0.025g of Captan added, cut seeds without Captan and cut seeds with ca. 0.025g of Captan added. Each treatment contained 20 seeds and was replicated five times. A standard germination test at 27°C with light was used, and root lengths were recorded 14 days after the germination test began.

RESULTS

Effects of Captan and cutting on seedling development

Captan had a negative effect on root growth when applied to seeds (Figure B-1). Seed germination rate and extent were not affected (data not shown), but the roots were < 0.5 cm in

length when Captan was applied. In contrast, when no fungicide was added to intact seeds, the roots averaged ca. 3.0 cm in length. Cutting also had a negative effect on root growth. When the fungicide was added to cut seeds, the roots were short nubs that protruded from the seed coat. When no fungicide was added, the average root length for the cut seeds was ca. 0.75 cm, which is significantly reduced from the root lengths of intact seeds.

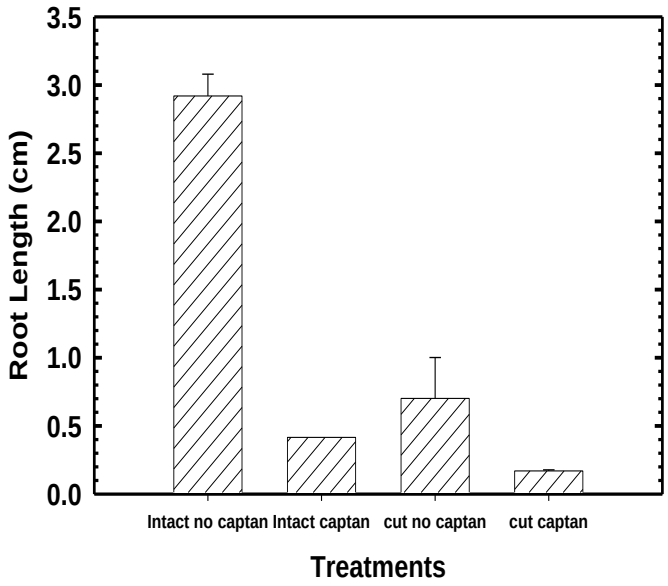

Figure B-1. Effect of Captan on *Spartina alterniflora* seedling root growth. Average root length of non-dormant Vermillion seeds for intact seeds without fungicide, intact seeds with fungicide, cut seeds without fungicide and cut seeds with fungicide. Seeds stored in submergence conditions at 2^0C for three months. Seeds were germinated at 27^0C with light for 14 days. Databook 3: 63-66; databook 5: 25-26.

What Effect Does Fungicide and Cutting Have on Seedling Vigor?

In the previous experiment, Captan severely stunted *S. alterniflora* root growth. If this fungicide was applied to seeds used in restoration projects, the stunted root growth could seriously impact seedling establishment. Such injury may also occur with other chemical pretreatments. Therefore, it was prudent to test several other fungicides for inhibition of root and shoot growth of germinating *S. alterniflora* seeds.

METHODS

Effects of Fungicide and Cutting Treatment on Seedling Root Length

S. alterniflora seeds harvested in 2003, and stored submerged at 2^0C for six months, were place on a backlight to check for full seeds. Twenty seeds per replication were used, and three replications per treatment were used. The treatments were: intact seeds treated with fungicides Captan (Voluntary Purchasing Group, Bonha, USA), Vitavax (Gustafson, Mkinney, USA), Kocide (Gustafson, Mkinney, USA), Allegiance (Mkinney, Gustafson, USA), Apron (Gustafson, Mkinney, USA), Maxim (Gustafson, Mkinney, USA) and a control with no fungicide. Also, cut seeds were treated with the same fungicides. All fungicides, with the exception of Captan, were added at concentrations of 0.1 ml/L up to 500 ml/L. Approximately, 0.025g of Captan, which is a powder, was added to each Petri dish of seeds. The solutions of fungicides were made up fresh from the commercial liquid formulations, and seeds were dipped in the fungicide for three seconds (M.C. Rush, personal communication). The seeds were dipped, rather than germinated in continuous contact with the fungicide, to mimic field conditions. In the field, the seeds will be coated with fungicide before they are planted. After the seeds were dipped, they were patted dry with a Kimwipe. Seeds were placed in a 9 x 9 x 2 cm square plastic Petri dishes that contained two pieces of moist (8 ml of H_20) Anchor paper. The seeds were placed in four rows of five seeds each, and then covered with a Kimwipe. Covered Petri dishes were placed in a moist germination box to maintain humidity. The germination box was placed at 27^0C in the light for 14 days. At the end of 14 days, the Petri dishes were removed, and germination percentage, shoot length and root length were scored.

RESULTS

Fungicide applications to *S. alterniflora* did not inhibit germination (root or shoot emergence)(data not shown). However, at higher concentrations of fungicide, the root lengths were greatly reduced. Also, the root lengths were affected much more than shoot lengths (Figures B-2 to B-6). Neither root nor shoot growth was greatly affected when treated with low doses of fungicides. However, as the fungicide concentrations increased, the root lengths become shorter than the average root length for the control seeds, which was 3.6 cm. Maxim appeared to have the most adverse effect on root growth of the seedlings (Figure B-4), causing root length to drop from an average root length of 3.6, when no fungicide was added, to less than 2 cm when 25 ml/L was added. Apron appeared to have the least effect on root growth, only causing the root growth to decrease to just above 2 cm, even at concentrations as high as 500 ml/L (Figure B-3). Vitavax did not have a great effect on root growth either, only causing the root length to decrease to just above 2cm at 100 ml/L of fungicide (Figure B-6). Allegiance (Figure B-2) and Kocide (Figure B-5) appeared to have a strong detrimental affect on root growth; both causing root lengths to drop to around 1cm when 100ml/L fungicide was added. At the lower concentrations, the fungicides did not appear to significantly affect shoot and root lengths. It appears that root growth was not severely affected until concentrations were increased to 25ml/L and above.

When seeds were cut, a decrease in both root and shoot lengths was observed. The control for the cut seeds had root and shoot lengths of 1.5 and 2.4 cm, respectively. The fungicides also affected root lengths at a much lower concentration in cut seeds than intact seeds. For example, the roots of cut seeds treated with 10ml/L of Allegiance were below 1 cm in length; however, intact seeds with the same treatment had root lengths greater than 3 cm (Figure B-2B). Cut seeds treated with Maxim had very stunted root growth; at only 5ml/L, the root lengths had already been reduced to 0.1 cm (Figure B-4). Vitavax did not have as great an impact on root

lengths as the other fungicides. The average length was reduced, but the length remained just below 1 cm in length, even when treated with 500 ml/L of Vitavax (Figure B-6).

