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Development of an efficient sea oats breeding program for coastal restoration

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DEVELOPMENT OF AN EFFICIENT SEA OATS BREEDING PROGRAM FOR COASTAL RESTORATION

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This dissertation is dedicated to my adorable nieces and nephews; Lynnette, Laureate, Lytton and Don Michael. Let this be your inspiration to value and treasure education. With God's mercy and determination everything is possible. I also dedicate this work to my lovely mum, dad, Susan, Tony and to all my family members, who have encouraged and provided me moral support throughout the entire course.

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ABSTRACT

Uniola paniculata (sea oats) has been used extensively to build and stabilize dunes along the southern Atlantic and Gulf of Mexico coasts of United States. A breeding program could enhance coastal restoration by developing improved plants for beach restoration. The goal was to initiate a successful breeding program for sea oats adapted to low dune profiles, with high seed yield and germination, and superior vegetative biomass essential. The specific objectives were to: 1) examine effect of storage environment on sea oats seed germination; 2) determine time necessary for sea oats seeds to germinate; 3) determine sea oats seed moisture content; 4) determine survival and performance of vegetative sea oats plants and sea oats seedlings at beach environments with shallow dune profiles; 5) develop efficient methods to identify saturation tolerant sea oats lines; 6) determine sea oats seed yield in natural and artificial environments and 7) identify fungal and bacterial pathogens of sea oats seed. Sea oats seed stored in hermetically sealed jars at room temperature had highest average germination and seed germination was highest 21 days after germination. Sea oats seed moisture content, ranged from 6 to 16 %, and was negatively correlated with germination. Small sea oats seedlings had highest mortality however, seedling cost significantly less than vegetative plants. Increasing seedling densities could reduce production costs and result in acceptable survival rates accompanied with genetic diversity. We found that small seedlings flooded continuously to 14 cm depth in greenhouse for 3 months could predict sea oats survival in saturated beach conditions after 6 months. In 2007, 2009, 2010, and 2011 we determined sea oats seed yield in natural and artificial environments. Consistent seed yields were not obtained for either environment; however, sea oats seed were possible to be produced in artificial production nurseries. Finally, to determine seed pathogens colonizing sea oats seed, bacteria and fungi were isolated from sea oats seed harvested in 2011

and identified using both morphological and molecular techniques. The dominant bacterial genera colonizing sea oats seed were *Bacillus* and *Enterobacter*; while the dominant fungal genera were *Fusarium* and *Curvularia.* Additional research needs to be done to establish the pathogenicity or endophytic status of these species.

CHAPTER 1: INTRODUCTION

The health of Louisiana's coast plays a vital role in the economics of the United States. This region produces 30% of the nation's sea food, provides wintering habitats for migratory waterfowls and serves as the entry point for 18% of America's foreign and domestic energy supply (Restore or Retreat 2007). Sand dunes play a large role in the protection of coastal areas (O'Connell 2003). Coastal regions characterized by dune formations face less damage during hurricanes than do coastal regions lacking such features. Stabilization of exposed dunes is attempted by planting with native dune species such sea oats (*Uniola paniculata* L.) (Westra and Loomis 1966; Woodhouse 1978). Sea oats are planted throughout the northern Gulf of Mexico coast on beaches to reduce coastal erosion. Sea oats is a perennial dune grass extremely valuable in dune restoration projects. Sea oats is regarded as a pioneer species for beach restoration endeavors (Johnson 1990). The result of sea oats vegetative growth and sand burial is development of sand dunes that protect coastal communities, industries, infrastructures and residents (Wagner 1964).

Sea oats can be propagated in three ways: vegetatively using rhizome material, sexually using seeds, or by using micropropagation techniques (Lonard et al. 2011). While sea oat propagation via rhizome material is comparatively the easiest of three methods, one consequence is elimination of genetic diversity, because every plant has an identical genotype. Genetic diversity is necessary for established sea oats to be able to adapt to environmental changes (Huenneke 1991; Kutner and Morse 1996; Ledig 1996). Sexually reproducing sea oats from seeds preserves genetic diversity; however there is limited access to sea oats seed. Reproducing sea oats using micropropagation techniques can maintain genetic diversity (Valero-Aracama et al. 2007; Valero-Aracama et al. 2010; Lonard et al. 2011) if care is taken to include many

genotypes. However, it requires specialized equipment and trained personnel. Therefore, few are able to micropropagate sea oats.

Among the major challenges faced by those engaged in coastal restoration is the insufficient production and availability of planting material for restoration projects due to limited seed production, seed dormancy, poor germination, poor seed storage and low seedling survival. Sea oats spread in the Northern Gulf of Mexico coast may also be impeded by shallow dune profiles. Many beaches in the Northern Gulf of Mexico coast have very shallow dunes. When dunes are shallow, sea oats grow at or near the water-table, which then contributes to plant death (Hester and Mendelssohn 1987). Improved cultivars that can adapt to the low dune profiles are necessary, especially for Louisiana with the lowest dune profiles (Monteferrante et al. 1982; Hester and Mendelssohn 1987). Improved cultivars are genetically distinct, which mimics undisturbed environments; have increased seed production and germination, which is necessary for self-sustainability; and have superior vegetative biomass essential for reducing coastal erosion. Field trials are needed to identify populations that are adapted to the low dunes of Louisiana and have increased seed yield, to accelerate utilization of sea oats in beach restoration projects.

The objectives of this study were to: 1) examine the effect of storage environment, specifically, storage temperature and container on sea oats seed germination, 2) determine the length of time necessary for sea oats seeds to germinate, 3) examine sea oats seed moisture content, 4) determine seed pathogen incidence during germination, 5) determine if survival and performance differed for large vegetative sea oats plants and small sea oats seedlings at beach environments with low dune profiles, 6) develop greenhouse protocols to identify saturation

tolerant sea oats lines, 7) determine sea oats seed yield in natural and artificial environments and 8) identify fungi and bacteria colonizing sea oats seed.

CHAPTER 2: LITERATURE REVIEW

2.1 Sea oats

2.1.1 Plant description, morphology, and taxonomy

Sea oats is a semi-tropical, rhizomatous perennial, C4 grass dominating many beach and dune environments (Wagner 1964; Sylvia 1986; Hester and Mendelssohn 1989; Bachman and Whitwell 1995). It is a tall and erect plant, which grows as tall as 1-2 meters with leaves measuring up to 20-40 cm in length and approximately 0.6 cm (1/4 inch) in width and have pointed tips hence in many ways resembling common agronomic oats (Wagner, 1964; Westra and Loomis, 1966; Harper and Seneca, 1974). Leaves are thick, sturdy, and deeply furrowed at the abaxial surface (Kearney 1900). The inflorescence is a panicle of many laterally compressed spikelets each with 10-20 florets and the lower 4-6 florets are normally empty (Hitchcock and Chase 1971). Flowering spikelets are flat and measure about 20-50 cm (Radford et al. l968). Seed heads are large, and become yellow brown and straw colored in late summer (Amos 1997). Sea oats trap wind-blown sands that eventually mound to form dunes (Johnson 1990). Sea oats have dense surface roots and deep penetrating roots (Hester and Mendelssohn 1987). Rhizomes are elongated and extensively creeping in habit. They readily root upon burial in sand (Hitchcock 1951; Clewell 1985; Duncan 1987). Rhizomes produce extensive lateral growth, which stabilizes continuous dune ridges (Duncan 1987). Sea oats is an herbaceous plant with buds arising from the internodes. These buds are formed randomly around the circumference of the stem within the nodal region and sand deposition somehow stimulates elongation of the internodes and growth of more buds (Hester 1985).

Sea oats belongs to kingdom: Plantae, subkingdom: Tracheobionta (vascular plants), class: Liliopsidia (monocotyledons), order: Cyperales, family: Poaceae (grass family), Genus:

Uniola, species: paniculata.(http://plants.usda.gov). The genus *Uniola* L. is a tetraploid with chromosome number 2*n*=40 (Watson 1992; Radford et al. l968).

2.1.2 Sea oats geographical distribution and range of habitants

Sea oats have an extensive range from temperate shorelines on the Atlantic Coast of the United States (e.g. North Hampton County, Virginia) southwards into Dos Bocas, Tabasco, Mexico (Wagner 1964). Sea oats are also widely distributed in the Bahama lslands and some sandy areas of Cuba's northwestern coast, although it is absent from the remainder of the West Indies (Wagner 1964). Throughout a large portion of its range, sea oats is a major component of the dune vegetation. Along the southeastern Atlantic coast sea oats is often the dominant foredune sand binding grass (Wagner 1964; Woodhouse et al. 1967).

Sea oats has a coastal zone distribution and is limited to a sand dune habitant (Seneca 1972). Sea oats is the most widely recognized plant species on the coastal pioneer and frontal dune sites throughout the Gulf and the southern Atlantic coastal region (Craig and Service 1984; Duncan and Duncan 1987; Barnett 1997) (Figure 1.1). Only a few plant species can tolerate the stresses of a dune environment, particularly frontal dune sites. Foredune plants must be able to survive being buried by blowing sand, sand blasting, salt spray, salt water flooding, drought, heat, and low nutrient supply (Salmon et al. 1982; Craig and Service 1984; Barnett 1997; Hill 2001). In Louisiana, sea oats is absent in coastal regions where the water table is raised to the soil surface (Hester and Mendelssohn 1989).

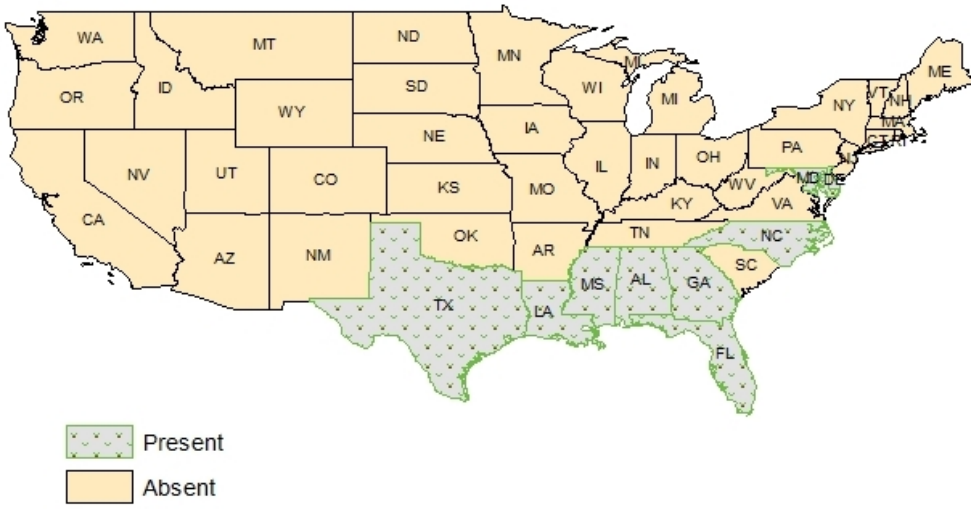

Figure 1.1 Geographical distribution of sea oats in the United States

Coastal dunes generally have 3 vegetation zones based on soil salinity (Salmon et al. 1982; Craig and Service 1984). Landward of the highest tides, pioneer or frontal zone sites are stabilized by sand trapping action of various rhizomatous grasses like sea oats that are tolerant to salt spray. Landward of the frontal zone area, the backdune zone supports less salt tolerant grasses and forbs as well as shrubs and some trees. Farthest from the ocean is the forest zone vegetation in this zone is transition from maritime to non-maritime species.

2.2 Coastal sand dunes

Coastal sand dunes are typically formed through the trapping of sand by dune vegetation. The type of vegetation that grows on dunes has special adaptation characteristics that allow the vegetation to establish, grow, and trap sand in the harsh conditions of coastal areas. In the absence of such vegetation, the wind can act on the exposed sand, forming migrating dunes that move back and forth with the wind (Chapman 1976). The vegetation of coastal dunes usually becomes established when seeds or plants are trapped along shorelines during very high tides

(Chapman 1976). Trapping of seeds or plants is the first step in the sequence of events and circumstances necessary for the development of coastal dune vegetation. The next crucial factor in the process is that the seeds or plants must be suitable for establishment in the area. The establishment of suitable seeds or plants must then be followed by one or two years of favorable growing conditions. However, on most sites the complete sequence does not occur. The early phases occur frequently on many sites, but the embryo dunes are usually destroyed by storm activity before sufficient establishment occurs (Woodhouse et al. 1967; Woodhouse 1978). Consequently, the sequence of events can be bolstered if the area can be stabilized for a long enough period of time with different stabilization techniques. Thus, one of the major functions of coastal dunes is to serve as a reservoir of sand for beaches (Salmon et al. 1982; Rogers et al. 2003).

2.2.1 Physical environment of the sand dunes

The sand dune environments are subject to selective forces on vegetation both temporally and spatially. Extreme episodic storm events can immediately effect the vegetation community through complete destruction, burial and/or exposure from sediment displacement and storm surge over-wash, mechanical damage from increased wind speeds, and periodic inundation by saltwater (Costa et al. 1996). The vegetation is subject to high incident sunlight due to lack of a plant canopy (Oosting 1954). In addition, soil surface temperatures can exceed 50°C during the growing season, and air temperatures often exceed 32°C at midday. The topography of the sand dune creates a mosaic pattern of soil water content, wind patterns, and exposure to salt spray deposition (Oosting 1954). High winds may cause sand abrasion and mechanical damage to plants as well as cause blowouts that expose roots and rhizomes to desiccation. Soil (i.e. sand) has a low water holding capacity, thus soil nutrients are quickly leached out, and soil evaporation

rates are relatively high due to high wind and temperatures. Although sand dune systems are usually not deficient in plant micronutrients (e.g. calcium, magnesium, and sodium), receiving many of these cations through salt spray, they are characteristically deficient in plant macronutrients (i.e. nitrogen, phosphorous, and potassium) essential for growth and carbon allocation.

2.2.2 Physical tolerances of sea oats

Sea oats tolerate high temperature conditions; they can withstand surface soil temperatures as high as 52-53 °C (125-127 °F) and air temperature around 35-38 °C (95-100 °F) (Oosting 1954). They are highly tolerant to inundation by sea water for short periods, however, they cannot tolerate prolonged periods of inundation (Hester and Mendelssohn 1989). Sea oats can thrive under salt spray conditions. It is assumed that salt spray provides a source of micronutrients in the heavily leached soils of beach stands (Hester and Mendelssohn 1989; Stalter 1993). Sea oats can withstand harsh environmental conditions that include drought. Stomata in sea oats close when soil moisture reaches 8.5% (Hester and Mendelssohn 1989). Sea oats do not tolerate water logging of roots (Hester and Mendelssohn 1987; Hester and Mendelssohn 1989). Tolerable soil pH for *Uniola* species ranges from 6.9 to 7.9 (Oosting 1954). Sea oats are colonized with beneficial microorganisms such as VAM (Vesicular-Arbuscular Mycorrhizal) fungi, which increase the surface area for nutrient absorption to plant roots. The hyphae of these fungi also help in binding sand grains into aggregates and aid in stabilizing substrata (Sylvia 1986).

2.3 Growth seasons of sea oats

The growing season of sea oats depends upon the geographic location. In North Carolina, germination of seeds occurs from late May to mid-June and growth the season occurs from May to September (Tyndall R.W 1987). In Florida, sea oats require three growing seasons

to flower and set seed (Wunderlin 1982). In Texas, flowering and seed formation occurs from April through November (Gould 1978). Sea oats are dormant during winter from the onset of cooler weather in early fall until spring when it warms up. Sea oats respond to lower winter temperatures by synthesis of anthocyanins which color the younger leaves light pink to a dull purplish green making it difficult on occasion to determine whether individuals are living or dead. The first indication of growth renewal is the development of new leaves which soon replace those of the preceding year. Later on, elongation of the flowering culm begins.

2.3.1 Sea oats phenology

Floral initiation is believed to be temperature controlled, beginning earliest in the south and progressing northwards (Harper 1973; Colosi 1979). By early July the spikelet primordial begin emerging from the protecting leaf sheaths. Rapid elongation of the culm internodes soon exposes the remainder of the panicle. The spikelets at this stage are rather small, composed of the two basal glumes, a series of sterile lemmas, and then a variable number of embryonic florets. These develop rapidly and by late July to early August, the spikelets are almost fully expanded. The spikelets are described to comprise a variable number of florets (ranging from 5-20 florets), which are typically hermaphroditic, unisexual, or sterile. Within each spikelet, the fertile florets are subtended by 3-6 sterile or neuter florets (Crewz et al. 1987). Florets develop and open (anthesis) from the base of the spikelet up toward the developing tip (Wagner 1964). At anthesis, the lemma and palea of the floret separate slightly, allowing the three anthers to be exposed to the wind and the two plumose stigmas to be exposed, one on each side of the floret through the lateral overlap of the lemma and palea margins (Wagner 1964). Florets open and close in the early morning and open only once per season (Colosi 1979). Seed development potentially begins with anthesis of the lowest fertile floret of the spikelet. Pollination may occur

at that time and the fertilized ovule may then begin to develop into a seed (Colosi 1979). Walsh (1994) noted that sea oats is wind pollinated and cross-pollination may be required for sea oats to produce large quantities of seeds.

The floret is composed of a large lemma and slightly smaller palea nested together, spoon fashion, including between them three stamens and ovary (Wagner 1964). At anthesis the lemma and palea separate slightly allowing the three anthers to be exposed to the wind and the two plumose stigmas to be exposed, one on each side of the floret through the lateral overlap of the lemma and the palea margins. By mid to late August all fertile florets have undergone anthesis. A typical spikelet has 2 glumes above which are 4 sterile lemmas followed by 6 to 8 fertile florets. In a terminal position are two or more incompletely developed florets. Despite the production of 6 to 8 fertile florets per spikelet few of these ever set seed. Although apparently viable a great number of ovaries subsequently abort.

In Louisiana, typically seed numbers range from 0.00-9.53 per culm depending on the population (Walsh 1994). Spikelets are rapidly disseminated by wind, and are usually buried by sand accretion. Ordinarily, sea oats spikelets after dispersal lie dormant until spring when all viable seeds germinate. Apparently some dormancy mechanism prevents germination in fall even if the environment is favorable (Woodhouse et al. 1967). Germination is prevented in the fall by the seed coat that, though permeable to liquids and gases, resists the expansion of the embryo referred to as thermally-sensitive physiological block (Wagner 1964). By late May, all viable seeds germinate in few weeks and then greatest concentration of seedlings can be found on the upper beach where sand accretion is light (Wagner 1964). Grisebach (1864) describes the grass having some flowers that are fertile and 3- androus while Hitchcock (1950) mentioned that the spikelets are apparently sterile, with neither caryopses nor stamens found (Wagner 1964).

Although the spikelets of sea oats are deciduous, falling from the panicle over late fall and early winter, the florets remain attached to the rachis of the spikelet so that whatever seeds are contained are distributed as a unit (Westra and Loomis 1966). Spikelets falling on sites of sand accretion are quickly buried and if this burial is not excessive (endosperm reserves allow the coleoptile to elongate no more than 6 inches), viable seeds will produce seedlings the following spring (Tyndall R.W 1987).

Self-pollination fails to increase genetic variability and therefore stunts a species' abilitiy to adapt (Roalson and McCubbin 2003). Most plant species would tend to self-pollinate when both male and female gametes are present in a single flower (Roalson and McCubbin 2003). Self-incompatibility (SI) is considered to be one of the most important strategies used by flowering plants to prevent self-fertilization and consequent inbreeding depression caused by homozygosity. Members of the grass family, the Poaceae, exhibit a gametophytic self-incompatibility (GSI) system which is controlled by at least two multiallelic and independent loci, S and Z (Lundqvist 1954; Hayman 1956).

Crewz (1987) observed that sea oats is a plant having a hermaphroditic flowering system, thus it could be an obligate outcrosser and possess a degree of self-compatibility, *i.e.,* pollen transfer would be probably among flowers of the same plant. He further pointed out that Wagner's (1964) observation that sea oats did not show apomixis could be attributed to having sea oats samples that were not under proper environment induction conditions. Under controlled condition experiments on sea oats where fruit set reduction was deduced to self-incompatibility, low fruit set could be due to the experimental set-up itself that inhibits pollination (Crewz et al. 1987). Hester and Mendelssohn (1987) proposed that this may be a consequence of limited

cross-pollination. If cross-pollination is limited, then the seed set that does occur may be a result of selfing, and self incompatability would explain the poor seed set that results.

2.4 Sea oats propagation methods

Sea oats plants exhibit both sexual and asexual modes of reproduction. It reproduces asexually by forming buds around the stem and sexually via seeds.

2.4.1 Vegetative propagation

The primary natural method of reproduction in the dune habitat is through vegetative means. Buds are formed around the stem base and the formation angle of the buds determines whether a shoot or lignified rhizome develops. Acutely angled buds become tillers and right angled buds become rhizomes. Internodal portions of the rhizomes decay leaving the nodal regions with associated culms to root and become new plants. As sand is deposited around the base of the plant, these rhizomes establish roots in dune sand. Vegetative reproduction for dune propagation is reportedly expensive (Lewis *et al.,* 1986), although it is the primary means of reproduction in the dune habitat (Bachman and Whitwell 1995). This means of reproduction only creates larger stands of sea oats and colonization of new areas that are spatially distant is accomplished primarily by seed (Holmes, 1983). Survival of transplants from one dune stand to another dune has been shown to be low. So nursery or greenhouse production of planting stock is essential for large-scale production of high quality transplants (Seneca 1972).

