CHAPTER II

MATERIALS AND METHODS

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Various *in vivo* and *in vitro* methods employed in the present investigation are described here.

(A) PLANT MATERIALS

Nine varieties of *Cajanus cajan* (L) Millsp (Pigeonpea) viz. T-15-15, GAUT 82-90, GAUT 82-99, AGS-498, Bandapalera, NP (WR) 15, BDN-2, Pusa Ageti, ICP 7336 were tried in the present studies. The seeds of above varieties were obtained from the Pulse Research Station, Gujarat Agricultural University, Model Farm, Baroda. The seeds of *Atylosia lineata* and *A. cajanifolia* were procured from Genetic Resources Unit, ICRISAT, Patancheru (AP). The seeds of *Atylosia lineata* and natural disease resistant as well as susceptible varieties of pigeonpea were grown at the Model Farm, Baroda for hybridization and ovule and embryo culture studies. Some of the seeds of all the varieties were grown in pots containing vermiculite for pathological studies.

Aseptic seedlings grown from above seeds served as the primary source of explants.

(B) CULTURE MEDIUM

b-1 Chemicals

The chemicals used in experimentation were of the highest purity available and obtained from Qualigen (BDH, AR grade), E. Marck (GR grade) and Sigma Chemical Company (USA).

b-2 Culture vessels

Depending upon the type of culture, test tubes (125 x 25 mm) or Erlenmeyer flasks (100 ml or 150 ml) were used. All the glasswares used in the present study were of Corning/Borosil make. Before use, they were cleaned with 40% chromic acid (potassium dichromate in sulphuric acid). The acid was washed off by thorough rinsing with running tap water. The glasswares were again cleaned with detergent teepol (BDH) and washed in running tap water. Finally, they were rinsed with double distilled water and dried in an oven at 80°C.

b-3 Preparation of Coconut milk

Coconut milk was derived from green coconuts purchased from the local market. The milk obtained was deproteinated by repeated boiling, cooling and filteration till all precipitates were removed. Aliquotes of 100 ml were placed in Erlenmeyer flasks and autoclaved. The sterile coconut milk was later stored in a deep-freeze.

b-4 Preparation of Media

In the present study, six different salt formulations were tested:

(1) White's medium (White 1954)

(2) MS medium (Murashige and Skoog's 1962)

(3) LS medium (Linsmaier and Skoog 1965)

(4) B_5 medium (Gamborg et al 1968)

(5) Modified B_5 medium (Mante and Boll 1975)

(6) EC_6 medium (Maheswaran and Williams 1984)

Vitamins of B_5 were used as vitamin source in all the salt formulations. The compositions of the media are presented in Table-1. Of these, modified MS (MMS) medium (salt of MS and Vitamins of B_5) was found the most favourable and was therefore used in all the subsequent experiments. Additions or modifications were done as and when required and are indicated in the text.

Separate stock solutions were prepared of macro and micro element salts in double glass distilled water and were stored in a refrigerator at 5-10°C. Final medium was prepared with double distilled water using these stocks. Supplements to the basal medium were added prior to final adjustment of the volume. pH was adjusted to 5.8 using GP Electronics pH meter with the help of 0.1 N NaOH or 0.1 N HCl. Medium was solidified by adding 0.8% (w/h) bacteriological agar (Qualigen) and boiled till dissolution with constant stirring.

