# IMPROVEMENT OF THE WHEAT (*Triticum aestivum* L.)

# TRANSFORMATION SYSTEM

By

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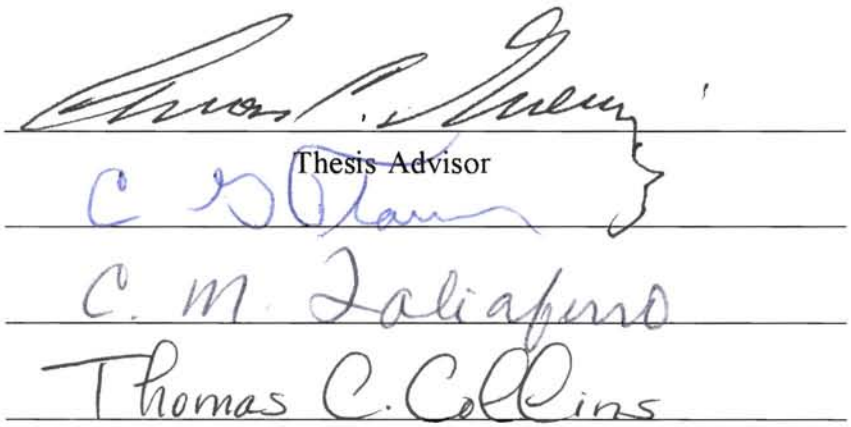

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# INTRODUCTION

The chapters in this thesis conform to the Publications Handbook and Style Manual of the American Society of Agronomy. Chapters will be submitted for publication in Crop Science, a Crop Science Society of America publication.

# CHAPTER 1

# EVALUATION OF THE EFFECT OF MANNITOL TREATMENTS ON THE TRANSFORMATION OF WHEAT (*Triticum aestivum* L.) EMBRYO-DERIVED CALLUS

## ABSTRACT

Biolistic™ transformation of wheat (*Triticum aestivum* L.) has been well established. The objective of this research was to determine osmotic treatments maximizing the transformation frequency. Immature wheat embryos were placed on media with 0, 0.2, 0.4, 0.6 M mannitol six days after callus initiation. Tissues were bombarded with DNA-coated microprojectiles 5 h after being transferred to treatment media. Tissues were transferred to 1 mg $L^{-1}$ bialaphos media 16 h after bombardment. All tissues were transformed with the pAHC25 plasmid containing *bar* and *uidA* genes driven by the *ubi-1* promoter. Frequencies of recovering embryogenic calli were; 0.6% with no mannitol, 1.2% with 0.2 M mannitol, 5.5% with 0.4 M mannitol, and 8.3% with 0.6 M mannitol. The optimum pre- and post-bombardment treatment for obtaining regenerable callus tissue in the presence of bialaphos was 0.6 M mannitol.

## INTRODUCTION

The methodology for transformation of wheat (*Triticum aestivum* L.) has been well developed making regeneration of viable plants from callus an actuality (Weeks et al., 1993). Recent evidence suggests that mannitol can be used to enhance biolistic transformation. It is believed that mannitol reduces the osmotic pressure in the target cells. This decreases injury to the tissue by the actual penetration of the particles of gold during bombardment and increases transformation frequency (Vain et al., 1993).

The objective of this research was to optimize the procedure for transforming wheat as described by Weeks et al. (1993). The parameter selected to optimize was the osmotic pressure in the embryonic tissue before, during, and after bombardment. Osmotic pressure was regulated by varying mannitol concentrations in pre-bombardment and post-bombardment tissue culture media. The success of the various treatments was determined by the number of embryos surviving to the callus stage and the number of plants regenerated from these tissues.

## LITERATURE REVIEW

### Methods of gene transfer

There are many methods of transforming plants today, but with the exception of rice, the only proven efficient way to transform cereals is with Biolistics (Klien *et al.*, 1992; Armaleo *et al.*, 1990). The best method for plant transformation is a method designed by nature using *Agrobacterium tumefaciens*. These bacteria transform plant cells to form tumors that produce opines that are secreted into the intercellular regions where the bacteria live. The plants are not able to metabolize opines which makes them available for the bacteria as a rich carbon and nitrogen source. The major limitation of *A. tumefaciens* is that it has a narrow host range and transformation frequencies range from 100% (potato) to 1% (sunflower). So far wheat has not been transformed with *A. tumefaciens* but recent improvements in the rice transformation system may warrant further investigation using the same Ti plasmid in wheat (Hiei *et al.*, 1994). There are several methods of direct gene transfer including microinjection delivery into whole plants, mixing pollen with DNA, embryo inbibition, electroporation, and the particle gun (Potrykus, 1990). Microinjection is commonly used to transform animals but has not yet been as successful with plants. There has been limited success with microinjection of protoplasts (Neuhaus and Spangenburg, 1990). It has been reported that using a mixture of DNA and pollen to pollinate has successfully transformed progeny , but as yet this process has not produced a

