NEM 451 Commercial Production of Nematode Antagonistic bio-agents (1+1)

Lecture 1	**Introduction – Economic yield loss caused by plant parasitic nematodes in agricultural and horticultural crops, Concepts and definition of bio control agents**

Introduction

Nematodes are soft-bodied animals generally called as round worms or eel worms or thread worms. The word "nematode" derives from a Greek word "nema" means "thread". They belonged to the phylum "Nematoda" which is extremely diverse and ubiquitous in nature. Globally species richness is estimated between 500,000 and 1 million and among them more than 28,000 nematode species have been described. Nematodes can be found in fresh water, marine and terrestrial environments. They are free-living as well as parasitic to plants and animals including human beings. Among them, plant parasitic nematodes are microscopic in size ranging from 0.5 to 2.0 mm in length and generally attack on roots, stem, foliage and flowers of plants. They possess all the organs similar to higher animals, but lack of circulatory and respiratory systems. The presence of a characteristic needle-like stylet is the key diagnostic feature for differentiating plant parasitic nematodes from all other types of nematodes. At present, about 4,100 species of plant parasitic nematodes have been described. Globally, their distribution varies greatly. Among them, some are cosmopolitan, and some species restricted in particular geographical condition or some are highly host specific.

Major Types of Plant Parasitic Nematodes based on feeding strategies

Based on mode of feeding, plant parasitic nematodes are classified into ectoparasites, semi- endoparasites, endoparasites and foliar nematodes. Ectoparasitic nematodes remain outside of the plant and use its stylet to feed from the cells without entering into roots. Ectoparasites are most commonly observed plant parasitic nematode species under field condition, but they are considered less damaging species, for example, stunt nematode (*Tylenchorhynchus* spp.) lance nematode (*Hoplolaimus* spp.) and spiral nematode (*Helicotylenchus* spp.). Semi-endoparasitic nematodes are able to partially

penetrate the plant roots and feed cell contents by developing permanent feeding cells or syncytia on plant roots, for example, reniform nematode (*Rotylenchulus reniformis*); citrus nematode (*Tylenchulus semipenetrans*). Endoparasitic nematodes enter the roots completely. They are classified in to two types a). Migratory endoparasites which migrate through root tissues by destructively feeding on plant cells, for example, lesion nematode (*Pratylenchus* spp.) and burrowing nematode (*Radopholus similis*); b). Sedentary endoparasitic nematodes are the most damaging plant parasitic nematodes genera in all over the world. They establish within the roots by developing specialized feeding sites for example cyst nematodes (*Heterodera* and *Globodera*) and the root-knot nematodes (*Meloidogyne* spp.). Foliar nematodes normally feed above-ground plant parts such as stem, leaves, inflorescence for example Ufra nematode (*Ditylenchus angustus*) and white tip nematode (*Aphelenchoides besseyi*) on rice.

Economic Importance

Plant parasitic nematodes are of major significance inflicting substantial damage to agriculture. In the tropical and sub-tropical climates, yield losses attributable to nematodes were estimated at 14.6% in the developing countries compared to 8.8% in developed countries with an average of 12.3%. In India, the annual estimated crop losses due to major plant parasitic nematodes are estimated to the tune of Rs. 242.1 billion

Table 1 : *Summary of estimated annual yield losses due to damage by plant-parasitic nematodes worldwide*

Life sustaining crops	Loss (%)	Economically important crops	Loss (%)
Banana	19.7	Cacao	10.5
Barley	6.3	Citrus	14.2
Cassava	8.4	Coffee	15.0
Chickpea	13.7	Cotton	10.7
Coconut	17.1	Cowpea	15.1
Corn	10.2	Eggplant	16.9
Field bean	10.9	Forages	8.2
Millet	11.8	Grape	12.5
Oat	4.2	Guava	10.8
Peanut	12.0	Melons	13.8
Pigeon pea	13.2	Miscellaneous	17.3
Potato	12.2	Okra	20.4
Rice	10.0	Ornamentals	11.1
Rye	3.3	Papaya	15.1
Sorghum	6.9	Pepper	12.2
Soybean	10.6	Pineapple	14.9
Sugar beet	10.9	Tea	8.2
Sugar cane	15.3	Tobacco	14.7
Sweet potato	10.2	Tomato	20.6
Wheat	7.0	Yam	17.7
Average	**10.7**	**Average**	**14.0**

Overall average 12.3 %

Table 2 : *Estimated crop losses due to major plant-parasitic nematodes in India*

