SYSTEM DEVELOPMENT FOR *IN VITRO* REGENERATION AND GENE DELIVERY INTO COMMON BEAN (PHASEOLUS VULGARIS)

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ABSTRACT

SYSTEM DEVELOPMENT FOR *IN VITRO* REGENERATION AND GENE DELIVERY INTO COMMON BEAN (PHASEOLUS VULGARIS)

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Common bean is an important staple food source for many people worldwide. Given the social economic and nutritional importance of this crop, the research presented in this dissertation focused on development of a novel system for *in vitro* regeneration using apical shoot meristem primordia explant and a gene delivery system for common bean. The research investigated methods for reducing recalcitrance of common bean towards *in vitro* regeneration. The results showed that a ratio of 2.5 mg L^{-1} benzyladenine (BA) to 0.1 mg L^{-1} of naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) promoted robust multiple shoot regeneration. The addition of 30 mg L^{-1} of silver nitrate reduced the inhibitory effect of phenolic compounds.

Standardized conditions for gene delivery into apical shoot meristem primordia were developed using both Biolistic™ bombardment and *Agrobacterium tumefaciens* with two marker genes, *bar* and *gus*. Results showed that transformation efficiency of the *bar* transgene with particle bombardment method was 8.4% using 7584 kPa helium pressure with a concentration of 1.5 µg of plasmid DNA per bombardment and bombarding the explants twice at a 24 hour interval. Effect of co-cultivation period for different strains of *A. tumefaciens* (EHA105, LBA4404 and GV3301) and genotypes of common bean were

assessed. Transient and stable expression of the *gus* gene showed *'*Sedona*'* to be more amenable to *Agrobacterium* transformation than 'Matterhorn'. A co-cultivation period of 15 days with *Agrobacterium* strain GV3301was most effective in producing the highest transient expression of 81% and stable expression of 0.68%. The above results show that the Biolistic^{TM} gun delivery system is more efficient than *Agrobacterium* system for generating stable transgenes into common bean.

Testing was conducted for stable integration and expression of two major agronomically valuable transgenes. The first was the barley (*Hordeum vulgare)* late embryogenesis abundant protein (*HVA1*) gene, which confers drought tolerance. Significant resilience of transformed plants versus wild type towards drought stress was observed with a corresponding increase in root length for transgenic genotypes 'Matterhorn' and 'Sedona'. The second gene tested was the wheat (*Triticum aestivum*) germin gene (*gf2.8*) that produces an oxalate oxidase that reduce pathogenicity of *Sclerotinia sclerotiorum* the causal agent of white mold of common bean. Transfer of this gene delayed the onset of lesions caused by *S. sclerotiorum* for a period of 72 hours in leaves of transgenic 'Matterhorn'.

In conclusion, the goals and objectives of the research were achieved by demonstrating the applicability of novel protocols that were developed for *in vitro* regeneration followed by gene delivery of two marker genes and two agronomically important genes into common bean. The novelty of this research is the utilization of apical shoot primordia cells that are actively dividing. The delivery of transgenes into these cells followed by their selection and regeneration resulted into stable transgenic common bean plants.

DEDICATION

I dedicate this work to my parents, Moses and Dorothy Kwapata for their encouragement and tireless support throughout my studies.

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KEY TO SYMBOLS AND ABBREVIATIONS

ABA: Abscisic Acid

Act1: Actin Rice Promoter

BAP: Benzylaminopurine

CamV35S: Cauliflower Mosaic Virus (CaMV) 35S Promoter

CWDE: Cell Wall degrading Enzymes

DREB: Dehydration Responsive Binding Element

gf2.8: wheat germin gene

HR: Hypersensitive response

HSP: Heat Shock Protein

HVA1: *Hordeum vulgare* late embryogenesis abandant protein gene

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric Acid

LB: Luria Bentani media

MS: Murashige and Skoog media

NAA: Naphthalene Acetic Acid

nptII: neomycin phosphotransferase

pBY520: plasmid containing *bar* and *HVA1* gene

pCAMBIA3301: Binary vector containing *gus* gene

PCR: Polymerase Chain Reaction

PGIP: Polygalacturonase Inhibiting Protein

pinIIt: potato proteinase inhibitor terminator

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcription- PCR

TDZ: Thidiazuron

tnos: nopaline synthase terminator

UidA: β-glucuronidase

CHAPTER I

LITERATURE REVIEW OF *IN VITRO* REGENERATION AND GENETIC TRANSFORMATION OF COMMON BEAN (*PHASEOLUS VULGARIS*)

1.0 Introduction

Common bean (*Phaseolus vulgaris*) is one of several crop species belonging to the Fabaceae family, commonly known as grain legumes or pulses. In total, there are about 650 genera and 18,000 species in the legume family (Hymowitz 1990). Common bean is a very important source of vegetable protein, especially in those regions of the world in which animal and fish protein is scarce. Common bean satisfy 22 % of the total protein requirement worldwide (Delgado-Sanchez et al. 2006) and account for over 50 % of all legumes consumed globally (Blair et al. 2006, McClean et al. 2004). Like most grain legumes, common bean is rich in the essential amino acid lysine. They are deficient, however, in methionine and cysteine, which are the sulphur-containing amino acids. These essential amino acid deficiencies alter the dietary protein balance (Babaoglu et al. 2000, Popelka et al. 2004). However, cereals that have relatively higher concentrations of these amino acids (Hymowitz 1990) can supplement these nutritional requirements.

The need for the continuous improvement of traits in crop species remains an ongoing effort for crop scientists and farmers. Different plant species have their own set of

phenotypes that need to be improved in order to both add nutritional values and enhance economic gains for humankind. Common bean also present an array of traits which need improvement. In general, common bean like most grain legumes are lower yielding in comparison to cereals. This low yield is caused by three main factors. The first factor is photorespiration which consumes 30% of photo assimilates in grain legume crops. The second is nitrogen fixation which diverts 10% of carbohydrates fixed to be used by *Rhizobium* bacteria. The third and last factor is the photosynthetic energy relationship which takes more energy to produce oil and protein products than starch (Hymowitz 1990).

These factors, which contribute to low yield production of common bean and other grain legumes, are also implicated in making these crops more susceptible to drought stress when compared to cereals (Dita et al. 2006). Despite these limitations, common bean and other grain legumes have a number of advantages over cereals, including the high protein content in their seeds and their ability to fix nitrogen. Further research for common bean improvement should focus on enhancing the positive traits while simultaneously addressing and improving the aforementioned limitations.

Conventional breeding has contributed singularly to the improvement of cultivated common bean. Whilst plant breeding has contributed to the much needed genetic variation necessary for trait improvement, certain genes that can add to the value of agronomic traits in common bean do not exist naturally in its gene pool. Due to this limitation of plant breeding, new trait improvement approaches such as interspecific

horizontal gene transfer via genetic engineering, need to be utilized in order to complement the limitations encountered by conventional breeding (Aragão et al. 1996, 1998, 2001).

1.1 *In vitro* Regeneration of Common Bean

A reliable and efficient plant *in vitro* regeneration system is a prerequisite to the development of an efficient genetic transformation system. A general feature of common bean genotypes is their recalcitrance to regenerate *in vitro*. This is because they produce significant amounts of phenolic compounds *in vitro* which inhibit their regeneration.

A successful *in vitro* regeneration depends on three major factors.

1) The type of media formulation is crucial to creating a balance between levels of cytokinin and auxin. In general, a higher concentration ratio of cytokinin to auxin is required to promote shoot development. In the case of common bean, the ratio of 2.5 mg L^{-1} benzyladenine (BA) to 0.1 mg L^{-1} of naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) promotes robust *in vitro* regeneration (Kwapata et al. 2009).
2.) The explant type also plays a role in either assisting or hindering *in vitro* regeneration. Grain legume explants that have been tested for *in vitro* regeneration include embryonic axes (Shroeder et al. 1993, 1995); cotyledonary nodes, stem nodal segments (de Kathen and Jacobsen 1990, Nauerby et al. 1991, Davies et al. 1993); and apical meristem (Pickardt et al. 1991, Russell et al. 1993). *In vitro*

regeneration of common bean using callus is extremely difficult and minimal success has been reported (Zambre et al. 1998, Arellano et al. 2009).

3.) The age of the explant is also critical. Young explants from emerging buds or growth points are most favorable to *in vitro* regeneration because they are actively dividing totipotent cells (Veltcheva et al. 2005). A summary of research on *in vitro* regeneration of common bean and tepary bean (*P. acutifolius*), a closely related genotype, is shown in Table 1.

Table 1: A summary of *in vitro* regeneration of common and tepary bean

Genotype	Explant	Shoot Regeneration Media	Rooting Media	References
P. vulgaris 'Flor de Junio', 'Flor de Mayo- Anita'	Shoot buds and embryo for multiple shoot regeneration	MS salt, 100mg/l myo-inosital, 1mg/l thiamin HCl, 3% sucrose, pH 5.8, 1NKOH, 6.8g/l agar BAP 10mg/l, Adenine hemisulfate 20mg/l	MS but without any growth regulators	Delgado-Sanchez et al. 2006.
P. acutifolius 'A. Gray', *P.vulgaris* 'XAN-159'	Callus from embryonic axis and cotyledon	MS Salt, 20g/l sucrose, 8g/l bacto Agar, pH5.7 0.1mg/l Thidiazuron (TDZ), 0.05mg/l IAA BM with 10% coconut H2O, 1mg/l BA	MS with no growth regulators	Zambre et al. 1998
P. vulgaris 'Fonix' and 'Maxidor'	Multiple Shoots from Intact seedlings (IS) and Cotyledonary nodes (CN)	MS, BA 1mg/l, 0.1mg/l NAA, pH5.7	plantlets were germinated on full MS, B5vit, no hormones, pH5.7	Ahmed et al. 2002
P. vulgaris 'Olathe'	Multiple shoots from apical meristems with primary leaves removed.	Full MS media, plus 44.3 uM BAP	MS with no growth regulators	Aragão et al. 1998

Table 1: Continue…..

P.vulgaris 'Jalo', 'Costa Rica', 'Carioca', 'GL11'	Apical meristems with embryonic axis and root tips	Full MS media no growth regulators or hormones.	MS without growth regulators	Ana et al. 1996
P. polyanthus 'Greenman' (Year bean)	Callus from buds without scales , Leafs, stem nodal segments	MS Salt, 20g/l sucrose, 8g/l bacto Agar, pH5.7 0.1mg/l Thidiazuron (TDZ), 0.05mg/l IAA	BM with 1mg/l BAP, 100ml/l coconut water,	Zambre et al. 2001

1.2 Common Bean Genetic Transformation

Common bean is not only known to be recalcitrant towards *in vitro* regeneration, but also genetic transformation (Estrada-Navarrete et al. 2007). Although inefficient, approximately 90% of stable transformation of common bean has been achieved through biolistic™ gene delivery system (Veltcheva et al. 2005). This is in contrast to other grain legumes such as soybeans, chickpeas, pigeon peas and peas that have demonstrated to be more amenable to *Agrobacterium*-mediated transformation system (Popelka et al., 2004). The drawback of Biolistic™ method, also known as gene gun bombardment, is that it often causes multiple gene insertions, which are sometimes fragmented and result into instability of transgenes and low gene expression. A review article (Veltcheva et al. 2005), cites early attempts by several researchers from 1989-1997 who have reported transient expression, (rather than stable expression) of transgenes when *Agrobacterium* mediated transformation system was used. They further state that many of the *Agrobacterium* genetic transformation techniques of common bean have not been reproducible in other labs. Therefore, many researchers have abandoned *Agrobacterium* as a vehicle for gene delivery and have instead opted to use the biolistic™ bombardment method.

Early attempts to develop a gene delivery system for common bean involved the use of electrical-discharge to accelerate DNA plasmid into meristems (Rech et al. 1991, McCabe and Christou., 1993, Aragão et al. 1993). Only Russell et al. (1993) were able to show stable transformation of the *bar* herbicide resistance gene using electrical-discharge

technique. However the recovery of transgenic plants was very low (0.03 %) to an extent that the technique used was rendered impractical for future work on the genetic transformation of common bean. Other researchers have unsuccessfully attempted to use DNA uptake by protoplast, either via polyethylene glycol or electroporation (Veltcheva et al. 2005).

The sole report on the successful use of *Agrobacterium*-mediated transformation comes from Liu et al. (2005). They describe a procedure of transforming kidney bean with a group 3 *LEA* (late embryogenesis abundant protein) gene from *Brassica napus*. Their technique bypassed the tissue culture stage, due to poor *in vitro* regeneration, and directly transformed the beans with *Agrobacterium* using sonication and a vacuum infiltration system. Although the transformation efficiency was low, the transgenic plants exhibited a high growth rate under salt and water stress. Since then there has been no other reports of this procedure ever being repeated or of any other successful transformation technique developed using *Agrobacterium*.

Using Biolistic^{TM} bombardment, Aragão et al. (2002) developed transgenic common bean carrying the *bar* gene which conferred resistance to the herbicide glufosinate ammonium at concentrations of 500 g ha^{-1} in greenhouse and 400 g ha^{-1} in the field. Common bean have also been engineered to express viral antisense RNA, which results in a delay and attenuation of symptoms of Bean Golden Mosaic Gemini Virus (BGMGV) Aragão et al. 1998). A different approach was performed by Bonfim et al. (2007) using

RNAi-hairpin construct to silence the *AC1* region of the viral genome of BGMGV. However, their transformation efficiency was so low that, out of 2,706 plants, only 18 putative transgenic lines were obtained, representing 0.66% transformation efficiency. Of the 18 putative transgenic plants, only one plant exhibited resistance to the virus. Field trials of the progenies of the putative transgenic plants showed partial resistance to BGMGV in the field (Aragão and Faria, 2009).

The nutritional improvement of common bean was enhanced by a plasmid construct containing the fusion of the *neo* and *gus* genes which had been co-bombarded with the Brazilian nut *methionine-rich 2S albumin* gene. When the methionine expressing embryonic axes were also co-transformed with anti-sense sequences of *AC1, AC2, AC3* and *BC1* genes from the BGMGV, the co-transformation efficiency of unlinked genes was 40-50% (Aragão et al. 1996) and the methionine expression increased to 14 and 23% in two different transgenic lines (Aragão et al. 1999).

A combination of both the *Agrobacterium* and Biolistic^{TM} bombardment methods were used by Brasileiro et al. (1996) to stabilize the transformation of *P. vulgaris* 'Jalo' that was bombarded with tungsten microprojectiles and inoculated with *A. tumefaciens* wild type (Ach5). The results showed that tumors were produced in 50-70% of the transgenic plants. When the bombarded meristems were also inoculated with the disarmed *A. tumefaciens* (LBA4404/p35SGUSINT), 44% of plants showed *gus* expression. Vianna et al. (2003) developed a novel approach of transforming the transgene assembly as

fragment pieces of DNA, as opposed to the entire plasmid. The transformation efficiency of using either an entire plasmid or a fragment of DNA has shown to be in the range of 0.2 to 0.8%, depending on plant genotypes.

Genetic engineering of common bean has remained a challenge due to the inefficient and recalcitrant nature of the species towards *in vitro* regeneration. Low regeneration efficiency and frequency of multiple shoots ranging from 4 to 8 per explant (Ahmed et al. 2002) are common. A recent report by Kwapata et al. (2009) shows that common bean, cultured *in vitro*, could produce as many as 20 multiple shoots per explant. Though this is a relatively higher number, it is still very low from the desired numbers regenerated in other crop species such as corn and other cereals (Oraby and Sticklen, 2005).

A closely related species to common bean, the tepary bean (*P. acutifolius*), has been shown to be more readily transformable with *Agrobacterium* inoculation of callus. This is due to high efficiency of *in vitro* regeneration in tepary bean. Tepary bean callus lines were co-cultivated with *A. tumefaciens* strain C58CIRif (pMP90) harboring a binary vector with neomycin phosphotransferase II (*nptII*) and β-glucuronidase (*uidA*) marker genes (DeClerq et al. 2002). In this experiment, the GUS activity was detected transiently in 5 of 6 genotypes tested. In another experiment, transgenic callus lines of genotype *P. acutifolius* 'N1576' was transformed with a marker gene and the genomic fragment encoding the *arcelin-5a* protein from *P. vulgaris,* which confers resistance to *Zabrotes subfaciatus* pest (Dillen et al. 1997). This research was followed by Goosens et al.

(1999), who reported *acelin-5* expression levels of the transgenic plants to be 25% of total soluble proteins. Optimization of the *Agrobacterium* transformation method in tepary bean has been explored using different selection methods under different temperatures, photoperiods, light conditions, *Agrobacterium* growth phases, co-cultivation periods and using various concentrations of acetosyringones (DeClercq et al. 2002, Zambre et al. 2003, 2005).

While tepary bean has been demonstrated to be more amenable towards *in vitro* regeneration and genetic transformation, they are of less value economically when compared to common bean. As a result, several researchers have proposed using tepary bean as a bridge of introducing foreign genes into the economically more valuable common bean (Dillen et al. 1997, Veltcheva et al. 2005). They suggest that this can be achieved by grafting the scion of common bean onto the root stock of tepary bean. This approach partially solves the problem of *in vitro* rooting, which is problematic in many grain legumes and especially in common bean (Krishnamurthy et al. 2000, Sarmah et al. 2004, Tewari-Singh et al. 2004, Sanyal et al. 2005). A summary of genetic transformation systems for grain legumes including common bean is presented in Table 2.

Table 2: Grain legume transformation systems using different explants, genes and gene delivery methods

Grain Legume	Explant	Transgenes	Transformation method	Reference
Arachis hypogaea (Peanut)	embryo axis	*gus*	*Agrobacterium*	Epen and George 1994
	embryo axis	*gus*		Mckently et al. 1995
	embryo axis	*gus*		Cheng et al. 1996
	embryo	*gus*	bombardment	Egnin et al. 1998
		uidA, bar		Brar et al. 1994
		uidA, bar		Christou 1997
		cry1Ac, hph		Singst et al. 1997
	callus	*uidA, bar*		Livingstone and Birch, 1998
		TSWV		Yang et al. 1998
		oxalate oxidase		Livingsone et al. 2005
		Ara2 protein gene		Dodo et al. 2005, 2008
		DREB1A		Bhatnagar-Mathur 2007
Cajanus cajan (Pigeon pea)	shoot apices,	*uidA, nptII,*	*Agrobacterium*	Geetha et al. 1999
	cotyledonary node,	*nptII*		Lawrence and Koundal 2001
	embryonic axis,	*nptII*		Satyavathi et al. 2003
	embryonic axis &	*hemagglutinin*		Dayal et al. 2003
	cotyledonary node	*protein* gene		Thu et al. 2003
	leaf	*uidA, nptII*		Mohan and Krishnamurthy, 2003
	cotyledonary nodes	*uidA*		Kumar et al. 2004
	decapitated embryo	*GFP, uidA,*		Singh et al. 2004
	axis, axillary shoot	*nptII*		Prasad et al. 2004
	cotyledonary node	*hpt, rice*		Surekha et al. 2005
	shoot apices	*chitnase*		Verma and Chand, 2005
	cotyledonary node	*hpt, uidA*		Sharma et al. 2006
	embryonal segment	*HN* gene		Surekha et al. 2007
	decapitated embryonic axis	*PPRV, nptII*	bombardment	Thu et al. 2003
	axillary bud of germinating seed	*nptII , cryI E-C, uidA, cryIAb*		
	nodal segment of embryos, plumule, cotyledon, shoot nodes	*cryIAb* *uidA* *hpt, uidA*		
	embryo axis epicotyl	*gus, nptII*		

Table 2: Continue….

Cicer arietinum (Chickpea)	leaf and stem	*nptII*	*Agrobacterium*	Srinivasan et al. 1988, 1991
	embryo	*nptII,uidA,*		Fontana et al. 1993
	embryonic axis	*cryIAc,*		Kar et al. 1996
	embryonic axis	*nptII,uidA*		Krishnamurthy et al. 2000
	embryonic axis and cotyledonary node	*uidA*		Sanyal et al. 2003
	plumule	*bar,*		Senthil et al. 2004
	half embryo with one cotyledon	*α-amylase inhibitor*		Sarmah et al. 2004
	embryo	*aspartate kinase* gene		Tewari-Singh et al. 2004
	embryo slices	*nptII,uidA*		Polowick et al. 2004
	embryonic axis	*cryI Ac* gene		Sanyal et al. 2005
	embryo	*gus, hpt*	bombardment	Ignacimuthu and Prakash, 2006
	embryo axis	*uidA, nptII*		Pathak and Hamzahm, 2008
	seeds	*pmi*		Akbulut et al. 2008
	shoots	*α -amylase inhibitor1*		Patil et al. 2009
	epicotyl	*nptII*		Shivani et al. 2007
		cry1Ac, nptII, uidA		
Glycine max (Soybean)	zygotic embryos	*hph*	bombardment	Christou et al. 1989
	immature embryos	*BPMV-pCP*		Santarem and Finer, 1999
	somatic embryos	*hpt and GFP*		Reddy et al. 2001
	embryos	*bar*		Frutani and Hidaka, 2004
	immature embryos	*hpt*		Parrott et al. 1989
	cotyledonary node	*ahas (imazapyr)*		Zhang et al.1999
	immature cotyledon	*CP4*	*Agrobacterium*	Yan et al. 2000
	embryonic axis	*Roundup*		Aragão et al. 2000
	cotyledonary node	*gus*		Clemente et al. 2000
	cotyledonary node	*G-OXO*		Donaldson et al.2001
	cotyledonary node	*hph*		Olhoft et al. 2003
	somatic embryos	*oleosin RNAi*		Schmidt et al. 2008
	apical shoot meristem	*gus*		Govindarajulu et al. 2008
Lathyrus sativus (Grass pea)	epicotyl	*uidA, nptII*	*Agrobacterium*	Barik et al. 2005

Table 2: Continue….

Lens culinaris (Lentil)	cotyledonary node and decapitated embryo	*nptII, uidA*	*Agrobacterium*	Sarkar et al. 2003
	cotyledonary node	*uidA*		Mahmaudian et al. 2002
	decapitated embryo	*uidA*	bombardment	Hassan et al. 2007
	cotyledonary node	*uidA, nptII*		Akcay et al. 2009
	cotyledonary node	*Als* (Lou Gehrig's disease)	electroporation	Gulati et al. 2002
	intact axillary bud	*uidA*		Chowrira et al. 1996
Lupinus angustifolius L.(Lupin)	embryonic axis slices	*2S albumin*	*Agrobacterium*	Molvig et al. 1997
	shoot apices	*bar*		Pigeaire et al. 1997
Phaseolus acutifolius (Tepary bean)	callus	*arcelin-5a*	*Agrobacterium*	Dillen et al. 1997
	callus	*uidA, nptII*		De Clerq et al. 2002
	callus	*uidA, nptII*		Zambre et al. 2005
Phaseolus vulgaris (Common bean)	seed	*lea protein* gene	*Agrobacterium* vaccum-sonication	Liu et al. 2005
	embryo axis	*uidA, bar,*	bombardment	Russell et al. 1993
	embryo axis meristems	*uidA, nptII, methionine rich albumin* gene		Aragao et al. 1996
	embryo axis	*Ach5*		Brasileiro et al. 1997
	embryo axis	*methionine rich albumin* gene	Agrobiolistics	Aragão et al. 1999
	embryo axis	*bar*	bombardment	Aragão et al. 2002
		BGMV		Bonfim et al. 2007
Pisum sativum (Pea)	shoot cultures	*hptII, bar,*	*Agrobacterium*	Puonti-Kaerlas et al. 1990
	immature embryo slices	*α-amylase inhibitor1*		Schroeder et al. 1993
	cotyledon epicotyls	*SAF8*		Schroeder et al. 1994,195
	immature embryo slices	*uidA,*		de Kathen and Jacobsen, 1995
	cotyledonary node		injection	Perrin et al. 2000
	embryo	*Fv antibody Cahin, bar*	electroporation	Grant et al. 1995, 2003
	embryonic segments	*nptII, uidA*		Svabova et al. 2005
	axillary meristem	*bar, PGIP,*		Richter et al. 2007
	intact axillary bud	*VST1*		Krejci et al. 2007
		uidA, bar		Chowrira et al. 1998
		PEMV , *uidA*		Chowrira et al. 1996

Table 2: Continue…

Vicia faba (Faba or broad bean)	stem stem segments embryo axis	Ti plasmid *uidA, lys C, methionine rich sunflower 2S albumin* gene *SFA8* gene, *bar, lysC*	*Agrobacterium*	Jelenic et al 2000 Bottinger et al. 2001 Hanafy et al. 2005
Vicia narbonensis (Narbon bean)	embryogenic callus embryogenic callus	*hpt* *2S albumin* *PAT*	*Agrobacterium*	Pickardt et al. 1991 Saalbach et al. 1994; Pickardt et al. 1998
Vigna aconitifolia (Moth bean)	protoplast protoplast hypocotyl	*nptII* *nptII* *cryIAc, nptII,* *uidA*	*Agrobacterium* PEG + electroporation bombardment	Eapen et al. 1987 Kohler et al. 1987a,b Kamble et al. 2003
Vigna angularis (Azuki bean)	epicotyl epicotyl	*hpt* *bar, hpt, GFP*	*Agrobacterium*	EL-Shemy et al. 2002 Khalafalla et al. 2005
Vigna mungo (Blackgram)	cotyledonary nodes shoot apex cotyledonary nodes	*uidA, nptII* *uidA, nptII* *uidA, nptII*	*Agrobacterium*	Saini et al. 2003 Saini and Jaiwal, 2005 Saini and Jaiwal, 2007
Vigna radiata (Mung bean)	cotyledons hypocotyl cotyledonary node, primary leaves callus leaf, cotyledonary node	*uidA., nptII* *uidA, nptII* *nptII, uidA* *bar,* *α-amylase inhibitor1*	*Agrobacterium*	Pal et al. 1991 Jaiwal et al. 2001 Tazeen and Mirza, 2004 Sonia et al. 2007
Vigna sesquipedalis (Asparagus bean)	cotyledonary node	*nptII, uidA*	*Agrobacterium*	Ignacimuthu, 2000
Vigna unguiculata (Cowpea)	cotyledonary node cotyledonary node cotyledonary node intact axillary bud embryonic axis embryonic axis	*uidA, nptII* *bar* *α-amylase inhibitor 1* *uidA* *uidA,* *bar*	*Agrobacterium* electroporation bombardment	Chaudhury et al. 2006 Popelka et al. 2006 Solleti et al. 2008a, 2008b Chowrira et al. 1996 Ikea et al. 2003 Ivo et al. 2003

CHAPTER II
DROUGHT, SALT STRESS AND WHITE MOLD PATHOGENESIS RELATED TO COMMON BEAN

2.0 Drought and salt stress

Abiotic stresses, including drought, salinity and high temperatures, pose a major obstacle for crop yield and production, with more than 90% of arable land experiencing one or more of these stresses (Dita et al. 2006). In an effort to overcome or reduce these stress factors, plants have evolved to adapt by synthesizing low molecular weight osmolytes. Drought and salt stresses share a similar pathway (Figure 1). When drought occurs or high salt content is present, ionic and osmotic homeostasis of cells becomes inbalanced. As a result, plants lose cellular turgidity, followed by the aggregation and misfolding of proteins (Zhu 2002).

