PAT 421. BIOCONTROL AGENTS FOR CROP DISEASES (0+2)
(1999 syllabus)

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PRACTICAL

Introduction to biocontrol agents – *Trichoderma, Pseudomonas* and *Bacillus* – Presence of biocontrol agents in nature – soil, rhizosphere, phyllosphere – preparation of media – common and special media – sterilization – autoclave, hot air oven, fermenter, etc., - isolation and purification of biocontrol agents – identification of different biocontrol agents – maintenance of pure cultures – evaluation of biocontrol agents against different plant pathogens – dual culture technique – preparation of media for mass multiplication – use of fermenter – estimation of spore load, formulation of biocontrol agents – packing and storae, quality control of products, delivery syster – seed, soil and foliar – evaluation of formulation under glass house and field.

Cost analysis and project preparation – principles of enterprise management – preparation of Agricultural project reports – project analysis and financial management – Agricultural finance – source of finance – acquisition – ratio analysis – principles of costing – economics of farm enterprise.

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I. INTRODUCTION

According to the estimate of FAO, the annual losses to crop production due to plant diseases alone are about 12 per cent. The losses are going to increase with change in agricultural practices and our country, which is tropical with warm and humid climate, provides ideal conditions for development and spread of diseases of crops cultivated under good management systems. Therefore, control of plant diseases assumes greater importance. Although several methods are followed for this purpose, disease control by chemicals is the promising one especially under intensive cropping programmes. However, their use poses certain problems of residues left over on crops, which has gained considerable importance in recent years, not only in India but also throughout the world. Besides, chemical fungicides and bactericides employed now in plant disease management are becoming costlier and they also leave non-biodegradable toxic complexes. When the doses are exceeded, they become phytotoxic. They also pollute the environment. Seed treatment chemicals, when used give protection only in the early stages of crop growth. Chemicals used to control soil-borne diseases are uneconomical, less effective and leave residues in the soil and plants. Further, they are toxic to beneficial microorganisms in the soil. In certain cases, the plant pathogens develop resistance to fungicides and bactericides. Under the above circumstances, it becomes inevitable to develop a bio-based, eco-friendly, biodegradable plant derived pesticides or microbial pesticides in order to control plant pathogens. Biological control or biocontrol using antagonistic microorganisms offers a practical and economical alternative for the management of plant pathogens.

Garrett (1970) defined biological control of plant diseases as any condition or practice, which reduced the survival or activity of a pathogen through the agency of any other living organisms other than man resulting in reduced disease incidence. Baker and Cook (1974) defined the term as the reduction of inoculum density or disease producing activities of a pathogen or a parasite in its active or dormant state by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonists or by mass introduction of one or more antagonists. Later, Cook and Baker

(1983) shortened the definition that it is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man.

Biological control is nothing but ecological management of community of organisms. It involves harnessing disease-suppressive microorganisms to improve plant health. Disease suppression by use of biological agents is the sustained manifestation of interactions among the plant (host), the pathogen, the biocontrol agent (antagonist), the microbial community on and around the plant and the physical environment. The biological control of plant diseases differs from insect biocontrol in following ways (Table 1).

Table 1. Differences between disese biocontrol and insect biocontrol.

Sl. No.	Disease biocontrol	Insect biocontrol
1.	Disease control is largely achieved by antibiosis, competition and comparatively less by hyperparasites.	Largely by parasites and predators.
2.	Antagonists are largely passive and are not mobile. Contact of pathogen is accidental.	Parasites are active, mobile and seek their prey.
3.	It is a mass effect. For a single species of pathogen a large number of antagonists / competitors available.	Single predator / parasite for single prey.
4.	This method relies mainly on native organisms.	Introduction of parasites / predators from other countries are normally followed.
5.	Pathogen free seeds and planting materials are widely used.	Pest free seeds are not used.

The biological control has many advantages than any other methods employed in the management of diseases. The advantages are as follows:

Advantages

1. Biological control is less costly and cheaper than any other methods.

2. Biocontrol agents give protection to the crop throughout the crop period.
3. They are highly effective against specific plant diseases.
4. They do not cause toxicity to the plants.
5. Application of biocontrol agents is safer to the environment and to the person who applies them.
6. They multiply easily in the soil and leave no residual problem.
7. Biocontrol agents can eliminate pathogens from the site of infection.
8. Biocontrol agents not only control the disease but also enhance the root and plant growth by way of encouraging the beneficial soil microflora. It increases the crop yield also. It helps in the volatilization and sequestration of certain inorganic nutrients. For example *Bacillus subtilis* solubilizes the element, phosphorous and makes it available to the plant.
9. Biocontrol agents are very easy to handle and apply to the target.
10. Biocontrol agents can be combined with biofertilizers.
11. They are easy to manufacture.

Although biological control is advantageous in many aspects, it has the following disadvantages.

1. Biocontrol agents can only be used against specific diseases.
2. They are less effective than the fungicides.
3. Biocontrol agents have slow effect in the control of plant diseases.
4. At present, only few biocontrol agents are available for use and are available only in few places.
5. They are unavailable in larger quantities at present.
6. This method is only a preventive measure and not a curative measure.
7. Biocontrol agents should be multiplied and supplied without contamination and this requires skilled persons.
8. The shelf life of biocontrol agents is short. Antagonists, *Trichoderma viride* is viable for four months and *Pseudomonas fluorescens* is viable for 3 months only.
9. The required amount of population of biocontrol agents should be checked at periodical interval and should be maintained at required level for effective use.

10. The efficiency of biocontrol agents is mainly decided by environmental conditions.
11. A biocontrol agent under certain circumstances may become a pathogen.

II. BIOLOGICAL CONTROL – PRINCIPLES AND PRACTICES

Soil is the source of many microorganisms, including bacteria, fungi, nematodes, protozoa, viruses, insects and mites, which are pathogenic to plants. The rhizosphere is the narrow zone of soil surrounding plant roots that contains root exudates, sloughed root remains and large populations of soil microorganisms of various nutritional groupings. Bulk soil, outside the rhizosphere, has relatively fewer populations of microorganisms. Biological control is usually operative in the rhizosphere but can occur in other habitats. Because soil changes less rapidly than the atmosphere above it, soil microorganisms have become adapted to a relatively stable habitat. Populations of antagonists, pathogens and host plants fluctuate within certain limits in response to abiotic and biotic interactions. The environment also affects host susceptibility and pathogen activity. Slight changes in soil environmental conditions may have profound effects on populations of the pathogen and antagonists. Changes produced by agricultural practices tend to be varied, rapid and on-going, which permits little time for re-establishment of the biological equilibrium. Biological equilibrium in crop disease situations can be manipulated through the integrated use of chemical controls, such as fungicides and nematicides; cultural controls such as sanitation or crop rotation; and biological control methods, such as plant resistance or introduction of antagonists. Usually biological controls are more subtle and operate more slowly but are generally more stable and long lasting than chemical and cultural control measures. However, biological controls should be integrated with other control measures because different methods are effective at different times and locations under varying conditions. Approaches to biological control of plant diseases with microbial antagonists are listed below.

Biological control with resident antagonists

- pathogen-suppressive soils
- general suppression
- specific suppression

Biological control with introduced antagonists

- rhizosphere competence
- root colonization process
- introduction with other soil treatments to favor the antagonist
- seed treatments
- composites of antagonists

Biological control with resident antagonists

Generally, biological control of plant pathogens is achieved with resident antagonists. They are managed or fostered by cultural practices. As a result of certain crop or soil management practices, some field or green house soils are naturally suppressive, even though a susceptible host and soil populations of the pathogen are present. Although the development of biological control systems is often poorly understood, pathogen suppressive soils have been associated consistently with these crop or soil management practices.

- Crop rotations with non-susceptible plant hosts or fallowing (no crop) which provide time for resident antagonists to displace soil-borne pathogens or for pathogen propagules to die

- Soil tillage, which accelerates displacement of certain pathogens in crop residues by resident antagonists

- Incorporation of composts and other organic materials which stimulate the activities of resident antagonists or suppress the activities of pathogens

- Selective soil treatments with steam, biocides or solarization which suppress or eliminate the pathogen but not resident soil saprophytes and

- Management of certain cultivars of a host which select specific rhizosphere antagonists

Suppressive soils

In each case, suppression of the activities of the pathogen by resident antagonists occurs prior to infection of the host root. Pathogen-suppressive soils are defined by Cook and Baker (1983) as soils in which the pathogen does not establish or persist, the pathogen establishes but causes no damage or the pathogen causes some disease damage, but the disease becomes progressively less severe even though the pathogen persists in soil. Most soils suppress soil-borne pathogens to some degree. Soil may suppress pathogens in a general or specific manner. General suppression of a pathogen is directly

related to the total amount of microbial activity in the soil or plant at a critical time in the life cycle of the pathogen. The general antagonism or general soil suppressiveness is non-specific. It operates against most, if not all pathogens. It involves the activites of many resident soil organisms and can be enhanced by addition of composted materials. Specific suppressiveness operates in addition to general soil suppressiveness but against only certain types of pathogens. Specific suppressiveness has been described for *Fusarium* wilts, *Gaeumannomyces graminis* var. *tritici, Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani* and *Thielaviopsis basicola.* In all cases, a particular pathogen causes significantly less disease in suppressive soils than in other soils(conducive soils); the effect is lost when soil is treated with biocides, indicating the involvement of microorganisms. The initial objective of biological control is to maximize soil suppressiveness through the manipulation of resident antagonists. These microorganisms probably resided in equilibrium with pathogens and plants before the intervention of agriculture. However, the residents may not be present at sufficient levels for effective disease control at specific vulnerable sites on the plant or at specific vulnerable stages of crop growth. In these circumstances, the population has to be enhanced with inoculant formulation, knowing that the site conditions are favourable for the control agents. Among many examples, *Phlebia gigantea* needs to be added to freshly cut pine stumps because the natural invasion of stump surfaces by this fungus is too low and unpredictable to guarantee control of *Heterobasidion*. Similarly, *Agrobacterium radiobacter* is applied to seeds or bare rooted transplants because the resident antagonistic population may be insufficient to give complete protection.

Biological control with introduced antagonists

First attempt biological control with introduced antagonists to the soil was made by Hartley in 1921 against damping-off of coniferous seedlings, Millard and Taylor in 1927 against common scab of potato and Sanford and Broadfoot in 1931 against take-all disease of wheat. An advantage of introduced antagonists over chemical protectants is that the microbial populations can grow from a small quantity of inoculum applied to seeds or cuttings or as in-furrow application. Although the first attempts at biological control were with introduced antagonists, they were generally ineffective unless the soil

had been steamed or large quantities of organic matter had been added to support the introduced antagonists. Thus, the concept developed that the soil environment must be modified to permit establishment of an *alien* microorganism. There are few examples of successful commercial use of introduced organisms. To achieve protection of subterranean plant parts, the biocontrol organism needs to be rhizosphere competent (Ahmad and Baker, 1987). Rhizosphere competent organisms can proliferate along the root as it develops. Certain mutants of the fungus *Trichoderma* that produce more cellulase than their wild-type parent strains are also more rhizosphere competent. The effectiveness of introduced antagonists for biological control depends on survival, establishment or proliferation in the infection site on the root. If a biocontrol agent introduced on seed multiplies around the site of application but not along the root surfaces away from the cotyledon attachment, it may suppress pathogens causing seed rot and seedlings diseases. However, root diseases are not typically suppressed by seed treatments. Control of *Agrobacterium tumefaciens* (crown gall of roses and fruit trees) using non-pathogenic strain, K 84 of *A. radiobacter* is another example of biological control achieved with introduced antagonists. The antagonist produces antibiotic-like substances, which are effective against the pathogen. Control of *Heterobasidion annosum*, the cause of pine root rot, by inoculation of freshly cut stumps with *Peniophora gigantea* is also an example, where an introduced antagonist has been used as a biological agent for disease control.

Introduction of antagonists in conjunction with other soil treatments

The microbiological balance is altered by various environmental factors such as heat treatments, pesticides and other chemical treatments that eliminate groups of microorganisms. Such treatment favours introduced strains of fungal species of *Trichoderma* or *Gliocladium* antagonistic to plant pathogens. Introduced *Trichoderma* spp. have a greater tolerance of broad-spectrum biocides than many other soil microorganisms and colonize treated soils more rapidly than other soil competitors. Species of *Trichoderma* predominate in soils after fumigation with dilute formaldehyde or carbon disulfide or after application of sodium azide to soil. Biocides can weaken pathogen propagules and render them more susceptible to attack by antagonists.

Armillaria spp., the cause of mushroom root rot of many woody crops, are susceptible to biocontrol with *Trichoderma* spp. when sublethal doses of soil fumigants interfere with defense mechanisms of the pathogen resting structures. *Trichoderma* spp. can also be introduced into soils treated by solarization to control soil-borne diseases. Biocontrol agents generally provide less effective control of seed and seedling root diseases and are more variable in their performance than are chemical pesticides. The antagonist must establish and grow well at the infection site after introduction on seed. Solid- matrix priming with vermiculite and an osmoregulant provides a suitable medium that is nontoxic, has a high water holding capacity, and has a favourable chemical and physical environment for the biocontrol agent. Application of *T. harzianum,* combined with solid-matrix priming, provides an effective and reliable biological seed treatment for a number of crops to protect them from several pathogens.

Biological control with composites of antagonists

Mixtures of biocontrol agents are particularly effective when they have different modes of action or different ecological attributes so that they function at different times or locations. This approach requires compatibility among composites of antagonists. Composites of antagonists

- should operate at different sites, such as in the rhizosphere or in organic residues
- should effect biocontrol by different mechanisms(by competition or antibiosis, etc.)
- should require different substrates for activity, such as mucigel metabolism by rhizosphere-competent fungi as compared to use of root exudates by pseudomonads and
- should be compatible with the soil environment and enhanced by cultural manipulations.

Composites of microorganisms can control several plant diseases. Fox example, a composite of fungal antagonists, selected for their ability to establish high populations in the rhizosphere of tomato, can reduce incidence of *Fusarium* crown rot of tomato. Combination of fluorescent pseudomonads and *Trichoderma koningii* were more

effective in control of take-all disease of wheat. Combination of pseudomonads and non-pathogenic *Fusarium* spp. was more effective in the control of *Fusarium* wilt of cucumber than either antagonist alone.

Factors involve in biological control

Antagonist, pathogen and the host are the major factors involved in the biocontrol of plant diseases. Besides, certain miscellaneous factors are also found responsible for biocontrol of plant pathogens. The influence of aforesaid factors in the biological control of plant pathogens are discussed hereunder.

1. Antagonist

The vast majority of antagonists are saprophytes. Saprophytes are more broadly adapted to the environment and are able to use a great range of nutrients than plant parasites, a situation favourable for biological control. Besides, they are more tolerant to extremes of temperature, desiccation, acidity, alkalinity, anaerobiosis and high CO_2. Exceptions occur, but in general the survival rate under these various environmental extremes is higher among saprophytes than among parasites. Saprophytes have broader distribution than parasites, presumably because of their more aggressive nature and their greater tolerance of environmental extremes. Parasites may be less competitive because of their specialized ability to derive food from living tissue. The more specialized the parasite, the more restricted the conditions under which it can cause disease and the more susceptible it is apt to be to antagonistic microorganisms. The antagonists in biological control of plant pathogens include fungi, bacteria, actinomycetes, viruses, higher plants and predatory microfauna such as protozoa, nematodes, collembola and mites.

Fungi: Fungi rank third as potential antagonists in biological control. This does not imply that they are unimportant. Because of their ability to make rapid growth on organic substrates in dry, acid, coarse textured soil they outperform bacteria and actinomycetes. *Aspergillus, Penicillium* and *Trichoderma* are able to solubilize insoluble phosphates in soil in addition to their antagonistic activity. Plant pathogens in straw,

wood, leaves, fruit, roots or other cellulosic materials are more apt to be replaced by saprophytic fungi than other organisms. Fungi, therefore appears to be important in biological control of plant pathogens during host-free period.

Bacteria: Bacteria are extremely important in biological control of plant pathogens. They exceed the number and weight of any other group of microorganisms in soil because of their rapidity of growth and ability to utilize different forms of nutrients under widely different conditions. Bacteria are especially important as antagonists of pathogens such as *Fusarium* and certain others that produce a germ tube and attack roots rapidly by multiple infections. *Bacillus* and *Clostridium* produce endospores, hence they survive longer at higher temperature than others. Some pseudomonads and *Bacillus* especially *B. subtilis* produce antibiotics, with activity against all types of soil microflora including plant pathogens. It is now generally accepted that some of the bacteria (*Aerobacter cloacae, Bacillus megaterium, Bacterium globiformae* and PGPR) also occur in healthy internal tissues of plants as endophytes and offer protection against plant pathogenic fungi especially vascular pathogens like *Fusarium, Verticillium,* etc.

Actinomycetes: As resident antagonist, actinomycetes are probably second only to bacteria in importance in maintaining a satisfactory biological balance in soil largely because of their widespread ability to produce antibiotics. Many have the ectoenzyme systems necessary to breakdown proteins, cellulose and chitin, which may have potential in biological control of microorganisms with chitinous walls. Actinomycetes grow slowly and are poor competitors hence they are potentially valuable as antagonist during host-free period, where time is not critical and where organic amendments can be used to encourage their activities. Actinomycetes do well in dry situations of high organic matter and high temperature.

Viruses: Viruses occur in a number of fungi including mushrooms and cause commercial loss. Virus infection of fungi increases hyphal leakage and decreases growth rate and sometimes the virulence of the fungi. No commercial use of viruses in biological control has so far been attempted.

Antagonists exert their influence through competition, parasitism, antibiosis and lysis. Competition normally exists for oxygen, water, nutrients and space. In parasitism, mycoparasites attack the fungal structures by penetrating the mycelia directly or growing within the host mycelium. Sometimes they may coil around the mycelium of the host with or without penetration. Antibiosis is the inhibition of one organism by a metabolite of another and lysis is the complete or partial destruction of fungal cell by enzymes. Among the various forms of antagonism, antibiosis has certain advantages over other forms. The toxic substances produced may diffuse in water films and water-filled pores through soil or on substrates or through air-filled pores in the case of a volatile and thus actual physical contact between antagonist and the pathogen is not necessary for effect.

It is suggested that soil bacteria are generally effective as scavengers and are thus important in competition; *Bacillus, Pseudomonas* spp. and some others are also producers of antibiotics. Actinomycetes are poor as scavengers and in competition, but are excellent antibiotic producers. Fungi are effective in competition (possession) and hyperparasitism and some effectively produce antibiotics (possession).

2. Pathogen

Plant pathogens are necessarily adapted to their biotic and abiotic surroundings and may not be easily subdued or extinguished by manipulation of environment. They exploit every advantage in amazingly better ways. This versatility of the pathogen is the real reason for difficulty in biological control. There are three general ways by which plant pathogens can overcome antagonism and thus nullify a potential system of biological control. Higher plants evade disease and pathogens avoid antagonists in the same way, *i.e.,* escape, resistance and tolerance.

By having knowledge of the biology of the pathogen and by using every minor advantage, the success in biological control can be achieved. In addition, most pathogens have certain obvious weaknesses where attempts at biological control should be concentrated. There is a possibility that an inactive pathogen has lesser defense against exploitation or some other antagonism than one with active growth. For some plant pathogens, the intermediate period of spore germination or zoospore release may be the

most vulnerable to antagonists. Short-term crop rotations are the practical means of biological control of those pathogens that depend on host tissue for survival and cannot form resistant survival structures of its own. Plant pathogens that survive by means of sclerotia are amenable for control during host-free periods. Host residues may protect sclerotia and lengthen their life in soil, but on the other hand, management practices that create an unfavourable physical or biotic environment can weaken sclerotia and hasten their rate of death and decomposition.

Most soil-borne pathogens make some growth outside the host before they penetrate. The time spent by the pathogen in the rhizosphere or on the rhizoplane before penetration is important to the success of an antagonist there. For example, the estimated time of 20 to 30 h spent by *Fuarium solani* f. sp. *phaseoli* is forming a thallus on a bean root are sufficient for its control if conditions of soil environment, exudation and the microflora are favourable to the development of large population of rhizosphere bacteria. The population density of soil-borne plant pathogens to cause disease in a field is also important and based on the population density, soil-borne pathogens are grouped in to 3 major groups (Table 2)

Table 2.Threshold population densities for selected soil-borne pathogens to cause disease.

Group	**Population density (units/g)**	**Pathogen**
I	Less than 1 / g	*Gaeumannomyces graminis, Phymatotrichum omnivorum, Rhizoctonia solani, Sclerotium rolfsii*
II	1 – 100 / g	*Plasmodiophora brassicae, Phytophthora cinnamomi, Verticillium albo-atrum, V. dahliae*
III	More than 100 / g	*Fusarium* spp., *Pythium ultimum, Thielaviopsis basicola*

In group I, a single infection is likely to cause severe plant damage, because these pathogens have extensive ectotrophic mycelial growth. The fungi belonging to group II produce secondary inoculum from primary survival structures. All fungi in the group III survive as thick-walled resting spores that apparently do not give rise to secondary

inoculum before infection. Knowledge of the threshold population densities for soil-borne plant pathogens is important in biological control in two ways:

i. Where elimination of the propagules by predation, lysis or digestion is the objective, population counts are necessary as a test for effectiveness.

ii. The population estimates can be used to determine when a suppressive flora is operative, by revealing whether inoculum is adequate, even though disease does not occur.

3. Host

The host plays a double role in the biological control of plant pathogens. It provides a meeting ground for the pathogen and its antagonists and it reduces the incidence or disease producing activities of the pathogen directly, through physiological processes that constitute host resistance. The ability of host roots to adapt to the environment plays a role in susceptibility of plants to disease. The various features of root system *viz.,* root structure, root hairs, mycorrhizal relationship, soil moisture and oxygen also play role in biological control. Root exudation stimulates pathogens as well as many other microorganisms, some of which facilitate nutrient uptake by the plant and some of which suppress pathogens. Several workers have demonstrated the feasibility of modifying root exudates through foliar application of chemicals.

Crop rotation: One of the benefits of crop rotation is the stimulation of organisms that partially or totally suppress the pathogen during its parasitic or saprophytic phases. Crop sequence to manipulate the microbiological balance is more difficult in areas of established monoculture, where microbiota are stabilized than in virgin soils brought under cultivation.

Plant residues: The role of crop residues in the biological control of plant pathogens has been recognized and studied very elaborately. The role of plant residues is basically one of the important factors in stimulating microorganisms antagonistic to plant pathogens. The greater the variety and number of microorganisms supported by the organic amendments / crop residues, the greater the probability of suppressing the pathogen. Plant residues also control soil-borne diseases and lower the populations of pathogens by propagules germination-lysis by the production of volatile substances such as

acetaldehyde, isobutyraldehyde, isovaleraldehyde, methanol and ethanol. Disease control by volatiles may also depend on time of planting relating to time of amendment *i.e.*, disease control is best achieved if planting is done within a few days after amendment, when germination-lysis is complete and microbial activity and antagonism is still intense.

When the host functions as reservoir of pathogen inoculum, it may be an impediment to the application of biological control. Pathogens that depend on host tissue for survival can be controlled by accelerated decomposition of the plant remains. Internally seed-borne pathogens safely perpetuate within their hosts and are unlikely to be controlled biologically but they can be controlled through host resistance. They offer little or no opportunity for suppression by external antagonists as they can persist indefinitely within their host. Weeds and wild hosts also act as important reservoirs of pathogen inoculum in cultivated crops.

Host resistance: Some forms of host resistance to microorganisms result from mechanical barriers such as thick cell wall or cuticle. Other forms of host resistance are associated with suberin and lignins; fungitoxic phenolics such as chlorogenic acid, caffeic acid, catechol or tannins and glucosides. Plants may also synthesize fungitoxic compounds (phytoalexins) in response to attempted invasion by microorganisms. There are several evidences to suggest that some microorganisms function as antagonists of pathogen by stimulating resistance in the host. Many microbial metabolites including actinomycin D, mitomycin C, phytoactin B, gliotoxin, cycloheximide, puromycin, chloramphenicol and culture filtrates of several fungi are considered as potent inducers of physiological processes associated with the resistance response within the plant cell including the production of phytoalexin.

In addition to the aforesaid factors, the following physical environment and miscellaneous factors also play role in biological control of plant pathogens. They are:

Environment: Environment controls the outcome of all host-pathogen-antagonist interactions. Environment in biological control operates by increasing the ability of the host to resist, tolerate or escape the pathogen through increasing the capacity of the antagonist as a competitor, parasite, or antibiotic producer or through weakening the

ability of the pathogen to affect the host. Abiotic factors such as soil type, soil moisture, pH, and temperature, inorganic and organic constituents of soil, quantity and type of pesticides applied to soil and components of soil atmosphere influence the efficacy of biocontrol in soil.

Soil: The soil rich in montmorillonite clay favour the development of antagonistic microorganisms, which in turn suppress the pathogenic activity in suppressive soils. The mechanism of suppression of soil includes antibiosis, competition, parasitism and predation. Important plant pathogens suppressed by these soils include *Fusarium* sp., *Phytophthora cinnamomi, Pythium* sp.and *Rhizoctonia solani.*

Soil moisture / irrigation: Irrigation methods are the best means of environmental manipulation because the daily mean and minimum water potential of a field soil can be raised or lowered with relative ease. Proper timing and frequency of irrigation controls scab of potatoe through a shift in the microbiological balance. Drier soils of raised beds prevents infection by root pathogens especially *Pythium* spp. Soil moisture is also found to affect the growth of antagonists. The population of bacteria drops rapidly when soil moisture is falling below 15 to 20 per cent, while that of fungi and actinomycetes increase. A quick shift of dominant flora from actinomycetes and fungi to bacteria is observed when the soil is moistened.

Soil solarization: Solar heating is effective especially in the control of soil-borne plant diseases. Death of the pathogen is caused by a combination of predisposing physical environment and the action of various soil microorganisms. Solarization increases the activity of microbes towards pathogen and also increases vulnerability of pathogens to soil microorganisms (Katan, 1981). Solarization affects the survivability of the pathogen for a longer period.

Drought and flood: Barley grown in dry soil develops adult plant resistance to powdery mildew than barley grown in wet soil. Conidial germination and formation of aspersoria on upper leaves were inhibited when adult plants were grown in dry soil. Flooding of

soil is helpful in biological control of many diseases. The resting propagules of pathogen are eliminated by excess of water. *e.g*., Panama wilt of banana.

pH: Hydrogen ion concentration plays a major role in the adaptability of organisms to their nutrient environment. Soil pH influences strongly the development and severity of certain diseases caused by soil-borne organisms. Manipulation of soil pH has been used successfully to control some of the important crop diseases. Low pH levels or acid soil favours the activities of *Trichoderma* spp. thereby it suppresses the soil-borne plant pathogens.

Inorganic nutrients: Effect of inorganic nutrients on soil-borne pathogens and biocontrol agents has been studied in detail by several workers. Some forms of fertilizers cause much greater pH changes than others. In general, ammoniacal form of nitrogen has the greatest acidifying effect on soil compared to nitrate forms, which has the tendency to raise the soil pH, atleast initially. Lime application is a well known means of counteracting pH drop, or of increasing the pH of acid soils and it has been used to control club root of crucifers, *Fusarium* wilt of tomato and certain other diseases suppressed by alkalinity or enhanced by acidity. Diseases caused by *Aphanomyces*, *Fusarium* and *Rhizoctonia* are increased in severity by ammoniacal form of nitrogen and decreased by the nitrate form.

Organic amendment: Addition of organic amendment is more advantageous than the use of any chemical in the biological control of soil-borne plant pathogens. Organic compounds have long lasting effect and multiple pathogen suppression effect. Its use involves lower cost, fewer hazards than the chemicals. It also improves soil fertility. Green manure controls potato scab disease (*Streptomyces scabies*). Heavy doses of organic matter controls basal stem rot of coconut, root rot of pulses, oilseeds and cotton. The process of fungistasis in soil is also enhanced by the organic amendments with alfalfa, oat-meal and linseed meal and thereby plays an important role in biological control of plant diseases.

