3. MATERIALS AND METHODS

3.1 EXPERIMENTAL PLANTS AND SOIL

The crop plants used for the present study were *Abelmoschus esculentus* L. (Moench.) variety 'Pusa Savani' (Okra), *Momordica charantia* L. (Bittergourd) and *Helianthus annuus L. (Sunflower), belonging to the family Malvaceae*, *Cucurbitaceae* and *Compositae* respectively. The seeds of the experimental plants were obtained from the germplasm collection of the Department of Botany, University of Kerala, Karyavattom, Thiruvananthapuram.

Surface soil (0-15 cm) was collected from 24 locations in the botanic garden of the department, air-dried, sieved through 2.0 mm sieve, mixed thoroughly and then used for the study. The soil was reddish brown sandy loam, acidic in reaction (pH 5.2-5.5) and low in total soluble salts (E.C.0.1-0.2 mm hos/cm). It contained low organic carbon (0.15-0.46%) but high levels of available phosphorus (30-42 kg P_2O_5/ha) and potassium (120-540 kg K_2O/ha). Thiruvananthapuram has tropical climate with an average temperature of 30 $\pm$ 3°C and mean annual rainfall of 1600 mm.

3.2 RHIZOSPHERE SOIL MICROFLORA

Soil was filled in twenty earthenware pots of 22 cm diam and 30 cm ht. No manure was added. About 10-15 seeds, after thorough washing in tap water, were sown in each pot and five pots were used for each species. The pots were kept in greenhouse and watered once daily with a definite quantity of water. Seven days after germination, the seedlings were thinned to six plants per pot.

When the seedlings were three-week-old, they were carefully removed from each pot and the adhering soil was removed by shaking well. The roots of all plants in one pot were cut at the collar region and they constituted a replicate. The rhizosphere and control soil microflora were determined by adopting soil-dilution and plate-count method (Timonin, 1940a).

On the basis of dry weight of the soil, serial dilutions were made to get a final dilution of 1:1,000,000 and from the appropriate dilution, 1.0 ml each was transferred to the sterile petri dishes. Three different media, namely, Soil Extract Agar (Allen, 1957), Kuster's medium

(Kuster and Williams, 1964) and Rose Bengal Agar medium (Martin, 1950)* were used for enumerating the bacterial, actinomycete and fungal populations respectively. Ten plates were used for each medium and per replicate. The plates were incubated at room temperature ($30^{\circ}C \pm 1^{\circ}C$) Bacterial and fungal colonies were enumerated after seven days incubation and the actinomycetes after ten days incubation, using a colony counter and the total population was calculated based on the mean value of 50 petri dishes.

3.3 ISOLATION AND MORPHOLOGICAL CHARACTERIZATION OF ACTINOMYCETES

The actinomycete colonies were isolated from Kuster's medium and maintained as pure cultures on Yeast extract-malt extrat agar slants. Characterization of the streptomycete cultures was done following the methods suggested by the collaborators of the International Streptomyces Project (ISP), co-sponsored by the Sub-committee on Taxonomy, American Society of Microbiology and the Sub-committee on the Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature (Shirling and Gottlieb, 1966). Species from other genera were characterized based on Bergey's Manual of Determinative Bacteriology (8th edition) (Buchanan and Gibbons, 1974). However, in addition to Bergey's Manual of Determinative Bacteriology (8th edition) (Buchanan and Gibbons, 1974). Bergey's Manual of Systematic Bacteriology, Vol. IV (Williams *et al.*, 1989) and Ray Fungi Higher Forms Vols. I-III (Krassilnikov, 1981a, b, c) were also referred to wherever found necessary for characterisation of actinomycetes.

Four standard culture media, namely, Tryptone-yeast extract broth (Pridham and Gottlieb, 1948), Yeast extract-malt extract agar (Pridham *et al.*, 1956-57) Oat-meal agar (Kuster, 1959) and Inorganic salts-starch agar (Kuster, 1959), all suggested by ISP collaborators were tried as media for growth and sporulation of actinomycetes. Among these, the Yeast extract-malt extract agar medium was found to be the best. The isolates were maintained on agar slants of Yeast extract-malt extract agar medium.