The key input signal for drought is believed to be the loss in turgor pressure due to water loss of the cells. The input signal for salt stress is the high concentration of Na^+ having similar effect on the cells. These input signals are recognized by a plant's primary sensors, such as receptor-like kinases (RLK) and ion channels. The immediate effect of sensing input signals by the primary sensors, such as those related to high levels of Na^+, is the activation of the Salt Overly Sensitive (SOS) pathway in which high amounts of Ca^{2+} is released into the cytosol. Na^+ influx into cells is via non-selective cation channels known as cyclic nucleotide-gated ion channels (CNGCs). Ca^{2+} plays a role in

inhibiting CNGC from allowing excessive entry of Na^+ into the cells. *SOS3* codes for a myristoylated calcium binding protein, which senses the Ca^{2+} and interacts with a serine/ threonine protein kinase, *SOS2*. The interaction between *SOS3* and *SOS2* regulates the transport activity and expression levels of *SOS1*, which is a salt tolerant effector gene that codes for a plasma membrane Na+/H+ antiporter (Zhu 2002).

Once secondary messenger elements, such as Ca^{2+}, ROS or ABA, have accumulated to a threshold level in hyperosmotic stressed cells, the protein phosphorylation cascade is triggered in association with the MAP Kinase cascade. In tobacco, a MAPK called salicylic acid induced protein kinase (SIPK) is activated by osmotic stress (Xiong et al. 2002). This group also explains that, due to the elicitation of Ca^{2+} during osmotic stress, calcium dependent protein kinases (CDPK) link the calcium signal to downstream responses. Phospholipid signaling, induced by osmotic stress, is catalyzed by phospholipases that cleave phospholipid messengers and generate a number of lipid messengers. These messengers function in guard cells to release more Ca^{2+} from internal stores and cause stomatal closure, while some other messengers function to activate protein kinase C.

The signaling mediated by phospholipid messengers is believed to be a double-edged sword: when the lipid signaling molecules are at low levels, they trigger downstream adaptive responses by activating transcriptional factors (TF's). On the other hand, when these signals increase due to drought or salt stress, they damage the cellular integuments.

Dehydration responsive transcription factor (*DREB*) and C-repeat binding factor (*CBF*) bind to the dehydration response element (*DRE*) and C repeat terminal (*CRT*) *cis* acting elements. Family members of these two TF's include *CBF1*, *CBF2* and *CBF3* or *DREB1B*, *DREB1C* and *DREB1A*, which are activated upon being induced by stress (Wang et al. 2003) The *DREB* or *CBF* are coded by certain multi-gene families and mediate the transcription of four groups of dehydration response genes.

- Detoxification Genes: Serve to reduce the concentration of reactive oxygen species (ROS), such as O_2, H_2O_2 and OH^-, playing the role of antioxidants and preventing damage to membranes and macromolecules.
- Osmoprotection Genes: Maintain turgor pressure of cells by driving the water gradient upwards towards the inside of the cell. These osmolytes fall into three categories:
    - Amino acids, e.g., proline
    - Quartery amines, e.g., glycine, betaine, and dimethylsulfoniopropionate
    - Polyol sugars, e.g., mannitol and trehalose
- Antiporter genes: Mediate the exchange between H^+ and Na^+ across the cellular membrane. The last group are
- Heat shock proteins (HSP): Regulated at the transcriptional level, *trans* acting heat shock factors (HSF) bind to *cis* acting elements (HSE's). HSP's are chaperones classified into one of five categories: HSP100, HSP90, HSP70, HSP60 or sHSP. These function to protect the protein from denaturing, misfolding and aggregation during osmotic stress (Wang et al. 2003). Late embryogenesis abundant (*LEA*) proteins are a class of HSP that are extremely

hydrophylic and resilient towards heat, such that they don't coagulate at boiling temperatures. These proteins may play a role in water binding, ion sequestration and macromolecule and membrane stabilization. *HVA1* is a gene from barley that encodes a type III *LEA* protein (Xu et al. 1996).

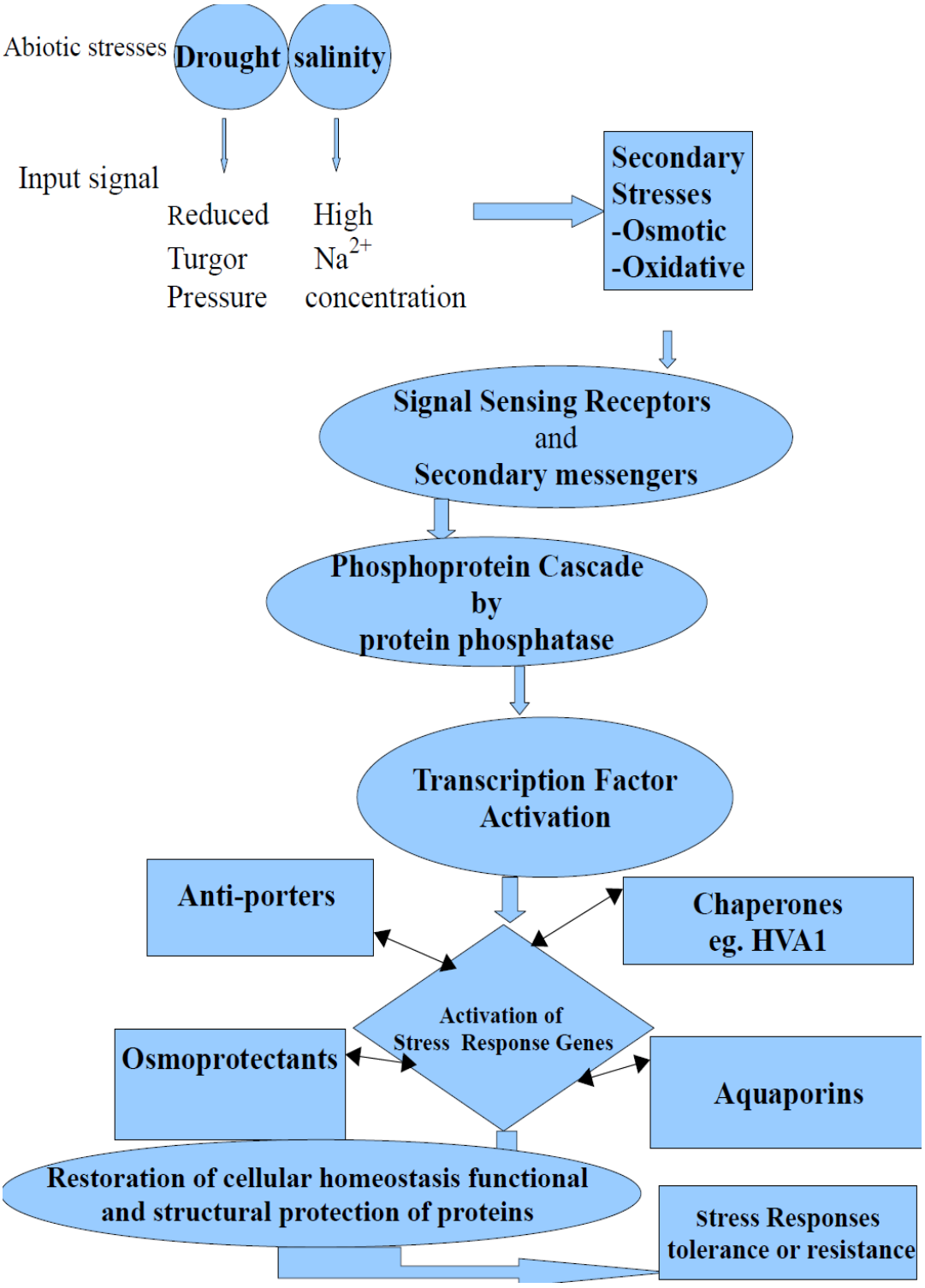


Figure 1: Drought and salt stress biosynthetic pathway. (modified from Wang et al. 2003)

2.1 White Mold (*Sclerotinia sclerotiorum*)

Sclerotinia sclerotiorum is the most devastating necrotrophic soil borne pathogen in the temperate region. Species of this pathogen cause both southern stem rot and white mold which result in the rotting of both seedling and pod in most grain legumes, especially in common bean, soybean, sunflower, lentils and peanut (Donaldson et al. 2001, Livingstone et al. 2005). This pathogen accounts for annual agricultural losses in the United States alone of more than $200 million (Bolton et al. 2006).

The characteristics of *S. sclerotiorum* include its hyaline, septate, branched and multinucleate hyphae. It produces white mycelium with no asexual conidial. For prolonged survival under unfavorable growth conditions, it relies on the production of sclerotia, which is a compact mass of mycelium. This pathogen infects host plants primarily by producing ascospores from its apothecia (Steadman et al. 1983). The disease symptoms characterized by this fungus are water soaked lesions on leaves which spread to the petiole and stem. These lesions develop into necrotic tissue with fluffy white mycelia, which is the most obvious sign of the fungus infection and successful colonization (Bolton et al. 2006). For successful colonization to occur, cool temperatures of about 10^{o}C, coupled with damp and moist conditions, are required. Due to their preference of moist conditions, the pathogen establishes more readily under irrigation or during rainfall season (Clarkson et al. 2004).

Pathogenic fungi of plants produce a wide array of cell wall degrading enzymes (CWDEs), which include, pectinases, β-1,3-glucanases, glycosidases, cellulases, xylanases and cutinases (Annis and Goodwin, 1997). Carbon and nitrogen sources, as well as ambient pH, are the precursors for the activation of these CWDEs at the transcriptional level. *S. sclerotiorum* secrete pectinases in particular polygalacturonases (PGs) that are induced by the presence of galacturonic acid, which is a pectin monomer. This then degrades pectin to allow the fungus to penetrate the cell wall and feed on its carbon source (Fraissinet-Tachet and Fevre, 1996, Riou et al. 1992).

Plants have an innate defense mechanism at the molecular level that can deter invading pathogens. Cell-wall-associated glycoproteins, such as polygalacturose-inhibiting protein (PGIP), have been shown to be effective in slowing down pathogen growth (Zuppini et al. 2005). Also, the secretion of endoPG's by pathogens elicits the hypersensitive response (HR), e.g., a rapid plant cell death aimed at stopping further invasion of the pathogen. Although HR has been shown to be more effective against biotrophic pathogens (pathogens that grow on living tissue), it promotes the growth and development of necrotrophic pathogens (pathogens that live on dead plant tissue) such as *S. sclerotiorum* (Govrin and Levine, 2000, Thomma et al. 2001). As a consequence, this necrotrophic fungal pathogen is more difficult to control than the biotrophic pathogens.

Oxalic acid (ethanedioic acid), has been implicated as the main pathogenicity factor of *S. sclerotiorum*. During the early stages of pathogenesis, oxalic acid accumulates in host plant infected tissue. As the oxalic acid concentration increases, it lowers the pH to 4 or 5

(Bolton et al. 2006). It is this low pH produced by oxalic acid that allows *S. sclerotiorum* to escape the inhibitory action of plant defense PGIP's. The low pH also weakens the plant's defense system (Favaron et al. 2004). In addition oxalic acid chelates calcium and pectic material, which in turn allows polygalacturonase to hydrolyze pectates and disrupt the integrity of host cell walls (Smith et al. 1986, Kurian and Stelzig 1995). As a consequence calcium dependent plant defense response, production of polyphenol oxidases and the oxidative burst are compromised due to the action of oxalic acid (Cessna et al. 2000, Bolton et al. 2006). Oxalic acid also induces the wilting of leaves by preventing guard cells from closing the stomata and inhibiting ABA induced stomatal closure. Strong evidence implicating oxalic acid as the main pathogenicity factor of *S. sclerotiorum* derives from the fact that it has been recovered from host infected tissue (Ferrar and Walker 1993). The most compelling evidence is the fact that mutant strains of *S. sclerotiorum* that are incapable of producing oxalic acid and yet posses all the CWDE arsenal are non-pathogenic (Godoy et al. 1990). In view of this, an opportunity exists to develop transgenic plants that can inhibit the effect of oxalic acid.

2.2 Germin Gene (*gf2.8*)

The germin gene (*gf2.8*) is a pepsin-resistant, homohexameric glycoprotein that is water soluble with a monomer molecular mass of ~22 kDa and an oligomer molecular mass of ~130 kDa (Lane, 2002). Germin protein is believed to promote plant cell hydration. Germin protein has been shown to correlate with germinating and maturing of embryos in wheat (Lane et al. 1992, 1993). Germin is an oxalate oxidase (G-OXO) which degrades

oxalic acid into CO_2 and H_2O_2 (Schmitt 1991). H_2O_2 promotes localized hypersensitive response (HR) cell death, but most importantly, H_2O_2 is toxic to the oxalate producing pathogens such as *S. sclerotiorum*. H_2O_2 also promotes lignification and cross linking of cell walls, which provides a barrier against invading fungal pathogens. Germin-OXO is able to free-up chelated Ca^{2+} bounded by oxalic acid (Luttrell et al. 1993, Apostol et al. 1989). As a consequence, the germin gene helps to promote plant defense against fungal pathogens such as *S. sclerotiorum*. Isoforms of the germin gene are present in all cereals, including wheat (Lane 2002). This is why most cereals are not susceptible to oxalate producing fungi such as *S. sclerotiorum*. While breeding initiatives are progressing to develop resistance to *S. sclerotiorum*, transgenic approaches have been attempted in a number of crops. Table 3 shows a summary of transgenic work performed in various crops using the germin gene to develop resistance to *S. sclerotiorum*.

Table 3: Summary of transgenic research using the germin gene in different crops

Crop	Method of Transformation	Method to Confirm Transgenes	Method Used to Test Transgene Activity	References
Helianthus annuus (Sunflower)	*Agrobacterium*: Plants were transformed with the *gf2.8* cDNA regulated by supermass promoters	Northern Blot showed a high level of transcription	*1.Sclerotinia sclerotiorum* infection assay; Description: 6 -wk old plants were infected with mycelia of the fungus. 2. Analysis of SA accumulation; Description; Total SA was extracted from 0.6g leaves tissue and samples were analyzed using liquid chromatography and UV light adsorption was measured using a photodiode array detector (model: 996 waters). 3. Histochemistry and microscopy Assay; Description; 5-wk leaf tissue was boiled in ethanol lactophenol 2:1 and viewed under an epifluorescence microscope. 4. Detection of oxalate oxidase (OXO) activity and H_20_2 accumulation; Description: OXO activity was detected by analyzing H_20_2 accumulation in the leaves in the presence of Oxalic Acid (OA)	Hu et al. 2003

Table 3: Continue…

Populus fastigiata (Poplar tree)	*Agrobacterium*: Plants were transformed with *gf2.8* regulated by CAMV 35S promoter	PCR SDS-Page gel showing the expression of germin gene (130 kDa).	Oxalate Oxidase (OXO) Assay; Description: Histochemical assay was done to localize the OXO in tissue. This was done by incubating 2.5mM OA in succinate buffer (25mM succinate acid, 3.5mM EDTA pH4.0 plus 4-chloro-1-naphthol 0.6g mg/ml. OXO activity was measured at an extinction coefficient of A350. 2. Oxalic Acid tolerance assay; Description; Samples were treated with 200mM OA for 2h. Infected areas were measured using NIH image 1.61 program. Control samples were treated with HCl pH 1.15 with 5 replicates. 2. Detection of OA produced by fungus; Description; OA calculations were done as described by Boehringer Mannheim. 3. *In vitro* pathogen resistance test; Description; leaf discs 15 mm diameter were inoculated in 20 ul of 10^6 conidia/ml and the severity of necrosis was analyzed.	Haiying et al. 2001

Table 3: Continue…

Glycine max (Soybean)	*Agrobacterium*: Plants were transformed with *gf2.8* regulated by CAMV 35S promoter	Southern Blot was used as well as western blot with a polyclonal germin antisera antibody. The level of activity was significant.	Histological screen for OXO activity; Description; leaf tissue were placed in detection solution as described above and samples with dark blue or purple color were designated positive. 2.Microscopy of tissue stained for OXO activity ; Description; Zeiss Axiphot microscope with Ektacheme 64T tungsten film set at 100 ASA. 3. Quantitative OXO assay on pellet and supernatant fraction. Description; Protein fractions were incubated in OXO developing solutions and absorbance of 555nm was used to determine the OXO activity. 4.Innoculation of samples with pathogen; Description; plant leaves and stems were wounded and the pathogen was inoculated on the wound sites.	Donaldson et al. 2001
Arachis hypogaea (Peanut)	Biolistic: Callus was bombarded with *gf2.8* regulated by CAMV 35S promoter.	Southern blot was used northern blots showed significant expression.	Oxalate Oxidase (OXO) activity Assay; Description; method same as one described above. 2.H_20_2 was measured using the Amplex Red Kit as per manufacturers instruction. 3. Oxalic Acid (OA) Bio-assay; Description: protocol same as described above used to assess level of tissue tolerance in the presence of OA. 4.Fungal Bio-assay; Description: same protocol as described above was used to assess level of tissue tolerance in the presence of fungal pathogen.	Livingstone et al. 2005

Table 3: Continue…

Solanum lycopersicum (Tomato)	The binary vector pGPTV driven by the CAMV35S promoter was used with the *Agrobacterium* strain LB4404	PCR, SDS- poly-acrylamide gel. The expected band size was 124kD	1.Histochemical Assay; Description: leaf tissue was placed in detection solution as described above and samples with dark blue or purple color were designated positive. 2.Wilting assay of leaf discs; Description: leaves were subjected to various levels of AO, 5,10, 20 and 30mM for a period of 24hrs. 3. Inoculation of samples with pathogen; Description; plant leaves and stems were wounded and the pathogen was inoculated on the wound sites. Pathogen concentration was 10^6 conidia/ml and the severity of necrosis was analyzed.	Walz et al. 2008

CHAPTER III

GOALS, OBJECTIVES AND METHODOLOGY

3.0 Goals

The overall goals of this research were to develop novel protocols for an *in vitro* regeneration and gene delivery system for common bean (*P. vulgaris*).

3.1 Specific Objectives

- **Objective I:** Develop an efficient and reproducible *in vitro* regeneration protocol for common bean.

- **Objective II:** Develop a gene delivery system for common bean using *gus* screenable marker gene, *bar* herbicide resistance selectable marker gene, *HVA1* drought/salt tolerance gene and germin-OXO (*gf2.8*) gene which confers resistance to fungal disease caused by *S. sclerotiorum.*

- **Objective III:** Evaluate putatively transgenic plants for transgene integration and expression

- **Objective IV:** Evaluate transgenic plants for their biological activity and function.

3.2 Materials and Methods

3.2.1 *In vitro* regeneration protocol for common bean

3.2.1.1 Plant material

Ten genotypes of common bean were used in this research. They were provided by Dr. James D. Kelly of Michigan State University. These ten genotypes were used in order to explore the different genetic diversities and the different potential for *in vitro* growth and regeneration. These genotypes represent the nine main commercial classes, the two main gene pools and the four main races of common bean that are grown in North America. Table 4 is a summary of the genotypes used. Complete details on each genotype can be found at http://www.css.msu.edu/bean/Variety.cfm

Table 4: Ten genotypes of *P. vulgaris* representing the ten different commercial classes grown in Northern America

GENOTYPE	**Commercial Class**	**Gene Pool**	**Race**
'Beluga'	White kidney	Andean	Nueva Granada
'Condor'	Black	Middle American	Mesoamerica
'Jaguar'	Black	Middle American	Mesoamerica
'Matterhorn'	Great northern	Middle American	Durango
'Merlot'	Small red	Middle American	Jalisco
'Montcalm'	Dark red kidney	Middle American	Nueva Granada
'Olathe'	Pinto	Middle American	Durango
'Redhawk'	Dark red kidney	Andean	Nueva Granada
'Seahawk'	Navy	Middle American	Mesoamerica
'Sedona'	Pink	Middle American	Jalisco

3.2.1.2. Seed sterilization and explant preparation

Seeds were rinsed twice with sterile distilled water; immersed in 75% ethanol for 3 min; rinsed thrice with sterile distilled water; and immersed for 20 min in a solution of 25% commercial Clorox, 5 ml L^{-1} Tween20 and 10 ml L^{-1} of 0.02% $HgCl_2$. Following sterilization, the seeds were rinsed five times in sterile distilled water and soaked for 20 hours. After soaking, the seeds were dissected and the embryos were excised. The hypocotyl and cotyledons were removed, leaving the epicotyl with apical meristem primordia. The excised epicotyl with apical meristem primordia were incubated *in vitro* for 5 days at 25^{o}C with 16 hours photoperiod and light intensity of 45-70 µmol/m^2/sec in the culture media described below.

3.2.1.3. *In vitro* multiple shoot regeneration media

Regeneration culture media contained 4.43 g L^{-1} MS (Murashige and Skoog 1962), 3% sucrose, 100 mg L^{-1} casein hydrolysate and 2.5 g L^{-1} gelrite, 2.5 mg L^{-1} benzyladenine (BA) and 0.1 mg L^{-1} indole-3-acetic acid (IAA). Silver nitrate 30 mg L^{-1} was added as an anti-oxidant to get rid of the phenolic compounds. After three weeks of visible shoot primordia growth the explants were transferred to shoot development media containing the above ingredients, excluding silver nitrate and adjusting BA and IAA to 1 mg L^{-1} each. Explants were kept on this medium for seven weeks before being transferred to rooting media, which contained all ingredients of the shoot development media excluding

BA and adjusting IAA to 0.1 mg L^{-1} supplemented with 4 mg L^{-1} of glufosinate of ammonium for selection. Shootlets were kept on this media for five weeks until firm roots developed. Growth regulators were added after autoclaving the media for 25 min at 120°C and 690 kPa. The final media combinations were then poured into 100 × 25 mm Petri dishes and solidified under a laminar flow hood. *In vitro* cultures were incubated at 25°C with 16 hours photoperiod and light intensity of 45–70 µmol m^{-2} s^{-1}.

Beacuse phenolic compounds were being produced in *in vitro* cultures of common bean, an experiment was designed to negate the inhibitory effects of these compounds. The experiment was conducted only with the most-phenolic producing genotype, 'Condor'. The apical meristem shoot multiplication experiment was repeated in media containing 4.43 mg L^{-1} of MS salts and vitamins, 2.5 mg L^{-1} BAP and 0.1 mg L^{-1} IAA and four different antioxidants which included ascorbic acid (2 mg L^{-1}), silver nitrate (30 mg L^{-1}), activated charcoal (15 mg L^{-1}) and glutathione (5mg L^{-1}) based on modification of published data (Abdelwahd et al. 2008).

3.2.1.4. Statistical and experimental design for shoot regeneration

The statistical design was a three-way factorial in a completely randomized design (CRD). In this design, ten genotypes were evaluated using nine levels of cytokinin (BAP and TDZ) and seven levels of auxin (NAA and IAA). The 10x9x7 factorial experiment

with 630 treatments was replicated three times. Each experimental unit (Petri dish) consisted of five *P. vulgaris* embryonic axes apical meristem primordia explants. After regeneration, three out of five samples were randomly selected for analysis.

A total of 1,890 experimental units with 5,670 data points were analyzed using PROC GLM (SAS version 9.1.3). An Analysis of Variance (ANOVA) was used to test the statistical significance at an alpha level of 0.001.

3.2.1.5. *In vitro* rooting of regenerated shootlets

The cut end of the regenerated shoots (2 cm long) were dipped (treated) for 30 s in different concentrations (0.0 1.0, 5.0, or 10 mg L^{-1}) of indole-3-butyric acid (IBA). The treated shoots were then cultured in 4.43 mg L^{-1} MS medium containing different concentrations (0.0, 0.05, 0.1 or 1.0 mg L^{-1}) of naphthalene acidic acid (NAA), indole-3-acetic acid (IAA) or IBA to examine rooting potential.

3.2.1.6. Statistical and experimental design for rooting

The statistical design for the *in vitro* rooting was a two factorial experiment in a CRD with the first factor being IBA dipping solution at four levels, and the second factor being auxin concentrations at ten levels. The 4x10 factorial experiment with 40 treatments was replicated in space three times. From each experimental unit (Petri dish), five explants where cultured and three plantlets were randomly selected for analysis. A total of 360

experimental units were analyzed using PROC GLM (SAS version 9.1.3). Analysis of Variance (ANOVA) was used to test the statistical significance at an alpha level of 0.001.

3.2.1.7. Morphogenesis studies via scanning electron microscopy

Samples of *in vitro* multiplied shoot apices, grown from apical shoot primordia explants, were fixed, dehydrated and dried as described by Klomparens et al. (1986). These samples were then coated with gold particles and microphotographed with a JEOL JSM 31 (Tokyo, Japan) scanning electron microscope.

3.2.2 Genetic transformation

3.2.2.1 Gene Constructs

pACT1F: The construct depicted below (Figure 2) was used as a selectable marker for transformation of β-glucuronidase (*gus*) into common bean.

