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**TRANSFORMATION AND IN VITRO CULTURE STUDIES TO
ENHANCE WHITE MOLD RESISTANCE IN DRY BEANS
(*Phaseolus vulgaris* L.)**

presented by

ANN ROSELLE ORILLO ARMENIA

has been accepted towards fulfillment
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M.S. degree in PLANT BREEDING AND
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**TRANSFORMATION AND IN VITRO CULTURE STUDIES TO ENHANCE
WHITE MOLD RESISTANCE IN DRY BEANS (*Phaseolus vulgaris* L.)**

By

Ann Roselle Orillo Armenia

A THESIS

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ABSTRACT

TRANSFORMATION AND IN VITRO CULTURE STUDIES TO ENHANCE WHITE MOLD RESISTANCE IN DRY BEANS (*Phaseolus vulgaris* L.)

By

Ann Roselle Orillo Armenia

White mold, caused by *Sclerotinia sclerotiorum*, is a serious yield limiting disease in dry beans. Based on evidence that identifies oxalic acid as the major pathogenicity factor for white mold, this study aimed to engineer enhanced white mold resistance in dry beans by overexpressing the wheat oxalate oxidase gene, *gf-2.8*, using particle bombardment and electrotransformation approaches; and to evaluate effects of cytokinin and particle bombardment on the survival and growth of three bean cultivars *in vitro*. A total of 1,150 Matterhorn and Olathe bean plants were putatively transformed using the electrotransformation procedure. Screening for *bar* identified herbicide resistant plants (T₁) and PCR analysis confirmed the integration of *gf-2.8* in 18 Matterhorn and 11 Olathe plants, the majority of which resulted from Hormone (identity preserved) pretreatments of the apical meristems prior to electrotransformation. Four T₂ *gf-2.8* PCR positive plants were generated and the expression of *gf-2.8* was confirmed in 3 T₂ plants through RT-PCR. Indirect tests of T₂ and T₃ plants showed increased H₂O₂ production and reduction in lesion size suggesting enhanced white mold resistance. *In vitro* culture studies of three dry bean cultivars showed that 6-benzylaminopurine in the shoot regeneration media caused a reduction in shoot growth and lengthened the culture time requirement. Bombardment injuries to the cultivar Olathe causes a significant decrease in shoot growth and longer culture time. Red Hawk was observed to be a more suitable cultivar for *in vitro* culture and transformation efforts of dry beans with particle bombardment.

to
mom, dad, nonoy and bordi

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CHAPTER I

LITERATURE REVIEW

White mold, caused by the fungal pathogen *Sclerotinia sclerotiorum*, is one the most devastating diseases that seriously limit dry bean (*Phaseolus vulgaris* L.) production in temperate regions. Major genes conferring resistance to white mold have not been found in dry beans, however, minor genes providing partial resistance to the pathogen have been identified in various QTL (Quantitative Trait Loci) analyses (Ender and Kelly, 2005; Kolkman and Kelly, 2003, Park et al., 2001; Miklas et al., 2001). Thus, breeding efforts to improve partial resistance to white mold have mainly focused on the incorporation of physiological resistance and architectural traits that provide avoidance mechanisms to the disease (Kolkman and Kelly, 2002). To further enhance the partial resistance found in current cultivars, this study attempted to genetically engineer enhanced resistance to white mold in dry beans by introducing and overexpressing an oxalate oxidase gene from wheat. This chapter will review the importance of dry beans, the biology of *S. sclerotiorum*, the status of white mold resistance breeding in dry beans, the nature and significance of oxalate oxidases in disease resistance, plant transformation, and the current status of transformation and tissue culture in *Phaseolus*.

Dry Beans

Dry beans are one of the most important grain legumes for human consumption in the world. Total bean production in 2004 was 18.4 M metric tons (MT) of which 8.4M MT were produced in Latin America and Africa (FAO, 2004). In 2004, 548,000 hectares were planted to beans in the United States with about 1.8M metric tons total production

(NASS, USDA, 2004). In Michigan, the area planted to beans was 76,900 hectares in 2004 and 95,100 hectares in 2005 (NASS, USDA, 2005). Following North Dakota, Michigan is the second highest dry bean producing state of the US, producing 15% of the national output in 2002-2004.

Beans play a significant role in human nutrition because they are excellent and inexpensive sources of dietary proteins and essential vitamins and minerals. Most beans, when uncooked, contain about 20-25% protein making them a good substitute for animal-based protein sources (Haytowitz and Matthews, 1986). In addition to having only about 1% fat content, beans provide the important minerals calcium, iron, magnesium, phosphorus, potassium and zinc (Geil and Anderson, 1994). Studies have shown that consumption of dietary fiber, isoflavones and antioxidants present in dry beans may provide cardiovascular benefits (Anderson et al., 1999). Low glycemic indexes and high soluble dietary fiber in beans may contribute to the prevention and improvement of diabetic stages, could aid in weight control and may reduce risk of heart disease (Anderson et al., 1999; Bennink, 2005). Consumption of dry beans has also been linked to inhibition of various types of cancer including breast, prostate and colon cancers which have been attributed to their low glycemic acid and phytonutrient contents. Epidemiological studies have suggested that beans reduce the likelihood of certain types of cancers, these predictions are supported by a reduction in prostate and colon cancers in animal studies (Singh and Fraser, 1998; Bennink, 2002; Hangen and Bennink, 2003).

Major breeding objectives for genetic improvement in dry beans are focused on improving resistance to various diseases. White mold caused by *S. sclerotium* is the most serious disease that affects dry bean production. In Michigan, white mold is

considered by dry bean growers as the most important problem compared to other diseases that affect dry beans (Webster and Kelly, 2000). Although chemical control currently provides the best control strategy, often treatments are ineffective. Compared with resistance to other major bean diseases, white mold resistance in dry beans is still inadequate (Singh, 2001), hence, the necessity to breed for enhanced resistance to the white mold pathogen.

Sclerotinia sclerotiorum

Sclerotinia sclerotiorum is an aggressive fungal pathogen that is capable of infecting a wide range of hosts (Purdy, 1979; Bolton et al., 2006) including soybean, sunflower, peanut, canola and dry beans. In dry beans, the cottony mycelium of by *S. sclerotiorum* has led to the common name, white mold.

Life Cycle. *S. sclerotiorum* is a necrotrophic ascomycete that produces large sclerotia and lacks a functional conidia or other sexual spores. The formation of sclerotia, the perpetuating structures of *S. sclerotiorum*, is a very important survival mechanism for the fungus. A sclerotium is a compact mass of fungal mycelium capable of surviving many years in the soil (Steadman, 1983). Studies have shown that environmental factors such as light, moisture and temperature affect apothecial development in *S. sclerotiorum*. Sclerotial germination requires adequate soil moisture and cool temperatures which usually occur during partial to complete row cover of bean crop (Schwartz and Steadman, 1978). Germination of the sclerotia leads to the production of apothecial stalks bearing ascospores (Figure 1). An increase in relative humidity in the environment then triggers mature ascospores to be released, creating a “puffing” phenomenon which assists in spore dispersal (Steadman, 1983). The germinating ascospores require an exogenous energy

source such as injured or senescent plant tissues (e.g. flowers) which provide the necessary nutrients required for the development of fungal mycelia in order to successfully colonize a bean plant. Following colonization, the fungus can then begin to infect green pods, leaves, and stems. Sclerotia are then produced from diseased plant tissue.

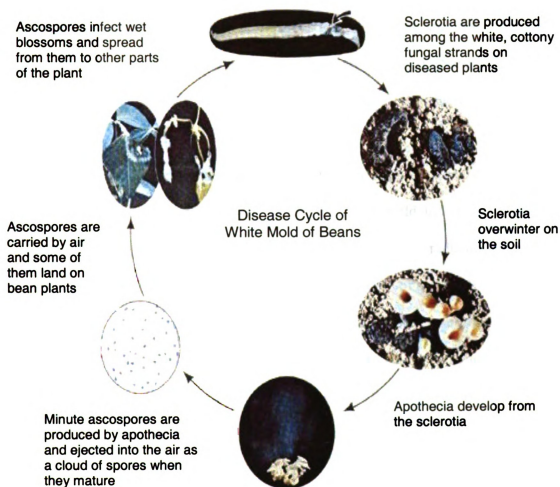


Figure 1. Life cycle of *S. sclerotiorum* on beans (Abawi and Hunter, 1979).

Signs and Symptoms. The penetration of host tissues by the fungal pathogen and subsequent enzymatic processes that occur during infection result in rapid disorganization of tissues (Purdy, 1979). The first symptoms observed in most hosts of the pathogen are watersoaked spots. These watery spots or lesions tend to enlarge and become rotted and are later covered by fungal mycelia. Signs of the fungus, in association with almost all hosts, are copious amounts of white cottony mycelium and the subsequent production of black sclerotia. Infection of stems and branches leads to wilting and in some cases plant death with stems and branches appearing dry and bleached (Steadman, 1983). *S. sclerotiorum* causes major yield losses in dry beans as a consequence of the reduced number of seeds and seed weight (Kerr et al., 1978).

Role of Oxalic Acid on Pathogenicity. Oxalic acid production by *S. sclerotiorum* has been implicated as a pathogenicity factor for white mold infection (Maxwell and Lumsden, 1970) and is thought to aid in the fungal virulence by reducing the apoplastic pH to levels more suitable for pectolytic enzyme degradation of plant cell walls (Bateman and Beer, 1965; Bateman and Millar, 1966). Mutants of *S. sclerotiorum* deficient in oxalic acid production were used to confirm that oxalic acid is a major pathogenicity factor for white mold infection (Godoy et al., 1990). Oxalic acid also has been found associated with chloroplast degeneration caused by the rupturing of chloroplast membranes (Tariq and Jeffries, 1985; Tu, 1989).

Tolerance of the white bean cultivar Ex Rico 23 to *S. sclerotiorum* was noted to be associated with tolerance to oxalic acid and to restricted movement of oxalic acid from veins to interveinal tissues (Tu, 1985). In a study comparing white mold resistant and susceptible dry bean cultivars, chloroplast and plasma membrane stability was associated

with tolerance to oxalic acid (Tu, 1989). Foliar dehydration has been attributed to oxalic acid due to its capability to deregulate guard cells and inhibit abscisic acid-induced stomatal closure (Guimaraes and Stotz, 2004). Oxalic acid also suppresses the active oxygen generation of host plants (Cessna et al., 2000) thereby compromising major host defense responses.

Therefore, an alternative breeding strategy to develop dry bean cultivars with resistance to white mold would be to incorporate genes coding oxalic acid degrading enzymes, whether from existing dry bean germplasm or from novel sources, through plant transformation.

Oxalate Oxidase (OXO)

Oxalate oxidases (OXO) are enzymes that are capable of oxidizing oxalic acid into hydrogen peroxide (H_2O_2) and carbon dioxide possibly leading to a reduction of the toxic effects of oxalic acid-producing pathogens. A small group of homologous proteins (Lane, 2002), with peroxide-generating and oxalate oxidase activity, are found in the cereals: barley, maize, oat, rice, rye, and wheat. These proteins are often referred to as germins (G-OXO) and have been known as a marker of growth onset in germinating seeds of cereals (Thompson and Lane, 1980; Lane, 1994). Two full-length germin sequences (*gf-2.8* and *gf-3.8*) have been cloned from wheat and *gf-2.8* was found to have apoplastic (Lane et al., 1986) and protease resistance properties (Grzelczak and Lane, 1984). A thermostable, protease-resistant oxalate oxidase with high homology to the wheat germin protein also has been isolated from barley (Woo et al., 1998; Dumas et al., 1993).

Role in Plant defense. The induction of oxalate oxidase and oxalate oxidase-like proteins have been implicated in plant defense against a variety of pathogens (Ramalingam et al., 2003; Zhou et al., 1998; Hurkman and Tanaka, 1996). Gel-based and quantitative spectrophotometric enzyme assays on germin OXOs and germin-like proteins (GLPs) have provided evidence of a superoxide dismutase activity which is involved in the response of plants to biotic and abiotic stresses (Woo et al., 2000). Berna and Bernier (1999) have previously shown upregulation of expression of the wheat OXO (*gf-2.8*) by biotic and abiotic stresses including heavy metals, polyamines, wounding and infection with Tobacco Mosaic Virus (TMV) in transgenic tobacco. Studies of wood-rotting basidiomycetes suggested that the breakdown of oxalic acid by oxalate oxidases delays wood decay by neutralizing the pH around the infection sites (Shimada et al., 1994). This capability to neutralize apoplastic pH could possibly lead to inhibition or delay of degradation of plant cell walls by pectolytic enzymes.

In addition to its ability to degrade oxalic acid, oxalate oxidases subsequently generate hydrogen peroxide (H_2O_2). H_2O_2 is a major participant in the oxidative burst, the production of reactive oxygen species related to host defense response, thereby making oxalate oxidase a vital agent for disease resistance. The production of H_2O_2 promotes lignification and cross linking in host cell wall proteins providing a generalized barrier against fungal penetration. In addition, studies have shown that H_2O_2 contributes to host plant resistance through a hypersensitive response by promoting a localized cell necrosis in resistant plant cultivars, thus forming a barrier that limits fungal invasion to sites of infection (Bolwell and Wojtasek, 1997; Bolwell et al., 2002; Levine et al., 1994; Tenhaken et al., 1995). Moreover, H_2O_2 is involved in the induction of systemic acquired

resistance (SAR) by acting as a diffusible signal (Alvarez and Lamb, 1997; Lamb and Dixon, 1997) and has also been suggested to trigger downstream components of the defense pathway and induce expression of defense related genes associated with SAR (Chen et al., 1993). Increased endogenous production of H₂O₂ in transgenic potato and tobacco expressing a peroxide-generating glucose oxidase gene resulted in accumulation of salicylic acid and expression of PR proteins which are commonly associated with the induction of SAR during plant defense (Kazan et al., 1998).

Breeding for White Mold Resistance

Resistance of dry beans to white mold may be achieved by combining disease avoidance and physiological defense mechanisms. Disease avoidance can limit fungus establishment and disease development and is primarily influenced by plant architecture, phenological traits, and agronomic management practices (Kolkman and Kelly, 2002). These traits include erectness, canopy elevation and porosity and late or early flowering. In a previous study, Fuller et al. (1984) showed that the elevation of canopy in a highly susceptible indeterminate great northern bean lead to a reduced white mold infection, whereas, increased plant height is associated with lower white mold levels in the field in dry bean Recombinant Inbred Line (RIL) populations (Miklas et al., 2001; Park et al., 2001). Moreover, the development of favorable microclimates within dense canopies is associated with higher white mold severity (Park, 1993; Coyne, 1980; Coyne et al., 1974). Physiological resistance, however, is controlled by biochemical factors and may be conditioned by increased activities of plant defense related enzymes (Miklas et al., 1993) and accumulation of phytoalexins (Sutton and Deverall, 1984).

Various QTL analyses have been conducted to identify possible QTL contributing to physiological resistance and avoidance traits to white mold in dry beans. In the study by Miklas et al. (2001), QTL conferring physiological resistance and avoidance mechanisms have been identified in the A55 x G122 dry bean RIL population. A QTL contributing 38% of phenotypic variation for disease score in greenhouse tests was identified on linkage group B7. The identified QTL on linkage group B7 and another on B1 conditioned 26% and 18% of variability for field resistance, respectively. QTL for white mold resistance conditioned by both physiological resistance as well as disease avoidance have also been identified in a PC-50/XAN-159 RIL population (Park et al., 2001). In this study, QTL conferring white mold resistance in the field were mapped on B4, B7, B8 and B11 linkage groups whereas the major QTL on B7 accounted for 12% of the phenotypic variation. QTL for avoidance traits such as plant height and canopy porosity were also identified on linkage groups B2, B5, B7, B8 and B10. In the Bunsu x Newport and Huron x Newport RIL populations, markers linked to QTL conferring partial white mold resistance were identified on B2 and B7 (Kolkman and Kelly, 2003). In both linkage groups, the markers were associated with QTL for reduced Disease Severity Index (DSI) while, on B7, the markers were associated with QTL for oxalate resistance, yield, days to flowering, branching pattern, lodging and seed size. Moreover, QTL that accounted for 9 to 15% of phenotypic variation were mapped to B2, B5, B7 and B8 using a Middle American bean RIL population (Ender and Kelly, 2005).

Breeding strategies for improving white mold resistance in dry beans have therefore focused on combining architectural avoidance mechanisms with physiological resistance. Currently, minor genes identified in various QTL analyses provide partial

resistance to the white mold pathogen in dry beans. Studies in the scarlet runner bean (*P. coccineus*), however, have reported a single dominant gene controlling white mold resistance in the species (Abawi et al., 1978) suggesting a possible source of resistance to white mold for possible introgression into *P. vulgaris*. Although this resistance was reported in the hybrid *P. vulgaris* x *P. coccineus*, F₂ and back cross populations, the resistance was not confirmed in later generations. Consequences of genetic background and difficulty in crossing between secondary gene pools of *Phaseolus* have proved to be hindrances to this breeding strategy. Therefore, in the enhancement of white mold resistance in current dry bean cultivars, breeders should consider novel sources of resistance such as those provided by oxalate oxidases.

Plant Transformation

Plant transformation technology has provided various benefits in the study of gene function as well as in crop improvement. With genetic engineering, it is now possible to incorporate novel genes and DNA sequences into diverse organisms. Plant transformation serves as an experimental tool in the genetic analyses of cell signals in addition to studies on enzyme and hormone function in development. Transformation has also been used to introduce economically desirable traits into crop plants (reviewed by Birch, 1997). Since the establishment of reproducible transformation protocols in many crop plants, the number of transgenic crops produced commercially and acreage of planting has increased. In 2005, a total of 90 million hectares was approved for planting to genetically modified (GM) crops in over 20 countries (James, 2005). The increase in growth rate of global GM production areas since its commercialization in 1996 implies

improved productivity leading to economic, environmental and social benefits provided by GM crops.

There are various methods through which desirable genetic sequences can be transferred to plants, most of which require a tissue culture stage. Totipotency, a single cell's ability to carry out all the stages of development, underlies the success of the major transformation techniques developed to date. Somatic embryogenesis and organogenesis are the two important developmental pathways in the production of transgenic plants (Hansen and Wright, 1999). The three most commonly used transformation systems are protoplast transformation, *Agrobacterium*-mediated transformation and particle bombardment.

