REPRODUCTIVE BEHAVIOR OF SEASHORE PASPALUM

(*Paspalum vaginatum* Sw.)

by

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(Under the Direction of Paul L. Raymer)

ABSTRACT

A clear understanding of the reproductive biology of seashore paspalum (*Paspalum vaginatum* Sw.) is needed to design improved production systems for seeded cultivars. A series of studies were conducted to determine the impact of environmental parameters on floral initiation; evaluate pollen release patterns, pollen viability, and longevity; as well as to determine of sexual compatibility levels among genotypes of seashore paspalum. The primary result of flowering habit studies of the seashore paspalum germplasm collection indicated that in general, in Griffin, GA, flower initiation increases while the day length approaches the longest day of the year. Flowering response to solar radiation showed significant reductions in flowering when radiation levels were reduced to 41% or less of the unshaded control. Seashore paspalum pollen was released between 0700 h and 1100 h with a very sharp release peak around 0800 h. The quantity of pollen released during this period was positively related to the solar radiation at 0800 h. An optimized liquid germination medium was developed to effectively evaluate pollen viability and longevity for seashore paspalum. Pollen collected at 0730 h from seven genotypes of seashore paspalum had viabilities that ranged from 60 to 88%. Pollen longevity of plant material from greenhouse and field environments was 80 min and 100 min, respectively at room

temperature. Pollen longevity was negatively impacted by temperatures of 30˚C or more. Studies of pollen-stigma interactions indicated that seashore paspalum is a self-incompatible and cross-fertile species. Results from a six by six reciprocal crossing experiment indicated that all cross pollinations produced seeds but varied in percent seed set from 7.7 to 66.3 %. Seed germination rates of seed produced in this crossing experiment ranged from 56.7% to 98.1% and 47% of all hybrid seeds had greater than 90% germination. The findings of this research provide useful information for future seashore paspalum breeding programs.

INDEX WORDS: Floral initiation, Pollen release pattern, Pollen viability, Pollen longevity, Environmental parameters, Sexual compatibility, Seashore paspalum

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DEDICATION

To my parents, brother and sister – thanks for the greatest love that you give me. It is your infinite support and encouragement that allowed me to make my way to a country abroad and complete this work.

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CHAPTER 1

INTRODUCTION

Turfgrasses are the vegetative covers that are primary used for aesthetic and athletic purposes. They are also used for improving environmental conditions and for soil stabilization. The estimated size of the Georgia turf industry is 1.6 million acres with an estimated maintenance cost of $1.56 billion per year (www.georgiaturf.com). Turfgrass is considered as one of the largest agricultural products in the state.

Seashore paspalum (*Paspalum vaginatum* Sw.) is a wind-pollinated, perennial warm-season grass that grows in large areas from tropical to warm temperate regions (Morton, 1973). Tolerance to multiple environmental stresses, high nutrient uptake efficiency, adaptation to a wide range of water quality, and desirable morphological traits make it popular as a turfgrass (Duncan and Carrow, 2000). It is considered as an emerging turfgrass that will be widely utilized on golf courses, athletic fields, home lawns, and commercial areas (Duncan, 1999).

Sseashore paspalum cultivars are primarily produced by clonal propagation and sold as plugs, sod, or sprigs. The first seeded cultivar of seashore paspalum, named ‘Sea Spray’, was developed by Fricker et al. (2007) and marked by Scotts Company, LLC. (Gervais, OR). Turf established with seashore pasplum seed rapidly forms a dense turf, sometimes taking less time for full establishment than sprigs. When establishment rates were compared with plugs from ‘Salam’, ‘SeaIsle1’ and ‘SeaIsle 2000’ seashore paspalum, Sea Spray seed provided greater percent soil coverage after 62 days (Fricker et al., 2007). Using seeds costs less in transportation

and establishment expenses and is not as time-consuming or labor intensive as using sod or sprigs. In addition, seeds can be stored for extended periods of time and could avoid many international shipping restrictions imposed upon live vegetative plant material like sprigs and sod.

Sea Spray is produced in Oregon, USA, in a region that is known for its ability to produce high-quality seed of cool-season turf species. Considerable expertise and infrastructure for grass seed production exists in this region, but the climate is cool to temperate and production of seed from warm-season species such as seashore paspalum is challenging. Limited seed set is usually associated with environmental and/or genetic factors. It involves complex mechanisms for a pollen grain to go through the complete processes of development, pollination, fertilization, and eventually result successful production of a seed. Situations that can cause a failure during reproduction and result in reduced set seed vary greatly. Limited flowerings, non-functional reproductive parts, lack of pollen release, poor pollen movement, non-viable pollen, short pollen longevity, incompatibility, and various environmental factors that can impact these aspects all can potentially influence seed yield (Elleman et al., 1992; Franklin-Tong, 2002). Development of additional seeded cultivars and identification of optimum production regions for seeded cultivars is limited by our lack of understanding of the reproductive biology of seashore paspalum and the environmental interactions that influence reproduction and seed production.

The long-term goal for this project was to identify the optimum seed production environment for seashore paspalum and to select suitable parental lines for development of more seeded cultivars. The short-term objectives were to gain information regarding the reproductive behavior of seashore paspalum and identify the major limitations associated with seed production in the southern United States. Studies presented in this thesis were focused on these short-term objectives.

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CHAPTER 2

LITERATURE REVIEW

Knowledge regarding the reproductive behavior of seashore paspalum (*Paspalum vaginatum* Sw.) is crucial for understanding the seed production process. Many experiments have been conducted exploring the reproductive behavior of various plants including members in the *Poaceae* family. However, none have been accomplished with seashore paspalum. Flower induction, anther dehiscence, pollen release pattern, pollen viability and longevity, stigma receptivity, pollen-stigma interactions, and their relationship with various environmental parameters are critical aspects to consider when studying the reproductive behavior of a crop. This review aims to provide a summary of knowledge and research results addressing these areas.

Environmental impacts on flower initiation

Successful reproduction in flowering plants requires functional flowers that effectively provide functional male and female organs to produce viable seeds. The timing of flowering is one of the major factors concerning breeders. A recent study indicates genes that control the time of flowering also affect hybrid vigor, hence impacting yield hence (Ni et al., 2009). The seed production industry requires selection of parental lines that not only produce functional flowers but also produce flowers in large quantities in order to produce large amounts of seed necessary for profitability. Limited flowering could be caused by genetic and/or environmental factors. Both *in vitro* and *in vivo* studies concerning floral induction have been conducted on many plant

species. Photoperiod and temperature are primary parameters of concern when studying the external factors that induce flowering (Rogers, 1950).

It is known that plants present different behaviors involving complicated mechanisms in response to environmental stimulations. Charles Darwin first described movement in plants stimulated by environmental factors such as light, temperature, and gravity (Darwin, 1880). Plant behavior has been defined as "a response to an event or environmental stimulation during its lifetime" (Silvertown and Gordon, 1989; Silvertown, 1998). Garner and Allard (1920; 1930) illustrated the impact that day length had on determination of floral initiation. Garner and Allard introduced the term of 'photoperiodism' to describe the response to the changing length of day or night in plants. For flowering plants (angiosperms), the time for flowering is critical for their reproductive success. Photoperiodism results in a synchrony of flower initiation within a population and promotes genetic recombination by outcrossing (Heide, 1985). Seasonal changes in photoperiod provide a signal that triggers plants to complete their life cycle. Hamner (1940) determined that flowering is regulated by exposure of plants to specific number of hours of uninterrupted darkness rather than the length of the light. Photoperiodic flowering plants are classified as long-day plants, short-day plants, and day-neutral plants (Thomas and Vince-Prue 1999). Plants flower when the day length shorter that their critical photoperiod are classified as short day plant. Most summer and fall flowering plants are short-day plants, such as, cotton (*Gossypium* spp.) and rice (*Oryza sativa)*. In contrast, long-day plants initiate flowering when the day length becomes longer that their critical photoperiod. Most spring flowering plants such as Ryegrass (*Lolium* spp.), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) are long-day plants. Some plants form flowers regardless of day length and are referred to as day-neutral plants (e.g. cucumbers and tomatoes) (Thomas and Vince-Prue, 1997). Even though specific

photoperiods appear twice during a year at a given latitude (Fig 2-1), the mechanism of sensing the increase or decrease of the day length assists plants in recognizing whether it is spring or fall (Nelson et al., 2010; Thomas and Vince-Prue, 1997).

Since Garner and Allard (1920) discovered that the photoperiod is a key factor for the floral initiation, photoperiodism has been studied extensively on many plants. In most light-dominate plants, the flowering is not only stimulated by the length of photoperiod but also the intensity of the light (Quedado and Friend, 1978). The flowering of *Saxifraga rivularis* L., a high arctic plant, requires nearly continuous light exposure (23 hours or more) with 0.3 ly min^{-1} irradiance in the growth chamber; while a daily darkness longer than six hours or a daily low intensity irradiance (0.1 ly min^{-1}) was found to prevent the flowering (Teeri, 1974). Inductive photoperiods must be maintained for a continuous 7-9 days to accomplish complete the induction of *Saxifraga rivularis* L. (Teeri, 1976). It seems likely a supply of assimilates from either current photosynthesis or the stored assimilates is prerequisite for the flower initiation (Thomas and Vince-Prue, 1997). High intensity light supplementary may response or affect the phytochrome action or the biosynthesis of the substances that promoting flower (Smith, 1975). Low light intensity can be compensated with sucrose or glucose in some long-day plant. However, the irradiance dependence/effect cannot be totally substituted by external energy (Brulfert et al. 1985).

Photoperiod influence and regulate plant development in both vegetative and reproductive growth, such as formation of storage organs, leaf development, flower initiation and seed germination (Thomas and Vince-Prue, 1984). Knight (1955) reported that optimum seed production of Dallisgrass (*Paspalum dilatatum* Poir.) occurred at 14h photoperiod with a high night temperature of 18.3 to 21.1˚C. No seed formed under 8h photoperiod, and erratic seed

heads and incomplete flowering were observed under12h photoperiod (Knight, 1955). Photoperiod studies conducted with *Kalanchoe* spp. (*K. glaucescens, K. manginii, and K. uniflora*) indicated that less than 12h photoperiod result in a increasing of flower with fewer nodes (Currey and Erwin, 2010). Slafer and Rawson (1996) reported increases in both photoperiod and temperature reduced time to heading of wheat. Most cool-season perennial grasses, such as perennial ryegrass (*Lolium perenne L.*), have a dual induction requirement for flowering (Heide, 1994). Such plants must undergo a low temperature effect before they respond to their critical day lengths and flower. After primary induction by exposure of the plants to low temperature (vernalization) and/or short days, secondary induction can occur by shifting the plant to long days and higher temperature environment (Evans, 1964; Heide 1994; Ofir and Koller, 1972).

Cyril et al. (2002) conducted a cold tolerance experiment for seashore paspalum using 28°C/22°C (day/night) at 250µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) over a 10 h photoperiod as optimum growth condition for seashore paspalum. However, no research has been done to determine the critical photoperiod of seashore papalum.

Pollen release

Due to the random nature of pollen travel, large quantities of pollen are needed to achieve effective pollination for most wind-pollinated species. Once the pollen grains are produced, pollen sac dehiscence, pollen dispersal, and deposition are impacted by environmental factors (Laaidi, 2001; Mesa et al., 2005). The time of pollen release, quantity or density of the pollen, and the impact of environmental factors on pollen viability and longevity are all important aspects to investigate for a better understanding of the reproductive behavior of seashore

paspalum.

The effect of environmental factors on pollen release and pollen density has been studied for numerous species. Most pollen release and pollen density related studies use Hirst-style volumetric spore traps to estimate airborne pollen concentrations while Tauber traps are routinely used to measure pollen deposition (Levetin et al., 2000). The Hirst-style volumetric spore trap (Gregory, 1973; Hirst, 1952) is a suction device in which an adhesive tape is mounted on a drum. Airborne particles which adhere to the tape while the air is drawn in at 10 liters min^{-1} are analyzed to estimate the concentrations, size, and composition of the airborne particles. Tauber traps are sedimentary samplers that are often used for to estimate long-term sedimentation of airborne particles (Moore et al., 1991). By conducting experiments in six locations in southern Spain and the United Kingdom, rainfall and maximum temperatures were observed by Sánchez-Mesa et al. (2003) as important factors that control the amount of grass pollen release. The total counts for grass pollen were from 1000 to over 8000 pollen m^{-3} during the pollen season and high daily pollen counts were observed with lower daily average temperature (Schäppi et al., 1998). The densities of grass pollen in the atmosphere were variable from day to day and season to season as well as year to year in response to a series of meteorological factors (Ong et al., 1995; Schäppi et al.,1998;). Sen and Adhikari et al. (2003) showed bimodal diurnal periodicity of pollen release (0800 h to 1200 h and 1400 h to 1600 h) in rice with a daily range of 0 to 386 pollen m^{-3} and a monthly average pollen concentration of 95 pollen m^{-3}.

Viability and longevity of pollen

Viability and longevity are two important parameters to evaluate the functionality of

pollen. Pollen viability has been defined and determined by many different approaches. Pollen grains were considered as viable if they are capable of being stained by certain vital stains (Rodriguez-Riano and Dafni, 2000), germinate *in vitro* (Stanley and Linskens, 1974), germinate on the stigma (Sun et al., 1991), and effectively participate in pollination that results in seed set (Cruzan, 1990). Pollen longevity was defined by Stanley and Linskens (1974) as "in the period in which pollen is viable". However, Kumar et al. (1995) for practical concerns defined "pollen viability duration" as duration that pollen grains remain more than 50% viable. Assessment of initial pollen viability and longevity is also crucial for understanding the progression of pollination and seed set of a plant (Stone et al., 1995). Numerous experiments of pollen viability and longevity have been completed for different purposes, such as crop/fruit development in breeding programs (Aylor, 2004; Luna et al, 2001; Khan and Perveen, 2009; Song et al., 2001; Tuinstra and Wedel, 2000), incompatibility and fertility studies (Huang et al., 2004), and pollen dispersal and gene flow (Fei and Nelson, 2003; Halsey et al., 2005; Kang et al., 2009; Wang et al., 2004).

Pollen viabilities of grass species, such as tall fescue, perennial ryegrass, zoysiagrass, and creeping bentgrass were reported to range from of 65% to over 90% (Ahloowalia, 1973; Fei and Nelson, 2003; Kang et al., 2009; Teare et al. 1970; Wang, et al, 2004). However, pollen viability studies have not been done with seashore paspalum. Short pollen longevities have also been demonstrated with zoysiagrass, switchgrass, bentgrass and tall fescue; most of these species have only a few hours longevity (Fei and Nelson, 2003; Ge et al., 2011; Kang et al., 2009; Wang, et al., 2004). Fei and Nelson (2003) examined pollen that was collected between 1000 and 1100 hours from individual plants of 'Crenshaw' creeping bentgrass and reported that pollen from different progeny differed in initial germination rate as well as in longevity. Pollen viability of creeping

grass rapidly decreased within 1.5 hours and completely lost viability after 3 hours storage. The environmental impact on pollen viability, such as temperature, ultraviolet-B irradiation (UV-B), and relative humidity have been studied for many crops including members of the Poaceae family. Pollen longevity tests for zoysiagrass indicated that the germination rates under natural conditions (>25˚C) dropped to 1% within 40 min; whereas the germination of pollen under 25˚C conditions dropped to 25% at 60 min and germination ability was not completely lost until after 180 min (Kang et al., 2009). High temperature (36˚C and 40˚C) and high levels UV-B irradiation (900-1500 μW cm^{-2}) reduced pollen viability, while relative humidity did not influence the pollen viability of tall fascue (Wang et al., 2004). Ge et al. (2011) reported similar results with switchgrass. Pollen viability of switchgrass was negatively impacted by high temperatures and high UV-B irradiation. Relative humidity had only limited effects on the pollen viability.

Viability test methods

Various *in vivo* and *in vitro* methods have been developed for evaluating pollen viability. These include *vital* stains, germination tests, stigma staining, and seed set. Currently there is no standard method that can be applied to all crops. Development of a suitable method for the crop being studied is often accomplished by adjusting techniques that have been applied for other species. Seed set is the most accurate *in vivo* method to test the pollen viability (Smith-Huerta and Vasek, 1984). Successful seed set indicates pollen viability; however, lack of seed set does not necessarily mean the pollen is not viable. The seed set method is time consuming and only evaluates pollen viability at a qualitative level. In contrast, *in vitro* measurements are fully quantitative and take less time (Dafni and Firmage, 2000). Vital staining using Alexander's stain, aniline blue, X-gal, Baker's reagent, fluorochromatic reaction (FCR), 2,3,5-triphenyl tetrazolium chloride (TTC) or 2,5-diphenyl monotetrazolium bromide (MTT), a solution of

iodine and potassium iodide (IKI), Evan's blue and others can provide an expedient method to determine the viability of cells or tissue (Table 2-1). Vital staining is not always accurate since some stains also dye aborted pollen as well (Rodriguez-Riano and Dafni, 2000). Vital stains usually determine live pollen by indicating the presence of cytoplasm or enzyme activity. Plant species vary in reaction to these stains; hence, there is not one universal stain for all the plant species. Limited information is available regarding suitable pollen staining methods for seashore paspalum, however, several stain techniques have been used for other turfgrass species. Wang et al. (2004) used X-gal, aniline blue, TTC, MTT, Lugol solution, and FDA for evaluating pollen viability of tall fescue. Unfortunately, none of these were effective in distinguishing viable and non-viable pollen. In another study, both fresh and heat-killed zoysiagrass pollen were stained by using IKI and TTC (Kang et al., 2009). MTT and peroxidase have been suggested for use since they do not normally dye dead pollen (Rodriguez-Riano and Dafni, 2000).

Another commonly used *in vitro* method for measuring pollen viability is the pollen germination test. Pollen germination medium composition depends on the species to be studied. Optimum artificial media (solid) has been successfully established for several *Poaceae* species including Kentucky bluegrass (Teare et al., 1970), tall fescue (Wang et al., 2004), zoysiagrass (Kang et al., 2009), and creeping bentgrass (Fei and Nelson, 2003). Optimization of medium specifically for evaluation of seashore paspalum pollen has not been done. In most cases, a simplified germination medium consisting of sucrose, boron, and calcium is sufficient for assessing pollen viability (Kearns and Inouye, 1993; Steer and Steer, 1989). Sucrose serves as an energy source and also aids in balancing the osmotic potential. Boron supports sugar uptake and maintains cell membrane function and pollen tube wall synthesis. Calcium assists in pollen germination, tip growth, pectin synthesis, and control of osmotic conditions (Khatun and

Flowers, 1995; Kearns and Inouye, 1993; Steer and Steer, 1989). Viability tests for tall fescue pollen conducted at 24 °C in the dark showed the highest level of germination on medium with 0.8 mol L^{-1} sucrose, 1.28 mmol L^{-1} boric acid (H_3BO_3), and 1.27 mmol L^{-1} calcium nitrate (Wang et al., 2004). Kang et al. (2009) found that medium containing 20% (w/v)(0.58 mol L^{-1}) sucrose and 50 ppm H_3BO_3 performed best for zoysiagrass pollen under fluorescent light at 25°C. For creeping bentgrass, the highest pollen germination percentage was obtained on a medium containing 1.0 mol L^{-1} sucrose, 1.0 mmol L^{-1} H_3BO_3, and 2.0 mmol L^{-1} $CaCl_2$ after 30 min germination under room temperature (Fei and Nelson, 2003).

Sexual compatibility

The self-incompatible (SI) system is widespread in plants and plays a major role for successful evolution of flowering plants (Brewbaker, 1957). SI mechanisms help prevent inbreeding, hence promote outcrossing bringing more genetic variability to the population (Brewbaker, 1957). Without this genetic variability, a species does not have the resources to adapt to new and changing growing conditions. Inbreeding reduces variation in the genome making plants less fit when challenged with environmental stresses. To avoid inbreeding, plants have developed several physical and genetic controls to prevent self-fertilization (Darwin,1877; Darwin, 1878)

Self-incompatibility systems can be classified as homomorphic or heteromorphic based on the morphological similarity (De Nettancourt, 1977; Lewis, 1949). Heteromorphic incompatibility is based on the different length of anthers and pistils which increases the distance between male and female floral parts in bisexual flowers thus prevents self-pollination. Homomorphic means all flowers have exactly the same structure which does not help to avoid

self-fertilization by morphological differences. Self-incompatibility of homomorphic plants is controlled by genetic mechanisms. Homomorphic SI can be further divided into two categories, gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI). Gametophytic self-incompatibility is widespread and has been found in *Solanaceae, Rosaceae, Ranunculaceae, Leguminosae, Plantaginaceae, Fabaceae, Onagraceae, Campanulaceae, Papaveraceae and Poaceae*. In contrast, sporophytic self-incompatibility is less distributed and is known to occur in the families of *Brassicaceae, Asteraceae, Convolvulaceae, Betulaceae, Caryophyllaceae, Sterculiaceae and Polemoniaceae* (Brewbaker, 1957; Franklin, et al.1995; Goodwillie, 1997; Igic and Kohn, 2001).

In the GSI system, SI is controlled by the genotype of the pollen, while in the SSI system SI is conditioned by the genotype of the pollen's parent (Hiscock, 2002). This type of SI relies on mechanisms involving protein to protein interactions between pollen and stigma (McClure and Franklin-Tong, 2006; Nasrallah and Nasrallah, 1993). Each SI system is controlled by a single locus known as the S-loci which may include numerous alleles (De Nettancourt, 1977; Newbigin et al., 1993; Ockendon, 1974). Currently the mechanisms for SI systems in *Brassicaceae, Solanaceae,* and *Papaveraceae* have been most extensively addressed (Takayama and Isogai, 2005).

Seashore paspalum has been reported as a sexually reproducing self-incompatible diploid species (Duncan and Carrow, 2000; Quarin, 1992). Studies of the self-incompatibility in *Paspalum simplex, Paspalum chaseanum* and *Paspalum plicatulum* indicted these species were highly self-incompatible but cross fertile (Espinoza and Quarin, 1997). However, no *in vitro* experiment has been done on seashore paspalum to reveal the nature of self-incompatibility or to

determine the extent of cross-compatibility among different genotypes within the species. Seed set provides the most convincing evidence for the self-incompatibility/compatibility.

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Table 2-1. Vital stains for evaluating pollen viability.

