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Micropropagation through somatic embryogenesis and cotyledonary nodal culture in sea oats (Uniola paniculata L.)

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MICROPROPAGATION THROUGH SOMATIC EMBRYOGENESIS AND COTYLEDONARY NODAL CULTURE IN SEA OATS (*UNIOLA PANICULATA* L.)

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ABBREVIATIONS

2, 4-D- 2, 4 Dichloro pheonoxyacetic acid

2ip- 2-isopentyladenine

AFLP- Amplified Fragment Length Polymorphism

BAP- 6-Benzylaminopurine

CIF- Callus induction frequency

CIM- Callus Induction Medium

CMM- Callus Multiplication Medium

GA_3- Gibberelic acid

IAA- Indole-3-acetic acid

IBA- Indole-3-butyric acid

ISSR- Inter-Simple Sequence Repeats

Kn- Kinetin

LS- Linsmaier and Skoog

MS- Murashige and Skoog

NAA- α-naphthalene acetic acid

PCR- Polymerase Chain Reaction

PGR- Plant Growth Regulator

RAPD- Random Amplified Polymorphic DNA

RFLP- Restriction Fragment Length Polymorphism

RM- Rooting Medium

SCR- Shoot Callus Regeneration

SE- Somatic Embryo

SH- Schenk and Hildebrandt

SMM- Shoot Multiplication Medium

SNP- Single Nucleotide Polymorphism

SSR- Simple Sequence Repeats

STS- Sequence Tagged Site

TDZ- Thidiazuron

USGS- United States Geological Survey

VAM- Vesicular-Arbuscular Mycorrhizal

LIST OF SPECIES WITH THEIR LATIN AND COMMON NAMES

Arachis retusa - Peanut

Actinidia deliciosa - Kiwi fruit

Bambusa balcooa, Bambusa bambos, Bambusa tulda – Bamboo

Bouteloua eriopoda - Black grama

Brassica oleracia - Mustard

Cynodon dactylon, C. transvaalensis - Bermuda grass

Eleusine indica - Goose grass

Gypsophila paniculata - Baby's breath

Ipomoea pes-caprae - Railroad-vine

Macadamia tetraphylla - Macadamia nut

Mucuna pruriens -Velvet bean

Musa spp**. -** Banana

Nyctanthes arbor - Night jasmine

Panax notoginseng - Tienchi ginseng

Panicum virgatum - Switch grass

Paspalum dilatatum - Dallisgrass

Paspalum notatum - Bahia grass

Phragmites australis - Reed grass

Picea mariana - Black spruce

Populus deltoides - Eastern cottonwood

Porteresia coarctata - Wild relative of rice

Prunus dulcis - Almond

Psoralea corylifolia - Malay tea

Rauvolfia tetraphylla - Devil-pepper

Saccharum officinarum – Sugarcane

Sorghum bicolor and *S. sudanense* - Sorghum

Spartina alterniflora - Smooth cord grass

Stenotaphrum secundatum - St. Augustine grass

Typha latifolia - Cattail

Vanilla planifolia - Vanilla

Zingiber officinale - Ginger

ABSTRACT

Uniola paniculata, commonly known as sea oats, is a C4 perennial grass capable of stabilizing sand dunes. Although this species is extensively used in beach restoration projects, production and availability of sea oats seedlings is seriously constrained due to disappearance of the natural stands. Other limitations are poor seed production, seed dormancy, and low seedling survival. With increasing interest in dune restoration, an efficient micropropagation technique is essential to generate sea oats seedlings in mass scale.

In this study an efficient, rapid and reproducible plant regeneration system was successfully established for sea oats through somatic embryogenesis and cotyledonary nodal culture. Six sea oats accessions, collected from Alabama, Florida, Louisiana, Mississippi, North Carolina, and Texas were tried for embryogenic callus induction and plant regeneration using mature seeds. Two accessions, one each from Louisiana and North Carolina, were tested for shoot multiplication using cotyledonary nodes as explants. The frequency of callus induction was studied using modified Murashige and Skoog (MS) medium with a variety of combinations of 2, 4-D and Kinetin. Transferring the callus to a lower concentration of 2, 4-D (9.05 µM) in combination with Kn (2.32 µM) increased the embryogenic callus mass. Callus was regenerated in MS medium supplemented with BAP, NAA and Kn and 83.26 mM maltose. Similarly, for cotyledonary nodal culture MS medium supplemented with different concentration of BAP with 87.64 mM sucrose and MS medium in combination with BAP (4.44 µM), NAA (2.69 µM), Kn (2.32 µM) with 83.26 mM maltose were tested. Presence of Kn and NAA enhanced the process of multiplication but the maximum number of plantlets were regenerated in presence of BAP only. All the plantlets were rooted in MS medium supplemented with NAA (5.38 µM) and Kn (0.46 µM). In Texas and Florida accessions, 8 % and 10 % of the plants were albinos,

respectively, but no morphological aberration was observed. RAPD (Random Amplified Polymorphic DNA) analysis using 5 arbitrary oligonucleotide decamers revealed genetic uniformity among the regenerants of each accession of sea oats developed via somatic embryogenesis and cotyledonary nodal culture, suggesting that this protocol can be used for clonal propagation of sea oats on a commercial basis.

CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Both natural disasters and man-made activities have led to destabilization and erosion of coastal beaches and dunes. The dunes are a unique ecosystem, and their conservation and preservation is critical for plant and animal habitat protection (Valero-Aracama et al., 2002). Reversal of coastal erosion is usually accomplished by beach nourishment through the addition of sand followed by dune stabilization (Westra and Loomis, 1966; Woodhouse et al., 1968; Dahl and Woodard, 1977). Stabilization is attempted by planting the exposed dunes with native dune species like sea oats (*Uniola paniculata*) (Woodhouse et al., 1968; Woodhouse, 1982). Sea oats, a perennial and semitropical C_4 grass species, is regarded as a pioneer species for beach restoration endeavors (Johnson and Barbour, 1990). Its underground extensive fibrous rooting system, highly lignified rhizome and capacity to withstand wide range of adverse environmental conditions make it a valuable species for coastal restoration. However, production and availability of large amounts of planting materials for restoration planting projects is restricted due to limited seed production, seed dormancy, poor germination, and low seedling survival. In this scenario, micropropagation could be an ideal technology to overcome this problem.

1.2 Background Study

Sea oats are hardy plants found along the Atlantic and Gulf coasts. They constitute an important barrier protecting the beach dunes from wind and wave erosion. Scientists have been looking for a way to produce sea oats in mass scale to protect critical dune sites. The success rate of growing sea oats in their natural habitat using seed scattering or clump transplant technique is low (Sea Grant study at University of Florida,

http://www.flseagrant.org/library/publications/fathom_magazine/volume-6_issue-3/sea_oats.htm). Greenhouse and field trials indicated that the best revegetation occurred only when the plants were grown in containers in a nursery and then transplanted back into their natural environment. Plants grown from seeds collected from the wild may not have desirable characteristics for survival in a particular site. Therefore, there is a need to create a workable system utilizing micropropagation technique for producing sea oats plants. Several studies have been conducted on sea oats and similar dune species to establish a technology to generate plants under laboratory conditions. Seed germination in the field condition is unpredictable as it is dependent on weather conditions but studies have shown that the germination rate is high in *in vitro* culture (Burgess et al., 2001). Moreover, application of specific hormones at a desired level is reported to enhance the regeneration (Valero-Aracama et al., 2002). Somatic embryogenesis, where a large number of somatic embryos are induced *in vitro* leading to development of multiple clones from a single explant has already been tried on several grass species like reed grass, switch grass, and cord grass (Jain et al., 2005). Sea oats has not been explored with this technique. On the other hand, successful protocols are available on nodal culture of several grasses. Sea oats being a grass species should be amenable for propagation through nodal culture in order to achieve a year round production. Tissue culture in plants generates a wide range of genetic variation, known as somaclonal variation (Larkin and Scowcroft, 1981). Plant tissue cultures isolated from a single cell can show variation after repeated subculture. Somaclonal variation generated from the tissue cultures can be heritable. It provides an additional source of novel variation for exploitation by plant breeders. At the same time, somaclonal variation is not desirable when the objective is to mass propagate a particular clone. Different molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA

(RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR) are used to analyze the genetic diversity within populations and the relationship among the related species. A wide range of genetic variation was found in field grown samples of sea oats (Parami, 2003; Subudhi et al., 2005).

1.3 Objectives

This research will focus on the development of an efficient micropropagation technique by using mature seeds of sea oats collected from several coastal states of USA: Alabama, Florida, Louisiana, Mississippi, North Carolina and Texas. A clear genetic variation has been established within and among the accessions of sea oats collected from these states (Parami, 2003; Subudhi et al., 2005). With the use of RAPD markers, this study aims to determine if there is any genetic variation induced during tissue culture in the micropropagated sea oats seedlings. Specifically, the objectives of this study are:

1) To develop an efficient micropropagation technique for rapid and large-scale production of sea oats seedlings and subsequent establishment in soil,

2) To compare response of different accessions to tissue culture, and

3) To investigate genetic variation in sea oats seedlings generated through somatic embryogenesis and cotyledonary nodal culture using RAPD markers.

1.4 Methodology

Our major goal is to develop a micropropagation protocol for mass scale production of sea oats seedlings. Somatic embryogenesis facilitates generation of multiple plants from calli, generated from a single seed. It is assumed that somaclonal variation is not induced in plants developed from somatic embryos. Molecular marker analysis of these plants will clarify the question of genotypic instability generated through somatic embryogenesis. Successful plant

production using micropropagation technology is usually achieved in four sequential steps: establishment of the propagule in sterile culture, multiple shoot induction, root initiation in the sterile medium, and transfer of plants to plug trays for establishment in soil.

Precisely, the seeds collected from different regions will be tested for germination and viability. Using MS (Murashige and Skoog, 1962) medium, different concentrations of 2, 4-dichloro pheonoxyacetic acid (2, 4-D) will be tested for callus (undifferentiated mass of cell) induction. Subsequent somatic embryo development, multiplication, and shoot and root regeneration will be done using different concentrations and combinations of plant growth regulators such as, α-naphthalene acetic acid (NAA), kinetin (Kn), and 6-benzylaminopurine (BAP). Similarly, shoot multiplication will be tested with cotyledonary nodal explants with different concentrations of BAP. The plants produced from a single callus/cotyledonary nodal explant will be analyzed using RAPD markers to determine the genetic uniformity among the micropropagated sea oats seedlings.

1.5 Long-term Goals

Frequent natural disasters like hurricanes Katrina and Rita have reduced the natural stands of sea oats in coastal areas. This study will provide an opportunity to propagate sea oats on a large scale in the laboratory condition so as to ensure steady supply of sea oats plants to the growers all-round the year. Since there is ecological concern about planting of the nonadapted ecotypes in new areas, sea oats plants are needed in large number from few available native Louisiana accessions for ongoing coastal restoration projects. This comprehensive study will establish a protocol for micropropagation independent of location-specific genotypes.

1.6 Chapter Overview

Subsequent chapter (chapter 2) will cover the literature review followed by materials and methods in chapter three, result and discussion in chapter four, which will describe the protocol and its significance, and summary and conclusion in chapter five will include the salient findings, their relevance and further research scope in this area.

CHAPTER 2

LITERATURE REVIEW

2.1 Coastal Erosion

In states bordering the Gulf of Mexico and the Atlantic Ocean, vast areas of coastal land have been destroyed since the mid 1800s as a result of natural processes and human activities (http://pubs.usgs.gov/of/2003/of03-337/). To many people, coastal land loss is synonymous with beach erosion. Coastal land loss includes beach erosion, but it is a much broader term because it also includes land losses along bluffs and losses of wetlands around interior bays and estuaries. Walsh (1994) reported that there is an approximately 50 meters coastal land loss per year in the Gulf Coast west of Mississippi delta to Texas. In Louisiana, the loss is around 5 to 50 meters per year (Penland and Boyd, 1981). After the hurricane Katrina and Rita in 2005, the land area loss in coastal Louisiana is approximately 562 square kilometers (United States Geological Survey, 2007, http://pubs.usgs.gov/ds/2007/281/). According to the USGS and the U.S. Army Corps of Engineers survey (2003), Louisiana lost 4,921 square kilometers from 1932 to 2000 and could lose another 1,813 square kilometers over the next 50 years if no new restoration takes place (http://www.nwrc.usgs.gov/releases/pr03_004.htm).

2.2 Coastal Restoration

In response to the high rate of coastal land loss, researchers are working on several indigenous dune plant species for restoration and stabilization of the coastal lands. Although nature recovers the loss in due course, favorable conditions must prevail to regain the eroded coastal areas. Frequent occurrences of natural disasters further restrict the recovery and restoration effort (McBride et al., 1991).

2.3 Biology of *Uniola paniculata*

2.3.1 Description

Uniola paniculata, commonly know as sea oats, is perhaps the most abundant plant species among beach dune communities (Sylvia, 1986; Hester and Mendelssohn, 1989, 1991; Bachman and Whitwell, 1995). This grass extends along the Atlantic and Gulf coasts from south eastern Virginia to the state of Tabasco, Mexico (Wagner, 1964). 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 taper into pointed tips. Leaves are thick, sturdy, and deeply furrowed at the abaxial surface, which become involute upon drying (Kearney, 1900). The inflorescence is a panicle of many laterally compressed spikelets each with 10-20 florets where the lower 4-6 florets are normally empty (Hitchcock, 1951). Flowering spikelets are flat and measure 20-50 cm (Radford et al., 1968). Seed heads are large, and become yellow brown and straw colored in late summer (Amos and Amos, 1997). It traps wind-blown sands that eventually mound to begin dune formation (Johnson and Barbour, 1990). Sea oats forms dense surface roots and penetrating deep 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 and Duncan, 1987). Rhizomes produce extensive lateral growth, which stabilizes continuous dune ridges (Duncan and 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). Vegetative reproduction is then enhanced by its C_4 photosynthetic ability, i.e., fixing carbon and allowing growth in low nutrient, low water and high light environment (Zelitch,

1982; Rodichaux and Pearchy, 1984). A relatively large dune complex is potentially made up of a single plant or fragments from the plant due to asexual propagation (Wagner, 1964). This provides an avenue for large scale sea oats propagation through nodal culture *in vitro*.

2.3.2 Physical Tolerances

Uniola paniculata tolerates high temperature conditions. It can withstand surface soil temperature as high as 52-53 ºC (125-127 ºF) and air temperature around 35-38 ºC (95-100 ºF) (Oosting, 1954). It is highly tolerant to inundation by sea water for short periods, and thrives under salt spray conditions. It is assumed that salt spray provides a source of micronutrients as it grows in the heavily leached soils of beach stands (Hester and Mendelssohn, 1990; Stalter and Odum, 1993).

Sea oats can withstand a wide variety of harsh environmental conditions including drought. Stomata in sea oats close when soil moisture reaches 8.5 % (Hester and Mendelssohn, 1987). Sea oats do not tolerate water logging of roots (Hester and Mendelssohn, 1987; Hester and Mendelssohn, 1989). Soil pH for *Uniola* species ranges from 6.9 to 7.9 (Oosting, 1954). Sea oats colonizes with beneficial microorganisms such as VAM (Vesicular-Arbuscular Mycorrhizal) fungi. Will and Sylvia (1990) studied the effect of these organisms and observed that they 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).

2.3.3 Reproductive Characteristics

Sea oats plants exhibit both sexual and asexual modes of reproduction. It reproduces asexually by forming buds around stem and sexually reproduces via seeds. It is not a prolific seed producer and the reason behind this might be the lack of cross pollination (Hester and

Mendelssohn, 1987; Bachman and Whitwell, 1995). Hester and Mendelssohn (1987) observed that the sea oats plant produced an average of 2.24 and 0.6 seeds per spikelets in North Carolina and Florida, respectively. High incidence of fungal invasion causing aborted ovules has been reported (Bachman and Whitwell, 1995). Sea oats florets open and close during early morning and open only once. Wind is the main factor for the pollination and seeds may be carried long distances by winds, storms and ocean currents (Oostings, 1954).

2.3.4 Growing Seasons

The growing season of *Uniola paniculata* is dependant upon the geographic location. Tyndall et al. (1987) studied the growth season of sea oats in North Carolina. They reported germination of seeds from late May through mid-June and growth season was from May to September. But in Florida, *U. paniculata* needed three growing seasons to flower and fruit set (Wunderlin, 1982), while flowering and fruiting occurred from April through November in Texas (Gould, 1978). Hester and Mendelssohn (1987) studied the response of temperature and moisture on seed production and germination of the *U. paniculata* population from the Chandeleur islands. Seed germination was high (65-88 %) at 18.3-35.0 ºC alternating thermoperiod after exposure of the seeds to a variety of pregermination moisture and temperature conditions. Temperature exposure of 4.4 ºC during imbibition did not alter the percentage of germination, but did increase the rate of germination. Stratification (moist-prechilling) stimulated the germination in comparison to the non-stratified seeds (Seneca, 1972). The highest germination (70 %) was observed for sea oats seeds stratified for 15 days followed by germination at an 8/16 hr thermoperiod at 35/25 ºC (95/77 ºF) (Burgess et al., 2001). Regardless of photoperiod, the influence of light was nonsignificant ($P = 0.45$) and stratification and

temperature were highly significant (P = 0.0001) on seed germination (Hester and Mendelssohn, 1987).

