APPENDICES

B Preparation of polyacrylamide gel (PAGE)

a) Preparative gel solution (7.1 % (w/v))

Acrylamide	6.81 g
N,N-methylene bisacrylamide	0.29 g

The mixture was mixed with gel buffer (0.375 M trizma base pH 8.8). The volume was made up to 100 ml.

b) Polymerising agents per 100 ml gel solution

Ammonium persulphate (2 % (w/v))	2.5 ml
N,N,N',N'-tetramethyl-ethylene diamina (TEMED)	60.0 µl

Ammonium persulphate was added to gel solution and degased for 10 minutes. TEMED was added and the solution was thoroughly mixed. The mixture were dispensed into each plate, the combs were placed into position and the gel was left to set.

c) Bromophenol blue.

Extraction buffer	1.9 ml
Bromophenol blue 1 % (w/v)	200.0 µl
1 drop glycerol	

C Staining Solution

Malate Dehydrogenase (Vallejose 1983)

Reaction: L-Malate + NAD → Oxaloacetate + NADH

Stain: Tetrazolium system.

Staining solution:

Tris 0.1 M	100 ml pH 7.5
DL-Malate 1 M	3 ml pH 7.5
NAD^+	30 mg
MTT	20 mg
PMS	4 mg

Procedure: Gels were incubated in the dark at 30° C for 15 to 16 min or until blue bands appeared.

Glutamate Oxaloacetate Transaminase (Aspartate Aminotransferase)

Reaction: L-Aspartate + 2-Oxaloglutarate → Oxaloacetate + L-Glutamate

Stain: diazonium system

Staining solution:

Tris HCl 0.2 M	50 ml pH 8.0
Pyridoxal 5-phosphate	1 mg
L-aspartic acid	100 mg

α-ketoglutaric acid	50 mg
Fast Violet B Salt	50 mg

Procedure:

Fast violet B was added to the rest of the ingredients just before use. Gels were incubated at 37° C until red orange bands appeared.

Esterase (Aryl Esterase)

Reaction:

Aryl acetate + H_2O → Aryl alcohol + Acetate

Stain: Diazonium system

Staining solution:

Na Phosphate 0.2 M	60 ml pH 6.0
α-Naphthyl acetate	50 mg
(in 1 ml acetone)	
Fast Blue RR salt	50 mg
(in 40 ml H_2O)	

Procedure:

Diazonium salt was mixed with the naphthyl acetate first, then buffer was added. This solution was prepared just before the start of incubation. The gels were immersed in it and quickly transfered to a dark cabinet. Incubation was done at 30° C for 4 to 8 hours.

Peroxidase

Staining solution:

Acetate 0.2 M	45 ml pH 5.0
3-Amino-9-ethyl carbazole	40 mg
(in 2.5 ml N,N-dimethylformamide)	
0.1 M $CaCl_2$	1 ml
30 % H_2O_2	100 ml

Procedure:

Gels were incubated in solution in the dark at 30° C until the blue bands appeared.

Acid Phosphatase (Vallejose, 1983)

Staining solution:

Na Acetate 50 mM	100 ml pH 5.5
$MgCl_2.6H_2O$ 1 M	1 ml
Fast Black K salt	100 mg
β-Naphthyl acid phosphatase 1 %	3 ml
(in 50 % acetone)	

Procedure:

Diazonium salt was dissolved in the buffer, then the naphthyl phosphate was added. At this point a suspension of particles appeared. The gels were incubated in the dark at

30° C for 1 to 5 hours or until purple or red bands appeared. The solution was discarded and rinsed with tap water .

Laccase (Polyphenol Oxidase or Phenol Oxidase)

(Vallejose 1983)

Reaction:

4 Benzediol + O_2 → 4 Benzosemiquinone + $2H_2O$

Stain: Redox dye, formation of azine.

Staining solution:

Na Phosphate 0.1 M	100 ml pH 6.8
Catechol	15 mg
Sulfanilic Acid	50 mg

Procedure:

The gels were incubated at 30° C for 30 min or until bands activity appeared.

APPENDIX 2

Phosphate buffer (0.05 M) pH 7.2 was prepared by diluting 25 ml Phosphate buffer to a total of 100 ml.

Preparation of Phosphate Buffer.

A = 0.2 M solution of monobasic sodium phosphate

(27.8 g in 1000 ml)

B = 0.2 M solution of dibasic sodium phosphate

(53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of

$Na_2HPO_4.12H_2O$ in 1000 ml)

14 ml of A + 36 ml of B and diluted to a total of 100 ml.

Extraction solution for inhibition of browning :

1) 2 % (w/v) insoluble PVP
2) 10 mM Potassium metabisulphite (inhibitor)
3) 5 mM Potassium metabisulphite + 1 % (w/v) insoluble PVP
4) 0.5 mM Mercaptobenzothiazole
5) 20 mM Diethyldithiocarbamate (DIECA) (inhibitor)
6) 20 mM L-Cystein HCl

All of the compounds were weighed and dissolved in water.

APPENDIX 3

Sterilisation technique

I Sterilisation of media and equipment

IA By steam

All equipment were sterilised in an autoclave, with a standard sterilisation cycle of 20 min at 121°C

IB By filtration

Filtration was used as an alternative method for plant growth regulators (GA_3, IAA) that would be damaged by steam sterilisation. The solutions were sterilised by passing through a 0.22 μm Swinnex-25 filter (Millipore), used in conjunction with a syringe (for small volumes).

APPENDIX 4

Murashige and Skoog media (1962). A commercial powder was being used, Murashige and Skoog medium without sucrose and agar (Flowlab, Sydney, Australia). All media was adjusted to pH 5.8 using NaOH and HCl.

Macronutrient

	mg/l
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 . 2H_2O$	440
$MgSO_4 . 7H_2O$	370
KH_2PO_4	170

Micronutrient

	µg/l
$MnSO_4 . 4H_2O$	22300
$ZnSO_4 . 7H_2O$	8600
H_3BO_3	6200
KI	830
$CuSO_4 . 5H_2O$	25
$Na_2MoO_4 . 2H_2O$	250
$CoCl_2 . 6H_2O$	25

$FeSO_4$. $7H_2O$	27850
Na_2EDTA. $2H_2O$	37250

Vitamin

	µg/l
Thiamine HCl	100
Nicotinic acid	500
Pyridoxine HCl	500

	mg/l
Glycine	20
Inositol	100

The following additions are added to the basic solidified (0.8 % (w/v) agar) Murashige and Skoog medium to make each medium.

		mg/l
MSO	No additions	
MS1	NAA	1.0
	Kinetin	1.0
MS2	NAA	1.0
	Kinetin	1.0
MS24	2,4-D	1.0
MS25	NAA	1.0
	6-BAP	5.0
MS26	6-BAP	5.0
MS27	IAA	2.0
	6-BAP	4.0
MS28	IAA	2.0
	6-BAP	4.0
	GA_3	1.0
MS29	NAA	1.0
	2,4-D	0.5
	6-BAP	0.5
MS30	NAA	1.0
	2,4-D	0.5
	Kinetin	0.5