Vital stain	Detection target
Alexander's stain (Alexander, 1969)	Intactness of plasma membrane
Aniline blue (Hauser and Morrison, 1964)	Callose in pollen walls and pollen tubes
Baker's reagent (Dafni, 1992)	Alcohol dehydrogenase activity
Evan's blue (Taylor and West, 1980)	Intactness of plasma membrane
Fluor chromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison and Heslop-Harrison, 1992)	Intactness of plasma membrane and the activity of esterase
Tetrazolium dyes 2,3,5-triphenyl tetrazolium chloride (TTC) or 2,5-diphenyl monotetrazolium bromide (MTT) (Shivana and Johri, 1989; Norton, 1966)	Dehydrogenase
X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) (Trognitz ,1991; Singh et al., 1985)	Galactosidase

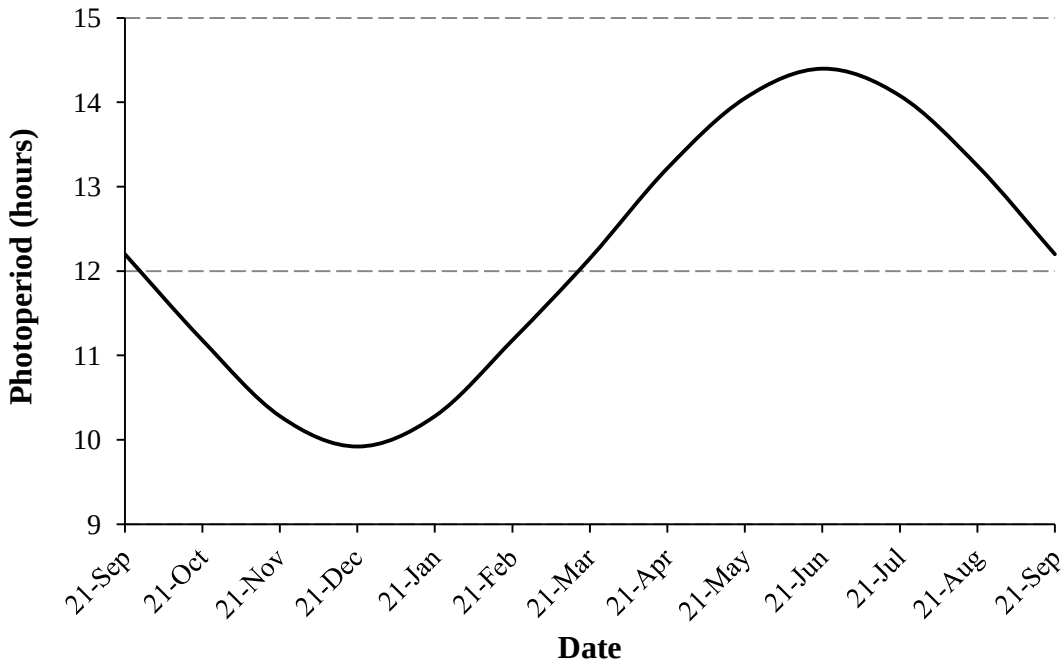

Fig 2-1. Seasonal changes in photoperiod at 33° 14' 48" N/84° 15' 51" W (Griffin, GA).

CHAPTER 3

METHODS FOR TESTING POLLEN VIABILITY OF SEASHORE PASPALUM

(*Paspalum vaginatum* Sw.) [1]

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ABSTRACT

Better knowledge of the reproductive behavior of seashore paspalum is needed for developing new seeded cultivars. Development of methods for evaluating pollen viability is part of the parcel of essential tools necessary to further our understanding of the pollination biology of plants. Four different approaches for pollen viability testing were used to determine the viability of seashore paspalum pollen. Four vital stains were evaluated for their potential to detect viable pollen. An *in vitro* liquid germination medium consisting of sucrose, calcium, and boron was evaluated to assess pollen function. *In vivo* pollinations and fluorescence microscopy using aniline blue staining were utilized to observe pollen tubes development within the stigma and style. A seed set test was conducted by making reciprocal crosses of 'SeaIsle 1' and 'SeaIsle 2000' to qualitatively confirm the viability of seashore paspalum pollen. Vital stains have potential to provide a quick assay for identifying viable pollen; unfortunately, none of the vital stains used in this experiment could effectively estimate the viability of seashore paspalum pollen. A germination medium was optimized for seashore paspalum consisting with 21% (w/v) sucrose, 0.62 g L^{-1} $Ca(NO_3)_2 \cdot 4\ H_2O$, and 0.24 g L^{-1} H_3BO_3. This medium allowed maximum pollen germination with little pollen tube rupture. Examination of pollinated and stained stigmas showed that pollen readily germinated and grew throughout the length of the style. Seed set from reciprocal crosses of SeaIsle 1 × SeaIsle 2000 and SeaIsle 2000 × SeaIsle 1 averaged 46.6% and 42.6%, respectively. *In vivo* pollen tube staining and seed set methods were reliable as a qualitative confirmation of pollen viability of seashore paspalum. Our results indicated that pollen germination tests using liquid germination medium can provide accurate and consistent quantitative estimates of pollen viability.

Abbreviations: TTC, 2,3,5-triphenyl tetrazolium chloride; FDA, fluorescein diacetate. MTT, 2,5-diphenyl monotetrazolium bromide; IKI, iodine and potassium iodide; K_3PO_4, tripotassium phosphate.

INTRODUCTION

Seashore paspalum (*Paspalum vaginatum* Sw.) is a wind-pollinated perennial warm-season grass that is widely distributed worldwide from tropical to warm temperate regions (Morton, 1973). As an emerging turfgrass, seashore paspalum has been successfully used on golf courses, athletic fields, and home lawns to enhance the aesthetic value as well as for soil stabilization and site reclamation (Duncan and Carrow, 2000). Most commercial cultivars of seashore paspalum are vegetatively propagated. Only one seeded cultivar of seashore paspalum, 'Sea Spray', is currently marketed (Fricker et al., 2007). A better understanding of the reproductive behavior of seashore paspalum is needed for the development of additional seeded cultivars. Various techniques for determining pollen viability have been used as essential tools in the study of reproductive biology of plants. Knowledge gained through these techniques has been used to improve plant breeding strategies (Aylor, 2004; Khan and Perveen, 2009; Song et al., 2001), determine fertility and compatibility (Huang et al., 2004), and study pollen dispersal and gene flow (Fei and Nelson, 2003; Halsey et al., 2005; Kang et al., 2009; Wang et al., 2004).

Pollen viability has been defined and determined using many different approaches. Pollen grains were considered as viable if they were capable of being stained by certain vital stains (Rodriguez-Riano and Dafni, 2000), germinate *in vitro* (Stanley and Linskens, 1974), germinate on the stigma (Sun et al., 1991), and effectively participate in pollination that results in seed set (Cruzan, 1990). The choice of methods used depends upon the crop under study and the focus of the study.

Vital stains such as aniline blue, 2,3,5-triphenyl tetrazolium chloride (TTC), fluorescein diacetate (FDA) and Evan's blue have the potential to provide a quick assay of pollen viability based on their ability for indicating the integrity of cytoplasm membranes or enzyme activity. Aniline blue-lactophenol staining solution notes the callose in the living pollen wall and pollen tubes (Hauser and Morrison, 1964). Tetrazolium chloride (i.e.,TTC) is a redox indicator of the presence of dehydrogenases which are enzymes that are essential for metabolism of living tissue (Norton, 1966; Khatun and Flowers, 1995). Fluorescein diacetate was used to confirm the integrity of the plasma membrane and the presence of esterase (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984; Khatun and Flowers, 1995; Evan's blue detects the intactness of the plasma membrane (Taylor and West, 1980). Vital stains have been used in attempts to evaluate pollen viability of tall fescue (*Festuca arundinacea* Shreb.) and zoysiagrass (*Zoysia japonica* Steud.) (Kang et al., 2009; Wang et al., 2004).

In vitro pollen germination assays are alternative methods to assess pollen viability and can provide added information on pollen function. A simplified germination medium consisting of sucrose, boron, and calcium is usually sufficient for assessing pollen viability for most species (Steer and Steer, 1989; Kearns and Inouye, 1993). Germination medium has been used successfully to estimate pollen viability in many turfgrass species including tall fescue, ryegrass (*Lolium spp.*), creeping bentgrass(*Agrostis stolonifera* L.), Kentucky bluegrass (*Poa pratensis* L.), and zoysiagrass (Ahloowalia, 1973; Fei and Nelson, 2003; Kang et al., 2009; Teare et al. 1970; Wang et al., 2004). The germination media established for these grasses were all solid medium with agar or agar substitute in Petri-dish plates.

Stigma staining is a commonly used technique for *in vivo* germination tests that estimates the capability of pollen to germinate on the stigma (Sun et al., 1991; Yi et al., 2006). Aniline

blue has been used to assess pollen tube growth within the style; when viewed under ultraviolet light, aniline blue illuminates callose plugs in pollen tubes (Dumas and Knox, 1983; Yi et al., 2006). Pollen viability and function can be determined using aniline blue stain which allows visualization of pollen germination and pollen tube growth within the stigma and style (Martin, 1959).

Seed set is considered as the most accurate *in vivo* method to test the pollen viability (Shivanna and Johri, 1989; Stanley and Linskens, 1974). An effective seed set certainly indicates pollen were viable. However, seed set determinations are extremely time consuming. Furthermore, results can be influenced by self-incompatibility, other pollen-pistil interactions, or pollen source effects.

Suitable methods for estimating pollen viability differ depending on the crop under investigation. There are no published reports that discuss pollen viability techniques suitable for use on seashore paspalum. The objective of this study was to evaluate and adapt existing methods for assessing pollen viability of seashore paspalum.

MATERIALS AND METHODS

Plant material

Two commercial cultivars ‘SeaIsle 1’, ‘SeaIsle 2000’ and two experimental breeding lines from The University of Georgia turfgrass breeding program, SIPV15-2 and 05-202B-20 were used as pollen sources of seashore paspalum for the different viability tests conducted in this study. Plant materials were established clonally from stolon nodes. Plants were maintained under greenhouse growth conditions (28 ±5/20 ±5˚C, day/night) with natural day length. Plants

were irrigated twice daily and fertilized monthly with a water soluble N-P-K (28-7-14) fertilizer (LESCO MacroN, Cleveland, Ohio). All experiments were conducted from June to September in 2010, during the most vigorous flowering period of seashore paspalum in Griffin, Georgia. Pollen samples were collected from fresh flowers in the early morning after sunrise, generally between 0630 h to 0830 h.

In vitro germination tests

Pollen from two genotypes, SIPV15-2 and 05-202B-20 were used in experiments aimed at optimizing germination medium for testing pollen viability of seashore paspalum. Preliminary tests using liquid germination media consisting of 0.62 g L^{-1} $Ca(NO_3)_2.4H_2O$, 0.24 g L^{-1} H_3BO_3 and with sucrose concentrations of 5, 10, 12, 15, 20, 25, or 30% (w/v) were conducted. Based on the results of the preliminary test, secondary tests were conducted to further refine the optimum sucrose concentration. The procedure was modified from the pollen germination test for almond pollen (Yi et al., 2003). Pollen was collected onto glass Petri dishes at 0730 h. Pollen grains were immediately dispensed onto 200 µl of germination medium into microplate wells (BD Falcon™, BD Biosciences, Franklin Lakes, NJ) using a fine texture brush in adequate amounts to provide a single layer of pollen grains in the bottom of the wells. After 60 minutes incubation at room temperature (22°C), 20 µl of fixative (HistoChoice, Amresco, Solon, Ohio) was added to each well to halt pollen tube development. An inverted microscope (Fisher Scientific, Pittsburgh, PA) was used to examine pollen germination under 20X magnification. Pollen grains were considered as germinated if the pollen tubes extended longer than the diameter of the pollen grain (Tuinstra and Wedel, 2000). Each sucrose concentration of both genotypes was replicated three times and 100 pollen grains were counted for each replication. Observations regarding the intactness and rupture of the plasma membrane of pollen tubes were also noted. Data from the experiments

described above were analyzed as a split plot design with pollen sampling dates serving as replications, genotypes as main plots, and sucrose levels as sub-plots. Genotypes and sucrose treatment effects were tested using the genotype × sucrose interaction mean square as the error term (Table 3-1).

Vital stains

Pollen collected from SeaIsle 1 and SeaIsle 2000 was used for a series of vital staining tests. Four vital stains (i.e., Aniline blue-lactophenol, FDA, TTC, Evan's blue) were selected to evaluate their abilities to assess pollen viability of seashore paspalum at room temperature (22°C).

Newly emerged anthers were collected onto glass Petri dishes in the early morning around 0730 h. Pollen grains from each sample were collected and dispensed onto a microscope slide in a drop of staining solution and followed with a cover slip. Slides were examined at 40X magnification using a light microscope (Olympus BX 60F5, Olympus, Pittsburgh, PA). Different filters and transmitted or fluorescent light were used as required by some staining techniques. Three slides were made for each staining method. A total of 100 pollen grains were counted from each slide and each staining test was repeated three times on different dates.

Aniline blue-lactophenol staining solution consisted of 0.05g aniline blue, 20 ml phenol, 20 ml lactic acid, 40 ml glycerine, and 20 ml distilled water (Hauser and Morrison, 1964; Wang et al., 2004). Wet mounts of pollen grains in aniline blue staining solution were held for 15 min prior to examining assessments. Pollen grains that stained blue were counted as viable. A 1% solution of Evan's blue was used to detect the intactness of the plasma membrane. Pollen grains were stained for 15 min before being examined. Non-stained pollen was considered as viable.

For viability staining using FDA, a slight modification of Heslop-Harrison and Heslop-Harrison (1970) was used. A stock solution was made by dissolving 2 mg fluorescein diacetate in 1 ml acetone. The FDA-sucrose working solution used for pollen staining was made by adding FDA stock solution dropwise, into a 21% (w/v) sucrose solution until a persistent milkiness appeared. Pollen grains were stained with FDA working solution for 10 min and examined under a fluorescence microscope using a U-MNIBA2 filter (Olympus, Pittsburgh, PA). Pollen exhibiting a green fluorescence was viable. Slides were examined 10min after the staining. For staining using TTC, a 1% solution was used to stain pollen for 30 min prior to observation under a light microscope. Deep pink or brick-red stained pollen grains were counted as viable.

The pollen viability of SeaIsle 1 and SeaIsle 2000 was also evaluated using the optimized liquid germination media discussed above. The results of the vital staining techniques were compared with results obtained using liquid germination medium for their ability to assess pollen viability. Data collected from these tests were analyzed as a split plot design with methods as main plots, genotypes as sub-plots and pollen sampling dates serving as replications.

In vivo germination tests

Pollen tube growth within styles was also determined by staining the hand pollinated pistils with aniline blue stain in order to visualize pollen tube growth within the stigma and style (Martin, 1959). Whole racemes with emerging florets of SeaIsle 1 and SeaIsle 2000 were collected separately in the early morning around 0730 h. At the same time, anthers with fresh pollen from SeaIsle 1 and SeaIsle 2000 were also collected separately. Florets were carefully emasculated before the anthers emerged. Emasculated florets of SeaIsle 1 and SeaIsle 2000 were then immediately hand-pollinated reciprocally with fresh pollen of the other parent. Pistils were

removed and fixed two hours after hand-pollination. The staining procedure was as described by Yi et al. (2006) with slight modification. Pistil tissues were fixed with 3:1 (v/v) 95% ethanol: glacial acetic acid for three hours to preserve the development of the pollen tubes. Then tissues were softened and cleared by autoclaving 15 min in 10% sodium sulfite solution. Pistils were then stained with aniline blue staining solution (0.01% aniline blue in 0.1 M K_3PO_4) overnight mounted on slides, then examined using an Olympus BX51 light microscope (Olympus America, Center Valley, PA) under ultraviolet fluorescent light to determine the extent of pollen tube development within the style. Pollen grains were considered as germinated if the pollen tube extended longer than the diameter of the pollen grain (Tuinstra and Wedel, 2000). Ten pistils from each cross were mounted and examined.

Seed set by hand-pollination

Ten pollinations each of SeaIsle 1 × SeaIsle 2000 and SeaIsle 2000 × SeaIsle 1 (reciprocals) were made by hand in the greenhouse for a total of 20 cross pollinations. Potential flowering racemes were covered with transparent polypropylene tubes (55 × 12 mm) (Sarstedt, Nümbrecht, Germany) prior to stigma emergence to prevent pollen contamination. Pollen collected from the pollen parent around 0730 h was immediately dispensed onto the stigmas of the female parent with a small artist brush. Freshly pollinated flowers were then immediately re-covered with the tubes for seven days. Mature racemes were carefully harvested 28 days after pollination. The number of florets present and seeds produced on each raceme were recorded. Percentage seed set was calculated as the (number of seed set)/(number of pollinated florets) × 100.

Statistical analysis

Data collected from each experiment were analyzed using SAS (SAS Institute, Cary, North Carolina). Analysis of variance was performed using the general linear model routine and subsequent statistical separation of treatment means was carried out using pairwise t-tests equivalent to Fisher's least significant difference (LSD) test at $\alpha=0.05$.

RESULTS

In vitro germination test

Results of the preliminary tests for optimizing the sucrose concentration of pollen germination medium showed that sucrose concentrations of 15% and below resulted in cytoplasmic leaking (Fig 3-1a) while sucrose concentrations of 25% and higher inhibited pollen germination (Fig 3-1b). Among the sucrose concentrations tested, 20% was identified as the best (Fig 3-1c). Fig 3-1d shows germination of an individual pollen grain with an intact pollen tube at the early extension stage. Based on these results, additional experiments to further optimize sucrose concentration was conducted using sucrose levels of 19, 20, 21, and 22 %. Results of the analysis of variance are presented in Table 3-1. Pollen germination was significantly impacted ($p < 0.05$) by sucrose level. No statistically significant differences in pollen germination were observed between pollen sampling dates or genotypes tested. The pollen viability of 05-202B-20 on 21% sucrose concentration is significant higher than on germination medium with 19, 20, and 22 % sucrose. While for SIPV15-2, no differences among 19, 20 and 21% and pollen incubated on 19, 20 and 21% sucrose germination medium has a higher percentage of germinated pollen than on 22% sucrose (Table 3-2).

When the data were combined over genotypes, no significant difference was observed in pollen germination among 19, 20, 21% sucrose treatments (data not shown). However, visual observations indicated that pollen tubes of both genotypes were most intact when germinated on media with 21% sucrose. Hence, an optimum germination medium consisting of 0.62 g L^{-1} $CaN_2O_6.4H_2O$, 0.24 g L^{-1} H_3BO_3 and 21% sucrose was selected as for evaluating pollen viability of seashore paspalum. Pollen germination rates of 65% to 93% were observed on the selected medium at room temperature. Most pollen grains germinated within 10 min of incubation.

Vital stains

The four vital stains tested produced very divergent values for pollen viability of seashore paspalum. TTC did not stain any of the pollen (indicating no viable pollen) while aniline blue stained all pollen grains (indicating 100% viable pollen). Hence, TTC and aniline blue were not able to distinguish between viable and non-viable pollen and results from these treatments were excluded from data analysis. Pollen viability estimates from FDA, aniline blue, and germination medium were compared using analysis of variance and subsequent mean separation tests. The summary of the combined analysis of variances are presented in Table 3-3. Estimates of pollen viability varied with sampling date ($p < 0.01$), method ($p < 0.001$), and genotype ($p < 0.01$). When averaged across all methods, pollen collected from SeaIsle 1 had higher estimates of viability than pollen from SeaIsle 2000. The method by genotype interaction was not significant. Data were also analyzed separately for method effects within each genotype (Fig 3-2). Estimates of pollen viability obtained using germination medium were highest while viability estimates obtained using Evan's blue were lowest in both genotypes.

In vivo germination test

Observations of the stained stigmas showed that in crosses of both SeaIsle 1 × SeaIsle 2000 (Fig 3-3) and SeaIsle 2000 × SeaIsle 1 (not shown), pollen was able to adhere to the stigma papillus, hydrate, germinate, extend a tube that entered the style and eventually reach the micropyle of the ovule. Callose plugs formed periodically in the pollen tube while it grew through stylar tissue. In conclusion, pollen of SeaIsle 1and SeaIsle 2000 is functional *in vivo*.

Seed set

Large variations in numbers of seed set within reciprocal crosses of SeaIsle 1 and SeaIsle 2000 were observed. When SeaIsle 1 was used as the female, percentage of seed set ranged from 25.0 to 66.7% with an average of 46.6 ±15.2%. Seed set varied from 20.0 to 66.7% and average 42.6 ±15.1% when SeaIsle 2000 was used as the female. This large variation in seed set may be attributed to variation in raceme size and/or variation in the determinacy among florets on individual racemes that occurred over the duration of this experiment.

DISCUSSION

Various *in vivo* and *in vitro* methods have been developed for evaluating pollen viability. Suitable methods for the crop being investigated can be developed by adjusting protocols developed for other species.

Vital staining is accepted as a rapid assay to evaluate pollen viability. However, vital stains do not always give reliable results for many plant species (Mulugeta et al., 1994; Rodriguez-Riano and Dafni, 2000). Wang et al. (2004) stained tall fescue pollen with X-gal (5-bromo-4-chloro-3-indoyle-b-galactoside), aniline blue, TTC, 2,5-diphenyl monotetrazolium

bromide (MTT), Lugol solution, and FDA; none of which clearly distinguished viable and dead pollen. Iodine and potassium iodide (IKI), TTC and FDA were used for evaluating pollen viability of zoysiagrass(Kang et al., 2009). In their study, both fresh pollen and heated-killed pollen grains responded similarly indicating those stains are not appropriate for estimating the pollen viability of zoysiagrass. Ge et al. (2011) stained switchgrass pollen with Lugol solution and aniline blue but neither of them could effectively distinguish viable and non-viable pollen. In the current study aniline blue and TTC were not able to distinguish between viable and non-viable pollen. FDA and Evan's blue did produce differential staining and apparently were able to distinguish aborted and non-aborted pollen grains. Problematic and characteristic of vital stains is that pollen grains often exhibit variations in staining intensity. This requires that a staining threshold be defined in order to classify pollen as viable or dead. Enzymatic activity and membrane integrity, which are the bases for most viability stains will vary in degree among viable pollen grains. Some of the pollen grains were "semi-stained". Furthermore, with fluorescence microscopy fading of signal can occur. Fluorescence faded quickly in the FDA test with seashore paspalum which may have affected results. The data presented in Fig 3-2 for FDA and Evan's blue represent the number of stringently-stained versus non-stained pollen grains, while "semi-stained" pollen was not counted. In order to obtain consistent and reproducible results using these methods, defining standards of viability need to be strictly defined. FDA and Evans blue do show utility in evaluating the pollen viability of seashore paspalum. However, estimates of viability using these methods were lower than viability estimates obtained using an optimized liquid germination medium.

Germination medium is the most commonly accepted method and is believed to be the most reliable approach for testing pollen viability (Ahloowalia, 1973; Stone et al., 1995). A

simplified sucrose-boron-calcium germination medium has been successfully used for evaluating the pollen viability for many species. A summary of published literature on pollen germination media used for pollen viability assessment of species closely-related to seashore paspalum is presented in Table 3-4. Previous research indicates that required levels of calcium and boron were similar among species, yet the sucrose levels can vary from 13% to 34%. Sucrose is the essential ingredient and has the most impact on pollen germination. The sucrose concentration varies with species and should be determined empirically. All germination media discussed in Table 3-4 except medium for seashore paspalum were solid medium in Petri dishes. Compared to solid media, liquid media are space and resource saving and most tests can be done in a single microplate. Inoculation of pollen in liquid medium facilitates consistent pollen density, with pollen forming an even layer in each microwell, instead of inoculations with clumps of pollen as often occurs on solid media. The single even layer of pollen creates a clear back ground for microscopic observation of pollen germination and avoids unwanted differences in pollen density or population effects as can occur on solid media. Brewbaker and Majumder (1961) described the "population effect" of pollen germination as when small populations of pollen tend to germinate less than large populations under optium germination conditions. Innoculation of uniform populations of pollen in a single layer is necessary to obtain reproducible results.