Protoplast Transformation. Protoplast transformation requires the production of protoplasts through mechanical or enzymatic processes from immature embryos, immature inflorescences, mesocotyls, immature leaf bases or anthers (Hansen and Wright, 1999; Maheshwari et al., 1995). Successful transformations have been achieved with this method in rice (Abdullah et al., 1986), *Arabidopsis* (Damm et al., 1989), and tobacco (Hu et al., 1996). However, limitations of this method in many other crops include problems associated with plant regeneration and transient gene expression (Rakoczy-Trojanowska, 2002).

***Agrobacterium*-mediated Transformation.** *Agrobacterium*-mediated transformation system is a complex method where success is based on finding genetic determinants in the bacterium and host cell. The vector used in this system is a tumor inducing (Ti) plasmid which contains a T-DNA, the portion of the plasmid that can be constructed to specifically contain the gene of interest and transferred from the bacterium

into the host cell with the aid of virulence genes expressed in wounded plant cells (Gelvin, 2000; Hansen and Wright, 1999). This system has been used to successfully transform crops like rice (Hiei et al., 1994), *Arabidopsis* (Valvekens et al., 1988), corn (Gould et al., 1991), and soybean (Donaldson et al., 2001). *Agrobacterium*-mediated transformation is the major method used due to its capability to insert single copies of the transgene into the host plant thereby avoiding the possibilities of suppression or changes of transgene expression. However, problems associated with genotype dependency of *Agrobacterium* strains create some limitations to the application of this method in all crops.

Biolistic Transformation. The biolistic transformation technique, also known as particle bombardment, involves the bombardment of plant cells or tissues with microparticles in the form of gold or tungsten which are coated with the genetic sequence(s) of interest. Delivery of the particles is achieved by acceleration provided by gunpowder, helium gas or electric discharge (Hansen and Wright, 1999). Successful and highly efficient transformation with this method has been obtained in crops like corn and wheat (Wright et al., 2001). Biolistic transformation can also be conducted on a broad range of cell and tissue types and in certain cases do not require a plant regeneration step from single cell or callus tissues, thus making it a highly suitable system for plants without established regeneration protocols. Bombardment of apical meristems excised for mature dry bean embryos has led to production of transgenic beans although with very low efficiency (Aragao et al., 2002). Due to the physical nature of the transformation procedure, this method is not limited by plant genotype (Christou, 1992), however, the

complex and unpredictable patterns of transgene integration often results in integration of multiple copies leading to transgene silencing (Reddy et al., 2003).

Transformation for Disease Resistance

Plant disease is a major constraint of crop production. Disease infection in an agricultural field can result in minor crop loss or even total crop failure. Thus, many breeding programs include breeding for disease resistance to major plant pathogens. With the advent of biotechnology, plant breeders have turned to plant transformation approaches to engineer disease resistance.

Antifungal and antibacterial proteins produced by a wide variety of fungi and bacteria provide excellent sources of transgenes for disease resistance breeding. Genetic studies on the antifungal properties of *Trichoderma* spp. have led to the purification of enzymes with various inhibitory roles to a number of plant pathogens (Lorito et al., 1998). Transformation work with the endochitinase gene (*ThEn-42*) from *T. harzianum* resulted in a high and broad range of resistance to the pathogens (*Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and *Rhizoctonia solani*) in potato and tobacco. Overexpression of a chimeric endochitinase gene in oilseed rape (*Brassica napus* var. *oleifera*) increased tolerance to the fungal pathogens *Cylindrosporium concentricum*, *Phoma lingam*, and *S. sclerotiorum* (Grison et al., 1996). Other microbial genes that have been expressed in plants for enhanced resistance to pathogens include glucose oxidase from *Aspergillus niger*, ribonuclease (*pac1*) from *Schizosaccharomyces pombe*, trichodiene synthase (*Tri5*) from *Fusarium sporotrichioides*, killer toxin from *Ustilago maydis*, and *Bt* toxin from *Bacillus thuringiensis* (reviewed by Lorito and Scala, 1999).

Disease resistance breeding through transformation has also employed genes involved in the successful infection of plant pathogens. Transformation of tobacco with the *expI* gene which encodes a signal involved in quorum sensing of *Erwinia carotovora* led to increased resistance to the pathogen (Mäe et al., 2001). Coat protein-mediated resistance has been used to provide enhanced host plant resistant to viruses (Gonsalves and Slightom, 1993). Transgenic tomato expressing the coat protein of the U1 strain of TMV exhibited partial resistance to infection and disease development of TMV and tomato mosaic virus (ToMV) (Nelson et al., 1988). Similarly, transformation in papaya with the papaya ringspot virus (PRSV) coat protein gene resulted in a broad range of resistance to PRSV isolates from Hawaii, Thailand and Mexico (Bau et al., 2003).

Another mode for breeding disease resistance into plants has focused on genes that are capable of inactivating toxins produced by plant pathogens or improving the host's tolerance to the pathogen. For instance, the gene encoding a tabtoxin acetyltransferase from *Pseudomonas syringae* pv. *tabaci* (*pst*) detoxifies tabtoxin, a toxin produced by *pst* pathogen. The transgene was shown to provide resistance to *pst* in transgenic tobacco plants (Anzai, 1989). Moreover, transformation of tobacco with *argK*, an enzyme involved in arginine biosynthesis, provided resistance to *Pseudomonas syringae* pv. *phaseolicola* through detoxification of the phaseolotoxin produced by the fungal pathogen (de la Fuente-Martinez et al., 1992).

Plant Transformation with OXOs

Following the isolation and cloning of oxalate oxidases from wheat and barley, efforts have been made to transform various crop plants in order to enhance resistance to oxalic acid-generating pathogens like *S. sclerotiorum*. Improved resistance to exogenous

application of oxalic acid, as exhibited by resistance to wilting, has been demonstrated in transgenic canola expressing a barley oxalate oxidase gene (Thompson et al., 1995). Donaldson et al. (2001) reported improved resistance to white mold, relative to wild type, against leaf and stem inoculation of *S. sclerotiorum* in soybean using a wheat G-OXO (*gf-2.8*) gene. The report established that transgenic soybean had greatly reduced disease progression and lesion length after inoculation with *S. sclerotiorum*. Transformation of the hybrid poplar clone, *Populus x euramericana* cv. 'Ogy' with wheat OXO resulted in increased tolerance to oxalic acid and a more efficient increase in apoplastic pH to neutral levels compared to the wild type. In addition, improved resistance to the oxalic acid-producing poplar pathogenic fungus, *Septoria musiva*, was observed in the OXO-transformed plants (Liang et al., 2002). Studies of sunflower transformed with *gf-2.8* have shown increased accumulation of H₂O₂, salicylic acid, and defense gene transcripts which were associated with hypersensitive response-like lesions. Moreover, this accumulation was closely associated with a progressive increase of oxalate oxidase activity during plant development and the transgenic sunflower plants produced exhibited enhanced resistance to white mold (Hu et al., 2003). Recently, transformation of peanut (*Arachis hypogaea*) with the barley oxalate oxidase gene resulted in enhanced resistance to the *Sclerotinia* blight pathogen, *S. minor*. Detached leaf assays against oxalic acid and fungal mycelia resulted in as much as 97% reduction of lesion size (Livingstone et al., 2005). Due to the success of OXOs in providing enhanced resistance to oxalic acid-producing pathogens like *S. sclerotiorum* using plant transformation, this study aims to improve white mold resistance in dry beans using a similar approach.

Transformation in *Phaseolus*

Currently, there are only a few reports on successful transformation within the genus *Phaseolus*. Early efforts to transform of *P. vulgaris* and *P. acutifolius* (tepany bean) employed *Agrobacterium*-mediated systems, electroporation and PEG-mediated protoplast transformation (Aragao, 2001). However, these attempts either failed to provide molecular evidence for genetic transformation or progeny analysis or were unable to produce transgenic plants due to regeneration problems. Recently, modest success has been attained in *P. acutifolius* using *Agrobacterium*, and in *P. vulgaris* using particle bombardment/biolistic methods (Zambre et al., 2005; Aragao et al., 2002).

***Agrobacterium*-mediated transformation.** *Agrobacterium*-mediated transformation has long been attempted in dry beans but no successful reports have been published to date. However, stable transformation has been successfully achieved only in tepary beans (*P. acutifolius*) (Zambre et al., 2005). Successful transformation with *Agrobacterium*-mediated systems was obtained using *Agrobacterium* strain C58C1Rif^R in six tepary bean genotypes. Transient expression of *uidA* was detected in calli explants for five out of the six genotypes tested (Dillen et al., 1997). These procedures were optimized at 22°C, 16-8 hr photoperiod in acidic medium with co-cultivation in the presence of acetosyringone, and molecular tests confirmed the stable integration and expression of *gus* in primary transgenes and T1 progenies (De Clercq et al., 2002). Recently, Zambre et al. (2005) developed a reproducible transformation system for cultivated tepary bean with different *arcelin* genes for resistance to the Mexican bean weevil.

Biolistic Transformation. Transgenic bean plants were first obtained from navy bean (*P. vulgaris* cv. Seafarer) transformed using an electrical particle acceleration device (Russel et al., 1993). However, the frequency of transgenic plants obtained was very low (0.03% transformation efficiency) and the tissue culture protocol involved was time-consuming, required several temperature treatments and media transfers of the bombarded embryos prior to achieving transgenic shoots.

Embryonic axes of the French bean (*P. vulgaris* cv. Goldstar) were transformed using gold particle bombardment (Kim and Minamikawa, 1996). Unfortunately, most shoot apices obtained were chimeric showing both transformed and non-transformed sectors and only 0.5% of the explants produced transgenic seeds. The biolistic or particle bombardment method involving tungsten microparticles has also been utilized to transform apical meristems of the dry bean cv. Olathe (Aragao et al., 1996, 1998). Transformation was performed with neomycin phosphotransferase II (*neo*) and β -glucuronidase (*gus*) marker genes, the methionine-rich 2S albumin gene from Brazil nut and the antisense sequence of genes from the bean golden mosaic geminivirus (BGMV). In a co-transformation study 40% to 50% co-transformation efficiency was obtained with unlinked genes and 100% with linked genes (Aragao et al., 1996). Introduction of resistance to BGMV with Rep-TrAP-REN and BC1 antisense viral genes resulted in the production of 16 independently transformed bean plants which when challenged-inoculated with BGMV exhibited delayed and attenuated symptoms (Aragao et al., 1998).

Transgenes encoding a methionine-rich storage albumin from the Brazil nut (*Bertholletia excelsa* H. B. K.) was introduced in dry beans using the biolistic approach mentioned earlier (Aragao, 1999) in an effort to correct a deficiency for essential amino

acids like methionine and cysteine in beans. Stable transformation and expression exhibited by significantly increased methionine content (14% and 23%) was observed in two of the five transgenic lines. Recently, Aragao et al. (2002) transformed dry bean cvs. Olathe and Carioca for resistance to the herbicide glufosinate ammonium using a biolistic approach. Only 0.6% of the regenerated plants from Olathe were resistant to the herbicide, and of these 24 plants, only two were tolerant in the first sexual generation (T_1). Nevertheless, these plants were resistant to herbicide in both glasshouse and field trials and have now been incorporated in the bean breeding program in Brazil.

Electroporation. Electroporation is a gene transformation technique which involves brief, high intensity electrical pulse(s) to create transient pores in the cell membrane thereby facilitating the entry of exogenous DNA (Prassana and Panda, 1997). Electroporation has been successfully used in crops like rice (Muniz de Padua et al., 2001; Li et al., 1991) and corn (Sawahel, 2002). This transformation method is dependent on efficient callus production and regeneration systems whereby, transient or stable expression of exogenous genes have been obtained from electroporation of cells from suspension cultures, protoplasts, and pollen in various crop plants. However, successful *in planta* transformation involving *in vivo* transformation of plants as they are developing normally has been achieved in a number of crops using electroporation. *In planta* transformation by electroporation has been achieved in pea, lentil, cowpea and soybean nodal meristems (Chowrira et al., 1995, 1996). Transient transformation of kidney bean (*P. vulgaris* cv. Giza3) was also obtained using electroporation of callus derived from shoot tips (Saker and Kuhne, 1997).

Tissue Culture in *Phaseolus vulgaris*

A reproducible and efficient *in vitro* regeneration protocol is one of the vital factors for successful transformations, particularly for methods such as *Agrobacterium*-mediated transformation and electroporation which rely on shoot induction from cell suspensions or callus. Due to the absence of a reliable *in vitro* regeneration protocol in beans, transformation in this economically important species has been difficult to achieve to date. However, transgenic plants have been obtained from callus with acceptable efficiency in other grain legume plants including *Glycine max* (Donaldson et al., 2001), *Pisum sativum* (Schroeder et al., 1993), and *P. acutifolius* (Dillen et al., 1997). Among the early reports on *in vitro* shoot production in *P. vulgaris* (McClean and Grafton, 1989; Franklin et al., 1991; Malik and Saxena, 1992; Mohamed et al., 1993), only one report (Mohamed et al., 1993) involved production of shoots from regeneration competent callus obtained from pedicels of *P. vulgaris*.

Using mature embryo explants on medium containing thidiazuron and indole-3-acetic acid, regeneration competent callus was obtained from *P. vulgaris* and *P. acutifolius* (Zambre et al., 1998). The *in vitro* produced plantlets from *P. vulgaris*, however, required *in vitro* grafting with epicotyls and hypocotyls of 4-day old seedlings due to hardening problems in the greenhouse. Plant regeneration has been achieved using intact seedling and cotyledonary node explants of *P. vulgaris* cvs. Fönix and Maxidor on MS media supplemented with benzylaminopurine and naphthaleneacetic acid (Ahmed et al., 2002). Recently, successful *in vitro* cultivation and regeneration was achieved from petioles explants of *P. vulgaris* Bulgarian cvs. Plovdiv 10, Plovdiv 11M and Dobroudjanski 7 (Veltcheva and Svetleva, 2004).

Transformation in crops without a well established regeneration system

Currently, difficulties in transformation still exist in crops where reproducible and efficient transformation and tissue culture protocols have yet to be established. Even when successful regeneration approaches have been reported, problems relating to reproducibility and efficiency of the protocols for *in vitro* shoot regeneration as well as genotype-dependency of developed systems provide difficulties in producing transgenic plants using methods relying on regeneration procedures. Thus, breeding strategies to introduce foreign DNA into dry beans using plant transformation should consider approaches that do not involve a tissue culture or regeneration step. Due to these difficulties, various alternative transformation techniques which include infiltration, electroporation of cells and tissues, electrophoresis of embryos, microinjection, pollen-tube pathway, silicon carbide-mediated transformation (Rakoczy-Trojanowska, 2002) and the Ac/Ds transposable elements transposition system (Lebel et al., 1995) have been developed. This study aims to enhance white mold resistance in dry beans using the novel electrotransformation protocol (Allison, personal communication), a transformation approach that targets cells in the apical meristem that are destined for meiosis and does not involve a tissue culture step.

CHAPTER II

ELECTROTRANSFORMATION AND BIOLISTIC APPROACHES FOR INTRODUCING *gf-2.8* INTO DRY BEANS (*Phaseolus vulgaris* L.) FOR ENHANCED WHITE MOLD RESISTANCE

INTRODUCTION

Production of dry beans has been constantly threatened by various biotic and abiotic stresses resulting in major crop losses. One of the diseases that seriously affects dry bean productivity is white mold caused by *Sclerotinia sclerotiorum*, an aggressive fungal pathogen that infects hundreds of plant species worldwide (Steadman, 1983; Purdy, 1979). Current breeding strategies for white mold resistance in dry beans involves combining physiological resistance conditioned by Quantitative Trait Loci or QTL (Miklas et al., 2001; Park et al., 2001; Kolkman and Kelly, 2003 Ender and Kelly, 2005) with architectural avoidance mechanisms (Coyne, 1980; Kolkman and Kelly, 2002; Kelly, 2000). However, low and variable QTL effects for resistance to white mold have limited breeding efforts to combine QTL for resistance. Thus, breeders need to go beyond the traditional genetic approaches and consider transgenic breeding methods to enhance resistance to white mold in dry beans.

Studies of *S. sclerotiorum* oxalic acid-deficient mutants have identified oxalic acid as an important pathogenicity factor in white mold (Godoy et al., 1990). Oxalic acid, which is a strong chelator of Ca^{2+} , induces wilting as a consequence of lowering the apoplastic pH to a value suitable for enzymatic degradation of plant cell walls (Bateman and Beer, 1965; Maxwell and Lumsden, 1970). In addition, oxalates suppress the oxidative burst of the host plant thereby compromising one of the host's natural defense responses (Cessna et al., 2000).

Genetic engineering techniques have been utilized to introduce exogenous genes for desirable agronomic traits in crop plants. An obvious breeding strategy for white mold resistance is the development of a transgenic dry bean which produces an enzyme that degrades oxalic acid. Lane (2002) has demonstrated that oxalate oxidases (OXO's) and the germin OXO (G-OXO) gene are involved in defense responses to white mold in cereals. The product of the germin gene is an oxalate oxidase that degrades oxalic acid into water and hydrogen peroxide (H_2O_2). By transducing the hypersensitive response, H_2O_2 promotes a localized cell necrosis in resistant cultivars, thereby limiting the site of fungal invasion (Bolwell and Wojtaszek, 1997). The production of H_2O_2 is a key in plant defense as an inducer of cellular protection genes and a hypersensitive response (Levine et al., 1994; Tenhaken et al., 1995). Since the identification and cloning of germin genes from wheat and barley, a few crops have been successfully transformed with oxalate oxidase including canola, peanut, soybean and sunflower (Thompson et al., 1995; Livingstone et al., 2005; Donaldson et al., 2001; Hu et al., 2003).

Only a few reports appear in the literature on the successful transformation of *Phaseolus*. Earlier efforts to produce transgenic bean plants have failed due to inefficient transformation systems and regeneration problems (Aragao, 2002). In contrast, researchers have successfully optimized stable transformation of the tepary bean (*P. acutifolius*) using *Agrobacterium* (Dillen et al., 1997, Zambre et al., 2005). The use of tepary bean as a 'bridging species' to introduce transgenes into economically more important common bean species has been suggested, but no successful reports have surfaced to date. Due to lack of success in regeneration and *Agrobacterium*-mediated transformation of *P. vulgaris*, other methods have been pursued. Biolistic methods have

been used to transform dry beans that have generated successful transformants. Russel et al. (1993) employed electrical-discharge particle acceleration, however, the tissue culture regeneration protocol involved was tedious, time consuming, and genotype dependent. Separate transformations for resistance to bean golden mosaic gemini virus and the herbicide glufosinate ammonium have also been reported in dry beans using particle bombardment method (Aragao et al., 1998, 2002). Stable integration of a transgene coding for a methionine rich storage albumin from Brazil nut was obtained by Aragao et al. (1999) using biolistic methods. However, the frequencies of transgenic plants obtained from these studies were quite low.

