STUDIES ON VARIABILITY AMONG THE ISOLATES OF *Sclerotium rolfsii* Sacc.

By

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CERTIFICATE

This is to certify that **Miss. R. RAJALAKSHMI,** has satisfactorily prosecuted the course of research and that the thesis entitled **""STUDIES ON VARIABILITY AMONG THE ISOLATES OF *Sclerotium rolfsii* Sacc."** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

Date : 03.01.03

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON VARIABILITY AMONG THE ISOLATES OF *Sclerotium rolfsii* Sacc."** submitted in partial fulfilment of the requirements for the degree of **'Master of Science in Agriculture'** of the Acharya N.G.Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried out by **Miss. R. RAJALAKSHMI** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

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DECLARATION

I, **Miss. R. RAJALAKSHMI,** hereby declare that the thesis entitled **"STUDIES ON VARIABILITY AMONG THE ISOLATES OF *Sclerotium rolfsii* Sacc."** submitted to Acharya N.G. Ranga Agricultural University, Hyderabad for the degree of **Master of Science in Agriculture** is the result of original research work done by me. I also declare that the materials contained in this thesis has not been published earlier.

R. Rajalakshmi

Date : 3.1.03

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ABSTRACT

Variability among three isolates of *Sclerotium rolfsii* in cultural, morphological, physiological characters and in pathogenicity and toxicogenicity was studied. Variability among isolates in protein and esterase banding pattern was also studied using polyacrylamide gel electrophoresis (PAGE).

Potato Dextrose Agar among solid media and Richard's solution among liquid media supported good growth and sclerotial production in all the three isolates. Crossandra isolate produced maximum growth (84.2 mm) and mycelial dry weight (387.6 mg) in solid media and liquid media, respectively, followed by tomato isolate (76.0 mm and 336.9 mg) and groundnut isolate (76.6 mm and 260.6 mg).

Crossandra isolate stood first in utilizing carbon sources tested registering the maximum growth (77.3 mm) followed by tomato (73.3 mm) and groundnut (72.4mm) isolates.

Among nitrogen sources, nitrate and sulphate forms were equally preferred by three isolates for growth and sclerotial production. Nitrite form proved detrimental to all the isolates.

The sclerotial number and size varied among the isolates throughout the cultural studies. Tomato isolate produced more but small sized sclerotia whereas crossandra isolate produced very few but bigger sclerotia. Groundnut isolate appeared intermediary to these isolates.

Dual culture studies indicated that all the three isolates were equally sensitive to the antagonists tested viz., *Trichoderma viride* and *T. harzianum.*

Virulence studies revealed that crossandra and tomato isolates were highly virulent where as groundnut isolate moderately virulent.

Toxins elaborated by all the isolates caused considerable reduction in both shoot and root growth of the host plants. Crossandra isolate caused maximum reduction in shoot (35.1%) and root (73.79%) growth in the presence of its toxin.

The PAGE analysis reflected variations in protein fractions among the isolates. In tomato isolate, 9 bands were resolved of which 5 bands were common to all the isolates.

Esterase analysis revealed the presence of 5 bands each in crossandra and groundnut isolates and 3 bands in tomato isolate with different Rm values. Crossandra isolate had two additional bands with Rm values of 0.35 and 0.77.

Recent occurrence of *S. rolfsii* on crossandra an important crop in Chittoor district of Andhra Pradesh, its greater virulence than other two isolates suggests more detailed study on this isolate that must evolve a useful management package.

LIST OF ABBREVIATIONS

%	=	Per cent
°C	=	Degree Celsius
CD	=	Critical difference
SEM	=	Standard Error Mean
et al	=	Co-workers
Fig	=	Figure
g	=	Gram
h	=	Hour(s)
mg	=	Milligram
mm	=	Millimetre
ml	=	Millilitre
p.s.i	=	Pounds per square inch
spp.	=	Species
viz.,	=	Namely
cm	=	Centimeter
µm	=	Micrometre
N	=	Normality
M	=	Molar
Kg	=	Kilogram
µl	=	Microlitre
V	=	Voltage
w/v	=	Weight/Volume

Chapter I

Introduction

CHAPTER- I

INTRODUCTION

Sclerotium rolfsii Sacc. is a serious soil borne plant pathogen of world wide importance with a host range of over 500 species belonging to hundred plant families. It is common in tropical and subtropical regions of the world where high temperature coupled with high humidity is prevalent during the rainy season.

The pathogen induces a variety of symptoms such as seed rot, seedling blight, collar rot, stem rot, wilt etc. in different host plants.

In nature, plant pathogens exist in different strains that exhibit variation in their morphological, cultural, physiological characters, pathogenicity and virulence. To understand present plant disease situations and to predict the possible future development, it is essential to learn as much as possible about the variability in fungi pathogenic to plants.

Morphogenic and pathogenic variations are known in many fungal pathogens (Koech *et al.,* 1994; Kumar *et al.,* 1995).

Isolates of *S. rolfsii* originating from different geographical areas and different hosts exhibited variation in their morphological, cultural and pathological characters (dela-Cueva, 1994; Ansari and Agnihotri, 2000). In the present studies, the variability among three isolates of *S. rolfsii* one each from crossandra, groundnut and tomato was investigated. Variability in *S. rolfsii* from these hosts has not been studied earlier.

The first occurrence of *S. rolfsii* on crossandra has recently been reported by Harinath Naidu (2000). Crossandra is an economically important ornamental flower plant and is commercially grown in Andhra Pradesh. Considerable damage (40-50%) of the crop has been noticed by *S. rolfsii* in the farmers fields at Bhimavaram in Chittoor district of Andhra Pradesh.

Prior to detailed investigation of this new isolate of *S. rolfsii,* a preliminary research on its behaviour was carried out in comparison with other two isolates from groundnut and tomato which are also very potential in this area.

The three isolates were compared in their morphological, cultural, physiological characters, and in their pathogenicity and virulence. Supporting evidence to the variability among isolates was also provided

through protein and esterase analysis coupled with toxicogenicity of the isolates. The present investigation has been taken up with the following objectives :

1. To study variability among *S. rolfsii* isolates in cultural and morphological characters.
   a. Cultural and morphological variability on different solid media
   b. Cultural and morphological variability in different liquid media
2. To study variability among *S. rolfsii* isolates in physiological characters.
   a. Utilization of carbon sources by *S. rolfsii* isolates
   b. Utilization of nitrogen sources by *S. rolfsii* isolates
3. Sensitivity of *S. rolfsii* isolates to fungal antagonists
4. Variability among *S. rolfsii* isolates in pathogenicity and virulence
5. Variability among *S. rolfsii* isolates in toxicogenicity
6. Variability among *S. rolfsii* isolates in proteins and esterase banding pattern.

Chapter II

Review of Literature

CHAPTER - II

REVIEW OF LITERATURE

Sclerotium rolfsii Sacc. is a soil borne plant pathogen of world - wide importance with a host range of over 500 species. Its host range comprises of dicotyledonous plants and a few monocotyledonous species on which the pathogen produces symptoms like crown and root rot, damping off, wilting.

The available literature on *S. rolfsii* pertaining to the aspects under present investigation is presented in this chapter. However, where the literature on particular aspect of *S. rolfsii* was scanty, it was amply supplemented with and supported by relevant literature from other fungi.

Thus, the present review has a direct or indirect bearing on the investigation carried out in this study.

2.1 THE PATHOGEN

Sclerotium rolfsii was first reported by Rolfs in 1892 as being parasitic on tomatoes in the United States. Saccardo (1911) named the fungus as *Sclerotium rolfsii* sp. nov. In India Shaw and Ajrekar (1915) isolated an organism from rotten potatoes and identified it as *Rhizoctonia destruens*. Later, Ajrekar found that *R. destruens* was identical to *S. rolfsii.*

Higgins (1927) worked in detail on physiology and parasitism of *S. rolfsii*. This was the first detailed and comprehensive study in USA which prompted other workers to investigate different aspects of the pathogen.

The teleomorph of *S. rolfsii* Sacc. was first described by Goto (1930) who placed it in *Corticium centrifugum* (Lev.) Bres. Later Curzi (1931) proposed that *C. rolfsii* should be designated as the basidial state of *S. rolfsii*. In India, Mundkur (1934) has successfully isolated the perfect state of *S. rolfsii*. Then the teleomorph was transferred to *Pellicularia* by West (1947) and to *Botryobasidium* by Venkatarayan (1950). In 1973, Talbot suggested that the basidial state of *S. rolfsii* belonged to the genus *Athelia*.

There are many reports regarding morphological characters of *S. rolfsii* (Taubenhaus, 1919; Ramakrishnan, 1930; Subramanian, 1964; Mahmood *et al.*, 1976; Boonthong and Sommart, 1985; Singh, 1987; Mirza and Aslam, 1993; Mohan *et al.*, 2000 and Harinath Naidu, 2000). The fungus *S. rolfsii* produces abundant white fluffy, branched, septate mycelium with clamp connections only on the main hyphae, spreading in strands giving a fan like appearance. Small white tufts were formed on aerial mycelium which later become smooth, hard and dark brown mature sclerotia. Sclerotia spherical to irregular in shape and attached to the mycelium. Mature sclerotia resembled the mustard seed.

The structure of sclerotia was studied by several workers using Scanning Electron Microscopy (SEM) (Tu and Kimbrough, 1975 ; Tsuno *et al.,* 1990; Punja and Damiani, 1996; and Chen *et al.,* 1998). It comprised of three layers of an outer rind, a middle cortex and inner medulla. The cortical layer was very thick and proteins were also distributed in both cortex and medulla region. Flora Zarani and Christias (1997) documented three stages in sclerotial development i.e. sclerotial initial, development and maturity. Sclerotial size varied with isolates and ranged from 0.1 mm to 3.0 mm (Om Prakash and Singh, 1976, Nagarajan and Ramjilal, 1979 ; Ansari and Agnihotri, 2000 ; Mohan *et al.,* 2000 and Anahosur, 2001).

2.2 DISTRIBUTION AND ECONOMIC IMPORTANCE

Sclerotium rolfsii has a world - wide distribution and has been recorded from the United States of America, Australia, China, Ceylon, Bangladesh, numerous islands of Pacific and Atlantic Oceans, Africa, India and countries surrounding Mediterranean region (Aycock, 1966). It is common in tropical and subtropical regions of the world where high temperature coupled with high humidity is prevalent during the rainy season.

The pathogen attacks more than 500 species of plants including vegetables, flowers, cereals, forage plants and weeds. Some of the most

common hosts include legumes, crucifers, cucurbits, potato, carrot, celery, sweet corn, egg plant, lettuce, okra, onion, pepper, sweet potato, tomato, amaryllis, chrysanthemum, tulip, alfalfa, cotton, peanuts and tobacco and cause foot rot or root rot of plants (Anahosur, 2001).

The pathogen causes a great economic loss in various crops. In groundnut, it caused 25 per cent of seedling mortality in the cultivar JL-24 at Parbhani (Ingale and Mayee, 1986). In Northern Karnataka 1-40 per cent wilting of potato plants and upto 25 per cent tuber infection in storage have been recorded indicating the extent of its economic importance (Anahosur *et al.,* 1997). Thiribhuvanamala *et al.,* (1999) observed that 30 per cent of crop loss in tomato was due to *S. rolfsii.* Harinath Naidu (2000) reported that *S. rolfsii* caused 40-50 per cent mortality in crossandra in Chittoor district of Andhra Pradesh.

2.3 SYMPTOMATOLOGY

The fungus *S. rolfsii* induces a variety of symptoms such as seed rots, seedling blight, collar rot, stem rot, wilt etc. in different host plants.

The major symptoms on certain important crops are presented below.

Crop	Symptoms	References
Arachis hypogaea L.	Seedling blight, collar rot, wilt, root rot, stem rot and pod rot	Mayee and Datar (1988), Narain and Kar (1990), Agarwal (1991)
Canavalia ensiformis	Water soaked brown lesions appeared at the collar region of the mature plants, white fungal mycelium with sclerotia on the affected regions	Kavitha *et al.* (2001)
Crossandra infundibiliformis	Shrunken and brown coloured dark roots, yellow to pink discolouration of leaves and drying of plants (collar rot)	Harinath Naidu (2000)
Carthamus tinctorius L.	Yellowing of leaves and collar rot	Vyas *et al.* (1983)
Eleusine coracana L.	Drying of leaves, disintegration of roots, rotting of tissues at collar region	Narain (1972)
Ficus auricularia	Fruit rot	Mohan *et al.* (2000)
Glycine max	Seed and seedling rot	Khare (1979), Akem and Dashiell (1991)
Hordeum vulgare L.	Wilting, chlorosis and necrotic lesions at the base of stem	Esnard and Hepperly (1995)
Helianthus annuus L.	Damping off, collar rot and lesions at stem base	Chakravarty and Bhowmik (1983), Okoli *et al.* (1991)
Ipomoea batatus L.	Rotting at collar region	Sivaprakasam and Kandaswamy (1983)
Lycopersicon esculentum	Stem rot	Thiribhuavan-amala *et al.* (1999)

Phaseolus vulgaris	Wilting and chlorosis	Montealegre and Esterio (1989)
Solanum pseudocapsium	Stem blight and basal rot	Garibaldi *et al.* (2000) Minuto *et al.* (2000)
Solanum tuberosum	Dark brown lesions appear on the stem at collar region and wilting of plants	Anahosur (2001)
Triticum aestivum	Damping off, chlorosis of leaves and lesions at the stem base	Kilpatrick and Merkle (1967)
Zea mays	Wilt, collar rot, deep cracks near collar region, shredding of roots, white mycelial growth on surface of freshly infected areas	Ahmad *et al.* (1988)

2.4 VARIABILITY STUDIES ON *Sclerotium rolfsii*

Cultures of *S. rolfsii* originating from different geographical areas and hosts have been grouped into distinct isolates by many workers based on morphological and physiological characters.

2.4.1 Studies on morphological and cultural characters of *S. rolfsii* isolates

dela - Cueva and Natural (1994) noted difference in cultural and morphological characters of different isolates of *S. rolfsii* originating from different hosts. They observed that an isolate from tomato produced highest number of sclerotial bodies while mungbean isolate produced the least. Very

few but big sclerotial bodies were observed from pepper isolate while cowpea isolate produced numerous but small sclerotial bodies.

Ansari and Agnihotri (2000) observed variation in morphological characters among *S. rolfsii* isolates of soybean obtained from different localities. The isolates were grouped into six categories on the basis of difference in sclerotial size (less than 750 µm as small, 750-1200 µm as medium and more than 1200 µm as big), colour and their arrangement.

Kim (1974) grouped *Magnolia kobus* isolates of *S. rolfsii* into two biologic strains on the basis of difference in morphology and mode of growth in culture. Radwan *et al.* (1987) observed variation in mycelial colour and sclerotial production in five isolates of *S. rolfsii* collected from different fields.

Prithiviraj *et al.* (1996) studied the variation in sclerotial morphology of *S. rolfsii* using scanning electron microscopy (SEM). The sclerotia were of two types viz., small and large and the difference in size of sclerotia was due to difference in the volume of medullary region.

Hernandez Medina and Ysla (1997) found variation among *Phaseolus vulgaris* and sunflower isolates of *S. rolfsii* especially in their mycelial growth rate, number and diameter of sclerotia.

Chen *et al.* (1998) classified the isolates of *S. rolfsii* according to sclerotial formation types, mycelial compatibility groups and sclerotial fine structure. Punja and Grogan (1983) showed that *S. rolfsii* can be placed in mycelial compatibility groups (MCG) based on mycelial interactions.

Nalim *et al.* (1995) grouped the isolates of *S. rolfsii* into different mycelial compatibility groups based on the formation of an antagonism zone between incompatible mycelia of paired isolates. Hernandez and Aleman (1997) grouped *S. rolfsii* from black bean (*Phaseolus vulgaris*) and sunflower into five isolates based on antagonistic capacities or showing compatibility groups.

2.4.1.1 Morphological and cultural characters of *S. rolfsii* isolates on solid media

Ramakrishnan (1930) reported that French bean and Oat juice agar media supported the best growth of Zinnia isolate of *S. rolfsii*. The average size of sclerotia was 1.7 mm in Oat juice agar medium.

Narain and Mishra (1979) found that malt extract agar supported the largest number and size of sclerotia of ragi isolate of *S. rolfsii* out of four solid media tested.

Sharma and Kaushal (1979) reported that among different solid media tested, Pea Meal Agar (PMA) and Potato Dextrose Agar (PDA) supported good growth of sunflower isolate of *S. rolfsii*. They obtained the highest number and bigger size of sclerotia on PDA medium.

While working on groundnut isolate of *S. rolfsii*, Hari *et al.* (1991) observed that PDA, Corn Meal Agar and Coon's Agar supported maximum radial growth of the fungus. PDA supported maximum sclerotial production followed by groundnut shoot extract agar, Corn Meal Agar and Coon's Agar.

Mahmood *et al.* (1976) also recorded good vegetative growth and sclerotial production of *S. rolfsii* on Corn Meal and Potato Dextrose Agar media.

Rawn (1991) observed different pattern of sclerotia production of *S. rolfsii* isolate on two agar media, predominantly at the colony centre on one medium (glucose / yeast extract) but at the dish wall on the other (glucose / NH_4CO_3).

Islam *et al.* (1994) studied variation in growth of *S. rolfsii* isolates from six different hosts. Among the four test media, the best one for sclerotial germination was Potato Carrot Straw Agar (PCSA) followed by Potato Dextrose Agar (PDA) and Potato Agar (PA) for all isolates. The maximum mycelial growth occurred in PDA followed by PCSA and PA.

Punja and Damiani (1996) compared isolates of *S. coffeicola, S.delphinii* and *S. rolfsii* for difference in growth response to different media and found that V8 agar and PDA were best for colony growth and sclerotial production, respectively for *S. rolfsii.*

Matsumoto *et al.* (2000) conducted studies on three isolates of *S. rolfsii* (two from beans and one from cucumber) on six vegetable substrates with regard to mass production of sclerotia. Two of the isolates produced higher number of sclerotia on autoclaved rice while the third isolate produced more sclerotia in the crude rice substrate.

2.4.1.2 Morphological and cultural characters of *S. rolfsii* isolates in Liquid media

When grown in liquid medium *S. rolfsii* produced an extracellular viscous polysaccharide (Kritzman *et al.,* 1979) but did not produce any sclerotia (Okon *et al.,* 1974).

Mathur and Sarbhoy (1976), while working on sugarbeet isolate of *S. rolfsii* recorded maximum growth (dry weight) on Richard's medium followed by Modified Dox Medium, Czapek's, Modified Brown's and Asthana and Hawker's media.

Sharma and Kaushal (1979) observed that the four liquid media used for the growth of sunflower isolate of *S. rolfsii* varied greatly in supporting vegetative growth of fungus and its sclerotial formation. Coon's medium followed by Czapek's sucrose nitrate medium were found superior.

Hari *et al.* (1991) found that among six liquid media, groundnut shoot extract supported maximum growth of groundnut isolate of *S. rolfsii* followed by Czapek's solution and Richard's solution.

2.5 PHYSIOLOGICAL STUDIES ON *S. rolfsii* ISOLATES

It is well known that carbon and nitrogen are the main essential elements required by fungi for functional and structural processes.

2.5.1 Utilization of carbon sources by *S. rolfsii* isolates

The isolates of *S. rolfsii* varied in their ability to utilize carbon compounds for growth. Ramakrishnan (1930) reported that maltose and sucrose were best carbon sources for linear growth of Zinnia isolate of *S. rolfsii.*

Mishra and Haque (1962) got maximum number of sclerotia of an isolate *S. rolfsii* when sucrose and glucose were the carbon sources.

Synchronization of sclerotium formation could be induced by the addition of lactose to the growth medium (Okon *et al.*, 1972). The formation of sclerotia required an increased supply of carbohydrates and energy which were mainly provided by glyoxalate pathway (Kritzman *et al.* 1976).

Bahadur *et al.* (1976) studied the effect of carbon source on wheat isolate of *S. rolfsii*. He found that in the presence of rhamnose and arabinose, the colour of the colony was white, the sclerotial formation started after 192 hrs in rhamnose and 432 hrs in arabinose and sclerotial arrangement was scattered. Maximum number of sclerotia were produced in rhamnose compared to arabinose.

Mathur and Sarbhoy (1976) recorded maximum colony growth and sclerotial formation of sugarbeet isolate of *S. rolfsii* on sucrose followed by raffinose, maltose, L-glucose, lactose, starch, dextrin and poor growth on fructose, inositol, sorbitol and mannitol.

While studying the physiological aspects of sunflower isolate of *S. rolfsii*, Sharma and Kaushal (1979) observed that starch was the best carbon source for the growth and sclerotia formation of fungus followed by sucrose. Poor growth and sclerotial formation were observed on mannitol and arabinose.

The suitability of different carbon sources for mycelial growth and sclerotial formation of three isolates of *S. rolfsii* was studied by Zoberi (1980). Mature sclerotia were formed on all sources tested except on the medium lacking any carbon source. Glucose, maltose and sucrose were the best sources, glucose being the most outstanding.

Hadar *et al.* (1983) suggested that the cultures of *S. rolfsii* were capable of producing sclerotial initials when glucose exhausted. In the presence of glucose, the culture was only able to produce undifferentiated mycelium.

Prasad *et al.* (1986) while studying the influence of nutritional factors on growth of *S.rolfsii* isolated from tomato fruit found that sucrose was the best carbon source for the growth.

Nwufo and Fajola (1986) found that dextrin was the best carbon source for the growth of *S. rolfsii* infecting *Colocasia esculenta.*

Hari *et al.* (1991) recorded maximum dry weight and sclerotial production of groundnut isolate of *S. rolfsii* in starch and dextrin, among different carbon sources tested.

Jyoti - Mishra *et al.* (1996) reported that glucose as a carbon source supported maximum growth and sclerotial formation of indigenous isolates of *S. rolfsii* tested as mycoherbicides against Parthenium.

2.5.2 Utilization of nitrogen sources by *S. rolfsii* isolates

Utilization of nitrogen sources by different isolates of *S. rolfsii* has been studied by many workers. Mahmood *et al.* (1976) observed best growth of *S. rolfsii* on peptone.

Among different nitrogen sources tested, Mathur (1977) recorded maximum growth of *S. rolfsii* on ammonium sulfate followed by sodium nitrate, ammonium chloride, calcium nitrate and maximum sclerotial formation on sodium nitrate, ammonium nitrate and ammonium chloride.

Among six nitrogen sources tried, Sharma and Kaushal (1979) found that ammonium nitrate was best for vegetative growth of sunflower isolate of *S. rolfsii* followed by sodium nitrate, potassium nitrate and ammonium sulfate. Sodium nitrite proved very poor nitrogen source for growth of the fungus.

Zoberi (1980) obtained the highest yield of sclerotia of three isolates of *S.rolfsii* on ammonium sulfate.

In vitro studies on effect of nitrogenous fertilizers on physiological activities of sugar beet isolate of *S. rolfsii* revealed that mycelial growth was reduced on Richard's Agar Medium supplemented with calcium nitrate and also in Potato Dextrose Pectin broth containing ammonium sulphate, calcium nitrate or urea but was largely enhanced by these N-compounds in Glucose Peptone broth (Upadhyay and Mukhopadhyay, 1985).

Brenneman (1990) found that ammonium bicarbonate inhibited the formation of sclerotia of groundnut isolate of *S. rolfsii in vitro.*

Among different nitrogen sources tested, Hari *et al.* (1991) recorded maximum dryweight of groundnut isolate of *S. rolfsii* in peptone and potassium nitrate. In sclerotial production rating also peptone ranked first (numerous) followed by sodium nitrate (excellant) and glutamic acid (very good). In the presence of ammonium sulphate, sodium nitrite and valine no sclerotia were formed.