For morphological studies of actinomycetes, the isolates were transferred on agar plates, two plates per isolate, and incubated at room temperature for one to two weeks.

* Composition of all media used is given in Appendix I.

3.3.1 Colour determinations

i. **The mass colour of the sporulating aerial growth**: The Tresner- Backus colour series (Tresner and Backus, 1963) was the basis for the determination of the colour of spore mass and the aerial mycelium.

ii. **The colour of the substrate mycelium as viewed from the reverse side**: For observing the colour of the substrate mycelium, a bit of the agar plug along with the mature culture was cut and removed using a cork borer of 1.0 cm diam. Excess medium was carefully removed from the reverse side so that the substrate mycelium was clearly visible. Change in colour, if any, on reverse side on addition of 0.05 N NaOH or 0.05 N HCl was also noted.

iii. **Melanin production**: All isolates, both coloured and non-coloured were tested for melanin production in Peptone-yeast extract iron agar (Tresner and Danga, 1958). About 10 ml of the medium was dispensed in 10 x 150 mm culture tubes, sterilized and solidified as slants. Each culture was inoculated on two agar slants and incubated at room temperature. The culture tubes were examined for melanin production two days after the inoculation. Cultures forming a greenish brown to brown and black diffusible pigment or a distinct brown pigment modified by other colours were recorded as 'melanin positive', and the non-producers of such diffusible pigments were designated as 'melanin negative'.

iv. **The diffusible soluble pigment other than melanins**: The secretion of pigments other than melanoids was studied by observing the colours imparted to the medium. Such isolates were regarded as the soluble pigment producers.

3.3.2 Characteristics of spore-bearing hyphae

Spore chain and spore morphology were studied by observing sporulated cultures. Coverslip cultures (Williams *et al.*, 1968) were grown to observe substrate and aerial mycelium simultaneously. A sterilized square coverslip was carefully inserted at an angle of about 45^{o} in Yeast extract-malt extract Agar medium in a petri dish until about half of the coverslip was in the medium. The actinomycete isolate was then inoculated along the line where the medium met the upper surface of the cover slip. During the growth, the colony extends over the coverslip also providing aerial mycelium. When the colony was sufficiently mature, the coverslip was carefully removed, its orientation in the medium being noted and placed upwards on a slide and examined under phase contrast microscope.

The nature, appearance and morphological features of the spore chains were observed and the isolates were grouped into four sections namely, *Rectiflexibiles* (straight to flexuous), *Retinaculiaperti* (open-loops), *Spirales* (Spirals) and *Verticillati* (all verticillate forms) as

suggested by Shirling and Gottlieb (1968a). The approximate number of spores in spore chains, the size and nature of spores, premitiveness of the open loops, tightness of coils of spira, etc. were also observed and noted.

For preservation and photography, the actinomycete growth was fixed on the coverslip with a few drops of absolute methanol and left undisturbed for 15 minutes. The coverslip was washed with distilled water and blotted to dry. It was then stained with crystal violet for a minute, washed off the excess stain and blotted to dry. The coverslip was then placed over a microscope slide with the mycelium upwards, examined under the microscope and photographs were taken from selected slides.

3.3.3 Surface morphology of spores

Surface morphology of the spores were studied using Transmission Electron Microscope (Carlzeiss). Formvar coated copper grids of 100 mesh were gently pressed to the aerial surface of a colony with mature spores. The spore chains which adhere to the coated surface of the grids were observed and photographed at a magnification of about 20,000X. The spore surface was characterized as smooth, spiny, hairy and warty (Shirling and Gottlieb, 1966).

3.4 PHYSIOLOGICAL CHARACTERISTICS

3.4.1 Carbon utilization

For studying the capability of actinomycetes in utilizing the various carbon sources, 1.0 per cent carbon source was incorporated with a basal mineral salt agar medium (Shirling and Gottlieb, 1966 - modified from Pridham and Gottlieb, 1948).

Simple carbohydrates: The carbon compounds recommended by collaborators of International Streptomyces project (ISP), were used in this study. Thus, *L*-arabinose, *D*-xylose, *D*-glucose, *D*- fructose, sucrose, mannose, lactose, maltose, rhamnose, raffinose, *I*-inositol and *D*-mannitol were the carbon compounds incorporated with the basal medium.