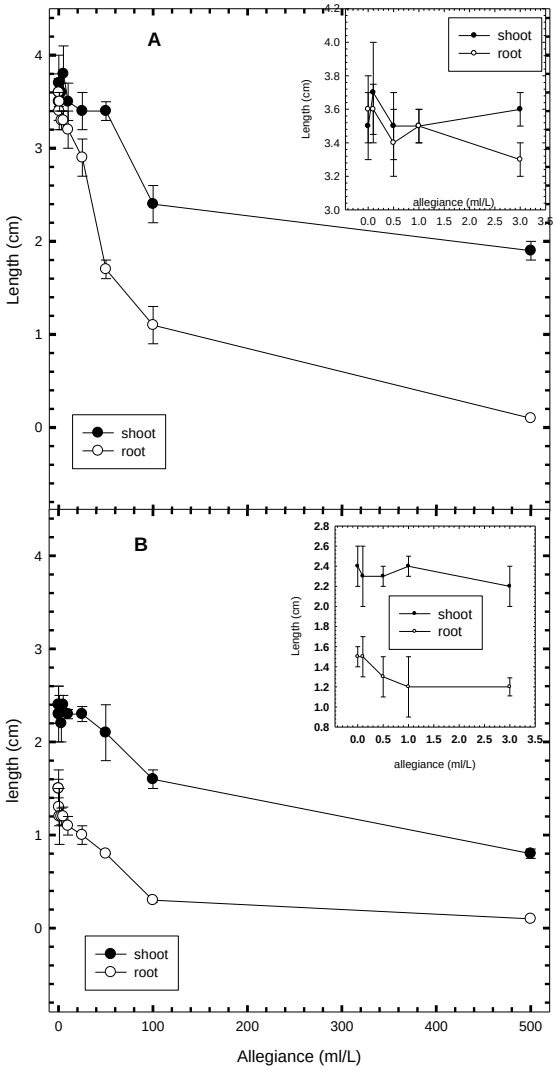

Figure B-2. The effect of Allegiance fungicide on root and shoot lengths of (A) intact non-dormant *S. alterniflora* seedlings (Port Fourchon, 2003 harvest) (B) Average root and shoot lengths of non-dormant cut *S. alterniflora* seeds treated with Allegiance (Port Fourchon, 2003 harvest). Seeds were stratified for 5 months submerged at 2^0C. Seeds were germinated at 27^0C with light for 14 days. This graph represents two runs. Insets represent concentrations 0-3ml/L of fungicide. Databook 5: 19-20, 33-34; databook 6: 5-6, 50-68.

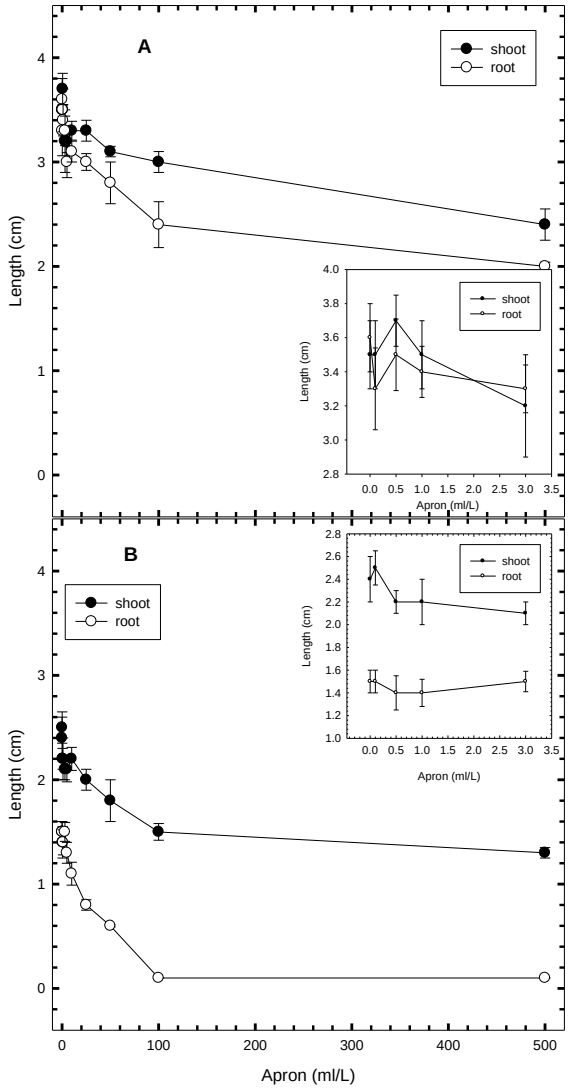

Figure B-3. The effect of Apron fungicide on root and shoot lengths of (A) intact non-dormant *S. alterniflora* seeds (Port Fourchon, 2003 harvest); (B) Average root and shoot lengths of non-dormant cut *S. alterniflora* seeds (Port Fourchon, 2003 harvest). Seeds were stratified for 5 months submerged at 2^0C. Seeds were germinated at 27^0C with light for 14 days. This graph represents two runs. Insets represent concentrations 0-3ml/L of fungicide. Databook 5: 37-38, 43-49, 72-100; databook 6: 8, 69-88

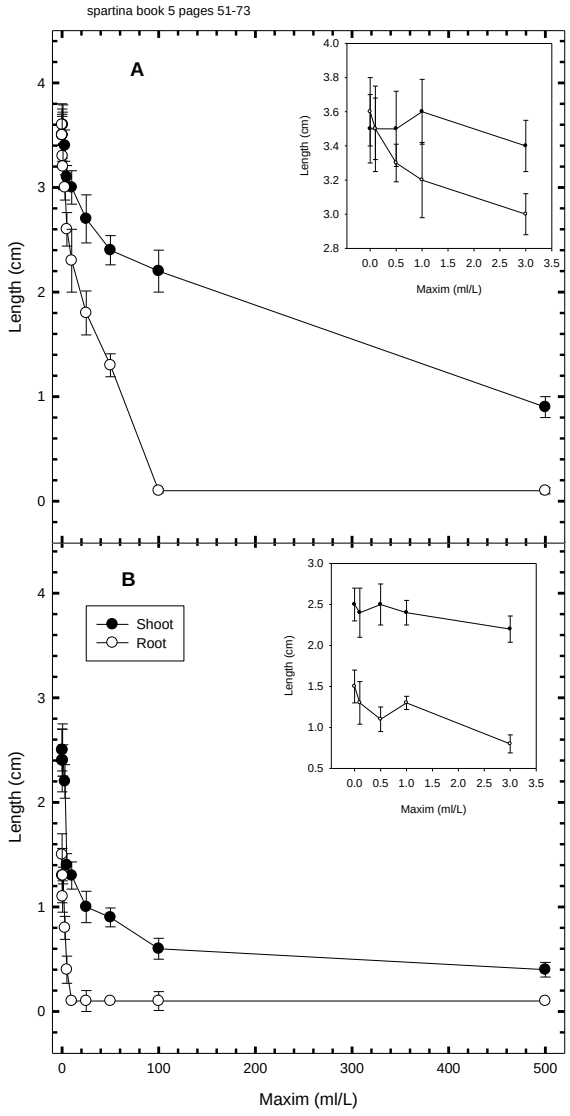

Figure B-4. The effect of Maxim fungicide on root and shoot lengths of (A) intact non-dormant *S. alterniflora* seeds (Port Fourchon, 2003 harvest), and (B) Average root and shoot lengths of non-dormant cut *S. alterniflora* seeds treated with Maxim (Port Fourchon, 2003 harvest). Seeds were stratified for 5 months submerged at 2^0C. Seeds were germinated at 27^0C with light for 14 days. This graph represents two runs. Insets represent concentrations 0-3ml/L of fungicide. Databook 5: 41-42, 71-88; databook 6: 9, 89-100.