Electrotransformation is a novel transformation procedure developed in the Allison Laboratory (Plant Biology, MSU) that involves long-time exposure of plants to electrical charge. This procedure has shown potential in transformation of large-seeded legumes. In unpublished data, *gus* and *bar* transformed plants have been obtained using this method with cowpea (Richard Allison, personal communication). Electrotransformation targets cells in the apical meristem of a seedling including those that are destined for meiosis. Transformation of pre-meiotic cells insures that the introduced trait will be inherited by the progeny. One feature that makes electrotransformation a more useful system particularly in crops like dry beans where efficient plant regeneration protocols have yet to be developed, is the lack of a tissue culture requirement.

The study aims to investigate the use of electrotransformation and biolistic methods to transform dry beans using the wheat G-OXO gene (*gf-2.8*) to enhance resistance to white mold. Specific objectives of the study were to: a) develop a

transformation plasmid with *gf-2.8* and *bar* as the reporter gene, b) develop a reproducible protocol for transformation in dry beans; c) evaluate effects of culture and transformation conditions in efficiency of transformation; and c) evaluate white mold resistance in putative transgenic plants.

MATERIALS AND METHODS

Plant Material

The dry bean (*Phaseolus vulgaris* L.) cvs. Matterhorn and Olathe were used. Matterhorn is a white medium-seeded, high yielding, widely adapted great northern cultivar (Kelly et al., 1999) while Olathe is a semi prostrate indeterminate pinto (Wood and Keenan, 1982). Both cultivars are highly susceptible to white mold.

Plasmid Construction

The 1.7 kb *EcoRI/HindIII* fragment, consisting of *gf-2.8* linked to the cauliflower mosaic virus (CaMV) 35S enhanced promoter and 3' terminator region, from pRD400/35S-*gf-2.8* (Donaldson et al., 2001) was excised and ligated into pCAMBIA3300 (CAMBIATM) that had been linearized with *EcoRI/HindIII*. The 4.8 kb *SspI/HindIII* fragment, including the *bar* gene linked to the 35S promoter and *nos* terminator was excised from pCAMBIA3300 (CAMBIATM) and subcloned to pBluescriptKSII(-) (Stratagene) previously linearized with *SspI/HindIII*. The *bar* gene encodes a phosphinothricin acetyl transferase known to confer tolerance to the herbicide glufosinate ammonium. The resulting plasmid, pBKS*bar/gf-2.8* carried the enh35S-*gf-2.8* transgene and the selectable marker gene *bar* (Figure 2).

Electrotransformation

Electrotransformation was conducted using the protocol developed by Richard Allison (Department of Plant Biology, Michigan State University). This procedure involved the use of electrotransformation units designed and built to MSU specifications by the Hyland Seed Company (Blenheim, Ontario).

Dry bean seeds were planted in pots containing autoclaved soil (BACCTO High Porosity Professional Planting Mix, Michigan Peat Company, Houston, TX). After 5 to 8 days, the seedlings were removed slowly from the soil, washed and placed in a transformation tube constructed from a 50ml polypropylene tube. One microliter of SspI/HindIII digested plasmid DNA (25 ng/ μ l) was mixed with 49 μ l of 1% TAE agarose and allowed to set at the tip of a 200 μ l pipette tip with a widened tip. Prior to electrotransformation various pretreatments of the apical meristem were performed (Table 1). The terminal apical meristem of the seedling was then inserted in the widened tip. Both the pipette tip and the transformation tube were filled with 1X TAE transformation buffer, connected to a power source and allowed to run at 125 V and 0.15 mA for 15 minutes of direct current and 30 seconds of alternating current.

Following transformation, the seedlings were rinsed with distilled water, transplanted directly into pots containing the potting material stated previously and kept in the laboratory growth room for 24-48 hrs at 22-28°C with a 16/8 hr light/dark photoperiod. The transformed plants (T_0) were grown to maturity in the greenhouse and T_1 seeds were harvested.

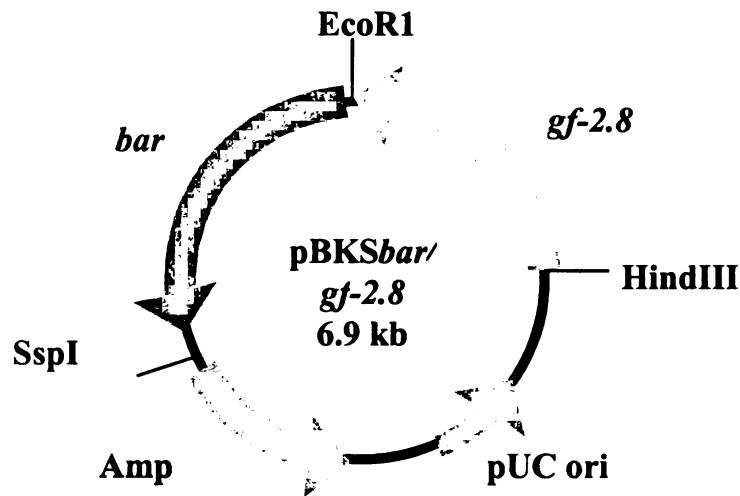


Figure 2. Diagram of the transformation plasmid pBKSbar/gf-2.8.

Table 1. Pre-transformation treatments performed on seedling apical meristems prior to electrotransformation.

Treatment	Concentration
Control	-
Meristems pierced with needle	-
Hormone (identity preserved)	1mM
Hormone	5mM
Hormone	10mM
Hormone	15mM
Hormone	30mM
Hormone	50mM
Hormone	75mM
Hormone	100mM
Lipofectin	20 µg/ml
Lipofectin	50 µg/ml
Lipofectin	100 µg/ml
Lipofectin	150 µg/ml
Lipofectin	200 µg/ml
Hormone + Lipofectin	1mM + 50 µg/ml Lipofectin
Hormone + Lipofectin	10mM + 50 µg/ml Lipofectin
Ascorbic Acid	30mM
Ascorbic Acid	50mM

Hormone identity preserved at the request of Richard Allison.

Biolistic method

A. Tissue preparation

Mature seeds of cvs. Matterhorn and Olathe were surface sterilized with 20% bleach and soaked in sterile distilled water for 16-18 hours at 22-28°C. The embryonic axes were then excised from the seeds and the primary leaves and radicles were removed to expose the apical meristems. The meristems were sterilized with 2% bleach and inserted in 9-cm diameter petri dishes containing solidified MS medium (Murashige and Skoog, 1962) with the apical region directed upward.

B. Preparation of microparticles

Preparation of microparticles was done as described by Aragao et al. (1996) with some modifications. In a 1.5 ml eppendorf tube, 50 µl of 1.2µm-diameter tungsten microparticles (M10, Biorad, Munich, Germany), 5 µl (1µg/µl) plasmid DNA, 50 µl CaCl₂ (2.5 M) and 20 ml spermidine free base were mixed sequentially while vortexing. The DNA coated microparticles were then centrifuged at 15,000 g for 10 seconds. Afterwards, the supernatant was removed and the pellet was washed with 250 µl of 100% ethanol. The final pellet was then resuspended in 60 ml 100% ethanol and vortexed for 1 minute before use. Aliquots of 10 µl were spread on macrocarrier membranes (Biorad, Munich, Germany) and allowed to dry.

C. Bombardment

Transformation was done as previously described by Aragao et al. (1996) with modifications to be amenable to the PDS-100 Helium Particle Delivery System (DuPont, Wilmington, DE) used. These modifications included: distance from rupture disk to macrocarrier – 1.5 cm; distance from macrocarrier to stopping screen (Biorad, Munich,

Germany) – 2 cm; distance from stopping screen to the target – 9 cm; vacuum in the chamber – 27 inches of Hg; and rupture disc (Biorad, Munich, Germany) pressure levels 1100 psi. The bombarded shoots were kept in the bombardment medium (basal MS media) for one week and subsequently subcultured into MS medium supplemented with 44.3 μ M 6-benzylaminopurine (BAP) as reported by Aragao et al. (1996). The shoots were cultivated at 25°C with a 16-hr photoperiod. After 3-4 weeks in culture, elongated shoots of about 4-6 cm in length and with roots produced from the bombarded apical meristems (T_0) were transferred to 15-cm diameter pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX) and covered with a plastic bag for 1-2 week to acclimatize in the greenhouse. The plants were grown to maturity and T_1 seeds were harvested.

Herbicide Screening for *bar*

A dose curve for the herbicide glufosinate ammonium of 50 mg/l to 300 mg/l was conducted to determine the minimum level of herbicide appropriate for screening. A subset of the T_1 seeds were sown and maintained in the greenhouse. Screening of the T_1 generation was done by spraying plants about 7-10 days after planting with an herbicide application rate determined from the dose curve conducted prior to each herbicide screen (150 to 250 mg/l). Tolerant plants were transplanted into 15-cm diameter pots and grown to maturity.

PCR Analysis

The PCR reaction was carried out in 25 μ l reactions containing 200 μ M of each dNTP, 2.5 mM $MgCl_2$, 1X PCR Buffer (Invitrogen, Carlsbad, CA), 0.2 μ M of each

primer, 1 U of *Taq* polymerase (Invitrogen, Carlsbad, CA) and 20 ng of genomic DNA. PCR amplification of the *bar* gene was conducted using the primers P2510 (5'GAAGTCCAGCTGCCAGAAAC3') and P2958 (5'GGTCTGCACCATCGTCAACC3') (Aragao et al., 2002), while amplification of *gfp-2.8* was performed with the primers 5'GCCTGTTCGCAATGCTGTTA3' and 5'ACCGACGTTGAACTGGAAGTG3'. The reaction mixture was denatured for 2 minutes at 94°C and amplified with 35 cycles (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute) with a final 5 minute cycle at 72°C using a PTC-100 Thermal Cycler device (MJ Research, Inc., Watertown, MA).

Oxalate Oxidase Assay: Fluorescence Assay

The fluorescent assays were done with the Amplex Red kit (Molecular Probes, Eugene, OR). The Amplex Red assay allowed the measurement of H₂O₂ generation. In the presence of peroxidase (supplied as Horseradish peroxidase) the Amplex Red Reagent (ARR) reacts with H₂O₂ to produce a red fluorescent oxidation product, resofurin. This assay relies on the detection of H₂O₂ produced by the activity of oxalate oxidase on oxalic acid. Two 4-mm leaf discs from 208 putative transgenic T₂ plants were incubated in microtiter plates with the oxalate oxidase assay buffer (18 mg oxalic acid in 100 ml of 2.5 mM succinic acid, pH 4) for 20 minutes at 37°C. After incubation of the leaf discs with the oxalic acid buffer, a 20 µl aliquot of each sample was transferred to a fresh plate using a multichannel pipettor and brought to 50 µl volume with the kit reaction buffer. For the detection reaction, 50 µl Amplex Red horseradish peroxidase reagent was added to each well and the plates were incubated in the dark for 1 hr. Fluorescence was detected with a plate reader, using a 531 nm excitation filter and 572

nm emission filter at 30 min and one hr after incubation. A standard curve allowed the calculation of the total H₂O₂ released.

Oxalic Acid Assay

Detached leaf assays were performed to assess the ability of oxalate oxidase expression to prevent leaf damage in response to application of oxalic acid to plant tissue. Detached leaflets were arranged on inverted plastic weighing boats in 15-cm petri dishes containing moist filter papers. The primary trifoliates were used in the assay for leaves from 208 T₂ plants, with each leaflet representing a sample. Each leaflet was wounded in two locations with an 18-gauge needle, and 20 µl of oxalic acid (200mM) was applied to each wound and incubated for 6 hrs (Figure 3a). To quantify lesion size, leaflets were scanned using a flatbed scanner and images were analyzed for lesion size using ASSESS: Image Analysis for Plant Disease Quantification (Lamari, 2002).

Fungal Bioassay

Fungal assays were performed using detached leaflets inoculated with an agar plug of *S. sclerotiorum* mycelia as described by Livingstone et al. (2005) with some modifications. *S. sclerotiorum* was isolated from infected dry bean plants and grown at room temperature on potato dextrose agar (Difco, VWR, Montreal, Quebec, Canada). For inoculum source in fungal bioassays, fungal mycelia plugs were inoculated on Petri dishes in the same medium composition and stored in the incubator at 25°C. Agar plugs (5.5 mm diameter) were taken from the actively growing edge. Leaflets were wounded with an 18-gauge needle near the midvein and the plugs were placed over the wounded area. Three leaflets from the second trifoliolate were inoculated for each plant line tested

using a minimal quantity of agar in each plug (Figure 3b). The inoculated leaflets were then incubated for 30 hrs at 25°C, and lesion areas were measured as previously described in the oxalic acid assays.

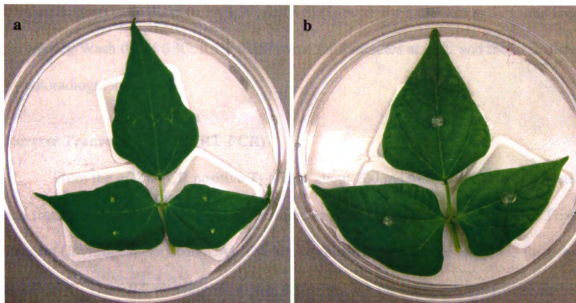


Figure 3. Set-up of assays conducted on T₂ and T₃ plants. a, oxalic acid assay. b, fungal bioassay.

Southern Blot Analysis for *bar* and *gf-2.8*

Total DNA was isolated from the *gf-2.8* PCR positive plants. Fifteen μ g of DNA was then digested with *EcoRI*, electrophoresed through 1% agarose and transferred to nylon membranes (Southern, 1975). The primers 5'GAAGTCCAGCTGCCAGAAAC3' and 5'GGTCTGCACCATCGTCAACC3' were used for the generation of a 445 bp probe for *bar* while 5'GTGAAGGTCAAGTTCAGCCA3' and 5'GCCTGTTCTGAATGCTGTTA3' were used to generate a 470 bp probe for *gf-2.8* in 35

cycles of 94°C – 1 min, 60°C – 1 min, 72°C 1 min. The probes were then labeled with ³²P using The Megaprime Labelling Kit (Amersham Biosciences, Piscataway, NJ). The prehybridization blots were hybridized with each probe overnight at 65°C. The blots were then washed in Low Stringency wash (2X SSC, 0.1% SDS) twice for 15 min at 25°C, Moderate Stringency Wash (0.2X SSC, 0.1% SDS) twice for 5 minutes at 37°C and High Stringency Wash (0.1X SSC, 0.1% SDS) twice for 5 minutes at 65°C and then visualized by autoradiography.

Reverse Transcription-PCR (RT-PCR)

From the *gf-2.8* PCR positive T₂ plants, about 200 mg of leaf tissue was collected and frozen on liquid nitrogen. The tissue samples were homogenized with 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA). The homogenized samples were incubated for 5 min at 22°-25°C. Then, 0.2 ml chloroform was added to each tube and the tubes were shaken by hand for 15 sec. The tubes were incubated at 22°- 25°C for 3 min and were centrifuged at 12,000xg for 15 min at 2-8°C. The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added. The samples were incubated at 22°- 25°C for 10 min and centrifuged at 12,000xg for 10 min at 2-8°C. The supernatant was then removed and the RNA pellet was washed with 1 ml 75% ethanol. The samples were vortexed briefly and centrifuged at 7,500xg for 5 min at 2-8°C. The RNA pellets were then allowed to dry for 10-15 min, dissolved in RNase-free water and stored at -80°C.

RT-PCR was performed using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) using the primers 5'GCCTGTTCGCAATGCTGTTA3' and 5'ACCGACGTTGAACTGGAAGTG3' to amplify the *gf-2.8* transcripts. KBACT-N and KBACT-C (Hamada et al., 2002) primers

were used to amplify the bean actin transcripts. The reaction was carried out in 25 μ l reactions containing 200 μ M of each dNTP, 2.5 mM MgCl₂, 1X PCR Buffer (Invitrogen, Carlsbad, CA), 0.2 μ M of each primer, 1 U of *Taq* polymerase (Invitrogen, Carlsbad, CA) and 20 ng of cDNA. The reaction mixture was denatured for 2 min at 94°C and amplified with 35 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) with a final 5 min cycle at 72°C using a PTC-100 Thermal Cycler device (MJ Research, Inc.).

Note: Images in this thesis are presented in color.

RESULTS

Study 1: Particle Bombardment

Eight batches of embryos were bombarded with the circular pBKS*bar/gf-2.8* DNA for the experiment for a total of 760 dry bean embryos. Using the shooting medium containing 10 mg/l of the cytokinin, 6-benzylaminopurine (BAP), all of the explants in the first two batches of bombardment were lost due to shoot death. Shoot growth and leaf development in the growing embryos was observed during the first week in the shooting media, however, growth of the plantlets appeared stunted, root formation was absent or inferior and shoots eventually turned brown and died after 3 to 4 weeks in the media (Figure 4). Due to these observations, it was suspected that the BAP concentration used in the shooting media had affected normal growth of embryos and plantlet development and survival *in vitro*. Therefore, succeeding embryo batches were either transferred to or grown initially on the shooting medium containing BAP levels less than 10 mg/l. In the last 6 batches of transformations conducted, 42 Matterhorn and 84 Olathe plants (T_0) were produced. The T_0 plants were allowed to set seed and a subset consisting of 6 seeds from each T_0 plant were screened for herbicide tolerance. The screen of 594 T_1 plants generated 4 Matterhorn and 5 Olathe herbicide tolerant putative transformed plants. Genomic DNA extracted from these 9 herbicide survivors were screened for integration of both *bar* and *gf-2.8* using PCR, however, no amplification was observed.

Table 2. Summary of results from particle bombardment experiments with *bar* and *gf-2.8* genes.

