BIOCONTROL POTENTIAL AND MICROHABITAT BEHAVIOR OF SOME MUTANT ISOLATES OF *Gliocladium virens* Miller *et al.*

A Thesis

Submitted to the

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in

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By

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DEPARTMENT OF PLANT PATHOLOGY

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MOHANPUR, NADIA, WEST BENGAL

2002

Dedicated

To My

Beloved Parents

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Certificate

This is to certify that the work incorporated in the thesis entitled " **Biocontrol potential and microhabitat behavior of some mutant isolates of *Gliocladium virens* Miller *et al.*** " submitted by Sri Ayon Roy in partial fulfillment of the requirements of the **Degree of Doctor of Philosophy in Agriculture (Plant Pathology)** of the Bidhan Chandra Krishi Viswavidyalaya, is a faithful record of *bona fide* research work carried out under my supervision and guidance. Results of the thesis have not been so far submitted for any other degree or diploma. Assistance and help received during the course of investigation have been duly acknowledged.

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The viva-voce of **Sri Ayon Roy** on his dissertation entitled "**Biocontrol potential and microhabitat behavior of some mutant isolates of *Gliocladium virens* Miller *et al.***" submitted in the partial fulfillment of the requirements of the **Degree of Doctor of Philosophy in Agriculture (Plant Pathology)** was held on ...10th June, 2003..... and it was recommended that the thesis be accepted for the award of the degree.

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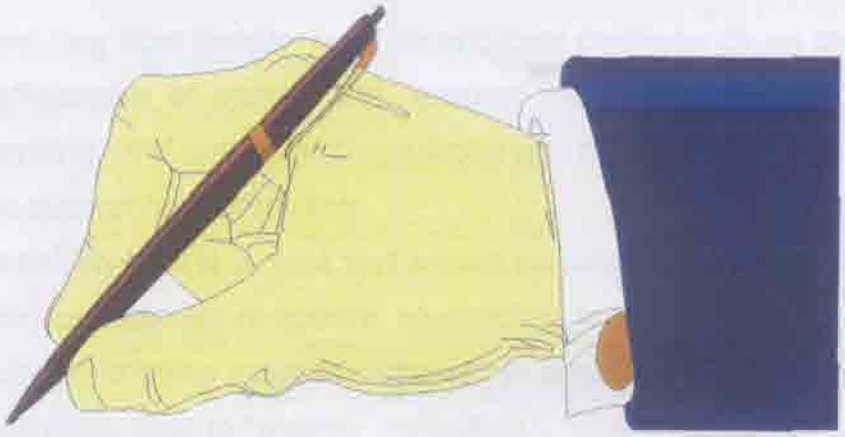
CHAPTER 1



Introduction

1. INTRODUCTION

" Biological control is a great renaissance of interest and research in microecological balance in relation to soil borne disease, and in the development of more enduringly profitable and wiser farming system"
Boswell, 1965.

The growing interest in non-chemical methods of disease control, pest management and weed control are in part a reaction to the environmental and health hazards resulting from modern agriculture's enormous dependence on chemical inputs. Some of the striking problems associated with pollution and negative health effects resulting from toxicity, overuse and over dependence on chemical pesticides, large inefficiencies of energy and resource use, interruption of natural ecological nutrient cycling, land and water degradation and destruction of biological balance that otherwise support crop protection.

Sustainability now is a quite well known paradigm for modern agriculture and the design and assessment of specific approaches to achieve a goal of sustainability is necessarily an ongoing process. Although in sustainable agriculture, they are by no means identical, next to "organic agriculture", "biological control" typifies mostly for the people the notion of non-chemical pest and disease management. Introduced as a specific term in the early twentieth century, the definition of "Biological control" has undergone several modifications (Garrett, 1965; Baker and Cook, 1974; Baker, 1985).

Sanford (1926) was probably the first among those to comprehend the theme of biological control of plant diseases with *Bacillus subtilis*. Pioneering work of Weindling on *Trichoderma* and *Gliocladium* during 30s added a new dimension in the population of antibiotic producing microorganisms and established them in the domain of potential biocontrol agent. But despondent outcome of these bioagents under field condition virtually desisted from further progress during middle of the century. A turning point for research on biological control of plant pathogens occurred in 1963 when at Barkley, California, an international symposium was held on "Ecology of Soil Borne Plant Pathogens – Prelude to Biological Control" (Baker and Snyder, 1965). This animated convention followed by indomitable efforts to exploit

Trichoderma and *Gliocladium* as potential biocontrol agents during past few decades have created a new milestone in non-chemical disease management system and organic agriculture in particular.

Before 1980, much works on *Trichoderma* and *Gliocladium* spp. were concentrated on their relative abundance and specific needs in varied ecological niches (Danielson and Davey, 1973 a,b,c,d; Davet, 1979a), mechanism of action (Baker and Cook, 1974, Krupa and Dommergues, 1979, Tu, 1980) and indirect enhancement of indigenous population by manipulation of environment (Cook and Baker, 1983) which involved a lot of speculation.

Being the member of class Deuteromycetes, *Trichoderma* and *Gliocladium* posess great genetic variabilities. Cells of these two genera are heterokaryotic while conidia are homokaryotic (Stasz *et.al.,* 1988). Therefore, it seems reasonable that the progeny derived from the single nuclei may represent the more stable strains than that of heterokaryotic progeny (Harman and Hayes, 1993).

The first requirement for successful biological control still remains a highly effective strain to be identified together with the development of suitable production and delivery system permitting high activity of the bioprotectant under condition of substantial microbial competition (Harman, 1990). A ineffaceable investigations during last two decades on this aspects have not only enriched our knowledge but also laid down the platform for developing superior strains through genetic modifications like protoplast fusion or transformations, rather than selection of natural strains (Pe'er and Chet, 1990; Chet and Barak, 1993; Harman and Hayes, 1993). However, such fascinating biotechnological opportunities need a lot of skill and patience due to lack of appropriate marker to identify the genetically modified strains.

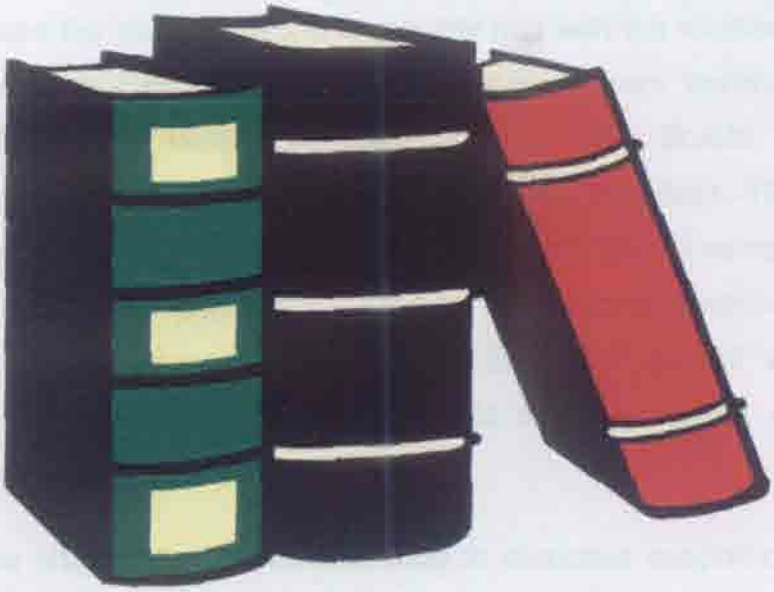
Protoplast fusion gives rise to wide range of progeny characteristics but as perhaps the parasexuality does not involve, this approach seems not to result in the incorporation of any large portion of DNA from the non prevalent strain into the progeny. Thus this limits the importance of the process to easily recombine parental traits of interest as expected (Stasz and Harman, 1990; Harman and Stasz, 1991). Moreover, in at least some cases single sporing indicates that the fusant progenies are greatly imbalanced heterokaryons (Stasz *et al.,* 1988; Stasz and Harman, 1990). Transformation of specific genes, which are known to have biocontrol activity may improve the biocontrol capability of organisms that are deficient in these particular

genes. Along with, it enhances the antagonistic potentiality by producing either greater amounts of the enzyme(s) in question or a product with a similar function but complementary properties (Chet and Barak, 1993). In spite of having great prospects, certain obstacles like cost-involvement, complexity of procedure, etc. restrict its spectrum of expansion in the field of biocontrol research. Mutation, on the other hand, is comparatively cheaper and easily accessible but it requires a marathon screening to find out a genetically improved mutant isolate.

Earlier, induction of mutation of *Trichoderma* and *Gliocladium* spp. have been attempted by exposing the conidia to physical (UV / γ-rays) or chemical mutagens with varied success (Troutman and Matejka, 1978; Papavizas and Lewis, 1983; Howell and Stipanovic, 1983; Ahmed and Baker, 1987, 1988; Howell, 1987; Papavizas *et al.*, 1990; Mukherjee and Mukhopadhyay, 1993; Roy and Pan, 1998; Dutta, 1998). The achievements were mostly directed towards improvement of some desirable traits like enhancement of biocontrol potential, increased enzyme production, fungicide tolerance and greater rhizosphere competence. But as a random phenomenon, induced mutation not only modifies the targeted character(s) but also some other traits that may cause ample alterations of the important biological properties in the mutants (Cook, 1993; Kumar and Gupta, 1999).

In attempts to explore the extent of accomplishment of the mutants as impressive biocontrol agents, potential antagonist strains of *Gliocladium* sp. had been exposed to five different doses of γ-radiation and the mutants generated from each dose were tested for any higher degree of biocontrol potential over the parental type. The domain of the present investigation revolved round the following aspects (I) screening for efficient wild isolates of *Gliocladium* spp. against some soil borne Deuteromycetes, (II) production of mutants by exposing the most efficient wild isolates to gamma radiation, (III) selection of stable mutants based on their antagonistic potential, (IV) phenotypic and physiological characterization of the selected mutants, (V) germinability of the spores of mutants in different substrates, (VI) fungistatic effect of soil and FYM on different spore types of the mutants, (VII) competitive parasitic ability of the spore types on sclerotia forming pathogens (VIII) rhizosphere colonization ability of the mutants (IX) tolerance of selected mutants to some seed treating fungicides and (X) determination of *in vivo* efficacy of the mutants for management of soil borne diseases.

CHAPTER 2



Review of Literature

Every invention demands confirmed logic and adequate supporting evidences to convince scientist community for its ultimate recognition. Fawcett's (1931) revolutionary concept on "the importance of investigations on the effects of known mixture of organisms" followed by Weindling's (1932, 1934, 1937, 1941) classical research publications on *Trichoderma viride* created an opportunity to commence a new venture in plant disease management namely "Biological control". Since then the phenomenon has gained immense popularity and remarkable increase of interest and researches have been witnessed during last few decades that make it one ecologically safe alternative of chemical control.

Perhaps the scientific world is already rich with the information on the subject of biological control by antagonists, as is evident from several classical reviews (Baker and Snyder, 1965; Toussoun *et al.*, 1970; Bruehl, 1975; Krupa and Dommergues, 1979; Schippers and Gams, 1979; Papavizas, 1981; Thresh, 1981). But one who is working on biological control should always remember the optimistic comment from Garrett (1965) on the future of biological control. However, he also opined that there were no shortcuts to biological control and only thorough elucidation of microbiological situation could make sure that control would work consistently.

In this chapter the author has tried to elucidate supporting evidences briefly and in an organized manner so far as possible with a view to provide basic information and guidance on the results obtained in the present investigation.

2.1. Biological control: Definition

Before going through a brief discussion on the available literatures, a better conception should be made about the term "Biological control". Since the evolutionary evidences by Potter (1908) and Sanford (1926), a number of definitions of "Biological control" were proposed by different scientists (Snyder, 1960; Park, 1960) where they emphasized on interruption of host parasite relationship through biological means and modification of crop culture with the aim to avoid or reduce

infection by utilizing the action of other microorganisms against the pathogen that invokes antagonism. First unanimous definition of biological control was proposed by Garrett (1965) during the First International Symposium on "Factors determining the behaviour of plant pathogens in soil" held at Barkley, California in April, 1963 where he defined biological control of plant disease as "any condition under which or practice whereby the survival or activity of a pathogen is reduced through the agency of any other living organism (except man himself) with the result that there is a reduction in incidence of the disease caused by the pathogens". This definition was more or less agreed by Sewell (1965) but a garbling fuzziness about the term still remained. Taking into account the preceding inclusions and exclusions an unified concept was given by Baker and Cook (1974), who defined biological control as the "reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state by one or more microorganisms accomplished naturally or through manipulation of the environment, host or antagonist or by mass-introduction of one or more antagonists". Finally Baker (1985) have shortened the definition by slight modification and described the biological control as the "prevention of diseases by manipulation of biological and physical environment so as to reduce indirectly the quantity and activity of the pathogen through effects on host or on accompanying microorganisms" and this definition is now universally accepted.

2.2. History

Weindling's (1934, 1937) pioneering work on *Trichoderma* and *Gliocladium* led to a new dimension in the area of biological control and a blurred research efforts were made to promote these potential bioagents both at commercial and field level. However, initial momentum of rasearch on *Gliocladium* (Webster and Lomas, 1964) previously described as *Trichoderma lignorum* (Weindling, 1932) along with different species by Rifai (1969) and subsequently by Papavizas (1985) was not up to the degree of satisfaction compared to that with *Trichoderma*. Most of the information during that decades were based on isolation, evaluation of *in vitro* antagonistic potential against several plant pathogenic fungi and production of antibiotics by different species of *Gliocladium* (Rudakov, 1970; Muromtsev *et. al.*, 1971; Davet, 1979a; Papavizas, 1985).

The systematic study on *Gliocladium* had been dramatically increased from 80's onward (Papavizas, 1985; Chet, 1987; Howell, 1987; Mukhopadhyay, 1987; Mukhopadhyay *et al.*,1992) and some research findings indicated that *G. virens* Miller *et al.* was more effective than the much studied *Trichoderma* spp. (Lumsden and Locke, 1989; Papavizas and Lewis, 1989; Wilcox and Harman, 1991; Mukherjee, 1992).

2.3. Biosystematics of *G. virens*: confusion with the genus *Trichoderma*

With increasing popularity of *Gliocladium* as a potential biocontrol agent, their accurate classification gained utmost importance. The comprehensive taxonomic and morphological studies of the genus *Gliocladium* had been discussed by Morquer *et al.*,(1963), Domsch *et al.*, (1980), Kubicek and Harman (1998). However, the genus *Gliocladium* is still not satisfactorily delimited and most satisfactory species concept within it is not yet determined.

A great dialemma still exists among scientists regarding the biosystematics of *G. virens*. This species is often misidentified as *T. viride*. Due to notable resemblance some scientists indicated this species as *Trichoderma* (= *Gliocladium*) *virens* to overrule the confusion, however they did not explain the genesis of such nomenclature (Rehner and Samuels, 1994; Highley, 1997; Highley *et al.*, 1997; Baek *et al.*, 1999; Prasad and Rangeshwaran, 1999; Howell *et al.*, 2000; Kullnig *et al.*, 2000; Meyer *et al.*, 2000; Singh *et al.*, 2001; Howell, 2002).

In this connection, Bissett (1991a,b) defined the genus *Trichoderma* previously placed in *Gliocladium* having elongate phialides and irregularly branched conidiophores. He included *T.virens* in the section Pachybasium. Earlier Scanning Electron Microscopy (SEM) expelled the disparities to some extent on the basis of echinulation of spores and presence of mucilage sheaths than on length: breadth ratios traditionally used (Hashioka, 1973; Mayer and Plaskowitz, 1989; Choudhuri and Sen, 1999). Recently developed Random Amplified Polymorphic DNA (RAPD) technique and combination of UP-PCR based approach and ribotyping were proved to be useful tools for clarifying the species distinction in *Trichoderma* and *Gliocladium* (Bulat *et al.*, 1998; Lexova *et al.*, 1998).

Regardless the confusion, *G. virens*, being included in the class Hyphomycetes, produced fast-growing colonies with densely penicillate conidiophores bearing appressed phialides. Conidia were smooth walled, short ellipsoidal, often irregularly curved with somewhat truncate base, rather large, measuring 4.5 – 6.0 x 3.5 – 4.0 µm. A large drop of slimy green conidia were produced on each whorls of phialide (Domsch *et al.*, 1980). The teleomorphic stage of this species was identified as *Hypocrea sublutea* (Doi, 1972).

2.4. Ecological habitat

A careful analysis of the ecological factors affecting the distribution of *Trichoderma* and *Gliocladium* in their natural habitat leads to an understanding of the population dynamics in soil and other habitats, their survival and proliferation in soil and the plant rhizosphere.

In general, *Gliocladium* is widely distributed all over the world (Domsch *et al.*,1980), occurring nearly in all soils and other natural habitats, especially in those containing or consisting of well decomposed organic matter (Papavizas, 1985). The ecological preference of *Trichoderma* and *Gliocladium* were discussed in the comprehensive reviews of Danielson and Davey (1973 a,b) and Davet (1979a) where physical and chemical properties of the soil were suggested to be the determinant factors of the heterogeneity of species aggregates. Mukhopadhyay (1987) in his presidential address delivered to the Annual Conference of the Indian Society of Mycology and Plant Pathology discussed the process of monitoring the population dynamics of the bioagents in soil.

2.5. *In vitro* antagonistic potential of *Gliocladium* spp. against soil borne plant pathogens

Since the demonstration by Weindling (1934), the antagonistic potentiality of *Trichoderma* and *Gliocladium* against various pathogenic fungi (both aerial and soil borne) had been proved repeatedly in *in vitro* studies and lots of research publications still come out making inroads into the exploitation of these two bioagents under field condition. Some selected references of *in vitro* antagonistic effect of *G. virens* against soil borne plant pathogens have been cited in the following table.

Table 2.1. : *In vitro* antagonistic potential of *G.virens* against some selected soil borne plant pathogens.

Pathogenic fungus	*Gliocladium* spp.	Reference(s)
Sclerotinia sclerotiorum	*G. virens*	Tu, 1980; Muller *et al.*,1985; Phillips, 1986; Chakrabarti *et al.*, 1998
Sclerotium rolfsii	*G. virens*	Hennis and Papavizas, 1983; Phillips, 1986; Papavizas and Lewis, 1989; Ristaino *et al.*, 1991; Maiti *et al.*, 1991; Mukhopadhyay *et al.*, 1992; Iqbal *et al.*, 1995; Mukherjee *et al.*, 1995; Jomduang and Sariah, 1997.
S. oryzae	*G. virens*	Puri *et al.*, 1998
S. cepivorum	*G. virens*	Jackson *et al.*, 1991
Sarocladium oryzae	*Gliocladium* sp.	Panneerselvam and Saravanamurthu, 1996
Rhizoctonia solani	*G. virens*	Mukherjee *et al.*, 1995; Dubey, 1998; Pan *et al.*, 2001.
Pythium ultimum	*G. virens*	Howell, 1982; Wolffhechel, 1992; Shahriary *et al.*, 1996.
P. aphanidermatum	*G. virens*	Sharma, 1998
Phytopthora cactorum	*G. virens*	Smith *et al.*, 1991
P. cinnamomi & P. citricola	*G. virens*	Chambers and Scott, 1995
P. colocasiae	*G. virens*	Pan and Ghosh, 1997
Fusarium oxysporum f sp. *cucumerinum, F. solani, F. oxysporum* f. sp. *lycopersici*	*G. virens*	Larkin *et al.*, 1998
Macrophomina phaseolina	*G. virens*	Entheshamul *et al.*, 1991; Shahida and Gaffar, 1991; Kanakamahalakshmi *et al.*, 1998, Pan *et al.*, 2001
Botryodiplodia theobromae	*G. virens*	Gupta *et al.*, 1999
Wood destroying white rot and brown rot fungi	*Trichoderma (?Gliocladium) virens*	Highley, 1997

2.6. Production of mutants with enhanced antagonistic potential

Successful biological control needs highly effective strain that would be more competitive in comparison to other microflora together with the ability to produce appropriate propagules with appropriate physiological properties for biocontrol (Harman, 1990; Jin *et al.*, 1991). For this, superior strain together with delivery system that enhances biocontrol potential must be developed (Harman *et al.*, 1989).

The sexual stages of *Trichoderma* and *Gliocladium* however are rare or entirely absent (Stasz *et al.*, 1988), so possibility of sexual recombination is either remote or not possible. Accordingly induced mutation using physical and chemical mutagens was proved to be one of the most promising approach to modify the genetic composition of these antagonists with an aimed at producing mutants with enhanced biocontrol potential (Alikhanian, 1962, Ball, 1980; Papavizas, 1987). But absence of suitable markers created some difficulties and the approach needed extensive selection procedure to obtain stable improved mutants. During 80's several successful attempts had been made to improve the biocontrol potential of *Trichoderma* spp by exposing the conidia to UV – radiation or chemical mutagens (Papavizas and Lewis, 1981a, 1983; Ahmed and Baker, 1987, 1988). Two rhizosphere competent mutants (T95 and T-12B) of *T. harzianum* were developed by Ahmed and Baker (1987,1988) that exhibited higher cellulase and biomass production than the wild counterpart.

Repeated exposure of *G. virens* to UV–rays failed to generate biotypes resistant to benomyl (Papavizas, 1987) until the success of Papavizas *et al.* (1990) by alternate exposure of the conidia to UV-rays and ethyl methane sulphonate (a chemical mutagen). However, mutants of *G. virens* developed by UV-radiation for altered antibiosis and mycoparasitism had been reported earlier (Howell and Stipanovic, 1983; Howell, 1987).

Troutman and Matejka (1978) first used γ - (gamma) radiation to develop mutants of *T. viride* resistant to benomyl. However, they did not report on the biocontrol potential of the mutants (Papavizas and Lewis, 1983). Strauss and Kubicek (1990) isolated a γ radiated mutant of *T. reesei* [*T. longibrachiatum*] QM

9414 M(8) capable of secreting β - glucosidase upon growth on cellobiose while parent isolate was unable to do it. Mukherjee and Mukhopadhyay (1993) successfully developed stable mutants of *G. virens* with enhanced biocontrol potentiality by exposing the conidia to γ-radiation. In continuation to this trend, increased inhibitory properties of mutants of *G. virens* obtained by γ-radiation had also been shown by Roy and Pan (1998) and Dutta (1998).

2.7. Mechanisms of antagonism

In the context of mechanisms of action the term antagonism, hyperparasitism, direct parasitism and interfungus parasitism are used interchangeably to refer the phenomenon of one fungus parasitic on another. Hornby (1983) stated that " a major problem that besets the subject of microbial hyperparasitism in soil is that many of the mechanisms discussed are presumptive and proof is difficult to come by". The suggested mechanisms to presumptively explain the biocontrol of plant pathogens by *Trichoderma* and *Gliocladium* proposed during last few decades include mycoporasitism, competition, antibiosis and lysis (Ayres and Adams, 1981; Cook and Baker, 1983; D'Ercole *et al.*, 1984; Fravel and Keinath, 1991; Howell *et al.*, 1993; Pieta, 1997.)

Tu (1980) showed that sclerotia formation by *S. sclerotiorum* was inhibited by *G. virens* but not by other fungi and bacteria. Scanning electron microscopy (SEM) revealed that mycoporasite formed appressorium like structure and presumably infected by active penetration. Transmission Electron Microscopy (TEM) demonstrated internal parasitism of sclerotial cells. *G. virens* effectively inhibited sclerotia formation of *R. solani* and when hyphae of *G. virens* touched those of *R. solani* following occurred (i) contact with host cell (ii) formation of appressoria (iii) penetration of host cell (iv) formation of intercellular hyphae (Tu and Vartaja, 1981). *S. rolfsii* was found to produce multihyphal strands when growing over *T. harzianum* colony *in vitro* and the aggregated strands enabled the pathogen to grow through soil over an inhospitable surface (Hadar *et al.*, 1981). Two five days growth of *G. virens* caused cytoplasmic leakage of *Rhizoctonia* mycelium, prevented secondary branching of mycelium and occasionally coiled around *Rhizoctonia* hyphae (Harris and Lumsden, 1997).

Various mechanisms of *in vitro* antagonism of an isolate of *T. harzianim* in suppressing *S rolfsii* (*Corticium rolfsii*) and *R. solani* was compared with *G. virens* by Mukherjee *et al.* (1995) & Jomduang and Sariah (1997). *G. virens* and *T. harzianum* were equally effective in parasitizing the hyphae of *R. solani*. Only *T. harzianum* parasitized the hyphae of *C. rolfsii* and two antagonists were comparable with respect to antibiosis on the test pathogen. However, *G. virens* readily parasitized the sclerotia of both pathogens and appeared to be more effective than *T. harzianum* in destroying the sclerotia. Under SEM *G. virens* was found to colonize, penetrate and sporulate inside the sclerotia of test pathogens. Hence parasitism of sclerotia was suggested as principle mechanism of biocontrol of *S. rolfsii* and *R. solani* by *G. virens*.

The physical mechanism of hyperparasitism on sclerotia of *S. rolfsii* had been well studied in case of *T. harzianum* (D'Ambra and Ferrata, 1984). However strain specificity of antagonists resulted in no clear relationship between penetration capacity and its effect on sclerotial germination. The production of lectin by *S. rolfsii* might explain the possible basis for specific recognition in the interaction of *Trichoderma* and *S. rolfsii* (Barak *et al.*, 1985). SEM studies revealed that hyphae of *T. harzianum* isolates capable of colonizing the sclerotia of *S. rolfsii*, grew abundantly on the sclerotial surface forming a dense branched mycelium that appeared to establish contact with host cells through thin mucilage followed by multiplication of antagonist hyphae and displayed the ability to penetrate rind and cortex (Henis *et al.*, 1983). Growth of antagonist on the rind was mainly achieved by formation of costricted hyphae leading to extensive host cell alterations. Cell breakdown occurred frequently in inner rind cells adjacent to medullary cells. Ingress of *T. harzianum* hyphae in the medulla was characterized mainly by a change in the mode of growth from intra to intercellular region. Although hyphae of *Trichoderma* did not penetrate the medullary cells yet the later showed pronounced alteration (Benhamou and Chet, 1996).

Apart from physical interaction a complex biochemical mechanism in biological control had been investigated extensively. Production of secondary metabolites including antibiotics like gliotoxin, gliovirin and fungal cell wall degrading enzymes like chitinase, glucanases, cellulase were considered to be the main biocontrol mechanism of *G. virens*. Recent evidence proved that these cell wall degrading

enzymes may act synergistically with antibiotics (Pierto *et al.*,1995). Physical interaction followed by enzymatic digestion of cell walls of sclerotia forming pathogens like *R. solani* and *S. rolfsii* had been demonstrated (Elad *et al.*, 1983 a,b). TEM observation suggested that extracellular fibrillar material was deposited between interacting cells and parasitic organelles like mitochondria, vesicles; dark, osmophilic inclusions accumulated in the parasitizing cells. In response to invasion the host produced a sheet matrix which encapsulated the penetrating hyphae leading to emptying of host cell cytoplasm. The phenomenon of physical contact followed by disorganization of the host cells and concomitant changes in the cells of the parasite had been unequivocally established for *Gliocladium* (Tu, 1980;Howell, 1982).

Trichodermas like *T. viride, T. harzianum, T. (= Gliocladium) virens* are well known to produce mycolytic enzymes such as β- 1,3 glucanase, β - 1,6 glucanase, β - 1,4 endoglucanase, chitinases, cellulase, protease, etc (Chet and Elad, 1982; Kubicek, 1983; Cruz *et al.*, 1995; Harman *et al.*, 1993, 1995; Lora *et al.*, 1995; Baek *et al.*, 1999; Yedidia *et al.*, 2000). These enzymes play an important role in degradation of target pathogen and consequent mycoparasitism .

High β - 1,3 glucanase and chitinase activities were detected in dual culture when *T. harzianum* parasitized *R. solani* and *S. rolfsii* compared with low levels found with either fungus alone (Chet and Baker, 1980; Elad *et al.*, 1983a). Culture filtrate of *G. virens* grown on chitin containing medium was strongly inhibitory to mycelial growth of *P. ultimum*, *P. megasperma*, *B. cinerea*, *R. solani* and different types of chitonolytic enzyme activities including endochitinase, chitin 1,4-β chitobiosidase and glucan – N acetyl β – D glucosamidase, as well as glucan 1,3 glucosidase were suggested to act synergistically with the antibiotic gliotoxin (Pietro *et al.*, 1993).

The chitinolytic system of *T. harzianum* resembled that of most chitinolytic organisms and consist of two types of hydrolases : chitinase and chitobiase. Both enzymes were produced when *T. harzianum* was grown on a chitin containing medium and repressed when mycelium was provided with an easily metabolized carbon sources such as glucose or N – acetyl glucosamine (Ulhoa and Perberdy, 1993). *G. virens* when grown on wheat straw and brown bread as C-sources a relatively high amount of xylase was produced in addition to cellulolytic enzymes (Todorovic *et al.*, 1988). In submerged culture supplemented with 2 % straw as C-sources mutant of *T. harzianum* yielded 13.8 U of cellobiohydrolase, 890.0 U of

carboxy methyl cellulase and 145.0 U of β - glucosidase/g of straw (Marcis *et al.*, 1989). Baker and Dickman (1993) found high enzyme activity in susceptible pathogen mycoparasite co-cultures thus rendering the conclusion that the production of different enzymes by *Trichoderma* and *Gliocladium* was greatly influenced by the nature of growth substances (Elad *et al.*, 1982; Senior and Saddler, 1990; Noronha *et al.*, 2000).

A 110 kDa and 29 kDa extracellular β - 1,3 exoglucanase were purified from *T. harzianum* grown with laminarin or in dual culture with host fungi (Cohen – Kupiec *et al.*, 1999; Noronha and Ulhoa, 2000). These results suggested that each β - 1,3 glucanase was different and was probably encoded by different genes. *T. koningii* produced two endo chitinase (Rf 0.15 and 0.24) and two exoacting chitinolytic enzymes (Rf 0.46 and 0.62) during degradation of crabshell chitin and *S. cepivorum* cell walls. The Rf 0.24 and 0.46 proteins were detected when *T. koningii* colonized *S. cepivorum* infected roots and were likely to be a component of the antagonism process (Metcalf and Wilson, 2001).

In recent times genetic improvement of *Trichoderma* and *Gliocladium* has been attempted successfully for increased enzyme production (Baker, 1991; Witkowska and Bien, 1991; Papavizas *et al.*, 1990). Three benomyl tolerant mutants of *G. virens* were developed by Papavizas *et al.* (1990) produced more cellulose (β - 1,4 glucosidase, carboxy methyl cellulase, filter paper cellulase) than their wild type strain. Recently, Kumar and Gupta (1999) studied the enzyme activity of UV – and γ-radiated mutants of *T. viride* and demonstrated significant variation of β - 1,4 endoglucanase, β - 1,3 glucanase and chitinase in culture filtrates of wild and mutant isolates grown in both inducible and noninducible condition.

Earlier direct parasitism was proved to be an unimportant phenomenon compared to antibiosis in the antagonism of *G. virens* to *C. rolfsii* (Aluko and Hering, 1970). Culture filtrates of *G. virens containing gliotoxin and viridin* were active against hyphae and killed the sclerotia even at low concentration. A reverse correlation between hyperparasitism and antibiosis was also reported in studies with isolates of *T. aureoviride*, *T. harzianum* and *G. virens* tested against *F. oxysporum*, *F. solani*, *Pythium* sp, *R. solani* and *S. sclerotiorum* causing root rot of pea (Velikanov *et al.*, 1994).

Changes in hyphal structure like abnormal granulation, cytoplasmic vacuolation and coagulation side by side production of volatile and nonvolatile metabolites suppressed the pathogenic growth of *P. aphanidermatum* when hyperparasitized by *Trichoderma* spp and *G. virens* (Usman *et al.*,1997). The antifungal activity of *T. lignorum* [*T. viride*] and *G. catenulatum* against *F. sambucinum* [*Gibberella pulicaris]* was due to volatile metabolites and enzymes (Pavlovskaya *et al.*, 1998). Studies on ultrastructure of parasitism of *G. virens* on *Botryodiplodia theobromae* suggested that in contrast to physical interaction by coiling and penetration it produced wall lytic enzymes or antifungal substances after coming in contact and finally resulting in wrinkling, bursting and collapsing of the pathogen mycelium (Gupta *et al.*, 1999).

Nutrient availability on various organs of raspberry was suggested to be a possible mode of antagonism of *G. roseum* against *Botrytis cinerea* (Yu *et al.*, 1997). But limited information on this aspect raised question about the degree of significance regarding competition for nutrients as one of the principle mechanism of biocontrol (Whipps and Lumsden, 1991).

Therefore it can be concluded that any single mechanism may not be responsible for a particular antagonistic action. Whatever be the mechanism involved the ultimate fate for obtaining substantial disease management had been achieved in most of the instances.

2.8. Microhabitat behavior of *Gliocladium* spp.

For effective biological control of soil borne plant pathogens, a major consideration is antagonist proliferation after introduction in to the soil or rhizosphere. Among the desirable attributes of a successful antagonists are its ability to produce inoculum in excess amount and to survive, sporulate, grow and proliferate in soil and in the plant rhizosphere (Baker and Cook, 1974). Ecologically this has been difficult because of the general inability of introduced alien microorganisms to survive in soils for a variety of reasons (Garrett, 1956).

Despite the fact, research in this area has increased significantly during recent years. Genetic manipulation of *Trichoderma* and *Gliocladium* through induced mutation have been attempted to make them more adaptive and efficacious against

various soil edaphic factors (Jeyarajan *et al.*, 1993). On the other hand as a random phenomenon, mutation not only modifies the targeted character(s) but some other traits also that may or may not be desirable for its biology (Kumar and Gupta, 1999). Any abolishment of important biological property of the wild isolate makes the mutants less fit to its parental ecosystem. This may be due to extra metabolic load in mutants toward a single or a few desirable characters (Cook, 1993). Perhaps due to these reasons several mutants effective *in vitro* were not much successful as biocontrol agents *in vivo* (Kommedahl and Windels, 1978). So it is necessary to assess some biological properties of the mutants that may increase their ecological fitness in soil. Due to paucity of information on these aspects literatures have been cited upon wild isolates of *Gliocladium* along with *Trichoderma* as and when required.

2.8.1. Germination behavior of spores of *Gliocladium* spp.

Trichoderma and *Gliocladium* are known to produce phialo- and chlamydospores (Rifai, 1969; Domsch *et al.*, 1980). However, unfortunately the ecological importance and potential role of chlamydospores in biological control have been overlooked repeatedly.

Biocontrol agents when applied as spore-suspension required to be germinated to exert the desired effect. A large number of physical, chemical and biological factors obviously interact to determine the germinability of spores in nature. The effect of several pre-identified pertinent factors affecting the germination of spore of *T. lignorum* had been discussed (Martin and Nicolas, 1970). Very little was known about the factors affecting germination of both spore forms of *Gliocladium* other than they germinated well on various substrates (Papavizas, 1985).

Nutrients are one possible factor that can be manipulated to enhance spore germination. As expected with saprophytic soil fungi *Trichoderma* and *Gliocladium* use a wide range of compounds as sole carbon and nitrogen source. Compounds which in general served as the sources of carbon for *Trichoderma* equivalent to dextrose included fructose, mannose, galactose, xylose, ribose, maltose, melibiose, trehalose, etc. (Danielson and Davey, 1973c; Bonlea and Sesan, 1980). Subsequently, Papavizas *et al.* (1990) confirmed that out of 10 carbon sources tested cellobiose, xylose and xylan were best for growth while galactose and glucose

were intermediate and arabinose, ribose, rhamnose were poor sources of carbon. Recently, Monga (2001) proved that sporulation of *G. virens* was excellent in its growth on all carbon sources except maltose.

Beside carbon sources growth of *Trichoderma* in buffered media was decidedly superior when supplemented with NH_4^+ - N as compared to NO_3 – N. However, some previous experiments in buffered media indicated that NO_3 – N was better sources of N than NH_4^+ (Hacskaylo *et al.*, 1954; Ward and Henry, 1961) probably due to production of H – ion concentration in cultures with NH_4^+- N (Racle, 1965). Furthermore, certain amino acids were found to be in general superior to both NH_4^+ or NO_3 -nitrogen (Hacskaylo *et al.*, 1954; Aube and Gagnon, 1969). The best sources of nitrogen that favoured the sporulation of *Trichoderma* were mostly found to be peptone, urea, ammonium salts, lysine, tryptophan, DL-asparagine, L-aspertic acid, L-alanine, L-glutamic acid and cashamino acid (Danielson and Davey, 1973c; Sesan, 1981). This was in agreements with general pattern of amino acid utilization by a wide variety of fungi (Cochrane, 1958). Ascorbic acid alone and in combination with glucose increased germination of phialo- and chlamydospores of *G. virens* (Saha and Pan, 1998a). Recently Dawson and Lovell (2000) showed that while sugar alcohols and inorganic nitrogen compounds showed no selective effect on phialospore germination some of the amino acids like L-phenyl alanine and beta-alanine selectively enhance germination of *G. roseum.*

Even less is known about the germination of chlamydospores *in vitro*. Although fresh chlamydospores germinated well (approx 75%) on nutrient agar while only 13 to 31% of chlamydospores from air dried preparation germinated perhaps due to the fact that dried chlamydospore might be dormant but became germinable under appropriate conditions (Papavizas, 1985).

In addition to several biochemical factors a large number of biotic factors such as temperature, pH, CO_2 level of atmosphere and water potential were found to be most determining factors for germination of both spore forms of *Trichoderma* and *Gliocladium*. The optimum temperature for both conidial germination and mycelial growth of *G. roseum* was found between 27^0 and 30^0C (Kohl *et al.*, 1999). Kang *et al.* (1989) claimed that the germination of phialospores of *G. virens* was most profoundly influenced by temperature optimum being 30^0C and

physiology related to growth suitably operated at pH range between 4.0 to 5.0. In a nutrient depleted system germination was greater in acid than in neutral condition. The older spores required more nutrients and became more sensitive to neutral condition. Experimental evidences showed that if CO_2 level of the atmophere increased from 2 to 10 % growth of *Trichoderma* spp. reduced on acid medium (pH 4.4) and increased on an alkaline medium of pH 7.5 (Danielson and Davey, 1973d). Spores of *G. virens* germinated maximum at 35^0C and pH range between 4.6 to 6.8. Conidial germination reduced with reducing water potential over the range between – 0.7 to – 14.0 Mpa (Sreenivasaprasad and Manibhusanrao, 1990; Jackson *et al.*, 1991). Recent evidence showed that pH had significant role on chlamydospore germination and 70 – 84 % germination at pH 4.5 was observed for dry and fresh chlamydospores of both wild and mutant isolates of *G. virens*. Soil leachets at its original pH (6.5) showed very poor germination (2-5 %) however, the germination percentage was raised to 50 – 70% when pH of the soil leachets was adjusted to 4.5 (Bag *et al.*, 2001).

2.8.2. Fungistasis

The term soil fungistasis or soil mycostasis describes the phenomenon whereby (a) viable propagule not under the influence of endogenous or constitutive dormancy do not germinate in soil under conditions of temperature and moisture favourable for germination or (b) growth of fungal hyphae is retarded or terminated by conditions of soil environment other than temperature and moisture (Watson and Ford, 1972). The phenomenon of soil fungistasis may be biotic or abiotic in origin and was generally attributed to microbial competition for energy substances and / or to inhibitory compounds (Lockwood, 1977). Direct or indirect approaches to annul or enhance soil fungistasis were suggested to have a considerable impact on survival and population dynamics of natural and introduced species of *Trichoderma* and *Gliocladium* in soil (Caldwell, 1958; Papavizas and Lumsden, 1980).

Conidia of *Gliocladium* were found to be relatively insensitive to soil fungistasis as compared to that of *Trichoderma* and sensitivity was more pronounced in acid than in alkaline soil. In addition, chlamydospores and hyphae were expected to be less sensitive to soil fungistasis than the conidia (Lockwood, 1977). The rapid formation of both conidia and chlamydospores during growth of *Trichoderma* on

natural organic matter in soil had been observed previously (Lewis and Papavizas, 1983). However, very little is known about the behavior of chlamydospores in soil.

The involvement of inhibitory volatile substances like ammonia was attributed as one of the most intriguing aspect of soil fungistasis in relation to *Trichoderma* and *Gliocladium* conidia (Schippers *et al.,* 1982). A high percentage of germination of spores of *Trichoderma* spp. in sterile soil had been demonstrated while it was only 1.7 – 7.3 % in natural soil (Kim *et al.,*1992). Lack of germination in nonsterile soil in absence of susceptible host was attributed to be the effect of soil fungistasis (Linderman and Gilbert, 1973; Lockwood, 1977).

With a view to achieve enhanced biocontrol potential it is fairly obvious that the prospects of control by manipulating the soil environment to reduce or to annul fungistasis to the antagonists is more promising. The annulment was suggested to be possible by addition of external nutrients to the soil (*Linderman* and Gilbert, 1973; Lockwood, 1977) or by root exudates of host plants stimulatory to the germination of antagonist propagule (Sullia, 1973). Although C-sources had no significant effect on sensitivity of soil fungistasis but profoundly affected the theoretical colonization index (TCI) of different spore population of *T. viride* (Mitcheli and Dix, 1975).

As with the stimulatory effect of root exudates, stimulation of germination of fungal propagules by organic materials without supporting formation of new resistant propagules and increasing sensitivity towards the attack by antagonists may provide the basis for biological control. The review has already focussed an idea on various factors affecting spore germination of *Trichoderma* and *Gliocladium* (section 2.8.1) yet sufficient knowledge regarding the role of soil fungistasis is very meager in general on parental (wild) isolates of *G. virens* and particularly on their mutant counterparts.

2.8.3. Competitive parasitic ability

The importance of competitive saprophytic ability of a pathogen determining its ability to survive in soil as a saprophyte had been discussed in detail by Garrett (1956). Since soil and soil habitat do not provide pure culture but are open to colonization by numbers of microorganisms in the soil population, organisms with high competitive parasitic ability was suggested to be most effective for successful

biological control (Henis *et al.*, 1983). Relatively small environmental variation may reverse the outcome of soil antagonism and this sort of information could be very relevant to the theory and practice of biological control (Griffiths and Siddiqui, 1961).

