DEVELOPMENT OF PLANT REGENERATION AND TRANSFORMATION TECHNIQUES TOWARDS REDUCING GLUCOSINALBIN BIOSYNTHESIS IN FIELD PEPPERWEED (*Lepidium campestre* L.)

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ABSTRACT

Field pepperweed (*Lepidium campestre* L.) is a cruciferous winter annual wild edible plant. It has potential medicinal properties as it contains a considerable level of glucoraphanin, which is the precursor for sulforaphane, a phase 2 protein inducer. Phase 2 proteins either directly or indirectly promote the scavenging of strong oxidants, and thus decrease the risk of many complex disorders such as atherosclerosis and Alzheimer's disease. However, field pepperweed plants also contain glucosinalbin, an antinutritional compound. For field pepperweed to become a green vegetable crop or source of functional food, it is desirable to reduce or eliminate glucosinalbin. The biosynthesis of glucosinalbin may be down-regulated through biotechnology. To that end, in the present studies, experimental protocols for plant regeneration and *Agrobacterium*-mediated transformation have been developed for field pepperweed. Establishment of such methods represents a vital first step in the process of engineering field pepperweed for enhanced nutritional value.

The effect of explant type and various combinations of growth regulators on regeneration were evaluated in three accessions of field pepperweed (Ames 13179, 13180 and 15718). Among the three genotypes, accession Ames 13179 had the highest regeneration frequency under several conditions. Regeneration from hypocotyl explants was more rapid and prolific than regeneration from either mature leaf or cotyledonary explants. Segments from the acropetal end of the hypocotyls were more regenerable than those from the basipetal end. Evaluation of different hormonal combinations and concentrations identified an optimal growth regulator combination (3 mg L^{-1} thidiazuron / 0.1 mg L^{-1} naphthalene acetic acid) for shoot induction.

The plant regeneration system established was adopted for field pepperweed transformation using the acropetal segments of hypocotyls as explants. Two plant expression

constructs were tested for down-regulating by RNA interference with the expression of a field pepperweed cytochrome P450 gene named LcCYP79B2. This gene may be involved in biosynthesis of glucosinalbin. Conditions for transformation such as pre-culture, co-cultivation time, and antibiotic concentration were evaluated. Transgenic plants were obtained and confirmed by histochemical staining of the reporter β-glucuranidase activity and PCR (polymerase chain reaction) analysis of the *NPTII* gene. The current study has established efficient plant regeneration and transformation protocols for field pepperweed. They should be useful for future molecular biology studies and biotechnological applications in this species.

ACKNOWLEDGMENTS

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2, 4-D	-	2, 4-dichlorophenoxy acetic acid
B5	-	Gamborg's salts and vitamins
BA	-	6-benzylaminopurine
GA_3	-	Gibberellic acid
GUS	-	β-glucuronidase
IBA	-	Indole-3-butyric acid
MES	-	2-N-morpholinoethane sulfonic acid
MS	-	Murashige and Skoog's salt
NAA	-	Naphthalene acetic acid
NPT II	-	Neomycin phosphotransferase II
PCR	-	Polymerase chain reaction
TDZ	-	Thidiazuron
X-Gluc	-	5-bromo-4-chromo-3-indolyl-glucuronidase

1.0 INTRODUCTION

Field Pepperweed (*Lepidium campestre* L.) is a winter annual weed plant and belongs to the family *Brassicaceae* (Thiede and Augspurger, 1996). Field pepperweed has been reported as an edible plant (Szczawinski and Turner, 1978; Elias and Dykeman, 1990). Fresh young leaves, shoots or seeds of field pepperweed could be added as a stimulating addition to soups, sauces, salads, and casseroles.

The constituent composition and content of the field pepperweed have been studied (Andersson *et al*., 1999; Windsor *et al*., 2005). Windsor *et al*., (2005) have reported leaves of field pepperweed contain certain types of glucosinolates such as glucoraphanin, glucosinalbin, glucoalyssin, glucohesperin, and glucobrassicin. Among those glucosinolates, glucoraphanin and glucosinalbin are the two major glucosinolates in leaves of field pepperweed (B. H. J. Juurlink, personal communication).

The glucosinolate glucoraphanin is a precursor for isothiocyanate sulforaphane (4-methylsulfinylbutyl isothiocyanate), a phase 2 protein inducer. Several studies have shown that isothiocyanates derived from the corresponding glucosinolates are beneficial components in the human diet, as they reduce the risk of cancer and other chronic and degenerative diseases by inducing phase 2 proteins (Fahey *et al*., 1997; Nestle, 1997; Faulkner *et al*., 1998; Jones and Brooks, 2006). In cultured human cells and rats, sulforaphane was able to induce several phase 2 proteins (mainly enzymes) and blocked the formation of 9, 10-dimethyl-1, 2-benzanthracene induced mammary tumors and induces apoptosis (Zhang *et al*., 1994). Juurlink (2001) suggested that one of the consequences of phase 2 protein induction is decreased oxidative stress and such an effect should retard or even prevent atherosclerotic changes,

thereby reducing the incidence of heart and brain attacks, while also slowing the progression of Alzheimer's disease.

While sulforaphane, a hydrolyzed product of glucoraphanin, is considered as an efficient inducer of phase 2 proteins, glucosinalbin in contrast is considered a toxic and anti-nutritional compound (Zukalová and Vašák, 2002). Some of the isothiocyanate breakdown products are capable of inhibiting the synthesis of the thyroxine and tri-iodine-thyronine thyroidal hormones which may lead to develop goiter (Zukalová and Vašák, 2002).

As leaves of field pepperweed contain a significant amount of glucoraphanin this plant could be converted into a functional food source. However, field pepperweed also contains a high level of glucosinalbin, which has many antinutritional properties. Therefore, the level of glucosinalbin needs to be reduced or eliminated in the field pepperweed plant for it to become a functional food crop. One possible approach to reducing this antinutritional compound glucosinalbin is to block or reduce its synthesis utilizing *Agrobacterium*-mediated transgenic technology.

The first objective of this project is to establish a reliable plant regeneration system for field pepperweed using potential explants such as hypocotyls, leaf and cotyledonary petiole explants. Three accessions of field pepperweed (Ames 13179, 13180 and 15718) were evaluated for *in vitro* shoot regeneration response. The second objective of this study is to develop an efficient *Agrobacterium*-mediated transformation method for field pepperweed.

2.0 LITERATURE SURVEY

2.1 Field pepperweed (*Lepidium campestre* L.)

Field Pepperweed (*Lepidium campestre* L.) is a winter annual plant (Figure 1A) (Thiede and Augspurger, 1996) belonging to the Brassicaceae (mustard) family. This plant species is native to Europe, but commonly found in North America (Claypole, 1886; Martindale, 1877) as an invasive weed. It is most abundant in the North East to the North Midwest. It grows on disturbed sites, waste grounds and roadsides (Martindale, 1877; Miller and Werner, 1987).

At early growth stages, the field pepperweed plant consists of a rosette of basal leaves. These leaves are round but taper to the base and the leaf margins may be lobed, toothed, or entire. Upper stem leaves are sessile, alternate and arrow-shaped. One or more flowering stems develop from the rosettes that are more or less erect. The stems reach 30 cm in height and are covered with short trichomes. Each stem terminates in a raceme of white flowers (Figure 1B). The flowers bloom toward the apex of the raceme, while the seedpods (siliques) develop below. Individual flowers are 2 mm long, have four white or greenish-white petals, and occur on 4-8 mm long stalks (pedicels). The root system consists of a stout taproot (Ontario weeds: Field pepper grass, 2003).

Field pepperweed germinates in the fall and forms an over-wintering basal rosette of leaves. The plant blooms in early spring and disperses its seeds in July and August. Each seedpod is about 5-6 mm long and 4 mm wide, with a “winged” structure at the apex (Figure 1C) (Ontario weeds: field peppergrass, 2003). The relatively small “winged” seed pods can be easily distributed by the wind or by moving vegetation (Thiede and Augspurger, 1996).

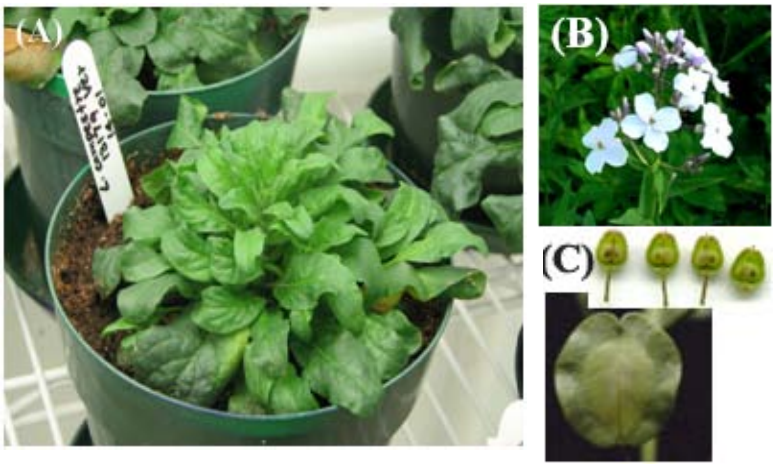

Figure 1. Growth chamber grown field pepperweed.
(A) Six-week old plant.
(B) Flowers.
(C) Seed pods.

2.1.1 Edible uses

Plants in the genus *Lepidium* are considered as edible wild plants by several authors (Fernald *et al*., 1958; Szczawinski and Turner, 1980; Duke, 1992). Field pepperweed is a very good substitute for zesty watercress. The pods and seeds have a taste in between mustard and pepper. The ground seeds can be mixed with vinegar and salt and used as a seasoning for meats. The fresh leaves are excellent in salads and sandwiches. Young shoots or leaves are a stimulating addition to soups, sauces and casseroles (Szczawinski and Turner, 1978; Elias and Dykeman, 1990).

Garden cress (*L. sativum* L.) is used as a green vegetable in Europe and America. The seeds of *L. sativum* are considered as a functional food in India and possess a number of medicinal properties like galactogogue, aperient, diuretic, alterative, tonic, demulcent, aphrodisiac, carminative and emmenagogue (Gokavi *et al*., 2004).

Macca (*Lepidium meyenii*) is a traditional food plant cultivated since 1600 B.C. on the high plateaus of the Andean mountain range in Peru. Tuberous roots of macca are used for production of salads, jams, bread, coffee substitutes and beer. Macca has many nutritional properties including anti-oxidative activity and macca is recommended as a medicinal plant for malabsorption syndrome, alcoholism and constipation, and to increase fertility (Večeřa *et al*., 2007).

2.1.2 Chemical composition of field pepperweed plant

The chemical composition and content of field pepperweed have been studied. Andersson *et al*. (1999) have reported that the seeds of field pepperweed contained dietary fibre (400 g kg^{-1}), crude fat (200 g kg^{-1}) and protein (190 g kg^{-1}). The main amino acids found in the seed were glutamic acid, aspartic acid and arginine. Linolenic acid was the most abundant fatty acid in the seed followed by erucic acid and oleic acid. The glucosinolate content in field pepperweed seed varied from 123 to 138 µmol g^{-1}. Glucosinalbin (4-hydroxybenzyl) was found to be the major glucosinolate in the seeds (Andersson *et al*., 1999). Glucoraphanin (4-methylsulfinylbutyl), glucosinalbin, glucoalyssin (5-methylsulfinylpentyl), glucohesperin (6-methylsulfinylhexyl) and glucobrassicin (4-methoxy-indol-3-yl-methyl) were also detected in the leaves of field pepperweed (Windsor *et al.,* 2005). Juurlink and colleagues demonstrated that the major glucosinolates in leaves of field pepperweed in the accession they examined were glucosinalbin and glucoraphanin (unpublished results). Bandara *et al.* (2008) have reported that the glucoraphanin content in leaves of eight different accessions of field pepperweed varied from 3.1 to 180.8 µg FW g^{-1}.

2.2 Glucosinolates

Glucosinolates, also known as mustard oil glucosides, are natural plant products that have received much attention due to their role in interactions between the host plant and pests. When consumed, the glucosinolates are flavor precursors, phase 2 protein inducer precursors and have some anti-carcinogenic effects. Glucosinolates are amino acid-derived secondary plant products containing a sulfate and a thioglucose moiety (Figure 2). Glucosinolates are found mainly in the order Capparales. To date more than 120 different glucosinolates have been identified (Hansen *et al*., 2001; Wittstock and Halkier, 2002; Zukalová and Vašák, 2002; Windsor *et al.*, 2005).

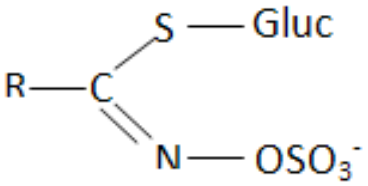

Figure 2. General structure of glucosinolates.

Glucosinolates are grouped into a number of chemical classes according to their structural similarities. Based on the amino acid precursors, glucosinolates are mainly grouped as aliphatic, aromatic and indolyl types (Chen and Andreasson, 2001; Fahey *et al.*, 2001). Secondary modifications of the glucosinolate side chain (oxidation, hydroxylation and esterification) also contribute to the enormous diversity of this class of phytochemicals (Wittstock and Halkier, 2002; Windsor *et al*., 2005).

2.2.1 Biosynthesis of glucosinolates

Glucosinolates are synthesized from only eight amino acids (alanine, methionine, valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan) and a number of chain-elongated homologues (Chen and Andreasson, 2001; Fahey *et al*., 2001; Windsor *et al*., 2005). The biosynthesis of glucosinolates comprises three independent stages: (1) chain elongation of amino acids, (2) followed by oxidative decarboxylation of the amino acid to its corresponding aldoxime, and (3) conversion of the oxime into basic glucosinolate structure and secondary modifications (Chen and Andreasson, 2001; Fahey *et al*., 2001; Hansen *et al*., 2001).

Biosynthesis of the glucosinolate core structure is initiated by the conversion of amino acids or chain elongated amino acids. In cyanogenic glucosides, cytochromes P450 belonging to the CYP79 family catalyze the conversion of amino acids to aldoximes. Homologues of these cytochromes P450 were identified in the *Arabidopsis* genome, and the heterologously produced proteins were characterized for their substrate specificity (Hansen *et al*., 2001, Wittstock and Halkier, 2002; Schuler and Werck-Reichhart, 2003; Windsor *et al*., 2005).

To date, six different members of the CYP79 family have been shown to be involved in glucosinolate biosynthesis. CYP79F1 and CYP79F2 convert chain elongated methionine derivatives respectively with 1-6 and 5-6 additional methylene groups in the side chain CYP79A2 metabolizes phenylalanine, while the substrate for CYP79B2 and CYP79B3 is tryptophan. CYP79A1 converts tyrosine into the relevant aldoxime. The members of the CYP79C subfamily have not yet been assigned a function. The cytochrome P450 belonging to the CYP83 family metabolizes aldoximes into relevant glucosinolates. CYP83A1 metabolizes the aliphatic aldoximes, whereas CYP83B1 metabolizes the indole and aromatic aldoximes (Wittstock and Halkier, 2002).

2.2.2 Biosynthetic pathways of glucoraphanin and glucosinalbin

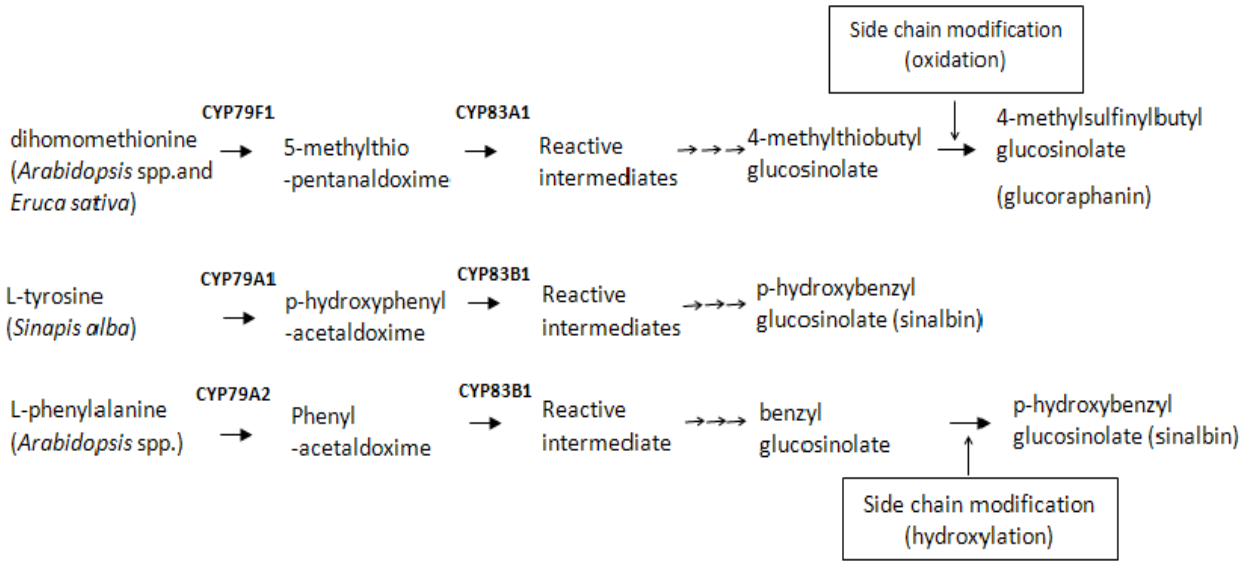

Figure 3. Cytochrome P450-dependent monooxygenases involved in the biosynthesis of the glucoraphanin and glucosinalbin core structures. CYP79F1 reacts on dihomomethionine and converts it into 5-methylthiopentanaldoxime. The 5-methylthiopentanaldoxime is converted further through an intermediate into 4-methylthiobutyl glucosinolate. Finally, a side chain modification takes place and produces 4-methylsulfinylbutyl glucosinolate (glucoraphanin). Glucosinalbin biosynthesis can be initiated from either tyrosine or phenylalanine. Both tyrosine and phenylalanine are further converted into their aldoxime forms by CYP79A1 and CYP79A2. The aldoximes then undergo several reactions through intermediates to derive glucosinalbin.

In Arabidopsis glucoraphanin is derived from methionine (Hansen *et al*. 2001) while glucosinalbin is derived from either tyrosine (Koch *et al*., 1995) or phenylalanine (Wittstock and Halkier, 2000) (Figure 3). In each of the pathways, the first two steps (the amino acid conversion via an aldoxime to a reactive intermediate) are catalyzed by cytochrome P450-dependent monooxygenases and the reactions are substrate-specific. It has been shown that the cytochrome CYP79F1 is responsible for the biosynthesis of glucoraphanin (Hansen *et al*., 2001), while two cytochromes CYP79A1 (Koch *et al*., 1995) and CYP79A2 (Wittstock and Halkier, 2000) are responsible for converting tyrosine and phenylalanine into their aldoxime

forms respectively (Figure 3). Most of the reactive intermediates in the biosynthetic pathways of glucosinolates have been identified and they are *N*-hydroxy amino acids, aldoximes, thiohydroximic acids and desulfoglucosinolates. Finally, different side chain modifications take place to produce glucoraphanin (Giamoustaris and Mithen, 1996) and glucosinalbin (Mithen *et al*., 2000) (Figure 3).

2.2.3 Hydrolysis of glucosinolates by plant and microbial myrosinase

Generally glucosinolates are biologically inactive. When the plant tissues are crushed or chewed, glucosinolates are hydrolyzed into biologically active isothiocyanates by the enzyme myrosinase (Fahey and Talalay, 1999). Myrosinase is present in both plant cells and human gut microflora (Fahey *et al*., 2001). A wide range of degradation products are formed by the hydrolysis of glucosinolates via glucose and an unstable aglycone moiety (Figure 4), which gradually rearranges to form different products. At neutral pH isothiocyanate is the dominant product while acidic pH nitrile derivatives are more prominent (Chen and Andresson, 2001). Glucosinolates which contain terminal double bonds produce an epithionitrile when they are being degraded in the presence of epithiospecifier proteins (ESP) and ferrous ions. If the glucosinolate side chain is hydroxylated, its degradation will produce oxazolidine-2-thiols through a spontaneous cyclization process (Chen and Andresson, 2001; Rouzaud *et al*., 2004). Some of these isothiocyanates are important class of phase 2 enzyme inducers. The isothiocyanate sulforaphane, a hydrolyzed product of glucoraphanin, has reported as the most potent naturally-occuring inducer of phase 2 enzymes (Prochaska *et al*., 1992; Zhang *et al*., 1992)

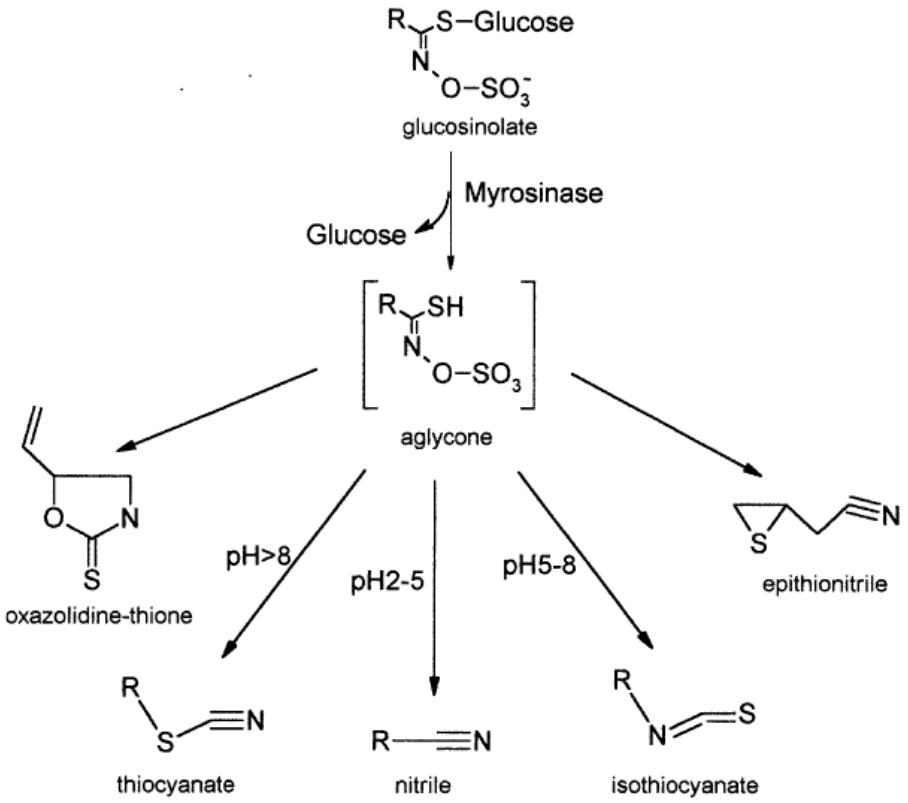

Figure 4. Names and structures of products from glucosinolate hydrolysis by the enzyme myrosinase (Chen and Andreasson, 2001).