Soil fungistasis: Fungistasis favours the germination of propagules even in the presence of susceptible host. Manipulation of soil environment inactivates the propagules even in the presence of root exudates by increasing the toxicity level. In nature, large amount of organic matter and various sources of energy nullify fungistasis (Lockwood, 1977).

Tillage: The method of tillage and of handling crop residue affects the temperature and water potential of soil and thus constitutes another practical means of changing the environment and perhaps altering the biological balance in a desired direction. Seeds unable to germinate in dry soil are commonly destroyed by *Aspergillus* spp., *Penicillium* spp. and other weak pathogens capable of growing in it. Seeds in wetter soil may germinate and resist or escape decay. Hence, conserving the seed-zone moisture is very important to seedling establishment.

Climate: The microclimate at the plant surface and variations in the seasons change the microhabitat of the plant surface. The microbes naturally present on the plant surface affects the incidence of disease on the aerial surfaces of plants. Manipulation of the environment of the plant surface enhances the activities of antagonists and is effective in the control of diseases. The architecture of the plant surface and its nutrition status can be changed to influence microbial population. Application of appropriate nutrients to the plant helps in the manipulation of microbial population on the plant surface.

III. BIOCONTROL AGENTS

A biological agent that reduces the number or disease producing activities of the pathogen is called as antagonist or biocontrol agent. The important biocontrol agents used in plant disease management includes fungi, bacteria, actinomycetes, mycorrhizal fungi, viruses, protozoa, bdellovibrios, etc. Among them, fungal and bacterial antagonists especially species of *Trichoderma, Gliocladium, Pseudomonas* and *Bacillus* are most widely used against plant diseases. They act on the pathogen through one or more of the following mechanisms like competition, parasitism, antibiosis, lysis, etc.

Ideal biocontrol agent

An ideal biocontrol agent should have the following features / characteristics.

1. It should not be pathogenic to plants, human beings, animals and microorganisms.
2. It should have broad spectrum of activity in controlling many types of diseases and must be genetically stable.
3. It should have fast growth and sporulation.
4. It must be cultured under artificial media.
5. The inoculum must be capable of abundant production using conventional methods such as liquid fermentation and withstand long term storage until application.
6. It should be amenable for inexpensive mass multiplication and economically viable.
7. It should have long shelf life.
8. It must be efficacious under different environmental conditions.
9. It should be compatible with biofertilizers.
10. It should have least susceptibility to the action by the seed treating chemicals.
11. It should not be toxic to beneficial organisms in or on the target area.
12. It should be easily formulated and methods of application must be convenient and compatible with common cultural practices.
13. It should easily establish in the soil with high persistence and survival capacity.
14. It should be biologically competitive with other microorganisms.

Diseases of roots, stems, aerial plant parts, flowers and fruits are caused by wide variety of pathogens. Similarly, the antagonistic species of these pathogens are also quite varied.

Root pathogens: Root diseases are caused by a wide variety of fungi and by some bacteria in many crops. The biocontrol agents involved are largely antagonists, who can occupy niches similar to the pathogens and either naturally or through manipulation outcompete the pathogens in these niches. Antibiotic production, mycoparasitism and induced resistance are the general mechanisms involved. Among the antagonistic fungi, *Trichoderma* spp. and *Gliocladium virens* are important and were studied widely to control root rot, damping off and wilt diseases incited by *Fusarium* spp., *Macrophomina phaseolina (Rhizoctonia bataticola), Pythium* spp., *Rhizoctonia solani* and *Sclerotium rolfsii,* in crops like beans, chickpea, citrus, cotton, maize, peanut, peas, potato, soybean, sugarbeet, sugarcane, sunflower, tobacco, tomato, etc. Efficacy of mycoparasitic fungi, *Pythium nunn* against *P. ultimum* and *P. vexans* and *Talaromyces flavus, Coniothyrium minitans* and *Sporidesmium sclerotivorum* against fungal pathogens producing sclerotia is well documented. Saprophytic *Fusarium* spp. are able to suppress population of pathogenic *Fusarium* spp. through competition for nutrients. *Streptomyces scabies,* the causative agent of potato scab is suppressed by naturally occurring populations of *Bacillus subtilis* and saprophytic *Streptomyces* spp. Species of *Pseudomonas* and *Bacillus* are reported to suppress many fungal diseases. *Pseudomonas* spp. possess properties particularly suited for effective suppression of root infecting pathogens in soil by means of antibiotic production and competition for ferric (Fe^{+++}) ions. *Agrobacterium radiobacter* effectively controls the crown gall pathogen, *Agrobacterium tumefaciens,* largely through the action of antibiotic, agrocin 84.

Fluorescent pseudomonads (FPs) are the bacteria belonging to the genus, *Pseudomonas,* which produce water-soluble fluorescent pigments in media supplemented with glycine. FPs have received overwhelming attention because of their capacity to inhibit a wide range of soil-borne and foliar pathogens. The root / soil pathogens which are inhibited by FPs include *Rhizoctonia solani* (causing root rot of cotton and sheath blight of rice), *Gaeumannomyces graminis* var. *tritici* (take-all disease of wheat), *Pythium*

spp. (damping off of beans, cotton, cucumber, maize, sugarbeet, tobacco and wheat), *Fusarium oxysporum* (wilts of banana, beans, carnation, chickpea, cotton, cucumber, flax, pigeonpea, radish and tomato), *Ralstonia solanacearum* (Moko wilt of banana, bacterial wilt of tomato and potato), *Sclerotium rolfsii* (stem and pod rot of groundnut, root rot of bean and sunflower), *Aphanomyces euteiches* (pea root rot), *A. cochlioides* (sugarbeet root rot) and *Theilaviopsis basicola* (tobacco root rot). FPs should be applied as seed treatment as these bacteria establish well in rhizosphere when they are introduced at germinating stage.

Stem pathogens: Stem diseases cause decay and cankers especially in forest and orchard trees. In many stem diseases, biological control warrants rapid colonization by non-pathogenic antagonistic competitors. Among them fungi like *Cladosporium*, *Fusarium* and *Trichoderma* and bacteria like *Agrobacterium* spp. and *Bacillus subtilis* are important. In the case of chestnut blight, hypovirulent strains of the pathogen, *Cryphonectria parasitica* itself are crucial in bringing about biological control. In this case, hypovirulence is transmitted cytoplasmically to virulent strains already infecting trees and disease symptoms decline and disappear.

Leaf pathogens: Phyllosphere is the three-dimensional space of the leaf surface. The growth of microorganisms on leaves is normally restricted by environmental factors. Nutrient levels are generally low on leaf surfaces and are not favourable for development of microbes. There are many factors that may influence microorganisms in the phyllosphere. The resident microorganisms of leaf surface use leaf resources actively to sustain their growth. As nutrients fluctuate, there is an ecological succession in colonization of bacteria, yeasts and filamentous fungi. Microclimate variables like leaf surface moisture, temperature and radiation are unfavourable for microbial development. In temperate and arid tropical regions, water will be intermittent on leaf surfaces. Water may be continuously present in humid tropical regions. Temperatures on leaf surfaces exposed to direct radiation may rise to several degrees above ambient level.

The phyllosphere microorganisms most frequently recorded on surfaces of crop plants include *Aureobasidium pullulans, Cladosporium* spp., *Cryptococcus* spp.,

Gliocladium spp., *Sporobolomyces* spp. and *Trichoderma* spp. in fungi and *Chromobacterium, Erwinia, Klebsiella*, *Pseudomonas*, and *Xanthomonas*, in bacteria.

Botrytis leaf spot in onion was suppressed by *Gliocladium roseum*. Leaf and flower blight of many ornamental plants and fruit crops caused by *Botrytis* was effectively controlled by *Trichoderma* spp. Powdery mildew disease in grapevine and cucumbers can be controlled by *Ampelomyces quisqualis*, *Tilletiopsis* and *Sporothrix* yeasts. Experiments with fluorescent pseudomonads have shown their ability to suppress leaf pathogens like *Alternaria* sp. (tobacco leaf spot), *Botrytis cinerea* (apple grey mold), *Cercospora moricola* (mulberry leaf spot), *Colletotrichum lagenarium* (cucumber anthracnose), *Phaeoisariopsis personata* (late leaf spot of groundnut), *Phoma betae* (sugarbeet leaf spot), *Puccinia carthami* (safflower rust), *Puccinia recondita* (wheat brown rust), *Pyricularia oryzae* (rice blast), *Rhizoctonia solani* (sheath blight of rice), *Septoria tritici* (*Septoria* blotch of wheat), *Erwinia amylovora* (fire blight of apple), *Erwinia carotovora* subsp. *carotovora* (soft rot of vegetables), *Xanthomonas axonopodis* pv. *cyamopsidis* (bacterial blight of clusterbeans), *Xanthomonas axonopodis* pv. *malvacearum* (bacterial blight of cotton) and *Xanthomonas oryzae* pv. *oryzae* (bacterial leaf blight of rice), cucumber mosaic virus (cucumber mosaic), rice tungro virus (tungro) and tobacco necrosis virus (Tobacco necrosis).

Flower and fruit pathogens: Fire blight of rosaceous plants caused by the bacterium, *Erwinia amylovora* is an important flower disease. Biological suppression of this disease has been achieved through use of non-pathogenic species of *Erwinia herbicola* and sometimes in combination with *Pseudomonas syringae*. *E. herbicola* was used by spraying aqueous suspensions on the flowers just before the time of potential infection. The mode of action is primarily competition. It competes with the pathogen for a growth-limiting resource and induced cessation of nectar secretion or accumulation of a host toxin. Sprays with spore suspension of the antagonist, *Trichoderma harzianum* were effective in suppressing the grey mould rot (*Botrytis cinerea*) incidence in grapevine. *Gliocladium roseum, Penicillium* sp. and *Trichoderma viride* were as effective as fungicides in suppressing *B. cinerea* on strawberries.

Post-harvest pathogens: Suppression of post-harvest diseases in fruit crops by species of *Aureobasidium, Debaryomyces, Sporobolomyces and Trichoderma,* (fungi), *Bacillus, Enterobacter,* and *Pseudomonas* (bacteria) has been reported by many workers.

The list of biocontrol agents and the pathogens controlled by each are given in Table 3.

Table 3. List of antagonistic organisms and plant pathogens controlled by them.

Sl. No.	Antagonist	Pathogens controlled
I	**Fungi**	
1.	*Acremonium persicianum*	*Puccinia arachidis*
2.	*Alternaria* sp.	*Alternaria porri*
3.	*Ampelomyces quisqualis* (syn. *Cicinnobolus sesatti*)	*Erysiphe graminis, Sphaerotheca fuliginea, Uncinula necator*
4.	*Aphanocladium album*	*Puccinia recondita*
5.	*Chaetomium globosum*	*Fusarium roseum, Pythium* spp., *Venturia inaequalis*
6.	*Cladosporium* spp.	*Venturia inaequalis*
7.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum, Sclerotium cepivorum*
8.	*Darluca filum* (syn. *Eudarluca caricis*)	*Puccinia arachidis*
9.	*Debaryomyces hansenii*	*Geotrichum candidum, Penicillium digitatum, P. italicum*
10.	*Fusarium equiseti*	*Sclerospora graminicola*
11.	*Fusarium proliferatum*	*Plasmopara viticola*
12.	*Gliocladium catenulatum*	*Sclerotinia sclerotiorum*

13.	*G. roseum*	*Phytophthora* spp., *Verticillium dahliae*
14.	*G. virens* (syn. *Trichoderma virens*)	*Botrytis cinerea, Pythium* spp., *Rhizoctonia solani, Sclerotium rolfsii*
15.	*Hansfordia* spp.	*Phaeoisariopsis personata*
16.	*Laetisaria arvalis*	*Pythium* spp., *Rhizoctonia* spp.
17.	*Myrothecium verrucaria*	*Rhizoctonia solani*
18.	*Penicillium islandicum*	*Puccinia arachidis*
19.	*P. oxalicum*	*Pythium* spp.
20.	*Phialophora radicicola*	*Gaeumannomyces graminis* var *tritici*
21.	*Phlebia gigantea* (syn. *Peniophora gigantea*)	*Fomes annosus*
22.	*Pythium nunn*	*Phytophthora cinnamomi, Pythium aphanidermatum, P. ultimum, Rhizoctonia solani*
23.	*Scytalidium* sp.	*Cronartium quercum, Endocronartium harknessii, Poria carbonica*
24.	*Sphaerellopsis filum*	*Puccinia recondita*
25.	*Sporidesmium sclerotivorum*	*Botrytis cinerea, Sclerotinia minor, S. sclerotiorum, S. trifoliorum, Sclerotium cepivorum*
26.	*Stilbella aciculosa*	*Rhizoctonia solani*
27.	*Talaromyces flavus*	*Rhizoctonia solani, Verticillium dahliae*
28.	*Trichoderma hamatum*	*Pythium* spp., *Rhizoctonia solani*
29.	*T. harzianum*	*Botrytis cinerea, Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotium rolfsii*
30.	*T. koningii*	*Sclerotinia sclerotiorum*
31.	*T. polysporum*	*Phytophthora cinnamomi*

32.	*T. viride*	*Armillaria mellea, Botrytis cinerea, Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani, Sclerotium rolfsii*
33.	*Trichothecium plasmoparae*	*Plasmopara viticola*
34.	*T. roseum*	*Venturia inaequalis*
35.	*Tuberculina costaricana*	*Cronartium* spp., *Puccinia arachidis, Urediniopsis mirabilis*
36.	*Verticillium hemileiae*	*Hemileia vastatrix*
37.	*V. lecanii*	*Puccinia arachidis, Uromyces appendiculatus*
II	**Bacteria**	
38.	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*
39.	*Azotobacter chrococcum*	*Rhizoctonia solani*
40.	*Bacillus subtilis*	*Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotium rolfsii, Streptomyces scabies, Verticillium* spp.
41.	*Bacillus thuringiensis*	*Alternaria alternata, Hemileia vastatrix*
42.	*Erwinia herbicola* pv. *herbicola*	*Erwinia amylovora*
43.	*Pseudomonas fluorescens*	*Fusarium* spp., *Macrophomina phaseolina, Pyricularia oryzae, Pythium* spp., *Rhizoctonia solani*
44.	*P. cepacia*	*Cercospora* sp.
45.	*Streptomyces diastaticus*	*Pythium aphanidermatum*
46.	*S. griseoviridis*	*Alternaria brassicola, Fusarium oxysporum* f. sp. *dianthi, Rhizoctonia solani*

IDENTIFICATION CHARACTERS

The important species of biocontrol agents in fungi and bacteria with their characteristic features are furnished

1. *Trichoderma* Persoon

(Deuteromycotina, Hyphomycetes, Moniliales, Moniliaceae)

Harz first clearly delimited the genus *Trichoderma* in 1871. Oudemans and Koning in 1902 first reported it in soil, where it is now known to be ubiquitous. In 1927, Gilman and Abbott considered that there were four species in the genus and it was accepted by Gilman in 1957, Bisby (1939) confirmed the connection between the Pyrenomycete, *Hypocrea rufa* and *T.viride* and considered that the conidial state of *H. gelatinospora* was a *Trichoderma. Trichoderma* spp. are more common in soil. Rifai (1969) revised the genus *Trichoderma* to include nine species and this has now generally been accepted.

Trichoderma spp. commonly inhabit soils having high moisture content, although strains probably grow down to –80 bars. Because of the extremely rapid growth and copious production of spores, *Trichoderma* spp. rapidly colonize substrata in soil after chemical or thermal treatment of the soil. Steamed and fumigated soils are quickly colonized by this fungus. *T. harzianum* survived in soil without a food base for at least 130 days but did not survive well or increase in the rhizosphere of bean or pea seedlings. *Trichoderma* spp. breakdown the cellulosic material by the production of cellulolytic enzymes. Cellulolytic enzyme produced by *T. reesei* help the sugar industry to dispose of sugar by-products, molasses and sugarbeet pulp by transferring sugar to protein. *T. harzianum* possesses oxidation system which is capable to degrade the organochlorine pesticide, endosulfan. Chitinolytic enzymes from *T. harzianum* and *T. virens* are responsible for degradation of fungal cell wall and are effective biocontrol agents to *Botrytis cinerea.*

Trichoderma spp. are mycoparasites and aggressive competitors with pathogens. Mycelia of *Trichoderma* spp. grow along and coil around hyphae of host fungi. Penetration of host mycelia may or may not occur, but susceptible hyphae become vacuolated, collapse and finally disintegrate. The mycoparasite then grows on the hyphal

contents. Much of the literature attributed for the biological control by *T.viride* is the production of the antibiotics *viz*., gliotoxin and viridin.

Chet and Baker (1980) showed that *T. harzianum* and *T. hamatum,* acting as mycoparasites of *Rhizoctonia solani* and *Sclerotium rolfsii*, produced β-1,3 glucanase and chitinase that caused exolysis of the host hyphae; antibiotics were not observed. The optimal pH levels for these enzymes are 4.5 and 5.3 respectively. β - 1, 3 glucanase produced by *T. viride* solubilizes mycelia of *Sclerotinia sclerotiorum*. *Trichoderma hamatum* also produces cellulase, which perhaps explains it ability to parasitize *Pythium* spp. *Trichoderma harzianum* is not cellulolytic. *T. koningii* and *T. reesei* produce cellulase enzymes. *T. viride* is an active antagonist in moist soil but is inhibited under very wet conditions when in soils at pH 5.4 or above. *T. harzianum* was most active as an antagonist in soil at pH 6.5 or lower. The efficiency of *T. harzianum* in control of *S. rolfsii* on bean decreased as the soil temperature rose above 22^{o}C.

Generic characters

The important characters for identifications are as follows:

Colonies: Mostly grow rapidly, no aerial mycelium, diffusible in shades of yellow or absent, while deep yellow pigments is characteristic to section longibrachiatum.

Mycelium: Smooth, hyaline and septate.

Chlamydospores: Globose to subglobose, intercalary, rarely terminal, smooth, green and less than 15 µm in dia.

Conidiophores: Long, thick, with or without branches, may or may not bear whip-like sterile hyphae (or elongation).

Phialides: On hyphae within aggregates is characteristic, but virtually impossible to define, flask-shaped.

Conidia: Less than 5 µm long and wide, globose to subglobose, ovoid, ellipsoidal to oblong, some shades of greenish yellow or colourless, green shades may range from deep green to nearly green (olive green) in mass.

T. hamatum (Bon.) Bain. (Perfect state: *H. semiorbis*)

Colonies: Slow growing and aerial mycelium is floccose.

Mycelium: Hyaline, smooth and 2 to 3 µm in dia

Chlamydospores: Abundant, globose to sub-globose, smooth and 7 to 12 µm in dia

Conidiophores: Highly ramified from very compact tuft. Lower portion with thick, fertile side branches, short, 2 to 3 barrel-shaped or shortly cylindrical cell, upper part typically sterile.

Phialides: Smooth, hyaline to pale green, short, plumpy, pear-shaped, ovoid, narrower at base than at middle, attenuate towards apex, short, conical neck and 46.5 x 3.0 – 4.0 µm in size.

Conidia: Globose, conical, pale green, smooth, rounded at the apex, attenuated slightly below into truncate base and 3.8 to 6.0 x 2.2 µm.

T. harzianum Rifai

Colonies: Grows rapidly, aerial mycelium floccose, whitish green, bright green to dull green.

Mycelium: Septate, hyaline, smooth and 1.5 to 2.5 µm in size.

Chlamydospores: Mostly globose, smooth, intercalary and terminal and 6 to 12 µm in dia.

Conidiophores: Hyaline, smooth, loose tuft, main branch produces numerous side branches especially in the lower portion.

Phialides: Arise in false verticillate up to five in number; short skittle-shaped, narrow at the base, attenuate, abruptly sharp, pointed neck and 25 to 75 x 3 to 4 µm.

Conidia: Sub-globose to short obovoid, often broad truncate base, smooth, pale green, much darker in mass, 2.8 to 3.2 x 2.5 to 2.8 µm.

T. koningii Oud.

Colonies: Fast growing, greenish white, dull green to dark green.

Mycelium: Hyaline, highly ramified and 2 to 5 µm.

Chlamydospores: Formed in submerged hyphae, globose, ellipsoid to barrel-shaped and up to 12 µm in dia.

Conidiophores: Much branched, compact or in loose tuft, main branch produces several side branches, in groups of 2 to 3, at wide angles.

Phialides: Pin-shaped, narrower at the base, attenuate towards apex, 7.5 to 12.0 x 2.5 to 3.5 µm.

Conidia: Elliptical or oblong, truncate base and rounded apex, smooth, pale green and appear much darker in mass and 3 to 5 x 1 to 2 µm in size.

T. piluliferum Webster & Rifai (Perfect state: *H. pallida* and *H. pilulifera*)

Colonies: Slow growing and white mycelial mat is formed over the surface.

Mycelium: Smooth, colourless and up to 10 µm in dia.

Chlamydospores: Infrequently globose or ellipsoidal.

Conidiophores: Highly ramified, compact tuft, main branches 5 to 7 µm in dia and arise in groups of 2 or 3.

Phialides: Typically short, plumpy, 4 to 6 x 2.0 to 3.5 µm, terminal, attain 10 µm dia, irregular whorls, short, flask-shaped, almost pyriform, narrower at base, truncate, apiculus and 2.5 to 3.5 µm in size.

Conidia: Produced singly and accumulated at the tip, globose to sub-globose, smooth, colourless, base truncate, apiculus and 2.5 to 3.5 µm in size.

T. polysporum (Link ex Pers.) Rifai (Syn. *Spototrichum polysporum* Link ex Pers.)

Colonies: Slow growing and watery white.

Mycelium: Ramified, hyaline, smooth and 2 to 3 µm.

Chlamydospores: Infrequently intercalary.

Conidiophores: Irregular, close together, tuft, long, cylindrical apex, elongate into sterile hyphal elongation.

Phialides: Short, plumpy, pear-shaped, wider above the middle, short, conical neck, crowded, whorl-like arrangement.

Conidia: Ellipsoidal to elongate, hyaline, smooth and 2.8 to 3.8 x 1.8 to 2.0 µm.

T. pseudokoningii Rifai (Perfect state: *H. schweinitzii*)

Colonies: Grows rapidly with very poor aerial growth.

Mycelium: Septate, hyaline, smooth and 1 to 5 µm.

Chlamydospores: Globose, hyaline, smooth and 7 to 10 µm in dia.

Conidiophores: Loosely tuft, may appear hairy at maturity, somewhat powdery with numerous long branches. Branches are irregularly formed, single or in opposite pairs or group of three.

Phialides: In false whorls, opposite pair in group of four in apical portion, skittle-shaped or pin-shaped, narrower at the base than middle, attenuated distinctly, obovate, spindle-shaped and 5.5 to 8.0 x 2.5 to 3.5 µm in size.

Conidia: Short, sub-cylindrical, almost oblong, ellipsoidal, usually rounded, and distally attenuate below, short, truncate base, green mass and 3.5 x 2.2 µm.

T. virens (Miller *et al*,) v. Arx. (Perfect state: *H. virens; Gliocladium virens* Miller *et al.*)

Colonies: Grows rapidly, aerial mycelium floccose.

Mycelium: Septate, hyaline, smooth, white to greyish

Chlamydospores: Globose, thin-walled and 6 to 8 µm in dia.

Conidiophores: Sub-hyaline, 30 to 300 x 2.5 to 4.5 µm, towards base, frequently unbranched for about half of the length, towards the apex, branching irregularly with each branch terminated by a cluster of 3 to 6 closely appressed apical flask-shaped phialides, branches arise at right angles are reflexed towards the apex, primary branches arising singly or in opposite pairs immediately beneath the septa, rebranched irregularly once or twice, ultimate branches one or two celled.

Phialides: Ampulliform to lageniform, 4.5 to 10.0 x 2.8 to 5.5 µm, swelling in the middle, mostly arising in closely appressed verticils of 2 to 5 or terminal branches.

Conidia: Broadly ellipsoidal to obovoid, dark green and 4.4 x 3.5 µm.

T. viride Pers. ex Gray [Perfect state: *Hypocrea rufa* (Pers. ex Fr.) Fr.; *H. gelatinospora* (Tode) Fr.]

Colonies: Fast growing, surface smooth, become hairy; dark green and typical coconut odour is emitted.

Mycelium: Hyaline, smooth, septate and much branched.

Chlamydospores: Globose, rarely ellipsoid, intercalary and 10 to 15 µm in dia.

Conidiophores: Arise in compact or loose tuft, main branches produce several side branches, in groups of 2 to 3 and all branches stand at wide angles.

Phialides: In false whorls beneath each terminal phialides, usually more than 2 to 3 phialides and 8 to 15 x 2 to 3 µm.

Conidia: Globose or short obovoid, broadly ellipsoidal, at distant apiculus like base, minute ruffling of their walls, pale green, smooth, 3.5 to 4.5 µm in size and accumulated at the tip of each phialides.

2. *Bacillus* Cohn.

(Kingdom – Procaryotae; Division – Firmicutes; Class – Firmibacteria)

Spore forming rods, mesophilic with maximum growth at 30-45^{o}C. Usually Gram-positive. It uses a wide range of simple organic compounds; growth-factor requirements also simple. Resistant endospores formed in cells may remain dormant for long periods. Selectively isolated by treating aqueous soil suspensions in hot water at 80^{o}C for 10 min or by treating soil with aerated steam (60^{o}C for 30 min). Motile by peritrichous flagella; aerobic or facultatively anaerobic. When inoculated to onion seeds, it gives protection against *Sclerotium cepivorum.* It inhibited the germination of sclerotia in the soil, perhaps due to the production of antibiotics. It also controls damping-off caused by *Pythium ultimum* and *R. solani.* It protects wounds in apple trees against infection by *Nectria galligena.*

Bacillus cereus Frankland & Frankland

It is most abundant in soil. Rods, 1.0-1.2 x 3.0-5.0 µm, Gram-positive, non-capsular, aerobic, motile and in chains. Endospores are central, form in 18 - 48 h and 1.0 x 1.5 µm. Colonies are large, usually rough, flat, whitish, form wavy arrangement of chains of cells that may develop into whip-like outgrowths.

Bacillus subtilis (Ehrenberg) Cohn

Rods and seldom in chains. Endospores are oval, 0.8 x 1.5 - 1.8 µm, thin-walled, located centrally in the cell but does not distend the walls. Cells are relatively small and 0.7 - 0.8 x 2.0 – 3.0 µm in size. Flagella are found laterally. Colonies are round or irregular.

3. ***Pseudomonas*** Migula

(Kingdom – Procaryotae; Division – Gracilicutes; Class – Proteobacteria; Family - Pseudomonadaceae)

Common inhabitants of soil, plant debris and especially in the rhizosphere. It is efficient producers of potent antibiotics. *Pseudomonas* spp. are favoured by moist soil high in organic matter and are especially prevalent in the rhizosphere and on the rhizoplane. They are capable of growing over a wide temperature range, including at near freezing temperatures and at 35 - 37^{o}C, depending on the strain. The action of the fluorescent pseudomonads in plant growth enhancement is thought to result from their production in the rhizosphere of a siderophore, pseudobactin that deprives pathogens of iron, thereby permitting the plant to grow better. *P. fluorescens* was inhibitory in culture to *Phymatotrichum omnivorum, Phytophthora megasperma, Pythium aphanidermatum Rhizoctonia solani* and *Sclerotinia sclerotiorum* and that this effect could be overcome by adding iron to the medium, which prevented fluorescent pigment formation.

Cells are single, straight or curved rods, Gram-negative and 0.5 - 1.0 x 1.5 – 4.0 µm in size; motile by polar monotrichous or multitrichous flagella. No sheaths or prothecae are produced; no resting stages known. Metabolism respiratory, never fermentative; strict aerobes. Catalase positive. Frequently develop fluorescent, diffusible pigments of green, blue, violet, lilac, rose or yellow, particularly in iron-deficient media; many species develop no pigment.

Pseudomonas cepacia Ballard *et al.* (Syn. *P. multivorans).*

No fluorescent pigments. Flagella multitrichous. Highly omnivorous; uses more than 100 organic compounds as carbon sources including cellobiose, D-arabinose and D-fructose.