In vivo germination test and seed set tests are naturally stimulated and more valid than *in vitro* tests (Shivanna and Johri 1989). Pistil or stigma staining with aniline blue provides more information other than pollen viability, such as pollen-pistil compatibility, pollen vigor, pollen competition, and even the morphology of the pollen tube development. Seed set used for comform the pollen viability, however, it is labor and time consuming (Stone et al., 1995). It is noteworthy that the percentage of seeds set is not indicative of the percentage of pollen viability.

The development of all florets on the same raceme ofter occurs over a two to three day period. Hand pollination should be made on the day when most stigmas have emerged and repeated the next day to ensure late emerging florets are pollinated. Pollen grains of seashore paspalum are very small with diameters of 32 µm on average and the pollen can lose their viability in a very short time. Also, there might have been many pollen garins per ovule, only the most competitive one set seed. For these reasons, it was difficult to quantify the number of pollen grains despensed onto each floret of each raceme. Hence for seashore paspalum, *in vivo* pollen viability tests performed using stigma staining and actual seed set can only be used to qualitatively verify the pollen viability. In contrast, *in vitro* measurements are rapid, easy to deliver, and fully quantitative (Dafni and Firmage, 2000). The results presented in this paper provide several options for pollen viability test. The optimum pollen germination medium was developed as the most suitible and efficeit method for evanluating the pollen viability of seashore paspalum.

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Table 3-1. Analysis of variance of the effects of genotype, media sucrose concentration, and their interaction on seashore paspalum pollen viability.

Source	df	Mean square	Pr > F
Replication/Date (D)	1	764.91	0.3678^{ns}
Genotype (G)	1	915.29	0.3422^{ns}
Error a: D × G	1	325.19	
Sucrose (S)	3	106.00	0.0280*
G × S	3	151.05	0.0061**
Error b	38	31.38	
CV = 7.5%			

*, ** Significant at 0.05 and 0.01 probability levels, respectively.

Table 3-2. Effects of sucrose concentration on germination of pollen from two genotypes of seashore paspalum in boron-calcium-sucrose liquid germination medium[†]

Genotype	Sucrose	Pollen germination	Range of values	SD
	% (v/w)	%	%	
05-202B-20	19	73.94[b]	63.59 - 87.68	±8.55
	20	77.88[ab]	70.83 - 86.96	±2.17
	21	83.80[a]	73.12 - 92.68	±3.99
	22	78.78[ab]	67.28 - 90.85	±4.32
SIPV 15-2	19	73.63[a]	63.69 - 88.89	±10.42
	20	71.93[a]	68.45 - 75.00	±6.23
	21	71.62[a]	65.28 - 76.43	±8.38
	22	62.28[b]	53.92 - 66.06	±11.25

[†]Germination medium consisting 0.62 g L^{-1} $CaN_2O_6.4H_2O$, 0.24 g L^{-1} H_3BO_3 and sucrose.
[a] Values with the same superscript letter within genotypes are not significantly different ($p < 0.05$)

Table 3-3. Analysis of variance of the effects of viability testing method, genotype, and their interactions on estimates of pollen viability in seashore paspalum.

Source	df	Mean square	Pr > F
Replication/Date (D)	2	918.58	0.0033**
Method (M)	2	1816.32	0.0009***
Error a: D × M	4	28.21	
Genotype (G)	1	216.54	0.0029**
M × G	2	23.53	0.3477ns
Model	11	531.48	
Error b	42	21.72	
CV = 7.86%			

, * Significant at 0.01and 0.001 probability levels, respectively.

Table 3-4. Optimum germination medium for testing pollen viability in various grasses.

Variety	Germination medium				Pollen viability	Reference
	Sucrose	H_3BO_3	Ca^{+2}	Gelling agent		
	% (m/v)	mmol L^{-1}	mmol L^{-1}	(m/v)	%	
Tall fescue (*Festuca arundinacea* Shreb.)	27	1.28	1.27 $Ca(NO_3)_2$	1% agar	68-75	Wang et al., 2004
Perennial ryegrass (*Lolium perenne* L.) Italian ryegrass (*Lolium multiflorum*Lam.) Meadow fescue (*Festuca pratensis* Huds.)	27	1.28	1.27 $Ca(NO_3)_2$	1% agar	69-83	Wang et al., 2004
Zoysiagrass (*Zoysia japonica* Steud.)	20	0.81	–	0.3% Phytagel	maximum > 90	Kang et al., 2009
Creeping bentgrass (*Agrostis stolonifera* L.)	34	1	2.0 $CaCl_2$	0.3% Phytagel	< 90	Fei and Nelson, 2003
Kentucky bluegrass (*Poa pratensis* L.)	13	0.081	0.042 $Ca(NO_3)_2.4H_2O$	0.75% granular agar	80-90	Teare et al., 1970
Seashore paspalum (*Paspalum vaginatum* Swa.)	21	3.88	2.63 $Ca(NO_3)_2.4H_2O$	– †	65- 93	
Switchgrass (*Panicum virgatum* L.)	27	1.28	1.27 $Ca(NO_3)_2$	1% agar	70-80	Ge et al., 2011; Wang et al., 2004

†Pollen germination medium for seashore paspalum is a liquid medium.

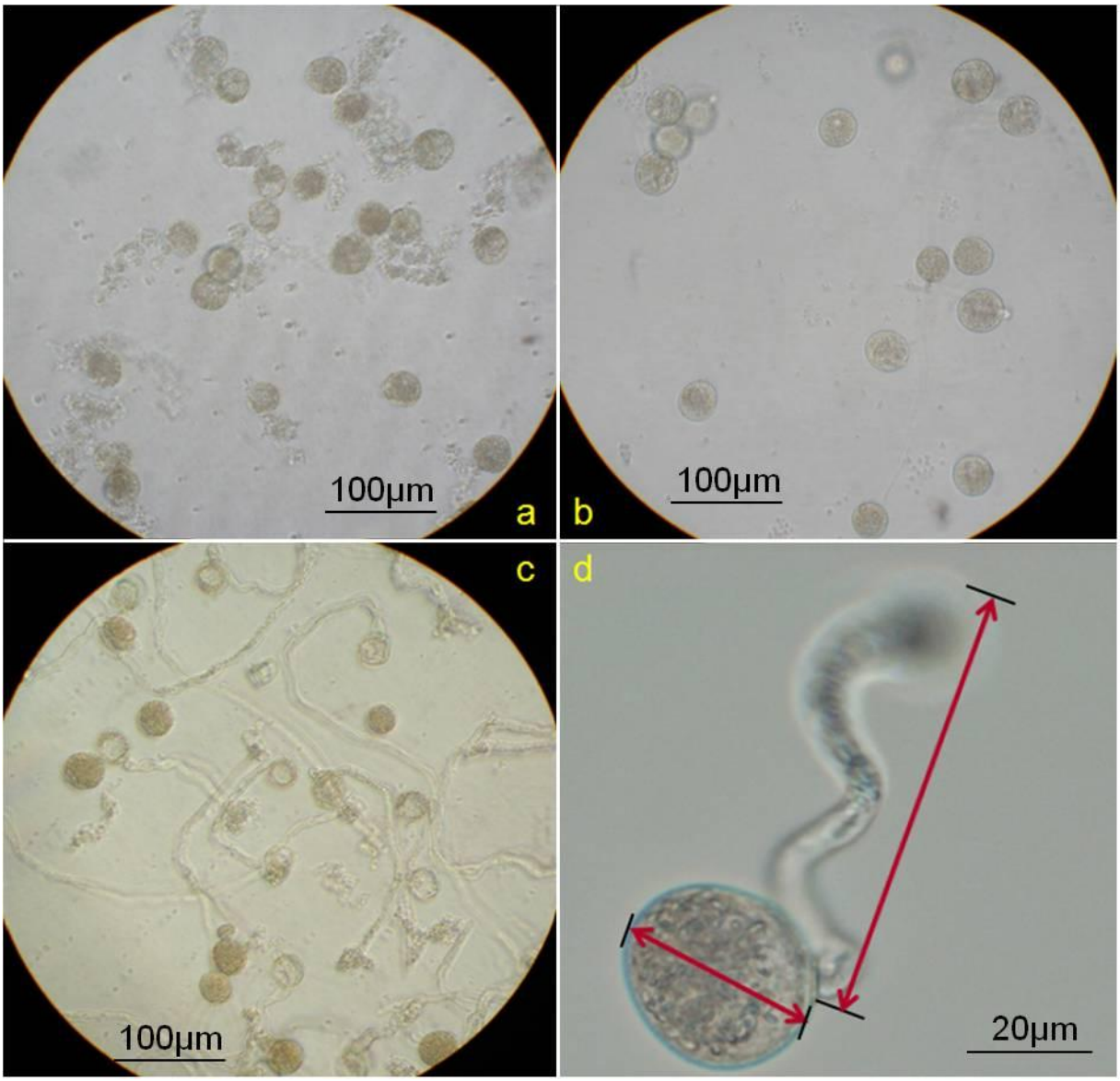

Fig 3-1. Microscopic images of pollen tube growth at different levels of sucrose. (a) Pollen grains ruptured on the germination medium with 10% sucrose. (b) Pollen germination was inhibited on the germiantion medium with 25% sucrose. (c) Twenty percent sucrose, resulted in the highest rate of pollen germination and the lowest rate of ruptured pollen grains. (d) Viable pollen grain germinating with a pollen tube at early extension stage.

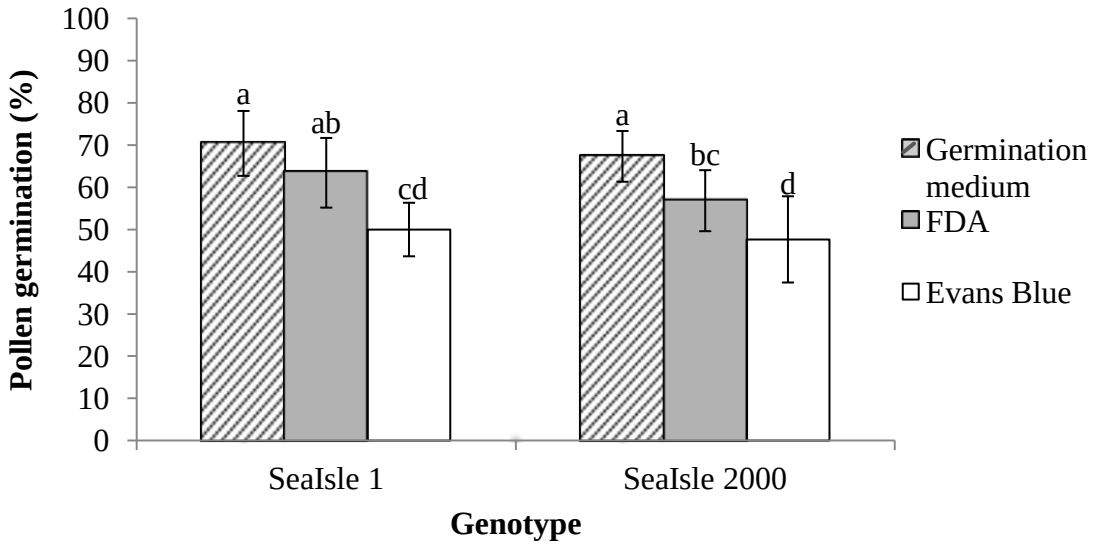

Fig 3-2. Mean pollen viability of two seashore paspalum genotypes estimated using vital stains and germination medium. Means within genotypes followed by the same letters are not considered different according Student's t-test at α=0.05.

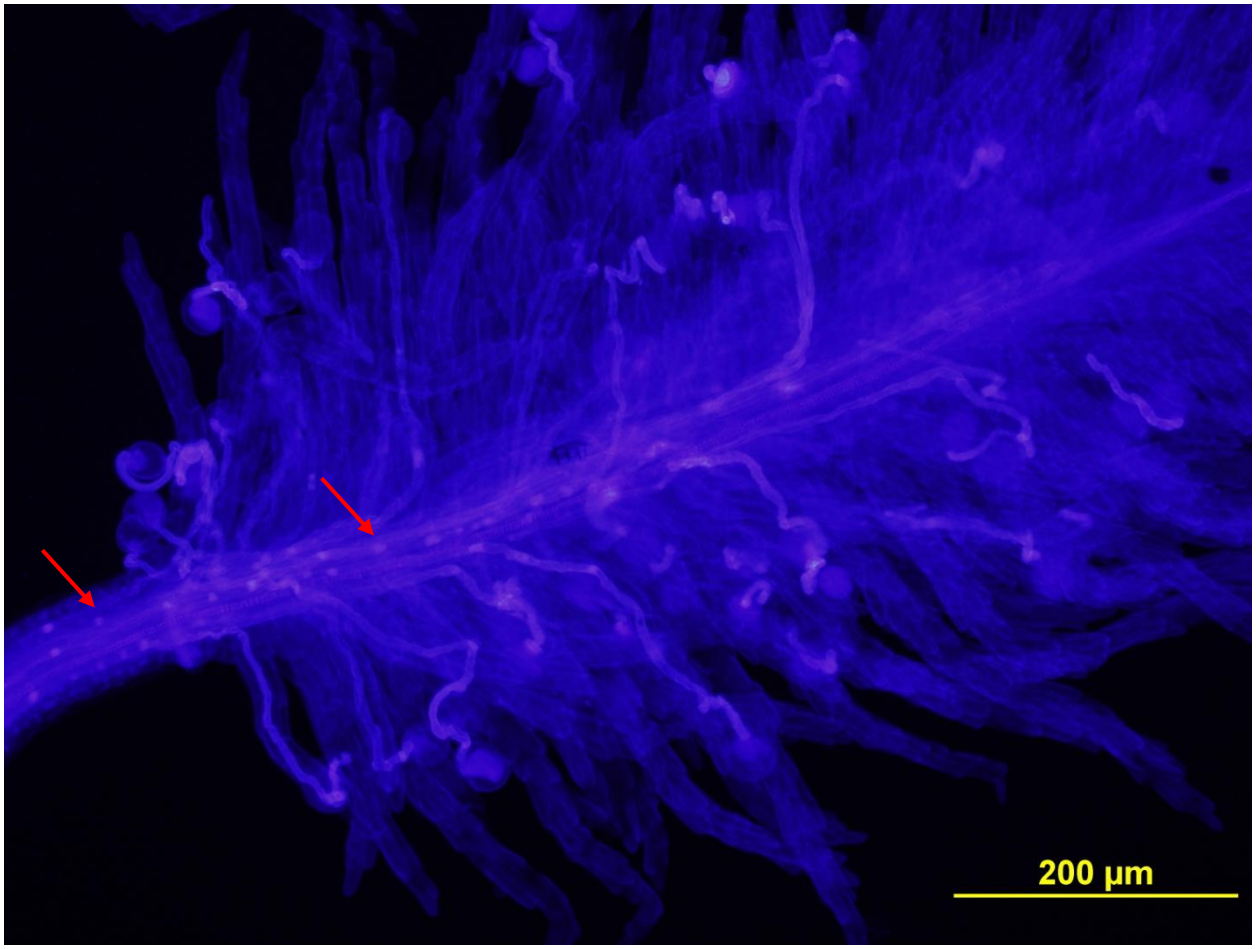

Fig 3-3. Fluorescent photomicrographs of aniline blue staining of a pollinated stigma (SeaIsle 1 × SeaIsle 2000) showing pollen tubes germinating and penetrating the papilla tissue and elongating through the style tissue. Callose plugs (arrows) formed periodically as pollen tubes extended through stylar tissue.

CHAPTER 4

POLLEN RELEASE PATTERN, VIABILITY, AND LONGEVITY OF SEASHORE PASPALUM (*Paspalum vaginatum* Sw.) [2]

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ABSTRACT

A clear understanding of the reproductive biology of seashore paspalum (*Paspalum vaginatum* Sw.) is needed to design improved production systems for seeded cultivars. A series of greenhouse and field studies were conducted to determine the pollen release pattern, viability, and longevity of seashore paspalum. An airborne particle counter was used to monitor pollen release in a field setting for 67 days. Meteorological data collected from a nearby weather station were compared with pollen release data in an attempt to identify environmental factors that influence pollen release. Additionally, pollen viabilities of seven genotypes of seashore paspalum were assessed using i*n vitro* germination medium. Longevity of seashore paspalum pollen at room temperature was determined by testing the germination rate at 20 min intervals over a 180 min period using bulk pollen from both field and greenhouse grown plants. The effect of temperature on pollen longevity was also determined by evaluating the pollen longevity under storage temperatures of 15, 20, 25, 30, 35 or 40˚C. Pollen release generally occurred between 0700 h and 1100 h with a very sharp release peak around 0800 h. The airborne pollen density varied greatly from day to day. Of the 25 meteorological variables studied only solar radiation at 0800 h was found to be associated with total pollen count between 0600 h to 1100 h. Pollen from all seven genotypes tested was viable and viability ranged from 60 to 88% when tested at room temperature. Pollen collected from both greenhouse and field environments had only half-life of 45 and 60 min, respectively, after release. Greenhouse and field-collected pollen failed to germinate after 80 and 100 min, respectively. The longevity of seashore paspalum pollen was dramatically reduced when stored at temperatures of 30˚C and above. Our results indicate that pollen release of seashore paspalum varied greatly from day to day and was released during a very short period in the early morning with an initial viability of 60-88%. Pollen remained viable

for only 60 to 100 min at room temperature and was negatively impacted by temperatures of 30˚C and above.

INTRODUCTION

Seashore paspalum (*Paspalum vaginatum* Sw.) is a wind-pollinated perennial warm-season grass that is widely distributed worldwide from tropical to warm temperate regions (Morton, 1973). It is rapidly gaining popularity for use as a fine turf by the golf industry around the world and has also been successfully utilized for soil stabilization and site reclamation. Seashore paspalum is considered the most salt tolerant warm-season turf grass and has often been used on salt-affected sites and where irrigation with saline or brackish water is necessary (Casler and Duncan, 2003; Duble, 1996). Most commercially available cultivars of seashore paspalum are clonally propagated. Only one seeded cultivar of seashore paspalum, 'Sea Spray', is currently marketed (Fricker et al., 2007). Development of additional seeded cultivars and identification of optimum production regions for seeded cultivars is limited by our lack of understanding of the reproductive biology of seashore paspalum and the environmental interactions that influence seed production. Studies to document pollen release pattern, pollen viability, pollen longevity, and their interactions with environmental parameters are needed to identify factors limiting seed production of seashore paspalum.

Understanding pollen dispersal and the relevance of environmental factors is important for studying the physiological and ecological behavior of a plant. For wind-pollinated species, pollen grains subsequent to anther dehiscence follow the aerobiological processes of emission, dispersion, and deposition which are all influenced by environmental factors (Laaidi, 2001; Mesa et al., 2005). It has been reported that air temperature, relative humidity, solar radiation level,

and wind can impact anthesis and anther dehiscence of grassy species (Emecz, 1962; Liem and Groot, 1973). Low temperatures prior to flowering can result in immature pollen and indehiscence of anthers thus reducing pollen shedding and the number of viable pollen grains (Satake and Hayase, 1970; Tuinstra and Wedel, 2000).

Hirst-style volumetric spore traps have been traditionally used as a measurement tool for most airborne pollen release and pollen concentration related studies (Hirst, 1952). Information regarding air borne pollen/spore concentration can be obtained by analyzing the sticky tapes that mount on the drum in the trap. Pollen release pattern has also been determined by testing changes in pollen germination rates over time (Fei and Nelson, 2003; Kang et al., 2009). Recently, a new type of laser-based automatic particle counter has been used for counting pollen in studies of pollen allergies and transgenic gene flow (Delaunay et al., 2007; Kawashima et al., 2007; Krug et al., 2003). These laser-based particle counters count and categorize airborne particles into size classes as the particles pass through a laser beam. This device provides an opportunity to obtain continuous pollen counts on real time basis.

Viability and longevity are two important parameters used to evaluate the functionality of pollen. One of the approaches used to define pollen viability is the capability to germinate *in vitro* (Stanley and Linskens, 1974). Pollen longevity was defined by Stanley and Linskens (1974) as “the period in which pollen is viable". However, Kumar et al. (1995) proposed that "pollen viability duration" should be defined as duration that pollen grains remain greater than 50% viabile. Pollen viability and longevity of different crops have been evaluated for different purposes, such as plant breeding (Aylor, 2004; Stone et al., 1995; Song et al., 2001), pollen dispersal and gene flow (Fei and Nelson, 2003; Halsey et al., 2005; Kang et al., 2009; Luna et al., 2001; Wang et al., 2004), and incompatibility and fertility studies (Huang et al., 2004). Among

numerous pollen viability evaluation techniques, germination medium has been shown to be a reliable method. It has been used successfully to estimate pollen viability and longevity in many crops, including turfgrass species, such as tall fescue (*Festuca arundinacea* Shreb.), ryegrass (*Lolium spp.*), creeping bentgrass(*Agrostis stolonifera* L.), Kentucky bluegrass (*Poa pratensis* L.), and zoysiagrass (*Zoysia japonica* Steud.) (Ahloowalia, 1973; Fei and Nelson, 2003; Kang et al., 2009; Teare et al., 1970; Wang et al., 2004). Short pollen longevity and negative impact of high temperature on the pollen longevity have also been reported (Ge et al., 2011; Wang et al., 2004).

A series of studies were designed to 1) document the pollen release pattern of seashore paspalum and to determine the impact of major environmental factors on pollen release, 2) estimate pollen viability and longevity, and 3) evaluate the effect of temperature on pollen longevity.

MATERIALS AND METHODS

Plant material

Two commercial cultivars ‘SeaIsle 1’ and ‘SeaIsle 2000’ and five breeding lines (Tyb2, PI 647892, SIPV15-2, 05-202B-20, 05-202D-04) from The University of Georgia seashore paspalum breeding program were used for this series of studies. Both greenhouse and field grown plant material were established clonally from stolon nodes in Griffin, Georgia. Greenhouse grown plants materials were maintained at 28 ±5/20 ±5˚C (day/night) with natural day length. Plants were irrigated twice daily and fertilized monthly with 28-7-14 (NPK) fertilizer (MacroN, Lesco, OH). Field grown plant materials were irrigated to supplement natural rainfall as needed and fertilized every three weeks with 24-4-11(NPK) fertilizer (MacroN, Lesco, OH).

All pollen used in the experiments was collected from June to September 2010 during the most vigorous flowering period of seashore paspalum in Georgia. Pollen samples were collected from fresh flowers in the early morning shortly after sunrise, generally between 0630 h to 0830 h.