All the carbon compounds were sterilized by using ether. The required amount of carbon compound was weighed and placed as a thin layer in a sterilized 250 ml Erlenmeyer flask plugged with a slightly loose cotton plug. Sufficient amount of acetone-free ethyl ether [$(C_2H_5)_2O$] was added to it to cover the carbohydrate. The flask was kept at room temperature under a ventilated fumehood until all the ether was evaporated. Sterile distilled water was added asceptically to the carbon compound to make a 10 per cent W/V solution of the carbon compound.

The solution of sterile carbon compound under study was then added to the sterilized basal medium at about 60°C so as to give a final concentration of 1 per cent. The liquid medium was thoroughly mixed and poured in to petri dishes.

Inoculum of each isolate was prepared as suggested by ISP (Shirling and Gottlieb, 1966). A wire loop full of inoculum was placed at the centre of each petri dish and four replicates were maintained for each isolate. In addition to this, the inoculum was also placed on petri dishes containing carbon-free medium in order to compare the difference in growth between carbon deficient and carbon enriched medium. After fourteen days of incubation the diameter of the colony was measured at right angles to one another and the average reading was recorded. If the average diameter of the colony exceeded 10 mm, the carbon utilization is termed 'efficient', if the diameter ranged between, 5 mm to 10 mm, the utilization is termed 'moderate' and, if the diameter ranged upto 5 mm, the utilization is poor.

The results obtained for all the twelve carbon tests for the four isolate groups, viz., rhizosphere actinomycetes of okra, bittergourd and sunflower and isolates from garden soil, were considered as from a factorial experiment and analysed accordingly.

Complex carbohydrates: The hydrolysis of starch and cellulose was studied by employing the methods adopted by Abraham and Herr (1964). Czapek-Dox Agar (Raper and Thom, 1949) with 10.0 g. of soluble starch substituted for sucrose was the medium used for starch hydrolysis test. Four plates were used for each isolate. After ten days incubation at room temperature, the petri dishes were flooded with potassium iodide solution (Stahly and Weiser, 1951) and left for a minute. Excess iodine solution was then poured off. The size of the cleared zone around the colony was obtained by measuring the zone width from the margin of the colony to the margin of the zone for each isolate. Two readings at right angles were taken for each colony and the mean was recorded. Width of the clear zone 1-5 mm is considered poor hydrolysis, 5-10 mm moderate and more than 10 mm efficient hydrolysis.

For the study of hydrolysis of cellulose, the method adopted by Abraham (1962) using carboxy-methyl cellulose (CMC) was employed. Five grams of CMC (Oxoid) was dissolved in a litre of hot nutrient broth. Ten ml of the solution was then poured into the test-tubes and autoclaved. Four tubes were inoculated with each isolate and incubated for ten days at room temperature (30°C $\pm$ 1°C). Four uninoculated tubes with medium alone served as control. The hydrolysis of CMC was observed by measuring the viscosity change in the broth. For this, the culture was first filtered through glass wool and then poured into a Cannon-Fenske

Viscometer (size No 300), kept in a water bath of constant temperature of 35°C. The control tubes were also filtered and the rate of flow in the Viscometer was noted. The time taken for the passage of the culture filtrate between the two markings in the viscometer was observed as the rate of flow. The difference between the rate of flow of the culture filtrates and that of the control in terms of seconds was recorded. A reduction of 0.1 to 1 sec was considered as poor utilization, 1.1 to 2 sec as moderate and 2.1 and above as efficient.

3.4.2 Hydrolysis of Gelatin

For gelatin liquefaction, the method adopted by Abraham (1962) was employed in this study. The medium was prepared by dissolving 1.6 g nutrient broth and 8.0 g of gelatin per litre. Each isolate was inoculated into four plates. After ten days of incubation at room temperature, each plate was flooded with mercuric chloride (15 per cent) solution. After a few minutes, the excess solution was poured out and the radius of the clear zone was measured and efficiency of gelatin hydrolysis was assessed as described for starch hydrolysis.

