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Improved resistance to insects in maize (*Zea mays* L.) and cowpea (*Vigna unguiculata* L.)

Jingqin Mao

Thesis submitted to the
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Abstract

A novel alteration in secondary metabolism of maize transformed with wheat oxalate oxidase gene (OXO) was determined using HPLC and microscopy. Phenolic concentration was significantly increased, but DIMBOA synthesis was down - regulated. The high levels of soluble phenolic acids, in particular free ferulic acid, most likely contributed to the insect resistance in the OXO maize. To facilitate future cowpea transformation with the OXO, a stable *in vitro* regeneration system was established in blackeye cowpea via shoot organogenesis. The optimal initiation medium comprised MS salts, B_5 vitamins, 2 mg l^{-1} BAP, 3% sucrose and 0.8% agar at pH 5.8. The highest initiation frequency and shoot number were obtained from the shoot apices of 3 - 5 days old seedlings. For shoot elongation, 0.5 - 5.0 mg l^{-1} GA_3 was required. Rooting medium was MS salts supplemented with B_5 vitamins, 3% sucrose and 0.8% agar.

Résumé

Une nouvelle altération dans le métabolisme secondaire du maïs transformé avec le gène oxalate oxydase (OXO) du blé a été déterminé par HPLC et par des études microscopiques. Le contenu phénolique était significativement augmenté, mais la synthèse de DIMBOA était "down-régulée". Les hauts niveaux en acides phénoliques solubles, en particulier l'acide férulique libre, contribuait favorablement à la résistance du maïs OXO contre les insectes. Pour faciliter la transformation future du dolique à œil noir par le gène OXO, un système stable de régénération *in vitro* a été développé par l'organogenèse des plantules. Le milieu initiateur optimal comprenait des sels MS, des vitamines V_5, 2 mg l^{-1} de BAP, 3% de sucrose et 0,8% d'agar à un pH de 5,8. La plus haute fréquence d'initiation et le plus grand nombre de pousse étaient obtenus à partir des apex des pousses âgées de 3-5 jours. Pour l'élongation des plantules, 0,5-5,0 mg l^{-1} de GA_3 était nécessaire. Le milieu propice au développement des racines comprenait des sels MS supplémentés avec des vitamines V_5, 3% de sucrose et 0,8% d'agar.

Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
BM	Basal Medium from Pellegrineschi et al. (1997)
B_5	Medium from Gamborg et al.(1968)
Bt	*Bacillus thuringiensis* gene
CRSP	Collaborative Research Support Program
DFA	Diferulic acid
DIMBOA	2, 4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one acid
ECB	European corn borer, *Ostrinia nubilalis*
DW	Dry weight
EtoAc	Ethyl acetate
2, 4-D	2, 4-Dichlorophenoxyacetic acid
FAO	Food and Agriculture Organization of the United Nations
FA	Ferulic acids
GA	Gibberellic acid
gl - OXO	Germen like oxalate oxidase
GLP	Germin-like protein
GM	Genetic modification
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid

IBA	Indole-3-butyric acid
IITA	International Institute of Tropical Agriculture
ISAAA	International Service for the Acquisition of Agri-Biotech Applications
IPM	Integrated Pest Management
KN	Kinetin
MBOA	6-Methoxybenzoxazolin-3-one acid
ROS	Reactive oxygen species
MS	Murashige and Skoog (1962)
NAA	1-Naphthalene acetic acid
NGICA	Network for the Genetic Improvement of Cowpea for Africa
OXO	Oxalate oxidase
PAL	phenylalanine ammonia-lyase
PLG	Plant growth regulators
2, 4, 5-T	2, 4, 5-Trichlorophenoxy acetic acid
2, 4, 5-TP	2, 4, 5-Trichlorophenoxy propionic acid
ZN	Zeatin

Chapter 1

General introduction

Both maize (*Zea mays* L.) and cowpea (*Vigna unguiculata* L.) are important staple foods for millions of people in Africa, Asia and Latin America. Maize is the world's third largest cereal crop after rice and wheat. The Food and Agriculture Organization (FAO) of the United Nations estimates that more than 700,000,000 metric tons of maize was produced globally in 2004. Cowpea, also called black eye bean, black eye pea, southern pea, black-eyed pea and Crowder pea, originates from Sub-Saharan Africa, particularly Kenya. Currently, it is an important crop for people in Western and Central Africa. It is estimated that about two hundred million people consume cowpea daily, and therefore, cowpea is regarded as a staple food of the poor in the developing countries (FAO data, presented in 2001). It is a principal source of dietary protein and a secondary source of carbohydrate. Cowpea can be consumed as different plant parts and at different stages of growing - green leaves, immature snapped pods, green pod seeds and dry mature seeds.

However, the production of the two crops, especially in developing countries, has been far below potential due to major insect damage. Both maize and cowpea are very susceptible to insects and suffer heavily from a wide range of insect pests. In the case of maize, loss of the world's maize production from insects, diseases and other pests accounts for 10-15% of the crop every year (FAO data). For instance, the European corn borer (ECB), *Ostrinia nubilalis* (Lepidoptera: Pyralidae), has been a serious problem to maize production for centuries. Sometimes, the economic losses caused by ECB are as high as 30% in certain areas (Scott and Pollak, 2005). As for the cowpea, a related insect, the cowpea pod borer *Maruca vitrata* (Lepidoptera: Pyralidae) is the main field insect pest. Depending on the location, year and cultivars, the yield reduction can reach as high as 95% (IITA data, presented in 2002).

Developing countries are often resource-poor zones, and farmers cannot afford either insecticides or proper grain storage facilities. They also lack skills for application of dangerous insecticides. Economically and technically, conventional insecticides are not an optimal answer to insect problems. Integrated Pest Management (IPM) of maize and cowpea has been attempted for many years. In the case of cowpea, the following reasons can explain this situation. First, no cowpea line has been identified to possess the necessary major resistance genes to insect pests, and wild species, such as *V. vexillata*, have been found to possess such genes. Second, interspecific crossing between cultivated *V. unguiculata* species and wild *Vigna* species failed due to the abortion of fertilized ovules, and no hybrids were obtained (Barone et al., 1992; Fatokun, 1991). Other IPM cultural practices, e.g. natural enemies, manually picking off insects and botanical insecticides, are either impractical or unavailable in cowpea production.

Genetic modification of maize and cowpea with wheat germin gene oxalate oxidase (OXO) was proposed as a means of providing more effective insect resistance in these important crops without the use of exotic bacterial or arthropod genes. Previous work in our laboratory showed that transgenic maize lines with the OXO gene, which expresses a protein generating hydrogen peroxide (H_2O_2), were highly resistant to the European corn borer (ECB) and were protected from insect damage (Ramputh et al., 2002). The present research was initiated to determine the mode of action of the insect resistance. It is thought that H_2O_2 signals defense processes through the induction of the naturally occurring shikimic acid pathway, leading to the accumulation of phenolic resistance factors. Using the advanced HPLC and fluorescence microscopy facilities, the nature of the induced biochemicals and mechanism of the improved resistance were determined. Cowpea has not yet been transformed with OXO due to the lack of an *in vitro* regeneration system. Therefore, in the second part of the project, a study of *in vitro* regeneration in cowpea *Vigna unguiculata* L. cv. blackeye was conducted. By systematically screening and optimizing the factors affecting plant tissue culture, such as explant sources, medium formulations, pH value and plant growth regulators, a productive *in vitro* regeneration protocol via shoot organogenesis was established. Currently, this protocol is being used in cowpea transformation with wheat OXO gene and *Bacillus thuringiensis* (Bt) gene.

1.1 Biotechnological approach to prevent insect damage

Advanced biotechnology makes it possible to modify metabolic pathways in host plants via introduction of exotic genes that have the potential to improve host plant resistance to insects. Genetic modification in host plants often results in alteration of their secondary compound concentrations. Since the first commercialization of genetically modified (GM) crops, the global production of such crops has increased in area every year. In 2004, total worldwide GM crop area was 81 million hectares, which is 20% higher than it was in 2003. In the U.S., GM maize (Bt/herbicide) accounted for 50% of the total adoption. This number is predicted to increase both in the U.S. and in other countries. South Africa planted approximately 0.5 million hectares of GM crops in 2004, which were mainly maize and another legume crop, soybean (ISAAA data presented in 2004). The benefits of GM crops utilizing the *Bacillus thuringiensis* (Bt) genes in increasing plant resistance and reducing insecticide application have been demonstrated in GM maize, cotton and rice (Cheng et al., 1998; High, et al., 2004; Huang et al., 2005). However, the crystalline insecticidal proteins, which confer resistance to insect pests, come from the spores of the soil bacterium *Bacillus thuringiensis* (Bt). Like any other insecticides, fast multiplying insect generation can theoretically evolve resistance to this Bt toxin (Baxter et al., 2005). Thus, it becomes important to explore alternatives to Bt as resistance mechanisms, so breeders can pyramid successful genes for sustainable crops.

The wheat oxalate oxidase gene was isolated from the genomic clone gf -2.8. It is an enzyme that catalyzes the oxidative breakdown of oxalate into H_2O_2 and carbon dioxide (CO_2) (EC 1.2.3.4) (Dumas et al., 1995):

$$\text{Oxalate} + O_2 \xrightarrow{\text{OXO}} H_2O_2 + 2\,CO_2 \qquad (1)$$

This protein (EC 1.2.3.4) exhibits both enzyme activity and germin properties as an apoplastic glycoprotein and exhibits high water-solubility, protease-resistance, and thermostability (Lane, 2002). Oxalate is regarded as a dynamic source of H_2O_2 in plants. H_2O_2 plays a key role in plant development and defence mechanisms. Recent studies on extra-cellular H_2O_2 production by OXO showed that H_2O_2 is related to cell wall cross-linking reactions, which stiffens cell walls through lignification (Olson and Varner, 1993; Lane et al., 1993; Lane, 1994). It was demonstrated that accumulation of OXO transcripts and protein, and its enzyme activity during wheat seed germination occurred specifically in cells and tissues in which cellular expansion is restricted. These previous results revealed that OXO has a significant role in mediating cell wall modification, in controlling cell wall extensibility and in participating in programmed cell death. It was suggested that the biological function of OXO was to terminate cell growth by local provision of H_2O_2 for cross-linking of cell wall components (Caliskan and Cuming, 1998). Recently, some other biological roles for this glycoprotein have been put forward, including as a defense mechanism to disease (Zhang et al., 1995; Zhou et al., 1998), and insect pests (Ramputh et al., 2002), and as a stress response to heavy metals (Delisle et al., 2001), salts (Hurkman et al., 1994) and drought (Ozturk et al., 2002).

Due to the significant biological function of gl-OXO in plant reaction to various stresses, attention has been paid to plant genetic engineering using the OXO genes. To date, transgenic plants with wheat germin gf-2.8 and barley HvOXO have been demonstrated to possess oxalate oxidase activities and improved resistance to pathogens and insects in the following plants: oilseed rape *(Brassica napus),* (Thompson et al., 1995), tobacco (*Nicotiana tabacum*) (Zaghmout et al., 1997), poplar (Liang et al., 2001), soybean (*Glycine max*) (Donaldson et al., 2001), maize (Ramputh et al., 2002), sunflower (*Helianthus annuus*) (Hu et al., 2003), rice (*Oryza sativa*) (Kachroo et al., 2003), potato (*Solanum tuberosum*) (Turhan, 2005), peanut (*Arachis hypogaea*) (Livingstone et al., 2005) and *Arabidopsis thaliana* (Liang et al., 2005).

Genetic modification of maize and cowpea with wheat OXO gene is proposed as a means of providing an effective alternative for insect resistance to the *Bacillus* *thuringiensis* (Bt) genes. Thus the OXO gene from wheat replaces a bacterial gene, which may be more publicly acceptable and provide a different mechanism of resistance. The germin gene OXO was originally of interest as a protein marker during wheat seed germination (Lane et al., 1993). The extensive investigation on germin and germin-like protein (GLP) led to the discovery of a large germin family. The family members are structurally related but functionally diverse. Their functions have been recognized in enzymatic activity, structural proteins and receptors (Bernier and Berna, 2001). OXO is one of the three different proteins that are involved in the enzymatic activities (Lane et al., 1993). The germins with OXO activity are called germin-like oxalate oxidase (gl-OXO). These genes can be expressed in almost all the plant organs such as seeds, cotyledons, roots, leaves, stems and flowers. A broad range of external stimuli, such as salt and drought stress, fungal infection, insect attack and endogenous auxins as well as germination can regulate their expression. Through GenBank of the National Center for Biotechnology Information (NCBI), 61 germin genes and proteins were found in major cereals, including 18 in wheat, 21 in barley, 19 in rice and 2 in maize (Appendix 1). At present, only wheat gf-2.8 (M63223) and barley HvOXO (L15737) have been demonstrated to be germination-specific genes with oxalate oxidase activity (Lane et al., 1993). Wheat gf-2.8 is a 2.8 kilobase pair fragment from one genomic clone, which encodes a complete sequence for germin mRNA. This germin mRNA has 1075 nucleotides, in which a 603 nucleotide sequence codes for a 201 residue mature protein (Dratewka-Kos et al., 1989) (Appendix 2).

1.2 Host plant resistance and secondary metabolites

The use of insect resistant varieties of maize and cowpea has been suggested to be a cornerstone of IPM methods to protect these crop plants against insects and stabilize grain production. Plants are attacked by a large number of insects during their life cycle. It is estimated that 50% of insect species are phytophagous (herbivorous). Each plant species can be eaten by more than one insect species. For example, insects that can feed on maize plants number as high as 300 different species.

However, plants have evolved a large array of heritable defense systems to protect them from insect pest attack through millions of years of co-evolution with insects. These defense systems include physical and phytochemical factors, which influence insect settling, feeding, oviposition, growth, fecundity, and fertility (Wallin, 2000). Physical factors, i.e. morphological resistance, are related to plant structures, such as thickened cell wall and special tissue shapes like thorns, spines and leaf hair. Phytochemical factors refer to plant secondary metabolites.

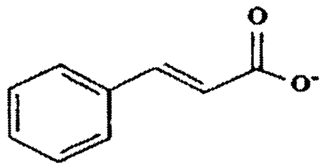
Plant secondary compounds (also called metabolites and natural products) are a class of metabolites in plants. That does not directly function in the processes of plant growth and development. But, plant secondary compounds contribute to their environmental fitness and adaptability. They are responsible for plant flavor, aroma and color, and sources of numerous medicinal, toxic, and industrial chemicals. Defending plants against a variety of herbivores and pathogenic microbes is an important function of the secondary compounds. By screening extracts from various plants against different types of insects, the bioactive compounds contained in selected extracts have been isolated and purified. Thus far, hundreds of thousands of plant secondary compounds have been identified, including phenols, alkaloids, terpenoids, flavonoids, and other minor secondary chemical compounds. Among these compounds, some have been demonstrated to be toxic to insects and serve as plant defenses against many herbivores.

Hydroxamic acids are major compounds in phytochemical defense against insect pests and pathogens in cereals. The defensive role of DIMBOA (2,4 - dihydroxy - 7 - methoxy -1,4 - benzoxazin-3-one) and its degradation product MBOA (6- Methoxybenzoxazolin-3-one acid) is well documented in maize. Generally, the leaf content of DIMBOA and MBOA across maize genotypes was negatively correlated with ECB larval growth and pupal weight (Campos 1989; Reid, 1990, 1991; Bergvinson, 1993). The high concentration in young leaves confers plant resistance to the first - brood ECB, and therefore DIMBOA is known as a defense factor for young leaves (Bergvinson, 1993). Campos (1989) demonstrated that in the diet of the ECB, 0.05 - 0.5 mg l^{-1} fresh weight diet DIMBOA and 0.5 - 4.0 mg l^{-1} MBOA prolonged all ECB developmental stages, and reduced pupal and adult weights. A study showed that DIMBOA acted as a digestive toxin by inhibiting the activity of the insect gut proteolytic enzyme, chymotrypsin (Houseman et al., 1992). The toxic effect of MBOA occurred after digestion. Biosynthesis of DIMBOA in maize has been elucidated recently (Desai et al., 1996; Frey et al., 1997; Rad et al., 2001). DIMBOA is stored in the vacuole as an inactive and less toxic glycoside (Sicker et al., 2000). Following wounding from insect attack, the toxic aglycone DIMBOA and subsequently MBOA are released because a glycosidase is released, which removes the sugar from DIMBOA glycoside. In maize, enzymatic release of DIMBOA is completed in 30 min after insects attack (Rad et al., 2001). Once released, DIMBOA decomposes to MBOA spontaneously.

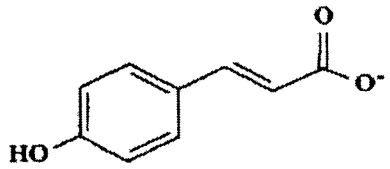
However, high DIMBOA concentration is not always associated with insect resistance in maize. For example, in the case of reduced light intensity, plants became very susceptible to ECB attack although they contain higher levels of DIMBOA (Manuwoto and Scriber, 1985). It was thought that higher levels of nitrogen and nutrients under reduced light treatments became favorable for ECB development. Moreover, DIMBOA could not explain resistance to tropical insects such as sugarcane borer and southwestern corn borer in tropical and subtropical maize varieties (Scriber et al., 1975).

Another important group of phytochemical compounds in plant resistance are the hydroxycinnamic acids, especially in cereals. Their defense function has been attributed to their ability to function as antifeedants and toxins, as well as to their role in leaf toughness (Classen et al., 1990; Bergvinson et al., 1993). The important simple hydroxycinnamic acids include cinnamic acid (CA), *p*-coumaric acid (PA), ferulic acid (FA), and caffeic acid (Fig. 1). They are also called phenylpropanoids because they contain a benzene ring and the three-carbon side-chain. The common biosynthetic pathway of hydroxamic acids and hydroxycinnamic acids in higher plants is the shikimate pathway (Fig. 2). This pathway converts simple primary carbohydrates, phosphoenol pyruvate (PEP) and erythrose - 4-phosphate (E - 4P) derived by plant photosynthesis to three aromatic amino acids: tryptophan, tyrosine, and phenylalanine. The most important step of the pathway is deamination of phenylalanine. The reaction, catalyzed by phenylalanine ammonia lyase (PAL), produces one key hydroxycinnamic acid, cinnamic acid, the precursor of all the plant phenolics. It also represents the point of change from primary metabolism to secondary metabolism.

