SEED DORMANCY, SMOKE-STIMULATED GERMINATION AND HARVEST TIMING OF PILI GRASS (*HETERPOGON CONTORTUS*), A NATIVE HAWAIIAN GRASS WITH POTENTIAL FOR EXPANDED RE-VEGETATION USE

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By

Orville C. Baldos

Dissertation Committee:

Joseph DeFrank, Chairperson
Richard Criley
Kent Kobayashi
Robert Paull
Curtis Daehler

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ABSTRACT

Pili grass (*Heteropogon contortus* (L.) P. Beauv. ex Roem. & Schult.) is a culturally and ecologically important native species in Hawaiʻi that has been used for re-vegetation of severely degraded land. Currently, it is being evaluated for expanded use on roadside and streambank stabilization, water efficient landscaping and agricultural buffer strip plantings. Despite efforts to increase its use on a variety of re-vegetation applications, planting materials such as seeds are still in limited supply. In order to develop large scale seed production protocols, studies on seed dormancy and seed harvest timing need to be conducted. The three main objectives of this dissertation were to: 1) elucidate the mechanisms involved in pili grass seed dormancy loss through the use of scarification and germination stimulants (i.e., gibberellic acid [GA], fluridone, food grade liquid smoke [FGLS], pili grass smoke infused water, xylose smoke infused water, karrikinolide and cyanide); 2) assess the effect of storage factors (i.e., seed moisture content and temperature) on pili grass seed dormancy loss and viability; and 3) determine an average cumulative growing degree unit (ACGDU) correlated indicator which has potential use for determining the optimum seed harvest timing.

Studies on seed scarification, germination with stimulants and dormancy loss under different storage conditions support the placement of pili grass under the non-deep physiological dormancy class of seeds. The observed partial germination with scarification (i.e., exposure of the embryo) suggests the presence of both physical and physiological dormancy controls.

Germination assays with the plant growth regulators, fluridone and GA, as well as with FGLS suggests that the abscisic acid (ABA):GA balance plays an important role in
pili grass seed dormancy. Seed germination response to chemical stimulants across a gradient in dormancy relief supports the theory of increased effective levels of GA as storage duration increases.

Short term seed storage studies (i.e., 1 year or less) show that dormancy and seed viability loss is affected by storage temperature and equilibrium relative humidity (eRH) (i.e., seed moisture content). Storage at 10°C, regardless of eRH, maintained seed dormancy and viability for one year. To optimize dormancy loss while maintaining seed viability, fresh seeds must be stored at either 12% eRH (6% seed moisture content, dry weight basis) at 30°C for 12 months or at 50% eRH (11% seed moisture content, dry weight basis) at 30°C for 9 months. As storage temperature was increased from 20 to 30°C and storage relative humidity was increased from 50 to 75% eRH (11 to 14% seed moisture content, dry weight basis), loss in seed viability became more pronounced over time. The viability of pili grass seeds, relieved of dormancy, can be maintained for at least 6 months with storage at 12% eRH (6% seed moisture content, dry weight basis) and 5°C.

FGLS, pili grass smoke infused water and xylose smoke infused water were effective in stimulating germination of dormant pili grass seeds. FGLS and pili grass smoke infused water provided consistent germination stimulation in two batches of seed that represented two levels of dormancy relief. Xylose infused smoke water was less effective since its ability to stimulate germination was not consistent across the two seed batches. Assays which evaluated two bioactive compounds found in smoke (i.e., karrikinolide and cyanide) indicated that only cyanide stimulated germination (20 to 29% germination) of dormant pili grass seeds. Cyanide stimulated germination suggests the
role of reactive oxygen species in dormancy loss of pili grass seeds under warm, dry conditions. The presence of cyanide was confirmed in pili grass smoke, but not in FGLS or xylose smoke infused water. Germination observed in these non-cyanide containing smoke sources indicates the presence of other bioactive compounds in smoke.

Germination assays conducted throughout this dissertation indicated differences in depth of dormancy with time of year harvest. March seeds exhibited the lowest levels of dormancy compared to seeds harvested in June, July and October. Seasonal dormancy in pili grass seeds can be attributed to differences in growing conditions during seed development.

Finally, harvest timing studies identified ACGDU, spike moisture content and the onset of seed head tangling as useful harvest timing indicators for pili grass seed production. Results indicate that maximum seed harvests can be obtained between 768 to 778 ACGDUs (79 to 82 days after cutting) under irrigated conditions. Spike moisture for optimum seed harvest timing was determined to be between 0.68 to 0.72 grams H$_2$O per gram of dry weight. The onset of seed head tangling provided a visual cue which coincided with the optimum seed yield and range of spike moisture content. A decline in seed production over four harvest cycles (spanning two years) was recorded with possible causal factors such as stand age, cutting height and thatch accumulation.
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CHAPTER 1

INTRODUCTION

Restoration and re-vegetation of altered land is an important activity for mitigating human impacts on the environment. While past efforts have involved planting a few species that were often non-native, there has been growing awareness that restoring ecosystem function and biological diversity requires the use of native plants (Lesica and Allendorf, 1999). In recent years, restoration and re-vegetation projects using native plants have greatly increased. Native grasses, in particular, have been extensively utilized not only for ecological restoration, but also for other uses such as pasture improvement, roadside and post-fire re-vegetation and urban landscaping (Knapp and Rice, 1994).

In Hawai‘i, applications for native grasses are currently expanding. Interest has grown in the past decade to incorporate more native Hawaiian plants, including native grasses (Eickhoff, 2013), in built landscapes. State laws such as the Hawai‘i Administrative Rules Pertaining to Acts 73 and 236 were instrumental in promoting the use of native Hawaiian plants in state funded projects (Tamimi, 1999). These state laws have resulted in increased utilization of native Hawaiian plants by landscape architects (Tamimi, 1999). State agencies such as the Hawai‘i Department of Transportation have also adopted the use of native Hawaiian plants in roadside re-vegetation projects (Bornhorst, 2010). Research efforts to develop planting and establishment protocols for a limited number of species have also been ongoing for the past five years (Baldos, 2009, Baldos et al., 2010) in support of an initiative to increase use, supply and awareness of native Hawaiian plants (Dacus, 2009).
Pili grass (*Heteropogon contortus* (L.) P. Beauv. ex Roem. & Schult.) is one of several native Hawaiian grasses currently being evaluated for expanded use in Hawai‘i. Pili grass is a perennial C4 bunchgrass found on all main islands of the Hawaiian Archipelago and throughout the tropics (Wagner *et al.*, 1999). In Hawai‘i, it is usually found on dry rocky cliffs, ledges or slopes near the ocean and from elevations ranging from sea level to 700 m (Wagner *et al.*, 1999).

Pili grass is an important re-vegetation and restoration species in Hawai‘i due to its cultural and ecological significance (Daehler and Goergen, 2005) and ability to grow in low resource conditions (i.e., low rainfall and low fertility soils) (USDA-NRCS, 2007). Over the past decade, pili grass has been extensively utilized in the restoration and re-vegetation of Kaho‘olawe (USDA-NRCS, 2007) as well as some offshore islands around O‘ahu (Smith, 2006). In recent years, its use has been expanded for water efficient landscaping (Board of Water Supply, 2004, USDA-NRCS, 2007, Aldridge *et al.*, 2009), streambank stabilization (Crago and Puttock, 2008), native species roadside re-vegetation (DeFrank and Lukas, 2012) and buffer strip plantings (Fares, personal communication June 17, 2010).

Despite efforts to increase utilization of pili grass in a wide variety of re-vegetation applications, planting materials especially seeds, are still in limited supply. This is due to the lack of information available on seed treatment and seed production technologies for the species. Seed dormancy and seed harvest timing are two important aspects that need to be studied in order to develop large scale seed production protocols. Seed dormancy is a major constraint to the expanded use of pili grass since freshly harvested seeds do not germinate or have very low germination. Dormancy relief requires
a dry storage period of 6 to 12 months (Tothill, 1977, Pater, 1993, Daehler and Goergen, 2005, USDA-NRCS, 2007), an exogenous application of 1% gibberellic acid (Tothill, 1977, Goergen and Daehler, 2001, USDA-NRCS, 2007) or application of aerosol smoke (Campbell, 1995, Campbell et al., 1996). Although dry storage is effective for dormancy relief in pili grass seeds, storage conditions (i.e., temperature and relative humidity/seed moisture content) to optimize dormancy loss while maintaining seed viability have yet to be examined in detail. In addition, the mechanisms involved in dormancy of pili grass seeds have not been studied. The contribution of seed coats (i.e., husk and endosperm) in germination inhibition (Bewley and Black, 1994, Baskin and Baskin, 2001c) and the stimulatory effects of gibberellic acid (Ali-Rachedi et al., 2004, Feurtado et al., 2007), fluridine (Grappin et al., 2000, Ali-Rachedi et al., 2004, Chae et al., 2004, Feurtado et al., 2007), smoke (Nelson et al., 2012) and the smoke derived compounds, karrikinolide (Chiwocha et al., 2009, Nelson et al., 2009, Nelson et al., 2012, Flematti et al., 2013) and cyanide (Flematti et al., 2011b, Nelson et al., 2012, Flematti et al., 2013) may provide insights on factors involved in pili grass seed dormancy.

Seed harvest timing is another aspect of pili grass seed production that needs further study. It is an important concern for seed producers since it affects yield and quality of seeds (Andersen and Andersen, 1980, de Andrade, 1993, Desai et al., 1997). Timely harvest is important for maximizing mature seed yield and minimizing losses due to immature seed (Desai et al., 1997, Bedane et al., 2006), seed shattering (Berdahl and Frank, 1998, Bedane et al., 2006, Wang et al., 2006) as well as losses from pest and diseases (Desai et al., 1997).
Harvest maturity indicators used to determine seed crop maturity may have potential utility for estimating the optimum harvest time for pili grass seeds. Flowering stages, degree of shattering, seed moisture content and seed weight are harvest indicators that have been evaluated and used in a number of grass and forage species (Andersen and Andersen, 1980, Berdahl and Frank, 1998, Garcia-Diaz and Steiner, 2000, West and Pitman, 2001, Lemke et al., 2003, Wang et al., 2006, Wang et al., 2008). These indicators are often related to growing degree units (GDU) to estimate optimum seed harvest timing (Berdahl and Frank, 1998, Wang et al., 2006). For example, spike water and seed mass was related to GDU after peak anthesis to determine harvest timing in the cool season grasses, *Agropyron desertorum*, *Psathyrostachys juncea*, *Thinopyrum intermedium* and *Pascopyrum smithii* (Berdahl and Frank, 1998). GDUs are the accumulated heat units or thermal time above a constant crop-specific base temperature (Hodges, 1991). It has often been used to describe and predict growth and development of plants (McMaster and Wilhelm, 1997) and has been useful for predicting optimum seed harvest timing in a number of species (Lemke et al., 2003, Bedane et al., 2006, Wang et al., 2006, Berti and Johnson, 2008). Finding an indicator which can be correlated to GDU can help increase yield and efficiency of pili grass seed harvests.

The three main objectives of this dissertation were: 1) to elucidate the factors involved in pili grass seed dormancy loss through the use of scarification and germination stimulants; 2) assess the effect of storage factors (i.e., seed moisture content and temperature) on pili grass seed dormancy loss and viability; and 3) to identify an average cumulative GDU (ACGDU) correlated indicator which has potential use for determining
the optimum seed harvest timing for pili grass. The specific objectives of the study were to:

1. Assess the effects of scarification and nicking on germination of dormant pili grass seeds.
2. Evaluate the effects of storage temperature and seed moisture content on dormancy loss and viability of pili grass seeds.
3. Examine the role of gibberellic acid (GA) and abscisic acid (ABA) on dormancy loss of pili grass seeds.
4. Evaluate the germination stimulation capacity of three different smoke sources (i.e., food grade liquid smoke, pili grass smoke infused water and xylose smoke infused water) and two recently isolated germination stimulants in smoke (i.e., cyanide and karrikinolide).
5. Relate ACGDU (after cutting) to the development of spike and culm components (e.g., moisture content, number of spikes, seed weight, seed number etc.).
6. Examine the effects of growing season on spike and culm development.

This dissertation is organized into 8 chapters. The current chapter (Chapter 1) provides the background, significance and objectives of the study. Chapter 2 focuses on the contribution of seed coats (i.e., endosperm and husk) on pili grass seed dormancy by assessing the effect of nicking different parts of the seed. Chapter 3 investigates the effects of storage temperature, storage relative humidity/seed moisture content and storage duration on non-deep physiological dormancy and viability of pili grass seeds. Chapter 4 is a follow up study on seed storage designed to characterize storage protocol...
for optimizing dormancy loss while maintaining seed viability. Seeds dried to 6% moisture were stored at the optimum dormancy loss temperature (30°C) for 6 months. Following storage to promote dormancy loss, seeds were stored for an additional 6 months at three temperatures (5°C, 20°C and 30°C) to determine the impact of prolonged storage on seed viability. Chapter 5 examines the impact of GA and ABA in dormancy loss of pili grass seeds. In this study, the ABA levels in the imbibed seeds were reduced using fluridone, a carotenoid biosynthesis inhibitor. Besides elucidating the role of GA and ABA, this study also examined the effect of food grade liquid smoke on the germination of pili grass seeds. Chapter 6 expands on the results obtained with food grade liquid smoke. Aqueous smoke derived from three sources (i.e., food grade liquid smoke, combustion of xylose and pili grass to produce smoke infused water) as well as two recently identified compounds in smoke (i.e., karrikinolide and cyanide) were evaluated for efficacy in stimulating germination of dormant pili grass seeds. The smoke sources were tested for the presence of cyanide. In Chapter 7, the association between ACGDU and spike/culm components was examined to identify potential harvest maturity indicators for pili grass. Harvest indicators were tracked across two years and 4 harvest cycles. Chapter 8 summarizes the key findings of the dissertation and its ecological implications, provides recommendations for further research and incorporates the findings with a suggested protocol for pili grass seed production and establishment.
CHAPTER 2
ELUCIDATING SEED DORMANCY MECHANISMS IN PILI GRASS
[HETEROPOGON CONTORTUS (L.) P.BEAUV. EX ROEM. & SCHULT.]: THE
EFFECT OF CUTTING AND NICKING ON SEED GERMINATION

Introduction

Seed dormancy is an important mechanism that plants utilize to distribute germination in space and in time (Bewley and Black, 1994, Li and Foley, 1997). It ensures the seed’s survival by preventing uniform germination under conditions which might prove unsuitable for establishment (Chen and Maun, 1998). According to Baskin and Baskin (2004), seed dormancy can be classified into five major classes. These are: 1) physiological dormancy; 2) morphological dormancy; 3) morphophysiological dormancy; 4) physical dormancy and 5) combinatorial dormancy. Among these dormancy classes, physiological dormancy (i.e., non-deep level) is the most common form and is found in gymnosperms and all major angiosperm clades (Baskin and Baskin, 2004, Finch-Savage and Leubner-Metzger, 2006). Seeds possessing non-deep physiological dormancy fail to germinate at any temperature or possess a very narrow range of germination temperatures (Baskin and Baskin, 2001b). Excised embryos from these seeds usually grow to produce normal seedlings. Treatments to break non-deep physiological dormancy include cold and warm stratification, scarification, exogenous application of gibberellic acid and dry after-ripening (i.e., a period of dry storage) (Baskin and Baskin, 2004).
Pili grass (*Heteropogon contortus*) is a drought tolerant native grass that is currently being evaluated in Hawaii for expanded re-vegetation use. It is a perennial bunchgrass that grows to approximately 0.4 to 1 m high; exhibits pale bluish green leaf blades measuring 10 to 30 cm long by 3 to 7 mm wide and bears solitary racemes up to 7 cm long (Wagner *et al.*, 1999). Each raceme can carry up to 18 spikelet pairs. Up to six pairs at the base contain either male or sterile spikelets while up to 12 spikelet pairs above contain a fertile female floret and a male or sterile floret. Caryopsis of this species are dark brown, 8 to 10 mm long by 1 mm wide and are equipped with a sharp callus at the base (attached to the floret) and a hygroscopic awn (up to 12 cm long) at the tip to facilitate seed dispersal and burial.

Pili grass is considered as an important native Hawaiian re-vegetation/restoration species because it has both cultural and ecological value; and has been in rapid decline in recent years (Daehler and Goergen, 2005). Seed dormancy is a limiting factor for large scale re-vegetation with pili grass. Freshly harvested seeds exhibit poor or no germination. Pili grass seed dormancy can be partially broken by repeated wetting and drying and by removal of enclosing glumes (Tothill, 1977). Complete removal of seed dormancy can be accomplished either by exogenous application of 1% gibberellic acid (Tothill, 1977, Goergen and Daehler, 2001, USDA-NRCS, 2007) or by dry after-ripening (Tothill, 1977, Daehler and Goergen, 2005). Based on these characteristics, pili grass can be classified under the non-deep physiological class of seed dormancy.

Physiological dormancy is determined by the sum and interactions of dormancy imposed by the embryo and coat (i.e., any embryo covering structures such as the testa, endosperm and/or pericarp) (Finch-Savage and Leubner-Metzger, 2006). Embryos can
change dormancy status of seeds by regulating plant hormones (i.e., abscisic acid, gibberellic acid and ethylene) (Baskin and Baskin, 2004, Finch-Savage and Leubner-Metzger, 2006), removing or absorbing products of enzyme hydrolysis and regulating endosperm inhibitors (Baskin and Baskin, 2001c). Coats or covering structures can prevent germination by interfering with water uptake, mechanically restraining the embryo, interfering with gas exchange, preventing the exit of inhibitors in the embryo and supplying inhibitors to the embryos (Bewley and Black, 1994, Baskin and Baskin, 2001c). While most studies on breaking pili grass seed dormancy have focused on the “whole” seed (i.e., combined effects of embryo and coat), little is known about the dormancy contributions of the seed’s covering structures. To gain a better understanding of the mechanisms involved in pili grass seed dormancy, it is essential to test whether coats inhibit seed germination. This study evaluated the efficacy of four physical scarification modes (i.e., naked caryopses, top cut, spear cut and spear side nicked seeds) on promoting germination in dormant pili grass seeds.

**Materials and methods**

**Seed source**

Pili grass seeds (Kaho‘olawe source identified germplasm) were obtained from irrigated, open-field plantings at the Magoon Research Station inside the University of Hawaii at Manoa campus (21° 18' 18.7698", -157° 48' 37.4508"). Collection and harvesting of seeds were done by hand on March 22 (first experimental run) and June 21, 2012 (second experimental run). The harvested seeds were of similar maturity, since they...
were collected 92 (March harvested seeds) and 91 (June harvested seeds) days after cutting the grass stand. After harvesting, the seeds were air dried for 22 days under ambient temperature and relative humidity and were passed through an air blast seed cleaner (Almaco, Nevada, IA) to remove most of the chaff, awns and empty seeds. The seeds were equilibrated for 46 days inside dessicators with saturated lithium chloride solutions. This saturated salt solution was used to maintain a relative humidity of 12% at 25°C (Winston and Bates, 1960, Commander et al., 2009). After equilibration, the seeds were placed in foil barrier packets (Seed Savers Exchange, Decorah, IA). The packets were sealed using a heat impulse sealer (Uline, Pleasant Prairie, WI) and stored in the freezer (-18°C) until used.

**Tetrazolium test**

Viability of the March and June harvested seeds was assessed using tetrazolium tests. The tetrazolium test is a widely recognized and accurate means of estimating seed viability and is a routine test in many seed testing facilities (Copeland and McDonald, 2001). It identifies the presence of dehydrogenase activity in actively respiring viable seed tissues (Elias et al., 2012). During respiration, dehydrogenase enzymes react with substrates and release hydrogen ions (Copeland and McDonald, 2001, Elias et al., 2012). The hydrogen ions react with the colorless tetrazolium chloride solution to produce a red dye called formazan (Copeland and McDonald, 2001, Elias et al., 2012). The staining pattern formed on the seeds with this reaction allows for the assessment of seed viability. Four replicates of 50 seeds were obtained from each seed harvest date and placed in 20 ml scintillation vials filled with distilled water. The seeds (pre-stored at 12% relative
humidity and frozen at -18°C) were incubated in water overnight to allow imbibition and activation of enzymes. After soaking, the imbibed seeds were placed in a petri dish and cut longitudinally through the middle of the embryonic axis (Peters, 2000). Half of the cut seed was returned back to the vial and the other half was discarded. After a batch of 50 seeds had been cut, the water in the vials was replaced with 3 ml of 1% tetrazolium chloride (Sigma Aldrich, St. Louis, MO) solution. The vials were incubated overnight in the dark at room temperature to allow staining. The stained seeds were washed with distilled water, arranged in a petri dish lined with moistened filter paper and observed under a dissecting microscope. Seeds were counted as viable based on specific staining patterns (Figure 1). Fully stained embryos (without endosperms stained) were considered as viable seed while partially stained embryos or stained whole seeds (i.e., both the endosperm and embryo is stained, caused by fungal contamination) were considered as non-viable. Viable seed numbers per replicate were expressed as a percent of the total number of seeds (i.e., 50). Mean percent seed viability was calculated for both seed batches.

**Mechanical scarification treatments**

Four types of mechanical scarification (naked caryopses, top cut, spear cut and spear side nicked seeds) were applied to different areas of the seeds to determine which part contributes most to the promotion of germination (Figure 2). Scarification was done at 75 (March harvested seeds) and 97 (June harvested seeds) days after harvest or 28 (March harvested seeds) and 51 (June harvested seeds) days after cold storage. Naked caryopses were obtained by gently removing the hull using a scalpel. Top cut seeds had
approximately 25% of seed removed from the awn side to expose a cross section of the endosperm. Spear cut seeds had the spears removed to expose a small part of the endosperm without exposing the embryo. Spear side nicked seeds had spears removed and exposed the embryo by removing approximately half of the endosperm and seed coat surrounding it. Besides the four scarification treatments, a control treatment consisting of intact seeds was also prepared.

**Germination assay**

Germination assays were used to evaluate the efficacy of the scarification treatments. Scarified and intact seeds (control) were sown on 100 x 15 mm round polystyrene petri dishes (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA) lined with 1 layer of filter paper (Whatman #3, Whatman International, Piscataway, NJ). The filter paper was pre-moistened with deionized water prior to sowing. Each treatment was replicated four times with 50 seeds sown in each petri dish. After sowing the seeds, the petri dishes were sealed on the sides with parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent drying. The petri dishes were incubated under 30°C constant temperature (Tothill, 1977) with 12 hours of supplemental light. Lighting was provided by a 60 watt incandescent grow light bulb (Philips Lighting Company, Somerset, NJ). The petri dishes were observed regularly for germination and re-wetted with deionized water as needed. Final percent germination was collected 20 days after sowing. Seeds were counted as germinated when at least 1 mm of the radicle had emerged.
Experimental setup and statistical analysis

The germination studies were set up as a randomized complete block with 4 replicates. Due to a large number of treatments that yield a zero value for percent germination, two types of statistical testing were conducted. Fisher’s exact tests were conducted to compare the number of germinated and non-germinated seeds across and within seed batches (Curtis Daehler, personal communication, November 15, 2013). Treatments with non-zero germination (at least 1 replicate exhibiting germination) within an experimental run were logit transformed and modeled using a generalized linear mixed model with a binomial distribution (i.e., PROC GLIMMIX in SAS version 9.3, SAS Institute Inc., Cary, NC). Generalized linear mixed models allow modeling of data exhibiting a non-normal distribution or heterogeneous variance and also include both fixed and random effects (Sileshi, 2012). The binomial response distribution has been found to be appropriate for studies involving proportion based germination data (Willenborg et al., 2005, Pendleton et al., 2012). Treatments with all replicates exhibiting zero germination result in zero values for both mean and variance. According to Reeve and Strom (2004), treatments that have zero or low variance can reduce the magnitude of the mean square error, which is the denominator for F tests and also a component of multiple comparison procedures. Removal of these treatments in the analysis would ensure that error rates are near their specified values (i.e., mean square error is not reduced due to zero variance treatments). In addition, this would also facilitate analysis with generalized linear mixed models. Software developed for these models, such as PROC GLIMMIX, can have numerical problems if zeros (i.e., all replicates are zero) are present in the data (Reeve and Strom, 2004). F tests were calculated based on Wald
statistics and means (on the logit scale) were separated using conservative Tukey-Kramer

grouping (P <0.05). For ease of interpretation, mean percent germination and viability in

Figure 3 were back transformed from the logit scale using the equation:

\[
a = 100 \left( \frac{e^x}{1 + e^x} \right)
\]

Where \( x \) is the logit estimate from the model, and \( a \) is the approximate

ergination or viability percentage after back transformation.

**Results**

Tetrazolium tests indicate that both seed batches exhibited a high degree of seed

viability. The average percent viability of the March 2012 seeds was 94%. June 2012

harvested seeds exhibited an average percent viability of 99%.

Consistent non-germination and consistent germination across seed batches were

recorded from specific scarification treatments (Figure 3). Non-germination was observed

in both experimental runs of intact as well as spear cut seeds. In contrast, seeds with spear

sides nicked consistently exhibited germination regardless of seed batch.

Naked caryopses and top cut seeds exhibited erratic germination across the seed

batches. Germination in these treatments occurred in the March 2012 seeds but exhibited

very little to no germination in the June 2012 seeds. Due to the presence of treatments

exhibiting zero and erratic germination in the two experimental runs, Fisher’s exact tests

were conducted to compare treatments between seed batches as well as between zero and

non-zero germinated treatments within an experimental run.
Fisher’s exact tests comparing the March and June germination of each scarification treatment (i.e., treatments exhibiting consistent germination across seed batches) indicated a significant difference in germination with seed batch (Table 1). Naked caryopses and spear side nicked seeds harvested in March exhibited significantly more germination than the June harvested seeds (Table 1 and Figure 3).

Fisher’s exact tests of the first experimental run (i.e., March harvested seeds) showed significant differences between scarification treatments exhibiting high levels of germination and those exhibiting non-germination (Table 2). Significant germination was observed in naked caryopses \((P = 0.0000)\) and seeds with spear side nicked \((P = 0.0000)\) when these were compared with those observed in intact seeds and in seeds with spear cut. Seeds with top cut off exhibited germination that was not significantly different from either intact seeds or seeds with spears cut \((P = 0.1231)\).

In the second experimental run, spear side nicked seeds and naked caryopses were the only scarification treatments which exhibited germination (Table 3). Fisher’s exact tests indicate that only the spear side nicked seeds exhibited significant germination \((P = 0.0000)\) when compared to treatments exhibiting no germination (i.e., spear cut, intact seeds and top cut off). Although germination was observed in seeds with naked caryopsis, the proportion of seeds that germinated was very small and did not significantly differ from treatments exhibiting non-germination \((P = 0.4987)\).

Significant differences in percent germination between treatments were recorded in the GLIMMIX analysis of each seed batch (Table 4). In the first experimental run (i.e., March 2012 seeds), spear side nicked seeds exhibited the highest percent germination (30%) followed by naked caryopses (16.5%) and seeds with top cut (2%) (Figure 3). In
the second experimental run (i.e., June 2012 seeds), spear side nicked seeds also exhibited the highest percent germination (19.5%). This was followed by seeds with naked caryopses (1%).

**Discussion**

Results of the study indicate that coats can contribute to the germination inhibition of dormant pili grass seeds. Both the husk and the endosperm surrounding the embryo play a role in imposing dormancy since removal of these seed parts stimulated germination. The observed germination inhibition appears to be caused in part by physical restrictions of embryo growth rather than restricting water uptake of the seed (i.e., physical dormancy). This is supported by the germination observed in spear side nicked seeds (i.e., embryo exposed). In this scarification treatment, percent germination was moderate (i.e., 20 to 30%) and was consistent across seed batches. In contrast, the scarification treatments designed to improve water uptake/break physical dormancy (i.e., naked caryposes and seeds with top and spear ends cut) resulted in poor (<17%) and inconsistent germination across seed batches.

According to Baskin and Baskin (2004), seeds possessing non-deep physiological dormancy can also be induced to germinate with scarification. Dormancy break by scarification appears to be related to the weakening of the seed layer (i.e., testa, endosperm or both) covering the embryo (Baskin and Baskin, 2004). In seeds of many species where coat imposed dormancy is present, the seed envelope tissue enforces a physical constraint, thereby preventing radicle protrusion (Kelly *et al.*, 1992, Bewley,
This physical restriction on the embryo can be overcome either by increasing embryo growth potential or by enzymatically weakening the tissues surrounding the embryo (Bewley and Black, 1994, Baskin and Baskin, 2001c, Koornneef et al., 2002). In the case of nicking, tissues surrounding the embryo were artificially removed, allowing physically restricted embryos to germinate.