3.4.3 Nitrate reduction

Each isolate was tested for its capability to reduce nitrate on duplicate slants of modified Asthana and Hawker's medium 'A' (Reddy, 1971). The inoculated culture tubes and the control tubes (uninoculated culture tubes) were incubated at room temperature for ten days. The culture was tested for the reduction of nitrate to nitrite by adding 2 ml each of solution I and II of, Griess Ilosroy's reagents. Presence or absence of red colour was noted and recorded.

3.4.4 Effect of temperature on the growth of isolates

The actinomycete isolates were grown in Yeast extract-malt extract broth and incubated at 10, 20, 30. 40, 50 and 60°C for two weeks. The cultures were filtered on previously weighed filter paper. dried uniformly at 60°C for 24 hr and the weight of dry mycelium and spores was determined. Comparing the dry weight thus obtained with that of the control (at room temperature), the actinomycetes were grouped as psychrophiles (growing at temperature below 20°C) mesophiles (growing at temperatures between 20 and 40°C) and thermophiles (with a range of optimum temperatures 40-60°C).

3.5 IDENTIFICATION OF ACTINOMYCETE ISOLATES

The actinomycete isolates were characterized, based on the results of various tests carried out, adopting the test methods recommended by collaborators of the International Streptomyces Project for descriptions of type and neotype strains of the genus *Streptomyces* (Shirling and Gottlieb, 1966, 1968a, 1968b, 1969, 1972). However, modification of ISP test methods and additional tests were also carried out wherever found necessary and suitable. Classification of the isolates up to generic level was made following the characteristics given in Bergey's Manual of Determinative Bacteriology, Eighth Edition (Buchanan and Gibbons, 1974). For identifying *Streptomyces* up to species level, diagnostic keys furnished by Nonamura (1974) was adopted in this study.

3.5.1 Similarity index of actinomycete isolates

An estimate of similarity in species composition among identified *Streptomyces* spp. isolated from the four sources viz. rhizosphere soils of okra, sunflower and bittergourd and control soil, were made using Sorensen's index of similarity by using the formula $S = [2c/(a+b)] \times 100$ (Sorensen, 1948), where;

a is the number of species at one source,
b is the number of species at the other source, and
c is the number of species common to both the sources.

3.6 ANTAGONISTIC ACTIVITY OF ACTINOMYCETES

3.6.1 Cross-streak method

The actinomycetes were tested for antagonistic activity in agar culture against ten root pathogenic fungi using cross-streak method. Each actinomycete species was streaked over Yeast extract-malt extract agar medium at the periphery of the Petri dish. The test fungus was streaked at right angles to the original streak of the actinomycete (Pridham *et al.*, 1956) (see Fig. 24). The following were the test fungi used:

i. *Fusarium moniliforme* Sheld.
ii. *F. oxysporum* Schl.
iii. *F. oxysporum* Schl. f. *melonis* Snyder & Hansen
iv. *F. roseum* Lk. emend. Snyder & Hansen
v. *F. solani* (Mart.) Sacc.
vi. *Pythium aphanidermatum* (Edson) Fitzp.
vii. *Rhizoctonia bataticola* (Taub.) Butler
viii. *R. solani* Kuehn

ix. *Sclerotium rolfsii* Sacc.

x. *Verticillium dahliae* Kleb.

In the case of *S. rolfsii*, as mycelium was found to be overgrowing the actinomycetes, sclerotia were serially placed against the actinomycetes.

The actinomycete that retarded the growth of the fungus either completely or partially was regarded as an antagonistic isolate. The zone of inhibition developed between the two colonies was measured. In doubtful cases the test was repeated by streaking the fungus parallel to the streak of the antagonist for confirmation (see Fig. 25).