2.4 Micropropagation

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.

2.4.1 Shoot Multiplication

Micropropagation of sea oats aims at two things: production of large number of plantlets and propagation of the selected genotypes without inducing any genetic variation. A lot of research work has been done on nodal culture of several plants (Sanjaya et al., 2005; Siddique et al., 2006). Faisal et al. (2006) developed an efficient plant regeneration system for *Mucuna pruriens* L. using cotyledonary node explants. They observed that cotyledonary node explants from 7 day old aseptic seedlings induced maximum shoots in MS medium with 5 µM BAP. Maximum shoot proliferation was observed in 5 µM BAP and 0.5 µM NAA in half strength MS medium at pH 5.8. For rooting, they used 2.0 µM IBA with half-strength MS medium. Lin and Chang (1998) also did similar experiment in *Bambusa edulis* with 0.1 mg/l of Thidiazuron

(TDZ). Alexandrova and Denchev (1996) produced approximately 500 plantlets from one parent of *Panicum virgatum* L. in 12 weeks by using nodes on MS media containing 12.5 µM BAP.

Das et al. (2005) produced genetically uniform regenerants of *Bambusa balcooa* using axillary meristem. Liquid MS medium supplemented with 2 mg/l BAP, 1 mg/l Kn and 8 % coconut water resulted in axillary bud-break and multiple bud induction. Successful shoot regeneration and proliferation occurred in liquid MS medium with 3 mg/l IBA. For rooting, they tried half strength liquid MS media supplemented with 0.2 mg/l IBA.

Kane and Bird (1992) reported one protocol for *in vitro* propagation of *Ipomoea pes-caprae* using nodal segments. In their study, 2-isopentyladenine (2iP) at 5 mg/l in MS medium gave the maximum shoot regeneration but did not promote axillary branching of the shoots.

Kapoor and Rao (2006) reported rhizome induction and plantlet formation in *Bambusa bambos* using embryogenic axes of caryopsis. Multiple shoots were initiated in MS medium supplemented with 5.0 µM BAP and 2% sucrose. 2.5 µM BAP together with GA_3 (0.1 µM) and NAA (50 µM) formed 100 % rhizome. Latha et al. (1998) used woody plant medium supplemented with 5.5 µM BAP and 2.3 µM Kn for micropropagation of *Porteresia coarctata.* This combination gave the greatest response to initiation and multiplication. The multiplication rate was 11 shoots/explant after 8 weeks of culture period.

2.4.2 Somatic Embryogenesis

Poor seed production in *U. paniculata* (Bachman and Whitwell, 1995) is the main reason for the unavailability of sufficient amount of plant materials for restoration planting projects. Somatic embryogenesis involves a process of development of embryos in callus cultures derived from the somatic explant *in vitro* and their subsequent development to full plants. Little information is available on the application of micropropagation technique for multiplication of

dune and marsh species (Straub et al., 1988; Cook et al., 1989). In the Poaceae family, somatic embryogenesis has been studied extensively in largest number of species (Vasil, 1987; Krishnaraj and Vasil, 1995). Several studies have shown the comparison of morphogenetic ability with varying media, auxin-cytokinin combinations and the effect of induction and growth of calli on type of explant.

The only report on callus culture in sea oats was that of Hovanesian and Torres (1986), who reported callus growth and complete plant formation from mature caryopses. MS medium supplemented with 22.5 µM 2, 4-D, 4.4 µM BAP, and 87.6 mM sucrose gave the maximum callus. Shoots were produced in the same medium without 2, 4-D and rooting was accomplished in half strength MS medium with sucrose (43.8 mM) and 14.7 µM IBA. An average of 37 shoots per callus colony and a total of 384 plants were obtained within an 8 month period. However, the detail of the protocol was lacking. This subsection will focus on similar studies conducted in other grass species.

Lauzer et al. (2000) used immature inflorescences of *Phragmites australis* for *in vitro* propagation by somatic embryogenesis. Highest percentage of embryogenic calli was produced in media containing 1 mg/l, 2 mg/l and 1 mg/l 2, 4-D with 100 mg/l inositol. Callus regeneration was done in growth regulator free MS medium. The plantlets produced through somatic embryogenesis in their study did not show any morphological variation like the seed-grown plants.

Yemets et al. (2003) produced an efficient method of callus formation and plant regeneration of *Eleusine indica* L. N_6 basal salts and B_5 vitamins supplemented media with 2, 4-D (1-2 mg/l), glycine (2 mg/l), asparagines (100 mg/l), casein hydrolysate (100 mg/l), sucrose (30 g/l) and 0.6 % agar were optimum for the development of morphogenic calli. They also

reported that embryogenic cell suspension culture was a better source of *E. indica* protoplast than callus or mesophyll tissue.

In *Tylophora indica*, Jayanthi and Mandal (2001) reported best response for callus induction in MS medium containing 2 mg/l 2, 4-D with 0.01 mg/l Kn. Presence of 2-isopentyladenine produced the maximum number of mature somatic embryos. In rice, addition of tryptophan to different combination of auxins and cytokinins increased the embryogenic calli mass (Ilahi et al., 2005). In *Cynodon dactylon,* MS medium containing 3 % (w/v) sucrose, 3 mg/l 2, 4-D and 1 % agar produced the maximum embryogenic callus from the basal segment of young leaves (Ramgareeb et al., 2001). They were able to produce 40 plants/explant in 14 weeks. In *Stenotaphrum secundatum,* Li et al. (2006) studied the effect of addition and concentration of BAP in the callus induction and subculture medium. In their study, addition of BAP in the callus induction/subculture medium enhanced the callus regeneration ability and didn't harm callus induction from immature embryos. Addition of BAP in the medium promoted callus induction but was not required for callus maintenance in *Phragmites australis* (Cui et. al., 2002).

Grando et al. (2002) used MS medium containing 30 µM dicamba (3, 6-dichloro-o-anisic acid) and 5 µM BAP for callus induction and growth from seed explants of *Paspalum notatum.* They observed 65.7 % germination of seeds and 21.4 % produced embryogenic callus. Shoot regeneration was best in MS medium with 5 µM BAP and 1 µM Gibberilic acid. They successfully produced 1640 plantlets per gram fresh weight of callus tissue. For rooting they tried hormone-free SH (Schenk and Hildebrandt) medium. For all the media they used 3 % (w/v) sucrose as the carbohydrate source.

Khan and Khatri (2006) obtained embryogenic callus by culturing young leaves of *Saccharum officinarum* on MS medium containing 4 mg/l 2, 4-D. Maximum number of embryos

were obtained when the callus was transferred to medium with lower concentration of 2, 4-D (0.5 mg/l). For somatic embryo regeneration, they supplemented MS medium with Kn (2 mg/l), IAA and IBA and 2 % sugar. Rooting was successful in MS medium supplemented with 1 mg/l IBA and 6 % sugar.

Osuna and Barrow (2004) induced somatic embryogenesis in a perennial forage grass *Bouteloua eriopoda* using the embryonic shoots excised from the germinated seeds for callus induction. They used both 2, 4-D and dicamba but somatic embryo induction was greatest on 4.52 µM dicamba in light, after transferring to an auxin-free medium.

Avci and Can (2006) developed somatic embryos from immature inflorescences of *Paspalum dilatatum* on LS (Linsmaier and Skoog, 1965) medium supplemented with 7.5 mg/l picloram. In *Typha latifolia*, picloram induced more callus in comparison to 2, 4-D (Rogers et al., 1998). In *Sorghum bicolor* and *S. sudanense*, a combination of 2, 4-D and Kn with MS basal medium induced good callus, and IAA with Kn gave optimum result in regeneration (Gupta et al., 2006).

Sarma and Rogers (2004) studied callus regeneration and shoot multiplication in *Juncus accuminatus*. Calli were induced in MS medium supplemented with 5 mg/l Picloram. Callus regeneration was best observed in MS medium supplemented with 5 mg/l BAP. They also reported significant effect of medium pH (3.8-7.8) and source of callus grown in dark or continuous light on regeneration.

In *Spartina alterniflora*, callus was induced from 6 day old seedlings and coleoptile-covered segments in MS medium supplemented with 1 mg/l 2, 4-D and 1 mg/l IAA, whereas shoot regeneration was accomplished on MS medium with BAP and TDZ (Wang et al., 2003). Thomas and Puthur (2004) reported that a combination of TDZ and NAA gave 100 % shoot

organogenesis in callus from *Kigelia pinnata* L. Similarly, Siddique et al. (2006) also reported TDZ to be an effective growth regulator for induction of maximum shoots and combination of BA and NAA for shoot multiplication in *Nyctanthes arbor*.

Wang et al. (2004) regenerated plants through somatic embryogenesis in *Scirpus robustus*, a brackish wetland monocot. They used MS medium supplemented with 1 mg/l 2, 4-D for callus induction. Thirty one percent of the calli became embryogenic upon transfer to MS media supplemented with 2, 4-D @ 0.25 mg/l and the presence of BAP (optimum 3 mg/l) increased the number of regenerated shoots. Rooting was done on transferring the regenerated shoots to MS medium free of any plant growth regulator.

In hybrid Bermuda grass (*Cynodon dactylon x C. transvaalensis),* Jain et al. (2005) reported young and immature inflorescence as the best source of explants for embryogenic callus, as opposed to apical meristem and nodal segments. Callus induction was optimum in MS medium supplemented with 4 mg/l 2, 4-D, 0.01 mg/l BAP, 200 mg/l Casein hydrolysate and 30 g/l sucrose. Regeneration and rooting of embryogenic calli were obtained on hormone-free MS basal medium containing 30 g/l sucrose.

All these literature citations show that auxin is the most important hormone in regulating somatic embryogenesis. 2, 4-D, NAA, Picloram, Dicamba were the best sources of auxin and the concentration of their requirement varied from species to species. During callus multiplication, transfer of the callus to media with low concentration of 2, 4-D resulted in significant increase in the amount of embryogenic calli (Wang et al., 2004, Khan and Khatri, 2006). Presence of BAP enhanced the number of shoots (Cui et al., 2002; Grando et al., 2002; Wang et al., 2004). Literature citations on micropropagation either through somatic embryogenesis or shoot multiplication through nodal culture in dune species (sea oats) are limited.

2.5 Molecular Markers

Molecular markers are now routinely used for characterization of genetic diversity, DNA fingerprinting, genome mapping, genome evolution, ecology, taxonomy, and plant breeding. DNA-based markers are abundant, highly polymorphic and independent of environment or tissue type. Most DNA-based markers can be classified into three categories depending on the technique used (Karp and Edwards. 1997): Hybridization-based DNA markers, arbitrarily primed Polymerase Chain Reaction (PCR)-based markers, and Sequence targeted and single locus DNA markers. Restriction fragment length polymorphism (RFLP) is an hybridization-based markers in which DNA polymorphism is detected by digesting DNA with restriction enzymes followed by DNA blotting and hybridizations with probes. Arbitrarily primed PCR-based markers are employed in organisms for which no genome sequence is available. These markers are RAPD and AFLP. Sequence tagged sites (STS), SSR, Single nucleotide polymorphisms (SNP) markers belong to sequence targeted and single locus PCR-based DNA markers. In our study, we used RAPD (Williams et al., 1990) to fingerprint the micropropagated sea oats seedlings to determine genetic variation induced during tissue culture process. RAPD has many advantages: non-radioactive detection, multiple loci detection in a single reaction, requirement of small quantity of DNA, no requirement of prior sequence information, quick, inexpensive and technical simplicity.

2.5.1 Molecular Marker Studies in Plants Regenerated through Tissue Culture

Variation in both morphology and genotype has been reported to occur during *in vitro* regeneration processes (Kaeppler et al., 2000). In plants regenerated via somatic embryogenesis, the quality of somatic embryos determines the production of true-to-type plants. Molecular tools are more reliable than phenotypic observation for evaluating tissue culture induced variations

(Leroy et al., 2000). Many authors have reported that dedifferentiation of plant tissues leads to genetic modifications (Taylor et al., 1995; Hashmi et al., 1997; Rani et al., 2000), but on the contrary, several reports also confirmed genetic integrity of tissue culture derived plants (Dale et al., 1981; Haydu and Vasil, 1981; Hanna et al., 1989; Jayanthi and Mandal, 2001; Gagliardi et al., 2004).

In *Arachis retusa,* Gagliardi et al. (2004) evaluated 90 genomic regions using 5 random primers which generated an average of 18 loci per clone. All RAPD loci were monomorphic in the plantlets regenerated from both apical segments and embryo axes. A recent report on *Macadamia tetraphylla* by Mulwa and Bhalla (2007) concluded that RAPD analysis could establish the clonal integrity of tissue cultured generated plants. In this study, they could not detect any polymorphism when the banding patterns of stock plants were compared to their *in vitro*-derived progeny. An AFLP study in neem plants revealed no variation among the tissue cultured regenerants and the mother tree (Singh et al., 2002). In *Actinidia deliciosa,* Prado et al. (2005) found a good correlation of similarity between the regenerants and the field-grown mother plant within a genotype. They also reported that somaclonal variation was independent of the *in vitro* culture period which contradicted the report of Orton (1985) and Hartmann et al. (1989). In *Chlorophytum arundinaceum,* RAPD analysis revealed similar banding profile between micro propagated plants and the mother plant (Lattoo et al. 2006). Martins et al. (2004) studied the genetic stability of micropropagated *Prunus dulcis* plantlets using RAPD and ISSR markers. Sixty four RAPD and 10 ISSR primers produced 326 distinct, reproducible, and monomorphic bands across all the regenerants. No variation was also observed among the regenerants of *in vitro* cultured *Bambusa balcooa* (Gillis et al., 2007). Another recent report on somaclonal variation in *Vanilla planifolia* (Reddampalli et al., 2007) reported genetic uniformity

among the micropropagated plants within one genotype. No variation was observed among the regenerants using both RAPD and ISSR markers. Rout et al. (1997) compared the genetic fidelity among *in vivo* and *in vitro* plant materials using RAPD markers but could not notice any variation among the micropropagated plants of *Zingiber officinale* cv. $V_3S_{18.}$

Jayanthi and Mandal (2001) did RAPD analysis of somatic embryo-derived plants of *Tylophora indica* to determine genetic homogeneity and the true-to-type nature of the regenerants. They tested 20 primers out of which 18 produced monomorphic bands confirming the genetic homogeneity among the regenerants. Shoyama et al. (1997) suggested somatic embryogenesis could be used for clonal propagation of *Panax notoginseng* as they found genetic homogeneity among all the micropropagated plants using RAPD markers. Twenty-one RAPD primers produced monomorphic bands all across the 17 regenerants produced through somatic embryogenesis. Isabel et al. (1993) did the RAPD analysis of the somatic embryogenesis-derived populations of *Picea mariana* where no variation was detected within clones. Similarly, genetic integrity among the somatic embryo-derived regenerants within the cultivar of *Brassica oleracia* was confirmed by ISSR analysis (Leroy et al., 2000). The lack of variation in somatic embryo-derived regenerants could be due to the stringent internal genetic controls throughout embryo formation causing selection pressure against abnormal types (Leroy et al., 2000).

In contrast to the above reports demonstrating clonal fidelity in tissue culture regenerants, several studies reported tissue culture induced variation in a variety of plant species. In *Gypsophila paniculata,* Rady (2006) detected a very low variation at the DNA level among the intact plant and the regenerants using RAPD. Ray et al. (2006) studied the genetic stability among micropropagated *Musa* cultivars. Using a total of 21 RAPD and 12 ISSR primers that amplified 5330 RAPD and 2741 ISSR loci, they could detect genetic stability in one cultivar

while the plants derived from meristem culture from the other two cultivar were not true to-type. This kind of somaclonal variation was also observed in *Musa* by Smith (1988) and Vuylsteke et al. (1991). Lakshmanan et al. (2007) reported complete uniformity among the regenerants and also between the regenerants and field grown mother clone in *Musa* spp. This contradictory result could be explained by Vendrame et al. (1999) who reported that when all the *in vitro* conditions were same during the culture of micropropagated plants, genetic variation in a culture line could be affected by the genotype more than the period in culture.