stable transformation event (Hess, 1977; Ohta, 1986). Embryo inbibition is a process where seeds are dried then imbibed in a water DNA mixture thus producing a transformed plant, but this method has so far not been reproducible (Ledoux *et al.*, 1974). Electroporation requires protoplasts as a target tissue and therefore requires species that can regenerate from the protoplast stage. This works very well with rice but the viability of wheat protoplasts is low and fertile transgenic wheat has not been regenerated from protoplasts (Becker *et al.*, 1994). Delivery into plants by microinjection has not been successful in cereals because of unstable DNA integration (Rogers and Rogers, 1992). The only reliable transformation system for wheat uses the particle gun, making it possible for the regeneration of fertile transgenic wheat plants (Weeks *et al.*, 1993; Vasil *et al.*, 1993; Vasil *et al.*, 1992). Having a more efficient transformation system will allow the rapid deployment of agronomic genes of interest. Wheat has been transformed by several labs for herbicide resistance (Vasil *et al.*, 1992; Weeks *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Blechl and Anderson, 1996). Optimization of this system is necessary to explore the routine germplasm enhancement of wheat such as expressing seed storage proteins that affect the bread making quality (Blechl and Anderson, 1996).

**Osmotic treatments in wheat transformation**

Few papers have been published describing the effect of osmotic treatments on plant transformation frequency. Mannitol is a sugar alcohol which occurs naturally in celery (*Apium graveolens* L.) providing a high salt tolerance due to its role as an osmoprotectant (Williamson *et al.*, 1995). To better understand the role of mannitol in plant transformation, one must first look at water potential and its direct relationship to osmotic adjustment. Plants can continue to absorb water as soil begins to dry as long as they can maintain a lower water potential than the source (Taiz and Zeiger, 1991). The plants accomplish this by increasing their cellular solute level, decreasing the water potential in the plant cells and achieving a positive water flow into the cells. An increased extracellular concentration of mannitol will induce plasmolysis in the callus tissue. This strategy reverses the flow of water out of the cells by decreasing the water potential in the medium. Plasmolized cells that have been penetrated by particles are less prone to extrude their protoplasm than cells under normal osmotic pressure (Armaleo *et al.* 1990; Sanford *et al.* 1992). Presently there are two papers have been published that mention using osmotic treatments on wheat calli. The first experiment was limited to three osmotic treatments of 0.0M, 0.25M, and 0.5M mannitol (Perl *et al.*, 1992). The optimum treatment for this experiment was 0.25M mannitol, however they did not regenerate plants from these cultures. Further optimization of the mannitol concentration is necessary due to the wide

range between 0.25M and 0.5M, and the possibility of the optimum being cultivar specific. Blechl and Anderson (1996) reported using 0.4 M mannitol for their 4 h pre-bombardment and 20 h post-bombardment treatments. Their experiments showed that post-bombardment treatments were only beneficial when pre-bombardment treatments are less than 24 h.

Other cereals have been transformed with the particle gun, but only maize has had osmotic conditioning of the target cells optimized (Vain *et al.*, 1993). Embryonic callus cultures were grown in liquid media [MS salts (Murashige & Scoog 1962), B5 vitamins (Gamborg *et al.*, 1968), 2% sucrose and 1.5 mg/L 2,4-D, at pH 5.7]. The embryonic cells were evenly dispersed on 7 cm filter discs (Whatman #4) forming a thin layer of cells and then placed on a solidified medium. The influence of osmotic treatments on transformation was tested by incorporating various concentrations of mannitol and/or sorbitol in the solidified medium. The plasmid pBARGUS was used to coat particles for bombardment using the particle gun. Each bombardment included a 4 h pre-treatment and a 16 h post-treatment on medium containing one of the osmotic treatments. The osmotic treatments were 0.0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 M (mannitol and sorbitol, 1:1). After assaying for GUS activity it was determined that 0.4 M (0.2 M mannitol and 0.2 M sorbitol) was the optimum treatment resulting in a 2.7-fold improvement of the number of transient foci. There was also a 6.8-fold improvement in the recovery of stably transformed maize plants. Other uses of osmotic treatments include a 7- to 10-fold enhancement of stable transformation of microorganisms (Armaleo *et al.*, 1990; Shark *et al.*, 1991) and nonembryogenic plant cells (Russel *et al.*, 1992). Mannitol may also play a role in plants other than osmoprotection; such as free radical scavenging, or carbon storage. Plants that produce endogenous mannitol are able to utilize it as an additional source of energy (Williamson *et al.*, 1995).