Jyoti - Mishra *et al.* (1996) reported that potassium nitrate supported maximum growth and sclerotial formation of different isolates of *S. rolfsii.*

Yu *et al.* (1996) suggested that different nitrogen sources had different effects on mycelial growth of *S.rolfsii.* They also observed that sclerotia did

not germinate on Czapek agar amended with sodium nitrite or ammonium nitrite and sclerotia were not formed on Czapek agar deprived of nitrogen or magnesium source.

2.6 SENSITIVITY OF *S. rolfsii* ISOLATES TO ANTAGONISTS

The potential for the use of fungal antagonists as a bio-control agent of plant diseases was suggested more than 50 yrs ago by Weindling (1932), who was the first to demonstrate the parasitic activity of *Trichoderma* spp. against pathogens such as *Rhizoctonia solani* and *S.rolfsii. In vitro* antagonism of *Trichoderma harzianum* against the isolates of *S. rolfsii* has been reported (Wells *et al.*, 1972; Chet *et al.*, 1978).

Singh and Dwivedi (1987) found that *T. viride* and *T. harzianum* inhibited the mycelial growth of barley isolate of *S. rolfsii* by 88 and 86 per cent, respectively, in dual culture. Henis *et al.* (1983) found that isolates of *Trichoderma* differed in their ability to penetrate the sclerotia of *S. rolfsii.* Among different isolates of *T. harzianum* tested. Elad *et al.* (1984) observed that only *T. harzianum* isolate 203 could attack sclerotia of *S. rolfsii.*

The hyphal interaction between *S. rolfsii* and *T. harzianum* was studied by several workers using scanning electron microscopy (SEM) (Elad *et al.*, 1983 ; Ferrota and Dambra, 1985; Lim and Teh, 1990; Meon, 1991 ; and

Benhamou and Chet, 1996). They observed hyphal coiling, entry through haustoria like structures and direct entry into the hyphae and sclerotia of *S.rolfsii* by *T. harzianum* in dual culture.

The studies conducted by Hari *et al.* (1988) on the interaction between *T. harzianum* and groundnut isolate of *S. rolfsii* by dual inoculation technique revealed strong antagonistic effect of *T. harzianum* against *S. rolfsii.*

Muthamilan and Jeyarajan (1992) studied *in vitro* effect of different isolates of antagonistic fungi on groundnut isolate of *S. rolfsii. Trichoderma viride* (isolate 2) was superior to all other isolates in arresting the mycelial growth and sclerotial production of *S. rolfsii.*

Iqbal *et al.* (1995) found that among different antagonists tested, *T. harzianum, T. koeningii* and *T. viride* overlapped the pathogen and suppressed the growth of lentil isolate of *S. rolfsii.*

Anitha Chowdary (1997) found that native isolate of *T. viride* was superior in suppressing the growth of bell pepper isolate of *S. rolfsii in vitro* than exotic isolates of *T. viride* and *T. harzianum.*

Mukherjee (1997) found that *Trichoderma* sp. was effective in preventing the growth of an isolates of *S. rolfsii* from beet root, carrot and bitter gourd.

Suseelendra Desai and Schlosser (1999) found that the isolates of *Trichoderma* differed significantly in their ability to infect, macerate and kill the sclerotia of *S. rolfsii.*

Among *Trichoderma* species, *T. harzianum* isolates effectively inhibited the mycelial growth of sunflower isolate of *S. rolfsii.* Complete inhibition of sclerotial germination was obtained with the culture filtrates of *T. harzianum* and *T. pseudokoeningii* (Prasad *et al.,* 1999).

In dual culture among 11 isolates of *T. harzianum* tested, Biswas and Sen (2000) observed only three isolates viz., T_8, T_{10} and T_2 were effective against *S. rolfsii* the causal agent of stem rot of groundnut and they over grew the pathogen upto 92, 85 and 79 per cent, respectively, *in vitro.*

Das *et al.* (2000) reported that *T. harzianum* was the most effective in inhibiting the mycelial growth of tomato isolate of *S. rolfsii.*

Triochoderma viride was highly effective in suppressing the growth of *S. rolfsii* but its effect on sclerotia formation was found negligible (Saikia *et al.,* 2000). The growth of potato isolate of *S. rolfsii* was completely overgrown and suppressed by the species of *Trichoderma* (Anahosur, 2001).

2.7 HOST RANGE OF *S. rolfsii*

There are differences in virulence of *S. rolfsii* isolates from same host (Kim, 1974 ; Akem and Dashiell, 1991) as well as from various hosts (Cooper, 1961 ; Punja, 1985).

Ramakrishnan (1930) reported that Zinnia isolate of *S. rolfsii* could establish pathogenicity on groundnut, cowpea, lucerne, bengal gram, linseed, maize, potato and cotton.

Narain and Mishra (1979) studied the host range of ragi isolate of *S.rolfsii* which could infect brinjal, chilli, groundnut, sunflower and tomato.

The host range of *S. rolfsii* within *Stylosanthes* species was studied by Bhaskar and Ahmad (1991) and found that it could infect *S. guanensis, S. humilis* and *S. scabra* but it could not infect *S. viscosa.*

Islam *et al.* (1994) tested the pathogenicity of isolates of *S. rolfsii* from six different hosts. The isolates of the fungus were more pathogenic to soybean and gram where they caused 100 per cent pre-emergence death. They also found that mustard isolate was the most while wheat isolate was the least pathogenic to the hosts tested.

dela - Cueva and Natural (1994) studied the pathological variation among the isolates of *S. rolfsii* from different crops. The isolates of cowpea and soybean caused wilting in peanut while the isolate of cotton caused wilting in wheat. Cowpea isolate was considered as the most aggressive and the most virulent which could infect all the crop species tested except wheat.

Harinath Naidu (2000) reported that crossandra isolate of *S. rolfsii* was pathogenic to tomato and chillies. The host range of potato isolate of *S. rolfsii* was studied by Anahosur (2001). Among different hosts, studied, it caused 100 per cent wilting on potato and beetroot and 86.6 per cent wilting on Palak, Tomato and Chilli.

2.8 ELABORATION OF TOXIC METABOLITES

It is well established that in many plant diseases the host pathogen interactions are associated with the synthesis of host invading substances like toxins, enzymes and other metabolites (Daly and Deverall, 1983; Collmer and Keen, 1986).

The synthesis of such substances including growth inhibitors was also demonstrated *in vitro* in cultural filtrates of many plant pathogenic fungi and their inhibitory action has been demonstrated by shoot / root inhibition bio assay (Anahosur, 1976; Chandrika, *et al.* 1984; Ali and Singh, 1992).

Many plant pathogenic fungi produce metabolites which reduce the germination of the seed and affect the seedling vigour (Jhutha Ram *et al.*, 1997).

Agarwal *et al.* (1986) while studying the effect of culture filtrates of *Fusarium oxysporum* f.sp *lentis, Rhizoctonia bataticola* and *Sclerotium rolfsii* on the mortality of lentil seedlings, found that culture filtrate of *S.rolfsii* caused 70% seedling mortality.

Parthasarathy Prasad and Hiremath (1983) observed 100% reduction in seed germination of fenugreek in the presence of toxin elaborated by *Rhizoctonia solani.*

Chandrika *et al.* (1984) demonstrated the toxin production in two isolates of *Colletorichum falcatum.* The toxin activity of culture filtrate was demonstrated by seed germination inhibition and plumule and radical growth inhibition of tested plants.

The culture filtrate of 15 isolates of *Sclerotium oryzae* showed variation in inhibiting the radical and plumule elongation of paddy (Ali and Singh, 1992).

The culture filtrates of two isolates of *Colletotrichum capsici* differed in their efficacy in inhibiting germination of seeds of chilli (Kamalesh Mathur, 1995).

Lakpale *et al.* (1996) reported that the toxin in the culture filtrate of *Rhizoctonia solani* inhibited the germination of seeds of several crop plants.

Kalaimani (1997) established toxin proudction of six isolates of *C. falcatum* by plumule and radical inhibition of both cereals and non-cereals.

It is well documented that *Sclerotium rolfsii* produces oxalic acid (Bateman and Beer, 1965; Maxwell *et al.,* 1968; Punja, 1985; Ansari and Agnihotri, 2000), Pectic enzymes (Punja, 1985; Upadhyay and Mukhopadhyay, 1985), Cellulolytic enzymes (Dasgupta, 1990) and Phosphatidases (Sellman, 1982). The role of these metabolites in tissue maceration and killing was well established (Bateman and Beer, 1965; Punja and Jenkins, 1984; Dasgupta, 1990; Ohazurike and Arinze, 1992).

2.9 ISOZYME AND PROTEIN ANALYSIS OF *S. rolfsii*

Isozyme analysis on polyacrylamide gel electrophoresis provides a well - established and efficient tool for revealing genetic variability in fungal population (Micales *et al.,* 1986). The presence of protein polymorphism reflect directly to the genetic background of the isolates of plant pathogens.

In spite of this advantage, the application of isozyme techniques in studies of phytopathogenic fungi has been limited until now. It is more so in the case of *S. rolfsii.* The relevant literature on other plant pathogenic fungi is also presented here.

Prithiviraj *et al.* (1996) made isozyme studies on two types of sclerotia viz., small and large of an isolate of *S. rolfsii.* The isozyme pattern of GPI-1 of both sclerotial types was the same but significant difference was observed in the relative mobility of PEP-1.

Reynolds *et al.* (1983) stated that electrophoretic protein patterns are useful means for distinguishing anastomosis of *Rhizoctonia solani.*

Isozyme analysis of esterase and peroxidase could be used to separate *Cryphonectria cubensis* isolates differing in their virulence pattern (Alfenas *et al.,* 1984).

Nygaard *et al.* (1989) studied isozyme variability among isolates of *Phytophthora megasperma*, using starch gel electrophoresis. Zambino and Harrington (1989) found esterase isozyme variation in seventy six isolates representing three taxonomic varieties of *Leptographium wageneri* through electrophoresis.

Fifty five enzymes and protein strains of *Uromyces appendiculatus* were screened in three horizontal gel electrophoresis system by Linde *et al.* (1990). Greater diversity was found for virulence than for isoenzyme.

Chen *et al.* (1991) compared soluble proteins and isozyme for seven homothallic species of *Pythium* by SDS-PAGE and starch gel, respectively. Similarities in soluble protein banding patterns and isozyme phenotypes were detected for morphologically distinct species.

The isolates of *Drechslera graminea* from barley have been divided into two or more groups on the basis of their virulence analysis as well as protein and isozyme patterns (Gatti *et al.*, 1992).

Simcox *et al.* (1992) studied differences in isozyme polymorphism between races of *Cochliobolus carbonum*, using starch gel electrophoresis.

Polyacrylamide gel electrophoresis gives more quantitative results and produces better resolved banding patterns for isozymes, since both net change and molecular weight affect the separation of proteins in this system (Rosendahl and Sen, 1992).

Damaj *et al.* (1993) characterized fifty isolates of binucleate *Rhizoctonia* by isozyme electrophoretic patterns. Of the 23 enzyme system screened, hexokinase and malate dehydrogenase displayed polymorphic banding patterns.

Welz *et al.* (1994) studied isozyme variation among isolates of *Cochliobolus carbonum* by starch gel electrophoresis. Of eight enzyme systems studied in detail, only phosphogluconate dehydrogenase was monomorphic, malate dehydrogenase and esterase were highly polymorphic.

Isozyme pattern for hexokinase and malate dehydrogenase in *Stenocarpella maydis* could differentiate US strains from South African strains (Dorrance *et al.,* 1995).

Mattila *et al.* (1996) compared differences in isozyme and RAPD-PCR polymorphisms among 33 isolates of *Fusarium avenaceum* using native polyacrylamide gel electrophoresis and agarose gel electrophoresis.

Boshoff *et al.* (1996) studied isozyme variability among bean isolates of *Phaeoisariopsis griseola* in Southern Africa.

Sudhakar (1996) reported that electrophoretic studies of proteins and esterases of *Rhizoctonia solani* expressed a narrow genetic distances between the isolates obtained from geographically nearer locations and vice-versa.

Isozyme analysis provides an effective means of identifying *Pythium* spp. that do not readily produce oospores in culture (Barr *et al.*, 1997).

Hussain and Barz (1997) analysed different isozyme patterns of 15 isolates of *Ascochyta rabiei*. Of the seven enzymes tested, only esterase and acid phosphatase showed qualitative and quantitative differences among the isolates of *A. rabiei*. They also found that there was no correlation between isozyme patterns and pathogenic group on the basis of aggressiveness.

Elaah and Nagdy (1999) used electrophoretic analysis of esterase and acid phosphatase isozymes to study the differences between eight species of *Achlya*.

Dorrance *et al.* (1999) compared the isolates of *Stenocarpella maydis* for isozyme pattern. Isozyme polymorphism were detected for α-esterase, hexose kinase and malate dehydrogenase of the enzymes assayed.

Isozyme variability among isolates of *Ascochyta rabiei* was studied by Uma Devi *et al.* (2001) using native polyacrylamide gel electrophoresis. Esterase, malate dehydrogenase and acid phosphatase gave a polymorphic banding pattern whereas peroxidase; alkaline phosphatase and glutamate dehydrogenase resulted in monomorphic banding pattern. Identical protein profiles for all the isolates with respect to major bands and differences in the minor bands were observed in SDS-PAGE for total proteins.

Chapter III

Materials and Methods

CHAPTER - III

MATERIALS AND METHODS

The laboratory and pot culture experiments pertaining to the present research work were conducted in the Department of Plant Pathology, S.V.Agricultural College, Tirupati. The materials used and techniques adopted are described in this chapter.

The general laboratory techniques employed in the research work were those described by Dhingra and Sinclair (1993) and Rangaswami and Mahadevan (2001) for media preparation, sterilization, isolation, purification and maintenance of fungal cultures with slight modifications wherever necessary.

3.1 MATERIALS

3.1.1 Glassware

All glassware used throughout the present investigation were of Corning or Borosil make.

Initially, all glassware were thoroughly washed with a detergent and rinsed in running tap water.

Subsequently they were kept in potassium dichromate cleaning solution for 24 h and finally rinsed with distilled water for 3-4 times. They were then air dried before use :

The composition of cleaning solution

Potassium dichromate	:	60 g
Concentrated sulphuric acid	:	60 ml
Distilled water	:	1000 ml

3.1.2 Chemicals

Chemicals used in the present study were of analytical reagent (AR) and guaranteed reagent (GR) grades of standard make. The pH of the medium was adjusted to required level with 0.1 N NaOH or 0.1 N HCl.

3.1.3 Media used

Different culture media and their composition used during the course of present investigation are given below.

Solid Media

a. Potato Dextrose Agar (PDA) medium

Peeled potato slices	:	200 g
Dextrose	:	20 g
Agar	:	17 g
Distilled water	:	1000 ml

pH (6.0)

b. Oat Meal Agar Medium

Oat Meal	:	30 g
Agar	:	15 g
Distilled water	:	1000 ml
pH (6.0)		

c. Czapek's Dox agar medium

Sodium nitrate	:	2 g
Dipotassium hydrogen phosphate:		1 g
Magnesium sulfate	:	0.5 g
Potassium chloride	:	0.5 g
Ferrous sulphate	:	0.01 g
Sucrose	:	30 g
Agar	:	20 g
Distilled water	:	1000 ml
pH (6.0)		

LIQUID MEDIA

a. Richard's Medium

Potassium nitrate	:	10 g
Potassium dihydrogen phosphate	:	5 g
Magnesium sulphate	:	2.5 g

Ferric chloride	:	0.02 g
Sucrose	:	50 g
Distilled water	:	1000 ml
pH (6.0)		

b. Coon's Medium

Sucrose	:	7.2 g
Dextrose	:	3.6 g
Magnesium sulphate	:	1.23 g
Potassium dihydrogen phosphate	:	2.72 g
Potassium nitrate	:	2.02 g
Distilled water	:	1000 ml
pH (6.0)		

c. Potato broth

Peeled potato slices	:	200 g
Dextrose	:	20 g
Distilled water	:	1000 ml
pH (6.0)		

d. Groundnut host-extract medium

Groundnut roots and stem	:	200 g
Dextrose	:	20 g
Distilled water	:	1000 ml
pH (6.0)		

e. Crossandra host-extract medium

Crossandra roots and stem	:	200 g
Dextrose	:	20 g
Distilled water	:	1000 ml
pH (6.0)		

d. Tomato host extract medium

Tomato roots and stem	:	200 g
Dextrose	:	20 g
Distilled water	:	1000 ml
pH (6.0)		

3.1.4 Seeds

Groundnut seed of the variety TMV-2 was used in the present work. The seeds of Crossandra and Tomato were obtained from local market.

3.2 METHODS

3.2.1 Sterilization of glassware, media and soil

Glassware properly wrapped in paper or aluminium foil were sterilized in hot air oven at 160°C for 90 minutes, while the culture media were sterilized in autoclave at 15 p.s.i for 20 minutes. For pot culture studies, the soil was sterilized in autoclave at 15 p.s.i for 20 minutes for 2 consecutive days.

3.2.2 Statistical analysis

Wherever necessary, the data were analysed statistically following the procedure given by Gomez and Gomez (1983).

3.2.3 Isolates of *Sclerotium rolfsii*

In the present investigation, three isolates of *S. rolfsii* were used one each from crossandra, groundnut and tomato and they were designated as C, G and T.

3.2.3.1 Isolation of the fungus

Sclerotium rolfsii was separately isolated from infected plants of crossandra, groundnut and tomato brought by the farmers to plant health clinic of S.V.Agricultural College, Tirupati for diagnosis.

Small pieces of tissue from infected stem or roots (3 mm) along with some healthy tissue were cut with sterile scalpel. The pieces were surface sterilized with 0.1 per cent mercuric chloride solution for 30 sec. The tissue pieces were subsequently washed in three changes of sterile distilled water to eliminate mercury ions. The surface sterilized pieces were transferred on to PDA in petri dishes and incubated at 28 ± 2°C and growth was observed periodically.

3.2.3.2 Maintenance of the isolates

All isolates of *S. rolfsii* were purified by single hyphal tip culture method and maintained separately on PDA in slants and petri plates.

3.3 VARIABILITY STUDIES AMONG THE ISOLATES OF *S. rolfsii* IN MORPHOLOGICAL AND CULTURAL CHARACTERS

A study was conducted to document the variation among the isolates of *S. rolfsii* in morphological and cultural characters like colony growth, number, size, colour and arrangement of sclerotia on different media. Both solid and liquid media were used for theis purpose.

3.3.1 Solid media

Three solid media viz., Potato Dextrose Agar, Oat Meal Agar and Czapek's Dox Agar media were used to assess the differential growth if any, of three isolates of the fungus.

3.3.1.1 Preparation of plates and inoculation

Fifteen millilitres of autoclaved and cooled medium was poured in each petri plate and allowed to solidify at room temperature. Later mycelial disc of 5 mm diameter was cut using sterilized cork borer from the periphery of an actively growing three days old culture of the fungus grown on PDA and transferred aseptically to the centre of each plate. Each treatment was replicated thrice and the plates were incubated at 28 ± 2°C.

3.3.1.2 Measurement of growth

For determining the variation among different isolates of *Sclerotium rolfsii* in colony growth, the colony growth of the fungus in each Petri plate was measured when entire Petri plate was covered by the fungus in some of the plates. The colony growth was measured along two diameters at right angles and averaged.

3.3.1.3 Study of sclerotial characters

For each isolate of *S. rolfsii*, observations for various cultural and morphological characteristics of sclerotia viz., time taken for sclerotial initiation, maturation, the number, colour and size of sclerotia and their arrangement on solid medium were recorded 15 days after incubation. The

mature sclerotia in each Petri plate were harvested separately using fine camel brush and counted. The sclerotial population was categorized as poor, fair, good and excellent as given below.

Categories	Rating
Poor	+ (1 - 100)
Fair	++ (101 - 250)
Good	+++ (251 - 500)
Excellent	++++ (> 500)

Sclerotial size of each isolate of *S. rolfsii* was measured by micrometry method. The microscope was calibrated with stage and ocular micrometer at a magnification of 100 x (eye piece : 10 X : objective : 10 X). The size of 30 sclerotia were measured in each replication and averaged.

3.3.2 Liquid media

The variation among the isolates of *S. rolfsii* with respect to their growth (dry weight) was determined in six liquid media viz., Richard's medium, Coon's medium, Potato broth, Crossandra host extract medium, Groundnut host extract medium and Tomato host extract medium.

3.3.2.1 Growing of the fungus in liquid media

Mycelial disc of 5 mm diameter was cut with sterile cork borer from peripheral growth of three days old culture and transferred aseptically into

3 ml of sterilized medium in 100 ml flasks and incubated at 28 ± 2°C for 12 days.

3.3.2.2 Measurement of growth in liquid media

The resultant growth of fungus was harvested and filtered through previously weighed Whatman No. 44 filter paper. It was washed thoroughly with distilled water. The fungal mat was dried at 40°C for two days in hot air oven and dry weight was recorded.

3.4 VARIABILITY AMONG THE ISOLATES OF *S. rolfsii* IN PHYSIOLOGICAL CHARACTERS

An experiment was carried out to know whether the isolates of *Sclerotium rolfsii* differ in their utilization of different carbon and nitrogen sources.

3.4.1 Utilization of different carbon sources by the isolates of *S. rolfsii*

In this investigation, the isolates of *S. rolfsii* were grown on different carbon sources using Czapek's Dox Agar medium as a basal medium. The carbon sources used were glucose, fructose, sucrose, maltose and mannitol. The carbon compound (sucrose) in the basal medium was substituted at a time with each carbon source on an equivalent of available carbon in sucrose (Bahadur *et al.*,1976). Medium without carbon served as control.

Mycelial disc of 5 mm diameter was cut using sterilized cork borer from the periphery of an actively growing three days old culture and transferred to the centre of each plate containing 15 ml of autoclaved and cooled PDA medium substituted with different carbon sources mentioned earlier. Each isolate was replicated thrice for a given carbon source.

3.4.2 Utilization of different nitrogen sources by the isolates of *S. rolfsii*

In this context, all the isolates of *S. rolfsii* were grown on different nitrogen sources substituted in Czapek's dox agar medium in place of $NaNO_3$. The different compounds used as nitrogen sources were, sodium nitrate, sodium nitrite, potassium nitrate, calcium nitrate and ammonium sulphate. The Nitrogen compound ($NaNO_3$) in Czapek's Dox Agar medium was substituted at a time with each nitrogen source on an equivalent of available Nitrogen in $NaNO_3$ (Bahadur *et al.*,1976).

Five millimeter diameter of mycelial disc was cut using sterilized cork borer from the periphery of an actively growing three days old culture and transferred to the centre of each plate containing 15 ml of autoclaved and cooled Czapek's Dox Agar medium substituted with different nitrogen sources mentioned earlier. Three replications were maintained for each nitrogen source.

3.4.3 Measurement of growth

The linear growth of the fungus on each Petri plate containing different carbon and nitrogen sources was recorded when the entire Petri plate was covered by the fungus in some of the plates. The colony growth was measured along two diameter at right angle and averaged.

3.4.4 Study of Sclerotial characters

The variation in sclerotial characters among the isolates of *S. rolfsii* with respect to their utilization of different carbon and nitrogen sources was determined. The sclerotial characteristics viz., time taken for sclerotial initiation, maturation, the number, colour and size of sclerotia and their arrangement on the medium were recorded 15 days after incubation. The mature sclerotia in each Petri plate were harvested separately using fine camel brush and counted. The sclerotial population was categorized as poor, fair, good and excellent.