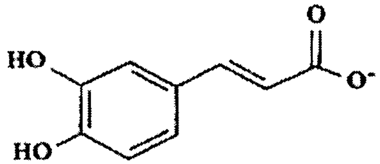
Fig. 1 Four important simple hydroxycinnamic acids and the structural relationship with shikimic acid, phenylalanine and anthranilic acid



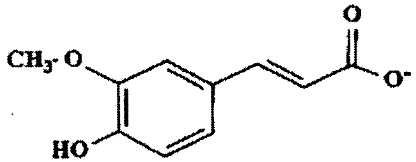
Cinnamic acid



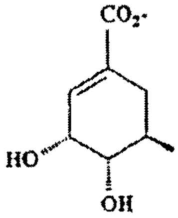
p-Coumaric acid



Caffeic acid

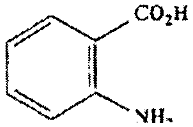


Ferulic acid



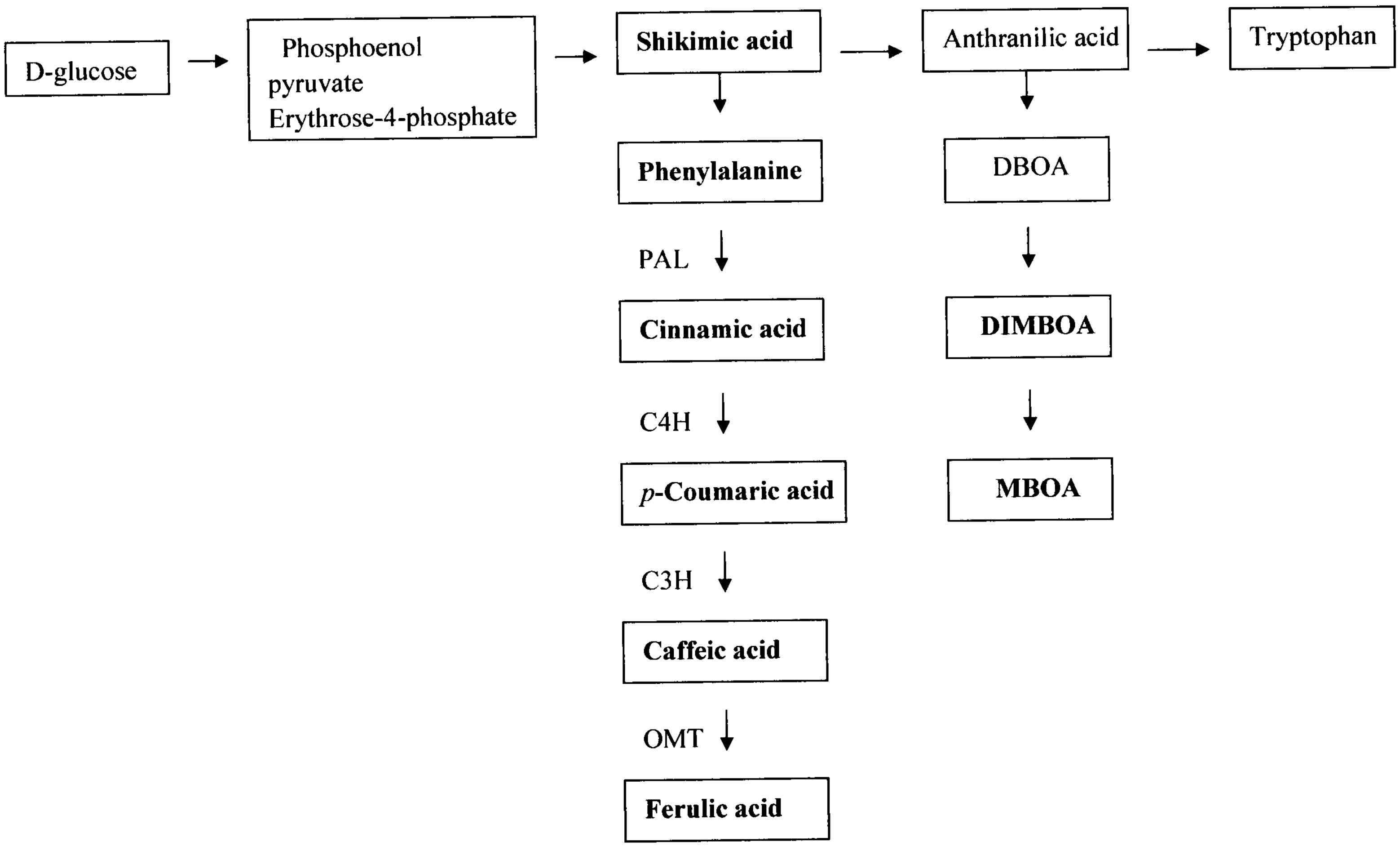
Shikimic acid

Phenylalanine



Anthranilic acid

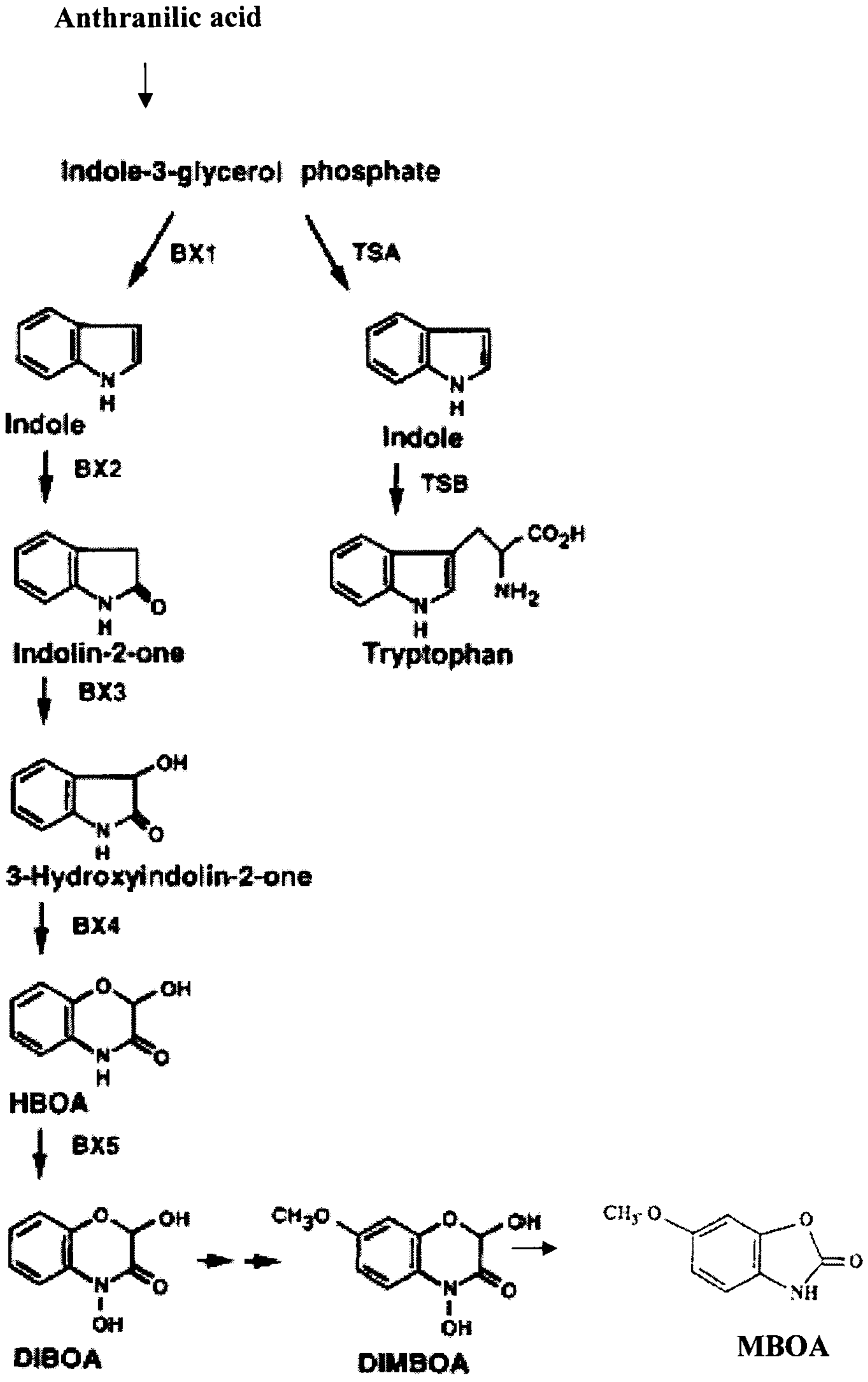
Fig. 2 Shikimic acid pathway of phenolic acid synthesis and the relationship with DIMBOA synthetic pathway in maize. PAL: Phenylalanine ammonia lyase;
C4H: Cinnamate 4-hydroxylase; C3H: p-Coumarate 3-hydroxylase;
OMT: Caffeic acid O-methyl transferase


D-glucose
Phosphoenol pyruvate Erythrose-4-phosphate
Shikimic acid
Anthranilic acid
Tryptophan
Phenylalanine
PAL
Cinnamic acid
C4H
p-Coumaric acid
C3H
Caffeic acid
OMT
Ferulic acid
DBOA
DIMBOA
MBOA

The DIMBOA biosynthetic pathway shares certain intermediates with the synthesis of tryptophan, one of the three aromatic amino acids in the shikimic acid pathway of phenolic synthesis (Desai et al., 1996; Frey et al., 1997; Rad et al., 2001) (Fig. 3). The formal branch point between the two pathways occurs at the intermediates, indole 3 - glycerol phosphate, which follows anthranilic acid in the pathway. Anthranilic acid, precursor of tryptophan, is incorporated into DIMBOA with the indole. The formation of DIMBOA in maize involves five genes, *bx*1 - *bx*5, in which the gene *bx*1 controls the rate determining the step for production of DIMBOA (Fig. 3) (Desai et al., 1996; Frey et al., 1997; Rad et al., 2001).

The reaction catalyzed by PAL is the most important regulatory step. PAL activity regulates the amount of phenolics in plant tissue because it is a key factor in controlling the flux from primary metabolism to phenolic production. The biosynthetic relationship of shikimic acid, phenylalanine and phenolic acids is described in Fig. 2. From cinnamic acid, a series of specific hydroxylation and methylation reactions take place, which results in the production of three other important hydroxycinnamic acids: *p*-coumaric, caffeic, and ferulic acids. Besides PAL, three other regulatory genes encoding three biosynthetic enzymes are involved, i.e. cinnamate 4 - hydroxylase (C4H) for *p*-coumaric acid, *p*-coumarate 3 - hydroxylase (C3H) for caffeic acid, and caffeic acid O - methyl transferase (OMT) for ferulic acid (Fig.2) (Martin et al., 2000). These end products are then used to synthesize an array of phenolic and polyphenolic products with putative defensive functions. For example, peroxidases can catalyze the oxidative coupling between phenols. The oxidative coupling between two ferulic acids (FA) produces a diferulic acid (DFA).

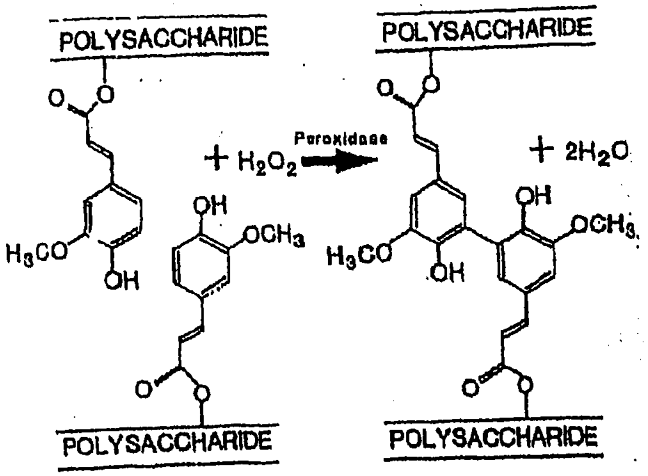
Fig. 3 Structure of DIMBOA and MBOA and their biosynthetic pathways in maize (based on Frey et al., 1997). At least five genes, *bx*1 - *bx*5 are required for DIMBOA formation.


Anthranilic acid
Indole-3-glycerol phosphate
BX1
TSA
Indole
Indole
BX2
TSB
Indolin-2-one
Tryptophan
BX3
3-Hydroxyindolin-2-one
BX4
HBOA
BX5
DIBOA
DIMBOA
MBOA

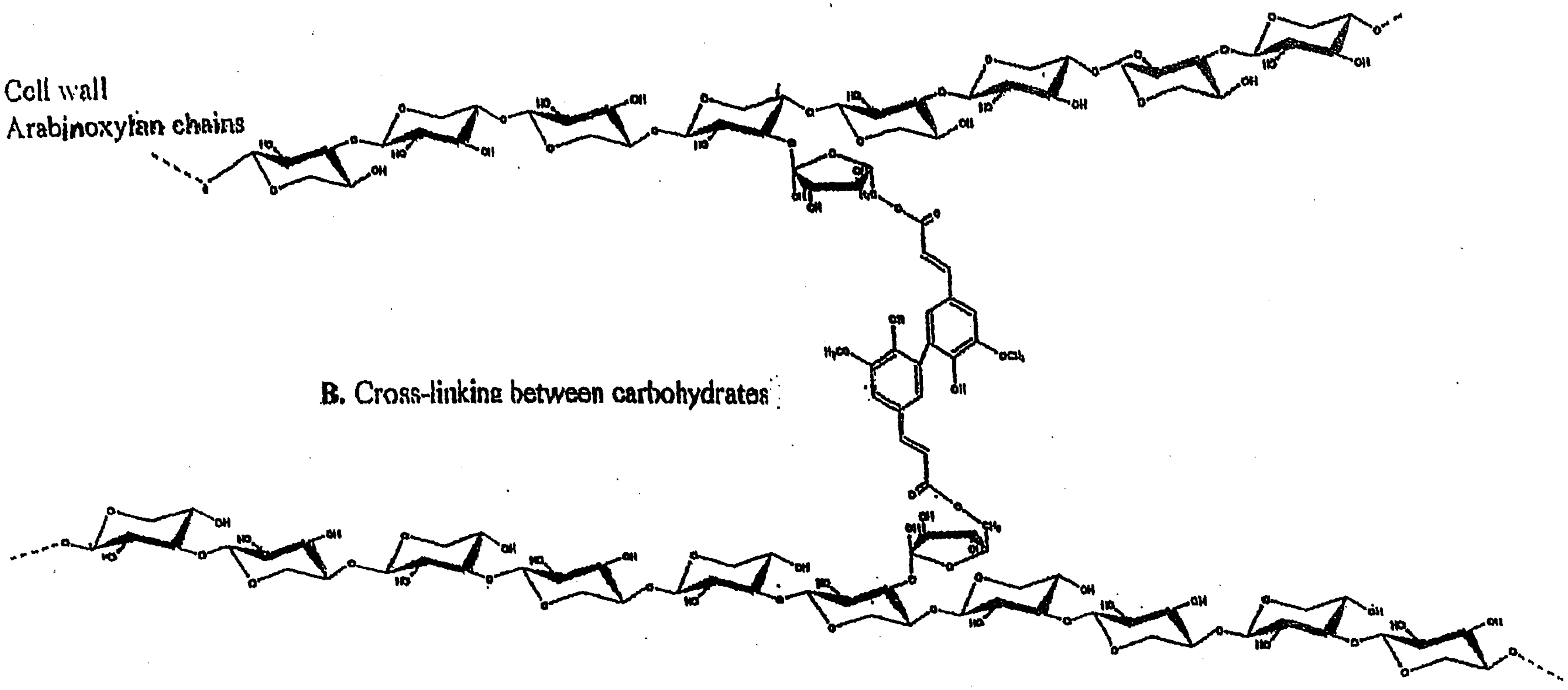
$$FA + H_2O_2 \xrightarrow{\text{Peroxidase}} DFA + 2H_2O \qquad (2)$$

Diferulic acid is an example of a secondary compound that functions in conferring structural reinforcement and hence resistance in the maize cell wall. The oxidative coupling results in cross - linking of the cell wall hemicellulose, arabinoxylan by diferulate, which strengthens the cell wall and impedes insect colonization and ingression (Fig. 4A - B).

Fig. 4A - B Oxidative coupling and cross-linking between cell wall carbohydrates



A. Oxidative coupling



B. Cross-linking between carbohydrates

1.3 Hypothesis and objectives

Part 1

Hypothesis 1: The genetic modification of maize with wheat oxalate oxidase will alter secondary metabolism in the biosynthetic pathway of phenolic acids and DIMBOA. Higher oxalate oxidase activity and higher levels of hydrogen peroxide are predicted to result in higher concentrations of phenolic acids in the leaf tissue of OXO maize. Levels of hydroxamic acids such as DIMBOA are probably also modified due to the diversion of metabolism. The altered secondary metabolism may be a major factor in insect resistance of transformed maize.

Objective 1: Determine response of phenolic acids and hydroxamic acids to OXO transformation in maize using high performance liquid chromatography method and fluorescence microscopy.

Part 2

Hypothesis 2: Significantly improved resistance to European corn borer was previously obtained in transgenic maize lines with OXO gene. Soybean, one major legume crop, has been successfully transformed with the OXO gene. Therefore, the genetic engineering of cowpea with the OXO is feasible and the putative OXO cowpea plants will likely be resistant to cowpea pod borer. However, an effective *in vitro* regeneration system is an initial requirement for plant transformation. It is hypothesized that certain cowpea genotype(s) and explanted plant tissue(s) have strong responses to shoot organogenesis and somatic embryogenesis. It is predicted that the regeneration system can be manipulated by alternation of plant growth regulators and mineral elements and growth conditions in culture media.

Objective 2: Develop an effective *in vitro* regeneration protocol for cowpea transformation via shoot organogenesis or somatic embryogenesis based on systematic media modification.

Chapter 2

Mechanism of improved resistance to European corn borer (*Ostrinia nubilalis* H.) in maize (*Zea mays* L.) transformed with wheat oxalate oxidase (OXO)

2.1 Introduction

The European corn borer (ECB) *Ostrinia nubilalis* (Lepidoptera: Pyralidae) is a major insect pest in maize production. The economic losses caused by ECB are as much as 30% in certain areas (Scott and Pollak, 2005). The use of insect resistant varieties of maize is considered to be the most practical way to reduce the losses from insect damage. Recently, maize transformed with wheat oxalate oxidase (OXO) was shown to be resistant to ECB (Ramputh et al., 2002). In all ECB infested transgenic lines, leaf consumption and stalk - tunneling damage was reduced by 34% and 40%, respectively. ECB larval growth and development were also delayed. However, the mode of action of the improved insect resistance to ECB in the OXO maize was not elucidated. Modification of metabolic flow in secondary metabolism by OXO transformation is a possible mechanism of the improved resistance.

The OXO protein, isolated from the wheat genomic clone gf - 2.8 (Dratewka - Kos et al., 1989), is an enzyme (EC 1.2.3.4) that can catalyze the oxidative reaction of oxalate into H_2O_2 and CO_2 (Dumas et al., 1995). Studies on extracellular expression of OXO showed that the H_2O_2 produced was involved in cell wall cross - linking reactions, which stiffens cell walls through lignification (Olson and Varner, 1993; Lane et al., 1993; Lane, 1994). Recently, its roles in defense mechanism to disease (Zhang et al., 1995; Zhou et al., 1998), and as a stress response to heavy metals (Delisle et al., 2001), salts (Hurkman et al., 1994) and drought (Ozturk et al., 2002) have been described.

The hydroxamic acid 2,4 - dihydroxy - 7 - methoxy - 1,4 - benzoxazin - 3 - one (DIMBOA) and its degradation product 6 - methoxybenzoxazolin - 3 - one (MBOA) are known as plant defense phytochemicals to ECB in young leaves (Bergvinson, 1993). Another resistance factor in maize is hydroxycinnamic acids. The important hydroxycinnamic acids include cinnamic acid, *p*-coumaric acid, ferulic acid, and diferulic acid. The defense functions of DIMBOA and phenolic acids in maize plants have been well elucidated in ECB (Bergvinson et al., 1993; 1994b), southwestern corn borer and sugarcane borer (Ramputh, 1997), and maize weevil *Sitophilus zeamais* (Arnason et al., 1997), maize stem borer *Sesamia nonagrioides* (Lefebvre) (Santiago et al., 2005).

In plant resistance, several signaling pathways exist to activate gene expression in plant cells. A rapid production of reactive oxygen species (ROS) is one of the signal pathways (Wallin, 2000). We hypothesized that a novel resistance mechanism was initiated by OXO and H_2O_2 activity, which ultimately leads to responses in levels of hydroxamic acids and hydroxycinnamic acids.

