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MICROPROPAGATION OF WIREGRASS AND CREEPING BLUESTEM, AND PROPAGATION OF GOPHER APPLE

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MICROPROPAGATION OF WIREGRASS AND CREEPING BLUESTEM, AND PROPAGATION OF GOPHER APPLE

FINAL REPORT

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PERSPECTIVE

There is a shortage of seeds and planting stock of native Florida grasses and other herbaceous plants for use in restoration or reclamation of native upland habitats on mined lands or other lands. Seeds of some species, such as wiregrass, can be collected from native habitats if prior arrangements are made to manage the land to promote seed production and if permission can be obtained to collect the seed. Even if permission is obtained and proper management is applied, unfavorable weather, insects, or disease, etc., can result in a poor harvest. Some important native grass species, such as creeping bluestem, produce very little seed under the best of conditions.

An alternative method of mass plant propagation is through cell and tissue culture. The general techniques for tissue culture have been developed for many species, but the specific protocols for native Florida grasses have not. The usual product of tissue culture and plant regeneration is a potted plant that can be planted in the field. A new method that has only been tried for a few species is the production of synthetic seeds. In this method, a tiny plant or embryo regenerated from tissue or cell culture is encapsulated in a gel containing the proper nutrients and hormones.

The goals of the project were to:

- Develop protocols for micropropagation (tissue culture) of two native grasses, wiregrass *(Aristida berychiana)* and creeping bluestem *(Schizachyrium scoparium* var. *stoloniferum*), through shoot multiplication.
- Test the establishment and growth of these micropropagated plants in the greenhouse and in the field compared to conventionally propagated plants.
- Examine the feasibility of synthetic seed (gel-encapsulated somatic embryo) production and greenhouse and field establishment of these grasses from synthetic seeds.
- Examine the propagation of gopher apple (*Licania michauxii*) by stem cuttings as well as micropropagation.

The reader is also referred to:

USDA Natural Resources Conservation Service. *Development of Seed Sources and Establishment Methods for Native Upland Reclamation*. FIPR Publication 03-120-189. The purpose of this research was to develop quality seed sources of Florida native upland plant species and to develop methodology for seed production and for field establishment of plants from seeds.

Steven G. Richardson
FIPR Director of Reclamation Research

ABSTRACT

Insufficient quantities of high quality plant material have limited the use of native species in phosphate mine reclamation. Micropropagation was evaluated as a means of producing two keystone native grasses – *Aristida beyrichiana* (wiregrass) and *Schizachyrium scoparium* var. *stoloniferum* (creeping bluestem). Preliminary work was also conducted on *Licania michauxii* (gopher apple), a species of extremely limited supply but which seems to be a prime candidate species for use in mine reclamation. Shoot multiplication cultures of creeping bluestem were established from somatic embryos. Acclimatized tubelings exhibited good to excellent survival on sand tailings and overburden. Somatic embryos were also encapsulated in an alginate gel to form synthetic seed. While the number of synthetic seed produced was limited, it was clear that best germination under greenhouse conditions was when growth regulators were incorporated in the gel. Establishment and maintenance of embryogenic callus of wiregrass was very difficult and hence results were limited. However, wiregrass plants were regenerated from embryogenic callus and grown to maturity. For gopher apple, methods were developed for establishing clean in vitro cultures. Ninety to 100 percent of stem tip cuttings rooted when cuttings were taken in April or May.

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EXECUTIVE SUMMARY

Creeping bluestem (*Schizachyrium scoparium* var. *stoloniferum*) and wiregrass (*Aristida beyrichiana*) are two keystone native grasses in areas of phosphate mining and hence highly desired for use in reclamation. Direct seeding of these species is potentially the most cost-effective means for revegetating mined lands. However, they are unreliable, and frequently poor, producers of viable seed. For those that want to direct seed wiregrass, they must harvest a "seed hay" from nearby land and spread the "seed hay" within a day or so of harvest. Otherwise, those wanting to use wiregrass must rely on a limited supply of tubelings. Those who currently use creeping bluestem in reclamation typically must harvest propagules from natural stands that occur near the reclamation site. At the time this study was initiated, the best opportunity for a consistent and widespread supply was for creeping bluestem and wiregrass propagated by tissue culture. Tissue culture techniques have the advantage of offering a rapid method of generating large quantities of propagules. Prior to this study, little work had been conducted involving micropropagation of these species.

Micropropagation, i.e., propagating plants via tissue culture, was evaluated as means of mass-producing creeping bluestem and wiregrass. The overall approach was to use somatic embryogenesis because plants regenerated by this method will probably look the same and be genetically identical to the mother plants. It is the most common method of regenerating grasses via tissue culture (Vasil IK. 1987. Developing cell and tissue culture systems for the improvement of cereal and grass crops. J. Plant Physiol. 128:193-218.). In this technique, a special type of callus tissue (which is basically an undifferentiated mass of cells) called embryogenic callus, is induced to form embryos. These embryos are identical to embryos in seeds that are normally generated by fertilization of eggs by the male gametes from pollen – zygotic seed. (Note: Seed in plants can also be formed without fertilization through a process called parthenocarpy.) Somatic embryos can be encapsulated to produce synthetic seed, plants can be directly regenerated from the embryos, or embryo cultures can be used to develop shoot multiplication cultures.

Our initial work focused on production of synthetic seed because if seed could be economically produced in large quantities then seeding of mined land would be possible. The base tissue culture media used in this study was the Murashige and Skoog basal medium (MS) supplemented with coconut milk from immature or mature coconuts. Supplementing the medium with 2,4-dichlorophenoxyacteic acid at 2-3 mg/L resulted in embryogenic callus formation from immature inflorescences and leaf tissue (creeping bluestem only). Embryogenic callus growing on solid media was also used to initiate liquid suspension cultures in which finely divided embryogenic callus was induced to form pre-embryos (also called embryoids). Pre-embryos were plated out onto solid media designed to allow development of the pre-embryos into embryos. Mature embryos on solid media were then mixed in alginate (a seaweed derivative) gel. Embryos in the gel were then dropped into a calcium chloride solution (one to three embryos in a

droplet). The droplet containing the embryo became hardened after several minutes in the calcium chloride solution thereby forming a synthetic seed.

Germination of creeping bluestem synthetic seed germination in vermiculite was good to excellent in vitro and fair under greenhouse conditions at the NFREC. Best germination was for seed that contained growth regulators in the gel. Preliminary results of greenhouse testing of creeping bluestem synthetic seed germination in Myakka and mine soil were inconclusive. Thirty-six wiregrass synthetic seed were produced but only three germinated in vitro. Contamination problems and difficulty in reestablishing suspension cultures (a critical step in synthetic seed production) limited our work with synthetic seed.

Moreover, the original embryogenic callus of wiregrass was lost due to contamination. After extensive evaluation of cultural methods to obtain clean cultures, the best method to obtain clean callus cultures was to germinate wiregrass seed in vitro and then use these seedlings to initiate callus. Numerous attempts to induce this callus to become embryogenic were not successful. Efforts to directly regenerate wiregrass in vitro without involving somatic embryogenesis were not successful either. Repeated attempts to generate new embryogenic callus from immature inflorescences as well as other types of tissue, were not successful. However, shoots were regenerated from the embryos growing on solid media and eventually rooted. Plants regenerated from somatic embryos developed normally after being transplanting to containers.

Because of the critical need to develop an efficient method of micropropagating creeping bluestem, it was decided to focus our efforts on shoot multiplication culture. Clumps of embryos from solid media were used to generate shoot multiplication cultures. Starting with a shoot cluster of 3-4 shoots, it took 32 weeks to establish shoot multiplication cultures for production (15-16 shoots per cluster per jar). Once established, number of shoots nearly doubled every 8 weeks. At this rate, about 1700 plants (per initial 3-4 shoot cluster) could be produced in about 1 year once shoot multiplication cultures are established at 32 weeks.

Rooting these shoots in vitro and then acclimatizing them in the greenhouse resulted in a greater percentage of surviving tubelings than when shoots were both rooted and acclimatized under greenhouse conditions. In greenhouse tests, tissue-cultured plants grown in pots on overburden and sand tailings were similar to plants by propagated by division with respect to shoot, root and rhizome mass, but tiller number per plant was greater on tissue-cultured plants compared with division plants.

In field trials on mined land, tissue-cultured plants established well when planted in January, July, or October; however, survival was less on overburden for the October planting, which was due to typical low rainfall at this time of the year. Plant diameter as measured by rhizomes expansion was good and averaged 24 cm over the three planting dates. On the mine site, a large soil seed bank resulted in much weed competition. Hence, good establishment of creeping bluestem on mined land will require a weed

management program that minimizes increases in the weed seed bank and weed competition during establishment.

The other part of our work involved preliminary propagation experiments with gopher apple (*Licania michauxii*). It is a drought-tolerant woody plant native to Florida uplands, is locally abundant, and can function as a groundcover. Gopher apple seems to be consistently demanded for in a variety of restoration and mitigation projects, and it is a prime candidate for use in mine reclamation. At a FIPR RTAC meeting, it was suggested that demand for gopher apple exceeds supply due to difficulty in adequate propagation methods, which is now via seed only. There have been no studies on commercial propagation of gopher apple except for informal work conducted by local nursery personnel. The main objective of our preliminary work was to provide empirical data that could be used in future studies for determining the most effective means of propagating gopher apple.

Gopher apple seed collected from a north Florida population (Leon County) were germinated in vitro (86% germination) primarily for generating plant material that was unlikely to be contaminated, at least externally. Terminal and nodal explants from these in vitro grown seedlings were cultured on MS supplemented with cytokinins to induce shoot formation. Abundant axillary shoot production was not achieved; however, terminal explants cultured on MS + CPPU (0.5 or 1.0 mg/L) developed an average of 2.5 or more shoots per explant compared to only one axillary shoot per explant on MS without any cytokinin. Basal callus formed on 10 and 100% of the terminal explants on MS + CPPU at 0.5 and 1.0 mg/L, respectively. Callus is generally not desired in this type of micropropagation culture designed for axillary shoot production. Seeds that germinated but where contaminated and not used as an explant source were successfully planted in soilless medium and grown in vivo. Explants derived from containerized plants grown outdoors had 100% contamination despite use of a broad-spectrum systemic fungicide about a week prior to harvesting of the explants.

Terminal stem cuttings were harvested in April, May, June, July, October, and November from a north Florida population (Wakulla County). Cuttings (10-13 cm; 3-4 leaves) with two 1-cm scores on opposite sides of the base were dipped in 0.1, 0.3, 0.8, 1.6, 3.0, or 4.5% IBA (Hormex) and then placed in propagation cups containing MetroMix 200. Cuttings were rooted under intermittent mist in a greenhouse. One month after sticking, cuttings were lightly fertilized. Gopher apple cutting harvest date seemed to have the most influence on rooting 2 months after cuttings were stuck. Cuttings taken in May rooted the best based on quantitative and visual assessments; IBA did not improve rooting. Cuttings harvested in April had acceptable rooting and benefited from IBA, but no more than 0.1% was necessary to improve rooting. Rooting ability of stem cuttings steadily declined for cuttings harvested in June, July, October, and November, with no rooting or callus formation on October and November cuttings after 2 months. The lack of any root/callus formation may have been due to inadequate heating of the greenhouse during late fall and winter. The rooting medium temperature was only about 10C.

Based on the results of our work, shoot multiplication culture seems to offer a practical method of producing large numbers of creeping bluestem for use in mine reclamation. For wiregrass, additional work needs to be conducted to determine if micropropagation is commercially practical. Our preliminary work with gopher apple revealed that stem cutting propagation is an alternative to propagating this species by seed, the only method currently used. We also developed a method for establishing clean in vitro cultures, which will facilitate future micropropagation studies of gopher apple.

CHAPTER 1

INTRODUCTION

Jeffrey G. Norcini, Tarak N. Chakravarty, Robert S. Kalmbacher, and James H. Aldrich

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PURPOSE

Mined mesic upland forests are recognized as vital ecosystems that need to be restored. Currently, most phosphate mine reclamation work involves reshaping the landscape and returning overburden, followed by establishment and management of introduced forage crops for pasture. Planting pines on reclaimed land is also practiced, but to a lesser extent. Herbaceous native plants endemic to Florida have been used but primarily on a small experimental basis.

Direct seeding of native herbaceous plants is potentially the most cost-effective means for revegetating mined lands. However, due to insufficient quantities of plant material, cost of seeding or sprigging native plants would be high. Also, use of these plants has been further limited because establishment is frequently poor due to the inferior quality of plant material, and seedlings lack vigor and compete poorly with weeds.

Switchgrass (*Panicum virgatum*) is the only herbaceous species native to Florida for which seed can be purchased in quantities suitable for seeding on phosphate mined land, and that is switchgrass (*Panicum virgatum*). This is because switchgrass is used as a forage crop in many other states. There are sources of seed for forage grass from western states, but they are not adapted to Florida conditions. Many of Florida's native grasses are poor and unreliable seed producers. Moreover, these grasses usually are rhizomatous, thus relying on vegetative reproduction.

If reclamation of phosphate land is to include native grass species, then the few grasses that produce seed will have to be commercially cultivated. The USDA Plant Materials Center (PMC) in Brooksville, Florida has worked on increasing seed of Florida ecotypes of native grasses and will soon be releasing some of these. They have also published a seed production guide (Pfaff and others 2002). Currently, however, there is no commercial native grass seed production industry in Florida. Some native grasses and forbs are being harvested from donor sites as a "seed hay" and being applied to mined land (Bissett 1996). Some Florida farmers are increasing and/or producing for sale seed of Florida ecotypes of native wildflowers but the industry is very small. Hopefully, the imminent availability of seed from Florida's PMC and the PMC seed production guide will facilitate commercial production of native grass seed.

Desirable native grasses that are not reliable seed producers will have to be propagated by division or tissue culture (e.g., shoot multiplication culture). Tissue culture techniques that are developed will have the advantage of offering a rapid method of generating large quantities of propagules.

Gopher apple (*Licania michauxii*) seems to be consistently demanded for in a variety of restoration and mitigation projects, and it is a prime candidate species for use in mine reclamation. At a FIPR RTAC meeting, it was suggested that demand for gopher apple exceeds supply due to difficulty in propagating it by seed, the only method

currently used. This conclusion is supported by two Florida growers – Nancy Bissett of The Natives (who annually produces 10,000 seedlings and sells out) and Terry Godts of Green Isle Gardens. Dr. Andre Clewell also has stated that supply exceeds demand.

The large demand-supply gap is due to problems associated with gopher apple production. It is only produced by seed and there are problems associated with seed propagation:

- Limited seed sources – Seeds are not regularly produced in most populations; good quality populations are not always accessible; future decline in accessible populations due to development; superior genotypes are hard to find
- Seed collection/processing – seed collectors cannot collect a lot of other species concurrently; processing is labor intensive
- Germination – typical rates are 20-30%
- Production – seedlings easily rot in the nursery without proper acclimatization
- Price – 5-cm liners are $1.25 each; up to $8 for a 30.5-cm tall plant in a 3.8-L container

Propagation of gopher apple by traditional asexual methods (stem cuttings or by rhizome root pieces) or via micropropagation has not been investigated based on a search of the literature. These methods could prove to be cost-effective but need to be investigated.

Another issue to consider regarding gopher apple is that its seed is dispersed (at least in part) by gopher tortoise (*Gopherus polyphemus*), a state species of concern (Myers 1990). Gopher tortoise has an impact on other species in its range, principally through sharing its burrow: over 300 species of obligate or facultative vertebrates and arthropods including three scarab beetles (candidates for federal listing), crawfish gopher frog (species of concern in Florida), and eastern indigo snake (federally threatened species) (Myers 1990). McCoy and Mushinsky (1992) list gopher tortoise as having a wide geographic distribution with restricted habitat specificity and at times, a large local population. They also refer to gopher apple as a species with a similar geographic distribution and broad habitat specificity and with locally large populations. There may be a symbiotic or possibly sympathetic relationship between gopher tortoise and gopher apple given the overlapping historical range of both species and since gopher apple is a food for the gopher tortoise.

An additional issue to consider is that gopher apple root extract has been found to be cytotoxic to human hepatoma and colon carcinoma in culture (Bashsa and others 2000).

HISTORICAL PERSPECTIVE

CREEPING BLUESTEM AND WIREGRASS

Prior to the work we conducted from 1997 through the end of the project in 2002, little work had been conducted on creeping bluestem and wiregrass that involved micropropagation. Chun Hua Wan conducted some preliminary unpublished work on in vitro culture of wiregrass (plant material provided by Dr. Andre Clewell). When young leaves were cultured on a Murashige and Skoog (1962) medium, swelling was first seen after 3 to 4 days at the cut edge of the segment, followed by callus growth. White compact callus was seen in 3 weeks. Somatic embryos could be found on the callus one month after culture initiation. His preliminary results were the main impetus for our project with wiregrass and creeping bluestem.

Timothy Croughan successfully produced synthetic seeds of smooth cordgrass (*Spartina alterniflora*), a native grass primarily found in salt marshes along the Gulf Coast (Croughan and Materne 1994). He also studied the use of synthetic seeds in lieu of the costly transplanting of seedlings in tidal marsh reclamation (Croughan and Materne 1994). Regeneration rate of smooth cordgrass is about 20%, although Dr. Croughan felt that this rate could be improved by using embryos that are more vigorous and by increasing the size of the embryo root system (T. Croughan, pers. comm.).

Rob Kalmbacher has been working for many years with native plant communities as a source of forage for cattle. Much of his research has focused on creeping bluestem: effects of defoliation on yield, persistence, and nutritive value; effects of disturbance (such as fire and mechanical brush control); and effects of soil fertility. In order to improve yield and nutritive value of Florida range, an effort was made to develop a creeping bluestem cultivar with high nutritive value which could be seeded on range. About 200 ecotypes were collected throughout Florida and were established in a replicated nursery at Ona, Florida in 1982 and 1983. These ecotypes were evaluated over a 3-year period for seed production and vegetative reproduction potential (Kalmbacher and others 1991) and for in vitro organic matter digestion (IVOMD) (Brown and Kalmbacher 1988). Two cycles of poly-crossing ecotypes with greatest seed production potential and greatest IVOMD were carried out from 1986 to 1990. Improvement in seed production and IVOMD were made, but variable environmental conditions and unknown factors within the plant made commercial seed production highly undependable and unrealistic.

GOPHER APPLE

To the best of our knowledge, no work has been published regarding the propagation of gopher apple. The only work that has been conducted is by a local nursery owner. Gopher apple is propagated by seed but germination rates are only 20 to 30% and seedlings can easily rot during production.

LITERATURE REVIEW

MICROPROPAGATION OF GRASSES

The grass family (Poaceae) includes more than 10,000 species and covers a great percentage of ecosystems. The earliest attempts to initiate and regenerate plants from tissue cultures of grasses relied largely on experience with dicotyledonous species that most often require combinations of auxins and cytokinins for optimal results. This resulted in only transient, and at best sporadic, plant regeneration. Since the early 1980's, recognition of factors such as the use of strong auxins and suitable explants has produced a dramatic improvement in regeneration. Plant regeneration has been reported for a great number of species in this important group of plants (Vasil and Vasil 1994).

SOMATIC EMBRYOGENESIS

Callus culture is the most efficient method to regenerate plants of grasses. Two pathways of plant regeneration have been described: **organogenesis**, which leads to formation of shoot primordia with open vascular systems, and **somatic embryogenesis**, which leads to formation of somatic embryos with closed vascular systems (Morrish and others 1987, I.K. Vasil and Vasil 1986). Since 1980 when somatic embryogenesis was first convincingly demonstrated in the Gramineae (Brettell and others 1980, Dale 1980, Vasil and Vasil 1980), it has been found to be the most common pathway in vitro plant regeneration for this group of plants (Vasil 1987). For example, somatic embryogenesis has been used to regenerate the native grass little bluestem (*Schizachyrium scoparium*) (Songstad and others 1986).

Somatic embryogenesis has a significant advantage over organogenesis in that, unlike adventitious shoot meristems formed in organogenesis, the somatic embryos, like their zygotic counterparts, arise from single cells either directly or after the formation of a mass of proembryogenic cells, as found in studies of dicots (McWilliam and others 1974, Tisserat and others 1979) and monocots (Conger and others 1983, Ho and Vasil 1983, Lu and Vasil 1985, Vasil and Vasil 1982a). Hence **genetic and phenotypic integrity of the parent plant are usually maintained**, as has been shown for napiergrass (*Pennisetum purpureum*) (Shenoy and Vasil 1992), sugarcane (*Saccharum* spp.) (Chowdhury and Vasil 1993), meadow fescue (*Festuca pratensis*) (Valles and others 1993), and lemongrass (*Cymbopogon flexuosus*) (Nayak and others 1996).

The use of plant growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D), and culture of young and immature tissues/organs such as leaves, inflorescences, and embryos readily allowed the induction of embryogenic callus in most grass species (Morrish and others 1987, I.K. Vasil and Vasil 1986). Production of such callus was rare and inefficient from mature tissues (Abe and Futsuhara 1985, Nabors and others 1983). Two types of calli are commonly produced in tissue culture of grasses: embryogenic and nonembryogenic. The embryogenic callus originates from specific foci in the explants.

In immature embryos it is initiated from cells adjoining the procambial strand in the scutellum (Lu and Vasil 1985, Vasil and Vasil 1982a), in young inflorescences from meristematic cells in the floral primordia (Botti and Vasil 1984) or parenchymatous cells near the vascular tissue, and in the basal portion of young leaves from cells of the lower epidermis and mesophyll near vascular bundles (Conger and others 1983, Haydu and Vasil 1981, Ho and Vasil 1983, Lu and Vasil 1981a). It is not known how and why these cells selectively express somatic embryogenesis.

The two types of calli are morphologically distinguishable. The nonembryogenic callus is soft, unorganized, and contains sparsely cytoplasmic, vacuolated, and large cells devoid of any prominent metabolic reserves. The embryogenic callus is most often compact, organized, and white to pale white in color, and contains large numbers of small, richly cytoplasmic, starch-containing meristematic cells (Botti and Vasil 1983, Ho and Vasil 1983, Karlsson and Vasil 1986, Lu and Vasil 1985, Vasil and Vasil 1981a, b). It is not uncommon to see isolated sectors of embryogenic callus arising in the masses of nonembryogenic callus. These sectors of embryogenic callus possibly originate from a few original groups of embryogenic-competent cells scattered amongst nonembryogenic cells (Vasil 1987), rather than from the conversion of nonembryogenic cells (Nabors and others 1983). Indeed, conversion of nonembryogenic cells into embryogenic cells has never been convincingly demonstrated. Embryogenic callus proliferates more slowly, especially during initial phases of growth, than nonembryogenic callus, requiring selective transfer of embryogenic callus at each subculture in order to maintain its embryogenic nature. A friable and fast growing embryogenic callus type is produced either directly from explants or after selection from white compact embryogenic callus in some species including maize (Green 1982), oat (Bregitzer and others 1989), sorghum (Wei and Xu 1990), wheat (Redway and others 1990), and teosinte (*Zea diploperennis*) (Pedrosa 1993).

Factors Controlling Induction of Somatic Embryogenesis

Growth and development of embryogenic cells is greatly influenced by the nature and amount of plant growth regulators, particularly 2,4-D, in the nutrient medium (Vasil 1987). At low levels of 2,4-D (1-2 mg/L), the meristematic activity of competent cells is maintained and stimulated, resulting in the formation of embryogenic callus. Increased concentrations (e.g., 5 mg/L or higher) are frequently toxic (V. Vasil and Vasil 1986). Reducing the 2,4-D level in the medium to about 0.1 mg/L allows formation of many somatic embryos, but also causes irreversible conversion of some embryogenic cells to enlarged and vacuolated nonembryogenic cells that may serve as nurse cells (Karlsson and Vasil 1986, Vasil and Vasil 1982b). Such a dynamic feature of growth and development of the embryogenic callus permits manipulation of somatic embryogenesis in vitro. For instance, Morocz and others (1990) used a 3-4 week subculture interval to maintain freshly isolated embryogenic callus cultures that contained embryoids at all stages of development (from green and/or whitish scutellar-like structures to small shoots and roots). However, when the callus was subcultured more frequently (every 10-15

days), the resulting cultures became highly friable and homogenous, with only early developmental stages of embryoids.

Besides 2,4-D-like growth regulators and immature tissue/organs as explants (see the previous section), genotype, developmental stage of explants, and environmental influence of donor plants are other important factors regulating initiation of embryogenic callus.

Genotypic variation in culture is a well-known phenomenon. Genetic control of plant regeneration from tissue cultures has been indicated in several studies. For example, a few nuclear genes were reported to be responsible for the control of maize regeneration (Hodges and others 1986, Tomes and Smith, 1985), while Ma and others (1987) found that the ability to form regenerable callus of sorghum was inherited as a dominant trait among different genotypes. Reports such as these indicate the possibility of improving tissue culture response by plant breeding (e.g., Morocz and others 1990). However, it does not explain the fact that genotypes once considered nonregenerable (e.g., maize, Green and Phillips 1975) were later found regenerable (Biswas and Zapata 1992, Duncan and others 1985).

The use of explants at defined stages of development has been identified as a critical factor in establishing totipotent cultures from embryos (Lu and others 1983, 1984, Vasil and Vasil 1981a), inflorescences (Rangan and Vasil 1983, Botti and Vasil 1984, Boyes and Vasil 1984), and leaves (Conger and others 1983, Haydu and Vasil 1981, Lu and Vasil 1981a, Wernicke and Brettell 1980). Response of explants from well-nourished plants was different in culture from those of nutrient-deficient plants (Duncan and others 1985). Cultures from plants grown in summer were known in some cases to behave differently than those collected from plants grown in the cool season (Hanzel and others 1985, Ma and others 1987, Rines and McCoy 1981). Many genetic differences in culture response could be circumvented by varying nutrients and growth regulators in the culture medium (Duncan and others 1985).

The difference in tissue culture response observed among genotypes, at various stages of development, and donor plants from different environmental conditions has been suggested to be a physiological phenomenon caused by varied hormonal status of the explants (Bhaskaran and Smith 1990). This viewpoint is supported by several lines of evidence. In tissue culture of young leaves of napiergrass, Rajasekaran and others (1987a) showed that the younger parts of leaves, which are competent for somatic embryogenesis, contain higher levels of endogenous indoleacetic acid (IAA) and abscisic acid (ABA), while the more mature parts of the leaves, which do not form embryogenic calli, contain a substantially lower level of endogenous IAA and ABA. The relationship of hormonal metabolism to competence for somatic embryogenesis was further demonstrated by Rajasekaran and others (1987b) who found that treatment of plants with fluridone, an inhibitor of ABA biosynthesis, inhibited somatic embryogenesis in leaf cultures. Such inhibition could be overcome by exogenous ABA in the culture medium. The varying degrees of embryogenic competence along the length of napiergrass leaves were not found to be associated with changes in cell cycle or DNA content (Taylor and

Vasil 1987), or levels of DNA methylation (Morrish and Vasil 1989). Dolezelova and others (1992) also reported no variations in the cell cycle and nuclear DNA content among maize tissues differing in embryogenic competence. These findings enhance the concept that expression of somatic embryogenic competence is largely a physiological phenomenon (Bhaskaran and Smith 1990). It is inferred, therefore, that all genotypes are capable of producing totipotent cultures, and that the correct meristematic explants (i.e., right developmental stage from right donor plants) plus initial exposure to correct in vitro culture conditions (i.e., nutrients, growth regulators, incubation methods) are critical.

Friable embryogenic callus (type II) can be maintained in culture for long periods of time; however, the potential of long-term culture of compact embryogenic callus (type I) is generally limited (Chandler and Vasil 1984, Lu and others 1982, 1983, Redway and others 1990). Friable callus is produced at very low frequency and is described only in maize (Green, 1982, Armstrong and Green 1985, Vasil and others 1984, Tomes and Smith 1985, Kamo and Hodges 1986, Prioli and Sondahl 1989), oat (Bregitzer and others 1989), wheat (Redway and others 1990), and sorghum (Wei and Xu, 1990).

Production of friable callus in grasses is influenced by various factors, including nutrition (Armstrong and Green 1985, Kamo and Hodges 1986, Vasil and others 1984, V. Vasil and Vasil 1986), developmental stage of explants (Armstrong and Green 1985, Redway and others 1990), environmental influence of donor plants (Redway and others 1990), and genotype (Bregitzer and others 1989, Kamo and Hodges 1986, Redway and others 1990). Furthermore, friable callus more often develops from compact callus over a long period of time by selective subculture. Vasil and others (1984) reported that the addition of casein hydrolysate and reduction of sucrose to 2% in the maintenance medium caused a gradual morphological transformation during subculture of the opaque and white compact callus produced from cultured immature maize embryos. They found that about 25-30% of the callus pieces became increasingly less opaque and white and appeared soft and somewhat transparent. This soft callus culture maintained its capacity for plant regeneration over a year. Kamo and Hodges (1986) found in maize that the friable callus that was embryogenic and fast growing could only be identified 3 months after selective subculture, and suggested that one of the inbred lines might contain genes that contribute to long-term maintenance in culture. In wheat, 5 months of subculturing immature embryo-derived callus was required before a friable embryogenic callus was obtained in a few cultures (Redway and others 1990). After further subculture, a fast-growing culture that consisted almost entirely of friable callus only was developed. Such a culture maintained its capacity for plant regeneration for over 19 months. An interaction between seasonal/environmental and genotypic factors in controlling the induction of the friable embryogenic callus was also suggested in this study.