Pseudomonas fluorescens Migula

Cells have multitrichous flagella. Gelatin liquified; pynocyanine absent; oxidase positive; arginine dihydrolase positive. Produces soluble fluorescent pigments. No growth-factor requirements; uses glucose and aromatic compounds but not starch. Lysed

by ethylene diamine tertra acetic acid (EDTA). Maximum growth is observed at 35 - 37^{o}C. Seven biotypes have been recognized.

Pseudomonas putida *(*Trev.) Migula

Some cells are more than 4 µm long. Gelatin not liquified; produces no pyocyanin; oxidase positive; arginine dehydrolase positive. Maximum growth is at 35 - 37^{o}C.

METHODS OF IDENTIFICATION

Fungal antagonists

Fungal antagonists are isolated from soil, purified by adopting specific technique and identification characters of different fungal species are given in the previous chapter.

Bacterial antagonists

Identification characters of different bacterial species used as biocontrol agents in disease management are given in previous chapter. Specific methods used to test different genera of bacterial antagonists are described below.

A. Staining methods

The chemical substances commonly used to stain bacteria are known as **dyes**. Dyes are classified as natural and synthetic. The natural dyes are used mainly for histological purposes while the synthetic ones are used for the bacterial stain preparations. Chemically a **dye** (stain) is defined as an organic compound containing a benzene ring plus a chromophore and auxochrome group. Such dyes are acidic, basic or neutral. Acidic dyes (*e.g.,* picric acid, acid fuchsin and eosin) are anionic and more alkaline in nature and stain the cytoplasmic components of the cells. On the other hand the basic dyes (*e.g*., methylene blue, crystal violet and safranin) are cationic and combine with those cellular elements, which are acidic in nature *(e.g*., nucleic acids). Neutral stains are formed by mixing the aqueous solutions of certain acidic and basic dyes. Staining solutions are prepared by dissolving a particular stain in either distilled water or alcohol. The stain is applied to smears for 30 - 60 sec, washed, dried and examined under the microscope.

There are two kinds of staining procedures, simple and differential. Simple stains involve a single dye (*e.g.*, methylene blue, crystal violet or carbol fuchsin) and cells and structures within each cell will attain the colour of the stain. Differential stains require more than one dye and distinguish between structures within a cell or types of cells by staining them with different colours. The various staining procedures involved in the identification of bacterial antagonists are given below.

Simple staining

In simple staining, the cells (smear) are stained by the application of a single staining reagent. A simple stain that stains the bacteria is a direct stain. The purpose of the simple staining technique is to determine cell shape, size and arrangement of bacterial cells. Simple staining is performed by basic stains, which has different exposure times (*e.g.*, crystal violet for 30 - 60 sec, carbol fuchsin for 15 - 30 sec and methylene blue for 60 sec).

Procedure

- Take a clean glass slide and mark the smear area on the lower side of the slide with a marking pencil.
- Prepare bacterial smear on the upper side of the slide and heat fix.
- Keep the slide on the staining tray and apply about 5 drops of any of the above stains for the designated period.
- Remove the excess stain and wash the smear gently with slow running tap water.
- Blot dry the slide using blotting paper and observe under a microscope.

Observation

Cells will appear blue or violet or red for the stains, methylene blue, crystal violet and carbol fuchsin respectively.

Negative staining

A stain that darkens the background and does not stain the bacteria is called a **negative stain**. An acidic dye (nigrosine) or indian ink is used in this method. It carries a negative charge, is repelled by the bacteria which too carry a negative charge on their surface, therefore, bacterial cells appear transparent and unstained upon examination. Negative staining is advantageous for two reasons: i. cells appear less shriveled or distorted because no heat fixing is done and ii. capsulated bacteria which are difficult to stain can be observed by this technique.

Procedure

- Place a drop of nigrosine at one end of a clean glass slide.
- With the help of a sterile inoculating loop, transfer a loopful of bacterial inoculum from the broth culture in the drop of stain and mix gently with the loop. When the inoculum is taken from an agar medium, mix a drop of water in the nigrosine.
- Take another clean slide, place it against the drop of suspended organism at an angle of 30^{o} and allow the droplet to spread across the edge of the top slide.
- Spread the mixture of the stained inoculum out a thin wide smear.
- Allow the smear to air dry and observe preparation under oil-immersion objective.

Observation

Cells will be colourless or transparent against a blue background.

Gram staining

The Gram stain was developed by a Danish physician, Dr. Hans Christian Gram in 1884. It is a very useful stain for identifying and classifying bacteria into two major groups *viz*., the Gram-positive and Gram-negative. In this process the fixed bacterial smear is subjected to four different reagents in the order listed: crystal violet (primary stain), iodine solution (mordant), alcohol (decolourizing agent) and safranin (counter stain). The colour changes that occur in bacterial cells at each stage due to these four reagents in the Gram staining process are given in Table 3. The bacteria which retain the

primary stain (appear dark blue or violet) (*i.e.,* not decolourized when stained with Gram's Method) are called **Gram-positive**, whereas those that lose the crystal violet and counter stained by safranin (appear red) are referred to as **Gram-negative** (Table. 4).

Table 4. Reaction of Gram-positive and Gram-negative bacteria to various reagents used in Gram staining.

Reagent	Gram-positive	Gram-negative
None (Heat-fixed cells)	Colourless	Colourless
Crystal-violet	Purple	Purple
Gram's Iodine	Purple	Purple
Ethyl alcohol	Purple	Colourless
Safranin	Purple	Red (pink)

The differences in staining responses to the Gram-stain can be related to chemical and physical differences in their cell walls. The Gram-negative bacterial cell wall is thin, complex, multi-layered structure and contains relatively high lipid contents, in addition to protein and mucopeptides. The higher amount of lipid is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall, which do not close appreciably on dehydration of cell-wall proteins, thus facilitating the leakage of crystal violet-iodine (CV-I) complex and resulting in the decolourization of the bacterium, which later takes the counter stain and appears red. In contrast the Gram-positive cell walls are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides. When treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of (CV-I) complex and cells remain blue.

Procedure

- Make thin smears of bacterial culture on the glass slide and air dry.
- Heat the smears.
- Cover each smear with crystal violet for 1 min and then wash with distilled water for few seconds.

- Cover each smear with iodine solution for 1 min and wash off the iodine solution with 95 per cent ethyl alcohol. Add ethyl alcohol drop by drop, until no more colour flows from the smear. (The Gram-positive bacteria are not affected while all Gram-negative bacteria are completely decolourized).
- Immediately wash the slides with distilled water and drain.
- Apply safranin to smears for 30 – 60 sec (counterstaining) and wash with distilled water and blot dry.
- Let the stained slides air dry and examine the slides microscopically using oil-immersion objective.

Observation

Those bacteria that appear violet are referred as Gram-positive and those appearing red (pink) are described as Gram-negative.

Flagella staining

Flagella are extremely thin, hair-like appendages that protrude through the cell wall and apparently originate from granular body just beneath the cell wall in the cytoplasm. Many species of bacilli possess flagella. Number and position of flagella, like monotrichous, multitrichous, amphitrichous, lophotrichous and peritrichous help in bacterial classification. It helps in movement and vital functions. It is made primarily of protein. Some carbohydrates and possibly some lipids are present. Individually protein molecules are called flagellins. They are antigenic. As the size is so small usually mordants are used for thickening and later stained with dye during flagella staining. There are two procedures used in flagella staining.

Procedure I

- Dip the slides in dichromate solution (cleaning solution) and thoroughly rinse in distilled water and then rinse in 95 % alcohol. Dry with clean towel or tissue paper.
- Pipette out 3 ml sterile distilled water into slants of bacterial culture. Gently agitate and let it stand for 10 min.

- Use a capillary pipette to transfer one drop from the bacterial suspension to the slide and allow it to run across the rigid surface of the slide. Air dry but do not fix with heat.
- Flood the slide with flagella mordant (Loeffler's flagella mordant) for 10 min.
- Wash with slide gently with distilled water and flood with carbol fuchsin (Loeffler's flagella stain) for 5 min.
- Wash the slide gently with distilled water and air dry.
- Examine under oil-immersion objective.

Observation

Bacterial cells will appear blue and the flagella will appear as red.

Precautions: i. Always use cleaned, grease-free slides.
ii. Do not heat fix the smear.
iii. Do not blot dry the smear.
iv. Always use reduced illumination to see the flagella clearly.

Procedure II

- Take clean, grease-free slide and mark the slide into two halves with glass marking pen / pencil.
- Add 2 ml of sterile water to 24 old bacterial slant culture and keep undisturbed for 10 to 20 min to allow the bacteria to swim out into the water.
- Transfer a drop of bacterial suspension to microscopic slide and tilt the slide till it comes to the central line.
- Dry in air and do not heat fix.
- Cover the smear with 1 ml of Leifson's staining solution and allow to act until a very fine rusty precipitate is obtained in about 10 min.
- Wash in tap water without first pouring off the stain.
- Counterstain with methylene blue for 5 – 10 min.
- Wash with water, air dry and examine under microscope.
- Place the slide over a rack. Pour the mordant and heat until the steam arise.

- Allow to react for 5 min and rinse it with distilled water.
- In the same way treat the smear with Leifson flagella stain.
- Dry in air and observe the colour of bacterium and flagella.

Observation

Without counterstain, the cells and the flagella stain pinkish red; with the counterstain, the cells stain blue and the flagella red.

Spore staining

Under conditions of inadequate nutrition and unfavourable factors to growth, specialized structures called spores or endospores are formed in certain Gram-positive cells such as aerobic rods (Bacilli), anaerobic rods (Clostridia) and a few cocci (Sporosarcinae). These spores have no metabolic activity and are resistant to heat, drying, freezing, toxic compounds and radiation. The spores have a high Ca^{++} content and dipicolinic acid which form 5-15 per cent of their dry weight.

Procedure

- Prepare a smear of 24 h old bacterial culture on a clean slide, air dry and heat fix.
- Flood the slide with Malachite green and place a cut piece of paper to wetting over the smear, so that it completely covers the smear and soaks up most of the stain. The toweling should be saturated with the stain throughout the staining process.
- Gently heat the slide until the stain begins to evaporate and keep it for 5 min.
- Remove the paper towel and wash the slide gently with water until all the excess stain is removed.
- Counterstain with basic fuchsin for 30 sec.
- Wash with water, blot it and air dry.
- Examine the slide using oil-immersion objective.

Observation

Observation under microscope will show red coloured cells containing green spores.

Composition of stains and solutions

Disinfectant (for surface sterilization of plant materials and inoculation chamber)

Mercuric chloride	-	20 g
Conc. HCl	-	100 ml

(Dilute 5 ml of the stock solution to 1.0 lit of water)

Methylene blue

Methylene blue (90 % dye content)	-	0.3 g
Distilled water	-	100.0 ml

Crystal violet (Hucker's modified)

Solution A

Crystal violet (90 % dry content)	-	2 g
Ethyl alcohol (95 %)	-	20 ml

Solution B

Ammonium oxalate	-	0.8 g
Distilled water	-	80.0 ml

Mix solutions A and B

Ziehl's Carbol-Fuchsin (acid-fast stain)

Solution A

Basic fuchsin (90 % dye content)	-	0.3 g
Ethyl alcohol (95 %)	-	10.0 ml

Solution B

Phenol	-	5.0 g
Distilled water	-	95.0 ml

Mix solutions A and B

Loeffler's alkaline methylene blue

Solution A

Methylene blue (90 % dye content)	-	0.3 g
Ethyl alcohol (95 %)	-	30.0 ml

Solution B

Dilute potassium hydroxide (0.01 %)	-	100 ml

Mix solutions A and B

Dorner's Nigrosin solution (Negative staining)

Nigrosin (water soluble)	-	10.0 g
Distilled water	-	100.0 ml
Formalin	-	0.5 ml

Dissolve nigrosin in distilled water and immerse the mixture in boiling water bath for 30 min. Then add formalin (as preservative). Filter the solution twice through double filter paper and store in small tubes.

Gram's iodine / Lugol's solution (Mordant)

Iodine	-	1.0 g
Potassium iodide	-	2.0 g
Distilled water	-	300.0 ml

Ethyl alcohol (95 %)

Ethyl alcohol (100 %)	-	95.0 ml
Distilled water	-	5.0 ml

Safranin (Gram staining and spore staining)

Safranin (2.5 % solution in 95 % ethyl alcohol)	-	10.0 ml
Distilled water	-	100.0 ml

or

Safranin O	-	0.25 ml

Ethyl alcohol (95 %)	-	10.0 ml
Distilled water	-	100.0 ml

Loeffler's flagella mordant (Flagella stain)

Aqueous tannic acid (20 %)	-	100.0 ml
Ferrous sulphate	-	20.0 g
Basic fuchsin (10 %) in alcohol	-	10.0 ml
Distilled water	-	40.0 ml

Dissolve ferrous sulphate crystals in distilled water by warming and add the remaining ingredients.

Loeffler's flagella stain

Basic fuchsin (1 %) in alcohol	-	20.0 ml
Aniline water (3 %)	-	80.0 ml

Leifson's stain (Flagella staining)

Solution A

Basic fuchsin	-	1.2 g
Absolute alcohol	-	100.0 ml

Shake vigorously to dissolve. Store in a tightly stoppered bottle.

Solution B

Tannic acid	-	3.0 g
Distilled water	-	100.0 ml

Add 0.2 % phenol, if stored for long.

Solution C

Sodium chloride	-	1.5 g
Distilled water	-	100.0 ml

Mix solutions A, B and C and store in a tightly stoppered bottle in a refrigerator. Shake well after bringing out from the deep freezer.

Counterstain (Flagella staining)

Methylene blue	-	1.0 g
Distilled water	-	100.0 ml

Saline / Normal saline solution

Sodium chloride	-	8.5 g
Distilled water	-	1.0 lit

Malachite green (Spore staining)

Malachite green	-	5.0 g
Distilled water	-	100.0 ml

Fungal stains

i. Cotton blue lactophenol

Lactic acid	-	20.0 ml
Phenol crystals	-	20.0 g
Glycerol	-	40.0 ml
Distilled water	-	20.0 ml
Cotton blue (1 % aqueous)	-	2.0 ml

Add lactic acid and glycerol / glycerine to the distilled water and mix thoroughly. Add phenol crystals and heat gently in hot water with frequent agitation until the crystals completely dissolve. Add the dye and mix thoroughly. Store the stain in brown bottle.

ii. Water – iodine solution

Gram's iodine (as Gram's staining)	-	10.0 ml
Distilled water	-	30.0 ml

iii. Congo Red (for bacteria and fungi)

Congo Red (80 % dye content)	-	2.0 g

Distilled water - 100.0 ml

B. Biochemical methods

Biochemical tests for *Bacillus subtilis*

i. Starch hydrolysis

- Prepare starch agar plates
- Streak bacterial culture on agar plates by dividing the plate into different sections
- Incubate the plates for 48 h at 37^{o}C
- Flood the agar plates with Gram's iodine solution, allow for 30 sec contact and pour off the excess solution
- Examine the cultures for the blue black colour surrounding the growth of each test organism

Observation

B. subtilis exhibits a clear zone, since it produces amylase enzyme

ii. Catalase test

- Apply 3 to 4 drops of 3 % hydrogen peroxide to flow over the entire surface of the culture
- Examine for the presence or absence of bubbling

Observation

B. subtilis is catalase positive

iii. Nitrate reduction test

- Prepare nitrate broth medium (nutrient broth + 0.1 % KNO_3 + 0.1 % agar)
- Inoculate the test bacterium and incubate for 24 – 48 h at 37^{o}C
- Add 5 drops of sulfanilic acid solution and 5 drops of methyl-alpha napthylamine solution

Observation

Examine for the appearance of red colour.

iv. Acid and gas production from glucose

- Prepare phenol red glucose broth and take it in a test tube with Durham's tube
- Inoculate the test organism and incubate for 24 – 48 h at 37^{o}C
- Examine for the presence of acid and gas

Observation

B. subtilis is acid positive and gas negative.

IV. MECHANISIM OF ACTION OF BIOCONTROL AGENTS

Understanding the mechanisms by which biocontrol agents suppress the plant pathogens is essential for improvement and wider use of biological methods. Researchers working in biological control were able to establish the involvement of certain metabolites particularly antibiotics, siderophores, ammonia, hydrogen cyanide, enzymes and stress-induced plant proteins in the mechanisms of biological control.

Biological control is principally achieved through antagonism which involves

1. Competition
2. Mycoparasitism / Hyperparasitism
3. Antibiosis and lysis
4. Induced Systemic Resistance (ISR)

Microorganism, which fulfils all the above criteria, will have a better chance to be a successful biocontrol agent. Antagonists of plant pathogens may be resident or introduced. Resident antagonists are part of the natural microbiota in soil or on roots, leaves or other plant parts, which provide biological control of a plant pathogen through any of the aforesaid mechanisms. Introduced antagonists are applied as cultures or prepared products to soil or to the plant for the purpose of controlling a pathogen through one or more forms of antagonism.

1. Competition

Microorganisms compete for space and minerals and organic nutrients to proliferate and survive in their natural habitats. This has been reported in both rhizosphere as well as phyllosphere. The term **rhizosphere** was proposed by Hiltner (1904) for the zone of soil around plant roots characterized with intense microbial activity. Microhabitat of the root surface itself is designated as **rhizoplane**. The term **phyllosphere** was introduced by Last (1955) and Ruinen (1956) more or less simultaneously for the microhabitat adjacently around the living leaves. Since the microbes are isolated from the living leaves the term **phylloplane** was suggested to be more appropriate than the phyllosphere. In all these cases, there is opportunity to exploit competition by biocontrol agents so that

pathogens are deprived of essential elements for the completion of the infection process or life cycle. Elements for which microorganisms generally compete are carbon, nitrogen and iron, which are essential for germination and penetration of host tissue by pathogens.

Competition has been suggested to play a role in the biocontrol of species of *Fusarium* and *Pythium* (causing wilt and rot of several plants) by some strains of fluorescent pseudomonads. Competition for substrates is the most important factor for heterotrophic soil fungi. Success in competition for substrate by any particular fungal species is determined by competitive saprophytic ability (CSA) and inoculum potential of that species. Those fungi with highest number of propagules or the greatest mass of mycelial growth (biomass) have the greatest competitive advantage. **Competitive saprophytic ability** is the summation of physiological characteristics that make for success in competitive colonization of dead organic substrates. The suggested four characteristics, which are likely to contribute to the competitive saprophytic ability, are

a. rapid germination of fungal propagules and fast growth of young hyphae towards a source of soluble nutrients
b. appropriate enzyme equipment for degradation of carbon constituents of plant tissues
c. excretion of fungistatic and bacteriostatic metabolites including antibiotics and
d. Tolerance of fungistatic substances produced by competitive microorganisms.

Inoculum potential of a fungus is described as the energy of growth of a fungus available for colonization of a substrate to be colonized. Possession of any of the four characteristics and inoculum potential is sufficient for a microbe to get success in microbial competition.

Successful biological control of root and butt-rot of conifers caused by *Heterobasidion annosum* by the fungal antagonist, *Peniophora gigantea* operates on the basis of competition. Competition is more effective in yeasts and bacteria and the filamentous fungi have been found relatively less effective. In the phylloplane, yeasts and bacteria are known to inhibit the leaf pathogens solely by competing for nutrients.

Leakage of solutes from spores and germ tubes of pathogens provides an additional source of nutrients for bacteria on plant surfaces. This results into enhanced populations of bacteria in the vicinity of fungal spores. When bacteria were present around the conidia of *Botrytis cinerea*, the leaked nutrients were preferentially absorbed by the bacteria, making them unavailable to conidia. In the absence of bacteria, leaked nutrients were reabsorbed by conidia, enhancing its germination.

Majority of necrotrophic plant pathogens require exogenous nutrients derived from plant leachates and from plant surface for their temporary saprophytic phase on the plant surface prior to penetration. Yeasts and filamentous fungi by actively consuming nutrients on plant surface are able to restrict the infection by necrotrophic pathogen such as *Cochliobolus sativus.* Yeasts, *Sporobolomyces* and *Cryptococcus* reduce the infection of maize leaves by *Colletotrichum graminicola* by reducing the availability of nutrients to germinating aspersoria. Since biotrophic pathogens such as rusts and powdery mildews do not consume any exogenous substrate for their spore germination, nutrient competitors are ineffective in inhibiting their germination and germ tube.

Some examples of successful competitors competing for nutrients with other microorganisms are bacteria vs. *Streptomyces scabies* (for oxygen), soil amoebae vs. *Gaeumannomyces graminis* var. *tritici* (wheat roots), *T. viride* vs. *Fusarium roseum* (wheat straw), *Chaetomium* spp. vs. *Cochliobolus sativus* (wheat straw), *Arthrobacter globiformis* vs. *Fusarium oxysporum* f. sp. *lini* (glucose and nitrate).

Siderophores

Siderophores are the extra cellular, low molecular weight (500 to 1000 Daltons) iron (III)-transport agents, which selectively make complex with iron (III) (Fe^{+++} ions) with very high affinity. Most aerobic and facultative anaerobic microorganisms respond to low iron stress by producing siderophores. It is a particular form of nutrient competition involving iron and this has been proposed as one of the mechanisms of biological control. On the basis of their chemical structure, siderophores are divided into

two distinct types *viz.,* catecholate type *i.e.,* with catechol residue and hydroxymate type *i.e.,* with hydroxymic acid residue. The general characteristics of siderophores are

1. They act as iron chelating agents (proteins).
2. They possess low molecular weight.
3. They are produced by microbes and plants.
4. The siderophores produced by one organism can be utilized by another organism although there is a great deal of specificity in their uptake mechanism.
5. Siderophores are utilized specifically *i.e.,* the producer organisms have special receptors for their utilization.
6. Under condition of low iron solubility in soil, iron chelators are extremely important for mobilizing iron and increasing its availability to plants and microbes.

The siderophore system of iron assimilation is very widely distributed in the microbial world and is known to operate in Gram-negative and Gram-positive bacterial species, animal and plant pathogens, diverse types of aerobic bacteria and fungi, symbiotic and free-living N_2-fixing bacteria and others.

Table. 5. Distribution of siderophores among microbial species.

Microorganism	**Siderophore**
A. Fungi	
Aspergillus spp. and *Penicillium* spp.	Ferrichromes
Neurospora spp. and *Ustilago* spp.	Copnogen
Rhodotorula sp.	Rhodotorulic acid
Ectomycorrhizal species	Hydroxymate type
B. Bacteria	
Actinomyces sp.	Ferrioxamines

Agrobacterium tumefaciens	Agrobactin
Anabaena sp.	Schizokinen
Arthrobacter sp.	Arthrobactin
Bacillus megaterium	Schizokinen
Enteric sp.	Agrobactin, Enterobactin
Pseudomonas sp	Pseudobactin, Pyochelin, Pyoverdine, Terribactin
Mycobacteria	Mycobactins

Rhizobacteria, the soil fluorescent pseudomonads, particularly specific strains of the *Pseudomonas fluorescens* and *Pseudomonas putida* group rapidly colonize plant roots of several crop plants. These bacteria generally produce fluorescent, yellow-green water-soluble siderophores with both hydroxymate and phenolate groups. These siderophores have been classified as either pyoviridins or pseudobactins. Different siderophores differ in their affinity for iron (and other cations) so that there can be competition between siderophores and those with the highest affinity will sequester all or most of the iron. If an antagonist produces a better siderophore than the pathogen, then the latter could be deprived of iron and therefore grow less well.

Kloepper *et al*. (1980) were the first to demonstrate the importance of siderophore in the biological control of plant pathogens. The function of siderophore is to supply iron to the cell. The concentration of soluble ferric ion at pH 7 is very low (10^{-17} M) to sustain microbial growth. Soil borne-microorganisms capable of producing siderophores are very active in low iron condition. In the field, these iron helating compounds of Plant Growth Promoting Rhizobacteria (PGPR) such as fluorescent pseudomonads, are thought to suppress the Deleterious Rhizosphere Microorganism (DRMO) since it creates artificial scarcity of iron for phytopathogen. The siderophore produced by PGPR has higher affinity for iron than those produced by phytopathogens or DRMOs. The siderophore producing cell in PGPR possess a specific receptor in the outer cell membrane, which helps in picking up the iron-siderophore complex from the soil

environment. Further PGPR also have the ability to utilize the ferri-siderophore complex produced by the DRMOs. On the contrary, DRMOs or the plant pathogens lack the receptor protein for ferri-siderophore complex of PGPR.

Stimulation of plant growth and yield increase by siderophore-producing pseudomonads are likely to be due to suppression of plant growth inhibiting microorganisms, which are not necessarily plant pathogens. Apart from suppression of non-parasitic harmful microorganisms, it also reduced the infection by various plant pathogens *viz*., *Gaeumannomyces graminis* var *tritici* (take-all disease of wheat and barley), *Fusarium oxysporum* f. sp. *lini* (wilt of flax), *Pythium* and *Rhizoctonia* spp. (seedling blight and wilt of cotton), *Erwinia carotovora* (tuber decay of potato), *Phytophthora* root rot of soybean and *Pythium* root rot of wheat. The yellow-green, fluorescent siderophore produced by Pseudomonas spp. is called as **pseudobactin**. Iron starvation (due to competition) prevents the germination of spores of fungal pathogens in rhizosphere as well as rhizoplane.

Apart from their role in iron chelation, siderophore may act as growth factor while some are potent antibiotic exhibiting both fungicidal and bactericidal effects under condition of low iron. The various compounds of siderophores produced by fluorescent pseudomonads are ferribactin, ferrichrome, ferrioxamine, phytosiderophores, pseudobactin B 10, pyochelin, pyoverdine, *etc*. In phyllosphere, siderophores may originate from the plant **(phytosiderophores)** or from the colonizing microorganisms. Phytosiderophores, found only in some grasses are produced under iron-limiting conditions. These are mugineic acid from barley, avenic acid-A from oat and 2-deoxymugineic acid from wheat. Phytosiderophores appear to have less affinity than microbial siderophores for ferric ion.

2. Mycoparasitism or Hyperparasitism

Mycoparasitism or hyperparasitism occurs when the antagonist invades the pathogen by secreting enzymes such as chitinases, cellulases, glucanases and other lytic enzymes. Mycoparasitism is the phenomenon of one fungus being parasitic on another

fungus. The parasitizing fungus is called **hyperparasite** and the parasitized fungus as **hypoparasite**. Mycoparasitism commonly occurs in nature. In mycoparasitism, two mechanisms operate among involved species of fungi. This may be hyphal interaction or destruction of propagules. As a result of inter-fungus interaction *i.e.*, fungus-fungus interaction, several events take place, which lead to predation *viz.*, coiling, penetration, branching, and sporulation, resting body production, barrier formation and lysis.

In coiling (A), an antagonist, the hyperparasite (a) recognizes its host hyphae *i.e.*, the hypoparasite (h) among the microbes and comes in contact and coils around the host hyphae. The phenomenon of recognition of a suitable host by the antagonists has been discovered in recent years. Cell wall surface of host and non-host contains D-galactose and N-acetyl D-galactosamine residues as lectin binding sites. With the help of lectins present on the cell wall, an antagonist recognizes the suitable sites (residues of lectins) and binds the host hypha (e). As a result of coiling, the host hyphae loose the strength. If the antagonist has capability to secrete cell wall degrading enzymes, it can penetrate the cell wall of host hyphae and enter in lumen of the cells. The event of entering in lumen of host cell is known as penetration (B). Several cell wall degrading enzymes, such as cellulase, β-1, 3 - glucanase, chitinase, proteases, etc. have been reported.

Sometimes host develops a resistant barrier (b) to prevent the penetration inside the cell. Cytoplasm accumulates to form a spherical, irregular or elongated structure, so that the hypha of antagonist could not pass towards the adjacent cells of the hypha (C). Depending upon nutrition, the antagonist forms branch and sporulates (s) inside the host hypha (D). Until the host's nutrients deplete, the antagonist produces resting bodies, the survival structures, for example chlamydospores (c) inside the host hypha (E). Finally post-infection events lead to lysis of the host hypha (F) due to loss of nutrients and vigour for survival. The examples of parasitism and post-infection events are given in Table.5.