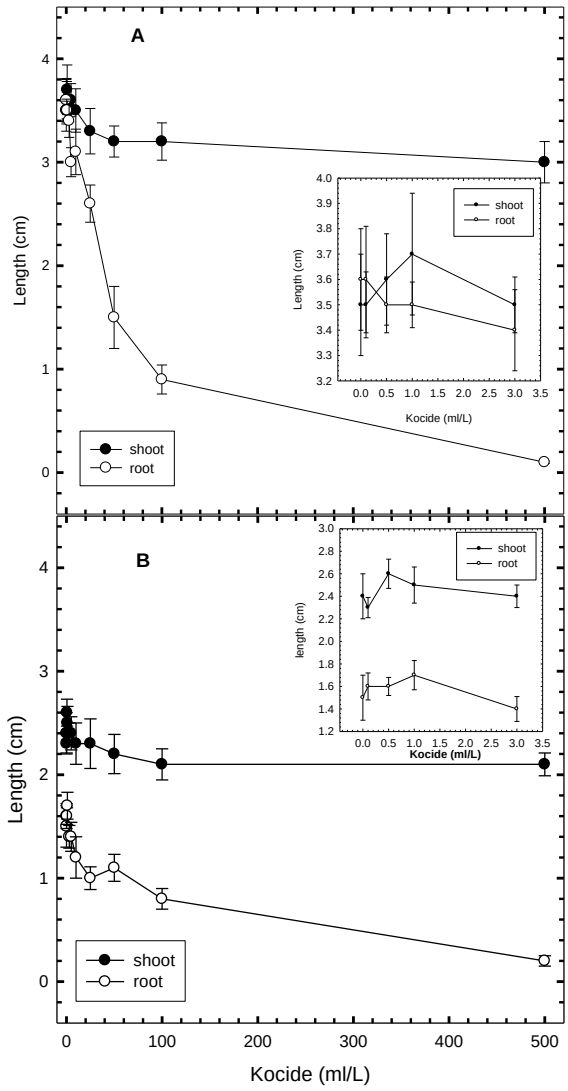

Figure B-5. The effect of Kocide fungicide on root and shoot lengths of (A) intact non-dormant *S. alterniflora* seeds (Port Fourchon, 2003 harvest), and (B) Average root and shoot lengths of non-dormant cut *S. alterniflora* seeds treated with Kocide (Port Fourchon, 2003 harvest). Seeds were stratified for 5 months submerged at 2^0C. Seeds were germinated at 27^0C with light for 14 days. This graph represents two runs. Insets represent concentrations 0-3ml/L of fungicide. Databook 5: 23-24, 35-36, 59-70; databook 6: 10-28.

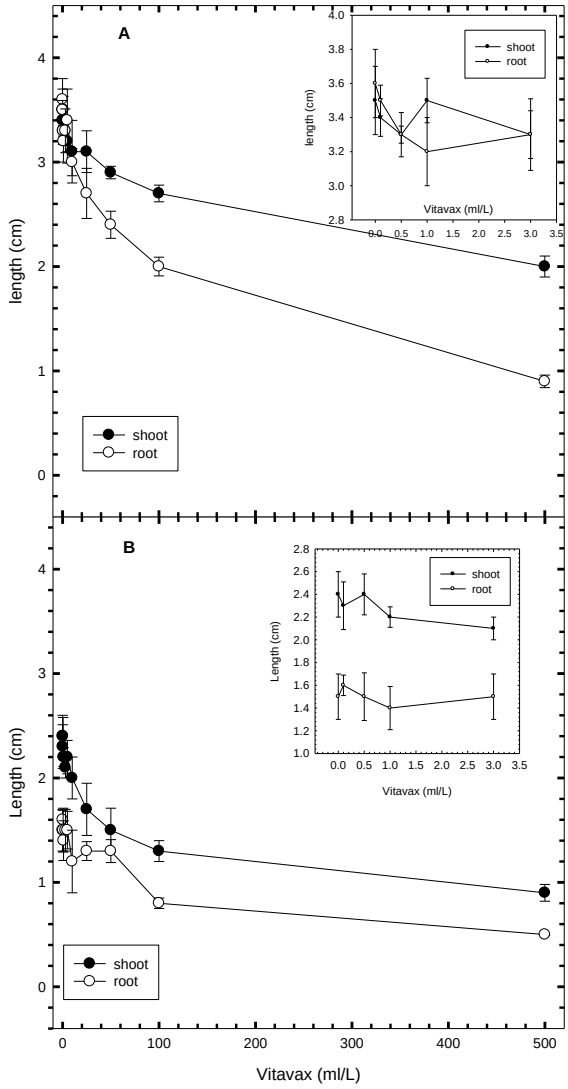

Figure B-6. The effect of Vitavax fungicide on root and shoot lengths of intact non-dormant *S. alterniflora* seeds (Port Fourchon, 2003 harvest), and (B) average root and shoot lengths of non-dormant cut *S. alterniflora* seeds treated with Vitavax (Port Fourchon, 2003 harvest). Seeds were stratified for 5 months submerged at 2^0C. Seeds were germinated at 27^0C with light for 14 days. This graph represents two runs. Insets represent concentrations 0-3ml/L of fungicide. Databook 5: 21-22, 39-40, 50-58; databook 6: 29-48.

DISCUSSION

At high concentrations, all of the fungicides tested had a detrimental effect on root growth. Shoots were also affected, but to a much lesser extent that roots. Roots may be more affected for a number of reasons. The first reason is the shoots may have a higher water content

than the roots, so the chemical is more diluted, hence causing less damage. Also, the roots are taking up water once they emerge, and even though the seeds were only dipped in the fungicides, there might still a halo of fungicide present around the seeds during germination. This may have caused the roots to absorb additional fungicide to further stunt their growth. This may also be the cause of more severe root stunting when Captan was applied. For the Captan experiments, Captan was placed in the Petri dish so it was present throughout germination. This may have allowed more Captan to be taken up by the roots and have a greater impact on root growth.

Cutting also causes root length to decrease, compared to intact seeds. The cut seeds in the control had root lengths that were less than half the root lengths in the intact control. The shoot lengths of the cut seed control were only slightly shorter than the intact control seeds. It is unclear why cutting would cause shorter root lengths; however, when the seeds are cut, sugars and amino acids vital to growth and metabolism leak out, leaving less energy for root growth.

When cut seeds were treated with fungicides, root and shoot growth decreased more than intact seeds, which could be caused by increased fungicide uptake. The extra fungicide that is allowed to enter should cause more stress to the seed, hence causing shorter root and shoot growth. Because a combined treatment of cutting and fungicides caused severe stunting of roots and slight decrease in shoot length, cutting seeds to break dormancy may not be used if a fungicide is applied. Further tests are required to determine whether or not fungicide concentrations that do not reduce seedling growth are sufficient to prevent fungal growth in a soil environment.

Because visible fungal growth was never observed on viable seeds during germination tests throughout all of the investigations reported in this dissertation, it was not possible to determine the effective fungistatic dose in relation to fungicide concentrations that inhibited

seedling growth. Non-viable seeds routinely supported endogenous fungal growth. The natural fungistatic properties of *Spartina alterniflora* seeds merit further investigation.

APPENDIX C
CORRESPONDING TABLES FOR FIGURES IN THE BODY OF THE DISSERATION

Table C-1. Data corresponding to Figure 2.2. Effect of temperature on time to first visible protrusion for shoots (solid bars) and roots (hatched bars) of *S. alterniflora* (Vermillion 2001, 2002 and 2004). Databook 3: 69-79; Databook 4: 15-18; Databook 8: 94-99.

