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Genetic engineering of Brassicas for pest and disease control and improved storage

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Final report for HAL project VG98085

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Purpose of the report:
This project (VG98085) was directed towards:

1. the development of methods to genetically modify vegetable brassicas

2. the preliminary assessment of genetically modified vegetable brassicas with agronomically useful genes

Funded by:
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Text by:
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1.0 Introduction

During the last decade, genetic modification has become a reality, with the first crops being commercialised. World-wide in excess of 44 million hectares of genetically modified plants worth about US $ 3 billion were grown in 2000. This is anticipated to increase to 50 million hectares in 2001 (www.isaaa.org). While vegetables represent, only a small portion of these figures (< 100,000 h), potato cultivars with resistance to Colorado beetle and Potato Leaf Roll Virus (PLRV), tomatoes with delayed ripening and squash with virus resistance have been commercialised in the USA.

This report summarises research to develop and use genetic modification to transfer agronomically useful genes to vegetable brassicas and describes four aspects of our research:

- the development of tissue culture regeneration systems
- the development of appropriate gene transfer systems
- the preparation of gene constructs and
- the preliminary assessment of genetically modified plants for there response to clubroot and diamondback moth

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1.1 Media summary

Gene technology has been used and evaluated with vegetable brassicas to increase their resistance to insect pests and diseases and improve shelf-life.

In Australia and world-wide, the insect pest diamondback moth and the fungal disease clubroot are major problems, reducing both the yield and quality of produce. The vegetable industry in partnership with the Institute for Horticultural Development - Knoxfield and Horticulture Australia Ltd are developing integrated crop management (ICM) programmes for diamondback moth and clubroot. For the past three years, researchers at IHD – Knoxfield have been developing ways to genetically modify vegetable brassicas with genes that may be useful to control insects and diseases to complement the ICM programmes.

Methods have been developed to transfer genes into a number of brassica crops. The necessary tissue culture systems for about 40 cultivars have been developed and the important factors that influence the regeneration of adventitious shoots identified. Gene transfer systems using *Agrobacterium* have been developed for the broccoli cultivar Marathon, the cauliflower cultivars Atlantis and Plana and the Chinese cabbage Pak Choi.

A population of genetically modified plants has been produced with different types of genes for insect and disease control and to increase shelf life. These plants are being assessed in glasshouse trials to find suitable lines for more detailed study. To date genetically modified lines with an insect control gene and a disease control gene have been screened and some promising lines identified.

1.2 Technical summary

The insect pest diamondback moth and fungal disease clubroot are major problems world-wide with vegetable brassicas, resulting in reduced yield and produce quality. Integrated crop management systems for both organisms are being developed to reduce reliance on chemical control. For crops such as broccoli, improved shelf life is also a desirable attribute.

Breeding for resistance to diamondback moth and clubroot and improved shelf life are difficult, as known sources of resistance are either not available or difficult to incorporate into cultivated lines using sexual hybridisation. Gene technology is able to overcome these barriers and provides a new, novel and powerful tool to study these problems.

Tissue culture methods have been developed to reliably and reproducibly regenerate adventitious shoots from broccoli (8 cultivars), Brussels sprout (3 cultivars), cabbage (2 cultivars) and cauliflower (15 cultivars). There is considerable variation in the way individual cultivars regenerate with an order of magnitude difference between the worst and best cultivars.

Gene transfer systems have been developed for a number of cultivars. Research using a construct with the *gusA* gene has not been particularly useful to develop a transformation system. Despite this difficulty, a population of genetically modified vegetable brassicas has been produced with potentially useful genes. In excess of 21,000 explants were processed resulting in 105 transgenic lines. This transformation frequency is very low and requires improvement.

A number of gene constructs have been produced, with different anti-microbial genes, a proteinase inhibitor gene and a gene associated with cytokinin biosynthesis. These have been transferred to a number of cultivars, including Marathon (broccoli), Atlantis and Plana (cauliflower) and Pak Choi (Chinese cabbage). These transgenic lines are in various stages of assessment in glasshouse trials.

Transgenic material has been screened in the glasshouse for their response to clubroot and diamondback moth and a number of lines with promise identified.

Future work will characterise these further and screen the remaining transgenic lines.

2.0 Tissue culture regeneration

2.1 Introduction

Brassica oleracea is a highly polymorphic species and includes a number of cultivated forms of vegetables such as broccoli (var. *italica*), Brussels sprout (var. *gemmifera*), cauliflower (var. *botrytis*) and cabbage (var. *capitata*).

Reliable adventitious shoot regeneration systems are an essential requirement if gene technology is to be applied to crop improvement. Adventitious shoots have been regenerated from various explants for important *Brassica* species. These include cotyledons, hypocotyls and roots (Bhalla and Smith 1998), hypocotyls (Pua *et al.* 1999), and peduncles (Eapen and George 1997).

With *Brassica* species, incorporation of silver, which inhibits ethylene action, has been shown to enhance shoot regeneration (Chi & Pua 1989, Palmer 1992) and improve androgenesis (Dias and Martins 1999). Similarly De Block *et al.* (1989) reported that silver was essential for shoot recovery of transformed *B. napus* explants.

Results presented here evaluate the influence and form of silver, seedling age and the influence of cultivar on adventitious shoot regeneration from hypocotyls of *Brassica oleracea*. Our long-term objective is to use the regeneration system for *Agrobacterium*-mediated gene transfer. For meaningful studies with transgenic plants it will be necessary to evaluate clonal lines and for this reason, factors were studied that influence shoot proliferation from adventitious shoots and shoot tips.

2.2 Methods and materials

2.2.1 Seed germination

Seeds were rinsed in 70% ethanol for five minutes, surface sterilised in 2% available chlorine with 0.1% Tween 20 for 15 minutes and rinsed three times in sterile water. Seeds were germinated in 250 mL polycarbonate tubs containing MS (Murashige and Skoog 1962) basal medium with 3% sucrose and solidified with 0.8% agar (Difco). The pH was adjusted to 5.7 prior to autoclaving at 100 kPa for 20 minutes. Cultures were incubated at 22 ^{0}C under a 16 hour : 8 hour (light : dark) photoperiod. The light source was cool-white fluorescent tubes providing 50 µmol m^{-1} s^{-1} at the culture level.

2.2.2 Regeneration experiments

2.2.2.1 Media and explants

Regeneration experiments were conducted in 90-mm Petri dishes containing 20 mL of B5 (Gamborg *et al.* 1968) basal medium with 10 µM BAP and solidified with 0.25% Gelrite. Modifications are indicated in the text.

Hypocotyl explants, five to seven mm long were prepared from seven-day old seedlings and placed horizontally on the medium. Unless indicated otherwise, only two hypocotyl explants (nearest to the cotyledons) from each seedling were used. Petri dishes were sealed with Micropore™ surgical tape (3M) and incubated as above. Explants were transferred to the fresh medium after three weeks. After six weeks, the number of adventitious shoots per explant were counted and the percentage regeneration determined and the mean shoot number for responding and all explants calculated. For simplicity, the only data presented in this section is for the regeneration index (RI). The RI is the product of the percentage shoot regeneration and the mean shoot number of responding explants and represents the number of adventitious shoots that can be regenerated from 100 explants. Data for percentage regeneration and mean shoot number for each experiment can be found in Appendix 1.

Each treatment was replicated five times, using Petri dishes, each containing 10 explants.

2.2.2.2 Effect of silver on regeneration of eight vegetable brassica cultivars

The effect of 50 µM silver supplied as ether silver nitrate or silver thiosulphate (STS) was tested with B5 basal medium with 10 µM BAP.

2.2.2.3 Effect of seedling age and silver concentration on regeneration of the cauliflower cultivar Atlantis

Hypocotyl explants harvested from three, seven and 14 day old seedlings were placed on B5 basal medium with 10 µM BAP supplemented with 0, 10, 25, 50, 75, 100 and 250 µM silver nitrate in factorial combination.

2.2.2.4 Cultivar screening

The response of 28 cultivars, representing broccoli, Brussels sprout, cabbage and cauliflower were screened on B5 basal medium with 10 µM BAP and 50 µM silver nitrate.

2.2.3 Miscellaneous regeneration experiments

2.2.3.1 Green Coronet

The cabbage cultivar Green Coronet performed poorly during our cultivar screening and the question was asked, "if it was important to have a better regeneration system for this cultivar, what factors could influence regeneration". Two experiments were done. The cytokinin BAP at 5, 10 and 20 µM was tested in factorial combination with or without 1 µM NAA. In a separate experiment, the cytokinins BAP and zeatin were each tested at 20 µM and a combined treatment using of 10 µM of each cytokinin.

2.2.3.2 Explant position

Instead of using only the hypocotyl explants nearest to the cotyledons, the whole hypocotyl was used.

2.2.3.3 Seedling age

Hypocotyl explants from seven and 14-day-old seedlings were used.

2.2.4 Shoot proliferation

Seeds of Atlantis (cauliflower) were germinated as described and shoot tips isolated and transferred to MS basal medium with BAP or kinetin at 1, 5 and 10 µM. Basal medium alone or with 10 µM GA_3 (as potassium salt) were also tested.

Adventitious shoots regenerated from hypocotyl explants were also tested as an explant source on MS basal medium with kinetin at 1, 5 and 10 µM.

Each treatment comprised five replicate 250-mL tubs with five explants per tub. Data was collected after six weeks.