These studies clearly demonstrated that DNA amplification techniques like RAPD, ISSR, and AFLP could be used to detect somaclonal variation in different micropropagated plants as well as genetic diversity among the cultivars. In this study, we are going to use RAPD which has been proved to be a potential DNA marker to determine the genetic variability among the regenerants.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Profile

The taxonomic classification of *Uniola paniculata* L. as provided by PLANT Database of USDA-NRCS (http://plants.usda.gov) is given below (Table 3.1). Likewise, a summary of its characteristics is presented (Table 3.2).

Table 3.1 *Uniola paniculata* L. as classified by USDA-NRCS PLANT Database.

Kingdom	Plantae
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Liliopsida (monocotyledons)
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae (grass family)
Genus	*Uniola*
Species	*paniculata*

Watson and Dallwitz (1992) reported the genus *Uniola* L. as a tetraploid with chromosome number of 2n=40 and haploid chromosome number of x=10.

3.2 *Uniola paniculata* L. Distribution and Source Sites

Populations of *U. paniculata* are documented to naturally thrive in southeast and south plains region of continental United States. Under the wetland indicator status, *U. paniculata* is classified as facultative upland type indicator, i.e., it usually occurs in non-wetlands, but

occasionally found on seasonally and semi-permanently flooded wetlands (US Fish and Wildlife Services, 1988).

Table 3.2 Summary of *U. paniculata* L. characteristics as detailed by USDA-NRCS PLANT Database (2007).

A. Morphology/Physiology			
Active Growth Period	Spring and Summer	Fruit/Seed Color	Brown
After Harvest regrowth rate	Slow	Growth Form	Rhizomatous
Allelopath	No	Growth Rate	Slow
Height, Mature (feet)	6.0	Nitrogen Fixation	None
Leaf Retention	No	Resprout Ability	No
Lifespan	Long	Shape and Orientation	Erect
Foliage Color	Green	Toxicity	None
Foliage Texture	Medium	C: N ratio	High
B. Growth Requirements			
Anaerobic Tolerance	Medium	pH	6.0-7.5
$CaCO_3$ Tolerance	Medium	Precipitation	35-65
Cold Stratification Required	Yes	Temperature (Min °F)	7
Drought Tolerance	High	Salinity Tolerance	Medium
Fertility Requirement	Medium	Shade Tolerance	Intolerant
Fire Tolerance	Medium	Frost Free Days (Min)	180
C. Reproduction			
Bloom Period	Early Summer	Fruit/Seed Abundance	Low
Seed per Pound	4500	Seed Spread Rate	Slow
Fruit/Seed Period Begin	Summer	Seedling Vigor	Low
Fruit/Seed Period End	Fall	Fruit/Seed Persistence	Yes
D. Suitability/Use			
Palatable Browse Animal	High	Nursery Stock Product	Yes
Palatable Graze Animal	Medium	Palatable Human	No
Fodder Product	No	Post Product	No
Fuel Wood Product	None	Protein Potential	Low

For this study, *U. paniculata* seeds harvested during September to December 2001 from six states in the southeastern and Gulf coast states of United States were used (Parami, 2003). These states include Alabama, Florida, Louisiana, Mississippi, North Carolina and Texas (Figure 3.1). For each state one accession was selected. An accession was identified as a group of harvested panicles within a natural population of *U. paniculata* in a state (Table 3.3).

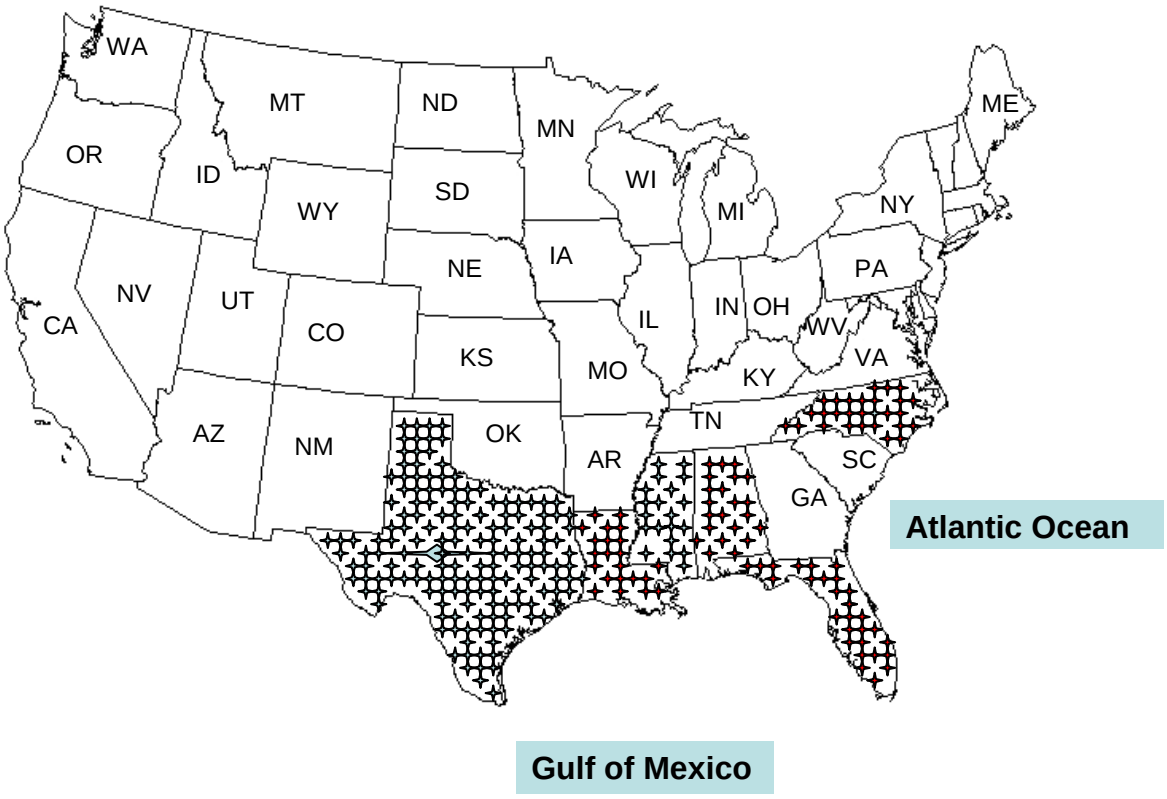

Figure 3.1 Map of continental United States showing the collection sites [Region 2 (southeast) = North Carolina, Florida, Alabama, Mississippi, Louisiana; Region 6 (south plain) = Texas] of *Uniola paniculata* L. seed materials and its natural distribution along the Atlantic and Golf coasts.

Table 3.3 *U. paniculata* L. accessions collected from southeastern Atlantic and Gulf coast areas and ID assignments.

Accession	Collection ID	Collection State	Collection site
UP01LA-19	JCH3	Alabama	Dauphin Island
UP01LA-39	GUIS-03-PK	Florida	W End Perdido Key Unit
UP01LA-15	68262-21	Louisiana	Fourchon Beach
UP01LA-41	GUIS-04B WSI	Mississippi	Westship Island
UP01NC-5	NCCB 01	North Carolina	Unknown
UP01LA-17	JCH1	Texas	Port Arthur

3.3 *In vitro* Culture Technique

3.3.1 Sterilization of Equipments and Glasswares

All operations for *in vitro* culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass materials and chemicals. A horizontal laminar flow cabinet (Envirco Corporation, Foster City, California, USA) with HEPA filter was used. The hood surface was wiped clean with paper towel soaked in 70 % ethanol (Figure 3.2) and sterilized by germicidal ultraviolet light for at least 10 min prior to use. All surgical instruments, glasswares and other accessories were sterilized in autoclave at 121 ºC with 15 psi for 30 min and then dried in oven. Surgical instruments like scalpel, forceps, and scissors were sterilized by dipping in 100 % ethyl alcohol and flaming prior to use.

3.3.2 Culture Room

The explants were incubated in a culture room where the temperature was maintained at 25-26 ºC, humidity at 85 % and either under continuous dark or under a photoperiod of 16 h light (25 µmol $s^{-1}m^{-1}$) and 8 h dark.

3.4 Preparation of Culture Media

MS (Murashige and Skoog 1962) inorganic salts, organic supplements, and vitamins were used as basal media for seed germination, callus induction, callus multiplication, and shoot and root induction. The formulation and composition of MS medium is given in Appendix 3.1.

Figure 3.2 Disinfection of the laminar flow hood with 70 % ethanol prior to use.

3.5 Preparation of Stock Solution

Stock solutions of the major components, such as macronutrients, micronutrients, vitamins, and plant growth regulators of the media were prepared and stored in refrigerator (Appendix-3.1).

3.6 Growth Regulators

Auxin and cytokinins were the two major phytohormones used in different concentrations and combinations in various media for induction and growth of callus, root and shoot.

3.6.1 Auxin

Powders of auxin (Sigma, St Louis, MO, USA) were dissolved in 1N NaOH and made up the volume with sterilized distilled water and then used or stored in freezer as stock for further use. The two auxins used in the present study were

1. 2, 4-Dichlorophenoxyacetic Acid (2, 4-D) and
2. α-Naphthalene Acetic Acid (NAA)

Four different concentrations (9.05, 13.58, 18.10 and 22.63 µM) of 2, 4-D were tested in MS medium, whereas NAA was used at 2 different concentrations (2.69 and 5.38 µM).

3.6.2 Cytokinins

The cytokinins (Sigma, St Louis, MO, USA) were dissolved in 1N NaOH and then used or stored as stock for further use. The two cytokinins used were

1. 6-Benzyl amino purine (BAP) and
2. Kinetin (Kn)

Six concentrations of BAP (2.22, 3.33, 4.44, 5.55, 6.66 and 7.77 µM) and three concentrations of Kn (0.46µM, 2.32 µM and 9.28 µM) were used in MS medium for somatic embryogenesis and cotyledonary nodal culture.

3.7 Preparation of Working Media

The required amount of salts, vitamins, and growth regulators from respective stock solutions were added into a conical flask with distilled water. Sucrose (87.64 mM) or Maltose (83.26 mM) (Sigma, St Louis, MO, USA) was added as carbon source and the final volume was made up to the required level with distilled water. The pH was adjusted within a range of 5.6 to 5.8 and agar (St Louis, MO, USA) was added to the solution at a rate of 8 g/l (0.8 % w/v). The

flask was covered with aluminum foil and sterilized by autoclaving at 121 ºC and 15 psi for 15 min.

3.8 Raising of Explants

3.8.1 Surface Sterilization of Seeds

Fully matured, healthy and well dried seeds were presterilised with 70 % ethanol for two min and washed with sterile distilled water. The seeds were then surface sterilized with 50 % (v/v) Clorox (commercial bleach) for 30 min with constant shaking at 220 rpm in Environ shaker (Lab-line, Melrose Park, Illinois, USA). The seeds were subsequently washed thoroughly (four to five times) with sterile distilled water inside the laminar flow cabinet until the trace of Clorox was gone.

3.8.2 Seed Germination

The sterilized seeds were given a cut at the non-embryo side and placed in petri dishes embryo side up in a hormone-free MS medium solidified with agar 0.8 % (w/v). For shoot multiplication two accessions were used (NCCB 01 from North Carolina and LA 68262-21 from Louisiana). Based on the previous seed germination experiment data (NCCB 01- 93 % and LA 68262-21- 50 %), 151 seeds of accession NCCB 01 were used in 6 replications and 338 seeds of LA 68262-21 were used in 14 replications. Individual petri dishes were wrapped with paraffin film to maintain it free from contamination. The seed cultures were incubated under dark at 26 ºC.

3.9 Inoculation of Explants

3.9.1 Inoculation of Explants for Shoot Multiplication

The shoot (coleoptile) including the hypocotyl was cut from 7-day-old seedlings and placed in the shoot multiplication media. Special care was taken not to damage the hypocotyl

region. Two explants were placed in a single bottle containing the medium. The bottles were put in the culture room [temperature - 25-26 ºC, humidity at 85 % and photoperiod of 16 h light (25 µmol $s^{-1}m^{-1}$) and 8 h dark].

3.9.2 Plant Growth Regulator Combination in the Medium

In a preliminary experiment, one accession (NCCB 01) was tested with several media for studying the optimum response. The media and their formulations are provided below.

1) SMM1 = MS + 2.22 µM BAP

2) SMM2 = MS + 3.33 µM BAP

3) SMM3 = MS + 4.44 µM BAP

4) SMM4 = MS + 5.55 µM BAP

5) SMM5 = MS + 6.66 µM BAP

6) SMM6 = MS + 7.77 µM BAP

7) SCR1 = MS + NAA (5.38 µM) + Kn (9.28 µM)

8) SCR2 = MS + NAA (2.69 µM) + BAP (6.66 µM) + Kn (2.32 µM)

The carbohydrate source was sucrose (87.64 mM) for all the SMM media and maltose (83.26 mM) for SCR1 and SCR2 media.

In our preliminary experiment, explants cultured in SMM1, SMM2 and SCR1 had no response to shoot multiplication (only vertical growth), whereas there was very slow response in SMM4, SMM6 (Initiation of multiplication in 1-2 explants after 12 weeks of culturing) to shoot multiplication (data not provided). Therefore, only three media (SMM3, SMM5, and SCR2) were used in the subsequent studies.

3.10 Shoot Multiplication and Maintenance

The explants were subcultured onto fresh media every 15 days. When the explants started to multiply, well grown axillary shoots were separated with the help of a sterile scalpel under the hood and put in the same media for further multiplication. The shootlets derived from each seed were tracked individually to determine the total number of plants produced from single seed and their subsequent genetic identity.

3.11 Rooting Media

Eight-to-10-cm long shoots with or without roots were cultured onto following two media for root induction.

1) RM1 = MS media with no plant growth regulator

2) RM2 = MS + NAA (5.38 µM) + Kn (0.46 µM)

The magenta culture vessels (Sigma, St. Louis, MO, USA) were maintained in the culture room under identical conditions as mentioned for shoot multiplication. Individual shoots from shoot clump derived from a single seed were tracked by proper numbering and maintained to get the complete plant.

3.12 Explants for Callus Induction

For callus induction, the sterilized seeds were also given a cut at the non-embryo side and placed in petri dishes embryo side up touching the callus induction medium. In this study, six accessions were tested. Fifty seeds from each accession were placed in two replications (25 each) in each of the five media. All the petri dishes were wrapped with parafilm to prevent from contamination. The seed cultures were incubated in the dark at room temperature. After 3 weeks, the tiny creamy yellow callus appearing near the junction of the root and shoot (embryonic axis) was subcultured on fresh but same medium and dark-incubated for callus multiplication. Two

types of calli were formed: 1) off-white or pale yellow, compact and some what nodular, and 2) soft, granular and translucent. Of these, only the first type that exhibits embryogenic differentiation (Vasil and Vasil, 1991) was selected for the next step.

3.13 Media Formulation

3.13.1 Callus Induction

For callus induction, the following combinations of plant growth regulators with MS medium were tested.

1) CIM2 = MS + 2, 4-D (13.58 µM) + Kn (2.32 µM)

2) CIM3 = MS + 2, 4-D (18.10 µM)

3) CIM4 = MS + 2, 4-D (18.10 µM) + Kn (2.32 µM)

4) CIM5 = MS + 2, 4-D (22.63 µM)

5) CIM6 = MS + 2, 4-D (22.63 µM) + Kn (2.32 µM)

3.13.2 Callus Multiplication

One month old callus with clump of somatic embryos (SE) was transferred (Figure 3.3) to callus multiplication medium and were maintained under dark at room temperature to obtain sufficient amount of somatic embryos.

Callus Multiplication Medium (CMM) = MS + 2, 4-D (9.05 µM) + Kn (2.32 µM)

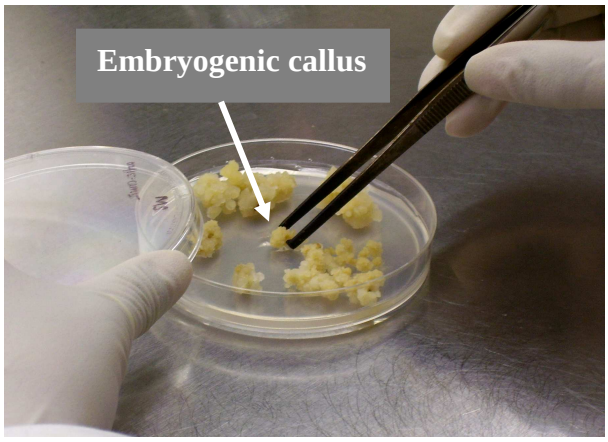

Figure 3.3 Selected embryogenic calli being transferred under aseptic condition.

3.13.3 Callus Regeneration

The cluster of mature somatic embryos were put for plant regeneration in magenta culture vessels (Sigma, St. Louis, MO, USA) containing the following two media.