2.4.2 Micropropagation of sea oats

Micropropagation has a great commercial potential due to the speed of propagation, decreased production space requirement, and the ability to multiply elite clones exhibiting superior growth and enhanced stress tolerance (Garton and Mosses, 1985; Kane et al., 1989).

Micropropagation also can be used to establish and maintain virus-free plant stock. This is done by culturing the plant's apical meristem, which typically is not virus-infected. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants (http://www.biotech.iastate.edu/lab_protocols/AV_Micropropagation.html). Micropropagation can be achieved by using different parts of the plant as the primary 'explant' (synonym of 'propagule' in situ) such as, the apical meristem, nodal bud, shoot buds, axillary buds, or through production of somatic embryos, a process commonly known as somatic embryogenesis.

2.4.3 Seed propagation

Genetic diversity during sexual reproduction results from independent assortment of chromosomes and crossing over during meiosis and fusion of gametes during fertilization. Plants grown from seed will therefore be genetically diverse compared to plants from cuttings. Genetic diversity is a goal in many restoration projects. Using direct seeding methods for projects also eliminates the production process in the nursery. This may reduce labor costs and other expenses as maintenance of mature plants would be unnecessary.

Seed production is rather low compared with its potential (Hester and Mendelssohn, 1990). Wagner (1964) found 2.24 caryopses per spikelet, Westar and Loomis (1964) found 2 caryposes per spikelet and Hester and Mendelssohn (1987) found a range from 0.091 to 0.002 caryopses per spikelet in Louisiana populations of *Uniola paniculata*, but few of these ever set seed (Holmes, 1983). Although fertile pollen is produced, about 70% of the ovules are aborted and thus sea oats are not considered as a heavy seed producer (Westra and Loomis, 1966).

2.4.4 Sea oats seed dormancy

In efforts to overcome the germination issues of sea oats that early scientists were facing, Wagner (1964) studied the seed dormancy both on dunes and in the greenhouse. Gibberellic acid

treatment on seeds was effective in breaking seed dormancy (Woodhouse and Hanes, 1966). However, Westra and Loomis (1966) disagreed with gibberellic acid treatment and instead recommended thiourea with a pre-chilling treatment of alternating high and low temperatures. Seneca (1972) reported his results on the germination and seedling response of three populations of sea oats distributed in Virginia and North Carolina region, Atlantic coast Florida, and the Gulf coast. He found out that seeds from Virginia and North Carolina required cold treatment while its seedlings showed homogenous vegetative potential. In the Atlantic coast Florida region, seeds did not require cold treatment and seedlings were found to have a low potential for vegetative growth. Gulf coast seeds showed response to cold treatment and the seedlings had the highest potential for vegetative growth (Seneca, 1972). In the succeeding years, Hester and Mendelssohn (1987) in a study involving Louisiana sea oats concluded that cold treatment did not enhance seed germination and was not required to break dormancy. It was noted, however, that room-temperature treatment yielded 88.8% germination without prior cold treatment suggesting that moist cold condition had done a great deal in reducing the time required to achieve 50% germination (Hester and Mendelssohn, 1987). Meanwhile (Bachman and Whitwell 1995) presented their results on the nursery production of sea oats utilizing preplant treatments of seeds. Thiourea was not effective in improving seed germination but gibberellic acid treatment at 100 ppm increased and accelerated germination of freshly harvested seeds (Bachman and Whitwell, 1995). The authors concluded that poor seed germination could not be attributed to dormancy.

2.4.5 Seed pathogens

Most of the wildly grown grass seeds have been found contaminated with various fungi, i.e., *Fusarium* spp., *Ulocladium* spp, *Alternaria* spp., and *Cuvularia* spp., and *Bipolaris*.spp

(Han-Mo Koo et al., 2003). These seed borne fungi can reduce not only seed quality but also cause yield loss (Neergaard 1979). Also, Neergaard (1979) described that reduction of yield could be caused by seed borne fungi that lead to poor seed development, which is not necessarily seed transmitted. Halfon-Meiri (1970) reported that seed borne fungi such as *Alternaria. brassicola* and *Phoma ligam* in crops belonging to family Gramineae could shrink and reduce seeds. Many seed borne fungi could cause seed rot either on plant or during germination (Neergaard 1979). Many species in *Fusarium* are major plant pathogens and many are seed borne (Neergaard 1979). *Fusarium. oxysporum*, an agent of *Fusarium* wilt of many hosts, is the most important plant pathogenic species of *Fusarium*, having a wide host range, and including numerous *formae speciales*, some of which contain two or several pathogenic races causing devastating wilt disease (Neergaard 1979). Many of these *Fusarium. oxysporum* are seed borne (Noble M. and Richard 1968), and the Anderson (1974) index includes hosts such as *Allium* spp., *Glycine max, Oryza. sativa, Solanum melongena, Sorghum. vulgare, Cucumis sativa*, and *Pisum sativum*. *Fusarium. semitectum* is predominantly a secondary invader of plant tissues of many hosts including *Oryza. sativa*, *Zea. mays*, and *Sorghum. vulgare,* and prevailed in tropical and subtropical regions (Neergaard, 1979). *Fusarium. solani* is a ubiquitous pathogen and occurs on seed of a wide range of hosts such as *Capsicum annuum*, *Coriandrum sativum*, *Cuminum cyminum*, *Lycopercon esculentum*, *Oryza. sativa*, *Phaseolus vulgaris,* and *Sorghum. vulgare* (Ram Nath 1970). Noble and Richardson (1968) also listed *Allium*, *Cucurbita*, *Trifolium pratense,* and *Zea mays*. The pathogen causes root rot in many hosts such as broad bean, *Phaseolus* bean, and pea (Neergaard, 1979).

Bipolaris. spicifera (teleomorph: *Cochliobolus spicifer* Nelson) is distributed worldwide and occurs mainly in tropical and subtropical regions (Sivanesan and Holliday, 1981; Alcorn,

1988). This fungus was first named as *Brachucladium spoiciferum* Bainier in 1908 (Sivanesan and Holliday, 1981), renamed as *Curvularia spicifera* (Bainier) Boedijin in 1933 (Sivanesan and Holliday, 1981), then *Helminthosporiun spiciferum* (Bainier) Nicot in 1953 (Sivanesan and Holliday, 1981; Alcorn, 1988), followed by *Drechslera spicifera* (Bainier) Arx in 1970 (Sivanesan and Holliday, 1981; Alcorn, 1988), and renamed again as *Bipolaris. spicifera* (Bainier) by Subramanian in 1971 (Holliday 1981). *Bipolaris. spicifera* has a wide host range infecting more than 77 plant species including 51 grass genera and could be isolated from soil and air (Domsch. K. H 1980).

CHAPTER 3: EFFECT OF SEED STORAGE ENVIRONMENT ON *UNIOLA PANICULATA* GERMINATION[1]

3.1 Introduction

Beach restoration is essential in the northern Gulf of Mexico coast due to high rates of coastal erosion. Sea oats (*Uniola paniculata*), a native perennial grass of the southern Atlantic and northern Gulf of Mexico coasts of the United States builds and stabilizes sand dunes, which buffer storm surge and protect coastal communities and infrastructure (Wagner 1964; Seneca 1972). To produce sea oats plants for beach restoration, seeds are often collected from natural populations and grown in greenhouses until large enough for planting. Significant work has investigated the effects of light, temperature, and stratification on sea oats seed germination. Alternating temperatures yield the highest germination (Seneca 1969, Seneca 1972, Hester and Mendelssohn 1987) and light is not required for germination (Westra and Loomis 1966, Burgess et al. 2002). Response to cold stratification varies. Sea oats seed produced in Virginia and North Carolina had higher germination after cold stratification (Seneca 1969, Seneca 1972, Burgess et al. 2002), while seed collected from Florida's Atlantic Coast were unaffected by cold stratification. Seneca (1972) also found that seeds collected from the northern Gulf of Mexico coast had an intermediate response while Hester and Mendelssohn (1987) found that seeds collected from Louisiana germinated faster after cold stratification.

To support sea oats restoration efforts, reduce the frequency of seed harvests that cause physical disruptions to natural ecosystems, and strengthen sea oats genetic conservation additional work is needed. Seed germination is known to be affected by seed storage conditions, moisture content, and pathogens (Copeland and McDonald 2001, Elias et al. 2007, Elias et al.

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2012); however there are only limited accounts of sea oats seed pathogens (Hester and Mendelssohn 1987, Burgess et al. 2002). Information on how long seeds must remain in germination conditions to achieve high germination percentages is also limited; most studies investigated germination for specific time periods (Seneca 1969, Seneca 1972, Hester and Mendelssohn 1987, Burgess et al. 2002). Germination period is essential for planning and implementing beach restoration projects. The objectives of this study were to: (1) examine effect of storage environment, specifically, storage temperature and container on sea oats seed germination, (2) determine the length of time necessary for sea oats seeds to germinate, (3) examine sea oats seed moisture content; and (4) determine seed pathogen incidence during germination.

3.2 Materials and methods

We harvested mature sea oats panicles in September 2010 from a seed production nursery established in a natural beach environment at Long Beach, MS. Seeds were threshed from panicles with a forage belt thresher (Agriculex Inc., Guelph, Ontario) and placed into six storage environments (Table 3.1) in December 2010.

Table 3.1. Storage environments and conditions of sea oats seed harvested in September 2010 from a seed production nursery at Long Beach, MS.

Environment	Temperature	Container
RT Ambient	Room Temperature (20-25°C)	None
RT Sealed Jar	Room Temperature (20-25°C)	Hermetically sealed jar
4°C Ambient	4°C	None
4°C Sealed Jar	4°C	Hermetically sealed jar
-20°C Ambient	-20°C	None
-20°C Sealed Jar	-20°C	Hermetically sealed jar

Environments were selected to simulate commercial storage. From January 2011 to December 2011, 200 seeds, 8 replicates of 25 seeds, were removed from each environment every 28 days.

We used the oven method to determine seed moisture content (Elias et al. 2012). We surface sterilized sea oats seeds with 25% Clorox for 15 minutes (Woodhouse et al. 1968) prior to placement onto germination paper moistened with distilled water (ISTA 2008). We germinated seeds in the dark at 18.3°C / 35°C for 17 / 7 hours (Woodhouse et al. 1968).

Every 7 days we examined seed germination and pathogen incidence for 35 days after planting. A seed was considered germinated when the radicle was greater than 2 mm; seed infected with pathogens were removed. Percentage germination was calculated for 21, 28, and 35 days after planting as: (number of germinated seed at d_χ / (number of seed planted – number of infected seed)) x 100, where d_χ is the number of days seeds were in the germination environment. Total percentage seed germination was calculated as: (total number of germinated seed/ (total number of seed planted – total number of infected seed) x 100. Percentage pathogen incidence was calculated as: (number of infected seed / number of seed planted) x 100.

3.3 Data Analysis

We analyzed data with analyses of variance (ANOVA) as randomized complete block designs (PROC MIXED, SAS Institute version 9.1.3). Percentage seed moisture had 2 replicates; total seed germination and pathogen incidence had 8 replicates; and seed germination at 21, 28 and 35 days had 96 replicates. Storage environment was specified as fixed effect; replicates were considered random effects. LSMEANS were separated using pdmix800 at $p < 0.05$ level (Saxton 1998). We found significant time by environment interactions; therefore separate analyzes were conducted.

3.4 Results and Discussion

Sea oats seeds stored in hermetically sealed jars at room temperature consistently had the highest total germination from 4 to 15 months after harvesting and ranged from 28-71% and seed

stored in ambient conditions at room temperature had high total germination except for 4 and 7 months after harvesting (Table 3.2). Other investigators reported high sea oats germination from Louisiana seed when stored at room temperature for 6 months (Hester and Mendelssohn 1987). This is not surprising because northern Gulf of Mexico coast typically remains above freezing year round; therefore northern Gulf of Mexico sea oats seeds are adapted to warm temperatures. Seed stored in ambient conditions at 4°C had the lowest total germination throughout the experiment and ranged from 12-32 % (Table 3. 2). Total germination of seed stored in hermetically sealed jars at 4°C, ambient conditions at -20°C and hermetically sealed jars at -20°C were typically among the lowest (Table 3.2). This suggests that low temperatures cause chilling injury prior to imbibition (Bewley and Black 1982), which reduces sea oats seed germination.

Seed moisture content is critical for seed storage, however specific reports of sea oats seed moisture are lacking. We found seeds stored at -20°C storage environments (ambient and hermetically sealed jars) had high seed moisture contents that ranged from 12-16% and 10-16% respectively (Table 3.2). Seeds stored at room temperature ambient conditions consistently had the lowest seed moisture contents ranging from 6-13% (Table 3.2). Typically seed moisture contents below 13% are recommended for grass seed storage (Elias et al. 2007). We also found a negative correlation between seed moisture content and germination ($p < 0.05$; r = -0.40); which is consistent with orthodox seed (Hong and Ellis 1996).

We were also interested in pathogen incidence because pathogens are known to affect seed quality and reduce germination. We found seed stored at -20°C ambient consistently had the highest pathogen incidence; 4°C ambient had high pathogen incidence except at 4 and 12 months after harvesting (Table 3.2). Seed stored in hermetically sealed jars at room temperature had the

lowest pathogen incidence (Table 3.2). Increased pathogen incidence at low temperature may have resulted from chilling injury (Hong and Ellis 1996) that allowed opportunistic pathogens to

Table 3.2. Average total percentage seed germination, moisture content, and pathogen incidence for sea oats seed stored for different lengths of time and in different environments. Averages followed by different letters within each row are significantly different (*t* test, $p < 0.05$).

Months after Harvesting	Storage Environment							
	RT Ambient	RT Sealed Jar	4°C Ambient	4°C Sealed Jar	-20°C Ambient	-20°C Sealed Jar	Significance Level	
			Seed Germination (%)				F Value	P Value
4	25 ^{bc}	33 ^{ab}	15 ^{c}	43 ^{a}	34 ^{ab}	33 ^{ab}	2.81	0.0307
5	28 ^{a}	28 ^{a}	12 ^{b}	23 ^{ab}	26 ^{a}	23 ^{ab}	2.68	0.0376
6	32 ^{abc}	40 ^{a}	21 ^{c}	38 ^{ab}	30 ^{abc}	28 ^{bc}	2.74	0.0341
7	34 ^{b}	53 ^{a}	19 ^{c}	19 ^{c}	27 ^{bc}	28 ^{bc}	11.37	<0.0001
8	32 ^{a}	37 ^{a}	32 ^{a}	44 ^{a}	34 ^{a}	37 ^{a}	0.90	0.4946
9	43 ^{a}	52 ^{a}	27 ^{b}	29 ^{b}	27 ^{b}	24 ^{b}	8.11	<0.0001
10	45 ^{a}	45 ^{a}	13 ^{c}	26 ^{b}	20 ^{bc}	24 ^{bc}	11.10	<0.0001
11	35 ^{a}	36 ^{a}	18 ^{b}	21 ^{b}	13 ^{b}	20 ^{b}	6.09	0.0004
12	50 ^{a}	58 ^{a}	30 ^{b}	32 ^{b}	33 ^{b}	27 ^{b}	6.28	0.0003
13	62 ^{a}	71 ^{a}	31 ^{b}	29 ^{b}	26 ^{b}	24 ^{b}	16.88	<0.0001
14	49 ^{a}	45 ^{a}	19 ^{b}	38 ^{a}	20 ^{b}	36 ^{a}	6.55	0.0002
15	52 ^{a}	52 ^{a}	29 ^{b}	28 ^{b}	26 ^{b}	24 ^{b}	6.55	0.0002
			Moisture Content (%)					
4	9 ^{c}	13 ^{b}	10 ^{c}	13 ^{b}	14 ^{a}	14 ^{ab}	48.02	0.0003
5	9 ^{d}	13 ^{bc}	10 ^{d}	12 ^{c}	15 ^{a}	14 ^{ab}	53.58	0.0002
6	8 ^{d}	13 ^{b}	11 ^{c}	13 ^{b}	14 ^{a}	12 ^{b}	67.90	0.0001
7	9 ^{c}	12 ^{b}	14 ^{ab}	13 ^{ab}	14 ^{a}	14 ^{a}	14.12	0.0057
8	12 ^{a}	13 ^{a}	11 ^{a}	12 ^{a}	13 ^{a}	13 ^{a}	0.64	0.6803
9	9 ^{b}	11 ^{ab}	11 ^{ab}	12 ^{ab}	13 ^{a}	13 ^{a}	3.97	0.0784
10	9 ^{d}	12 ^{c}	12 ^{c}	14 ^{ab}	14 ^{b}	16 ^{a}	29.60	0.0010
11	9 ^{c}	11 ^{bc}	12 ^{bc}	13 ^{ab}	16 ^{a}	13 ^{ab}	7.34	0.0237
12	13 ^{c}	16 ^{a}	13 ^{c}	13 ^{bc}	15 ^{ab}	10 ^{d}	14.10	0.0057
13	9 ^{b}	12 ^{a}	11 ^{ab}	12 ^{a}	12 ^{a}	13 ^{a}	4.19	0.0709
14	6 ^{d}	7 ^{cd}	9 ^{bc}	11 ^{ab}	13 ^{a}	13 ^{a}	17.96	0.0033
15	10 ^{b}	12 ^{ab}	12 ^{ab}	13 ^{ab}	15 ^{a}	15 ^{a}	3.66	0.0903

(Table 3.2. continued)

Months after Harvesting	Storage Environment							
	RT Ambient	RT Sealed Jar	4°C Ambient	4°C Sealed Jar	-20°C Ambient	-20°C Sealed Jar	Significance Level	
	Pathogen Incidence (%)							
4	36 [ab]	29 [bc]	12 [d]	25 [c]	40 [a]	42 [a]	13.95	<.0001
5	34 [a]	13 [b]	36 [a]	25 [ab]	32 [a]	29 [a]	4.06	0.0052
6	32 [ab]	28 [b]	31 [ab]	36 [ab]	32 [ab]	39 [a]	1.15	0.3513
7	30 [c]	49 [a]	41 [ab]	7 [d]	38 [abc]	31 [bc]	14.53	<0.0001
8	34 [b]	22 [c]	45 [a]	45 [a]	41 [ab]	34 [b]	6.90	0.0001
9	25 [bc]	23 [bc]	36 [a]	25 [bc]	30 [ab]	19 [c]	4.11	0.0049
10	47 [a]	48 [a]	39 [a]	47 [a]	47 [a]	41 [a]	1.02	0.4191
11	25 [bc]	18 [c]	32 [ab]	33 [a]	36 [a]	25 [bc]	6.24	0.0003
12	27 [b]	31 [ab]	29 [b]	29 [b]	41 [a]	28 [b]	1.82	0.1337
13	27 [a]	31 [a]	39 [a]	35 [a]	31 [a]	31 [a]	0.91	0.4887
14	39 [ab]	33 [b]	45 [a]	46 [a]	41 [ab]	48 [a]	1.87	0.1252
15	40 [a]	35 [a]	36 [a]	39 [a]	43 [a]	36 [a]	0.66	0.6530

invade weak seed coats and deteriorate the embryo (Klich 2007).

Restoration practitioners are extremely interested in length of time required for sea oats seeds to germinate because it directly affects production costs and project planning. We found the highest germination 21 days after planting and lowest germination 28 and 35 days after planting for all environments (Table 3. 3). In addition seeds stored in hermetically sealed jars at room temperature had highest germination, while seed stored at 4°C ambient conditions had lowest germination (Table 3.3). We expected seeds stored at low temperatures to germinate faster than seeds stored at room temperature based upon previous studies (Seneca 1972, Hester and Mendelssohn 1987, Burgess et al. 2002); however they had lower germination percentages in comparison to room temperature storage (Table 3.3). We also found highest pathogen incidence 21 days after germination and when seed were stored at -20°C ambient (Table 3.3). More work is needed to determine why both seed germination and pathogen incidence are highest 21 days after germination. Burgess et al. (2002) identified numerous pathogens associated with sea oats seed decay during germination. This study and previous studies (Hester and Mendelssohn 1987, Burgess et al. 2002) suggest that measures to reduce pathogen incidence including chemicals and plant resistance would improve sea oats plant yields for beach restoration.

3.5. Conclusion

In conclusion, we found sea oats seed stored in hermetically sealed jars at room temperature consistently had the highest average germination for seed harvested from the northern Gulf of Mexico coast. We also were the first to report sea oats seed moisture content, which ranged from 6 to 16 %, and was negatively correlated with germination. Finally, we found seed germination was highest 21 days after germination.

Table 3.3. Average percentage seed germination and pathogen incidence after 21, 28, and 35 days in germination environment for sea oats seed stored in six environments. Averages within a row for each variable (seed germination, pathogen incidence) followed by different capitalized letters are significantly different (t test, $p < 0.05$). Averages within a column followed by different superscripts are significantly different (t test, $p < 0.05$).

