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Tissue culture and recombinant DNA technology: Developing protocols for potentially higher yielding switchgrass cultivars

Stephen Michael Foulk
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To the Graduate Council:

I am submitting herewith a thesis written by Stephen Michael Foulk entitled "Tissue culture and recombinant DNA technology: Developing protocols for potentially higher yielding switchgrass cultivars." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Janice Zale, Major Professor

We have read this thesis and recommend its acceptance:

Dennis West, Feng Chen

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Tissue culture and recombinant DNA technology: Developing protocols for potentially higher yielding switchgrass cultivars

A Thesis
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Master of Science
Degree
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Stephen Michael Foulk
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Abstract

The purpose of this thesis project was to, firstly, establish and optimize protocols for high-throughput callus induction and plant regeneration for the new, higher yielding switchgrass (*Panicum virgatum* L.) cultivars NSL and SL93. Secondly, to subclone the complementary DNA (cDNA) of the anthocyanin transcription factor, *C1*, from pBECKS.red into pUC18 and pBSL15 for downstream use.

For the first part of this project the cultivars, NF/GA992, NF/GA993, NSL, and SL93, were tested for callus induction by plating whole dehusked caryopses on callus induction media (CIM) containing 8.5u*M* of the auxin dicamba. NF/GA992 responded best to the treatment with 44% of plated seeds producing callus.

For the second experiment seeds of the cultivars NSL, SL93, and Alamo were plated on CIM containing various molar concentrations of dicamba (0u*M*, 8.5u*M*, 17u*M*, and 34u*M*) and 6-benzylamino purine (BAP) (0u*M*, 5u*M*, 15u*M*, and 45u*M*). This research revealed that the presence of BAP in CIM plates did not promote callus induction in Alamo, SL93, and NSL, but elevated concentrations (34u*M*) of dicamba significantly increased callus formation in all three cultivars. It was also found that the SL93 callus derived from CIM plates containing 34u*M* dicamba and 15uM BAP regenerated the most shoots, 27 shoots were regenerated from 3 calluses.

Seed pretreatments were evaluated to determine their impact on callus induction and subsequent plant regeneration. For this experiment, seeds of NSL, SL93, and Alamo were plated on CIM containing 34u*M* of dicamba. Seeds were subjected to one of three treatments before plating: a) dehusked with H_2SO_4, b) chilled for two weeks at 4°C then plated, and c) sterilized with sodium hypochlorite and ethanol. This research revealed that

seed pretreatment significantly increased callus induction amongst the three tested cultivars. The second part of this experiment compared the shoot regeneration efficiencies of seed-derived calluses to inflorescence-derived calluses. Analysis showed that SL93 calluses induced from inflorescences produced significantly more shoots than any of the other explants.

The final arm of this thesis project focused on sub-cloning the *C1* anthocyanin regulatory cDNA. The *C1* cDNA isolated from pBECKS.red was sub-cloned into pUC18 and pBSL15 using a sticky-end ligation.

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Introduction

1.1 Switchgrass Background/Overview

Switchgrass (*Panicum virgatum* L.) is a perennial, warm season C4 grass that is native to North America (Talbert, Timothy et al. 1983). It is suited for establishment in both warm and cool climates (Vogel and Moore 1993) and has been used for soil conservation, as a fodder crop, and as an ornamental plant (Barnes, Miller et al. 1995). Switchgrass species are designated as being either upland or lowland ecotypes. Upland and lowland varieties are genetically distinct, with lowland cultivars being tetraploid and upland cultivars being primarily octoploid (Barnett and Carver 1967). Additionally, lowland cultivars are taller, have thicker stems, and are more tolerant of poor drainage, whereas upland cultivars are shorter, have narrower stems, and are more drought resistant (Cassida, Muir et al. 2005).

Switchgrass has been chosen by the U.S. Department of Energy (DOE) as the model herbaceous monocot for cellulosic ethanol production (Vogel and Jung 2000). Switchgrass was selected because as an energy crop it has been observed to require relatively low amounts of fertilizer, little additional irrigation, and it produces reliable yields with high yield potentials (Wright 2007). One area of vital importance for establishing a biomass industry is feedstock research and development. Maximizing feedstock potential will require both traditional agricultural practices as well as utilizing and developing plant biotechnologies (U.S. Department of Energy 2008). Genomic and agronomic research will focus on increasing crop yields, manipulating cell wall structure

and composition, inducing plant expression of enzymes and cellulases, and improving the overall quality of energy crops (U.S. Department of Energy 2008).

1.2 Tissue Culture and Plant Regeneration

The successful genetic transformation of switchgrass will require the development of a reliable plant regeneration system. Micropropagation allows the researcher to produce many plants from a single initial plant over a short period of time (Chawla 2003). Plant micropropagation techniques generally require four stages, the first being plant culture initiation wherein an appropriate explant is selected, sterilized, and placed on growth media. This is followed by a shoot formation stage, then by shoot elongation and a root development phase. Finally, the plant is transferred to a normal growing environment such as a greenhouse (Kane 2005).

Plant micropropagation can be attained through either organogenesis or somatic embryogenesis (Chawla 2003). Organogenesis can be further subcategorized as being indirect or direct, in reference to the plant's developmental sequence. Indirect organogenesis is characterized by the presence of a callus phase, which is a state of unorganized cell growth. Direct organogenesis lacks a callus phase and the adventitious organs emerge directly from the explant (Hicks 1994). Of these two methods direct organogenesis from axillary buds is preferred because plant regeneration from callus has been known to increases the likelihood of somaclonal variation (Gaba 2005).

Regardless of which growth sequence, either indirect or direct, both are induced by the manipulation of exogenous auxin and cytokinin growth hormone levels. Plant organ development is directed by the manipulation of these plant growth regulators

(PGR's). Auxins at high concentrations coupled with low levels of cytokinins can induce callus formation in some plant species. Shoot organogenesis can be induced by decreasing auxin concentrations whilst simultaneously increasing cytokinin concentrations. Most plant species will develop roots upon transfer from a medium containing high cytokinin concentrations, to a medium with both low auxin and cytokinin levels. Some common auxins used for plant regeneration media are: 2,4-Dichlorophenoxy acetic acid (2,4-D), Indole-3 acetic acid (IAA), Indole-3 butyric acid (IBA), a-Napthalene acetic acid (NAA), and 3,6-dichloro-*o*-anisic acid (dicamba). Examples of commonly used cytokinins include BAP, Thidiazuron (TDZ), kinetin, and zeatin (Gaba 2005).

Like indirect organogenesis, somatic embryogenesis requires a callus induction phase but is distinguishable from indirect organogenesis in that somatic embryogenesis requires only one hormonal signal to produce a bipolar embryo capable of generating a whole plant. Indirect organogenesis requires two separate signals, one to initiate shoot growth and another to induce root development (Chawla 2003).

Seminal research of switchgrass explored tissue culture procedures wherein 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) where used to initiate callus from whole caryopses (cv. Alamo). This research concluded that 45u*M* BAP and 22.5 u*M* 2,4-D elicited optimal results, where ≈65% of caryopses produced callus (Denchev and Conger 1994). It was also determined that caryopses were the best choice of explant because of their ease in handling and their ability to readily induce calluses (Denchev and Conger 1994). The major disadvantage of utilizing switchgrass seed for tissue culture is that switchgrass is almost entirely self-incompatible (Martinez-

Reyna and Vogel 2002). Therefore, each seed is a different genotype, making it difficult to maintain clonal fidelity amongst regenerated plants (Denchev and Conger 1994).

Later research sought to minimize genotype variability by developing callus and regenerating plants from immature inflorescences of the cultivar Alamo (Alexandrova, Denchev et al. 1996 a). This procedure required the harvesting of E2 to E4 stage tillers from which the top node, containing the immature inflorescence, was excised, split longitudinally, and plated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962)supplemented with 30g maltose and 5 u*M* BAP (Alexandrova, Denchev et al. 1996 a).After 20-30 days, the generated spikelets were separated from the panicles and plated on MS media containing 30g maltose, 22.5 u*M* 2,4-D and 5 u*M* BAP for four weeks. The resultant calluses were transferred to MS media without PGR's (MS-0), embryogenic calluses produced roots and shoots, whereas non-embroygenic calluses continued to grow without cellular differentiation (Alexandrova, Denchev et al. 1996 a). Nodal segments cultured on MS media with 30g maltose and 5 u*M* BAP produced 2062 spikelets, each spikelet cultured on MS media containing 30g maltose, 22.5 u*M* 2,4-D and 5 u*M* BAP produced on average 0.95 regenerates (Alexandrova, Denchev et al. 1996 a).

Tissue culture was further refined by using nodal segments for plant micropropagation. Nodes of the cultivar Alamo were harvested from tillers possessing four to six visible nodes (Alexandrova, Denchev et al. 1996 b). Nodal segments, excluding the top node, were sterilized, split longitudinally, and cultured on MS media containing 30g maltose for eight weeks. Split nodes were also plated on MS media supplemented with 0.0, 5.0, 12.5, or 25.0 u*M* BAP and cultured for eight weeks. Nodal

segments and shoots were then transferred to MS-0 and incubated in 16 h of light per day for four weeks to induce rooting. Nodes cultured on 12.5 u*M* BAP showed the best results producing on average seven shoots per node. It was reasoned that this procedure could produce approximately 500 plants from a single parent with 15, 6 node tillers in a 12 week period (Alexandrova, Denchev et al. 1996 b). This method also mitigates the problem of induced chromosomal and genetic variation associated with dedifferentiation and redifferentiation from callus cultures (Larkin and Scowcroft 1981).

Further research was done to determine optimal concentrations and types of auxins and cytokinins for effective tissue culture and subsequent plant regeneration of switchgrass, cv. Alamo(Denchev and Conger 1995).. This study compared the efficacy of callus induction and regeneration from young seedlings and mature caryopses cultured on concentrations (11.3 u*M*, 22.5 u*M,* or 45.0 u*M*) of the auxins 2,4-D or picloram*,* combined with concentrations of BAP ranging 0.0 u*M,* 5.0 u*M*, 15.0 u*M,* or 45.0 u*M.* Explants were dark incubated at 29° C for 4 weeks on MS media containing 30g maltose and a combination of the aforementioned auxin/cytokinin. Cultures were then transferred onto MS-0 after 4 weeks, and resultant calluses were subcultured every 30 days. Combinations of 11.3-45.0 u*M* of 2,4-D and 15.0 or 45 u*M* BAP yielded the best results, with 22.3 u*M* 2,4-D and 5.0 u*M* BAP generating more than 45 shoots per callus (Denchev and Conger 1995).

Later research focused on pairing the auxin 2,4-D with the cytokinin, thidiazuron (TDZ) to study its effects on multiple shoot formation (Gupta and Conger 1998). Mature caryopses of the Alamo, Trailblazer, and Blackwell cultivars were dehusked and cultured on MS medium containing 3% maltose and a combination of 2,4-D (4.5 u*M,* 9.0 u*M,* or

22.6 u*M*) and TDZ (4.5 u*M,* 9.1 u*M,* or 18.2 u*M*) for 3 weeks at 29° C. Cultures were then exposed to a 16 h photoperiod per day for four weeks, multiple shoot clumps were transferred to MS-0 for an additional four weeks. Best results for all three cultivars were observed with the combination 4.5 u*M* 2,4-D and 18.2 u*M* TDZ, which produced a mean number of shoots per explant for Alamo, Trailblazer, and Blackwell of 39.6, 29.3 and 24.4 shoots respectively (Gupta and Conger 1998).

Dicamba , a synthetic auxin similar to 2,4-D, has been shown to be an effective auxin for tissue culture in various monocots such as, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*), Orchardgrass (*Dactylis glomerata* L.) and Kentucky bluegrass (*Poa pratensis* L.)(Conger, Hanning et al. 1983; Duncan, Singletary et al. 1989; Griffin and Dibble 1995; Bahieldin, Dyer et al. 2000; Przetakiewicz, Orczyk et al. 2003). Besides 2,4-D and picloram, dicamba was also evaluated for its effectiveness as a PGR for switchgrass callus induction and plant regeneration. However, it yielded poor results in initial trials but the concentrations of dicamba that were used were never published nor were the results of these trials (Denchev and Conger 1995; Denchev and Conger 1994).

1.3 Recombinant DNA Technology in Switchgrass

As previously stated, switchgrass plant modification will not only require traditional plant breeding techniques, but will also utilize recombinant DNA (rDNA) technology, and tissue culture. Transformants have been produced using several different methods (Potrykus 1991) regardless of which, all employ the same basic methodology: a) isolation of a gene of interest b) recombination of the gene into a vector c) transformation

of target cells d) regenerating transformants e) screening of putative transformants (Birch 1997). Methods commonly used to transform plant cells include *Agrobacterium*-mediated transformation, directed gene transfer to protoplast, biolistics, and microinjection (Hansen and Wright 1999). To date, *Agrobacterium*-mediated transformation, directed gene transfer to protoplast, and biolistics have been successfully used to introduce recombinant DNA into switchgrass (Mazarei, Al-Ahmad et al.2008. Somleva, Tomaszewski et al. 2002, Richards, Rudas et al. 2001).

