HOST-PATHOGEN-ANTAGONIST INTERACTIONS IN RELATION TO BIOLOGICAL CONTROL OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS* IN *BRASSICA JUNCEA*

By

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CERTIFICATE I

This is to certify that this dissertation entitled "Host-pathogen-antagonist interactions in relation to biological control of Xanthomonas campestris pv. campestris in Brassica juncea" submitted for the degree of Ph.D., in the subject of Plant Pathology, of CCS Haryana Agricultural University, is a bonafide research work carried out by Indu Jalali (nee Kachru) under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the dissertation entitled "Host-pathogen-antagonist interactions in relation to biological control of Xanthomonas campestris pv. campestris in Brassica juncea" submitted by Mrs. Indu Jalali (nee Kachru) to CCS Haryana Agricultural University in partial fulfilment of the requirements for the degree of Ph.D., in the subject of Plant Pathology, has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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(INDU JALALI)

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CHAPTER I

INTRODUCTION

Oilseeds occupy a prime position in Indian economy since edible oils are an essential component of our diet. Since the establishment of Oilseed Commission in 1986, the oilseed production in the country has shown a progressive increase. However, the per capita availability of oils and fats in India (22 g/day) is far below the minimum requirement and also far below the current fat consumption of Western Countries (60 g/day), (Vles and Gottenbos, 1989).

Rapeseed-mustard cultivation is believed to have been in existence in India from times immemorial. References to mustard have been found in ancient Greek, Roman, Indian literature and Indian Ayurvedic scripts. The estimated area and production of rapeseed-mustard in India is about 5.7 million hectares and about 5.39 million tonnes, respectively (Anonymous, 1990). Average yield in India is around 942 kg/ha (Rao, 1990) whereas in many Western European Countries it ranges between 20-30 q/ha.The major rapeseed-mustard growing regions in India are Uttar Pradesh, Rajasthan, Madhya Pradesh, Punjab, Haryana and Assam. It requires a cool temperature for satisfactory growth and hence is mostly grown in temperate and warm temperate zones of Asia and Europe. In India, it is grown during rabi season from September/October to February/March and thus can be cultivated in wide range of agroclimatic conditions. It thrives best in light to heavy loamy soils of pH 7.

Considering all the major oilseed producing crops, the quantity lost, on world wide basis due to different diseases is 13.5 million tons/year - amounting to about 16 million US dollars. Black rot of crucifers caused by

Xanthomonas campestris pv. campestris (Pammel, 1895) Dowson 1939 occurs in crucifer growing areas throughout the world (Williams, 1980). The pathogen is seed borne and thus has contributed to its wide distribution. Occurrence of this disease has been reported from Brazil (Chaves, 1947), Canada (Conners and Savile, 1946), Germany (Klemm, 1938), Sweden (Loof and Appleqvist, 1972) and U.S. (Bain, 1952). In India Patel et al. (1949) first observed black rot symptoms in Brassica juncea under natural conditions. This disease appeared in severe form during 1972-73 in the State of Haryana (Satyavir et al., 1973; Gandhi and Parashar, 1977). It can be effectively controlled by using aureomycin (chlorotetracycline), captafol or copper oxychloride foliar spray (Aveling and Robbertse, 1990; Shah et al., 1985). However, application of antibiotics has its own limitations. Streptomycin resistant mutants have arisen in nature as a result of its continuous application (Schroth et al.,1974). Over and above indiscriminate and unjudicious use of bactericides which are mutagenic in both prokaryotes and eukaryotes will upset the biological balance that can lead to severe outbreak of disease(s). Application of excessive and non-specific chemicals destroys the ecological niche of biological bacterial flora on plant milieu, and thus alter the disease severity due to elimination of antagonist(s).

Oilseed crops have a low yield genetic potential unlike that of cereals. Therefore, the least expensive control measures, such as the use of host resistance,cultural and biological control find favour with growers to improve oilseed production. There is no information available on checking or minimizing black rot of crucifers through biological methods. In a few host pathogen combinations, antagonists have been successfully used to control bacterial plant diseases. The mode of action of these antagonists has been reported to be antibiosis, pH changes, competition, etc. In the present studies Brassica

juncea and Xanthomonas campestris pv. campestris were selected as a host-pathogen model to identify an effective antagonist present in the phylloplane and to find out the mode of action in effective disease control. The present investigations were conducted with the following objectives:

A. To study the phylloplane microflora of Brassica juncea and identification of antagonistic micro-organisms against black rot pathogen both in vitro and in vivo.

B. To study the mode of antagonism.

CHAPTER II

REVIEW OF LITERATURE

Black rot is an important world wide disease of crucifers, attacking all cultivated brassica, radish and numerous cruciferous weeds (Williams, 1980). Since Russell's publication in 1898, there has been a growing recognition of the seriousness of black rot on cruciferous crops. The pathogen Xanthomonas campestris pv. campestris (Pammel) Dowson colonizes the vascular system after gaining entry through the hydathodes at the leaf margins or through wounds (Sutton and Williams, 1970a). The pathogen and its extracellular polysaccharide (Xanthan) plug the xylem vessels, restricting the water flow resulting in characteristic V-shaped lesions originating from the margins of the leaves. It leads to blackening and rotting of vascular tissues and dwarfing of affected plants.

Satyavir et al. (1973) reported rotting in Brassica junea upto 60 per cent in certain varieties, due to an unidentified species of Xanthomonas. Patel et al. (1949), in a cross inoculation experiment with X. campestris, reported Brassica juncea as one of the hosts, however this could not be confirmed further (Mathur and Swarup, 1965). Severe outbreak of bacterial rot of raya in Haryana was reported to be caused by a strain of X. campestris (Gandhi and Parashar, 1977).

2.1 Phylloplane microflora

The aerial plant surface provides a habitat for epiphytic micro-organisms, many of which are capable of influencing the growth of pathogens (Blakeman and Fokkema, 1982). Bacteria, yeast and filamentous fungi may form resident population on leaves. Some of the earliest investigations of the phylloplane were carried out by Potter (1910) who demonstrated the presence of fungi and bacteria on Solanum and Helianthus leaves. Massey

and Hattersley (1929) reported that a saprophytic yellow bacterium was constantly associated with X. malvacearum infection in cotton and overgrew the pathogen when both were cultured together. The activity of both saprophytes and pathogen on leaves is dependent on the micro-climatological conditions at the plant surface as well as on the chemical environment (Blakeman, 1973). These microorganisms live on nutrients exuded by the leaves, on dust or pollen deposits and on insect excreta. Fokkema and Schippers (1986) isolated about 150 microorganisms per cm^2 leaf surface of wheat. The most commonly isolated Gram-negative bacteria from the leaf surface are Erwinia, Pseudomonas and Xanthomonas while gram-positive bacteria such as Lactobacillus and Bacillus are isolated less frequently.

Dickinson et al. (1975) reported Erwinia herbicola, Flavobacterium, Klebsiella aeroginosa, Micrococcus, Sarcina, Pseudomonas, Lactobacillus and Bacillus to be common on leaf surface of Lolium perenne. Occurrence of Flavobacterium, Lactobacillus, Pseudomonas, Aerobacter, Aeromonas and Micrococcus as non parasitic epiphytes has been amply demonstrated (Leben, 1965; Crosse, 1971; Klincare et al., 1971; Chowdhury and Verma, 1980; Verma et al., 1983). Erwinia sp., Bacillus sp. B. pumilus, B. brevis, Clavibacter sp. and Xanthomonas sp. have been isolated from various parts of cotton plants (Misaghi and Donndelinger, 1990). Although actinomycetes abound in the soil they have rarely been detected in the phyllosphere (Last and Warren, 1972).

2.2 Pathogens as epiphytes

Phytopathogenic bacteria have been established to live in a non-pathogenic epiphytic phase on the foliar surfaces (Blakeman and Bordie, 1976; Blakeman, 1982; Mew and Cruz, 1988).

Pseudomonas syringae pv. syringae has been reported as an epiphyte on a range of different host plants (Leben et al., 1970; Ercolani et al., 1974) both on susceptible and resistant bean lines (Daub and Hagedorn, 1981). Varvaro and Surico (1984) showed the presence of P. syringae pv. savastanoi on olive in resident phase.

Xanthomonas campestris pv. vesicatoria (Leben, 1963; McGuire et al., 1991), P. cepacia (Kawamoto and Lorbeer, 1972), Erwinia amylovora (Miller and Schroth, 1972), X. campestris pv. phaseoli (Cafati and Saettler, 1980; Ishimar et al., 1991), X. campestris pv. campestris (Kuan et al., 1986) and X. campestris pv. glycines (Groth and Braun, 1989) have been found in the resident phase of phylloplane microflora on their respective hosts. Cotton and cassava leaves have also been reported to harbour X. campestris pv. malvacearum (Chowdhury and Verma, 1980) and X. campestris pv. manihotis (Persley, 1978) as residents, respectively. Hirano and Upper (1983) presented a list of epiphytically growing plant pathogenic bacteria and suggested that epiphytism is a general rather than an exceptional phenomenon existing in nature.

2.3 Biological control

Biological control provides an important route to environmentally harmless plant protection (Mendgen et al., 1992). The use of non-pathogenic microorganisms to control plant diseases was demonstrated as early as 1910 by Potter. Control of crown gall with Agrobacterium radiobacter is the most notable example of an antagonistic bacterium preventing infection of phytopathogenic bacterium (Kerr, 1980; Vidaver, 1981). Strain 84 of A. radiobacter is used in Japan commercially against crown gall of roses (Makino and Marita, 1986). Adetuyi (1989) isolated a gram-negative bacterium

from rice seed-coat, which produced diffusible toxins inhibiting Pythium ultimum (damping off) and Pyricularia oryzae (rice blast). Pseudomonas solanacearum has been successfully suppressed by a bacterium designated as BC-8 to control potato wilt (Ciampi-Panno et al., 1989). P. fluorescens was antagonistic to X. campestris pv. citri (Unnamalai and Gnanamanickam, 1984; Sakthivel et al., 1988) and its strain CHAO was found to protect plants from diseases caused by various soil borne fungi (Haas et al., 1991). Pseudomonas sp. obtained from rice showed a broad spectrum antibacterial activity against Clavibacter michiganensis pv. sepedonicus, C. michiganensis pv. michiganensis and X. campestris pv. citri (Hirayae and Wakimoto, 1987). Pseudomonas putida strain (PP 22) from fruits of bell pepper and tomato inhibited the growth of broad spectrum of phytopathogenic bacteria including 24 strains of soft rot bacteria (Colyer and Mount, 1984; Liao, 1989). Use of an avirulent mutant of P. solanacearum has also been found to be effective for biocontrol of bacterial wilt of tomato (Trigalet and Trigalet, 1990).

Bacterial blight of cotton (X. campestris pv. malvacearum) was reduced by the application of Flavobacterium sp. isolated from its phylloplane (Habish, 1968; Verma et al., 1983). Shekhawat and Chakravarti (1977) isolated a yellow bacterium (B_2) with a dark tinge from chilli leaves which was antagonistic to X. campestris pv. vesicatoria and controlled leaf spot of chillies on coinoculation. Microflora from cowpea seeds could control the seed borne infection of X. campestris pv. vignicola (Jindal and Thind,1990a). Seed borne infection of X. campestris pv. vignaeradiatae has also been reported to be controlled by the isolated seed microflora (E. herbicola, Penicillium oxalicum and P. citrinum of green gram (Thind and Jindal, 1988). Erwinia herbicola a common saprophyte has been reported to suppress

X. campestris pv. oryzae (Hsieh and Buddenhagen, 1974), P. syringae pv. morsprunorum causing bacterial canker of stone fruits (Crosse, 1965), E. amylovora (Chatterjee et al., 1969; Riggle and Kloss, 1972; Thomson et al., 1976), X. campestris pv. citri (Goto et al., 1979).

Many plant pathogenic fungi and bacteria are sensitive to Bacillus spp. or their culture filtrates (Chang and Kommendahl, 1968; Cubeta et al., 1985; Tschen, 1987). Bacillus subtilis, B. licheniformis, B. cereus and B. polymyxa have been reported to be highly antagonistic to a number of bacterial plant pathogens, viz., P. solanacearum (Farag et al., 1980; Aspiras et al., 1985; Anuratha and Gnanamanickam, 1990), X. campestris pv. malvacearum (Singh and Singh, 1981), X. campestris pv. manihotis (Musere and Ikotun, 1983) and P. syringae pv. tabaci (Kalsadia and Ampova, 1987).

Bacillus species have been commonly cited in literature to be effective biofungicides (Spurr, 1978; Pusey and Wilson, 1984; Pusey, 1989). B. cereus and B. thuringiensis are known to provide fair protection against rust in leek (Doherty and Preece, 1978) and coffee rust (Roveratti et al., 1989), respectively. B. pumilus controlled cereal rust by causing lysis of the germtubes of Puccinia (Morgan, 1963). B. cereus var. mycoides effectively controlled the tobacco Alternaria leaf spot (Fravel and Spurr, 1977). B. subtilis provided protection against invasion by the apple canker pathogen Nectria galligena (Swinburne, 1973). B. subtilis strain A-13 isolated by Broadbent et al. (1971) was inhibitory to several plant pathogens and as seed treatment, it increased the yield of carrots by 48 per cent and oats by 33 per cent (Merriman et al., 1974). Ferreira and his associates (1991) reported that pre-inoculation spray of B. subtilis significantly reduced die back infection in grapevines (Eutypa lata). B. subtilis T-99, as substrate

drainage before planting and cuttings resulted in a significant decrease of Fusarium wilt of carnations caused by F. oxysporum f. sp. diantti (Obieglo, 1991). Pre-treatment of bean blossoms with B. polymyxa or its cell free culture filtrate prevented Sclerotinia sclerotiorum infection on stem and pods of bean plants (Godoy et al., 1991).

2.4 Mode of action of antagonists

Biological systems are very specialized and controlling the plant pathogens by biological means still has not taken any measurable strides commercially. This failure may reside in the lack of understanding of the mode of action of antagonists, their ecology as well as an inadequate knowledge of method of production and formulation for commercial use (Whipps, 1986). Classical mechanisms of antagonism are antibiosis, competition, exploitation and stimulation of host defense mechanism.

2.4.1 Antibiosis

Antibiotics are known to play a major role in plant disease suppression (Weller, 1988). Antibiotic negative mutants have provided considerable evidences that antibiosis is involved in disease control (Kloepper and Schroth, 1978). P. fluorescens Hv 37a produces an antifungal compound (Afu) inhibitory to Pythium ultimum (James and Gutterson, 1986; Paulitz and Loper, 1991). Howell and Stiponovic (1980) demonstrated that the purified antibiotic pyoluteorin and pyrrolinitrin from P. fluorescens Pf-5, provided protection of cotton seedlings against damping-off caused by P. ultimum or Rhizoctonia solani as did the bacterium. Phenazines produced by some fluorescent pseudomonads was suppressive to take-all disease of wheat when applied as seed treatment (Weller and Cook, 1983; Thomashaw and Weller, 1988). Gurusiddaiah et al. (1986) reported this antibiotic to be a dimer of

phenazine-1-carboxylate. P. cepacia produced an active substance, pyrrolinitrin that controls Botrytis cinerea (Pusey, 1989). Teliz-Ortz and Burkholder (1960) reported the antagonistic activity of P. fluorescens to E. hyacinthi, X. campestris pv. campestris, Corynebacterium michiganensis and P. syringae pv. phaseolicola. This antibiotic was reported to be transported upwards in the bean plants. Some of the pseudomonads (Gamliel and Katan, 1991) and Bacillus spp. (Broadbent et al., 1977) antagonistic to pathogens have also been reported to stimulate plant growth.

Bacillus subtilis produces two antifungal antibiotics which cause germ tube of Nectria galligena to swell and burst (Swinburne et al., 1975). Antibiotic production has been reported as the mode of antagonism against R. solani (Podile et al., 1988), Sclerotium cepivorum (Utkhede and Rahe, 1983) and Monilinia fructicola (Mckeen et al., 1986). As early as 1948 Landy and his co-workers showed the production of bacillomycin, an antibiotic from B. subtilis. Besson and his associated (1976) and Pusey(1989) characterised antibiotic, iturin-A produced by various strains of B. subtilis.

Agrocin-84, an antibiotic (bacteriocin) from A. radiobacter strain K-84 was found to suppress the growth of A. tumefaciens (Kerr, 1980). These specific protein containing antibiotic substances have also been reported to be produced by leaf inhabiting bacteria, namely, Erwinia, Corynebacterium and Pseudomonas (Vidaver, 1976, 1983; Beer and Rundle, 1980). Xie and his associates (1989) have designated a bacteriocin solanacearicin M2 produced by P. solanacearum. The involvement of bacteriocin from P. solanacearum strains in the protection of tomato wilt caused by P. solanacearum was confirmed by using non-bacteriocin producing strains, which were unable to check the wilt (Tsai et al., 1985).

2.4.2 Competition

The phenomenon that microorganisms compete for nutrients and space in soil is well studied (Clark, 1963; Paulitz and Baker, 1987). However, only the overall outcome of interaction in soil is observed, not the competition for materials or space per se and most of the studies thus are from circumstantial evidence (Lewis et al., 1988).

