CHAPTER 3
MATERIALS AND GENERAL METHODS

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MATERIAL AND METHODS - GENERAL

This chapter describes the general procedures for initiation and maintenance of plant and fungal cultures used by me. Specific methods related to individual experiments are described in each of the individual chapters.

1. PLANT AND FUNGAL MATERIAL:

Tobacco seeds of commercial bidi varieties A2, GT4, A23, A3, A3430 (=GT5) and A119 were obtained from Gujarat Agricultural University, Anand. Plants were raised in the Botanical Garden of M.S. University, Baroda. Virulent Strain of Fusarium oxysporum f.sp. nicotianae was isolated from diseased tobacco plant and cultured on modified Richard's solution (Hendrix and Nielson, 1958). Commercially important variety of Sugarcane CO219 and the virulent strain of Red-rot pathogen Colletotrichum falcatum were collected from Regional sugarcane research centre, Navsari, Gujarat.

2. IN VITRO CULTURE PROCEDURES

a. Chemicals:

The chemicals were of high purity grade and were obtained from following suppliers:

British Drug House (B.D.H.), E. Merck, Sarabhai Chemicals, and Sigma Chemicals Co. (USA).

b. Culture Vessels:

Depending upon the type of culture, Erlenmeyer conical flasks of different capacities, test tubes and petridishes were used as culture vessels. They all were made up of corning glass. Before use they were cleaned with chromic acid (potassium dichromate 100g, sulfuric acid 500 ml and water 1000 ml; Alexopoulos and Behnke, 1955). The acid was removed by prolonged rinsing in tap water. Later the glassware were washed with detergent Teepol (B.D.H.) which was then removed completely by tap water. They were then finally rinsed with double glass distilled water and dried in a hot air oven.

c. Composition and preparation of the culture media:

The composition of different media used in the investigation is given in Table 8. The basal media for plant cultures were prepared from concentrated stock solutions which is stored at 5 to 10°C. Double glass distilled water was used for the preparation of stock solutions as well as the media.

The constituents of the medium were added in the order

shown in the Tables 8 to 10. All the supplements to the basal medium were added prior to the final adjustment of volume. After making the volume, pH of the medium was adjusted with the help of Digichem 8201 pH meter (India). For the semi-solid media 0.8% w/v of Difco-Bacto or E Merk agar was added to the pH adjusted medium and heated gently with constant stirring and then was distributed equally into the culture vessels.

d. Sterilization of media and culture vessels:

The mouth of the culture vessels was closed with non-absorbent cotton plugs wrapped with a double layered gauze cloth. Brown papers were covered around these bungs. Media were sterilized by autoclaving at a pressure of 15 p.s.i. and a temperature of 120°C for 20 min.

3. Aseptic procedures

a. Inoculation chamber:

All aseptic manipulations were carried out in a horizontal Laminar air flow cabinet (Klenzaids, India). The cabinet bench was wiped with cotton squab soaked in 70% ethanol. Media containing vessels and the stainless steel tools, viz., anatomical scissors, scalpels,

spatula, needles and forceps were transferred to the table used for the inoculation. While working, the UV was put off and the airflow was switched on. The pressure in the manometer attached to the hood was always maintained between 10 to 15 mm. The tools were sterilized frequently by flaming with absolute alcohol over the flame of spirit lamp during course of aseptic operations.

b. Surface sterilization of plant material

Tobacco Young leaves of tobacco plants from the field grown plants were collected. They were thoroughly washed with tap water and then with distilled water. They were surface sterilized with 80% ethanol for 2-3 min. They were finally washed, copiously with sterile glass distilled water. The same procedure was followed to surface sterilize the diseased portions of tobacco to isolate the endogenous pathogen F. oxysporum.