Although the genera *Trichoderma* and *Gliocladium* had been considered to be weak saprophytes under natural soil condition (Danielson and Davey, 1973a) yet they were shown to attack sclerotia of various pathogens (Coley-Smith and Cooke, 1971; Kakoti and Saikia, 1998). Increased activity of soil microorganisms near dried sclerotia of *S. rolfsii* had been demonstrated (Gilbert and Linderman, 1971) although they did not examine the behavior of fresh sclerotia. Fresh sclerotia produced on PDA or on natural soil were found neither to germinate or nor to degrade by *T. harzianum* (WT-6) in field soil (Henis and Papavizas, 1983), whereas dried sclerotia of *S. rolsfii* rotted within 2-3 week in moist soil because they leaked nutrients which provided nutrient rich microsites for soil microbiota to overcome sclerotial defense (Smith, 1972).

G. virens colonized and decayed sclerotia of *S. sclerotiorum* , *S. minor, B. cinerea, S. rolfsii, M. phaseolina* and caused a reduction in survival ability of these pathogens in soil (Tu, 1980, Phillips, 1986; Mukherjee *et al.*,1993). The antagonist was found to be effective when applied to the soil @ 10^4 conidia / kg soil and active under a broad range of moisture level (optimum 80 % field capacity) but the main limiting factor was temperature for its effectiveness (Phillips, 1986; Saha and Pan, 1998a). The sclerotial germination of *Claviceps fusiformis* causing ergot of pearl millet was significantly reduced by *T. viride, T. harzianum* and *G. virens* after 8 wk and about 94.37% inhibition of sclerotial germination by *T. viride* and *G. virens* was observed even after 15 wk (Mohan and Jeyarajan, 1990).

The influence of *G. virens* on germination and infectivity of sclerotia of *S. rolfsii* was elaborately studied by Papavizas and Collins (1990). They developed a system to (1) test the ability of sclerotia of *S. rolfsii* to infect bean plants or hypocotyls after their incubation in soil amended with *G. virens* (G 1-3) (2) relate infectivity to colonization of sclerotia by the antagonist and (3) evaluate germination of antagonist infected sclerotia on agar. A positive correlation was found between the percentage of colonization and reduction of infectivity and germination of sclerotia by *G. virens* in

soil whereas *S. rolfsii* tested from control soil were 100% viable and from 80 – 100 % infective.

In recent study, on competitive parasitic ability of different wild and mutant isolates of *G. virens* to colonize sclerotia of *R. solani* and *S. rolfsii*, better colonization of sclerotia by all isolates was observed in sterilized soil over natural soil at 30^0C and at pH 5.5 mutant isolates performed more efficiently than the wild isolate (Pan *et al.*, 2002).

2.8.4. Rhizosphere colonization

Biocontrol agents differ fundamentally from chemical fungicides that upon application they must grow and proliferate for exerting effective disease control (Nelson *et al.*, 1994). Therefore, the effective antagonists must become established in crop ecosystem and remain active against target pathogen(s) during periods favourable for plant infection (Lo *et al.*, 1998). Although *Trichoderma* and *Gliocladium* have been isolated from plant roots and numerous attempts have been made so far to use them for biocontrol of root diseases (Cook and Baker, 1983; Papavizas and Lewis, 1981b), quantitative studies on the survival, establishment and proliferation in crop rhizosphere by the two antagonists is extremely limited.

The abundance of *Trichoderma* spp. and, to a lesser extent, of *Gliocladium* spp. in various soils coupled with their ability to degrade various organic substances in soil, their metabolic versatility and their resistance to microbial inhibitors suggested that they might possess the ability to survive in many ecological niches depending on the prevailing conditions of the habitat (Papavizas, 1985).

The effectiveness of *Trichoderma* and *Gliocladium* for seed treatment is probably determined not only by their biocontrol qualities but also by their abilities to multiply in the rhizosphere. In general, antagonist applied to seed was known not to establish or proliferate in the rhizosphere (Kommedahl and Windels, 1981; Papavizas, 1982). The inability might be due to lack of proper nutrients or due to presence of toxic substances in the root exudates or for the presence of competing microorganisms (Chao *et al.*, 1986). Papavizas (1985) suggested that if antagonist inoculum multiplied around the site of application, but not along the root surfaces away from the cotyledon attachment, it probably suppressed pathogens causing

seed rot and seedling diseases but not those causing root diseases. However, Alstrom (2000) showed that rape seedlings raised from seeds when exposed to *Trichoderma* and *Gliocladium* were colonized by their hyphae in gnotobiotic conditions and the emergence and root length was significantly affected by their presence.

Proliferation and subsequent establishment of *Trichoderma* and *Gliocladium* in rhizosphere largely depend upon age of inoculum with relation to food base concentration of inoculum applied, soil properties and age of the plant. Bruehl's (1975) substrate possession principle might explain the relatively unimpeded proliferation of the antagonist in soil. Addition of 3 days old mycelial preparation of *G. virens* to provide as little as 0.01 % bran and $10^1 - 10^2$ propagules /g resulted in 10^5-10^6 fold increase in population dynamics within the first 3 wk with the rate of increase peak in first week (Lewis and Papavizas, 1984a). In contrast conidia of various *Trichoderma* spp. did not survive when introduced into soil and less than 50% of the added number retrieved after 35 days (Papavizas, 1982) indicating increased sensitivity of conidia towards soil fungistasis (Lockwood, 1977). Increased population of *T. harzianum* in triadimefon treated rhizosphere soil was found at the inoculum dose of $1x10^3$ cfu/g than that of $1x10^6$ cfu/g soil. In clay-loam soil with good texture and high C:N ratio the antagonist population was higher than in sandy-loam or clay soils (Yan *et al.*, 2000). In addition, rhizosphere colonization by the antagonist was found to be inversely proportional with soil depth (Kim *et al.*, 1992).

Trichoderma spp. was found to be dominant in both the rhizosphere and rhizoplane and associated with the roots throughout the life of the plant (Wright, 1956). The number of active fungal propagules in the rhizosphere was highest in the seedling stage and decreased until about 50 days after which there was a gradual rise culminating in another peak at nearly 100 days (Odunfa and Oso, 1979). The rise in microbial population during later part of the plant growth was ascribed to the breakdown of the cortex, the epidermal cells and root hairs and this decomposing cells provided suitable carbon and nitrogen sources for the growth of the fungi.

The effect of cotton roots on the inoculum dynamics of *G. virens* was investigated by Park *et al.* (1992) and it was claimed that inoculum densities differed significantly between *G. virens* inoculated soil with or without cotton seedlings. In soil

without plants inoculum density increased 2 fold during first 10 days and then remained constant for next 50 days, while in soil with cotton seedlings inoculum density increased 10-fold during the first 30 days which was correlated with root-length of seedlings. Colonization of cotton roots by *G. virens* increased linearly with time in infested soil and the rate of colonization was significantly higher on primary roots than on secondary and tertiary roots. However, previously it was shown that rhizosphere competent *G. virens* (G872 B) colonized lateral as well as main root (Bae *et al.*, 1990).

The host root exudates, play significant roles stimulatory or inhibitory stimulatory or inhibitory towards rhizosphere colonization by the antagonist. The frequency of occurrence of *Trichoderma* and *Penicillium* in both the rhizosphere and rhizoplane showed remarkable variation. The stimulatory factor might be due to abundance of sugars and amino acids exuded from roots of cowpea seedlings (Odunfa, 1975).

The role of host age in this respect could not be ignored. Increased rate of oxygen uptake by spores of *T. aureoviride* during early stages of cotton, maize, broad bean was suggested due to exudation from plant roots (Afifi *et al.*, 1977). Aging of maize plant resulted in production of exudates stimulatory to spores of *T. aureoviride* young plants were also reported by Naim *et al.* (1977). Detection of growth promoters indicated the presence of indole and gibberellin like substances which exerted stimulatory effect on spore germination.

Chemical treatment of soil before planting has a significant impact on rhizosphere colonization by *Trichoderma* and *Gliocladium* (Evans, 1955; Kruetzer, 1965). Any alteration mediated by heat or chemical undoubtedly create a partial vacuum in soil (Baker, 1981) that favors indigenous or introduced antagonist to rapidly recolonize the rhizosphere of newly sown crop. The antagonists not only predominate in soil for three weeks after fumigation with dilute formaldehyde or carbon disulfide but also persist for at least six months after fumigation.

A purposeful approach to obtain benomyl tolerant mutants of *Trichoderma* spp. with greater rhizosphere competence had been done successfully by Ahmed and Baker (1988). However, tolerance to benomyl was not seen to be necessary attribute for rhizosphere competence. In contrary, a perfect correlation

was observed between increased cellulase production by mutants and the rhizosphere colonization (Baker, 1991). It was suggested that the mutants use the mucigel on roots as a substrate and induced increased growth responses from seed treatment than wild type parents. Although some studies have been conducted recently for production of genetically modified *Trichoderma* spp. with enhanced rhizosphere competence (Lo *et al.*, 1998; Migheli *et al.*, 1998) information on this aspect with respect to *G. virens* is totally absent.

2.9. Fungicide tolerance

The indiscriminate use of potentially hazardous fungicides in modern agriculture system resulting in exclusive environmental pollution of resistant strains of the pathogens has been the course of worldwide concern. The possibility of controlling pathogenic fungi with antagonistic microorganisms introduced as a substitute for or in combination with low (sub-lethal) doses of fungicides has long been considered to be an alternative approach as well as special interest of integrated disease management. There are two basic ways to apply antagonistic micro organisms in combination with various chemicals (1) a strain of antagonist resistant to chemical could be applied simultaneously with the chemical, the advantage being that the chemical may then be applied at a sub-lethal dose reducing the problem of ecological hazards and (2) the antagonists may be introduced into soil just after soil treatment with some biocides to prolong the effects of these treatments (Chet, 1990).

The literature on non-target effects of soil fumigants upon *Trichoderma* and *Gliocladium* is extremely voluminous (Evans, 1955; Ohr *et al.*, 1973; Rodriguez-Kabana and Curl,1980; Kelly and Rodriguez-Kaabana, 1981; Cook and Baker,1983; Lewis and Papavizas, 1984b) and mostly suggested that the probable dominance of these antagonists following application of soil biocides was due to their inherent resistance to most biocides and to its ability to rapidly colonize substrates in absence of significant competition from other microorganisms (Papavizas, 1985).

In general, *Trichoderma* and *Gliocladium* appeared to be very much sensitive to benzimidazole group of fungicides (benomyl, thiabendazole, thiophanate methyl) in contrast to metalaxyl captan, thiram, chlorothalonil, chlorneb, PCNB, iprodione,

procymidone, vinclozolin, mancozeb and organo phosphorus fungicides (Davet, 1979b, Abd – El Moity *et al.*, 1982; Papavizas, 1985; Malathi and Jeyarajan, 1995; Viji *et al.,* 1997). High insensitivity of *G. virens* to vinclozolin rendered the possibility for selective isolation of this antagonist by addition of 2.5g of this fungicide per litre of selective medium (Davet, 1979a). Captan and dichloran were found less inhibitory up to 50 ppm compared to thiram at 5 ppm and verdasan at 10 ppm (Kuthubutheen and Pugh, 1978). Recently similar trends of tolerance limit was found where captaf, captafol, metalaxyl, chlorothalonil, mancozeb had significantly low inhibition while thiram and carbendazim were highly inhibitory to *T. harziarum* and *G. virens* (Sharma and Mishra, 1995; Singh *et al.,* 1995; Dubey and Patel, 2001; Akbari and Parakhia, 2001; Sharma *et al.,* 2001).

The normal sensitivity to benzimidazole fungicides was proved to be an inherent trait of *Trichoderma* spp (Davet, 1981). Benomyl even at a concentration of 0.5 ppm was found inhibitory to *Trichoderma* in culture (Mirkova, 1982). However, Yokomizo *et al.* (1980) isolated 6 isolates of *Trichoderma* spp. tolerant to 50 ppm benomyl indicating that possible natural mutation might led to the development of more tolerant biotypes. Production of benomyl tolerant mutant of *T. harzianum* which was rhizosphere competent too suggested that in course of producing benomyl mutant through induced mutation a rhizosphere incompetent strain became competent because of the same genetic change that made it tolerant to benomyl or because of different one (Ahmed and Baker, 1987; Baby, 1998).

2.10. *In vivo* antagonistic performances of *G. virens*

It is now widely recognized that biological control of plant pathogens is a distinct possibility for the future and can be successfully exploited inn modern agriculture, especially within the frame work of integrated disease management system which is needed to hold disease below economic threshold without damaging the agro ecosystem (Papavizas and Lewis, 1981b). Redundant evidences of *in vitro* hyperparasitism followed by simultaneous *in vivo* results proved *G. virens* to be an effective bioagent for protecting a number of crop plants from damage induced by several soil borne plant pathogens (Howell, 1982; Papavizas, 1985; Cho et al., 1989; Harman *et al.*, 1989; Papavizas and Lewis, 1989;, Lumsden and Locke, 1989; Tu and Vartaja, 1989; Kim *et al.*, 1990; Tjamos *et al.*,1992; Mukhopadhyay *et al.,* 1992).

In spite of such voluminous information some erratic performances might be ascribed to the failure in devicing suitable delivery systems (Backman and Rodriguez-Kabaana, 1975; Papavizas *et al.*, 1984). A tremendous endeavor have been made through out the world during last few years but lack of knowledge in certain areas particularly the recognition phenomenon and its ecology restricted the perception and ingenuity to capitalize on this apparent asset. Following table has been presented to elucidate some selected achievement made during last five years using *G. virens* as potential bioagent against several soil borne plant diseases under glass house and field conditions, exclusively from the Indian point of view.

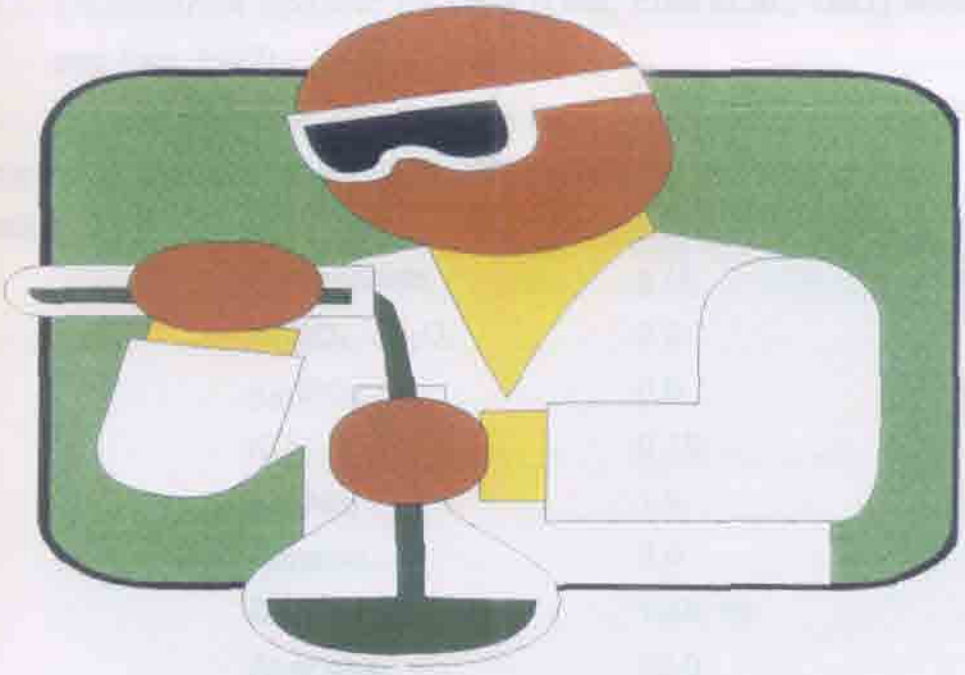
Table 2.2 : *In vivo* antagonistic performances of *G. virens* against soil borne plant diseases.

	Pathogen	Disease	Host	Mode of application	Formulation	Dose	Extent of control(%)	Reference(s)
1	*Thanatephorus cucumeris*	Web blight	Horse gram	SoT	Wheat bran saw dust (WBSD)	0.2 -0.8%	100.00	Dubey, 1998
2	*R. solani*	Root rot	French bean	SoT	WBSD	10g / pot	81.74	Abraham and Gupta, 1998
3.	*F. udum*	Wilt	Pigeonpea	ST / ST + Carboxin	-	1g kg^{-1} / 1g + 1.5g kg^{-1}	43.10 – 82.90	Chaudhury and Kumar, 1999
4	*M. phaseolina*	Dry root rot	Mungbean	SoT	Wheat bran / saw dust	10g / kg of soil	48.32	Rajeswari *et al.*, 1999
5	*R. solani*	Seedling blight	Chickpea	ST	Granular formulation	$3x10^7$ cfu/g	23.00	Prasad and Rangeshwaran, 1999
6	*S. rolfsii*	Root rot and collar rot	Sunflower	ST	Spore suspension	2.6 – 8.4 x 10^7 cfu/ml	48.60	Prasad *et al.*, 1999
7	*R. solani*	Web blight	Groundnut	SoT	WBSD	0.2%	76.71	Dubey, 2000
8	*S. rolfsii*	Wilt	Jasmine	SoT	Talc	10g / pot	49.15	Ramamoorthy *et al.*, 2000
9	*P. aphanidermatum*	Damping off	Tomato	ST or SoT	Spore suspension or wheat bran culture containing $5.2x10^8$ cfu/g	ST with $3.4x10^8$ cfu/ml. SoT – 50g/kg of soil	ST – 73.00 SoT –60.80	Hazarika *et al.*,2000

	Pathogen	Disease	Host	Mode of application	Formulation	Dose	Extent of control(%)	Reference(s)
10	*Hypochnus sasakii*	Banded leaf and sheath blight	Maize	Foliar application	Spore suspension	-	-	Sharma and Saxena, 2000
11	*R. solani, S. rolfsii, F. oxysporum* f.sp *lentis*	Wilt complex	Lentil	ST & SoT	WBSD & Spore suspension	100-200kg ha^{-1} or 10^7 – 10^8 conidia or chlamydospore / ml suspension	-	Singh and Mukhopadhyay, 2000
12	*S. rolfsii, R. solani, Fusarium* spp, *M. phaseolina*	Seed and seedling rot complex	Soybean	ST with sorghum grain	Powdered sorghum grain	0.2% + 0.1% carboxin (vitavax)	-	Pant and Mukhopadhyay, 2001
13	*Colletotrichum falcatum*	Red rot	Sugarcane	Sett dip treatment	Spore suspension	-	-	Bhatt and Sabalpara, 2001
14	*S. rolfsii, R. solani, F oxysporum* f.sp *phaseoli*	Wilt complex	French bean	ST with contaf	Talc	0.1% + 0.025% contaf	-	Mukherjee *et al.*, 2001

ST – Seed Treatment SoT – Soil Treatment

CHAPTER 3



Materials and Methods

3. MATERIALS AND METHODS

In this chapter the brief features of the materials used and techniques adapted for achieving the objectives are described. For routine phytopathological and analytical techniques suitable references have been cited.

3.1. Media used

Media used for the present series of investigations are described below.

3.1.1. Trichoderma Specific Medium (TSM, Elad *et al.*, 1981; Modified by Saha and Pan, 1997)

Used for selective isolation of wild and mutant isolates of *Gliocladium* spp. The competitive parasitic ability and rhizosphere colonization were also studied on modified TSM.

Components	g / L
$MgSO_4$, $7H_2O$,	0.2
K_2HPO_4	0.9
KCl	0.15
NH_4N0_3	1.0
Glucose	3.0
Distilled water to	1000 ml
Agar agar	20.0

pH – 5.0 to 5.5

Medium was sterilized at 15 lb psi for 15 min. After autoclaving following components were added

Chloramphenicol	250 mg / L
Methyl orange	300 ,,
Rose Bengal	150 ,,
Captan 50% WP	25 ,,

3.1.2. Potato Dextrose Agar (PDA, Riker and Riker, 1936)

Used for routine culture of wild and mutant isolates of *Gliocladium* spp. and other pathogenic fungi.

Components	g / L
Peeled potato (used for making decoction)	200.00
Dextrose	20.00
Agar agar	20.00
Distilled water to	1.0 L

Medium was sterilized at 15 lb psi for 15 min. Potato Dextrose Broth (PDB) when required was prepared using same composition without agar agar.

3.1.3. Glucose Tartarate Medium (GT, Brian and Hemming, 1950)

Used for the production of chlamydospores of wild and mutant isolates of *Gliocladium* sp. under test.

Components	g / L
Glucose	20.00
KH_2PO_4	2.0
$MgSO_4$, $7H_2O$	1.0
Ammonium tartarate	1.0
$FeSO_4$	0.001
Distilled water to	1.0 L

pH 5.0 to 5.5

Medium was sterilized at 15 lb psi for 15 min.

3.1.4. Czapek Dox Broth (CDB, Raper and Thom, 1949)

Used for assay of enzyme production by wild and mutant isolates of *Gliocladium* sp.

Components	g / L
$NaNO_3$	2.0
K_2HPO_4	1.0
$MgSO_4, 7H_2O$	0.5
KCl	0.5
$FeSO_4, 7H_20$	0.01
Sucrose	30.0
Distilled water to	1.0 L

pH 5.0 to 5.5

Medium was sterilized at 15 lb psi for 15 min.

To investigate the effect of induction on enzyme production, partial substitution of sucrose with following combinations were made keeping other ingredients unchanged.

(i) 10.0 g sucrose + 10.0 g mycelial powder of *M. phaseolina* per litre.

(ii) 10.0 g sucrose + 10.0 g mycelial powder of *Pythium ultimum* per litre.

(iii) 10.0 g sucrose + 42.0g colloidal chitin (contain 12% chitin) per litre as mentioned by Pietro *et. al.* (1993).

(iv) 10.0 g sucrose + 10.0 g carboxy methyl cellulose (CMC) per litre.

Mycelial powder of *M. phaseolina* and *P. ultimum* were prepared by culturing the organisms separately in PDB for 7 days at 28±1^{0}C. Mycelial mats were harvested by filtering through double layered muslin cloth and dried in oven at 60^{0}C for 2 days. The dried mycelial mat was ground in a grinder and used for medium preparation.

3.1.5. Oat meal agar (OMA, Johnson and Curl, 1972)

Used for cultural and morphological characterization of selected mutant and wild isolates of *Gliocladium* sp.

Components	g / L
Oat meal	17.0
Agar agar	20.0
Distilled water to	1000.0 ml

Medium was sterilized at 15 lb psi for 15 min.

3.1.6. Other media used

Other media used for mass multiplication of antagonist include sterilized rice husk containing 50-60 % moisture for mass multiplication of wild and mutant isolates of *Gliocladium* sp., sterilized sand maize meal medium (1:2 w/w; distilled water 1:3 v/w) for mass multiplication of *M. phaseolina*, sterilized paddy straw for mass production of sclerotia of *R. solani* and sterilized wheat grain for mass multiplication of *F. oxysporum* f.sp. *udum*.

3.2.1. Biocontrol agents

Biocontrol agents (*Gliocladium* spp.) were isolated from soil of different ecological niches containing highly decomposed organic matter, using soil dilution plate method (Waksman and Fred, 1922) on modified TSM. and subsequently identified as isolates of *G. virens* Miller *et al.*, *G. roseum* Bain, *G. penicillioides* Corda. Their code, crop canopy and location of source are presented in table 3.1.

Table: 3.1. Description of isolates of *Gliocladium* spp and their sources.

Sl No.	Isolate code	Crop canopy	Location
1.	Gv_1*	Betelvine Boroj	BCKV Farm, Kalyani
2.	Gv_2	Casurina plantation	BCKV, Mohanpur, Nadia
3.	Gv_3	Soil around manure peat	Mohanpur, Nadia
4.	Gr**	Do	Do
5.	Gp***	Betelvine Boroj	Incheck Farm, BCKV, Kalyani, Nadia.

* Gv : *Gliocladium virens*

** Gr : *Gliocladium roseum*

*** Gp : *Gliocladium penicillioides*.

3.2.2. The pathogens:

The plant pathogenic fungi used throughout the experiment included mostly soil borne fungal organisms causing seedling blight, root rot and wilt pathogens like *Rhizoctonia solani* Kuhn. (isolated from sheath blight infected rice plants), *Macrophomina phaseolina* Tassi (Goid.) [isolated from stem rot infected jute plants], *Sclerotium rolfsii* Sacc. (isolated from foot rot infected tube rose plants and *Fusarium oxysporum* f.sp. *udum* Butler. (isolated from wilt infected pigeonpea plants).

3.2.3. The host plant

The host plant used for study of rhizosphere colonization was ground nut (*Arachis hypogea* L.) var. TMV 2. In addition to groundnut, jute (*Corchorus capsularis* L.) var. JRC. 321, green gram (*Vigna radiata* L.) var. S-12 and pigeonpea (*Cajanus cajan* L.) var. Local were used for the study of *in vivo* efficacy of wild and mutant isolates of *Gliocladium* sp. The seeds were obtained from the local market.

3.3. Sterilization of soil, FYM and planting material

Soil and FYM, used in low volume in the experiments like study of fungistasis (section 3.9.5) and competitive parasitic ability (section 3.9.6), were sterilized twice in the consecutive days at 30 lb psi for 1 hr. For sterilization of soil and FYM, used in bulk quantity in the experiments like rhizosphere colonization (section 3.9.7) and management of diseases caused by soil borne plant pathogens under green house condition (section 3.11), soil and FYM mixture (2:1 v/v) were fumigated with 5% formaldehyde solution @ 1L/cft followed by covering with polythene sheet for 7 days. For isolation of pathogenic fungi, surface sterilization of the diseased plant material was done by soaking the soft tissues in 1% sodium hypochlorite solution for 2-3 min. and hard tissues for 4-5 min.

3.4. *In vitro* screening of antagonistic potential of different wild isolates of *Gliocladium* spp. against the soil borne plant pathogens

The antagonistic potential of different wild isolates of *Gliocladium* spp was evaluated *in vitro* against four soil borne plant pathogens using dual culture plate method (Dennis and Webster, 1971) on the basis of relative growth rate of antagonist

and pathogen. Both antagonist and pathogen were inoculated at opposite ends in sterilized Petriplates (90 mm diam) containing 20 ml sterilized PDA medium. Inoculation was done in such a way (by staggering the day of inoculation) that the point of contact occurred somewhere in the middle of the plate. The inoculated plates were incubated in a BOD incubator at 28±1^{0}C. The antagonistic potential was rated by taking daily observation on a class of scale proposed by Bell *et al.* (1982) with slight modification suggested by Saha and Pan, 1997. The modified Bell's scale was as given below.

S_1: When antagonist overlaps the total growth of pathogen

S_2: When antagonist overlaps 2/3 growth of the pathogen.

S_3: When antagonist overlaps 1/2 of the pathogen growth.

S_4: When pathogen growth is restricted at the point of contact.

S_5: When pathogen starts overlapping the antagonist growth.

The best two isolates of *Gliocladium* spp. showing greater antagonistic potential over the others against at least any two of the test pathogens were selected for further experiments. In the present investigation among the isolates of *Gliocladium* spp., two isolates of *G. virens* were selected for their greater antagonistic potential.

3.5. Production of mutant isolates of *G. virens*

Four-day-old culture of selected wild isolates of *G. virens* in PDA slants were exposed to non-particulate electromagnetic γ-radiation of wavelength 10^{-11} to 10^{-7} cm from ^{60}Co source at Central Research Institute for Jute and Allied Fibre (CRIJAF), Nilganj, Barrackpore, West Bengal. Each isolate was separately exposed to five different doses of γ-radiation viz. 50 KR, 75 KR, 100 KR, 125 KR, 150 KR and the irradiated cultures were kept at 4^{0}C for 24 hrs.

3.5.1. Isolation and purification of phenotypic mutants

Twenty-four hours after irradiation spore suspension with a concentration of 20 x 10^3 spores / ml was prepared in sterilized distilled water separately from each of the five doses. A drop of teepol was added to avoid

aggregation of spores. One ml of this spore suspension was plated on modified TSM to get discrete colonies. The plates were incubated at 28±1^{0}C for 7 days. The colonies showing distinct phenotypic variations on color of the colony, their texture and growth patterns over wild isolate were selected. The young hyphal tips of selected colonies were transferred to PDA slants and were coded according to dose of radiation, respective wild isolate followed by a Roman number.

3.6. Selection of the mutants with enhanced biocontrol potential by *in vitro* rapid screening method :

In vitro evaluation of the antagonistic potential of mutant isolates against four above mentioned test pathogens was made through rapid screening test developed in this laboratory. In this method, each Petriplate containing PDA medium was divided into four quadrants. Each quadrant was inoculated with one each mutant isolate where as the test pathogen was inoculated centrally. The inoculated plates were incubated at 28± 1^{0}C. Antagonistic potential was rated by modified Bell's scale as mentioned earlier (section 3.4). Mutants showing greater antagonistic potential against any two of the test pathogens over their wild counterpart were selected for subsequent studies.

3.7. Stabilization of selected isolates through generation studies

To study the stability of selected mutant isolates, they were subcultured for 10 successive generations through serial transfer in PDA plates. The antagonistic potential against the four test pathogens as well as the colony characters on 2nd, 6th and 10th generation were compared with their respective first generation culture. The mutant isolates, those maintained the original phenotypic characters and antagonistic potentiality were considered as stable mutants and were used in the subsequent experiments.

3.8. Phenotypic and physiological characterization of wild and selected mutant isolates

3.8.1. Cultural characterization

Twenty ml of molten OMA medium was poured in each Periplate (90 mm diam.) and was allowed to gel. These plates were inoculated with 6.0mm diam mycelial disc taken from the periphery of actively growing 3/4 days old culture(s) of selected mutant isolates and their respective wild counterparts. The inoculated plates were incubated at 28±1^{0}C for 5 days. The cultural characters like color, texture, growth pattern, etc. of the colonies were recorded.

3.8.2. Morphometry

Semi-permanent mounts were prepared from 4 days old cultures of selected mutants and their wild counterparts grown on OMA medium using 0.1% lactophenol-cotton blue solution. Micrometric measurements of phialospores and phialides were recorded at 100 X magnification under a calibrated light microscope.

3.8.3. Scanning Electron Microscopy (SEM)

To perceive critically the distinct sporophore and spore morphology of the selected mutants and their wild counterparts, Scanning Electron Microscopy (SEM) was done at Scientific Instrumentation Center, University of Burdwan, W.B. following the procedures described by Maitra (2000). For this purpose, selected mutants and wild isolates were cultured in test tubes containing 10 ml PDB for 10 days at 28±1^{0}C. Fungal mats were collected by decanting the medium and the mats were repeatedly washed with sterilized distilled water. After thorough washing primary fixation of the fungal mats was made with 2% gluteraldehyde in 0.I M sodium cacodylate buffer (pH 7.0) for 2 hrs at 4^{0}C followed by repeated washing with the same buffer. Secondary fixation of the fungal mats was done with 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer at pH 7.0 for 2 hrs at 4^{0}c which again was followed by repeated washing with the same buffer. Each isolate was then passed through increasing concentrations of ethyl alcohol series (30% ethyl alcohol for 5 min, 50% ethyl alcohol for 5 min, 70% ethyl alcohol for 10min, 90% ethyl alcohol

two times each for 30 min and finally absolute alcohol two times each for 30 min). The dehydrated fungal mats were vacuum-dried and coated with gold. Sporophore and spore morphology of the wild and mutant isolates was compared and distinct characters were photographed.

3.8.4.1. Assay for enzyme production :

Quantification of extracellular hydrolytic enzymes produced by test mutants and their wild isolates of *G. virens* was assayed in liquid medium. Indirect quantification of four hydrolytic enzyme viz., β-1,3 glucanase, β-1,4 glucanase, chitinase and cellulase was made by the methods as described below.

3.8.4.1.1. Composition of buffers and reagents

3.8.4.1.1.1. McIlvaines buffer

For every 200 ml (A) 2.84 g Na_2HPO_4 was dissolved in 100 ml distilled water.

(B) 1.92g citric acid was dissolved in 100ml distilled water.

(A) and (B) were mixed and the pH was adjusted to 4.0 using 1N NaOH.

3.8.4.1.1.2. Citrate buffer

For every 200 ml (A) 0.1M solution of citric acid was prepared by dissolving 2.10g citric acid in 100ml distilled water.

(B) 0.1 M solution of sodium citrate was prepared by dissolving 2.94g sodium citrate in 100ml distilled water.

To get 200ml citrate buffer of pH 4.8, 46ml of (A) and 54ml of (B) were mixed thoroughly in a volumetric flask. The volume was made up to 200ml with 100ml distilled water.

3.8.4.1.1.3. Potassium ferricyanide reagent

Components	Quantity / 250 ml
Na_2CO_3	13.25g
Potassium ferricyanide	125.0 mg
Distilled water to	250 ml

3.8.4.1.1.4 Dinitrosalicylic acid reagent

Components	Quantity / 100 ml
Dinitrosalicylic acid	1.0g
Phenol	0.2g
Sodium sulfite	0.05g
Sodium hydroxide	1.0g
Sodium potassium tartarate (Rochelle salt)	18.0g
Distilled water to	100 ml

3.8.4.1.1.5. Preparation of colloidal chitin

The colloidal chitin was prepared from crude crab shell chitin following the stepwise procedure described below.

i) The crude chitin was grinned in a grinder and 30g crude chitin was washed in 500ml distilled water.

ii) The water was decanted and the residue was extracted in 505ml acidified ethanol-ether mixture (ethanol 250ml diethyl ether 250ml and 5ml concentrated HCl).

iii) The mixture was centrifuged (model Remi C 24) at 12,000 rpm for 15 min and the residue was bleached by 500ml of 0.2 M sodium hypochlorite solution at 75^0C for 1hr with continuous stirring.

iv) The mixture was centrifuged at 12,000 rpm for 15 min at 5^0C and residue was collected. Ten-ml acetone was added to it followed by concentrated HCl at 0^0C until most of the residues dissolved.

v) The mixture was centrifuged at 12,000 rpm for 15 min at 5^0C and the supernatant was collected.

vi) Distilled water (1.5 L) and ice was added to the collected supernatant and the chitin was allowed to precipitate over 2hr at 0^0C.

vii) The chitin was washed 3 times with 1L distilled water and was centrifuged after each wash. The final residue was collected and this colloidal form of chitin was preserved in an air- tight container at 0^0C for subsequent use.

3.8.4.1.2. Preparation of enzyme source

The selected mutant and wild isolates of *G. virens* were separately inoculated in 250 ml conical flasks containing 100 ml of CDB and media with other substitution as mentioned in section 3.1.4. with a 6 mm mycelial disc from 9 day-old culture. The flasks were incubated at 28±1^{0}C for 10 days with a shaking for 5 min at 125 rpm twice in a day. After incubation culture filtrate of each isolate was filtered through double layered Whatman filter paper no. 1 and the collected culture filtrate was preserved at (–)5^{0}C after adding sodium azide (0.02% w/v) as preservative. This culture filtrate was assayed for enzyme production and electrophoretic study of extracellular proteins was made (section 3.8.4.2.3.). The total protein content in the culture filtrates was estimated (Lowry *et al.*, 1951).

3.8.4.1.3.1. Assay for β-1,3 glucanase:

(I) Reaction mixture

(a) 0.5 ml laminarin*(concentration 3.2 mg/ml distilled water).

(b) 1.0 ml of 0.05 M citrate buffer (pH 4.8)

(c) 0.5 ml culture filtrate.

[*Laminarin isolated from *Laminaria digitata* was supplied by Fluka, Switzerland]

(II) The reaction mixture was incubated at 40^{0}C for 60 min and the reaction was stopped by boiling. The amount of reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). In this method equal volume of dinitrosalicylic acid reagent was added to the reaction mixture and warmed in boiling water bath for 15 min. The absorbance of the reaction mixture was measured at 575 nm in a spectrophotometer (model Elico India SL 171) and compared with the standard graph drawn by following the same procedure but using different concentrations of glucose instead of culture filtrate.

(III) One unit of enzyme activity was expressed as release of 1μmol glucose/ml. of culture filtrate / min.

3.8.4.1.3.2. Assay for β-1,4 glucanase (endoglucanase)

(I) Reaction mixture (a) 1.0 ml of 1% carboxy methyl cellulose (CMC)
(b) 2.0 ml of 0.05 M citrate buffer (pH 4.8).
(c) 1.0 ml of culture filtrate.

The reaction mixture was incubated at 55^0C for 30 min in a water bath with periodical shaking. The reaction was stopped by boiling.

(ii) The amount of glucose released in the reaction was estimated by dinitrosalicylic acid method and enzyme activity was expressed same as mentioned in section 3.8.4.1.3.1.

3.8.4.1.3.3. Assay for chitinase :

(1) Reaction mixture : (a) 0.5ml of 0.5% colloidal chitin
(b) 1.0 ml McIlvaines buffer (pH 4.0)
(c) 0.5 ml of culture filtrate

The reaction mixture was mixed thoroughly and was incubated at 37^0C for 20 min in a water bath with periodical shaking. The reaction was stopped by boiling for 3 min in heated water bath.

(II) In the reaction mixture 3ml potassium ferricyanide reagent was added and was heated in boiling water bath for 15 min.

(III) The amount of N-acetyl glucosamine (NAG) released was estimated from the absorbance of the reaction mixture at 420 nm comparing with the standard graph drawn by performing the same procedure but using different concentration of commercial NAG suspension instead of culture filtrate. One unit of enzyme activity had been expressed as release of 1μmol NAG / ml culture filtrate / min.

3.8.4.1.3.4. Assay for β-1,4 exoglucanase (cellulase)

The method was similar to that for estimation of endoglucanase (section 3.8.4.1.3.2.) but the only difference was addition of 1% cellulose was added in reaction mixture instead of CMC as the substrate of enzyme production.

For assay of all enzymes control/standard was prepared following same procedure but only replacing culture filtrate with concerned sterilized media in the reaction mixture. Though 10 day-old culture filtrate was used for assay, actually the enzyme production initiated as early as the growth of antagonist started. So, the residual NAG or glucose/reducing sugars was expected to interfere in quantification of enzymes. To avoid this difficulty a separate set of treatments was arranged without adding substrate but keeping the total volume of the mixture unchanged by addition of required volume of sterile distilled water.

3.8.4.2. Variation in extracellular protein production by SDS-PAGE

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was performed to examine the variation in production of extracellular protein by different isolates (both mutants and their wild counterparts) of *G. virens*.

3.8.4.2.1. Materials used

3.8.4.2.1.1. Stock acrylamide solution

Acrylamide	30.0g
Bisacrylamide	0.8g
Distilled water to	100ml

3.8.4.2.1.2. Separating gel buffer

Tris HCl	22.7g
Distilled water to	100.0ml

pH was adjusted to 8.8 using concentrated HCl.

3.8.4.2.1.3. Stacking gel buffer:

Tris HCl	7.26g
Distilled water to	100.0ml

pH was adjusted to 6.8 using concentrated HCl.

3.8.4.2.1.4. Polymerising agent :

(a) Ammonium persulphate : 0.5g /10ml, prepared freshly before use.

(b) TEMED : used fresh from refrigarator.

3.8.4.2.1.5. Electrode buffer:

Tris	12.0g
Glycine	28.8g
SDS	2.0g
Distilled water to	2.0 L

pH – 8.2 to 8.4 (No adjustment was required and was used 2-3 times).

3.8.4.2.1.6 Sample buffer : (5x Conc.)

Sucrose	5g
Tris HCl buffer (pH 6.8)	5ml
SDS	0.5g
Marcaptoethanol	0.25ml
Bromophenol blue	1.0ml

(0.5%w/v solution in warter).

Stored frozen in small aliquouts and diluted to 1xconc. before use. Sodium dodecyl sulphate (SDS) 10% solution was prepared in distilled water and stored at room temperature.

3.8.4.2.2. Gel casting

(i) The glass plates (16cm x 14 cm) and spacers were cleaned thoroughly, assembled with bulldog clips and clamped in upright position. 2% water agar was then applied at the edge of the spacers to seal the chamber.

(ii) A sufficient volume of 15% separating gel mixture (40ml) was prepared by mixing the following.

Stock acrylamide solution	20.0ml
Tris HCl (pH 8.8)	8.0ml
Ammonium persulphate solution	0.2ml
10% SDS	0.4ml
TEMED	20.0µl

The ingredients were mixed gently and poured in glass chamber with enough caution for avoiding formation of air bubble within the gel. Top 5cm of the chamber was left for stacking gel and allowed to gel for 30 – 60 min.

(iii) Ten ml of 4% stacking gel was prepared by mixing the following components

Stock acrylamide solution	1.35ml
Tris HCl (pH 6.8)	1.0ml
Distilled water	7.5ml
Ammonium persulphate solution	50µl
10% SDS	0.1ml
TEMED	10.0µl

The ingredients were mixed and poured on separating gel. The comb was placed carefully in stacking gel and the gel was allowed to set for 30-60 min.

(iv) After polymerization of the stacking gel, the comb, clips, agar etc were removed and gel was fitted in electrophoresis apparatus. The electrode buffer was poured in both upper and lower tank connected to a DC-power supply unit.

3.8.4.2.3. Sample loading and gel running

(i) The samples were prepared using the culture filtrate mentioned in section 3.8.4.1.2. The protein concentration in each sample was adjusted to 25 µg/100µl by mixing sample buffer of 1x Conc. with required amount of culture filtrate. The samples were mixed thoroughly in effendorf tube and injected separately in the individual wells of stacking gel by a micropipette.

(ii) The power supply unit was switched on and regulated to 15 mA for few min until the samples traveled through the stacking gel. Then the run was continued at 25 mA for about 2hrs until the bromophenol blue

reached the desired distance. The gel was then taken out from glass plates and subjected to stain.

3.8.4.2.4. Silver staining

As the protein concentration in the samples used for electrophoresis was very low, silver staining process was followed to detect the band in spite of using coomassie brilliant blue.

3.8.4.2.4.1. Materials used

3.8.4.2.4.1.1. Washing solution

Formaldehyde solution (37%)	1.0ml
Methanol	40.0ml
Distilled water to	600ml

3.8.4.2.4.1.2. Sodium thiosulphate solution :200 mg sodium thiosulphate was dissolved in 1L of distilled water.

3.8.4.2.4.1.3. Silver nitrate solution : 0.1g silver nitrate was dissolved in 100ml of distilled water.

3.8.4.2.4.1.4. Developer :

Sodium carbonate	3g in 80 ml distilled water
Sodium thiosulphate solution (0.2%)	1.0ml
Formaldehyde solution (37%)	1.0ml

Final volume was made up to 100ml with distilled water.

3.8.4.2.4.1.5. Stopper : 5g citric acid was dissolved in 100 ml distilled water.

3.8.4.2.4.2. Procedure :

(i) After electrophoresis the gel was transferred in a clean plastic container and was washed by the washing solution for 10 min with slow shaking.

(ii) The washing solution was discarded and the gel was rinsed with plenty of distilled water for 2 min.