Nutrient competition is one of the most difficult biocontrol strategy (Handelsman and Parke, 1989). However, not much information is available on the phyllosphere interactions of microorganisms in relation to nutrient and space competition (Morris and Rouse, 1985). Bordie and Blakeman (1976) have correlated that inhibition of germination of conidia of Botrytis cinerea by Pseudomonas sp. (isolate 14) on leaf surface was due to the uptake of aminoacids from a mixture of aminoacid/glucose solution. Antagonistic bacteria can compete for endogenous reserve lost from conidia, also contribute towards the inhibitory effect (Bordie and Blakeman, 1975). Biological control of frost damage against ice nucleating bacteria by P. syringae has been demonstrated to be the result of competition, rather than antibiosis (Lindow, 1985). Wilson and his associates (1987) obtained partial control of peach fruit rot caused by Rhizopus stolonifer with Enterobacter cloacae and nutrient competition was suggested as a major factor in the interaction between these two organisms(Wisniewski et al., 1989).

The ability to sequester iron provides a competitive advantage to microorganisms (Kloepper et al., 1980a; Leong, 1986). Different siderophores differ in their affinity for iron, so there can be competition between siderophores and those with highest affinity will sequester all or most of the iron. Apart from their role in transport of iron (III), siderophores may also act as growth factors and some are potent antibiotics (Neilands, 1981).

Kloepper and his associates (1980b, 1983) reported increased growth and yield of potato when P. fluorescens and P. putida were applied to potato seed pieces. The enhanced growth was attributed to siderophere production by the antagonist which sequester iron away from E. carotovora. Siderophores have been chemically characterized in the recent years (Smith et al., 1985; Meyer et al., 1989). They have been involved in the suppression of Fusarium oxysporum (Scher and Baker, 1982; Sneh et al., 1984), Gaeumannomyces graminis var. tritici (Weller et al., 1988; Wong and Baker, 1984), Pythium spp. (Loper,1988) and E. carotovora sub. sp. carotovora (Liao, 1989) produced by flourescent pseudomonads strains.

2.4.3 pH-shift

Newhook (1951) found that colonization of woundsby nearly all the saprophytic bacteria and fungi tested, raised the pH to 7.8 - 8.4 from 7.0, whereas in control lesions with Botrytis alone maximum pH was 6.8 - 7.2. Bhatt and Vaughan (1962) reported that Cladosporium (a successful antagonist on young flowers) raised the pH of the medium upto 8.0 and thus considered the change in pH as the possible mechanism of antagonism. E. herbicola has been reported to create acid conditions on the aerial surface of fruit trees (Farbee and Lockwood, 1958; Riggle and Klos, 1972). Inhibition of X. campestris pv.oryzae on rice leaves by E. herbicola has been attributed to its ability to lower the pH of the medium from 7.0 to 4.3, unfavourable for the growth of pv. oryzae (Hsieh and Buddenhagen, 1974).

2.4.4 Stimulation of host defences

Antagonists have also been reported to stimulate protective responses in host tissues and enhancing its resistance to pathogen invasion (Goodman, 1967). Resistance to a virulent strain of P. solanacearum was induced in

potato by seed piece treatment with avirulent or incompatible strains of P. solanacearum or P. fluorescens (Kempe and Sequeira, 1983). Co-inoculations of live or attenuated X. campestris pv. carotae cells has been reported by Cook and Robeson (1986) to provide protection against black rot pathogen X. campestris pv. campestris in cabbage leaves. Similar incompatible plant response resulted in soybean when co-inoculated with mixture of incompatible and compatible races of living P. syringae pv. syringae (Keen and Kennedy, 1974; Keen, 1982). Alfalfa leaflets infiltered with suspensions of avirulent mutants of Corynebacterium insidiosum, when challenged 6 to 12 h before pathogen inoculation, were protective against bacterial wilt disease (Carrol and Lukezic, 1972). Leaf inoculation of cucumber with Colletotricum lindemuthianum gave protection against C. cucumerinum (scab.) and anthracnose pathogen C. lagenarium (Kuc', 1981; Dean and Kuc', 1986).

2.4.5 Electrolyte leakage

Permeability changes are very common in initial or early events of infection process during disease development (Wheeler, 1978). Nema (1988) studied the permeability alterations in betelvine inoculated with leaf spot pathogen X. campestris pv. beticola. Electrolyte conductivity of the inoculated tissues increased with the disease development. The faster the development of disease, the greater was the electrolyte leakage (Gupta et al., 1983). Some toxins or enzymes produced by the pathogen bring about rapid changes in the permeability of plant tissue membranes; as a consequence, water balance is altered, cells become leaky and loose electrolytes and other materials (Lewis and Papavizas, 1987). Elad and his associates (1982) have demonstrated that Trichoderma harzianum excreted lytic enzymes (β-1,3-glucanase and chitinase) which destroyed the membrane permeability of

Sclerotium rolfsii. Inoculation of P. syringae pv. pisi in tobacco foliage resulted in typical hypersensitive reaction only in leaves kept in a normal room, and not in a humid chamber. The delaying symptoms in humid chamber however, did not delay electrolyte leakage and membrane damage.

2.5 Multiplication of phytopathogenic and phylloplane bacteria in plants

It is generally accepted that disease symptoms due to bacterial infection are correlated rather closely with the bacterial multiplication in the intercellular space. Allington and Chamberlain (1949) reported the population trend of pathogenic X. campestris pv. phaseoli and P. syringae pv. glycinea within leaf tissues of susceptible and immune species of bean and soybean respectively. Multiplication was initiated equally (Stall and Cook, 1966) until the third day after inoculation in both immune and susceptible hosts. However, the population in susceptible host continued to increase logarithmically irrespective of the initial inoculum levels (Cook and Robeson, 1986). Further studies on inoculation of mixture of compatible X. campestris pv. campestris and incompatible X. campestris pv. carotae resulted in restricted pathogen multiplication and provided protection against black rot infection, whereas the density of pv. carotae was constant and apparently unaffected by the presence of pv. campestris. Robinson and Callow (1986) observed that in homologous interactions pv. campestris multiplication reached maximum (10^8 cfu cm^{-2}) in 20 days and it was distributed throughout the host leaves.

Dowler (1972) demonstrated that a saprophytic bacterium (unidentified) isolated from peach trees along with P. syringae pv. syringae when infected into green pods, decreased the pathogen multiplication to 10-fold as compared to inoculation with pathogen alone. Young and Paton (1972) studied the population of pathogenic (P. syringae pv. phaseolicola) and saprophytic

(P. fluorescens, P. putida and E. herbicola) bacteria in plant tissues of bean and recorded a slow decline in the population of saprophytic bacteria. When the population of saprophytic bacteria were studied together with the pathogen, the population of the former was stimulated while there was no affect on the pathogen population, suggesting that pathogen induced the release of nutrients.

CHAPTER III

MATERIAL AND METHODS

3.1 Collection, isolation, purification and identification of the test pathogen

Diseased Brassica juncea plants showing typical black rot symptoms on leaves and stems (stem cracking) were collected from different locations at CCS HAU, Hisar experimental research area.

The samples were examined under a microscope for bacterial ooze. The diseased leaves were washed and surface sterilized in 0.1 per cent mercuric chloride solution for 1 min. followed by four washings in sterile distilled water. A small portion cut from the surface sterilized leaf was placed on a sterile glass microslide along with a drop of sterilized distilled water and under aseptic conditions, sharp, clean cut was given with sterilized razor. After two minutes, with the help of sterilized Pasteur pipette a small water drop containing the bacterial ooze was sucked from the vein ending and streaked on the agar surface on the petri plates containing yeast-extract-peptone-glucose agar medium (Yeast extract, 5.0 g; Bacto peptone, 5.0 g; glucose 10.0 g; agar, 20 g and water to make 1 litre: pH 7.2). The plates were incubated at 25±1°C for 3-4 days.

Slow growing, yellow, circular, mucoid, glistening convex bacterial colonies with entire margins (suspected to be Xanthomonas campestris pv. campestris) were selected and transferred to YPGA slants. These colonies were further purified and grown on a new semiselective medium of Randhawa and Schaad (1984). The colonies on this medium appeared after 3-5 days, 3-5 mm in diameter as transluscent, mucoid, entire, colourless to slightly green and surrounded by starch digestion zone of 6-10 mm.

Finally, three purified suspected isolates of the pathogen were obtained. All these isolates were maintained in mineral oil on glucose yeast calcium carbonate agar slants at 5 to 10°C.

For identification of the selected isolates, various biochemical tests viz., 3-ketolactase production, Hugh and Leifson test, Oxidase test, urease production, starch hydrolysis, aesculin hydrolysis, geletin hydrolysis, nitrate reduction, phenylalanine deaminase test, catalase activity test, H_2S production and Gram reaction of each isolate were performed as previously described by Schaad (1980), Fahy and Persley (1983), Lelliot and Stead (1987). Pathogenicity of all the isolates was tested on Brassica juncea (cv. RH-30).

3.2 Screening for resistant source

While studying the interaction of antagonist and pathogen on contrasting genotypes (resistant and susceptible) it became imperative to evaluate the source of resistance for black rot pathogen hitherto unknown. The brassica germplasm (59 cvs.) was raised in the field and forty day old leaves were clip inoculated with the test pathogen suspension (10^7 cfu ml^{-1}). In each of the three replications, five plants were randomly selected in which four leaves per plant were inoculated. The disease was assessed with the scale given below and the cultivars designated as resistant, moderately resistant, moderately susceptible, susceptible and highly susceptible.

3.2.1 Enumeration of black rot infection and determination of necrosis rating

Black rot symptoms on the leaf tissue surrounding each inoculation site was examined visually. The sites which developed black rot infection were characterised by 'V-shaped' chlorotic lesions extending outwards from the point of inoculation towards the leaf margin and in addition many of the veins were blackened. The host reaction was recorded by using a scale

of 0-5 as given below:

Scale	% leaf area infected	Reaction
0	0	Infection free
1	1-5	Resistant
2	6-10	Moderately resistant
3	11-25	Moderately susceptible
4	26-40	Susceptible
5	More than 40	Highly susceptible

3.3 Studies on antagonistic phylloplane microflora

3.3.1 Isolation and identification of epiphytic microflora

Phylloplane microorganisms were isolated by the leaf washing technique (Voznyakovskya and Khudakov, 1960) and leaf grinding technique (Sleesman and Leben, 1976). Ten healthy leaves from each cultivar of B. juncea were collected at random (including lower, middle and upper leaves) at three different stages of plant growth (viz., 30, 60 and 90 day old plants). Two hundred discs (0.5 cm diameter) were cut with the help of sterile cork borer from each cultivar. One hundred discs from each cultivar were transferred to 20 ml distilled sterile water in Erleneyer flask (100 ml) and stirred vigorously for 20 min to dislodge the surface micrlfora. In case of leaf grinding technique, 100 leaf discs were ground in sterilized pestle and mortar. One ml of each preparation was transferred to a test tube containing 9 ml sterile distilled water. It was further serially diluted to 10^{-6}. An aliquot of 0.1 ml of each dilution was transferred on to the surface of YPGA and PDA containing 100 µg/ml cycloheximide and streptocycline respectively in petriplates. The inoculum was spread with a sterile glass spreader. Plates

were incubated at 25±1°C for 2-3 days. All morphologically different discrete colonies were selected and transferred to YPGA (for bacteria) and PDA (for fungi) slants. They were purified further on plates and maintained on respective media for further studies.

Fungal and bacterial cultures isolated from phylloplane of Brassica juncea were identified by using standard procedures and their identity was got confirmed from ITCC, IARI, New Delhi and CAB, International Mycological Institute, U.K.

3.3.2 Evaluation of phylloplane microorganisms for antagonism to X. campestris pv. campestris

All the fungal and bacterial isolates obtained from the phylloplane of B. juncea were screened for their antagonistic activity against X. campestris pv. campestris in vitro. Five hundred ml of YPGA medium was dispensed in 11 flasks, autoclaved and cooled to 42-45°C. Bacterial suspension (24 h old) of X. campestris pv. campestris was prepared in distilled sterile water (10^9 cfu ml^{-1}), 10 ml of this suspension was added to each flask. The pathogen seeded medium was poured in sterilized plates and allowed to solidify. These were then refrigerated for 4 h at 7°C. The plates were spot inoculated with 5 mm diameter discs (24 h old) from the lawn culture of the test phylloplane bacterium with four replications.

In case of phylloplane fungi, the mycelial agar discs cut from the margin of 4-5 day old culture were transferred to the pathogen seeded plates. These plates were incubated for 48 h at 25+1°C. The inhibition of test organism resulted in clear zone around the inoculated spot/mycelial disc which was measured crosswise by a mm scale.

3.4 Preparation of standard bacterial suspension

A young actively growing (24 h old) culture of X. campestris pv. campestris and the antagonist (HSb-19) were used for the bacterial count. Suspension of both the bacterial culture in sterilized distilled water were serially diluted to 0.1, 0.2, 0.5 and 1.0 OD with the help of Spectronic-20 at 640 nm. The suspensions with different OD were serially diluted (10^{-9}) and 0.1 ml of 10^{-5} to 10^{-9} dilutions were plated on YPGA plates with three replications.

3.5 Isolation of mutants

Incorporation of antibiotic resistant markers into the test pathogen and antagonist is a pre-requisite for quantitative estimation of their population when re-introduced in nature for biocontrol studies. To develop resistant mutants, at first, both the cultures (pathogen and antagonist) were evaluated for their sensitivity to different antibiotics. Finally the minimum inhibitory concentration (MIC) of the antibiotic was determined for raising the antibiotic resistant mutants.

3.5.1 Sensitivity of X. campestris pv. campestris and antagonist (HSb-19) to antibiotics

The sensitivity of X. campestris pv. campestris and HSb-19 to fourteen antibiotics viz., kanamycin, tetracycline, neomycin, chloramphenicol, nalidixic acid, cephaloridine, streptomycin, ampicillin, ledramycin, erythromycin, gentamycin, penicillin, carbenicillin and trimethoprim were tested in vitro by paper disc method (Thornberry, 1950). YPGA medium in Erlenmeyer flask was autoclaved and cooled to 40-45°C. Bacterial suspensions (OD 0.1/10^{8} cfu ml^{-1}) of X. campestris pv. campestris and HSb-19 were prepared in sterile distilled water and 2.5 ml of it was added to each flask

separately. The bacteria seeded medium was poured into sterilized petri-plates and allowed to solidify. Pre-loaded antibiotic disc was placed in the centre of the plate with three replications. Sterilized distilled water discs served as controls. The plates were incubated at 25±1°C for 48 h. The bacterial growth inhibition was measured and inhibition annulus obtained by Smale and Keils (1966) equation:

$$\text{Inhibition annulus} = \pi (R_1-R_2) (R_1+R_2)$$

Where, R_1 = radius of zone of inhibition + radius of assay disc

R_2 = radius of assay disc

The inhibition zone annulus thus calculated was compared with standard zone size interpretative chart of Bryant (1981).

3.5.2 Development of antibiotic resistant mutants

Mutant resistant to chloramphenicol and tetracycline of the pathogen and antagonist respectively were selected by incorporating an increased dosage of antibiotics serially in the growing medium. The antibiotic concentration was raised from MIC by plating 1.0 ml thick actively growing bacterial suspension. Isolates which grew after 4-5 days of incubation at 25±1°C on antibiotic-containing media were repeatedly transferred to media with next higher concentration of the antibiotics, till mutants resistant to a concentration of 20-25 $\mu g\ ml^{-1}$ were obtained. Single cell resistant isolates thus developed, were transferred to antibiotic containing media and rechecked for resistance. These antibiotic-resistant clones were maintained on slants without antibiotic. The antibiotic resistant mutants of antagonist and pathogen were further tested for their antagonistic and pathogenic potential, respectively.

