CULTURAL TECHNIQUES TO INCREASE SURVIVABILITY AND VIGOR IN *ZEA MAYS L.* DOUBLED HAPLOIDS

by

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TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES viii
ABSTRACT ix
CHAPTER 1: LITERATURE REVIEW 1
1.1 Maize hybrid production 1
1.2 Doubled haploid breeding 5
1.3 Embryo rescue 9
1.4 Nitrogen form and rate 10
1.5 Foliar mist application 12
CHAPTER 2: INTRODUCTION TO THE EXPERIMENTS 14
CHAPTER 3: GENERATING PLANTLETS VIA TISSUE CULTURE 17
3.1 Introduction 17
3.2 Materials and methods 18
3.2.1 Seedling production in the greenhouse 18
3.2.2 Transplanting seedlings to a screenhouse 20
3.2.3 Pollination of female inbred with male inducer pollen 20
3.2.4 Embryo rescue 21
3.2.5 Plantlet generation from the rescued embryos 22
3.2.6 Transplanting of plantlets to the field 25
3.2.7 Pollination 28
3.2.8 Maturation and harvest 29
3.3 Results 29
3.3.1 Fungicide treatment of seed 29
3.3.2 Germination 31
3.3.3 Embryo length 32
3.3.4 Post *in vitro* plantlet development 35
3.3.5 Plantlet growth 36
CHAPTER 4: NITROGEN FORM AND RATE TREATMENTS OF POST *IN VITRO* PLANTLETS 40
4.1 Introduction 40
4.2 Materials and methods 41
4.3 Results 43
4.3.1 Plantlet growth 43
4.3.2 Doubled haploid return 48
4.4 Conclusions 49

CHAPTER 5: MIST TREATMENTS OF POST *IN VITRO* PLANTLETS 50
5.1 Introduction 50
5.2 Materials and methods 51
5.3 Results 53
5.3.1 Plantlet growth 53
5.3.2. Doubled haploid return 56
5.4 Conclusions 57
CHAPTER 6: EMBRYO LENGTH EFFECT ON GERMINATION AND PLOIDY 58
6.1 Introduction 58
6.2 Materials and methods 58
6.3 Results 60
6.4 Conclusions 61
CHAPTER 7: TEMPERATURE EFFECTS ON EMBRYO GERMINATION AND GROWTH 62
7.1 Introduction 62
7.2 Materials and methods 62
7.3 Results 63
7.4 Conclusions 65
CHAPTER 8: CONTROLLING *IN VITRO* MICROBIAL CONTAMINATION 66
8.1 Introduction 66
8.2 Materials and methods 67
8.3 Results 67
8.4 Conclusions 68
CHAPTER 9: OVERALL DISCUSSION 69
CHAPTER 10:OVERALL CONCLUSIONS 75
APPENDIX 76
REFERENCES 80

LIST OF TABLES

Table 1.1 Products within the food, seed and industrial categories for US maize usage..... 2

Table 3.1 Summary of peat pellet analysis. 24

Table 3.2 Weight and volume of peat pellets before and after hydration. 25

Table 3.3 Number of plantlets transplanted to the field for each genotype, and treatment. 27

Table 3.4 Germination percentage of MI seeds in response to Quadris fungicide pretreatment. 30

Table 3.5 Germination percentages of Non Stiff Stalk (NSS), Stiff Stalk (SS) and Male Inducer (MI) seeds at the time of the five weekly plantings. 31

Table 3.6 *In situ* embryo length as a function of week of planting, days after pollination and maize line [Non Stiff Stalk (NSS) and Stiff Stalk (SS)]..... 33

Table 3.7 Accumulated heat units for the weekly planting at days after pollination..... 34

Table 3.8. *In vitro* germination percentage and its angular transformation as affected by inbred maternal line (Non Stiff stalk [NSS] and Stiff Stalk [SS]) and week of planting. 36

Table 3.9 Height, width, and height divided by width of NSS and SS plantlets (germinated embryos) at the end of the 7-day *in vitro* period in response to week of planting. 38

Table 4.1 Concentrations of the fertilizer salts to yield 100, 200 or 400 mg $N \cdot L^{-1}$ solutions of 100% nitrate, 100% ammonium, or 50% nitrate plus 50% ammonium, and the pH and electrical conductivity (EC) of these solutions. 42

Table 4.2 Effects of nitrogen form and rate on the height and width of NSS and SS maize plantlets measured at 2, 4 or 6 days during a 7-day post *in vitro* culture period in peat pellets. 44

Table 4.3 Effects of nitrogen form and rate on the survival of NSS and SS plantlets at the end of the 7-day post *in vitro* culture in peat pellets. 47

Table 4.4 The effect of nitrogen form and rate during 7-day post *in vitro* plantlet growth in peat pellets on the number of NSS or SS plants bearing DH seeds following transplanting to the field. 48

Table 5.1 Effects of water- or micronutrient-mist on the height and width of NSS and SS maize plantlets measured at 2, 4 and 6 days after beginning post *in vitro* culture in the greenhouse..... 55

Table 5.2 Effects of mist (straight water or trace element solution) on the survival of NSS and SS plantlets at the end of the 7-day post *in vitro* culture in peat pellets. 56

Table 5.3 The effect of straight water mist or trace nutrient mist during the 7-day post *in vitro* plantlet growth in peat pellets on the number of NSS or SS plants bearing DH seeds following transplanting to the field..... 57

Table 6.1 Phytatray © grouping for embryo length categories..... 59

LIST OF FIGURES

Figure 1.1 Inbred depression reducing vigor and yield. 4

Figure 1.2 Phenotypic variations between diploids and haploid maize kernels. 7

Figure 2.1 Regenerated plantlet 15

Figure 2.2 Process flow chart 16

Figure 3.1 "Germinated" vs. "non-germinated" embryo classification. 23

Figure 3.2 Average weekly NSS embryo length at day after pollination (DAP) and selected accumulated HU values 35

Figure 5.1 Mist system schematic. 53

Figure 6.1 Germination and diploid escape percentages as a function of embryo length. 61

Figure 7.1 Effect of temperature on percentage germination of NSS/SS hybrid plantlets (roots and shoots ≥ 2.5 cm long). 64

Figure 7.2 Effect of temperature on the number of days needed for plantlets to reach maturity (shoots and roots ≥ 2.5 cm long). 64

Figure 8.1 Percentage of plates with microbial contamination in response to Plant Protective Mix (PPM) included in the medium. 68

ABSTRACT

The embryo rescue technique in combination with doubled haploids can be a valuable tool to hasten inbred creation and thus reduce the generational time. The process for obtaining doubled haploid maize (*Zea mays* L.) lines using embryo rescue techniques, however, does not address embryo survival and doubled haploid return percentages. I subjected these tissue-cultured plantlets ("germinated" embryos) to several treatments (nitrogen form and rate, water vs. trace nutrient mist) in an attempt to improve doubled haploid return.

Solution fertilization via sub-irrigation with 200 mg $N \cdot L^{-1}$ from nitrate nitrogen compared with 100 to 400 mg $N \cdot L^{-1}$ ammonium-N or 1 nitrate-N : 1 ammonium-N, resulted in the greatest plantlet height and width after 6 days of post *in vitro* growth in peat pellets. Nitrate- and ammonium-N, compared to the 1:1 combination, increased plantlet survival 5.2 percentage points. Nitrogen form or rate during the 7-day post *in vitro* period failed to affect the return of plants bearing doubled haploid kernels. The presence of mist (water or trace nutrient solution) failed to increase plantlet growth, plantlet survival or the return of plants bearing doubled haploid kernels.

Ancillary studies showed embryo length was associated with accumulated heat units and that optimal *in vitro* conditions for plantlet production were: selection of

embryos 4.0 to 4.5 mm long, constant 30° C, and the use of an antimicrobial solution (Plant Protective Mix) in the agar medium.

Chapter 1

LITERATURE REVIEW

1.1 Maize hybrid production

In 1908, when George Harrison Shull, then at the Cold Spring Harbor Laboratory, introduced the concept of hybrid corn (*Zea mays* L.), few people realized its potential to increase the world's agricultural productivity. The early innovative research by Dr. G.H. Shull outlined the procedures that later became the standard in corn-breeding programs. Schull's idea of cross breeding inbred lines soon led to the four-way cross and ultimately, by the contributions of Henry A. Wallace, the birthing the Hi-Bred Corn Company in 1926 (Crow and Dove, 1998).

The hybridization of maize improves farmers' productivity and helps ensure a reliable and sustainable food supply. This phenomenon that captured Henry Wallace's attention makes hybrid maize valuable to growers today and supports the multi-billion dollar business of developing, producing and marketing maize hybrids (Duvick, 1977).

The advent of hybrid-directed breeding in concert with the green revolution contributions of fertilizer and pesticide inputs, and the genetic engineering revolution, continue to increase the yields and acreage committed to maize production.

Three hundred four million tons (twelve billion bushels) were harvested in 2008 in the United States with an average yield of 9.6 metric tons hectare $^{-1}$ (153 bushel acre^{-1}) compared with 1 ton hectare^{-1} (39 bushel acre^{-1}) in 1930 (Duvick, 1977).

Just as maize yields have increased over the last century, so too have the uses for this commodity. The U.S. Department of Agriculture divides total US maize usage into three major categories: Feed/Residual (45.9%), Food/Seed/Industrial (35.2%) and Export (18.9%). Presently, there are over 3,500 uses for maize other than food/feed, with ethanol, high fructose corn syrup and starch leading the processed uses (Table 1.1).

Table 1.1 Products within the food, seed and industrial categories for US maize usage.

Category	Percentage of Total U.S. Corn Usage, 2007-08
Ethanol	24.7%
High Fructose Corn Syrup	3.9%
Starch	2.1%
Sweeteners	1.8%
Cereal/Other	1.5%
Alcohol	1.0%
Seed	0.2%

*Table-Nebraska Corn Board 2008

As the demands for maize use and yield continue to grow, the breeder's task of delivering hybrids containing traits for their intended use such as digestibility (silage) and lower lignin content (ethanol), are constantly coupled with native and foreign genetics that enhance yield. Herbicide resistance, insect resistance, disease resistance or drought tolerance are incorporated by either backcrossing or inserting genes into elite lines. Such improvements both reduce pesticide use and promote higher yields.

Hybridization is necessary to eliminate the deleterious effects of inbreeding known as "inbreeding depression" (Figure 1.1). Inbreeding depression is the reduction in yield and vigor if seeds from hybrid progeny are planted the next year. The limited genetic shuffling associated with inbred depression results in smaller plants and lower vigor and ultimately yield. While this depression is not a desirable path for a farmer to follow, it is the very path sought by breeders to recover inbreds from hybrids.

*P_1: Parent 1, P_2: Parent 2, F_1-F_8: Subsequent selfed generations

Photo-D.F. Jones

Figure 1.1 Inbred depression reducing vigor and yield.

Even in the competitive marketplace of hybrid maize research and development, the traditional breeding strategies, the crossing of elite inbred lines from two heterotic groups would be easily recognized by breeders from 50 years ago (Duvick, 2001). An example of this would be the creation of hybrid A/B, where vowels and consonants are two different heterotic groups. The process needed to identify A would consist of making breeding crosses of E/I, I/U etc. and then converting them back into inbreds through years of self-pollination and selection to fix homozygosity. Typically, seven to eight generations of selfing are required to produce a new inbred line (A) with predictable genetics. The new inbred line is then ready for hybridization with other new inbred line(s) (B) derived in the same manner from the

other heterotic group. Given this cycle, breeders today are estimated to be working with only 5% of the total maize germplasm in the world (Blanco, 2008).

While the seven to eight selfing generations needed for recovering homozygous lines in the past translated into eight years of inbred development, (one generation per growing season), the advent of winter nurseries (two cycles per year) removed four years from this process. Still, resources such as labor, land, and input costs remained constant even though the recovery time was reduced. To eliminate both time and cost in inbred recovery, the implementation of doubled haploid breeding has been an accepted form of setting homozygousity and thereby producing inbred lines.

1.2 Doubled haploid breeding

Doubled haploid (DH) breeding enables breeders to produce genetically uniform lines within one generation. This bypasses the lengthy process of self-pollination and selection normally required to produce inbred parents. Through the concerted use of DH, molecular markers and off-season nurseries, maize breeders have unprecedented capabilities to create progeny quickly and precisely (Smith et al., 2008).

Maize has two sets of 10 chromosomes making it a diploid (2n). The rare occurrence of haploid (1n) progeny was less than 0.1% prior to the discovery of inbred line Stock 6 by Ed Coe (1959). He found Stock 6 to induce maternal haploids at 1-2%. Besides the obvious genetic differences between haploids and diploids, the

phenotypes between the two are radically different. Haploids exhibit smaller stature, more pointed leaves, and are generally less vigorous than diploids (Chase, 1964). Another major difference between the two is that haploids are sterile.

Through selective breeding, a number of new inducers with higher haploid-induction rates (~10%) have been created such as WS14 (Lashermes et al., 1988), ZMS (Chalyk, 1994), KMS (Tyrnov and Zavalishina, 1984), MHI (Eder and Chalyk, 2002), and RWS (Rober et al., 2005).

In maternal haploid production, the haploid inducer (male) is crossed to a hybrid (future inbred line; female). The resulting ear may contain kernels that are approximately 10% haploid kernels, 89% diploid and 1% germless.

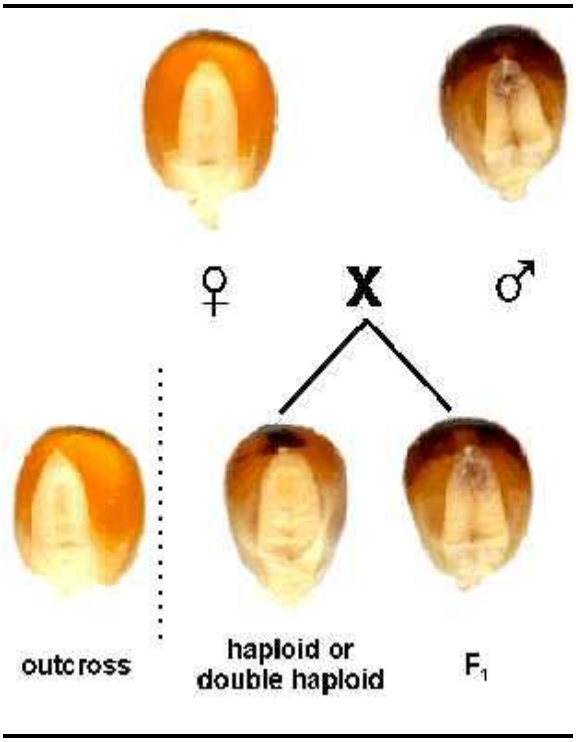


*Photo-University of Hohenheim

Figure 1.2 Phenotypic variations between diploids and haploid maize kernels.