**Transformation of Monocots**

Monocotyledonous plants transformed to date are wheat, oat (*Avena sativa* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), tritordeum (*Hordeum x Triticum)* , creeping bentgrass (*Agrostis palustris* Hudds.), tall fescue (*Festuca arundinacea* Schreb.), and orchardgrass(*Dactylis glomerata* L.). Wheat has been transformed producing fertile plants with stable transgenes since 1992 (Vasil *et al.*, 1992; Weeks *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Blechl and Anderson, 1996). Barley has been transformed using transgenes for *bar, uidA,* and *nptII* (neomycin phosphotransferase II) like most other monocots (Wan and Lemaux, 1994; Ritala *et al.*, 1994). Oat has also been transformed but initially there were problems with a high incidence of male sterility (about 50%). There was also a concern about out crossing with wild oat causing a potential weed problem when using bialaphos as a selection agent (Somers *et al.* 1992). Since then the *nptII* gene has used with paromomycin as the selection agent reducing the ecological risk associated with the *bar* gene (Torbert *et al.*, 1995). The first fertile maize also used *bar* and *uidA* genes for transformation (Spencer *et al.*, 1990; Gordon-Kamm *et al.*, 1990). This transformation system was later improved with the addition of osmotic treatments (Vain *et al.*, 1993). Rice has been transformed by *Agrobacterium*, electroporation, and Biolistics$^{TM}$(Hiei *et al.*, 1994; Zhang *et al.*, 1988; Duan *et al.*, 1996;). Rice was also the first cereal to be transformed by *Agrobacterium* (Chan *et al.*, 1993; Hiei *et al.*, 1994). Tritordeum is a hybrid transformed by microprojectile bombardment of inflorescence tissue (Barcelo *et al.*, 1994). Rye, tall fescue, orchardgrass, and creeping bentgrass have all been transformed by biolistic direct gene transfer using explants ranging from protoplasts to immature embryos (Castillo *et al.*, 1994; Wang *et al.*, 1992; Horn *et al.*, 1988; and Hartman *et al.*, 1994).

## MATERIALS AND METHODS

This research was modeled after the protocol used in the optimization of osmotic treatments in maize (Vain *et al.*, 1993).

**Plant tissue preparation**

Bobwhite wheat plants, chosen for their high regenerative capacity (Fellers *et al.*, 1995), were grown in the greenhouse with 14 hour days and a 22°/18°C day/night temperature. Callus cultures were established on MS media as described by Weeks *et al.* (1993). All of the caryopses were harvested about 10 to 18 days post-anthesis and surface sterilized (Weeks *et al.* 1993). Embryos 1 mm to 2 mm in length were plated onto the callus initiation medium (wheat MS medium with no selection reagent added). The embryo-derived callus was grown for 5 to 10 days and was then transferred to mannitol treatment plates (Vain *et al.*, 1993). Mannitol was added to the wheat MS media at concentrations of 0, 0.2, 0.4, or 0.6 M. Calli were placed in the center of the plate in an area about 2.2 cm diameter (about 10 to 15 embryos per plate) and were allowed to equilibrate for 5 h. This pretreatment was necessary to reduce the intracellular osmotic pressure prior to bombardment. All treatments were bombarded once with gold microprojectiles that had been coated with the pAHC25 plasmid (Christensen and Quail, 1996) using a procedure modified from Weeks *et al.*(1993). The post-treatment was for 16 h on the same plates as pretreatments and bombardments. The osmotic treatment media contains 3.5 g $L^{-1}$ of Phytagel as the gelling agent instead of 2.5 g $L^{-1}$ that is contained in all other media. Treatment medium was of higher density to protect against evacuation of the medium during bombardment. After bombardments, calli were transferred to selection media which consists of normal wheat MS media with 1 mg $L^{-1}$ bialaphos as a selection agent (used in all media except initiation medium). Calli were transferred to fresh selection medium every 2 wk. As somatic embryos become well developed (usually by the fourth transfer) they were transferred to regeneration medium containing 0.5 M dicamba (Hunsinger *et al.* 1987) to encourage shoot development. When shoots were at least 1 cm long they were transferred to rooting medium to encourage root development. The rooting medium was like the initiation medium with half the concentration of all the ingredients and the complete omission of plant growth regulators. When roots became well established the plantlets were transferred to soil and placed in a plastic bag and were slowly acclimated in a controlled environment room. In about two weeks the plants were taken to the greenhouse where they were grown to maturity. These plants were designated $T_0$ plants and their progeny were designated $T_1$ plants.

**Plasmid DNA**

The plasmid of choice for this project was pAHC25 (Christensen and Quail, 1996) maintained in host *Escherichia coli*(strain DH-5α). The plasmid contains the *bar* gene (Thompson *et al.*, 1987) and the *uidA* gene (Jefferson *et al.*, 1987). Both genes are under the control of the maize ubiquitin *Ubi1* promoter (Christensen and Quail, 1996). The *bar* gene sequence comes from *Streptomyces hygroscopicus,* and it encodes phosphinothricin acetyl transferase which breaks down phosphinothricin (the active ingredient of bialaphos and basta) thus conferring resistance to the herbicide bialaphos (Murakami *et al.*, 1986). The *uidA* gene is used as a marker gene because it encodes the enzyme β-glucuronidase which turns tissue blue in the presence of certain substrates.