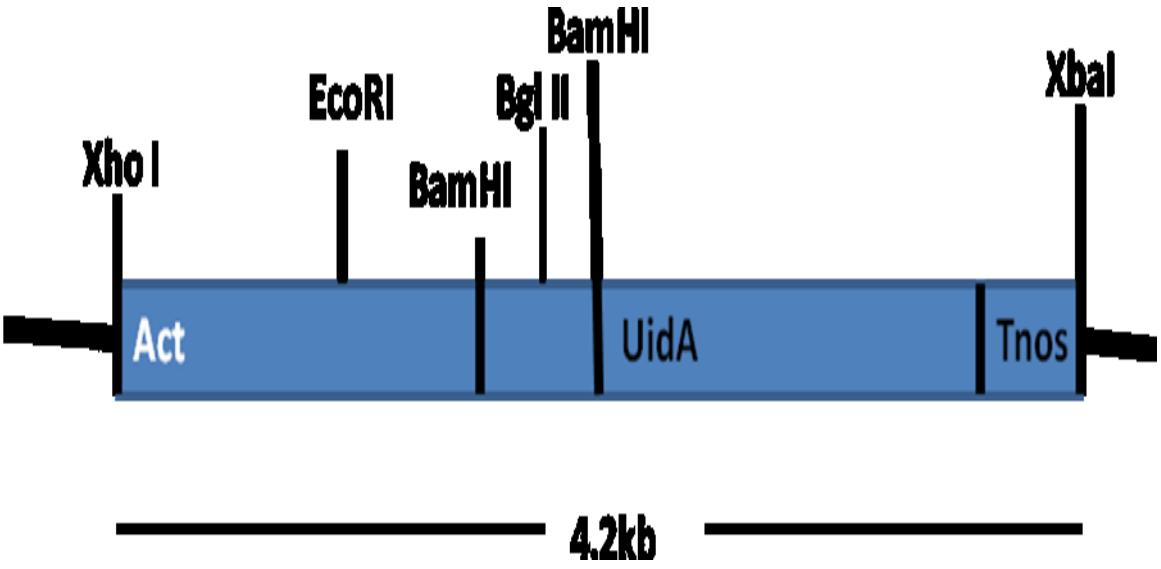
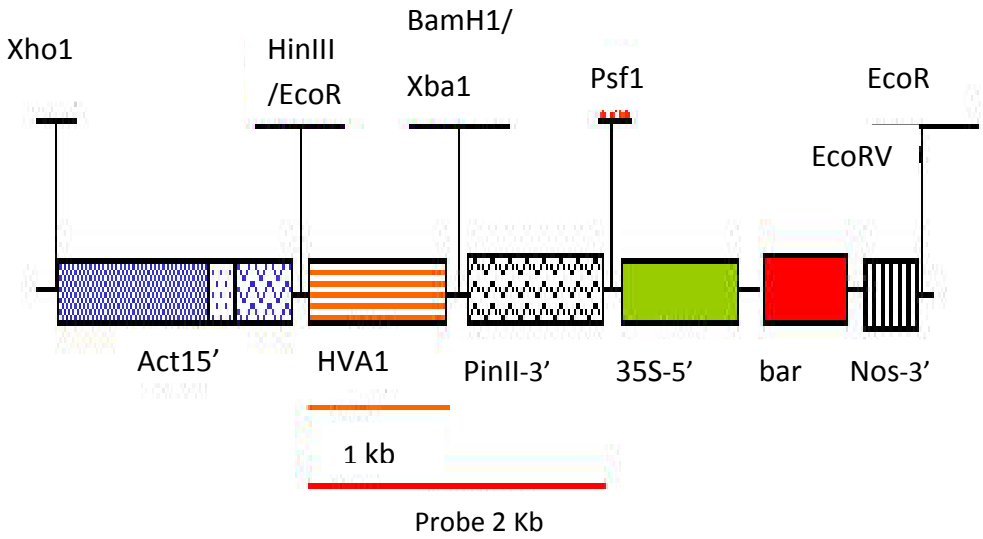


Figure 2: Linear map of pACT1F cassette (not drawn to scale). Rice actin promoter (Act), *gus* gene (*UidA*), and nopaline synthase terminator (*Tnos*)

pBY520: The construct depicted below (Figure 3) was used for the transformation of common bean with the *HVA1* gene conferring drought and salt stress tolerance. This construct also contains the *bar* gene as a selectable marker.



pBY520 (8.1kb) Xu et al. 1996

Figure 3. Linear map of pBY520 cassette (not drawn to scale). Rice actin promoter (Act1), Barley or *Hordeum vulgare* (*HVA1*) LEA 3 gene, Cauliflower Mosaic Virus 35S promoter, *bar* gene and nopaline synthase terminator (*Tnos*)

pBKS*bar*/*gf2.8*: The construct depicted below (Figure 4) was used for the transformation of common bean with the germin gene (*gf2.8)* that produces oxalate oxidase. This construct also contains the *bar* gene as a selectable marker.

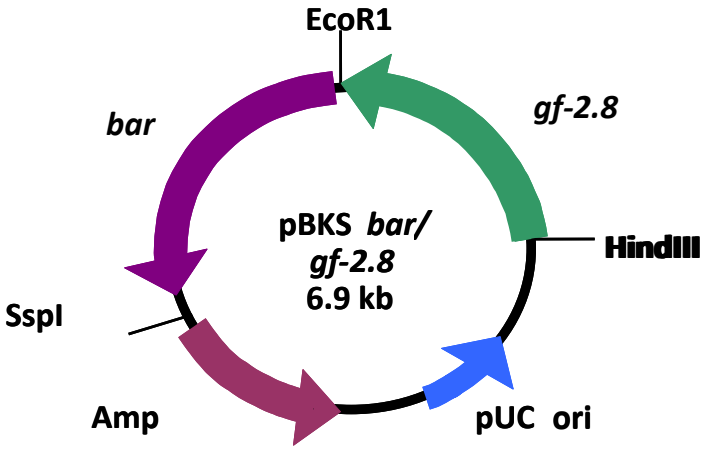


Figure 4**.** Circular map of the 6.9 Kb of pBKS*bar*/*gf2.8*, not drawn to scale. *Amp*= ampicilin resistant marker, *bar*=herbicide selectable marker, pUC ori= origin of replication of the pUC 18 plasmid vector.

pCAMBIA3301: The binary vector depicted below (Figure 5), not drawn to scale, was used for *Agrobacterium* transformation of common bean with the *gus* and *bar* gene as a selectable marker.

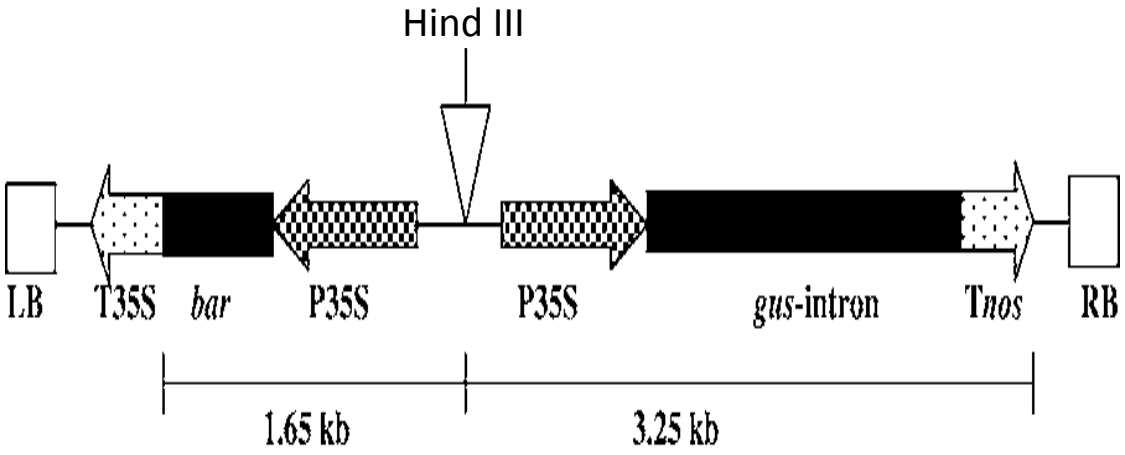


Figure 5. Linear map of pCAMBIA3301 T-DNA cassette. LB/RB – left/right T-DNA border sequences; P35S/T35S – CaMV 35S promoter/terminator; *bar* – coding region of the phosphinothricin resistance gene; *Tnos* – nopaline synthase terminator; *gus*-intron – *gusA* gene coding region with intron sequence

3.2.2.2. Transformation of plasmid vectors into *E.coli* competent cells

All plasmid vectors of interest were transformed into *E. coli* for multiplication. In order to transform *E. coli,* 50 µl of DH5α *E. coli* competent cells (Sigma) were used which were mixed with 2 µl of plasmid DNA. The mixture was placed on ice for 25 min and then heat-shocked for 45 s in a water bath at a temperature of 42^0C. Immediately thereafter, it was placed on ice for 2 minutes. Luria Bertani (LB) medium 950 µl was added to the tubes containing the transformed *E.coli*. This was then incubated for 2 hours at 37^0C with 150 rpm shaking. After two hours the cultures were plated on solid LB media with the appropriate antibiotic, kinamycin or ampicilin at a concentration of 15 mg L^{-1} for bacterial colony selection. Colonies that grew on the LB selection media were putative transformants and single colony PCR was performed to see the presence of the gene(s) of interest. Upon confirming gene presence, the single colony was then placed into glass flask containing 50 ml of LB media which was then incubated at 37^0C with 280 rpm shaking for 48 hours. Thereafter, the plasmids where purified using Qiagen plasmid purification kit (Cat.No.12123) as per manufacturers instruction. Purified plasmid was used for either Biolistic^{TM} bombardment or for *Agrobacterium* transformation.

3.2.2.3 Biolistic^{TM} bombardment

Apical shoot meristems of mature embryos were excised and then bombarded with the helium particle delivery System (gene gun), model PDS-1000 (DuPont, Wilmington, DE). The plasmid DNA was coated onto 50 µg L^{-1} of 10 µm tungsten particles with 2.5 M calcium chloride and 0.1 M spermidine suspended in a solution of 1:1 (v/v) of 75% ethanol and 50% glycerol. Three levels of pressure were applied (3447, 6895 and 7584 kPa) to assess the most effective pressure. The concentration of plasmid DNA per bombardment was varied at 1.5 µg and 3.0 µg in order to see which concentration was most favorable. Three levels of bombardment frequency (1, 2 and 3) were used and plant tissues were kept for 24 hours before re-bombarding tissue. The plasmid vector pACT1F (Figure 2), containing the *gus* marker gene, was used in a mixture of 1:1 (v/v) with the plasmid vector pBY520 (Figure 3) containing the *bar* herbicide resistant selection marker gene. The plasmid vector *gf2.8* (Figure 4) for white mold resistant was transformed independently.

3.2.2.4 *Agrobacterium* transformation

Three strains of *A. tumefaciens* (EHA105, GV3301 and LBA4404) were used, transformed with the pCAMBIA 3301 binary vector (Figure 5) containing *gus* gene driven by the 35S promoter with or without *bar* gene. These were cultured in 50 µl LB media in the dark at 37°C in a rotator at 280 rpm for 48 hours; OD_{600}=1. These strains were co-cultivated with the explants for 1, 5, 10 and 15 days. The regeneration media

described above was supplemented with 500 mg L^{-1} of Timentin to kill the *A. tumefaciens* after the appropriate co-cultivation period.

3.2.3 Confirmation of transgene integration and expression

3.2.3. 1. Polymerase Chain Reaction (PCR) analysis

Polymerase chain reaction (PCR) analysis for the detection of *HVA1, gf.2.8* and *bar* genes was conducted on T0-T3 plants. Genomic DNA was obtained from leaf disks with diameters the size of the lid of a 1.5 ml Eppendorf tube. Extraction of DNA was done using REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, MO, Cat No. XNA-P), as per manufacturer's instruction. The primers used were: *bar* F, 5`-ATG AGC CCA GAA CGA CG-3` (forward primer); *bar* R, 5`-TCA CCT CCA ACC AGA ACC AG-3` (reverse primer); and *HVA1* F, 5`-TGG CCT CCA ACC AGA ACC AG-3` (forward primer); *HVA1* R, 5`-ACG ACT AAA GGA ACG GAA AT-3` (reverse primer); *gf2.8* F, 5`-ATG GGG TAC TCC AAA ACC CTA G-3` (forward primer); *gf2.8* R, 5`-CTA GAA ATT AAA ACC CAG CG-3`(reverse primer). The thermocycler (PerkinElmer/ Applied Biosystem, Forster City, CA) was used for DNA amplification. Optimized PCR conditions were 94°C for 3 min for initial denaturation; 35 cycles of 50 s at 94°C; 50 s at 56°C, 1 min at 72°C and a final 10 min extension at 72°C. The PCR product was loaded onto a 1% (w/v) agros, gel stained with 2 µl ethidium bromide and visualized under UV light.

3.2.3. 2. Southern Blot Hybridization Analysis

The Southern blot hybridization analysis was conducted to determine the stability of the transgenic event and determine the gene copy numbers of *HVA1, gf2.8* and *bar* gene. The DIG High Prime DNA Labeling and Detection Starter Kit (Roche Co., Cat. No. 1 585 614), was used as per manufacturer's instructions. Transgenic and non-transgenic genomic DNA was isolated using the methods described by Saghai-Maroof et al. (1984). Hind III or BamHI restriction enzymes were used to digest 20 µg of genomic DNA, which was electrophoresed at 70 v on 1% agarose gel and transferred to a Hybond-N+ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA) at an energy level of 2,000 J. The DIG labeled probes that were used for *bar, HVA1* and *gf2.8* were synthesized using the primers for the specific gene as described above.

3.2.3.3. Reverse Transcription-PCR (RT-PCR)

PCR positive plants of *gf2.8* and *HVA1* were used in the RT-PCR analysis. Leaf tissue weighing 200 mg was ground using liquid nitrogen. Trizol Reagent 1 ml (Invitrogen, Carlsbad, CA) was applied to homogenize samples. In each tube 0.2 ml chloroform was added and vortexed for a few seconds. The tubes were placed into a centrifuge and spun at 12,000 xg for 15 min at 4^{o}C. In fresh tubes containing 0.5 ml of isopropanol, 0.2 ml of aqueous phase was transferred. Samples were incubated at room temperature for 5 min and centrifuged at 12,000xg for 10 min at 4^{0}C. The supernatant was discarded, leaving

the RNA pellet. The pellet was washed with 1 ml 75% ethanol and flicked to better wash it and later spun in a centrifuge at 7,500xg for 2 min at a temperature of 4^{o}C. The RNA pellets were immediately dissolved in RNase-free water and quantified using a spectrophotometer. The RNA obtained was used for cDNA synthesis using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The same primers and PCR conditions for *gf2.8* and *HVA1* as described above were used but the number of cycles were reduced from 35 to 25 in order to detect differences in banding intensity.

3.2.3.4. Northern Blot

Northern blot analysis was conducted using the DIG labeled Northern Starter Kit (Roche Co., Cat.No. 12 039 672 910), as per manufacturer's instructions, in order to assay the gene expression of the transgenic plants harboring the *bar, HVA1* and *gf2.8* transgenes. Total RNA from the leaves of transgenic and non-transgenic plants was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO), as per manufacturer's instructions. A total of 30 µg of RNA per sample was loaded onto a 1.2% (m/v) agarose-formaldehyde denaturing gel as described by Sambrook et al. (1989) and transferred to a Hybond-N+ membrane (Amersham-Pharmacia Biotech) and fixed with a UV crosslinker (Stratalinker UV Crosslinker 1800, Stratagene, CA) at an energy level of 200 J. An RNA or DNA DIG labeled probe, containing the coding region of the gene of interest, was used for detection of transcripts.

3.2.4 Biological activity tests

3.2.4.1. Histochemical *gus* assay

Gus activity was tested on transgenic and non transgenic seeds and embryos using histochemical staining with 5-bromo-4-chloro-3-indoyl-β-D-glucuronicacid salt (X-gluc). Plant samples were dipped into *gus* substrate buffer, according to Jefferson et al. (1987), and incubated at 37°C for 24 hours. The tissue samples were washed with 100 percent ethanol to remove all coloration.

3.2.4.2. Biological assay for *bar*

The herbicide Liberty (Aventis, Strasboug, France), with the active ingredient ammonium glufosinate, was used in both multiple shoot and rooting media, and applied to determine which plants were transgenic as well as to score the segregation ratios of the transgenic progeny. Plants were sprayed at different stages of growth and development ranging from three-week-old young seedlings to two and three-month-old plants. Different foliar application rates of the herbicide were assessed ranging from 50, 100, 250, and 350 mg L^{-1} of the Liberty herbicide.

3.2.4.3. Drought tolerance test

Seedlings were raised in the growth chamber for three weeks or until trifoliate leaves appeared. They were then transferred to the greenhouse into 15 cm diameter clay pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX). The plants were watered daily for three weeks, after which moisture was withheld for 21 days. Observations were recorded on plant survival, degree of leaf wilting, root length, plant growth and height. After the 21 days, moisture was applied to the plants continuously for 14 days in order for them to recover from the drought. The percentage of plants recovered was recorded.

3.2.4.4. Salt tolerance stress test

Seedlings were raised in the growth chamber for two weeks. They were then transferred to the greenhouse into 8 cm diameter plastic pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX). The plants were watered daily with normal tap water for one week in the greenhouse after being transferred from the growth chamber. Thereafter, moisture was withheld for a week to induce drought symptoms, after which five levels (0, 50, 100, 150 and 200 mM) of NaCl concentration was applied to the plants for 10 days. Observations were made and recorded on plant survival, degree of leaf wilting, root length, plant growth and height. After 10 days, water application without NaCl was applied daily for one week in order to recover plants injured by salt stress.

3.2.4.5 Pathogen resistance test: Fungal bio-assay

Two methods were used to conduct the fungal bioassay. The first was the straw test, in which an agar plug of *S. sclerotiorum* mycelia was inserted into a straw and placed over the cut stem of the transgenic beans growing in the greenhouse. The second was the detachment of trifoliate leaves that were inoculated with *S. sclerotiorum* mycelia by placing a 6mm diameter agar plug with inoculum on center of the detached leaf. The inoculation was conducted either in a Petri dish or in a glass tray covered with a plastic paper containing agar or wet paper towel placed at the bottom to keep the leaves and the fungus moist during the infection process. The source of the *S. sclerotiorum* was obtained from Dr. J. Kelly's lab at Michigan State University. The fungal pathogen was grown at room temperature in the dark for 72 hours in medium containing potato dextrose agar (Difco, VWR, Montreal, Quebec, Canada). The protocol used was modified from Livingstone et al. (2005).

CHAPTER IV

RESULTS AND DISCUSSION : SYSTEMS DEVELOPMENT FOR *IN VITRO* REGENERATION OF COMMON BEAN

4.0 *In vitro* regeneration

In vitro regeneration of *P. vulgaris* poses the greatest obstacle and challenge limiting potential for an efficient genetic transformation system of common bean. Attempts have been made towards developing various *in vitro* regeneration protocols for *P. vulgaris*. Intact seedling (IS) and cotyledonary node (CN) tissue, cultured on full MS medium with 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA resulted into buds and shoots being produced more from IS than CN (Ahmed et al., 2002). Cotyledon explants for *P. vulgaris* 'XAN–159', regenerated successfully as opposed to embryonic axis explants which failed. In contrast both explants of *P. acutifolius* genotypes 'NI574' regenerated successfully with embryonic axis giving the best results. *P. vulgaris* had difficulty acclimatizing in the greenhouse due to poor *in vitro* rooting ability. However, *P. acutifolius* established easily in the greenhouse. *In vitro* grafting to harden *P. vulgaris* was done as a means to overcome this problem (Zambre et al., 1998). Arellano et al (2009) developed an *in vitro* regeneration protocol for *P. vulgaris* 'Negro Jamapa' black bean using indirect organogenesis with 50% regeneration frequency. Apical meristems and cotyledonary nodes explants were used for callus induction on medium containing 1.5 µM 2,4 Dichlorophenoxyacetic acid and shoot development on medium containing 22.2 µM 6-

benzylaminopurine. Delgado-Sanchez et al. (2006) used embryonic axes of *P. vulgaris* 'Flor de Junio Marcela' (FJM) and 'Flor de Mayo Anita' (FMA) to regenerate whole plantlets with 83% and 50% regeneration efficiency respectively when cultured on MS supplemented with 5 or 10 mg L^{-1} benzylaminopurine (BAP).

An efficient and repeatable *in vitro* plant regeneration protocol is the most important requirement for successful genetic transformation. In addition, *in vitro* regeneration is also important for the recovery of certain valuable germplasm which naturally have low germination potential. Furthermore, certain *in vitro* regenerations such as shoot apical meristem culture is also important for mass propagation of virus free and true-to-type plants which can be distributed to farmers in regions of the world where there is high prevalence of seed borne viral diseases (Delgado-Sanchez et al., 2006).

The objective of this study was to develop a highly efficient and reproducible *in vitro* shoot apical meristem multiplication and somatic embryogenesis protocols for different genotypes of *P. vulgaris* that are commonly grown in the U.S.A using different combinations of concentration of cytokinin, auxin and antioxidants for optimization of efficient apical shoot meristem multiplication.

4.1 Organogenesis and embryogenesis

The statistical model for the experiment was significant with an R-square value of 98% and a coefficient of variation of 17.5 with a root mean square of 77% (appendix 1). Statistically significant differences were observed for *in vitro* regeneration performance of different *P. vulgaris* genotypes (appendix 2). The separation of means for the different genotypes (appendices 3 and 4) showed that there were no statistically significant differences for the different genotypes within the races except for genotypes belonging to the race Durango which showed a significant difference (Table 5).

The effect of growth regulators is also significant. For example, there are statistically significant differences among different cytokinin and cytokinin concentration levels (appendix 6). There are also significant differences among different auxins and auxin levels (appendix 8). As a result of interaction between growth regulators and genotype, the number of multiple shoot regeneration varies from genotype to genotype depending on the growth regulator combination used. Overall the most efficient growth regulator combination for shoot multiplication was a combination of BAP 2.5 mg L^{-1} and IAA 0.1 mg L^{-1} which produced an average of 12 multiple shoots per explant in all genotypes tested (Figure 6). Table 6, shows the genotypic specific growth regulator combination that gave the highest number of multiple shoots. The result in figure 7 further show that 'Olathe' produced the highest number of multiple shoots followed by 'Sedona', 'Merlot', 'Matterhorn', 'Seahawk', 'Jaguar', 'Redhawk', 'Beluga', 'Montcalm' and 'Condor'.

Table 5: Effect of genetic origin as represented by gene pool and race on the efficiency of apical shoot meristem multiplication of 10 contrasting genotypes of common bean (*Phaseolus vulgaris*)

Genotype	Gene Pool	Race	Mean Number of Multiple Shoots
'Montcalm'	Andean	Nueva Granada	3A
'Redhawk'	Andean	Nueva Granada	3A
'Beluga'	Andean	Nueva Granada	3 A
'Condor'	Middle American	Mesoamerica	4 B
'Jaguar'	Middle American	Mesoamerica	4 B
'Seahawk'	Middle American	Mesoamerica	4 B
'Matterhorn'	Middle American	Durango	5 C
'Merlot'	Middle American	Jalisco	6 D
'Sedona'	Middle American	Jalisco	6 D
'Olathe'	Middle American	Durango	7 F

Means with same letter are not different $LSD_{0.001}$ was used to separate the means

Table 6: Genotypic specific growth regulator combination promoting highest number of multiple shoots

Genotypes	Growth regulator combination
'Beluga'	BAP 5 mg L^{-1} and NAA 0.1 mg L^{-1}
'Condor'	TDZ 1 mg L^{-1} and IAA 0.1 mg L^{-1}
'Jaguar'	TDZ 2.5 mg L^{-1} and IAA 0.05 mg L^{-1}
'Matterhorn'	BAP 5 mg L^{-1} and IAA 0.1 mg L^{-1}
'Merlot'	TDZ 2.5 mg L^{-1} and NAA 0.1 mg L^{-1}
'Montcalm'	BAP 5 mg L^{-1} and NAA 0.05 mg L^{-1}
'Olathe'	BAP 2.5 mg L^{-1} and IAA 0.1 mg L^{-1}
'Redhawk'	TDZ 2.5 mg L^{-1} and NAA 0.1 mg L^{-1}
'Seahawk'	TDZ 1 mg L^{-1} and IAA 0.1 mg L^{-1}
'Sedona'	BAP 2.5 mg L^{-1} and IAA 0.1 mg L^{-1}

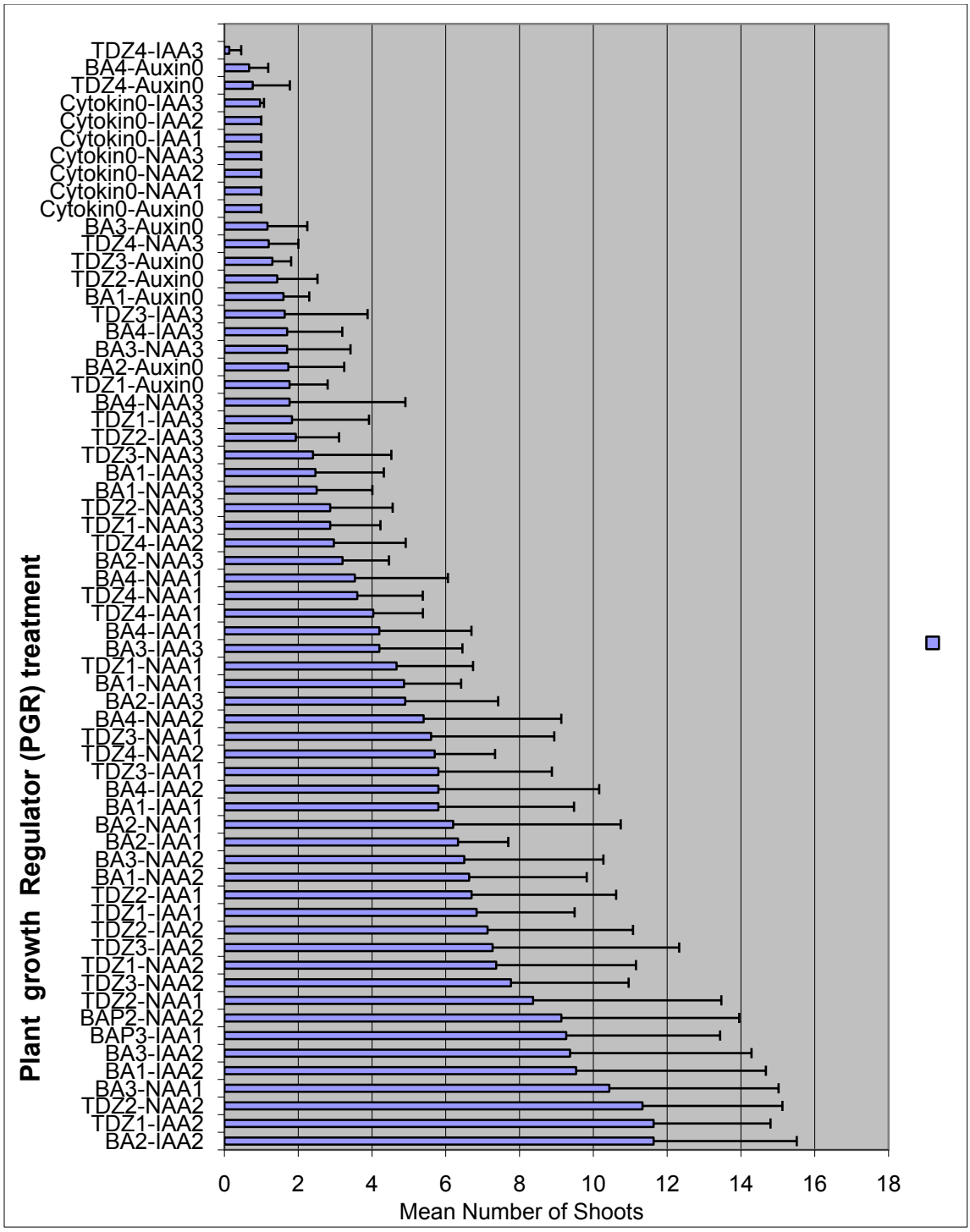


Figure 6: Effect of cytokinin-auxin combinations on apical shoot meristem multiplication of 10 *P. vulgaris* genotypes. Note: BAP/TDZ 1,2,3&4=1, 2.5, 5,10 mg L^{-1}; NAA/IAA 1, 2, 3= 0.05, 0.1, 1 mg L^{-1} (for supplemental data see appendix 9 and 10)

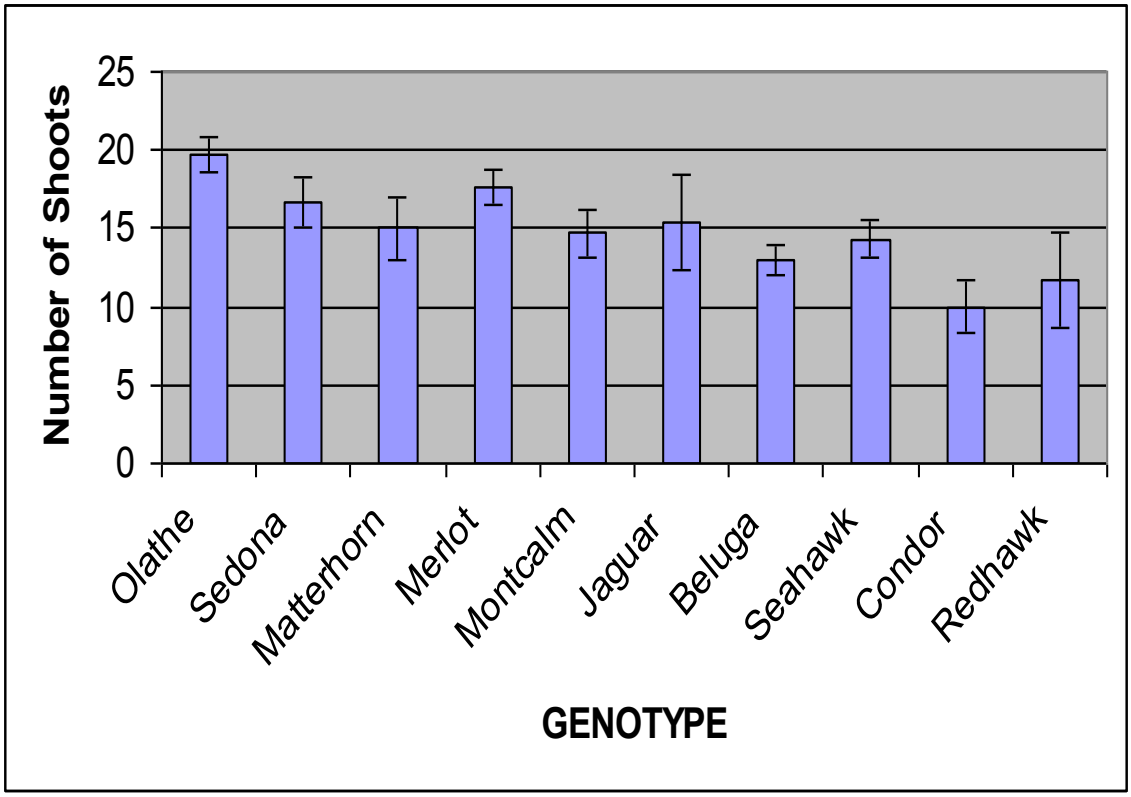


Figure 7: In vitro apical shoot meristem multiplication performance of 10 *P. vulgaris* genotypes

*Overcoming phenolics from culture*s: Phenolic compounds exuding from the excised site of the embryonic axis gave a characteristic browning and black color, which hindered normal growth and development of multiple shoots *in vitro*. To overcome this problem the MS media with growth regulators was supplemented with various antioxidants as described in the materials and methods. The qualitative comparison of control treatment that had no antioxidants and treated tissue with antioxidants, showed that silver nitrate (30 mg L^{-1}) and activated charcoal (15 mg L^{-1}) produced better quality multiple shoots (Figure 8) with reduced degree of browning and weight of secondary callus tissue (Table 7).