The observed germination stimulation with artificial removal of the endosperm (i.e., nicking to expose the embryo) may suggest the involvement of gibberellic acid and abscisic acid in pili grass seed dormancy. Endosperm weakening is promoted by gibberellic acid and inhibited in part by abscisic acid through the abscisic acid:gibberellic acid ratio (Finch-Savage and Leubner-Metzger, 2006). Hydrolytic enzymes responsible for endosperm weakening such as expansin (Koornneef et al., 2002), endo-β-mannase (Bewley and Black, 1994, Finch-Savage and Leubner-Metzger, 2006) and class I β-1,3-glucanase (Koornneef et al., 2002, Finch-Savage and Leubner-Metzger, 2006) are mediated by gibberellic acid (Finch-Savage and Leubner-Metzger, 2006).

Despite observing consistent germination, nicking the seeds to expose the embryos did not completely relieve seed dormancy of pili grass. Although average seed viability of the two seed batches were high (94 and 99%), percent germination of spear side nicked seeds was only between 19.5 to 30%. This observation, together with the findings on the contribution of pili grass coats (i.e., endosperm), confirms the presence of physiological factors involved in pili grass seed dormancy. Past studies by Tothill (1977) and Campbell et al. (1996) as well as studies conducted in chapters of this dissertation...
(Chapters 3, 4, 5 and 6) have shown that pili grass seed dormancy is primarily physiological.

Besides partial germination stimulation through nicking (i.e., removal of endosperm to expose the embryo), differences in the depth of dormancy between seasons were also observed. March harvested seeds generally exhibited more germination (i.e., less depth of dormancy) than June harvested seeds. Evidence of time of year harvest affecting dormancy status was also presented in Chapters 3 and 6 of this dissertation. In general, March harvested seed appear to exhibit less dormancy than seeds harvested in June (current chapter), July (Chapter 6) or October (Chapter 3). Seasonal differences in depth of seed dormancy can be attributed to the growing conditions seeds received during its development. Environmental conditions which can affect depth of dormancy include daylength, light quality, mineral nutrition, soil moisture, temperature and physiological age of mother plants (Simpson, 1990, Baskin and Baskin, 2001a).

In summary, the findings obtained from this study indicate that the endosperm surrounding the embryo can partly contribute to the germination inhibition of dormant pili grass seeds. Coat-imposed germination inhibition likely appears to be a physical restriction to the embryo rather than a restriction to water uptake. The literature cited here indicates that this physical restriction can be overcome by hydrolytic enzymes which soften the endosperm. Gibberellic acid and abscisic acid appears to be involved in pili grass seed dormancy since these hormones also control endosperm weakening. Scarification to expose the embryo was partially effective in relieving seed dormancy. Percent germination of spear side nicked seeds was between 19.5 to 30% despite having high percent seed viability (94 to 99%). This observation, together with the findings on
the contribution of pili grass coats (i.e., endosperm), indicate that physiological factors
are involved in pili grass seed dormancy. Aside from the confirmation that pili grass seed
dormancy involves physical and physiological factors, the germination data from this
study also suggests that dormancy status in pili grass seeds is affected by time of year that
seeds are harvested. March harvested seeds exhibited more germination with the
scarification treatments than June harvested seeds (naked caryopsis: 19.5% in March vs.
1% in June; spear side nicked seeds: 30.0% in March vs. 19.5% in June), indicating a
reduced depth of dormancy of the former.
**Table 2.1.** Fisher’s exact tests comparing the total number of seeds germinating (in parenthesis, pooled across replicates) in a scarification treatment applied on the March and June seed batches. The total sample size for each comparison was 400.

<table>
<thead>
<tr>
<th>Scarification treatment</th>
<th>Seed batch</th>
<th>Fisher’s Exact test, Two-tail P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked caryopsis</td>
<td>March (33) vs. June (2)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Spear side nicked</td>
<td>March (60) vs. June (39)</td>
<td>0.0202*</td>
</tr>
</tbody>
</table>

For each comparison, significant differences were denoted: *P<0.05; **P<0.01.

**Table 2.2.** Fisher’s exact tests of the March 2012 harvested seeds comparing the scarification treatments exhibiting germination (naked caryopsis, top cut and spear side nicked) and those exhibiting no germination (spear cut and intact seeds). The total number of seeds germinating in each scarification treatment (pooled across replicates) is indicated in parentheses. The total sample size for each treatment comparison was 400.

<table>
<thead>
<tr>
<th>Treatment comparison (total number of germinated seeds)</th>
<th>Fisher’s Exact test, Two-tail P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked caryopsis (33) vs. Spear cut (0)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Naked caryopsis (33) vs. Intact (0)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Top cut (4) vs. Spear cut (0)</td>
<td>0.1231ns</td>
</tr>
<tr>
<td>Top cut (4) vs. Intact (0)</td>
<td>0.1231ns</td>
</tr>
<tr>
<td>Spear side nicked (60) vs. Spear cut (0)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Spear side nicked (60) vs. Intact (0)</td>
<td>0.0000**</td>
</tr>
</tbody>
</table>

For each comparison, significant differences were denoted: ns not significant; **P<0.01.

**Table 2.3.** Fisher’s exact tests of the June 2012 harvested seeds comparing the scarification treatments exhibiting germination (naked caryopsis and spear side nicked) and those exhibiting no germination (spear cut, intact seeds and top cut). The total number of seeds germinating in each scarification treatment (pooled across replicates) is indicated in parentheses. The total sample size for each treatment comparison was 400.

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>Fisher’s Exact test, Two-tail P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked caryopsis (2) vs. Spear cut (0)</td>
<td>0.4987ns</td>
</tr>
<tr>
<td>Naked caryopsis (2) vs. Intact (0)</td>
<td>0.4987ns</td>
</tr>
<tr>
<td>Naked caryopsis (2) vs. Top cut (0)</td>
<td>0.4987ns</td>
</tr>
<tr>
<td>Spear side nicked (39) vs. Spear cut (0)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Spear side nicked (39) vs. Intact (0)</td>
<td>0.0000**</td>
</tr>
<tr>
<td>Spear side nicked (39) vs. Top cut off (0)</td>
<td>0.0000**</td>
</tr>
</tbody>
</table>

For each comparison, significant differences were denoted: ns Not significant; *P<0.05; **P<0.01.
Table 2.4. Generalized linear mixed model analysis for germination of March and June harvested pili grass seeds applied with different mechanical scarification treatments.

<table>
<thead>
<tr>
<th>Season</th>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 2012</td>
<td>Scarification treatment</td>
<td>2</td>
<td>6</td>
<td>18.9</td>
<td>0.0026**</td>
</tr>
<tr>
<td>June 2012</td>
<td>Scarification treatment</td>
<td>1</td>
<td>3</td>
<td>18.8</td>
<td>0.0226**</td>
</tr>
</tbody>
</table>
Figures

Figure 2.1. Staining patterns used for evaluating viable (A) and non-viable (B) seeds in the tetrazolium test: fully stained embryo (A1 and A2), infected with fungi (B1), dead seed (B2) and partially stained seed (B3).

Figure 2.2. Mechanical scarification treatments applied to pili grass seeds (L-R): Intact seed (untreated), naked caryopses, top cut (~25% of awn side of seed cut), spear cut and spear side nicked seeds (spears removed and nicked on the spear side to expose part of the embryo).
Figure 2.3. Percent germination of March and June 2012 harvested pili grass seeds treated with the different modes of seed scarification. Treatments with asterisks (*) indicate zero or not significantly different from treatments exhibiting zero germination based on Fisher’s exact tests. Treatments with the same letters within a chart (i.e., seed batch) are not significantly different at $P=0.05$. 

![Graph showing germination percentages for March and June 2012 seeds with different treatments marked with letters and asterisks. The graph includes columns for intact, naked coropese, tip cut, and spear cut treatments, with germination rates ranging from 0.0 to 50.0 percent.]
CHAPTER 3

EFFECTS OF STORAGE HUMIDITY AND TEMPERATURE ON DORMANCY LOSS OF PILI GRASS \(Heteropogon contortus\) (L.) P. BEAUV. EX ROEM. & SCHULT. ] SEEDS

**Introduction**

Seed dormancy is an inherent adaptation in plants that hinders the germination of newly dispersed seed (Tarasoff et al., 2007). It allows germination to be temporally spread, thereby increasing a plant species’ chances of survival. In restoration and re-vegetation with native groundcovers, seed dormancy can both be an advantage and a disadvantage. While seed dormancy can help in long term proliferation and persistence of plantings, it can be a hindrance during planting and establishment. If directly seeded native groundcovers possess dormancy, it can delay establishment and canopy fill-in, thereby making the site more prone to erosion and weed invasion.

In Hawai‘i, there has been recent interest in the use of native species for restoration, roadside re-vegetation/erosion control and urban landscaping. A number of native groundcovers have been identified and evaluated as potential re-vegetation species. However, there is a dearth of knowledge in seed production and germination biology of these plants, which hinders use in large scale re-vegetation projects. Pili grass \(Heteropogon contortus\) is one of several native Hawaiian re-vegetation species which needs further study in these areas.
Pili grass is a drought tolerant, perennial C4 bunchgrass that grows to approximately 0.4-1 meters high (Wagner et al., 1999). It is a widely distributed grass species throughout the dry tropical and subtropical grasslands of the world (Tothill, 1968, Carino and Daehler, 1999, Carino, 1999). In Hawai‘i, it is found on all main islands, usually on dry rocky cliffs, ledges or slopes near the ocean and from elevations ranging from sea level to 700 m (Wagner et al., 1999). Due to its adaptability to low rainfall, low fertility soils (USDA-NRCS, 2007) and inherent cultural and ecological value (Daehler and Goergen, 2005), pili grass is considered as an important re-vegetation and restoration species in Hawai‘i.

A major constraint to the use of pili grass in restoration and re-vegetation is its seed dormancy. Freshly harvested seeds do not germinate or have very low (<10%) germination. To break seed dormancy, seeds must be kept in dry storage (i.e., dry after-ripening) for 6 to 12 months (Tothill, 1977, Pater, 1993, Daehler and Goergen, 2005, USDA-NRCS, 2007). Although dry after-ripening following drying is known to remove seed dormancy and subsequently improve germination in pili grass, storage conditions to optimize this process have yet to be determined.

Temperature and relative humidity/seed moisture status are important aspects of storage conditions that affect the rate of seed dormancy loss as well as seed viability loss (Baskin and Baskin, 1979, Foley, 1994, Steadman et al., 2003, Foley, 2008, Commander et al., 2009, Bazin et al., 2011). Studies that have been done in a number of species indicate that, in general, increasing the storage temperature and seed moisture content accelerates dormancy loss of physiologically dormant seeds (Baskin and Baskin, 1979, Steadman et al., 2003, Commander et al., 2009, Bazin et al., 2011). The increase in the
rate of dormancy loss with higher temperatures operates at a species-specific range of seed moisture contents. Above or below this species-specific critical moisture threshold value, dormancy loss can be either inhibited or delayed (Bewley and Black, 1994, Finch-Savage and Leubner-Metzger, 2006, Iglesias-Fernandez et al., 2011). While storage at low temperatures and low seed moisture content can maintain seed longevity, storage at high temperatures and high seed moisture content facilitates seed viability loss (Copeland and McDonald, 2001). The objective of this study was to evaluate the effects of time of year (i.e., season) harvest, storage equilibrium relative humidity (eRH), storage temperature and incubation period on loss of seed dormancy and seed viability in pili grass.

**Materials and methods**

**Seed source**

Freshly harvested seeds (1 month old or less) of a Kaho‘olawe source identified natural germplasm were utilized for the study. Seeds were combine harvested from irrigated field plantings maintained at the US Department of Agriculture-Natural Resource Conservation Service (USDA-NRCS) Plant Materials Center (PMC) on the island of Moloka‘i (21° 8’ 33.7842”; -157° 6’ 3.369”). After harvesting, the seeds were immediately transported back to the University of Hawai‘i at Mānoa campus on O‘ahu for further processing and cleaning. Seeds were air dried for 9 days and passed through an air blast seed cleaner (Almaco, Nevada, IA) to remove most of the chaff, awns and empty seeds.
Two seed batches were harvested and prepared for the study. Seeds that were harvested on March 22, 2011 were used for the first run, while seeds harvested on October 27, 2011 were utilized for the 2\textsuperscript{nd} experiment. During eRH conditioning (see next section for details) 2 grams of seeds were placed in each of the 252 unsealed, foil barrier packets (Seed Savers Exchange, Decorah, IA) used in each experiment (i.e., seed batch). After eRH conditioning (see next section for details), foil packets were sealed and incubated in 1 of 3 temperature-controlled chambers for 1, 3, 6, 9 and 12 months.

**Experimental design and treatment application**

The experimental design was a split-split-split-split plot. The main effect plots were the experimental runs/seed batches (i.e., March and October harvested seeds). The sub plot effect was equilibrium relative humidity (i.e., 75\% eRH, 50\% eRH and 12\% eRH). The sub-sub plot effect was storage temperature [10\^\circ C (cold storage temperature), 20\^\circ C (air conditioned laboratory temperature) and 30\^\circ C (incubator temperature)] and the sub-sub-sub plot effect was the length of storage/incubation (0, 1, 3, 6, 9 and 12 months of incubation). The eRH treatments were the first to be applied to the seeds by storing the unsealed packets (already containing the seeds) for 28 days in 3 desiccators containing different saturated salt solutions (Commander \textit{et al.}, 2009, Turner \textit{et al.}, 2009). Saturated salt solutions of lithium chloride [LiCl\cdot H\textsubscript{2}O], calcium nitrate [Ca(NO\textsubscript{3})\textsubscript{2}] and sodium chloride [NaCl] were used to maintain humidities of 12\%, 50\% and 75\% eRH, respectively at 25\^\circ C (Winston and Bates, 1960, Turner \textit{et al.}, 2009). After the seed moisture content was equilibrated, the packets were sealed using a thermal impulse heat sealer (Uline, Pleasant Prairie, WI) to maintain the designated humidity/seed moisture
treatment. Prior to the application of the three storage temperature treatments, 36 sealed packets were randomly sampled to determine the seed moisture, viability (i.e., tetrazolium test) and germination at month 0. The rest of the packets were portioned and incubated at 10°C (refrigerator), 20°C (ambient laboratory temperature) or 30°C (incubator) for 1, 3, 6, 9 and 12 months.

**Seed moisture determination, germination and seed viability tests**

Percent seed moisture (dry weight basis), germination and tetrazolium tests were conducted at the end of each incubation period to monitor seed moisture, dormancy loss and seed viability/deterioration. Seed moisture determination was done using the gravimetric method. A one gram seed sample from a seed packet (n=4) was dried for 17 hours in a drying oven (Quincy Lab, Inc., Chicago, IL) with temperature set at 103°C (ISTA, 1996, Commander et al., 2009). Seed moisture (as a proportion) was calculated using the formula:

$$\text{Percent seed moisture (dry weight basis)} = \frac{\text{Fresh weight} - \text{Oven dry weight}}{\text{Oven dry weight}} \times 100$$

Note: fresh and oven dry weights exclude the weights of the container.

Germination tests were conducted with four replicates of 50 seeds sown in Petri dishes lined with two layers of moistened filter paper (Whatman #3, Whatman International). Edges of the petri dish were sealed with parafilm (SPI Supplies, West Chester, PA). The petri dishes were germinated in a 30°C (Tothill, 1977) incubator under dark conditions. To prevent drying, the petri dishes were moistened as needed with distilled water. Seed germination was observed regularly under lighted conditions for 20
days. The total number of seeds that germinated at 20 days was recorded. Seeds were counted as germinated when at least 1 mm of the radicle had emerged.

Tetrazolium tests for seed viability were also conducted at the end of each incubation period. This stain-based test is a widely recognized and accurate means of estimating seed viability and is a routine test in many seed testing facilities (Copeland and McDonald, 2001). It detects the presence of dehydrogenase activity in actively respiring viable seed tissues (Elias et al., 2012). During respiration, dehydrogenase enzymes react with substrates and release hydrogen ions (Copeland and McDonald, 2001, Elias et al., 2012). The hydrogen ions react with the colorless tetrazolium chloride solution to produce a red dye called formazan (Copeland and McDonald, 2001, Elias et al., 2012). The staining patterns formed on the seeds with this reaction allows for the assessment of seed viability.

Four replicates of 50 seeds were soaked in distilled water overnight to activate seed enzymes, facilitate softening and cutting. After soaking, the imbibed seeds were cut longitudinally through the middle of the embryonic axis (Peters, 2000). Half of the cut seed was placed in a 20 ml scintillation vial. The vial was filled with 3 ml of 1% tetrazolium chloride (Sigma Aldrich, St. Louis, MO) solution and incubated in the dark at 27°C for 5 hours to allow staining. The stained seeds were washed with distilled water, arranged in a petri dish lined with moistened filter paper and observed under a dissecting microscope. Seeds were counted as viable based on specific staining patterns (Figure 1). Fully stained embryos were recorded as viable seed while partially stained embryos or stained whole seeds (caused by fungal contamination) were considered as non-viable. The percentage of viable seeds was recorded.
Statistical analysis

Moisture content

Analysis of variance (PROC GLM in SAS 9.3, SAS Institute Inc., Cary, NC) was conducted on the seed moisture data to monitor for any significant changes in seed moisture during the experiment. Before analysis, moisture content values (i.e., grams H$_2$O/dry weight in grams) were square root transformed. Square root transformation was determined to be the appropriate transformation since the range of seed moisture values was between 0 and 30% (Gomez and Gomez, 1984). A conservative Tukey-Kramer grouping (P <0.05) was used to separate the means of significant treatment factors or interactions. For ease of interpretation during the discussion of the results, the means were back transformed to percent moisture (dry weight basis) using the equation:

$$\text{Percent moisture (dry weight basis) } = 100(p^2)$$

Where $p$ was the square root transformed proportion-based seed moisture (i.e., grams H$_2$O/dry weight in grams).

Germination data

Due to a large number of treatments that yield a zero value for percent germination in the seed storage experiment, Fisher’s exact tests and a generalized linear mixed model were used to analyze the data. Monthly differences in the proportion of seeds that germinated from the March and October seed harvests were compared using Fisher’s exact tests (Curtis Daehler, personal communication, November 15, 2013). The total number of germinated and non-germinated seeds in each seed batch and in each incubation month was recorded. The association between the incidence of germination
and seed batch at each incubation month was examined using a 2 x 2 contingency table (Pagano and Gauvreau, 2000).

A generalized linear mixed model with a binomial distribution was used to analyze treatment combinations exhibiting consistent germination for both seed batches (i.e., seeds stored at 12% and 50% eRH; at 20 and 30°C and incubated from 6 to 12 months) (Matthew Kramer, Statistician General, USDA-ARS, personal communication, January 16, 2013). Logit transformation and modeling of the data was accomplished using PROC GLIMMIX (SAS version 9.3, SAS Institute Inc., Cary, NC). Generalized linear mixed models allow modeling of data exhibiting a non-normal distribution or heterogeneous variance and also include both fixed and random effects (Sileshi, 2012). The binomial response distribution has been found to be appropriate for studies involving proportion based germination data (Willenborg et al., 2005, Pendleton et al., 2012).

Treatment combinations exhibiting zero and inconsistent germination (i.e., germination on one batch and none in the other) in both seed batches were excluded in the GLIMMIX analysis. This included all low temperature (10°C) treatment combinations, all treatment combinations less than six months in incubation and all high humidity (75% eRH) treatment combinations at 20 and 30°C. Treatments with all replicates exhibiting zero germination result in zero values for both mean and variance. According to Reeve and Strom (2004), treatments that have zero or low variance can reduce the magnitude of the mean square error, which is the denominator for $F$ tests and also a component of multiple comparison procedures. Removal of these treatments in the analysis ensures that error rates are near their specified values (i.e., mean square error is not reduced due to zero variance treatments). In addition, this facilitates analysis with
generalized linear mixed models. Software developed for these models, such as PROC GLIMMIX, can have numerical problems if zeros (i.e., all replicates in a treatment exhibit zero germination) are present in the data (Reeve and Strom, 2004).

*Seed viability data*

A generalized linear mixed model was also used to analyze the tetrazolium viability data. Treatment combinations exhibiting zero means were also removed prior to statistical analysis (Reeve and Strom, 2004). For this dataset, only the October 2011, 75% eRH/30°C/9 month treatment combination was removed. Logit transformation and modeling of the data was accomplished using PROC GLIMMIX (SAS version 9.3, SAS Institute Inc., Cary, NC).

*F* tests for both the germination and tetrazolium viability tests were calculated based on Wald statistics. Means on the logit scale were separated using conservative Tukey-Kramer grouping (P <0.05). For ease of interpretation, mean percent germination and viability prior to entry in tables and figures were back transformed from the logit scale using the equation:

\[ a = 100 \left( \frac{e^x}{1 + e^x} \right) \]

Where \( x \) was the logit estimate from the model, \( e \) was the exponential function and \( a \) was the approximate germination or viability percentage after back transformation.
**Results**

**Seed moisture, viability and percent germination after equilibration**

Moisture content determination at month 0 (i.e., end of equilibration phase, start of temperature application) indicated levels of seed moisture depending on the storage eRH. Seed moisture content of the March 2011 seed batch prior to the application of the temperature treatments were 5.7, 11.1 and 14.1 percent (dry weight basis) for 12% eRH, 50% eRH and 75% eRH, respectively. For the October 2011 seeds, the percent seed moisture content prior to the application of the temperature treatments was 5.7, 11.8 and 14.9 percent (dry weight basis) for 12% eRH, 50% eRH and 75% eRH, respectively. The initial/base percent viability for both batches was similar, ranging from 87.5 to 90 percent. Percent germination data prior to the application of the temperature treatments indicated that the seeds were still dormant. Percent germination ranged from zero to less than 0.3 percent for both seed batches.

**Percent moisture content**

Analysis of seed moisture content indicated significant interactions between storage month and storage temperature ($P=0.0006$) and between storage month, seed batch and storage humidity ($P=0.0000$) (Table 1). Mean comparisons of monthly seed moisture content pooled over eRH indicate that seeds stored at 10°C and 20°C maintained mean moisture content of 10 percent over the 12 month incubation period (Table 2). Moisture content of seeds stored at 30°C during the first 6 months was also maintained at
mean of 10 percent. However, a slight but significant loss of moisture was observed at 9 and 12 months in incubation at this storage temperature.

Mean comparisons of monthly seed moisture content pooled over all storage temperatures indicated maintenance of seed moisture content at the 12% eRH storage treatment (Table 3). Both March and October harvested seeds stored at 12% eRH maintained a seed moisture content of 6 percent (dry weight basis) over the 12 month incubation period. Moisture content of seeds stored at 50% eRH was generally maintained in both seed batches at 11 percent (dry weight basis) except for the March harvested seed stored at 6 months (Table 3). At this incubation period, seed moisture significantly increased to 12.6 percent.

March harvested seeds stored at 75% eRH exhibited the same seed moisture content (14 percent) during the 12 month incubation period (Table 3). In contrast, the October harvested seeds exhibited a slight but significant decrease in seed moisture content during the storage period. Seed moisture content slightly decreased from 14.9 percent (dry weight basis) in month 0 to 13.3 percent (dry weight basis) after 12 months of storage. Loss of seed moisture during storage was an indication of loss in packaging integrity. The data on seed moisture content indicated that the levels obtained during the pre-storage moisture conditioning phase were generally maintained throughout the storage intervals used here.

**Percent germination over time**

Fisher’s exact tests conducted at each storage period detected significant differences in the germination between the March and October 2011 harvested seeds
Differences in the proportion of germinated seeds between the two seed batches were detected from 3 to 12 months in incubation. March harvested seeds exhibited 96, 368, 403 and 433 germinated seeds after 3, 6, 9 and 12 months in incubation, respectively. In contrast, October harvested seeds exhibited a significantly lower number with 7, 202, 309 and 382 seeds germinating after 3, 6, 9, and 12 months in incubation, respectively. This difference in the proportion of germinated seeds can be an indication that the March 2011 batch had a reduced depth of dormancy compared to the October 2011 batch.

Analysis of germination data indicated a significant 4-way interaction between seed batch, equilibrium relative humidity, storage temperature and storage period (Table 5). For the March 2011 seeds, the highest improvements in germination among all treatment combinations were recorded in seeds stored either at 12% eRH/30°C or at 50% eRH/30°C (Table 6). In these treatments, a significant improvement in germination was observed as early as after 6 months of storage. Maximum germination was observed and maintained in these treatment combinations for up to 12 months. Optimum germination of seeds stored at 12% eRH/30°C ranged from 73.2 to 81.1 percent while seed stored at 50% eRH/30°C exhibited a range of 65.7 to 69.1 percent (Table 6). Seeds stored at 50% eRH/20°C also exhibited improvement in mean percent germination over time. However, this was significantly lower than the percent germination observed in seeds stored at 12% eRH/30°C and 50% eRH/30°C. Little improvement in germination over time (<10%) was observed in seeds stored at 12% eRH/20°C. Seeds stored at 10°C, regardless of eRH, maintained dormancy as it exhibited zero to less than 3 percent germination. Therefore,
these and other treatment combinations with little to no germination were not included in the statistical analysis (Table 6).

The best storage temperature and relative humidity combinations observed in the October 2011 seed batch were consistent with that observed in the March 2011 seeds (Table 6). Seeds stored at either 12% eRH/30°C or 50% eRH/30°C exhibited the highest improvement in germination followed by seeds stored at 50% eRH/20°C (Table 6). However, it can be observed that the October 2011 seeds had to be stored for 12 months at 12% eRH/30°C to achieve maximum germination. Seeds stored at 50% eRH/30°C achieved maximum germination at 9 months in storage. However, storage for 12 months resulted in a significant decline in percent germination. Storage at 10°C, regardless of eRH, maintained dormancy of October 2011 harvested piligrass seeds. Since very little (0.5-3.0%) to no germination occurred at these treatment combinations, these were not included in the GLIMMIX analysis. Based on percent germination data obtained from both seed batches, piligrass seeds can be stored either for 9 months at 50% eRH/30°C or for 12 months 12% eRH/30°C to optimize dormancy loss.

**Percent viability over time**

The generalized linear mixed model for the tetrazolium seed viability test indicated a significant 4-way interaction between seed batch, storage eRH, storage temperature and period of incubation (Table 7). Most storage eRH and temperature treatment combinations in both seed batches maintained a high percentage of viable seed over time (Table 8). At 10°C, seed viability was maintained at or near the maximum level regardless of seed batch, storage eRH and length of storage period. At ambient
temperature (20°C), no significant deterioration in viability was observed in the low (12% eRH) to medium (50% eRH) relative humidity combinations. At 30°C, loss in seed viability was most pronounced at 75% eRH. Pili grass seed viability is best preserved when eRH levels are set at either 12 or 50%.

**Discussion**

Results of the study indicated that a specific range of storage temperature and eRH (i.e., seed moisture content) affects the rate of dormancy loss and viability in pili grass seeds. Dormancy loss was hastened by increasing the storage temperature from ambient (20°C) to high (30°C) at a narrow range of seed moisture content (6 to 11%, dry weight basis). This range of seed moisture content was within the minimum and maximum values [2.5 to 20% (dry weight basis)] that have been reported in a number of species (Foley, 1994, Steadman et al., 2003, Bazin et al., 2011). Increasing storage temperatures alleviated seed dormancy at seed moisture contents between 2.5 to 12% in *Helianthus annuus* (Bazin et al., 2011), 5 to 20% in *Avena fatua* (Foley, 1994) and 6 to 18% in *Lolium rigidum* (Steadman et al., 2003).

When seed moisture contents are below the species-specific threshold, dormancy loss is halted under dry, unimbibed conditions (i.e., dry after-ripening) (Iglesias-Fernandez et al., 2011). In the current study, the range of seed moisture contents evaluated did not indicate a minimum threshold for a loss in pili grass seed dormancy. Pili grass seed dormancy was maintained by low temperature (10°C). Low storage temperatures are known to prevent or delay seed dormancy loss of dry stored seeds.
(Simpson, 1990, Steadman et al., 2003, Bazin et al., 2011). The temperature at which dormancy loss is prevented is species specific. A number of studies have identified the temperature at which seed dormancy of certain species is maintained (i.e., critical base temperature). In red rice \( (Oryza sativa) \), storage at -15°C with seed moisture contents of 11 to 12% maintained dormancy for up to a year (Cohn and Hughes, 1981). Thermal after-ripening time models for dormancy loss in \( Helianthus annuus \) L., cv. LG5665 and \( Lolium rigidum \) calculated the critical base temperature at 8°C with moisture contents above 10% \( (0.1 \text{ g H}_2\text{O g}^{-1} \text{ dry weight}) \) (Bazin et al., 2011) and 5.4°C with moisture contents between 6 and 18% \( (\text{dry weight basis}) \) (Steadman et al., 2003), respectively.