Size of seashore paspalum pollen

Pollen grain diameter was determined using pollen from greenhouse grown SeaIsle 1, SeaIsle 2000 and from a mixed stand of field grown SeaIsle 1 and SeaIsle 2000. Fresh anthers with pollen grains were collected onto a glass Petri dish in the early morning around 0730 h. Pollen grains from each sample were mounted onto a microscope slide in a drop of 21% (w/v) sucrose solution. The diameters of pollen grains were measured immediately at 40X magnification using a light microscope with a calibrated optical eyepiece graticule (Fisher Scientific, Pittsburgh, PA). The actual sizes of pollen grains were then calculated according to the calibration table for the microscope. Twenty pollen grains were measured from each sample.

Pollen release pattern

The test field used for monitoring pollen release had an area of approximately 900 m^2 and was located at The University of Georgia Griffin Campus in Griffin, GA. This field was established in 2008 by inter-planting sprigs of SeaIsle 1 and SeaIsle 2000. An airborne laser particle counter (Handheld 5016, Lighthouse Worldwide Solutions, Fremont, CA) located near the center of the test field was used to quantify pollen released on a real-time basis. Prior microscopic measurements of seashore paspalum pollen from multiple cultivars from the field ranged from 27 to 39 µm in diameter. Therefore, the counter was programmed to count particles greater than 25 µm in size as seashore paspalum pollen and record the average number m^{-3} of air sampled over each 10 min period. Data of meteorological parameters were obtained from an

adjacent automated weather station maintained as part of the Georgia Automated Weather Monitoring Network (www.georgiaweather.net) established by The University of Georgia. Pollen release and associated weather data were collected a total of 67 days during the period of July to October, 2010 and 2011. Twenty-five meteorological variables either directly recorded by the weather station or calculated from recorded data, were summarized for each of the 67 days on which pollen release data was collected. Variables included temperature, humidity, dew point, and their changes from hour to hour; as well as wind speed, solar radiation, and rainfall 12h prior to 0800 h. Statistical analysis using Pearson's Product-Moment Correlation was performed to determine if total pollen release from 0600 h to 1100 h had a linear relationship with any of the meteorological variables.

Assessment of pollen viability

The pollen viability of seven genotypes (SeaIsle 1, SeaIsle 2000, Tyb2, PI 647892, SIPV15-2, 05-202B-20, and 05-202D-04) were tested. A liquid germination medium consisting of 21% sucrose, 0.24 g L^{-1} boric acid, and 0.62 g L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ was developed for assessing pollen viability of seashore paspalum. The procedure used was a modification of the pollen germination test used by Yi et al. (2003). Samples of fresh pollen from each genotype were collected in glass Petri dishes around 0730 h. Pollen grains were then immediately inoculated onto 200 µl of germination medium in microplate wells (BD Falcon™, BD Biosciences, Franklin Lakes, NJ) to provide a single layer of pollen grains and incubated at room temperature (22°C). Pollen from each genotype was placed into three separate wells and tests were repeated on two different dates. Twenty µl of fixative (HistoChoice, Amresco, Solon, Ohio) was added to each well after 60 min to halt pollen tube development. An inverted light microscope (Fisher Scientific, Pittsburgh, PA) was used to examine pollen germination under

20X magnification. A total of 100 pollen grains were counted from each well. Pollen grains were considered as germinated if the pollen tube extended longer than the diameter of the pollen grain (Tuinstra and Wedel, 2000). Pollen viability was calculated as number of germinated pollen per 100 pollen grains. Analysis of variance was calculated to determine genotypic differences in pollen germination. The experiment was considered as a randomized complete block design with sample dates serving as replications.

Estimation of pollen longevity

Pollen longevity tests were performed using pollen from greenhouse grown SeaIsle 1 and pollen collected from field grown mixed material of SeaIsle 1 and SeaIsle 2000. Bulk quantities of fresh pollen of each pollen source were collected in glass Petri dishes around 0730 h. A small portion of the fresh pollen from each sample was immediately dispensed onto germination medium to establish the initial viability rates. The remaining pollen from each sample was maintained at room temperature (22˚C) in desiccators. A small portion of the bulk samples of pollen was removed from the desiccators and dispensed onto the germination medium every 20 min over a period of 180 min. Three wells were inoculated at each time interval for each pollen sample. A total of 100 pollen grains from each well were examined using an inverted light microscope (Fisher Scientific, Pittsburgh, PA) under 20X magnifications. Pollen grains were considered as viable if the pollen tube extended longer than the diameter of the pollen grain (Tuinstra and Wedel, 2000). Pollen germination rate of each well was calculated and recorded. All tests were repeated on two different dates. Data were analyzed using analysis of variance as a split-plot design with dates serving as replications, environments as main plots, and time as the sub-plot factor. Maximum pollen longevity was considered as the period of storage time required

for pollen germination percentage to reach zero. Pollen t_{50} was estimated by interpolation and defined as the duration of time at which 50% of the pollen remained viable.

Temperature effect on longevity

The effect of temperature on pollen longevity was tested using fresh pollen of greenhouse grown SeaIsle 1. Fresh pollen was collected onto glass Petri dishes around 0730 h. The initial germination percentage of the pollen was established by inoculating a small portion of fresh pollen onto the germination medium immediately. Pollen was then divided into six samples and placed in different incubators with temperature treatments of 15, 20, 25, 30, 35, 40˚C in the dark. Relative humidity of the incubators was maintained around 65% by using $NaNO_2$ saturated solution (Winston and Bates, 1960). In order to determine the longevity under each temperature treatment, a small portion of pollen stored at each temperature treatment was removed from the incubators and dispensed onto germination medium every 20 min over a period of 180 min. Three wells containing germination medium were inoculated with pollen from each temperature treatment at each time interval. All temperature treatments were repeated on three different dates. Data obtained from this experiment were analyzed as a split plot design with pollen sampling dates serving as replications, temperature as main plots, and time as sub-plots.

Statistical analysis

Data collected from each experiment were analyzed by using the SAS statistical package (SAS Institute, Cary, NC). Following analysis of variance, statistical separation of treatment means was carried out using pairwise t-tests at α=0.05.

RESULTS

Size of seashore paspalum pollen

Microscopic measurement of the pollen diameter indicated that SeaIsle 1 pollen collected from the greenhouse ranged from 30.0 to 39.0 µm in size, with an average of 34.4 ±2.9 µm. Greenhouse collected pollen of SeaIsle 2000 was slightly smaller and ranged from 30.0 to 36.0 µm with an average size of 33.2 ±2.9 µm. Mixed pollen collected from the field varied in size from 27.0 to 39.0 µm and averaged of 32.4 ±2.9 µm.

Pollen release pattern

Using an airborne particle counter we were able to monitor pollen release in a field setting for a total of 67 days over a two-year period. Total pollen release varied greatly from day to day and ranged from 9 to 13282 pollen grains m^{-3} during the period of 0600h to 1100h. Pollen release was inconsistent from day to day and distinct release peaks that were readily discernible from background were only detected on 17 of the 67 days monitored. Fig 4-1 illustrates the day-to-day variability in pollen release over a 7-day period as recorded by the airborne laser particle counter on a real-time basis. On days with distinct pollen release peaks, pollen release generally occurred between 0700 h and 1100 h with a very sharp release peak around 0800 h.

Meteorological data collected from a nearby weather station were compared with pollen release data in an attempt to identify environmental factors that influence pollen release. Of the 25 weather-related variables studied, only solar radiation at 0800 h was found to be weakly associated with pollen desity between 0600 h to 1100 h ($p < 0.05$) and $r = -0.27$ (Table 4-1).

Pollen viability

Pollen viability estimated by initial pollen germination rate of seven genotypes averaged 72.7%. Pollen viability of the genotypes tested ranged from 60 to 88% (Fig 4-2). Analysis of variance indicated significant ($p < 0.001$) genotypic differences for pollen germination rate. Genotype 05-202B-20 had the highest pollen germination rate of 88%. PI 647892 had the lowest germination rate of 60%, although it was not significantly different from that of 05-202D-04. Pollen germination was initiated within 10 min after being dispensed onto the germination medium and germinating pollen had elongated tubes after 60 min incubation. Fig 4-3 shows pollen tube development over time during the first 100 minutes.

Pollen longevity

Fig 4-4 shows pollen longevity of pollen that collected from both greenhouse and field grown plant materials and maintained at room temperature. Initial pollen viability was 72% and 66% for field collected and greenhouse collected pollen, respectively. Pollen viability declined rapidly when stored at room temperature. Greenhouse and field collected pollen reached their $t_{50\%}$ in 45 min and 60min, respectively. The average maximum pollen life was 80 min for greenhouse pollen and 100 min for field collected pollen. Pollen longevity response curves for greenhouse and field collected pollen were similar, however significant differences in pollen germination occurred at 60 and 80 min when field collected pollen had significantly higher germination rates than greenhouse collected pollen. Field collected pollen did show greater variation in pollen germination as indicated by larger standard errors associated with the means (Fig 4-4).

Temperature effect on pollen longevity

The overall analysis of variance showed that pollen germination rates were not only affected by storage time ($p < 0.0001$) but also by storage temperature ($p < 0.0001$) (Table 4-2). The effects of storage temperature on pollen longevity are presented in Fig 4-5. Among the temperatures tested, pollen stored at 15˚C had the best survival with a maximum longevity of approximately 140 min. Pollen longevities at 20˚C and 25˚C were similar and pollen remained viable for up to 120 min. When pollen was stored at 30˚C, pollen germination rate dropped rapidly. Pollen germination rate decreased to 23.8% within 20 min, only 3.3% of the pollen remained viable after 40 min, and maximum longevity was less than 60 min. When exposed to temperatures of 35˚C and above, viability of pollen rapidly dropped to 0% within 20 min. Our results indicate that pollen longevity was best at 15˚C and rapidly became nonviable at temperatures of 30˚C and above. Pollen half-life ($t_{50\%}$) of pollen store at 15, 20, and 25˚C were similar with a $t_{50\%}$ value of 63, 67, and 65min, respectively. Pollen halflife decreased from 65 min at 25˚C to 15 min at 30°C. At temperature 35 and 40˚C, half-life cannot be calculated due to the lack of data points. Results of the t-test (data not shown) indicated that there were no differences in initial germination rates at 0 min prior to initiation of temperature treatments. However, significant differences in pollen germination were observed between temperature treatments at 25˚C and below when compared to 30˚C and above after only 20 min. By 20 min, the negative impact of temperatures was readily apparent.

DISCUSSION

The efficiency of pollination and resulting seed set of wind-pollinated plant species can be impacted by the concentration of the air borne pollen available. More detailed information

289 regarding pollen release, such as quantity of pollen released, time of release, and their
290 relationships with environmental factors, not only contribute to our basic understanding of
291 pollination biology but also is of great interest to those in the seed industry seeking to improve
292 seed production efficiency.

293 Hirst-style volumetric spore traps, traditionally, have been used for most airborne pollen
294 release and pollen concentration related studies (Hirst, 1952; Levetin and Rogers, 2000). Schäppi
295 et al. (1998) reported that the daily grass pollen counts varied from 0 to over 400 pollen grains
296 m^{-3} during the pollen season and pollen counts were positively associated to the daily average
297 temperature. However, Hirst-style volumetric spore traps require visual assessment or
298 photographic software assistance and only provide estimates over broad periods of time (daily or
299 hourly).

300 Some scientists intended to estimate pollen shedding pattern by testing for changes in
301 pollen viability or pollen germination rate at different times of the day. In previous research
302 using such methods, pollen was observed to be primarily released around 1000 h for zoysiagrass
303 (Kang et al., 2009), and two pollen release peaks at 0900 h and 1400 h were observed in creeping
304 bentgrass (Fei and Nelson, 2003). However, this method is more valid when used to provide
305 information on the pollen viability at different times of the day rather than the pollen
306 shedding/release pattern since it provides no information on the real time of pollen release or on
307 pollen concentration.

308 The laser-based automatic particle counter used in this study allows counting and
309 categorization of airborne particles into differential size classes. This device provided us with an
310 opportunity to obtain pollen counts on real time basis. By using this device, we were able to

311 determine that quantity of pollen released was highly variable from day to day, and on those days
312 with obvious release peaks, pollen release often occurred during a very short time period of
313 approximately 10 to 30 minutes. This detailed understanding of pollen release in seashore
314 paspalum would not have been possible using traditional pollen counting techniques.

315 In this series of studies we compared pollen release data with meteorological data
316 collected from a nearby weather station in an attempt to identify environmental factors that
317 influence pollen release. Emecz (1962) found temperature and light positively impacted the
318 process of anthesis in an experiment that estimated environmental effects on anthesis of five
319 grass species (*Alopecurus, Dactylis, Festuca, Lolium and Phleum*). According to Sharma et.al
320 (1998), anthesis and anther dehiscence in *Pinus roxburghii* was greatly affected by air
321 temperature and relative humidity but not by light intensity. In our study, the analysis of
322 meteorological factors correlating pollen release of seashore paspalum indicated that only solar
323 radiation at 0800 h was weakly and positively associated with total pollen counts from 0600 h to
324 1100 h. The laser particle counter appeared to reliably monitor pollen release, however, we
325 monitored only 67 days in total. In other plant species, meteorological factors have been often
326 shown to have a substantial impact on pollen release patterns and pollen density. Perhaps
327 monitoring pollen release and meteorological variables for a longer period of time would help to
328 create a stronger data set for statistical analysis to reveal the environmental influences on pollen
329 release of seashore paspalum.

330 The results of pollen viability and longevity tests presented in this paper indicate that
331 pollen of seashore paspalum had an initial viability of 60% to 91% across seven different
332 genotypes. This data provides firm evidence that seashore paspalum is male fertile. Pollen
333 viability tests for other grass species, such as tall fescue, perennial ryegrass, zoysiagrass, and

creeping bentgrass demonstrated comparable pollen viabilities that ranged from 65% to over 90% (Ahloowalia, 1973; Fei and Nelson, 2003; Kang et al., 2009; Teare et al. 1970; Wang, et al, 2004). However, the pollen (greenhouse) of seashore paspalum remains viable for less than 2 h and only half of the pollen remains viable after 45 min at room temperature. Short pollen longevities have also been reported with zoysiagrass (40-180 min), bentgrass (120 min), switchgrass (20-150 min), creeping bentgrass (<180 min) and tall fescue (45 min to 22 h) (Fei and Nelson, 2003; Ge et al., 2011; Kang et al., 2009; Wang et al., 2004).

During the short duration after pollen release, pollen viability was very sensitive to temperature and was negatively impacted by high temperatures. Wang et al. (2004) reported that pollen viability of tall fescue was greatly reduced when the pollen stored at temperature 36˚C and above. Pollen viability and longevity of swichgrass were decreased with the increasing temperature and half-life of the pollen was less than 10 min at temperature 32˚C and above. A dramatic decline of pollen viability was observed when pollen was exposed to temperatures over 30˚C. According to Kumar et al. (1995), the time period of pollen with greater than 50% viability is more critical and practical for seed production. The estimated pollen half-life of SeaIsle 1 seashore paspalum was range from 63 to 67min at 15˚C to 25˚C and dramatically dropped to 15min at 30˚C. High temperature in the morning could be a negative impact for the seashore paspalum seed set in Georgia.

Our research indicates that under Georgia conditions, pollen release from seashore paspalum was erratic from day to day and pollen was released over a very brief period of time. Although pollen viability was initially relatively high, its longevity was short and negatively impacted by high temperatures. In Georgia winds are typically very calm especially during the early morning when seashore paspalum pollen is released. It is possible that production fields

could benefit from additional air movement during pollination. The strongly negative impact of air temperatue on pollen longevity implies that seed production of seeded cultivars of seashore paspalum may be better suited to environments with cooler daytime temperatures during the flowering season. However, it may be possible to select cultivars that flower earlier in the year when temperatures in Georgia are lower.

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Table 4-1. Summary of meteorological variables and their Pearson product-moment correlations with total number of pollen released from 0600 h to 1100 h.

Variable	df	Mean	Std. dev.	Min.	Max.	Correlation coef.	Prob. of signif.
Day of Year	66	240	24.1	201	281	-0.04	0.77
Temperature(T) 6am	66	20.3	4.7	5.5	24.9	0.07	0.56
T 7am	66	20.5	4.8	6.9	25.1	0.06	0.61
T 8am	66	22.5	4.3	8.5	27.9	0.04	0.76
T dif 7-6am	66	0.2	0.5	-0.8	1.4	-0.07	0.59
T dif 8-7am	66	2.0	1.6	-0.1	7.3	-0.08	0.49
Dew point(DP) 6am	66	17.7	5.1	4.0	23.4	0.10	0.44
DP 7am	66	17.9	5.2	3.7	23.4	0.09	0.46
DP 8am	66	18.3	5.1	3.6	23.5	0.08	0.50
DP dif 7-6am	66	0.1	0.4	-1.2	0.7	-0.03	0.80
DP dif 8-7am	66	0.4	0.6	-1.0	2.1	-0.09	0.49
T&DP dif at 6am	66	2.6	1.5	0.8	8.8	-0.10	0.41
T&DP dif at 7am	66	2.6	1.5	0.8	9.2	-0.12	0.35
T&DP dif at 8am	66	4.2	2.4	0.8	11.3	-0.11	0.38
Humidity(H) 6am	66	85.5	7.7	57.6	95.2	0.11	0.37
H 7am	66	85.1	7.6	56.1	95.3	0.13	0.30
H 8am	66	77.6	11.2	49.1	95.3	0.13	0.29
H dif 7-6am	66	-0.4	2.8	-9.8	6.1	0.04	0.73
H dif 8-7am	66	-7.5	6.0	-22.1	0.3	0.08	0.51
Wind speed(WS) 6am	66	0.9	0.6	0.0	2.4	-0.20	0.10
WS 7am	66	1.1	0.7	0.0	2.7	-0.18	0.15
WS 8am	66	1.4	0.7	0.0	2.6	-0.11	0.36
Solar Radiation(SR) 6am	66	1.6	2.8	0.0	11.7	-0.08	0.52
SR 7am	66	52.9	37.2	2.2	150.3	-0.16	0.20
SR 8am	66	218.0	76.8	8.6	339.2	-0.27	0.027*
Rainfall 12h before 8am	66	1.0	3.5	0.0	19.6	-0.07	0.59

* Significant at 0.05 probability level.

442

Table 4-2. Summary of analysis of variance of the effect of storage temperature on pollen longevity of SeaIsle 1seashore paspalum

Source	df	Mean Square	Pr > F
Temperature (T)	5	10261.97	< 0.0001***
Error a	12	67.66	
Minutes (M)	8	29588.90	< 0.0001***
M × T	40	1349.26	< 0.0001***
Error b	420	5.94	
CV = 12.6%			

*** Significant at 0.001 probability level.

443

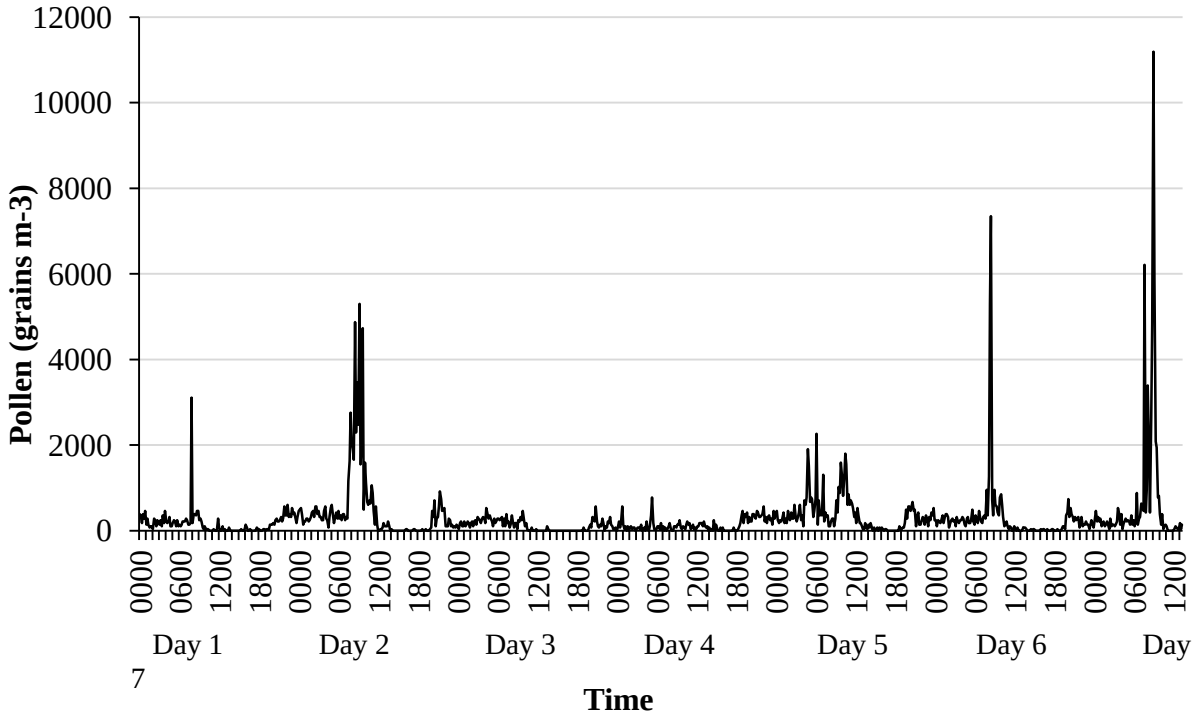


Fig 4-1. Pollen release pattern in a field-grown mixture of SeaIsle 1 and SeaIsle 2000 seashore paspalum. Data were collected over a seven day period from Sep 9th to 15th, 2010 at Griffin, Georgia.

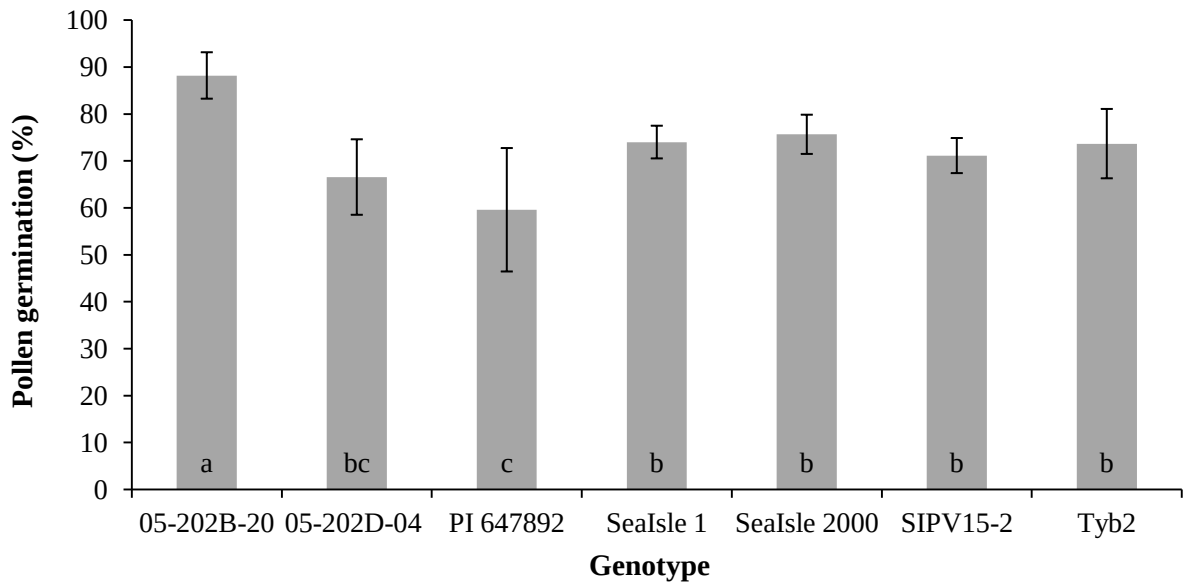


Fig 4-2. Initial pollen germination rates of seven genotypes of seashore paspalum. Genotypes with the same letters are not considered statistically different at α=0.05 according to Student's t-test.

