APPENDIXES

Appendix A

Culture media

1- Murashige and Skoog's (MS) medium (1962) nutrient formulations.

Constituent	MS medium (mg/l)
Macronutrients	
$CaCl_2.2H_2O$	440.0
NH_4NO_3	1,650.0
KNO_3	1,900.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
Micronutrients	
KI	0.33
$CoCl_2.6H_2O$	0.025
H_3BO_3	6.20
$Na_2MoO_4.2H O_2$	0.25
$MnSo_4.4H_2O$	22.30
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.60
Iron	
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Vitamins	
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Thiamine.HCl	0.1
Myo-inositol	100.0

Sucrose 3% (w/v) and agar 0.8% (w/v) were added to the media before steam autoclaved.

2-Murashige and Tucker (MT) medium (1969).

Constituent	MS medium (mg/l)
Macronutrients	
$CaCl_2.2H_2O$	440.0
NH_4NO_3	1,650.0
KNO_3	1,900.0
KH_2PO_4	170.0
$MgSO_4.7H_2O$	370.0
Micronutrients	
KI	0.33
$CoCl_2.6H_2O$	0.025
H_3BO_3	6.20
$Na_2MoO_4.2H O_2$	0.25
$MnSo_4.4H_2O$	22.30
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.60
Iron	
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Vitamins	
Nicotinic acid	5.0
Pyridoxine.Hcl	10.0
Thiamine.HCl	10.0
Myo-inositol	100.0

Sucrose 3% (w/v) and agar 0.8% (w/v) were added to the media before steam autoclaved.

3-Phytohormones tried

Culture media used contained MT basal media and the addition of NAA and BAP as an individuals or in combinations.

MT with NAA or BAP as individual phytohormones.

NAA at (0, 0.1, 0.5, 1, 2, 3, 5, and 10 mg/l) and BAP at (0, 1, 2, 3, 5, 10 mg/l)

MT with (NAA, BAP) in combinations at.

(0.5, 1), (1, 1), (1, 2), (1, 3), (2, 1), (2, 2), (2, 3), (5, 1), (5, 2), (5, 3)

Appendix B
Sterilisation methods

The types of sterilisation methods employed for the various apparatus and solutions depend on the nature of the substance to be sterilissed. Liquids can only be autoclaved but if they contain thermolabile compounds, they should be filter sterilised.

1. By steam

All glassware's, equipment and media were sterilised in an autoclave, with a standard sterilisation cycle of 20 minutes at 121 ^{o}C (1 Kg/cm ^{2}).

2. By filtration

Filtration was used as an alternative method of sterilisation for media containing components that would be damaged by steam sterilisation. All enzyme and some phytohormones were sterilised by passing through a 0.22 μm Millex-GU Millipore.

Appendix C

Composition of media used for the washing of protoplasts

CPW salts:

Components	Concentration (mg/l)
$KH_2.PO_4$	27.2
KNO_3	101.0
$CaCl_2.2H_2O$	1480.0
$MgSO_4\ 7H_2O$	246.0
KI	0.16
$CuSO_4.5H_2O$	0.025

Solutions were prepared by adding 21% (w/v) sucrose to the CPW salts and 13% (w/v) mannitol for CPW 13M pH was adjusted to 5.7 - 5.8 using 1 N NaOH and HCl before sterilisation and stored in refrigerator at 10 C.

Appendix D

Enzyme composition

All the enzymes were stored as dry powder, at 0 °C. Enzyme mixture made by dissolving the respective dry weight of each enzyme in CPW13M, filter sterilised and stored in 10 ml aliquots in centrifuge tubes, identified by enzyme code. The mixtures were thawed before use.

Enzyme code	Enzyme mixture % (w/v)
E_1	Cellulase R-10 3.00 % Macerozyme R-10 0.30 %
E_2	Cellulase R-10 3.00 % Macerozyme R-10 0.30 % Driselase 0.10 %
E_3	Cellulase R-10 3.00 % Macerozyme R-10 0.35 % Driselase 0.10 %

Appendix E

Leaf anatomy

Preparation of plant material for microtme sectioning Jhonsen, (1940).

1-Fixing

The specimen were cut to the required size and then fixed in FAA solution.

Preparation of FAA.

70% ethyl alcohol	90 ml
glacial acetic acid	5 ml
Formaldehyde	5 ml

The specimens were kept in the fixative at 10 ^{o}C for at least 48 hours.

2-Washing

The specimens were washed in 70% alcohol for a few minutes.

3-Dehydration

The specimens were dehydrated through a series of Tertiary Butyl Alcohol (T. B. A.) as follows :-

step	95 % alcohol (ml)	100 alcohol (ml)	T. B. A. (ml)	Distilled water (ml)	Time interval (hours)
1	50	-	-	50	4
2	50	-	10	40	4
3	50	-	20	30	4
4	50	-	35	15	4
5	50	-	50	-	4
6	-	25	75	-	12
7	-	-	100	-	12
8	-	-	100	-	12
9	-	-	100	-	12

4. Infiltration (Modified Technique)

(a) Specimens were transferred into another fresh 100% T.B.A. and suction at 25-30 atmospheric pressure was applied at 60C for 10 minutes.