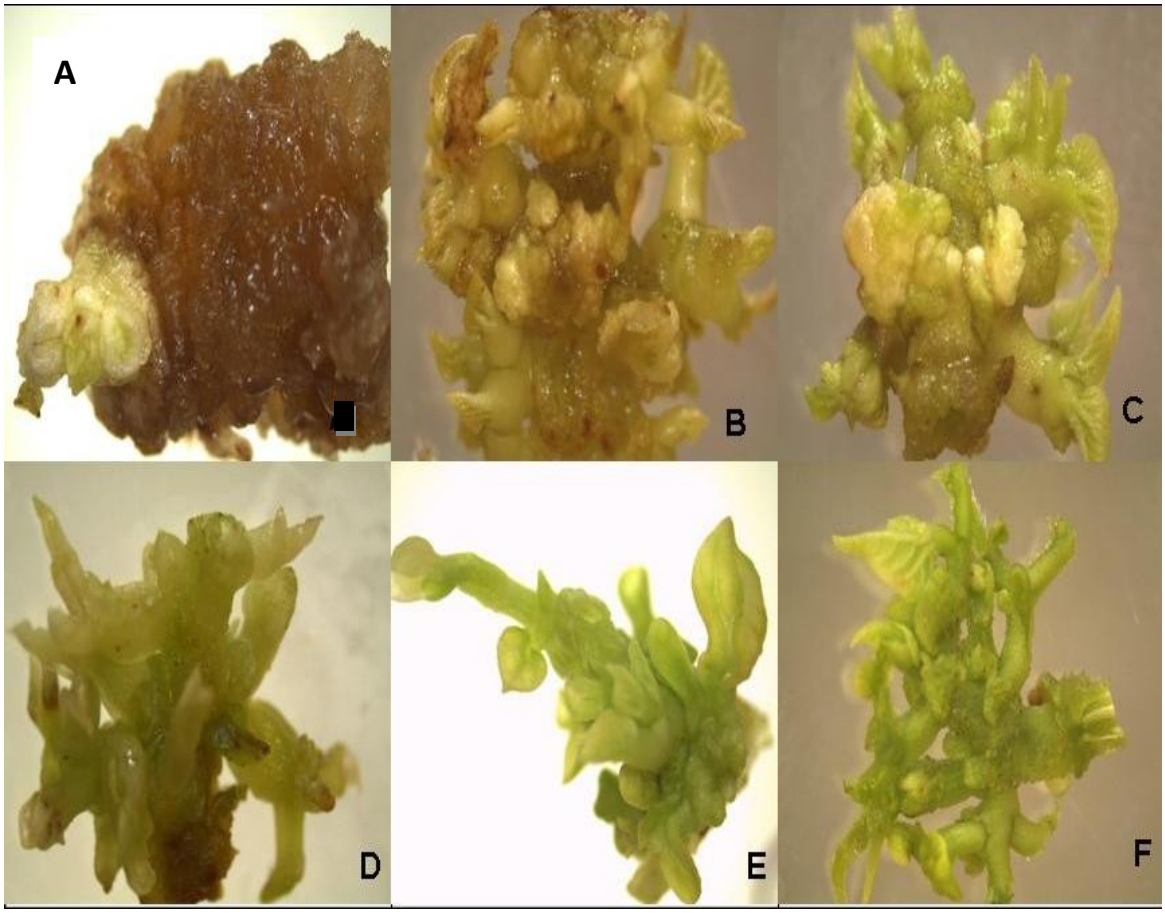


Figure 8: Effect of 4 antioxidant treatments on the quality of multiple shoots

- A an extreme case of an untreated explant failing to regenerate due to phenolics
- B control treatment with no antioxidant
- C treatment with ascorbic acid
- D treatment with glutathione
- E treatment with activated charcoal
- F treatment with silver nitrate

Table 7: Effect of anti-oxidants on apical shoot meristem qaulity in common bean genotype, 'Condor'

Antioxidants	Percent Browning of Multiple Shoots	Weight (mg) of Secondary Callus at the Multiple Shoot Base
No antioxidant	67±7	6.5±2
Ascorbic Acid	54±5	4.5±1
Silver Nitrate	24±4	1.2±0.6
Activated Charcoal	22±5	0.9±0.4
Glutathione	48±3	2.3±0.8

Morphogenesis studies via scanning electron microscopy. Direct adventitious shoot primordia were formed (Figure 10A) after 3 weeks of culturing the embryonic axes in culture media A (Figure 9). Direct somatic embryogenesis (Figure 10B) occurred after the same duration of culturing the embryonic axis in culture medium B (Figure 9). Shoots developed from clumps of adventitious shoot primordia after 4 weeks on culture medium D (Figure 10C and D) and from direct somatic embryos after 7 weeks on culture medium D (Figure 10C). Rooted *P. vulgaris* plantlets were obtained 5 weeks by culturing of 2-3 cm long shootlets on culture medium E (Figure 10E). The type and concentration of growth regulators were the key factors in determining the morphological pathway of *in vitro* regeneration of *P. vulgaris*. Cytokinin, in particular BAP resulted into organogenesis, whereas, less cytokinin in particular TDZ resulted into initiation of embryogenesis (Figure. 9).

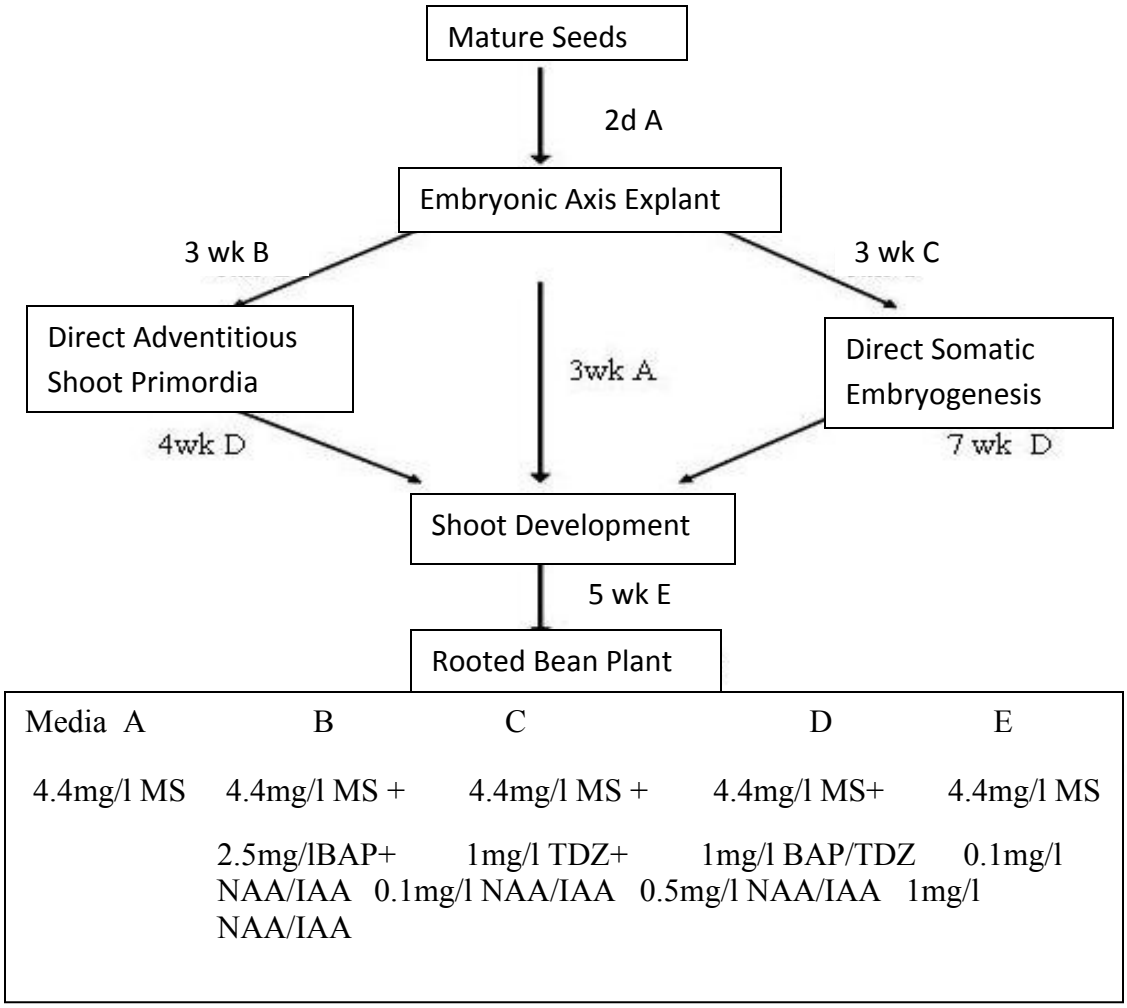


Figure 9: Effect of growth regulator combinations on morphogenesis pathway of in vitro cultures of *P. vulgaris*

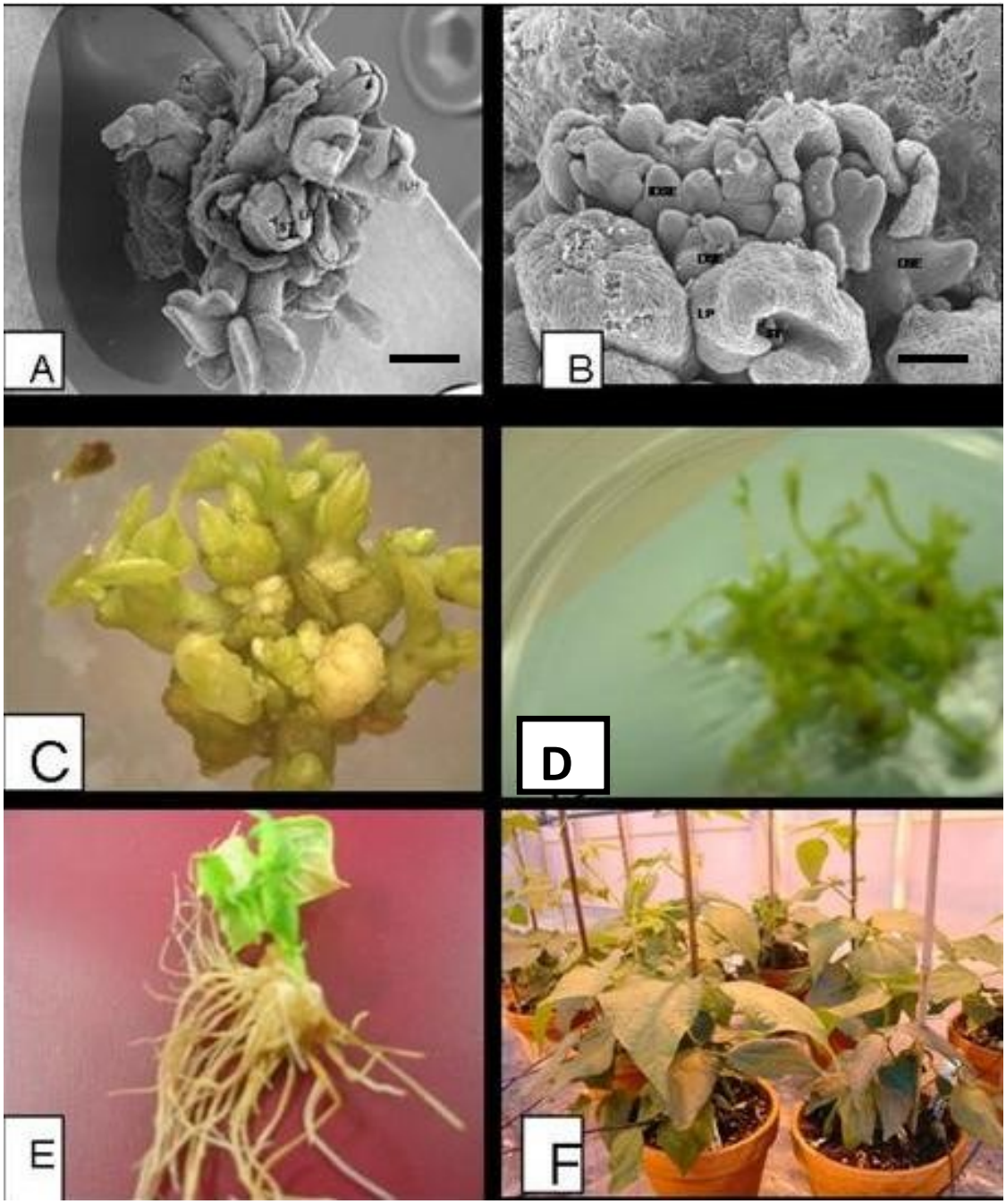


Figure 10: Differentiation of somatic embryos, multiple shoots and regenerated mature greenhouse grown rooted 'Olathe' common bean plants.

A. Scanning electron micrograph of a section of a multiple shoot clump 3 wk after *in vitro* culture of an embryonic axis. AvS, adventitious shoot; LP, leaf primordia; ST, shoot tip; LH, leaf hair. x 20; *bar*= 200µm

B. Scanning electron micrograph of a mixture of somatic embryos and organogenesis resulting 6 wk after *in vitro* culture of excised apical meristem of an embryogenic axis explant. IDSE, indirect somatic embryo; DSE, direct somatic embryo; LP, leaf primordia and ST, shoot tip resulting from organogenesis. . x 40; *bar*= 200µm

C. An advanced regenerated shoot clump and embryogenic tissues 6 wk after *in vitro* culture of an embryonic axis. x 8

D. An advanced apical multiple shoot clump regenerated through organogenesis 10 wk after *in vitro* culture of an embryonic axis.

E. Effect of 30 s dipping of the cut end of a single *in vitro* regenerated shoot in 1.0 mg/l IBA followed by 5 wk of culture in 0.1 mg/l NAA.

F. Greenhouse grown mature plants produced from rooted shoots.

Rooting. The higher level of auxin and lower levels of cytokinin had the greatest effect on root establishment. Auxin level of 0.1 mg L^{-1} gave the best results while lesser amount (0.05 mg L^{-1}) led to poor or no root development (Figure 11). High amounts of auxin 1 mg L^{-1} in the presence of low amount of cytokinin 1 mg L^{-1}, gave many roots with little or no shoots while the same high amount of auxin in the presence of high concentration of cytokinin (5-10 mg L^{-1}) gave no roots and a few short shoots with many large leaves. There were no statistically significant differences among different auxin types based on the number and length of roots produced. However, there were significant differences among different concentration levels used on the root length and number of roots produced. The effect of dipping shootlets in IBA was also significant (Figure 12). Overall the best treatment that produced strong multiple root establishment was dipping in 1 mg L^{-1} IBA solution and then culturing of the IBA treated shootlets in media containing 0.1 mg L^{-1} of NAA, IAA or IBA. This resulted in the number of roots ranging from 1 to 28 and the root length ranging from 4 to 48 cm (for statistical and supplemental data on rooting see appendices 12, 13, 14, 15 and 16).

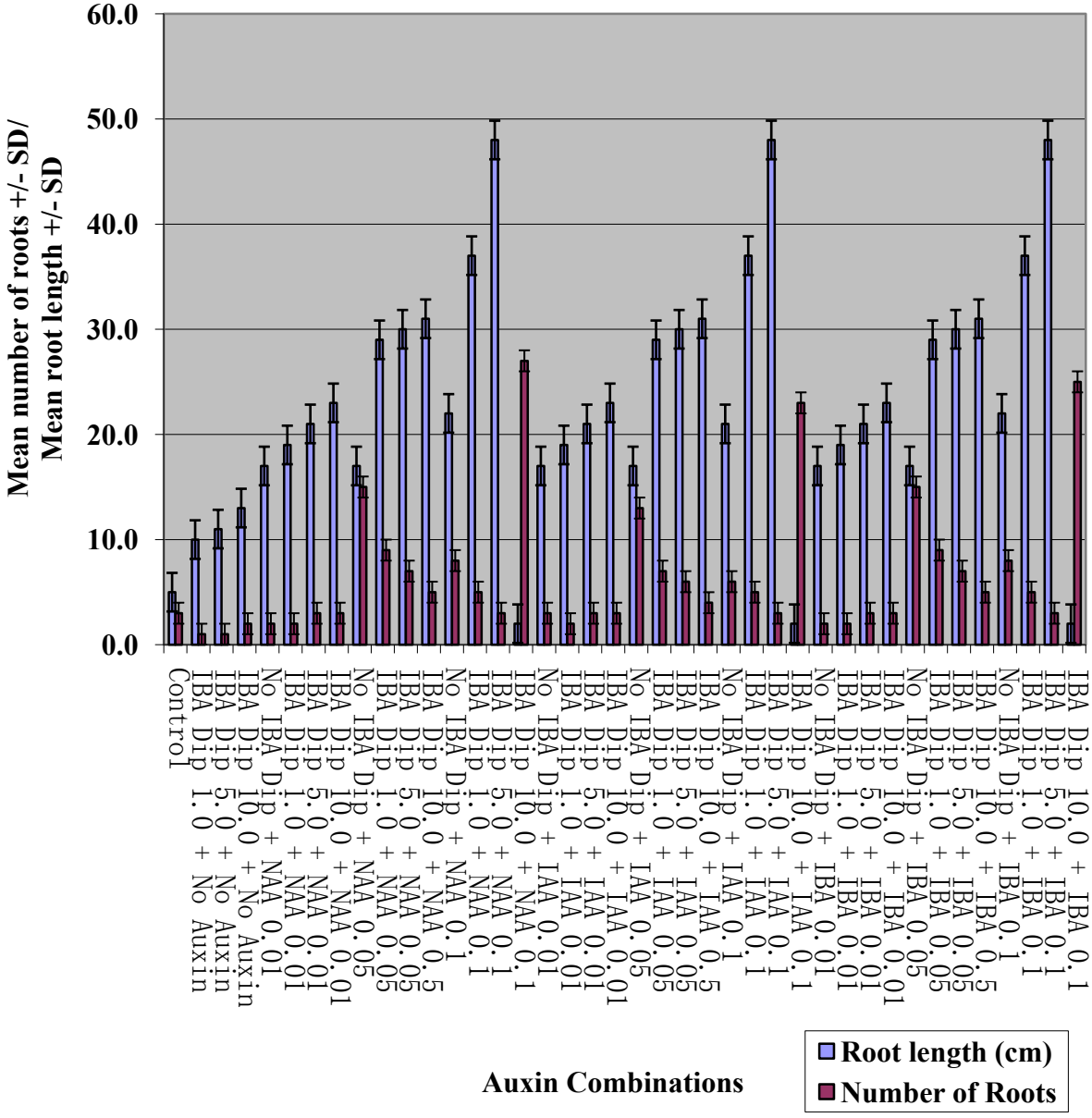


Figure 11: Effect of dipping shootlets in IBA and culturing of shoots in different auxins on the number and the length of regenerated roots five weeks after transfer of shoots into rooting media.

Figure 12. *In vitro* response of rooting ability using different levels of IBA dipping system

Figure legend for figure 12

Label	Treatment
A	Plain MS media without any hormones
B	1 mg L^{-1} IBA dipping solution
C	5 mg L^{-1} IBA dipping solution
D	10 mg L^{-1} IBA dipping solution
E	0.05 mg L^{-1} of NAA, IAA or IBA
F	0.1 mg L^{-1} of NAA, IAA or IBA
G	1 mg L^{-1} of NAA, IAA or IBA
H	1 mg L^{-1} IBA dipping solution plus 0.05 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
I	1 mg L^{-1} IBA dipping solution plus 0.1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
J	1 mg L^{-1} IBA dipping solution plus 1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
K	5 mg L^{-1} IBA dipping solution plus 0.05 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
L	5 mg L^{-1} IBA dipping solution plus 0.1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
M	5 mg L^{-1} IBA dipping solution plus 1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
N	10 mg L^{-1} IBA dipping solution plus 0.05 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
O	10 mg L^{-1} IBA dipping solution plus 0.1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media
P	10 mg L^{-1} IBA dipping solution plus 1 mg L^{-1} of either NAA, IAA or IBA in 4.4 mg L^{-1} MS media

Acclimation of rooted plantlets and transfer to greenhouse. Rooted plantlets were removed from Petri dishes, the agar media was removed from the roots by direct rinsing under running tap water, and the washed rooted plantlets were transferred into small pots containing BACTO potting soil. The pots were covered with plastic bags to eliminate evaporation resulting in high humidity around the potted plantlets to mimic the high humidity in Perti-dishes. Potted covered plantlets were maintained under fluorescence light for three weeks or until new leaves emerged on the plants. Holes of approximately 3 mm in diameter, were punched in the plastic bag covers every other day to gradually reduce humidity and to eventually acclimate plantlets to the low humidity in the greenhouse. Acclimated plants were transferred into larger pots and kept in the greenhouse where they were grown to maturity and seeds were produced (Figure 10F).

4.2 Discussion

This experiment has demonstrated that it is possible to efficiently regenerate multiple shoot meristems from excised apical shoot meristem primordia of embryonic axes of *P. vulgaris*. This is achievable through optimization of appropriate combinations and concentrations of cytokinin and auxin. Genotype played a significant role in apical shoot multiplication of *P. vulgaris*. Similar genotypic effects were also demonstrated in cereals by Sticklen and Oraby (2005). The results clearly show that closely related genotypes (belonging to the same race) perform similar as opposed to distantly related genotypes. Observations showed that genotypes that were less recalcitrant towards *in vitro* regeneration were those that were able to heal faster from the wounding caused by excising the hypocotyl and the cotyledonary nodes and those that produced less secondary callus tissue at the excision site.

Cytokinin more than auxin was the key in accelerating wound healing of explants *in vitro* and reducing the amount of callus produced on the wounded explant, since auxins are well known to induce callus tissue *in vitro*. Moderate levels of cytokinin ranging from 2.5 to 5 mg L^{-1} favored the acceleration of wound healing and reduction of callus tissue which resulted in an increase in the number of multiple shoots. On the other hand high levels of cytokinin (10 mg L^{-1}) delayed wound healing and inhibited the production of multiple shoots.

It was observed that *in vitro* plantlet development from somatic embryos in *P. vulgaris* is more difficult with low regeneration efficiency and takes a longer time as compared to shoot development from adventitious shoots followed by rooting. However if one should succeed, somatic embryogenesis has an advantage over adventitious shoots in that potentially more plantlets per explant can be regenerated *in vitro*.

In this study, rooting of *in vitro* grown shoots was a challenge mainly because the base of the shootlets developed phenolic compounds *in vitro* causing blackening and death of cells which prevented rooting. Similar problems were encountered by other researchers (Mohamed et al. 1991, Santalla et al. 1998, Zambre et al. 1998). In order to overcome this problem, the effect of phenolic compounds was reduced by supplementing the growth media with 15 mg L^{-1} activated charcoal or 30 mg L^{-1} silver nitrate and dipping the base of the explant tissue in IBA solution for 30 sec. The rooting media lacked cytokinin as cytokinin was observed to delay root establishment.

As had been indicated by other researchers (Ozyigit et al., 2008), callus combined with phenolic compounds are naturally produced following wounding to aid in healing of plant tissue and to prevent entry of microbes. However in the rooting studies, the greatest limiting factor for *in vitro* regeneration of *P. vulgaris* was its propensity to produce high amounts of callus tissue that blocked root formation and phenolic compounds that caused death of tissues due to oxidation of the tissue (Arnaldos et al., 2001). These oxidized phenols prevent multiple shoot development, rooting or

regeneration of the explants (Ozyigit et al., 2008). In this study, the anti-oxidants, activated charcoal and silver nitrate were able to effectively reduce the oxidative effect of the phenolic compounds resulting into more and better quality multiple shoots with increased *in vitro* regeneration efficiency.