Storage Environment	Days after Planting									
	21	28	35	Significance level		21	28	35	Significance level	
	Seed Germination (%)			F Value	P Value	Pathogen Incidence (%)			F Value	P Value
RT Ambient	29 B^a	5 F^b	1 G^c	309.70	<0.0001	22 AB^a	8 AB^b	3 D^c	188.37	<0.0001
RT Sealed Jar	31 A^a	6 F^b	3 G^c	207.10	<0.0001	18 C^a	7 B^b	6 B^b	57.99	<0.0001
4°C Ambient	12 E^a	6 F^b	2 G^c	126.30	<0.0001	19 C^a	8 AB^b	7 A^b	39.61	<0.0001
4°C Sealed Jar	19 C^a	6 F^b	2 G^c	133.62	<0.0001	20 BC^a	9 A^b	4 C^c	81.90	<0.0001
-20°C Ambient	14 D^a	6 F^b	3 G^c	70.12	<0.0001	24 A^a	10 A^b	4 C^c	147.34	<0.0001
-20°C Sealed Jar	15 D^a	6 F^b	3 G^c	86.35	<0.0001	19 C^a	9 A^b	5 B^c	70.03	<0.0001

CHAPTER 4: EFFECT OF *UNIOLA PANICULATA* PLANT SIZE ON SURVIVAL AND PERFORMANCE AT BEACHES WITH LOW DUNE PROFILES[2]

4.1 Introduction

In the United States, northern Gulf of Mexico sand dunes are important for reducing the effects of tropical storms and hurricanes on coastal communities and infrastructure (Claudino-Sales et al. 2008). Dunes accumulate more quickly when vegetation is present because vegetation traps windblown sediments (Synder and Boss 2002; Claudino-Sales et al. 2008; Houser et al. 2008). Most dune restoration projects along the northern Gulf of Mexico coast utilize sea oats (*Uniola paniculata*), a perennial dune grass native to the southern Atlantic and northern Gulf of Mexico coasts in the United States (Wagner 1964; Judd et al. 1977). The primary habitat of sea oats is coastal dunes that are subject to high winds, salt spray, tropical storms and hurricanes, sand burial, low soil nutrients, xeric conditions, and extremely high soil temperatures (Lonard et al. 2011).

The size of sea oats planted for beach restoration projects along northern Gulf of Mexico beaches varies widely. In Louisiana, most sea oats are large, established container plants that are vegetatively reproduced for at least 1 year. Every plant is genetically identical, which eliminates genetic diversity necessary for plants to adapt to environmental changes (Huenneke 1991; Kutner and Morse 1996; Ledig 1996). In Mississippi and Florida, most sea oats plants used for beach restoration are produced from seeds and can vary significantly for plant age and size. This preserves genetic diversity however most seeds are harvested from natural beach environments, a practice that disrupts natural ecosystems.

[2] This chapter originally appears as "Nabukalu, P. and C.A. Knott. 2013. Effect of *Uniola paniculata* plant size on survival and performance at beaches with low dune profiles. Ecological Restoration 31(1): 12-16.". Reprinted with permission of Ecological Restoration Journal.

Investigations to determine whether large, vegetative sea oats plants survive and perform better than seedlings have not been documented to our knowledge. This is critical information needed to plan and implement successful beach restoration projects. The objective of this study was to determine if survival and performance differed for large vegetative sea oats plants and small sea oats seedlings at beach environments with low dune profiles.

4.2 Materials and Methods

Sea oats seeds collected from 8 states (TX, LA, MS, AL, FL, SC, NC, VA) were germinated, transplanted to natural beaches, and evaluated for plant performance at beaches with low dune profiles (Bertrand-Garcia et al. 2012). In 2005, rhizomes were harvested from plants selected for superior performance in low dune profile beaches (Bertrand-Garcia et al. 2012), planted into 1-gallon containers and maintained in controlled greenhouses. We divided rhizomes of greenhouse propagated material, replanted them into 1-gallon containers, and allowed plants to grow in controlled greenhouses for 1 year to provide vegetative plants for the study; each plant had at least 5 stems. In 2009 we harvested sea oats seeds from beach field trials described above. We germinated seeds in controlled greenhouses. Large seedlings were 8 months old and more than 30 cm tall. Small seedlings were 3 months old and 10 to 15 cm tall.

We established a preliminary study in May 2008 at Biloxi, MS, to evaluate sea oats seedling performance; 384 vegetative plants, 25 large seedlings, and 25 small seedlings were included. Larger trials established in March 2009 and June 2010 at Long Beach, MS, included 365 vegetative plants, 664 small seedlings and 64 large seedlings and 140 vegetative plants, 720 large seedlings, and 113 small seedlings, respectively.

In 2008, plant survival and vigor were measured for each plant in August, September, and October (3, 4, and 5 months after transplant). We considered plants with any green color as alive

while plants without any green color were considered dead. Plant vigor was measured with a 0-10 scale: 0 was a dead plant and 10 was a plant with numerous dark green leaves and stems, densely distributed stems, and aggressive vegetative spread. Heavy machinery unintentionally destroyed seedlings after month 5 evaluations. In 2009, plant survival at a second site was measured for each plant in May and June (2 and 3 months after transplant) at Long Beach. Heavy equipment also destroyed these seedlings prior to the 3 month assessments. In 2010, another field site was established at Long Beach; sea oats seedlings' locations were communicated to equipment operators to avoid plant destruction. Plant survival was measured for each plant in August, September, October, 2010, and March, April, and November, 2011: 2, 3, 4, 9, 10, and 17 months after transplant. Plant vigor was measured 9, 10, and 17 months after transplant. Total number of stems per plot was measured 4, 9, 10 and 17 months after transplant. Stem density was measured as number of stems per 100 cm^2 at 9, 10, and 17 months after transplant.

4.3 Data Analysis

We conducted individual analyses of variance (ANOVA) for each environment (Biloxi, 2008; Long Beach, 2009; Long Beach, 2010) as completely random design. Data were analyzed as repeated measures for each environment using PROC MIXED in SAS version 9.1 (Institute 2002). Plant size was specified as fixed effect while replicates were considered random effects. Least squares means were used for means separation at $P < 0.05$.

4.4 Results and Discussion

In the 2008 preliminary trial, average plant survival ranged from 88 to 100% (Figure 4.1, Graph A) 3 months after transplanting. Vegetative sea oats plants and large seedlings had significantly higher survival (*F= 4.49, P= 0.0117*) 3 months after transplant than small sea oats

seedlings (Figure 4.1, Graph A). We also found that large seedlings were 20% more vigorous (*F= 9.33, P= 0.0001*) than vegetative plants or small seedlings (Figure 4.1, Graph B).

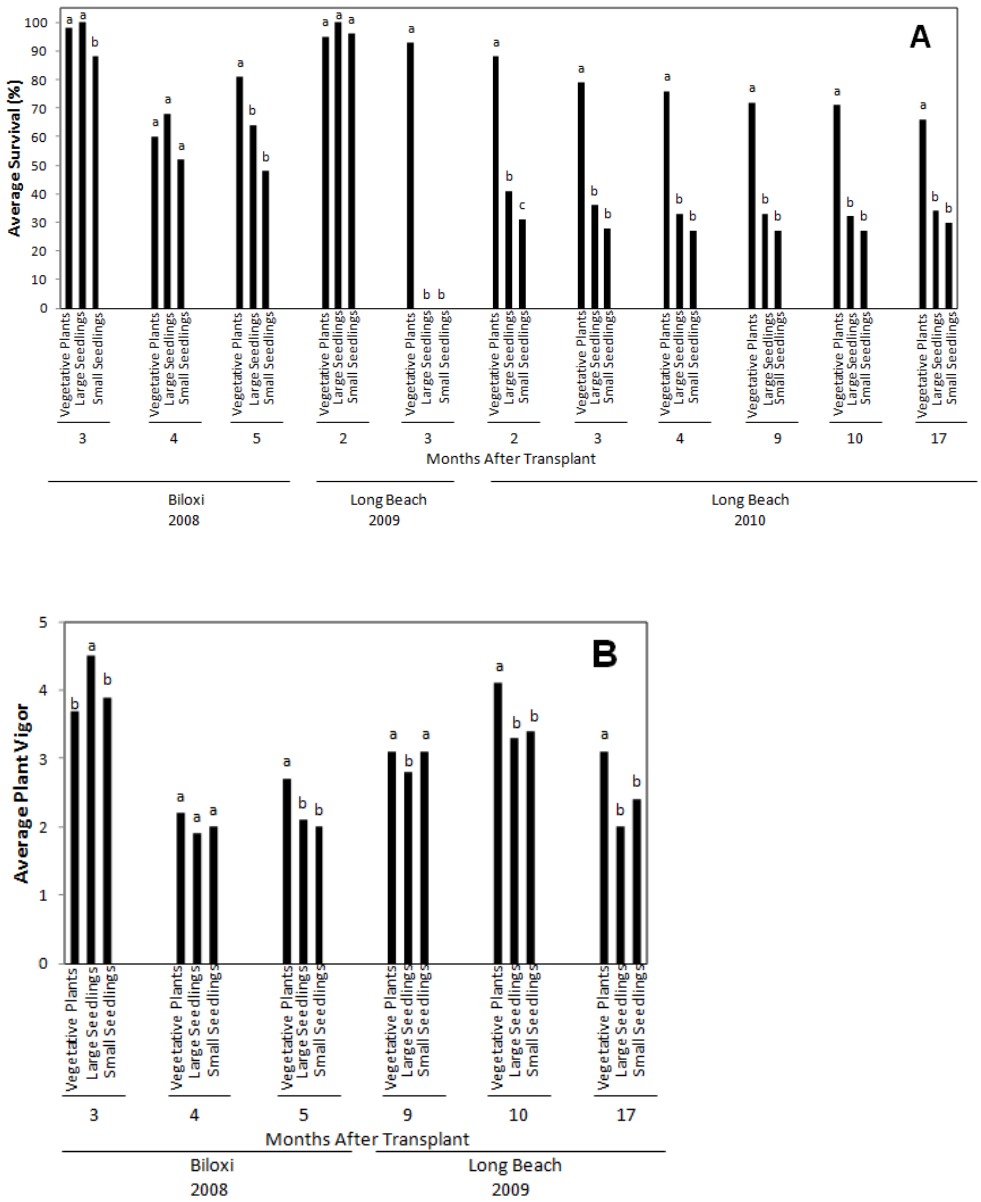

Figure 4.1. Average plant survival (A) and vigor (B) of vegetative sea oats plants, 8 month old sea oats (large) seedlings and 3 month old sea oats (small) seedlings at three field trials in natural beach environments. Columns labeled with different letters for each assessment date and location are significantly different at $p < 0.05$ level.

Approximately 3.5 months after transplant, Hurricane Gustav made landfall near Cocodrie, LA, 260 km southwest of the experiment. The beach was flooded by approximately 2 m of storm surge for 7 days. After surge receded, debris was removed from plots. Plant survival and vigor were measured, which corresponded with 4 months after transplant. Surprisingly, survival and plant vigor were similar ($F= 0.66, P= 0.5151$) for all sea oats plant sizes (Figure 4.1, Graph A and B). Five months after transplant, we found that vegetative plants had significantly higher plant survival ($F= 9.04, P= 0.0001$) and plant vigor ($F= 4.44, P= 0.0125$) than seedlings (Figure 4.1, Graph A and B). Unfortunately, we could not complete further evaluations because heavy machinery removing storm debris from Hurricane Gustav unintentionally removed the seedlings.

In 2009, plant survival 2 months after transplant was similar ($F= 1.64, P= 0.1939$) for vegetative plants, large and small seedlings (Figure 4.1, Graph A), which supports our findings in the small preliminary trial in 2008 that survival is similar for all sea oats plant sizes in normal conditions. Seedlings were again removed accidentally by heavy machinery cleaning the beach.

In 2010, survival was consistently higher for vegetative plants than seedlings ($P< 0.0001$ for each evaluation date; Figure 4.1, Graph A). This was most likely due to significant storm surge generated by Tropical Storm Alex, which flooded the newly established field trial 1 week after transplant. Despite these harsh conditions, approximately 30% of small sea oats seedlings, 40% of large seedlings, and 80% of vegetative plants survived. We also found that vegetative plants had a higher survival rate than seedlings 1 month after storm surge in the 2008 preliminary trial. These findings suggest that seedlings are less tolerant to salt water flooding than vegetative plants.

In 2010, plant vigor was significantly higher ($F= 5.72, P= 0.0036$) for vegetative plants and small sea oats seedlings 9 months after transplant while at 10 months ($F= 23.28, P< 0.0001$)

and 17 months ($F= 21.48, P< 0.0001$) after transplant vegetative plants were more vigorous than seedlings (Figure 4.1, Graph B). Plant vigor was not measured for the first 8 months because all plants had a similarly low vigor due to storm surge. We also measured the number of stems produced by each plant in 2010. We found that small sea oats seedlings consistently produced the most stems while large seedlings produced the fewest stems ($P< 0.0001$ for all evaluation dates; Figure 4.2, Graph A). We also found that stem density was greatest for vegetative plants 9 months ($F= 5.03, P= 0.0071$) and 17 months ($F= 28.10, P< 0.0001$) after transplant (Figure 4.2, Graph B) and that 10 months after transplant stem density was similar ($F= 2.37, P= 0.0953$) for all sea oats plant sizes (Figure 4.2, Graph B). These results suggest that sea oats seedlings spread further from the original planting site because they consistently produced the most stems and lowest stem density. In theory, sea oats seedlings are ideal for beach restoration projects because they provide significantly more genetic diversity than vegetative plants (Rauf et al. 2010) and are more cost effective because seedlings can be produced for about 25% of the cost of large vegetative plants. In this study we found that survival of sea oats seedlings was similar to the survival of vegetative plants in normal beach conditions. However, after storm surge events vegetative plants survived better and had higher stem densities than seedlings, while seedlings produced more stems than vegetative plants.

4.5 Conclusion

Our results indicate that large vegetative plants are most appropriate for beach environments that are vulnerable to frequent storm surge events and that seedlings would survive better in environments with less frequent storm surge. However, if sea oats seedlings are preferred in beach restoration projects for increased genetic diversity and reduced cost,

restoration planners should require 3 times as many sea oats seedlings as large vegetative plants to compensate for higher mortality rates of sea oats seedlings in unfavorable conditions.

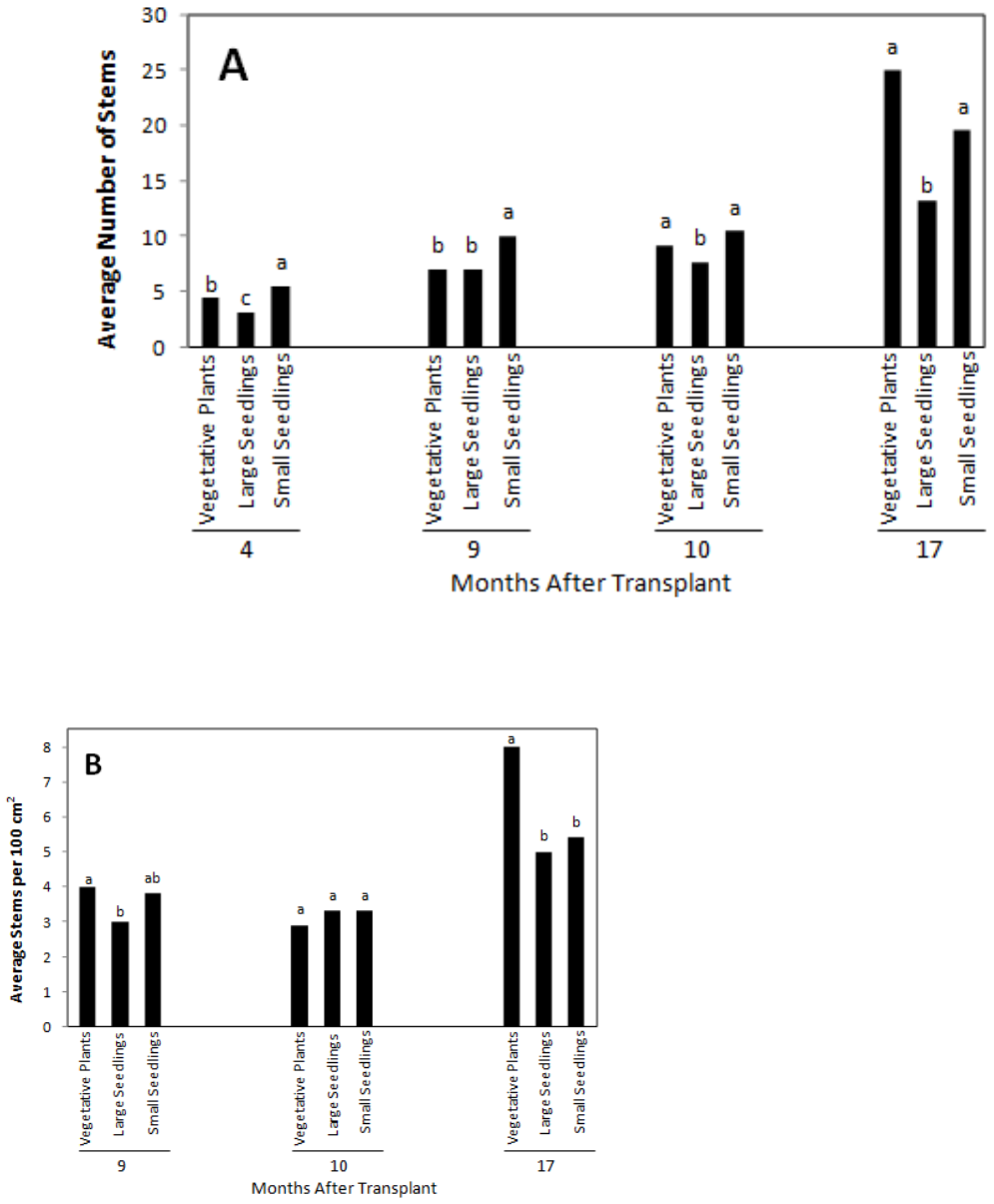

Figure 4.2. Average number of stems (A) and stem per 100cm2 (B) of vegetative sea oats plants, 8 month old sea oats (large) seedlings and 3 month old sea oats (small) seedlings at a large field trial, Long Beach, MS, 2010. Columns labeled with different letters for each assessment date are significantly different at $p < 0.05$ level.

CHAPTER 5: DEVELOPMENT AND EVALUATION OF SEA OATS LINES FOR BEACHES WITH SHALLOW DUNES

5.1 Introduction

Sea oats (*Uniola paniculata* L.), an ecologically important perennial dune grass, is planted on beaches in the northern Gulf of Mexico coast to reduce coastal erosion and restore natural ecosystems. Sea oats stabilize and build sand dunes, which protect coastal communities, industries, infrastructure and residents (Wagner 1964). It is native to beach environments throughout the mid and southern Atlantic and northern Gulf of Mexico coasts of the United States (Wagner 1964; Judd et al. 1977). Beaches are subject to numerous harsh conditions such as high winds, salt spray, tropical storms and hurricanes, sand scouring, burial, low soil nutrients, xeric conditions, and extremely high soil temperatures (Lonard et al. 2011). Sea oats typically spread in beach environments with underground rhizomes (Wagner 1964; Hester and Mendelssohn 1987; Lonard et al. 2011; Lonard et al. 2012), which are stimulated to grow by sand burial (Wagner 1964; Lane et al. 2008). In natural environments sea oats commonly produce large areas of genetically identical sea oats plants (Frank W. Judd 1977; Hester and Mendelssohn 1987; Subudhi et al. 2005), because sea oats rarely become established from seeds (Wagner 1964). Rare seedling establishment is likely due to environmental factors such as sand burial, high soil temperature, and low sand moisture on beaches (Lonard et al. 2012). Limited sea oats seed production, which has been reported to range from complete sterility to 2.4 seeds per spikelet (Wagner 1964; Westra and Loomis 1966; Hitchcock and Chase 1971; Hester and Mendelssohn 1987), may also contribute to low sea oats seedling establishment in natural environments.

Sea oats spread in the Northern Gulf of Mexico coast may also be impeded by shallow dune profiles. Many beaches in the Northern Gulf of Mexico coast have very shallow dunes

(Monteferrante et al. 1982; Hester and Mendelssohn 1987) due to shoreline erosion (Morton et al. 2004; Couvillion et al. 2011), overwash events (Hester and Mendelssohn 1987), low sediment supply (Davis Jr. 1994; Houser et al. 2008), persistent onshore winds (Davis Jr. 1994), and narrow widths of beaches that do not allow significant sand accumulation (Claudino-Sales et al. 2008). When dunes are shallow, sea oats grow at or near the water-table, which can contribute to plant death (Hester and Mendelssohn 1987). Soil saturation has long been identified as a major abiotic stress, which markedly affects plant growth and development. As water saturates the soil pores, gases are displaced, a reduction in gas diffusion occurs and phytotoxic compounds accumulate as anaerobic conditions prevail (VanToai et al. 2001). Various methods have been used to identify saturation tolerance in several plant species. For agricultural crop species where yield is the ultimate judging factor (Rosielle and Hamblin 1981; VanToai et al. 1994) the approach is to select varieties with highest yield regardless of stress conditions and/or morphological adaptations for tolerance to soil saturation (Bacanamwo and Purcell 1999a; 1999b). Several other traits have also been used to make determinations about saturation tolerance: leaf color, plant height, chlorophyll content, and biomass of roots and shoots (VanToai et al. 2001).