The first reported transformation of switchgrass used the plasmid psGFP-BAR, which contained the green fluorescent protein (*sgfp*) gene behind the rice actin (*Act*1) promoter, and the *uidA* (*gus*) gene and the bialaphos/Basta resistance gene (*bar*) behind the maize ubiquitin (*Ubi*1) promoter (Richards, Rudas et al. 2001). Embryogenic calluses derived from inflorescences (cv. Alamo) were bombarded with tungsten microparticles coated with psGFP-BAR. The bombarded calluses were then transferred to MS-0 medium containing bialaphos, and screened healthy plantlets were eventually transferred to soil. T_1 offspring continued to display resistance to Basta, indicating inheritance of the *bar* gene. Southern blot hybridization revealed the presence of the *bar* in two transgenic plants, while a separate Southern blot for *sgfp* confirmed its presence in all five tested transgenic plants (Richards, Rudas et al. 2001).

Agrobacterium-mediated transformation of switchgrass has also been documented. The vector pDM805 containing the *bar* gene behind the *Ubi*1 promoter and *gus* gene behind the *Act*1 promoter was transformed into Alamo using the *A. tumefacians* strain AGL1 (Somleva, Tomaszewski et al. 2002). Putative transformants were screened with Basta, genomic DNA Southern blots of ten T_0 plants showed cotransformation of the

gus and *bar* genes. T_1 plants were screened by PCR analysis for the transgenes, and also confirmed by Southern blots of the PCR products (Somleva, Tomaszewski et al. 2002).

As mentioned transient expression from directed gene transfer to protoplasts has also been established with switchgrass. Protoplasts from young Alamo and Alamo2 leaves and roots were isolated and transfected with pBI221 and pAHC24, both of which contain the *gus* gene but behind *CaMV* 35S and *Ubi*1 promoters, respectively. *GUS* activity was observed to be greater in root derived protoplasts of the cv. Alamo2, and when driven by the *Ubi*1 promoter (Mazarei, Al-Ahmad et al.2008).

All three of the aforementioned transformation experiments focused on the cultivar Alamo or a hybrid thereof. Recently, new synthetic lines designated Northern Lowland (NL) and Southern Lowland (SL) have been introduced and shown to have a higher biomass yield potential than Alamo, Kanlow, and Cave-in-Rock in field trials conducted at Chickasha, OK, Booneville, AK, and Orange, VA (Taliaferro 1998).

All three transformation experiments described the effects of GFP, BAR, and GUS gene expression in switchgrass. These three genes are of course scorable markers, that through visual observation can confirm or deny the co-expression of other genes of interest that may not display a readily discernable phenotypic change in the target plant tissue. The GFP gene is stimulated to emit green light by energy released from the oxidation of coelentrazine (Prasher 1995). The gus gene, isolated from E. coli, encodes a β-glucuronidase that when combined *in situ* with the substrate 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) produces diX-indigo that can the be assayed by light microscopy (Guivarc'h, Caissard et al. 1996). The *bar* gene, isolated from Streptomyces hygroscopicus, encodes for phosphinothricin acetyl transferase (PAT)

which inactivates the herbicide, phosphinothricin (PPT). Putative transformants are screened by exsposure to herbicides like Basta, Liberty, and Herbiace which contain PAT (Rathore, Chowdhury et al. 1993). However, there are limitations associated with all three markers. Light emitted by the GFP gene can be masked by plant auto- fluorescence, screening for *bar* gene activity can take several weeks or more, and staining for the gus gene activity requires a destructive tissue assay and is limited to endogenous activity (Elliott, Campbell et al. 1999; Mantis and Tague 2000; Richards, Rudas et al. 2001).

Anthocyanins, more specifically the maize derived *R, C1, and β-peru* anthocyanin regulatory genes, are another group of genes that have been used as scorable markers for plant transformation (Bower, Elliott et al. 1996; Nelson and Bushnell 1997; Chawla, Cass et al. 1999). Belonging to the flavanoid class of molecules, anthocyanins play a diverse role in plant functions. Along with other flavanoids, their pigmentation attracts pollinators, acts to deter feeding, and protect the plant from UV damage (Holton and Cornish 1995). The study of anthocyanins helped lay the foundation of modern genetics with Mendel's study of pea flower color and McClintock's research of transposable elements in maize (Winkel-Shirley 2001). Since anthocyanins avoid the complications associated with GUS, BAR, and GFP markers, they warrant a closer look for future switchgrass transformation. It should be noted however, that the use of anthocyanins as visual markers is not totally without drawbacks. Research has shown that gene expression may be limited by tissue specific promoters, or even prove to be toxic to damaged cells and developing plants (Bower, Elliott et al. 1996; Selinger, Lisch et al. 1998).

1.4 Summary/Rationale for Studies

To date all published research of switchgrass has focused on the cultivars Alamo, Kanlow, Cave-in-Rock, Blackwell, and Shelter. Research detailing tissue culture for newer, and possibly higher yielding synthetics SL and NL has not yet been described.

The efficacy of tissue culture techniques should be explored with the newer cultivars. The main objectives of this research were to first, investigate the effects of the auxin dicamba on callus induction from whole caryopses of the cultivars NF/GA992, NF/GA993, NSL, and SL93. Second, optimize tissue culture plant growth regulator requirements and concentrations for the cultivars NSL and SL93. Third, examine the effects of explant pretreatments on callus induction and subsequent plant regeneration for callus derived from NSL, SL93, and Alamo caryopses along with callus produced from NSL, SL93, and Alamo inflorescences. Lastly, subclone the *C1* anthocyanin regulatory cDNA into an intermediate vector for future recombinant DNA research.

2

Callus induction efficiencies of Mature NF/GA992, NF/GA993, NSL, and SL93 caryopses on 8.5 µM dicamba

2.1 Introduction

Tissue culture is a requisite component to most plant transformation protocols to regenerate and eventually propagate transformants. Published research has focused on the switchgrass cultivars Alamo, Kanlow, Cave-in-Rock, Blackwell, and Shelter. Two synthetic switchgrass lines developed by Oklahoma State University, Southern Lowland (SL) and Northern Lowland (NL), have been shown in field trials to have a higher biomass yield potential than Alamo, Kanlow, and Cave-in-Rock (Taliaferro 1998). NF/GA992 and NF/GA993, developed by the University of Georgia and licensed by the Noble Foundation, was produced from an Alamo x Kanlow cross for decreased seed dormancy (J. Zale, personal communication, July 2008), and has also shown a high yield capacity, with NF/GA993 averaging a 23% higher yield than Alamo (Bouton 2006). As with the SL and NL ascensions, research detailing NF/GA992 and NF/GA993 tissue culture has not yet been published.

When this research was undertaken there were not a sufficient number of mature switchgrass plants available to harvest inflorescences and generate calluses. However there was an abundance of seed. An efficient protocol has been established to induce callus from switchgrass caryopses (cv. Alamo) using the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) (Denchev and Conger 1994).The growth regulator dicamba has been shown to be an effective auxin for tissue culture in various monocots such as, maize (*Zea mays* L.), wheat (*Triticum*

aestivum L.), barley (*Hordeum vulgare* L.), Orchardgrass (*Dactylis glomerata* L.) and Kentucky bluegrass (*Poa pratensis* L.)(Conger, Hanning et al. 1983; Duncan, Singletary et al. 1989; Griffin and Dibble 1995; Bahieldin, Dyer et al. 2000; Przetakiewicz, Orczyk et al. 2003). This research focused on callus induction efficiency from caryopses of NF/GA992, NF/GA993, NSL, and SL93 using Callus Induction Media (CIM; Horvath et al. 2002) supplemented with the auxin dicamba.

2.2 Materials and Methods

Caryopses Preparation

Four new populations of high yielding switchgrass seeds were obtained from the Nobel Foundation and Oklahoma State University. These seeds were plated on Callus Induction Medium (CIM; Horvath et al. 2002) containing 8.5 u*M* dicamba (Figure 2-1). One gram of whole NF/GA992, NF/GA993, NSL, and SL93 caryopses were put into separate 50 ml Falcon™ (Becton Dickinson; Franklin L, NJ) tubes, which were then filled with 60% H_2SO_4 and placed on a rotary shaker for 30 minutes at 350 rpm. The H_2SO_4 was decanted and the seeds were washed once with autoclaved distilled H_2O. The Falcon™ tubes were filled with 50% commercial bleach solution containing 0.1% Triton-100X and placed on the rotary shaker at 350 rpm for 30 minutes. The bleach was aseptically removed and the caryopses were rinsed five times and soaked for ten minutes with sterile distilled H_2O. The rinsed caryopses were then transferred to CIM. The caryopses were incubated in the dark for four weeks at 29 ±2°C (Denchev and Conger 1994). After four wks the number of calluses for each cultivar was recorded and transferred to fresh CIM plates supplemented with 8.5 u*M* dicamba. Calluses were

maintained in the dark at 29±2°C for 14 months, being transferred every four to five weeks.

Data Analysis:

The results were analyzed using descriptive statistics (Minitab14). A single-factor completely random design (CRD) analysis of variance (ANOVA) was used to test for significant differences amongst the number of calluses produced from each cultivar: NF/GA992, NF/GA993, NSL, and SL93 (SAS9.13.). A CRD is often used to analyze results when an experiment is lacking an equal number of replications (Gomez and Gomez 1984). A Tukey-Kramer procedure was used to make a multiple comparison of means. The Tukey-Kramer procedure has been shown to always give conservative results when comparing unequal sample sizes (Wilcox 1987).

2.3 Results and Discussion

All tables and figures can be found in the appendix following the results and discussion section. A total of 266 calluses were produced from 1400 seeds for an overall efficiency of 19%. The NF/GA lines were the highest yielding, with NF/GA993 (Figure 2-1) and NF/GA992 (Figure 2-2 and 2-3) yielding 46 and 160 calluses respectively (Figure 2-6). While the OSU lines yielded the least with SL93 (Figure 2-4) producing 35 calluses and NSL (Figure 2-5) producing 25 (Figure 2-6). The amount of callus produced was significantly different between the four cultivars (Table 2-1). The NF/GA992 seeds produced significantly more calluses than the other three (Table 2-2).

Approximately 44% of NF/GA992 produced callus. It has been reported that ≈65% of Alamo seeds plated on MS medium supplemented with 45 u*M* BAP and 22.5

u*M* 2,4-D generate callus (Denchev and Conger 1994). It is unknown whether the difference in callus induction efficiency between Alamo and NF/GA992 is solely due to genotype or to the use of different PGR's. However, our initial callus induction experiments on the NF/GA lines using 2,4-D failed to produce results (data not shown). It has also been reported that higher molar concentrations of dicamba than 2,4-D are needed to induce callus in wheat (Carman, Jefferson et al. 1988). The u*M* concentration of 2,4-D used to generate callus from Alamo seeds was nearly three times higher than the concentration of dicamba used in this experiment.

As stated previosly callus production is an extremely useful technique for downstream plant biotechnology applications (Dixon and Gonzales 1994). This research on new switchgrass cultivars, along with seminal research of callus production from various switchgrass explants (Denchev and Conger 1995), is useful for creating a high-throughput method for switchgrass callus production

Hitherto published switchgrass tissue culture research has focused on the use of 2,4-D as the primary auxin for callus production and subsequent plant regeneration (Denchev and Conger 1994; Denchev and Conger 1995; Alexandrova, Denchev et al. 1996; Gupta and Conger 1999; Mazarei, Al-Ahmad et al. 2008). The auxin dicamba has been shown to be highly effective at producing callus in various plant species and this research has shown that is also an effective growth regulator for switchgrass tissue culture especially in respect to the NF/GA992 cultivar. Further comparisons are clearly needed, such as comparing the effects of differing molar concentrations of dicamba and assessing the effects of the combination of dicamba and BAP on callus induction and plant regeneration.

3

Tissue culture response of mature NSL, SL93, and Alamo caryopses to different concentrations of dicamba and BAP

3.1 Introduction

The previous chapter documented research pertaining to callus induction efficiency of the relatively new switchgrass cultivars NF/GA992, NF/GA993, NSL, and SL93, initiated on callus induction medium (CIM) with 8.5 u*M* dicamba. This chapter examines the effects of different concentrations and combinations of the auxin dicamba, and the cytokinin 6-benzylaminopurine (BAP) on callus induction from NSL, SL93, and Alamo caryopses.