Plant Regeneration from Embryogenic Cell Suspensions

Embryogenic cell suspension cultures have been described as suspensions which are finely dispersed, free of any callus pieces, or organized tissues, meristems and meristemoids, fast growing, and are comprised mostly of groups of small, richly

cytoplasmic and starch-containing meristematic cells that form somatic embryos upon decrease of the 2,4-D concentration (IK Vasil and Vasil 1986). The importance of such cultures in relevance to the synthetic seed technology is that unlike callus cultures, embryogenic cells are to a great extent separated from each other, thereby making possible the encapsulation of individual embryos.

The general method for establishment of embryogenic cell suspensions remains the same as originally proposed (Ho and Vasil 1983, Lu and Vasil 1981b, Vasil and Vasil 1980, Vasil and Vasil 1981b). It involves careful selection for small embryogenic cells at each subculture. It applies well to the white compact type of embryogenic callus as starting material for establishment of an embryogenic suspension (Vasil and Vasil 1984a). Dalton (1988) and Yan and others (1991) adapted the method for fescue and ryegrass, and barley, respectively, by directly culturing embryos in liquid medium and hence reducing the time required for establishing cell suspensions. In maize and wheat, the use of homogenous, fast growing, friable, and embryogenic callus has been found to be critical (maize: Green 1983, V Vasil and Vasil 1986; wheat: Ahmed and Sagi 1993, Redway and others 1990).

The time needed to develop a cell suspension varies. With the friable embryogenic callus as the starting material, it can be from a few weeks to a few months. For instance, in maize the time period from placing the friable callus in liquid medium to an established stable embryogenic cell suspension was 2 weeks by Morocz and others (1990) and 5 weeks by V. Vasil and Vasil (1986). With compact embryogenic callus, 2-9 months (an average of 4-5 months) is needed, as reported for napiergrass (Vasil and others 1983), orchardgrass (Horn and others 1988), rice (Wang and others 1989), and barley (Jahne and others 1991). During this period, selective enrichment of the cultures by smaller groups of small, round, richly cytoplasmic and starch-containing cells is enforced at each subculture of 3-7 days. The enrichment is accomplished by slowly draining off old liquid medium and replacing it with fresh medium at certain dilution ratios at each subculture (Vasil and Vasil 1984b). The draining of the supernatant medium removes most of the large, vacuolated, and nondividing cells which are sloughed off into the medium along with other debris. However, in some cases it was also possible to produce embryogenic cell suspensions by filtering with sieves for certain sizes of cell groups and subculturing only the selected ones in fresh liquid medium (Abdullhah and others 1986, Qiao and others 1992).

It is common to initiate a cell suspension by placing calli in a liquid medium of the same composition as the callus initiation or maintenance medium (Wei and Xu 1990) or placing them in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 2,4-D (1.0-2.5 mg/L), sucrose (3%), and inositol (100 mg/L) with or without 5-10% coconut milk (V Vasil and Vasil 1984b). However, there may be special nutrient requirements for suspension cultures. For example, Ozawa and Komamine (1989) found that rice calli which were initiated and maintained on N6 medium or R-MS medium (a modified MS medium) did not proliferate in liquid R-MS medium but grew vigorously when the R-MS medium was supplemented with 300 mg/L casein hydrolysate

(CH) or 300 mg/L CH and 25 mM L-proline. By contrast, the calli grew vigorously in liquid N6 medium even in the absence of proline and/or CH.

After a long period (often 4-5 months) of careful selection and manipulation, embryogenic cell suspensions that are finely dispersed and fast-growing can be established in all major cereals and grasses (Vasil and Vasil 1992). These cell suspensions, often referred to as cell lines, are generally maintained by subculture at a 1:5 or 1:7 dilution (suspension inocula: fresh medium) every 4-5 days in liquid media containing 1.0-2.0 mg/L 2,4-D (V Vasil and Vasil 1984b). However, the embryogenic nature of the cell lines is lost over time, often in a few months (Jahne and others 1991) and sometimes in 1-2 years (Biswas and Zapata 1992, Funatsuki and others 1992, Ozawa and Komamine, 1989).

Established embryogenic cell suspensions consist predominantly of small clusters (ca. 10-100 cells) of densely cytoplasmic cells, as originally described for pearl millet (Vasil and Vasil 1982b) and later for orchardgrass (Horn and others 1988), maize (Prioli and Sondahl 1989), barley (Jahne and others 1991), and wheat (Redway and others 1990). Sometimes, these small cell clusters tended to grow into larger aggregates and selective subculture and filtering were used to maintain a high frequency of small aggregates of embryogenic cells (Prioli and Sondahl, 1989). The established cell suspension can contain other cell types (Redway and others 1990, V Vasil and Vasil 1986), and occasionally somatic embryos up to the globular or the early scutellar stage in established cell suspensions of some grass species (Lu and Vasil 1981b, Vasil and Vasil 1981b). Further development of these embryo structures did not occur in liquid medium.

Transfer of embryogenic cell suspensions to agar-media containing low concentrations of 2,4-D is a general method for plant regeneration (V Vasil and Vasil 1986). In some cell lines, special conditions such as alternate subculture intervals, changes in the composition and concentration of salts and growth regulators are required. For instance, Biswas and Zapata (1992) obtained only a few green shoots when rice cells were transferred to low 2,4-D media. In comparing different media, they found that although MS-based media did not allow plant regeneration to occur, N6-based media supported shoot regeneration. The regeneration of shoots could be further improved when the basal N6 medium was reduced to half strength and both auxin (1-napthaleneacetic acid; NAA) and cytokinin (benzylaminopurine; BA) were included in the regeneration medium. These results were in agreement with those of Ozawa and Komamine (1989), who also reported that their rice cell suspensions could regenerate plants only when subcultured every 3 days (not 7 days).

Genetic fidelity of plants regenerated in culture is a basic requirement for clonal propagation and genetic transformation. Although variability, both epigenetic and genetic, has been widely reported in plant tissue culture (Bayliss 1980), it is believed that there is a strong selection in favor of normal cells during the process of somatic embryogenesis with the result that only normal or nearly normal plants can be regenerated (Swedlund and Vasil 1985, IK Vasil and Vasil 1986).

Recent surveys at biochemical and molecular levels have failed to detect any variation among grasses regenerated via embryogenesis. Shenoy and Vasil (1992) and Pedrosa (1993) showed absence of variation in different isozymes in plants regenerated via somatic embryogenesis of napiergrass and teosinte, respectively. By restriction fragment length polymorphism (RFLP) analysis of mitochondrial, plastid, and nuclear genomes, no variability could be found in plants from embryogenic cultures of napiergrass (Shenoy and Vasil 1992), sugarcane (Chowdhury and Vasil 1993), or meadow fescue (Valles and others 1993). Variations seen in plants derived from somatic embryos were clearly transient in nature and were considered to have resulted from epigenetic changes rather than genetic changes. For instance, teosinte plants regenerated via somatic embryogenesis showed considerable amounts of variation in morphology, including dwarfing and conversion of male flowers in the tassel to females (Pedrosa, 1993). Such variability disappeared completely in the sexual progenies or after treatment of the abnormal plants with gibberellic acid.

Depending on the system, regeneration of somatic embryos into plants typically averages 80 to 100% for cereal and turf species (I Vasil, pers. comm.). Also, Rajasekaran and others (1987c) reported that yields of vegetative tissue of napiergrass were greater from somatic embryo-derived plants than from asexually-derived plants. They attributed the increased yield to a greater number of tillers.

Synthetic Seed

The concept of synthetic seed, i.e., a single quiescent or nonquiescent somatic embryo that may or may not be encapsulated in an artificial medium such as a gel, was proposed in the late 1970's (Murashige 1978). Very little research has been conducted on synthetic seed technology for grasses because 1) it is not cost effective to utilize this technology for relatively low value crops such as forage grasses, and 2) traditional methods of seed production are the most cost effective for high value grain crops. Most of the synthetic seed research on grasses has centered on orchardgrass (*Dactylis glomerata*) (e.g., see Gray and others 1987). Synthetic seed technology with orchardgrass has focused on use of desiccated quiescent embryos. (However, we encapsulated nonquiescent embryos in our project.)

It is important that the embryos selected for encapsulation be mature and vigorous enough to break through the encapsulation material and grow into a plant. Ghosh and Sen (1994) made a direct comparison of encapsulated and nonencapsulated synthetic seed for *Asparagus cooperi*. They determined that the optimum stage for embryo encapsulation was for the embryo to have distinct shoot and root meristems. The best conversion rate for encapsulated seed germinated in vitro was 34% as compared to 45% for nonencapsulated seed. For the male bamboo, *Dendrocalamus strictus*, embryoids without visible shoots or roots were encapsulated, but nutrients previously determined to stimulate shoot and root growth were included in the encapsulation matrix (Mukunthakumar and Mathur 1992). Conversion rate in vitro and 'in soil' was 96% and 45%, respectively.

Redenbaugh and others (1986) showed that calcium alginate was the optimum gel to use for encapsulating somatic embryos. Embryos are suspended in alginate and then dropped one at a time (i.e., each embryo is in a bead of alginate) in to a solution of calcium chloride ($CaCl_2 \cdot 2H_2O$). Bead characteristics, which will affect the conversion frequency of the synthetic seed to a plantlet, are influenced by the alginate and calcium chloride concentrations, alginate source (guluronic:mannuronic acid ratio), and by the amount of time the bead is stirred in the calcium chloride solution. Sodium alginate will complex when mixed with di- and trivalent metal cations to form calcium alginate via formation of ionic bonds between carboxylic acid groups on the guluronic acid molecules of alginate. The size of the capsule can be controlled by the viscosity of the sodium alginate and by the inside diameter of the nozzle used to form the drops.

Two major problems with hydrogel encapsulation, which may result in a low germination efficiency of synthetic seeds, are embryo respiration and seed storage. Encapsulated embryos were reported to have a lower in vitro conversion frequency than naked embryos. Poor embryo respiration due to restricted gas flow through the alginate gel was considered one of the possible reasons. Additional activated charcoal in the capsules may enhance gas diffusion and improve respiration. The storage of encapsulated seeds at 4C was considered to improve germination. However, it was observed that this storage also resulted in water loss of capsules. It was suggested that dehydration and freezing in liquid nitrogen might improve the survival of encapsulated embryos. For instance, Ghosh and Sen (1994) tested different sources and concentrations of alginate as well as different concentrations of calcium chloride and found that the best conversion frequency of asparagus embryos (34%) was obtained with 3.5% alginate (Sigma) and 50 mM $CaCl_2 \cdot 2H_2O$. They also speculated that harder gels obtained with higher rates of alginate might have inhibited the emergence of shoots and roots. In contrast, excellent conversion frequencies for male bamboo synthetic seed were obtained using 6% alginate and 100 mM $CaCl_2 \cdot 2H_2O$ (Mukunthakumar and Mathur 1992).

Inclusion of nutrients in the calcium alginate bead seems to be important for successful synthetic seed production, at least of monocots. Ghosh and Sen (1994) dipped asparagus embryos into MS medium supplemented with 1 mg zeatin per liter before placing them in the alginate solution. The calcium alginate used to encapsulate male bamboo embryoids contained MS basal salts supplemented with NAA and kinetin (Mukunthakumar and Mathur 1992); this nutrient solution was added to both the alginate and calcium chloride solutions. They also found that heavy grade mineral oil reduced the tackiness of the calcium alginate bead, but even more significant was that the mineral oil coating increased the germination rate from 45% to 56%.

Shoot Multiplication Culture

An alternative to encapsulating somatic embryos to produce artificial or synthetic seed production is to establish shoot multiplication cultures from clumps of somatic embryoids and embryos. Shoot multiplication culture using embryoids has several well-

defined steps adapted from Murashige (1974): Stage II – shoot proliferation from embryoids/embryos, Stage III – rooting, if necessary, and Stage IV – acclimatization. (Note – Stage I is the establishment of a sterile culture). This method is generally used to propagate commercially or scientifically important monocot and dicot species in which traditional methods are inefficient or rapid large-scale propagation is not possible. For example, Saxena and Dhawan (1999) developed a protocol for micropropagating the economically important monocot male bamboo (*Dendrocalamus strictus*) via somatic embryo multiplication culture because it is difficult to propagate by seed. Small clumps of somatic embryos were placed on media on which they multiplied two to five fold over 5-6 weeks. Rooted plantlets were acclimatized to ambient conditions over several weeks, with 80% survival. Over 100,000 plants were produced.

For proper growth and development, plants depend on light for photosynthesis and photomorphogenesis (Smith 1982). Plants cultured in vitro (somatic embryos or plants derived from somatic embryos) require less light, mainly to regulate morphogenetic processes (Murashige 1974; Seibert and Kadkade 1980). Economou and Read (1987) stated that success of micropropagation is influenced by light not only by photoperiod and light intensity, but by spectral quality as well. Although cool-white, warm-white, and Gro-Lux fluorescent light are the most commonly used light sources for tissue culture (Economou and Read 1987), researchers continue to look for more efficient light sources (Fang and Jao 2000). Kodym and Zapata-Arias (1999) evaluated the use of natural light to reduce tissue culture costs and reported reported that banana cultures produced more shoots under natural light than in a growth chamber; however, light intensity was also greater under the natural light conditions. Blue, red, and far red light seem to have the greatest positive effect on shoot and root formation (Economou and Read 1987), but enhancement of shoot and root formation in response to these spectra varies by species (Kadkade and Jopson 1978, Economou and Read 1986a, 1986b, Kadkade and Seibert 1977, Chée and Poole 1989).

ROOTING AND ACCLIMATIZATION

Shoots from multiplication culture can be rooted in vitro or in vivo. Shoots rooted in vivo are simultaneously rooted and acclimatized under greenhouse conditions. Acclimatization is necessary for plantlets to develop normal root systems and stomatal function. Initially, high humidity is established in the greenhouse either by misting or by covering the plantlets with clear plastic for one or more weeks; then misting frequency/humidity levels are gradually decreased. Once acclimatized to greenhouse conditions rooted shoots (i.e., plantlets) are acclimatized to ambient conditions. *Pennisetum* and *Cortaderia* plantlets were successfully acclimated under intermittent mist (Meliza and Khatamian 1998, Robacker and Corley 1992), whereas *Spartina*, *Sporobolus*, and *Brachypodium* were all acclimatized under clear plastic (Bablak and others 1995, Li and Gallagher 1996, Straub and others 1992).

In order for tissue-cultured plantlets to acclimate to high light conditions, some shading may be necessary initially. *Cortaderia* plantlets were placed for 1 week under

65% shade before being subjected to ambient greenhouse light (Robacker and Corley 1992). For 7 to 10 days, in vitro plantlets of *Cymbopogon* were placed in a plastic tent under 40% shade before repotting and exposure to field conditions; final survival was 60% (Patnaik and others 1997). *Brachypodium* plantlets on the other hand were acclimatized and hardened off under natural greenhouse light conditions for 14 to 28 days in a clear plastic bag (Bablak and others 1995).

GOPHER APPLE

Description

Gopher apple is in the Coco family (Chrysobalanaceae). Chrysobalanaceae is a family of woody dicots and has a worldwide distribution in tropical and subtropical regions.). Most *Licania* species inhibit moist forests, with gopher apple being the only species to be found beyond the tropics of North America. Gopher apple is distributed from Louisiana to Florida, northward to South Carolina (Prance and White 1988, Radford and others 1968, U.S. Dept. Agric. Nat. Res. Cons. Serv. 2001) concentrated among the dry sandy pinelands, oak barrens, and sand dunes. It can be locally abundant (Prance and White 1988), covering graded roadsides with hundreds of stems (Ward and Taylor 1999) and as well as natural areas (Pessin 1933). Dispersal of some *Licania* species is by fish, some by bats, fruit pigeon or squirrel. Dispersal of gopher apple is thought to be, at least in part, by the gopher tortoise.

It has an extensive subterranean stem up to 8 cm thick, 1 to 10 cm deep and to 30 m in length, and was described as an "underground tree" by Prance and White (1988). The erect aerial portions (which may be hundreds from one plant) are less than 5 mm in diameter, with simple, alternate leaves 4 to 10 cm long and 1 to 3.5 cm wide. Inflorescences develop terminally with a panicle of cymes in May and June. The petals are white (Radford and others 1968) or yellow-green (Clark 1971). One or two sweet-fleshed ellipsoid drupes (2 to 3 cm long) per aerial stem mature between September and October. Although gopher apple is the most familiar common name, sweet-bush, ground oak, and deer plum suggest additional characteristics or uses of this species.

Habitat loss in Florida was noted by Richardson (1989) when estimating that only 5 to 10% of the sand pine scrub communities in Palm Beach, Broward, and Dade counties remain. Polk and Highland counties of central Florida have 35% of this habitat remaining. Gopher apple is only one of some 300 vascular plant species originally found in this scrub. Although part of the past impact of gopher apple has been revealed, it is the current and possible future use of gopher apple that compels it to be considered in this study. Seedlings, liners, and larger containerized plants are primarily marketed for restoration and mitigation projects. Additionally, use by wildlife managers and landscape personnel as a ground cover is possible. The drought tolerance of this "underground tree" and its influence on other species are of paramount importance.

Propagation

No information about how to propagate this species via traditional methods (such as seed or cuttings) or by micropropagation could be found in a review of published literature. It is only propagated by seed as discussed in ***Purpose****.*

Propagation of woody plants via stem cuttings is commonly practiced for an innumerable number of woody species. Many factors can affect the rooting response including maturity (softwood, semi-hardwood, hardwood), time of year when cuttings are harvested, and the auxin (type, formulation, and application method) used to stimulate or enhance rooting (Dirr and Heuser 1987). The two factors we focused on were auxin type and season of harvest.

There are several auxins used to stimulate rooting of woody plant stem cuttings, the most common of which are NAA and indolebutyric acid (IBA). These are available in water and water insoluble forms, a factor that can influence rooting. As Dirr and Heuser (1987) point out, auxins dissolved in water may not be able to penetrate and be taken up by the cutting as effectively as an auxin dissolved in an organic solvent like alcohol. In contrast, cuttings of some species are sensitive to organic solvents. Another alternative is to use talc formulations of rooting compounds.

Timing is another factor that can significantly affect the degree of rooting. Many species can be rooted equally well regardless of the time of year cuttings are taken but propagation of some species is very time specific. Bottlebrush buckeye (*Aesculus parviflora*) is a woody species native to the southeast. Like gopher apple, it was propagated by seed (Fordam 1987) but was in short supply at one time because of problems associated with seed propagation (Fordam 1987). Dirr (1987) observed that this species could be propagated by stem cuttings; however, timing was very critical – cuttings had to be taken within the first 6 weeks after bud break. Timing is also an important factor for propagating southern wax myrtle (*Myrica cerifera*) (Pokorny and Austin 1982) and native azaleas (Nienhuys 1980).

Micropropagation of woody plants is not as common as traditional propagation methods. It is frequently attempted in cases where traditional methods are slow or inefficient. The successful tissue culture of a species (Stage 0 to IV; Kane 2000) is related to many factors including genotype, donor plant health and developmental stage, explant tissue, medium, components, and plant growth regulators in the media (Merkle and others 1995). Stage 0 is the selection and preparation of the donor plant. Stage I is the surface sterilization to obtain uncontaminated tissue(s). Stage II is the abundant development of axillary shoots. Stage III is the eventual rooting of the axillary shoots although this stage is done ex vitro at times. Stage IV is the acclimatization of the plantlets, generally in a greenhouse, before moving them outside.

Selection and preparation of the donor plant for tissue culture is closely related to Stage I – obtaining uncontaminated cultures. The goal is to have an explant that is contaminant free. The less infested an explant is prior to surface-sterilization the better,

hence the general recommendation, as pointed out in Dirr and Heuser (1987), is to use plants grown in the greenhouse or an incubator. Better yet is the use of in vitro grown plant material from contaminant-free cultures such as germinating seed in vitro and using the seedling as an explant source (Dirr and Heuser 1987). Use of in vitro seedlings as explants is highly recommended because surface-sterilization practices can negatively affect tender explants (Dixon and Gonzales 1994).

Production of axillary shoots (Stage II) – shoot multiplication culture – is the most common method of woody plant micropropagation. Dirr and Heuser (1987) summarize numerous examples in which axillary shoot production is used for woody plant species. One of the most important factors affecting shoot production is the plant growth regulator in the medium. A high cytokinin to auxin ratio is the generally accepted method for axillary shoot production. However, determining which cytokinin (or cytokinin precursor) to use for optimal shoot production, and at what concentration, can require much study. The most commonly used cytokinins are BA, 6-(γ,γ-dimethylallylamino)-purine riboside (2-iP), 6-furfurylaminopurine (kinetin), thidiazuron (TDZ), and 9-(β-D-ribofuranosyl)-trans-zeatin (zeatin).

PROJECT APPROACH AND LIMITATIONS

CREEPING BLUESTEM AND WIREGRASS

In this study, micropropagation, i.e. propagating plants via tissue culture, was evaluated as means of mass-producing creeping bluestem and wiregrass. The overall approach was to regenerate plants via somatic embryogenesis. In this technique, a special type of callus tissue (which is basically an undifferentiated mass of cells) called embryogenic callus is induced to form embryos. These embryos are identical to embryos in seeds that are normally generated by fertilization of eggs by the male gametes from pollen – zygotic seed. (Note: Seed in plants can also be formed without fertilization through a process called parthenocarpy.) Somatic embryos can be encapsulated to produce synthetic seed, plants can be directly regenerated from the embryos, or embryo cultures can be used to develop shoot multiplication cultures. No mater the method used to regenerate plants, grasses produced from somatic embryos usually maintain their genotypic and phenotypic integrity. Embryos generated in culture were 1) separated and encapsulated to form synthetic seed, and 2) induced to germinate, with the resulting shoots used to generate shoot multiplication cultures (creeping bluestem only). Shoot multiplication culture is a method in which large numbers of shoots are produced in a short amount of time. These shoots are then rooted and acclimatized to ambient conditions. The acclimatized shoots can then be planted or grown to maturity in containers.

Our plan was to first develop sustainable somatic embryogenic cultures of these species, regenerate plants (including via the use of synthetic seed), and then evaluate these tissue-cultured plants under greenhouse, field and mined land conditions. Developing these cultures presented more of a challenge than anticipated. Out of the numerous attempts to initiate embryogenic callus, only a few lines could be established. Moreover, for wiregrass, embryogenic callus could only be initiated from one immature inflorescence out of the hundreds that were cultured. While we were able to successfully regenerate plants from this line (both from encapsulated [synthetic seed] and nonencapsulated somatic embryos), this callus line eventually was lost. Numerous attempts to initiate new embryogenic callus lines were severely hampered by contamination problems associated with the plant material, and eventually it was decided to forego these efforts and to concentrate our efforts on creeping bluestem.

Despite contamination issues that plagued us after the first year of the project, we had much better success with creeping bluestem than with wiregrass. Three lines of embryogenic callus were initiated. While the initial line and a second line were lost due to widespread contamination, the third line of embryogenic callus was used to establish shoot cultures from which we were able to produce numerous acclimatized tubelings, most of which were for evaluation under field conditions and at the CFI mine site near Ona, FL. Survival of tissue-cultured plants on sand tailings and over burden has been fair to good. The last 2 years of the project focused not only on the field work but also in refining the micropropagation and acclimatization protocol.

Overall, there were two major limitations to this portion of the project – contamination and establishing embryogenic callus cultures. Overcoming contamination problems consumed an enormous amount of time and effort. Fortunately we were able to replicate establishment of creeping bluestem embryogenic callus. Establishing embryogenic callus is clearly the rate-limiting step in regenerating these grasses via somatic embryos. We were not successful in establishing wiregrass embryogenic callus more than once.

GOPHER APPLE

Work with gopher apple began in late 2001, with the long-term goal of finding the most efficient method of propagating gopher apple. The main objective of our work in 2001 and 2002 was to determine how to obtain clean explants for use in future micropropagation work, to conduct some preliminary studies on shoot multiplication cultures, and examine the effect of time of harvest on rooting response in stem cuttings rooted in vivo under a traditional mist bed system.

Unlike the native grass micropropagation work, contamination was not a major limitation. The main limitation was obtaining seed that was germinated in vitro to produce clean explants. Obtaining good quality stem cuttings was also a problem once gopher apple had flowered. Overall though, work proceeded quite smoothly and we were able to establish clean cultures. We were not able to establish rapidly multiplying shoot multiplication cultures, although we only had time to evaluate a limited number of media supplements. Timing of taking stem cuttings seemed to have a major effect on rooting in vivo.

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CHAPTER 2

MICROPROPAGATION OF CREEPING BLUESTEM

Jeffrey G. Norcini, Tarak N. Chakravarty, Robert S. Kalmbacher, and Wusi Chen

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EXECUTIVE SUMMARY

Creeping bluestem (*Schizachyrium scoparium* var. *stoloniferum*) is one of the keystone native grasses in areas of phosphate mining and hence is highly desired for use in reclamation. Direct seeding of this species is potentially the most cost-effective means for revegetating mined lands. However, it is a poor seed producer that primarily relies on its rhizomatous nature to spread and reproduce. Those who currently use creeping bluestem in reclamation typically must harvest it from natural stands that occur near the reclamation site. If use of creeping bluestem is to become more widespread in phosphate mine reclamation, then production methods will have to be developed that result in sufficient quantities of good quality tubelings, or even seed.

In this study, micropropagation, i.e. propagating plants via tissue culture, was evaluated as means of mass-producing creeping bluestem. The overall approach was to use somatic embryogenesis because plants regenerated by this method will probably look the same and be genetically identical to the mother plants. It is the most common method of regenerating grasses via tissue culture (see I.K. Vasil, 1987, "Developing cell and tissue culture systems for the improvement of cereal and grass crops," J. Plant Physiol. 128:193-218.). In this technique, a special type of callus tissue (which is basically an undifferentiated mass of cells) called embryogenic callus, is induced to form embryos. These embryos are identical to embryos in seeds that are normally generated by fertilization of eggs by the male gametes from pollen – zygotic seed. (Note: Seed in plants can also be formed without fertilization through a process called parthenocarpy). Somatic embryos can be encapsulated to produce synthetic seed, plants can be directly regenerated from the embryos, or embryo cultures can be used to develop shoot multiplication cultures.

Our initial work focused on production of synthetic seed because if seed could be economically produced in large quantities then seeding of mined land would be possible. The base tissue culture media used in this study was the Murashige and Skoog basal medium supplemented with coconut milk from immature or mature coconuts. Supplementing the medium with 2,4-dichlorophenoxyacteic acid at 2-3 mg/L resulted in embryogenic callus formation from immature inflorescences and leaf tissue. Embryogenic callus growing on solid media was also used to initiate liquid suspension cultures in which finely divided embryogenic callus was induced to form pre-embryos (also called embryoids). Pre-embryos were plated out onto solid media designed to allow development of the pre-embryos into embryos. Mature embryos on solid media were then mixed in alginate (a seaweed derivative) gel. Embryos in the gel were then dropped into a calcium chloride solution (one to three embryos in a droplet). The droplet containing the embryo became hardened after several minutes in the calcium chloride solution thereby forming a synthetic seed.

Synthetic seed germination in vermiculite was good to excellent in vitro and fair under greenhouse conditions at the NFREC. Best germination was for seed that contained growth regulators in the gel. Preliminary results of greenhouse testing of

synthetic seed germination in Myakka and mine soil were inconclusive. Contamination problems and difficulty in reestablishing suspension cultures (a critical step in synthetic seed production) limited our work with synthetic seed. Because of the critical need to develop an efficient method of micropropagating creeping bluestem, it was decided to focus our efforts on shoot multiplication culture.

Clumps of embryos from solid media were used to generate shoot multiplication cultures. Starting with a shoot cluster of 3-4 shoots, it took 32 weeks to establish shoot multiplication cultures for production (15-16 shoots per cluster per jar). Once established, number of shoots nearly doubled every 8 weeks. At this rate, about 1700 plants (per initial 3-4 shoot cluster) could be produced in about 1 year once shoot multiplication cultures are established at 32 weeks.

Rooting these shoots in vitro and then acclimatizing them in the greenhouse resulted in a greater percentage of surviving tubelings than when shoots were both rooted and acclimatized under greenhouse conditions. In greenhouse tests, tissue-cultured plants grown in pots on overburden and sand tailings were similar to plants by propagated by division with respect to shoot, root and rhizome mass, but tiller number per plant was greater on tissue-cultured plants compared with division plants.