**Microprojectile bombardment**

The heart of the transformation system is the helium-driven DuPont Biolistic$^{TM}$ Delivery System (Model PDS-1000). To prepare for a bombardment the first step was to coat the gold microprojectiles with plasmid DNA. The same procedure Weeks (1993) detailed was used with the following modifications; all centrifuge steps have been reduced to a ten second pulse on a microfuge, TOMY mixer time is extended to a minimum of 15 minutes (A. L. Shook and A. C. Guenzi. 1994. unpublished results). After the DNA is precipitated onto the gold, the supernatant is removed and 36 μL of absolute ethanol was added to each microfuge tube of gold/DNA mixture. Ten microliters of this mixture was applied to macro carriers and inserted into the particle gun. The plated calli were uncovered and placed in the vacuum chamber which was then brought down to a negative pressure of 25 inches Hg. The pressure chamber was then pressurized to 1100 p.s.i. which caused the rupture disc to explode causing a shock wave to throw the macrocarrier into the stopping screen which then accelerated the DNA coated gold microprojectiles into the cells of the calli. If the projectile arrives in the nucleus the DNA can possibly be incorporated into the genomic DNA. If these transformed cells develop into somatic embryos then it is possible to recover a transgenic plant as previously described.

**Data collection**

At 2 wk intervals calli were transferred to fresh medium and visually rated. A score of '1' designates calli containing somatic embryos, green sectors and relatively vigorous growth, '2' indicates calli with good growth and healthy tissue, and '3' indicates calli with poor or no growth. When transferring calli, all calli rated '3' were discarded. Calli that were rated '1' throughout the entire experiment were considered transformation events even if plants were not recovered. Not all calli produce recoverable plants in the presence of bialophos, and healthy growth of tissue under intense selection pressure was a positive indicator that the osmotic treatment was beneficial. A total of 2,125 embryos were bombarded across all

treatments. Each treatment was replicated 15 times with ca. 35 embryos bombarded per replication. Data were analyzed with the Statistical Analysis System (SAS) using the General Linear Models (GLM) procedure and variance was tested using the Least Squares Means (LSM) method.

**Progeny assays**

Approximately 20 immature embryos from a single wheat head ($T_1$ plants) were germinated on selection media containing 3 mg $L^{-1}$ bialaphos and no synthetic auxin to determine probable transformants. The embryos were excised at 20-25 days post anthesis and were placed scutellum side down to promote germination. The explants were kept in the dark until germination or cessation of growth occurred. After germination plantlets were transferred to a G.A.-7 box with the same media to allow the plantlets to grow to a survivable size. The surviving plantlet was transferred to soil but did not survive to maturity.

## RESULTS AND DISCUSSION

The results demonstrated that the mannitol treatments increased the number of embryogenic calli (Fig. 1). The number of bombarded embryos producing a regenerated plant increased at the 0.4 M and 0.6 M mannitol treatments (Fig. 2). The number of fertile regenerated plants increased at 0.4 M and 0.6 M mannitol treatments (Fig. 3). A prelimenary experiment indicated that 0.8 M mannitol was equivalent to no mannitol.

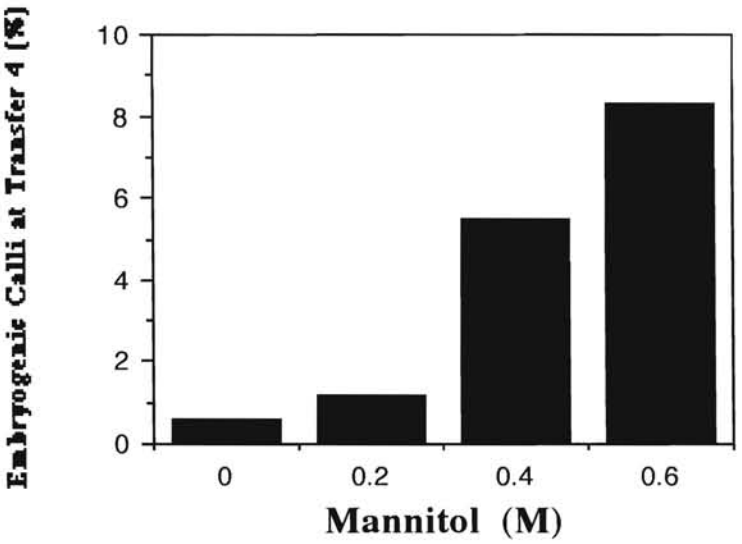


**Figure 1.** The percent of embryogenic calli recovered at the fourth transfer (8 weeks) were; 0.6% with no mannitol, 1.2% with 0.2 M mannitol, 5.5% with 0.4 M mannitol, and 8.3% with 0.6 M mannitol. LSD (0.05) = 3.7