Nearly all current plant transformation protocols that require tissue culture include a callus phase to produce transformed cells and to regenerate plants (Hansen and Wright 1999). There are two main switchgrass transformation protocols, and plant regeneration occurs after a callus phase in each of these (Denchev and Conger 1995; Alexandrova, Denchev et al. 1996; Gupta and Conger 1998; Gupta and Conger 1999; Richards, Rudas et al. 2001; Somleva, Tomaszewski et al. 2002). The previous chapter reported that only ≈6% of NSL and ≈10% of SL93 seed produced callus when plated on medium containing 8.5 u*M* dicamba. In contrast, it has been shown that MS medium supplemented with 45 u*M* BAP and 22.5 u*M* 2,4-D can induce callus from ca. 65% of Alamo seeds (Denchev and Conger 1994). Other research compared the efficacy of callus induction and plant regeneration from young Alamo seedlings and mature caryopses cultured on 11.3 u*M*, 22.5 u*M,* or 45.0 u*M* of the auxins 2,4-D or picloram, in combination with 0.0 u*M,* 5.0 u*M*, 15.0 u*M,* or 45.0 u*M* of BAP.

The objective of this study was to determine the optimal concentrations of dicamba and BAP on callus induction and subsequent plant regeneration from caryopses of the switchgrass cultivars SL93, NSL, and Alamo. The information gathered will be useful for conducting future tissue culture experiments with the OSU cultivars.

3.2 Materials and Methods

One gram each of NSL, SL93, and Alamo caryopses were put into separate 50 ml Falcon^{TM} (Becton Dickinson; Franklin L, NJ) tubes, which were filled with 70% ethanol and placed on a rotary shaker for ten minutes at 350 rpm. The ethanol was decanted, and the Falcon^{TM} tubes were filled with 70% commercial bleach solution containing 0.1% Triton-100X, and placed on the rotary shaker for 30 minutes. The bleach was aseptically removed and the caryopses were rinsed five times, at ten minutes per rinse, with sterile distilled water. The caryopses were transferred to Callus Induction Media (CIM Table 3-1; Horvath et al. 2002) containing 0.2% Plant Preservation Mixture (PPM^{TM}; Plant Cell Technology; Washington D.C.), and all permutations of dicamba levels (0 u*M*, 8.5 u*M*, 17 u*M*, and 34 u*M*) and BAP levels (0 u*M*, 5 u*M*, 15 u*M*, and 45 u*M*).

A completely randomized design was employed with 20 caryopses per Petri dish, and five replications (Petri dishes) per treatment for each cultivar NSL, SL93 and Alamo. The caryopses were incubated in the dark for six weeks at 29° ±2 C and afterwards the number of calluses for each cultivar was recorded. The calluses were transferred to CIM(Green) (Table 3-1) plates supplemented with 443.9 n*M* 6-BAP l^{-1} and 8.5 u*M* dicamba $l^{-1}$and incubated at 22^{o} ±4C under indirect light for two weeks and transferred to fresh CIM(Green) for another two weeks (Figure 3-1). The calluses were transferred to

shoot growth media (SGM; Table 3-1) supplemented with 4.44 u*M* BAP and incubated under direct light for four weeks (Figure 3-2 and 3-3). Following four weeks on SGM, shoot clusters were transferred to root growth media (RGM; Table 3-1) and incubated for another four weeks at 22^{o} ±4C under 60 watt cool white fluorescent bulbs (Philips, Rock Hill, SC) providing light at 60 μmol $m^{-2}s^{-1}$for a 24 hour photoperiod (Figures 3-4 and 3-5). After four weeks, seedlings were transferred to fresh RGM and shoots were counted for each callus.

3.3 Results and Discussion:

In wheat, research has shown that higher levels of dicamba compared to 2,4-D are required to produce callus (Carman, Jefferson et al. 1988). It has also been noted that the inclusion of a cytokinin such as BAP in the tissue culture medium greatly increases plant regeneration efficiencies in switchgrass (Denchev and Conger 1994). Such findings were also observed to be true in this experiment.

All tables and figures can be found in the appendix following the results and discussion section. An analysis of variance for callus induction from seed of the three cultivars showed significant effects for the plant growth regulator (PGR) treatment and for the interaction between PGR treatment and cultivar (Table 3-2). Of all of the treatments only three were shown to be significant, 17 u*M* dicamba and 0 u*M* BAP, 34 u*M* dicamba and 0 u*M* BAP, and 34 u*M* dicamba and 5 u*M* BAP (Table 3-4). Of those three, the combination 34 u*M* dicamba and 0 u*M* BAP induced the most calluses from all three cultivars with 26%, 23%, and 21% of SL93, NSL, and Alamo caryopses, respectively (Table B-3). Clearly, as the molarity of dicamba increased, callus induction

increased. However, increased concentrations of BAP are not correlated with any increase in callus production from caryopses of any cultivar (Table 3-4). Seeds plated on medium containing high concentrations of BAP exhibited precocious root development and were incapable of regenerating shoots. Similar results were reported with 2,4-D, BAP, and Alamo caryopses (Denchev and Conger 1995).

The calluses that were induced on CIM(0) were then used to regenerate shoots via indirect organogenesis by passage over three subsequent media types CIM(Green), SGM, and RGM (Table 3-1). There were two main causes for lost samples in this experiment. Contamination was the major contributor. The other stemmed from transferring calluses to media containing too high of a concentration of BAP. It is believed that this caused the callus to turn necrotic, and die. Because of the lost samples, it was impossible to conduct meaningful data analyses or cross comparisons for shoot regeneration. Nevertheless, shoots were successfully regenerated from two treatments, Alamo plated on CIM supplemented 34 u*M* dicamba and 0 u*M* BAP and SL93 plated on CIM supplemented with 34 u*M* dicamba and 15 u*M* BAP (Table 3-5). The Alamo on 34 u*M* dicamba and 0 u*M* BAP produced 12 shoots from 17 calluses, and SL93 on 34 u*M* dicamba and 15 u*M* BAP produced 27 shoots from six calluses. Future research could explore whether differences in multiple shoot production were due to a genotype effect or to the presence of the cytokinin in the medium.

The addition of a higher molarity of dicamba did indeed yield significant increases of callus for all three cultivars tested, but not are at a level previously reported, of ≈65% (Denchev and Conger 1994). And from the results of the last experiment, it appears that even at a lower concentration of dicamba (8.5 u*M*), NF/GA-992 was much

more efficient at producing callus. However, callus induction was greatly increased from the results reported in that experiment with both of the OSU cultivars. It should also be noted for future research, that higher concentrations of auxins in tissue culture media can inhibit plant regeneration and development (Murata 1989; Bahieldin, Dyer et al. 2000). However, this can be avoided by transferring the calluses to media containing a lower concentration of auxin after induction (Dixon and Gonzales 1994).

4

Effects of seed pretreatments on tissue culture and comparison of shoot regeneration from calluses derived from seed vs. inflorescences

4.1 Introduction

The last two chapters explored the effects of various auxin and cytokinin treatments on callus induction and plant regeneration of different switchgrass cultivars, whereas this section examines explant choice and preparatory caryopses treatments on seed germination and callus induction. Comparisons amongst SL93, NSL and Alamo cultivars are presented for callus induction and plant regeneration from seed derived versus inflorescence derived calluses.

As stated previously there are both advantages and disadvantages to using switchgrass caryopses for tissue culture. Switchgrass seed is useful for tissue culture because of its small size, and capacity to potentially induce callus, (Denchev and Conger 1994). It has also been suggested that the use of seeds as an explant increased transformation efficiency in regards to *Agrobacterium*-mediated transformation (Somleva 2006). Conversely, the highly variable genotype between individual caryopses poses the largest obstacle to maintaining generational clonal fidelity (Denchev and Conger 1994). To date, publications utilizing switchgrass caryopses for callus induction have employed the use of H_2SO_4 for dehusking seeds (Denchev and Conger 1994; Denchev and Conger 1995; Richards, Rudas et al. 2001; Somleva, Tomaszewski et al. 2002). But, it is unclear whether this step provides any significant increase in germination, callus production, or any other aspect of tissue culture. The effects of stratification on switchgrass seed germination has been well documented (Wolf and Fiske 1995). Research has shown that

prechilling can break dormancy in Alamo, Blackwell, and Cave-in-Rock cultivars (Zarnstorff, Keys et al. 1994; Douglas and Grabowski 1995); the effect on callus induction has yet to be determined. The objectives of these experiments are to compare the effects of various seed treatments on callus induction and plant regeneration for NSL, SL93 and Alamo. Comparisons are also made amongst these cultivars for callus induction and plant regeneration from seed-derived callus versus inflorescence-derived callus.

4.2 Materials and Methods

Caryopses Preparation and Tissue Culture

Whole caryopses underwent one of three treatments prior to plating. Treatment one consisted of placing one half gram of whole NSL, SL93, and Alamo caryopses into separate 50 ml Falcon™ (Becton Dickinson; Franklin L, NJ) tubes, which were then filled with 60% H_2SO_4 and placed on a rotary shaker for 30 minutes at 350 rpm . The H_2SO_4 was then decanted, and the seeds were washed once with autoclaved distilled H_2O. The Falcon™ tubes were filled with 50% commercial bleach solution containing 0.1% Triton-100X, then placed on the rotary shaker for 30 minutes. The bleach was aseptically removed and the caryopses were rinsed five times, ten minutes per wash, and soaked for ten minutes with sterile distilled H_2O.

For treatment two, one half gram of whole NSL, SL93, and Alamo caryopses were put into separate 50 ml Falcon™ tubes, filled with 70% ethanol and placed on a rotary shaker for ten minutes at 350 rpm. The ethanol was decanted, and the Falcon tubes were filled with 70% commercial bleach solution containing 0.1% Triton-100X, then

placed on the rotary shaker for 30 minutes. The bleach was aseptically removed and the caryopses were rinsed five times and soaked for ten minutes per rinse with sterile distilled H_2O. After sterilization the Falcon^{TM} tubes, containing the seeds, were refrigerated at 4°C for 14 days. Treatment three followed the same sterilization technique as treatment two except the seeds were not refrigerated after sterilization.

The experiment was set up as factorial arrangement of three seed treatments, acid washed, chilled, and sterile, and the three cultivars, SL93, NSL, and Alamo in a complete randomized design. Following the treatments, 20 seeds of each cultivar were plated five times for each treatment on Petri dishes containing callus induction medium (CIM; Horvath et al. 2002) supplemented with 34u*M* dicamba and 0.2% Plant Preservation Mixture (Table 4-1). The Petri dishes were then incubated in the dark at 29°C. The number of germinated seeds was recorded after ten days, and the number of calluses was recorded after six weeks. After six weeks the calluses were transferred to CIM(Green) plates with 40ul l^{-1} BAP and 375 ul l^{-1} dicamba (Horvath et al. 2002) and placed under indirect light at 22^{o} ±4C temp for two weeks. Thereafter a modificiation of the protocol of Horvath et al. (2002) was implemented by incubating the calluses under indirect light for an additional two weeks. After four weeks on CIM(Green) calluses were transferred to Shoot Growth Medium (SGM) supplemented with 400 ul l^{-1} BAP (Table 4-1) and incubated under direct 60 watt cool white fluorescent bulbs (Philips, Rock Hill, SC) providing light at 60 μmol $m^{-2}s^{-1}$for a 24 hour photoperiod at 22^{o} ±4C temperature for four weeks. Thereafter, shoot clusters were transferred to Root Growth Medium (RGM; Table 4-1) and incubated for another four weeks, after which the shoot cultures were transferred to fresh RGM and the number of shoots were counted for each callus.

Inflorescence Preparation and Tissue Culture

SL93, NSL, and Alamo tillers were harvested at the E2 to E4 stage according to the protocol by Alexandrova, Denchev et al. (1996). The top node, containing the immature inflorescence, was excised, split longitudinally, and plated on inflorescence media supplemented 5 u*M* BAP and 0.2% PPM (Alexandrova, Denchev et al. 1996; Table 4-2). After six weeks, spikelets initiated in *vitro* from the half nodes. The spikelets were separated from the panicle, and plated on CIM supplemented with 34 u*M* dicamba and 0.2% PPM (Table 4-1). After two weeks the number of calluses were recorded. The calluses were transferred to CIM(Green) with 40 ul l^{-1} BAP and 375 ul l^{-1} dicamba (Table C-1; Horvath et al 2001)

Calluses were incubated under indirect light for two weeks, transferred to fresh CIM(Green), and incubated another two weeks under indirect light. The calluses were transferred to SGM supplemented with 400ul l^{-1} BAP (Table 4-1) and incubated under direct light direct 60 watt cool white fluorescent bulbs (Philips, Rock Hill, SC) providing light at 60 μmol $m^{-2}s^{-1}$for a 24 hour photoperiod at 22^{o} ±4C temperature for four weeks. Following four weeks on SGM, shoot clusters were transferred to RGM (Table 4-1) and incubated under direct light for another four weeks. If the shoots were still not rooted, they were transferred to fresh RGM. Shoot clusters and multiple shoots were divided, and the number of shoots produced was recorded for each callus.