In conclusion, it is possible to successfully regenerate *in vitro* apical meristem primordia into multiple shoots and/or somatic embryos of *P. vulgaris*. *In vitro* regeneration of *P. vulgaris* is genotypic sensitive and therefore the media formulation has to be made specific for a particular genotype in order to obtain the maximum *in vitro* grown multiple shoots and/or somatic embryos. The excretion of phenolic compounds from wounds associated with high production of callus tissue is the greatest obstacle to *in vitro* regeneration of *P. vulgaris.* Supplementation of anti-oxidants to the culture media significantly improves the quality and increases the regeneration efficiency as well as the numbers of multiple shoots and the rooting ability of *P. vulgaris* plantlets *in vitro*.

CHAPTER V

RESULTS AND DISCUSSION: GENETIC TRANSFORMATION SYSTEM DEVELOPMENT IN COMMON BEAN

In this chapter results of experiments that were conducted in order to develop a gene delivery system for common bean are presented. The figures below show the optimization of Biolistic^{TM} and *Agrobacterium tumefaciens* transformation systems followed by confirmation of transgene integration and expression in different common bean genotypes. In order to demonstrate and provide proof of concept for the genetic transformation system that was developed, the *bar* and *gus* genes were used as selectable and screenable markers respectively.

5.1. Optimizing conditions for the Biolistic^{TM} bombardment method using stable integration of the *bar* gene

In the bombardment method, conditions were optimized in order to obtain the maximum efficiency of transformation. The optimized conditions involved varying the pressure of the gene gun at three levels (3447, 6895 and 7584 kPa); varying the plasmid DNA concentration at two levels (1.5 and 3.0 μg); and finally, the number of times that embryonic tissue was bombarded varied at three levels (1, 2 and 3 times), each 24 hours apart.

To optimize the transformation efficiency of common bean, using the particle bombardment method, results suggest bombarding the plant twice, using a pressure setting of 7584 kPa with a concentration of 1.5 μg of plasmid DNA per bombardment. Such conditions yielded a transformation efficiency of 8.4% (Table 8).

Table 8: Different treatment combinations (gene gun pressure, DNA plasmid concentration and bombardment frequency) used for optimizing Biolistic^{TM} bombardment conditions

Bombardment Pressure (kPa)	Concentration of plasmid DNA (ug)	Bombardment Frequency	Mean Transformation Percent
3447	1.5	1	0.1±0.04
3447	1.5	2	0.2±0.10
3447	1.5	3	0.4±0.30
3447	3	1	0.1±0.04
3447	3	2	0.6±0.32
3447	3	3	0.7±0.32
6895	1.5	1	2.9±0.67
6895	1.5	2	3.9±1.4
6895	1.5	3	5.1±1.2
6895	3	1	5.6±1.0
6895	3	2	8.1±0.3
6895	3	3	7.4±1.0
6895	1.5	1	7.2±0.70
7584	1.5	2	8.4±0.74
7584	1.5	3	8.2±0.50
7584	3	1	7.5±0.69
7584	3	2	4.8±0.93
7584	3	3	3.3±0.92

The gene gun pressure was the greatest determining factor for successful integration of transgene. Low gene gun pressure yielded very low and poor transformation efficiencies while increased frequency of bombardment damaged the explants. The transformation efficiency that was obtained was higher than those that have been reported by other researchers who have bombarded explants only once or used different gene gun pressures (Somers et al., 2003, Popelka et al., 2004).

5.2. Optimization of conditions used in developing *Agrobacterium*-mediated transformation method for transient expression of *gus* gene

A. tumefaciens was used to evaluate its potential as a vehicle for gene delivery. Many researchers have failed to use *A. tumefaciens* as a vector for delivery of foreign genes into common bean (Velchelva et al. 2005). The approach used in this research was to optimize conditions for *A. tumefaciens*-mediated transformation. Different co-cultivation periods were assessed as well as different strains of *A. tumefaciens*, which included EHA105, LBA4404 and GV3301. The relative transformation efficiencies were also compared among 'Sedona' and 'Matterhorn' genotypes

The results indicated that, for both transient and stable expression of *gus* gene, 'Sedona' is more amenable to *Agrobacterium* transformation than 'Matterhorn'. The results also show that, for both transient and stable expression of *gus* gene, the *Agrobacterium* strain GV3301 is the most effective when compared to EHA105 or LBA4404. The most favorable co-cultivation period for high transformation frequency is 15 days. It was noted

that there was a significant discrepancy between transformation efficiencies of tissues that were transiently being expressed as compared to those with stable transformation. With a co-cultivation period of 15 days, using GV3301, transient expression efficiencies of *gus* were 51% with 'Matterhorn' and 81% with 'Sedona'. Using the same co-cultivation period and with the strain EHA105, transient expression efficiencies for *gus* of 66% and 69% were achieved for 'Matterhorn' and 'Sedona' respectively. Under the same conditions using LBA4404, 18% and 50% transient expression efficiencies were achieved for 'Matterhorn' and 'Sedona', respectively (Figure 13).

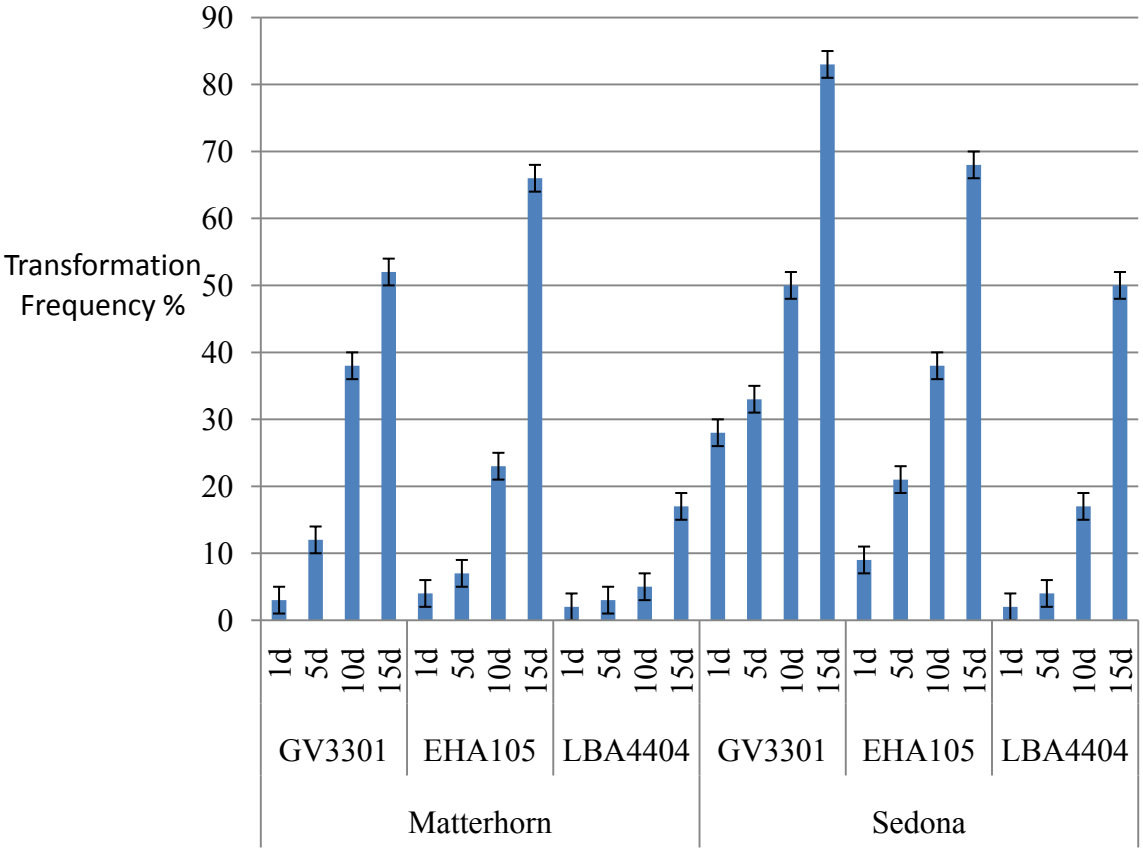


Figure 13. Effect of co-cultivation period (1, 5, 10 and 15 d) on the transformation frequency of two genotypes of common bean, 'Matterhorn' and 'Sedona', using three different strains of *A. tumefaciens* (EHA105, GV3301 and LBA4404).

Despite having these relatively high frequencies of transient expression of the *gus* gene using the *Agrobacterium* method of transformation, there was much lower stable transformation frequencies. For example, with a 15 day co-cultivation period and using *Agrobacterium* strains GV3301, EHA105 and LBA4404, the efficiencies of stable transformation were 0.38, 0.31 and 0.1 percent, in that order, for the genotype 'Matterhorn'. The frequencies for the genotype 'Sedona' were 0.68, 0.52 and 0.36 percent for the strains GV3301, EHA105 and LBA4404, in that order (Figure 14). The reasons why transient expression showed higher frequencies are most likely because (1) not every transiently expressed gene is stably integrated into the transgenic plants, and (2) the GUS staining can diffuse into plant tissues even if the plasmid expression vector is not integrated on the chromosome. Fifteen days of co-cultivation was more effective than fewer days of co-cultivation. In order to improve upon the relative frequencies of stable transformation, future research has to explore increasing the co-cultivation period beyond 15 days and also the use of chemicals such as acetosyringones or tobacco extract to increase the virulence of the *Agrobacterium*.

Stable transformation of common bean with *gus* gene:

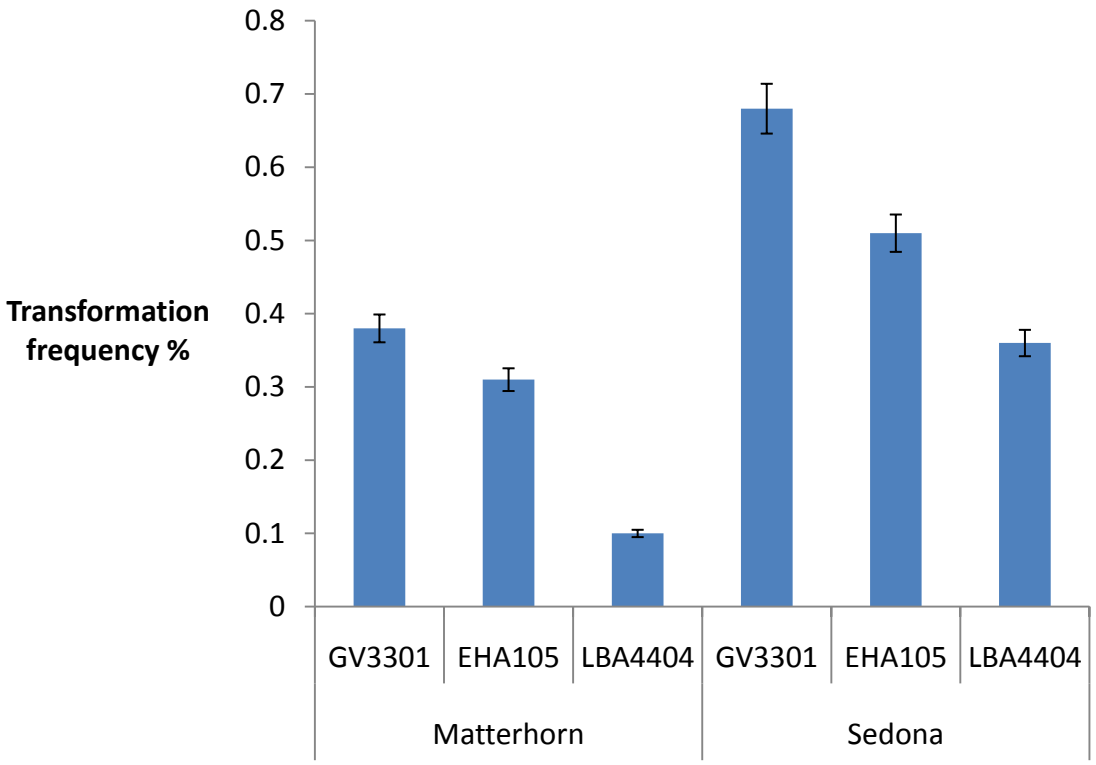


Figure 14. Effect of using different strains of *A. tumefaciens* (EHA105, GV3301 and LBA4404) with two common bean genotypes, 'Matterhorn' and 'Sedona', on the relative stable transformation frequency of T1 (second generation) plants after 15 days of co-cultivation.

5.3 Confirmation of stable gene integration using PCR and Southern blot.

PCR of *bar* transgene integration of T1 plants

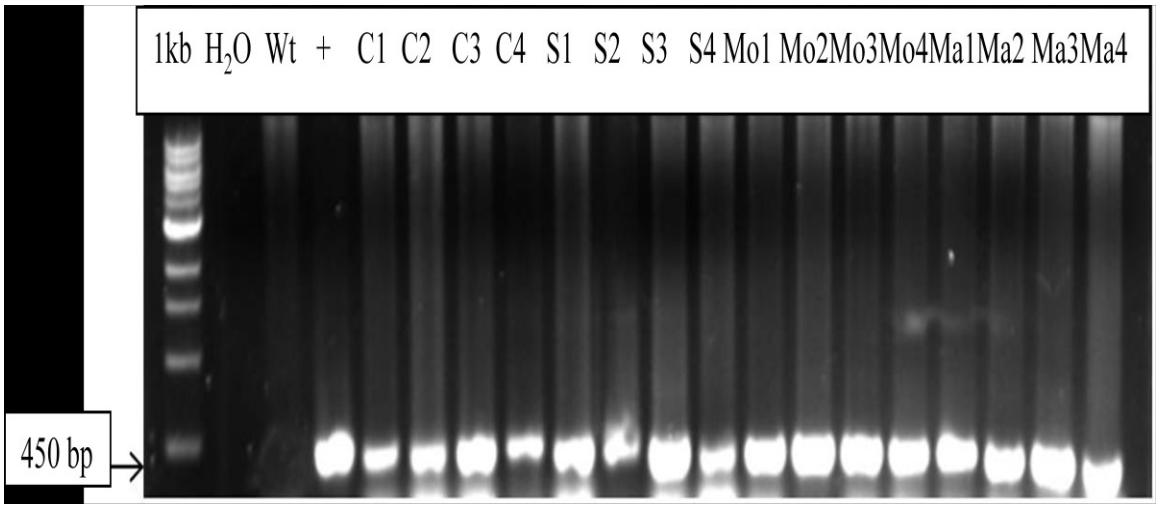


Figure 15: PCR T1 plants of genotypes; 'Condor'(C), 'Sedona'(S), 'Montcalm' (Mo) and 'Matterhorn' (Mat). Expected band size is 450 bp

Segregation of T2 plants confirming the stability of *bar* transgene

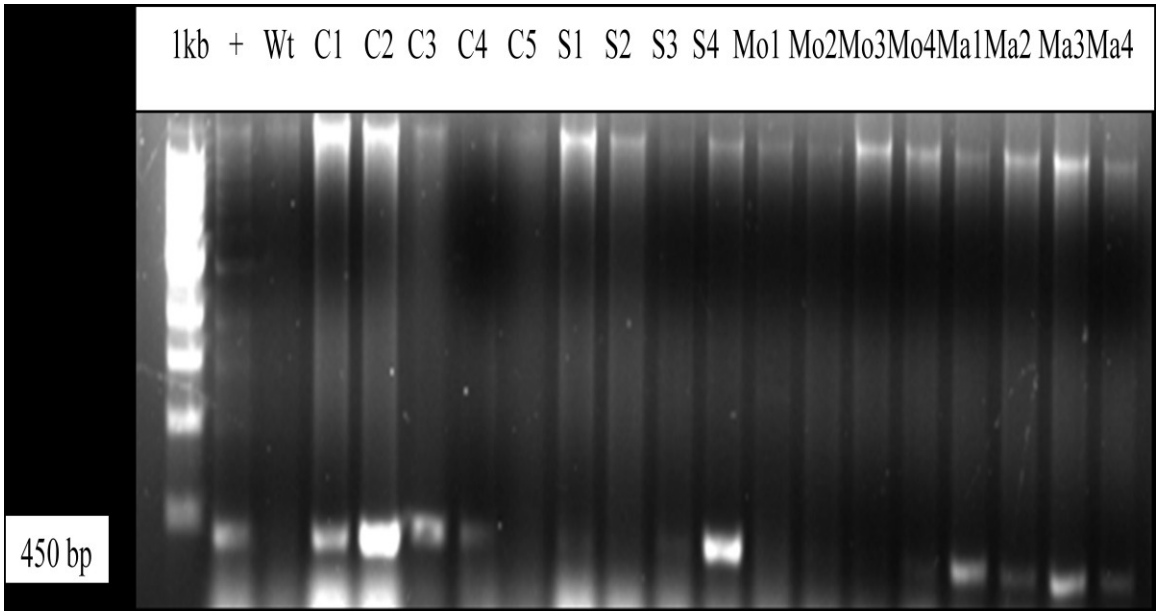


Figure 16: PCR T2 plants of genotypes: 'Condor'(C), 'Sedona'(S), 'Montcalm' (Mo) and 'Matterhorn' (Mat). Expected band size is 450 bp.

Segregating T3 plants confirming the stability of *bar* transgene

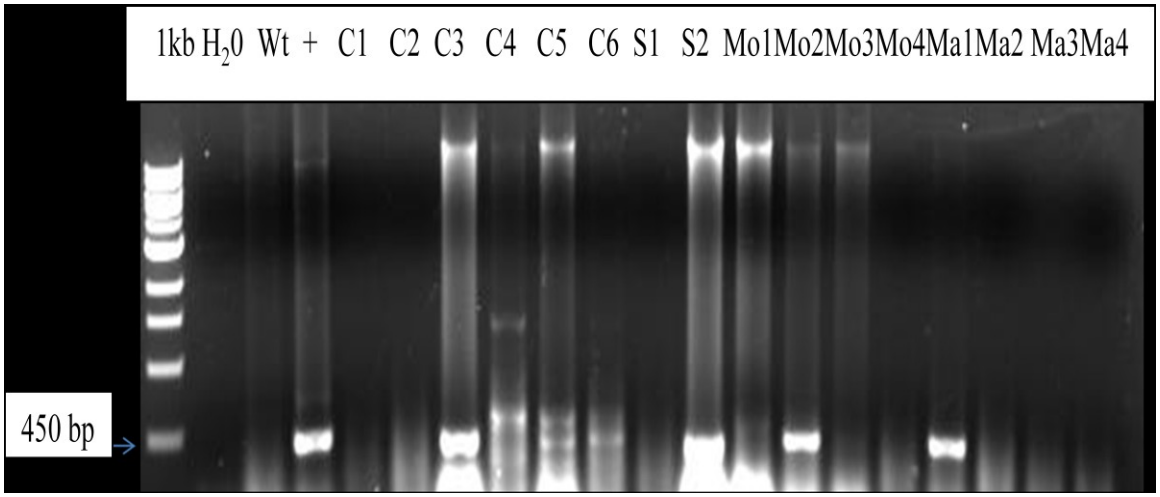


Figure 17: PCR T3 plants of genotypes: 'Condor'(C), 'Sedona'(S), 'Montcalm' (Mo) and 'Matterhorn' (Mat). Expected band size is 450 bp

Southern blot analysis confirming a single gene copy integration of *bar* transgene in T2 'Condor' plants

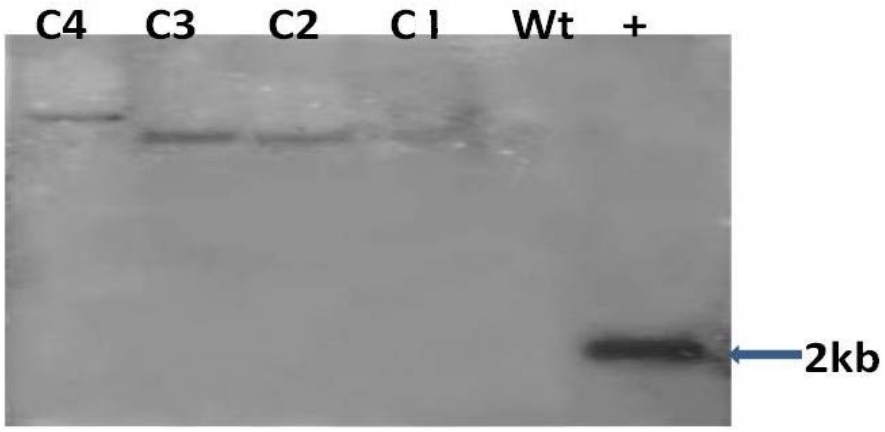


Figure 18: Southern blot showing integration of *bar* gene in genotype 'Condor'. The (+) represents the plasmid DNA; Wt: Wild type non transgenic leaf DNA; C1 to C3: DNA taken from leaves of three transgenic plants of the same transgenic line; C4: DNA taken from leaves of a different independent transgenic line.

Southern blot analysis confirming a single gene copy integration of *bar* transgene in T2 'Matterhorn' plant.

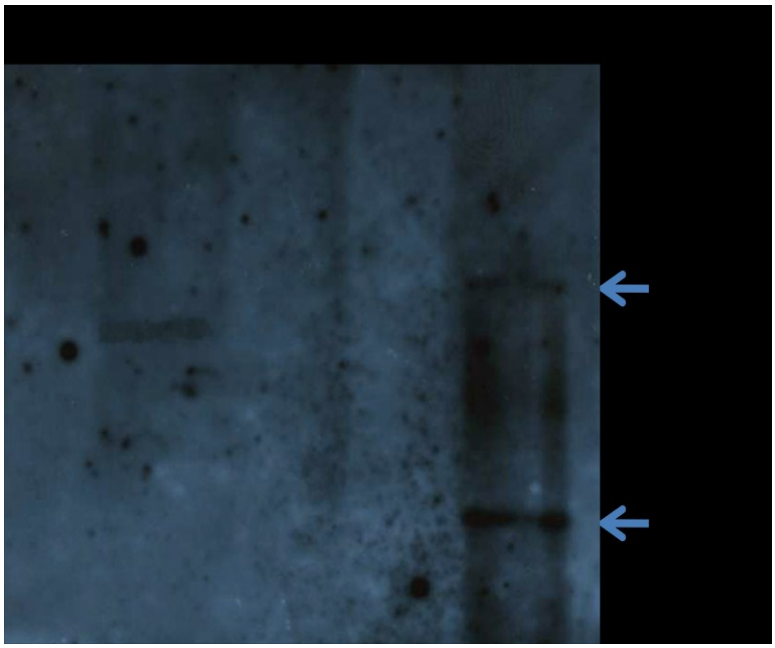


Figure 19: Southern blot showing integration of *bar* gene in genotype 'Matterhorn' line 2 (M2), digested with BamH1, the other line (M1) shows no integration. The results indicate that there is a single gene integration: Wt= Wild type; (+) = plasimid

Southern blot analysis confirming different copy numbers of integration of *bar* transgene in T2 of different common beans genotypes

Figure 20. Southern blot showing integration of *bar* gene in genotypes, Mat = 'Matterhorn', Sed = 'Sedona'and Mont = 'Montcalm', Wt = wild type, (+) = plasmid. Digestion was done with Hind III. The results indicate that there are four copies of the gene in 'Matterhorn', three copies in 'Sedona'and two copies in 'Montcalm'.

5.4 Confirmation of gene expression using Northern blot analysis

Northern blot analysis of *bar* transgene expression in T2 of 'Condor' genotype

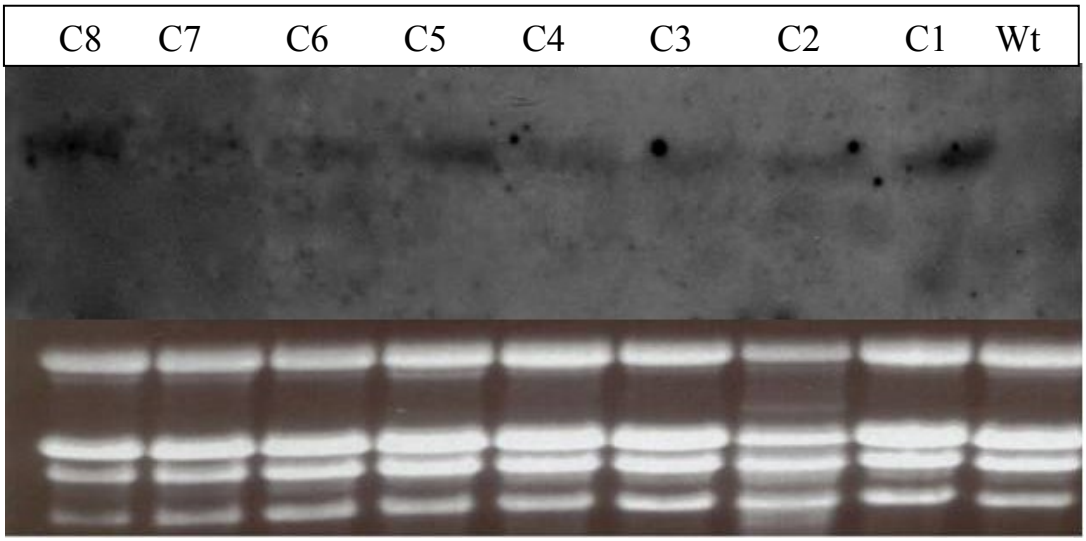


Figure 21. Northern blot expression of *bar* gene in T2 plants; genotype C1-8 = 'Condor', Wt= Wild type.

Northern blot analysis of *bar* transgene in T3 of ‘Condor’ and ‘Matterhorn’ genotypes

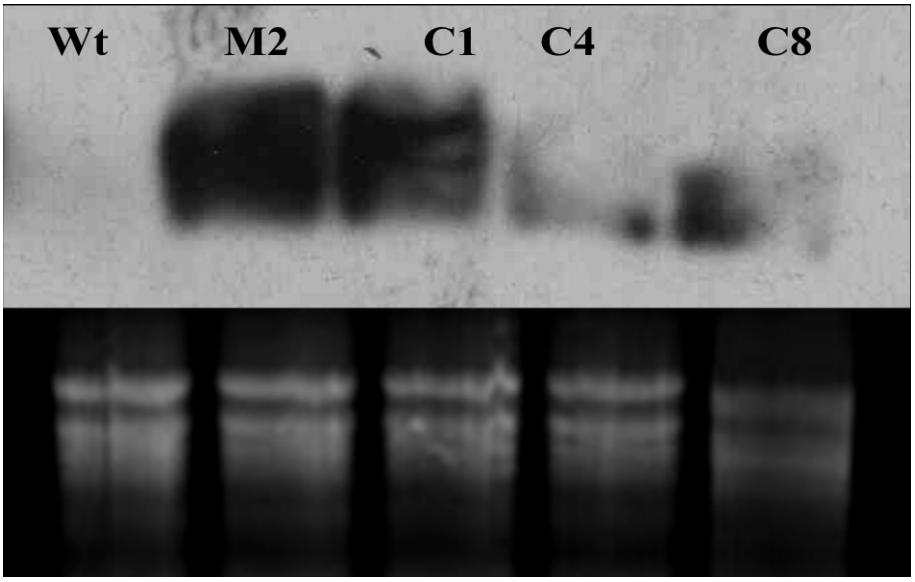


Figure 22. Northern blot expression of *bar* gene in T3 plants: genotype ‘Matterhorn’ (M2), ‘Condor’ lines C1, C4, and C8. ‘Matterhorn’ seems to have a higher expression than the ‘Condor’ lines (C1-C8).