In field trials on mined land, tissue-cultured plants established well when planted in January, July, or October; however, survival was less on overburden for the October planting, which was due to typical low rainfall at this time. Plant diameter as measured by rhizomes expansion was good and averaged 24 cm over the three planting dates. On the mine site, a large soil seed bank resulted in much weed competition. Hence good establishment of creeping bluestem on mined land will require a weed management program that minimizes increases in the weed seed bank and weed competition during establishment.

Because it is a major component of central and south Florida native plant communities, creeping bluestem needs to be included in restoration of mined lands. Since the grass produces little seed, tissue culture offers a practical way to produce plants that can establish and grow well on phosphate-mined land.

METHODOLOGY

Plant incubator conditions throughout all studies in this chapter, except as noted: 25-26C; 16-hr photoperiod; light provided by cool white fluorescent bulbs, with light level at 40μE at Petri plate level.

STUDY 1

Callus Initiation from Young Leaves

- Creeping bluestem from south central Florida; propagated by division of original three plants (B-1)

All creeping bluestem were grown in 3.8- or 11.4-liter pots under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

Young leaves were sterilized as follows: 70% ethanol for 30 sec; shaking in 1% sodium hypochlorite + few drops of Tween 20 at 100 rpm for 20 min; rinse 5X with sterile distilled water (SDW).

The older external portion of leaves was removed; 1- to 3-cm long segments from the basal portion of the leaves was used as starting material. The inner young leaf segments were cut into 1-mm sections and cultured on MS medium with different concentrations and combinations of plant growth regulators. **Unless otherwise mentioned, all media mentioned throughout this chapter was at pH 5.8 and contained 3% sucrose (w/v).**

a. MS + 2,4-D (2 mg/L) + 5% CM
b. MS + 2,4-D (2.5 mg/L) + 5% CM
c. MS + 2,4-D (3.0 mg/L) + 5% CM
d. MS + 2,4-D (2.5 mg/L) + BA (0.2 mg/L) + 5% CM

2,4-D	2,4-dichorophenoxyacetic acid
BA	6-Benzylaminopurine (aka benzyladenine)
CM	Coconut Milk (mature coconuts; % is v/v) (*Milk from fresh, mature coconuts was extracted, heated to 70C for 60 min, and then filtered to remove particulate matter. It was stored at -20C.*)
MS	Murashige and Skoog Basal Medium (Murashige and Skoog 1962)

Callus Initiation, Maintenance and Embryogenesis from Immature Inflorescences

- Creeping bluestem from south central Florida; propagated by division of original three plants (B-1)

All creeping bluestem were grown in 3.8- or 11.4-liter pots under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

Young, unemerged inflorescences were surface sterilized, as follows, while still enclosed by several whorls of leaves: 70% ethanol for 30 sec; shaking in 1% sodium hypochlorite + few drops of Tween 20 at 100 rpm for 20 min; rinse 5X with SDW. The sterilization procedure was the same as above. The inflorescence was exposed (by removal of the surrounding leaves), cut into 1- to 2-mm thick segments, placed on MS medium (see below) and cultured at 25C in the dark.

a. MS + 2,4-D (2 mg/L) + 5% CM
b. MS + 2,4-D (2.5 mg/L) + 5% CM
c. MS + 2,4-D (3.0 mg/L) + 5% CM
d. MS + 2,4-D (2.5 mg/L) + BA (0.2 mg/L) + 5% CM
e. MS + 2,4-D (3.0 mg/L) + BA (0.2 mg/L) + 5% CM
f. MS + 2,4-D (3.5 mg/L) 5% CM

Initiation, Maintenance and Embryoid Induction of Cell Suspension Cultures

- Creeping bluestem from south central Florida; propagated by division of original three plants (B-1)

Embryogenic callus was dissected into small pieces. About 100 mg fresh weight of callus was placed in 25 ml liquid Medium *g* or *h*, and in 125-ml flasks on a gyrating shaker (150 rpm) to initiate cell suspension cultures. The suspensions were routinely sub-cultured on the same medium every 4 days. The cultures were grown in darkness at 27C. Three weeks after initiation, the cultures were transferred to Medium *j* or *k* to induce pre-embryoid formation.

g. MS + 2,4-D (1.5 mg/L) + 2.5% CM
h. MS + 2,4-D (2.5 mg/L) + 2.5% CM
j. MS + 2,4-D (0.5 mg/L) + BA (0.2 mg/L) + 2.5% CM
k. MS + 2,4-D (1.5 mg/L) + BA (0.2 mg/L) + 2.5% CM

Embryoid Formation and Plant Regeneration on Solid Media

- 4- to 5-week-old embryogenic callus (B-1)
- 6- to 8-week-old cell suspension cultures (B-1)

Four methods of embryoid formation and plant regeneration were evaluated:

- Four to 5-week-old embryogenic callus was transferred onto regeneration Medium *n*. After embryos matured on this medium and tiny plantlets were observed, the callus clumps were transferred onto MS medium without plant growth regulators. Further development and plant regeneration was carried out in larger containers; later, plantlets were transplanted in a soilless medium (5.7 cm x 5.7 cm x 5.7 cm plastic cups containing pine bark:Canadian sphagnum peat:sand, 3:1:1 [by vol.] amended [per m^3] with 2.4 kg Osmocote 18N-2.6P-10K [18-6-12], 942 g Micromax, and 106 g sublimed sulfur) and placed in "acclimation chambers" (clear, plastic shoe boxes) in a greenhouse.

- Suspension cultures were mixed with equal amount of 1.0% MS agar medium and poured into Petri dishes.

- 1-ml suspension cultures were spread on Medium *m, o,* or *p*, or MS medium without plant growth regulators in Petri dishes.

- Cell suspension cultures were selected by passing through meshes of 0.7 mm and 0.2 mm in diameter. Cell clumps larger than 0.7 mm and between 0.2 and 0.7 mm were cultured on Medium *m*, *o*, or *p*, or MS medium without plant growth regulators in Petri dishes.

Four weeks after being planting on these semi-solid media, embryoids were separated and transferred onto MS medium without plant growth regulators. Further development and plant regeneration were carried out in larger containers. Seedlings were transplanted in a soilless medium (see above) and then placed in an "acclimation chamber" in a greenhouse.

m. MS + 2,4-D (0.2 mg/L) + 5% CM
n. MS + NAA (0.05 mg/L) + BA (0.2 mg/L) + 5% CM
o. MS + ABA (0.264 mg/L) + 5% CM
p. MS + TDZ (0.2 mg/L) + 5% CM

ABA	Abscisic acid
NAA	1-Naphthaleneacetic acid
TDZ	Thidiazuron

Encapsulation of Somatic Embryos and Germination of Synthetic Seeds

- Somatic embryoids of creeping bluestem (B-1)

Somatic embryos were isolated from polyembryoid clumps. One hundred milliliters sterile $CaCl_2$ solution was placed in a sterile 250-ml beaker. A sterile disposable plastic pipette was placed into the opened flask of sterile sodium alginate, and alginate was drawn into the pipette. Alginate was then slowly dropped from the tip that was positioned above the $CaCl_2$ salt bath. Isolated somatic embryos were inserted into the alginate drop by using forceps. The alginate drop containing one to three somatic embryos then fell into the $CaCl_2$ salt bath. The capsules were complexed after shaking in an incubator for 30 to 45 min. Then the $CaCl_2$ solution was decanted and the beads were washed in 100 ml sterile distilled for 5 min (3X). Capsules containing somatic embryos were stored at 4C for 2 to 3 weeks before planting in vitro on MS medium, on sterile vermiculite in vitro, or in nonsterile vermiculite in the greenhouse. Germination was assessed 2 to 3 weeks after planting. In vitro germination was in an incubator at 25C and 16-hr photoperiod (cool white fluorescent light; 40 μE at Petri plate level).

Solutions

- 50 mM $CaCl_2$ (used for 3% and 3.5% alginate)
- 100 mM $CaCl_2$ (used for 2% alginate)
- 2%, 3% or 3.5 % sodium alginate dissolved in
  - distilled water
  - 50% MS medium
  - 50% MS + ABA (10 μM)
  - 50% MS + 2,4-D (10 μM)
  - 50% MS + GA_3 (10 μM)

Germination Media

- Solid MS medium without growth regulators
- Solid MS + 2,4-D (1 μM)
- Solid MS + ABA (1 μM)
- Solid MS + GA_3 (1 μM)
- Sterilized vermiculite soaked with sterilized water
- Non-sterile vermiculite soaked with tap water

STUDY 2

This study was conducted to determine repeatability of the methods of Study 1, to further optimize those methods, develop a shoot multiplication protocol, and to evaluate the use of tissue-cultured bluestem under field conditions.

Embryogenic Callus Initiation

At the end of July 1998, there was severe fungal and bacterial contamination and many cultures were lost. Contamination may have been due to environmental conditions – excessive rains during the summer of 1998 season may have lead to an increase in ambient spore levels. Several steps were taken to control the contamination, including consultation with experts and sending out the contaminated cultures for identification of the bacteria and fungi. Also there was poor embryogenic callus formation (from the experiment described above), so modification of the media was done to try and improve embryogenic callus initiation. These procedures are outlined below.

Various sterilization procedures for individual immature inflorescences were evaluated as noted below based on our experience and the advice of Dr. Tim Schubert (Division of Plant Industry) and Dr. Kenneth C. Torres (Phytotechnology Labs):

- Creeping bluestem from south central Florida (B-1); propagated by division of original three plants
- Tissue-cultured progeny of creeping bluestem (B-1): planted 2/18/98 and repotted 4/13/98
- Creeping bluestem from Apalachicola Bluffs and Ravines Preserve (collected during the first week of October 1998)

All plants were grown in 3.8- or 11.4-liter pots in full sun or under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

- Explant kept in distilled water – 0 or 24 hr
- Wash in tap water for 45 or 60 min
- Surface-sterilize with 90% EtOH for 30 sec instead of 1 min; 70% EtOH used previously
- Soak in 0.3% or 0.5% mercuric chloride for 5 min; 1% or 5.25% sodium hypochlorite used previously
- Wash with sterile distilled water 5X @ 10 min
- Replace filters in laminar flow hood; purchase fresh chemicals
- Add Plant Preservative Mixture (PPM) to media – various concentrations
- Immature inflorescence explant size: 1-2 mm or 2-3 mm

"Sterile" immature inflorescences were cut into 1-2 mm or 2-3 mm long segments from the basal portion of the inflorescence and cultured on in the dark at 25C on solid media listed below for embryogenic callus initiation.

A. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.8% Phytagel
B. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + 10% GCM + PPM (2 ppm) + 0.8% Phytagel

C. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.1% AC + PPM (2 ppm) + 0.8% Phytagel
D. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.1% AC + PPM (2 ppm) + 0.8% Phytagel
E. MS + 2,4-D (3.0 mg/L) + $ZnSO_4$ (5.2μM) + 10% GCM + 0.8% Phytagel
F. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + $ZnSO_4$ (5.2μM) + 10% GCM

GCM	Milk from immature green coconuts (% is v/v)
CH	Casein Hydrolysate
PPM	Plant Preservative Mixture ((Phytotechnology Labs)
AC	Activated Charcoal (% is w/v)
$ZnSO_4$	Zinc Sulfate
	Phytagel (Sigma Chemical)

[Note: All media in Study 2 (i.e., post May 1998) are in capital letters so as to differentiate them from media used in Study 1 (June 1997 through May 1998).]

Starting in August 1998, several batches of immature creeping bluestem inflorescences were frozen at -80C to determine if stored ultra frozen tissue could be used to initiate embryogenic callus. Several batches from the plants listed above were frozen at -80C from August 1998 to December 1998. Inflorescences were packed in plastic quart-sized freezer bags (ZipLock™), labelled, and placed in a freezer at -80°C. The inflorescences were packed in several batches on different dates. When these tissues were transported from Quincy to Monticello, they were kept frozen by storing them on dry ice.

Since we observed slow or no callusing of creeping bluestem explants in the Study 2 experiments conducted previously, we questioned whether the vigor of the stock plants had an effect. There are reports that vigor is increased in these plants after burning, and our plants were last burned in 1994.

On March 19, April 12, and May 19, 1999 several containerized creeping bluestem plants were burned (burn conditions: plants – sunk in soil to top of pot; air temp. – 3-19: 24.9C; 4-12: 29.4C; 5-19: 24.7C; RH – 3-19: 39%; 4-12: 20%; 5-19: 61%; wind – 3-19: 7 mph; 4-12: 11 mph; 5-19: 2.9 mph; type of fire – back fire; length of time of burn – 3-19: 5 min; 4-12: 7 min; 5-19: 9 min.; moisture content of oak leaves and or pine needles used as supplement fuel – 3-19: 8%; 4-12: 13%; 5-19: 20%). The burned plants were transferred to the greenhouse the same day. After 48 hr, new shoots began to emerge.

All creeping bluestem were grown in 3.8- or 11.4-liter pots in full sun or under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended with (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

Plants that were burned:

- Creeping bluestem from south central Florida; propagated by division of original three plants
- Tissue-cultured progeny of creeping bluestem: planted 2/18/98 and repotted 4/13/98

Explants were excised from the basal 1-cm portions of young shoots that emerged after plants were burned.

Studies were initiated to determine optimal type of tissue explant to use for embryogenic callus initiation, but first a new sterilization procedure needed to be worked out because soaking the explants in 0.5% mercuric chloride for 5 min (the sterilization protocol that resulted in the least contamination as determined from the previous study) apparently killed the explants based on results of tetrazolium (TZ) tests. The use of TZ salt solutions is used to testing seed viability (Grabe). The TZ salt is an oxidation-reduction indicator. Tissues that are living and respiring react with TZ to form a reddish pink compound. After the TZ solution (pH range 6-8) is imbibed by the tissue it interacts with, the reduction processes of living cells and accepts hydrogen from the dehydrogenases. By hydrogenation of the 2,3,5- triphenyl tetrazolium chloride, a red, stable substance, triphenyl formazan, is produced in living cells. This makes it possible to distinguish the red-colored living parts of the tissue/seed from the colorless dead ones (Moore 1966).

Tetrazolium is rarely used in tissue culture to assess explant or cell viability, and a literature search revealed no instances in which TZ was used to assess tissue viability after surface sterilization. However, Nyange and others (1997) used the TZ dye MTT in cell suspension cultures of *Coffea arabica* to assess susceptibility of *Coffea arabica* genotypes to coffee berry disease.

While evaluating the sterilization methods, microbial contamination was assessed by incubating treated explants in three selective media – AC Broth (wide variety of microorganisms), YE Broth (bacteria), and SA Broth (molds/yeasts). The three controls were no tissue in tube, untreated tissue in tube, and dead sterile tissue (treated with 90% EtOH for 30 min) in tube.

Tissues evaluated:

- Leaf tissue from the basal 1-cm portions of young shoots that emerged after plants were burned
- Shoots were excised and rinsed in water. Outer leaf sheaths were removed to gain access to the cream-colored tissue at the shoot/root interface. Three pieces of each of three types of explants (2-3 cm leaf tissue just above interface, 4-6 mm section at interface, 2-3 cm rhizome section [1-2 mm diam.] excised from just below shoot/root interface) were collected.
- Immature inflorescences (August 1999 – October 1999)

- Mercuric chloride treatments:

0.5% $HgCl_2$	1 min
0.5% $HgCl_2$	0.5 min
0.4% $HgCl_2$	1 min
0.4% $HgCl_2$	0.5 min
0.3% $HgCl_2$	1 min
0.3% $HgCl_2$	0.5 min
0.2% $HgCl_2$	1 min
0.2% $HgCl_2$	0.5 min
0.1% $HgCl_2$	1 min
0.1% $HgCl_2$	0.5 min
100% Clorox	5 min
4-6% NaOCl	5 min

In separate studies:

- Shoots were excised and rinsed in water tap water for 60 min. Outer leaf sheaths were removed to gain access to the cream-colored tissue at the shoot/root interface. Three pieces of each of three types of explants (2-3 cm leaf tissue just above interface, 4-6 mm section at interface, 2-3 cm rhizome section [1-2 mm diam.] excised from just below shoot/root interface) were collected.
- Immature inflorescences (from August 1999 – October 1999); rinsed in tap water for 60 min
- 'Tucker-grade', a clone provided by R. Kalmbacher (CBS TG 001B) – root-shoot interface (5-6 mm at interface) (plant CBS TG 00 1B)

Explants were immersed in 90% ethanol for a few seconds and then immersed in 100% Clorox (containing 2 drops of Tween 20 [Fisher Scientific, St. Louis, MO]) for 30 min. The explants were then washed with sterile distilled water 5X @ 5 min.

The media evaluated for callus initiation are listed below. There were five plates (replicates) per treatment, with three to five explants per plate. Percentage was calculated as an average of the replicates. Explants were cultured in the dark at 25C. Tucker grade root-shoot explants were evaluated in Media *H* to *M* because of limited plant material.

G. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + $ZnSO_4$ (5.2 μM) + CH (500 mg/L) + 10% GCM + 0.3%
H. MS + 2,4-D (0.5 mg/L) + 10% GCM + 0.3% Phytagel
I. MS + 2,4-D (1.0 mg/L) + 10% GCM + 0.3% Phytagel
J. MS + 2,4-D (1.5 mg/L) + 10% GCM + 0.3% Phytagel
K. MS + 2,4-D (2.0 mg/L) + 10% GCM + 0.3% Phytagel
L. MS + 2,4-D (2.5 mg/L) + 10% GCM + 0.3% Phytagel
M. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.3% Phytagel
N. MS + 2,4-D (0.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
O. MS + 2,4-D (1.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel

P. MS + 2,4-D (1.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
Q. MS + 2,4-D (2.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
R. MS + 2,4-D (2.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
S. MS + 2,4-D (3.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel

Reinitiation of Cell Suspension Cultures

- Embryogenic callus of B-1 creeping bluestem in culture since March 1998

Embryogenic callus (initiated from immature inflorescence) growing in the dark at 25^0C, which was maintained on MS + 2,4-D (3.0 mg/L) + 5% (by vol.) coconut milk (CM) + 0.7% Phytagel since 1998, was used to initiate suspension cultures. Pieces of embryogenic callus, each *ca.* 200-mg (subdivided 1-g pieces), were placed in separate 125-ml flasks containing 40 ml of one of the 19 media listed below. Flasks were shaken at 150 rpm in darkness at 25C. Subculturing was done every fifth day. Finely dissociated embryogenic cells were selected for embryoid formation cultures by filtering the suspension through a sterile nylon sieve (100 μm pore size). One ml of the resultant filtered suspension that contained these finely divided embryoid cells was added to 40 ml of fresh medium (per flask) for growth and further selection. Cell suspensions containing embryoids were maintained by subculture at a 1:4 dilution (suspension inocula:fresh medium) using the same methods.

V. MS + 2,4-D (0.5 mg/L) + 10% GCM
W. MS + 2,4-D (1.0 mg/L) + 10% GCM
X. MS + 2,4-D (1.5 mg/L) + 10% GCM
Y. MS + 2,4-D (2.0 mg/L) + 10% GCM
Z. MS + 2,4-D (3.0 mg/L) + 10% GCM
AA. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + 10% GCM
BB. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
CC. MS + 2,4-D (2.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
DD. MS + 2,4-D (3.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
EE. MS + 2,4-D (0.5 mg/L) + zeatin (0.2 mg /L) + 10% GCM
FF. MS + 2,4-D (0.2 mg/L) + BAP (0.2 mg/L) + ABA (1.0 mg/L) + 10% GCM
GG. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + ABA (2.0 mg/L) + 10% GCM
HH. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + ABA (3.0 mg/L) + 10% GCM
II. MS + 2,4-D (1.5 mg/L) + zeatin (2.0 mg/L) + ABA (0.2 mg/L) + 10% GCM
JJ. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
KK. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
LL. MS + 2,4-D (2.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
MM. MS + 2,4-D (3.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
NN. MS + 2,4-D (1.5 mg/L) + BAP (0.2 mg/L) + GA_3 (0.2 mg/L) + zeatin (0.5 mg/L) + 10% GCM

Plant Regeneration on Solid Media

- Reinitiated suspension cultures (see above) derived from embryogenic callus (derived from immature inflorescence) of creeping bluestem B-1 growing on solid media culture since March 1998
- Embryogenic callus derived from leaf tissue of plant PB 98 3A
- Callus derived from rhizome tissue
- Callus derived from root-shoot tissue of Tucker grade

Determining If Callus Was Embryogenic

Evaluating media for plant regenerative ability served a dual purpose – plant regeneration as well as to determine if callus was embryogenic. If callus was embryogenic, then coleoptile formation would be observed.

For suspension cultures, 1 ml of finely divided cells (putative embryoids) from Medium *NN* was plated out onto one of the solid medium listed below. Callus was checked twice per week for embryo formation (as evidenced by coleoptile formation observed under a dissecting scope). Clumps of callus (approx. 100 mg) growing on solid media and derived from leaf tissue (PB 98 3A) were also subcultured onto these media and checked as above for embryo formation. For each type of culture (suspension/solid), there were five single plate replicates per culture medium.

H. MS + 2,4-D (0.5 mg/L) + 10% GCM + 0.3% Phytagel
I. MS + 2,4-D (1.0 mg/L) + 10% GCM + 0.3% Phytagel
J. MS + 2,4-D (1.5 mg/L) + 10% GCM + 0.3% Phytagel
K. MS + 2,4-D (2.0 mg/L) + 10% GCM + 0.3% Phytagel
M. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.3% Phytagel
OO. MS + 2,4-D (0.2 mg/L) + 10% GCM + 0.3% Phytagel
PP. MS + ABA (0.264 mg/L) + 10% GCM + 0.3% Phytagel
QQ. MS + TDZ (0.2 mg/L) + 10% GCM + 0.3% Phytagel
RR. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + 10% GCM + 0.3% Phytagel
SS. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + 10% GCM + 0.3% Phytagel
TT. MS + 2,4-D (2.0 mg/L) + BAP (0.1 mg/L) + 10% GCM + 0.3% Phytagel
UU. MS + 2,4-D (3.0 mg/L) + BAP (0.1 mg/L) + 10% GCM + 0.3% Phytagel
VV. MS + 2,4-D (0.5 mg/L) + zeatin (0.2 mg /L) + 10% GCM + 0.3% Phytagel
WW. MS + 2,4-D (1.5 mg/L) + BAP (0.2 mg/L) + GA_3 (0.2 mg/L) + zeatin (0.5 mg/L) + 10% GCM + 0.3% Phytagel

Scanning Electron Microscopy (SEM). Callus in which coleoptile formation was observed was subjected to SEM to confirm its embryogenic nature. Callus was fixed in FAA for 48 hr. The processing started in 50% ethanol and continued through 75%, 95% and 100% ethanol, with 10 min at each step. Samples were then placed in fresh 100% ethanol overnight at 4C. Samples were brought back to room temperature and critical point dried by CO_2 on a Balzers CPD 0-30 (Balzers, Germany). They were

mounted and sputtered with gold (20 nm gold sputtered) on an Eiko Engineering IB-4 sputter coater. The samples were viewed on a Hitachi S-4000 Field emission scanning electron microscope (Tokyo, Japan) to check for the presence of scutella and/or coleoptiles.

Plant Regeneration and Development

Clumps of mature embryos were transferred to MS + 10% CM + 0.3% Phytagel and grown in the dark at 25C. Some shoots and roots formed after 2 to 3 weeks. Further plantlet development was carried out on different media in Petri plates (25 plates per medium, with five replicates of five plates each per medium; one clump of mature embryos was subdivided into 5 pieces for each replicate) in a growth chamber (25C; 16-hr photoperiod; light provided by cool fluorescent bulbs, with light level of 40μE at Petri plate level).

K. MS + 2,4-D (2 mg/L) + 10% GCM + 0.3% Phytagel
M. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.3% Phytagel
OO. MS + 2,4-D (0.2 mg/L) + 10% GCM + 0.3% Phytagel
WW. MS + 2,4-D (1.5 mg/L) + BAP (0.2 mg/L) + GA_3 (0.2 mg/L) + zeatin (0.5 mg/L) + 10% GCM + 0.3% Phytagel
XX. MS + 2,4-D (3 mg/L) + zeatin (0.5 mg/L) + 10% GCM + 0.3% Phytagel
YY. MS + 2,4-D (3 mg/L) + zeatin (0.5 mg/L) + CH 500 mg/L + 10% GCM + 0.3% Phytagel
ZZ. MS + 2,4-D (1.5 mg/L) + zeatin (2.0 mg/L) + ABA (0.2 mg/L) + 10% GCM + 0.3% Phytagel
AAA. MS + 2,4-D (0.2 mg/L) + TDZ (0.2 mg/L) + 10% GCM + 0.3% Phytagel
BBB. MS + 2,4-D (0.2 mg/L) + TDZ (0.2 mg/L) + 10% GCM + 0.3% Phytagel + 0.1% AC
CCC. MS + 2,4-D (0.2 mg/L) + BAP (0.2 mg/L) + ABA (1 mg/L) + 10% GCM + 0.3% Phytagel
DDD. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + ABA (2.0 mg/L) + 10% GCM + 0.3% Phytagel
EEE. MS + 2,4-D (1.0 mg/L) + BAP (0.5 mg/L) + ABA (2.0 mg/L) + 10% GCM + 0.3% Phytagel
FFF. MS + 2,4-D (0.2 mg/L) + BAP (0.2 mg/L) + NAA (2 mg/L) + 10% GCM + 0.3% Phytagel
GGG. MS + 2,4-D (0.2 mg/L) + ABA (1 μM) + 0.1% AC + 10% GCM + 0.3% Phytagel
HHH. MS + 2,4-D (1.5 mg/L) + BAP (0.2 mg/L) + GA_3 (0.2 mg/L) + zeatin (0.5 mg/L) + 10% GCM + 0.3% Phytagel
III. MS + 2,4-D (3.0 mg/L) + BAP (1.0 mg/L) + GA_3 (1.0 mg/L) + CH 500 mg/L + 10% GCM + 0.3% Phytagel
JJJ. MS + 2,4-D (3.0 mg/L) + BAP (1.0 mg/L) + GA_3 (1.0 mg/L) + NAA (0.5 mg/L) + CH 500 mg/L + 10% GCM + 0.3% Phytagel

Production via Shoot Multiplication Culture

When regenerated plantlets filled the Petri plates (2-3 weeks), they were transferred to glass jars (diameter - 6.2 cm; height - 10.8 cm; vol. - 237 ml) and placed in a growth chamber growth chamber (25C; 16-hr photoperiod; light provided by cool white fluorescent light).

To determine the best medium for shoot multiplication culture, regenerated plantlets were cultured on Medium *III* in Petri plates for further development. When the tiny shoot clusters filled the Petri plate (~3weeks), clusters were subcultured on the following five media (15 jars per media) to evaluate shoot multiplication rates.

M. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.3% Phytagel
KKK. MS + 2,4-D (3 mg/L) + 10% CM + 0.1% AC + 0.3% Phytagel
LLL. MS + 2,4-D (3 mg/L) + zeatin (0.5 mg/L) + kinetin (3 mg/L) + 10% CM + 0.1% AC + 0.3% Phytagel
MMM. MS + 2,4-D (3 mg/L) + BA (1.0 mg/L) + GA3 (1 mg/L) + kinetin (2 mg/L) + zeatin (0.5 mg/L) + ZnSO4 (5.2 μM) + CH 500 mg/L + 10% GCM + 0.3% Phytagel
NNN. MS + zeatin (0.5 mg/L) + NAA (1 mg/L) + 10% CM + 0.3% Phytagel

There were one to two clusters of shoots per 237-ml jar. Shoot multiplication rate was recorded every 4 weeks over a 20-week period, and the clusters of shoots subdivided into two or more clumps to maintain 15 shoots in a larger jar (diameter 7.8 cm., height 13 cm., vol. 472 ml; Ball, U.S.A).

Effect of Light Quality on Shoot Multiplication Rate

Shoot clusters (3-4 cm high, 2-3 tillers per clump) in this experiment had been maintained on Medium *MMM* (subcultured every 4 weeks) in 237-ml jars. Each jar had two clumps and there were 10 replicates per light treatment. The light treatments were:

- Fluorescent Cool White bulbs (two 20W tubes per shelf; Color Rendering Index (CRI)* = 68; spectrum 500-600 nm; Phillips Lighting Company, Somerset, N.J.)
- Verilux F20T12 SUN (two 20W tubes per shelf; CRI = 94.5; spectrum 400-700 nm; Verilux Inc., Stamford, CT)
- Agrosun BUAG (two 20W tubes per shelf; CRI = 93; spectrum 430-660 nm; Hydrofarm Gardening Products, Petaluma, CA)
- Phillips F20T12 AGRO-INFLR (two 20W tubes per shelf; CRI = 97; spectrum 550-660 nm; Phillips Lighting Company, Somerset, N.J.)

* CRI for natural light = 100.

Cultures were positioned 6 cm from the incubator wall and 12 cm from the back of the incubator on each shelf. The light intensity maintained per shelf was 65µE, with a 16-hr photoperiod. The effect of light quality on shoot multiplication rate was evaluated after 16 weeks. The temperature inside the incubator was 25 ± 1^{0}C. Cultures were subcultured on fresh medium every 4 weeks.