2.2.4 Nutritional and toxic effects of glucosinolates

Several studies have shown that various isothiocyanates derived from the corresponding glucosinolates are beneficial components in the human diet as they reduce the risk of cancer (Fahey *et al*., 1997; Nestle, 1997; Faulkner *et al*., 1998; Jones and Brooks, 2006). In cultured human breast cancer cells and rats, sulforaphane could induce several phase 2 proteins (mainly enzymes) (Zhang *et al*., 1994). Futhermore, sulforaphane has been shown to block the formation of mammary tumors induced by the carcinogen 9, 10-dimethyl-1, 2-benzanthracene and trigger (or promote) apoptosis (Zhang *et al*., 1994). Juurlink (2001)

suggested that one of the consequences from phase 2 protein induction is decreased oxidative stress, which is suggested to retard or even prevent atherosclerotic changes, leading to reduced incidence of heart and brain attacks, while also slowing the progression of Alzheimer's disease. Subsequent studies by Noyan-Ashraf *et al*. (2006) have indicated that intake of sulforaphane does decrease oxidative stress in the spontaneously hypertensive stroke-prone rat (SHRsp) resulting in better health (Noyan-Ashraf *et al*., 2005; Noyan-Ashraf *et al*., 2006) .

Zukalová and Vašák (2002) have pointed out that some of the isothiocyanate breakdown products may prevent the uptake of iodine by the thyroid gland. The isothiocyanates derived from hydroxy-glucosinolates are not stable and subsequently re-arrange to form oxazolidinethionse. 5- vinyl-2-oxazolidinethione or goitrine is a strongly goitrogenic substance and may lead to develop goiter (Zukalová and Vašák, 2002).

2.2.5. Health impacts of dietary glucoraphanin

An imbalance between production and scavenging of oxidants such as superoxide anion, in favor of the oxidants leads to damage which is termed "oxidative stress". This oxidative stress and its consequences, hypertension and inflammation are causal factors in many complex disorders such as atherosclerosis and Alzheimer's disease (Juurlink, 2001). Phase 2 proteins either directly or indirectly promote the scavenging of strong oxidants, thus decrease the oxidative stress.

Wu *et al*. (2004) have reported that feeding glucoraphanin rich broccoli sprouts to male SHRsp rats (200 mg of dried broccoli sprouts) resulted in decreased oxidative stress and inflammation in the kidneys and cardiovascular system. Treated rats also had significantly lower blood pressures than rats on the control diet. Their results also showed that the expression of phase 2 protein genes was upregulated by the high-glucoraphanin diet.

Oxidative stress should also increase activation of NF-κB, a DNA binding transcriptional factor complex that interacts with promoter elements in pro-inflammatory genes (Juurlink, 2001). Wu *et al.* (2004) found that rats on the high-glucoraphanin diet showed decreased activation of NF-κB, decreased expression of pro-inflammatory genes and, therefore decreased inflammation in kidneys and the cardiovascular system.

Noyan-Ashraf *et al*. (2005) have shown that intake of broccoli sprouts rich in glucoraphanin also decreased inflammation in the Central Nervous System similar to that observed in the kidney and cardiovascular system. Further studies by Noyan-Ashraf *et al*. (2006) demonstrated that a glucoraphanin-rich diet induced the expression of phase 2 proteins, and decreased oxidative stress and associated hypertension and inflammation in female SHRsp rats. One of the most interesting findings was that if pregnant and lactating female rats were maintained on the glucoraphanin containing diet, their adult offspring had better blood pressures and less tissue inflammation regardless the diet they were on than the parental generation on control diet (Noyan-Ashraf *et al*., 2005; Noyan-Ashraf *et al*., 2006). These results collectively suggest that the consumption of a diet which includes phase 2 protein inducers such as sulforaphane may ameliorate or even prevent many complex diseases which are associated with oxidative stress.

Fahey *et al*. (2002) have reported that consumption of broccoli and broccoli sprouts which are abundant with sulforaphane can prevent the development of gastric cancer. According to their results sulforaphane is a potent bacteriostaic agent against antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo(a)pyrene induced fore-stomach tumors in ICR (Imprinting Control Region) mice. In addition, anticancer effects of the dietary isothiocyanate sulforaphane on pancreatic tissues have been reported by Pham *et al*. (2004). Their results have shown that daily sulforaphane intake (375 μmol/kg/d for 3 weeks) in severe

combined immunodeficient mice with PANC-1 (human pancreatic carcinoma, epithelial-like call line) had a 40% decrease in tumor growth compared to controls.

2.2.6 Induction of phase 2 proteins by sulforaphane

As previously explained oxidative damage plays a major role in developing cancer, ageing and a number of chronic diseases. Antioxidants are believed to be able to retard the development of these diseases or conditions. There are two types of antioxidants; *direct* and *indirect*. Direct antioxidants such as vitamin E, thioredoxin-dependent peroxidases, glutathione-S-transferases, and superoxide dismutase scavenge free radicals directly. Indirect antioxidants do not scavenge free radicals directly, but induce or boost the activity of the phase 2 enzymes, which can detoxify free radicals. These enzymes act as a defense mechanism, triggering a broad spectrum of antioxidant activities that neutralize free radicals (Juurlink, 2001). The isothiocyanate sulforaphane is the most potent naturally-occurring inducer of phase 2 proteins such as quinone reductase (Fahey *et al*., 1997) and glutathione-S-transferase (Maheo *et al*., 1997). It also inhibit the phase 1 protein cytochrome P450 2E1 (Barcelo *et al*., 1996).

Two major proteins are involved in the transcriptional activation of phase 2 gene expression; NF-E2 related factor 2 (Nrf2) and Keap1, a cytoplasmic protein (Chui *et al.*, 1995; Hayes *et al*., 1999; Itoh *et al.*, 1999). Generally, inactive Nrf2 is bound in the cytoplasm to Keap 1. Albena *et al*. (2002) reported that sulforaphane is highly reactive with cysteine thiols in Keap 1, and this interaction releases Nrf2 from the Nrf2-Keap 1 heterodimer. After the release, the active Nrf2 translocates from the cytoplasm to the nucleus. In the nucleus, the Nrf2 forms heterodimers with other transcription factors such as small Maf and the Nrf2-Maf complex binds the antioxidant response element (ARE) in the promoters of phase 2 protein

genes, triggering their transcription. Figure 5 illustrates how sulforaphane promotes transcription of phase 2 protein genes.

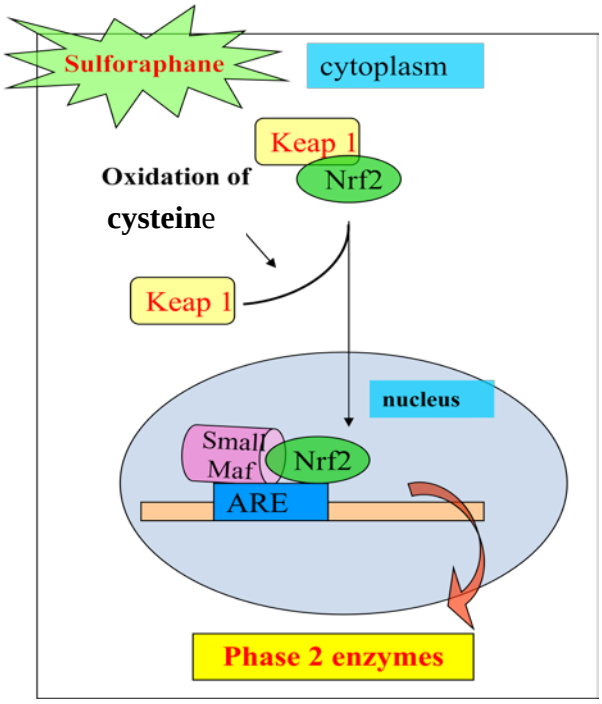

Figure 5. Activation of phase 2 enzymes by sulforaphane. Inactive Nrf2 is bound in the cytoplasm to Keap 1. Sulforaphane is highly reactive with cysteine thiols in Keap 1, and this interaction releases Nrf2 from the Nrf2-Keap 1 heterodimer. Then Nrf2 forms a heterodimer with small Maf, which binds to the Antioxidant Response Element in the DNA and triggers the phase 2 gene expression.

2.3 *In vitro* plant regeneration

In vitro plant regeneration facilitates germplasm conservation, and mass propagation of important crops and medicinal plants as well as crop improvement through genetic transformation (Rout *et al.*, 2000; Kintzios *et al.*, 2004; Thomas *et al.*, 2004). Therefore, there is always a need to develop reliable regeneration systems for use in plant biotechnology.

Plant morphogenesis consists of a series of complex developmental events. *In vitro* morphogenesis can occur two ways, either somatic embryogenesis or shoot organogenesis,

followed by root organogenesis (Phillips, 2004). Both pathways can occur either directly or indirectly. Direct or adventitious organogenesis takes place from the explant without going through a callus stage, the proliferation of undifferentiated tissue. Indirect or *de novo* organogenesis occurs through an unorganized and dedifferentiated callus tissue before organ formation (Gamborg and Phillips, 1995).

The response of excised explants to *in vitro* culture conditions and morphogenesis of organs from the explants depends on a range of different factors, including the donor plant genotype, the type of explants, the physiological stage of the explant source, levels of various plant growth regulators and environmental conditions under which the explants are grown (e.g. light and temperature) (Rout *et al*., 2000; Almeida *et al*., 2003). Furthermore, many genotype-dependent effects are also affected by the interactions between plant genotypes and the growth regulators. Consequently, in order to develop an optimal procedure for *in vitro* regeneration of a new plant species, various factors such as plant genotype, explant type, growth regulator and culture condition need to be evaluated (George, 1993). Some of the important factors will be reviewed in the following sections.

2.3.1 Genotype

The ability of plant tissues to regenerate *in vitro* is to a large extent controlled genetically. For example in wheat, some genotypes develop shoots from explants cultured on different media, while little success is achieved with other varieties on any medium (Lazar *et al.*, 1983). Similarly, several studies have shown that regeneration responses of melon (*Cucumis spp*.) are genotype-dependent (Orts *et al*., 1987; Oridate *et al*., 1992; Gray *et al*., 1993). Even though sufficient levels of regeneration have been achieved for most commercial apple genotypes, regeneration of transformed apple plants remains difficult and seems to be

strongly genotype-dependent (Yepes and Aldwinckle, 1994; Sriskandarajah and Goodwin, 1998). Akasaka-Kennedy *et al*. (2005) reported over 97% of the apple genotypes tested showed shoot regeneration ability, but the frequency of bud formation ranged widely from 1.3% to 100% among varieties, suggesting that genetics is one major factor for the shoot regenerability.

2.3.2 Physiological stages of source plants and tissues

Several studies have shown that the age and developmental stage of the source plants and explants are important factors in the success of *in vitro* regeneration. It has been reported that young source plants are more regenerative than old stock plants and juvenile plant tissues are more regenerative than mature tissues. In many woody plants, only juvenile explants can be established in culture (Geier, 1986; Stamp *et al*., 1990; Nugent *et al*., 1991). Shoot tip explants of the apple cultivars 'Redspur' and 'Goldspur' could be established more readily if they were taken from newly grafted shoots, rather than directly from orchard grown trees (Jones *et al*., 1985). Nodal segments obtained directly from 10 year-old *Fagraea fragrans* trees did not grow in culture, but the same explants taken from rooted cuttings of a 10-year-old tree established without difficulty (Lee and Rao, 1986). Kathal *et al*. (1988) documented that a higher proportion of *Cucumis melo* tissue cultures produced adventitious shoots [the development of shoots from tissues lacking preformed buds (Cassells and Gahan, 2006)] when they were initiated from small leaves (0.3 – 0.5 mm) of young seedlings than from leaves of the same size taken from older seedlings. One important reason why young tissues have better regenerability is that they are less differentiated and contain cells which could be more easily de-differentiated and become proliferative.

Although explants from many organs are capable of producing adventitious shoots, explants excised from different organs or from different tissues within an organ can vary greatly in their morphogenic capacity. According to Jaiswal *et al*. (1987), in *Brassica carinata*, 86% of cotyledons, 74% of the hypocotyl segments and 26% of root segments produced shoots. In *Pennisetum* and *Populas*, a gradient of embryogenic and organogenic ability is found within a single leaf such that explants taken from the base of the lamina are the most regenerative in cultures (Rajasekaran *et al*., 1987; Lee-Stadelmann *et al*., 1991). Also, regeneration from melon hypocotyl explants is more rapid than from proximal cotyledon explants (Gaba *et al*., 1999).

In vitro regeneration can be influenced by the way the source plants are treated and by the environment in which they have been grown. Explants taken from tissue-culture derived source plants have higher *in vitro* regeneration capacity than those from field- or greenhouse – grown source plants (Read, 1990). For some plants, the results from tissue cultures can also vary according to the time of year at which explants are excised or according to the environmental conditions under which the source plants were grown. Shoot regeneration from greenhouse grown *Peperonia* leaf segments was greatest when leaves were taken in summer, less successful in spring and poor in winter (Kukulczanka *et al*., 1977). Results from *in vitro* culture of *Larix occidentalis* showed that bud explants of mature trees gathered in June produced multiple axillary buds more rapidly than those taken at other times of the year (in 3-4 weeks compared to 3-4 months at other times) and a higher proportion of the explants responded to *in vitro* culture (Chesick *et al*., 1990).

2.3.3 Plant growth regulators (PGR)

Plant growth and *in vitro* morphogenesis are controlled by the interaction between naturally-occurring endogenous growth regulators and exogenous growth regulators in the culture media. There are several classes/types of plant growth regulators, including auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Among these classes, auxins and cytokinins are widely used for regulating growth and organogenesis in plant tissue and organ cultures. A balance between auxin and cytokinin growth regulators is a very important factor for the formation of shoots and roots (George, 1993). In 1957, Skoog and Miller showed that shoot formation could be induced from tobacco callus using low levels of auxin and a high level of cytokinin in the shoot induction media. A number of growth regulator combinations and concentrations need to be tested when designing *in vitro* culture protocols for a new plant species or new varieties (Valdés *et al*., 2001; Singh *et al*., 2003).

The most common natural auxin is indole-3-acetic acid (IAA). There exist many chemical or synthetic analogues of IAA such as 2, 4- dichlorophenoxyacetic acid (2,4-D), IBA (3- indole butyric acid) and 1- naphthalene acetic acid (NAA), which have similar biological properties but are more stable than IAA. NAA and IBA are extensively used for shoot cultures while 2,4-D is often used to initiate callus cultures. There are about 25 naturally occurring cytokinins. Zeatin (4 hydroxy-3- methyl-trans-2-butenylamino purine) and 2-iP [6-(3-methyl-2-butenylamino purine)] are the most commonly used cytokinins in tissue culture. Synthetic cytokinins widely used in plant tissue culture work include kinetin (6-furfuryl amino purine), 6-benzyl amino purine (BAP) and N-phenyl-1, 2, 3 thidiazol – 5- ylurea (TDZ).

It is known that the formations of adventitious shoots from explant tissues are regulated by the auxin to cytokinin ratio rather than the absolute hormone levels. Skoog and Miller (1957) reported that shoot formation could be induced using relatively low levels of

auxin and a high level of cytokinin in the culture media. Figure 6 illustrates the relative concentrations of auxin and cytokinin required for various types and states of growth and differentiation in tissue culture.

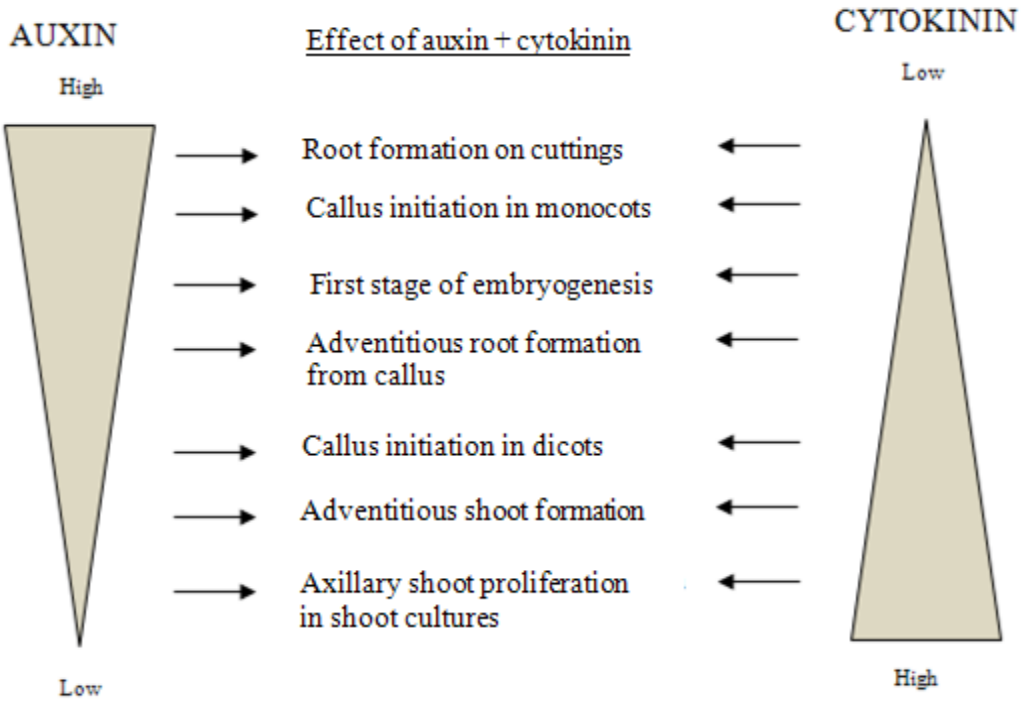

Figure 6. Relative concentrations of auxin and cytokinin required for growth and organogenesis (George, 1993).

2.3.4 Environmental factors

Light and temperature conditions also affecting the *in vitro* plant regeneration. Pre-culture light intensity and quality may alter the endogenous hormone levels in the donor plants (Read, 1990). Shepard and Totten (1977) reported light intensity and temperature during culture as critical factors in potato (*Solanum tuberosum* L.) shoot morphogenesis. Healthy

green calli were able to initiate more shoot buds under a light intensity 4,000 lux, but shoot bud development was increasingly inhibited when the temperature was above 24°C.

Generally, callus formation takes place in the dark while shoots regeneration occurs in the presence of light. However, Caillot *et al*. (2009) reported that the percentage of shoot initiation from flax (*Linum usitatissimum)* calli was significantly higher when high light intensity (150 µmol photon m^{-2} s^{-1}) was used. Furthermore, the high light intensity doubled the percentage of callus formation as well as their organogenic potential. A high light intensity has also been reported to stimulate the organogenic ability of garlic (*Allium sativum*) (Martin-Urdiroz *et al*., 2004).

Conversely a high level of light intensity does not always increase shoot regeneration. High photosynthetic photon flux (PPF) (100 – 125 µmol m^{-2} s^{-1}) reduced the regeneration of apple varieties such as 'Empire' and 'McIntosh' from 93% to 33% and 98% to 41%, respectively (Yepes and Aldwinckle, 1994). Furthermore, it has been shown that the number of shoots developed per explant was reduced from 6 or 8 when treated with low PPF (15 – 30 µmol m^{-2} s^{-1}) to 1 or 2 when treated with high PPF. However, once regeneration had taken place, PPF from 40 to 70 µmol m^{-2} s^{-1} increased shoot growth and development.