Aside from the maintenance of seed dormancy, storage at low temperature \( (10°C) \), regardless of seed moisture content also preserved viability of pili grass seeds for at least a year. Similar studies have also observed the maintenance of seed viability with storage at low temperatures. In \( Euphorbia esula \), storage of seeds at 5°C for 24 weeks did not result in loss of seed viability even at 88% relative humidity or 13% seed moisture content \( (\text{dry weight basis}) \) (Foley, 2008). Seed viability was also preserved in wild \( Euphorbia pulcherrima \) seeds stored for 9 months at 5°C with 7.7% or less seed moisture content \( (\text{dry weight basis}) \) (Bannon et al., 1978). Increasing the seed moisture content to 18.6%, however, resulted in full viability loss by 6 months in storage. For most crop seeds, temperatures at 4 to 10°C can be considered safe for short term storage as long as the relative humidity does not exceed 70% (Copeland and McDonald, 2001). If long term storage is considered \( (\text{i.e., } 10 \text{ years or longer}) \), seeds must be placed at a relative humidity lower than 50% and temperatures lower than 5°C (Toole, 1950, Copeland and McDonald, 2001). Although the current study indicated that at 10°C, pili grass dormancy and seed
viability was maintained regardless of storage relative humidity, longer term studies (> 1 year) are recommended to determine the optimum long-term storage conditions.

Seed moisture contents above the threshold value can also inhibit dormancy loss (Bewley and Black, 1994). Higher seed moisture levels can induce secondary dormancy (Bewley and Black, 1994) and it can also increase loss of seed viability (Baskin and Baskin, 1979, Bewley and Black, 1994, Probert, 2000, Foley, 2008). In the current study, loss of seed viability was observed in seeds stored in both ambient and high temperatures at 75% eRH (14% seed moisture content) and in seeds stored at ambient temperature at 50% eRH (11% seed moisture content). Storage at these conditions resulted in a drastic decline in seed viability over time. Increased incidence of mold growth on seeds was also observed with storage at 75% eRH (14% seed moisture content).

Aside from temperature and seed moisture effects on loss of seed dormancy and seed viability, results also indicated that the two seed batches exhibited differences in the depth of dormancy. Despite having similar percent seed viability, Fisher’s exact tests performed at each incubation period showed differences in the proportion of seeds that germinated in the March and October seed batches. From month 3 to month 12 of incubation, the March harvested seeds consistently exhibited more germination than the October harvested seeds.

In addition to the differences observed in the total number of seeds germinated from each seed batch, differences in the depth of dormancy can also be detected in the time period wherein optimum dormancy loss was achieved. In the March 2011 seed batch, maximum germination was observed as early as 6 months with storage at 30°C in either 12% eRH (6% moisture, dry weight basis) or 50% eRH (11% moisture, dry weight
basis). For the October 2011 seed batch, the maximum germination was recorded after 9 months in storage at 30°C and 50% eRH (11% seed moisture, dry weight basis) and after 12 months with storage at 30°C and 12% eRH (6% seed moisture, dry weight basis).

Seasonal differences in depth of seed dormancy can be attributed to the growing conditions seeds received during its development and can include daylength, light quality, mineral nutrition, soil moisture, temperature and physiological age of mother plants (Simpson, 1990, Baskin and Baskin, 2001a). Climate data obtained for both experimental runs indicated distinct differences in daylength, average temperatures, solar radiation, total precipitation and growing degree days (Table 9). There was also a difference in the length of time needed to reach seed maturity (Table 9). The March 2011 seeds were harvested 111 days after cutting while the October 2011 seeds were harvested 94 days after cutting. While these differences in growing conditions might have affected the dormancy status of each seed batch, more in-depth studies focusing on each of these environmental factors are needed in order to confirm and quantify these effects.

In addition to practical applications in restoration, re-vegetation and seed storage, the temperature and relative humidity effects described in this study may also help explain the phenology and seed dormancy loss of pili grass under natural conditions in Hawaii. The dry leeward side of the islands where pili grass thrives is seasonally dry and receives an annual rainfall of 1,200 mm or less (Wagner et al., 1999, Goergen and Daehler, 2001). Much of the annual rainfall in these areas occurs primarily during the winter months (October to April) (Goergen and Daehler, 2001). Rains during the winter season provide adequate soil moisture for pili grass to flower and set seeds. Seeds produced during the winter months are shed and undergo a drying period as the summer
season approaches (May to September). During the summer season, the shed seeds are likely exposed to hot and dry conditions which are conducive for dormancy loss. By the time the next winter rains arrive, the seeds have already lost dormancy and are ready to germinate.

In summary, this study showed that storage temperature and relative humidity impacted dormancy loss and viability of pili grass seeds. For optimum dormancy loss, seeds must be stored either at 30°C and 12% eRH (6% seed moisture, dry weight basis) for 12 months or at 30°C and 50% eRH (11% seed moisture, dry weight basis) for 9 months. Storage at 10°C, regardless of eRH maintained dormancy and viability for up to a year. Seed viability was maintained for up to a year with storage at ambient (20°C) and high (30°C) temperatures as long as the relative humidity was maintained at 12% (6% seed moisture content).
Tables

Table 3.1. Analysis of variance (ANOVA) table for percent moisture content of pili grass seeds harvested at different dates (i.e., March and October 2011) and incubated for 0, 1, 3, 6, 9 and 12 months at different equilibrium relative humidities (12%, 50% and 75%, resulting in seed moisture contents of 6%, 11% and 14%, dry weight basis) and storage temperatures (10°C, 20°C and 30°C).

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<td>0.0000</td>
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<tr>
<td>Month x Seed batch</td>
<td>5</td>
<td>0.00156</td>
<td>0.00031</td>
<td>2.78</td>
<td>0.0182</td>
</tr>
<tr>
<td>Month x Humidity</td>
<td>10</td>
<td>0.00474</td>
<td>0.00047</td>
<td>4.23</td>
<td>0.0000</td>
</tr>
<tr>
<td>Month x Temp</td>
<td>10</td>
<td>0.00363</td>
<td>0.00036</td>
<td>3.21</td>
<td>0.0006</td>
</tr>
<tr>
<td>Month x Seed batch x Humidity</td>
<td>10</td>
<td>0.00453</td>
<td>0.00045</td>
<td>4.04</td>
<td>0.0000</td>
</tr>
<tr>
<td>Month x Seed batch x Temp</td>
<td>10</td>
<td>0.00158</td>
<td>0.00016</td>
<td>1.41</td>
<td>0.1757</td>
</tr>
<tr>
<td>Month x Humidity x Temp</td>
<td>20</td>
<td>0.00173</td>
<td>0.00009</td>
<td>0.77</td>
<td>0.7477</td>
</tr>
<tr>
<td>Month x Seed batch x Temp x Humidity</td>
<td>20</td>
<td>0.00273</td>
<td>0.00014</td>
<td>1.22</td>
<td>0.2399</td>
</tr>
<tr>
<td>Error (d)</td>
<td>269</td>
<td>0.03015</td>
<td>0.00011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>430</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Monthly percent seed moisture content (dry weight basis, based on 1 gram samples) of pili grass seeds pooled over storage temperatures.

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>10°C</td>
<td>10.3 a</td>
<td>10.0 ab</td>
<td>10.3 a</td>
<td>10.3 a</td>
<td>10.0 ab</td>
<td>10.1 a</td>
</tr>
<tr>
<td>20°C</td>
<td>10.3 a</td>
<td>10.0 ab</td>
<td>10.3 a</td>
<td>10.6 a</td>
<td>10.0 ab</td>
<td>10.3 a</td>
</tr>
<tr>
<td>30°C</td>
<td>10.2 a</td>
<td>10.2 a</td>
<td>10.1 ab</td>
<td>10.6 a</td>
<td>9.3 c</td>
<td>9.4 bc</td>
</tr>
</tbody>
</table>

*Means across columns and rows followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05. Seed moisture contents presented are back transformed means.
Table 3.3. Monthly percent seed moisture content (dry weight basis, based on 1 gram samples) of pili grass seeds pooled over equilibrium relative humidities and seed batches.

<table>
<thead>
<tr>
<th>Seed batch</th>
<th>Storage Humidity</th>
<th>Months in incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>March</td>
<td>12</td>
<td>5.9 g</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11.3 ef</td>
</tr>
<tr>
<td>Oct</td>
<td>12</td>
<td>5.7 g</td>
</tr>
<tr>
<td>October</td>
<td>75</td>
<td>14.0 ab</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>11.8 de</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>14.9 a</td>
</tr>
</tbody>
</table>

*Means across columns and rows followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05. Seed moisture contents presented are back transformed means.

Table 3.4. Contingency table at each month of incubation comparing the pooled number of germinated and non-germinated pili grass seeds in the March and October 2011 seed batches.

<table>
<thead>
<tr>
<th>Seed batch</th>
<th>Germination</th>
<th>Months in incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>March 2011</td>
<td>No</td>
<td>1797</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>October 2011</td>
<td>No</td>
<td>1800</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0</td>
</tr>
<tr>
<td>Fisher’s Exact test, Two-tail P value</td>
<td>0.1249\textsuperscript{ns}</td>
<td>1.000\textsuperscript{ns}</td>
</tr>
</tbody>
</table>

\textsuperscript{ns} The proportion of seeds that germinated are identical between the two seed batches.
* The proportion of seeds that germinated are different between the two seed batches (P=0.05).
** The proportion of seeds that germinated are different between the two seed batches (P<0.01).
Table 3.5. Generalized linear mixed model analysis for germination of pili grass seeds harvested at different dates (i.e., March and October 2011) and incubated for 0, 1, 3, 6, 9 and 12 months at different equilibrium relative humidities (12%, 50% and 75%, resulting in seed moisture contents of 6%, 11% and 14%, dry weight basis, respectively) and storage temperatures (10°C, 20°C and 30°C).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humidity</td>
<td>1</td>
<td>33</td>
<td>49.82</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Temp</td>
<td>1</td>
<td>33</td>
<td>432.16</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Temp</td>
<td>1</td>
<td>33</td>
<td>104.21</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Month</td>
<td>2</td>
<td>33</td>
<td>29.97</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Month</td>
<td>2</td>
<td>33</td>
<td>2.7</td>
<td>0.0821</td>
</tr>
<tr>
<td>Temp x Month</td>
<td>2</td>
<td>33</td>
<td>16.6</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Temp x Month</td>
<td>1</td>
<td>33</td>
<td>5.88</td>
<td>0.021</td>
</tr>
<tr>
<td>Seed batch</td>
<td>1</td>
<td>33</td>
<td>45.72</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Seed batch</td>
<td>1</td>
<td>33</td>
<td>0</td>
<td>0.9761</td>
</tr>
<tr>
<td>Temp x Seed batch</td>
<td>1</td>
<td>33</td>
<td>7.88</td>
<td>0.0083</td>
</tr>
<tr>
<td>Humidity x Temp x Seed batch</td>
<td>1</td>
<td>33</td>
<td>0.02</td>
<td>0.8765</td>
</tr>
<tr>
<td>Month x Seed batch</td>
<td>2</td>
<td>33</td>
<td>10.96</td>
<td>0.0002</td>
</tr>
<tr>
<td>Humidity x Month x Seed batch</td>
<td>2</td>
<td>33</td>
<td>6.79</td>
<td>0.0034</td>
</tr>
<tr>
<td>Temp x Month x Seed batch</td>
<td>2</td>
<td>33</td>
<td>1.32</td>
<td>0.282</td>
</tr>
<tr>
<td>Humidity x Temp x Month x Seed batch</td>
<td>1</td>
<td>33</td>
<td>19.04</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Table 3.6. Percent germination of pili grass seeds harvested at different dates (i.e., March and October 2011) and incubated for 0, 1, 3, 6, 9 and 12 months at different equilibrium relative humidities (12%, 50% and 75%, resulting in seed moisture contents of 6%, 11% and 14%, dry weight basis) and storage temperatures (10°C, 20°C and 30°C).

<table>
<thead>
<tr>
<th>Seed batch</th>
<th>Storage Temperature (°C)</th>
<th>Equilibrium relative humidity</th>
<th>Months in incubation**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>March 2011</td>
<td>10°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.5*</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>20°C</td>
<td>12</td>
<td>1.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>30°C</td>
<td>12</td>
<td>0.0*</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0*</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>October 2011</td>
<td>10°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>20°C</td>
<td>12</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.5*</td>
</tr>
<tr>
<td>30°C</td>
<td>12</td>
<td>0.5*</td>
<td>0.5*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.0*</td>
<td>0.5*</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
</tbody>
</table>

* Germination data was mostly zero or inconsistent across the seed batches, therefore were not included in the GLMM analysis.

** Means across columns and rows followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05. Seed moisture contents presented are back transformed means.
Table 3.7. Generalized linear mixed model analysis for seed viability of pili grass seeds harvested at different dates (i.e., March and October 2011) and incubated for 0, 1, 3, 6, 9 and 12 months at different equilibrium relative humidities (12%, 50% and 75%, resulting in seed moisture contents of 6%, 11% and 14%, dry weight basis) and storage temperatures (10°C, 20°C and 30°C).

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed batch</td>
<td>1</td>
<td>6</td>
<td>0.97</td>
<td>0.3622</td>
</tr>
<tr>
<td>Humidity</td>
<td>2</td>
<td>6</td>
<td>58.76</td>
<td>0.0001</td>
</tr>
<tr>
<td>Humidity x Seed batch</td>
<td>2</td>
<td>6</td>
<td>9.41</td>
<td>0.0141</td>
</tr>
<tr>
<td>Temp</td>
<td>2</td>
<td>6</td>
<td>43.24</td>
<td>0.0003</td>
</tr>
<tr>
<td>Temp x Seed batch</td>
<td>2</td>
<td>6</td>
<td>5.11</td>
<td>0.0507</td>
</tr>
<tr>
<td>Humidity x Temp</td>
<td>4</td>
<td>24</td>
<td>54.28</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Temp x Seed batch</td>
<td>4</td>
<td>162</td>
<td>2.16</td>
<td>0.0764</td>
</tr>
<tr>
<td>Month</td>
<td>4</td>
<td>24</td>
<td>9.15</td>
<td>0.0001</td>
</tr>
<tr>
<td>Month x Seed batch</td>
<td>4</td>
<td>162</td>
<td>20.83</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Month</td>
<td>8</td>
<td>36</td>
<td>9.55</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Month x Seed batch</td>
<td>8</td>
<td>162</td>
<td>4.38</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Temp x Month</td>
<td>8</td>
<td>24</td>
<td>8.25</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Temp x Month x Seed batch</td>
<td>8</td>
<td>162</td>
<td>4.54</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Temp x Month</td>
<td>16</td>
<td>162</td>
<td>10.02</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Humidity x Temp x Month x Seed batch</td>
<td>15</td>
<td>162</td>
<td>3.31</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
Table 3.8. Percent seed viability of pili grass seeds harvested at different dates (i.e., March and October 2011) and incubated for 0, 1, 3, 6, 9 and 12 months at different equilibrium relative humidities (12%, 50% and 75%, resulting in seed moisture contents of 6%, 11% and 14%, dry weight basis) and storage temperatures (10°C, 20°C and 30°C).

<table>
<thead>
<tr>
<th>Seed batch</th>
<th>Storage Temperature (°C)</th>
<th>Equilibrium relative humidity</th>
<th>Months in incubation**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>March 2011</td>
<td>10°C</td>
<td>12</td>
<td>78.2 abcdefg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>84.4 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>82.8 abcede</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>12</td>
<td>80.4 abcdefg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>85.0 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>81.1 abcede</td>
</tr>
<tr>
<td></td>
<td>30°C</td>
<td>12</td>
<td>84.7 abcd</td>
</tr>
<tr>
<td>October 2011</td>
<td>10°C</td>
<td>12</td>
<td>82.2 abcde</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>80.5 abcdef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>84.8 abcd</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>12</td>
<td>79.7 abdcef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>87.8 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>87.1 ab</td>
</tr>
<tr>
<td></td>
<td>30°C</td>
<td>12</td>
<td>78.1 abcdefg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>88.0 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>84.1 abcd</td>
</tr>
</tbody>
</table>

* Mean percent germination was zero, therefore was not included in the GLMM analysis.
** Means across columns and rows followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05. Seed moisture contents presented are back transformed means.
Table 3.9. Climate data/trends recorded during the growing conditions of the March and October 2011 seed batches.

<table>
<thead>
<tr>
<th>Climate component</th>
<th>March 2011</th>
<th>October 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daylength</td>
<td>Increasing</td>
<td>Decreasing</td>
</tr>
<tr>
<td>Average temperature</td>
<td>21.5°C</td>
<td>24.2°C</td>
</tr>
<tr>
<td>Solar radiation</td>
<td>Increasing (41 to 598 cal/cm²)</td>
<td>Decreasing (227 to 653 cal/cm²)</td>
</tr>
<tr>
<td>Total precipitation</td>
<td>25.9 cm</td>
<td>1.2 cm</td>
</tr>
<tr>
<td>Total precipitation*</td>
<td>25.9 cm</td>
<td>1.2 cm</td>
</tr>
<tr>
<td>Total growing degree</td>
<td>760.6 GDU</td>
<td>935.0 GDU</td>
</tr>
<tr>
<td>units Maturity</td>
<td>111 days</td>
<td>94 days</td>
</tr>
</tbody>
</table>

*In addition to total precipitation, supplemental irrigation was also applied in the field.
Figure 3.1. Staining patterns used for evaluating viable (A) and non-viable (B) seeds in the tetrazolium test: fully stained embryo (A1 and A2), infected with fungi (B1), dead seed (B2) and partially stained seed (B3).
CHAPTER 4

THE EFFECT TEMPERATURE ON DORMANCY LOSS AND SHORT-TERM DRY STORAGE OF PILI GRASS [Heteropogon contortus (L.) P.BEAUV. EX ROEM. & SCHULT.] SEEDS

Introduction

Seed dormancy is a hindrance to the use of native plants in re-vegetation and erosion control projects. It prevents uniform and timely establishment, making the site more prone to erosion and weed invasion. Pili grass (Heteropogon contortus) is one of several native Hawaiian species which have potential use in large scale re-vegetation, but is hampered by seed dormancy. Freshly harvested pili grass seeds cannot be used since these exhibit poor or no germination (see Chapter 3). According to Baskin and Baskin (2001b), pili grass seeds exhibit the physiological type of seed dormancy (i.e., non-deep physiological dormancy). To break this dormancy type, seeds must be dry-stored for 6 to 12 months (i.e., after-ripening) (Tothill, 1977, Pater, 1993, Daehler and Goergen, 2005, USDA-NRCS, 2007) or treated with 1% gibberelic acid (Tothill, 1977, Goergen and Daehler, 2001).

Dormancy loss through the manipulation of storage conditions is an effective and scalable method for improving pili grass seed germination. Results from Chapter 3 indicated that the rate of dormancy loss in pili grass seeds is affected by equilibrium relative humidity (eRH) (i.e., seed moisture content), temperature as well as storage duration. Dormancy loss was optimized by storing the seeds for 12 months at 30°C and at
12% eRH (i.e., 6% seed moisture, dry weight basis) or for 9 months at 30°C and at 50% eRH (i.e., 11% seed moisture, dry weight basis). While optimum conditions for breaking pili grass seed dormancy were identified, storage temperatures for holding the seeds that have after-ripened (i.e., stored for a period of time at the optimum eRH/seed moisture content and temperature) have not been explored. In this study, the effects of storage temperature on viability and germination of pili grass seed relieved of dormancy through warm, dry storage were evaluated.

**Materials and methods**

**Seed source**

Pili grass seeds of a Kaho‘olawe source identified natural germplasm (accession #: 9079683, HA-5748) were utilized for this study. Seeds were harvested on October 27, 2011 from irrigated field plantings at the US Department of Agriculture-Natural Resource Conservation Service (USDA-NRCS) Plant Materials Center (PMC) on the island of Moloka‘i (21° 8’ 33.7842"; -157° 6’ 3.369”). Studies in Chapter 3 indicated that a high level of dormancy was present with this seed batch. Harvesting was accomplished using a combine. Immediately after harvest, the seeds were transported to the UH Mānoa Campus on O‘ahu, where they were air dried under ambient temperature and humidity for 10 days to 14.2% moisture (dry weight basis). After drying, the seeds were passed through an air blast seed cleaner (Almaco, Nevada, IA) to remove most of the chaff, awns and empty seeds. The seeds were placed in plastic zipper bags (Hefty Slider Storage Bags, Reynolds Consumer Products Inc., Richmond, VA) and stored at 5°C until used.
Optimized dormancy release conditions and application of storage temperature treatments

Seeds (54 days after harvesting) were equilibrated first at 12% eRH at ambient laboratory conditions (~20°C) for 52 days. The optimum eRH and temperature identified from Chapter 3 (i.e., 12% eRH/30°C) was used to relieve dormancy of the seeds. This was accomplished by placing the seeds in desiccators with an aqueous saturated solution of lithium chloride (LiCl·H₂O) (Winston and Bates, 1960, Commander et al., 2009). After 52 days, 2.5 grams of seeds were placed in foil barrier packets (Seed Savers Exchange, Decorah, IA) and then sealed to maintain the seed moisture content of 5.6% (dry weight basis). Packets were incubated at 30°C for six months to relieve dormancy. After storage at the optimum dormancy loss conditions, the packets were portioned into the three storage temperature treatments (5°C, 20°C and 30°C). The packets were stored under these temperatures for six months to observe differences in seed viability and germination. This storage experiment was not repeated.

Germination assay

Germination tests were used to provide a pre-storage treatment baseline (i.e., after dormancy loss conditions were applied, before storing at the three temperature treatments) and to evaluate the effects of the storage temperature treatments. The test was conducted on 100 x15 mm round polystyrene petri dishes (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA) lined with one layer of filter paper (Whatman #3, Whatman International, Piscataway, NJ). Prior to sowing, the filter papers were moistened with approximately 3 ml of deionized water. Fifty seeds were sown on each
filter paper-lined petri dish. Treatments were replicated four times. After sowing, the petri dishes were sealed on the sides with parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent drying. These were then incubated under 30°C (Tothill, 1977) without light. The petri dishes were observed regularly under lighted conditions and re-moistened with treatment solution as needed. Final germination percentage was recorded 20 days after sowing the seeds. Seeds were counted as germinated when at least 1 mm of the radicle had emerged.

**Seed viability test**

A 50-seed tetrazolium viability test (replicated four times) was conducted concurrently with the germination tests to monitor changes in seed viability before and after the application of dormancy loss conditions and after storage at the different temperature treatments. This stain-based test is a widely recognized and accurate means of estimating seed viability and is a routine test in many seed testing facilities (Copeland and McDonald, 2001). It detects the presence of dehydrogenase activity in actively respiring viable seed tissues (Elias et al., 2012). During respiration, dehydrogenase enzymes react with substrates and release hydrogen ions (Copeland and McDonald, 2001, Elias et al., 2012). The hydrogen ions react with the colorless tetrazolium chloride solution to produce a red dye called formazan (Copeland and McDonald, 2001, Elias et al., 2012). The staining patterns formed on the seeds with this reaction allow for the assessment of seed viability.

Seeds were pre-imbibed with deionized water for 24 hours to activate enzymes and facilitate cutting. After soaking, the seeds were individually cut in half through the
middle of the embryonic axis (Peters, 2000). Half of the cut seed was placed in a 20 ml scintillation vial. About 3 ml of a 1% aqueous solution of tetrazolium chloride was pipetted into the vials. These were then incubated in the dark at 27°C for 5 hours to allow staining. After the staining time has elapsed, the seeds were washed with deionized water and arranged cut side facing up in a petri dish with moistened filter paper (Whatman #3, Whatman International, Piscataway, NJ). A dissecting microscope was used to help determine whether the seeds were viable or not (Figure 1). Fully stained embryos were considered as viable seed while partially stained embryos or completely stained seeds (i.e., endosperm and embryo, caused by fungal contamination) were considered as non-viable. The total number of viable seeds per petri dish was counted and percent viability was recorded.

**Experimental setup and statistical analysis**

The tetrazolium viability and germination tests were laid out as a completely randomized design. Analysis of variance (ANOVA) was conducted on both the tetrazolium and germination data. No data transformation was required since the assumptions of the ANOVA were met. Tetrazolium viability data before and after dormancy loss as well as after storage at the different temperature treatments were included in the ANOVA. For germination, data subjected to an ANOVA were germination after dormancy loss and germination after storage for 6 months at the different temperature treatments. Germination data before controlled dormancy loss was excluded in the analysis since the mean and variance was zero. According to Reeve and Strom (2004), treatments that have zero or low variance can reduce the magnitude of the
mean square error, which is the denominator for $F$ tests and also a component of multiple comparison procedures. Removal of these treatments in the analysis would ensure that error rates are near their true values (i.e., mean square error is not reduced due to zero variance treatments). Tukey HSD test was used to separate the treatment means.

**Results**

Tetrazolium viability tests indicated a significant effect of storage temperature imposed after the dormancy loss conditions ($P=0.0443$) (Table 1). Seeds stored at 5°C exhibited significantly higher viability (88.6%) compared to those stored at 20°C (78.9%) (Figure 2). Seed viability at 30°C was not significantly different (86.0%) between the two storage temperature treatments. When seed viability at each storage temperature treatment was compared with those recorded prior to and after the application of controlled dormancy loss conditions, no significant differences were observed.

Data for germination tests indicated a significant effect of storage temperature ($P=0.0114$) (Table 2). Pili grass seeds stored at 30°C exhibited the highest percent germination (72.5%) (Figure 3). Germination of seeds stored at 5°C (56.5%) was not significantly different from germination immediately after the dormancy loss treatment (52.5%) and germination from seeds stored at 20°C (63.5%).
Discussion

Results obtained from this study indicate that dormancy status of dry stored pili grass seeds (i.e., 12% eRH or 6% seed moisture) can be maintained or relieved depending upon storage temperature. Storage at 30°C promoted dormancy loss while storage at 5°C maintained dormancy status. Storage for six months at the different temperatures did not cause significant deterioration in seed viability. These results were similar to those reported in Chapter 3 as well as in studies with red rice (Cohn and Hughes, 1981). Results from Chapter 3 indicated that dormant dry seeds (12% eRH) of the same seed batch (i.e., harvested in October 2011) fully lost seed dormancy after one year of storage at 30°C. In contrast, dry seeds (12% eRH) stored at 10°C maintained seed dormancy with no substantial loss in seed viability. In red rice (11 to 12% seed moisture, fresh weight basis), the rate of dormancy loss was faster with storage at 30°C than 20°C (Cohn and Hughes, 1981). Storage at 5°C significantly slowed dormancy loss. However, dormancy loss did occur if storage was prolonged (i.e., 11 months).

Although storage at 30°C for one year was beneficial for continuing the loss of pili grass dormancy in this study, it is recommended that seeds relieved of dormancy be transferred to a low storage temperature (i.e., 5°C or lower) for long term storage. In general, lower temperatures slow down loss of seed viability (Harrington, 1972, George, 2011). High storage temperatures impact seed viability loss by increasing the rate of metabolic reactions, increasing non-enzymatic deteriorative processes such as lipid oxidation (Basu, 1995) and by promoting the proliferation of fungus/mold (as viewed during viability measurements).
Based on the findings of this study, a protocol is suggested for relieving pili grass seed dormancy and for maintaining seed viability in post dormancy relief storage. The first step is to air dry freshly harvested seeds for 10 to 20 days to achieve 14% moisture content (dry weight basis). After air drying, store freshly harvested seeds for approximately 28 days at 12% eRH (i.e., storage at 25°C in desiccators with 50 grams of saturated lithium chloride solution per 250 grams of seed) to develop a moisture content of 6% (dry weight basis). Optimum dormancy relief is obtained with storage for 1 year in an airtight container (to prevent rehydration or additional moisture loss) at 30°C. Seeds relieved of dormancy can have viability maintained with storage at 5°C for at least 6 months. Maintenance of seed viability for longer periods may be possible but confirmation of longer storage times will need to be determined.
### Tables

Table 4.1. Analysis of variance (ANOVA) table for the tetrazolium viability tests of seeds dry-stored at different temperatures.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>202.727</td>
<td>50.6817</td>
<td>3.18</td>
<td>0.0443</td>
</tr>
<tr>
<td>Error</td>
<td>15</td>
<td>238.915</td>
<td>15.9277</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>441.642</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2. Analysis of variance (ANOVA) table for the percent germination of seeds dry-stored at different temperatures.

<table>
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<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>923</td>
<td>307.667</td>
<td>5.73</td>
<td>0.0114</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>644</td>
<td>53.667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>1567</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4.1. Staining patterns used for evaluating viable (A) and non-viable (B) seeds in the tetrazolium test: fully stained embryo (A1 and A2), infected with fungi (B1), dead seed (B2) and partially stained seed (B3).
Figure 4.2. Percent viability of seeds before and after controlled dormancy loss and after storage for six months at 5°C, 20°C and 30°C. Percent viability data presented are original means of four 50-seed replications. Means followed by the same letters are not significantly different as determined by Tukey HSD Test (P<0.05).