3.6.2 Assay of sterile culture filtrates of actinomycetes for antagonistic activity

The actinomycete isolates which were found to be antagonists by cross-streak method were further tested by cylinder-plate method using culture filtrate. The cylinder-plate method of Pratt and Dufrenoy (1953) and Waksman (1945) as given in Johnson and Curl (1972), were used for the study. To get the culture filtrate of the actinomycete, 125 ml Erlenmeyer flasks containing sterilized liquid Yeast extract-malt extract medium were inoculated with a uniform quantity of agar culture of the 'antagonistic' isolates and incubated for 10 days. The flasks were periodically shaken by placing over a shaker to avoid heavy sporulation. At the end of the incubation period, the culture was first filtered through a thick pad of glass wool and the filtrate was then further filtered by passing through sintered glass filter. Nutrient agar was used as the assay medium. A base layer of 2 to 3 mm deep of nutrient agar was poured into the petri dishes and kept overnight. About 0.1 ml of a 1:10 dilution of conidial and mycelial suspension of the test fungus was added to 5 ml of sterilized, cooled agar medium and mixed thoroughly. This mixture was poured over the base layer and allowed to solidify. A sterile glass ring (cylinder) of 1 cm ht and 1.3 cm inner diam was placed on the surface of the seeded agar medium at the centre of the plate and filled with previously prepared filter- sterilized culture filtrate of the actinomycetes (Johnson and Curl, 1972) (see Fig. 26). Two plates were used for each fungus and incubated for 7 days. The size of the zone of inhibition was measured 7 days after the addition of the culture filtrate.

The antagonistic study was also made by substituting the cylindrical reservoir with a well, bored in the centre of the agar plate by removing a plug of agar using a cork borer of 1 cm diam. The well was then filled with the filter-sterilized culture filtrate and incubated for 7 days at room temperature (see Fig. 27).

3.7 STUDIES ON THE SUPPRESSION OF SOIL-BORNE PATHOGENIC FUNGI BY ANTAGONISTIC ACTINOMYCETES IN THE SOIL

3.7.1 Test with seeds in soil (Johnson and Curl, 1972)

Three antagonists ie., *S. vastus, S. luteogriseus*, and *S. endus* isolated from the rhizosphere of okra, sunflower and garden soil respectively, were tried against *R. solani* in steam sterilized soil. Surface sterilized seeds of the above plants were soaked in a blended suspension of mycelia and spores of the antagonists for 24 hr. The seeds along with the actinomycete inoculum adhering on the surface were sown in pots of 15 cm diam size, contianing steam-sterilized soil infested with *R. solani*. The soil was infested three days prior to sowing. Four pots were used for each plant species and six seeds were sown in each pot. Similarly, in another experiment seeds were treated with the test pathogen and sown in soil previously infested with the antagonist. Three types of control treatments were also maintained simultaneously, namely;

i. sowing untreated seeds in sterilized soil,

ii. sowing untreated seeds in pathogen-infested soil, and

iii. sowing antagonist-treated seeds in sterilized soil.

The pots were kept in a closed room provided with fluorescent lamps. The pots were watered with a minimum quantity of sterilized tap water. The germination and development of the seedlings were observed every day and the percentage of survival of seedlings assessed after 3 weeks.

3.7.2 Test with seedlings in soil

As it was done for seed inoculation studies, sterilized soil in garden pots of 15 cm diam, was first infested with the test pathogen. After 3 days, the infested soil was then thoroughly mixed with antagonist inoculum and incubated for 10 days. Seedlings of okra and sunflower, grown in garden soil for ten days, were uprooted carefully and their roots were washed first in tap water and then in sterile distilled water. These seedlings were then carefully transplanted in four garden pots containing the above infested soil. Six seedlings were transplanted in each pot. *S. vastus* and *S. endus* were tested against *F. oxysporum* with sunflower seedlings and *S. luteogriseus* and *S. endus* were used against *R. bataticola* using okra seedlings, as the host plants. The plants were maintained at room condition as in the previous experiment.

Similarly, for a comparative study and as a check, the following tests were also carried out with certain modifications and alterations in the procedure, with replications as above.

i. Seedlings were transplanted in non-sterilized soil without any antagonist or pathogen.

ii. Seedlings were transplanted in sterilized soil without any antagonist or pathogen.

iii. Seedlings were transplanted in antagonist treated soil.

iv. Seedlings were transplanted in fungus-inoculated soil.

v. Seedlings, treated (root dipping) with antagonist suspension were transplanted in sterilized soil, inoculated with the test pathogen, three days earlier.