Temperature (C°)	Shoot Emergence (h) ± SE	Root Emergence (h) ± SE
17	8 ± 2	30 ± 2
22	14 ± 4	28 ± 2
27	11 ± 2	23 ± 2
32	13 ± 1	19 ± 4
34	7 ± 1	11 ± 1
37	11 ± 3	44 ± 5

Table C-2. Data corresponding to Figure 2.3. Time to 50% germination for shoots and roots of *S. alterniflora* (Vermillion 2001 and 2002) seeds germinated at a range of temperatures. Databooks: 3: 69-79; Databook 4: 15-18, 22-31; Databook 8: 94-99.

Temperature	Shoot Emergence (h) ± SE	Root Emergence (h) ±SE
17	47 ± 3	60 ± 3
22	29 ± 3	45 ± 2
27	25 ± 2	38 ± 1
32	20 ± 1	34 ± 2
34	24 ± 1	28 ± 1
37	35 ± 4	82 ± 2

Table C-3. Data corresponding to Figure 2.4. Relationship between time of cold-stratification and germination for *S. alterniflora* seeds (wild type, 2006). Databook 7: 31, 44, 48

Months	% Germination ± SE
0	7 ± 2
1	17 ± 3
2	33 ± 6
3	82 ± 2
4	98 ± 2

Table C-4. Data corresponding to Figure 2.5 (A). Initial viability of *S. alterniflora* accessions after harvest. Databook 1: 59; Databook 3: 6; Databook 4: 55; databook 7: 46-47; databook 10: 30-31; databook 12: 20.

	26-13		Vermillion		7-14		7-8		26-18		27-2		WT		10C-WT	
Year	% V	SE	%V	SE	V	SE	%V	SE	%V	SE	%V	SE	%V	SE	%V	SE
2001	97	1.9	90	7.1	94	1.4	94	2.4	96	2.9	96	1.4	xxx	xxx	xxx	xxx
2002	88	3.8	87	8.0	96	2.0	88	4.3	96	1.6	82	6.9	xxx	xxx	xxx	xxx
2003	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	92	2.3	xxx	xxx
2004	96	1.8	96	1.4	96	3.0	93	2.4	97	2.4	98	1.3	xxx	xxx	xxx	xxx
2005	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	98	2.0	93	5.0
2006	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	95	1.2	97	2.0

Table C-5 . Data corresponding to Figure 2.5 (B). Stratification time required for 50% germination of *S. alterniflora* accessions harvested in 2001 (closed bars), 2002 (light grey hatched bars), and 2004 (dark grey hatched bars) from Ben-Hur Experimental Station, Baton Rouge, Louisiana and wild type *S. alterniflora* seeds harvested in 2003 (black with grey checks), 2005 (light grey with grey checks), and 2006 (dark grey with grey checks) from Port Fourchon, Louisiana; and wild type seeds (Port Fourchon, LA) harvested in 2005 (black bar with horizontal stripes) and 2006 (white bar with horizontal stripes) stratified at 10°C. Databook 4: 6-8, 23-24, 51-52, 61-62; databook 1: 59-71; databook 7: 44-47, 72, 92-93; databook 8: 40, 56, and 73; databook 10: 30-31, 58, 78; databook 12: 20-21, 35, 85, 97, 100.

Year	**26-13**	**Vermillion**	**7-14**	**7-8**	**26-18**	**27-2**	**WT**	**10C-WT**
2001	2.1	2.1	2.2	3.1	2.6	2.4	xxx	xxx
2002	2.3	1.3	2.1	2.1	1.2	2.4	xxx	xxx
2003	xxx	xxx	xxx	xxx	xxx	xxx	1.6	xxx
2004	2.5	2.1	3.0	3.1	1.6	2.8	xxx	xxx
2005	xxx	xxx	xxx	xxx	xxx	xxx	1.8	2.2
2006	xxx	xxx	xxx	xxx	xxx	xxx	2.3	3.0

Table C-6. Data corresponding to Figure 2.6. Three different methods of drying for recalcitrant *S. alterniflora* seeds. Air dried (●) and rapid dried (△) seeds were desiccated at room temperature (22-24°C) and seeds dried at 33% RH (■) were dried at 27°C. Databook 12: 36-46, 45-57,71-84, 86-90, 93-99.

% MC vs. Drying Time					
33% RH		**Air Dry**		**Rapid Dry**	
Days	%MC	Days	%MC	Days	%MC
0.0	120.0	0.0	120.0	0.000	115.0
1.0	41.4	1.0	47.2	0.020	59.1
2.0	43.8	2.0	42.4	0.020	52.9
3.0	34.3	5.0	41.6	0.020	61.7
5.0	26.7	7.0	31.3	0.042	53.6
6.0	26.0	10.0	27.9	0.042	53.2
7.0	23.5	12.0	28.7	0.083	50.6
10.0	14.3	15.0	24.0	0.083	50.0
15.0	12.6	17.0	23.6	0.125	48.6
20.0	8.6	20.0	19.6	0.280	33.0
		21.0	14.6	0.830	42.2
		25.0	9.4	1.0	44.7
				1.0	45.0
				2.0	39.3
				2.0	39.7
				2.0	37.2
				2.0	38.0
				5.0	21.5
				5.0	28.0
				5.0	27.0
				7.0	19.4
				10.0	7.5
				13.0	7.1
				14.0	6.5

Table C-7. Data corresponding to Figure 2.7. Viability of *S. alterniflora* seeds as a function of moisture content. Databook 13: 21-27, 33-38, 45-51, 60-68, 55-60, 80-85. (Rapid drying from Carbonyl and TAR experiments was used).

Drying Method					
Air		**33% RH**		**Rapid**	
% MC (DWB)	% Viability	% MC (DWB)	% Viability	%MC (DWB)	%Viability
122	97	125	93	120	100
118	98	122	97	119	100
115	100	119	100	118	100
51	90	118	98	115	100
50	90	44	100	115	95
50	85	41	95	115	95
49	75	40	50	114	90
49	85	35	40	110	100
46	80	35	60	54	100
46	100	30	45	53	90
43	60	29	30	46	95
43	75	28	60	45	80
43	70	26	30	44	85
39	100	26	50	43	95
39	35	23	15	42	80
38	50	18	10	37	55
36	75	9	0	25	15
36	40	7	0	21	10
29	70	7	0	14	5
28	45	6	0	7	0
26	45			7	5
25	0				
24	5				
24	0				
23	0				
23	5				
21	40				
21	0				
20	15				
18	35				
18	0				
18	0				
17	30				
17	0				
17	20				

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Table C-7 Continued

Drying Method					
Air		**33% RH**		**Rapid**	
% MC (DWB)	% Viability	% MC (DWB)	% Viability	%MC (DWB)	%Viability
17	5				
16	25				
14	20				
13	0				
13	5				
13	0				
13	0				
12	0				
10	0				

Table C-8. Data for dormant seeds corresponding to Figure 2.8. Viability of dormant *S. alterniflora* seeds as a function of moisture content. Shoot viability was scored after three different drying regimes: flash dry (Δ), 33 % relative humidity (RH) (■), and air dry (●). (Note: Non-dormant data in Figure 2.8 is identical to the data in Figure 2.7). Databook 12:36-46,45-57,71-84, 86-90, 93-99.