In some situations, darkness also favors growth and morphogenesis of plant tissue cultures. Dark conditions were used to encourage elongation of hypocotyls or epicotyls before explants were excised (Jagannathan and Marcotrigiano, 1986). Some species like *Picea pungens* did not produce adventitious buds unless the source plant was given a 8-day dark treatment followed by a 16 h photoperiod with 1000 lux illumination for shoot development (George, 1993). Furthermore, Kowalczyk *et al.,* (1983) observed that leaf disc and protoplast derived callus of *Solanum khasianum* produce shoots 21-28 days after the dark treatment. Bach, (1987) reported that for morphogenesis of species such as *Freesia hybrida,* the

requirement for light or dark depended on the plant genotype. Calli from some cultivars were able to produce shoots in dark, while calli from other cultivars required light. These observations collectively indicate that optimal light intensity is highly dependent of plant species, genotype, and explant type.

2.4 Plant transformation

2.4.1 Methods of plant transformation

Plant transformation refers to the introduction of desired foreign DNA into plant cells or tissues. Various methods have been developed for plant transformation. The most commonly used method is plant transformation mediated by the soil phytopathogen *Agrobacterium tumefaciens,* the causative agent of crown gall disease (Zupan *et al*., 2000).

Agrobacterium tumefaciens is attracted to phenolic compounds (acetosyringone), sugars, and organic acids that are released from wounded plant tissues (Winans, 1992; Tzfira and Citovsky, 2002). *Agrobacterium* has the ability to transfer a portion of its tumor-inducing (Ti) pasmid to the plant genome. There are two major regions in the Ti plasmid, the T-DNA region which is responsible for the crown gall disease and the region containing virulence (*vir*) genes which encode proteins required for T-DNA transfer (Winans, 1992; Zupan *et al*., 2000). For laboratory use in plant transformation, the gall-forming sequences (oncogenes) have been removed from the T-DNA region of *Agrobacterium* strains. The most commonly used method to inactivate the disease-causing ability is to remove the T-DNA by an *in vitro* recombination event and replace it with an engineered expression cassette. The resulting *Agrobacterium* strains retain the *vir* genes and as such are suitable hosts for plant transformation vectors. Also instead of a single Ti plasmid, most of the current *Agrobacterium* strains used in plant

transformation contain a binary system consisting of two plasmids, one plasmid having the *vir* region and the other having the desired T-DNA region containing one or more gene expression cassettes (Hoekama *et al*., 1983).

Agrobacterium-mediated transformation has become the most common method for *Brassica* transformation. Compared to other transformation methods, *Agrobacterium*-mediated transformation is simple, less expensive and does not need any specialized equipments (Bhalla and Singh, 2008). Relatively large segments of DNA can be accommodated within the T-DNA region and transferred to the plant. Generally only one or a few copies of the target gene(s) are integrated into the plant genome (Trigiano and Gray, 2005). Despite being the most common method, *Agrobacterium*-mediated transformation has several disadvantages. The main disadvantage is its ineffectiveness in transforming monocot plants (Songstad *et al*., 1995). Also, although it has been generally believed that during *Agrobacterium*-mediated transformation only a region between the right and left border repeats of T-DNA is transferred into the plant genome, several studies have shown that fragments of vector DNA can be either transferred as an attachment to the T-DNA or transferred independently from the T-DNA (Permyakova *et al*., 2009). Another approach of plant transformation, similar to *Agrobacterium*-mediated transformation, uses *Agrobacterium rhizogenes* (Christey and Sinclair, 1992; Poulsen, 1996). However, in *A. rhizogenes*-mediated transformation, transformed plants are regenerated via hairy roots. Several studies have reported some undesirable abnormal phenotypes such as altered flower morphology, reduced apical meristem and wrinkled leaves arising from *A. rhizogenes*-mediated transformation (Metz *et al*., 1995; Henzi *et al*., 2000; Puddephat *et al*., 2001).

Other two commonly used transformation methods are protoplast-mediated transformation and microprojectile bombardment (Trigiano and Gray, 2005). These two

methods are widely used as alternatives to *Agrobacterium*-mediated transformation due to their ability to transform both monocot and dicot plants (Songstad *et al*., 1995). Protoplasts (cells without cell walls) are capable of direct uptake of macromolecules such as DNA. Electroporation and polyethylene glycol (PEG) are the two major methods for delivering DNA into protoplasts (Songstad *et al*., 1995). The transformation frequency of protoplasts by electroporation depends on several factors including electroporation voltage, current and pulse duration, DNA concentration, and buffer type (Trigiano and Gray, 2005). PEG can chemically induce the formation of membrane pores and thus PEG concentration is most critical in PEG-mediated protoplast transformation (Trigiano and Gray, 2005). High concentrations of PEG could reduce the protoplast viability while low concentrations would not be effective in DNA uptake (Hayashimoto *et al*., 1990). Microprojectile bombardment is widely used for transformation of species that are difficult to transform using *Agrobacterium*-mediated transformation and protoplast-mediated transformation (Songstad *et al*., 1995). In this technique tungsten or gold coated DNA molecules are blasted into the plant cells using high pressure helium gas or propulsion of an explosive charge at a high velocity. The DNA molecules are released from their carriers and integrated into the chromosome by cellular components (Trigiano and Gray, 2005).

2.4.2 Selectable markers

Selectable markers are usually necessary for efficient production of transgenic plants as these selectable marker genes allow preferential growth of transformed cells in the presence of the corresponding selective agent. As an example, the aminoglycoside antibiotics, such as kanamycin, neomycin, paromomycin and geneticin (G418) kill bacterial cells by inhibiting protein translation. The gene *NPTII* (neomycin phosphotransferase II), which was originally

isolated from *Escherichia coli,* encodes for the protein neomycin phosphotransferase. This enzyme inactivates the aminoglycoside antibiotics by phosphorylation, thus allowing growth of transformed plant cells on media containing these selection agents (Kiernan *et al*., 1989; Masson *et al*., 1989; Wu *et al*., 2006). Another selectable marker is *aph IV*, which encodes for hygromycin phosphotransferase. This enzyme inactivates hygromycin through phosphorylation and thereby allows growth of resistant transformed shoots on media containing hygromycin. Several studies have shown that hygromycin can be used for efficient transformation of a number of plant species such as *Oryza sativa ssp. Indica* L.(Lin and Zhang, 2005), *Medicago sativa* L.(D'Halluin *et al*., 1990) *Glycine max* L.(Finer and McMullen, 1991). Gentamycin, streptomycin and spectinomycin can also be used as selectable agents (Hayford *et al*., 1988; Svab *et al*., 1990). Phosphinothricin (PPT) is a herbicide which inhibits glutamine synthetase (GS), a key enzyme for ammonium assimilation and the regulation of nitrogen assimilation in plants. The *bar* gene encoding for phosphinothricin acetyltransferase can inactivate PPT and confer resistance to PPT (DeBlock *et al*., 1989). Thus, it can also be used as a selectable marker.

2.4.3 Pre-culturing

Pre-culturing or preconditioning is the enhancement of culture growth by prior treatment of the source plant or explant (Cassells and Gahan, 2006). Several studies have shown that pre-culturing explants prior to incubation in *Agrobacterium* solution helps to reduce necrosis and increased the transformation frequency. Cardoza and Stewart (2003) reported *Brassica napus* hypocotyl segments tended to turn necrotic within a week after transformation. This problem was overcome by pre-culturing the hypocotyl explants for 3 days on 2,4-D prior to transformation. Similar observations were reported by Ovesná *et al*.

(1993) for *B. napus*. They have shown that pre-culture of hypocotyl explants on 2,4-D and kinetin for 7 days helped to overcome the lethal effect of toxins from the *Agrobacterium*. However there was excessive growth of callus due to the 2,4-D in the medium. Improvement in transformation frequency due to the pre-culturing of the explants has been reported for several plant species such as *Arabidopsis thaliana* (Schmidt and Willmitzer, 1988; Sangwan *et al.*, 1992), sugarbeet (*Beta vulgaris* L.) (Jacq *et al*., 1993), tobacco (*Nicotiana tabacum*) (Sunilkumar *et al*., 1999), watermelon (*Citrullus lanatus*) (Choi *et al*., 1994) and *Populus nigra* (Confalonieri *et al*., 1994).

Pre-culture of *Arabidopsis thaliana* L. Heynh explants on media containing plant growth regulators before the *Agrobacterium* infection can enhance the initiation of cell division in specific cell types which are prone to transformation (Sangwan *et al*., 1992). These newly synthesized cell walls produce compounds which induce *vir* genes in the *Agrobacterium* (Stachel *et al*., 1985; Spencer and Towers, 1991). Sunilkumar *et al*. (1999) also reported that production of *vir* gene inducers by the tobacco leaf explants during the pre-culturing period is an import contributing factor to increase transformation efficiency.

In contrast to the above findings, Costa *et al*. (2002) reported pre-culture of Duncan grapefruit (*Citrus paradise*) epicotyl segments on co-cultivation medium prior to incubation significantly reduced regeneration. Their results showed genetic transformation efficiency was much higher in the treatment without pre-culture (5.9%) than when the explants pre-cultured for 1 or 2 days. Cervera *et al.* (1998) have shown for citrus, that transformation frequency was drastically decreased in explants pre-cultured on co-cultivation medium compared to explants directly inoculated and co-cultivated with *Agrobacterium* after explants were excised.

2.4.4 Inoculation and co-cultivation

The *Agrobacterium* infection process is basically divided into two stages: (1) inoculation and (2) co-cultivation. The inoculation stage takes place for a short period, typically a few minutes, by immersion of explants in an *Agrobacterium* suspension. The excess bacterial suspension is then removed and the explants are co-cultivated for a period of 1 to 7 days on a co-cultivation medium to allow for DNA transfer and integration. The duration of stage 1 can influence efficiency of transformation. Costa *et al*. (2002) have shown that more shoots were produced from *Citrus paradise* (Macf) explants which had been incubated in *Agrobacterium* solution for 5, 10 or 20 min than explants incubated for 1 min. For another citrus species *C. sinensis*, epicotyl explants incubated longer than 10 min showed a decrease in the transformation efficiency (Bond and Roose, 1998). Wu *et al*. (2006) reported that 8-10 min was the best inoculation period for tomato transformation. For *Brassica* species, Cardoza and Stewart (2003) observed that a co-cultivation period of 48 hrs for *B. napus* gave the best transformation efficiency, while a longer period like 72 hrs resulted in a low number of transgenic plants due to necrosis. Ovesná *et al*. (1993) also indicated that a longer co-cultivation period for *B. napus* induced necrosis. In contrast, Zhang *et al*. (2000) reported 72 hrs as the best co-cultivation period for transformation of Chinese cabbage (*Brassica campestris* L. ssp. Pekinensis).

Sometimes, inoculated explants are incubated upon a piece of sterile filter paper that covers cultured cells to improve transformation frequency by secreting *vir* gene inducers (Horsch *et al*., 1985). In addition, inclusion of 200 µM acetosyringone, a *vir* gene inducer, in the co-cultivation medium significantly increased the T-DNA delivery (Wu *et al*., 2003).

3.0 MATERIALS AND METHODS

3.1 Materials

Seeds of three accessions of field pepperweed (*L. campestre* L.): Ames 13179, 13180, and 15718 were provided by Dr. Manjula Bandara of the Crop Diversification Centre South, Alberta Agriculture and Food, Alberta, Canada. These three accessions were shown to have relatively high levels of glucoraphanin among the seven accessions and a local variety analyzed by Bandara *et al*., (2008). The tissue culture laboratory facilities and chemicals required in this project were kindly provided by Dr. Alison Ferrie, National Research Council, Plant Biotechnology Institute, Saskatoon, SK, Canada. Plant tissue culture laboratory training was provided by Joe Hammerlindl a researcher well experienced in *Brassica* transformation in the Plant Biotechnology Institute of the National Research Council, Saskatoon, SK, Canada. All the protocols used in this study were based on standard *Brassica* transformation protocols use in the plant transformation laboratory.

3.1.1 Seed sterilization and germination

Seeds of the field pepperweed accessions Ames 13179, 13180 and 15718 were surface-sterilized prior to use. Approximately 2.0 mL volume of seeds (0.5 g) was placed into a 15 mL falcon tube and 10 mL of 50% commercial Javex bleach (sodium hypochlorite 5.25 %) was added to the tube. Two drops of Tween 20 were added as a wetting agent. The tube was then placed in a rotating mixer for 20 min to insure adequate sterilization of the seeds. After 20 min, the seeds were poured into a sterile funnel lined with Miracloth (placed into the neck of a

large flask) and thoroughly rinsed with 500 mL sterile distilled water. The seeds were then transferred onto a piece of sterile filter paper in a sterile Petri dish.

The germination medium consisted of ½ strength Murashige and Skoog 1962 (MS) medium with 1% sucrose in 15 x 70 mm Petri dishes filled nearly to the top (90%) with medium. Thirty five to forty seeds were placed on the surface of the medium. Then the seeded plates, with lids removed, were placed onto the upturned lids of sterile Magenta boxes and covered with the transparent magenta boxes. The seeds were allowed to germinate and grow, first under dark conditions in a closed wood cabinet at room temperature (24 ± 2 °C) for 3 days and then under growth chamber conditions (25 ± 2 °C**,** 16 h/8 h light/dark) for another 3-4 days.

3.1.2 Leaf sterilization

All three accessions of field pepperweed were grown in 6 inch pots containing Sunshine Mix 3 (SUN GRO Horticultural) to obtain young healthy leaves for use as explants. Eight plants from each accession were grown in a chamber supplemented with cool white fluorescent light with a photoperiod of 16 h/8 h day/night. The light intensity was approximately 120 µmol m^{-2} s^{-1}. Temperature was 25° ± 2 °C. Plants were watered everyday and fertilized once with 20-20-20 (N: P: K) slow release fertilizer.

To obtain leaf explants, young healthy leaves were excised from field pepperweed plants (6 weeks or older) growing in pots that continuously produced new leaves (the plants do not flower until after a vernalization treatment). For surface sterilization five to eight whole leaves were collected into a 500 mL Pyrex beaker filled with water. After collecting leaf samples, water was removed and 10% commercial Javex bleach (5.25 % of sodium

hypochlorite) was added to cover all leaf materials. Two drops of Tween 20 were added as a surfactant. The beaker was covered with a plastic wrap and placed on a shaker for 15 min (~ 60 rpm). The leaves were then rinsed three times consecutively by using a pair of sterile forceps to dip the leaf pieces into sterile Petri dishes filled with sterile distilled water each for 3-5 minutes. Leaf pieces of about 4.0 x 4.0 mm size were then cut using a sterile scalpel and placed on pre-culture media.

3.2 Media preparation

The basal medium used to culture explants in this study was: Murashige and Skoog (1962) inorganic salts with B5 vitamins as specified by Gamborg *et al.* (1968) (referred to as MS/B5 thereafter). All media were solidified with Phytoagar and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

3.2.1 Seed germination medium

The seed germination medium (SGM) consisted of hormone-free ½ strength MS/B5, 1% sucrose, and 0.8% phytoagar. SGM was poured into 15 x 70 mm Petri dishes, approximately 30 mL per plate.

3.2.2 Pre-culture/co-cultivation medium

All the explants were pre-cultured on pre-culture/co-cultivation media before transfer to shoot induction medium. Two plant growth regulators 2,4-D (1 mg L^{-1}) and kinetin (1 mg L^{-1}) were added to MS/B5 basal medium containing 3% sucrose and 0.7 % Phytoagar. Autoclaved medium was poured into 15 x 70 mm sterile Petri dishes, approximately 25 mL per plate.

3.2.3 Shoot induction media (SIM)

The shoot induction media (SIM) consisted of MS/B5 basal medium containing 3% sucrose, 0.8% phytoagar, MES 500 mg L^{-1} and combinations of different concentrations of auxins (NAA) and cytokinins (BA, TDZ and Zeatin). Hypocotyls, leaf and cotyledonary petiole explants were initially screened on MS/B5 medium with 21 different growth regulator combinations as shown in Table 1 to identify suitable concentration ranges for shoot induction of field pepperweed. These hormonal combinations were based on suggestions from Mr. Joe Hammerlindl with his experience with *Brassica* plant regeneration.

Table 1. Concentrations of the auxin NAA and cytokinins used in initial experiments.

NAA/BA		**NAA/TDZ**		**NAA/Zeatin**	
mg L^{-1}	**µM**	**mg L^{-1}**	**µM**	**mg L^{-1}**	**µM**
0.0 /1.0	0.0/4.0	0.0 /3.0	0.0/13.6	0.0 /1.0	0.0/4.5
0.0 /2.0	0.0/8.0	0.0 /10.0	0.0/45.4	0.0 /2.0	0.0/9.0
0.0 /4.0	0.0/16.0	0.1 /3.0	0.53/13.6	0.1 /1.0	0.53/4.5
0.1 /1.0	0.53/4.0	0.1 /10.0	0.53/45.4	0.1 /2.0	0.53/9.0
0.1 /2.0	0.53/8.0	1.0 /3.0	5.3/13.6	1.0 /1.0	5.3/4.5
0.1 /4.0	0.53/16.0	1.0 /10.0	5.3/45.4	1.0 /2.0	5.3/9.0
1.0 /1.0	5.3/4.0	-	-	-	-
1.0 /2.0	5.3/8.0	-	-	-	-
1.0 /4.0	5.3/16.0	-	-	-	-

NAA = Naphthalene acetic acid (FW – 186.21g)
BA = 6-benzylaminopurine (FW – 225.25g)
TDZ = Thidiazuron (FW – 220.25g)
Zeatin (FW – 219.25g)

3.2.4 Shoot elongation medium (SEM)

After 2-3 weeks, explants with regenerating shoots were transferred onto shoot elongation medium (SEM), consisting of MS/B5 medium with 2% sucrose, 0.5 mg L^{-1} benzyladenine (BA), 1.5 mg L^{-1} gibberellic acid (GA_3) and 0.9% phytoagar. They were

incubated for 3 weeks on this media in a tissue culture room at 25 ± 2 °C**,** 16 h/8 h light/dark and ~ 120 µmol m^{-2} s^{-1} of light intensity.

3.2.5 Root induction medium (RIM)

Elongated shoots were rooted on root induction medium (RIM) consisting of ½ strength MS/B5 medium supplemented with 0.5 mg L^{-1} indole-3-butyric acid, 1% sucrose, 500 mg L^{-1} MES and 0.8% agar. Shoots were maintained in a tissue culture room at 25 ± 2 °C**,** 16 h/8 h light/dark and ~ 120 µmol m^{-2} s^{-1} of light intensity. After about 4 weeks, plantlets with roots were transferred directly to 6-inch pots containing Sunshine Mix 3 and covered with a transparent plastic cup for a week to maintain the humidity. Plants were grown in a chamber supplemented with cool white fluorescent light with a photoperiod of 16 h/8 h day/night. The light intensity was approximately 120 µmol m^{-2} s^{-1}. Temperature was 25° ± 2 °C.

3.3 Evaluation of explants for shoot induction.

Hypocotyl explants were obtained from 7-day-old seedlings. Whole hypocotyls without sectioning were placed on a sterile filter paper on pre-culture media for 4 days. They were then cut into pieces approximately 5 mm in length and placed horizontally on 21 different plant growth regulator treatment combinations (Table 1) for initial screening.

Sterilized leaf explants were obtained as described earlier and incubated on a pre-culture medium for 7 days. They were tested with the same growth regulator combinations. Cotyledon explants were excised without the apical meristem but with the petiole, as described by Moloney *et al.* (1989). They were pre-cultured for 4-days and then inserted into the SIM in a way that the cut surface of the explant was in contact with the medium.

All the cultures were maintained in a tissue culture room at 25 ± 2 °C with a photoperiod of 16 h/8 h light/dark. Shoots formation, root formation, and appearance of callus were surveyed and noted after four weeks.

3.3.1 Further optimization of *in vitro* shoot regeneration

Based on the initial series of experiments to assess growth regulator combinations, two sets of experiments were conducted. First, green compact callus (after 2 subcultures every 2-3 weeks) from leaf explants were divided into four groups and cultured on these following different plant growth regulator conditions: (1) SIM medium as specified in Section 3.2.3 but containing 3.0 mgL^{-1} TDZ and 0.1 mgL^{-1} NAA, (2) SIM containing a low cytokinin level (0.3 mgL^{-1} TDZ with 0.1 mgL^{-1} NAA or 0.1 mgL^{-1} BA with 0.1 mgL^{-1} NAA), (3) SIM containing a low auxin level (0.01 mgL^{-1} NAA with 3.0 mgL^{-1} TDZ or 2.0 mgL^{-1} BA), and (4) hormone free SIM containing no plant growth regulators.

Secondly, since the various regions of the hypocotyl may differ in their ability to regenerate shoots, hypocotyls were divided into three major segments (Figure 7): acropetal (segment 1), middle (segment 2), and basipetal (segment 3). After pre-cultivation on pre-culture media, each segment was cut into pieces of about 5 mm in length and cultured using the most promising growth regulator combination (3.0 mg L^{-1} TDZ with 0.1 mg L^{-1} NAA) identified in the initial round of experiments. The experiments were performed twice. In each experiment, for each treatment, two replicating plates each containing about 16 explants were used.