Table 6. Parasitism and predation by biocontrol agents

Mode of antagonism	**Plant pathogens**	**Antagonists (hosts)**	**Type of parasitism**
Mycoparasitism	*Botrytis alli*	*Gilocladium roseum*	Penetration of hyphae
	Cephalosporium purpurea	*Fusarium roseum*	Parasitism of sclerotia
	Cochliobolus sativus	*Epicoccum purpurascens Myrothecium verrucaria*	Antibiosis and penetration
	Gaeumannomyces graminis var. *tritici*	*Didymella exitialis*	Cell wall penetration and breakdown
	Phytophthora megasperma var. *sojae*	*Cephalosporium* sp., *Dactylella spermatophaga, Humicola fuscoatra, Pythium* sp.	Oospore parasitism and destruction
	Rhizoctonia solani	*Corticium* sp., *Fusarium oxysporum, Penicillium vermiculatum*	Hyphal coiling and penetration
	Rhizoctonia solani, Fomes annosus	*Trichoderma viride*	Coiling and cytoplasm coagulation
	Sclerotium rolfsii	*T. harzianum*	Coiling, penetration and lysis
Mycophagy	*Cochliobolus sativus*	Soil amoebae	Perforation in conidia
	Gaeumannomyces graminis var *tritici*	Soil amoebae	Penetration and lysis of hyphae

The mycoparasites of important plant pathogens are listed below in Table 7.

Table. 7. Important plant pathogens controlled by different mycoparasites.

Pathogen	Mycoparasite
Alternaria brassicae	*Nectria inventa*
Botrytis cinerea	*Pythium oligandrum,* *Trichoderma harzianum, T. viride*
Erysiphe graminis	*Ampelomyces quisqualis*
Hemileia vastatrix	*Verticillium hemileiae*
Plasmopara viticola	*Trichothecium plasmoparae*
Puccinia arachidis	*Acremonium persicinum* *Penicillium islandicum, Sphaerellopsis filum (=Darluca filum) (*Syn. *Eudarluca caricis),* *Tuberculina costaricana, Verticillium lecanii*
Puccinia recondita	*Aphanocladium album, Bacillus pumillus*
Pythium spp.	*Laetisaria arvalis*
Rhizoctonia solani	*Laetisaria arvalis, Trichoderma hamatum,* *Trichoderma lignorum*
Sclerotinia sclerotiorum	*Coniothyrium minitans, Pythium oligandrum,* *Sporodesmium sclerotivorum,* *Teratosperma oligocladium*
Sclerotium cepivorum	*Trichoderma harzianum*
Sclerotium rolfsii	*Trichoderma harzianum*
Sphaerotheca fuliginea	*Acremonium alternatum,* *Ampelomyces quisqualis (*Syn. *Cicinobolus sesatii),* *Tilletiopsis minor* (yeast)
Uromyces appendiculatus	*Verticillium lecanii*
Uromyces phaseoli	*Bacillus cereus* subsp. *mycoides, Bacillus subtilis,* *Bacillus thuringiensis*

3. Antibiosis and lysis (Amensalism)

Antibiosis

Antibiosis is defined as antagonism mediated by specific or non-specific metabolites of microbial origin, by lytic agents, enzymes, volatile compounds or other toxic substances. Antibiosis plays an important role in biological control. **Amensalism** is a phenomenon where one population adversely affects the growth of anothe pathogens are secreted by roots of clover, lentil, maize (glycine, phenylalanine) and other legumes, flax (hydrocyanic acid), pine (volatile mono-and sesquiterpenes) and by other plant roots. Other plant residues are the sources of phenolic and non-volatile compounds, which are toxic to microbes. Changes in microbial structures (cell wall, hyphae, conidia, etc.) may occur when microorganisms lack resistance against the attack by deleterious agents or unfavourable nutritional conditions. A chemical substance (*i.e.,* melanin) is present in their cell walls to resist the lysis. Cell wall constituents like xylan or xylose containing hetero-polysaccharides protect fungal cells from lysis. There are volatile antibiotics and also gaseous products like ethene (ethylene) and hydrogen cyanide that affect microbial growth, which are active at low concentrations but are not generally considered as antibiotics.

Antibiotics are generally considered to be organic compounds of low molecular weight produced by microbes and at low concentrations they are deleterious to the growth or other metabolic activities of other microorganisms. Antibiotic production as mechanism of biological control has largely been demonstrated for biocontrol agents used against soil-borne plant pathogens. Antibiotic production requires substantial substrates whereas the life style of soil microbes is characterized by starvation with brief periods of activity in the saprophytic and/or parasitic condition. Infact, antibiotics function within an ecosystem in a defensive manner to maintain possession of a relatively rich substrate. Therefore, the challenging strategy in biological control involves manipulation of such antagonists so that they not only produce antibiotics for their own survival, but also interfere with pathogenic activity in the infection court by reducing inoculum potential or by interfering with saprophytic increase.

The fungi are known to produce a wide variety of toxic substances. Antibiotics may diffuse in water, air or substrate to other microbes and thus direct contact between the two is not necessary. *Cephalosporium graminearum* produces a wide spectrum antibiotic that enables it to retain a substrate for 2 to 3 years. Colonization of pea seeds by *T. viride* resulted in the accumulation of significant amount of the antibiotic, gliotoxin in the seeds and thus *Trichoderma* as seed-coat inoculant controlled *Pythium ultimum*. Among the bacterial agents, fluorescent pseudomonads (*Pseudomonas fluorescens*) and among fungal ones, *Trichoderma* and *Gliocladium* have been largely exploited for their potential in biocontrol of soil-borne diseases, involving antibiosis (antibiotic production) alone or additional mechanisms of biological control. Antibiotic production by some strains of fluorescent pseudomonads is now recognized as an important mechanism in disease control. A range of compounds are known to be produced by them. These include phenazines, pyoluteorin, tropolone, pyocyanin, 2, 4 – diacetyl phloro - glucinol (DAPG) and pyrrolnitrin. Some of these, like tropolone have broad spectrum activity against many bacteria and fungi. Some are specific in their activity towards a particular pathogen.

Bacteriocins are "antibiotic-like compounds with bactericidal specificity, which restrict bacterial strains closely related to the bacteriocin-producer". For example avirulent non-bacteriocin-producing strains of *Pseudomonas solanacearum* were unable to protect tomato plants from infection by *P. solanacearum* in greenhouse tests, whereas the bacteriocin-producing avirulent strains gave protection against the virulent *P. solanacearum*.

In phyllosphere, antibiotic production is characteristic of some leaf-inhabiting filamentous fungi. There are relatively a few leaf bacteria, which produce antibiotics. Though there are so many reports of production of antibiotic or similar compounds *in vitro* by antagonistic microbes in phyllosphere, there is lack of correlation of such *in vitro* antibiosis with biocontrol in the field. Species of *Bacillus* have been shown to affect fungal pathogens of aerial plant parts by antibiotic production. Germination of

uredospores of the rust fungus, *Puccinia allii* on leek leaves was inhibited by *Bacillus cereus* and it may be due to an unstable or volatile inhibitory factor. An isolate of *B. subtilis* also reduced the pustules of the rust fungus, *Uromyces phaseoli* on snap beans and dry beans by reducing uredospore germination and preventing normal development of germ tubes. Two antibiotics from *B. subtilis viz*., bacilysin and fengymycin were proved to be active against *Rhizoctonia solani* on rice. This bacterium also reduced the incidence of apple canker pathogen, *Nectria galligena* and brown rot of peach and plum caused by *Monilinia fructicola* through inhibition of spore germination and early germ tube development by production of two antifungal antibiotics. *Pseudomonas cepacia* also produced an antibiotic, which is effective in the control of *Drechslera maydis* (leaf blight of corn), *Cercospora* leaf spots on groundnut and *Alternaria* leaf spot on tobacco. Though antagonism by yeasts generally thought not to involve antibiotics, a fungistatic substance has been isolated from *Sporobolomyces ruberrimus*. Of the filamentous fungi associated with the aerial plant parts, *Botrytis cinerea* is known to produce at least three antimicrobial substances *viz.,* oxalic acid, botrycine and botrydial. *Trichoderma* spp., although not strictly phylloplane inhabitants can often be isolated from leaves especially after heavy rains when spores are splashed on to plant foliage from soil. Both non-volatile and volatile antibiotics are produced by *Trichoderma* spp. *Chaetomium globosum* produced the potent antibiotic, chaetomin, *in vitro* and was effectively correlated against the apple scab pathogen (*Venturia inaequalis)* on apple seedlings.

Species of *Trichoderma* produce the following fungitoxic metabolites *viz.,* gliotoxin, gliovirin, viridin, viridiol, trichodermin, trichozianin A and B harzianolide, pyrones, volatile compounds of lactones, alcohols, terpenes and few more antibiotics that are yet to be identified. They are not only good sources of various toxic metabolites and antibiotics but also of various enzymes such as exo-and endo-glucanases, cellobiase, chitinase, laminarinase, etc. Role of antibiotics in disease suppression by fluorescent pseudomonas (FPs) has also received considerable attention. Besides siderophores, FPs produce several other secondary metabolites with antimicrobial properties notably hydrogen cyanide, 2, 4 - diacetyl phloroglucinol, pyoluteorin, pyocyanine, pyrrolnitrin, phenazine - 1 - carboxylic acid (PCA), oomycin A, fenpiclonil, fludioxonil, pterines,

indoles, indole - 3 - acetic acid, ammonia, amino acids, peptides, alginate, lipids, byocyanin, tropolene, pyocompounds, pseudomonic acid, salicylic acid, chitinase, β -1, 3 - glucanase, laminarinase *etc*. (Table 8). Fluorescent pseudomonads also produce plant growth promoting substances *viz*., auxins, gibberellins and cytokinins and increase plant growth and yield. Several isolates produce indole acetic acid (IAA) and increase early germination and seedling growth although it has not directly been involved in disease control. Thus, fluorescent pseudomonads are collectively called as Plant Growth Promoting Rhizobacteria (PGPR). *Agrobacterium radiobacter* 84 was found to produce an antibiotic called Agrocin 84, *Bacillus subtilis* is known to produce subtilin, zwittermycin A, kanosamine.

Table. 8. Specific microbial metabolites implicated in the control of plant diseases.

Pathogen	**Disease**	**Effective metabolites**
Agrobacterium tumefaciens	Crown gall of fruit trees	Agrocin 84
Fusarium oxysporum	Flax wilt	Pseudobactin B 10
Gaeumannomyces graminis var. *tritici*	Take-all of wheat	Phenazines, c-acetyl phloroglucinols, pyochelin and or salicylic acid
Pyrenophora tritici-repentis	Tan spot of wheat	Pyrrolnitrin
Pythium spp.	Damping off of wheat/ maize	Ammonia
Pythium spp.	Pre-emergence damping off of cotton	Oomycin A

Pythium ultimum	Damping off of wheat/ maize	Pyochelin and or salicylic acid
Pythium ultimum	Pre-emergence Damping off of sugar beet	Pyoluteorin, 2,4-diacetyl phloroglucinol
Thielaviopsis basicola	Black root rot of tobacco	2,4-diacetyl phloroglucinol
Thielaviopsis basicola	Wheat	Hydrogen cyanide

Lysis

Lysis is the complete or partial destruction of a cell by enzymes. It has been much studied in relation to the destruction of invading organisms by defense mechanisms in the blood of animals, but for our purposes we may distinguish two types, endolysis, and exolysis. **Endolysis** (also called **autolysis**) is the breakdown of the cytoplasm of a cell by the cell's own enzymes following death, which may be caused by nutrient starvation or by antibiotics or other toxins. Endolysis does not usually involve the destruction of the cell wall. Secondly, there is **exolysis** (also called **heterolysis**), which is the destruction of cell by the enzymes of another organism. Typically exolysis is the destruction of the walls of an organism by chitinases, cellulases, *etc.* and this frequently results in the death of the attacked cell. In exolysis the death is caused by the lysis, but in endolysis the death is the cause of the cell's own lysis. There can be some overlap between the terms. For example, when a bacterium colonizing a hypha, produces an antibiotic that causes endolysis and at the same time produces a chitinase that destroys the fungal wall so that both forms of lysis occur at the same time, and it may be difficult to determine exactly what is happening. Endolysis may be caused by normal death from old age or the use of all nutrients in that part of a resource. It may also be caused by an untimely death brought about by toxins from another organism. These toxins are often antibiotics (which operate at low concentrations: less than 10 ppm) and should be distinguished from such things as production of hydrogen ions to change pH or the production of ethanol, which is required in comparatively high concentrations to be toxic or to inhibit growth.

Chitin is a linear polymer of β-1-4 linked N-acetylglucosamine and is a major component of the cell walls of fungi (except those in class Oomycetes). It is also a chief constituent of exoskeleton of arthropods. Vascular plants and mammals do not contain chitin. All organisms that contain chitin and also the plants have been found to contain chitinase. In the plants they are produced in response to microbial infections or other injuries and thus chitinases are part of the "pathogenesis-related proteins". It has been postulated that plants produce chitinase to protect themselves from chitin containing parasites such as fungi and insects (Boller 1985). Bacteria are also producers of chitinases (exo- and endo) and if the bacterial strain used as a biological control agent secretes chitinase it would hydrolyse the chitin in the cell wall of pathogens. The involvement of chitinase in the control of *R. solani* has been considered as a possible mechanism. *Sclerotium rolfsii* is another pathogen that contains chitin in its cell wall and may be controlled by chitinase secreted by a bacterial antagonist. If a strain of bacterium would have the combined ability to secrete both antibiotic(s) and chitinase(s), the level of protection it affords against plant pathogens such as *R. solani* is greatly enhanced.

The potent antagonists *e.g., Trichoderma harzianum* and *T.viride* are known to secrete cell wall degrading enzymes *viz.*, β-1, 3-glucanase, chitinase and cellulase. However, production of chitinase and β-1, 3-glucanase by *T. harzianum* inside the attacked sclerotia of *Sclerotium rolfsii* has also been reported.

Volatile substances

Antibiosis mediated by volatile substances has received less attention than antibiosis through the production of non-volatile antibiotics or siderophores. *Enterobacter cloacae* inhibits radial growth of fungal pathogens, including *Pythium ultimum, Rhizoctonia solani* and *Verticillium dahliae*. A volatile fraction responsible for inhibition was identified as ammonia in the culture filtrate of the bacterium. Volatile metabolites identified as alkyl pyrones have been reported from *Trichoderma harzianum*. These compounds are fungistatic to most fungi, but they are shown to act as paramorphogens (*i.e.,* they altered the spatial distribution of the fungus biomass) for somefungi,particularly *R. solani*. Volatiles may also be involved in protection of plants from pathogens by some ectomycorrhizal fungi. Volatile organic metabolites of *Boletus*

variegatus include ethanol, isobutanol, iso-amyl alcohol and isobutyric acid. All these, except ethanol inhibited the pathogen, *Fomes annosus* and *Phytophthora cinnamomi*.

Enzymes

The involvement of enzymes in biological control blurs the distinction between parasitism and antibiosis. For example, production of a cell wall degrading enzyme by an antagonist would likely be involved simultaneously in parasitism and antibiosis. Other enzymes may cause only antibiosis. For example *Talaromyces flavus* is a good competitor in soil and this antagonist produces antibiotic-like compound that kills microsclerotia of *Verticillium dahliae* both *in vitro* and in soil. This compound has infact enzymatic properties and it has been identified as glucose oxidase. Glucose or glucose oxidase alone is not inhibitory to pathogen. The reaction product of glucose and glucose oxidase, hydrogen peroxide is quite toxic to germination of microsclerotia of the pathogen. The antibiotic lactobacillin produced by lactobacilli has also been identified as hydrogen peroxide.

4. Induced Systemic Resistance (ISR)

The phenomenon of induced systemic resistance is at present widely accepted and even exploited for the biocontrol of plant diseases. **Induced Systemic Resistance** is the ability of an agent (a fungus, bacterium, virus, chemical, *etc*.) to induce plant defense mechanisms that lead to systemic resistance to a number of pathogens. Inoculation of plants with weak pathogens or non-pathogens leads to induced systemic plant resistance against subsequent challenge by pathogens. The mechanisms remain largely unknown but typically the induced resistance operates against a wide range of pathogens and can persist for 3 - 6 weeks. Then a booster treatment is required. Rhizobacteria mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria and viruses in *Arabidiopsis*, bean, carnation, cucumber, radish, tobacco and tomato under conditions in which the inducing bacteria and the challenging pathogen remain spatially separated. Rhizobacteria known to induce resistance can be found among *Pseudomons aeruginosa, P. aureofaciens, P. corrugata, P. fluorescens, P. putida,* and *Serratia marcescens.* Also some fungi, including *Trichoderma harzianum* T 39 are able to induce

resistance. Induced resistance is thought to be the principal means by which non-pathogenic strains of *Fusarium oxysporum* protect sweet potato cuttings from attack by pathogenic strain. This technique is used commercially in Japan as pre-plant treatment of the bases of sweet potato cuttings. Induction of systemic resistance by selected strains of PGPR against plant diseases has been proved by spatially separating the pathogen and PGPR in plants. PGPR bring about ISR through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defense chemicals against a challenge pathogen. PGPR induce cell wall structural modification in response to pathogenic attack. Seed treatment of PGPR in bean induces lignification of cell wall. In tomato, seed bacterization has brought about cell wall thickening, deposition of phenolic compounds and formation of callose resulting in restricted growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* to the epidermal cell and outer cortex in the root system in the treated plants. Such a rapid defense reaction at sites of fungal entry delays the infection process and allows sufficient time for the host to build up other defense reactions to restrict the pathogen growth to the outer most layer of root tissue. Application of PGPR results in biochemical or physiological changes in the plants. Normally ISR by PGPR is associated with accumulation of PR proteins like chitinase, glucanase, proteinase inhibitor and peroxidase, synthesis of phytoalexin and other secondary metabolites. Recently pseudomonad strains expressing the lacZY genes survived endophytically when introduced into rice stem and induced a systemic resistance response. In rice tissues showing ISR response, salicylic acid levels doubled. In summary, induction of systemic resistance by PGPR especially against soil-borne pathogens is associated with ultrastructural cell wall modification in such a way to prevent the invasion of mycelium of the pathogen in the vascular stele followed by the biochemical changes *viz*., accumulation of PR-proteins and / or phytoalexins.

There are indications that phyllosphere organisms, potent plant pathogens as well as saprophytes can not only reduce the development of pathogens by direct antagonism, but are also able to stimulate host plant resistance to disease. The reduction of powdery mildew of wheat on induced resistant plants was higher in field experiments compared to

glasshouse experiments. The mechanisms involved in such systems are complex. Those suggested include, recognition phenomena, protection with lectins, enhanced peroxidase activity and lignification and/or production of phytoalexins.

Apart from the aforesaid mechanisms, hydrogen cyanide, stress induced plant proteins and hypovirulence are also the beneficial microbes also, such as resident competitors, other antagonists, rhizobia and other mycorrhizal fungi. Chaetomin, produced by *Chaetomium globosum*, a biocontrol agent for apple scab pathogen (*Venturia inaequalis)* has been shown powerful broadspectrum mycotoxin acting against non-target microflora.

ii. Stress-induced plant proteins: It has also been recently demonstrated that plant cells are induced to produce greater amounts of pathogenesis-related proteins when attacked by the pathogens. Initially these are present inside cell and in intercellular spaces in trace amount but their production accelerate after attack. These proteins are said to confer resistance to the plant. Besides these, more or less similar proteins are also produced by plants under stress of climate, chemicals etc. These proteins produced under stress are called **stress-induced plant proteins (SIPP).** They are also involved in protecting plants against pests and pathogens and the effect of SIPPs is shown to be antibiotic-mediated. These are classified according to the induced stress factor e.g. cold shock or heat shock proteins etc. These proteins have been reported to completely block the growth of *Agrobacterium tumefaciens, Botrytis cinerea, Erwinia amylovora, Fusarium oxysporum* and *Xanthomonas campestris*.

iii. Hypovirulence: The term hypovirulence has varying definitions. Simply stated, a culture is hypovirulent when the capacity to cause disease is reduced from the expected. This was first observed in chestnut blight fungus, *Endothia parasitica*. The reduced virulent or hypovirulent isolate induced less canker development when co-inoculated with viruent isolates of *E. parasitica*.

V. ISOLATION, PURIFICATION AND STORAGE OF BIOCONTROL AGENTS

Pure culture techniques are used for isolation, identification, storage and multiplication of microorganisms. All the above require sterile conditions. Sterilization of apparatus and working areas involves inactivation or physical elimination of all living cells from the environment. It does not include the destruction or elimination of constitutive enzymes, metabolic by-products or removal of dead cells.

STERILIZATION TECHNIQUES

Sterilization is achieved by exposing materials to lethal agents, which may be chemical, physical, or ionic in nature or in the case of liquids, physical elimination of cells from the medium. Selection of method depends upon the desired efficiency, its applicability, toxicity, ease of use, availability and cost and effect on the properties of the object or the material to be sterilized. The methods commonly used for sterilization are:

1. Heat sterilization

Heat is the most reliable method of sterilization when the material to be sterilized is not modified by high temperature. High temperature can be attained by using either dry or moist heat. The chief mechanism of death is oxidation or coagulation of proteins.

a. Dry heat

Dry heat is used for the sterilization of glasswares, metal instruments, certain plastics and heat-stable compounds. Dry heat requires higher temperatures for longer duration than moist heat for sterilization. This is because sterilization by heat is primarily a process of coagulation of proteins. Sufficient moisture must be present for protein coagulation. Dry heat removes water from microorganisms while moist heat adds water to them. In addition, moist heat has greater penetrating power than the dry heat.

Hot-air oven: It is equipment used for dry heat sterilization. It is most commonly used for sterilizing glassware like Petri dishes, test tubes, pipettes and metal instruments that can tolerate prolonged heat exposure. Oils, powders, waxes and other articles are either spoiled or not effectively sterilized by the moist heat. Sterilization is accomplished by exposure of materials / articles usually at 160^{o}-180^{o}C for 20 min to 1 h. An oven consists of an insulated cabinet, which is held at a constant temperature by means of an electric heating mechanism and thermostat. It is fitted with a fan to keep the hot air circulating at a constant temperature and thermometer for recording the temperature of the oven. For proper circulation of the hot air, shelves are provided and they are perforated. For normal sterilization work, the oven should be operated at 160 ^{o}C and most glassware will require

a period of one hour for total sterilization. A schedule of time and temperature for sterilization with dry air using oven is given in Table 9.

Table 9. Schedule of time and temperature used in hot air oven.

Temperature (ºC)	**Time**
120	8 h
140	3 h
160	**1 h**
180	**20 min**

Exposure time is counted from when objects to be sterilized have reached the desired temperature inside the oven. Glassware should be perfectly dried with clean cloth before placing in a hot air oven since wet glassware may break. Objects, such as a glass culture plate, should be placed in sealable metal or other heat-resistant container to prevent recontamination during cooling, transport or storage. After the sterilization process, the oven and its contents should be allowed to reach ambient temperature before opening the doors to prevent breakage and recontamination by cool air rushing into the chamber. Sterilized material may remain in the oven until used or stored in a dry area free of air currents, but should be used within a short time and not stored for longer periods.

b. Moist heat

Moist heat is usually provided by saturated steam under pressure in an autoclave or pressure cooker and it is the most reliable method of sterilization for most of the materials. The saturated steam heats an object about 2.5 times more efficiently than does hot air at the same temperature. It is not suitable for materials damaged by moisture or high temperature or culture media containing compounds hydrolyzed or reactive with other ingredients at high temperature. Moist heat has advantages over dry heat in which heat conduction is rapid and has greater penetrating power and the temperature required for sterilization is lower and the duration of exposure is also shorter. The process is

usually carried out in an autoclave or a kitchen type pressure cooker equipped with pressure gauges, thermometer, automatic pressure control valves and exhaust valves. The temperature and length of time for sterilization with steam are different from that of dry heat. The time required for sterilization at temperatures ranging from 100 to 130 ^{o}C. For most purposes 15 min at 121 ^{o}C or 30 min at 115 ^{o}C are recommended.

Autoclave: It is an apparatus in which saturated steam under pressure effects sterilization (autoclaving). The increased pressure increases the boiling point of water and produces steam with a high temperature. Cells are destroyed by the higher temperature and not by the pressure. Most of the microorganisms are killed at 121^{o}C (*i.e*., 15 lbs/in^{2}) in 15 min. Autoclave is a double-walled cylindrical metallic vessel, made of thick stainless steel or copper, one end of which is open to receive the material to be sterilized. Autoclave lid is provided with pressure gauge for noting the pressure, steam cock (exhaust valve) for exhaustion of air from the chamber. Autoclave is provided with controls for adjusting the pressure and temperature and a safety valve to avoid explosions. The articles to be sterilized are kept loosely in a basket, provided with holes all around, for the free circulation of the steam. To increase the area for the materials to be sterilized, metallic separators, having holes or perforations can be used. A cross section of a simple autoclave is diagrammatically represented in Fig. The relationship between pressure and temperature of steam in an autoclave is furnished in Table 10.

Table 10. Relationship between pressure and temperature of steam in an autoclave.

Pressure in pounds per square inch (psi)	**Temperature in ^{o}C**
Free flowing steam *i.e.*, 0	100.0
5	109.0
10	**115.0**
15	**121.0**
20	**126.0**
25	130.0

30	135.0

Pressure cooker: It is a suitable alternative to an autoclave. It is a closed vessel usually made of stainless steel or aluminium in which sterilization is accomplished with saturated steam under pressure. The built-in pressure inside the vessel increases the temperature of water and vaporizes it to steam at temperature above $100^{o}C$.

Fermentor: Largescale multiplication of microorganisms often requires large vessels, commonly called **fermentors.** Industrial fermentors are designed to provide the best possible growth. These vessels must be strong enough to withstand the pressures of large volumes of aqueous medium, but at the same time, the materials from which they are fabricated must not be corroded by the fermentation product nor contribute toxic ions to the growth medium. Provisions must be made for rapid incorporation of sterile air into the medium as the fermentation of microorganism is to occur aerobically. The oxygen of the air is dissolved in the medium and readily available to microorganisms and at the same time carbon dioxide resulting from microbial metabolism is flushed out from the medium. Some form of stirring should be available in the fermentor. The fermentor should provide for the intermittent addition of antifoam agents as demanded by the foaming status of the medium. A mechanism for detecting pH values of the culture medium and for adjusting these values during growth is often required. There must also be a drain in the bottom of the fermentor or some provisions for removing the compelled fermentation broth from the tank and access must be had to the inside of the fermentor so that it can be thoroughly cleaned between fermentation runs.

Fermentors are available in varying sizes. These sizes are usually stated based on the total volume/capacity of the fermentor. However, the actual operating volume in a fermentor is always less than that of the total volume, because a “head space” must be left at the top of the fermentor above the liquid medium to allow for splashing, foaming and aeration of the liquid. This headspace usually occupies one fifth to one fourth or more of the volume of the fermentor. Small laboratory fermentors have a total volume of one to two litres of medium with a maximum of about 12 to 15 lit pilot plant fermentors,

which are used in large scale studies of fermentations. Pure culture fermentation usually requires that the medium be sterilized. In small laboratory fermentors, the medium is placed directly in the fermentor and the fermentor is then autoclaved. Fermentation utilizing fungi are conducted as aerated submerged fermentation. This method is followed for the mass multiplication of the antagonistic *Trichoderma* spp.

c. Flame sterilization

Flame sterilization is used for metal objects, such as transfer needles and the tips of forceps and glass objects, such as the lips of flasks and culture tubes, microscope slides and cover slips and the surface of certain plastic materials. The object to be sterilized is held at a 45^{o} angle in the upper portion of a flame from a Bunsen burner or alcohol (spirit) lamp. Tempered metal can be heated to "red hot" and remains sterile as long as it is hot. Glass objects are passed through the flame several times and should not be placed immediately on a cool surface otherwise they will crack.