Discerning haploids from diploids is a tedious process of visual selection using the dominant R-*nj* (R-*Navajo)* gene that the inducer carries. With the help of the R-*nj* gene, diploids will exhibit a phenotype of purple endosperm and embryo, while haploids will have purple endosperm, but a colorless embryo. Out-crosses are distinguishable by the absence of pericarp coloration (Figure 1.2). While the R-*nj* gene is helpful, ambiguity exists across female genotypes leading to screening accuracy of about 85% (personal observation).

Deimling et al. (1997) showed that soaking haploid seedlings in 0.06% (w/v) colchicine (a mitotic inhibitor) for 8 hours, digested spindle fibers at metaphase. This inability of the cell to separate doubled chromosomes results in the cell ploidy being "doubled" back to 2n. Consequently, the 1n to 2n chromosomal conversion results in instant homozygosity of the maternal genome.

After colchicine treatment, seedlings are grown in either greenhouse or shade house conditions until they are ready to transplant to the field. At anthesis, DH are selfed to increase seed. Breeders then are able to use these seeds as an inbred parent for hybridization.

While homozygosity has been achieved in one generation using DH rather than seven, DH lines have two unfortunate problems. From personal observation, colchicines firstly reduces survival considerably (63% survival). Secondly, the localized doubling of tassel cells results in only small regions of the tassel being fertile. Such reduced fertility makes self-pollination difficult. So difficult, that seed set on selfed DH usually has a mode of 1 kernel and mean of 21. These two detriments combined usually result in <12% of the initially treated haploids setting seed. This level of return is not economical for the large scale production of a commercial breeding program.

1.3 Embryo rescue

Tissue culture (*in vitro*) embryo rescue techniques in synthetic maize breeding have been used for several years (Chang and Coe Jr., 2009). Embryo rescue is useful when both destructive analysis of endosperm and seed germination are required. Mutants that show limited germination due to defective endosperms can be propagated this way. Embryo rescue can also be used to identify transformed progeny by plating embryos onto medium containing the appropriate selection agent. In 1993, Weymann et al. concluded that although labor intensive, the techniques can find use for the rapid cycling of transgenic maize progeny in segregating populations. On a smaller scale, embryo rescue can be a valuable tool to hasten both the selection and the selfing of plants and thus reduce generation time. In this process, ears are harvested 10-14 days after pollination (DAP) and under sterile conditions embryos are excised and plated on agar-based germination media. The regenerated plantlets then are planted for the next selfing cycle. The time saved by not having to wait for kernels to mature, dry, and then germinate, is about 6 weeks. In a year, embryo rescue can net an additional generation compared to regular cycles that depend on mature seed for subsequent plantings.

The combination of embryo rescue and haploid induced germplasm led to a 2008 patent application by Pioneer Hi-Bred International Incorporated entitled "Doubling of Chromosomes in Haploid Embryos: United States Patent Application

20080216191" (Gordon-Kamm et al., 2008). It was by this process that haploid lines used in this research were induced, isolated, exposed to mitotic inhibition through chemical means, and recovered.

This process does not address the possibilities for improvement in embryo survival and DH line return percentages (plants bearing DH kernels). There is a strong correlation between plant/embryo lethality and mitotic inhibitor concentration (personal observation). Thus the doubling concentrate must be potent enough to achieve 2n cellular division and yet provide for acceptable survival and return. In addition, most *in vitro* tissue culture propagants are tender and require a transitional environment to increase survival in the greenhouse or field.

The objective of my research was to increase the survival and DH return by modifying the post *in vitro* environment in two independent ways. The first study examined nitrogen fertilization form and rate, and the second examined the foliar application of a trace nutrient solution via misting.

1.4 Nitrogen form and rate

The two forms of nitrogen fertilizer available for plant growth are cationic ammonium (NH_4^+) and nitrate (NO_3^-). Given that NH_4^+ and NO_3^- account for 70% of the total cation and anions taken up by plants (Zou et al., 2001), limited supply of nitrogen is second only to drought stress in limiting growth. Although plants can

utilize either form of nitrogen, nitrate is the form more often found because ammonium will quickly oxidize to nitrate (especially in warm, well-aerated soils) due to the microbial process of nitrification.

Pill and Lambeth (1977) demonstrated, with tomato, the limitations of NH_4^+-N. Compared to nitrate, ammonium applications resulted in reduced shoot and root concentrations of Ca, Mg, K, P and NO_3^-. Furthermore, NH_4^+ increased leaf and root resistances to water flux and decreased water use efficiency as compared to plants cultured with NO_3^-. Plant fresh weight and leaf area increased with time under nitrate solutions, but remained constant (no growth) under NH_4^+ nutrition.

Hydroponic studies have shown that mixed N form can optimize growth and yield (Smiciklas and Below, 1992) Shortenmeyer (1993) confirmed these observations with a split root seedling experiment with maize; with a 1 : 1 nitrate and ammonium mixture giving greater DM than either form alone.

The efficacy of either N form or N rate on DH embryo rescue maize tissue-culture plantlets as they transition from the *in vitro* environment to the field has not been studied. This study seeks to determine the effects on survival of maize DH seedlings post *in vitro* when liquid fertilizer 100% NH_4-N, 100% NO_3-N, or a 50% NH_4-N /50% NO_3-N is applied at 100, 200, and 400 mg $N \cdot L^{-1}$.

1.5 Foliar mist application

From personal observation, embryo-rescued DH plantlets have limited root mass, and by extension, limited ability to absorb water. During the transition from the 100% relative humidity (RH) environment of the test tube to the lower RH of the greenhouse, desiccation can occur within the first 48 hours (personal observation). Through the use of a mist system, the aerial RH can be maintained higher. In commercial plant propagation, during the rooting of cuttings, creating a 100% aerial RH micro-environment prevents tissue and ultimately plant desiccation. This is achieved with the higher aerial RH, lowering the vapor pressure deficits (VPD) between leaf and air, thereby decreasing transpirational loss. It is my hypothesis that plant water stress during the greenhouse phase can be reduced using mist, thereby increasing DH survival.

Leach and Hameleers (2001) found that a critical time for maize seedlings is from germination to the 5-leaf stage where small root-soil interface can restrict the uptake of P and Zn. Foliar applications of P and Zn at 36 days after sowing increased shoot dry matter and yield. The optimal rate of zinc foliar spray for achieving significant grain yield response was between 0.0054 to 0.0082 mg $Zn \cdot L^{-1}$. Grain yield increase was 18% (mean of three years) as compared to the treatment fertilized only with NPK. Plants fertilized with 0.0054 mg $Zn \cdot L^{-1}$ increased both total N uptake and grain yield.

Furthermore, because of the limited root-soil interface at the time of transition from *in vitro* to *in situ*, I further hypothesize that micro-nutrients would be absorbed to a greater extent by foliar application rather than by root uptake.

Given the probability of increasing survival and vigor of DH during the transition between the *in vitro* and *in situ* phases of ontogeny, an objective of my research was to determine the effects of increasing aerial humidity with overhead mist containing water or a micronutrient solution on plantlet survival and growth.

Chapter 2

INTRODUCTION TO THE EXPERIMENTS

Given the novelty of the technology and the lengthy process by which I generated DH for the nitrogen and mist experiments (Figure 2.1), an introduction to the experiments would be beneficial. Chapter 3 outlines the generation of the haploid embryos for experimentation which was a substantial part of my work. It outlines the pre-treatment processes; seeding, transplanting to the screenhouse, induction pollinations, and embryo rescue and regeneration. Chapter 3 also contains results on embryo "germination", tracking embryo size and regeneration survival. I included these statistics because I go into greater detail in Chapters 6-8 regarding the improvements of this upstream process to procure embryos for future experimentation.

After the embryo regeneration (plantlet) stage (Figure 2.1), the nitrogen form/rate and mist treatments were applied. The treated plantlets then followed the same post-treatment procedures also outlined in Chapter 3; transplanting to the field, pollination, maturation and finally harvest.

Figure 2.1 Regenerated plantlet

Chapters 6-8 explore embryo size, temperature, and controlling contamination, all procedural components that would help to establish a more successful protocol base from which to explore further cultural enhancements such as nitrogen fertilization and mist treatment.

For each experiment, I provide the following: introduction, materials and methods, results and conclusions. In Chapter 9, I provide an overall discussion on all the experiments and a subjective evaluation on the entire process.

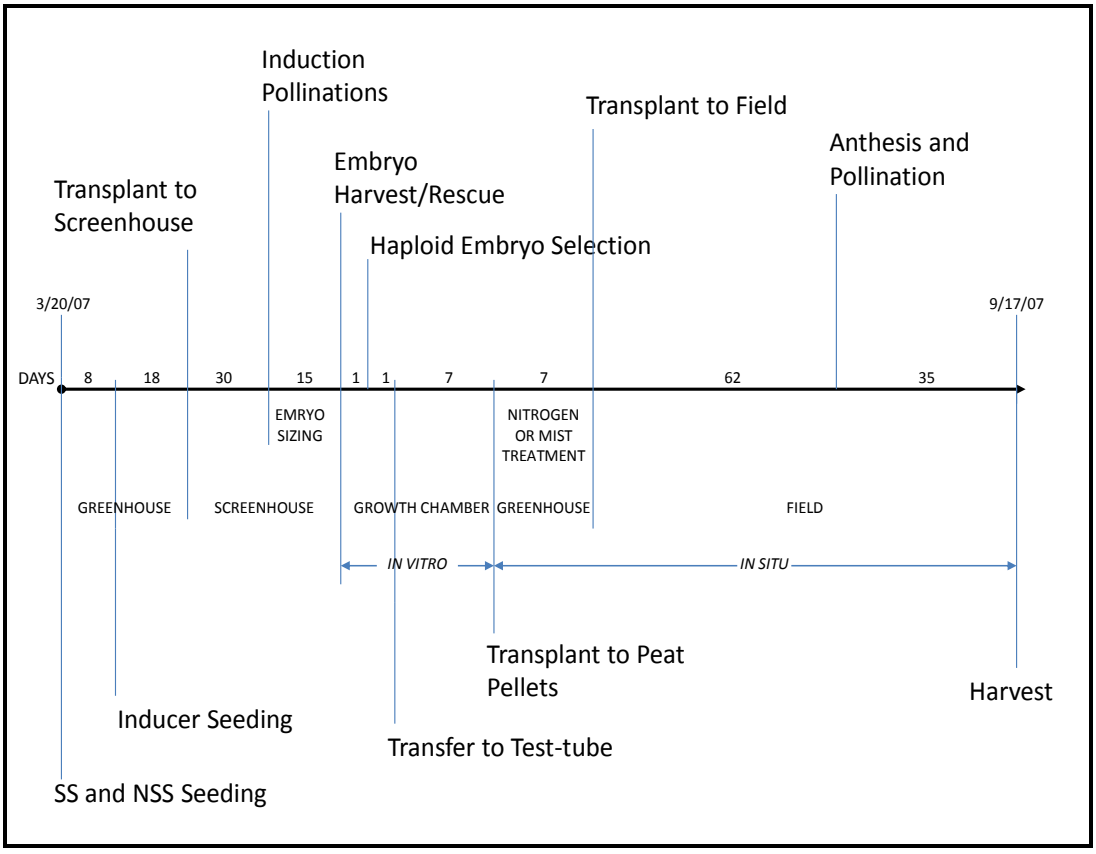


Figure 2.2 Process flow chart

Chapter 3

GENERATING PLANTLETS VIA TISSUE CULTURE

3.1 Introduction

This chapter outlines the overall process and conditions to create haploid embryos, their chromosomal doubling, their regeneration into plantlets, and the transplanting of the DH plantlets to the field.

Two elite Pioneer inbred lines were selected for maternal haploid induction, a Stiff Stalk (SS) and a Non Stiff Stalk (NSS). Though breeding crosses are the preferred target of induction and would give a more accurate picture of the direct application of the cultural improvements to the current process of embryo rescued DH, inbred lines were selected in an effort to minimize genetic variance, thereby providing a less varied response to cultural treatments imposed on the plantlets (Chapters 4 and 5).

Seeds of the two inbred female lines and a male inducer (MI) line were sown weekly for five weeks in plug trays in a greenhouse. Three weeks after planting, seedlings were transplanted into larger pots and transferred to a screenhouse. The female lines were hand-pollinated with MI pollen bulk, and resultant ears were harvested 12-14 DAP for embryo rescue.

After rescuing the embryos under sterile conditions, putative haploid embryos (PHE) were identified and transferred to germination media and allowed to "germinate" in a growth chamber. After 7 days, plantlets were transferred to peat pellets in the greenhouse where they subjected to either forms and rates of nitrogen fertilization via sub-irrigation (Chapter 4) or foliar mist with micronutrients (Chapter 5) for seven days before being transplanted to the field. At anthesis, plants were hand-pollinated and kernels were harvested 35 days later.

DH lines derived from the process were then grown in the greenhouse to confirm homozygosity through visual uniformity.

3.2 Materials and methods

3.2.1 Seedling production in the greenhouse

Starting on 20/3/2007, 75 kernels of the inbred lines (SS and NSS) treated with Maxim XL (fludioxonil and mefenoxameach, Syngenta, Greensboro, NC) were sown (1kernel per cell), in 50-cell trays (5.0 x 5.0 cm x 3cm deep, 75cm^3; 50 Prop-Tray T.O. Plastics, Clearwater, MN). The trays were filled with Metromix 360 (Sun-Gro, Bellevue, WA) and watered with 1% (v/v) Aquagro (Aquacontrols, Paulsboro, NJ) wetting agent, no fertilizer being applied. Plants were kept in a greenhouse (39°39'45.22 N, 75°47'10.11W, 113' elevation) in Newark DE at 30°C/21°C (D/N) for 3 weeks. The greenhouse was equipped with supplemental lighting [3:1 mix of Metal Halide and High Pressure Sodium providing an average photosynthetically

active radiation (PAR) of 2.1 µmoles m^{-2} s^{-1} (not including background solar] during 16 hour photoperiods. This planting process was repeated every week for five additional weeks.

The MI kernels were treated with the label rate of Maxim XL fungicide and were rolled in germination paper (SD3815L, Anchor Paper Co, St. Paul, MN) moistened with 0.05% (v/v) Quadris fungicide (Syngenta, Greensboro, NC) then placed in a plastic bag (one end open) within a growth chamber for 3 days at constant 80% RH and 27° C.