Drying Method					
Air		**33% RH**		**Rapid**	
% MC (DWB)	% Viability	% MC (DWB)	% Viability	%MC (DWB)	%Viability
111	100	115	100	111	95
108	90	110	95	111	100
106	95	100	100	108	100
102	95	41	90	102	100
48	80	40	80	100	100
48	85	38	85	73	85
47	95	36	75	61	90
47	95	26	50	59	85
47	85	26	50	54	90
46	95	25	75	53	100
46	95	21	30	53	95
44	80	21	35	52	95
43	80	12	2	51	90
43	85	10	2	50	90
42	80			49	95
42	95			45	95
41	100			42	80
40	90			40	80
38	80			39	70

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Table C-8 continued

Drying Method					
Air		33% RH		Rapid	
% MC (DWB)	% Viability	% MC (DWB)	% Viability	%MC (DWB)	%Viability
34	65			39	95
34	40			37	70
33	80			36	70
33	55			34	70
32	65			33	60
31	45			27	30
31	35			26	25
29	65			22	35
29	65			17	15
29	50			13	0
28	75			13	20
28	55				
27	40				
24	30				
24	25				
24	50				
24	35				
22	30				
21	40				
20	30				
20	10				
19	35				
15	0				
14	5				
14	10				
11	0				
10	0				
10	5				
9	0				
9	0				
8	0				

Table C-9. Data corresponding to Figure 2-9. Viability of non-dormant (closed symbols) and dormant (open symbols) *S. alterniflora* seeds as a function of moisture content when dried at 27°C at different relative humidities. Databook 12: 36-46, 58-70.

Non-Dormant					
KCl (93% RH)		**NaCl (75% RH)**		**$MgCl_2$ (33% RH)**	
% MC (DWB)	% Viability	% MC (DWB)	% Viability	% MC (DWB)	% Viability
110.0	100.0	110.0	100.0	110.0	100.0
105.0	100.0	105.0	95.0	105.0	95.0
60.4	95.0	51.6	100.0	51.5	80.0
55.7	90.0	51.0	70.0	44.1	80.0
50.9	90.0	50.8	90.0	40.4	90.0
50.2	90.0	49.6	85.0	39.6	85.0
49.5	90.0	47.3	55.0	38.7	60.0
47.3	65.0	46.2	65.0	38.6	80.0
47.2	85.0	45.8	80.0	38.2	80.0
46.8	65.0	43.9	65.0	28.7	70.0
46.7	70.0	43.3	90.0	26.9	55.0
46.0	50.0	43.2	60.0	26.8	55.0
44.9	85.0	42.8	50.0	26.3	55.0
44.8	55.0	41.0	65.0	26.1	50.0
44.4	65.0	40.6	70.0	25.5	45.0
42.6	65.0	38.8	80.0	24.1	55.0
42.5	60.0	38.3	50.0	23.2	60.0
41.6	60.0	37.1	40.0	21.5	45.0
38.9	65.0	35.2	55.0	12.9	15.0
38.0	70.0	33.2	65.0	10.1	0.0

Table C-10. Data corresponding to Figure 2.9. Viability of non-dormant (closed symbols) and dormant (open symbols) *S. alterniflora* seeds as a function of moisture content when dried at 27°C at different relative humidities. Databook 13:32-37, 45-51, 59.

Dormant					
KCl (93% RH)		**NaCl (75% RH)**		**$MgCl_2$ (33% RH)**	
%MC	%Viability	%MC	%Viability	%MC	%Viability
110	95.0	105.0	100.0	110	100.0
105	100.0	101.0	95.0	105	95.0
53	90.0	49.6	50.0	40	45.0
51	60.0	47.8	80.0	35	15.0
43	55.0	44.1	55.0	30	15.0
41	75.0	41.3	55.0	28	45.0
38	70.0	35.7	30.0	26	25.0
35	75.0	33.7	35.0	18	5.0

Table C-11. Data corresponding to Figure 2.11. Representative dry down curve for rapidly dried orthodox *S. pectinata* (▲) and *S. alterniflora* (△). Databook 13:55-63; databook 11: 51-59.

Time of Rapid Drying			
S. pectinata		***S. alterniflora***	
Days	**% MC**	**Days**	**%MC**
0.000	105	0.000	115
0.000	95	0.020	59
0.000	118	0.020	53
0.000	105	0.020	61
0.042	73	0.042	54
0.063	44	0.042	53
0.075	48	0.083	51
0.075	15	0.083	50
0.080	41	0.125	49
0.080	46	0.280	33
0.125	37	0.830	42
0.830	13	1.000	45
1.040	14	1.000	45
		2.000	39
		2.000	37
		5.000	22
		5.000	27
		7.000	19
		10.000	8
		13.000	7
		14.000	7

Table C-12. Data corresponding to Figure 3.1. TBA-reactive substances in non-dormant *S. pectinata* seeds (WNS-03A, lot 6042, Coaldale, CO) when the seeds were freeze- clamped in liquid N_2 before extraction or extracted at 4°C. Databook 9: 81-98.

Non-dormant *S. pectinata*				
% Moisture Content	4°C extraction	%MC	Freeze-clamped	% Viability
130	0.1510	142	0.1380	100
128	0.1500	140	0.1160	100
84	0.1400	139	0.1050	100
78	0.1500	72	0.1350	100
72	0.1400	71	0.1400	100
70	0.1490	67	0.1450	100
63	0.2160	60	0.1480	100
61	0.2180	59	0.1230	100
48	0.2200	58	0.1005	100
46	0.2580	57	0.1369	100
43	0.2570	55	0.1490	100
41	0.2500	51	0.1510	100

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Table C-12 continued from previous page

		Non-dormant		
% Moisture Content	4◦C extraction	%MC	Freeze-clamped	% Viability
39.0	0.2540	40.8	0.1470	100.0
36.0	0.2700	41.2	0.1360	100.0
35.0	0.2885	41.6	0.1410	100.0
31.0	0.2781	39.1	0.1345	100.0
28.0	0.2800	38.4	0.1504	98.0
21.0	0.2750	37.7	0.1600	100.0
15.0	0.1590	33.0	0.1432	98.0
11.0	0.1535	35.0	0.1209	100.0

Table C-13. Data corresponding to Figure 3.2. TBA-reactive substances (TBARS) in non-dormant *S. alterniflora* (Vermillion, 2004) when the seeds were freeze-clamped in liquid nitrogen before extraction (○), and when extraction was carried out at 4°C (●). Databook 9: 65-72, 76-78.

		Non-dormant *S. alterniflora*	
% Moisture Content	**4◦C extraction**	**%MC**	**Freeze-clamped**
160.0	0.1800	164.0	0.2100
155.0	0.2070	163.7	0.2120
108.0	0.1840	163.5	0.2440
82.2	0.2750	161.0	0.2400
78.0	0.3400	158.1	0.2600
76.1	0.3200	158.0	0.2500
66.9	0.2040	109.0	0.2200
48.6	0.2220	87.0	0.2400
48.1	0.2190	83.0	0.2720
37.4	0.2300	81.9	0.2320
33.2	0.2607	81.4	0.2600
31.0	0.2800	81.0	0.2400
29.5	0.3207	72.0	0.2320
18.0	0.3500	69.0	0.2400
14.2	0.3409	69.0	0.2280
13.0	0.2900	69.0	0.2520
		68.6	0.2640
		68.6	0.2040
		68.1	0.2640
		68.0	0.2560
		65.1	0.2800
		64.9	0.2600
		64.2	0.2800
		61.0	0.2680

Continued, next page

Table C-13. Continued from previous page

% Moisture Content	4◦C extraction	Non-Dormant %MC	Freeze-clamped
		60.6	0.2600
		60.0	0.2800
		57.7	0.2520
		57.0	0.2640
		55.0	0.2560
		54.0	0.2640
		54.0	0.2320
		52.3	0.2520
		51.0	0.2280
		50.0	0.2560
		46.0	0.2440
		45.0	0.2560
		45.0	0.2320
		44.0	0.2480
		40.8	0.2520
		40.0	0.2560
		39.8	0.2800
		39.4	0.2560
		35.0	0.2640
		31.0	0.2745
		27.0	0.2550
		20.0	0.2650
		17.0	0.2430

Table C-14. Data corresponding to Figure 3.3 panel C (non-dormant). The effect of drying upon viability and TBARS values, calculated according to Heath and Packer (1968), Du and Bramlage (1992) and Hodges et al (1999). Databook 8: 36, 41-44, 46-55, 57-66.