3.5 DIFFERENTIAL SENSITIVITY / TOLERANCE OF *S. rolfsii* ISOLATES TO ANTAGONISTS

Two fungal antagonists viz., *Trichoderma viride* and *T. harzianum* were used to test whether the isolates of the pathogen differ in their sensitivity / tolerance to these antagonists. Dual culture technique (Muthamilan and Jeyarajan, 1992) was employed for this purpose.

Fifteen millilitres of autoclaved cooled PDA was poured into 9 cm petri plates and allowed to solidify. Mycelial discs of 5 mm diameter of the antagonists as well as the test pathogen were cut with sterile cork borer from the periphery of an actively growing three days old cultures and then placed on opposite sides of Petri plate. The distance between inoculum blocks was 7 cm. The inoculated petri plates were incubated at 28 ± 2°C for three days. The Petri plates inoculated with pathogen alone served as control.

The per cent growth reduction of the pathogen was calculated by using the formula given below.

Per cent growth reduction =

$$\frac{\text{Linear growth of } \textit{S. rolfsii} \text{ in control} - \text{Linear growth of } \textit{S. rolfsii} \text{ in presence of antagonist}}{\text{Linear growth of } \textit{S. rolfsii} \text{ in control}} \times 100$$

3.6 PATHOGENIC VARIABILITY AMONG THE ISOLATES OF *S. rolfsii*

Cross inoculation experiments for testing pathogenic variability among the isolates of *S. rolfsii* were conducted in pot culture in the glass house. The pathogenic potential of each isolate of *S. rolfsii* was tested on three hosts viz., crossandra, groundnut and tomato.

3.6.1 Mass Multiplication of the inoculum

Each isolate of *S. rolfsii* was multiplied in Sand - Maize meal medium. Sand and Maize meal (roughly ground maize seed) were mixed in the ratio of 9:1. One hundred grams of the mixture was transferred to 250 ml conical flasks moistened to the saturation and autoclaved 15 p.s.i for 20 minutes. Five sclerotia of the isolate were aseptically transferred into the flask. The inoculated flasks were incubated at 28 ± 2°C. After five days of incubation, the inoculated medium was aseptically mixed with glass rod for uniform distribution of inoculum throughout the medium. The flasks were incubated for another 10 days, by which time the medium was entirely covered with profuse brown sclerotia and scanty white mycelium.

3.6.2 Raising of the host plants in pots for inoculation

Earthen parts of 15 cm diameter each containing 2 kg of sterilized soil were used for raising the host plants.

Groundnut seedlings from directly sown seed were used for inoculation whereas for crossandra and tomato, transplanted seedlings were used. Ten plants were maintained for each pot and all agronomic practices were followed for growing of plants.

3.6.3 Inoculation

Plants were inoculated when they attained the age of 40 days. Each host was inoculated with each isolate of the pathogen. Five sclerotia were placed around the stem of each plant on the soil surface. The pots were then judicially watered. The un-inoculated plants of each host served as controls. Each treatment was replicated thrice. The inoculated plants were periodically observed for symptoms and final disease incidence was calculated as follows:

$$\text{Per cent disease incidence} = \frac{\text{No. of infected plants}}{\text{Total number of plants inoculated}} \times 100$$

3.7 ELABORATION OF TOXINS BY *S. rolfsii* ISOLATES

This experiment was conducted to test whether the isolates of *S. rolfsii* differ in the elaboration of toxins. The per cent inhibition of shoot and root growth of crossandra, groundnut and tomato in the presence of elaborated toxin was taken as a measure of toxicogenicity of each isolate. Each isolate of the fungus was grown in Richard's medium. Mycelial disc of 5 mm diameter was cut with sterile cork borer from peripheral growth of three days old culture and dropped aseptically into 100 ml of sterilized Richard's medium in 250 ml conical flask. After incubation for 10 days, the mycelial mat was separated by passing the filtrate through cheese cloth. The culture filtrate was diluted 1:1 with distilled water, passed through seitz bacteria proof filter. Petri plates with three layers of filter paper inside were sterilized in hot air

oven and cooled. The filter paper layers were sufficiently moistened with culture filtrate of the isolate. Surface sterilized seeds were placed on moistened filter paper and incubated for germination. Seeds kept on filter paper moistened with sterile distilled water served as control. Each treatment was replicated thrice.

The shoot and root growth in each treatment was measured when they attained sufficient growth in control plate. Per cent inhibition of shoot and root growth was calculated with the following formula.

$$= \frac{\text{Growth of shoot or root in control} - \text{Growth of shoot or root in the presence of toxin}}{\text{Growth of shoot or root in control}} \times 100$$

3.8 PROTEIN AND ISOZYME VARIABILITY AMONG ISOLATES OF *Sclerotium rolfsii*

The variability among three isolates of *S. rolfsii* in protein and esterase patterns were studied using sodium - dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE, respectively, following the procedure of Sambrook *et al.*, 1989.

3.8.1 Fungus cultures

Each isolate of the fungus was grown in Richard's solution for eleven days at 28 ± 2°C and the resultant mycelial mat was used for electrophoretic studies.

3.8.2 Protein and esterase extraction

For extraction of proteins and enzyme from the mycelial mats, the following extraction buffers were used.

A. Extraction buffer for proteins :

Tris (Hydroxy methylene amino methane)	:	1.21 g
1% SDS (Sodium dodecyl sulphate)	:	1 ml
Mercaptoethanol	:	1 ml
Distilled water	:	100 ml
pH	:	7.5

B. Extraction buffer for esterase

(0.1 M Tris HCl, pH 7.5)

Tris	:	1.21 g
Distilled water	:	100 ml

3.8.2.1 Procedure for extraction

Mycelial mats were harvested, washed thoroughly with distilled water and homogenized in cold extraction buffer in pre-chilled mortar and pestle. These homogenates were centrifuged at 10,000 rpm for 20 minutes using eppendorf centrifuge 5415 R. The clear supernatant was collected in eppendorf tubes which served as test solutions.

3.8.3 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was followed for separation of esterase and protein fractions. Native PAGE-system was used to study esterase and SDS-PAGE system was employed for protein studies.

3.8.3.1 Composition of different solutions used in the preparation of slab gels are furnished below

A. Acrylamide solutions

Acrylamide	:	30.0 g
N,N' methylene bisacrylamide	:	0.8 g

Dissolved in 80 ml of distilled water and volume made upto 100 ml. The solution was filtered and stored at 4°C in an amber coloured bottle.

B. Resolving gel buffer : 1.5 M Tris-HCl, pH 8.8)

Tris : 18.15 g

Dissolved in 80 ml of distilled water and pH was adjusted to 8.8.

C. Stacking gel buffer (0.5 M Tris - HCl, pH 6.8)

Tris : 6 g

Dissolved in 80 ml of distilled water and pH was adjusted to 6.8.

D. 10% Sodium dodecyl sulphate

SDS : 1 g

Distilled water : 10 ml

E. Polymerising agent :

Ammonium per sulphate (10%) - Catalyst

(0.1 g/ml distilled water, freshly prepared)

N, N, N', N', tetramethyl ethylenediamine (TEMED) - Chain initiator.

F. Electrode buffer

i. For proteins

Tris : 1.8 g

Glycine : 8.64 g

SDS : 0.6 g

Distilled water to make 600 ml

ii. For isozymes

Tris : 1.8 g

Glycine : 8.64 g

Distilled water to make 600 ml

3.8.3.2 Gel composition

Stock solution	12% resolving gel	4% stacking gel
30% Acrylamide	8.0 ml	2.7 ml
Resolving gel buffer	6.0 ml	—
Stacking gel buffer	—	2.0 ml
**10% SDS	0.3 ml	0.05 ml
Distilled water	16 ml	1.5 ml
*10% Ammonium per sulphate	0.2 ml	0.2 ml
TEMED	0.05	0.05

** for proteins
* Freshly prepared before use

3.8.3.3 Preparation of gels

Gel plates were washed thoroughly with cleaning solution followed by distilled water and dried. Appropriate spacers were placed between the glass plates on sides. Then these glass plates were sealed using a special adhesive tape in order to prevent leakage of gel solution. Resolving gel solution was prepared by mixing the stock solution in quantities given in the above table. The solution was poured into the sandwich to a level of 2 cm from the top.

Distilled water was added gently along the wall of the sandwich to form uniform gel surface and allowed for polymerization. After polymerization, the water on the resolving gel was poured off and wiped off with filter paper. Stacking gel was prepared and overlayed on the resolving gel. The comb was inserted into the stacking gel and allowed to polymerize.

3.8.3.4 Preparation of samples

Proteins and isozyme samples were separately prepared in respective sample buffers.

A. Sample buffer for proteins

0.1 M Tris HCl	:	1 ml
Glycerol	:	0.8 ml
10% SDS (w/v)	:	1.6 ml
Mercaptoethanol	:	0.4 ml
Bromophenol blue (w/v)	:	0.2 ml
Distilled water	:	4 ml

Fifty microlitres of purified protein was mixed with an equal volume of sample buffer in an eppendorf tube and boiled for 5 minutes in a water bath and cooled rapidly by keeping on ice.

B. Sample buffer for esterase

0.1 M Tris HCl	:	1 ml
Glycerol	:	0.8 ml
Mercaptoethanol	:	0.4 ml
Bromophenol blue (w/v)	:	0.2 ml
Distilled water	:	4 ml

Seventy microlitres of sample was mixed with 20 µl of sample buffer in an eppendorf tube.

3.8.3.5 Loading of samples

The inserted comb was gently removed from the gel after polymerization. The air bubbles, if any, were removed by rinsing with distilled water. The lower and upper chamber of electrophoretic apparatus were filled with electrode buffer. About 70 µl of sample was loaded in each well.

3.8.3.6 Running of gels

The electrophoretic unit was connected to power pack and a regulated electric power supply of 60 V, slowly raised to 100 V was supplied for the separation of proteins and isozymes. The electrophoresis was run till the day front reached the bottom of the gel which took 4 h. Then the power supply was switched off. The gel was carefully dismantled after electrophoresis and incubated in respective staining solution.

3.8.4 Staining of the gels

A. Staining Solution for proteins

Coomassie brilliant blue R 250	:	0.1 g
Methanol	:	40 ml
Acetic acid	:	10 ml
Distilled water	:	50 ml

B. Destaining solution for proteins

Methanol	:	40 ml
Acetic acid	:	10 ml
Distilled water	:	50 ml

The gel was incubated in staining solution for 12 hrs and then gel was taken out and properly destained by keeping it in destaining solution. The destaining solution was changed every four hours and destaining was hastened by gently rotating the trays.

C. Staining solutions for esterase

0.15 M sodium phosphate buffer	:	pH 7.2
Sodium dihydrogen phosphate	:	2.8 g
Disodium hydrogen phosphate	:	1.1 g

dissolved in 200 ml of distilled water.

b. α-naphthyl acetate	:	0.05 g
c. Fast blue RR salt	:	0.2 g

(Fast blue RR and α-naphthyl acetate were dissolved in 50% acetone in separate vials)

After electrophoresis, gel was incubated in staining solution for 30-45 minutes and destained with water : methanol : acetic acid : (5:4:1).

The zymograms were prepared indicating the relative mobility of isozyme and protein bands. Photographs were taken immediately after the appearance of bands. Separate runs were made to record the banding pattern of proteins and esterase enzyme.

The difference among the isolation of *S. rolfsii* in protein and isozyme pattern was determined by relative mobilities of different isozymes.

$$\text{Relative mobility (Rm) value} = \frac{\text{Distance travelled by the protein or isozyme front (cm)}}{\text{Distance travelled by dye front (cm)}}$$

Chapter IV

Results

CHAPTER - IV

EXPERIMENTAL RESULTS

Three isolates of *S. rolfsii*, one each from crossandra, groundnut and tomato were used in the present investigation.

Results of laboratory experiments on variability of the isolates in morphological, cultural and physiological characters and pot culture experiments on variability of isolates in pathogenicity and virulence are presented in this chapter.

4.1 VARIABILITY AMONG THE ISOLATES OF *S. rolfsii* IN MORPHOLOGICAL AND CULTURAL CHARACTERS

Both solid media and liquid media were used to study morphological and cultural variability among *S. rolfsii* isolates.

4.1.1 Growth on solid media :

The isolates of *S. rolfsii* were grown on three solid media viz., Potato Dextrose Agar (PDA), Oat Meal Agar (OMA) and Czapek's Dox Agar (CDA) media at room temperature. The cultural and morphological characters taken into account for assessing the existence of variation are colony growth, number, size, colour and arrangement of sclerotia.

A significant variation was noticed among the isolates of *S. rolfsii* with respect to colony growth, number and size of sclerotia on different solid media. The colony growth of three isolates on different solid media is presented in Table 1 and Fig. 1.

Among the solid media tested, PDA supported maximum colony diameter (90.0 mm) to all the three isolates followed by OMA (87.2 mm) while least growth was observed on CDA medium (59.6 mm).

Among the isolates, crossandra isolate recorded maximum colony diameter (84.2 mm) and is significantly superior to other two isolates. The colony diameter of groundnut (76.6 mm) and tomato (76.0 mm) isolates are on par with each other.

The pattern of growth of the individual isolates on different solid media indicated that crossandra isolate recorded maximum growth on PDA (90.0 mm) followed by OMA (86.7 mm) which are at par and significantly superior to the growth in CDA (76.0 mm).

The same pattern of growth was observed in both groundnut and tomato isolates also.

Table 1 : Growth and sclerotial population of *S. rolfsii* isolates on different solid media

Media	*Colony diameter (mm), *Sclerotial population (+)			
	Isolates			
	Crossandra	Groundnut	Tomato	Mean radial growth
Potato Dextrose Agar	90.0 +	90.0 ++	90.0 ++++	90.0
Oat Meal Agar	86.7 +	88.0 ++	87.0 +++	87.2
Czapek's Dox Agar	76.0 +	51.7 ++	51.0 ++	59.6
Mean radial growth	84.2	76.6	76.0	

*Mean of three replications

Sclerotial population

-	No sclerotia
+	Poor (1-100)
++	Fair (101-250)
+++	Good (251-500)
++++	Excellent (>500)

Colony growth

		SEM at 5%	CD at 5%
Isolates	=	0.64	1.91
Media	=	0.64	1.91
Isolates x Media	=	1.10	3.31

Fig. 1 : Growth of *S. rolfsii* isolates on different solid media

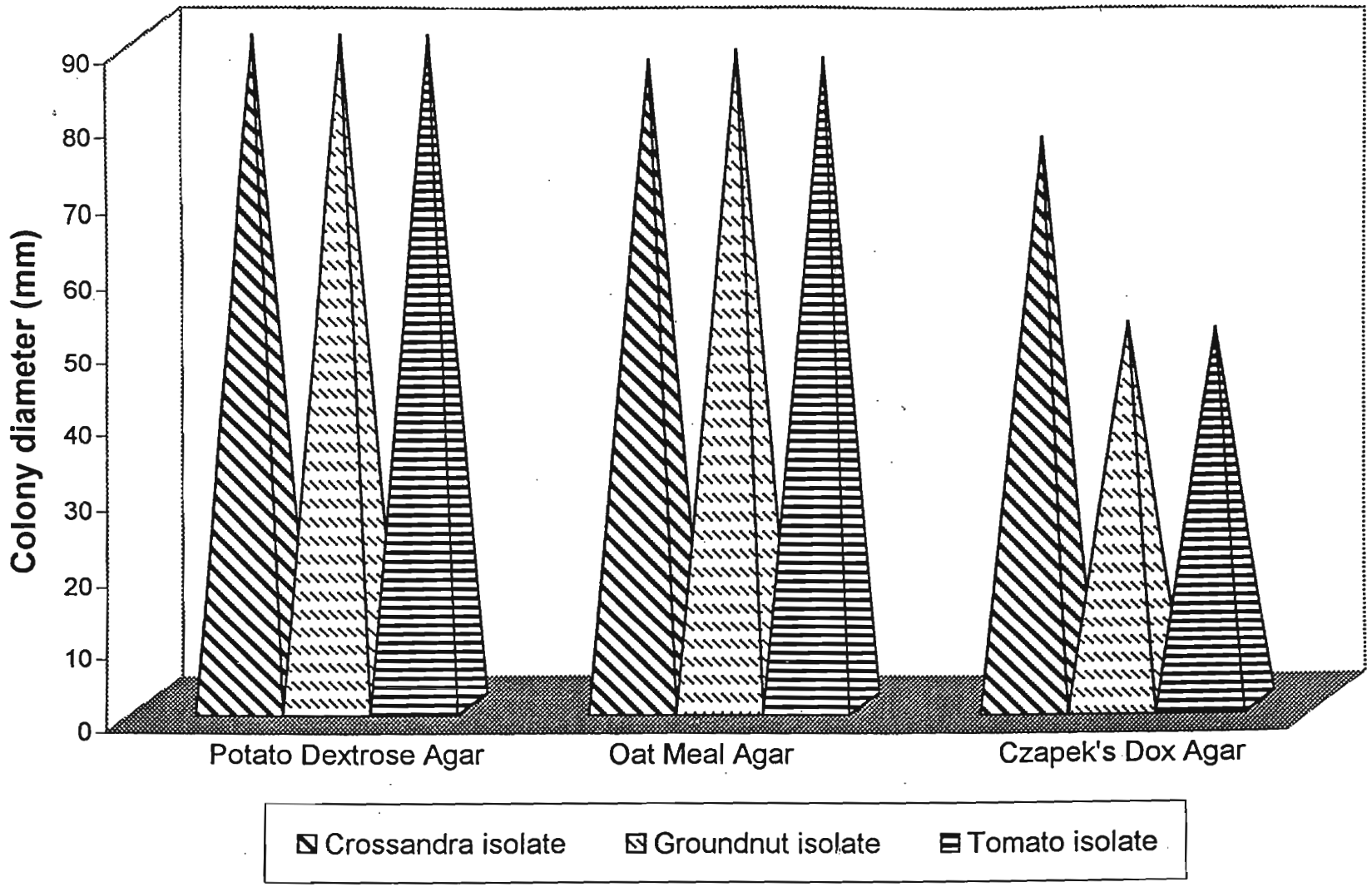
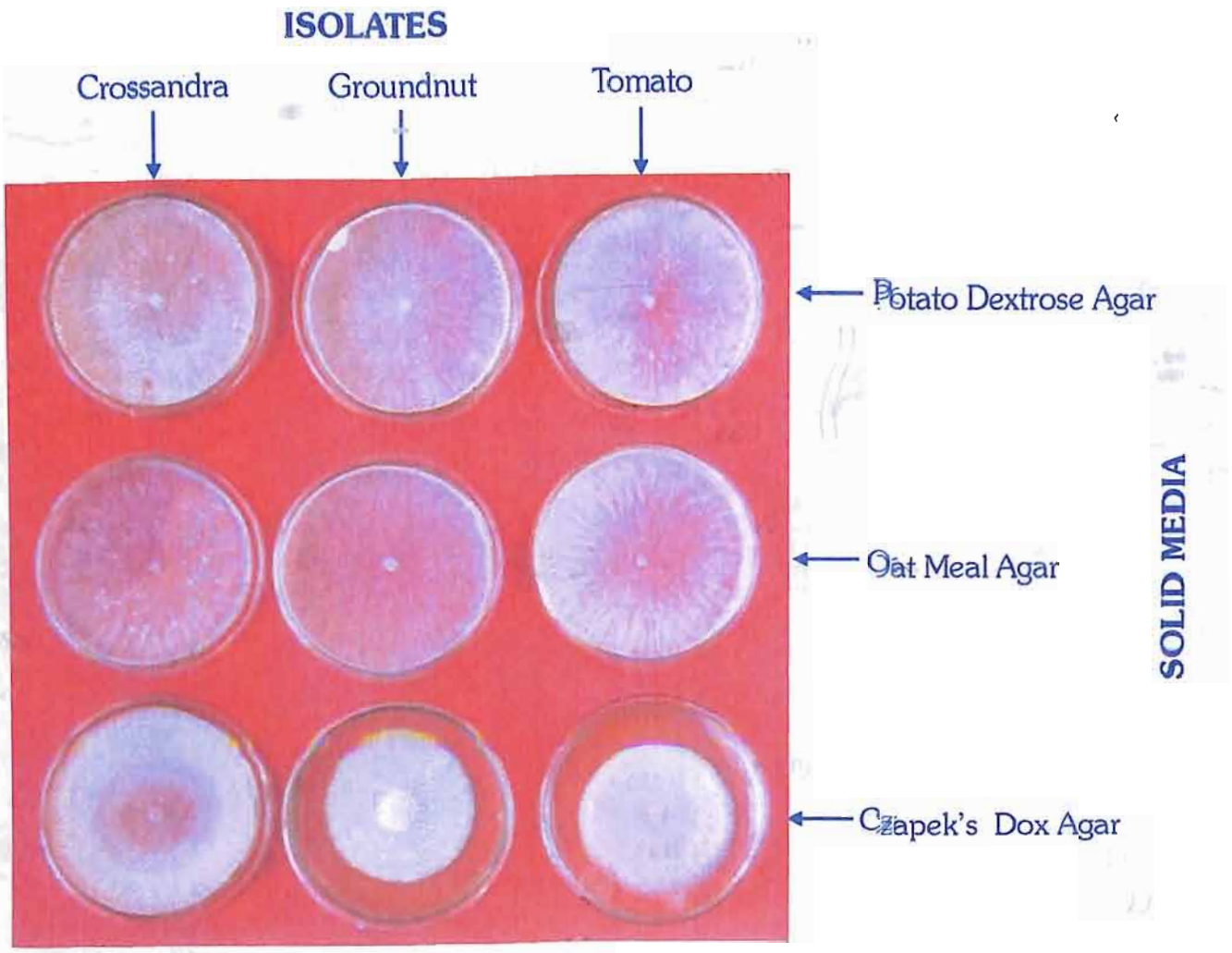


Plate 1: Colony growth of *S.rolfsii* isolates on solid media



4.1.1.2 Sclerotial morphology on solid media

In all the three isolates, when the growth was completely covered the surface of the media in the petri plate, small mycelial masses began to form but their appearance was more conspicuous 24 hrs after their formation. The mycelial masses appeared as white tiny knots whose size gradually increased and finally smooth, shiny and hard sclerotia were formed. Large drops of shiny liquid material were noted on the sclerotial bodies during period of maturation. The mature sclerotial bodies were easily detachable from the mycelium. The sclerotial characters of all the three isolates on different solid media are described in Table 2.

The sclerotial population of three isolates of *S. rolfsii* on different solid media is presented in Table 1 and Fig. 2 . Among the three isolates, tomato isolate produced more sclerotia (fair to excellent) followed by groundnut isolate (fair) and crossandra isolate (poor), irrespective of the solid medium used.