Figure 4.3. Percent germination of seeds after controlled dormancy loss and after storage for six months at 5°C, 20°C and 30°C. Percent germination data presented are original means of four 50-seed replications. Means followed by the same letters are not significantly different as determined by Tukey HSD Test (P<0.05). Mean percent germination recorded before the application of the controlled dormancy loss treatment was zero (*), hence was not included in the statistical analysis.
CHAPTER 5
ELUCIDATING THE SEED DORMANCY LOSS MECHANISMS IN PILI GRASS
(Heteropogon contortus): GERMINATION STIMULATION WITH
GIBBERELLIC ACID, FLURIDONE AND FOOD GRADE LIQUID SMOKE

Introduction

Pili grass [Heteropogon contortus (L.) Beauv. ex Roem and Schult.] is a culturally and ecologically important perennial bunchgrass native to the main Hawaiian islands (Daehler and Carino, 1998). In recent years, interest has increased in utilizing this drought tolerant species for restoration, erosion control, roadside re-vegetation and landscaping. To increase availability of planting materials for these applications, improvements on pili grass seed production and seed treatments to relieve dormancy are necessary.

Although pili grass readily produces seeds, it possesses a non-deep physiological dormancy that prevents germination of freshly harvested mature seed. Non-deep physiological dormancy in pili grass can be relieved with dry, warm storage (Tothill, 1977, Daehler and Goergen, 2005). Data reported in Chapters 3 and 4 have determined that storage temperature, duration of storage and seed moisture content affect the loss of dormancy and viability of pili grass seeds. To optimize dormancy loss and minimize loss of seed viability, seeds must be dry stored (12% equilibrium relative humidity) for 12 months at 30°C (see Chapter 3). Alternatively, non-deep physiological dormancy in pili grass can also be completely relieved with exogenous application of gibberellic acid
Dormant seeds can be partially relieved either by nicking to expose the embryo (see Chapter 2), and exposing imbibed seeds to aerosol smoke (Campbell, 1995, Campbell et al., 1996). Studies reported in this dissertation have also shown that germination of dormant seeds can be improved with aqueous solutions containing cyanide, food grade liquid smoke or water infused with smoke (i.e., combustion products) of pili grass tissue and xylose (see Chapter 6).

While the studies mentioned above have focused on finding effective treatments for relieving pili grass seed dormancy, research on dormancy loss mechanisms involved in this species have not been conducted. A number of studies have pointed out that seed dormancy is regulated in part, by GA and abscisic acid (ABA) (Ali-Rachedi et al., 2004, Cadman et al., 2006, Finch-Savage and Leubner-Metzger, 2006, Huarte and Benech-Arnold, 2010). The hormone balance theory suggests that GA and ABA simultaneously and antagonistically regulate the onset, maintenance and termination of dormancy (Wareing and Saunders, 1971, Ali-Rachedi et al., 2004, Baskin and Baskin, 2004, Finch-Savage and Leubner-Metzger, 2006). Rather than the actual hormone contents, seed dormancy and germination is regulated both by the ratio between ABA and GA and by the seed’s sensitivity to each hormone (Kucera et al., 2005, Finch-Savage and Leubner-Metzger, 2006, Foley and Chao, 2008).

The ABA:GA ratio and hormone sensitivity is manipulated by environmental signals that regulate the expression of each hormone’s biosynthetic and catabolic enzymes (Finkelstein et al., 2008). Dry after-ripening is an example of an environmentally mediated mechanism that can transition seeds from a dormant to a non-dormant state (Iglesias-Fernandez et al., 2011). Dry after-ripening effectively shifts the
ABA:GA balance towards higher levels of GA by a combination of the following: 1) decreasing ABA seed content (Grappin et al., 2000, Ali-Rachedi et al., 2004); 2) decreasing ABA sensitivity (Grappin et al., 2000, Romagosa et al., 2001); 3) increasing GA biosynthesis (Cadman et al., 2006); and 4) increasing GA sensitivity (Leubner-Metzger, 2002).

While it is confirmed that GA application relieves pili grass seed dormancy (Tothill, 1977), the contributions of ABA in seed dormancy maintenance of this species have not been fully elucidated. To investigate the role of ABA, researchers have used chemical agents to alter ABA biosynthesis during seed germination. Fluridone (1-methyl-3-phenyl-5-(3-trifluoromethyl-(phenyl))-4-(1H)-pyridinone) has been employed as a metabolic inhibitor to demonstrate the role of ABA in seed dormancy and germination as well as in other growth and developmental processes (Webb et al., 2009, Thammina et al., 2012). Fluridone is marketed as an aquatic herbicide and can prevent ABA production by inhibiting phytoene desaturase, a critical enzyme in the biosynthesis of the ABA precursor, carotene (Chae et al., 2004, Feurtado et al., 2007, Webb et al., 2009, Thammina et al., 2012).

The objectives of this study were to: 1) determine the germination response of pili grass seeds with decreasing levels of dormancy (i.e., imposed by increasing time of storage under conditions optimized for dormancy relief) to fluridone, GA, fluridone + GA and food grade liquid smoke (FGLS); 2) quantify the efficacy of these germination stimulants; and 3) elucidate the role of ABA in seed dormancy maintenance of pili grass.
Materials and methods

Seed source

Pili grass seeds used were of a Kahoʻolawe source identified natural germplasm (accession #: 9079683, HA-5748). Seeds were obtained from irrigated field plantings at the US Department of Agriculture-Natural Resource Conservation Service (USDA-NRCS) Plant Materials Center (PMC) on the island of Molokaʻi (21° 8' 33.7842"; -157° 6' 3.369`). Collection and harvesting of seeds were done on March 18, 2011 using a combine harvester. The seeds were immediately transported to the UH Mānoa Campus on Oahu, where they were air dried for 9 days to 10.3% moisture (dry weight basis). After drying, the seeds were passed through an air blast seed cleaner (Almaco, Nevada, IA) to remove most of the chaff, awns and empty seeds. The seeds were placed in plastic zipper bags (Hefty Slider Storage Bags, Reynolds Consumer Products Inc., Richmond, VA) and stored at 10°C until used. This storage condition was confirmed to preserve dormancy status of pili grass seeds for up to a year (see Chapter 3).

Controlled dormancy release conditions

Controlled dormancy release of the pili grass seeds was initiated on May 22, 2012 (431 days after harvest, 420 days storage at 10°C) using the optimum dormancy loss conditions (12% equilibrium relative humidity [eRH], 30°C, described in in Chapter 3). Prior to storage at 30°C, the seeds were portioned into 16 unsealed, foil barrier packets (Seeds Savers Exchange, Decorah, IA). Seeds (already placed in unsealed packets) were equilibrated in desiccators containing a saturated aqueous solution of lithium chloride
(LiCl·H₂O). This salt solution was used to maintain a humidity of 12% at 25°C (Winston and Bates, 1960, Commander et al., 2009, Turner et al., 2009). The seeds were stored under these conditions for 28 days to allow equilibration of seed moisture content. After equilibration, the packets were sealed using a thermal impulse sealer (Uline, Pleasant Pairie, WI) to maintain the designated humidity/seed moisture treatment. The sealed packets were placed inside heat-sealed plastic bags and then stored at 30°C constant temperature for 0, 2, 4, and 6 months to produce seeds with increasing levels of dormancy relief. A separate batch from the same harvest date (March 18, 2011) was eRH-equilibrated and incubated separately (i.e., in time) and used for the second run of the experiment.

**Germination assay**

The response of pili grass to GA, fluridone, fluridone + GA and FGLS was assessed using germination tests conducted after 0, 2, 4 and 6 months storage at 12% eRH and 30°C. A 50-seed germination test replicated four times was used to evaluate the treatments for each study. The test was conducted in square petri dishes (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA) lined with 2 layers of filter paper (Whatman P8, Whatman International, Piscataway, NJ). Prior to sowing, the filter papers were premoistened with approximately 3 ml of treatment solution or deionized water (control). Fifty seeds were sown on each filter paper-lined petri dish. After sowing, the petri dishes were sealed on the sides with parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent drying and incubated under 30°C constant temperature (Tothill, 1977) with 12 hours of light. The light was supplied by a 60 watt incandescent plant light bulb.
(Philips Agro-Lite A/9, Philips, Andover, MA). The petri dishes were observed regularly and re-moistened with treatment solution on an as needed basis. Final germination percentage was recorded 20 days after sowing the seeds. Seeds were counted as germinated when at least 1 mm of the radicle had emerged. The germination experiment was repeated using independently prepared treatment solutions and a second seed batch.

**Treatment solution preparation**

*Food grade liquid smoke*

A 1% by volume solution of FGLS (Colgin Liquid Smoke Natural Mesquite, The Colgin Companies, Dallas, TX) was prepared from bottles purchased in Hawaii in 2012. This dilution was based on the optimum concentration obtained from the dose rate studies conducted in Chapter 6. To prepare a 100 ml solution, 1 ml of FGLS was diluted with 99 ml of deionized water. Fresh solutions were used in the initial treatment application. The same solution was stored in clear bottles at 5°C and re-used for subsequent re-wetting of the petri dishes.

*Gibberellic acid*

Commercial grade GA (Falgro 20 SP, Fine Agrochemicals, Walnut Creek, CA) was purchased and prepared by dissolving 2.5 grams of product in 100 ml deionized water (0.5 percent by weight). This effective dilution of GA was based on studies conducted by Daehler and Goergen (unpublished manuscript) to induce germination of dormant pili grass seeds. Fresh solutions were used in the initial treatment application. The same solution was stored in clear bottles at 5°C and re-used for subsequent re-wetting of the petri dishes.
**Fluridone**

Analytical grade fluridone was purchased from Sigma Aldrich and prepared using a modified procedure by Zhang and Gusta (2010). For this study, a 40 μM solution was used and prepared from a 200 μM stock solution. This concentration was determined as optimal for improved germination of pili grass seeds (Baldos, unpublished data). To make a 200 μM stock solution, 13.17 mg of fluridone was dissolved in 400 μL of methanol. Once dissolved, the solution was added dropwise to 199.6 mL of deionized water to obtain a 200 mL 200 μM stock solution. Fresh solutions were used in the initial treatment application. The same solution was stored in clear bottles at 5°C and re-used for subsequent re-wetting of the petri dishes.

**Gibberellic acid and fluridone**

A solution containing 0.5 percent (by weight) GA and 40 μM fluridone was prepared by dissolving 2.5 g of Falgro 20SP in 100 ml of 40 μM fluridone. The 100 mL solution of 40 μM fluridone was prepared from the 200 μM fluridone stock solution previously described. Fresh solutions were used in the initial treatment application. The same solution was stored in clear bottles at 5°C and re-used for subsequent re-wetting of the petri dishes.

**Seed viability test**

Tetrazolium tests were conducted concurrently with the germination assays to monitor changes in viability of seeds in storage at the optimized dormancy relief condition. This stain-based test is a widely recognized and accurate means of estimating seed viability and is a routine test in many seed testing facilities (Copeland and
McDonald, 2001). It detects the presence of dehydrogenase activity in actively respiring viable seed tissues (Elias et al., 2012). During respiration, dehydrogenase enzymes react with substrates and release hydrogen ions (Copeland and McDonald, 2001, Elias et al., 2012). The hydrogen ions react with the colorless tetrazolium chloride solution to produce a red dye called formazan (Copeland and McDonald, 2001, Elias et al., 2012). The staining patterns formed on the seeds with this reaction allows for the assessment of seed viability.

Fifty seeds were obtained from each of the opened seed packets (n=4) used in the germination tests. Seeds were soaked in distilled water overnight to activate enzymes and facilitate cutting. After soaking, the imbibed seeds were longitudinally cut through the middle of the embryonic axis. Half of the cut seed was placed in a 20 ml scintillation vial and filled with 3 ml of 1% tetrazolium chloride (Sigma Aldrich, St. Louis, MO) solution. The vials were incubated in the dark at 27°C for 5 hours to allow staining. The stained seeds were washed with distilled water, arranged in a petri dish lined with moistened filter paper and observed under a dissecting microscope. Seeds were counted as viable based on specific staining patterns (Figure 1). Fully stained embryos were recorded as viable seed while partially stained embryos or stained endosperm and embryo (caused by fungal contamination) were considered as non-viable.

**Experimental setup and statistical analysis**

The germination study was setup as a split split plot with four replicates. The main effect plots were the experimental runs. The sub plot effect was the chemicals/germination stimulants used and the sub-sub plot effect was incubation period
under the optimum dormancy loss conditions. Total percent germination after 20 days was modeled using analysis of variance (ANOVA) in Statistix 9 (Analytical Software, Tallahassee, FL). No data transformation was necessary since the assumptions of the ANOVA were met (i.e., homogeneity of variance and normality). Tukey’s range test was used to separate the means of significant factors or interactions.

The tetrazolium seed viability test was setup as a split plot with four replicates. The main effect plots were the experimental runs and month of incubation was the sub plot effect. ANOVA (in Statistix 9) was also used to model percent seed viability. No data transformation was necessary since the assumptions of the ANOVA were met (i.e., homogeneity of variance and normality). Tukey’s range test was used to separate the means of significant factors or interactions.

**Results**

Analysis of variance for percent germination did not indicate a significant three-way interaction between treatment, month and experiment (P=0.134) (Table 1). A significant interaction between month and experiment (P=0.0022) as well as treatment and month (P<0.01) was recorded. Means for the month by experimental run interaction were pooled across germination stimulant treatments (Table 2). The pooled mean percent germination data indicated that dormancy relief increased with length of storage. Means for the month by germination stimulant treatment interaction were pooled across experimental runs (Table 3). These data indicated that the response to the germination stimulant treatments was dependent upon the degree of dormancy relief, i.e., storage time.
under optimized dormancy relief conditions. At zero months of storage, all germination stimulant treatments increased percent germination above the distilled water control. Fluridone + GA was additive in its effect on germination in comparison to either chemical alone. The germination stimulation in seeds treated with FGLS was comparable to those in seeds treated with fluridone + GA or GA alone. Seeds treated with FGLS also exhibited significantly higher percent germination in comparison to seeds incubated in distilled water or fluridone alone. At two months in storage, only the fluridone + GA and FGLS treated seeds exhibited percent germination that were greater than those recorded in distilled water. At four and six months of storage, dormancy relief of the seeds was near its maximum level. Thus, the germination stimulants did not increase germination levels beyond those obtained with distilled water.

Analysis of variance for the tetrazolium viability test neither indicated a significant experimental run by storage month interaction nor a significant effect of storage time. Only the factor of experimental runs had a significant effect on seed viability (Table 4). Seed viability, pooled across all storage months was 74.2% in run 1 and 83.0% in run 2. Seed viability was not affected by storage for 6 months under the optimum dormancy loss conditions of 12% eRH and 30°C.

**Discussion**

In this study, full relief of pili grass seed dormancy was established at the maximum level of germination obtained at six months of storage (i.e., 78.5% in distilled water). Full dormancy relief was confirmed since the average seed viability of the
experimental runs was 78.6%. Since percent germination data at four and six months of storage indicated no significant differences between treatments, elucidation of the impact of GA and ABA on pili grass seed dormancy relief was focused on data obtained at zero and two months of storage.

At zero months of storage, the application of either fluridone or GA alone improved pili grass seed germination above distilled water. Germination stimulation observed in the fluridone only treatments suggests that de novo ABA biosynthesis was prevented, thereby shifting the ABA:GA ratio towards GA (i.e., endogenous GA). Fluridone stimulated germination may indicate the role of de novo ABA synthesis in dormancy maintenance (Grappin et al., 2000) of pili grass.

Exogenous application of GA on pili grass seeds shifted the ABA:GA ratio towards GA by supplementing endogenous GA levels. Kucera et al (2005) and Finch-Savage and Leubner-Metzger (2006) indicate that GA can promote germination of dormant seeds through endosperm weakening and by increasing embryo growth potential. The observed germination stimulation with exposure of the embryo (see Chapter 2) suggests a mode in which GA acts to relieve dormancy in pili grass. Aside from supplementing endogenous levels, GA application may have also reduced ABA levels in the seed through catabolisis (Grappin et al., 2000, Gonai et al., 2004). Germination studies with Nicotiana plumbaginifolia and Lactuca sativa cv. ‘Grand Rapids’ seeds may support this claim since a decline in ABA content was reported when exogenous GA was applied to dormant seeds of these species (Grappin et al., 2000, Gonai et al., 2004).
Application of either fluridone or GA alone exhibited similar levels of germination stimulation for pili grass at 0 months of storage. In other reports the effectiveness of fluridone and GA was shown to be species dependent. The application of 10 µM fluridone on seeds of *Arabidopsis thaliana* (Cape Verde Islands ecotype) was more efficient in stimulating seed germination than exogenous applications of 100 µM GA (Ali-Rachedi *et al.*, 2004). In contrast, GA (100 µM) applications were slightly more stimulatory than fluridone (100 µM) for *N. plumbaginifolia* seeds (Grappin *et al.*, 2000).

Treating the seeds stored for zero months with fluridone + GA resulted in an additive effect, significantly improving germination above the levels observed when either chemical was applied alone. This additive effect of GA and fluridone was consistent with studies in *A. thaliana* (Cape Verde Islands ecotype) (Ali-Rachedi *et al.*, 2004), *N. plumbaginifolia* (Grappin *et al.*, 2000), *Orobanche* spp. (Song *et al.*, 2006) and cultivars of *L. sativa* (Gonai *et al.*, 2004, Argyris *et al.*, 2008, Dong *et al.*, 2012). The ABA:GA ratio in the fluridone + GA treatment may have strongly shifted in favor of GA since endogenous GA levels were supplemented (GA effect), ABA catabolism may have been promoted (GA effect) and *de novo* ABA biosynthesis may have also been inhibited (fluridone effect).

At two months of storage, dormancy relief of the seed batch had increased. The observed improvement in germination in the distilled water treatment suggested that the ABA:GA ratio of the seeds has changed to favor higher levels of GA. The shift in the ABA:GA ratio during storage may be due to decreasing endogenous ABA levels and/or sensitivity and/or increasing endogenous GA levels and/or sensitivity. In either case, the impact would be to provide an effective increase in GA levels and relief of dormancy.
Since fluridone alone did not increase germination above the levels recorded in distilled water, it was concluded that endogenous ABA levels or ABA sensitivity may have been reduced to the point where blockage of de novo ABA no longer had a significant stimulation effect. Studies on *N. plumbaginifolia* (Grappin et al., 2000) provides support to this conclusion since the same responses to fluridone was also observed in dormant and non-dormant seeds of this species. Quantification of ABA in dormant and non-dormant (i.e., after-ripened) *N. plumbaginifolia* seeds indicated ABA degradation during after-ripening due to a decrease in ABA content following one year of storage. Fluridone induced germination observed in non-dormant *N. plumbaginifolia* was attributed to a decrease in ABA levels (Grappin et al., 2000). Grappin et al. (2000) also showed that dormant and non-dormant *N. plumbaginifolia* seeds exhibited differences in sensitivity to ABA. Germination studies with exogenous ABA showed that dormant *N. plumbaginifolia* seeds were 4-fold more sensitive to ABA than non-dormant seeds. Dormant *N. plumbaginifolia* seeds required 2 µM of exogenous ABA to obtain 50% germination inhibition while non-dormant seeds required 8 µM of exogenous ABA to obtain the same effect (Grappin et al., 2000).

The GA alone impact on germination of two month stored pili grass seeds was similar to fluridone alone and distilled water. Besides decreases in ABA level or sensitivity, dormancy relief at two months of storage may have also increased endogenous GA levels or sensitivity to the point where exogenous GA application did not result in significant germination stimulation. Studies on *A. thaliana* (Cape Verde Islands ecotype) (Cadman et al., 2006) and in *Nicotiana tabacum* (Leubner-Metzger, 2002) support this conclusion since changes in GA biosynthesis (i.e., expression of gene
transcripts) and GA responsiveness with dry storage (i.e., after-ripening) were recorded in these experiments. Cadman et al. (2006) recorded a 40-fold higher expression of the gene encoding GA 3-betadioxygenase (GA3ox2) in after-ripened A. thaliana Cape Verde Islands ecotype seeds exposed to light than in dormant seeds. GA3ox2 increases GA levels in the seeds by converting inactive precursors to the active GA1 and GA4.

Leubner-Metzger (2002) observed a decreased GA requirement for dark germination of after-ripened N. tabacum seeds. Fresh, photodormant N. tabacum seeds required a higher amount of GA (0.73 μM) to achieve 50% dark germination than non-photodormant seeds (0.02 to 0.2 μM).

Although the application of fluridone + GA on two month stored seeds exhibited significant germination stimulation above the level recorded in distilled water, its additive effect over GA and fluridone alone has diminished. Percent germination in the fluridone + GA treatment was not significantly different from those recorded in the fluridone only or GA only treatments.

The germination stimulation observed in zero and two month stored seeds incubated with FGLS were comparable to those incubated in GA + fluridone. Both treatments relieved seed dormancy completely since percent germination in these treatments were not significantly different from those observed in the non-dormant control seeds (i.e., incubated at 4 and 6 months and germinated in distilled water). Studies by Schwachtje and Baldwin (2004) suggests that the dormancy breaking ability of FGLS appears to affect ABA pools and GA sensitivity. ABA levels of N. attenuata seed increased within two hours of FGLS (House of Herbs Inc., Passaic, NJ) treatment and then decreased significantly to levels lower than in seeds incubated in distilled water after
22 hours (Schwachtje and Baldwin, 2004). Besides changes in levels of ABA, seed sensitivity to exogenous GA under light and dark conditions also increased dramatically with FGLS application (Schwachtje and Baldwin, 2004).

Of the two recently discovered smoke-derived active compounds (i.e., karrikinolide [KAR₁] and cyanide), only cyanide was observed as stimulatory to pili grass seeds (see Chapter 6). When quantified for cyanide content (i.e., using the Cyantesmo paper), FGLS tested negative, indicating that compounds other than KAR₁ or cyanide are responsible for the observed germination stimulation with FGLS.

In summary, results of this study supported the theory that GA, ABA and the ratio between their levels in the seed play an important role in dormancy maintenance of imbibed pili grass seeds. Germination stimulation with food grade liquid smoke was observed to be as effective as fluridone + GA. The literature cited here supports the theory that FGLS works to reduce ABA levels and increase tissue sensitivity to GA.
### Tables

**Table 5.1.** Analysis of variance (ANOVA) table for percent germination of pili grass seeds stored under the optimum dormancy loss conditions (12% relative humidity and 30°C for 0, 2, 4 and 6 months) and incubated in solutions of gibberellic acid, fluridone, gibberellic acid + fluridone and food grade liquid smoke.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>3</td>
<td>183.6</td>
<td>61.2</td>
<td>0.66</td>
<td>0.4767ns</td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0.4767ns</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>45.6</td>
<td>15.2</td>
<td>18.17</td>
<td>0</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>7138.8</td>
<td>1784.71</td>
<td>0.66</td>
<td>0.4767ns</td>
</tr>
<tr>
<td>Experimental run x Treatment</td>
<td>4</td>
<td>342.3</td>
<td>85.56</td>
<td>0.87</td>
<td>0.4956ns</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>24</td>
<td>2357.3</td>
<td>98.22</td>
<td>121.85</td>
<td>0</td>
</tr>
<tr>
<td>Incubation month</td>
<td>3</td>
<td>26504.6</td>
<td>8834.87</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Experimental run x Incubation month</td>
<td>3</td>
<td>1138.2</td>
<td>379.4</td>
<td>5.23</td>
<td>0.0022</td>
</tr>
<tr>
<td>Treatment x Month</td>
<td>12</td>
<td>8343.1</td>
<td>695.26</td>
<td>9.59</td>
<td>0</td>
</tr>
<tr>
<td>Experimental run x Treatment x Incubation month</td>
<td>12</td>
<td>1316.5</td>
<td>109.71</td>
<td>1.51</td>
<td>0.134ns</td>
</tr>
<tr>
<td>Sub-sub plot error</td>
<td>90</td>
<td>6525.5</td>
<td>72.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>159</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ns** Not significant.

**Table 5.2.** Percent germination of pili grass seeds combined within experimental runs and months in incubation (0, 2, 4 and 6 months) under the optimum dormancy loss conditions (12% relative humidity and 30°C). Percent germination data presented are original means. Means across columns and rows followed by the same letters are not significantly different as determined by Tukey Range Test (P<0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Months in incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; experimental run</td>
<td>48.4 bc</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; experimental run</td>
<td>40.9 c</td>
</tr>
</tbody>
</table>

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Table 5.3. Percent germination of pili grass seeds stored for 0, 2, 4 and 6 months under the optimized dormancy loss conditions (12% relative humidity and 30°C) and incubated in germination stimulants. Means were pooled over two experimental runs. Means in columns and rows followed by the same letters are not significantly different as determined by Tukey Range Test (P<0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Months in incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control (distilled water)</td>
<td>18.5 g</td>
</tr>
<tr>
<td>40 μM Fluridone</td>
<td>39.0 f</td>
</tr>
<tr>
<td>Gibberellic acid (0.5%)</td>
<td>43.3 ef</td>
</tr>
<tr>
<td>Fluridone + Gibberellic acid</td>
<td>63.3 abc</td>
</tr>
<tr>
<td>Food grade liquid smoke (1% v/v)</td>
<td>59.3 bcde</td>
</tr>
</tbody>
</table>

Table 5.4. Analysis of variance (ANOVA) table for percent seed viability of pili grass seeds stored under the optimum dormancy loss conditions (12% relative humidity and 30°C for 0, 2, 4 and 6 months) and incubated in solutions of gibberellic acid, fluridone, gibberellic acid + fluridone and food grade liquid smoke.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>3</td>
<td>170.34</td>
<td>56.781</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>621.28</td>
<td>621.28</td>
<td>12.65</td>
<td>0.0379</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>147.34</td>
<td>49.115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Month</td>
<td>3</td>
<td>196.09</td>
<td>65.365</td>
<td>0.91</td>
<td>0.4556ns</td>
</tr>
<tr>
<td>Experimental run x Month</td>
<td>3</td>
<td>646.09</td>
<td>215.365</td>
<td>3</td>
<td>0.0579ns</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>18</td>
<td>1292.56</td>
<td>71.809</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ns Not significant.
Figure 5.1. Staining patterns used for evaluating viable (A) and non-viable (B) seeds in the tetrazolium test: fully stained embryo (A1 and A2), infected with fungi (B1), dead seed (B2) and partially stained seed (B3)
CHAPTER 6

GERMINATION RESPONSE OF DORMANT PILI GRASS (*Heteropogon contortus*) SEEDS TO SMOKE-INFUSED WATER AND TWO SMOKE-ASSOCIATED STIMULATORY COMPOUNDS

**Introduction**

Fire is an important and well documented form of ecological disturbance. It provides a recruitment opportunity for plants since fires greatly modify the local environment by altering the soil, increasing light, reducing allelochemicals and removing plant competition (Nelson *et al.*, 2012, Flematti *et al.*, 2013). Besides modifying the microenvironment, fires also produce heat, smoke and/or charred plant material which can serve as physical and chemical cues for triggering seed germination (Bond and Keeley, 2005). Smoke-stimulated germination has become an active area of research since it was first reported by De Lange and Boucher (1990) in the early 1990s. A recent Web of Science literature search (August 12, 2013) using the keywords smoke, germination and seed yielded approximately 106 articles evaluating germination stimulation by smoke or components in smoke in a number of plant species. It is estimated that about 1200 species from 80 genera are smoke responsive under field conditions (Chiwocha *et al.*, 2009, Dixon *et al.*, 2009, Flematti *et al.*, 2011b, Flematti *et al.*, 2013). Smoke-stimulated germination has been observed in both fire and non-fire adapted species (Flematti *et al.*, 2013) such as lettuce (*Lactuca sativa*) (Drewes *et al.*, 1995), corn (*Zea mays*) (Sparg *et al.*, 2006), tomato (*Solanum lycopersicon*) (Taylor and...
van Staden, 1998), celery (*Apium graveolens*) (Thomas and van Staden, 1995) and papaya (*Carica papaya*) (Chumpookam *et al*., 2012).