	Non-dormant- *S. alterniflora*		
% Moisture Content	Heath and Packer	Du and Bramlage	Hodges
164.0	0.2100	0.1801	0.0600
163.7	0.2120	0.1840	0.0600
163.5	0.2440	0.1840	0.0720
161.0	0.2400	0.1920	0.0480
158.1	0.2600	0.2160	0.0480
158.	0.2500	0.2000	0.0560
109.0	0.2200	0.1920	0.0520
87.0	0.2400	0.1760	0.0800
83.0	0.2720	0.1920	0.0640
81.9	0.2320	0.1920	0.0680

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	Non-dormant- *S. alterniflora*		
% Moisture Content	**Heath and Packer**	**Du and Bramlage**	**Hodges**
81.4	0.2600	0.2200	0.0720
81.0	0.2400	0.2080	0.0680
72.0	0.2320	0.2000	0.0520
69.0	0.2400	0.1760	0.0600
69.0	0.2280	0.2040	0.0680
69.0	0.2520	0.2000	0.0560
68.6	0.2640	0.2200	0.0720
68.6	0.2040	0.2160	0.0520
68.1	0.2640	0.2160	0.0440
68.0	0.2560	0.2400	0.0660
65.1	0.2800	0.2240	0.0640
64.9	0.2600	0.2400	0.0480
64.2	0.2800	0.2600	0.0560
61.0	0.2680	0.2400	0.0440
60.6	0.2600	0.2200	0.0440
60.0	0.2800	0.2360	0.0600
57.7	0.2520	0.2120	0.0560
57.0	0.2640	0.2240	0.0520
55.0	0.2560	0.2040	0.0560
54.0	0.2640	0.2160	0.0640
54.0	0.2320	0.1880	0.0360
52.3	0.2520	0.2400	0.0480
51.0	0.2280	0.1960	0.0520
50.0	0.2560	0.2280	0.0400
46.0	0.2440	0.1800	0.0520
45.0	0.2560	0.2160	0.0400
45.0	0.2320	0.1840	0.0640
44.0	0.2480	0.2040	0.0360
40.8	0.2520	0.1960	0.0400
40.0	0.2560	0.1880	0.0520
39.8	0.2800	0.2400	0.0600
39.4	0.2560	0.2040	0.0600
35.0	0.2640	0.2200	0.0400
31.0	0.2745	0.2304	0.0700
27.0	0.2550	0.2105	0.0650

Table C-15. Data corresponding to Figure 3.3 panel D. The effect of drying upon viability and TBARS values, calculated according to Heath and Packer (1968), Du and Bramlage (1992) and Hodges et al (1999). Databook 8: 22-40

	Non-dormant *S. pectinata*		
% Moisture Content	**Heath and Packer**	**Du and Bramlage**	**Hodges**
142.0	0.1380	0.0950	0.0210
140.0	0.1160	0.1010	0.0478
139.0	0.1050	0.1120	0.0401
72.0	0.1350	0.1060	0.0400
70.5	0.1400	0.1100	0.0350
67.0	0.1450	0.1210	0.0250
60.0	0.1480	0.1100	0.0401
59.1	0.1230	0.1150	0.0365
58.6	0.1005	0.1200	0.0430
57.0	0.1369	0.1250	0.0520
55.0	0.1490	0.1340	0.0350
51.0	0.1510	0.1260	0.0460
40.8	0.1470	0.1160	0.0499
41.2	0.1360	0.1201	0.0601
41.6	0.1410	0.1120	0.0402
39.1	0.1345	0.1280	0.0376
38.4	0.1504	0.1190	0.0563
37.7	0.1600	0.1260	0.0490
33.0	0.1432	0.1150	0.0330
35.0	0.1209	0.1070	0.0526
32.0	0.1490	0.1170	0.0301
26.0	0.1410	0.1201	0.0401

Table C-16. Data corresponding to Figure 3.4 (dormant). Comparison of TBARS values between dormant and non-dormant *S. alterniflora* seeds (Vermillion, 2004 and Port Fourchon, 2005). The upper panel represents the viability of dormant (□) and non-dormant (■) *S. alterniflora* seeds as they are flash-dried. Non-dormant data is presented in Table C-15. Databook 10: 40-46.

	Dormant *S. alterniflora*	
% MC	**Heath and Packer**	**Hodges**
123.4	0.2491	0.0784
113.8	0.2408	0.0518
111.8	0.2379	0.0517
111.4	0.2370	0.0755
82.9	0.2310	0.0795
69.8	0.2565	0.0575
49.1	0.2236	0.0782
49.0	0.2234	0.0594
39.0	0.2931	0.0709
37.0	0.2446	0.1048

Table C-17- Data corresponding to Figure 3.5 (A) (non-dormant, 2004 harvest). Ferrous xylenol orange (FOX) assay to measure lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. alterniflora* harvested in 2004 and 2005 from Ben-Hur Experimental Station, Baton Rouge, Louisiana and Port Fourchon, Louisiana. Databook 9: 16-51, 60-64, 73, 79.

	***S. alterniflora*, Non-dormant 2004 harvest**	
% Moisture content (DWB)	**FOX products (nanomoles seed^{-1})**	**% Viability**
161.0	0.560	100.00
102.0	0.600	100.00
84.9	0.580	100.00
84.0	0.590	100.00
81.3	0.560	100.00
81.2	0.600	100.00
81.0	0.730	100.00
79.5	0.880	100.00
79.4	0.930	100.00
79.2	0.950	100.00
79.2	0.950	100.00
75.9	1.010	100.00
72.0	1.180	100.00
71.0	0.920	100.00
70.0	1.110	98.00
69.8	1.030	100.00
69.8	1.010	100.00

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Table C-17. Continued from previous page

S. alterniflora, Non-dormant 2004 harvest		
% Moisture content (DWB)	**FOX products (nanomoles seed^{-1})**	**% Viability**
69.6	1.010	100.00
69.0	0.880	100.0
60.4	0.760	100.0
58.5	0.590	100.0
54.1	0.550	100.0
52.8	0.680	100.0
45.2	0.580	100.0
44.9	0.600	80.0
43.6	0.600	85.0
43.6	0.600	75.0
41.2	0.660	80.0
39.2	0.640	65.0
38.9	0.630	60.0
33.0	0.610	45.0

Table C-18. Data corresponding to Figure 3.5(A) (non-dormant, 2005). Ferrous xylenol orange (FOX) assay to measure lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. alterniflora* harvested in 2004 and 2005 from Ben-Hur Experimental Station, Baton Rouge, Louisiana and Port Fourchon, Louisiana. Databook 10: 48-57.

