MATERIALS AND METHODS

3. MATERIALS AND METHODS

'Damping-off' is an important disease of greenhouse tomato, causing important losses in nurseries where young susceptible transplants are produced. This disease is mainly caused by *Pythium ultimum* and *Pythium aphanidermatum* which are responsible for seed decay as well as pre- and post-emergence damping-off of tomato seedlings (Gravel *et al.*, 2005). This disease is usually identified in nurseries by which use fungicides. Regular use of fungicides leads to environmental pollution. To overcome this pollution, alternative methods can be utilized for the disease management, like biological control which is ecology conscious and environment friendly. *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp., are the biological control agents which are being used successfully. In the present study, the effect of talc-based formulations of *Pseudomonas fluorescens* on damping - off disease in tomato plant under green house conditions and the activity of the defense enzymes in diseased conditions of the plant and biometric parameters of the plant were analysed.

The experimental procedure for the study entitled **"Induction of Defense Related Enzymes and Growth Promoting Traits in Tomato (*Lycopersicon esculeantum*) Plant"** is discussed under the following headings.

3.1. Collection and maintenance of plant growth promoting rhizobacteria (PGPR)

3.2. Collection and maintenance of fungal pathogens

3.3. Development of talc-based formulation of *P. fluorescens*

3.4. Efficacy of talc-based formulations of *P. fluorescens* isolates against damping – off disease under green house conditions

3.5. Induction of defense mechanism in tomato plant

3.5.1. Sample collection

3.5.1.1. Estimation of phenylalanine ammonia lyase activity

3.5.1.2. Estimation of peroxidase

3.5.1.3. Estimation of polyphenol oxidase

3.5.1.4. Estimation of total phenol

3.5.1.5. Native polyacrylamide gel electrophoresis analysis

3.6. Estimation of Growth hormones

3.6.1. Estimation of Indole acetic acid

3.6.2. Estimation of Gibberellic acid

3.7. Biometric parameters

3.7.1. Root length

3.7.2. Shoot length

3.7.3. Estimation of chlorophyll content

3.1. Collection and Maintenance of Plant Growth Promoting Rhizobacteria (PGPR)

Plant Growth Promoting Rhizobacteria which is used as biocontrol agents were collected from the Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore and pure cultures were maintained on King's B (Appendix I) agar slants at 4°C.