3.6 Bioefficacy of the antagonist (HSb-19)

3.6.1 Standardization of inoculation methods

A number of methods have been employed for the inoculation of host plants with phytopathogenic bacteria for the purpose of laboratory, green house and field studies (Dhingra and Sinclair, 1985). In the present studies four methods as demonstrated in Fig. 1 were compared to standardize a suitable technique to obtain the maximum infection of X. campestris pv. campestris with least physical injury to the host. For this purpose, Brassica juncea (cv. RH-30) plants were raised in 9 inch pots under green house conditions for 40 days. These plants were inoculated with X. campestris pv. campestris (10^7 cfu ml^{-1}) by the following methods:

a) Leaf infiltration: Leaves of the test plants were infiltrated by hypodermic syringe with the bacterial suspension from lower surface of the leaf while supporting the leaf on the opposite side (Klement, 1963). A maximum of three infiltrations were made per leaf; for the control plants the leaves were infiltered with sterilized mineral salt solution (Cook and Robeson,1986) and sterilized distilled water.

b) Wound inoculation: Wound inoculation was made by directly dipping a sterile needle into bacterial colony (24 h growth) and jabbing the needle into leaf petioles and leaf lamina at three sites (Shaw and Kado, 1988). In control, the jabbing was done with sterilized needle.

c) Hydathode inoculation: Forty day old plants were shifted from green house to a mist chamber at 27°C and 100 per cent humidity for 3 h to induce the formation of guttation droplets (Robeson et al., 1989). An aliquot of inoculum suspension (1 µl) was delivered into each targeted guttation droplet with a hypodermic syringe. After inoculation, the plants

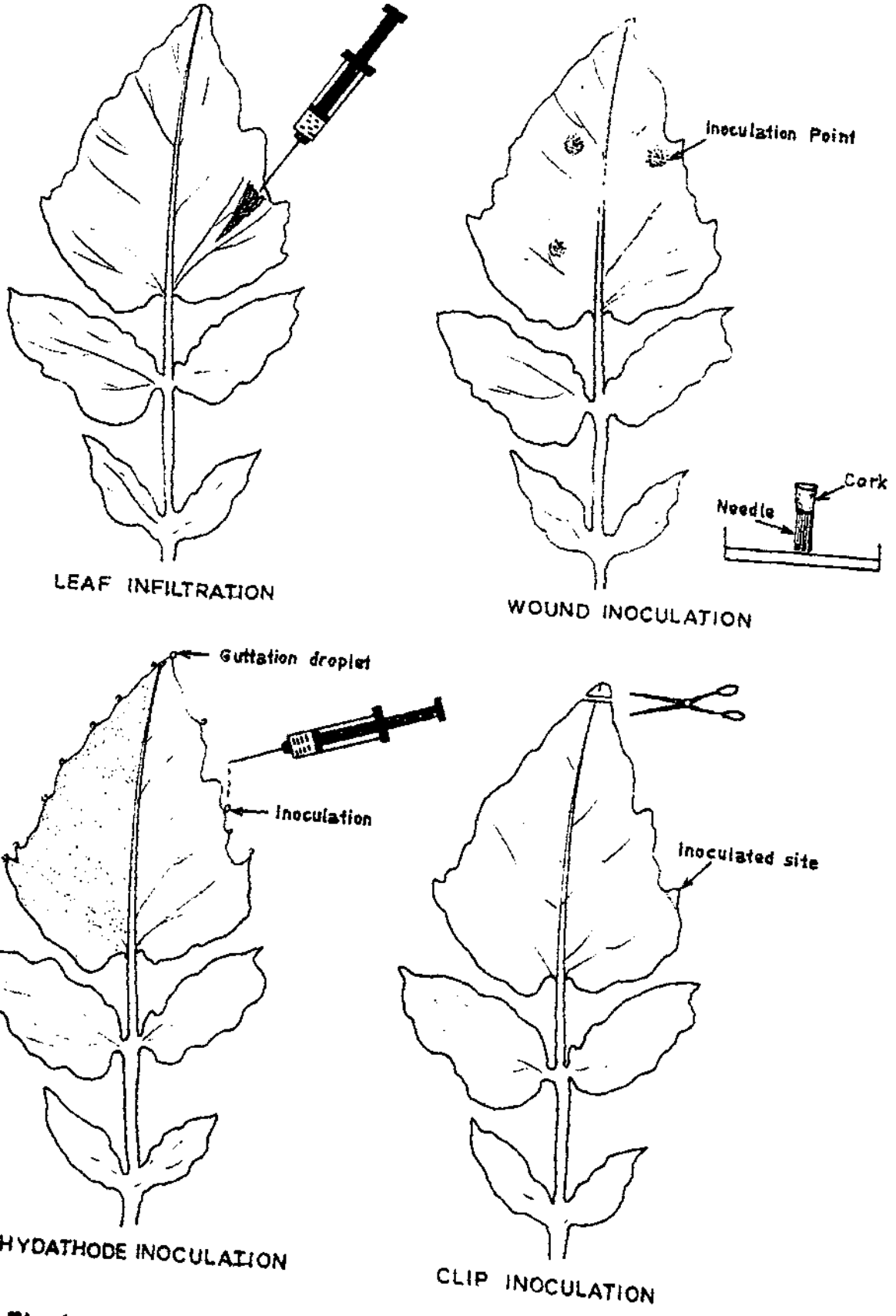


Fig. 1. HOST INOCULATION METHODS

were allowed to remain in the dew chamber without the mist for 2 h in order to facilitate the entry of the pathogen into the plants. In check plants, sterile mineral salt solution was delivered into guttation droplet.

d) Clip inoculation: Leaves were inoculated by the method of Sutton and Williams (1970a). Notches were cut about 2 mm into the leaf margin at the endings of large veins by scissors dipped in cell suspension (Kaufman et al., 1973). Control leaves were notched with scissors dipped in sterile mineral salt solution.

e) Grading: The extent of chlorosis and necrosis proximal to the inoculated end of leaves was recorded 2 days after inoculation till the 15th day by adopting the following grading scale:

- \- no symptom
- \+ chlorosis
- ++ chlorosis + necrosis
- +++ chlorosis + necrosis + vein blackening

3.6.2 Bacterial inoculum

The bacterial inoculum was prepared by growing bacteria on YPGA medium in Roux bottles. The bacteria (X. campestris pv. campestris/antagonist (HSb-19) were harvested after 24 h in sterile mineral salt solution (Cook and Robeson, 1986). The suspensions were adjusted by appropriate dilution to a turbidity of 0.1 OD at 640 nm to contain Ca. 10^8 cfu ml^{-1} Inoculum for co-inoculation experiments was prepared by mixing suspensions of X. campestris pv. campestris and antagonist (HSb-19) at various ratios according to their A_{640} values.

3.6.3 Determination of optimum antagonist population for black rot control

Studies were conducted to evaluate the optimum cell numbers of the antagonist to give effective control of black rot pathogen. Brassica plants were raised in 9" pots and forty day old plants were used for inoculation by three methods viz., spray, clip and infiltration. Dual inoculation of pathogen and antagonist in three ratios and cell free culture filtrate of antagonist were used under green house conditions. The antagonist suspension was made by adjusting the optical density of 0.1, 0.2 and 0.5 at 640 nm to give 10^8, 10^9 and 2×10^9 cfu ml^{-1}. The X. campestris pv. campestris inoculum was prepared as described earlier. The suspensions were mixed in appropriate ratios to get the desired number of pathogen : antagonist bacterial cells for inoculation viz., 10^7:10^7 cfu ml^{-1} (1:1), 10^7:10^8 cfu ml^{-1} (1:10) and 10^7 : 2×10^8 cfu ml^{-1} (1:20).

3.6.4 Evaluation of antagonist for disease control under field conditions

The antagonist (HSb-19) was evaluated for its potential to serve as a biocontrol agent of X. campestris pv. campestris. Brassica juncea (cv. RH-30) plants were raised in field in plots of 3.2 x 1.6 m in a randomized block design, replicated thrice with fourteen treatments given below.

1.	Pathogen (Path.)	
2.	Antagonist cell (C)	
3.	Antagonist culture filtrate (CF)	
4.	Path. + Antagonist (C)	Simultaneous spray
5.	Path. + Antagonist (CF)	
6.	Antagonist (C) — Path. (24 h)	Sequential
7.	Antagonist (C) — Path. (72 h)	
8.	Path. — Antagonist (C) (24 h)	
9.	Path. — Antagonist (C) (72 h)	

10. Streptocycline 250 ppm
11. Dithane M-45 0.2%
12. Cu-oxychloride 0.2%
13. Seed treatment [Antagonist(C)]
14. Control (no treatment)

The plants (40 day old) were spray inoculated with pathogen and antagonist (10^7 : 10^8 cfu ml^{-1}) simultaneously and sequentially. Seed treatment with antagonist bacterium (live cells) and cell free culture filtrate as foliar sprays were also included besides commonly recommended fungicides/antibiotics.

Ten plants in each plot were randomly selected for disease rating. The host reaction was observed and disease scored from lower, middle and upper leaves (5) of each plant by the scale given earlier at 10 day interval till 60 days. The disease index was calculated by using the following formula:

$$\text{Disease index (\%)} = \frac{\text{Sum of all the numerical ratings x 100}}{\text{Total number of leaves observed x 5}}$$

3.7 Mode of action of antagonist in inhibition of the pathogen

The primary objective of carrying out these investigations was to understand the mode of antagonism. This knowledge of the mechanism of antagonism will be helpful for future possibilities of genetic manipulation and the 'de novo' selection of desirable traits (Lewis et al., 1988). Information of this type can be obtained from in vitro studies, although such studies have their own limitations and may not provide information on their in vivo modes of action, particularly within plants. These inhibitory effects of antagonists may be antibiosis, pH change, nutrient competition etc.

3.7.1 Studies on pH alterations during antagonism

a) pH optima for the growth of antagonist and pathogen: Double strength YPGA was prepared and 50 ml of this was distributed in 100 ml conical flask. To each flask 30 ml of distilled water was added to make the volume to 80 ml. The media were adjusted to pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 by using 0.1N HCl or 0.1N NaOH. After adjusting the pH, 20 ml of buffer of each pH was added so as to make the total volume 100 ml. Citrate phosphate buffer (pH 4.5 to 7.0) and phosphate buffer (pH 7.5 to 8.5) were used (Gomari, 1955). The media were then autoclaved and transferred to plates. Five replications for each pH range were taken. Each plate was inoculated with 0.1 ml of cell suspension prepared from 24 h growth. The colony size was measured after 48 h of inoculation at 25±1°C.

b) Multiplication of the pathogen in presence of the antagonist in buffered and unbuffered media:

The antagonist alone and in combination with X. campestris pv. campestris were grown in buffered and unbuffered yeast peptone glucose (YPG) broth.

The unbuffered YPG broth was prepared with pH 7.0 (adjusted with 0.1N HCl or 0.1N NaOH). The buffered YPG broth of pH 7.0 was prepared with citrate phosphate buffer (4 parts broth: 1 part buffer) and sterilized.

The inoculum of X. campestris pv. campestris and antagonist were prepared as described earlier. Inoculation of each treatment was done in bulk. Ten ml of each bacterial inoculum suspension was added to 750 ml broth separately and 50 ml of the inoculated broth was distributed aseptically into previously sterilized flasks. Similarly for simultaneous inoculations, both the organisms (pathogen and antagonist) were added together before distribution of broth into smaller sterilized 100 ml flasks to get more or less identical population of the test organism in each flask. However, for sequential

inoculations, the antagonist was added after 24 h of the growth of X. campestris pv. campestris (0.5 ml/50 ml broth) and vice versa. The inoculated flasks were incubated at 25±1°C on a rotary shaker.The flasks were withdrawn at different intervals viz., 24, 48, 72 and 96 h after inoculation to record the change in pH and viable counts of pathogen and antagonist. The viable count was recorded by serial dilution technique. Three dilutions were made for each treatment at each time interval. Every dilution was replicated three times. An aliquot of each dilution (0.1 ml) was transferred to the surface of YPGA medium containing chloramphenicol (25 $\mu g\ ml^{-1}$) and tetracycline (20 $\mu g\ ml^{-1}$) plates for X. campestris pv. campestris and antagonist, respectively. The change in pH of the inoculated broth was recorded by an Eltop digital pH meter. In sequential inoculations, first observation was recorded after 24 h of inoculation of the second test organism.

3.7.2 Competition

a) Effect of inoculum density: The test pathogen and the antagonist were coinoculated in YPG broth with varying ratios (cell number) viz., 1:1, 1:2, 1:5, 1:10, 2:1, 5:1, 10:1. The procedure of inoculum preparation and inoculation was as described earlier. Pathogen and antagonist inoculated singly served as controls. The inoculated flasks were incubated on a rotary shaker at 25±1°C and were withdrawn at different intervals (0, 24, 48, 72 and 96 h) to record the population of the two test organisms by viable count on antibiotic containing plates.

b) Nutrients: To examine if nutrients are a limiting factor in antagonism of X. campestris pv. campestris, it was grown in the cell free culture filtrate of antagonist. The antagonist was inoculated and incubated in YPG broth and at different time intervals (viz., 24, 48, 72 and 96 h)

the culture filtrate was subjected to centrifugation (10,000 g for 15 min.) to obtain cell free culture filtrate. One half of the cell free culture filtrate obtained at each time interval was subjected to autoclaving (15 lb psi for 15 min.) and the other half was filter sterilized with Whatman membrane filter (pore size 0.45 μm). Each part was further divided into three parts. The first part was supplemented with equal volume of double strength YPG broth, the second part was not supplemented, both were then inoculated with X. campestris pv. campestris. The remaining third part (unsupplemented and uninoculated) served as control. Inoculated (pathogen) YPG broth (same volume) served as reference control. The viable count of X. campestris pv. campestris was determined by dilution plate technique after 48 h of incubation at 25±1°C.

3.7.3 Membrane permeability-electrolyte leakage

The method of Vasquez-Tello et al. (1990) with slight modification was followed for studying the electrolyte leakage. Forty day old plants were spray inoculated with pathogen or antagonist alone or in combination. The alterations in membrane integrity were based on the measurement of the electrolyte efflux. Fresh leaves (4th leaf from top) were sampled, washed gently with distilled water and dried with filter paper. Five mm discs cut with the help of a punching machine in distilled water were immersed (@ 6 discs/ml) in streptocycline solution (20 μg ml^{-1}) in small beakers. The beakers were covered with aluminium foil to minimize evaporation and incubated at 25 ± 1°C. The leaf discs were removed after 24 h and the electrolyte leakage of the resultant solution measured by a digital conductivity meter. The electric conductivity was expressed as μmhos cm^2. The same leachate was used for various other biochemical estimations viz., amino acids,

sugar and proteins. The methods described by Yemn and Willis (1954), Lowry et al. (1951) and Swain and Hill (1959) were followed for the estimation of total sugars, proteins and amino acids, respectively.

3.7.4 Antibiosis

a) Antimetabolite production: Antibiotic production by the test antagonist was investigated on two media: YPG broth and Watanabe broth (Cook and Robeson, 1986). The antagonist was inoculated in YPG broth (for antibiosis), Watanabe broth minus Fe^{+++} (for siderophore) in 150 ml conical flasks and incubated for 96 h at 25±1°C on rotary shaker. The culture filtrate was centrifuged at 10,000 g for 15 min. and the cell free supernatant obtained. It was then subjected to different treatments viz., boiling (at 100°C for 10 min.), autoclaving (121°C at 15 lb psi for 15 min.), filter sterilization with membrane filter (pore size 0.45 µm) and incineration (600°C for 30 min.). These were bioassayed for their inhibition of the test pathogen by measuring the inhibition zone. Each plate received 50 ml of X. campestris pv. campestris inoculated YPGA medium. On solidification, wells (5 mm diameter) were made with sterilized cork borer and each well was sealed at the bottom with 5 µl of 2 per cent sterilized molten water agar. Into these wells the treated cell free culture filtrate 20-80 µl was added. After 48 h at 25±1°C, the inhibition zones were measured.

b) Siderophores: To detect siderophore production the standard method of Schwyn and Neilands (1987) was used. The medium prepared was rich blue in colour. The 10 µM Fe (III) tints the agar with a rich blue colour, while the concentration of siderophore excreted by iron-starved microorganisms generally exceeds this level. A strong legand L (e.g., a siderophore) is added to a highly coloured iron dye complex. When the iron

legand complex (FeL) is formed, the release of free dye is accompanied by a colour change. These dye plates were spot inoculated with the test organisms. Observation of any colour change around the colonies was observed after 4 days of incubation at 25±1°C.

3.8 Monitoring of multiplication and progression of the pathogen and antagonist in the host

Antibiotic resistant mutants of X. campestris pv. campestris and antagonist were used for leaf inoculation (1×10^7 cfu ml^{-1}) using clip inoculation technique. Pathogen and antagonist separately, or in combination and their culture filtrate were used for inoculation. Leaves of the test plants (same age and stage) were taken at different time intervals. The progression and multiplication of the test organisms were determined at different time intervals by dilution plate method (Robinson and Callow,1986). Washed leaf portions (20 x 10 mm) were cut along the inoculated mid vein with a sterilized scissors after 1, 2, 4, 6, 8, 10, 12, 24, 48, 72, 96, 120 and 240 h of inoculations. It was further divided into 10 smaller parts of 2.5 x 10 mm each starting from the point of inoculation (Fig.2). After 120 h the inoculated leaf was divided into 10 parts of 1 cm each and 2.5 x 10 mm terminal end of each segment (Fig.3) was sampled. After 30 days of leaf inoculation, the multiplication of X. campestris pv. campestris at the end of leaf petiole, xylem and phloem of the stem between the two internodes of the inoculated leaf was examined. It was divided into 1 cm segments and the terminal (2.5 mm) end of each segment was sampled. The phloem and xylem of each part were sampled separately.