	Matterhorn	Olathe
embryos bombarded	360	400
plants produced	42	84
screened with herbicide	187	407
herbicide tolerant	4	5
PCR positives (<i>bar</i> and <i>gf-2.8</i>)	0	0

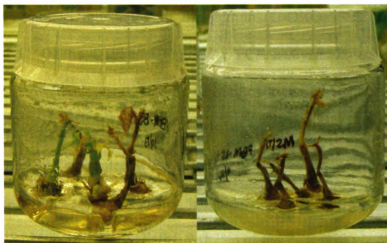


Fig 4. Olathe and Matterhorn shoots growing in 10 mg/L BAP after 3 weeks in culture.

Study 2: Electrotransformation

Using the electrotransformation protocol, a total of 1,150 dry bean seedlings were transformed with the linearized *SspI/HindIII* fragment of pBKS*bar/gf-2.8* (Table 3 and 4). About 84% and 92% seedling survival was noted on Matterhorn and Olathe plants, respectively. Using a sample of T₁ seeds from each T₀ plant, 4,250 Matterhorn and 2,888 Olathe plants were screened for herbicide tolerance. A total of 92 Matterhorn and 71 Olathe plants survived the herbicide screenings.

PCR Analysis on T₁ and T₂

Polymerase chain reaction (PCR) from herbicide tolerant plants using the *gf-2.8* specific primers showed integration of the germin (G-EXO) gene in a total of 18 Matterhorn and 11 Olathe T₁ plants (Figure 5). PCR analysis, however, with the *bar* specific primers showed integration in only 12 Matterhorn and 8 Olathe T₁ plants (Figure 6). Sixteen (89%) of the *gf-2.8* PCR positive plants from the cv. Matterhorn were progenies of T₀ plants pretreated with various levels of Hormone (identity preserved) prior to transformation. Of these plants, ten (62.5%) were from T₀ plants that were pretreated with 5mM Hormone. In Olathe, 9 (82%) of the PCR positive plants were from T₀ seedlings pretreated with various Hormone concentrations. In contrast to Matterhorn, the majority (63.6%) of the PCR positive plants of Olathe were from the 30mM Hormone pretreatment. Due to earlier results on the generation of *bar* and *gf-2.8* PCR positives from pretreatments with Hormone, additional electrotransformations were performed on Matterhorn plants pretreated with 5, 30, 75 and 100mM Hormone. However, transformation and herbicide screens did not produce additional *bar* or *gf-2.8* PCR positive plants. In Figure 7, integration of the germin gene was confirmed through PCR

in only 4 T₂ plants, three of which are Matterhorn (D2M1-5, E13M1-3 and E13M2-1) and one was Olathe (D23O1-5).

Southern Hybridization

Several attempts were conducted to verify gene integration in *gf-2.8* PCR positive T₁ and T₂ plants using Southern hybridizations with probes for both the *bar* and *gf-2.8* genes without success.

Table 4. Results of herbicide (glufosinate ammonium) and PCR screening of Olathe plants using the electrotransformation protocol.

Treatment	batch name	number of plants transformed (T ₀)	number of survivors	number of plants sprayed (T ₁)	Herbicide survivors	PCR + for <i>gf-2.8</i>
No treatment	A, B	50	50	198	0	0
Meristems pierced with needle	I	30	29	112	8	0
1mM Hormone	C	25	24	88	0	0
5mM Hormone	D	25	21	221	1	1
10mM Hormone	E	25	22	233	4	1
15mM Hormone	F	25	25	109	5	0
30mM Hormone	G	35	34	327	18	7
50mM Hormone	H	35	33	316	13	0
20 ug/ml Lipofectin	J	30	28	130	9	0
50 ug/ml Lipofectin	K	30	27	154	3	0
100 ug/ml Lipofectin	L	30	29	167	6	1
150 ug/ml Lipofectin	M	30	30	153	0	-
200 ug/ml Lipofectin	N	30	13	65	0	-
1mM Hormone + 50 ug/ml Lipofectin	O	30	30	152	1	0
10mM Hormone + 50 ug/ml Lipofectin	P	30	27	147	0	0
30mM Ascorbic Acid	R	30	27	149	1	1
50mM Ascorbic Acid	S	30	29	167	2	0
Total		520	478	2888	71	11

Treatment		batch name	number of plants transformed (T ₀)	number of survivors	number of plants sprayed (T ₁)	Herbicide survivors	PCR + for <i>gfp</i> -2.8
No treatment		A, B	50	49	255	0	0
Meristems pierced with needle		I	30	30	159	1	0
1mM Hormone		C	25	24	135	0	0
5mM Hormone		D	50	25	521	19	10
10mM Hormone		E	25	25	294	3	3
15mM Hormone		F	25	23	101	3	0
30mM Hormone		G	65	35	677	18	2
50mM Hormone		H	35	34	352	8	1
75mM Hormone		T	25	24	254	9	0
100mM Hormone		U	30	26	287	7	0
20 ug/ml Lipofectin		J	30	29	157	4	0
50 ug/ml Lipofectin		K	30	29	172	1	0
100 ug/ml Lipofectin		L	30	26	142	6	1
150 ug/ml Lipofectin		M	30	29	151	0	0
200 ug/ml Lipofectin		N	30	24	114	1	0
1mM Hormone + 50 ug/ml Lipofectin		O	30	22	121	0	0
10mM Hormone + 50 ug/ml Lipofectin		P	30	26	113	1	0
30mM Ascorbic Acid		R	30	23	113	0	0
50mM Ascorbic Acid		S	30	25	132	11	1
Total			630	528	4250	92	18

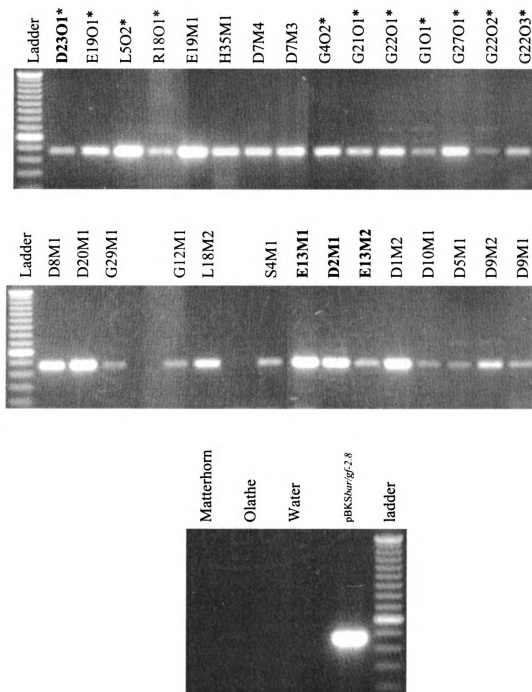
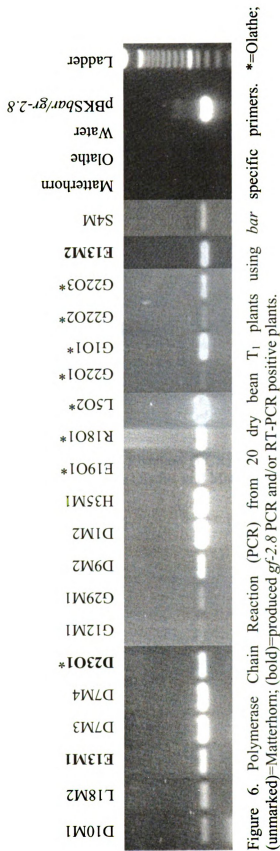


Figure 5. Polymerase Chain Reaction (PCR) results in 29 dry bean T₁ plants using *gf-2.8* specific primers. *=Olathe; (unmarked)=Matterhorn; (bold)=produced *gf-2.8* PCR and/or RT-PCR positive plants.



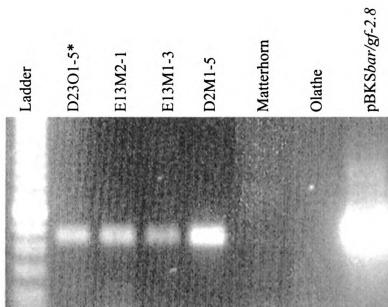


Figure 7. Polymerase Chain Reaction (PCR) from four T_2 plants using *gf-2.8* specific primers. Genotypes with * are Olathe, and Matterhorn if unmarked.

RT-PCR

Since the Southern analyses were unsuccessful, RT-PCR was performed on the 4 *gf-2.8* PCR positive T₂ plants. Positive RT-PCR results were obtained from two T₂ Matterhorn lines, D2M1-5 and E13M2-1, and the Olathe line, D23O1-5, indicating the production of the *gf-2.8* transcript which confirms the expression of the *gf-2.8* gene (Figure 8).

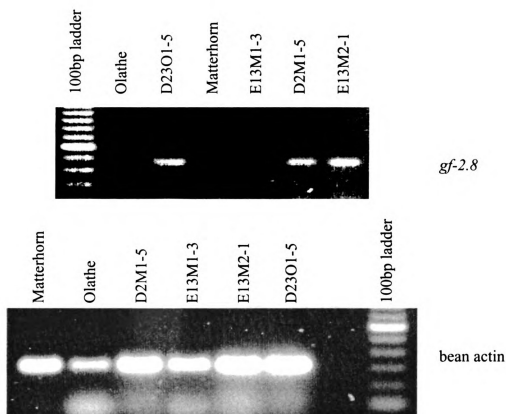


Figure 8. RT-PCR with *gf-2.8* and bean actin specific primers on four T₂ plants previously confirmed for *gf-2.8* gene integration with PCR.

Oxalate Oxidase: Fluorescence Assay

The assay was performed on 208 T₂ plants from 23 T₁ *gf-2.8* PCR positive plants (Table 3A). However, fluorescence results are only reported for four T₂ PCR and RT-PCR positive plants. The four plants were obtained from experiments performed on different dates; thus, respective Matterhorn and Olathe untransformed control plants for each experimental batch are reported for comparisons.

In comparison to controls, higher fluorescence readings were noted from the 4 T₂ plants after both 30 min and 1 hr incubation. A higher mean fluorescence reading was also consistently observed in the T₂ plant D2M15-1 compared to D23O1-5, E13M1-3 and E13M2-1 (Figure 9).

A consistently higher H₂O₂ concentration was observed in all of the four *gf-2.8* PCR positive T₂ plants relative to the control (Table 5). Statistical analysis confirmed significant increase in H₂O₂ concentration in some of the T₂ plants over the respective batch controls in both incubation durations. Among the four plants, the Matterhorn D2M1-5 was observed to have the highest amount of H₂O₂ produced relative to the untransformed control. As much as 37% and 54% significant increases in H₂O₂ concentration over the control was recorded for D2M1-5 after 30 min and 1 hr incubations, respectively. The second highest H₂O₂ concentration was noted from the Olathe, D23O-5 with about 32% increase in H₂O₂ amounts over the control after 1 hr incubation.

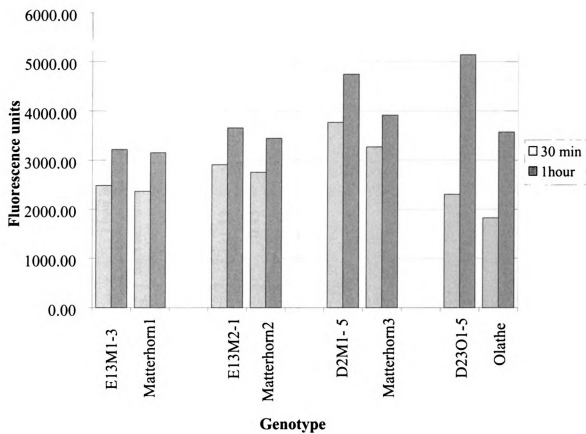


Figure 9. Mean fluorescence units of the PCR positive T_2 plants relative to the batch untransformed control.

Table 5. Mean H₂O₂ production in four T₂ PCR positive plants and percent increase in H₂O₂ concentration over the batch untransformed control.

T ₂ plant ID	30 min incubation				1 hour incubation			
	Average H ₂ O ₂ conc. (μM)	Batch untransformed control concentration (μM)	p-value (t-test) ^a	% increase in H ₂ O ₂	Average H ₂ O ₂ conc. (μM)	Batch untransformed control concentration (μM)	p-value (t-test) ^a	% increase in H ₂ O ₂
D2M1-5	0.55	0.40	0.0476*	37.07	0.75	0.49	0.0477*	53.89
D23O1-5	0.94	0.79	0.0521	18.64	1.76	1.33	0.0031**	31.98
E13M1-3	0.33	0.31	0.7824	6.30	0.45	0.43	0.9263	2.65
E13M2-1	0.57	0.53	0.5139	8.62	0.48	0.41	0.5947	15.97

^a * = significant at <0.05; ** = significant at <0.01; *** = significant at <0.001

Evaluation for White Mold Resistance

The oxalic acid and fungal bioassays were conducted on 208 T₂ plants on different dates, and the results from the 4 *gf*-2.8 PCR and/or RT-PCR positive T₂ plants are reported with respect to controls (Table 1A and 2A). The four T₂ PCR positive plants had a lower average lesion size in comparison to controls following inoculation, however, significant reduction in mean lesion size was noted only in D23O1-5 and E13M2-1 (Table 6). The highest reduction in lesion size was obtained from E13M2-1 with as much as 36% reduction over the untransformed Matterhorn. This was closely followed by about 28% reduction in lesion size from D23O1-5.

Fungal bioassays with mycelial plugs of *S. sclerotiorum* were also performed to evaluate enhanced white mold resistance. Similar to the oxalic acid assay, only results from the 4 *gf*-2.8 PCR and/or RT-PCR positive T₂ plants are reported with respective batch controls for comparisons. Enhanced resistance in this case was suggested by a reduction in lesion size in comparison to controls. Fungal bioassays showed a smaller lesion area in the four PCR positive T₂ plants compared to controls implying a minor enhancement of resistance to *S. sclerotiorum*. Statistical analysis showed a significant reduction in mean lesion size in the three T₂ lines: D2M1-5, E13M1-3 and E13M2-1 (Table 7). In contrast to oxalic acid assay results, E13M1-3 had the lowest mean lesion area recorded (381 mm²) and highest reduction in mean lesion area (24%) among the T₂ plants screened. D2M1-5 closely followed with about 19% reduction in lesion area. Although the T₂ plant, D23O5-1, which previously showed promising resistance based on fluorescent and oxalic acid assays, was observed to have a relatively low lesion size reduction (10%); not significantly different from the control.

Table 6. Average lesion size in the four PCR positive T₂ plants and the percent reduction in lesion area from the untransformed control using the oxalic acid assay.

T ₂ plant ID	Average lesion area (mm ²)	Batch untransformed control lesion area (mm)	p-value (t-test) ^a	% reduction from batch control
D2M1-5	61.13	72.09	0.3775	15.21
D23O1-5	84.12	116.16	0.0174*	27.59
E13M1-3	107.93	119.59	0.3484	9.75
E13M2-1	74.06	114.85	0.0038**	35.51

^a * = significant at <0.05; ** = significant at <0.01; ***significant at <0.001

Table 7. Average lesion size in the four PCR positive T₂ plants and the percent reduction in lesion area from the untransformed control using fungal bioassay.

T ₂ plant ID	Average lesion area (mm ²)	Batch untransformed control lesion area (mm)	p-value (t-test) ^a	% reduction from batch control
D2M1-5	432.33	533.48	0.0072**	18.96
D23O1-5	553.15	617.20	0.0693	10.38
E13M1-3	381.36	502.54	0.0020**	24.11
E13M2-1	396.58	474.70	0.0304*	16.46

^a * = significant at <0.05; ** = significant at <0.01; ***significant at <0.001

Bioassays on T₃ Plants

T₃ plants derived from the 4 PCR positive T₂ plants and 3 T₂ plants with lowest lesion size in the two assays conducted (Table 4A) were used to assess activity of the oxalate oxidase gene. Eight seeds from each plant were planted in the greenhouse; however, oxalic acid and fungal bioassays were conducted only on plants that germinated. Similar to the assays conducted on T₂ plants, the genotypes were assessed on different dates, hence, comparisons were done based on respective controls (Table 5A and 6A).

Table 8 shows the genotypes selected based on a 20% or higher reduction in lesion area relative to batch controls. Among the progenies from the 4 PCR and/or RT-PCR positive T₂ plants evaluated with oxalic acid assay, E13M13-8 had the highest significant reduction in mean lesion size at 39%. This reduction is considerably higher than what was previously observed from the T₂ parent (E13M1-3). The T₂ plant, E13M2-1 which had the highest reduction in lesion size (35%) among the PCR confirmed plants produced the T₃ progeny E13M21-3 with a significant mean lesion size reduction of 29%. The T₃ plants D23O15-4 and D23O15-7, progenies of the T₂ plant D23O1-5 previously exhibiting moderate improvements in white mold resistance in majority of the indirect tests performed, had 23 and 20% reduction in lesion size, respectively.

Results from fungal bioassays showed that the T₃ plant, D23O15-2, was observed to have the highest reduction (39%, $p < 0.0001$) in lesion size (Table 9). This reduction is about 4-fold over that observed in the T₂ parent. Three T₃ progenies from the T₂ line E13M1-3 grouped among the progenies with highest reduction in lesion size (27, 25 and 19% reduction). It should be noted that T₃ plants D2M15-3 and D2M15-4 had both high

lesion size reduction relative to the control in both oxalic acid and fungal bioassays. These observations indicate potential improved gene activity and expression from progenies as well as more stable transgene transfer to succeeding generations than previously noted among T₂ progenies. However, verification of transgene integration through PCR analysis in the T₃ lines has yet to be conducted.

Table 8. Lesion area and lesion size reduction over batch controls in selected T₃ plants following oxalic acid assays with mean lesion size reduction of at least 20%.

T ₃ plant ID	Average lesion area (mm ²)	Batch untransformed control (mm ²)	p-value (t-test) ^a	% reduction from batch control
E13M13-8	64.32	105.11	0.0015**	38.81
E13M21-3	73.50	103.63	0.0180*	29.07
E13M13-7	73.88	103.63	0.0194*	28.70
D23O15-4	92.94	120.20	0.0319*	22.68
D2M15-4	59.28	75.93	0.1655	21.93
E13M13-4	71.44	90.74	0.1086	21.26
D23O15-7	82.83	103.88	0.0963	20.27
D2M15-3	62.20	77.70	0.2197	19.95

^a * = significant at <0.05; **= significant at <0.01; ***significant at <0.001

Table 9. Lesion area and lesion size reduction over batch controls in selected T₃ plants following fungal bioassays with mean lesion size reduction of at least 15%.