Typically saturation tolerance research has been conducted in controlled greenhouse and growth chamber environments for agronomic crops. Field evaluations have also been utilized, but to a lesser extent due to lower levels of control that can be achieved in field environments. Plant death is rare when saturation tolerance is evaluated in controlled conditions (Oosterhuis et al. 1990; Tara 2010) because plants in the greenhouse usually produce aerenchyma and adventitious roots closer to the soil surface. In addition greenhouse investigations typically use coarse-textured soils, which have a low water- holding capacity (Oosterhuis et al. 1990). When

saturation tolerance is evaluated in field conditions plant death can occur due to prolonged saturation periods (Sullivan et al. 2001; Tara 2010). To our knowledge the only reported study that investigated sea oats saturation tolerance was by Hester and Mendelssohn (1989). They examined the growth responses of sea oats to 3 watering regimes and 4 controlled water-table depths. They observed that excessive water from either inundation or shallow water-table depth negatively affected sea oats plant growth and survival. This may explain the decline of sea oats populations in Louisiana. It also demonstrates the need to develop and incorporate genetically diverse saturation tolerant sea oats lines into northern Gulf of Mexico beach restoration projects.

The overall goal of this study was to develop a sea oats breeding program for saturation tolerance. The specific objectives were to: (1) develop greenhouse protocols to identify saturation tolerant sea oats lines and (2) determine sea oats seed yield in natural and artificial environments.

5.2 Materials and methods

5.2.1 Sea oats saturation tolerance trials

Sea oats seeds were collected in 2009 from beach trials at Holly Beach, LA, and Long Beach, MS (Bertrand-Garcia et al. 2012). Seed was germinated and seedlings were produced in controlled greenhouse conditions maintained at 25°C with natural photoperiod.

In March 2010, a greenhouse study with 7 treatments was initiated which had 3 water depths (6, 10, 14 cm), 2 flood regimes (static and cyclic) and a non-flooded control (Table 5.1). Flooded treatments were accomplished in plastic-lined wood-framed tanks that were 2.1 m x 1.25 m x 0.4 m. A total of 160 seedlings, which were approximately 6 months old and at least 12 cm tall, were placed into each treatment. Water in the flooded treatments was mechanically circulated with submersible fountain pumps (Quanzhou Yuanhua Electronic Technology Co.,

Ltd, Fujian, China). The static flood regime (S) continuously flooded sea oats plants. Water in the S treatments was drained every 28 days and immediately refilled. Sea oats plants in the cyclic flood regime (C) were flooded for 14 days followed by 14 days that were non-flooded. Sea oats plants in the control treatment were grown on a dry greenhouse bench and watered every 3 days. All sea oats plants were fertilized every 14 days with Peter's peat lite 20-10-20 water soluble fertilizer (11.98 g L^{-1}; Scott's Sierra Horticultural Products Co., Marysville, Ohio). Sea oats survival, plant height, number of leaves per plant, root length, and fresh weight were measured in April, May, and June, 2010. Sea oats plants with any green color were considered alive while plants without any green color were considered dead. Plant height was measured from the soil level to the tip of the plant. The number of green leaves was counted to determine number of leaves per plant. Forty sea oats seedlings per treatment, 10 per replication, were randomly selected and measured for total fresh-weight biomass and root length every 28 days. Roots were carefully removed from soil, washed with tap water, and dried with paper towels. Total fresh-weight biomass was measured by weighing seedlings and root length was measured from the shoot base to root tips.

In July 2010, a beach trial with 360 sea oats seedlings, which were approximately 3 months old and at least 12 cm tall, was established at an extremely saturated beach with shallow dunes at Holly Beach, LA. Sea oats seedling survival was measured in August, September and October, 2010.

In December 2011, a second greenhouse study was initiated; 4 treatments, including 2 salinity levels (0 and 35 ppt; parts per thousand) and 2 flood levels (0 and 14 cm), were examined. A total of 720 sea oats seedlings, which were 4 months old and at least 12 cm tall, were included: 180 seedlings for each treatment (Table 5.3). Salinity levels were accomplished

with Instant Ocean® salt (Aquarium Systems, Mentor, Ohio) according to manufacturers' directions. Flooded treatments were accomplished as described above. Sea oats seedlings in the 0 cm flood treatment were watered every 3 days. Water in the flooded treatments was drained and immediately replaced every 28 days. Sea oats survival, plant height, and number of leaves per plant were measured as described above every 28 days. In March 2011, fresh-weight biomass and root length were measured as described above.

In 2012, a beach trial and a field trial were initiated. In May 304 seedlings, which were approximately 8 months old and at least 30 cm tall, were transplanted to an extremely saturated beach with shallow dunes at Holly Beach, LA. In August 304 seedlings, which were approximately 10 months old and at least 30 cm tall, were transplanted to saturated fields at Burden Museum and Gardens, Baton Rouge, LA. Sea oats survival, plant height, and number of leaves per plant were measured as described above every 2 days. Above-ground fresh-weight biomass was measured 6 months after transplanting.

5.2.2 Sea oats seed yield

Sea oats seeds were collected from 8 states (TX, LA, MS, AL, FL, SC, NC, VA) and germinated in 2003 (Bertrand-Garcia et al. 2012). Seedlings were transplanted and evaluated for performance at saturated beaches with shallow dunes: Holly Beach, LA, and Long Beach, MS (Bertrand-Garcia et al. 2012). In 2005, rhizomes and stem material of 75 plants from 6 states (TX, LA, AL, FL, NC), which performed well in saturated beach conditions, were harvested and transported to Baton Rouge, LA, where they were established in an artificial breeding nursery (Bertrand-Garcia et al. 2012). Plants were fertilized once per month with approximately 500 L of all-purpose water soluble plant food (24-8-16; The Scott's Company, LLC.). Mature sea oats panicles were harvested September 2007, 2009, 2010, and 2011 from the artificial breeding

nursery and beaches with shallow dunes: Holly Beach, LA, and Long Beach, MS. Seeds were threshed from panicles with a forage belt thresher (Agriculex Inc., Guelph, Ontario). Number of seed spikelet^{-1}, number of seed plant^{-1}, number of spikelets plant^{-1}, average number of florets spikelet^{-1}, 100 seed weight, number of seed g^{-1}, percent seed set, and seed viability were measured. Percent seed set was calculated as: (number of seed spikelet^{-1} ÷ number of florets spikelet^{-1}) x 100. In February 2013, seeds were soaked in a 1% solution of 2, 3, 5-triphenyl tetrazolium chloride to test for embryo viability (Baskin 1998).

5.3 Statistical analyses

5.3.1 Sea oats saturation tolerance trials

Analyses of variance (ANOVA) were conducted as repeated measures for sea oats percent survival, plant height, number of leaves per plant, and root length using PROC MIXED in SAS® version 9.1 (Institute 2002). Percent sea oats survival was arcsine square root transformed. Non-transformed data are reported; data interpretations were based upon transformed data. ANOVA were conducted for fresh weight biomass using PROC MIXED in SAS® (SAS Institute, 2002). In 2010 the greenhouse environment was a randomized complete block factorial design (3 flood depths, 2 flood regimes, and the control) with 4 replications; each replication had 40 plants. In 2010 the beach environment was a completely randomized design with 360 total seedlings; 4 replications of 80 plants. The 2011 greenhouse experiment was a randomized complete block factorial design (2 salinity and 2 flood levels) with 720 seedlings; 4 replications of 45 seedlings. In 2012 the beach and field trials were completely randomized design with 304 total seedlings; 4 replications of 76 plants. Least squares means were used for means separation at the $p < 0.05$ level with *pdmix* (Saxton 1998). Pearson correlation coefficients were calculated using PROC CORR in SAS® version 9.1 (SAS Institute 2002) for

percent sea oats survival between 2010 greenhouse treatments and Holly Beach and 2011 greenhouse treatments and 2012 beach and field trials.

5.3.2 Sea oats seed yield

Analyses of variance (ANOVA) were conducted for number of seed spikelet^{-1}, number of seed plant^{-1}, number of spikelets plant^{-1}, average number of florets spikelet^{-1}, 100 seed weight, number of seed g^{-1}, percent seed set, and seed viability using PROC MIXED in SAS version 9.1 (Institute 2002). Least squares means were used for means separation at the $p < 0.05$ level with *pdmix* (Saxton 1998).

5.4 Results and discussion

5.4.1 Sea oats saturation tolerance trials

Saturated beaches with shallow dune profiles are present along the northern Gulf of Mexico coast. The most saturated beaches are considered to be in Louisiana because of extremely high erosion rates within the state (Hester and Mendelssohn 1989; Couvillion et al. 2011). Consequently, sea oats populations are limited within Louisiana. To prevent the extinction of sea oats within Louisiana and to improve performance of sea oats throughout the northern Gulf of Mexico coast, saturation tolerant sea oats lines should be developed. In this study, we examined methods to identify and develop saturation tolerant sea oats lines for use in beach restoration projects throughout the northern Gulf of Mexico coast and to produce sufficient numbers of genetically different sea oats seeds to support a breeding initiative.

In 2010 greenhouse and beach trials were established. Significant ($p < 0.05$) treatment by time interactions were detected for survival, plant height, leaf numbers, fresh weight and root length (data not shown); therefore data were analyzed separately for each month. One month after transplant (MAT), survival of sea oats seedlings ranged from 47-100% with the non-

flooded greenhouse control having the highest survival, 100%, and Holly Beach having the lowest survival, 47% (Table 5.1). In greenhouse environment, sea oats seedling survival was

Table 5.1 Average sea oats survival in a controlled greenhouse trial, Baton Rouge, LA, and saturated beach trial, Holly Beach, LA, in 2010

		Survival (%)					
		1 MAT[a]		2 MAT		3 MAT	
Treatment[b]	F-Value[c]	50.67***		44.15***		83.34***	
Control		100.0	a[d]	99.0	a	98.0	a
06C		98.0	a	100	a	98.0	a
06S		98.0	a	96.0	ab	92.0	a
10C		98.0	a	95.0	ab	92.0	a
10S		83.0	b	78.0	cd	69.0	b
14C		91.0	ab	88.0	bc	80.0	b
14S		81.0	b	70.0	d	41.0	c
Holly Beach		47.0	c	46.0	e	25.0	d

[a] MAT = months after transplanting.
[b] Control indicates sea oats plants were grown on dry bench and watered every 3 days; 06, 10, and 14 indicate water depths were 6, 10, and 14 cm, respectively; C indicates cyclic flood where sea oats plants were cyclically flooded for 14 days followed by 14 days of dry conditions; S indicates static flood where sea oats plants were continuously flooded.
[c] *** indicates $p < 0.001$.
[d] Averages within a column followed by different letters are statistically different according to the *t* test on difference of least square means ($p = 0.05$).

lowest for seedlings constantly flooded at 10 cm (10S) and 14 cm (14S), 83% and 81%, respectively (Table 5.1). Sea oats seedling survival 2 and 3 MAT, was highest for the control, cyclic flood at 6 cm (06C), static flood at 6 cm (06S) and cyclic flood at 10 cm (10C) and lowest at Holly Beach (Table 5.1). The lowest sea oats survival amongst greenhouse treatments was found in 14S, with 70% and 41% at 2 and 3 MAT, respectively. Sea oats survival in 2010 greenhouse conditions was consistently lowest when sea oats plants were constantly flooded in 14 cm of water. We also found that survival in saturated beach conditions at Holly Beach, LA, was always lowest when compared to greenhouse treatments.

The combination of multiple extreme environmental conditions likely resulted in high ($p < 0.05$) sea oats mortality at Holly Beach in 2010. Approximately two weeks prior to sea oats plantings at Holly Beach Hurricane Alex made landfall at Soto la Marina, Tamaulipas, Mexico, approximately 800 km southwest of Holly Beach. Hurricane Alex generated a tremendous amount of storm surge that flooded Holly Beach. In addition, 22 cm rainfall occurred within the first 28 days after transplant, which is approximately 9 cm above average. Finally, the average low air temperature at Holly Beach one month after transplant was 24.7° C while the average low temperature in the greenhouse trial was 20° C. We have observed low temperatures exceeding 20°C reduce vegetative growth of sea oats. Others investigators have also reported that as low daily temperatures increase, vegetative growth of some plants species decreases (Abdul-Razack 2011). It is possible that when all these factors were combined they resulted in extreme sea oats plant stress and ultimately much higher sea oats mortality than the greenhouse environment.

Although mean sea oats survival differed between greenhouse and beach environments we wanted to determine whether survival in greenhouse treatments could predict survival in saturated beach conditions. Three significant ($p < 0.05$) correlations between greenhouse and beach environments were detected: 06S one MAT and Holly Beach one MAT ($r = 0.9643$; $p = 0.0364$); 06S two MAT and Holly Beach one MAT ($r = 0.9636$; $p = 0.0364$); and 06S three MAT and Holly Beach two MAT ($r = 0.9688$; $p = 0.0312$). Although significant correlations were detected for one greenhouse treatment and the beach environment, the correlations were not meaningful. Sea oats survival when flooded with 6 cm of water for 1, 2, and 3 months correlated with survival at Holly Beach after 1, 1, and 2 months, respectively. Our goal was to develop a

greenhouse protocol that could reduce the time not increase the time necessary to identify saturation tolerant sea oats.

Sea oats plant height, leaf numbers, total fresh weight biomass and root length were also measured in the 2010 greenhouse trial to determine the response of sea oats to saturated growing conditions. We were unable to measure these traits for sea oats at Holly Beach due to the extremely time consuming and laborious nature of the measurements. In the greenhouse, plant height varied among treatments. AT 1 MAT, plant height ranged from 16.66 to 22.18 cm (Table 5.2). Plants grown in 14C had the tallest plants while plants grown in 10C had the shortest plants (Table 5.2). At 2 MAT, plants under 14C had the tallest plants, whereas, the control had the shortest plants (Table 5.2). At 3 MAT, plants grown in 10C and 10S had the tallest plants and the control had the shortest plants (Table 5.2). It appears that sea oats seedlings respond to saturation stress by increasing in plant height. Similar findings (Sman et al. 1991; Banga et al. 1995; Vartapetian and Jackson 1997; Voesenek et al. 2004; Striker et al. 2005) have been reported in several plant species. Increased plant height is thought to restore contact of shoots with the atmosphere for increased access to aerated and illuminated zones above or close to the water surface, which increases oxygen uptake (Armstrong 1979; Laan and Blom 1990a; Laan et al. 1990b).

We also found that the number of sea oats leaves differed significantly among experimental treatments. At 1 MAT, the control had the most leaves per plant and 14S had the least leaves per plant (Table 5.2). At 2 MAT, 06S and 10C had the most leaves per plant while the control and 14S had the least leaves per plant (Table 5.2). At 3 MAT, 06C and 10C had the most leaves and the control had the least leaves (Table 5.2). When grown in saturated conditions sea oats seedlings produced more leaves than plants grown in the control treatment, which were

Table 5.2 Average plant height, number of leaves per plant, total fresh weight biomass, and root length for sea oats grown in greenhouse conditions, Baton Rouge, LA, 2010

Treatment[a]	1 MAT[b]	2 MAT	3 MAT
Plant Height (cm)			
F-Value[c]	9.71***	6.52***	22.92***
Control	18.13 cd[d]	18.11 cd	19.05 d
06C	18.84 bc	22.82 ab	28.11 b
06S	19.95 b	21.04 b	24.26 c
10C	16.66 d	21.46 b	33.19 a
10S	17.84 cd	21.73 b	33.70 a
14C	22.18 a	23.94 a	30.63 ab
14S	19.85 b	20.72 b	30.99 ab
Number of Leaves			
F-Value	9.90***	36.87***	17.90***
Control[b]	2.46 a	2.33 d	2.36 c
06C	2.34 ab	3.61 a	3.59 a
06S	2.33 ab	2.95 c	2.84 b
10C	2.31 ab	3.66 a	3.88 a
10S	2.12 bc	2.95 c	2.85 b
14C	2.07 c	3.27 b	2.97 b
14S	1.68 d	2.34 d	3.07 b
Total Fresh Weight Biomass (g)			
F-Value	2.14*	4.94***	6.07***
Control[b]	0.37 abc[c]	0.43 d	0.53 c
06C	0.35 c	0.54 cd	0.75 b
06S	0.44 a	0.71 a	0.66 bc
10C	0.35 bc	0.69 ab	0.82 ab
10S	0.37 abc	0.57 bc	0.95 a
14C	0.44 a	0.72 a	0.97 a
14S	0.33 c	0.52 cd	0.88 ab
Root Length (cm)			
F-Value	3.72**	3.01**	16.29***
Control[b]	24.42 a	30.27 a	32.98 a
06C	19.86 bc	18.58 b	17.63 c
06S	16.16 c	21.28 b	18.38 c
10C	19.63 bc	19.70 b	17.43 c
10S	17.56 c	19.30 b	19.86 bc
14C	23.49 ab	20.38 b	23.48 b
14S	19.59 bc	21.88 b	21.68 bc

[a] Control indicates sea oats plants were grown on dry bench and watered every 3 days; 06, 10, and 14 indicate water depths were 6, 10, and 14 cm, respectively; C indicates cyclic flood where sea oats plants were cyclically flooded for 14 days followed by 14 days of dry conditions; S indicates static flood where sea oats plants were continuously flooded. [b] MAT = months after transplanting. [c] * indicated $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$. [d] Averages within a column followed by different letters are statistically different according to the *t* test on difference of least square means ($p = 0.05$).

non-saturated conditions; a similar observation was made by Grimoldi et al (1999). It has been suggested that increased leaf numbers under saturated conditions increase the surface area making contact with oxygen, light and carbon dioxide, which are major features contributing to survival in saturated conditions (Grimoldi et al. 1999).

Finally we measured total fresh weight biomass and root length for sea oats seedlings grown in greenhouse in 2010. At 1 MAT, biomass was highest for sea oats seedlings grown at 06S and 14C and lowest for 06C and 14S (Table 5.2). At 2 MAT, sea oats plants under 06S and 14C still had the most biomass while the control had the least (Table 5.2). At 3 MAT, 10S and 14C had the highest biomass while the control had the lowest biomass. Root length also varied significantly among different treatments. Sea oats seedlings grown under normal conditions (control treatment) had the longest roots 1, 2, and 3 MAT (Table 5.2). One MAT, 06S and 10S had the shortest roots; 2 MAT all the flooded sea oats had short roots; and 3 MAT 06C, 06S, and 10C had the shortest roots (Table 5.2). From this study it appears that saturated conditions reduce normal root development in sea oats. We also observed sea oats plants grown in saturated conditions developed more adventitious roots at the soil level (data not shown). This mechanism has been reported to occur to replace roots that have been killed by anoxia (Vartapetian and Jackson, 1997). These roots usually emerge from the shoot base into surface layers to enhance oxygen transport (Visser et al., 1996) and reduce saturation injury.

In 2011 a second greenhouse trial was initiated to determine if greenhouse conditions could be used to predict saturated field conditions. We examined 4 treatments: 2 salinity levels (0 and 35 ppt) and 2 flood levels (0 and 14 cm). Saline treatments were added to simulate beach conditions after an extreme episodic storm event accompanied with salt water intrusion. We chose 14 cm constant flooding because this treatment provided the highest mortality, tall plants,

and high plant biomass in the 2010 greenhouse trial. We also established a beach trial at Holly Beach in 2012 and a field trial at Burden Museum and Gardens in 2012. The field trial was a saturated agronomic field.