4.3 Results and Discussion

The seeds of the three cultivars, SL93, NSL, and Alamo, produced different numbers of calluses and shoots in response to the three treatments. Although SL93

yielded the greatest total number of calluses, overall, due to lost samples, Alamo generated the highest percentage with ≈ 32%, SL93 produced 24%, and only ≈ 17% of NSL seed developed callus (Table 4-3). An analysis of variance for callus induction is presented in Table 4-4. Both treatment and cultivar had a significant effect on callus induction, as well as cultivar x treatment interaction. It is interesting to note that all three cultivars responded differently to each treatment. Whereas SL93 and Alamo responded well to the acid wash, NSL did not. Similarly, Alamo responded well to chilling, whilst the other two cultivars produced less callus. These observations are true for seed germination as well since seed germination is a prerequisite for callus induction. Cultivar, treatment, and the cultivar x treatment interaction had a significant effect on seed germination (Table 4-5). Yet, no single treatment elicited a consistent response amongst all three cultivars (Table 4-3). Thus there appears to be significant differences amongst the switchgrass varieties.

The number of plants regenerated from calluses derived from infloresences suffered from unequal sample sizes. That is, an unequal number of nodes were initially tested for *in vitro* floral initiation and subsequent callus induction from SL93, NSL, and Alamo. Therefore, unequal callus production and plant regeneration shown in this analysis is a reflection of that fact. SL93 produced the most callus with 52 calluses derived from 31 spikelets originating from 18 half nodes. Alamo produced 32 calluses derived from 36 spikelets originating from 12 half nodes. Finally, NSL generated 19 calluses derived from 32 spikelets originating from 14 half nodes (Table 4-6). In terms of a callus to spikelet ratio SL93 was the most efficient cultivar while NSL was the least. The apparent incongruence of the number of nodal segments for each cultivar and

number of spikelets produced probably is the result of nodes that were harvested lacking an immature inflorescence or the nodes were cut unevenly, leaving the inflorescence in just one half.

The callus cultures in this experiment, like the last one, were not tested for embryogenesis. The plants that were generated used indirect organogenesis to generate shoots. Of the three cultivars tested, Alamo responded the best, averaging 5.00 (± 4.47) shoots per callus for the refrigeration treatment and 1.20 (± 2.68) shoots per callus for the surface sterilization treatment (Tables 4-7 and 4-8). An analysis of variance showed that cultivar and the combination of cultivar and treatment had a significant effect on shoot generation from switchgrass seed. However, treatment alone did not have a significant effect on shoot development (Table 4-9). Of the three cultivars that nodes were harvested from to regenerate shoots only SL93 showed any results producing an average 7.17 (± 4.80) shoots per callus (Table 4-7). The results of an analysis of variance for shoots regenerated from inflorescence-derived callus are presented in Table 4-10. Figure 4-1 depicts a one-way analysis of means for shoots derived from seed based callus or inflorescence based callus for each cultivar. Surprisingly none of the explants that produced shoots had a mean number of shoots per callus that were significantly different from one another.

So what does it all mean? One conclusion would be that SL93 inflorescences NSL, Alamo, and SL93 seeds are all equally regenerative and equally suited for tissue culture. Also, an acid treatment appears to significantly increase callus production from SL93 and Alamo caryopses. The seed pretreatments employed in this experiment have also been researched specifically for their effects on switchgrass seed germination, those

experiments concluded that a factorial combination of chilling, acid scarification, and bleaching greatly increased germination (Haynes, Pill et al. 1997). It is possible that same reasons for increased germination are related to increased callus induction, i.e. low dormancy in switchgrass would increase seed germination and hence callus production. Shoot regeneration was modest compared to that observed from using MS supplemented with 2,4-D and BAP (Denchev and Conger 1995). The use of a cytokinin such as BAP, absent in this experiment, might help to increase shoot regeneration as evidenced from the experiment, and other research conducted on switchgrass (Denchev and Conger 1995; Alexandrova, Denchev et al. 1996; Gupta and Conger 1998). Furthermore, although all three cultivars generated shoots from seed-derived calluses, none approached the number of shoots produced by SL93 inflorescence derived callus. The addition of a cytokinin may increase overall efficiency from either explant.

5

Sub-cloning the anthocyanin transcriptional regulator C1 into pUC18, and pBSL15

5.1 Introduction

This chapter focuses on experiments to ascertain the utility of anthocyanin transcriptional regulators, specifically the *C1* cDNAs, as a visual marker of transformation events. It was originally intended to clone this cDNA behind the rice actin promoter:intron and use them in biolistics of switchgrass explants as cell autonomous markers of the transformation event (McCormac, Elliott et al. 1997). Unfortunately, optimization of consistent microprojectile bombardment protocols using pBECKS.red and the β-Peru biolistics plasmid in switchgrass proved problematic.

Anthocyanins are flavonoids which produce red and purple pigments in fruits, seeds, flowers, and leaves; they aid in signaling, recruiting pollinators, deterring predators, and providing protection from ultra violet radiation (Holton and Cornish 1995; Winkel-Shirley 2001). Of the twelve known anthocyanin genes in maize (*Zea mays* L.), ten have been extensively analyzed at the molecular level (Scheffler, Franken et al. 1994). The *C1* allele from maize is specifically expressed in the aleurone and scutellum, and has been shown to function as transcriptional factor regulating expression of the *A1* and *Bz1* anthocyanin genes (Chen and Coe 1977; Cone, Burr et al. 1986; Klein, Roth et al. 1989). Along with *C1*, the *B*, and *R* regulatory genes have successfully been used as reporter systems in numerous plant species (Lloyd, Walbot et al. 1992; Nelson and Bushnell 1997; Chawla, Cass et al. 1999). The use of an anthocyanin based reporter

system eliminates the problems associated with other reporter genes such as; plant autofluorescence obscuring *GF*P expression, the lengthy screening period necessary for selection of *bar* gene transformants, and the destructive tissue assays required for screening *gus* gene activity (Elliott, Campbell et al. 1999; Mantis and Tague 2000; Richards, Rudas et al. 2001). This section describes the research performed to sub-clone the maize *C1* cDNA isolated from pBECKS.red (McCormac, Elliott et al. 1997) for subsequent recombinant DNA experiments.

5.2 Methods and Materials

The pBECKS.red plasmid contains the *C1* and *Lc* cDNAs (McCormac, Elliott et al. 1997). Both are driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the nopaline synthase (NOS) terminator. The *C1* expression cassette also includes the maize *Adh1* intron 1 behind the promoter for better gene expression in monocots (McCormac, Wu et al. 1998). The *C1* cDNA was excised from pBECKS.red with *E*coRI, and was purified with a QIAquick™ Gel Extraction Kit (Qiagen; Germantown, MD). The pBSL15 plasmid (Alexeyev 1995) contains a kanamycin (kan) resistance gene flanked by mirror repeats of the pUC18 Multiple Cloning Sequence (MCS) and an ampicillin resistance gene in a pK18 (Pridmore 1987) backbone. The kan gene was excised with *Eco*RI and discarded, the rest of the digested vector backbone was purified with a QIAquick Gel Extraction Kit (Qiagen). The pBSL15 ends were then dephosphorylated using Shrimp Alkaline Phosphatase (SAP). The excised *C1* fragment was ligated into the linearized pBSL15 with *Eco*RI sites on its ends. The ligation was transformed using Invitrogen MAX efficiency® DH10B (Carlsbad, CA) chemically

competent cells. The DH10B cells plus plasmid transformation mixture was streaked onto ampicillin plates and the colonies were screened for recombinants. Colonies were picked, grown overnight in broth with antibiotics, and plasmid DNA was extracted with a QIAprep Spin Miniprep Kit (Qiagen; Pittsburgh, PA). The extracted DNA was then digested with *Eco*RI, separated with gel (1%) electrophoresis and compared to a positive control, *Eco*RI digested pBECKS.red. The new construct will be referred to hereafter as pBSL15.red.

The pUC18 (Vieira and Messing 1982) plasmid was digested with *Eco*RI and its ends were dephosphorylated with SAP. The purified *C1* fragment was ligated into the *Eco*RI sites using directional cloning. The ligation was transformed into Invitrogen MAX efficiency® DH10B chemically competent cells. The DH10B cells plus plasmid were streaked onto ampicillin plates and screened for transformants. Colonies were picked and DNA was extracted with a FastPlasmid™ Mini-prep Kit (Eppendorf; Gaithersburg, MD). The extracted DNA was digested with *Eco*RI, separated with gel electrophoresis and compared to *Eco*RI digested pBECKS.red. The resultant construct will be referred to as pUC.red.

5.3 Results and Discussion

Because of its mirrored pUC18 Multiple Cloning Sites (MCS), the pBSL15 plasmid is an effective tool for designing recombinant DNA constructs. With it a gene of interest can be ligated into the plasmid and excised out with a considerable range of restriction site/enzyme options. For this reason it was chosen to ligate the *C1* cDNA into pBSL15. The utility of the *C1* anthocyanin regulatory cDNA as a visual marker has been

previously mentioned in the introduction of this chapter. Figure 4-1 is a gel image of the pBECKS.red, pBSL.red, and pUC.red plasmids digested with *Eco*RI next to the HyperLadder1 (BIOLINE). The 1140 bp *C1* cDNA is present in all three lanes (Figure 5-1).

The initial objectives of this project were to blunt-end clone the *C1* cDNA behind the rice Actin promoter and intron and use this construct for transient expression in biolistics. That project was not without difficulties, but these difficulties were further compounded by the failure to produce consistent transient expression of anthocyanins in switchgrass explants after biolistics. While trying to optimize the parameters for biolistics, the use of switchgrass callus in biolistics produced too few foci. In contrast, transient expression in caryopses proved to be even more variable, and switchgrass stem and leaf explants produced too great of a wounding response to determine the degree of anthocyanin expression. Gene expression may have been inhibited by the inability of damaged cells to accumulate anthocyanin pigment, or from cell and tissue specific expression of anthocyanins directed by promoter elements (McElroy 1994; Bower, Elliott et al. 1996; Selinger, Lisch et al. 1998). It has also been suggested that the constitutive expression of anthocyanin genes may be debilitating to the targeted tissue (Bower, Elliott et al. 1996). Given the possible causes for reduced anthocyanin expression, perhaps *Agrobacterium*-mediated transformation using another marker system might be a more amenable system for future switchgrass transformation.

6

Summation and Conclusions

Interest in the development of alternative fuel sources is unlikely to diminish in the near future and it is possible that switchgrass will play a pivotal role when fossil fuel sources become less available. The development of switchgrass as an alternative fuel source will require a combination of biotechnology and traditional agronomic practices. It is to this end that I have examined tissue culture protocols for Alamo, and the possibly higher yielding NF/GA-992, NF/GA-993, NSL, and SL93 cultivars in this work. As stated previously, tissue culture is a requisite component to most plant transformation protocols to regenerate and eventually propagate recombinants.

The first experiment examined the callus induction efficiencies of NF/GA-992, NF/GA-993, NSL, and SL93 seeds when treated with 8.5u*M* dicamba. All four of the cultivars produced callus. But of the four cultivars, NF/GA-992 responded best to the treatment. Other research suggested that higher concentrations of dicamba were needed to induce callus formation.

Plant growth regulator concentrations were more closely investigated in a multi-level study. This experiment shifted from the NF/GA lines to Alamo and the Oklahoma State University developed NSL and SL93 cultivars, and analyzed their responses to different concentrations and combinations of dicamba and 6-benzylaminopurine (BAP). This study was envisioned not only produce calluses but to regenerate shoots as well. The use of a cytokinin such as BAP has long been known to be required for plant regeneration. All three cultivars produced the most calluses when treated with 34 u*M* dicamba. The inclusion of BAP did not play a significant role in callus induction. What

effect the dicamba and BAP combinations had on shoot regeneration is unknown since sample loss made it impossible to conduct a meaningful data analysis.

The last tissue culture experiment examined explant choice, preparatory caryopses treatments, as well as evaluated plant germination, callus induction, and shoot regeneration from the SL93, NSL and Alamo cultivars. Even though inflorescences regenerated a greater quantity of shoots, this study revealed that switchgrass seed was a more reliable source for calluses and regenerating shoots than inflorescences. Alamo seed-derived calluses produced on average 2.07 ± 3.56 shoots per callus. Despite producing shoots via indirect organogenesis it is possible that some of the Alamo calluses were embryogenic. Perhaps future experiments could examine this observation in depth.

The last chapter focused on the utility of anthocyanins, specifically the *C1* regulatory complementary DNA (cDNA), as a visual marker of transformation. Optimization of consistent microprojectile bombardment protocols using pBECKS.red and the β-Peru biolistics plasmid in switchgrass was not realized over the course of this research. However, the *C1* cDNA was recombined into two new plasmids pBSL15.red and pUC.red that will be useful in future research.

References

Alexandrova, K. S., P. D. Denchev, et al. (1996). "In vitro development of inflorescences from switchgrass nodal segments." Crop Science **36**(1): 175.

Alexandrova, K. S., P. D. Denchev, et al. (1996). "Micropropagation of switchgrass by node culture." Crop Science **36**(6): 1709.

Alexeyev, M. F. (1995). "Three kanamycin resistance gene cassettes with different polylinkers." Biotechniques **18**(1): 52-54.

Bahieldin, A., W. E. Dyer, et al. (2000). "Concentration effects of dicamba on shoot regeneration in wheat." Plant Breeding **119**(5): 437-439.