Two active ingredients in smoke responsible for improving seed germination have been isolated and characterized. The butenolide compound, 3-methyl-2H-furo[2,3-c]pyran-2-one or karrikinolide (KAR₁), was first described in 2004. The discovery of KAR₁ was made by two independent research teams from Australia (Flematti *et al*., 2004) and South Africa (van Staden *et al*., 2004). KAR₁ is a highly active, heat stable and long lasting compound (van Staden *et al*., 2004) capable of stimulating germination in lettuce (*Lactuca sativa*) and in a large number of smoke-responsive species (Flematti *et al*., 2011b, Nelson *et al*., 2012). KAR₁ is a very potent germination stimulant. Species sensitive to KAR₁ can be stimulated to germinate at very low concentrations (1 ppb; 1μg/L; 10⁻⁹ M) (Flematti *et al*., 2004, Flematti *et al*., 2013). KAR₁ can be isolated from smoke-infused water prepared from the combustion of plant material, cellulose or simple carbohydrates (Flematti *et al*., 2011a). A number of synthetic analogues have also been prepared (Scaffidi *et al*., 2011) with some detected in smoke (Chiwocha *et al*., 2009).

Based on combustion experiments with pure xylose, glucose or cellulose, it was proposed that KAR₁ is derived from a pyranose sugar (Flematti *et al*., 2011a, Nelson *et al*., 2012).

Glyceronitrile is another compound recently isolated from plant-derived smoke. It was first collected and characterized in 2011 from smoke-infused water prepared from the combustion of oaten hay as well as from fresh and dried bushland shoot materials (Flematti *et al*., 2011b). Research that led to the isolation and characterization of glyceronitrile was spurred by the inactivity of KAR₁ on seeds of *Anigozanthos manglesii*, a smoke responsive species (Flematti *et al*., 2011b). Glyceronitrile itself does not cause
the observed germination stimulation in A. manglesii seeds. Flematti et al. (2011b) found that, in the presence of water, glyceronitrile slowly hydrolyzes to release cyanide which in turn stimulates seed germination. This observation was later confirmed by germination assays using a number of cyanohydrins (i.e., mandelonitrile, acetone cyanohydrin, glycolonitrile and 2,3,4-trihydroxybutyronitrile). Cyanide-stimulated germination is not a new observation (Flematti et al., 2013). It has been reported in a wide variety of plant species, including grasses (Roberts, 1973, Taylorson and Hendricks, 1973, Cohn and Hughes, 1986, Siegień and Bogatek, 2006, Flematti et al., 2013). The novelty of discovering the presence of cyanohydrins in smoke is that it establishes cyanide as an important germination stimulant in post-fire environments (Flematti et al., 2013).

Pili grass (Heteropogon contortus) is a drought and fire tolerant native Hawaiian bunchgrass that is currently being used in conservation area restoration and has potential applications for roadside re-vegetation, agricultural buffer strips, forage and urban landscaping. A major hindrance to the immediate and large scale use of pili grass is its seed dormancy. Freshly harvested seeds exhibit zero to less than 10% germination (see Chapters 2 and 3). To remove dormancy and improve seed germination, an after-ripening period (i.e., dry storage) of about 6 to 12 months is required (Tothill, 1977, Pater, 1993, Daehler and Goergen, 2005, USDA-NRCS, 2007). Alternatively, the dormancy of pili grass can be relieved by soaking the seeds in 0.5% (Daehler, unpublished data) to 1% gibberellic acid (Tothill, 1977). This practice, however, can be costly and results in elongated seedlings (Baldos, personal observation).

Smoke-infused water and food grade liquid smoke can offer a cheaper and more practical alternative to gibberellic acid application to enhance pili grass seed germination.
Since pili grass is a fire-adapted species (Goergen and Daehler, 2001), it is assumed that smoke applications may improve the germination of dormant seeds. Campbell et al. (1996) confirmed this hypothesis through germination studies of seeds treated with cool aerosol smoke from combusted pili grass. Assays indicate that smoked seed exhibited more than twice the germination of untreated seed. While Campbell et al. (1996) confirmed the smoke responsiveness of pili grass seeds, follow-up studies have yet to be conducted to further explore other smoke sources and assay the recently identified germination stimulants found in smoke (i.e., KAR\textsubscript{1} and cyanide). By examining the response of pili grass seeds to these smoke and smoke-derived compounds, one may be able to elucidate the mechanisms that control its dormancy and develop a basis for applied uses on direct seeded plantings. The objectives of this study are to: 1) identify the optimum concentrations of smoke-infused water derived from burned xylose, burned pili grass and food grade liquid smoke; 2) confirm the stimulatory capability and identify the optimum concentrations of KAR\textsubscript{1} and cyanide; 3) compare the germination stimulation capability of the smoke sources against KAR\textsubscript{1} and cyanide and 4) estimate the amount of cyanide in each smoke source using the Cyantesmo paper (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

**Materials and methods**

Germination assays to assess the stimulatory capability of smoke and its known components on pili grass were divided into several interrelated studies. Individual dose response experiments were conducted on three differently sourced smoke-infused water
(i.e., food grade liquid smoke, xylose derived smoke-infused water and pili grass derived smoke-infused water), KAR$_1$ and cyanide. After the optimum concentrations were determined in the dose response experiments, studies comparing the stimulatory capability of each smoke source and known component/s in smoke were assessed.

**Seed source**

Seeds used for the assays were of a Kaho‘olawe source identified natural germplasm (accession #: 9079683, HA-5748). Seed batches were obtained from irrigated field plantings at the US Department of Agriculture-Natural Resource Conservation Service (USDA-NRCS) Plant Materials Center (PMC) on the island of Moloka‘i (21° 8' 33.7842"; -157° 6' 3.369"). Collection and harvesting of seeds were done in March 2011 and July 2012 using a combine harvester. The seeds were transported to the University of Hawai‘i at Mānoa campus on O‘ahu, where they were air dried for 9 days to 10.3% moisture (dry weight basis). After drying, the seeds were passed through an air blast seed cleaner (Almaco, Nevada, IA) to remove most of the chaff, awns and empty seeds. The seeds were placed in plastic zipper bags (Hefty Slider Storage Bags, Reynolds Consumer Products Inc., Richmond, VA) and stored at 5°C until used to prevent dormancy loss.

**Germination assay**

A 50-seed per experimental unit germination test was used to evaluate the treatments for each study. The test, which was replicated four times, was conducted on 100 mm x 15 mm Petri dishes (Fisherbrand) lined with a layer of filter paper (Whatman #3, Whatman International, Piscataway, NJ). Prior to sowing, the filter papers were pre-
moistened with approximately 3 ml of treatment solution. Fifty seeds from a specified batch (i.e., harvest date, storage temperature and storage duration were noted) were sown on each filter paper-lined petri dish. After sowing, the petri dishes were sealed along the sides with parafilm (Bemis Flexible Packaging, Neenah, WI) to prevent drying. These dishes were then incubated at 30°C (Tothill, 1977) and 12 hours of light. The light was supplied by a 60 watt incandescent plant light bulb (Philips Agro-Lite A/9, Philips, Andover, MA). The petri dishes were observed daily and re-moistened with treatment solution on an as needed basis. Total percent germination in each petri dish was recorded after 20 days of incubation. Seeds were counted as germinated when at least 1 mm of the radicle or shoot had emerged. The germination experiment was repeated using independently prepared treatment solutions and the same seed batch.

Dose rate studies with smoke-infused water derived from three different sources

Food grade liquid smoke (Colgin Liquid Smoke Natural Mesquite, The Colgin Companies, Dallas, TX), xylose smoke-infused water and pili grass smoke-infused water were prepared and evaluated for their effectiveness in stimulating germination of dormant pili grass seeds. The experiments were conducted between September and December 2012 as separate dose rate studies for each smoke source. The seeds used for the experiments were harvested in March 2011 and stored at 5°C (525 days for food grade liquid smoke and xylose smoke-infused water or 608 days for pili grass smoke-infused water) to preserve dormancy until used.
**Food grade liquid smoke**

The dose rate study with food grade liquid smoke was conducted between September and October 2012. Two bottles from different lots (i.e., 2 different expiration dates: 5/4/2013 and 7/7/2014) were purchased for the experiment. Four dilution treatments (undiluted, 1/10, 1/100 and 1/1000 by volume) and a control solution (distilled water) were prepared to determine the optimum concentration for maximum germination.

**Xylose smoke-infused water**

The dose rate study with xylose smoke-infused water was conducted between September and October 2012. The dilution series used in this study was the same as that used in the food grade liquid smoke dose rate experiment. Methods for producing smoke-infused water from xylose were based on a modified procedure by Flematti *et al.* (2011a). Briefly, combustion and smoke production was accomplished by placing a 2.4 gram sample of D-xylose (Sigma Aldrich, St. Louis, MO) into a preheated 3-necked 250 mL round bottom flask. The flask, which was connected to a positive air source and a 250 mL Erlenmeyer flask, was preheated for approximately five minutes with an open flame. After preheating, D-xylose was added to the flask to combust (Figure 1). The smoke produced from combustion was bubbled into 100 ml of deionized water (in the 250 mL Erlenmeyer flask) at 30 mL/min. After 10 minutes of heating, the D-xylose was carbonized and did not produce further smoke. Condensates that have accumulated inside the connector tubes and joints attaching the Erlenmeyer flask and the round bottom flask were rinsed using the smoke-infused water. The resulting smoke-infused water was yellow and exhibited a characteristic smell of burnt/caramelized sugar (Baldos, personal observation). Immediately after the first smoke solution was made, a second solution of
xylose-derived smoke was independently prepared. The smoke solutions were stored in clear glass bottles at 5°C prior to use.

**Pili grass smoke-infused water**

The dose rate study for evaluating pili grass smoke-infused water was conducted between November and December 2012. The treatment dilution series was the same as previously described. Pili grass smoke production followed the same procedure described in the xylose smoke dose rate study. Briefly, 2.4 grams of air-dried pili grass (i.e., leaves, stems and seedheads cut into 1 cm pieces) was combusted and the smoke produced was bubbled through 100 mL of distilled water at 30 mL/min. After 10 minutes of heating, the plant material was carbonized and did not produce additional smoke. Condensates that accumulated inside the connector tubes and joints attaching the Erlenmeyer flask and the round bottom flask were rinsed using the smoke-infused water and filtered through a layer of Whatman #3 filter paper. Smoke-infused water derived from the dried pili grass plant material exhibited a light yellow color and a distinctive burnt, acrid smell (Baldos, personal observation). Immediately after the first batch of pili grass smoke-infused water was made, a second smoke solution was independently prepared for the second run of the experiment. The pili grass smoke solutions were stored in clear glass bottles at 5°C prior to use.

**Dose rate studies with recently identified stimulatory compounds in smoke**

**Karrikinolide (KAR1)**

The response of dormant pili grass seeds to different concentrations of KAR1 was evaluated using March 2011 harvested seeds stored at 5°C. The experiment was first
conducted in May 2012 (seeds held 381 days at 5°C) and repeated in September 2012 (seeds held 534 days at 5°C) using the same seed batch. KAR₁ was kindly provided by Dr. Gavin Flematti, University of Western Australia. A 66.7 μM (10 ppm) KAR₁ stock solution was used to prepare the 0.0067, 0.067, 0.67, 6.67 and 66.7 μM dilution series. This range of concentrations was based on dilutions tested by Flematti et al. (2004). The stock solution was prepared by weighing exactly 1 mg of KAR₁ and dissolving it in a beaker filled with 100 ml distilled water. To facilitate the dissolution of KAR₁, the water was slightly heated using a hotplate stirrer (Corning PC-351, Acton, MA).

*Mandelonitrile and potassium cyanide*

The cyanide-generating compounds, mandelonitrile and potassium cyanide were evaluated for efficacy in stimulating germination of pili grass seeds. Low rate studies of mandelonitrile and potassium cyanide were based from concentrations used by Flematti et al. (2011b) (i.e., 0, 1, 5, 10, 20 and 50 μM).

The low rate mandelonitrile studies were conducted from October to November 2012. For potassium cyanide, the low rate studies were done from October to December 2012 while the high rate studies were conducted between January and February 2013. Both the low rate mandelonitrile and low rate potassium cyanide studies used March 2011 harvested seeds stored at 5°C (554 to 575 days in storage). The high rate potassium cyanide studies used seeds harvested in July 2012 and stored at 5°C (138 days in storage). Both rate studies were repeated using the same seed batch but with freshly prepared solutions.

Technical grade mandelonitrile (Sigma Aldrich, St. Louis, MO) was diluted to a 100 μM (13.3 ppm) stock solution. To make the stock solution, 2.66 mg (2.384 μL) of
mandelonitrile was diluted with distilled water to a volume of 200 mL. Mandelonitrile at 1, 5, 10, 20 and 50 μM were prepared using the 100 μM stock solution. In addition to the dilution treatments, a control treatment (distilled water) was also included.

Since the decomposition of mandelonitrile produced both free cyanide and benzaldehyde, a germination assay for benzaldehyde was also conducted. Benzaldehyde (Sigma Aldrich, St. Louis, MO) was evaluated at 100 μM and at the same concentrations as the mandelonitrile experiment (1, 5, 10, 20 and 50 μM). The 100 μM (10.61 ppm) stock solution was made by dissolving 2.038 μL of technical grade benzaldehyde in distilled water and bringing it to a volume of 200 mL. The controls were distilled water (control) and 50 μM mandelonitrile.

To determine if cyanide contributed to germination stimulation of smoke-infused water in pilic grass, assays with potassium cyanide were also carried out. Two independent dilution studies were conducted. The low concentration studies (1, 5, 10, 20 and 50 μM) evaluated dilution treatments similar to that of the mandelonitrile study. The high concentration studies evaluated concentrations at 50, 100, 250 and 500 μM. This range contains the maximum amount typically used in germination studies (50 μM) and the maximum concentration of cyanide detected in smoke-infused water (300 μM, Flematti et al., 2011b).

Dilution treatments in the low rate studies (1, 5, 10, 20 and 50 μM) were prepared from a 100 μM (6.51 ppm) stock solution. The stock solution was prepared by dissolving 1.30 mg of technical grade potassium cyanide (Sigma Aldrich, St. Louis, MO) in distilled water and bringing the solution to a volume of 200 mL. Dilution treatments for the high rate studies (50, 100, 250 and 500 μM) were made from a 500 μM (32.55 ppm) stock
solution. To make the stock solution, 6.51 mg of technical grade potassium cyanide was dissolved in distilled water and brought to a volume of 200 mL. A distilled water treatment (control) was also included in each experiment.

Germination studies with potassium chloride were conducted immediately following the potassium cyanide experiments. These follow-up studies were done to determine whether the potassium ion had an effect on pili grass germination. The levels of potassium chloride tested included the potassium concentrations used in each potassium chloride experiment, concentrations which were double the highest amount tested (i.e., 100 μM for the low rate study and 1000 μM for the high rate study), the highest potassium cyanide concentration tested for each rate study (i.e., 50 μM and 500 μM) and a control treatment (distilled water).

**Germination response to the optimum concentrations of smoke-infused water and cyanide**

The optimum concentrations of smoke-infused water and cyanide obtained from the previous dose rate studies were evaluated using seeds harvested in July 2012 and stored at 5°C for 175 days. Germination assays were performed to compare the response of the stored seeds to food grade liquid smoke (1% v/v dilution), xylose smoke-infused water (undiluted) and pili grass smoke-infused water (undiluted) against the high rate of potassium cyanide (500 μM). Smoke-infused water and potassium cyanide solutions were prepared three days prior (stored at 5°C) to the germination test according to the methods previously described. The control was distilled water.
Quantification of cyanide in the different smoke-infused waters using the Cyantesmo paper

Smoke and cyanide solutions used in the previously described germination assays were tested for the presence of cyanide. This was accomplished using the Cyantesmo qualitative test paper (CQTP) (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Manufacturer’s instructions were followed and involved placing 10 ml of treatment solution in 20 mL scintillation vials. The solution in each vial was then acidified by adding one drop of concentrated sulfuric acid. CQTP was cut into 3 cm strips and immediately placed inside the vials with 1 cm of the strip submerged in the solution and remaining portion exposed to the vapor phase above the test solution. The vials were then sealed to allow accumulation of hydrocyanic acid (HCN), a product of the acidification process which reacts with the test paper. When cyanide generating compounds are present, HCN will be produced; turning the color of the test strip in the vapor phase (of the solution) from light yellow to varying shades of blue (depending on cyanide concentration). According to manufacturer’s instructions, the minimum sensitivity limit of the CQTP is 7.4 μM (0.2 ppm) HCN after 15 minutes of reaction.

Smoke solutions that tested positive for cyanide underwent further examination to estimate the amount they contained. Using the CQTP, the presence of cyanide in the smoke-infused water dilutions was compared to the potassium cyanide solutions (0, 50, 100, 250, 500 μM). An estimation of cyanide content was determined by comparing the intensity of the blue shading of the test paper in the smoke solutions with those produced by the known potassium cyanide dilutions.
Experimental setup and statistical analysis

The germination studies were setup as a split plot with four replicates. The main effect plots were the experimental runs and the sub plot effect was treatment concentration/stimulant type. Total percent germination after 20 days, except for the higher rate potassium cyanide datasets, was transformed to conform the data to the analysis of variance (ANOVA) assumptions (i.e., homogeneity of variance and normality). Arcsine square root transformation was done for the dose response studies with smoke (i.e., food grade liquid smoke, xylose smoke and piligrass smoke), KAR₁, mandelonitrile and benzaldehyde as well as the potassium cyanide/smoke datasets. Square root transformation was conducted for the lower rate potassium cyanide and potassium chloride studies. ANOVA was conducted using Statistix 9 (Analytical Software, Tallahassee, FL). Tukey’s range test was used to separate the means in all datasets except for the benzaldehyde and potassium chloride studies. Mean separation in these datasets used Dunnett’s Test since the objective for these two studies was to compare the germination of seeds in the control treatment (distilled water) with those incubated in compounds with or without cyanide. Dunnett’s test is designed specifically for this type of planned comparisons (Sileshi, 2012).

Results

Dose rate studies with different smoke sources

Food grade liquid smoke

A significant interaction existed between food grade liquid smoke concentration and experimental run (P =0.0000) (Table 1). Germination in both experimental runs of
the food grade liquid smoke treatments consistently increased when concentrations were raised from 1/1000 to 1/100 (Figure 2). At the 1/10 dilution level of food grade liquid smoke, the germination response between experimental runs were significantly different. Percent germination was 9.4% in the first run and 38.4% in the second run. Increasing the concentration to undiluted resulted in 0% germination in both experimental runs. Based on these results, the optimum food grade liquid smoke dilution for maximum germination of pili grass seed was determined to be 1/100. This dilution exhibited the highest and most consistent percent germination among the concentrations tested. Percent germination with this treatment dilution ranged between 43 and 48%. These means were not significantly different between the two runs. Percent germination in the control treatments ranged from 0.7% to 9.1%.

*Xylose smoke-infused water*

A significant interaction occurred between experimental run and xylose smoke treatments (P=0.0469) (Table 2). Except for the second experimental run of the 1/10 dilution, there was a consistent improvement in germination with increasing concentrations of xylose smoke with maximum germination in the undiluted solution treatment (Figure 3). Percent germination with the undiluted solution was 43% for the first run and 51% for the second run. Percent germination in the control treatments ranged from 5.1% to 12.0%.

*Pili grass smoke-infused water*

Significant germination stimulation was found with pili grass smoke (P=0.0000) but not in the experimental run (P=0.6046) or the experimental run by treatment interaction (P=0.3631) (Table 3). Therefore, percent germination of treatments between
experimental runs was pooled. Increasing concentrations of pili grass smoke generally improved germination of dormant seeds (Figure 4). Undiluted solutions exhibited the highest percent germination (59%) followed by the 1/10 dilution treatment (51%) and the 1/100 dilution treatment (31%). Percent germination at the 1/1000 dilution treatment (11%) was not significantly different from those observed in the distilled water treatment (5.7%).

**Dose rate studies with recently identified stimulatory compounds in smoke**

*Karrikinolide (KAR)*

No effects of experimental run (P=0.0599), treatment (P=0.2839) and experimental run by treatment interaction (P=0.8969) were observed (Table 4). Pili grass seeds in the KAR treatments exhibited little to no germination (<3%) (Figure 5), indicating that pili grass is not responsive to KAR at the concentrations tested.

*Mandelonitrile and potassium cyanide*

Results for both the low rate mandelonitrile study and the low and high rate potassium cyanide studies indicated a significant treatment concentration effect but no significant experimental run or interaction effects (Tables 5 to 7). Therefore, percent germination of treatments between experimental runs was pooled.

Low rate dose response studies with mandelonitrile and potassium cyanide indicated that these cyanide-generating compounds significantly stimulated germination of pili grass seeds. Percent germination was consistently improved at mandelonitrile concentrations between 5 to 50 μM. Effective concentrations of mandelonitrile produced percent germination rates between 11% (5 μM) and 36% (50 μM) (Figure 6). Percent
germination observed in 1 μM mandelonitrile treatment (1.7%) was not significantly different from the control treatment (1.4%)

In the potassium cyanide studies, significant improvements in germination were observed at 5 and 50 μM in the low rate study (Figure 7). Concentrations at 1, 10 and 20 μM were not significantly different from the control treatment (1.7%). Higher rates of potassium cyanide produced significant improvement in germination between 100 and 500 μM (Figure 8). Percent germination observed in these concentrations was between 20 (250 μM) to 29% (500 μM).

Analysis of percent germination recorded in the benzaldehyde study indicated a significant effect of experimental run (P=0.012) and concentration (P<0.01) but no significant interaction between experimental run and concentration (P=0.0832) (Table 8). Percent germination of treatments between experimental runs was therefore pooled. Comparisons with the control (i.e., distilled water) using Dunnett’s test indicated that benzaldehyde concentrations above 50 μM were stimulatory to pili grass seeds (Figure 9). Seeds incubated in 50 μM mandelonitrile or in 50 and 100 μM benzaldehyde produced significantly higher percent germination in contrast with those incubated in the control treatment (10.2%). Seeds incubated in 50 and 100 μM of benzaldehyde had 25% and 31% germination, respectively. Germination recorded in 50 μM mandelonitrile was 43%.

The benzaldehyde and low rate potassium cyanide studies confirmed that the mandelonitrile stimulated germination was caused by both the products of its decomposition (i.e., cyanide and benzaldehyde). Interestingly, percent germination in both 50 μM cyanide (Figures 7 and 8) and 50 μM benzaldehyde (Figure 9) revealed an
additive effect, corresponding to the percent germination observed in 50 μM mandelonitrile.

The potassium chloride dose rate studies showed a significant treatment effect (P<0.01) but no significant experimental run (P=0.6443) or experimental run by treatment interaction (P=0.0709) (Table 9). Percent germination of treatments between experimental runs was therefore pooled. Percent germination of seeds incubated in 50 to 1000 μM potassium chloride was not significantly different from those observed in the control (distilled water) treatment (Figure 10). Germination at these concentrations was below 5%. In contrast, seeds incubated in 500 μM potassium cyanide produced significantly higher germination percentages (18%). The study indicated that the potassium ion was not responsible for the observed potassium cyanide germination stimulation. Therefore, it can be concluded that the observed germination stimulation by potassium cyanide was caused by cyanide.

**Germination response to the optimum concentrations of smoke-infused water and cyanide**

A significant interaction was found between experimental run and germination stimulants (P=0.0116) (Table 10). Undiluted pili grass smoke-infused water, 500 μM potassium cyanide and 1% (v/v) food grade liquid smoke significantly improved germination of pili grass seeds (Figure 11). Mean comparisons between the percent germination responses of these stimulants indicated similar levels of efficacy. Seeds incubated in undiluted pili grass smoke-infused water exhibited percent germination between 37 to 44%. Seeds incubated in 500 μM potassium cyanide exhibited percent
germination between 24 to 26%. Percent germination of seeds incubated in 1% (v/v) food grade liquid smoke was between 18 to 23%. Incubation in xylose smoke-infused water did not improve percent germination of pili grass seeds (Figure 11). Percent germination in this treatment ranged from 6 to 0%, for runs 1 and 2, respectively.

**Cyanide quantification of the different smoke-infused waters using the Cyantesmo paper**

Colorimetric testing of the smoke-infused water samples using CQTP indicated the presence of cyanide in pili grass smoke-infused water but not in food grade liquid smoke or xylose smoke-infused water (Figures 12 and 13). The intensity of blue shading in pili grass smoke water was lighter compared to that observed in the 500 μM potassium cyanide solution. This suggests that pili grass smoke contains less than 500 μM of cyanide. Further testing with CQTP and known dilutions of potassium cyanide indicated that undiluted pili grass derived smoke-infused water contained approximately 100 μM of cyanide (Figure 13).

**Discussion**

**Dose rate studies with different smoke sources**

Dose response experiments with different smoke sources confirmed that smoke-infused water stimulates germination of dormant pili grass seeds. Smoke-infused water from burned xylose, burned pili grass as well as from food grade liquid smoke was effective, with germination maximized at specific dilution levels. For xylose and pili
grass smoke-infused water, undiluted solutions were the most stimulatory. For food grade liquid smoke, more diluted solutions (i.e., 1/100 v/v) provided the highest levels of germination. These differences in the optimum dilutions in smoke-infused water can be due to factors such as the type of combustion material and combustion temperature (Jäger et al., 1996b, Brown and van Staden, 1997).

Jäger et al. (1996b) observed that optimum dilutions of smoke-infused water for germination stimulation depended on the type of starting material and combustion temperatures for preparing the smoke-infused water solutions. Smoke solutions derived from the combustion of different leaves (i.e., Acacia mearnsii, Eucalyptus grandis, *Hypoxis colchifolia* and *Pinus patula*) and tissue paper exhibited maximized germination stimulation of Grand Rapids lettuce (*Lactuca sativa*) between 1:100 or 1:10 dilution of the undiluted solutions. Smoke-infused water from the combustion of *E. grandis* or tissue paper (5 g, dry weight) provided maximum germination at the 1:100 dilution. For smoke-infused water derived from *A. mearnsii, H. colchifolia* and *P. patula* leaves (5 g, dry weight), maximum germination was recorded at the 1:10 dilution. Smoke-infused water concentrations greater than the optimum dilutions resulted in significant germination inhibition. Besides differences in germination response with starting material, Jäger et al. (1996b) also reported that increasing combustion temperature from 140 to 200°C increased the stimulatory effects of smoke-infused water. Smoke-infused water derived from dried *Themeda triandra* leaves (5 g, dry weight) burned at 200°C resulted in the highest germination (~60%) of Grand Rapids lettuce. (Jäger et al., 1996b). Combustion temperatures exceeding 200°C reduced the stimulatory effect of smoke-infused water.
Dose rate studies with recently identified stimulatory compounds in smoke

Dose rate studies with known stimulatory compounds in smoke showed that pili grass was responsive to cyanide but not to karrikinolide. Significant germination stimulation of pili grass was observed with potassium cyanide at concentrations between 5 to 500 μM. Assays with potassium chloride confirmed that cyanide and not the potassium ion was responsible for observed germination stimulation with potassium cyanide. The cyanide amounts evaluated in the current study covered the range of concentrations naturally found in smoke-infused water and stimulatory to a number of species. Studies by Flematti et al. (2011b) estimated the amount of cyanide in undiluted smoke-infused water to be between 199 to 300 μM. Concentrations of cyanide that were observed as stimulatory to test plants (i.e., A. manglesii and Rhodocoma arida) were between 5 to 50 μM (Flematti et al., 2011b).

The cyanide-stimulated germination observed in this study was consistent with observations in other grass species which exhibit physiological dormancy. Cyanide-stimulated germination has been observed in Avena fatua (Simpson, 1990), Panicum virgatum (200 μM, Sarath et al., 2006), Aristida contorta (100 to 1000 μM, Mott, 1974) and Oryza sativa (1000 μM, Cohn and Hughes, 1986, Cohn et al., 1989). The mode of action for cyanide-stimulated germination is believed to involve reactive oxygen species (Siegień and Bogatek, 2006, Oracz et al., 2007, Oracz et al., 2009, Iglesias-Fernandez et al., 2011, Nelson et al., 2012) and ethylene (Oracz et al., 2008, Gniazdowska et al., 2010, Nelson et al., 2012). Oracz et al. (2009) hypothesized that the dormancy relieving effect of cyanide is due to the accumulation of reactive oxygen species (ROS) in seeds. Results from their study support this link, since there was an observed increase in the generation
of hydrogen peroxide and superoxide anions in cyanide treated dormant sunflower 
(*Helianthus annus*) embryonic axes (Oracz *et al.*, 2009, Flematti *et al.*, 2013). The increase in ROS inhibited catalase and superoxide dismutase activities and activated NADPH oxidase, an ROS generating enzyme that has been implicated to play a role in seed germination in rice and warm season grasses (Oracz *et al.*, 2009). An increase in ROS following cyanide treatment or after-ripening, can trigger carbonylation of proteins that are specifically associated with seed germination (Oracz *et al.*, 2007, Oracz *et al.*, 2009, Iglesias-Fernandez *et al.*, 2011). In addition to ROS production, cyanide can also activate the expression of ERF1, a transcription factor component of the ethylene signaling pathway (Oracz *et al.*, 2008, Flematti *et al.*, 2013). Although ethylene biosynthesis is not required for cyanide-stimulated germination, its functional receptors have been observed to play a role in the cyanide signaling pathway (Oracz *et al.*, 2008, Flematti *et al.*, 2013).