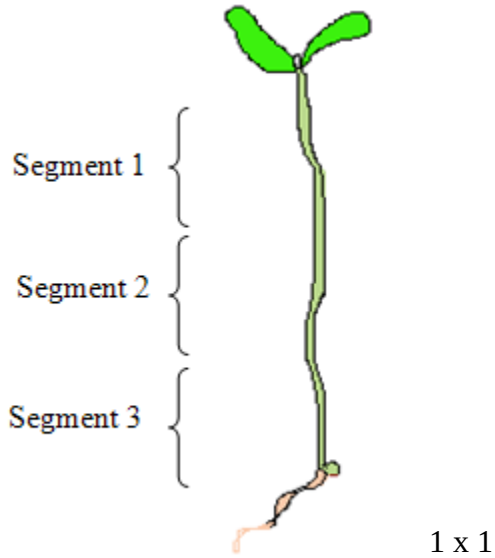

Figure 7. Schematic representation of acropetal (segment 1), middle (segment 2) and basipetal (segment 3) segments of the hypocotyl of field pepperweed.

Results from the above experiments showed that the hypocotyl has the highest regeneration frequency among the three types of explants. Furthermore, the acropetal segment of hypocotyls had the highest regeneration frequencies among the three segments. Based on these results, another set of experiments were performed to optimize the SIM for hypocotyl explants of field pepperweed. The acropetal end explants were further tested on 16 different growth regulator combinations: TDZ at four concentrations (1.0, 3.0, 5.0 and 7.0 mg L^{-1}) in combinations with four NAA concentrations (0.0, 0.1, 0.2, and 0.5 mg L^{-1}). These concentrations were selected based on the results of initial experiments testing different plant growth regulator combinations (Table 1).

After 2-3 weeks, explants with regenerating shoots were transferred to shoot elongation medium (SEM) for another 3 weeks. Elongated shoots were rooted on RIM and incubated for 4 weeks in a tissue culture room at 25 ± 2 °C**,** 16 h/8 h light/dark and ~ 120 µmol m^{-2} s^{-1} of light intensity. Plantlets with roots were transferred to pots containing

Sunshine Mix 3 and covered with a transparent plastic cup for one week to maintain the humidity. Plants were grown in a chamber supplemented with cool white fluorescent light with a 16h photoperiod. The light intensity was approximately 120 µmol m^{-2} s^{-1}. Temperature was 25° ± 2 °C.

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3.4 *Agrobacterium*-mediated transformation

Based on results from previous experiment hypocotyl explants from seven-day-old *in vitro*-grown seedlings were used for transformation experiments. These explants were pre-cultured on pre-culture media which contained 2,4-D (1 mg L^{-1}) and kinetin (1 mg L^{-1}) for 4 days before the transformation experiments.

3.4.1 Bacterial strains and plasmids

The *Agrobacterium* strain GV3101::pMP90 (Koncz and Schell, 1986) was used in transformation experiments. The binary vector pHS723 was obtained from Dr. Gopalan Selvaraj' laboratory (National Research Council-Plant Biotechnology Institute, Saskatoon, SK, Canada) and used in the initial experiments to optimize transformation conditions. It contains the *GUS* reporter gene fused with the selectable marker gene *NPTII* for kanamycin resistance driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter.

The RNAi construct (named S25C) targeting a field pepperweed P450 gene was prepared by Dr. Xianzong Shi (Department of Biochemistry, University of Saskatchewan, Saskatoon, Canada) using pBBI-PI-ISH (Zhang *et al*., 2006a), which is a derivative of pBin19. The P450 cDNA fragments were inserted in the sense and anti sense orientations in the plasmid pBBI-PI-ISH (Figure 8). For the control construct (named S25D), only the sense P450 fragment was inserted.

Figure 8. Expression cassette in RNAi construct targeting a field pepperweed P450 gene. The construct was prepared using the vector pBBI-PI-ISH.

3.4.2 *Agrobacterium* transformation of hypocotyl explants of field pepperweed

Acropetal end hypocotyl segments were harvested from 7-day-old *in vitro*-grown seedlings. These segments were placed onto sterile filter papers (Whatman #1) which were then placed on the surface of pre-culture/co-cultivation medium and incubated for 4 days in a tissue culture room at 25 ± 2 °C, 16 h/ 8h (light/dark) photoperiod.

An *Agrobacterium* culture was prepared by growing *Agrobacterium* (GV3101::pMP90) in 5 mL Luria broth (LB) containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} gentamycin at 28°C for 24 hrs. After about 24 hrs the culture was spun down at 822 x g for 10 min, and the supernatant discarded. The pellet was resuspended in 5 mL hormone-free MS containing 3% sucrose.

Hypocotyls were cut into small pieces (approximately 5-mm long) and collected in a 15 x 70 mm Petri dish containing 3.6 mL of hormone-free MS medium. *Agrobacterium* suspension of 0.4 mL was then added to the Petri dish and mixed well by pipetting and swirling by hand to ensure all tissue pieces were inoculated. After 5-8 min of inoculation, excess liquid was removed by pipette and discarded. The inoculated explants were then placed onto plates of pre-culture/co-cultivation medium covered with a sterile filter paper and incubated for 2 days in a tissue culture room at 25 ± 2 °C, 16 h/ 8 h (light/dark) photoperiod.

After the co-cultivation period, explants were transferred to fresh SIM containing kanamycin (20 mg L^{-1}) as the selection agent and 300 mg L^{-1} timentin to inhibit *Agrobacterium* growth. Plates were kept in a tissue culture room for 2 weeks at 25 ± 2 °C, 16 h/ 8 h (light/dark) photoperiod. Inoculated explants were sub-cultured every 2-3 weeks on to fresh SIM until shoots started to develop. Healthy green shoots with leaves were excised on to SEM containing 25 mg L^{-1} kanamycin. The shoots were sub-cultured onto fresh SEM every 3-4 weeks. Well developed green shoots were separated from each other and transferred on to RIM containing 25 mg L^{-1} kanamycin. After 1-3 weeks, root development had started. Rooted shoots were kept for a further 2 weeks until a good root system was developed and then transferred to pots containing Sunshine Mix 3.

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3.4.3 Evaluating optimum kanamycin concentration for *Agrobacterium* transformation

Acropetal end hypocotyl explants from field pepperweed accession Ames 13179 were cultured on kanamycin-containing media to determine the optimum kanamycin concentration for selection of transformed explants. Both control and transformed explants treated with *Agrobacterium* containing pHS723 were tested on different kanamycin concentrations (0, 20, 25 and 30 mg L^{-1}). The hypocotyl cultures were grown in a tissue culture room 25 ± 2 °C with a 16 h/ 8 h (light/dark) photoperiod. After 3 weeks, the explants were sub-cultured onto fresh SIM and shoot regeneration frequencies were obtained after another 3 weeks.

Since the vector pBBI-PI-ISH was used to prepare the RNAi construct, the kanamycin concentration was also tested to compare pHS723 with the RNAi construct, to ensure that the kanamycin concentration used was also appropriate for transformation using the RNAi construct. The acropetal hypocotyl explants of field pepperweed, accession Ames 13179 were

inoculated with *Agrobacterium* harboring either pHS723 or the RNAi construct (S25C). After 2 days of co-cultivation the explants were placed separately on SIM containing 0, 20, 25 or 30 mg L^{-1} kanamycin. After 3 weeks explants were sub-cultured onto fresh SIM with the same range of kanamycin concentrations. Shoot formation was recorded after 3 weeks on this media.

3.4.4 Co-cultivation

Acropetal hypocotyl explants of accession Ames 13179 were inoculated with *Agrobacterium* strain GV3101::pMP90 containing plasmid pHS723. The explants were placed on SIM containing 20 mg L^{-1} kanamycin for different lengths of time (0, 2, 4 and 6 days) to determine the optimal co-cultivation time.

3.5 Analysis of transgenic plants

3.5.1 GUS assay

The GUS assay was conducted with minor modifications according to the method described by Jefferson *et al.* (1987). Pieces of healthy green leaves (approximate 4 x 4 mm) from putative transgenic shoots were collected in a 96 well plate and X-Gluc (5-brom-4-chloro-3-indolyl glucuronide) solution was added to cover the leaf pieces. Occasionally, a whole leaf (about 2 centimeters in length) was used. It was placed in a plastic weighing boat containing the X-Gluc solution and covered with a piece of plastic. The leaf samples were incubated overnight at 37°C. They were then bleached using 10% commercial Javex bleach (10% Javex contained 0.525% sodium hypochlorite) to remove the chlorophyll. Samples were

observed under the light microscope. Development of a blue color was an indicator of *GUS* gene expression.

3.5.2 DNA extraction

Genomic DNA was extracted from leaves of root-developing plantlets using the protocol described by Edwards *et al.* (1991) with minor modifications. Leaf tissue samples (about 5 mm diameter) were collected into 1.5 mL pellet Pestle Tube on ice. Edwards extraction buffer (200 µl) was added to the tube and the tissue was ground with a pellet pestle. An additional 200 µl of extraction buffer was added and the tissue was further ground to make sure it was thoroughly fragmented and dispersed. The tubes were then centrifuged at 13,000 rpm (eppenforf centrifuge) for 5 min and 300 µl of the supernatant was removed to a fresh tube. To this fresh tube, 300 µl of iso-propyl alcohol was added and mixed well by inversion and kept at room temperature for 15 minutes. The tube was centrifuged again at 13,000 rpm for 5 min. After the supernatant was discarded, the DNA pellet was washed with 200 µl of 70% ethanol and centrifuged at 13,000 rpm for 1 min. The supernatant was removed and the DNA pellet was allowed to dry in the flow hood. When dry, 100 µl of Tris EDTA buffer (10 mM Tris pH 8.0, and 1 mM EDTA) was added to dissolve the pellet. The solution was then centrifuge at 13,000 rpm for 5 min. The supernatant was used for polymerase chain reactions.

3.5.3 PCR amplification of *NPTII* gene

Genomic DNA extracted from the leaf samples of putative transformants of *L. campestre* was used to detect the presence of the *NPT II* gene by polymerase chain reaction (PCR). The primers, forward primer JH 1 5`-ATCTCCTGTCATCTCACC and reverse primer JH 2 5`-AAGAAGGCGATAGAAGGC, were used to amplify a 500-bp fragment of the *NPT*

II gene. For PCR, the following temperature profile was used: a 2 min initial denaturation step at 94°C was followed by 35 amplification cycles of 94° C for 30 sec, 55° C for 30 sec and 72° C for 30 sec), and then the final extension step at 72°C for 5 min. Amplified fragments were analyzed using a 1.2 % agarose gel containing 0.5 μg mL^{-1} ethidium bromide. After electrophoresis, DNA bands were visualized using a UV illuminator.

4.0 RESULTS

4.1 Evaluation of genotypes, explant types and plant hormonal combinations for shoot induction

The first objective of this study was to establish a system for regeneration of field pepperweed. A literature survey indicated that there has been no published information available on any aspect of tissue culture and plant regeneration for this species. Consequently, several initial experiments were conducted to determine the basic conditions for effective shoot induction of this plant species using genotype, explant type and combinations of plant hormones, particularly auxin and cytokinin as variables. Our strategy was to test broad conditions first and the range of conditions which show promise in shoot regeneration would be further tested and optimized.

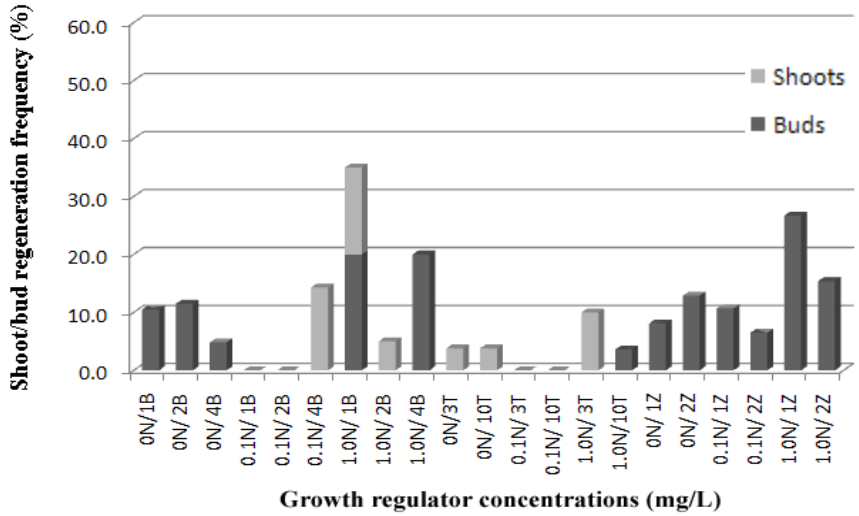
Initially, three explant types and 21 hormonal combinations, as described in Table 1, were investigated using one genotype - accession Ames 15718. Cotyledonary petiole and hypocotyl explants from 7-day-old seedlings and leaf explants from plants growing in pots were incubated on the pre-culture medium for 7 days before being transferred onto the SIM. Among the explants tested, it was observed that hypocotyls had the highest regeneration frequency, followed by leaf explants, while cotyledonary petiole explants did not respond to any of the growth regulator combinations. To further confirm the preliminary observations, the same three explant types and 21 hormonal combinations were tested using all three pepperweed genotypes, namely accessions Ames 13179, 13180 and 15718. The results will be discussed in the following according to the explant type tested.

4.1.1 Leaf explants

At the end of one-week incubation on the pre-culture medium, leaf explants started expanding. After transferring these leaf explants to SIM, they started to form callus at the cut ends. After four weeks, almost all the leaf explants developed green compact callus, which was not seen in hypocotyl explants (data not shown). Light green bud-like smooth outgrowths could be seen on some calli, and most of these developed into buds. The frequency of this bud development was genotype-dependent, and is indicative of shoot regenerability. Among the 21 hormonal combinations evaluated, the number of hormonal combinations showing any bud/shoot formation was 17 for accession Ames 13179 (Figure 9A), 8 for accession Ames 13180 (Figure 9B), and 4 for accession Ames 15718 (Figure 9C). In this experiment, buds or shoots were all counted as a positive response of shoot induction. For accessions Ames 13180 and Ames 15718, only buds were observed while shoots were observed with Ames 13179. These results suggest that accession Ames 13179 was more responsive to the culture conditions than the other two genotypes.

While most hormonal combinations produced buds when applied to Ames 13179, only three hormonal combinations (0.1NAA/4BA, 1.0NAA/1BA and 1.0NAA/3TDZ) produced more than one shoot (Figure 9A). For accession Ames 13180, eight hormonal combinations produced buds and one of them developed shoots. For accession Ames 15718, only four hormonal combinations showed some signs of bud development, with one or two buds each (Figure 9C). In general, the leaf explants did not produce many shoots, and furthermore these shoots appeared succulent and slender-shaped compared to shoots generated from hypocotyl explants.

(A)


Shoot/bud regeneration frequency (%)
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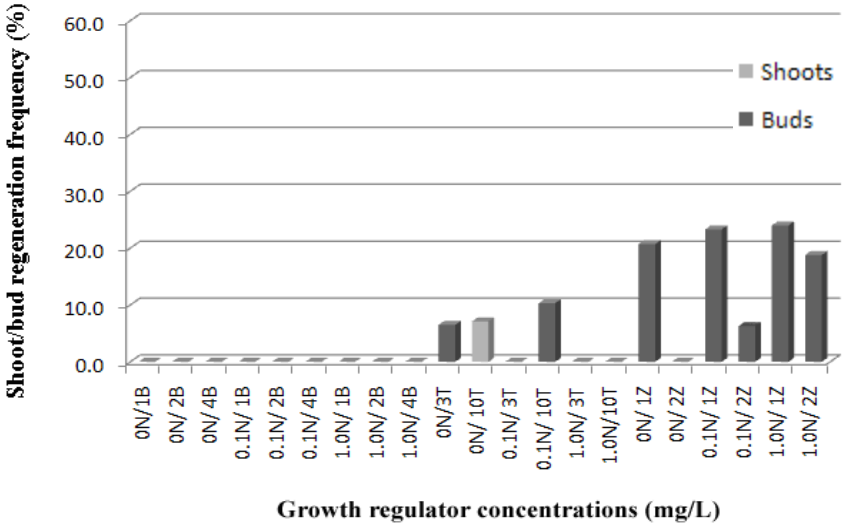
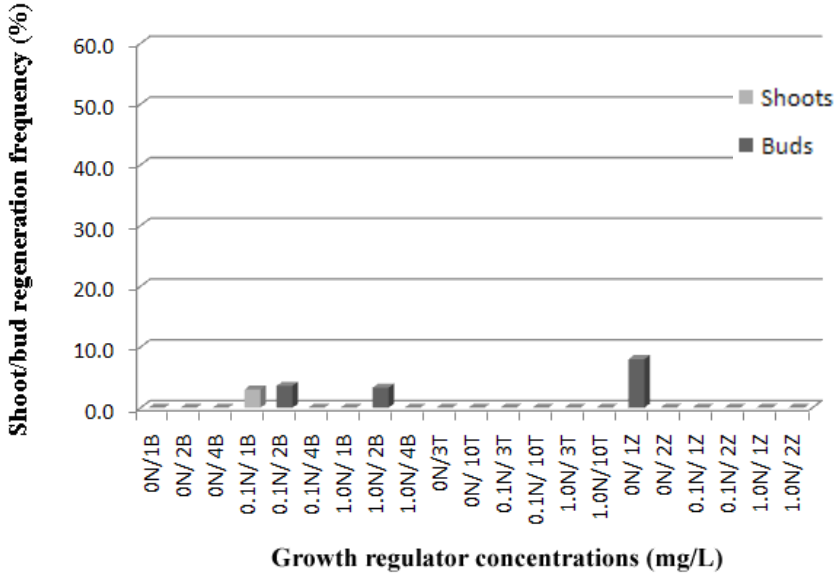
50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(B)


Shoot/bud regeneration frequency (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(C)


Shoot/bud regeneration frequency (%)
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50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(D)

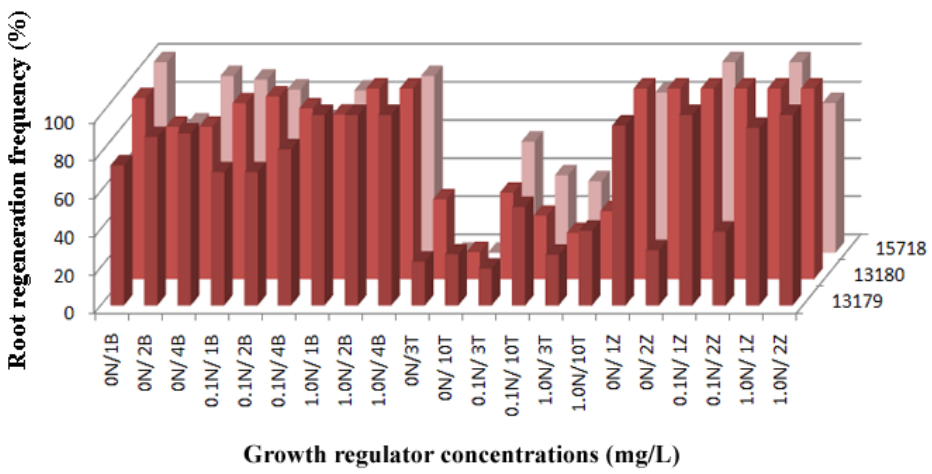


Figure 9. Effects of genotypes and hormonal combinations on shoot and root induction from leaf explants of field pepperweed. Accession Ames 13179, 13180 and 15718 were used in the general survey of explants and hormonal combinations. Leaf explants from mature plants were incubated on pre-culture medium for 7 days before transfer onto the shoot induction medium (SIM). Observations were made after 4 weeks of incubation on SIM for shoot and root formation, with the percentages of regeneration shown. The hormonal combinations are indicated as abbreviations in the order of auxin/cytokinin. The numbers stand for concentrations in mg L^{-1} and the letters following the numbers for hormones.

N- NAA, B-BA, T-TDZ and Z- zeatin

(A) Shoot/bud regeneration frequency of leaf explants of *L. campestre* accession Ames 13179.

(B) Shoot/bud regeneration frequency of leaf explants of *L. campestre* accession Ames 13180.

(C) Shoot/bud regeneration frequency of leaf explants of *L. campestre* accession Ames 15718.

(D) Root regeneration frequencies of leaf explants of *L. campestre* accession Ames 13179, 13180 and 15718.

In contrast to the low frequency of shoot development, leaf explants showed a high frequency of root development (Figure 9D and 10D). For accession Ames 13179, eight hormonal combinations resulted in root development in more than 90% of the explants. Among the hormonal combinations 1.0 NAA/1BA, 1.0 NAA/2BA, 1.0 NAA/4BA, 0.1 NAA/1Z and 1.0 NAA/2Z had 100% root development frequency (Figure 9D).