2.3 Results and discussion

2.3.1 General comment

The first morphogenic change observed was the formation of callus at one end of hypocotyl within seven days. Shoot primordia were visible within 14 days at either one or both ends of the explant. To begin with, shoot primordia were purple to green in colour. After about 28 days the shoot primordia elongated and developed into green shoots. Our data clearly shows that hypocotyl explants can regenerate shoots on media with 10 µM BAP (Figure 2.1 and 2.2). Elongated shoots readily produced roots within 21 to 28 days (Fig. 2.3), and could be transferred to the glasshouse (Fig. 2.4 and 2.5).

2.3.2 Effect of silver on regeneration

In general, incorporation of silver into the media improved regeneration, with silver nitrate being better than STS. The promotive effect of silver on regeneration was observed on all four crops, with the exception of the cauliflower cultivars Chaser and Prestige. With the broccoli cultivars Green Belt and Marathon, and the Brussels sprout cultivar Ambitus, silver, irrespective of form, improved regeneration. This interaction between cultivar and silver is also evident with the cabbage cultivars (Table 2.1). Based on these results, B5 basal medium with 10 µM BAP and 50 µM silver nitrate was chosen for screening other cultivars.

Table 2.1 Effect of silver on the regeneration index of eight vegetable brassica cultivars

Crop	Cultivar	Basal[1]	Silver nitrate[2]	STS[3]
Broccoli	Green Belt	99	258	261
	Marathon	204	486	224
	Maverick	101	168	54
Brussels sprout	Ambitus	162	422	408
Cabbage	Green Coronet	79	158	24
	Savoy King	460	486	230
Cauliflower	Atlantis	423	537	218
	Chaser	345	243	283
	Prestige	516	352	180

(1) B5 with 10 µM BAP
(2) B5 with 10 µM BAP and 50 µM silver nitrate
(3) B5 with 10 µM BAP and 50 µM silver thiosulphate

See Appendix 1.1 for further details.

2.3.3 Effect of seedling age and silver concentration on regeneration of the cauliflower cultivar Atlantis

There was little effect of silver concentration on the regeneration index, up to 50 µM. At higher silver concentrations regeneration declined. There was little effect of seedling age on regeneration (Table 2.2).

From this experiment a concentration of 50 µM silver and seedlings between seven and 14 days were considered the best combination for shoot regeneration.

Table 2.2 Effect of seedling age and silver concentration on the regeneration index of the cauliflower cultivar Atlantis

Age (d)	Silver concentration (µM)						
	0	10	25	50	75	100	250
3	430	381	328	346	342	232	227
7	341	333	335	381	396	355	165
14	452	460	248	461	221	194	301

Main effects

a) Silver concentration

0	408
10	391
25	304
50	396
75	320
100	260
250	231

b) Seedling age

3	327
7	329
14	334

See Appendix 1.2 for further details.

2.3.4 Cultivar screening

Twenty eight cultivars were screened for shoot regeneration on B5 basal medium with 10 µM BAP and 50 µM silver nitrate. All cultivars regenerated adventitious shoots, however the response varied considerably, depending on cultivar (Table 2.3) and crop (Table 2.4). Cauliflower cultivars had the highest overall regeneration with a regeneration index of 422, followed by broccoli with 325, cabbage with 322 and Brussels Sprouts with 192 (Table 2.4). Within a crop, there was considerable cultivar difference. For example, with the broccoli cultivars there was nearly a four-fold difference with TB185 having a regeneration index of 637 and Maverick of 168. The greatest difference was found with the Brussels sprout cultivars screened, where there was a 40-fold difference, with Ambitus having a regeneration index of 422 and Roger of only 10 (Table 2.3).

This difference in crop and cultivar response suggests that the variation may be strongly influenced by genetics.

Table 2.3 Regeneration index for 28 vegetable brassica cultivars

Crop	Cultivar	Regeneration index
Broccoli	TB185	637
	Marathon	486
	TB254	296
	TB234	260
	Green Belt	258
	HS279	252
	TB243	240
	Maverick	168
Brussels sprout	Ambitus	422
	Oliver	144
	Roger	10
Cabbage	Savoy King	486
	Green Coronet	158
Cauliflower	Omeo	640
	Marba	600
	HS5393	547
	Atlantis	537
	Holis	520
	HS5263	500
	Calisa	461
	Sirente	420
	HS1640	410
	Plana	387
	Prestige	352
	White Rock	252
	Virgin	243
	Chaser	243
	Dova	230

See Appendix 1.3 for further details.

Table 2.4 Regeneration index based on crop

Crop	Regeneration Index
Broccoli	325
Brussels sprout	192
Cabbage	322
Cauliflower	423

See Appendix 1.4 for further details.

2.3.5 Miscellaneous regeneration experiments

2.3.5.1 Green Coronet

Altering BAP concentration, irrespective of presence or absence of NAA did not greatly improve regeneration of Green Coronet (Table 2.5).

Table 2.5 Effect of BAP and NAA on regeneration of Green Coronet

BAP (µM)	NAA (µM)	Regeneration index
5	0	16
10	0	10
20	0	10
5	0.5	8
10	0.5	0
20	0.5	12

See Appendix 1.5 for further details.

The best way to improve regeneration of Green Coronet was to use BAP and zeatin in combination, which was superior to each cytokinin individually (Table 2.6).

Table 2.6 Effect of BAP and zeatin on regeneration of Green Coronet

BAP (µM)	Zeatin (µM)	Regeneration index
20	0	10
0	20	132
10	10	251

See Appendix 1.6 for further details.

2.3.5.2 Explant position

There was no influence of explant position within the hypocotyl on regeneration. For consistency, all regeneration experiments only used the explants nearest the cotyledons, but for transformation, the complete hypocotyl was used.

2.3.5.3 Seedling age

With the broccoli cultivar Maverick and the cauliflower cultivar Atlantis there was an improvement in the regeneration index with 14-day-old seedlings (Table 2.7).

Table 2.7 Effect of seedling age on regeneration

Crop	Cultivar	Seedling age (days)	Regeneration index
Broccoli	Maverick	7	266
		14	326
Cauliflower	Atlantis	7	537
		14	855

See Appendix 1.7 for further details.

2.3.6 Shoot proliferation

Shoot tips from seedlings successfully proliferated using the growth regulators BAP and kinetin. While BAP at 5 and 10 µM resulted in the highest number of shoots, they were fasciated and vitrified (Fig. 2.6). Shoots regenerated using kinetin at the same concentrations formed an average of three shoots, all of which looked normal and readily initiated roots. Therefore, 5 µM kinetin was selected as a suitable cytokinin for shoot proliferation. Gibberellic acid (GA_3) at 10 µM did not induce shoot proliferation and was less effective than the control treatment for shoot elongation (Table 2.8).

Table 2.8 Effect of cytokinin type and concentration on shoot proliferation of shoot tips derived from seedlings of Atlantis

Treatment (µM)		Shoot number	Shoot length (mm)	Leaf number
Kinetin	1	2.1	16	6
	5	3.0	6	11
	10	3.1	5	14
BAP	1	4.4	1	12
	5	7.2	2	20
	10	8.2	1	17
GA_3	10	1.0	39	6
Control		1.0	47	7

When shoot tips derived from adventitious shoots were used, proliferation increased with increasing kinetin concentration (Table 2.9). These shoots could be separated and ultimately acclimatised in potting medium in the glasshouse.

Table 2.9 Effect of kinetin concentration on shoot proliferation of adventitious shoots derived from hypocotyl explants of Atlantis

Treatment		Shoot number	Shoot length (mm)	Leaf number
Kinetin	1	2.1	27	8
	5	3.1	23	9
	10	4.3	12	11
Control		1.2	27	8

This series of experiments result in a workable system to clone shoots from transformation experiments.

2.4 Conclusions

A reliable and reproducible shoot regeneration system has been developed for a range of cultivars representing broccoli, Brussels sprout, cabbage and cauliflower. A suitable medium based on the formulation of Gamborg *et al.* (1968) supplemented with 10 µM BAP and 50 µM silver nitrate is a suitable medium to screen cultivars. Those cultivars with an unacceptable regeneration index can their specific requirements determined.

Other factors, which appear to have less influence on regeneration, include explant position and seedling age.

Adventitious shoots can be proliferated using kinetin and be readily acclimatised in potting medium and grown in the glasshouse.