Phenolic compounds are present in plant cells in four different forms: insoluble cell wall - bound, soluble ester - bound, soluble ether - bound and free phenolics. Previous studies on maize resistance mechanism were concentrated on metabolism of bound phenolics (Bergvinson et al., 1994b; Ramputh, 1997; Fry et al., 2000; Ramputh et al., 2002). The roles of soluble phenolics in enhancing OXO maize resistance to insects have not been reported to date. In transgenic maize with OXO, Ramputh et al. (2002) found that there was little change in cell wall bound phenolics between transformed and null lines. Therefore, in the present study, changes of metabolism in maize hydroxamic acids DIMBOA and MBOA and soluble hydroxycinnamic acids CA, FA and DFA were examined to determine if they were factors in the improved resistance.

2.2 Materials and methods

2.2.1 Plant materials

Maize transformation with wheat OXO was previously conducted by Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada via particle bombardment armed with vector pact - OXO on the embryogenic tissue. The *in vitro* somatic embryogenesis was obtained from the explanted immature embryos of an A188 × B73 derivate. The primary transformed lines were then used to backcross to corn inbred lines CK44 and B73. Stable transgene expression of the OXO transgenic maize lines was demonstrated in seven generations of backcross. Simultaneously, OXO homozygous and OXO null lines were identified. Tests on hydroxamic acids involved OXO homozygous CK44 line and the corresponding null line. Two OXO lines, homozygous CK44 and B73, and their corresponding null controls were investigated for phenolic acid tests. For OXO activity assay and ECB field test, three OXO maize lines were used: homozygous line CK44, hemizygous line CO286 line and hemizygous line CO328 line and their corresponding lines. Maize plants were grown in a controlled chamber at 25 ± 1°C with 16:8 h photoperiod and light intensity of 50 μE m^{-2} s^{-1} from cool white fluorescent tubes.

Each transformed line and null control was arranged randomly in a growth chamber (20 plants per line). The sixth leaf was collected at the Stage V7 (the seventh leaf emerged) from 2 week- old seedlings. Top and bottom parts were removed, and middle parts were collected, and stored at - 80°C for later testing. For localization of phenolics via fluorescence microscopy, sample leaves were freshly prepared.

2.2.2 Oxalate oxidase quantification (performed by A. I. Ramputh)

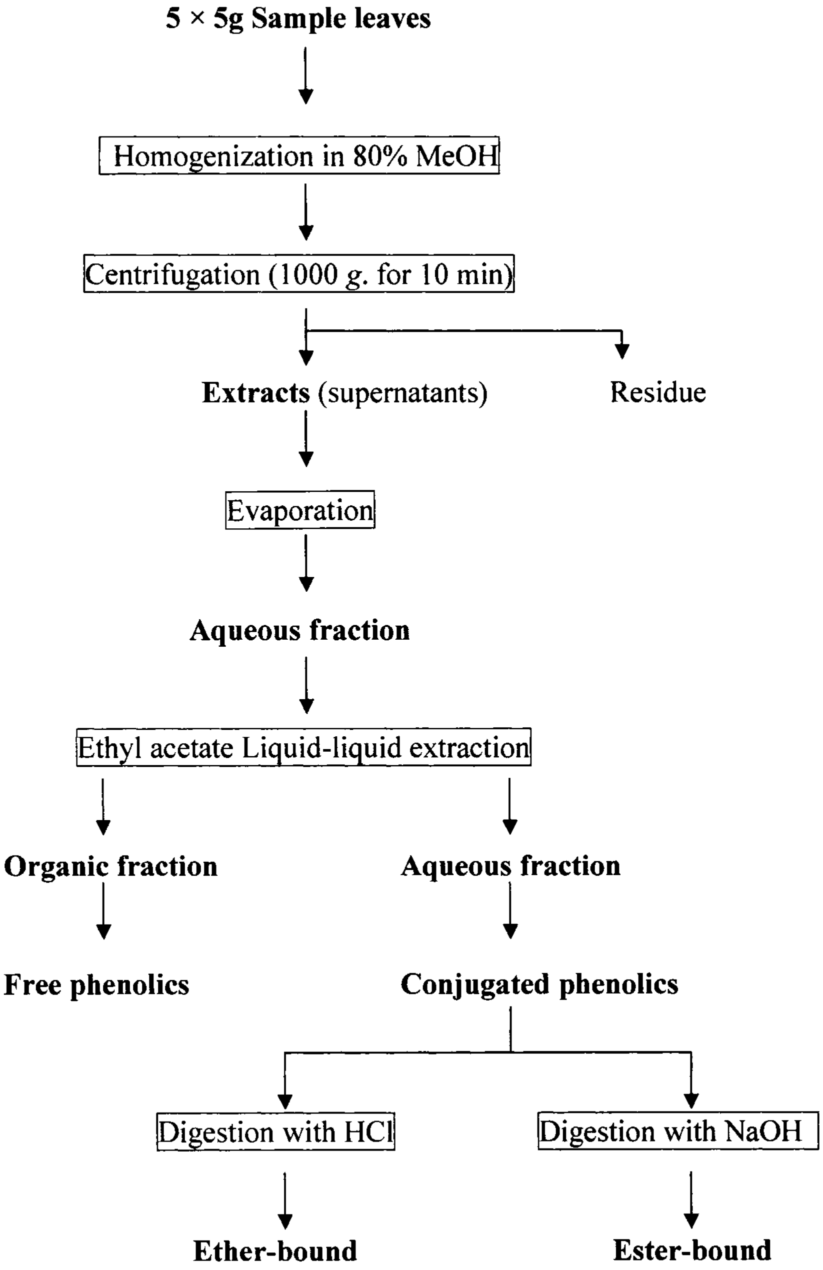
The protocol for OXO quantification was from Zhang et al. (1996) with minor modifications. Following grinding in liquid nitrogen, the leaf tissue (0.05 g) was placed in a microfuge tube on dry ice. To each tube was added 500 μl of dH_2O and vortexed for a few seconds. After centrifuging at 1000 xg for 10 minutes, the pellet was collected, and 1 ml of dH_2O was added and vortexed for a few seconds. To inactivate other endogenous enzymes, the samples were heated for 5 min in an 80°C water bath. The slurry was centrifuged at 1000 xg for 10 min, and the remaining pellet was harvested, on which oxalate oxidase assay was conducted. A 100 ml developing solution was prepared as follows: 40 ml of 0.1 M succinate buffer (pH 3.5), 60 ml of 100% ethanol, 8 mg of 4 - aminoantipyrine, 8 mg of soybean peroxidase (60 U/mg), 20 μl of *N, N* - dimethylaniline, and 13 mg of oxalic acid. Oxalic acid was omitted from the control developing solution. Every 1 ml of developing solution was added to the tissue pellet while 1 ml of control developing solution was added to the control tissue pellet and vortexed for a few seconds. Standards, samples and control tubes were placed on a shaker at room temperature for 2 hour and supplemented with 20 μl of 1 N NaOH to stop the reactions. After being centrifuged at 1000 xg for 5 min, the supernatant was transferred to a cuvette and absorbance at 555 nm was recorded using a spectrophotometer. A standard curve of OXO activity was generated using barley OXO (Sigma). One unit of enzyme activity was defined as the amount of the enzyme, which formed 1 μM of H_2O_2 per min in the standard assay system.

2.2.3 Soluble phenolic quantification by HPLC analysis

FA and *p*-CA, the most prevalent phenolic acids in maize leaves, were analyzed as well as four DFA isomers (DFA 5 - 5 and DFA 8 - 5 linear form, DFA 8 - 0 - 4 and DFA 8 - 5 benzofuran). For each compound, the free phenolic acid, ester conjugate and ether conjugate were determined.

Methods of extraction of soluble phenolics were adapted from Weidner et al. (1996) and Bily et al. (2004) with minor modification (Fig. 5). Frozen maize leaves were collected in 50 ml flasks with 5 g per flask and 5 replicates. Soluble phenolic compounds were extracted using 80% methanol, 30 ml per flask, and then homogenized with a Polytron mixer (Brinkman, Westbury, NY, USA). Homogenized samples were shaken in darkness for 1 hour at 220 rpm, and then centrifuged for 10 min at 1000 x*g*. The supernatants were collected while the pellets were extracted twice more using 30 ml 80% methanol. Pooled supernatants were evaporated to 30 ml in a Savant SpeedVac (Bio101, Vista, CA) at 45 °C in order to remove the majority of the methanol. The solutions were acidified to pH 2 using 2 N HCl and filtered through Whatman paper No.1. Free phenolic compounds were extracted into an equal volume (30 ml) of ethyl acetate (EtOAc). In order to extract ester - and ether - conjugated phenolics, the aqueous fractions were

Fig. 5 Procedure for extraction of soluble phenolic acids (adopted from Weidner et al., 1996 and Bily et al., 2004)


5 × 5g Sample leaves
Homogenization in 80% MeOH
Centrifugation (1000 g. for 10 min)
Extracts (supernatants)
Residue
Evaporation
Aqueous fraction
Ethyl acetate Liquid-liquid extraction
Organic fraction
Aqueous fraction
Free phenolics
Conjugated phenolics
Digestion with HCl
Digestion with NaOH
Ether-bound
Ester-bound

divided into two equal parts (10ml per part). Part I was treated with 10 ml 2 N NaOH and digested under a nitrogen atmosphere in darkness for 3 hour to hydrolyze the esterified phenolic acids. Part II was added with 10 ml 2 N HCl and incubated at 4 °C for 1 hour to release ether - conjugated phenolic acids. Both parts I and part II were adjusted to pH 2 with 2 N HCl (Part I) and 1 N NaOH (Part II). EtOAc (20 ml) was added to extract the released phenolic acids. The above three fractions were evaporated under vacuum. All the dried samples were dissolved in 3 ml of methanol and stored at -18 °C until HPLC analysis. HPLC analysis followed the method of Bily et al. (2004). A Hewlett - Packard (Agilent, Palo Alto, CA, USA) ChemStation series1100 LC with a Diode Array Detector (DAD) was used. Separations were obtained with a Waters YMC ODS - AM narrow bore column (100 x 2 mm i. d.; 3 µm particle size). The mobile phase system consisted of acetonitrile (solvent A) and 0.05% aqueous trifluoroacetic acid buffer (pH 2.4) (solvent B). The gradient was as follows: initial conditions 10:90 (A: B); 30:70 in 3.5 min; 32:68 in 6.5 min; 100:0 in 4 min; hold 100:0 for 4.5 min; and then returned the initial conditions 10:90 (A: B) by 3 min. The elution profiles were monitored at 325 and 280 nm. Standard *p*-CA and FA were purchased (Sigma, St. Louis, MO, USA) while 5 - 5 DFA was synthesized by the group of G. H. N. Towers (University of British Columbia, Vancouver, Canada). Samples (1.00 ml each) were filtered through a 0.22 µm PTFE membrane (Chromatographic Specialties, Brockville, ON) and injected into the HPLC column. The mobile phase rate was 0.3 ml/min and the total analysis was completed in 21.5 min. The peak identity of each phenolic was confirmed by retention time and UV spectrum comparisons with the commercial standard solutions and known spectra.

2.2.4 DIMBOA quantification by HPLC analysis

The method for extraction of DIMBOA was from Melanson et al. (1997) and Bily et al. (2004). The sample leaves stored at - 80 °C were removed and immediately treated with liquid nitrogen. The frozen leaves were ground to a fine powder and put into five flasks with 1.0 g per flask. When ground leaves were warmed up to room temperature, 5 ml of water was added and then incubated at room temperature for 1 hour to allow for the release of the aglycones. After centrifugation, the supernatant was collected and extracted four times using an equal volume of EtOAc. Pooled extracts were evaporated to remove solvent under vacuum with a Savant SpeedVac (Bio101, Vista, CA). The solid residue was dissolved in 1.0 ml methanol for HPLC analysis. Analyses were performed using a Hewlett - Packard ChemStation series1100 HPLC equipped with a Waters YMC ODS - AM narrow bore column (100 x 2 mm i.d.; 3 µm particle size). The mobile phase system of methanol (solvent A) and trifluoroacetic acid (0.05%) in water (pH 2.4; solvent B) was used as follows: initial conditions 25:75 (A: B), changing to 40: 60 in 10 min, then to 100: 0 in 5 min, 100% methanol is held for 2 min, then returned to initial conditions in 3 min. The column was allowed to equilibrate for 5 min. The mobile phase flow rate was 0.2 ml/min and the total analysis time was 25 min. The sample injection volume was 4 µl, and the elution profiles were monitored at 210 and 265 nm. DIMBOA eluted at 4.25 min and MBOA at 7.25 min. Peak identities were confirmed by matching retention time and absorbance spectra with purified standards.

2.2.5 Specimen preparation for fluorescence microscopy

Sample leaves were freshly collected from plants grown in a chamber with controlled environment as described above. The middle of the leaves (0.5 cm wide) with midrib veins were isolated and immediately frozen using Tissue - Tek O.C.T. compounds (Miles Inc., USA) at - 20 °C. The tissue was freshly sectioned with microtome at - 20°C. To locate soluble phenolics, the cryosections (nominally 8 microns) were gently moved to slides and then mounted in glycerol. The images were taken at an excitation wavelength of 360 nm (UV) and exposure of 23 microseconds, using a10 x objective. In the case of localization of insoluble cell wall - bound phenolics and lignin, three histochemical reagents for staining were used, including chlorine - sulfite, phloroglucinol - HCl, and toluidine blue. These are three standard stains for lignin. Their characteristic reactions with phenolic compounds make direct observation of location of phenolic compounds in plant tissue possible. Chlorine - sulfite was freshly made by mixing 20 g l^{-1} sodium sulfite (aqueous) with bleach (6% sodium hypochlorite) at 8°C. The pH value was adjusted to pH 1 by 6 N HCl with constant stirring. Phloroglucinol was made by adding 60 g l^{-1} saturated phloroglucinol (aqueous) to 6 M HCl by a proportion 4:1. Toluidine blue was made by adding 0.05% toluidine blue (aqueous) into 20% $CaCl_2$. The fresh cryosection slides were submerged into stain reagents and incubated for 2 min. After drained of excess stain reagent, slides were mounted in glycerol.

2.2.6 Meridic diet test

A 4 days growth reduction and feeding assay was carried out to test the effects of FA on ECB larval growth using the bioassay method presented in Ewete et al. (1996). Commercially available FA (Sigma) was used. Meridic diet was made from agar, methylparaben solution, propionic - phosphoric acid, formaldehyde and fumagillin (Guthrie et al., 1989). Based on the concentration of FA in OXO transgenic maize leaves, the experimental treatments were designed as: control with 1% (v/w) of ethanol; FA 30, 100, 300, 1000, and 3000 μg/g FW (fresh weight of diet). Compounds dissolved in ethanol were incorporated into the diet before the agar was solidified. Ethanol concentration was 0.5% in all diets. The bioassay was conducted under the same environmental conditions as the ECB was maintained. Second instar larvae reared on the defined culture diet were exposed to each treatment and negative controls, twenty larvae per vial. Each larva was weighed and transferred to an individual glass vial (5 dram) provided with an appropriately treated or control diet cube with twenty larvae per treatment and control. The vials were plugged with cotton and kept in a growth chamber at 28°C / 19°C (day / night), 85% R.H. and an 18:6 hour (light/dark) photoperiod. On day 4, the larva and diet were weighed before incubation. Three nutritional indices were calculated on a wet weight basis according to the methods presented in Ewete et al. (1996):

Relative growth rate (RGR) = (final wt – initial wt) / [(final wt – initial wt)/2] (1)
Consumption index (CI) = ingested food wt/larval wt (2)
Conversion efficiency of ingested food (ECI) = larval wt gained/ing food wt ×100 (3)
(wt: weight; ing: ingested)

2.2.7 Field feeding testing (performed by A. I. Ramputh)

ECB egg masses were maintained in a controlled chamber at 80% RH with 18 (26 °C):6 (19 °C) hour photoperiod in the laboratory. Three OXO maize lines were used in the field test: CK44 OXO homozygous, CO286 hemizygous, and CO328 hemizygous line. The test plants were grown in a randomized block design with 15 plants per row, 1 row per replicate, and 3 replicates per test (*n* = 45 plants for each line). Insect field feeding testing was conducted at mid - whorl stage (40 cm in height) of maize plants. Egg masses were manually placed in the plant whorl using long forceps with two egg masses per plant and 25 eggs per mass. Leaf damage rating was evaluated eight weeks after infestation following the 1 - 9 scale method (from no damage to most leaves with long lesions) developed by Guthrie et al. (1960). Damage extent on stalks was recorded after mature plants were harvested and stalk was split with a knife. The cumulative lengths of borer tunnels were measured in a 90 cm section of stalk, which was isolated at 45 cm above plus below the primary ear node.

2.2.8 Statistical analysis

All the data were subjected to either analysis of variance (ANOVA) or linear regression analysis using Microsoft Excel - General vsn.1.71 (1997 - 2000 Analysis - it Software Ltd, UK).

2.3 Results

2.3.1 Oxalate oxidase activity

All the OXO lines showed significant OXO activity in leaf tissue (Table 1). No OXO activity could be detected in the corresponding OXO null lines. Homozygous CK44 line showed the highest level of OXO expression, which was 3 - fold greater than that in hemizygous CO286 line, and 1.5 - fold higher than that in hemizygous CO328 line.

Table 1 Oxalate oxidase (OXO) activity
in leaf tissue of OXO maize lines and their corresponding null lines

Maize lines	Oxalate oxidase ($U \times 10^{-4}$/mg FW)*
CK44 homozygous line	5.74 ± 0.20a
CK44 null line	0
CO286 hemizygous line	1.84 ± 0.08c
CO286 null line	0
CO328 hemizygous line	3.92 ± 0.30b
CO328 null line CK44++	0

* The testing was performed by A. I. Ramputh.

Maize plants are designated as homozygous, hemizygous, and null for the OXO transgenic maize. Means ± S.E. within the same column followed by the same letters were not significantly different, and with different letters they were significantly different [SNK (Student-Newman-Keuls) test, $p < 0.05$)]. One unit of OXO activity forms 1 nM H_2O_2 per min from oxalate at pH 3 at 37°C.

2.3.2 Concentrations of soluble phenolic acids

In CK44 homozygous line, levels of both total soluble phenolics and each individual compound tested were significantly higher in transgenic lines than they were in the corresponding null lines (Fig. 6). Concentrations of total phenolics, FA, *p*-CA and DFA in OXO lines were 3.2, 3.6, 2.8 and 2.0 times higher than that in responding null line, respectively (Table 2). The highest level (2091 μg/g DW) was recorded for FA content, in which trans- FA was 2.0 - fold higher than cis- FA (Table 3). Total FA content accounted for 80.4 % of total soluble phenolics in CK44 homozygous line (Table 2). Total DFA was 365 μg /g DW (Table 2), in which DFA 5 - 5 gave the highest concentration; followed by DFA 8 - 5, DFA 8 – 0 - 4, and DFA 8 - 5 benzofuran (Table 3). A lower level occurred with *p*-CA (144.4 μg/g DW) (Table 2). Analysis of variance (ANOVA) showed that the content differences of total soluble phenolic acids and total FA were highly significant between OXO and null lines ($p < 0.01$) (Appendix 3A - B; Appendix 4A - B). The content differences of DFA and *p*-CA were significant between OXO and null lines at a lower level ($p < 0.05$) (Appendix 5A - B; Appendix 6A - B). Except for DFA 5 - 5, two ferulic acid isomers and three DFA isomers had significantly higher contents in OXO line than those in null line (Fig. 6).

Free phenolic acids accounted for 80.8% of total soluble phenolic acids in homozygous transgenic line of CK44. In contrast, the ester conjugate was 5.1%, while the ether conjugate was only 0.7% (Table 4). The major form of soluble phenolic acids was free phenolics in transgenic maize lines. Ferulic acid was the prominent intracellular soluble phenolic acid for transgenic OXO maize lines.