After one-week incubation on the pre-culture medium, leaf explants started expanding (Figure 10A) and developed compact green calli (Figure 10B) within four-weeks. Leaf explants were sub-cultured three times for every 2-3 weeks. Although the shoots increased in size, the morphology for most of them remained abnormal in that the leaf blades did not expand and leaves appeared succulent and slender-shaped (Figure 10E). During this period, the calli grew but few additional shoots developed (Figure 10C). Following three subcultures on SIM, to further determine whether it was possible to generate any more shoots, explants having greenish compact calli as shown in Figure 10B were each divided into four equal pieces, and placed onto the following four media: (1) SIM medium containing 3.0 mgL^{-1} TDZ and 0.1 mgL^{-1} NAA, (2) low cytokinin SIM (0.3 mg L^{-1} TDZ and 0.1 mg L^{-1} BA with 0.1 mgL^{-1} NAA) in SIM, (3) low auxin SIM (0.01 mg L^{-1} NAA with 3.0 mg L^{-1} TDZ or 2 mg L^{-1} BA) in SIM, and (4) hormone-free SIM. No additional shoot regeneration was observed on any of the media.

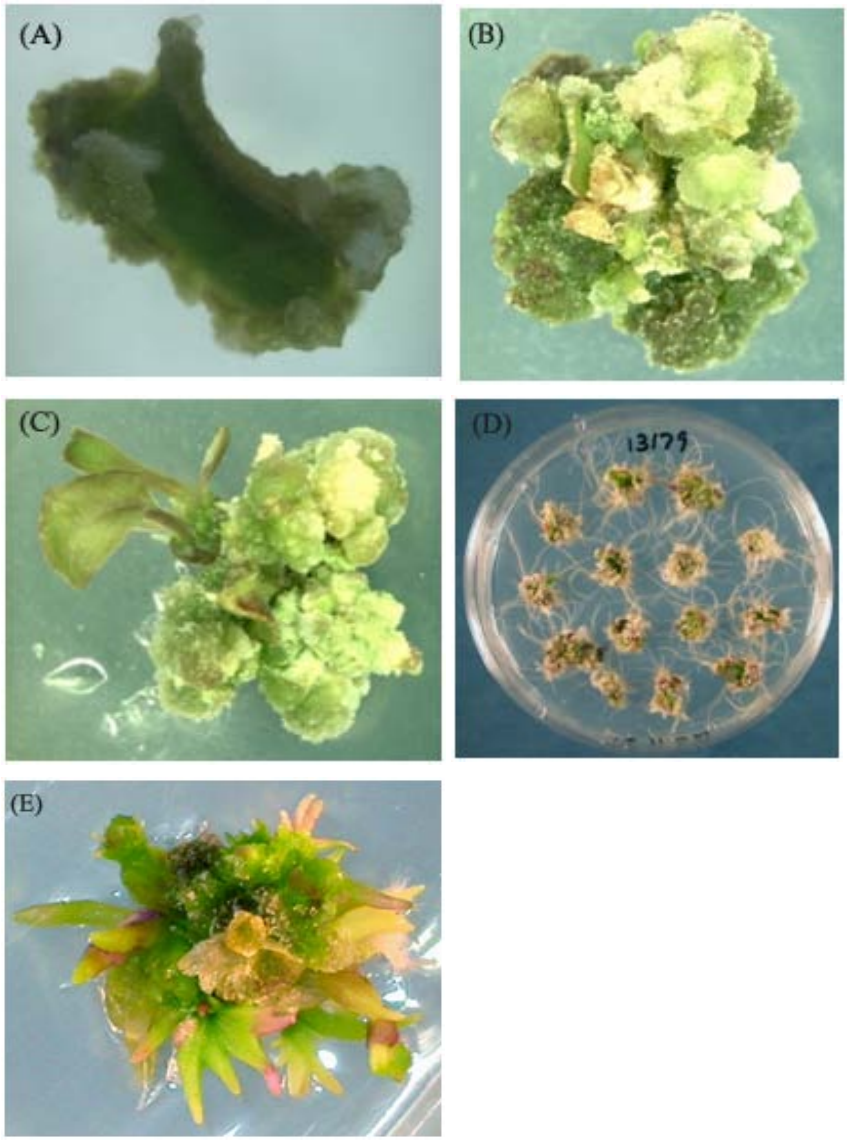


Figure 10. Shoot regeneration from leaf explants of field pepperweed. (A), (B) and (C) are from accession 15718 while (D) and (E) are from accession 13179.

(A) One week-old leaf explant on pre-culture media, showing callus growth around the cut edges of the explant.

(B) Four week-old leaf explant showing green compact calli, which was the common callus type for leaf explants.

(C) Occasionally shoots developed from leaf explants on shoot induction medium containing 1.0 mg L^{-1} zeatin and 0.1 mg L^{-1} NAA .

(D) Leaf explants on 0.1 mg L^{-1} NAA and 2 mg L^{-1} BA produced more roots than shoots.

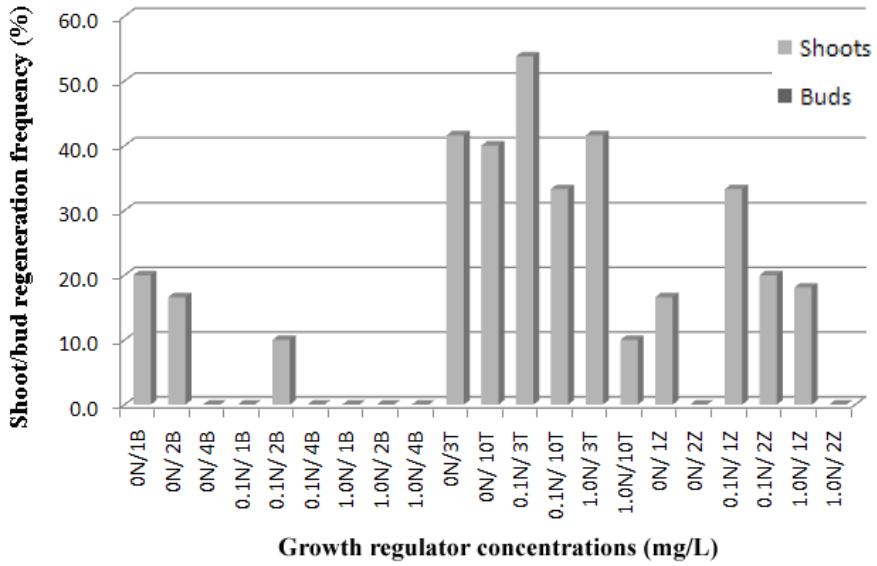
(E) Abnormal shoot development from leaf explants. Shoots developed from leaf explants were generally succulent and slender-shaped compared to shoots from hypocotyl explants.

4.1.2 Hypocotyl explants

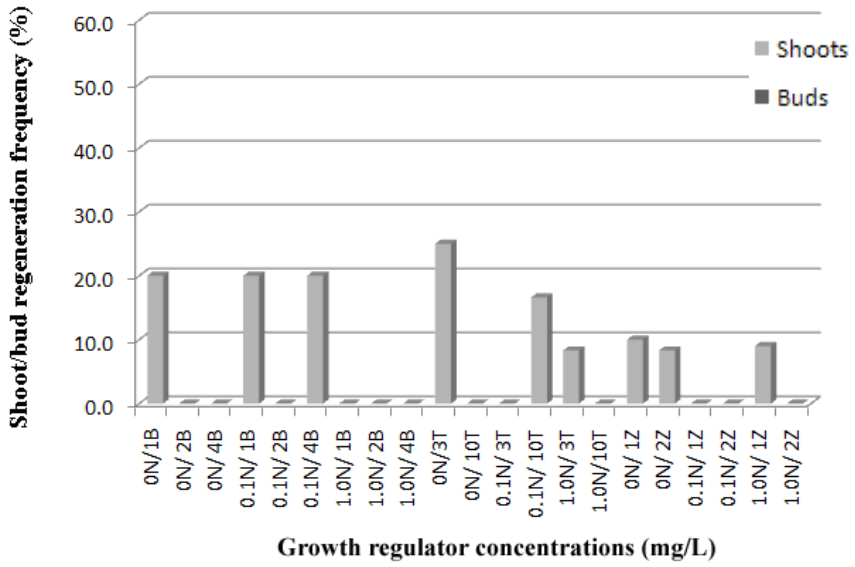
Before the transfer to SIM combinations, all hypocotyl explants were placed on pre-culture medium for 7 days. Once on SIM, there was slight swelling of the explants, and then the growth of small green calli was observed at the both cut ends. By contrast, for leaf explants callus growth covered most of the explant. Among the three genotypes, the accession Ames 13179, as in the case of leaf explants, showed a strong response for shoot regeneration (Figure 11A). For this accession, the hormonal combinations using NAA and TDZ resulted in the higer frequencies of shoot induction (Figure 11A). Among six hormonal combinations using NAA and TDZ, five of them had a shoot-to-explant ratio of over 30%, with the combination of 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ showing 50% of shoot regeneration (Figure 11A and 12).

The frequency of shoots was lower for accession Ames 13180 and was even worse for the accession Ames 15718 (Figures 11B and 11C). Root development from the hypocotyl explants of all accessions was much less frequent in general compared to leaf explants (Figure 11D vs 9D). Compared to leaf explants, hypocotyl explants produced less callus, and when shoots were generated, they were not succulent and leaves showing similar morphology to the leaves of control plants.

(A)


Shoot/bud regeneration frequency (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(B)


Shoot/bud regeneration frequency (%)
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40.0
30.0
20.0
10.0
0.0
Shoots
Buds
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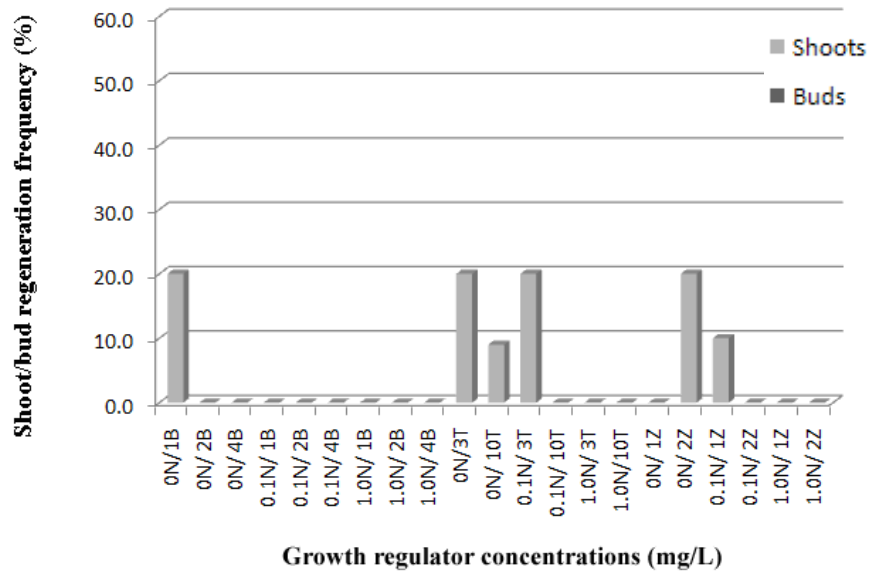
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(C)


Shoot/bud regeneration frequency (%)
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50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(D)

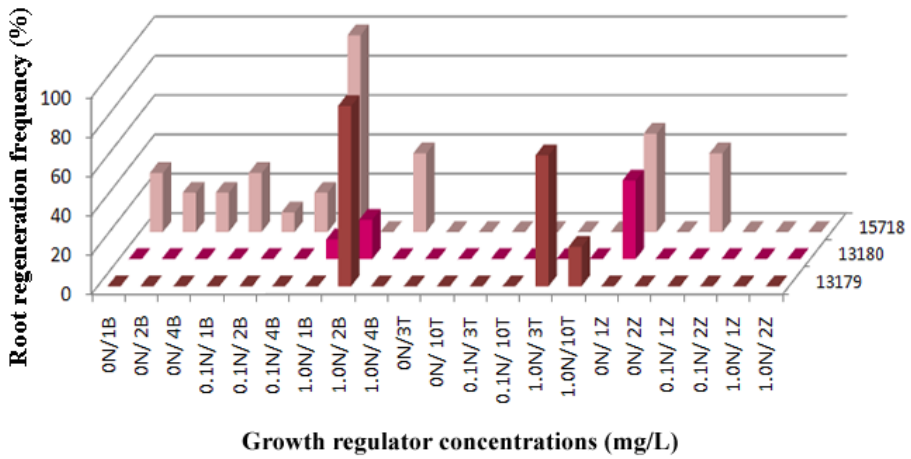


Figure 11. Effects of genotypes and hormonal combinations on shoot and root induction from hypocotyl explants of field pepperweed. Accession Ames 13179, 13180 and 15718 were used in the general survey of explants and hormonal combinations. Hypocotyl explants from 7-day old seedlings were incubated on pre-culture medium for 7 days before transfer onto the shoot induction medium (SIM). Observations were made after 4 weeks of incubation on SIM for shoot and root formation, with the percentages of regeneration shown. The hormonal combinations are indicated as abbreviations in the order of auxin/cytokinin. The numbers stand for concentrations in mg L^{-1} and the letters following the numbers for hormones.
N- NAA, B-BA, T-TDZ and Z- zeatin

(A) Shoot/bud regeneration frequency of hypocotyl explants of *L. campestre* accession Ames 13179.

(B) Shoot/bud regeneration frequency of hypocotyl explants of *L. campestre* accession Ames 13180.

(C) Shoot/bud regeneration frequency of hypocotyl explants of *L. campestre* accession Ames 15718.

(D) Root regeneration frequencies of hypocotyl explants of *L. campestre* accessions Ames 13179, 13180 and 15718.

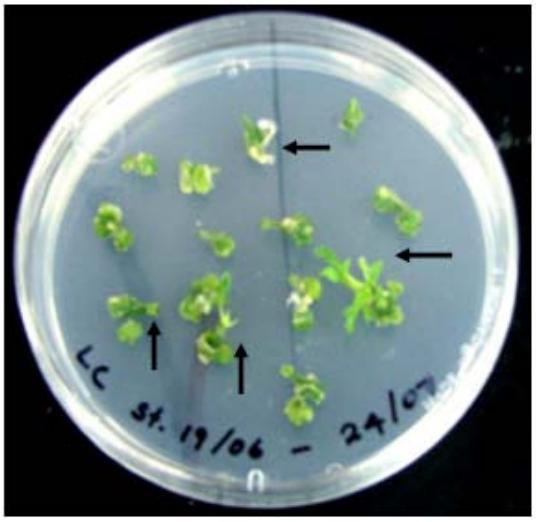
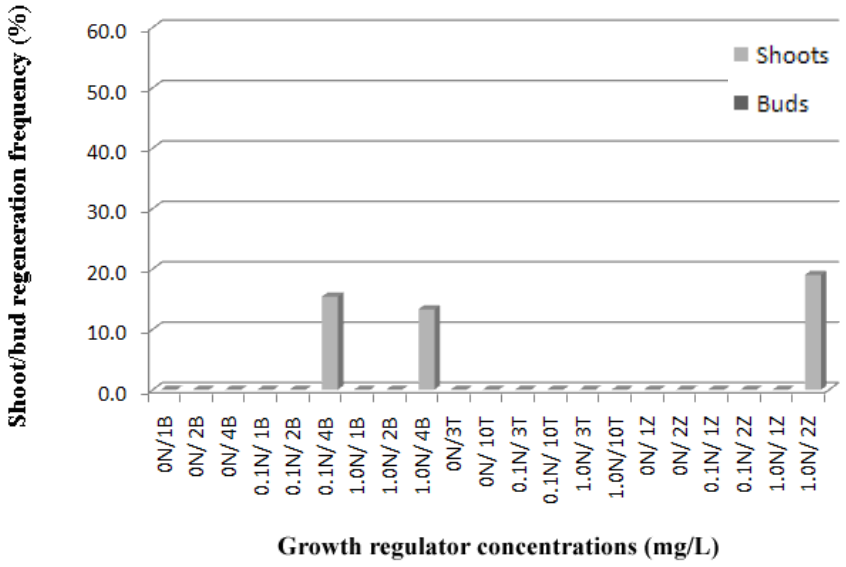


Figure 12. Shoot regeneration of hypocotyl explants from accession Ames 13179 of field pepperweed. Hypocotyl explants were pre-cultured for seven days and transferred to shoot induction medium (SIM) containing 3.0 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA. The photo was taken after four weeks on SIM, showing a high frequency of shoot regeneration.

4.1.3 Cotyledonary petiole explants

There was only rare occurrence of bud or shoot development from cotyledonary petiole explants under any hormonal combination (Figure 13A to C). The accessions Ames 13179 and 15718 showed a low frequency of bud development under 3 hormonal combinations (Figure 13A and B) while the accession 13180 showed bud/shoot development only under 1 hormonal combination (Figure 13C). The low frequency of bud/shoot development suggests that cotyledonary petiole is not a suitable explant for shoot regeneration from field pepperweed under the conditions tested. In addition, root development was also infrequent for cotyledonary petiole explants (Figure 13D).

(A)


Shoot/bud regeneration frequency (%)
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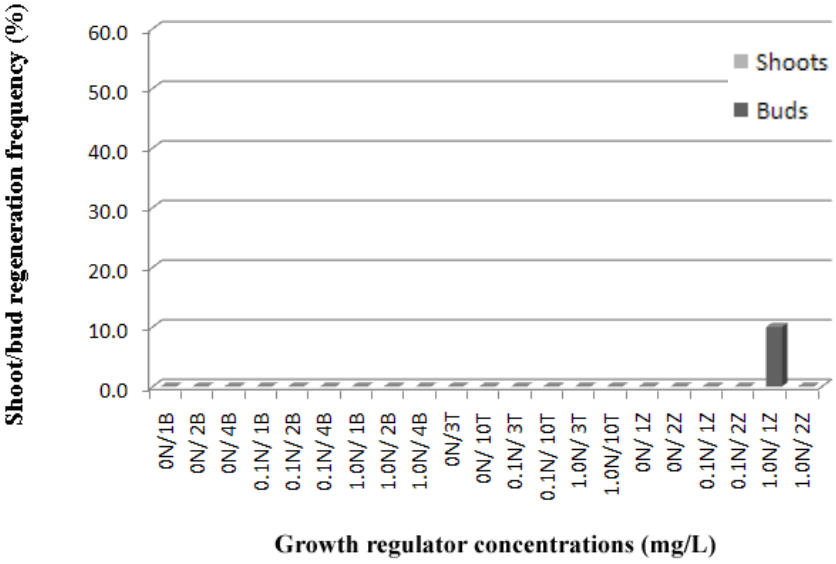
50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(B)


Shoot/bud regeneration frequency (%)
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50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
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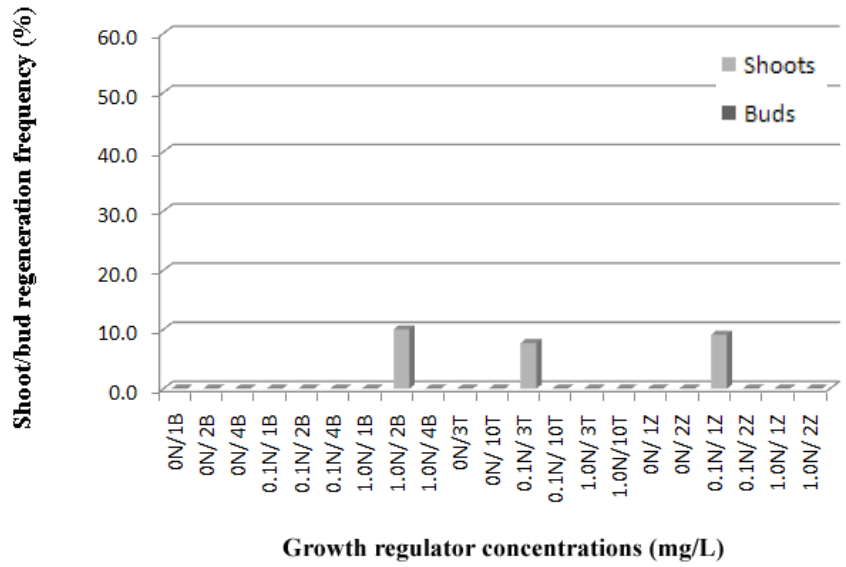
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(C)


Shoot/bud regeneration frequency (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Shoots
Buds
0N/1B
0N/ 2B
0N/ 4B
0.1N/ 1B
0.1N/ 2B
0.1N/ 4B
1.0N/ 1B
1.0N/ 2B
1.0N/ 4B
0N/3T
0N/ 10T
0.1N/ 3T
0.1N/ 10T
1.0N/ 3T
1.0N/10T
0N/ 1Z
0N/ 2Z
0.1N/ 1Z
0.1N/ 2Z
1.0N/ 1Z
1.0N/ 2Z
Growth regulator concentrations (mg/L)

(D)

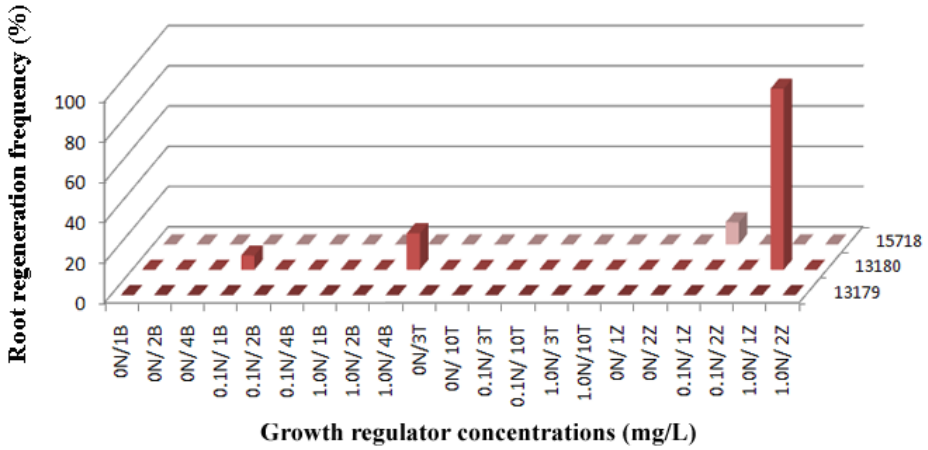


Figure 13. Effects of genotypes and hormonal combinations on shoot and root induction from cotyledonary petiole explants of field pepperweed. Accession Ames 13179, 13180 and 15718 were used in the general survey of explants and hormonal combinations. Cotyledonary petiole explants from 7-day old seedlings were incubated on pre-culture medium for 7 days before transfer onto the shoot induction medium (SIM). Observations were made after 4 weeks of incubation on SIM for shoot and root formation, with the percentages of regeneration shown. The hormonal combinations are indicated as abbreviations in the order of auxin/cytokinin. The numbers stand for concentrations in mg L^{-1} and the letters following the numbers for hormones.