Acclimatization to Greenhouse and Ambient Conditions

- Shoots of bluestem regenerated via somatic embryogenesis (new line of embryogenic callus developed in Study 2; origin of callus was immature inflorescence embryogenic callus from Study 1).

Extensive studies were conducted to determine methods of rooting shoots generated from multiplication culture (except as noted), and then acclimatizing these rooted plantlets to greenhouse and ambient (outdoor) conditions. Shoots were rooted in vitro, and then acclimatized, or they simultaneously were rooted and acclimatized under greenhouse conditions before being acclimatized to ambient conditions.

Rooting In Vitro and Acclimatization In Vivo

Experiments were conducted on three occasions (experiments started on Jan. 25, March 15, and October 8, 2001) to evaluate in vitro rooting of shoot clusters (2-3 tillers per cluster; 3-4 cm tall) followed by acclimatization under greenhouse conditions. There were four clusters per 275-ml jar (replicate) and five replicates per medium. There was no coconut water in any media.

OOO. MS1/2 (1/2 =half strength) + 0.3% Phytagel
PPP. MS + 0.3% Phytagel
QQQ. MS1/2 + NAA (0.05 mg/L) + 0.3% Phytagel
RRR. MS + NAA (0.05 mg/L) + 0.3% Phytagel
SSS. MS1/2 + NAA (0.1 mg/L) + 0.3% Phytagel
TTT. MS + NAA (0.1 mg/L) + 0.3% Phytagel
UUU. MS1/2 + NAA (0.2 mg/L) + 0.3% Phytagel
VVV. MS + NAA (0.2 mg/L) + 0.3% Phytagel
WWW. MS1/2 + NAA (0.4 mg/L) + 0.3% Phytagel
XXX. MS + NAA (0.4 mg/L) + 0.3% Phytagel
YYY. MS1/2 + IBA (0.05 mg/L) + 0.3% Phytagel
ZZZ. MS + IBA (0.05 mg/L) + 0.3% Phytagel
AAAA. MS1/2 + IBA (0.1 mg/L) + 0.3% Phytagel
BBBB. MS + IBA (0.1 mg/L) + 0.3% Phytagel
CCCC. MS1/2 + IBA (0.2 mg/L) + 0.3% Phytagel
DDDD. MS + IBA (0.2 mg/L) + 0.3% Phytagel
EEEE. MS1/2 + IBA (0.4 mg/L) + 0.3% Phytagel
FFFF. MS + IBA (0.4 mg/L) + 0.3% Phytagel

IBA - indolebutyric acid

The cultures were grown on a light stand [two 20W cool white fluorescent lights (Phillips Lighting Company) per shelf; 65μE; ~25C]. Percent survival and in vitro rooting were evaluated at 4 weeks, immediately prior to acclimatization in the greenhouse.

Surviving shoot clusters were then acclimatized in root trainer folding trays (78 ml/well, Stuewe and Sons, Inc. Corvallis, OR) containing only MetroMix 200 (Scotts) amended with (per m^3) 1362 g 18N-2.6P-10.6K (Osmocote 18-6-12, 8-9 month formulation; Scotts), 942 g Micromax (Scotts), and 61 g sublimed sulfur (Fisher). Root trainer folding trays are very similar to tubeling trays in dimensions except that root trainer trays can be opened like the page of a book to study the root system without disturbing it. The root trainers were placed on propagation mat (56 x 152 cm; Hummert International, Earth City, MO) and the temperature was maintained at 28C. For the first week, mist frequency 6.5 sec every 5 min, and for the next 3 weeks mist frequency was 2.5 min once per day. Light level in the greenhouse was 30% of ambient. After 4 weeks, misting was halted. Root systems of at least 10 plants (remaining plants used for field evaluation in some cases) were evaluated 4 weeks later for number of tillers, length of longest shoot, basal clump diameter, presence or absence of root ball, shoot dry weight, root dry weight, presence or absence of root ball, whether the root ball held together when removed from the tray, and percent survival; some parameters were not recorded in all experiments. We evaluated whether the root ball held together since that would affect transplanting to the field. In Experiment 1, lateral root systems were rated on a 1 to 5 scale as follows:

1 = No lateral roots
2 = One or two short lateral roots
3 = More than two lateral roots, but none long
4 = Some long lateral roots
5 = Many long lateral roots

Rooting In Vivo /Acclimatization In Vivo

Tissue-Cultured Shoots Vs. Divisions of Container-Grown Plants. Tissue-cultured shoots regenerated from somatic embryos and divisions from containerized plants (same clone as tissue-cultured shoots) were treated with Rootone F® (0.2% 1-naphthaleneacetamide, 4.04% tetramethylthiuramidsulfide [Thiram]; Rhône-Poulenc, Research Triangle Park, NC) and planted in 160-ml cell packs (height 6 cm, width 6 cm, depth 5.5 cm) in a medium comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol), or pine bark:Canadian sphagnum peat, 1:1 (vol/vol). Propagules were placed under mist (6.5 sec every 5 min, 24 hr/day for 3 days) in a greenhouse under 30% light exclusion cloth (~600 μE at 1200 HR). The greenhouse temperature ranged from 21 to 32°C. After 2 weeks under mist in the greenhouse, plants were repotted in standard 1.5-liter plastic nursery pots (The Lerio Corp., Mobile, AL) containing a soilless medium comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol) amended with (per

m^3) 1362 g 18N-2.6P-10.6K (Osmocote 18-6-12, 8-9 month formulation; Scotts, Marysville, OH), 942 g Micromax (Scotts), and 61 g sublimed sulfur (Fisher), and moved to a shadehouse (30% light exclusion). Acclimatization experiments were run three times, with 15 replicates per propagule type per medium at each time. Percent survival was recorded 2 weeks after the plants were placed under mist in the greenhouse and 2 weeks after being transferred to the shadehouse.

Effect of Medium, Tiller Height, Rooting Growth Regulator, and Fertilizer on Rooting and Survival. Rooting and survival of in vitro grown shoot clusters (2-4 tillers per cluster) – 1-2 or 3-5 cm tall – were evaluated under greenhouse conditions in a factorial experiment arranged in a completely random design. There were five replicates per treatment.

- MetroMix 200
- MetroMix 200 + Rootone F
- MetroMix 200 + Fertilizer
- MetroMix 200 + Fertilizer + Rootone F
- Pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol)
- Pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol) + Rootone F
- Pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol) + Fertilizer
- Pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol) + Rootone F + Fertilizer

Fertilizer: (per m^3 of medium; incorporated) 3560 g Osmocote 18N-2.6P-10K (18-6-12, 8-9 month formulation) + 1411 g Micromax + 159 g sublimed sulfur

Shoot clusters harvested from in vitro cultures were planted in tubeling trays (one cluster per 98-ml cell; 5 X 5 cm, depth 4.5 cm; 96 cells/tray) in one of the media listed above. The trays of clusters were placed under mist (6.5 sec/5 min, 24 hr/day) for 2 weeks. Environmental conditions in the greenhouse were: 30% shade (~776 μE at 1200 HR); natural light; 21 to 32°C. After 2 weeks, the clusters were removed from the mist bed but remained in the greenhouse. Two weeks later, survival, rooting (number of primary roots; lateral root rating) and vigor of the shoot clusters were recorded. Lateral root systems were rated on a scale of 1 to 5, where

1 = No lateral roots
2 = One or two short lateral roots
3 = More than two lateral roots, but none long
4 = Some long lateral roots
5 = Many long lateral roots

Plant vigor was rated as follows:

- D = Plant is dead
- P = Growth is poor, looks lanky and not healthy
- F = Growth is fair

- G = Growth is good, looks healthy and vigorous

Effect of Tiller Number, Growth Regulators, and Mist Frequency on Rooting and Survival. Rooting and survival of in vitro grown shoot clusters (3-4 cm tall) – 1-2 or 3-4 tillers per cluster – were evaluated under greenhouse conditions in a factorial experiment that was repeated one month later. There were five replicates per treatment.

Unrooted shoots clusters composed of 1-2 tillers or 3-4 tillers were dipped (base of shoot cluster) into one of the rooting powders listed below and then planted in tubeling trays containing Metromix 200 amended with (per m^3) 3560 g 18N-2.6P-10.6K (Osmocote 18-6-12, 8-9 month formulation), 1411 g Micromax, and 159 g sublimed sulfur. Growth regulator x tiller density treatments were completely randomized within a tray. Trays were placed under one of two mist frequencies - 6.5 sec/5 min or 6.5 sec/10 min. Shoot clusters were under the mist for 24 hr/day for the first 2 weeks, and then from 6 AM to 8 PM and 11 PM to 2 AM for the second 2 weeks.

- None
- Rootone F
- Hormex rooting powder #1 (0.1% Indole-3-Butyric acid)
- Hormex rooting powder #3 (0.3% Indole-3-Butyric acid)
- Hormex rooting powder #8 (0.8% Indole-3-Butyric acid)
- Hormex rooting powder #16 (1.6% Indole-3-Butyric acid)
- Hormex rooting powder #30 (3.0% Indole-3-Butyric acid)
- Hormex rooting powder #45 (4.5% Indole-3-Butyric acid)

Environmental conditions in the greenhouse were as follows: 30% shade (~776 μE at 1200 HR); natural light; 21 to 32C. After 4 weeks, rooting (number of primary roots, lateral root rating, length of longest root), number of tillers, length of longest shoot, and percent survival were recorded. Lateral root systems were rated on a 1 to 5 scale as follows:

1 = No lateral roots
2 = One or two short lateral roots
3 = More than two lateral roots, but none long
4 = Some long lateral roots
5 = Many long lateral roots

Longest shoot and root length were recorded in one experiment.

Effect of Fertilizer Type on Rooting and Survival. Rooting and survival of in vitro grown shoot clusters (3-4 cm tall; 1-2 tillers per cluster) were evaluated under greenhouse conditions in an experiment arranged in a completely random design.

Fertilizer treatments

- None
- (per m^3) 2.4 kg Osmocote 18N-2.6P-12K (18-6-12, 8-9 month formulation)

- (per m^3) 2.4 kg Osmocote 15N-9P-12K (15-9-12, 5-6 month formulation)
- (per m^3) 2.4 kg Osmocote 14N-14P-14K (14-14-14, 3-4 month formulation)
- Water-soluble 21N-5P-20K (0.476 g/L yielded 100 ppm Nitrogen)
- Water-soluble 24N-8P-16K (0.416 g/L yielded 100 ppm Nitrogen)
- Water-soluble 15N-30P-15K (0.665 g/L yielded 100 ppm Nitrogen)

Shoot clusters were transplanted in tubeling trays containing MetroMix 200, some of which was amended with controlled release fertilizer noted above. The water-soluble fertilizer (50 ml/plant) (Peters Professional, U.S.A.) was applied once per week. Shoot clusters were placed under a mist frequency of 6.5 sec/5 min for 24 hr/day for the first 2 weeks. For the second 2 weeks, mist frequency was 6.5 sec/10 min from 6 AM to 8 PM and 11 PM to 2 AM. There were 12 replicates per treatment.

Environmental conditions in the greenhouse were as follows: 30% shade (~776 μE at 1200 HR); natural light; 21 to 32C. After 6 weeks, rooting (number of primary roots, lateral root rating), number of tillers, and survival were recorded. Lateral root systems were rated on a 1 to 5 scale as follows:

1 = No lateral roots
2 = One or two short lateral roots
3 = More than two lateral roots, but none long
4 = Some long lateral roots
5 = Many long lateral roots

Greenhouse and Field Evaluation of Tissue-Cultured Plants

Growth Under Greenhouse Conditions

(Edited excerpt from manuscript submitted to Native Plants Journal)

On Aug. 23 2000, ~50 kg each of sand tailings and overburden soils (upper 15 cm) were collected from CF Industries in Ft. Green, FL (areas DB3 and DB6, respectively) and taken to the Range Cattle REC (RCREC) in Ona. At the RCREC, 50 kg of the A1 horizon of a Myakka fine sand (sandy siliceous hyperthermic aeric Alaquods) was collected from a soil characterization site (Robins and others 1984). Soils were treated with methyl bromide to eliminate weed seeds and any possible mycorrhizal contributions from the native Myakka soil. On Sept. 27, pots were filled with each soil. A sample of each soil was saved for texture analyses (Soil Characterization Lab. Univ. of Florida, Gainesville), and for analysis of organic matter, pH, P, K, Ca, Mg, Cu, Fe, Mn, and Zn at the Univ. of Florida, Analytical Research Lab.

On Oct. 10, 30-40 creeping bluestem inflorescences were collected from a native area at the RCREC. A relatively large amount of material was needed to obtain sufficient caryopses, which were separated from the lemmas and paleas with a Woodward

Laboratory air-seed cleaner (Aaron's Engineering, Fargo, OK). On Nov. 6, caryopses were germinated on each of the three soils, which had been moistened, in Petri plates (15 7.5-cm diameter dishes; 10 caryopses per plate). Germination was under a 12-hr photoperiod – 28C (dark)/20C (light). After 28 days, seedlings were counted, and the soil and rooted seedlings were lifted intact from the Petri dishes and placed in pots with the three soils. Pots with seedlings received 6 sec mist every 5 min for about 2 wk until they were established. They then were irrigated three to four times daily from an overhead water trolley. Seedlings were not fertilized.

On Jan. 24, 2001, rhizome sections (40-50 cm long), each containing some roots and several internodes, were placed in containers (98 mL Ropak Multi-pots, Stuewe & Sons, Inc. Corvallis, OR) filled with 4P Fafard mix (40% sphagnum, 30% perlite, 30% pine park). This ecotype had been collected in Polk County in 1984 and had been growing in a nursery at the RCREC (Kalmbacher and others 1991). It was noted to be a strongly rhizomatous, robust type with no observed seed production. These plants were referred to as division plants, and during development were not fertilized but were watered on the same regime as seedlings.

On March 5, tissue-cultured (rooted and acclimatized plantlets from shoot multiplication culture) and division plants were transplanted in individual 15-cm pots, and seedlings were thinned to a single plant in each pot. The 45 pots (five replicates of the three soils each with the three sources of creeping bluestem) were randomized on a greenhouse bench. Pots were overhead irrigated until July 27 when inflorescences were tall enough to interfere with the trolley. After July 27, pots were placed in saucers and bottom watered. They were not fertilized.

On Oct. 23 (232 days after transplanting), reproductive and vegetative tillers were counted. Plants were cut near the soil surface (2-3 cm), and top growth was dried (60C for 72 hr) and weighed. Dried vegetative tillers of similar size were composited over replications because of the limited amount of dry matter. Tissue was ground and analyzed for minerals at Waters Agricultural Laboratory, Camilla, GA. Two cores (2.5-cm diam. x 15-cm deep) were taken from each pot and analyzed for pH and minerals as described above. Soil was washed from roots and rhizomes, and they were dried and weighed.

Seed germination, tiller number, and plant mass data were analyzed with using a generalized linear model procedure of SAS for a completely randomized design. Means for main effects and interactions were separated with the PDIFF option.

Growth and Survival Under Field Conditions

An upland field plot (10.4 m x 6.7 m) at the NFREC-Monticello was treated with Roundup 2 weeks prior to planting. The experimental design was completely randomized, with 32 tiller number X rooting compound treatments from a previous in vivo rooting study (*Effect of Tiller Number, Growth Regulators, and Mist Frequency on*

Rooting and Survival) and five replicates per treatment. Creeping bluestem were planted (61 cm on-center) on Aug. 28, 2000. Although 160 plants were initially acclimatized in the greenhouse, 50 died and only 110 were field planted. The plot was mulched with pine straw (2.5 to 3.0 cm deep). Except for 1.9 cm of irrigation applied immediately after planting, the plants did not receive any additional supplemental irrigation. The plants were evaluated 3 months after planting for percent survival, shoot length, clump diameter, and number of tillers. Fourteen months after planting, number of tillers, shoot length, and clump diameter were recorded. Dry biomass was determined for main shoot and rhizomes at 14 months.

A similar evaluation was started at the RCREC in Ona on July 16, 2000. Ninety creeping bluestem were transplanted 25 cm on-center in a flatwoods range in 1.2 m X 1.2 m cages. No Roundup was used prior to planting. The experimental design was completely randomized, with the same 32 treatments as at the NFREC and five replicates per treatment. No mulch was used and no supplemental irrigation was provided (because it was a flatwoods a site). At 4 and 12 months after planting, number of tillers, shoot length, and clump diameter were recorded. Dry biomass was determined for main shoot and rhizome at 12 months.

Growth and Survival Under Mine Site Conditions

(Edited excerpt from manuscript submitted to Native Plants Journal)

Sand tailings and overburden sites were selected at CFI mine in Ft. Green, FL. These were the same mining areas (DB3 and DB8), but not the exact locations, where soils were collected in August 2000. Sand tailings had not been reclaimed. Plant cover on sand tailings was sparse, with scattered drought-tolerant plants like natalgrass (*Melinis repens*), Mexican tea (*Chenopodium ambrosioides*), and rough hairy indigo (*Indigofera hirsuta*) on higher areas, and southern crabgrass (*Digitaria ciliaris*), broomsedge (*Andropogon virginicus*), dogfennel (*Eupatorium capillifolium*), shyleaf (*Aeschynomene americana*), coffeebean (*Sesbania emerus*) where it graded into a lower adjacent area. The overburden site had been reclaimed over a 2-year period by direct planting of containerized native species or by seeding from a donor site (John Kiefer, CF Industries, personal communication, 2002). Aside from a few scattered native plants that were present, most of the dense ground cover consisted of hairy indigo, bahiagrass, (*Paspalum notatum* Fluegge), guineagrass (*Urochloa maxima*), crabgrass, etc. Both sites were prepared by mowing and spraying with glyphosate (Roundup) at 9.4 L/ha. Areas were resprayed with the same rate of glyphosate on the day of planting.

Within each site, tissue-cultured creeping bluestem was planted on July 27 and October 16 2001, and January 24, 2002. Both sites had ~3% slope which provided a moisture gradient. At each date, there were six rows, 1-m apart, and each row was 50-m long and oriented from top to bottom of the slope. Double rows were planted, with plants spaced 0.5, 1.0, or 1.5-m apart in the rows. Variable spacing was used to provide insight on planting density through evaluation of long-term plant coverage, which is beyond the

scope of this report. The July 2001 planting was fertilized on August 10, 2001 with the equivalent of 56 kg N/ha applied as ~50 mL of an ammonium nitrate solution to individual plants. All plantings received 56 kg N/ha on June 11and August 6, 2002.

Chemical weed control was essential on both sites – Weedmaster (WM) (0.12 kg/L dicamba + 0.34 kg/L 2, 4-D) at 2.3 L/ha, Plateau (P) (imazapic) at 0.3 or 0.4 L/ha, and Remedy (R) (triclopyr) at 0.9 L/ha. On sand tailings, the July planting was sprayed on April 9 (WM) and August 6, 2002 (P). The October planting was sprayed on December 3, 2001 (P), and both the October and January plantings were sprayed on April 9 (WM), June 11 (P), and August 6, 2002 (P). On overburden, the July planting was sprayed on December 3, 2001 and April 9 (WM), June 6 (WM, P), August 6 (P), and September 23, 2002 (R). The October planting was sprayed on December 3, 2001 (P) and January 25, 2002 (WM), and both the October and January plantings were sprayed on April 9 (WM), June 11 (P), August 6 (WM), and September 23, 2002 (R).

Ten soil cores were taken (2.5 cm diam. x 15-cm depth) and composited from the upper and lower portions of each site on the days of planting. Soil was analyzed for pH, P, K, Ca, Mg, Cu, Fe, Mn, and Zn (Mehlich-I extractable) at the Univ. of Florida, Analytical Research Laboratory (Hanlon and Devore 1989). Soil organic matter contents were determined on samples taken at planting.

Plant survival for each date was determined at 100 days after planting. On October 28, 2002, plant diameter, as indicated by extent of rhizome (tiller) extension, and survival was measured on all plants. On the October planting, which was selected because it represented 1-year growth, vegetative and reproductive (inflorescences) tiller numbers were counted at each of the 1-m spaced plants. There, every third plant was cut at the soil surface, dried (60C for 120 hr), and weighed.

On October 25, 2001, vegetative tillers of uniform size in the July planting were cut at the soil surface, dried, and ground for mineral analyses. For comparison, similar tillers were clipped on the same day at the RCREC on the native Myakka fine sand site. Vegetative tillers of uniform size from the 1-m spaced plants sampled on October 28, 2002 were composited to form five samples that were ground and analyzed for minerals (Water's Lab., Camilla, GA).

On April 24, 2002, penetrometer resistance was determined at 5-cm increments into the soil starting at the soil surface down to 45-cm deep at each of the 1-m spaced plants (n=100) in the October planting on both sites. Penetrometer data were analyzed with the MIXED procedure in a model that included site, depth, and site x depth interaction (SAS 1999).

Plant survival from October 2002 was analyzed with the CATMOD procedure (SAS 1999), which models categorical data by fitting linear models to functions of response frequencies. The model for plant survival included site, date of planting, and their interaction. Plant diameter and tiller number were analyzed with the MIXED procedure in a model that included site, date of planting and their interaction (SAS 1999).

In analyses of plant survival, tiller number, and penetrometer resistance, blocks were established by assigning observations based on position from the top to bottom of the gradient as described above. Thus, randomization was restricted because at each planting date a row, which was sectioned into blocks, ran continuously from top to bottom of the gradient.

Production of Synthetic Seed

- Creeping bluestem embryos – derived from embryogenic callus generated from immature inflorescences of south central Florida ecotype (B-1)
- Creeping bluestem – unknown ecotype; seed

Embryos at various stages of development (1or 2 leaves barely visible, or leaves 2-3 mm long) were put into a 50-ml beaker containing a sterile solution of sodium alginate (prepared with 50% MS media + 10 μm GA_3 [cold sterilized; added after autoclaving]). Embryos were dropped one by one into 50 mM calcium chloride using a plastic disposable pipette with the lower 1.5 cm cut off to make a large bead (about 3-4 mm). The exposure time in the $CaCl_2$ solution was 35 to 40 minutes. The encapsulated embryos were rinsed four times (20 min per rinse) in sterile distilled water. After the final rinse, the synthetic seeds were stored in a sterile Petri plate (plus a few drops water) at 4C in the dark for 10-15 days.

Greenhouse Evaluation of Synthetic Seed

- Creeping bluestem synthetic seed – derived from embryogenic callus generated from immature inflorescences of south central Florida ecotype (B-1); rhizome pieces of B-1
- Creeping bluestem – unknown ecotype; seed

Synthetic seed and 25-mm long rhizome pieces from the same ecotype were compared with sexual seed (naked caryopses which had a Petri dish germination of 50%) of an unknown creeping bluestem ecotype (collectively referred to as propagules). Two trials were repeated in a greenhouse on May 6 and June 8, 1998 at the RCREC. Greenhouse temperatures ranged from 21 to 32C, with a light level of about 600 μE at 1200 HR (30% shade cloth over greenhouse). Synthetic seed in the June 8 trial were maintained in a growth chamber at 20C. Experiments were watered by daily spraying at 1000, 1200, 1300, 1400, 1500, 1600, and 1800 HR. At each date, the three propagules were placed in three media – potting mix (a peat-based mix with added sand); Myakka fine sand (0-7-cm depth) from a native area; and mine soil from a reclaimed phosphate mine. At each date, there were three flats of each media, one for each for the three propagules (nine total).

Direct Regeneration of Creeping Bluestem

Studies were conducted to try and directly regenerate multiple creeping bluestem plantlets in vitro using different types of explants (nodes and leaves from plants that were cut back or burned and then placed in greenhouse; nodes, leaves, and inflorescences from ultra frozen tissues). There were at least three Petri plates per treatment with at least five explants per plate. The surface sterilization procedure included the use of mercuric chloride (0.5% for 1 min).

WW. MS + myoinositol (10 mg/L) + NAA (1.0 mg/L) + zeatin (0.5 mg/L) 0.1% AC + 10% GCM

XX. MS + 2,4-D (1.0 mg/L) + GA3 (1.0 mg/L) + kinetin (1.0 mg/L) + 0.1%AC + 10% GCM

YY. MS + myoinositol (10 mg/L) + NAA (1.0 mg/L) + zeatin (0.5 mg/L) + 10% GCM

RESULTS

STUDY 1

Callus Initiation from Young Leaves

All explants turned brown and died after 3 to 4 weeks.

Callus Initiation, Maintenance and Embryogenesis from Immature Inflorescences

Creeping bluestem is a good species to initiate callus. Tissue swelling was observed only 3-5 days after culturing. Callus formation was observed 2 weeks later. More white and compact embryogenic callus was formed on the medium containing 2,4-D and BA (Media *d* and *e*) than on the medium containing 2,4-D only (Media *a*, *b*, *c*, and *f*).

Embryogenic callus was maintained on medium containing 3 mg/L 2,4-D and 0.2 mg/L BA (Medium *e*) since embryogenic callus proliferated much more rapidly when BA was in the medium. Using only 2,4-D favored production of non-embryogenic callus.

Initiation, Maintenance and Embryoid Induction of Cell Suspension Cultures

Suspension cultures were comprised of two distinct types of cells: (1) non-embryogenic cells – large, vacuolated and elongated cells, with sparse cytoplasm, and devoid of any storage starch, and (2) embryogenic cells – small, generally rounded cells, that were richly cytoplasmic and contained a prominent nucleus and plastids with starch. These cells were in compact groups of many cells without intercellular spaces. At the early stage of cell suspension culture, the culture consisted predominantly of embryogenic cells. These cells divided rapidly and there was frequent fragmentation of the compact clumps into smaller units. Creeping bluestem cultures were relatively easy to maintain in this embryogenic state. Increasing the concentration of 2,4-D in 4-week-old suspension cultures to 2.5 mg/L appeared necessary for maintaining the embryogenic nature of cell suspension cultures. Cells grew more slowly but remained embryogenic in this medium.

Formation of embryoids in cell suspension cultures was observed in all four media (*g, h, j, k*). A low concentration of 2,4-D (0.5 mg/L) and additional 0.2 mg/L BA induced the cell clumps to form more embryoids. However, non-embryogenic callus grew rapidly under this condition. A higher concentration of 2,4-D (3.5 mg/L) in the medium slowed nonembryogenic callus formation. Although the higher 2,4-D concentration also reduced embryoid formation, the higher concentration seemed necessary for cell clump dissociation and maintenance of embryogenic capacity.

Embryoid Formation and Plant Regeneration on Solid Media

From Suspension Culture

More globular embryos formed when embryogenic cultures grew rapidly without subculturing, or after being transferred to growth regulator-free or reduced 2,4-D media. It was thought that a low concentration of 2,4-D was required for the organization of globular embryoids. Such a concentration (0.2 mg/L) was achieved in the first instance by allowing the culture to grow and thereby metabolize the 2,4-D present to low intra- as well as extra-cellular levels of free 2,4-D, and then by subculturing onto a medium with a reduced 2,4-D level. However, further development of embryos was inhibited by the presence of 2,4-D. Plant regeneration carried out on MS medium without growth regulators.

The development of embryoids was carried out by planting suspension cultures onto semi-solid media with or without growth regulators. It was better to plant suspension cultures without air or vacuum drying, which resulted in cell clumps turning brown and dying after planting. Abscisic acid (ABA) was found to be essential for organized growth of planted cell suspension cultures as it induced the development of embryoids. In the presence of ABA (0.26 mg/L), within 3 to 4 weeks the organized structures developed into embryoids consisting of a pale-yellow and cup-shaped scutellum with a tubular coleoptile. It was found that 2,4-D (0.2 mg/L) promoted the embryoid development to maturity. Some mature embryoids were obtained from cell suspension cultures planted on MS medium without growth regulators.

From Embryogenic Callus on Solid Media

Two weeks after embryos of creeping bluestem were planted on semi-solid media, numerous tiny plantlets were obtained from all four regeneration media. The development of plantlets on MS medium without plant growth regulators took another 6 weeks. More than 40 regenerated plants were successfully grown in a soilless medium under greenhouse conditions

Embryoid Development

Development of embryoids into plantlets of creeping bluestem was achieved on MS basal medium with coconut milk. However, some of embryoids showed abnormal development. They formed roots but no leafy shoots. Both the normal and abnormal embryoids often showed precocious germination. Cell proliferation from the peripheral potions of the developing embryoids was observed, resulting in adventive cleavage polyembryony. Our results suggested that slight changes in culture composition caused by the combination and concentration of growth regulators in culture medium, the stage of inoculum at the time of sub-culture, or other unknown factors, had a marked effect on

the embryogenic potential of cultures, and hence on the number of embryoids produced per planted culture.

Regenerated plants typically had multiple shoots. This may have resulted for several reasons:

- direct development from typical globular embryoids
- the presence of a shoot-root axis
- a single coleorhizae with one or two root meristems.