(iii) The gel was soaked in sodium thiosulphate solution for 1-2 min and was washed with distilled water twice for 1-2 min.

(iv) The gel was soaked in silver nitrate solution for 10 min with gentle shaking and was washed as earlier.

(v) The developer was poured to the plastic container and the gel was shaken slowly. The protein reduced silver nitrate to silver and yellow to dark brown bands appeared in the gel. When the sufficient intensity of band was developed the reaction was stopped by stopper.

3.9. Microhabitat behavior

Microhabitat behavior of selected mutants and their wild counterparts was examined through different experiments, like (i) germinability of different spore forms like phialospores and chlamydospores in different sources of water (ii) fungistasis in soil and decomposed farm yard manure (iii) rhizosphere colonization and (iv) competitive parasitic survival using sclerotia as live baits.

3.9.1. Production of phialospores and chlamydospores

The phialospores of mutants and their wild isolates were collected from 9 day-old culture in PDB at 28±1^{0}C with a thin camel hair brush and the spore suspension was prepared in sterilized distilled water. For mass production of chlamydospores selected mutants and their wild isolates were grown in GT broth for 21 days at 28±1^{0}C. The mycelial mat was harvested using Whatman filter paper no. 1 and macerated in a blender using sterile distilled water. The suspension was repeatedly (3-4 times) centrifuged at 4000 X g for 10 min. The supernatant containing mycelial fragments were carefully decanted. The sediment containing chlamydospores were used in following experiments by resuspending in sterilized distilled water.

3.9.2. Mass multiplication of antagonists

The selected mutants and their wild counterparts were mass multiplied in sterilized rice husk with 50 – 60% moisture in 500 ml conical flasks each containing about 150 g of medium. Each flask was inoculated with three mycelial discs and incubated at 28±1^{0}C for 15 days with periodic stirring after inoculation. The colony forming unit (cfu) per g of final products were estimated on modified TSM using dilution plate method.

3.9.3. Mass production of sclerotia of *R. solani* and *S. rolfsii*

For mass production of sclerotia of *R. solani* chopped paddy straw was soaked overnight in water. Excess water was drained out and sterilized twice in autoclave at 15lb psi for 15 min in double layered polypropelene bags. Inoculated paddy straw was incubated for 15 days at 28±1^{0}C and sclerotia produced were separated by floating the final product. For mass production of sclerotia of *S. rolfsii,* sterilized PDB was inoculated with the fungus and incubated at 28±1^{0}C for 25 days. The sclerotia of both *R. solani* and *S. rolfsii* were collected, washed, air-dried, and surface sterilized with 1% sodium hypochlorite and stored at 5^{0}C in screw cap test tubes for future use.

3.9.4. Germinability of spores in water from different sources

The *in vitro* germinability of phialospores and chlamydospores of the mutants and their wild counterparts was studied in sterilized distilled water (SDW), pond water (PW), tap water (TW) and soil leachet (SL). The pH of each sources of water was measured by a digital pH meter (model Systronics 335). To prepare soil leachets 500 g freshly collected, air-dried, powdered sieved soil was thoroughly stirred with 500 ml distilled water, free steamed for 30 min and allowed to settle overnight. The supernatant was gently decanted, filtered through filter paper and used for the experiment as soil leachet. Phialospore and chlamydospore suspension of each selected mutants and their wild biotypes were prepared in different sources of water at a concentration of 1x10^3 spores / ml. Now one drop of spore suspension was pipetted on to the each cavity of a groove slide separately with sufficient replications. The slides containing spore suspension were incubated at 28±1^{0}C for 18 hrs. After incubation one drop of lactophenol-cotton blue solution was added in each cavity to

fix and to observe under microscope. The percentage of spore (phialospore or chlamydospore) germination in different sources of water was recorded.

3.9.5. Fungistasis in soil and in decomposed farm yard manure :

To study the fungistatic effect by agar disc method (Jackson, 1958), 6 mm diam of 2% water agar blocks were placed on 1 cm^2 sterilized Whatmann filter paper no. 1 lying in the surface of moist sterilized and nonsterilized soil and decomposed farm yard manure in 200ml plastic cup. The entire arrangements were placed overnight at 4°C for activation of the agar blocks. On next day one drop each of phialo and chlamydospore suspensions of wild and mutant isolates prepared in SDW (section 3.9.1) with a concentration of $1x10^3$ spores/ml was pipetted on to each agar block. Three cups were arranged in this way for a particular isolate and for each of the sterilized and nonsterilized substrate with a specific spore type. After incubation at 28±1°c for 18hr one drop of 0.1% lactophenol-cotton blue stain was added in each agar block to fix the fungus and observations were made under microscope. The percent germination of phialo and chlamydospores on sterilized and nonsterilized soil and decomposed farm yard manure was recorded.

3.9.6. Competitive parasitic ability on sclerotia of the pathogen

The competitive parasitic ability of selected mutants and their wild type isolates was studied by using sclerotia of *R. solani* and *S. rolfsii* as live baits. Phialo- and chlamydospore suspension of each isolate were mixed with 100g air- dried, powdered, soil (sterilized and nonsterilized) separately in such a way that the soil had 10^2, 10^4 and 10^6 cfu per g and the mhc of the soil became adjusted around 50%. The augmented soil was poured in 100ml plastic cups and three replications were maintained for each treatment. Ten sclerotia each of *R. solani* and *S. rolfsii* were buried in soil for each replication at a specific depth of 0.5-2.0 cm. The mouth of the plastic cups was covered with perforated aluminum foil and the cups were incubated at 28±1°C for 7 days. The sclerotia of *R. solani* or *S. rolfsii* were then harvested by floatation and sieving method (Rodriguez- Kabana *et al.*,1974) and were plated on modified TSM after surface sterilization with 1% sodium hypochlorite solution for a definite duration (for *R. solani* surface sterilization was done for 2 min and that for *S.*

rolfsii it was 3 min). The Petriplates seeded with sclerotia were incubated at 28±1°C for 7 days and the sclerotia yielding isolate of *G. virens* for each treatment were recorded.

3.9.7. Rhizosphere colonization

Rhizosphere colonization ability of selected mutants and their wild counterparts was studied in both sterilized and nonsterilized soil. Two-kg potting mixture of sterilized or nonsterilized soil and FYM.(2:1 v/v) was potted separately and supplemented with 5g of compound fertilizer (N:P:K = 10:26:26). Mass multiplied antagonist was then added in required amount so that the whole preparation contained 10^5 cfu per g potting mixture. Ten groundnut seeds (var. TMV 2) were sown per pot in replicated treatment for each isolate. The pots were irrigated frequently whenever required to maintain the mhc of the potting mixture above 20%. Rhizosphere soil was collected at two weeks interval by gently uprooting the plants and the soil adhered with the roots was collected by brushing. Ten mg of these soil samples of each isolate was suspended separately in 10 ml sterilized distilled water and 1 ml each of these suspension was plated on modified TSM in replicated treatments. After uniform horizontal shaking to distribute the suspension on the surface of TSM-medium the Petriplates were incubated at 28 ±1°C for 5 days and the number of colony forming units per g of rhizosphere soil for each isolates were enumerated.

3.10. Fungicide tolerance

The degree of fungicide tolerance of some selected mutants and their wild isolates of *G. virens* towards six popular seed treating fungicides viz. Benomyl, Bavistin, Thiram, Captan, Iprodione and Mancozeb was evaluated through poisoned food technique (Dhingra and Sinclair, 1995). For each fungicide four concentrations (eg., for Benomyl and Bavistin 0.5, 1.0, 2.0 and 5.0 ppm; for Thiram and Iprodione 5.0, 10.0, 20.0, 50.0 ppm; for Captan 1.0, 2.0, 5.0 and 10.0 ppm and for Mancozeb 20.0, 50.0, 100.0 and 250.0ppm) were incorporated in sterilized PDA medium. The fungicide amended medium was poured in 90 mm Petriplates and the plates were inoculated centrally with 6 mm diam, 3-4 day old mycelial discs of each isolate

maintaining five replications for each treatment. Inoculated plates were incubated at 28±1^{0}C for 4 days and the radial growth of the antagonists was measured in opposite directions. The ED_{50} value of the individual fungicide for each selected isolate was also computed.

3.11. Study on *in vivo* disease management :

In vivo efficacy of different test mutants and wild isolates of *G. virens* was evaluated against four soil borne diseases viz., seedling blight of greengram (c.o. *R. solani*), stem rot of jute (c.o. *M. phaseolina*) wilt of pigeonpea (c.o. *F. oxysporum* f.sp *udum*) and root rot of groundnut (c.o. *S. rolfsii*), through green house trial.

3.11.1. Doses of pathogenic inoculum :

(i) Mass multiplied *R. solani* and *S. roflsii* (as mentioned in section 3.9.3) were used@ 10 and 25 mature sclerotia/kg of potting mixture respectively.

(ii) *M. phaseolina* mass multiplied in sterilized sand maize meal medium at 28±1^{0}C for 21 days was applied @ 2.5g/kg of potting mixture.

(iii) *F. oxysporum* f.sp *udum* mass multiplied in wheat grain medium at 28±1^{0}C for 21 days was applied @ 2.5g / kg of potting mixture.

3.11.2 Preparation of pots, sowing of seeds and disease scoring

The pots were filled with both sterilized and nonsterilized potting mixture separately as mentioned in section 3.9.7. The only difference was application of pathogenic inoculum in their respective doses (mentioned in section 3.11.1) before addition of antagonists. Seeds of susceptible cultivar of crops as mentioned in section 3.2.3 were sown (25 seeds of jute and 10 ten seeds for other three crops / pot) in replicated treatments. Same dose of compound fertilizer (10:26:26) i.e., 5g/pot was applied once during sowing and another after 45 days of sowing not only to supply nutrients to the pot bound plants but also to increase the antagonist

population as well. It was visually observed that where ever the fertilizer granules dissolved and mixed with soil antagonist isolates rapidly colonized that loci [Plate 1(a-c)]. The pots were irrigated whenever required to maintain the mhc of the potting mixture above 20%. Percent plant mortality due to attack by different pathogens was scored at definite time interval depending upon the duration of the crop and appearance of disease symptoms.

PLATE - 1(a)

PLATE - 1(b)

PLATE - 1(c)

CHAPTER 4

Results

The preliminary objective of the present investigation was to isolate and collect wild isolates of *Gliocladium* spp. effective against a number of soil borne plant pathogens. To meet the necessity, soil samples from different ecological niches were collected and used for isolation of the antagonist isolates as described in section 3.2.1.

4.1. Isolation of *Gliocladium* spp.

Five isolates of *Gliocladium* spp. were isolated on modified *Trichoderma* Specific Medium (TSM) from soils of different ecological niches. Tentative identification was made at 100X magnification under calibrated microscope. The microscopic characters of the isolates were compared with Rifai's (1969) monograph and subsequently designated in accordance with concerned capital and small alphabets in order for genus, species followed by a subscripted number if belongs to same species.

Three isolates among the five produced densely penicillate conidiophores, bearing one celled brightly pigmented, smooth walled conidia in heads; conidia formed green slimy masses. The conidia were often slightly curved with a somewhat truncated base or short ellipsoidal in shape measuring 3.39 – 5.09 x 3.39 – 3.96 μm. Phialides were broadly ampuliform measuring 5.65 – 10.17 x 1.70 – 3.39 μm. These three isolates were subsequently identified as *G. virens* and designated as Gv_1, Gv_2, Gv_3 (Fig.1.i).

One isolate produced abundant aerial mycelium erect, septate conidiophores arose as side branches. Phialides were slender, measuring 8.48 – 12.43 x 1.70 – 3.96 μm, produced more convergently giving a broom shaped appearance. Conidia were in definite chains, elongate elliptical to bacillate, smooth walled, measuring 2.83 – 3.39 x 2.26 – 2.83 μm. This isolate was identified as *G. penicillioides* (Fig.1.ii).

The last isolate, produced granular to felty white colonies. Conidia were asymmetrically navicular measuring 2.26 – 3.95 x 1.70 – 2.83 μm. Phialides were aculeate, measuring 8.48 – 11.30 x 1.13 – 2.83 μm. This isolate was tentatively identified as *G. roseum* (Fig.1.iii).

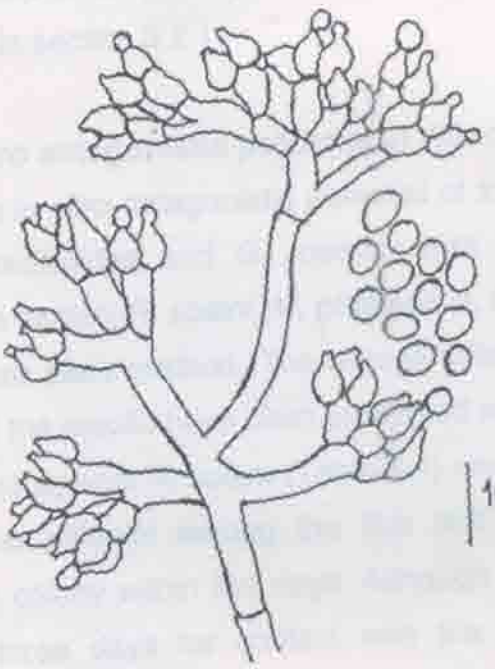


Fig.1.i.- *Gliocladium virens*

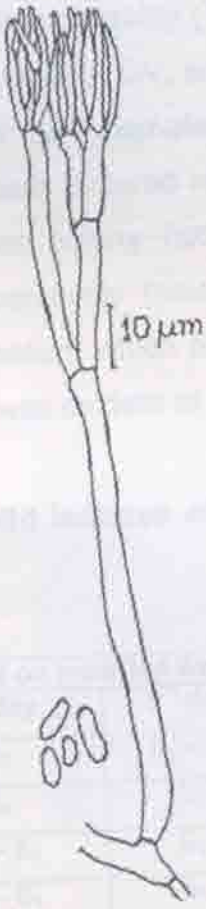


Fig.1.ii.- *G. penicillioides*

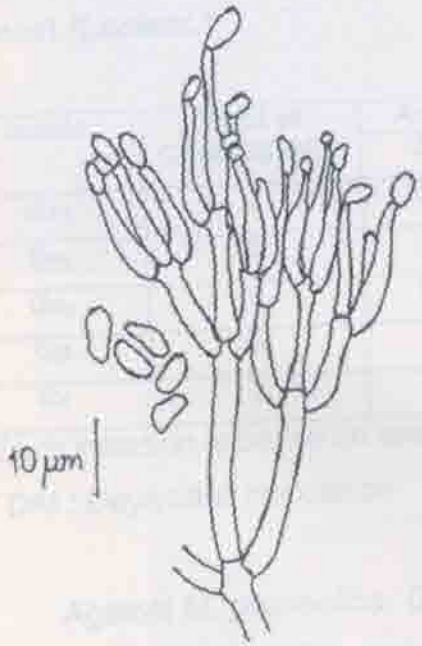


Fig.1.iii.- *G. roseum*

A table of isolated *Gliocladium spp*, their native echological niches have been presented in section 3.2.1.

4.2. *In vitro* antagonistic potential of *Gliocladium* spp.

The *in vitro* antagonistic potential of three biotypes of *G. virens* and one each of *G. penicillioides* and *G. roseum* was evaluated against four selected plant pathogens namely *R. solani, M. phaseolina, F. oxysporum* f.sp. *udum* and *S. rolfsii* by dual culture plate method. The antagonistic potential was rated on modified Bell's scale and the results have been presented in the following tables (Table 4.1 to 4.4).

Test against *R. solani* (Table 4.1) revealed that Gv_2, Gv_3 and *G. penicillioides* were most efficient among the five test isolates and completely overgrew the pathogen colony within five days. Although Gv_2 initially showed reduced growth and needed three days for contact with the pathogen colony but after contact the antagonist isolate (Gv_2) reached S_1 stage comparatively faster. Apparently less antagonistic potential was shown by Gv_1 and *G. roseum* which attained S_1 stage on 6^{th} day of inoculation. Formation of inhibition zone was evident at the point of contact between colony of *G. roseum* and *R. solani*.

Table 4.1 : *In vitro* antagonistic potential of wild isolates of *Gliocladium* spp. against *R.solani*.*

Isolate	Point of Contact(DAI)**	Antagonistic potential on modified Bell's scale rating			
		3^{rd} day	4^{th} day	5^{th} day	6^{th} day
Gv_1	2	$S_3 - S_2$	S_2	$S_2 - S_1$	S_1
Gv_2	3		S_2	S_1	
Gv_3	2	$S_3 - S_2$	$S_2 - S_1$	S_1	
Gp	2	$S_3 - S_2$	$S_2 - S_1$	S_1	
Gr	3		$S_3 - S_2$	S_2	S_1

* Each insertion is based on average of five replications.

** DAI : Days after inoculation

Against *M. phaseolina*, Gv_1 and *G. penicillioides* reached S_1 stage on 5^{th} day of inoculation whereas Gv_3 and Gv_2 required 6 and 7 days respectively. Although Gv_1 and *G. penicillioides* required same days to attain the S_1 stage but after contact Gv_1 overgrew the pathogen more rapidly than *G. penicillioides*. *G. roseum* showed

PLATE - 2

PLATE - 3(a)

PLATE - 3(b)

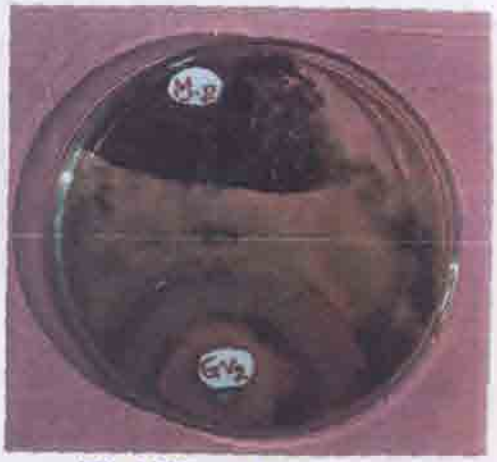


PLATE - 3(c)

PLATE - 3(d)

highest antagonistic potential and completely overgrew the pathogen colony within four days after inoculation (Table 4.2).

Table 4.2 : *In vitro* antagonistic potential of wild isolates of *Gliocladium* spp. against *M. phaseolina*.*

Isolate	Point of Contact(DAI) **	Antagonistic potential on modified Bell's scale rating				
		3rd day	4th day	5th day	6th day	7th day
Gv_1	3		S_2	S_1		
Gv_2	4			$S_3 - S_2$	$S_2 - S_1$	S_1
Gv_3	3		$S_3 - S_2$	$S_2 - S_1$	S_1	
Gp	2	$S_3 - S_2$	$S_2 - S_1$	S_1		
Gr	3		S_1			

* Each insertion is based on average of five replications.

** DAI : Days after inoculation

Antagonistic potential against *F. oxysporum* f.sp. *udum* revealed that Gv_1 was most efficient (attaining S_1 stage on 5th day of inoculation). Intermediate potentiality was shown by Gv_3, *G. penicillioides* and *G. roseum* (reached S_1 stage on 6th day of inoculation) and Gv_2 was least effective exhibiting full lysis of pathogen colony 10 days after inoculation (Table 4.3). A yellow to dark brown zone at the interacting face was visible from the reverse side of the culture plates of all antagonist-pathogen combinations indicating the production of pigment(s) by the pathogen upon invasion by the antagonist.

Table 4.3: *In vitro* antagonistic potential of wild isolates of *Gliocladium* spp. against *F. oxysporum* f sp. *udum*.*

Isolate	Point of Contact (DAI)**	Antagonistic potential on modified Bell's scale rating							
		3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
Gv_1	2	$S_3 - S_2$	$S_2 - S_1$	S_1					
Gv_2	4			$S_3 - S_2$	$S_3 - S_2$	S_2	$S_2 - S_1$	$S_2 - S_1$	S_1
Gv_3	3		$S_3 - S_2$	$S_2 - S_1$	S_1				
Gp	3		$S_3 - S_2$	$S_2 - S_1$	S_1				
Gr	3		$S_3 - S_2$	$S_3 - S_2$	S_1				

* Each insertion is based on average of five replications.

** Days after inoculation

PLATE - 3(e)

PLATE - 3(f)

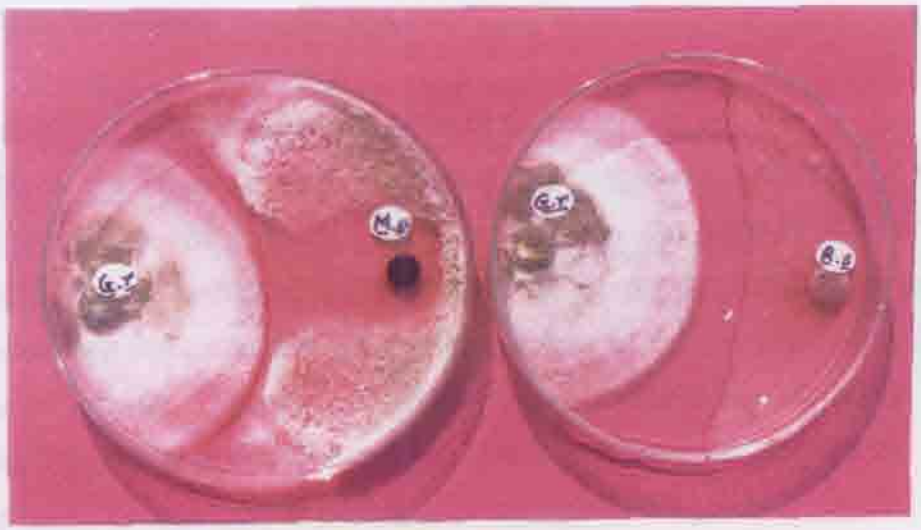


PLATE - 3(g)

In *in vitro* dual culture of *Gliocladium* spp and *S. rolfsii* all the antagonist isolates restricted the pathogen growth by coiling followed by gradual lysis of pathogen mycelium. Incidental formation of small sclerotia by *S. rolfsii* was observed around the pathogen disc. This was soon inhibited with the progress of lytic zone towards the disc. Gv_1 that completely lysed *S. rolfsii* within 7 days of inoculation exhibited most effective antagonistic potential (Table 4.4). Gv_2 and *G. roseum* were intermediate in ratings (9 days was required for lysis) whereas Gv_3 and *G. penicillioides* were least effective (reached S_1 stage 10 days after inoculation). Gv_3, however, touched the pathogen colony (point of contact) much faster than *G. penicillioides* but thereafter made slow progress over the pathogen colony. On the other hand Gv_2 and *G. roseum* required same days for contact with *S. rolfsii* colony as occurred in case of Gv_1 but required longer duration to reach the S_1 stage than Gv_1.

Table 4.4 : *In vitro* antagonistic potential of wild isolates of *Gliocladium* spp. against *S. rolfsii**

Isolate	Point of Contact (DAI)**	Antagonistic potential on modified Bell's scale rating						
		4th day	5th day	6th day	7th day	8th day	9th day	10th day
Gv_1	3	$S_3 - S_2$	S_2	$S_2 - S_1$	S_1			
Gv_2	3	$S_3 - S_2$	$S_3 - S_2$	S_2	$S_2 - S_1$	$S_2 - S_1$	S_1	
Gv_3	3	$S_3 - S_2$	$S_3 - S_2$	S_2	S_2	$S_2 - S_1$	$S_2 - S_1$	S_1
Gp	4		$S_3 - S_2$	$S_3 - S_2$	S_2	$S_2 - S_1$	$S_2 - S_1$	S_1
Gr	3	$S_3 - S_2$	$S_3 - S_2$	S_2	$S_2 - S_1$	$S_2 - S_1$	S_1	

* Each insertion is based on average of five replications.

** Days after inoculation

4.3. Selection of efficient wild isolates of *Gliocladium* spp.

Above results on *in vitro* evaluation of antagonistic potential of wild isolates revealed that there was a marked variation amongst the isolates of *Gliocladium* spp. The variation was estimated on the basis of duration required by an isolate to overgrow or lyse the pathogen colony. In addition, individual isolates differed among themselves against different pathogens. A comparative analysis on overall antagonistic potential of *Gliocldium* spp. (Table 4.5) revealed that all the five isolates exhibited more or less similar potential of antagonism against *R. solani* ($5S_1$ to $6S_1$)

and that varied a little against *M. phaseolina* ($4S_1$ to $7S_1$) under condition of time required for contact between antagonist and pathogen colony. The marked variation was observed against *F. oxysporum* f.sp. *udum* and *S. rolfsii* where Gv_1 and Gv_3 showed efficient antagonistic ability ($5S_1$ and $6S_1$ respectively against *F. oxysporum* f.sp. *udum*, $7S_1$ and $10S_1$ respectively against *S. rolfsii*) as compared to Gv_2 and *G. roseum*. *G. penicillioides*, however, showed apparently better antagonistic potential against all the pathogen tested but due to erratic growth habit in culture plate it became difficult to work with further. Two isolates namely Gv_1 & Gv_3 were therefore aptly selected for further studies and exposed to five different doses of γ-radiation as the method described in section 3.5 for the purpose of mutation.

Table 4.5: Overall view on the antagonistic potential of wild isolate of *Gliocladium* spp. against *R. solani*, *M. phaseolina*, *F. oxysporum* f.sp. *udum* and *S. rolfsii*.

Isolate of *Gliocladium spp*	Pathogen tested (Days required for attaining S_1 stage)			
	R. solani	*M. phaseolina*	*F.oxysporum f sp. udum*	*S. rolfsii*
Gv_1	$6S_1$	$5S_1$	$5S_1$	$7S_1$
Gv_2	$5S_1$	$7S_1$	$10S_1$	$9S_1$
Gv_3	$5S_1$	$6S_1$	$6S_1$	$10S_1$
Gp	$5S_1$	$5S_1$	$6S_1$	$10S_1$
Gr	$6S_1$	$4S_1$	$6S_1$	$9S_1$

4.4. Viability of irradiated conidia of *G. virens*

In vitro evaluation on viability of γ-radiated conidia of *G. virens* showed that more than 99% mortality had been occurred in most of the cases and the reduction in viability was increased with increasing doses of radiation. However, Gv_3 showed comparatively better resistance to 50 KR of γ-radiation permitting 97% reduction in viability after exposure (Table 4.6).

Table 4.6: Viability of selected wild isolates of *G. virens* upon exposure to different doses of γ -radiation.

Isolate of *G. virens*	Dose of radiation applied (Krad)	Concentration used (per ml)	Viable population on TSM ($X10^3$ / ml)	% Reduction in viability over no exposure
Gv_1	No exposure	20×10^3	3.8	
	50	"	0.02	99.47
	75	"	0.003	99.92
	100	"	0.00025	99.99
	125	"	0.000036	99.99
	150	"	0.000012	99.99
Gv_3	No exposure	20×10^3	1.31	
	50	"	0.04	96.95
	75	"	0.0065	99.50
	100	"	0.006	99.54
	125	"	0.0032	99.70
	150	"	0.00086	99.94

4.5. Isolation of mutant isolates of *G. virens*

Irradiated cultures of selected wild isolates (Gv_1 & Gv_3) of *G. virens* resulted in production of more than hundred of mutant isolates from which twenty-five mutant isolates were short-listed. These isolates showed distinguishable phenotypic variation with respect to their color (from dark green to albino), texture of the colony (from nodular to fluffy and scanty to profuse mycelial growth) and growth pattern (from submerged to raised colony) [Table 4.7]. Among these apparently confirmed mutant isolates, 4 isolates, namely $50Gv_1V$, $50Gv_1VI$, $75Gv_1IX$ and $100Gv_3I$ deserved special interest for their unique behavior to produce albino colony instead of normal green as exhibited by their wild counterparts.

4.6. *In vitro* rapid screening for selection of mutant isolates of *G. virens* with enhanced antagonistic potential

In vitro rapid screening on antagonistic potential of mutant isolates of *G. virens* revealed varied degree of antagonism. The overall perusal of result highlighted comparatively better antagonistic potential of most of the mutant isolates particularly against *R. solani* and *M. phaseolina* whereas some mutant isolates were equally or

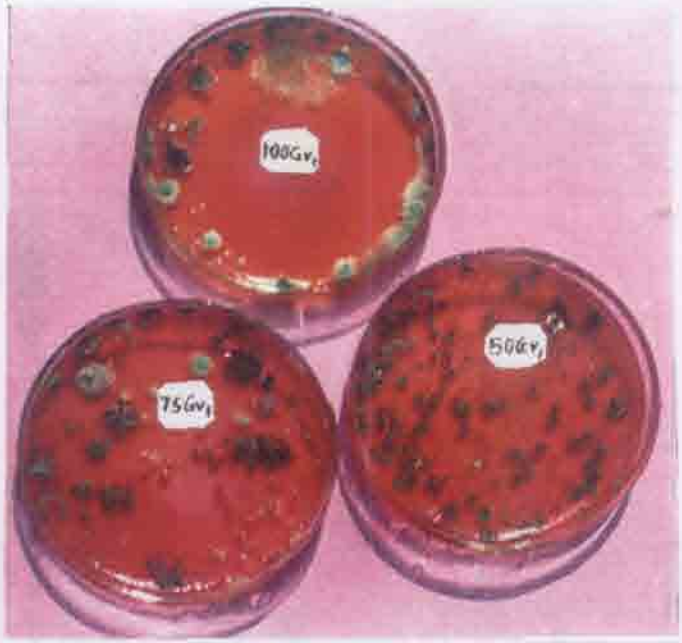


PLATE - 4(a)

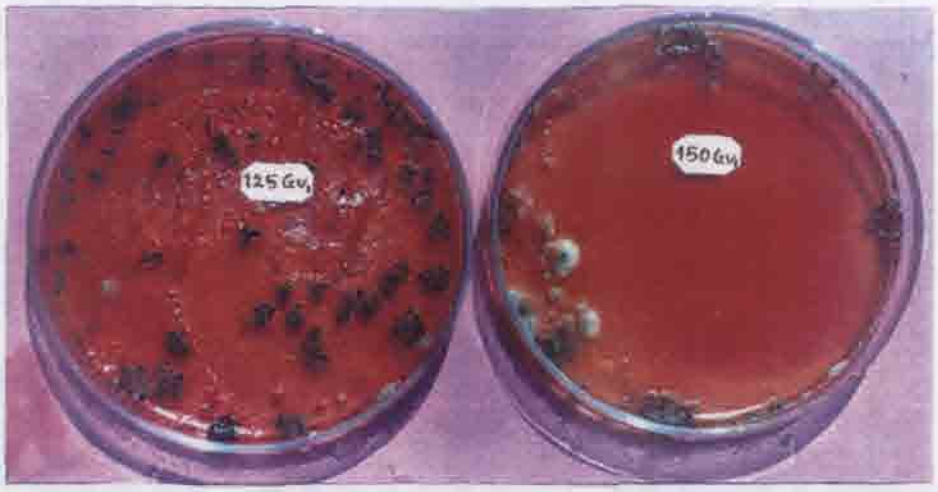


PLATE - 4(b)

PLATE - 4(c)

PLATE - 4(d)

Table 4.7 : Cultural characterization of mutant isolates of *G. virens*

Wild isolate	Mutant isolate	Cultural characteristics exhibited on PDA
Gv_1	$50Gv_1I$	Dark green, submerged colony.
	50 Gv_1II	Dark green, submerged colony with white mycelial growth at the margin.
	50 Gv_1III	Green raised colony with profuse mycelial growth.
	50 Gv_1IV	Yellowish green, nodular, submerged colony with scanty mycelial growth.
	50 Gv_1V	Albino (white), nodular ,submerged colony with scanty mycelial growth.
	50 Gv_1VI	Albino (white), nodular, submerged colony with comparatively better mycelial growth over 50 Gv_1V.
	75 Gv_1I	Dark green, raised colony with white mycelial growth at the margin.
	75 Gv_1II	Dark green, raised colony.
	75 Gv_1III	Green, raised colony with white mycelial growth at the margin.
	75 Gv_1IV	Dark green raised colony.
	75 Gv_1V	Dark green raised nodular colony.
	75 Gv_1VI	Yellowish green fluffy colony with profuse mycelial growth.
	75 Gv_1IX	Albino (buff white), submerged colony with scanty mycelial growth.
	100 Gv_1I	Green, fluffy colony with profuse mycelial growth.
	150 Gv_1II	Green fluffy colony with white mycelial growth at the margin.
	150 Gv_1III	Light green, fluffy colony with mycelial growth.
Gv_3	$50Gv_3I$	Dark green, raised, nodular colony with white mycelial growth at the margin.
	50 Gv_3II	Dark green, raised colony.
	50 Gv_3III	Dark green, raised colony with white mycelial growth at the margin.
	50 Gv_3IV	Yellowish green, submerged colony with scanty mycelial growth.
	75 Gv_3I	Greyish white, raised colony.
	75 Gv_3II	Dark green, raised, nodular colony with white mycelial growth at margin.
	75 Gv_3III	Yellowish green, submerged colony with scanty mycelial growth.
	100 Gv_3I	Albino (white), raised colony.
	100 Gv_3II	Yellowish green, submerged colony with scanty mycelial growth.

Table 4.8 : Rapid screening of antagonistic potential of mutant isolates of *G. virens* against *R. solani*, *M. phaseolina*, *F. oxysporum* f. sp. *udum* and *S. rolfsii*.

Mutant isolate	Antagonistic potential on modified Bell's scale rating*			
	R. solani	*M. phaseolina*	*F. oxysporum* f. sp. *udum*	*S. rolfsii*
50Gv_1I	$4S_1$	$4S_1$	$6S_1$	$4S_1$
50 Gv_1II	$4S_1$	$4S_1$	$6S_1$	$5S_1$
50 Gv_1III	$4S_1$	$4S_1$	$6S_1$	$5S_1$
50 Gv_1IV	$5S_1$	$3S_1$	$5S_1$	$5S_1$
50 Gv_1V	$4S_1$	$4S_1$	$6S_1$	$4S_1$
50 Gv_1VI	$3S_1$	$4S_1$	$6S_1$	$5S_1$
75 Gv_1I	$4S_1$	$6S_1$	$7S_1$	$6S_5$
75 Gv_1II	$4S_1$	$3S_1$	$6S_1$	$7S_1$
75 Gv_1III	$4S_1$	$4S_1$	$6S_1$	$7S_1$
75 Gv_1IV	$3S_1$	$6S_1$	$7S_1$	$7S_5$
75 Gv_1V	$3S_1$	$4S_1$	$6S_1$	$6S_1$
75 Gv_1VI	$3S_1$	$3S_1$	$5S_1$	$4S_1$
75 Gv_1IX	$5S_1$	$7S_1$	$7S_1$	$7S_1$
100 Gv_1I	$5S_1$	$3S_1$	$6S_1$	$6S_5$
150 Gv_1II	$4S_1$	$3S_1$	$5S_1$	$4S_1$
150Gv_1III	$5S_1$	$4S_1$	$7S_1$	$5S_5$
50 Gv_3I	$4S_1$	$6S_1$	$6S_1$	$7S_1$
50 Gv_3II	$4S_1$	$7S_1$	$6S_1$	$6S_1$
50 Gv_3III	$4S_1$	$5S_1$	$7S_1$	$7S_1$
50 Gv_3IV	$4S_1$	$5S_1$	$6S_1$	$7S_1$
75 Gv_3I	$4S_1$	$3S_1$	$5S_1$	$5S_1$
75 Gv_3II	$5S_1$	$3S_1$	$7S_1$	$4S_1$
75 Gv_3III	$5S_1$	$5S_1$	$6S_1$	$6S_1$
100 Gv_3I	$4S_1$	$3S_1$	$5S_1$	$4S_1$
100 Gv_3II	$4S_1$	$5S_1$	$6S_1$	$6S_1$

* An average of five individual observation.

PLATE-5(a)

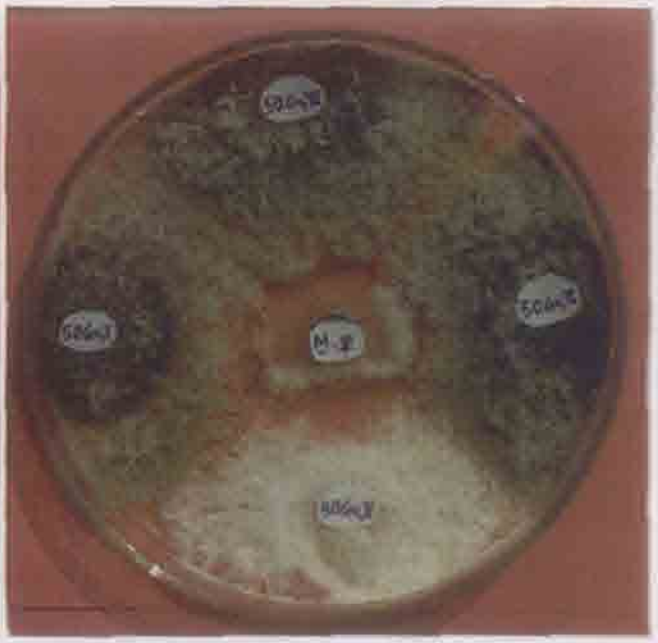


PLATE-5(b)

PLATE-5(c)

PLATE-5(d)

poorly effective as compared to their wild counterparts (Table 4.8). Total loss of antagonistic potential, particularly against *S. rolfsii*, was also evident for some mutant isolates (75Gv_1I, 75Gv_1IV, 100Gv_1I and 150Gv_1III) where *S. rolfsii* started to overgrow the antagonist colony and hence rated as S_5. Comparatively greater antagonistic potential was shown by 50Gv_1I, 50Gv_1II, 50Gv_1III, 50Gv_1IV, 50Gv_1V, 50Gv_1VI, 75Gv_1II, 75Gv_1III, 75Gv_1V, 75Gv_1VI, 100Gv_1I, 150Gv_1II, 50Gv_3I, 50Gv_3III, 50Gv_3IV, 75Gv_3I, 100Gv_3I, 100Gv_3II against both *R. solani* and *M. phaseolina*. Against *F. oxysporum* f.sp. *udum* two mutant isolates (75Gv_3I, 100Gv_3I) showed higher antagonistic potential ($5S_1$) whereas 3 mutant isolates (50Gv_1IV, 75Gv_1VI, 150Gv_1II) appeared equally effective ($5S_1$) as compared to their wild biotype. Significant increase in antagonistic potential against *S. rolfsii* was shown by 50Gv_1I, 50Gv_1V, 75Gv_1VI, 150Gv_1II, 75Gv_3II, 100Gv_3I ($4S_1$). Based on this result, six mutants from two biotypes viz., 50Gv_1I, 50Gv_1V, 75Gv_1VI, 150Gv_1II, 75Gv_3I and 100Gv_3I showing greater antagonistic potential over their wild counterparts (Gv_1 and Gv_3) were selected for subsequent studies.

4.7. Stability of selected mutant isolates through generation studies

Stability of characters of the selected mutant isolates was tested by simultaneous comparison of antagonistic potential and phenotypic characters on culture plates in different generation. This study was continued up to 10^{th} generation and finally stability of the mutant characters was confirmed by comparing with that expressed in first generation. Antagonistic potential of selected mutant isolates revealed that all the mutants except 100Gv_3I performed in more or less similar way even up to 10^{th} generation. Slight fluctuation, however, was very much relevant for each mutant isolates against a particular pathogen depending upon the duration required for contact between antagonist and pathogen colony. Although 100Gv_3I appeared exceptionally well and effective against all the test pathogens but unfortunately lost its viability after 2^{nd} generation and therefore was excluded from the study. The other mutants showed an overall 15.00–37.50 % increase in antagonistic potentiality against *R. solani* and *M. phaseolina*, while it was 28.50–45.00% in case of *S. rolfsii*. Against *F. oxysporum* f.sp. *udum* only 75Gv_3I exhibited 12.50% increase in antagonistic potential in comparison to its wild counterpart (Gv_3) [Table 4.9].

Table 4.9 : Antagonistic potential of mutant isolates of *G. virens* in different generations.

Isolate	Pathogen tested	Antagonistic potential on modified Bell's scale*					% increase (+) or decrease (-) in antagonistic potential of mutants over their wild biotype
		Wild biotype	Generation of mutant isolates				
			Ist	2nd	6th	10th	
Gv_1	*R. solani*	$6S_1$					
	M. phaseolina	$5S_1$					
	F. oxysporum.udum	$5S_1$					
	S. rolfsii	$7S_1$					
$50Gv_1I$	*R. solani*		$4S_1$	$4S_1$	$4S_1$	$5S_1$	(+)29.17
	M. phaseolina		$4S_1$	$4S_1$	$5S_1$	$4S_1$	(+)15.00
	F. oxysporum.udum		$6S_1$	$7S_1$	$6S_1$	$6S_1$	(-)25.00
	S. rolfsii		$4S_1$	$5S_1$	$5S_1$	$6S_1$	(+)28.57
$50Gv_1V$	*R. solani*		$4S_1$	$5S_1$	$4S_1$	$5S_1$	(+)25.00
	M. phaseolina		$4S_1$	$5S_1$	$4S_1$	$4S_1$	(+)15.00
	F. oxysporum.udum		$6S_1$	$7S_1$	$6S_1$	$5S_1$	(-)20.00
	S. rolfsii		$4S_1$	$4S_1$	$4S_1$	$5S_1$	(+)39.29
$75Gv_1V$	*R. solani*		$3S_1$	$4S_1$	$4S_1$	$4S_1$	(+)37.50
	M. phaseolina		$3S_1$	$4S_1$	$4S_1$	$4S_1$	(+)25.00
	F. oxysporum.udum		$5S_1$	$6S_1$	$6S_1$	$6S_1$	(-)15.00
	S. rolfsii		$4S_1$	$5S_1$	$5S_1$	$4S_1$	(+)35.71
$150Gv_1II$	*R. solani*		$4S_1$	$4S_1$	$5S_1$	$5S_1$	(+)25.00
	M. phaseolina		$3S_1$	$4S_1$	$5S_1$	$4S_1$	(+)20.00
	F. oxysporum.udum		$5S_1$	$6S_1$	$6S_1$	$6S_1$	(-)15.00
	S. rolfsii		$4S_1$	$4S_1$	$5S_1$	$7S_1$	(+)28.57
Gv_3 (wild)	*R. solani*	$5S_1$					
	M. phaseolina	$6S_1$					
	F. oxysporum.udum	$6S_1$					
	S. rolfsii	$10S_1$					
$75Gv_3I$	*R. solani*		$4S_1$	$4S_1$	$4S_1$	$5S_1$	(+)15.00
	M. phaseolina		$3S_1$	$4S_1$	$4S_1$	$4S_1$	(+)37.50
	F. oxysporum.udum		$5S_1$	$5S_1$	$6S_1$	$5S_1$	(+)12.50
	S. rolfsii		$5S_1$	$5S_1$	$5S_1$	$7S_1$	(+)45.00
** $100Gv_3I$	*R. solani*		$4S_1$	$4S_1$	**		
	M. phaseolina		$3S_1$	$4S_1$			
	F. oxysporum.udum		$5S_1$	$6S_1$			
	S. rolfsii		$4S_1$	$6S_1$			

* Antagonistic potential was screened by Dual plate culture on Potato Dextrose Agar medium, each insertion is based on an average of five replications.