N- NAA, B-BA, T-TDZ and Z- zeatin

(A) Shoot/bud regeneration frequency of cotyledonary petiole explants of *L. campestre* accession Ames 13179.

(B) Shoot/bud regeneration frequency of cotyledonary petiole explants of *L. campestre* accession Ames 13180.

(C) Shoot/bud regeneration frequency of cotyledonary petiole explants of *L. campestre* accession Ames 15718.

(D) Root regeneration frequencies of cotyledonary petiole explants of *L. campestre* accessions Ames 13179, 13180 and 15718.

The experiments described above were designed to determine conditions that could facilitate shoot regeneration from explants of field pepperweed. Several general conclusions could be drawn from the data. First, the accession Ames 13179 has a strong potential for shoot regeneration. Second, hypocotyl explants in general have a higher frequency of shoot regeneration. Third, the combinations of low concentration of NAA (0 or 0.1 mg L^{-1} NAA) with TDZ had a higher chance of shoot regeneration, particularly with accession Ames 13179. These data provided a good lead for further testing the hormone combinations and concentrations for shoot induction of accession Ames 13179.

4.2 Optimization of shoot induction from hypocotyl explants

From the above experiments, the highest responsive conditions for shoot induction were as follows: hypocotyl explants of accession Ames 13179 cultured on SIM containing 0.1 mg L^{-1} NAA/3.0 mg L^{-1} TDZ (Figure 12). Two tests using these conditions were thus performed to verify the observation. Although shoot regeneration was observed, but the frequency was lower than the 50% observed previously. It was hypothesized that this variation might be in part due to the differential regenerability in different regions of the hypocotyl. Accordingly, experiments were performed using three accessions to determine whether different segments of the hypocotyl explants differed in their ability to regenerate shoots.

The hypocotyl explants were cut into three pieces: top acropetal segment (segment 1), middle segment (segment 2) and bottom basipetal segment (segment 3), as shown in Figure 7. Each segment was then cut into smaller pieces. After pre-culture on pre-culturing media, the explants were transferred onto SIM containing 0.1 mg L^{-1} NAA/3.0 mg L^{-1} TDZ. As shown in Figure 14 and 15A, the acropetal segments (segment 1) for the three genotypes showed a higher degree of shoot induction than the other two segments in terms of both the frequency

of explants with shoots and the average number of shoots per explant (Figure 14 and Figure 15B). Among the three accessions, once again accession Ames 13179 showed the highest frequency of regeneration. The difference among the three genotypes appeared to be less for the first segment, with all of them having a frequency of more than 65% for the explant with shoots (Figure 15A). The frequency of root regeneration was relatively low for all the three segments (Figure 15B).

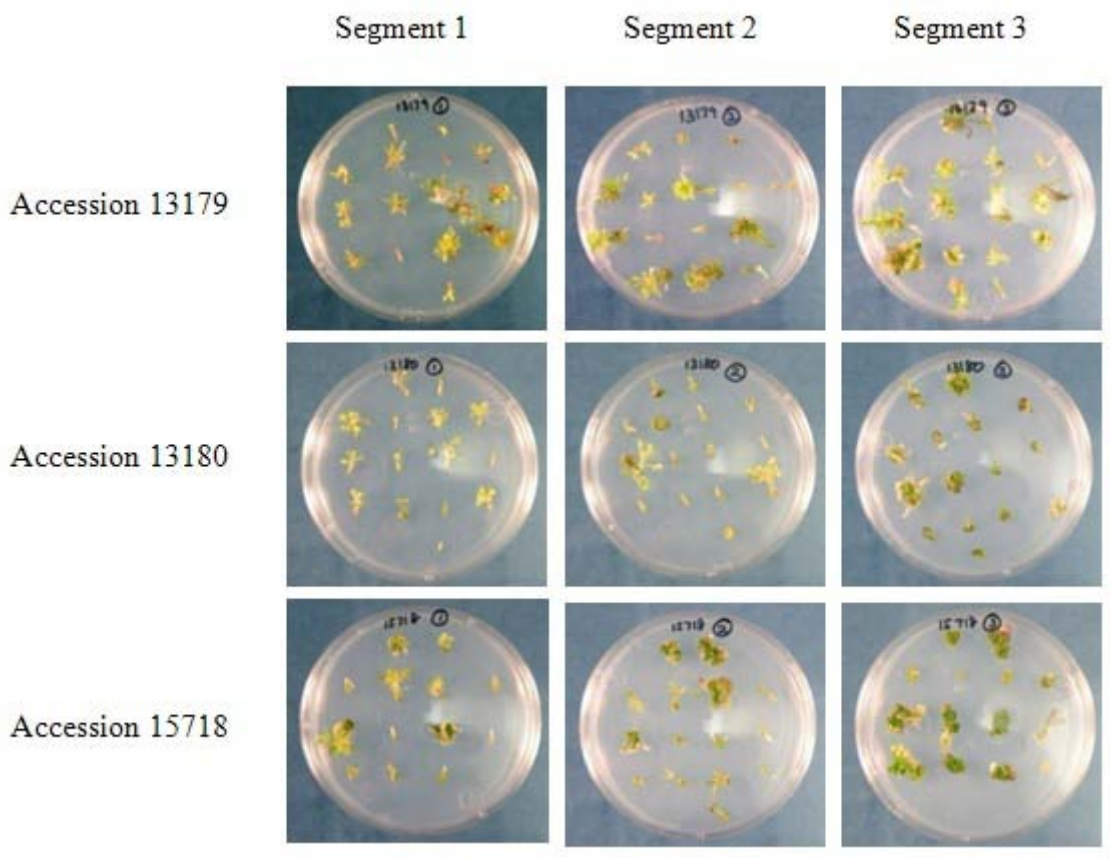
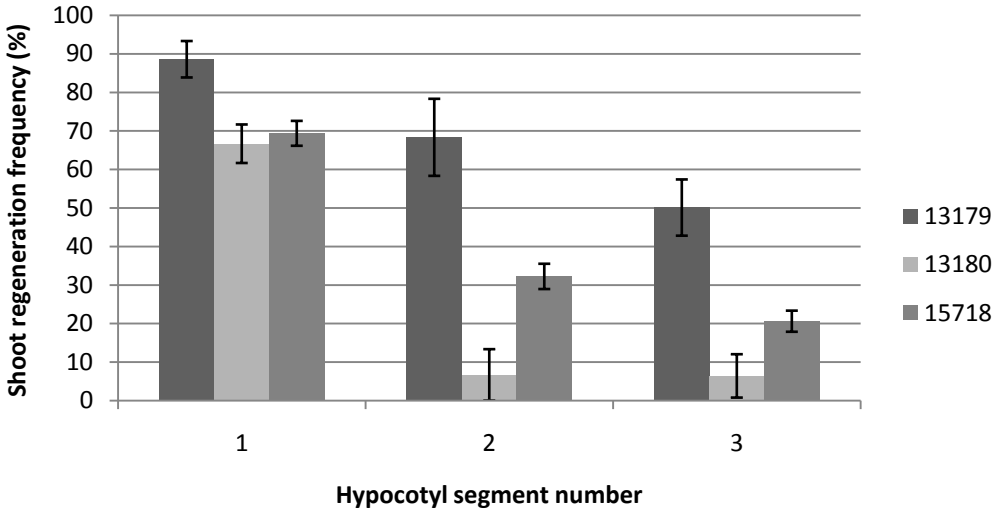


Figure 14. Shoot regeneration from hypocotyl explants of accessions Ames 13179, 13180 and 15718 on shoot induction medium containing 3.0 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA. Hypocotyls of accessions 13179, 13180 and 15718, shown from the top to bottom row, were cut into segments 1, 2 and 3. After pre-culture, the explants were placed on the shoot induction medium, and photos were taken after 6 weeks of incubation.

All the explants taken from the acropetal portion of the hypocotyl developed shoots directly from the explant tissue without much callus growth, and we will refer to these as adventitious shoots from here on. In contrast, explants from the basipetal portion of the hypocotyl (segment 3) usually developed large mass of green compact callus. During shoot regeneration, buds which do not have well developed leaf morphology and a “base” or stem-like structure appeared first. The development of a typical adventitious shoot is briefly described here. Under a dissecting microscope, adventitious bud formation could be observed 14 days after incubation on SIM. Adventitious buds appeared first as “nodule”-like structures with light green glossy surface (Figure 16A and 16B). They developed into multiple shoots (Figure 16C). With further development, multiple leaves were formed and these leaves were attached to the same base (Figure 16D). The number of shoots continued to increase after four weeks. Although most of shoots on hypocotyl explants developed without much callus growth (Figure 16B), some explants of segment 1 and 2 did have callus development and shoot regeneration at both ends of the explants (Figure 17A), and the callus mass was smaller compared to calli developed on explants of segment 3. Occasionally, shoots developed in the middle of the explant where wounding or crack occurred due to the use of forceps during transfers (Figure 17B).

(A)



(B)

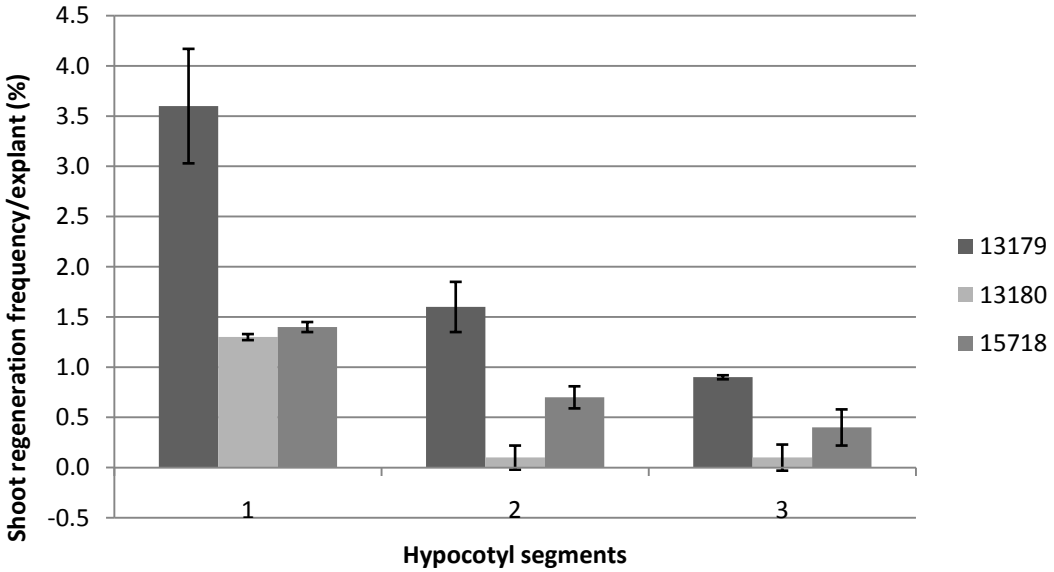


Figure 15. Shoot regeneration frequencies of acropetal, middle and basipetal segments of hypocotyl explants of field pepperweed. The hypocotyl explants of three accessions (shown at the right of graphs) were cut into the top acropetal segment (segment 1), middle segment (segment 2) and bottom basipetal segment (segment 3), and cultured separately on shoot induction medium containing 3.0 mg L^{-1} TDZ/0.1 mg L^{-1} NAA (mg L^{-1}). After four weeks, the percentages of shoot regeneration and root regeneration were obtained. Standard errors based on two experiments are shown above the graph bars.
(A) Shoot regeneration
(B) Shoot regeneration per explant

Figure 16. Shoot regeneration from hypocotyl explants of accession 13179 of field pepperweed. Hypocotyl explants were harvested from the acropetal end of seven day-old seedlings. Explants were pre-cultured for seven days and transferred to shoot induction medium containing 3.0 mg L^{-1} TDZ/0.1 mg L^{-1} NAA. After 14 days, shoot regeneration had started.
(A) A hypocotyl explant showing adventitious shoots with callus development.
(B) A hypocotyl explant showing adventitious shoots without much callus development.
(C) A hypocotyl explant showing multiple shoot development.
(D) A hypocotyl explant showing multiple shoots with many leaflets.

The above observations confirm that the hypocotyl is a good source of explants and furthermore the acropetal segment of the hypocotyl possesses the greatest potential for shoot and root induction. Subsequently further studies were conducted to optimize hormone concentrations for shoot induction using accession Ames 13179 and explants taken from the acropetal section of the hypocotyl segment 1. The accession 13179 was selected based on the frequency of shoot regeneration from the hypocotyl explants and the consideration that it has a good level of glucoraphanin (about 165.5 µg FWg^{-1}; Bandara *et al*., 2008).

The previous results showed that the hormonal combination of 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ was good for shoot regeneration of *L. campestre*. To better define the optimum concentrations of plant growth regulators, smaller increments around 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ were selected, resulting in 16 NAA/TDZ combinations (Figure 18). After pre-culture, the explants were transferred onto SIM containing the corresponding NAA/TDZ concentrations (Figure 18A). In general, combinations containing 3.0 mg L^{-1} TDZ produced a higher percentage of shoot regeneration than the other three concentrations of TDZ, with the frequency of explants having shoot development ranging from 41% to 83% (Figure 18A). The 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ combination resulted in the highest frequency of explants with shoots. These results are consistent with the observation from earlier experiments, confirming that 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ combination is a good hormonal combination for shoot regeneration. The average shoot number per explant for this plant growth regulator combination was also one of the highest among the sixteen hormonal combinations (Figure 18B). Despite the wide variation in shoot development frequency of explants among the hormonal combinations, all the NAA/TDZ hormone combinations evaluated were able to induce shoot regeneration from explants taken from the acropetal section of the hypocotyl (segment 1) explants, indicating that this hormone

combination is favorable for shoot induction. Based on these results, SIM containing 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ used on acropetal hypocotyl explants of segment 1 would be used in future experiments.

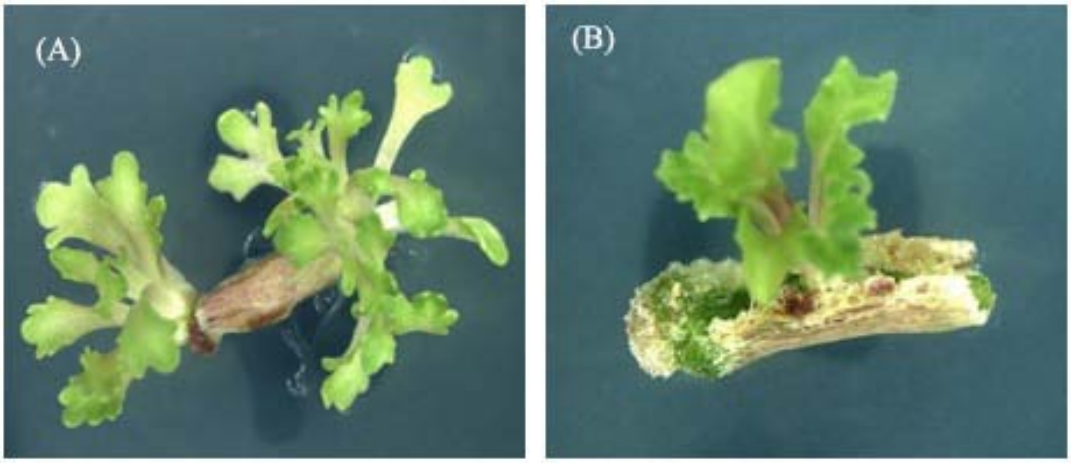


Figure 17. Shoot development on hypocotyl explants of accession Ames 13179 of field pepperweed. Hypocotyl explants from acropetal ends of the seedlings were pre-cultured and then transferred to SIM containing 3.0 mg L^{-1} TDZ / 0.1 mg L^{-1} NAA. Three week-old hypocotyl explants are shown here.
(A) Usually multiple shoots were developed from the ends of the hypocotyl explant.
(B) Occasionally some shoots developed at the middle of the explant that was wounded or cracked with the forceps during transfers.

4.3 Shoot elongation

After 3 weeks on SIM containing 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ, regenerating shoots were transferred to SEM containing basic MS/B5 medium plus 0.5 mg L^{-1} BA and 1.5 mg L^{-1} GA_3. As described above, most of the hypocotyl explants taken from segment 1 produced multiple shoots. These explants were transferred to SEM without being separated. The shoots were allowed to grow and develop further for 3-4 weeks. In general, shoots grew larger, but stems were shorter. Leaves grew larger and exhibited a shape typical of field pepperweed.

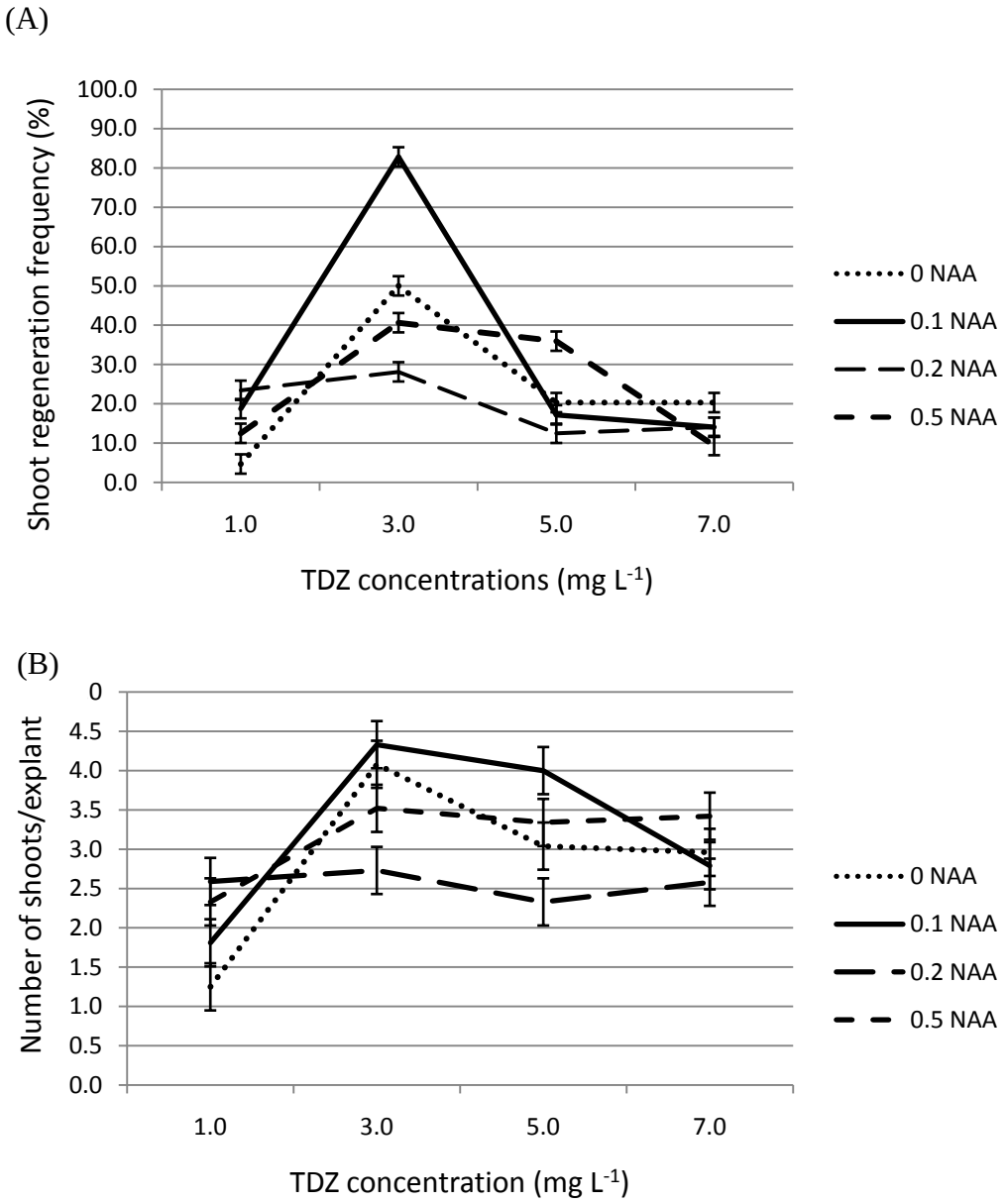


Figure 18. Further optimization of hormone concentrations for shoot induction from hypocotyl explants of field pepperweed. Hypocotyl explants from acropetal ends of seedlings were tested on shoot induction medium containing 0, 0.1, 0.2 and 0.5 mg L^{-1} NAA and 1.0, 3.0, 5.0 and 7.0 mg L^{-1} TDZ. For each treatment, four plates each containing 16 explants were used Standard errors are shown on the graph lines.