Bunsen burner: The Bunsen burner (named after R. W. Bunsen) is a type of gas burner with which a very hot, practically non-luminous flame, the temperature of which reaches 1,870^{o}C at its hottest point, is obtained by allowing air to enter at the base and mix with the gas. The Bunsen burner or spirit lamp is used to sterilize inoculating loops/ needles before they are inserted into cultures. It is also used for flaming the mouths of test tubes, flasks containing media and other glass apparatus that the process can be carried out at low temperatures and relative humidity. Objects can be sterilized in their containers since most gases will diffuse out of most containers with time. The process can be carried out using simple equipment such as rubber or plastic bags and metal or plastic drums. Major disadvantages are that a longer time is required for sterilization over that of heat. Materials used are flammable and highly toxic and the cost is higher than heat. Some gases used for gas sterilization are ethylene oxide, propylene oxide, formaldehyde, methyl bromide, β- propiolactone and ozone.

Formaldehyde: It is an excellent microbicide and viricide but its use is restricted because of its pungency, poor penetration and diffusion ability and toxicity. It is used

generally as surface sterilant although a thin film of organic matter can restrict its activity. Formaldehyde gas boils at –20^{o}C, but in aqueous solution at 90^{o}C. The usual concentration required for gas sterilization ranges from 3 to 10 mg/l of chamber space with a relative humidity of 75 to 90 % at 55 to 60^{o}C. Formalin (5 to 10 %) solution is a powerful and rapid disinfectant when applied directly to contaminated surfaces. A variety of chambers can be used for gas sterilization if they are air tight, non-reactive with formalin and non-permeable.

3. Physical method

Gamma rays, X-rays, cosmic rays, ultraviolet rays and visible light are all forms of radiation. Radiation differs in wavelength and energy. Radiation with the shortest wavelengths has the greatest energy and is most lethal. Both ionizing radiation (X-rays and gamma-rays) and non-ionizing radiation (Ultraviolet rays) are used in microbial control. High-energy ionizing radiation is an effective sterilizing agent and is commonly employed as an alternative to the autoclave for sterilizing plastic Petri dishes and other heat-sensitive materials.

Ultraviolet rays: It has lower energy content than ionizing radiation and is capable of producing a lethal effect in cells exposed to the low penetrating wavelength in the range of 210 nm to 300 nm. The most lethal wavelength is 265 nm, which corresponds to the optimal absorption wavelength of DNA. UV light induces aberrant chemical bonds between adjacent thymine nucleotide bases in the nucleic acid, which results in a deletion mutation. Because of the low penetration capacity, the UV light is used as a disinfecting agent and has a very limited application as a sterilizing agent. It is effectively used to sterilize the air (wavelengths of 250-265 nm) of operating rooms in hospitals. In the pharmaceutical industries and the food industries, ultraviolet radiation is important in reducing the number of microorganisms present in air. UV is present in natural day-light. Concentrated UV rays are obtained from special low-pressure mercury vapour lamps, which have a high output (90%) of 253.7 nm, which are considered as very effective bactericidal lamps.

4. Chemical method

Disinfectants are chemical agents (usually liquids), which are used on the surfaces of non-living (inanimate) materials to lower the level of microbes on that surface. The chemical used to wipe down laboratory work area contains a strong disinfectant. The chemicals that are applied to skin or other living tissues to decrease the number of microbes are called **antiseptics**.

Mercuric chloride / Sodium or calcium hypochlorite: Chemicals such as mercuric chloride (0.1%) sodium or calcium hypochlorite (0.1%) or 80% ethyl alcohol are used for surface sterilization of the infected tissues before isolation of the pathogen. It enables killing of all microorganisms including the pathogen on the surface but ensures growth of the pathogen inside the tissue without being inhibited by the surface microflora.

Alcohols: Ethanol (ethyl alcohol) and isopropanol (isopropyl alcohol) are the alcohols used to reduce the number of microbes. Alcohols are colourless hydrocarbons with one or more -OH functional groups. Ethanol is the most widely used skin antiseptic because of its relatively germicidal, non-toxic, non-irritating and inexpensive characteristics. Solutions of 70-90% ethanol are routinely used as skin de-germing agents because the surfactant action removes skin oil, soil and even some microbes lying in deeper skin layers. The effectiveness of alcohol as an antiseptic depends upon its concentration. Concentrations of 50% and above dissolve lipids from membranes disrupt cell surface tension and compromise membrane integrity. Alcohol that has entered the protoplasm denatures proteins through coagulation, but only in alcohol-water solutions of 50-95%. Absolute alcohol (100%) dehydrates cells and inhibits their growth, but is generally not a protein coagulant. The top of the working table and the palms of the workers may also be sterilized with a cotton swab soaked in rectified spirit.

Dettol: Normally one per cent or two per cent dettol is used for sterilization. Take 10 ml of dettol and add 90 ml of distilled water to make up one litre to get one per cent dettol. This is an antiseptic solution to disinfect the culture room and working places like the table tops.

Inoculation room: It should be completely sealed and provided with a double-door having provisions to put on a sterile apron before entering into the main room. Such rooms should also be air-cooled, preferably dehumidified and provided with an inverted exhaust with a glass wool filter to create positive air pressure. The inoculation rooms are occasionally sprayed with disinfectants such as dettol, phenol, lysol, aerosol, formalin, *etc*., or equipped with UV lamps.

Transfer chamber: It is a chamber with two front doors enabling the hands of workers to be taken inside and a slanted front with glass fittings all around. Often, an inverted exhaust, a fluorescent tube lamp and a UV lamp (17 watt) are fitted inside.

Laminar flow work station or hood or chamber

One can work openly and easily for a longer period on the table of laminar airflow. This maintains the atmosphere essentially free of air-borne particles when operated in an uncontrolled area. Clean atmosphere is achieved by placing HEPA (high efficiency particulate air) filter across the entire back or top of the bench and moving a large volume of air in the room uniformly through these filters and across the bench. It also helps to control the air-borne contamination provided by the work process since it moves on air-borne materials generated away from the work surface. Laminar flow workstation may be of horizontal or vertical. The ambient air primarily passes through a double stage pre-filter and finally through a HEPA filter. Such filters remove particles larger than 0.3 μm. The ultra clean air, which is free from fungal and bacterial contaminants flows at the velocity of about 27 $\pm$ 3 m / min through the working area. All contaminants are blown away by the ultraclean air thereby an aseptic environment is maintained over the working area. The flow of air does not put out the flame of a spirit lamp. Therefore, a spirit lamp can be used conveniently during the work.

CULTURE MEDIA PREPARATION

In nature, microorganisms exist as mixed populations of many widely differing types. They require suitable nutrients as well as favourable environment for their survival and multiplication. To grow these microorganisms under laboratory conditions, we require culture medium, which contains a great variety of substances. To be suitable for most heterotrophic microorganisms, culture media must provide a source of energy, usually a carbohydrate and source of organic nitrogen, vitamins and minerals. Most microorganisms require about one per cent of a carbon source in the form of sugar, less than 0.5 per cent of a nitrogen source as salt or yeast extract and small quantities of phosphate, sulphur, potassium, magnesium and traces of calcium, iron, zinc, manganese and molybdenum. These nutrients are supplied to the organisms in different inorganic forms, depending upon their capacity to utilize them. The culture media commonly used in the laboratory are classified into non-synthetic (natural) and synthetic or organic or inorganic media. Non-synthetic media include organic substances with complex or simple molecules, while synthetic media consist mostly of chemicals of known molecular structure and composition. Such media may be in liquid, solid or semi-solid form. In order to solidify the liquid medium containing the various ingredients 1-2 per cent agar (a substance obtained from marine algae and available in dried purified form as shreds or powder) or 10-20 per cent gelatin is added. For semi-solid media about half the quantity of agar or gelatin is added. The procedure commonly adopted for preparing the laboratory media is to dissolve weighed quantities of chemicals in measured quantity of water, adjust the pH to the required level by adding 0.1 N NaOH or HCl, filter the contents through absorbent cotton wool or a double layer of cheese cloth, dispense into test tubes or flasks, plug with cotton and sterilize in an autoclave at the right temperature and steam pressure. For preparing solid agar medium, half the quantity of water is used for dissolving the chemicals and the remaining half for dissolving the agar shreds or powder. The agar is dissolved in water by slow heating with constant stirring. The melted agar is added to the other half of the medium containing the chemicals. Usually the pH is adjusted before mixing with agar. The mixture is then dispensed into tubes or flasks as desired, the mouths plugged with cotton wool and autoclaved.

The following are some of the important culture media generally used for culturing fungi, bacteria and actinomycetes.

1. Potato Dextrose Agar (PDA) medium (for fungi)

Peeled potato	-	250.0 g
Dextrose	-	20.0 g
Agar	-	15.0 g
Water	-	1000.0 ml
pH	-	6.0 to 6.5

Peeled potato is made in to thin slices, boiled in 500 ml of water and extracted. To this extract, weighed quantity of dextrose is added. The agar is melted in another half of water and mixed in potato dextrose solution and the volume is made up to a litre before sterilizing.

***2. Trichoderma* Selective medium (TSM)**

Magnesium sulphate	-	0.20 g
Di-potassium hydrogen phosphate	-	0.90 g
Ammonium nitrate	-	1.00 g
Potassium chloride	-	0.15 g
Glucose	-	3.00 g
Dexon 50 WP or Apron (Metalaxyl)	-	0.30 g
PCNB	-	0.20 g
Rose Bengal	-	0.15 g
Chloromphenicol	-	0.25 g
Agar	-	15.0 g
Distilled water	-	1000 ml

3. King's B Medium (selective medium for *Pseudomonas fluorescens*)

Peptone	-	20.0 g
Di-potassium hydrogen phosphate	-	1.50 g

Magnesium sulphate	-	1.50 g
Glycerol	-	10.0 ml
Cycloheximide	-	100 ppm
Ampicillin	-	50 ppm
Chloramphenicol	-	12.5 ppm
Agar	-	15.0 g
Distilled water	-	1000 ml
pH	-	7.0 to 7.2

(King's broth is prepared without the addition of agar and antibiotics)

4. Nutrient Agar (NA) medium (for bacteria and actinomycetes)

Bactopeptone	-	5.0 g
Beef extract	-	3.0 g
Sodium chloride	-	5.0 g
Agar	-	15.0 g
Water	-	1000.0 ml
pH	-	6.8 to 7.2

ISOLATION AND PURIFICATION TECHNIQUES

A. ISOLATION METHODS

In order to learn more about the commercial biocontrol agents they have to be cultured *in vitro*. A culture containing a single unadulterated species of cells is called a **pure culture**. Several different techniques are applied to isolate and study microorganisms in pure culture. The biocontrol agents are in general saprophytic in nature and they are present in the soil, root surface and in phyllosphere regions. The methods of isolation of these organisms vary considerably. In the case of plant pathogens, generally the tissue segment is used for isolating the organisms that grow in artificial media. But the antagonistic organisms can be isolated from three main zones *viz.*, a. rhizosphere/ rhizoplane b. phyllosphere/ phylloplane and c. endosphere or

endophytic. For isolation, several **media** (solutions / solids containing all the nutrients required for the growth of microorganisms) are employed.

Though various methods are available to isolate and enumerate microorganisms, the **serial dilution-agar plate method** or **viable plate count method** is the one, most commonly used method for the isolation and enumeration of fungi, bacteria and actinomycetes. This method is based upon the principle that when material containing microorganisms is cultured each viable microorganism will develop into a colony; hence the number of colonies appearing on the plates represents the number of living organisms present in the sample.

In serial dilution agar-plate method, a known amount (10 ml or 10 g) of material is suspended or agitated in a known volume of sterile water blank (90 ml or so to make the total volume to 100 ml i.e., 10^{-1} dilution) to make a suspension of microbes. Serial dilutions of 10^{-2}, $10^{-3,}$ ……..10^{-7} is made by pipetting measured volumes (usually 1 ml or 10 ml) into additional dilution blanks (having 9 ml or 90 ml sterile water). Finally 1 ml aliquot of various dilutions are added to sterile Petri dishes (triplicate for each dilution) to which 15 ml (approximately) of the sterile, cool, molten (45^{o}C) media (nutrient agar and soil extract agar for bacteria, KenKnight's agar or nutrient agar for actinomycetes and Martin's Rose bengal agar or Czapek-Dox agar for fungi) are added. The dilutions 10^{-2} to 10^{-5} are selected for enumeration of fungi, 10^{-3} to 10^{-6} for actinomycetes and 10^{-4} to 10^{-7} for bacteria relative to their proportion in soil. Upon solidification, the plates are incubated in an inverted position for 2 to 7 days at 25 - 30^{o}C. The number of colonies appearing on dilution plates are counted, averaged and multiplied by the dilution factor to find number of cells/ spores per g or ml of the sample.

$$\text{No. of cells /ml or g} = \frac{\text{Number (average of 3 replicates) of colonies x Dilution factor}}{\text{Dry weight of soil}}$$

Dilution factor = Reciprocal of the dilution (*e.g.,* $10^{-7} = 10^{7}$)

1. Isolation of rhizosphere microflora

The term **rhizosphere** was introduced in 1904 by the German scientist Hiltner to denote the region of soil, which is under the influence of plant roots. It is defined as the region of the soil immediately surrounding the roots of a plant together with the root surfaces. Operationally, the rhizosphere can be defined as the region, extending a few millimetres from the surface of each root, where the microbial population of the soil is influenced by the chemical activities of the plant. The region provides certain characteristic conditions for the increased occurrence of microflora in it, which is attributed to the rich food materials provided by sloughed off portions of root tissues and root exudates. Root exudates contain sugars, amino acids, vitamins and other growth factors, which serve as nutrients for microorganisms in the rhizosphere. The rhizosphere effect is beneficial to the plants in two ways:

i. It helps in providing nutrition to the plants and
ii. It helps the plants in combating root diseases; the differences are both quantitative and qualitative. However, the major effect observed is a quantitative one.

The number of microorganisms in the rhizosphere usually exceeds the number in the neighbouring non-rhizosphere soil by a factor of 10 and often by a factor of several hundreds. The rhizosphere to soil (R: S) ratio called **rhizosphere effect**, calculated by dividing the number of microorganisms in the rhizosphere soil by the number in the soil free from plant growth. Greater rhizosphere effect is seen with bacteria (R: S values from 10 to 100 or more) than with actinomycetes and fungi. With regard to protozoa and algae there are only negligible changes. The rhizosphere effect increases with the age of the plant and normally reached its maximum at the stage of greater vegetative growth. Following the death of the plant, the microbial population reverted gradually to the level of that in the surrounding soil. Microorganisms which can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens. Pathogens encounter antagonism by the rhizosphere microorganisms before and during primary infection and also during secondary spread on the root.

Serial dilution- agar plate method

Soil sampling

- It is generally advised to draw rhizosphere soil from healthy plants growing in soil where in disease is present or was seen earlier. In other words, the soil samples are collected from the suppressive soils, where the disease occurrence is low. Areas where a pathogen was introduced but not established and areas of monoculture of crops where disease intensity has decreased after a few years with a susceptible crop provide excellent chances of finding a suitable antagonist. For each field, samples are taken from a minimum of 5 to 20 points along a diagonal transect. These will be 5 paces apart, starting 10 paces into the field.
- At each sampling site, carefully dig out the plant or portion of the plant hill to include tillers, roots and soil down to a depth of 15 - 20 cm using the narrow bladed sampling tool. Soil adhering to the roots is left intact.
- The plant portion and soil is placed in a polythene bag and transferred to the laboratory for further analysis. Do not leave plants in plastic bags for long periods. If laboratory assessment cannot be initiated immediately on return from field sites, remove plants and leave on top of bags to prevent moisture build-up and rot.
- From each plant manually remove a portion of adhering soil from roots using a brush or knife.
- For each field, combine all the collected sub-samples to form one composite and mix well.
- Powder the soil sample finely and sieve through 9-mesh (per sq.inch) sieve. Take a portion of soil and determine the moisture percentage by drying at 105 - 110^{o}C for 24 h.

After collection and before attempting the microbial analysis for antagonists, it is desirable to test the soil for its suppressive effect. These tests give an indication of the soil microflora rather than individual antagonists. The suppressiveness of a soil may not be due to an individual antagonist but a group of antagonists acting together and belonging to different taxonomical groups. When tested individually, these organisms

may not show any significant suppressive effect. On the other hand, a non-suppressive soil may contain a single strong antagonist in a low population or dormant form. The suppressiveness of soil is tested by simple methods. A soil sample is divided into five portions: one used directly, the second treated with aerated steam for 30 min at 60^{o}C and the other three portions gas sterilized, autoclaved or pasteurized with steam at 100 ^{o}C for 30 min. In the last three portions, one is amended with one per cent unsterilized soil and another with 10 per cent of unsterilized soil and the third is the control to determine if the suppressiveness is of biological nature.

Procedure

- Prepare 90 ml and 9 ml sterile water blanks
- Weigh representative soil samples of 10 g from the composite soil sample collected from the experiment site (field)
- Add 10 g of finely pulverized soil samples to 90 ml of water blank to make dilution of 1:10 (10^{-1})

Note: For the isolation of *Bacillus subtilis* one gram of representative soil samples drawn from composite soil is mixed with 9 ml of sterilized nutrient broth in a test tube. Keep the tubes on a boiling water bath at 80^{o}C for 10 min and then incubate it at room temperature for 24-48 h. From this, serial dilutions of 10^{-2}, $10^{-3,}$, 10^{-4}, 10^{-5} and 10^{-6} dilutions are prepared.

- Vigorously shake the dilution (on a magnetic shaker) for 20-30 min to obtain uniform suspension of microorganisms.
- Transfer 1 ml of suspension from 10^{-1} dilution into 9 ml water blank with a sterile pipette under aseptic condition to make 1:100 (10^{-2}) dilution and shake it well for about 5 min.
- Prepare another dilution of 1:1000 (10^{-3}) by pipetting one ml of 10^{-2} dilution into 9 ml water blank, using a fresh sterile pipette and shake well.
- Make further dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} by pipetting one ml of suspension into additional water blanks (9 ml) as prepared above.

- Transfer 1 ml of aliquot from the desired dilution as given in the table 11 to each Petri dish (3 replicates for each dilution) and add 15-20 ml of cooled medium (45^{o}C) to each Petri dish and mix by gentle rotation of Petri dishes.

Table. 11. Dilutions and media used for isolation of microorganisms.

Microorganism to be isolated	Dilution range	Medium to be used
Fungi		
General	10^{-2} to 10^{-5}	Rose Bengal Agar
Trichoderma spp. and *Gliocladium* spp	10^{-2} to 10^{-5}	*Trichoderma* Selective Medium (TSM)
Bacteria		
General	10^{-4} to 10^{-7}	Soil Extract Agar (SEA)
Pseudomonas fluorescens	10^{-4} to 10^{-7}	King's B medium (KB medium)
Bacillus subtilis	10^{-4} to 10^{-7}	Nutrient Agar (NA) medium
Actinomycetes	10^{-3} to 10^{-6}	KenKnight's Agar (KKA) / Dextrose nitrate agar

- Upon solidification of media, incubate the plates in an inverted position at 25- 30^{o}C for 2 – 7 days.

Observations

- Count the colonies with a colony counter. Then compute the number of colonies/ g of the soil by multiplying the number obtained with dilution factor.

$$\text{No. of colony forming units (cfu) / g of soil} = \frac{\text{Mean no.of colonies / plate x dilution factor}}{\text{Dry weight of soil taken}}$$

The colonies are then purified and identified for further study.

2. Isolation of root surface (rhizoplane) microflora

- Take root pieces from the first dilution in screw cap bottle with 100 ml sterile water and shake it well.
- Serial washings (10-20) are given to the above roots with sterilized water until clear root surface is exposed.
- Plate the washed roots on Czapek-Dox agar and nutrient agar plates.
- Incubate the plates for 2-7 days at 25^{o}C in an inverted position and observe for the colonies.

3. Isolation of microorganisms from non-rhizosphere soil

- Collect the soil sample from non-rhizosphere bulk soil
- Isolate microorganisms from the bulk soil by the serial dilution technique as followed in rhizosphere soil.
- Follow the steps given in isolation of rhizosphere microflora.

4. Isolation of microorganisms from phyllosphere / phylloplane

The term phyllosphere and phylloplane are interchangeably used in literature. Phylloplane is a natural habitat on leaf surface, which supports heterogeneous population comprising both pathogen and non-pathogens. The phylloplane microbes cover a wide variety of microorganisms including yeasts, filamentous fungi, bacteria, actinomycetes, blue green algae and even ferns. The phylloplane have antagonistic action against fungal parasites, degrade plant surface wax and cuticles, produce phytoalexins, decompose plant material, activate plants to produce phytoalexins, act as a source of allergic air-borne spores and influence growth behaviour and root exudation of plants.

Several methods are employed to study the phylloplane microflora. These are classified as direct (direct observations, impression films, clearing, scanning microscopy, phase contrast, fluorescent antibody and infra-red microscopy) and cultural (spore fall, plating, moist chamber, surface sterilization, leaf maceration, leaf impression) methods.

Of these, serial dilution plate method and leaf impression method are the two commonly employed techniques in the isolation of phyllosphere microorganisms.

Procedure

- Collect fresh, healthy leaves (of all ages) in fresh sterile polythene bags and bring it to the laboratory.
- Cut five discs each of 6 mm dia from every leaf using sterile cork borer.
- Transfer 50 discs to 100 ml water blank
- Stir the discs for 20 min using magnetic stirrer.
- Transfer 10 ml of the suspension to 90 ml sterile water blank (labeled 2) using sterile pipette diluting the original sample to10 times (10^{-1})
- Shake the contents for 2-3 min
- Transfer 10 ml suspension while in motion to another 90 ml sterile water blank (labeled 3) using another fresh pipette (10^{-2})
- Shake the contents for the uniform distribution of the cells / spores
- Transfer 1ml aliquots from 10^{-1} and 10^{-2} dilutions to sterile Petri plates (six replicates for each dilution, 3 for fungi and 3 for bacteria and actinomycetes).
- Pour the melted and cooled (45-50^{o}C) Martin's Rose Bengal agar medium and Nutrient agar medium (3 plates per dilution) to above plates
- Incubate the plates at 25-30^{o}C in an inverted position for 7 days

Observations

Observe the nutrient agar plates after 24-72 h and Martin's Rose Bengal agar plates after 2-7 days of incubation for the appearance of colonies of bacteria and actinomycetes and fungi respectively.

Microbial population /cm^2 can be calculated by applying the following formula.

$$\text{Propagules /cm}^2 = \frac{\text{Total no.of spores/ cells in one ml}}{\text{Total area of 50 discs x 2}} \times 100$$

Area of one leaf disc = r^2 (where r is the radius of disc in cm)

5. Isolation of pathogenic microorganisms from infected tissues

- Take small bits of tissue, preferably from around the margins of the infected regions of plant, approximately about 1 to 4 mm x 1 to 2 mm.
- Surface sterilize the tissues in 0.1% mercuric chloride solution for 30 sec to 2min and wash immediately in sterile distilled water for 2 to 3 times.
- Place the surface sterilized tissue bits aseptically with the help of sterile forceps on sterile Petri dishes containing agar medium or selective medium.
- Incubate the plates at room temperature for 2 to 5 days and observe for the fungal growth arising from the tissue. As soon as the tissues show mycelial growth, isolate the fungus carefully, prove its pathogenicity and maintain the culture in slants for further study.

B. PURIFICATION OR PURE CULTURE TECHNIQUES

A culture that contains one kind of microorganism is called a **pure culture**. A culture, which contains more than one kind of microorganisms is called a **mixed culture**. If it contains only two kinds of microorganisms that are deliberately maintained in association with one another, it is called **two-member culture**. Pure cultures are essential in order to study any of the following: colony characteristics, biochemical properties, morphology, staining reactions and immunological reactions or the susceptibility to antimicrobial agents of a particular strain of fungus and bacterium or actinomycetes. Pure cultures of microorganisms that form discrete colonies on solid media may be most simply obtained by one of the modifications of the plating method. Each viable organism gives rise, through growth to a colony from which transfer can be readily made.

The most commonly used methods for obtaining pure cultures (purification) of microorganisms are

A. Fungi
   i. Single spore isolation
   ii. Single hyphal tip culture

B. Bacteria

i. Streak-plate method

ii. Pour-plate method

iii. Spread-plate method

Fungi

Single spore isolation

This method is especially followed when a fungus is found mixed with other fungi.

- Prepare spore suspension of the given sample till 1 ml of suspension contains not more than 5-10 spores. Pour the spore suspension (0.5 – 1.0 ml) aseptically in a Petri plate.
- Prepare and pour warm plain agar (2%) into the Petri plate and mix it thoroughly.
- Incubate the plate at optimum temperature for 4 h.
- Invert the Petri plate and examine for single germinating spores under the microscope and such spores are marked by glass marker on the back of the Petri dish.
- Cut the marked area containing a single spore along with some medium using a sterile cork borer.
- Transfer it with the help of a sterile inoculation needle to agar slant and incubate to obtain a single-spore culture.

Single hyphal tip culture

This method is employed to purify the fungus when it is found mixed with bacteria. In this method, the growth of spores is allowed on a plain agar as done in single spore isolation to obtain pure culture.

- Prepare plain agar (2 %) medium and pour into the Petri dish.
- Inoculate the mixture culture in the centre of the Petri dish and incubate at the room temperature. The fungus grows and put forth hyphae quickly to the periphery of the Petri dish in search of nutrition.
- Invert the Petri dish and examine under the microscope for single hyphal tip in the periphery of the Petri dish.

- Mark the single hyphal tip with a glass marker and transfer to suitable medium as done in single spore isolation for obtaining pure culture.

Bacteria

Streak-plate method

The streak-plate method offers a most practical method of obtaining discrete colonies and pure cultures. It was originally developed by two bacteriologists *viz.*, Loeffler and Gaffkey, in the laboratory of Robert Koch.

- Use nutrient agar plates.
- Sterilize the inoculation needle by flaming it red hot and allow it to cool.
- Hold the culture tube in left hand and take the tube near flame.
- Remove the cotton plug with right hand and flame the mouth of the tube for a few seconds.
- Touch the culture with the needle loop and take the culture.
- Make a series of parallel non-overlapping streaks on the nutrient agar medium.

Observations

Observe individual colonies and make transfers from the individual colonies.

Pour-plate method

This method was developed in the laboratory of the famous bacteriologist, Robert Koch.

- Add one ml of successive dilutions of the inoculum (serially diluting the original specimen) into sterile Petri plates.
- Add melted and cooled (42-45^{o}C) nutrient agar medium to it.
- Rotate the plates for thorough mixing of the contents and allow it to solidify.
- Incubate the plates at room temperature for 24 to 48 h.
- Examine for the presence of individual colonies.

Observation

The pure colonies which are of different size, shape and colour may be isolated / transferred into test tube containing culture media for making pure culture.

Spread-plate method

The spread-plate technique is used for the separation of a dilute, mixed population of microorganisms so that the individual colonies can be isolated. The theory behind this technique is that as the Petri dish spins, at some stage single cells will be deposited with the bent glass rod on to the agar surface. Some of the cells will be separated from each other by a distance which is sufficient to allow the colonies that develop to be free from each other. In this technique microorganisms are spread over the solidified nutrient agar medium with a sterile L-shaped glass rod while the Petri dish is spun on a turn table.

- Use the nutrient agar plates.
- Serially dilute the given culture.
- Label the nutrient agar plates corresponding to the dilutions.
- Place one ml from each dilution in separate nutrient agar plates.
- Sterilize the “L” rod with alcohol by flaming it.
- Cool the rod and place it gently on the agar surface.
- Spread the sample over the surface of the agar medium by rotating the Petri plate manually or placing it on the rotating plate disc.
- Incubate the Petri plates in inverted position at room temperature for 24 to 48 h.
- Observe the well-separated colonies.
- Using sterile inoculation needle pick up the isolated colonies and inoculate it into the liquid medium and incubate at room temperature.

Observation

After sufficient growth in the liquid medium, make wet mount of the culture and observe under the microscope. If the cultures are pure (axenic) they may be individually transferred to agar slants and labeled.