** Mutant characters of the isolate ($100Gv_3I$) gradually started to degenerate after 2nd generation. Hence the study on the particular mutant could not be continued further.

Comparison of cultural characteristics of selected mutants of Gv_1 and Gv_3, on PDA, through different generations confirmed that all the mutants, except $100Gv_3I$, maintained their original phenotypic characters as mentioned previously (Table 4.7) even at 10th generation. These five mutant isolates were therefore considered as stable mutants with enhanced biocontrol potentiality and were subjected to subsequent studies for determining their response towards different ecological parameters.

4.8. Phenotypic and physiological characterization of stable mutant isolates of *G. virens*

Phenotypic and physiological characterization of stable mutant isolates of Gv_1 and Gv_3 was urgently needed to establish the fact that they were truly mutant isolates. Cultural variation on PDA has already partially proved that the isolates were phenotypic mutants but such apparent confirmation demanded further clarification to substantiate the mutative nature of those isolates. Morphometry, scanning electron microscopy and physiological characterization were therefore done to observe the variability among the mutant isolates and their corresponding wild isolates as well.

4.8.1. Cultural characterization

As most of the previous identifications of *G. virens* by different scientists were made on Oat meal agar medium, cultural characterization of both wild and stable mutant isolates of *G. virens* was also done here on the same medium.

Table 4.10 : Cultural characterization of the wild & mutant isolates of *G. virens* in Oat Meal Agar (OMA) medium.

Isolate	Cultural and other characteristics in OMA
Gv_1	Dark green, submerged colony with radiating hyphal growth, texture granular.
$50Gv_1I$	Dark green, submerged, little raised mycelial growth at the edge of the colony
$50Gv_1V$	Albino (white), submerged mycelial growth, nodular, radiating mycelial growth.
$75Gv_1VI$	Greyish green, nodular, mycelial growth submerged.
$150Gv_1II$	Deep green, granular, submerged mycelial growth.
Gv_3	Dark green, submerged with little raised white mycelial growth at the edge of the colony.
$75Gv_3I$	Dark green submerged colony with little raised white mycelial growth at the centre.

Distinctive variation in colony characteristics (Table 4.10) was more or less similar as exhibited in PDA. Mutant isolate 75Gv_1VI, however, produced yellowish green, fluffy colony with profuse mycelial growth in PDA but here on OMA it produced greyish green, nodular and submerged colony. Radiating hyphal growth produced by Gv_1, 50Gv_1V were very much distinctive from that observed in PDA.

4.8.2. Morphometry

Morphometry of phialide and conidia of different wild & mutant isolates of Gv_1 and Gv_3 showed that there was no significant variation in phialide length / breadth measurement however, apparent differences among the isolates were clear (Table 4.11). Dimensions of conidia of 150Gv_1II differed significantly from its wild isolate (Gv_1) whereas 50Gv_1I and 75Gv_3I showed slight variation from their corresponding wild counterparts (Gv_1 and Gv_3).

Table 4.11: Micrometry of wild and selected mutant isolates of *G. virens*

Isolate	Phialides (µm)		Conidia (µm)*	
	Length (Mean) x Breadth (Mean)		Length (Mean) x Breadth (Mean)	
Gv_1	8.52 – 17.08 (10.54) x 2.13 – 6.39 (4.47)		4.26 (4.26) x 4.26 (4.26)	
50 Gv_1I	8.52 – 17.08 (11.18) x 2.13 – 6.39 (4.58)		2.13 – 6.39 (4.15) x 2.13 – 4.26 (4.05)	
50 Gv_1V	6.39 – 12.78 (9.48) x 4.26 – 6.39 (4.58)		4.26 (4.26) x 4.26 (4.26)	
75Gv_1VI	4.26 – 17.04 (11.72) x 2.13 – 4.26 (4.15)		4.26 (4.26) x 4.26 (4.26)	
150Gv_1II	6.39 – 14.91 (10.44) x 2.13 – 4.26 (4.05)		2.13 – 4.26 (2.98) x 2.13 – 4.26 (2.98)	
Gv_3	6.39 – 10.65 (8.63) x 4.26 (4.26)		4.26 – 6.39 (4.69) x 4.26 (4.26)	
75Gv_3I	6.39 – 17.04 (10.65) x 4.26 – 6.39 (5.31)		2.13 – 6.39 (4.37) x 2.13 – 4.26 (4.08)	
	Length	Breadth	Length	Breadth
SEM±	0.56	0.17	0.16	0.12
CD (P=0.01)	2.05(NS)	0.62(NS)	0.60	0.44

* An average of fifty individual observation.

4.8.3. Scanning electron microscopy

Scanning electron microscopy of wild and mutant isolates of Gv_1 and Gv_3 to observe the distinguishable spore morphology revealed that the mutant isolates could be differentiated from their respective wild type on basis of size and degree of echinulation on conidia. Formation of compact large spore ball by Gv_1 was the

Plate 6(a-g) Scanning electron micrographs on spore morphology of different wild and mutant isolates of *G. virens*

(a) Smooth walled, subglobose to oval spore of Gv_1; formation of compact large spore ball

(b) Subglobose to oval, echinulated spore of $50Gv_1I$

(c) Smooth walled, spherical to oval spore of $50Gv_1V$

(d) Subglobose to oval spore with single deep depressions in case of $75Gv_1VI$

See next page

(e) Subglobose to oval, echinulated spore of $150Gv_1II$

(f) Subglobose to oval, echinulated spore of Gv_3

(g) Subglobose to oval, echinulated, smaller sized spore of $75Gv_3I$

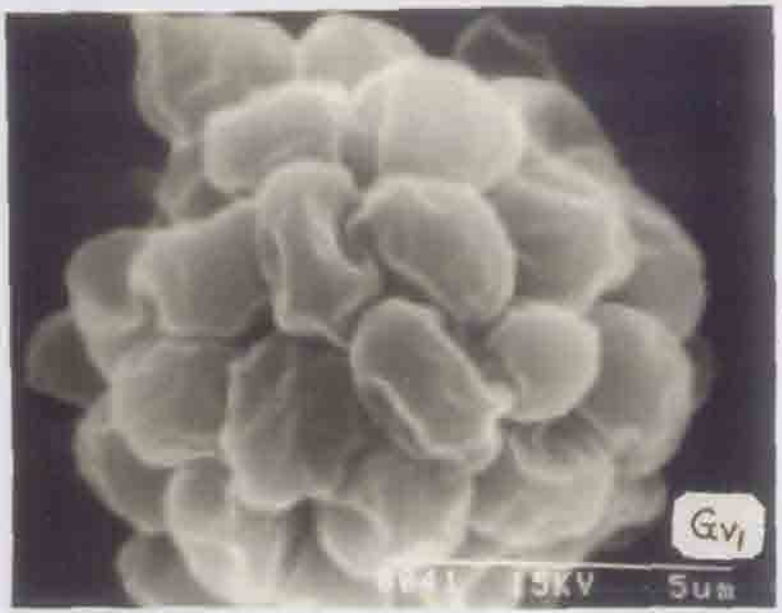


PLATE-6(a)

PLATE-6(b)

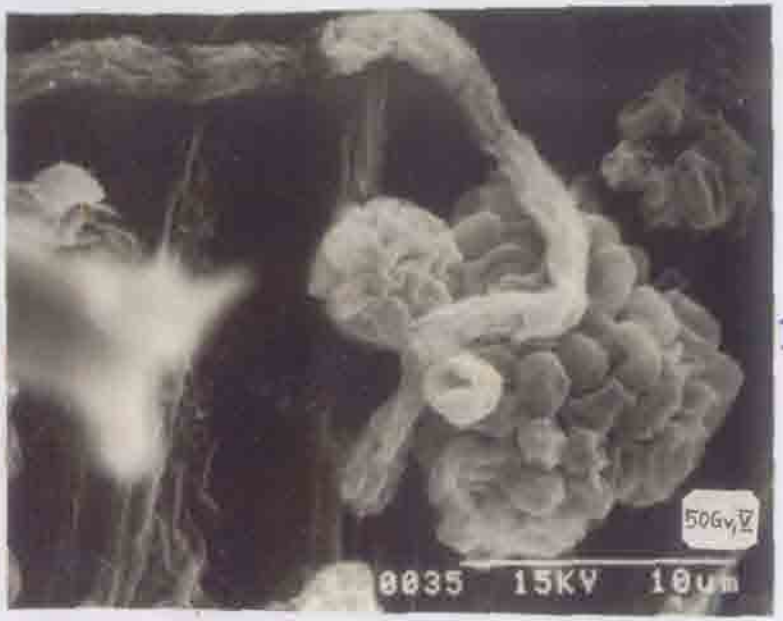


PLATE-6(c)

PLATE-6(d)

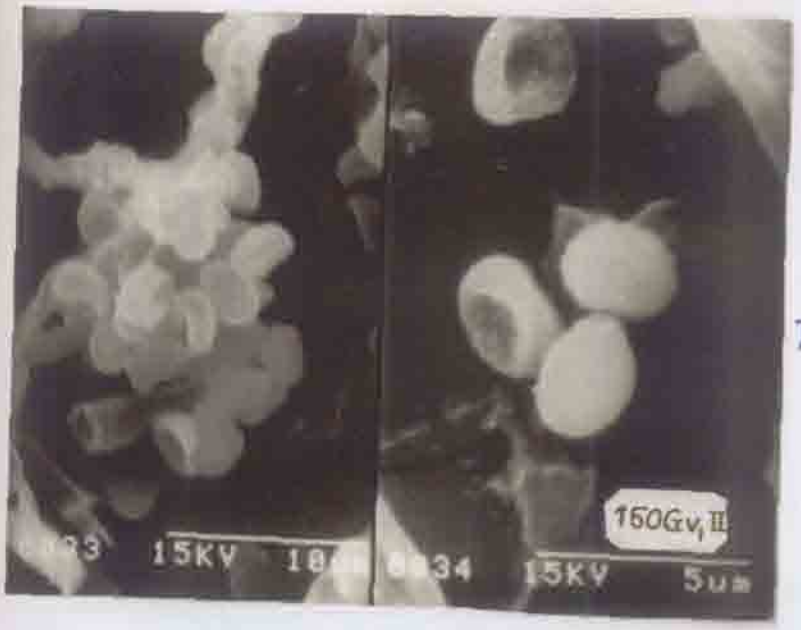
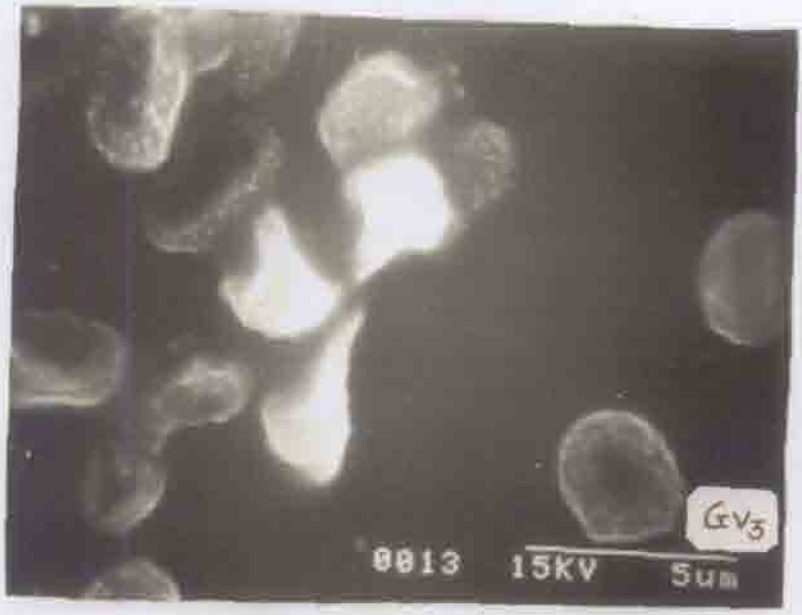


PLATE-6(e)

PLATE-6(f)



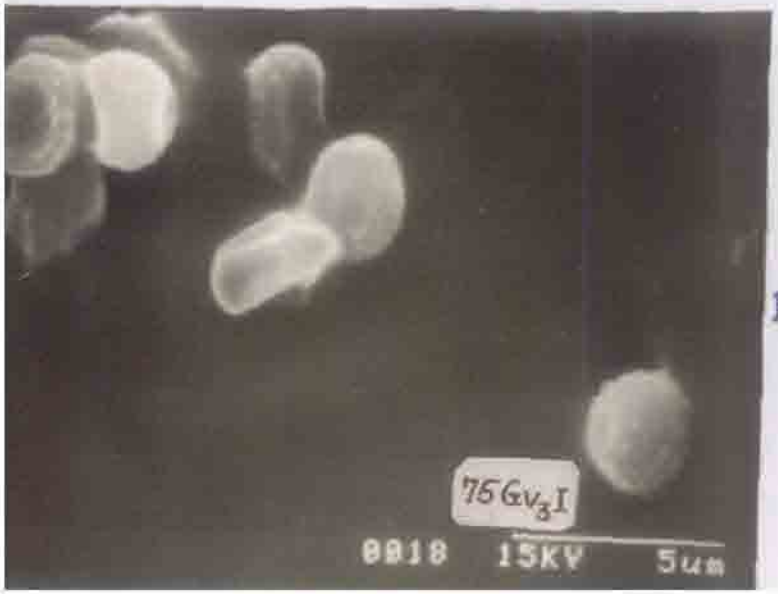


PLATE-6(g)

distinctive character of the genus. A clear variation was evident by the presence of echinulation on the conidial surface of all the mutant isolates of Gv_1, but the conidia of Gv_1 appeared smooth walled. Mutant isolate $75Gv_3I$ was similar to Gv_3 but the only difference was the production of smaller sized conidia by this mutant in particular (Table 4.12).

Table 4.12: Spore morphology of different wild and mutant isolates of *G. virens* studied under Scanning Electron Microscope (SEM).

Isolate	Differences in spore morphology identified through SEM
Gv_1	Subglobose to oval with few deep and light depressions smooth walled, formed compact large spore ball, size rather larger.
$50Gv_1I$	Subglobose to oval with single deep (giving plate like appearance) and few light depression, echinulation on surface is the most distinctive to the isolate (Gv_1).
$50Gv_1V$	Spherical to oval with several depressions, smooth walled, size much smaller than other isolates.
$75Gv_1VI$	Subglobose to oval, plate like appearance due to single deep depressions, distinct echinulation on the surface, size nearly same to the wild isolate.
$150Gv_1II$	Similar to $75Gv_1VI$, but size is smaller.
Gv_3	Subglobose to oval with single deep and few light depressions, echinulated, larger than Gv_1.
$75Gv_3I$	Similar to wild isolate (Gv_3) but size is much smaller than the wild.

4.8.4. Physiological characterization of wild and mutant isolates of *G.virens*

4.8.4.1. Enzyme production

Assuming that the variation in enzyme production might be an efficient and indirect approach for confirmation of genetic diversity among wild and mutant isolates of *G. virens,* an experiment was conducted on the ability to produce different kinds of hydrolytic enzymes viz, β - 1,3 glucanase, β -1,4 glucanase, chitinase and cellulase, by the isolates (wild vis-à-vis mutants) in various sucrose supplemented media as the method described in section 3.8.4.1.

In Czapek Dox broth, where sucrose was used as sole carbon source significantly higher quantity of β-1,3 glucanase (78.67 U) was produced by mutant isolate $150Gv_1II$ than others (8.74 – 19.00 U). Similar variation was observed when CDB was partially substituted with chitin (29.33 U produced by $150Gv_1II$ while 7.50 –

Table 4.13 : Production of β-1,3 glucanase * by wild and mutant isolates of *G. virens* in Czapek-Dox broth with partial substitutions in carbon source.**

Isolate	CDB	S+*M.p*[a]	S+Chitin[b]	S+CMC[c]	S+*P.u*[d]
Gv_1	19.00±6.19	78.33±4.66	8.67±2.81	33.00±3.47	108.33±4.77
$50Gv_1I$	16.67±2.90	73.05±5.33	8.33±1.24	31.00±2.42	231.69±6.44
$50Gv_1V$	12.83±2.57	49.17±8.03	8.33±1.20	72.50±4.35	137.84±3.25
$75Gv_1VI$	10.17±2.61	158.33±15.53	7.50±1.66	46.66±2.65	263.33±4.78
$150Gv_1II$	78.67±6.37	123.33±4.01	29.33±4.09	96.67±6.17	282.50±8.67
Gv_3	12.84±2.63	70.00±4.01	9.66±1.46	31.66±2.69	291.34±4.38
$75Gv_3I$	8.74±3.07	89.17±3.59	9.17±1.78	28.33±2.79	123.00±3.71
SEm±	2.36	4.37	1.30	2.15	3.13
CD(p=0.01)	9.94	18.40	5.47	9.05	13.18

* Unit = Release of 1μmol of glucose from substrate / ml of culture filtrate / min.

** 30 gm of sucrose in CDB partially substituted per litre as follows:

(a) S+*M. p* → 10gm sucrose + 10gm mycellial powder of *M. phaseolina*

(b)S+chitin → 10gm sucrose + 42 gm colloidal chitin (contain 12% crude chitin)

(c) S+CMC → 10gm sucrose + 10 gm carboxymethyl cellulose.

(d) S+*P . u* → 10gm sucrose + 10gm mycellial powder of *P. ultimum*

9.66 U of β-1,3 glucanase produced by others) and carboxy methyl cellulose (96.67 U produced by $150Gv_1II$ while 28.33 – 72.50 U of β-1,3 glucanase produced by others). In CDB partially supplemented with CMC, beside $150Gv_1II$, mutant isolates $50Gv_1$ V and $75Gv_1VI$ produced significantly higher quantity of β-1,3 glucanase (72.50 U and 46.66 U respectively) as compared to their wild counterpart Gv_1 (33.00 U). Several fold increase in β-1,3 glucanase production (1.5 – 15 fold) by both wild and mutant isolates was observed when CDB was partially substituted with mycelial powder of *M. phaseolina*. More over the induction of enzyme production was predominant in medium supplemented with mycelial powder of *P. ultimum*. (3.5 – 25 fold increase in β-1,3 glucanase production). In *M. phaseolina* supplemented CDB medium mutant isolates $75Gv_1VI$, $150Gv_1II$ and $75Gv_3I$ produced significantly higher amount of β-1,3 glucanase (158.33 U, 123.33 U and 89.17 U respectively) than their corresponding wild counterparts (78.33 U and 70.00 U produced by Gv_1 and Gv_3 respectively). All the mutant isolates of Gv_1 were found to produce significantly higher amount of β-1,3 glucanase (137.84 – 282.50 U) than the wild biotype (108.33 U) in *P. ultimum* supplemented CDB medium, however, highest production was noticed in case of Gv_3 (191.34 U) [Table 4.13].

Variation in production of β-1,4 glucanase by different wild and mutant isolates of *G. virens* was almost similar to that of β-1,3 glucanase (Table 4.14). Mutant isolate $150Gv_1II$ appeared most effective in producing significantly higher quantity of β-1,4 glucanase in CDB (12.67 U) and CMC supplemented CDB media (61.67 U) than others (2.67 – 5.67 in CDB and 11.33 – 36.19 U in CMC supplemented CDB medium). In chitin supplemented CDB medium the variation however was inadmissible. In *M. phaseolina* supplemented CDB medium 3-7 fold increase in β-1,4 glucanase production by the wild and mutant isolates was observed whereas in *P. ultimum* supplemented CDB medium it was 12.5 – 91 fold. Significantly higher amount of β-1,4 glucanase in *M. phaseolina* supplemented CDB medium was produced by mutant isolate $150Gv_1II$ (69.51 U) as compared to others (12.33 –-26.94 U). In *P. ultimunm* supplemented CDB medium significantly high quantity of β-1,4 glucanase was produced by $50Gv_1I$ and $150Gv_1II$ (396.67 U and 180.82 U respectively) than their wild biotype Gv_1 (150.16 U).

Table 4.14 : Production of β-1,4 glucanase * by wild and mutant isolates of *G. virens* in Czapek-Dox broth with partial substitutions in carbon source**.

Isolate	CDB	S+*M.p*[a]	S+Chitin[b]	S+CMC[c]	S+*P.u*[d]
Gv_1	4.00±1.17	24.33±2.59	13.50±0.74	12.33±2.09	156.16±6.47
$50Gv_1I$	4.33±0.82	20.02±4.86	9.50±2.02	17.33±2.13	396.67±9.00
$50Gv_1V$	4.50±0.83	26.94±4.83	12.66±2.04	36.19±7.02	60.34±8.60
$75Gv_1VI$	5.67±1.80	16.44±2.06	8.67±1.18	18.33±3.03	71.33±5.26
$150Gv_1II$	12.67±2.42	69.51±5.80	13.50±2.00	61.67±4.03	180.82±7.30
Gv_3	2.67±1.00	14.04±1.34	10.04±1.66	11.33±3.28	116.66±4.73
$75Gv_3I$	4.44±0.63	12.33±1.81	8.67±1.71	28.75±4.51	108.34±5.13
Sem±	0.79	2.15	0.97	2.34	3.94
CD(p=0.01)	3.33	9.05	4.08	9.85	16.59

* Unit = Release of 1μmol of glucose from substrate / ml of culture filtrate / min.

** 30 gm of sucrose in CDB partially substituted per litre as follows:

(a) S+*M. p* → 10gm sucrose + 10gm mycellial powder of *M. phaseolina*

(b)S+chitin → 10gm sucrose + 42 gm colloidal chitin (contain 12% crude chitin)

(c) S+CMC → 10gm sucrose + 10 gm carboxymethyl cellulose.

(d) S+*P . u* → 10gm sucrose + 10gm mycellial powder of *P. ultimum*

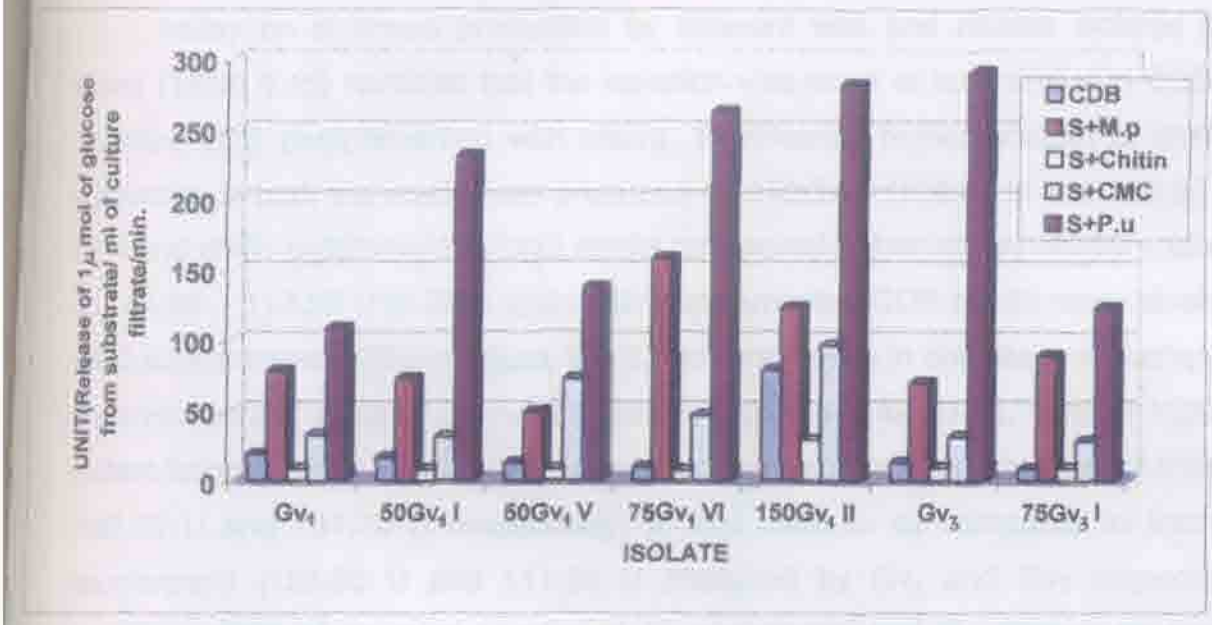


Fig. 4.2: Production of β -1,3 glucanase by wild and mutant isolates of *G.virens* in Czapek Dox broth (CDB) and CDB with partial substitution in different carbon source

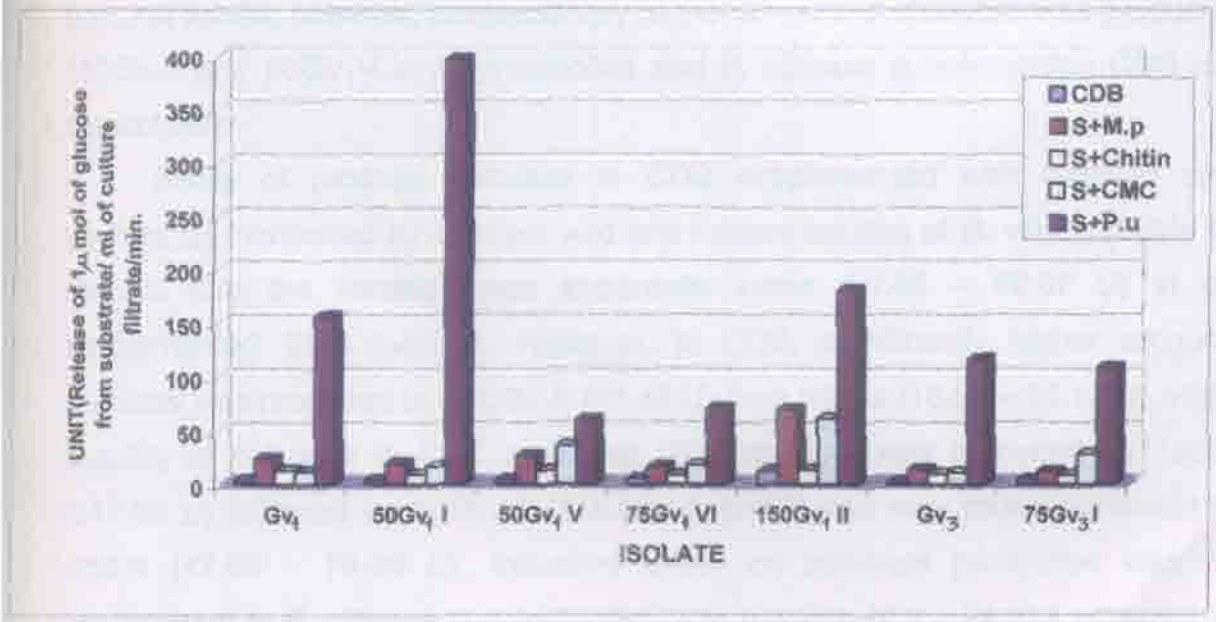


Fig. 4.3: Production of β -1,4 glucanase by wild and mutant isolates of *G.virens* in Czapek Dox broth (CDB) and CDB with partial substitution in different carbon source

Assay on chitinase production by different wild and mutant isolates of *G. virens* (Table 4.15) revealed that the variation was more or less similar in CDB and modified CDB (supplemented with chitin). Significantly higher amount of chitinase production in both the media was produced by $150Gv_1II$ (179.00 U and 193.80 U in CDB and chitin supplemented CDB media respectively) than others (46.30 – 62.50 U and 64.30 – 117.30 U in CDB and chitin supplemented CDB media respectively). In CMC supplemented CDB medium, 2 – 3.5 fold increases in chitinase production was observed except in case of mutant isolate $150Gv_1II$ (182.50 U). Beside $150Gv_1II$, mutant isolates $50Gv_1V$ and $75Gv_3I$ produced significantly higher amount of chitinase (188.20 U and 151.20 U respectively) in that medium as compared to their wild counterparts (126.20 U and 111.30 U produced by Gv_1 and Gv_3 respectively). Inductive effect of mycelial powder of *M. phaseolina* and *P. ultimum* on enzyme production was repeated here also with 1.5 – 3.5 fold increase in chitinase production except in case of mutant isolate $150Gv_1II$ (196.69 U and 154.50 U in *M. phaseolina* and *P. ultimum* supplemented CDB media respectively). None of the mutant isolates appeared superior in chitinase production as compared to their wild counterparts in both the media, however, comparatively higher amount of chitinase was produced by $150Gv_1II$ and $50GV_1V$ in *M. phaseolina* and *P. ultimum* supplemented CDB media respectively.

Ability of produce cellulase in CDB supplemented with different carbon sources as mentioned by different wild and mutant isolates of *G. virens* (Table 4.16) showed that the variation was apparently inane (20.66 – 29.67 U) in chitin supplemented CDB medium. However, in CDB, significantly higher amount of cellulase was produced by $150Gv_1II$ (57.43 U) than others (16.67 – 22.17 U). Highest quantity of cellulase in CMC amended CDB medium was produced by $150Gv_1II$ (547.56 U) followed by $75Gv_1VI$ (118.32 U) and it was very much significant than others (47.09 – 73.86 U). Inductive effect on cellulase production was more predominant in *P. ultimum* supplemented CDB medium (2.5 – 24 fold increase) than in *M. phaseolina* supplemented CDB medium (1.0 – 2.5 fold increase). In *P. ultimum* supplemented CDB medium mutant isolate $50Gv_1I$ produced highest quantity of cellulase (415.00 U) followed by $75Gv_3I$ (170.68 U), $150Gv_1II$ (149.96 U) and these productions were significantly higher in comparison to their wild counterparts (100.83 U and 143.00 U of cellulase produced by Gv_1 & Gv_3 respectively).

Table 4.15 : Production of chitinase* by wild and mutant isolates of *G. virens* in Czapek-Dox broth with partial substitutions in carbon source.**

Isolate	CDB	S+*M.p*[a]	S+Chitin[b]	S+CMC[c]	S+*P.u*[d]
Gv_1	62.50±5.36	193.85±7.32	73.00±3.30	126.20±6.19	173.00±6.18
$50Gv_1I$	61.50±8.42	138.11±8.33	66.00±5.94	139.00±6.81	161.20±5.25
$50Gv_1V$	52.60±7.56	87.89±7.38	64.30±4.34	188.20±6.47*	174.39±5.00
$75Gv_1VI$	46.30±7.17	144.34±8.89	69.60±13.26	136.50±3.42	173.20±7.13
$150Gv_1II$	179.00±10.77*	196.69±7.18*	193.80±5.45*	182.50±7.45*	154.50±4.43
Gv_3	50.30±5.18	164.21±3.75	65.20±3.60	111.30±5.38	174.20±1.73
$75Gv_3I$	52.70±6.00	106.30±5.64	117.03±66.03	151.20±5.30*	170.80±3.09
SEm±	4.29	4.11	14.87	3.46	2.88
CD(p=0.01)	18.06	17.30	62.60	14.57	12.13

* Unit = Release of 1µmol N-acetyl glucosamine from substrate / ml of culture filtrate / min.

** 30 gm of sucrose in CDB partially substituted per litre as follows:

(a) S+*M. p* → 10gm sucrose + 10gm mycellial powder of *M. phaseolina*

(b)S+chitin → 10gm sucrose + 42 gm colloidal chitin (contain 12% crude chitin)

(c) S+CMC → 10gm sucrose + 10 gm carboxymethyl cellulose.

(d) S+*P . u* → 10gm sucrose + 10gm mycellial powder of *P. ultimum*

Table 4.16 : Production of cellulase* by wild and mutant isolates of *G. virens* in Czapek-Dox broth with partial substitutions in carbon source.**

Isolate	CDB	S+*M.p*[a]	S+Chitin[b]	S+CMC[c]	S+*P.u*[d]
Gv_1	20.67±2.09	47.33±5.27	29.67±3.23	51.86±5.48	100.83±4.00
$50Gv_1I$	16.67±3.01	41.33±5.34	27.67±2.31	53.03±2.99	415.00±7.54
$50Gv_1V$	21.00±1.46	56.55±6.35	21.50±1.78	73.86±3.55	88.00±2.31
$75Gv_1VI$	22.17±3.59	44.07±5.76	23.65±3.08	118.32±4.80	76.66±4.54
$150Gv_1II$	57.43±5.52	113.21±3.84	20.66±3.00	547.56±10.96	149.96±5.02
Gv_3	20.67±3.02	47.26±4.18	22.50±2.92	47..09±3.22	143.00±5.48
$75Gv_3I$	19.90±1.22	25.33±3.27	21.83±2.35	57.41±4.25	170.68±8.12
Sem±	1.82	2.87	1.56	3.26	3.24
CD(p=0.01)	7.66	12.08	6.57	13.72	13.64

* Unit = Release of 1µmol of glucose from substrate / ml of culture filtrate / min.

** 30 gm of sucrose in CDB partially substituted per litre as follows:

(a) S+*M. p* → 10gm sucrose + 10gm mycellial powder of *M. phaseolina*

(b)S+chitin → 10gm sucrose + 42 gm colloidal chitin (contain 12% crude chitin)

(c) S+CMC → 10gm sucrose + 10 gm carboxymethyl cellulose.

(d) S+*P . u* → 10gm sucrose + 10gm mycellial powder of *P. ultimum*

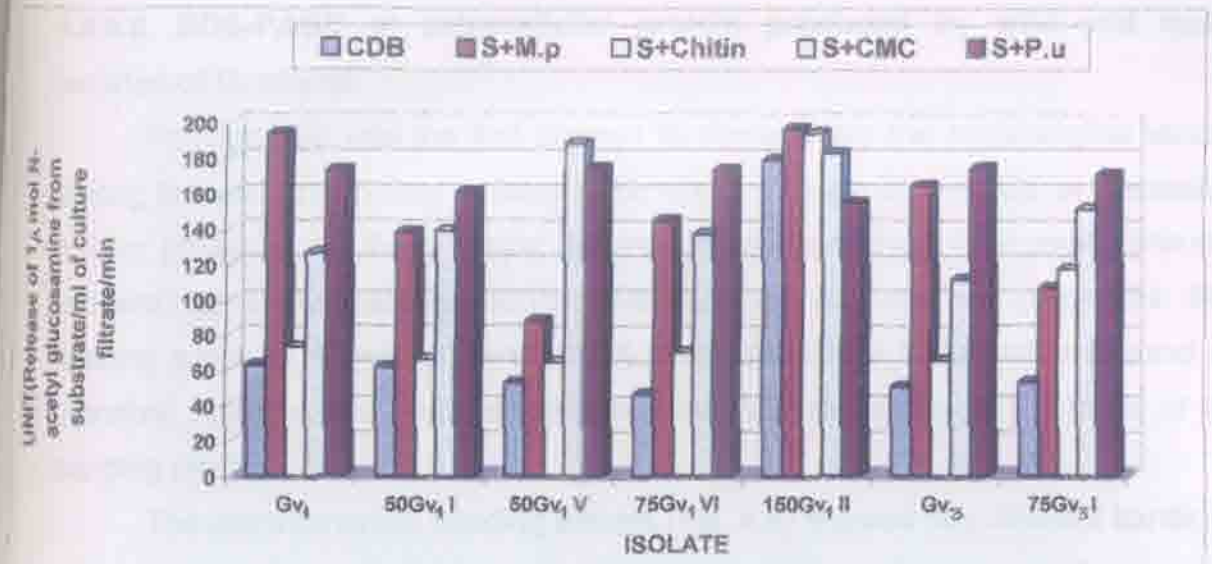


Fig.4.4 : Production of chitinase by wild and mutant isolates of *G.virens* in Czapek Dox broth (CDB) and CDB with partial substitution in different carbon source

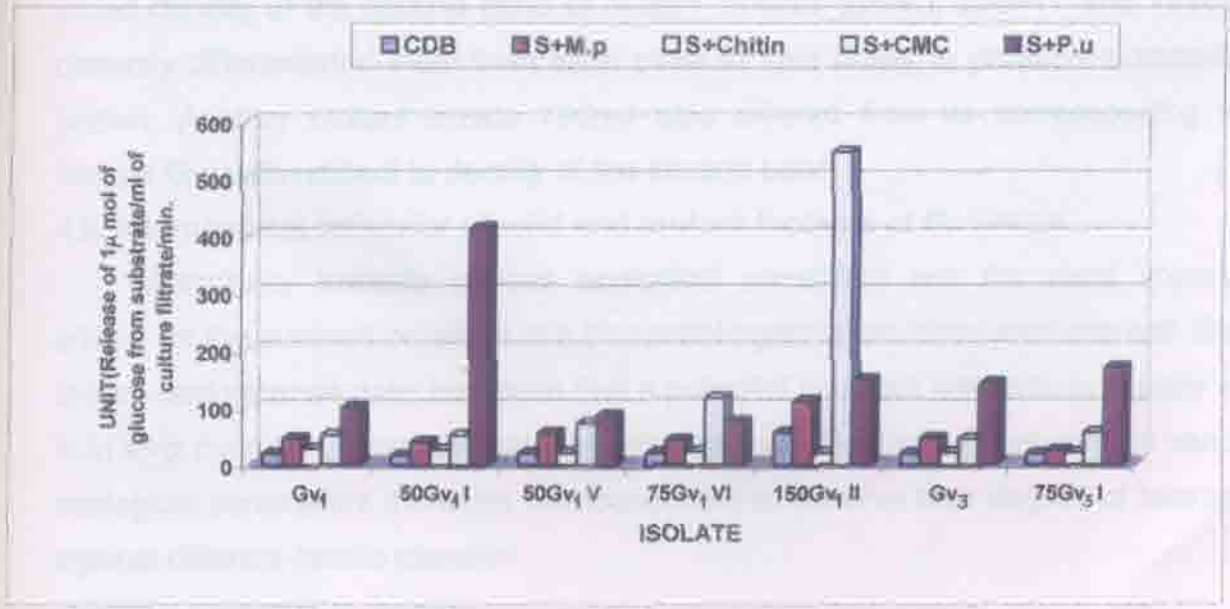


Fig.4.5 : Production of cellulase by wild and mutant isolates of *G.virens* in Czapek Dox broth (CDB) and CDB with partial substitution in different carbon source

4.8.4.2. SDS-PAGE of extracellular protein produced by wild and mutant isolates of *G. virens*

Perhaps this was the first attempt to demonstrate the physiological variation among the wild and mutant isolates of *G. virens* through SDS-PAGE of extracellular protein produced by them. Since culture filtrates contained very small amount of proteins, generally practiced coommasie blue staining was not successful. Silver staining which is known to have 100 % more sensitivity in detection of band was therefore followed and the test isolates were differentiated on the basis of their banding pattern.

The electrophoretic banding pattern (Fig. 4.6) showed two different bands (Rm 0.69 and 0.81) for each isolate indicating the production of two types of extracellular proteins with different molecular weight. However, the bands differed in intensity and density. Greater intensity and density of both bands in wild isolate Gv_1 signified that the isolate was capable of producing more quantity of the extracellular proteins. The second band (Rm 0.81) of $75Gv_1VI$ was however similar but the low intensity of first band (Rm 0.69) pointed out the variation with its wild isolate Gv1. On the other hand, varied density of the second band of mutant isolates $50Gv_1I$, $50Gv_1V$ and $150Gv_1II$ distinctly differentiated them from each other in their ability to produce extracellular protein. Another mutant isolate $75Gv_3I$ also differed from its corresponding wild biotype Gv_3 with respect to density of the second band.

4.9. Microhabitat behavior of wild and mutant isolates of *G. virens*

Sensitivity towards various ecological constrains are the most important criteria for the success or failure of a biocontrol agent when introduced into soil. Good *in vitro* performance does not mean that a potential bioagent will perform equally well in *in vivo* condition under several overriding factors. A set of experiments on various ecological parameters therefore was conducted to observe their degree of tolerance against different hostile situation.

4.9.1. Germinability in different sources of water

Surface and sub-surface water are frequently used as irrigation water but the effect of these sources of water on germination of different spore forms of *G. virens* has been repeatedly overlooked. The *in vitro* germinability of phialo- and chlamydospores of different wild and mutant isolates of *G. virens* was therefore

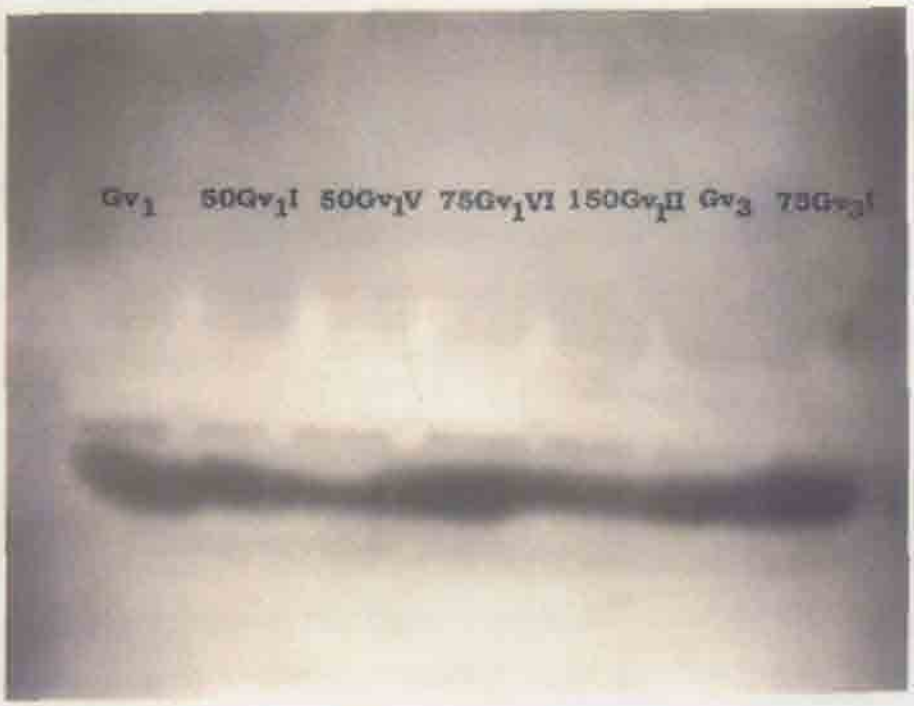


Plate 7: Variation in extracellular protein production by different mutant isolates of *G. virens* examined by SDS-PAGE

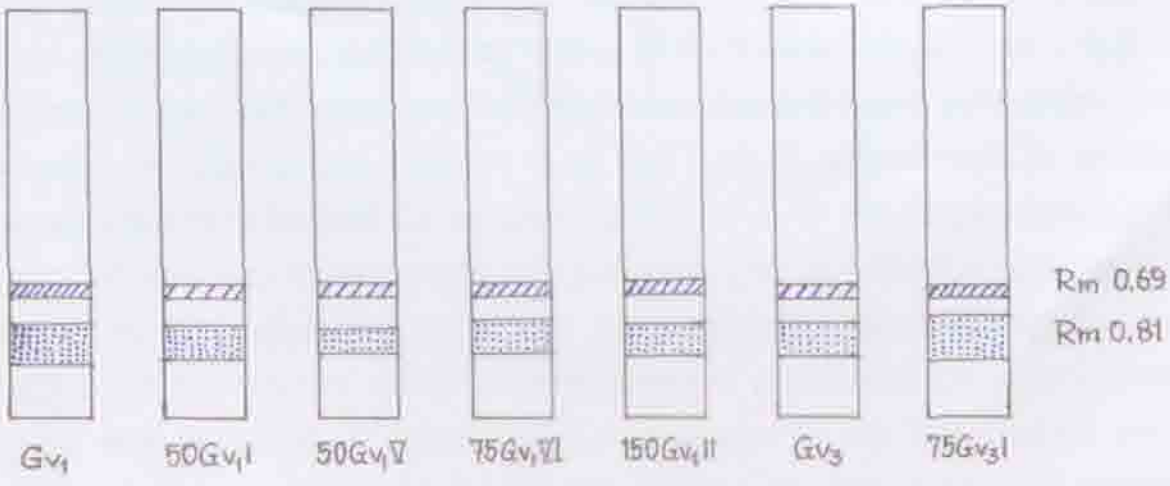


Fig 4.6: Zymogram of SDS-PAGE of extracellular protein produced by wild and mutant isolates of *G. virens*

examined where pond water and tap water in addition to sterilized distilled water and soil leachet were used as substrates for germination.