Non-Dormant *S. alterniflora* (2005 harvest)		
% MC (DWB)	**FOX products (nanomoles seed^{-1})**	**% Viability**
155.0	0.5790	100
154.0	0.5050	90
85.0	0.5350	95
70.0	0.5470	95
65.0	0.6060	100
59.0	0.5520	100
55.0	0.5560	95
48.0	0.5320	100
46.0	0.5450	100
46.0	0.5260	100
38.0	0.7240	80

Table C-19. Data corresponding to Figure 3.5 (A) (dormant 2005 harvest). Ferrous xylenol orange (FOX) assay to measure lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. alterniflora* harvested in 2004 and 2005 from Ben-Hur Experimental Station, Baton Rouge, Louisiana and Port Fourchon, Louisiana. Databook 10: 49-57.

Dormant *S. alterniflora* 2005 harvest	
% Moisture Content (DWB)	**FOX Positive Products (nanomoles seed^{-1})**
155.0	0.5880
154.0	0.5531
85.0	0.6942
77.0	0.6240
74.0	0.6339
65.0	0.6740
59.0	0.6321
56.0	0.6140
46.0	0.6070
43.0	0.6400
33.0	0.8000

Table C-20. Data corresponding to Figure 3.5 (B) (dormant). Ferrous xylenol orange (FOX) assay to measure lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. pectinata* purchased from Western Native Seed (WNS 03A, Lot # 6042). Databook 11: 87-99.

Dormant *S. pectinata*		
%MC (DWB)	**FOX products (nanomoles seed^{-1})**	**% Viability**
86.0	0.7960	100
85.0	0.8014	95
84.0	0.7600	95
78.0	0.7220	100
70.0	0.7677	100
61.0	0.7680	100
55.0	0.7500	95
45.0	0.7300	100
40.0	0.7740	100
38.0	0.7150	100
31.0	0.8100	100
13.0	0.7830	100
8.0	0.7800	100

Table C-21. Data corresponding to Figure 3.5 (B) (non-dormant). Ferrous xylenol orange (FOX) assay to measure lipid hydroperoxides as (A) dormant (open symbols) and non-dormant (closed symbols) *S. pectinata* purchased from Western Native Seed (WNS 03A, lot # 6042). Databook: 14: 13-16, 23-24, 36-38.

Non-dormant *S. pectinata*	
%MC (DWB)	**FOX products (nanomoles seed^{-1})**
104.0	0.3460
100	0.5490
95.0	0.5599
93.0	0.7800
85.0	0.9498
83.0	0.8430
79.0	0.8000
68.0	0.4450
65.0	0.5430
58.0	0.4990
52.0	0.0561
43.0	0.5327
41.0	0.5990
28.0	0.5560
25.0	0.5490

Table C-22. Data corresponding to Figure 3.6 (non-dormant, 2005). Organic and inorganic leachates amounts as *S. alterniflora* (Port Fourchon) seeds were desiccated. Databook: 10: 83-87.

***S. alterniflora* (whole seeds), Non dormant 2005 harvest**		
% MC (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates Absorbance (280 nm)**
112.0	XXX	0.275
79.5	XXX	0.335
62.7	XXX	0.290
56.4	XXX	0.288
17.7	XXX	0.285
15.3	XXX	0.301

Table C-23. Data corresponding to Figure 3.6 (non-dormant, 2006).). Organic and inorganic leachates amounts as *S. alterniflora* (Port Fourchon) seeds were desiccated. Databook: 13: 5-11, 32.

Non-dormant *S. alterniflora* 2006 Harvest		
% MC (DWB)	**Inorganic umhos ml^{-1}**	**Organic Leachates (A 280)**
116	80	0.225
115	71	0.265
114	67	0.356
110	71	0.299
54	74	0.277
53	71	0.297
44	68	0.315
43	68	0.236
37	71	0.258
21	65	0.276
14	73	0.341

Table C-24. Data corresponding to Figure 3.6 (dormant, 2006). Organic and inorganic leachates amounts as *S. pectinata* seeds were desiccated. Databook 12: 22-24.

Dormant *S. alterniflora* 2006 Harvest		
% MC (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates (A280)**
105	86	0.288
101	79	0.265
39	83	0.281
26	78	0.298

Table C-25.Data corresponding to Figure 3.7 (non dormant). Organic and inorganic leachates amounts as *S. pectinata* seeds were desiccated. Databook 10: 46-55.

	Non-dormant *S. pectinata*	
% MC (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates (A280)**
98.0	59	0.275
94.0	64	0.301
66.0	57	0.300
58.0	62	0.255
41.0	69	0.266
38.0	65	0.288
22.0	64	0.254
14.0	70	0.277
8.0	58	0.312

Table C-26. Data corresponding to Figure 3.7 (dormant). Organic and inorganic leachates amounts as *S. pectinata* seeds were desiccated. Databook 14: 32-41.

	Dormant *S. pectinata* whole seeds	
% MC (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates (A280)**
101.0	63	0.254
91.0	67	0.299
55.0	64	0.297
50.0	69	0.243
38.0	59	0.301
31.0	57	0.265
20.0	63	0.289
14.0	64	0.262

Table C-27. Inorganic (solid bar) and organic (hatched bar) leachates from the seeds of dried, aged *S. alterniflora* (Vermillion, 2004). This experiment was repeated twice Databook 10: 89; 12: 23.

	Dried, Aged Seeds	
	Inorganic Leachates umhos ml^{-1}	**Organic Leachates (A280)**
Dried, Aged Seeds	525	1.36

Table C-28. Data corresponding to Figure 3.9 (A). Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated after drying. Databook 14: 19-22.

Embryos of *S. alterniflora* isolated after desiccation		
% Moisture content (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates Absorbance at 280 nm**
123.9	112	0.797
112.9	100	0.699
47.4	125	1.069
43.0	136	1.120
18.0	245	1.550
12.9	220	1.516

Table C-29. Data corresponding to Figure 3.9 (B) (*S. pectinata*). Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated after drying.Databook 13:88-93.

Embryos of *S. pectinata* isolated after desiccation		
% Moisture content (DWB)	**Inorganic Leachates umhos ml^{-1}**	**Organic Leachates Absorbance at 280 nm**
98.0	104.0	0.730
15.0	230.0	1.200
13.0	240.0	1.400
38.0	128.0	1.100

Table C-30. Data corresponding to Figure 3.10 (A) (*S. alterniflora*). Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated before drying. Databook 14: 7-12.

Embryos of *S. alterniflora* isolated before desiccation		
% **Moisture content (DWB)**	**Inorganic Leachates uMhos ml^{-1}**	**Organic Leachates Absorbance at 280 nm**
123.0	112	0.797
113.0	129	0.683
75.0	118	0.687
68.0	130	0.650
42.0	147	0.801
38.0	151	0.830
22.0	213	1.250
18.0	225	1.280
16.0	231	1.300

Table C-31. Data corresponding to Figure 3.10 (B) (*S. pectinata*). Relationship between desiccation and leachate amounts from non-dormant *S. alterniflora* and *S. pectinata* embryos isolated before drying. Databook 14: 39-44.

Embryos of *S. pectinata* isolated before desiccation		
% **Moisture content (DWB)**	**Inorganic Leachates uMhos ml^{-1}**	**Organic Leachates Absorbance at 280 nm**
90	130	0.645
88	135	0.660
78	136	0.621
73	141	0.651
46	148	0.777
43	147	0.799
13	205	1.080
10	210	1.170

Table C-32. Data corresponding to Figure 4.1 (A) (non-dormant, 2005). Data corresponding to Figure 4.1 (A) (dormant, 2005). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook 10: 59-66.