Sea oats seedling survival in 2011 greenhouse and 2012 beach and field trials ranged from 59 to 100 % one MAT. Seedling survival was highest when sea oats plants were grown in greenhouse on dry bench and watered regularly every 3 days (control), flooded to 14 cm with 35 ppt saline solution (14Sal) and at Holly Beach (Table 5.3). Sea oats survival was lowest when sea oats seedlings were grown flooded to 14 cm with fresh water (14Fr; Table 5.3), which was similar to what we observed in 2010 trial (Table 5.1). At 2 MAT, sea oats survival ranged from 7.8 to 97.7%; the control and Holly Beach had the highest survival while plants grown on dry bench regularly watered with 35 ppt saline solution (BSal) had the lowest survival (Table 5.3). At 3 MAT, sea oats seedling survival ranged from 0.3 to 98%; the control had the highest survival while plants under BSal had the lowest survival (Table 5.3). This indicates that in non-saturated conditions, sea oats are very sensitive to saline conditions. Others have documented that although sea oats tolerate salt spray (Boyce 1954; Valk 1977) they are not salt-tolerant plants (http://www.plant-materials.nrcs.usda.gov/pubs/lapmcpg7412.pdf). It is also very interesting to note that survival of sea oats plants in 14Sal was consistently higher than survival of sea oats plants grown in 14Fr. Other investigators have reported that sea oats grow larger and are more vigorous when salt spray is present on leaves or when washed into root zone (Boyce 1954; Valk 1977). A remarkable difference in sea oats survival was found between Holly Beach in 2010 and 2012. Sea oats survival 3 MAT was 76% in 2012 while only 25% in 2010. In 2010,

Table 5.3 Average sea oats survival, plant height, number of leaves, fresh weight and root length of sea oats plants evaluated in 2011 in controlled greenhouse (Baton Rouge, LA) and in 2012 in saturated beach (Holly Beach, LA) and field (Baton Rouge, LA) conditions

Treatment [a]	1 MAT [b]	2 MAT	3 MAT
		Survival (%)	
F-Value [c]	52.31***	219.75***	190.99***
14Fr	59.0 c[d]	28.3 d	14.5 d
BSal	81.9 b	7.8 e	0.3 e
14Sal	94.9 a	73.0 b	40.5 c
Control	100.0 a	97.7 a	98.0 a
Burden	78.5 b	56.7 c	45.8 c
Holly Beach	96.1 a	93.8 a	76.0 b
		Plant Height (cm)	
F-Value	313.18***	292.47***	192.69***
14Fr	19.7 c	21.1 c	23.9 cd
BSal	16.1 d	18.7 cd	26.0 bcd
14Sal	15.9 d	16.8 d	19.2 d
Control	17.6 cd	20.5 c	25.2 c
Burden	50.7 b	49.8 b	52.9 b
Holly Beach	59.8 a	65.7 a	67.7 a
		Number of Leaves	
F-Value	124.24***	47.80***	45.12***
14Fr	1.79 d	1.48 c	2.52 c
BSal	1.68 d	1.62 c	1.66 bc
14Sal	2.31 cd	1.81 c	2.06 c
Control	2.46 cd	2.33 c	2.34 c
Burden	11.09 a	16.06 a	23.83 a
Holly Beach	5.66 b	11.71 b	13.28 b
		Fresh Weight Biomass (g)[e]	
F-Value			39.75***
14Fr	- -	- -	0.26 b
BSal	- -	- -	0.13 b
14Sal	- -	- -	0.19 b
Control	- -	- -	0.47 b
Burden	- -	- -	5.63 a
Holly Beach	- -	- -	6.72 a
		Root Length (cm)	
F-Value			5.88**
14Fr	- -	- -	7.13 b
BSal	- -	- -	6.42 b
14Sal	- -	- -	6.97 b
Control	- -	- -	20.47 a
Burden	- -	- -	- -
Holly Beach	- -	- -	- -

[a] Control indicates sea oats plants were grown on dry bench and watered every 3 days; 14Fr indicates sea oats plants were grown flooded to a depth of 14 cm with fresh water; BSal indicates that sea oats plants were grown on dry bench and water every 3 days with 35 ppt saline solution; 14Sal indicates sea oats plants were grown flooded with 35 ppt saline solution at a flood depth of 14 cm. [b] MAT = months after transplanting. [c] * indicated $p < 0.05$; ** indicates $p < 0.001$; *** indicates $p < 0.001$. [d] Averages within a column followed by different letters are statistically different according to the *t* test on difference of least square means ($P = 0.05$). [e] Total biomass was measured for the controlled greenhouse trial and above-ground biomass was measured for the beach and field trials.

Hurricane Alex resulted in significant storm surge at Holly Beach just prior to transplanting; 22 cm rainfall within the first 28 days after transplant, and the average low temperature was 24.7°C. In 2012, Holly Beach was not affected by any major storm event prior to or within the first 28 days after transplant. It also received 8 cm rainfall and the average low temperature was 20.3°C. This suggests that in 2010 beach conditions were much more saturated and had higher night time temperatures which may have resulted in higher mortality. We also measured plant survival 6 MAT for Holly Beach and Burden (data not shown). Sea oats survival was higher at Holly Beach, 74%, than at Burden, 40%. This is likely due to higher water holding capacity of the soils at Burden, 31 cm rainfall within the first 28 days after transplant, and low temperatures at Burden were 22°C within the first 28 days after transplant.

To further investigate the morphological responses of sea oats seedlings to saturated conditions, we measured plant height, leaf numbers, and fresh weight biomass for greenhouse treatments and field environment and root length for the greenhouse trial. Significant ($p < 0.05$) treatment by time interactions were detected for plant height, leaf numbers, fresh weight and root length (data not shown); therefore data were analyzed separately for each month. Sea oats plant height was significantly different for the different treatments. Throughout the trial Holly Beach had the tallest plants (Table 5.3). This was not surprising because sea oats seedlings used at Holly Beach field trial were about 8 months old compared to seedlings used in greenhouse treatments, which were about 3.5 months old. We specifically examined young seedlings in the greenhouse trials and older plants in beach and field trials to determine whether small seedlings evaluated in greenhouse conditions could predict survival of large sea oats plants at saturated beaches. We also found that sea oats at Burden consistently had the highest number of leaves, while greenhouse treatments had lowest number of leaves (Table 5.3). This is most likely

because seedlings used in field environments were older than seedlings used in greenhouse treatments.

To determine fresh weight biomass we measured total biomass for plants under greenhouse treatments and above-ground biomass for plants under beach and field environments. Accurately measuring below-ground biomass in field and beach conditions would have been nearly impossible due to aggressive spread of sea oats roots and rhizomes from neighboring plants. When we analyzed biomass, the beach and field environments had the highest sea oats biomass compared to greenhouse treatments; all greenhouse treatments had similar total biomass (Table 5.3). The difference in age of seedlings used in greenhouse and field environments could have contributed to increased biomass at field and beach environments. In the greenhouse, roots were longest for control plants (Table 5.3) an observation similar to what was observed in 2010.

Pearson correlation coefficients were used to determine whether sea oats survival in greenhouse treatments could predict sea oats survival in saturated beach and field environments. Sea oats survival 3 MAT when grown in greenhouse with constant flooding at 14 cm with fresh water was significantly correlated with survival of sea oats 6 MAT at Holly Beach ($r = 0.9696$; $p = 0.0304$) and Burden ($r = 0.9943$; $p = 0.0057$). The ability to predict sea oats survival in greenhouse conditions has significant implications for a saturation tolerance breeding program. Selection of saturation tolerant sea oats lines after only 7 months (4 month old plants and 3 month assay) instead of 14 months (8 month old plants and 6 month assay) would reduce the time to select sea oats saturation tolerant lines, thus increasing the efficiency of the breeding program. It is noteworthy that the selected greenhouse treatment appears to be a good predictor of sea oats survival under saturated beach conditions in the absence of storm surge. However, after a significant storm event that produces storm surge, such as occurred at Holly Beach in

2010, significant correlations between greenhouse treatments and beach environments were not found. This suggests that additional work would be needed to identify greenhouse conditions if selection for storm surge tolerance in the greenhouse is desired.

5.4.2 Sea oats seed yield

In addition to effective selection, genetic diversity is also an essential component for a successful saturation tolerant sea oats breeding program. Seed is a readily available source of genetic diversity in sea oats because it is an open-pollinated heterozygous plant (Wagner 1964). To determine whether large quantities of sea oats seeds could be produced outside of beach environments, we compared sea oats seed yield, seed yield components, and seed viability in natural low dune profile beach environments and artificial nurseries at Baton Rouge, LA. Significant ($p < 0.05$) time by treatment interactions were detected for number of seed spikelet^{-1}, number of seed plant^{-1}, number of spikelets plant^{-1}, average number of florets spikelet^{-1}, percent seed set, and seed viability therefore individual analyses were conducted.

The number of seed spikelet^{-1} ranged from 0.38-1.23 for natural environments and 0.61-2.19 for the artificial environments (Table 5.4). More sea oats seeds spikelet^{-1} were produced in artificial environments in 2007 and 2010, while more seeds spikelet^{-1} were produced in natural beach environments in 2009 and 2011 (Table 5.4). The number of seeds plant^{-1} ranged from 92.6 to 470.3 (Table 5.4). More seeds plant^{-1} were produced at artificial nursery than natural beach environments in 2007 while in 2009, more seeds plant^{-1} were produced in natural beach environments. In 2010 and 2011, there were no significant differences in number of seeds plant^{-1} among environments. In 2007, higher seed yield in artificial environments could be explained by increased soil fertility, because plants were continuously fertilized from 2005 to 2012.

Table 5. 4 Average number of seed spikelet^{-1}, number of seed plant^{-1}, number of spikelets plant^{-1}, number florets spikelet^{-1}, percent seed set, and percent viable seed for sea oats harvested from natural (Holly Beach, LA, and Long Beach, MS) and artificial (Baton Rouge, LA) environments in 2007, 2009, 2010 and 2011

Environment		2007	2009	2010	2011
		Number of Seed Spikelet^{-1}			
	F-value	141.54***[a]	8.61**	9.46**	46.44***
Natural		0.38 b[b]	0.98 a	1.23 b	1.14 a
Artificial		2.19 a	0.61 b	1.56 a	0.53 b
		Number of Seed Plant^{-1}			
	F-value	76.49***	216.10***	8.65**	2.31$^{n.s.}$
Natural		92.6 b	219.3 a	171.5 b	177.3 a
Artificial		470.3 a	107.0 b	215.8 b	129.8 a
		Number of Spikelets Plant^{-1}			
	F-value	3.42$^{n.s.}$	19.14***	15.29***	4.48*
Natural		241.4 a	227.4 a	184.4 b	192.3 a
Artificial		215.9 a	202.3 b	221.3 a	135.9 b
		Number Florets Spikelet^{-1}			
	F-value	6.38*	92.98***	0.00$^{n.s.}$	12.32**
Natural		12.7 a	7.0 b	9.6 a	11.7 b
Artificial		11.2 b	8.6 a	9.6 a	13.7 a
		Seed Set (%)			
	F-value	217.73***	43.29***	49.36***	48.61***
Natural		3.2 b	14.6 a	10.7 b	10.2 a
Artificial		20.0 a	6.5 b	16.3 a	4.3 b
		Viable Seed (%)			
	F-value	0.00$^{n.s.}$	1.09$^{n.s.}$	47.60***	1.04$^{n.s.}$
Natural		0.0 a	36.0 a	68.0 a	66.0 a
Artificial		0.0 a	24.0 a	16.0 b	56.0 a

[a] * indicates $p < 0.05$; ** indicates $p < 0.001$; *** indicates $p < 0.001$; n.s. indicates non-significant difference.

[b] Averages within a column followed by different letters are statistically different according to the *t* test on difference of least square means ($P = 0.05$).

Crewz (1987) found that increased soil fertility improved sea oats seed yield. The higher seed yields at natural environments in 2009 may be due to the effects of Hurricane Gustav, which made landfall at Cocodrie, Louisiana in 2008. Storm surge may have resulted in introduction of nutrients. In addition we have observed increased panicle and seed production following storm events that destroy dunes and above-ground plant material to ambient beach elevations. We have also observed that as above-ground sea oats material and rhizomes densities increase panicle and seed yields decrease. These observations suggest a physiologic plant response to quantities of above- and below- ground plant material that affect seed yield.

Average number of florets spikelet^{-1} varied for plants in natural and artificial environments with the exception of 2010 (Table 5.4). In 2007, plants in natural environment had more florets spikelet^{-1} than plants in artificial environment (Table 5.4). In 2009 and 2011, sea oats plants in artificial environments had more florets spikelet^{-1} than plants in natural environments (Table 5.4). In 2009 and 2011, sea oats plants in natural environment had more spikelets plant^{-1} than plants in artificial environment (Table 5.4). In 2010, sea oats plants in artificial environments had more spikelets plant^{-1} than plants in natural environment (Table 5.4). Based on our data, environments with high number of spikelets plant^{-1} were observed to have high average number of seed spikelet^{-1} and number of seed plant^{-1}. Seed set varied for plants in natural and artificial environments. In 2007 and 2010, sea oats plants in artificial environment had a higher percent seed set than plants in natural environments (Table 5.4). In 2009 and 2011 sea oats plants in natural environments had a higher seed set than plants in artificial environments (Table 5.4). It was surprising that environments with higher average number of florets spikelet^{-1} had low seed set. These data indicate that most of the florets formed were not fertile. The number of fertile florets in each spikelet determines the absolute capacity of seed

that can be formed per spikelet. Although only a small fraction of this potential is realized, the number of fertile florets in each spikelet may serve as an indicator of the reproductive potential of spikelets. Finally we measured 100 seed weight and seeds g^{-1} because seed weight is known to influence seed germination, seedling vigor and establishment (Copeland and MacDonald 2001). The 100 seed weight was similar for natural and artificial environments (Table 5.5). When we analyzed the number of sea oats seed per gram, the artificial environments were found to have more seeds per gram than the natural environments (Table 5.5).

Table 5.5 Average weight of 100 sea oats seeds and number of seeds g^{-1} in natural (Holly Beach, LA and Long Beach, MS) and artificial environments (Baton Rouge, LA) in 2007, 2009, 2010, and 2011

Environment		
	100 seed wt.(g)	
	F-value[a]	0.54[n.s.]
Natural		0.30 a[b]
Artificial		0.44 a
	Number of Seed g^{-1}	
	F-value	10.88**
Natural		237.13 b
Artificial		721.18 a

[a] n.s. indicates non- significant difference.
[b] Averages within a column followed by different letters are statistically different according to the *t* test on difference of least square means ($p = 0.05$).

In 2007, sea oats seed germination was measured (data not shown). Germination ranged from 0 to 100% with an average of 38% and the mode was 32% for seed harvested from natural environments. The seed germination of the artificial seed production nursery also ranged from 0 to 100%; however the average was only 20% and the mode was 0%. We speculate the lower germinations found in the artificial environment were due to seed maturity. The panicles harvested from natural environments had already begun to shatter; most of the panicles had already shed the top 5 cm of spikelets at the time of harvest. In contrast, panicles from the

artificial environment were harvested when the first spikelet was shed, which was <1 cm. This would suggest that seeds harvested from natural environments were more mature and therefore had higher germination. Panicle harvests in 2009, 2010, and 2011 were delayed until approximately 5 cm spikelets had been shed. Due to time constraints we were unable to measure seed germination within 5 months of harvest. Instead we measured seed viability for all harvested seed in 2013. In 2007, no viable seeds were found (Table 5.4). This is not surprising because in previous studies we found that sea oats seed viability decreases as storage time increases (Nabukalu and Knott, 2013). Viability of seeds harvested in 2009 was similar for natural and artificial production environments, 36 and 24%, respectively (Table 5.4). Sea oats seed viability was higher for seeds harvested from natural environments in 2010, 68 versus 15% (Table 5.4). In 2011, seed viability was similar for both environments: 66% for natural environments and 56 % for artificial environments (Table 5.4).

We found that sea oats seeds can be produced in artificial environments. Establishing seed production nurseries could support coastal restoration efforts by minimizing sea oats seed harvests from natural environments. Minimizing seed harvests from natural ecosystems will also reduce disruption of the natural ecosystems, which can result in sea oats stand decline and resultant land loss. Establishing artificial seed production nurseries also support breeding programs by producing a source of readily available genetic diversity. However, our findings clearly demonstrate the need for additional research to optimize sea oats seed yield, viability, and storage.

5.5 Conclusion

We identified a greenhouse protocol that could predict sea oats survival in half the time of saturated beach conditions. We also found that seed can be produced in artificial production

nurseries, but that additional research is required to optimize seed yields, viability and storage conditions.

CHAPTER SIX: IDENTIFICATION OF FUNGI AND BACTERIA COLONIZING SEA OATS SEEDS

6.1 Introduction

Seeds play a vital role in species preservation and maintaining biodiversity. Seed quality is widely accepted as essential for plant production and conservation efforts (Guan 2009), but seed lots often fall short of this ideal. Plant seeds are not only reproductive organs (Guan 2009), they are also carriers of various beneficial microbes as well as pathogens. The composition of microbial communities on and within seeds tends to affect their storage and field performance. Many studies have confirmed that the surface and interior of seeds bear a variety of microbial organisms (Nelson 2004) residing in the rhizosphere, in the phyllosphere, and inside the tissues of healthy plants (Liu et al. 2012).

Among the bacterial species colonizing barley seeds during the early stages of germination are *Acinetobacter*, *Bacillus*, *Burkholderia*, *Pantoea* and *Pseudomonas* (Normander 2000). Cotton seeds were colonized by species of *Enterobacter*, *Microbacterium*, *Curtobacterium* etc. (McKellar 2003). Germinating seeds are colonized by indigenous microbial populations within a few hours of sowing and populations may reach densities of 10^5–10^7 cells/seed (Liu et al. 2012). The nature and activities of the organisms colonizing germinating seeds would be expected to significantly affect the performance of microbial strains artificially introduced to seeds for the purpose of nitrogen fixation, plant growth promotion or biological disease control.

The association of various fungi with sea oats seeds has been reported to have either significant harmful effect on seed germination and plant growth or to be beneficial to seed performance under particular field conditions (Sylvia 1986). An obvious effect of seed borne pathogenic fungi is the deterioration of seed quality and the significant decrease in germination

rates. Some of the seed-borne fungi have been found to be very destructive, causing seed rot, decreased seed germination and pre and post germination death. In other plant species, seed borne pathogens have been reported to affect the seed quality by damaging external or internal seed tissues and cause the important seed diseases like seed rot, seed necrosis, and seedling damage through the local or systemic infection (Bateman and Kwasna 1999).

A range of new and traditional methods are available for detection of microbial organisms surviving in seed. As the majority of plant diseases are transmitted through contaminated seed or propagative materials, detection and identification of pathogens in seed is of paramount importance required for formulating disease control mechanisms. The objectives of the present study were to: 1) determine the effect of seed pathogens on sea oats seed germination and 2) identify fungal and bacteria species colonizing sea oats seed.

6.2 Materials and methods

6.2.1 Germination assay

Seed used in this study was harvested in 2011 from 2 natural beach environments (Long Beach, MS and Holly Beach, LA), and 2 artificial breeding nurseries (Louisiana State University Agriculture Center's Central Research Station, Baton Rouge, LA and Burden Museum and Gardens, Baton Rouge, LA).

6.2.2 Seed treatment and experimental designing

Sea oats seeds were first surface sterilized with a 25% Clorox solution for 15 minutes (Woodhouse et al, 1968) to reduce the incidence of surface contaminants. Disinfected seeds were rinsed thoroughly with distilled water. The disinfected seeds were then placed on moist germination paper towel (ISTA 2003) saturated with distilled water. Two hundred seeds from each study environment were germinated. One hundred seeds were placed on each germination

paper and they were arranged into 4 replicates of 25 seeds. The seeds were then incubated in the dark at 18.3°C for 17 hours and 35°C for 7 hours (Woodhouse et al, 1968). Once placed in alternating thermoperiod, seeds were kept moist with sterilized deionized water and checked every 7 days for 28 days after germination. A seed was considered germinated when the radical was greater than 2 mm in length. Seeds were considered infested with fungi or bacteria when mycelia or bacterial discharge (ooze) was visible, respectively. Seeds infested with fungi and bacteria were counted and removed to prevent spread of pathogens to adjacent seeds.

6.2.3 Data collection and analysis

Percentage seed germination was calculated as follows: (number of germinated seeds / total seed) x 100. Percentage infected seed was calculated as (number of removed seed / number of seeds planted) x 100. The cumulative percent germination and pathogen infestation were determined on a weekly basis. All statistical analyses were performed in SAS 9.3. Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure. A critical significance level of $\alpha = 0.05$ was chosen for all statistical tests. Least squares means were used for means separation at $P < 0.05$.

6.3 Bacteria identification

6.3.1 Bacteria isolation

Bacteria were isolated from sea oats seed harvested in 2011 from natural beach environment Long Beach, MS and artificial breeding nursery; Louisiana State University Agriculture Center's Research Station, Baton Rouge, LA BenHur. From each location a 1.0 g batch of seed was processed. In a laminar-flow hood, seeds were partially crushed (until approximately 80% of the seed was broken) with a sterilized mortar and pestle and suspended in 100 ml of sterile phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 0.01 M

Na_2HPO_4, 1.8 mMKH_2PO_4, pH 7.4) with 0.025% Tween 20 (Sigma-Aldrich, St.Louis). The seed suspension was incubated for 2 h at 4°C. Then, 100 µl of 10-fold serial dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of the suspension in sterile saline (0.85% NaCl) was plated in duplicate on Nutrient agar (NA; Difco™, Sparks, MD) supplemented with 0.01% cycloheximide (Sigma-Aldrich). NA was prepared by adding 23 g of nutrient agar (Difco™, Sparks, MD) to 1 L distilled water and autoclaving at 121 °C for 25 min. The serial diluted plates were incubated at 22 °C under 12 h light and 20 °C with 12 h darkness. The plates were observed for development of bacterial colonies daily for 5 days. A loop-full of bacterial cells from the edge of each colony was streaked onto another NA plate for single-cell purification and identification of the bacteria. The isolates were further purified on nutrient agar (NA; Difco Laboratories, Detroit) and maintained at -80°C in 15% glycerol.

6.3.3 DNA extraction

Total DNA was extracted from bacterial colonies using QIAprep Spin Miniprep kit (Qiagen, Inc.,Valencia, CA) according to the manufacturer's instructions. DNA concentrations were measured using a nanodrop spectrophotometer (Thermo Scientific Inc., Waltham MA) and DNA quality was verified using a 1% agarose gel in 1× Tris-acetate EDTA (TAE) buffer stained with ethidium bromide. The agarose gel was visualized under UV light and documented after electrophoresis. DNA concentration of each sample was adjusted to 50 ng/µL. The DNA was stored at -20 ˚C.