These seeds were planted on weeks 2-6 so that their tassel anthesis would complement the maturity of the inbred females' ears. Both female inbred lines, SS and NSS, have a documented Silking Heat Unit (SLKHU) maturity of 1400, while the MI has a documented Shedding Heat Unit (SHDHU) of 1200. Average daily heat unit (HU) accumulation in the greenhouse was 25. Daily HU was calculated using the following equation from the 2007 Purdue Corn and Soybean Field Guide (50°F base temperature = 10°C):

Daily HU = {{(24hr High[if >85F,85][if <50,50]) + (24hr Low[if <50,50])}/ 2}-50

Irrigation was with Newark city water with a pH of 6.5-7.0 and electrical conductivity (EC) of < 0.5 dS m^{-1} (Myron L AG6/pH Agri-Meter Model AG-6, Myron L Co, Carlsbad, CA).

3.2.2 Transplanting seedlings to a screenhouse

After three weeks, when seedlings reached the V2 stage (collar of the 2nd leaf is visible; McWilliams et al., 1999) the NSS, SS and MI seedlings were transplanted (2 per pot) into 36-cm diameter pots containing Metromix 360. The media in each pot was top-dressed with 50 g of Osmocote 3-4 month slow-release fertilizer (15 N-9 P_2O_5-12 K_2O Plus Minors, Scott's Co, Marysville, OH), 14 g of Marathon 1% granular pesticide (Olympic, Mainland, PA) and 4 g of Orthene TTO insecticide (Valent, Walnut Creek, CA).

The pots were then transferred outside to a hoop-house covered with 30% black shade fabric (Thor Tarp, Oconomowoc, WI). A gravel floor was covered with black weed fabric (Thor Tarp, Oconomowoc, WI) which had been sprayed with a label rate of Roundup Ultra Max herbicide (Monsanto, St. Louis, MO) one week prior to plant occupation for weed control. The pots were arranged to simulate a 76 cm (30 inch) between-row and 15 to 20 cm (6 to 8 inch) in-row spacing. Irrigation with Newark water was delivered via weighted microtubing for 45 minutes at 0600 and 1800 h every other day.

3.2.3 Pollination of female inbred with male inducer pollen

Just before anthesis, ears of NSS and SS were covered with Lawson 250 shoot bags (Seedburo, Chicago, IL) and the plants were detassled by pruning to prevent self-pollination. Upon silk emergence, silks were cut back to 1 cm above the

ear tip and recovered with shoot bags. About 20-24 h later, pollen was bulk collected from the dehiscing MI plants and within 10 minutes poured over the new "brush" of silks on the SS and NSS plants. Ears were then covered with the Lawson 402 pollination bags (Seedburo, Chicago, IL). All SS and NSS inductions for each week's series were pollinated with MI pollen within 1400-1450 HU maturity. An average of 50 ears of each female line were induced over 2 days each week, with inductions of the NSS the first day and SS the second. The yields from these crosses are 10% haploid, 89% diploid, and 1% germless (personal observation).

A sampling of embryos during maturation of the induced ears was conducted daily beginning 10 DAP. Embryos were considered mature enough for rescue upon reaching 2.0 mm length. When 75% of the sampled embryos reached this size, ears pollinated from that date were harvested.

Ears were harvested and their husks and silks were removed on the morning of the embryo rescue process. Ears were transported from Newark, DE to New Holland, PA in a cooler with blue ice packs. Upon arrival, the ears were stored in a refrigerator at 2°C until embryo rescue was performed.

3.2.4 Embryo rescue

Due to the sensitive nature of the embryo rescue process and the trade secrets involved, the embryo rescue protocol is limited to the publically available

patent application submitted by Pioneer Hi-Bred International Inc. (Gordon-Kamm et al., 2008).

3.2.5 Plantlet generation from the rescued embryos

Embryos excised from the kernels were placed in doubling medium containing a mitotic inhibitor. After PHEs were selected (based on coloration) from the doubling medium, they were transferred under sterile conditions to 272M Maize germination medium (DuPont Experimental Station, Wilmington DE) prepared no earlier than 1 week prior to transfer. Embryos were transferred (one per tube) using forceps to the surface of 12 ml of medium contained within 25mm X 150mm clear glass test tubes (VWR 47729-586, VWR Int'l, West Chester, PA) which then were covered with corresponding caps (VWR 73660-25) and sealed with Gas Permeable Fiber Tape (Scotch - 199708 – Carolina Biological, Burlington, NC). Tubes were grouped in sets of 24 in autoclavable Test Tube Racks (Bel-Art - 60915-422 – VWR) and placed in a growth chamber with 24 hr lighting (0.52 µmole m^{-2} s^{-1} PAR from Phillips cool white fluorescent tubes), constant 28°C and 40% R.H. After 7 days in the chamber, plantlets were transferred to peat pellets. A plantlet is an embryo that "germinated" to develop both a root and shoot system. Embryos were classed as "non-germinated" if severe callusing limited root or shoot initiation (Fig. 3.1). Root and shoot lengths for NSS and SS DH germinated embryos (plantlets) were measured with vernier calipers.

Figure 3.1 "Germinated" vs. "non-germinated" embryo classification.

Peat Pellets (42 mm diameter x 327 mm tall, cylindrical horticultural pellet 327420, Jiffy Products of America, Lorain, OH) were soaked in water 1 hour before use, with each pellet absorbing about 175 ml. Table 3.1 shows the analysis (University of Delaware Soil Testing Lab) of the peat pellets.

Table 3.1 Summary of peat pellet analysis.

TEST	MEAN	TEST	MEAN
pH	5.5	TN(%)	1.0
Buffer pH	7.7	TC(%)	48.3
OM (%) by LOI	88.9	M3-S (mg/kg)	291.8
EC(dS·m-1)	0.6	M3-Al (mg/kg)	432.3
M3-P(mg/kg)	111.2	NH_4-N (mg/kg)	583.2
M3-K(mg/kg)	347.6	NO_3-N (mg/kg)	247.6
M3-Ca(mg/kg)	9499	Exch Ca (meq/100g)	17.9
M3-Mg(mg/kg)	5073	Exch K (meq/100g)	0.5
M3-Mn(mg/kg)	33.5	Exch Mg (meq/100g)	16.1
M3-Zn(mg/kg)	8.5	Exch Na (meq/100g)	0.6
M3-Cu(mg/kg)	9.8	CEC @ pH7 (meq/100g)	85.4
M3-Fe(mg/kg)	291.6	CEC @ pH7 (meq/L)	115.4
M3-B(mg/kg)	11.0		

The peat pellets also were evaluated (n=10) for uniformity in size and hydration. Dry volume prior to hydration was 39.3 cm^3 (Table 3.2). While the standard deviation in dry weight was 0.73, their standard deviations for hydrated weight and volume were 3.33 and 7.69, respectively. I believe this level of variation following hydration to be due to the variation in composition of the peat within each pellet and thought to be normal and of no consequence.

Roots of the plantlets were rinsed twice in distilled filtered water to remove any excess media that might accelerate pathogen growth. Each plantlet was inserted into a lengthwise cut in the peat pellet in an attempt to minimize damage to

roots. Plantlets were planted so that the embryo was approximately 2.5 cm below the surface of the peat pellet. The pellets were grouped in sets of 36 and contained in 30.5 cm square by 10.2 cm high Monarch trays (Zipset-4 Propagation Tray, Monarch Manufacturing, Salida, CO). The plantlets were now ready for cultural treatments (Chapters 4 and 5).

Table 3.2 Weight and volume of peat pellets before and after hydration.

Sample number	Dry weight (g)	Dry volume (cm^3)	Wet weight (g)	Wet volume (cm^3)
1	26.29	39.28	187.91	192.49
2	24.16	39.28	180.63	172.86
3	24.45	39.28	188.9	176.89
4	24.25	39.28	186.04	176.89
5	24.06	39.28	179.35	186.61
6	25.72	39.28	188.58	176.79
7	25.11	39.28	182.6	180.71
8	25.07	39.28	185.91	196.43
9	24.64	39.28	182.84	180.71
10	25.3	39.28	184.37	176.79
Mean	**24.91**	**39.28**	**184.73**	**181.72**
SD	**0.73**	**0.00**	**3.32**	**7.69**
CV	**2.93**	**0.00**	**1.79**	**4.23**

3.2.6 Transplanting of plantlets to the field

After cultural treatments (Chapters 4 and 5), plantlets (V2 to V3) in peat pellets were transferred from the greenhouse in New Holland to the field

(40°03’41.07N, 76° 04’10.39”W, elevation 160 m) and transplanted in a randomized block design to the field. Tests of the soil, a Duffield silt loam, revealed a consistent soil texture and nutrient composition from a composite mixture of samples from each of the four 46 m x 46 m (150’x 150’) quadrants (Appendix). The field was tilled and disced 1 week before transplanting, and then was “basket rolled” on the morning of transplanting. The plantlets in peat pellets were planted at 76 cm (30 inch) between-row and 20 cm (8 inch) in-row spacing. There were four weekly field plantings (replication over time) of plantlets that received N form/rate (Chapter 4), and three weekly plantings (replication over time) of plantlets that received mist treatments (Chapter 5). The number of plantlets in each replication varied depending on the greenhouse survival (Table 3.3).

A modified tobacco transplanter (Mechanical Transplanter Co., Holland MI) was used to transplant the peat pellet-plantlet units. The depth of the transplant was deep enough such that the top of the peat pellet was flush with the soil surface. Press wheels backfilled and closed the furrow. Onboard water tanks delivered approximately 0.5 L of water to each transplant. Drip tape irrigation was applied immediately after transplanting and as needed during maturation. Inbred borders were planted around the experimental block to avoid border effects on the experimental plants.

The field was monitored weekly for weeds and pests. Weeds were hoed by hand and there were insufficient disease and pest populations to necessitate

pesticide application. Plants were side-dressed with urea ammonium nitrate (28-0-0) at 224 kg N·/ha^{-1} (200 lbs·acre^{-1}) at the V4-V5 stage (collar of leaf 4 or 5 is visible; McWilliams et al., 1999). There were no signs of nutrient deficiency or toxicity in maturing plants. Random tissue samples analyzed at the University of Delaware also confirmed the absence of nutrient deficiencies.

Table 3.3 Number of plantlets transplanted to the field for each genotype, and treatment.

	Nitrogen form and rate		**Surviving Plants per Week**			
Genotype	**NO3:NH4[a] (%)**	**Rate (mg N·L^{-1})**	**Wk 1**	**Wk 2**	**Wk 3**	**Wk 4**
NSS	50 : 50	100	7	8	5	3
SS	50 :50	100	3	2	0	2
NSS	50 :50	200	6	8	1	4
SS	50 : 50	200	2	2	3	4
NSS	50 : 50	400	4	4	8	3
SS	50 : 50	400	2	3	1	4
NSS	100 : 0	100	7	8	6	5
SS	100 : 0	100	2	2	8	3
NSS	100 : 0	200	5	8	10	12
SS	100 : 0	200	7	2	0	2
NSS	100 : 0	400	7	7	10	12
SS	100 : 0	400	5	5	3	2
NSS	0 : 100	100	10	8	9	9
SS	0 : 100	100	2	2	2	4
NSS	0 : 100	200	9	7	10	12
SS	0 : 100	200	3	5	2	4
NSS	0 : 100	400	4	11	9	12
SS	0 : 100	400	6	1	2	4
NSS	n/a	Control	0	3	7	11
SS	n/a	Control	7	7	4	2

[a] Fertilizer analyses: 50 : 50 = 10-30-20, 100 : 0 = 13-0-44, 0 : 100 = 21-7-7.

Table 3.3 continued

Mist, water vs trace nutrient				
		Surviving Plants per Week		
Genotype	**Mist**	**Wk 1**	**Wk 2**	**Wk 3**
NSS	Water mist	15	19	17
SS	Water mist	11	9	14
NSS	Trace nutrient mist	18	18	16
SS	Trace nutrient mist	12	15	12
NSS	No mist	23	17	17
SS	No mist	11	11	9

3.2.7 Pollination

Just before anthesis, plants were shoot-bagged to ensure emerging silks were not accessible to foreign pollen and self-pollinated. The silks were cut to 1 cm above the cob tip and tassel-bagged pollen was poured over the silks 24 h later. At this time, a phenotypic assessment of whether plants were DH or diploid escapes was recorded. DH plants were significantly smaller and with more upright leaves than diploid escapes. Diploid escapes resembled typical hybrids with larger internodes. An additional help in classifying the DH from the diploid escapes was the presence of anthocyanin coloration on the glumes, an indication of the male-inducer genetic contribution, thereby confirming the plantlet was a diploid escape and not a maternal haploid. Bags were dated and color coded to relay information about glume color. Red striped bags were used to indicate glumes with purple anthocyanin coloration,

while those with no glume coloration received plain pollination bags to denote a DH. Pollinated plants were allowed to mature 35 days before harvesting.

3.2.8 Maturation and harvest

At time of harvest (R6), the number of plants bearing DH kernels was recorded. DH plants normally have a lower seed set than that of diploid escape plants. Also recorded was the presence or absence of purple anthocyanin coloration in the seed. Since both inbreds, NSS and SS, were devoid of the MI anthocyanin marker, the presence of anthocyanin in the seed confirmed a male contribution and therefore was evidence of a diploid escape that had been misclassified as a haploid at the embryonic stage. Seeds were dried at 35°C for 5 days. Seeds from DH then were grown in the greenhouse and plants allowed to mature to confirm uniformity and homozygosity within their ears.

3.3 Results

3.3.1 Fungicide treatment of seed

Prior to sowing, all seeds received a labeled rate of MaximXL fungicide seed treatment. However, due to the discovery of *Aspergillus flavus* and *Penicillium oxalicum* (identified by DuPont pathologist Adriana Tomas) on the MI seed prior to harvest, an additional pre-treatment of Quadris (Syngenta, Greensboro, NC) was thought to be necessary to protect the MI inducer during germination.

The pretreatment consisted of soaking germination paper (SD3815L, Anchor Paper Co, St. Paul, MN), in a 0.05% (v/v) solution of Quadris. Four replications, 100 kernels per germination sheet, were sown, rolled and secured with a rubber band at the top and bottom. Likewise, four replicates of MI seed without the Quadris pretreatment (only Maxim XL) were rolled in germination paper soaked with autoclaved water. Germination rolls were then placed in a plastic bag (one end open) within a growth chamber for 3 days in darkness at constant 80% RH and 27° C. After 3 days, rolls were removed from the growth chamber and germinated seeds were counted. It was observed that the Quadris pretreatment increased MI germination from 71.7% to 95.3% (Table 3.4).

Table 3.4 Germination percentage of MI seeds in response to Quadris fungicide pretreatment.