T ₃ plant ID	Average lesion area (mm ²)	Batch untransformed control (mm ²)	p-value (t-test) ^a	% reduction from batch control
D23O15-2	348.59	541.12	<0.0001***	35.58
E13M13-1	219.03	311.51	0.0044**	29.69
E13M21-7	312.19	424.58	0.0006***	26.47
E13M13-6	265.41	352.70	0.0010**	24.75
D2M15-4	323.41	418.57	0.0034**	22.74
E13M21-8	258.87	330.90	0.0258*	21.77
E13M13-3	269.52	330.90	0.0567	18.55
D2M15-3	272.52	330.90	0.0698	17.64
E13M21-1	292.08	352.70	0.0203*	17.19
E13M21-3	292.57	352.70	0.0213*	17.05
D2M15-5	279.76	330.90	0.1117	15.45

^a * = significant at <0.05; **= significant at <0.01; ***significant at <0.001

DISCUSSION

Particle Bombardment

The failure to produce PCR positive bean plants via particle bombardment led us to terminate further bombardment efforts and focus on producing transformants with the eletrotransformation protocol. Early efforts with bean transformation using particle bombardment by Allavena and Bernacchia (1991) had transformed embryo axis of *P. vulgaris* and *P. coccineus* with the *gus* and *nptII* genes. However, no molecular confirmation of gene integration was reported.

In the study by Aragao et al. (2002), biolistic transformation of over 11,000 dry bean embryos for the *bar* gene resulted in a 39% plantlet production efficiency and 0.53% transformation rate. Because of the extremely low plantlet production and transformation efficiencies in dry beans associated with the biolistic procedure, the failure to produce transformants in this study could be attributed to the small number of embryos bombarded. Although, the physical nature of the particle bombardment approach eliminates genotype-dependency, Aragao et al. (1997) found that morphological factors of the apical meristem are vital for the transformation of dry bean embryos. Thus future studies with beans using this transformation procedure should consider the use of dry bean cultivars with exposed apical meristems. Plant growth hormones have been used in tissue culture to influence growth and development of plant tissues. Optimizing the growth hormone concentrations in the shooting medium may also help improve the low plantlet production efficiency in this study and could possibly help in the recovery of transformed shoots in the future.

Due to the difference in the apparatus for explant bombardment used, certain changes in the transformation conditions used by Aragao et al. (1996), were implemented in the study. These modifications included various longer flying distances of microparticles to target and lower pressure level. Moreover, the limited capability for bombardment pressure levels in the PDS-Helium System available for use in the study allowed only for bombardment of embryos at 1100 psi instead of 1200 psi as used by Aragao et al. (1996, 2002). Thus, use of other bombardment pressure levels for bombardment could be investigated.

Electrotransformation

Previously, cowpea plants were transformed with the electrotransformation protocol and integration of the *bar* gene was confirmed through PCR (Allison, personal communication). This study is the first report of the successful transformation with the novel protocol of electrotransformation that provides PCR, RT-PCR and bioassay evidences of transformation beyond the T₀ generation in dry beans. Moreover, in comparison to the work on cowpea (Allison, personal communication) where transfer of the *bar* gene to the T₂ progeny failed, we have provided evidence through PCR and/or RT-PCR for the successful inheritance of the *gf-2.8* gene in four T₂ plants.

PCR with T₂ genomic DNA indicates inheritance following reproduction. However, the rate of 1.9% transfer to the next generation was lower than expected. It is speculated that in almost all of the T₂ plants screened, both the *bar* and *gf-2.8* were not successfully transferred to the next generation. The loss of the transgene can be attributed to a possible mode for gene insertion in the genome that occurs during electrotransformation suggesting instability in gene integration, and subsequent

elimination that could be associated with the transformation approach. Further studies need to be conducted to determine mode of transgene integration with the electrotransformation procedure.

The majority of the *bar* and *gf-2.8* PCR positive T₁ plants were progenies from plants pretreated with Hormone. The Hormone is commonly associated with signaling in gram-negative bacteria, and in bacterial conjugation which has been suggested to share a similar mechanism with T-DNA transfer using *Agrobacterium*-mediated plant transformation (Binns and Thomashow, 1988). Effective transformation, as evidenced by PCR, was achieved using 5mM Hormone in Matterhorn and 30mM Hormone in Olathe.

The fluorescence assays performed showed a considerable increase in H₂O₂ concentration following incubation with oxalic acid and subsequently peroxidase. As much as 54% and 32% increases in H₂O₂ concentration were observed in D2M1-5 and D23O1-5, respectively. This data supports the RT-PCR results indicating positive transgene expression in these two lines. The fluorescence units observed in this study, where twice as much tissue was utilized for the assay, was about 3 to 10 times higher than obtained in fluorescence assays reported by Livingstone et al. (2005).

This study has demonstrated a slight increase in resistance of detached dry bean leaves to exogenous application of 200mM oxalic acid. Oxalic acid is a very strong organic acid and can cause extensive damage to plants tissues (Bateman and Beer, 1965). The levels of oxalic acid used for exogenous application in the study is about 20-fold higher than levels normally found in infected plant tissue. In this study, as much as 36% reduction in lesion size was observed in the line, E13M2-1. This reduction in lesion size however, was lower than what was previously observed in peanut transformed with the

barley oxalate oxidase gene wherein 65% to 89% reduction was observed (Livingstone et al., 2005). However, the reduction observed in this study was considerably higher than the 17% to 18% lesion reduction in transgenic hybrid poplar leaf disks exposed to 200 mM oxalic acid (Liang et al., 2002). The differences in the resistance to oxalic acid observed between this study and the peanut and poplar studies could be due to differences in plant species, gene expression levels and the assay conditions used.

Enhanced resistance to the application of *S. sclerotiorum* fungal mycelia was also exhibited by the four T₂ transgenic plants. In this study, the greatest reduction in lesion size was noted from E13M1-3, with a 24% reduction in lesion size. This value, however, is lower than both the oxalate oxidase transformed peanut and hybrid poplar. In the case of the barley OXO transformed peanut, inoculation with the fungus *S. minor* resulted in a mean lesion size reduction of 75 to 97%, whereas a lesion size reduction of 63% was observed in the wheat OXO transformed hybrid poplar (Livingstone et al., 2005; Liang et al., 2001). The differences in the indirect test for resistance observed in this study in comparison to transgenic peanut and hybrid poplar studies may have resulted from the different pathogens and assay conditions used.

The results from the fluorescence assay, however, do not appear to correspond with those observed in the oxalic acid and fungal bioassays. The variable results in the three assays conducted could be due to different reasons. Indirect tests to evaluate improvement in white mold resistance were chosen due to lack of prior knowledge on the activity of this transgene in dry beans as well as the apparent lack of an *in vitro* culture system that would allow for the generation of multiple clones from a single T₂ plant for all the different assays. The detached leaf method is one of the methods used to evaluate

bean genotypes for resistance to white mold. However, several reports have shown that findings in detached leaf assays often do not correlate with field results (Steadman et al., 2001). Moreover, Kull et al. (2003) found that the detached leaf assay was less sensitive in evaluating white mold resistance in dry beans compared to the cut stem inoculation methods.

Oxalic acid and fungal bioassays were conducted on a sample of T₃ plants from the 4 PCR positive T₂ plants. From these assays, higher reductions in average lesion size were obtained from a few T₃ plants compared to the T₂ parents. However, these differences in enzyme activity could be due to the varying planting dates and environmental conditions despite the similar incubation conditions maintained during the assays. Based on the results of the various assays conducted on T₂ and T₃ progenies to indirectly assess gene activity and white mold resistance, further evaluation of the remaining progenies from the four PCR positive T₂ parents and the T₃ parents, D23O15-2, D2M15-3, D2M15-4, E13M13-8 and E13M21-3, is suggested.

CONCLUSIONS

From the herbicide screens on T₁ and subsequent analyses through PCR, four T₂ plants were confirmed to contain the *gf-2.8* transgene. RT-PCR was used to further establish gene integration in only 3 of the 4 T₂ *gf-2.8* PCR positive plants. Indirect tests that quantify gene activity showed a slight improvement for potential white mold resistance in the four T₂ PCR positive and T₃ lines. Increased H₂O₂ production over controls, as well as decreased lesion size following fungal mycelia or oxalic acid inoculations indicated minor gene activity toward oxalic acid degradation. The four PCR positive T₂ lines as well as T₃ plants from these lines, however, still need further molecular confirmation either by Southern or Northern Hybridizations.

This study established the applicability of the electrotransformation protocol for plant transformation particularly in dry beans. This approach will have wide applications particularly to crops where reproducible and reliable plant regeneration systems have not yet been established.

Based on the assays conducted in this study, only a slight improvement in white mold resistance was attained in the transformed plants. In the white mold infection process, multiple factors are involved besides the production of oxalic acid by the fungal pathogen. Establishment of fungal disease and infection involves hydrolytic enzymes and other cell wall degrading enzymes. A better and more durable resistance to a very complex disease as white mold may require expression of other endogenous resistance genes in combination with the oxalate oxidase gene.

CHAPTER III

EFFECT OF CYTOKININ AND PARTICLE BOMBARDMENT ON *IN VITRO* GROWTH OF *Phaseolus vulgaris* SEEDLINGS

INTRODUCTION

Genetic transformation of plants has been used as an essential breeding tool to successfully transfer novel genes for the purpose of improving such traits as biochemical properties, quality, disease resistance, and yield in crops. One of the vital factors for the success of genetic transformation as a breeding tool for crop improvement is a reproducible and efficient *in vitro* regeneration protocol in the species of interest (Hansen and Wright, 1999; Birch, 1997) particularly for transformation methods that rely on shoot regeneration from cell suspension or callus cultures.

Efficient and reproducible tissue culture and transformation procedures are presently available for a number of leguminous species. Successes in both vital aspects have lead to the production of transgenic plants from soybean, pea, and tepary bean (Donaldson et al., 2001; Shade et al., 1994; Dillen et al., 1997). Studies on *in vitro* culture and transformation of dry beans (*Phaseolus vulgaris* L.) are still limited and only two groups have successfully produced regeneration competent callus tissues (Mohamed et al., 1993; Zambre et al., 1998).

Plant growth regulators (PGRs) are biologically active molecules naturally produced by plants that influence such physiological processes as growth, differentiation and development (Gaspar et al., 1996). PGRs are routinely used in the tissue culture of plants for various purposes. Two of the major PGR groups used in plant tissue culture media are auxins and cytokinins. Auxins influence such developmental processes as

apical dominance, abscission, root formation, and delay of leaf senescence and fruit ripening (reviewed by Gaspar et al., 1996). In tissue culture, auxins are generally used for root formation, induction of somatic embryos, and callus formation and growth (Hangarter et al., 1980). The major auxins used in plant culture media include α -naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D). In contrast, cytokinins are PGRs discovered as early as the 1950s that influence aspects of plant growth and physiology such as seed germination, apical dominance, and flower and fruit development (Haberer and Keiber, 2002; Jameson, 2000). Cytokinins such as 6-benzylaminopurine (BAP), kinetin, zeatin and 2-isopentenyl-adenine (2ip) may be supplemented in plant tissue culture media to enhance shoot formation, inhibit root formation, promote cell division, and callus initiation and growth (Werner et al., 2001).

In *P. vulgaris*, limited information exists on successful and efficient tissue culture protocols. Zambre et al. (1998) obtained regeneration competent callus from both *P. vulgaris* and *P. acutifolius* from media supplemented with 0.1 mg/l of the cytokinin thidiazuron and 0.05 mg/l IAA. Plant regeneration has also been achieved in *P. vulgaris* cvs. Fönix and Maxidor on MS medium supplemented with 1 mg/l BAP and 0.1mg/L NAA (Ahmed et al., 2002). Shoot production from particle bombarded mature bean embryos was successfully achieved in MS medium supplemented with 44.3 μ M BAP (Aragao et al., 1996). Successful shoot elongation was obtained in three Bulgarian dry bean varieties on MS containing 22.2 μ M BAP and 0.057 μ M IAA, and plant recovery was established on MS with 4.4 μ M BAP and 0.58 μ M GA₃ (Veltcheva and Svetleva, 2005).

A limitation, however, to the adoption of the type and amount of plant growth regulators stated in current literature that have shown success in tissue culture experiments is irreproducibility. Concentrations of PGRs that have worked in previous studies can produce variable results in other laboratories even under similar culture conditions. Moreover, varietal differences in response to various culture conditions can limit applicability and suitability of certain media formulations and growth hormone concentrations in other laboratories.

Successful transformation system of dry beans has only been reported with the particle bombardment process (Aragao et al., 1996, 1998, 2002). Using this procedure it is now possible to transform shoot apical meristems of bean embryonic axes and produce transgenic plants directly without requiring callus production and regeneration. However, production of transgenic plants by particle bombardment still requires efficient transformation and *in vitro* plantlet production procedures. The production of dry bean plantlets from bombarded meristems in plant transformation has been extremely inefficient due to the slow shoot production and failure of majority of the meristems to grow and develop in the cytokinin levels reported by Aragao et al. (1996). Therefore, the evaluation of the effects of cytokinin on *in vitro* growth of bean embryos as well as the determination of the optimum level of cytokinin for shoot production in dry bean cultivars would greatly improve the efficiency of producing transgenic plants. Moreover, analysis on possible injury effects of the transformation procedure and subsequent reduction of shooting efficiency and shoot growth needs to be evaluated.

Objectives

This study specifically aimed to: a) evaluate the effect of various cytokinin levels on the growth of dry bean embryos *in vitro*, b) compare differences in the growth of meristems between three dry bean cultivars *in vitro* c) determine the optimum cytokinin level for growth of meristems, and d) evaluate the effects of the particle bombardment process on shoot production.

MATERIALS AND METHODS

Preparation and plating of meristems

Mature seeds of dry bean cvs. Matterhorn, Red Hawk and Olathe were surface sterilized with 20% bleach for 15 min and soaked in sterile distilled water for 16-18 hrs at 22-28°C. The embryonic axes were then excised from the seeds, and the primary leaves and radicles were removed to expose the apical meristem. The embryos were sterilized with 2% bleach for 10 min and inserted on 9-cm Petri dishes containing MS medium (bombardment media) with the apical region directed upward (Murashige and Skoog, 1962). After a week, the developing meristems were transferred to shooting media (MS + varying levels of 6-benzylaminopurine, BAP) (Table 11). The shoots were cultured at 25°C with a 16-hr photoperiod. Plantlets were transferred to fresh shooting medium after 3 weeks in culture.

Shoot length measured from the base of the shoot to the shoot tip was taken weekly starting from the week after transfer to the shooting medium with varying levels of BAP. Measurements were taken until the second week after transfer. This parameter allowed for the measurement of the increase in shoot length (ISL). The number of plantlets produced as well as the number of weeks required *in vitro* culture from transfer to shooting medium to size ready for transfer to the greenhouse (NWS) was determined. Shoot lengths of above 3 cm with ample branching, and good leaf development were among the visual criteria used to classify a plantlet as ready for transfer to planting medium in the greenhouse. Plantlet production efficiency (PPE) was computed:

$$\text{Plantlet Production Efficiency (PPE)} = \frac{\text{number of shoots produced}}{\text{number of embryos plated}} \times 100$$

Bombardment of meristems

Transformation of Matterhorn, Red Hawk and Olathe meristems using particle bombardment was done as described by Aragao et al. (1996) with the following modifications: distance from rupture disk to macrocarrier – 1.5 cm; distance from macrocarrier to stopping screen (BioRad, Munich, Germany) – 2 cm; distance from stopping screen to the target – 9 cm; vacuum in the chamber – 27 inches of Hg; and rupture disc (BioRad, Munich, Germany) pressure levels 1100 psi to be amenable to the PDS-100 Helium Particle Delivery System (Dupont, Wilmington, DE). The bombarded shoots were kept in the bombardment medium (without cytokinin) for one week and subsequently subcultured to MS supplemented with various levels of BAP (Table 1). The shoots were cultivated and measurements for shoot length, number of weeks in culture and number of shoots produced were taken as previously described.

Statistical analysis

The number of weeks in shoot induction media (NWS), increase in shoot length (ISL) and plantlet production efficiency (PPE) were analyzed using proc glm.

Table 10. Summary of experiments conducted and the media treatments used for each experiment.

Experiment number	Treatment name	BAP (mg/l)
1	Control (MS0)	0
	MS1	1
	MS3	3
	MS5	5
	MS10	10
	MS15	15
2	Control (MS0)	0
	MS1	1
	MS3	3
	MS5	5

RESULTS AND DISCUSSION

Aragao et al. (1996) successfully produced bean plantlets from bombarded Olathe embryos within 3 weeks in culture using 44.3 μM (9.98 mg/l) BAP. However, in a biolistic transformation experiment previously conducted, growth of embryos and later shoots were greatly affected causing longer culture time, poor shoot and root growth and even shoot death. So far, successful efforts to transform dry beans have only been achieved with the particle bombardment procedure (Aragao et al., 1996, 1998, 2002). An efficient and reproducible *in vitro* culture procedure for dry beans needs to be established prior to efforts in transformation using the particle bombardment approach. Thus, a study which looked at wider concentration ranges of BAP was performed. Three dry bean genotypes each belonging to different market classes were selected. Matterhorn, a great northern cultivar (Kelly et al., 1999), and Olathe, a pinto bean (Wood and Keenan, 1982), are cultivars involved in a transformation study for enhanced white mold resistance while Red Hawk is a red kidney bean cultivar (Kelly et al., 1998). Both Red Hawk and Matterhorn are successful high yielding cultivars in Michigan and would be suitable genotypes for trait improvement using plant transformation. Olathe was included as it was the bean cultivar successfully transformed by Arago et al. (1998, 2002). The selection of different dry bean market classes was designed to investigate possible genotypic differences in performance of growth *in vitro*.

Experiment 1: Effect of Cytokinin on Shoot Production

Among the three dry bean cultivars, plantlets were recovered only from Olathe and Red Hawk. No plantlets were produced from Matterhorn, as the majority of the Matterhorn meristems plated in the control (no BAP) and 1 mg/l BAP were lost due to

bacterial contamination. The few remaining Matterhorn embryos on 3-15 mg/l BAP treatment exhibited elongation of the shoot tip during the first week of culture but the shoot apices later turned brown and eventually died. Due to these problems, results from Matterhorn were not considered in the statistical analyses.