Fig. 2 : Sclerotial population of *S. rolfsii* isolates on different solid media

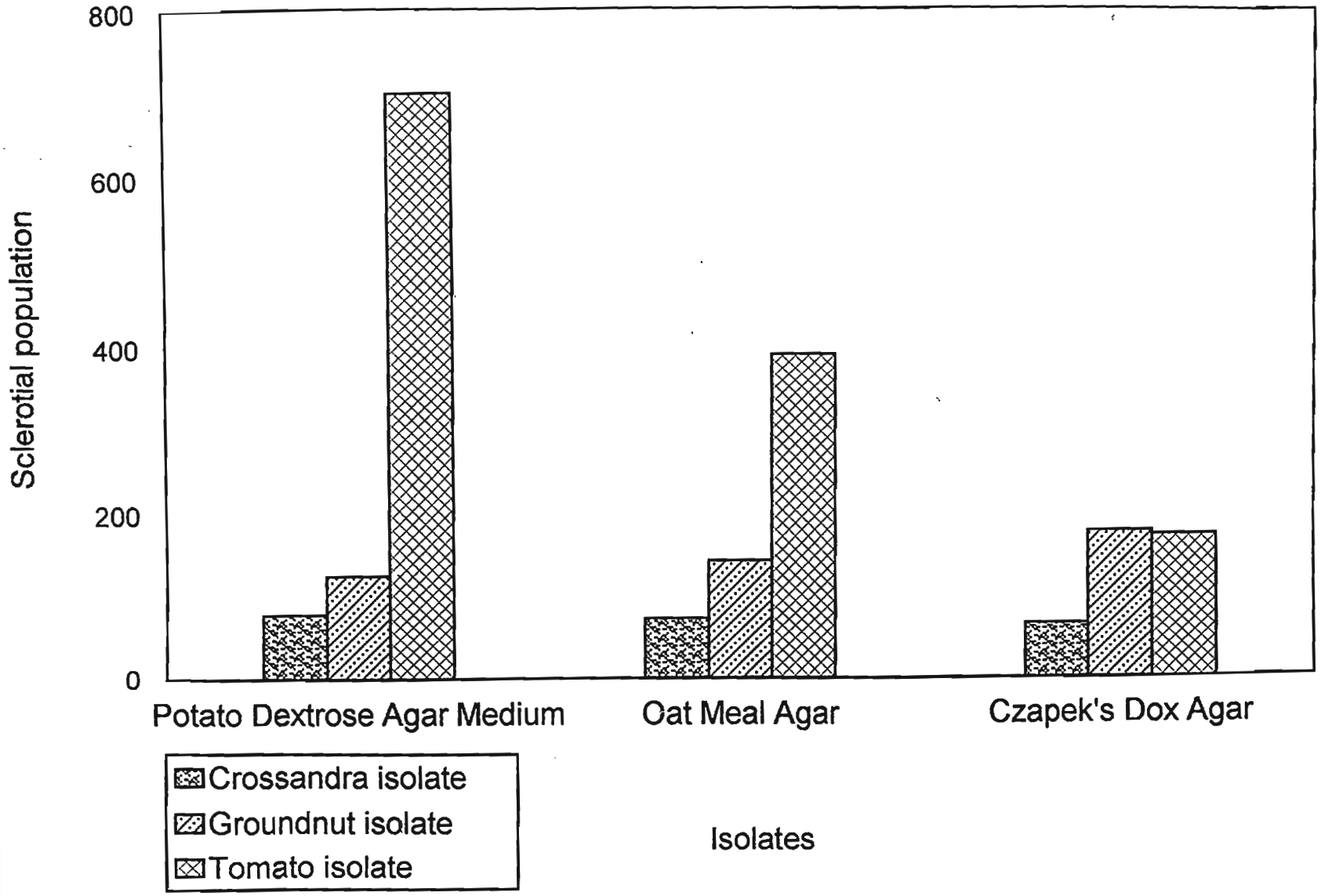
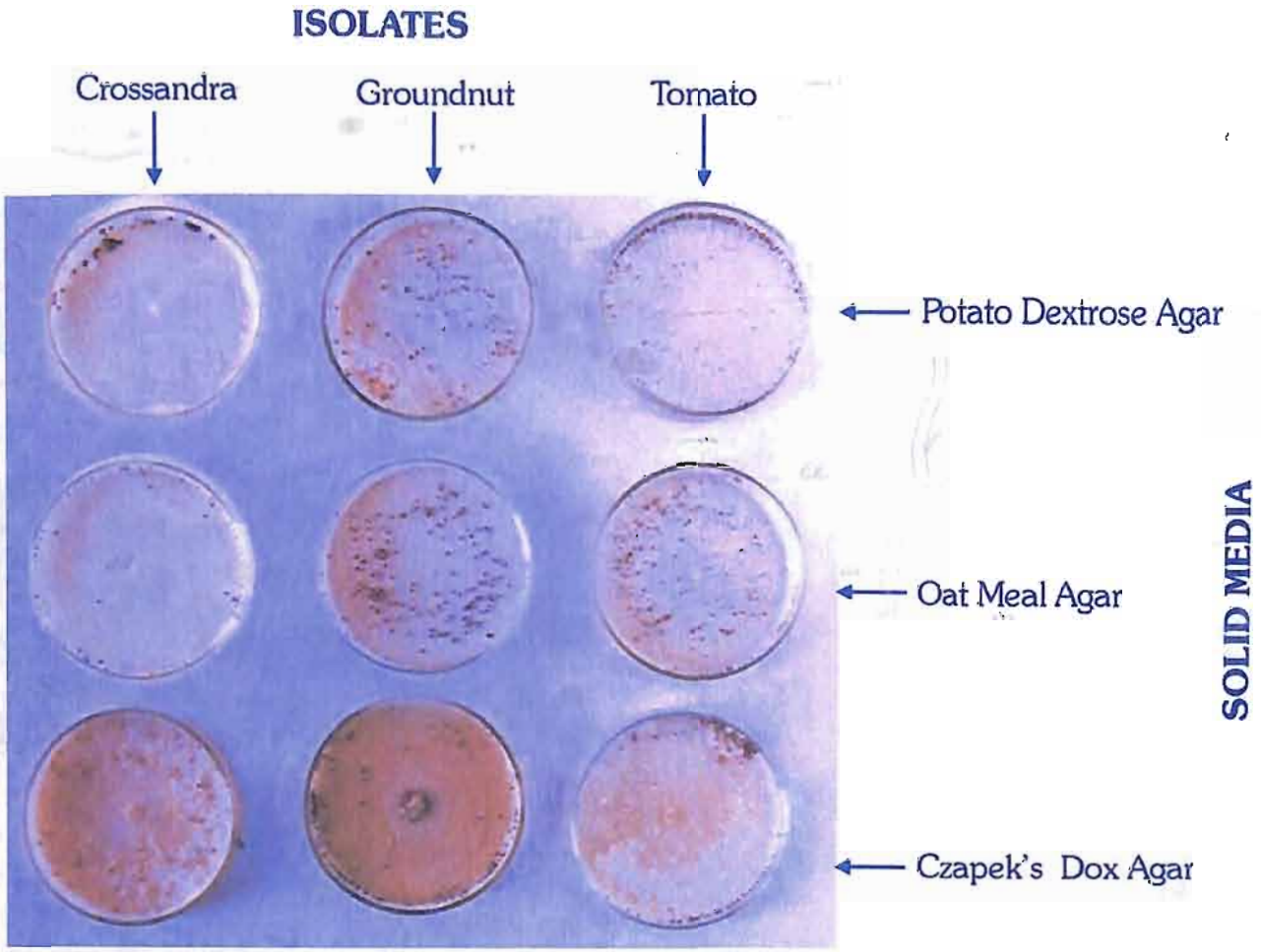


Table 2 : Sclerotial characters of *S. rolfsii* isolates on solid media.

Media	Crossandra isolate	Groundnut isolate	Tomato isolate
PDA	Dark brown, 1.2-1.3 mm, round sclerotia formed 192 h after inoculation, spread all over the medium	Brown, 1.0-1.1 mm, round sclerotia formed 168 h after inoculation and concentrated only in the periphery of the medium	Brown, oval to round, 0.8-1.00 mm sclerotia scattered all over the medium preferably in the margin. Sclerotia were observed 144 h after inoculation
Oat Meal Agar medium	Brown, 1.2-1.3 mm, round sclerotia spread all over the medium preferably in center, formed 192 h after inoculation	Light brown, oval to round, 1.1-1.2 mm sclerotia formed only in the periphery of the medium, sclerotia were formed 168 h after inoculation	Light brown, 0.9-1.0 mm, mostly round, a few ellipsoidal sclerotia scattered all over the medium preferably in centre, 144 h after inoculation
Czapek's Dox Agar medium	1.2-1.3 mm, brown, round sclerotia scattered throughout the medium. Sclerotia were formed 216 h after inoculation	Dark brown, 1.1-1.2 mm, round sclerotia distributed all over the medium, sclerotia were observed 192 h after inoculation	Light to medium brown, 0.9-1.0 mm, oval to round sclerotia arranged only in the margin of the medium. Sclerotia were formed 190h after inoculation

Plate 2 : Sclerotial production in *S.rolfsii* isolates on solid media



4.1.2 Liquid Media

4.1.2.1 Growth of *S. rolfsii* isolates in different liquid media

The isolates of *S. rolfsii* were grown in different liquid media and their growth (mycelial dry weight) is presented in Table 3 and Fig .3

It was evident from the data that there was a significant difference between isolates, media and their interaction.

Growth of all the three isolates was maximum in Richard's medium with mean mycelial dry weight of 532.6 mg followed by potato broth (461.1 mg) and groundnut host extract medium (420.1 mg). Coons' medium was found to support poor growth in all the three isolates (83.9 mg).

Among the isolates, crossandra isolate registered maximum mycelial mean dry weight of 387.6 mg followed by tomato isolate (336.9 mg) and groundnut isolate (260.6 mg).

The pattern of growth in different liquid media by the individual isolates indicated that crossandra isolate recorded maximum growth in Richard's medium (615.7 mg) which was significantly higher compared to all other media. The next in order were potato broth (538.0 mg), groundnut

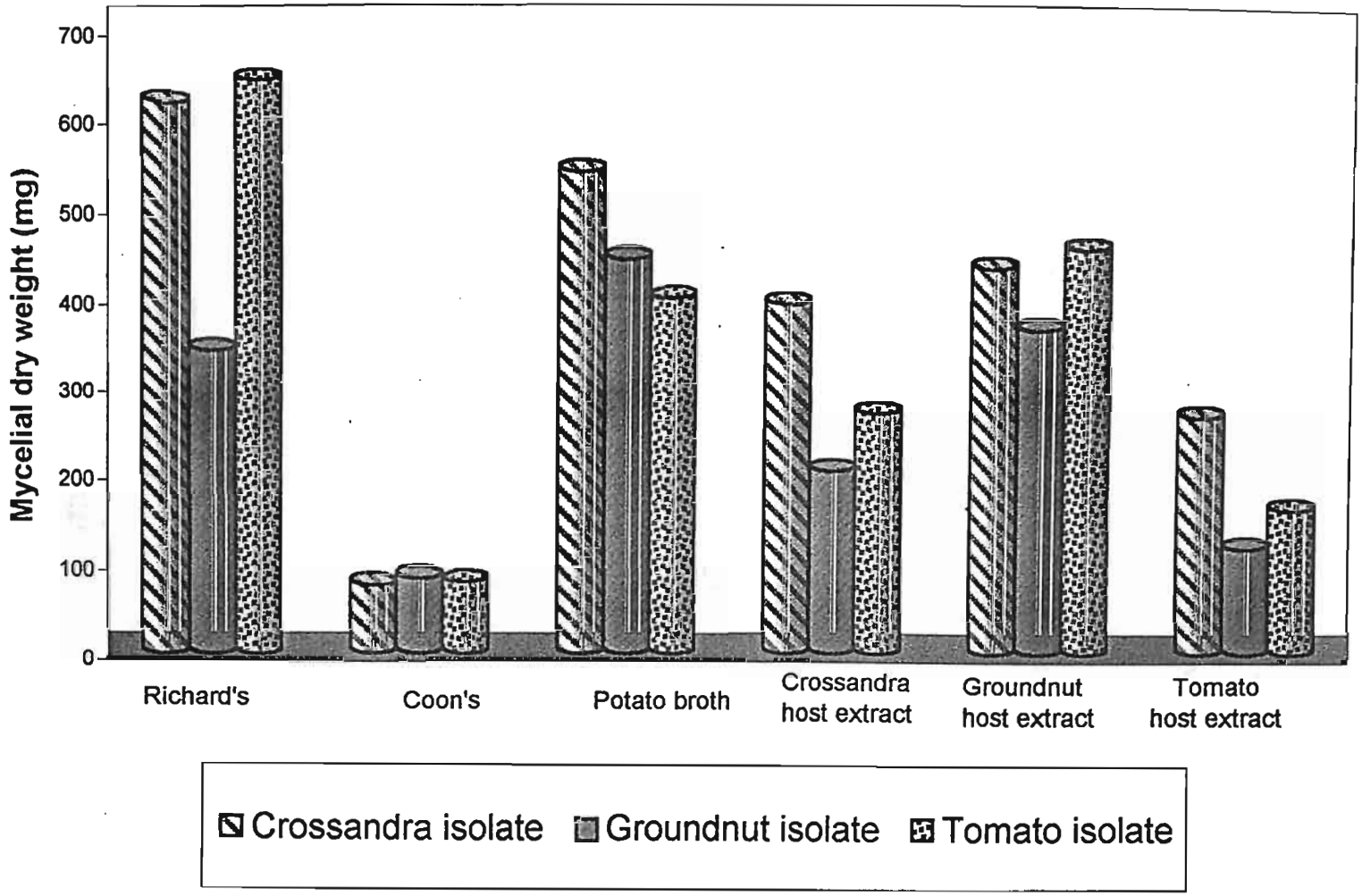
Table 3 : Growth of *S. rolfsii* isolates in different liquid media

Media	Mycelial dry weight* (mg)			
	Isolates			
	Crossandra	Groundnut	Tomato	Mean
Richard's	615.7	341.7	640.3	532.6
Coon's	76.3	85.0	90.3	83.9
Potato broth	538.0	444.7	400.7	461.1
Crossandra host extract	394.3	204.0	269.0	289.1
Groundnut host extract	435.3	367.7	457.3	420.1
Tomato host extract	266.0	120.7	163.7	183.4
Mean	387.6	260.6	336.9	

*Mean of three replications

		SEM at 5%	CD at 5%
Isolates	=	1.95	5.62
Media	=	2.76	7.95
Isolates x Media	=	4.78	13.8

Fig. 3 : Growth of *S. rolfsii* isolates in different liquid media



host extract (435.3 mg) , crossandra host extract (394.3 mg) and tomato host extract (266.0 mg) media while the least growth was observed in Coon's medium (76.3 mg).

Among the other two isolates, groundnut isolate recorded its best growth in potato broth medium (444.7 mg) followed by groundnut host extract (367.7 mg), Richard's medium (341.7 mg) and crossandra host extract(204.0 mg). The least growth was recorded in tomato host extract (120.7 mg) and Coon's medium (85.0 mg). All the media significantly differed from each other in supporting the growth of the isolate.

In case of Tomato isolate, Richard's medium was found to be superior in supporting maximum growth (640.3 mg) followed by groundnut host extract medium (457.3 mg). Potato broth (400.7 mg) and crossandra host extract (269.0 mg) and tomato host extract (163.7 mg) media were next in order while the least growth was registered in Coon's medium (90.3 mg).

4.2.1 Utilization of different carbon sources by *S. rolfsii* isolates

4.2.1.1 Growth in the presence of carbon sources

The colony growth of three isolates in the presence of various carbon sources is presented in Table 4 and Fig.4.

Table 4 : Effect of carbon sources on growth and sclerotial population of *S. rolfsii* isolates

Carbon sources	*Colony diameter (mm), *Sclerotial population (+)			
	Isolates			
	Crossandra	Groundnut	Tomato	Mean Radial Growth
Glucose	82.0 +	80.7 ++	81.0 +++	81.2
Fructose	60.0 +	36.3 ++	43.0 +	46.4
Sucrose	90.0 +	90.0 ++++	90.0 ++++	90.0
Maltose	90.0 +	90.0 ++++	90.0 ++++	90.0
Mannitol	90.0 +	90.0 +++	90.0 +++	90.0
Control	51.7 —	47.7 —	46.0 —	48.44
Mean Radial Growth	77.3	72.4	73.3	

*Mean of three replications

		SEM	CD at 5%
Isolates	=	0.27	0.82
Carbon sources	=	0.40	1.16
Isolates x Carbon sources	=	0.70	2.02

Sclerotial population

- No sclerotia
+ Poor (1-100)
++ Fair (101-250)
+++ Good (251-500)
++++ Excellent (>500)

Fig. 4 : Growth of *S. rolfsii* isolates on various carbon sources

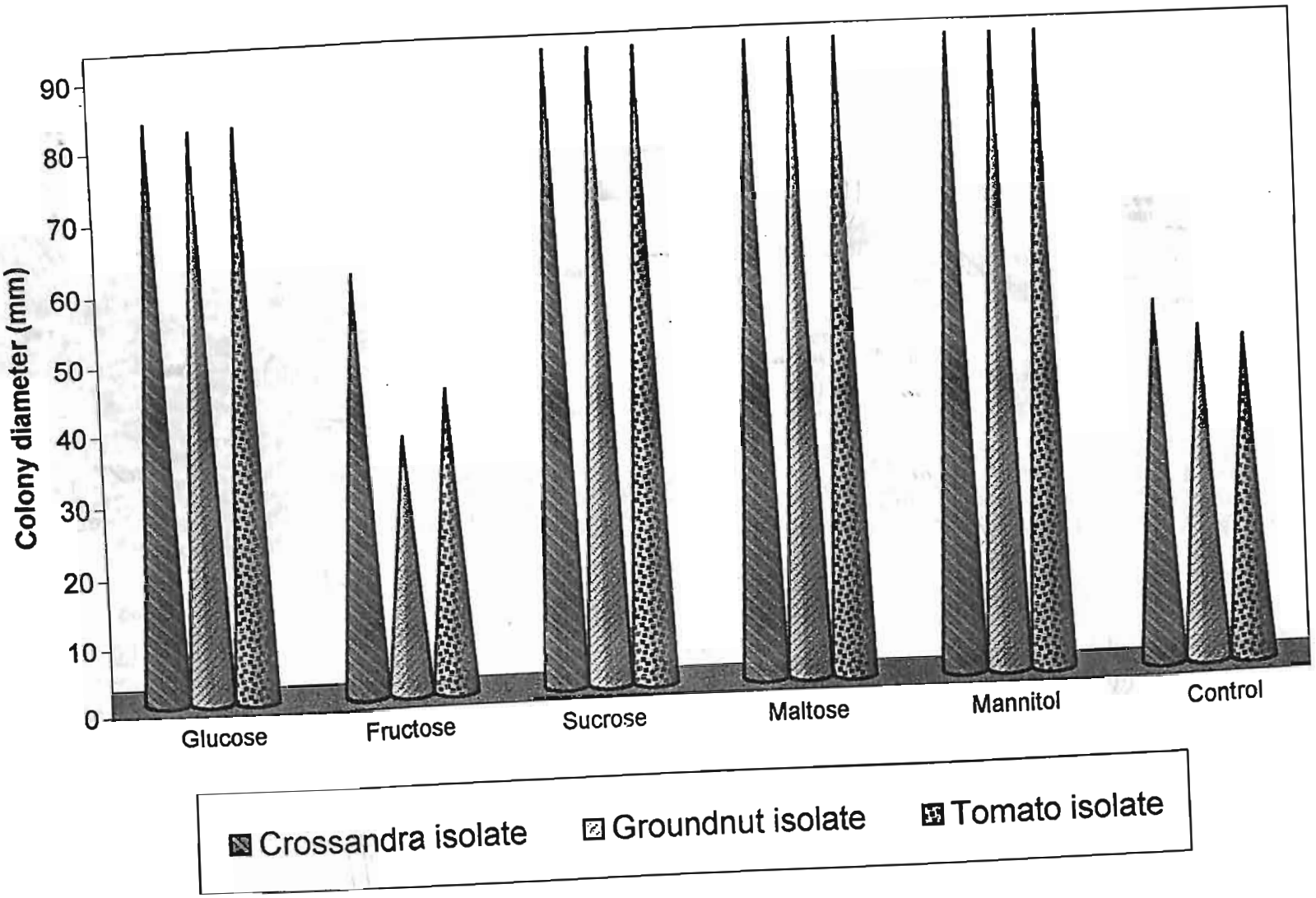
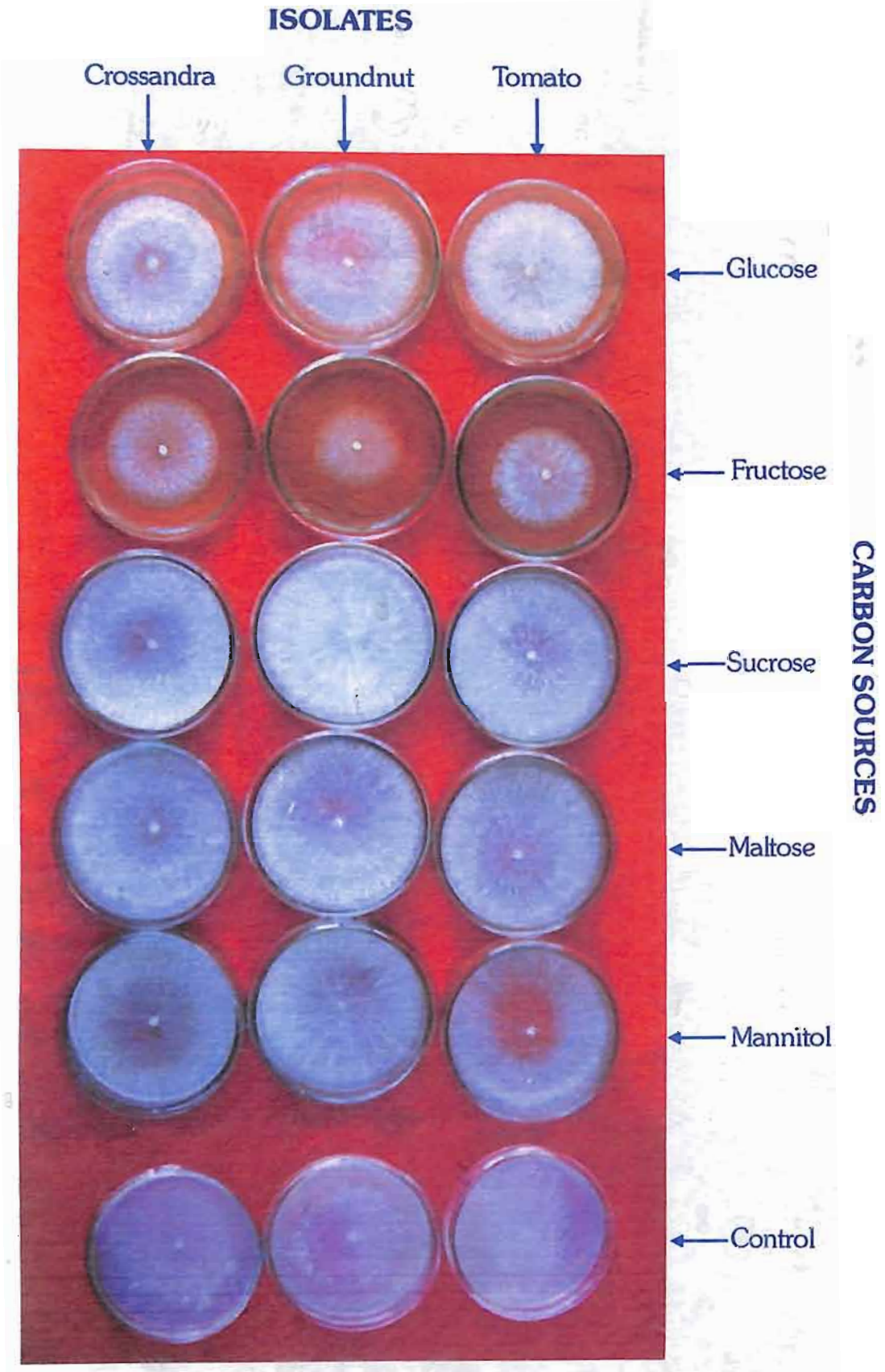


Plate 3 : Colony growth of *S.rolfsii* isolates on various carbon sources



The data revealed that all the three isolates of the pathogen registered significant increase in growth in the presence of any of the carbon sources compared to control. Sucrose, maltose and mannitol supported maximum growth (90.0 mm) in all the three isolates followed by glucose (81.2 mm) and fructose (46.4 mm).

Among the isolates, crossandra isolate recorded maximum mean radial growth (77.3 mm) in the presence of carbon sources. The growth of tomato isolate (73.3mm) and groundnut isolate (72.4mm) were at par.