1) SCR1 = MS + NAA (5.38 µM) + Kn (9.28 µM)

2) SCR2 = MS + NAA (2.69 µM) + BAP (6.66 µM) + Kn (2.32 µM)

The cultures were maintained in the culture room [temp. 25-26 ºC, humidity at 85 % and photoperiod of 16 h light (25 µmol $s^{-1}m^{-1}$) and 8 h dark]. At 15 days interval, two subcultures were done. The regenerated shoots were separated individually from the clump and cultured in separate vessel for optimum growth. In each case, the number of shoots from a single callus derived from a single seed was tracked.

3.13.4 Rooting Media

Eight to 10 cm long shoots with or without roots were transferred onto two media same as the ones for cotyledonary nodal culture for root induction. The cultured vessels were maintained in the culture room under identical conditions with the plants derived from somatic embryos.

3.14 Hardening and Establishment of Tissue Culture Generated Plants in the Greenhouse

Healthy plants with well developed roots (5-7 cm) from both the shoot multiplication and somatic embryogenesis experiments were taken out of the culture room and kept for hardening at room temperature under diffused sunlight for a period of 2 days. The plants were then removed from the culture vessels, roots were washed thoroughly in running tap water to ensure removal of traces of agar, and plants were planted in 7.5 x 7.5 cm^2 plastic containers filled with sterilized sand and jiffy mix (2:1). The pots were transferred to greenhouse inside an artificially created mist chamber covered with plastic sheets and fitted with a ReliOn® ultrasonic humidifier (Kansas

City, USA). The pots received 6 times mist spray of 10 min each during the day time. The plants were watered at 3-4 days interval and a slow releasing fertilizer Osmocote® (Scotts, Marysville, Ohio, USA) was applied to each pot at 15 days interval. After one month, the plants were taken out of the mist chamber and grown inside the greenhouse, which was maintained at 29 ºC during day with a light intensity of 50-70 µmol $m^{-2}s^{-1}$ and 22 ºC during night. At weekly interval, a nutrient solution Miracle-Gro® (Scotts, Marysville, Ohio, USA) was sprayed once on the leaf surface.

3.15 Molecular Marker Analysis of Micropropagated Plants

3.15.1 Genomic DNA Extraction

Total genomic DNA was isolated from young leaf tissue samples of all the plants derived through nodal shoot multiplication and somatic embryogenesis from each accession. Leaf tissues were ground into a fine powder in liquid nitrogen using a mortar and pestle. Up to 100 mg of the powdered tissue sample was transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Genomic DNA was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich®, Saint Louis, MO, USA). The kit contains all the reagents, columns, and tubes necessary to isolate genomic DNA. Following the instructions provided by the manufacturer, leaf tissue cells were lysed with 350 µl of lysis solution A and 50 µl of lysis solution B in the microcentrifuge tubes. After vortexing, the samples were incubated at 65 ºC for 10 min with occasional inversion to dissolve the precipitate. No RNA treatment was given since the kit is designed to selectively isolate DNA. A precipitation solution (130 µl) was added to the mixture and mixed thoroughly by inversion and incubated on ice for 5 min. The samples were centrifuged (13,200 rpm) for 5 min. The supernatant was pipetted onto the filtration column without disturbing the debris and centrifuged at 13,200 rpm for 1 min. The flow through liquid

was collected and added with 700 µl of binding solution. The column membrane was conditioned by adding 500 µl of the column preparation solution and centrifuging (13,200 rpm) for 1 min. The DNA in the binding solution was transferred to the column and the bound DNA was washed twice with ethanol mixed wash solution and centrifuged at 13, 200 rpm for 3 min. Pure genomic DNA was eluted into the collection tube by adding 50 µl of prewarmed (65 ºC) elution buffer (10 mM Tris, 1mM EDTA, pH 8.0) to the column and centrifuged at 13,200 rpm for 1 min. A second elution was made by adding another 25 µl of the prewarmed elution buffer into the column followed by centrifugation (13,200 rpm for 1 min). The DNA samples were stored at -20 ºC for further use.

The DNA quality was determined by running 2 µl aliquot of DNA on 1 % agarose gel along with a λ plasmid DNA of known concentration (50 and 100 ng/µl) as standard (Figure 3.4). The agarose gel image allowed inspection of the integrity of the DNA. The concentration of the genomic DNA was determined by using a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA samples were diluted to 25 ng/µl concentration for use in RAPD analysis.

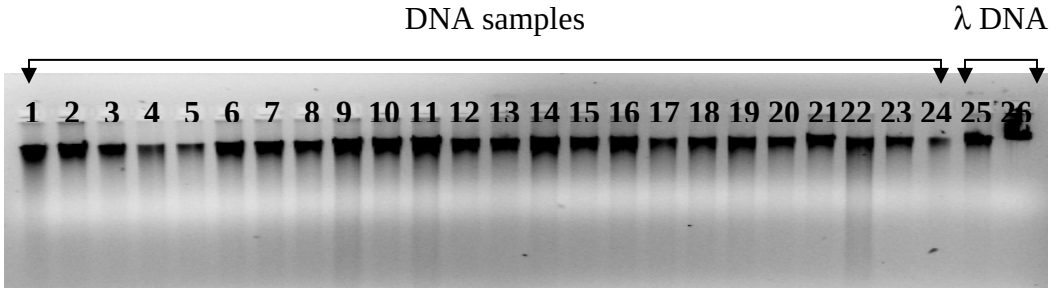

Figure 3.4 An agarose gel showing good quality DNA suitable for RAPD analysis [Lane #1-24 - sea oats DNA samples, lane #25 and 26 - λ plasmid DNA of concentration 50 and 100 ng/µl, respectively].

3.15.2 RAPD Assays

Optimization of RAPD protocol was done by using DNA samples from two seed propagated sea oats plants and a total of 66 primers from Operon RAPD primer kits (OPB 1-20, OPX 1-5, OPX 10-20, OPD-5, 13, 16 and 20, OPJ 1, 6, 9 and 17, OPT 1, 8, 12, 14, 15, 17, OPA 7, OPC 1, 10, 12, OPF 4, 10, 14, 18, OPG 2, 12, OPH 7, 12 and OPY 17, Operon Technologies, Alameda, CA) to identify the optimum DNA concentration and the primers yielding multiple, clear and reproducible amplification products. The DNA amplification was carried out on a PTC-100 Programmable Thermal Cycler (MJ Research, MA, USA) using the following profile: one cycle of 2 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C, followed by one cycle of 7 min at 72 °C

Seventy five ng of DNA samples from 125 plants i.e., ninety plants of five accessions (from somatic embryogenesis) and thirty five plants of two accessions (from cotyledonary node culture) were run using the selected primers following the same PCR profile.

The final volume for the PCR reaction was 25 µl containing 2.5 µl of 10X PCR reaction buffer (Promega, Madison, Wisconsin, USA), 2.5 µl of 25 mM $MgCl_2$, 200µM of each dNTP, 2.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 50 ng/µl primer (Appendix 3.2). Three µl of 5X loading dye was added to the PCR amplification product and twenty five µl of PCR product was electrophoresed on 1.5 % agarose gel in 1X TAE buffer, stained with ethidium bromide, and visualized and documented under UV light in a KODAK Gel Logic 100 (Kodak, New Haven, Connecticut, USA). Four hundred nanograms of a DNA size ladder (Hi-Lo™ DNA marker; www.mnmolecular.com) was loaded in the gel along with the PCR products.

CHAPTER 4

RESULTS AND DISCUSSION

The present study was conducted to investigate optimal concentrations and combinations of plant growth regulators in the medium for efficient micropropagation in sea oats via nodal shoot culture and somatic embryogenesis (through callus culture). Further, the genetic identity of the tissue culture propagated plants was verified by using RAPD markers. The results obtained are presented and discussed in this chapter.

4.1 Shoot Multiplication

4.1.1 Seed Germination

Seeds of eight accessions of sea oats were used for germination test (Table 4.1). The seeds were placed in MS medium without any plant growth regulator. When a cut was given to the seed at the opposite end to the embryo, there was remarkable increase in the percentage of germination. For example, 93 % of cut seeds of NCCB 01 germinated after two weeks, whereas only 12 % germination was noticed for uncut seeds (Figure 4.1). Similar kind of result was observed in a seed dormancy study of sea oats where 100 % of seeds germinated after giving a cut at the non-embryo side of the seeds (Westra et al., 1966).

Table 4.1 Germination percentage of eight accessions of sea oats (cut seeds).

Accession	Germination percentage
NCCB01	93±1.7
LA 68262-21	50±5.7
GUIS-03-PK	67±3.3
LP4	25±5.0
JCH1	47±1.7
GUIS 04 BW01	68±1.7
SDN 042	67±1.7
JCH5	37±3.3

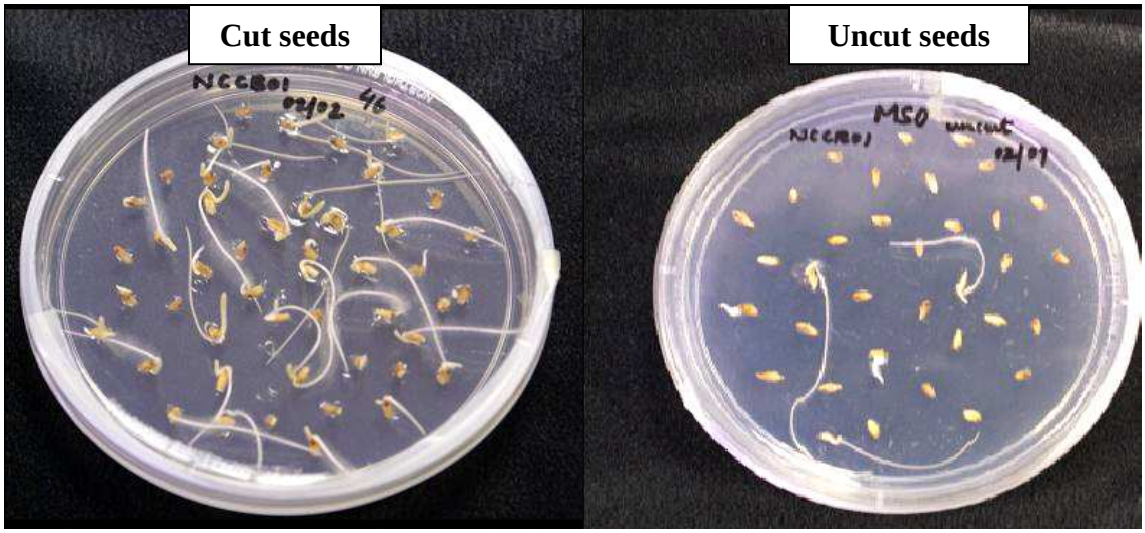

Figure 4.1 Comparison of seed germination in cut and uncut seeds of sea oats accession NCCB 01.

4.1.1.1 Comparison of Seed Germination in NCCB 01 and LA 68262-21

Two accessions, NCCB 01 and LA 68262-21 were selected for the cotyledonary nodal culture study. A comparative germination test between accessions NCCB 01 and LA 68262-21 revealed that there was 43 % more germination in NCCB 01 than LA 68262-21 (Figure 4.2). NCCB 01 had 63 % germination after 10 days, whereas only 20 % of seeds germinated in LA 68262-21 after 16 days.

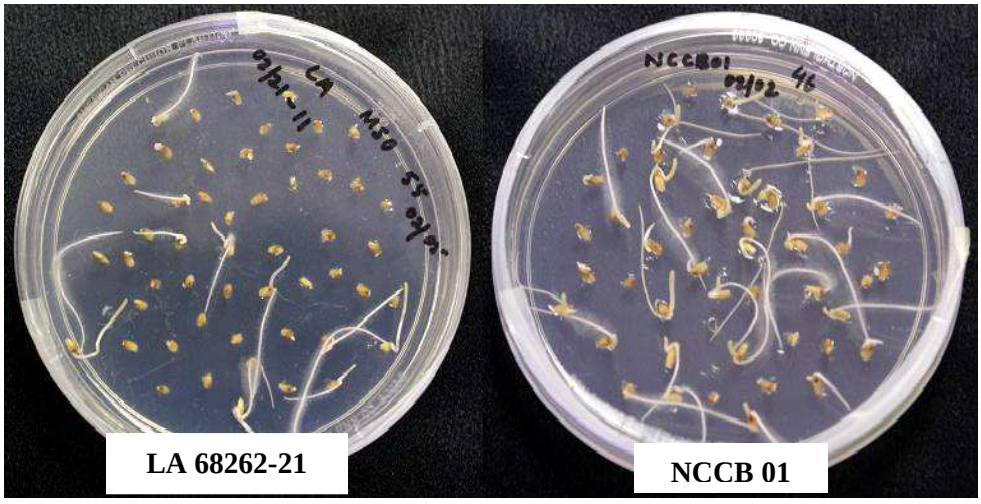

Figure 4.2 Comparison of germination between LA 68262-21 (left plate) and NCCB 01 (right plate).

4.1.2 Shoot Multiplication in NCCB 01 and LA 68262-21

In a preliminary experiment of shoot multiplication from cotyledonary nodal explant, seven accessions were tested with eight different medium-PGR combinations. There was no response in SMM1, SMM2, SMM4, SMM6 and SCR1 (detailed in material and methods), where only vertical growth was observed. On the other hand, SMM3, SMM5 and SCR2 media responded well to shoot multiplication (data not shown).

Eventually, two accessions (NCCB 01 and LA 68262-21) were used for shoot multiplication in three media i.e., SMM3, SMM5, and SCR2.

4.1.2.1 Response of NCCB 01 to Shoot Multiplication

A total of 151 seeds were used for germination of which 136 germinated. Two-week-old embryonic shoots were placed in SMM3, SMM5, and SCR2 media in three replications each. Early response was observed in SCR2 medium, where axillary bud initiation started on the 24th day of transferring the hypocotyls to the shoot multiplication medium. In both SMM3 and SMM5, the multiplication started 4 days later than SCR2 i.e., on the 28th day (Table 4.2). Out of all the explants placed in shoot multiplication media, shoot multiplication was observed in 74 % of explants in SCR2, whereas this value was only 40 % and 37 % for SMM3 and SMM5 respectively (Table 4.4).

Table 4.2 Number of days taken for initiation of shoot multiplication.

Medium\Accession	NCCB 01	LA 68262-21
SCR2	24	19
SMM3	28	24
SMM5	28	34

4.1.2.2 Response of LA 68262-21 to Shoot Multiplication

For the shoot multiplication study, 338 seeds of LA 68262-21 were placed for germination of which 146 (43.2 %) germinated. The seedlings were very weak and 31 died while

transferring to the shoot multiplication media. Although SCR2 medium responded to early multiplication (Table 4.2), SMM5 showed maximum percentage (61 %) of embryonic shoot multiplication (Table 4.4). The multiplied explants were carefully separated with the help of a sterile scalpel into several plantlets depending on the number of axillary shoots produced.

4.1.3 Effect of Media on Multiple Shoot Induction

In both accessions, NCCB 01 and LA68261-21, SCR2 medium (Figure 4.3) responded early with regard to shoot multiplication (Table 4.2), but the number of axillary shoots from a single embryonic seedling was higher in SMM3 (Figure 4.4) and SMM5 medium (Figure 4.5) (Table 4.3). For each accession 10 embryonic seedlings were maintained in individual medium to count the number of shoots per cotyledonary node. In NCCB 01, it ranged from 3 to 8 in SCR2, 7 to 11 in both SMM3 and SMM5 medium. In LA 68262-21, 4 to 10 plants were produced from a single seedling where as it was 3 to 12 in SMM3 medium and 6 to 15 in SMM5 medium.

Table 4.3 Number of plantlets from a single embryonic seedling in accessions NCCB 01 and LA 68262-21.

Accession	# shoots/nodal explant					
	SCR2		SMM3		SMM5	
	Mean	Range	Mean	Range	Mean	Range
NCCB 01	6.0 ± 0.56[$]	3-8	9.3 ± 0.42	7-11	8.8 ± 0.42	7-11
LA 68262-21	3.0 ± 0.58	4-10	7.1 ± 1.10	3-12	9.4 ± 0.86	6-15

[$] **Mean ± standard error of means over 10 hypocotyls**

Table 4.4 Comparison of shoot multiplication response in sea oats accessions NCCB 01 and LA 68262-21.

Accession	# seed inoculated	# seed germinated	# hypocotyls inoculated in different media			# explants dead	# contaminated	% hypocotyls showing multiplication*		
			SCR2	SMM3	SMM5			SCR2	SMM3	SMM5
NCCB 01	151	136	39	30	33	12	22	74±6.8	40±5.8	37±4.5
LA 68262-21	338	146	39	12	39	31	25	44±6.8	42±8.3	61±4.4

*** Mean ± SE (m)**

Figure 4.3 Shoot multiplication in SCR2 medium from a single sea oats seedling of NCCB 01.