Encapsulation of Somatic Embryos and Germination of Synthetic Seeds

Encapsulated somatic embryos of creeping bluestem, which were stored at 4C for 2 weeks, were planted in vitro, or in vermiculite that was irrigated with 50% MS medium. Germination of encapsulated embryos was greater in vermiculite than in vitro possibly because of increased light intensity, lowered humidity, or partial microbial breakdown of the alginate matrix (Table 2.1).

Table 2.1. Germination of Creeping Bluestem Synthetic Seed.

Alginate	Alginate Gel Type	Germination Medium	No. Germ. For Diff. Expts.[1]
2%	50% MS	MS w/o PGRs	0/10
2%	50% MS	MS + 1μM 2,4-D	1/14, 0/4, 1/10, 5/10
2%	50% MS	MS + 1μM ABA	3/10, 0/40, 6/10
2%	50% MS + 10μM ABA	MS w/o PGRs	0/10[2]
3.5%	50% MS	MS + 1μM ABA	8/10, 0/10
3.5%	50% MS + 10 μM 2,4-D	MS w/o PGRs	1/10
2%	50% MS	Sterile vermiculite[3]	0/50[4]
3.5%	50% MS	Sterile vermiculite[3]	0/50[4]

Table 2.1 (Cont.). Germination of Creeping Bluestem Synthetic Seed.

2%	50% MS + 10 μM 2,4-D	Sterile vermiculite	24/50, 4/10
2%	50% MS + 10 μM ABA	Sterile vermiculite	16/40, 5/10
2%	50% MS + 10 μM GA_3	Sterile vermiculite	29/70, 19/40, 7/10
3%	50% MS + 10 μM 2,4-D	Sterile vermiculite	6/10
3%	50% MS + 10 μM ABA	Sterile vermiculite	7/10
3%	50% MS + 10 μM GA_3	Sterile vermiculite	8/10
2%	Water	Nonsterile verm.	0/50[4]
2%	50% MS + 10μM 2,4-D	Nonsterile verm.	1/24, 4/14, 2/32, 4/9
2%	50% MS + 10 μM ABA	Nonsterile verm.	2/32, 2/24
2%	50% MS + 10 μM GA_3	Nonsterile verm.	2/24, 4/14, 4/32, 4/9
3%	50% MS + 10 μM 2,4-D	Nonsterile verm.	6/32, 4/9
3%	50% MS + 10 μM GA_3	Nonsterile verm.	23/48, 4/9
3.5%	50% MS + 10 μM ABA	Nonsterile verm.	5/24

[1] Germination as of April 9, 1998; planting date varied from March 29 to April 3, 1998.
[2] Planted on same date as encapsulation (March 16).
[3] Petri plate placed in greenhouse instead of incubator.
[4] Germination on March 26, 1998.

The three factors that appeared to influence germination of our synthetic seeds were the developmental stage of embryoids when they are encapsulated, percentage of sodium alginate, and the growth regulators in the synthetic seed coat. It was observed that the stage for encapsulating the embryo for obtaining best germination was when the first leaf just started to form and the embryo turned green. Embryos encapsulated at a younger developmental stage results in delayed or a poor germination.

Associated with the percentage of sodium alginate used for encapsulation, the longer the embryos were covered by gel, the poorer the germination would be because the respiration of embryoid in alginate gel is poor, which in turn influenced the embryoid development and germination of seeds. Two percent alginate gel was considered the best for the germination of synthetic seeds in vitro (either on MS medium or sterilized vermiculite). However, under greenhouse conditions the gel lost water faster resulting in poor germination. Three to 3.5% alginate gel used as seed coat yielded a greater germination rate. Additional growth regulators in alginate MS gel significantly improved seed germination. Best results were when the gel contained GA_3. It is widely known that GA can stimulate seed germination. ABA and a low concentration of 2,4-D have a similar function. Seeds coat without growth these regulators resulted in poor germination of synthetic seeds. Cold storage (4C) did not seem to improve seed germination.

STUDY 2

Embryogenic Callus Initiation

Contamination was nearly eliminated when we followed the protocol listed below for immature inflorescence explants that were 1-2 mm long.

1. Explant kept in distilled water for 24 hr
2. Wash in tap water for 60 min
3. Surface-sterilize with 90% EtOH for 30 sec
4. Soak in 0.5% mercuric chloride for 5 min
5. Wash with sterile distilled water 5X @ 10 min
6. Add 2 ppm Plant Preservative Mixture to media

However, we later discovered (via tetrazolium [TZ] testing) that this protocol was lethal even though explants remained green. Tissues that are living and respiring react with TZ to form a reddish pink compound. We performed a preliminary TZ test (1% solution; pH 6.7) test on fresh and dead (boiled or soaked in ethanol for a few minutes) leaf explants of wiregrass similar to ones that were being cultured in vitro. A pink color developed on fresh explants; no color developed on the dead explants. We then ran TZ tests on some nonresponsive explants (that were still green) that were being cultured. No pink or red color formed, so we assumed the explants were dead.

As a result of TZ testing to evaluate the effect of mercuric chloride, we observed that the tissues were alive in the center, and in most cases the basal and distal ends were killed (> 1 mm) by mercuric chloride. One to two millimeters of the basal and distal ends of the explants was removed so as to eliminate any dead tissue that might hinder callus initiation. Also, 0.1-0.5% mercuric chloride for greater than 1 min was usually lethal to immature leaf explants.

We observed that treating creeping bluestem explants with 100% Clorox for 30 min kept explants clean and was not lethal (Table 2.2). Given the relative success with Clorox and the problems associated with mercuric chloride, the use of mercuric chloride for callus initiation experiments was discontinued and is not recommended.

The best method for surface sterilization was as follows:

1. Wash in running tap water for 60 min
2. Surface sterilize with 95% EtOH for 30 sec
3. Surface sterilize in 100% Clorox (+ two drops Tween 20) for 30 min
4. Wash with sterile distilled water 5X @ 10 min

Of the thirteen media evaluated (Media *G* to *S*), callus initiation only occurred on three media (Medium *G*, *M*, and *S*) for PB 98 3A, and only in Medium *K*, *L*, and *M* for root-shoot interface tissue from Tucker grade (CBS TG 001B) (Table 2.3). In postburn bluestem leaf tissues, swelling and initiation of callus was seen about 8 days after the cultures were started (60% response) in Medium *G*. Since we had some success in generating callus from PB 98 3A on this media, we evaluated callus formation (PB 98 3A only) on various combinations of 2,4-D concentrations ± CH at 500 mg/L. The response was almost same (60% response; callus in 8 days) as in Medium *M*. For Tucker grade, callus initiation was first observed after 3 weeks. The lower concentrations of 2,4-D did not result in callus formation.

No callus was initiated from frozen immature inflorescences harvested in late summer and fall of 1998. This may have been due to the use of mercuric chloride (as noted before) and/or related to the vigor of the stock plants.

Reinitiation of Cell Suspension Cultures

Embryoid formation in suspension culture occurred in only four of the 19 suspension culture media (Table 2.4). Embryoid formation was best in Medium *NN*. Although the embryoids were compact and globular, they were not in the expected heart-shaped form that we observed in Study 1. In Study 2, the embryogenic clumps were small and generally globular, with cells that were richly cytoplasmic, contained plastids with starch, and had no intercellular spaces. It was concluded that 2,4-D and zeatin at low concentrations promoted embryoid formation. These cell suspensions were maintained by subculturing at a 1:4 dilution (suspension inocula:fresh medium) every 4 to 5 days in liquid Medium *NN*.

Plant Regeneration on Solid Media

Determining If Callus Was Embryogenic

Callus growing on solid media since March 1998 (callus derived from immature inflorescences) and then placed in suspension culture to generate embryoids was

determined to be embryogenic. Coleoptile formation was observed in 44 to 64% of clumps derived from the putative embryoids after 18 weeks in culture on solid Media *I*, *K*, *M*, *PP*, *QQ*, and *WW*. Best coleoptile formation was on Medium *M*, which only was amended with one PGR – 2,4-D at 3 mg/L.

Callus derived from leaf tissue of plant 98 PB 3A was also determined to be embryogenic based on coleoptile formation. After 18 weeks, coleoptile formation was observed on 52 to 80% of the clumps, with best coleoptile formation on Medium *M*.

Scanning Electron Microscopy (SEM). Horseshoe-shaped scutella surrounding coleoptiles were observed in callus derived from immature inflorescences (Figure 2-1) and leaf tissue. Hence, callus from both these tissues was embryogenic.

Table 2.2. Surface Sterilization and Callus Initiation of Creeping Bluestem.

Sterilization Method		100% Clorox; 5 min	100% Clorox; 30 min
Plant type	Explant type	% Contam.	% Contam.
Container; Postburn	Root/shoot Interface	50	30
	Immature Inflorescence	70	20
	Rhizome	90	20
Container; Tissue-Cultured; Postburn	Root/Shoot Interface	50	10
	Immature Inflorescence	60	20
	Rhizome	10	5

Table 2.3. Callus Initiation of Creeping Bluestem.

Plant	Explant	Medium	% Callus Initiation	Time
Container; Postburn	Leaf tissue just above root/shoot interface	*G*	60	8 days
		M	50	8 days
Container; Tissue-Cultured; Postburn	Leaf tissue just above root/shoot interface	*G*	60	8 days
		M	60	8 days
	Rhizome tissue	*S*	50	4 days
Container; Tucker grade	Root/shoot interface	*K*	80	21 days
		L	70	21 days
		M	60	21 days

Table 2.4. Reinitiation, Maintenance, and Embryoid Formation in Cell Suspension Cultures of Creeping Bluestem.

Medium	Embryoid Formation
FF	+
GG	++
II	+++
NN	++++

+	Fair embryoid formation
++	Good embryoid formation
+++	Very good embryoid formation
++++	Excellent embryoid formation

Plant Regeneration and Development

Of the 16 media evaluated for plantlet development, only six Media – *M*, *XX*, *YY*, *III*, and *JJJ* – yielded positive results after 3 to 4 weeks. The best medium for plant regeneration and development was Medium *JJJ* (mean of 75% of cultures). This percentage

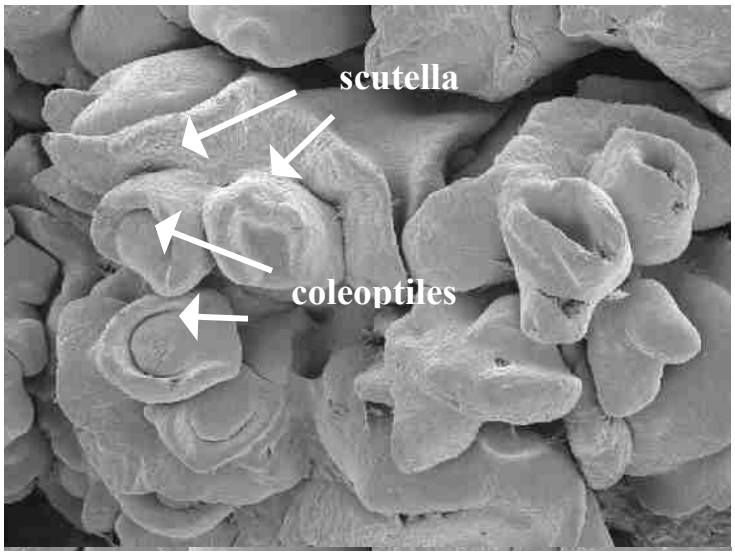

Figure 2.1. Creeping Bluestem Embryos with Horseshoe-Shaped Scutella Surrounding the Coleoptiles (70X).

was significantly greater (as determined by Duncan's MRT, α=0.05) than regeneration/development in Medium *M* (45%), Medium *XX* (46%), Medium *YY* (49%), and Medium *III* (54%), all of which were statistically equal. Medium *JJJ* was used for all subsequent plant regeneration and plant development.

Production Via Shoot Multiplication Culture

Of the five media evaluated, shoot multiplication was only observed on Medium *MMM* (mean percent multiplication rate of 46.6 ± 6.3% over two 4-week periods).

It took 32 weeks to establish shoot multiplication cultures for production starting with shoot clusters of 3-4 shoots that were regenerated in Petri plates as we described in the previous section. Smaller jars (80 ml medium in a 237-ml jar) were used in the establishment phase of shoot clusters because growth in small jars was initially faster than in larger jars (120 ml medium in 472-ml jars). Once the cluster starts growing (after about 8-12 weeks), better growth rates seemed to occur in 472-ml jars. The average rate of multiplication during establishment in 237-ml jars was about 20.8 ± 12.8% (n-=15). The average rate of multiplication in 472-ml jars was 40.7 ± 14.4% (n=15).

After 32 weeks, shoot clusters (one per 472-ml jar) were subcultured every 4 weeks on Medium *MMM*. After 8 weeks, with one subculture at 4 weeks, there were 30 to 32 shoots per cluster. These clusters were divided in half to generate two new 15-16 shoot clusters, and so forth. Hence every 8 weeks, the number of shoots per cluster was approximately doubled.

At the 41% multiplication rate, about 1700 plants (per initial 3-4 shoot cluster) could be produced in just less than 1 year once shoot multiplication cultures are established at 32 weeks.

Effect of Light Quality on Shoot Multiplication Rate

No significant differences were observed in the multiplication rates after 16 weeks of exposure to different spectrums of light at 65µE, although number of tillers and length of longest shoot in clusters tended to be greater under Verilux light (Table 2.5). Hence, there was no distinct advantage of using a light source other than cool white fluorescent light. Moreover, cool white fluorescent lights were the least expensive of the four light sources.

Table 2.5. Effect of Light Quality on Shoot Multiplication Rate After 16 Weeks.

Light Source[1]	CRI[2]	Spectrum (nm)	No. of Tillers	Longest Shoot Length (cm)
Cool White	68	500-600	24 a	6.4 a
Verilux	94.5	400-700	35.5 a	15.8 a
Agrosun	93	430-660	15.2 a	9.2 a
Phillips	97	550-660	16.0 a	10.2 a

[1]CRI - Color Rendering Index; CRI for natural light = 100.
[2]Treatment means within columns separated by Duncan's Multiple Range Test, 5% level.

Acclimatization to Greenhouse and Ambient Conditions

Rooting In Vitro /Acclimatization In Vivo

In Experiment 1, best rooting in vitro occurred with full strength MS + 0.05 mg/L IBA (Medium *ZZZ*) (Table 2.6). Ninety percent of these plants survived the entire rooting/acclimatization process but only 39% of these plantlets had a root ball that held together after 8 weeks (Table 2.7). Rooted plantlets in which the root ball held together was no greater than 75% for any of the treatments. Extending the length of time plantlets were in liner trays should overcome this problem. While there were few differences in the effects of in vitro rooting media on acclimatization, the final yield of rooted acclimatized plants trended to be greater among shoots rooted in vitro with IBA (Table 2.7).

In Experiment 2, best rooting in vitro occurred with full strength MS + 0.05 mg/L NAA Medium *RRR*) (Table 2.8). Eighty percent of these plantlets survived the entire rooting/acclimatization process and 82% of these plantlets had a root ball that held together after 8 weeks (Table 2.9). Also, in Experiment 2, in vitro rooting tended to be poorer but tiller production and percentage of root balls that held together were greater (Tables 2.8, 2.9). As in Experiment 1, there were few differences in the effects of in vitro rooting media on acclimatization (Table 2.9).

The third experiment was run in hopes of clarifying differences observed between the first two experiments. Unfortunately, results of this third experiment were different from those of the first two experiments (Tables 2.10, 2.11). In vitro rooting improved over that in Experiment 2 but there were few differences among treatments. The in vitro

Table 2.6. Experiment 1 – Rooting and Survival of Shoots After 4 Weeks In Vitro[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Longest Shoot Length (cm)	Number Primary Roots	Primary Root Length (cm)	% Survival[2]
½	None	2.5 bcde	8.1 b	0.5 c	0.4 bcd	75
½	IBA (0.05)	3.9 a	11.3 ab	4.2 a	1.0 ab	100
½	IBA (0.1)	2.7 bcde	11.8 ab	3.8 ab	1.1 ab	80
½	IBA (0.2)	2.5 bcde	10.1 ab	3.9 ab	1.4 a	90
½	IBA (0.4)	2.6 bcde	11.3 ab	3.8 ab	0.9 abc	80
½	NAA (0.05)	3.1 abcd	8.7 b	1.0 c	0.1 cd	95
½	NAA (0.1)	3.3 ab	8.1 b	2.3 abc	0.4 bcd	75
½	NAA (0.2)	2.5 bcde	9.1 ab	0.5 c	0.1 cd	60
½	NAA (0.4)	3.3 ab	8.2 b	0.0 c	.	55
Full	None	2.2 de	8.2 b	0.9 c	0.3 bcd	75
Full	IBA (0.05)	3.1 abcd	12.5 a	4.4 a	1.5 a	95
Full	IBA (0.1)	2.5 bcde	8.9 ab	2.5 abc	0.7abcd	90
Full	IBA (0.2)	2.3 cde	8.6 b	2.2 abc	0.9 abc	70
Full	IBA (0.4)	2.1 e	9.1 ab	2.1 abc	1.0 ab	55
Full	NAA (0.05)	2.5 bcde	10.5 ab	1.5 bc	0.3 bcd	50
Full	NAA (0.1)	2.8 bcde	8.4 b	0.4 c	0.1 cd	85
Full	NAA (0.2)	2.7 bcde	8.1 b	0.4 c	0.1 cd	70
Full	NAA (0.4)	2.8 bcde	10.1 ab	2.6 abc	0.7 abcd	40

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.
[2] Percent survival of all 20 shoot clusters (five replicates, with four clusters/replicate).

Table 2.7. Experiment 1 – Acclimatization of Shoots Rooted In Vitro After 8 Weeks of Acclimatization in a Greenhouse[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Longest Shoot Length (cm)	Number Primary Roots	Longest root (cm)	Lateral Root Rating[z]	% Intact Root Balls	% Surv. Acclim.	Total % Survival
½	None	5.7 abc	24.0 f	15.5 fgh	13.8 a	4.3 ab	56	93	70
½	IBA (0.05)	9.1 a	33.6 abc	28.2 ab	12.9 ab	4.5 a	58	95	95
½	IBA (0.1)	7.2 ab	36.1 a	24.7 abcd	12.9 ab	4.0 abc	62	100	95
½	IBA (0.2)	5.4 abc	29.3 cdef	22.2 bcdfg	12.3 ab	2.8 cd	53	94	85
½	IBA (0.4)	4.4 bc	30.0 bcde	23.0 abcdf	12.5 ab	2.8 cd	75	88	70
½	NAA (0.05)	4.9 bc	29.2 cdef	16.8 fgh	13.6 ab	2.9 cd	21	100	95
½	NAA (0.1)	4.86 c	29.5 bcde	17.6 cdfgh	13.4 ab	2.3 d	67	100	75
½	NAA (0.2)	4.6 bc	25.5 ef	17.0 dfgh	13.1 ab	3.2 abcd	0	100	60
½	NAA (0.4)	6.5 ab	31.3 abcd	22.1 bcdfg	13.7 ab	4.0 abc	22	100	55
Full	None	2.3 c	26.2 def	12.5 h	13.7 ab	3.5 abcd	50	67	50
Full	IBA (0.05)	7.4 ab	29.6 bcde	25.0 abc	12.8 ab	3.7 abcd	39	95	90
Full	IBA (0.1)	5.9 abc	34.9 ab	22.1 bcdfg	13.3 ab	3.2 abcd	35	94	85
Full	IBA (0.2)	5.3 abc	29.6 bcde	28.1 ab	12.2 ab	3.2 abcd	57	79	55
Full	IBA (0.4)	5.8 abc	30.7 bcde	30.0 a	14.0 a	4.5 a	75	73	60
Full	NAA (0.05)	3.6 bc	31.7 abcd	14.6 gh	13.1 ab	2.7 cd	11	90	45
Full	NAA (0.1)	4.6 bc	28.6 cdef	21.1 bcdfg	13.5 ab	3.5 abcd	25	82	70
Full	NAA (0.2)	4.9 bc	29.2 cdef	17.2 dfgh	11.9 b	3.0 bcd	33	64	45
Full	NAA (0.4)	6.0 abc	36.2 a	24.2 abcd	13.0 ab	4.5 a	13	100	40

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.
[2] Lateral root systems were rated on a scale of 1-5 as follows: 1 = No lateral roots, 2 = One or two short lateral roots, 3 = More than two lateral roots, but none long, 4 = Some long lateral roots, 5 = Many long lateral roots.

rooting treatments that yielded the greatest number of acclimatized plants were shoots rooted on full strength MS supplemented with 0.1 or 0.2 mg/L IBA or NAA (Medium *TTT*, *VVV*, *BBBB*, *DDDD*).

There were very few significant linear and quadratic effects for IBA and NAA, with no consistency among these significant effects for the three experiments (results not shown).

Table 2.8. Experiment 2 – Rooting and Survival of Shoots After 4 Weeks In Vitro[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Longest Shoot Length (cm)	No. Primary Roots	Primary Root Length (cm)	% Survival[2]
½	None	4.1 abcd	11.06 b	1.3 ab	0.9 b	95
½	IBA (0.05)	2.9 de	9.4 bcd	1.0 abc	1.0 b	40
½	IBA (0.1)	3.7 abcde	7.2 bcd	0.0 e		70
½	IBA (0.2)	3.0 de	5.7 cd	0.0 e		95
½	IBA (0.4)	4.2 abcd	8.6 bcd	0.2 cde	0.5 b	80
½	NAA (0.05)	4.5 abcd	8.5 bcd	0.7 bcde	0.6 b	100
½	NAA (0.1)	3.2 cde	6.2 cd	0.8 bcde	0.5 b	70
½	NAA (0.2)	4.3 abcd	7.4 bcd	0.2 cde	1.0 b	75
½	NAA (0.4)	3.3 bcde	9.9 bc	0.2 cde	0.5 b	95
Full	None	4.9 abc	8.7 bcd	0.9 bcd	0.8 b	70
Full	IBA (0.05)	4.0 abcd	6.6 cd	0.3 cde	1.0 b	80
Full	IBA (0.1)	3.0 de	7.2 bcd	0.0 e		85
Full	IBA (0.2)	2.9 de	5.3 d	0.0 e		95
Full	IBA (0.4)	2.7 de	15.1 a	1.7 a	0.7 b	95
Full	NAA (0.05)	5.3 a	9.0 bcd	0.8 bcde	0.7 b	100
Full	NAA (0.1)	5.1 ab	6.0 cd	0.1 de	0.8 b	100
Full	NAA (0.2)	3.3 bcde	7.1 bcd	0.4 cde	1.1 b	80
Full	NAA (0.4)	2.0 e	6.7 bcd	0.1 de	2.5 a	60

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.
[2] Percent survival of all 20 shoot clusters (five replicates, with four clusters/replicate).

Table 2.9. Experiment 2 – Acclimatization of Shoots Rooted In Vitro After 8 Weeks of Acclimatization in a Greenhouse[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Longest Shoot Length (cm)	Basal Diameter (cm)	Shoot Dry Mass (g)	Root Dry Mass (g)	% Intact Root Balls	% Surv. Acclim.	Total % Survival
½	None	8.0 bcd	5.7 cd	15.6 b	1.5 bcd	0.5 a	71	100	95
½	IBA (0.05)	9.0 bcd	62.9 abc	11.5 bc	2.0 abc	0.4 ab	83	100	40
½	IBA (0.1)	7.4 cdef	62.9 abc	11.5 bc	1.6 bc	0.4 cd	73	86	60
½	IBA (0.2)	10.7 abcd	42.1 e	9.9 bc	0.8 de	0.1 cd	70	84	80
½	IBA (0.4)	11.2 abc	47.6 de	10.3 bc	1.4 bcd	0.3 bc	71	81	65
½	NAA (0.05)	8.9 bcd	65.3 abc	14.6 bc	1.9 abc	0.6 a	80	90	90
½	NAA (0.1)	7.9 bcde	69.6 ab	11.4 bc	1.7 bc	0.4 ab	86	100	70
½	NAA (0.2)	14.4 a	61.4 abc	12.0 bc	1.7 bc	0.4 ab	88	100	75
½	NAA (0.4)	7.4 cde	54.1 cd	12.9 bc	1.6 bc	0.4 ab	67	90	85
Full	None	9.6 abcd	62.9 abc	13.7 bc	1.6 bc	0.5 a	60	93	65
Full	IBA (0.05)	11.9 ab	65.6 abc	14.5 bc	2.7 a	0.6 a	75	88	70
Full	IBA (0.1)	7.4 cde	54.8 cd	10.3 bc	1.2 cd	0.3 bc	57	94	80
Full	IBA (0.2)	4.3 f	37.6 e	9.9 bc	0.5 e	0.2 d	80	63	60
Full	IBA (0.4)	7.0 cdef	56.0 cd	12.2 bc	1.8 bc	0.5 a	80	100	95
Full	NAA (0.05)	10.7 abcd	62.7 abc	13.6 bc	2.26 ab	0.6 a	82	80	80
Full	NAA (0.1)	13 ab	59.2 bcd	12.8 bc	1.68 bc	0.4 ab	90	85	85
Full	NAA (0.2)	4.6 f	60.0 abcd	22.3 a	1.62 bc	0.4 ab	75	94	75
Full	NAA (0.4)	7.0 cdef	72.9 a	12.46 bc	1.8 bc	0.4 ab	67	100	60

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.

Table 2.10. Experiment 3 – Rooting and Survival of Shoots After 4 Weeks In Vitro[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Longest Shoot Length (cm)	No. Primary Roots	Longest root (cm)	% Survival[2]
½	None	2.2 bcd	8.4 bcd	1.3 a	0.8 b	65
½	IBA (0.05)	2.1 bcd	12.8 abc	2.5 a	1.1 a	70
½	IBA (0.1)	3.6 ab	10.5 bcd	2.0 a	0.9 b	15
½	IBA (0.2)	2.3 cd	7.8 cd	2.4 a	0.7 b	70
½	IBA (0.4)	2.1 bcd	8.1 cd	2.5 a	0.8 b	50
½	NAA (0.05)	2.0 cd	10.1 bcd	2.2 a	0.6 b	55
½	NAA (0.1)	4.6 a	5.0 d	.	.	15
½	NAA (0.2)	2.0 cd	10.4 bcd	3.0 a	0.9 a	70
½	NAA (0.4)	2.1 bcd	11.4 bcd	2.0 a	0.8 b	80
Full	None	1.7 cd	8.8 bcd	2.0 a	1.0 a	90
Full	IBA (0.05)	1.6 cd	18.1 a	2.9 a	1.0 a	45
Full	IBA (0.1)	2.4 bcd	7.2 cd	2.8 a	0.9 a	80
Full	IBA (0.2)	3.2 bc	7.9 cd	2.3 a	0.9 a	95
Full	IBA (0.4)	2.4 bcd	9.3 bcd	2.1 a	0.7 b	55
Full	NAA (0.05)	2.6 bcd	6.5 cd	2.5 a	0.7 b	90
Full	NAA (0.1)	1.4 d	14.8 ab	2.4 a	1.0 a	80
Full	NAA (0.2)	2.5 bcd	12.7 abc	2.3 a	0.9 a	85
Full	NAA (0.4)	1.8 cd	12.3 abc	2.0 a	0.8 b	80

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.
[2] Percent survival of all 20 shoot clusters (five replicates, with four clusters/replicate).

Table 2.11. Experiment 3 – Acclimatization of Shoots Rooted In Vitro After 8 Weeks of Acclimatization in a Greenhouse[1].

Strength of MS Basal Medium	PGR (mg/L)	No. Tillers	Basal Diameter (cm)	Shoot Dry Mass (g)	Root Dry Mass (g)	% Surv. Acclim.	Total % Survival
½	None	4.4 cd	21.1 abcd	4.4 f	0.204 de	92	60
½	IBA (0.05)	5.5 bcd	24.3 abc	6.3 cdef	0.271 de	71	70
½	IBA (0.1)	3.8 d	14.6 cd	4.3 f	0.255 de	100	15
½	IBA (0.2)	4.4 cd	13.8 d	5.8 def	0.241 de	93	65
½	IBA (0.4)	9.3 ab	16.7 bcd	7.7 cdef	0.313 cde	80	40
½	NAA (0.05)	5.0 bcd	24.4 abc	7.2 cdef	0.442 bcd	55	30
½	NAA (0.1)	6.5 bcd	25.2 ab	8.7 bcdef	0.322 cde	100	15
½	NAA (0.2)	8.7 abc	26.8 a	10.3 abcd	0.566 bc	86	60
½	NAA (0.4)	5.4 bcd	23.9 abc	6.8 cdef	0.379 cde	75	60
Full	None	4.7 cd	20.0 abcd	9.5 abcd	0.410 cde	56	50
Full	IBA (0.05)	9.3 ab	28.8 a	13.5 a	0.866 a	100	45
Full	IBA (0.1)	6.6 bcd	25.7 ab	9.2 abcde	0.572 bc	100	85
Full	IBA (0.2)	11.7 a	28.5 a	12.3 ab	0.650 ab	95	90
Full	IBA (0.4)	4.6 cd	12.7 d	5.9 def	0.149 e	100	55
Full	NAA (0.05)	7.6 abcd	21.2 abcd	8.4 bcdef	0.283 de	50	45
Full	NAA (0.1)	3.7 d	15.8 bcd	4.9 ef	0.141 e	100	80
Full	NAA (0.2)	6.3 bcd	22.3 abcd	9.0 bcde	0.248 de	100	85
Full	NAA (0.4)	8.7 abc	27.9 a	10.5 abc	0.331 cde	69	55

[1] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level.