Sl.No.	Crop	Nematode	Estimated loss (Rs)/Avoidable field loss
1.	Barley	*Heterodera avenae*	Rs. 30 million
2.	Black gram	*Meloidogyne incognita*	8.7%
3.	Brinjal	*M.incognita*	33.7%
4.	Citrus	*Tylenchulus semipenetrans*	15%
5.	Coffee	*Pratylenchus coffeae*	Rs. 20 million
6.	Cotton	*M.incognita*	17.7-19.9%
7.	Cowpea	*M.incognita*	28.6%
8.	Finger millet	*M.incognita*	4.8%
9.	French bean	*M.incognita*	43.5%
10.	Groundnut	*M.arenaria*	51%
11.	Maize	*Rotylenchulus reniformis*	6%
		M.incognita	6%
12.	Okra	*M.incognita*	28.1%
13.	Pigeonpea	*Heterodera cajani*	14.2%
14.	Pea	*M.incognita*	20%
15.	Potato	*Globodera rostochiensis*	Total failure of the crop
16.	Rice	*Aphelenchoides besseyi*	12.2%
		Hirschmaniella oryzae	30-87%
		H.macronata	43%
17.	Tobacco	*M.incognita*	50%
18.	Wheat	*Heterodera avenae*	Rs. 40 million

Biological control

Biological control may be defined as reduction of nematode population that is accomplished through the action of living organisms other than the nematode resistant host plants, which occurs naturally or through the manipulation of the environment or the introduction of the antagonists (Stirling, 1991).

In pest management, biological control usually refers to the action of parasites, predators or pathogens on a nematode population which reduces its numbers below a level causing economic injury.

Concepts of bio control agents

1. Introduction = Importation

It has been used most for introduced or “exotic” pests. The origin of the pest is determined and then a search for natural enemies in its native habitat is conducted. Potential biocontrol agents are imported to the new location of the pest and released. Generally, the hope is for permanent establishment of the natural enemy.

2. Augmentation = Mass Culture or Collection and Release

Inundative Release – a single release of large numbers of a natural enemy; release can be in a small or large area; natural enemy does not become established and reproduce; goal is a one-time reduction in pest numbers.

Inoculative Release – smaller releases of a natural enemy over a period of time; natural enemy is expected to colonize and spread in the area of release.

3. Conservation and Enhancement

Utilization of practices that protect, maintain and enhance already existing natural enemy populations. Such practices could include habitat diversification to provide additional shelter or food for a natural enemy, provision of artificial food supplements, use of pesticides that are selective for target pests and have minimal effects on natural enemies, avoiding cultural practices that disturb or destroy natural enemies, etc.

Desirable Attributes of Bio-agents

An ideal bio control agent should have the following features or characteristics.

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

ECO FRIENDLY MANAGEMENT OF PLANT PARASITIC NEMATODES

Once nematodes are established in the field, it is virtually impossible to eradicate. For the effective management in infested field, it is imperative to disturb the harmonious relationship between nematode and host plant through altering soil ecosystem. Although chemical nematicides play an important role in the management of plant parasitic nematodes in modern agriculture, they are persistent and have long-term effect on non-target organisms. The need for alternatives to nematicides has stimulated research focusing on sustainable and eco-friendly tactics for management of plant parasitic nematodes.

COMPONENTS OF ECOFRIENDLY NEMATODE MANAGEMENT

Cultural methods

The principles behind cultural practices are

a). Prevention of new area from pest infestations

Root-knot nematodes are easily spread through infested soil, crop residues, vegetative propagules, human activities and irrigation water. Dissemination of root knot nematodes to newer areas is due to unawareness of nematode infestation, limitation in cleanliness standards in nurseries and also not adopting any phytosanitary measures while transporting to different parts of the country. Therefore, exclusion of infested materials is the only solitary principle to prevent newer area infestations.

b). Reduction of secondary soil inoculum once nematode is infested

Once the root knot nematodes are established in vegetable field, it is virtually impossible to eradicate. Since eradication of nematode population is neither feasible nor desirable unless there are quarantine and regulatory requirements of nematode control. Hence, nematologists advised the concept of 'living with the nematodes' by reducing inoculum level below the economic threshold level to derive maximum profits out of the management cost incurred. The following techniques may be followed to suppress root knot nematodes.

Summer ploughing

Two or three deep summer ploughing during the hot summer months expose the nematodes and infected tissue to solar heat and dehydration. This practice not only reduces the nematode population densities, but also reduces weeds, soil borne pathogenic fungi and bacteria. Three deep summer ploughing in nematode sick field with an interval of two

weeks period during May - June significantly reduced (48%) of *M. incognita* population. The efficiency of summer ploughing can be improved by soil solarisation, which helps in trapping and retaining more heat under polyethylene mulching than the direct exposure alone.