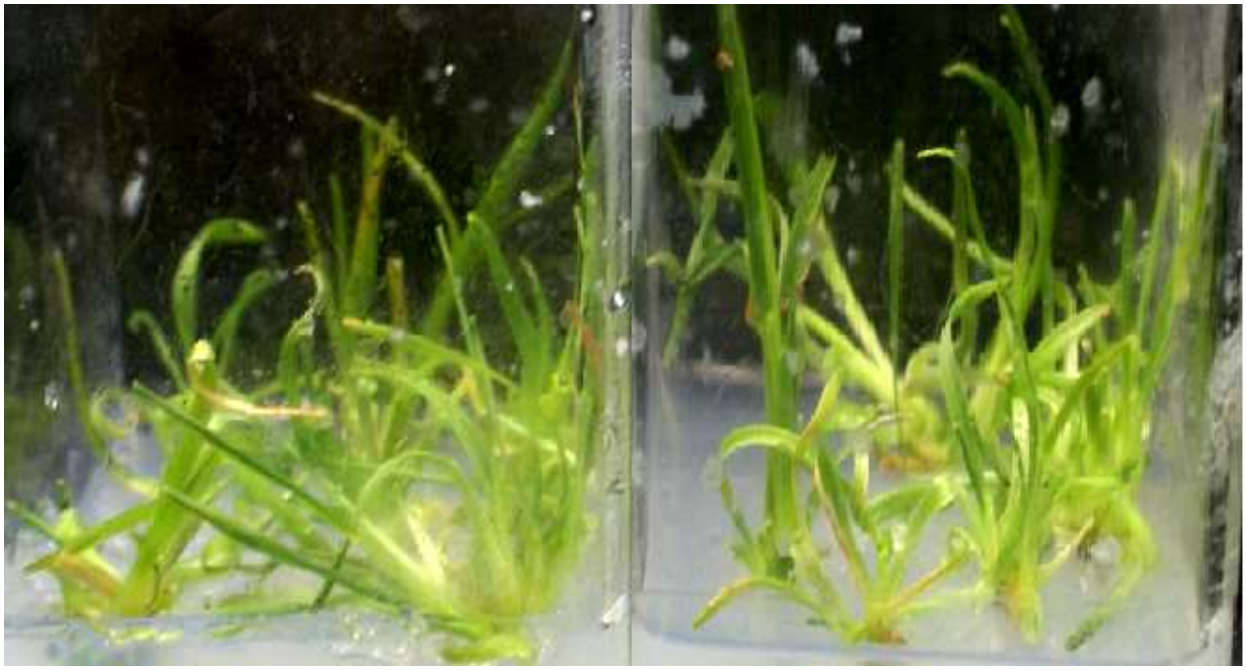

Figure 4.4 Shoot multiplication in SMM3 medium from a single sea oats seedling of NCCB 01.

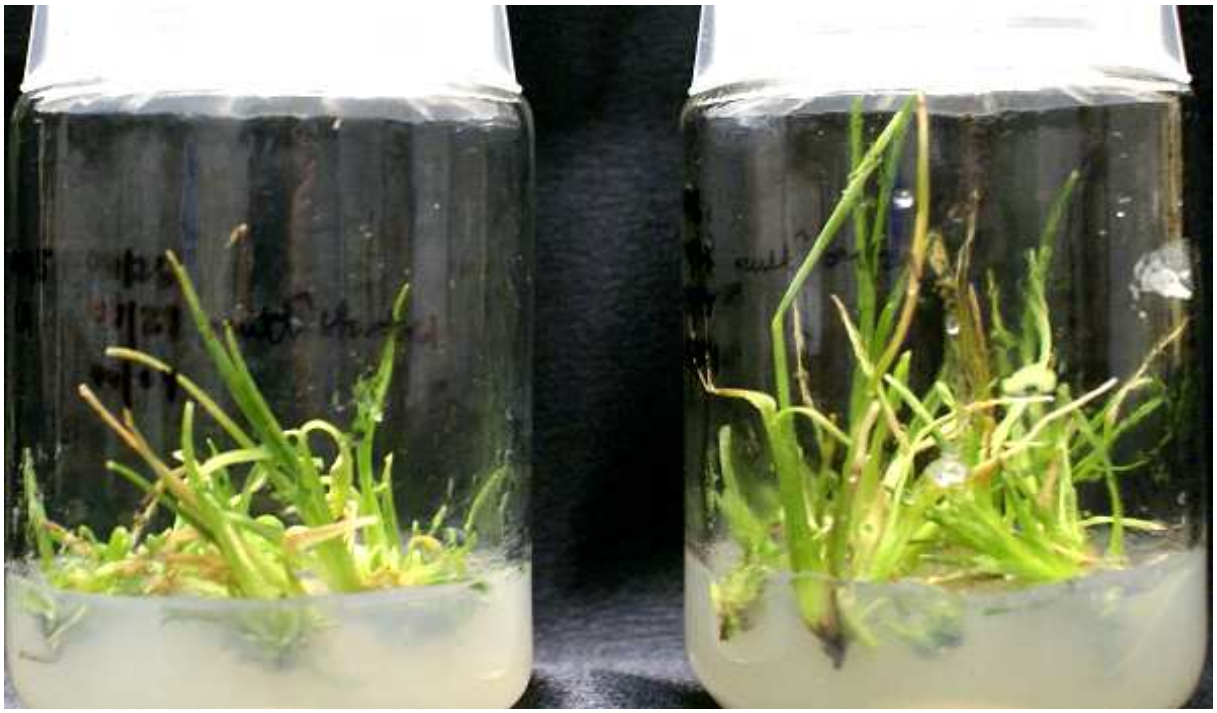

Figure 4.5 Shoot multiplication in SMM5 medium from a single sea oats seedling of LA 68262-21.

4.1.4 Comparison of Media for Different Accessions to Shoot Multiplication

A total of 241 and 238 plantlets were regenerated through cotyledonary nodal culture in NCCB 01 and LA 68262-21, respectively. There was no difference between SMM3 and SMM5 medium for the North Carolina accession NCCB 01. NCCB 01 produced 93 and 88 plantlets in SMM3 and SMM5 medium, respectively. But, difference was observed between SMM3 and SMM5 medium for LA 68262-21 accession. Total 94 plantlets were regenerated from 39 explants in SMM5 medium, whereas there were 71 plantlets from 12 explants in SMM3 medium. Based on quantitative and visual assessments, the optimal BAP concentrations could be 4.44-6.66 µM for shoot multiplication. Necessity and effects of different concentrations of BAP on shoot multiplication rates in sea oats genotypes have been studied by Valero-Aracama et al. (2002). Philman and Kane (1994) reported *in vitro* propagation of *Uniola paniculata* from terminal and lateral shoot tip culture in LS medium supplemented with 2.22 µM BAP. However,

there is no report of a high shoot multiplication rate in sea oats genotypes as obtained in the present study. This result is consistent with the findings of Arya et al. (1999) in *Dendrocalamus asper* and Saxena et al. (1992) in *Arachis hypogaea* where BAP induced direct shoot regeneration from intact seedlings.

SCR2 medium, which has the combination of BAP, Kn and NAA produced 60 plantlets in NCCB 01 accession and 73 plantlets in LA 68261-21 accession. Combination of BAP and NAA was most effective for shoot regeneration in *Psoralea corylifolia* (Saxena et al., 1997) and *Rauvolfia tetraphylla* (Faisal and Anis, 2002). In comparison to SMM3 and SMM5 media, SCR2 produced less number of shoots as presence of NAA increased basal callusing (Table 4.3). Higher concentration of NAA reduced regeneration frequency and number of shoots in *Mucuna pruriens* (Faisal et al., 2006).

These results and the preliminary experiment with different media revealed that an optimum level of BAP concentration was needed for shoot multiplication. Absence or low level of BAP increased the leaf length without shoot multiplication (Arya et al., 1999). The present study confirmed that MS medium fortified with 6.66 µM BAP, designated as SMM5 medium (independent of accession) was optimum for shoot multiplication in sea oats.

4.1.5 Rooting of Multiple Shoots

The multiplied shoots were initially tested for rooting in MS medium free of any plant growth regulator (RM1) and observation was taken weekly. After two weeks, no root initiation was observed in any of the two accessions. But root primordia were observed within two and half to three weeks upon transfer of the shoots to another medium RM2 (MS + 5.38 µM NAA + 0.46 µM Kn). The roots were thick and strong in this medium (Figure 4.6). There was no difference in the root morphology of the plants derived from different multiplication media.

However, the plants derived from SCR2 medium exhibited relatively quick response to rooting (13 - 23 days) in comparison with the SMM3 (20-27 days) and SMM5 (15-35 days) (Table 4.6). It could be due to presence of NAA already in the shoot multiplication SCR2 medium. NAA has been reported to have a stimulatory effect on root induction in species including *Panax notoginseng* (Shoyama et al., 1996), *Bambusa tulda* (Saxena, 1990), and *Porteresia coarctata* (Latha et al., 1998). A total of 43 plantlets from NCCB 01 and 30 plantlets from LA 68262-21 multiplied in SCR2 medium were tried for rooting. The rooting response was 85 % and 70 % for NCCB 01 and LA 68262-21, respectively. Similarly, regenerants from each accession multiplied in different media were placed in the rooting medium and the observations were shown in Table 4.5. There was better response to rooting in NCCB 01 in comparison to LA 68262-21 (Table 4.5). Also, shoots multiplied in SMM5 medium had higher percentages (95 % and 75 %) of rooting in both accessions (Table 4.5).

Table 4.5 Response to rooting in RM2 medium of plants multiplied in different media.

	# Plants transferred Plant regeneration medium			Rooting response (%) Plant regeneration medium		
Accession	SCR2	SMM3	SMM5	SCR2	SMM3	SMM5
NCCB 01	43	20	39	85	75	95
LA 68262-21	30	21	20	70	67	75

Table 4.6 Number of days for initiation of rooting.

Media	Number of days to rooting
SCR2	13-23
SMM3	20-27
SMM5	15-35

Figure 4.6 Complete plants multiplied from cotyledonary nodes with well developed roots (NCCB 01 accession in RM2 medium).

4.2 Somatic Embryogenesis

4.2.1 Callus Induction

Seeds from six sea oats accessions were used for callus induction in five different callus induction media (Table 4.7). A total of 1388 seeds were put for callus induction of which 7 got contaminated, 314 germinated with no callus induction, and the remaining 1067 seeds showed callus induction. In CIM2 medium, callus induction was maximum (53 %) in JCH3 and the minimum (26.3 %) was observed in LA 68262-21. In CIM3, best response was noticed in NCCB 01 (54 %) which was the maximum value of the entire callus induced media (Table 4.7). In CIM4, GUIS 03 PK was the best in callus induction (50 %). The callus initiation was noticed as creamy white swelling (pre-embryogenic masses or pre-globular proembryos near the root-shoot junction after two weeks of seed inoculation (Figure 4.7 A). Subsequent subculture of these embryos in the same medium for one month resulted in their multiplication (induction phase as termed by Dodeman et al., 1997) and transferring them to a callus multiplication medium with

lower concentration of 2, 4-D for one month resulted in maturation of somatic embryos (Figure 4.7 B). This indicated an indirect somatic embryogenesis pathway (intermediary step of callus culture) as pre-embryogenic masses were further developed after long cultures on auxin-supplemented media (Emons, 1994). Exclusion or reduced level of 2, 4-D from the culture medium triggers the expression (maturation, the second phase in somatic embryogenesis pathway) of somatic embryogenesis (Jimenez, 2001). Lower concentration of 2, 4-D enhanced the number of mature somatic embryos in *Saccharum officinarum* (Khan and Khatri, 2006) and in *Scirpus robustus* (Wang et al., 2004).

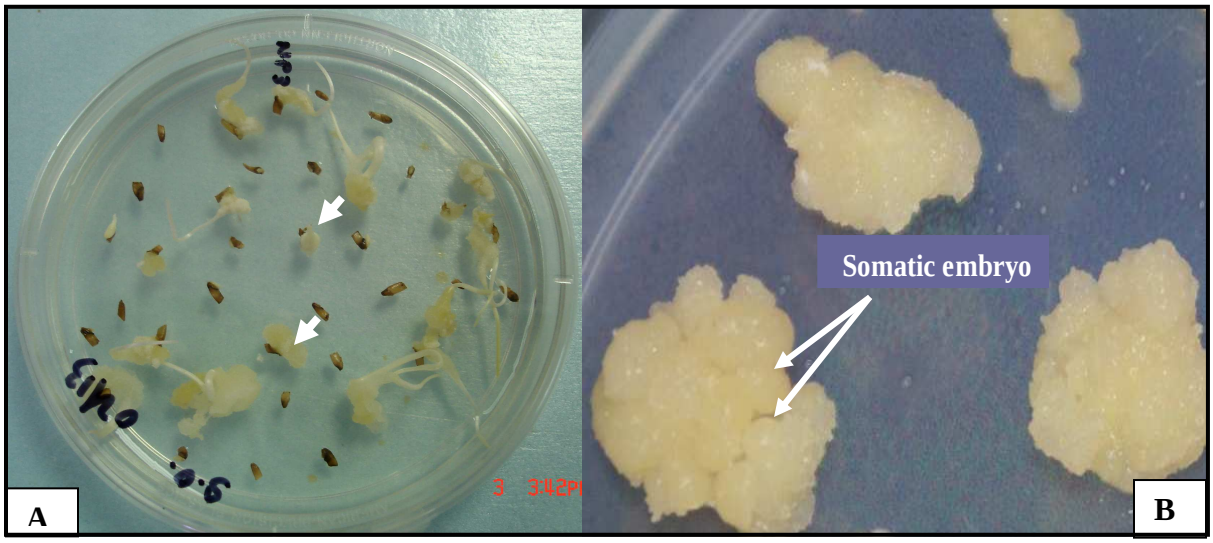

Figure 4.7 Callus induction in sea oats. (A) Primary callus induced from the root-shoot junction (arrow marked), (B) Embryogenic callus with clusters of somatic embryos (arrow marked) multiplied from a seed-derived primary callus.

4.2.2 Response of Different Sea oats Accession to Callus Induction (Independent of Medium)

Among six accessions used for callus induction frequency (CIF), NCCB 01 (North Carolina) was found to be the best accession with 45 % CIF followed by JCH3 (Alabama) with 41 %, GUIS 03 PK (Florida) with 40 %, GUIS 04 BW 01 (Mississippi) with 35 % and JCH1 (Texas) with 29 % CIF (Fig.4.8). LA 68262-21 responded poorly to callus induction (22 % CIF).

As was observed in this study, genotypic differences in the response to *in vitro* propagation has also been reported in sea oats (Valero-Aracama et al., 2002).

Table 4.7 Response of six accessions of sea oats to different media.

Media\Accession	JCH1	JCH3	NCCB01	LA 68262-21	GUIS 04 BW 01	GUIS 03 PK
CIM2	32.0±5.0*	53.0±3.4	48.0±1.0	26.3±2.3	28.3±5.0	38.1±4.4
CIM3	27.0±2.0	38.0±4.4	54.0±3.6	26.0±3.5	45.7±2.4	42.0±5.5
CIM4	32.0±1.7	36.0±5.6	34.0±7.5	20.0±3.7	28.3±5.0	50.0±3.0
CIM5	36.0±6.4	44.0±6.5	48.0±2.7	20.0±4.2	49.0±5.0	40.0±1.2
CIM6	16.3±2.5	34.0±1.3	40.0±5.4	20.0±3.7	23.0±2.2	30.0±3.0

- Mean ± SE(m)

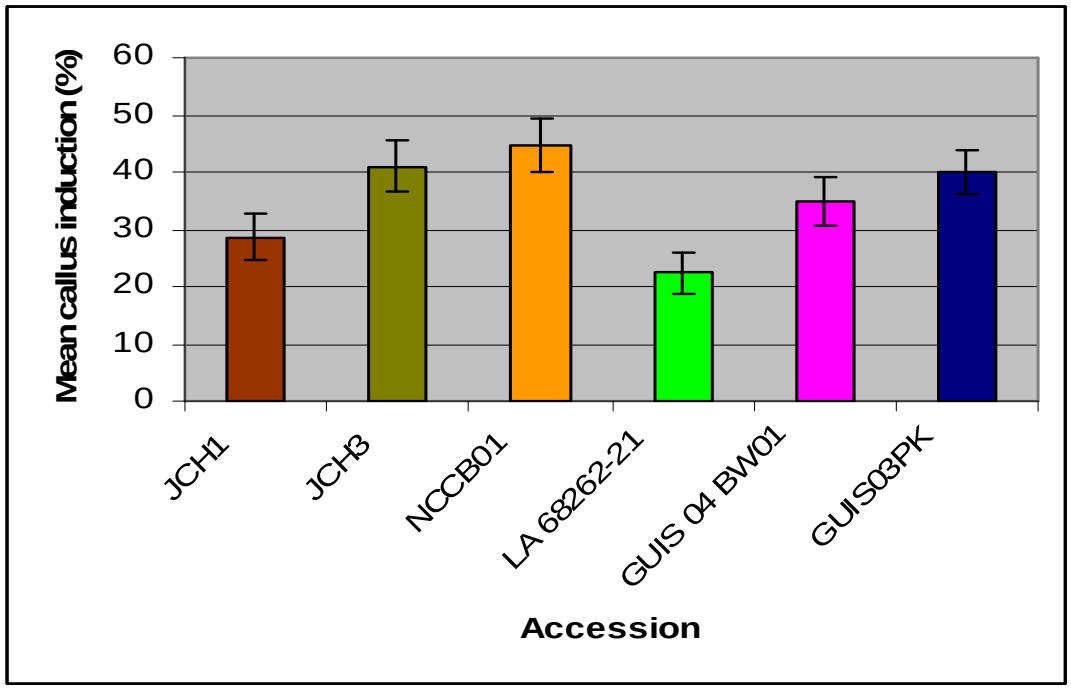

Figure 4.8 Comparison of mean callus induction percentage in different accessions pooled over media.