STORAGE OF CULTURES

Once a microoganism is located in a pure form, it is subcultured on plates or agar slants (a tube containing solid medium prepared by keeping tube tilted as agar solidifies, the resultant slope surface provides more area and is easier to streak than a horizontal surface) at regular intervals to maintain viability. The interval between subculturing varies from four weeks to months depending on the storage conditions and on the growth rate of organisms. The organism can be subcultured on maintenance media especially designed to allow low growth rates and extend the culture's life. Storing cultures in a refrigerator, at a temperature of 4^{o}C, which slows growth, protects from damage due to evaporation of medium and preserves the culture. Subculturing of refrigerated cultures is to be carried out at regular fortnightly intervals. It is advisable to maintain two slants, one as the **working culture** to be used as a source for routine laboratory work and the other as **stock culture** from which new working cultures are prepared whenever required. Such a system decreases the chances of contaminating the stock culture. Maintenance of stock cultures requires great care. To preserve microorganisms, it is necessary to reduce their metabolism to a minimum. As a result, the processes that lead to ageing and death are slowed down and the microorganism can be maintained in its inactive state for several years. There is no universal method for storing the cultures. There are several methods available for maintenance of pure cultures but the choice of the method to be followed depends upon the purpose, size of collection and the laboratory. Some of the commonly used methods are as follows:

Periodical transfer

The transfer of organisms to fresh media at regular intervals consumes more time and laborious. Many bacteria and fungi become adapted to saprophytic growth and lose virulence and / or fail to sporulate. The interval between transfers depends upon storage temperature and humidity. Conditions permitting rapid culture dehydration dictate transfers at shorter time intervals. Tube cultures stored in moisture proof containers, which allow air exchange, in a refrigerator at 5 to 8^{o}C, need transferring every 3-4 months. To avoid contamination use absorbent cotton plugs and apply few drops of 1%

mercuric chloride solutions to the plugs. Sealing the culture tubes while the fungus is still metabolically active, may result in slow growth and changes in colony characteristics due to accumulation of metabolic by-products and CO_2 and reduced O_2.

To seal the cultures with cigarette paper, push the cotton plug into the culture tube so that it is just below the rim, warm the rim in a flame and press it gently in a rotating motion on the surface of a jelly (20 % gelatin plus 2 % copper sulphate in water) then press it hard against a cigarette paper. After drying, burn off the excess paper.

Use of refrigerator or cold room storage

Live cultures on a culture medium can be successfully stored in refrigerators or cold rooms maintained at 4^{o}C. Generally the metabolic activities of the microorganisms will be greatly slowed down at this temperature, but it is not low enough to stop metabolism completely. Thus growth will occur slowly, nutrients will be utilized and waste products produced, which will eventually kill the microorganisms. So regular subculturing is necessary, for bacteria it ranges from 2-3 weeks and for fungi from 3-4 months.

Mineral oil or Paraffin oil

Fungi and bacteria actively growing on agar media in tubes remain viable for long periods when covered with oil. Some fungi continue growth, though slow, under oil. This method is widely adopted to many fungi, bacteria and actinomycetes. Fungi that produce acid or liquefy media are not suitable for storage under oil.

Grow fungal or bacterial cultures on suitable agar medium slants until acceptable growth and or sporulation has occurred. Under aseptic conditions, cover the cultures with sterile mineral oil to 1 cm above the edge of the agar. Oxygen diffusion through a thick layer is unfavourable. If the tip of the agar slant is not covered with the oil, it will dry out. Use medicinal grade oil of specific gravity 0.86 to 0.89 and autoclave on 2 successive days followed by drying for 1 to 2 h at 170^{o}C. To prevent contamination of

oil from spores blown out from culture, while pouring oil into the cultures, sterilize small quantities of the oil in individual containers. Seal the culture tubes and store either in a refrigerator or at room temperature. To revive culture under oil, remove all mass of mycelium with spores, drain off the excess oil and streak on a suitable medium. The first subculture is slow growing due to the pressure of the oil. Two or three transfers are needed to restore the original growth rate. Oil can be removed by washing the culture mass in sterile water in a culture tube or plate or by making a transfer to an agar slant and incubating upright allowing the oil to drain to the bottom.

Soil

Test tubes are half-filled (5 – 10 g) with fine sieved garden loam soil (20 % moisture) and sterilized at 15 lbs pressure for 20 min on two successive days. A spore suspension (2 to 3 ml) of fungal culture is poured into the sterile soil and incubated for about 10 days (for *Fusarium* only 2 to 3 days) and stored in a refrigerator or cool place. Sprinkling a few soil grains on to an agar plate retrieves cultures.

Freeze drying or Lyophilization

It is a long-term preservation of microorganisms. Cultures are dried from the frozen state by withdrawal of water vapour under reduced pressure. The dried cultures or spore suspensions are sealed and stored in glass ampoules. This is best suited to healthy sporing cultures. The final cultures are completely sealed in glass and hence there is no risk of cross infection or mite infestation.

Liquid nitrogen

Cultures, tissues or spore suspensions are treated with a protective medium such as 10 per cent glycerol, placed in ampoules and frozen at ultra low temperatures such as in liquid nitrogen. At these low temperatures metabolism is suppressed, so if the initial shock of freezing is survived, the culture should remain viable indefinitely. Revivals are said to be best when the cooling is done slowly. This technique requires a large and expensive apparatus and a reliable source of liquid nitrogen.

VI. SCREENING OF ANTAGONISTS

Various mechanisms have been reported to be involved in the biocontrol activities of the antagonists. The antagonistic activity of the biocontrol agents may be the result of the production of various compounds produced during their interaction with target pathogens. The determination of the type of mechanisms will enhance our understanding of the biocontrol agents and also assist in the selection of efficient bioagents for mass multiplication and field application. *In vitro* screening methods of biocontrol agents with reference to various mechanisms of action are given below.

A. Studying the mycoparasitic activity

i. Screening for antagonism by dual culture technique

Microorganisms obtained from dilution plates are to be purified and these can be tested individually for their efficacy against plant pathogens *in vitro* as well as for their antibiotic/ toxic metabolite production in agar medium. **Bi-cultures** or **dual cultures** or **paired cultures** or **cross cultures** are the techniques generally followed to test their efficacy. This method can be used for testing antibiotic production as well as to study the mycoparasitism and lysis of pathogen's propagules by bioagents. The culture media used for dual culture should favour the growth and antibiotic production of potential antagonists as well as growth of the pathogens.

Procedure

- Pour 15 ml of melted and cooled (45-50^{o}C) potato dextrose agar (PDA) medium to each Petri plate and allow them to solidify since the thickness of the medium in culture plates influences the size of zone of inhibition. To obtain large and distinct zone, not more than 15 ml of medium should be used in 9 cm dia culture plates.
- After cooling, place an 8 mm mycelial disc cut from the margin of the actively growing colony of test pathogen near the periphery on the one side of the PDA plate.
- Place a disc of antagonist on the other side of the PDA plate just opposite to the test pathogen i.e., at an angle of 180 ^{o}.
- Incubate the inoculated plates at room temperature for 2 to 7 days.
- If antagonism is to be tested between the bacteria, make a streak across the centre of nutrient agar plate with the antagonistic bacteria. About 1 - 2 cm on each side makes a parallel streak of test pathogen.
- If the antagonism is tested between bacteria/ actinomycetes and fungi, actinomycetes/ bacteria are spotted or streaked and the test fungus is inoculated as culture discs.

(**Note**: If both the organisms are fast growing then each is seeded opposite each other near the periphery of the plate. If both are slow growing, they are placed 2 to 3 cm apart. If the test organism is fast growing such as *Macrophomina phaseolina*, *Rhizoctonia solani* and *Pythium* spp., the antagonist is seeded near the periphery of the plate 1 or 2 days prior to seeding with the test pathogen. If antagonism is tested between fast growing

antagonists (*i.e.*, *Bacillus subtilis*) and slow growing fungal pathogen, the test pathogen should be seeded 1 or 2 days prior to inoculation of antagonist).

The antagonism is also tested by the following methods.

a. The test pathogen is seeded at 3 to 4 equidistant points near the periphery of the culture plate and the antagonist is seeded in the centre of the plate or vice versa.
b. Three or four potential antagonists are placed at equidistant sites 1 cm away from the periphery of Petri plate and the pathogen is seeded in the centre. This method is useful for screening a large number of antagonists.
c. A pathogen spore or mycelium suspension is prepared in the agar medium and 15 ml is poured into each plate. The antagonist is either streaked or spot-seeded at 3 to 5 points on the hardened agar surface and observed for zone of inhibition or vice versa.

Observation

Observe the plates at every 24 h post-inoculation for the antagonism and continue the observations for 5 - 7 days. In the above tests, as the two organisms grow towards each other, the reduced growth rate of the pathogen at a distance from the periphery of the potential antagonist indicates the production of antibiotic. In certain cases, growth may be arrested due to nutrient deprivation. Other reactions that can occur in paired cultures are that both organisms stop growing upon contact with a small but clearly marked space between them. Antagonism between two organisms also is indicated when the pathogen stops growing upon contact with the antagonist and its mycelium begins to lyse backwards and the antagonist may continue its growth over the colony of the test fungus (mycoparasitism). To see the hyphal integration, small bits of mycelium can be taken from interaction zone and observed under Scanning Electron Microscope (SEM). Hyphal interaction in dual culture can also be studied by growing the fungi on a cellophane membrane in Petri dish with water-agar plus glucose (0.5% w/v). After 4 to 5 days, pieces of cellophane membrane from the contact zone are cut and prepared for microscopic observation. Antagonism is also indicated when the aerial fluffy mycelium of the test pathogen is appressed when growing near the antagonist.

When the efficacy of *Trichoderma* spp.is tested against *Macrophomina phaseolina in vitro*, the inhibition of mycelial growth, production of sclerotia, number, size and their germination are also to be observed by following specific procedures.

ii. Sclerotial number: Take four mycelial discs (8 mm dia) of the pathogen from the dual culture plate where the pathogen and the antagonist interact after five days of inoculation. Place the discs in a beaker containing 10 ml of sterile distilled water and stir well for 30 min to separate the sclerotia from the medium. Squeeze the entire contents through muslin cloth and wash in several changes of distilled water and transfer to a glass vial containing 2.5 ml of 2.5 % ammonium sulphate. The sclerotia will float after 10 min. Filter these sclerotia through a filter paper, rinse them in distilled water and count with a stereo zoom microscope.

iii. Sclerotial size: For each dual plate, harvest 25 sclerotia after 7 days of inoculation, dry for 2 h in shade and measure the size with an ocular micrometer calibrated microscope. The procedure for measuring the size of fungal spore/ other structures is given below.

Determination of size of a microorganism is one of the properties useful for identification in the laboratory. The study of measurement of microorganisms is called **micrometry**. Since the microorganims are small, their dimensions are usually expressed in units smaller than millimetre *viz*., micrometre. The size of the viruses are still smaller, hence they are expressed as nanometre and angstrom.

One micrometre (μm)	-	one thousandth (10^{-3}) of a millimeter (or) one millionth (10^{-6}) of a metre.
One nanometre (nm)	-	one billionth (10^{-9}) of a metre (or) one millionth (10^{-6}) of a millimetre.
One angstrom (Å)	-	one-tenth (10^{-1}) of a nanometre (or) one-ten billionth (10^{-10}) of a metre

Since microorganisms can be seen only under a microscope, their size can be measured by equipping the microscope with an ocular micrometer, which is then calibrated against a stage micrometer. The ocular micrometer is simply a glass disc with

etched lines on its surface. It has 100 equally spaced divisions, marked 0-10. The distance between the graduations of an ocular micrometer does not have any standard value and varies depending on the objective used; the latter determines the size of the field. This distance is found out by calibrating it with a known scale, the stage micrometer. Stage micrometer is a special glass slide having in its centre a known distance one millimeter, which is encircled and mounted by a cover glass (Fig.). This one mm distance is etched into 100 equally spaced divisions. Since there are 1000 micrometres in one millimetre, one division of stage micrometer equals to 10 μm or 0.01 mm. The distance of each stage micrometer's division becomes correspondingly enlarged under high-power and oil-immersion objectives of the microscope.

Ocular micrometer after putting inside the eye-piece is calibrated by superimposing the graduations of ocular micrometer over graduations of the stage micrometer, which is accomplished by rotating the ocular lens. By determining the number of ocular micrometer divisions coinciding with the number of divisions on stage micrometer, the calibration factor for one ocular division (O.D) is calculated for the particular combination of objective and ocular lens used by applying the formula:

$$\text{One ocular division (in } \mu\text{m)} = \frac{\text{Number of divisions on stage micrometer}}{\text{No. of divisions on ocular micrometer}} \times 10$$

After calibrating, an ocular micrometer can be used to determine the size of an organism/structure of an organism in terms of length, breadth and diameter by the formula:

Size (in μm) = No. of ocular divisions x calibration factor of the objective used.

Calibration of an ocular micrometer for different objectives (low-power, high power and oil-immersion) of a microscope

Procedure

1. Remove the eyepiece (ocular) lens from your microscope, unscrew it and insert the ocular micrometer disc on the circular shelf (metal diaphragm) with the engraved side down into it. Screw the eyepiece back and insert it in the microscope. When observed, ocular micrometer divisions will be seen in sharp focus and there will be no changes in lines and distances under different objectives.
2. Place the stage micrometer on the microscope stage and bring its scale in the microscope's field centre under a sharp focus first using the low-power objective, and thereafter with high-power and oil-immersion objective.
3. Turn the ocular lens until the parallel lines of the ocular micrometer to be parallel with those of the stage micrometer.
4. Make the lines to coincide with the left end and find another set of lines which coincide.
5. Now count the number of distances (divisions), in both the ocular and stage micrometers, between the two coinciding lines.
6. Take at least six readings at random repeating the steps 3-5.
7. Repeat procedure (steps 3-6) with the high-power (40x) and oil-immersion (100x) objectives.

Results: Record your observations in the form of a table. Take the means of the 5-6 readings taken for the ocular micrometer coinciding with the stage micrometer divisions each for low-power, high-power and oil-immersion objectives. Determine the value of calibration factor for different objectives.

a. Low-power objective (10x X 10x *i.e.*, 100x magnification)

   Suppose 20 ocular micrometer divisions = 30 stage micrometer divisions

$$\text{One ocular division} = \frac{30}{20} \times 10\ \mu m\ (1 \text{ stage division} = 10\ \mu m) = 15\mu m$$

b. High-power objective (10x X 40x *i.e.*, 400x magnification)

   Suppose 24 ocular divisions = 9 stage divisions

$$\frac{9}{}$$

$$\text{One ocular division} = \frac{\text{-----}}{24} \times 10 = 3.75\ \mu m$$

c. Oil-immersion objective (10x X 100x *i.e.,* 1000x magnification)

Suppose 20 ocular divisions = 3 stage divisions

$$\text{One ocular division} = \frac{3}{20} \times 10 = 1.5\ \mu m$$

Measurement of microorganisms by use of an ocular micrometer

Procedure

- Find out the calibration factor for one ocular micrometer division for the low-power, high-power and/or oil-immersion objective, depending upon the size of the microorganism, whose measurement is to be taken, as outlined in the previous experiment 3, or use a calibrated micrometer.
- After calibration, replace the stage micrometer with the prepared/provided slide of the microorganism.
- Count the number of ocular micrometer divisions a single cell occupies.
- By rotating the ocular lens determine the division/divisions of several individual cells/ conidia/ fructifications and take at least 10 readings (if organism is not round take 10 readings each for length and width of the microorganism).

Result

Based on your observations determine the size of a microorganism as follows:

$$\text{Size of microorganism} = \text{Number of ocular micrometer divisions occupied} \times \text{Calibration factor for one ocular division (for the objective used)}$$

For example, a bacterial cell occupies 5 divisions of ocular micrometer when examined under oil-immersion, and then the dimension of a cell would be:

5 x 1.5 μm (one ocular division for oil-immersion objective = 1.5 μm) = 7.5 μm.

iv. Sclerotial germination: Take 25 sclerotia from each dual culture plate and test their germination by cavity slide germination method. Incubate the cavity slide in a moist chamber for 24 h. The number of germinated sclerotia and the hyphae putforth by each sclerotium is counted using a microscope.

B. Testing antibiotic production in culture filtrate

Cell-free culture filtrates of antagonists that cause growth inhibition of the test pathogen in agar media can be tested to confirm the production of antibiotics. The organisms are grown on a suitable liquid medium in stationary or shake culture. After sufficient growth has occurred a majority of the microbial cells are removed by filtering through filter paper or by centrifugation. The almost clear liquid is sterilized by filtering through membrane or sintered glass filters. Seitz asbestos pad filters should not be used because antibiotic may be adsorbed to it. The cell free filtrate obtained is added to the sterile growth medium on which pathogen is to be grown, in desired concentration. The selection of a suitable assay medium that supports growth of the test pathogen and will not inactivate or mask the activity of the antibiotic is very essential. The amount of agar medium per culture plate should be standardized for comparative tests.

i. Filter paper disc method

Pour 15 ml of a culture medium incorporated with test pathogen (3 ml of the spore suspension is mixed with 12 ml of the culture medium) uniformly to 9 cm flat-bottomed culture plates. After solidification, filter paper discs of 1 to 2 cm dia are autoclaved, dried and then soaked in the culture filtrate. They are dried separately on a wire net and then placed on the seeded agar medium at least 1 to 1.5 cm from the periphery of the plate. Four to six discs can be placed on a single plate. At least ten discs from each concentration of the culture filtrate and two culture plates should be used. After incubation, the zones of inhibition around the filter paper discs are measured.

ii. Well-in-agar method

Culture plates are prepared as above. Using a flamed cork borer of 1 to 2 cm dia, agar plugs are removed at a distance of 1 to 2 cm from the periphery of the plate and these wells filled with a standardized quantity of the culture filtrate.

iii. Spore germination method

In the well of a depression slide, place 0.2 to 0.5 ml of the culture filtrate (use potato dextrose broth for fungi and King's B broth for *P. fluorescens*) and dry at room temperature. The same amount of spore suspension (5×10^3/ml) prepared in sterile water or nutrient solution, is added over the dried culture filtrate and the slides incubated in a humid chamber at 25 to 28^{o}C. After 24 hours spore germination and characteristics of the germ tube are determined and compared to that of the control.

iv. Inhibition of mycelial growth

In another method, sterile culture filtrate in desired concentration is added to sterile culture medium and plated. The mycelial disc of test pathogen is inoculated at the centre of the plate. Medium without incorporation of culture filtrate is served as control. The inoculated plates are incubated at room temperature for 2-7 days. Reduction in colony growth in filtrate amended medium compared to control indicates the secretion of antibiotic compounds into culture by antagonists.

v. Assay in liquid medium

The culture filtrate of the antagonist is amended with nutrients suitable for the growth of the test organism and sterilized through filters. If the dilution of the culture filtrate is desired, the nutrient solution of different concentration is prepared so that when the culture filtrate is added, the concentration of the nutrient in each becomes identical. It may be desirable to dry the culture filtrate and the amount of solid material obtained from a lit of culture filtrate is calculated and then a weighed quantity of solid material is added to a standardized pre-autoclaved nutrient solution. The test can be done in culture tube or conical flasks. The latter is better suited for fungal pathogens. The medium is seeded with a standardized amount of inoculum of the test pathogen, that is a loopful of bacterial

suspension (10^7 / ml) or a 5 mm disc from an agar culture of the test fungus. After incubation (shaken or stationary) and until sufficient growth has occurred in the control the growth of bacteria is determined photometrically. The growth of fungi is determined by dry weight.

C. Production of non-volatile metabolites

The non-volatile metabolites are produced in the medium in which the biocontrol agent is grown. The pathogen is grown on the same media containing the metabolites of the biocontrol agent. The growth of the pathogen on that media will indicate the effectiveness of the metabolites in checking the growth in comparison with the growth in control plate.

Procedure

- Cut the cellophane papers to the size of the lower lid of Petri plate and autoclave
- Pour the molten and cooled potato dextrose agar (15 ml) in sterilized Petri plates under aseptic conditions
- Place the sterilized cellophane paper over the surface of solidified agar medium aseptically
- Incubate the plates for 24 h.
- Inoculate 8 mm actively growing mycelial disc of antagonistic fungus at the centre of the cellophane paper
- Incubate the plates at room temperature for 7 days
- Maintain control plates without inoculation of antagonistic fungus
- Remove the cellophane paper along with the fungal mat after incubation
- Inoculate 8 mm mycelial disc of pathogen culture at the centre of the Petri plate and incubate
- Plates incubated earlier only with the cellophane paper without antagonistic fungus serve as control
- Observe for the mycelial growth in all the Petri plates

Observation

If the metabolites produced by the fungal biocontrol agent are inhibitory towards the pathogen, there will be less growth in the treatment plate as compared to control plates.

D. Production of volatile metabolites

Many volatile metabolites are reported to be produced by various biocontrol agents in the process of their interaction with the target pathogens. The volatile metabolites are less inhibitory to the pathogen *in vitro* when compared to the effect of the non-volatile metabolites, as there is no direct contact.

Procedure

- Place 8 mm mycelial disc of actively growing colony of test antagonistic fungus in the center of the Petri plate containing PDA medium
- Inoculate another Petri plate of the same diameter with actively growing mycelial disc (8 mm) of the pathogen in the same manner as done above
- Invert the pathogen inoculated basal Petri plate (after removing the upper lid) over the first plate (test antagonistic fungus inoculated) after removing the lid
- Seal the junction of both plates with parafilm and incubate at 28±2^{o} C for 7 days
- Maintain control plates with pathogen disc inverted and sealed over the plates with uninoculated medium
- Observe and measure the growth of pathogen in all the plates and assess the per cent inhibition of mycelial growth

Observation

If the volatile metabolites produced by the biocontrol agent are inhibitory towards the pathogen then there will be less growth in the treatment plates as compared to control plates.

E. Production of siderophores

One of the modes of inhibition of the pathogen by the antagonist is considered to be the production of siderophores by the bacterium. Siderophores are the iron-chelating agents, which deprive, the surrounding pathogens, of iron. This will cease the growth of the pathogen. The methodology for detection of siderophore is given below.

Detection of siderophore

A simple qualitative test can be run to screen for production of siderophores in the test organism based on Chrom Azurol S (CAS) agar assay. This molecule contains chomplexometric titration dye chrom Azurol S, which comes closest to a universal chemical probe for siderophore structure.

Screening in CAS agar medium

Solution A. Dye solution

Chrom-Azurol S	-	60.5 mg
Distilled water	-	50.0 ml

Solution B. 1 m M $FeCl_3$. 6 H_2O in

10 mM HCl	-	10.0 ml

Solution C. Hexadecyl trimethyl

ammonium bromide	-	72.9 ml
Distilled water	-	40.0 ml

Solution A is added to solution B and the resulting solution is added to solution C slowly. The resultant dark blue solution is autoclaved and added to nutrient agar before pouring the plates. CAS agar plates are then inoculated with active culture of the test microorganism and plates are incubated at optimum temperature for 48 h.

Observation

Appearance of an orange zone against the dark blue background suggests siderophore production.

Another method of siderophore detection in *P. fluorescens* is done by the following method.

Procedure

- Prepare the inoculum of the antagonistic bacteria in King's B broth and incubate at 28±2^{o} C for 18 h.
- Prepare succinate medium
  (The composition includes K_2HPO_4 – 6.0 g; KH_2PO_4 – 3.0 g; $(NH_4)_2 SO_4$ – 1.0 g; Mg SO_4. 7 H_2O – 0.2 g; succinic acid – 4.0 g and distilled water – 1000.0 ml)
- Inoculate one ml of bacterial inoculum produced in King's broth to 200 ml of succinate medium in a 500 ml conical flask
- Incubate the inoculated flask at 28±2^{o} C on a rotary shaker (120 rpm) for 48 h.
- Centrifuge the culture at 10,000 g for 20 min.
- Collect the cell-free supernatant and examine for absorption spectrum between 200 and 600 nm using UV-visible spectrophotometer.

Observation

Presence of a peak at or near 405 nm will indicate the production of siderophore. The effective strain, producing the siderophore will give a peak at 405 nm wavelength.

F. Production of HCN

Production of HCN is one of the factors that can suppress the growth of pathogen by fluorescent pseudomonads. It is known that cyanogenesis from glycine results in the production of HCN, which is volatile. The HCN produced reacts with picric acid in the presence of ammonium carbonate [$(NH_4)_2 CO_3$] to give orange colour. Negative reaction shows no colour change, where the yellow colour of the substrate remains unchanged.

Procedure

- Prepare medium by amending King's medium with glycine @ 4.4 g/lit.
- Pour 20 ml of medium in each Petri plate
- Streak the selected isolates of bacterial antagonist on plates separately
- Soak Whatman No. 1 (9 cm dia) filter paper in a solution of 0.5 per cent picric acid containing 2.0 per cent ammonium carbonate

- Place each filter paper disc in the under side of the upper lid of Petri plate
- Seal the plates with parafilm and incubate at 28±2^{o} C for 4 days
- Maintain uninoculated plates as control and observe for colour change

Observation

In the positive reactions, the colour of the filter paper disc will turn orange from yellow indicating the production of HCN by the test bacterium.

VII. MASS MULTIPLICATION

A. MASS MULTIPLICATION

For successful biological control, technologies including the production, formulation and delivery system of antagonists play an important role. In addition, these aspects must be implemented to be compatible with industrial and commercial development methods and field application. With the increasing interest in developing alternatives to chemical fungicides, mass production of biocontrol agents for use as bioprotectant has become a focus of industrial research and development. Methods and techniques were also devised for industrial production of biocontrol agents, which were actually adopted from the existing methods for mass culture of microorganisms in pharmaceutical industries.

The following are the methods generally employed for the mass production of biocontrol agents. They are

1. Mass multiplication in solid substrates (solid state fermentation using crop grains, agricultural waste materials, by-products, *etc*.)
2. Mass multiplication in liquid media
    i. Through still culture technique
    ii. Through shake culture technique
    iii. Through liquid fermentation technology

The first method of solid-state fermentation is followed only for fungal biocontrol agents. The second method is followed to both fungal and bacterial biocontrol agents.

Solid state fermentation

Solid-state fermentation is a very common method of mass production of antagonists in laboratory experimentation. The antagonistic fungus is grown in Petri plates, spores and other propagules are then harvested and formulated. Other types of solid-state fermentation are the use of various cheap agriculture wastes and by-products for mass production of biocontrol agents. Various substrates being used in solid-state fermentation are listed below in Table 12. They are well decomposed farm yard manure,

gobar gas slurry, well decomposed pressmud, well decomposed coconut coir pith, paddy chaff, wheat bran, rice bran, sand-maize medium, tapioca thippi, tapioca rind, mushroom spent bed, groundnut shell, sugarcane bagasse, wheat straw, sheep manure, shelled maize cob, paddy straw, Bengalgram husk, poultry manure, *etc*. This type of fermentation results in a product that is generally used as such rather than being formulated further. These products are likely to obstruct agricultural machinery and thus are probably not feasible for commercial use.

Table 12. Substrates successfully used to produce antagonistic organisms by solid-state fermentation.

Substrate	**Reference**
FYM, coconut coir pith and neem cake	Dinakaran, 1997
FYM, wheat-bran, rice-bran, peat soil, rice straw	Mehta *et al.*, 1995; Sangeetha, 1988
Grain bran	Wells *et al*., 1972
Groundnut shell medium	Raguchander *et al*., 1993
Peat soil	Jeyarajan *et al.*, 1994
Peat-bran	Maplestone *et al.*, 1991
Sand-Corn meal	Lewis and Papavizas, 1980
Sand and sorghum medium	Padmanabhan and Alexander, 1987
Sorghum grain	Upadhyay and Mukhopadhyay, 1986
Tapioca rind and refuse, FYM, pressmud	Kousalya and Jeyarajan, 1988
Wheat bran	Henis *et al*.,1978; Lewis and Papavizas,1993
Wheat bran, kaolin clay	Lewis and Papavizas, 1985.
Wheat bran, peat or combination of these	Howell, 1982; Papavizas, 1985; Sivan *et al*., 1984
Wheat bran - sawdust	Elad *et al*., 1980
Wheat bran - sawdust modified medium	Mukhopadhyay *et al*., 1986

Liquid state fermentation

During the past 30 years liquid state fermentation has been developed extensively by industry for the production of antibiotics, enzymes, organic acids and other microbial products. Hence, it appears feasible for industry to produce various beneficial microbes against plant pathogens through liquid fermentation. In this method, biomass of a bioagent must be produced in a timely and cost-effective manner. The biomass is usually dried to avoid microbial contamination. Maximum biomass of bioagent can be realized in short-time by using appropriate medium in a fermentor with aeration, agitation, temperature, pH and antifoam controls than in shake-cultures and will be more suitable for industrial production of bioagent. Two types of fermentors *viz*., batch type and continuous type are available. In batch type fermentors the organism is grown in the medium for a definite period of time. Then the biomass is separated or concentrated. In the continuous type fermentors, the inflow of the ingredients into the medium is regulated with the withdrawal of fermented product of biomass in such a manner as to maintain the fermentation process continuously at a given level of operation. Various media used in liquid fermentation are listed below in Table 13.