The general trend of phialo- and chlamydospore germination of the test isolates was very inconsistent and rather confusing where an individual isolate germinated well in a particular source of water others responded differently.

Test on phialospore germination (Table 4.17) revealed that significantly higher germination in pond water. (pH 8.68) was exhibited by $75Gv_1VI$, $50Gv_1V$ and $50Gv_1I$ (19.29, 11.22 and 10.22 % germination respectively) than others (2.25 – 6.84 % germination). Except $150Gv_1II$ all the mutant isolates showed greater germinability than their wild counterparts. But in tap water (pH 7.88), mutant isolate $150Gv_1II$ only showed significantly higher germination (10.88 %) while 2.54 – 5.08 % germination was recorded for rest of the isolates. In sterilized distilled water and soil leachet the germination was comparatively better than in tap water. In sterilized distilled water (pH 7.0) significantly higher germination was recorded for $50Gv_1V$ (15.95 %) in comparison to the wild biotype Gv_1 (10.00 %) whereas in soil leachet (pH 7.1) all the mutants of Gv_1 showed significantly higher germination (10.05 – 18.97 %) than Gv_1 (6.16 % germination). Mutant isolate $75Gv_3I$, on the other hand, germinated poorly in comparison to its wild counterpart Gv_3 in all the substrates tested except in pond water.

More or less similar trend was observed in case of chlamydospore germination of the antagonist isolates however the overall germination was apparently low in comparison to phialospore germination (Table 4.18). In pond water comparatively better germinability was exhibited by $50Gv_1I$ (7.78 %) than others (1.48 – 5.26 %) whereas in tap water maximum and minimum chlamydospore germination were recorded for $75Gv_1VI$ and $150Gv_1II$ (5.88 and 0.14 % respectively). In sterilized distilled water all mutants of Gv_1 showed significantly lower rate of germination (2.90 – 4.62 %) than their corresponding wild biotype (11.11 %), whereas reverse result (13.40 – 16.18 % germination for mutants of Gv_1 and 5.65 % germination of Gv_1) was observed in soil leachet. Although chlamydospore germination of mutant isolate $75Gv_3I$ was comparatively better than phialospores in sterilized distilled water (5.14 %) and tap water (6.58 %) but the germination was less in soil leachets (2.61 %) and the reduction in germination was significant than the chlamydospore germination of its wild counterpart Gv_3 (9.77 %).

Table 4.17 : *In vitro* germinability of phialospores of wild and mutant isolates of *G. virens* in different sources of water.

Isolate	Sources of water							
	SDW		PW		TW		SL	
	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype
Gv_1	10.00±1.40		6.84±1.53		5.08±1.49		6.16±2.18	
$50Gv_1I$	12.58±2.13	(+)25.80	10.22±2.13	(+)49.41	4.20±1.66	(-)17.32	10.05±2.91	(+)63.15
$50Gv_1V$	15.95±9.50	(+)59.50	11.22±1.18	(+)64.03	2.54±0.63	(-)50.00	14.35±3.22	(+)132.95
$75Gv_1VI$	10.59±4.17	(+)5.90	19.29±6.46	(+)182.01	3.46±0.79	(-)31.89	18.97±7.54	(+)207.95
$150Gv_1II$	11.01±2.14	(+)10.10	2.25±2.02	(-)67.10	10.08±4.12	(+)98.42	13.69±2.31	(+)122.24
Gv_3	9.41±4.85		2.50±0.67		4.30±1.57		5.26±2.95	
$75Gv_3I$	3.69±3.27	(-)60.79	3.89±1.00	(+)55.60	4.12±0.48	(-)4.19	2.47±0.83	(-)53.04

SEm±		CD (p = 0.01)	
Isolate	0.96	Isolate	3.64
Sources of water	0.73	Sources of water	2.36
Isolate x sources of water	1.93	Isolate x sources of water	7.28

Table 4.18 : *In vitro* germinability of chlamydospores of wild and mutant isolates of *G. virens* in different sources of water.

Isolate	Sources of water							
	SDW		PW		TW		SL	
	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype
Gv_1	11.11±4.16		5.26±0.80		1.37±0.76		5.65±2.06	
$50Gv_1I$	3.48±1.66	(-)68.68	7.78±1.89	(+)47.91	3.41±2.14	(+)148.90	13.75±4.73	(+)143.36
$50Gv_1V$	4.62±3.29	(-)58.42	5.13±0.52	(-)2.47	4.50±1.71	(+)228.47	14.30±7.20	(+)153.10
$75Gv_1VI$	3.29±2.03	(-)70.39	4.17±3.53	(-)20.72	5.88±1.28	(+)329.20	13.40±4.85	(+)137.17
$150Gv_1II$	2.90±1.28	(-)73.90	2.38±0.71	(-)54.75	0.14±0.13	(-)89.78	16.18±6.35	(+)186.37
Gv_3	6.10±3.74		3.61±1.52		3.73±1.31		9.77±1.16	
$75Gv_3I$	5.14±2.45	(-)15.74	1.48±1.19	(-)59.00	6.58±0.90	(+)76.41	2.61±1.28	(-)73.28

SEm±	
Isolate	0.83
Sources of water	0.62
Isolate x sources of water	1.65

CD (p = 0.01)	
Isolate	3.12(NS)
Sources of water	2.36
Isolate x sources of water	6.23

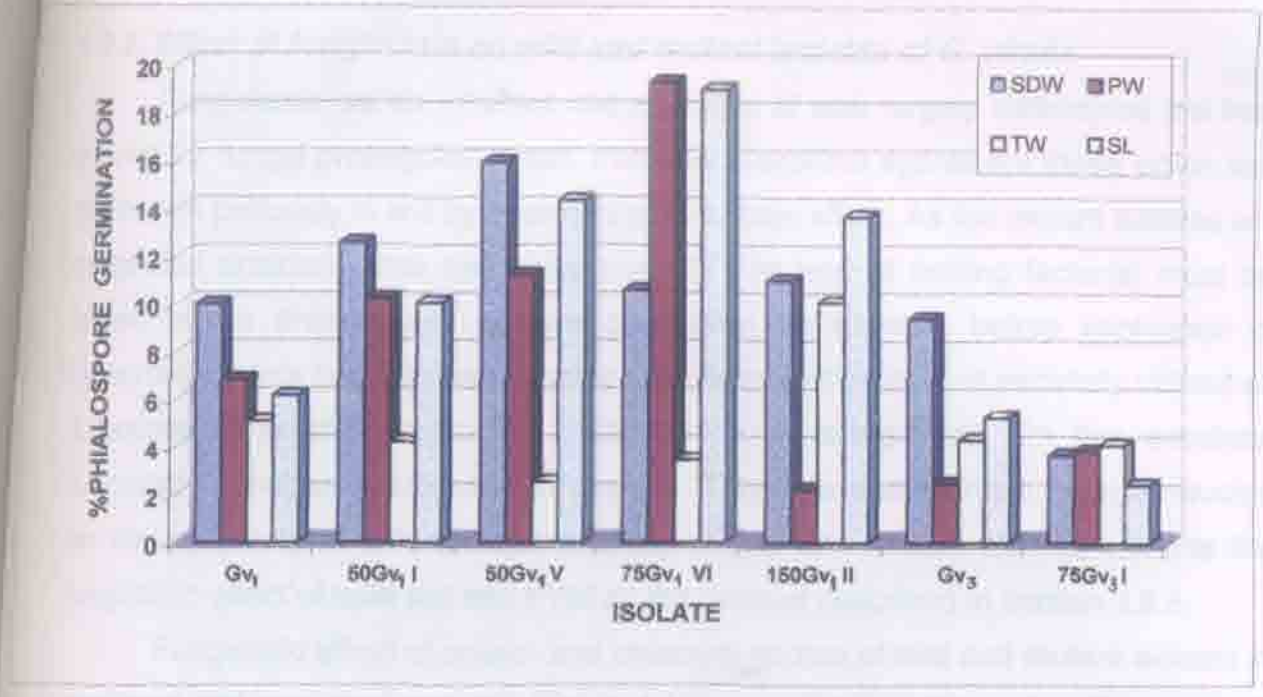


Fig. 4.7 : *In vitro* germinability of phialospores of wild and mutant isolates of *G.virens* in different sources of water

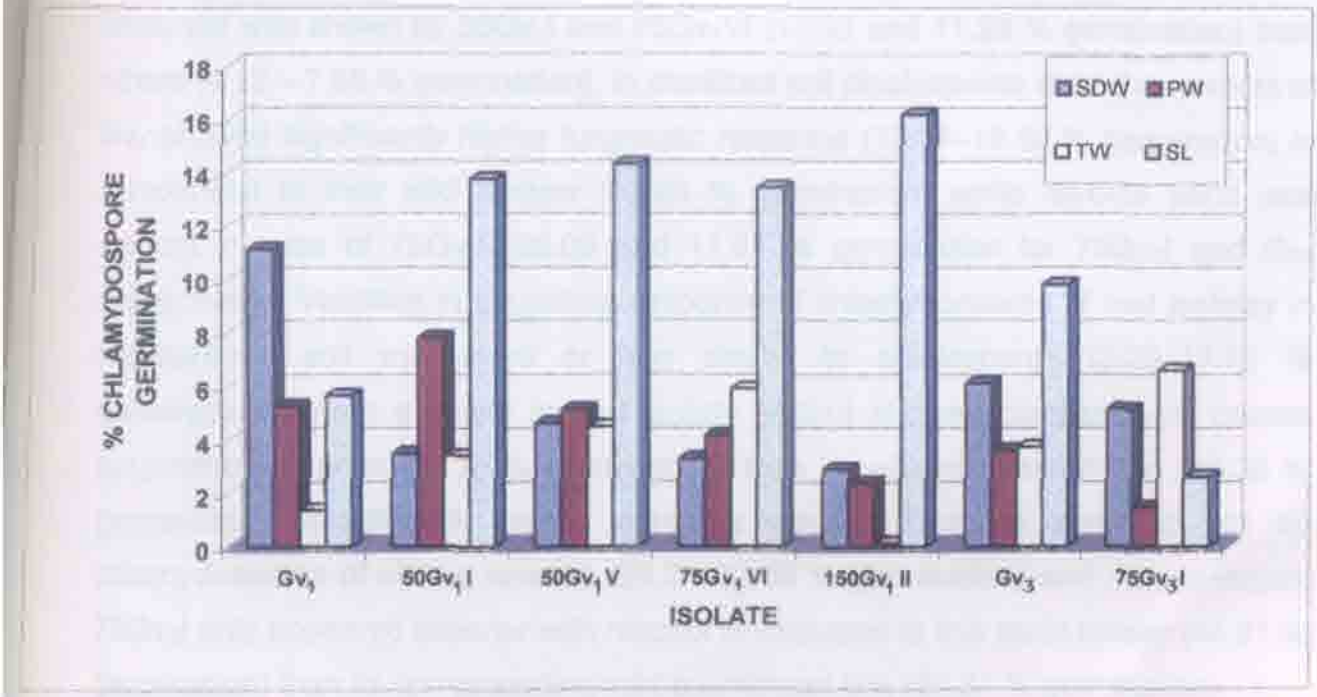


Fig. 4.8: *In virto* germinability of chlamydospores of wild and mutant isolates of *G.virens* in different sources of water

SDW=Sterilized distilled water ; PW= Pond water; TW=Tap water ; SL= Soil leachet

4.9.2. Effect of fungistasis on wild and mutant isolates of *G. virens*

Fungistasis, as an inherent characteristic of soil, largely determines the free survival of fungal propagules in soil. Potential biocontrol agents are those which can germinate profusely in soil by overcoming this static effect. As the mutant isolates are generated artificially their sensitivity towards that kind of limiting factor(s) must be tested before drawing any positive conclusion. In addition, before application of biocontrol agents into soil, well decomposed farm yard manure is popularly utilized as a source of organic matter for multiplication of antagonists. On this substrate fungistatic principal is supposed to operate. Therefore studies have been conducted on the sensitivity of different spore forms of wild and mutant isolates towards the fungistatic effect of both soil and FYM as the method described in section 3.9.5.

Fungistatic effect of phialo- and chlamydospores of wild and mutant isolates in sterilized and non sterilized soil (Table 4.19) revealed that the static effect on both spore forms was more predominant in nonsterilized soil and particularly on phialospores as usual. Phialospore of none of the isolates showed significantly greater fungistatic tolerance in nonsterilized soil, however, comparatively better tolerance was shown by $50Gv_1I$ and $75Gv_1VI$ (10.93 and 11.23 % germination) than others (4.12 – 7.58 % germination). In sterilized soil phialospores of all the mutants of Gv_1 showed significantly higher fungistatic response (17.37–19.32 % germination) in comparison to their wild biotype (38.20 % germination) while reverse trend was evident in case of $75Gv_3I$ (38.08 and 11.87 % germination for $75Gv_3I$ and Gv_3 respectively). Variation in fungistatic response of chlamydospores of test isolates in nonsterilized soil was more or less similar to phialospores (2.23–17.75 % germination) where a single mutant isolate $50Gv_1I$ showed comparatively greater fungistatic tolerance (17.75 % germination) than its wild counterpart Gv_1 (13.35 % germination). Significantly better tolerance was exhibited in sterilized soil by chlamydospores of all test isolates (24.31–79.68 % germination) and mutant isolate $75Gv_3I$ only appeared superior with respect to tolerance to this static barrier (44.21 % germination) than its corresponding wild counterpart Gv_3 (24.31 % germination).

Test in farm yard manure (FYM) revealed that both spore forms of the antagonist isolates were predominantly sensitive to fungistatic barrier in nonsterilized FYM (Table 4.20) and the variation was insignificant in case of phialospores (2.71 – 8.64 % germination). However, in case of chlamydospore apparently greater

Table 4.19 : Fungistatic effect of sterilized and nonsterilized soil on phialo- and chlamydospores of wild and mutant isolates of *G. virens*.

Isolate	Phialospores				Chlamydospores			
	Sterilized soil		Nonsterilized soil		Sterilized soil		Nonsterilized soil	
	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype
Gv_1	38.20±7.59		6.39±0.79		79.68±3.44		13.35±5.08	
$50Gv_1I$	19.29±6.02	(-)49.50	10.93±3.78	(+)71.85	48.78±9.77	(-)38.78	17.75±7.44	(+)32.96
$50Gv_1V$	19.32±5.19	(-)49.42	5.03±2.60	(-)20.91	43.15±20.89	(-)45.84	10.09±4.56	(-)24.42
$75Gv_1VI$	17.37±2.75	(-)54.53	11.23±3.63	(+)76.57	50.19±6.24	(-)37.01	7.31±0.68	(-)45.24
$150Gv_1II$	17.76±2.53	(-)53.51	6.80±2.44	(+)6.92	54.26±6.22	(-)31.90	6.78±1.84	(-)49.21
Gv_3	11.87±1.57		4.12±1.35		24.31±1.75		4.87±1.20	
$75Gv_3I$	38.08±29.38	(+)220.56	7.58±1.42	(+)83.98	44.21±12.38	(+)81.86	2.23±0.48	(-)54.21

For phialospore

SEm±		CD(p=0.01)	
Isolate	3.66	Isolate	14.28(NS)
Soil type	1.95	Soil type	7.63
Isolate x soil type	5.17	Isolate x soil type	20.20(NS)

For chlamydospore

SEm±		CD(p=0.01)	
Isolate	3.25	Isolate	12.68
Soil type	1.74	Soil type	6.78
Isolate x soil type	4.59	Isolate x soil type	17.94

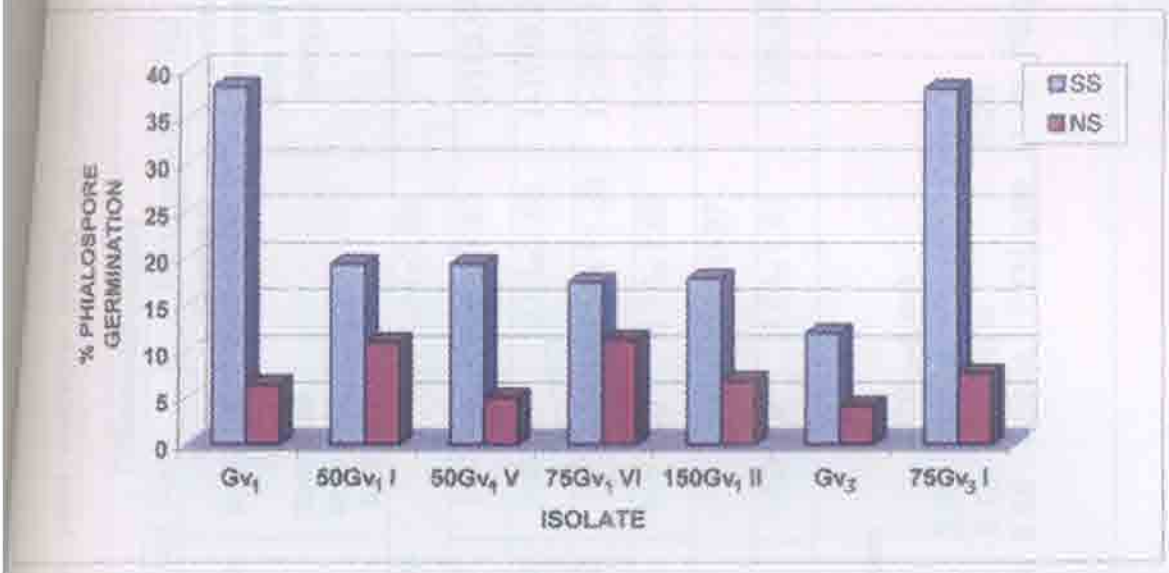


Fig.4.9: Fungistatic effect of sterilized soil (SS) and nonsterilized soil (NS) on phialospores of wild and mutant isolates of *G.virens*

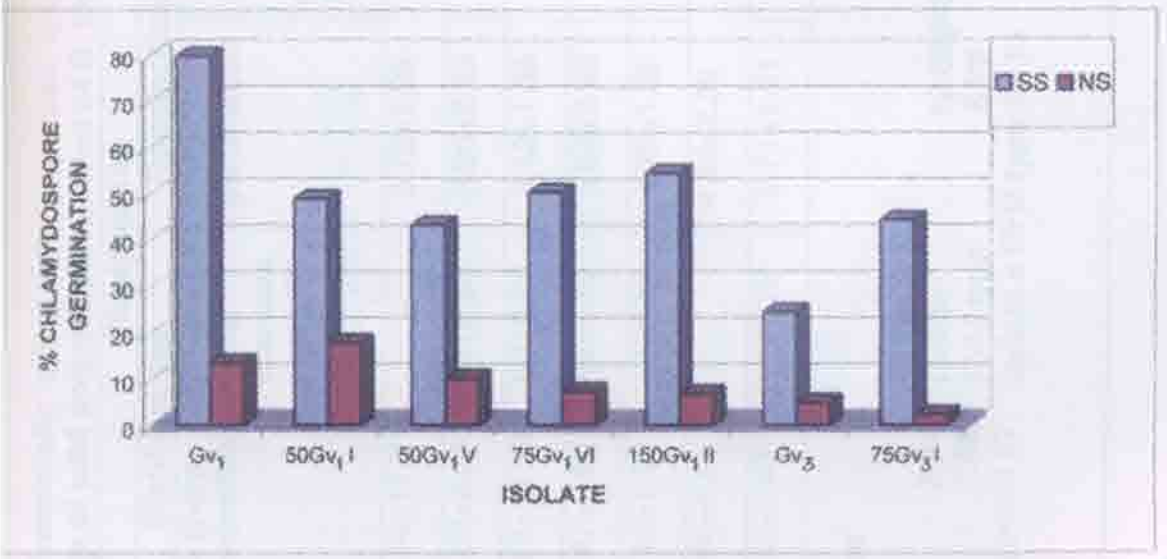


Fig. 4.10: Fungistatic effect of sterilized soil (SS) and nonsterilized soil (NS) on chlamydospores of wild and mutant isolates of *G.virens*

Table 4.20 : Fungistatic effect of sterilized and nonsterilized Farm yard manure (FYM) on phialo- and chlamydospores of wild and mutant isolates of *G. virens*

Isolate	Phialospores				Chlamydospores			
	Sterilized soil		Nonsterilized FYM		Sterilized soil		Nonsterilized FYM	
	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype	% spore germination	% increase or decrease over wild biotype
Gv_1	19.74±11.91		3.72±1.99		32.53±24.04		4.24±3.62	
$50Gv_1I$	17.60±2.40	(-)10.84	3.98±2.53		34.41±5.47	(+)5.78	4.81±3.20	(+)13.44
$50Gv_1V$	21.73±10.50	(+)10.08	6.42±1.35		25.97±4.59	(-)20.17	12.01±1.50	(+)183.25
$75Gv_1VI$	14.47±2.65	(-)26.70	8.64±2.92		30.33±19.92	(-)6.76	16.67±11.33	(+)293.16
$150Gv_1II$	13.16±1.68	(-)33.33	4.39±1.38		13.97±7.14	(-)57.06	6.78±1.99	(+)59.90
Gv_3	11.86±5.31		4.26±2.39		12.29±0.96		6.43±0.27	
$75Gv_3I$	8.33±6.43	(-)29.76	2.71±1.61		9.08±6.31	(-)26.12	2.85±1.45	(-)55.68

For phialospore

SEm±		CD(p=0.01)	
Isolate	2.74	Isolate	12.69(NS)
FYM type	1.46	FYM type	5.72
Isolate x FYM type	3.87	Isolate x FYM type	15.13

For chlamydospore

SEm±		CD(p=0.01)	
Isolate	3.93	Isolate	15.34
FYM type	2.10	FYM type	8.20
Isolate x FYM type	5.55	Isolate x FYM type	21.70 (NS)

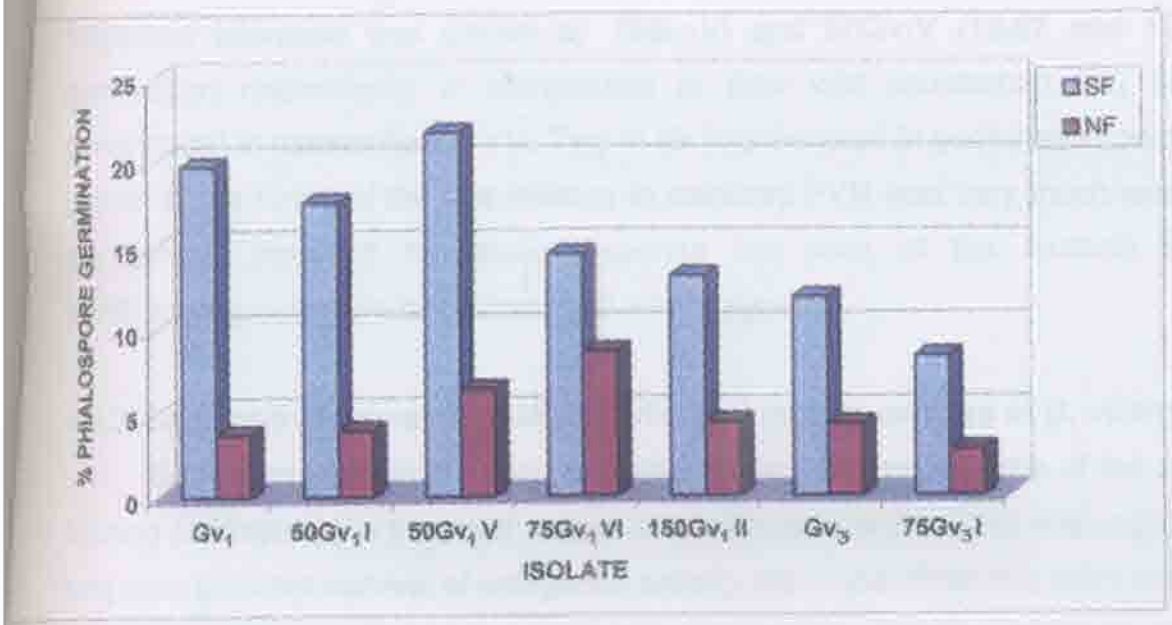


Fig.4.11: Fungistatic effect of sterilized FYM (SF) and nonsterilized FYM (NF) on phialospores of wild and mutant isolates of *G.virens*

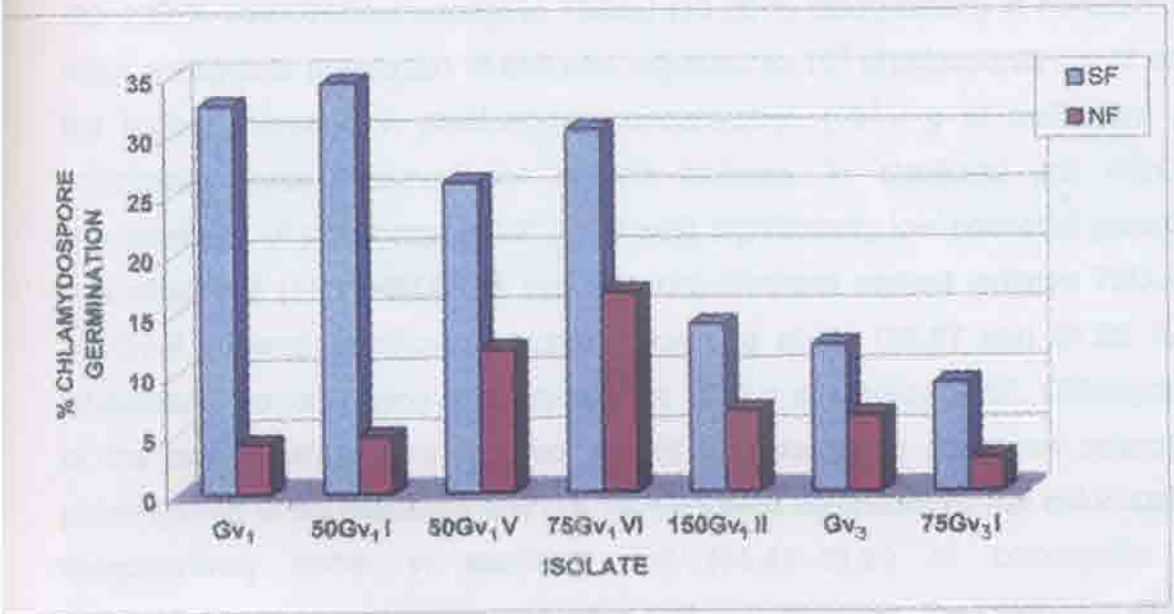


Fig.4.12: Fungistatic effect of sterilized FYM (SF) and nonsterilized FYM (NF) on chlamydospores of wild and mutant isolates of *G.virens*

fungistatic tolerance was shown by $75Gv_1VI$ and $50Gv_1V$ (16.67 and 12.01 % germination respectively) in comparison to their wild counterpart Gv_1 (4.24 % germination) in nonsterilized FYM. Two to six fold increase in germination percentage of both spore forms of the test isolates in sterilized FYM was very much relevant to the general trend of fungistatic response but none of the mutants showed significantly greater tolerance than their wild biotypes.

4.9.3. Competitive parasitic ability of wild and mutant isolates of *G. virens*

Beside saprophytic survival, parasitic survival on live sclerotia of the sclerotia forming pathogen(s) is the most unique characteristics of *G. virens* that may provide long term dormant survival of antagonist propagules in soil. From this point of view an experiment was conducted to study the competitive parasitic ability of wild and mutant isolates of *G. virens* on sclerotia of *R. solani* and *S. rolfsii* as the method described in section 3.9.6.

Competitive parasitic ability on sclerotia of *R. solani* (Table 4.21) revealed that phialospores of the test isolates were more efficient in colonizing pathogen's sclerotia (90–100 % colonization) except in $75Gv_3I$ (10.00 % colonization) in nonsterilized soil when antagonist population in soil was adjusted to 10^2 phialospores / g of soil. With the further increase in phialospore concentration (10^4 / g of soil) cent percent colonization was observed for all the isolates. In sterilized soil with lowest concentration of phialospore (10^2 / g of soil) significantly low sclerotial parasitization was observed (11.10–63.00 % colonization) whereas mutant isolates $75Gv_1VI$ and $150Gv_1II$ showed significantly higher colonizing ability (86.67 and 81.82 %) when phialospore concentration was adjusted to 10^6 / g of sterilized soil. Chlamydospores of the test isolates were less competent in parasitizing pathogen sclerotia than phialospores in nonsterilized soil (18.18 – 61.54% colonization) but colonization was comparatively better in sterilized soil (44.44–88.89 % colonization) when chlamydospore concentration was 10^2 / g of soil. However, the colonizing ability was found to increase with increasing concentration of chlamydospores in soil. Cent percent colonization in both sterilized and non sterilized soil was exhibited by chlamydospores of mutant isolates $75Gv_1VI$ and $150Gv_1Ii$ at 10^6 cfu / g of soil.

Competitive parasitic survival of the test isolates on live sclerotia of *S. rolfsii* was quite different from that on *R. solani*. In nonsterilized soil 0 – 52. 80 % and in

Table 4.21 : Competitive colonization on sclerotia of *R. solani* by phialo and chlamydospores of wild and mutant isolates of *G. virens* in sterilized and nonsterilized soil.

Isolate	Soil type	Percent colonization of sclerotia by *G. virens*.					
		Phialospore			Chlamydospore		
		Phialospore conc. / g of soil			Chlamydospore conc. / g of soil		
		10^2	10^4	10^6	10^2	10^4	10^6
Gv_1	NS	100.00	100.00	100.00	62.50	88.89	88.89
	ST	20.00	23.07	40.00	61.54	62.50	80.00
$50Gv_1I$	NS	100.00	100.00	100.00	88.89	90.00	90.00
	ST	25.00	40.00	50.00	18.18	50.00	81.82
$50Gv_1V$	NS	90.00	100.00	100.00	44.44	44.44	81.82
	ST	11.10	20.00	46.15	22.22	44.44	50.00
$75Gv_1VI$	NS	100.00	100.00	100.00	72.65	100.00	100.00
	ST	11.43	14.28	86.67	50.00	88.89	100.00
$150Gv_1II$	NS	100.00	100.00	100.00	60.00	81.82	100.00
	ST	30.77	81.82	81.82	27.27	50.00	100.00
Gv_3	NS	90.00	100.00	100.00	46.15	50.00	100.00
	ST	63.00	77.78	91.67	22.22	40.00	70.00
$75Gv_3I$	NS	10.00	100.00	100.00	57.50	87.50	88.89
	ST	25.71	40.00	57.69	40.00	70.00	88.89

For phialospore

	SEm±	CD(p=0.01)
Isolate	7.14	30.84(NS)
Soil type	3.57	15.42
Phialospore conc/g of soil	4.37	18.88
Isolate x soil type	10.09	43.61(NS)
Soil type x phialospore conc/g of soil	6.18	26.71(NS)
Isolate x phialospore conc /g of soil	12.36	53.42(NS)

For chlamydospore

	SEm±	CD(p=0.01)
Isolate	4.43	19.14
Soil type	8.29	35.82
chlamydospore conc/g of soil	6.77	29.25
Isolate x soil type	4.43	19.14
Soil type x chlamydospore conc/g of soil	3.25	14.04
Isolate x chlamydospore conc /g of soil	6.77	20.86

Plate 8 Growth of Gv_1, $50Gv_1V$ and $150Gv_1II$ in PDA

Plate9 Competitive parasitic ability of $50Gv_1V$ on sclerotia of *R. solani* in nonsterilized soil

Plate10 Mass multiplied cultures of different wild and mutant isolates of *G. virens*

PLATE - 8

PLATE - 9

PLATE - 10

Fig. 4.13: Competitive colonization on sclerotia of *R. solani* by phialospores of wild and mutant isolates of *G. virens* in nonsterilized soil

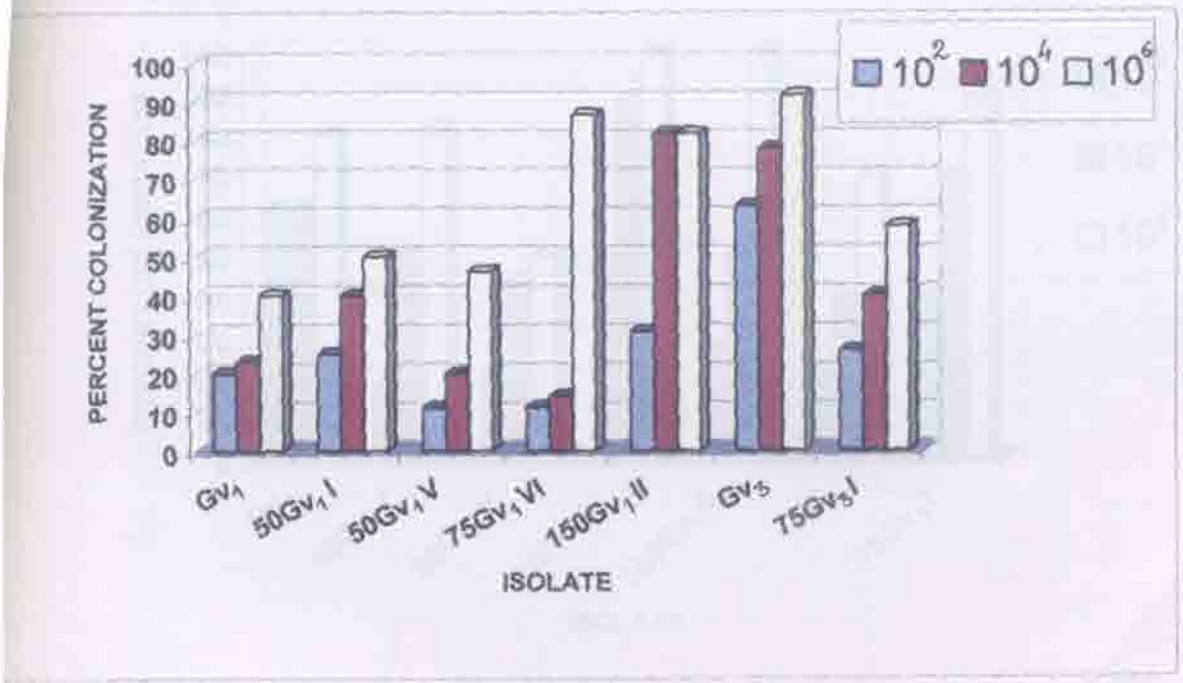


Fig. 4.14: Competitive colonization on sclerotia of *R. solani* by phialospores of wild and mutant isolates of *G. virens* in sterilized soil

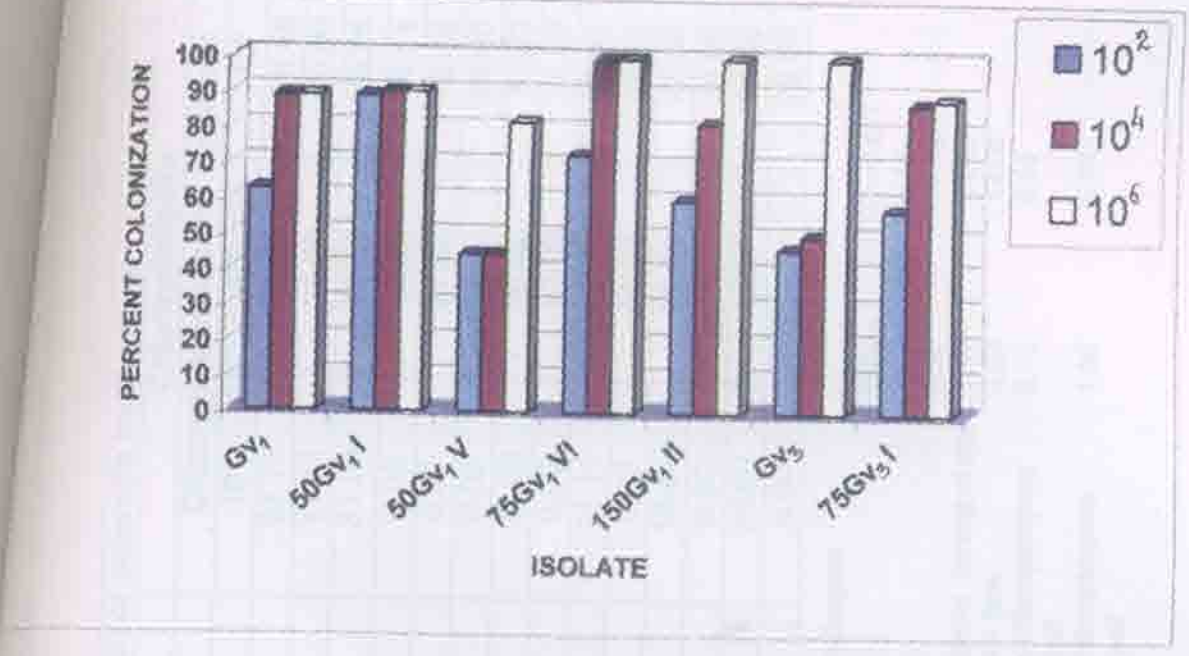


Fig. 4.15: Competitive colonization on sclerotia of *R. solani* by chlamydospores of wild and mutant isolates of *G. virens* in nonsterilized soil

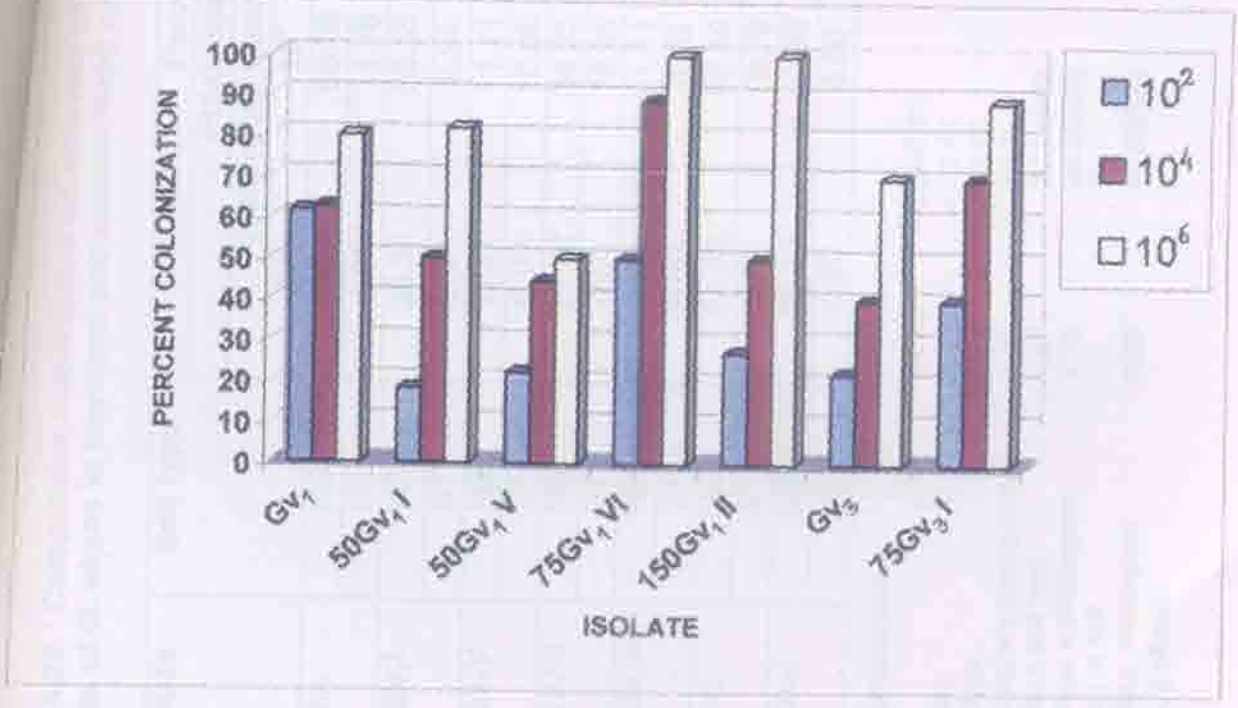


Fig. 4.16: Competitive colonization on sclerotia of *R. solani* by chlamydospores of wild and mutant isolates of *G. virens* in sterilized soil

Table 4.22 : Competitive colonization on sclerotia of *S. rolfsii* by phialo- and chlamydospores of wild and mutant isolates of *G. virens* in sterilized and nonsterilized soil.