2.5 References

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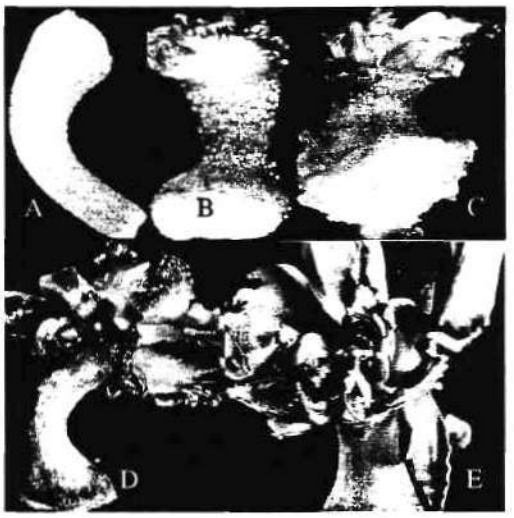

Fig 2.1 Shoot regeneration from hypocotyl explants occurs over a five week period

A = + 7 days, B = + 14 days, C = + 21 days, D = + 28 days, E = + 35 days

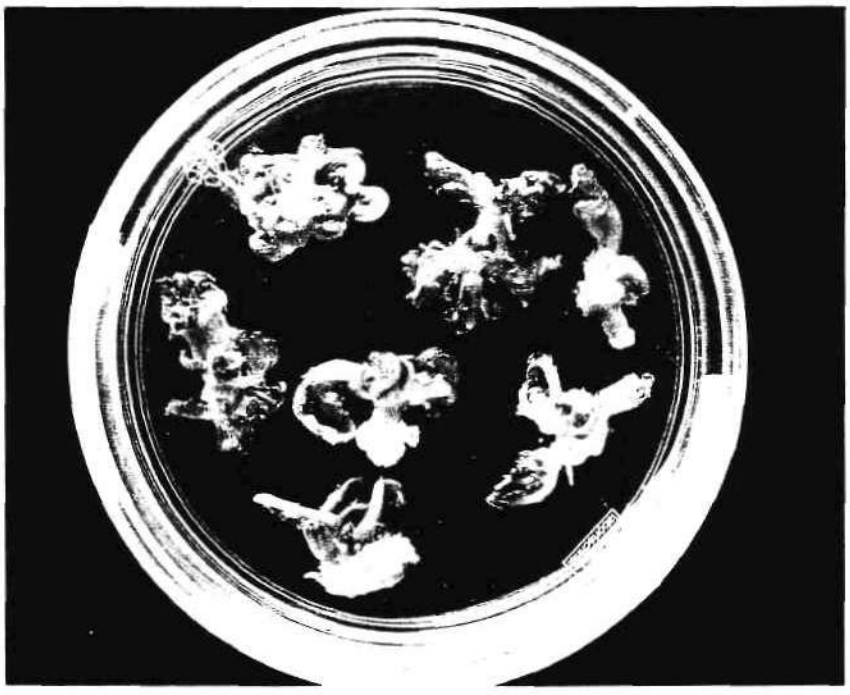

Fig 2.2 Adventitious shoot regeneration from cauliflower, 28 days after establishing culture

Fig. 2.3 Plantlet derived from adventitious shoot growing in culture and ready for transfer to the glasshouse

Fig. 2.4 Acclimatised plants from tissue culture growing in potting medium

Fig. 2.5 Plants derived from tissue culture growing in the containment glasshouse

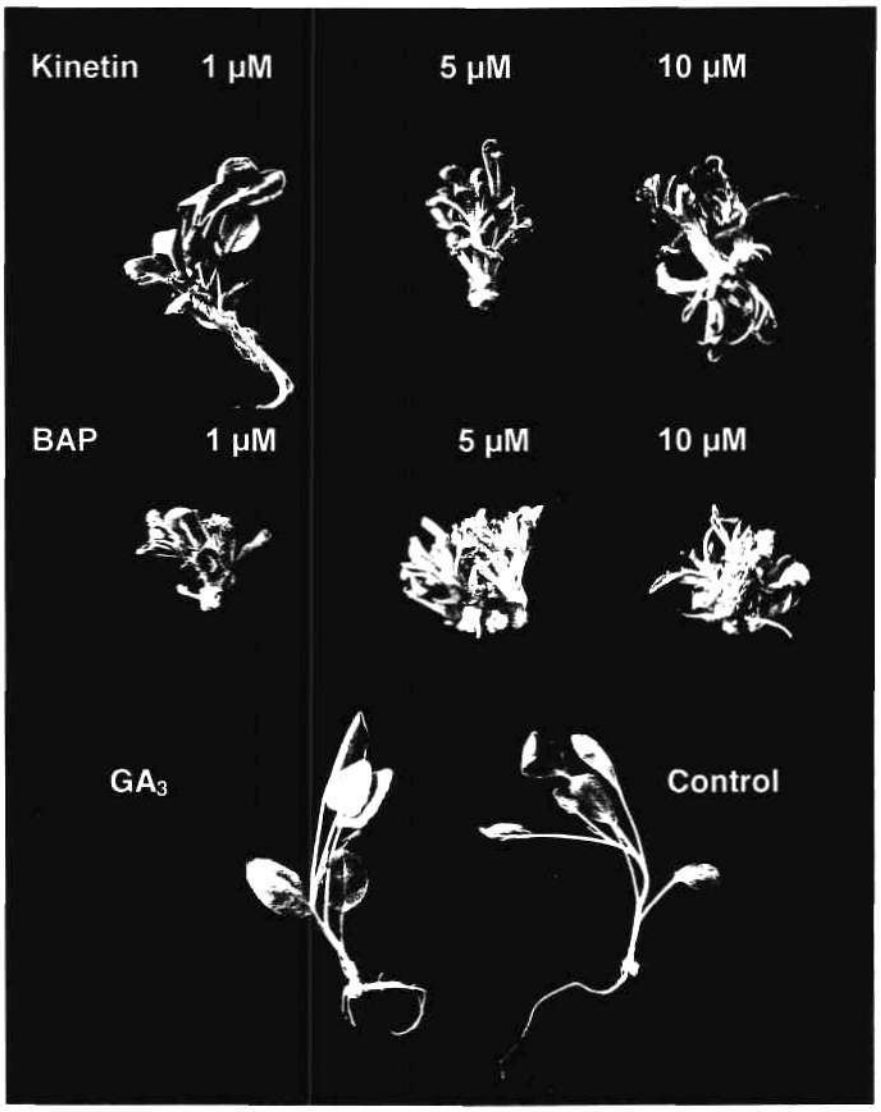

Fig. 2.6 Effect of BAP, kinetin and GA_3 on shoot proliferation from shoots derived from seedlings

3.0 Gene transfer system

3.1 Introduction

The production of genetically modified plants is a time consuming and complex process. When using *Agrobacterium* as a gene transfer system, a cascade of events occur. Briefly, phenolic compounds produced by wounded plant cells initiate the transfer of T-DNA from the *Agrobacterium* through the induction of the bacterial *vir* genes. The T-DNA, as a single stranded molecule, moves through the bacterial membrane and is transferred to the plant cell where it moves to the nucleus and is integrated at random (Galun and Galun 2001). Once this has occurred it is then necessary for the transgenic cells to regenerate to shoots thence plants. From the original inoculation of bacterial and plant cells to the eventual development and growth of a transgenic plant can take from four to six months.

From a practical point, the transfer of genes, when using *Agrobacterium*, involves three distinct stages, (1) the manner in which the bacterial cells are inoculated with an explant, (2) the duration of the co-cultivation period of the bacterial cells with the plant tissue and (3) the selection and regeneration of transgenic cells. During the last 10 years, the *gusA* gene has become a valuable tool to study factors that influence gene transfer (Martin *et al.* 1992). It is easy to detect; a substrate, x-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) is reacted with plant tissue and GUS positive tissue turns blue (Jefferson 1987). The main problems with using GUS is the assay is destructive and results are obtained some 24 hrs after setting up. Recently, a new reporter gene system based on the green fluorescent protein (GFP), isolated from the jellyfish, *Aequorea victoria* has been developed (Chalfie and Kain 1998). The main advantage of GFP are that no substrate is required, expression can be monitored with fluorescence microscopy and it is non-destructive.

Some of the data presented in this section results from a series of experiments to evaluate factors that could influence gene transfer, using the *gusA* gene. The *gusA* gene used has been modified to prevent expression in *Agrobacterium* through a portable plant intron, so that the gene expression can be attributed to the treatment imposed (Vancanneyt *et al.* 1990). Information is also provided about the other gene constructs used in the project (see section 4.0 Preparation of gene constructs for further details).

3.2 Methods and materials

3.2.1 Agrobacterium growth

The *gusA* gene construct (pBECKS$_{400}$ GUSintron. nptII. hph) (M^{c} Cormac *et al.* 1997) (Fig. 4.1) used to develop the gene transfer system was mobilised to

Agrobacterium strains using tri-parental mating or electroporation. Details of the other gene constructs used can be found in Figs. 4.2 to 4.7.

For gene transfer, *Agrobacterium* cultures were grown in liquid YEP medium with appropriate antibiotics and shaking at 28 °C for 24 hours, spun down and washed in 0.9% NaCl. Cells were resuspended in 100 mL of induction medium and grown for 16-18 hours. Induction medium comprises AB salts (Gartland 1995), 2 mM phosphate buffer, 30 µM MES, 0.5% glucose and 100 µM acetosyringone.

3.2.2 Transformation

Plant material was grown as described (see section 2.2.2.1 Media and explants). Hypocotyls were removed from the seedlings and submerged in the *Agrobacterium* solution and one cm explants cut. Explants were blotted dry and transferred to B5 basal medium (Gamborg *et al.* 1968) to co-cultivate for three days, then transferred to pre-selection medium (B5 basal medium with 10 µM BAP, 50 µM silver nitrate, 150 µg mL^{-1} timentin [or 250 µg mL^{-1} cefotaxime] and solidified with 0.25% Gelrite). After seven days, explants were transferred to selection medium (pre-selection medium plus appropriate antibiotics (kanamycin [25-50 µg mL^{-1}], hygromycin [10 µg mL^{-1}] or gentamycin [25-50 µg mL^{-1}]). Explants were transferred to fresh selection medium at three-week intervals until shoots appeared.

To determine if the shoots were transgenic they were screened twice. Initially for the ability of shoots to initiate and grow roots in the presence of the appropriate antibiotic and for the presence of the antibiotic resistance gene using PCR (Polymerase Chain Reaction). Plants 'passing' both tests were considered transgenic, given an unique number and called a line. For studies using *gusA*, a histochemical test was also used, which results in a blue colouration after reaction with x-gluc.