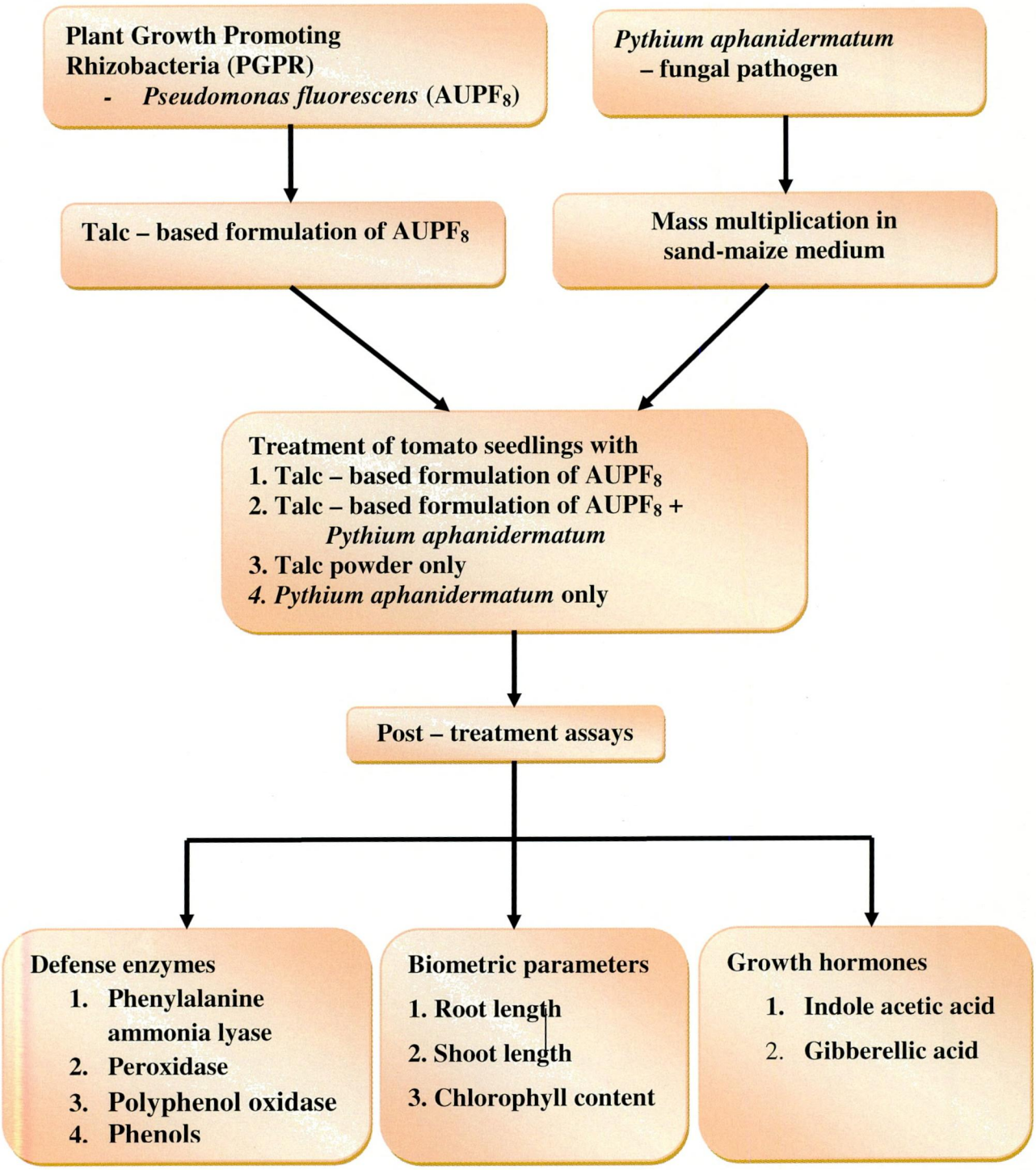
3.2. Collection and Maintenance of Fungal Pathogens

The fungal pathogen *Pythium aphanidermatum* which is responsible for damping – off disease in tomato seedlings were collected from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and the cultures were maintained on Potato Dextrose Agar medium (Appendix I)

3.3. Development of Talc-based Formulation of *P.fluorescens*

Pseudomonas fluorescens strain $AUPF_8$ was found effective in the management of damping-off of tomato plant. This strain was formulated by using talc as the carrier material which is depicted in Appendix II.

EXPERIMENTAL DESIGN



3.4. Efficacy of Talc based Formulations of *Pseudomonas fluorescens* against Damping-off Disease under Green House Conditions

The *Pseudomonas fluorescens* strain $AUPF_8$ was selected for reducing the damping-off incidence in tomato plant under green house conditions. The efficacy of talc-based formulation of $AUPF_8$ was recorded by potting the tomato plantelets with *Pythium aphanidermatum* which mass multiplied in sand – maize medium Appendix III.

3.5. Induction of Defense Mechanism in Tomato Plant

Induction of defense mechanism by *P.fluorescens* $AUPF_8$ was assessed in response to infection by *Pythium aphanidermatum* in tomato plant. The following treatments were carried out.

1. Treatment of tomato seedlings with *P.fluorescens* $AUPF_8$
2. Treatment of tomato seedlings with *P.fluorescens* $AUPF_8$ + *Pythium aphanidermatum*
3. Treatment of tomato seedlings with talc powder
4. Treatment of tomato seedlings with *Pythium aphanidermatum*
5. Control

Three replications were maintained for each treatment.