Each leaf portion thus collected were thoroughly crushed separately in 1 ml sterile mineral salt solution in a small test tube (10 x 75 mm) using a sterile glass rod with one end moulded in the form of a pestle with

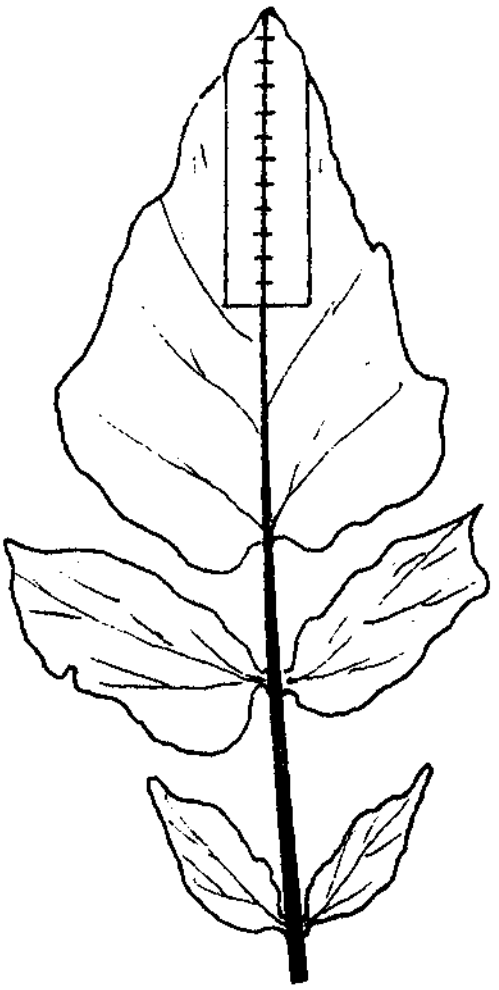


Fig. 2. LEAF SAMPLING FOR PATHOGEN MULTIPLICATION AND PROGRESSION (96h)

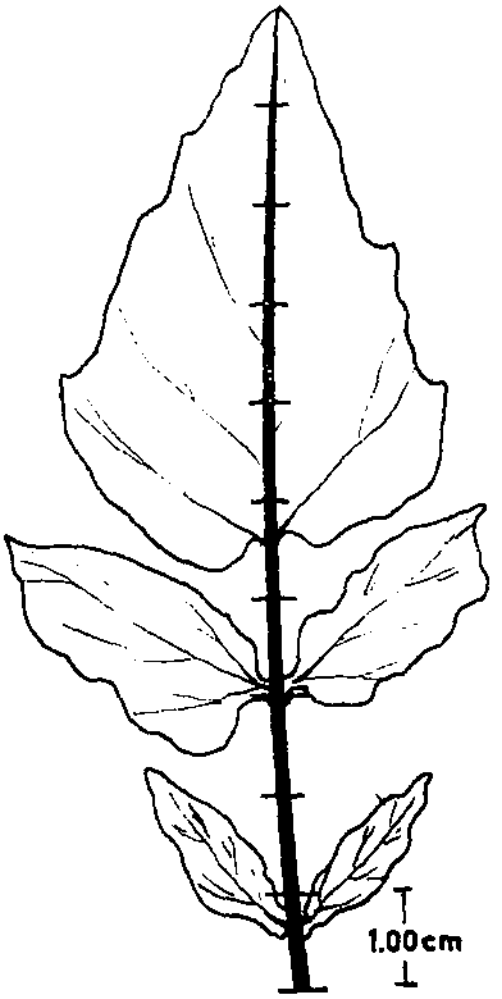


Fig.3. LEAF SAMPLING FOR PATHOGEN MULTIPLICATION AND PROGRESSION (120 h)

rough surface. The macerate was mixed for 1 min using a vortex mixture and dilutions were prepared to determine the population of the test organisms by viable count on antibiotic containing plants.

3.9 Nutritional status of inoculated and healthy leaves

The nutritional status of the leaves of B. juncea was studied by estimation of total of NPK sugars, proteins and phenol content at 10 and 20 day interval after spray inoculation of leaves with the pathogen alone or antagonist alone or their co-inoculation. The leaves were dried at 60°C in an oven for 8 h and the dried samples were ground to a fine powder.

For analysis of N, P and K, 200 mg of the dried powdered plant material was digested in diacid mixture of H_2SO_4 and $HClO_4$ (4:1). Nitrogen and phosphorus was determined spectrophotometrically using the method of Lindner (1944) and Koeing and Johnson (1942), respectively. Potassium was determined flame photometrically (Jackson, 1958). To determine the total water soluble sugars the extraction procedure of Cerning and Guilbot (1973) was used. The total soluble sugars and reducing sugars were estimated by the method of Yemin and Willis (1954) and Somogyi (1945), respectively. The content of non-reducing sugars was calculated from the difference between total sugars and reducing sugars.

CHAPTER IV

RESULTS

4.1 Isolation and identification of the test pathogen

Three isolates similar to Xanthomonas campestris pv. campestris were obtained from diseased leaf samples of Brassica juncea (Plate I) in axenic culture. The cultures were maintained in mineral oil for further studies. All the three purified isolates of the bacterium were identified as the strains of X. campestris pv. campestris based on morphological, biochemical and pathological tests. The test isolates were Gram negative, oxidase, urease, nitrate reduction, 3-keto lactose production and phenyl alanine deaminase positive. They hydrolysed starch, tween-80, aesculin and gelatin. All the isolates were catalase positive.

4.2 Screening for source of resistance in Brassica for black-rot disease

Fifty nine Brassica genotypes were evaluated against black rot pathogen under artificial disease stress conditions (clip inoculation). None of the cultivars screened was found to possess high degree of resistance (Table 1). Six genotypes were moderately susceptible, 50 were susceptible and three were highly susceptible to the test pathogen.

4.3 Studies on antagonistic phylloplane microflora

4.3.1 Isolation of epiphytic microflora

The leaf surface microorganisms were isolated from eleven cultivars of Brassica at three stages of plant growth. In all 25 bacterial and 12 fungal isolates were obtained (Table 2). The fungal flora was found to be more abundant in the late stages of host growth. These isolates were maintained in the laboratory during the course of investigations.

PLATE I

CHARACTERISTIC SYMPTOMS OF BLACK ROT ON LEAVES AND STEM

Table 1. Evaluation of Brassica genotypes against black rot under artificial disease stress conditions

Reaction	Genotypes				
	B. juncea	B.campestris	B.napus	B.carinata	B.alba
Resistant	-	-	-	-	-
Moderately susceptible	DIRA-313, PHR-1, DOMO	-	ISN-7-4, Gullivar	-	B. alba
Susceptible	PR-8805, NDR-872, NDR-873 YRT-3, RH-8544, CSR-448, NDR-8601, WRR-3-1, CSR-1-42, CSR-416, RC-781, RH-8545, DIRA-326, PYSR-3, PHR-2, BJ-2, RWARS-9, DIR-247, Kranti, Krishna, Zem-1, Zem-2, RSK-10, RH-7859, EC-126745, EC-129126, Varuna,RLM-619, RH-30, RLM-514, RH-8113	PYS-841,PYS-843, T-9, SSK-1, NDYS-2,YSK-8502, SPAN, PT-303, PYS-842, DYS-7-1	GSL-1501, GSB-7006, GSB-7027, GSL-1,HNS-3, HNS-4,Tower, MIDAS	HC-1	-
Highly susceptible	NDR-871, RWARS-3	-	HNS-8	-	-

Table 2. Phylloplane microflora isolated from Brassica genotypes

Cultivar	Bacteria Isolate No.	Fungi Isolate No.
B. campestris		
cv. PT-303	HSb-1, HSb-2, HSb-3, HSb-4, HSb-5, HSb-6, HSb-7, HSb-12, HSb-19, HSb-33, HSb-39, HSb-44	HSf-57, HSf-67, HSf-68, HSf-71, HSf-74, HSf-75, HSf-77
cv. T-9	HSb-1, HSb-3, HSb-4, HSb-8, HSb-9, HSb-12, HSb-19, HSb-31, HSb-35	HSf-57, HSf-64, HSf-67, HSf-68, HSf-69, HSf-71, HSf-74, HSf-81
B. juncea		
cv. Krishna	HSb-1, HSb-2, HSb-3, HSb-4, HSb-8, HSb-9, HSb-12, HSb-19, HSb-31, HSb-36	HSf-57, HSf-64, HSf-68, HSf-71, HSf-73
cv. RH-781	HSb-1, HSb-2, HSb-3, HSb-4, HSb-5, HSb-8, HSb-12, HSb-19, HSb-44	HSf-57, HSf-61, HSf-64, HSf-67, HSf-69, HSf-72, HSf-74
cv. Kranti	HSb-1, HSb-2, HSb-3, HSb-12, HSb-19, HSb-20, HSb-39	HSf-67, HSf-68, HSf-71, HSf-72, HSf-74
cv. RH-7859	HSb-1, HSb-2, HSb-3, HSb-4, HSb-12, HSb-18, HSb-19, HSb-20, HSb-31, HSb-39	HSf-57, HSf-68, HSf-71, HSf-72
cv. Varuna	HSb-1, HSb-2, HSb-3, HSb-4, HSb-5, HSb-10, HSb-12, HSb-15, HSb-19, HSb-20, HSb-33, HSb-39	HSf-67, HSf-71, HSf-72
cv. RLM-619	HSb-1, HSb-2, HSb-3, HSb-4, HSb-5, HSb-11, HSb-12, HSb-19, HSb-20, HSb-31, HSb-39	HSf-57, HSf-63, HSf-67, HSf-68, HSf-71, HSf-72, HSf-73, HSf-81
cv. RH-30	HSb-1, HSb-2, HSb-3, HSb-4, HSb-12, HSb-19, HSb-21, HSb-33, HSb-39, HSb-44	HSf-57, HSf-63, HSf-67, HSf-72, HSf-73
cv. RLM-514	HSb-1, HSb-2, HSb-3, HSb-4, HSb-5, HSb-12, HSb-15, HSb-19, HSb-21, HSb-27, HSb-31, HSb-39	HSf-57, HSf-63, HSf-64, HSf-67, HSf-68, HSf-71, HSf-72, HSf-74
cv. RH-8113	HSb-1, HSb-3, HSb-4, HSb-5, HSb-12, HSb-19, HSb-24, HSb-28, HSb-31, HSb-33, HSb-36	HSf-57, HSf-63, HSf-65, HSf-67, HSf-69, HSf-71, HSf-72, HSf-72, HSf-73, HSf-74, HSf-81

Contd.....

Table 2 contd.....

Arthrobacter	HSb-4	Cladosporium cladosporoides, HSf-57; Aspergillus niger, HSf-61; Curvularia lunata, HSf-63; Cephalosporium acremonium, HSf-64; Fusarium chlamydosporum, HSf-65; F. oxysporum, HSf-67; Penicillium oxalicum, HSf-68; Unidentified, HSf-69; F. pallidoroseum, HSf-71; Alternaria alternata, HSf-72; Aspergillus fumigatus, HSf-73; P. funiculosum, HSf-74; Drechslera tetramara, HSf-75; Cladosporium oxysporum, HSf-77; A. brassicae, HSf-78; A. brassicicola, HSf-80; Unidentified, HSf-81
Bacillus spp.	HSb-5, HSb-6, HSb-8, HSb-9, HSb-11, HSb-15, HSb-19, HSb-21, HSb-27, HSb-33, HSb-35, HSb-36, HSb-39, HSb-44	
Erwinia spp.	HSb-2, HSb-10, HSb-12	
Pseudomonas spp.	HSb-1, HSb-3, HSb-28	
Unidentified	HSb-18, HSb-7	
X. campestris pv. campestris	HSb-31	
Xanthomonas sp.	HSb-24	

4.3.2 Identification of epiphytic microorganisms

The fungal isolates (listed in Table 2) were identified as Alternaria alternata, Aspergillus fumigatus, A. niger, Cephalosporium acremonium, Cladosporium cladosporoides, C. oxysporum, Curvularia lunata, Drecheslera tetramara, Fusarium clamydosporum, F. oxysporum, F. pallidoroseum, Penicillium funiculosum and P. oxalicum. C. cladosporoides, A. alternata, Aspergillus fumigatus and F. oxysporum were found to be the most predominant fungi at later stages (90 days) of plant growth.

Among the bacterial isolates (Table 2) 14 were Bacilli. Pseudomonas, Erwinia, Arthrobacter and Xanthomonas spp. were also identified. The most frequently occurring bacterium was HSb-19. This was found invariably on all the cultivars during all the stages of plant growth. The black-rot pathogen X. campestris pv. campestris(HSb-31) was also isolated from B. campestris var. T-9; B. juncea var. RLM-619, Krishna, RH 78-59, RLM-514 and RH-8113 at the 3rd stage (90 day) as a symptomless epiphyte (Resident phase).

4.3.3 In vitro evaluation of phylloplane microorganisms for antagonism to X. campestris pv. campestris

All the phylloplane fungal isolates were evaluated for their antagonistic potential against black-rot pathogen. None of these was found to be antagonistic to the test bacterium.

Thirteen epiphytic bacterial isolates namely HSb-5, HSb-6, HSb-8, HSb-9, HSb-11, HSb-15, HSb-19, HSb-21, HSB-33, HSb-35, HSb-36, HSb-39 and HSb-44 produced inhibition zones of varying degrees (Table 3). On the basis of the above observations and interpreting these with standard zone size interpretation chart (Appendix I) they were considered to be antagonistic to X. campestris pv. campestris. The isolates HSb-8, HSb-11,

Table 3. Screening of phylloplane bacteria for *in vitro* inhibition of X. *campestris* pv. *campestris*

Isolate	Inhibition annulus(mm^2)	Isolate	Inhibition annulus(mm^2)
HSb-1	0	HSb-18	0
HSb-2	0	HSb-19	905
HSb-3	0	HSb-21	1002
HSb-4	0	HSb-24	0
HSb-5	549	HSb-27	0
HSb-6	603	HSb-28	0
HSb-7	0	HSb-31	0
HSb-8	779	HSb-33	477
HSb-9	650	HSb-35	458
HSb-10	0	HSb-36	490
HSb-11	725	HSb-39	593
HSb-12	0	HSb-44	791
HSb-15	644		

Phylloplane bacteria showing inhibition against the test bacterium belong to the *Bacillus* group

HSb-19, HSb-21 and HSb-44 produced comparatively larger inhibition zones (Plates II and III). From these studies bacterial isolate HSb-19 was selected for the present investigations as a biocontrol agent because of its consistent presence as an epiphyte on most of the Brassica cultivars examined during all the stages of crop growth. HSb-19 was Gram positive, small celled, endospore forming member of the genus Bacillus.

4.4 Isolation of antibiotic resistant mutants

4.4.1 Antibiotic sensitivity

Sensitivity of the test pathogen and antagonist against the antibiotics evaluated is presented in Appendix II. It was found that both the test organisms were sensitive to tetracycline, kanamycin, chloramphenicol, nalidixic acid, ledramycin and erythromycin. Therefore, chloramphenicol and tetracycline were selected for developing resistant mutants of pv. campestris and antagonist (HSb-19) respectively.

4.4.2 Antibiotic resistant mutants

The minimum inhibitory concentrations (MIC) of chloramphenicol and tetracycline against pv. campestris and antagonist were 2 $\mu g\ ml^{-1}$ and 4 $\mu g\ ml^{-1}$ respectively. Taking the MIC as the bench mark the strains of pv. campestris resistant to chloramphenicol (25 $\mu g\ ml^{-1}$) and antagonist (HSb-19) to tetracycline (20 $\mu g\ ml^{-1}$) were isolated. Both these resistant mutants developed were similar in all the characters to their parental types.

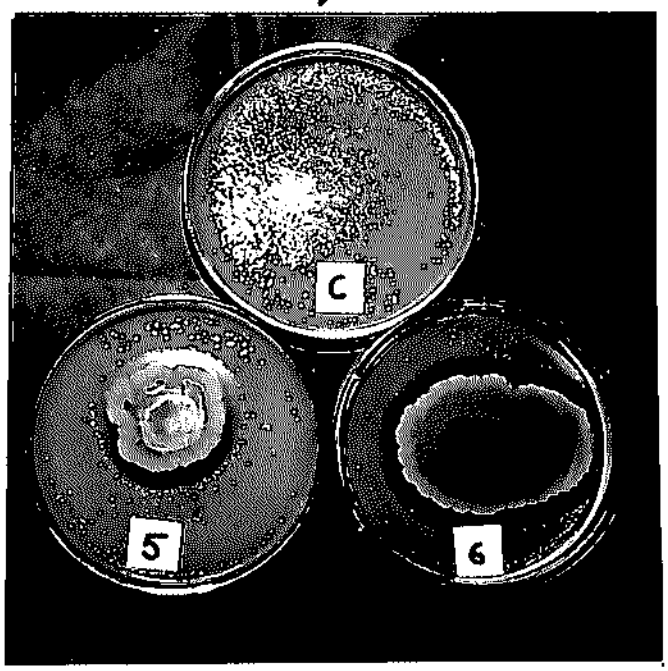
4.5 Bioefficacy of the antagonist

4.5.1 Evaluation of host inoculation methods

The progress of disease development by all the inoculation techniques used is presented in Table 4. The perusal of the data indicates that leaf

PLATE II

1



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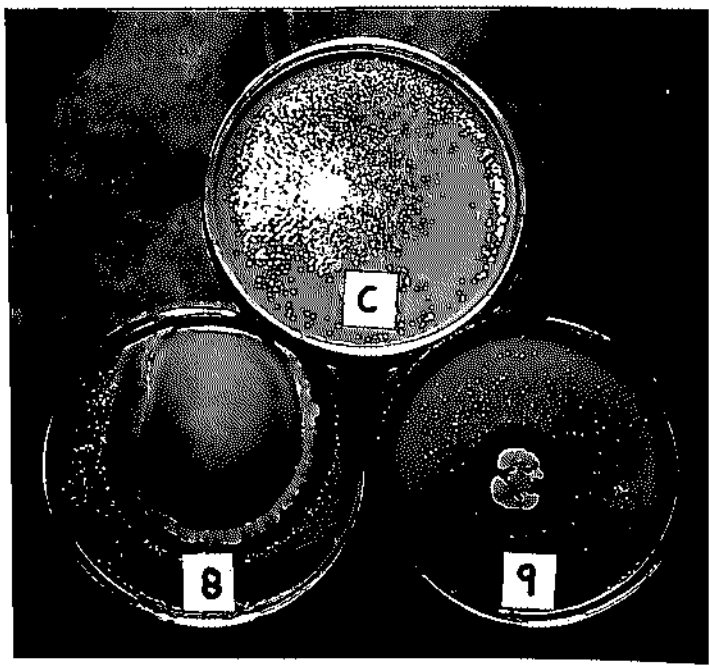


3

INHIBITION OF X. CAMPESTRIS PV. CAMPESTRIS BY ANTAGONISTS

C = CHECK

1 = Hsb-5, Hsb-6

2 = Hsb-8, Hsb-9

3 = Hsb-11, Hsb-15

Table 4. Evaluation of host inoculation methods

Inoculation technique	Symptoms*				
	Days after inoculation				
	2	5	10	15	20
Leaf infiltration	+	+	++	+++	+++
Clip inoculation	-	+	++	+++	+++
Wound inoculation	-	-	-	-	-
Hydothode inoculation	-	-	-	-	-

*No symptom -; Chlorosis +; Chlorosis+necrosis ++; Necrosis+vein blackening +++

infiltration and clip inoculation induced leaf chlorosis, necrosis and vein blackening within 15 days (Plate IV). Hydathode and wound inoculations were not successful in B. juncea as there was no expression of symptoms till 15 days or later. Clip inoculation technique was thus selected for further studies.