	Germination (%)	
Treatment	Mean	S.D.
MI seed with Quadris	95.3	0.6
MI seed without Quadris	71.7	3.2
Difference	23.7	

3.3.2 Germination

MI seed sown for the experiment received both Maxim XL and Quadris treatment, while the NSS and SS seed, (not previously afflicted with ear mold(s) at harvest) only received the Maxim XL fungicide and was directly sown. Each week (1-5), 100 kernels of NSS and SS were planted, while 75 kernels (only a pollen donor) of MI seed were planted each week (1-6). Inbreds NSS and SS were of high quality with 99% and 100% germination, respectively, while MI seed had 94% germination (Table 3.5). The fungicide treatments may have aided in deterring possible pathogen (*Aspergillus*, *Fusarium*, *Penicillium*, *Pythium*) attack on the kernels.

Table 3.5 Germination percentages of Non Stiff Stalk (NSS), Stiff Stalk (SS) and Male Inducer (MI) seeds at the time of the five weekly plantings.

		Germination (%)		
Week of planting	Planting date	NSS[x]	SS[x]	MI [y]
1	3/20/2007	100	100	95
2	3/27/2007	100	100	91
3	4/3/2007	98	100	91
4	4/10/2007	100	100	94
5	4/17/2007	97	100	95
6	4/24/2007	*	*	95
	MEAN	99	100	94

[x]NSS and SS were treated with only MaximXL fungicide.
[y] MI was treated with MaximXL and Quadris fungicides.
*Only MI was planted on week 6 to make the nick with NSS and SS from week 5.

3.3.3 Embryo length

Due to the large number of embryos being harvested, a sampling system was established to monitor embryo growth as a function of DAP to anticipate the number of days from pollination that would yield embryos suitable for rescue. For each of the 5 weeks of pollinated plants, 10 randomly selected embryos were sampled from 10 random ears (1 embryo/ear, randomly selected from the middle of the ear). This sampling was done daily from 10 DAP until 15 DAP.

The DAP and week after planting interaction and line affected embryo length (Table 3.6). Embryo length increased from about 0.6 mm at 11 DAP to >2.4 mm by 15 DAP. Within any given DAP, embryo length underwent little or no increase in weeks 1-4; but week 5 was significantly larger at each DAP interval.

Accumulated HUs for the five weekly plantings were affected by the interaction of week of planting with DAP (Table 3.7). Particularly with 12 to 15 DAP, accumulated HUs were markedly higher with the fifth week of planting than with the previous four weekly plantings which, although sometimes different from each other, had a fairly small range of values. Thus, embryo length appears to correlate with accumulated HU values.

Table 3.6 ***In situ*** **embryo length as a function of week of planting, days after pollination and maize line [Non Stiff Stalk (NSS) and Stiff Stalk (SS)].**

	Embryo length (mm) Days after pollination				
Week of planting	11	12	13	14	15
1	0.54 n[z]	1.01 l	1.26 ij	1.78 ef	2.38 d
2	0.55 n	1.03 kl	1.31 g	1.80 e	2.38 d
3	0.56 n	1.04 k	1.28 hi	1.80 e	2.53 b
4	0.56 n	1.01 l	1.24 j	1.76 f	2.51 b
5	0.59 m	1.30 gh	1.80 e	2.43 c	3.00 a
	$LSD_{0.05}$ =0.02				

Significances[y]			
Week (WK)	***		
Days after pollination (DAP)	***	Line	Embryo length (mm)
WK x DAP	***	NSS	1.48
Line (L)	*	SS	1.51
WK x L	NS		
DAP x L	NS		
WK x DAP xL	NS		

[Z] Means followed by the same letter cannot be considered different according $LSD_{0.05}$.

[y] NS,*,*** Not significant or significant at $P \leq 0.05$ or $P \leq 0.001$, respectively.

Table 3.7 Accumulated heat units for the weekly planting at days after pollination

Week of planting	Accumulated heat units Days after pollination 11	12	13	14	15
1	249 r[z]	274m	293 j	303 h	319 e
2	220 v	244 s	271 n	297 i	319 e
3	221 u	239 t	265 o	293 j	322 d
4	253 q	271 n	284 k	305 g	327 c
5	254 p	280 l	308 f	338 b	367 a
	$LSD_{0.05} = 0.47$				

Significances[y]	
Week (WK)	***
Days after pollination (DAP)	***
WK x DAP	***

[Z] Means followed by the same letter cannot be considered different according to $LSD_{0.05}$.

[Y] ***Significant at $P \leq 0.001$.

In Figure 3.2, the significant difference in embryo length between week 5 and weeks 1-4 can be observed. At a given embryo length (e.g 1.75 mm) the DAP varies between 13 for week 5 and 14 for weeks 1-4, however the accumulated HUs are similar, 307.5 and 304.5, respectively. Accumulated HU would be a better determinate of embryo length than DAP. At 14 DAP, for instance, embryo length increased from 1.75 mm with 304.5 accumulated HU to 2.4 mm with 338 accumulated HU.

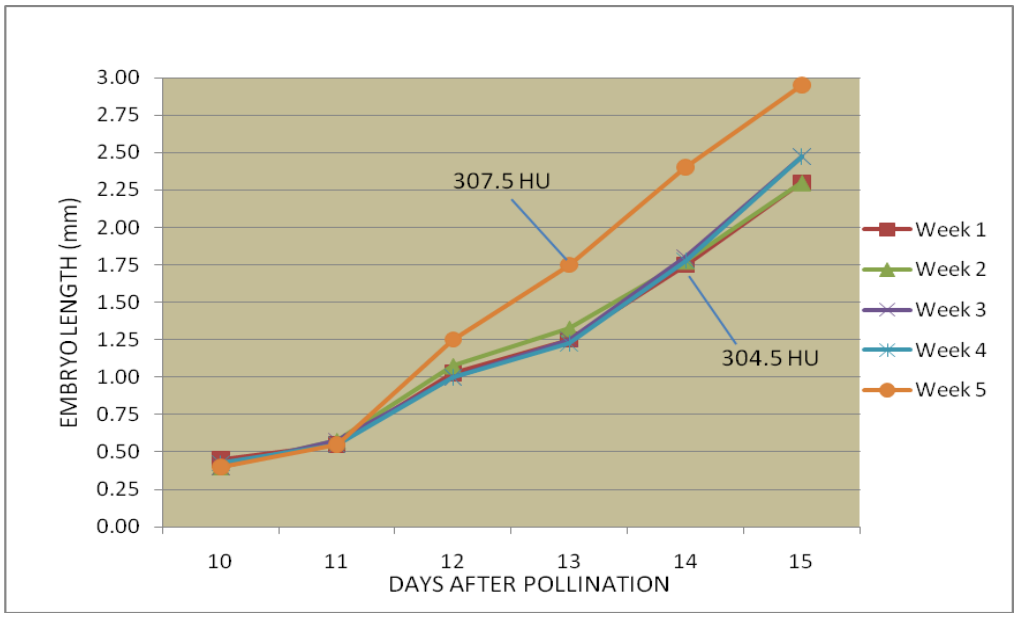


Figure 3.2 Average weekly NSS embryo length at day after pollination (DAP) and selected accumulated HU values.

3.3.4 Post *in vitro* plantlet development

At the end of the 7-day tissue culture period, the embryos were evaluated as to whether "germinated". The presence of both a root and shoot at 7 DAT (days after transfer) to *in vitro* culture constituted a germinated embryo, while the absence of one or both developmental organs was termed a non-germinated embryo.

Germination percentage was affected by line, but not by week of planting or its interaction with line (Table 3.8). The SS inbred line had 83.1% germination while the NSS line had 68.9% germination.

Table 3.8. *In vitro* germination percentage and its angular transformation as affected by inbred maternal line (Non Stiff stalk [NSS] and Stiff Stalk [SS]) and week of planting.

	Germination			Germination	
	(%)	(degrees)		(%)	(degrees)
Week of planting			Maize line		
1	75.0	60.2 a[z]	NSS	68.9	56.2 b
2	81.8	64.8 a	SS	83.1	65.9 a
3	77.5	62.1 a			
4	76.8	62.0 a			
5	68.8	56.2 a			
Significances[y]					
Week of planting (WK)	NS				
Line	**				
WK x L	NS				

[Z] Means followed by the same letter cannot be considered different according to $LSD_{0.05}$.

[Y NS,**] Not significant or significant at $P \leq 0.01$, respectively.

3.3.5 Plantlet growth

After transfer of germinated embryos (plantlets) to the peat pellets, their shoot heights and widths were measured. These measurements provide a baseline growth measurement just before the plantlets were subjected to N form and rate treatments (Chapter 4) or to mist with micronutrient treatment (Chapter 5). Plantlet shoot height was measured from the base of the first leaf collar to the unmanipulated tip of the plant, and shoot width was measured at the first leaf collar.

All three factors, plantlet height, width and height/width were affected by line and by week of planting, but not by the interaction of the two (Table 3.9). Shoots of SS were taller (81.7 mm) and wider (0.80 mm) than those of NSS (74.1 and 0.74 mm, respectively). This response would be expected since the SS embryos were longer and therefore more advanced than those of NSS (Table 3.6). The height/width ratio of SS (108), however, was smaller than that of NSS (140). Thus, even though SS shoots were taller than those of NSS, SS shoot widths were proportionately greater than those of NSS. Based on this evidence, SS was more vigorous than NSS. The decrease in height/shoot with week after planting may have reflected an increase in solar radiation from the last week of March thru April that would reduce plant stretching as the proportion of red radiation in the visible spectrum decreased.

Table 3.9 Height, width, and height divided by width of NSS and SS plantlets (germinated embryos) at the end of the 7-day *in vitro* period in response to week of planting.

Factor	Plantlet height (mm)	Plantlet width (mm)	Embryo height/width
Line			
NSS	74.1 b	0.74 b	140 a
SS	81.7 a	0.80 a	108 b
Week after planting			
1	81.4 b	0.68 b	183 a
2	72.0 b	0.90 a	158 a
3	71.4 b	0.92 a	95 b
4	77.7 b	0.96 a	93 b
5	94.4 a	0.67 b	92 b
$LSD_{0.05}$	12.6	0.13	19
Significances[y]			
Week of planting (WK)	**	***	***
Line	*	**	*
WK x L	NS	NS	NS

[z] Means followed by the same letter within a column and a factor cannot be considered different according to $LSD_{0.05}$.

[y] NS, *,**, *** Not significant or significant at $P \leq 0.05$, $P \leq 0.01$, or $P \leq 0.001$, respectively.

3.4 Conclusions

Treating MI seeds with Quadris (in addition to MaximXL) increased germination by 23.7 percentage points from 71.7% with MaximXL alone to 95.3%. Kernels of the two inbred lines (NSS and SS) treated with only MaximXL had 99-100% germination.

At 15 DAP, SS embryos were longer than those of NSS. Embryo length was little affected by week of planting for the first four weeks, but increased at the fifth week of planting, which was associated with a substantial increase in accumulated heat units.

Week of planting did not affect embryo "germination" (embryo development into a plantlet that contained a root and shoot system). SS had a higher percentage of germination than NSS.

Plantlets of the SS line were taller and wider than those of NSS, and yet they had a lower height/width ratio.

Chapter 4

NITROGEN FORM AND RATE TREATMENTS OF POST *IN VITRO* PLANTLETS

4.1 Introduction

While it has long been known that the application of nitrogen to maize increases yields, the response of post *in vitro* DH plantlets to N form and rate are unknown. There is limited understanding of the nutritional needs of tissue cultured plantlets undergoing the embryo rescue process. Post *in vitro* plantlets were transplanted to peat pellets which have limited buffering capacity. The peat pellet is essentially a hydroponic medium capable of transitioning the plantlet from an *in vitro* environment to the field. The time for this transition is about seven days during which the plantlets must extract water and nutrients to become robust, thereby increasing the likelihood of survival in the field. The plantlet must be able to adapt to a much lower RH in the greenhouse than the 100% RH of the *in vitro* environment. Regulation of plant water relations and conversion from heterotrophy to autotrophy are just a few of the conditions with which the plantlet must adapt during its post *in vitro* ontogeny. During this transition period, the embryonic plantlets are also recovering from treatment with the mitotic inhibitor during the *in vitro* stage.

This experiment examined the effect of nitrogen form (NH_4 : NO_3) ratios (100 : 0, 50 : 50 or 0 : 100, respectively) at 100, 200 or 400 mg $N \cdot L^{-1}$ over a 7-day period on the growth in length of the first true leaf of plantlets in the greenhouse.

4.2 Materials and methods

Plantlets of NSS or SS were transferred to longitudinal cuts within the peat pellets. These transplanted plantlets were randomly assigned a treatment number 1-10 (Table 4.1) and grouped in 40 cm x 40 cm x12 cm trays (Zipset-4 Propagation Tray, Monarch Manufacturing, Salida, CO.) at 36 per tray, which have large openings in their sides and bottoms. Each tray of 36 plantlets represented a single replication of the ten fertilizer solution treatments. Each tray was placed in a 45 cm x 45 cm x 7 cm plastic tub into which the appropriate fertilizer solution was poured to a 2.5 cm depth. The peat pellets were allowed to sub-irrigate for one hour on alternate days, with a total of four irrigations during the 7-day duration of the study. We determined that the average uptake by each peatlite was 175 ml of solution, enough for the peat pellets to achieve container capacity. The trays were arranged in randomized complete blocks with 4 replications within a greenhouse set at 30°/23°C (day/night) with natural light. The maximum and minimum temperatures recorded were 30° and 18°C, respectively.

The fertilizers and their analyses (percentage by weight of N, P_2O_5 and K_2O) used for preparing the solutions (all from Griffin Greenhouse and Nursery Supplies, Morgantown, PA) were: Peters Acid Special (21-7-7; 100% ammonium),

Peters Pro Blossom Booster (10-30-20; 50% ammonium, 50% nitrate), and Scott's Champion (13-0-44; 100% nitrate). The weights of fertilizer per liter of double-distilled water to provide the 100, 200, or 400 mg $N \cdot L^{-1}$, as well as the pH and E.C values of the solutions are shown in Table 4.1.

Twenty-four hours after each irrigation treatment, plantlet shoot height was measured from the base of the first leaf's collar to the unmanipulated tip of the plant and shoot width was measured at the first leaf collar. Both measurements were taken with vernier calipers.

Table 4.1 Concentrations of the fertilizer salts to yield 100, 200 or 400 mg $N \cdot L^{-1}$ solutions of 100% nitrate, 100% ammonium, or 50% nitrate plus 50% ammonium, and the pH and electrical conductivity (EC) of these solutions.