(A) Effect of TDZ and NAA hormonal combinations on shoot regeneration

(B) Average number of shoots per explant.

4.4 Rooting

After about 4 weeks on SEM, individual shoots were transferred to RIM containing basic MS/B5 media plus 0.5 mg L^{-1} indole-3-butyric acid. Fourteen days later small roots became visible. Usually, after 4-5 weeks on RIM, the root system had developed and at this stage plantlets were ready for transfer to soil (Figure 19)

Figure 19. Plantlet of field pepperweed with a fully developed root system on root induction medium (RIM). Shoots were transferred from shoot elongation medium to RIM and maintained on RIM for four weeks.

4.5 Optimizing kanamycin concentration for transoformation of field pepperweed.

The second major objective of this study was to establish a plant transformation protocol for field pepperweed, utilizing the established regeneration system. For this purpose, several factors, including conditions for the selectable marker, co-cultivation conditions for *Agrobacterium*-mediated transformation and analysis of putative transformants were examined.

Since the vectors in this study used kanamycin as a selective agent, the effect of kanamycin on shoot regeneration of hypocotyl segment 1 explants was determined. Segment 1 hypocotyl explants of field pepperweed accession Ames 13179 were pre-cultured for four days, before the explants were co-incubated with *Agrobacterium* solution. After co-cultivation, (usually two days unless specified), the explants were transferred onto SIM containing 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} TDZ, plus various concentrations of kanamycin (0, 20, 25 or 30 mg L^{-1}) as well as 300 mg L^{-1} timentin (Figure 20).

Shoot regeneration was monitored over the next 8 weeks. Without kanamycin, over 75% of explants produced shoots for both the control (without *Agrobacterium*) and the treatment with *Agrobacterium* harboring the plant expression vector pHS723 (Figure 21). With 20 mg L^{-1} or more kanamycin, there was a dramatic decrease in the frequency of explants with shoots (the explants were gradually bleached and died), in the control there was still a portion of explants capable of producing shoots, representing the frequency of shoots which could grow at the particular concentration of kanamycin without being transformed. However, treatment with pHS723-carrying *Agrobacterium* produced had a higher frequency of explants with shoots at each of the kanamycin concentrations (Figure 21). It appears that at 20 mg L^{-1} kanamycin there was a larger difference between the *Agrobacterium* treated explants and the control, and also the frequency of explants with shoots for the *Agrobacterium* treatment was higher than that at higher concentrations of kanamycin, the 20 mg L^{-1} kanamycin was considered to be a good balance in terms of shoot regeneration frequency and probability of transformation and thus was used in SIM for all subsequent transformation studies. However, in SEM, 25 mg L^{-1} kanamycin concentration was used to reduce the number of escapes.

4. 6 Co-cultivation

Co-cultivation is another important factor for explant-based *Agrobacterium*-mediated transformation. Explants were pre-cultured on the medium containing 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} kinetin for 4 days and then they were inoculated with *A. tumefaciens* and co-cultivated for 0, 2, 4 or 6 days, to determine the optimal length of the co-cultivation period. Following co-cultivation, the explants were transferred onto SIM modified as described above. After 3 weeks on SIM, for 0-day co-cultivation treatment, young shoots were visible whereas for other treatments the development of shoots was delayed. However with time, shoots of 0-day co-cultivation control gradually became pale in color and died due to the continued exposure to the 20 mg L^{-1} kanamycin. After 8 weeks on SIM (including one subculture), the highest frequency of shoot regeneration (number of shoots/number of explants) was observed with 2-day co-cultivation treatment among the four treatments tested (Figure 22). Based on this result, 2-day co-cultivation period was selected for subsequent plant transformation experiments.

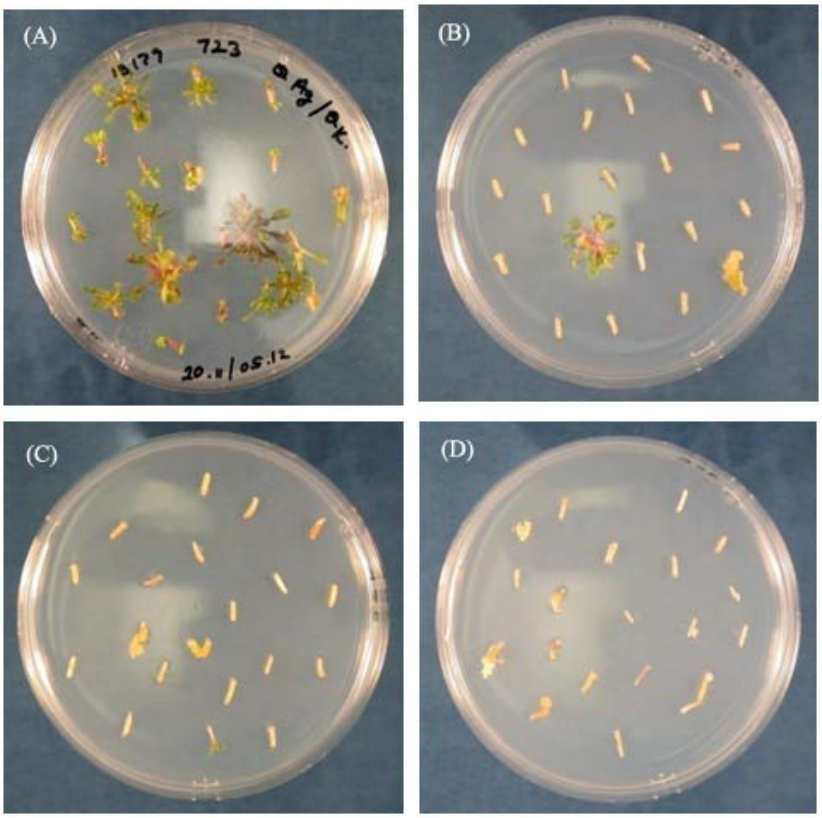


Figure 20. Shoot induction from hypocotyl explants of accession Ames 13179 of field pepperweed on different concentrations of kanamycin. Hypocotyl explants (segment 1) were inoculated with *Agrobacterium* containing the plasmid pHS723. After inoculation, explants were co-cultivated for three days and transferred to SIM with different kanamycin concentrations. Following co-cultivation, the explants were transferred onto shoot induction medium. The photos were taken after 8 weeks on shoot induction medium.
(A) Hypocotyl explants on 0 mg L^{-1} kanamycin.
(B) Explants on 20 mg L^{-1} kanamycin.
(C) Explants on 25 mg L^{-1} kanamycin.
(D) Explants on 30 mg L^{-1} kanamycin.

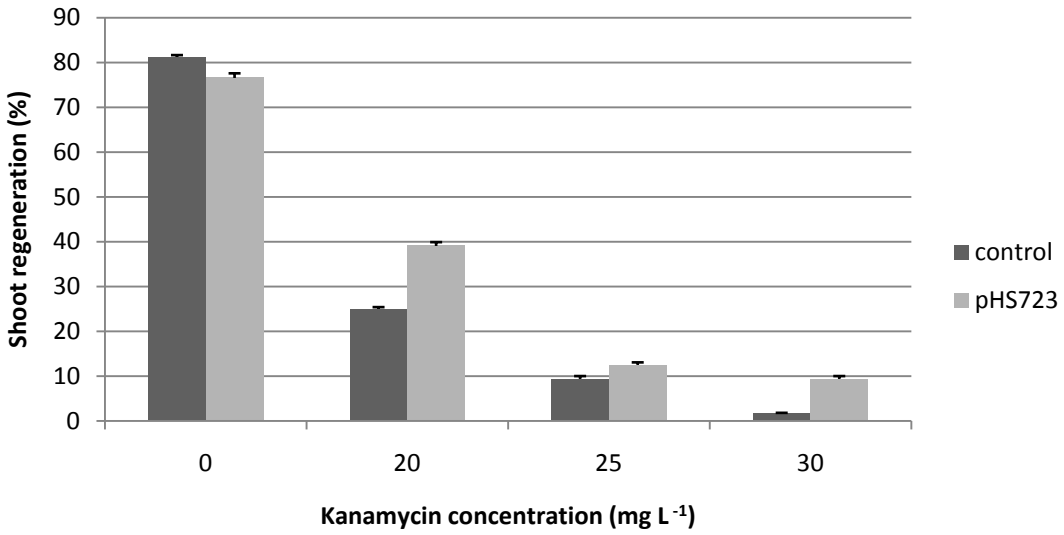


Figure 21. Effect of of kanamycin concentration on shoot regeneration frequency of the hypocotyl explants of field pepperweed accession Ames 13179. Explants were pre-cultured, and then were either infected with pHS712-containing *Agrobacterium* or not inoculated. They were transferred onto SIM and sub-cultured once. The frequency of explants with shoots was determined eight weeks after the transfer to SIM. For each treatment, four plates each containing 16 explants were used. Standard errors are shown on each of the graph bars.

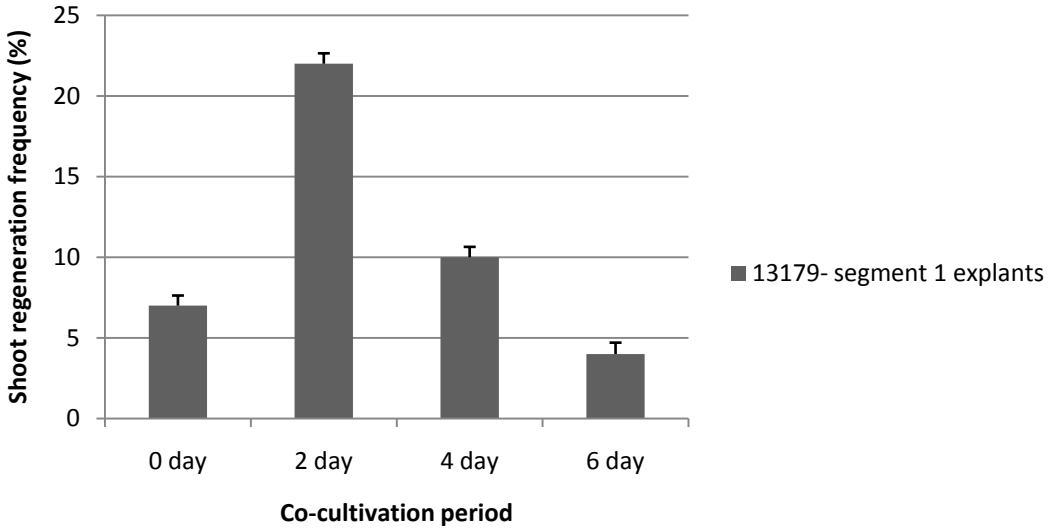


Figure 22. Effect of co-cultivation period on shoot regeneration frequency of the hypocotyl explants of accession Ames13179 of field pepperweed. *Agrobacterium* containing plasmid pHS723 were used to inoculate hypocotyl explants. Inoculated explants were co-cultivated for 0, 2, 4 or 6 days. They were then transferred to SIM containing 3.0 mg L^{-1} TDZ/0.1 mg L^{-1} NAA and 20 mgL^{-1} kanamycin. After 8 weeks culture on SIM, the frequency of shoot regeneration was recorded. For each treatment, four plates each containing 16 explants were used. Standard errors are shown on each of the graph bars.

In the previous experiment, the plasmid pHS723 was used to establish the optimum kanamycin concentration and co-cultivation period. The final objective for this study was to down-regulate the cytochrome P450 gene involved in glucosinalbin biosynthesis. Accordingly, an RNAi construct (S25C) based on the vector pBBI-PI-ISH (Zhang *et al*., 2006a) was prepared. The construct S25C contains the sense and anti-sense fragments of a field pepperweed P450 gene named *LcCYP79B2* (X. Shi and H. Wang, unpublished data). The construct was compared with plasmid pHS723 for kanamycin selection. Conditions were similar to those described above. The hypocotyl explants of field pepperweed accession Ames 13179 were inoculated with *A. tumefaciens* strain (GV3101::pMP90) harboring either the

pHS723 or S25C. Following 2-days of co-cultivation, the explants were transferred to SIM containing 300 mg L^{-1} timentin and either 20 or 25 mg L^{-1} kanamycin. The frequency of explants with shoots was determined after 8 weeks on SIM (including one subculture). As shown in Figure 23, the co-cultivation with SC25 appeared to produce a slightly higher frequency of explants with shoots than plasmid pHS723 at both kanamycin concentrations. Since the frequency was higher at 20 mg L^{-1} for both constructs, this concentration was selected for future transformation with the *LcCYP79B2* RNAi construct S25C.

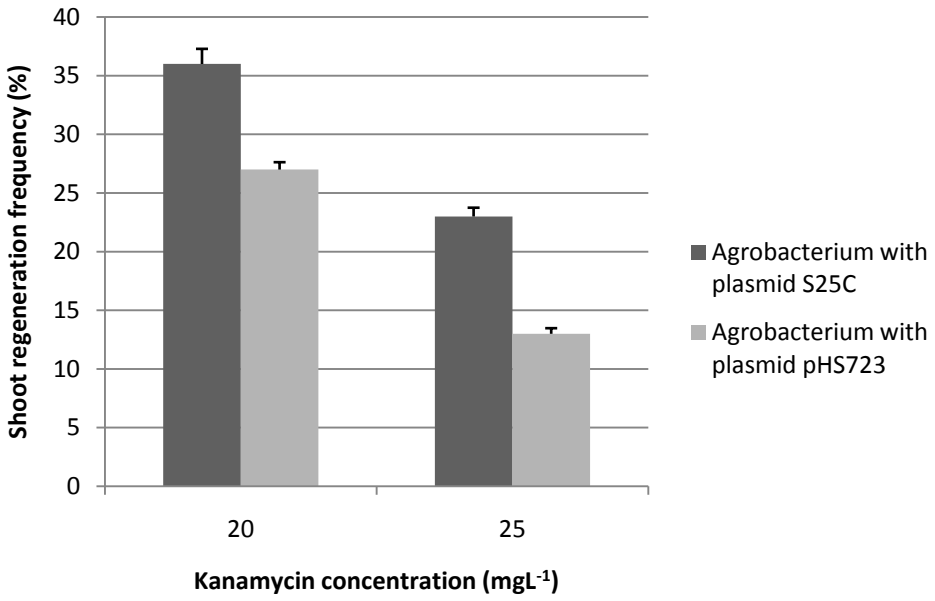


Figure 23. Comparison of two constructs on shoot regeneration of field pepperweed accession Ames13179 in the presence of kanamycin. Hypocotyl explants were inculated with *Agrobacterium* harboring plasmid pHS723 or S25C, co-cultivated for 2 days and then cultured on shoot induction medium containing 20 or 25 mg L^{-1} for 8 weeks. The percentage of shoot regeneration was based on 4 plates, each having 16 explants. Standard errors are shown on each of the graph bars.

4.7. Analysis of putative transformants

4.7.1 Plant transformation

Transformation of field pepperweed was carried out with the *LcCYP79B2* RNAi construct S25C for explants taken from the acropetal end of the hypocotyls of accession Ames 13179. Hypocotyl explants were pre-cultured for 4 days, and then placed on sterile filter paper overlying the co-cultivation medium for 2 days. Co-cultivation of explants on filter paper helped reduce over-growth by *Agrobacterium* and associated tissue necrosis, thereby improving the transformation frequency. Co-cultivated explants were then transferred onto SIM containing 20 mg L^{-1} kanamycin and 300 mg L^{-1} timentin. After about 8 weeks culturing on SIM green shoots appeared at the cut ends of the hypocotyl explants. Most of the time, they grew in a small cluster consisting of 3-5 shoots of varying sizes. Shoots continued to form up through 10 weeks on SIM.

For selection of transformed explants, 20 mg L^{-1} kanamycin was used in SIM, allowing recovery of more putatively transformed shoots than if a higher level of kanamycin (25 mg L^{-1}) was used. All shoots able to grow in the presence of 20 mg L^{-1} kanamycin were transferred (at about 10 weeks) to SEM containing 25 mg L^{-1} kanamycin. Shoots developing in the presence of kanamycin had lighter green color than shoots without kanamycin. Non-transformed shoots gradually became pale in color and died. Based on results of this study, it was concluded that reliable selection could be achieved through a long exposure (4 weeks or more) of transformed explants to 25 mg L^{-1} kanamycin on SEM. After 4-6 weeks dark green elongated shoots were transferred to RIM from SEM. With a further 2-3 weeks on RIM, a normal root system had developed.

4.7.2. GUS assay

In the experiments to determine conditions for plant transformation, the plasmid pHS723 was used, which contain a GUS (β-glucuronidase) reporter. GUS histochemical staining was thus used to determine whether the kanamycin-resistant seedlings were true transformants. In the initial analysis, 15% of the seedlings that were "transformed" based on kanamycin selection, process the GUS positive. GUS activity was not detected in any of seedlings from the control without co-cultivation with pHS723-containing *A. tumefaciens*, demonstrating that a certain percentage of the kanamycin-resistant seedlings from pHS723 treatment were true transformants. Usually, the entire leave of the transformant showed GUS activity with intense blue color (Figure 24).

(A) (B)

Figure 24. Histochemical analysis of leaves of field pepperweed plants transformed with the plasmid pHS723.
(A) Beta-glucuronidase positive leaf from a transformed plantlet.
(B) Control leaf of field pepperweed.

4.7.3 Confirmation of transformants by polymerase chain reaction (PCR)

Since no GUS marker was present in the kanamycin-resistant seedlings produced from the *LcCYP79B2* RNAi construct, PCR was used to verify the presence of the transgene. Leaves of green seedlings that survived the kanamycin selection were selected for DNA extraction. Thirty-three seedlings were subjected to the analysis. PCR was carried out to amplify the *NPTII* gene present in the plasmid vector. Among the 33 seedlings analyzed, 29 showed the presence of the *NPTII* gene (Figure 25). If the initial number of explants used was taken as the base, transformation frequency was about 11%. Transgenic plantlets were transferred directly to soil (Figure 26).

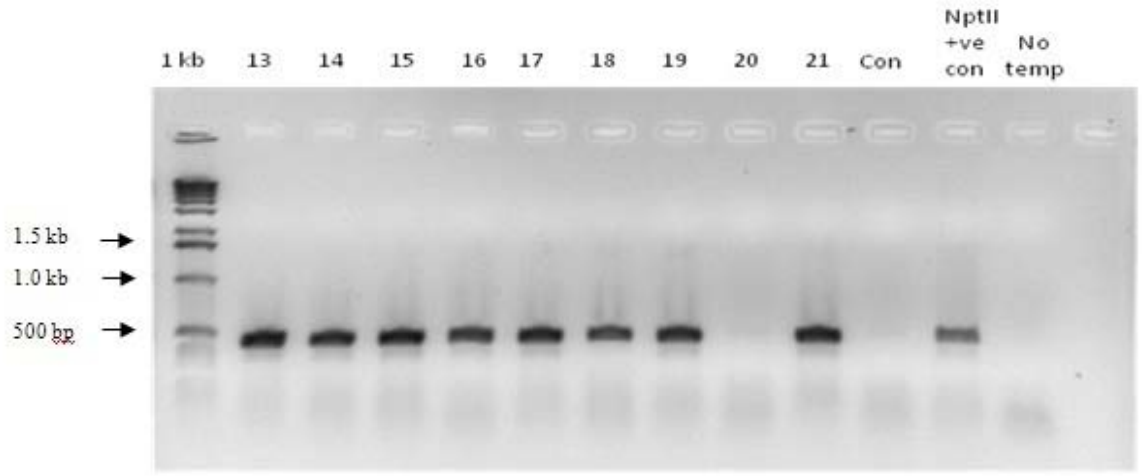


Figure 25. PCR (polymerase chain reaction) analysis of kanamycin-resistant field pepperweed plants. DNA was extracted from leaf samples of plantlets on rooting media containing 25 mg L^{-1} kanamycin. PCR reaction was carried out with primers specific for *NPTII* to amplify a fragment of about 500 base pairs. Control DNA of field pepperweed (wt) and a plasmid containing *NPTII* gene was used as a positive control (NptII +ve con) and ddH_2O (No temp) were used as negative control. Amplified PCR products were subjected to electrophoresis using 1.2% agarose gel. Results from sample numbers 13 to 21 are shown here.