Table 1 : Compositions of the different media

	Macro salts	Murashige and Skoog (MS) mg/l	Gamborg (B_5) mg/l	EC$_6$ mg/l	Linsmaier and Skoog (LS) mg/l	White mg/l	Modified B_5 mg/l
a.	$(NH_4)_2SO_4$	-	134	-	-	-	150
b.	NH_4NO_3	1650	-	600	1650	-	-
c.	KNO_3	1900	2500	950	1900	80	2500
d.	$Ca(NO_3)_4H_2O$	-	-	-	-	300	-
e.	$CaCl_2.2H_2O$	440	150	166	440	-	250
f.	$MgSO_4.7H_2O$	370	250	185	370	720	250
g.	KH_2PO_4	170	-	170	170	-	-
h.	$NaH_2PO_4.H_2O$	-	150	-	-	16.5	150
i.	KCl	-	-	-	-	65	-
j.	Na_2SO_4	-	-	-	-	200	-
k.	Na_2EDTA	37.35	37.35	37.35	37.35	-	40
l.	$FeSO_4.7H_2O$	27.85	27.85	27.85	27.85	-	27.85
m.	$Fe_2(SO_4)_3$	-	-	-	-	2.5	-
	Micro						
a.	KI	0.83	0.75	0.083	0.83	0.75	0.75
b.	H_3BO_3	6.20	3.00	0.62	6.20	1.50	3.00
c.	$MnSO_4.H_2O$	22.30	-	22.30	22.00	5.00	10.00
d.	$ZnSO_4.7H_2O$	8.60	3.00	0.86	8.60	3.00	3.00
e.	$Na_2MoO_4.2H_2O$	0.25	0.25	0.025	0.25	-	0.25
f.	$CuSO_4.5H_2O$	0.25	0.25	0.0025	0.025	0.001	-
g.	$COCl_2.6H_2O$	0.025	0.25	0.0025	0.025	0.01	0.25
h.	$MnSO_4.H_2O$	-	10.00	-	-	-	-
	Vitamins and others						
	Thiamine HCl	0.10	10.0	-	0.4	0.1	1.0
	Nicotinic acid	0.50	1.0	-	-	0.5	1.0
	Pyridoxine HCl	0.50	1.0	-	-	0.1	1.0
	Glycine	2.00	-	-	-	3.0	20.0
	Myo-Inositol	100	100	100	-	-	45.0
	Glutamine	-	-	-	-	-	60.0
	Glutamic acid	-	-	-	-	-	7.5
	Asparagine	-	-	-	-	-	10.0
	Aspartic acid	-	-	-	-	-	7.5
	Urea	-	-	-	-	-	45.0
	Arginine	-	-	-	-	-	10.0
	Adenine sulphate	-	-	-	-	-	2.5
	Folic acid	-	-	-	-	-	0.1
	Biotin	-	-	-	-	-	0.1
	Riboflavin	-	-	-	-	-	0.1

For culture and maintenance of the fungus Fusarium udum liquid modified Richard's medium (Hendrix and Nielson 1958) was employed.

b-5 Sterilization of Media and Culture Vessels

Fixed volumes of hot medium were transferred to culture vessels. The culture vessels were plugged with non-absorbent cotton wool plugs wrapped with a double layered gauze. Brown paper coverings were used to protect them from condensed water during autoclaving. Petridishes and other glass vessels used in experimentation were wrapped in brown paper and sterilized by autoclaving. The instruments like scalpels, spatula, forceps, scissors etc. were sterilized by flaming with alcohol inside the chamber. While not in use, these instruments were kept immersed in alcohol. The glassware and media were sterilized by autoclaving at a pressure 15 lb/in sq. for 15m (121°C).

C. ASEPTIC TECHNIQUES

All inoculations and aseptic manipulations were carried out in a Laminar flowhood (Kleinzaids, Bombay). The working table was cleaned with 70% alcohol. Ultra violet light and air flow was kept

on for 30 m before use to reduce any chances of contamination.

c-1 Surface sterilization of seeds (for imbibition and raising of seedlings)

Seed of C. cajan (Viz. Bandapalera) were thoroughly washed first with mild detergent teepol solution and cleaned with tap water. Later they were rinsed with 80% ethanol for 4-5 m followed by washing with distilled water. Finally, surface disinfaction was done in the laminar hood with 0.1% (w/v) mercuric chloride solution for 3 m and rinsed with sterile distilled water at least three times. The seeds were soaked in sterile distilled water for imbibition on a gyrotary shaker for 3-4 h. They were then introduced aseptically into sterile culture vessels containing solid medium, 2-3 seeds per each vessel. The culture vessels were kept under 8-16 h light-dark regime.

In case of other five genotypes [T-15-15, GAUT 82-90, BDN-2, Bandapalera, NP (WR) 15], the sterilized seeds were incubated in sterile water-containing flask on a gyrotary shaker for 16-18 h for imbibition before inoculation on the culture medium. Differential imbibition periods were given

for aseptic seed germination (3-4 h) and for obtaining cotyledon explants (16-18 h) respectively.

c-2 Surface sterilization of pods

Different cultivars of pigeonpea, grown in the field at Model Farm, Baroda were selfed. Crossed flowers were tagged on the day of pollination and pods were excised at different intervals. These pods were thoroughly washed with running water till they became free of adhered dust. They were surface sterilized in 80% ethanol for 4 m and 0.1 % (w/v) mercuric chloride for 5 m followed by 3 rinses in sterile distilled water under aseptic conditions.