**Table 1.** Single degree of freedom contrasts for data of Fig. 1 and Fig. 2 using LS Means method.

| | Embryogenic Calli at Transfer 4 | Bombarded Embryos w/ Regenerated Plant |
|---|---|---|
| Comparison | P | P |
| 0 vs. 0.2 | 0.6915 | 0.9767 |
| 0 vs. 0.4 | 0.0208 | 0.3096 |
| 0 vs, 0.6 | 0.0018 | 0.0284 |
| 0.2 vs. 0.4 | 0.0501 | 0.2884 |
| 0.2 vs. 0.6 | 0.0051 | 0.0243 |
| 0.4 vs. 0.6 | 0.3502 | 0.2091 |

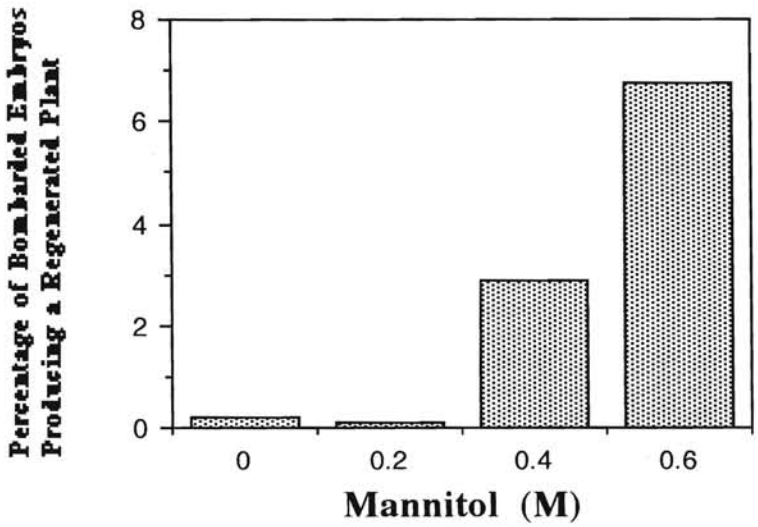


**Figure 2.** The percentage of bombarded explants of calli producing a regenerated plant were; 0.2% with no mannitol, 0.1% with 0.2 M mannitol, 2.9% with 0.4 M mannitol, and 6.7% with 0.6 M mannitol. LSD (0.05) = 5.6

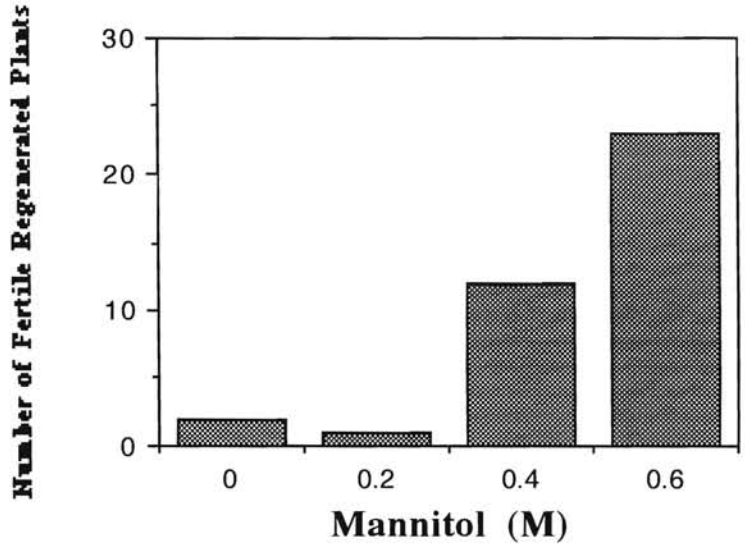


**Figure 3.** The number of fertile regenerated plants were; 2 plants for no mannitol, 1 plant for 0.2 M mannitol, 12 plants for 0.4 M mannitol, and 23 plants for 0.6 M mannitol.

A significant difference ($p < 0.05$)for the 0.6 M mannitol treatment when compared to 0 or 0.2 M mannitol treatments was observed for embryogenic calli at transfer four. The 0.4 M mannitol treatment when compared to 0 or 0.2 M mannitol treatments was significant to the no mannitol treatment only. However, due to the high variability that is intrinsic to Biolistics™, no significant differences could be detected between the 0.4 M and 0.6 M mannitol treatments. The bombarded embryo producing a regenerated plant analysis was significant for the 0.6 M mannitol treatment when compared to 0 or 0.2 M mannitol treatments. The 0.4 M mannitol treatment was not significantly different to any of the treatments when comparing bombarded embryos producing a regenerated plant. By comparing the means of the 0.4 M and 0.6 M treatments, it is evident that 0.6 M treatment produces the greatest number of regenerated plants. Although conclusive evidence proving genetic transformation was not shown, using mannitol treatments as previously described does enhance recovery of plants following bombardment, which is an important aspect of a transformation system.