Northern blot analysis of T3 transgenic ‘Sedona’and ‘Montcalm’ genotypes

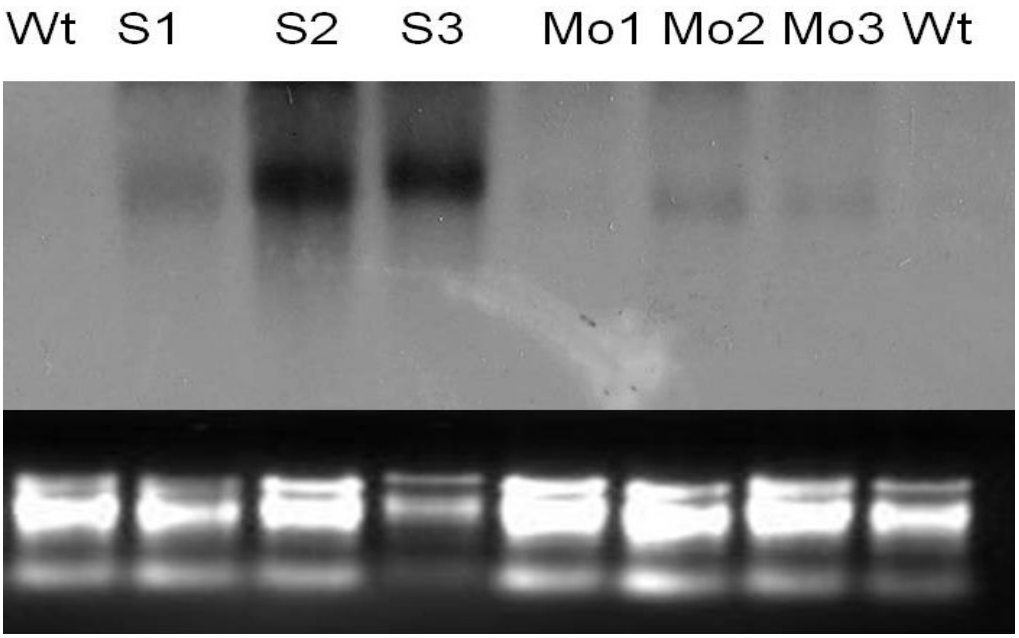


Figure 23. Northern blot expression of *bar* gene in T3 plants; genotypes ‘Sedona’ (S1-S3) and ‘Montcalm’ (Mo1-Mo3). ‘Sedona’ plants S2 and S3 have a higher expression than either ‘Montcalm’ plants Mo2 and Mo3. The expression of ‘Montcalm’ is extremely low.

5.5 Liberty herbicide resistance test of T2 and T3 of transgenic plants

Figure 24: *Bar* tested T2 plants at a concentration of 150 mg L^{-1}. 'Condor' (A), 'Matterhorn' (B), 'Montcalm' (C) and 'Sedona'(D). 'Matterhorn' seems to be better expressed. Transgenic plants are not 100% resistant, some leaves are scorched, and exhibit stunted growth. However, their survival is better than the wild type (Wt) plants

Liberty herbicide resistance test of T3 of transgenic plants

Figure 25: T3 plants showing partial resistance to Liberty herbicide the genotypes used are ‘Condor’, ‘Matterhorn’, ‘Montcalm’ and ‘Sedona’. The tray on the left represents wild type non-transformed plants while that on the right are transformed plants. Each plastic container contains the four genotypes mentioned above. The spray rate of the herbicide liberty was 200 mg L^{-1}

5.6 Confirmation of *gus* transgene expression using the GUS assay in T3 'Matterhorn' genotype

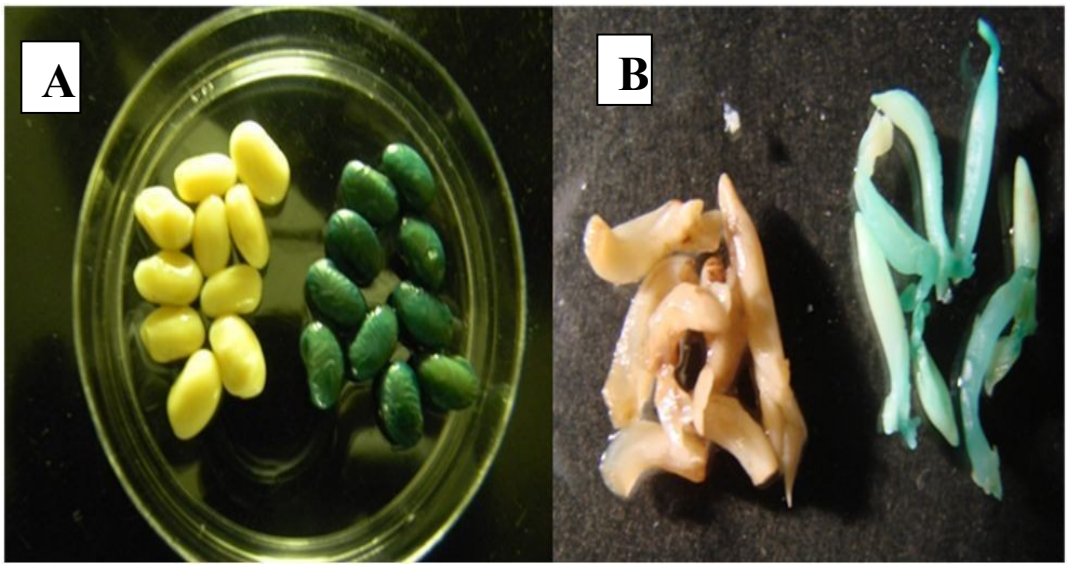


Figure 26: *Gus* expression in 'Matterhorn'. All genotypes transformed, namely, 'Matterhorn', 'Condor', 'Sedona', 'Olathe', and 'Montcalm', showed *gus* positive for both plants transformed using bombardment and *Agrobacterium*. However 'Matterhorn' had the best expression; panel (A) in seed and (B) embryo.

The integration of *bar* gene was demonstrated across four generations (T0-T3). The expected band size of this gene product is 450 bp, which confirms that there is successful *bar* gene integration in the genotypes transformed, which were 'Condor', 'Sedona', 'Montcalm' and 'Matterhorn'. However, the Chi-square test of T2 and T3 plants reveal that the segregation of the *bar* gene does not follow Mendelian inheritance. The most probable explanation for this observation is that the transgenic plants are still chimeric, meaning that not all cells in the plant contain the *bar* transgene. Also, as another possibility, some of the introduced foreign plasmids could be residing in the cytoplasm as opposed to being integrated on the chromosomes. This scenario would still enable the plasmids to be transmitted from one generation to the next vegetatively, like organelle genomes or like viruses and bacteria that have symbiotic relationships with plant cellular systems. In order to solve this problem more generations of selfing are required in order for all the cells to acquire the transgene.

The Southern blot analysis of T2 plants bombarded with a construct containing the *bar* gene shows integration of four different transgenic 'Condor' plants with a single gene copy insert (Figure 18). Two 'Matterhorn' independent transgenic lines showed successful integration of the *bar* gene (Figures 19 and 20) with a single and four copy numbers of the *bar* transgene. 'Sedona'and 'Montcalm' showed three and two copy number of *bar* transgene respectively (Figure 20).

Northern blot analysis of T2 and T3 plants confirms the transcription of *bar* transgene (Figures 21, 22 and 23). The expression levels are not very high except for 'Matterhorn' and 'Sedona'which shows a higher expression level than the others. This might result from differences in the integration site of the *bar* gene in the plant genome rather than the genotypes used.

Resistance to Liberty herbicide was tested on two months old T2 plants and showed that they were still chimeric because certain portions of the leaves got burnt by the herbicide three days after being sprayed with 150 mg L^{-1} of Liberty herbicide (Figure 24). Testing of T3 plants was conducted to see if their level of resistance towards the herbicide had improved. The foliar application of the herbicide was increased to 250 mg L^{-1}. The observation noted is that the transgenic plants were still chimeric because some of the leaves got scorched by the herbicide (Figure 25).

Stable expression of *gus* transgene is shown in seeds and zygotic embryos of T3 plants (Figure 26). The expression levels of *gus* in seeds and embryos of transgenic plants obtained from bombardment or *Agrobacterium*-mediated method was the same. This led to the conclusion that expression levels are not a function of method of transformation but maybe other factors such as genotype or promoter construct. Even though we had good expression of GUS protein (Figure 26) there were still a few small spots on the seeds and embryos not showing the expected blue color. These results suggest that the

transgenic plant material were still chimeric. This observation also helps us to correlate the previous observation made on Liberty treated transgenic plants that showed that some spots on the leaves were scotched while others were not. This clearly indicates chimeric expression.

CHAPTER VI

RESULTS AND DISCUSSION: DROUGHT AND SALT STRESS TOLERANCE OF TRANSGENIC COMMON BEAN

With the transformation protocol that was developed and optimized, 2,000 embryonic axis tissues were bombarded with tungsten microprojectile particles containing the plasmid DNA with *HVA1* gene. The PCR results showed that 161 plants were positive which is about 8% transformation efficiency for putative transgenic material. However even though the PCR showed 8% positive in the T0 only five plants showed positive integration of transgene in T2 generation using southern blot analysis. The number of integrated transgenes ranged from one to two (Figures 28 and 29). PCR positives for these plants was demonstrated in the T3 population (Figure 27). RT-PCR analysis for T2 transgenic plants of 'Montcalm', 'Condor', 'Matterhorn' and 'Sedona' were positive and all four genotypes showed similar expression levels (Figure 30). However the relative expression levels changed in the T3 generation with 'Montcalm's' expression declining significantly when compared to the T2 generation and 'Condor' completely losing its expression (Figure 31). Northern blot analysis was done on T3 plants and only 'Matterhorn' and 'Sedona' showed some expression, whilst no expression was detected for 'Montcalm' and 'Condor' (Figure 32).

6.1. Confirmation of *HVA1* transgene integration in plants using PCR and Southern blot analysis

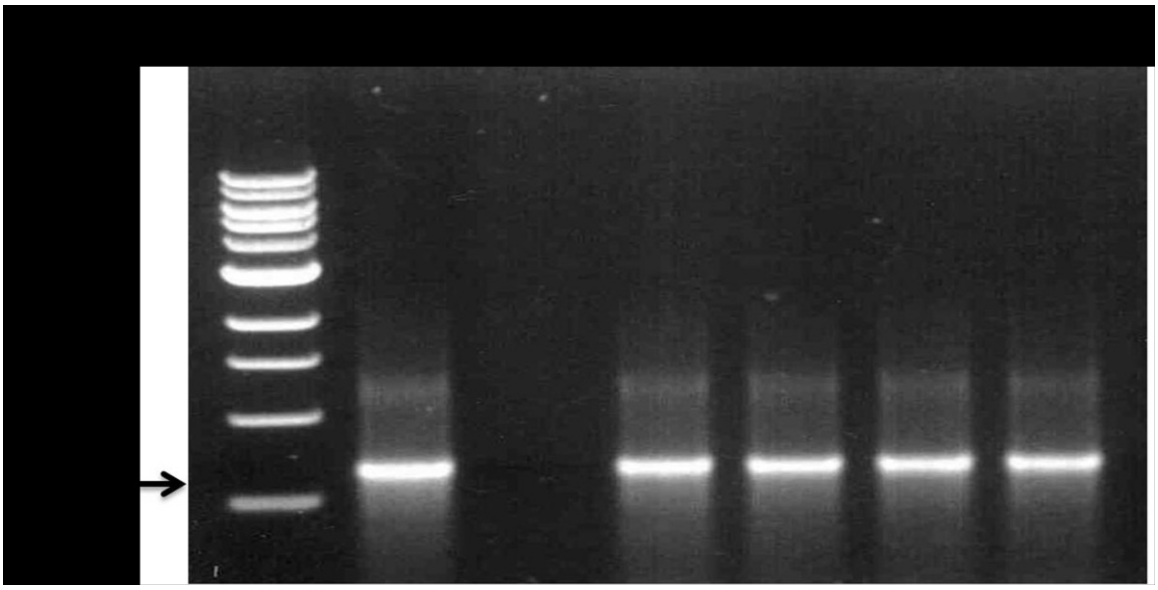


Figure 27. PCR results of T3 transgenic plants of 'Montcalm', 'Condor', 'Sedona'and 'Matterhorn' confirms the stability of *HVA1* transgene integration. The expected band size is 670 bp.

Southern Blot Analysis confirming the copy number of *HVA1* gene that have been integrated into 'Condor' plants.

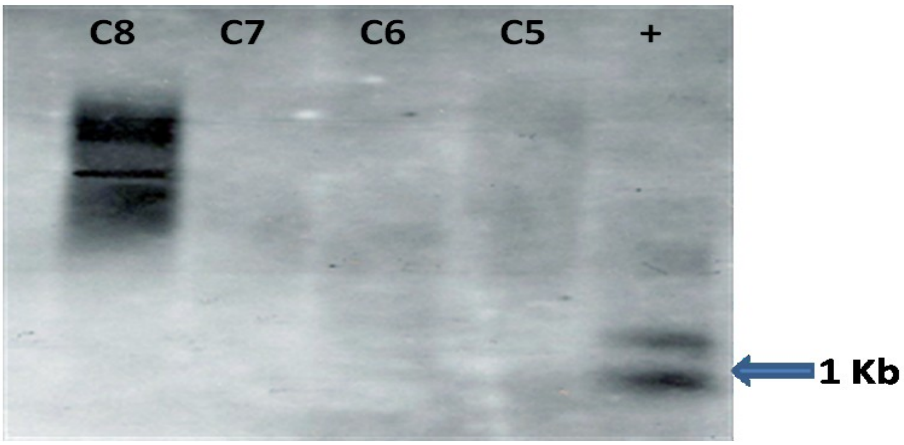


Figure 28: Southern blot showing integration of *HVA1* gene in genotype 'Condor'(C8), digested with BamH1, the other lines show no integration. The results indicate that there is a double gene integration.

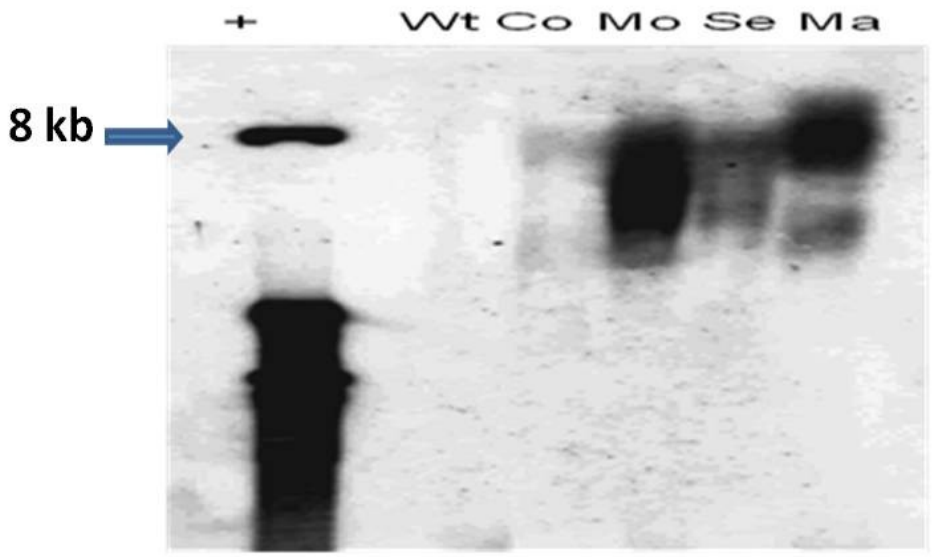


Figure 29. Southern blot showing integration of *HVA1* gene in genotypes Co = 'Condor', Mo = 'Montcalm', Se = 'Sedona'and Ma = 'Matterhorn' digested with BamH1. The results indicate that there is a double gene integration in all genotypes except 'Montcalm' which has a single copy number. The Wt= wild type shows no transgene integration.

6.2. Confirmation of *HVA1* transcription in plants using Reversed Transcription PCR, followed by Northern blotting

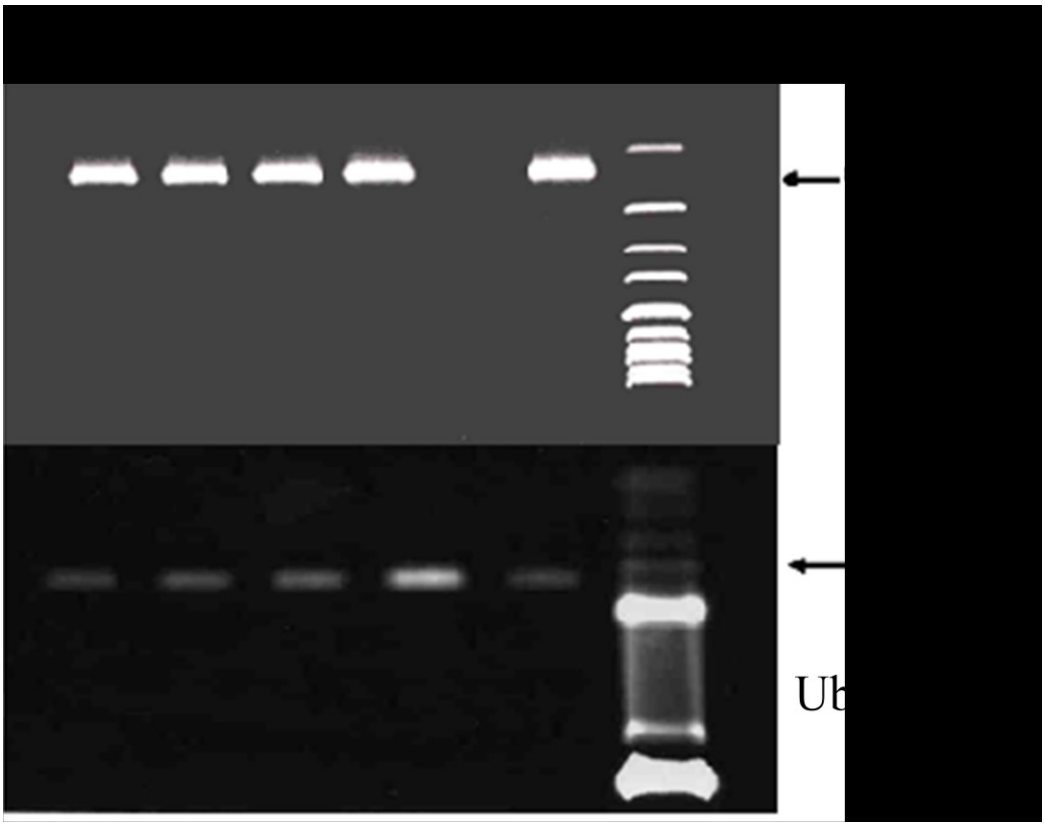


Figure 30. RT-PCR of *HVA1* expression for T2 transgenic plants of S = 'Sedona', C = 'Condor', Mo = 'Montcalm' and Ma = 'Matterhorn'. Expected band size is 670 bp for *HVA1*. The expression levels are the same for all four plants. Below is the cDNA loading control showing the expression of ubiquitin with an expected band size of 450 bp.

RT-PCR

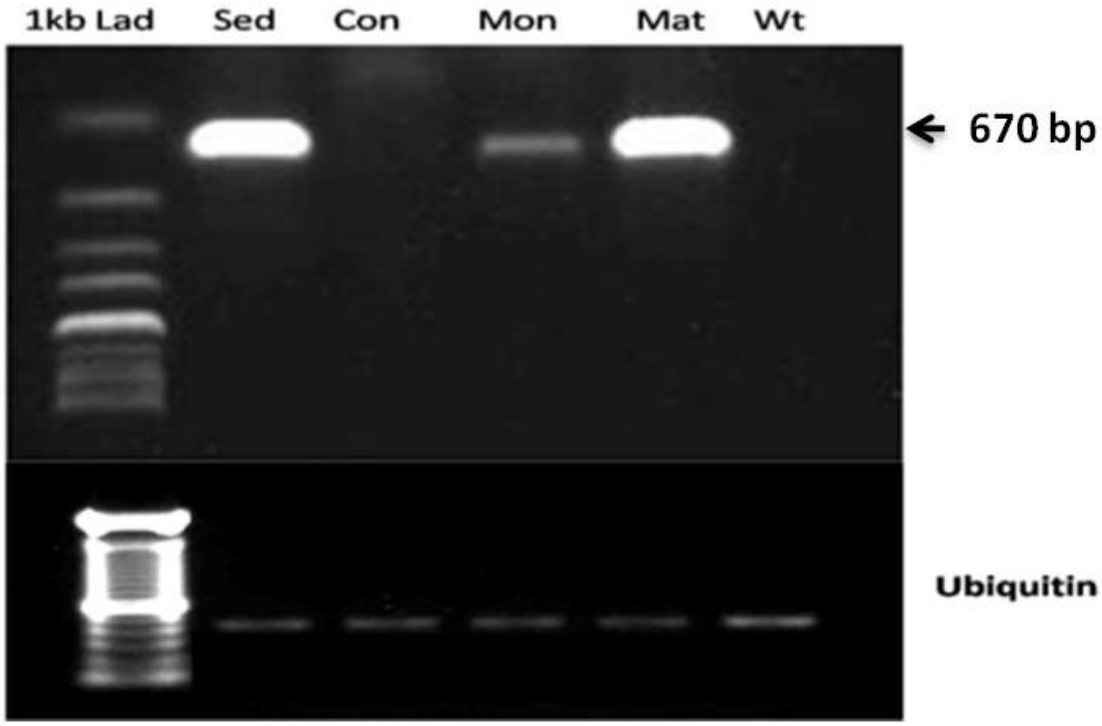


Figure 31. RT-PCR showing expression of *HVA1* T3 transgenic plants with expected band size of 670 bp. 'Condor' completely lost its expression that was previously detected in the T2 generation. The expression of 'Montcalm' declined while that of 'Sedona' and 'Matterhorn' remained stable. Below is the cDNA loading control showing the expression levels of ubiquitin the expected band size is 450 bp.

Northern Blot

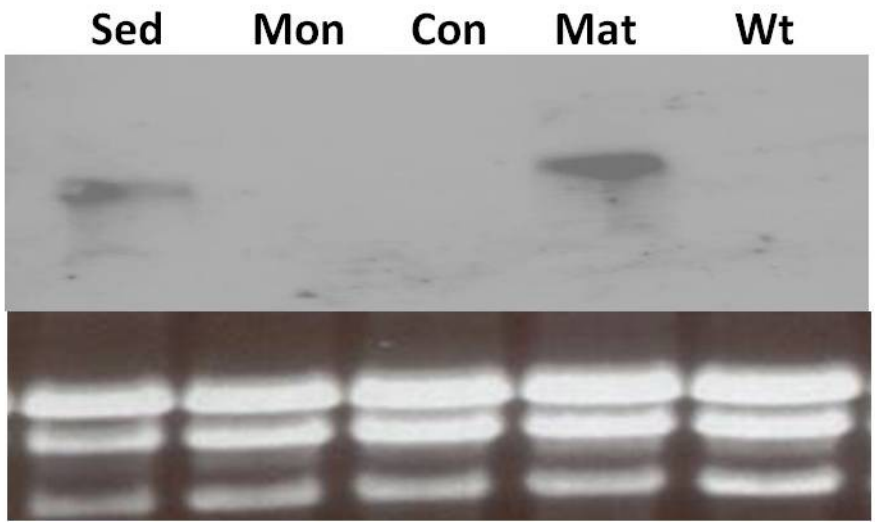


Figure 32. Northern blot expression of *HVA1* gene from T3 transgenic plants subjected to drought stress. Mat = 'Matterhorn' and Sed = 'Sedona' showed some expression. The remaining lanes, Wt = Wild type, Mon = 'Montcalm' and Con = 'Condor' showed no expression at all**.**

6.3. Drought tolerance tests of transgenic versus control non-transformed plants.

When water was withheld for 21 days continuously, all the 'Condor' plants regardless whether they were transgenic or non-transgenic wild types died within 12 days of treatment. No differences could be distinguished between the transgenic and the wild types. Similar results were also obtained for 'Montcalm' plants that died within 16 days of treatment with no clear distinction between transgenic plants and wild types. On the other hand 'Sedona' and 'Matterhorn' transgenic plants persisted for 21 days without water. They showed symptoms of drought stress but soon recovered after three days when moisture application resumed. The wild types showed more severe symptoms of drought stress with most of the leaves wilted and dehisced (Figure 33B). Out of 30 plants that were planted for each genotype in the experiment, 15 were wild types and the other 15 were transgenic. Survival of wild type 'Sedona' plants was only 2 out of 15 and the transgenic plants were 5 out of 15. Survival of 'Matterhorn' wild type plants was 3 and transgenic plants were 8 out of 15.

The percent leaf abscission was used as an indirect measure of the degree of plant wilting. Wilting was defined as the difference of ratios between the number of leaves on plant before 21 days of moisture withdraw and the number of green leaves on plant remaining after 21 days of moisture withdraw. The percent leaf abscission for transgenic 'Sedona' plants was 78% and wild type was 91% and for 'Matterhorn' it was 72% and 88% for transgenic and wild type respectively. It appears that 'Matterhorn' possesses a

genotypic advantage over ‘Sedona’in terms of tolerating drought as indicated by the results of the performance of their wild types (Singh 2007).

The mean height or growth rate of transgenic versus non-transgenic plants did not differ significantly. For example before the experiment was conducted plants of uniform height (20 cm) were selected. After the treatment period height measurement was taken again. The results showed that the mean height for ‘Sedona’ transgenic plants was 23 cm and that for wild type plants was 22 cm. There was a net growth after 21 days of treatment of three and two centimeters for transgenic and wild type plants. The mean height for ‘Matterhorn’ transgenic plants was 24 cm and for wild type plants it was 23 cm. The net growth after 21 days was four and three centimeters respectively for transgenic and wild type plants. In contrast, the control normal watered plants grew to a height of 33 cm and had a net growth of 13 cm. This is an average of three-fold increase in growth compared to the plants under drought stress.

The rooting ability was also examined and showed that the root growth of transgenic plants was more robust than wild type plants under stress but less developed than wild type plants under normal moisture regime (Figure 33D). The average root length measured after 21 days of treatment for ‘Sedona’ transgenic plants was 15 cm and for wild type plants was 11 cm. For ‘Matterhorn’ the average root length measured after the same treatment application was 17 cm for transgenic plants and 13 cm for wild type plants. In contrast, for control plants under normal irrigation the average root length was

28 cm. From the results of this experiment it was shown that transgenic plants engineered with *HVA1* utilize their energy in developing and growing their root system as opposed to the above ground stem and canopy which exhibited little growth under drought stress conditions and showed no significant phenotypic difference between transgenic plants and wild types. (Figure 33C and D). A summary of the results of the drought experiment is shown in Table 9.