c-3 Sterilization of disease infacted material

Infected root portions of pigeonpea were collected from the field at Model Farm, Baroda. The infected roots were cut into small segments and thoroughly washed with running water till they became free of adhered dust. After surface sterilization in 0.1 % (w/v) mercuric chloride solution for 3 m, they were rinsed with sterile distilled water for three to four times.

c-4 Subculture and growth measurement

i. Subculture

The explants were transferred on to freshly made media of same compositions as and when required depending on the experiments and explant responses. Culture ressels with experimental cultures were maintained in the culture room.

ii. Fresh and dry weights

Growth measurements were made as a function of increase in fresh and dry weights of culture tissues. Of the total number of replicates in culture, 5 were harvested at fixed intervals of time till the end of culture period. Standard error was calculated to ascertain statistical significance of growth in a particular treatment. The cultured tissues grown on solid medium were carefully removed from the culture vessels and freed from specks of agar which was adhered at the point of contact. Accumulated water was blotted out gently, without squeezing the tissues. The tissue was transferred onto preweighed aluminium foil and the weight was determined on a single pan balance. After recording the fresh weights, the tissues were oven dried at

60°C to a constant weight on the same foils for determination of their dry weight.

(D) CULTURE TECHNIQUES

d-1 Inoculation of the seedling explants

In vitro grown seedlings (7 days old) of *C. cajan*, (var. Bandapalera) were used as the source material. Various parts viz., epicotyl, leaf and cotyledons of these seedlings were used as explants. The explants were cultured on modified MS (MMS) medium. Hormones were supplemented in different combinations and conc. as required. Two or three explants, measuring 1.0 - 1.5 cm in length, were inoculated in each culture vessel. Six replications were kept for each of the treatments. The cultures were incubated as stated above.

d-2 Somatic embryogenesis from cotyledonary explant

Different genotypes were tried to study the variation in *in vitro* response. The sterilized seeds were incubated in sterile water on a gyratory shaker for different periods of time. After incubation for optimal period, the seed-coats were removed and the cotyledons detached from embryo axis. The cotyledons were cut transversely and only the distal halves of the

cotyledons were used for different experiments to rule out any shoot regeneration from the axillary buds.

d-3 In vitro embryo rescue method

For obtaining viable hybrid embryos and plants, the above said method was employed.

Seeds of natural disease resistant and susceptible varieties of pigeonpea were sonw simultaneously in the field at Model Farm, Baroda, during 1989. Seeds of Atylosia lineata were also sown to synchronize its flowering with that of Cajanus species. Flower buds of appropriate size on the female parents were emasculated by hand between 8.00 to 12.00 a.m. and were immediately pollinated with the male parent. Records were kept of the commencement of bud drop or pod set. Pods were excised 16 to 20 days after pollination for embryo culture.

Flowers of some of the field grown pigeonpea cultivars were tagged on the day of anthesis for pod development studies and for standardization of embryo culture conditins. After the pods were excised as stated above, the immature embryos were dissected aseptically from sterilized pod with the help of needles and small pointed

forceps. The dissected embryos were transferred to culture vessels, each containing 15 ml agar medium. Like all other cultures, the embryos were incubated at 26 ± 2°C with 16 h photoperiod at 2000 lux.

d-4 Ovule culture method

Four to twenty days old pods were collected from the field grown T-15-15 variety. They were sterilized as described above. The ovules were dissected aseptically. Three to four ovules were transferred to each culture vessels, containing 15 ml media and incubated in the culture room.

(E) PATHOLOGICAL STUDIES

e-1 Collection of wilt infected pigeonpea plant and isolation of the pathogen

Infected root portions of pigeonpea, var. T-15-15 were collected from a cultivated plot at the Model Farm, GAU, Baroda. The fungus Fusarium udum, Butler, was isolated on Potato Dextrose Agar medium (PDA) after surface sterilization of infected roots and incubation at room temperature. The fungus was also grown in liquid modified Richard's (MR) medium (Hendrix and Nielson 1958) for maintenance as suspension culture and for use as inoculum.

The taxonomical identification of the fungus was confirmed by Dr. A. K. Sarbhoy, Chief Mycologist, Mycology and Plant Pathology Division, IARI, New Delhi.

For varietal screening for resistance against the pathogen and for selection pressure, the fungus was used either as (1) homogenate of the mycellium, or as (ii) culture filtrate of the medium in which the fungal mycellium grew.