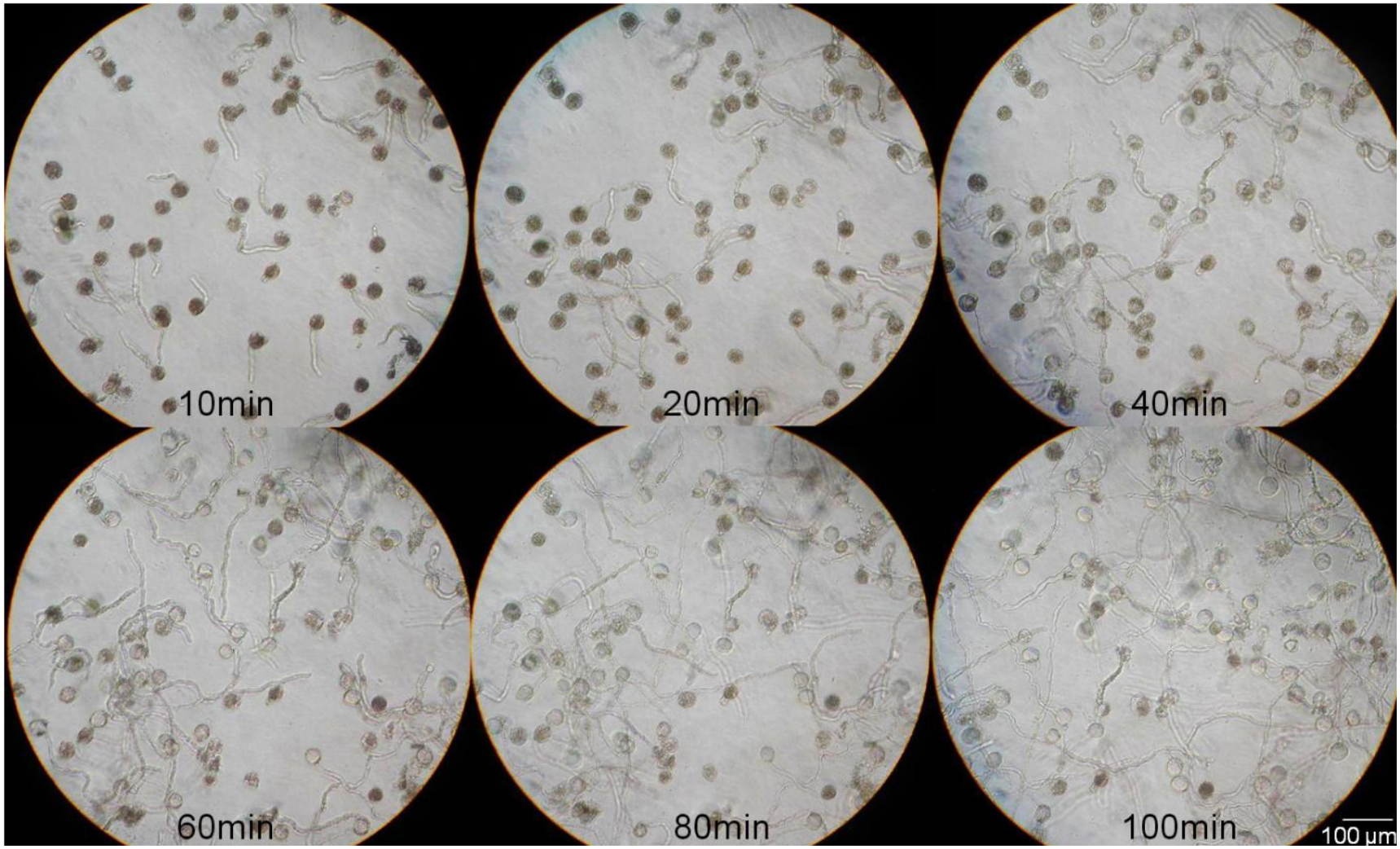
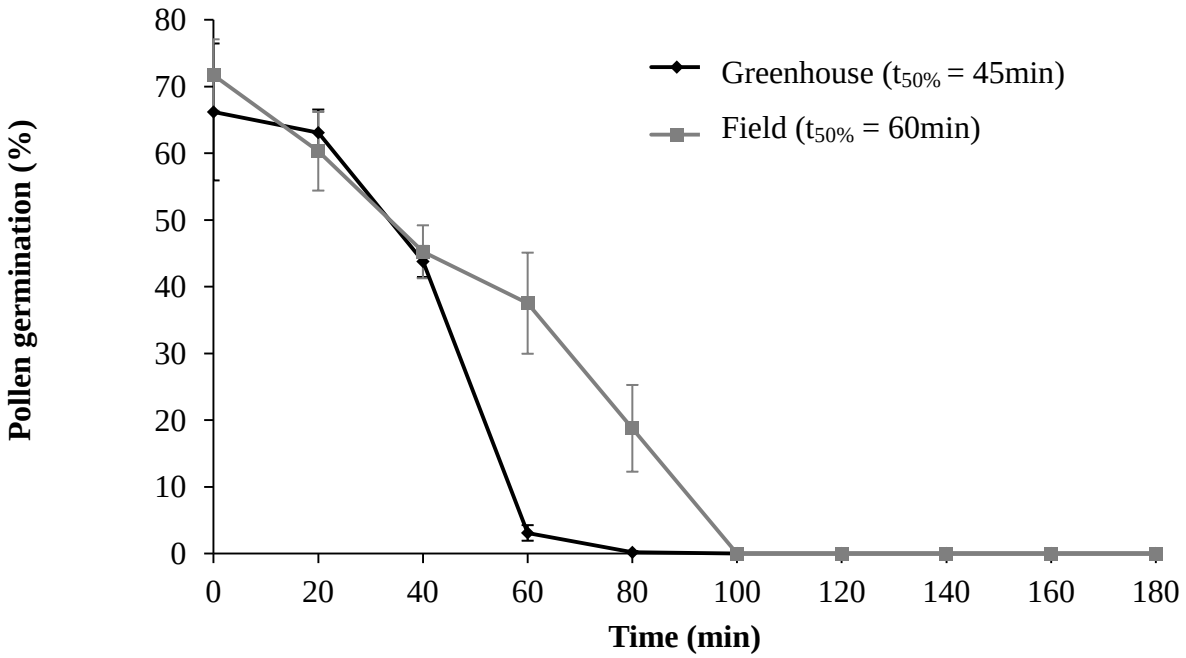


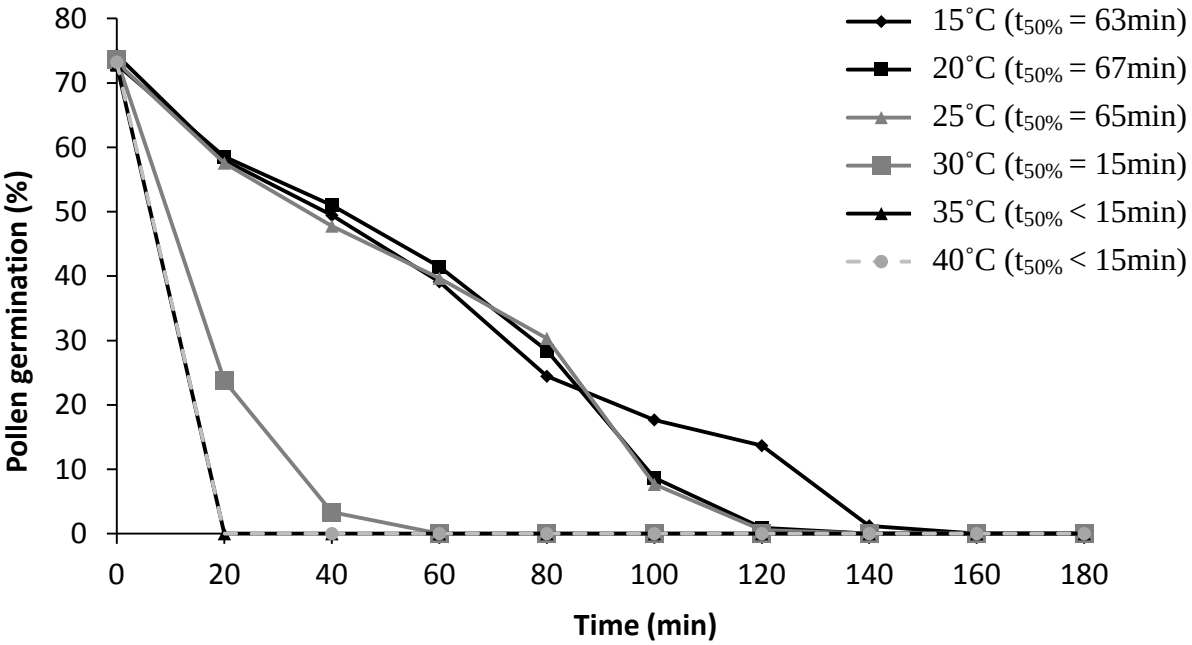
Fig 4-3. Pollen germination and pollen tube development of SeaIsle 1 seashore paspalum after 10, 20, 40, 60, 80, and 100 min on a liquid germination medium at room temperature.



457

458 **Fig 4-4**. Pollen longevities of greenhouse-grown SeaIsle 1 and field-grown mixed plant material
459 of SeaIsle 1 and SeaIsle 2000. Time of pollen half-life indicated as $t_{50\%}$.

460



461

462 **Fig 4-5.** Effects of storage temperature on pollen viability and longevity of SeaIsle 1 seashore
463 paspalum.
464

CHAPTER 5

POLLEN-STIGMA COMPATIBILITY AND SEED SET AMONG DIFFERENT GENOTYPES OF SEASHORE PASPALUM (*Paspalum vaginatum* Sw.) [3]

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3 Na Wang, Hazel Y. Wetzstein, Brian M. Schwartz, Paul L. Raymer*. To be submitted to Crop Science.

ABSTRACT

Selection of parental lines with high levels of sexual compatibility is essential when developing new seeded seashore paspalum cultivars. Pollen-pistil interactions and sexual compatibility are crucial in the process of fertilization and thus essential to maximize seed yield. However, little is known about these aspects of the reproductive biology of seashore paspalum. Experiments were conducted to investigate pollen-stigma interactions, tube growth through the style, and compatibility among six genotypes as well as to evaluate the viability of hybrid seeds. The nature of pollen-pistil interactions was studied using aniline blue staining as a technique to visualize pollen tube growth within the stigma and style. Pollen tubes exhibited extensive growth only in pistils that were cross-pollinated; pollen tube growth was inhibited in early stages of elongation with self-pollinated pistils. A six by six diallel crossing experiment was conducted to determine the level of sexual compatibility among different parental combinations. Results showed that every cross-pollinated parental combination set seeds with seed set ranging from 7.7% to 63.1%. Seeds produced in the crossing study were then tested for viability using germination tests. Seed germination ranged from 56.7% to 98.1% with average of 78.2%. Our results indicate that seashore paspalum is self-incompatible but cross-fertile with differing levels of compatibility among genotypes. Seed from the crosses were viable with germination rates up to 98.1%.

Abbreviations: SI, self-incompatibility; GSI, gametophytic self-incompatibility; SSI, sporophytic self-incompatibility.

INTRODUCTION

Seashore paspalum (*Paspalum vaginatum Sw*.) is a perennial warm-season grass that grows in large areas from tropical to warm temperate regions (Morton, 1973). Many desirable physiological and morphological traits such as salt tolerance, water logging tolerance, dark green color, and fine leaf texture make it popular as a turfgrass worldwide (Duncan, 1999; Duncan and Carrow, 2000). Seashore paspalum is mainly vegetatively propagated. Selection of parental lines with high levels of compatibility and efficient fertilization is essential for the development of new seeded seashore paspalum cultivars. Low seed set is often associated with low flowering intensity, low pollen density, non-functional reproductive organs, incompatibility, and many other environmental or genetic factors (Elleman et al., 1992).

Seashore paspalum has been reported as a sexually reproducing self-incompatible diploid species (Duncan and Carrow, 2000; Quarin, 1992). Espinaza and Quarin (1997) reported that *Paspalum simplex, Paspalum chaseanum* and *Paspalum plicatulum* Michx. were self-incompatible but cross-fertile. In hybridization studies among three paspalum species conducted by Burson (1987), *Paspalum intermedium* was reported as self-incompatible while *Paspalum jurgensii* and *Paspalum dilatatum* were highly self-fertile. However, no experiment has been done to reveal the nature of self-incompatibility or to determine the extent of cross-compatibility among different genotypes of *Paspalum vaginatum*. Seed setting provides the most convincing evidence for the self-incompatibility/compatibility, yet only at qualitative level.

It has been reported interspecific crosses with other Paspalum species have shown to be difficult to accomplish (Carpenter, 1958; Duncan and Carrow, 2000). The first seeded cultivar of seashore paspalum ‘Sea Spray’, which was released in 2003 is the hybrid of ‘Q36313’ and ‘Hyb7’ (Fricker et al., 2007).

Self-incompatibility is mainly genetically controlled (Pandy, 1960). Fertilization could fail due to an incompatibility barrier even though the plant produces functional male and female reproductive organs (Newbigin el al, 1993; Takayama and Isogai, 2005). Prerequisites for successful sexual reproduction in higher plants include a sequence of critical interactions occurring between pollen and stigmatic surfaces which include pollen capture, pollen hydration, germination and tube growth (Franklin-Tong 2002; Edlund et al, 2004; Hiscock and Allen 2008). Investigation of pollen-pistil interactions is the essential way to determine compatibility. Non-compatible pollen development can be halted at any stage of the process due to different types of incompatibility mechanisms and result in no seed setting (Hiscock, 2002). Aniline blue stains callose in pollen walls and tubes and fluoresces under ultraviolet light (Dumas and Knox, 1983; Martin, 1959). It has been used to observe pollen tube development within the styles in many plants (Shafer et al., 2000; Yi et al., 2006).

Seed set is the most accurate and practical method to assay successful fertilization. It provides information about pollen viability, stigma receptivity, and pollen-stigma compatibility (Dafni and Firmage, 2000). The seed industry is most concerned about the resulting impact of seed set on seed yield. Factors reported to impact seed set include density (availability) of airborne pollen, density of the racemes, size of the raceme, number of fertile florets on the raceme, and other possible environment factors that impact these aspects. However the sexual compatibility of the parents is the primary concern when selecting parental lines for the production of seeded cultivars (Chastain and Young, 1998).

Seed germination is one important aspect for evaluating seed viability and quality. Environmental signals, such as temperature and photoperiod, are very critical for regulating seed germination. Carpenter (1958) reported that viable seed production from seashore pasaplum was

554 low. Germination tests of seeds produced at multiple locations in Georgia showed that seashore
555 paspalum seeds had germination rates of less that 12% (Duncan, 1999). Shin et al. (2006)
556 reported that germination rates up to 77% were achieved for seashore paspalum seeds at 25/30˚C
557 night/day temperatures following treatment with KNO_3.

558 The objectives of this study were to: (1) determine the nature of pollen-stigma
559 compatibility among different seashore paspalum genotypes, (2) determine the level of cross-
560 compatibility among different seashore paspalum genotypes and, (3) evaluate germination rates
561 of seashore paspalum hybrid seeds

562 MATERIALS AND METHODS

563 *Plant material*

564 Two commercial cultivars 'SeaIsle 1'and 'SeaIsle 2000' and four experimental breeding
565 lines from The University of Georgia turfgrass breeding program, Hyb7, Q36313, Q36315, PI
566 647892 were selected for this study based on previous work. Plants were maintained under
567 greenhouse growth conditions (28 ±5/20 ±5˚C, day/night) with natural day length. Plants were
568 irrigated twice daily and fertilized monthly with water soluble 28-7-14 (NPK) fertilizer (LESCO
569 MacroN, Cleveland, OH). Experiments were conducted from June to September in 2011, during
570 the most vigorous flowering period of seashore paspalum in Georgia. Pollen used for hand
571 pollination was collected from fresh flowers in the early morning shortly after sunrise and
572 generally between 0630 h to 0830 h.

573 *Pollen-stigma compatibility*

574 A six by six full-diallel crossing experiment was conducted by hand pollination in the lab.

Freshly emerged racemes were collected in the early morning from the greenhouse onto glass Petri dishes and emasculated before the anthers emerged. Donor pollen from each of the six genotypes was collected around 0730 h. Donor pollen was dispensed onto the receptive stigmas to accomplish each of the 36 cross combinations. Pollen-pistil compatibility among the 36 different cross combinations was determined using aniline blue stain that allows visualization of pollen tube growth within the stigma and style (Martin, 1959). Pollinated racemes were maintained at room temperature (22˚C) for three hours, at which time florets on the racemes were dissected to remove the pistils. Pistil staining was conducted as described by Yi et al. (2006) with a few modifications. After dissection, pistils were submerged in a fixative [(95% ethanol: glacial acetic acid, 3:1 (v/v)]. Tissues were softened and cleared by autoclaving for 15 min at 120˚C, in 10% sodium sulfite solution. Pistil tissues were then stained with aniline blue staining solution (0.01% aniline blue in 0.1 M K_3PO_4) overnight. Whole pistils were mounted in a drop of stain, and gently squashed with a coverslip. Slides were examined using an Olympus BX51 light microscope (Olympus America, Center Valley, PA) under ultraviolet light to determine the extent of pollen tube development within the style (Yi et al., 2006). The lengths of the pollen tubes were measured with AutoCAD software (Autodesk Inc., San Rafael, CA). Hyb7 and Q36313 are the parents of 'Sea Spray' and known to be cross-compatible (Fricker et al., 2007); therefore their reciprocal crosses were used as controls. Three slides (replications) were made for each cross combination.

Hand pollination and seed set

Both self- and cross-compatibility among the six genotypes was also determined using seed set as an indicator of compatibility level. A six by six full-diallel crossing experiment was conducted using hand pollinations in a greenhouse. Potential flowering racemes were covered

with transparent polypropylene tubes (55 × 12 mm) (Sarstedt AG & Co., Nümbrecht, Germany) prior to stigma emergence to minimize pollen contamination. Each of the 36 cross combinations were accomplished by collecting pollen from fresh flowers of the male donor around 0730 h and immediately dispensing it onto freshly emerged stigmas of the female parent using a small artist brush. Freshly pollinated flowers were then immediately recovered with the tubes and kept covered for seven days. Ten crosses were made for each of the 36 cross combinations. Racemes were carefully harvested 28 days after pollination. The number of florets and seeds produced on each raceme were recorded. The percentage seed set was calculated as the (number of seed)/(number of florets pollinated) × 100%. Analysis of variance was performed using SAS (SAS Institute, Cary, North Carolina). Analysis of variance and pairwise Student's t-tests for mean separation were performed using the JMP. 8.0.2 statistical software package (SAS Institute, Cary, NC).

S*eed germination*

Seeds from each of the 36 cross combinations were carefully collected into coin envelopes and stored in a desiccator at room temperature (22˚C). Seed germination tests were conducted using the procedure described by Shin et al. (2006). Transparent polystyrene boxes (11×11×3.5 cm) with lids (Hoffman Manufacturing, Inc., Albany, OR) were used as germination boxes. Germination boxes were prepared with two layers of germination paper (Anchor Paper, St. Paul, Minn). Seventeen ml of 0.2% KNO_3 was added to the box, and seeds were then carefully placed onto the germination paper. Germination boxes were then placed in a seed germinator (Model 12, Stults Scientific Engineering Corp., Springfield, IL) at 27/35˚C (12/12 h), 8/16 h (light/dark), and 100% relative humudity. Seed was considered germinated if "both plumule and radicle protruded through the testa" (Shin et al., 2006). Numbers of germinated seeds were

recorded weekly for five weeks. Germination of seeds from each raceme was calculated independently as the (number of germinated seed)/(total number of the seed) × 100%. Data collected for all of the crosses were subjected to analysis of variance and mean separation was performed using Student's t-tests at α=0.05 (JMP. 8.0.2, SAS Institute, Cary, NC)

RESULTS

Pollen-stigma compatibility

The six by six reciprocal crossing block resulted in 36 cross combinations with six self-pollinated and 30 cross-pollinated crosses. Major differences between self- and cross-pollinated stigmas were noted. Fig 5-1 shows stained pistils of self and reciprocal crosses of Hyb7 and Q36313. These crosses were used for reference since these two genotypes are known to be compatible. Pistils of self and reciprocal crosses of SeaIsle 1 and SeaIsle 2000 are shown in Fig 5-2 as one example of the staining results from the six by six reciprocal crossing tests. In self pollinations, pollen grains attached on the stigma were able to hydrate and germinate, but the pollen tubes only extended a short distance after penetrating the stigma papilla. Measurements of pollen tube lengths of selfed crosses showed tube extension ranged from 89 to 452 µm, about 3 to 14 times the pollen diameter. The pollen tubes appeared tortuous in shape near the pollen grain and swollen at the tip (Fig 5-1a and b; Fig 5-2a and b). In contrast, stigmas pollinated with non-self pollen had pollen that not only adhered to stigmatic papillae, hydrated, germinated, and penetrated the papilla surface, but also exhibited tube elongation into the style with growth toward the ovary. For all non-self crosses in this experiment, pollen tubes reached the micropyle of the ovule within three hours. The length of the random sampled advanced pollen tubes ranged from 1332 to 2335 µm, which were 41-69 times the pollen diameter. Callose plugs formed

643 periodically in the pollen tube as they it grew through stylar tissue (Fig 5-1c and d; Fig 5-2c and
644 d).