Results of the current KAR₁ dose rate study places pili grass on the list of grass species reported to be unresponsive to this smoke-derived germination stimulant. Literature searches and germination assays by Long *et al.* (2011) found KAR₁ stimulates only 7 out of the 19 grass species assayed. The KAR₁ response in grasses was observed to be dependent on a number of factors including germination temperature, concentration and after-ripening/dormancy status. In the current study, the germination assays tested a range of concentrations typically observed as stimulatory in most species (0.0067 to 66.7 μM). However, the results still did not indicate significant germination stimulation with KAR₁.
Assays comparing smoke-infused water and cyanide and cyanide quantification of smoke-infused water

Pili grass smoke-infused water significantly improved germination of pili grass seeds. Colorimetric tests and subsequent quantification with the CQTP indicated that approximately 100 μM of cyanide was present in undiluted piligrass smoke-infused water (2.4 g, dry weight of combustion material per 100 ml of infused water). Although the dose rate studies confirmed that cyanide can provide significant germination stimulation, the smoke-infused water and cyanide comparison studies suggested that other compounds in smoke worked in combination with cyanide to promote germination. Interestingly, germination studies that included mandelonitrile confirmed benzaldehyde as stimulatory to pili grass at concentrations between 50 and 100 μM. Benzaldehyde has been observed to improve germination of select weed and crop species (French and Leather, 1979, French et al., 1986, Kokalis-Burelle et al., 2002) and it has been detected in tar from food grade liquid smoke (Hruza et al., 1974) and smoke from the combustion of a number of plant products (Kleindienst et al., 1986, Edye and Richards, 1991, Kataoka et al., 1997, Guillén and Manzanos, 1999, Hedberg et al., 2002). Although benzaldehyde has been detected in smoke, information on its quantity in these smoke sources is meager, making it difficult to establish whether the amounts present is optimal for germination stimulation to occur. Further research on quantifying the amount of benzaldehyde in smoke-infused water is therefore recommended.

In contrast to pili grass smoke infused water and food grade liquid smoke, xylose smoke infused water was not consistent in its stimulatory capability. Although the dose rate studies with seeds harvested in March 2011 exhibited significant germination
stimulation with increased concentrations, the assay that used July 2012 harvested seeds exhibited a non-significant germination response. This inconsistency in germination response may be due to differences in the quality of xylose smoke infused water solutions prepared or due to dormancy status of seeds. As noted in other chapters, seeds harvested in March had a reduced depth of dormancy compared to seeds harvested from other dates (see Chapters 2 and 3).

Differences in quality of smoke infused water samples produced may be caused by subtle differences in preparation which may have affected the production of the germination stimulant. As mentioned earlier, combustion temperature can greatly influence the final products formed (Jäger et al., 1996b). Since the preparation of xylose smoke infused water used an open flame, there was no direct control of the combustion temperature for each smoke infused water sample produced. Differences in combustion temperatures may have increased or decreased the amount of germination stimulant in the smoke infused water samples.

In addition to the subtle preparation differences that may have possibly affected xylose smoke infused water solutions, the inconsistent germination observed in seeds incubated in xylose smoke infused water may also be due to differences in seed dormancy status between batches. In the dose rate study where the March 2011 seeds were used, significant germination stimulation in the undiluted xylose smoke infused water was observed. In contrast, no significant germination was observed in the same xylose smoke infused water concentration where July 2012 seeds were used (i.e., the smoke and cyanide comparison studies). Upon looking at the storage histories of both seed batches, the March 2011 seeds were stored longer at 5°C (525 days) than the July
2012 harvested seeds (175 days). Although storage at low temperature can maintain dormancy of pili grass seeds (see Chapter 3), the longer holding period (i.e., > 1 year) probably reduced the dormancy status of the seed batch to some degree. Besides differences in the length of storage of both seed batches, seasonal differences can also impact the depth of seed dormancy. Evidence of time of year harvest affecting dormancy status was reported in Chapters 2 and 3. The seeds harvested in March appeared to have less depth of dormancy compared to seeds harvested in June (see Chapter 2) and October (see Chapter 3).

The effects of dormancy status on the responsiveness of seeds to smoke derived germination stimulants (i.e., smoke and KAR₁) have also been reported in a number of species. According to Nelson et al. (2012), the efficacy of smoke or KAR₁ depends upon the dormancy state, which is in turn affected by storage conditions. For example, storage under laboratory conditions or burial in soil increased smoke responsiveness of Stylidium affine, Stylidium crossocephalum and A. manglesii seeds (Tieu et al., 2001, Nelson et al., 2012). In seeds of Eragrostis curvula, KAR₁ responsiveness increased when seeds were dark stratified at 20/10°C (Long et al., 2011). Inconsistencies in germination response between seeds collected at different growing seasons were also observed in Brassica tournefortii (Stevens et al., 2007). In that study, seeds collected from the same localities for two consecutive years (i.e., Perth metropolitan region and Shark Bay in 2005 and 2006) exhibited differences in response to KAR₁. Less than 30% germination was recorded in the 2005 collected seeds treated with KAR₁. In contrast, seeds harvested in 2006 exhibited complete germination (i.e., 100%) with KAR₁ application.
The results observed highlight the complexity of the mechanisms behind smoke stimulated germination in dormant pili grass seeds. The efficacy of smoke in stimulating seed germination has been shown to be affected by source material, combustion temperature and seed batch (i.e., sensitivity to the germination stimulant). Cyanide was confirmed as a germination stimulant present in aqueous pili grass smoke. Its mode of action is believed to be related to ROS and ethylene, both of which are also related to dormancy loss through dry after-ripening (Iglesias-Fernandez et al., 2011). Assays with non-cyanide containing smoke sources also indicate that there are other, possibly described (i.e., benzaldehyde) or undescribed compounds capable of stimulating germination in pili grass. Further research is therefore recommended to isolate, quantify and test these compounds and confirm whether these stimulants may work additively or synergistically with cyanide.
### Tables

Table 6.1. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different rates of food grade liquid smoke. Percent germination data used for the analysis were arcsine square root transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>P</th>
</tr>
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<td>12.84</td>
<td>4.28</td>
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<tr>
<td>Experimental run</td>
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<td>535.28</td>
<td>27.38</td>
<td>0.0136</td>
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<td>3</td>
<td>58.65</td>
<td>19.55</td>
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<td></td>
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<tr>
<td>Food grade smoke</td>
<td>4</td>
<td>8804.97</td>
<td>2201.24</td>
<td>168.34</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x Food grade smoke</td>
<td>4</td>
<td>638.11</td>
<td>159.53</td>
<td>12.2</td>
<td>0.0000</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>24</td>
<td>313.83</td>
<td>13.08</td>
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</tr>
<tr>
<td>Total</td>
<td>39</td>
<td></td>
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</tbody>
</table>

Table 6.2. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different rates of xylose smoke-infused water. Percent germination data used for the analysis were arcsine square root transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<td>120.22</td>
<td>40.074</td>
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<td>Experimental run</td>
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<td>Main plot error</td>
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<td>57.628</td>
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<tr>
<td>Xylose</td>
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<td>3238.71</td>
<td>809.678</td>
<td>18.89</td>
<td>0.0000</td>
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<tr>
<td>Experimental run x Xylose</td>
<td>4</td>
<td>485.31</td>
<td>121.329</td>
<td>2.83</td>
<td>0.0469</td>
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<tr>
<td>Sub plot error</td>
<td>24</td>
<td>1028.84</td>
<td>42.868</td>
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<tr>
<td>Total</td>
<td>39</td>
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</table>

Table 6.3. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different rates of pili grass smoke-infused water. Percent germination data used in the analysis were arcsine square root transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<td>Rep</td>
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<td>87.21</td>
<td>29.07</td>
<td>0.33</td>
<td>0.6046</td>
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<td>Experimental run</td>
<td>1</td>
<td>22.41</td>
<td>22.41</td>
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<tr>
<td>Main plot error</td>
<td>3</td>
<td>202.2</td>
<td>67.4</td>
<td></td>
<td></td>
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<tr>
<td>Pili grass smoke</td>
<td>4</td>
<td>8063.36</td>
<td>2015.84</td>
<td>82.4</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x Pili grass smoke</td>
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<td>111.21</td>
<td>27.8</td>
<td>1.14</td>
<td>0.3631</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>587.16</td>
<td>24.47</td>
<td></td>
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<tr>
<td>Total</td>
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</table>
Table 6.4. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different dilutions of karrikinolide. Percent germination data used for the analysis were arcsine square root transformed to conform data to the ANOVA assumptions.

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<tr>
<th>Source</th>
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<td>4.858</td>
<td>1.619</td>
<td>8.72</td>
<td>0.0599&lt;sup&gt;ns&lt;/sup&gt;</td>
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<td>Experimental run</td>
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<td>142.077</td>
<td>142.077</td>
<td>49.24</td>
<td>0.0001</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>48.892</td>
<td>16.297</td>
<td>4.73</td>
<td>0.118</td>
</tr>
<tr>
<td>Karrikinolide</td>
<td>5</td>
<td>137.64</td>
<td>27.528</td>
<td>1.32</td>
<td>0.2839&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>Experimental run x Karrikinolide</td>
<td>5</td>
<td>33.477</td>
<td>6.695</td>
<td>0.32</td>
<td>0.8969&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>30</td>
<td>627.492</td>
<td>20.916</td>
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<tr>
<td>Total</td>
<td>47</td>
<td></td>
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</table>

<sup>ns</sup> Not significant.

Table 6.5. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different dilutions of mandelonitrile. Percent germination data used in the analysis were arcsine transformed to conform data to the ANOVA assumptions.

<table>
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<td>94.39</td>
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<td>0.118</td>
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<td>Experimental run</td>
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<td>122.91</td>
<td>122.91</td>
<td>4.73</td>
<td>0.118</td>
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<td>26.01</td>
<td>4.73</td>
<td>0.118</td>
</tr>
<tr>
<td>Mandelonitrile</td>
<td>5</td>
<td>5253.07</td>
<td>1050.61</td>
<td>28.22</td>
<td>0.0000</td>
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<td>Experimental run x Mandelonitrile</td>
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<td>Total</td>
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Table 6.6. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in low rate dilutions (<50 μM) of potassium cyanide. Percent germination data used in the analysis were square root transformed to conform data to the ANOVA assumptions.

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<td>0.04</td>
<td>0.856</td>
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<td>Experimental runs</td>
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<td>0.05326</td>
<td>0.04</td>
<td>0.856</td>
</tr>
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<td>1.36368</td>
<td>0.04</td>
<td>0.856</td>
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<td>Potassium cyanide 50</td>
<td>5</td>
<td>23.1286</td>
<td>4.62536</td>
<td>7.53</td>
<td>0.0001</td>
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<td>Experimental run x Potassium cyanide 50</td>
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<td>1.114</td>
<td>0.22229</td>
<td>0.36</td>
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<td>Total</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.7. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in the higher rate dilutions (>50 μM) of potassium cyanide. Original percent germination data was used in the analysis since this conformed to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
<td>3</td>
<td>55.6</td>
<td>18.533</td>
<td>0.48</td>
<td>0.5374</td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>14.4</td>
<td>14.4</td>
<td>0.48</td>
<td>0.5374</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>89.6</td>
<td>29.867</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCNhigher</td>
<td>4</td>
<td>2160</td>
<td>539.9</td>
<td>15.75</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x KCNhigher</td>
<td>4</td>
<td>65.6</td>
<td>16.4</td>
<td>0.48</td>
<td>0.7513</td>
</tr>
<tr>
<td>Subplot error</td>
<td>24</td>
<td>823</td>
<td>34.283</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.8. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in dilutions of benzaldehyde. Percent germination data used in the analysis were arcsine transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
<td>3</td>
<td>72.5</td>
<td>24.168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>159.46</td>
<td>159.46</td>
<td>29.89</td>
<td>0.012</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>16.01</td>
<td>5.335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>7</td>
<td>6529.35</td>
<td>932.76</td>
<td>41.48</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x Benzaldehyde</td>
<td>7</td>
<td>309.15</td>
<td>44.164</td>
<td>1.96</td>
<td>0.0832</td>
</tr>
<tr>
<td>Subplot error</td>
<td>42</td>
<td>944.36</td>
<td>22.485</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 6.9. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in potassium chloride dilutions (>50 μm). Percent germination data used in the analysis were square root transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
<td>3</td>
<td>4.0334</td>
<td>1.34447</td>
<td>0.26</td>
<td>0.6443</td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>0.0766</td>
<td>0.07657</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>0.878</td>
<td>0.29268</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl_high</td>
<td>6</td>
<td>33.5456</td>
<td>5.59093</td>
<td>12.37</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x KCl_high</td>
<td>6</td>
<td>5.8409</td>
<td>0.97348</td>
<td>2.15</td>
<td>0.0709</td>
</tr>
<tr>
<td>Subplot error</td>
<td>36</td>
<td>16.2771</td>
<td>0.45214</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.10. Analysis of variance (ANOVA) table for percent germination of pili grass seeds incubated in different smoke-infused water solutions including 500 μM cyanide. Percent germination data used in the analysis were arcsine transformed to conform data to the ANOVA assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
<td>3</td>
<td>102.85</td>
<td>34.28</td>
<td>1</td>
<td>0.3909</td>
</tr>
<tr>
<td>Experimental run</td>
<td>1</td>
<td>39.67</td>
<td>39.67</td>
<td>1</td>
<td>0.3909</td>
</tr>
<tr>
<td>Main plot error</td>
<td>3</td>
<td>118.94</td>
<td>39.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>5894.29</td>
<td>1473.57</td>
<td>51.12</td>
<td>0.0000</td>
</tr>
<tr>
<td>Experimental run x Treatment</td>
<td>4</td>
<td>470.04</td>
<td>117.51</td>
<td>4.08</td>
<td>0.0116</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>24</td>
<td>691.81</td>
<td>28.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>391.81</td>
<td>28.83</td>
<td></td>
<td></td>
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</tbody>
</table>
Figures

Figure 6.1. Setup and details of the combustion chamber for producing smoke-infused water derived from either dried piligrass plant material or xylose.
Figure 6.2. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 525 days) to dilutions of food grade liquid smoke. Germination percentages are back transformed means. Means followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.

Figure 6.3. Mean percent germination response of piligrass seeds (harvested March 2011, stored at 5°C for 525 days) to dilutions of xylose smoke-infused water. Germination percentages are back transformed means. Means within a graph followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.
Figure 6.4. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 608 days) to dilutions of pili grass smoke-infused water. Germination percentages are back transformed means combined across experimental repeats. Means followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.

Figure 6.5. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 381 and 534 days) to dilutions of karrikinolide (KAR₁). Germination percentages are backtransformed means combined across experimental repeats. Analysis of variance indicated no significant differences in percent germination between treatment concentrations.
Figure 6.6. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 554 and 575 days) to dilutions of mandelonitrile (<50 μM). Germination percentages are back transformed means combined across experimental repeats. Means followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.

Figure 6.7. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 554 and 575 days) to dilutions of potassium cyanide (<50μM). Germination percentages are arcsine back transformed means combined across experimental repeats. Means followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.
Figure 6.8. Mean percent germination response of pili grass seeds (harvested July 2012, stored at 5°C 138 days) to higher concentrations of potassium cyanide (>50μM). Germination percentages are original means combined across experimental repeats. Means followed by the same letters are not significantly different as determined by Tukey’s range test at P<0.05.

Figure 6.9. Mean percent germination response of pili grass seeds (harvested March 2011, stored at 5°C for 554 and 575 days) incubated in dilutions of benzaldehyde and mandelonitrile. Germination percentages are arcsine backtransformed means combined across experimental repeats. Means with asterisks are significantly different from the control (i.e., distilled water) as determined by Dunnett’s test at P<0.05.
Figure 6.10. Mean percent germination response of pili grass seeds (harvested July 2012, stored at 5°C for 154 days) to dilutions of potassium chloride and potassium cyanide. Germination percentages are backtransformed means combined from the two experimental runs. Means with an asterisk are not significantly different from the control (i.e., distilled water) as determined by Dunnett’s Test at P<0.05.

Figure 6.11. Mean percent germination response of pili grass seeds (harvested July 2012, stored at 5°C for 175 days) incubated in potassium cyanide, food grade liquid smoke, pili grass smoke-infused water and xylose smoke-infused water. Percent germination data are arcsine backtransformed means. Means followed by the same letters are not significantly different as determined by Tukey Range Test P<0.05.
Figure 6.12. Colorimetric cyanide detection in the different smoke-infused water sources used for germinating dormant pili grass seeds stored at 5°C. The presence of cyanide, as indicated by the blue shading of the Cyantesmo test strip, was detected in the pili grass smoke-infused water but not in food grade liquid smoke and xylose smoke-infused water.
Figure 6.13. Using the Cyantesmo test strips to quantify the concentration of cyanide in pili grass smoke-infused water. A) Shading produced by known concentrations of potassium cyanide were used to estimate the amount of cyanide contained in pili grass smoke. B) Pili grass smoke-infused water was the only smoke water which tested positive for cyanide. Pili grass smoke-infused water contained approximately 100 μM of cyanide.
CHAPTER 7
EVALUATION OF SPIKE AND CULM CHARACTERISTICS AS A TOOL FOR
OPTIMIZING PILI GRASS (*Heteropogon contortus* (L.) P.BEAUV. EX
ROEM. & SCHULT.) SEED HARVEST TIMING

Introduction

Determining the optimum time to harvest seed is an important concern for seed
producers in their efforts to maximize yield and quality of seeds (Andersen and
Andersen, 1980, de Andrade, 1993, Desai *et al.*, 1997). Timely harvest of seeds is
essential to maximize mature seed yield and minimize losses due to immature seed
(Desai *et al.*, 1997, Bedane *et al.*, 2006), seed shattering (Berdahl and Frank, 1998,
Bedane *et al.*, 2006, Wang *et al.*, 2006) as well as losses from pest and diseases (Desai *et
al.*, 1997). For many native grasses, determining an appropriate time to harvest seed can
be a challenge since these plants exhibit indeterminate flowering and seed production

Pili grass (*Heteropogon contortus*) is a native, perennial bunchgrass that has been
used extensively for restoration and erosion control in Hawai‘i. It is considered as an
important re-vegetation species in the state since it is drought tolerant; adaptable to low
nutrient conditions (USDA-NRCS, 2007) and has both cultural and ecological value
(Daehler and Carino, 1998, Carino and Daehler, 1999, Goergen and Daehler, 2001). In
recent years, there has been increased interest in using pili grass for a number of
applications such as roadside re-vegetation, riparian buffer strips and urban landscaping.
As uses for pili grass begin to expand, it is imperative to develop seed harvest protocols to produce a sufficient volume of seeds for large scale seeding/re-vegetation projects.

Several harvest maturity indicators that have been evaluated in a number of crop species have the potential for determining the optimum harvest time for pili grass seeds. These include, calendar date, growing degree days, flowering stages (i.e., days after flowering, days after a certain number of seed head appears), degree of shattering as well as seed moisture content and weight (Andersen and Andersen, 1980, Berdahl and Frank, 1998, Garcia-Diaz and Steiner, 2000, West and Pitman, 2001, Lemke et al., 2003, Wang et al., 2006, Wang et al., 2008). Calendar date is the least reliable indicator for seed harvesting since maturity will depend on the environmental factors experienced (e.g., season, temperature, rainfall, pests, soil fertility) and management practices employed throughout the seed production/growing period (West and Pitman, 2001). Growing degree days or growing degree units (GDU) have often been used in place of calendar days as a measure of harvest maturity for a number of species including C3 grasses (Berdahl and Frank, 1998, Wang et al., 2006), forage crops such as birdsfoot trefoil (*Lotus corniculatus*) (Garcia-Diaz and Steiner, 2000) and industrial crops such as guayule (*Parthenium argentatum*) (Bedane et al., 2006) and cuphea (*Cuphea viscosissima × lanceolata, PSR23*) (Berti and Johnson, 2008). A GDU-based index provides a more accurate estimation of seed harvest timing since it considers temperature (i.e., accumulated heat units above a constant base temperature) as a factor in seed development and maturity. To estimate the optimum seed harvest timing, GDUs are often related to flowering or seed shedding stages as well as seed yield and quality characteristics (Berdahl and Frank, 1998, Wang et al., 2006).
Stages during flowering such as days after flowering, days after peak anthesis and maximum inflorescence number can be useful indicators for estimating the optimum timing of seed harvest. In order for these stages to be effective indicators, experience must be gained in relating these plant development stages to seed yield and quality (West and Pitman, 2001). Flowering stages have been used by Berdahl and Frank (1998) to estimate seed maturity in the tetraploid perennial grass species, crested wheatgrass (*Agropyron desertorum*) Russian wildrye (*Psathyrrostachys juncea*), intermediate wheatgrass (*Thinopyrum intermedium*) and western wheatgrass (*Pascopyrum smithii*). To characterize flowering and seed production, Lemke *et al.* (2003) used anthesis counts of seed tillers, cupule counts and seed harvests. Wang *et al.* (2006) related days after peak anthesis (50% flowers per spike opening) with seed weight per spike, seed number per spike, thousand seed weight, percent germination, speed of germination and electrical conductivity of seed leachate.

Seed characteristics such as dry weight and moisture content can provide an accurate estimate of seed harvest timing. Seed dry weight has been used to determine physiological maturity of the seed [i.e., maximum dry weight and seed quality is reached (Copeland and McDonald, 2001)]. While seeds can be harvested at physiological maturity, moisture content is often too high for mechanical harvesting and threshing (Siddique and Wright, 2003). Mechanical harvesting of seeds with high moisture content increases the risk of damaging the seeds, negatively impacting seed viability (Andersen and Andersen, 1980). To make mechanical harvesting permissible, seeds are allowed to dry to a lower moisture content (usually 15-20%, fresh weight basis) [i.e., harvest maturity (Copeland and McDonald, 2001)]. For grass seed crops, seed moisture content is
the most dependable indicator of maturity and harvest timing (Silberstein et al., 2010). Careful monitoring of seed moisture content after physiological maturity is essential in order to optimize mature seed yield while minimizing seed loss from shattering. In this study, cumulative growing degree units (CGDUs) were related to number of spikes (i.e., inflorescence) and culm (i.e., grass stem) components (number of spikes per culm, seed yield per culm and seed number and weight extracted from 10 spikes) as well as spike moisture content to predict the optimum seed harvest timing in pili grass. The effect of sequential harvest season on all spike and seed yield components was also determined.

**Materials and methods**

**Site characteristics and land preparation**

The study was conducted from March 2011 to December 2012 at the Magoon Research and Teaching Facility of the University of Hawai‘i Mānoa Campus in Honolulu, Hawaii (21° 18’ 18.77” N, 157° 48’ 38.91”W, ~45.6 m.a.s.l.). Soils in the study site were composed of Makiki stony clay loam (fine, mixed, isohyperthermic Andic Ustic Humitropept). Site preparation prior to planting was initiated in December 2010 and involved a spray to wet application of 1% glyphosate solution (Roundup Pro, Monsanto) to kill existing vegetation. After two weeks, the area was cleared of dead vegetation, stones and boulders and then rototilled. Lime (Dolomite 65 AG Agricultural Liming Material, Chemical Lime Company) and fertilizer (11-52-0, Crop Production Services Inc.) were applied at a rate of 2,242 kg per ha and 196 kg per ha (196 kg N and 45 kg P per ha), respectively. The field was subsequently rototilled to incorporate the
lime and fertilizer, break soil clods and further remove rocks and other debris. To prevent weed growth after field preparation, the pre emergence herbicide, oxyfluorfen (Goal Tender®, Dow AgroSciences) was applied at a rate of 0.56 kg active ingredient per hectare 22 days prior to planting pili grass transplants.

**Plug preparation, planting and establishment**

Pili grass plugs were grown from seed 56 days prior to planting. Seeds of the Kaho‘olawe source identified germplasm (accession #: 9079683, HA-5748) were presoaked for 15 minutes in 1% food grade liquid smoke solution (Colgin Natural Mesquite, Colgin Inc., Dallas, TX) to relieve any remaining dormancy and enhance germination (see Chapter 6). The pretreated seeds were planted in plastic trays filled with a commercially prepared mixture of potting media (Pro-mix ‘BX’, Premier Horticulture). Seeds were allowed to germinate and grow under greenhouse conditions for one week before they were individually transplanted into dibble tubes filled with a mixture of potting mix (Pro-mix ‘BX’, Premier Horticulture) and fertilizer (10-4-16, Best Microgreen 10, applied at 456 grams per 2 cubic feet of potting mix). The plugs were allowed to grow under overhead-irrigated, glasshouse conditions for 49 days.

Two days prior to field planting, foliage of the plugs were cut to a height of approximately 17 centimeters to minimize transplant shock and encourage tillering. Plugs were planted on January 7, 2011 in 6 rows, spaced 1.1 meters apart, with an in-row spacing of 1.0 meter. A total of 156 plants were transplanted into the 171.6 square meter plot. After transplanting, a broadcast application of granular oxadiazon (Ronstar G, Bayer
Environmental Science) was applied at a rate of 2.24 kg active ingredient per hectare to control weeds emerging from seeds.

A drip irrigation system was installed in the field at the time of planting. The drip tape (Chapin Deluxe Turbulent Flow Twin-Wall Drip Tape, Jain Irrigation Inc.), which was manufactured to apply 774 liters per hour (per 100 meters) at 0.69 bar, was placed along the rows of the planted plugs. Irrigation was set to deliver approximately 186 liters of water daily over the entire field.

**Seasonal mowing schedule and fertilization**

In preparation for inflorescence counts and culm harvests conducted during the spring-summer and fall-winter growing seasons, the field was routinely cut every 3 months and fertilized every 6 months. Seasonal mowing started during the 2011 spring equinox (i.e., March 20, equivalent to 72 days after transplanting the plugs) and continued in subsequent equinoxes and solstices of 2011 and 2012. During the initial cutting (2011 spring equinox), the plants were trimmed to about 17 centimeters from the ground to avoid killing the newly established plant. After this season, the stands were cut every three months to a height of about 3 to 4 centimeters from the ground using a hedge trimmer (Shindaiwa Articulating Hedge Trimmer, Shindaiwa, Lake Zurich, IL). The cut stems were not removed from the field and eventually produced a dense surface mulch. Fertilizers, in the form of calcium nitrate (YaraLiva Calcinit, Yara North America Inc.) and ammonium sulfate (Yara Ammonium Sulfate, Yara North America Inc.), were applied at a rate of 112 kg N per hectare prior to cutting during the fall-winter 2011, spring-summer 2012 and fall-winter 2012 growing seasons.
Determining the initial date to harvest culms

To determine the initial date of data collection (i.e., culm harvests) and to determine peak flowering, weekly inflorescence counts were conducted within the first six weeks after mowing. Inflorescence counts were done on 10 randomly selected plants within the four inner rows of the field (i.e., the outer row of plants served as a buffer). All inflorescences exhibiting anthesis (i.e., exerted anthers or stigmas) were counted using a handheld tally counter. The number of days after the initial mowing to reach peak flowering (57 days) was determined by plotting the number of new inflorescences per week. This time interval was used to set the start date for conducting culm harvests during the spring 2011 season. In the subsequent seasons, the time interval used to initiate data collection (i.e., harvesting) were 57 (spring 2011, fall 2011 and fall 2012) and 59 (spring 2012) days after mowing. CGDUs were then calculated from heat units accumulated during these time intervals and were plotted against spike and seed development measurements for each season.

Harvesting of culms to characterize seed development

To characterize stages of flowering, seed production and seed maturity, representative culms from individual plants were harvested every 3 days during the spring-summer and fall-winter growing seasons of 2011 and 2012. For each season, culm harvests were initiated at 57 (spring 2011, fall 2011 and fall 2012) and 59 (spring 2012) days after mowing and ended at 90 (spring 2011, fall 2011, fall 2012) or 92 (spring 2012) days after mowing. Within each harvest period, two representative culms from 20 plants in each row were collected. Out of these 40 culms harvested per row (replicate), 10 culms
were randomly selected for inflorescence counts. For each culm, inflorescences were classified into three distinct stages: unopened, intact (i.e., at or past anthesis, but not shed) and shed (i.e., at least one seed has shed on the spike). The mean numbers of unopened, intact as well as shedding inflorescences were calculated for each row (replicate).

Spike moisture content, number and weight of filled and empty seeds were recorded using 10 randomly selected inflorescences from bulked row samples (i.e., flowering/flowered or shedding spikes bulked per row). Spike moisture content was expressed as grams of water per gram of dry weight (see equation below). Selected inflorescences had their awns removed before recording the fresh weight. Dry weights were recorded after the spikes were oven dried at 103°C for 17 hours (ISTA, 1996).