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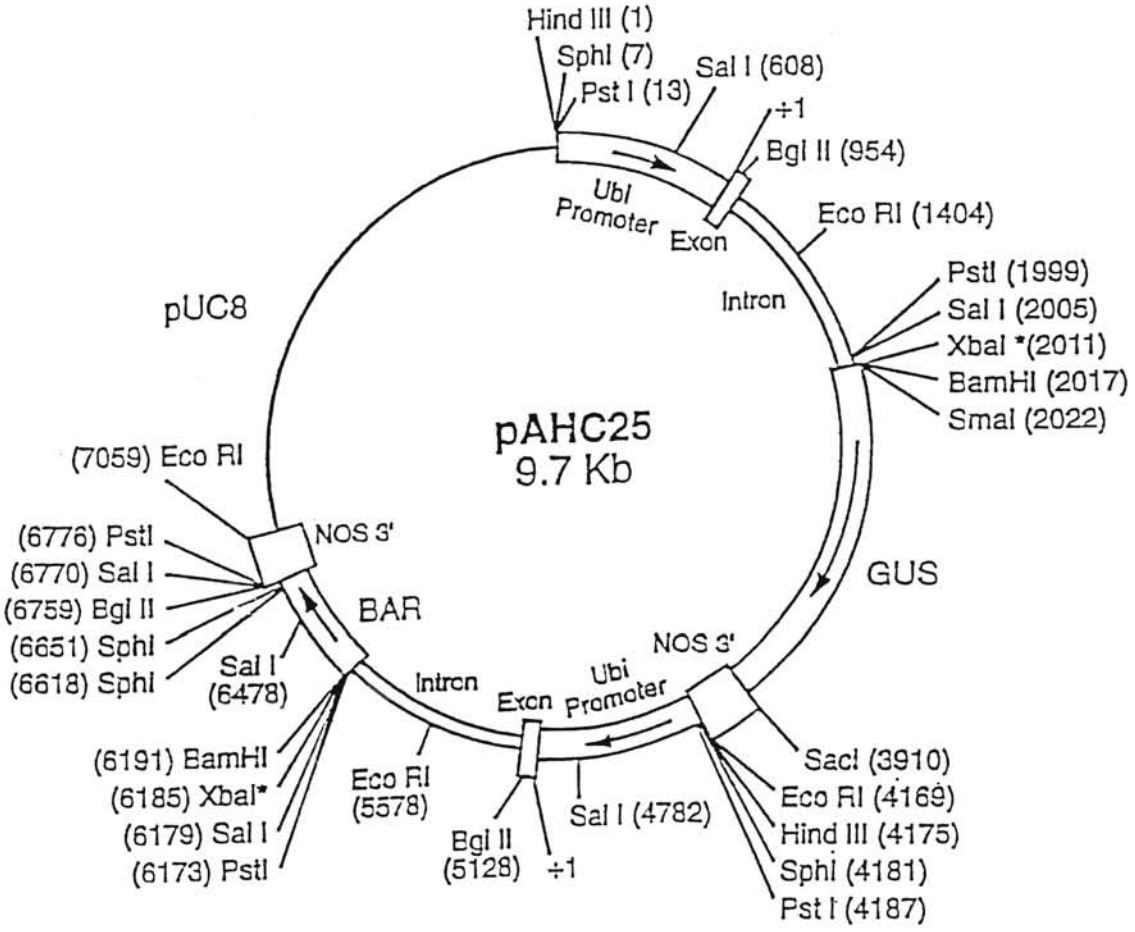
# APPENDIX




This plasmid map for pAHC25 (Christensen and Quail, 1996) illustrates the placement of genes and restriction sites, with pUC8 representing the bacterial DNA nescessary for replication in the host bacteria.

# CHAPTER II

## PLANT REGENERATION FROM WHEAT (*Triticum aestivum* L. cv Bobwhite) CALLUS CULTURES IMPROVED BY INCREASING COPPER CONCENTRATION

# Plant regeneration from wheat (*Triticum aestivum* L. cv Bobwhite) callus cultures improved by increasing copper concentration

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## ABSTRACT

The ability to regenerate shoots determines the usefulness of a tissue culture system. The objective of this experiment was to determine if a highly regenerative wheat cultivar could have an increased shoot and root regeneration capacity. Shoot regeneration from wheat calli was significantly enhanced by increasing the concentration of cupric sulfate. Immature embryos from 'Bobwhite' wheat were cultured on MS medium containing 0, 0.1, 1, 2, 6, 10 μM $CuSO_4$. More calli produced green plantlets on MS medium containing 2 μM $CuSO_4$ and no significant difference was observed between 0 and 0.1 μM $CuSO_4$.There was no significant difference between treatments on root growth due to the high variability intrinsic to tissue culture. The 2 μM $CuSO_4$ mean shoot weight was significantly different than 6 μM and the 10 μM $CuSO_4$ treatments (Tab. 2) The 2 μM level of $CuSO_4$ is the best treatment for shoot regeneration and shoot growth in rooting medium. Approximately 20 plantlets per treatment were transferred to rooting medium containing the same concentration of $CuSO_4$. After 6 wk, plants were removed from the medium and their roots and shoots were weighed separately. Plants grown on medium containing 10 μM $CuSO_4$ achieved greatest root mass. The shoot mass peaked at 2 μM $CuSO_4$ and sharply decreased as concentration was increased. For plant regeneration from Bobwhite callus cultures 2 μM $CuSO_4$ should be utilized.