One week after transfer to the shooting media with varying cytokinin concentrations, increase in growth of shoots was significantly affected by the BAP level ($p=0.009$). The highest increase in shoot length of 0.75 cm was observed from the control without BAP, however, this was not significantly different from the increase observed in the MS + 1 and 3 mg/l BAP which had mean shoot length increases of 0.65 cm and 0.52 cm, respectively (Figure 10). The lowest increase was noted in the shoots produced from the treatments with 5, 10 and 15 mg/l BAP indicating that BAP levels of 5 mg/l or higher can significantly reduce the growth of dry bean shoots *in vitro*. The effect of genotype on shoot growth during the first week of culture was not significant ($p=0.4198$) for the two dry bean cultivars, Olathe and Red Hawk.

Two weeks after transfer to the shooting media, the increase in shoot length of the plantlets produced was significantly affected by BAP ($p<0.0001$). The highest increase in shoot length was observed from plantlets grown in the control medium, with a mean ISL of 2.33 cm (Figure 10). Plantlets from MS + 3 to 15 mg/l BAP have the least mean ISL of 0.71 cm to 0.76 cm, respectively. These results suggest that after two weeks of growth, cytokinin levels of 3 mg/L BAP or higher have a negative effect on shoot growth inhibiting growth compared to those grown on much lower BAP concentrations. The significant reduction in shoot growth at 3 mg/l BAP indicates that prolonged exposures of

embryos to levels of cytokinin as low as 3 mg/l can significantly reduce growth of plantlets *in vitro*.

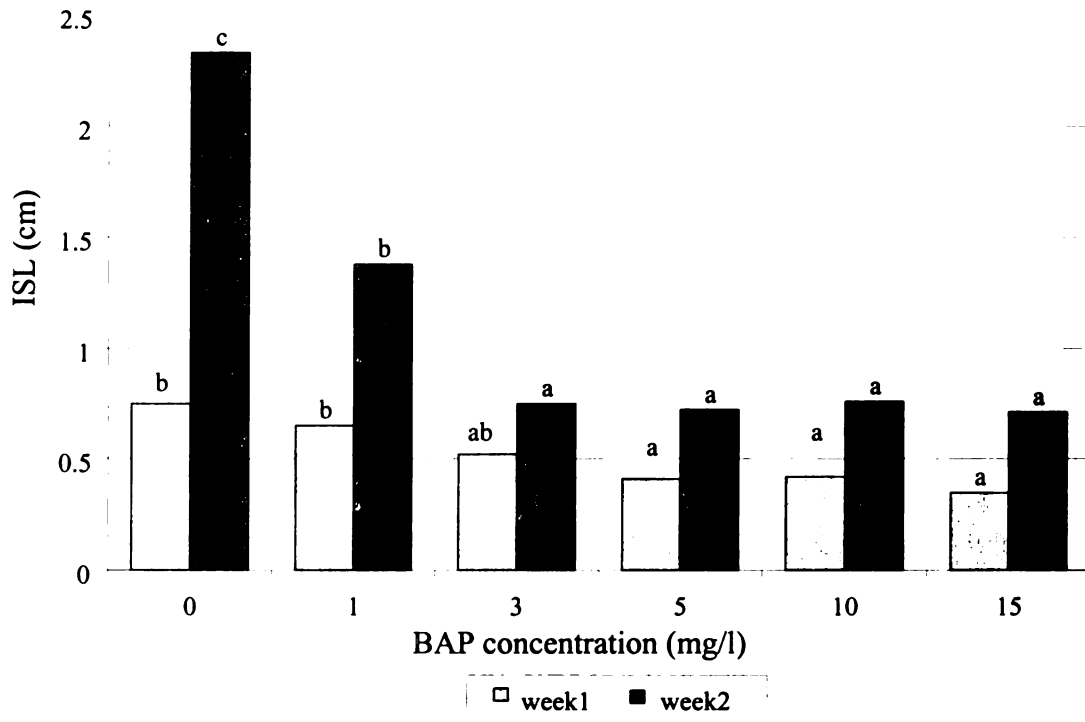


Figure 10. Mean increase in shoot length (ISL) of dry bean plantlets as affected by BAP concentration in the shooting media. Means followed by the same letter designation are not significantly different ($\alpha=0.05$).

Statistical analyses shows that BAP concentration did not significant affect PPE in the three bean cultivars used ($p=0.9252$). However, A generally higher PPE (77% mean efficiency) was observed from Red Hawk compared to Olathe (47% mean efficiency) ($p=0.0003$). This observation suggests a better *in vitro* performance of Red Hawk in terms of plantlet production as well as possible bombardment injury susceptibility of Olathe embryos. In Red Hawk, highest shoot production efficiency (93%) was obtained in shooting media with 1 mg/l BAP level with a decrease in

efficiency in treatments with 5 mg/l BAP or higher (Figure 11). In Olathe, highest shoot efficiency was produced from 0 mg/l and 5 mg/l (60% and 62%). Reduced efficiencies in Olathe at 1 and 3 mg/l BAP treatments could be due to injuries imparted to the embryo during handling.

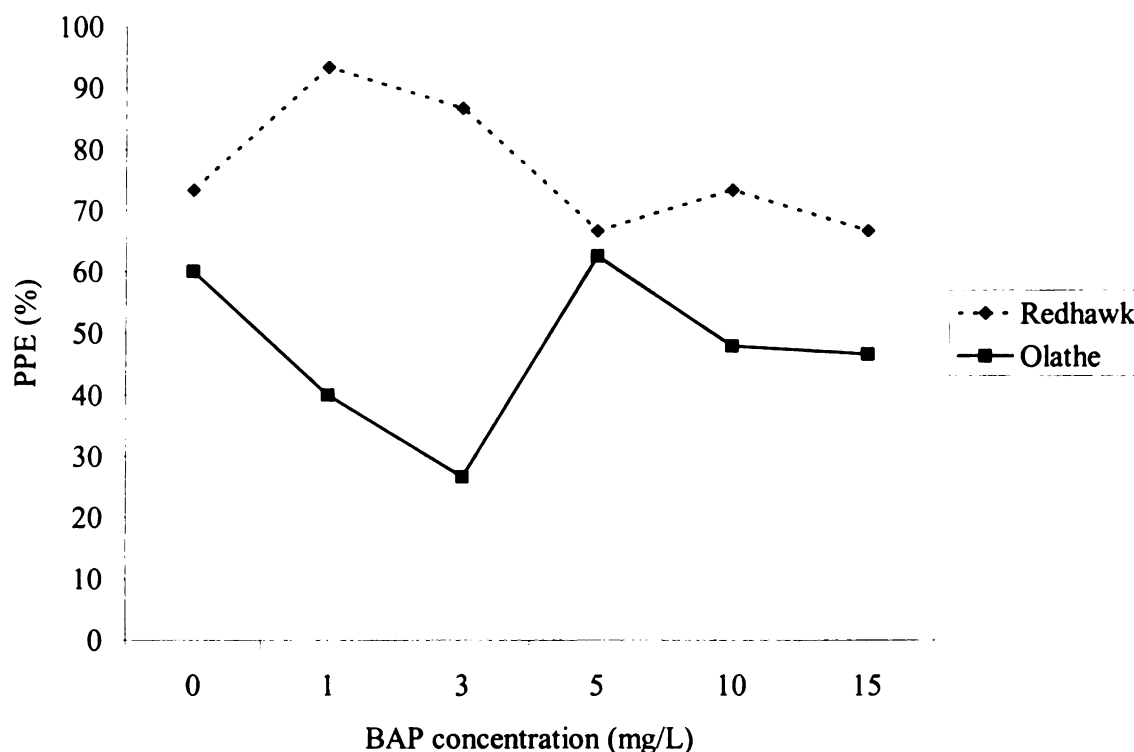


Figure 11. Plantlet production efficiency (PPE) in Olathe and Red Hawk in various BAP levels.

Although high efficiencies of plantlet production were obtained at BAP levels of 5 mg/l for Olathe as well as in 1 and 3 mg/l for Red Hawk, the rate of growth of the plantlets produced was considerably lower than the control plantlets. This suggests that although the amendment of the MS with BAP can results in slight increase of plantlets produced *in vitro*, BAP can lead to significant reductions in the shoot growth.

General observations noted during the course of the experiment included better root development from the plantlets produced at levels less than 3 mg/l BAP for Red Hawk and less than 1 mg/l BAP for Olathe. In both genotypes superior root development was consistently achieved by control plantlets (Figure 12b). Finally, shoots produced by both bean genotypes grown in higher cytokinin levels (>3 mg/l) were stunted whereas those in lower BAP levels appeared normal with better shoot growth and leaf development (Figure 12a). Although more plantlets were obtained in certain treatments containing BAP in both genotypes, the slow shoot growth, limited leaf development, general stunting and poor root development of the plants led to more culture time and possibly additional hardening problems upon transfer of the plants to the greenhouse.

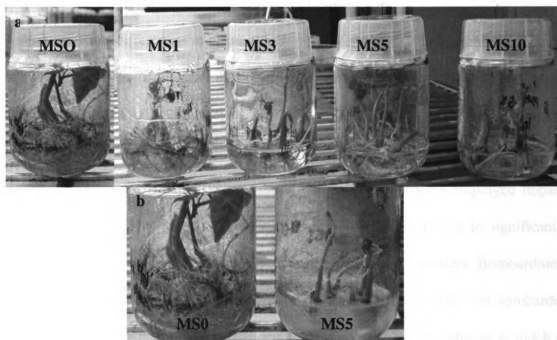


Figure 12. Growing Olathe plantlets. a, plantlets grown in 0 mg/L to 10 mg/L at three weeks after transfer to the shooting media; b, comparison of root and shoot development without BAP and in high BAP concentration (MS5).

Experiment 2: Effect of cytokinin and bombardment on shoot production in three dry bean genotypes

Based on the analysis of variance, number of weeks in the shooting media (NWS) was significantly affected by the growth media ($p < 0.0001$). The least mean NWS (3.11 weeks) was obtained from embryos grown in the control medium followed by embryos cultured on MS + 1 mg/l BAP (3.39 weeks). Embryos grown on media with 3 and 5 mg/l BAP had the highest number of weeks (3.96 weeks) required for culture in shooting media (Figure 13). These observations indicated that BAP levels used in this study delayed shoot and leaf development by almost 2 weeks compared to embryos grown without BAP. The delay in the *in vitro* culture requirement for plantlets grown in any media with BAP were primarily due to slow and poor shoot growth in combination with inferior leaf development as will be shown and discussed later.

Significant differences in mean NWS were noted among the bombarded and non-bombarded embryos from the three varieties ($p = 0.0018$). Bombarded Matterhorn embryos required significantly fewer weeks than those which were not bombarded (Figure 14). No difference was seen between bombarded and non-bombarded Red Hawk embryos indicating that for this genotype, the bombardment process employed imparts less injury effects on the growth of embryos and plantlet development to significantly affect the time required for *in vitro* culture and production of plantlets. Bombardment appeared to have a significantly negative effect on Olathe, causing non-bombarded embryos to produce plantlets which were ready for transfer to the greenhouse as much as one week earlier than those which were bombarded. The higher NWS in bombarded Olathe embryos could possibly suggest the susceptibility of Olathe to injuries from the bombardment procedure leading to a slightly longer culture time. Olathe is the bean

cultivar consistently used by Aragao et al. (1996, 1998, 2002). Although the particle bombardment procedure can deliver DNA into any target cells and is not limited by genotype, morphology of the apical meristem is a vital factor for successful transformation of bean embryos through particle bombardment. Transgenic dry beans of the Carioca type which have exposed shoot apices were obtained in a study by Aragao and Rech (1997). The presence of leaf primordia partially covering the meristem apex reduces the number of meristematic cells that can be reached by the microparticles. Olathe is a cultivar similar to the Carioca cultivars in Brazil and the longer culture period requirement for this cultivar could be due to excessive injuries to the exposed apical meristems during bombardment.

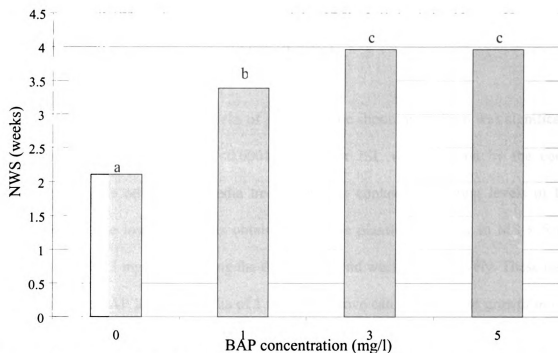


Figure 13. Mean number of weeks (NWS) required for culture in shooting media of three dry beans cultivars as affected by BAP concentration. Means followed by the same letter designation are not significantly different ($\alpha=0.05$).

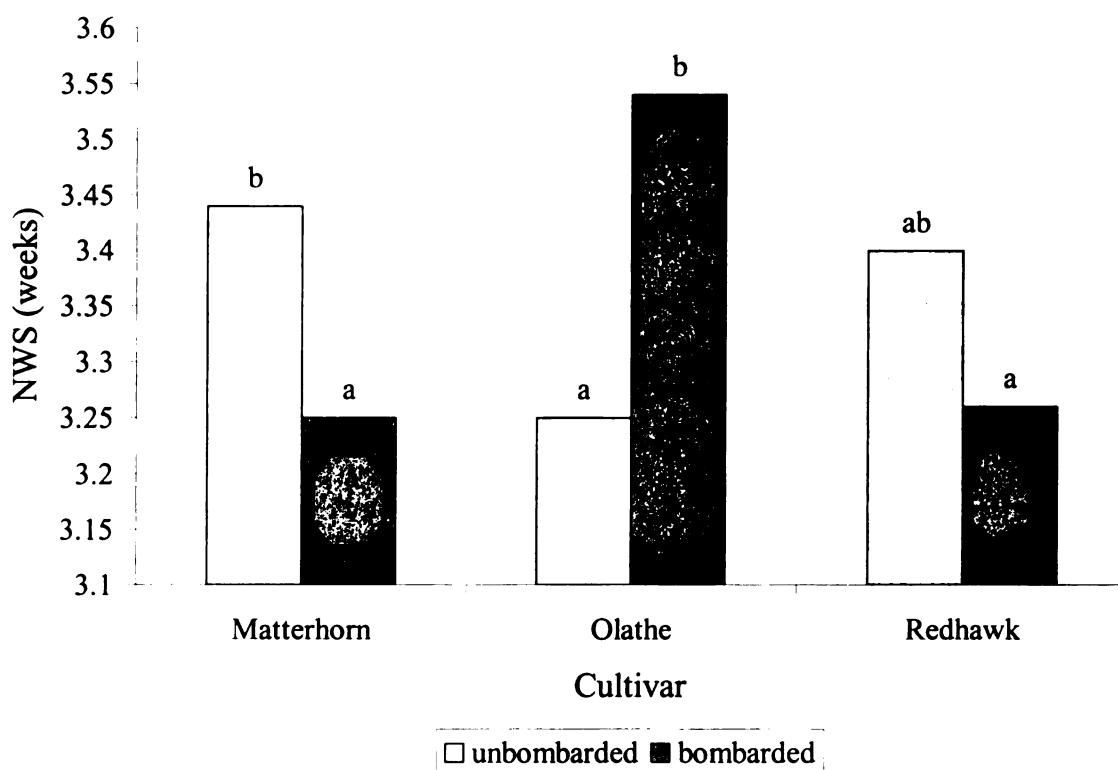


Figure 14. Mean number of weeks (NWS) required for culture in shooting media by three dry bean varieties as affected by bombardment. Means followed by the same letter designation are not significantly different ($\alpha=0.05$).

The ISL after 1 and 2 weeks of growth in the shooting medium was significantly affected by BAP ($p<0.0001$; $p<0.0001$). A higher ISL was obtained by the control compared to the other three media treatments that contained different levels of BAP (Figure 15). The lowest ISL was obtained from the plantlets growing in MS + 5 mg/l BAP and MS + 3 mg/l BAP during the first and second weeks, respectively. These results indicated that BAP levels in media of 1 mg/l and above can inhibit shoot growth *in vitro*. Similar to the first experiment, the highest ISL during the first week in culture was noted for the control, however, during that study significant reduction in shoot growth was observed only with BAP levels of 5 mg/l or higher. A slightly larger sample size for each

treatment was used in the second study which could account for the variable results between the second and the first experiment.

Individual effects of genotype were not significant ($p=0.3143$). The three dry bean cultivars used in this study each belong to different market classes, pinto, great northern and kidney, suggesting that even among different dry bean market classes a possibly similar *in vitro* culture response in terms of shoot growth of embryos may be observed.

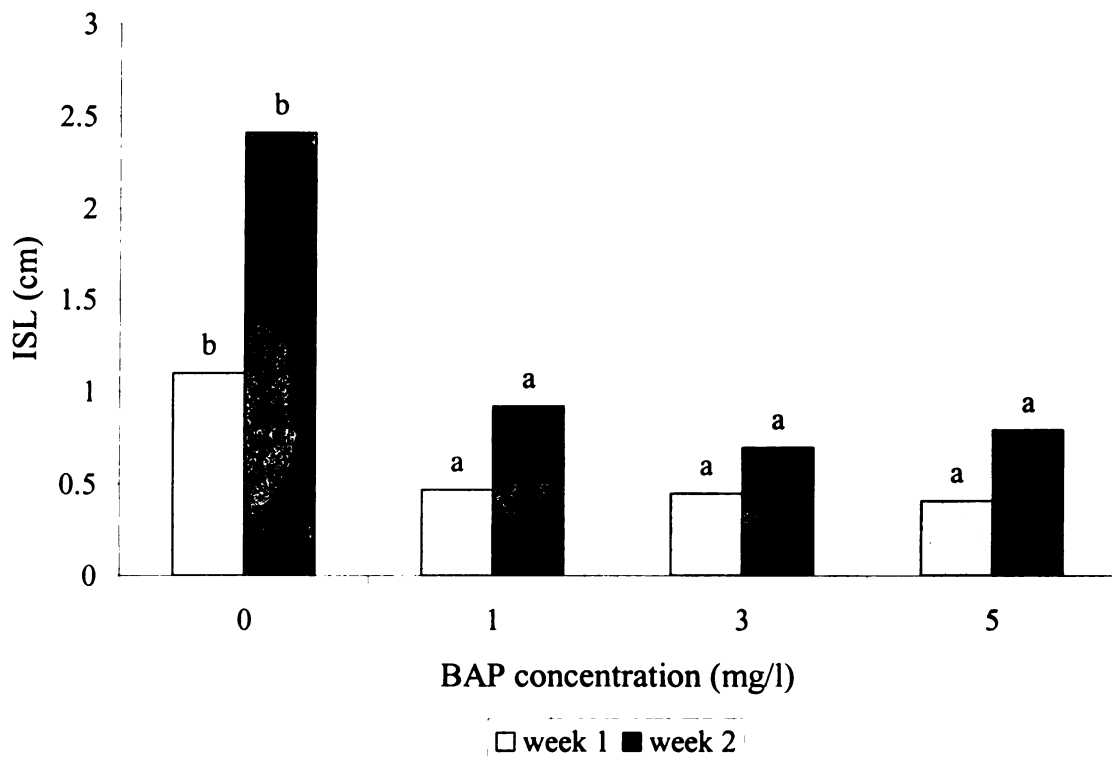


Figure 15. Mean increase in shoot length (ISL) of dry bean as affected by BAP concentration in the shooting media. Means followed by the same letter designation are not significantly different ($\alpha=0.05$).