4.5.2 Determination of optimum number of cells of pathogen: antagonist against black rot control

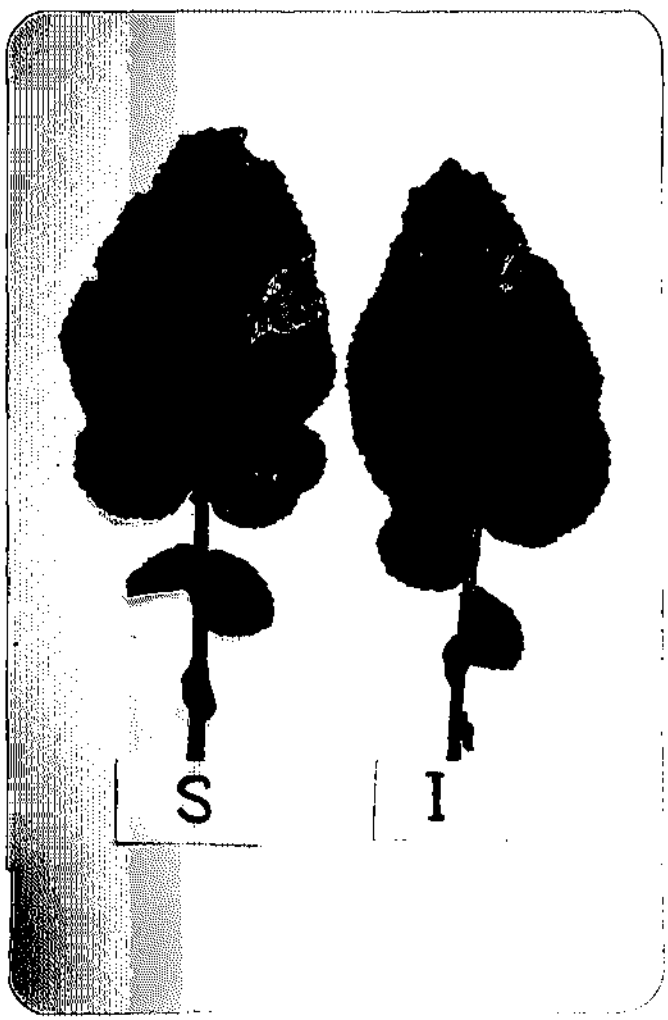
Data in Table 5 indicate that there was considerable difference in disease intensity (% leaf area infected) and the ratio of pathogen:antagonist. Minimum disease was observed with 1:20 (pathogen:antagonist) ratio; 1:10 ratio was also equally effective in controlling the disease by all the three methods of inoculation used. No disease control was obtained when both the test organisms were inoculated in equal proportion (1:1). The 48 h old cell free culture filtrate of antagonist also was ineffective.

4.5.3 In vivo evaluation of antagonist

The biocontrol potential of the antagonist (HSb-19) was compared with standard recommended chemicals/antibiotics and the results are presented in Table 6. It is evident from the data that the antagonist used could serve as a successful biocontrol agent. Although there was no significant difference in the disease incidence in the first 30 days, thereafter the differences were significant among various treatment.

Antagonist cell suspension spray 24 and 72 h prior to pathogen inoculation (pre-inoculation) gave 82.42 and 83.15 per cent disease control over pathogen inoculated check, respectively. The per cent disease index of the above treatments was significantly lower (8.0%) than streptocycline (17.0%), Dithane M-45 (29.83%) and copper oxychloride (24.16%). The antagonist as post-inoculation spray (30-43%) was less effective when

PLATE IV



HOST INOCULATION TECHNIQUES

S = CLIP INOCULATION

I = LEAF INFILTRATION

Table 5. Determination of optimum pathogen:antagonist ratio for black rot control

Method of inoculation	Leaf area infected (%)								
	Inoculation								
	Spray			Clip			Infiltration		
Treatment ratio	Days after inoculation								
	2	10	25	2	10	25	2	10	25
Xcc	0	5	25	0	20	38	2	19	30
Xcc+Ant (1:1)	0	5	7	0	11	30	3	14	31
Xcc+Ant (1:10)	0	0	0	0	0	5	0	5	8
Xcc+Ant (1:20)	0	0	0	0	0	3	0	5	5
Xcc+Ant (CF)	0	8	21	0	15	28	0	18	35
Control (water sterile)	0	0	0	0	0	0	0	0	0

Table 6. Biocontrol efficacy of antagonist (HSb-19) against black rot of B. juncea

Treatment		Disease index (%) Days after inoculation 30	40	50	60	Disease control (%)
Xcc		3.99	28.16	38.00	47.50	-
Ant		4.50	5.50	5.50	8.00	83.15
Ant (CF)		5.00	22.66	26.00	34.33	27.72
Xcc+Ant (Cells)	Sim	6.00	10.66	12.83	19.33	59.30
Xcc+Ant (CF)	Sim	6.00	25.33	33.66	40.83	14.04
Ant (Cell)⟶ Xcc (24 h)	Seq	5.00	6.00	7.50	8.00	83.15
Ant (Cell)⟶Xcc (72 h)	Seq	5.00	5.50	8.00	8.00	83.15
Xcc ⟶Ant (24 h)	Seq	6.00	12.83	17.83	33.00	30.52
Xcc ⟶Ant (72 h)	Seq	5.00	15.66	22.83	26.83	43.51
Seed treatment (Ant cell)		5.00	10.66	16.83	25.33	46.67
Streptocycline (250 ppm)		5.00	12.83	16.00	17.00	64.21
Dithane M-45 (0.2%)		4.50	16.83	25.83	29.83	37.20
Copper oxychloride (0.2%)		4.00	12.83	15.50	24.16	49.13
Check (No treatment)		6.00	24.66	29.16	36.16	-
CD at 5%		NS	4.88	5.29	7.16	

CF = Culture filtrate; Sim = Simultaneous inoculation;
Seq = Sequential inoculation

compared to pre-inoculation (82-83%) and simultaneous inoculation (59%) in checking the disease. Cell free culture filtrate of antagonist on co-inoculation was ineffective in reducing the disease. Seed treatment with antagonist cell suspension also provided reduction in disease by 46 per cent.

4.6 Mechanism of action of antagonist in the control of black-rot pathogen

4.6.1 Studies on pH alteration during antagonism

a) pH optima of antagonist and pathogen: The observations on the growth of both the test organisms on media of various pH are presented in Table 7. The results reveal that the maximum growth of X. campestris pv. campestris occurred at pH 6.0 to 7.5. However, its growth was retarded below and above the said range of pH. Whereas, the antagonist was capable of growing at a wider range of pH (5.5 - 8.5).

b) Influence of antagonist on the multiplication of pathogen in buffered and unbuffered media

Perusal of the data (Table 8) indicate that both the test organisms increased the pH of the buffered and unbuffered media. X. campestris pv. campestris increased the pH from 6.7 to 8.27 and 8.22 and antagonist from 6.68 to 8.14 and 8.21 of buffered and unbuffered broth, respectively. There was parallel increase in pH in dual and sequential inoculations of pathogen and antagonist.

Bacterial populations taken at different intervals during the growing period are presented in Table 9. The population of X. campestris pv. campestris and antagonist (inoculated separately) increased with time continuously in buffered as well as unbuffered media. However, in dual inoculations (simultaneous) the population of pv. campestris exhibited a decreasing pattern after 24 to 72 h. No viable cells (Xcc) could be

Table 7. Effect of pH on growth of X. campestris pv. campestris and antagonist (HSb-19)

pH	Colony growth (72 h)*	
	Xcc	Ant
4.5	0	+
5.0	+	++
5.5	+	+++
6.0	+++	+++
6.5	+++	+++
7.0	+++	+++
7.5	+++	+++
8.0	++	+++
8.5	+	+++

*Colony size - 0, No growth; +, 1 mm; ++, 2 mm; +++, 4 mm.

Table 8. *In vitro* effect of antagonist (HSb-19) and pathogen (Xcc) on pH in buffered and unbuffered media

Treatment	pH at time interval (h)				
	0	24	48	72	96
Buffered					
Xcc	6.70	6.76	7.07	7.62	8.27
Ant	6.68	6.53	6.44	6.39	8.14
Xcc+Ant (0 h)	6.70	6.53	6.09	7.58	7.85
Xcc+Ant (24 h)	6.78	6.98	7.54	8.32	8.55
Ant+Xcc (24 h)	5.63	5.81	6.38	7.65	8.52
Unbuffered					
Xcc	6.71	7.13	7.11	7.56	8.22
Ant	6.68	7.11	6.46	7.08	8.21
Xcc+Ant (0 h)	6.66	5.94	5.70	8.14	8.25
Xcc+Ant (24 h)	6.97	7.05	7.75	7.87	8.66
Ant+Xcc (24 h)	5.00	6.58	6.38	8.03	8.56
CD at 5%	NS	NS	NS	NS	NS

Table 9. In vitro effect of antagonist (HSb-19) on pathogen (Xcc) population in buffered and unbuffered media

Treatment	Organism	Population (cfu ml^{-1})				
		Time intervals(h)				
		0	24	48	72	96
Buffered						
Xcc	Xcc	$1.0x10^7$	$2.4x10^9$	$1.0x10^{11}$	$4.0x10^{11}$	$2.5x10^{12}$
Ant	Ant	$8.0x10^5$	$1.4x10^6$	$8.0x10^7$	$1.8x10^8$	$4.0x10^8$
Xcc+Ant (0 h) Sim	Xcc	$1.0x10^7$	$6.0x10^3$	$3.0x10^5$	$1.0x10^4$	0
	Ant	$8.0x10^5$	$1.6x10^7$	$3.0x10^7$	$7.0x10^7$	$3.9x10^8$
Ant→Xcc (24 h) Seq	Xcc	-	$2.0x10^2$	0	0	0
	Ant	$8.0x10^5$	$3.0x10^7$	$1.0x10^8$	$2.4x10^8$	$4.0x10^8$
Xcc→Ant (24 h) Seq	Xcc	$1.0x10^7$	$8.0x10^{10}$	$1.0x10^{11}$	$1.3x10^{12}$	$3.2x10^7$
	Ant	-	$5.0x10^4$	$2.0x10^6$	$5.0x10^7$	$6.6x10^7$
Unbuffered						
Xcc	Xcc	$1.2x10^7$	$4.8x10^9$	$9.8x10^{10}$	$2.4x10^{11}$	$1.0x10^{12}$
Ant	Ant	$8.5x10^5$	$1.6x10^6$	$7.0x10^7$	$1.0x10^8$	$1.4x10^8$
Xcc+Ant (0 h) Sim	Xcc	$1.2x10^7$	$2.0x10^4$	$1.8x10^3$	$2.2x10^2$	0
	Ant	$8.5x10^5$	$9.5x10^5$	$3.7x10^6$	$8.0x10^6$	$2.0x10^7$
Ant→Xcc (24 h) Seq	Xcc	-	$1.0x10^3$	0	0	0
	Ant	$8.5x10^5$	$5.0x10^6$	$6.0x10^7$	$1.4x10^8$	$1.6x10^8$
Xcc→Ant (24 h) Seq	Xcc	$1.2x10^7$	$9.6x10^{10}$	$3.0x10^{11}$	$1.2x10^{12}$	$2.5x10^5$
	Ant	-	$3.3x10^5$	$1.0x10^6$	$1.4x10^7$	$2.0x10^7$

recovered/retrieved at 96 h from the growing medium. In sequential inoculation (pathogen inoculated 24 h after antagonist) pv. campestris could not be detected after 48 h in the growing medium. However, its population continued to increase in the initial phase of 72 h before decreasing when the pathogen was inoculated 24 h before the antagonist. The antagonist population increased in all the treatments irrespective of the sequence of inoculation.

4.6.2 Competition

a) Effect of inoculum density: It is evident from the data (Table 10) that the co-inoculation of pathogen with antagonist in varying ratios resulted in the restriction of pathogen multiplication at the early stage of growth (24-48 h) and at later stages (72-96 h) its (Xcc) complete elimination. No viable cells of pathogen were recovered after 72 h when the antagonist population was half or more than the pathogen population. However, when the pathogen population was 5 to 10 folds higher than the antagonist population, the inhibition of pathogen was delayed. The antagonist population in all the co-inoculations demonstrated a continuous increase to 10^8 - 10^9 cfu ml^{-1} as in control, irrespective of the pathogen population at the time of inoculation.

b) Pathogen growth in culture filtrate of antagonist: Results on nutrient competition and multiplication of the pathogen in the cell free culture filtrate of antagonist obtained at different intervals of its growth are presented in Table 11. The antagonist culture filtrate (autoclaved and sterilized) obtained after 24, 48, 72 and 96 h did not support the multiplication of X. campestris pv. campestris. Similar observations were observed in filter sterilized supernatant supplemented with YPG medium.

Table 10. In vitro effect of antagonist (HSb-19) inoculum on the growth of X. campestris pv. campestris

Treatment ratio Xcc : Ant	Viable cell count of	Population (cfu ml^{-1}) Time intervals (h) 0	24	48	72	96
1:1	Xcc	$1.3x10^6$	$3.0x10^6$	$5.3x10^4$	0	0
	Ant	$1.8x10^6$	$2.4x10^6$	$8.2x10^7$	$1.0x10^8$	$4.0x10^8$
1:2	Xcc	$1.4x10^6$	$4.0x10^4$	$3.0x10^3$	0	0
	Ant	$3.0x10^6$	$4.9x10^6$	$9.2x10^7$	$1.9x10^8$	$4.1x10^8$
1:5	Xcc	$1.5x10^6$	$2.0x10^4$	$1.6x10^3$	0	0
	Ant	$6.6x10^6$	$7.8x10^6$	$2.1x10^8$	$7.9x10^9$	$8.0x10^9$
1:10	Xcc	$1.2x10^6$	$1.8x10^4$	$1.1x10^2$	0	0
	Ant	$8.2x10^6$	$9.7x10^6$	$4.4x10^8$	$1.2x10^{10}$	$1.8x10^{10}$
2:1	Xcc	$2.8x10^6$	$1.1x10^7$	$5.6x10^4$	0	0
	Ant	$1.4x10^6$	$9.8x10^6$	$7.4x10^7$	$8.0x10^7$	$5.4x10^8$
5:1	Xcc	$8.4x10^6$	$2.8x10^7$	$1.9x10^6$	$1.7x10^4$	0
	Ant	$1.8x10^6$	$2.6x10^6$	$3.4x10^7$	$7.5x10^8$	$8.0x10^8$
10:1	Xcc	$1.0x10^7$	$5.2x10^7$	$4.1x10^6$	$3.3x10^4$	0
	Ant	$1.5x10^6$	$2.1x10^6$	$6.1x10^7$	$8.9x10^8$	$9.0x10^8$
Check	Xcc	$1.8x10^6$	$2.2x10^7$	$3.3x10^8$	$1.1x10^9$	$3.0x10^{10}$
	Ant	$1.7x10^6$	$4.1x10^6$	$7.2x10^7$	$8.1x10^8$	$1.0x10^9$

Table 11. Multiplication of X. campestris pv. campestris in the cell free culture filtrate (CF) of antagonist (HSb-19)

Treatment of culture filtrate of antagonist	Status of inoculation 2×10^7 cfu ml^{-1}	Population (cfu ml^{-1}, 48 h) Culture filtrate obtained at different time interval (h)			
		24	48	72	96
Autoclaved					
CF	Uninoculated	0	0	0	0
CF	Xcc	1.5×10^6	1.8×10^5	1.6×10^5	1.6×10^5
CF+YPG	Xcc	2.7×10^7	2.2×10^7	2.5×10^7	8.5×10^6
YPG (check)	Xcc	2.0×10^9	-	-	-
Filter sterilized					
CF	Uninoculated	0	0	0	0
CF	Xcc	1.4×10^6	3.6×10^5	2.6×10^5	2.9×10^5
CF+YPG	Xcc	1.6×10^6	7.1×10^6	2.6×10^6	1.2×10^5
YPG (check)	Xcc	2.0×10^9	-	-	-

However, the pathogen could multiply with a marginal increase (from 2 x 10^7 to 2.2 x 10^7 cfu ml^{-1}) in autoclaved supplemented culture filtrate obtained after 24, 48 and 72 h but not in 96 h culture filtrate.