4.2.3 Effect of Plant Growth Regulators in Medium to Callus Induction (Independent of Accession)

Callus induction frequency was determined for different media by pooling the values over different accessions. Of the five media tested, highest percentage of callus induction was observed in CIM5 (39.5 %) medium followed by CIM3 (38.8 %) over all the accessions (Figure 4.9). A comparative callus growth from different accessions from a single seed in CIM5 medium is shown in Figure 4.10. The medium CIM6 was the poorest (27.2 %) among all in inducing callus from all six accessions. Highly embryogenic callus with small preglobular somatic embryos were maintained and multiplied in MS medium with reduced (9.05 µM) 2, 4-D (Figure 4.7). The differential growth and development of calli from different accessions of sea oats in different callus induction media is shown in Figure 4.12. It has been demonstrated in many cases that 2, 4- D is usually the choice of auxin for callus induction and subculture of grasses (Bhaskaran and Smith, 1990; Chaudhury and Qu, 2000).

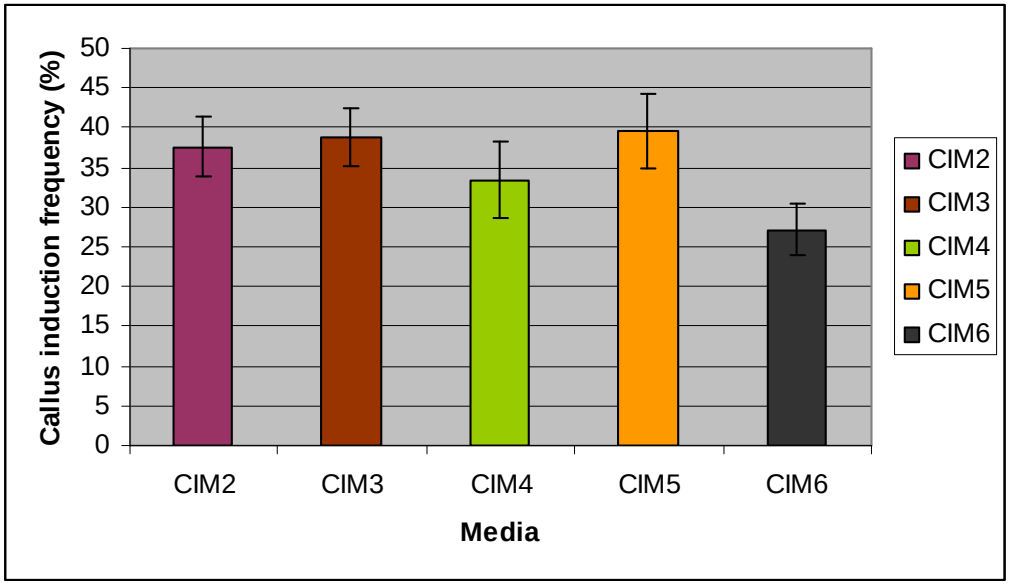

Figure 4.9 Comparison of callus induction frequency in different media (pooled over accessions).

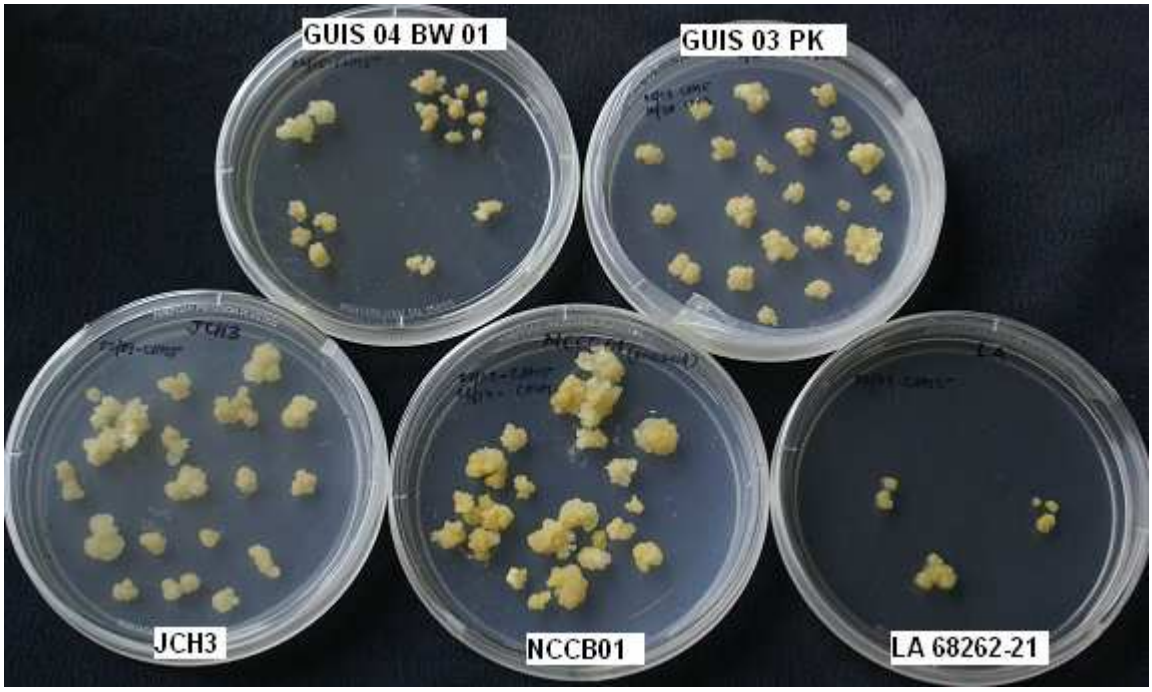

Figure 4.10 Growth of the embryogenic callus generated from a single seed of sea oats in the best medium CIM5.

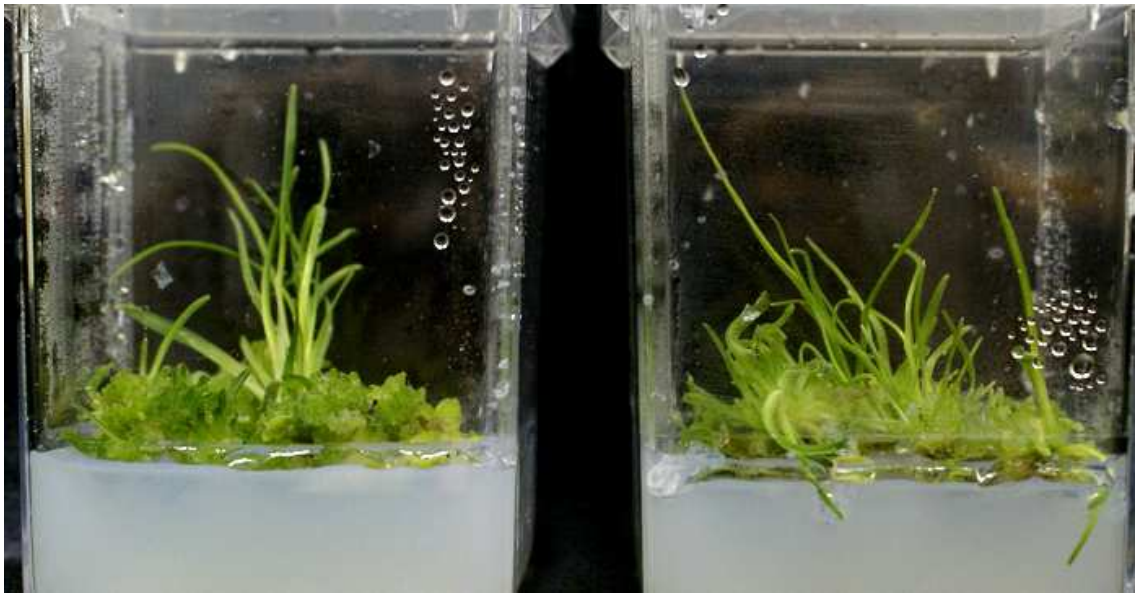

Figure 4.11 Shoot regeneration from embryogenic callus of sea oats in two regeneration media.

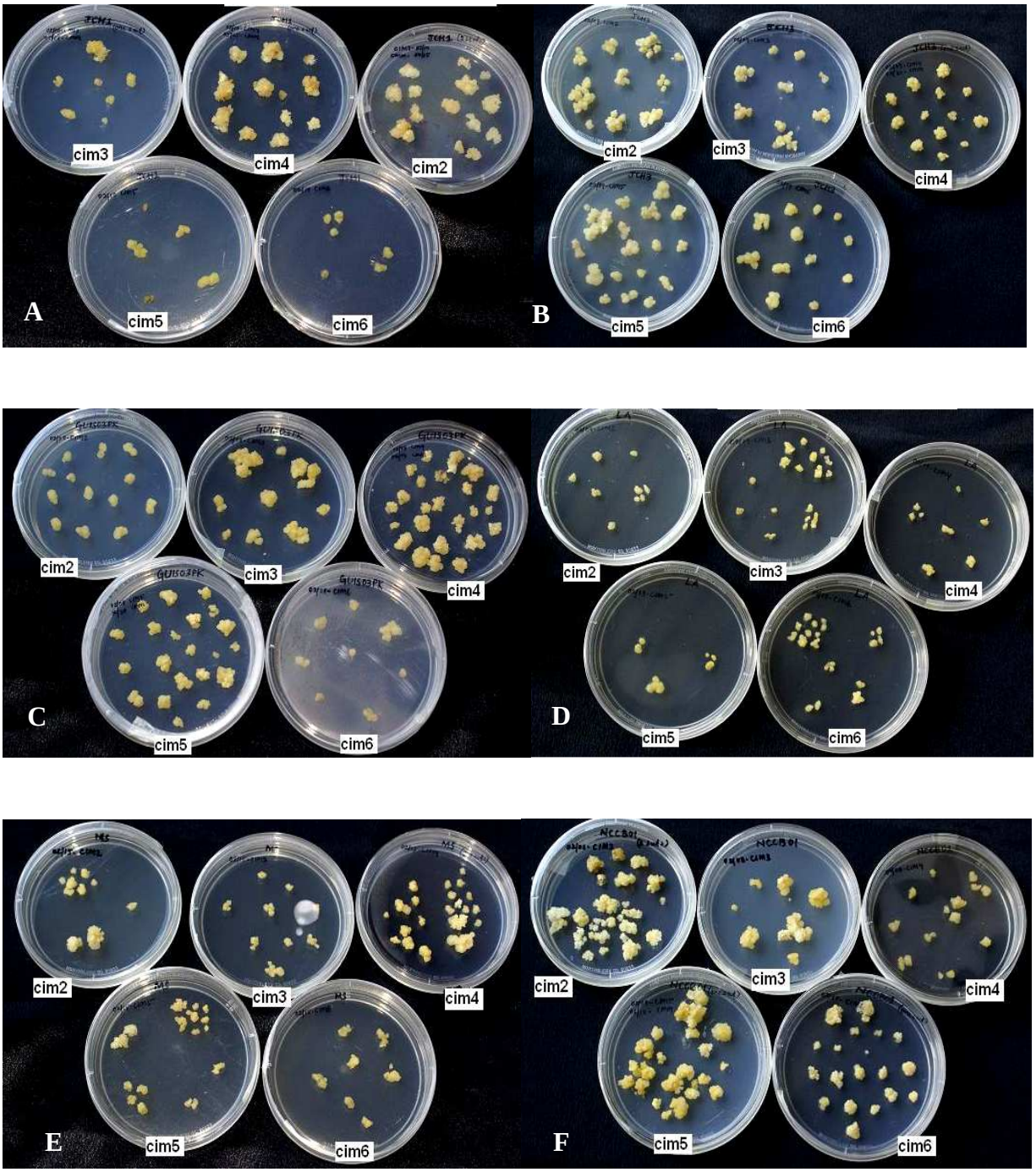

Figure 4.12 Growth of callus from six different accessions of sea oats in different media. Each plate contains embryogenic callus developed from a single seed.
A- JCH1, B- JCH3, C- GUIS 03 PK, D- LA 68262-21, E- GUIS 04 BW01 and F- NCCB 01.

4.2.4 Regeneration of Callus with Somatic Embryos

The MS medium-plant growth regulator combinations designated as SCR1 (5.38 µM NAA + 9.28 µM Kn) and SCR2 (2.69 µM NAA + 6.66 µM BAP + 2.32 µM Kn) were tested for regeneration of the somatic embryos. After placing the calli with clusters of preglobular proembryos in callus multiplication media with reduced 2, 4-D for one month sufficient amount of calli with mature somatic embryos were developed and subsequently transferred to regeneration media. The regenerability of the somatic embryos was clearly visible as green tiny shoot primordia were observed within 3-4 days of transfer and after a week the green shoots emerged out of the callus. Albino plants were observed only in Florida (10 % of plants) and Texas (8 % of plants) accessions.

It was observed that regeneration of callus was faster in SCR1 medium in comparison with SCR2 media (data not shown). However, the number of shoots regenerated in SCR2 medium was higher than the SCR1 medium (Table 4.8; Figure 4.11). It could be due to presence of BAP in SCR2 medium which is necessary for shoot induction as well as lateral shoot multiplication (Grando et al., 2002; Cui et al., 2002; Sarma and Rogers, 2004).

Application of specific hormones at a desired level could enhance the callus regeneration (Valero-Aracama et al., 2002). Multiple shooting was observed in presence of BAP, NAA and Kn in the cotyledonary nodal culture study. Some of the somatic embryos developed into shoot and root, while most developed into shoots only (Figure 4.11), an observation similar to Shoyama et al. (1997). This suggested the heterogeneity (asynchronous nature) of the somatic embryos with regard to their development and maturity (Toonen et al., 1994).

Table 4.8 Comparison of shoot regeneration of embryogenic calli of different sea oats accessions in two different regeneration media.

Accession\Media	SCR1			SCR2		
	# calli	# plants regenerated	#plants/callus	# calli	# plants regenerated	#plants/callus
JCH1	21	90	4.3	16	96	6.0
JCH3	20	76	3.8	13	67	5.2
NCCB 01	15	48	3.2	30	182	6.1
GUIS 04 BW 01	34	124	3.7	10	59	5.9
LA 68262-21	8	17	2.1	4	13	3.1
GUIS 03 PK	23	157	6.8	13	113	8.7

Table 4.9 Effect of callus induction medium on callus regeneration.

Callus induced on medium	# calli placed for regeneration	# calli regenerated (SCR1 and SCR2 combined)	Response (%)
CIM2	23	8	35
CIM3	50	40	80
CIM4	50	36	72
CIM5	50	37	74
CIM6	34	25	72

4.2.5 Comparison of Callus Regeneration in Different Accessions

The influence of accessions was obvious in this study when regeneration and number of shoots from clump of somatic embryos in different accessions were compared. Shoot regeneration was faster in the accession GUIS 03 PK compared to other accessions. Number of shoots per cluster of somatic embryos was also more (8.7/callus) in this accession (Table 4.8). NCCB 01 accession showed the best response in callus induction and callus regeneration. On an average six shoots were obtained per callus (Table 4.8). Calli from JCH1, JCH3 and GUIS 04

BW 01 accessions developed 5-6 shoots/callus in SCR2 medium. There was poor response to callus induction, maturation of somatic embryos and callus regeneration in case of the Louisiana accession LA 68262-21. In all the accessions, presence of BAP produced multiple shoots. Altogether, 1042 plants were regenerated from somatic embryos of six accessions (14 seeds) in two regeneration media. Valero-Aracama et al. (2002) studied two sea oats genotypes and reported that presence of BAP was beneficial for regeneration. As expected, the shoot-forming efficiency varied with accession. Such genotypic differences for response to shoot regeneration were well documented in *Poa pratensis* (Hu et al., 2006) and *Cenchrus ciliaris* (Colomba et al., 2006).