Sugarcane: The upper portions of the stem were collected from the field grown plants. Upper broad green leaves and lower stem portion were cut and stem tip of 5-7 cm was taken as explant. This portion was surface sterilized with 0.1% Hgcl2 for 8-10 min. and

was rinsed thrice in sterile distilled water. Young leaves from innermost whorle were sliced into 2-3 mm pieces and were inoculated.

c. Inoculation procedure:

The materials were transferred to sterile petriplate and dissected into pieces with the help of sharp scalpel. They were then placed on the culture media with appropriate orientation.

d. Incubation of the culture tissues:

After inoculation onto the medium, the explants were incubated for growth at 25 ± 2°C for 16 hr. photo period. The intensity of light given was close to 3000 lux, provided by cool fluorescent (Philips) tubes.

Healthy and fast growing host and pathogen cultures were maintained on fresh medium by sub-culturing every two to three week intervals. Subculturing involved aseptic transfer of a suitable size inoculum to fresh medium using sterile spatula, nicrome-loop or sterile pipettes. Care was taken to minimise the degree of variability in the present experimental setup. The factors taken into consideration included environmental uniformity as well as homogeneity, age and size of the experimental

materials. In order to confirm and minimise the experimental variation, the experiments were repeated twice and the average reading was taken.

Table 8:

MURASHIGE AND SKOOG'S (1962) MEDIUM

		Concentration in 1 litre of complete medium expressed, as mg of hydrated salt.
I. Inorganic Salts		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_2O$	440.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Potassium dihydrogen orthophosphate	KH_2PO_4	170.00
II. Microelements and vitamins		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Sodium Molybdate	Na_2MoO_4	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese sulphate	$MnSO_4.4H_2O$	22.3
Zinc Sulphate	$ZnSO_4.7H_2O$	8.6
Copper sulphate	$CuSO_4.5H_2O$	0.025
* Ferric sulphate	$FeSO_4.7H_2O$	27.85
* Ethylenediaminetera-acetic acid disodium salt	Na_2EDTA	37.35
Thiamine hydrochloride		0.1
Nicotinic acid		0.5
Pyridoxine hydrochloride		0.5
Glycine		2.0
III. Supplements		
Sucrose		20.0 gms
Myo-inositol		100.0 mg
Kinetin		
Auxin		-
Water (double glass distilled)		1 litre

* A stock solution of Ferric sulphate and Na2EDTA was prepared in following way:

5.57g of FeSO4.7H2O was dissolved in approximately 200 ml of double glass distilled water. The Na2EDTA solution was dissolved in 200 ml of double glass distilled water, heated and mixed (under continuous stirring) with the Ferrous sulphate solution. After cooling the volume was adjusted to 1000 ml. Heating and more stirring resulted in a more stable FeEDTA complex. 5 ml of this stock solution was used to prepare 1 l of medium.

pH of the medium was adjested to 5.8

Table 9:

POTATO - DEXTROSE - AGAR MEDIUM
(JOHNSTON AND BOOTH, 1983)

Constituents	Amounts
Potato extract	
Potatoes	1,800 g
Water	4,500 ml
Medium	
Potato extract	500 ml
Dextrose	20 g
Agar	2 g
Water	500 ml

Peeled and cut potatoes, were suspended in muslin cloth in water and boiled for 10 min. The potato pieces were discarded and the sterile liquid was stored in refrigerator. When required, the extract was mixed with sucrose and water. pH was adjusted to 6.5. Agar was added to it and autoclaved at 15 lbs. P.S.I. pressure for 20 min.

Table 10:

MODIFIED RICHARD'S LIQUID MEDIUM

Constituents	Molarity in Medium	Amount of constituents added (g l^{-1})
NH_4NO_3	125 X 10^{-3}	10.0
KH_2PO_4	37 X 10^{-3}	5.0
$MgSO_4.7H_2O$	2 X 10^{-2}	2.5
$FeCl_3.6H_2O$	1 X 10^{-4}	0.02
SUCROSE	15 X 10^{-3}	50
pH	6.2	