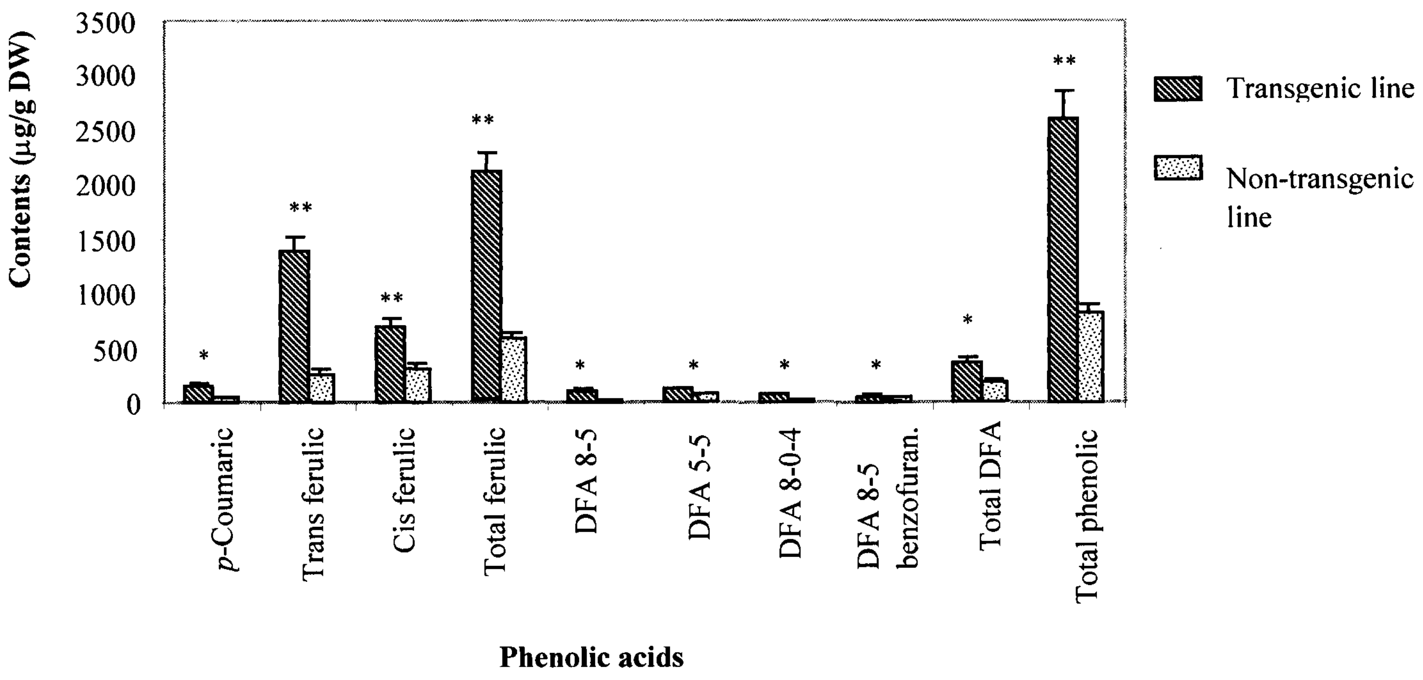

Contents (µg/g DW)
3500
3000
2500
2000
1500
1000
500
0
*
**
**
**
*
*
*
*
*
**
p-Coumaric
Trans ferulic
Cis ferulic
Total ferulic
DFA 8-5
DFA 5-5
DFA 8-0-4
DFA 8-5 benzofuran.
Total DFA
Total phenolic
Phenolic acids
Transgenic line
Non-transgenic line

Fig. 6 HPLC profiles of soluble phenolic acids derived from leaves of CK44 OXO homozygous transgenic line and the corresponding null line (DW: dry weight; ** significant at $p < 0.01$; * significant at $p < 0.05$). Leaf extracts were obtained from the middle parts with veins of 6th leaves (V6) of plant at V7 stage (7th leaf emerged). Concentrations of total FA and DFA were calculated by their isomers. Total phenolic content was the sum of *p*-CA, total FA and total DFA.

Table 2 Concentrations (μg/g DW) of soluble phenolic compounds in leaf tissue of CK44 OXO homozygous line and the corresponding null control

Corn lines	Total soluble	Total ferulic	*p* –Coumaric	Total DFA
OXO line	2600.1 ± 267	2090.6 ± 200	144.4 ± 32	365.0 ± 56
Null line	819.1 ± 83	585.3 ± 69	52.3 ± 6	181.5 ± 14
OXO lines / Null lines	3.2 ± 3.2	3.6 ± 2.9	2.8 ± 5.3	2.0 ± 4.0
Percentage in total phenolics (%)		80.4	5.6	14.0

Concentrations were assessed by the means ± S.E. (μg/g DW)

Table 3 Concentrations (μg /g DW) of different isoforms of ferulic and diferulic acids in leaf tissue of CK44 OXO homozygous line and the corresponding null control

Corn lines	Trans-ferulic	Cis-ferulic	DFA 5-5	DFA 8-5	DFA 8-0-4	DFA 8-5 benzofuran
OXO line	1387.7 ± 25	702.9 ± 9	120.1 ± 9	111.6 ± 4	71.5 ± 4	61.8 ± 5
Null line	269.6 ± 34	315.6 ± 35	80.6 ± 6	33.0 ± 4	19.6 ± 9	42.5 ± 5

Concentrations were assessed by the means ± S.E. (μg / g dry weight (DW)

Table 4 Concentrations (μg/g DW) of four different present forms in leaf tissue of CK44 OXO homozygous line (S.E. in parenthesis) and ratios in total soluble phenolics

Phenolics	Free		Ester - bound		Ether - bound		Total (μg / g)
	Concentrations (μg / g)	Percentage (%)	Concentrations (μg / g)	Percentage (%)	Concentrations (μg / g)	Percentage (%)	
Ferulic	1774.7 (106.8)	84.8	106.5 (32.4)	5.1	15.1 (5.2)	0.7	2090.5 (199.7)
Diferulic	242.3 (3.4)	66.4	27.3 (9.2)	7.5	2.9 (1.0)	0.8	365.1 (56.4)
p-Coumaric	84.9 (11.7)	58.8	37.0 (10.2)	25.6	7.3 (7.2)	5.1	144.5 (32.4)
Total	2101.9 (154.1)	80.8	170.7 (46.6)	6.5	25.3 (14.9)	1.0	2600.1 (267.4)

The HPLC results from another OXO transgenic line, B73 homozygous line showed the same trend as CK44 homozygous line. The levels of total phenolic acid and each individual compound were obviously higher in transgenic lines than those of the null lines (Fig.7). The free phenolic acids accounted for 77.1% while ester - and ether - bound phenolics are only 8.5% and 1.0%, respectively (data not shown).

2.3.3 Results of fluorescence microscopy

From the leaf section staining by toluidine blue, extensive lignification of phenolic acids in the cell walls of vascular and epidermal tissue in OXO line was observed (Appendix 8). Fluorescence was located through the cell walls of vascular and epidermal tissue. However, there were no obvious differences in colour brightness between the OXO and null lines. It might suggest that the contents of insoluble cell wall bound phenolics, lignin and cell wall thickness did not show obvious differences between OXO and null lines in high resolution micrographs. Autofluorescence and the two histological stains, chlorine - sulfite and phloroglucinol, gave similar results (Appendix 7). This was consistent with the previous HPLC result from Ramputh et al. (2002) that indicated there was no difference in bound phenolic contents between OXO and null lines.

In the case of the leaf sections for the analyzing soluble phenolics, apparent differences in blue fluorescence were observed between OXO and the null lines. The leaf cross - sections from CK44 homozygous line produced a brighter blue color than its null line (Fig. 8). In addition, a fluorescent halo outside the cut section was

Fig. 7 HPLC profiles of soluble phenolics acids derived from leaves of B73 OXO homozygous transgenic maize line and the corresponding null line (DW: dry weight). Leaf extracts were obtained from the middle parts with veins of 6th leaves (V6) of plant at V7 stage (7th leaf emerged). Concentrations of total FA and DFA were calculated by their isomers. Total phenolic content was the sum of *p*-CA, total FA and total DFA.

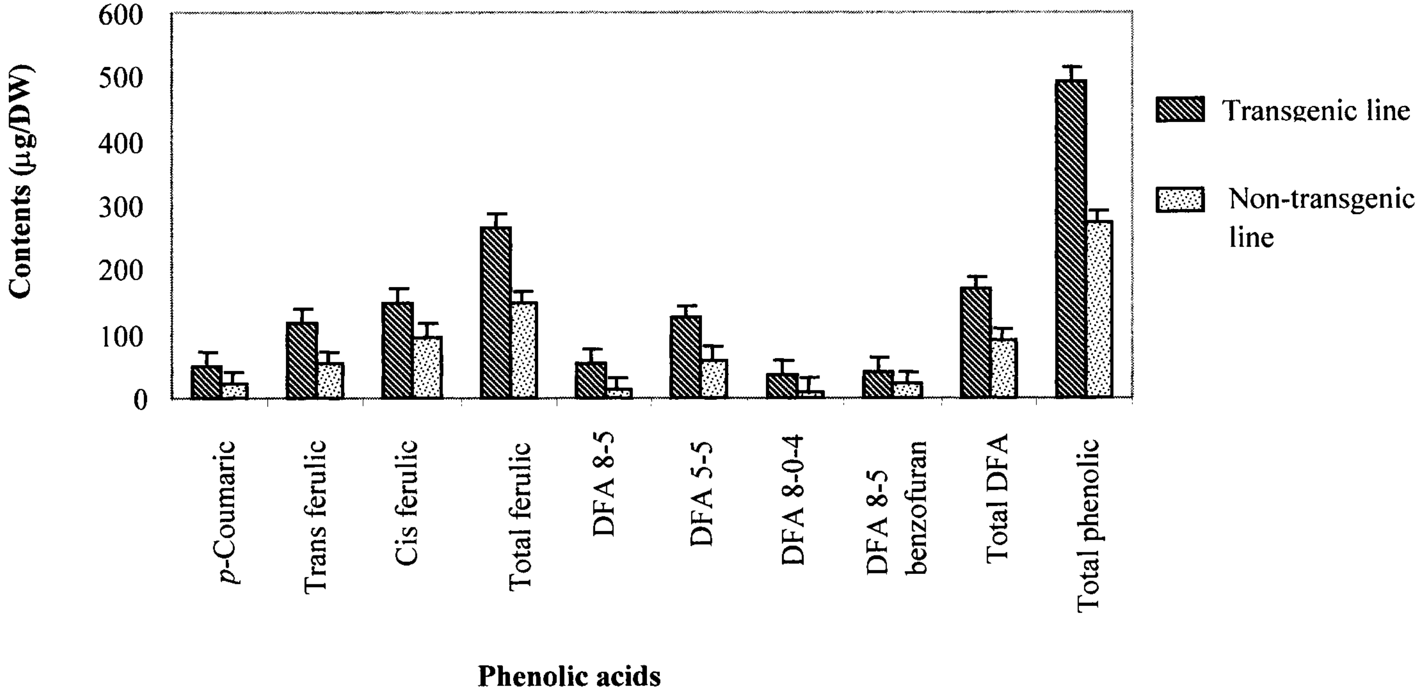
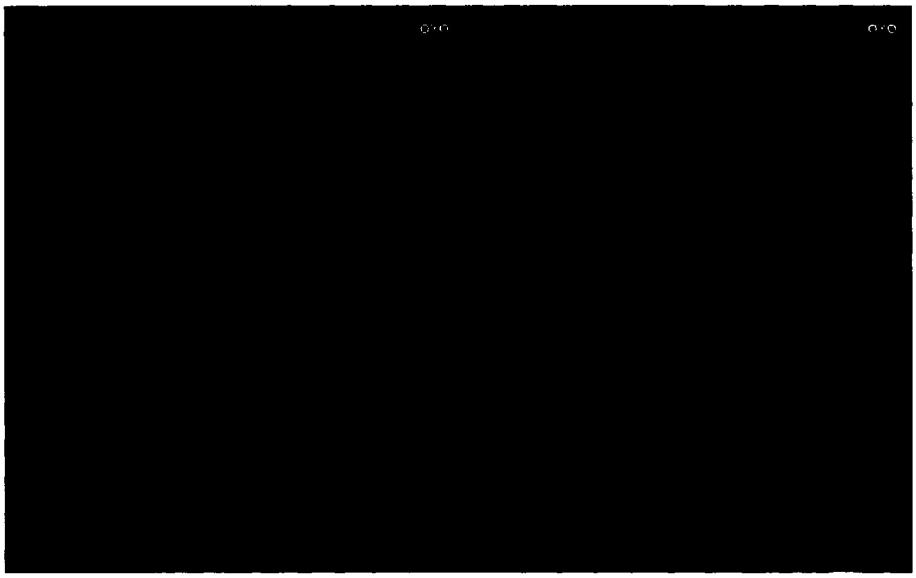
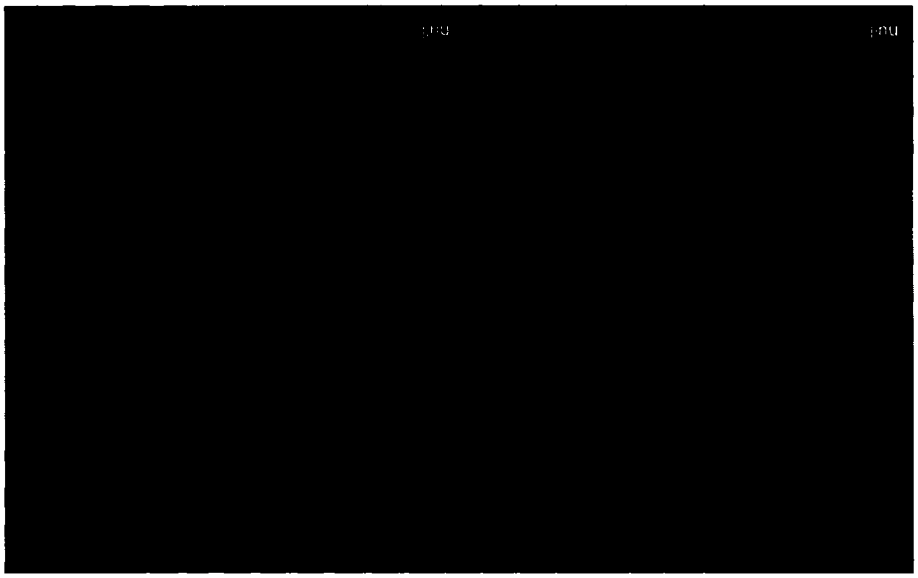

Contents (µg/DW)
600
500
400
300
200
100
0
p-Coumaric
Trans ferulic
Cis ferulic
Total ferulic
DFA 8-5
DFA 5-5
DFA 8-0-4
DFA 8-5 benzofuran
Total DFA
Total phenolic
Phenolic acids
Transgenic line
Non-transgenic line

Fig. 8 Autofluorescence of leaf cross-section of CK44 OXO homozygous transgenic and the corresponding null lines under ultraviolet light (Wave length: 360nm; Exposure: 23 microseconds; and magnification: 10 X).

Upper two sections are from null control lines, and the bottom two from OXO lines. The halos around the sections are the phenolic compound diffused from leaf tissues during sectioning.



0.0
0.0

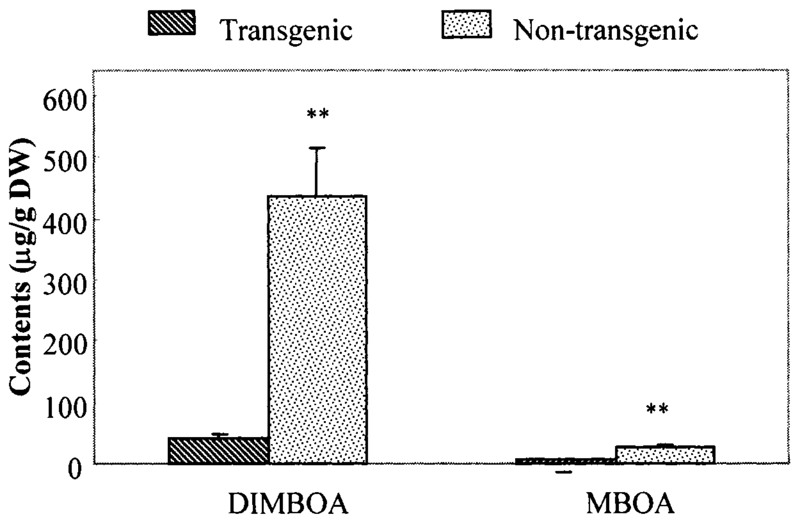


observed. This halo was from compounds that were diffusing out of leaf tissues after sectioning. The OXO line section gave a very bright halo (Fig. 8). In contrast, less fluorescence was seen from the two leaf sections of null line (upper). Therefore, the observed colour difference suggested that there was a difference in the contents of soluble phenolics between OXO and its null lines. The brighter blue color emitted from OXO maize leaf sections implied that the contents of soluble phenolic compounds in OXO maize leaf tissue were much higher than null line. Phenolics are a class of UV absorbing compounds that can be visualized by autofluorescence under UV excitation. Absorbance of ultraviolet light by plant tissue is a good indicator of the presence of phenolic compounds. However, the blue fluorescence emitted by the target compounds is a more sensitive indicator that can be used to investigate the tissue localization and relative distribution of the phenolic compounds (Hutzler et al., 1998). The microscopy results visually confirmed the conclusions from HPLC.

2.3.4 Contents of DIMBOA and MBOA

Significant differences in DIMBOA and MBOA contents were observed between transgenic line CK44 and its corresponding null line (Fig. 9). Unlike soluble phenolic acids, the higher levels of DIMBOA and MBOA occurred with non-transgenic null line, which are respectively10.6 and 4.1 fold higher than those of transgenic line (Table 5). DIMBOA content was 437.9 μg/g DW, and was close to the level of the mature leaf tissue (middle part of V8) of regular maize inbred lines (Bergvinson, 1993). The content of DIMBOA was significantly reduced to 41.4 μg/g DW in transgenic line due to the insertion of the OXO gene.

Fig. 9 HPLC profiles of DIMBOA and MBOA derived from leaves of CK44 OXO homozygous transgenic maize line and its corresponding null line (DW: dry weight; ** significant at $p < 0.01$). Leaf extracts were obtained from the middle parts of 6th leaves (V6) of plant at the V7 stage (7th leaf emerged).



DIMBOA and MBOA

Table 5 Contents (μg/g DW) of DIMBOA and MBOA in leaf tissue of CK44 OXO homozygous line and the corresponding null control line

Corn lines	DIMBOA (μg/g)	MBOA (μg/g)
OXO line	41.4 ± 6.0	6.7 ± 0.6
Null line	437.9 ± 75.3	27.1 ± 5.0
Null lines/ OXO lines	10.6	4.1

Contents were assessed by the means ± S.E. (μg/g DW)

2.3.5 Leaf consumption and stalk tunneling of ECB on maize field plants

Visual leaf damage ratings, recorded eight weeks after field infestation, were significantly reduced by 35 - 42% in the OXO transgenic lines (Table 6). From null line to hemizygous line until homozygous line, plant resistance was increased and leaf damage cause by ECB larvae went down gradually. Stalk tunneling damage, measured at plant harvest, was also significantly reduced in all OXO transgenic lines (Table 6). Reductions in stalk tunneling ranged from 40.2% in CO328 hemizygous line to 45.4% in CO286 hemizygous line and 46.3% in CK44 homozygous line. This implied that the presence of the wheat OXO gene significantly reduced ECB damage on leaves and stalks in all cases.