Rooting In Vivo /Acclimatization In Vivo

In preliminary work, shoots and roots regenerated in the dark from embryogenic callus were etiolated. When these plantlets were transplanted to pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol) in plastic liner cups and placed under greenhouse conditions (30% shade), all etiolated plantlets turned green within 2-3 days and nearly 100% survived. Subsequent growth appeared normal.

Tissue-Cultured Shoots Vs. Divisions of Container-Grown Plants. Only potting mix affected the rooting/acclimatization procedure. For propagules growing in pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol), average survival after 2 weeks in the greenhouse and after 2 weeks in the shadehouse was 91% and 90%, respectively (Table 2.12). Percent survival values in this mix were significantly greater (P = 0.05) than survival in pine bark:Canadian sphagnum peat, 1:1 (vol/vol), which was only 64% and 56% for greenhouse and shadehouse, respectively. About 78% of all plants survived the entire acclimatization procedure when using the pine bark/peat/sand mix but only about 33% survived acclimatization when potted in the pine bark/peat mix.

Table 2.12. Cumulative Survival of Creeping Bluestem Plants Propagated by Division and Tissue Culture and Then Acclimatized to Greenhouse and Nursery (30% Shade) Conditions. [1]

	Divisions		Tissue-Cultured	
Potting Medium	% Survival: G'house (2 weeks)	% Survival: Nursery (2 weeks)	% Survival: G'house (2 weeks)	% Survival: Nursery (2 weeks)
Pine Bark: Canadian Sphagnum Peat, 1:1	64 b[2]	56 b	62 b	58 a
Pine Bark: Canadian Sphag. Peat: Sand, 3:1:1	87 a	84 a	91 a	91 a

[1] Plantlets acclimatized to greenhouse conditions before being acclimatized to nursery conditions.

[2] Means with the same letter within a column are not significantly different; Duncan's Multiple Range Test, 5% level. Percent survival was calculated as the mean of experiments (15 reps per treatment) conducted on three occasions; percentage data were transformed (arsine sq root) prior analysis but nontransformed means are presented.

Effect of Medium, Tiller Height, Rooting Growth Regulator, and Fertilizer on Rooting and Survival. Shoot cluster height appeared to affect survival and rooting. Fifty-five percent of clusters that were 3-5 cm tall survived in contrast to the 30% survival rate of clusters 1-2 cm tall. Lateral root systems and plant vigor were also rated higher for 3-5 cm clusters. Twelve of the 22 surviving 3-5 cm clusters had lateral root systems rated as 4 or 5 whereas no lateral roots systems of the 1-2 cm clusters were rated 4 or 5. For plant vigor, 35% of 3-5 cm clusters were rated Good and 20% rated Fair; for 1-2 cm clusters, only 10% were rated Good and 15% rated Fair. None of the factors affected primary root formation; root formation of 3-5 cm and 1-2 cm clusters averaged 7.6 and 7.4, respectively.

For 3-5 cm shoot clusters, there were significant interactions between medium and fertilizer for lateral root rating (P=0.0148) and plant vigor (P=0.0177). Fertilizer significantly increased (5% level) lateral root rating (2.9 vs. 1.2) and plant vigor (2.3 vs. 1.0) of 3-5 cm clusters rooted in MetroMix; however, fertilizer had no effect on these parameters when clusters were rooted in the pine bark medium.

While shoot clusters could be rooted in the pine bark mix without fertilizer, we felt it was better to use MetroMix 200 because the product is widely available and batches of the pine bark mix could vary depending on the type and quality of the pine bark and sand. Hence, MetroMix 200 was used in all subsequent acclimatization studies.

Effect of Tiller Number, Growth Regulators, and Mist Frequency on Rooting and Survival. In Experiment 1, tiller number, and secondarily mist frequency, were the two factors that affected rooting. Shoot clusters having 1-2 tillers had an average of 6.2 primary roots while clusters having 3-4 tillers had an average of 10.9 primary roots (P = 0.0022). Number of tillers after 4 weeks was also affected by the initial number of tillers as expected (P = 0.0001) – 4.5 and 2.1 tillers for 3-4 and 1-2 initial tillers, respectively. Mist frequency affected longest root length (P = 0.0012) – 10.6 cm and 7.5 cm for 6.5 sec/5 min and 6.5 sec/10 min, respectively. For longest shoot length, mist frequency was somewhat significant (P=0.0934) – 12.6 cm and 10.0 cm for 6.5 sec/5 min and 6.5 sec/10 min, respectively. No factor affected survival (56%); however, percent survival tended to be inversely related to IBA concentration. Nontreated clusters had the greatest survival (75%), with percent survival gradually declining to a low of 38% for Hormex 45 (4.5% IBA). Use of a growth regulator did not affect rooting, survival, or plant vigor. There were many significant two- and three-way interactions, but none was of any practical significance. Averaged over all treatments, lateral root rating was 3.2.

In Experiment 2, the initial tiller number affected number of primary roots and number of tillers after 3 weeks (P = 0.0005 and P = 0.0001, respectively). Shoot clusters having 1-2 tillers had an average of 7.0 primary roots while clusters having 3-4 tillers had an average of 10.4 primary roots. Number of tillers after 4 weeks was 4.0 and 1.9 tillers for 3-4 and 1-2 initial tillers, respectively. There were no other significant main or interactive effects. Averaged over all treatments, percent survival was 71%, lateral root rating was 3.2, longest root length was 10.1 cm, and longest shoot length was 14.7 cm.

Effect of Fertilizer Type on Rooting and Survival. After 6 weeks, many of the 1-2 shoot clusters had died. Best survival was for shoots in the medium containing Osmocote 14-14-14 (58%); for this treatment, the average number of primary roots was 4.9 and lateral root rating was 3.7. For all incorporated fertilizer treatments, survival was only 38% compared to only 14% for the water-soluble fertilizer treatments. Part of the reason for the poor results may have been that we only had 1-2 tiller clusters available, which was not the optimum as we have previously observed. Since this study was conducted in the fall, photoperiod may also have affected rooting.

Greenhouse Evaluation of Tissue-Cultured Plants

(Edited excerpt from manuscript submitted to Native Plants Journal)

All three soils contained >90% sand, but sand particle size differed (Table 2.13). Myakka had more fine sand, and the sand tailings contained more coarse sand, with overburden intermediate. Myakka had more silt than sand tailings and overburden. Myakka and sand tailings had little clay compared with overburden.

Sand tailings and overburden were very high in P, Ca, and pH compared with Myakka soil (Table 2.14). Mineral concentrations, pH, organic matter, and soil separates used in this study compare well to that reported for sand tailings in another phosphate mine reclamation study (Mislevy and Blue 1981, 1985). Overburden seems to be quite variable as Mislevy and Blue (1985) reported concentrations of P, Ca, and pH in overburden was 390, 930, and 6.0, respectively, which was 17 and 15 % of the P and Ca, respectively, found in our overburden.

Soil type did not affect 28-day germination and emergence of creeping bluestem (P=0.57). Germination on Myakka was 49%, 54% on sand tailings, and 53% on overburden. These percentages were typical for germination of creeping bluestem on blotter paper in Petri plates (Kalmbacher and others 1991). When moisture and temperature are not limiting, sand tailings and overburden are a favorable environment for germination.

On March 5, when division and tissue culture plants were started in pots and 119 days after seedlings were transplanted from Petri plates to pots, seedlings in Myakka were 11-cm tall compared with 1 and 2-cm tall seedlings in sand tailings and overburden, respectively. Division and tissue culture plants were both 15-cm tall. Seedlings and division plants consisted of a single tiller, while tissue culture plants averaged 1.9 tillers/plant. Seedlings in sand tailings and overburden were yellow while all others were green.

At 56 days after transplanting (April 30), there was a plant source x soil interaction for plant height (data not shown) primarily because of the inability of seedlings

Table 2.13. Particle Size Distribution in Soils at the Start (March) of the Greenhouse Evaluation.

Soil Separates	Myakka Fine Sand	Sand Tailings	Overburden
	----------% Sand ----------		
V. Coarse (1-2 mm)	0	1.6	1.4
Coarse (1-0.5 mm)	1.6	12.6	9.6
Medium (0.5-0.25 mm)	21.6	51.2	42.2
Fine (0.25-0.1 mm)	53.2	31.4	38.4
Very Fine (0.1-0.005 mm)	5.6	0.8	2.8
Total	92.0	97.6	94.4
Silt	6.9	1.3	1.4
Clay	11	1.1	4.2

to grow on sand tailings and overburden. They remained stunted (mean = 1 cm tall and chlorotic. Plants from division on sand tailings (mean = 29 cm tall) were taller than plants from division on Myakka (mean = 21 cm tall), with plants from division on overburden (mean = 25 cm tall) intermediate and not different from the previous two. There was no difference in height of plants growing on Myakka (mean = 21 cm tall). There was no difference in height of tissue-cultured plants growing on the three soils (mean = 20 cm tall), but all were shorter than plants from division on sand tailings (mean = 29 cm tall).

There was a plant source x soil interaction for tiller number on 30 April (data not shown) primarily due to profusion of tillers on tissue-cultured plants grown on Myakka, which had 14 tillers per plant. This was greater than that on overburden or sand tailings (mean = 4 per plant). Tiller numbers on plants from division growing on Myakka and overburden were not different (mean = 3 tillers per plant), but they were greater than division

Table 2.14. Mineral Concentrations, pH, and Organic Matter Contents of a Native Myakka Soil and Two Soils from a Reclaimed Phosphate Mine and Mineral Concentrations in Creeping Bluestem Vegetative Tillers (Composited Over Replications) at the End of the Study on Oct. 23, 2001.

Soil:	Myakka		Overburden		Sand Tailings	
Item	Soil	Tissue	Soil	Tissue	Soil	Tissue
N %	-[1]	0.52	---	0.58	---	0.52
P %	10.2 c[2]	0.05	2160 b	0.06	2280 a	0.08
K %	14.9.a	0.48	5.7 a	0.78	11.1 a	0.77
Ca %	504 c (177)[3]	0.17	5943 b (5900)	0.21	6244 a (6300)	0.18
Mg %	289 b (40)	0.15	291 b (176)	0.16	476 a (320)	0.13
S %	---	0.08	---	0.07	---	0.09
Cu, ppm	0.13 a	1.07	0.12 a	0.97	0.13a	1.59
Fe, ppm	22.2 c	108	43.7 a	78.5	41.1 a	88.3
Mn, ppm	0.18 c	20.0	5.56 a	72.8	4.80 b	64.1
Zn, ppm	3.7	12.0	0.85	14.4	2.32	13.3
pH	6.0 b (4.2)	na[4]	8.9 a (6.9)	na	8.9 a (7.6)	na
O.M. %	2.4	na	0	na	0	na

[1] Not determined in soil.
[2] Means within a line followed by the same letter are not different (P>0.05).
[3] Values for Ca, Mg, and pH in parenthesis are values at the start of the study.
[4] Not applicable to tissue.

plants on sand tailings (mean = 1 tiller per plant). It seems that division plants on sand tailings concentrated growth in height rather than expansion via tillers.

Seedling plants may not have been able to develop a secondary root system in sand tailings or overburden. These soils were extremely high in P and Ca compared with Myakka, and pH was high as well (Table 2.14), which may have reduced availability of Cu and perhaps Zn. Both tissue culture and division plants had a well-developed root system when they were transplanted. However, at CF mine we observed broomsedge, which came from seed, growing on lower sand tailings and throughout the overburden site. Four *Andropogon* spp. were reported growing on overburden, but these sites were mostly 50-years old (Craig and Smith 1980).

At the termination of the study on October 23, 2001, there was a significant plant source x soil interaction for shoot mass, root and rhizome mass, and vegetative tiller number (Table 2.15). Seedling plants died on sand tailings and overburden, but these plants on Myakka were green and healthy. Seedlings plants had less shoot mass than plants from division with tissue culture plants intermediate. Much of the mass came from reproductive tillers (five per plant) on plants from division grown on Myakka. The only other plants with reproductive tillers were seedling plants on Myakka, which averaged one per plant. There were no differences between plant source or soil for shoot mass.

Tissue-cultured plants had the greatest root and rhizome mass compared with plants from seed or division, which were not different (Table 2.15). There were no differences in root and rhizome mass for plants from seed or division on sand tailings or overburden.

On Myakka, tissue-cultured plants had more vegetative tillers than plants from seed, with division plants intermediate (Table 2.15). Compared with tissue-cultured plants on Myakka, tissue-cultured plants on sand tailings and overburden had fewer tillers. Plants from division grown on Myakka had the same number of tillers as those on sand tailings or overburden.

Calcium and Mg concentrations and pH increased in soil from the start to end of the study because our irrigation water is high in Ca and Mg carbonates (Table 2.14). While this would have little effect on the soils from the mine, it could have had an influence on Myakka soil because these values are inherently low in the native condition. In spite of the relatively great differences in P and Ca concentrations in the three soils, tissue P and Ca were relatively uniform. Most other minerals were similar in tissue grown on the three soils. Iron seemed to be high in tissue from Myakka compared with the other soils, while Mn in tissue grown on Myakka was comparatively low. Concentrations of minerals in creeping bluestem grown in Myakka soil in pots was very similar to respective concentrations of vegetative tillers of creeping bluestem grown from March to October on a native Immokalee fine sand (arenic Alaqouds) (Kalmbacher and Martin 1981). In that study, Fe (32 ppm) and Mn (40 ppm) were a little closer to the values for these minerals in plants grown on mine soils.

Table 2.15. Creeping Bluestem Shoot, Root and Rhizome Dry Mass, and Vegetative Tiller Density from the Pot Study at Termination on October 23, 2001.

	Soil		
Plant Source	Myakka soil	Overburden	Sand Tailings
Shoot Dry Mass (g/pot)			
Seed	20.8 c[1] A[2]	0 b B	0 b B
Division	41.6 a A	4.6 a B	3.6 a B
Tissue Culture	29.8 b B	4.0 a B	3.2 a B
Root and Rhizome Dry Mass (g/pot)			
Seed	24.2 b A	0 b B	0 b B
Division	23.0 b A	5.2 a B	5.2 a B
Tissue Culture	36.4 a A	2.8 a B	2.6 a B
Vegetative Tillers (No./pot)			
Seed	15.4 b A	0 b B	0 b B
Division	3.0 c A	3.0 a A	2.0 a A
Tissue Culture	21.0 a A	4.2 a B	4.0 a B

[1] For a given response, means within a column followed by the same lower case letter are not different; $P > 0.05$.
[2] Means within a row followed by the same upper case letter are not different; $P>0.05$.

Field Evaluation of Tissue-Cultured Plants

***Note**: The acclimatized tissue-cultured plants used in this field study were the plants left over from one of the Rooting In Vivo/Acclimatization In Vivo studies. The*

variable survival of plants for the tiller number x PGR x mist frequency treatments, and the lack of at least three reps from all treatments precluded any meaningful statistical analyses. Hence, data were pooled by tiller number treatment (only factor of the three that had a substantial effect on rooting and survival of tissue-cultured shoots) to obtain some preliminary indication of growth and survival under field conditions.

Sixty days after acclimatized tissue-cultured plantlets were transplanted, overall survival at Monticello (upland field site; fine sand) average 77% (data not shown) but by 90 days survival was only 39% (Table 2.16). Eleven months later, survival had declined to 20%. In contrast, survival of acclimatized tissue-cultured plantlets at Ona (flatwoods) was substantially better (Table 2.17). Ninety days after planting, nearly 90% of plantlets survived and by 12 months 76% of plantlets had survived. Differences among other parameters were minimal for surviving transplants (although not statistically analyzed because of differences among transplant origin used at both sites). Survival differences among the sites highlighted the importance of soil moisture level for survival of these tissue-cultured transplants. In Monticello, September 2000 was a very wet month (23.9 cm) while October total rainfall was only 2.3 cm. While this lack of October rain did not manifest itself by 60 days after transplanting, the effect of lack of rain during October was evident by the end of November (3 months after transplanting). Octobers in Monticello typically are characterized by relatively high evapotranspiration rates because of warm temperatures and progressively lower relative humidities. As a result, plants that are becoming established can be very stressed.

Table 2.16. Growth and Survival of Rooted and Acclimatized Tissue-Cultured Creeping Bluestem Plantlets on an Upland Site Under Low Input Conditions.

No. of Tillers of Original Tissue-Cultured Clump Prior to Rooting	3 Months after Transplanting to Field				14 Months after Transplanting to Field			
	No. of Tillers[1]	Longest Shoot Length (cm)[1]	Shoot Clump Diam. (cm)[1]	% Survival[1]	No. of Tillers[1]	Longest Shoot Length (cm)[1]	Shoot Clump Diam. (cm)[1]	% Survival[1]
1-2	5.0	21.5	3.8	33	25.1	53.9	29.5	18
3-4	5.0	18.6	2.8	44	26.1	52.1	31.4	22

[1] There were no significant differences between the two treatments after 3 or 14 months; survival data analyzed by Chi-square.

Despite the better survival of plants under flatwoods conditions, shoot and rhizome dry mass of surviving plants appeared to be substantially greater under the

upland conditions at Monticello (Table 2.18). No analyses of site differences were conducted because of differences in the origin of the transplants and different ending dates.

Table 2.17. Growth and Survival of Rooted and Acclimatized Tissue-Cultured Creeping Bluestem Plantlets on a Flatwoods Site Under Low Input Conditions.

No. of Tillers of Original Tissue-Cultured Clump Prior to Rooting	4 Months After Transplanting to Field				12 Months After Transplanting to Field			
	No. of Tillers[1]	Longest Shoot Length (cm)[1]	Shoot Clump Diam. (cm)[1]	% Survival[1]	No. of Tillers[1]	Longest Shoot Length (cm)[1]	Shoot Clump Diam. (cm)[1]	% Survival[1]
1-2	11.1	33.2	11.5	89	28	41.2	27.5	71
3-4	12.8	33.6	9.5	90	24	37.6	23.4	80

[1] There were no significant differences between the two treatments after 4 or 12 months; survival data analyzed by Chi-square.

Table 2.18. Main Shoot and Rhizome Dry Mass of Tissue-Cultured Creeping Bluestem 12 or 14 Months After Transplanting.

Site	Dry Mass Main Shoot (g)	Dry Mass Rhizome (g)
Monticello[1]	30.9	10.7
Ona[2]	11.3	1.4

[1] Upland site; 14 months after transplanting.
[2] Flatwoods site; 12 months after transplanting.

Growth and Survival Under Mine Site Conditions

(Edited excerpt from manuscript [Kalmbacher and others] submitted to Native Plants Journal)

Survival. Survival was very good in the July 2001 planting on both sites 100 days after planting (Table 2.19). During this post-planting period, there was 77 cm of rain, which was relatively high but still typical for the rainy season in central Florida. By October 28, 2002, survival remained high on sand tailings, but had declined to 55% survival on overburden. For the October 2001 planting, survival in the first 100 days was 81% on sand tailings and 58% on overburden (Table 2.19). During this period, 6.2 cm rain was recorded, which reflects the fact that October to December are some of the driest months in central Florida. During this period soils usually carry a reserve of moisture from summer rain. At 1 year after planting, survival on both sites was relatively unchanged. In the January 2002 planting, survival in the first 100 days was 95% on both sand tailings and overburden. During this post-planting period, 23.3 cm rain was received. During the cooler months, evapotranspiration was less than during summer or fall.

Table 2.19. Creeping Bluestem Survival at Two Dates after Planting on Sand Tailings (ST) and Overburden (OB) and Plant Diameter Least Square Means on October 28, 2002 for the Month of Planting X Site Interaction.

Planting Date:	----- July 2001 -----		---October 2001 ---		--- January 2002 ---	
Survival (%)	ST	OB	ST	OB	ST	OB
1st 100 Days	100	78	81	58	95	95
Oct. 28, 2002	98	55	82	58	83	88
Plant Diam. (cm)	30.8A[1]	27.9a ns[2]	29.1A	22.9b **	16.9B	18.0c ns

[1] For comparing dates within sites. Sand tailings means followed by the same capital letter are not different over month of planting and overburden means followed by the same lower case letter are not different (P>0.05).
[2] For comparing sites within dates. NS and ** indicate that sand tailings and overburden are not different from each other (P>0.05) or different (P<0.01) within a month of planting, respectively.

It appeared that survival during the first 100 days was related to rainfall, with the poorest survival from the drier October planting. We purposely avoided the most difficult period for any plant growing on mined soil, which would be April to early June. This is historically the most severe drought period in central Florida (Kalmbacher and Linda 1993), and a period when cattlemen suspend pasture establishment. Our research showed that creeping bluestem planted from July to January, thus representing three plant ages of established plants going into the critical dry spring period, survived very well.

On the mid- to lower end of the sand tailings, tunneling by mole cricket (*Scapteriscus vicinus*) was very apparent in the spring 2002, but there were no signs, such as wilting, that crickets were eating creeping bluestem. Missing plants were scattered on

sand tailings and appeared to be random. On overburden, missing plants were clustered. We found up to 14 missing plants in a row, and adjacent rows often contained additional spaces once occupied by plants. This suggested that clay pockets or other soil-borne problems might occur on overburden.

Weed control was viewed as the greatest challenge to establishment of creeping bluestem on these mined sites. Weeds were a much greater problem on overburden than on sand tailings, and this was a contributing factor to the difference in creeping bluestem survival. On overburden, the January, July, and October plantings were sprayed four, five and six times, respectively, compared with three, two, and four times, respectively on sand tailings. A large soil seed bank had been allowed to build up after overburden and sand tailings were deposited and leveled. Legumes (hairy indigo, shyleaf, coffeebean, and showy crotalaria (*Crotalaria spectabilis*) (in this order) seemed to be the major broadleaf weed problems due to their abundance and difficulty to kill. No single herbicide was effective on all weed species. Plateau was relatively effective on most broadleaf species and grasses (except *Brachiaria* spp.), but Weedmaster was needed for pusley (*Richardia* spp.) and most legumes. Only Remedy was effective on hairy indigo.

Penetrometer Resistance. There was a site x depth interaction ($P<0.0001$) for penetrometer resistance. Resistance over depth on sand tailings was linear, whereas it was quadratic on overburden. There low values for R^2, which reflect the extreme variability especially for overburden. In overburden, the sampling variance was six-times as large as the error variance compared with two and a half on sand tailings. Resistance on overburden reached near a maximum of 45 kg/cm^2 at 20-cm depth, then resistance leveled-off. When resistance measurements were made (April 24), soil was very dry throughout the profile on sand tailings (not quantified) whereas on overburden there was some moisture associated with clay lenses. We feel that the difference in resistance between sites was due to moisture. When survival measurements were made at 100 days post planting, we suspected that hard clay areas on overburden would be associated with plant loss. Greater resistance at the site of a missing plant on overburden was not associated with plant presence or absence.

Plant Diameter. There was an interaction between month of planting and site (Table 2.19). On October 28, 2002, there were no differences in plant diameter between sites at the January 2002 and July 2001 planting dates, but overburden had smaller plants compared with sand tailings at the October 2001 planting. Within the overburden site, plants from July 2001 were larger than plants from the October planting, and plants from the January planting were smaller than the former. Within sand tailings, plant diameter from the July and October plantings was not different, but these plants were larger than plants from the January planting. These differences were a function of plant age.

The mean plant diameter in the July and October plantings (27.7 cm) was greater than creeping bluestem plant diameter at 1-year after planting (~20 cm) in an earlier study (Kalmbacher and others 1986). The smaller diameters in the January planting date were probably not due to age alone, otherwise diameter in July planting should have exceeded that of October with little difference between October and January due to

cooler temperatures. The January plants at the time of planting were noticeably smaller (no data) than those from previous planting dates. We attributed this to the fact that January plants were cultured and developed under short days and cooler temperatures (September to December) than earlier plants.

Tiller Number and Plant Mass. There were more reproductive and total tillers per plant on sand tailings compared to plants on overburden (Table 2.20). Mean tiller density (56.8) of these 1-year-old plants was similar to ungrazed creeping bluestem (63 tillers/plant) at 2-years after planting (Kalmbacher and others 1986). In the pot study, the tissue-cultured plants had greater tiller number than plants from division. It appears this was a characteristic of tissue-cultured plants.

Table 2.20. Creeping Bluestem Tiller Number and Above-Ground Dry Matter Mass on the October Planting at 1 Year After Planting on Sand Tailings and Overburden.

	Site		
Tillers (No./Plant)	Sand Tailings	Overburden	*P*
Reproductive	16.7	11.4	0.01
Total	63.5	50.1	0.02
Plant Mass (g/Plant)[1]	116.7	106.9	0.55

[1] Plants on 1-m centers in rows 1 m apart; kg/ha =grams/plant x 10.

There was no difference in above-ground plant mass between sites (Table 2.20). Above-ground biomass of these 1-year-old plants averaged 1120 kg/ha. Annual growth of unburned creeping bluestem on a native Spodosol was 1350 kg dry matter/ha (Kalmbacher and others 1985). In another study, annual yield of unfertilized creeping bluestem was 1400 kg dry matter/ha (Kalmbacher and others 1993). These latter references for yields of creeping bluestem were yields of vegetative growth. Undisturbed in the native condition, creeping bluestem will flower very little unless physically disturbed, burned, or fertilized. Yields of the present study contained a great portion of reproductive growth. We have noticed that creeping bluestem seedlings transplanted to the field in spring or summer will flower prolifically in the fall.

Production of Synthetic Seed

Over 500 seed of bluestem were produced. Synthetic bluestem seed were successfully germinated in vermiculite in the greenhouse at the NFREC in Monticello.

Some of these germinated seeds were repotted and successfully acclimatized to outdoor conditions.

Greenhouse Evaluation of Synthetic Seed

Synthetic seed germination in vermiculite was good to excellent in vitro and fair under greenhouse conditions at the NFREC (Table 2.1), but seed germination at Ona (Table 2.21) was poor for several possible reasons: overhead watering system buried seed, high greenhouse temperatures, media effects, desiccation. Since germination in vermiculite in vitro has been good to excellent, most seeds are viable. The problem we need to resolve is how to optimize ex vitro germination, first in the greenhouse (vermiculite, and then other media) and then in the field.

We conducted some preliminary work using embryos that were better developed (embryos with leaves of 2-3 mm) but germination did not improve despite the larger bead size (Table 2.22).

Table 2.21. Percentage of Cells in Greenhouse Flats that Contained a Living Creeping Bluestem Plant at 4 and 9 Weeks After Initiation on May 6, 1998.

	Medium		
	Potting Mix	Myakka Soil	Mine Soil
Artificial Seed			
4 wk	3	0	0
9 wk	1	0	0
Sexual Seed[1]			
4 wk	43	33	29
9 wk	41	38	33
Rhizome Piece			
4 wk	12	19	9
9 wk	13	18	4

[1] One seed per cell; seed had 50% germination in Petri-plate incubation.

Table 2.22. Percentage of Cells in Greenhouse Flats that Contained a Living Creeping Bluestem Plant at 4 Weeks after Initiation on June 8, 1998.

Medium:	Potting Mix	Myakka Soil	Mine Soil
Artificial seed	0	2	0
Sexual seed[1]	65	42	44
Rhizome piece	11	11	1

[1] Two seeds per cell; seed had 50% germination in Petri-dish incubation.

Direct Regeneration of Creeping Bluestem

In two separate studies, no shoots, roots, or callus formed after 3 months in cultures of nodes and leaves from plants that were cut back or burned and then placed in the greenhouse, or nodes, leaves, and inflorescences from ultrafrozen tissues. The lack of any response was probably due to the use of mercuric chloride.

Observations of Growth of Tissue-Cultured Plants

Growth of acclimatized tissue-cultured plants appeared normal except initially they seemed to grow as fast or faster than plants propagated by division. Phenotypically, there were no differences between the mother plants and the tissue-cultured plants except the tissue-cultured plants were slightly shorter. This difference in height may have been because the tissue-cultured plants flowered earlier than those propagated by division. Studies in other grasses confirm that plants regenerated by somatic embryogenesis show high uniformity of regenerants and avoidance of somaclonal variation. There is a high probability that our regenerated plants via somatic embryogenesis do not have any genetic variation and are true-to-type.

CONCLUSIONS AND RECOMMENDATIONS

Generation of embryogenic callus was the rate-limiting step in micropropagating creeping bluestem via somatic embryogenesis. Obtaining embryogenic callus was further complicated by commonly occurring bacterial and fungal contamination. The use of mercuric chloride should be avoided as it was lethal to the young tissue. Surface sterilization protocols utilizing Clorox for 30 min substantially reduced contamination but were not lethal. Contamination also hindered our efforts to produce synthetic seed as embryogenic suspension cultures became contaminated and became difficult to reestablish.