## INTRODUCTION

An efficient tissue culture system is essential for transformation of plants. Bobwhite wheat has been used because it can produce many regenerable plantlets, but even with Bobwhite many of the potentially transformed calli never become embryogenic (Fellers *et al.*, 1995). In some cultivars, particularly winter wheats, embryogenisis is so low that transformation is nearly impossible. Wheat tissue culture medium is based on tobacco tissue culture medium developed by Murashige and Skoog (1962). Tobacco has been easily transformed numerous times but wheat was only recently transformed (Weeks *et al.* 1993; Vasil *et al.* 1993). Recovery of transformed wheat plants is still below 10% and improvement is necessary to make the process an economical and efficient tool for wheat breeding programs. Increasing $CuSO_4$ has been demonstrated to be beneficial in plant regeneration in wheat (*Triticum aestivum* L. and *Triticum turgidum* L.), triticale (*X Triticosecale* Wittmack), tobacco (*Nicotiana plumbaginifolia* Viv.), melon (*Cucumis melo* L.), and barley (*Hordeum vulgare* L.) (Purnhauser 1991; Purnhauser and Gyulai 1993; Ghaemi *et al.* 1994; Garcia-Sogo *et al.* 1991; Dahleen 1995).

The molecular basis of how copper affects somatic embryogenesis and shoot and root formation has not been established but, there is evidence to suggest that there are at least two possible pathways involved in the process. Copper is a necessary component for electron transfer, polyphenol metabolism, carbohydrate and protein biosynthesis, lignification, and production of peroxidases. There are three different forms of proteins which contain copper (Sandman and Boger, 1983). "Blue proteins" have no oxidase activity and function in a one-electron transfer. Plastocyanin is an example of a "blue protein". "Non-blue" proteins produce peroxidases and oxidize monophenols to diphenols. The "multi-copper proteins" such as laccase and ascorbate oxidase, act as oxidases and contain at least four copper atoms per molecule. The level of peroxidase

activity in callus has been shown to be an indicator of somatic embryogenesis in pumpkin callus lines (Krsnik-Rasol, 1991). When the levels of peroxidase activity in embryogenic and non-embryogenic calli were compared the embryogenic calli had an enzyme activity 20 times that of the non-embryogenic calli. Krsnik-Rasol (1991) also observed that two of the ten culture flasks had an increased level of peroxidase activity and a day later embryoids were noticed. It has been reported that a rise in specific peroxidase activity in carrot cell suspension cultures was also seen a day before globular embryoids appeared.

Lignification is highly regulated by copper because of its role in producing *p*-coumaric acid, one of the precursors of lignin biosynthesis. The multicopper enzymes phenolase and laccase oxidize phenols including *p*-coumaric acid (Marschner, 1995). Structural differences between embryogenic calli and non-embryogenic calli of wheat (Fellers *et al.* 1995) are similar to differences found in stem sections of copper sufficient and deficient sunflower plants (Marschner, 1995). In sunflower stem sections that were copper sufficient the walls of the sclerenchyma were thick and lignified but in stem sections that were copper deficient the walls of the sclerenchyma were thin and nonlignified (Marschner, 1995). Embryogenic wheat calli become predominately nodular and compact retaining their embryogenicity in excess of a year (Redway *et al.* 1990). Nonembryogenic wheat calli are usually pale white, sometimes almost translucent, with a soft loose structure possibly because of nonlignified cell walls but lignin content has not been compared between the two. The objective of this research was to determine what concentration of copper would significantly increase somatic embryogenisis for an already highly embryogenic wheat cultivar such as Bobwhite.

## MATERIALS AND METHODS

Bobwhite wheat plants were grown in the greenhouse with 14 h days at 21-26$^0$ C day and 15-20$^0$ C night temperatures. Caryopses were collected 10-12 days after pollination and were surface sterilized by immersing them in 70% ethanol for 5 m, 20% bleach (5.25% sodium hypochlorite) for 20 m, and two separate rinses in sterile reverse osmosis and deionized water. Immature embryos 1.0-1.5 mm long were excised with sterile forceps and placed on treatment medium embryonic axis down. The treatment media consisted of MS medium, 1.5 mg $L^{-1}$ 2,4-D and 20 g $L^{-1}$ sucrose with six different concentrations of $CuSO_4$ (0, 0.1, 1, 2, 6, and 10 μM). Approximately 10 embryos were placed on each petri dish with 5 petri dishes per treatment medium. A total of 300 embryos were plated and numbered to maintain their identity throughout the study. Subsequent to this, calli were transferred to their corresponding fresh medium every 2 wk and were grown in a controlled environment room at 20-22$^0$ C under cool white florescent lights for 12 h per day for 2 m. Approximately 20 shoots were selected from each treatment and were placed on rooting medium consisting of half-strength MS medium containing the same concentration of $CuSO_4$ as the plants were derived from, and no 2,4-D. The selected shoots were grown for 4 wk on rooting medium and plants were removed for weighing of the roots and shoots. The percent of calli with green shoots was determined at 17, 28, and 41 days after initiation to compare how and when the different concentrations of $CuSO_4$ affects plant regeneration Data were analyzed with the Statistical Analysis System (SAS) using the General Linear Models (GLM) procedure and variance was tested using the Least Squares Means (LSM) method.