3.8 STUDIES ON THE CHEMICAL NATURE OF THE ROOT EXUDATES OF EXPERIMENTAL PLANTS

Surface sterilized seeds were germinated and placed in specially prepared root exudate collection apparatus for the collection of root exudate to study the presence of amino acids and carbohydrates in it.

3.8.1 Preparation of the apparatus for collection of root exudate

The apparatus used for growing seedlings and designed for collection of root exudate in asceptic condition was prepared as suggested by Kandaswami *et al.* (1973) with minor modifications. The apparatus consisted of an inverted 250 ml corning Erlenmayer flask provided with a stop-cock at the bottom (Fig. 1). One hole (2.5 cm diam) at the centre and three holes (0.5 cm diam) on the sides were made at the top (base of the flask). Through the central hole, a glass tube (7 cm in length) was fused so that about 4 cm length of the tube remained inside the flask forming a well. Through the other 3 holes, bent glass tubes of varying length were fused for passing sterile air (T_1), for adding nutrient solution (T_2) and for the exit of air (T_3). The flask was then filled with a layer of quartz at the bottom, a mixture of sand and quartz above it and then sand alone on the top, each layer about 3 cm in thickness. The sand and quartz used for the experiment were thoroughly acid-washed and then rinsed with distilled water several times. The exit of the tubes were plugged with non-absorbent cotton, covered with aluminium foil and sterilized at 15 lbs/in^2 for an hour (Fig.2).

3.8.2 Growing seedlings for collection of root exudates

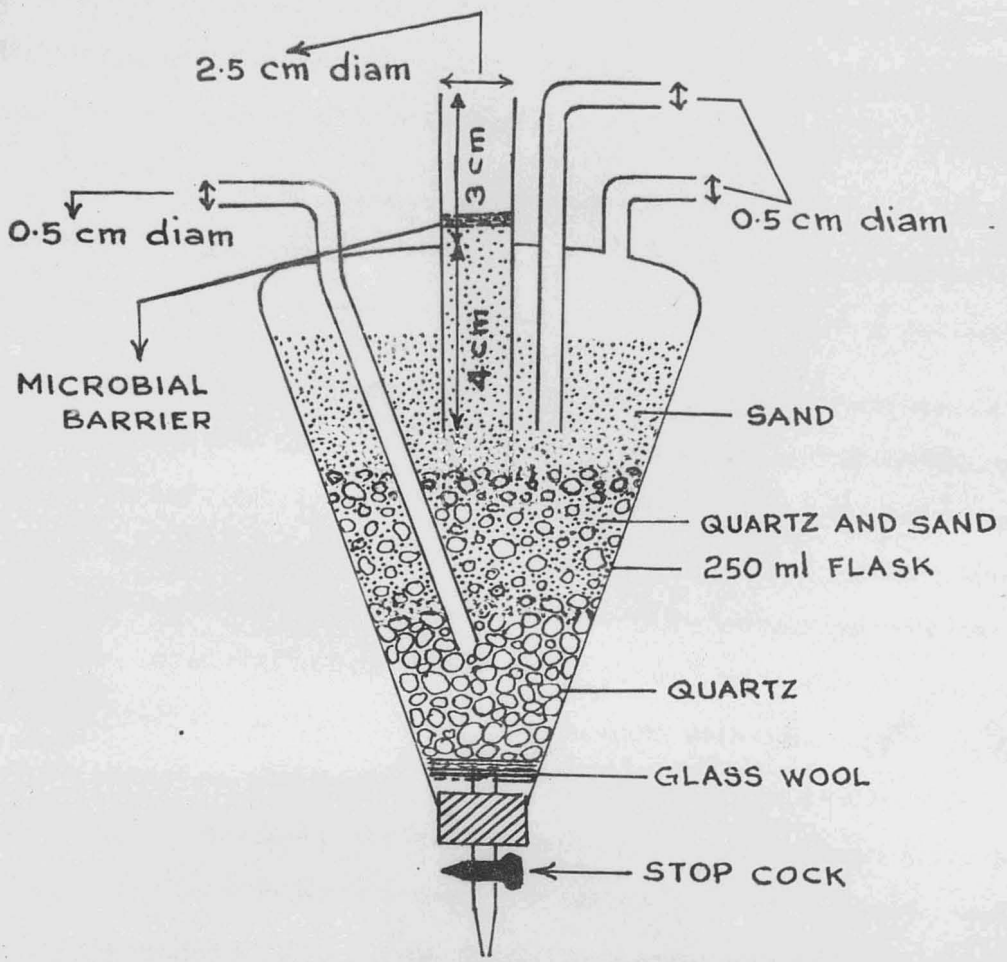
The seeds of okra var. *Pusa savani*, bittergourd and sunflower were used for the collection of root exudates. The seeds were thoroughly washed first in tap water and then