Barnes, R. F., D. A. Miller, et al. (1995). "Forages Volume I An introduction to grassland agriculture." Publisher: Iowa State University Press, Ames, Iowa, USA: 205-473.

Barnett, F. L. and R. F. Carver (1967). "Meiosis and rollen stainability in switchgrass, Panicum virgatum L." Crop Science **7**(4): 301.

Birch, R. G. (1997). "Plant transformation: problems and strategies for practical application." Annual Reviews in Plant Physiology and Plant Molecular Biology **48**(1): 297-326.

Bower, R., A. R. Elliott, et al. (1996). "High-efficiency, microprojectile-mediated cotransformation of sugarcane, using visible or selectable markers." Molecular Breeding **2**(3): 239-249.

Carman, J. G., N. E. Jefferson, et al. (1988). "Induction of embryogenicTriticum aestivum L. calli. II. Quantification of organic addenda and other culture variable effects." Plant Cell, Tissue and Organ Culture **12**(1): 97-110.

Cassida, K. A., J. P. Muir, et al. (2005). "Biofuel component concentrations and yields of switchgrass in south central US environments." Crop Science **45**(2): 682.

Chawla, H. S. (2003). Plant Biotechnology: A Practical Approach, Enfield, NH: Science Publishers.

Chawla, H. S., L. A. Cass, et al. (1999). "Expression of anthocyanin pigmentation in wheat tissues transformed with anthocyanin regulatory genes." Current Science **76**: 1365-1370.

Chen, S. M. and E. H. Coe (1977). "Control of anthocyanin synthesis by the C locus in maize." Biochemical Genetics **15**(3): 333-346.

Cone, K. C., F. A. Burr, et al. (1986). "Molecular analysis of the maize anthocyanin regulatory locus C1." Proceedings of the National Academy of Sciences of the United States of America **83**(24): 9631.

Conger, B. V., G. E. Hanning, et al. (1983). "Direct embryogenesis from mesophyll cells of Orchardgrass." Science **221**(4613): 850-851.

Denchev, P. D. and B. V. Conger (1994). "Plant regeneraton from callus cultures of switchgrass." Crop Science **34**(6): 1623.

Denchev, P. D. and B. V. Conger (1995). "In vitro culture of switchgrass: Influence of 2, 4-d and picloram in combination with benzyladenine on callus initiation and regeneration." Plant Cell, Tissue and Organ Culture **40**(1): 43-48.

Dixon, R. A. and R. A. Gonzales (1994). Plant Cell Culture: A Practical Approach, Oxford University Press.

Douglas, J. L. and J. Grabowski (1995). "Seed Germination of Alamoswitchgrass as influenced by age of seed and prechill." SEED **11**(4).

Duncan, D. R., G. W. Singletary, et al. (1989). "Increased induction of regenerable callus cultures from cultured kernels of the maize inbred FR27rhm." Plant Cell Reports **8**(6): 350-353.

Elliott, A. R., J. A. Campbell, et al. (1999). "Green-fluorescent protein facilitates rapid in vivo detection of genetically transformed plant cells." Plant Cell Reports **18**(9): 707-714.

Gaba, V.P. Plant growth regulators in plant tissue culture and developmeny. Ed. Trigiano, R. N. and D. J. Gray (2005). Plant Development and Biotechnology, CRC Press

Gomez, K. A. and A. A. Gomez (1984). Statistical Procedures for Agricultural Research, John Wiley and Sons (WIE).

Griffin, J. D. and M. S. Dibble (1995). "High-frequency plant regeneration from seed-derived callus cultures of Kentucky bluegrass (Poa pratensis L.)." Plant Cell Reports **14**(11): 721-724.

Guivarc'h, A., J. C. Caissard, et al. (1996). "In situ detection of expression of thegus reporter gene in transgenic plants: ten years of blue genes." Transgenic Research **5**(5): 281-288.

Gupta, S. D. and B. V. Conger (1998). "In vitro differentiation of multiple shoot clumps from intact seedlings of switchgrass." In Vitro Cellular & Developmental Biology-Plant **34**(3): 196-202.

Gupta, S. D. and B. V. Conger (1999). "Somatic embryogenesis and plant regeneration from suspension cultures of switchgrass." Crop Science **39**(1): 243-247.

Hansen, G. and M. S. Wright (1999). "Recent advances in the transformation of plants." Trends in Plant Science **4**(6): 226-231.

Haynes, J. G., W. G. Pill, et al. (1997). "Seed treatments improve the germination and seedling emergence of switchgrass (Panicum virgatum L.)." HortScience **32**(7): 1222-1226.

Hicks, G. S. (1994). "Shoot induction and organogenesis in vitro: A developmental perspective." In Vitro Cellular & Developmental Biology-Plant **30**(1): 10-15.

Holton, T. A. and E. C. Cornish (1995). "Genetics and biochemistry of anthocyanin biosynthesis." Plant Cell **7**(7): 1071-1083.

Horvath, H., J. Huang, et al. (2002). "Of transgenic plants." Barley Science: Recent advances from molecular biology to agronomy of yield and quality.

Klein, T. M., B. A. Roth, et al. (1989). "Regulation of anthocyanin biosynthetic genes introduced into intact maize tissues by microprojectiles." Proceedings of the National Academy of Sciences of the United States of America **86**(17): 6681.

Larkin, P. J. and W. R. Scowcroft (1981). "Somaclonal variation—a novel source of variability from cell cultures for plant improvement." TAG Theoretical and Applied Genetics **60**(4): 197-214.

Lloyd, A. M., V. Walbot, et al. (1992). "Arabidopsis and Nicotiana anthocyanin production activated by maize regulators R and C1." Science **258**(5089): 1773-1775.

Mantis, J. and B. W. Tague (2000). "Comparing the utility of beta-glucuronidase and green fluorescent protein for detection of weak promoter activity in Arabidopsis thaliana." Plant Molecular Biology Reporter **18**(4): 319.

Martinez-Reyna, J. M. and K. P. Vogel (2002). "Incompatibility systems in switchgrass." Crop Science **42**(6): 1800.

Mazarei, M., H. Al-Ahmad, et al. (2008). "Protoplast isolation and transient gene expression in switchgrass, Panicum virgatum L." Biotechnol **3**(3): 354-9.

McCormac, A. C., M. C. Elliott, et al. (1997). "pBECKS. A flexible series of binary vectors for Agrobacterium-mediated plant transformation." Mol Biotechnol **8**(3): 199-213.

McCormac, A. C., H. Wu, et al. (1998). "The use of visual marker genes as cell-specific reporters of Agrobacterium-mediated T-DNA delivery to wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.)." Euphytica **99**(1): 17-25.

McLaughlin, S., J. Bouton, et al. (1999). "Developing switchgrass as a bioenergy crop." Reprinted from: Perspectives on new crops and new uses: 282-299.

Murashige, T. and F. Skoog (1962). " A revised medium for rapid growth and bioassays with tobacco tissue cultures." Physiol. Plant. I **5**: 473-497.

Murata, M. (1989). "Effects of auxin and cytokinin on induction of sister chromatid exchanges in cultured cells of wheat (Triticum aestivum L.)." TAG Theoretical and Applied Genetics **78**(4): 521-524.

Nelson, A. J. and W. R. Bushnell (1997). "Transient expression of anthocyanin genes in barley epidermal cells: potential for use in evaluation of disease response genes." Transgenic Research **6**(3): 233-244.

Paz-Ares, J., D. Ghosal, et al. (1987). "The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators." EMBO J **6**(12): 3553-3558.

Potrykus, I. (1991). "Gene transfer to plants: assessment of published approaches and results." Annual Reviews in Plant Physiology and Plant Molecular Biology **42**(1): 205-225.

Prasher, D. C. (1995). "Using GFP to see the light." Trends in Genetics **11**(8): 320-323.

Pridmore, R. D. (1987). "New and versatile cloning vectors with kanamycin-resistance marker." Gene **56**(2-3): 309-12.

Przetakiewicz, A., W. Orczyk, et al. (2003). "The effect of auxin on plant regeneration of wheat, barley and triticale." Plant Cell, Tissue and Organ Culture **73**(3): 245-256.

Rathore, K. S., V. K. Chowdhury, et al. (1993). "Use of bar as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts." Plant Molecular Biology **21**(5): 871-884.

Richards, H. A., V. A. Rudas, et al. (2001). "Construction of a GFP-BAR plasmid and its use for switchgrass transformation." Plant Cell Reports **20**(1): 48-54.

Scheffler, B., P. Franken, et al. (1994). "Molecular analysis of C1 alleles in Zea mays defines regions involved in the expression of this regulatory gene." Molecular and General Genetics MGG **242**(1): 40-48.

Selinger, D. A., D. Lisch, et al. (1998). "The Maize Regulatory Gene B-Peru Contains a DNA Rearrangement That Specifies Tissue-Specific Expression Through Both Positive and Negative Promoter Elements." Genetics **149**(2): 1125-1138.

Somleva, M. N., Z. Tomaszewski, et al. (2002). "Agrobacterium-mediated genetic transformation of switchgrass." Crop Science **42**(6): 2080.

Talbert, L. E., D. H. Timothy, et al. (1983). "Estimates of genetic parameters in switchgrass." Crop Science **23**(4): 725.

Taliaferro, C. M. (1998). "Breeding and selection of new switchgrass varieties for increased biomass production." 1997 Annual report from Oklahoma State Univ. to Oak Ridge National Laboratory, March.

U.S. Department of Energy. Biomass Multi Year Program. March 2008. Accessed: July 8 2008.
<http://www1.eere.energy.gov/biomass/pdfs/biomass_program_mypp.pdf>

Vieira, J. and J. Messing (1982). "The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers." Gene **19**(3): 259-68.

Vogel, K. and K. Moore (1993). Native North American grasses. In'New crops'.(Eds J Janick, JE Simon) pp. 284–293, John Wiley and Sons, Inc: New York.

Vogel, K. P. and H. G. Jung (2000). "Genetic improvement of switchgrass and other herbaceous plants for use as biomass fuel feedstock." Oak Ridge National Laboratory Environmental Sciences Division: 1-31.

Vogel, K. P. and R. A. Masters (1998). "Developing switchgrass into a biomass fuel crop for the midwestern USA." Proceedings of BioEnergy 98-Expanding Bioenergy Partnerships: 4-8.

Wilcox, R. R. (1987). "New designs in analysis of variance." Annual Reviews in Psychology **38**(1): 29-60.

Winkel-Shirley, B. (2001). "Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology." Plant Physiology **126**(2): 485.

Wolf, D. D. and D. A. Fiske (1995). "Planting and managing switchgrass for forage, wildlife, and conservation." Virginia Cooperative Extension Publication: 418-013.

Wright, L. "Historical perspective on how and why switchgrass was selected as a "model" high-potential energy crop."

Zarnstorff, M. E., R. D. Keys, et al. (1994). "Growth regulator and seed storage effects on switchgrass germination." Agronomy Journal **86**(4): 667.

Appendix

Table 2-1. Callus Induction Medium (CIM)/Shoot Growth Medium (SGM) and Root Growth Medium (RGM) Protocol (for 1liter)*

CIM/ RGM Requirements:

1) MS (5524): 4.3 g
2) Maltose: 30 g
3) Myo inositol:0. 25 g
4) Casein: 1.0 g
5) L-proline:0. 69 g
6) Thiamine-HCl (4 mg ml^{-1}): 250 ul
7) CuSO4 (50 mM): 100 ul
8) Distilled water: 1l final volume
   Adjust pH to 5.8 with KOH
9) Phythagel: 3.5 g

Autoclave add hormones after autoclaving

10) *CIM*: add 375 ul dicambal^{-1} (stock: 5mg ml^{-1} DMSO).
11) *RGM:* Same as above except no dicamba or BAP

CIM (Green): same as above

12) add 40ul 6-BAP l^{-1} (0.1mg, stock: 2.5mg ml^{-1})
: 375ul dicamba per liter

SGM Requirements:

1) MS (M2909): 2.7g
2) Maltose: 62 g
3) Casein: 1.0 g
4) NH4NO3:0. 165 g
5) Glutamine:0. 75 g
6) Myo inositol:0.1 g
7) Thiamine-HCl (4mg ml^{-1}): 100 ul
8) CuSO4: 100 ul
9) ddH_20: till 1liter
   Adjust to pH 5.6 with NaOH
11) Add phytagel
   Autoclave add hormones after autoclaving
12) Add 400 ul 6-BAP l^{-1} (stock: 2.5mg ml^{-1})
11) Do not add dicamba

* Horvath et al, 2002.

Table 2-2. The GLM Procedure CRD ANOVA table for callus induction for the cultivars NF/GA993, NSL, NF/GA992, SL93.

Source	DF	Mean Square	F Value
Cultivar	3	217.66	97.26 **
Error	67	2.24	

** Indicates significant at $\alpha = 0.01$

Table 2-3. Mean number of calluses produced per replication plus standard deviation for the cultivars NF/GA993, NSL, NF/GA992, and SL93.