645 **Seed set**

646 Results of seed set from the 36 cross combinations generated by the six by six dialle are
647 presented in Table 5-1. Major differences were observed in seed set between self-pollinated and
648 cross-pollinated crosses according to the result of the mean separation tests performed. Mean
649 seed set from cross-pollinations ranged from 7.7% to 66.3%. Significant differences in percent
650 seed set were found among different cross combinations ($p < 0.001$). Student's t tests indicated
651 that differences ($p < 0.001$) in seed set existed among female parents when averaged across all
652 possible males, but differences in seed set were not observed among males when averaged across
653 all possible females. When used as female parents, SeaIsle 1, Hyb7, and Q36315 set significantly
654 higher percentages of seed than SeaIsle 2000, Q36313, and PI 647892. Approximately 32.3% of
655 all cross-pollinated racemes had seed set greater than 50% indicating high levels of cross
656 compatibility among the six genotypes tested. A comparison of reciprocal crosses showed the
657 reciprocals of the same parental combinations differed statistically in seed set percentage in eight
658 of 15 parental combinations. For example, the cross of Q36315 × PI 647892 resulted in 54%
659 seed, while PI 647892 × Q36315 produced only 12.5 % seed set. SeaIsle 1 × Q36315 had the
660 highest seed set percentage of 66.3% and the reciprocal cross yielded over 50% seeds as well.

661 About 75% of self-pollinated crosses of seashore paspalum yielded no progeny, but
662 occasionally a single crossed raceme produced a few seeds. When calculated over all selfed
663 crosses, the rate of seed set from self pollinations was only 0.02%.

664

Seed germination

Results of germination tests conducted on seeds produced from the six by six diallel crossing experiment are presented in Table 5-2. Germination rates of seeds produced as a result of cross-pollinations ranged from 56.7% to 98.1% with an average of 78.2%. Seeds produced by 14 of the 30 cross-pollinated parental combinations had germination percentages over 80%. Analysis of variance of these data indicated there was no female effect or male effect on seed germination rate

DISCUSSION

Our results indicated that seashore paspalum is highly self-incompatible but cross-fertile within the species. Levels of cross compatibility varied greatly with parental combination.

Self- compatibility/incompatibility have been reported for other species of Paspalum (Burson, 1987; Espinaza and Quarin, 1997). However, no research has determined whether the type of self-incompatibility in *P. vaginatum* is gametophytic, sporophytic (Duncan and Carrow, 2000). Visual observations of pollen tube development in pollinated-pistils of 36 cross combinations indicated that the six genotypes of seashore paspalum used in this study are self-incompatible but cross-fertile. Self-incompatibility (SI) is an evolutionary mechanism that maintains genetic variability by promoting outcrossing. Self-incompatibility is generally categorized as either gametophytic self-incompatibility (GSI) or sporophytic self-incompatibility (SSI) (Brewbaker, 1957). The inhibition of pollen grains in GSI plants generally occurs during some stage of pollen development within the style while the inhibition of SSI plants usually occurs on the surface of the stigma (Brewbaker, 1957; Hiscock, 2002). Observations of pollen tube development visualized using stained pistils showed that self pollen of seashore paspalum

687 was able to germinate, but pollen tubes only extended a short distance after penetrating the
688 surface of stigma. These observations strongly indicate that seashore paspalum is a species with
689 gametophytic self-incompatibility.

690 Non-self pollen tubes successfully reached the micropyle within three hours which was
691 the time of our observations. It has been reported that two to four hours is needed for pollen
692 tubes to reach the micropyle in both buffelgrass × birdwood grass and most of the interspecific
693 crosses among *Paspalum* species reported (Shafer, 2000; Burson, 1987).

694 Visual observations of extensive pollen tube development within the pistil do not provide
695 complete certainty on the actual occurrence of fertilization. Visualization of the pollen tubes
696 growing toward to the micropyle only implies that pollen were accepted by the stigma and do not
697 necessarily indicate successful fertilization (Franklin et al., 1995). For these reasons, a seed set
698 experiment was conducted at the same time in order to confirm the compatibility among
699 genotypes of seashore paspalum.

700 Results from the seed set study indicated that all non-self parental combinations set seed,
701 with the percentage of seed set ranging from 7.7% to 63.3% depending upon the parental
702 combination. There were only a few seeds produced from some self-pollinated crosses of some
703 genotypes. Seed production as a result of self-pollination was very erratic and rate of seed set
704 was only 0.02%. The production of selfed seed in seashore paspalum seems unlikely considering
705 our observations from the pistil staining experiments where we noted minimal pollen tube
706 development from all genotypes when self pollinated. The seed set experiment was conducted in
707 a greenhouse cooled with evaporative pads requiring high levels of air movement and where
708 other genotypes were flowering at the same time as our hand pollinations were made. Efforts

were taken to minimize the possibility of unintended cross pollination by covering racemes prior to stigma emergence and re-covering pollinated racemes for seven days following hand pollination. The few seeds produced with self pollinations are most likely a result of pollen contamination rather than a low level of self-compatibility.

The seeds viability tests conducted by Duncan (1999) indicated that seed germination rates of field harvested seashore paspalum were extremely low with mean germination rates less than 12%. Our results show that the germination rates of hybrids of seashore paspalum produced by hand pollinations were much higher and ranged from 56.7% to 98.1%. The seeds viability tests conducted by Shin et al. (2006) indicated that seed germination of Sea Spray seashore paspalum under optimum germination conditions was 77%.

Information gained from these studies provides encouragement that selection of highly-compatible parental combinations of seashore paspalum for future breeding efforts should not be difficult.

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Table 5-1. Mean percentage of seed set observed from reciprocal crosses made among six seashore paspalum genotypes.

	Female						Male seed set averaged over all females
	SeaIsle 1	SeaIsle 2000	Hyb 7	Q36313	Q36315	PI 647892	
Male	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
	%	%	%	%	%	%	
SeaIsle 1	−	42.6 ±15.1	57.6 ±12.8	30.2 ±25.2	50.0 ±12.7	16.7 ±4.8	39.4 ±20.9a[†]
SeaIsle 2000	46.6 ±15.2	−	43.0 ±16.2	14.9 ±11.9	63.2 ±17.8	23.5 ±7.2	38.2 ±22.1a
Hyb 7	60.9 ±15.2	34.4 ±12.8	−	36.8 ±25.0	57.9 ±19.5	24.1 ±10.5	42.8 ±22.0a
Q36313	38.4 ±13.8	34.0 ±12.8	49.8 ±23.3	−	47.1 ±17.9	7.7 ±7.0	35.4 ±21.5a
Q36315	66.3 ±21.3	28.9 ±12.6	52.2 ±14.3	10.8 ±4.2	−	12.5 ±2.7	34.1 ±25.4a
PI 647892	41.7 ±11.7	25.5 ±14.5	41.3 ±17.3	31.5 ±26.2	54.6 ±21.4	−	38.9 ±20.7a
Female seed set averaged over all males							
Mean ±SD	50.8 ±18.7a	33.1 ±14.3b	48.8 ±17.5a	24.8 ±22.2c	54.5 ±18.3a	16.9 ±9.2d	

[†] Means followed by the same letter within the same column or row are not considered different according Student's t-test at α=0.05.

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Table 5-2. Mean percentage of seed germination observed from seeds produced as a result of reciprocal crosses made among six seashore paspalum genotypes.

	Female						Male seed set averaged over all females
	SeaIsle 1	SeaIsle 2000	Hyb 7	Q36313	Q36315	PI 647892	
Male	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
	%	%	%	%	%	%	
SeaIsle 1	–	74.9 ±23.0	68.1 ±24.6	64.0 ±42.4	88.3 ±11.0	95.0 ±15.8	78.1 ±27.4ab[†]
SeaIsle 2000	71.5 ±18.8	–	64.1 ±11.4	66.7 ±40.8	76.6 ±15.7	85.6 ±24.9	72.9 ±24.8b
Hyb 7	69.7 ±15.2	62.5 ±26.6	–	84.4 ±30.6	78.6 ±16.4	93.5 ±18.9	77.7 ±22.9ab
Q36313	93.3 ±21.1	88.9 ±12.8	87.5 ±7.3	–	82.8 ±10.7	56.7 ±49.8	81.8 ±27.8ab
Q36315	77.1 ±21.6	72.9 ±22.8	64.8 ±17.8	86.7 ±21.9	–	71.0 ±35.3	74.5 ±24.7ab
PI 647892	98.1 ±14.4	93.4 ±8.7	84.7 ±15.9	59.2 ±26.3	91.8 ±9.7	–	85.4 ±19.9a
Female seed set averaged over all males							
Mean ±SD	81.9 ±20.1ab	78.5 ±22.2ab	73.8 ±18.8ab	72.2 ±33.9b	83.6 ±13.7a	79.7 ±32.8ab	

[†] Means followed by the same letter within the same column or row are not considered different according Student's t-test at α=0.05.

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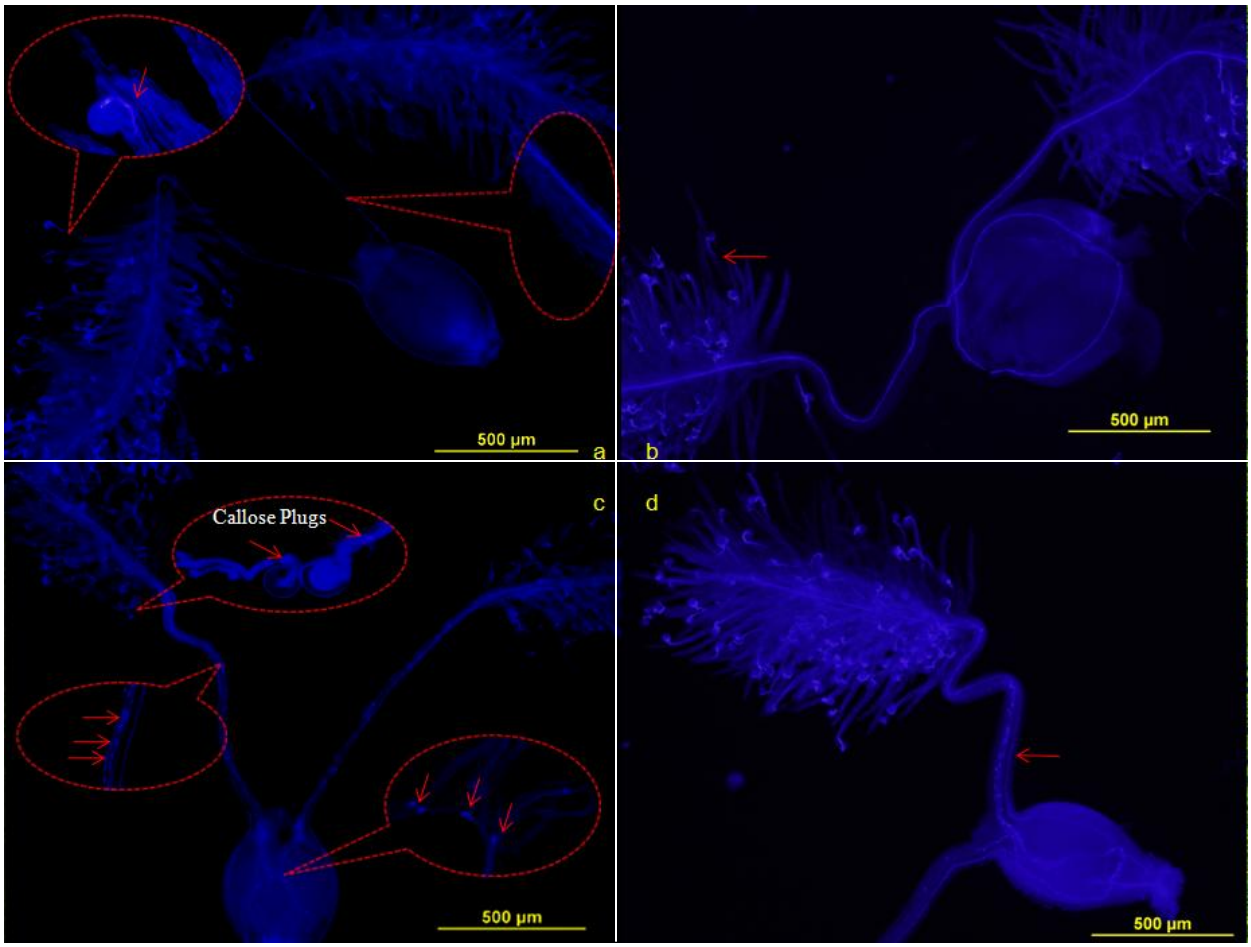

Fig 5-1. Fluorescent photomicrographs of pistils stained with aniline blue from self and reciprocal crosses of Hyb 7 and Q36313. The fluorescent photomicrographs illustrate major differences in pollen growth and development between self- and cross-pollinations. Self-pollinated stigmas of Q36313 (a) and Hyb7 (b) show pollen tube growth was halted soon after penetration (arrows). Cross-pollinated stigmas of Hyb7 × Q36313 (c) and Q36313×Hyb7 (d) show pollen tubes penetrated the papillae and elongated toward the micropyle (arrows show callose plugs).

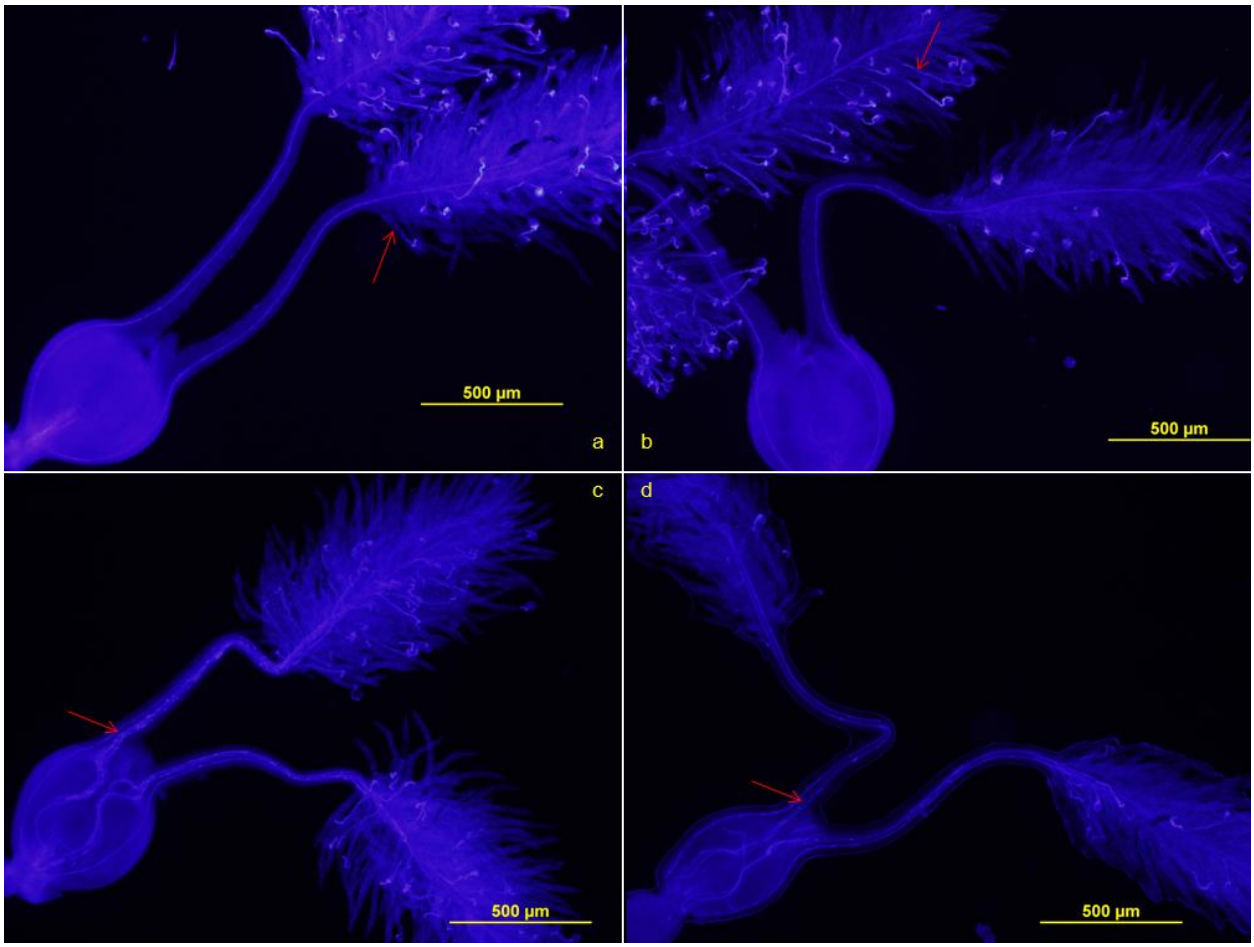

Fig 5-2. Fluorescent photomicrographs of pistils stained with aniline blue from self and reciprocal crosses of SeaIsle 1 and SeaIsle 2000. Self-pollinated stigmas of SeaIsle 1 (a) and SeaIsle 2000 (b) showing very short elongation of pollen tubes (arrows). Pistils from reciprocal crosses of SeaIsle 1 × SeaIsle 2000 (c) and SeaIsle 2000 × SeaIsle 1 (d) showing pollen tubes penetrating and elongating toward the micropyle (arrows).

CHAPTER 6

INFLUENCE OF SOLAR RADIATION AND PHOTOPERIOD ON FLOWER INITIATION OF SEASHORE PASPALUM (*Paspalum vaginatum* Sw.) [4]

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ABSTRACT

The timing and quantity of flowers play a pivotal role in the process of seed production. External factors of photoperiod and solar radiation were evaluated for their influences on flower initiation of seashore paspalum. The impact of solar radiation was revealed by evaluating flowering habit of 11 genotypes of seashore paspalum in response to radiation levels of 100, 41, 27, and 13% of the unshaded control in a greenhouse study. Eighty-nine genotypes from the USDA seashore paspalum germplasm collection were monitored for flowering habit for 15 weeks in a greenhouse receiving the natural photoperiod of Griffin, GA. In the radiation study, few flowers were produced at radiation levels of 27 and 13% of the unshaded control, while most genotypes flowered readily under the 100% radiation treatment. Only a few genotypes tested flowered in response to the 41% radiation level treatment. Plants receiving weekly cumulative PAR (photosynthetically active radiation) less than 90162 μmol m^{-2} did not flower. Flowering response to photoperiod varied greatly among genotypes. Flower initiation for the majority of the monitored genotypes increased dramatically as photoperiod reached 14 h and progressed to the longest photoperiod of 14.4 h. Information obtained from this work can be useful for future projects that involve flower induction of seashore paspalum.

Abbreviations: PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux density.

INTRODUCTION

Seashore paspalum (*Paspalum vaginatum* Sw.) is a perennial warm-season grass that has recently become popular as a turfgrass (Duncan, 1997; Morton, 1973). This species has great potential to enhance the aesthetic value of environments, and has been successfully used for soil stabilization and site reclamation (Duncan, 1997; Duncan and Carrow, 2000). As recently as 2000, a few researches have suggested that seashore paspalum must be propagated vegetatively from sod or sprigs since seeds were not reliable (Duncan and Carrow, 2000). After years of breeding research focused on development of seeded cultivars, the first seeded cultivar 'Sea Spray' was released in 2003 and is the only seeded cultivar currently commercially available (Fricker et al., 2007). Sea Spray is produced in Oregon, USA, in a region that is known for its ability to produce high-quality seed of cool-season turf species. Considerable expertise and infrastructure for grass seed production exists in this region, but the climate is cool to temperate and production of seed from warm-season species such as seashore paspalum is challenging. Oregon has a cool and very short growing season that typically limits the flowering period of seashore paspalum. Seed fields of seashore paspalum often suffer from winter injury which results in slow green-up in the spring. Delays in flowering push maturation of the seed crop closer to the onset of fall and winter rains typical of this region. Seed production trials conducted in multiple locations in the southern United States have typically resulted in much lower flowering intensity and lower seed yields than are achieved in Oregon in spite of its limited growing season. In order to produce profitable seed yields, parental lines chosen for use in production fields must not only produce functional and compatible reproductive organs but also must produce large numbers of flowers (Chastain and Young, 1998).

Changing environmental factors can affect and regulate flowering in plants. Photoperiod

and temperature are the primary external factors known to induce flowering (Roberts and Struckmeyer, 1938; Rogers, 1950; Thompson, 1944). Photoperiodic flowering plants are classified as long-day plants, short-day plants, and day-neutral plants (Thomas and Vince-Prue, 1997). Measurement of photosynthetically active radiation is important to determine the effect of light on plant growth and flower induction (McCree, 1972a; 1972b; 1981). Photosynthetically active radiation (PAR) is the range of light (400 to 700 nm wavelength) that can be used by plants for photosynthesis (Commission Internationale de l'Eclairage, 1970). Photosynthetically active radiation is quantified by photosynthetic photon flux density (PPFD) and is reported as micromoles of photon per square meter per second. Changing of PAR is according to the latitude, season, and time of day. It can be influenced by weather or objects that block direct sunlight.

A supply of assimilates from either current photosynthesis or the stored assimilates is prerequisite for flower initiation (Thomas and Vince-Prue, 1997). Hence, photosynthesis which is affected by duration, intensity and quality of light plays a significant role in flower induction. High intensity light may affect the phytochrome action or the biosynthesis of the substances that promote flowering (Smith, 1975). Low light intensity may be sufficient as a light breaks during the dark period for inducing the flower in some plant, such as *lolium* (Evans, 1958). Low intensity supplementary light can be compensated with sucrose or glucose in some long-day plants; however, the requirement for high irradiance levels cannot be totally substituted by external energy (Brulfert et al., 1985).

For flowering plants (angiosperms), the time of flowering is critical for their reproductive success. Photoperiodism results in a synchrony of flower initiation within a population which promotes genetic recombination by out crossing (Heide, 1985). Photoperiod is known to

influence and regulate many aspects of plant development including formation of storage organs, leaf development, seed germination, and flower initiation (Thomas and Vince-Prue, 1984). Knight (1955) reported that optimum seed production of dallisgrass (*Paspalum dilatatum* Poir.) occurred at 14 h photoperiod with a high night temperature of 18.3 to 21.1˚C. No seed formed under 8 h photoperiod, and erratic seed heads and incomplete flowering were observed under12 h photoperiod (Knight, 1955). Photoperiod studies conducted with *kalanchoe* sp. (*Kalanchoe glaucescens, K. manginii, and K.uniflora*) indicated that less than 12 h photoperiod results in increased flower number with fewer nodes (Currey and Erwin, 2010). An understanding of the flowering habits of germplasm lines is helpful to researchers who wish to select parents with synchronized flowering in order to promote crossing.

Limited information is currently available regarding the impact of photoperiod and solar radiation on flower induction of seashore paspalum. The objectives of this research were to (1) determine the impact of solar radiation levels on flower initiation of seashore paspalum and (2) evaluate the USDA seashore paspalum germplasm collection for flowering response under the natural photoperiod of Griffin, Georgia.