3.5.1. Sample collection

For all the experiments, plant samples were collected from treated, pathogen inoculated and un-inoculated control at 2 days interval starting from 0-8 days after inoculation of the pathogen.

3.5.1.1. Estimation of phenylalanine ammonia lyase activity

Phenylalanine ammonia lyase (PAL) that is involved in phytoalexins and phenolics synthesis. Phenylalanine ammonia lyase was estimated by the Method of Dickerson *et al.* (1984) which is elaborated in Appendix IV.

3.5.1.2. Estimation of peroxidase

Peroxidase catalyses the oxidation of a variety of electron donors with the help of hydrogen peroxide and thus scavenge the endogenous hydrogen peroxide. This was estimated by the Method of Hammerschmidt *et al.* (1982) as explained in Appendix V.

3.5.1.3. Estimation of polyphenol oxidase

The mechanism of action proposed for polyphenol oxidase is based on its capacity to oxidize phenolic compounds. When the tissue is damaged, the rupture of plastids, the cellular compartment where polyphenol oxidase is located, leads to the enzyme coming into contact with the phenolic compounds released by rupture of the vacuole, the main storage organelle of these compounds (Queiroz *et al.*, 2008). Polyphenol oxidase was estimated by the Method of Mayer *et al.* (1965) as shown in Appendix VI.

3.5.1.4. Estimation of total phenol

Phenolics present in healthy, uninfected plant tissues, as preformed antimicrobial compounds, that inhibit the growth of fungi may include simple phenols, phenolic acids, flavonols, and some isoflavones. Those that are induced in response to fungal infection include phenolic phytoalexins, isoflavonoids, pterocarpans, furocoumarins, flavans, stilbenes, phenanthrenes (Chérif *et al.*,2007). Total phenol was estimated by the Method of Zieslin and Ben-Zaken (1993) and the procedure is presented in Appendix VII.

3.5.1.5. Native polyacrylamide gel electrophoresis (Native- PAGE) analysis

The isoform profile of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis (Laemmli, 1970) which is elaborated in Appendix VIII.

3.6. Estimation of Growth Hormones

3.6.1. Estimation of Indole acetic acid

Indole-3-acetic acid (IAA) is the main auxin in plants controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (Shahab *et al.*, 2009). Indole acetic acid in the samples was estimated spectrophotometrically by the method of Gordon and Weber (1951), as recorded in Appendix IX.

3.6.2. Estimation of Gibberellic acid

Gibberellic acid is a plant growth regulator of economic and industrial importance. Phytohormones, mainly including auxins, cytokinins, abscisic acid, gibberellins, and ethylene, induce some important physiological responses at different stages of plant development at low concentrations. Various gibberellins are available and are associated with several plant growth and development processes, such as seed germination, stem elongation, flowering, and fruit development. Gibberellic acid in root exudates was estimated by spectrophotometrically by the method of Mahadevan and Sridhar, (1982) as explained in Appendix X.

3.7. Biometric Parameters

After one-month of treatment, the seedlings were removed and the plant growth parameters of the seedlings were recorded.

3.7.1. Root length

The root length was measured from the base of the plantlets where the rooting starts to the tip of the longest root and expressed as cm / plant (Raja *et al.*, 2007).

3.7.2. Shoot length

The shoot length was measured from the base of the plantlets to the tip of the growing point and expressed as cm / plant. (Raja *et al.*, 2007).

3.7.3. Estimation of chlorophyll content

The chlorophyll content was determined as described by Wintermans and Demots (1965). 1g of leaf sample was homogenized and extracted with 95% ethanol and centrifuged. The absorbance of the extract was measured at 663 and 645 nm in a Beckman DU-64 Spectrophotometer and the results were expressed as mg/g of tissue as shown in Appendix XI.

Above discussed parameters were done and the results of the tests were explained in detail in next chapter.