Treatment number	Fertilizer analysis	% N as NO_3	% N as NH_4	Nitrogen concentration ($mg \cdot L^{-1}$)	g fertilizer$\cdot L^{-1}$	pH	EC ($dS \cdot m^{-1}$)
1	None, control	0	0	0	0	7.2	0.0
2	21-7-7	0	100	100	0.476	5.2	0.5
3	21-7-7	0	100	200	0.952	5.0	1.0
4	21-7-7	0	100	400	1.905	5.0	2.0
5	10-30-20	50	50	100	1.000	4.2	0.8
6	10-30-20	50	50	200	2.000	4.2	1.6
7	10-30-20	50	50	400	4.000	4.2	8.2
8	13-0-44	100	0	100	0.769	5.6	0.5
9	13-0-44	100	0	200	1.538	5.8	1.4
10	13-0-44	100	0	400	3.077	6.0	3.4

4.3 Results

4.3.1 Plantlet growth

SS plantlets were taller and wider than NSS plantlets at day 2, but thereafter there was no difference in growth or growth rate between the two genotypes (Table 4.2). Nitrogen form affected plantlet height at days 2 and 4 but not after 6 days. Ammoniacal-N resulted in greater height than NO_3 + NH_4, with NO_3 giving values similar to the other N-form treatments. Plantlet width was unaffected by N form at days 2 and 4. By the sixth day, however, N form interacted with N rate in influencing plantlet height and width. With NO_3, 200 mg $N \cdot l^{-1}$ N resulted in greater leaf length and width than 100 mg $N \cdot l^{-1}$, and exceeding 200 mg $N \cdot l^{-1}$failed to increase leaf length or width. Plantlet height at day 6 was unaffected by N rate when the plantlets were supplied either as NH_4 or NO_3 + NH_4. Only 200 mg $NO_3 \cdot L^{-1}$ resulted in greater plantlet height than occurred in control plantlets. Plantlet width was unaffected by NH_4-N rate, but was affected by concentrations of both NO_3-N and NO_3 + NH_4. As with plantlet height, however, only 200 mg $NO_3 \cdot L^{-1}$ resulted in greater plantlet width than occurred in control plantlets

Table 4.2 Effects of nitrogen form and rate on the height and width of NSS and SS maize plantlets measured at 2, 4 or 6 days during a 7-day post *in vitro* culture period in peat pellets.

Treatment	Nitrogen Concn. ($mg\ N \cdot l^{-1}$)	Plantlet height (mm)[a] Days after *in vitro* culture 2	4	6	Plantlet width (mm)[a] Days after *in vitro* culture 2	4	6	Plantlet growth rate between 2 and 6 days ($mm \cdot d^{-1}$) Height		Width
Genotype										
NSS		85.2 a[b]	120.8	159.4	0.9 a	1.4	2.1	10.3		0.17
SS		73.9 b	111.5	156.9	0.8 b	1.4	2.2	10.2		0.18
Nitrogen form										
$NO_3 + NH_4$		74.1 b	104.3 b	148.1	0.8	1.3	2.0	9.6		0.16 b
NO_3		80.5 ab	118.6 ab	160.3	0.8	1.4	2.3	10.6		0.20 a
NH_4		87.4 a	127.0 a	148.1	0.9	1.4	2.1	10.5		0.16 b
								SS	NSS	
$NO_3 + NH_4$	100			146.2 bcd			2.1 bc	8.7 cde	9.1 bcde	0.16 b
	200			135.7 d			1.7 c	9.4 bcde	8.8 cde	0.14 b
	400			163.7 abcd			2.2 ab	13.2 ab †	9.8 bcde	0.19 ab
NO_3	100			142.1 cd			2.0 bc	8.1 de	10.2 bcde	0.16 b
	200			180.9 a†			2.6 a†	11.9 bcd †	12.4 abc†	0.24 a†
	400			154.7 abcd			2.2 ab	8.8 cde	10.2 bcde	0.19ab
NH_4	100			174.6 ab			2.2 ab	16.4 a †	10.6 bcde	0.18 b
	200			167.6 abc			2.1 bc	7.3 e	11.9 bcd	0.17 b
	400			149.9 bcd			1.9 bc	10.3 bcde	8.6 cde	0.14 b
Control		79.0	105.7	146.2	0.9	1.4	2.1	9.1	8.7	0.16

Table 4.2 continued

Table 4.2 continued

	Plantlet height (mm)[a] Days after *in vitro* culture			Plantlet width (mm)[a] Days after *in vitro* culture			Plantlet growth rate between 2 and 6 days ($mm \cdot d^{-1}$)	
	2	4	6	2	4	6	Height	Width
Significance[c]								
Genotype (G)	*	NS	NS	*	NS	NS	NS	NS
N form (NF)	*	*	NS	NS	NS	NS	NS	NS
G X NF	NS	NS	NS	NS	NS	NS	NS	NS
N rate (NR)	NS	NS	NS	NS	NS	NS	NS	NS
G X NR	NS	NS	NS	NS	NS	NS	NS	NS
NF X NR	NS	NS	**	NS	NS	**	**	**
G X NF X NR	NS	NS	NS	NS	NS	NS	*	NS

[a] Plantlet height was measured from the first leaf collar to the unmanipulated leaf tip. Plantlet width was measured at the first leaf collar.
[b] Means followed by the same letter within a column main effect or within an interaction cannot be considered significant by $LSD_{0.05}$.
[c] NS, *, ** Not significant or significant at $P \leq 0.05$ or 0.01, respectively.
[†] Significantly different from the control mean by one-way $LSD_{0.05}$

Although plantlet height growth rate ($mm \cdot d^{-1}$) between days 2 and 6 was affected by the 3-way interaction of genotype with N form and rate, only with NH_4 at 100 mg $N \cdot l^{-1}$ did SS plantlets have a greater height growth rate than did NSS (Table 4.2). Plantlet height growth rate of the NSS genotype was unaffected by N rate and concentration (mean of 10.3 $mm \cdot d^{-1}$ and range of 3.8 $mm \cdot d^{-1}$). The SS genotype had a similar plantlet height growth rate (10.2 $mm \cdot d^{-1}$) as the NSS genotype (10.3 $mm \cdot d^{-1}$) in response to N form and rate but had a much greater range (9.1 $mm \cdot d^{-1}$). Only three treatments (NO_3 + NH_4 at 400 mg $N \cdot L^{-1}$, NO_3 at 200 mg $N \cdot L^{-1}$ and NH_4 at 100 mg $N \cdot L^{-1}$) resulted in greater plantlet height growth rate of the SS genotype than occurred in control plants. Only NO_3-N at 200 mg $N \cdot L^{-1}$, gave greater plantlet height and width growth rates of NSS plantlets than was achieved in control plantlets.

At the end of the 7-day post *in vitro* period, the percentage of surviving plantlets was determined. Both genotype and nitrogen form affected survival, but nitrogen rate did not. The 26.6% of surviving NSS plantlets was more than the 15.7% of surviving SS plantlets (Table 4.3). Nitrate-N and ammonium-N similarly gave greater survival (22.9%) than the 50% nitrate: 50% ammonium nutrition (17.7%; Table 4.2).

Table 4.3 Effects of nitrogen form and rate on the survival of NSS and SS plantlets at the end of the 7-day post *in vitro* culture in peat pellets.

Treatment	Plantlet survival	
	Percentage (out of 36)	Arcsine √ percentage
Genotype		
NSS	20.7	26.6 a[a]
SS	8.2	15.7 b
Nitrogen form		
$NO_3 + NH_4$	10.3	17.7 b
NO_3	16.0	22.4 a
NH_4	17.0	23.3 a
Control		
NSS	5.0	21.4
SS	5.3	19.1
Significance		
Genotype (G)		***
N form (NF)		**
G X NF		NS
N rate (NR)		NS
G X NR		NS
NF X NR		NS
G X NF X NR		NS
Genotype (G)		***
1-way		NS
G x 1-way		NS

[a]Means followed by the same letter within a column main effect cannot be considered significant by $LSD_{0.05}$.

[b] NS, **, *** Not significant or significant at $P \leq 0.01$ or 0.001, respectively.

4.3.2 Doubled haploid return

Ultimately, attempts to improve the vigor and survival of embryo-rescued DH plantlets must result in increased number of DH plants with kernels (i.e. increased DH return).

Following the nitrogen form and rate treatments given plantlets during the 7-day post *in vitro* greenhouse culture period, the plantlets were transplanted to the field and given normal cultural care (see Chapter 3 for details). Plants were self-pollinated and the number of DH plants (based on both phenotypic appearance, anthocyanin coloration of the glumes, and colorless endosperm) were recorded.

The number of plants bearing DH kernels was unaffected by genotype, N form, N rate, and their interactions (Table 4.4).

Table 4.4 The effect of nitrogen form and rate during 7-day post *in vitro* plantlet growth in peat pellets on the number of NSS or SS plants bearing DH seeds following transplanting to the field.

Source of variation	DF	Chi-square value	$P \geq$ Chi-square value
Replication	3	14.3274	0.0025
Genotype (G)	1	1.0145	0.3138
Nitrogen form (NF)	3	2.7055	0.4393
Nitrogen rate (NR)	1	0.005	0.9436
G x NF	3	0.7495	0.8615
G x NR	1	0.0549	0.8148
G x NF x NR	2	0.6562	0.7203

4.4 Conclusions

The single combination of N form and rate that provided the greatest plantlet width and height with the least N was NO_3 at 200 mg $N \cdot l^{-1}$ during a 7-day post *in vitro* cultural period in the greenhouse. However, genotype and N form and rate had no effect on the numbers of plants with DH kernels or the number of kernels per plant following plantlet transplanting and maturation of the plants in the field.

Chapter 5

MIST TREATMENTS OF POST *IN VITRO* PLANTLETS

5.1 Introduction

The survival and growth of tissue-cultured plantlets is a common struggle in horticulture. The sensitivity of the young plantlet as it transitions from an *in vitro* environment of almost 100% RH to the lower RH of the greenhouse, demands that it be able to survive the stress of increased evapotranspirational demand and other stresses to survive. One common way to minimize the desiccation of tissue cultured plantlets is to subject them to cyclical misting which causes an average ambient RH of about 60%.

Likewise, the transplanting of the plantlet from the agar-based *in vitro* medium to a peat-based medium proves to be an additional hurdle in adjusting to post-*in vitro* life. The delicate roots that developed in agar are now required to absorb nutrients. Until existing or new root growth compensates for this transition and new environment, the plantlet is stressed by limited root uptake of micronutrients. Leach and Hameleers (2001) found that a critical time for maize seedlings is from germination to the 5-leaf stage where a small root-soil interface can restrict the uptake

of P. Their work found that foliar applications of P and Zn at 36 days after sowing resulted in increased shoot kernel growth.

The objectives of this study are to determine whether foliar application of micronutrients in a mist form can affect post *in vitro* survival and growth. A water-mist and no mist provided comparison treatments.

5.2 Materials and methods

Plantlets of NSS or SS were transferred to longitudinal cuts within the peat pellets. These transplanted plantlets were randomly assigned a treatment (Table 5.1) and grouped in 40 cm x 40 cm x 12 cm trays (Zipset-4 Propagation Tray, Monarch Manufacturing, Salida, CO.) at 36 per tray, which have large openings in their sides and bottoms. Each tray of 36 plantlets represented a single replication of the three mist treatments. Treatments included, soluble trace element mix (STEM; Griffin Greenhouse and Nursery Supplies, Morgantown, PA) mist supplied at 0.6 $mg \cdot L^{-1}$, water mist and no mist. The diluted STEM solution supplied as mist contains the following ($mg \cdot L^{-1}$): S (0.078), B (0.0081), Cu (0.0138), Fe (0.045), Mn (0.048), Mo (0.0024), and Zn (0.0023).

The trays were arranged in randomized complete blocks on the mist bench with 3 replications. The greenhouse was set at 30°/23°C (day/night) with natural light. The maximum and minimum temperatures recorded were 30° and 18°C, respectively. The mist bench was constructed in the greenhouse (40°03’41.07N, 76°04’10.39”W,

elevation 487') in New Holland, PA. The over head mist system (Fig. 5.1), with all components supplied by Griffin Greenhouse and Nursery Supplies, Morgantown, PA, was able to achieve uniform coverage of all plantlets without overlap or interference. This was achieved by using Plexiglas (Paul B. Zimmermans Hardware, Ephrata PA) to divide the treatment benches to prevent drift.

Well water from on-site was pH 6.5 to 6.8 for the duration of the experiment. The well water was filtered by a Netafim Arkal 120 µm disc filter. The micronutrient solution was injected into the water line using a Dosatron 42 $L \cdot min.^{-1}$ injector with an injection ratio set at 1 (stock):100 (water), whereas the water mist system excluded the injector. Two Nelson Pro 7900 Series solenoids were plumbed into the 1.9 mm (0.75 in.) Netafilm tubing. Solenoids were wired to a Gemini misting controller which in turn was wired to a Mist-a-Matic control device positioned at plant level on the mist bench. Since this device turns on the mist when a certain weight of liquid water has evaporated from a synthetic leaf, the misting is controlled by the rate of evaporation. Misting nozzles were arranged such that STEM mist, water mist and no mist were randomized within the greenhouse (Figure 5.1).

At 2, 4 and 6 days post *in vitro*, plantlet shoot height was measured from the base of the first leaf collar to the unmanipulated tip of the plant and shoot width was measured at the first leaf collar. Both measurements were taken with vernier calipers. The percentage of plantlets surviving at the end of the 7-day period was recorded.