3.2.3 Factors evaluated

Using the *gusA* gene, a range of factors were evaluated that could influence transformation. These included the way in which *Agrobacterium* cultures were grown, the value of a pre-selection stage, the *Agrobacterium* strain used and the antibiotic resistance gene used for selection.

3.2.4 Gene constructs used

Details of the gene constructs used during this project can be found in section 4.0. Preparation of gene constructs. A brief description of the potential use of the genes used is provided in Table 3.1.

Table 3.1 General information on the gene constructs and their potential use

Gene construct	Comment
pBECKS$_{400}$ GUSintron. nptII. hph	Contains the *gusA* gene, which is commonly used in transformation to study factors that influence gene transfer. Also contains two antibiotic selectable markers genes (*npt* II for resistance to kanamycin and *hph* for resistance to hygromycin). See also Fig. 4.1.
pNOV022	Contains the *mpi* gene, which allows mannose to be used as a selectable marker. This is an alternative to antibiotic resistant genes and may result in better consumer acceptance of GM foods. See also Fig. 4.2.
p35S glucose oxidase ***and*** pTobRB7 glucose oxidase	Glucose oxidase has anti-microbial activity and could be effective against clubroot. See also Figs. 4.3 and 4.4.
pJVG33a TpthII	Contains a thionin gene, which has anti-microbial activity and could be effective against clubroot. See also Fig. 4.5.
p35S AM8	Contains a proteinase inhibitor gene, which could be effective against diamondback moth. See also Fig. 4.6.
pSAG12 ipt	Contains a gene associated with cytokinin production, which could be useful to extend shelf life. See also Fig. 4.7.

3.3 Results and discussion

During the project, 21,286 explants were processed and 105 transgenic plants produced (Table 3.2).

Despite the poor transformation efficiency, research continued with the other constructs so that a population of transgenic vegetable brassicas with different genes was produced.

Table 3.2 Details of explants processed and transgenic plants produced for the gene contructs used

Construct	Explants	Transgenic plants
pBECKS$_{400}$ GUSintron. nptII. hph	3,010	8
pNOV022	2,177	31
p35S glucose oxidase	2,921	16
pTobRB7 glucose oxidase	4,517	9
pJVG33a TpthII	5,944	20
p35S AM8	1,200	12
pSAG12 ipt	1,517	9
Total	**21,286**	**105**

A total of 3,010 explants were screened using the *gusA* construct resulting in 8 GUS positive plants being produced (Table 3.3, Fig. 3.1). It was expected that studies using the *gusA* gene would have been useful to determine factors that influence gene transfer. Unfortunately, this was not the case and from these experiments, it was impossible to determine which factors have a major influence on gene transfer. While the results with *gusA* have been disappointing, it strongly indicates that gene transfer, at least for vegetable brassicas, is a complex and poorly understood process.

Table 3.3 Transgenic plants produced using pBECKS$_{400}$ GUSintron. nptII. hph

Crop	Cultivar	Explants	Transgenic plants
Broccoli	Marathon	1,535	3
Cauliflower	Atlantis	1,475	5
	Total	**3,010**	**8**

Details of the population of transgenic vegetable brassicas produced, using other gene constructs, can be found in Tables 3.4 to 3.9. These plants are systematically being assessed and details of plants containing p35S glucose oxidase and p35S AM8 can be found in sections 5.0 and 6.0 respectively.

Table 3.4 Transgenic plants produced using pNOV022

Crop	Cultivar	Explants	Transgenic plants
Cauliflower	Atlantis	1,250	20
	Plana	745	11
Cabbage	Green Coronet	182	0
	Total	**2,177**	**31**

Table 3.5 Transgenic plants produced using p35S glucose oxidase

Crop	Cultivar	Explants	Transgenic plants
Broccoli	Marathon	550	0
Cauliflower	Atlantis	1,515	14
	Chaser	116	0
	Plana	500	0
	Prestige	240	2
	Total	**2,921**	**16**

Table 3.6 Transgenic plants produced using pTobRB7 glucose oxidase

Crop	Cultivar	Explants	Transgenic plants
Cauliflower	Atlantis	2,890	6
	Chaser	133	0
	Plana	1,200	3
	Prestige	294	0
	Total	**4,517**	**9**

Table 3.7 Transgenic plants produced using pJVG33a TpthII

Crop	Cultivar	Explants	Transgenic plants
Broccoli	Marathon	2,920	18
Cabbage	Savoy King	1,877	0
Cauliflower	Atlantis	1,147	2
	Total	**5,944**	**9**

Table 3.8 Transgenic plants produced using p35S AM8

Crop	Cultivar	Explants	Transgenic plants
Cauliflower	Plana	1,200	12
	Total	**1,200**	**12**

Table 3.9 Transgenic plants produced using pSAG12 ipt

Crop	Cultivar	Explants	Transgenic plants
Broccoli	Marathon	1,093	3
Chinese cabbage	Pak Choi	424	6
	Total	**1,517**	**9**

If the transformation data is evaluated according to crop, it can be seen that cabbage is more difficult to transform than the other crops, as no plants were produced after processing 2,059 explants (Table 3.10). Of the other crops assessed, broccoli and cauliflower are more amenable to transformation. No transformation experiments were done with Brussels sprout

Table 3.10 Transgenic plants produced based on crop and cultivar

Crop	Cultivar	Explants	Transgenic plants
Broccoli	Marathon	6,098	24
Cabbage	Green Coronet	182	0
	Savoy King	1,877	0
Cauliflower	Atlantis	8,277	47
	Chaser	249	0
	Plana	3,645	26
	Prestige	534	2
Chinese cabbage	Pak Choi	424	6
	Total	**21,286**	**105**

3.4 Conclusions

Transformation of vegetable brassicas is a numbers game. It has been impossible to accurately determine important factors that influence gene transfer. Despite these problems, a population of transgenic plants has been produced. A better understanding of the factors that influence transformation of vegetable brassicas is clearly required.

3.5 References

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M^{c} Cormac AC, Elliot MC, Chen D-F (1997) pBECKS. A flexible series of binary vectors for Agrobacterium-mediated plant transformation. *Molecular Biotechnology* **8,** 199-213.

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Fig. 3.1 Leaf from transgenic cauliflower (Atlantis) expressing GUS

4.0 Preparation of gene constructs

4.1 Introduction

Gene technology has a total reliance on preparing genes in such a way they can be transferred to and expressed in plants. This requires the use of cloning vectors for the initial manipulation of DNA and binary vectors with appropriate promoters and terminators that can be grown in *E. coli* and *Agrobacterium.*

4.2 Methods and materials

All DNA manipulations were conducted according to the requirements of the Office of the Gene Technology Regulator (OGTR) in a PC2 containment laboratory, using methods as described (Sambrook and Russell 2001).

The gene constructs prepared are illustrated in Figs. 4.1 to 4.7.

4.3 References

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M^{c} Cormac AC, Elliot MC, Chen, D-F (1997) pBECKS. A flexible series of binary vectors for Agrobacterium-mediated plant transformation. *Molecular Biotechnology* **8,** 199-213.

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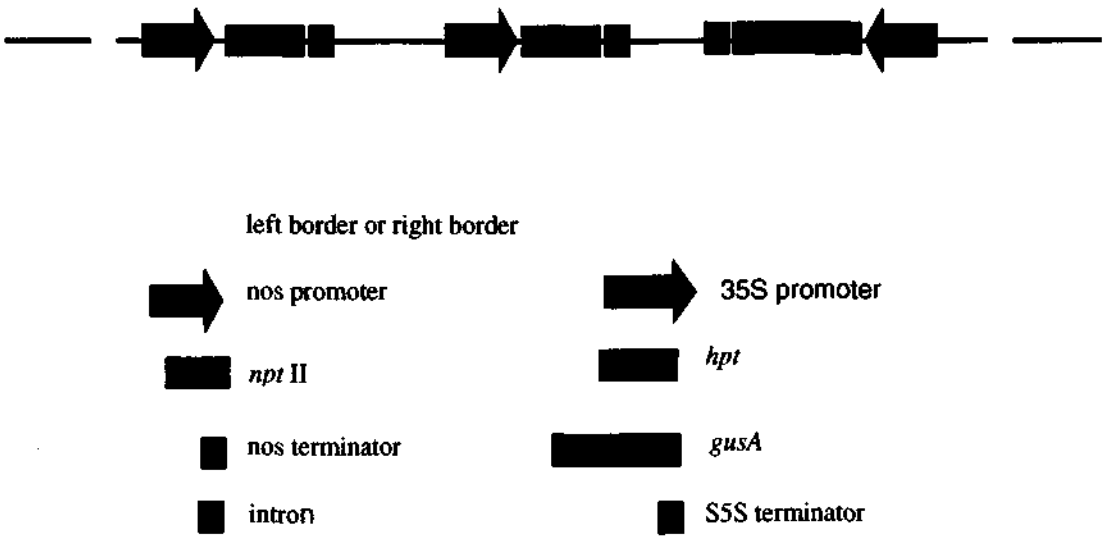
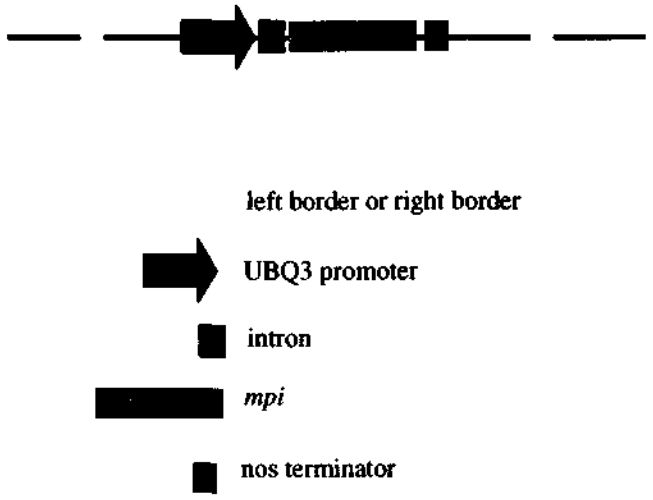
Fig. 4.1 pBECKS$_{400}$ GUSintron. nptII. hph