2.3.6 Effects of chemical ferulic acid in artificial diet on ECB

Insect growth was reduced as the FA concentration was increased when ECB second instar larvae were fed the artificial diet containing chemical FA (Table 7). The linear regression analysis (Fig. 10A - C) showed a very strong negative correlation between FA concentration and ECB relative growth rate ($r = -0.5$, $p < 0.001$) (Fig. 10 A). Higher FA concentrations were associated with lower RGR of ECB larvae. This trend in artificial diet was consistent with the observation that the higher levels of FA in OXO homozygous lines were correlated with the increased ECB resistance. However, consumption index rose (Fig. 10B), and efficiency of conversion of ingested food dropped (Fig. 10C).

Table 6 Field rating and stalk tunneling from ECB infestation in OXO maize lines and their null lines*

Maize Lines	Leaf damage rating (1–9)	Stalk tunneling (cm)
CK44 homozygous line	2.2 ± 0.1c	13.9 ± 1.5d
CK44 null line	3.8 ± 0.2b	26.0 ± 2.1c
CO286 hemizygous line	2.8 ± 0.2bc	27.7 ± 1.5c
CO286 null line	4.4 ± 0.5a	50.7 ± 2.2a
CO328 hemizygous line	2.5 ± 0.1bc	19.9 ± 1.2cd
CO328 null line	4.1 ± 0.3a	33.4 ± 1.9b

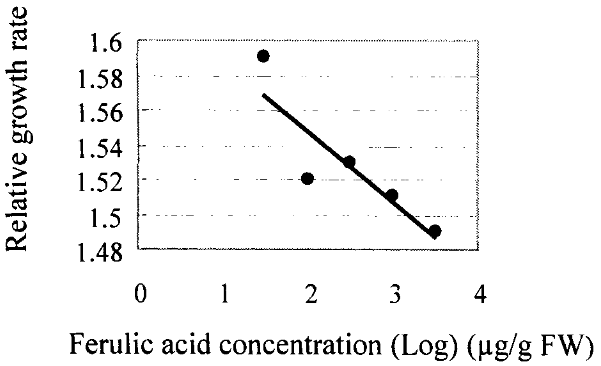
* The testing was performed by A. I. Ramputh.

Leaf damage rating was evaluated eight weeks after infestation of field plants following the 1-9 (from no damage to most leaves with long lesions) scale method developed by Guthrie et al. (1960). Damage extent on stalks was recorded after mature plants were harvested. The cumulative lengths of borer tunnels were measured in a 90 cm section of stalk, which was isolated at 45 cm above plus significantly different and different letters, significantly different (SNK test, $p < 0.05$).

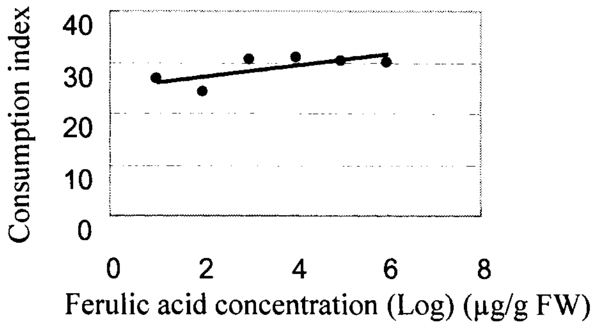
Table 7 Effects of chemical ferulic acid - supplemented diet on ECB larval growth

FA concentration (μg/g FW)	Control (0.5% ethanol)	30	100	300	1000	3000
Relative growth rate	1.55	1.59	1.52	1.53	1.51	1.49
Consumption index	26.6	24.1	30.51	31.00	30.0	30.6
Efficiency of conversion of ingested food	3.28	3.67	2.83	2.78	2.86	2.74

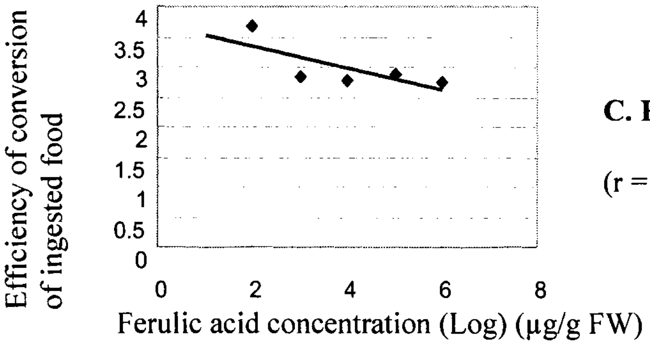
Fig. 10A - C Linear regression analysis of effects of FA-supplemented diet on larval growth of ECB. A. Relative growth rate; B. Consumption index; and C. Efficiency of conversion of ingested food.



A. Relative growth rate
($r = -0.5$, $r^2 = 0.25$, $p < 0.001$, $n = 100$)



B. Consumption index
($r = 0.4$, $r^2 = 0.04$, $p = 0.035$, $n = 100$)



C. Efficiency of conversion of ingested food
($r = -0.3$, $r^2 = 0.06$, $p = 0.010$, $n = 100$)

2.4 Discussion

Transgenic maize with wheat OXO gene showed significantly greater resistance to ECB compared with the null lines. Homozygous CK44 line showed the highest level of OXO expression, which was 3 - fold greater than that in the hemizygous CO286 line. No OXO activity could be detected in the corresponding OXO null lines. Thus, the genetic modification of maize with OXO increased OXO enzymatic activity in the OXO maize lines. The present study demonstrated that the transformed maize lines possessed an altered secondary metabolism in the biosynthetic pathways of hydroxamic acids and hydroxycinnamic acids. Thus, this suggests that the increased OXO activity triggered the alteration of biosynthesis pathways. The altered secondary metabolism was a factor in insect resistance of transformed maize.

Hydroxamic acids are major compounds in phytochemical defense against insect pests and pathogens in cereals. However, in the present study, a significant increase of resistance to ECB occurred with the significant drop of DIMBOA and MBOA levels. They were 10.6 and 4.1 fold lower respectively in transgenic line than null line. It suggested that biosynthesis of DIMBOA was down - regulated in the OXO transgenic maize due to the diversion of the metabolic pathway caused by the new OXO gene. The similar situation was observed in transgenic Bt maize (Nie et al., 2005). The introduction of the Bt gene had adverse effects on the biosynthesis and accumulation of DIMBOA in the maize plants. Under conditions of water or nitrogen stress, the accumulation of DIMBOA in the leaves of the Bt maize could be enhanced. The researcher suggested that Bt maize might have a weaker capacity to synthesize

DIMBOA in the leaves relative to traditional Chinese corn hybrids.

Unlike hydroxamic acids, biosynthesis of hydroxycinnamic acids was significantly enhanced by the elevated OXO activity in OXO transgenic maize. Total soluble phenolic level in the OXO maize line was increased by 3.2 times compared with the null line. The alteration in secondary metabolism pathway resulted in the significant increase of soluble phenolic acid contents. Usually, most simple phenolics in maize plants are bound to other moieties, the majority of which exist as esters (Newby et al., 1980). However, in the OXO maize, free phenolics accounted for 80.8% of soluble phenolics, which was 12 - fold higher than the ester - bound products. In particular, free FA was 16.6 - fold higher than its esterified coupling products. This situation suggested that the high contents of soluble phenolics acids, in particular, free FA, most likely contributed as defense factors to ECB in OXO transgenic maize lines. In artificial diet tests, reduction of larval growth was significantly and negatively related to FA content in the diet.

The present results showed that the secondary metabolism of maize plants was altered due to the insertion of the exotic gene OXO. The diversion in biosynthesis pathway of hydroxamic acids and hydroxycinnamic acids resulted in the increased phenolic acid level and decreased DIMBOA level. These results suggested that hydroxamic acids were not important resistance factors for the OXO maize. High concentrations of soluble phenolic acids, particularly ferulic acid probably became the new defense phytochemical factor, and H_2O_2 may play a secondary role.

Chapter 3

Plant *in vitro* regeneration of cowpea [V*igna unguiculata* (L.) Walp.] via shoot organogenesis and somatic embryogenesis

3.1 Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important food for people in Western and Central Africa. The analyses of the cowpea cultivars from Senegal and USA by Hall et al. (2003) showed that the crude protein in grain was 22% to 29%. The protein contents of tender leaves were between 27.1% and 34.7% on a dry weight basis (Ahenkora et al. 1998). Like other legumes, cowpea can fix atmospheric nitrogen and therefore can improve soil fertility (Hall et al. 2003). The most remarkable characteristic of cowpea is its tolerance to dry environments. For example, the cowpea variety Ein El Gazal produced 596 kg dry grain/ha in an area with an average 285 mm of annual rainfall (Elawad and Hall 2002). According to estimates in 2003, the total area under cowpea cultivation was 14 million hectares worldwide. West Africa alone accounted for 9.3 million hectares of this area with the annual dry grain production of 2.9 million tonnes (Singha et al. 2003).

Cowpea production and productivity have been far below their potential due to a number of biotic and abiotic stresses. Among biotic stresses, the cowpea pod borer *Maruca vitrata* (Lepidoptera: Pyralidae) is an important field insect pest that often causes a tremendous loss of grain yield. Genetic transformation of cowpea for insect resistance may show promise in protecting cowpea. Unfortunately, cowpea, like most legumes, is recalcitrant to *in vitro* regeneration, which limits its potential for improvement via genetic manipulations (Brar et al. 1999a). Cowpea *in vitro* regeneration via shoot organogenesis was first reported in 1981, but progress since then has been limited. To date, about 57 cultivars and breeding lines have been explored for organogenesis potential, such as Vita 5 - exiita (Kartha et al. 1981), C-152 (Muthukumar et al. 1995; Amitha et al. 1996), Ife Brown and Vita3 and IITA breeding lines (Pellegrineschi 1997), Coronet etc. and USA breeding lines (Brar et al. 1997; 1999a). However, shoot organogenesis was obtained from only a very few of the genotypes tested. In particular, the agronomically important cowpea variety blackeye has not been regenerated. Earlier studies indicated that cowpea *in vitro* regeneration responses are genotype specific (Appendix 9). Furthermore, the variability involved almost every aspect of the regeneration systems explored, such as optimal explant tissues, basal salt compositions, plant growth regulators (PGR) and sucrose concentrations.

Among many varieties and breeding lines of cowpea grown in the world, blackeye cowpea possesses high yield potential as well as early flowering and maturation. These characteristics are well suited to the natural climatic conditions in tropical Africa, including short rainy seasons, little available soil moisture and hot temperatures. At present, local African genotypes are grown under harsh conditions and are adapted to the arid tropical Savanna area but have low yield. Therefore, a breeding program crossing the local African genotypes with the blackeye cowpea has been designed in order to develop new, advanced lines (Hall et al. 2003). Thus far, this program has developed high - yielding cultivars and breeding lines for the arid zones in African as 'Ein El Gazal' (Elawad and Hall 2002).

The present study was aimed at achieving an effective and reproducible protocol for the regeneration of *V. unguiculata* cv. blackeye cowpea via shoot organogenesis. Such a regeneration system should enable the stable genetic transformation of this commercial genotype for insect resistance and other traits. To our knowledge, *in vitro* regeneration of *V. unguiculata* cv. blackeye cowpea has not been previously reported.

3.2 Materials and Methods

3.2.1 Plant materials

The explant sources explored were (1) mature cotyledons, embryo tips, hypocotyls and radicals from mature dry seeds; (2) immature cotyledons, embryo tips, hypocotyls and radicals from immature green pods; (3) shoot apices and primary leaf segments, leaf petioles and plant stem segments from 3 - 5 day old seedlings and older seedlings (>7 day old).

Mature seeds were surface sterilized in 70% ethanol for 1 min and shaken for 15 min in 15% commercial bleach containing 6% sodium hypochlorite. Two drops of Tween 20 per liter (Sigma Chemical Co.) were added to the solution. Seeds were rinsed 4 - 5 times in sterile distilled water and soaked in fresh distilled water for 4 - 24 hour. Mature cotyledons, embryo tips, hypocotyls and radicles were excised from imbibed seeds. To obtain seedlings, the surface sterilized seeds were grown in Petri dishes (60 × 20 cm). Shoot tips, leaf discs, primary leaf petioles and stem segments were detached from 3 - 5 day old (or >7 day old) aseptic seedlings. To obtain 3 - 5 mm long immature seeds, green pods were harvested about 7 days post - flowering from plants grown in the greenhouse. The immature seeds were surface sterilized using the same method as mentioned for mature seeds. Cotyledons, embryo tips, hypocotyls and radicles were excised from these seeds.

3.2.2 Medium formulations and culture conditions

Basal media for organogenesis initiation involved the use of MS (Murashige and Skoog 1962), BM (Pellegrineschi et al. 1997) and B_5 (Gamborg et al. 1968). For MS basal media, third strength MS (1/3 MS), half strength MS (1/2 MS), and full strength MS were evaluated. Either MS vitamin mix or B_5 vitamin mix was used in each basal medium (Table 8). Sucrose was added to the media at concentrations of 1.0%, 1.5%, 2.0%, 3.0% or 4.0%. All media were supplemented with 1 g l^{-1} casein hydrolysate (Sigma), 0.5 mg l^{-1} L - glutamine (Sigma) and 0.8% (w/v) Bacto agar prior to being autoclaved. Plant growth regulators (PGRs) N - benzylaminopurine (BAP), 6 - furfurylaminopurine (KN), 2,4 - dichlorophenoxyacetic acid (2,4 - D), naphthalene - acetic (NAA) and indole - 3 - acetic acid (IAA) were evaluated. A wide range of concentrations as well as various combinations of these regulators was used in each basal medium (Table 8). The pH of the media was adjusted to 5.8 and 7.0 by NaOH and / or HCl. All the media were autoclaved after adding phytagel. The shoot initiation medium was used for proliferation and maintenance of the organogenic tissue. Subcultures were performed biweekly. The effect of silver nitrate ($AgNO_3$)

Table 8 Possible permutations and combinations of medium formulations for initiation of cowpea regeneration tissues

Basal salt formulations	Vitamin formulations	PGR concentration (mg l^{-1})				
		BAP	2, 4 - D	NAA	KN	IAA
1/2MS	MS vitamin mix	0.1 - 20.0	0	0	0	0
1/3MS		0.1 - 2.0	0.5 - 5.0	0	0	0
MS		0.1 - 2.0	0.5 - 5.0	0	2.0	0
BM		0	0.5 - 40.0	0	0	0
B_5		0.1 - 2.0	0	0.1 - 0.5	0	0
		0	0	0.5 - 10.0	0	0
		0.5 - 2.0	0	0	0	0.5
MS	B_5 vitamin mix	0.1 - 10.0	0	0		
		0.1 - 2.0	0.5 - 5.0	0		
		0	0.5 - 40.0	0		
		0.1 - 2.0	0	0.1 - 0.5		
		0	0	0.5 - 10.0		

on cowpea regeneration was also investigated. Four levels of AgNO3 were assessed: 0 mg l^{-1}, 5 mg l^{-1}, 10 mg l^{-1} and 15 mg l^{-1}. Regenerated shoots (1 to 2 cm long) were transferred to shoot elongation medium made up of regeneration medium supplemented with 0.5 - 5.0 mg l^{-1} gibberellic acid (GA_3). Rooting medium was composed of MS basal salts supplemented with B_5 vitamin mix with or without the addition of 10 mg l^{-1} indolebutyric acid (IBA).

For shoot initiation culture, all the explants were incubated at 26 ± 2 °C under cool - white fluorescent light (10 μM m^{-2} s^{-1}) and a 16 -hour photoperiod for two weeks. The elongation and rooting of adventitious shoots were maintained under cool white fluorescent light (20 - 40 μM m^{-2} s^{-1}) and a 16 - hour photoperiod at 26 ± 2 °C. Plantlets with healthy root systems were transferred to the soil containing a mixture of peat, perlite and vermiculite in a proportion of 3.5:1:0.5. During the growing period, 50 ppm of 20 – 08 - 20 fertilizer (N - P - K) was applied weekly.

3.2.3 Experimental design and data analysis

Experiments screening the basal media optimal for shoot initiation were designed as a randomized complete block. Each treatment group and control was made up of 3 - 5 Petri dishes (60 ×15 mm) with 4 - 6 explants per dish and three replicates per treatment and control. Response of explants to the shoot initiation culture was monitored using a binocular microscope. Two parameters shoot initiation frequencies and shoot numbers per explant, were applied to evaluate the influence of different explant sources and media. The initiation frequency was obtained by calculating the numbers of explants with adventitious shoots relative to the total number of cultured explants. The shoot initiation frequencies and shoot numbers per explant were recorded 8 weeks after the shoot initiation culture. The data were subjected to analysis of variance (ANOVA) (Analysis - it for Microsoft Excel, 1997 - 2000). Multiple comparisons were applied in deciding optimal factors and corresponding concentrations.

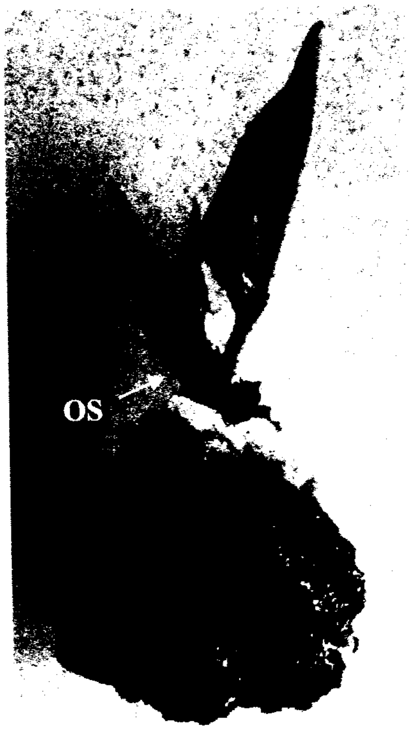
3. Results

3.3.1 Effects of explant sources on regeneration initiation

Two types of cell mass were produced after the plant tissues were explanted on media. Within 3 - 5 days of induction culture, a whitish and friable cell mass was first observed on the surface of some explants, with the exception of radicles and stem discs. This white callus did not exhibit regeneration potential. In contrast, a greenish and compact cell mass emerged from the bottom of other explants after 3 - 10 days (Fig. 11A). Under the microscope, adventitious shoot (AS) initials were observed on the callus at this stage. The elongated shoot buds became visible within 4 weeks (Fig. 11B).

Among an array of explant sources assessed, shoot apices and primary leaf petioles from 3 - 5 day old seedlings, mature hypocotyls and mature embryo tips formed adventitious shoots. A greenish and compact cell mass emerged from the bottom of other explants after 3 - 10 days. The elongated shoot buds became visible within 4 weeks (Fig. 11B). However, apart from seedling shoot apices, the other

Fig. 11A - B Explanted shoot tip and initiation of adventitious shoots. Shoot tips from 3-5 day-old seedlings were proved to be the optimal explant. Within 1 week, greenish callus occurred from the bottom of the explants (A). Original shoots (OS) continued to develop and primary leaves were elongated. Within 3 - 4 wk, adventitious shoots (AS) became visible on the surface of organogenic tissue (B).



A

B

three explant sources exhibited very poor initiation responses (Fig. 12A - B). Also, the potential of organogenesis was gradually lost during subsequent culture. Conversely, immature hypocotyls, leaf segments, petioles and mature hypocotyls produced non - organogenic white calli. The whitish and friable cell mass was first observed on the surface of some explants after 3 - 5 days on shoot induction. This white callus did not exhibit regeneration potential. The radicles, stem discs, cotyledons, on the other hand, did not exhibit any callus formation.