When the growth of each isolate in the presence of different carbon sources was compared, crossandra isolate exhibited maximum growth (90.0 mm) in the presence of sucrose, maltose and mannitol which is significantly superior when compared to control, followed by glucose (82.0 mm). The least growth of this isolate was observed in the presence of fructose (60.0 mm).

Groundnut and tomato also exhibited maximum growth (90.0 mm) in the presence of sucrose, maltose and mannitol followed by glucose which is significantly superior when compared to control. But the growth of the tomato isolate (43.0 mm) was significantly superior to the growth of groundnut isolate in the presence of fructose (36.3 mm).

4.2.1.2 Sclerotial morphology in the presence of carbon sources

Considerable variation in sclerotial characters was observed among the isolates, irrespective of different carbon sources used. The sclerotial population of three isolates of *S. rolfsii* in the presence of various carbon sources is presented in Table 4 and Fig. 5.

Among the three isolates, tomato isolate produced more sclerotia (good to excellent) in all carbon sources except in fructose (poor) followed by groundnut isolate (fair to excellent) and crossandra isolate (poor).

Sclerotial characters of *S. rolfsii* isolates on various carbon sources are described in Table 5.

Fig. 5 : Sclerotial population of *S. rolfsii* isolates on various carbon sources

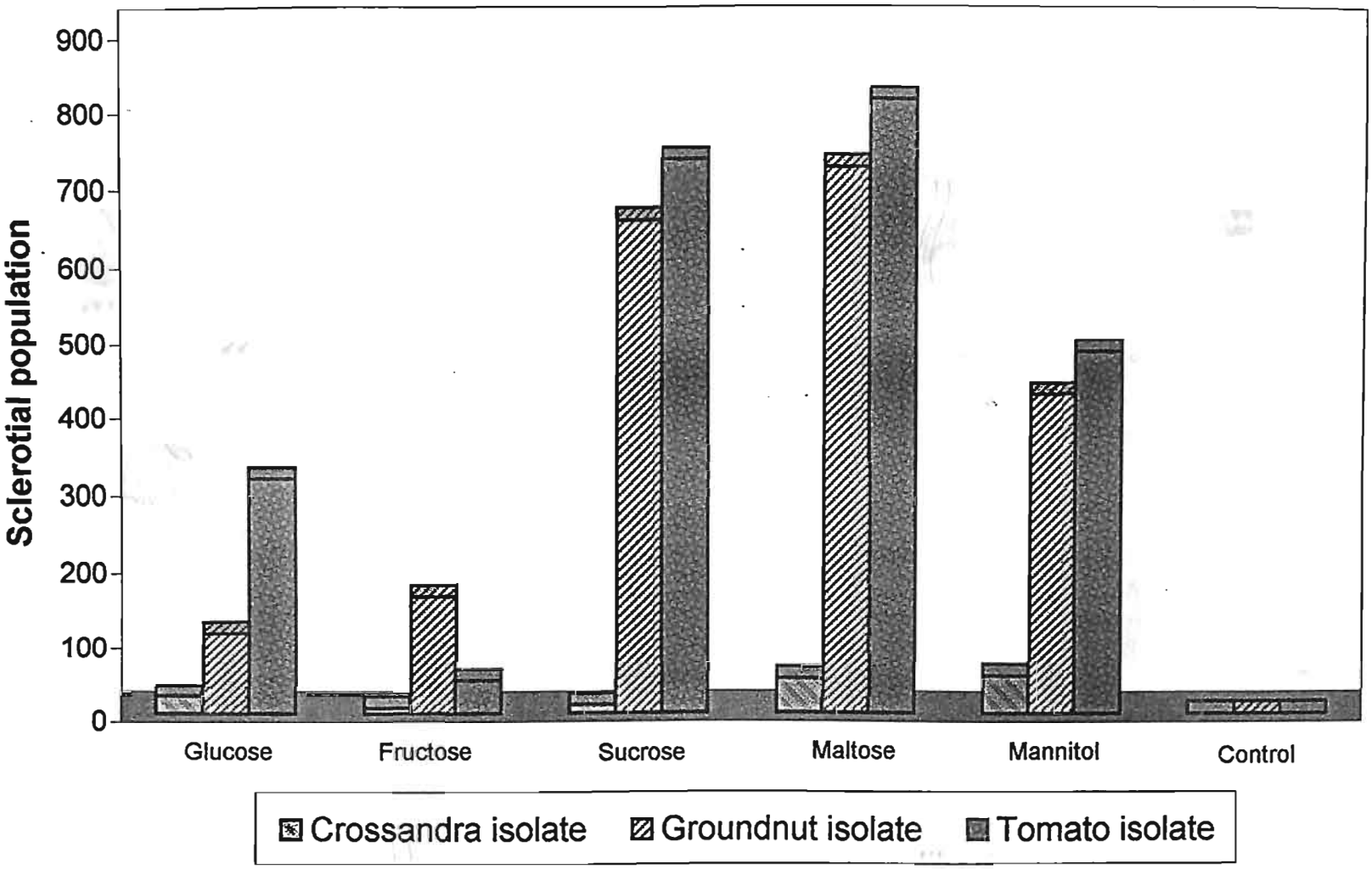


Plate 4 : Sclerotial production in *S.rolfsii* isolates on various carbon sources

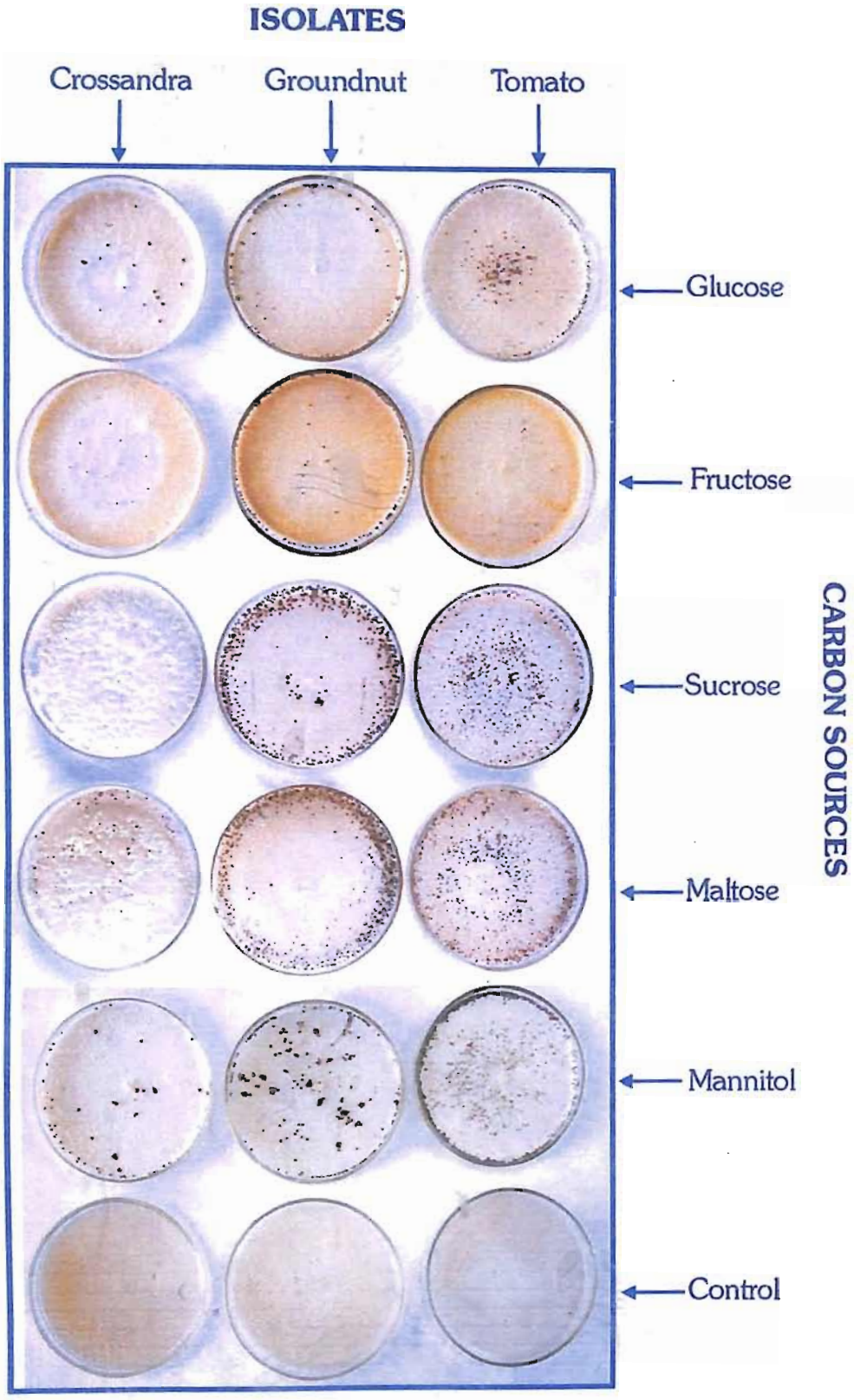


Table.5 : Sclerotial characters of *S. rolfsii* isolates on various carbon sources

Carbon sources	Crossandra isolate	Groundnut isolate	Tomato isolate
Glucose	Dark brown, 1.3-1.4 mm, round sclerotia formed 216 h after inoculation and concentrated in center	Dark brown, 1.1-1.3 mm, round sclerotia formed 176 h after inoculation and arranged in the margin	Brown, 1.0-1.1 mm, oval to round sclerotia distributed both in center and margin, formed 168 h after inoculation
Fructose	Brown, 1.2-1.3 mm, round sclerotia formed 264 h after inoculation in centre of the medium	Dark brown, 1.1-1.2 mm, round sclerotia observed 200 h after inoculation in the margin	Brown, round to irregular, 0.9-1.0 mm sclerotia spread all over the medium and formed 152 h after inoculation
Sucrose	1.2-1.3 mm, brown, oval to round sclerotia observed in middle of the medium, formed 336 h after inoculation	Dark brown, 1.0-1.1 mm round sclerotia formed 120h after inoculation and arranged peripherally	0.9-1.0 mm, oval to round sclerotia spread all over the plate and formed 120 h after inoculation
Maltose	Brown, 1.2-1.3 mm, round sclerotia spread all over the medium and formed 224 h after inoculation	Dark brown, 1.0-1.1 mm, round sclerotia observed 120 h after inoculation and arranged peripherally	Dark brown, 0.9-1.0 mm, oval to round sclerotia, spread all over the medium and formed 120 h after inoculation
Mannitol	Brown, 1.2-1.3 mm, round sclerotia spread all over the medium and formed 224 h after inoculation	Dark brown, 1.0-1.1 mm, round sclerotia spread all over the medium 144 h after inoculation	Dark brown, 0.9-1.0 mm, oval to round sclerotia formed 120 h after inoculation and arranged peripherally
Control	—	—	—

4.2.2 Utilization of different nitrogen sources by the isolates of *S. rolfsii*

4.2.2.1 Growth in the presence of nitrogen sources

The colony growth of three isolates of *S. rolfsii* is presented in Table 6 and Fig.6.

It is evident from the data that all the three isolates of the pathogen registered a significant increase in growth in the presence of any of the nitrogen sources compared to control except sodium nitrite where the growth of all isolates was zero. Excepting sodium nitrite remaining nitrogen sources appeared to be equally good for growth of all isolates.

Among the nitrogen sources, sodium nitrate, calcium nitrate, potassium nitrate supported maximum growth in all the three isolates (89.8-90.0 mm) which are significantly superior to ammonium sulphate and control (79.4 mm).

There is no significant difference among the isolates in their utilization of various nitrogen sources. All the three isolates exhibited maximum growth of 73.0 mm in the presence of nitrogen sources.

Table 6 : Effect of nitrogen sources on growth and sclerotial population of *S. rolfsii* isolates

Nitrogen sources	*Colony diameter (mm), *Sclerotial population (+)			
	Isolates			
	Crossandra	Groundnut	Tomato	Mean Radial Growth
Sodium nitrate	90.0 +	90.0 ++++	90.0 ++++	90.0
Sodium nitrite	0.0 –	0.0 –	0.0 –	0.0
Calcium nitrate	89.7 ++	89.7 ++++	90.0 ++++	89.8
Ammonium sulphate	88.7 +	89.3 ++++	88.7 ++++	88.9
Potassium nitrate	89.7 +	89.7 ++++	90.0 ++++	89.8
Control	80.0 -	79.0 -	79.3 -	79.4
Mean Radial Growth	73.0	72.9	73.0	

*Mean of three replications

		SEM	CD at 5%
Isolates	=	0.12	0.34
Nitrogen sources	=	0.17	0.48
Isolates x Nitrogen sources	=	0.29	0.83

Sclerotial population

- \- No sclerotia
- \+ Poor (1-100)
- ++ Fair (101-250)
- +++ Good (251-500)
- ++++ Excellent (>500)

Fig. 6 : Growth of S. rolfsii isolates on various nitrogen sources

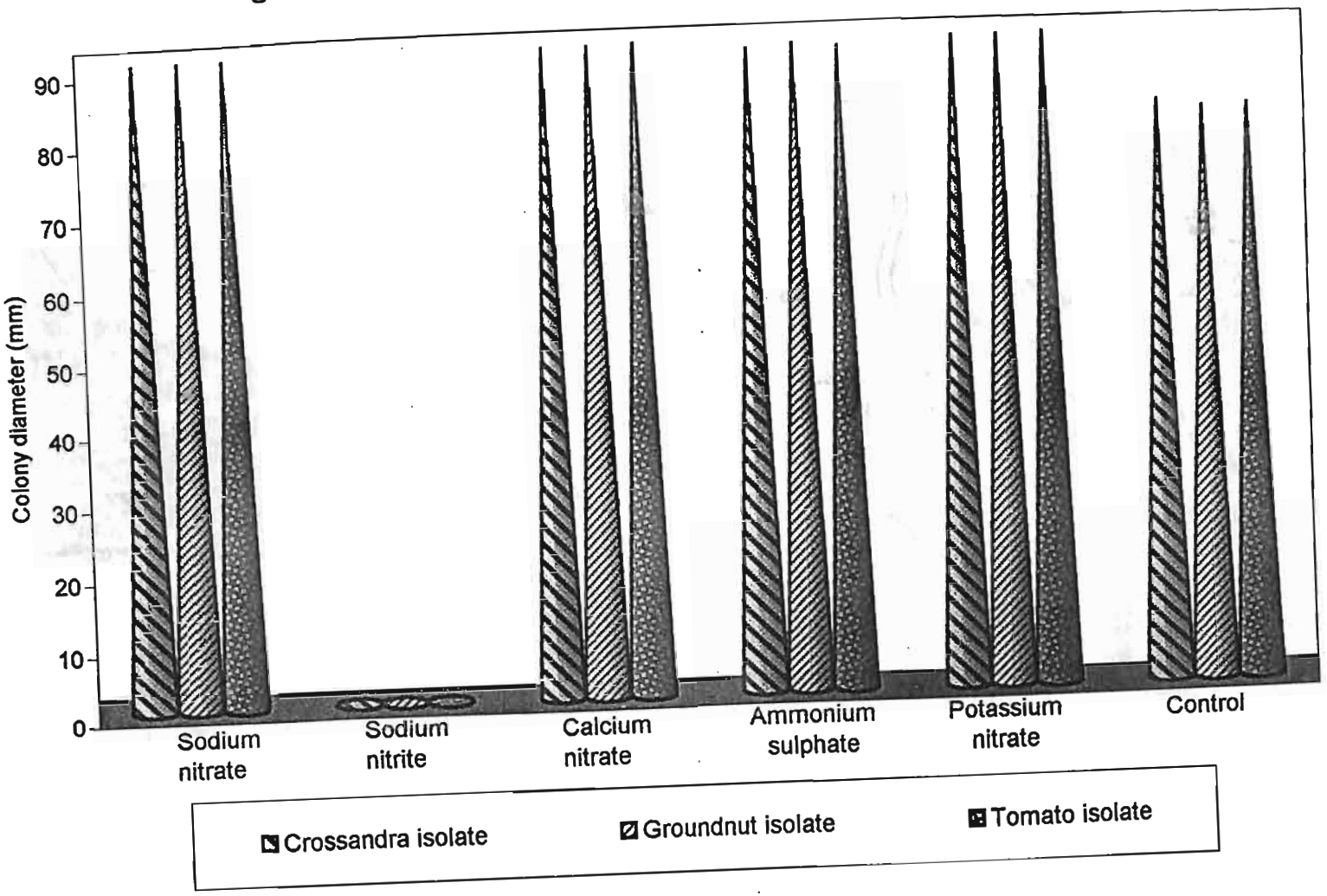
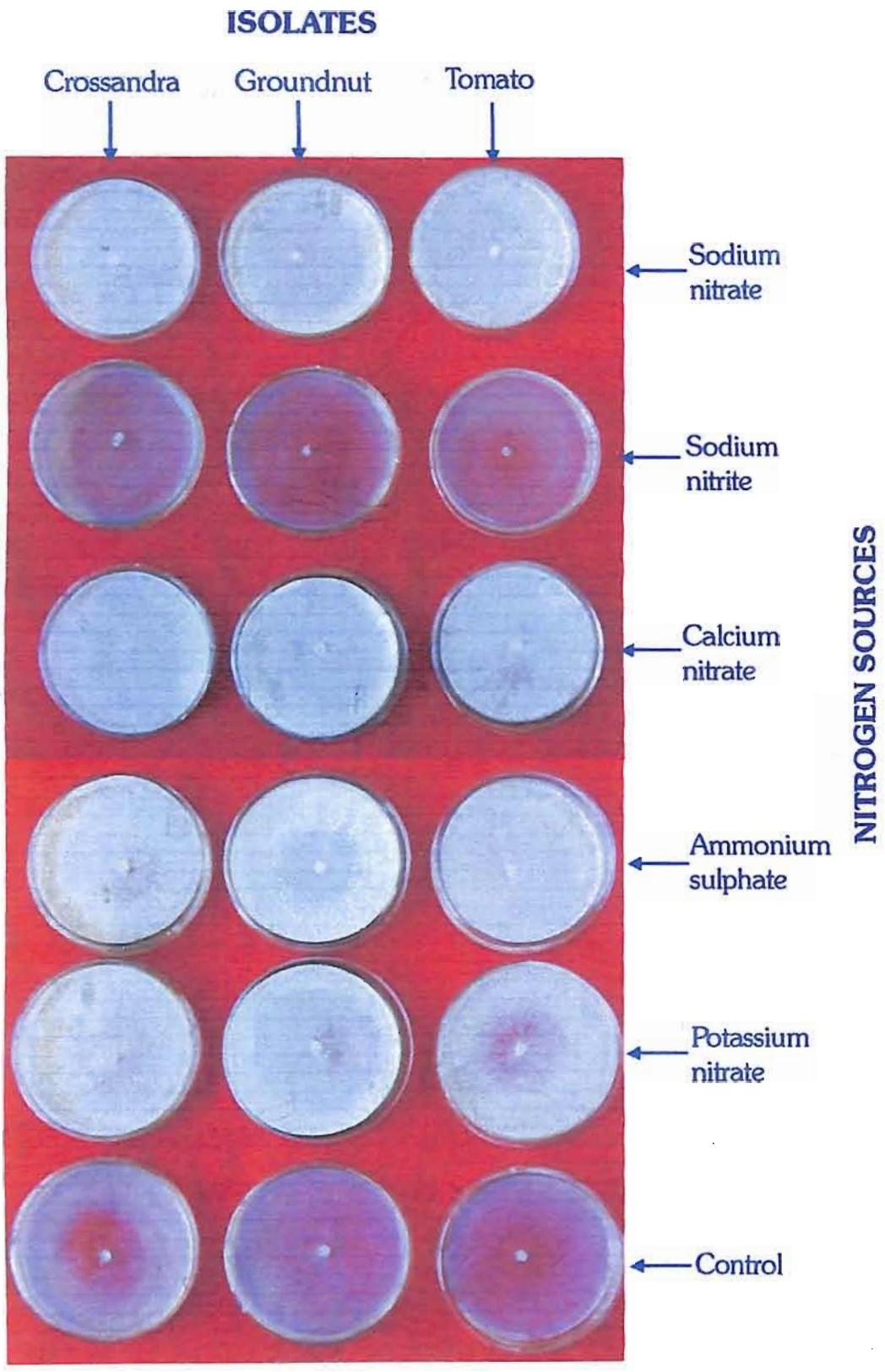


Plate 5 : Colony growth of *S.rolfsii* isolates on various nitrogen sources



When compared the growth of individual isolate in the presence of different nitrogen sources, crossandra and tomato isolates exhibited maximum growth (89.7-90.0 mm) in the presence of sodium nitrate, calcium nitrate and potassium nitrate which is significantly superior to the growth in the presence of ammonium sulphate (88.7mm) whereas groundnut isolate exhibited maximum growth in the presence of sodium nitrate (90.0 mm), , calcium nitrate, potassium nitrate (89.7 mm) and ammonium sulphate (89.3mm) which are all at par but significantly superior when compared to control.

4.2.2.2 Sclerotial morphology in the presence of nitrogen sources

Considerable variation in sclerotial characters was observed among the isolates, irrespective of different nitrogen sources used. The sclerotial characters of all the three isolates on various nitrogen sources are described in Table.7.

The sclerotial population of the three isolates in the presence of different nitrogen sources is presented in Table 6 and Fig. 7. Groundnut and tomato isolates recorded excellent sclerotial population in presence of all nitrogen sources whereas crossandra isolate recorded mostly poor except in the presence of calcium nitrate (fair). All the three isolates did not produce sclerotia in the absence of nitrogen source.