PLATE I

Fig. 1. Apparatus used for collection of root exudates. Diagrammatic sketch

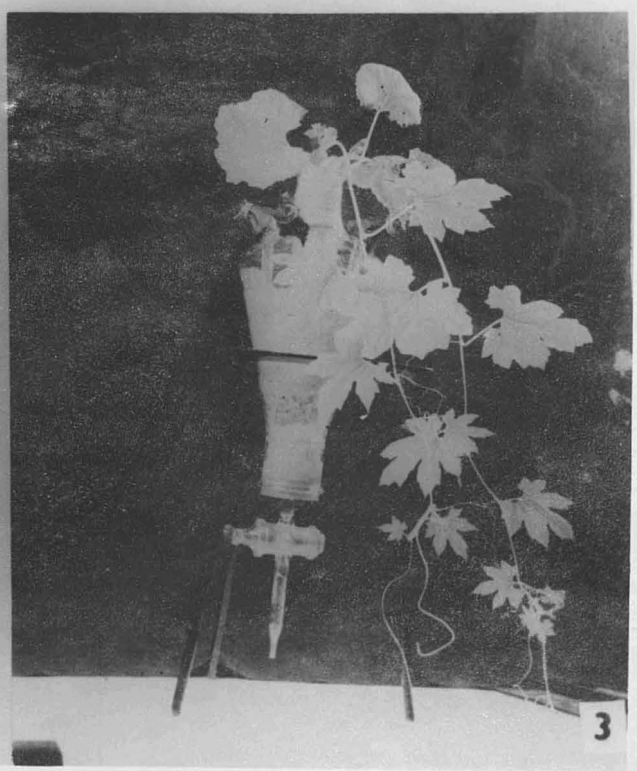
Fig. 2. The apparatus filled with acid-washed quartz and sand for autoclaving

Fig. 3. Three-week-old bittergourd plants growing in the apparatus


2·5 cm diam
3 cm
4 cm
0·5 cm diam
0·5 cm diam
MICROBIAL BARRIER
SAND
QUARTZ AND SAND
250 ml FLASK
QUARTZ
GLASS WOOL
STOP COCK

1

2



3

per cent mercuric chloride solution for 30 seconds. The seeds of okra were surface sterilized with the mercuric chloride solution for about 10 minutes with the seed coat on. All the seeds were then washed repeatedly in sterile distilled water.

The surface sterilized seeds were then placed on Water Agar in petri dishes for germination. When the radicle attained a length of 1.0 to 2.0 cm, uncontaminated germinated seeds were asceptically transferred to the flasks kept ready for collection of root exudate.

Two well-germinated seeds were transferred asceptically to the top layer of the sand in the central well of the apparatus. The mouth of the 'well' was plugged with sterilized cotton plug and the flasks were covered with black paper. When the plumule reached the bottom of the cotton plug, the cotton plug was removed and thin layer of sterilized cotton wool was placed over the surface of the sand so that the stems of the two seedlings were held erect through this cotton wool layer easily. Over this was placed about 1 cm thick layer of Dowex 50 (20-50 mesh) ion exchange resin which was previously washed with 0.1 N HCl followed by 0.2 N Mercuric chloride solution and then several times with sterile distilled water. Over this was kept another layer of sterile non absorbent cotton. All these were carried out in asceptic condition. Care was also taken not to cause any injury to the growing seedling. Minimum quantity of sterilized modified Van der Crone nutrient solution (Rovira, 1956a) was introduced into the flask through the inlet T_1 as and when the sand appeared dry. Before the nutrient solution was introduced, sterile air was passed through the inlet T_2. Twelve such replicates were used for each species.