Creeping bluestem can be successfully regenerated from somatic embryos, either by synthetic seed or by plants directly regenerated from the embryos. However, given the difficulties we experienced with initiating and maintaining cell suspension cultures – a critical step in synthetic seed production – and the poor germination of synthetic seed and/or poor survival of the resulting seedlings on mine soil under greenhouse conditions, much research needs to be conducted to make this a cost effective method of generating "seed" for planting on mine sites.

The best method we found for micropropagating creeping bluestem that could be used for mine reclamation was shoot multiplication culture under cool white, fluorescent light, with clumps of somatic embryos as starting material. Once established (in about 32 weeks), number of shoots in multiplication cultures nearly doubled every 8 weeks. At this rate, about 1700 plants (per initial 3-4 shoot cluster) could be produced in about 1 year once shoot multiplication cultures are established at 32 weeks. Rooting these shoots was best accomplished in vitro (4 weeks), although results of three experiments failed to provide us consistent evidence of which medium was optimum for rooting these shoots. Rooted shoots are then planted in MetroMix 200 in tubeling trays and acclimatized for 4 weeks in the greenhouse and 2 weeks under 30% shade. While a high percentage of tubelings had root balls that held together after 6 weeks of acclimation--an important consideration when outplanting--increasing the time of acclimation by 2 to 4 weeks should result in even a higher percentage of tubelings with sturdy root balls.

Production time can be decreased by 4 weeks by rooting and acclimatizing shoots simultaneously, although ultimate survival of acclimatized tubelings is less than if shoots are rooted in vitro. The two most important factors in rooting shoots in the greenhouse under mist are tiller number (3-4) and height (3-5cm); there is no benefit to treating shoots with Rootone F or IBA (powder forms for both) prior to sticking shoots in tubeling trays containing MetroMix 200.

Rooting these shoots in vitro and then acclimatizing them in the greenhouse resulted in a greater percentage of surviving tubelings than when shoots were both rooted and acclimatized under greenhouse conditions. In greenhouse tests, tissue-cultured plants grown in pots on overburden and sand tailings were similar to plants by propagated by

division with respect to shoot, root and rhizome mass, but tiller number per plant was greater on tissue-cultured plants compared with division plants.

In field trials on mined land, tissue-cultured plants established well when planted in January, July, or October; however survival was less on overburden when the planting occurred in October, which was due to typical low rainfall at this time. On our sites, a large soil seed bank resulted in much weed competition. We feel that weed control by preventing the build-up of weed seed after mining plus a diligent herbicidal weed control program after planting creeping bluestem will be very important to success. Because it is a major component of central and south Florida native plant communities, creeping bluestem needs to be included in restoration of mined lands. Since the grass produces little seed, shoot multiplication culture offers a practical way to produce plants that can establish and grow well on phosphate-mined land.

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CHAPTER 3

MICROPROPAGATION OF WIREGRASS

Jeffrey G. Norcini, Tarak N. Chakravarty, Robert S. Kalmbacher, and Wusi Chen

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EXECUTIVE SUMMARY

Wiregrass (*Aristida beyrichiana*) is a highly desired native grass for use in revegetating mined lands; however; it is an unreliable, and frequently poor, seed producer. Direct seeding of this species is potentially the most cost-effective means for revegetating mined lands. For those that want to direct seed, they must harvest a "seed hay" from nearby land and spread the "seed hay" within a day or so of harvest. Otherwise, those wanting to use wiregrass must rely on a limited supply of tubelings. At the time this study was initiated, the best opportunity for a consistent and widespread supply was for wiregrass propagated by tissue culture. Tissue culture techniques have the advantage of offering a rapid method of generating large quantities of propagules. Prior to this study, little work had been conducted involving micropropagation of wiregrass.

Micropropagation was evaluated as means of mass-producing wiregrass. The overall approach was to use somatic embryogenesis because plants regenerated by this method will probably look the same and be genetically identical to the mother plants. It is the most common method of regenerating grasses via tissue culture (I.K. Vasil, 1987, "Developing cell and tissue culture systems for the improvement of cereal and grass crops," J. Plant Physiol. 128:193-218.). In this technique, a special type of callus tissue (which is basically an undifferentiated mass of cells) called embryogenic callus, is induced to form embryos. These embryos are identical to embryos in seeds that are normally generated by fertilization of eggs by the male gametes from pollen – zygotic seed. (Note: Seed in plants can also be formed without fertilization through a process called parthenocarpy.) Somatic embryos can be encapsulated to produce synthetic seed, plants can be directly regenerated from the embryos, or embryo cultures can be used to develop shoot multiplication cultures.

Our initial work focused on production of synthetic seed because if seed could be economically produced in large quantities then seeding of mined land would be possible. The base tissue culture media used in this study was the Murashige and Skoog basal medium supplemented with coconut milk from immature or mature coconuts. Supplementing the medium with 2,4-dichlorophenoxyacteic acid at 2-3 mg/L resulted in embryogenic callus formation but embryogenic callus could only be induced to form from immature inflorescences of two plants. Repeated attempts to reproduce these results with the immature inflorescences from that plant and others, as well as other types of tissue, were not successful. However, shoots were regenerated from the embryos growing on solid media and eventually rooted. Plants regenerated from somatic embryos developed normally after being transplanting to containers.

Embryogenic callus growing on solid media was also used to initiate liquid suspension cultures in which finely divided embryogenic callus was induced to form pre-embryos (also called embryoids). Pre-embryos were plated out onto solid media designed to allow development of the pre-embryos into embryos. Mature embryos on solid media were then mixed in alginate (a seaweed derivative) gel. Embryos in the gel were then dropped into a calcium chloride solution (one to three embryos in a droplet).

The droplet containing the embryo became hardened after several minutes in the calcium chloride solution thereby forming a synthetic seed. Thirty-six synthetic seed were produced but only three germinated in vitro.

Contamination of in vitro cultures was a major problem and the original embryogenic callus of wiregrass was lost. After extensive evaluation of cultural methods to obtain clean cultures, the best method to obtain clean callus cultures was to germinate wiregrass seed in vitro and then use these seedlings to initiate callus. Numerous attempts to induce this callus to become embryogenic were not successful. Efforts to directly regenerate wiregrass in vitro without involving somatic embryogenesis were not successful either.

METHODOLOGY

Plant incubator conditions throughout this chapter, except as noted: 25-26C; 16-hr photoperiod; light provided by cool white fluorescent bulbs, with light level of 40μE at Petri plate level).

STUDY 1

Callus Initiation from Young Leaves

All wiregrass were grown in 3.8- or 11.4-liter pots under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

- Wiregrass North Florida (plant nos. 1321, 1521, 3221, 4521, 5121, 5221, 6121, 6421,7321, 7421, 7621, 72213, 47212, 47213, 47214, N-1, N-2, N-3, N-4, N-5)
- Wiregrass from Central Florida (plant nos. 1122, 1422, 3622, 3722, 4422, 5422, 61021
- Wiregrass from South Central Florida (plant no. R-1)

Young leaves were sterilized as follows: 70% ethanol for 30 sec; shaking in 1% sodium hypochlorite + few drops of Tween 20 at 100 rpm for 20 min; rinse 5X with sterile distilled water (SDW).

The older external portion of leaves was removed; 1- to 3-cm long segments from the basal portion of the leaves was used as starting material. The inner young leaf segments were cut into 2-mm sections and cultured in the dark at 25C on MS medium amended with different concentrations and combinations of plant growth regulators. **Unless otherwise mentioned, all tissue culture media used for wiregrass was at pH 5.8 and contained 3% sucrose (w/v), with solid media containing 0.8% Phytagel (Sigma Chemical) (w/v).**

a. MS + 2,4-D (2 mg/L) + 5% CM
b. MS + 2,4-D (2.5 mg/L) + 5% CM
c. MS + 2,4-D (3.0 mg/L) + 5% CM
d. MS + 2,4-D (2.5 mg/L) + BA (0.2 mg/L) + 5% CM

2,4-D	2,4-dichorophenoxyacetic acid
BA	6-Benzylaminopurine (syn. benzyladenine)
CM	Coconut Milk (mature coconuts; % is v/v) (*Milk from fresh, mature coconuts was extracted, heated to 70C for 60 min, and then filtered to remove particulate matter. It was stored at -20C.*)

MS Murashige and Skoog Basal Medium (Murashige and Skoog 1962)

Callus Initiation, Maintenance and Embryogenesis from Immature Inflorescences

All wiregrass were grown in 3.8- or 11.4-liter pots under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

- Wiregrass North Florida (1121, 1521, 2421, 2521, 3221, 3821, 4521, 5121, 6421, 6521, 7321, 7421, 7821)
- Wiregrass from Central Florida (3622, 61021)
- Wiregrass from South Central Florida (R-1)

Young, unemerged inflorescences were surface sterilized while still enclosed by several whorls of leaves. The sterilization procedure was the same as above. The inflorescence was exposed (by removal of the surrounding leaves), cut into 1- to 2-mm thick segments, placed on MS medium (see below) and cultured at 25C in the dark.

a. MS + 2,4-D (2 mg/L) + 5% CM
b. MS + 2,4-D (2.5 mg/L) + 5% CM
c. MS + 2,4-D (3.0 mg/L) + 5% CM
d. MS + 2,4-D (2.5 mg/L) + BA (0.2 mg/L) + 5% CM
e. MS + 2,4-D (3.0 mg/L) + BA (0.2 mg/L) + 5% CM
f. MS + 2,4-D (3.5 mg/L) 5% CM

Initiation, Maintenance, and Embryoid Induction of Cell Suspension Cultures

- Embryogenic callus of North Florida wiregrass 1521 and 3221

Callus was dissected into small pieces. About 100 mg (fresh weight) callus were placed in 25 ml liquid Medium *g* or *h* in 125-ml flasks on a gyrating shaker (15 rpm). Suspension cultures were subcultured on the same medium every 4 days. The cultures were grown in darkness at 27C. Four weeks after initiation, the cultures were maintained in Medium *i*.

g. MS + 2,4-D (1.5 mg/L) + 2.5% CM
h. MS + 2,4-D (2.5 mg/L) + 2.5% CM
i. MS + 2,4-D (2.5 mg/L) + BA (0.2 mg/L) + 2.5% CM
j. MS + 2,4-D (0.5 mg/L) + BA (0.2 mg/L) + 2.5% CM
k. MS + 2,4-D (1.5 mg/L) + BA (0.2 mg/L) + 2.5% CM

Embryoid Formation and Plant Regeneration on Solid Media

- Embryogenic callus initiated from immature inflorescence of wiregrass 1521 and 3221

Embryogenic callus was transferred from Medium *a* or *b* to different regeneration media (see below; 0.5% agar [w/v]). Embryos at different developmental stages were observed 2 weeks after culturing on those media. Mature embryos were then transferred to MS medium without plant growth regulators for plant regeneration. After inducing rooting and shooting, regenerated plantlets were transplanted into larger containers and placed in a growth chamber for further development.

Six to 8 weeks later, seedlings were transplanted into 5.7 cm x 5.7 cm x 5.7 cm plastic cups containing a medium comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur, and then acclimatized to greenhouse conditions. About 7 weeks later, seedlings were transplanted to 3.8-liter plastic pots (same soilless medium as above), some of which were placed in a shadehouse (30% shade).

(Nonconsecutive labeling of the media is due to media used for creeping bluestem but not wiregrass.)

m. MS + 2,4-D (0.2 mg/L) + 5% CM
n. MS + NAA (0.05 mg/L) + BA (0.2 mg/L) + 5% CM
o. MS + ABA (0.264 mg/L) + 5% CM
p. MS + TDZ (0.2 mg/L) + 5% CM

ABA	Abscisic acid
NAA	1-Naphthaleneacetic acid
TDZ	Thidiazuron

Encapsulation of Somatic Embryos and Germination of Synthetic Seeds

- Somatic embryos of wiregrass 1521 growing on solid medium

Somatic embryos were isolated from polyembryoid clumps. One hundred milliliters sterile 100 mM $CaCl_2$ solution was placed in a sterile 250-ml beaker. A sterile disposable plastic pipette was placed into the opened flask of sterile sodium alginate (2% [w/v] in half-strength MS medium), and alginate was drawn into the pipette. Alginate was then slowly dropped from the tip that was positioned above the $CaCl_2$ salt bath. Isolated somatic embryos were inserted into the alginate drop by using forceps. The alginate drop containing one to three somatic embryos then fell into the $CaCl_2$ salt bath. The capsules were complexed after shaking in an incubator for 30 to 45 min. Then the $CaCl_2$ solution was removed and the beads were washed in 100 ml sterile distilled for 5 min (3X).

Capsules containing somatic embryos were stored in the dark at 4C for 3 days before planting in vitro on solid MS + 2,4-D (0.2 mg/L) (12 synthetic seed in each of three Petri plates). Synthetic seed were germinated in an incubator at 25C (16-hr photoperiod).

STUDY 2

This study was conducted to determine if the methods of Study 1 were repeatable, and if not, to evaluate other methods of micropropagating wiregrass via somatic embryos.

Reevaluation of Surface Sterilization and Embryogenic Callus Initiation Protocols

All wiregrass were grown in 3.8- or 11.4-liter pots in full sun or under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

- Wiregrass from Central Florida (1122, 1422, 4422)
- Wiregrass from North Florida (1521, 3221, 47214, 47213, 47212)
- Progeny of wiregrass 1521: seeds, with 1521 as mother plant (could have been cross-pollinated); tissue-cultured (solid media) plants planted March 3, 1998 and repotted April 13, 1998

At the end of July 1998, there was severe fungal and bacterial contamination and many cultures were lost. Contamination may have been due to environmental conditions – excessive rains during the summer of 1998 may have led to an increase in spores in the atmosphere. Several steps were taken to control the contamination, including consultation with experts and sending out the contaminated cultures for identification of the bacteria and fungi. In addition, there was poor callus formation (on Media *a* to *f*), so the media were modified to improve embryogenic callus initiation. These procedures are outlined below.

Various sterilization procedures for individual immature inflorescences of wiregrass (stock plants and tissue-cultured plants) were evaluated based on our experience and the advice of Dr. Tim Schubert (Division of Plant Industry) and Dr. Kenneth C. Torres (Phytotechnology Labs):

- Explant kept in distilled water – 0 or 24 hr
- Wash in tap water for 45 or 60 min
- Surface-sterilize with 90% EtOH for 30 sec instead of 1 min; 70% EtOH used previously
- Soak in 0.3% or 0.5% mercuric chloride for 5 min; 1% or 5.25% sodium hypochlorite used previously

- Wash with sterile distilled water 5X @ 10 min
- Replace filters in laminar flow hood; purchase fresh chemicals
- Add Plant Preservative Mixture (PPM) to media – various concentrations
- Immature inflorescence explant size: 1-2 mm or 2-3 mm

"Sterile" immature inflorescences were cut into 1-2 mm or 2-3 mm long segments from the basal portion of the inflorescence and cultured on in the dark at 25C on solid media listed below for embryogenic callus initiation.

A. MS + 2,4-D (3.0 mg/L) + 10% GCM
B. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + 10% GCM + PPM (2 mg/L)
C. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.1% AC + PPM (2 mg/L)
D. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.1% AC + PPM (2 mg/L)
E. MS + 2,4-D (3.0 mg/L) + $ZnSO_4$ (5.2μM) + 10% GCM
F. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + BAP (1.0 mg/L) + CH (500 mg/L) + $ZnSO_4$ (5.2μM) + 10% GCM

GCM	Milk from immature green coconuts (% is v/v)
CH	Casein Hydrolysate
PPM	Plant Preservative Mixture ((Phytotechnology Labs)
AC	Activated Charcoal (% is w/v)
$ZnSO_4$	Zinc Sulfate

[Note: All media in Study 2 (i.e., post May 1998) are in capital letters so as to differentiate them from media used in Study 1 (June 1997 through May 1998).]

Starting in August 1998, several batches of immature wiregrass inflorescences were frozen at -80C to determine if stored ultrafrozen tissue could be used to initiate embryogenic callus. Several batches of wiregrass from the plants listed above were frozen at -80C from August 1998 to December 1998. In addition to the above plants, immature inflorescences of the following wiregrass plants were also frozen: 3821, 3921, 1622, 2214, 1351, 5422, 3422, 7421, 6821, 5431, 71121, 7521, 7821, 7721, ONF and ANF.

Inflorescences were packed in plastic quart-sized freezer bags (ZipLock™), labelled, and placed in a freezer at -80ºC. The inflorescences were packed in several batches on different dates. When these tissues were transported from Quincy to Monticello, they were kept frozen with dry ice.

Since we observed slow or no callusing of wiregrass explants in the Study 2 experiments described above, we questioned whether the vigor of the stock plants had an effect. Vigor of wiregrass is increased after burning, and our plants were last burned 5 years ago.

On March 19, April 12, and May 19, 1999 several containerized wiregrass plants were burned (burn conditions: plants – sunk in soil to top of pot; air temp. – 3-19: 24.9C; 4-12: 29.4C; 5-19: 24.7C; RH – 3-19: 39%; 4-12: 20%; 5-19: 61%; wind – 3-19: 7 mph; 4-12: 11 mph; 5-19: 2.9 mph; type of fire – back fire; length of time of burn – 3-19: 5 min; 4-12: 7 min; 5-19: 9 min.; moisture content of oak leaves and or pine needles used as supplement fuel – 3-19: 8%; 4-12: 13%; 5-19: 20%). The burned plants were transferred to the greenhouse the same day. After 48 hr, new wiregrass shoots emerged.

All wiregrass were grown in 3.8- or 11.4-liter pots in full sun or under 30% shade, some of which were transferred to a greenhouse. The potting medium ("low fertility mix") was comprised of pine bark:Canadian sphagnum peat:sand, 3:1:1 (by vol.) amended with (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur.

- Progeny of wiregrass 1521: from seeds, with 1521 as mother plant (could be cross-pollinated); tissue-cultured (solid media) plants planted 3/3/98 and repotted 4/13/98
- Wiregrass – ONF FWWG 1997 A5
- Wiregrass 1521 from solid cultures

Studies were initiated to determine optimal type of tissue explant to use for embryogenic callus initiation, but first a new sterilization procedure needed to be worked out because soaking the explants in 0.5% mercuric chloride for 5 min (the sterilization protocol that resulted in the least contamination as determined from the previous study) apparently killed the explants based on results of tetrazolium (TZ) tests. The use of TZ salt solutions is used to testing seed viability (Grabe 1970). The TZ salt is an oxidation-reduction indicator. Tissues that are living and respiring react with TZ to form a reddish pink compound. After the TZ solution (pH range 6-8) is imbibed by the tissue it interacts with the reduction processes of living cells and accepts hydrogen from the dehydrogenases. By hydrogenation of the 2,3,5-triphenyl tetrazolium chloride, a red, stable substance, triphenyl formazan, is produced in living cells. This makes it possible to distinguish the red-colored living parts of the tissue/seed from the colorless dead ones (Moore 1966).

Tetrazolium is rarely used in tissue culture to assess explant or cell viability, and a literature search revealed no instances in which TZ was used to assess tissue viability after surface sterilization. However, Nyange and others (1997) used the TZ dye MTT in cell suspension cultures of *Coffea arabica* to assess susceptibility of *Coffea arabica* genotypes to coffee berry disease.

While evaluating the sterilization methods, microbial contamination was assessed by incubating treated explants in three selective media – AC Broth (wide variety of microorganisms), YE Broth (bacteria), and SA Broth (molds/yeasts). The three controls were no tissue in the tube, untreated tissue in the tube, and dead sterile tissue (treated with 90% EtOH for 30 min) in the tube.

- Shoots were excised and rinsed in water. Outer leaf sheaths were removed to gain access to the tissue at the shoot/root interface. Three 4-6 mm sections at the interface were excised.
- Immature inflorescences (August 1999 – October 1999)
- Mercuric chloride treatments:

0.5% $HgCl_2$	1 min
0.5% $HgCl_2$	0.5 min
0.4% $HgCl_2$	1 min
0.4% $HgCl_2$	0.5 min
0.3% $HgCl_2$	1 min
0.3% $HgCl_2$	0.5 min
0.2% $HgCl_2$	1 min
0.2% $HgCl_2$	0.5 min
0.1% $HgCl_2$	1 min
0.1% $HgCl_2$	0.5 min
100% Clorox	5 min

In a separate study:

- Shoots were excised and rinsed in water. Outer leaf sheaths were removed to gain access to the tissue at the shoot/root interface; three 4-6 mm sections were collected.
- Immature inflorescences (August 1999 – October 1999)
- Seeds – 2 seed coat layers were removed

Explants were immersed in 90% ethanol for a few seconds and then immersed in 100% Clorox (containing 2 drops of Tween 20 [Fisher Scientific, St. Louis, MO) for 30 min. The explants were then washed with sterile distilled water 5X @ 5 min.

The media evaluated for callus initiation are listed below. There were five plates (replicates) per treatment, with three to five explants per plate. Percentage was calculated as an average of the replicates. Explants were cultured in the dark at 25C.

G. MS + 2,4-D (3.0 mg/L) + GA_3 (1.0 mg/L) + $ZnSO_4$ (5.2 μM) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
H. MS + 2,4-D (0.5 mg/L) + 10% GCM + 0.3% Phytagel
I. MS + 2,4-D (1.0 mg/L) + 10% GCM + 0.3% Phytagel
J. MS + 2,4-D (1.5 mg/L) + 10% GCM + 0.3% Phytagel
K. MS + 2,4-D (2.0 mg/L) + 10% GCM + 0.3% Phytagel
L. MS + 2,4-D (2.5 mg/L) + 10% GCM + 0.3% Phytagel
M. MS + 2,4-D (3.0 mg/L) + 10% GCM + 0.3% Phytagel
N. MS + 2,4-D (0.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
O. MS + 2,4-D (1.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
P. MS + 2,4-D (1.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
Q. MS + 2,4-D (2.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
R. MS + 2,4-D (2.5 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel

S. MS + 2,4-D (3.0 mg/L) + CH (500 mg/L) + 10% GCM + 0.3% Phytagel
T. MS+ 2,4-D (3.0 mg/L) + Kn (2.0 mg/L) + GA_3 (1.0 mg/L) + zeatin (0.5 mg/L) + $ZnSO_4$ (5.2μM) + 10% GCM + 0.3% Phytagel
U. MS + 2,4-D (4.0 mg/L) + 10% GCM + 0.3% Phytagel

Reinitiation of Cell Suspension Cultures

- Embryogenic callus of North Florida wiregrass 1521 and 3221 in culture since March 1998

Embryogenic callus growing in the dark at 25C, which was maintained on MS + 2,4-D (3.0 mg/L) + 5% (by vol.) CM + 0.7% Phytagel since 1998, was used to initiate suspension cultures. Pieces of embryogenic callus, each *ca.* 200-mg (fresh weight; subdivided 1-g pieces), were placed in separate 125-ml flasks containing 40 ml of one of the 19 media listed below. Flasks were shaken at 150 rpm in darkness at 25^0C. Subculturing was done every fifth day. Finely dissociated embryogenic cells were selected for by filtering the suspension through a sterile nylon sieve (100 μm pore size). One ml of the resultant filtered suspension that contained these finely divided embryoid cells was added to 40 ml of fresh medium (per flask) for growth and further selection.

V. MS + 2,4-D (0.5 mg/L) + 10% GCM
W. MS + 2,4-D (1.0 mg/L) + 10% GCM
X. MS + 2,4-D (1.5 mg/L) + 10% GCM
Y. MS + 2,4-D (2.0 mg/L) + 10% GCM
Z. MS + 2,4-D (3.0 mg/L) + 10% GCM
AA. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + 10% GCM
BB. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
CC. MS + 2,4-D (2.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
DD. MS + 2,4-D (3.0 mg/L) + BAP (0.1 mg/L) + 10% GCM
EE. MS + 2,4-D (0.5 mg/L) + zeatin (0.2 mg /L) + 10% GCM
FF. MS + 2,4-D (0.2 mg/L) + BAP (0.2 mg/L) + ABA (1.0 mg/L) + 10% GCM
GG. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + ABA (2.0 mg/L) + 10% GCM
HH. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + ABA (3.0 mg/L) + 10% GCM
II. MS + 2,4-D (1.5 mg/L) + zeatin (2.0 mg/L) + ABA (0.2 mg/L) + 10% GCM
JJ. MS + 2,4-D (0.5 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
KK. MS + 2,4-D (1.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
LL. MS + 2,4-D (2.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
MM. MS + 2,4-D (3.0 mg/L) + BAP (0.1 mg/L) + GA_3 (0.1 mg/L) + 10% GCM
NN. MS + 2,4-D (1.5 mg/L) + BAP (0.2 mg/L) + GA_3 (0.2 mg/L) + zeatin (0.5 mg/L) + 10% GCM

Plant Regeneration on Solid Medium

- Embryos generated from embryogenic callus of North Florida wiregrass 1521 and 3221 in culture since March 1998

A limited number of wiregrass embryos were cultured (in a lighted incubator) on MS medium containing 2,4-D at 3 mg/L (Medium *M*) for plant regeneration.

Direct Regeneration of Wiregrass

Studies were conducted to try and directly regenerate multiple wiregrass plantlets in vitro using different types of explants (nodes and leaves from plants that were cut back or burned and then placed in greenhouse; nodes, leaves, and inflorescences from ultrafrozen tissues). There were at least three Petri plates per treatment with at least five explants per dish. Cultures were incubated in the dark at 25C. The surface sterilization procedure is below.

- Explant kept in distilled water – 24 hr
- Wash in tap water for 45 min
- Surface-sterilize with 90% EtOH for 30 sec
- Soak in 0.5% mercuric chloride for 1 min
- Wash with sterile distilled water 5X @ 10 min

OO. MS + myoinositol (10 mg/L) + NAA (1.0 mg/L) + zeatin (0.5 mg/L) + 0.1% AC + 10% GCM

PP. MS + 2,4-D (1.0 mg/L) + GA3 (1.0 mg/L) + Kn (1.0 mg/L) + 0.1%AC + 10% GCM

QQ. MS + myoinositol (10 mg/L) + NAA (1.0 mg/L) + zeatin (0.5 mg/L) + 10% GCM

RESULTS

STUDY 1

Callus Initiation from Young Leaves

Almost all explants turned brown and died after 3 to 4 weeks. Only a few explants – four sections of wiregrass 3622 and one section of 1521 – formed callus 3 weeks after culture. All of the calli formed from leaves was non-embryogenic.

Callus Initiation, Maintenance, and Embryogenesis from Immature Inflorescences

Tissue swelling at the cut edge of some explants was observed 1 week after culturing. After 4 to 5 weeks, two types of calli were formed from explants of plant 1521 on Media *b*, *c*, and *d* – soft, wet and unstructured non-embryogenic callus, and compact, nodular and pale-white embryogenic callus. Explants from most of the other donor plants did not form any callus. Some to little callus was formed from explants of wiregrass 4521, 3221, 6421, 6521 and 7321. Globular and heart shape pre-embryoids were observed 5-6 weeks after callus initiation, primarily from explants of plant 3221.

Embryogenic callus was maintained on MS medium containing 3 mg/L 2,4-D with or without BA (Medium *c* and *e*, respectively). Callus of wiregrass 1521 started rooting on the medium with BA 6-8 weeks after initiation.

Initiation and Maintenance of Cell Suspension Cultures

Suspension cultures were comprised of two distinct types of cells: (1) non-embryogenic cells – large, vacuolated and elongated cells, with sparse cytoplasm, and devoid of any storage starch, and (2) embryogenic cells – small, generally rounded cells, richly cytoplasmic, that contained a prominent nucleus and plastids with starch. These cells were in compact groups of many cells without intercellular spaces.

Callus turned brown and grew slowly for the first 2 weeks in suspension culture. Three to 4 weeks after initiation, callus remained white and grew rapidly. A low concentration of 2,4-D (1.5 mg/L) in liquid medium (Medium *g*) resulted in fast growth of embryogenic callus. Additional BA in the medium caused fast growth of both embryogenic and non-embryogenic callus. Some pre-embryos (embryoids) were observed in all of those media, except Medium *i*, 5 to 6 weeks after initiation. The best result was obtained from Medium *j*. Four weeks after initiation, the cultures were characterized by a mucilaginous consistency. Many embryogenic cells enlarged, had few starch grains, and appeared less dense due to increased vacuolation. Eight weeks after initiation, the culture contained many embryogenic clumps interspersed with large

numbers of loose, elongated and vacuolated cells. Ten to 12 weeks after initiation, the suspension cultures became extremely thick because of heavy accumulation of a mucilaginous substance. Cell enlargement predominated rather than cell division, with the majority of cells being thick-walled, elongated and non-dividing. At this last stage, it was difficult to return the cultures back to an embryogenic state.

This has been one of the problems with wiregrass suspension cultures. They have proceeded to this last stage and we were unable to return the suspension cultures back to an embryogenic state. Increasing the concentration of 2,4-D to 3.5 mg/L in 4-week-old suspension cultures appeared necessary for maintaining the embryogenic nature of cell suspension cultures.