Field pepperweed transformants confirmed by PCR were grown in pots in a growth chamber held at 25 ± 2 °C with a 16 h/ 8 h (light/dark) photoperiod. The plants appeared morphologically similar to the control plants (Figure 26).

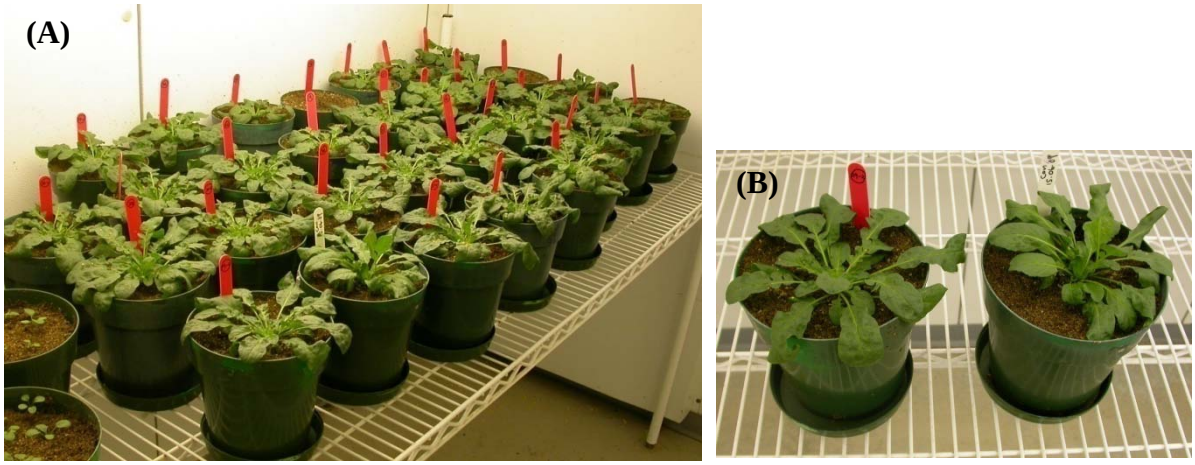


Figure 26. Transformed plants of field pepperweed.
(A) Transformed plants of field pepperweed were grown in a plant growth chamber.
(B) Phenotype of transformed (left) and control (right) plants.

Therefore, based on results of the present study, procedures for plant regeneration and transformation of field pepperweed using accession 13179 have been established. The main conditions used are summarized in Figure 26.

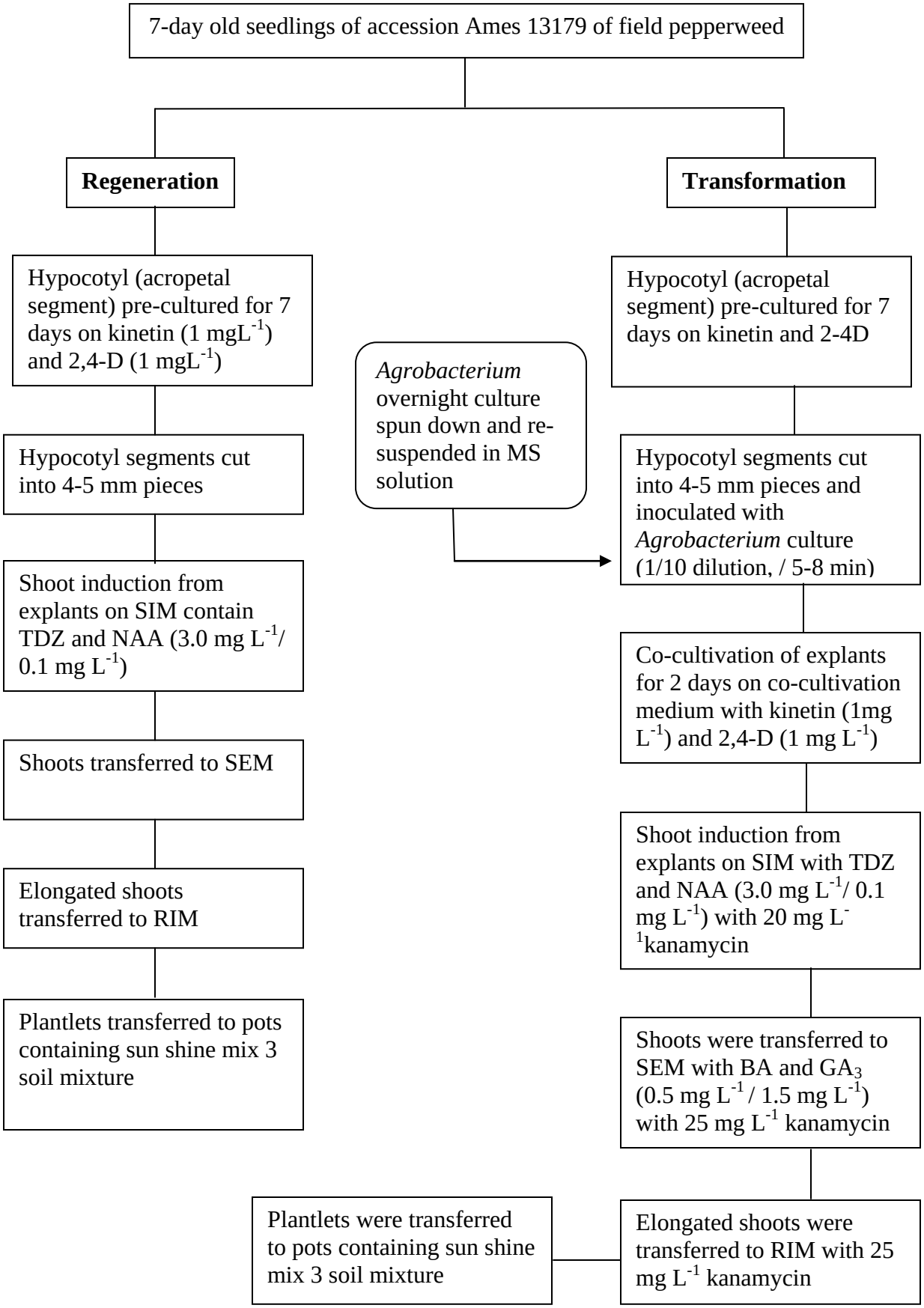

7-day old seedlings of accession Ames 13179 of field pepperweed
Regeneration
Transformation
Hypocotyl (acropetal segment) pre-cultured for 7 days on kinetin (1 mgL-1) and 2,4-D (1 mgL-1)
Hypocotyl segments cut into 4-5 mm pieces
Shoot induction from explants on SIM contain TDZ and NAA (3.0 mg L-1/ 0.1 mg L-1)
Shoots transferred to SEM
Elongated shoots transferred to RIM
Plantlets transferred to pots containing sun shine mix 3 soil mixture
Agrobacterium overnight culture spun down and re-suspended in MS solution
Hypocotyl (acropetal segment) pre-cultured for 7 days on kinetin and 2-4D
Hypocotyl segments cut into 4-5 mm pieces and inoculated with Agrobacterium culture (1/10 dilution, / 5-8 min)
Co-cultivation of explants for 2 days on co-cultivation medium with kinetin (1mg L-1) and 2,4-D (1 mg L-1)
Shoot induction from explants on SIM with TDZ and NAA (3.0 mg L-1/ 0.1 mg L-1) with 20 mg L-1kanamycin
Shoots were transferred to SEM with BA and GA3 (0.5 mg L-1 / 1.5 mg L-1) with 25 mg L-1 kanamycin
Elongated shoots were transferred to RIM with 25 mg L-1 kanamycin
Plantlets were transferred to pots containing sun shine mix 3 soil mixture

Figure 27. Summary of the plant regeneration and transformation procedures developed in this study for field pepperweed accession Ames 13179. Plant regeneration uses acropetal hypocotyl explants. After pre-culture, the explants are cultured sequentially on shoot induction (SIM), shoot elongation (SEM) and root induction (RIM) media as shown. For plant transformation, acropetal hypocotyl explants are inoculated and then co-cultivated with *Agrobacterium*. The transformed plants are then cultured sequentially for shoot and root regeneration as for plant regeneration.

5.0. DISCUSSION

Glucoraphanin which is one of the major glucosinolates present in field pepperweed, is the precursor of sulforaphane. Sulforaphane is a well known inducer of phase 2 enzymes. The incentive for initiating this study was the long-term goal to develop and improve field pepperweed as a vegetable crop plant. Plant tissue culture techniques could be used to manipulate plant secondary products. For that purpose, it is very important to establish a reliable regeneration and *Agrobacterium*-mediated transformation system for field pepperweed. This study presents the first report of plant regeneration from and plant transformation of field pepperweed. In this study, various explants and auxin/cytokinin combinations were evaluated to optimize shoot/root regeneration.

It has been widely known that genotype is one of the major factors affecting plant regeneration. Ono *et al.* (1994) evaluated 100 cultivars of *B. napus* for shoot regeneration using cotyledonary explants and reported wide variation in shoot regeneration frequency ranging from 0% to 97% among the 100 cultivars tested. In another study, *B. napus* cultivar GSL-1 showed better regeneration efficiency than cultivar Westar (Phogat *et al.*, 2000). Pental *et al*. (1993) tested hypocotyl explants of twelve cultivars of *B. juncea* from different regions of the world for their *in vitro* regeneration and found that cultivars of Indian origin were more responsive than the cultivars of CIS (Commonwealth of Independent States) and Australian origin. Zhang *et al.*, (1998) also observed large variation (from 0% to 95%) in regeneration frequency among 123 genotypes of Chinese cabbage (*B. campestris* L. ssp. pekinensis). In the present study regeneration rates of three different genotypes of field pepperweed (accessions Ames 13179, 13180 and 15718) were tested for a range of hormonal concentrations. It was found that accession Ames 13179 was the most responsive in terms of shoot regeneration,

likely due to genetic differences. The field pepperweed accession Ames 13179 also has a higher biomass production than Ames 13180 and Ames 15718 and also a good level of glucoraphanin (165.5 µg FWg^{-1}) (Bandara *et al*., 2008). A high level of biomass production is desirable if the plant is to be developed as a nutritional vegentable crop as the source of glucoraphanin.

The frequency of shoot regeneration of field pepperweed was found to be higher from hypocotyl explants than from cotyledonary petiole and leaf explants. This result was similar to that reported by Pande *et al.* (2002) who compared hypocotyl segments to leaf explants, shoot apices, nodal segments, cotyledonary leaves and radicles of *L. sativum* and observed higher shoot regeneration rates in hypocotyl explants. Similarly, several other authors obtained the highest frequencies for shoot regeneration from hypocotyl explants of *Brassica* species (Lazzeri and Dunwell, 1986; Jonoubi *et al*., 2005; Munshi *et al*., 2007; Ravanfar *et al*., 2009). Curuk *et al.* (2002) observed rapid *in vitro* regeneration from the proximal zone of the hypocotyl of melon (*Cucumis melo* L.). The reason for this greater frequency of regeneration may be due to the presence of more young undifferentiated or less differentiated cells in the proximal zone as well as accumulation of naturally- occurring growth regulators in this zone. When the acropetal segment of hypocotyl explants were exposed to hormones in the SIM, due to the presence of more young undifferentiated cells, they have a stronger ability to redirect their developmental program thereby producing new shoot apical meristems.

During plant regeneration through organogenesis, in general the first objective is to induce shoots from explants, since shoot regeneration is often more difficult to achieve than root regeneration. It was observed in the present study that leaf explants of field pepperweed tended to produce more roots compared to hypocotyl and cotyledonary petiole explants, making the leaf a less ideal source of explants for this species. Since the presence of auxin

seems to be necessary for root induction (Trigiano and Gray, 2005), a higher frequency of root formation from leaf explants may be associated with a higher endogenous auxin level (George, 1993). Also, the uptake of auxin from the medium might have been enhanced by wounding. Dunwell (1981) reported that root production in three *Brassica* spp (*B. oleracea, B. campestris* and *B.* napus) from leaf explants depended upon the size of the explants, with larger explants (> 3 mm) having more roots. In addition, among the growth regulator combinations tested, NAA and TDZ combinations seem to have the lower root regeneration frequencies on the shoot induction media for leaf explants. This observation may be due to the inhibitory effect by TDZ on root formation (George 1993).

The combination of NAA and TDZ has been reported by different authors as important for shoot regeneration in many plant species (Suezawa *et al*., 1988; Fasolo *et al.,* 1989; Matsuta and Hirabayashi, 1989; Leblay, *et al*., 1991). Guo *et al*. (2005) reported 5.37 µM NAA with 4.54 µM TDZ as the best combination for shoot regeneration (52.4%) from leaf explants of *Brassica juncea* var. tsatsai. They also observed that a higher concentration (11.35 µM) of TDZ dramatically decreased the shoot induction frequency.

Thidiazuron (TDZ), a substituted phenylurea (N-phenyl-1, 2, 3 thidiazol – 5- yl-urea) is a potent regulator of *in vitro* morphogenesis (Murthy *et al*, 1998). The presence of TDZ, either alone or in combination with other growth regulators, is important for shoot organogenesis in a wide variety of plant species, including several woody plants (Huetteman and Preece, 1993), peanut (*Arachis hypogaea* L.) (Matand and Prakash, 2007), herbaceous medicinal plants (Murch *et al.*, 2000; Liu *et al.*, 2003), pigeon pea (*Cajanus cajan* L. Millsp) (Singh *et al.,* 2003) and *Brassica napus* (Jonoubi *et al.,* 2005). TDZ has been found to be more effective than 6-benzyladenine (BA) in the promotion of shoot proliferation (Kern and Meyer, 1986; Dennis, 2003). Studies by Capelle *et al.* (1983) have suggested that TDZ promotes the

conversion of cytokinin ribonucleotides to the biologically more active ribonucleosides in the callus tissue of *Phaseolus lunatus* L. Victor *et al*., (1999) have observed that the addition of TDZ into the media for peanut embryogenic culture increased the levels of endogenous adenine, adenosine, dihydrozeatin and zeatin, and decreased the endogenous level of 2iP. Due to the inhibitory role of TDZ on cytokinin oxidase (Hare and van Staden, 1994), TDZ can modify the cytokinin biosynthetic pathway (Victor *et al*., 1999). Also, TDZ has the ability to increase the levels of purines which are required for maintaining rapid cell growth and divisions in plant regeneration (Victor *et al*., 1999), as well as the level of auxin (Hutchinson *et al*., 1996). In addition, TDZ may affect regeneration frequency by altering the levels of abscisic acid (Li and Yang, 1988), ethylene (Yip and Yang, 1986) and proline (Murch and Saxena, 1997).

This study has established a tissue culture protocol for efficient shoot organogenesis and plant regeneration from hypocotyl explants of field pepperweed. *In vitro* regeneration frequency and the number of shoots produced by each regenerant are important parameters in determining the success of transformation experiments. Based on present studies, some key conditions have been identified for shoot regeneration, including SIM containing 3 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA and the use of acropetal portion of the hypocotyl as explants. Under such conditions, the hypocotyl explants of accession Ames 13179 showed a frequency of over 50% shoot regeneration. Therefore the procedure established here offers a reliable plant regeneration system for applications such as *Agrobacterium*-mediated transformation.

Based on the successful use of *Agrobacterium*-mediated transformation in many plant species including the *Brassica* species, experiments were conducted to establish an *Agrobacterium*-mediated transformation protocol for field pepperweed. The plant regeneration system established from this study was utilized in this transformation process, with hypocotyls

as the explants. Hypocotyl explants have been reported to be highly sensitive to co-cultivation with *Agrobacterium* and tend to become necrotic during subsequent cultivation (Cardoza and Stewart, 2003). This difficulty can be overcome by pre-cultivation of the plant material on callus induction medium (Cardoza and Stewart, 2003) or by delaying the antibiotic selection step (Ovesná *et al.*, 1993). Pre-conditioning explants before *Agrobacterium* inoculation and co-cultivation can also increase the cell competence for *Agrobacterium* transformation (Radke *et al*., 1988, Misra, 1990; Ovesná *et al.*, 1993) and DNA integration through increased DNA synthesis and cell divisions (Kartzke *et al*., 1990). However, Janssens *et al.* (1995) reported that pre-conditioning of *Brassica napus* explants with 2,4-D induced excessive callus growth during the shoot regeneration phase and also increased the frequency of transformants with multiple copies of the transgene. In the present study, a pre-culturing period of 4 days was used and this resulted in no significant overgrowth of the callus. This is likely because the particular combination of NAA/TDZ was selected based on the evaluation of many auxin/cytokinin combinations and the hypocotyl explants on this hormonal combination produced only a minimal amount of callus. The period of co-cultivation can also make a significant difference in the transformation efficiency. A co-cultivation period of 48 h gave high transformation efficiency for *B. napus,* while co-cultivation for 72 h resulted in necrosis (Cardoza and Stewart, 2003). However, Zhang *et al.* (2000) reported a 72 h co-cultivation period to be optimal for Chinese cabbage (*B. campestris)*. For *B. oleracea* 2-day co-cultivation was the optimal period for cotyledon and hypocotyl transformation, while longer periods such as 4, 5 and 7 days resulted in tissue necrosis and lower shoot regeneration (Bhalla and Smith, 1998). Results of the present study suggest that 2-day co-cultivation for *Agrobacterium* inoculation is optimal among the different periods tested for field pepperweed.

Various antibiotic resistance genes have been used as selectable markers in the production of transgenic plants (Yoder and Goldsbrough, 1994). The *NPTII* gene is one of the most commonly used selectable markers for many plant species (Fraley *et al*., 1986), including *Brassica* plants (Christey and Sinclair, 1992; Cardoza and Stewart, 2003; Jonoubi *et al*., 2005; Bhalla and Singh, 2008; Zhang *et al*., 2006b). Inclusion of an appropriate level of the selection agent (kanamycin) into the shoot induction medium and the media used for any subsequent developmental stages is needed to select out putative transgenic shoots. Transformed tissues remained green while untransformed tissues gradually become pale and died due to exposure to the kanamycin. (Radke *et al*., 1988). For field pepperweed, under current experimental conditions, 25 mg L^{-1} kanamycin reduced the shoot regeneration frequency of field pepperweed. Therefore, 20 mg L^{-1} kanamycin was used in SIM. Since some untransformed shoots could also survive the 20 mgL^{-1} kanamycin selection, it is important that once the explants develop shoots they are transferred onto fresh medium every 2-3 weeks to maintain the selection pressure. In addition, 25 mg L^{-1} kanamycin was used with lower sucrose concentrations in SEM and RIM to reduce the frequency of untransformed shoots (escapes). Usually, the kanamycin resistant shoots remained green and developed a root system whereas non-transformed shoots did not produce roots in the root induction medium.

The plasmid pHS723 which was used to optimize the transformation conditions for field pepperweed contains a *GUS* reporter gene. It was thus easy to screen the putative transformants using PCR and GUS assays. After optimizing the transformation conditions the plasmid S25C was used. Since the plasmid S25C does not contain the *GUS* reporter gene, plantlets resistant to 25 mg L^{-1} kanamycin and showing root development on the root induction medium were screened to identify transformed field pepperweed plantlets by a PCR assay. Twenty-nine out of thirty-three plantlets tested for *npt II* gene were positive, suggesting

that a high proportion of the plantlets surviving the kanymycin selection were true transformants.

Due to time limitations, it was not possible to characterize the transformed plants further. In further studies, we need to analyze whether the *LcCYP79B2* gene targeted by the RNAi construct is down-regulated. For instance, RT-PCR could be used to screen for plants the transformed for a reduced level of *LcCYP79B2* expression. Transformed plants with clearly reduced *LcCYP79B2* expression could then be subjected to glucosinolate analysis. Glucosinolates can be extracted from leaf tissues (preferably at several different stages) of wild type plant, transformed plants with a vector alone, and transformed plants with a reduced level of LcCYP79B2. The amount of glucosinalbin can be determined as described with a known sample. Although this is an exciting long term goal, it was not possible to conduct all the work within the time frame of a master thesis research.

In summary, an efficient system for regeneration of field pepperweed has been established for field pepperweed using the accession 13179 as indicated in Figure 27. Although the regeneration procedure was optimized based on field pepperweed accession 13179, these conditions can be the basis for developing regeneration procedures for other genotypes, if a particular genotype needs to be used. Similarly, a procedure was succesfully established for plant transformation of field pepperweed based on the established plant regeneration procedure (Figure 27). The regeneration and transformation procedures developed from hypocotyl explants of field pepperweed should provide one of the fundamental tools essential for molecular biology studies of this species. In the future, the methods developed here should be useful in efforts to convert field pepperweed into a glucoraphanin-rich green vegetable crop free of anti-nutritional constituents such as glucosinalbin.

6.0 References

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