Small quantities of the nutrient solution percolated through the sand and gravel was periodically collected through the stop-cock to test for any microbial contamination. Contaminated flasks were discarded. An average of 4 to 5 uncontaminated flasks were obtained. After three weeks of growth (Fig. 3) the stems of the seedlings were cut a few centimeters above the mouth of the well. Resin and cotton layer were carefully removed. Nutrient solution present in the apparatus was collected and the stop-cock along with the rubber stopper was removed. The whole contents of the apparatus (sand and the root system) was then allowed to fall into a 500 ml sterile flask containing 250 ml sterile distilled water without causing any injury to the root system. The liquid was then decanted into a sterile 500 ml flask. The contents of each flask (sand-gravel-root mixture) was then washed with 50 ml sterile water. This was also decanted into above flask. The liquid thus collected from the uncontaminated apparatus was kept in deep freeze for further studies.

3.8.3 Analysis of Root-exudate

Desalting the solution: The root and sand washings were filtered and centrifuged at 4000 rpm. The supernatant was concentrated under vacuum to about 50 ml. It was again centrifuged at 4000 rpm. The supernatant solution was desalted passing through the ion-exchange resins. Zeo-Karb (SRC 41) 226 (Permutit) - 14 + 52 mesh was used as cation exchange resin and De-acedite FF (SRA 69) - 14 + 52 mesh was used as the anion exchange resin. Purification and preparation of the resins were done according to Smith (1969). The purified resins were suspended in adequate quantity of distilled water and then filled in columns.

The concentrated exudate solution was slowly passed first through cation exchange resin column (Zeo-karb 226) and the filtrate was collected. The residual solution in the column was also washed with 15 ml of distilled water and collected along with the first filtrate. The anions, proteins and neutral molecules were washed through the column. The amino acids were displaced from the resin by passing 50 ml of 2N NH_3 solution through the column. The filtrate was then concentrated to dryness under vacuum.

The solution obtained after passage through Zeo-karb 226 was then passed slowly through a column filled with De-acedite. The filtrate containing neutral sugar was then concentrated under vacuum to dryness.

Amino acid analysis: The amino acid fraction collected in ammonia solution was evaporated to dryness under vacuum and redissolved in 10 per cent isopropanol. The amino acids were separated by two-dimensional descending paper chromatography on Whatman No.1 filter paper using butanol: acetic acid: water (50:10:10) as the solvent for the first run and phenol: water (80:20) for the second run. Ninhydrin and Isatin as 0.2 per cent solution in acetone were used as location reagents for detection and identification of amino acids (Smith, 1969). The colour reactions and Rf values were noted and they were compared with that of the standard map prepared simultaneously for identification using a loopful of pure amino acid solution made by dissolving 5 mg of the amino acid in 1 ml of 10 per cent isopropanol.

Sugar analysis: The neutral fraction containing sugar was concentrated to dryness under vacuum and redissolved in adequate quantity of 10 per cent isopropanol. For separation of sugars, unidirectional chromatography was used on Whatman No.1 filter paper with the solvent butanol: acetic acid: water (120:30:50) run for 42 hr. Standard maps were prepared by spotting a loopful of individual pure sugars (glucose, fructose, arabinose, xylose, rham-

nose, raffinose, sucrose, mannose, maltose, inositol and mannitol) in 10 per cent isopropanol and running for 42 hr.

The chromatogram was first sprayed with aniline hydrogen phthalate prepared by dissolving 0.93 g of aniline and 1.66 g of phthalic acid in 100 ml of water-saturated butanol (Patridge, 1949). After drying the butanol, the chromatogram was heated for 5 minutes at 105°C and examined under UV light for aldopentoses, aldohexoses and deoxysugars. The chromatogram was then sprayed with 2 per cent orcinol in 2N HCl which was allowed to dry at room temperature and then heated at 90°C for 2 minutes (Menzies and Seakins, 1969). On the basis of the development of various colours on the chromatogram, and also by comparing the Rf values with those of standard maps, the various carbohydrates namely, aldopentoses, aldohexoses, and ketoses were identified.