Fig. 7 : Sclerotial population of *S. rolfsii* isolates on various nitrogen sources

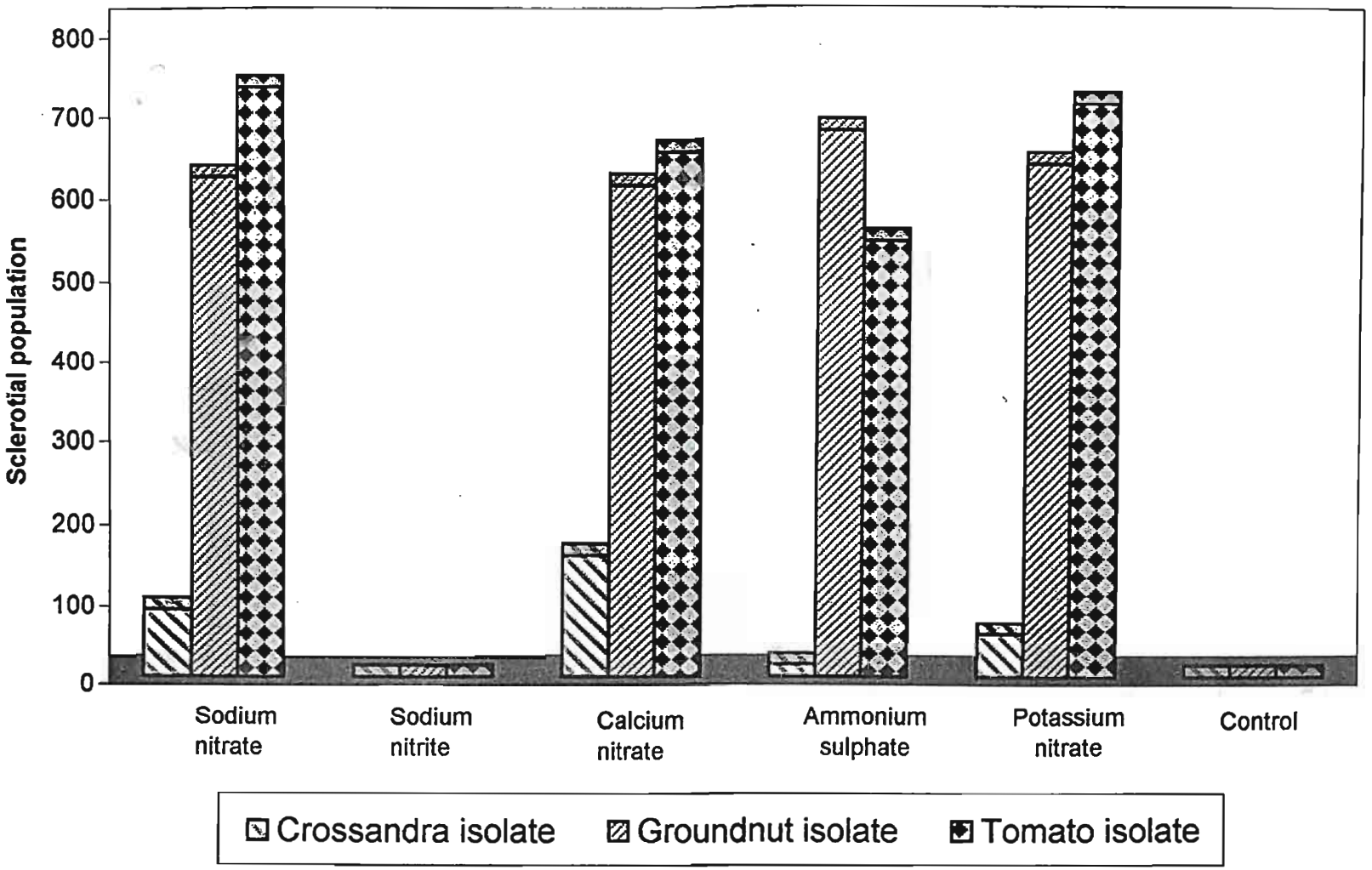


Table. 7 : Sclerotial characters of *S. rolfsii* isolates on various nitrogen sources

Nitrogen Sources	Crossandra isolate	Groundnut isolate	Tomato isolates
Sodium nitrate	Light brown, 1.2-1.3 mm, round sclerotia produced 204 h after inoculation in the peripheral region of the medium	Dark brown, 1.1-1.2 mm, brown sclerotia formed 1.44 h after inculcation in peripheral region of the medium	Dark brown, 0.9 - 1 mm, oval to round sclerotia formed on margin of the medium after inoculation of 120 h
Sodium nitrite	-	-	-
Calcium nitrate	Dark brown, 1.2-1.3 mm, round sclerotia concentrated mostly in centre and a few distributed on margin . Sclerotia were formed 168 h after inoculation	Dark brown, 1.1-1.2 mm, round sclerotia formed 144 h after inoculation and concentrated in centre and margin	Brown, 0.9 - 1.0 mm, oval to round sclerotia formed 120 h after inoculation and concentrated in both centre and margin
Ammonium sulfate	Brown, 1.3-1.35 mm, round scleroita formed 228 h after inoculation and scattered throughout the medium	Pale brown, 1-1.2 mm, round sclerotia formed 168 h after inoculation in periphery region of the medium. Sclerotia arranged in chain	Pale brown, 0.9 to 1.0 mm, oval to round sclerotia formed 168 h after inoculation only in periphery as cluster
Potassium nitrate	Light brown, 1.2 - 1.3 mm, round sclerotia formed 228 h after inoculation in the periphery of medium	Dark brown, 1.0-1.1 mm, round sclerotia formed 144 h after inoculation and concentrated only in peripheral region and arranged in chain	Dark brown, 0.9 - 1.0 mm, oval to round sclerotia produced 144 h after inoculation only in peripheral region of the medium
Control	-	-	-

Plate 6 : Sclerotial production in *S.rolfsii* isolates on various nitrogen sources

Plate 7 : Size difference in sclerotia of three isolates of *S.rolfsii* observed throughout the investigation

4.3 SENSITIVITY / TOLERANCE OF *S. rolfsii* ISOLATES TO ANTAGONISTS

Two fungal antagonists viz., *Trichoderma viride* and *T. harzianum* were used to test whether the isolates of the pathogen differ in their sensitivity/ tolerance to the antagonists, by dual culture technique.

It was evident from the data (Table 8 and Fig.8) that there was no significant difference between isolates, antagonists and their interaction.

All the three isolates of the pathogen were equally sensitive to the antagonists. Similarly both the antagonists had an equal effect in reducing the mycelial growth and sclerotial production of the isolates of the pathogen but the reduction was significant when compared to control.

The per cent growth reduction of all the isolates of pathogen ranged from 37.5 to 41.3.

The sclerotial number was more in tomato isolates (449.7 and 443.3, in the presence of *Trichoderma viridae* and *T. harzianum,* respectively). In groundnut isolate it was 107.7 and 104.0, whereas in crossandra isolate it was 66.0 and 64.0.

Table 8 : *Growth and sclerotial production of the isolates of *S rolfsii* in presence of fungal antagonists

Fungal antagonists	Isolates								
	Crossandra			Groundnut			Tomato		
	Mycelial growth (mm)	Per cent inhibition over control (%)	Sclerotial number	Mycelial growth (mm)	Per cent inhibition over control (%)	Sclerotial number	Mycelial growth (mm)	Per cent inhibition over control (%)	Sclerotial number
Trichoderma viride	53.7	39.7 (39.05)	66.0	52.0	40.9 (39.76)	107.7	53.3	38.7 (38.44)	449.7
T. harzianum	52.6	40.80 (39.7)	64.0	51.66	41.3 (39.97)	104.0	54.66	37.5 (37.780	443.3
Control	89.0		92.3	88		151.67	87.0		593.3

*Mean of three replications
Figures in parantheses are angular transformed values

		SEM at 5%	CD at 5%
Isolates	:	0.42	1.31
Antagonists	:	0.34	1.07
Isolates x Antagonists	:	0.59	1.85

Fig. 8 : Growth and sclerotial production of the isolates of *S. rolfsii* in presence of fungal antagonists

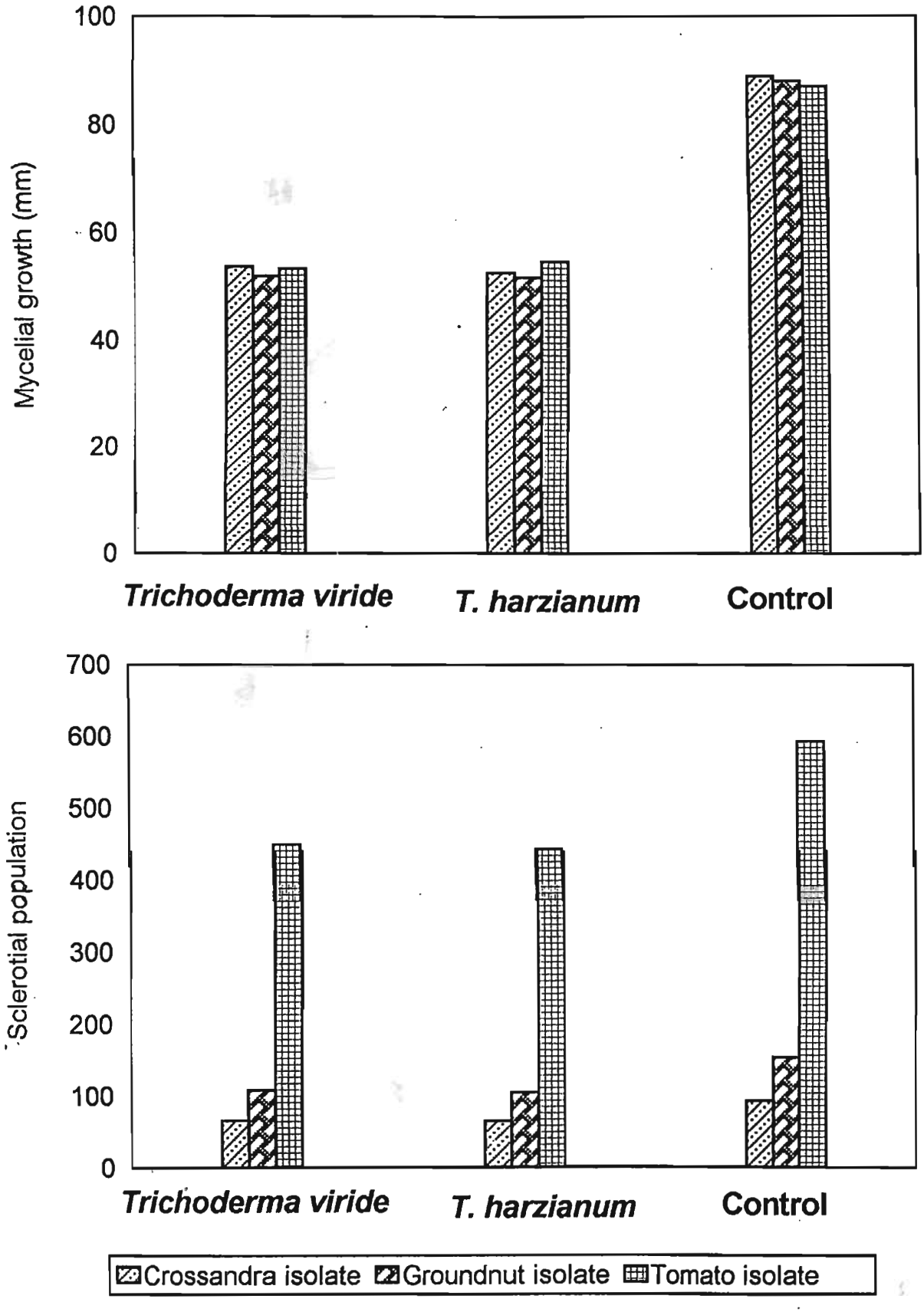
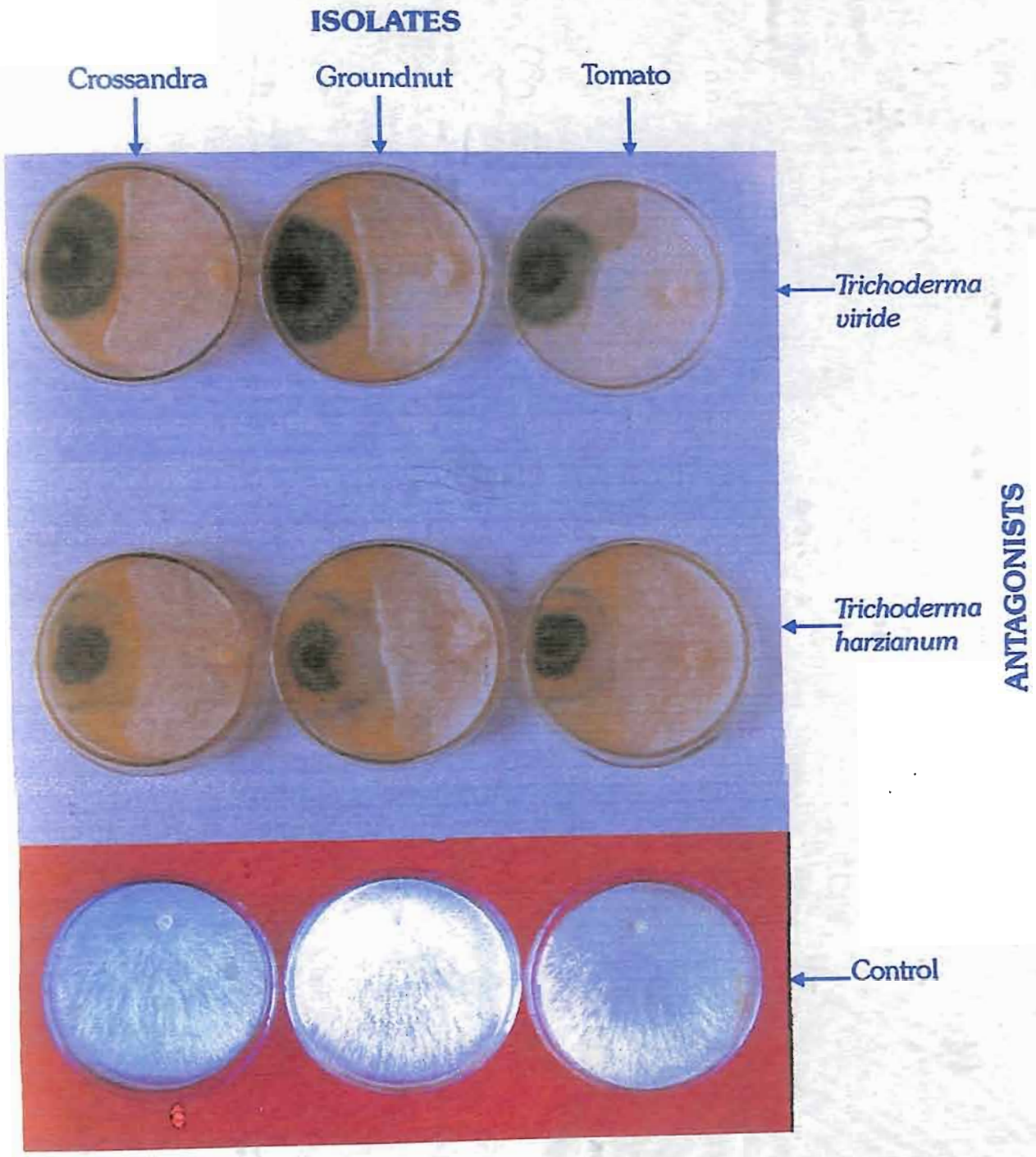


Plate 8 : Growth of *S.rolfsii* isolates in presence of antagonists



4.4 PATHOGENIC VARIABILITY AMONG THE ISOLATES OF *S. rolfsii*

The pathogenic potential of each isolate of *S. rolfsii* was tested on three hosts viz., crossandra, groundnut and tomato in pot culture.

The data (Table 9 and Fig. 9) revealed that there was no significant difference between isolates and hosts except their interaction.

All the isolates of the pathogen were equally pathogenic on all the host plants tested and all the host plants were equally susceptible to the isolates of the pathogen.

When the virulence of each isolate on different host plants was compared, crossandra isolate exhibited maximum incidence of disease on crossandra host (40.0%) followed by groundnut host (36.7%) and tomato host (33.3%) which are on par with each other.

Groundnut isolate recorded maximum per cent of disease incidence on crossandra plants (33.3%) followed by groundnut host (30.0%) and tomato host (26.7%) which are also on par with each other where as tomato isolate caused maximum incidence of disease (50.0%) on tomato plants which was significantly superior to disease incidence on other two host plants. Crossandra and groundnut plants showed 30.0 and 26.7 per cent incidence of disease which are at par.

Table : Pathogenicity of *S. rolfsii* isolates on different host plants

Isolates	Infected plants (%)*			
	Hosts			
	Crossandra	Groundnut	Tomato	Mean
Crossandra	40.00 (39.15)	36.70 (37.14)	33.30 (35.22)	36.66 (37.28)
Groundnut	33.3 (35.22)	30.0 (33.00)	26.7 (30.99)	30.0 (33.07)
Tomato	30.0 (33.00)	26.7 (30.99)	50.0 (45.0)	35.56 (36.33)
Mean	34.43 (35.78)	31.13 (33.71)	36.66 (37.07)	

*Mean of three replications

Figures in parantheses are angular transformed values

		SEM at 5%	CD at 5%
Isolates	=	1.74	5.23
Hosts	=	1.74	5.23
Isolates x Hosts	=	3.02	9.05

Fig. 9 : Pathogenicity of *S. rolfsii* isolates on different host plants

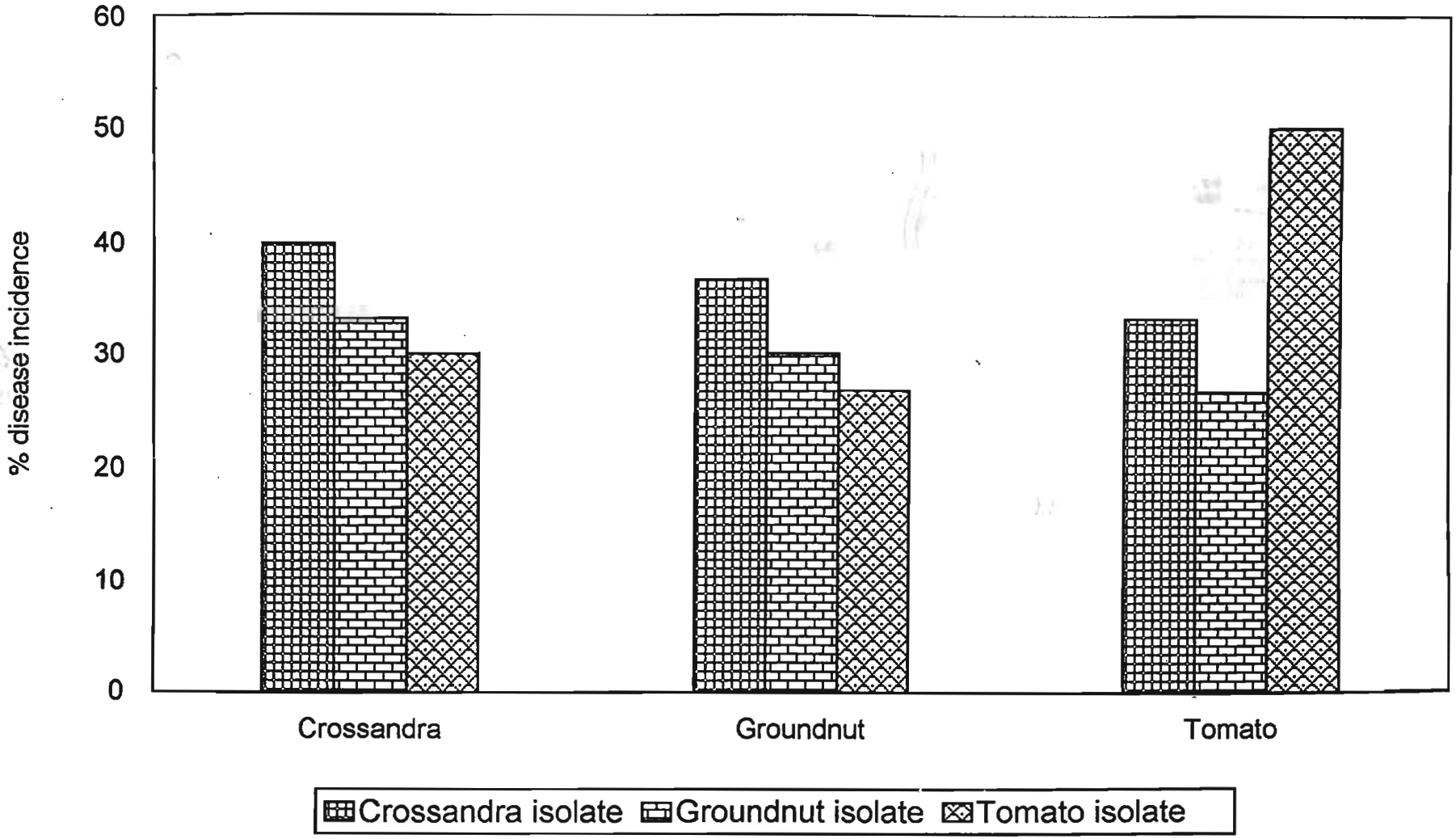
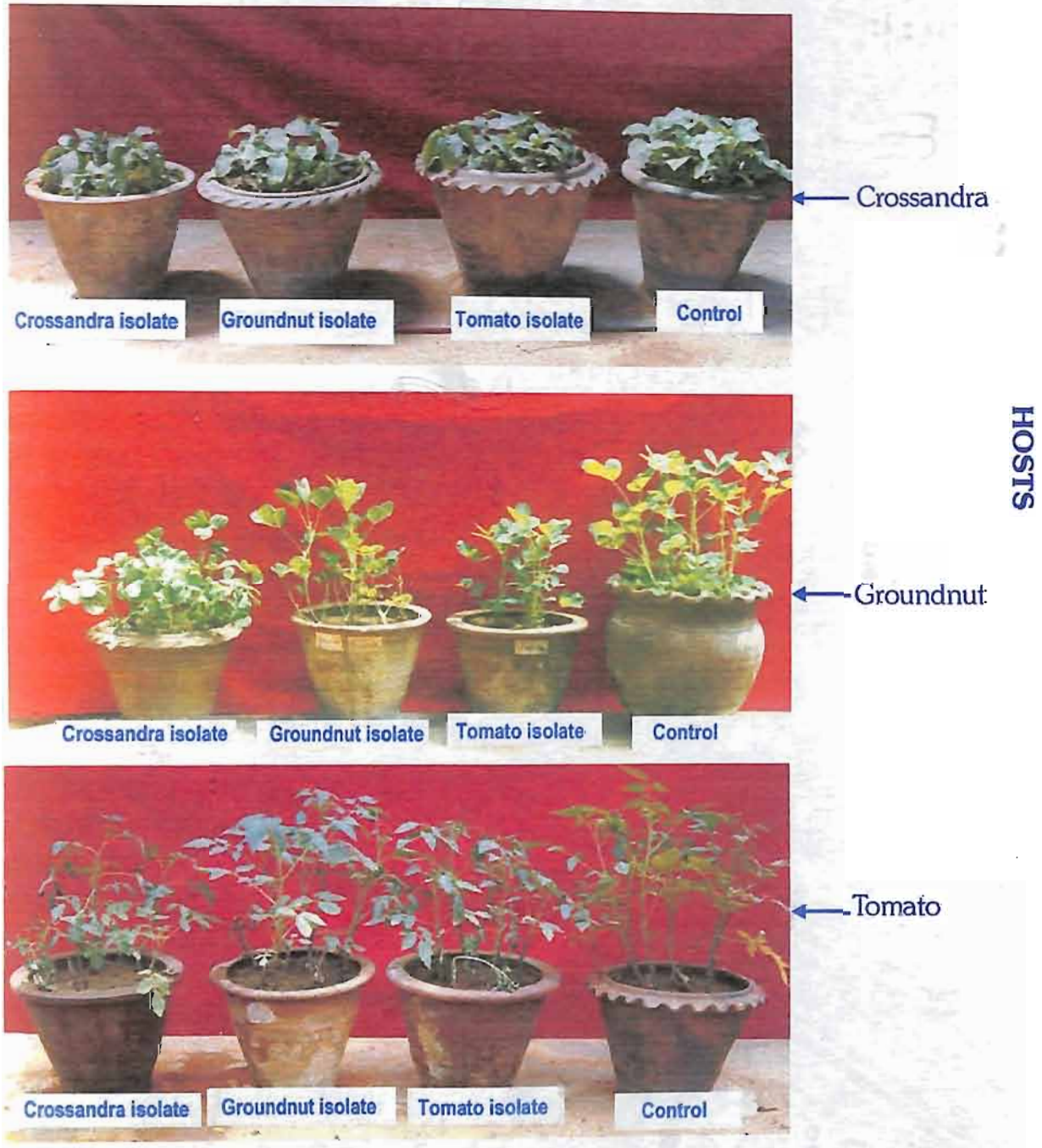


Plate 9 : Pathogenicity and virulence of *S.rolfsii* isolates on different host plants



In general crossandra isolate was found to be more virulent on all the host plants tested, followed by tomato and groundnut isolates but all on par with each other.

Crossandra and tomato isolates of the pathogen produced maximum disease incidence on their respective hosts whereas groundnut isolate produced more incidence of disease on crossandra host.

4.5 ELABORATION OF TOXINS BY *S. rolfsii* ISOLATES

The per cent inhibition of shoot and root length of crossandra, groundnut and tomato in the presence of toxin elaborated by each isolate was studied.

The results indicated that the inhibitory effect of toxins elaborated by all the three isolates is more on root length than on shoot length.