Table. 13. Growth media used for production of bioagents in liquid state fermentation

Growth medium	**Reference**
Czapek-Dox broth and V 8 broth	Harman *et al*., 1991; Lorito *et al*., 1993
King's B broth	Vidhyasekaran and Muthamilan, 1995
Molasses and brewer's yeast	Papavizas *et al*., 1984
Molasses-Soy medium	Prasad and Rangeshwaran, 2001
Molasses yeast medium	Ramakrishnan *et al*., 1994
Modified Richard broth medium	Lorito *et al*., 1994
Nutrient broth	Jeyarajan *et al*., 1994

B. ESTIMATION OF POPULATION OF ANTAGONISTS

The population estimation of antagonists is very essential to maintain the quality of the end product. The population is assessed in liquid culture before mixing with talc, the substrate by using haemocytometer and in the product after formulation by serial dilution-agar plating technique.

a. Estimation of cells / spores of microorganisms in liquid culture

The density of cells, spores/conidia of microorganisms can be measured in the laboratory by several methods either by direct or indirect counts. In the direct microscopic count, a known volume of liquid is added to the slide and the number of microorgansims is counted by examining the slide with the bright field microscope. For direct microscopic counts **Neubauer or Petroff-Hausser counting chamber**, blood smears or electronic cell counter (as coulter counter) are used. Population density may be determined by observing some property that provides indirect evidence of microbial number in a sample. Various methods for indirect counts are: determining cell mass (dry weight determination) or cellular constituents (DNA and protein), oxygen uptake, carbon dioxide production, turbidimetric measurements for increase in cell number (spectrophotometric or colorimetric analysis), membrane-filter count and the serial dilution-agar plate (plate count) method. Excepting the last two methods, which are used to determine the viable cells, the major disadvantage common to all these are that the total count includes both the dead as well as living cells. The two most common indirect methods are plate count and turbidity measurements.

Measurement by counting chamber

The haemocytometer (because it was originally devised for counting blood cells) is used for counting the fungal spores in liquid suspension. It is a special microscopic slide with an H-shaped trough forming two counting chamber. It is more commonly used in various laboratories. The specifications for depth and size of the squares are mentioned on the slides. The counting chamber consists of 9 big squares, each of 1 mm x 1 mm engraved over it. But only one big square per field is visible under 100x

microscopic magnification (10x ocular and 10x objective). Each of these big squares is divided in to 25 medium sized squares (0.2 x 0.2 mm each) and each one is separated by triple lines, the middle one act as the boundary. Each medium size square is further subdivided into 16 small squares (0.05 x 0.05 mm each). The depth of the counting chamber is 0.1 mm.

Area of one big square	= 1 mm x 1mm = 1 mm^2
Volume of one big square	= 1 mm x 1 mm x 0.1 mm
	= 0.1 mm^3
	= 0.0001 ml (1 ml = 1000 mm^3)
Area of one medium size square	= 0.2 mm x 0.2 mm = 0.04 mm^2
Volume of one medium size square	= 0.2 mm x 0.2 mm x 0.1 mm
	= 0.004 mm^3
	= 0.000004 ml (4 x 10^{-6} ml)
Area of one small square	= 0.05 mm x 0.05 mm = 0.0025 mm^2
Volume of one small square	= 0.05 mm x 0.05 mm x 0.1 mm
	= 0.00025 mm^3
	= 0.00000025 ml (2.5 x 10^{-7}ml)

Procedure

- Place a drop of conidial suspension made from liquid culture (filtered through one or more layers of muslin cloth depending on the size and morphology of conidia) on the engraved grid and let the preparation stand for 1 to 2 min to allow the conidia to settle at the bottom.

- Put the cover glass (special cover glass of counting chamber) over the grid carefully so that no air bubble enters between the slide and cover glass.

- Slide the cover glass backwards and forwards until coloured rings are visible as the two surfaces of cover glass and slide come into close contact.

- Count the spores of fungus (if they are small in size and more in number in the middle square (small squares) which consists of 25 groups of 16 small squares in each group with 0.2 mm square or 0.04 mm^2. The size of each small square is 0.0025 mm^2.

- For larger spores or fewer spores count spores in 4 corner large squares (medium size squares). The size of the square is 0.04 mm^2.

Results

Calculate the number of cells or spores per ml of the suspension mathematically as follows:

For larger cells or spores counted in medium sized squares

$$\text{Cells or spores / ml} = \frac{\text{Average number of spores counted per medium size square}}{\text{Volume of fluid}}$$

If one cell or spore / square is observed

$$\text{Cells or spores /ml} = \frac{1}{0.000\ 004} = 2,\ 50,000$$

$$= 0.25 \times 10^6 \text{ spores /ml}$$

Factor : 0.25×10^6 spores /ml

For smaller cells or spores counted in small squares

$$\text{Cells or spores / ml} = \frac{\text{Average number of spores counted per small size square}}{\text{Volume of fluid}}$$

If one cell or spore / square

$$\text{Cells or spores /ml} = \frac{1}{} = 4,000,000$$

0.000 000 25

$= 4 \times 10^6$ spores /ml

Factor: 4×10^6 spores /ml

Precautions

- Always count cells or spores on top and left touching middle line of the perimeter of each square and do not count cells or spores touching the middle line at bottom and right sides.
- Do not overfill or under fill the chambers of haemocytometer.
- A minimum of 200-250 spores (*i.e.*, 20-25 cells or spores /square) should be counted to avoid error.

Disadvantages

Although counting of cells or spores by this method are rapid but disadvantage lies in counting both dead and living cells. Moreover this method is not sensitive to populations of fewer than one million cells or spores.

b. Enumeration (counting) of microorganisms in formulations by plate count or serial dilution–agar plating technique

In the biocontrol formulations, determination of the viable propagules in a given sample and purity of the formulation without contamination is a perquisite for assessing the efficacy. The plate count technique is one of the most routinely used procedures because of the enumeration of viable cells by this method. This method is based on the principle that when material containing bacteria / fungi are cultured, every viable bacterial cell or fungal spore/ mycelial fragment / viable units develops into a viable colony on a nutrient medium. The number of colonies therefore is the same as the number of cells or **colony forming units (cfu)** contained in the sample. In this procedure a small measured volume (or weight) is mixed with a large volume of sterile water called

the diluent or dilution blank. Dilutions are usually made in multiples of ten. A single dilution is calculated as follows:

$$\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Total volume of the sample and the diluent}}$$

Serial dilutions are later prepared by transferring a known volume of the dilution to second dilution blank and so on. Once diluted, the specified volume of the dilution sample from various dilutions is added to sterile Petri plates (in triplicate for each dilution) to which molten and cooled (45-50^{o}C) suitable agar medium is added. The colonies are counted on a colony counter. The number of colonies developed on the plates after an incubation period of 2-7 days is multiplied by the dilution factor, which is the reciprocal of the dilution to obtain the population per ml. To facilitate calculations, the dilution is written in exponential notation. For example, 1:1000 dilution would be written as 10^{-3}.

$$\text{Number of cfu / ml} = \frac{\text{Number of colonies}}{\text{Amount plated x dilution}}$$

Procedure

- Label the dilution blanks as 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7}
- Prepare the initial dilution by adding 1 ml or 1 g of the sample into a 9 ml dilution blank labeled 10^{-1} thus diluting the original sample 10 times (1/10 and is written 1:10 or 10^{-1})
- Mix the contents by rolling the tube back and forth between your hands to obtain uniform distribution of organisms (cells)
- From the first dilution transfer 1 ml of the suspension while in motion, to the dilution blank 10^{-2} with a sterile and fresh 1 ml pipette diluting the original specimen /suspension to 100 times (1/10 x 1/10 = 1/100 or 10^{-2})
- From the 10^{-2} suspension, transfer 1 ml of suspension of 10^{-3} dilution blank with a fresh sterile pipette thus diluting the original samples to 1000 times (1:1000 or 10^{-3}).

- Repeat this procedure till the original sample has been diluted 10,000,000 time (10^{-7}) or desired dilutions using every time a fresh sterile pipette.
- From the appropriate dilutions (10^{-4} to 10^{-7}) transfer 1 ml of suspension while in motion, with the respective pipettes, to sterile Petri dishes with three replications for each dilution.
- Add approximately 15 ml of the melted and cooled nutrient medium to each Petri dish containing the diluted sample. Mix the contents of each dish by rotating gently to distribute the cells throughout the medium.
- Allow the plates to solidify.
- Incubate these plates in an inverted position for 2 - 7 days at room temperature.

Observation

1. Observe all the plates for the appearance of fungal or bacterial colonies.
2. Count the number of colonies in the plates, by placing each plate one by one in colony counter.

Results

Calculate the number of bacterial cell /cfu of fungi per ml of the original suspension/ sample as follows:

$$\text{Cells or cfu / ml or g of the sample} = \frac{\text{Number of colonies (average of 3 replicates)/plate}}{\text{Amount of sample plated x dilution}}$$

For example, if 60 colonies were counted on a $1:10^5$ dilution

$$\frac{60 \text{ colonies}}{1 \text{ ml} \times 10^{-5}} = 6{,}000{,}000 = 6 \times 10^{6} \text{ cfu / ml or g of sample.}$$

By this method, the population load of bio-formulations can be estimated before packing and also at different intervals during storage period to know the viability of organisms at storage.

VIII. FORMULATIONS AND DELIVERY SYSTEMS

After identification of effective isolate of antagonistic microorganisms, they are multiplied in large scale in suitable and cheaper substrate available locally. The biocontrol agents, mass multiplied in different substrates can not be applied as such. Hence they are to be formulated in suitable carriers and delivered to control target organisms. The various formulations and delivery systems employed in the use of biocontrol agents are discussed.

A. FORMULATIONS

A formulated product for agricultural application should possess several desirable characters, which are given below.

- Propagules should be of appropriate type and desiccation tolerant. *e.g.*, chlamydospores of *Trichoderma*
- Production must be economical.
- Biomass should be preserved against contamination.
- High percentage of propagules must be germinable and effective (sustained efficacy).
- Long shelf-life.
- Should have good stability during transportation and storage.
- Easy to prepare and apply.
- Safe to plants, animals and microorganisms.
- Should give effective and consistent results under a variety of environmental conditions.
- Should have adequate market potential.

The composition and concentration of substrate are important in improving the performance of antagonists. The carrier for the formulation may be inert (talc, gypsum, saw dust, vermiculite, alginate and diatomaceous earth), nutrients (rye grass seed, molasses-yeast, wheat bran, rice bran, sugarcane bagasse, *etc*.) or combination of both

(wheat bran – saw dust, rice bran – saw dust, *etc*.). The commonly used carrier materials are talc (magnesium trisilicate), kaolinite clay, bentonite clay, peat, lignite, *etc*. Various carrier materials, which are commonly used in the formulation of bioagents are listed Table 14.

Table. 14. Carrier / food base materials used in the formulation of bioagents.

Carrier	**Reference**
Diatomaceous earth granules-molasses	Backman and Rodriguez-Kabana, 1975
Lignite and stillage	Jones *et al*., 1984
Pyrax	Papavizas *et al*., 1984; Papavizas and Lewis, 1989
Shelled maize cob, blackgram shell	Gandhikumar *et al*., 2001
Sodium alginate, wheat flour and $CaCl_2$	Fravel *et al*.,1985;Lewis and Papavizas,1985
Talc	Ramakrishnan *et al*., 1994
Talc, gypsum, lignite, kaolin, peat	Ranganathan *et al*., 1995
Talc, peat, vermiculite, lignite, kaolin	Vidhyasekaran *et al*., 1997
Vermiculite and wheat-bran	Lewis *et al*., 1991; Nakkeeran *et al*., 1997
Wheat-bran, kaolin	Lewis and Papavizas, 1985
Wheat-bran	Prasad and Rangeshwaran, 2001

In general, product formed from solid or semi-solid state fermentation does not require sophisticated formulation procedures prior to use. For example, grain or other types of organic matter upon which antagonists are grown are simply dried, ground and added to the area to be treated. The various solid substrates used for the mass multiplication of fungal bioagents are given below:

i. Diatomaceous earth granules (Backman and Rodriguez - Kabana, 1975)

Diatomaceous earth granules are added into a broth consisting of 100 ml black strap molasses (pH 5.0), 900 ml of water, 3 g each of KNO_3 and KH_2PO_4 till the level of saturation. It is autoclaved at 121^oC for 15 min and spread in shallow pans to a height of 3-5 cm and autoclaved again. The fungal antagonist (3 day old culture) is homogenized in a waring blender for 3 seconds and mixed with sterilized granules in shallow pans and incubated at 25^oC for 4 – 7 days. Clumps are broken up and granules, air dried with frequent stirring and used as an inoculum. The inoculum is mixed at 1:1 (v/v) with sterilized diatomaceous earth granules impregnated with 10 per cent molasses solution.

ii. Wheat bran: saw dust formulation (Elad *et al*., 1980)

Wheat bran: saw dust: tap water mixture (3: 1: 4 v/v) is taken in polypropylene bags and autoclaved for 1 hr at 121^oC for two successive days. The bags are inoculated with fungal bioagent and incubated in illuminated chambers for 14 days at 30^oC. It is applied at the time of sowing and mixed with the soil to a depth of 7 – 10 cm with a rotary hoe.

iii. Wheat bran: peat formulation (Sivan *et al*., 1987)

Wheat bran: peat mixture (1:1 v/v) is autoclaved for 1 h. Substrate moisture is adjusted to 50 % (w/w) with sterile water, medium was inoculated with 0.1 ml of a conidial suspensions containing 2 x 10^4 conidia /ml and incubated for 7 days at 30^oC and used at 10 % (v/v) level.

iv. Vermiculite - wheat bran formulation (Lewis *et al*., 1991)

The fungal bioagent is multiplied in molasses-yeast medium for 10 days. Vermiculite (grade 4) and milled wheat bran (250 mesh) are heated in hot air oven at 70^oC for 3 days using metal pans, vermiculite (100 g), wheat bran (3.3 g), liquid culture (14 ml) and 0.05 N HCl (17.5 ml) are mixed and packed. This can be used immediately for soil application.

v. Alginate pellets (Lewis and Papavizas, 1985)

Sodium alginate (20 g) is dissolved in 750 ml water at 40^{o}C on a stirring hot plate. Wheat bran ground to pass through a 0.425 mm mesh screen was placed in a glass blender container with distilled water (50 g / 250 ml). Kaolin is also used in place of bran at the same rate. It is autoclaved for 30 min and cooled. Depending upon the extent of fungal growth, wet fermentor biomass (FB) is added at 16 – 21 g and dried at 2.8 – 4.2 g / lit. The fungal biomass containing mycelium and spores are added to provide 7 x 10 ^{6} propagules / lit. The mixture containing fungus, alginate and bran or kaolin is added drop wise into 500 ml gellant solution (0.25 M $CaCl_2$, pH 5.4). As it enters, each droplet gelled and a distinct spherical bead is formed. After 20 min in the gellant, beads are separated from the solution by gentle filtration, washed and dried for 24 h in a stream of air at 25^{o}C. Pellets of uniform and required size can be prepared and is less bulky than most organic matter. Alginate pellets can be formed with the inert matter or with food base or with the combination of both as bulking agents.

The methodology given by Fravel *et al*. (1985) is as follows:

Twenty five g of sodium alginate is dissolved in 750 ml of distilled water and 50 g of wheat flour (food base) is suspended in 250 ml of distilled water. These preparations are autoclaved at 15 lbs for 30 min and when cooled are blended together with 200 ml of fermentor biomass. The mixture is added drop by drop into calcium chloride solution to form spherical beads, which are air-dried and stored at 5^{o}C.

The other substrates found to support the growth of fungal bioagents are farm yard manure, gobargas slurry, pressmud, paddy chaff, rice bran, groundnut shell, *etc*.

There are several problems with solid-state fermentation, which may make the system inappropriate for commercial product development. The preparations are bulky, they may be subject to a greater risk of contamination and they may require extensive space for processing, incubation and storage. The liquid state fermentation is devoid of such problems and large quantities of biomass can be produced with few days. The spores / mycelial biomass or together with the liquid broth are homogenized and population adjusted to required level (through centrifugation or dilution). The inoculum suspension is mixed with the carrier material and it should be inert and non-deleterious to

the viability of the propagules during storage. The normal ratio of inoculum to the carrier is 1:2. The suspension is thoroughly mixed with the carrier so as to get a homogenic mass. The resulting clumps are to be broken and shade dried. It may be either air dried or dried in a spray drier. The resulting fine powder formulation is immediately packed in polyethylene / polyproylene bags in required quantities and sealed carefully. The cell or spore count of the sample of the formulation should be at least $x10^8$ / g of the formulation. The packed product can be readily used for inoculation of crops. The packet should contain a label with the following particulars.

Name of the biocontrol agent :
Strain / species :
Spore or cell count :
Net weight :
Date of manufacture :
Date of expiry :
Batch number :
Address of the manufacturer :
Other details about the method of inoculation, dosage, crops, etc. may also be printed.

Generally powder formulations are suitable for easy application in the control of plant diseases. The following powder formulations viz, lignite, lignite-fly ash, lignite + lignite-fly ash, peat, talc of *Trichoderma, Bacillus* and *Pseudomonas* are made and are being currently used for the control of plant diseases. *Trichoderma* spp. can be formulated as pellets (Fravel *et al*., 1985; Lewis and Papavizas, 1985), dusts and powders (Nelson and Powelson, 1988) and fluid drill gels (Conway, 1986; Hadar *et al*., 1982). Among the various formulations, talc based formulation is considered advantageous because of the following characteristics.

- Not harmful to animals and human beings
- Do not affect environment
- No residue in food and ground water
- No risk of the pathogens developing resistance

- Easy to apply by farmers
- High cost- benefit ratio

Quality control

Quality control in the production of biocontrol agents need to be adhered strictly by the producer and inspected by regulatory authorities. The main criteria for quality control are biological activity, purity, physical features and safety to vertebrates and non-target organisms. These criteria are to be considered strictly for getting better results under field conditions and this will give more faith on the biopesticides.

Development of formulations

The methods of development of formulations of important biocontrol agents are given below:

1. Trichoderma viride

Trichoderma viride is a potential biofungicide used to control many of the soil- and seed-borne plant pathogens. The procedure for mass multiplication and preparation of talc based formulation of *T.viride* developed by Tamil Nadu Agricultural University, Coimbatore is given below.

Small scale

Grow the antagonistic fungus, *Trichoderma* in potato dextrose agar medium. Transfer mycelial discs grown in PDA medium to 250 ml conical flasks each containing 100 ml of sterilized molasses yeast medium (molasses – 30 g; baker's yeast granules – 5 g; distilled water – 1000 ml sterilized at 15 lbs psi for one hour) and incubate for 10 days. The medium can also be prepared in polypropylene bags instead of conical flasks. Homogenize the mycelial mat along with the broth, mix with talc powder (500 meshes) @ 1:2 v/w and shade dry until it attains 20 per cent moisture level. Before mixing with talc powder, assess the spore load of the broth culture using the haemocytometer and it should be between 10^8 and 10^9 spores/ml. After mixing, break the clumps and homogenize uniformly. To this mixture, add the sticker, carboxy methyl cellulose (CMC)

@ 5 g per kg and pack in polythene bags of 100 g capacity. Talc powder is differently named as French chalk powder or soap stone powder (magnesium trisilicate). The shelf life of the product is 120 days.

The required population level at the time of packing should be 20 x 10^7 cfu per g of the product and it should not go below 20 x 10^6 cfu per g during storage. The population (colony forming units) in the product is assessed by serial dilution method.

Large scale

Prepare the molasses yeast medium in 500 ml conical flasks and sterilize (at 15 psi for 1 h). Inoculate with mycelial disc of *T. viride* and incubate for 10 days. In a fermentor of 250 lit capacity, prepare 200 lit of molasses yeast medium and sterilize. Add the inoculum grown in conical flasks to the fermentor at one lit/ 200 lit of medium. Incubate for 10 days and provide aeration in the fermentor for 4 – 8 h / day by agitation. Then agitate the entire medium to get a broth culture containing fungal mats, spores, enzymes and antibiotics. Assess the spore concentration by drawing samples from the fermentor using haemocytometer. A minimum of 10^8 – 10^9 conidia / ml should be present in the sample. Mix the broth cultures (200 lit) with 400 kg of talc powder (1:2 ratio), air dry to less than 20 per cent moisture level and pass through a sieve to get a fine powder. To this, add 2 kg of CMC and mix thoroughly. Pack in polythene bags, heat seal and store at room temperature. This talc-based product can be used for seed treatment and soil application.

Quality control

- Take 1g of *Trichoderma* product and mix with 10 ml of sterile water (1:10) and shake well. Make serial dilutions to get 10^6 or 10^7.
- Transfer 1 ml of the desired suspension to sterilized Petri plates.
- Add 15 ml of melted and cooled *Trichoderma* selective medium in the same Petri plates.
- Rotate the plates gently and allow the medium to solidify.
- Incubate at room temperature (5-7 days).
- Observe the development of colonies.

- Count the number of colonies and calculate the colony forming units present in 1 g of the product.
- The population of the formulated product should not be less than 20 x 10^6 cfu /g (Fresh product should contain more than 28 x 10^6 cfu / g).
- At the time of mixing the broth with the talc powder, the broth should contain a minimum of 10^8 spores / ml.
- The moisture content of the fresh product should not exceed 20 per cent.
- The product should be packed in white polyethylene bags and stored.
- The product should be used before 4 months since the viability of the propagules is reduced drastically after 4 months of storage. Population should be maintained at not less than 20 x 10^6 cfu after 4 months of storage.
- The size of the talc powder used for product preparation should be of 500 microns in dia.

2. Pseudomonas fluorescens

TNAU has identified effective strains of *P. fluorescens* which control chickpea wilt, groundnut root rot, pigeonpea wilt and rice blast and sheath blight. A product has been developed and it is for seed treatment, soil application and foliar spray. Capsule formulations have also been developed for the control of banana wilt.

Small scale

Transfer a loopful *P. fluorescens* culture to 100 ml of sterilized King's B broth in a 250 ml conical flask under aseptic condition and incubate on a rotary shaker (150 rpm) at room temperature (28 $\pm$ 2^0C) for 48 h. Assess the population of *P. fluorescens* in the broth after the incubation period is over (A minimum of 9 x 10^9 / cfu ml is necessary). Adjust pH of the substrate (talc powder) to 7 by addition of 150 g calcium carbonate / kg of talc powder and sterilize it in an autoclave at 15 psi for 30 min on two successive days. Transfer 1000 g of the sterilized substrate (talc powder) and 10.0 g of sticker, carboxy methyl cellulose into a polythene bag or any sterile container under aseptic condition and add 400 ml of *P. fluorescens* suspension. Mix thoroughly and shade dry until it reaches moisture content of less than 20 per cent. The formulation can be stored in milky white

polythene bags for 3-4 months. The bio-formulation should contain a minimum of 2.5 x 10^8 cfu /g.

Large scale

The bacterium can also be multiplied in a fermentor. Prepare King's B broth in 500 ml conical flasks and sterilize at 15 psi for 30 min. Inoculate with a loopful of bacterial culture and incubate for 48 h. In a fermentor of 250 l capacity, prepare 200 l of King's B broth and sterilize. Add the inoculum, grown in conical flasks to the fermentor at one lit/ 200 l and incubate it for 48 h. Assess the population by drawing samples from the fermentor and it should contain a minimum of 9 $\pm$ 2 x 10^9 cfu / ml. Adjust pH of the substrate (talc powder) to 7 by addition of 150 g calcium carbonate / kg of talc powder. Sterilize it in an autoclave at 15 psi for 30 min for two successive days. Take 500 kg of the sterilized substrate (talc powder) after mixing with 2.5 kg of carboxy methyl cellulose in any sterile container under aseptic condition and add 200 l of P. *fluorescens* broth culture. Mix thoroughly and shade dry until it reaches moisture content of less than 20 per cent. The formulation can be stored in polythene bags. The product should contain a minimum of 2.5 x 10^8 cfu /g and it has a shelf life of 3-4 months under room temperature.

Quality control

- Product should be packed in white polythene bags.
- Moisture content of the product should not be more than 20 % in the final product.
- At the time of mixing population per ml of the broth should be 9 $\pm$ 2 x 108^8 cfu.
- The pH of the talc before mixing should be 7.0 (This can be achieved by addition of calcium carbonate @ 150 g / kg of talc)
- Fresh product should contain 2.5 x 10^8 cfu /g.
- After three months of storage at room temperature, the population should be 8-9 x 10^7 cfu /g.
- Storage period is 3 – 4 months.
- Minimum population load for use is 1.0 x 10^8 cfu /g.

3. Bacillus subtilis

Seed treatment with *Bacillus subtilis is* effective in controlling many soil-borne plant pathogens. This treatment increases plant yield, growth and dry matter production. A mass production technology developed by TNAU is given below.

Large scale

Prepare nutrient broth in 100 ml in 250ml conical flasks and sterilize. Inoculate with one loopful of *B. subtitlis* culture and keep it for incubation for 24 h. This serves as a mother culture. Transfer 1000 ml of mother culture to 100 l sterilized medium in a fermentor. Harvest the broth culture after 72 h and mix it with 250 kg of sterilized, amended peat soil (Peat soil can be amended by adding 15 % $CaCO_3$). Pack in white polythene bags and use. This peat-based formulation can be stored up to 6 months under room temperature.

Quality control

- Peat based culture should contain initial population of 10^8 cfu / g.
- At the time of mixing, broth culture should contain 10^9 cells / ml.

Commercial formulations

The biocontrol agents are mass multiplied in suitable media and mixed with the carrier material before they are used for the control of plant diseases. Commercial formulations of biocontrol agents are available only in few countries like Australia, Bulgaria, France, Germany, India, Israel, New Zealand, Russia, Sweden, USA, etc. (Table 15)

Table. 15. List of commercial preparations of biocontrol agents available worldwide.