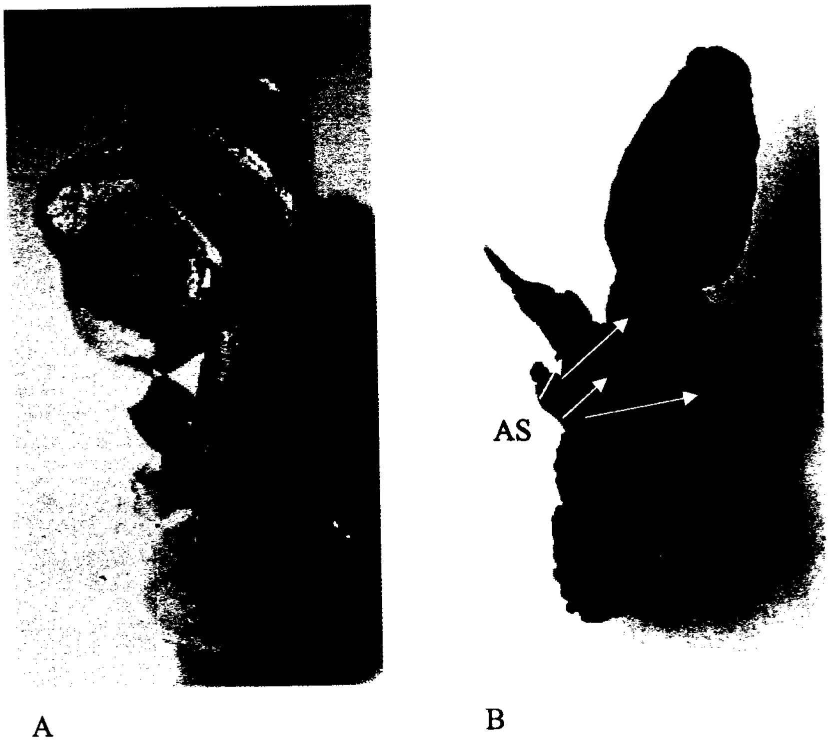
3.3.2 Effects of basal medium formulations on regeneration initiation

Among the five basal salt formulations investigated - 1/3 MS, 1/2 MS, MS, BM and B_5, shoot formation occurred when MS salt mix was combined with B_5 vitamin mix. MS salt mix supplemented with MS vitamin mix favored non - organogenic white calli. Similarly, B_5 basal salts with B_5 vitamin mix produced white calli. One third MS, 1/2 MS and BM with either MS vitamin mix or B_5 vitamin mix also produced white calli.

3.3.3 Effects of plant growth regulators and pH values on regeneration

The PGRs KN, 2,4 - D, NAA or IAA failed to initiate shoot organogenesis. KN was found toxic to cowpea shoot formation. Explants on media containing KN differentiated into adventitious roots rather than shoots. 2,4 - D favored the formation of non - organogenic calli. In contrast, the two auxins, NAA and IAA, were effective in the formation of greenish compact calli, which produced abundant hairy adventitious roots instead of shoots. No shoot organogenesis was observed by

Fig. 12A - B Primary leaf petiole explanted and initiation of adventitious shoots. Primary leaf petiole (A) also gave rise to shoot formation. Within 3 - 4 week, adventitious shoots were induced (B). But, initiation frequency and shoot number per explant were low. Also, the initiation potential was easily lost during subculture.


AS
A
B

using KN, 2,4 - D, NAA or IAA in combination with BAP. BAP played a key role in shoot formation as only those media containing it formed adventitious shoots. Different BAP levels resulted in a strong variability of initiation frequencies of AS and AS numbers per explant (Fig. 13 and Fig. 14). ANOVA results revealed that the differences among BAP levels on initiation frequencies were significant ($p < 0.01$) (Table 9). At BAP 0.5 mg l^{-1}, shoot apices produced green calli with no subsequent shoot formation. As BAP concentration increased from 0.5 to1.0 mg l^{-1}, adventitious shoots began to grow from the green calli. The highest initiation frequency (76.64%) was obtained at 2 mg l^{-1} BAP (Table 10). Multiple comparison tests verified that the effect of BAP at 2.0 mg l^{-1} was significantly different from the other five BAP levels (0.5, 1.0, 3.0, 5.0 and 10.0 mg l^{-1}) (Table 10). The influence of varying BAP levels on AS numbers was similar to that of BAP's impact on initiation frequencies. For example, in both cases, the highest shoot number per explant was achieved at 2.0 mg l^{-1} (Table 10). Analysis of variance indicated that the difference in the numbers of adventitious shoots from varying BAP levels was statistically significant ($p<0.05$) (Table 10). Multiple comparisons showed that the effect on the numbers of adventitious shoots per explant from 2.0 mg l^{-1} BAP was significantly different from the other three levels (Table 10). Therefore, it is concluded that the optimal BAP level for the initiation of AS is 2.0 mg l^{-1}, at which concentration shoot tips give the highest initiation frequency (76.64 %) and shoot number per explant (8.1).

The pH value of the medium was another factor contributing to AS initiation. A

Fig. 13 Initiation frequency (means ± S.E.) of adventitious shoots from shoot tips of 3 - 5 days old seedlings on MS + B5 basal medium formulations with different BAP levels and pH values.

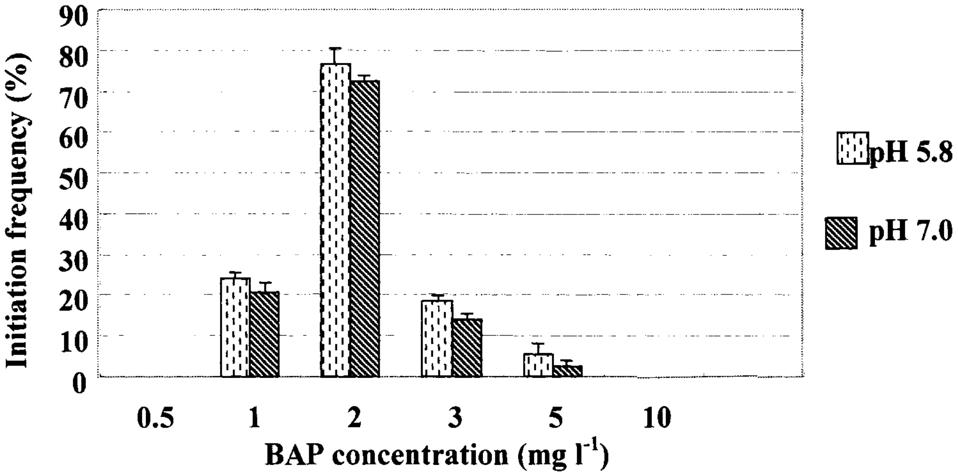

Initiation frequency (%)
90
80
70
60
50
40
30
20
10
0
0.5
1
2
3
5
10
BAP concentration (mg l-1)
pH 5.8
pH 7.0

Fig. 14 Number (means ± S.E.) of adventitious shoots per explant from shoots tips of 3 - 5 day old seedlings on MS + B5 basal medium formulations with different BAP levels and pH values.

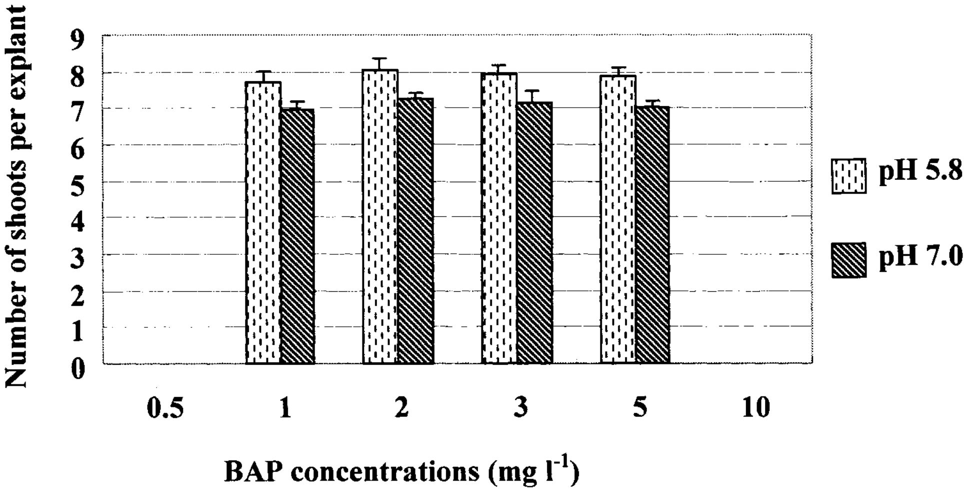

Number of shoots per explant
9
8
7
6
5
4
3
2
1
0
0.5
1
2
3
5
10
BAP concentrations (mg l^{-1})
pH 5.8
pH 7.0

Table 9 Analysis of variance on initiation frequencies of adventitious shoots from shoot apices of 3 - 5 day old seedlings as affected by BA levels at pH 5.8

Variation	SSq	DF	MSq	F	p
BA levels	12455.8	5	2491.2	240.8*	<0.0001
Error	124.1	12	10.3		
Sum	12579.9	17			

* The above ANOVA employed the T test, significant at $p < 0.01$.
SSq: sum of square
DF: degree of freedom
MSq: mean square

Table 10 Multiple comparisons of initiation frequencies of adventitious shoots and shoot numbers per explant as affected by BA levels at pH 5.8

BA concentrations (mg l^{-1})	Initiation frequencies (%)	Numbers of shoots per explant
1.0	23.84 ± 0.90 b	7.68 ± 1.03 db
2.0	76.64 ± 2.71 a	8.01 ± 0.56 a
3.0	18.45 ± 1.24 c	7.92 ± 0.67 bc
5.0	5.46 ± 1.39 db	7.84 ± 1.25 c
10.0	2.05 ± 0.86 ed	1.43 ± 0.49 e

Means ± S.E. within the same column followed by different letters were significantly different; otherwise, the difference was not significant.

medium pH of 5.8, as compared to pH 7.0, resulted in noticeably higher shoot numbers. The difference of shoot numbers as affected by pH values was significant (Table 11 and Fig. 12). The highest numbers of ASs (8.01 per explant) occurred with medium pH 5.8 partnered with 2.0 mg l^{-1} BAP. Shoot formation and AS numbers per explant were significantly enhanced on media with pH 5.8. In contrast, pH 7.0 was favorable to the initiation of non - organogenic white calli.

3.3.4 Effects of sucrose and silver nitrate concentrations on regeneration

Few calli were formed when explants were cultured on media with 1.0, 1.5 and 4.0% sucrose. Shoot formation was obtained only with 3.0% sucrose. The addition of silver nitrate ($AgNO_3$) inhibited calli from browning (Fig. 15A - D). After about 2 weeks, calli cultured on the control medium (without silver nitrate) turned brown. In contrast, calli on the medium supplemented with 10 mg l^{-1} silver nitrate remained green and shoot formation occurred 3 - 5 day earlier than in the case of the control. No obvious difference in initiation frequencies or in numbers of shoots per explant was observed (Data not shown).

3.3.5 Proliferation and rooting of AS and plantlet growth in greenhouse

Have transferred to the same initiation medium, the organogenic tissue with AS initials was proliferated, and clusters of shoots were developed (Fig. 16A - B). However, shoots grew slowly on this initiation medium (Fig. 16A). Addition of 5.0 mg l^{-1} of GA_3 promoted elongation of shoot initials. Elongated shoot buds with primary leaves and branches became visible in 4 - 5 weeks, (Fig. 16B). At this point,

Table 11 Analysis of variance of numbers on adventitious shoots per explant from shoot tips of 3 - 5 day old seedlings as affected by BAP levels and pH values

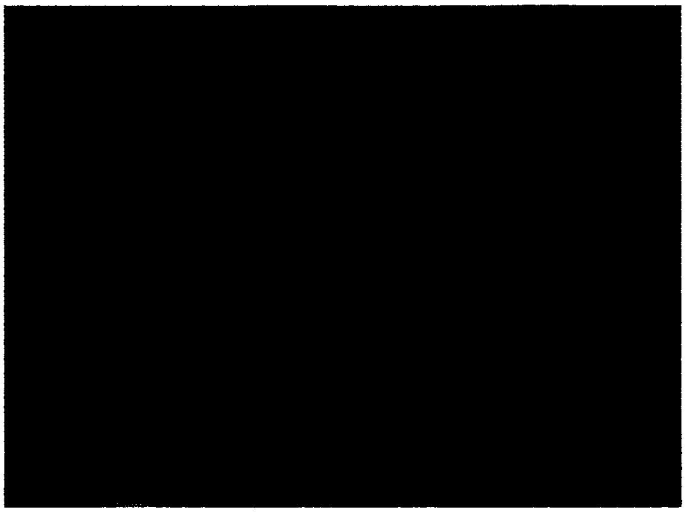
Variation	SSq	DF	MSq	F	p
BA levels	0.1347	3	0.0449	23.6*	< 0.05
pH values	1.5955	1	1.5955	839.7**	< 0.01
Error	0.0058	3	0.0019		
Sum	1.7360	7			

The above ANOVA employed the T test.
SSq: sum of square; DF: degree of freedom; and MSq: mean square.
* Significant at $p < 0.05$ for difference from different BA levels
** Significant $p < 0.01$ from different pH levels

Fig. 15A - D Effects of silver nitrate on inhibition of callus from browning. **A and B**. Callus on the medium without the addition of $AgNO_3$ turned brown within 2 weeks. **C and D**. Callus on the medium with 10 mg l^{-1} silver nitrate stayed green and fresh within 2 weeks.

Fig. 16A - B Elongation of adventitious shoots in cowpea. A. ASs grew slowly on the regeneration medium without gibberellic acid; B. ASs were elongated obviously in 4 - 5 weeks by adding gibberellic acid (0.5 - 5.0 mgl^{-1}) in the regeneration medium.

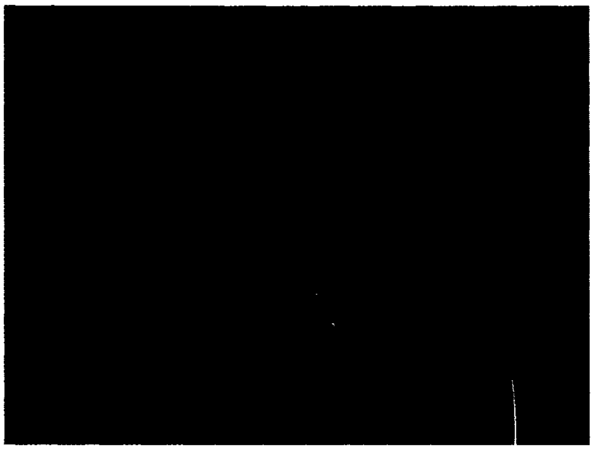
A



B

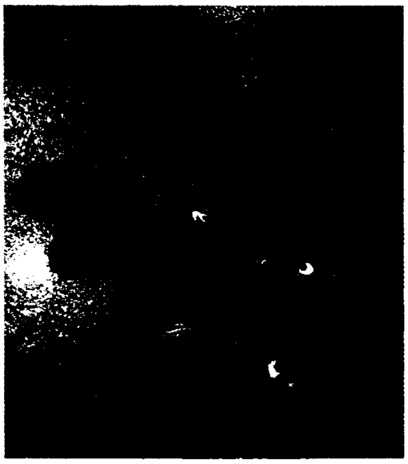
the elongated shoots were ready for root induction. Within 1-1.5 weeks, the excised shoots produced white healthy roots on medium supplemented with 10 mg l^{-1} IBA or in the absence of PGRs (Fig. 17A - B). Rooted plantlets with healthy roots achieved a 100% survival rate subsequent to being transplanted into soil (Fig. 17C). The regenerated plants were demonstrated to be fertile with high yields of mature, dry seeds (Fig. 17D).

Fig. 17A - D Rooting and growing of cowpea plantlets. **A - B**. White healthy roots appeared on excised ASs in 2 weeks after transplanting to the rooting medium. **C - D**. Plants transplanted into the soil. Regenerated plants in greenhouse produced flowers and green pods within 6 weeks.

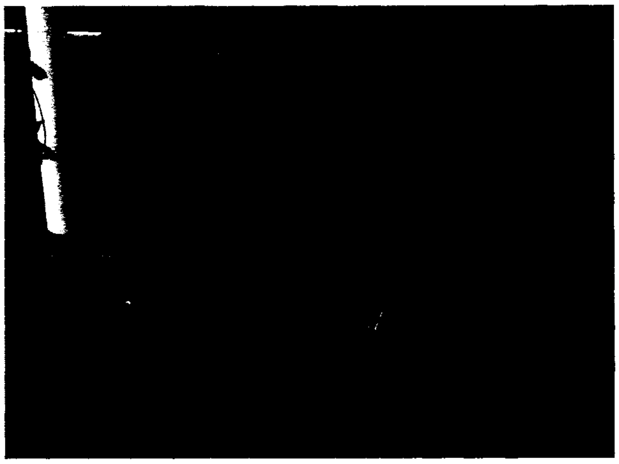


A

B



C



D

3.4 Discussion

In the present study, a wide range of explant sources was evaluated for their response to shoot organogenesis. A stable shoot organogenesis was observed only in shoot apices of 3 - 5 days old seedlings. Leaf petioles of 3 - 5 day old seedlings, mature hypocotyls and mature embryo tips, showed regeneration potential but their responses were weak and unstable. Other plant sources either formed non-organogenic calli or poorly reacted to regeneration conditions. The strong variability in response to *in vitro* regeneration among different plant tissues was consistent with previous reports on cowpea regeneration in other *V. unguiculata* species (Appendix 9). For instance, the optimal explant tissues from different genotypes evaluated varied from shoot meristems and root apices (Kartha et al. 1981; Kulothungan et al. 1993; Brar et al. 1997), to primary leaves (Pandey and Bansal 1989; Muthukumar et al. 1995), cotyledons and hypocotyls (Amitha and Reddy 1996; Pellegrineschi 1997; Brar et al. 1999a). Brar et al. (1999a) evaluated 36 cowpea genotypes for *in vitro* regeneration. Only 47% of those genotypes favorably responded with regeneration frequencies between 1% and 11%. Earlier reports on cowpea regeneration have indicated that a wide range of basal media have been used depending on the genotypes and explants used. Shoot organogenesis and embryogenesis were achieved using 1/3 MS (Brar et al. 1999a), 1/2MS (Kulothungan et al. 1995; Anand et al. 2000), full strength MS (Kulothungan et al. 1995; Amitha et al. 1996; 1998; Anand et al. 2000), BM (Pellegrinesch 1997) and B_5 (Muthukumar et al. 1995). All the media exhibited a wide range of macro-element concentrations (Table 12). For instance, full strength MS has a 2 - 3 fold higher level of macro-elements than 1/2 MS and 1/3 MS. B_5 medium does not contain ammonium nitrate (NH_4NO_3) and potassium phosphate (KH_2PO_3). However, the concentration of

Table 12 Comparison of macro-elements

of MS, B_5 and BM media used for cowpea regeneration

Components	Concentration (mg l^{-1})				
	1/3 MS	1/2 MS	MS	BM	B5
NH_4NO_3	550.0	825.0	1650.0	264.0	
KNO_3	666.3	900.0	1900.0	684.0	3000.0
KH_2PO_3	56.7	85.0	170.0	6.80	
$CaCl_2{\cdot}2H_2O$	110.7	166.6	332.2	23.25	150.0
$MgSO_4{\cdot}7H_2O$	60.3	90.35	180.7	18.07	500.0
$NaH_2PO_4 H_2O$					150.0

potassium nitrate (KNO_3) in B_5 medium is as high as 3000.0 mg l^{-1}, compared to 1900.0 mg l^{-1} in MS. Pellegrineschi et al. (1997) observed that cowpea zygotic embryos have very low levels of macro-elements (N, P, K, Mg, and Ca). Based on this observation, Pellegrineschi et al. (1997) formulated BM in which macro-element contents were reduced by 1/3 - 1/24 from the MS formulation. By using such a BM basal medium with the addition of MS vitamin mix, shoot organogenesis was successfully achieved from hypocotyls and cotyledons of green pods (Pellegrineschi 1997). The difference in vitamin composition between MS and BM is evident (Table 13). Thiamine content in B_5 medium is over 10 times higher than in MS. In the present report, adventitious shoot formation only occurred with the MS medium when combined with B_5 vitamin mix. MS salts with MS vitamins and B_5 salts with B_5 vitamins were unfavorable for shoot organogenesis. Thus, in order to form adventitious shoots, the blackeye cowpea has a specificity for macro-elements requiring 2 - 3 fold higher concentrations of nitrogen, phosphate and potassium as compared to other cowpea genotypes previously reported by Kulothungan et al. (1995), Muthukumar et al. (1995), Pellegrinesch (1997), Brar et al. (1999) and Anand et al. (2000). The blackeye cowpea's specific demand for vitamin nutrition resulted in enhanced cell division and differentiation during shoot formation with higher levels of thiamine HC1 (10 mg l^{-1}).