6.3.4 Polymerase chain reaction (PCR) amplification

For identification of the bacterial cultures, Polymerase chain reaction (PCR) was performed to amplify the 16S rRNA genes from the extracted DNA using the primer set 27f (5-AGA GTT TGA TCM GGC TCA G-3) and 1492 r (5-GGT TAC CTT GTT ACG ACT T-3)

(Lane, 1991). The 50µL PCR reaction mixture contained 50ng of DNA extract, 1.5mM $MgCl_2$, 1X Reaction buffer (without $MgCl_2$) (Promega Corporation, Madison, WI), 200µM of each dNTP, 0.20µM of each primer and 1.0U Taq polymerase (Promega). PCR was performed in BioRad MyCycler™ Programmable thermal cycler (BioRad, Hercules, CA). After initial denaturation at 94°C for 5min, each thermal cycling was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1min. At the end of 30 cycles, the final extension step was at 72°C for 10min. PCR products were run on 1% agarose in TBE buffer (445mM Tris, 445mM Boric acid and 10mM EDTA (pH8.0) to confirm that the correct product approximately 1500bp in size was produced.

6.3.5 DNA sequencing and phylogenetic analysis

The PCR products were purified using the QIAGEN PCR purification kit (Qiagen, Inc.,Valencia, CA) following the manufacturer's instructions. Purified PCR products were sequenced in an automated sequencer at the Beckman Coulter Genomics DNA Sequencing Facility (http://www.beckmancoulter.com). Sequencing was performed using the same forward and reverse primer sets which were used for PCR amplification.

Sequences were edited and their similarities determined against known sequences in the NCBI Genbank database using the Basic Local Alignment Search Tool (BLAST) algorithm21. For phylogenetic analysis, multiple sequence alignment of concatenated ITS sequence was constructed in MEGA5 using a MUSCLE algorithm (http://www.megasoftware.net/; Tamura et al. 2011) and the resulting alignment checked and the sequences used for phylogenetic tree construction. Gene phylogenies were inferred by maximum likelihood (ML) in RAxML (Stamatakis, 2006) using a GTR model of evolution in the CIPRES Gateway Science portal

(Miller *et al*., 2010). Support for the branching topologies was evaluated by bootstrap analysis derived from 1000 replicates with 10 random additions replicated.

6.4 Identification of fungi

6.4.1 Seed Samples

Fungi were isolated from visually healthy sea oats seed harvested in 2010 from 2 artificial nurseries; Louisiana State University Agriculture Center's research stations (Ben Hur research station, Baton Rouge and Burden Rural life research station, Baton Rouge) and 2 natural environments; Long Beach, MS and Holly Beach, LA. A working sample of 200 seeds was taken at random from the seed harvested from each location.

6.4.2 Fungal isolation

In a laminar flow hood, seeds were surface sterilized by placing the seeds in sterilized strainers and immersing them for 1 minute in a solution of 70% ethyl alcohol and then for 15 minutes in 25% sodium hypochlorite solution and then thoroughly rinsed with sterilized distilled water. Following sterilization the seeds were blotted with sterilized blotter paper prior to being placed onto potato dextrose agar (PDA) in Petri plates. PDA was prepared by adding 39 g of dehydrated PDA (Difco™, Sparks, MD) to one liter of distilled water in a conical flask. After mixing it thoroughly, the medium was autoclaved for 15 min at 121˚C, cooled before being poured into Petri plates. The seeds were placed individually on PDA in Petri dishes using sterile forceps. Ten sterilized seeds were placed on each plate. The plated seeds were incubated at 22-24 ˚C with 12 h light/12 h darkness. The incubated plates were observed for fungal growth at 2, 4, 6, 8, and 10 days after plating. Plaques on culture medium (2mm in diameter) with hyphal tips of the developing fungal colonies were cut and transferred onto PDA for purification.

6.4.3 Morphological identification

Cultures were examined periodically and identified when they sporulated. The cultures were separated into groups based on classical morphological features including growth pattern, colony texture, pigmentation, and growth rate of the colonies on PDA (Promputtha et al., 2005). When fungal colonies sporulated on PDA, small plaques from the edge and the center of each growing colony were transferred onto glass slides, and then were examined using a compound light microscope (Zeiss immersion 518 N microscope). Specimens for light microscopy were mounted in sterile distilled water for observation for characteristics of their vegetative and reproductive structures such as hyphal color and structures, shape and size of conidia, conidiophores, and microsclerotia. Genus identification was made by examining the isolated fungi under with the help of identification keys (Raper and Fennell, 1965; Booth, 1971; Ellis et al., 1980; Barnett and Hunter, 1972; Sivaesan, 1990).

6.4.4 DNA extraction

Total DNA was extracted from fungal mycelia using QIAprep Spin Miniprep kit (Qiagen, Inc.,Valencia, CA) according to the manufacturer's instructions. DNA concentrations were measured using a nanodrop spectrophotometer (Thermo Scientific Inc., Waltham MA) and DNA quality was verified using a 1% agarose gel in 1× Tris-acetate EDTA (TAE) buffer stained with ethidium bromide. The agarose gel was visualized under UV light and documented after electrophoresis. DNA concentration of each sample was adjusted to 50 ng/µL. The DNA was stored at -20 ˚C.

6.4.5 Polymerase chain reaction (PCR) amplification

For identification of the fungal isolates, DNA of each isolate was amplified by polymerase chain reaction (PCR). Two different Internal Transcribed Spacer (ITS) primers sets;

ITS1 (5' TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') (White et al., 1990) were used for amplification of the fungal ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions 1 and 2 of all isolates. These primers produce an amplicon that is between 600 and 800 bp. The 50µL PCR reaction mixture contained final concentrations of 10x PCR buffer, 2.0 mM MgCl2, 2.5 mM dNTPs, 10 µM of each primers, 2 units of Taq polymerase (Promega Corp., Madison, WI), and 2 µl of DNA extract. The PCR amplifications were conducted using a BioRad MyCycler™ Programmable thermal cycler (BioRad, Hercules, CA). The PCR cycles included initial denaturing for 2 min at 95 °C; 35 cycles of denaturation at 94 °C for 45 sec, annealing at 54 °C for 1 min and extension at 72 °C for 1.5 min; followed by final extension at 72 °C for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide, and visualized with a UV transilluminator. The lengths of the amplification products were estimated by comparing with a 100-bp DNA ladder.

6.4.6 DNA sequencing and phylogenetic analysis

The PCR products were purified using the QIAGEN PCR purification kit following the manufacturer's instructions. Purified PCR products were sequenced in an automated sequencer at the Beckman Coulter Genomics DNA Sequencing Facility (http://www.beckmancoulter.com). Sequencing was performed using the same forward and reverse primer sets which were used for PCR amplification. Sequences were edited and their similarities determined against known sequences in the NCBI Genbank database using the Basic Local Alignment Search Tool (BLAST) algorithm21. For phylogenetic analysis, multiple sequence alignment of concatenated ITS sequence was constructed in MEGA5 using a MUSCLE algorithm (http://www.megasoftware.net/; Tamura et al. 2011) and the resulting alignment verified and the sequences used for phylogenetic tree construction. Gene phylogenies were inferred by

maximum likelihood (ML) in RAxML (Stamatakis, 2006) using a GTR model of evolution in the CIPRES Gateway Science portal (Miller *et al*., 2010). Support for the branching topologies was evaluated by bootstrap analysis derived from 1000 replicates with 10 random additions replicated. Out-groups were selected based on sister relationships relative to each group (Millanes, *et al.*, 2011).

6.5 Results and discussion

6.5.1 Germination Assay

To determine the effect of seed borne pathogens on sea oats seed germination, a germination assay was conducted. We monitored seed germination and pathogen infestation every 7 days for 28 days from the day of planting. During week 1, no sea oats seeds were germinated; however seeds infested with either fungi or bacteria were observed. Sea oats seeds harvested from Burden were observed to have the highest pathogen incidence in week 1(13%; Figure 6.1). In week 2, 3 and 4 all locations had high pathogen incidence varying from 17-46.5%. Beginning from week 2 and continuing until week 4, Holly Beach and Long Beach had high seed germination (Figure 6.1). The seed produced at the artificial nurseries: Burden and Ben Hur had very low germination percentages (Figure 6.1). Possible explanations for natural locations having significantly high germination percentages are environmental differences and seed maturity levels at harvest. Sea oats seeds harvested from natural environments; Holly Beach and Long Beach could have attained physiological and harvest maturity prior to harvesting and were fully capable of germination when dormancy. Sea oats seed harvested from artificial nurseries: Burden and Ben Hur were harvested early, and they may have not reached harvest maturity. There was no significant difference in cumulative pathogen incidence for seed harvested from the different locations in week 2, 3 and 4 (Figure 6.1).

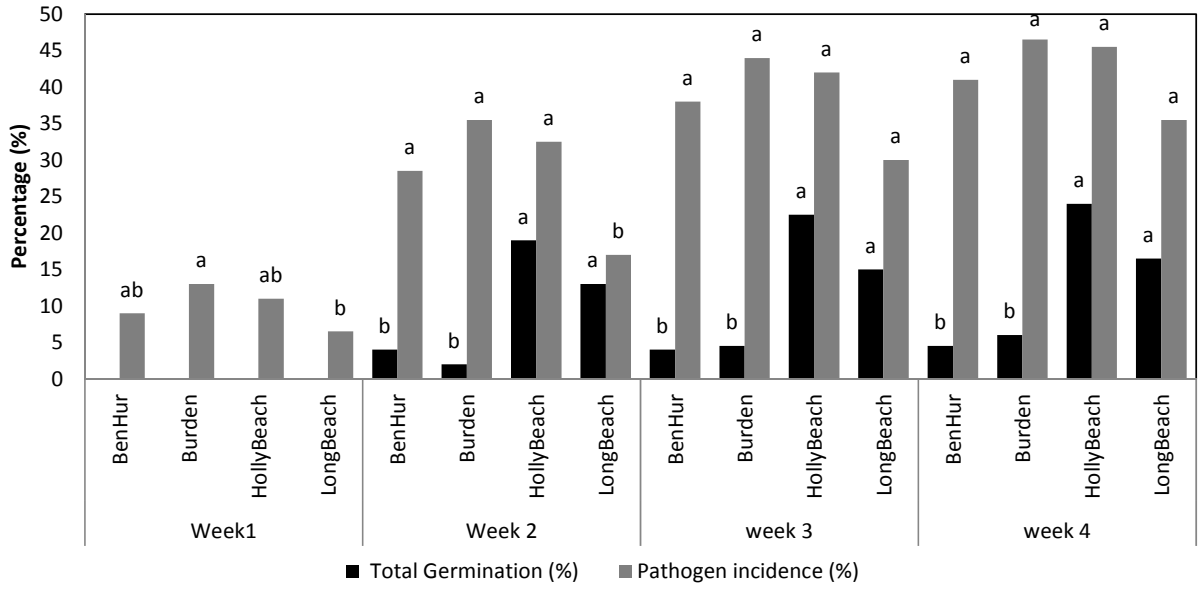

Figure 6.1 Cumulative percent seed germination and cumulative pathogen incidence of sea oats seeds harvested from Ben Hur, Burden, Holly Beach and Long Beach. Columns labeled with different letters for each assessment week and location are significantly different at $p < 0.05$ level.

However, each location consistently had a higher cumulative pathogen incidence than cumulative percent germination by week 2, 3 and 4 (Figure 6.1). This clearly demonstrated how pathogen incidence can deteriorate sea oats seed quality and decrease germination rates. Seed-borne fungi are very destructive; they cause seed rot, decreased seed germination and pre- and post- germination death. In other plant species, seed borne pathogens have also been reported to affect the seed quality by damaging external or internal seed tissues, causing seed diseases like seed rot, seed necrosis, and seedling damage through the local or systemic infection (Bateman and Kwasna 1999).

In order to identify control measures to these seed borne pathogens, it is very necessary to know the exact species inhabiting the seeds and identify seed treatments to decrease pathogen

incidence. Fungicide seed treatments protect seed viability and inhibit the invasion of fungal pathogens causing seed rot and seedling blight.

6.5.2 Identification of Bacteria colonizing sea oats seed

To identify seed borne bacteria colonizing sea oats seeds, bacteria were isolated from sea oats seed, purified to single-cell isolates, and identified using molecular species identification. The PCR products were sequenced and putative species identifications of isolates were made by comparing consensus sequences to other isolates in the NCBI Genbank database using a Blastn search (http://www.ncbi.nlm.nih.gov/). Species identifications were based primarily on 16S rDNA sequence identity. Cultures which shared 97% or more 16S rDNA sequence identity with Genbank sequences were considered conspecific and any sequence variation of 3% or less was assumed to be intraspecific.

Based on 16S rDNA sequence analysis 9 bacteria genera were represented: *Bacillus* (29.16 %), *Paenibacillus* (7.29 %), *Cronobacter* (9.38 %), *Enterobacter* (30.21 %), *Erwinia* (4.17 %), *Escherichia* (2.08 %), *Pantoea* (9.37 %), *Pectobacterium* (1.04 %), *Pseudomonas* (7.29 %; Figure 6.2). Among the dominant bacteria genera detected were *Bacillus* and *Enterobacter* (Figure 6.2). *Bacillus* and *Enterobacter* are known to be plant growth- promoting bacteria (PGPB; Sturz 1995; Videira et al.2009; Lucy et al.2004; Liu et al.2011), which may directly or indirectly affect the growth and development of plants (Feng and Song 2001).

The most dominant bacterial species were *Bacillus amyloliquefaciens* (Table 6.1). *Bacillus amyloliquefaciens* is an important plant growth-promoting rhizobacteria (PGPR) which can produce secondary metabolites antagonistic to several plant pathogens (Jun-Yuan et al.2013). Environment-friendly applications of PGPR in agriculture have gained more importance, in particular in horticulture and nursery production. Considerable progress has been

achieved in the area of PGPR biofertilizer technology. It has been also demonstrated and proved that PGPR can be very effective and are potential microbes for enriching the soil fertility and enhancing the plant yield. Recent studies confirm that the treatment of seeds with non-pathogenic bacteria, such as *Agrobacterium, Bacillus, Streptomyces, Pseudomonas, Alcaligenes,* etc. induce root formation in some plants because of natural plant growth promoting substances produced by the bacteria. It appears that more work is needed to determine if identified bacteria are putative pathogens or beneficial endophytes.

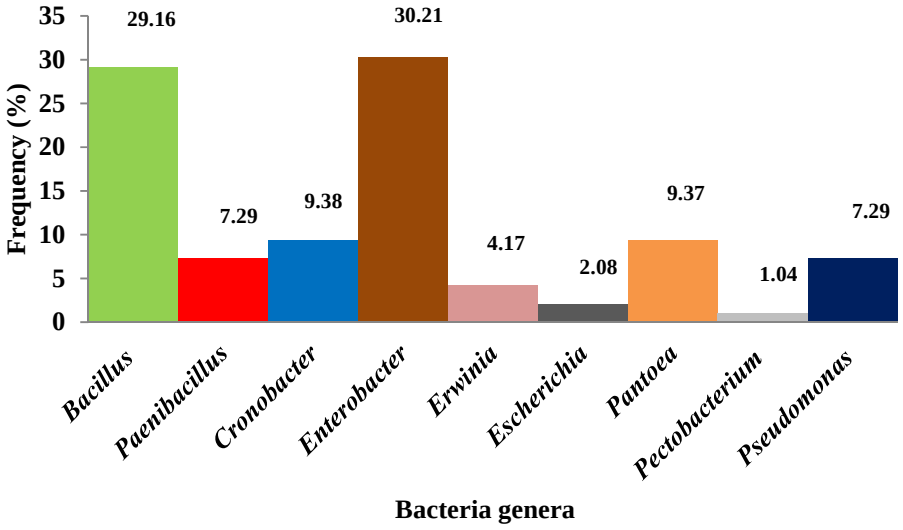

Figure 6.2 Bacteria genera represented basing on 16S rRNA sequences obtained from sequencing bacteria colonies obtained from sea oats seed.

6.5.3 Morphological Identification of fungi colonizing sea oats seed

A total of 435 pure fungal cultures were obtained; 105, 141, 99, 90 from Ben Hur, Burden, Long Beach and Holly Beach respectively. The cultures were separated into groups based on classical morphological features including growth pattern, colony texture, pigmentation, and growth rate of the colonies on PDA

Table 6.1 Distribution of 16S rRNA sequences obtained from sequencing bacteria colonies obtained from sea oats seed

Phylum	Genus	Closest NCB1 match	Location*	No of isolates	% Total isolates	% identity
Firmicutes	*Bacillus*	*Bacillus aerophilus*	LB	1	1.04	98
		Bacillus amyloliquefaciens	LB, BH	13	13.54	100
		Bacillus aryabhattai	LB	1	1.04	100
		Bacillus cereus	BH	2	2.08	100
		Bacillus circulans	LB	1	1.04	100
		Bacillus megaterium	LB, BH	4	4.17	100
		Bacillus sp.	BH	3	3.13	99
		Bacillus subtilis	BH, LB	2	2.08	99
		Bacillus tequilensis	LB	1	1.04	99
	Paenibacillus	*Paenibacillus hunanensis*	LB	2	2.08	99
		Paenibacillus polymyxa	LB	3	3.13	99
		Paenibacillus sp.	LB	2	2.08	99
Proteobacteria	*Cronobacter*	*Cronobacter dublinensis*	LB, BH	5	5.21	98
		Cronobacter sakazakii	BH, LB	4	4.17	99
	Enterobacter	*Enterobacter cloacae*	LB	1	1.04	99
		Enterobacter cowanii	BH	1	1.04	99
		Enterobacter sp.	LB, BH	27	28.13	99
	Erwinia	*Erwinia persicina*	LB	1	1.04	99
		Erwinia sp.	LB	3	3.13	99
	Escherichia .	*Escherichia* sp.	LB, BH	2	2.08	99
	Pantoea	*Pantoea agglomerans*	LB	2	2.08	99
		Pantoea ananatis	LB	2	2.08	98
		Pantoea gaviniae	BH	1	1.04	99
		Pantoea sp.	LB, BH	3	3.13	99
		Pantoea stewartii	LB	1	1.04	99
	Pectobacterium	*Pectobacterium cypripedii*	LB	1	1.04	99
	Pseudomonas	*Pseudomonas oryzihabitans*	BH	1	1.04	99
		Pseudomonas sp.	LB, BH	6	6.25	99

* location: BH= Ben Hur and LB= Long Beach

When fungal cultures from Ben Hur were examined under compound light microscope, 12 genera were observed including: *Alternaria* (3.81 %), *Aspergilus* (1.90 %), *Bipolaris* (7.72 %), *Curvularia* (17.14 %), *Fusarium* (43.81 %), *Penicilium* (1.90 %), *Pestalotia* (1.90 %), *Pithomyces* (0.95 %), *Sarocladium* (0.95 %), *Colletotrichum* (2.86 %), *Epicoccum* (4.0 %), *Setosphaeria* (7.62%), and non-sporulated fungi (4.92 %). *Fusarium* was observed to be the most common genera colonizing sea oats seed harvested from Ben Hur as shown in Figure 6.3.1.

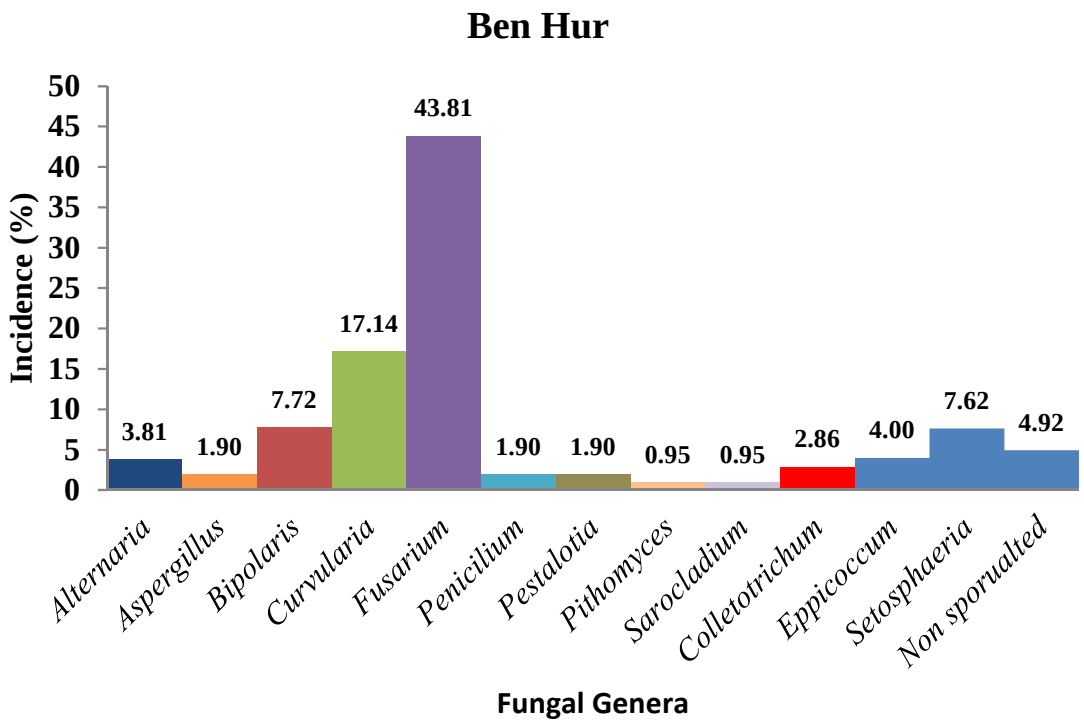

Figure 6.3.1 Fungi isolated from sea oats seed harvested from Ben Hur in 2011 basing on morphological characterization as observed under compound light microscope.