Non-dormant *S. alterniflora* 2005 Harvest	
% MC (DWB)	**% Dianisidyl Radical Suppression**
127.0	30.0
120.0	28.0
98.0	28.0
93.0	26.0
65.0	24.0
56.0	20.0
51.0	24.0
47.0	23.0
40.0	22.0
35.0	16.0
27.0	14.0
26.0	11.0
18.0	20.0
12.0	13.0

Table C-33. Data corresponding to Figure 4.1 (A) (dormant, 2005). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook: 10: 59-66.

Dormant *S. alterniflora* 2005 Harvest	
% MC (DWB)	**% Dianisidyl Radical Suppression**
140.0	23.0
133.0	27.0
69.0	31.0
68.0	23.0
56.0	19.0
54.0	24.0
40.0	25.0
33.0	18.0
30.0	12.0
18.0	19.0
8.0	13.0

Table C-34. Data corresponding to Figure 4.1(A) (non dormant, 2006). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook 13: 12-19.

Non-dormant *S. alterniflora* 2006 Harvest	
% MC (DWB)	**% Dianisidyl Radical Suppression**
114.0	31.0
111.0	31.0
108.0	28.0
61.0	26.0
55.0	21.0
42.0	25.0
40.0	29.0
33.0	21.0
24.0	18.0
11.0	17.0

Table C-35. Data corresponding to Figure 4.1 (A) (dormant, 2006). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook 12: 28-34.

Dormant *S. alterniflora* 2006 Harvest	
% MC (DWB)	**% Dianisidyl Radical Suppression**
111.0	25.0
108.0	24.0
73.0	26.0
61.0	25.0
52.0	23.0
42.0	25.0
36.0	23.0
27.0	21.0
17.0	20.0
13.0	11.0
13.0	16.0

Table C-36. Data corresponding to Figure 4.1 (B) (Non-dormant). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook 11: 48-60.

Non-dormant *S. pectinata*	
% MC (DWB)	**% Dianisidyl Radical Suppression**
105.0	11.1
100.0	13.0
75.0	12.0
60.0	11.0
50.0	11.6
38.0	10.5
30.0	10.0
24.0	12.0
21.0	14.0
15.0	10.7

Table C-37. Data corresponding to Figure 4.1 (B) (dormant). Total water-soluble antioxidant capacity of *S. alterniflora* and *S. pectinata* seeds during rapid drying. Databook 11: 72-77.

Dormant *S. pectinata*	
% MC (DWB)	**% Dianisidyl Radical Suppression**
97.0	14.4
95.0	13.1
81.0	13.9
78.0	14.7
69.6	9.7
54.6	10.1
53.4	8.5
40.0	10.7
35.0	8.0
25.9	11.5
14.2	10.0
5.7	9.1

Table C-38. Data corresponding to Figure 4.2 (A) (*S. pectinata*) Total water-soluble antioxidant amounts in maturing (A) *S. pectinata* and (B) *S. spartinae*. Databook 13: 95-98.

Maturing *S. pectinata* (South Dakota)	
% **Moisture Content (DWB)**	**Dianisidyl Radical Suppression $\pm$ SE**
58	23.1 $\pm$ 2
40	21.4 $\pm$ 6
26	8.2 $\pm$ 2

Table C-39. Data corresponding to Figure 4.2 (B) (*S. spartinae*). Total water-soluble antioxidant amounts in maturing (A) *S. pectinata* and (B) *S. spartinae*. Databook 13: 87-94.

Maturing *S. spartinae* (Port Fourchon)	
% **Moisture Content (DWB)**	**Dianisidyl Radical Suppression $\pm$ SE**
129	16.5 $\pm$ 2.5
62	11.7 $\pm$ 5.4
13	8.5 $\pm$ 3.3
8	1.1 $\pm$ 1.4

Table C-40. Data corresponding to Figure 4.3 (non-dormant, 2005 harvest). Protein carbonyl assay with (DNPH) as an indicator of protein oxidation. Databook 10: 88-99; Databook 11: 3-11.

Non-dormant *S. alterniflora* 2005 Harvest	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
130	2.4
125	2.1
122	1.6
120	2.0
110	0.9
85	1.7
81	1.8
63	2.2
61	1.4
50	1.9
46	2.6
44	2.4
42	3.0
40	4.1
39	3.9
38	3.6
38	3.4
36	3.3
30	3.5
25	2.9
9	3.8
7	5.1
6	5.2
5	4.6

Table C-41. Data corresponding to Figure 4.3 (non-dormant, 2006 harvest). Protein carbonyl assay with (DNPH) as an indicator of protein oxidation. Databook 13: 55-62.

Non-dormant *S. alterniflora* 2006 Harvest	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
115.0	2.3
112.0	2.1
65.0	1.9
55.0	2.2
45.8	2.0
42.0	2.4
38.0	2.9
11.0	4.3
8.0	4.1
6.7	3.7

Table C-42. Data corresponding to Figure 4.3 (A) (dormant, 2006 harvest). Protein carbonyl assay with (DNPH) as an indicator of protein oxidation. Databook 12: 25-27, 70-71.

Dormant *S. alterniflora* 2006 Harvest	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
113.0	2.1
110.0	1.9
60.0	1.4
55.0	2.6
43.0	3.5
41.0	3.3
36.0	2.6
31.0	3.6
22.0	3.5
14.0	5.0
13.0	4.8
10.0	4.2

Table C-43. Data corresponding to Figure 4.3 (B) (non-dormant, *S. pectinata*). Protein carbonyl assay with (DNPH) as an indicator of protein oxidation. Databook 11: 26-27,33,39.

Non-dormant *S. pectinata*	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
98.0	4.8
91.0	5.1
65.0	5.4
58.0	5.0
38.0	4.7
36.0	5.5
13.0	5.3
9.0	5.5
8.0	5.7

Table C-44. Data corresponding to Figure 4.3 (B) (dormant, *S. pectinata*). Protein carbonyl assay with (DNPH) as an indicator of protein oxidation. Databook 12: 7-15.

Dormant *S. pectinata*	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
95.0	5.3
93.0	5.7
71.0	5.5
65.0	5.1
53.0	4.9
43.0	5.8
35.0	5.6
10.0	5.5
8.0	5.6

Table C-45. Data corresponding to Figure 4.4 (A) (*S. pectinata*, South Dakota). Protein carbonyl amounts during the first dry down (maturation dry) of (A) *S. pectinata* (South Dakota, 2007 harvest) in the field and (B) *Spartinae spartinae* (Port Fourchon, 2007). Databook 14: 25-31.

Maturing *S. pectinata* (South Dakota)	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
58.0	4.3 ± 0.4
40.0	4.4 ± 0.001
26.0	4.5 ± 0.1

Table C-46. Data corresponding to Figure 4.4 (B) (*S. spartinae*). Protein carbonyl amounts during the first dry down (maturation dry) of (A) *S. pectinata* (South Dakota, 2007 harvest) in the field and (B) *Spartinae spartinae* (Port Fourchon, 2007). Databook 14: 33-38.

Maturing *S. spartinae* (Port Fourchon)	
% Moisture Content (DWB)	**Protein Carbonyl amounts (nmoles mg-1 protein)**
129	7.4 ± 0.3
62	7.9 ± 1.2
13	11.8 ± 0.3
8	12.7 ± 0.2

VITA

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