Cultivar	Mean	S.E.
NF/GA993	2.71	±1.76
NSL	1.32	±1.06
NF/GA992	8.90 **	±1.71
SL93	2.19	±1.42

** Indicates significant at $\alpha = 0.01$

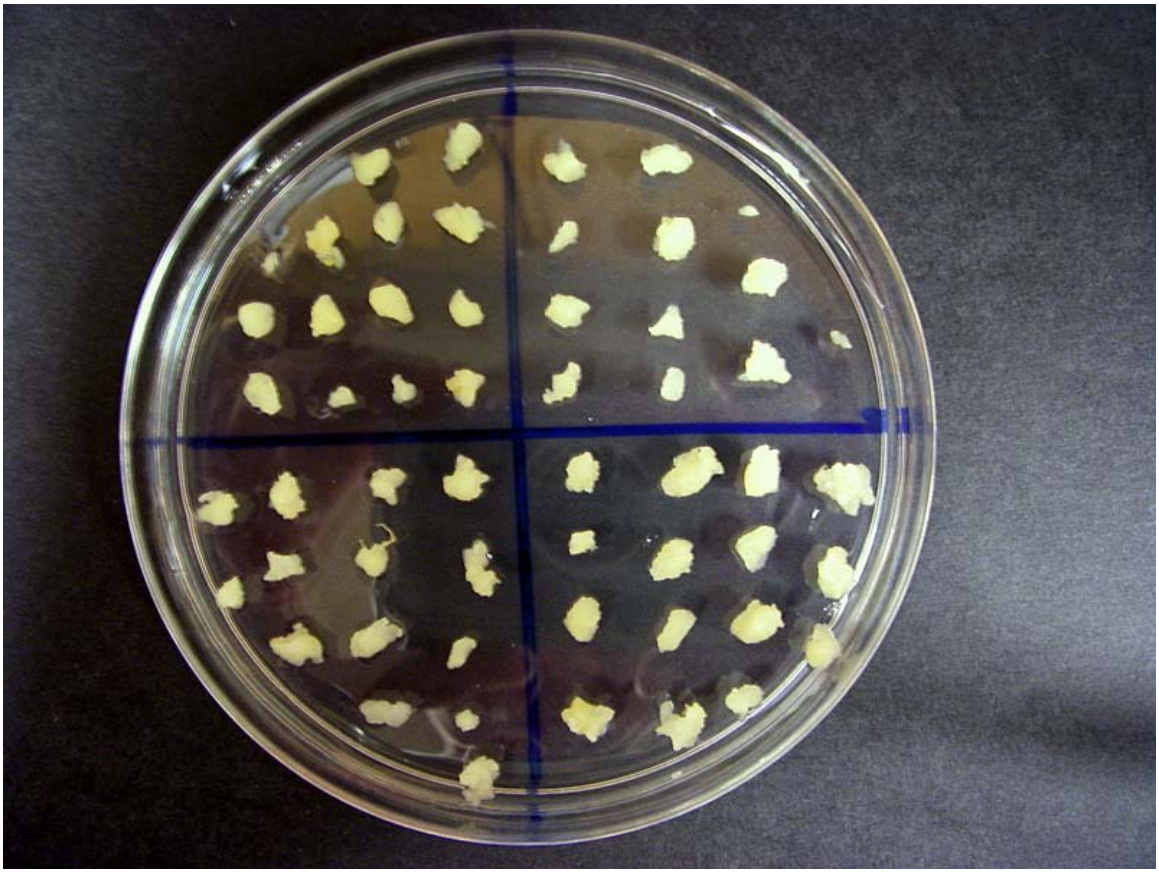

Figure 2-1. NF/GA993 seed derived callus on callus induction media.

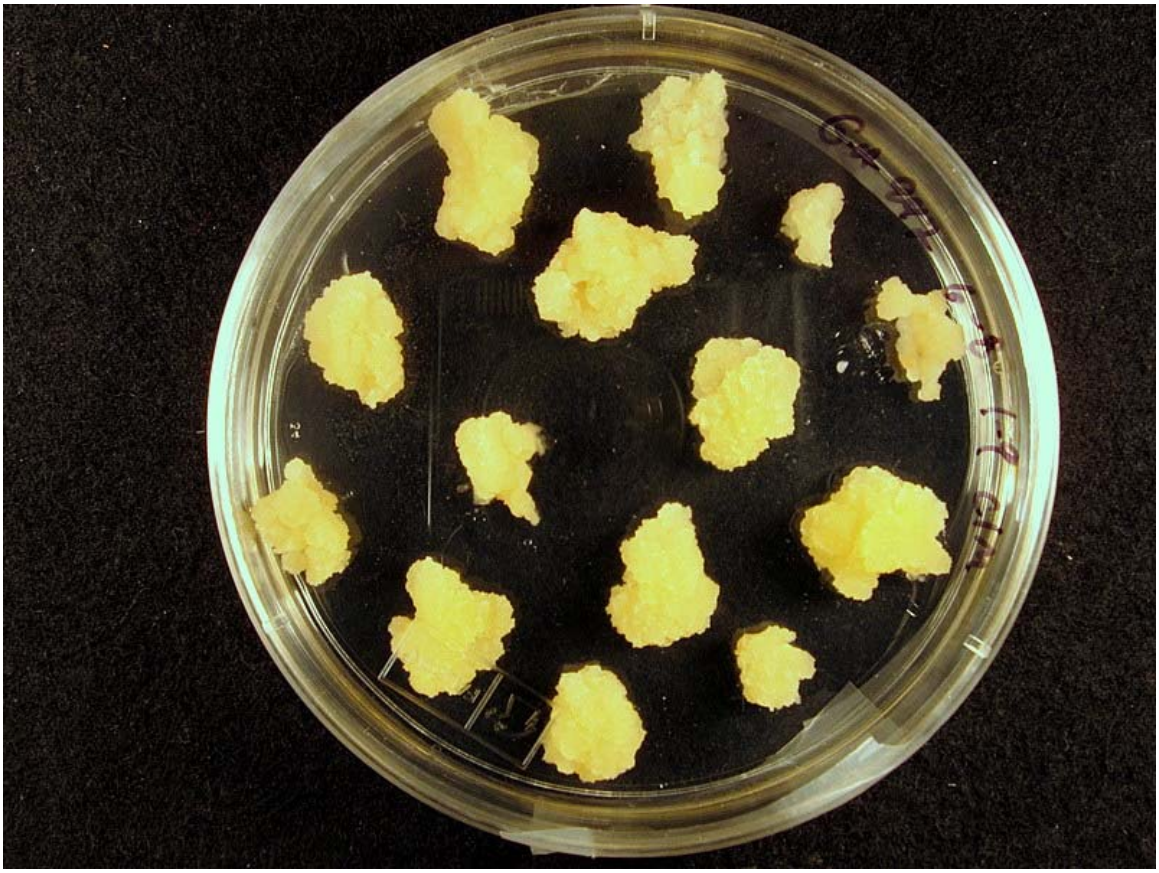

Figure 2-2. NF/GA992 seed derived callus on callus induction media.

Figure 2-3. NF/GA992 seed derived callus on callus induction media.

Figure 2-4. SL93 seed derived callus on callus induction media.

Figure 2-5. NSL seed derived callus on callus induction media.

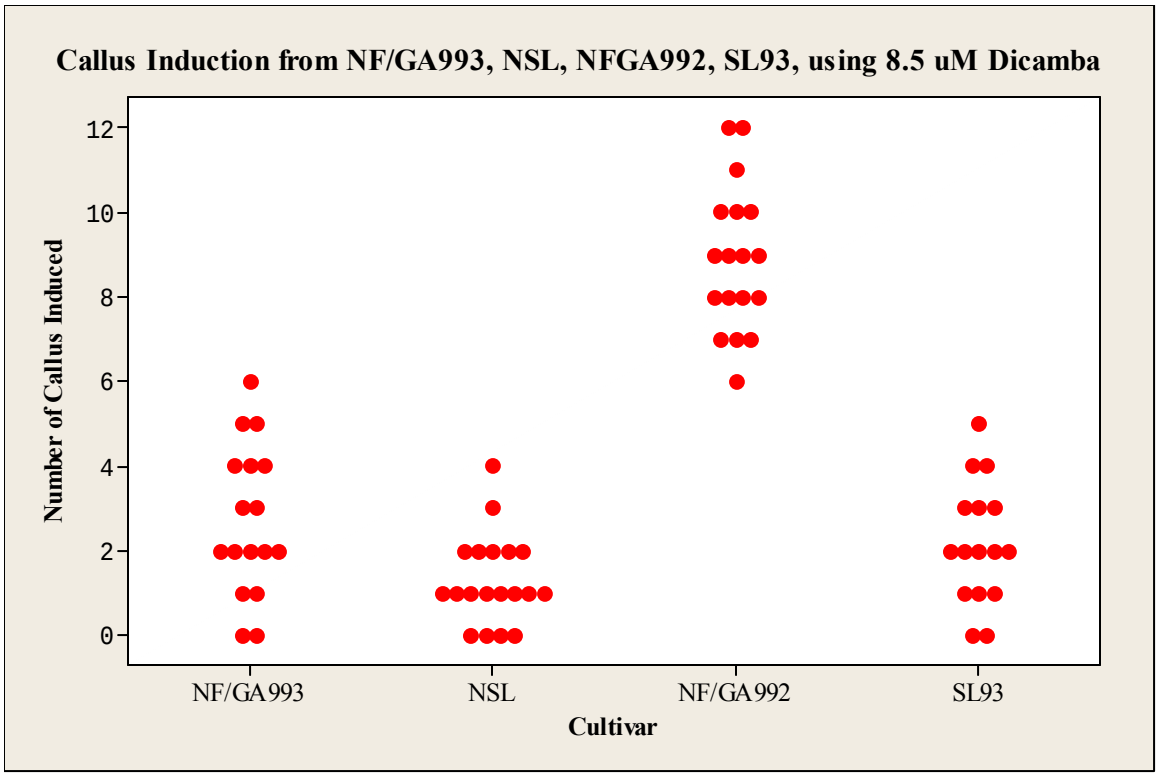

Figure 2-6. Number of calluses induced per petri dish using 8.5 u*M* dicamba for the cultivars NF/GA993, NSL, NF/GA992, and SL93.

Table 3-1. Callus Induction Medium (CIM)/Shoot Growth Medium (SGM) and Root Growth Medium (RGM) Protocol (for 1liter)*

CIM/ RGM Requirements:

1) MS (5524): 4.3 g
2) Maltose: 30 g
3) Myo inositol:0. 25 g
4) Casein: 1.0 g
5) L-proline:0. 69 g
6) Thiamine-HCl (4 mg ml^{-1}): 250 ul
7) CuSO4 (50 mM): 100 ul
8) Distilled water: 1l final volume
   Adjust pH to 5.8 with KOH
9) Phythagel: 3.5 g

Autoclave add hormones after autoclaving

12) *CIM*: add 375 ul dicambal^{-1} (stock: 5mg ml^{-1} DMSO).
13) *RGM:* Same as above except no dicamba or BAP

CIM (Green): same as above

12) add 40ul 6-BAP l^{-1} (0.1mg, stock: 2.5mg ml^{-1})
: 375ul dicamba per liter

SGM Requirements:

1) MS (M2909): 2.7g
2) Maltose: 62 g
3) Casein: 1.0 g
4) NH4NO3:0. 165 g
5) Glutamine:0. 75 g
6) Myo inositol:0.1 g
7) Thiamine-HCl (4mg ml^{-1}): 100 ul
8) CuSO4: 100 ul
9) $_{d}dH_20$: till 1liter
   Adjust to pH 5.6 with NaOH
11) Add phytagel
   Autoclave add hormones after autoclaving
12) Add 400 ul 6-BAP l^{-1} (stock: 2.5mg ml^{-1})
11) Do not add dicamba

* Horvath et al, 2002.

Table 3-2. Factorial ANOVA table of callus induction with varying concentrations of dicamba and BAP for three cultivars

Source	DF	Mean Square	F Value
rep	4	0.48	1.79
cultivar	2	0.49	1.80
PGR treatment	11	22.05	81.54 **
rep*cultivar	7	0.41	1.53
rep*treatment	44	0.27	1.01
cultivar*treatment	21	0.55	2.03 *
error	57	0.27	

* significant at $\alpha = 0.05$ and ** significant at $\alpha = 0.01$ as determined by F-test.

Table 3-3. Number of SL93, NSL, and Alamo caryopses derived callus produced from each treatment with 5 plates of 20 seeds per treatment unless otherwise noted.

Treatment		Callus per cultivar (# of plates)		
Dicamba uM	BAP uM	SL93	NSL	Alamo
8.5	0	1	3	3 (3)
8.5	5	0	0	0 (2)
8.5	15	2	0	0 (4)
8.5	45	0	0	0 (1)
17	0	6	13	0 (0)
17	5	0	0	1 (2)
17	15	2	0	0 (1)
17	75	0	1	1 (3)
34	0	26	23	17 (4)
34	5	6	6	0 (1)
34	15	3	0	0 (2)
34	45	0	0	0 (4)

Table 3-4. Mean number of calluses produced ± standard error for each treatment.