Destruction of crop residues

Root-knot nematodes can survive in residues of infected plants. These crop residues enhance the rate of survival of nematodes by slowing the rate of desiccation and providing mechanical protection during adverse conditions. Therefore, after harvest removal of infected plants and their destruction by burning helps in reducing inoculum densities.

Weed management

Weeds such as *Chenopodium album*, *Solanum nigrum*, *Tithonia rotundifolia* and other unknown weeds act as alternate hosts for root knot nematodes for the perpetuation of life cycle. Thus, removal of these weeds is an essential strategy to reduce the inoculum level under field condition.

Crop rotation

Root knot nematodes are known to infest >3000 plant species, however, they have delineated host ranges. This practice includes certain non-hosts, graminaceous poor hosts and certain antagonistic crops for one or two years have been found effective to bring down inoculum level of nematodes. Considering to certain cropping sequences, including non-preferred hosts like sesame, mustard, wheat, maize, etc. are found to suppress the nematode population in vegetable crops. Rotation of non-host crops such as mustard, garlic, onion and cereals at least for 2 to 3 years in a suitable cropping system helps in minimizing inoculum level of the nematodes.

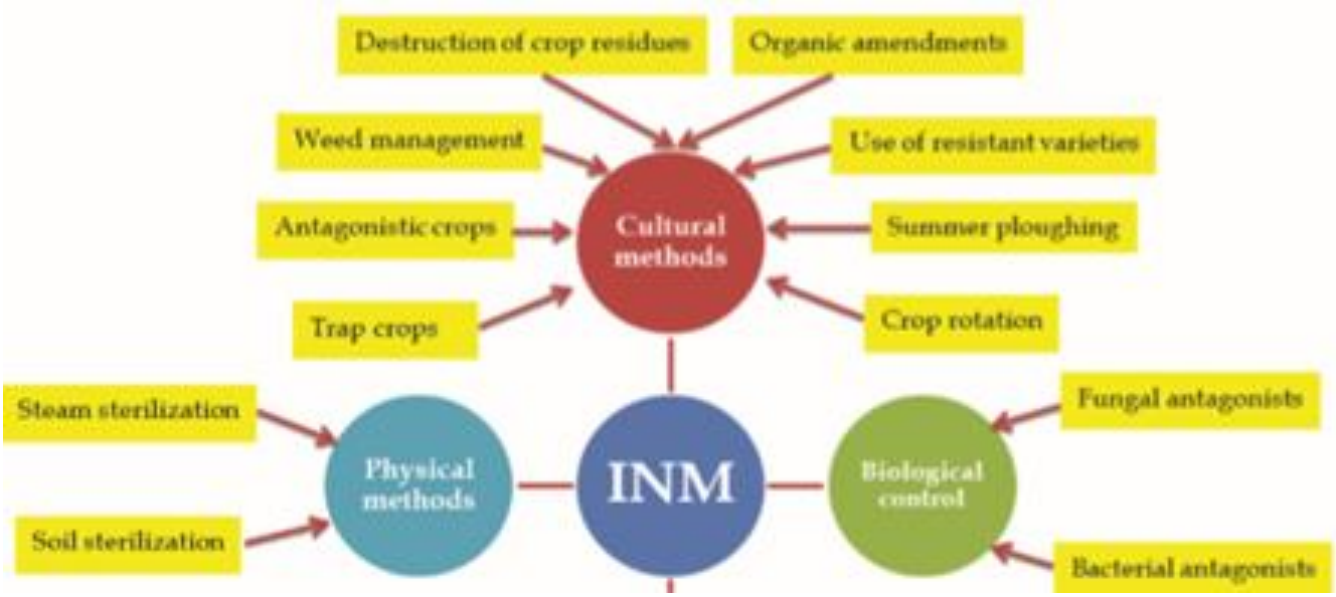
Trap crops

Trap crops are highly susceptible crops normally grown in root knot nematode infested fields. These crops which allow invasion by root knot nematodes, but do not support their development However, crops should allowed to grow over a time period to trap the infective juveniles of root knot nematodes in the roots and should be destroyed prior to reach its maturity. For example crop like *Crotalaria spectabilis*.

