CHAPTER 2

MATERIALS AND GENERAL METHODS

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2-A PLANT MATERIAL

2-B IN VITRO CULTURE PROCEDURE

- 2-B.1. Chemicals
- 2-B.2 Culture vessels
- 2-B.3 Composition and preparation of the media
- 2-B.4 Sterilization of media and culture vessels

2-C ASEPTIC TECHNIQUES

- 2-C.1 Inoculation chamber
- 2-C.2 Surface sterilization of plant material
- 2-C.3 Inoculation procedure
- 2-C.4 Incubation of the culture tissues

2-D MEASUREMENT OF GROWTH

2

MATERIALS AND GENERAL METHODS

This chapter describes the general procedures for initiation and maintenance of cultures used throughout the course of research. Detailed methods for specific experiments are described in individual chapters.

2-A PLANT MATERIAL

Present studies were carried out using a) diploid suspension cultures derived from leaf callus of Nicotiana tabacum var. Anand 2, initiated and maintained on Murashige and Skoog's (MS) medium (1962), b) haploid plants obtained from N. tabacum anthers cultured on Nitsch's medium (1969) and c) tobacco wilt pathogen Fusarium oxysporum f. sp. nicotianae cultured on modified Richard's solution (Hendrix and Nielson, 1958).

Table 2.2 : Nitsch's (1969) medium

Constituent		Molarity in medium	Conc. in stock solution (g l^{-1})	Vol. of stock (ml l^{-1})
A.	KNO_3	9.39×10^{-3}	95.00	10
B.	NH_4NO_3	8.94×10^{-3}	72.00	10
C.	$MgSO_4.7H_2O$	1.54×10^{-3}	18.5	10
D.	$CaCl_2$	1.49×10^{-3}	16.6	10
E.	KH_2PO_4	4.99×10^{-4}	6.8	5
F.*	$Na_2EDTA.2H_2O$ /	1.11×10^{-4}	7.45	5
	$FeSO_4.7H_2O$	1.83×10^{-4}	7.57	
G.	$MnSO_4.4H_2O$	1.65×10^{-4}	5.00	5
	H_3BO_3	1.62×10^{-4}	2.00	
	$ZnSO_4.7H_2O$	6.19×10^{-5}	2.00	
	$Na_2MoO_4.2H_2O$	1.20×10^{-6}	0.05	
	$CuSO_4.5H_2O$	1.00×10^{-7}	0.005	
H.Ø	Myo-inositol	5.55×10^{-4}	20.00	5
	Nicotinic acid	4.06×10^{-5}	1.00	
	Glycine	2.66×10^{-5}	0.4	
	Pyridoxine HCl	2.40×10^{-6}	0.1	
	Thiamine HCl	1.40×10^{-6}	0.1	
	Folic acid	1.10×10^{-6}	0.1	
	Biotin	2.00×10^{-7}	0.01	
	Supplements			
	Coconut milk	-	-	150
	Sucrose	5.85×10^{-2}	-	20(g l^{-1})

* Preparation as mentioned in Table 2.1

Ø 'H' stock was filter sterilized and freeze preserved.

Table 2-3 : Potato-sucrose-agar medium (Johnston and Booth, 1983).

Constituents	Amounts
Potato extract	
Potatoes	1,800 g
Water	4,500 ml
Medium	
Potato extract	500 ml
Sucrose	20 g
Agar	20 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 surcose 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 2-4 : Modified Richard's solution

Constituents	Molarity in medium	Amount of constituent added (g l^{-1})
NH_4NO_3	$125X10^{-3}$	10.0
KH_2PO_4	$37X10^{-3}$	5.0
$MgSO_4.7H_2O$	$2X10^{-2}$	2.5
$FeCl_3.6H_2O$	$1X10^{-4}$	0.02
Sucrose	$15X10^{-3}$	5.0

The constituents of the medium were added in the order shown in the Tables 2-1 to 2-4. All supplements to the basal medium were added prior to the final adjustment of volume. After making the volume, pH of the medium was measured and adjusted according to the need of different culture growths, with the help of Digichem 8201 pH meter (India). For the semi-solid media 0.8% w/v of Difco-Bacto or E Merck agar was added to the pH adjusted medium and heated gently with constant stirring and then was distributed equally into the culture vessels.

2-B.4 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 lbs. p.s.i. (125°C) for 20 min.

2-C ASEPTIC TECHNIQUES

2-C.1 Inoculation Chamber :

All aseptic manipulations were carried out in a horizontal Laminar air flow cabinet (Klenzaids, India). The cabinet was saturated with an aerosol of 2% thymol in 90% ethanol and the work bench was wiped with a cotton squab soaked in 70% ethanol. Media containing vessels and the stainless tools, viz., anatomical scissors, scalpels, spatula, needles and forceps

were transferred to the table used for inoculation. While working, the UV was put off and the air flow was switched on. The tools in aseptic operation were sterilized by flaming with absolute alcohol inside the hood.

2-C.2 Surface sterilization of plant material :

Young leaves of tobacco seedlings and floral buds from the mature plants were collected from the greenhouse. The materials were throughly washed with tap water and then with distilled water. They were surface sterilized with 80% ethanol for 2 min. followed by 0.1% (w/v) mercuric chloride 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.

2-C.3 Inoculation procedure :

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

2-C.4 Incubation of the culture tissues :

After inoculation onto the medium, the explants were incubated for growth at 25 ± 2°C in 16 hr photoperiod (1,200 lux light intensity) by day light flourescent (philips) tubes.

Healthy and fast growing host and pathogen cultures were maintained on fresh medium by sub-culturing every two to four weeks. Cell suspension cultures usually require regular subculture at more intervals than the callus cultures from which they were derived. Subculturing involved aseptic transfer of a suitable size inoculum to fresh medium using either pipettes, or autoclavable metal syringes. These cultures served as inocula for the experiments.

2-D MEASUREMENT OF GROWTH

Growth was measured by determining the increase in fresh and dry weights of the total number of replicates inoculated. Four to 6 replicate cultures were harvested at fixed intervals of time for growth measurements. Standard dev.: was calculated to ascertain statistical significance of the growth data.

Care was taken to minimise the degree of variablity in the present experimental set up. The factors taken into consideration included environmental uniformity as well as homogenity, 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. Furthermore, to reduce variability amongst treatments, and to reduce sampling error, many replicates were harvested at random at a time, pooled together and then analysed.