A range of PGRs including BAP, KN, ZN, 2,4 - D, 2,4,5 - T, 2,4,5 - TP, NAA, IAA, and IBA has been reported in cowpea regeneration in different concentrations. Successful regeneration was obtained from 5.0μM BAP+80.0μM 2, 4, 5 - T (for 20 day treatment) (Muthukumar et al. 1995), 66.6μM BAP (for 5 day treatment) (Muthukumar et al. 1995), 111.0 μM (for 20 day treatment) and 2.0μM BAP+ZN 0.1

Table 13 Comparison of vitamin composition of MS and B_5 media used for cowpea regeneration

Components	Concentration (mg l^{-1})	
	MS	B_5
Myo-inositol	100	100
Nicotinic Acid	0.5	1.0
Pyridoxine HC1	0.5	1.0
Thiamine HC1	0.5	10
Glycine	2	-

mg l^{-1} (for another 20 day treatment) (Pellegrineschi et al. 1997), 6.78 μM 2,4 - D (Anand et al. 2000), 9.05 μM 2,4 - D (Kulothungan et al. 1995), 2.0 - 5.0 μM 2,4 - D and 0.5 - 1.5μM 2,4,5 - T (Amitha et al. 1998). Kulothungan obtained mature somatic embryos in liquid MS medium by decreasing the concentration of 2,4 - D from 9.05 μM to 4.50 μM (Kulothungan et al. 1995). However, Anand et al. (2000) suggested that 2,4 - D level should be lowered to 0.05 μM when adding ABA (5 μM) and mannitol (3%) during embryo maturation. In the present study, PGR type and concentration in media strongly influenced cowpea regeneration responses. The best regeneration result was obtained by using 2 mg l^{-1} BAP among the five PGRs tested. KN, 2,4 - D, NAA and IAA each produced non - organogenic calli.

It was observed that cowpea organogenesis initiation was sensitive to sucrose levels. Kulothungan (1995) and Anand (2000) obtained regeneration tissue from P152 young leaves on MS solid medium with 3% (w/v) sucrose. A report on the C-152 variety, on the other hand, showed that no regeneration response was observed when sucrose levels surpassed 1.5% (w/v) on solid MS (Amitha and Reddy 1998). Our study on blackeye cowpea was in agreement with the results from Kulothungan et al. (1995) and Anand et al. (2000) on the P152 cowpea line. Shoot formation needed as high as 3.0% (w/v) of sucrose. We observed that freshly regenerated calli readily turned brown and lost viability, inhibiting callus growth during the callus - induction stage. In fact, about one third of calli were lost due to severe browning. One of the factors causing browning of the callus is the presence of ethylene. Ethylene is a plant growth regulator produced by the callus during tissue culture process. Silver nitrate, an ethylene inhibitor, can improve regeneration in various species (Tiainen 1992; Mederos-Molina and Trujillo 1999). The present study showed that the addition of 8.48 mg l^{-1} $AgNO_3$ was helpful in minimizing callus browning.

Chapter 4

General conclusion and discussion

4.1 General conclusion

In previous research from our group, maize was transformed with OXO and the OXO maize was highly resistant to ECB. However, the mechanism of insect resistance was unknown. In this thesis, new information about the mechanism in the OXO maize was obtained. Prior to this investigation, cowpea had not yet been transformed with OXO due to lack of an *in vitro* regeneration system. In the second part of the thesis, a stable regeneration system for blackeye cowpea was developed. The established protocol is now being used in cowpea transformation with the OXO gene and the Bt genes for enhanced insect resistance. The bioassay of insect resistance and field test of transgenic cowpea will be initiated once the OXO or Bt cowpea is achieved.

4.2 Improved resistance to ECB in transgenic maize with OXO gene

The genetic modification of maize with wheat oxalate oxidase increased OXO enzymatic activity in OXO maize lines, and the transformed lines were more resistant to insects in field tests. Alteration of secondary metabolism was observed in two biosynthetic pathways: phenolics and DIMBOA. The enhancement of phenolic synthesis resulted in the significant increase of soluble phenolic acid contents, especially free phenolics. In the CK44 OXO homozygous line, the levels of total soluble phenolic acids, FA, DFA, and *p*-CA were 3.2-, 3.6-, 2.8- and 2.0- fold higher than they were in the corresponding null line, respectively. FA was the most prominent intracellular soluble phenolic acid in OXO maize, which accounts for 80.4% (trans-: cis- ≈ 2:1) of the total soluble phenolics. The soluble phenolic acids evaluated were present mostly as free phenolics (84.8 %) in leaf tissue. In contrast, ester - bound and ether - bound phenolic conjugates were only 5.1% and 0.7 %. In transgenic line B73, free phenolic acids accounted for 77.1% while ester - and ether - bound phenolics were only 8.5% and 1.0% respectively, which were consistent with the conclusion from the homozygous line CK44. Localization of phenolic compounds in maize leaf upper epidermis by fluorescence microscope confirmed visually that there was a higher content of soluble phenolics in OXO lines than the corresponding null line. The increase in soluble phenolic contents negatively corresponded with lower insect damage. Visual leaf damage ratings were significantly reduced by 35 - 42% in the OXO transgenic lines. Reductions in stalk tunneling ranged from 40.2% in CO328 hemizygous line to 45.4% in CO286 hemizygous line and 46.3% in CK44 homozygous line. Clearly, the presence of the wheat OXO gene significantly reduced ECB damage on leaves and stalk in all cases. The bioassay of FA in artificial diet on ECB showed that ECB larval growth was reduced as the FA concentration was increased. The experiment demonstrated the sensitivity of ECB to FA but significant effects were found at higher concentrations than that measured in OXO maize. The HPLC analysis of OXO maize was a crude total leaf measurement, and the microscope clearly showed that most of the free FA was located in epidermis and parenchyma, the preferred feeding substrate for the larvae. The contents in the tissues where the insects were feeding may be much higher than the HPLC data suggested.

Generally, in conventional maize, free phenolic acid content is low in comparison to phenolic acids that are conjugated through ester linkages to sugars (Bergvinson, 1993). Most simple phenolics are bound to other moieties, the majority of which exist as esters (Newby et al., 1980). However, in the OXO maize, free phenolics accounted for 80.8% of soluble phenolics, which was 12 - fold higher than the ester - bound products. In particular, free ferulic acid was 16.6 - fold higher than its esterified coupling products. The result suggested that high contents of free phenolics might be a source for other defensive phenolic compounds.

Conversely to the phenolics, the biosynthesis of DIMBOA was down - regulated, and the DIMBOA level dropped, possibly due to diversion of metabolism to the phenolics. The hydroxamic acid DIMBOA is known as a plant defense phytochemicals. The role of the hydroxamic acids DIMBOA and MBOA in resistance to the ECB has been well demonstrated (Campos 1989; Reid, 1990, 1991; Houseman et al., 1992 and Bergvinson, 1993). The reduced DIMBOA level also implied hydroxamic acids were not resistance factors for OXO maize, and suggested that another novel mechanism is possibly operating.

The hyperacidification of insect gut from high contents of free phenolic acids is a direct way for secondary compounds to discourage insects from consuming tissue since ECB maintains a basic gut for optimal protease activity (Houseman et al., 1992). Alternatively, H_2O_2 produced by OXO has direct effects on ECB growth (Ramputh et al., 2002), and the combination of high FA and H_2O_2 may be significant (not tested).

4.3 *In vitro* regeneration of cowpea cv. blackeye

A wide range of explant sources were evaluated for their response to shoot organogenesis. A stable shoot organogenesis, and maximum initiation frequency and numbers of adventitious shoots per explant were obtained only in shoot apices of 3 - 5 day old seedlings. Other plant sources either formed non - organogenic calli or poorly reacted to regeneration conditions. The strong variability in response to *in vitro* regeneration among different plant tissues was consistent with previous reports on cowpea regeneration in other *V. unguiculata* varieties.

Full strength MS combined with B_5 vitamin mix produced adventitious shoots. Other treatments, either MS salts (1/3 MS, 1/2 MS and MS) combined with MS vitamins or B_5 salts with B_5 vitamins were unfavorable for shoot formation whereas non - regenerating calli was produced. It suggested that the shoot formation of blackeye cowpea required higher (2 - 3 fold) concentrations of macro-elements such as nitrogen, phosphate and potassium, and higher levels of thiamine HC1 (10 mg l^{-1}). PGR types and concentrations in media strongly influenced cowpea regeneration responses. Among the plant growth regulators evaluated, BAP levels significantly influenced shoot initiation frequencies. Other regulators, KN, 2,4 - D, NAA and IAA either produced massive numbers of fibrous roots or a non-functional cell mass.

The pH values significantly influenced numbers of adventitious shoots. The highest numbers of ASs (8.01 per explant) occurred with medium pH 5.8 while pH 7.0

was favorable to the initiation of non - organogenic white calli. The organogenesis initiation of the blackeye cowpea was sensitive to sucrose levels. Shoot formation needed as high as 3.0% (w/v) sucrose. Therefore, the optimal medium for maximum shoot initiation was comprised of MS salts, B_5 vitamins, BAP 2 mg l^{-1}, sucrose 3%, and agar 0.8%, adjusted to a pH of 5.8. The highest shoot initiation frequency (76.64%) was obtained using this most favorable medium, reaching a maximum numbers of shoots per explants of 8.1.

For shoot elongation, 0.5 - 5.0 mg $l^{-1}GA_3$ was required. Rooting of regenerated plants was obtained through a medium containing MS salts, B_5 vitamins, sucrose 3% and agar 0.8%, at a pH of 5.8. Presence of IBA in the rooting medium had a certain effect on root induction. The regenerated plants proved to be fertile and developed normally.

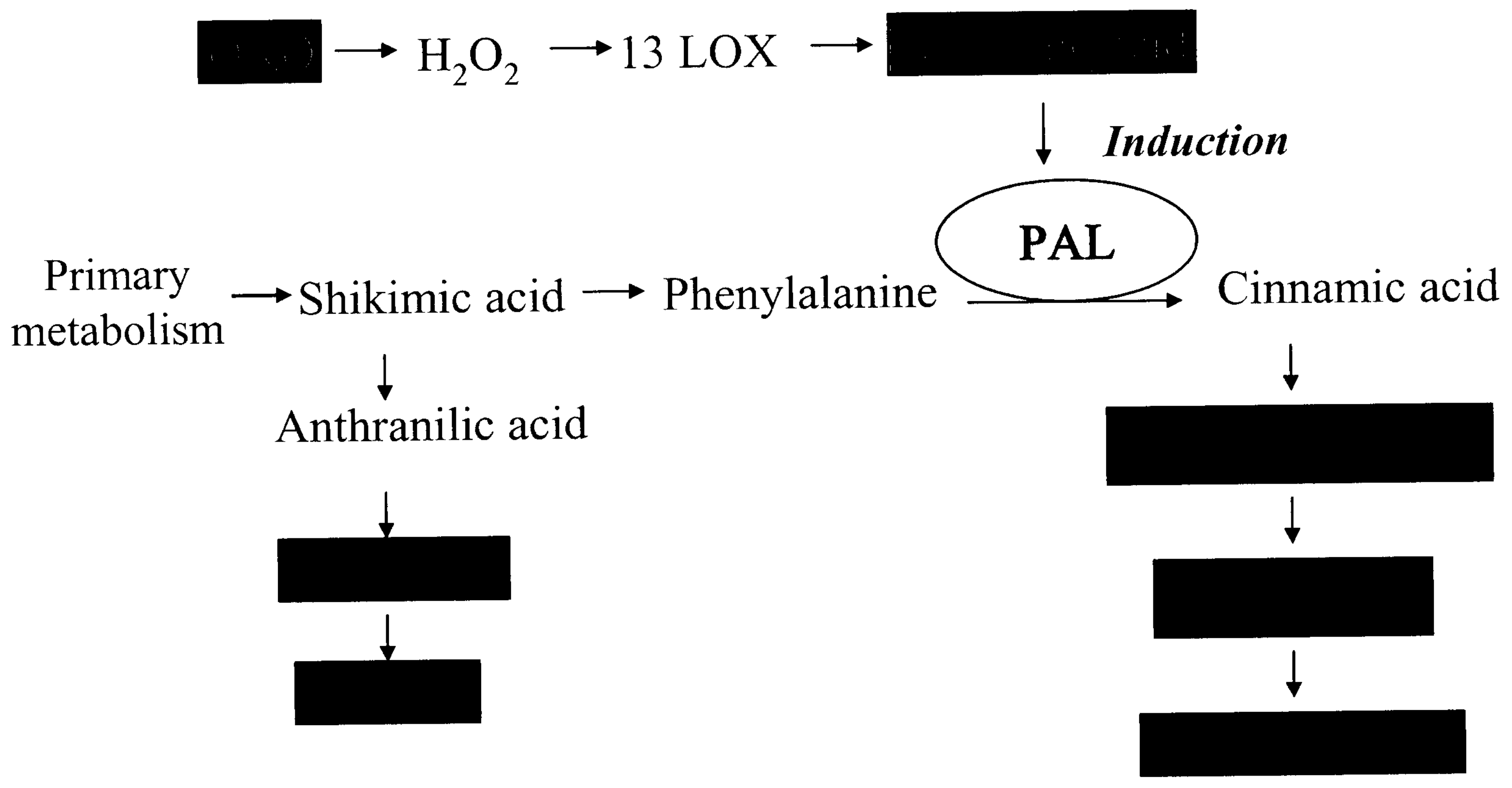
4.4 Implications

The genetic modification of traditional maize and cowpea varieties with wheat OXO was desired to improve host plant resistance. The alteration of metabolic pathways that was observed in this thesis was not entirely predictable due to the poor understanding of the metabolic networks involved (De Carolis and De Luca, 1993). The present study focuses our attention on the distribution of the metabolic precursors of the branch point in biosynthesis of hydroxamic acids and hydroxycinnamic acids in the shikimic acid pathway. This will help us to understand the metabolic networks that regulate the shikimic acid pathway, and more importantly the redirection of the flow of the key defense metabolites in maize and cowpea genetic engineering.

In higher plants, most of the phenolics are synthesized at least in part from the shikimic pathway. The formation of cinnamic acid by eliminating an ammonia molecule from phenylalanine is an important regulatory step in the synthesis of many phenolic compounds. This important reaction is catalyzed by PAL. Therefore, the activity of PAL plays a key role in controlling the flux through the pathway toward phenolic compounds. The activity of PAL can be increased by many factors. Jasmonic acid is a signaling compound involved in plant resistance to chewing insects. It was demonstrated that 13 - lipoxygenase (13 - LOX), the starting point for jasmonic acid biosynthesis, was significantly up - regulated in the OXO line [Simmonds et al., 2005 (personal communication)]. It was suggested that the significantly increased OXO activity in OXO maize lines triggers the octadecanoic acid pathway. The elevated octadecanoic acid pathway leads to JA synthesis, which activates PAL accumulation, a key step for phenolic synthesis. The increased activity and amount of PAL then stimulates the synthesis of phenolic compounds in the OXO maize. Thus, the metabolic flux to phenolics by diversion metabolism could reduce the synthesis of DIMBOA. The suggested biosynthesis of hydroxycinnamic acids and hydroxamic acids DIMBOA and MBOA in OXO transgenic maize lines can be demonstrated as shown by Fig. 18.

A very similar result involving the metabolic alteration of the shikimic acid pathway was obtained in transformed *Brassica napus* cv. Westar (Chavadej et al.,

Fig. 18 Suggested novel biosynthesis of hydroxycinnamic acids and hydroxamic acids DIMBOA and MBOA in OXO transgenic maize lines.


[redacted]
H_2O_2
13 LOX
[redacted]
Induction
PAL
Primary metabolism
Shikimic acid
Phenylalanine
Cinnamic acid
Anthranilic acid
[redacted]
[redacted]
[redacted]
[redacted]
[redacted]

1994). In oil- producing crops like *Brassica napus* (canola), the presence of indole glucosinolates in seedcake decreased meal palatability and limited their value as animal feed. A gene encoding tryptophan decarboxylase (TDC) was transformed in canola plants. The inserted exotic gene redirected tryptophan into tryptamine and diverted metabolism away from indole glucosinolates (Appendix 8).

A common concern regarding genetically modified foods is their safety to human beings and to the environment. The present study showed that the OXO transgenic maize had high level of soluble phenolic acid, in particular, ferulic acid. Elsewhere, the beneficial effects of ferulic acid have been well established both in human and animal studies. Many common foods, such as grains, seeds and leafy vegetables and fruits, contain large amounts of ferulic acid. Recently, ferulic acid has been described as an important nutriceutical due to its antioxidant properties, including application in anti-aging, diabetes, cardiovascular disease, cancer, neuroprotection, bone degeneration, menopause, immunity, and athletic performance. The effective dosage of FA is 250 mg twice per day (Wang and Ou-Yang, 2005). The FA concentration in OXO maize leaves is 2090.6 μg/g DW, and the metabolic change in other tissue like seeds and roots has not been tested. It is unlikely that ferulic acid levels in grain will threaten human safety but further testing of levels is required. The exotic OXO gene is from an agricultural crop, wheat. Taking together the beneficial effects of ferulic acid to human health, and the crop origin of the transgene, it is likely that the transgenic OXO maize or cowpea will be more readily accepted by the public than GMO's containing the Bt gene. The effects on ecological environments of OXO maize need further observation.

4.4 Future work

The understanding of how the OXO gene increases plant resistance to herbivores is still in its early stages. To date, what has been demonstrated is that the genetically modified maize with wheat OXO has resulted in a significant increase in soluble phenolics. More information is required on signaling pathways such as the role of the octadecanoic acid in elevated phenolic levels. Additionally, we know that two enzymes, PAL in the shikimic acid pathway of phenolic synthesis and *bx*1 in the DIMBOA synthetic pathway, play important roles in controlling the biosynthesis flux of the two pathways, and furthermore, the contents of phenolic acids and DIMBOA. In future experiments, we need to determine how the elevated OXO activity and increased H_2O_2 concentration affect accumulations of JA. In addition, the role JA plays in controlling the key enzyme activities could lead to a better understanding of the flux of two pathways.

The significantly improved resistance to insects in transformed OXO maize line and the achievement of the productive *in vitro* regeneration protocol of cowpea is a great encouragement for the future transformation of cowpea with insect resistance genes. Future projects in this field may provide insect resistant maize and cowpea genotypes that are sustainable for worldwide use.