4.5.1 Shoot length inhibition

The data (Table 10 and Fig. 10) indicated that toxins of all the three isolates significantly inhibited the shoot length of crossandra, groundnut and tomato.

Among the hosts, the mean per cent inhibition of shoot length was maximum in crossandra (34.29%) and tomato (34.86%) which are significantly more than shoot length inhibition in groundnut seedlings (17.49%).

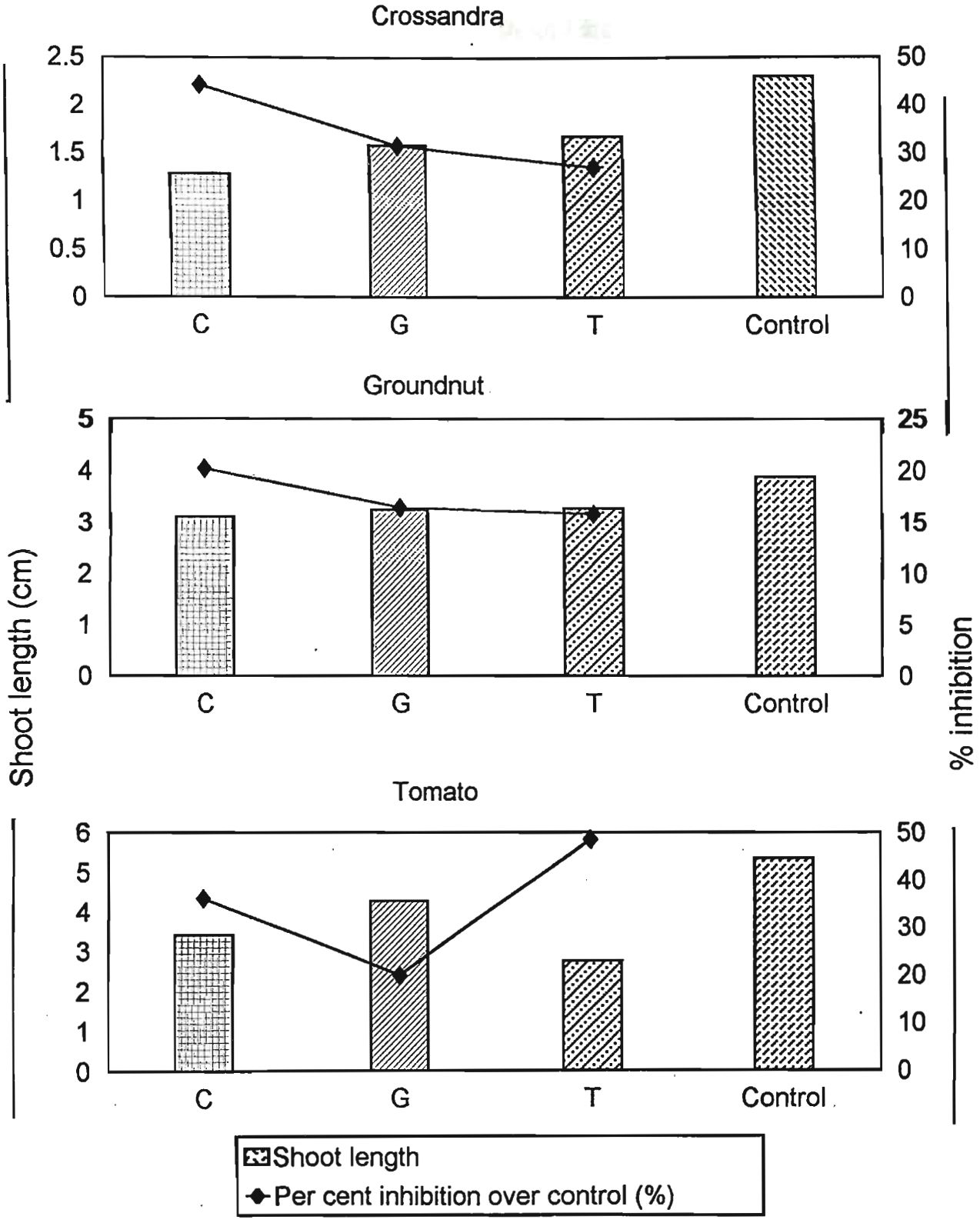
Table 10 : Toxicogenic effect of *S. rolfsii* isolates on *shoot growth of different host plants

Isolates	Hosts						
	Crossandra		Groundnut		Tomato		Mean % inhibition
	Shoot length (cm)	Per cent inhibition over control (%)	Shoot length (cm)	Per cent inhibition over control (%)	Shoot length (cm)	Per cent inhibition over control (%)	
Crossandra	1.28	44.23 (41.63)	3.10	20.2 (26.68)	3.41	36.2 (37.0)	33.54 (35.10)
Groundnut	1.58	31.53 (34.16)	3.25	16.46 (23.89)	4.28	19.9 (26.48)	22.63 (28.18)
Tomato	1.68	27.1 (31.31)	3.27	15.8 (23.29)	2.75	48.5 (44.14)	30.46 (32.92)
Control	2.30	-	3.89	-	5.35	-	
Mean % inhibition		34.29 (35.7)		17.49 (24.62)		34.86 (35.88)	

*Mean of three replications
Figures in parantheses are angular transformed values

		SEM at 5%	CD at 5%
Isolates	:	0.72	2.16
Hosts	:	0.72	2.16
Isolates x Hosts	:	1.25	3.74

Fig. 10 : Toxicogenic effect of *S. rolfsii* isolates on shoot growth of different host plants



C = Crossandra isolate
G = Groundnut isolate
T = Tomato isolate

In general, maximum inhibition of shoot length of all the hosts was observed in the presence of toxin of crossandra isolate (33.54%) followed by tomato isolate (30.46%) which are at par while groundnut isolate significantly recorded minimum shoot length inhibition (22.63%).

While comparing the effect of toxins elaborated by each isolate of the pathogen on shoot length of different hosts, crossandra isolate recorded maximum per cent inhibition of shoot length on crossandra in the presence of toxin (44.23%) followed by on tomato (36.2%) which are at par and significantly superior to shoot length reduction in groundnut host (20.2%).

Among the other two isolates, toxin elaborated by groundnut isolate caused significant reduction (31.53%) in shoot length of crossandra. The shoot length reduction in groundnut (16.46%) and tomato (19.9%) was on par with each other.

The toxin elaborated by tomato isolate recorded significant reduction in shoot length of all the seedlings. Maximum reduction was recorded in tomato (48.5%) followed by crossandra (27.1%) and groundnut (15.8%).

Plate 10 : Inhibition of shoot and root length of host plants by *S.rolfsii* isolates

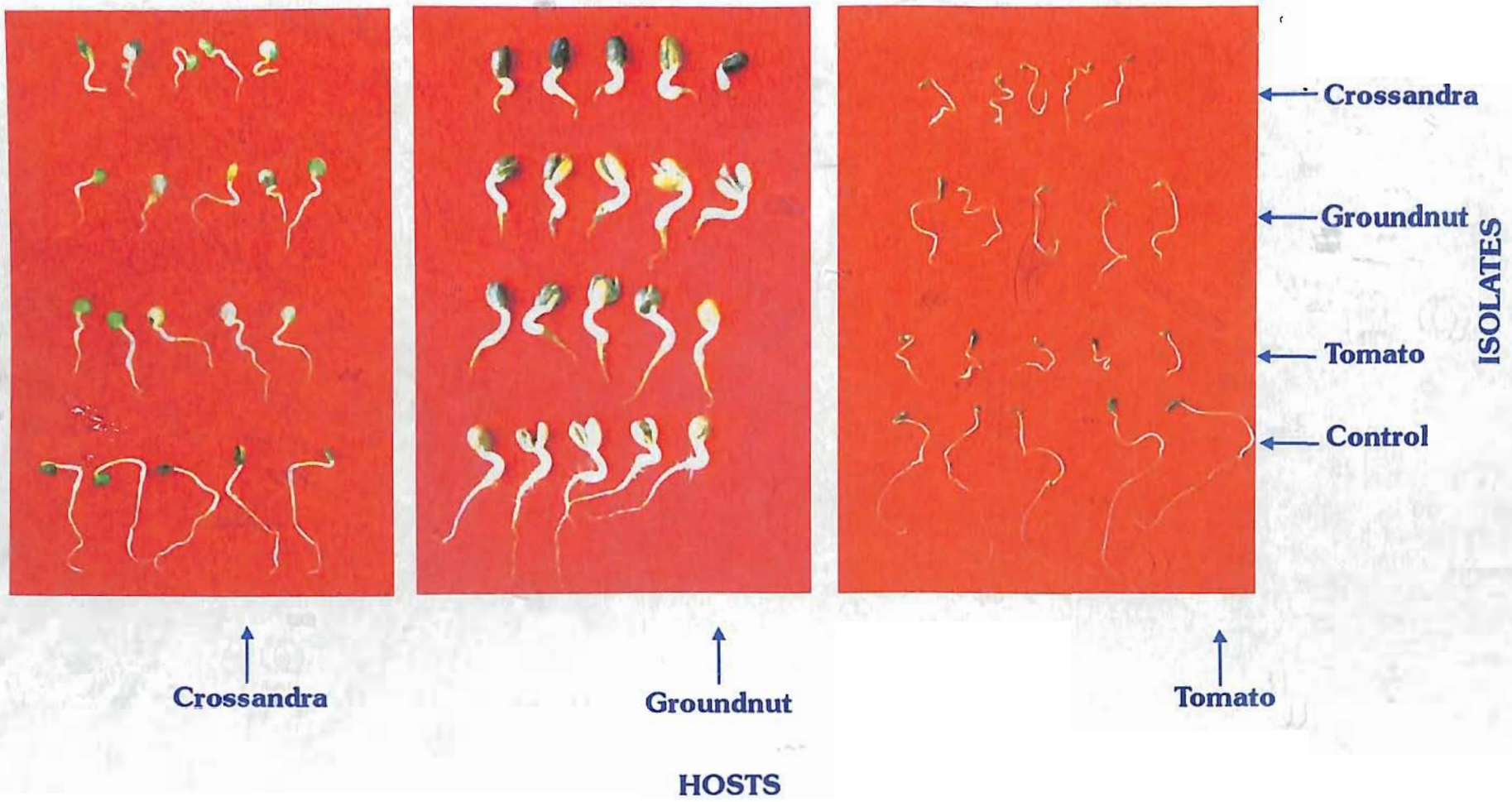


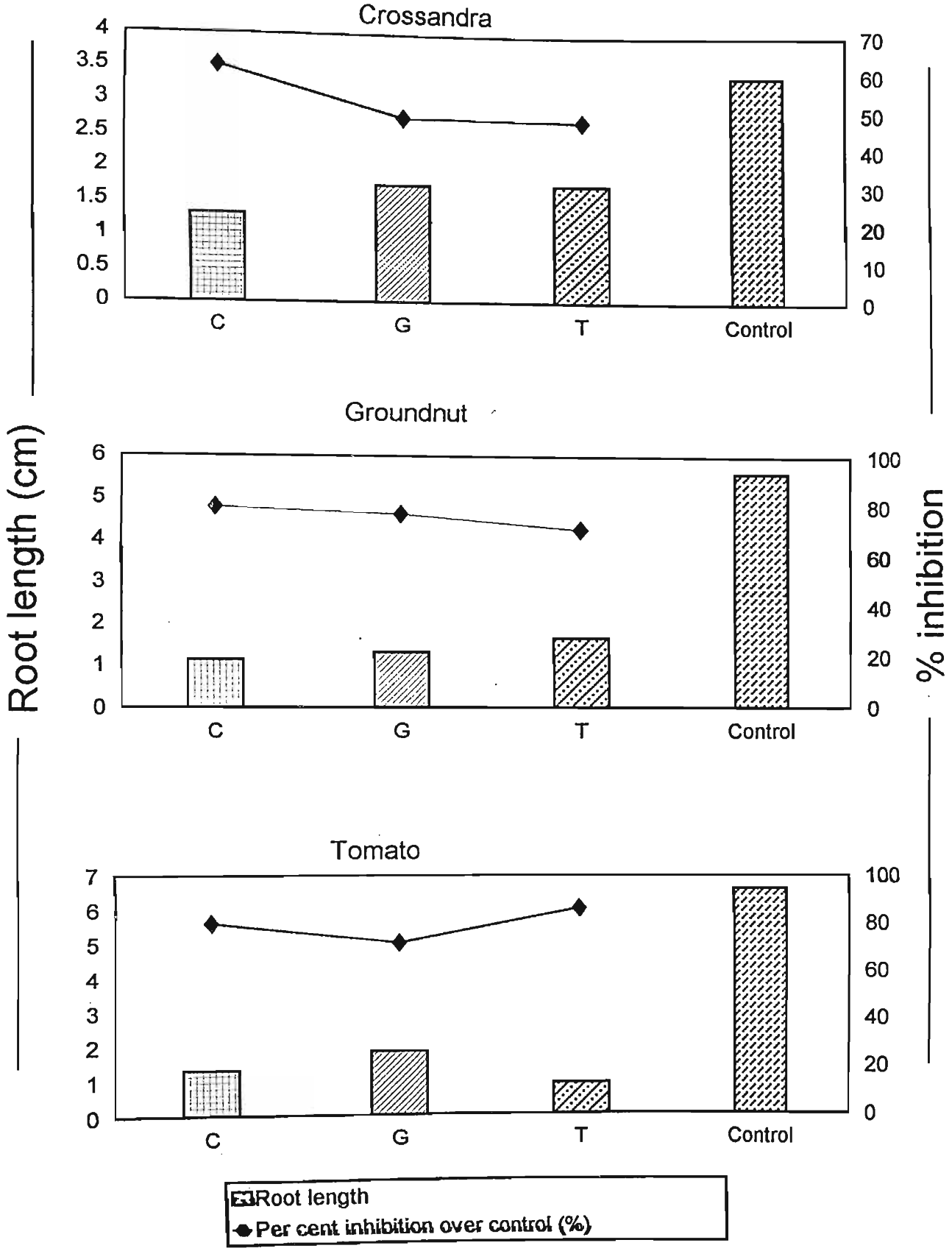
Table 11 : Toxicogenic effect of *S. rolfsii* isolates on *root growth of different host plants

Isolates	Hosts						
	Crossandra		Groundnut		Tomato		Mean % inhibition
	Root length (cm)	Per cent inhibition over control (%)	Root length (cm)	Per cent inhibition over control (%)	Root length (cm)	Per cent inhibition over control (%)	
Crossandra	1.31	61.60 (51.71)	1.13	79.73 (63.27)	1.32	80.06 (63.49)	73.79 (59.49)
Groundnut	1.75	48.43 (44.10)	1.30	76.8 (61.22)	1.85	72.10 (58.12)	65.78 (54.48)
Tomato	1.77	47.86 (43.78)	1.64	70.7 (57.23)	0.90	86.4 (68.37)	68.32 (56.46)
Control	3.40		5.60		6.62		
Mean % inhibition		52.63 (46.53)		75.74 (60.57)		79.52 (63.33)	

*Mean of three replications
Figures in parantheses are angular transformed values

		SEM at 5%	CD at 5%
Isolates	:	0.28	0.83
Hosts	:	0.28	0.83
Isolates x Hosts	:	0.48	1.44

Fig. 11 : Toxicogenic effect of *S. rolfsii* isolates on root growth of different host plants



C = Crossandra isolate
G = Groundnut isolate
T = Tomato isolate

4.5.2 Root length inhibition

The data (Table 11 and Fig.11) indicated that among the hosts, the mean per cent inhibition of root length was maximum in tomato (79.52%) followed by groundnut (75.74%) and crossandra (52.63%).

Among the isolates, toxin elaborated by crossandra isolate was found to cause significantly maximum reduction in root length (73.79%) followed by tomato (68.32%) and groundnut (65.78%) isolates.

The effect of toxin elaborated by each isolate on different hosts revealed that crossandra isolate caused maximum reduction in root length of tomato (80.06%) and groundnut (79.73%) which are at par but significantly more than the root length reduction in crossandra host (61.6%).

The toxin elaborated by groundnut isolate showed significant reduction in root length of all the hosts. Maximum reduction was observed in groundnut (76.8%) while minimum on crossandra seedlings (48.43%).

Tomato isolate recorded significant maximum reduction in root length of tomato seedlings (86.4%) in the presence of its toxin followed by groundnut seedling (70.71%) while the least reduction was observed in crossandra seedlings (47.86%).

4.6 PROTEIN AND ISOZYME BANDING PATTERN OF *S. rolfsii* ISOLATES

The three isolates of *S. rolfsii* were subjected to variability studies involving proteins and esterase enzyme analysis in PAGE.

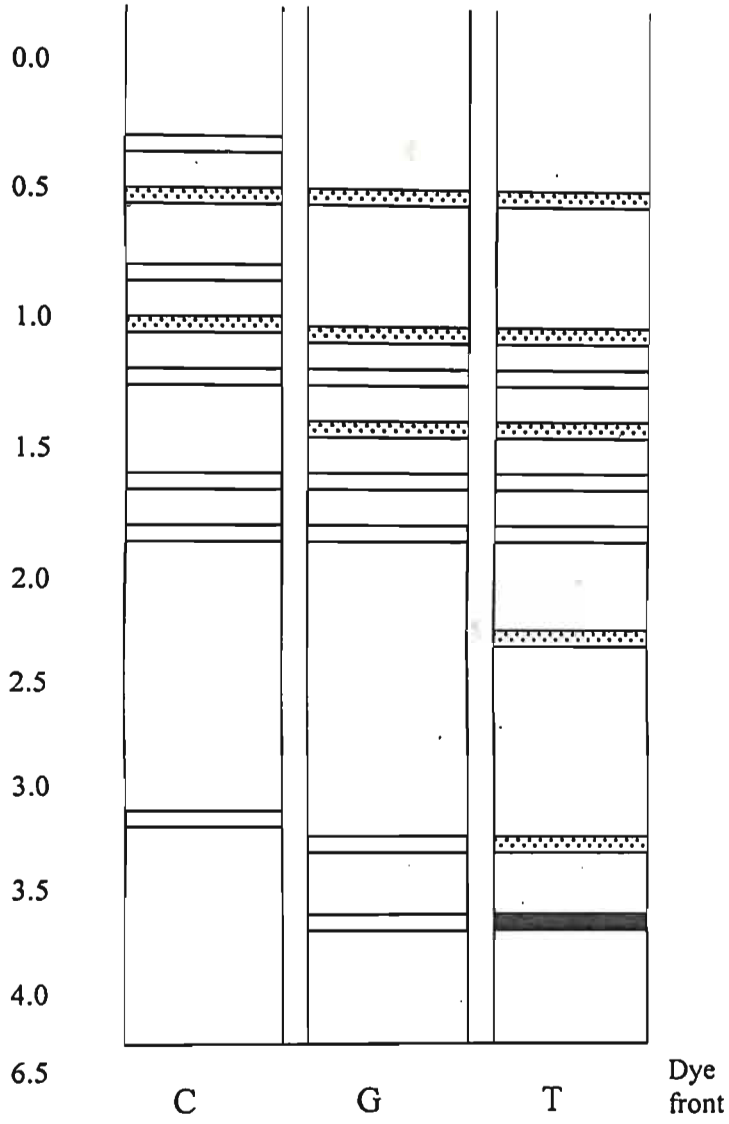
4.6.1 Protein profiles

The protein banding patterns of three isolates of *S. rolfsii* is presented in the form of photograph (Plate 11) and zymogram (Fig.12). The differences in proteins among the isolates were expressed in terms of relative mobility (Rm) values of bands at different loci (Table 12).

The results revealed that there was a considerable difference among the isolates in banding loci of proteins.

Crossandra and groundnut isolates exhibited protein pattern with eight banding loci each and tomato isolate with nine banding loci.

Figure 12. **Zymogram of protein banding patterns of *S. rolfsii* isolates**



□ Light ▨ Medium ■ Dark

C Crossandra isolate

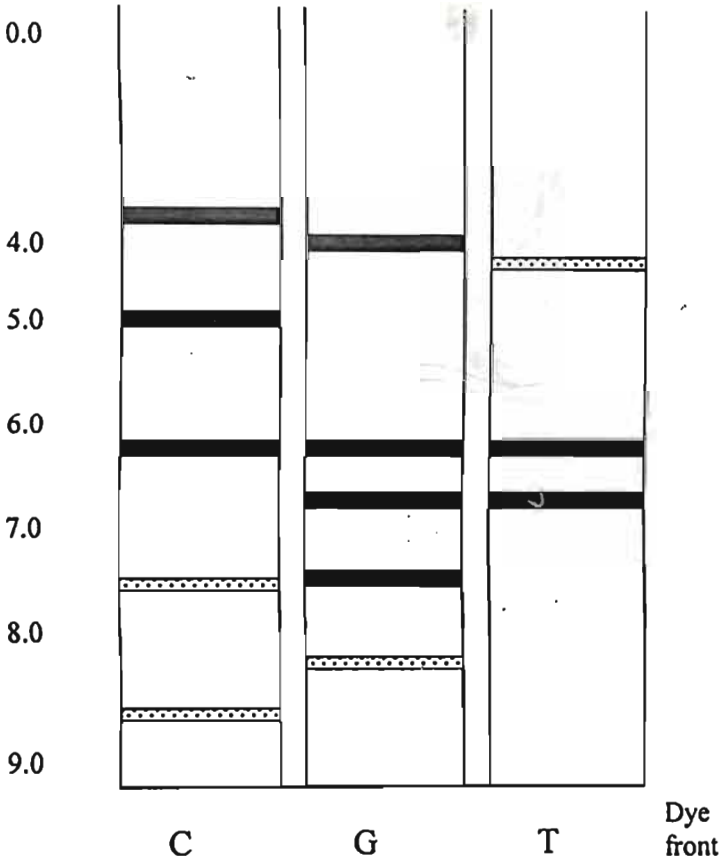
G Groundnut isolate

T Tomato isolate

Table 12 : Relative mobility (Rm) values of protein banding patterns of *S. rolfsii* isolates

C	G	T
0.06	—	—
0.09	0.09	0.09
0.14	—	—
0.17	0.17	0.17
0.2	0.2	0.2
—	0.23	0.23
0.26	0.26	0.26
0.29	0.29	0.29
—	—	0.37
0.51	—	—
—	0.54	0.54
—	0.6	0.6

Figure 13 : Zymogram of esterase isozyme banding pattern of *S. rolfsii* isolates



Medium ■ Dark

C Crossandra isolate

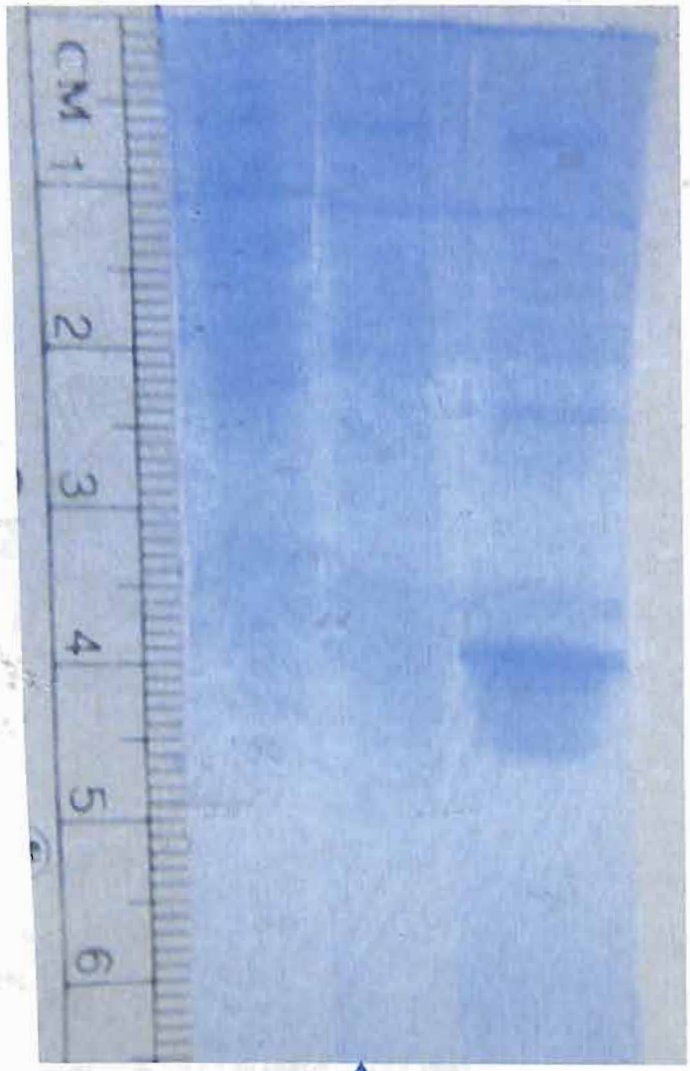
G Groundnut isolate

T Tomato isolate

Table 13 : Relative mobility (Rm) values of esterase isozyme banding pattern of *S. rolfsii* isolates