Spike moisture content was calculated as:

\[
\text{Spike moisture content (g} \ H_2O \cdot g^{-1} \ \text{dry weight)} = \frac{\text{fresh weight} - \text{oven dry weight}}{\text{oven dry weight}}
\]

The seeds of the oven-dried inflorescences were manually extracted and separated by palpation into filled and empty seed categories. The number of each extracted seed type (i.e., filled and empty) and their combined number and weight (i.e., total and average weight of seeds) were recorded.

**Extraction of seeds from harvested culms**

All inflorescences from the remaining 30 culms collected per row at each harvest date were air dried at outdoor temperature and humidity for three weeks. Culms were threshed using a plastic round comb and a rubber pet brush to separate the seeds and awns from the inflorescences. After threshing, the seeds were passed through an air blast
seed cleaner (Almaco, Nevada, IA) to remove awns and associated debris. Extracted seed yield in grams from the bulked culms (per row) at each harvest date were recorded.

**Plotting inflorescence and seed yield data in growing degree units**

CGDUs during each harvest period (starting from the date of cutting to the last harvest) were calculated using daily maximum and minimum temperatures (°C) recorded at the site and at the Honolulu International Airport. Temperature data from April 7 to 12, 2012 used airport data since the on-site sensors did not log at these dates. CGDUs at each harvest period were calculated using a modified growing degree day model (Russelle et al., 1984, Horak and Loughin, 2000):

\[
\text{Cumulative growing degree units} = \sum M \text{ for the growth period considered where:}
\]

\[
M = \frac{\text{Maximum temperature} + \text{Minimum temperature}}{2} - Tb
\]

Where \( M \) is the degree (heat) unit for a given day, \( Tb \) is the base temperature at which little or no growth occurs. Based on studies conducted by Tothill (1966) and Novelly (1986), pili grass has a base temperature of 15°C. Flowering and seed yield components were analyzed and plotted against average CGDU (ACGDU) across seasons to facilitate statistical analysis through the analysis of variance.

**Field photographs of the 2012 growing season harvest dates**

Photographs of the pili grass field were recorded at the time of data collection during the 2012 growing season. The first two harvest dates in spring 2012 are missing due to operator error. A digital camera (Canon Powershot SX1 IS, Canon USA Inc.,
Melville, NY) was used, taking pictures of the field on the same location and perspective. Pictures were used to provide visual harvest timing cues which were related to the spike and seed yield data collected.

**Statistical analysis**

Analysis of variance (ANOVA) was conducted for all potential harvest indicators (i.e., spike water content, total spike production per culm, number of intact spikes, shed and unopened spikes per culm, number and weight of filled and empty seeds extracted from 10 spikes, total number and weight of seeds extracted from 10 spikes, average weight of individual seeds extracted from 10 spikes and seed yield extracted from 30 culms) to determine significant treatment effects (i.e., ACGDU) or interactions. All potential harvest indicators were analyzed as a split plot, with growing season as the main plot and ACGDU as the subplot. Regression analysis (i.e., polynomial or linear) was performed for all potential harvest indicators. Tukey HSD Pairwise Comparisons were used to separate the average number of seeds extracted for 10 spikes recorded across ACGDUs. Both ANOVA and regression analyses were performed using Statistix® 9 (Analytical Software, Tallahassee, FL). Polynomial regression lines were plotted using Microsoft Excel (Microsoft Corporation, Redmond, WA). Square root transformation was determined as the appropriate transformation for conforming the data to the assumptions of the ANOVA or regression (Gomez and Gomez, 1984). Square root transformation of count data with many values less than 10 or zero used \((Z + 0.5)^{1/2}\) instead of \(Z^{1/2}\), where \(Z\) is the original data (Gomez and Gomez, 1984). For ease of interpretation, predicted values \((X)\) and equations for all data presentations were back
transformed using \((X^2 - 0.5)\) for count data with many values less than 10 or zero; otherwise \(X^2\) was used.

**Results**

**Spike moisture content**

Analysis of variance for spike moisture content indicated a significant interaction between growing season and ACGDU (P<0.01) (Table 1). Regression models of spike moisture content indicated decreasing trends in all seasons with increasing ACGDU (Figure 1). Significant negative linear trends were recorded for spring 2011 (P<0.01), fall 2011 (P<0.01), and fall 2012 (P=0.0001) growing seasons while a significant positive quadratic trend was recorded for the spring 2012 (P<0.01) growing season. Spikes exhibited different initial moisture contents for each harvest season and year. Spikes harvested in spring 2012 exhibited the highest initial moisture content (1.26 g g\(^{-1}\) dry weight). Spikes harvested in spring 2011 exhibited the lowest initial moisture content (0.80 g g\(^{-1}\) dry weight). On-plant drying rates of spikes harvested during each growing season were different, but by 759 ACGDU spike moisture contents exhibited a narrow range of values (0.69 to 0.74 g g\(^{-1}\) dry weight). The \(R^2\) values for each growing season indicate varying strengths of association between spike moisture content and ACGDU (Figure 1). The spring 2012 regression model exhibited the highest \(R^2\) value (0.83) followed by the fall 2011 (0.63), fall 2012 (0.33) and spring 2011 (0.17) models.
**Spike numbers**

Analyses of variance for total, intact, shed and unopened spikes indicated a significant growing season by ACGDU interaction (P<0.01 for each spike component) (Tables 2 to 5). Regression models of total spike production per culm exhibited a significant positive linear trend with ACGDU during the spring 2011 (P<0.01), fall 2011 (P<0.01) and spring 2012 (P<0.01) growing seasons (Figure 2). The regression model for the fall 2012 growing season was not significant (P=0.2200), indicating that total spike production per culm was not significantly affected by increasing ACGDU (Figure 2). Total number of spikes showed a decrease with each successive harvest from 2011 to 2012 (Figure 2). The spring 2011 growing season produced the most spikes per culm. The fewest spikes were produced in the fall 2012 growing season. $R^2$ values for each season indicated varying strengths of association between total number of spikes per culm and ACGDU. The fall 2011 regression model exhibited the highest $R^2$ (0.79), followed by spring 2012 (0.54) and spring 2011 (0.40) (Figure 2).

Regression models of intact spike production indicated different trends and peaks for each growing season with increasing ACGDU (Figure 3). Significant negative quadratic trends were recorded in the regression models for spring 2011 (P=0.0004), fall 2011 (P=0.0001) and fall 2012 (P<0.01). The regression model for spring 2012 exhibited a significant cubic trend (P<0.01). The ACGDU when the peak number of intact spikes was recorded was different for each growing season. The peaks were at 667 to 671 ACGDU (13 intact spikes per culm) for spring 2011, 728 ACGDU (6 spikes per culm) for fall 2011, 778 to 779 ACGDUs (6 spikes per culm) for spring 2012 and 652 to 654 ACGDU (5 spikes per culm) for fall 2012. The peak number of intact spikes was reduced
after the spring 2011 growing season. The strength of association between the number of intact spikes and ACGDUs also varied with growing season. The spring 2012 regression model exhibited the highest $R^2$ value (0.85) followed by the fall 2012 (0.50), fall 2011 (0.35) and spring 2011 (0.29) models.

Regression models for the number of shed spikes per culm indicated increasing trends for all growing seasons as ACGDU increased (Figure 4). Regression models for spring 2011 and fall 2012 exhibited significant positive linear trends ($P<0.01$ for both seasons) while regression models for fall 2011 and spring 2012 exhibited positive quadratic trends ($P<0.01$ for both seasons). In general, the number of shed spikes decreased after the spring 2011 growing season. There is a strong association between the number of shedding spikes per culm and ACGDU for all growing seasons. The fall 2011 regression model exhibited the highest $R^2$ value (0.97) followed by the spring 2012 (0.92), the spring 2011 (0.80) and the fall 2012 (0.80) models.

Regression models of unopened spikes per culm with increasing ACGDU did not exhibit consistent trends across seasons (Figure 5). The regression model for spring 2011 was not significant ($P=0.1477$), indicating that there was no change in the number of unopened spikes per culm as ACGDU increased. The fall 2011 and fall 2012 regression models exhibited significant positive quadratic trends. An increasing quadratic trend was observed in the fall 2011 regression model ($P=0.0002$) while a decreasing quadratic trend was observed in the fall 2012 regression model ($P<0.01$). The spring 2012 regression model exhibited a significant negative linear trend ($P<0.01$). The strength of association between total number of unopened spikes and ACGDUs varied with growing season. The
spring and fall 2012 regression model exhibited high $R^2$ values (0.74 and 0.81, respectively) while the fall 2011 regression model exhibited a low $R^2$ value (0.31).

**Seed components sampled from 10 spikes**

Analysis of variance for the number of filled seeds extracted from 10 spikes showed a significant interaction between ACGDU and growing season (P<0.01) (Table 6). Regression models for each season did not exhibit consistent trends as ACGDU increased (Figure 6). For the spring 2011 growing season, the number of filled seeds exhibited a significant positive linear trend with increasing ACGDU (P<0.01). The number of filled seeds reached the maximum amount (38 seeds) at 857 ACGDUs (ACGDU at the end of data collection). The fall 2011 growing season exhibited a significant negative quadratic trend (P<0.01) with a distinct peak of 36 seeds between 797 and 798 ACGDUs. After 798 ACGDUs, the number of filled seeds begins to decline slightly. By 857 ACGDUs, the number was down to about 33 seeds. The spring 2012 growing season exhibited a significant cubic trend (P<0.01) with a distinct peak of 31 filled seeds at 795 ACGDUs. By 857 ACGDUs, the number of filled seeds decreased to 19. The regression model for the fall 2012 growing season was not significant (P=0.1156), indicating that the number of filled seeds produced did not change as ACGDU increased. The strength of association between the number of filled seeds extracted from 10 spikes and ACGDUs varied with growing season. The spring 2012 regression model exhibited the highest $R^2$ value (0.77) followed by the fall 2011 model (0.68). The spring 2011 regression model exhibited the lowest $R^2$ value (0.31).
Analysis of variance for the number of empty seeds extracted from 10 spikes exhibited a significant ACGDU by growing season interaction (P=0.0006) (Table 7). All growing seasons exhibited significant negative linear trends (P<0.01) as ACGDU increased (Figure 7). The strength of association between the number of empty seeds extracted from 10 spikes and ACGDUs varied with growing season. Regression models for the spring 2012 and fall 2012 growing season exhibited the highest $R^2$ values (0.71 and 0.74, respectively) followed by the fall 2011 regression model (0.66). The spring 2011 regression model exhibited the lowest $R^2$ value among the growing seasons (0.40).

Analysis of variance for the total number of extracted seeds from 10 spikes exhibited a significant ACGDU by growing season interaction (P<0.01) (Table 8). Regression models for fall 2011, spring 2012 and fall 2012 growing seasons exhibited significant negative quadratic trends (P<0.01) (Figure 8). The regression model for the spring 2011 exhibited a significant negative linear trend (P=0.0016) (Figure 8). The strength of association between the average number of extracted seeds from 10 spikes and ACGDUs varied with growing season. The fall 2012 regression model exhibited the highest $R^2$ value (0.76) followed by the fall 2011 and spring 2012 regression models (0.55 and 0.54, respectively). The spring 2011 regression model exhibited the lowest $R^2$ value (0.20).

Analysis of variance for the total weight of seeds extracted from 10 spikes exhibited a significant interaction between ACGDU and growing season (P<0.01) (Table 9). Regression models of the total weight of seeds indicated significant cubic trends for fall 2011, spring 2012 and fall 2012 growing seasons (P<0.01 for each season) (Figure 9).
The regression model for spring 2011 exhibited a significant positive linear trend (P=0.0103) (Figure 9). The maximum weight of seeds for each growing season was recorded at the following ACGDUs: 869 ACGDUs for spring 2011 (0.167 g); 743 to 751 ACGDUs for fall 2011 (0.179 g), 768 to 774 ACGDUs for spring 2012 (0.182 g) and 598 to 603 ACDGUs for fall 2012 (0.171 g). The strength of association between the total weight of seeds and ACGDUs varied with growing season. The spring 2012 regression exhibited the highest $R^2$ value at 0.65, followed by fall 2012 ($R^2 = 0.61$) and fall 2011 ($R^2 = 0.50$). Regression analysis of the spring 2011 dataset exhibited a mild association between the total weight of seeds and ACGDUs ($R^2 = 0.13$).

Analysis of variance for the average weight of individual seeds (i.e., total weight/total number of seeds extracted from 10 spikes) indicated a significant interaction between ACGDU and growing season (P<0.01) (Table 10). Regression models exhibited different trends for each growing season (Figure 10). The spring 2011 growing season exhibited a significant positive linear trend (P=0.0001) for individual seed weight with increasing ACGDU. The maximum individual seed weight for spring 2011 was 1.460 mg at 857 ACGDUs (91 days after cutting). The fall 2011 growing season exhibited a significant negative quadratic trend (P<0.01) for individual seed weight with increasing ACGDU. Peak individual seed weight for the fall 2011 growing season (1.440 mg) was reached between 795 and 800 ACGDU (82 to 85 days after cutting). In the spring 2012 growing season, the average weight of individual seeds exhibited a significant cubic trend (P<0.01) with increasing ACGDU. The maximum seed weight for the spring 2012 growing season (1.543 mg) was observed between 800 and 803 ACGDUs (82 to 85 days after cutting), close to the range that was observed in the fall 2011 growing season.
Regression analysis was not significant (P=0.1504) for the average individual seed weight recorded during the fall 2012 growing season. This indicated that average individual seed weight at this growing season did not change with increasing ACGDU.

The strength of association between average weight of individual seeds and ACGDUs varied with each growing season. The spring 2012 growing season exhibited the highest $R^2$ value (0.77) followed by fall 2011 ($R^2 = 0.58$) and spring 2011 ($R^2 = 0.29$).

**Seed yield from 30 representative culms**

Analysis of variance for seed yield extracted from 30 representative culms exhibited a significant interaction between ACGDU and growing season (P<0.01) (Table 11). A significant cubic trend was observed for the spring 2011, fall 2011 and fall 2012 regression models (P<0.01) (Figure 11). The regression model for the spring 2012 growing season exhibited a significant negative quadratic trend (P<0.001) (Figure 11). Distinct peaks in harvested seed yield were observed for each growing season. For the spring 2011, fall 2011 and spring 2012 growing seasons, the range of ACGDU values where maximum seed yield was observed fell between 767 to 777 ACGDUs (approximately between 79 and 82 days after cutting). The maximum seed yields and ACGDU ranges for each of the three growing seasons were as follows: 5.33 g at 767 to 770 ACGDU (spring 2011), 3.33 g at 774 to 777 ACGDU (fall 2011) and 2.36 g at 768 to 774 ACGDU (spring 2012). The fall 2012 growing season exhibited an earlier peak of seed yield (1.38 g at 621 to 629 ACGDUs) in contrast with the previous seasons. In general, seed yield harvested from 30 culms decreased with each subsequent growing season. The maximum seed yield decreased from 5.33 g (spring 2011) to 1.38 g (fall
2012). $R^2$ values of the regression models from each growing season exhibited varying strengths of association between seed yield and ACGDU. The fall 2011 dataset exhibited the highest $R^2$ value (0.83) followed by spring 2011 (0.69), spring 2012 (0.64) and fall 2012 (0.49).

Field photographs of the 2012 growing season harvest dates

The images recorded during the spring and fall 2012 growing season are presented in Figures 12 and 13, respectively. The inset image for each harvest date is a magnified section of representative seedheads. Minimal photo retouching was applied to these images to provide the most accurate representation of each harvest date.

Discussion

Results of the study indicate that ACGDU and spike water content provided the best indication of optimum harvest timing in pili grass. Seed yield from 30 culms, total weight of seeds from 10 spikes, number of filled seeds from 10 spikes and average weight of individual seeds exhibited their maximum values within a common ACGDU range in three of the four cropping cycles (i.e., spring 2011 through spring 2012). Seeds obtained from culms and the number of filled seeds exhibited a maximum yield at 767 to 778 ACGDUs (79 to 82 days after cutting) and 795 to 798 ACGDUs (84 to 87 days after cutting), respectively. Yield components describing physiological maturity (i.e., total weight of seeds and average weight of individual seeds) were also observed within this ACGDU range. The maximum values for the total weight of seeds and the average
weight of individual seeds were located between 743 and 807 ACGDUs or about 79 to 85 days after cutting. Harvest date photographs taken in spring 2012 (i.e., representative of the three cropping cycles) indicate the co-occurrence of the appearance of tangled seedheads with the ACGDU range where maximum values in seed yield were recorded (Figure 12).

Besides ACGDU, spike water content also appears to be a useful indicator for harvest timing in pili grass. Although the initial moisture content varied across seasons, spike water contents dried to a similar range (0.68 to 0.72 g H₂O per g of dry weight) by 767 and 778 ACGDU, the ACGDU range where maximum seed production and seedhead tangling were observed. This spike water content was higher compared with those observed at physiological maturity in most cool season grass species as well as in spring wheat. In crested wheatgrass (*Agropyron desertorum*), Russian wildrye (*Psathyrostachys juncea*), intermediate wheatgrass (*Thinopyrum intermedium*) and western wheatgrass (*Pascopyrum smithii*), spike water concentration at or near the maximum seed mass is 0.35 g H₂O per g of dry weight (350 g kg⁻¹ dry weight basis) (Berdahl and Frank, 1998). Spike water concentration in spring wheat (*Triticum aestivum* cv. Sinton) is 0.27 g H₂O per g of dry weight (270 g kg⁻¹ dry weight basis) at physiological maturity (Bauer *et al*., 1986).

Individual analysis of culm components such as the number of intact, shed and unopened spikes per culm did not show trends that would aid in identifying the optimum harvest time. Although peaks were observed in intact spike production per culm, they were independent of the seed yield-maximized ACGDU range. However, when both plots of the intact spike number (Figure 3) and shed spike number (Figure 4) were
considered, an ACGDU range where both are numbers equal (i.e., plots intersect) can be observed. The intersection between the plots of intact spike number and shed spike number represents the point at which 50 percent of the total productive spike population is intact or conversely shed. A productive spike population showing 50 percent shedding is an indication that the peak in seed yield (Figure 11) has been passed. This range was usually observed after the maximum seed yield was reached. The spring 2011 and fall 2012 growing seasons exhibited 50 percent spike shedding between 833 to 835 ACGDUs while the fall 2011 and spring 2012 growing seasons exhibited 50 percent spike shedding at >857 and 854 ACGDUs, respectively.

Aside from detecting an ACGDU range for optimum yield, there was also an observed decrease in productivity of the pili grass stand over time. This decline can be noted in both seed yield data obtained from 30 culms as well as total spike production per culm. In the seed yield data, there was a quantitative decrease in overall seed yield and a flattening of the yield curve with each successive harvest. There was also a shift in the ACGDU range where seed maturity was recorded. The fall 2012 seed crop required less ACGDU than in all previous harvest cycles to yield mature seed. Seed shedding in fall 2012, as measured by the total number of seed extracted from 10 spikes (Figure 8), occurred sooner than in all previous cropping cycles. The ACGDU range (667 to 700) where the total seed number for fall 2012 began to fall rapidly corresponded with the appearance of tangled seed heads (Figure 13).

In addition to seed yield components, total spike production also revealed the decline in productivity of the pili grass stand over time. Total spike production in response to ACDGU showed a positive linear trend in the first harvest cycle. As each
cropping cycle occurred, the slope of total spike production declined. Visual observations of the field also indicated that the plants in the spring 2012 growing season were taller and more vigorous than plants of the fall 2012 season (Figures 12 and 13, respectively). A combination of factors including stand age, cutting height as well as reduced amplitude in soil temperatures, growth inhibition imposed by pili grass straw accumulation (i.e., cut above ground portions of the plant remained at the base of plants and accumulated during the course of this study) and pest incidence may have contributed to the decreased yield and reduced ACDGU requirement for seed maturity.

Seed yield decline with increasing stand age is a common characteristic in a number of grass seed crops such as perennial ryegrass (*Lolium perenne*) (Hebblethwaite *et al.*, 1980), meadow bromegrass (*Bromus riparius*) (Loeppky and Coulman, 2002) and Kentucky bluegrass (*Poa pratensis*) (Canode and Law, 1975). Decreases in seed yield of these grasses can be observed after two to three or three to four seed crops (Canode and Law, 1975, Loeppky and Coulman, 2002). In Kentucky bluegrass, the decline in seed yield with stand age was attributed to decreased regrowth potential and accumulation of thatch in the older portions of the plants (Canode and Law, 1975). As plantings age, panicle production moves towards the outer circumference of the clump, causing a ‘ring’ or ‘dead center’ effect. This observation by Canode and Law (1975) was also observed in the pili grass plantings. Mowing after every growing season revealed that much of the vigorous regrowth were observed at the outer edge of each grass bunch.

Aside from stand age, cutting height can also influence seed yields of a grass crop. While grasses respond differently to cutting, low cutting heights can generally slowdown re-growth, thereby affecting tiller synchronization and delaying inflorescence
development (Dwivedi et al., 1999, Loch et al., 1999). In the current study, the high cutting height (17 cm) received during the spring 2011 growing season resulted in higher number of spikes and seeds per culm compared to the subsequent seasons in which the cutting heights were low (3 to 4 cm). This response of pili grass to low cutting height was similar to that observed in the tropical forage grass, *Chloris gayana* cv. Callide and *Setaria sphacelata* cv. Nandi. Severe cutting in *Chloris gayana* cv. Callide resulted in a decline in the number of inflorescences compared to a higher cutting height (Loch et al., 1999). Seed yields in *Setaria sphacelata* cv. Nandi were higher in uncut stands compared to clipped (45 cm above ground level) or severely cut (10 cm above ground level) plants (Dwivedi et al., 1999).

Thatch or straw left after harvesting was another factor that may have influenced the yield decline in the current study. In a number of cool season species, leaving crop residue can restrict tiller development thereby severely depressing seed production (Chilcote et al., 1980, Young et al., 1984, Chastain et al., 1997). Plant litter can affect crop growth by directly or indirectly altering the physical and chemical environment of the soil surface. Physical changes brought about by the accumulation of plant litter include increased shading, lowered soil temperatures and reduced evaporation (Rice and Parenti, 1978, Facelli and Pickett, 1991). Studies have shown that light penetration through the crowns and surface soil temperatures can affect productivity of grasses. Meijer and Vreeke (1988) attributed the increased production of fertile tillers in *Poa pratensis* and *Festuca rubra* with greater light penetration in treatments with lower plant litter. In tall grass prairie, removal of plant litter by mowing or burning resulted in higher soil temperatures compared with the unmowed control treatment (Rice and Parenti,
1978). This increase in soil temperature increased dry matter production of four dominant
tall grass prairie species.

Aside from manipulating light and temperature at the soil surface, plant litter can
also affect growth by chemically altering the soil environment. Decomposition of plant
litter can either withhold nutrients such as nitrogen (Mary et al., 1996) or release
that result in autotoxicity. In the current study, autotoxicity may have played a greater
part in the seed yield decline than nitrogen immobilization since the field was regularly
fertilized and the plants did not exhibit visible nutrient deficiency symptoms.

Autotoxicity is defined as the condition wherein a plant species releases toxic
substances that inhibit its germination and growth (Miller, 1996). It is a common
phenomenon in monocultures or sole cropping systems where plant residues are left after
harvest. A number of crop and forage species including alfalfa (Medicago sativa),
asparagus (Asparagus officinalis), rice (Oryza sativa), wheat (Triticum aestivum), corn
(Zea mays) and sugarcane exhibit autotoxicity (Chou and Lin, 1976, Miller et al., 1991,
Miller, 1996, Singh et al., 1999, Sánchez–Moreiras et al., 2003). Problems due to
autotoxicity can be mediated through proper management of crop residue. Singh et al.
(1999) suggests a number of cultural practices such as: providing a fallow period between
crop cycles, shifting agricultural patterns (i.e., relay cropping or double cropping) and
proper crop rotation.

Pest incidence may be another contributing factor to the observed yield decline of
pili grass in the fall 2012 season. Although there were no observed aphids feeding on the
inflorescences, most of the spikes harvested in fall 2012 exhibited sticky secretions
similar to honeydew (Figure 14). The honeydew-like secretions on spikes may be indicative of pest build up over time. A critical threshold of pests might have been reached, resulting in the widespread observance of the symptom in the fall 2012 season, but not in the previous seasons.

While the factors listed above may possibly explain the decline in pili grass seed production over cropping seasons, further studies are recommended to confirm these. Since it can be noted that the potential factors responsible for yield decline are interrelated, future research should focus on isolating each factor to determine the individual contributions of each. Additional research is also needed to test whether these factors impact time to harvest (i.e., seed maturity) within a growing season.

In conclusion, this study has identified potential indicators for harvest maturity in pili grass. ACGDUs, spike moisture content and the onset of seed head tangling can be useful tools since these exhibited some consistency in identifying an optimum range for maximizing seed yield. Based on observations in three of the four cropping cycles, maximum seed harvest was obtained between 768 to 778 ACGDUs (79 to 82 days after cutting). The range of spike moisture content for optimum harvest was between 0.68 to 0.72 g H₂O per g of dry weight. The onset of seed head tangling can also aid in determining harvest timing since this provided a visual cue which coincided with the optimum seed yield and range of spike moisture content. Aside from finding the peak seed yield, results also indicated a general decrease yield of pili grass over time. A number of factors such as, stand age, cutting height, thatch accumulation and increased pest incidence may explain this decline. However, further studies are recommended to
confirm and identify the contributions of each in the overall decline in seed yield over consecutive cropping cycles.
### Tables

**Table 7.1.** Analysis of variance (ANOVA) table for spike moisture content of pili grass harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
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<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Rep</td>
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<td>0.12273</td>
<td>0.04091</td>
<td>9.39</td>
<td>0.0039</td>
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<td>Season</td>
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<td>0.67601</td>
<td>0.22534</td>
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<td></td>
</tr>
<tr>
<td>Main plot error</td>
<td>9</td>
<td>0.21601</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACGDU</td>
<td>10</td>
<td>3.53332</td>
<td>0.35333</td>
<td>31.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
<td>30</td>
<td>1.02841</td>
<td>0.03428</td>
<td>3.1</td>
<td>&lt;0.01</td>
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<tr>
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<td>120</td>
<td>1.32632</td>
<td>0.01105</td>
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</tr>
<tr>
<td>Total</td>
<td>175</td>
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**Table 7.2.** Analysis of variance (ANOVA) table for total number of spikes of pili grass harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Total numbers of spikes were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<tr>
<td>Rep</td>
<td>3</td>
<td>0.251</td>
<td>0.0838</td>
<td>280.31</td>
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<tr>
<td>ACGDU</td>
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<td>7.312</td>
<td>0.6648</td>
<td>17.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
<td>33</td>
<td>4.733</td>
<td>0.1434</td>
<td>3.67</td>
<td>&lt;0.01</td>
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<tr>
<td>Sub plot error</td>
<td>132</td>
<td>5.152</td>
<td>0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>191</td>
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</tr>
</tbody>
</table>

**Table 7.3.** Analysis of variance (ANOVA) table for number of intact spikes of pili grass harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Numbers of intact spikes were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<td>0.1937</td>
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<td>Season</td>
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<td>58.5769</td>
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<tr>
<td>Main plot error</td>
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<td>0.6404</td>
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<tr>
<td>ACGDU</td>
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<td>3.7278</td>
<td>0.3389</td>
<td>10.84</td>
<td>&lt;0.01</td>
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<tr>
<td>Season x ACGDU</td>
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<td>7.4389</td>
<td>0.2254</td>
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<td>Sub plot error</td>
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<td>0.0312</td>
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<td>Total</td>
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Table 7.4. Analysis of variance (ANOVA) table for number of shed spikes of pili grass harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Numbers of shed spikes were square root transformed to conform data to the analysis of variance assumptions.