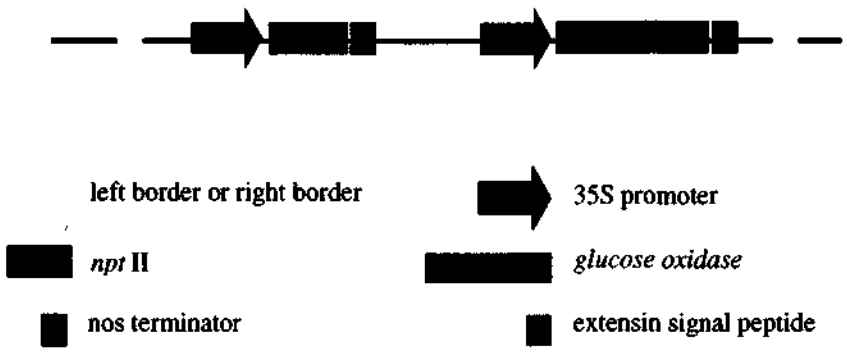
The pBECKS$_{400}$ GUSintron . npt II. hph construct has two selectable marker genes, *npt* II under the control of the nos promoter and *hph* under the control of the 35S promoter. Therefore, either kanamycin or hygromycin can be used as a selectable marker. It also contains the *gusA* gene, with an intron. Further details can be found in M^{C} Cormac *et al.* (1997).

Fig.4.2 pNOV022



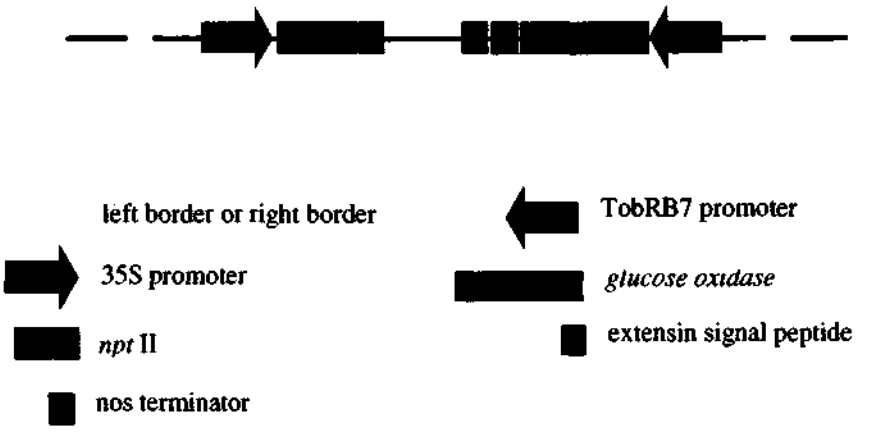
The pNOV022 construct contains the *mpi* gene (Miles and Guest 1984) under the control of the UBQ3 promoter (Norris *et al.* 1993). The *mpi* gene encodes for mannose-6-phosphate isomerase (MPI) and allows mannose to be used a selective agent. The selection strategy is based on the observation that transgenic plants expressing MPI are able to convert mannose-6-phosphate to fructose-6-phosphate, which can then be utilised as a carbon source.

Fig. 4.3 p35S glucose oxidase



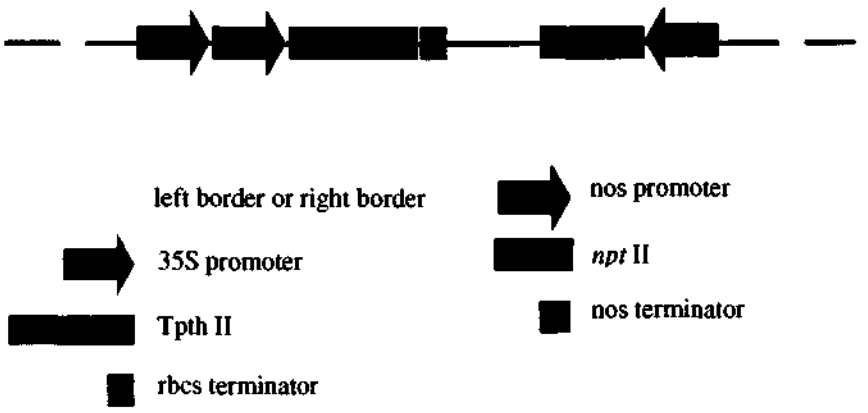
This construct has the 35S promoter regulating a *glucose oxidase* gene with an extensin signal peptide. The selectable marker gene is *npt* II under the control of the 35S promoter. This construct was obtained from D. Llewellyn (CSIRO Plant Industry).

Fig. 4.4 pTobRB7 glucose oxidase



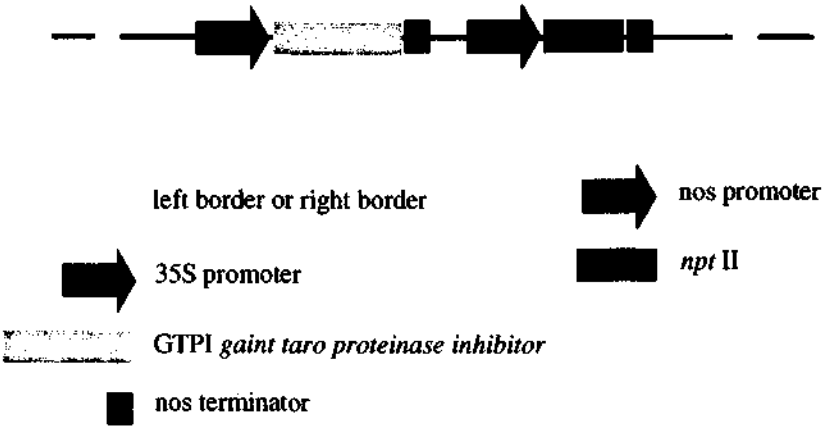
Similar to the previous construct except the *glucose oxidase* gene is under the control of the root specific promoter TobRB7 (Conkling *et al.* 1990). This construct was obtained from D. Llewellyn (CSIRO Plant Industry).

Fig. 4.5 pJVG33a TpthII



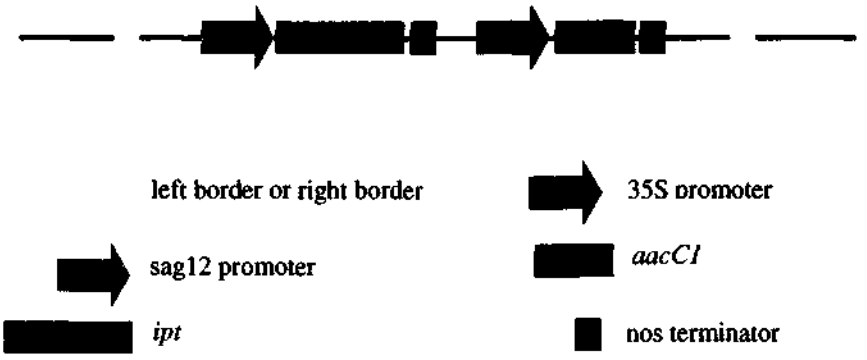
This construct uses a double 35S promoter to control a truncated thionin (*Tpth* II) gene. The selectable marker gene is *npt* II under the control of the nos promoter.

Fig. 4.6 p35S AM8



This construct uses the 35S promoter to control a gaint taro proteinase inhibitor (*GTPI*) gene (Mathews *et al.* 1996). The selectable marker gene is *npt* II under the control of the nos promoter.

Fig. 4.7 pSAG12 ipt



This construct contains a senescence associated promoter (sag12) (Gan and Amasino 1995) controlling the *ipt* gene. The selectable marker gene is *aacC1* (encoding gentamycin resistance) under the control of the 35S promoter.

5.0 Screening transgenic plants against clubroot

5.1 Introduction

Clubroot (*Plamodiophora brassicae*) is an important soil-borne pathogen of vegetable brassicas world-wide with a complex life cycle (Ingram and Tommerup 1972). Primary infection occurs in root hairs resulting in the development of uninucleate plasmodia. Secondary infection of cortical cells results in multinucleate plasmodia and the development of the characteristic galls.

Breeding for clubroot resistance is difficult, as it appears to be a recessive character controlled by more than one gene (Chiang and Crête 1970).

Plants are able to defend themselves against invading pathogens by a number of mechanisms. Specific host-pathogen responses involve the interaction between pathogen *avr* (avirulence) genes and the corresponding *R* (plant resistance) genes (Dangl and Jones 2001). Cysteine-rich and basic polypeptides have also been recognised as being involved with plant defense against pathogens. Such compounds include thionins (Bohlmann 1994), lipid transfer proteins (Garcia-Olmedo *et al.* 1995) and plant defensins (Terras *et al.* 1995).