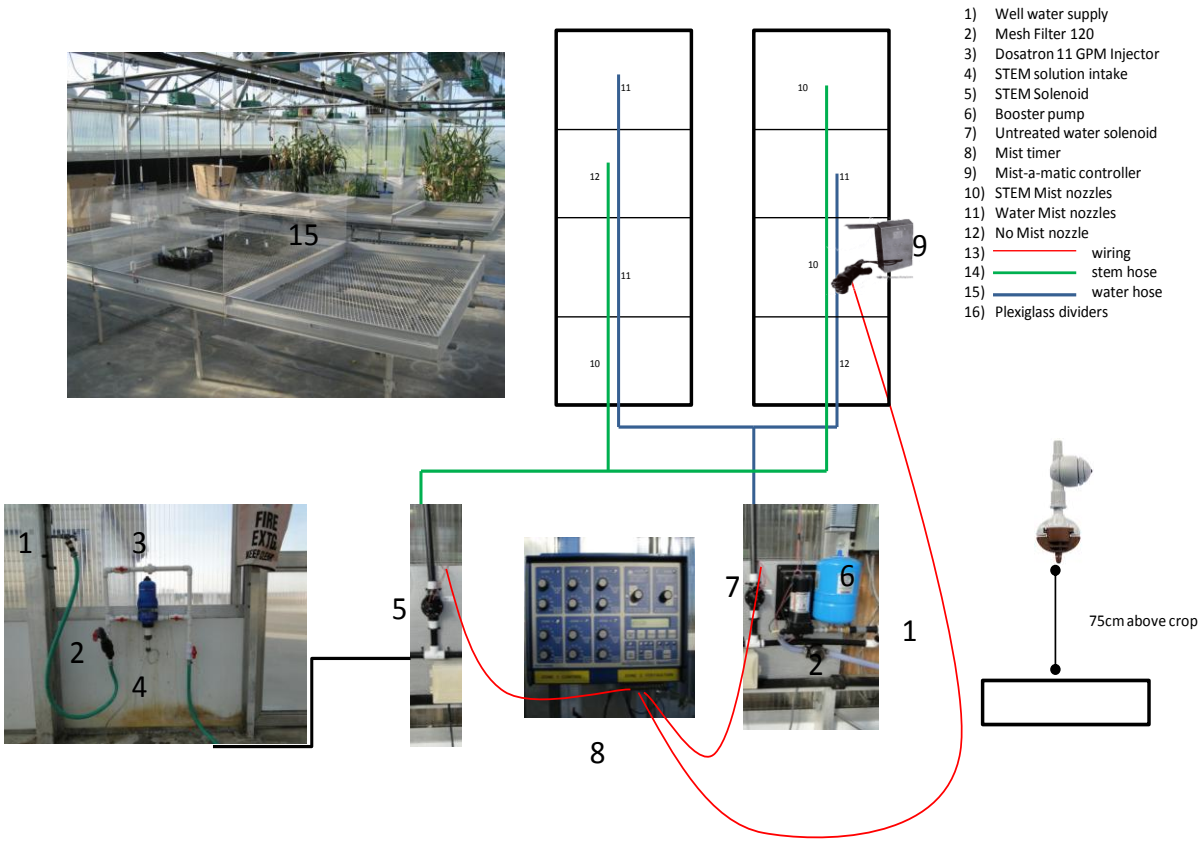


Figure 5.1 Mist system schematic.

5.3 Results

5.3.1 Plantlet growth

SS plantlets were taller and wider than those of NSS plantlets at 2, 4, and 6 days after plantlet transfer from *in vitro* culture (Table 5.1). While height growth

rate was unaffected by genotype, the width growth rate of SS plantlets was greater than that of NSS plantlets. Mist treatments affected plantlet height but not plantlet width, only by 6 DAT, with values in the order: control ≥ micronutrient mist ≥ water mist. This same order also applied to plantlet height growth rate. Genotype failed to interact with mist treatment in affecting plantlet growth.

The NSS genotype had 49.4% survival by the end of the 7-day post *in vitro* period compared with 32.1% for the SS genotype (Table 5.2). The mist treatments and their interactive effects with genotype had no effect on plantlet survival. NSS plantlets were smaller (height and width) than those of SS plantlets at the end of the post *in vitro* treatment (Table 5.1) and yet they had greater survival than the SS plantlets (Table 5.2).

Table 5.1 Effects of water- or micronutrient-mist on the height and width of NSS and SS maize plantlets measured at 2, 4 and 6 days after beginning post *in vitro* culture in the greenhouse.

Treatment	Plantlet height (mm)[a] Days after *in vitro* culture			Plantlet width (mm)[a] Days after *in vitro* culture			Plantlet growth rate ($mm \cdot d^{-1}$)	
	2	4	6	2	4	6	Height	Width
Genotype								
SS	98.0 a[b]	134.1 a	191.7 a	1.0 a	2.1 a	2.6 a	13.2	0.23 a
NSS	69.9 b	108.9 b	161.2 b	0.7 b	1.7 b	2.0 b	12.7	0.19 b
Mist								
Water			160.5 b			2.2	11.2 b	0.2
Micronutrient			172.6 ab			2.3	12.5 ab	0.2
Control (no mist)			186.6 a			2.5	15.2 a	0.2
Significance[c]								
Genotype (G)	***	***	***	***	*	***	NS	**
Mist (M)	NS	NS	*	NS	NS	NS	**	NS
G X M	NS	NS	NS	NS	NS	NS	NS	NS

[a] Plantlet height was measured from the first leaf collar to the unmanipulated leaf tip. Plantlet width was measured at the first leaf collar.

[b] Means followed by the same letter within a column main cannot be considered significant by $LSD_{0.05}$.

[c] NS, *, **,*** Not significant or significant at $P \leq 0.05$, 0.01 or 0.001, respectively.

[†] Significantly different from the control mean by one-way $LSD_{0.05}$

Table 5.2 Effects of mist (straight water or trace element solution) on the survival of NSS and SS plantlets at the end of the 7-day post *in vitro* culture in peat pellets.

Treatment	Plantlet survival	
	Percentage (out of 36)	Arcsine √percentage
Genotype		
NSS	49.4	44.7 a[a]
SS	32.1	34.4 b
Mist		
Water mist	39.4	38.7 a
Trace nutrient mist	42.1	40.4 a
None	40.7	39.5 a
Significance[b]		
Genotype		***
Mist		NS
Genotype x Mist		NS

[a]Means followed by the same letter within a column main effect cannot be considered significant by $LSD_{0.05}$.

[b] NS, *** Not significant or significant at $P \leq 0.001$, respectively.

5.3.2. Doubled haploid return

The ultimate goal was to improve the vigor and survival of embryo-rescued DH plantlets to increase the number of DH plants with kernels (i.e. increased DH return). Following the mist treatments given plantlets during the 7-day post *in vitro* greenhouse culture period, the plantlets in peat pellets were transplanted to the field and given normal cultural care (see Chapter 3 for details). Plants were self-pollinated and the number of DH plants (based on both phenotypic appearance,

anthocyanin coloration of the glumes, and colorless endosperm) was recorded. The number of plants bearing DH kernels was unaffected by genotype, mist treatments, or their interactions (Table 5.3).

Table 5.3 The effect of straight water mist or trace nutrient mist during the 7-day post *in vitro* plantlet growth in peat pellets on the number of NSS or SS plants bearing DH seeds following transplanting to the field.

Source of variation	DF	Chi-square value	$P \geq$ Chi-square value
Replication	3	9.0651	0.0108
Genotype (G)	1	0.0003	0.9869
Mist (M)	2	4.4943	0.1057
G x M	2	2.2270	0.3284

5.4 Conclusions

Water mist or trace nutrient mist failed to increase plantlet height, plantlet width, or plantlet survival compared to values achieved in control plantlets that received no mist. The NSS genotype had higher values for all these variables than the SS genotype. DH return was unaffected by genotype or mist treatments.

Chapter 6

EMBRYO LENGTH EFFECT ON GERMINATION AND PLOIDY

6.1 Introduction

While the embryo length for initiating the nitrogen and mist experiments was approximately 2.0 mm, this size may not be optimal for transfer to post *in vitro* experimentation. Embryo length affects both the reliability ofpurple coloration from the marker genes residing in the MI that express the ploidy level and the ability of the embryo to regenerate. Due to several occurrences of diploid escapes in both the nitrogen and mist treatment experiments, coloration from the marker genes was not optimal at the time of embryo rescue. Recent experiments conducted by our laboratory have demonstrated that a longer embryo (3.50-4.00 mm) provided less ambiguity in this classification. However, larger embryos have increased risk of callusing (and failure to "germinate") in the germination media and an increased failure to thrive.

6.2 Materials and methods

Methodology for germination and induction of embryos were as described above in Chapters 2 and 3, with the exception that the female for this experiment was

the hybrid of NSS and SS. The NSS/SS hybrid was induced with MI pollen in the fall of 2008 within a greenhouse in New Holland, PA. Embryos between 1 and 7 mm long were harvested and sorted by length (to the nearest 0.25 mm) with a sterilized metal ruler. Embryos (144 embryos within each length category; Table 6.1) were transferred to Phytatrays ©s, 11.5mm x 8.9mm x 6.3mm (Phytatray © P1552, Sigma-Aldrich, St. Louis, MO).

Table 6.1 Phytatray © grouping for embryo length categories.

Phytatray#	Embryo Length (mm)	n	Phytatray#	Embryo Length (mm)	n
1	1	144	14	4.25	144
2	1.25	144	15	4.5	144
3	1.5	144	16	4.75	144
4	1.75	144	17	5	144
5	2	144	18	5.25	144
6	2.25	144	19	5.5	144
7	2.5	144	20	5.75	144
8	2.75	144	21	6	144
9	3	144	22	6.25	144
10	3.25	144	23	6.5	144
11	3.5	144	24	6.75	144
12	3.75	144	25	7	144
13	4	144			

Identical procedures were performed as described in Chapter 3 for isolation, doubling, and *in vitro* regeneration. Embryo length was measured daily throughout the 7-day incubation period with a sterilized metal ruler. After 7 days,

embryos were transplanted to peat pellets and allowed another 7 days of growth before being transplanted into 36cm diameter pots containing Metro-mix 360 in the greenhouse. Potting and nutrition was the same as described in Section 3.2.2. Greenhouse temperatures were set at 30°/21°C (day/night) for 3 weeks. The greenhouse was equipped with supplemental lighting (3 Metal Halide: 1 High Pressure Sodium with a PAR of 2.1 µmoles m^{-2} s^{-1} (600 watts m^{-2}) to ensure a 16 hour photoperiod. Plants were allowed to mature until a positive phenotype of DH or diploid escape was observable in the glume coloration (DH = colorless; Diploid escape = purple).

6.3 Results

Germination, the presence of both root and shoot, peaked at 100% at the 4.00-4.25 mm embryo length range (Figure 6.1). Germination at ≤ 50% occurred in embryos with lengths < 1.75 mm or > 6.25 mm. Embryos shorter than the optimal range had no callusing while embryos longer than this range had extensive callusing.

While classification of ploidy level at all embryo sizes was possible with the MI reporter genes, there was better accuracy in embryos >4 mm long. At 4 mm, the diploid escapes decreased to 0 or 1%.

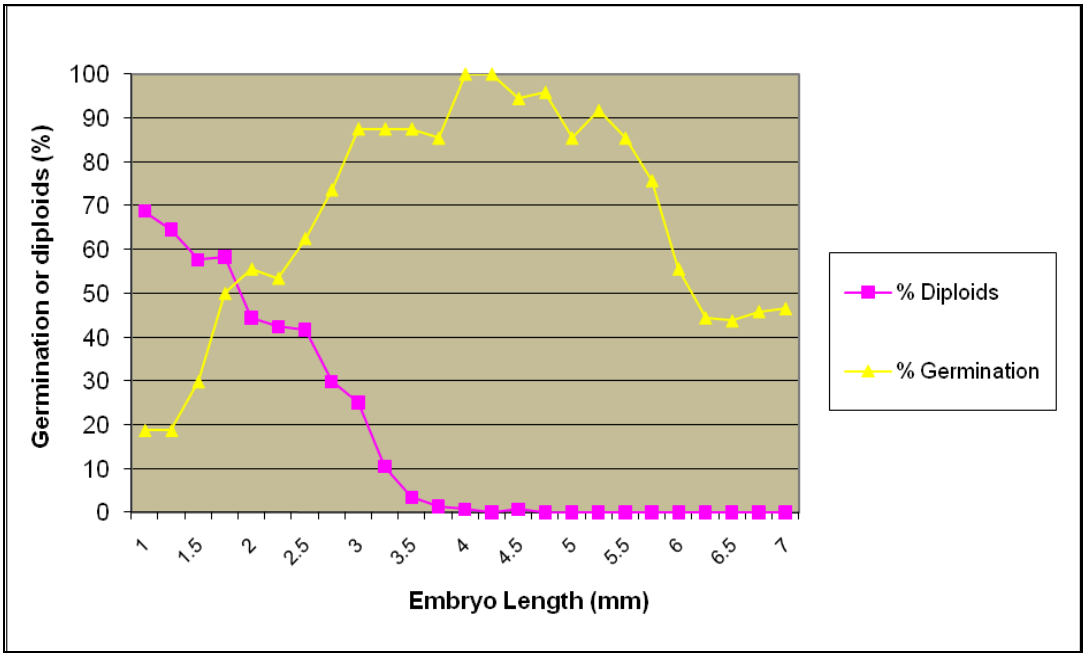


Figure 6.1 Germination and diploid escape percentages as a function of embryo length.

6.4 Conclusions

Embryos 4 to 4.5 mm long gave optimal germination and enabled optimal phenotyping of NSS/SS hybrid embryos.

Chapter 7

TEMPERATURE EFFECTS ON EMBRYO GERMINATION AND GROWTH

7.1 Introduction

As discussed in Chapter 6, embryo germination percentage could be increased by selecting embryos 4.00 - 4.25 mm long. Environmental factors such as temperature could also affect *in vitro* embryonic germination and growth. The time for plantlets to reach a size mature enough for transplanting to peat pellets could be shortened if temperature were optimized. This experiment examines temperature effects on embryo germination and growth.

7.2 Materials and methods

Following induction and embryo rescue protocols detailed in Chapter 3 and 6, the hybrid NSS/SS was induced to produce embryos 4.00 - 4.25 mm long. The putative haploid embryos (PHEs) were planted in Phytatrays ©s containing 272M Maize Germination Medium (Sigma-Aldrich, St. Louis, MO).

Phytatrays ©s were then transferred to a growth chamber with 24 hr lighting (Phillips cool white fluorescent lamps, 0.52 µmoles m^{-2} s^{-1}) and 40% R.H. Because of the limitation of only having one chamber, temperature treatments were

run sequentially. Temperatures ranged from 10° to 35° C in 5° C increments, with each increment having two replications of 84 embryos.

Embryo germination was recorded daily over a 7-day period. Plantlets also were observed for a maturity level suitable for transfer. Plantlets were considered mature enough for transfer to peat pellets when each organ, root and shoot, exceeded 2.5 cm in length. After 7 days of maturation in the growth chamber, plantlets that did not reach this maturation level were classified as immature.

7.3 Results

Germination percentage was greatest at 20° to 30° C (average 90%), but decreased to 0% with 10° C and to 62% with 35° C (Figure 7.1). The average number of days until plantlets were sufficiently mature for transplanting to peat pellets was reduced to 6 days with 30° or 35° C, from the 13 days at 15° C (Figure 7.2).