Isolate	Soil type	Percent colonization of sclerotia by *G. virens*					
		Phialospore			Chlamydospore		
		Phialospore conc. / g of soil			Chlamydospore conc. / g of soil		
		10^2	10^4	10^6	10^2	10^4	10^6
Gv_1	NS	50.00	60.00	70.00	27.73	69.21	76.46
	ST	0.00	11.76	48.29	11.11	25.00	40.00
$50Gv_1I$	NS	33.33	55.55	88.00	31.29	52.74	85.25
	ST	0.00	9.24	37.50	0.00	14.29	41.11
$50Gv_1V$	NS	0.00	11.11	12.50	0.00	21.22	48.65
	ST	0.00	10.00	50.00	0.00	10.00	26.00
$75Gv_1VI$	NS	22.22	33.33	80.00	23.33	50.00	70.00
	ST	0.00	12.39	37.50	0.00	12.00	38.00
$150Gv_1II$	NS	0.00	11.11	18.80	11.11	29.45	49.22
	ST	0.00	0.00	0.00	0.00	14.29	46.00
Gv_3	NS	52.80	62.50	83.33	16.67	56.65	76.59
	ST	5.61	19.72	46.39	0.00	11.11	39.00
$75Gv_3I$	NS	25.00	40.00	100.00	25.00	45.57	85.69
	ST	0.00	0.00	11.11	0.00	6.00	29.00

For phialospore

	SEm±	CD(p=0.01)
Isolate	5.42	23.43
Soil type	2.71	11.72
Phialospore conc/g of soil	3.32	14.35
Isolate x soil type	7.67	33.14(NS)
Soil type x phialospore conc/g of soil	4.70	20.29(NS)
isolate x phialospore conc /g of soil	9.39	40.58(NS)

For chlamydospore

	SEm±	CD(p=0.01)
Isolate	2.12	9.16
Soil type	3.97	17.15
chlamydospore conc/g of soil	3.24	14.00
Isolate x soil type	3.24	14.00
Soil type x chlamydospore conc/g of soil	2.12	9.16
isolate x chlamydospore conc /g of soil	1.56	6.74

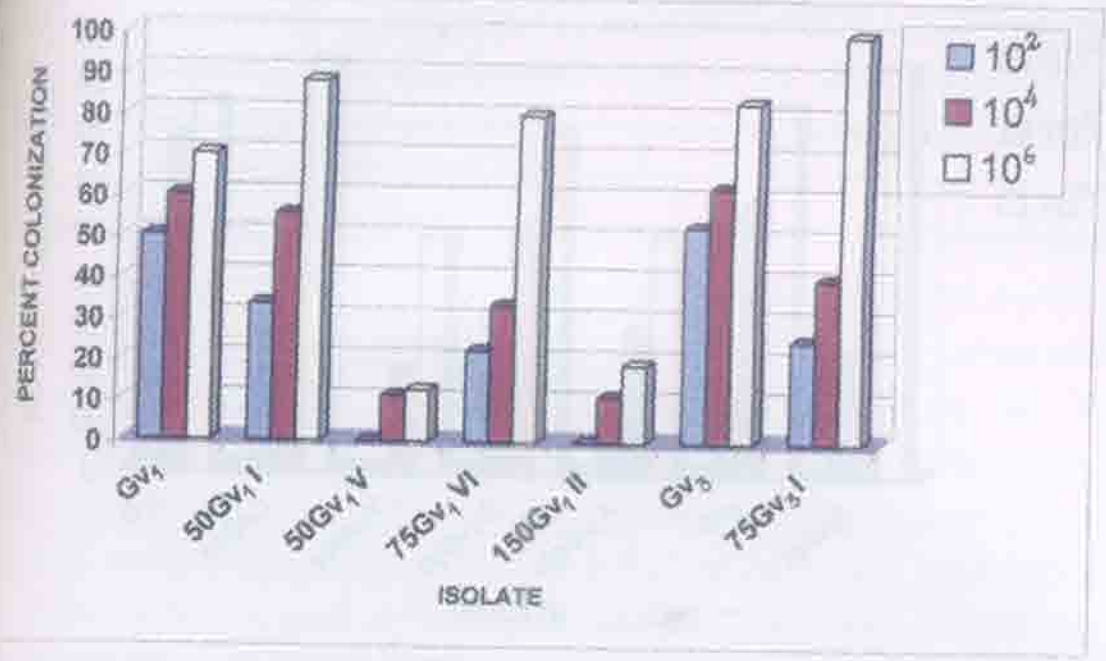


Fig. 4.17: Competitive colonization on sclerotia of *S. rolfsii* by phialospores of wild and mutant isolates of *G. virens* in nonsterilized soil

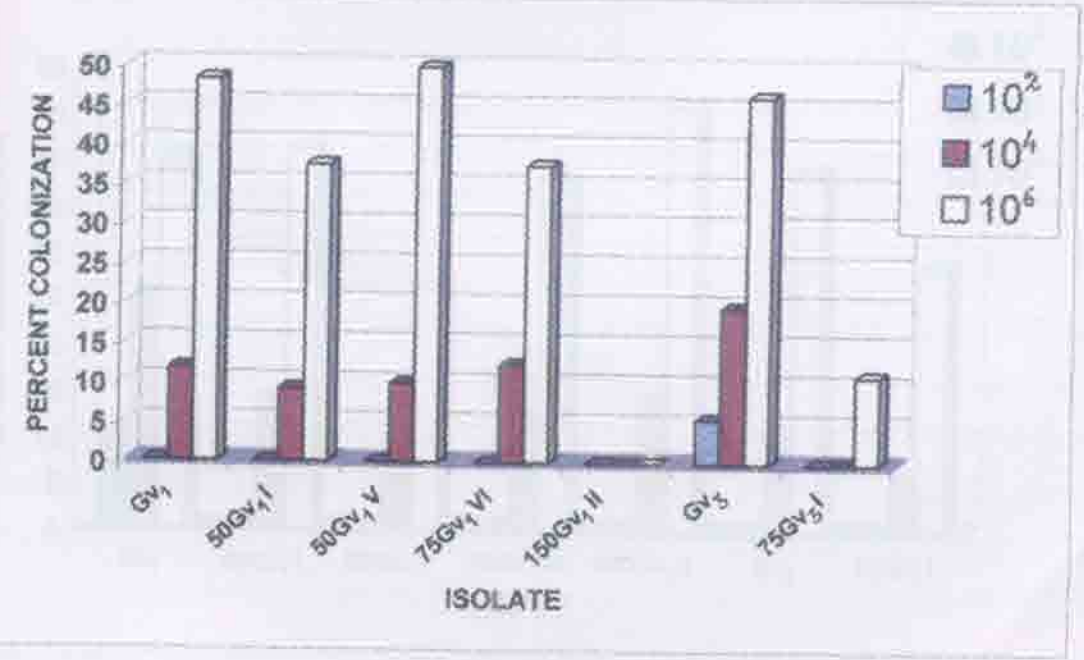


Fig. 4.18: Competitive colonization on sclerotia of *S. rolfsii* by phialospores of wild and mutant isolates of *G. virens* in sterilized soil

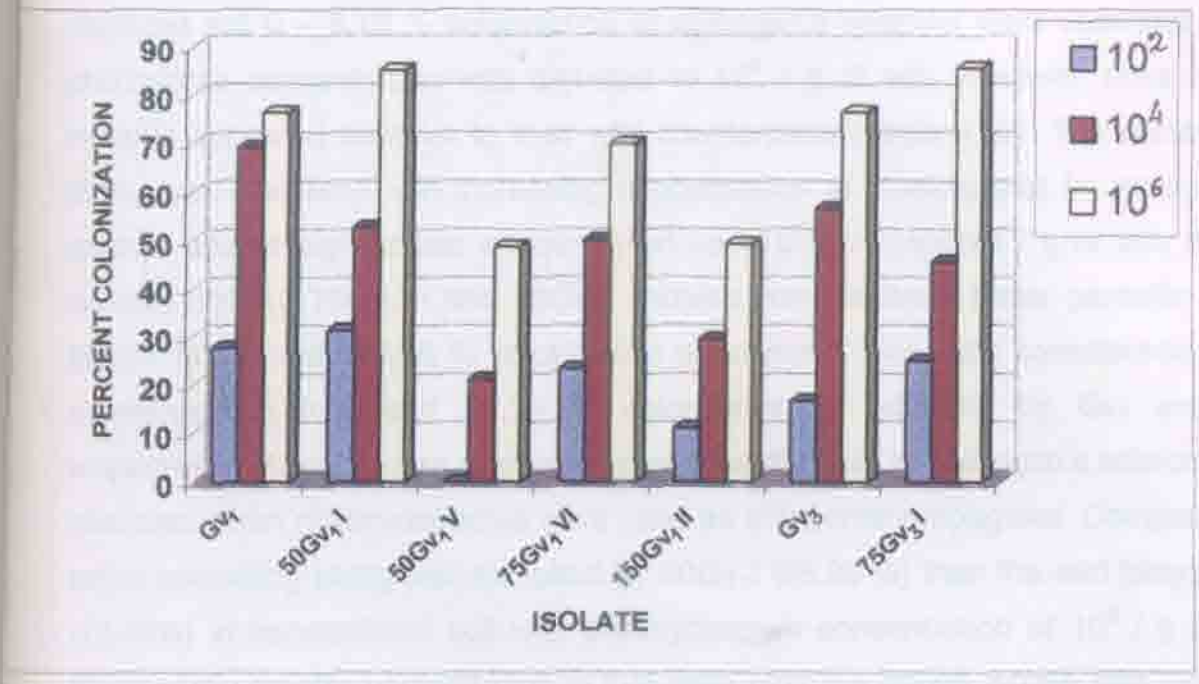


Fig. 4.19: Competitive colonization on sclerotia of *S. rolfsii* by chlamydospores of wild and mutant isolates of *G. virens* in nonsterilized soil

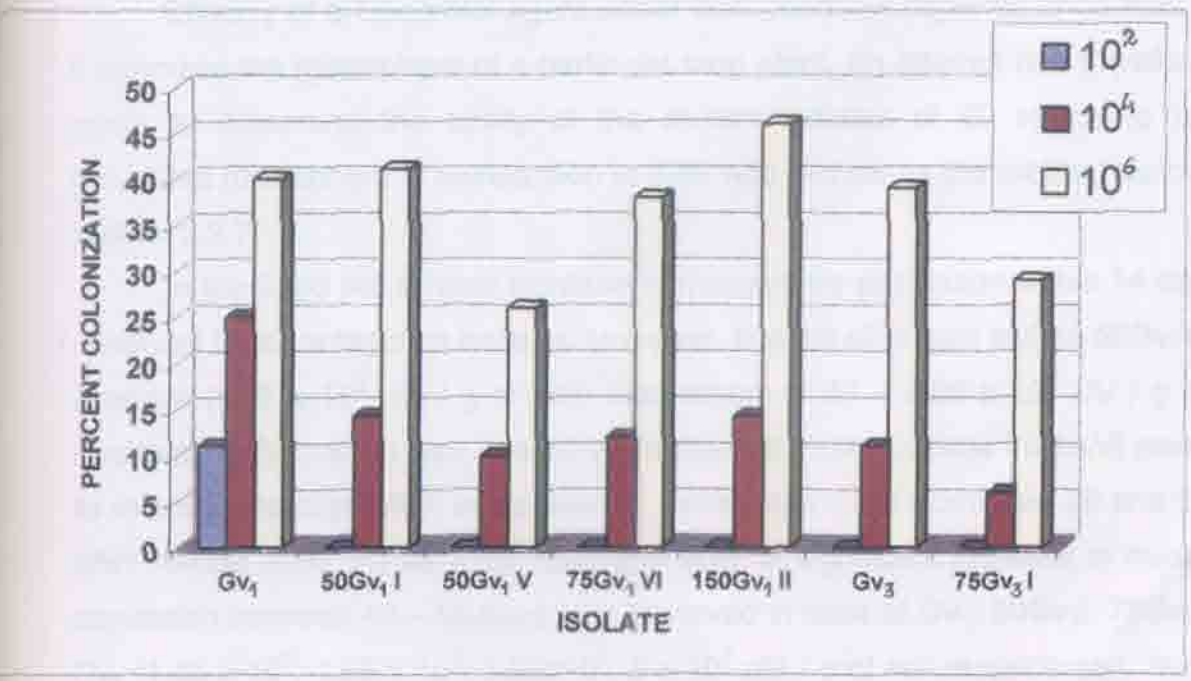


Fig. 4.20: Competitive colonization on sclerotia of *S. rolfsii* by chlamydospores of wild and mutant isolates of *G. virens* in sterilized soil

sterilized soil 0 – 5.16 % colonization of pathogen's sclerotia were observed when phialospore concentration was adjusted to 10^2 / g of soil, however, none of the mutants appeared superior to their wild counterparts (Table 4.22). The parasitizing ability was increased with increasing concentration of phialospores for most of the isolates and at highest test concentration i.e., 10^6 phialospores / g of soil, mutant isolates $50Gv_1I$, $75Gv_1VI$ and $75Gv_3I$ showed comparatively better parasitic ability (88.00, 80.00 and 100.00 % colonization of sclerotia) than their corresponding wild counterparts (70.00 and 83.33 % colonization of sclerotia by Gv_1 and Gv_3 respectively). More or less similar trend of parasitization of pathogen's sclerotia was observed when chlamydospores were used as antagonist propagules. Comparatively better colonizing ability was exhibited by $50Gv_1I$ (85.25 %) than the wild biotype Gv_1 (76.46%) in nonsterilized soil with chlamydospore concentration of 10^6 / g of soil. Significantly greater parasitization in that case over the parent isolate was exhibited only by $75Gv_3I$ (85.69 and 76.59 % colonization by $75Gv_3I$ and Gv_3 respectively).

4.9.4. Rhizosphere colonization by wild and mutant isolates of *G. virens*

Efficacy of a biocontrol agent under field condition depends upon how quickly it colonizes the rhizosphere of a particular crop plant. An attempt has therefore been made to determine the ability of the mutant isolates of *G. virens* to colonize groundnut rhizosphere in comparison to their wild biotype as the method described in section 3.9.7.

In sterilized soil a rapid increase in rhizosphere population within 14 days was observed for all antagonist isolates. However, in case of mutant isolate $50Gv_1I$, it was aberrant (1.05×10^8 cfu / g of soil) than others ($1.37 - 6.06 \times 10^7$ cfu / g of soil). Inconsistent fluctuation was noticed thereafter but mutant isolate $75Gv_1VI$ maintained its rhizosphere population comparatively better than others between 28 and 56 days after sowing ($6.92 - 1.93 \times 10^7$ cfu / g of soil). A significant increase in rhizosphere population between 42 – 56 days was observed in case of Gv_1, $50Gv_1I$, $75Gv_1VI$ and Gv_3 (1.25×10^7, 1.96×10^7, 1.93×10^7, 3×10^7 cfu / g of soil respectively). In spite of that none of the mutant isolates appeared more rhizosphere competent (3.4×10^6 – $1.98\ 10^7$ cfu / g of soil) than their corresponding wild counterparts (2.21×10^7 and 1.8×10^7 cfu / g of soil of Gv_1 and Gv_3 respectively) during the later part of the experiment (Table 4.23).

Table 4.23: Rhizosphere colonization of wild and mutant isolates of *G. virens* in sterilized soil.

Isolate	Colony forming units ($x10^5$) / g of soil					
	Initial population	14 DAS	28 DAS	42 DAS	56 DAS	70 DAS
Gv_1	1.00 ± 0.00	274.08±24.21^a	139.25±9.09^b	59.25±13.84^a	125.25±12.23^b	221.75±13.31^c
50 Gv_1I	1.00 ± 0.00	1050.70±134.08^c	130.00±21.28^b	54.72±18.54^a	196.00±25.96^c	198.00±35.81^b
50Gv_1 V	1.00 ± 0.00	155.33±19.44^a	50.00±5.49^a	24.89±4.87^a	58.50±12.46^a	34.17±12.99^a
75Gv_1VI	1.00 ± 0.00	200.25±21.75^a	692.85±41.73^c	128.74±14.50^b	193.75±30.56^c	174.62±13.74^b
150Gv_1II	1.00 ± 0.00	150.75±7.72^a	60.28±18.08^a	25.80±1.83^a	32.63±2.64^a	47.20±4.65^a
Gv_3	1.00 ± 0.00	606.00±73.62^b	41.53±5.51^a	44.88±8.67^a	300.50±27.59^d	179.74±30.45^b
75Gv_3I	1.00 ± 0.00	137.62±24.42^a	155.34±24.45^b	44.15±6.10^a	52.31±3.21^a	61.84±12.81^a

SEm±		CD (p = 0.01)	
Isolate	8.36	Isolate	31.23
Days	7.74	Days	28.91
Isolate X days	10.49	Isolate X days	76.49

Table 4.24 : Rhizosphere colonization of wild and mutant isolates of *G. virens* in nonsterilized soil.

Isolate	Colony forming units ($x10^5$) / g of soil					
	Initial population	14 DAS	28 DAS	42 DAS	56 DAS	70 DAS
Gv_1	1.00 ± 0.00	1.19± 0.04[a]	4.52 ± 0.51[a]	3.33 ± 0.87[a]	1.30 ± 0.07[a]	1.41 ± 0.25[a]
50 Gv_1I	1.00 ± 0.00	1.67 ± 0.38[a]	5.39 ± 0.34[a]	3.48 ± 0.38[a]	1.24 ± 0.13[a]	1.12 ± 0.12[a]
50Gv_1 V	1.00 ± 0.00	5.44 ± 0.73[a]	4.26 ± 0.47[a]	1.96 ± 0.15[a]	1.94 ± 0.46[a]	1.22 ± 0.31[a]
75Gv_1VI	1.00 ± 0.00	1.60 ± 0.43[a]	4.60 ± 1.29[a]	1.86 ± 0.27[a]	1.50 ± 0.01[a]	1.57 ± 0.18[a]
150Gv_1II	1.00 ± 0.00	2.71 ± 0.48[a]	3.08 ± 0.56[a]	1.10 ± 0.14[a]	1.50 ± 0.24[a]	1.71 ± 0.25[a]
Gv_3	1.00 ± 0.00	13.39 ± 1.87[a]	18.33 ± 2.42[b]	4.51 ± 0.60[a]	8.01 ± 0.56[a]	2.80 ± 0.47[a]
75Gv_3I	1.00 ± 0.00	82.21 ± 13.74[b]	81.29 ± 9.30[c]	21.75 ± 3.82[b]	34.89 ± 7.82[b]	9.39 ± 1.59[b]

SEm±		CD (p = 0.01)	
Isolate	0.86	Isolate	3.19
Days	0.79	Days	2.96
Isolate X days	2.10	Isolate X days	7.82

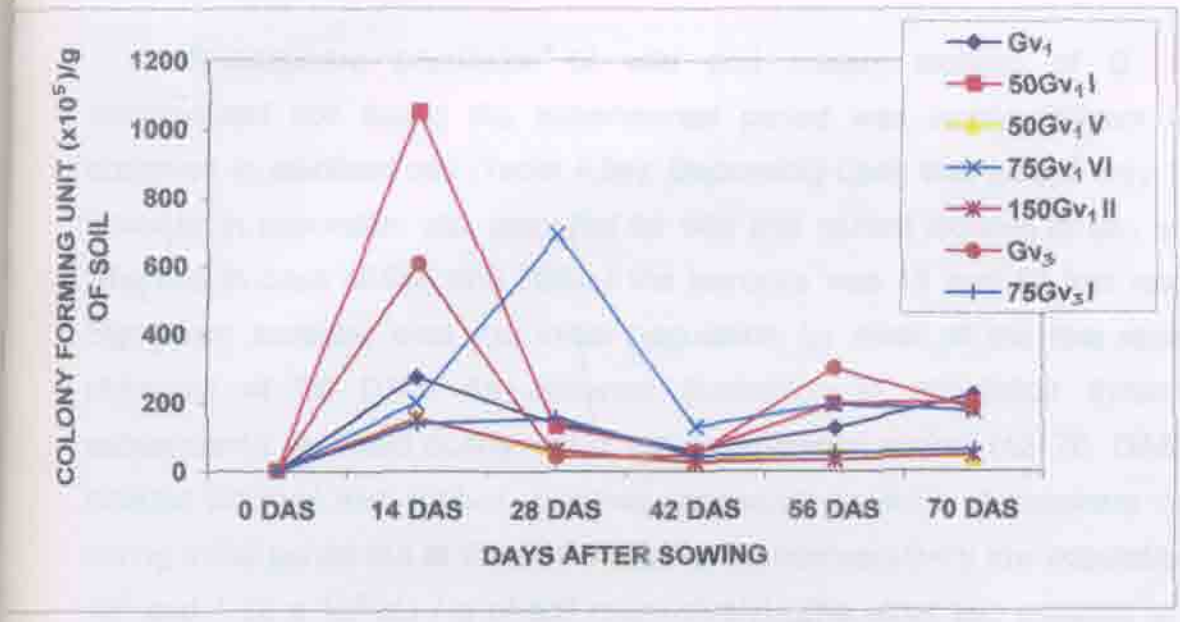


Fig. 4.21: Rhizosphere colonization of wild and mutant isolates of *G.virens* in sterilized soil

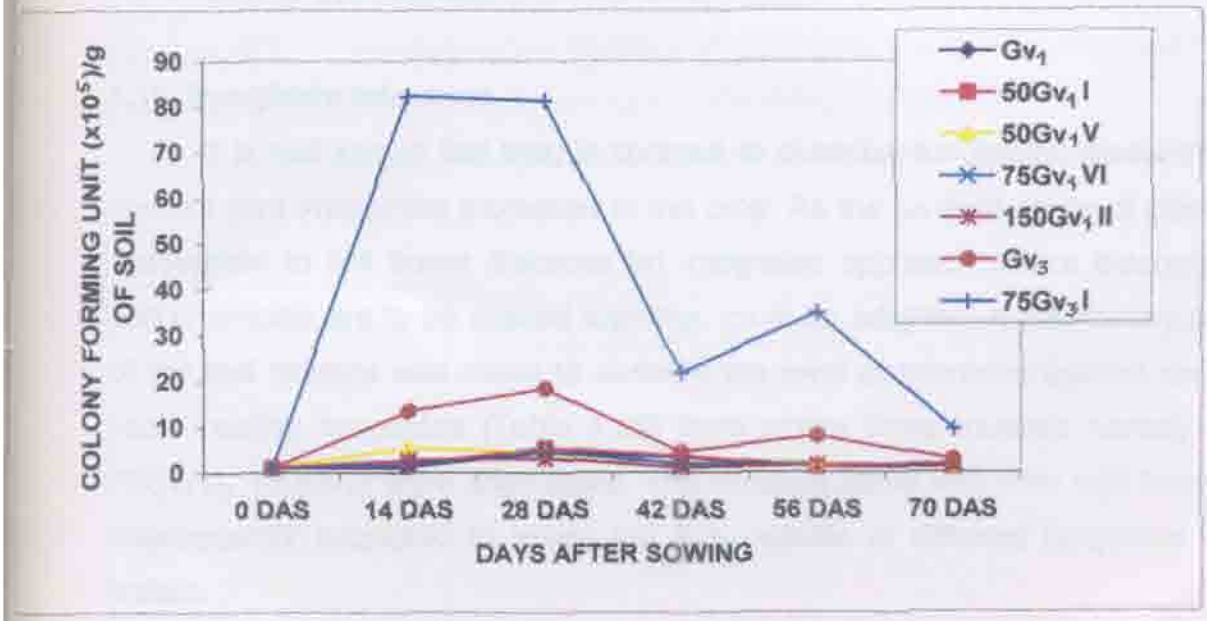


Fig. 4.22: Rhizosphere colonization of wild and mutant isolates of *G.virens* in nonsterilized soil

Rhizosphere population of wild and mutant isolates of *G. virens* in nonsterilized soil during the experimental period was vastly different from that observed in sterilized soil (Table 4.24). Depending upon that isolate only 1 – 5 fold increase in population was recorded for wild and mutant isolates of Gv_1 at 14 DAS whereas in case of Gv_3 and $75Gv_3I$ the increase was 13 and 82 fold respectively. Significant increase over the initial population for most of the test isolates was observed at 28 DAS. An apparent fluctuation in population dynamics was subsequently recorded during rest of the experimental period. (42–70 DAS). Mutant isolates $50Gv_1V$ and $50Gv_1I$, however, appeared potential rhizosphere competent during initial period but at the end they showed comparatively low population (1.22×10^5 and 1.12×10^5 cfu / g of soil respectively). The other two mutants of Gv_1 i.e., $75Gv_1VI$ and $150Gv_1II$ exhibited comparatively better rhizosphere colonizing ability (1.57×10^5 and 1.71×10^5 cfu / g of soil) as compared to their wild counterpart (1.41×10^5 cfu / g of soil) at the later part of the experimental period (70 DAS). Significantly greater rhizosphere colonizing ability throughout the experimental period was exhibited only by the mutant isolate $75Gv_3I$.

4.10. Fungicide tolerance

It is well known fact that, in contrast to chemical fungicides, biocontrol agents can not give immediate protection to the crop. As the juvenile stage of plant is most susceptible to soil borne diseases an integrated approach where biocontrol agent and chemicals are to be applied together, must be adapted. A preliminary screening of the test isolates was made to observe the level of tolerance against six different seed treating fungicides (Table 4.25) from where three mutants namely $50Gv_1V$, $75Gv_1VI$, $150Gv_1II$ were short listed. The mutants along with their wild biotype were subsequently subjected to assay the ED_{50} values of different fungicides for each isolate.

Table 4.25 : Rapid screening of fungicide tolerance of the mutant isolates of *G. virens*.

Isolate	Mean Colony diameter (mm) #					
	Fungicide Concentration (ppm)					
	Benomyl (2 ppm)	Bavistin (2 ppm)	Thiram (5 ppm)	Captan (5 ppm)	Iprodione (5 ppm)	Mancozeb (50 ppm)
Gv_1	21.80*	8.50	83.20	46.30	57.00	79.50
50 Gv_1 I	12.40	6.00	80.80	34.30	40.20	74.00
50 Gv_1 V	18.50	12.00	82.80	37.20	39.00	88.50
75 Gv_1 VI	21.30	11.75	86.40	40.00	41.80	87.00
150 Gv_1 II	20.10	13.50	88.50	47.50	47.40	90.00
Gv_3	12.70	6.00	78.50	45.80	28.20	77.50
75 Gv_3 I	12.10	6.00	83.30	40.50	31.40	81.00

* Each insertion is based on an average of 5 replications.

Mycelial disc of 6 mm diameter was inoculated in each replication.

Test against Benomyl (Table 4.26) showed that 75Gv_1VI was comparatively tolerant (ED_{50} 1.68 ppm) while 150Gv_1II and 50Gv_1V were sensitive (ED_{50} 1.32 and 0.69 ppm respectively) than their wild biotype (ED_{50} 1.48 ppm).

Table 4.26 : Tolerance to Benomyl by wild and selected mutant isolates of *G. virens*.

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Benomyl concentration (ppm)				
	0.5	1.0	2.0	5.0	
Gv_1	0.00*	30.00±9.11	71.48±13.91	94.81±7.34	1.48
50Gv_1V	41.67±21.62	46.30±28.81	73.71±25.41	90.19±0.26	0.69
75Gv_1VI	2.03±0.52	33.14±15.72	67.59±7.73	86.67±2.40	1.68
150Gv_1II	18.14±9.95	34.44±18.86	77.03±2.62	88.89±1.64	1.32

* An average of five replications.

All mutants and Gv_1 were much sensitive to Bavistin and 50% inhibition in growth was observed below 0.5 ppm (Table 4.27). However, 50Gv_1V showed comparatively higher level of tolerance (70.37 % inhibition) than to its wild counterpart (78.15 % inhibition).

Table 4.27: Tolerance to Bavistin by wild and selected mutant isolates of *G. virens*.

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Bavistin concentration (ppm)				
	0.5	1.0	2.0	5.0	
Gv_1	78.15±0.94*	82.59±0.52	90.74±0.69	100.00±0.00	<0.5
$50Gv_1V$	70.37±0.95	81.67±0.91	90.56±0.45	100.00±0.00	<0.5
$75Gv_1VI$	80.74±0.26	81.11±0.45	85.19±1.31	100.00±0.00	<0.5
$150Gv_1II$	78.70±2.77	82.59±2.50	86.11±2.08	100.00±0.00	<0.5

* An average of five replications.

Thiram appeared less toxic to the wild and mutant isolates of *G. virens*. Comparatively higher ED_{50} values were recorded for $150Gv_1II$ and $75Gv_1VI$ (41.89 and 41.81ppm respectively) than their wild biotype (37.48 ppm). Although $50Gv_1V$ was most sensitive to Thiram (ED_{50} 33.96 ppm) but at 10.0 ppm concentration mean percent inhibition was comparatively low (27.03 %) than Gv_1 (29.08 %) [Table 4.28].

Table 4.28 : Tolerance to Thiram by wild and selected mutant isolates of *G. virens*.

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Thiram concentration (ppm)				
	5.0	10.0	20.0	50.0	
Gv_1	2.41±1.83*	29.08±3.40	37.59±12.02	61.48±6.93	37.48
$50Gv_1V$	4.81±2.50	27.03±1.83	45.37±4.77	65.74±5.83	33.96
$75Gv_1VI$	0.00	12.97±4.19	22.78±2.27	59.81±3.40	41.81
$150Gv_1II$	0.00	15.92±0.52	18.70±1.83	60.74±0.26	41.89

* An average of five replications.

Tolerance to Captan revealed that wild isolate (Gv_1) was comparatively more tolerant (ED_{50} 9.56 ppm) than the mutants (ED_{50} 7.18–8.57 ppm). Among the mutants $150Gv_1II$ appeared best followed by $75Gv_1VI$ and $50Gv_1V$ (Table 4.29).

Table 4.29 : Tolerance to Captan by wild and selected mutant isolates of *G. virens*.

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Captan concentration (ppm)				
	1.0	2.0	5.0	10.0	
Gv_1	20.89±2.74*	28.23±4.23	40.67±5.19	48.89±1.26	9.56
$50Gv_1V$	27.78±2.69	32.69±1.38	52.34±0.61	56.19±2.58	7.18
$75Gv_1VI$	19.48±1.24	32.73±3.62	41.77±1.86	55.82±4.73	8.05
$150Gv_1II$	24.91±8.54	33.52±11.54	44.91±2.17	53.73±16.20	8.57

* An average of five replications.

Test against Iprodione showed that wild isolate (Gv_1) was more tolerant to Iprodione (ED_{50} 11.19 ppm) than the mutant isolates (ED_{50} 4.22 – 4.97 ppm). Like Captan, among the mutants, $150Gv_1II$ appeared best followed $75Gv_1VI$ and $50Gv_1V$, (Table 4.30).

Table 4.30 : Tolerance to Iprodione by wild and selected mutant isolates of *G. virens*.

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Iprodione concentration (ppm)				
	5.0	10.0	20.0	50.0	
Gv_1	41.11±2.76*	44.44±4.80	68.14±2.91	85.92±1.05	11.19
$50Gv_1V$	65.56±0.91	69.44±4.03	76.67±3.17	82.59±2.29	4.22
$75Gv_1VI$	64.44±3.96	68.52±1.38	74.44±2.40	81.86±9.18	4.35
$150Gv_1II$	49.63±0.69	58.89±11.45	70.19±12.94	82.78±2.36	4.97

* An average of five replications.

A high level of tolerance to Mancozeb had been exhibited by the mutant and wild isolates of *G. virens* (Table 4.31). All the mutants possessed comparatively high level of tolerance (ED_{50} 371.54–758.58 ppm) than their wild type Gv_1 (ED_{50} 331.13 ppm).

PLATE - 11 (a)

PLATE-11(b)

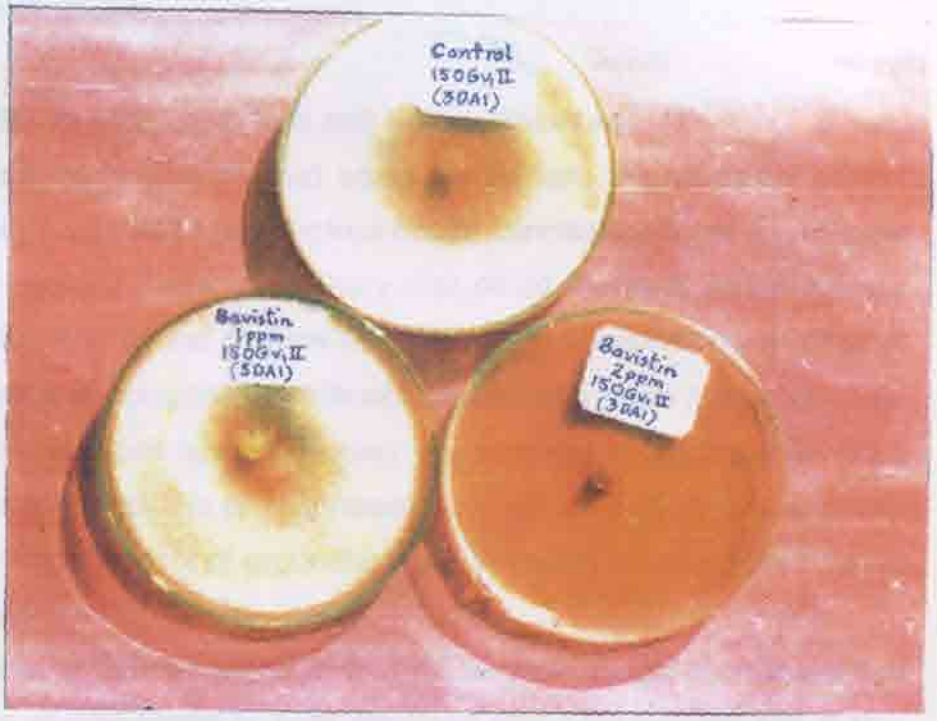


PLATE - 11 (c)

Table 4.31 : Tolerance to Mancozeb by wild and selected mutant isolates of *G. virens.*

Isolate	Mean percent inhibition over control				ED_{50} (ppm)
	Mancozeb concentration (ppm)				
	20.0	50.0	100.0	250.0	
Gv_1	6.67±2.83*	13.70±2.58	27.59±7.81	44.44±4.60	331.13
$50Gv_1V$	0.18±0.13	1.11±0.91	21.29±4.98	32.59±3.46	371.54
$75Gv_1VI$	0.18±0.13	2.22±0.78	8.52±7.03	22.96±3.53	575.44
$150Gv_1II$	0.00	0.00	1.67±1.34	17.78±4.37	758.58

* An average of five replications.

4.11. Green house trial on four important soil borne diseases

Before any recommendation for field scale application, the biological control agents initially required to be tested under glass-house condition against a particular pathogen-host combination so that a preliminary idea could be made about efficacy of the antagonist. At the end of the present investigation a comprehensive attempt therefore has been made to compare the efficacy of wild and mutant isolates of *G. virens* against four important soil borne diseases viz, seedling blight of green gram (c.o. *R. solani*), stem rot of jute (c.o. *M. phaseolina*), wilt of pigeon pea (c.o. *F. oxysporum* f.sp. *udum*) and root rot of groundnut (c.o. *S. rolfsii*) following the method described earlier (Section3.11).

A detailed perusal of the results on *in vivo* efficacy of test antagonist isolates against seedling blight of greengram in both sterilized and nonsterilized soil (Table 4.32 and 4.33) revealed that comparatively better germination of seeds was noticed in nonsterilized soil over the sterilized one (37.78–51.28 % and 33.33–46.02 % in nonsterilized and sterilized soil respectively). This was more prominent in case of some of the mutant isolates, viz., $50Gv_1V$, $75Gv_1VI$ and $150Gv_1II$. With the aging of plants from germination to 40 days age of the seedlings, percent seedling stands gradually reduced due to disease development irrespective of the condition of the soil (sterilized or nonsterilized). In case of control (without antagonist) the rate of seedling mortality gradually increased up to 40 DAS while in antagonist amended soil significant increase in disease incidence was obtained in between 10 to 20 days (except Gv_3) followed by gradual reduction in disease incidence which reached in

Table 4.32 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *R. solani* induced seedling blight of green gram in sterilized soil.

Isolate	Germination(%)	Present mortality caused by *R. solani*				% Reduction in disease incidence	SEm±	CD(p=0.05)
		10 DAS	20 DAS	30 DAS	40 DAS			
Gv_1	45.00±5.00	10.96±10.83	36.67±8.98	55.83±10.96	69.17±24.22	18.34	7.10	20.94
$50Gv_1I$	43.33±7.45	3.33±7.45	23.89±4.78	33.89±6.71	57.78±5.98	31.79	2.98	8.78
$50Gv_1V$	40.90±6.07	24.72±4.45	25.83±6.72	56.11±6.50	68.61±6.76	19.00	3.49	10.30
$75Gv_1VI$	45.13±4.48	22.50±5.42	53.89±11.29	57.22±9.51	57.22±9.51	32.45	4.34	12.79
$150Gv_1II$	46.02±4.99	11.27±8.14	37.42±7.98	57.86±13.11	66.35±8.97	21.67	4.61	13.60
Gv_3	35.00±9.57	38.61±9.15	57.22±7.56	73.89±12.79	73.89±12.79	12.77	4.84	14.27
$75Gv_3I$	40.00±8.16	36.94±7.72	59.72±11.64	79.45±10.21	79.45±10.21	6.21	4.14	13.98
Control	33.33±9.43	33.00±4.76	51.11±12.42	78.61±17.75	84.71±18.35		6.94	20.46
SEm±	3.20	3.51	4.34	5.13	6.01			
CD(p=0.05)	9.16	10.03	12.41	14.67	17.18			

Table 4.33 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *R. solani* induced seedling blight of green gram in nonsterilized soil.

Isolate	Germination(%)	Percent mortality caused by *R. solani*				% Reduction in disease incidence	SEm±	CD(p=0.05)
		10 DAS	20 DAS	30 DAS	40 DAS			
Gv_1	40.56±7.31	23.77±5.78	44.36±6.30	52.86±4.12	63.57±10.83	31.89	3.39	10.01
$50Gv_1I$	37.78±10.83	27.70±11.75	41.51±4.95	55.16±8.37	58.49±4.95	37.33	3.78	11.16
$50Gv_1V$	51.28±8.50	17.52±4.07	51.61±6.97	67.04±5.05	67.04±5.05	28.17	2.54	7.49
$75Gv_1VI$	51.28±8.50	19.90±5.43	32.96±5.05	45.06±5.18	51.31±8.69	45.02	2.96	8.72
$150Gv_1II$	51.28±16.42	18.24±4.24	34.81±8.60	42.96±5.94	44.63±6.27	52.18	4.12	12.16
Gv_3	46.67±13.74	43.25±7.46	59.52±14.79	74.40±4.99	78.57±10.80	15.81	4.56	13.45
$75Gv_3I$	46.67±8.39	24.60±7.33	38.39±6.22	63.00±7.90	68.16±4.00	26.97	3.12	9.20
Control	40.00±10.00	36.67±3.34	52.22±13.56	84.45±16.63	93.33±9.43		5.58	16.46
SEm±	4.86	2.98	4.47	3.67	3.55			
CD(p=0.05)	13.88	8.52	12.77	10.49	10.15			

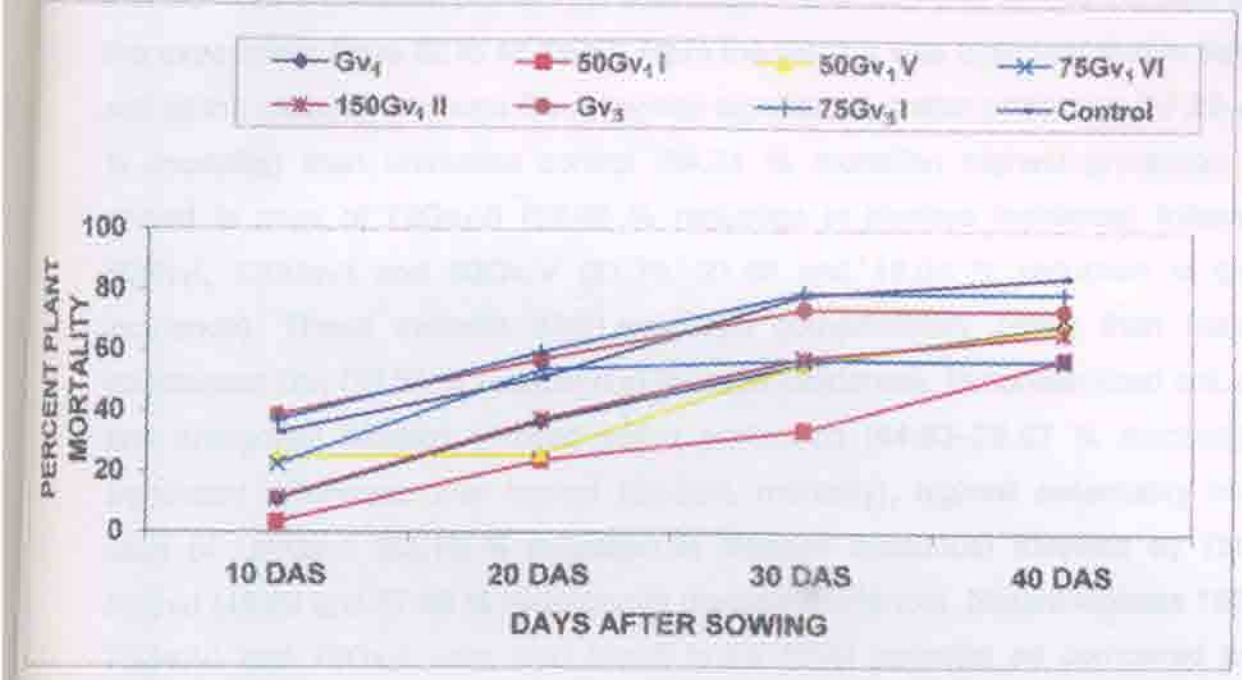


Fig. 4.23 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against seedling blight of green gram induced by *R. solani* in sterilized soil

Fig. 4.24: *In vivo* efficacy of wild and mutant isolates of *G. virens* against seedling blight of green gram induced by *R. solani* in nonsterilized soil

more or less a constant plateau for both natural and sterilized soil during later part of the experiment (from 20 to 40 days). From the result it was observed that in sterilized soil all the mutants of isolate Gv_1 provided significantly better protection (57.22–68.61 % mortality) than untreated control (84.71 % mortality) highest protection being shown in case of $75Gv_1VI$ (32.45 % reduction in disease incidence) followed by $50Gv_1I$, $150Gv_1II$ and $50Gv_1V$ (31.79, 21.67 and 19.00 % reduction in disease incidence). These mutants also appeared comparatively better than their wild counterpart Gv_1 (18.34 % reduction in disease incidence). In nonsterilized soil, all the test antagonist isolates showed better protection (44.63–78.57 % mortality) with significant difference over control (93.33% mortality), highest potentiality being in case of $150Gv_1II$ (52.18 % reduction in disease incidence) followed by $75Gv_1VI$, $50Gv_1I$ (45.02 and 37.33 % reduction in disease incidence). Mutant isolates $150Gv_1II$, $75Gv_1VI$ and $75Gv_3I$ were also found to be more potential as compared to their corresponding wild biotypes (31.89 and 15.81 % reduction in disease incidence in case of Gv_1 and Gv_3 respectively).