MATERIALS AND METHODS

Effect of PAR level on floral induction

Impact of solar radiation level on flower induction was determined by monitoring the number of flowers initiated under different solar radiation levels. Two commercial cultivars ‘SeaIsle 1’and ‘SeaIsle 2000’and nine breeding lines (Q36313, Hyb 7, PI 647920, PI 647892, PI647894, 03-501-46, 03-522-23, 03-528-126, 03-531-22) from The University of Georgia seashore paspalum breeding program were used in this study. Plant materials were established

clonally from stolon nodes. Plants were grown in 10 x 10 cm pots and were maintained in a greenhouse with a temperature of 28 ±5/20 ±5˚C (day/night) without any artificial light supplementation. During the period of study, plants were never trimmed. Plants were irrigated twice daily and fertilized monthly with 28-7-14 (NPK) fertilizer (MacroN, Lesco, Ohio). In the greenhouse study, four levels of solar radiation 100 (no shade cloth), 41, 27, and 13% were created by using commercially available shade cloth designated as 60, 40, and 20% shade. Radiation levels were imposed on plants by placing PVC frames fitted with shade cloth covers over greenhouse flats each containing one replicate of the 11 genotypes. Quantum sensors (LI-190, LI-COR Environment, Lincoln, NE) were used to measure PAR under each level of solar radiation. Data of PAR value was recorded with a Campbell Scientific 21X data logger (Campbell Scientific, Logan, UT) and summarized on a weekly basis. The number of flowers produced in each pot were recorded and removed weekly for 23 weeks. This experiment contained three replications and was repeated in time. Data were collected from the two trials (repetitions) from March 35th to August 26th and April 1st to September 2nd in 2011.

The experiment was statistically analyzed using analysis of variance (ANOVA) (SAS Institute, Cary, NC) as a split-plot design with the four radiation levels as main plots and the 11 genotypes as sub-plots. The flowering data collected at each of the 23 weeks (under different photoperiods) were considered as repeated measures.

Effect of cumulative PAR on flower initiation

The actual solar radiation data collected during this study were also examined in an attempt to determine if there was evidence to support a minimum radiation level necessary for flowering in seashore paspalum. Hierarchical cluster analysis was used to divide the weekly data

from all experimental units (reps/genotypes/radiation levels) into five discrete classes based on actual radiation level received. The numbers of flowers produced by each experimental unit were then graphically displayed by weekly sample dates for each radiation level class. Cluster analysis and graphic display of the results was accomplished using SAS JMP 8.0.2 (SAS Institute, Cary, NC).

Flowering response of USDA seashore paspalum germplasm collection

The flowering patterns of 88 genotypes from the USDA seashore paspalum germplasm collection were monitored for 15 weeks under natural photoperiods in a greenhouse experiment. Plant materials used in the study were established clonally from stolon nodes. All plants were grown in 10 x 10 cm pots and maintained without clipping in an air conditioned greenhouse maintained at 25 ±2/18 ±2˚C (day/night) temperature under natural photoperiod. Water was supplied twice daily by an automatic irrigation system and fertilizer applied monthly with a water soluble 28-7-14 (NPK) fertilizer (LESCO MacroN, Cleveland, Ohio). The experimental design was a completely randomized block and each genotype was replicated six times. The number of flowers occurring in each pot was recorded and flowers removed weekly for 15 weeks from May to September in 2011, the most vigorous flowering period of seashore paspalum in Georgia. Data were analyzed using analysis of variance by SAS (SAS Institute, Cary, NC) and graphically displayed using SAS JMP 8.0.2 (SAS Institute, Cary, NC).

RESULTS

Solar radiation effect on flower initiation

The results of the analysis of variance were summarized and are presented in Table 6-1.

The combined analysis indicated no differences between the two trials (repetitions) of this experiment. The four radiation treatments imposed created a wide range of radiation levels from 100% to 13% of the unshaded control and significant differences ($p < 0.001$) were found in the number of flowers produced in response to these radiation treatments. Genotypic differences in flower initiation ($p < 0.001$) among the 11 genotypes tested were noted. The numbers of flowers initiated were highly significantly different ($p < 0.001$) among dates indicating that changes in the natural day length over time significantly affected the flowering in seashore paspalum. The radiation by genotype interaction was also highly significantly different ($p < 0.001$) indicating that genotypes responded differently in response to the radiation treatments. Table 6-2 shows the average weekly flowering response of each genotype in response to the four radiation levels averaged over the duration of the experiment. Few flowers were produced at radiation levels of 13, 29, and 41%; for most genotypes flower production was low under any level of shade, with no significant differences observed among the three low irradiation treatments. In contrast for 10 out of 11 genotypes, plants grown under the highest irradiation (unshaded) produced significantly more flowers than plants under shade, In addition, PI 647920 flowered most intensively and produced significantly more flowers at 41% than at 29 or 13% radiation levels. Experimental line 03-501-46 produced very few flowers regardless of radiation level.

Effect of cumulative PAR on flower initiation

PAR values were ranged from 0 during the night and to over 2000 µmol photons m^{-2} s^{-1} around 1400 h. The weekly cumulative PAR over the entire period of the experiments were 226464, 93408, 61824, and 28896 µmol m^{-2} for the 100, 41, 27, and 13% radiation treatments, respectively. Cluster analysis (data not shown) divided mean weekly PAR into discrete classes of 11014-32787, 32787-64445, 64445-90162, 90162-189732, and189732-297736 µmol m^{-2}.

Experimental units receiving weekly cumulative PAR of less than 90162 µmol m^{-2} did not flower (Fig 6-1). However, flowering did occur when weekly cumulative PAR levels were between 90162-189732µmol m^{-2}. Greater flowering was observed at weekly cumulative PAR of 189732-297736 µmol m^{-2}. These results provide support to the concept that seashore paspalum is not able to respond to normally inductive photoperiods when grown under low light intensity conditions.

Genotypic flowering response to photoperiod

The mean number of flowers produced weekly over 15 weeks by each of 88 genotypes of the USDA seashore paspalum collection is presented in Appendix A. Statistical analysis of this data showed significant genotypic differences ($p < 0.001$). Fig 6-2 shows the overall response of the 88 genotypes in relation to photoperiod. Flowering increased rapidly as photoperiod increased above 13 h and peaked near the longest day of 14.4 h on Jun 21. Flowering intensity then declined rapidly and reached a plateau as the photoperiod dropped below 14 h. Flowering response to photoperiod varied greatly among genotypes. Cluster analysis (data not shown) classified the 88 genotypes into seven groups according to the similarity of the flowering habit (Fig 6-3). Flower initiation for the majority of the genotypes increased dramatically as photoperiod reached 14 h and progressed to the longest photoperiod of 14.4 h. Genotypes clustered as the group number 4 (Collier, Excalibur, Kai Luna, and Wai Lua Kauai) with peak flowering that corresponded to a shorter photoperiod before June 2nd. The genotypes classified together as the group 2 flowered the least with no obvious peaks in response to changing photoperiod. Genotypes classified into the group number 1 showed a second flowering peak in the middle of August; however flowering was less intense than during the initial peak in June.

DISCUSSION

The information gained from these two studies suggests that lower light intensity does inhibit the initiation of flowering of seashore paspalum. During this experiment, plants maintained under low light intensity remained vegetative, and increased growth of above ground biomass. Longer internodes and fewer nodes were observed. Similarly, Quedado and Friend (1978) reported that flowering of *Anagais arvensis* L. was increased by increasing irradiation up to 1900 µmol m^{-2} $s^{-1}$and that high photon density was required for flower induction. In the current study, it was difficult to separate the effects of solar radiation from photoperiod effects in a greenhouse radiation study since natural radiation levels increase or decrease with seasonal changes of photoperiod. Further studies with the ability to independently control of light intensity and photoperiod are needed to better define the impact of light intensity on flowering in seashore paspalum.

Most genotypes of seashore paspalum flowered most intensely as day length increased to the longest day of the year. The percentage of plants flowering was the greatest (90%) around the longest day of the year. As day length decreased, the number of flowers produced steadily decreased. This finding provides some support for the designation of seashore paspalum as a long-day plant, which initiates flowering when the day length is longer than their critical photoperiod (Thomas and Vince-Prue, 1999). Substantial genotypic differences in flowering habit were found in response to photoperiod among the 88 genotypes evaluated. This represents the first report of genotypic diversity in response to photoperiod and flowering time in seashore paspalum. Approximately 25% of the lines evaluated flowered very little when exposed to the natural photoperiod of Griffin, Georgia. It is unknown if these lines require even longer photoperiods than were presented in this study or if they simply do not flower. Based on our data,

four lines, ‘Collier’, ‘Excalibur’, ‘Kai Luna’, and ‘Wai Lua Kauai’ and possibly ‘Cloister’, ‘Adalayd’, and ‘HI 10’ could be classified as early flowering genotypes.

Nelson et al. (2010) proposed that induction of flowering in plants can be affected not only by length of the photoperiod, but also by the direction of change (increasing or decreasing) of the photoperiod. Photoperiodism affects both vegetative and reproductive growth (Hay and Heide, 1983). According to Slafer and Rawson (1996), increases in both photoperiod and temperature reduced time to heading of wheat. Torres and Lopez (2011) reported that *Tecoma stans* remained vegetative when grown under a 9 h photoperiod, only 30% of plants flowered when the photoperiod increased to 12 h, and all plants had visible buds and flowered under 14 and 16 h photoperiod. Peterson and Loomis (1949) reported that flower induction in Kentucky bluegrass was related to a combined effect of low temperature where short photoperiod and flowering was prevented by long photoperiod regardless of the temperature. No research on seashore paspalum has previously been conducted to determine if it is a long-day plant, short-day plant or day-natural plant. Flowering should be monitored for a broader period of time in order to accurately determine the flowering habit. Future experiments should be conducted with a wider range of photoperiods under controlled temperature with high light intensity to more accurately determine the critical day-length for flower induction of seashore paspalum.

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Table 6-1. Summary of analysis of variance showing effects of trials (repetitions), solar radiation, genotype, and date on flower number of seashore paspalum in a greenhouse experiment conducted under natural photoperiod from March to Sepember, 2011.

Source of variation	df	Mean square
Trial (T)	1	0.01
Error a: Rep (T)	2	1.39
Radiation (R)	3	18.71***
T × R	3	0.47
Error b: Rep × R (T)	6	0.42
Genotype (G)	10	2.85***
T × G	10	4.20***
R × G	30	1.84***
T × R × G	30	2.98***
Error c: Rep × G (T × R)	80	2.13
Date (D)	22	5.09***
T × D	22	1.33**
R × D	66	3.80***
G × D	220	0.60
T × R × D	66	0.86*
T × G × D	220	0.67
R × G × D	660	0.52
T × R × G × D	660	0.57
Error d	3960	0.63

*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively.

Table 6-2. Least square mean of flower number of 11 genotypes in response of radiation levels over 23 weeks.

	Radiation level (µmol m^{-2} s^{-1})			
Genotype	100%	41%	27%	13%
	flower number per week			
03-501-46	0.01a	0.03a	0.00a	0.00a[†]
03-522-23	0.04a	0.00b	0.00b	0.00b
03-528-126	0.71a	0.08b	0.05b	0.05b
03-531-22	0.17a	0.04b	0.02b	0.01b
Hyb 7	0.05a	0.00b	0.01b	0.00b
PI 647892	0.76a	0.04b	0.03b	0.00b
PI 647894	0.52a	0.06b	0.01b	0.00b
PI 647920	2.01a	0.49b	0.06c	0.01c
Q36313	1.23a	0.30b	0.09b	0.04b
SI 1	0.36a	0.04b	0.01b	0.00b
SI 2000	0.07a	0.00b	0.00b	0.00b

[†] Means in the same row followed by the same letter are not considered different according Student's t-test at α=0.05.

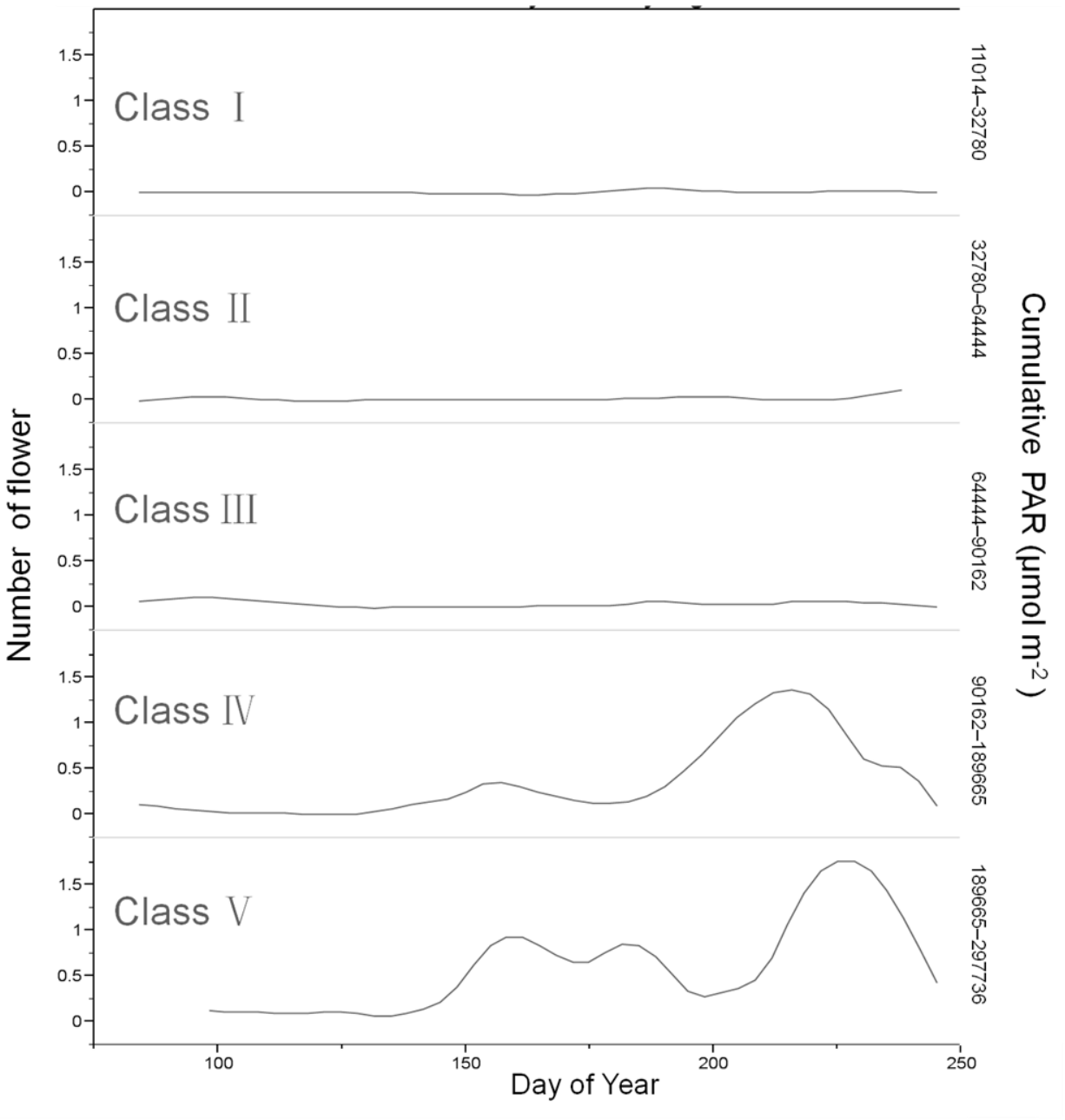

Fig 6-1. The effect of weekly cumulative PAR on flower production. PAR has been divided into five discrete classes with seasonal flower response shown.

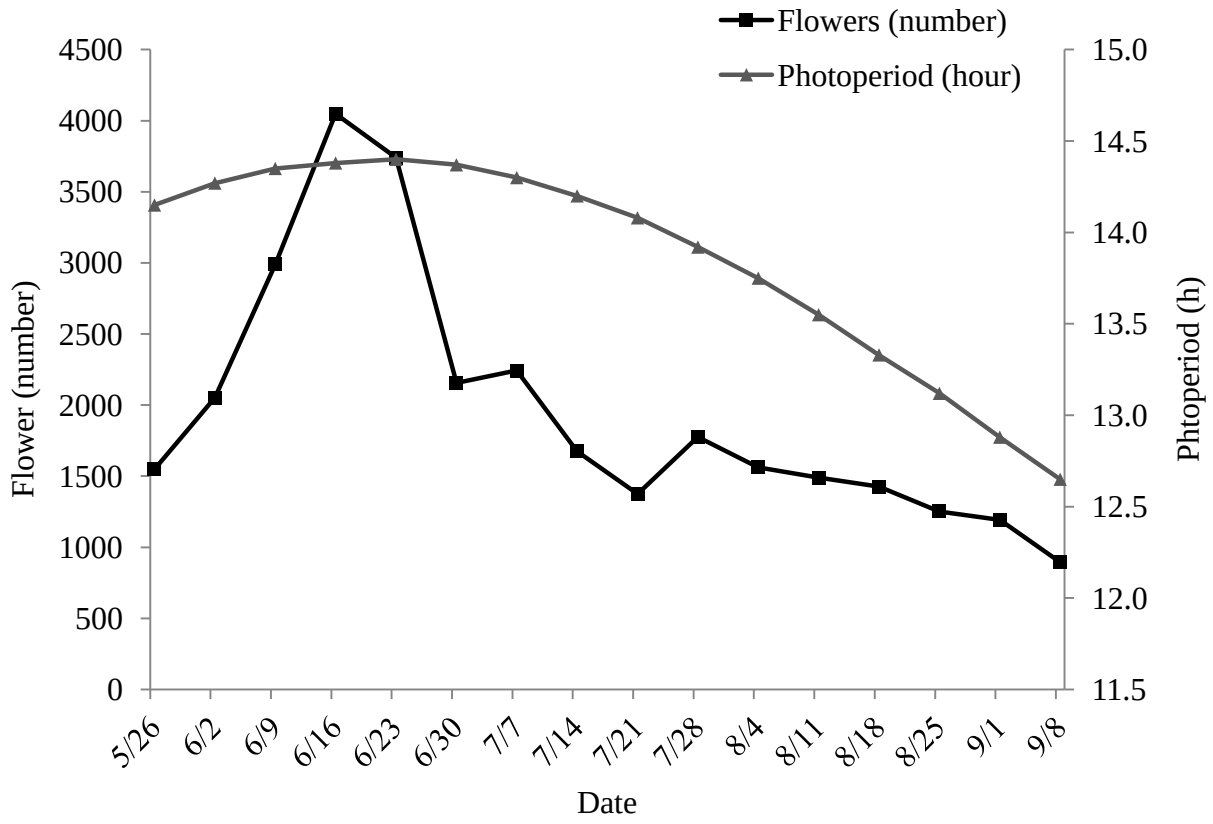

Fig 6-2. Total number of flowers initiated on 88 genotypes in response to natural changes in photoperiod occurring from May to September, 2011.

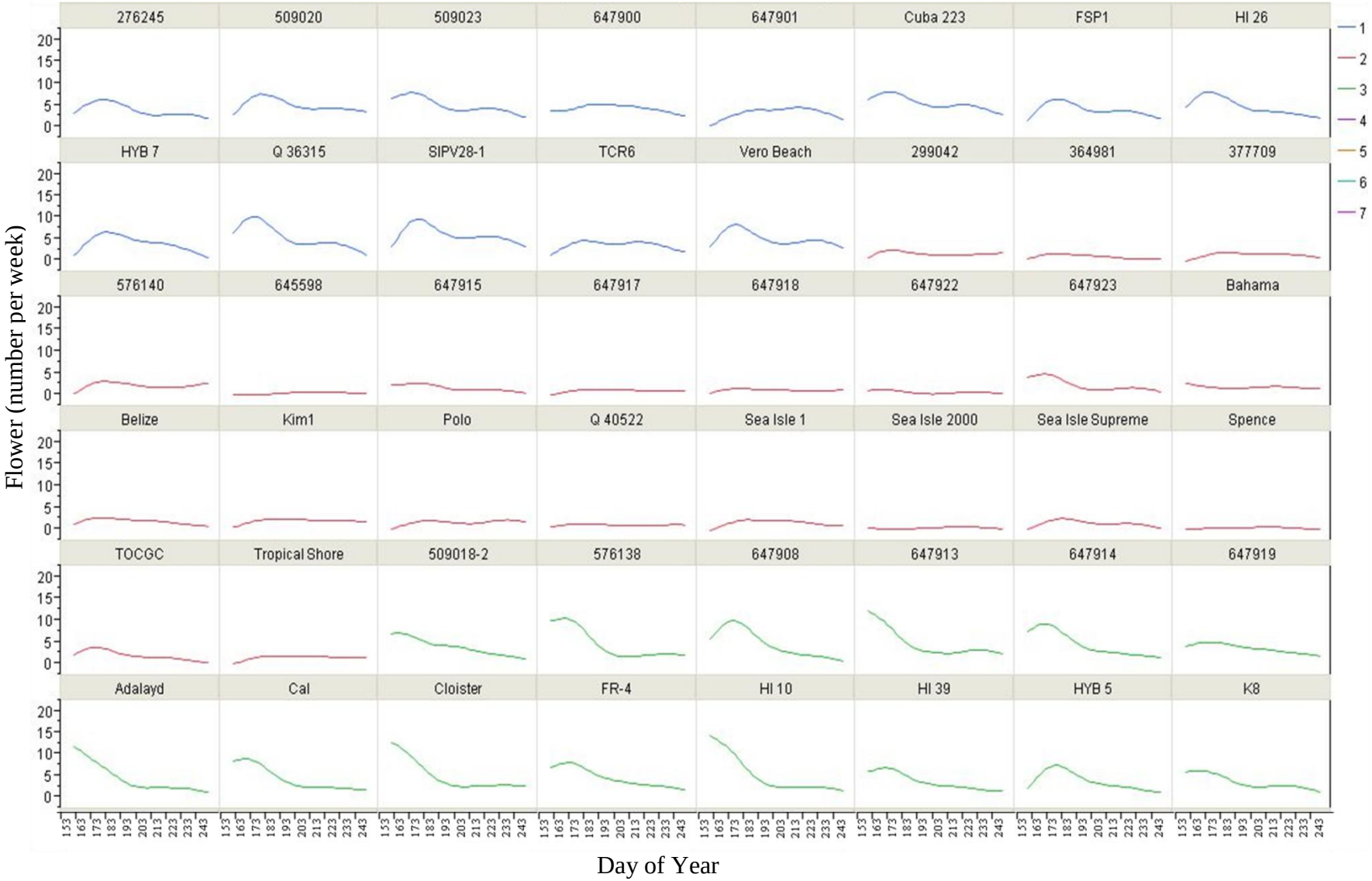

Fig 6-3. Genotypic differences in flowering habit of 88 genotypes in response to natural photoperiod changes during May to September, 2011. Genotypes with same color response curves have similar patterns of flowering response.