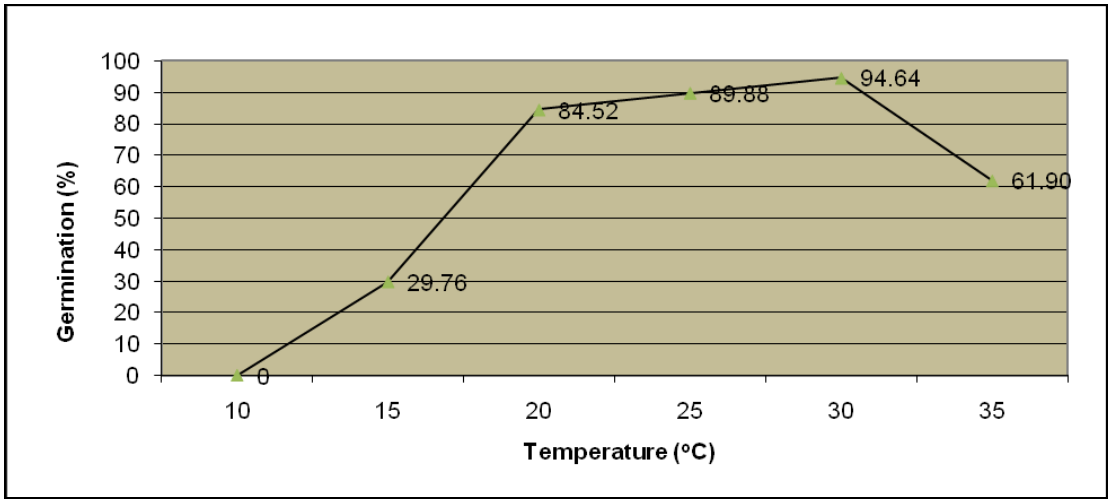


Figure 7.1 Effect of temperature on percentage germination of NSS/SS hybrid plantlets (roots and shoots ≥ 2.5 cm long).

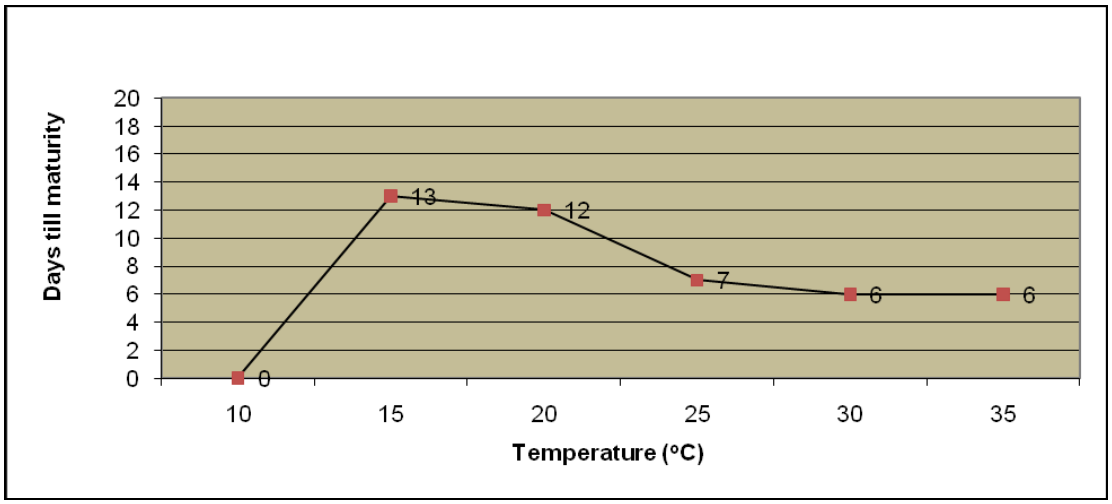


Figure 7.2 Effect of temperature on the number of days needed for plantlets to reach maturity (shoots and roots ≥ 2.5 cm long).

7.4 Conclusions

The higher "germination" percentage (95%) and the shortest time (6 days) for plantlets to mature (roots and shoots ≥ 2.5 cm long) occurred at 30° C. This would be the optimal *in vitro* temperature to produce plantlets suitable for transfer to peat.

Chapter 8

CONTROLLING *IN VITRO* MICROBIAL CONTAMINATION

8.1 Introduction

During tissue culture, microbial contamination can cause great loss. High humidity and the abundance of sugar and other nutrients in the germination medium can result in fast and overwhelming development of contamination. Given the large numbers of embryos in DH production, an individual test tube for each embryo was no longer an efficient option, although it did isolate contamination. Instead, embryos are now plated on germination media in a Phytatray © capable of holding 100+ embryos. Given the months of work required to induce and isolate embryos and then doubling their ploidy, microbial contamination can result in large losses in time and money.

Initial efforts to control this loss begin with ear surface sterilization. Ear sterilization involves stirring the ear in a 20% bleach (1.25% NaOCl) solution with $0.5ml \cdot L^{-1}$ of Tween 80 for 20 minutes. In an effort to reduce medium contamination, Plant Preservative Mix, (PPM, Plant Cell Technologies, Washington D.C.) was added to the germination media to reduce the contamination. This experiment sought to quantify effect of PPM in reducing microbial contamination and to verify that the additive was not phytotoxic to the germinating embryos.

8.2 Materials and methods

Four Phytatrays ©s of 42 embryos each were transferred over the course of 49 weeks, with the application of PPM starting in week 16. Isolated embryos were placed on germination media containing the manufacturer's recommendation of 1ml $PPM \cdot L^{-1}$. PPM was added to the liquid medium before it was poured into Phytatrays ©s. Embryos were transferred in all trials by the same technician in an effort to minimize variation due to sterile technique. Phytatrays ©s were placed in the growth chamber at constant 27° C for 7 days and plates were scouted daily for contamination. Confirmation of contamination within a Phytatray © was recorded as an observation. There was no need to count points of contamination within a plate since typical early infestation resulted in complete microbial coverage of the plate during the 7-day period.

8.3 Results

While the average contamination rate for the first 16 weeks without PPM was 30%, PPM in the media decreased contamination to 1.5% (Figure 8.1). As surviving plants from both PPM and no PPM experiments were transferred and pollinated there was no difference in either survival or DH return, both were around 80% and 20%, respectively. While the manufacturer does not specify the active

ingredients in PPM, there is some concern for unintentionally developing pathogen resistance.

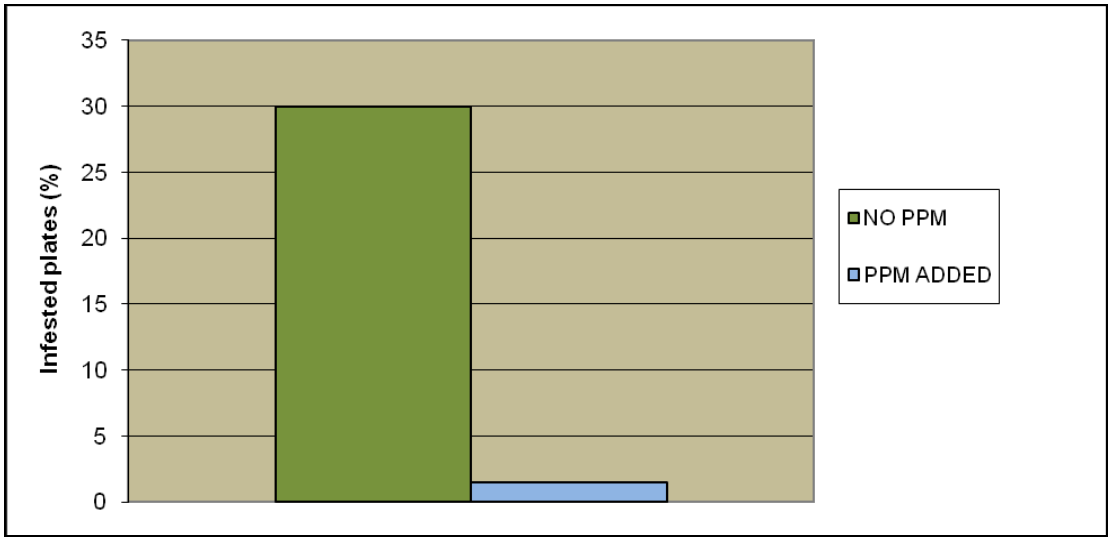


Figure 8.1 Percentage of plates with microbial contamination in response to Plant Protective Mix (PPM) included in the medium.

8.4 Conclusions

The presence of Plant Protective Mix at $1ml \cdot L^{-1}$ of cultural medium, reduced microbial contamination from 30% to 1.5%.

Chapter 9

OVERALL DISCUSSION

The embryo rescue technique can be a valuable tool to hasten the selfing of plants and thus reduce the generation time. The process for obtaining DH lines using embryo rescue techniques, however, does not attempt to address embryo survival and DH return percentages. I subjected these tissue-cultured plantlets ("germinated" embryos) to several treatments (nitrogen form and rate, water vs. trace nutrient mist) in attempt to improve DH return.

Prior to these treatments during post *in vitro* growth, I had established that greater embryo length was associated with greater accumulated heat units rather than the week of planting or the number of DAP (Table 3.7, Fig. 3.3). It would appear that maize embryo growth follows the same formula developed for whole plant growth.

After 2 and 4 days of treatment initiation, embryo length was influenced in the order: NH_4-N $\geq$ NO_3-N $\geq$ NO_3-N plus NH_4-N during a 7-day post *in vitro* cultural period in the greenhouse (Table 4.2). The literature on the effect of N form on graminaceous crops is mixed. For instance, Okomoto et al. (2004) established that nitrate-N gave greater seedling shoot and root growth than 1 NO_3:1 NH_4 fertilization. Others found, however, that mixed nitrate and ammonium fertilization resulted in greater maize seedling growth than either N form alone (Shortenmeyer, 1993;

Smiciklas and Below, 1992). In my study, I found that by 6 days after treatment initiation, the single combination of N form and rate that provided the greatest plantlet width and height, width growth rate, with the least N was NO_3 at 200 mg $N \cdot l^{-1}$ (Table 4.2). Nitrate-N, compared to ammonium-N increased the uptake of Ca, Mg, K, P and NO_3 in tomato plants and reduced plant water stress (Pill and Lambeth, 1977).

The peat pellets contained 583 and 248 $mg \cdot kg^{-1}$ of NH_4 and NO_3-N, respectively (Table 3.1). Since each peat pellet weighed only 25 g (dry weight), the NH_4-N and NO_3-N concentrations, respectively would only be 15 and 6 mg per pellet, very small values compared to those of the treatments (100, 200, or 400 mg $N \cdot L^{-1}$).

Had the plants been subjected to a longer period of nitrogen fertilization than the 1 week following *in vitro* culture, the effects of N form and rate may have been more pronounced. Future work should examine the effects of a longer post *in vitro* period. Greater root growth would likely increase uptake of nutrients from the fertilizer solutions. Greater root growth also would perhaps result in faster recovery from transplanting shock.

Nitrate- and ammonium-N, compared to the 1:1 combination, increased plantlet survival 5.2 percentage points to an average 16.5% from 10.3% (Table 4.3). This small but significant increase failed to translate into increased return of plants bearing DH kernels (Table 4.4). Unless the DH return is increased, nitrogen nutrition of the post *in vitro* plantlets is of little importance. Extending the time over which the N form and rate could influence plantlet growth and possibly DH return should be

examined in further work. In further work, there should be an effort to make pH, P and K concentrations equal among the N form and N rate treatments. The analyses of the fertilizers used in this study [(21-7-7; 100% ammonium), Peters Pro Blossom Booster (10-30-20; 50% ammonium, 50% nitrate), and Scott's Champion (13-0-44; 100% nitrate)], showed considerable variation existed in the concentrations of these primary macronutrients.

So few plantlets were transplanted to the field (Table 3.3) that statistical analysis of field results was problematic. Lack of normal population distributions necessitated the use of non-parametric statistics. Without greater plant numbers in the field, the real effects of the post *in vitro* N form and rate treatments on percentage DH return remains unknown.

Plantlet height and height growth rate were affected in the order: no mist (control) ≥ trace nutrient mist ≥ water mist (Table 5.1); but neither plantlet survival (average 40.7%, Table 5.2) nor DH return (Table 5.3) was affected by mist treatments. Since plantlets have limited root systems, it would seem logical that by raising the aerial relative humidity, transpiration would be reduced thereby lessening the need for water uptake through the rudimentary roots. This result would suggest that the plantlets were not under water stress, since reduced transpiration by raising aerial humidity by misting, failed to increase plant height.

Mist may be an efficient way to administer nutrients to plantlets. Leach and Hameleers (2001) found that during early maize growth (to the 5-leaf stage), P and

Zn , 0.0005 and .00054 $mg \cdot L^{-1}$, respectively, applied through mist led to increased shoot and economic yield. The soluble trace element mix (STEM) contained 0.0027 mg $Zn \cdot L^{-1}$; and yet plant height was greatest in the absence of mist (Table 5.2). That plantlet growth was not increased by misting with trace nutrients would suggest that foliar absorption of such nutrients was superfluous. Had the mist contained soluble P, as reported by Leach and Hameleers (2001) to promote seedling growth, perhaps plantlet growth could have been enhanced. As with the N-form and rate study, extending the post *in vitro* period beyond 7 days would permit more time for treatments to yield differential responses.

SS plantlets were bigger than NSS plantlets (Table 5.1) and yet they had lower survival (Table 5.2) by the end of the 7-day post *in vitro* period. Greater survival of NSS than SS plantlets could reside in other traits than plantlet size at the end of the 7-day post *in vitro* period.

As with the N form and rate study, so few plantlets were transplanted to the field that the true return of DH remains unknown.

While the N form and N rate and Mist studies were in progress, ancillary studies were conducted with the objective of increasing embryo "germination" into plantlets. By selecting embryos of different length ranges, we established that embryos 4 to 4.5 mm long gave the greatest germination (90 to 100%) and enabled optimal phenotype identification of NSS/SS haploid embryos (Figure 6.1). Presumably, longer embryos than those used in the earlier studies (2.0mm) have

greater capability to develop shoots and roots, i.e. greater capability to "germinate". Longer embryos presumably produce larger plantlets. Larger root systems probably would have increased the potential for differential responses to treatments imposed during the 7-day post *in vitro* period. Selecting longer embryos, along with extending the post *in vitro* period, may enhance plantlet responses to treatment.

Another study examined the effect of *in vitro* temperature on embryo "germination" and plantlet maturity (roots and shoots ≥ 2.5 cm long). Embryo "germination" (95%) and the shortest time (6 days) for plantlets to mature occurred at constant 30° C (Figure 7.1). Yet another study established that Plant Protective Mix at $1ml \cdot L^{-1}$ of cultural medium, reduced microbial contamination from 30% to 1.5%. These three ancillary studies helped to determine the optimal *in vitro* conditions for plantlet production. Namely embryos 4.0 to 4.5 mm long should be grown at constant 30° C in media containing antimicrobial Plant Protective Mix.