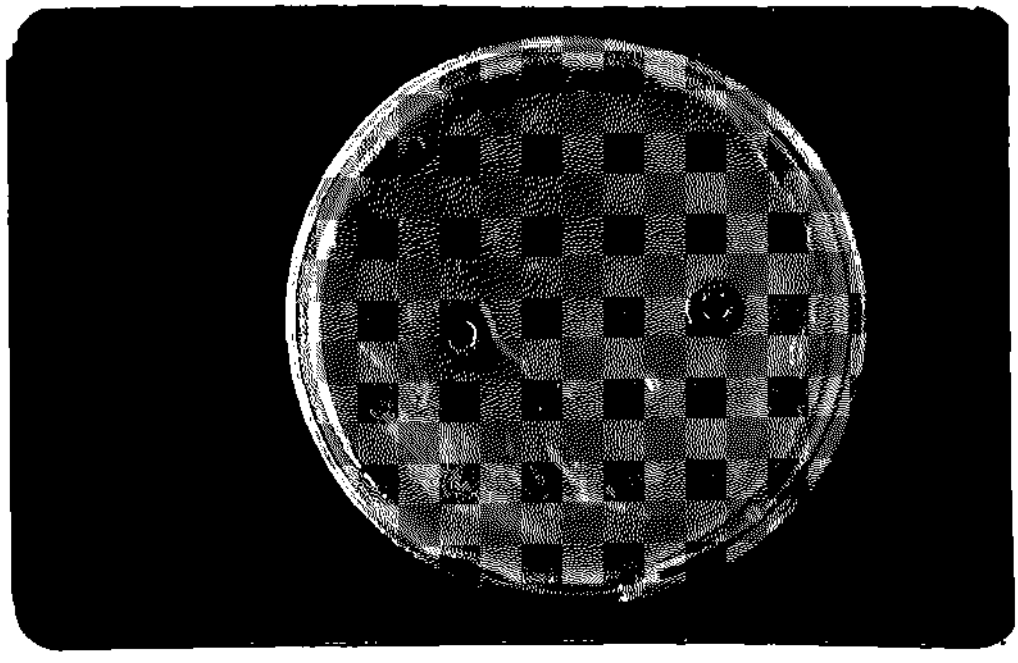
4.6.3 Antibiosis

a) Antimetabolite production: The inhibitory activity of the cell free culture filtrate of the antagonist was examined against X. campestris pv. campestris (Plates V and VI). The data presented (Table 12) indicate that with the increase in the volume of filter sterilized culture filtrate in each well, there was an increase in the inhibition annulus (8.6 and 157 mm^2) of the pathogen. On heating the culture filtrate (100°C) for 10 min. the inhibition was pronounced. This increased activity may be due to evaporation of the supernatant, resulting in increased concentration of antibacterial compound. These findings suggest that the antibacterial metabolite produced by the antagonist may be non proteinaceous. However, autoclaving and incineration of the culture filtrate destroyed the bactericidal property of the antagonistic compound. The culture filtrate from iron minus Watanabe medium was as effective in pathogen inhibition as the filter sterilized culture filtrate from iron containing medium but did not exhibit any increased inhibition. Thus siderophores, if produced by the antagonist (HSb-19), did not appear to have a role in antibacterial activity.

b) Siderophores: The ability of antagonist to produce siderphores in iron deficient conditions was tested on MM-9 medium modified with chrome azural-S agar plates. Orange halos were observed around the spot-inoculated antagonist colonies on the rich blue agar medium after 4 days (Plate VII). However, no change in colour was observed in pv. campestris colonies. The Bacillus (HSb-19) produced siderophores which formed an

PLATE V

1



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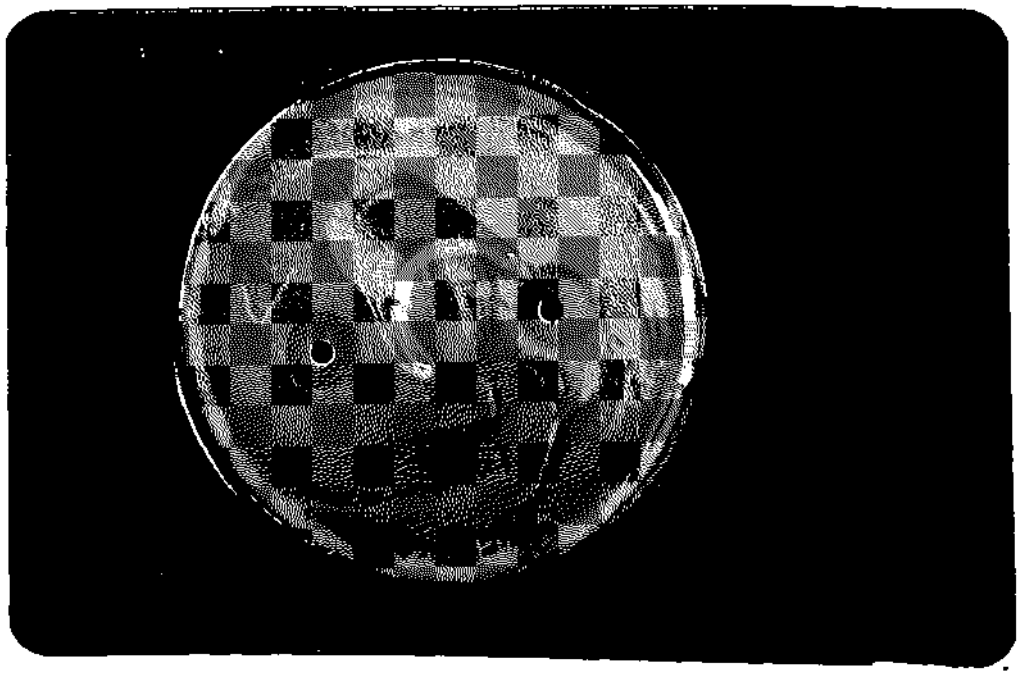


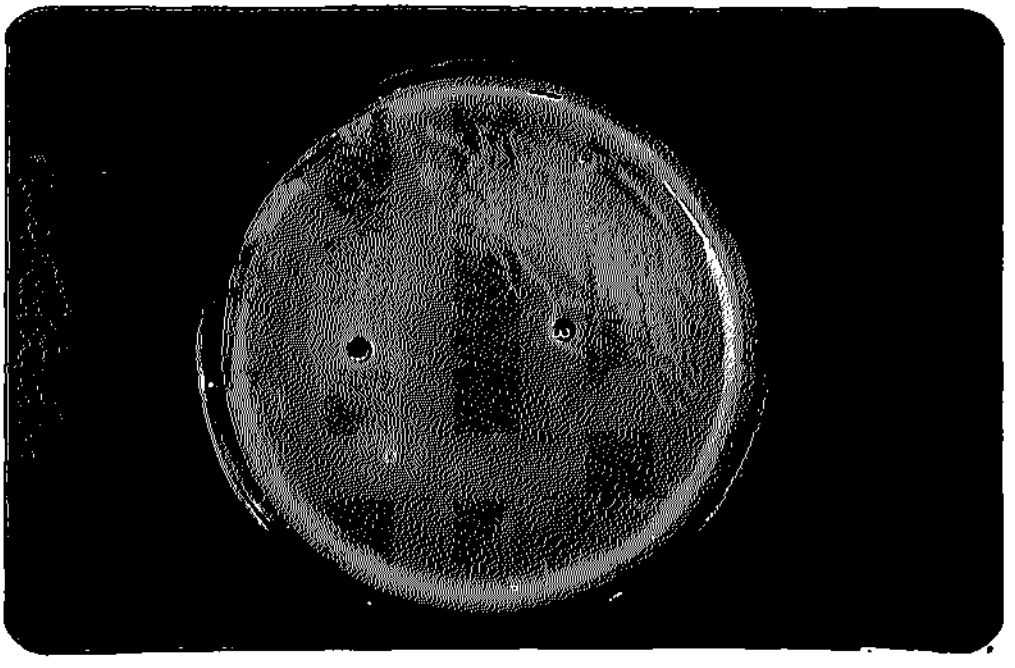
INHIBITORY ACTIVITY OF CELL FREE CULTURE FILTRATE OF ANTAGONIST (Hsb-19) AGAINST THE TEST PATHOGEN

1 FILTER STERILIZED

2 HEATED (100 °C)

PLATE VI

3



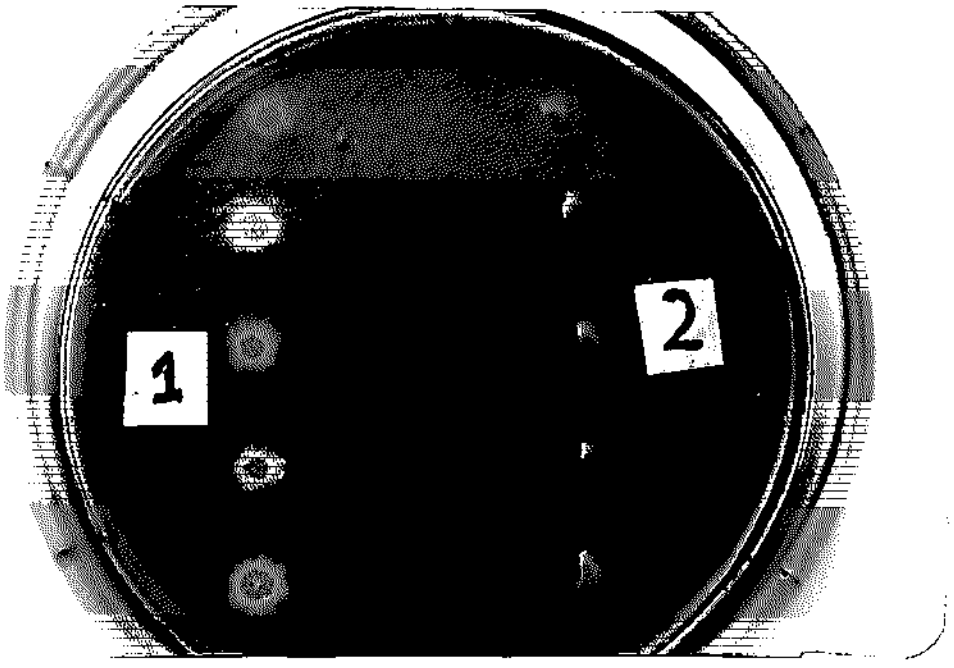
4

INHIBITORY ACTIVITY OF CELL FREE CULTURE FILTRATE OF ANTAGONIST (Hsb-19) AGAINST THE TEST PATHOGEN

3 = AUTOCLAVED

4 = IRON MINUS (SIDEROPHORES)

PLATE VII



PRODUCTION OF SIDEROPHORES

1 = ORANGE HALO FORMED AROUND SPOT-INOCULATED COLONIES OF BACILLUS STRAIN (Hsb-19)

2. = NO HALO FORMED AROUND SPOT-INOCULATED COLONIES OF X. CAMPESTRIS PV. CAMPESTRIS

Table 12. Inhibitory effect of cell free culture filtrate of antagonnist (HSb-19) on black rot pathogen (Xcc)

Treatment (CF)	Inhibition annulus (mm^2)			
	CF/well (µl)			
	20	40	60	80
Filter sterilized	8.60	75.39	154.30	157.07
Heated	43.50	157.07	181.42	207.34
Autoclaved	0	0	0	0
Incinerated	0	0	0	0
Fe^{-}	8.60	75.39	113.97	157.07

iron ligand complex from the blue coloured iron dye complex and the dye is released which results in an orange halo.

4.6.4 Membrane permeability-electrolyte leakage

The effect on cell permeability was determined by measuring electrolyte leakage. The data (Table 13) revealed that in 48 h there was an increased conductivity of leachates in pathogen inoculated (12.9 μ mhos cm^{-2}) leaf tissues than from untreated controls (8.4 μ mhos cm^{-2}). However, dual inoculation with antagonist cells, showed a decrease in conductivity (10.0 μ mhos cm^{-2}) during the said period. Similar corresponding increase was recorded at 240 and 260 h after inoculation. At 72 and 96 h, did not reveal much variation in electrolyte conductivity in any of the treatments from untreated controls.

The leaf leachate analysis at 10 and 15 days after inoculation for protein, amino acids and sugars is presented in Table 14. The total proteins leached out was significantly higher in pathogen inoculated (228.5 μg cm^{-2}) leaves than from untreated controls (109.0 μg cm^{-2}) and all other treatments. In pathogen inoculated leaves the maximum proteins (234 μg cm^{-2}) were leached in 10 days. Co-inoculation of pathogen with antagonist cells (148 μg cm^{-2}) or its cell free culture filtrate (194 μg cm^{-2}) significantly reduced the leaching of proteins than from pathogen inoculated samples. Leakage of aminoacid and total sugars also showed similar increasing trend as in proteins with more than 100 per cent increase in pathogen inoculated leaves than from untreated controls. Co-inoculation with antagonist cells, significantly reduced these leachates.

Table 13. Permeability alterations in Brassica juncea (RH-30) in response to pathogen (Xcc), antagonist (HSb-19), inoculations

Treatments	Conductivity (μ mhos cm^{-2}) Time after inoculation (h)					
	24	48	72	96	240	360
Pathogen	9.7	12.9	12.6	12.6	17.1	18.3
Pathogen+Antagonist	9.7	10.0	11.5	11.8	14.1	15.8
Pathogen+Culture filtrate of antagonist	9.7	12.4	12.3	12.4	16.9	16.8
Antagonist	9.6	9.7	10.0	12.2	14.8	15.8
Control	9.7	8.4	11.0	11.9	14.4	15.3

Table 14. Proteins, sugars and amino acids in leachates of leaf tissues, inoculated with pathogen (Xcc) and antagonist (HSb-19)

Time (days)	Proteins ($\mu g\ cm^{-2}$) Inoculated with					
	Xcc	Xcc+Ant	Xcc+Ant (CF)	Ant	Uninoculated control	Mean
10	234.0	148.0	194.0	157.0	91.0	162.00
15	223.0	186.0	200.0	187.0	127.0	182.60
Mean	228.5	167.0	192.0	172.0	109.0	
CD at 5%	Days = 7.07; Treatment = 11.18; Days x Treatment = 15.81					
	Sugars ($\mu g\ cm^{-2}$)					
10	101.5	51.5	74.2	57.0	49.1	66.60
15	114.0	69.9	106.6	49.9	42.7	86.40
Mean	107.0	60.7	90.4	53.4	54.9	
CD at 5%	Days = 5.20; Treatment = 8.22; Days x Treatment = 11.63					
	Amino acids ($\mu g\ cm^{-2}$)					
10	56.2	33.2	55.3	37.4	26.7	41.77
15	75.5	51.1	74.2	50.2	36.7	57.38
Mean	65.4	42.2	64.8	43.82	31.7	
CD at 5%	Days = 1.7; Treatment = 2.7; Days x Treatment = 3.8					

CF = Culture filtrate of antagonist

4.7 Monitoring of multiplication and progression of the pathogen and antagonist in the host

Influence of antagonist on the multiplication of X. campestris pv. campestris in the leaf tissues of Brassica juncea (cv. RH-30) was studied with a view to quantify the impact of the antagonist on the fate of the pathogen. Inoculation of pathogen suspension (10^7 cfu ml^{-1}) alone (Table 15) resulted in the entry of 5.5 x 10^3 cells/segment of the leaf tissues. The multiplication started after 1 h of inoculation, reaching 3 x 10^5 cfu/1st segment (1.2 x 10^6 cfu cm^{-2}) in 6 h. Thereafter until 72 h, the pathogen population in the first segment remained more or less constant, however, its linear spread along the midrib of the inoculated leaf was gradual until 120 h reaching upto 20 mm distance. The multiplication resumed and the pathogen population started increasing after 96 h in the distal ends also, reaching a maximum in 240 h, 1.6 x 10^7 cfu/segment (6.4 x 10^7 cfu cm^{-2}). During the said period, the pathogen had spread all along the mid vein of the leaf reaching the terminal end of petiole. With the progress of disease and appearance of black rot symptoms, the bacterial population in the zone of inoculation was less as compared to its number in the distal part of the leaf.