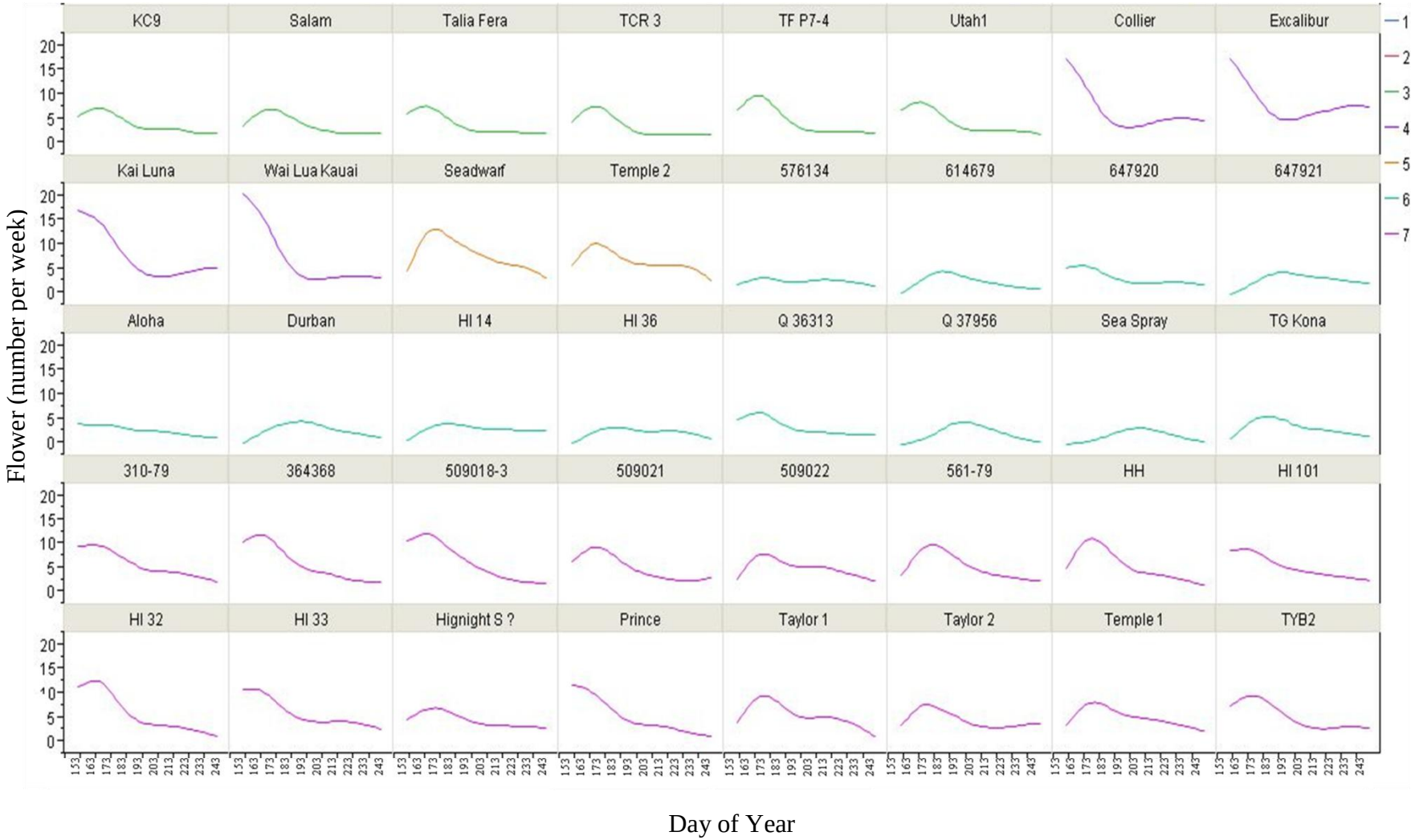

Fig 6-3. (Continue)

CHAPTER 7

CONCLUSIONS

Difficulties in seed production currently limit the development and future adoption of seeded cultivars of seashore paspalum. Results from the series of experiments described in this thesis provide useful information regarding the reproductive biology of seashore paspaloum. This information may prove useful in the development of production strategies to overcome many seed production related issues and perhaps even make it possible to produce seashore paspalum in the southern United States.

Many aspects of plant reproduction must be considered when for breeding for seeded cultivars. Every step of the reproductive cycle is critical. Photoperiod and temperature are the primary external factors that induce flowering. Results of experiments presented in chapter 6 indicate that flowering could only be induced under long day length and with high levels of solar radiation. The observation of flowering habit of 88 genotypes of *Paspalum vaginatum* under a natural photoperiod indicated that the majority of seashore paspalum genotypes flower most intensively when photoperiods reached their maximum of 14.4 h in Georgia. Characterization of the individual flowering habit of the seashore paspalum germplasm collection provided useful reference information necessary for selecting parental lines with synchronized flowering time. Selecting of parental lines with similar flowering habit should greatly improve the opportunity for cross pollination and increased seed production. Several genotypes were also identified that flowered earlier and more intensely under shorter photoperiods. These lines may hold potential as parents for use in southern seed production environments.

A laser particle counter was used to monitor the pollen release patterns in a field setting for 67 days (chapter 4). Pollen release from seashore paspalum occurred around 0800 h and usually lasted only 10 to 30 min. Pollen release was also found to be very inconsistent from day to day. Efforts to correlate pollen release with numerous meteorological variables were largely unsuccessful and only a weakly positive association with solar radiation around the time of pollen the peak release was noted. In future studies, longer periods of time for monitoring pollen release and various meteorological variables would help to create a stronger data set for statistical analysis to reveal other the environmental factors impacting pollen release.

The results presented in chapter 3 provide several options for evaluating the pollen viability of seashore paspalum. A liquid medium consisting with 0.62 g L^{-1} $Ca(NO_3)_2.4H_2O$, 0.24 g L^{-1} H_3BO_3 and 21% sucrose was selected as the optimum germination medium for evaluating the pollen viability of seashore paspalum. An *in vivo* staining technique was adapted and used to determine the nature of pollen-stigma interactions following pollination. These methods were used to confirm the functionality of the male and female reproductive organs of seashore pasaplum and to determine pollen viability, longevity, and pollen-stigma receptivity.

Initial pollen viability ranged from 60 to 88% at room temperature, half of the pollen lost viability about 45 min in the greenhouse and 60min in the field after the release. Due to its wind-pollinated nature, lack of air movement during early morning hours could result in a loss of pollen viability before landing on receptive stigmas. Pollen was also found to be highly sensitive to temperature. Pollen longevity was greatest at 15˚C and temperatures of 30˚C and above dramatically reduced longevity. Based on these results, seed production environments with sufficient air movement and morning temperatures less that 25˚C and would be most desirable

for production of seeded cultivars. Seed production fields in environments without adequate natural air movement may benefit from the use of tractor mounted fans in the early morning to enhance pollen movement.

The results in chapter 5 indicate that seashore paspalum is extremely self-sterile and the level of cross-compatibility among genotypes within the species varies greatly. Among the 30 possible cross-pollinated parental combinations generated from a six by six reciprocal crossing block, approximately 32.3% of the combinations had greater than 50% set seed, indicating high levels of cross compatibility. Hybrid seeds from this crossing experiment had germination rates ranging from 56.7% with to 98.1% and 47% of all hybrids had germination rates over 90%. These results indicate that selection of parental lines with high levels of sexual compatibility is possible and essential for producing maximum seed yields.

Information obtained from this series of studies on flower initiation, pollen release pattern, pollen viability and longevity, sexual compatibility, and the impact of various environmental variables on the reproductive success of seashore paspalum provides highly useful information for future breeding and seed production efforts. The results of this thesis indicate that the development of new seeded cultivars with improved seed yield is possible and that it may also be possible to produce seeded cultivars in southern environments by selecting earlier flowering parental lines. The techniques developed and approaches used to study the reproductive biology of seashore paspalum could also be applied for other grass species.

APPENDICES

APPENDIX A.

FLOWERING HABITS OF A GERMPLASM COLLECTION OF 88 GENOTYPES OF SEASHORE PASPALUM

APPENDIX A. Flowering response of 88 genotypes of seashore paspalum in response to photoperiod over 15 weeks in 2011. Numbers of flowers were summarized on a weekly basis.

	Mean number of flowers per week														
Genotype	Jun-2	Jun-9	Jun-16	Jun-23	Jun-30	Jul-7	Jul-14	Jul-21	Jul-28	Aug-4	Aug-11	Aug-18	Aug-25	Sep-1	Sep-8
276245	2.3	5.2	6.5	7.5	4.5	7.8	2.5	2.7	2.5	2.7	3.2	2.8	3.5	3.0	1.3
299042	0.0	1.2	5.2	2.0	0.8	1.6	1.7	1.2	1.2	0.8	1.7	1.3	1.7	1.2	2.0
364368	8.3	12.2	15.7	11.0	7.2	6.2	4.3	3.3	5.3	5.7	1.7	2.0	2.8	1.7	2.5
364981	0.3	0.3	2.3	2.2	1.5	0.5	1.0	1.5	1.2	0.3	1.0	0.0	0.2	0.3	0.5
377709	0.0	0.2	0.7	2.7	1.8	1.8	1.3	1.0	1.5	1.5	1.2	1.8	1.0	1.2	0.5
509020	2.2	2.5	11.8	7.8	5.2	7.7	4.8	3.2	4.5	3.5	6.0	3.0	5.0	3.7	3.5
509021	4.5	9.0	9.7	12.5	6.3	5.8	4.0	3.3	4.5	3.3	2.5	2.3	1.7	2.7	3.3
509022	0.5	6.0	10.5	9.7	5.0	5.3	4.2	4.7	6.3	6.3	3.5	4.0	3.5	3.0	2.2
509023	6.2	5.8	9.7	10.3	3.8	6.3	2.3	3.2	4.5	4.3	4.3	4.2	4.8	2.2	2.2
576134	1.5	2.2	3.2	5.2	1.8	1.7	1.8	2.5	3.0	3.7	1.5	3.7	1.3	2.0	1.5
576138	8.7	9.7	13.2	11.2	4.2	3.8	1.3	1.7	2.7	1.7	2.0	2.3	2.3	2.7	1.7
576140	0.2	0.0	5.3	3.2	2.0	3.2	2.5	1.3	1.7	2.5	1.8	1.0	2.3	2.3	2.8
614679	0.0	1.0	1.7	4.8	5.5	5.0	2.5	2.2	3.0	2.2	1.7	1.7	1.2	0.5	1.2
645598	0.0	0.2	0.0	0.0	0.0	0.5	0.7	0.2	1.0	0.8	0.7	0.3	0.5	0.5	0.2
647900	5.0	2.2	3.3	4.8	6.3	5.7	4.8	3.8	5.7	5.5	2.7	4.8	4.0	2.3	2.7
647901	0.2	1.2	3.0	2.2	4.7	4.7	3.2	3.0	4.0	5.3	5.0	3.5	3.5	2.5	1.7
647908	3.7	7.7	13.5	10.8	7.8	4.7	3.7	3.0	3.7	2.5	1.0	2.8	1.3	1.5	0.3
647913	11.5	10.3	12.5	6.7	1.7	3.3	4.0	2.7	2.8	1.3	2.5	4.7	3.5	2.5	2.2
647914	4.7	11.2	11.5	8.2	5.7	5.2	2.5	3.0	3.3	3.0	2.7	1.5	2.2	1.8	1.5
647915	2.5	1.5	2.8	3.0	3.0	2.2	0.8	0.7	1.2	0.8	2.0	1.0	0.8	1.0	0.2
647917	0.0	0.3	0.7	2.3	0.2	0.8	2.2	0.5	1.5	1.3	0.3	0.8	0.7	1.2	0.8

APPENDIX A. Continued

	Mean number of flowers per week														
Genotype	Jun-2	Jun-9	Jun-16	Jun-23	Jun-30	Jul-7	Jul-14	Jul-21	Jul-28	Aug-4	Aug-11	Aug-18	Aug-25	Sep-1	Sep-8
647918	0.0	1.3	1.7	1.5	1.0	1.2	1.3	1.2	1.5	0.3	1.7	0.5	0.8	1.0	1.3
647919	3.2	5.7	4.8	5.0	5.2	4.3	3.3	2.8	4.5	3.3	2.5	2.3	2.7	2.3	1.7
647920	4.2	6.0	7.0	5.5	2.5	3.7	1.8	1.5	2.5	2.7	1.7	2.7	1.3	3.5	0.8
647921	0.2	0.3	0.8	3.3	3.7	5.8	4.0	2.8	3.5	2.8	3.5	3.0	1.8	2.7	1.8
647922	0.0	3.0	0.3	1.3	0.5	0.2	0.2	0.2	0.3	0.2	0.7	1.7	0.2	0.2	0.5
647923	3.0	5.0	6.0	5.0	2.3	1.5	0.7	1.3	1.3	1.0	2.0	1.7	1.5	1.7	0.3
310-79	9.0	9.5	10.3	10.7	6.8	6.8	5.2	3.0	5.2	4.5	4.2	4.2	2.5	3.2	2.0
509018-2	5.3	9.2	7.8	4.3	4.0	2.8	5.5	4.7	3.5	2.2	2.2	2.7	1.3	2.7	0.5
509018-3	8.7	12.7	13.7	13.5	6.7	8.2	6.8	4.0	5.5	3.3	2.2	2.7	2.2	1.7	2.2
561-79	2.2	5.7	10.0	13.3	8.8	6.8	5.8	4.8	4.7	3.5	3.3	3.7	2.3	2.8	2.2
Adalayd	11.3	12.0	6.3	8.5	6.5	3.5	1.8	1.7	2.5	3.8	1.2	2.2	2.3	2.2	0.7
Aloha	5.2	1.5	3.7	6.2	2.8	2.8	1.7	3.0	2.8	2.3	2.2	1.3	1.2	1.7	1.0
Bahama	2.7	2.5	1.3	2.2	1.0	1.8	1.8	1.0	2.2	2.0	2.3	1.3	1.8	1.0	1.7
Belize	1.0	1.5	3.7	3.3	1.7	2.3	2.5	1.0	2.7	2.0	1.7	1.2	0.7	1.3	0.7
Cal	7.2	9.2	11.3	7.5	5.0	4.2	2.3	2.3	3.0	1.5	3.5	1.7	2.2	1.8	1.7
Cloister	11.3	14.0	9.0	8.7	4.0	3.8	2.7	2.3	2.8	3.2	2.2	2.7	3.8	2.2	2.7
Collier	15.0	19.2	10.8	8.2	3.7	2.8	3.7	4.2	3.0	4.8	5.0	5.3	5.5	4.5	4.5
Cuba 223	6.0	5.8	10.7	9.7	3.8	7.2	5.0	3.7	3.0	7.0	4.8	6.2	3.2	3.8	2.7
Durban	0.2	0.7	2.0	5.2	2.5	3.8	5.7	4.8	3.0	2.5	2.2	2.3	2.0	1.7	1.0
Excalibur	16.8	15.7	12.5	8.3	5.0	5.0	3.8	5.3	7.0	7.7	4.8	6.7	9.3	8.7	6.3
FR-4	5.5	8.0	10.3	8.7	4.3	4.2	4.5	3.8	3.8	3.2	2.2	3.0	2.7	2.7	1.3

APPENDIX A. Continued

	Mean number of flowers per week														
Genotype	Jun-2	Jun-9	Jun-16	Jun-23	Jun-30	Jul-7	Jul-14	Jul-21	Jul-28	Aug-4	Aug-11	Aug-18	Aug-25	Sep-1	Sep-8
FSP1	0.5	2.8	8.8	7.2	5.0	6.0	3.5	2.5	2.5	4.3	4.7	4.5	2.2	2.3	2.2
HH	2.3	7.8	16.0	13.0	5.8	8.8	4.5	2.2	5.5	3.8	3.8	3.2	1.8	2.3	1.3
HI 10	13.5	13.0	12.8	10.8	4.0	3.5	3.3	1.8	2.8	2.7	2.7	1.2	3.2	2.5	1.0
HI 101	8.2	7.2	13.3	6.8	6.0	5.2	4.3	4.7	4.7	4.0	2.5	4.0	3.2	2.0	2.8
HI 14	0.2	1.8	3.3	4.2	3.7	5.2	2.7	2.3	3.5	2.7	3.2	2.7	3.0	1.5	3.2
HI 26	2.3	7.7	10.8	7.5	6.3	6.3	4.0	2.2	4.5	4.0	3.5	3.2	3.0	2.2	2.2
HI 32	10.3	10.0	17.7	14.0	5.0	5.0	3.2	4.3	3.8	3.3	3.7	2.5	2.2	2.0	1.0
HI 33	9.0	12.5	12.3	8.7	6.0	5.5	4.0	4.8	4.0	3.5	5.3	4.7	3.0	3.3	2.7
HI 36	0.0	0.5	2.0	4.0	3.0	3.7	3.0	1.5	2.2	2.0	4.2	2.0	1.8	1.7	0.7
HI 39	5.0	6.8	8.0	8.2	3.0	3.7	3.2	3.0	2.3	2.7	3.2	2.0	1.2	1.2	2.0
Hignight 5	3.8	6.3	6.3	9.8	4.8	5.7	4.8	3.0	3.3	3.7	4.3	2.3	3.3	3.5	2.7
HYB 5	0.7	4.5	7.0	12.2	4.3	5.3	3.5	2.7	4.0	1.7	3.0	2.8	1.5	1.0	1.3
HYB 7	0.8	1.3	8.7	6.7	6.8	5.7	4.5	2.7	5.2	3.8	4.8	2.7	2.3	1.5	0.7
K8	5.2	6.7	6.8	5.5	5.0	3.8	2.2	1.5	3.0	2.7	3.0	3.0	2.3	1.8	1.2
Kai Luna	17.0	13.3	20.2	13.8	6.3	8.0	4.2	3.7	3.2	3.5	4.8	3.5	4.2	7.3	4.2
KC9	4.2	6.5	10.3	5.8	6.0	3.8	2.8	1.7	3.0	3.7	4.2	1.7	1.7	1.7	2.3
Kim1	0.7	0.3	2.7	2.3	2.3	2.3	2.2	2.5	2.7	1.3	1.7	2.5	1.7	2.5	1.3
Polo	0.2	0.3	1.7	2.5	2.0	1.8	1.7	1.3	1.2	1.0	1.5	2.5	2.7	2.2	1.3
Prince	10.0	13.8	9.5	10.7	5.3	4.0	4.5	2.7	4.2	3.7	3.7	1.7	1.8	1.2	1.5
Q36313	4.2	4.2	9.0	7.7	3.3	2.0	2.5	2.2	3.2	2.7	1.5	1.7	1.8	2.3	1.3
Q36315	3.5	10.7	12.8	11.8	5.3	5.8	3.5	2.7	5.0	2.8	6.2	3.5	3.5	2.2	1.2

APPENDIX A. Continued

	Mean number of flowers per week														
Genotype	Jun-2	Jun-9	Jun-16	Jun-23	Jun-30	Jul-7	Jul-14	Jul-21	Jul-28	Aug-4	Aug-11	Aug-18	Aug-25	Sep-1	Sep-8
Q 37956	0.0	0.2	0.8	1.0	1.0	6.7	4.2	4.2	3.8	2.2	2.7	1.5	0.7	0.5	0.5
Q 40522	0.5	1.2	0.8	1.8	1.0	1.5	0.8	0.5	1.3	1.3	0.8	0.5	1.0	1.8	0.7
Salam	2.0	6.8	5.7	10.3	4.8	4.7	4.5	2.0	3.7	2.0	2.3	1.5	2.2	2.5	1.8
Sea Isle 1	0.0	0.0	0.7	4.5	1.2	2.3	1.3	2.2	2.2	2.0	1.7	1.5	0.3	1.2	1.0
Sea Isle 2000	0.5	0.0	0.0	0.0	0.2	0.3	0.0	0.7	0.3	0.7	0.7	0.3	0.8	0.0	0.2
Sea Isle Supreme	0.0	0.2	1.7	3.5	2.7	2.3	1.2	0.8	1.3	1.3	1.0	2.2	1.2	0.3	0.3
Sea Spray	0.0	0.0	0.4	0.2	1.0	3.2	2.0	3.8	3.3	2.8	1.5	2.0	0.5	0.8	0.3
Seadwarf	1.8	7.8	18.3	16.2	7.5	10.2	9.8	6.7	10.3	4.3	5.7	6.0	6.2	4.8	2.3
SIPV28-1	1.3	5.3	11.8	14.7	4.5	6.7	4.8	5.0	5.2	5.2	6.7	6.2	4.3	4.3	3.2
Spence	0.0	0.2	0.2	0.2	0.7	0.5	0.0	0.0	1.2	0.8	0.2	0.5	0.2	0.0	0.2
Talia Fera	4.5	7.0	11.7	5.8	4.7	3.5	1.8	2.5	2.8	2.0	2.5	2.3	1.8	2.3	2.0
Taylor 1	1.7	7.8	11.7	10.8	8.2	5.7	4.8	2.5	7.0	5.8	4.5	4.8	2.8	3.2	0.7
Taylor 2	1.3	6.0	11.2	7.5	5.3	5.7	5.3	3.0	3.0	3.3	2.7	3.2	3.5	4.3	3.3
TCR 3	2.7	6.3	9.8	9.3	4.2	3.5	2.3	1.2	1.8	2.2	1.8	1.5	2.5	1.0	2.0
TCR6	1.3	1.5	4.2	6.0	4.5	4.8	2.2	2.3	5.3	5.2	3.3	4.8	2.7	2.2	2.2
Temple 1	2.2	5.7	9.3	10.8	6.2	5.3	4.8	4.7	5.8	5.0	3.7	3.7	3.3	3.0	2.2
Temple 2	4.2	8.2	14.3	10.8	5.5	5.2	6.0	4.5	6.3	4.8	5.3	5.2	4.8	5.0	1.5
Temple 2	4.2	7.3	13.7	12.3	6.7	5.8	6.2	5.3	7.2	5.2	5.3	5.8	5.7	5.7	1.3
TF P7-4	4.5	9.2	12.5	12.7	3.0	5.2	3.0	2.2	2.5	3.3	1.5	3.0	1.5	2.7	2.0
TG Kona	0.2	2.5	5.7	5.2	6.2	5.7	4.0	2.0	2.3	4.0	2.7	2.2	2.0	1.2	1.8
TOCGC	0.5	4.7	4.8	3.8	2.7	1.7	1.3	2.0	1.3	1.7	2.0	1.0	0.7	0.5	0.3

APPENDIX A. Continued

	Mean number of flowers per week														
Genotype	Jun-2	Jun-9	Jun-16	Jun-23	Jun-30	Jul-7	Jul-14	Jul-21	Jul-28	Aug-4	Aug-11	Aug-18	Aug-25	Sep-1	Sep-8
Tropical Shore	0.0	0.3	1.3	2.2	2.5	1.2	1.7	1.3	2.2	1.5	1.5	1.8	1.5	1.2	1.5
TYB2	5.7	10.3	9.2	12.2	6.3	6.3	4.7	2.8	4.2	1.7	2.7	3.7	3.3	3.3	2.5
Utah1	5.2	8.2	11.3	8.5	4.5	4.0	2.5	2.2	3.5	2.3	3.0	2.5	2.2	2.8	1.5
Vero Beach	1.5	5.5	10.7	11.5	4.3	5.2	4.5	4.0	3.0	4.3	4.3	5.8	4.5	4.0	2.5
Wai Lua Kauai	19.0	18.3	20.3	11.3	5.5	4.3	3.7	1.3	4.3	3.7	3.7	3.2	2.8	4.2	2.8