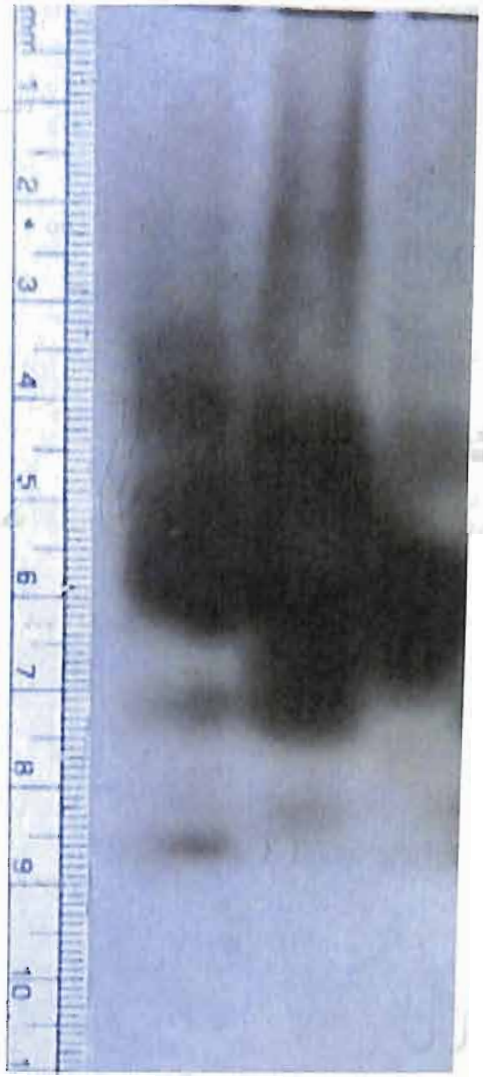
C	G	T
0.35	—	—
—	0.36	0.36
0.44	—	—
0.55	0.55	0.55
—	0.59	0.59
0.65	0.65	—
—	0.75	—
0.77	—	—

Plate 11 : SDS PAGE analysis of total fungal proteins in three isolates of *S.rolfsii*



Lanes from L-R represent Crossandra Groundnut and Tomato isolates respectively

Plate 12 : Esterase isozyme profiles of three isolates of *S.rolfsii*



Lanes from L-R represent Crossandra Groundnut and Tomato isolates respectively

All the three isolates shared five bands with Rm values of 0.09, 0.17, 0.2, 0.26 and 0.29. Three bands with Rm values of 0.54, 0.6 and 0.23 were common for groundnut and tomato isolates.

Crossandra isolate was found to be unique in having three additional bands (Rm 0.06, 0.14 and 0.51) that were absent in other two isolates. Crossandra isolate also lacked two bands (Rm 0.23, 0.45 and 0.6). Tomato isolate was found to be unique in having one band with Rm value of 0.37 which was absent in other two isolates.

4.6.2 Esterase enzyme profiles

The esterase isozyme banding pattern of three isolates of *S. rolfsii* is presented in the form of photograph (Plate 12) and zymogram (Fig.13). The difference in this enzyme among the isolates was expressed in terms of relative mobility (Rm) values of bands at different loci (Table 13).

Considerable variation among the isolates in banding loci of esterase enzyme was noticed.

Crossandra and groundnut isolates exhibited five banding loci each and tomato isolate with three banding loci.

All the three isolates shared only one band with Rm value of 0.55. Two bands with Rm values of 0.36 and 0.59 were common for groundnut and tomato isolates. A band with Rm value of 0.65 was common for crossandra and groundnut isolate.

Crossandra isolate was found to be unique in having three bands (Rm values, 0.35, 0.44 and 0.77) that were absent in groundnut and tomato isolates. A band with Rm value of 0.75 was found to be specific to groundnut isolate only.

Chapter V

Discussion

CHAPTER – V

DISCUSSION

Sclerotium rolfsii Sacc. is a soil borne plant pathogen of worldwide importance with a host range of over 500 plant species including many dicots and few monocots. The fungus induces a variety of symptoms such as root rots, seedling blights, collar rots, stem rots, wilts etc in different host plants.

Cultures of *S. rolfsii* originating from different geographical areas and hosts exhibit variations in their morphological, cultural and pathological characters. In the words of Dr. E.C. Stakman plant diseases are "Shifty enemies". This could be the simplest expression for variability in fungi causing plant diseases. The knowledge of variability in fungi is not only important for understanding pathogenesis, present or future, it is equally important for the success of any breeding programme for development of varieties resistant to the diseases.

But the information available on variability among the isolates of *S. rolfsii* is limited, which prompted to carry out the detail investigations on

the morphology, physiology, pathogenicity and isozyme variability of the isolates obtained from different host plants.

In the present investigation, the isolates were drawn from three host plants viz., crossandra, groundnut and tomato and used throughout the studies. First occurrence of *S. rolfsii* on crossandra has been recently reported by Harinath Naidu (2000). The importance of crossandra in this area and recent occurrence of *S. rolfsii* on this host that caused huge losses, also prompted to carry out preliminary work on this isolate in comparison with other equally potential isolates.

The results obtained from the present investigation given in the preceding chapter are discussed here under.

5.1 VARIABILITY STUDIES AMONG THE ISOLATES OF *S. rolfsii* ON SOLID MEDIA

Fungi respond differently to nutritional factors which to a great extent depends on function of the substrate.

In the present study, among different solid media, PDA supported maximum growth and sclerotial population in all the three isolates of *S. rolfsii* followed by OMA and CDA media. Similar results were obtained by several workers. Sharma and Kaushal (1979) reported that PDA

supported maximum growth and highest number of sclerotia in sunflower isolate of *S. rolfsii*. PDA supported maximum growth and sclerotial population in groundnut isolate of *S. rolfsii* (Hari *et al.*, 1991).

Crossandra and groundnut isolates produced less number but bigger size sclerotia whereas tomato isolate produced highest number of small sclerotia which is in agreement with the findings of dela-Cueva and Natural (1994). In the present study, the time taken for sclerotial formation was directly related to the size of sclerotia produced by the isolates. Crossandra isolate took more time for sclerotial formation when compared to other two isolates, may be due to the formation of bigger sclerotia.

5.2 GROWTH OF *S. rolfsii* ISOLATES IN DIFFERENT LIQUID MEDIA

Among the liquid media, Richard's medium supported maximum growth (mycelial dry weight) in all the three isolates followed by potato broth, groundnut host extract, crossandra host extract, tomato host extract and coon's medium.

Crossandra isolate recorded maximum dry weight when compared to other two isolates. Byadgi and Hegde (1985) reported that the most virulent bean isolate of *Macrophomina phaseolina* produced the maximum mycelial dry weight. Similar analogy can be drawn to crossandra isolate of present

investigation which was found to be more virulent as adjudged by toxicogenicity and virulence studies.

Mishra and Haque (1962) and Mathur and Sarbhoy (1976) reported that the Richard's medium supported maximum mycelial dry weight of the isolates of *S. rolfsii.*

In the present study, coon's medium was found to be the least in supporting the growth in all the three isolates. On the contrary, Sharma and Kaushal (1979) found that coon's medium supported maximum growth in sunflower isolate of *S. rolfsii.*

5.3 UTILIZATION OF DIFFERENT CARBON SOURCES BY THE ISOLATES OF *S. rolfsii.*

Studies on carbon utilization by the isolates of the pathogen indicated its versatility in utilizing a wide range of carbon sources used.

Among carbon sources, sucrose, maltose and mannitol supported maximum growth and sclerotial population in all the three isolates followed by glucose and fructose. The results are in agreement to those of Ramakrishnan (1930), Mishra and Haque (1962), Mathur and Sarbhoy (1976) and Zoberi (1980) who reported that sucrose and maltose as carbon

sources supported good growth and sclerotial production of the isolates of *S. rolfsii.*

In general, most of the fungi are unable to utilize sugar alcohols as efficiently as corresponding sugars. Mathur and Sarbhoy (1976) and Sharma and Kaushal (1979) found mannitol as a poor carbon source for the growth and sclerotia formation of sugar beet and sunflower isolates of *S. rolfsii,* respectively. Conversely, in the present study all the three isolates exhibited good growth and sclerotial population in the presence of mannitol.

In the presence of glucose all the three isolates recorded moderate growth and poor sclerotial formation. Hadar *et al.* (1983) suggested that the potential to produce sclerotial initials was affected by the presence of glucose in the culture medium. The cultures were capable of producing sclerotial initials when glucose was exhausted and when fungal biomass reached its maximum level. Hashiba and Ishikawa (1978) found that adenosine 3-5 cyclic mono-phosphate (cAMP) plays a role in inducing sclerotial formation in *Rhizoctonia solani.* Both sclerotial differentiation and cAMP level in the mycelium were affected by glucose. The level of cAMP sharply increased upon glucose depletion and decreased upon addition of glucose. In the present investigation the failure of isolates to

produce more scleortia might also be due to decreased level of cAMP in the presence of glucose.

In the present studies fructose supported poor growth and sclerotial production in all the three isolates. Similar results were obtained by Mathur and Sarbhoy (1976).

The formation of sclerotia required an increased supply of energy which was mainly provided by glyoxalate pathway (Kritzman *et al.* 1976). In the absence of carbon, the isolates failed to form sclerotia in the present study, which clearly indicated that due to lack of carbon source there was no supply of energy and hence sclerotia were not formed on basal medium lacking carbon source. The results obtained from the above study revealed that the isolates of the pathogen prefer complex sources (disaccharides and sugar alcohol) rather than readily available carbon sources (monosaccharides).

5.4 UTILIZATION OF DIFFERENT NITROGEN SOURCES BY THE ISOLATES OF *S. rolfsii*

Fungi utilize nitrogen for functional as well as structural purposes, some fungi are very specific in their nitrogen requirements, while others

are not. Every nitrogen source used in the culture medium is transformed in to a mixture of nitrogen complex during the growth of the fungus.

Studies on the utilization of various nitrogen sources by the isolates of *S. rolfsii* revealed that nitrate and ammonical sources were utilized very well for growth and sclerotial formation. Sodium nitrate, calcium nitrate, ammonium sulphate and potassium nitrate were found to support maximum growth and excellent sclerotial population in all the isolates of *S. rolfsii.* Poor growth and no sclerotia were observed in the absence of any nitrogen source in the medium. Sodium nitrite as a nitrogen source was found to be detrimental to the growth and sclerotial production in all the isolates.

The results are in accordance with the findings of Mathur (1977), Zoberi (1990), Hari *et al.* (1991), Yu *et al.* (1996) with different isolates of *S. rolfsii.*

The inability of the fungus to grow on nitrite source of nitrogen may be due to the toxicity of undissociated nitrous acid occurring in nitrite medium under acidic condition (Cochrane, 1958). In the present investigation, all cultural studies were carried out a pH of 6.0

Davis (1956) attributed the non-utilization of nitrite form of nitrogen to the lack of specific carrier enzyme systems required to transport organic as well as inorganic solutes (ions) across the living cell membrane. More over, the non-utilization of nitrite nitrogen may be ascribed to the lack of enzyme nitrite reductase.

5.5 SENSITIVITY OF *S. rolfsii* ISOLATES TO ANTAGONISTS

Bio-control through the use of resident and introduced antagonists has of late gained importance and is poised for successful application in modern agriculture (Cook, 1985).

Management of *S. rolfsii* a major soil borne plant pathogen, through application of fungicides in soil has been proved to be an enigma, as its broad host range and almost world wide distribution precludes such strategy. Biological control has been proved to be a promising disease management technology especially, against soil borne plant pathogens.

Trichoderma spp. have received considerable attention as possible bio-control agents of *S. rolfsii*. *In vitro* antagonism of *T. harzianum* against *S. rolfsii* has been reported long back (Wells *et al.*, 1972, Chet *et al.*, 1978).

In the present investigation, an attempt was made to know whether *S. rolfsii* isolates differ in their sensitivity to *T. viride* and *T. harzianum*. The results revealed that all the three isolates of the pathogen were equally sensitive to the antagonists tested. There were no significant difference between the antagonists in reducing mycelial growth and sclerotial population of the isolates. However, greater reduction in number of sclerotia of crossandra isolate in the presence of antagonists can suggest its greater sensitivity to the antagonists tested.

Mathur and Sarbhoy (1978) and Singh and Dwivedi (1987) found that *T. viride* and *T. harzianum* inhibited the mycelial growth of *S. rolfsii* isolates by 88 and 86 per cent respectively, in dual culture. But in the present investigation, it was found that both the antagonists reduced the growth upto 40 per cent. This difference may be due to difference in strains of antagonists or difference in isolates of the pathogen. It is well known that all the isolates of *T. viride* and *T. harzianum* are not equally antagonistic toward a species of pathogen.

Henis *et al.* (1983) and Suseelendra and Schlosser (1999) found that the isolates of *Trichoderma* differed in their ability to infect, macerate and

kill the sclerotia of *S. rolfsii*. In the present investigation, lysis of the pathogen with antagonists was observed.

5.6 VARIABILITY AMONG ISOLATES OF *S. rolfsii* IN PATHOGENICITY AND VIRULENCE

Sclerotium rolfsii has a very wide host range of plants comprising more than 500 species. The isolates of *S. rolfsii* differed in their virulence from same host (Kim, 1974 ; Aken and Dashiell, 1991) as well from various hosts (Cooper, 1961 and Punja, 1985). In the present studies a cross inoculation study was carried out to determine the pathogenic potential of *S. rolfsii* isolates, if any, that could be useful in supporting the morphological, cultural and physiological variability.

The results revealed that all three isolates of *S. rolfsii* established pathogenicity in all the host plants tested but they slightly differed in per cent disease incidence of different host plants.

Harinath Naidu (2000) reported that crossandra isolate of *S. rolfsii* was pathogenic to tomato and chillies. dela - Cueva and Natural (1994) found that the isolates of cowpea and soybean were pathogenic to peanut and an isolate from cotton to wheat. A strong saprophytic ability and a wide array of enzymes produced might be the reason for wide host - range

of *S. rolfsii* (Punja, 1985). On the contrary, Anahosur (2001) reported that potato isolate of *S. rolfsii* did not infect radish and egg plant. The isolate also showed different degrees of virulence on a variety of host plants tested.

5.7 ELABORATION OF TOXINS BY *S. rolfsii* ISOLATES

In the present study, culture filtrates of isolates of *S. rolfsii* differed in the degree of inhibiting the growth of root and shoot of the hosts tested. Culture filtrate of crossandra isolate was the most inhibitory followed by tomato isolate and groundnut isolate.

The variation in inhibiting shoot and root growth among the isolates may be due to the production of different amount of metabolites or other inhibitory substances in the culture filtrate.

It is well documented that in many plant diseases the host - pathogen interactions are associated with the synthesis of host invading substances like toxins, enzymes and other metabolites (Daly and Deverall, 1983 ; Collmer and Keen, 1986). *In vitro* synthesis of such substances and their action on shoot / root growth was also demonstrated (Chandrika *et al.*, 1984 ; Ali and Singh, 1992).

Agarwal *et al.* (1986) found that the culture filtrate of *S. rolfsii* caused 70% seedling mortality in lentil.

Not much work has been done on the elaboration of toxins by *S. rolfsii.* It is well known that *S. rolfsii* produces oxalic acid in culture filtrate (Punja, 1985 ; Ansari and Agnihotri, 2000) and some extra cellular enzymes like pectinase (Upadhyay and Mukhopadhyay, 1985) cellulase (Das Gupta, 1990) and Phosphatidases (Sellman, 1982) during pathogenisis. Besides tissue maceration and killing, whether these metabolites of *S. rolfsii* play a role in shoot and root length inhibition is not understood. Whether there are any non enzymatic metabolites in *S. rolfsii* that might play a role in shoot and root length inhibition is also not well established. However, in the present studies root and shoot inhibition by culture filtrates clearly indicates that *S. rolfsii* must be producing toxins and there was considerable variation among the isolates in the extent of toxin elaboration.

5.8 ISOZYME VARIABILITY AMONG THE ISOLATES OF *S. rolfsii*

Electrophoretic analysis of proteins and isoenzymes can be used as an adjunct to morphological, cultural and pathogenic variability of different isolates of a pathogen (Hall, 1967).

A considerable morphological, cultural, toxicogenic variation observed in the present investigation among the three isolates of *S. rolfsii* aroused interest to carryout protein and isozyme analysis as a supporting evidence for variability.

In the present investigation polymorphism was observed among the isolates both in protein and esterase activity. Crossandra and groundnut isolates exhibited protein pattern with eight bands each and tomato isolate with nine bands. Crossandra isolates was found unique in having three bands that lacked in other two isolate. Tomato isolate exhibited one extra band which was absent in other isolates.

In the esterase activity the number of bands ranged from three (tomato isolate) to five (crossandra and groundnut isolates) with one common band in all the isolates (Rm 0.55). These differences in protein and esterase activity indicates genetic variability among the isolates.

Protein and isozyme analysis on polyacrylamide gel electrophoresis provides a well established and efficient tool for revealing genetic variability in fungal populations (Micales *et al.*, 1986). When polymorphism is detected, it reflects directly to the genetic background of the isolates (Shaw, 1965).

The protein and esterase polymorphism observed in the present investigation among the three isolates could be traced back to their genetic variability. Several workers correlated proteins and isozyme banding pattern of the isolates to their pathogenic and virulence variation.

The isozyme banding pattern obtained in the isolates of *Drechslera graminea* from barley have been divided into two or more groups on the basis of their virulence analysis as well as protein and isozyme patterns (Gatti *et al.*, 1992)

Isozyme analysis of esterase and peroxidase, could be used to separate *Cryphonectria cubensis* isolates, differing in their virulence pattern (Alfenas *et al.*, 1984).

In the present studies, the three isolates did not differ much among themselves in virulence. However, the increased activity of proteins and esterase observed in crossandra isolate may in part reflect to its greater virulence when compared to other two isolates.

The hypothesis of Newton *et al.* (1985) that "the saprophytes are genetically more variable owing to their existence in much diverse habitats" supports the observed variation among *S. rolfsii* isolates in protein and esterase activity in the present investigation. The isolates in the present investigation were drawn from taxonomically distantly related plants from different areas. Sudhakar (1996) also reported that electrophoretic studies of proteins and esterases of *Rhizoctonia solani* expressed narrow genetic distances between the isolates obtained from geographically nearer locations and *vice-versa*.

Chapter VI

Summary

CHAPTER - VI

SUMMARY

Sclerotium rolfsii Sacc. is a serious soil borne plant pathogen of world wide occurrence and causing huge losses in about 500 host plants. In the present investigation, variability studies among three isolates of *S. rolfsii* obtained from crossandra, groundnut and tomato pertaining to their morphological, physiological and toxicogenic characters were carried out under laboratory condition. Variability in pathogenicity and virulence among the isolates was studied in pot culture experiment. Variability among the isolates in protein and esterase banding pattern was also studied using SDS-PAGE and native PAGE respectively. The results obtained in the present investigation are summarized here.

Among the solid media, PDA supported maximum growth (90.0mm) in all the three isolates followed by OMA (87.2 mm) and CDA (59.6 mm). Among the isolates, crossandra isolate recorded maximum colony diameter (84.2 mm) followed by groundnut isolate (76.6mm) and tomato isolate (76.0 mm).

Out of six liquid media tested, Richard's medium supported maximum growth (dry weight) of 532.6 mg, followed by potato broth

(461.1 mg), groundnut host extract medium (420.1 mg), whereas coon's medium supported the least growth (83.9 mg).

Crossandra isolate recorded maximum mycelial dry weight (387.6 mg) in liquid media. Tomato (336.9 mg) and groundnut isolates (260.6 mg) were next in order.

Among six carbon sources tested, sucrose, maltose and mannitol supported maximum growth (90.0 mm) and sclerotial production in all the isolates followed by glucose (81.2 mm). Fructose (46.4mm) was the least preferred carbon source by all the isolates. Crossandra isolate found to be superior in utilizing all carbon sources.

The three isolates did not differ significantly in utilizing the nitrogen sources tested. All the isolates exhibited maximum growth (73.0 mm) in the presence of sodium nitrate, calcium nitrate, ammonium sulphate and potassium nitrate. Except crossandra isolate, both groundnut and tomato isolates recorded excellent sclerotial population in the presence of above nitrogen sources.

The three isolates failed to produce any growth or sclerotia when medium was amended with sodium nitrite and the isolates also did not produce any sclerotia in the absence of nitrogen source.

The sclerotial number and size varied among the isolates throughout the cultural studies. Tomato isolate produced more but small sized (0.9-1.1mm) sclerotia whereas crossandra isolate produced very few but bigger (1.2-1.3 mm) sclerotia. Groundnut isolate appeared intermediary (1.1-1.2 mm) to these isolates.

In dual culture studies, all the three isolates of the pathogen were equally sensitive to the fungal antagonists tested viz., *Trichoderma viride* and *T. harzianum.* The per cent growth reduction of all the isolates of pathogen ranged from 37.5 to 41.3. The sclerotial number was also reduced considerably in the presence of fungal antagonists.

Pathogenicity and virulence studies of three isolates on different host plants showed that all the isolates of the pathogen were equally pathogenic on all the host plants tested and all the host plants were equally susceptible to the isolates of the pathogen. However, crossandra and tomato isolates caused maximum incidence of disease on their respective hosts (40.0% and 50.0% respectively) but groundnut isolate recorded maximum disease incidence on crossandra host (33.3%).

Toxin elaborated by crossandra isolate reduced the shoot and root growth of the hosts to the extent of 33.5 and 73.79 per cent, respectively,

followed by tomato isolate (30.46% and 68.32%) and groundnut isolate (22.63% and 65.78%).

Electrophoretic studies revealed that the isolates differed in their protein and esterase banding pattern.

SDS-PAGE profiles of proteins of three isolates revealed the presence of eight protein fractions in crossandra and groundnut isolate and nine in tomato isolate. Out of these, five were common to all the isolates. Crossandra isolate lacked three fractions with Rm value 0.23, 0.45 and 0.6.

Native PAGE analysis of esterase enzyme revealed the presence of five bands in crossandra and groundnut isolates and three in tomato isolate with different Rm values. All the three isolates shared only one band with Rm value 0.55. Bands with Rm values 0.35,0.44 and 0.77 were unique for crossandra isolate.

Recent occurrence of *S. rolfsii* on crossandra an important crop in Chittoor district of Andhra Pradesh, its greater virulence than other two isolates suggests more detailed study on this isolate that must evolve a useful management package.

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LITERATURE CITED

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***Original not seen**