(i) Preparation of fungal homogenate

The fungal mat was collected by filtering one month old suspension culture using Whatman filter paper. After washing the mat thrice with sterile water, it was weighed and homogenized in a known volume of sterile distilled water in a blender. Finally, the homogenate was diluted with sterile distilled water to make solutions of required strengths (w/v).

(ii) Preparation of fungal culture filtrate (CF)

The mycelial mat with conidiospores of the fungal isolate grown in liquid MR mediumm were removed by filtering through a fine nylon mesh. The CF was again filtered twice through Whatman No. 1 filter paper and finally filter-sterilized with 0.22 μ microbiological filter. The filter

sterilized cultures filtrate was used for varietal screening and was also used as selection pressure in *in vitro* experiments.

e-2 Varietal screening of pigeonpea at whole plant level

Various concentrations of fungal mat and CF prepared in sterile distil water as described above, were added to sterile corning test tube (125 mm x 25 mm). The roots of one month old plants of different varieties were surface sterilized and were transferred to tubes keeping their roots fully immersed in (i) the fungal suspension as well as in (ii) CF. Control plants were subjected to sterile distilled water and Richard's medium. The tubes were closed by sterile cotton plugs which did provide support to the aerial shoots and also prevented entry of other contaminants. The experimental set-up was incubated at normal room temperature.

Observations on morphological and anatomical changes were recorded at regular intervals. The inhibitory effect of the pathogen on healthy plant was expressed as the yellowing of lower foliage leaves, vein clearing in upper foliage

leaves, drying up of leaves, wilting of entire plant and wilt index (% wilt-score). The latter was calculated according to Ebells (1967) as follows:

$$= \frac{\text{Sum of wilt scores of all plant in the treatment}}{5 \times \text{number of plants per treatment}}$$

e-3 Varietal screening of pigeonpea using leaf-discs

Leaf discs (8 mm diameter) cut from surface sterilized young leaves of the susceptible variety T-15-15 were inoculated in liquid culture containing various conc. of CF for the determination of 50% Lethal Dose (LD 50). The inhibitory effect of the CF was expressed in terms of decrease in dry wt and chlorophyll degradation from cultured leaf discs. Chlorophyll estimation was carried out on wt. basis at an interval of 24 h upto 22 h. Chlorophyll was extracted with 10 ml of 80% aqueous acetone containing a pinch of $CaCO_3$ to prevent phytin formation. After centrifuging the extract at 2000 rpm for 5 m the O.D. of supernatent was determined at 643 and 663 nm using spectrophotometer. Chlorophyll value was calculated using following formula (Arnon, 1949).

Total chlorophyll (mgl^{-1}) = 17.3 A 663 + 7.18 A 643. Eight varieties of C. cajan and two spp. of Atylosia were screened based on the above experiments.

e-4 Use of culture filtrate as selection pressure in in vitro culture

One month old CF was mixed with molten agar (0.8%) based MMS medium to obtain various conc (v/v) 5, 10, 20, 30, 40, 60 and 80% CF in culture medium. No CF was added to the control medium. The CF was mixed with MMS medium in such a way that the strength of MMS medium remained constant. Alternatively, CF was varied with MR (50%) medium, while MMS (50%) medium remained constant. The medium was supplemented with various concentrations of growth hormones.

F. HISTOLOGICAL PREPARATION

f.1 Diseased plant parts

The root and stem excised from the infected plant were cut into 0.3 cm thick sections for fixation in FAA fluid (6% formalin, 25% acetic acid and 50% ethanol in water) as recommended by Johnston and Booth (1983). After 24 h in fixative, they were dehydrated by passing through

alcohol : xylene series and ultimately embedded in paraffin wax. Paraffin blocks were trimmed and transverse and longitudinal serial sections were cut at 6-10 µm thickness using Spencer's 820 rotary microtome. These sections were stained with 0.05% toluidine blue in 50 mM sodium phosphate buffer (pH 6.8) for 75 sec and then rinsed in water as suggested by Harling and Taylor (1984). Stained sections were dehydrated through alcohol: xylene series and finally mounted in Euperol or DPX.

f-2, Somatic embryoids

Histological preparations of somatic embryoids induced in cultures were also made as stated above.

(G) PHOTOGRAPHY

Exakta or Contax camera was used to photograph the cultures showing different morphogenetic responses under different treatments. ILFORD 125 ASA film was used for most of the exposures. For microphotography also ILFORD 125 ASP film was used standardizing the conditions for exposure, Xerography was used for line drawings.