Statistical analyses shows that BAP concentration did not significantly affect PPE in the three bean cultivars used ($p=0.0507$). However, significant differences in PPE were notes among the cultivars ($p=0.0004$). Similar to the first experiment, a generally higher

PPE was observed from Red Hawk compared to both Matterhorn and Olathe (Figure 16). Particularly for Matterhorn, 48% reduced efficiency over Red Hawk can be noted. Better *in vitro* performance of Red Hawk appeared consistent with the first experiment where Red Hawk had greater efficiencies over Olathe in the different media treatments.

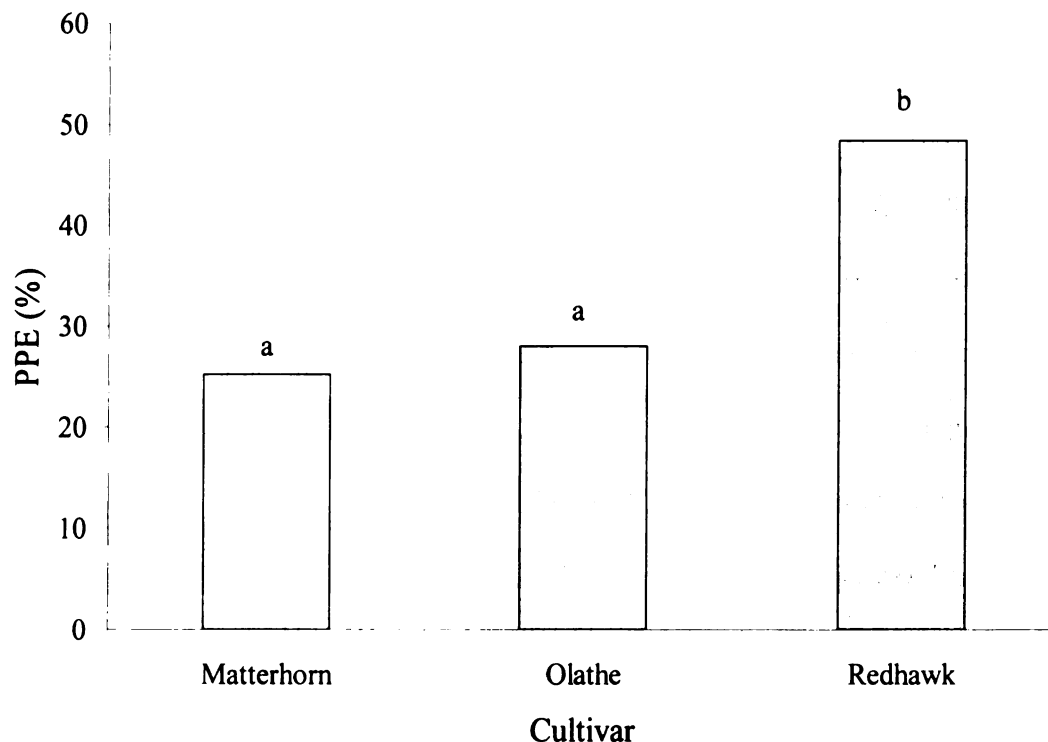


Figure 16. Plantlet production efficiency (PPE) of three dry bean cultivars *in vitro*.

Although plant regeneration and multiple shoot production have been achieved using higher cytokinin concentrations ($\geq 10\text{mg/l}$ BAP) in beans and other leguminous species, optimal levels involving lower BAP concentration (1 to 3 mg/l) have also been reported. In the study by Saam (1983), a medium supplemented with 1 and 3 mg/l BAP was found to be optimal for shoot multiplication in one pinto and three great northern bean cultivars. Plant regeneration studies in dry beans have shown optimal induction of multiple shoot formation using MS with 1 mg/l BAP and 0.1 mg/l NAA (Ahmed et al.,

2002). Avenido and Hattori (2000) have successfully regenerated the adzuki bean (*Vigna angularis*) with R media (MS basal medium with B5 vitamins) amended with 4.4 μM (≈ 1 mg/l) BAP. High frequency plant regeneration from soybean (*Glycine max*) hypocotyl explants was achieved using 0.5mg/l NAA and 3 mg/l BAP (Tripathi and Tiwari, 2003). These reports and results obtained from this study confirm that lower BAP concentrations are needed for *in vitro* culture of beans.

Saam (1983) also noted a sharp decrease in shoot production and internode elongation as well as the development of rosette-like cultures and multiple bud formation in growth media with a higher BAP concentration (above 10mg/l). In this study, the majority of plantlets produced from the three BAP treatments were observed to have reduced branching, rosette leaf formation and an overall stunting as suggested by the significantly lower ISL than the control (Figure 17). Also, the plantlets produced from media BAP treatments seemed to have an inferior root system compared to the control indicating that BAP levels used in the study may have severe effects on root development. Root growth from these treatments was observed during the first week following transfer to the shooting media but additional root formation was no longer observed. Plantlets from control clearly had better branching, leaf development and root growth.

Auxin is a growth hormone that is used in tissue culture experiments mainly to induce root development. Given the higher plantlet production efficiencies from the BAP treatments in the initial study, use of cytokinin in combination with auxin for plantlet production in dry beans can be evaluated. Root development was successfully achieved using low concentrations of IAA (0.3mg/L) and NAA (0.6mg/L) (Saam, 1983).

Additional studies could look into the use of low auxin concentrations either alone or in combination with cytokinin for root development.

In addition to inferior root formation in the cytokinin amended treatments (Figure 18c), enlargement of the shoot base was noticed in the majority of the Matterhorn and Olathe plantlets. Callus formation at the shoot base was also observed in some Olathe and Red Hawk plantlets (Figure 18 a and b). This suggests that the cytokinin levels used in this study were sufficient to promote callus growth and thus it would be interesting to look at the regenerative capabilities of the calli produced.

For both Olathe and Matterhorn, high plantlet production efficiency can be attained using MS medium without BAP. Moreover, MS alone resulted in faster shoot elongation and shorter culture duration. In Red Hawk, where generally higher PPE was noted compared to both Matterhorn and Olathe, no considerable differences were noted in PPE among the treatments. However, BAP concentration for this cultivar was found to affect rate of shoot growth as well as time required for *in vitro* culture.

The better *in vitro* performance observed in Red Hawk in terms of efficient production of plantlets as well as better shoot and root development compared to Matterhorn and Olathe suggests genotypic differences in performance among different dry bean cultivars. Genotypic effect on *in vitro* performance has been reported in a number of crops affecting such developmental processes as embryogenesis and plant regeneration (Bailey et al., 1993; McKently, 1995). In addition, the physiological state of the explant is also a vital factor for *in vitro* performance. Red Hawk belongs to the large-seeded Andean bean gene pool (Evans, 1980). Seed size affects biomass accumulation of bean cultivars, with larger seeds producing larger stem, root and leaf mass than smaller

seeded; thus providing larger seeded beans better growth early on in the season (Lima et al., 2005). This *in vivo* performance advantage of large seeded beans in terms of growth over small seeded cultivars could possibly explain the superior *in vitro* performance of Red Hawk over the medium seeded Olathe and Matterhorn. Red Hawk embryos were considerably larger than both Olathe and Matterhorn, possibly imparting more embryo vigor and initiating faster initial growth. The increased dry matter production in large seeded beans has also been linked to increased cell number and cell volume (Sexton et al., 1997). The larger cell size in tissues of Andean beans including the kidneys may also contribute to generally more vigorous plantlets possibly leading to the increased tolerance to bombardment injuries than both medium seeded beans, Matterhorn and Olathe.

Based on the results from this study, future transformation work with dry beans employing similar conditions for DNA delivery should consider targeting Red Hawk and generating plantlets in MS without BAP. Moreover, due to relatively high efficiencies obtained in Red Hawk and its apparent tolerance to injuries from bombardment, it is necessary to evaluate the morphological structure of its apical meristem for suitability to transformation using the particle bombardment approach.

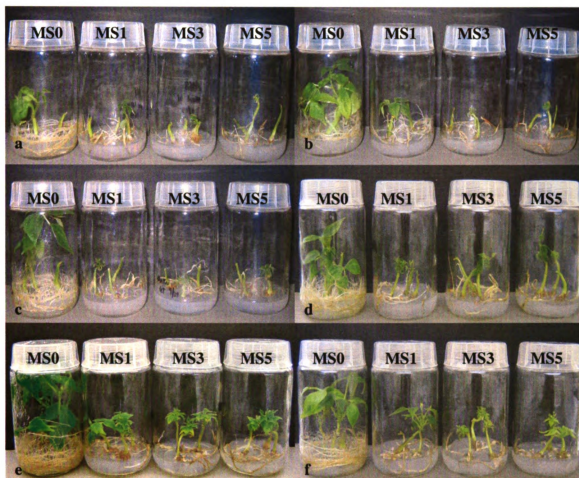


Figure 17. Growing plantlets of Matterhorn, Olathe and Red Hawk after two weeks of growth in shooting media. a, unbombarded Matterhorn, b) bombarded Matterhorn, c) unbombarded Olathe, d) bombarded Olathe, e) unbombarded Red Hawk, f) bombarded Red Hawk.

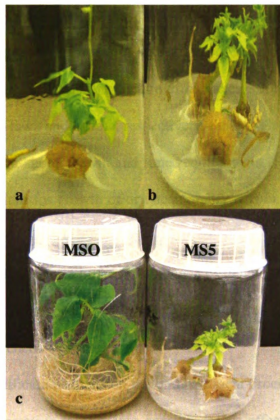


Figure 18. Plantlets growing *in vitro*. a, callus formation at shoot base in Olathe; b, callus formation at shoot base in Red Hawk; c, comparison of root formation of Red Hawk plantlets grown in control and 5 mg/L treatments.

CONCLUSIONS

Based on the results of this study, the presence of BAP in the growth medium for plantlet production from dry bean embryos caused an overall reduction in shoot growth, a slight decrease in plantlet production efficiency, longer *in vitro* culture period requirement prior to greenhouse transfer, and poor root development. Although, in the initial study slightly higher plantlet production efficiencies were produced in media with 1 and 5 mg/L for Red Hawk and Olathe, respectively, these high efficiencies have greatly compromised the rate of shoot growth and time requirements. Thus, our results indicate that the optimum medium for growth of dry bean embryos is basal MS medium without BAP.

No significant differences in the performance of the cultivars were detected in the two studies conducted. However, under specific media treatments and bombardment procedures some differences were noted.

Due to the modifications made on the bombardment process in the transformation experiment, it was imperative to look into possible injury effects. Results of the study, however, confirmed that the bombardment process does not impart injuries to the embryo that can significantly affect growth of bombarded embryos as well as efficiency of generating plantlets although it resulted in a slightly reduced shoot growth and longer culture time *in vitro* for Olathe.

APPENDICES

Table 1A. Mean lesion size from leaves of T₂ plants and batch controls following oxalic acid assay.

Genotype	Average Lesion Area
Batch 1	
D20M1-1	133.07
D20M1-10	103.59
D20M1-2	89.73
D20M1-5	98.57
D20M1-6	99.25
D20M1-9	84.41
D8M1-10	91.55
D8M1-2	99.46
D8M1-6	75.71
D8M1-9	77.19
E13M1-1	82.99
E13M1-10	96.18
E13M1-2	82.95
E13M1-3	107.93
E13M1-5	96.16
E13M1-8	93.77
E13M1-9	111.00
G12M1-1	97.64
G12M1-2	95.17
G12M1-3	98.02
G12M1-4	98.72
G12M1-6	85.26
G12M1-7	98.43
G12M1-8	120.54
G12M1-9	80.90
H35M1-3	96.12
H35M1-4	88.05
H35M1-5	91.57
H35M1-6	96.29
Matterhorn	119.59
Batch 2	
D20M1-3	95.10
D20M1-4	84.58
D20M1-7	97.20
D20M1-8	96.31
D8M1-1	87.44

Table 1A (cont-d).

D8M1-3	93.68
D8M1-4	80.03
D8M1-5	78.13
D8M1-7	90.87
D8M1-8	85.15
E13M1-7	100.73
G12M1-10	74.95
G12M1-5	84.12
H35M1-1	84.84
H35M1-2	83.38
H35M1-7	106.45
H35M1-8	80.67
Matterhorn	86.02
Batch 3	
G29M1-1	84.75
G29M1-3	86.61
G29M1-5	89.92
G29M1-6	72.83
G29M1-9	84.26
D9M1-1	81.72
D9M1-3	88.31
D9M1-5	75.88
D9M1-7	84.69
D9M1-9	87.04
G29M1-2	94.74
G29M1-4	90.57
G29M1-7	95.02
G29M1-8	96.88
G29M1-10	94.85
D9M1-2	90.91
D9M1-4	84.24
D9M1-6	86.30
D9M1-8	84.73
D9M1-10	90.74
E13M2-10	76.33
E13M2-2	94.02
E13M2-3	77.96
E13M2-4	76.73
E13M2-9	84.60
E19M1-7	75.65
E19M1-5	74.30

Table 1A (cont-d).

E19M1-3	73.62
E19M1-1	87.71
E13M2-5	87.69
E13M2-6	87.71
E13M2-7	88.05
E13M2-8	81.47
E13M2-9	93.98
E19M1-8	98.53
E19M1-6	112.12
E19M1-4	72.83
E19M1-2	103.63
D7M3-10	79.48
D7M3-9	66.53
D7M3-8	61.98
D7M3-7	69.45
D7M3-6	85.87
D7M3-5	81.00
D7M3-4	71.37
D7M3-3	55.35
D7M3-2	70.27
D7M4-2	93.30
D7M3-1	72.14
D7M4-10	79.18
D7M4-9	78.00
D7M4-8	89.20
D7M4-7	79.90
D7M4-6	75.48
D7M4-5	67.06
D7M4-3	59.33
D7M4-1	102.28
D7M4-4	82.70
D2M1-1	69.09
D2M1-10	66.29
D9M2-10	71.25
D9M2-6	67.69
D9M2-4	62.76
D9M2-2	61.26
D9M2-1	51.92
D2M1-7	77.66
D2M1-6	70.68
D2M1-8	69.53

Table 1A (cont-d).

D9M2-9	84.73
D2M1-2	64.24
D2M1-9	59.22
D9M2-7	56.01
D9M2-5	73.43
D9M2-3	66.99
D2M1-3	63.71
D2M1-5	61.13
D2M1-4	71.40
Matterhorn	72.09
Batch 4	
E13M2-1	74.06
Matterhorn	114.85
Batch 5	
D9M2-8	95.55
Matterhorn	73.03
Batch 6	
D5M1-9	96.35
D5M1-7	102.98
D5M1-10	96.82
D23O1-9	101.09
D23O1-3	94.66
D23O1-10	87.78
D1M2-1	85.77
Matterhorn	119.25
Olathe	80.31
Batch 7	
D10M1-1	89.51
D10M1-8	88.46
D1M2-10	95.80
D1M2-2	103.80
D1M2-3	119.40
D1M2-4	102.36
D1M2-5	87.88
D1M2-6	99.04
D1M2-7	106.24
D1M2-8	115.78
D1M2-9	92.14
D23O1-6	100.46
D23O1-7	79.90
D23O1-1	110.32

Table 1A (cont-d).

D23O1-2	111.46
D23O1-8	43.14
D5M1-2	79.84
D5M1-3	86.61
D5M1-4	90.02
D5M1-5	89.60
D5M1-6	84.46
D5M1-8	100.01
E19O1-2	93.03
E19O1-8	166.81
E19O1-9	125.37
G21O1-1	90.21
G21O1-2	70.10
G21O1-4	51.67
G21O1-5	95.17
G21O1-7	65.79
G21O1-10	93.45
G21O1-8	98.57
G21O1-9	116.44
G4O2-1	130.62
G4O2-2	115.80
G4O2-5	141.44
L18M2-2	112.61
L18M2-3	146.11
L18M2-5	104.39
L18M2-6	99.95
L18M2-8	123.49
L18M2-9	93.05
S4M1-1	95.61
S4M1-2	91.02
S4M1-3	94.19
S4M1-5	128.12
S4M1-7	101.18
S4M1-9	123.59
Olathe	82.21
Olathe	87.12
Matterhorn	82.85
Matterhorn	77.62
Batch 8	
D10M1-10	87.63
D10M1-2	76.67

Table 1A (cont-d).

D10M1-3	76.18
D10M1-4	82.42
D10M1-5	78.78
D10M1-6	73.96
D10M1-7	70.42
D10M1-9	81.53
D23O1-5	84.12
E19O1-1	120.33
G1O1-3	103.36
G21O1-3	125.92
G21O1-6	84.54
G4O2-10	121.84
G4O2-3	144.80
G4O2-4	106.51
G4O2-6	115.91
G4O2-8	103.72
G4O2-9	138.68
L18M2-1	113.11
L18M2-10	92.41
L18M2-4	176.78
S4M1-10	178.27
S4M1-4	90.61
S4M1-6	119.10
S4M1-8	98.32
Matterhorn	95.19
Olathe	116.16
Batch 9	
E19O1-3	143.34
E19O1-4	111.68
Olathe	110.72

Table 2A. Mean lesion size from leaves of T₂ plants and batch controls following fungal bioassay

Genotype	Average Lesion Area
Batch 1	
D20M1-1	408.50
D20M1-10	372.47
D20M1-2	401.32
D20M1-5	294.30
D20M1-7	445.37
D20M1-9	460.27
E13M1-1	430.91
E13M1-3	381.36
E13M1-7	366.23
E13M1-9	362.10
G12M1-1	433.15
G12M1-2	409.51
G12M1-3	412.98
G12M1-4	446.62
H35M1-3	360.32
Matterhorn	502.54
Batch 2	
D20M1-3	490.35
D20M1-4	507.58
D20M1-8	602.95
D8M1-1	456.14
D8M1-10	498.67
D8M1-2	570.63
D8M1-3	487.30
D8M1-6	562.74
D8M1-7	545.76
D8M1-8	522.03
D8M1-9	512.02
E13M1-10	551.05
E13M1-2	574.36
E13M1-8	535.14
G12M1-5	580.41
G12M1-6	567.33
G12M1-7	591.02
G12M1-8	536.00
H35M1-2	539.26
H35M1-4	524.95
H35M1-5	529.19

Table 2A (cont-d).