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<td>Season</td>
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<td>42.4062</td>
<td>14.1354</td>
<td>171.63</td>
<td>&lt;0.01</td>
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<tr>
<td>Main plot error</td>
<td>9</td>
<td>0.7412</td>
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<tr>
<td>ACGDU</td>
<td>11</td>
<td>49.73</td>
<td>4.5209</td>
<td>108.84</td>
<td>&lt;0.01</td>
</tr>
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<td>Season x ACGDU</td>
<td>33</td>
<td>6.7438</td>
<td>0.2044</td>
<td>4.92</td>
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<tr>
<td>Sub plot error</td>
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<td>5.4829</td>
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<tr>
<td>Total</td>
<td>191</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.5. Analysis of variance (ANOVA) table for number of unopened spikes of pili grass harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Numbers of unopened spikes were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
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<th>DF</th>
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<tbody>
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<td>Rep</td>
<td>3</td>
<td>0.0926</td>
<td>0.0309</td>
<td></td>
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</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>62.4014</td>
<td>20.8005</td>
<td>259.58</td>
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</tr>
<tr>
<td>Main plot error</td>
<td>9</td>
<td>0.7212</td>
<td>0.0801</td>
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</tr>
<tr>
<td>ACGDU</td>
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<td>3.3409</td>
<td>0.3037</td>
<td>16.11</td>
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</tr>
<tr>
<td>Season x ACGDU</td>
<td>33</td>
<td>4.5716</td>
<td>0.1385</td>
<td>7.35</td>
<td>&lt;0.01</td>
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<tr>
<td>Sub plot error</td>
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</tr>
<tr>
<td>Total</td>
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</tr>
</tbody>
</table>

Table 7.6. Analysis of variance (ANOVA) table for the number of filled seeds extracted from ten pili grass spikes harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Filled seed numbers were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
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<th>P</th>
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<tbody>
<tr>
<td>Rep</td>
<td>3</td>
<td>0.822</td>
<td>0.2739</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>65.496</td>
<td>21.8319</td>
<td>39.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Main plot error</td>
<td>9</td>
<td>5.012</td>
<td>0.5569</td>
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<td></td>
</tr>
<tr>
<td>ACGDU</td>
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<td>199.984</td>
<td>18.1804</td>
<td>22.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
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<td>3.7268</td>
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<td>&lt;0.01</td>
</tr>
<tr>
<td>Sub plot error</td>
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<td>0.8253</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>191</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7.7. Analysis of variance (ANOVA) table for the number of empty seeds extracted from ten pili grass spikes harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Empty seed numbers were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<th>P</th>
</tr>
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<tbody>
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<td>Rep</td>
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</tr>
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<td>Season</td>
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<td>25.202</td>
<td>8.4008</td>
<td>16.01</td>
<td>0.0006</td>
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<tr>
<td>Main plot error</td>
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<td>4.722</td>
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</tr>
<tr>
<td>ACGDU</td>
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<td>163.357</td>
<td>14.8506</td>
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<tr>
<td>Season x ACGDU</td>
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<td>41.933</td>
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<td>Sub plot error</td>
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<tr>
<td>Total</td>
<td>191</td>
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<td></td>
</tr>
</tbody>
</table>

Table 7.8. Analysis of variance (ANOVA) table for the total number of seeds extracted from ten pili grass spikes harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Total seed numbers were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
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<th>P</th>
</tr>
</thead>
<tbody>
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<td>Rep</td>
<td>3</td>
<td>0.7638</td>
<td>0.25461</td>
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<td></td>
</tr>
<tr>
<td>Season</td>
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<td>18.8595</td>
<td>6.28651</td>
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<td>0.0006</td>
</tr>
<tr>
<td>Main plot error</td>
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<td>ACGDU</td>
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<td>47.5735</td>
<td>4.32487</td>
<td>26.15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
<td>33</td>
<td>31.2536</td>
<td>0.94708</td>
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<td>&lt;0.01</td>
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<tr>
<td>Sub plot error</td>
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<tr>
<td>Total</td>
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</tbody>
</table>

Table 7.9. Analysis of variance (ANOVA) table for the total weight of seeds extracted from ten pili grass spikes harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons.

<table>
<thead>
<tr>
<th>Source</th>
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<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
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<td>0.00053</td>
<td>0.00018</td>
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<td></td>
</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>0.00635</td>
<td>0.00212</td>
<td>18.25</td>
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<tr>
<td>Main plot error</td>
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<td>0.00104</td>
<td>0.00012</td>
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<tr>
<td>ACGDU</td>
<td>11</td>
<td>0.02482</td>
<td>0.00226</td>
<td>5.48</td>
<td>&lt;0.01</td>
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<tr>
<td>Season x ACGDU</td>
<td>33</td>
<td>0.06676</td>
<td>0.00202</td>
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<td>&lt;0.01</td>
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<td>Sub plot error</td>
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<tr>
<td>Total</td>
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</tbody>
</table>
Table 7.10. Analysis of variance (ANOVA) table for the average weight of individual seeds extracted from ten pili grass spikes harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Weights of individual seeds were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
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</tr>
</thead>
<tbody>
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<td>Rep</td>
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<td>0.01276</td>
<td>0.00425</td>
<td>1.84</td>
<td>0.2109</td>
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<tr>
<td>Season</td>
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<td>0.02418</td>
<td>0.00806</td>
<td>1.84</td>
<td>0.2109</td>
</tr>
<tr>
<td>Main plot error</td>
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<td>0.00439</td>
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<td>ACGDU</td>
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<td>14.84</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
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<td>0.35249</td>
<td>0.01068</td>
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<td>&lt;0.01</td>
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<tr>
<td>Sub plot error</td>
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<tr>
<td>Total</td>
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</tr>
</tbody>
</table>

Table 7.11. Analysis of variance (ANOVA) table for the total weight of pili grass seeds extracted from 30 culms harvested over average cumulative growing degree day units (ACGDU) during the 2011-2012 spring and fall growing seasons. Seed weights were square root transformed to conform data to the analysis of variance assumptions.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep</td>
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<td>0.346</td>
<td>0.1152</td>
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</tr>
<tr>
<td>Season</td>
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<td>218.671</td>
<td>72.8902</td>
<td>461.09</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Main plot error</td>
<td>9</td>
<td>1.423</td>
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</tr>
<tr>
<td>ACGDU</td>
<td>11</td>
<td>35.851</td>
<td>3.2592</td>
<td>28.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Season x ACGDU</td>
<td>33</td>
<td>26.522</td>
<td>0.8037</td>
<td>7.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sub plot error</td>
<td>132</td>
<td>14.903</td>
<td>0.1129</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>191</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7.1. Regression of pili grass spike water content (g H$_2$O g$^{-1}$ dry weight) harvested during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The prediction equations and $R^2$ values are $Y_{Spring2011} = 1.08 - 0.00052X_{AGDD}$, $R^2 = 0.17$; $Y_{Fall2011} = 2.08 - 0.0018X_{AGDD}$, $R^2 = 0.63$; $Y_{Spring2012} = 5.30 - 0.011X_{AGDD} + 0.0000061X_{AGDD}^2$, $R^2 = 0.83$ and $Y_{Fall2012} = 1.60 - 0.0011X_{AGDD}$, $R^2 = 0.33$. 
Figure 7.2. Regression of pili grass total spike production per culm during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{Spring2011} = (3.16 + 0.0026X_{AGDD})^2 - 0.5$, $R^2 = 0.40$; $Y_{Fall2011} = (0.82 + 0.0034X_{AGDD})^2 - 0.5$, $R^2 = 0.79$ and $Y_{Spring2012} = (1.35 + 0.0021X_{AGDD})^2 - 0.5$, $R^2 = 0.54$. *F-test for the fall 2012 regression model was not significant (P=0.2200). This indicated that there was no change in the total number of spikes produced during the growing season.
Figure 7.3. Regression of intact spike production per culm of pili grass harvested during the spring and fall growing seasons of 2011 and 2012. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{Spring 2011} = (-2.35 + 0.01781X_{AGDD} - 0.000013X_{AGDD}^2)^2 - 0.5$, $R^2 = 0.29$; $Y_{Fall 2011} = (-3.70 + 0.01734X_{AGDD} - 0.000012X_{AGDD}^2)^2 - 0.5$, $R^2 = 0.35$; $Y_{Spring 2012} = (43.49 - 0.20364X_{AGDD} + 0.00032X_{AGDD}^2 - 0.0000016X_{AGDD}^3)^2 - 0.5$, $R^2 = 0.85$ and $Y_{Fall 2012} = (-3.34 + 0.01734X_{AGDD} - 0.000013X_{AGDD}^2)^2 - 0.5$, $R^2 = 0.50$. 

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Figure 7.4. Regression of shed spike production per culm of pili grass harvested during the spring and fall growing seasons of 2011 and 2012. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{Spring2011} = (-2.94 + 0.0074X_{AGDD})^2 - 0.5$, $R^2 = 0.80$; $Y_{Fall2011} = (7.17 - 0.0230X_{AGDD} + 0.000021X_{AGDD}^2)^2 - 0.5$, $R^2 = 0.97$; $Y_{Spring2012} = (4.70 - 0.0140X_{AGDD} + 0.000012X_{AGDD}^2)^2 - 0.5$, $R^2 = 0.92$ and $Y_{Fall2012} = (-1.54 + 0.0041X_{AGDD})^2 - 0.5$, $R^2 = 0.80$. 

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Figure 7.5. Regression of unopened spike production per culm of pili grass during the spring and fall 2011 and 2012 growing seasons. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are 

$Y_{\text{Fall}2011} = (4.36 - 0.00821X_{\text{AGDD}} + 0.0000063 X_{\text{AGDD}}^2)^2 - 0.5$, $R^2 = 0.31$; $Y_{\text{Spring}2012} = (3.58 - 0.0025X_{\text{AGDD}})^2 - 0.5$, $R^2 = 0.74$ and $Y_{\text{Fall}2012} = (9.39 - 0.0206X_{\text{AGDD}} + 0.000013 X_{\text{AGDD}}^2)^2 - 0.5$, $R^2 = 0.81$. *The regression model for the spring 2011 data was not significant (P=0.1477), indicating that there was no change in number of unopened spikes produced over average cumulative growing degree days.
Figure 7.6. Regression of filled seeds (number) extracted from 10 spikes of pili grass during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{\text{Spring}2011} = (-0.24 + 0.0075X_{\text{AGDD}})^2 - 0.5, R^2 = 0.31$; $Y_{\text{Fall}2011} = (-37.71 + 0.11X_{\text{AGDD}} - 0.000069X_{\text{AGDD}}^2)^2 - 0.5, R^2 = 0.68$ and $Y_{\text{Spring}2012} = (186.75 - 0.877X_{\text{AGDD}} - 0.00135X_{\text{AGDD}}^2 + 0.00000067X_{\text{AGDD}}^3)^2 - 0.5, R^2 = 0.77$. *The regression model for the fall 2012 data was not significant (P=0.1156). This indicated that there was no change in number of filled seeds produced over average cumulative growing degree days.
Figure 7.7. Regression of empty seeds (number) extracted from 10 spikes of pili grass during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{Spring2011} = (13.76 - 0.0060X_{AGDD})^2$, $R^2 = 0.40$; $Y_{Fall2011} = (16.55 - 0.0098X_{AGDD})^2$, $R^2 = 0.66$; $Y_{Spring2012} = (16.00 - 0.0085X_{AGDD})^2$, $R^2 = 0.71$ and $Y_{Fall2012} = (18.43 - 0.013X_{AGDD})^2$, $R^2 = 0.74$. 
Figure 7.8. Regression of total seed number extracted from 10 spikes of pili grass during the spring and fall 2011 and 2012 cropping seasons. The back transformed prediction equations and $R^2$ values are $Y_{Spring2011} = (12.04 - 0.0016X_{AGDD})^2$, $R^2 = 0.20$; $Y_{Fall2011} = (-0.871 + 0.038X_{AGDD} - 0.000029X_{AGDD}^2)^2$, $R^2 = 0.55$; $Y_{Spring2012} = (-4.78 + 0.048X_{AGDD} - 0.000036X_{AGDD}^2)^2$, $R^2 = 0.54$ and $Y_{Fall2012} = (1.553 + 0.036X_{AGDD} - 0.000033X_{AGDD}^2)^2$, $R^2 = 0.76$. 
Figure 7.9. Regression of total seed weight extracted from 10 spikes of pili grass during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The prediction equations are $Y_{\text{Spring 2011}} = 0.095 + 0.00084X_{\text{AGDD}}, R^2 = 0.13$; $Y_{\text{Fall 2011}} = 2.93 - 0.014X_{\text{AGDD}} + 0.000021X_{\text{AGDD}}^2 - 0.00000011X_{\text{AGDD}}^3, R^2 = 0.50$; $Y_{\text{Spring 2012}} = 5.44 - 0.024X_{\text{AGDD}} + 0.000037X_{\text{AGDD}}^2 - 0.00000018X_{\text{AGDD}}^3, R^2 = 0.65$ and $Y_{\text{Fall 2012}} = -4.43 + 0.020X_{\text{AGDD}} - 0.000027X_{\text{AGDD}}^2 + 0.000000012X_{\text{AGDD}}^3, R^2 = 0.61$. 
Figure 7.10. Regression of average weight of individual seeds extracted from 10 spikes of pili grass during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The prediction equations and $R^2$ values are $Y_{Spring2011} = (0.805 + 0.00047X_{AGDD})^2$, $R^2=0.29$; $Y_{Fall2011} = (-1.14 + 0.0059_{AGDD} - 0.0000368_{AGDD})^2$, $R^2=0.58$ and $Y_{Spring2012} = (13.16 - 0.056_{AGDD} + 0.0000837_{AGDD}^2 - 0.0000000407_{AGDD}^3)^2$, $R^2=0.79$. *The regression model for the fall 2012 dataset was not significant (P=0.1504). This indicates that there was no change in weight of individual seeds over average cumulative growing degree days.
Figure 7.11. Regression of seed yield extracted from 30 culms of pili grass during the spring and fall 2011 and 2012 cropping seasons. The 95% confidence intervals are depicted by dotted blue lines. The back transformed prediction equations and $R^2$ values are $Y_{\text{Spring}2011} = (40.19 - 0.177X_{\text{AGDD}} + 0.00027X_{\text{AGDD}}^2 - 0.00000013X_{\text{AGDD}}^3)^2$, $R^2=0.69$; $Y_{\text{Fall}2011} = (13.95 - 0.062X_{\text{AGDD}} + 0.0001X_{\text{AGDD}}^2 - 0.000000052X_{\text{AGDD}}^3)^2$, $R^2=0.83$; $Y_{\text{Spring}2012} = (-3.70 + 0.0139X_{\text{AGDD}} - 0.0000090X_{\text{AGDD}}^2)^2$, $R^2=0.64$ and $Y_{\text{Fall}2012} = (-15.43 + 0.070X_{\text{AGDD}} - 0.000097X_{\text{AGDD}}^2 + 0.00000043X_{\text{AGDD}}^3)^2$, $R^2=0.49$. 
Figure 7.12. Harvest date photographs of the pili grass field during the spring 2012 growing season. Detail of the tangled seed heads (inset picture), the actual cumulative growing degree units recorded and spike moisture content expressed in grams of water per grams of oven dry weight are also shown.
Figure 7.13. Harvest date photographs of the pili grass field during the fall 2012 growing season. Detail of the tangled seed heads (inset picture), the actual cumulative growing degree units recorded and spike moisture content expressed in grams of water per grams of oven dry weight are also shown.
Figure 7.14. Sticky secretions, resembling honeydew observed from pili grass spikes harvested during the fall 2012 season.
CHAPTER 8

CONCLUSIONS, ECOLOGICAL IMPLICATIONS AND SUGGESTED SEED PRODUCTION AND ESTABLISHMENT PROTOCOLS

Findings reported in this dissertation provided insights on both the mechanisms and factors involved in pili grass seed dormancy and pili grass seed development. The dormancy loss and germination studies confirmed that pili grass seeds exhibited non-deep physiological dormancy. The germination response of pili grass to scarification (i.e., nicking to expose the embryo) (see Chapter 2), GA application (see Chapter 5) and warm, dry storage (see Chapter 3) are consistent with the characteristics described by Baskin and Baskin (2004) for seeds expressing non-deep physiological dormancy. The response of pili grass seeds to the germination stimulants, fluridone, GA, smoke and cyanide (see Chapter 5) also provided support, since these studies elucidated the dormancy loss mechanisms involved under warm, dry storage (i.e., dry after-ripening).

Scarification studies (see Chapter 2) indicated the role of the seed coats (i.e., endosperm surrounding the embryo) in inhibiting germination of dormant pili grass seeds. The observed germination stimulation of seeds by nicking (to expose the embryo) suggests that pili grass endosperm physically restrict the embryo rather than restricting water uptake. This physical restriction can be overcome by GA and ABA mediated hydrolytic enzymes that soften the endosperm. Besides coat imposed germination inhibition, the observed partial germination stimulation with scarification also indicated that physiological factors, such as the hormonal balance between GA and ABA (see Chapter 5), are involved in the maintenance or loss of pili grass seed dormancy.
Seed storage studies (see Chapters 3 and 4) characterized the role of storage temperature and storage relative humidity (i.e., seed moisture content) on dormancy and viability loss in pili grass seeds. Low storage temperatures (5 to 10°C), regardless of storage relative humidity maintained seed dormancy and viability of pili grass seeds for one year. Increasing storage temperature from 10 to 30°C generally increased dormancy loss in pili grass seeds, provided that storage relative humidity is maintained at 12% eRH (6% seed moisture, dry weight basis). Loss in seed viability was more pronounced over time as storage temperatures were increased from 20 to 30°C and as storage relative humidity were increased from 50 to 75% eRH (11 to 14% seed moisture content, dry weight basis). To optimize dormancy loss while maintaining seed viability, it was recommended that fresh seeds be air dried for two weeks under ambient conditions and then stored at either 12% eRH (6% moisture, dry weight basis) at 30°C for 12 months or at 50% eRH (11% moisture, dry weight basis) at 30°C for 9 months. After storage in the optimized dormancy loss conditions, seeds can be safely stored at 12% eRH (6% moisture, dry weight basis) at 5°C for 6 months without significant loss in seed viability. Seed viability response to longer storage times (>6 months) were not included in these studies.

Germination assays with fluridone, GA and food grade liquid smoke (see Chapter 5) suggested that seed dormancy of pili grass was mediated by the balance between ABA and GA. Fluridone stimulated germination suggests that de novo ABA synthesis was necessary for maintaining dormancy of imbibed seeds. The observed GA stimulated germination through exogenous application was possibly due to the promotion of endosperm weakening (see Chapter 2) and lowering of ABA levels through ABA
catabolisis. During dry storage to relieve seed dormancy, the ABA:GA ratio was possibly shifted towards higher effective GA levels by lowering ABA levels and/or sensitivity and/or increasing GA levels and/or sensitivity. The germination response of food grade liquid smoke was as effective as shifting the ABA:GA ratio towards higher levels of GA.

Assays with smoke infused water (see Chapter 6) indicated that food grade liquid smoke, pili grass smoke infused water and xylose smoke infused water were effective in stimulating germination of dormant pili grass seeds. Pili grass smoke infused water and food grade liquid smoke appear to be better smoke sources for improved pili grass seed germination since a consistent response was observed across seed batches (with suspected differences in depth of seed dormancy). Of the two recently identified components in smoke (i.e., KAR$_1$ and cyanide), only cyanide was found to promote germination in pili grass seeds. Cyanide stimulated germination suggested a possible role for ROS in dormancy loss of pili grass seed under warm, dry storage. Increasing ROS levels, through cyanide treatment or warm, dry storage (i.e., dry after-ripening), can trigger carbonylation of proteins associated with seed germination (Oracz et al., 2007, Oracz et al., 2009, Iglesias-Fernandez et al., 2011). Aside from cyanide, there were probably other compounds in smoke, such as benzaldehyde, that promoted germination of pili grass seeds. Further research was recommended to isolate, quantify and test these compounds and confirm whether these stimulants may be additive or synergistic with cyanide.

Seasonal differences in the depth of dormancy were also observed in the germination studies throughout this dissertation. Seeds harvested in March exhibited less dormancy than seeds harvested in June (see Chapter 2), July (see Chapter 6) and October
(see Chapter 3). Seasonality of seed dormancy was attributed to differences in growing conditions during seed development. To confirm and quantify the effects of growing conditions on dormancy of pili grass, more in-depth studies should focus on evaluating specific environmental factors (i.e., daylength, light quality, mineral nutrition, soil moisture, temperature and physiological age of mother plants).

Harvest timing studies (see Chapter 7) indicated that ACGDUs, spike moisture content and the onset of seed head tangling were useful indicators for determining the harvest maturity of pili grass seeds. Based from the results in three of four cropping cycles, maximum seed harvest was obtained between 768 to 778 ACGDUs (79 to 82 days after cutting). The range of spike moisture contents for optimum harvest was between 0.68 to 0.72 grams H$_2$O per gram of dry weight. The onset of seed head tangling can also aid in determining harvest timing since this provided a visual cue that coincided with the optimum seed yield and range of spike moisture content. Aside from determining the optimum time (after cutting the stand) to harvest seed, seasonal harvests also indicated a general decrease in pili grass seed yield over time. This decline may be due to a number of factors such as stand age, cutting height, thatch accumulation and increased pest incidence. Further studies are needed to confirm and identify the contributions of each on the overall decline in seed yield over a number of cropping cycles are recommended.

**Implications of findings on the natural distribution and ecophysiology of pili grass**

Besides applications for restoration and re-vegetation, the studies conducted in this dissertation can also provide some explanation on the natural distribution and
ecophysiology of pili grass in Hawai‘i. According to Wagner et al. (1999) and Goergen and Daehler (2001), pili grass thrives on the dry leeward sides of the islands where annual rainfall is 1,200 mm or less. Much of the annual rainfall in these areas occurs primarily during the cooler months (October to April) (Goergen and Daehler, 2001). The pronounced wet and dry periods in these areas are favorable for the survival and persistence of pili grass since the warm drying period of summer allows seeds to lose dormancy. Under these climatic conditions, the annual life cycle of pili grass in Hawai‘i can be described as follows: Winter rains trigger pili grass stands to flower and set seeds. As the seeds are shed and the winter rains diminish, conditions (i.e., lower relative humidity and warmer soil temperatures, see Chapter 3) become more favorable for seeds to lose dormancy. The summer season (May to September) provides the optimum period for dormancy loss conditions to occur. By the time the next winter rains arrive, the seeds have already lost dormancy and are ready to germinate.

Fires during the summer season can also provide smoke cues for pili grass seeds to germinate (see Chapter 6). Smoke cues such as glyceronitrile are produced during fires and deposited in the soil. When the winter rains arrive, glyceronitrile is hydrolyzed and breaks down to release free cyanide which serves as a germination cue for pili grass seeds.

Aside from promoting seed germination, fires may also improve the vigor of pili grass stands. Results from Chapter 7 indicate the requirement for burning since a decline in seed production was observed with excessive accumulation of thatch. Both historical evidence and recent research studies support the invigorating effects of fire on pili grass stands. According to Cuddihy and Stone (1990) as cited by Daehler and Goergen (2005),
large tracks of pili grasslands in Hawai‘i were regularly burned by the ancient Hawaiians to stimulate new growth, which was used for thatching houses. Studies on pili grass dominated pastures in Australia by Campbell (1995) demonstrated that burning of stands increased basal area of clumps and helped restore degraded pastures better than management through mowing. Experimental restoration of pili grass on the island of Hawai‘i also indicated increased establishment and dominance with biennial burning (Daehler and Goergen, 2005).

**Suggested seed production and establishment protocol for pili grass**

Based on the findings obtained from this study, a protocol for pili grass seed production and establishment was developed.

**Site preparation**

Clear the re-vegetation/seed production site of trash and debris and any unwanted tall shrubs or grasses that might interfere with planting. Once cleared, install a temporary overhead irrigation to encourage active weed growth prior to systemic herbicide application. Conduct sequential flushing and killing of weeds for at least three to six months prior to planting to exhaust the weed seed bank and stored reserves of perennial species. To kill existing vegetation, conduct a spray to wet application of glyphosate (e.g., Roundup Pro, Monsanto) at a rate of 1.12 kg active ingredient (a.i.)/ha (DeFrank, personal communication, November 2013).
Planting and establishment

Establishment by plugs

Freshly harvested seeds need to be relieved of dormancy prior to the production of pili grass transplants (see section below on optimum storage conditions for dormancy loss). Presoak pure or raw seeds (i.e., loosened mass of awns and seed from bales) for 15 minutes in 1% food grade liquid smoke solution (e.g., Colgin Natural Mesquite, Colgin Inc., Dallas, TX) or neat pili grass smoke infused water (see Chapter 6 for preparation details) to relieve any remaining dormancy and enhance germination. Sow treated seeds in plastic trays filled with a commercially prepared mixture of potting media (e.g., Pro-mix ‘BX’, Premier Horticulture). After one week, transplant seedlings into dibble tubes filled with a mixture of potting media (e.g., Pro-mix ‘BX’, Premier Horticulture) and fertilizer (e.g., 10-4-16, Best Microgreen 10, applied at 456 grams per 2 cubic feet of potting mix). Allow the plugs to grow under overhead-irrigated conditions for at least two months. Cut the grass to 10 cm height before transplanting to prevent transplant shock and to remove flower heads that may have formed. The recommended minimum spacing for planting is 2.0 m between rows and 0.5 to 1.0 m in rows. Row planting is recommended to facilitate weed control during pili grass growth and establishment. Fertilize at a rate of 112 kg N/ha to stimulate rapid growth, generate weed suppressing mulch and canopy fill in.

Establishment by seeds

Freshly harvested seeds need to be relieved of dormancy prior to the production of pili grass transplants (see section below on optimum storage conditions for dormancy loss). Presoak pure or raw seeds (i.e., loosened mass of awns and seed from bales) for 15
minutes in 1% food grade liquid smoke solution (e.g., Colgin Natural Mesquite, Colgin Inc., Dallas, TX) or neat pili grass smoke infused water (see Chapter 6 for preparation details) to relieve any remaining dormancy and enhance germination. Sow pretreated seed by hand on a 60 cm band along the irrigation drip line at a rate of 258 live seeds per square meter (Lukas, 2011). The recommended between row spacing is 2.0 m. Cover the planted surface with a mixture of oxadiazon (Ronstar® 50 WP, 2.80 kg a.i. per ha to control germinating weeds) and hydromulch (8.6 kg/ha tackifier, 832 kg/ha straw mulch and 904 kg/ha paper mulch) at a rate of 61,118 liters/ha (Lukas, 2011). Irrigate the field with 10 cm of water daily for 48 days using supplemental overhead irrigation along with drip irrigation (Lukas, 2011). Reduce irrigation amount and frequency once plants have established. Fertilize at a rate of 112 kg N/ha to stimulate rapid growth and canopy fill in.

**Mowing**

Three to four months after planting, mow the pili grass plants to 10 cm height in order to promote upright growth and increased number of inflorescence bearing tillers. Use a hedge trimmer or sickle bar mower to make a clean cut to reduce plant shock and encourage stem proliferation.

**Pre- and postemergence weed control**

For pre-emergence weed control of newly transplanted plugs, DeFrank (2003) recommends an over the top spray of either oxadiazon (Ronstar® 50 WP) or oryzalin (Surflan 4 AS) at 2.24 kg a.i./ha. For post emergence weed control of broadleaf weeds, an over the top spray of triclopyr (Garlon 4) at 2.24 kg a.i./ha is recommended starting at 21
days after planting (DeFrank, 2003). For post emergence weed control of grassy weeds, DeFrank and Sakamoto (2007) recommends quizalofop p-ethyl (Assure II) at 0.98 kg a.i./ha applied as a directed spray to minimize injury to pili grass.

**Seed production and harvesting**

The pili grass seed production protocol discussed below is designed to harvest seeds with the lowest level of dormancy. Based on the seasonal differences observed with seed batch in this dissertation (see Chapters 2, 3 and 6), seed production targeting a March harvest is recommended for irrigated plantings. To prepare plantings for seed production, the grass stands must be cut to a height of no less than 10 cm during the winter solstice (December 21 or 22). Use a hedge trimmer or sickle bar mower to make a clean cut to the stand. After cutting, fertilize the plants at a rate of 112 kg N/ha to stimulate rapid growth and seed production. Regularly monitor spike and seed development as plants approach the 79th day after cutting (768 CGDUs). Seeds are ready for harvesting at 768 to 778 CGDUs (79 to 82 days after cutting), when extensive tangling of spikes is observed (Figure 1) and the spike moisture content (measured without the awn) reaches 0.68 to 0.72 grams H₂O per gram of dry weight.

**Storage to maximize dormancy loss and minimize seed viability loss**

Freshly extracted seeds must be dried under ambient conditions for two weeks before transferring into dessicators containing a saturated LiCl solution (see Chapter 3). Use about 50 grams of LiCl per 250 grams of seed to equilibrate seed moisture content with the relative humidity inside the dessicator. After 28 days of storage or after reaching
6% seed moisture content (dry weight basis), place the seeds in heat sealable foil barrier packets or airtight containers. To facilitate dormancy loss, store the sealed packets or containers at 30°C constant temperature for 12 months. After 12 months of storage at 30°C, transfer the seeds to a 5°C refrigerator for storage until use. Seeds will maintain viability under these conditions for at least 6 months.
Figure 8.1. Extensive appearance of tangled seed heads between 768 to 778 ACGDUs (79 to 82 days after cutting) indicates that seeds are ready for harvest.
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