Glucose oxidase (GO) has been found effective against fungal and bacterial pathogens *in vitro*, which is attributed to the production of hydrogen peroxide (H_2O_2) as a result of the oxidation of GO (Peng and Kuc 1992). Expression of a *glucose oxidase* gene in transgenic potato plants has resulted in enhanced disease resistance (Wu *et al.*1995).

Results presented here describe the preliminary analysis resulting from the challenge of 10 transgenic lines of the cauliflower cultivar Atlantis containing the *glucose oxidase* gene under the control of the 35S constitutive promoter.

5.2 Methods and materials

5.2.1 Production of transgenic plants

Transgenic plants were produced using methods described (see section 3.2.2 Transformation) with the gene construct p35S glucose oxidase (Table 3.1, Fig. 4.3). Ten transgenic lines of Atlantis (cauliflower) were clonally propagated *in vitro*, acclimatised and grown in the containment glasshouse at IHD - Knoxfield for three weeks prior to inoculation. Non-transgenic control plants were regenerated from hypocotyl explants.

5.2.2 Clubroot challenge

Transgenic lines were inoculated with 200 µL of spores (10^4 or 10^8 spores mL^{-1}) collected from an infected site at Trentham (Victoria) or water (zero inoculum) (Fig. 5.1). Control plants were treated as above. Each treatment was replicated five times.

Plants were grown for eight weeks prior to analysis. Soil was washed off the plants (Fig. 5.2) and the root system evaluated using the 'root galling assessment scale'. The shoot and root system of each plant was oven dried and weighed (Appendix 2).

5.3 Results and discussion

As expected the zero inoculum (water) treated plants, irrespective of whether transgenic lines or control plants, resulted in no galls. Plants inoculated with the lowest spore density (10^4 spores mL^{-1}), all had low ratings, with one or less galls per plant. This result indicates the level of inoculum is too low to induce symptoms. There were variable results with the highest inoculum density (10^8 spores mL^{-1}), ranging from 3.6 (lines 1152 and 1153) to five and above (lines 1213, 1214, 1217 and control (Table 5.1). This type of result is common with transgenic plants and demonstrates the importance of screening numerous plants before selecting those for more detailed analysis. It is encouraging that some of the tested lines (1152, 1153, 1154, 1215 and 1218) have lower gall ratings than the control. There is no apparent relationship between the plant dry weight data and the gall rating, which indicates that the interactions between transgenic lines, clubroot and plant response are likely to be complex. The response of plants from the clubroot challenge can be found in Fig. 5.3.

Table 5.1 Ratings of transgenic Atlantis lines inoculated with clubroot spores at two densities [1]

Line	Inoculum density (spores mL^{-1})		
	0	10^4	10^8
1152	1.0	1.4	3.8
1153	1.0	1.0	3.6
1154	1.0	1.0	4.4
1213	1.0	1.0	5.6
1214	1.0	1.0	5.6
1215	1.0	1.0	4.0
1216	1.0	1.2	4.8
1217	1.0	1.0	5.2
1218	1.0	1.0	4.0
1219	1.0	1.0	4.6
Control	1.0	1.2	5.0

[1] Plants were rated using the 'root galling assessment scale'.

1= no galls
2= single gall
3= several small galls
4= mild tap root infection
5= moderate tap root infection
6= severe tap root infection, many galls

5.4 Conclusions

A preliminary screen of transgenic plants with the *glucose oxidase* gene has identified some lines with reduced gall numbers. These transgenic lines need to be tested again, using only one inoculum density. In addition, the glucose oxidase activity needs to be determined, both before and after inoculation.

Additional transgenic lines with glucose oxidase (Tables 3.5 and 3.6) and a truncated thionin gene (Table 3.7) need to be assessed.

5.5 References

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Fig. 5.1 Inoculating transgenic plants with clubroot spores

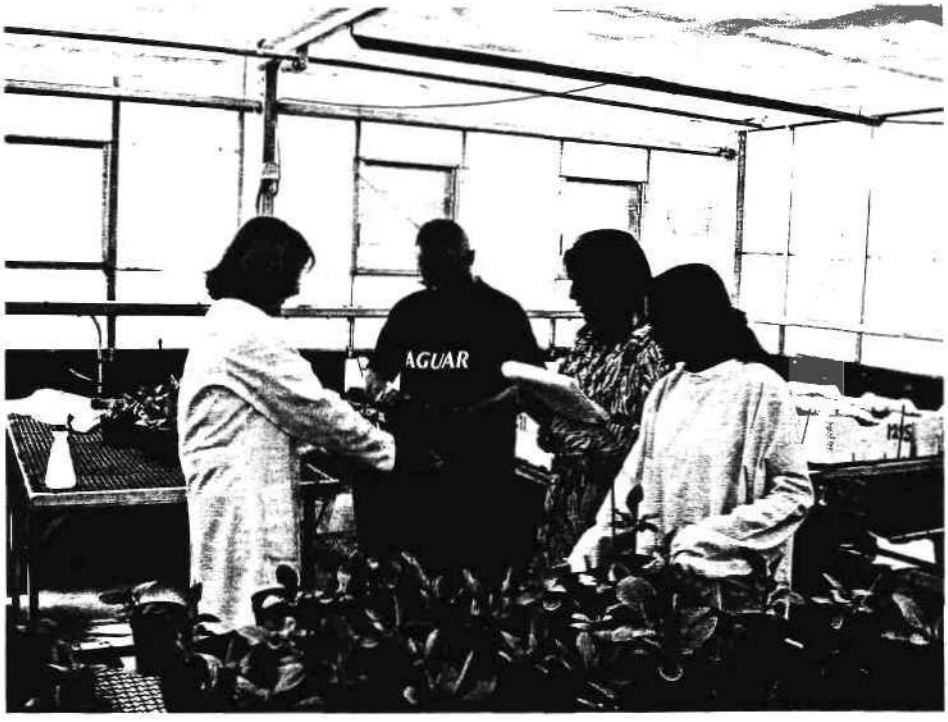

Fig. 5.2 Assessing transgenic plants after inoculation with clubroot

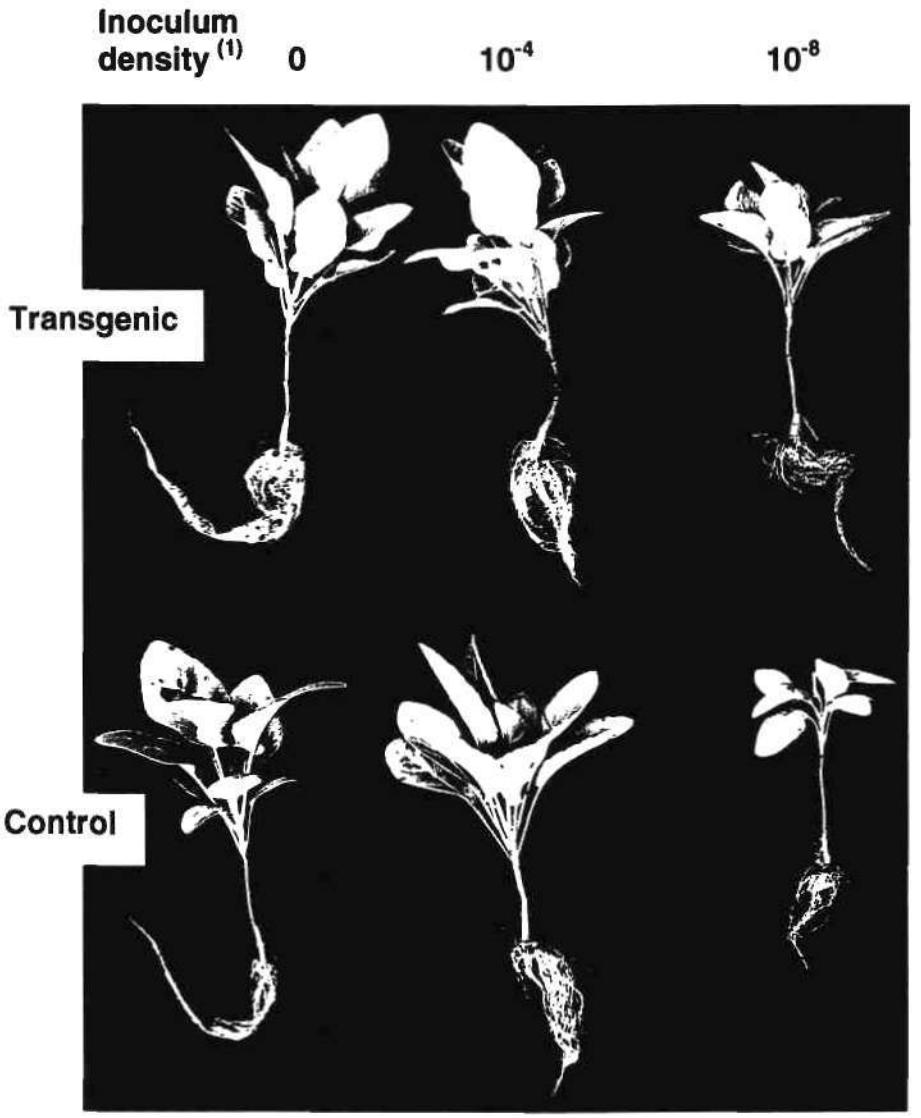

Fig. 5.3 Response of Atlantis plants to clubroot challenge

(1) spores mL^{-1}

6.0 Screening transgenic plants against diamondback moth

6.1 Introduction

Diamondback moth (*Plutella xylostella*) is the most important insect pest of vegetable brassicas world-wide. Control of this pest has been an issue for the Australian vegetable industry since the 1980's, when resistance to pyrethroid insecticides was identified. Consequently, integrated crop management (ICM) systems were developed to reduce the reliance on conventional insecticides. A desirable part of the solution for ICM programmes is the availability of cultivars with a level of tolerance (or ideally resistance) to the target.