Embryoid Formation and Plant Regeneration on Solid Media

Embryos at different developmental stages were observed 3 to 4 weeks after transfer onto regeneration media. A lower concentration of 2,4-D seemed to be required for maturity of the embryos. Plant regeneration was also carried out on MS medium without growth regulators; however, NAA and BA were required for root development. Total regeneration time for shoot and root development was 10 weeks. Also, only 12 of the embryos grew into seedlings and were successfully transplanted.

Seedlings were successfully transplanted into a pine bark:peat:sand medium, acclimatized to greenhouse conditions, and transplanted to 3.8-liter containers. Under irrigated shade house conditions, all plants developed normally. Flowering occurred within two months from the time they were transferred to the shade area; however, flowering occurred later than that of the stock plants. Tissue-cultured produced plants of wiregrass 1521 flowered in the greenhouse during September.

Encapsulation of Somatic Embryos and Germination of Synthetic Seed

Of the 36 synthetic seed germinated in vitro, only three germinated after 17 days; the remainder died.

STUDY 2

Reevaluation of Surface Sterilization and Embryogenic Callus Initiation Protocols

Contamination was nearly eliminated when we followed the protocol listed below for immature inflorescence explants that were 1-2 mm long.

1. Explant kept in distilled water for 24 hr
2. Wash in tap water for 60 min

3. Surface-sterilize with 90% EtOH for 30 sec
4. Soak in 0.5% mercuric chloride for 5 min
5. Wash with sterile distilled water 5X @ 10 min
6. Add 2 ppm Plant Preservative Mixture to media

However, we later discovered (via tetrazolium [TZ] testing) that this protocol was lethal even though explants remained green. Tissues that are living and respiring react with TZ to form a reddish pink compound. We performed a preliminary TZ test (1% solution; pH 6.7) test on fresh and dead (boiled or soaked in ethanol for a few minutes) leaf explants of wiregrass similar to ones that were being cultured in vitro. A pink color developed on fresh explants; no color developed on the dead explants. We then ran TZ tests on some nonresponsive explants (that were still green) that were being cultured. No pink or red color formed, so we assumed the explants were dead.

As a result of TZ testing to evaluate the effect of mercuric chloride, we have observed that the tissues were alive in the center, and in most of the cases the basal and distal ends were killed (>1 mm) by mercuric chloride. One to two millimeters of the basal and distal ends of the explants was removed so as to eliminate any dead tissue that could have hindered callus initiation. Also, exposure to 0.1-0.5% mercuric chloride for greater than 1 min was usually lethal to immature leaf explants of wiregrass.

We observed that treating explants with 100% Clorox for 30 min kept explants clean and initiation of callus was observed (Table 3.1). We sent contaminated wiregrass explant samples (after bacterial and fungal indexing conducted in June) to the Division of Plant Industry to determine the pathogens. The only bacterium that was identified was *Curtobacterium flaccumfaciens*; the three fungi identified were *Alternaria alternata*, *Epicoccum purpurascens*, and *Phoma* sp. Given the relative success with Clorox and the problems associated with the use of mercuric chloride, the use of mercuric chloride for callus initiation experiments was discontinued and is not recommended.

The best method for surface sterilization was as follows:

1. Wash in running tap water for 60 min
2. Surface sterilize with 95% EtOH for 30 sec
3. Surface sterilize in 100% Clorox (+ two drops Tween 20) for 30 min
4. Wash with sterile distilled water 5X @ 10 min

After 3 months, no callus was initiated from immature inflorescences in Media *A* to *F* or Media *G* to *U* (Table 3.1), or from tissue of the root-shoot interface (Media *G* to *U*, Table 1).

Wiregrass seeds germinated in vitro within 8 days only on MS + 2,4-D (3.0 mg/L) + 10% GCM (Medium *M*). Nonembryogenic callus formed at what appeared to be the root/shoot interface in less than 3 weeks (95% response) (Table 3.1). In addition to the callus, some roots formed as well. The optimum results for Medium *M* was for seed treated with 100% Clorox for 30 min.

No callus was initiated from frozen immature inflorescences harvested in late summer and fall of 1998. This may have been due to the use of mercuric chloride and/or related to the vigor of the stock plants.

Reinitiation of Cell Suspension Cultures

Several attempts to reinitiate embryogenic wiregrass suspension cultures from the original embryogenic callus of plants 1521 and 3221 (growing in the dark on solid media) in Media *g*, *h*, or *i*, or Media *V* to *NN* were not successful.

Table 3.1. Surface Sterilization and Callus Initiation of Wiregrass.

Clorox Exposure:		100% Clorox; 5 min		100% Clorox; 30 min	
Plant type	Explant type	Pct. Contam.	Pct. Callus Initiation	Pct. Contam.	Pct. Callus Initiation
Container-Grown; Mother Plant; Postburn	Root/shoot Interface	30	0	10	0
	Immature Inflorescence	20	0	5	0
	Seed	90	0	5	95
Container-Grown; Tissue-Cultured; Postburn	Root/Shoot Interface	40	0	10	0
	Immature Inflorescence	50	0	5	0

Plant Regeneration on Solid Media

Wiregrass embryos (derived from the original embryogenic callus line) cultured on MS medium containing 3 mg/L 2,4-D (Medium *M*) exhibited root and shoot formation in 25% of the cultures.

Direct Regeneration of Wiregrass

In two separate studies, no shoots, roots, or callus formed after 3 months in cultures of nodes and leaves from plants that were cut back or burned and then placed in

the greenhouse, or nodes, leaves, and inflorescences from ultrafrozen tissues. The lack of any response was probably due to the use of mercuric chloride.

Observations of Growth of Tissue-Cultured Plants

Growth of the acclimatized tissue-cultured plant appeared normal except initially they seemed to grow as fast or faster than plants propagated by division. Phenotypically there was no difference between the mother plant and the tissue-cultured plants except that tissue-cultured plants were slightly shorter. This difference in height may have been because the tissue-cultured plants flowered earlier than those propagated by division. Studies in other grasses confirm that plants regenerated by somatic embryogenesis show high uniformity of regenerants and avoidance of somaclonal variation. There is a high probability that our plants regenerated via somatic embryogenesis do not have any genetic variation and are true-to-type.

CONCLUSIONS AND RECOMMENDATIONS

It was very difficult to initiate embryogenic callus from immature leaf or inflorescence tissue. Although embryogenic callus was initiated on MS basal medium supplemented with 2,4-D at 2.5 or 3 mg/L, future researchers should evaluate the use of other common media. Obtaining embryogenic callus was further complicated by commonly occurring bacteria and fungi that contaminated cultures. The use of mercuric substantially reduced contamination but it should be avoided as it was lethal to the young tissue. In contrast, surface sterilization protocols utilizing Clorox for 30 min substantially reduced contamination but were not lethal.

The other major problem occurred with maintaining the embryogenic nature of suspension cultures. After 10 to 12 weeks, these cultures became extremely thick because of heavy accumulation of a mucilaginous substance. Cell enlargement predominated rather than cell division, and the majority of cells were thick-walled, elongated and non-dividing. At this stage, it was difficult to return the cultures back to an embryogenic state. If the generation of two-three embryo clumps is desired for production of synthetic seed (or for other purposes), future work will need to focus on methods of prolonging the embryogenic nature of suspension cultures. However, given the short-lived nature of embryogenic suspension cultures and poor germination of synthetic seed, strong consideration should be given to regenerating plants from clumps of embryos growing on solid media. These clumps of embryos could also be used to start shoot multiplication cultures.

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CHAPTER 3

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CHAPTER 4

PRELIMINARY STUDY: PROPAGATION OF GOPHER APPLE VIA MICROPROPAGATION AND STEM CUTTINGS

James H. Aldrich, Jeffrey G. Norcini, and Tarak N. Chakravarty

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EXECUTIVE SUMMARY

Gopher apple (*Licania michauxii*), a drought-tolerant woody native to Florida uplands, is locally abundant and can function as a groundcover. It seems to be consistently demanded for in a variety of restoration and mitigation projects, and it is a prime candidate for use in mine reclamation. At a FIPR RTAC meeting, it was suggested that demand for gopher apple exceeds supply due to difficulty in adequate propagation methods, which is now via seed only. There have been no studies on commercial propagation of gopher apple except for informal work conducted by local nursery personnel. The main objective of this study was to provide empirical data that could be used in future studies for determining the most effective means of propagating gopher apple.

Gopher apple seed collected from a north Florida population (Leon County) were germinated in vitro (86% germination) in 60-ml test tubes for the primary purpose of generating plant material that was unlikely to be contaminated, at least externally. Terminal and nodal explants from these in vitro grown seedlings were cultured on Murashige and Skoog basal medium (MS) supplemented with cytokinins (kinetin, 2-iP, zeatin, CPPU, thidiazuron, or benzyladenine) to induce shoot formation. Abundant axillary shoot production was not achieved; however, terminal explants cultured on MS + CPPU (0.5 or 1.0 mg/L) developed an average of 2.5 or more shoots per explant compared to only one axillary shoot per explant on MS without any cytokinin. Basal callus formed on 10 and 100% of the terminal explants on MS + CPPU at 0.5 and 1.0 mg/L, respectively. Callus is generally not desired in this type of micropropagation culture designed for axillary shoot production. Seeds that germinated but where contaminated and not used as an explant source were successfully planted in soilless medium and grown in vivo. Explants derived from containerized plants grown outdoors had 100% contamination despite use of a broad-spectrum systemic fungicide about a week prior to harvesting of the explants.

Terminal stem cuttings were harvested in April, May, June, July, October, and November from a north Florida population (Wakulla County). Cuttings (10-13 cm; 3-4 leaves) with two 1-cm scores on opposite sides of the base were dipped in 0.1, 0.3, 0.8, 1.6, 3.0, or 4.5% IBA (Hormex) and then placed in propagation cups containing MetroMix 200. Cuttings were rooted under intermittent mist in a greenhouse. One month after sticking, cuttings were lightly fertilized. Gopher apple cutting harvest date (which was related to cutting quality) seemed to have the most influence on rooting 2 months after cuttings were stuck. Cuttings taken in May rooted the best based on quantitative and visual assessments; IBA did not improve rooting. Cuttings harvested in April had acceptable rooting and benefited from IBA, but no more than 0.1% was necessary to improve rooting. Rooting ability of stem cuttings steadily declined for cuttings harvested in June, July, October, and November, with no rooting or callus formation on October and November cuttings after 2 months. The lack of any root/callus formation may have been due to inadequate heating of the greenhouse during late fall and winter. The rooting medium temperature was only about 10C (50F).

METHODOLOGY

MICROPROPAGATION

- Undamaged mature seeds from a natural stand of gopher apple along Tram Road in Leon County, FL (collected in October 2001)

Seed flesh was manually removed after seed were collected in October 2001. Thirty-seven seeds were surface sterilized on October 24, 2001 as follows:

1. Soak in 95% EtOH for 5 min
2. Soak in 100% bleach (2 drops Tween 20 / 100 ml) for 15 min
3. Rinse with sterile distilled water 5X

Bleach – Star White Bleach, Dolgencorp, Inc.
Tween 20 – Sigma Chemical

Surface-sterilized seeds were separately cultured in 60-ml tubes that contained Murashige and Skoog (MS) basal medium (1962). All media in this chapter was supplemented with 3% sucrose (w/v) and 0.3% Phytagel (w/v) (Sigma Chemical), and was at pH 5.8. Incubator conditions throughout this chapter were as follows: 25C; 16-hr photoperiod; a light intensity of 355μE provided by two 40W cool white fluorescent bulbs.

In vitro seedlings were used as explant sources to investigate axillary shoot production. Although 32 of the 37 seeds cultured in October 2001 had sprouted by April 26, 2002 (the other five seeds were contaminated), only 24 seedlings had 0.5-cm terminals or terminals with one or more 1-cm nodal sections. Terminal and nodal explants were cultured on 19 MS media (100 ml medium per 473-ml jar [pint mason jar, item #14400-61000; Alltrista Corp., Muncie, IN]) amended with one of six cytokinins.

a. MS + 0.3% Phytagel
b. MS + BA (0.25 mg/L) + 0.3% Phytagel
c. MS + BA (0.5 mg/L) + 0.3% Phytagel
d. MS + BA (1.0 mg/L) + 0.3% Phytagel
e. MS + CPPU (0.25 mg/L) + 0.3% Phytagel
f. MS + CPPU (0.5 mg/L) + 0.3% Phytagel
g. MS + CPPU (1.0 mg/L) + 0.3% Phytagel
h. MS + 2iP (0.25 mg/L) + 0.3% Phytagel
i. MS + 2iP (0.5 mg/L) + 0.3% Phytagel
j. MS + 2iP (1.0 mg/L) + 0.3% Phytagel
k. MS + kinetin (0.25 mg/L) + 0.3% Phytagel
l. MS + kinetin (0.5 mg/L) + 0.3% Phytagel
m. MS + kinetin (1.0 mg/L) + 0.3% Phytagel
n. MS + TDZ (0.25 mg/L) + 0.3% Phytagel
o. MS + TDZ (0.5 mg/L) + 0.3% Phytagel

p. MS + TDZ (1.0 mg/L) + 0.3% Phytagel
q. MS + zeatin (0.25 mg/L) + 0.3% Phytagel
r. MS + zeatin (0.5 mg/L) + 0.3% Phytagel
s. MS + zeatin (1.0 mg/L) + 0.3% Phytagel

BA – 6-Benzylaminopurine
CPPU – N-(2-chloro-4-pyridyl)-N-phenylurea
2iP – 6-(γ,γ-dimethylallylamino)-purine riboside
TDZ – Thidiazuron

There were three or four replications single jar replications of nodal sections, but for terminals, all 0.25 mg/L media were excluded as was Media r; there was one explant per jar. Explants were cultured for 8 weeks under the same incubator conditions as before. The original 32 seeds/seedlings were subcultured on April 27 or 28 on fresh MS in 473-ml jars to evaluate regrowth potential. At 8 weeks (June 8), all cultures were transferred to fresh MS without any cytokinins. Contamination, number of axillary shoots, length of each axillary shoot, regrowth of a terminal and its length, and callusing were evaluated on June 29, 2002. Seeds/seedlings were evaluated for regrowth of shoots, rooting, and callusing.

On May 19, 2002, Heritage fungicide (azoxystrobin; Syngenta) at 11.3g/m^2 was applied to containerized plants to evaluate its effectiveness in reducing contamination in tissue cultures. The soilless medium was composed of pine bark:Canadian sphagnum peat: sand, 3:1:1 (by vol.) amended (per m^3) with 2.4 kg Osmocote 18N-2.6P-10K (18-6-12), 942 g Micromax, and 106 g sublimed sulfur. Plants were growing in a shadehouse (30% shade) with overhead irrigation at the NFREC-Quincy. On May 25 and 26, explants from 19 of the sprayed plants were collected. One centimeter nodal explants and 0.5-cm terminal explants and were surface sterilized as described below, and then cultured on Media *a* to *s* in 473-ml jars.

1. Soak in 50% bleach (2 drops Tween 20 / 100 ml) for 5 min
2. Soak in 95% EtOH for 30 sec
3. Rinse with sterile distilled water 5X

All data were subjected to ANOVA and regression analyses using SAS (SAS 2000).

PROPAGATION VIA STEM CUTTINGS

- Gopher apple stem cuttings collected on or about April 10, May 10, June 10, July 10, October 10, and November 10, 2002, from a sandy location in full sun at Alligator Point, FL (Wakulla County). The cuttings were sealed in plastic bags with moisten paper towels, placed in a cooler with frozen Acool Paks®, and brought to the NFREC in Quincy, FL.

One day before stem cuttings were collected, propagation cups (5.4 x 5.4 x 7.8 cm; vol. 175 ml; Lerio Corp.) were filled with MetroMix 200 (Scotts) and placed on a bench under mist (6 sec/5min; 24 hr) in a greenhouse (45% shade). Terminal cuttings (10-13 cm; 3-4 leaves) with two 1-cm scores on opposite sides of the base were dipped in different Hormex formulations (Brooker Chemical Corp.); Hormex is the general name for all of Brooker Chemical's talc formulations of IBA. The Hormex formulations used in this experiment contained (by weight) 0.1, 0.3, 0.8, 1.6, 3.0, and 4.5% IBA. Nontreated and IBA-treated cuttings then stuck in MetroMix in cups and arranged in a completely randomized design; there were 10 replications per treatment. After 1 month, 29.6 ml/7.6 L (2 Tbsp/2 gal) of Peters 15-16-17 (Scotts) was applied over-the-top of the cuttings, and the cuttings were placed on a bench with a 24-hr mist frequency of 6 sec/10min. One month later, medium was washed from the cuttings and a visual rating of rooting (1=no, 2=slight, 3=some, 4=moderate, 5=heavy, or 6=extremely heavy) and number of primary roots was recorded. The roots were dried at 60C and the dry mass determined. Mass/primary root and percent cuttings rooted were calculated.

Regression and ANOVA analyses were performed using SAS (SAS 2000. Data for studies initiated in April, May, June, July, October, and November were analyzed separately.

RESULTS

MICROPROPAGATION

In vitro germination of gopher apple seed from a north Florida population (Leon was 86%. Generally, no cytokinin enhanced axillary shoot production on terminal or nodal explants derived from the in vitro seedlings (Tables 4.1-4.6). The one exception was for CPPU at 0.5 mg/L (Medium *f*) – axillary shoot production was two and a half times greater than that of the control (2.5 vs. 1.0). In addition, all cytokinins tended to lower percent regrowth and increase basal callusing, especially on nodal sections (Tables 4.1-4.6). Shoot regrowth occurred on 26% of the original seedlings in culture, with 2.5 shoots/seedling.

All of the cultures containing explants derived from fungicide-treated plants growing in the shadehouse were contaminated; no axillary shoot, regrowth, or callus data were recorded.

PROPAGATION VIA STEM CUTTINGS

Rooting of stem cuttings was affected by IBA concentration, and seemed to be affected by the date cuttings were taken (Table 4.7). Cuttings harvested in April benefited from IBA but no more than 0.1% was necessary to improve rooting. May cuttings seemed to root the best based on quantitative and visual assessment but IBA did not improve rooting. Rooting ability of stem cuttings steadily declined for cuttings harvested in June and July (Table 4.7), and November. For cuttings taken in June and July, IBA significantly improved rooting, with best rooting at 3 and 4.5% IBA. In contrast, October and November cuttings did not even callus after 2 months, with or without IBA. The lack of any root/callus formation on October and November cuttings may have been due to inadequate heating of the greenhouse during late fall and winter. The rooting medium temperature was only about 10C.

Table 4.1. Effect of 2-iP on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

2-iP (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
0.5	Term.	3	0	0.3		1.0		67	1.5		0
1.0	Term.	3	33	0.0		0.0		50	.0		67
Significance Linear Quad.			---	*P*>F NS NS	r^2 NS NS	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 NS NS	---
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	1.3		1.3		33	2.0		0
0.5	Nodal	4	0	2.3		4.7		75	4.0		0
1.0	Nodal	4	50	1.3		4.5		33	35		33
Significance Linear Quad.			---	*P*>F NS NS	r^2 --- ---	*P*>F NS NS	r^2 --- ---	---	*P*>F .0235 .0001	r^2 .6026 .9774	---

Table 4.2. Effect of CPPU on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

CPPU (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
0.5	Term.	3	33	2.5		5.0		0	0		10
1.0	Term.	3	0	2.7		5.2		0	0		100
Significance Linear Quad.			---	*P*>F .0433 NS	r^2 .5208 NS	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 NS NS	---
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	0.3		11		0	0		67
0.5	Nodal	4	0	1.3		6.0		0	0		100
1.0	Nodal	4	25	0		0		0	0		100
Significance Linear Quad.			---	*P*>F NS NS	r^2 --- ---	*P*>F NS NS	r^2 --- ---	---	*P*>F NS NS	r^2 --- ---	---

Table 4.3. Effect of Kinetin on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

Kinetin (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
0.5	Term.	3	0	2.7		3.5		67	6.0		33
1.0	Term.	3	0	1.0		3.0		33	5.0		0
Significance Linear Quad.			---	*P*>F NS NS	r^2 NS NS	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 NS NS	---
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	1.0		4.3		0	0		33
0.5	Nodal	4	0	1.7		2.0		0	0		67
1.0	Nodal	4	25	0.7		3.0		33	3.0		0
Significance Linear Quad.			---	*P*>F NS NS	r^2 --- ---	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 --- ---	---

Table 4.4. Effect of Zeatin on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

Zeatin (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
1.0	Term.	3	67	1.0		4.0		100	15.0		0
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	2.3		1.3		0	0		67
0.5	Nodal	4	33	1.0		6.0		50	10.0		0
1.0	Nodal	4	0	1.5		5.8		0	0		50
Significance			---	*P*>F	r^2	*P*>F	r^2	---	*P*>F	r^2	---
Linear				NS	---	NS	---		NS	---	
Quad.				NS	---	NS	---		NS	---	

Table 4.5. Effect of Thidiazuron (TDZ) on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

TDZ (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
0.5	Term.	3	67	0		0		0	0		100
1.0	Term.	4	25	1.6		1.3		0	0		100
Significance Linear Quad.			---	*P*>F NS NS	r^2 NS NS	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 NS NS	---
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	0.3		2.0		0	0		100
0.5	Nodal	4	25	0.3		3.0		0	0		100
1.0	Nodal	4	25	0.3		2.0		0	0		100
Significance Linear Quad.			---	*P*>F NS NS	r^2 --- ---	*P*>F NS NS	r^2 --- ---	---	*P*>F NS NS	r^2 --- ---	---

Table 4.6. Effect of Benzyladenine (BA) on Axillary Shoots and Terminal Regrowth of Terminal and Nodal Sections of Gopher Apple Cultured In Vitro on MS Medium.

BA (mg/L)	Explant	No. Reps	% Contam.	No. Axillary Shoots		Axillary Shoot Length (mm)		% Explants w/Terminal Regrowth	Regrowth Length (mm)		% Callused
0	Term.	4	25	1.0		5.5		67	3.8		0
0.5	Term.	3	0	2.0		6.3		0	0		33
1.0	Term.	3	0	1.0		2.8		25	12.0		50
Significance Linear Quad.			---	*P*>F NS NS	r^2 NS NS	*P*>F NS NS	r^2 NS NS	---	*P*>F NS NS	r^2 NS NS	---
0	Nodal	4	25	1.3		3.5		75	2.8		0
0.25	Nodal	3	0	0.3		3.0		0	0		0
0.5	Nodal	3	0	1.3		4.5		0	0		33
1.0	Nodal	4	0	1.8		1.9		0	0		67
Significance Linear Quad.			---	*P*>F NS NS	r^2 --- ---	*P*>F NS NS	r^2 --- ---	---	*P*>F NS NS	r^2 --- ---	---

Table 4.7. Rooting Response to IBA of Gopher Apple Stem Cuttings Harvested in April, May, June, and July 2002.

Stem Cuttings Harvested April 2002									
% IBA	Visual Rating[1]		No. Primary Roots		Root Dry Mass (mg)		Dry Mass (mg)/Root		% Rooted
0	3.6		5.7		36.7		5.9		90
0.1	4.8		9.7		44.4		4.6		90
0.3	4.1		7.6		38.7		5.1		100
0.8	3.7		6.5		30.5		4.8		100
1.6	3.9		8.5		36.1		5.8		100
3.0	4.8		10.7		43.1		5.4		100
4.5	4.9		11.0		53.5		5.3		100
Significance	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	---
Linear	NS	---	.0266	.0703	NS	---	NS	---	
Quadratic	NS	---	NS	---	NS	---	NS	---	
Cubic	NS	---	NS	---	NS	---	NS	---	
Stem Cuttings Harvested May 2002									
% IBA	Visual Rating[1]		No. Primary Roots		Root Dry Mass (mg)		Dry Mass (mg)/Root		% Rooted
0	4.3		11.3		50.3		5.0		90
0.1	5.0		7.2		54.1		8.6		100
0.3	4.2		10.5		36.4		5.7		100
0.8	4.3		13.2		38.0		3.9		100
1.6	4.8		12.2		58.1		6.3		100
3.0	4.0		10.2		26.8		3.1		100
4.5	4.9		9.7		48.5		5.6		100
Significance	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	---
Linear	NS	---	.0266	.0703	NS	---	NS	---	
Quadratic	NS	---	NS	---	NS	---	NS	---	
Cubic	NS	---	NS	---	NS	---	NS	---	

Table 4.7 (Cont.). Rooting Response to IBA of Gopher Apple Stem Cuttings Harvested in April, May, June, and July 2002.

Stem Cuttings Harvested June 2002									
% IBA	Visual Rating[1]		No. Primary Roots		Root Dry Mass (g)		Dry Mass (mg)/Root		% Rooted
0	2.8		5.9		20.5		2.3		70
0.1	2.4		3.0		27.4		5.2		50
0.3	2.7		4.7		10.9		1.8		90
0.8	4.3		8.8		27.7		3.4		100
1.6	3.6		5.6		34.5		5.9		90
3.0	4.5		10.1		47.3		4.0		100
4.5	4.3		10.9		36.9		3.5		90
Significance	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	---
Linear	.0004	.1676	.0002	.1805	.0069	.1211	NS	---	
Quadratic	.0003	.2136	.0012	.1827	.0062	.1662	NS	---	
Cubic	.0001	.2179	.0038	.1827	.0116	.1802	NS	---	
Stem Cuttings Harvested July 2002									
% IBA	Visual Rating[1]		No. Primary Roots		Root Dry Mass (mg)		Dry Mass (mg)/Root		% Rooted
0	1.2		1.1		4.9		0.9		20
0.1	1.3		0.3		0.6		0.6		30
0.3	1.1		0.7		3.2		0.5		10
0.8	1.3		0.9		25.1		2.8		10
1.6	1.6		1.3		10.6		3.2		40
3.0	1.3		1.0		7.9		4.3		30
4.5	2.1		5.1		11.0		1.5		70
Significance	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	*P*>F	r^2	---
Linear	.0143	.0851	.0060	.1058	NS	---	NS	---	
Quadratic	.0177	.1134	.0009	.1882	NS	---	NS	---	
Cubic	.0372	.1196	.0015	.2066	NS	---	NS	---	

[1] Visual rating of rooting was on a Scale of 1 To 5, with 1=None, 2=Slight, 3=Some, 4=Moderate, 5=Heavy, 6=Extremely Heavy.

CONCLUSIONS AND RECOMMENDATIONS

In this preliminary study of in vitro propagation of gopher apple, we successfully germinated seed in vitro and obtained clean cultures. Using 60-ml culture tubes, a high percentage of seeds can be germinated in vitro, with a large number of seeds per unit area of incubator space. Seeds that germinated but where contaminated and not used as an explant source were successfully planted in soilless medium and grown in vivo. While Stage II cultures--abundant development of axillary shoots--were not established, terminal explants cultured on MS + CPPU (0.5 or 1.0 mg/L) developed 2.5 or more shoots per explant compared to only one axillary shoot per explant on MS without any cytokinin. Basal callus formed on 10 and 100% of the terminal explants on MS + 4-CPPU at 0.5 and 1.0 mg/L, respectively. Callus is generally not desired in this type of micropropagation culture designed for axillary shoot production. Future work should focus on evaluating factorial combinations of CPPU with other growth regulators (especially auxins and gibberellic acids) and combinations of axillary shoot production. The reuse of seedlings in vitro as a continued source of sterile explants should be also evaluated. The potential for increased percent germination should be studied by comparing the in vitro method to the in vivo method.

Given that 100% contamination occurred for explants from containerized plants, future investigators should also evaluate the use of explants from new growth in the early spring or late summer/fall excised from greenhouse grown plants (watered via drip or sub-irrigation).

Based on the preliminary results for propagating gopher apple by stem cuttings, harvest date seemed to have the most influence on rooting. April was the first month the spring flush of growth was of the minimal size from which to harvest cuttings. In May, the quality of the cuttings was the best with mature healthy leaves and semi-softwood stems. By June, terminal flowering on the new growth was abundant and the cutting quality was lower. July cuttings were of mostly mature wood (no stems with fruit were used). Fruit set was very poor, perhaps 3%. Cuttings harvested in October and November were the late summer/fall flush of growth, which was similar to cuttings of April. Therefore, the optimal times to collect gopher apple for vegetative propagation are shortly after the spring growth flush but before flowering and perhaps after the late summer/fall growth flush. The use of IBA for rooting of healthy early spring cuttings does not seem necessary based on rooting observed under our conditions; however, IBA does improve rooting of cuttings harvested later in the year. Adequate heating of the propagation house might improve rooting of stem cuttings harvested in mid to late fall.

Given that this work was only conducted with one population of gopher apple, was only conducted for one year, and none of the cuttings were transplanted, further work needs to focus on the following:

- Gopher apple stem cuttings collected from several disjunct populations
- Use of other auxins, auxin combinations, and auxin formulations
- Reevaluation of effect of harvest date
- Effect of air and medium temperature on rooting
- Evaluation of rooted liners as transplants for container production, or for direct transplant into the field

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CHAPTER 4

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