In these studies, plantlets were transferred, using forceps, from the *in vitro* medium to a longitudinal slit made in the top of peat pellets. It is possible that plantlet survival and growth in the peat pellets could be enhanced by transferring the plantlets in a hydrophilic polymer following the fluid drilling technique (Pill, 1991). The fluid drilling technique typically involves mixing germinated seeds in the gel and then transferring the mixture to the seed bed, with the result that seedling emergence percentage and rate are increased compared to sowing dry seeds. Kitto and Pill (1991) used this technique with success for delivering somatic embryos of carrot to the seed

bed. While the embryos were in the hydroxyethyl cellulose gel, treatments could be imposed that enhanced conversion of the embryos to plantlets. Such treatments included the addition of fungicide and nutrients and adjustment of temperature.

Chapter 10

OVERALL CONCLUSIONS

•Embryo length was associated with accumulated heat units.

•Solution fertilization *via* sub-irrigation with 200 mg $N \cdot L^{-1}$ from nitrate nitrogen (compared with 100 to 400 mg $N \cdot L^{-1}$ ammonium-N or 1 nitrate : 1 ammonium, resulted in the greatest plantlet height and width after 6 days of post *in vitro* growth in peat pellets.

•Nitrate- and ammonium-N, compared to the 1:1 combination, increased plantlet survival 5.2 percentage points to an average 16.5% from 10.3%.

• Nitrogen form or rate during the 7-day post *in vitro* period failed to affect the return of plants bearing DH kernels.

• The presence of mist (water or trace nutrient solution) failed to increase plantlet growth, plantlet survival or the return of plants bearing DH kernels.

•Ancillary studies showed that optimal *in vitro* conditions for plantlet production were: selection of embryos 4.0 to 4.5 mm long, constant 30° C, and the use of a antimicrobial material (Plant Protective Mix) in the agar medium.

APPENDIX

SOIL TEST REPORT
UNIVERSITY OF DELAWARE — SOIL TESTING LABORATORY
NEWARK, DELAWARE 19717-1303

BACKGROUND INFORMATION: Grower copy

FIELD NAME OR NO.	ACRES	COUNTY	DATE SAMPLED	DATE RECEIVED	DATE COMPLETE	LAB NO.	BAG NO.
DM HAPLOIS # 1	1	OUT OF STATE	6/05/07	6/08/07	03/14/10	73854	156293

SOIL TEST FOR: GROWER

DANIEL MONGEAU
982 NEW HOLLAND RD.
NEW HOLLAND PA 17557

ADDITIONAL COPY TO:

COUNTY AGENT

SOIL NAME	SOIL DRAINAGE	SOIL COLOR	SOIL TEXTURE	SAMPLE DEPTH	TILLAGE	PRESENT COVER	IRRIGATION	INJ. PUMP
	WELL	NORMAL		0- 8	CONV PLOW		TRKL	NO

LAST CROP	YIELD OF LAST CROP	MANURE TYPE	MANURE T/A	MANURE WHEN	LAST FERTILIZER N	LAST FERTILIZER P_2O_5	LAST FERTILIZER K_2O	LAST LIME MOS. AGO	LAST LIME T/A	LAST LIME TYPE	OTHER NUTRIENTS
CORN CONV TILL	150 BU							18+	0.0	UNK	

SOIL TEST RESULTS:

		INDEX VALUE	LOW	MEDIUM	OPTIMUM	EXCESSIVE
	pH	6.1	**********			
PHOSPHORUS	P	225	**********	**********	**********	**********
POTASSIUM	K	83	**********	**********	*****	
MAGNESIUM	Mg	125	**********	**********	**********	**********
CALCIUM	Ca	122	**********	**********	**********	**********

0 25 50 75 100 150

B	Mn	Zn	SO_4-S	% ORGANIC MATTER	SOL. SALTS MMHOS/CM	BUFFER pH	% Phosphorus Saturation	CEC meq/100gm	% Base Saturation	ENCLOSURES
	LBS/ACRE									
1.8	246.6	9.0	36.6	3.4		7.43	46.5	12.4	63.3	1,2,5,14,18

SUGGESTED FERTILIZER PROGRAM:

CROP: CORN, CONVENTIONAL TILLAGE
YIELD GOAL: 200 BU/A

LIME T/A	LIME TYPE	N LBS/A	P_2O_5 LBS/A	K_2O LBS/	S LBS/A	B LBS/A
0.0		200	0	0		

1. Phosphorus level in soil is excessive. Application of phosphorus in fertilizers or manures, other than starter fertilizers, is NOT RECOMMENDED.
2. Apply 1/4 to 1/3 of the recommended N at planting, the balance to be sidedressed when corn is 15 inches tall.
3. Manganese level in the soil at this pH is adequate.
4. Zinc deficiency is unlikely at this pH, soil zinc and soil phosphorus levels.

SOIL TEST REPORT

UNIVERSITY OF DELAWARE — SOIL TESTING LABORATORY
NEWARK, DELAWARE 19717-1303

BACKGROUND INFORMATION: Grower copy

FIELD NAME OR NO.	ACRES	COUNTY	DATE SAMPLED	DATE RECEIVED	DATE COMPLETE	LAB NO.	BAG NO.
DM HAPLOID # 2	1	OUT OF STATE	6/05/07	6/08/07	03/14/10	73855	156294

SOIL TEST FOR: GROWER

DANIEL MONGEAU
982 NEW HOLLAND RD.
NEW HOLLAND PA 17557

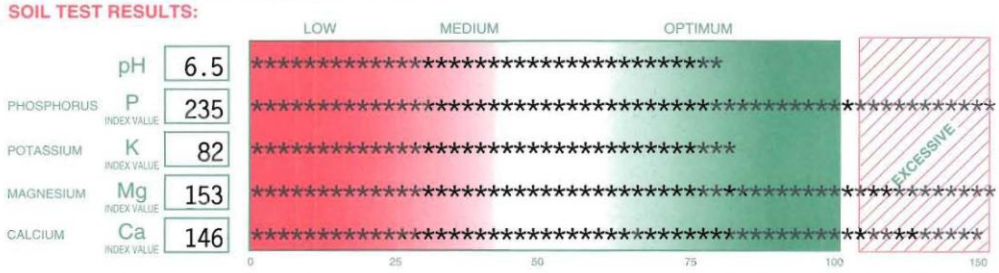
ADDITIONAL COPY TO:

COUNTY AGENT

SOIL NAME	SOIL DRAINAGE	SOIL COLOR	SOIL TEXTURE	SAMPLE DEPTH	TILLAGE	PRESENT COVER	IRRIGATION	INJ. PUMP
	WELL	NORMAL		0- 8	CONV PLOW		TRKL	NO

LAST CROP	YIELD OF LAST CROP	MANURE TYPE	MANURE T/A	MANURE WHEN	LAST FERTILIZER N	LAST FERTILIZER P_2O_5	LAST FERTILIZER K_2O	LAST LIME MOS. AGO	LAST LIME T/A	LAST LIME TYPE	OTHER NUTRIENTS
CORN CONV TILL	150 BU							18+	0.0	UNK	

SOIL TEST RESULTS:



B	Mn	Zn	SO_4-S	% ORGANIC MATTER	SOL. SALTS MMHOS/CM	BUFFER pH	% Phosphorus Saturation	CEC meq/100gm	% Base Saturation	ENCLOSURES
	LBS/ACRE									
2.1	236.6	9.9	30.8	2.8		7.53	52.5	13.1	71.3	1,2,5,14,18

SUGGESTED FERTILIZER PROGRAM:

CROP: CORN, CONVENTIONAL TILLAGE
YIELD GOAL: 200 BU/A

LIME T/A	LIME TYPE	N LBS/A	P_2O_5 LBS/A	K_2O LBS/	S LBS/A	B LBS/A
0.0		200	0	0		

1. Phosphorus level in soil is excessive. Application of phosphorus in fertilizers or manures, other than starter fertilizers, is NOT RECOMMENDED.
2. Apply 1/4 to 1/3 of the recommended N at planting, the balance to be sidedressed when corn is 15 inches tall.
3. Manganese level in the soil at this pH is adequate.
4. Zinc deficiency is unlikely at this pH, soil zinc and soil phosphorus levels.

SOIL TEST REPORT

UNIVERSITY OF DELAWARE — SOIL TESTING LABORATORY
NEWARK, DELAWARE 19717-1303

BACKGROUND INFORMATION: Grower copy

FIELD NAME OR NO.	ACRES	COUNTY	DATE SAMPLED	DATE RECEIVED	DATE COMPLETE	LAB NO.	BAG NO.
DM HAPLOID # 3	1	OUT OF STATE	6/05/07	6/08/07	03/14/10	73856	156295

SOIL TEST FOR: GROWER

DANIEL MONGEAU
982 NEW HOLLAND RD.
NEW HOLLAND PA 17557

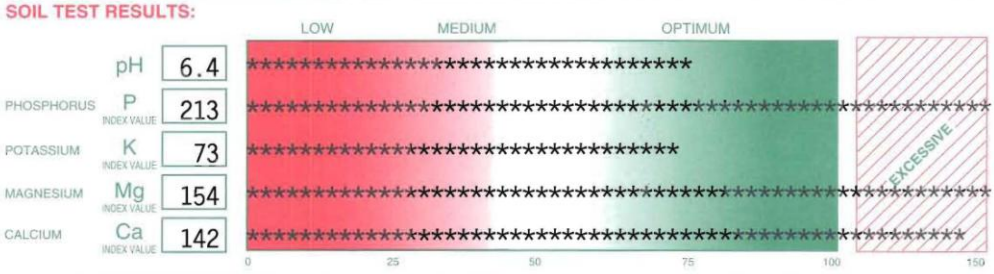
ADDITIONAL COPY TO:

COUNTY AGENT

SOIL NAME	SOIL DRAINAGE	SOIL COLOR	SOIL TEXTURE	SAMPLE DEPTH	TILLAGE	PRESENT COVER	IRRIGATION	INJ. PUMP
	WELL	NORMAL		0- 8	CONV PLOW		TRKL	NO

LAST CROP	YIELD OF LAST CROP	TYPE MANURE	T/A MANURE	WHEN MANURE	N LAST FERTILIZER	P_2O_5 LAST FERTILIZER	K_2O LAST FERTILIZER	MOS. AGO LAST LIME	T/A LAST LIME	TYPE LAST LIME	OTHER NUTRIENTS
CORN CONV TILL	150 BU							18+	0.0	UNK	

SOIL TEST RESULTS:



B	Mn	Zn	SO_4-S	% ORGANIC MATTER	SOL. SALTS MMHOS/CM	BUFFER pH	% Phosphorus Saturation	CEC meq/100gm	% Base Saturation	ENCLOSURES
	LBS/ACRE									
1.9	227.9	9.4	26.1	2.9		7.50	46.9	13.1	69.5	1,2,5,14,18

SUGGESTED FERTILIZER PROGRAM:

CROP: CORN, CONVENTIONAL TILLAGE
YIELD GOAL: 200 BU/A

T/A LIME	TYPE LIME	N LBS/A	P_2O_5 LBS/A	K_2O LBS/	S LBS/A	B LBS/A
0.0		200	0	0		

1. Phosphorus level in soil is excessive. Application of phosphorus in fertilizers or manures, other than starter fertilizers, is NOT RECOMMENDED.
2. Apply 1/4 to 1/3 of the recommended N at planting, the balance to be sidedressed when corn is 15 inches tall.
3. Manganese level in the soil at this pH is adequate.
4. Zinc deficiency is unlikely at this pH, soil zinc and soil phosphorus levels.

SOIL TEST REPORT

UNIVERSITY OF DELAWARE — SOIL TESTING LABORATORY
NEWARK, DELAWARE 19717-1303

BACKGROUND INFORMATION: Grower copy

FIELD NAME OR NO.	ACRES	COUNTY	DATE SAMPLED	DATE RECEIVED	DATE COMPLETE	LAB NO.	BAG NO.
DM HAPLOID # 4	1	OUT OF STATE	6/05/07	6/08/07	03/14/10	73857	156296

SOIL TEST FOR: GROWER

DANIEL MONGEAU
982 NEW HOLLAND RD.
NEW HOLLAND PA 17557

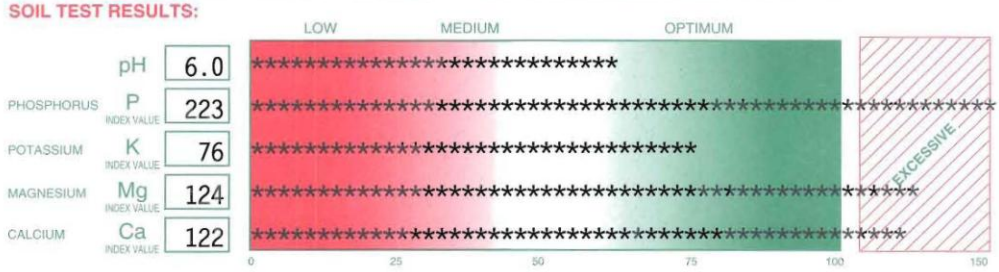
ADDITIONAL COPY TO:

COUNTY AGENT

SOIL NAME	SOIL DRAINAGE	SOIL COLOR	SOIL TEXTURE	SAMPLE DEPTH	TILLAGE	PRESENT COVER	IRRIGATION	INJ. PUMP
	WELL	NORMAL		0- 8	CONV PLOW		TRKL	NO

LAST CROP	YIELD OF LAST CROP	MANURE TYPE	MANURE T/A	MANURE WHEN	LAST FERTILIZER N	LAST FERTILIZER P_2O_5	LAST FERTILIZER K_2O	LAST LIME MOS. AGO	LAST LIME T/A	LAST LIME TYPE	OTHER NUTRIENTS
CORN CONV TILL	150 BU							18+	0.0	UNK	

SOIL TEST RESULTS:



B	Mn	Zn	SO_4-S	% ORGANIC MATTER	SOL. SALTS MMHOS/CM	BUFFER pH	% Phosphorus Saturation	CEC meq/100gm	% Base Saturation	ENCLOSURES
	LBS/ACRE									
1.8	221.4	8.4	32.4	3.0		7.44	47.2	12.3	63.5	1,2,5,14,18

SUGGESTED FERTILIZER PROGRAM:

CROP: CORN, CONVENTIONAL TILLAGE
YIELD GOAL: 200 BU/A

LIME T/A	LIME TYPE	N LBS/A	P_2O_5 LBS/A	K_2O LBS/	S LBS/A	B LBS/A
0.0		200	0	0		

1. Phosphorus level in soil is excessive. Application of phosphorus in fertilizers or manures, other than starter fertilizers, is NOT RECOMMENDED.
2. Apply 1/4 to 1/3 of the recommended N at planting, the balance to be sidedressed when corn is 15 inches tall.
3. Manganese level in the soil at this pH is adequate.
4. Zinc deficiency is unlikely at this pH, soil zinc and soil phosphorus levels.

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