Treatment		Average number of calluses per replication ± S.E.		
Dicamba uM	BAP uM	SL93	NSL	Alamo
8.5	0	0.2 ± 0.4	0.6 ± 0.8	1.0 ± 1.0
8.5	5	0.0	0.0	0.0
8.5	15	0.4 ± 0.5	0.0	0.0
8.5	45	0.0	0.0	0.0
17	0	1.2 ± 0.4*	2.6 ± 1.1*	N/A
17	5	0.0	0.0	0.5 ± 0.6
17	15	0.4 ± 0.5	0.0	0.0
17	45	0.0	0.2 ± 0.4	0.3 ± 0.6
34	0	5.2 ± 1.1*	4.6 ± 1.1*	4.3 ± 0.5*
34	5	1.2 ± 0.8*	1.2 ± 0.4*	0.0
34	15	0.8 ± 0.4	0.0	0.0
34	45	0.0	0.0	0.0

* significant at $\alpha = 0.05$ as determined by Tukey-Kramer test.

Table 3-5. Mean number of shoots produced per SL93 and Alamo callus.

Cultivar	Treatment	Mean # of shoots per callus ± SE
SL93	34 u*M* dicamba, 15 u*M* BAP	0.7 ± 0.8
Alamo	34 u*M* dicamba, 0 uM BAP	4.5 ± 1.1

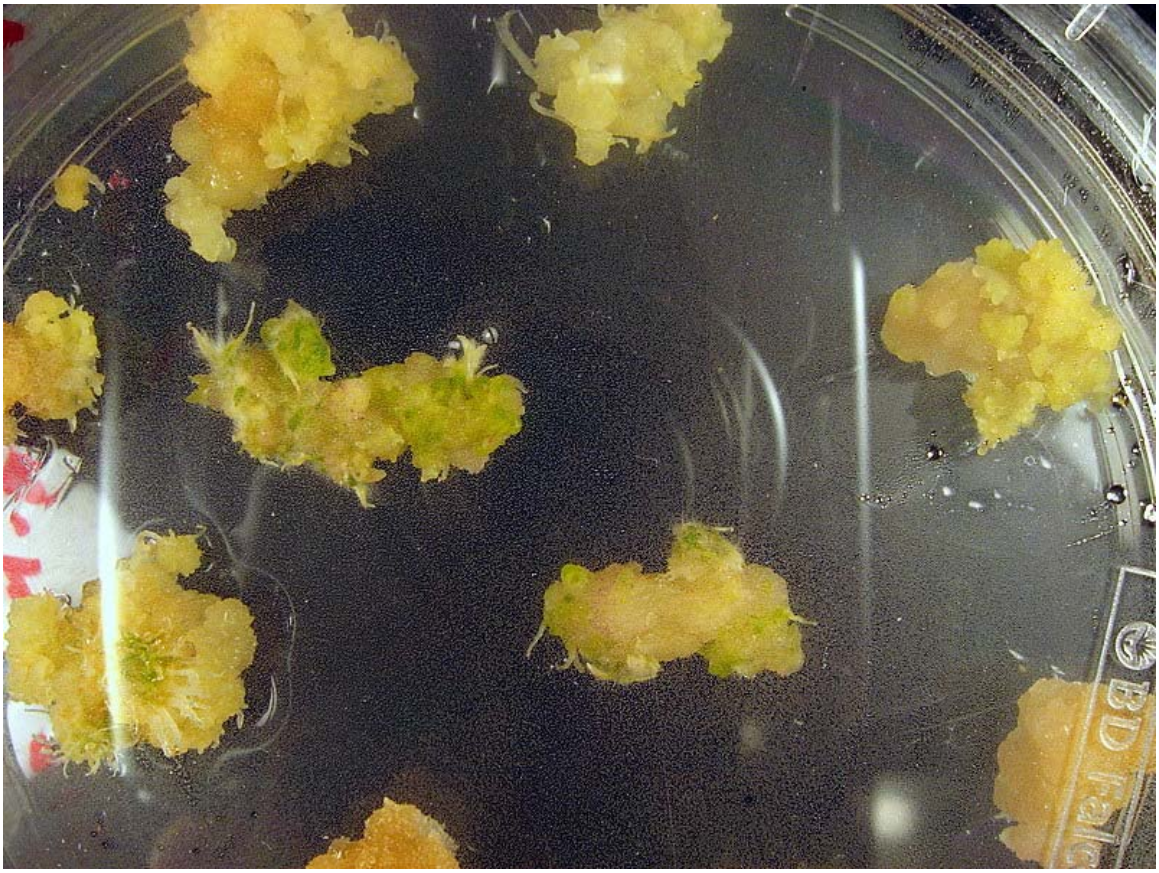

Figure 3-1. SL93 seed derived calluses on CIM(Green).

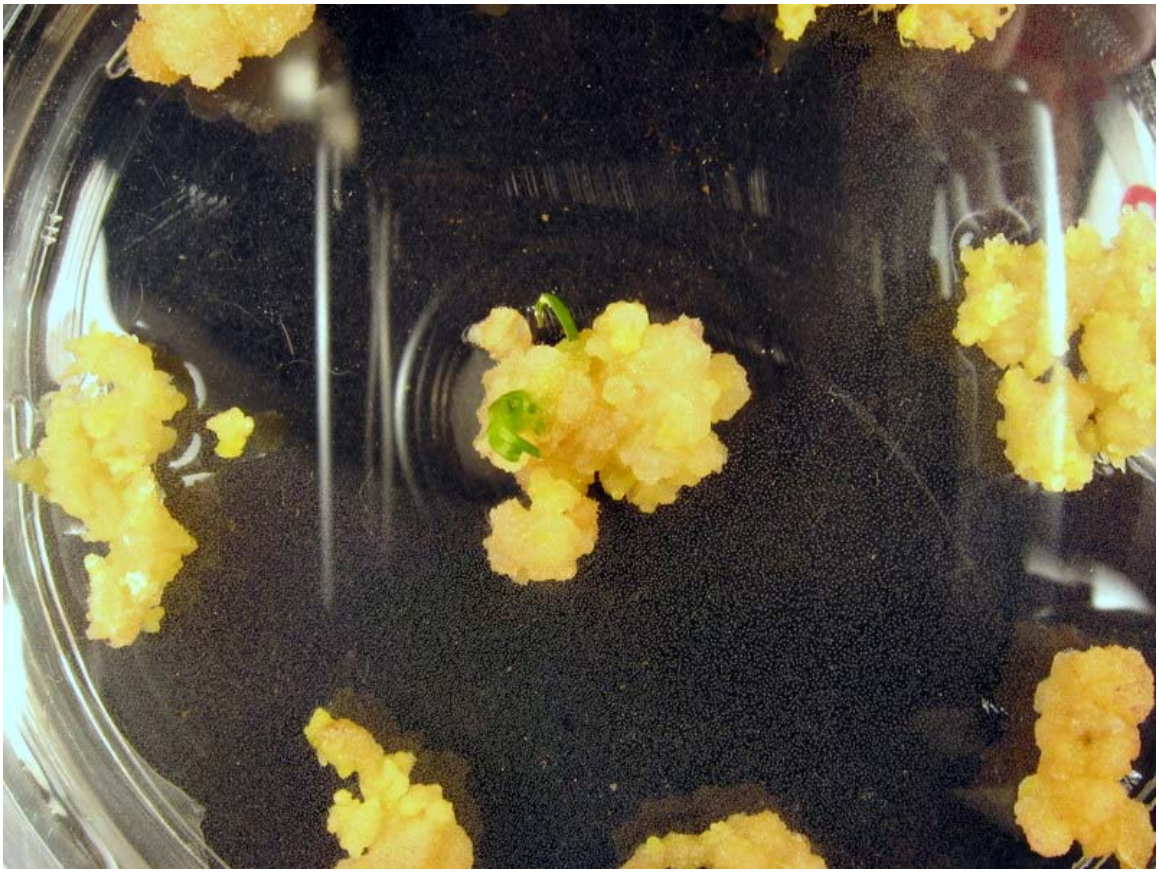

Figure 3-2. Alamo seed derived calluses after two weeks on shoot growth media (SGM).

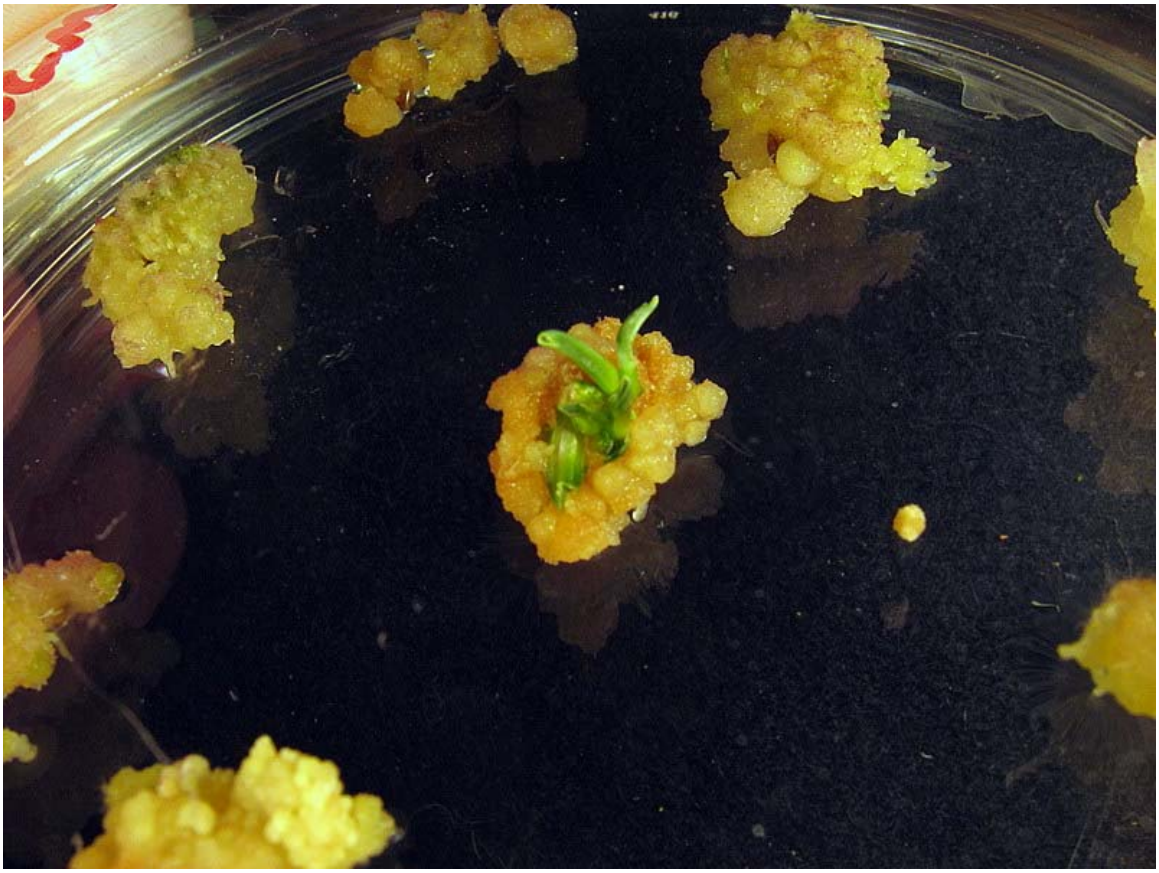

Figure 3-3. SL93 seed derived calluses developing shoots after two weeks on SGM.

Figure 3-4. Alamo plantlet on root growth media (RGM).