Test against stem rot of jute / *M. phaseolina* revealed that all the antagonist isolates supported comparatively better seedling stand of jute in nonsterilized soil. (Table 4.34 and 4.35). In case of control (without antagonist) a rapid increase in disease situation was observed between 14 – 28 days after sowing irrespective of condition of soil (sterilized or nonsterilized). A significant difference in disease incidence was noted in both soil types at 28 DAS where all antagonist amended treatments resulted in significantly less seedling mortality than untreated control. In sterilized soil after 56 days, all the isolates of Gv_1 except $50Gv_1V$ gave significantly better protection with 31.20-48.80 % reduction in disease incidence and 150 Gv_1 II appeared comparatively better than its wild biotype Gv_1 (48.02 % reduction in disease incidence). Mutant isolates $50Gv_1I$, $50Gv_1V$ and $75Gv_1VI$, however, initially (14 DAS) showed no plant mortality but apparently low antagonistic activity was visualized after 28 days as compared to Gv_1 and $150Gv_1II$. In nonsterilized soil after 56 days, all the antagonist isolates except $75Gv_3I$ were found to exert significantly better protection (43.12-75.60 % reduction in disease incidence) over the soil inoculated with *M. phaseolina* only (89.17% mortality) and mutant isolate $150Gv_1II$ provided comparatively better protection than its wild counterpart Gv_1 (71.01 % reduction in disease incidence).

Table : 4.34 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *M. phaseolina* induced stem rot of jute in sterilized soil.

Isolate	Germination(%)	Percent plant mortality caused by *M. phaseolina*				% Reduction in disease incidence	SEm±	CD(p= 0.05)
		14 DAS	28 DAS	42 DAS	56 DAS			
Gv_1	62.22±19.12	8.12±6.81	15.81±12.58	20.94±3.40	48.51±5.08	48.02	5.50	17.97
$50Gv_1I$	74.45±8.75	0.00	47.00±4.88	51.76±5.45	64.21±5.68	31.20	3.27	10.69
$50Gv_1V$	43.33±12.47	0.00	37.04±2.77	62.96±12.75	70.37±12.08	24.60	6.29	20.54
$75Gv_1VI$	83.33±11.86	0.00	45.56±11.20	57.63±15.20	62.37±8.54	33.17	7.33	23.93
$150Gv_1II$	64.45±3.14	3.33±2.72	24.07±4.29	30.74±13.65	47.78±11.00	48.80	6.12	20.00
Gv_3	43.33±20.55	0.00	22.62±17.58	53.59±5.07	70.24±14.96	24.74	8.36	27.30
$75Gv_3I$	58.83±13.43	4.76±1.40	40.71±13.13	83.33±23.57	83.33±23.57	10.71	12. 68	41.41
Control	60.00±21.60	13.22±2.32	74.08±18.88	88.89±15.71	93.33±9.43		9 .34	30.51
SEm±	10.66	1.95	8.56	9.52	8.90			
CD(p=0.05)	31.95	5.86	25.65	28.54	26.67			

Table 4.35 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *M. phaseolina* induced stem rot of jute in nonsterilized soil.

Isolate	Germination(%)	Percent plant mortality caused by *M. phaseolina*				% Reduction in disease incidence	SEm±	CD (p=0.05)
		14 DAS	28 DAS	42 DAS	56 DAS			
Gv_1	90.78±4.84	0.00	1.75±1.34	4.93±3.89	25.85±7.44	71.01	3.00	9.80
$50Gv_1I$	92.00±6.53	0.00	7.48±4.14	22.62±3.88	50.72±13.65	43.12	5.23	17.07
$50Gv_1V$	71.11±8.31	0.00	15.28±13.75	48.38±8.65	48.38±8.65	45.74	6.50	21.25
$75Gv_1VI$	97.00±2.16	1.67±1.36	6.84±4.84	34.57±16.58	49.66±9.84	44.31	7.04	23.01
$150Gv_1II$	88.33±8.50	2.78±2.08	6.96±1.02	6.96±1.02	21.76±9.66	75.60	3.53	11.54
Gv_3	64.45±16.63	9.52±6.79	25.76±7.59	50.02±7.11	50.02±7.11	43.90	5.06	16.53
$75Gv_3I$	68.69±20.61	2.56±2.04	40.60±6.97	86.54±10.30	89.10±10.46	0.08	5.79	18.91
Control	72.22±22.83	11.11±1.81	63.11±29.50	88.18±11.71	89.17±10.34		11.82	38.62
SEm±	9.47	1.93	8.69	6.49	6.95			
CD(p=0.05)	28.39	5.80	26.06	19.46	20.84			

Fig. 4.25: *In vivo* efficacy of wild and mutant isolates of *G. virens* against stem rot of jute induced by *M. phaseolina* in sterilized soil

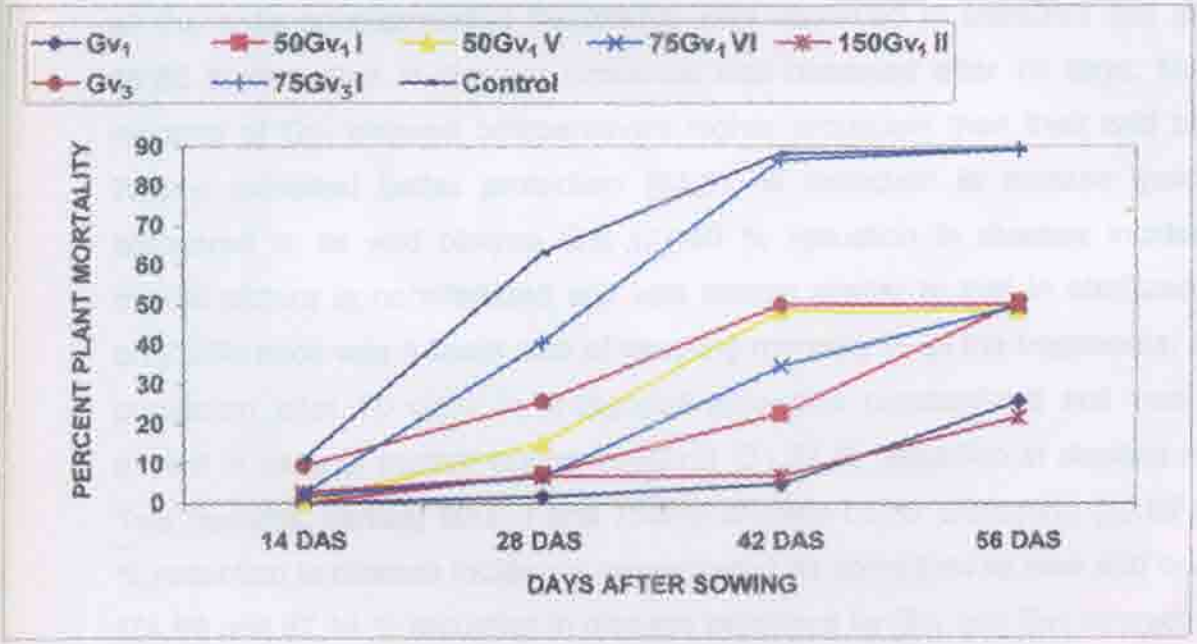


Fig. 4.26: *In vivo* efficacy of wild and mutant isolates of *G.virens* against stem rot of jute induced by *M. phaseolina* in nonsterilized soil

Apparently poor germination of pigeonpea seeds was found in both sterilized and nonsterilized soil, however, the germination was comparatively better in nonsterilized soil (Table 4.36 and 4.37). Most of the antagonist isolates were found partially ineffective in reducing wilt of pigeonpea irrespective of nature of soil (sterilized or nonsterilized) and significant increase in disease incidence was observed after 45 days. Wild isolate Gv_3 only gave significantly higher protection in both sterilized and nonsterilized soil (38.22 and 48.41 % reduction in disease incidence respectively) than in soil without antagonist (93.33 and 70.00% mortality respectively).

Test against root rot of groundnut / *S. rolfsii* revealed that the seedling stand of groundnut was comparatively better in nonsterilized soil (76.67 – 86.67%) than in sterilized soil (36.67–76.67 %). Wild isolate Gv_1 supported comparatively better germination (76.67 %) than untreated control (60.00 %) in sterilized soil (Table 4.38) but none of the isolates were found to be so effective in this respect in nonsterilized soil (Table 4.39). Significantly higher protection throughout the experimental period in all the antagonist-amended treatments was observed in sterilized soil and 58.50-85.60 % reduction in disease incidence was observed after 70 days. None of the mutants of Gv_1 showed comparatively higher protection than their wild biotype but $75Gv_3I$ exhibited better protection (83.21 % reduction in disease incidence) as compared to its wild biotype Gv_3 (77.60 % reduction in disease incidence). The overall picture in nonsterilized soil was almost similar to that in sterilized soil. The only difference was a lower rate of seedling mortality in all the treatments. Significant protection after 70 days in antagonist amended nonsterilized soil was observed except in case of mutant isolate $150Gv_1II$ (31.77 % reduction in disease incidence). Two mutants, namely $50Gv_1I$ and $75Gv_3I$ showed better protection (82.82 and 82.61 % reduction in disease incidence respectively) as compared to their wild counterparts (74.88 and 67.14 % reduction in disease incidence by Gv_1 and Gv_3 respectively).

Table 4.36 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *F. oxysporum* f. sp. *udum* induced wilt of pigeonpea in sterilized soil.

Isolate	Germination(%)	Percent plant mortality caused by *F. oxysporum* f. sp. *udum*.				% Reduction in disease incidence	SEm±	CD (p=0.05)
		15 DAS	45 DAS	75 DAS	105 DAS			
Gv_1	26.67±9.43	7.44±2.04	50.00±8.16	63.33±12.47	75.00±20.41	19.64	8.96	29.29
$50Gv_1I$	16.67±9.43	0.00	61.11±7.86	77.78±15.71	88.89±15.71	4.76	8.34	27.23
$50Gv_1V$	20.00±0.00	10.00±0.00	60.00±14.14	66.67±12.47	66.67±12.47	28.57	7.99	26.11
$75Gv_1VI$	36.67±20.55	8.89±1.57	68.89±1.57	72.22±5.66	88.89±15.71	4.76	5.96	19.46
$150Gv_1II$	36.67±9.43	9.56±3.27	55.56±11.33	55.56±11.33	68.89±24.55	26.18	10.51	34.33
Gv_3	43.33±9.43	0.00	6.67±1.18	43.33±12.47	57.66±3.30	38.22	4.58	14.96
$75Gv_3I$	33.33±4.71	8.33±3.37	61.11±8.31	80.56±14.16	88.89±15.71	4.76	8.12	26.54
Control	30.00±14.14	6.67±1.53	53.33±4.71	70.00±21.60	93.33±9.43		8.52	27.82
SEm±	7.91	1.40	5.87	9.85	11.26			
CD(p=0.05)	23.70	4.18	17.61	29.43	33.65			

Table 4.37 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *F. oxysporum* f. sp. *udum* induced wilt of pigeonpea in nonsterilized soil.

Isolate	Germination(%)	Percent mortality caused by *F. oxysporum* f. sp. *udum.*				% Reduction in disease incidence	SEm±	CD (p=0.05)
		15 DAS	45 DAS	75 DAS	105 DAS			
Gv_1	26.67±4.71	3.33±0.62	38.89±7.83	43.33±5.98	61.11±8.31	12.70	1.43	4.69
$50Gv_1I$	40.00±8.16	6.67±1.30	13.33±5.96	13.33±5.96	46.11±8.94	34.13	4.37	14.28
$50Gv_1V$	63.33±4.71	0.00	24.60±5.73	27.94±8.48	43.33±9.43	38.10	4.92	16.08
$75Gv_1VI$	40.00±8.16	15.00±4.08	33.89±13.63	33.89±13.63	63.33±10.27	9.53	7.85	25.66
$150Gv_1II$	33.33±12.47	6.67±0.85	36.11±10.39	36.11±10.39	61.11±7.86	12.70	5.90	19.28
Gv_3	30.00±8.16	11.11±2.57	27.78±5.66	27.78±5.66	36.11±10.39	48.41	4.73	15.45
$75Gv_3I$	36.67±12.47	15.00±0.41	36.67±15.13	43.33±9.43	68.33±13.12	2.39	7.83	25.58
Control	53.33±12.47	11.43±2.02	29.52±4.14	29.52±4.14	70.00±21.60		7.95	25.96
SEm±	6.67	1.37	6.62	5.99	8.49			
CD(p=0.05)	19.99	4.12	19.86	17.97	25.44			

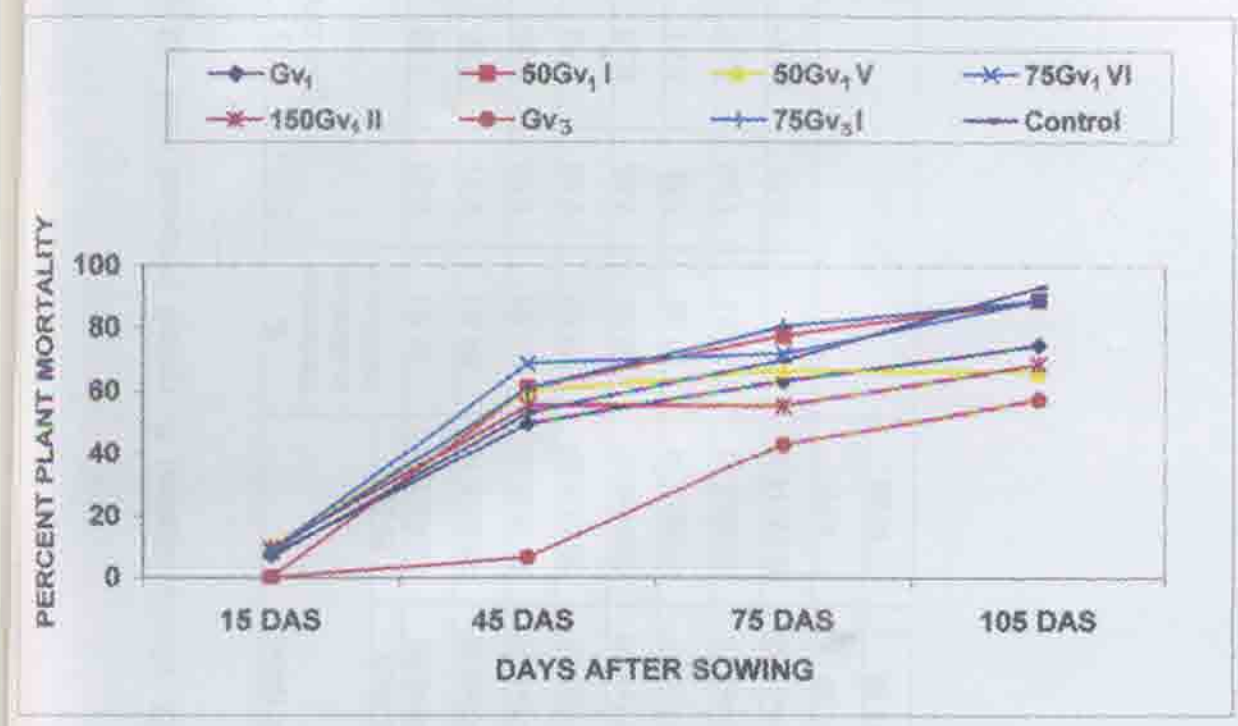


Fig. 4.27: *In vivo* efficacy of wild and mutant isolates of *G. virens* against wilt of pigeonpea induced by *F. oxysporum* f.sp. *udum* in sterilized soil

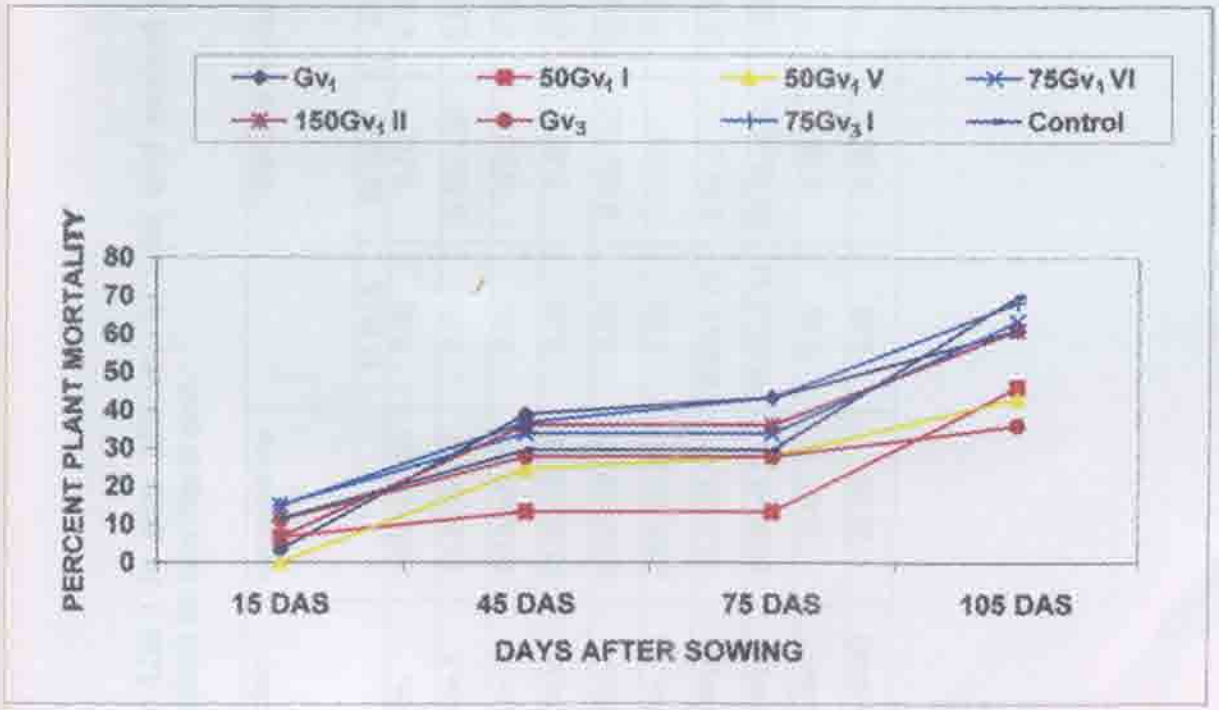


Fig. 4.28: *In vivo* efficacy of wild and mutant isolates of *G. virens* against wilt of pigeonpea induced by *F. oxysporum* f.sp. *udum* in nonsterilized soil

Table 4.38 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *S. rolfsii* induced root rot of groundnut in sterilized soil.

Isolate	Germination (%)	Percent mortality caused by *S. rolfsii*					% Reduction in disease incidence	SEm±	CD (*p*=0.05)
		14 DAS	28 DAS	42 DAS	56 DAS	70 DAS			
Gv_1	76.67±17.00	0.00	0.00	4.76±0.55	4.76±0.55	11.43±1.82	85.60	0.63	1.98
$50Gv_1I$	56.67±4.71	0.00	5.56±1.57	5.56±1.57	12.22±5.15	12.22±5.15	84.60	2.41	7.60
$50Gv_1V$	60.00±8.16	0.00	0.00	4.76±3.50	21.75±5.02	21.75±5.02	72.60	2.50	7.89
$75Gv_1VI$	53.33±18.86	0.00	0.00	4.17±3.12	4.17±3.12	20.83±5.89	73.76	2.33	7.34
$150Gv_1II$	56.67±12.47	0.00	8.22±1.50	13.09±2.21	23.41±4.99	32.94±6.20	58.50	2.65	8.36
Gv_3	36.67±9.43	0.00	0.00	0.00	6.67±1.36	17.78±3.14	77.60	1.08	3.41
$75Gv_3I$	43.33±9.43	6.67±2.72	6.67±2.72	13.33±2.72	13.33±2.72	13.33±2.72	83.21	1.92	6.07
Control	60.00±8.16	16.99±2.34	33.18±5.45	61.59±20.28	67.14±12.62	79.37±14.72		9.07	28.61
SEm±	8.42	0.90	1.62	5.29	4.00	4.77			
CD(p=0.05)	25.23	2.69	4.85	15.86	11.98	14.29			

Table 4.39 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against *S. rolfsii* induced root rot of groundnut in nonsterilized soil.

Isolate	Germination (%)	Percent mortality caused by *S. rolfsii*					% Reduction in disease incidence	SEm±	CD (p=0.05)
		14 DAS	28 DAS	42 DAS	56 DAS	70 DAS			
Gv_1	83.33±12.47	3.33±2.72	3.33±2.72	3.33±2.72	10.83±8.25	10.83±8.25	74.88	3.98	12.55
$50Gv_1I$	83.33±9.43	0.00	0.00	3.70±2.62	7.41±2.62	7.41±2.62	82.82	1.44	4.53
$50Gv_1V$	80.00±8.16	0.00	8.33±6.24	8.33±6.24	13.09±10.24	13.09±10.24	69.64	5.36	16.90
$75Gv_1VI$	86.67±12.47	0.00	7.04±5.00	11.80±1.82	11.80±1.82	11.80±1.82	72.63	1.87	5.89
$150Gv_1II$	76.67±18.86	0.00	3.70±2.62	10.37±3.66	24.66±13.37	29.42±19.94	31.77	7.72	24.35
Gv_3	83.33±12.47	6.67±2.72	6.67±2.72	10.83±6.49	10.83±6.49	14.17±11.20	67.14	4.74	14.94
$75Gv_3I$	80.00±16.33	4.17±1.31	4.17±1.31	4.17±1.31	7.50±1.78	7.50±1.78	82.61	1.07	3.39
Control	86.67±12.47	7.00±4.97	11.80±1.82	18.84±3.34	27.95±1.98	43.12±10.41		3.89	12.27
SEm±	9.36	1.61	2.38	2.79	5.07	7.15			
CD (p=0.05)	28.05	4.81	7.13	8.38	15.20	21.43			

Plate 12 *In vivo* efficacy of Gv_3 and $75Gv_3I$ for management of *F. oxysporum* f.sp.*udum* induced wilt of pigeonpea in nonsterilized soil

Plate 13 Sclerotia formation by *S. rolfsii* on the infected roots of groundnut in nonsterilized soil

Plate 14 *In vivo* efficacy of Gv_1 and $50Gv_1I$ for management of *S. rolfsii* induced root rot of groundnut in nonsterilized soil

PLATE - 12

PLATE - 13

PLATE - 14

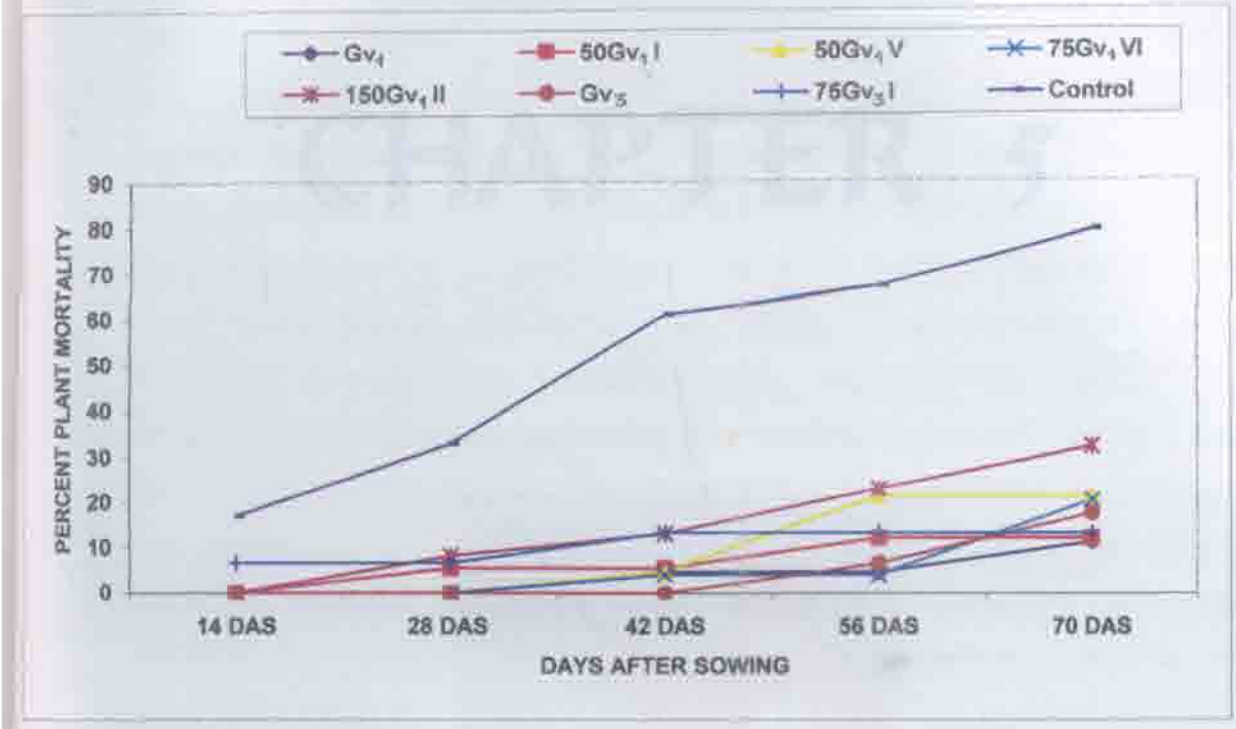


Fig. 4.29: *In vivo* efficacy of wild and mutant isolates of *G. virens* against root rot of groundnut induced by *S. rolfsii* in sterilized soil

Fig. 4.30 : *In vivo* efficacy of wild and mutant isolates of *G. virens* against root rot of groundnut induced by *S. rolfsii* in nonsterilized soil

CHAPTER 5

Discussion

Gliocladium virens has been identified as one of the most promising and potential biocontrol agent for several plant pathogenic fungi (Miller *et al.*, 1907). However, due to its great resemblance with *T. viride,* unexpected disparities still exist among the scientists regarding the nomenclature of this antagonist. In addition, two faced nomenclature as *Trichoderma* (=*Gliocladium*) *virens* without adequate evidences (Rehner and Samuels, 1994; Highley, 1997; Baek *et al.*,1999; Singh *et al.*, 2001;Howell, 2002) not only creates a confusion but also opens up a new direction for its accurate and detailed taxonomic position. Employing Random amplified polymorphic DNA (RAPD) and combination of UP PCR based approach like ribotyping, scientists have tried to overcome such diallema and confusing situation (Bulat *et al.*, 1988; Lexova *et al.*, 1998). But further confirmation is necessary for its acceptable taxonomic position in the genus *Trichoderma.* In the present dissertation the author has preferred to use the nomenclature of *G. virens* instead of *T. virens* for the fungus used with an amendable mind as per confirmation of its identity in future.

Three isolates of *G. virens* and one each of *G. roseum* and *G. penicillioides* were isolated from soils of different ecological niches containing well decomposed organic matter that proves the ecological preference of these bioagents (Papavizas, 1985). Such isolates were used in the present investigation. Although the physical and chemical properties of the soils from which they were obtained were not tested but such abiotic factors have been suggested to be the determinant factors of the heterogeneity of species aggregates in soil (Danielson and Davey, 1973a,b; Davet, 1979a).

Although the results of *in vitro* studies reflecting the antagonistic potential of the microorganisms are not always related to the degree of antagonism observed in the field yet such studies are important for screening the antagonists effective against soil borne pathogens (Bell *et al.*, 1982). Among the antagonist isolates *G. penicillioides,* however, exhibited satisfactory antagonistic potential against four test pathogens but its erratic growth habit and behavior on culture plates compelled to set it aside from further studies, whereas Gv_1 and Gv_3 were selected for their good antagonistic potential against at least two of the test pathogens. Gv_1 and Gv_3 completely overgrew and antagonized *R. solani, M. phaseolina, F. oxysporum* f.sp. *udum* within 5-6 days and *S. rolfsii* within 7-10 days depending upon the time

required for the contact between antagonist and pathogen colony. Mukherjee and Tripathi (2000) have observed that for complete overgrowth *G. virens* took 4,10 and 12 days for *R. solani* , *S. rolfsii* and *F. oxysporum* f.sp. *phaseoli* respectively. Considerably higher antagonistic potential of the native isolates of *G. virens* against *F. oxysporum* f.sp. *udum*, *R.solani*, *M. phaseolina* have been reported recently (Pan *et al.*,2001). Large number of evidences corroborated to a great extent with the present result. Higher growth rate and greater competitive ability of the selected isolates are the indicatives of their better antagonistic potential.

The isolates of *G. virens* were observed to readily interact with *R. solani* and *M. phaseolina*. Usually the mycoparasite *G. virens* grow towards host fungi, run parallel and coils around the host hyphae. Production of knob like haustorial structures with infection peg followed by penetration of host hyphae and finally coagulation, vacuolation and lysis of host protoplasm have been reported to be the principal mechanism in the mycoparasitism of *R. solani* by *G. virens* (Tu and Vartaja, 1981; Pant and Mukhopadhyay, 2001). The directed growth of *G. virens* towards *R. solani* and *M. phaseolina* indicates a positive chemotropism of the parasite towards its host (Chet, 1987). Mycoparasitism including both hyphal interaction and sclerotial parasitization as the most vital mechanisms of antagonism have been favored by many scientists (Elad *et al.*, 1981; Howell, 1982; Prasad and Rao, 1990; Dubey, 1998). Antibiosis mediated by specific or nonspecific metabolites of *G. virens* as the principal mechanism in biocontrol of cotton seedlings induced by *R. solani* has been reported (Howell, 1993). No direct hyperparasitism was observed between *Gliocladium* spp. and *S. rolfsii* or *F. oxysporum* f.sp. *udum*; however these pathogens could not be re-isolated from dual culture. After contact coagulation of protoplasm followed by shrinkage of pathogen hyphae leading to lysis reveals the capability of the antagonists to kill these fungi efficiently. Formation of multihyphal strands by *S. rolfsii* at the interaction site corroborated the earlier observation (Hadar *et al.*, 1981). Elad and Zimand (1991) and subsequently Singh (1994) and Tewari(1996) have observed that antagonists suppressed hyphal growth of the fungus. *Trichoderma* spp. antagonistic to a range of fungi has been reported to produce volatile and nonvolatile antibiotics (Roy, 1977; Dennis and Webster, 1971; Mukhopadhyay and Kaur, 1990; Upadhyay and Mukhopadhyay, 1983). Mukhopadhyay (1996) has claimed that culture filtrate of *Trichoderma* spp. produces volatile and nonvolatile antibiotics effective against *S. rolfsii* and *R. solani*. Volatile metabolites identified as

alkyl pyrones have been reported from *T. harzianum* and these compounds suppressed *R. solani* inducing damping off of lettuce (Fravel, 1988). In the present instance inhibition of mycelial growth and sclerotia formation of *R. solani* and *S. rolfsii* was noted. This provided strong evidences for the involvement of nonvolatile antibiotics in the biocontrol *R. solani* and *S. rolfsii* (Plate 15 and 16). Gliovirin and Gliotoxin are the principal substances released as nonvolatile compounds by *G. virens* and these two antibiotic substances caused inhibition of *F. oxysporum* f.sp. *phaseoli in vitro* (Mishra, 1996).

The varied antagonistic potential of different strains of the biocontrol agent has been ascribed to be due to their great genetic variabilities (Lumsden and Locke, 1989; Smith *et al.*, 1990; Harman, 1991). Multinucleate cells of *Gliocladium* are heterokaryotic while conidia are uninucleate (since conidia receive a single nucleus from phialide) and therefore homokaryotic. Consequently progeny derived from single nucleus may represent stable, improved biocontrol types. As the genetic material of nuclei is DNA, it is supposed that, any genetic information regarding biocontrol potentiality is carried in chromosome of at least two polydeoxynucleotide strands of one DNA molecule. One hundred nine alleles at sixteen putative loci have been identified using isozyme electrophoresis of seventy-one strains of *Trichoderma* and *Gliocladium* (Stasz *et al.*, 1989). Therefore, any single or multipoint alteration of mutational "hot spot" loci through the action of mutagens (physical and / chemical) followed by isolation of single spore led to production of different kinds of mutated base pair(s) which may have the possibility to obtain improved biocontrol strains (Harman and Hayes, 1993).

In the present investigation the selected wild isolates of *G. virens* were irradiated to five increasing doses of non particulate electromagnetic γ- radiation and subsequently twenty five mutant isolates were picked up from hundreds of randomly selected phenotypic mutants on the basis of their variation of phenotypic characters. Viability of irradiated isolates was found to reduce with increasing doses of γ-radiation. Prolonged exposure to γ- radiation have been reported detrimental to growth and aflatoxin production of *Aspergillus flavus* (Srinivas *et al.*, 1996). A direct correlation between dose of γ- radiation and mortality of conidia of *G. virens* has been explained and 125 Krad was found to be most mutagenic as compared to 50 and 75 Krad (Mukherjee and Mukhopadhyay, 1993).

Plate 15 Inhibition of sclerotia formation of *R. solani* by nonvolatile antibiotic of mutant isolates of *G. virens*

Plate 16 Inhibition of mycelial growth of *S. rolfsii* by nonvolatile antibiotic of wild and mutant isolates of *G. virens*

PLATE - 15

PLATE - 16

Among the twenty five selected mutants of *G. virens* (Gv_1 and Gv_3), four isolates produced albino colonies instead of normal green as exhibited by their wild *counterparts* and therefore have the possibility to act as phenotypic marker which seems to be a determinant factor for screening of the mutated spores of *G. virens*. Mukherjee and Mukhopadhyay (1993). Subsequently Kumar and Gupta (1999) have developed albino mutants of *G.virens* and *T. viride* through γ- radiation.

The principal approach for obtaining mutant isolates of *G. virens* with enhanced biocontrol potential was primarily justified by the evaluation of the antagonistic potential which showed that mutant isolates like their wild biotypes also varied in the efficacy of the antagonism ranging from comparatively better to even loss in potentiality particularly against *S. rolfsii*. This might be due to random alteration of DNA base pair(s) which regulate the biocontrol potentiality of these antagonists.

Among the six finally selected mutant isolates of *G. virens* (Gv_1 and Gv_3), showing enhanced biocontrol potential, five were stable with respect to their original phenotypic characters and antagonistic potentiality. One mutant isolate, $100Gv_3I$, was found unstable and could not be grown on PDA medium after 2nd generation. It was suspected that possible recessive lethal allele(s) segregate(s) after 2nd generation and expressed phenotypically due to achieving homozygous recessive state and thus rendering the conversion of the prototrophic mutant into auxotrophic one (Fincham and Day, 1971).

The simple and convenient use of γ- radiation for the production of improved biocontrol strains has been first demonstrated by Troutman and Matejka (1978), however, they didn't report the antagonistic potential of mutated *T. viride* isolates. Mukherjee and Mukhopadhyay (1993) have successfully developed seven stable mutant isolates of *G. virens*, which differed from the wild type and from each other in their ability to antagonize the test pathogens in dual culture. Roy and Pan (1998) and Dutta (1998) have also reported increased inhibitory properties of some γ- irradiated mutants of *G. virens*.

Scanning electron microscopy revealed that there was a distinctive variation among the wild and mutant isolates of *G. virens* with respect to conidial size and degree of echinulation on conidia. No direct evidences however were available but such approach might help in identifying the differences between mutant and wild isolates. Scanning electron microscopy on the basis of degree of echinulation on

spore and mucilage sheath has been suggested to be the simplest way in distinguishing *Trichoderma* and *Gliocladium* spp. (Hashioka, 1973; Meyer and Plaskowitz, 1989; Chowdhuri and Sen,1999).

Excretion of hydrolytic enzymes is one of the most essential character of fungal biocontrol agent to act as mycoparasite of fungal plant pathogens. Hydrolytic enzymes such as glucanase, chitinase, cellulase, xylanase, acid and alkaline phosphatase, esterase, lipase, leucinearylaminidase, α- and β galactosidase, α- and β glucosidase, N-acetyl glucosaminidase and protease are known to be produced by *Trichoderma* upon induction (Aziz *et al.*, 1993). Some of these enzymes are implicated in the degradation of fungal cell wall and hence the lysis of target plant pathogens (Elad *et al.*, 1982). In the present investigation a wide variation in β-1,3 glucanase, β-1,4 glucanase, chitinase and cellulase production by the wild and mutant isolates of *G. virens* was observed when grown under both inducible and non-inducible condition. However, all the enzymes were produced irrespective of combination of the carbon sources used reinforces the earlier observation made by Gomes *et al.* (1992). Mutant isolate 150Gv$_1$II had shown significantly higher amount of production of these enzymes while others differed considerably irrespective of the composition of the media. The nature of growth substances greatly influence the production of different enzymes (Elad *et al.*, 1982; Senior and Saddler, 1990; Ulhoa and Peberdy, 1993; Witkowska *et al.*, 1999; Noronha *et al.*, 2000). Fairly low quantity of the enzymes was detected when the test isolates (except in case of chitinase production by mutant isolate 150Gv$_1$II) were grown in CDB and CDB supplemented with chitin. Tronsmo and Harman (1992) have claimed that *T. harzianum* showed low enzyme activity in simple mineral salt solutions plus single carbon sources including chitin while addition of any of several complex materials substantially increased enzyme yield. Several fold increases in enzyme production by the isolates when grown in CDB supplemented with mycelial powder of *M. phaseolina* and *P. ultimum* indicated the greater capability of the antagonists for enzyme production under inducible condition. The cell wall of *M. phaseolina* is known to have β-1,3 glucan and chitin that should have resulted in the induction of β-1,3 glucanase and chitinase production in mycelial mat amended media (Kumar and Gupta, 1999). *P. ultimum* on the other hand contains β-1,4 endoglucan and cellulose in their cell wall. The antagonist isolates recognized these components, hydrolyzed them and utilized as substrates for the production of β-1,4 glucanase and cellulase.High enzyme activity in

susceptible pathogen – mycoparasite co-culture has been demonstrated (Baker and Dickman,1993).

Genetic modification for the development of improved strains producing higher quantity of lytic enzymes has been tried successfully (Mandels *et al.*,1971; Witskowska and Bien, 1991). Earlier evidences from industrial microbiology have shown the possibility to improve the yield and quality of enzymes synthesized by *Trichoderma* spp. by genetic manipulation (Montenecourt and Eveleigh, 1977,1979). Variation in lytic enzyme production among the single spore isolates of *T. koningii* has been reported (Worasatit *et al.*, 1994) which in turn certainly explain the variable degree of substrate utilization by the wild biotypes and their mutant isolates and genetic diversity among them as well.

At present we are well acquainted with the unlimited evidences of *in vitro* antagonism of the species of *Trichoderma* and *Gliocladium*. But information about the survival ability of free propagules of both *Trichoderma* and *Gliocladium* spp., in general, and *Gliocladium* spp. in particular are deficient. The abundance of these bioagents in various soil coupled with their ability to degrade various organic substances in soil, the metabolic versatility and their resistance to microbial inhibitors suggest that they may possess the ability to survive in various ecological niches depending upon the prevailing conditions of the habitat (Papavizas, 1985). Adaptability of genetically modified strain(s) to the diverse ecosystem is the predeterminant factor for its success or failure when such strain(s) are introduced into soil. Papavizas *et al.* (1982) have shown that in addition to appearance and growth habit the UV-induced biotypes of *T. harzianum* differed considerably from wild strain with respect to survival ability in soil, fungitoxic metabolite(s) production and ability to suppress various soil borne diseases. This may be due to random alteration(s) of other non-targeted trait(s) of the organism (Kumar and Gupta, 1999) which imposed extra metabolic load in the mutants towards a single or a few desirable characters (Cook, 1993). Perhaps due to this reason, a mutant isolate showing good antagonistic potential *in vitro*, perform differently when examine under *in vivo* condition.

A large number of biotic and abiotic factors obviously interact to determine the germinabilty of spores in nature (Martin and Nicolas, 1970). So far considerable information are available on the general aspects of conidial germination but little is known about both biotic and abiotic barriers that interact in a complex manner

particularly on chlamydospores during germination (Papavizas, 1985). In the present investigation inconsistent and erratic germination of phialo- and chlamydospores in different sources of water was ascribed to the diversified biochemical and physical properties particularly nutrient and pH status of the water sources. The variation in the germination among the wild and mutant isolates might be due to carbon and nitrogen utilization pattern in addition to adaptability to adverse pH status of the substrates. Nutrients as one of the most important factor for germination have been suggested by many scientists (Danielson and Davey, 1973c,d; Bonlea and Sesan, 1980; Saha and Pan, 1998a; Monga, 2001). A range of nutritional compounds stimulating the germination of *Trichoderma* spp. have been shown (Nelson *et al.*, 1988; Jackson *et al.*, 1991). Significantly higher germination of both spore forms in soil leachets reinforces the fact that various organic and inorganic substances favors the germination of the test isolates. However, Danielson and Davey (1973c) have conclusively suggested that germination of conidia were nutrient dependent while that of chlamydospores were relatively independent. Furthermore, temperature, pH, CO_2 level of the atmosphere and water potential have been identified to profoundly influence the germination of spores of *G. virens* (Danielson and Davey, 1973d; Kang *et al.*, 1989; Sreenivasaprasad and Manibhusanrao,1990; Jackson *et al.*,1991). Poor germination in tap water might be due to combined effect of alkaline pH (7.9) and presence of inhibitory metal ions (Danielson and Davey, 1973d). Although Martin and Nicolas (1970) and subsequently Jackson *et al.* (1991) have demonstrated lack of germination to very poor conidial germination of *T. lignorum* and *G. virens* in distilled water, fairly high percentage of phialospore germination in sterilized distilled water (except 3.69 % germination of $75Gv_3l$) explained that unwashed conidia contribute enough nutrients to initiate and support germination (Danielson and Davey, 1973c). Apparently low germination of phialospores and chlamydospores in particular conclusively suggested that (i) depending upon the situation, 18-36 hrs are needed for swelling of spores prior to germination and to overcome the barrier imposed by endogenous dormancy (Martin and Nicolas, 1970) (ii) appropriate condition is essential for germination of chlamydospores which otherwise remain dormant (Papavizas, 1985) and (iii) phialospores are physiologically ready for germination as compared to chlamydospores (Saha and Pan, 1998a).