(b) After 12 hours, the specimens were transferred into a mixture of 1:1 fresh T.B.A. and tissue prep.

(c) After 12 hours, the mixture was poured away and replaced with tissue prep. The specimens were left inside the tissue prep for a few hours in 60 ^{o}C oven. Suction at 25 - 30 atmospheric pressure was applied for 30 minutes and left to rest for one hour suction was once again applied for half an hour.

(d) After 12 hours, the specimens were ready for embedding.

5. The tissue prep wax along with the specimens was poured out into a paper boat. The specimens were arranged in proper position and then the wax block was left to cool for 45 minutes in a basin filled with water and cut into smaller blocks.

6. Sectioning

The sectioning process was carried out by using a hand microtome in an air-conditioned room. Leaf sections were cut at 8μm.

7. Mounting

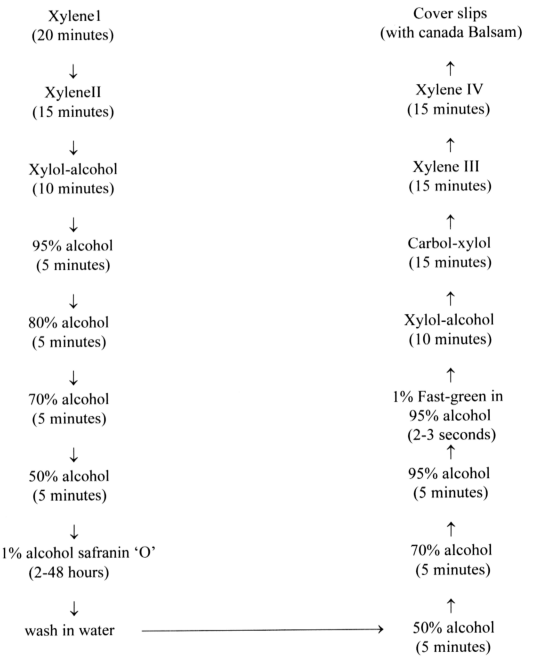
(a) The wax ribbon was cut into suitable length (about 4 cm). A drop of egg albumin was smeared on a clean slide. The wax ribbon was placed on top of the egg albumin and a few drops of distilled water were added .

(b) The slides were warmed at 10 ^{o}C on a hot plate so that the tissues in the wax ribbon became well spread out.

(c) The slides were then dried in the oven at 40 ^{o}C for a few days (4 days).

8. Staining the specimens in safranin-fast green

The generalized schedule for removing wax, redehydrating, staining and rehydrating sections are as follows:



9. Preparation for staining

(a) Xylol- alcohol

1: 1 mixture of xylene and 95% alcohol.

(b) Carbol-xylol

3 : 1 mixture of xylene and phenol crystal.

10. Drying

Slides were left to dry at 40 ^{o}C for at least 4 days before standing them up .

APPENDIX F

Isoenzyme analysis

Preparation of isoenzyme analysis using polyacrylamide gel electrophorasis (PAGE).

1- Buffer solutions

a) Extraction buffer

Trisma base (0.05 M)	500.0 ml (pH 8.0)
Dithiothereitol (DTT)	154.24 mg
Glycerol	20.0 ml
$MgCl_2$ (anhydrous)	76.2 mg
Antipain (1 mg/ml)	0.677 ml
Leupeptin (2 mg/ml)	0.25 ml

The volume was made up to 1000 ml with distilled water.

b) Gel buffer

Trizma base	0.375 M

Using 1N HCl , it was adjusted to pH 8.8 .

c) Running buffer /Top buffer

Trizma base	0.025 M
Glycine	0.129 M

Using 1N HCl, it was adjusted to pH 8.3 .

2- 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 was dispensed into each plate, the combs were placed into position and the gels were left to set.

c) Bromophenol blue.

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

+ 1 drop glycerol

3- Staining solutions

a. Glutamate Oxaloacetate Transminase (Aspartate Aminotransferase)

Reaction:

L-Aspartate + 2-oxaloglutarate ------> oxaloacetate + L-Glutamate

Staining solution:

Tris HCl 0.2 M	50 ml pH 8.0
Pyridoxal 5-phsphate	1 mg
L-aspartic acid	100 mg
∝-Ketoglutaric acid	50 mg
Fast Violet B Salt	50 mg

Procedure:

Fast violet B was added to the rest of ingredients just before use and the gels were incubated at 37 ^{o}C until red orange bands appeared.

b. Peroxidase

Staining solution:

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

Procedure:

The gels were incubated in the solution in the dark at 30 ^{o}C until the blue bands appeared.

c. Malate dehydrogenase (Vallejiose, 1983)

Reaction: L-Malate + NAD ------> oxaloacetate + NADH

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: The gels were incubated in the dark at 30 ^{o}C for 15 to 20 minutes.