Sl. No	Antagonist	Commercial product and source	Pathogen controlled
I	**Fungi**		
1.	*Trichoderma* spp.	Biofungus (Belgium)	*Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia solani*, *Sclerotinia* sp., *Verticillium* sp.
		Ecofit (India)	*Macrophomina phaseolina*, *R. solani*
		Trichoderma 2000 (Israel)	*Fusarium* sp., *Pythium* sp., *Rhizoctonia solani*, *Sclerotium rolfsii*
		Trichodermin (Bulgaria and Russia)	*Botrytis cinerea*, *Pythium* spp., *Sclerotinia sclerotiarum*, *Verticillium* spp.
2.	*Trichoderma harzianum*	BINAB –T (Sweden and UK)	*Armillaria mellea*, *Ceratocystis ulmi*, *Chondostereum purpureum*, *Endothia parasitica*, *Fusarium* spp., *Gaeumannomyces graminis* var *tritici*, *Rhizoctonia* spp.
		F-Stop (USA)	*Pythium* spp.
		Root-Pro (Israel)	*Fusarium* spp, *Pythium* spp., *R. solani*, *Sclerotium rolfsii*
		Root Shield (USA)	*Fusarium* spp., *Pythium* spp., *R. solani*
		Supresivit (Czech Republic, Denmark and USA)	*Fusarium* spp., *Pythium* spp., *R. solani*,

		Trichodex T 39 (Israel and USA)	*Botrytis cinerea*, *Colletotrichum* sp., *Erysiphe* spp., *Uncinula necator*
		Trichopel (New Zealand)	*Armillaria* sp., *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp
3.	*Trichoderma virens* (Syn. *Gliocladium virens* strain GL - 21)	Gliogard (USA)	*Pythium ultimum*, *Rhizoctonia solani*
		Soilgard (USA)	*Pythium* spp.
4.	*Trichoderma viride*	Antagon-TV (India)	*R. solani*, *M. phaseolina*
		Basderma (India)	*Fusarium* spp., *Rhizoctonia* spp.
		Bio-atom (India)	*Fusarium* spp., *Rhizoctonia* spp.
		Bioderma (India)	*Fusarium* spp., *Rhizoctonia* spp.
		Niprot (India)	*Fusarium* spp., *Rhizoctonia* spp.
		Sun-derma (India)	*Fusarium* spp., *Rhizoctonia* spp.
		Trichodowels, Trichoject, Trichoseal (New Zealand)	*Armillaria* sp., *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp.
		Tricontrol (India)	*Fusarium* spp., *Rhizoctonia* spp.
II	**Bacteria**		
5.	*Bacillus subtilis*	Epic (USA)	--
		Gus 4000 (USA)	*Gaeumannomyces graminis* var *tritici*
		Kodiak, Kodiak HB, Kodiak At (USA)	*Aspergillus flavus* *A. parasiticus*
		Quantum 4000 (USA)	*Fusarium* spp., *Rhizoctonia* spp., *Sclerotium rolfsii*

		Rhizo-Plus Rhizo-Plus-Konz (Germany)	--
		Serenade (USA)	--
6.	*Pseudomonas cepacia*	Intercept (USA)	*Cercospora* sp., *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp.
7.	*Pseudomonas fluorescens*	Blight Ban A 506	--
		Conqueror (Australia)	*Pseudomonas tolaasi*
		Dagger – G (USA)	*Pythium* spp., *Rhizoctonia solani*
		Pseudomonas (India)	--
		Sudo (India)	--
		Victus (USA)	--

B. DELIVERY SYSTEMS

The success in developing growth and delivery systems for introducing antagonists to soil was limited by several factors. Biocontrol agents should be in active state in the right place at the right time for successful biocontrol. A single delivery technique will not be suitable in all situations. Type of delivery system to be adopted depends upon the biocontrol agent employed for a pathogen in a crop situation. The strategies used for biocontrol of soil-borne plant pathogens include

a. protection of the infection court (rhizosphere and spermosphere),

b. growth of pathogen in the soil and

c. inactivation of overwintering inoculum.

Biocontrol agent that works through competition and antibiosis are used for protecting the infection court and impeding the progress of the pathogen in the soil, while mycoparasites are often used for destruction of overwintering inoculum. Biological

control with an organism introduced only once or occasionally would be the ideal bioagent since suppression of the target pathogen would be more or less permanent within the agro ecosystem where the organism is released. However, introduced microbial bioagents tend not to persist where they do not occur naturally or at populations higher than occur naturally. Hence, timely and inundative or augmentative releases are necessary to control target pathogens.

The most important techniques employed for application of bioagents are

1. Seed inoculation
2. Root inoculation
3. Soil inoculation
4. Aerial application / spot application / wound dressing
5. Fluid drill technology.

1. Seed inoculation

Application of biocontrol agents to seeds was suggested as an alternative approach to introducing them into soil. This method requires smaller amounts of biological material than in-furrow or broadcast applications. This method is more economical and effective. The inoculation of seeds with fast growing antagonists will help in controlling externally seed-borne and soil-borne plant pathogens. Spore suspensions as well as dry powder of the antagonists are used to coat the seeds. Examples of diseases controlled by seed treatment are furnished in Table 16. Seed priming, in which seeds are mixed with an organic carrier and then moisture content, is brought to a level just below that required for seed treatment has been used to deliver *T. harzianum* to control damping off of cucumber. The antagonists employed in the control of plant pathogens, causing diseases in various crops are given in Table. 16.

Table. 16. Antagonists employed in the control of plant pathogens by seed treatment.

Crop	**Pathogen**	**Antagonist**
Beans	*R. solani*	*T. koningii*
	S. rolfsii	*T. harzianum, T. viride*

	S. sclerotiorum	*T. koningii*
Cauliflower	*P. aphanidermatum*	*T. harzianum*
Chickpea	*F.oxysporum* f.sp. *ciceri* *R. solani* *S. rolfsii*	*T. virens* *T. harzianum* *T. harzianum* and *T. viride*
Cucumber	*Pythium* spp.	*T. harzianum*
Groundnut	*Aspergillus niger* *Macrophomina phaseolina*	*Trichoderma* sp. *Bacillus subtilis, T.viride*
Muskmelon	*F. oxysporum*	*T. harzianum*
Onion	*F. oxysporum* f.sp. *cepae*	*T. harzianum* and *T. viride*
Pea	*R. solani*	*T. hamatum*
Pigeonpea	*F. udum*	*T. harzianum,* *T. virens, T. viride*
Potato (Tuber)	*R. solani*	*T. harzianum, T. viride*
Radish	*R. solani*	*T. hamatum*
Sesame	*F. oxysporum* f. sp. *sesami* *R. solani, R. bataticola*	*T. viride* *T. viride, Bacillus subtilis*
Sugarbeet	*S. rolfsii*	*T. harzianum*
Sunflower	*M. phaseolina* *R. solani* *S. rolfsii*	*Bacillus subtilis, T. viride* *T. harzianum* *Trichoderma* spp.

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2. Root inoculation

The roots of seedlings / saplings / cuttings or propagative materials are dipped in a spore / cell suspension / slurry of the biocontrol agents for a stipulated period of time before planting. The root applications in flower crops such as carnation had controlled the root rot caused by *R. solani* by more than 70 per cent. This method of application is superior and economical wherever possible when compared to soil application of

inoculum. Seedling root dip of rice in *Pseudomonas fluorescens* for 30 min is found to control blast disease effectively.

3. Soil inoculation

Normally for soil inoculation / application, the fungal biocontrol agents are first multiplied in agricultural wastes or crop seeds and then incorporated into soil. With regard to bacterial bioagents, multiplication in solid substrates is not successful. Hence in this case the formulations (powder or peat based) are mixed with small quantity of well-decomposed FYM (powdered) or sand and then uniformly distributed in soil and thoroughly incorporated. Numerous attempts have been made to control several soil borne pathogens by incorporating natural substrates colonized by antagonists into the soil. Some examples of diseases controlled by soil amendment of bioagents are given below in Table 17.

Table 17. Antagonists employed in the control of plant pathogens by soil application

Crop	**Pathogen**	**Antagonist with carriers**
Beans	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
Carrot	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
Chickpea	*Rhizoctonia solani*	*T. harzianum* (wheat-bran powder granules)
Citrus	*Phytophthora* spp.	*T. harzianum*
Cotton	*Rhizoctonia solani*	*Trichoderma* spp. (Alginate prills)
Groundnut	*Aspergillus niger*	*Trichoderma* spp.
	Rhizoctonia solani	*T. longibrachiatum, T. virens* (wheat bran - saw dust medium)
Mustard	*Pythium aphanidermatum*	*T. viride, T. harzianum*
Pea	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
	Sclerotinia sclerotiorum	T. *harzianum* (wheat-bran preparation)

Pigeonpea	*Fusarium udum*	*T. harzianum*
Potato	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
	Rhizoctonia solani	*T. harzianum* (Supresivit)
Rice	*Sclerotium rolfsii*	*T. aureoviride*
Snapbean	*Sclerotium rolfsii*	*T. harzianum* (Pyrex formulation) *T. longibrachiatum, T. virens* (wheat-bran and saw dust medium)
Strawberry	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
Sugarbeet	*Sclerotium rolfsii*	*T. harzianum* (wheat-bran preparation)
Sugarcane	*Pythium graminicola*	*T. viride*
Tomato	*Rhizoctonia solani*	*T. harzianum* (wheat-bran preparation)
	Sclerotium rolfsii	*T. koningii*
Zinnia	*Rhizoctonia solani*	*T. hamatum, T. virens* (Alginate prills)

Though drenching of soil with aqueous suspensions of bioagents propagules are carried out, there will not be any even distribution of bioagents in the soil. Soil drenching with spore suspension of *T. viride* was very effective in reducing infection from *Colletotrichum truncatum* (brown blotch) infected cowpea seeds. Soil drenching with *T. harzianum* has given good control of stem rot of groundnut caused by *S. rolfsii*. An aqueous drench containing conidia of *T. harzianum* controlled wilt of chrysanthemum by preventing reinvasion by *F. oxysporum*.

4. Aerial or spot application / Wound dressing

Bioagents can be applied as foliar spray to control diseases affecting above ground parts. Biological control of foliar diseases is rarely practiced when compared to soil-borne diseases as it resulted in little success. The reasons being the poor survival of

antagonists on above ground plant parts (phyllosphere), availability of cheap and effective chemical fungicides and the ease of application to the foliage. However selected type of biocontrol agents are used even now for aerial application for the control of foliar and shoot diseases especially blossom and fruit diseases. Application of fluorescent pseudomonads (0.2 %) has given effective control of rice blast and grain discolouration. *Trichoderma* application to aerial plant parts is the biocontrol of wounds on shrubs and trees applied pruning, in advance of decay fungi. The effectiveness of *T. viride* against *Stereum purpureum*, the cause of silver leaf disease on plum has also been reported. Some examples of disease control achieved by foliar spray / wound dressing are given in Table 18.

Table 18. Antagonists employed in the control of plant pathogens affecting above ground plant parts by foliar application

Crop	**Pathogen**	**Antagonist**
Chickpea	*Botrytis cinerea*	*Trichoderma* spp.
Cowpea	*C. truncatum*	*T. viride*
Eggplant	*F. solani*	*T. hamatum*
Grapevine	*Botrytis cinerea*	*T. harzianum*
Jute	*Colletotrichum corchori*	*Aspergillus nidulans*
Kiwi fruit	*Botrytis cinerea*	*T. viride*
Pine	*F. oxysporum* *F. solani*	*T. harzianum* *T. viride*
Rose	*Botrytis cinerea*	*T. harzianum*
Strawberry	*Botrytis cinerea*	*T. viride*
Tomato	*Botrytis cinerea*	*T. harzianum*
	F. o. f. sp. *radicis lycopersici*	*T. harzianum*
Trees	*Stereum purpureum*	*T. polysporum, T. viride*

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5. Fluid drill technology

This delivery system involves the incorporation of biocontrol agents into fluid drill gels. In one study, vegetable and fruit tree seedlings were dipped into gels incorporated with antagonists so that the root area was surrounded by a thin layer of gel before the seedlings were planted. Fluid- drilling gels have been used to deliver *T. harzianum* for the control of *R. solani* and *S. rolfsii* on apple. This innovative approach utilizing the benefits derived from fluid drill technology offers considerable promise for the formulation and application of biocontrol microorganisms.

C. METHODS OF APPLICATION

Despite the availability of many potent biocontrol agents, practical methods of application of biocontrol agents are to be standardized. This may be due to

i. enough efforts are not made to study biocontrol *per se*
ii. theories do not adequately reveal the principles of production systems
iii. poor attention to the importance of the ecological complexity
iv. not understanding the economical feasibility
v. ignorance of modern tools for biotechnology and bioengineering

Biocontrol agents provide unique opportunities for crop protection because they grow, proliferate and can colonize and protect newly formed plant parts to which they were not initially applied. An antagonist applied to seed or in soil can grow on the planted seed, transfer to the emerging root and if rhizosphere competent, colonize and protect the entire root system against soil-borne pathogens. The various application methods of important biocontrol agents usually adopted under field conditions are presented below:

A. Trichoderma viride

The fungal bioagent, *Trichoderma viride* is widely used for the control of many soil-borne plant pathogens including *Aspergillus, Fusarium, Macrophomina, Rhizoctonia,*

Phytophthora, Pythium, Sclerotium, etc. This antagonist is applied to crops as either seed or soil or combination of both.

1. Seed treatment

Talc based formulation of *T. viride* was developed and tested as seed treatment for the control of root rot diseases of pulses, oilseeds, cotton etc. This product is used for treating the seeds as dry seed treatment at the rate of 4 g / kg of seed. The treated seeds should be sown immediately. *Trichoderma* product can also be mixed with biofertilizers like *Azospirillum* or *Rhizobium* and can be applied as single treatment by following the procedure for treatment with biofertilizer.

2. Soil application

Methods for soil application have been developed by multiplying the antagonistic organism in different substrates like FYM, decomposed coconut coir waste, groundnut husk, tapioca thippi, sugarcane bagasse, gypsum, and talc and peat soil. The antagonistic microorganisms multiply in the substrates and survive for longer period.

a. Vermiculite

For soil application, vermiculite – wheat bran medium was used. First *Trichoderma* was multiplied in molasses yeast medium for 10 days. Vermiculite (Grade 4) and milled wheat bran (250 meshes) were sterilized in an oven at 70^{o}C for 3 days using metal pan. For 1 kg of vermiculite, 33 g of wheat bran, 140 ml of liquid culture and 1750 ml of 0.05 N HCl were mixed and packed. This was immediately used for soil application at the required dose. This is applied at 125 kg / ha at the time of planting.

b. Coir pith

Trichoderma multiplied in well-decomposed coir pith can also be used for soil application at 5 per cent (w/w) level at the time of sowing.

c. Farm Yard Manure (FYM)

Well-decomposed FYM (100 g) was sun dried for one week and moisture content adjusted to 50 per cent. It was taken in polypropylene bags, heat sealed and sterilized at 1.4 kg / cm for 2 h for 2 consecutive days. It was inoculated and incubated for 15 days (Kousalya and Jeyarajan, 1988).

A quantity of 500 g of talc-based inoculum can be mixed with 100 kg FYM and heaped for 1 week and 50 per cent moisture is maintained. After three weeks, it can be applied as basal dose @ of 250 kg /ha. Gypsum based formulation have been developed. This formulation supported better survival of *Trichoderma* than talc or other carriers.

B. Pseudomonas fluorescens

The talc-based formulation of *Pseudomonas fluorescens* is applied to crops in various ways. They are

1. Seed treatment

Talc based *Pseudomonas* formulation is used as seed treatment for the control of wilt of chickpea and pigeonpea and blast of rice. This bioformulation is used to treat the seeds at 10 g / kg of seed.

2. Seedling root dip treatment

Two and half kg of talc-based *Pseudomonas* formulation is required to treat the roots of the seedlings for transplanting one ha. Make a plot of 2.5 sq.m and stagnate water to a depth of 2.5 cm. Mix 2.5 kg of talc based *Pseudomonas* formulation in the standing water and dip the roots of rice seedlings for 30 min after pulling out from the nursery and before transplanting.

3. Soil application

Talc based product of *Pseudomonas* can be applied to soil @ 2.5 kg/ha after mixing with 50 kg of organic manure or sand, 30 days after sowing. Five hundred gram of inoculum was mixed with 100 kg of FYM and heaped. Turning is given at weekly intervals and the moisture was adjusted by adding sufficient water. After three weeks, it will be ready for application as basal dose at 10 tonnes / ha.

4. Foliar spray

Spraying of talc based *Pseudomonas* formulation is generally used @ 0.2 % for the control of blast and grain discolouration of rice.

5. Capsule application

Capsule application is practiced for controlling Panama wilt in banana. Talc based *Pseudomonas* product is filled in empty gelatin capsule @ 50 - 60 mg per capsule and injected at the base of the pseudostem.

C. Bacillus subtilis

The peat-based formulation of *Bacillus subtilis* is mainly used as seed treatment for the control of root rots and wilt diseases of crop plants. The method of application and the precautions to be followed are given below.

1. Seed treatment

A quantity of 600 g of peat based *B. subtilis* product is required to treat the seeds required for one hectare.

Procedure

- Peat based culture is mixed with cooled rice gruel and a slurry is prepared.
- Seeds are gently mixed with the slurry to obtain uniform coating.
- Treated seeds are dried in shade and sown in the field.

(**Note**: *B. subtitlis* is compatible with biofertilizers *viz*., *Rhizobium* and *Azospirillum)*

Precautions

- Treated seeds should not be dried in the sun.
- Sticks or iron rods are not to be used for mixing the seeds with slurry
- Mixing should be very gentle
- Treated seeds should be sown within 24 hours in the field.

PRECAUTIONS WHILE USING BIOCONTROL AGENTS

The important precautionary measures to be followed while using the biocontrol agents are furnished below:

- Even when the biocontrol agents are applied beyond the recommended dose they will not cause harmful effects to the host. However, it is uneconomic. But when is applied at the rate lower than the recommended dose the biocontrol agent will not give better control of the disease.
- Biocontrol agents possess poor shelf life. Hence they should be used before expiry. Storage may reduce the viability.
- Presence of moisture during seed treatment period improves the efficiency.
- The biocontrol formulation should not be subjected to high temperature.
- Treated seeds should not be sun dried.
- Treated seeds should be sown immediately. It should not be stored for more than 48 hours. If it is not used immediately for sowing, the viability of propagules will decrease drastically.
- Treated seeds should not be further coated or treated with any other chemical agents (pesticide, fungicide, nutrient fertilizers, seed hardeners, *etc*.)
- No chemical should be mixed with biocontrol agents, as they are highly toxic.
- Application of higher quantities of organic manures in soil improves the efficiency of biocontrol agents.
- As the biocontrol agents are slow active in nature repeated application is necessary for successful results.
- During seed treatment, natural stickers such as rice starch, tapioca starch, gum arabic, *etc*. may be used instead of commercial synthetic stickers.
- It is not desirable to mix one type of biocontrol agents with other type (for example fungal and bacterial antagonists should not be mixed) as it may be antagonistic to each other resulting in poor efficiency.

IX. EVALUATION OF FORMULATIONS AND REGISTRATION

The formulated biological agents must be delivered in a proper manner into the cropping systems so as to achieve the effective control of diseases. There are several factors involved in the improvement of efficacy of biocontrol agents. These include

- the most likely system in which the applications of biocontrol agents will be successful.
- the methods of formulation and delivery
- reliable methods of monitoring activity and testing efficacy in agricultural horticultural applications.
- consideration of environmental fluctuations and variation in pathogen inoculum potential
- providing a formulation with reasonable shelf-life and marketing potential.

Crops and environments in glasshouses where a degree of control of physical factors can be maintained are more amenable to biocontrol than fluctuating field environments. Simplicity in an ecosystem also favours biocontrol by reducing competition of the soil microbiota with antagonists for available nutrients. Successful field testing requires careful consideration of several factors. Several representative locations should be used under standard conditions with the anticipated delivery system and formulation. Ideally, the soil should be naturally infested with the pathogen at sufficient inoculum density to ensure uniform disease development. When such situation is not available, artificial infestation is necessary. Consideration should be given in field-testing to proper choice of the responses and conditions to measure. Seedling emergence, plant stand, disease incidence and severity, plant biomass, flowering time, yield, and weight of marketable crop produce are to be evaluated. In addition, care must also be taken in expressing the amount of disease control by the biocontrol agent.

A. EVALUATION

The commercialized microbial products need to be tested scientifically before use so that the real use of bioproducts can be made. The important testing methods of bioformulations are discussed below:

1. Assessment of population (cfu) in the product

Take one g of the product and make it up to 10 ml with sterile distilled water and shake well (1:10). Take one ml from 1:10 dilution and transfer 9 ml sterile distilled water in next tube (1:100). Make further dilutions by transferring one ml of the suspension to the subsequent tubes to get $1:10^6$ or $1:10^7$ dilutions. Transfer one ml of the dilutions ($1:10^6$ or $1:10^7$) to sterile Petri plate and add 15 ml of molten and cooled medium, which is selective for the concerned antagonist in the same Petri plate. Rotate the plate gently and allow it for solidification. Incubate at room temperature for 2 to 7 days. Observe for the development of colonies. Count the average number of colonies per plate and calculate the number of colony forming units (cfu) present in one g of the product. The product should contain the required minimum population.

2. Dual culture bioassay method

Mix 15 mg of the product with 15 ml of molten and cooled agar medium and pour in a sterilized Petri plate. After solidification, transfer one 8 mm mycelial disc of any of the test fungus *viz., Fusarium, Macrophomina, Pythium, Rhizoctonia, Sclerotium, etc*. in the centre of the plate. Complete inhibition of the test fungus after 3 to 5 days of incubation indicates the bioefficacy of the product.

3. *In vitro* testing on the efficacy of antagonist in the seed

Prepare suspension of the test pathogen in sterile distilled water and mix with melted and cooled potato dextrose agar medium. Distribute 15 ml of the pathogen-amended medium in sterile Petri dishes. Allow it to solidify. Transfer the antagonist treated seeds to the centre of the medium. Incubate at room temperature for 5 to 6 days. Observe for the inhibition zone and measure.

4. Testing the efficacy of antagonists in glass house conditions

Fill the pots with sterilized garden land soil and incorporate the pathogen inoculum, multiplied in sand-maize medium, to the pot soil @ 5 per cent w/w depending on virulence (determined in pre-tests). After a week, sow the antagonist treated seeds in the pathogen incorporated pot soil and observe the growth of seedlings and disease incidence. Seeds sown without antagonists serve as control. Calculate the seedling vigour as well as the percentage disease control by the antagonist.

5. Field testing of biocontrol agents

Apply the biocontrol agents by any of the method suitable (seed, soil, root, foliar, etc.) and record the disease incidence (PDI or per cent disease) and yield in both antagonists treated and untreated control plots. The recommended chemical control measure can also be included as one of the treatments in the experimentation for comparison. The cost: benefit ratio should be worked out to know the practical utility and viability of the technology.

B. REGISTRATION REQUIREMENTS

The registration of microbial pesticides requires toxicological tests for oral, dermal, eye and other health hazards using test animals or fish. If these tests show non-toxic properties and when the biocontrol agent is not a plant pathogen, then that is registered as a biopesticide and marketed. The time to obtain registration is much less than chemical pesticides. In India, the Central Insecticides Board, Government of India has defined certain rules and regulations for registration of biopesticides. There are number of microorganisms identified by scientists as potential biocontrol agents but to reach the level of technical realization, the agents are expected to:

- reduce the population of pathogen below ETL (economic threshold level)
- prevent the pathogen from infecting the plant
- limit the disease development after infection specifications on target pathogens, target crops and environment where it will work and shelf life of the formulation are needed.

X. ECONOMICS

It has been estimated that in India about 54,000 metric tonnes of *Trichoderma* powder formulation is required for treating the seeds of pulses and oilseeds crops alone. In addition, this antagonist is also being utilized for several other crops to control many soil-borne diseases. Hence, there is a vast scope for commercial firms to enter in this venture and wipe the scourge of plant diseases off the face of our resource-poor farmers and to develop many entrepreneurs among the unemployed agricultural graduates.

The economic viability of commercial production of important biocontrol agents is given in the Tables 20, 21 and 22.

Table. 20. Production of ***Trichoderma viride*** formulation

(4000 kg /year in 50 batches of fermentation using 2 fermentors)

Sl. No.	Particulars	Quantity	Amount (Rs.)
I. Non-Recurring or Capital investment			
1.	Fermentor – 50 lit capacity	2	2,00,000.00
2.	Autoclave	1	15,000.00
3.	Hot air oven	1	15,000.00
4.	Laminar flow work station	1	40,000.00
5.	Gas connection deposit, cooker and burner		4,000.00
6.	Balance	1	5,000.00
7.	Racks and cabinets		20,000.00
8.	Plastic trays and glasswares		10,000.00
9.	Spray drier	1	10,000.00
10.	Bag sealer	1	2,000.00
11.	Research microscope	1	5,000.00
12.	Refrigerator	1	10,000.00
		Total	**3,36,000.00**

II. Recurring or Working expenditure			
1.	Talc powder (carrier material)	4000 kg	20,000.00
2.	Chemicals		20,000.00
3.	Polyethylene bags		20,000.00
4.	Electricity and gas charges		10,000.00
5.	Lab technician and casual labour		40,000.00
6.	Rent for the building		24,000.00
7.	Miscellaneous expenditure		10,000.00
	Total		**1,44,000.00**
III. Total cost of production			
1.	Working expenditure		1,44,000.00
2.	Interest on capital investment @ 15% p.a.		50,400.00
3.	Depreciation for capital investment @ 10 %		33,600.00
	Total expenditure		**2,28,000.00**
IV. Income			
1.	Two thousand lit of biomass produced in 50 batches are used to prepare 4000 kg of product and income by sale @ Rs. 100/kg	4000 kg	4,00,000.00
2.	Total expenditure		2,28,000.00
	Net profit / year		**1,72,000.00**

Table. 21. Production of ***Pseudomonas fluorescens*** formulation

(5000 kg /year in 50 batches of fermentation)

Sl. No.	Particulars	Quantity	Amount (Rs.)
I. Non-Recurring or Capital investment			
1.	Fermentor – 50 lit capacity	1	1,00,000.00
2.	Autoclave	1	15,000.00
3.	Hot air oven	1	15,000.00
4.	Laminar flow work station	1	40,000.00

5.	Gas connection deposit, cooker and burner		4,000.00
6.	Balance	1	5,000.00
7.	Racks and cabinets		20,000.00
8.	Plastic trays and glasswares		10,000.00
9.	Spray drier	1	10,000.00
10.	Bag sealer	1	2,000.00
11.	Research microscope	1	5,000.00
12.	Refrigerator	1	10,000.00
		Total	**2,36,000.00**
II. Recurring or Working expenditure			
1.	Talc powder (carrier material)	5000 kg	25,000.00
2.	Calcium carbonate	750 kg	7,500.00
3.	Chemicals for medium preparation		75,000.00
4.	Polyethylene bags		25,000.00
5.	Electricity and gas (fuel) charges		10,000.00
6.	Lab technician and casual labour		40,000.00
7.	Rent for the building		24,000.00
8.	Miscellaneous expenditure		10,000.00
		Total	**2,16,500.00**
III. Total cost of production			
1.	Working expenditure		2,16,500.00
2.	Interest on capital investment @ 15% p.a.		35,400.00
3.	Depreciation for capital investment @ 10 %		23,600.00
		Total expenditure	**2,75,500.00**
IV. Income			
1.	Two thousand lit of biomass produced in 50 batches are used to prepare 5000 kg of product and income by sale @ Rs. 100/kg	5000 kg	5,00,000.00
2.	Total expenditure		2,75,500.00
		Net profit / year	**2,24,500.00**

Table 22. Production of ***Bacillus subtilis*** formulation

(4000 kg /year in 50 batches of fermentation)

Sl. No.	Particulars	Quantity	Amount (Rs.)
I. Non-Recurring or Capital investment			
1.	Fermentor – 50 lit capacity	1	1,00,000.00
2.	Autoclave	1	15,000.00
3.	Hot air oven	1	15,000.00
4.	Laminar flow work station	1	40,000.00
5.	Gas connection deposit, cooker and burner		4,000.00
6.	Balance	1	5,000.00
7.	Racks and cabinets		20,000.00
8.	Plastic trays and glasswares		10,000.00
9.	Spray drier	1	10,000.00
10.	Bag sealer	1	2,000.00
11.	Research microscope	1	5,000.00
12.	Refrigerator	1	10,000.00
		Total	**2,36,000.00**
II. Recurring or Working expenditure			
1.	Peat soil or lignite (carrier materials)	4000 kg	8,000.00
2.	Calcium carbonate	750 kg	7,500.00
3.	Chemicals for medium preparation		25,000.00
4.	Polyethylene bags		20,000.00
5.	Electricity and gas (feul) charges		10,000.00
6.	Lab technician and casual labour		40,000.00
7.	Rent for the building		24,000.00
8.	Miscellaneous expenditure		10,000.00
		Total	**1,44,500.00**
III. Total cost of production			
1.	Working expenditure		1,44,500.00

2.	Interest on capital investment @ 15% p.a.		35,400.00
3.	Depreciation for capital investment @ 10 %		23,600.00
	Total expenditure		**2,03,500.00**
IV. Income			
1.	Two thousand lit of biomass produced in 50 batches are used to prepare 4000 kg of product and income by sale @ Rs. 75/kg	4000 kg	3,00,000.00
2.	Total expenditure		2,03,500.00
	Net profit / year		**96,500.00**