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Appendix 1 Germin family and their demonstrated or putative function in major cereals*

Cereal Species	Nucleotides or proteins	GenBank Acc.	Products	Function	Tissue type
Wheat (*Triticum aestivum*)	**gf-2.8**	**M63223**	**OXO**	**Germination**	**Germinating embryo**
	gf-3.8	M63224	G	Germination	Germinating embryo
	pSBGer1	Y09915	GLP	Germination	Germinating embryo
	pSBGer2	Y09916	GLP	Germination	Germinating embryo
	pSBGer3	Y09917	GLP	Germination	Germinating embryo
	pSBGer4	Y09918	GLP	Germination	Germinating embryo
	OXO	AF005084	GLP	Metal-stress	Root tip
	glp2b	AJ237943	GLP	Pathogen	Leaf
	glp2a	AJ237942	GLP	Pathogen	Leaf
	Precursor	M21962	GLP	Germination	Germinating embryo
	G	AAB24031	G	Germination	Germinating embryo
	Ger4	T06563	Pseudo G	Germination	Germinating embryo
	Ger3	T06561	GLP	Germination	Germinating embryo
	Ger1	T06559	GLP	Germination	Germinating embryo
	Pseudo-G	S27248	GLP	Germination	Germinating embryo
	Pseudo-G	S27247	Pseudo G	Germination	Germinating embryo
	Pseudo-G	P29532	Pseudo G	Germination	Germinating embryo
	Precursor	A33268	Pseudo G	Germination	Germinating embryo
Barley (*Hordeum vulgare*)	**HvOXO**	**L15737**	**OXO**	**Germination**	**Germinating embryo**
	HvOxOa	**Y14203**	**OXO**	**Pathogen**	**Leaf**
	HvGLP1P	X93171	GLP	Pathogen	Leaf
	HvGLP1	Y15962	GLP	Pathogen	Leaf
	Subunit	U01963	GLP	Salt-stress	Root
	GL8	AF493980	GLP	***	***

	GL12	AF493981	GLP	***	***
	Ger D	AF250936	GLP	Tissue-specific	Developing embryo
	Ger F	AF250935A	GLP	Tissue-specific	Developing embryo
	Ger B	F250934	GLP	Tissue-specific	Developing embryo
	Ger E	AF250937	GLP	Tissue-specific	Developing embryo
	Ger A	AF250933	GLP	Tissue-specific	Leaf
	glp2	AJ310534B	GLP	Pathogen	Root
	GLP	Q740170	GLP	Drought-stress	Root
	GLP	BQ739995	GLP	Drought-stress	Root
	GLP	BM816594	GLP	Drought-stress	Roots
	GLP	T05956	GLP	Pathogen	Leaf
	GLP	T05721	GLP	Pathogen	Leaf
	Precursor	P45851	GLP	Salt-stress	Root
	GS2	P28526	GLP	Salt-stress	Root
	GS1	P28525	GLP	Tissue--specific	Leaf
	Crystals	1FI2A	GLP		Root
Rice (*Oryza sativa*)	GER1	AF032971	GLP	Tissue-specific	Shoot
	GER2	AF032972	GLP	Tissue-specific	Shoots
	GER3	AF032973	GLP	Tissue-specific	Panicle
	GER4	AF032974	GLP	Tissue-specific	Shoot
	GER5	AF032975	GLP	Tissue-specific	Shoot
	GER6	AF032976	GLP	Tissue-specific	Shoot
	GLP	CA765798	GLP	Drought-stress	Flowering Panicle
	GLP	CA765366	GLP	Drought-stress	Flowering Panicle
	GLP	CA762843	GLP	Drought-stress	Flowering Panicle
	Chromosome3	AC120508	GLP	Tissue-specific	
	Chromosome5	AAO38848	GLP	Tissue-specific	
	Chromosome5	AAO38486	GLP	Tissue-specific	
	Chromosome5	AC135427	GLP	Tissue-specific	
	Chromosome1	NM189765	GLP	Tissue-specific	

	Chromosome1	NM1897654	GLP	Tissue-specific	
	Chromosome1	NM191639	GLP	Tissue-specific	
	Chromosome1	NM187793	GLP		
	Chromosome1	NM187721	GLP		
	Chromosome8	AP005531	GLP		Growing tissue
	GLP1	T02239	GLP		Growing tissue
	GLP2	T02241	GLP		Growing tissue
	GLP3	T02591	GLP		Growing tissue
	GLP4	T02658	GLP		Growing tissue
	GLP6	T02660	GLP		Growing tissue
	GLP7	T02923	GLP		Growing tissue
	GLP8	T02931			
Maize (*Zea mays*)	GLP1	AF261941	GLP	Tissue-specific	Seedling
	GLP2	AF261942	GLP	Tissue-specific	Root
	glp1	AY394010	GLP	Tissue-specific	Leaf

* Web-computerized gene bank search results; G: Germin isoform; and GLP: Germin-like protein

Appendix 2 Nucleotide sequence of wheat OXO (gf-2.8) mRNA

mRNA cap site: 1, 1st (capital lettering);
5'-UTR sequence: 104, 1-104 (italic lettering);
Signal-peptide sequence: 69, 105-173 (underlining lettering);
Mature protein sequence: 603, 174-776 (bold-face lettering).

1 Actcatccac cacagcttag *cagcagcaac aaccagtgcc atagacactc tccatcaaca*
*61 aactctagct gatcaatcct agctaagctt attacatagc aagc*atgggg tactccaaaa
121 ccctagtagc tggcctgttc gcaatgctgt tactagctcc ggccgtcttg gcc**accgacc**
181 **cagaccctct ccaggacttc tgtgtcgccg acctcgacgg caaggcggtc tcggtgaacg**
241 **ggcacacgtg caagcccatg tcggaggccg gcgacgactt cctcttctcg tccaagttgg**
301 **ccaaggccgg caacacgtcc accccgaacg gctccgccgt gacggagctc gacgtggccg**
361 **agtggcccgg taccaacacg ctgggtgtgt ccatgaaccg cgtggacttt gctcccggag**
421 **gcaccaaccc accacacatc cacccgcgtg ccaccgagat cggcatcgtg atgaaaggtg**
481 **agcttctcgt gggaatcctt ggcagcctcg actccgggaa caagctctac tcgagggtgg**
541 **tgcgcgccgg agagacgttc ctcatcccac ggggcctcat gcacttccag ttcaacgtcg**
601 **gtaagaccga ggcctccatg gtcgtctcct tcaacagcca gaaccccggc attgtcttcg**
661 **tgcccctcac gctcttcggc tccaacccgc ccatcccaac gccggtgctc accaaggcac**
721 **tccgggtgga ggccagggtc gtggaacttc tcaagtccaa gtttgccgct gggttt**taat
781 ttctaggagc cttccctgaa atgataatta tataattcca tatatgcatg ctagcaaaat
841 ttaataattc tcaccagaag acatgtattc aagtttcagg ttaatctcgc atgtagtcgt
901 gtaataagat tgaacaagtt agcctcatgg tgtagccttc gatcagaacc aatatgagga
961 attgaatgta ctacttttta ttgtcgtctt tgttcttttc actgaacgga atatataata
1021 agcattttcg t

Appendix 3A - B ANOVA* of total phenolic acid levels from OXO transgenic and non-transgenic maize lines CK44 leaf tissue

A

Total phenolic contents	n	Mean	S.D.	S.E.
OXO line	5	2600.1	597.9	267.4
Null line	5	819.1	185.9	83.1

B

Source of variation	SSq	DF	MSq	F	p
Sample	7929948.2	1	7929948.2	40.5	0.0002
Within cells	1567930.1	8	195991.3		
Total	9497878.3	9			

* The above ANOVA employed the t test, significant at $p < 0.01$.

n: replicates
SD: standard deviation
SE: standard error

SSq: sum of square
DF: degree of freedom
MSq: mean square

Appendix 4A - B ANOVA* of ferulic acid levels from OXO transgenic and non-transgenic maize lines CK 44 leaf tissue

A

Ferulic contents	n	Mean	SD	SE
OXO line	5	585.3	154.8	69.2
Null line	5	2090.6	446.4	199.7

B

Source of variation	SSq	DF	MSq	F	p
Sample	5664969.2	1	5664969.2	50.8	<0.0001
Within cells	893048.8	8	111631.1		
Total	6558018.1				

* The above ANOVA employed the t test, significant at $p < 0.01$.

n: replicates
S.D.: standard deviation
S.E.: standard error
SSq: sum of square
DF: degree of freedom
MSq: mean square

Appendix 5A - B ANOVA* of diferulic acid levels from OXO transgenic and non-transgenic maize lines CK 44 leaf tissue

A

Diferulic contents	n	Mean	S.D.	S.E.
OXO line	5	365.0	126.1	56.4
Null line	5	181.5	30.1	13.5

B

Source of variation	SSq	DF	MSq	F	p
Sample	84183.6	1	84183.6	10.0	0.0133
Within cells	67223.5	8	8402.9		
Total	151407.1	9			

* The above ANOVA employed the t test, significant at $p < 0.05$.

n: replicates
S.D.: standard deviation
S.E.: standard error

SSq: sum of square
DF: degree of freedom
MSq: mean square

Appendix 6A - B ANOVA* of *p*-coumaric acid levels from OXO transgenic and non-transgenic maize lines CK 44 leaf tissue

A

Ferulic contents	n	Mean	S.D.	S.E.
OXO line	5	144.5	72.51	32.4
Null line	5	52.3	13.1	5.9

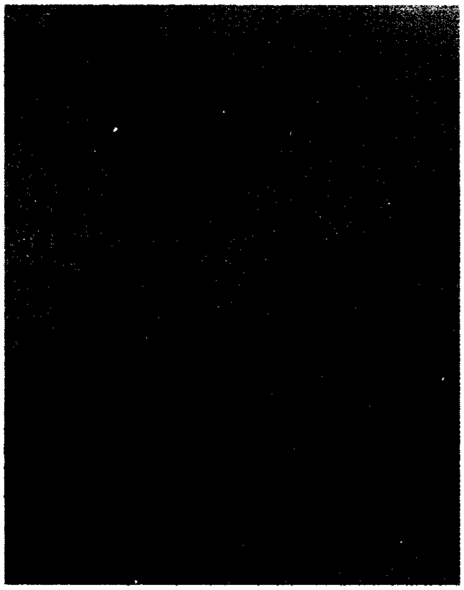
B

Source of variation	SSq	DF	MSq	F	p
Sample	21243.95	1	21243.8	7.9	0.0233
Within cells	21704.2	8	2713.0		
Total	42948.0	9			

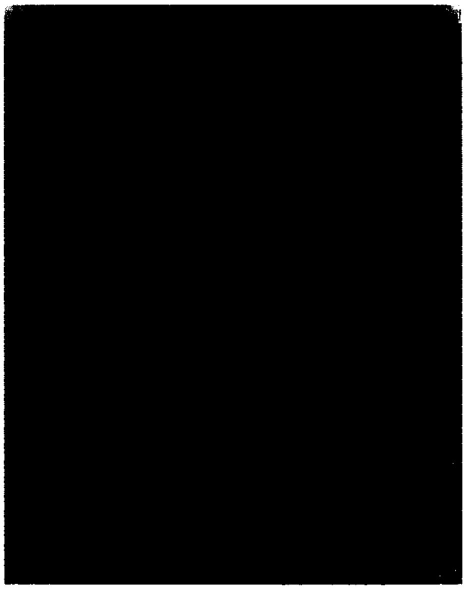
* The above ANOVA employed the t test, significant at $p < 0.05$.

n: replicates
S.D.: standard deviation
S.E.: standard error
SSq: sum of square
DF: degree of freedom
MSq: mean square

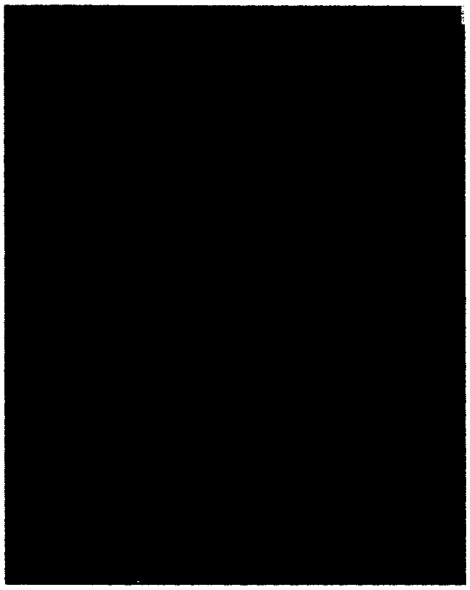
Appendix 7 Toluidine blue staining of leaf cross-sections (8 microns) of OXO maize line and its null line observed under white light with neutral density filters. Extensive lignification of phenolic acids in the cell walls of vascular and epidermal tissue in OXO line was observed. Fluorescence was located through the cell walls of vascular and epidermal tissue.



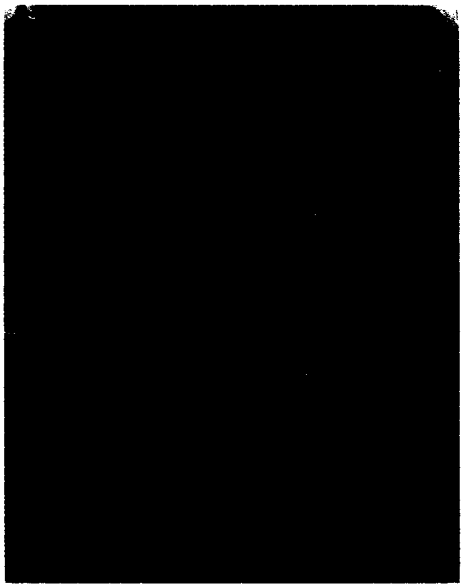
A. Middle leaf tissue (10 X)



B. Middle leaf tissue (20 X)



C. Middle vein (10 X)



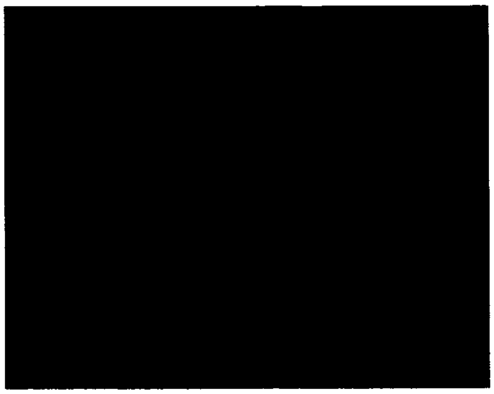
D. Middle vein (20 X)

Appendix 8 Autofluorescence, chlorine sulfite, and phloroglucinol staining of cross sections of OXO transgenic maize line and the corresponding null lines observed under ultraviolet light and white light with neutral density filters. There were no visible obvious differences on contents of insoluble cell wall bound phenolics and lignin, or on cell wall thickness between OXO and null lines.

A. OXO Autofluorescence (10X)

B. Null Autofluorescence (10X)

C. OXO Chlorine sulfite (10X)



D. Null Chlorine sulfite (10X)

E. OXO Phloroglucinol (20X)

F. Null Phloroglucinol (20X)

Appendix 9 Variables of cowpea *in vitro* regeneration

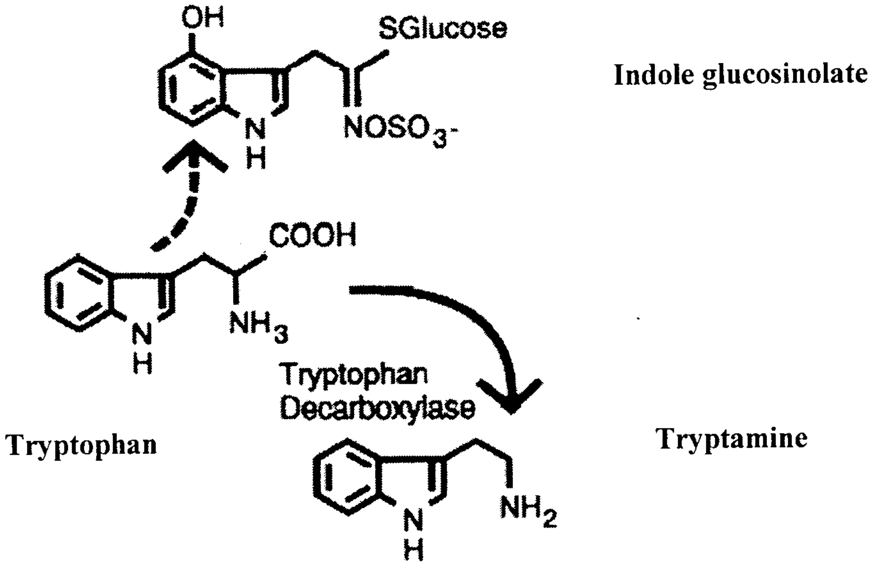
Genotype and explants	Medium formulation		Regeneration results		References
	Basal media* (mg l^{-1})	Plant growth regulators / levels (µM) / days	Developing stages	Regeneration frequencies (%) **	
P152 / Leaves	1. MS (solid) 2. MS (liquid) 3. MS (liquid) 4. 1/2 MS (solid)	2, 4-D / 6.78 2, 4-D / 2.26 2, 4-D + ABA / 0.05 + 5.0 ZN + ABA / 2.0 + 5.0	Germinated somatic embryos and plantlets	25.5	Prem Anand et al., 2000
P152 / Leaves	1. MS (solid) 2. MS (liquid) 3. 1/2MS (liquid)	2,4-D / 9.05 2,4-D / 4.5 ZN + ABA / 2.26 + 1.89	Cotyledonary-stage somatic embryos		Kulothungan et al., 1995
C-152 / Leaves, hypocotyls and mature cotyledons	1. MS (solid) 2. 1/2 MS (solid)	2,4-D / 2.0 - 5.0 or 2,4,5-T / 0.5 - 1.5	Globular stage somatic embryos	64.5 - 100.0	Amitha and Reddy, 1998
Chinese cowpea / Cotyledon protoplast	1. MS + B_5 vitamins (solid) 2. MS + B_5 vitamins (liquid) 3. MS + B_5 vitamins (solid)	2,4-D + ABA / 2.0 + 0.5 2,4-D + ABA / 2.0 + 0.5 IAA + KT / 0.1 + 0.5	Germinated somatic embryos and plantlets	Very low	Li et al., 1993

C-152 / Leaves and petioles	P. B_5 1. B_5 2. B_5 3. B_5	2,4,5-T / 80.0 / 20 BAP / 5.0 / 20	Rooting shoots	22.1	Muthukumar et al., 1995
Coronet etc. and USA breeding lines / Mature cotyledons	P. 1/3 MS (solid) 1. 1/3 MS (solid) 2. 1/3 MS (solid) 3. MS (solid)	BAP / 66.6 / 5 BAP / 66.6 / 5 BAP / 1.0	Rooting shoots	1.0 -11.0	Brar et al., 1999a and 1999b
Ife Brown etc. and IITA breeding lines / Hypocotyls and immature cotyledons	1. BM + MS vitamins 2. BM + MS vitamins 3. BM + MS vitamins	BAP / 111.0 / 20 + 2.0 / 20 ZN / 0.46	Rooting shoots	53.3	Pellegrineschi, 1997
Vita 5-exiita / Shoot meristem	MS + B_5 vitamins	0.001 - 0.05	Rooting shoots	92 - 100	Kartha et al., 1981

* Basal media were divided into five types according to different culture stages:
1. Induction media;
2. Proliferation media;
3. Maturation media for embryos and rooting media for shoots;
4. Germination media for embryos;
P. Pre-germination media for mature seed explant.

** Regeneration frequency: numbers of explants producing shoots (embryos) relative to numbers of overall explants cultured.

Appendix 10 Metabolic alteration of the shikimic acid pathway in transformed *Brassica napus* cv. Westar with TDC gene (Chavadej et al., 1994). Transformation of canola with the TDC gene is expected to redirect tryptophan into tryptamine rather than into the indole glucosinolates normally occurring in nontransformed plants.


OH
SGlucose
NOSO3-
N
H
Indole glucosinolate
COOH
NH3
Tryptophan
Tryptophan
Decarboxylase
Tryptamine
NH2