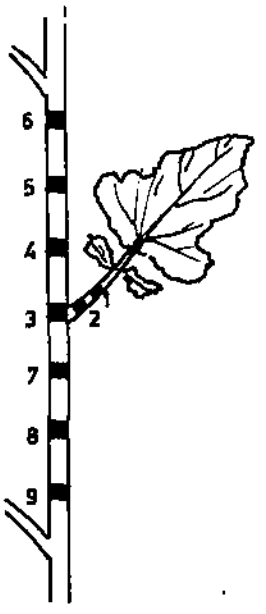
The pathogen spread and multiplication in the stem (upper and lower internode) from the inoculated leaf was further studied after 30 days and the data is presented in Table 16. The pathogen population was 1.6 to 4.8 x 10^7 cfu cm^{-2} in the distal end of leaf petioles. The xylem of the stem at the point of attachment of petiole showed higher (2.7 x 10^7 cfu cm^{-2}) bacterial number than the phloem (1.4 x 10^6 cfu cm^{-2}) of the same zone. Intriguingly no movement of the pathogen was observed in the upward direction (upper internode of the inoculated leaf). While it moved in the

Table 15. Multiplication and progression of X. campestris pv. campestris in B. juncea leaves

Time (h) after inoculation	Population of Xcc cfu/segment (2.5 mm)									
	Segment number									
	1	2	3	4	5	6	7	8	9	10
	Distance of each segment from the point of inoculation (mm)									
	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0		
0	5.5×10^{3}	0	0	0	0	0	0	0		
1	4.8×10^{3}	0	0	0	0	0	0	0		
2	1.1×10^{4}	8.0×10^{2}	4.0×10^{2}	3.4×10^{2}	0	0	0	0		
4	3.0×10^{4}	8.0×10^{2}	3.8×10^{2}	2.5×10^{2}	0	0	0	0		
6	3.0×10^{5}	7.5×10^{2}	2.0×10^{2}	1.1×10^{2}	2.3×10^{2}	0	0	0		
8	2.5×10^{5}	8.5×10^{2}	3.2×10^{2}	2.2×10^{2}	2.0×10^{2}	0	0	0		
12	1.0×10^{5}	6.0×10^{2}	9.3×10^{2}	2.1×10^{2}	2.4×10^{2}	1.4×10^{2}	0	0		
24	2.3×10^{5}	8.0×10^{2}	4.0×10^{2}	3.0×10^{2}	2.1×10^{2}	2.2×10^{2}	0	0		
48	2.5×10^{5}	7.0×10^{3}	2.7×10^{2}	1.3×10^{2}	2.1×10^{1}	2.0×10^{2}	0	0		
72	2.7×10^{5}	1.3×10^{5}	3.0×10^{4}	5.0×10^{3}	6.4×10^{3}	2.0×10^{2}	1.6×10^{2}	0		
96	3.9×10^{5}	2.0×10^{5}	3.0×10^{5}	3.0×10^{3}	4.0×10^{3}	4.5×10^{2}	3.0×10^{3}	0		
120	5.2×10^{5}	1.8×10^{4}	1.2×10^{5}	5.0×10^{3}	5.0×10^{3}	4.8×10^{2}	5.0×10^{2}	5.0×10^{2}		
	Distance of each segment from the point of inoculation (mm)									
	10	20	30	40	50	60	70	80	90	100
240	6.0×10^{4}	6.5×10^{4}	6.8×10^{5}	6.9×10^{5}	9.0×10^{5}	1.0×10^{6}	1.5×10^{7}	1.4×10^{7}	1.6×10^{7}	1.5×10^{7}
480	1.5×10^{4}	1.3×10^{7}	1.4×10^{7}	1.2×10^{7}	1.2×10^{7}	1.5×10^{7}	1.5×10^{7}	1.4×10^{7}	1.4×10^{7}	1.5×10^{7}

Table 16. Number of X. campestris pv. campestris cells recovered from xylem and phloem of the two internodes (above/ below) of the inoculated leaf after 30 days of inoculations

Segment No.	Xcc population (cfu cm^{-2})	
	Xylem	Phloem
1	1.6×10^7	Xylem+Phloem
2	4.8×10^7	
3	2.7×10^7	1.4×10^6
4	0	0
5	0	0
6	0	0
7	1.6×10^7	1.5×10^5
8	1.7×10^7	7.6×10^5
9	1.8×10^7	3.8×10^4



downward direction with higher population in xylem (1.7 x 10^7 cfu cm^{-2}) than in the corresponding phloem tissues.

It is clear from the data presented in Table 17 that co-inoculation of X. campestris pv. campestris with the cell suspension of antagonist (HSb-19) resulted in restriction of pathogen multiplication and its gradual decline from 4.5 x 10^3 cfu/segment until no viable cells could be recovered after 72 h either in the first segment or anywhere along the leaf length of the inoculated leaf. The pathogen and antagonist did not move beyond 5 mm in the early period of inoculation (6 h). The antagonist population reached a maximum (6.4 x 10^4 cfu/segment) in 8 h and thereafter remained static (4 x 10^3 cfu/segment) in the zone of inoculation. There were no symptoms on such leaves except for slight drying of the cut edge of the leaf. Co-inoculation with cell free culture filtrate of the antagonist was ineffective in restricting the multiplication and spread of pathogen (Table 18) in the host leaves. There was a brief period in the early phase (4 h) of inoculation when the multiplication and spread of pathogen was suppressed. After 6 h of co-inoculation, the pathogen multiplied and spread unhindered moving 15 mm in 120 h with a population of 6 x 10^5 cfu/first segment. In 480 h the pathogen population reached to 2.1 x 10^7 cfu/segment (8 x 10^7 cfu cm^{-2}) and it moved along the midvein to the entire leaf length.

4.8 Nutritional status of inoculated and healthy leaves

Nutritional status (N, P, K, total sugars, crude protein, phenols and Zn) of the leaves analysed at 50 and 70 days (10 and 30 days after spray inoculation) of plant growth is presented in Table 19. At 10 days, in pathogen inoculated plants the total nitrogen and crude protein decreased

Table 17. Multiplication and progression of the pathogen (Xcc) in *B. juncea* leaves on co-inoculation with antagonist (HSb-19)

Time (h)	Bacterial population (cfu/segment 0.25 cm^{-2})			
	Segment number			
	1		2	
	Xcc	Ant	Xcc	Ant
0	$4.5x10^3$	$1.0x10^2$	0	0
1	$4.1x10^3$	$2.0x10^3$	0	0
2	$5.5x10^2$	$2.5x10^2$	$1.4x10^2$	8.0x10
4	$4.8x10^2$	$5.0x10^2$	8.1x10	9.2x10
6	$3.9x10^2$	$7.9x10^3$	5.4x10	$1.0x10^2$
8	$2.0x10^2$	$3.1x10^4$	0	2.0x10
12	8.0x10	$1.0x10^4$	0	0
24	5.0x10	$6.4x10^4$	0	0
48	2.2x10	$8.0x10^3$	0	0
72	0	$6.9x10^3$	0	0
96	0	$4.0x10^3$	0	0
120	0	$4.0x10^3$	0	0
240	0	$1.0x10^3$	0	0
480	0	$2.0x10^3$	0	0

Table 18. Effect of co-inoculation of cell free culture filtrate of antagonist (HSb-19) on the pathogen (Xcc) multiplication and progression in B. juncea

Time (h)	Xcc population cfu/segment (0.25 cm^2)									
	Segment number									
	1	2	3	4	5	6	7	8	9	10
	Distance of each segment from the point of inoculation (mm)									
	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0		
0	4.9×10^3	0	0	0	0	0	0	0		
1	4.1×10^3	0	0	0	0	0	0	0		
2	2.0×10^3	0	0	0	0	0	0	0		
4	3.1×10^3	0	0	0	0	0	0	0		
6	1.4×10^5	5.0×10^2	4.0×10^2	2.6×10^2	0	0	0	0		
8	1.1×10^5	9.0×10^3	3.0×10^2	2.5×10^2	0	0	0	0		
12	4.5×10^5	8.0×10^2	6.0×10^2	6.6×10^2	3.1×10^2	0	0	0		
24	2.5×10^4	7.2×10^2	1.7×10^2	6.4×10^2	2.4×10^2	0	0	0		
48	1.2×10^5	3.2×10^3	7.8×10^2	2.3×10^2	2.0×10^3	2.4×10^3	0	0		
72	8.0×10^5	2.7×10^4	3.2×10^3	3.0×10^3	2.0×10^3	2.1×10^3	0	0		
96	7.0×10^5	5.0×10^4	3.2×10^3	3.0×10^3	2.2×10^3	1.1×10^3	0	0		
120	6.0×10^5	4.2×10^5	6.8×10^4	9.1×10^3	2.4×10^3	1.4×10^3	0	0		
	Distance of each segment from the point of inoculation (mm)									
	10	20	30	40	50	60	70	80	90	100
240	3.9×10^4	1.2×10^5	2.4×10^5	4.0×10^6	6.6×10^6	6.2×10^6	5.8×10^6	6.2×10^6	7.9×10^6	7.8×10^6
480	1.1×10^4	1.3×10^5	1.2×10^7	1.4×10^7	1.4×10^7	1.5×10^7	1.4×10^7	1.8×10^6	2.1×10^7	1.8×10^7

Table 19. Nutritional status of Brassica juncea leaves inoculated with pathogen (Xcc) and antagonist (HSb-19) (% dry weight basis)

Treatments		Days after inoculation*														
		N		Protein		P		K		Total sugar		Red sugar		Zn (ppm)		Phenol
		D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_2
Pathogen (P)		4.00	1.71	25.00	10.68	0.303	0.262	4.14	3.04	4.08	5.21	1.45	1.75	51.2	47.2	0.85
Ant. Cell (C)		4.28	4.15	26.75	25.93	0.375	0.357	3.59	2.77	4.20	6.35	1.50	1.62	50.8	48.3	0.85
Ant. culture filtrate (CF)		4.38	3.40	27.37	21.25	0.285	0.250	4.06	3.12	5.00	5.00	1.75	1.43	53.6	48.9	0.91
Path.+Ant.(C)	Sim.	4.46	3.18	27.87	19.87	0.339	0.285	4.06	3.04	4.68	6.00	1.62	2.00	58.2	52.1	0.83
Path.+Ant.(CF)	Sim.	4.10	1.90	25.62	11.87	0.303	0.250	4.21	3.04	5.12	3.50	1.75	1.29	48.8	48.2	0.96
Ant.↔P (24 h)	Seq.	5.31	3.40	33.18	21.25	0.339	0.250	4.53	3.12	4.15	6.22	1.54	2.10	56.3	52.3	0.86
Ant.(C)→P (72 h)	Seq.	4.28	3.18	26.75	19.87	0.321	0.250	3.82	3.04	4.20	6.45	1.52	2.24	55.6	52.0	0.93
P→Ant.(C) (24 h)	Seq.	4.28	2.75	26.75	17.18	0.464	0.303	4.06	3.04	4.25	6.40	1.57	2.21	53.8	49.2	0.90
P→Ant.(C) (72 h)	Seq.	4.14	2.00	25.87	12.50	0.339	0.267	3.82	3.04	4.50	5.12	1.34	2.00	51.2	47.0	0.84
Streptocycline (250 ppm)		4.56	2.62	28.50	16.37	0.303	0.285	4.14	2.77	4.68	6.00	1.60	2.10	50.1	47.4	0.95
Dithane M-45(0.2%)		4.28	2.34	26.75	14.62	0.285	0.275	3.82	3.28	5.12	6.12	1.80	2.10	50.8	48.2	0.94
Cu-Oxychloride(0.2%)		4.28	2.75	26.75	17.18	0.339	0.303	4.14	3.04	4.28	6.28	1.44	2.00	52.1	48.2	0.85
Seed treatment Ant. (C)		4.56	3.56	28.50	22.75	0.321	0.285	2.21	2.57	5.00	6.22	1.62	2.00	53.6	50.1	0.85
Control(untreated)		5.93	4.15	37.06	25.93	0.303	0.267	4.03	3.04	4.12	5.12	1.50	1.70	51.1	48.2	0.95

*Spray inoculation at 40 days of plant growth

D_1 = 10 days after inoculation

D_2 = 30 days after inoculation

appreciably in comparison to untreated controls. The decline was more pronounced at 30 days after inoculation from 4.00 to 1.71% N and 25.0 to 10.68 per cent proteins in pathogen inoculated plants. Similar reduction in N and proteins was recorded in co-inoculation of pathogen with culture filtrate of the antagonist. However, co-inoculation with live cells of the antagonist in various sequences and antagonist alone, did not express striking decrease in nitrogen and protein content.

There was no noticable change in P and K content of leaves in any of the treatments. However, with plant maturity their level decreased both in untreated controls and other treatments. Total sugars in untreated control leaves increased as the plant matured (4.12 to 5.12%). Inoculation of pathogen, antagonist alone, or co-inoculation resulted in an increased sugar content of leaves. However, the increase in sugar content was more pronounced in antagonist and fungicidal spray treatments. The reducing sugar also gave parallel increase as total sugars. The phenolic content of leaves estimated at 30 days after inoculation did not show any measurable change in any of the treatments. The Zn concentration in leaves showed a slight decrease (in Brassica) irrespective of its treatments. The variation in Zn concentrations were not indicative of any specific trend in respect to disease appearance or its control.

CHAPTER V

DISCUSSION

Black rot of crucifers, caused by Xanthomonas campestris pv. campestris (Pammel) Dowson is well known in most of the crucifer growing countries in the world (Williams, 1980). It affects several cruciferous crops, viz., cabbage (Brassica oleracea L. var. capitata), cauliflower (B. oleraea L. var. itilica) and Chinese cabbage (B. pekinensis Rupr.). Heavy incidence of this disease on raya (B. juncea) was reported in Haryana during 1972-1973 causing a severe crop damage to the extent of 60 per cent (Satyavir et al., 1973). Petrie (1975) pointed out that if this disease becomes established in rape seed it would probably be devastating.

Limitations of plant disease control by conventional chemicals have become apparent in recent years. A number of chemicals once effective are now ineffective due to the development of resistance by the target pathogens. In addition to this, several chemicals have been banned or withdrawn in many developed countries because of their recognized hazards to the humans and the environment (Anonymous, 1987).

Alternative disease control strategies are now in much more demand to reduce or to eliminate the need for environmentally hazardous pesticides (Nelson, 1991). Biological control of plant pathogens has thus generated much interest and has been a subject of intense investigations in recent years (Cook and Baker, 1983). Baker and Cook (1974) pointed out that antagonists are likely to be acting continuously against potential pathogens in majority of the situations. Thus, the biological control is in constant operation in nature. This natural biological control leads to ecological balance between the antagonists and the pathogens.

During the recent past, most of the attention in biocontrol research has been focused on root pathogens, since diseases caused by such pathogens are difficult to control by conventional methods. Biological control on aerial plant surfaces is much less investigated, however, it is highly desirable tactic to add to the currently used disease control strategies (Spurr, 1981).

The logical start of any investigations in biocontrol of phytopathogens is the search for potential antagonists in the habitat in which the pathogen is normally found (Skidmore, 1976). It is now well recognised that leaf surface is inhabited by a large number of different micro-organisms including bacteria, yeast and fungi. Thus leaf microflora is a good natural source to search for effective antagonists for foliar pathogens (Spurr, 1981). Keeping this in view, the composition of phylloplane microflora of eleven Brassica genotypes was studied in the present investigation to find potential antagonist(s) for black rot pathogen. The results obtained herein indicate the presence of 17 fungal species on the leaf surface. These fungal species belonged to genera Alternaria, Aspergillus, Cladosporium, Curvularia, Cephalosporium, Drechslera, Fusarium and Penicillium. Parallely, Sharma and Tripathi (1981) reported 22 fungal genera from the phylloplane of B. juncea and B. campestris. Cladosporium spp. were found to be predominant in all the Brassica genotypes except cvs Kranti and Varuna of B. juncea throughout the growing season. More or less parallel observations were recorded on rapeseed (Tsuneda and Skoropad, 1978; Sharma and Gupta, 1979). In the present study it was further observed that in the early stages of plant growth, bacterial species were isolated more frequently than the fungal epiphytes. Similar observations have also been documented by Blakeman and Fokkema (1982) and Jindal and Thind (1990b). The 23 bacterial isolates obtained from Brassica phylloplane belonged to genera Arthrobacter, Bacillus,

Erwinia and Pseudomonas. Black-rot pathogen X. campestris pv.campestris (HSb-31) was also intercepted as a symptomless epiphyte on some of the cultivars, viz., B. campestris var. T-9; B. juncea var. Krishna, RH 7859, RLM-619, RLM-514 and RH-8113. Epiphytic presence of X. campestris pv. campestris on Brassica has also been reported by Kuan et al. in 1986.

None of the epiphytic fungi isolated in the present studies from the phylloplane of Brassica genotypes was found to possess any antagonistic potential against black rot pathogen. Although Cladosporium has been reported in literature to antagonize fungal pathogens (Corke and Hunter, 1979; Blakeman and Bordie, 1976), none of the three Cladosporium spp. isolated showed antagonism to X. campestris pv. campestris in the present investigations. Similarly Fusarium spp. were ineffective in inhibiting the black rot pathogen. Fusarium spp. however, have been found to reduce the sclerotial formation by Claviceps perpurea and also degrade toxic alkaloid ergotamine (Mower et al., 1975). Penicillium citrinum, Aspergillus niger and a white sterile fungus of the phylloplane fungi from clusterbean have been reported to reduce the incidence of bacterial blight of clusterbean caused by X. campestris pv. cyamopsidis (Parashar et al., 1992). Beer and Rundle (1980) have concluded that application of antagonistic phylloplane bacteria to leaves has been more successful in the control of phytopathogenic bacteria than fungi.