Table 9: Summary of measurement parameters of drought stress test

	Number Of Plants Surviving Per 15 Plants		Percentage Of Leaf Abscission		Plant Height (cm) After 21 Days of Drought		Root Length (cm) After 21 Days of Drought	
Genotype	Transgenic	Wt	Transgenic	Wt	Transgenic	Wt	Transgenic	Wt
'Matterhorn'	8	3	72	88	24	23	17	13
'Sedona'	5	2	78	91	23	22	15	11
'Condor'	0	0	100	100	21	21	8	7
'Montcalm'	0	0	100	100	22	22	9	9

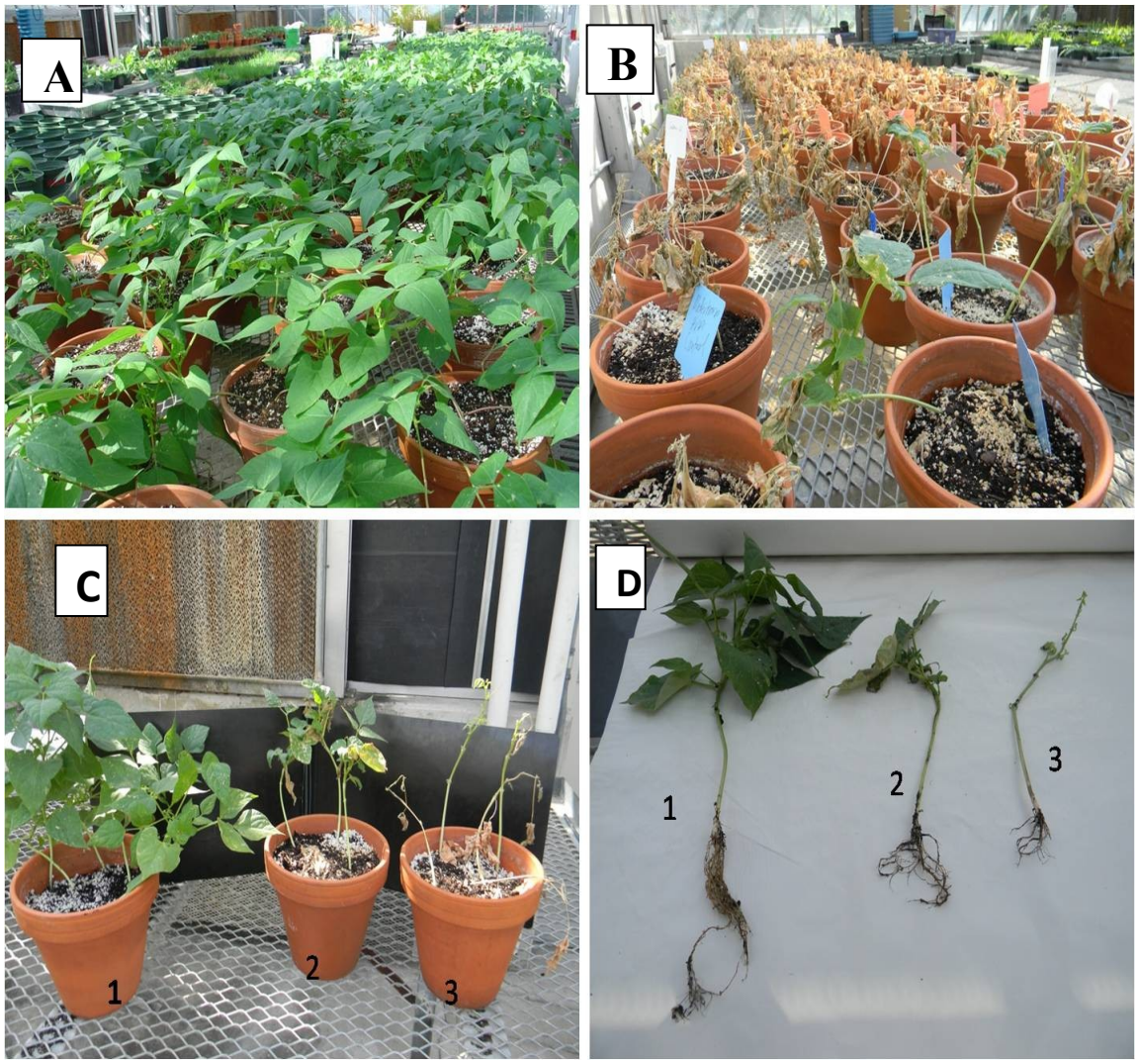


Figure 33. Panel (A): ‘Matterhorn’ plants before drought induction; (B) Plants after 21 days continuous no irrigation; (C) ‘Matterhorn’ drought recovered plants after 3 days of water re-application; 1= control non-transgenic plant that was watered throughout the experiment; 2= ‘Matterhorn’ transgenic plant after 21 days of no-irrigation, 3= Wild type non-transgenic plant after 21 days of no-irrigation; (D) root development of plants after 21 days of drought stress. 1: Control non-transgenic plant roots, these were watered daily, 2: Transgenic plant roots after 21 days of no-irrigation and 3: Wild type non-transgenic plant roots after 21 days of no-irrigation.

6.4. Salt stress test of *HVA1* of T3 plants

The salt stress test that was conducted did not distinguish the wild type plants from the transgenic plants. Five different regimes 0, 50, 100, 150 and 200 mM of NaCl solution were used. Beyond 150 mM all plants died and never recovered including the transgenic plants. At 100 mM there was partial recovery of both wild type, transgenic plants while at 50 mM both the transgenic, and wild type survived but showed symptoms of salt stress that were characterized by wilting and stunted growth (Figure 34).

Although salt and drought stress share a similar pathway, there was no observable significant phenotypic differences between transgenic and wild type plants under salt stress. Only one transgenic plant showed a significant resistant phenotype from the rest of the plants at 50 mM application of NaCl. With this low statistical power it is difficult to tell whether this is a genuine resistant transgenic plant or an escape plant, further tests are required to be conducted to verify this observation.

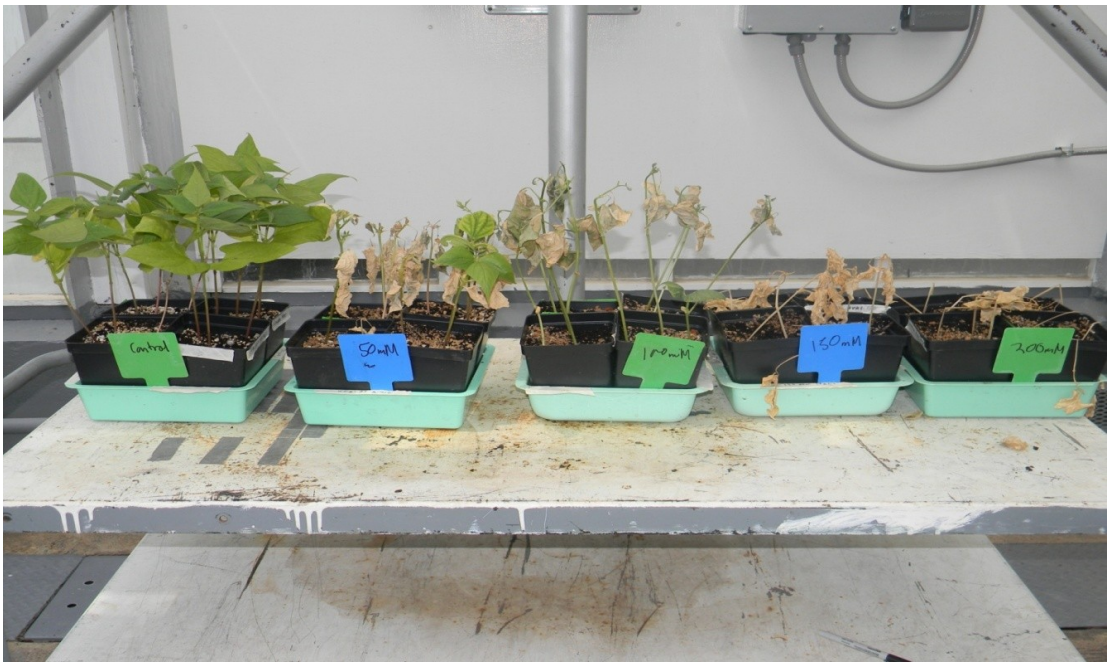


Figure 34. Salt stress test at 5 levels of concentration (0, 50, 100, 150 and 200 mM) on ‘Matterhorn’ plants 10 days after salt treatment. Note that the two plants on the left side of each flat are control non-transgenic and the two plants on the right side of each flat are Northern blot positive transgenic.

When comparing the salt stress test with the drought simulation test, very clear phenotypic differences were seen between transgenic and wild type plants, in particular 'Sedona' and 'Matterhorn', under drought stress test. A possible reason as to why there was an observable clear phenotypic difference between transgenic and non-transgenic plants under drought stress as opposed to salt stress maybe due to the functional class of *HVA1* gene. This gene belongs to the group 3 *LEA* proteins that are members of HSP chaperones that have been known to be more effective towards heat and drought stress rather than salt stress. Salt stress needs the addition of detoxification and Na^+ antiporter genes such as *SOS1*. Therefore in order to detect meaningful phenotypic differences in salt tolerance between transgenic and non-transgenic plants members of such gene families may need to be engineered into common bean together with *HVA1*.

In general, the plants under drought stress remained stunted and weak as compared to the control non-transgenic plants with normal watering. However, the transgenic plants, in particular 'Sedona' and 'Matterhorn' performed better than the rest of the other genotypes. In this experiment, *HVA1* has been shown to be more effective in common bean in alleviating drought stress symptoms and not so effective for conferring resistance towards salt stress.

CHAPTER VII

RESULTS AND DISCUSSION: WHITE MOLD STRESS TESTS OF TRANSGENIC COMMON BEAN

Application of the optimized transformation protocol was developed using 2,000 embryonic axis tissues that were bombarded with tungsten micro projectile particles coated with DNA plasmid containing the germin gene (*gf2.8*). The PCR showed that 138 plants (i.e. less than 7% of the bombarded plant material) contained the *gf2.8* insert in the T0 generation.

7.1. Confirmation of germin transgene integration using PCR and Southern blot analysis

Even though the PCR tests were positive for T1 and T2 plants, only three plants showed positive integration of transgene using Southern blot analysis. Therefore, this may mean that the other plants were chimerically transgenic or the transformed plasmid is resident in the cytoplasm and not on the chromosome in the nucleus. In Southern blot, the number of integrated transgenes ranged from 2 to 4 copies (Figure 36). PCR positives for these plants were demonstrated in the T3 population along with other plants that did not show Southern blot positive (Figure 35).

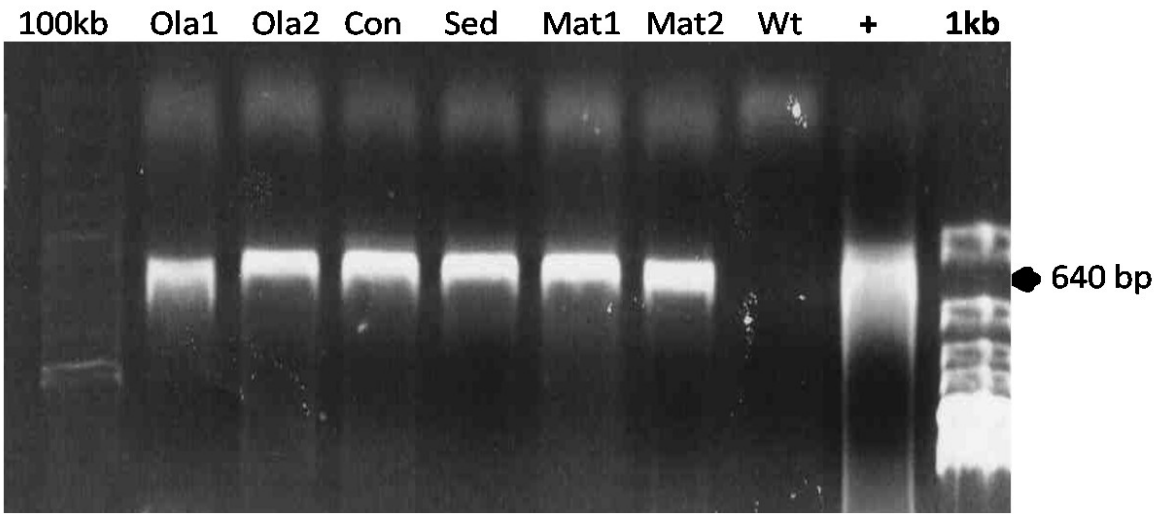


Figure 35: PCR results of T3 transgenic plants of Ola 1-2 = ‘Olathe’, Con = ‘Condor’, Sed =‘Sedona’and Mat 1-2 = ‘Matterhorn’. The expected band size is 640 bp.

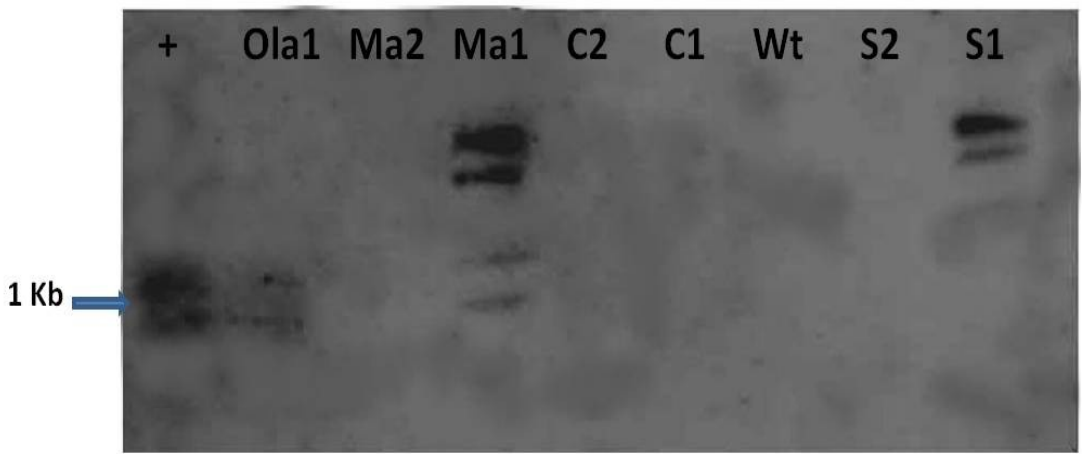


Figure 36: Southern blot analysis showing integration of transgene in the T2 plants. Positive lines are S1 = 'Sedona' with 2 gene inserts, Ma1 = 'Matterhorn' with four gene inserts and Ola1 = 'Olathe' with two gene inserts. The C1 or 2 = ''Condor',' did not show positive integration of transgene and Wt=Wild type is also negative.

7.2. Confirmation of germin gene expression using RT-PCR and Northern blot analysis

The RT-PCR analysis for PCR positive T2 plants of 'Olathe', 'Condor', 'Matterhorn' and 'Sedona' was positive. The expression levels of these genotypes appeared to be the same for T2 plants using RT-PCR (Figure 37). However, when northern blot analysis of *gf2.8* plants was carried out, none of the plants showed northern expression. This indicated that the expression levels were just too low to be detected by northern blot analysis, which is less sensitive than RT-PCR. When the same plants were inoculated with the fungal pathogen, northern blot analysis was done again using RNA collected from infected tissue. The results showed that only 'Matterhorn' had limited expression and no expression was detected at all in the other plants (Figure 38).

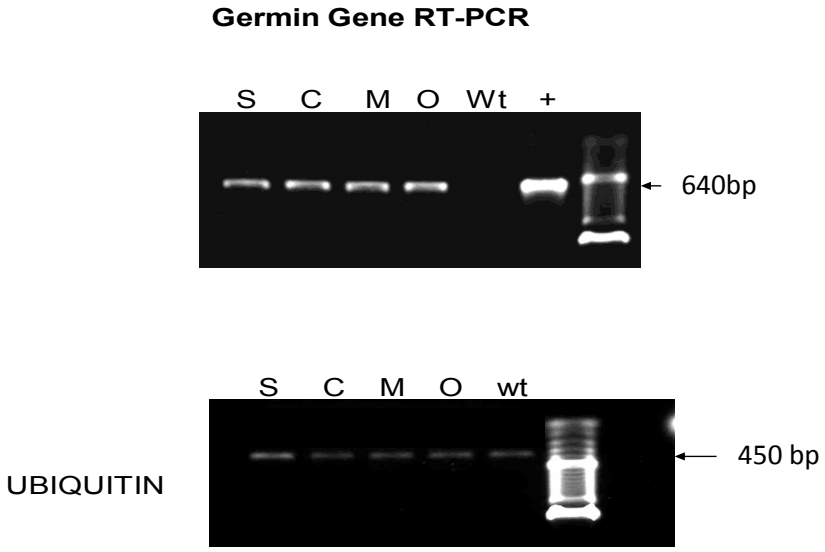


Figure 37:RT-PCR of the germin gene for T2 plants has an expected band size 640 bp. All four genotypes transformed, S= 'Sedona', C= 'Condor', M= 'Matterhorn' and O= 'Olathe' show expression which is less than the positive control. Wt=Wild type is negative. Below is the ubiquitin loading control which shows equal amount of cDNA loading, with an expected band size of 450 bp.

Northern Blot

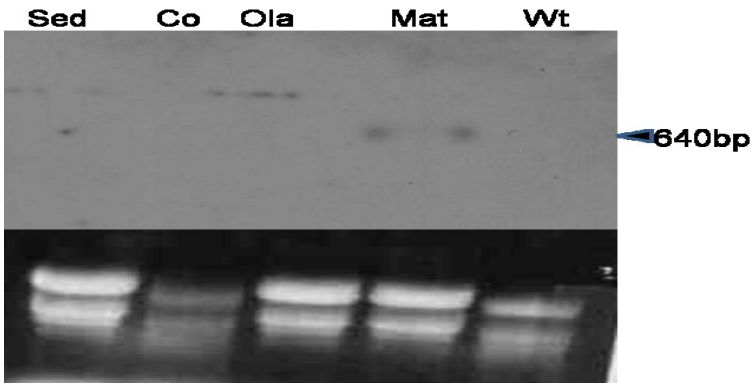


Figure 38:Northern blot of the germin gene from infected tissue of T3 plants. Only M= 'Matterhorn' showed positive results for the expected band size of 640 bp. The rest Wt=Wild type, Ola= 'Olathe', Co= 'Condor' and Sed= 'Sedona' have negative results.

7.3. Fungal biological assay of RT-PCR and Northern blot positive transgenic plants

Prior to inoculation, the fungal pathogen was grown on PDA at ambient room temperature. Only the tip of the growing fungus was used for inoculation and the fluffy white mycelia shown (Figure 39) was not utilized because its mycelia had stopped actively growing.

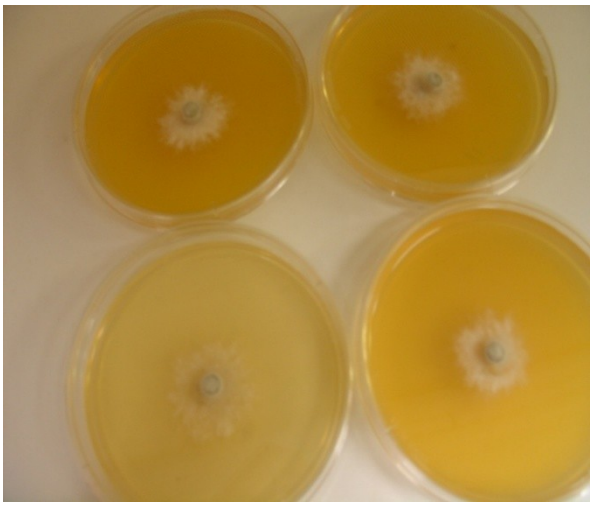


Figure 39. *S. sclerotiorum* fungus 72 hours after being grown on potato dextrose agar.

The fungal biological assay was carried out on all RT-PCR positive transgenic plants that were compared to the control non-transgenic plants. Three systems were experimented on for directly testing the biological activity of germin transgene product (oxalate oxidase). The first experiment was the straw test (lower panel of figure 40), in which the growing mycelia were wedged into a straw, and placed onto an excised shoot tip of the common bean plant growing in the greenhouse. This test did not work because the greenhouse was too hot for the survival of the pathogen. A different approach was used in which trifoliate leaves were placed in a Petri dish containing plain agar and a plug of mycelia was placed on top of the leaves (upper part of Figure 40). This system failed because the agar was unable to provide adequate moisture for the growth of the pathogen.

Figure 40. White mold pathological test. The upper two panels shows the trifoliate leaf detachment assay with plugged mycelia on leaf surface incubated in Petri dishes containing agar media. The two bottom panels show the straw test in the greenhouse, with the straws containing the fungus inserted into the shoot tip of common bean. These two tests did not work because the fungus was unable to infect neither the transgenic nor the wild type plants. In both cases, the humidity was not conducive for the growth of the pathogen.

The third technique involved placing wet paper towels into a sterile tray covered with a transparent plastic wrap. This technique worked well because it provided adequate moisture for the fungal pathogen to grow and infect host tissue. The level of resistance of different independent transgenic lines that were inoculated with the fungal pathogen was compared to the non-transgenic wild type plants that were used as control. The RT-PCR positive transformed plant leaves showed little resistance against the pathogen when compared to the wild-type non-transgenic leaves which showed no resistance to the fungal pathogen. Among RT-PCR positive transformed plant leaves, the genotype 'Matterhorn' performed the best. It displayed the longest delayed onset of lesions on the leaves. This was followed by 'Sedona' then 'Olathe' and finally 'Condor' (Figure 41). Despite observing delayed establishment of the pathogen on 'Matterhorn' transgenic plant leaves for a period of 72 hours (Figure 42), there were no observable differences between susceptibility of transgenic versus non-transgenic plants beyond the 72 hours time period.

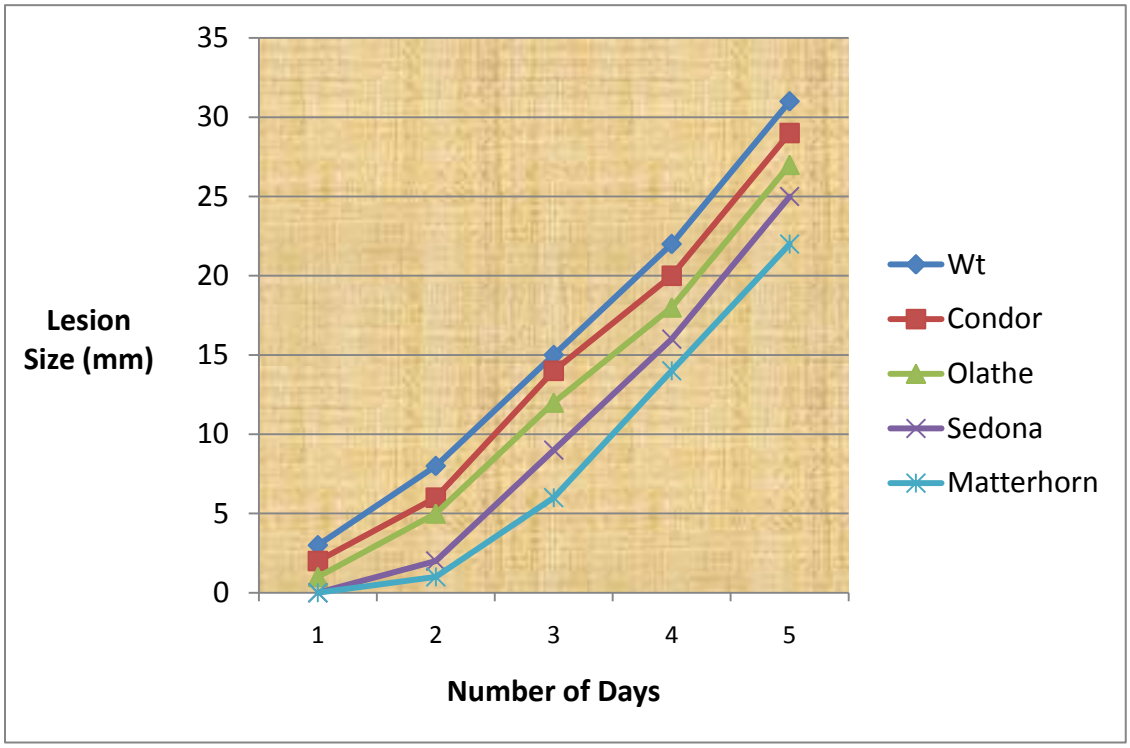


Figure 41. Relative rate of infection and development spread of pathogen as measured by lesion size on leaf surface of T2 'Matterhorn', 'Sedona', 'Olathe', 'Condor' and wild type plants.

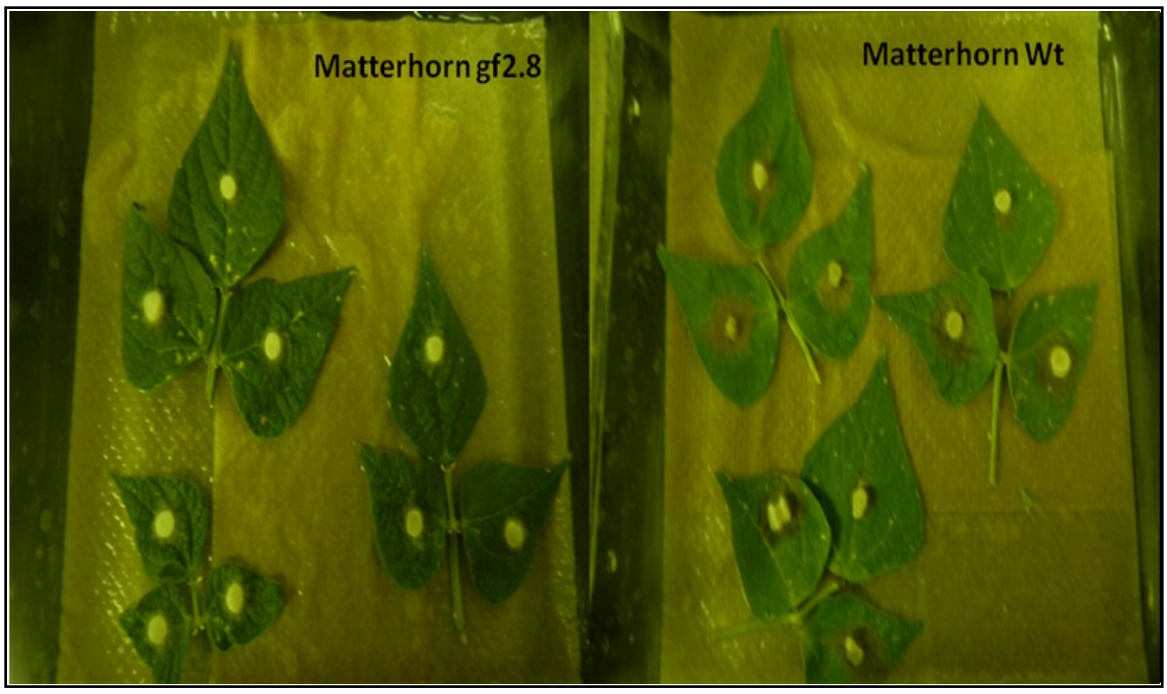


Figure 42. Fungal biological assay: trifoliate leaves placed on moist paper towel in a tray with plug mycelia on top of the leaves. Transgenic 'Matterhorn' (*gf2.8*), on the left, shows delayed infection after 72 hours of inoculation, compared to wild type on the right.