H35M1-6	538.80
H35M1-7	564.79
Matterhorn	580.54
Batch 3	
D20M1-6	449.18
D8M1-4	455.80
D8M1-5	464.54
G12M1-10	475.62
H35M1-1	493.73
H35M1-8	400.83
Matterhorn	512.21
Matterhorn	485.50
Batch 4	
D2M1-1	414.23
D2M1-5	432.33
D2M1-6	432.65
D7M3-7	422.97
D9M1-1	499.32
D9M1-5	434.26
D9M1-6	442.40
D9M1-9	496.27
D9M2-3	457.77
E13M2-2	419.80
E13M2-3	416.92
E13M2-7	457.86
E13M2-9	456.99
G29M1-6	452.23
Matterhorn	533.48
Batch 5	
D2M1-10	479.09
D2M1-2	403.42
D2M1-3	504.51
D2M1-4	453.6
D2M1-7	482.9
D2M1-8	485.99
D2M1-9	492.95
D7M3-1	477.75
D7M3-10	492.19
D7M3-4	482.66
D7M3-5	458.81

Table 2A (cont-d).

D7M3-6	513.55
D7M3-8	560.62
D7M3-9	500.72
D7M4-1	514.77
D7M4-10	512.11
D7M4-2	489.99
D7M4-3	525.17
D7M4-5	457.75
D7M4-6	548.92
D7M4-7	518.39
D7M4-8	490.2
D7M4-9	528.62
D9M1-10	465.62
D9M1-2	565.23
D9M1-3	494.22
D9M1-4	610.83
D9M1-7	442.15
D9M1-8	525.42
D9M2-1	431.25
D9M2-10	503.41
D9M2-2	475.06
D9M2-5	431.14
D9M2-6	527.79
D9M2-7	524.49
D9M2-9	561.59
E13M2-10	533.70
E13M2-4	520.64
E13M2-5	484.04
E13M2-6	431.82
E13M2-8	476.74
E19M1-1	465.88
E19M1-2	482.98
E19M1-3	494.69
E19M1-4	460.99
E19M1-5	507.81
E19M1-6	468.88
E19M1-7	482.92
E19M1-8	480.44
E19M1-9	482.43
G29M1-1	495.72

Table 2A (cont-d).

G29M1-10	493.20
G29M1- 2	481.33
G29M1-3	458.70
G29M1-4	455.38
G29M1-5	477.33
G29M1-7	482.11
G29M1-8	440.44
G29M1-9	440.14
Matterhorn	461.56
Matterhorn	535.26
Batch 6	
E13M2-1	396.58
D7M4-4	440.99
D7M3-2	359.73
D7M3-3	302.39
D9M2-4	400.07
D9M2-8	378.59
Matterhorn	474.70
Batch 7	
D10M1-1	428.12
D1M2-1	458.85
D1M2-2	487.02
D1M2-3	450.24
D1M2-4	509.48
D1M2-5	466.11
D1M2-6	469.35
D1M2-8	509.78
D1M2-9	403.08
D23O1-3	459.25
D23O1-10	538.16
D23O1-5	553.15
D23O1-6	496.02
D23O1-8	413.77
D5M1-1	362.08
D5M1-10	475.59
D5M1-3	387.67
D5M1-6	425.01
D5M1-7	422.34
D5M1-8	524.38
D5M1-9	422.87

Table 2A (cont-d).

G21O1-5	433.58
G21O1-7	441.58
G4O2-2	589.41
G4O2-5	522.50
S4M1-1	469.90
Olathe	617.20
Matterhorn	366.27
Matterhorn	474.56
Batch 8	
D10M1-10	352.93
D10M1-2	396.39
D10M1-3	359.62
D10M1-4	393.11
D10M1-5	370.48
D10M1-6	396.45
D10M1-8	404.28
D10M1-9	383.14
D1M2-10	466.09
D23O1-1	481.90
D23O1-2	417.26
D5M1-2	464.80
D5M1-4	372.70
D5M1-5	414.36
E19O1-8	466.89
E19O1-9	538.99
G21O1-8	552.28
G21O1-1	478.37
G21O1-10	396.41
G21O1-2	466.98
G21O1-3	529.89
G21O1-4	581.00
G21O1-9	484.89
G4O2-1	610.32
G4O2-3	610.74
G4O2-8	489.73
L18M2-2	471.72
L18M2-3	424.03
L18M2-5	412.62
L18M2-6	420.67

Table 2A (cont-d).

L18M2-8	440.94
L18M2-9	441.88
S4M1-2	442.49
S4M1-3	425.30
S4M1-5	389.19
S4M1-7	401.83
S4M1-9	373.89
Matterhorn	429.07
Matterhorn	462.81
Olathe	576.09
Batch 9	
D10M1-7	388.92
E19O1-2	517.69
G1O1-3	382.31
G21O1-6	385.34
G4O2-10	521.42
G4O2-6	588.39
G4O2-7	485.25
L18M2-1	470.77
L18M2-10	482.41
L18M2-4	450.41
S4M1-10	466.53
S4M1-4	479.04
S4M1-8	463.85
S4M1-6	446.93
Matterhorn	507.77
Olathe	655.17
Olathe	516.74

Table 3A. Average H₂O₂ production in T₂ plants and experimental batch control as assessed by the Amplex Red Fluorescence Assay.

ID	Average H ₂ O ₂ concentration	
	30 min	1 hour
Batch 1		
H35M1-1	0.35	0.47
H35M1-2	0.45	0.64
H35M1-3	0.38	0.53
H35M1-4	0.36	0.52
H35M1-5	0.39	0.57
H35M1-6	0.44	0.68
H35M1-7	0.37	0.54
H35M1-8	0.42	0.62
E13M1-1	0.34	0.45
E13M1-2	0.32	0.44
E13M1-3	0.33	0.45
E13M1-4	0.36	0.53
E13M1-5	0.37	0.52
E13M1-7	0.36	0.52
E13M1-8	0.34	0.46
E13M1-9	0.35	0.49
E13M1-10	0.31	0.46
D8M1-1	0.37	0.50
D8M1-2	0.37	0.55
D8M1-3	0.38	0.52
D8M1-4	0.37	0.52
D8M1-5	0.42	0.57
D8M1-6	0.36	0.51
D8M1-7	0.36	0.53
D8M1-8	0.39	0.56
D8M1-9	0.45	0.66
D8M1-10	0.45	0.60
D20M1-1	0.44	0.54
D20M1-2	0.50	0.64
D20M1-3	0.45	0.60
D20M1-4	0.47	0.63
D20M1-5	0.51	0.67
D20M1-6	0.49	0.64
D20M1-7	0.43	0.58
D20M1-8	0.48	0.64
D20M1-9	0.49	0.67

Table 3A (cont-d).

D20M1-10	0.47	0.62
G12M1-1	0.48	0.66
G12M1-2	0.80	1.06
G12M1-3	0.76	0.92
G12M1-4	0.47	0.64
G12M1-5	0.54	0.79
G12M1-6	0.54	0.76
G12M1-7	0.34	0.50
G12M1-8	0.50	0.64
G12M1-9	0.46	0.61
G12M1-10	0.51	0.75
Matterhorn	0.31	0.43
Batch 2		
E19M1-1	0.57	0.50
E19M1-2	0.60	0.59
E19M1-3	0.57	0.52
E19M1-4	0.54	0.48
E19M1-5	0.56	0.48
E19M1-6	0.53	0.45
E19M1-7	0.59	0.56
E19M1-8	0.52	0.45
E19M1-9	0.55	0.49
E13M2-1	0.57	0.48
E13M2-2	0.53	0.49
E13M2-3	0.53	0.36
E13M2-4	0.52	0.46
E13M2-5	0.50	0.41
E13M2-6	0.57	0.53
E13M2-7	0.47	0.37
E13M2-8	0.57	0.56
E13M2-9	0.49	0.43
E13M2-10	0.55	0.52
D9M1-1	0.45	0.31
D9M1-2	0.53	0.45
D9M1-3	0.52	0.47
D9M1-4	0.50	0.37
D9M1-5	0.46	0.32
D9M1-6	0.57	0.47
D9M1-7	0.52	0.43
D8M1-8	0.49	0.51

Table 3A (cont-d).

Matterhorn	0.53	0.41
Batch 3		
D8M1-9	0.30	0.36
D8M1-10	0.34	0.39
G29M1-1	0.42	0.60
G29M1-2	0.42	0.55
G29M1-3	0.34	0.37
G29M1-4	0.36	0.39
G29M1-5	0.28	0.28
G29M1-6	0.35	0.50
G29M1-7	0.34	0.33
G29M1-8	0.26	0.31
G29M1-9	0.31	0.35
G29M1-10	0.28	0.36
D7M4-1	0.33	0.35
D7M4-2	0.29	0.33
D7M4-3	0.27	0.35
D7M4-4	0.34	0.45
D7M4-5	0.34	0.41
D7M4-6	0.34	0.41
D7M4-7	0.27	0.35
D7M4-8	0.25	0.31
D7M4-9	0.32	0.42
D7M4-10	0.27	0.31
D7M3-1	0.18	0.27
D7M3-2	0.30	0.45
D7M3-3	0.28	0.36
D7M3-4	0.23	0.28
Matterhorn	0.28	0.36
Batch 4		
D7M3-5	0.55	0.72
D7M3-6	0.47	0.64
D7M3-7	0.57	0.77
D7M3-8	0.32	0.61
D7M3-9	0.39	0.45
D7M3-10	0.49	0.64
D2M1-1	0.50	0.67
D2M1-2	0.36	0.44
D2M1-3	0.36	0.54
D2M1-4	0.47	0.67
D2M1-5	0.55	0.75

Table 3A (cont-d).

D2M1-6	0.47	0.70
D2M1-7	0.37	0.45
D2M1-8	0.49	0.64
D2M1-9	0.62	0.85
D2M1-10	0.45	0.61
D9M2-1	0.39	0.56
D9M2-2	0.49	0.62
D9M2-3	0.60	0.72
D9M2-4	0.47	0.67
D9M2-5	0.34	0.46
D9M2-6	0.43	0.59
D9M2-7	0.60	0.79
D9M2-8	0.45	0.63
D9M2-9	0.35	0.46
D9M2-10	0.47	0.65
Matterhorn	0.40	0.49
Batch 5		
G21O1-1	0.98	1.23
G21O1-2	0.94	1.18
G21O1-3	1.09	1.33
G21O1-4	1.01	1.22
G21O1-5	0.97	1.27
G21O1-6	1.06	1.37
G21O1-7	1.07	1.38
G21O1-8	1.05	1.41
G21O1-9	0.91	1.08
G21O1-10	0.99	1.28
E19O1-1	1.07	1.38
E19O1-2	1.07	1.45
E19O1-3	1.02	1.32
E19O1-4	0.98	1.24
E19O1-8	1.06	1.41
E19O1-9	1.06	1.46
G4O2-1	0.94	1.18
G4O2-2	0.99	1.27
G4O2-3	0.97	1.29
G4O2-4	1.05	1.40
G4O2-5	1.03	1.32
G4O2-6	1.11	1.51
G4O2-8	1.33	1.87
G4O2-9	1.19	1.54

Table 3A (cont-d).

G4O2-10	1.13	1.47
G1O1-3	1.07	1.40
Matterhorn	1.34	1.86
Olathe	1.03	1.38
Batch 6		
D23O1-1	0.76	1.20
D23O1-2	0.81	1.47
D23O1-3	0.84	1.44
D23O1-5	0.94	1.76
D23O1-6	0.75	1.24
D23O1-7	0.95	2.00
D23O1-8	0.83	1.70
D23O1-9	0.94	1.78
D23O1-10	0.76	1.26
L18M2-1	0.98	2.00
L18M2-2	0.92	1.90
L18M2-3	0.86	1.51
L18M2-4	0.89	1.62
L18M2-5	1.10	2.13
L18M2-6	0.99	1.89
L18M2-8	0.88	1.52
L18M2-9	0.83	1.48
L18M2-10	1.11	2.15
S4M1-1	0.95	1.68
S4M1-2	0.83	1.30
S4M1-3	0.81	1.40
S4M1-4	0.86	1.73
S4M1-5	0.87	1.60
S4M1-6	0.82	1.29
S4M1-7	0.80	1.28
Olathe	0.79	1.33
Matterhorn	0.84	1.72
Batch 7		
S4M1-8	0.91	1.30
S4M1-9	0.78	1.15
S4M1-10	0.77	1.09
D1M2-1	0.85	1.24
D1M2-2	0.89	1.27
D1M2-3	0.86	1.20
D1M2-4	0.87	1.28
D1M2-5	0.85	1.19

Table 3A (cont-d).

D1M2-6	0.79	1.09
D1M2-7	0.81	1.18
D1M2-8	0.84	1.19
D1M2-9	0.88	1.25
D1M2-10	0.83	1.16
D5M1-5	0.98	1.43
D5M1-2	0.85	1.18
D5M1-3	0.84	1.32
D5M1-4	0.79	1.07
D5M1-6	0.80	1.20
D5M1-7	0.87	1.26
D5M1-8	0.99	1.46
D5M1-9	0.94	1.34
D5M1-10	0.95	1.40
Matterhorn	0.80	1.09
Batch 8		
D10M1-1	0.69	0.93
D10M1-2	0.70	1.00
D10M1-3	0.79	1.16
D10M1-4	0.75	1.07
D10M1-5	0.73	1.01
D10M1-6	0.69	0.99
D10M1-7	0.70	0.97
D10M1-8	0.66	0.90
D10M1- 9	0.70	0.95
D10M1-10	0.68	0.92
Matterhorn	0.64	0.87
Olathe	0.72	0.92

Table 4A. Lesion size in T₂ plants included in T₃ oxalic acid and fungal bioassays.

T ₂ plant ID	Oxalic acid assay		Fungal bioassay	
	Average lesion area (mm ²)	% reduction from batch control	Average lesion area (mm ²)	% reduction from batch control
D9M2-1	51.92	54.79	431.25	6.57
D20M-5	98.57	17.58	294.30	35.99
D23O1-8	43.14	50.49	413.77	19.49

Table 5A. Average lesion size from leaves of T₃ plants and experimental batch controls following oxalic acid assay.

Genotype	Average Lesion Area
Batch 1	
D20M15-6	84.42
D9M21-6	70.21
D2M15-3	62.20
E13M21-8	74.28
E13M21-4	64.00
E13M13-5	103.12
E13M13-3	78.02
D23O18-4	104.49
MATTERHORN	77.70
OLATHE	90.40
Batch 2	
D23O15-7	82.83
D20M15-1	50.96
D20M15-8	86.79
E13M21-3	73.50
D20M15-7	81.13
E13M21-5	95.08
D20M15-3	62.90
E13M21-2	89.70
D20M15-2	76.98
E13M21-1	99.66
E13M13-7	73.88
E13M13-6	94.53
E13M13-2	112.13
MATTERHORN	103.63
OLATHE	103.88
Batch 3	
E13M21-6	85.54
D9M21-8	74.16
E13M13-8	64.32
D2M15-5	89.91
D9M21-1	84.31
D23O15-2	103.07
E13M13-1	98.41
D23O18-6	143.38
MATTERHORN	105.11
OLATHE	118.70

Table 5A (cont-d).

Batch 4	
D20M15-4	64.28
D2M15-4	59.28
D20M15-5	88.02
D23O18-7	95.95
D9M21-3	62.41
D9M21-5	80.05
D9M21-2	81.37
D23O18-8	102.86
MATTERHORN	75.93
OLATHE	93.29
Batch 5	
D23O15-8	81.73
D23O18-2	78.63
E13M13-4	71.44
D9M21-4	104.88
D2M15-8	75.73
MATTERHORN	90.74
OLATHE	96.35
Batch 6	
E13M21-7	74.71
D23O15-4	92.94
MATTERHORN	77.15
OLATHE	120.20

Table 6A. Average lesion size from leaves of T₃ plants and experimental batch controls following fungal bioassays.

Genotype	Average Lesion Area
Batch 1	
D9M21-6	260.03
E13M21-4	304.99
E13M13-3	269.52
D2M15-3	272.52
E13M21-8	258.87
D2M15-5	279.76
MATTERHORN	330.90
Batch 2	
E13M21-2	477.01
D20M15-1	428.77
D20M15-8	406.51
E13M13-6	265.41
E13M21-3	292.57
E13M21-1	292.08
E13M13-2	334.14
E13M13-5	358.42
D20M15-6	344.38
D23O18-4	444.31
D23O15-7	334.29
D20M15-4	350.24
MATTERHORN	352.70
OLATHE	446.87
Batch 3	
E13M21-6	357.59
E13M21-5	342.86
E13M13-7	358.46
E13M13-8	352.17
D20M15-2	411.44
D9M21-1	210.54
D20M15-3	254.19
D9M21-8	195.60
E13M13-1	219.03
MATTERHORN	311.51
OLATHE	430.68
Batch 4	
D9M21-3	329.33
D20M15-5	346.69

Table 6A (cont-d).

D2M15-4	323.41
D23O18-7	389.49
D9M21-5	352.57
MATTERHORN	418.57
OLATHE	506.45
Batch 5	
D23O15-4	443.61
D9M21-2	437.24
MATTERHORN	316.55
OLATHE	513.82
Batch 6	
D23O18-2	424.84
D9M21-4	361.48
D23O18-8	430.97
D23O15-2	348.59
E13M13-4	399.29
D20M15-7	329.31
MATTERHORN	349.63
OLATHE	541.12
Batch 7	
D23O18-6	516.51
D2M15-8	413.26
D23O15-8	437.98
E13M21-7	312.19
MATTERHORN	424.58
OLATHE	512.23

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