The fungistatic effect being a combination of non-specific barriers hinders the germination of fungal propagules in soil. Although this phenomenon is not in general

desirable for proliferation of an antagonist isolate in soil. Information available on fungistatic effect of soil on different spore forms of *Gliocladium* is very meager and that on mutant isolates is totally absent. Several pertinent factors stimulating the germination of spores are known to operate in overcoming such static barriers. Poor phialospore germination in nonsterilized soil has been attributed to the presence of fungistatic effect (Lockwood, 1977; Kim *et al.*, 1992). Inhibitory volatile and nonvolatile substances (Schippers *et al.*,1982) and activity of different microbial population may also modulate the fungistasis under natural condition. Several fold increase in germination in sterilized soil and FYM on the other hand indicated that such barriers particularly the biotic barriers were partially eliminated when these substrates were sterilized. Partial annulment of fungistatic effect can also be brought about by addition of external sources of nutrients to the soil (Linderman and Gilbert, 1973; Lockwood, 1977) or root exudates of host plants stimulatory to the germination of antagonist propagules (Sullia, 1973). Greater germination of chlamydospores irrespective of type of soil and FYM suggested that chlamydospores are less sensitive to fungistasis (Lockwood, 1977). However, failure to overcome the fungistatic barrier by chlamydospores of mutant isolates $75Gv_1VI$ and $75Gv_3I$ in nonsterilized soil might be considered as an aberration of the general principal and a deviation from the normal behavior. Farmyard manure as a rich source of organic nutrients supported better germination however substrate quality and activity of numerous saprophytic microorganisms may inhibit the germination by elevating the fungistatic effect (Hoitink and Boehm, 1999).

Use of live baits (sclerotia) and their penetration / colonization by antagonist reflect much upon their parasitic ability rather than saprophytic attributes in the intensely competitive microbiotic environment in soil. *R. solani* and *S. rolfsii* are well known to produce sclerotia, the principal structure adapted for surviving adverse environmental condition. The basic structural difference between the sclerotia of these two pathogens has been clearly explained (Parmeter and Whitney, 1970; Talbot, 1973). Both the sclerotia possess melanin in their rind (Chet, 1969) that confers resistance to microbial attack. However, the extent of colonization varies since sclerotia of *R. solani* are formed of loosely woven hyphae rather than compact hyphae as in case of *S. rolfsii*. *Trichoderma* spp and other fungi were shown to attack sclerotia of various pathogensin soil (Coley-Smith and Cooke, 1971; Henis and Papavizas, 1983). Baker and Cook (1974) have suggested that the hyperparasites

are most effective against survival structures of pathogens because these are generally less mobile and do not multiply rapidly. In the present investigation greater colonization of sclerotia of *R. solani* by the isolates of *G. virens* corroborated the fact that sclerotia of *R. solani* are more susceptible than *S. rolfsii* to colonization by the antagonist. Dubey and Patel (2001) have observed that approximately cent percent moniloid cells of *R. solani* were colonized by *G. virens* after 14 days of incubation. Colonization of sclerotia of *R. solani* by *Trichoderma* spp. has also been documented (Roy, 1977; Rosales and Mew, 1982). As to how the antagonists, *Trichoderma* and *Gliocladium* availed the access inside sclerotia have been amply demonstrated (Elad *et al.*, 1983; Papavizas and Collins, 1990). There is some doubt about the ability of *Trichoderma* spp. to parasitize healthy sclerotia , but more evidence exists that it attacks those that are injured some how (Coley- Smith and Cooke, 1971). Smith (1972) has demonstrated that dried sclerotia rotted within 2-3 weeks in moist soil because they leaked nutrients (amino acids and sugars) which provided sufficient colonization potential permitting *Trichoderma* and *Gliocladium* to overcome sclerotial barrier. Increased activity of soil microorganisms near dried sclerotia of *S. rolfsii* has been reported (Gilbert and Linderman, 1971). In the present investigation the test isolates varied in their ability to colonize the sclerotia of *R. solani* and *S. rolfsii*. Henis *et al.* (1983) have shown that strains of *Trichoderma* spp. varied in their ability to colonize the sclerotia of *S. rolfsii*. The possible role of lectin in specific recognition between *Trichoderma* spp. and *S. rolfsii* has been discussed (Barak *et al.*, 1985). The recovery of viable sclerotia was declined with increased dose of antagonist application and whatever the sclerotia could be recovered from soil yielded only *G. virens* in germination tests. The results are in agreement with the earlier observation of Papavizas and Lewis (1989) and Sarmah and Mukhopadhyay (1999). Greater colonization of sclerotia by isolates of *G. virens* in nonsterilized soil suggested that existing soil fauna actively participate in the destruction of persistent survival propagules by combined perforation and lysis (Old, 1978). Phialospores of the test isolates were found to colonize the sclerotia more vigorously than the chlamydospores which indicated that the same factors involve in quick germination of phialospores may also be responsible for greater colonization of sclerotia of *R. solani* and *S. rolfsii*.

The changing ecological conditions greatly influence the survival, growth and population proliferation of an antagonist in soil and in the rhizosphere (Baker and

Cook, 1974). For maximum utilization of the antagonistic potential of an organism, it is necessary that the environment to which a prospective antagonist is added should be studied carefully (Bhatnagar, 1996). In the present investigation a rapid increase of antagonist population in sterilized rhozosphere soil was observed up to first 14 days which was subsequently followed by an inconsistent fluctuation during later part of the study. Similar trend in population proliferation was evident in nonsterilized soil although the rate of population increase was slower and the cfu/g of rhizosphere soil was much lower than that in sterilized soil. The initial increase in population might be due to germination of different spore forms and their subsequent proliferation with or without food base (Saha and Pan, 1998b). Poor population density in nonsterilized rhizospher soil could be attributed to widespread soil fungistasis (Linderman and Gilbert, 1973; Lockwood, 1977). Any kind of soil treatment either physical or chemical prior to antagonist application create a partial vacuum in soil that favors indigenous or introduced antagonist to rapidly recolonize and proliferate in the rhizosphere (Baker, 1981). The population density of *T. viride* (T-1-R_4) and *T. harzianum* (WT-6-24) have been reported to increase about 10^4 and 10^3 fold respectively in natural soil during first 3 weeks when introduced as mycelium inoculum in wheat bran- sand culture but conidial inoculum with or without wheat bran didn't behave so (Lewis and Papavizas, 1984a). However, Papavizas (1981) has previously shown that introduced *T. harzianum* failed to establish in the rhizosphere of bean and pea seedlings and resulted in subsequent reduction in population. It has been suggested that population densities in soil was governed by the factors like soil moisture, depth of sampling and total bacteria in soil (Wu, 1986). Remarkable increase of antagonist population in rhizosphere soil during initial stages could also be attributed to the abundance of sugars and amino acids exuded from roots that stimulated germination and proliferation (Odunfa, 1975) while the subsequent decrease could be ascribed to a reduction of or change in composition of root exudateswith the plant age. (Rovira, 1956; Odunfa and Oso, 1979). The population densities of the antagonists isolates were found to increase particularly in sterilized soil after 42 days. Odunfa and Oso (1979) have suggested that with the maturity of plants the normal process of breakdown of the cortex, the epidermal cells and the root hairs was started and these decomposing cells could provide suitable carbon and nitrogen sources for the growth of fungi. In spite of such elaborate discussion the aberrant increase in rhizosphere

population of mutant isolate $50Gv_1I$ in sterilized soil (14 DAS) was assumed to be due to faulty collection of rhizosphere soil sample.

The possibility of controlling pathogenic fungi with antagonistic microorganisms, introduced as a substitute for or in combination with low (sub-lethal) doses of fungicides, has long been considered beneficial (Chet, 1990). Integration of biocontrol agents with fungicides has been reported to give significantly higher disease control in several crops (sugarbeet, tobacco, tomato, potato, lentil, chickpea, etc.) than that obtained by the biocontrol agents or fungicide alone (Mukhopadhyay *et al.*, 1986; Upadhyay and Mukhopadhyay, 1986). Mukhopadhyay *et al.* (1992) have suggested that use of sub-lethal dose of chemical weakens the pathogens and the antagonist get more access to parasitize them. In the present investigation selected wild and mutant isolates of *G. virens* showed high sensitivity to Bavistin and Benomyl, moderately to Iprodione and Captan but Thiram and particularly Mancozeb appeared less sensitive. The results were in agreement with the earlier observations (Kuthubutheen and Pugh, 1978; Abd-El Moity *et al.*, 1982; Papavizas, 1985). Benzimidazole group of fungicides (Benomyl and Bavistin) have been found toxic to *T. harzianum* and *G. virens* (Viji *et al.*, 1997). Benomyl even at a concentration of 0.5 ppm was strongly inhibitory to *Trichoderma* spp. (Mirkova, 1982; Papavizas *et al.*, 1982). Pant and Mukhopadhyay (2001) have found that Thiram at 25 ppm inhibited the growth of both *T. harzianum* and *G. virens* while Bavistin was found inhibitory to both the antagonists and more than 90% inhibition was observed at 1 ppm concentration. Mancozeb at concentration of 100 ppm have been reported non inhibitory to *G. virens* (Mukherjee and Tripathi, 2000). Feasibility of induced mutation to produce new biotypes resistant to commonly used fungicides has been proved repeatedly a more promising approach to bio control since the initial work made by Troutman and Matejka (1978). In this study mutant isolate $75Gv_1VI$ showed comparatively higher level of tolerance to Benomyl, Thiram and Mancozeb while another mutant isolate $150Gv_1II$ was eclusively tolerant to to Mancozeb than their wild biotype (Gv_1). Mutants of *T. viride* and *T. harzianum* tolerant to high concentration of Benomyl have been reported (Papavizas, 1980; Papavizas and Lewis, 1981a; Papavizas and Lewis, 1983). Exposure of *G. virens* to UV-rays or γ-radiation failed to generate biotypes resistant to benomyl (Papavizas, 1987; Mukherjee and Mukhopadhyay, 1993) however, Papavizas *et al.* (1990) have successfully developed stable mutants of *G. virens* tolerant to 10 ppm Benomyl.

None of the test mutant isolates was more tolerant to Iprodione than the wild type (Gv_1) corroborated the earlier observation made by Mohamed and Fahmy (1990).

Laboratory or green house screening for *Trichoderma* and *Gliocladium* can not predict their performance in field (Kommedahl and Windels, 1978) but such studies are important for maximum utilization of the antagonistic potential of an organism. It is necessary that the environment to which a prospective antagonist is added should be studied carefully and modified accordingly to assist establishment and proliferation of antagonist in soil or plant rhizosphere (Bhatnagar, 1996). Physical factors like soil moisture and pH influence the activity of biocontrol agents; unfavorable temperature and changes in C/N ratio may even be more important limiting factors (Eastburn and Butler, 1988; Saha and Pan, 1998b).

The formulation of fungal antagonists is critical for the effective implementation of biocontrol of various crop diseases. Direct application of antagonists continues to be the principal method for introducing into soil for biological control of soil borne plant pathogens which can initiate seed rot, seedling damping off and root rots (Prasad and Rangeshwaran, 1999). In the present investigation rice bran based mass culture of wild and mutant isolates of *G. virens* were used to test their efficacy against four important soil borne diseases under green house condition. Plant growth promoting potential of *Trichoderma* and *Gliocladium* has been reported (Chang *et al.*, 1986; Windham *et al.*,1986; Pant and Mukhopadhyay, 2001; Mukherjee *et al.*, 2001). Comparatively lower germination of seeds in sterilized soil in the present experiment might be due adverse residual effect of formaldehyde and absence of plant growth promoting rhizobacteria (Nene and Thapliyal, 1993; Scroth and Hancock, 1982).

Green house trial against seedling blight of green gram caused by *R. solani* showed that with exception for Gv_1, $50Gv_1V$, Gv_3 and $75Gv_3I$, the rest of the isolates were significantly effective in reducing the disease in sterilized soil. In nonsterilized soil the situation was entirely different where all antagonist isolates significantly reduce the seedling mortality and a maximum of 52 % disease reduction over control was achieved by mutant isolate $150Gv_1II$. The potentiality of *G. virens* to control *R. solani* has been repeatedly mentioned (Lumsden *et al.*, 1995; Lewis and Larkin, 1997; Abraham and Gupta, 1998; Prasad and Rangeshwaran, 1999; Dubey, 2000; Sharma and Tripathi, 2001) provided the antagonist should be applied in soil with an effective food base so that it could grow better and had better access in the

rhizosphere competence by producing higher inoculum densities (Lewis and Papavizas, 1984a). Once established, being better rhizosphere competitors by virtue of their faster growth rate and potential antagonism these suppressed pathogens. In the present study a positive correlation between higher competitive parasitic survival and biocontrol potential of mutant isolates $50Gv_1I$, $75Gv_1VI$ and $150Gv_1II$ could be established in nonsterilized soil even at a very low level of inoculum. Comparatively lower rate of disease reduction in sterilized soil might be due to absence of other antagonistic microflora and better saprophytic ability of the pathogen which led to a rapid colonization of soil by the pathogenic organism. During investigation it was observed that *R. solani* rapidly formed new sclerotia on the surface of sterilized soil in all treatments including control within 10 days but was rarely found in nonsterilized soil. The reduction of this saprophytic growth might be due to increase in antagonistic population during later part of the investigation. The result reinforces the suggestion that the antagonistic potential of the biocontrol agent is more important than the population dynamics in soil. The cell walls of *R. solani* are composed mostly of glucans with only about 6-8 % chitin (Bartnicki – Garcia, 1973). It seems therefore that β-1,3 glucanase activity is more important in the degradation of cell walls of *R. solani* (Hadar *et al.*, 1979; Van Tilburg and Thomas, 1993). High β-1,3 glucanase production by $50Gv_1I$, $75Gv_1VI$ and $150Gv_1II$ justified the successful disease management in both types of soil.

Against stem rot of jute caused by *M. phaseolina*, mutant isolate $150Gv_1II$ again showed comparatively better potential to reduce the disease irrespective of soil type but the reduction was more in nonsterilized soil (75%). Higher glucanase(s) production and greater rhizosphere colonizing ability in nonsterilized soil obviously supported the present outcome. Successful biological control of *M. phaseolina* with *Trichoderma* and *Gliocladium* have been reported (Kousalya and Jeyarajan, 1991; Rajeshwari *et al.*, 1999; Pant and Mukhopadhyay, 2001) and the extent of reduction was quite similar to the present result.

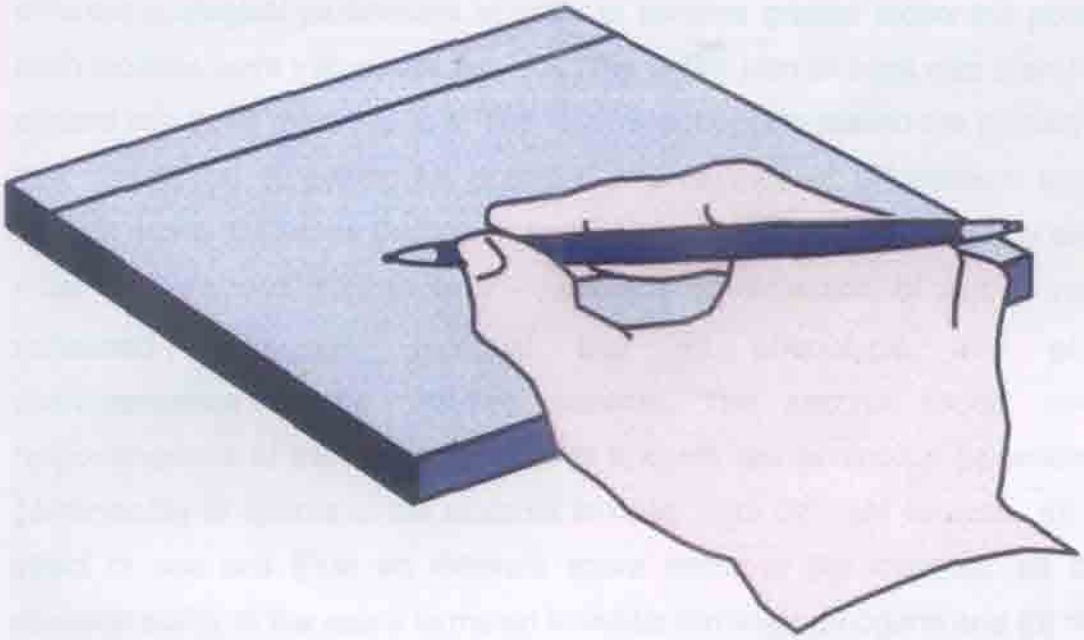
A wide variation was exhibited by the antagonist isolates against Fusarium wilt of pigeonpea and most promising result was shown by wild isolate Gv_3 in both types of soil. However in comparison to the other diseases the reduction was much less. Potentiality of *Trichoderma* and *Gliocladium* has been reported against Fusarium wilt of different crops in several other experiments (Mukhopadhyay, 1995; Castejon and Oyarzun, 1995; Godwin – Egein, 1997; Pandey and Upadhyay, 1999; Singh and

Mukhopadhyay, 2000). It could be suggested that higher doses of antagonist might be able to reduce the disease to a greater extent.

Most interesting result have been obtained against root rot of groundnut caused by *S. rolfsii*. Up to 85% reduction in disease incidence has been noted in both sterilized and nonsterilized soil. However, with the exception to the results of other disease management studies, comparatively low disease reduction in nonsterilized soil was due to slow plant mortality even in control treatment. This clearly indicated the dominant role of resident antagonistic microorganisms in control of root rot of groundnut. A positive correlation between improved *in vitro* antagonistic potential, competitive parasitic ability, rhizosphere colonization and biocontrol potential in green house experiment for some mutant isolates was discernible. To protect groundnut crop from *S. rolfsii* infection, finding out the optimum time of application appeared to be important. Subramanian (1960) reported that groundnut plants were susceptible to *S. rolfsii* at all stage of growth, but the younger ones were more susceptible than the mature plants. Application of antagonist's inoculum during sowing was therefore better than other stages of plant growth. Successful biological control of diseases incited by *S. rolfsii* with the soil application of *G. virens* have been reported by several workers (Punja, 1985; Papavizas and Lewis, 1989; Sarmah, 1990; Prasad *et al.*, 1999; Ramamoorthy *et al.*, 2000) and even with *T. harzianum* (Maity *et al.*, 1991)

The overall green house tests showed that the antagonist isolates varied in their efficacy to control different soil borne plant pathogens. It is well known phenomenon that species of *Trichoderm* and *Gliocladium* are differentially sensitive against different fungi (Mughogho, 1968; Dennis and Webster, 1971; Wells *et al.*, 1972; Papavizas, 1985) and the variation has been also reported in the comparative biocontrol potential of different species as well as isolates (Lumsden and Locke, 1989).

CHAPTER 6



Summary and Conclusion

6. SUMMARY AND CONCLUSION

The review of literature conclusively established that *G. virens* is a potentially important fungal antagonist effective against a number of soil borne plant pathogens. In agreement with the earlier workers, possibility to develop more efficient isolates of *G. virens* through mutation of the wild biotype has encouraged to set up a plan of investigation on this relatively new and promising approach in the field of biological control of fungal plant pathogens. The objectives of the present dissertation were to study the effects of mutation by use of non-particulate electromagnetic γ-rays on the enhancement of antagonistic potential of the mutants vis-a-vis their response to different ecological parameters in order to achieve greater biocontrol potential when such isolates were introduced into soil. The entire plan of work was therefore broadly divided into three main aspects. The first aspect encompassed the primary objectives that include (a) screening for potential wild isolates of *Gliocladium* spp. effective against some soil borne Deuteromycetes, (b) production of mutants by exposing the most efficient wild isolates to γ - radiation, (c) selection of stable mutants with enhanced antagonistic potential and (d) phenotypic and physiological characterization of the selected mutants. The second aspect covered the responsiveness of the selected mutants towards few ecological parameters like (a) germinability of spores of the mutants in water from different sources, (b) fungistatic effect of soil and FYM on different spore forms of the mutants, (c) competitive parasitic ability of the spore forms on sclerotia forming pathogens and (d) rhizosphere competence of the mutants. Finally, in the third aspect, an emphasis was given on (a) to find out level of fungicide tolerance of the selected mutants to some seed treating fungicides as an important component of integrated disease management and (b) determination of *in vivo* efficacy of the mutants for management of soil borne diseases. The concluding summary that could be drawn from the present experiments are a encouraging exposition of enhancement of biocontrol potential in the mutants produced by γ-radiation over the wild biotype in most of the case of trials.

1. Five isolates of *Gliocladium* spp. were isolated on modified *Trichoderma* Specific Medium (TSM) from soils of different ecological niches and subsequently compared

with Rifai's (1969) monograph for their accurate identification at species level. Three among them were identified as *G. virens* (Gv_1, Gv_2 and Gv_3) and one each as *G. penicillioides* and *G. roseum*.

2. *In vitro* antagonistic potential of the wild isolates of *Gliocladium* spp. evaluated against four important soil borne plant pathogens, viz., *R. solani, M. phaseolina, F. oxysporum* f.sp. *udum* and *S. rolfsii*. A marked variation in *in vitro* antagonistic potential amongst the isolates of *Gliocladium* spp. against different pathogens was very much evident under condition of time required for contact between antagonist and pathogen colony. Against *R. solani* most efficient antagonism was shown by Gv_2, Gv_3 and *G. penicillioides*, completely overgrowing the pathogen colony within 5 days of inoculation, whereas against *M. phaseolina, F. oxysporum* f.sp. *udum* and *S. rolfsii*, Gv_1 showed comparatively higher antagonistic potential attaining the S_1 stage in 5,5 and 7 days respectively. Next to Gv_1, Gv_3 was potentially intermediate against *M. phaseolina* and *F. oxysporum* f.sp. *udum*, however, against *S. rolfsii* it was least effective. From the overall comparison of *in vitro* antagonistic potential of *Gliocladium* spp, two efficient isolates of *G. virens* (Gv_1 and Gv_3) were selected for mutation.

3. The selected wild isolates of *G. virens* (Gv_1 and Gv_3) were exposed to five different doses of γ-radiation for the purpose of mutation. Viability of the exposed conidia from each doses, as measured on modified TSM, was found to reduce with increasing doses of γ-radiation and more than 99% mortality was recorded in most of the cases.

4. More than hundred of mutant isolates were identified when irradiated cultures were plated on modified TSM and subsequently transferred individually in PDA slants. Among them twenty-five mutants were short-listed for their distinctive phenotypic variations with respect to color, texture of the colony and growth pattern. Special attention had been drawn by four mutant isolates namely $50Gv_1V$, $50Gv_1VI$, $75Gv_1IX$ and $100Gv_3I$ for their unique ability to produce albino colony thus rendering the evidence for occurrence of mutation in the wild biotypes and the scope to utilize them as phenotypic markers.

5. *In vitro* rapid screening of antagonistic potential of the mutants was performed to screen out the isolates with enhanced biocontrol potential over their wild biotypes. Comparatively better antagonistic potential was shown by most of the mutant isolates particularly against *R. solani* and *M. phaseolina* whereas some mutants were equally

or poorly effective as compared to their wild counterparts. Against *S. rolfsii*, some mutant isolates namely $75Gv_1I$, $75Gv_1IV$, $100Gv_1I$ and $150Gv_1II$ completely lost the antagonistic potential whereas significant increase in antagonistic potential was shown by $50Gv_1I$, $50Gv_1V$, $75Gv_1VI$, $150Gv_1II$, $75Gv_3II$ and $100Gv_3I$. Against *F. oxysporum* f.sp. *udum* only two mutant isolates namely $75Gv_3I$ and $100Gv_3I$ showed higher antagonistic potential over the wild biotype (Gv_3). From the overall results six mutant isolates viz., $50Gv_1I$, $50Gv_1V$, $75Gv_1VI$, $150Gv_1II$, $75Gv_3I$ and $100Gv_3I$ along with their wild counterparts (Gv_1 and Gv_3) were selected for further studies.

6. Stability of the selected mutant isolates was examined by serial transfer and simultaneous comparison of antagonistic potential and phenotypic characters up to 10^{th} generation. Among the mutants, $100Gv_3I$, although showed exceptionally well antagonistic potential against all the pathogens tested but unfortunately lost its viability after 2^{nd} generation and therefore appeared unstable. The other mutants showed an overall 15.00 – 37.50 % increase in antagonistic potential against *R. solani* and *M. phaseolina* while it was 28.50 – 45.00 % for *S. rolfsii*. Against *F. oxysporum* f.sp. *udum* only $75Gv_3I$ exhibited 12.50 % increase in antagonistic potential as compared to its wild counter part (Gv_3). Comparison of cultural characteristics of the mutant isolates in different generations revealed that all the mutants except $100Gv_3I$ maintained their original phenotypic characters even at 10^{th} generation and therefore were considered as stable mutants with greater biocontrol potential.

7. Phenotypic characterization of the stable mutant isolates of Gv_1 and Gv_3 was done for further clarification of the mutative nature of these isolates. Cultural variation among the mutants in OMA was very much apparent as exhibited previously in PDA. No significant variation among the isolates was found with respect to phialide measurement, however, conidia of $150Gv_1II$ differed significantly from its wild counterpart (Gv_1) and that of $50Gv_1I$ and $75Gv_3I$ showed slight variation from their wild biotypes. Scanning electron microscopy in advance clearly differentiated the mutants from each other and from their wild biotypes on the basis of size of spore and degree of echinulation. Compact, large spore-ball produced by Gv_1 was the distinctive character of *Gliocladium*. Echinulation on conidial surface of all the mutants of Gv_1 clearly distinguished them from smooth walled conidia of wild Gv_1.

Mutant isolate $75Gv_3I$, on the other hand, produced small sized conidia in comparison to those by Gv_3.

8. Physiological characterization of the mutants and their wild biotypes was made by assaying the ability to produce different hydrolytic enzymes viz. β-1,3 glucanase, β-1,4 glucanase, chitinase and cellulase in CDB and CDB partially substituted with *different carbon* sources.

8.1. Significantly higher amount of β-1,3 glucanase was produced by $150Gv_1II$ in CDB (78.67U) and CDB partially substituted with chitin and CMC (29.33 and 96.67U respectively). Besides $150Gv_1II$, $50Gv_1V$ and $75Gv_1VI$ produced significantly higher amount of the enzyme (72.50 and 46.66 U respectively) than their wild biotype (33.00 U) when CDB was partially substituted with CMC. Inductive effect of mycelial powder of *M. phaseolina* and *P.ultimum*, when used as substituted C-sources, increased β-1,3 glucanase production irrespective of the isolates, however, wide variation was observed among them. In *M. phaseolina* supplemented CDB medium $75Gv_1VI$, $150Gv_1II$ and $75Gv_3I$ whereas in *P. ultimum* supplemented CDB medium all the mutants of Gv_1 produced significantly higher amount of β-1,3 glucanase. However, in *P. ultimum* supplemented medium highest production of the enzyme was exhibited by Gv_3 (291.34 U).

8.2. Variation in production of β-1,4 glucanase by different wild and mutant isolates of *G. virens* was almost similar to β-1,3 glucanase. In CDB and CMC supplemented CDB media $150Gv_1II$ produced significantly higher amount of β-1,4 glucanase (12.67 and 61.67 U) respectively than its wild biotytpe Gv_1 (4.00 and 12.33 U respectively). In chitin supplemented CDB medium the variation however was inadmissible. Several fold increases in β-1,4 glucanase production was detected when mycelial powder of *M. phaseolina* and *P. ultimum* were utilized as substituted C-sources. In *M. phaseolina* supplemented CDB medium mutant isolate $150Gv_1II$ and in *P. ultimum* supplemented CDB medium $50Gv_1I$ showed highest production of β-1,4 glucanase (69.51 and 396.67 U respectively).

8.3. Assay on chitinase production showed that the variation in this enzyme production by the wild and mutant isolates of *G.virens* was more or less similar in CDB and CDB supplemented with chitin. Mutant isolate $150Gv_1II$ produced significantly higher amount of chitinase in both the media (179.00 and 193.80 U

respectively) including CMC supplemented CDB medium (182.50 U). Several fold increase in chitinase production was detected when mycelial powder of *P. phaseolina* and *P. ultimum* were utilized as substituted C sources, but none of the mutants appeared significantly superior in chitinase production as compared to their wild counterparts in both the media.

8.4. Significantly higher amount of cellulase was produced by mutant isolate $150Gv_1II$ in CDB and CMC supplemented CDB media (57.43 and 547.56 U respectively) but the variation was apparently insignificant when CDB was partially substituted with chitin. Inductive effect of mycelial powder as a substituted C-source on cellulase production was more pronounced in *P. ultimum* supplemented CDB medium than in *M. phaseolina* supplemented CDB medium and mutant isolate $50Gv_1I$ produced highest amount of cellulase (415.00 U) in the former medium followed by $75Gv_3I$ (170.68 U).

From the overall study it could be concluded that the wild and mutant isolates of *G. virens* varied in their ability to produce different kinds of hydrolytic enzyme, implicated in the action of biological control, depending upon the nature of growth substances. Simple mineral salt solution resulted in less production of the enzymes as compared to when complex substances were added in the medium.

9. An attempt had also been made to demonstrate the physiological variation among the wild and mutant isolates of *G. virens* through SDS-PAGE of extracellular protein. Each isolate produced two different bands (Rm 0.69 and 0.81) indicating the production of two different kinds of extracellular protein in detectable quantities, however, the bands differed in their intensity and density. Greater intensity and density of both bands in wild isolate Gv_1 signified that the isolate was capable of producing more quantity of the extracellular proteins. Low intensity of second band (Rm 0.81) of $75Gv_1VI$ clearly differentiated it from its wild biotype (Gv_1). On the other hand varied density of the second band of mutant isolates $50Gv_1I$, $50Gv_1V$, $150Gv_1II$ and $75Gv_3I$ distinctly distinguished them from each other and from their wild counterparts as well.

10. The general trend of phialospore germination was very inconsistent where an individual isolate germinated well others responded differently. In sterilized distilled water and in soil leachet germination was comparatively better except in case of

$75Gv_3I$. In sterilized distilled water significantly higher germination was recorded for $50Gv_1V$ (15.95 %) than its wild biotype Gv_1 (10.00 %). In soil leachet phialospores of all the mutants of Gv_1 showed significantly higher germination (10.05-18.97 %) than Gv_1 (6.16 %). In pond water mutant isolates $50Gv_1I$, $50Gv_1V$ and $75Gv_1VI$ whereas, in tap water only $150Gv_1II$ showed significantly higher phialospore germination over their wild counterpart (Gv_1). In case of chlamydospore germination more or less similar trend was observed but the overall chlamydospore germination was low in comparison to phialospore germination. In sterilized distilled water all mutants of Gv_1 and Gv_3 showed significantly lower rate of germination than their corresponding wild biotypes but the opposite trend was detected when soil leachet was used as a substrate for germination was shown by $50Gv_1I$ (7.78 %) whereas in tap water $75Gv_1VI$ germinated well (5.88 %).

11. Fungistatic effect of soil on phialo- and chlamydospores of wild and mutant isolates of *G. virens* revealed that the static effect was more predominant in nonsterilized soil and particularly on phialospores as usual. Phialospores of none of the mutants showed significantly greater ability of fungistatic tolerance in nonsterilized soil, however, comparatively better tolerance was shown by $50Gv_1I$, $75Gv_1VI$ and $75Gv_3I$ than their corresponding wild biotypes. In sterilized soil, phialospores of all the mutants of Gv_1 showed significantly higher fungistatic response than Gv_1 but $75Gv_3I$ showed significantly greater fungistatic tolerance than Gv_3. Variation in fungistatic response of chlamydospores was more or less similar to phialospores in nonsterilized soil. Only $50Gv_1I$ showed comparatively greater fungistatic tolerance than the wild isolate Gv_1. As was obtained in case of phialospores, chlamydospores of $75Gv_3I$ only showed significantly greater fungistatic tolerance than its wild counterpart (Gv_3) in sterilized soil.

12.1. Phialospores of the test antagonist isolates were more efficient in colonizing the sclerotia of *R. solani* in nonsterilized soil except $75Gv_3I$ (10.00 %) when antagonist population in soil was adjusted to 10^2 phialospores / g of soil. With further increase, cent percent colonization was observed for all the isolates. In sterilized soil, mutant isolates $75Gv_1VI$ and $150Gv_1II$ showed significantly higher colonization of sclerotia (86.67 and 81.82 % respectively) than Gv_1 (40.00 %) when phialospore concentration in soil was 10^6/g of soil. Chlamydospores were less competent than phialospores in their ability to colonize sclerotia of *R. solani* in nonsterilized soil,

however, cent percent colonization of sclerotia in both sterilized and nonsterilized soil was exhibited by $75Gv_1VI$ and $150Gv_1II$.

12.2. In nonsterilized and sterilized soil 0-52.80 % colonization of sclerotia of *S. rolfsii* respectively were observed at phialospore conc. of 10^2 / g of soil. In sterilized soil, phialospores of $50Gv_1V$ showed comparatively higher colonization (50.00 %) when phialospore conc. was adjusted to 10^6 / g of soil. When chlamydospores (10^6/ g of soil) were used as antagonist propagules comparatively greater colonization over phialospore on pathogen sclerotia (*S.rolfsii*) was exhibited by $50Gv_1I$ and $75Gv_3I$in nonsterilized soil (85.25 and 85.69 % respectively) and $150Gv_1II$ in sterilized soil (41.11 and 46.00 % respectively).

13.1. In the experiment on rhizosphere competence 1-5 fold increase in rhizosphere population in nonsterilized soil was observed for the isolates of Gv_1, whereas in case of Gv_3 and $75Gv_3I$ it was 13 and 82 fold respectively at 14 DAS. Significant increase over initial population for most of the test isolates was observed at 28 DAS. An apparent fluctuation was recorded during later part of the experiment. Significantly greater rhizosphere colonizing ability was shown by $75Gv_3I$. Mutant isolates $75Gv_1VI$ and $150Gv_1II$ exhibited comparatively better colonizing ability as compared to their wild counterpart (Gv_1) at the later part (42 – 70 days).

13.2. In sterilized soil, a rapid increase in antagonist population in rhizosphere was observed within 14 days for all the antagonist isolates, however, in case of Gv_1 it was aberrant. Inconsistent fluctuation in spore population was recorded thereafter but mutant isolate $75Gv_1VI$ maintained its population comparatively better than others between 28-56 DAS. A significant increase in rhizosphere population between 42-56 days was observed in case of Gv_1, $50Gv_1I$, $75Gv_1VI$ and Gv_3 but none of the mutants appeared more competent than their corresponding wild counterparts during later part of the experiment.

14. Test on degree of fungicide tolerance of wild and mutant isolates of *G. virens* revealed that mutant isolate $75Gv_1VI$ was comparatively tolerant to Benomyl and Thiram (ED_{50} 1.68 and 41.81 ppm respectively) than its wild counterpart (ED_{50} 1.48 and 37.48 ppm respectively). Against Thiram highest level of tolerance was shown by $150Gv_1II$ (ED_{50} 41.89 ppm). High sensitivity towards Bavistin was exhibited by all the test isolates (ED_{50} < 0.5 ppm). None of the mutants appeared more tolerant to Captan and Iprodione than their wild biotype. All the mutants on the other hand

possessed comparatively high level of tolerance against Mancozeb (ED_{50} 371.54 – 758.58 ppm) as compared to their wild counterpart (ED_{50} 331.13 ppm).

15.1. *In vivo* trial on efficacy of wild and mutant isolates of *G. virens* against *R. solani* induced seedling blight of green gram revealed that the antagonists provided significant protection against the disease after 20 days irrespective of the condition of the soil (either sterilized or nonsterilized). In antagonist amended sterilized soil, 57.22 – 68.61 % mortality of plants were recorded and highest protection was given by $75Gv_1VI$ with 32.45 % reduction in disease incidence followed by $50Gv_1I$ and $150Gv_1II$ (31.79 and 21.67 % reduction in disease incidence respectively). These isolates were also more effective than their wild counterpart (18.34 % reduction in disease incidence). In nonsterilized soil highest potentiality was shown by $150Gv_1II$ (52.18 % reduction in disease incidence) followed by $75Gv_1VI$ and $50Gv_1I$ (45.02 and 37.33 % reduction in disease incidence respectively) and these isolates were also found to be more potential as compared to their wild counterpart.

15.2. Test against *M. phaseolina* induced stem rot of jute revealed that at 28 DAS all antagonist amended treatments resulted in less seedling mortality than untreated control irrespective of soil type (sterilized or nonsterilized). In sterilized soil, after 56 days all isolates of Gv_1 except $50Gv_1V$ provided significantly better protection (31.20 – 48.80 % reduction in disease incidence) and $150Gv_1II$ appeared comparatively more efficient over its wild biotype (48.02 % reduction in disease incidence). In nonsterilized soil, all the antagonist isolates except $75Gv_3I$ were found to provide significantly low plant mortality (21.76-50.72 % mortality) than only *M. phaseolina* treated control (89.17 % mortality) and high protection was found in case of $150Gv_1II$ (75.60 % reduction in disease incidence).

15.3. Apparently low antagonistic activity by all the antagonist isolates in both sterilized and nonsterilized soil was recorded against *F. oxysporum* f. sp. *udum* induced wilt of pigeonpea. Wild isolate Gv_3 only provided significantly better protection in both sterilized and nonsterilized soil (38.22 and 48.41 % reduction in disease incidence respectively).

15.4. *In vivo* trials on biocontrol ability of the wild and mutant isolates of *G. virens* in sterilized soil revealed that the antagonist amended treatments resulted in significantly lower seedling mortality throughout the experimental period and only the mutant isolate $75Gv_3I$ showed comparatively higher protection (83.21 % reduction in

disease incidence). The overall picture in nonsterilized soil was almost similar to that in sterilized soil but the only difference was lower rate of seedling mortality in all the treatments. Comparatively better protection in nonsterilized soil was provided by $50Gv_1I$ and $75Gv_3I$ (82.82 and 82.61 % reduction in disease incidence respectively) and these isolates were superior to their wild counterparts (74.88 and 67.14 % reduction in disease incidence by Gv_1 and Gv_3 respectively).

The salient derivation that could be made from results of the present series of experiments conducted are much varied but what could confirmatively inferred are:

(i) Some of the selected mutants, generated through γ-radiation, possessed enhanced biocontrol potential against at least one test pathogen *(R. solani, M. phaseolina, F. oxysporum* f.sp. *udum* and *S. rolfsii)* as compared to their wild biotype.

(ii) The mutants not only differed among themselves but also from their wild counterparts with respect to their macroscopic and microscopic characters studied through cultural characterization, morphometry and scanning electron microscopy.

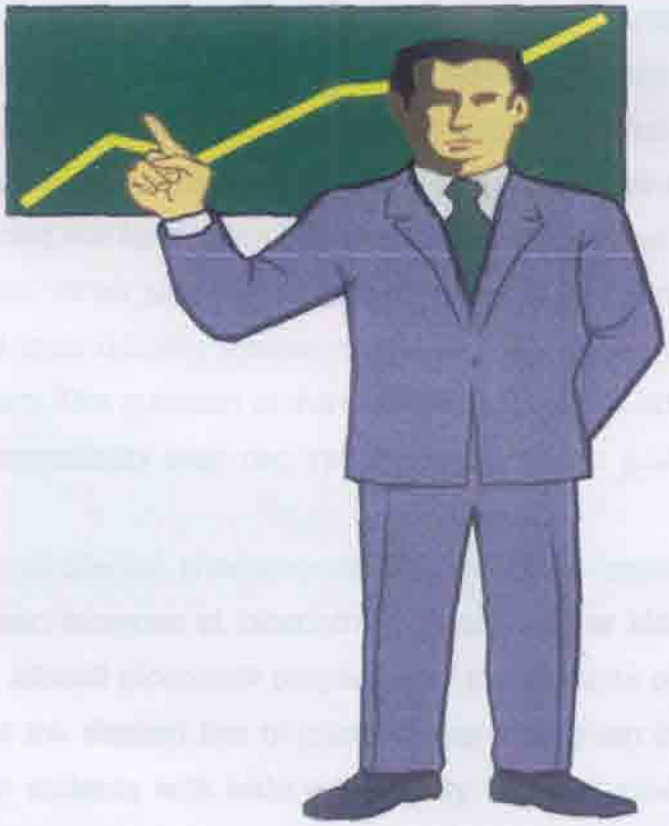
(iii) The ability for production of hydrolytic enzymes and variation in nature of extracellular proteins studied through SDS-PAGE also critically distinguished the mutants from their wild counterparts. Mutant isolates producing greater amount of the enzymes depending upon the nature of growth substances might have an important role for their success when tested under *in vivo* condition.

(iv) The mutants responded variably towards various ecological parameters considered to have reflected much through their improved biocontrol potential under *in vivo* condition.

(v) Higher tolerance to some seed treating fungicides, in some cases, created a promise for utilization of the mutants as an effective component in IDM system along with such fungicides.

(vi) The mutants differed in their efficacy to reduce the intensity of different soil borne diseases and in a few instances they were more effective over their wild biotypes. A direct correlation between greater *in vivo* efficacy of the mutants with higher *in vitro* antagonistic potential, greater mycolytic enzyme production and efficient adaptation to various ecological barriers could be drawn.

CHAPTER 7



Future Scope of Research

7. FUTURE SCOPE OF RESEARCH

It has been conceptualized as also could be visualized from the scan of the literature that mutation of living organism results in uncontrolled genetical alteration that lead to the development of traits with either desirable or undesirable characters. The information generated from the present series of experiments revealed that the desirable traits either wholly or in part may be grouped into or segregated into many of the mutant isolates, some times exhibiting better performance at least in few instance over the wild counterparts, subject to their adaptability under diverse ecosystems and varied environmental conditions. Under this situation the future scope of research on this aspects are definitely immense. Therefore, the extensive and sustained researches need to be carried out for solving the crux of the problem on the way of application of biocontrol agents, either singly or as a component of IDM, under field conditions. The development of ideal delivery system or systems would be the other important area of research in future. The question of the ecological fitness or adaptability and wide range of pesticidal compatibility also required to be answered justifiably through sustained researches.

It is an established phenomenon that mutation results in alteration of genetic sequences of wild biotypes of biocontrol agents. So the identification of the gene(s) responsible for altered biocontrol properties of the mutants over their wild counterpart definitely will be the desired line of future researches given the required facilities. The search for such mutants with wide adaptability to pH, temperature and soil moisture fluctuations vis-à-vis with the vigor for quick and greater rhizosphere competence coupled with long duration sustainability in soil will definitely add new dimension in the sphere of researches on biological control of plant disease under field conditions. Mutant isolates non responsive to soil fungistasis which will enhance the quick germination of spores either non-dormant (phialospores) or dormant (chlamydospores) resulting in massive biomass development thus increasing the vigor of the antagonist in soil ecosystem required to be searched for further advancement.

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