Among the isolated bacterial epiphytes from Brassica phylloplane, thirteen showed in vitro inhibition of the test bacterium. These antagonistic bacteria belonged to Bacillus spp. and the most commonly intercepted strain, HSb-19 was used as a test antagonist. Various species of Bacillus have been successfully reported to antagonize fungi in vitro and reduce their infection in vivo (Ferreira et al., 1991; Godoy et al., 1991). Bacillus

spp. have been demonstrated to inhibit many bacterial pathogens, viz., X. campestris pv. cyamopsidis (Saini and Parashar, 1991); X. campestris pv. manihotis (Musere and Ikotun, 1983) and P. syringae pv. tabaci (Kalsadia and Ampova, 1987). However, Podile et al. (1988) have shown that B. subtilis was ineffective to be antagonistic to X. campestris pv. citri.

Bacterial pathogens are incapable of penetrating intact plant surfaces unlike fungi. Staub and Williams (1972) successfully obtained the infection of guttating cabbage leaves by spray inoculation with X. campestris pv. campestris. Its infection has also been achieved by clip inoculation method (Sutton and Williams, 1970a). In the present study, leaf infiltration and clip inoculation techniques were found to be more effective. The efficacy, simplicity and reliability of clip inoculation method has been highly correlated with natural infection (Kaufman et al., 1973). In the present investigations, delivering the test bacterium into the guttation droplet did not lead to disease expression in B. juncea. Wallis and his associates (1973) were also unable to infect cabbage seedlings by inoculating into guttalion droplet. While, Robeson et al. (1989) obtained 75 per cent success by this method on cabbage leaves. The morphological characters of various Brassica species may be particularly responsible for the above failure.

None of the B. juncea, B. campestris and B. napus including some wild exotic genotypes showed any degree of resistance against X. campestris pv. campestris. Parallel observations have been recorded by Gandhi and Parashar (1977). Mithen et al. (1986) reported the presence of preformed antimicrobial compounds (glucosinolates) in various Brassica species. Dow and his coworkers (1991) correlated the susceptibility of most of the Brassica species against black rot pathogen to the ineffectiveness of these compounds (glucosinolates).

Furthermore, optimum concentration (cfu/ml), appropriate time of application and effective compatible combinations of the efficient antagonists were determined.

The strain (HSb-19) of Bacillus showed potential in control of black rot. Shekhawat and Chakravarti (1977) demonstrated 100 per cent control of X. campestris pv. vesicatoria by an antagonistic bacterium (B_2) when used in the ratio of 1:16 or more. Unless present in unusually high densities, epiphytic antagonists cannot respond before the pathogen penetrates the host and escapes (Cullen and Andrew, 1984). However, Spurr (1977) has shown that increased dosage of antagonist may not increase its disease control potential. Therefore, pathogen:antagonist ratio used in the present studies was 1:10. Pre-inoculation with antagonist (24 and 72 h) resulted in 82-83 per cent control of black rot in the field evaluation of the antagonist. The post-inoculation spray of antagonist and simultaneous spray inoculations (Pathogen + antagonist) were however, less effective. The culture filtrate of antagonist was ineffective in checking the disease, suggesting the presence of live antagonist cells prior to infection by the pathogen are essential for effective control of black rot disease. Similar observations have been documented in the literature (Riggle and Klos, 1972; Rao and Pavgi, 1976). Sheshagiri Rao and his associates (1978) have shown that bacterial leaf streak of rice (X. campestris pv. oryzicola) did not develop when the antagonist was spray inoculated 24 h before pathogen inoculation.

The question of mechanism is crucial for developing and improving biocontrol strategies (Cullen and Andrew, 1984). Studies presently conducted to find the mechanism(s) of antagonism of Bacillus (HSb-19) in vitro revealed that the change in pH (7 8) does not seem to be the mode of

antagonism, as parallel increase in pH was recorded in the culture media of antagonist and pathogen alone or in dual cultures. Moreover, buffering of the media also could not alter this trend. In the presence of the antagonist, the pathogen however, was not recovered after 96 h. The bactericidal effect of the antagonist was advanced or delayed, but was seen depending upon the time and sequence of inoculation with the antagonist bacterium. The population of the later continued to increase in time, irrespective of the inoculation sequence. This indicates that the shift in pH is not the mode of antagonism in the present model. A decrease or increase in pH of the growing media has been however, reported by various workers as one of the mechanisms of antagonism of E. herbicola against E. amylovora and Cladosporium sp. against Botrytis cinerea (Hsieh and Buddenhaggen, 1974; Bhatt and Vaughan, 1962).

Microorganisms are known to compete for space and nutrients (Isenbeck and Schulz, 1986). In the present investigations an attempt was made to study the competition for space between X. campestris pv. campestris and Bacillus and the results revealed that this mechanism may not be contributing to the antibacterial effect of Bacillus strain. There was no recovery of pv. campestris from the growing medium inoculated with the antagonist (cell number was 50% or more than the pathogen population). This adverse effect on pathogen was however, delayed when the antagonist population was less than 50 per cent. From these studies, it is concluded that competition for space may not be the mode of antagonism. This was further supported by the static growth of the pathogen in culture filtrate (supplemented with nutrients or unsupplemented) of antagonist obtained at different stages of its growth. This indicates that there is no competition

for space or nutrients. The bacteriostatic and not bactericidal property of the culture filtrate further strongly suggests that the presence of live bacteria is essential for the antagonistic effect. Similar observations have been reported by Isenbeck and Schulz (1986), that live cells were more effective in controlling fire blight of stone fruits caused by Erwinia amylovora.

Siderophores are reported to be involved in reduction of many fungal and bacterial plant diseases (Loper, 1988; Liao, 1989). The present findings revealed that the siderophore produced by the Bacillus strain was not responsible for the antagonistic activity against X. campestris pv. campestris in vitro because there was no increased inhibition of the pathogen by the culture filtrate of antagonist from iron minus medium.

Antibiosis has been reported as another mechanism of biological control (Weller, 1988). The findings of the present study suggest that the antibacterial activity of phylloplane Bacillus may be associated with the production of antibiotics. The results indicate that some antibacterial compounds are produced by the test antagonist as the cell free culture filtrate of the antagonist was inhibitory to the multiplication of the test pathogen. These extracellular, diffusable antimetabolites seem to be organic, non proteinaceous, thermostable (100°C) compounds as their bactericidal property was lost on incineration and autoclaving but not on boiling at 100°C. Several workers have reported that many phytopathogens are sensitive to Bacillus species which produce antibiotics (Parashar et al.,1992; Swinburne et al., 1975; Pusey, 1989). Cubeta and his associates (1985) have shown that chloroform soluble, autoclaved (heat stable) filtrates of B. subtilis were fungicidal to Penicillium spp. and fungistatic to six soybean pathogens. Similarly the bacterial metabolites produced by E. herbicola

were heat stable (Isenbeck and Schulz, 1986). Bactericidal activity of B. subtilis has been attributed to the production of antibiotics (Podile et al., 1988; Anuratha and Gnanamanickam, 1990). There is now sufficient evidence, mostly obtained by genetic methods, to indicate that antibiotics do function as a mechanism of biocontrol in nature (Howell and Stiponovic, 1983; Fravel, 1988).

It is a well known fact that electrolyte leakage of host cells is an important event during the course of infection. In the present investigations, the effect of antagonist on the electrolyte leakage of host cells by the pathogen was studied. A notable leakage of electrolytes was recorded in 48 h, when the X. campestris pv. campestris population in the leaves reached about 3×10^5 cfu ml^{-1}. It indicates a direct correlation between the multiplication of the pathogen and electrolyte leakage of host cells. Cook and Stall (1968) observed that the maximum leakage of electrolytes in 60 h was preceded by higher bacterial population in susceptible Capsicum annuum leaves inoculated with X. campestris pv. vesicatoria. This leakage of electrolytes was however, prevented in an atmosphere of close to 100 per cent relative humidity (Sutton and William, 1970b). In the present study this phenomenon probably may explain for no measurable loss of electrolytes in pathogen inoculated plants than the uninoculated controls immediately after rainfall. The suppression of pathogen multiplication within the leaf tissues by the Bacillus sp. may thus be the reason of the restricted leakage of electrolytes similar to untreated controls in co-inoculated leaf tissues.

The multiplication and spread of the pathogen in the presence of antagonist (co-coinoculation) was suppressed. The in vivo bactericidal action of the antagonist live cells corresponds with the in vitro experiments. In

contrast, the multiplication and spread of the pathogen was not adversely affected but continued unabated reaching maximum in 240 h when inoculated alone or with the cell free culture filtrate of antagonist. It further supports our earlier observations that the cell free culture filtrate of Bacillus is ineffective in controlling the disease, as it could not inhibit the multiplication and spread of the pathogen. The population of X. campestris pv. campestris reached a maximum in 240 h (throughout the host leaves)in compatible interactions (Robinson and Callow, 1986). The pathogen multiplication decreased to 10 fold on co-inoculation with an antagonistic bacterium (Dowler, 1972). Contrary to the earlier reports of (Sutton and Williams, 1970b; Sharon et al., 1982), where in, no living bacteria were isolated from the necrotic tissues, the results reported herein indicated considerable reduction in the pathogen's population, but it could nevertheless be recovered from the necrotic leaf tissues. It is supported by the observation of Robinson and Callow (1986) that the multiplication and spread of X. campestris pv. campestris continued after 20 days throughout the leaf tissue. The movement of the pathogen, though has been predicted to occur systemically into the plant via petiole (Sutton and William, 1970a). However, the present findings conclusively indicate its multiplication and movement both in xylem and phloem vessels via petiole of the inoculated leaf to the stem in the lower internode. Convincing evidences have demonstrated ultra-structurally that the initial sites of proliferation of E. amylovora and many other pathogenic bacteria are the xylem vessels and their spread laterally and distally in the inoculated leaf (Goodman and White, 1981; Willis et al., 1973). Lewis and Goodman (1965) have recorded bacterial presence in the phloem and its invasion in the entire length of the shoot in upward

direction. However, in the present investigations, the ceased movement of the pathogen in upward direction in the stem is intriguing and unexplained. It could possibly be due to plugging of the vessels at the point of petiole and stem attachment. It has been observed that X. campestris pv.campestris and its extracellular polysaccharide (EPS) along with the pectic substances of host plug the xylem vessels which lead to characteristic V-shaped lesions (Sutton and William, 1970b). To further elucidate this ceasation, ultra-structural studies coupled with inoculation of EPS⁻ mutants of the pathogen would give a better understanding and insight.

Foliar diseases have been reported to affect the crop quality adversely (Isawa, 1985). The nutritional status of the leaves inoculated with pathogen and antagonist individually and in combination reduced the nitrogen and protein content of the leaves in 20 days, while this reduction was lower than in the pathogen inoculated leaves. This indicates that the total nitrogen concentration decrease during the development of black rot. Parallel observations have been recorded in Alternaria leaf blight (Gupta and Gupta, 1991) and white rust (Gupta et al., 1984) on Brassica species. In contrast to nitrogen, the total sugar increased in general and further increased with antagonist and fungicidal spray in particular. A similar increase in the sugar content of leaf has been reported in infection of X. campestris pv. phaseoli in french bean and pea powdery mildew (Khatri et al., 1983; Rathi, 1984). However, Dhawan et al. (1981) recorded a decrease in sugar content with white rust infection in Brassica juncea. Since diseases have been classified into high and low sugar diseases (Horsfall and Dimond,1957) therefore, the high amount of sugar in infected leaves in present study indicates that X. campestris pv. campestris does not require high amount of

sugar. Antagonist and fungicidal foliar sprays may also be adversely affecting the phylloplane microflora (target and nontarget) resulting in further enhancement of sugar content in leaves. Application of Bacillus sp. due to its antagonistic activity against X. campestris pv. campestris, consequently leading to the latters elimination from the leaf (as has been demonstrated by in vitro and in vivo studies) may be responsible indirectly for the increase in sugar and lesser decline in nitrogen content of leaf.

Finally, it may be concluded from the present findings that the antagonism is operating in the phylloplane of Brassica spp. as 13 bacterial antagonist(s) were obtained from the leaf surface. Bacillus sp. (HSb-19) has been found to possess potential antagonistic activity against X. campestris pv. campestris both in vitro and in vivo.

The approach to the biological control by using specific antibiotic resistant markers of X. campestris pv. campestris and antagonist (HSb-19 - a Bacillus sp.) clearly indicated that black rot pathogen was unable to multiply and spread within its host leaves when co-inoculated with the antagonist's live cells. The presence of live antagonistic Bacillus cells is essential for the effective control of black rot. The mode of this antagonism seems to be antibiosis.

CHAPTER VI

SUMMARY

Black rot of crucifers caused by Xanthomonas campestris pv. campestris (Pammel) Dowson, is a world wide disease (Williams, 1980) causing moderate to heavy losses. There is a growing public awareness over indiscriminate use of pesticides and other agro-chemicals, which pose a serious threat to the humans and the environment (Anonymous, 1987). Biological control of plant diseases is gaining increasing attention as it is environmentally harmless and more economical than the conventional pesticides. Therefore in the present study, an attempt was made for controlling black rot disease by suitable antagonistic microbes selected from the phylloplane microflora of eleven Brassica gentoypes. The bacterial epiphytes were isolated more frequently than the fungal epiphytes in the early phase of crop growth. None of the isolated 17 fungal epiphytes exhibited any in vitro angatonism against X. campestris pv. campestris. Only 13 out of 25 bacterial epiphytes showed in vitro inhibition of the test pathogen. All these bacterial antagonists were identified as the members of the genus Bacillus. Strain HSb-19 of Bacillus was found to be most commonly present on all the Brassica cultivars. Black rot causing bacterium, X. campestris pv. campestris was also found to exist as a symptomless epiphyte (resident) on a few Brassica cultivars. 59 Brassica genotypes were screened for source of resistance against black rot disease. None of these genotypes possessed resistance, they showed a moderately susceptible to highly susceptible reaction under artificial disease stress conditions.

Specific antibiotic resistant markers of X. campestris pv. campestris to chloramphenicol (25 $\mu g\ ml^{-1}$) and antagonist (HSb-19) to tetracycline

(20 μg ml^{-1}) were selected for *in vivo* control of black rot. It was found that this antagonist could control black rot effectively. Pathogen:antagonist ratio of 1:10 as pre-inoculation spray was more effective (82-83%) in controlling black rot disease as compared to post-inoculation and simultaneous sprays of antagonist. The cell free culture filtrate of antagonist was ineffective in reducing the disease. The presence of live antagonist cells prior to infection by the pathogen are essential for effective biocontrol of the black rot pathogen. Seed treatment with antagonist cells also provided a moderate (46%) disease control.

The pathogen, *X. campestris* pv. *campestris* multiplied well in the inoculated leaves reaching maximum population (10^7 cfu ml^{-1}) in 10 days and in 20 days its spread was observed all along the leaf length and petiole. Its movement in the stem was observed both in xylem and phloem vessels in the lower internode of the inoculated leaf, but not in its upper internode. However, in the presence of live antagonist (HSb-19) cells the multiplication and spread of *X. campestris* pv. *campestris* was adversely affected and no viable cells of the latter could be recovered after 72 h in the leaf. The cell free culture filtrate of antagonist on co-inoculation with *X. campestris* pv. *campestris* was ineffective in inhibiting the multiplication of the test pathogen and thus did not control the black rot disease.

The biocontrol mechanism of the *Bacillus* sp. (HSb-19) operating to inhibit the multiplication and progression of black rot pathogen *in vitro* and *in vivo* seems to be antibiosis. Change in pH, competition for space and nutrients were not the mechanisms operating in the present antagonism.

Siderophores produced by the antagonist were also not responsible for the antibacterial activity. The antibacterial metabolites produced by HSb-19 were non-proteinaceous, heat stable (100°C) and organic in nature.

From the present investigations, it is quite apparent that black rot of Brassica juncea can be effectively checked by using a bacterial antagonist, Bacillus sp. (HSb-19).

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*Original not seen.

APPENDICES

APPENDIX I

Standard zone-size interpretation chart

Antibiotic	Disc potency μg	Inhibition zone annulus (mm^2)		
		Resistant	Intermediate	Sensitive
Chloramphenicol	30	85	104–199	226
Erythromycin	15	104	126–199	226
Streptomycin	10	67	95–126	148
Tetracycline	30	126	148–226	255

APPENDIX II

Sensitivity of X. campestris pv. campestris and antagonist (HSb-19) to antibiotics

Antibiotic	ug/disc	Inhibition zone annulus (mm^2)	
		Xcc	HSb-19
Ampicillin	25	0	0
Carbenicillin	100	0	0
Cephaloridine	30	0	43.98
Chloramphenicol	30	395.84	360.40
Erythromycin	10	687.22	888.28
Gentamycin	10	75.39	207.34
Kanamycin	30	207.84	360.40
Ledramycin	10	263.87	263.89
Nalidixic acid	30	552.92	735.13
Neomycin	30	30.60	157.07
Penicillin	10	0	0
Streptomycin	10	62.00	158.40
Tetracycline	30	471.20	395.80
Trimethoprim	100	0	784.61

APPENDIX III

Standard bacterial suspension: optical density vs viable count

Optical density	Population (cfu ml^{-1})	
	Xcc	Bacillus (HSb-19)
0.1	$1.0x10^8$	$1.3x10^8$
0.2	$6.5x10^8$	$7.4x10^8$
0.5	$1.0x10^{10}$	$6.4x10^9$
1.0	$6.2x10^{12}$	$7.8x10^{11}$