This experiment has demonstrated that, even though the expression levels of germin gene in transgenic plants was significantly low as shown by northern blot analysis, the potential of using *gf2.8* gene in common bean to confer resistance to white mold is feasible. Therefore, future research should use our transformation protocol and place more emphasis on exploiting the use of stronger *cis* and *trans* acting regulatory elements. These include different promoters or enhancer elements that have the capacity to increase expression levels.

CHAPTER VIII

CONCLUSIONS AND FUTURE PERSPECTIVES

The conclusions from these studies have confirmed that common bean still remains a crop species that is recalcitrant to *in vitro* regeneration and genetic engineering. Nevertheless, the research that has been presented here shows that great potential for efficient transformation of common bean can be further exploited and optimized by establishing suitable conditions for both *in vitro* regeneration and gene delivery into these plants. In this research effort emphasis was placed on resolving some of the obstacles associated with low transformation efficiencies, including the high rate of transgenic chimera as well as transgene loss in progressive generations.

A significant breakthrough of this research dealt with one of the major causes leading to recalcitrance of *in vitro* regeneration which is, the prolific excretion of phenolic compounds. It was demonstrated that the application of anti-oxidants, silver nitrate in particular, is very effective against oxidation of phenolic compounds that turn into toxic oxides that kill tissue that is growing *in vitro*. Another cause of recalcitrance of common bean in *in vitro* regeneration is the lack of totipotency of cells. In work recently reported (Kwapata et al., 2009), it was shown that totipotency can be established by balancing accordingly the ratios of cytokinin and auxin. Using this approach, the number of multiple shoots per explant was increased to 20. This was significantly higher than 4 to 8 multiple shoots per explants that have previously been reported by a number of

researchers (Ahmed et al. 2002). However, the number of multiple shoots that were obtained is still lower than the number produced from cereal explants. In view of this work, more effort is needed to achieve higher numbers of multiple shoots per explant. Therefore, continued efforts to find more suitable media formulations and better methods for the removal of phenolic compounds from the culture media should be investigated.

In vitro regeneration via direct organogenesis, using either cotyledonary nodes or embryogenic axis, are the current methods used by researchers. These methods have proven to be very inefficient (Popelka et al. 2004, Veltcheva et al. 2005). The system developed in this research of using multiple shoots from the apical meristem primordia offers a better alternative for an improved regeneration protocol that is amenable to a relatively efficient gene delivery system. An ideal alternative transformation protocol for common bean might involve the use of regeneratable embryogenic callus or cell suspension cultures. If successful, these explants can increase the efficiency of transformation and reduce the rate of chimeric transgenic plants based on the fact that somatic embryos originate from single cells while organogenesis originates from multiple cells.

Unfortunately, no report exists of successfully using embryogenic callus or cell suspension cultures for genetic transformation of common beans. This is because callus explants produce phenolic compounds *in vitro* at a rate faster than the cells are able to multiply. These phenolic compounds suffocate the cells and hinder their growth. Future

research can explore the possibility of using the system that has been developed for eliminating phenolic compounds of multiple shoots grown *in vitro* and applying it to callus or cell suspension cultures.

Another challenge associated with *in vitro* cultures of grain legumes, including common bean, is their prolonged regeneration period. This prolonged period invites opportunity for somaclonal variations that could adversely affect the phenotype and regeneration capacity. Developing protocols that expedite the regeneration processes may assist in alleviating these problems. Closely related to the problem of prolonged *in vitro* regeneration period of grain legumes including common bean, is the poor *in vitro* rooting, this presents a problem of establishing plantlets in soil and greenhouse. Due to the difficulty of regenerating roots of common bean *in vitro*, other researchers have opted to use grafting in order to solve this problem. However, this approach has been proven to be cumbersome and inefficient (Krishnamurthy et al. 2000, Sarmah et al. 2004, Tewari-Singh et al. 2004, Sanyal et al. 2005). In this research a better and more efficient method of rooting has been developed using an IBA dipping system. This method ensures healthy rooting, survival and establishment of *in vitro* grown plants in the greenhouse.

The *Agrobacterium*-mediated gene delivery system that was developed is still in its early development phase and needs to be improved. The results have shown that common bean receptiveness towards genetic transformation with *Agrobacterium* is poor compared to most other docotyledoneous plants (e.g. potato, tobacco, etc). Despite this, there are some

encouraging signs. Even though the system exhibited low transformation efficiencies, some stable transformed plants were obtained. It was determined from the study that the most promising strains for *Agrobacterium*-mediated transformation are EHA105 and GV3301, both of which showed better results for gene delivery than LBA4404. In addition to the type of strains used in the studies, it was shown that the co-cultivation period also significantly affects the efficiency of *Agrobacterium*-mediated transformation systems. In this particular study, longer co-cultivation periods were shown to increase the chances of gene delivery. The results that were obtained can be used as a base for future work of improving *Agrobacterium*-mediated transformation systems. In order to improve on this work, virulence conditions for promoting transgene delivery should be investigated, such as the use of chemicals like acetosyringones or physical means like sonication.

The results of the research have shown that Biolistic^{TM} bombardment of multiple shoot offered a relatively better way of delivering transgene than *Agrobacterium.* The results obtained in this research were slightly better than those reported by other researchers who used either the bombardment method (Bonfim et al. 2007), electroporation or polyethylene glycol (PEG) treatment of protoplast (Babaoglu et al. 2000). However, the method that has been developed as a result of this research for multiple shoot bombardment still needs further improvement. This can be achieved by optimizing bombardment conditions such as the rapture disk pressure, concentration of plasmid DNA and the size and type of DNA micro-carrier.

In the research conducted, the potential of using the *HVA1* transgene to alleviate symptoms of drought in common bean has been demonstrated. However, there was no distinct effectiveness of this gene for salt stress tolerance of transgenic plants when compared to wild type non-transgenic plants. The biochemical pathways for salt and drought stresses are similar, in the sense that common transcription factors and genes are switched on during the occurrence of these stresses. It has been shown, however, that salt related injuries to plants are more complex at a molecular level because many more genes are required to remove the salt toxicity, in addition to the restoration of homeostasis and turgor pressure of the cells (Kassem et al. 2004, Lee et al. 2004, Popelka et al. 2004, Sharma and Lavanya 2002).

The four classes of osmotic stress related genes mentioned earlier, which are osmoprotectants, antiporters, aquaporins and chaperones, are required to work in concert in order to bring about an effective drought and salt stress tolerant response. Chaperones like *HVA1* are more effective against heat and drought stress than they are to salt stress. This is why there were no observable phenotypic differences between transgenic and non-transgenic plants under salt stress. In order to fully optimize and develop high resistant genotypes of common bean towards drought and salt stresses, the other classes of osmotic stress related genes will need to be incorporated using a pyramiding or stacking scheme of transgenes. To further improve the resilience of the transgenic

common bean towards drought, future research needs to explore the genetic engineering of up regulation of drought-related transcription factors such as DREB1A.

The research conducted has also demonstrated the potential of using the germin gene which expresses an oxalate oxidase. This gene product confers white mold resistance to common bean by inhibiting the pathogenic effect of *S. sclerotiorum.* The northern blot analysis conducted on the plants that were transformed with this gene showed very low expression levels. However, 'Matterhorn' showed a relatively higher expression and the fungal bio-assay conducted on the transgenic plants showed that this genotype was able to delay the establishment of the fungus for a period of 72 hours when compared to the non-transformed wild type. However, beyond 72 hours, it was observed that the rate of fungal pathogen spread on the transgenic plants was as fast as the wild type. A possible explanation is that the necrotic tissue that developed on the plant leaf surface, as a result of plants HR cell death caused by the inoculated pathogen, conferred an advantage to this necrotrophic fungus to establish itself. In the previously chapters, it was discussed that HR cell death, despite being a natural defense mechanism for plants against invading pathogens, is only effective against biotrophic pathogens that need to keep the plant tissue alive for their survival. On the other hand, necrotrophs thrive on plants tissue that has died as a result of an HR plant cell death response.

In conclusion, the goals and objectives set in this research have been met. Through this research the development of an efficient and reproducible *in vitro* regeneration protocol

for common bean has been achieved. Gene delivery systems for both *Agrobacterium* and Biolistic^{TM} bombardment have been developed, although they still need further improvement. However dispite having achieved this the relative ease to which the two gene pools (Andean and Middle America) were able to regenerate *in vitro* and integrate the transgene significantly differed. Andean gene pool still remains more recalcitrant towards genetic engineering than Middle American gene pool. Better methods of manipulating the Andean gene pool to become more amenable towards genetic engineering need to be investigated in future research.

The main problem encountered in the transformation systems developed is low expression of transgenes. The reasons why the transgenes had low expression could be due to poor promoter performance, positional effect variegation, methylation of transgenes or some other endogenous silencing activity within the bean genome, such as activation of transposable elements. Future research should replicate the transformation systems that have been developed from this research and investigate the causes for low gene expression.

APPENDICES

Appendix 1

Table 10: Anova for statistical model

The SAS System

The GLM Procedure
Class Level Information

Class	Levels	Values
Genotype:	10	‘Beluga’, ‘Jaguar’, ‘Merlot’, ‘Montcalm’, ‘Olathe’, “Red hawk”, ‘Sedona’, ‘Condor’, ‘Matterhorn’, ‘Seahawk’
cytokinin	9	BAP1, BAP2, BAP3, BAP4, Cytokin0, TDZ1, TDZ2, TDZ3, TDZ4
Auxin	7	Auxin0, IAA1, IAA2, IAA3, NAA1, NAA2, NAA3

Number of Observations Read 1890
Number of Observations Used 1890
The SAS System 04:42 Tuesday, November 11, 2008 2

The GLM Procedure
Dependent Variable: Shoots

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	629	32766.02169	52.09224	88.30	<.0001
Error	1260	743.33333	0.58995		
Corrected Total	1889	33509.35503			

R-Square	Coeff Var	Root MSE	Shoots Mean
0.977817	17.51745	0.768080	4.384656

Appendix 2

Table 11:Anova for treatment and interaction of treatments for type i and type ii sum of squares

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Genotype	9	3307.873545	367.541505	623.01	<.0001
Cytokinin	8	5168.397884	646.049735	1095.10	<.0001
Auxin	6	9937.121693	1656.186949	2807.35	<.0001
Genotype*cytokinin	72	2141.655026	29.745209	50.42	<.0001
Genotype*Auxin	54	1852.433862	34.304331	58.15	<.0001
Cytokinin*Auxin	48	4032.268783	84.005600	142.40	<.0001
Genoty*Cytokin*Auxin	432	6326.270899	14.644146	24.82	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	9	3307.873545	367.541505	623.01	<.0001
Cytokinin	8	5168.397884	646.049735	1095.10	<.0001
Auxin	6	9937.121693	1656.186949	2807.35	<.0001
Genotype*Cytokinin	72	2141.655026	29.745209	50.42	<.0001
Genotype*Auxin	54	1852.433862	34.304331	58.15	<.0001
Cytokinin*Auxin	48	4032.268783	84.005600	142.40	<.0001
Genoty*Cytokin*Auxin	432	6326.270899	14.644146	24.82	<.0001

Appendix 3

Table 12: Least squares mean for the genotypic effect

The GLM Procedure

Genotype	LSMeans	Number
‘Beluga’	3.2	1
‘Redhawk’	3.1	2
‘Merlot’	5.7	3
‘Montcalm’	3.0	4
‘Olathe’	7.0	5
‘Jaguar’	3.7	6
‘Sedona’	6.0	7
‘Condor’	3.6	8
‘Matterhorn’	4.8	9
‘Seahawk’	3.7	10

Appendix 4

Table 13: Separation of means for genotypic effect

Least Squares Means for effect Genotype

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Shoots

i/j	1	2	3	4	5	6	7	8	9	10
1		0.2842	<.0001	0.0111	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	0.2842		<.0001	0.1409	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001		<.0001	<.0001	<.0001	0.0011	<.0001	<.0001	<.0001
4	0.0111	0.1409	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	0.3153	<.0001	0.6393
7	<.0001	<.0001	0.0011	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	0.3153	<.0001		<.0001	0.1409
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
10	<.0001	<.0001	<.0001	<.0001	<.0001	0.6393	<.0001	0.1409	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 5

Table 14 : Least squares mean for cytokinin effect

The GLM Procedure

Shoots

Cytokinin	LSMean	Number
BAP1	4.8	1
BAP2	6.0	2
BAP3	6.3	3
BAP4	3.3	4
Cytokin0	1.0	5
TDZ1	5.3	6
TDZ2	5.7	7
TDZ3	4.5	8
TDZ4	2.6	9

Appendix 6

Table 15: Least squares mean seperation for cytokinin effect

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Shoots

i/j	1	2	3	4	5	6	7	8	9
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0012	<.0001
2	<.0001		0.0019	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.0019		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001
8	0.0012	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 7

Table 16: Least squares mean for auxin effect

The GLM Procedure

Auxin	Shoots LSMean	Number
Auxin0	1.3	1
IAA1	5.6	2
IAA2	7.4	3
IAA3	2.1	4
NAA1	5.5	5
NAA2	6.7	6
NAA3	2.1	7

Appendix 8

Table 17: Least squares mean separation for auxin effect

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: Shoots

i/j	1	2	3	4	5	6	7
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		<.0001	<.0001	0.1045	<.0001	<.0001
3	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001		<.0001	<.0001	1.0000
5	<.0001	0.1045	<.0001	<.0001		<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
7	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix 9

Table 18: Means for the interaction between cytokinin and auxin

Cytokinin	Auxin	LSMean	Number
BAP1	Auxin0	1.6	1
BAP1	IAA1	5.8	2
BAP1	IAA2	9.6	3
BAP1	IAA3	2.5	4
BAP1	NAA1	4.9	5
BAP1	NAA2	6.6	6
BAP1	NAA3	2.5	7
BAP2	Auxin0	1.7	8
BAP2	IAA1	6.3	9
BAP2	IAA2	11.6	10
BAP2	IAA3	4.4	11
BAP2	NAA1	6.1	12
BAP2	NAA2	9.1	13
BAP2	NAA3	2.9	14
BAP3	Auxin0	1.2	15
BAP3	IAA1	9.3	16
BAP3	IAA2	9.4	17
BAP3	IAA3	4.2	18
BAP3	NAA1	11.7	19
BAP3	NAA2	6.5	20
BAP3	NAA3	1.7	21
BAP4	Auxin0	0.6	22

Table 18: Continued…

BAP4	IAA1	4.2	23
BAP4	IAA2	5.8	24
BAP4	IAA3	1.7	25
BAP4	NAA1	3.5	26
BAP4	NAA2	5.4	27
BAP4	NAA3	1.7	28
Cytokin0	Auxin0	1.0	29
Cytokin0	IAA1	1.0	30
Cytokin0	IAA2	1.0	31
Cytokin0	IAA3	0.97	32
Cytokin0	NAA1	1.0	33
Cytokin0	NAA2	1.0	34
Cytokin0	NAA3	1.0	35
TDZ1	Auxin0	1.8	36
TDZ1	IAA1	6.8	37
TDZ1	IAA2	11.5	38
TDZ1	IAA3	1.8	39
TDZ1	NAA1	4.7	40
TDZ1	NAA2	7.4	41
TDZ1	NAA3	2.9	42
TDZ2	Auxin0	1.4	43
TDZ2	IAA1	7.0	44
TDZ2	IAA2	7.1	45
TDZ2	IAA3	1.9	46
TDZ2	NAA1	8.4	47

Table 18: Continued…

Cytokinin	Auxin	LSMEAN	Number
TDZ2	NAA2	11.1	48
TDZ2	NAA3	2.9	49
TDZ3	Auxin0	1.3	50
TDZ3	IAA1	5.8	51
TDZ3	IAA2	7.3	52
TDZ3	IAA3	1.6	53
TDZ3	NAA1	5.6	54
TDZ3	NAA2	7.8	55
TDZ3	NAA3	2.4	56
TDZ4	Auxin0	0.8	57
TDZ4	IAA1	4.0	58
TDZ4	IAA2	3.0	59
TDZ4	IAA3	0.1	60
TDZ4	NAA1	3.6	61
TDZ4	NAA2	5.7	62
TDZ4	NAA3	1.2	63

Appendix 10

Table 19: Means of multiple shoots for the interaction between genotype and auxin

Genotype	Auxin	LSMean	Number
'Beluga'	Auxin0	1.2	1
'Beluga'	IAA1	3.9	2
'Beluga'	IAA2	5.2	3
'Beluga'	IAA3	1.3	4
'Beluga'	NAA1	3.7	5
'Beluga'	NAA2	5.6	6
'Beluga'	NAA3	1.7	7
'Jaguar'	Auxin0	0.9	8
'Jaguar'	IAA1	3.9	9
'Jaguar'	IAA2	4.3	10
'Jaguar'	IAA3	1.5	11
'Jaguar'	NAA1	3.6	12
'Jaguar'	NAA2	5.6	13
'Jaguar'	NAA3	2.1	14
'Merlot'	Auxin0	1.9	15
'Merlot'	IAA1	6.5	16
'Merlot'	IAA2	9.2	17
'Merlot'	IAA3	2.3	18
'Merlot'	NAA1	7.1	19
'Merlot'	NAA2	9.9	20
'Merlot'	NAA3	3.1	21
'Montcalm'	Auxin0	1.0	22

Table 19: Continued…

‘Montcalm’	IAA1	4.2	23
‘Montcalm’	IAA2	4.0	24
‘Montcalm’	IAA3	0.8	25
‘Montcalm’	NAA1	4.6	26
‘Montcalm’	NAA2	5.7	27
‘Montcalm’	NAA3	0.8	28
‘Olathe’	Auxin0	1.7	29
‘Olathe’	IAA1	9.2	30
‘Olathe’	IAA2	12.0	31
‘Olathe’	IAA3	4.4	32
‘Olathe’	NAA1	10.0	33
‘Olathe’	NAA2	8.2	34
‘Olathe’	NAA3	3.3	35
‘Redhawk’	Auxin0	0.7	36
‘Redhawk’	IAA1	6.6	37
‘Redhawk’	IAA2	5.4	38
‘Redhawk’	IAA3	0.8	39
‘Redhawk’	NAA1	5.2	40
‘Redhawk’	NAA2	6.3	41
‘Redhawk’	NAA3	1.0	42
‘Sedona’	Auxin0	1.8	43
‘Sedona’	IAA1	6.6	44
‘Sedona’	IAA2	11.5	45
‘Sedona’	IAA3	2.7	46
‘Sedona’	NAA1	7.6	4

Table 19: Continued…

Genotype	Auxin	LSMean	Number
‘Sedona’	NAA2	9.2	48
‘Sedona’	NAA3	2.3	49
‘Condor’	Auxin0	1.2	50
‘Condor’	IAA1	3.8	51
‘Condor’	IAA2	5.9	52
‘Condor’	IAA3	1.7	53
‘Condor’	NAA1	4.2	54
‘Condor’	NAA2	5.7	55
‘Condor’	NAA3	2.7	56
‘Matterhorn’	Auxin0	1.7	57
‘Matterhorn’	IAA1	6.0	58
‘Matterhorn’	IAA2	9.0	59
‘Matterhorn’	IAA3	2.7	60
‘Matterhorn’	NAA1	5.2	61
‘Matterhorn’	NAA2	6.1	62
‘Matterhorn’	NAA3	2.7	63
‘Seahawk’	Auxin0	0.6	64
‘Seahawk’	IAA1	5.3	65
‘Seahawk’	IAA2	7.0	66
‘Seahawk’	IAA3	3.1	67
‘Seahawk’	NAA1	3.4	68
‘Seahawk’	NAA2	5.1	69
‘Seahawk’	NAA3	1.7	70

Appendix 11

Table 20: Means of multiple shoot for the interaction between genotype and cytokinin

Genotype	Cytokinin	Lsmeans	Number
'Beluga'	BAP1	4.3	1
'Beluga'	BAP2	4.7	2
'Beluga'	BAP3	5.7	3
'Beluga'	BAP4	0.9	4
'Beluga'	Cytokin0	1.0	5
'Beluga'	TDZ1	3.7	6
'Beluga'	TDZ2	4.5	7
'Beluga'	TDZ3	2.7	8
'Beluga'	TDZ4	1.3	9
'Redhawk'	BAP1	4.0	10
'Redhawk'	BAP2	4.8	11
'Redhawk'	BAP3	3.0	12
'Redhawk'	BAP4	1.0	13
'Redhawk'	Cytokin0	1.0	14
'Redhawk'	TDZ1	3.4	15
'Redhawk'	TDZ2	5.4	16
'Redhawk'	TDZ3	3.6	17
'Redhawk'	TDZ4	1.9	18
'Merlot'	BAP1	5.4	19

Table 20: Continued…

‘Merlot’	BAP2	7.9	20
‘Merlot’	BAP3	7.9	21
‘Merlot’	BAP4	3.3	22
‘Merlot’	Cytokin0	1.0	23
‘Merlot’	TDZ1	7.5	24
‘Merlot’	TDZ2	8.2	25
‘Merlot’	TDZ3	6.6	26
‘Merlot’	TDZ4	3.6	27
‘Montcalm’	BAP1	1.7	28
‘Montcalm’	BAP2	4.3	29
‘Montcalm’	BAP3	7.3	30
‘Montcalm’	BAP4	2.8	31
‘Montcalm’	Cytokin0	1.0	32
‘Montcalm’	TDZ1	3.5	33
‘Montcalm’	TDZ2	1.9	34
‘Montcalm’	TDZ3	2.5	35
‘Montcalm’	TDZ4	2.1	36
‘Olathe’	BAP1	8.0	37
‘Olathe’	BAP2	10.0	38
‘Olathe’	BAP3	10.0	39
‘Olathe’	BAP4	8.8	40
‘Olathe’	Cytokin0	1.0	41

Table 20: Continued…

‘Olathe’	TDZ1	6.6	42
‘Olathe’	TDZ2	7.6	43
‘Olathe’	TDZ3	7.7	44
‘Olathe’	TDZ4	3.2	45
‘Jaguar’	BAP1	2.7	46
‘Jaguar’	BAP2	4.2	47
‘Jaguar’	BAP3	7.6	48
‘Jaguar’	BAP4	3.0	49
‘Jaguar’	Cytokin0	1.0	50
‘Jaguar’	TDZ1	5.2	51
“‘Jaguar’	TDZ2	4.3	52
‘Jaguar’	TDZ3	2.7	53
‘Jaguar’	TDZ4	2.6	54
‘Sedona’	BAP1	6.2	55
‘Sedona’	BAP2	9.0	56
‘Sedona’	BAP3	7.1	57
‘Sedona’	BAP4	5.0	58
‘Sedona’	Cytokin0	1.0	59
‘Sedona’	TDZ1	7.6	60
‘Sedona’	TDZ2	7.5	61
‘Sedona’	TDZ3	7.6	62
‘Sedona’	TDZ4	2.7	63

Table 20: Continued…

‘Condor’	BAP1	4.4	64
‘Condor’	BAP2	4.1	65
‘Condor’	BAP3	3.1	66
‘Condor’	BAP4	3.1	67
‘Condor’	Cytokin0	1.0	68
‘Condor’	TDZ1	4.2	69
‘Condor’	TDZ2	5.0	70
‘Condor’	TDZ3	4.3	71
‘Condor’	TDZ4	3.4	72
‘Matterhorn’	BAP1	6.6	73
‘Matterhorn’	BAP2	6.0	74
‘Matterhorn’	BAP3	6.3	75
‘Matterhorn’	BAP4	3.1	76
‘Matterhorn’	Cytokin0	1.0	77
‘Matterhorn’	TDZ1	5.9	78
‘Matterhorn’	TDZ2	7.1	79
‘Matterhorn’	TDZ3	4.3	80
‘Matterhorn’	TDZ4	2.9	81
‘Seahawk’	BAP1	4.6	82
‘Seahawk’	BAP2	5.3	83
‘Seahawk’	BAP3	4.5	84
‘Seahawk’	BAP4	1.9	85

Table 20: Continued…

‘Seahawk’	Cytokin0	1.0	86
‘Seahawk’	TDZ1	5.1	87
‘Seahawk’	TDZ2	5.3	88
‘Seahawk’	TDZ3	3.4	89
‘Seahawk’	TDZ4	2.6	90

Appendix 12

Table 21: Anova for rooting experiment

Dip*Hormone Effect Sliced By Hormone For Roots

Source of Var. Hormone	DF	Sum of Squares	Mean Square	F Value	Pr > F
Hormone0	3	6.000000	2.000000	1.79	0.1555
IAA1	3	0.666667	0.222222	0.20	0.8968
IAA2	3	147.000000	49.000000	43.88	<.0001
IAA3	3	758.250000	252.750000	226.34	<.0001
IBA1	3	1.583333	0.527778	0.47	0.7022
IBA2	3	172.666667	57.555556	51.54	<.0001
IBA3	3	775.333333	258.444444	231.44	<.0001
NAA1	3	3.000000	1.000000	0.90	0.4473
NAA2	3	206.250000	68.750000	61.57	<.0001
NAA3	3	753.583333	251.194444	224.95	<.0001

Appendix 13

Table 22: Least squares means for number of roots for dipping effect in IBA

IBA Dip	LSMEAN	Number
10mg/l	9.0	1
1mg/l	5.0	2
5mg/l	3.7	3
Dip0	7.2	4

Appendix 14

Table 23: Least squares means for the effect of different levels of hormone concentration in media on the number of roots

Hormone	LSMean	Number
Hormone0	1.7	1
IAA1	2.3	2
IAA2	7.8	3
IAA3	9.4	4
IBA1	2.3	5
IBA2	8.3	6
IBA3	9.7	7
NAA1	2.2	8
NAA2	8.3	9
NAA3	9.3	10

Appendix 15

Table 24 : Least squares means for the effect of IBA dipping on the length of roots (cm)

Dip	LSMeans	Number
10mg/l	18.32	1
1mg/l	26.35	2
5mg/l	30.85	3
Dip0	17.15	4

Appendix 16

Table 25: least squares means for the effect of growth regulators, NAA, IAA and IBA (hormone) on root length (cm)

hormone	LSMEAN	Number
Hormone0	9.93	1
IAA1	19.95	2
IAA2	26.66	3
IAA3	27.08	4
IBA1	20.09	5
IBA2	26.80	6
IBA3	27.32	7
NAA1	20.08	8
NAA2	26.63	9
NAA3	27.13	10

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