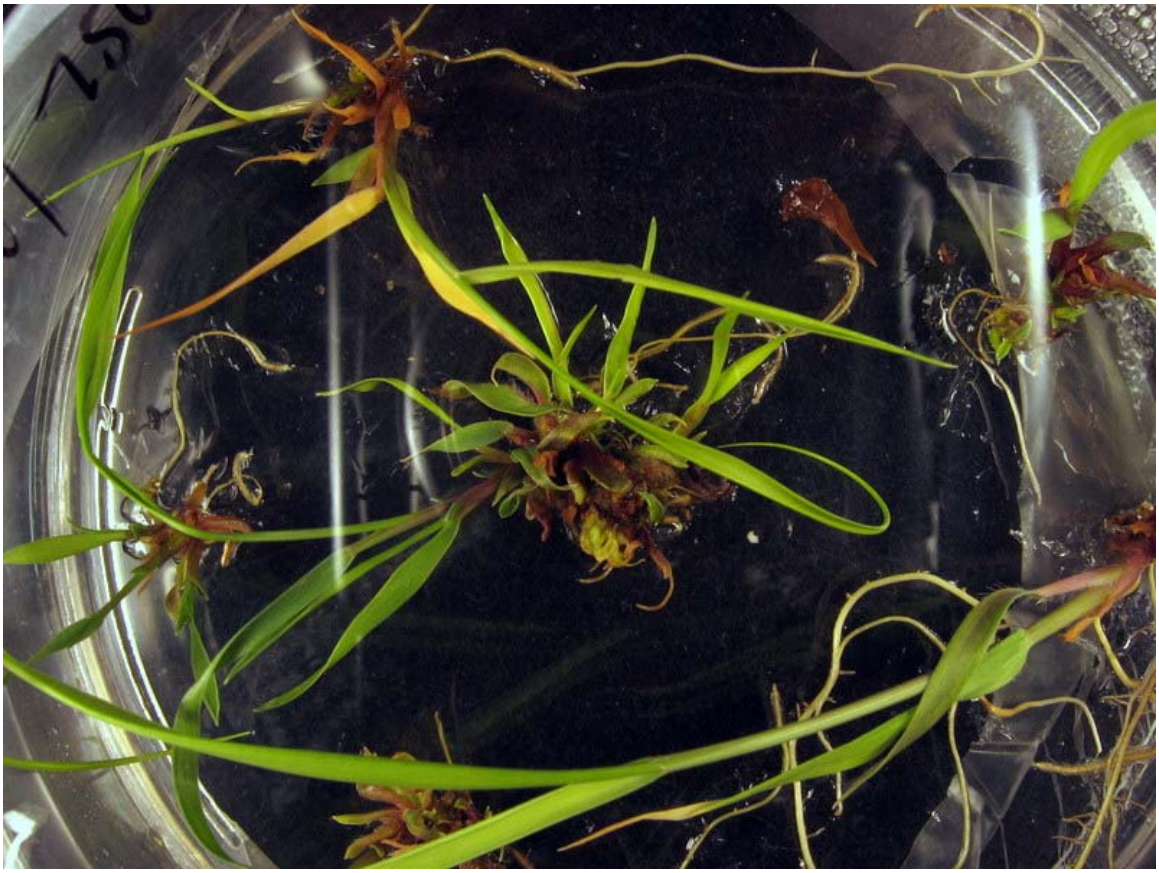

Figure 3-5. SL93 plantlets with multiple shoots on RGM.

Table 4-1. Callus Induction Medium (CIM)/Shoot Growth Medium (SGM) and Root Growth Medium (RGM) Protocol (for 1liter)*

CIM/ RGM Requirements:

1) MS (5524): 4.3 g
2) Maltose: 30 g
3) Myo inositol:0. 25 g
4) Casein: 1.0 g
5) L-proline:0. 69 g
6) Thiamine-HCl (4 mg ml^{-1}): 250 ul
7) CuSO4 (50 mM): 100 ul
8) Distilled water: 1l final volume
   Adjust pH to 5.8 with KOH
9) Phythagel: 3.5 g

Autoclave add hormones after autoclaving

14) *CIM*: add 375 ul dicambal^{-1} (stock: 5mg ml^{-1} DMSO).
15) *RGM:* Same as above except no dicamba or BAP

CIM (Green): same as above

12) add 40ul 6-BAP l^{-1} (0.1mg, stock: 2.5mg ml^{-1})
: 375ul dicamba per liter

SGM Requirements:

1) MS (M2909): 2.7g
2) Maltose: 62 g
3) Casein: 1.0 g
4) NH4NO3:0. 165 g
5) Glutamine:0. 75 g
6) Myo inositol:0.1 g
7) Thiamine-HCl (4mg ml^{-1}): 100 ul
8) CuSO4: 100 ul
9) $_{d}dH_20$: till 1liter
   Adjust to pH 5.6 with NaOH
11) Add phytagel
   Autoclave add hormones after autoclaving
12) Add 400 ul 6-BAP l^{-1} (stock: 2.5mg ml^{-1})
12) Do not add dicamba

* Horvath et al, 2002.

Table 4-2. Inforescence media recipe for 1liter.

Inflorescence Media	
H_2O	800 ml
MS Medium	4.33 g
Maltose	30 g
H_2O	up to 1l
Phytagel	3.5 g
After autoclaving**:**	
BAP l^{-1}	500 ul (5.0 uM) (stock: 2.25 mg $ml^{-1)}$

Table 4-3. Number of SL93, NSL, and Alamo germinated seedlings and caryopses derived calluses produced from each treatment with 5 plates of 20 seeds per treatment unless otherwise noted.

Treatment	Cultivar	Germinated (10 days)	Callus	Lost
H_2SO_4	SL93	31	33 *	
	NSL	12	16	
	Alamo	22	22*	(2 plates)
4°C for 14 days	SL93	14	18	
	NSL	6	9 *	
	Alamo	40	29	
Surface Sterilized	SL93	21	22	
	NSL	9	25	
	Alamo	4	6	(2 plates)

* significant at $\alpha = 0.05$ as determined by Tukey-Kramer test and Duncans means comparison.

Table 4-4. Factorial ANOVA of callus induction from seed for three cultivars and three seed treatments.

Source	DF	Mean Square	F Value
rep	4	2.63	1.73
cultivar	2	17.76	11.68 **
treatment	2	10.52	6.92 **
rep*cultivar	8	1.65	1.08
rep*treatment	8	1.37	0.90
cultivar*treatment	4	9.18	6.04 **
error	12	1.52	

** = significant at $\alpha = 0.01$

Table 4-5. Factorial ANOVA of seed germination for three cultivars and three seed treatments.

Source	DF	Mean Square	F Value
rep	4	3.12	2.72
cultivar	2	33.80	28.83 **
treatment	2	18.47	15.75 **
rep*cultivar	8	1.77	1.51
rep*treatment	8	1.19	1.01
cultivar*treatment	4	31.37	26.76 **
error	16	1.17	

** = significant at $\alpha = 0.01$

Table 4-6. Total number of spikelets plated and calluses produced from SL93, NSL and Alamo .

Cultivar	**Spikelets**	**Calluses**
SL93	31	52
NSL	32	19
Alamo	36	32

Table 4-7. Average number of shoots produced per callus for three cultivars.

Seed-derived			**Inflorescence-derived**		
Cultivar	Mean	StDev	Cultivar	Mean	StDev
SL93	2.50	± 2.12	SL93	7.17	± 4.80
NSL	6.00	± 0.00	NSL	0.00	± 0.00
Alamo	6.42	± 3.50			
			Alamo	0.00	± 0.00

Table 4-8. Average number of shoots produced per treatment for each cultivar.

Cultivar	Treatment	Mean	StDev
SL93	Sterilization	0.00	± 0.00
	H_2SO_4	0.00	± 0.00
	4°C	1.00	± 1.73
NSL	Sterilization	0.00	± 0.00
	H_2SO_4	1.20	± 2.68
	4°C	0.00	± 0.00
Alamo	Sterilization	1.20	± 2.68
	H_2SO_4	0.00	± 0.00
	4°C	5.00	± 4.47

Table 4-9. Factorial ANOVA table of the number of shoots produced from seed-derived calluses of three cultivars.

Source	DF	Mean Square	F Value
cultivar	2	14.47	3.48
treatment	2	12.80	3.08
cultivar*treatment	4	12.67	3.05
error	36	4.20	

Table 4-10. One-way ANOVA table for the number of shoots produced from inflorescence-derived calluses of three cultivars.

Source	DF	Mean Square	F Value
model	2	121.40	10.84
error	41	11.20	

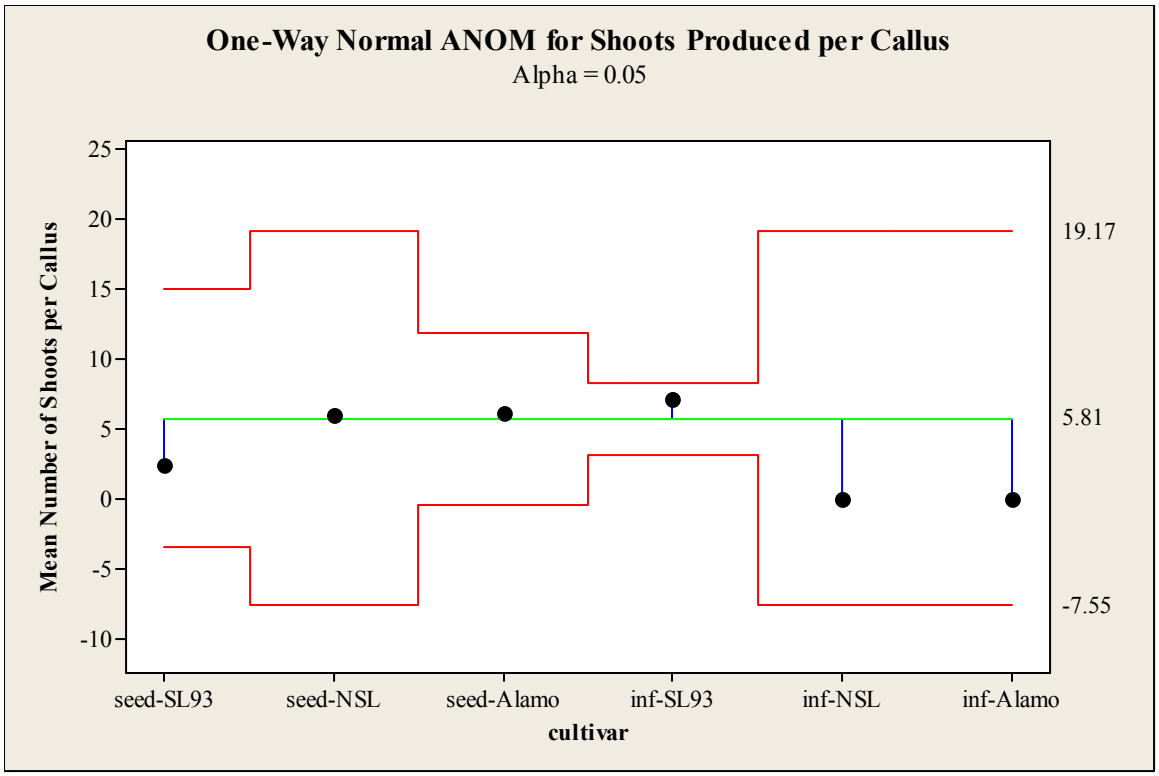

Figure 4-1. The mean number of shoots per callus produced for each cultivar from caryopses and inflorescences (note red line indicates the limits of $\alpha = 0.05$.)

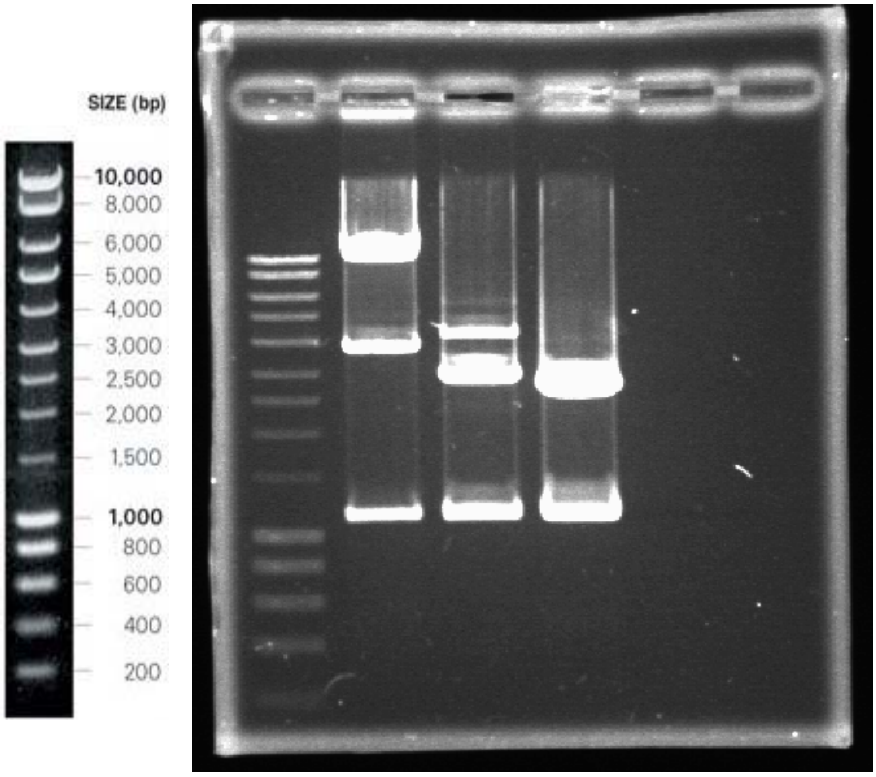

Figure 5-1. Gel image of a HyperLadder I (lane 1), pBECKS.red (lane 2), pBSL.red (lane 3), and pUC.red (lane 4) digested with *Eco*RI

Vita

Stephen Foulk was born in Moline, IL on August 21, 1976. He grew up and attended elementary school and John Deere Junior High there. Stephen graduated from Moline High School in 1995. He received his B.S. in biology from St. Ambrose University in Davenport, IA. Stephen received his M.S. in plant sciences from the University of Tennessee, and is interested in pursuing a career in biotechnology.