## RESULTS and DISCUSSION

### Shoot Regeneration

Most explants were able to initiate callus and regenerate green plants for all the $CuSO_4$ treatments.(Table 1). The response across the three time periods were highly correlated, therefore typical split plot approach to repeated measures analysis was not valid. Each transfer period was analysed separately.

**Table 1.** Shoot regeneration as influenced by $CuSO_4$.

| | Shoot Producing Calli | | |
|---|---|---|---|
| $CuSO_4$ | 17 days | 28 days | 41 days |
| –µM– | | % | |
| 0 | 4 | 21 | 23 |
| 0.1 | 3 | 15 | 28 |
| 1 | 11 | 40 | 51 |
| 2 | 14 | 42 | 58 |
| 6 | 10 | 37 | 51 |
| 10 | 5 | 27 | 49 |

LSM Comparisons see Table 3.

**Shoot and Root weights**

While the first measurements previously mentioned illustrate both the effects of increased $CuSO_4$ over time and how the differences between treatments above 1 μM $CuSO_4$ diminish over time, benefits other than the percentage of embryos capable of producing regenerable cultures needed to be addressed (Table 2).

Table 2. Growth of shoots and roots as influenced by $CuSO_4$ (14 wk after initiation of callus).

| | Fresh Weights | |
|---|---|---|
| $CuSO_4$ | Shoots | Roots |
| –μM– | g | |
| 0 | 0.3759 | 0.1619 |
| 0.1 | 0.3848 | 0.1687 |
| 1 | 0.3953 | 0.1579 |
| 2 | 0.4629 | 0.1945 |
| 6 | 0.3001 | 0.2073 |
| 10 | 0.3245 | 0.2308 |
| LSD(0.05) | 0.1270 | 0.1217 |

**Table 3.** Probabilities from a single degree of freedom comparisons of effects of $CuSO_4$ treatments on shoot regeneration of Bobwhite wheat at 28 and 41 days of tissue culture. Probabilities from the 17 day measurements were not included ($P > F = 0.2116$)

| | Day 28 | Day41 |
|---|---|---|
| Comparison | P | P |
| 0 vs. 0.1 | 0.4284 | 0.7222 |
| 0 vs. 1.0 | 0.1335 | 0.0268 |
| 0 vs. 2.0 | 0.0378 | 0.0004 |
| 0 vs. 6.0 | 0.1335 | 0.0059 |
| 0 vs. 10.0 | 0.7507 | 0.0138 |
| 0.1 vs 1.0 | 0.0194 | 0.0392 |
| 0.1 vs. 2.0 | 0.0027 | 0.0004 |
| 0.1 vs. 6.0 | 0.0194 | 0.0079 |
| 0.1 vs. 10.0 | 0.2167 | 0.0193 |
| 1.0 vs. 2.0 | 0.6169 | 0.1612 |
| 1.0 vs. 6.0 | 1.0000 | 0.5306 |
| 1.0 vs. 10.0 | 0.1747 | 0.9768 |
| 2.0 vs. 6.0 | 0.6169 | 0.4587 |
| 2.0 vs. 10.0 | 0.0438 | 0.1216 |
| 6.0 vs. 10.0 | 0.1747 | 0.5051 |

There was no significant difference between treatments on root growth due to the high variability intrinsic to tissue culture. The 2 μM $CuSO_4$ mean shoot weight was significantly different than 6 μM and the 10 μM $CuSO_4$ treatments (Table 2) The 2 μM level of $CuSO_4$ is the best treatment for shoot regeneration and shoot growth in rooting medium. This treatment consistently out performed the others in plant regeneration over time as indicated in Table 1. The 2 μM $CuSO_4$ treatment was also the only treatment that had a higher root growth than the normal MS medium (0.1 μM $CuSO_4$) and no inhibition of shoot growth. These results indicate that for plant regeneration 2 μM is the best

concentration of $CuSO_4$ in all tissue culture media for Bobwhite. Similar results have been reported for wheat (Purnhauser *et al.*, 1993) and barley (Dahleen, 1995) but inhibition of shoot growth at $CuSO_4$ concentrations greater than 2 μM has not been reported. There may be a possibility of increasing regenerability of recalcitrant winter wheat cultivars by manipulating $CuSO_4$ concentration as Dahleen (1995) reported in barley .

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# VITA

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