4.2.6 Effect of Callus Induction Media on Callus Regeneration

In this study, several concentrations of 2, 4-D alone and in combination with 2.32 µM Kn were tried for callus induction. It was mentioned earlier that the best response for callus induction was achieved in CIM5 medium followed by CIM3, CIM2, and CIM4 and the least response was in CIM6 independent of accessions (Figure 4.9). A study was conducted to compare the effect of callus induction medium on callus regeneration. Calli induced in CIM3 medium revealed 80 % regeneration upon transferring to callus regeneration medium (SCR1 and SCR2). Though CIM5 medium responded highest to callus induction, the regeneration percentage was 74 (Table 4.9). Positive effect of growth regulator levels in the callus induction medium on shoot development was observed in several grass species (Li et al., 2006). This result is consistent with earlier report (Grando et al., 2002; Li et al., 2006; Gupta et al., 2006) where high concentration of 2, 4-D present in callus induction reduced plant regeneration. Also CIM6 medium which responded least to callus induction, turned out to be better for shoot regeneration (72 %). This could be due to presence of Kn whose inclusion in callus induction medium

enhanced the shoot induction, similar to the study in sorghum (Gupta et al., 2006). Several other reports are also available where addition of low concentration of cytokinin in callus culture has been shown to enhance callus regeneration (Choudhury and Qu, 2000; Altpeter and Posselt, 2000).

4.2.7 Rooting of Somatic Embryo-derived Plantlets

Plants with 10-15 cm long leaves from different callus regeneration media were transferred to only one rooting medium RM2 (MS + 5.38 µM NAA + 0.46 µM Kn) because the shoots multiplied from cotyledonary nodes developed roots only in this medium. Altogether 382 somatic embryo-derived plants from five accessions were placed for rooting of which 336 responded to rooting. It took 16 to 20 days to develop the root primordia. However, the growth of the roots was faster afterwards. In another 9-10 days (i.e., 25 to 30 days after placing in root induction medium), complete plantlets with 5-6 cm long roots were developed (Figure 4.13). The plants developed more profuse rooting (25-30 roots per plant) in comparison with the ones developed through cotyledonary nodal explants (13-17 per plant). Furthermore, somatic embryo-regenerated plants responded to rooting earlier than the plants multiplied from cotyledonary nodes. A possible explanation for this is that the plants developed through somatic embryogenesis had residual auxin (NAA) retained as they were regenerated in NAA containing media, whereas the cotyledonary node-derived shoots were propagated only in BAP containing media.

4.3 Greenhouse Establishment of *in vitro* Propagated Sea Oats Plants

The plants regenerated from shoot multiplication and somatic embryogenesis experiments were initially transferred inside a mist chamber as described earlier in the materials and methods section (Figure 4.14). Damping off due to root rot fungus was a problem that

caused mortality of the seedlings after 4-5 days of establishment in mist chamber. This fungus was controlled by a fungicide 'Banrot' (Scotts-Sierra Crop Protection Company, Marysville, OH), which was sprayed at a rate of 400 mg/l at seven days interval. The survival rate was 80 % in callus regenerated plants and 90 % in plants multiplied from cotyledonary shoots. In sea oats, ex vitro establishment has been reported to be difficult and low in plants multiplied from BAP containing media (Valero-Aracama et al., 2006). This problem was successfully circumvented in this study with the use of a mist chamber in the early stages of their establishment inside the greenhouse.

4.4 Genetic Uniformity of Micropropagated Sea Oats

In general, plants regenerated through cotyledonary nodal culture from a single seed and somatic embryogenesis from calli of a single seed were morphologically the same. Molecular tools are more reliable than phenotypic observation for studying the genetic variation (Leroy et al., 2000). To confirm whether or not the sea oats plantlets regenerated through somatic embryogenesis and cotyledonary node culture were genetically similar, random amplified polymorphic DNA (RAPD) marker was used in this study. Plants generated from each of five accessions (one seed per accession independent of medium) through somatic embryogenesis and cotyledonary nodal culture from a single seed were tested for genetic uniformity. Of the 66 RAPD primers initially screened, five primers, OPA 7, OPB 6, OPB 12, OPB 15, and OPX 4 (Appendix 4.1) were selected on the basis of robustness of amplification, reproducibility and scorability of bands. Primers OPA 7 and OPX 4 were polymorphic in the preliminary screening experiment between two natural (not generated through tissue culture) sea oats genotypes. Five RAPD primers used in this analysis amplified a total of 25 bands (loci). RAPD markers have been used as tools for genetic identification of micropropagated plants (Rani et al., 1995).

Figure 4.13 Rooting of somatic embryo- generated plants of sea oats in RM2 medium.

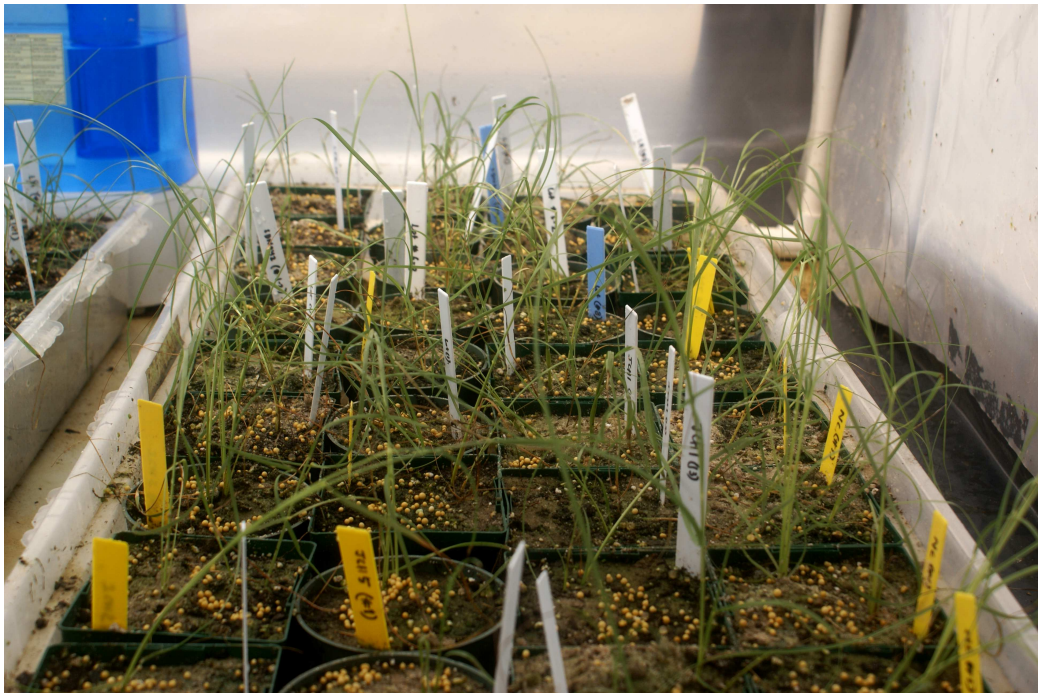

Figure 4.14 Regenerated plants established inside a mist chamber in the greenhouse.

4.4.1 RAPD Analysis of Cotyledonary Node-derived Plants

Twelve regenerants from each of the two accessions (NCCB 01 and LA 68262-21) were used for RAPD analysis. The two decamer RAPD primers (OPB 12 and OPB 15) generated 10 fragments ranging from 396 bp to 2036 bp in size. The number of bands for each primer varied from four (OPB 15) to six (OPB 12). In all the regenerants from both the accessions no polymorphic loci was observed (Figure 4.15), which suggested genetic homogeneity among the plants regenerated from a single seed. Genetic stability among tissue culture generated plants has been described in several earlier reports, namely, *Zingiber officinale* through meristem culture (Rout et al.,1998), micropropagated plants from hardwood cuttings of *Actinidia deliciosa* (Prado et al.,2005), clones regenerated from *Prunus dulcis* shoots (Martins et al., 2004). Rani et al. (1995) performed genetic analysis of micropropagated plants of *Populus deltoides* where 11 primers produced monomorphic bands across all the regenerants. It suggests that plants regenerated through cotyledonary nodal culture *in vitro* could be successfully used for clonal propagation with very little risk of somaclonal variation and RAPD could be a good molecular marker system to study the genetic similarity or variation among the tissue culture derived plants.

4.4.2 RAPD Analysis of Somatic Embryo-derived Plants

Eighteen somatic embryo-derived plants from each of the four accessions (except LA 68262-21, which did not have sufficient regenerants) were analyzed by RAPD: Accession NCCB 01 and GUIS WS 01 with five primers and Accession JCH1 and GUIS 03 PK with two primers OPB12 and OPB 15). The five primers produced 25 monomorphic loci among all the plants in NCCB 01 accession (Figure 4.16). Primer OPA 7 produced a maximum of seven and primer OPB 15 produced a minimum of four monomorphic bands.

The plantlets from JCH1 accession were tested for genetic uniformity with OPB 12 and OPB 15 (Figure 4.17). Twelve loci were amplified, which were all monomorphic among all eighteen regenerants analyzed. The number of bands varied from four in OPB 15 to six in OPB 12. Similarly RAPD analyses of GUIS 04 BW01 and GUIS 03 PK resulted in monomorphic bands among the micropropagated plants (Figure 4.18 and 4.19). Primer OPA 7 which was polymorphic among two non-tissue cultured plants was also tested in the studied accessions and as was expected no variation was found among the regenerants (Figures 4.16, 4.18). These results corroborated with earlier reports of genetic homogeneity among somatic embryo-derived plants (Shoyama et al., 1997; Jayanthi and Mandal, 2001). Jayanthi and Mandal (2001) compared the banding pattern in 14 regenerated plants and the mother plants with 18 OPB RAPD primers. All 18 primers produced monomorphic bands confirming the genetic homogeneity among the regenerants. In *Panax notoginseng* (Shoyama et al., 1997), monomorphic banding pattern was observed in all the regenerants using 21 random primers. The present study suggested that somatic embryogenesis could also be used as an alternative for clonal propagation of sea oats on a commercial basis.

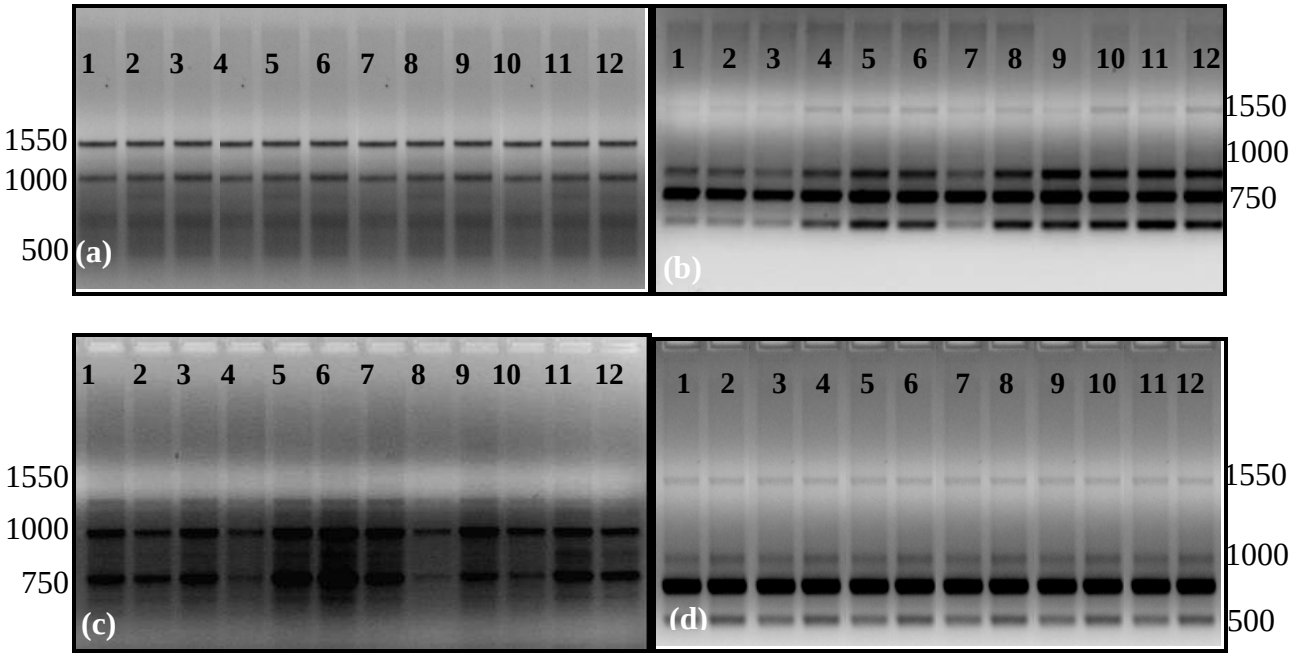

Figure 4.15 Gel electrophoresis showing amplification of micropropagated sea oats plants obtained from cotyledonary nodal cultures of NCCB 01 (a and b) and LA 68262-21 (c and d), using RAPD primers OPB-12 (a and c) and OPB-15 (b and d). The size of the fragments in bp is indicated on the left and right.

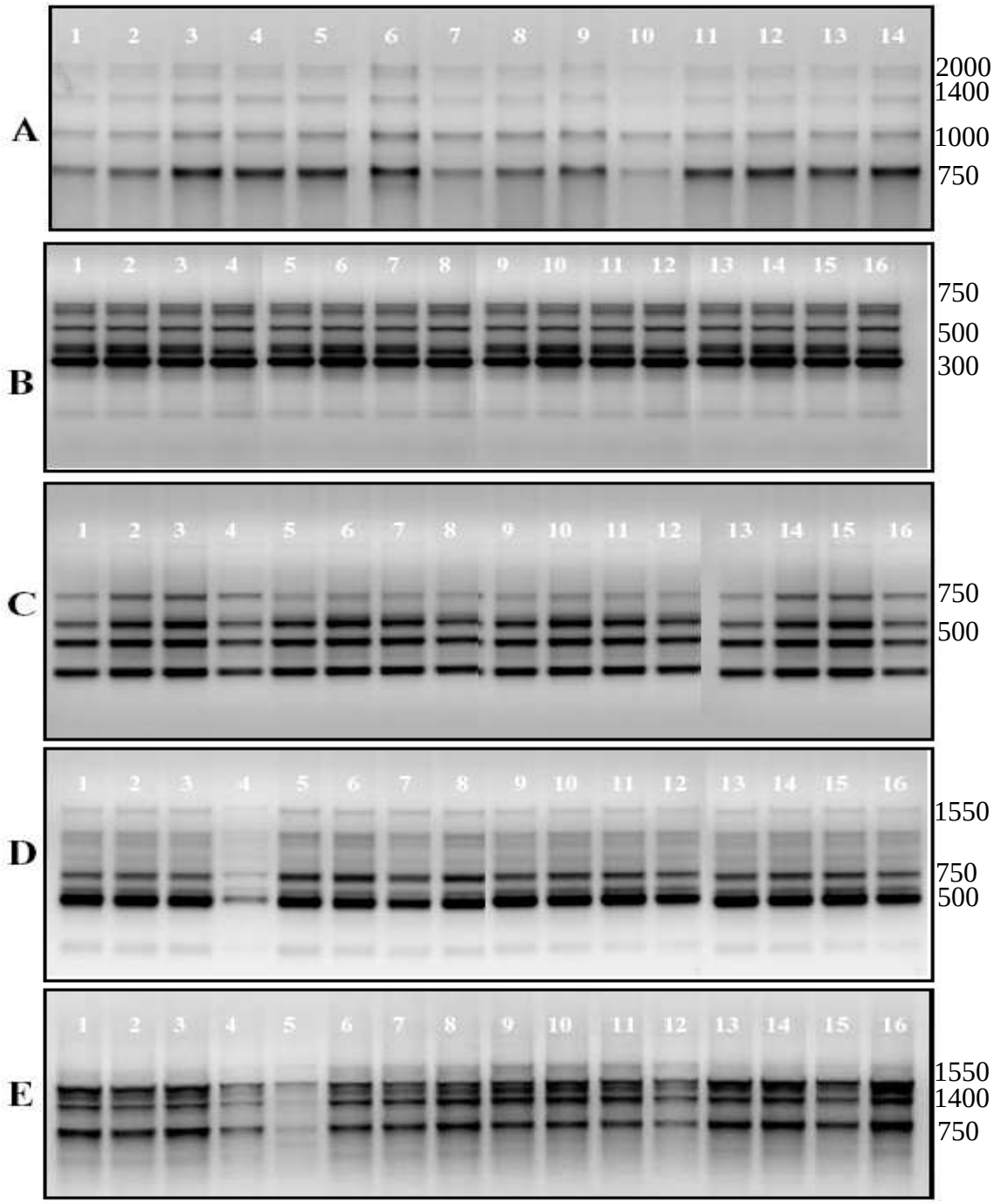

Figure 4.16 Gel electrophoresis showing amplification of micropropagated sea oats plants obtained from somatic embryogenesis of NCCB 01 with primer OPB 6 (A), OPB 12 (B), OPB 15 (C), OPA 7(D) and OPX 4 (E). Lanes lacking distinct bands were deleted. The size of the fragments in bp is indicated on the right.