Resistant cultivars can be produced by either conventional breeding or gene technology. Unfortunately, breeding for diamondback moth resistance is difficult due to the lack of appropriate germplasm. Gene technology has been proven to be effective, and two compounds, the proteinase inhibitors and the insecticidal proteins from *Bacillus thuringiensis*, have been identified and demonstrated to have wide application in controlling insect pests of plant species (Gatehouse *et al.* 1992, Peferoen 1992).

Proteinase inhibitors are proteins produced naturally in certain plants as a defence mechanism (Ryan 1990). The gene encoding for a proteinase inhibitor from the giant taro (*Alocasia macrorrhiza*) has been cloned and characterised and its effectiveness tested against cotton bollworm (*Helicoverpa armigera*) in transgenic tobacco lines (Wu *et al.* 1997). The giant taro proteinase inhibitor (GTPI) has two reactive sites, which are active against trypsin and chymotrypsin, the two major proteinases present in the insect larval midgut. Therefore, GTPI should have an impact on the digestive capacity of insects following consumption.

This section provides preliminary data on the growth and development of diamondback moth larvae fed leaf tissue from eight transgenic lines of the cauliflower cultivar Plana.

6.2 Methods and materials

6.2.1 Production of transgenic plants

Transgenic plants were produced using the methods described (see section 3.2.2 Transformation) with the gene construct p35S AM8 (Table 3.1, Fig. 4.6) and screened for the presence of transgenes with PCR. Lines were cloned *in vitro*, acclimatised and grown in the containment glasshouse at IHD - Knoxfield. Non-transgenic control plants were grown from seeds.

6.2.1 Diamondback moth challenge

A diamondback moth colony derived from insects collected at Berwick (Victoria) was reared in the laboratory at 25 °C on cabbage seedling leaves. Eggs were collected on a Parafilm® sheet and first instar larvae (within 24 hr of hatching) were used in the feeding trial. One-cm leaf discs from glasshouse grown plants were placed in small clear plastic cups (Solo® plastic portion cups, 28 mL) and used for the feeding trials (Fig 6.1). For the first three days of the feeding trial, a single leaf disc was used and subsequently two (or more) discs were used. Leaf discs were replaced every second day. Larvae were weighed after seven days and maintained until the adults emerged. Mortality was recorded at seven day intervals.

The experimental unit was an individual cup containing a larva. Four cups of each transgenic line and control material were placed in a closed 18 litre plastic container (a replication) lined with moistened paper towel (Fig 6.2). Three plastic containers were maintained at 25 °C and 80% relative humidity.

6.3 Results and discussion

Larvae fed on seven of the eight transgenic lines had a lower body weight than control fed larvae (Table 6.1). The greatest reduction in larval weight was found with line 1122, which was 12.5 times less than the control fed larvae. In contrast, larvae fed on line 1129 were heavier than control fed larvae. Lines 1122 and 1127 resulted in a higher larval mortality than other transgenic lines and the control. With lines 1123, 1127 and 1129, the percentage emergence of moths from pupae was reduced.

The observed slower growth, increased larval mortality and the failure of moths to emerge from pupae could be due the levels of GPTI expressed in transgenic lines. The mechanism of GTPI on growth retardation has not been studied, however it may have been due to a decrease or increase of gut proteiolytic activity, as observed by Broadway and Duffy (1986).

Table 6.1 Response of diamondback moth larvae after feeding on transgenic lines

Plant number	Larval body weight (mg) [1]	Laval mortality (%) [2]	Adults emerged from larvae (%)
1122	0.2	33.3	100
1123	1.1	8.0	82
1124	1.3	0.0	100
1125	1.2	8.3	100
1127	1.5	33.3	88
1128	1.0	0.0	100
1129	3.5	8.3	90
1130	1.4	8.3	100
Control	2.5	16.6	100

[1] at seven days
[2] at 21 days

6.4 Conclusions

This preliminary screen of transgenic lines with the GTPI gene has identified some lines with potential, due to reduced larval weight and increased mortality. Some of the transgenic lines need to be tested again in a more detailed study.

6.5 References

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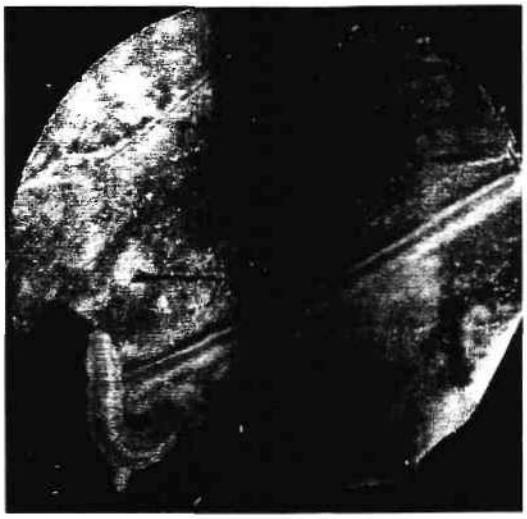

Fig. 6.1 Larva of diamondback moth feeding on leaf disc

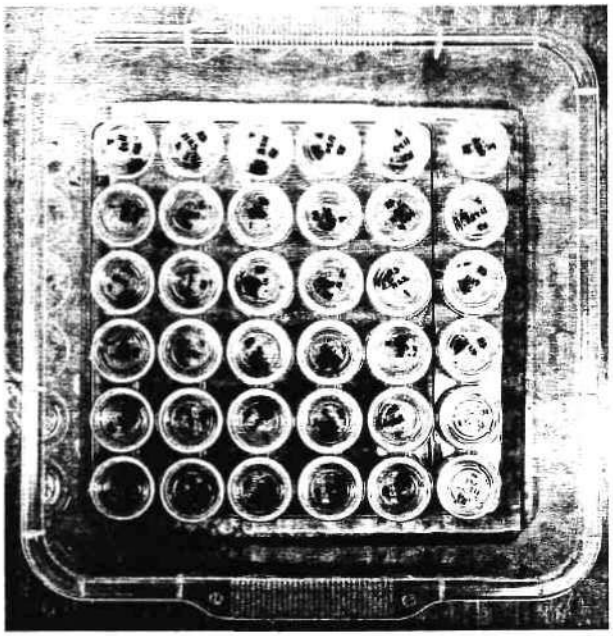

Fig. 6.2 System used for leaf disc bioassy to screen transgenic lines against diamondback moth larvae

7.0 Technology transfer

7.1 Publications/Conferences/Seminars

Members of the project team participated in the clubroot research team meeting and industry day at IHD-Knoxfield in October 1998.

Genetic engineering – will it help? Plutella Update (No. 37, May 1999)

Genetic engineering – can it help? Galls and All (No.8, July1999)

Department of Natural Resources and Environment, Horticultural Conference (6 and 7 September 2000)

Kaul V, Maheswaren G, Hutchinson JF. Genetic engineering of brassicas for pest and disease control and improved storage. 'Growing for Profit' field day, Gympie, Queensland. Queensland Fruit and Vegetable Growers and HRDC.

Genetic engineering for improved vegetable brassicas. Institute for Horticultural Development Annual Report 2000/01

Seminars at Crop and Food Research (New Zealand), University of Melbourne (School of Forestry and Institute of Land and Food Resources) on 'Biotechnology research with vegetable brassicas and potatoes at IHD'

7.2 Laboratory visits

Meeting organised by Patrick Ulloa, Industry Development Officer, Victoria on improving communication skills between growers and researchers (Melbourne Market, April 1999)

Werribee-Expo and discussed with the Young Growers Group their R&D priorities (May 1999)

Some members of the HRDC Brassica Commodity Group visited to see how the project was progressing. This laboratory visit was associated with the clubroot meeting organised by Caroline Donald and Ian Porter (23 and 24 August 2000)

Ontario Processing Vegetable Growers Association. Members included the Directors and six growers. (www.ovgmb.org) January 2001

Two delegations from the Biotechnology Inovation Centre, Democratic Peoples Republic of Korea. As part of ACIAR visit. March 20001

Patrick Ulloa, the Vegetable Industry Development Officer for Victoria, led a group of vegetable growers to IHD - Knoxfield in December 1999 to learn about the project. Growers were shown tissue culture and gene transfer systems and given a guided tour of the laboratory facilities. Much of the discussion was about safety issues associated with gene technology. This was a most beneficial exercise and additional visits from any sectors of the vegetable industry are welcome

This visit was reported in Vege Link (Issue 4, March 2000) Is genetic engineering the way to go?

Vegetable growers visit IHD - Knoxfield

8.0 Recommendations

1. Gene technology has considerable potential for the improvement of vegetable brassica germplasm and it is highly recommended that this research continue.

2. It is recommended that the technologies developed during the project be capatilised on. Genetic modification is an ideal way to conduct targeted breeding. We have developed an enabling technology that can be used to transfer any available gene to vegetable brassicas.

3. The transgenic lines generated from this project need to be fully evaluated in glasshouse trials. To date only some transgenic lines with glucose oxidase and GTPI have been screened in preliminary glasshouse trials. Further transgenic lines to be assessed include additional lines with *glucose oxidase* and all those with a truncated *thionin* and the *ipt* genes.