When fungal cultures from Burden were examined under compound light microscope, 12 genera were observed including: *Alternaria* (0.71 %), *Aspergilus* (2.84 %), *Bipolaris* (4.26 %), *Colletotrichum* (0.71 %), *Curvularia* (48.94 %), *Epicoccum* (0.71 %), *Fusarium* (29.79 %),

Penicilium (1.42 %), *Nigrospora* (1.0 %), *Helminthosporium* (4.31 %), *Pithomyces* (0.71 %), *Setosphaeria* (1.42. %), and non-sporulated fungi (3.20 %). *Curvularia* was observed to be the most common genera colonizing sea oats seed harvested from Burden as shown in Figure 6.3.2.

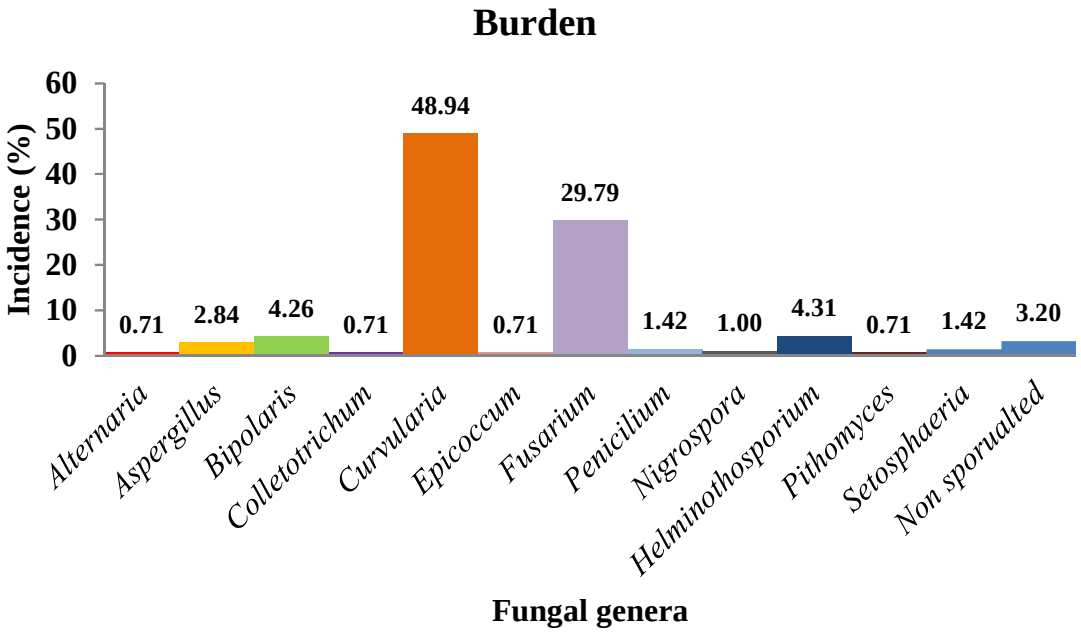

Figure 6.3.2 Fungi isolated from sea oats seed harvested from Burden in 2011 basing on morphological characterization as observed under compound light microscope.

When fungal cultures from Long Beach, MS were examined under compound light microscope, 13 genera were observed including: *Alternaria* (2.11 %), *Aspergilus* (2.11 %), *Bipolaris* (7.37 %), *Epicoccum* (1.05 %), *Curvularia* (46.32 %), *Fusarium* (11.58 %), *Penicilium* (1.05 %), *Pyricularia* (1.05 %), *Helminthosporium* (1.05 %), *Sclerotium* (7.37 %), *Rhizopus* (1.05 %), *Setosphaeria* (4.50. %), *Trichoderma* (2.11%) and non-sporulated fungi (11.29 %). *Curvularia* was observed to be the most common genera colonizing sea oats seed harvested from Long Beach, MS as shown in Figure 6.3.3.

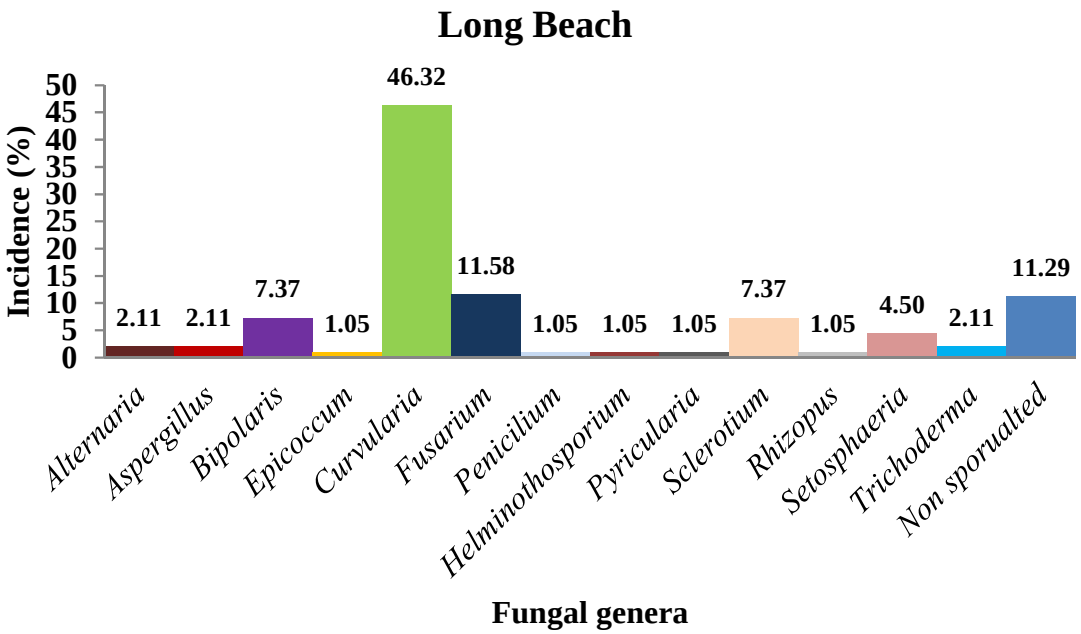

Figure 6.3.3. Fungi isolated from sea oats seed harvested from Long Beach, MS. basing on morphological characterization as observed under compound light microscope.

When fungal cultures from Holly Beach, LA were examined under compound light microscope, 12 genera were observed including: *Alternaria* (5.66 %), *Aspergilus* (5.66 %), *Bipolaris* (6.67 %), %), *Curvularia* (34.44 %), *Fusarium* (18.89 %), *Epicoccum* (5.20%), %), *Helminthosporium* (1.11 %), *Setosphaeria* (1.11 %), *Penicilium* (1.11 %), *Pithomyces* (4.44 %), *Sclerotium* (2.22 %), *Trichoderma* (1.11%) and non-sporulated fungi (12.58 %). *Curvularia* was observed to be the most common genera colonizing sea oats seed harvested from Holly Beach, LA as shown in Figure 6.3.4.

Curvularia was found to be the most dominant fungal genera at Long Beach, Holly Beach and Burden whereas, *Fusarium* dominated Ben Hur location. *Fusarium* tends to be an opportunistic pathogen causing diseases which are most damaging on plants that are debilitated.

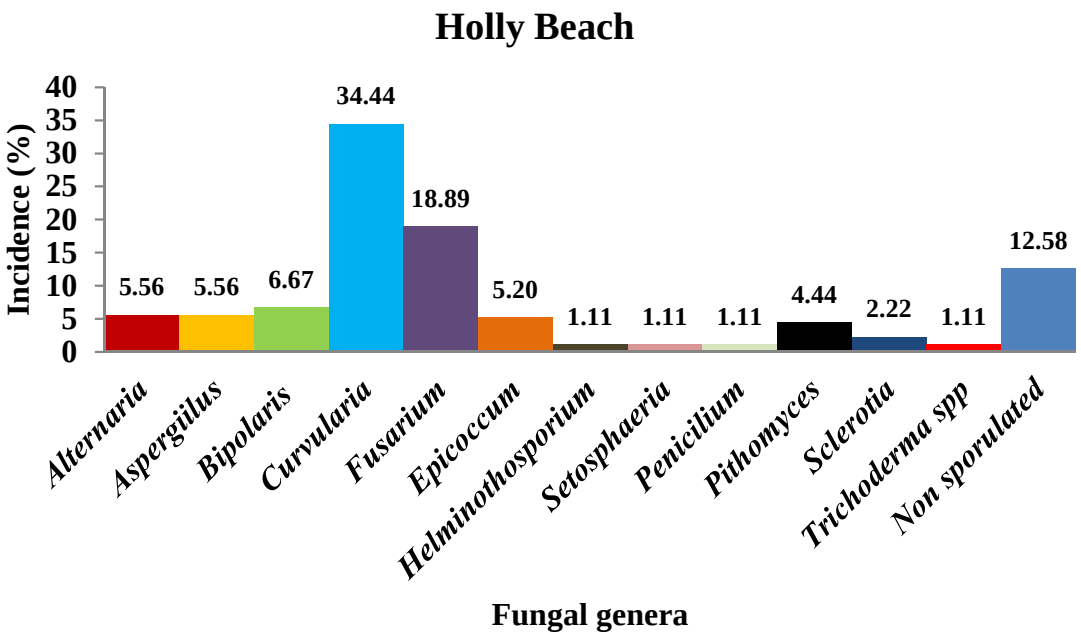

Figure 6.3.4. Fungi isolated from sea oats seed harvested from Holly Beach, LA in 2011 basing on morphological characterization as observed under compound light microscope.

This was interesting because Long Beach, Holly Beach and Burden sea oats production systems are all rainfed , whereas Ben Hur is under sprinkler irrigation which provides conditions favorable for pathogen infestation. Plants at Ben Hur location were observed to be stressed, most likely due to the excessive water from sprinkler irrigation. Sea oats are not tolerant to prolonged inundation. Ben Hur nursery production environment could have increased *Fusarium* incidence. Other fungal genera found to be common to all locations were: *Alternaria*, *Aspergillus*, *Bipolaris*, *Curvularia*, *Fusarium*, *Epicoccum*, *Setosphaeria*, and *Penicilium*. *Helminothosporium* were only found at Long Beach, Holly Beach and Burden. *Colletotrichum* was only found on sea oats seed harvested from artificial breeding nurseries, Ben Hur and Burden. *Sclerotia* and *Trichoderma* was only found on sea oats seed harvested from natural environments, Long Beach and Holly Beach. *Pyricularia* and *Rhizopus* were only identified on

sea oats seed harvested from Long Beach. *Pithomyces* were identified on sea oats seed harvested from Burden, Holly Beach and Ben Hur. *Nigrospora* was only found on sea oats seed harvested from Burden. *Sarocladium* was only identified on sea oats seed harvested from Ben Hur.

For species identification, 96 pure fungal cultures were randomly chosen for DNA extraction and PCR amplification. The PCR amplified DNA displayed only one distinct band, approximately 650 bp on agarose gel. The PCR products were sequenced, and putative species identifications of isolates were made by comparing consensus sequences to other isolates in the NCBI Genbank database using a Blastn search (http://www.ncbi.nlm.nih.gov/).

Based on ITS sequence analysis, 13 fungal genera were represented in the 96 pure fungal cultures selected for molecular analysis including; *Alternaria* (6.37 %), *Ampelomyces* (1.06 %), *Bipolaris* (2.12 %), *Curvularia* (26.59 %), *Fusarium* (31.92%), *Colletotrichum* (2.13 %), *Pestalotia* (1.06 %), *Epicoccum* (4.26 %), *Phoma* (7.44 %), *Setosphaeria* (11.7 %), and *Nigrospora* (2.12 %; Figure 6.4). The most commonly represented genera were still *Fusarium* and *Curvularia* (Figure 6.4).

Species identifications were based primarily on ITS sequence identity. Isolates which shared 97% or more ITS sequence identity with Genbank sequences were considered conspecific and any sequence variation of 3% or less was assumed to be intraspecific. Thirty nine fungal species were observed. *Curvularia lunata* and *Fusarium acuminatum* were the most frequently observed fungal species (Table 6.2). *Curvularia lunata* is a seed borne pathogen and causes leaf blight (Kim- Jisoo and Lee-DuHyung, 1998). Generally, a number of *Curvularia* species have been reported as causal agents of leaf spots, leaf blights, grain rot, root rot, seedling blights, grain discoloration, grain lesion and grain deformation (Benoit and Mathur, 1970). In rice,

Curvularia has been reported to be a seed borne pathogen and causes germination failure (Sisterna and Bello, 1998).

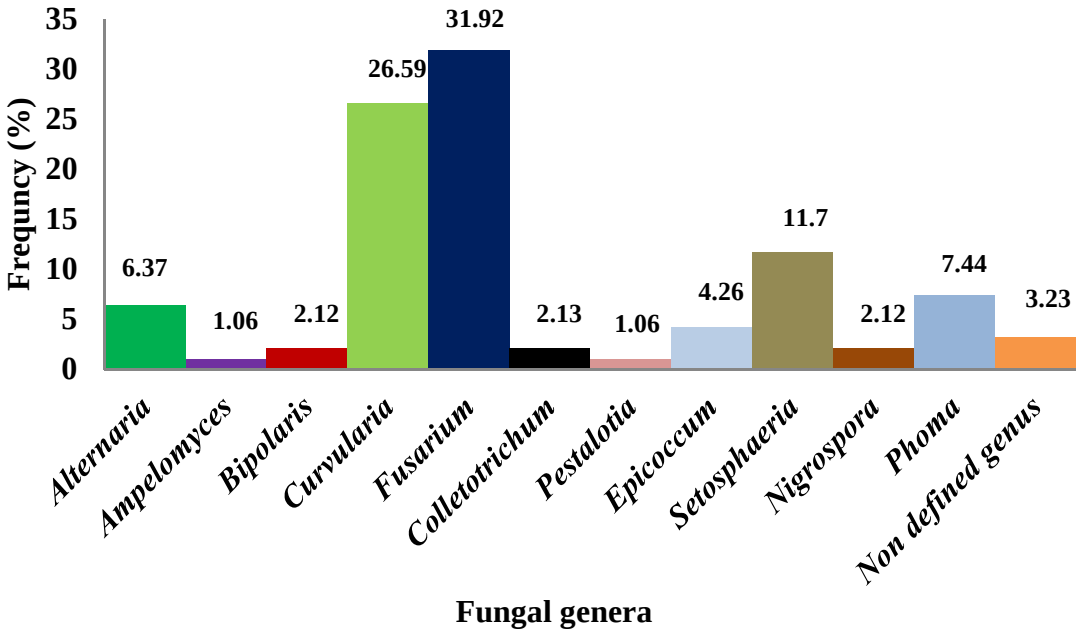

Figure 6.4 Fungal genera represented basing on ITS sequence analysis

Fusarium species are considered to be important plant pathogens. *Fusarium* species normally have numerous *formae speciales*, some of which contain two or several pathogenic races causing devastating wilt disease (Neergaard 1979). Many of the *Fusarium* species are seed borne (Noble M. and Richard 1968; Ram Nath 1970; Neergaard 1979; Domsch. K. H 1980) and occur on seed of a wide range of hosts such as *Capsicum annuum*, *Coriandrum sativum*, *Cuminum cyminum*, *Lycopercon esculentum*, *O. sativa*, *Phaseolus vulgaris,* and *S. vulgar*, *Allium*, *Cucurbita*, *Trifolium pratense,* and *Zea mays*, *Glycine max, Solanum melongena, S. vulgare, Cucumis sativa*, and *Pisum sativum*. Like many fungi, this genus is endowed with

several means of survival, amongst which is its quick capacity for change, both morphological and physiological, when faced with environmental changes (Booth, 1971). There are pathogenic and non–pathogenic forms. The latter can colonize the cortex of roots of plants without causing symptoms of disease (Appel & Gordon, 1994), and survive in live tissue, as also exercising antagonism between the pathogenic forms in the soil (Edel *et al*., 1997). The phytopathogenic species affect a wide range of hosts and cause root rot, vascular wilting, yellowing and foliar necrosis (Ramachandran *et al*., 1982; Nelson & Hansen,1997).

6.5.4 Conclusion

The high pathogen incidence observed in this study, could account for the decreased sea oats seed germination percentages. Among the most dominant bacteria genera colonizing sea oats seed were *Bacillus* and *Enterobacter*. With a few exceptions, most of them are known to be plant growth promoting bacteria and can be beneficial endophytes. In general, beneficial endophytes promote host plant growth, increase plant nutrient uptake, inhibit plant pathogen growth, reduce disease severity, and enhance tolerance to environmental stresses. These are very important for sea oats plants which grow in very harsh environmental conditions, thus enabling them to adapt and survive better. However, additional research needs to be done to establish the pathogenicity or endophytic status of these species. Fungal genera; *Fusarium* and *Curvularia*, which were observed to be the most dominant fungal genera are important plant pathogens and cause germination failure. More research is needed in formulating seed treatments that suppress pathogens during storage and germination.

Table 6.2 Distribution of ITS sequences obtained from sequencing fungal isolates obtained from sea oats seed

Phyllum	Class	Genus	Closest NCB1 match	Location	No. of Isolates	% Total isolates	% IDENTITY
Ascomycota	Ascomycetes	*Curvularia*	*Cochliobolus hawaiiensis*	LB	1	1.06	100
			Curvularia affinis	HB, BH	5	5.32	100
			Curvularia fallax	BH	1	1.06	100
			Curvularia geniculatus	LB, BH	2	2.13	100
			Curvularia lunata	LB, BH, BT	10	10.64	100
			Curvularia sp.	BT	1	1.06	99
			Curvularia spicifer	BH, LB	2	2.13	100
			Curvularia trifolii	HB	1	1.06	100
			Curvularia verruculosa	BH, LB	2	2.13	100
		Colletotrichum	*Colletotrichum gloeosporioides*	BH	2	2.13	100
		Epicoccum	*Epicoccum sorghi*	BH, HB, BT, LB	4	4.26	100
	Dothideomycetes	*Alternaria*	*Alternaria alternata*	BH	1	1.06	99
			Alternaria brassicae	HB, BT, LB	2	2.13	100
			Alternaria porri	BT	1	1.06	99
			Alternaria tenuissima	BH	1	1.06	100
			Alternaria triticimaculans	BH	1	1.06	100
		Ampelomyces	*Ampelomyces* sp.	LB	1	1.06	100
		Bipolaris	*Bipolaris papendorfii*	BT	1	1.06	99
			Bipolaris sp.	BT	1	1.06	100
		----------	*Dothideomycete* sp.	LB	2	2.13	100
		Phoma	*Phoma herbarum*	LB	1	1.06	99
			Phoma macrostoma	BH	1	1.06	100
			Phoma sp.	BT, LB, BH	5	5.32	100
		----------	*Pleosporales* sp.	HB	1	1.06	99
		Setosphaeria	*Setosphaeria rostrata*	BH, LB, BT	11	11.7	100
	Sordariomycetes	*Fusarium*	*Fusarium acuminatum*	BT, BH, HB	8	8.51	100
			Fusarium armeniacum	BH, HB	2	2.13	99

(Table 6.2. continued)

Phyllum	Class	Genus	Closest NCB1 match	Location	No. of Isolates	% Total isolates	% IDENTITY
			Fusarium chlamydosporum	HB, BH, BT	4	4.26	89
			Fusarium equiseti	BH, LB	4	4.26	99
			Fusarium fujikuroi	BH	1	1.06	100
			Fusarium langsethiae	BH	1	1.06	99
			Fusarium moniliforme	BT	2	2.13	99
			Fusarium oxysporum	BH, BT	2	2.13	99
			Fusarium purpureum	BH	1	1.06	100
			Fusarium sp.	LB	3	3.19	100
			Fusarium sporotrichioides	BT	2	2.13	95
		Nigrospora	*Nigrospora* sp.	BH	1	1.06	100
			Nigrospora sphaerica	BT	1	1.06	99
		Pestalotia	*Pestalotiopsis microspora*	LB	1	1.06	99

Where for location: BH= Ben Hur, BT = Burden Trial, LB= Long Beach and HB= Holly Beach

CHAPTER 7: SUMMARY AND CONCLUSIONS

Uniola paniculata (sea oats) is a coastal foredune grass that plays an essential role in dune stabilization. However, coastal developments and recent severe hurricanes have substantially reduced coastal vegetation throughout the Southeastern United States. Specifically, populations of sea oats have been considerably reduced; thereby having a direct impact on coastline stability. Currently, a significant need exists to re-establish damaged sea oats populations. A breeding program could enhance coastal restoration by developing improved sea oats plants for beach restoration. The goal was to initiate a successful breeding program for sea oats adapted to shallow dune profiles, with high seed yield and germination, and superior vegetative biomass essential for reducing coastal erosion.

The foundation of any successful breeding program relies on availability of germplasm diversity and efficient seed production methods. In addition, identification of important selectable traits and development of efficient selection methods is very necessary. Knowledge pertaining to seed storage, germination, plant production methods and agronomic practices are also very essential in establishing a successful breeding program.