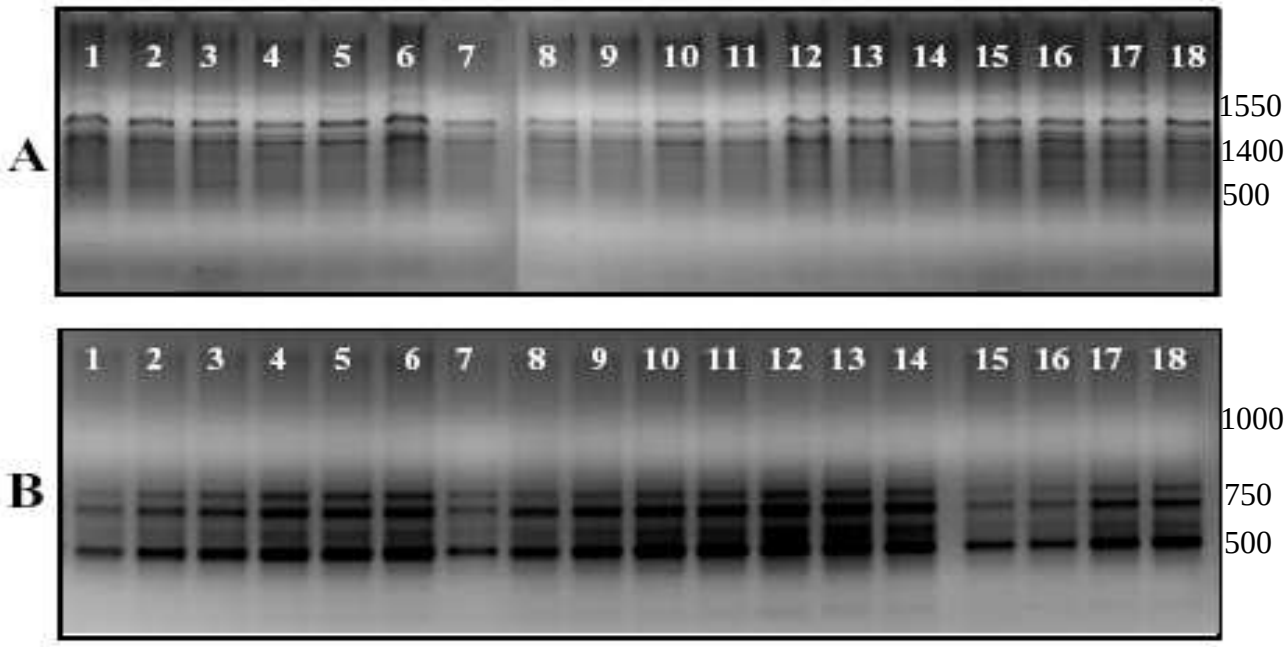

Figure 4.17 Gel electrophoresis showing amplification of micropropagated sea oats plants obtained through somatic embryogenesis of accession JCH1 with primer OPB 12 (A), OPB 15 (B). The size of the fragments in bp is indicated on the right.

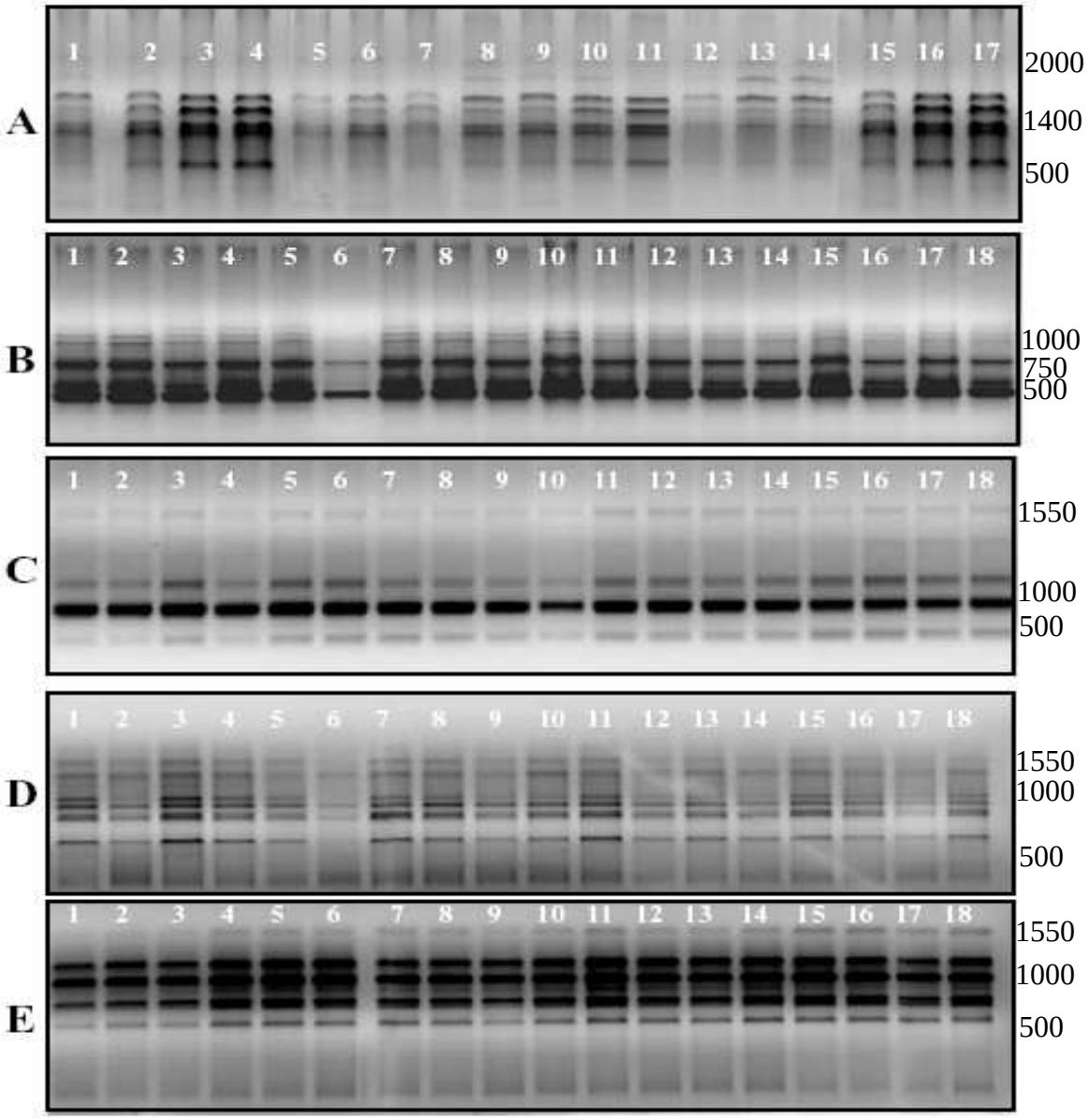

Figure 4.18 Gel electrophoresis showing amplification of micropropagated sea oats plants obtained from somatic embryogenesis of accession GUIS 04 BW01 with primer OPB 6 (A), OPB 12 (B), OPB 15 (C), OPA 7(D), and OPX 4(E). The size of the fragments in bp is indicated on the right.

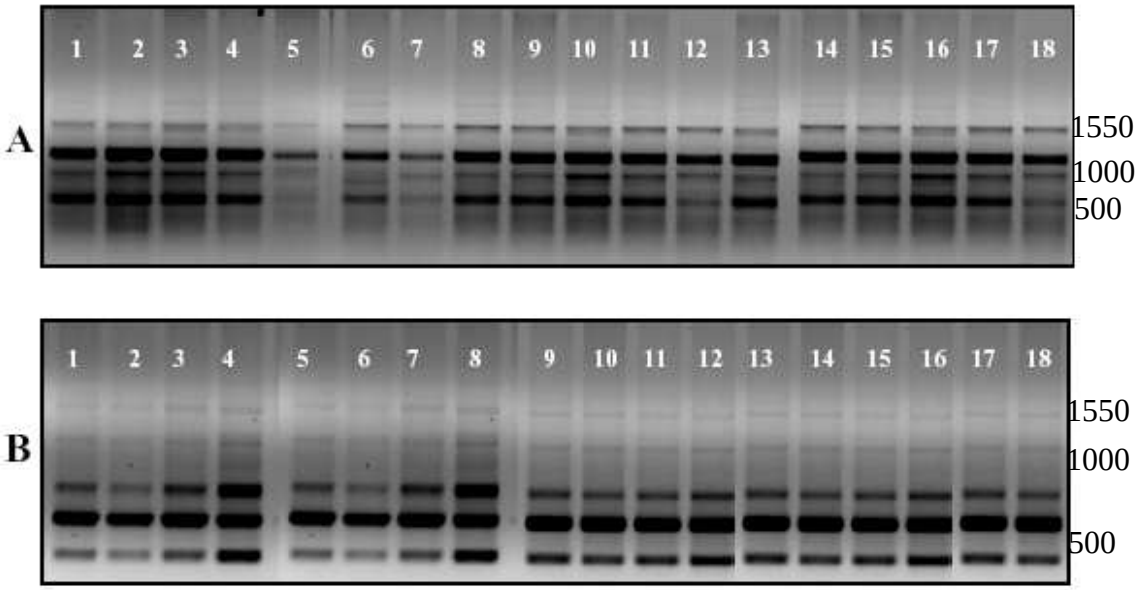

Figure 4.19 Gel electrophoresis showing amplification of micropropagated sea oats plants obtained from somatic embryogenesis of pattern of accession GUIS 03 PK OPB 12 (A), OPB 15 (B). The size of the fragments in bp is indicated on the right.

CHAPTER 5

SUMMARY AND CONCLUSION

Preservation and conservation of dune system is the call of the day for animal and plant habitat protection. Dune stabilization through sea oats planting needs a year round supply of clonally propagated plants. Production of sea oats via tissue culture is an alternative to conventional propagation (seed germination and vegetative propagation). Using tissue culture techniques, it is possible to produce large number of plants throughout the year under controlled growth conditions in a small space. In natural condition sea oats propagation is very slow, which leads to coastal erosion. In this pursuit, an efficient micropropagation protocol was developed for rapid and mass scale production of sea oats plants. This protocol can be successfully used in large scale commercial multiplication of elite sea oats clones.

By using the protocol developed through this effort, it is possible produce 130 to 150 plants from a single seed through somatic embryogenesis within 6 months of culture period. Higher number of plantlets could be produced if the mass of callus is multiplied for more time. It is estimated that the number of plants could be more than double if the primary somatic embryos are further multiplied for an additional month to get the viable secondary somatic embryos. In this effort, five accessions were tested and NCCB 01 accession was the best in terms of callus induction (45 %) with 6 shoots/callus in the regeneration medium. MS medium containing 22.63 µM 2, 4-D was found to be the best one for callus induction in all the accessions. More somatic embryos were obtained when the embryogenic calli were maintained in MS medium containing lower concentration (9.05 µM) of 2, 4-D. For callus regeneration, optimum result was obtained with MS medium containing growth regulators like BAP (6.66 µM), NAA (2.69 µM), Kn (2.32 µM) and 3 % (w/v) maltose. In this medium, the shoot multiplication was more in comparison to

medium lacking BAP. GUIS 03 PK accession produced the maximum number of shoots/callus (9) and a range of 3-6 shoots/callus was regenerated in other accessions. All the regenerated plantlets were rooted in MS medium containing NAA (5.38 µM) and Kn (0.46 µM). The survival rate was 80 % during the greenhouse establishment.

All the plants produced from a single seed were genetically identical. All the regenerated plants tested with the five RAPD primers produced monomorphic bands confirming that the somatic embryos, as expected, were genetically homogeneous in the tissue culture process. Primers OPX 4 and OPA 7 were polymorphic in naturally propagated sea oats plants but produced no polymorphic banding pattern when tested on somatic embryos-derived plants. This ruled out the general apprehension of somaclonal variation as a result of tissue culture-induced instability arising from normal pathway of organogenesis and/or caulogenesis.

There is no previous detail report on *in vitro* studies in sea oats except a short report on sea oats plant regeneration through callus culture (Hovanesian et. al., 1988). The present effort was successful in developing a rapid and reproducible *in vitro* micropropagation system from cotyledonary nodal explants through high frequency axillary bud differentiation and shoot proliferation, followed by greenhouse establishment. The medium-plant growth regulator recipe developed could produce 13-15 plants easily from one seed within 5-6 months of culture period. Again, this number can be raised higher (up to 50) if the time in culture is increased by another 2 months to allow one more cycle of splitting for shoot multiplication. Further, the plants derived from the shoot multiplication experiment of the two accessions did not show any genetic variation when tested with the RAPD markers. Study of two accessions from Louisiana and North Carolina revealed high shoot proliferation in MS medium containing BAP (4.44 µM and 6.66 µM). MS medium containing 6.66 µM BAP found out to be the best for both the accessions

in cotyledonary nodal culture. The plants regenerated through this medium also responded favorably (95 % - NCCB 01 and 75 % - LA 68262-21) to rooting and subsequent *ex vitro* establishment. North Carolina accession produced 241 plantlets from 10 hypocotyls, whereas Louisiana accession produced 238 plantlets. The survival rate in the greenhouse was 90 %. The protocol described here is simple in comparison with many earlier reports where the media have been manipulated with addition of special substances like activated charcoal, coconut milk, thidiazuron, silver nitrate etc. in addition to phytohormones.

In conclusion, the protocol developed through this present investigation will be useful for large-scale multiplication of sea oats and possibly other related dune species used for wetland restoration. This work could also be helpful to researchers attempting to use biotechnological approaches to improve this grass.

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APPENDIX: LIST OF CHEMICAL INGREDIENTS NEEDED FOR TISSUE CULTURE AND PCR, LIST OF RAPD PRIMER SEQUENCES

Appendix-3.1

STOCK SOLUTION PREPARATION MURASHINGE AND SKOOG MEDIA (MS MEDIA)				
MEDIA	**COMPONENT**	**STOCK SOLUTION (g/l)**	**AMOUNT (ml/l)**	**FINAL CONCENTRATION (mg/)**
MS I (50X)	NH_4NO_3	82.5	20	1650.0
	KNO_3	95.0		1900.0
MS II (100X)	$MgSO_4$ $7H_2O$	37.0	10	370.0
	$MnSO_4$ $4H_2O$	3.23		22.3
	$ZnSO_4$	0.86		8.6
	$CuSO_4$ $5H_2O$	0.0025		0.025
MS III (100X)	$CaCl_2$ H_2O	44.0	10	440.0
	KI	0.083		0.83
	$CoCl_2$ $6H_2O$	0.0025		0.025
MS IV (100X)	KH_2PO_4	17.0	10	170.0
	H_3BO_3	0.62		6.2
	Na_2MoO_4 $2H_2O$	0.025		0.25
MS V (100X)	$FeSO_4.7H_2O$	2.785	10	27.85
	Na_2EDTA	3.725		37.25
MS VI VITAMINS	Nicotinic Acid	0.25	2	0.5
	Pyridoxine HCL	0.25		0.5
	Thymine HCL	0.5		1.0
	Glycine	1.0		2.0
	Myoinositol	50		100

Appendix 3.2 PCR mix preparation for 1 reaction

Ingredients	**Amount (µl) for 1x reaction**
10x PCR buffer	2.50
25 mM $MgCl_2$	3.00
2 mM dNTP mix	2.00
Primer	1.25
Taq polymerase	0.20
Sterile water	14.05
Genomic DNA	2.00
Total volume	25.00

Appendix-4.1 Sequences of RAPD markers

RAPD Primer	Sequence
OPB 6	5′-TGCTCTGCCC-3′
OPB 12	5′-CCTTGACGCA-3′
OPB 15	5′-GAAGGGTGTT-3′
OPA 7	5′-GAAACGGGTG-3′
OPX 4	5′-CCGCTACCGA-3′

VITA

Diptimayee Sahoo was born in Bhubaneswar, India. She finished her bachelor of science in agriculture in Orissa University of Agriculture and Technology at Orissa on November 2004. She worked as a project officer at National Bank for Agriculture and Rural Development before joining LSU in fall 2005. She successfully completed the projects on identification of appropriate farming system and credit demand in a drought affected area in India.

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