4. Due to the low number of transgenic plants produced, it is important that ways to improve vegetable brassica transformation continue. Research using the green fluorescent protein maybe a better alternative than the frequently used *gusA* gene.

5. During this research project it was announced (December 2000) that the genome of *Arabidopsis thaliana* had been sequenced. *Arabidopsis* has become the 'model species' for plant research as it has a small genome, well-known genetics, a short life cycle (eight to 10 weeks) and can be easily transformed with *Agrobacterium*. Importantly for the vegetable brassica industry is that *Arabidopsis* is susceptible to clubroot and diamondback moth. This makes *Arabidopsis* an ideal experimental plant to study problems associated with vegetable brassicas. It is recommended that a small research programme with *Arabidopsis* proceed in parallel with the main brassica project.

6. The vegetable industry needs to be aware of the advantages and disadvantages of gene technology, so that meaningful decisions can be made about future investments. The internet is a powerful resource with a wealth of information. A number of URLs are suggested.

AgBio View (www.agbioworld.org) and
AgNet (www.plant.uoguelph.ca/safefood)

These sites provide daily updates on biotechnology and discuss the advantages and disadvantages.

Horticulture Australia GMO (Guiding Meaningful Opinions) newsletter (www.horticulture.com.au/)

Provides an overview of activities, with a horticultural slant.

Tomorrow's Bounty (www.tomorrowsbounty.org)

Put together by a number agricultural commodity organisations in the USA with the specific purpose of providing information to the agricultural community.

Appendix 1

Appendix 1.1 Effect of silver on the regeneration of eight vegetable brassica cultivars. (see also Table 2.1)

Percentage (%) regeneration

Crop	Cultivar	Basal	Silver nitrate	STS
Broccoli	Green Belt	33	60	58
	Marathon	51	81	64
	Maverick	46	84	36
Brussels sprout	Ambitus	44	88	85
Cabbage	Green Coronet	24	44	15
	Savoy King	78	76	50
Cauliflower	Atlantis	94	96	78
	Chaser	69	81	86
	Prestige	86	80	60

Mean shoot number for responding explants

Crop	Cultivar	Basal	Silver nitrate	STS
Broccoli	Green Belt	3.0	4.3	4.5
	Marathon	4.0	6.0	3.5
	Maverick	2.2	2.0	1.5
Brussels sprout	Ambitus	3.7	4.8	4.8
Cabbage	Green Coronet	3.3	3.6	1.6
	Savoy King	5.9	6.4	4.6
Cauliflower	Atlantis	4.5	5.6	2.8
	Chaser	5.0	3.0	3.3
	Prestige	6.0	4.4	3.0

Mean shoot number for all explants

Crop	Cultivar	Basal	Silver nitrate	STS
Broccoli	Green Belt	1.0	2.6	2.5
	Marathon	2.0	3.2	2.5
	Maverick	1.0	0.8	0.4
Brussels sprout	Ambitus	1.6	4.3	4.0
Cabbage	Green Coronet	0.8	1.0	0.3
	Savoy King	4.4	4.8	2.3
Cauliflower	Atlantis	4.3	5.3	2.1
	Chaser	3.0	2.4	2.8
	Prestige	5.0	3.5	2.0

Appendix 1.2 Effect of seedling age and silver concentration on the regeneration index of the cauliflower cultivar Atlantis (see also Table 2.2)

Percentage (%) regeneration

Age (d)	Silver concentration (µM)						
	0	**10**	**25**	**50**	**75**	**100**	**250**
3	86	93	78	96	90	86	84
7	62	90	93	93	88	96	66
14	78	100	92	98	92	88	86

Main effects

a) Silver concentration

0	75
10	94
25	88
50	96
75	90
100	90
250	79

b) Seedling age

3	88
7	84
14	91

Mean shoot number for responding explants

Age (d)	Silver concentration (µM)						
	0	10	25	50	75	100	250
3	5.0	4.1	4.2	3.6	3.8	2.7	2.7
7	5.5	3.7	3.6	4.1	4.5	3.7	2.5
14	5.8	4.6	2.7	4.7	2.4	2.2	3.5

Main effects

a) Silver concentration

0	5.4
10	4.1
25	3.5
50	4.1
75	3.5
100	2.8
250	2.9

b) Seedling age

3	3.7
7	3.9
14	3.7

Mean shoot number for all explants

Age (d)	Silver concentration (µM)						
	0	10	25	50	75	100	250
3	4.3	3.8	3.2	3.5	3.4	2.3	2.2
7	3.4	3.3	3.4	3.8	4.0	3.6	1.7
14	4.4	4.6	2.5	4.6	2.2	1.9	3.0

Main effects

a) Silver concentration

0	4.0
10	3.9
25	3.0
50	3.9
75	3.2
100	2.6
250	2.3

b) Seedling age

3	3.3
7	3.3
14	3.3

Appendix 1.3 Regeneration of 28 vegetable brassicas based on cultivar (see also Table 2.3)

Crop	Cultivar	Regeneration (%)	Mean shoot number	
			All explants	Responding explants
Broccoli	TB185	98	6.3	6.5
	Marathon	81	3.2	6.0
	TB254	74	3.0	4.0
	TB234	84	2.6	3.1
	Green Belt	60	2.6	4.3
	HS279	84	2.4	3.0
	TB243	80	2.3	3.0
	Maverick	84	0.8	2.0
Brussels Sprout	Ambitus	88	4.3	4.8
	Oliver	72	1.4	2.0
	Roger	4	0.3	2.4
Cabbage	Savoy King	76	4.8	6.4
	Green Coronet	44	1.0	3.6
Cauliflower	Omeo	100	6.4	6.4
	Marba	100	6.0	6.0
	HS5393	96	5.5	5.7
	Atlantis	96	5.3	5.6
	Holis	100	5.2	5.2
	HS5263	98	5.0	5.1
	Calisa	96	4.6	4.8
	Sirente	100	4.2	4.2
	HS1640	82	4.1	5.0
	Plana	90	4.0	4.3
	Prestige	80	3.5	4.4
	White Rock	84	2.6	3.0
	Virgin	90	2.4	2.7
	Chaser	81	2.4	3.0
	Dova	72	2.5	3.2

Appendix 1.4 Regeneration of vegetable brassica based on crop (see also Table 2.4)

Crop	Regeneration (%)	Mean shoot number	
		All explants	Responding explants
Broccoli	80	2.9	4.0
Brussels Sprout	54	2.0	3.0
Cabbage	60	2.9	5.0
Cauliflower	91	4.2	4.5

Appendix 1.5 Effect of BAP and NAA on regeneration of Green Coronet (see also Table 2.5)

BAP	NAA	Regeneration (%)	Mean shoot number	
			All explants	Responding explants
5	0	10	0.1	1.6
10	0	8	0.1	1.2
20	0	6	0.1	1.6
5	0.5	6	0.1	1.3
10	0.5	0	0	0
20	0.5	8	0.1	1.5

Appendix 1.6 Effect of BAP and zeatin on regeneration of Green Coronet (see also Table 2.6)

BAP	Zeatin	Regeneration (%)	Mean shoot number	
			All explants	Responding explants
20	0	8	0.1	1.2
0	20	40	1.3	3.3
10	10	66	2.5	3.8

Appendix 1.7 Effect of seedling age on regeneration (see also Table 2.7)

Crop	Cultivar	Seedling age (days)	Regeneration (%)	Mean shoot number	
				All explants	Responding explants
Broccoli	Maverick	7	92	2.6	2.9
		14	96	3.2	3.4
Cauliflower	Atlantis	7	96	5.3	5.6
		14	90	8.5	9.5

Appendix 2

Appendix 2.1 Shoot dry weight of transgenic Atlantis lines inoculated with clubroot spores at two densities

Line	Inoculum density (spores mL^{-1})		
	0	10^4	10^8
1152	1.40	0.92	1.26
1153	1.40	1.96	1.30
1154	1.43	1.33	1.02
1213	1.76	1.22	0.61
1214	1.51	1.24	0.73
1215	1.19	1.65	1.12
1216	1.52	1.17	0.67
1217	1.90	1.78	1.20
1218	1.59	1.43	1.12
1219	1.61	1.30	0.97
Control	0.88	1.77	0.76

Appendix 2.2 Root dry weight of transgenic Atlantis lines inoculated with clubroot spores at two densities

Line	Inoculum density (spores mL^{-1})		
	0	10^4	10^8
1152	0.50	0.37	0.40
1153	0.57	0.68	0.64
1154	0.54	0.50	0.50
1213	0.57	0.42	0.42
1214	0.51	0.37	0.41
1215	0.39	0.44	0.66
1216	0.39	0.39	0.53
1217	0.78	0.64	0.67
1218	0.46	0.48	0.41
1219	0.41	0.45	0.41
Control	0.27	0.58	0.32

Appendix 2.3 Shoot:root: ratio of transgenic Atlantis lines inoculated with clubroot spores at two densities

Line	Inoculum density (spores mL^{-1})		
	0	10^4	10^8
1152	2.80	2.49	3.15
1153	2.46	2.88	2.03
1154	2.65	2.66	2.04
1213	3.09	2.90	1.45
1214	2.96	3.35	1.78
1215	3.05	3.75	1.70
1216	3.90	3.00	1.26
1217	2.44	2.78	1.79
1218	3.46	2.98	2.73
1219	3.92	2.89	2.37
Control	3.26	3.05	2.38