In natural beach environments, seed yields are affected by several stochastic events like hurricanes. For reliable seed yields, alternative seed production environments are necessary. In this study we were interested in determining whether sea oats seeds could be produced away from natural beach environments. It was found that sea oats seeds can be produced in artificial environments. Establishing seed production nurseries could support coastal restoration efforts by minimizing sea oats seed harvests from natural environments. Minimizing seed harvests from natural ecosystems will also reduce disruption of the natural ecosystems, which can result in sea

oats stand decline and resultant land loss. Establishing artificial seed production nurseries also support breeding programs by producing a source of readily available genetic diversity.

To preserve sea oats seeds in storage, experiments were carried out to identify sea oats seed storage environments leading to maximum longevity. Sea oats seed stored in hermetically sealed jars at room temperature consistently had the highest average germination for seed harvested from the northern Gulf of Mexico coast. Identification of seed storage methods can help in ex situ seed storage necessary for sea oats conservation and also provide a source of genetically diverse germplasm for restoration.

These investigations found high seed pathogen incidence during germination. The most dominant bacteria genera colonizing sea oats seed were *Bacillus* and *Enterobacter*. *Bacillus* and *Enterobacter* are most commonly cited as plant growth promoting bacteria. Additional research needs to be done to establish the pathogenicity or beneficial endophytic status of these species. It would be interesting to identify beneficial endophytes as they are known to promote host plant growth, increase plant nutrient uptake, inhibit plant pathogen growth, reduce disease severity, and enhance tolerance to environmental stresses. *Fusarium* and *Curvularia*, were observed to be the most dominant fungal genera attacking sea oats seed. *Fusarium* and *Curvularia* are most commonly cited as pathogens. From these studies, it would appear that identifying seed treatments to reduce these fungi would enhance sea oats seed germination. There is still need in formulating seed treatments to suppress pathogens during storage and germination.

To determine minimal sea oats plant size needed for restoration, vegetative plants, large seedlings, and small seedlings were evaluated. Large vegetative plants are very costly to produce, and to reduce costs small seedlings would be preferred. When conditions were ideal 88% of the small seedlings survived. Despite the significant storm surge 1 week after

transplanting, at 3 months after transplanting approximately 30% of small sea oats seedlings survived. Therefore small sea oats seedlings can be used at greater densities to compensate for higher mortality rates in unfavorable conditions. Increasing seedling densities could reduce production costs and result in acceptable survival rates accompanied with genetic diversity.

A greenhouse protocol that could predict sea oats survival in half the time of saturated beach conditions was identified. Selection of saturation tolerant sea oats lines in greenhouse conditions in half the time of that required in saturated beach conditions would reduce the time to select sea oats saturation tolerant lines, thus increasing the efficiency of the breeding program. The ability to predict sea oats survival in greenhouse conditions has significant implications for a saturation tolerance breeding program; allowing multiple selection cycles to occur each year thus speeding up the breeding program.

This work has identified: alternative seed production environments, seed storage methods, putative seed pathogens, characteristics essential for selection of sea oats lines adapted to the Northern Gulf of Mexico Coast and minimal plant sizes to be used in beach restoration projects. All these were essential in developing a successful sea oats breeding program.

CHAPTER 8: LITERATURE CITED

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APPENDIX A: PHYLOGENETIC ANALYSIS OF FUNGI ISOLATED FROM SEA OATS SEED BASED ON ITS SEQUENCE ANALYSES

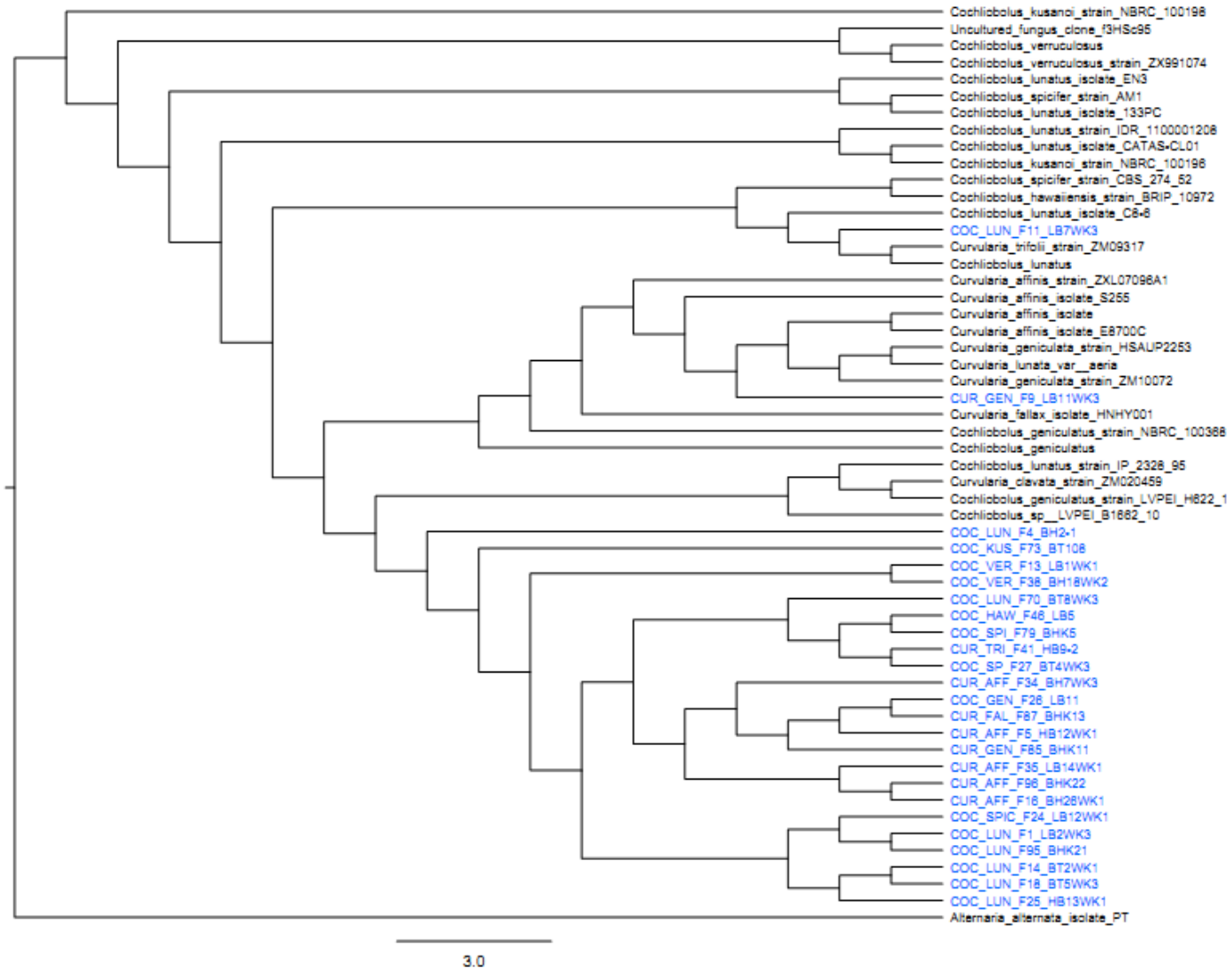

Figure A.1 Phylogenetic relationships in *Curvularia* isolates based on analyses of ITS sequences. ITS Genbank accession numbers are listed. Boostrap values above 50% are indicated. *Alternaria Alternata* was used as an outgroup.

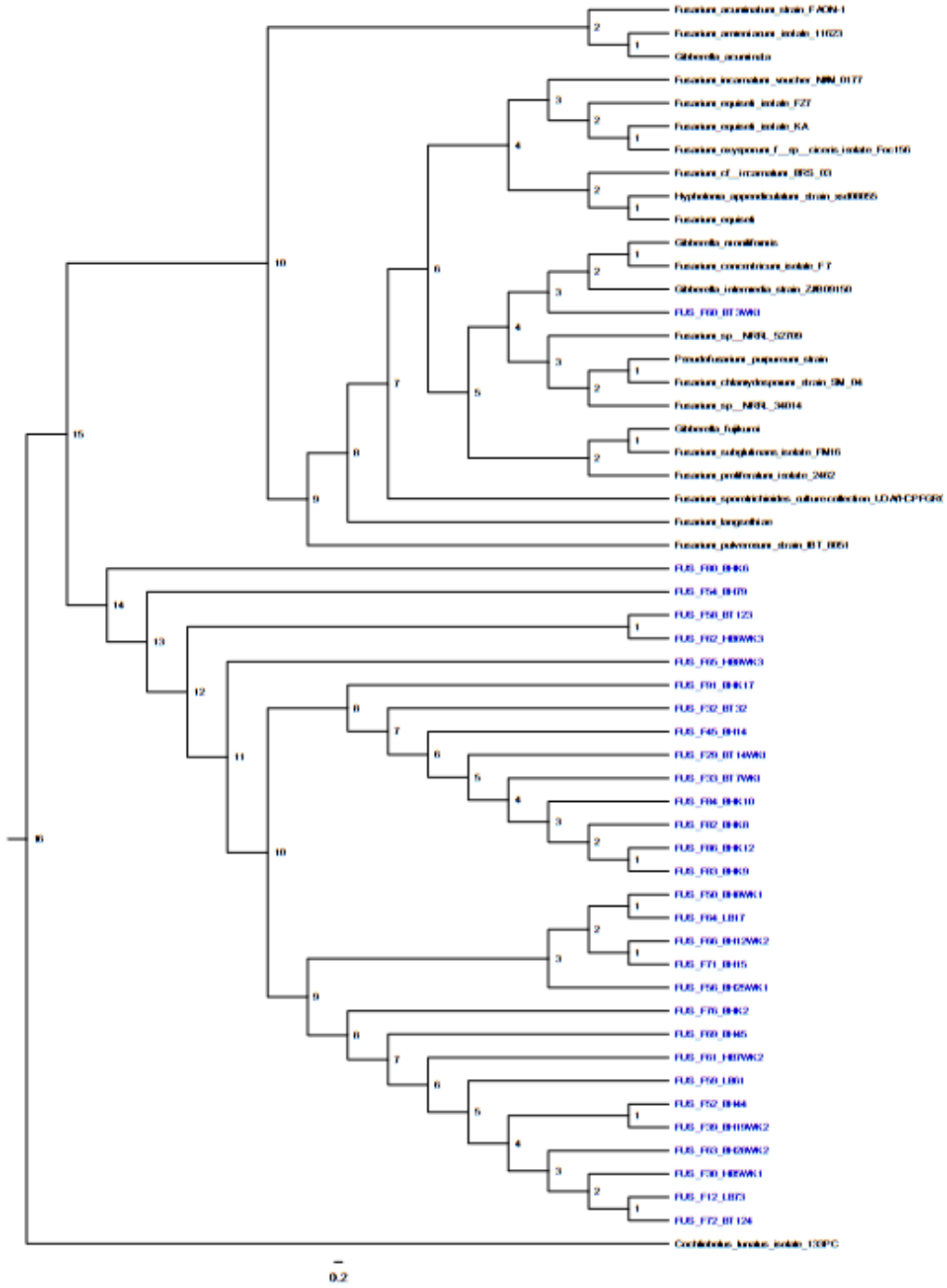

Figure A.2 Phylogenetic relationships in *Fusarium* isolates based on analyses of ITS sequences. ITS Genbank accession numbers are listed. Boostrap values above 50% are indicated. *Cochliobolus lunata* was used as an outgroup.

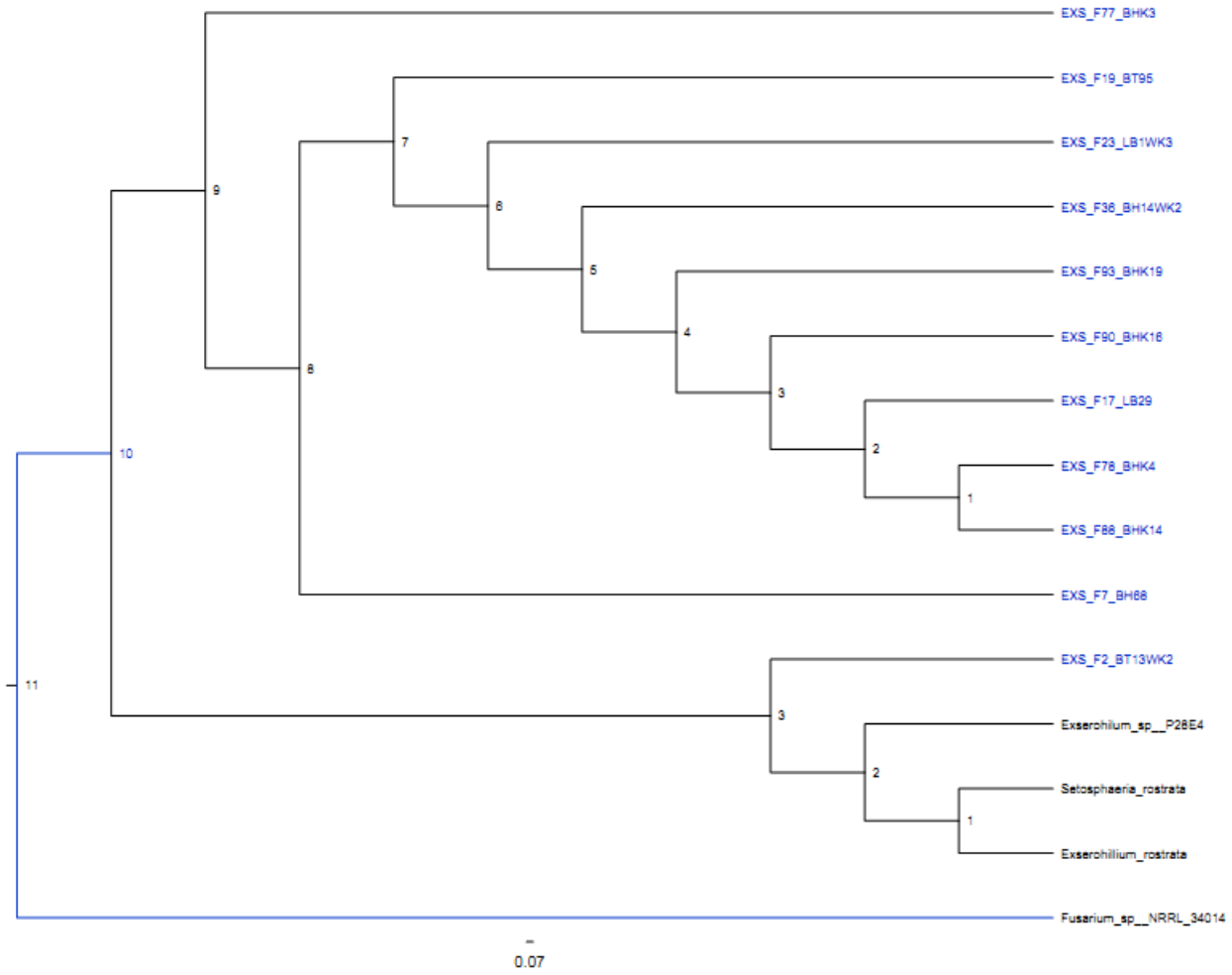

Figure A.3 Phylogenetic relationships in *Setospharia* isolates based on analyses of ITS sequences. ITS Genbank accession numbers are listed. Boostrap values above 50% are indicated. *Fusarium* was used as an outgroup.

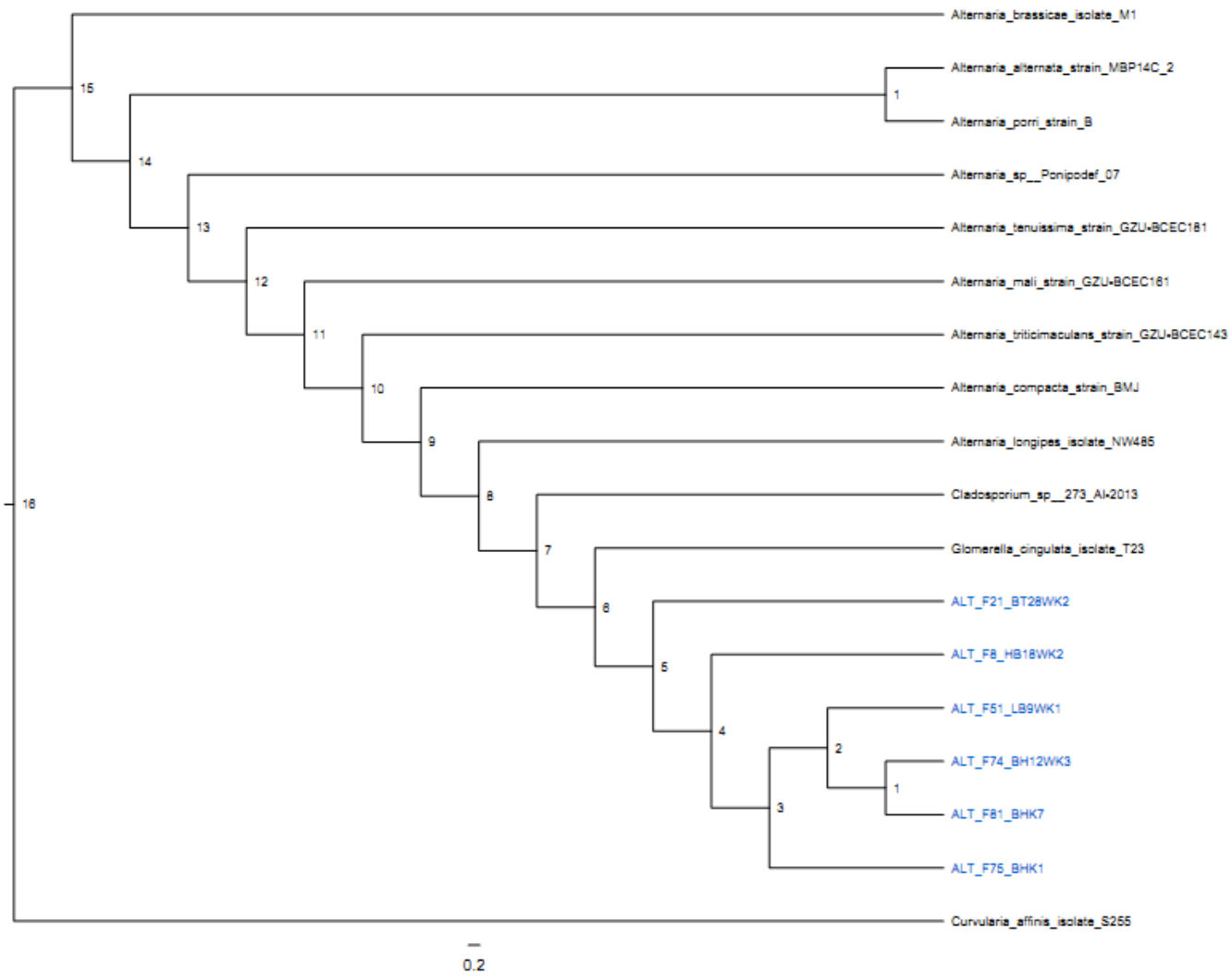

Figure A.4 Phylogenetic relationships in *Alternaria* isolates based on analyses of ITS sequences. ITS Genbank accession numbers are listed. Boostrap values above 50% are indicated. *Curvularia affinis* was used as an outgroup.

APPENDIX B: PERMISSION LETTER

From: Pheonah Nabukalu [mailto:pnabuk1@tigers.lsu.edu]
Sent: Thursday, May 23, 2013 3:17 PM
To: ERjournal@AESOP.Rutgers.edu
Cc: Knott, Carrie
Subject: Permission Letters

Dear Dr. Aronson,

I would like to request for permission letters to include all of the work that I have already published in Ecological Restoration Journal into my doctoral dissertation.
My publications are:

Nabukalu, P. and C.A. Knott. 2013. Effect of *Uniola paniculata* plant size on survival and performance at beaches with low dune profiles. Ecological Restoration 31(1): 12-16.
Nabukalu, P. and C.A. Knott. 2013. Effect of storage environment on *Uniola paniculata* germination. Ecological Restoration 31(1):16-19.

Thank you
Pheonah Nabukalu
Louisiana State University

From: ER Journal ERJournal@aesop.rutgers.edu

To: Pheonah Nabukalu <pnabuk1@tigers.lsu.edu>
Date: Thu, May 30, 2013 at 7:41 PMsubject:

RE: Permission Lettersmailed-by: aesop.rutgers.edu

Hi Pheonah,

You have permission to include the work mentioned below in your doctoral dissertation. This is how to cite the copyright:

[Article title] appears in Ecological Restoration [issue number]. Copyright 2013 by the Board of Regents of the University of Wisconsin.

If her thesis formatting requirements don't allow her to put this at the bottom of the first page of the particular article/chapter, then I assume she can included it on an acknowledgments page at the beginning of the manuscript.

Let me know if you have any more questions!

-Myla

Myla F.J. Aronson, Ph.D., managing editor

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VITA

Pheonah Nabukalu was born in Kampala, Uganda. After completing high school at Trinity College Nabbingo, she joined Makerere University Kampala and graduated in 2005 with a Bachelors of Science degree in Agriculture; majoring in crop science. After graduating, she got a scholarship from Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) to pursue her Masters of Science degree in crop science (Plant Breeding and Genetics). In 2009, she joined the coastal plant breeding program in the School of Plants, Environment and Soil Science at Louisiana State University to pursue a doctoral degree under the supervision of Dr. Carrie Knott. She will receive the Degree of Doctor of Philosophy during the summer commencement 2013.