Antagonistic crops

Inter cropping or green manuring of certain crops like asparagus (*Asparagus officinalis*), mustard, and African marigold as antagonistic crops in susceptible main crop

helps in suppression of root knot nematode population. Growing African marigold (*Tagetes erecta* or *Tagetes patula*) with susceptible crop helps in suppressing root knot nematode population by releasing nematotoxic compounds polyterthienyl (α-terthienyl) through root exudates respectively. Incorporation of Brassica spp. such as Indian mustard (*Brassica juncea*) and rapeseed (*B. napus*) as green manures into the soil limits the reproduction of nematodes. After decomposition, they release volatile compound like isothiocyanates produced from glucosinolates, which are highly toxic to root knot nematodes. This process generally termed as bio-fumigation.



Organic amendments

The use of soil amendments is an age old traditional agricultural practice for enhancing soil fertility. However, they have been evaluated for their potential against major plant parasitic nematodes including root knot nematodes. Organic amendments include plant products, organic manures such as poultry, farm yard manure and vermicompost.

The mechanisms of action of these amendments against plant parasitic nematodes are to

(a) Stimulate intense microbial activities including nematode antagonists, predators and parasites in the soil during decomposition because organic amendments act as nutrient source for these antagonists. Their survival and establishment (colonization) is considerably enhanced when applied to field

(b) Releases specific compounds after decomposition may be nematicidal

(c) Enhance the soil capacity to withhold nutrients, which improves plant vigor and plant tolerance to nematodes (Bridge, 1996; Oka, 2010; Akhtar and Mahmood, 1996).

However, nematode management potential of an organic amendment is directly related to its nitrogen (N) content. Organic amendments with C: N ratios between 12 and 20 were highly suitable to exhibit high nematicidal activity and even to avoid phytotoxicity on crops. Organic amendments classified in two categories. They are (I) plant products (II) organic manures

I. Plant products

Numerous crops and plant species representing 57 families have been exhibited nematicidal activity against number of plant parasitic nematodes. They release of nematotoxic compounds through volatilization, exudation from roots, leaching from plants or residues, and decomposition of residues. Among identified several plants, Neem (leaf, seed kernel, seed powders, seed extracts, oil, sawdust, and oilcake) has been extensively used against control of root knot nematodes including other major plant parasitic nematodes. Particularly oil cakes are widely studied amendments and recommended in nematode management programs.

Nematicidal action is due to release of chemical compounds from neem such as salanin, azadirachtin, nimbin, thionemone and various flavonoids. Besides the nematicidal effects, triterpene compounds in neem oilcake inhibit the nitrification process and increase available nitrogen for the same amount of fertilizer. In vegetable nursery, application of neem cake at 0.5 kg/m2 area followed by solarization of the raised nursery beds with polyethylene mulch and optimum 1-2 t/ha neem cake (*Azadirachta indica*) was efficient to manage root-knot nematodes under field condition. In addition to neem cake, other available oilcakes such as castor (*Ricinus communis)*, Mustard cake (*Brassica campestris*), Pongamia cake and Karanja cake have also been proved their efficacy against root knot nematode.

II. Organic manures

Application of organic manure such as poultry, farm yard manure, and vermicompost were efficient to increase plant growth parameters as well as to reduce the root knot nematode inoculum in vegetable crops. The combined application of FYM @ 10 t/ha + poultry manure @ 2.5 t/ha + bio-fertilizers (Rhizobium and Phosphate Solubilizing Bacteria)

effectively reduced root knot incidence along with other plant parasitic nematodes. The presence of organic manure and decomposed products also stimulate increased activity of biological antagonists of nematodes.

Host plant resistance

Breeding and developing nematode-resistant plant cultivars are of much significance as effective and environmentally safe alternative to chemical nematicides.

Crop varieties identified/developed resistant to plant parasitic nematodes

CROP	NEMATODE	RESISTANT VARIETIES
Tomato	Root-knot nematodes (*Meloidogyne incognita/ M. javanica*)	PNR-7, NT-3, NT-12, Hisar Lalit
Chilli	Root-knot nematodes (*Meloidogyne incognita/ M. javanica*)	NP-46A, Pusa Jwala, Mohini
Cowpea	Root-knot nematodes (*Meloidogyne incognita/ M. javanica*)	GAU-1
Mungbean	Root-knot nematodes (*Meloidogyne incognita/ M. javanica*)	ML-30, ML-62
Cotton	Root-knot nematodes (*Meloidogyne incognita*)	Bikereni Nerma, Sharda, Paymaster
Grapevine	Root-knot nematodes (*Meloidogyne incognita/ M. javanica*)	Khalili, Kishmish Beli, Banquabad, Cardinal, Early Muscat, Loose Perlett
Potato	Potato cyst nematode (*Globodera rostochiensis*)	Kufri Swarna

Biological Control

Biological control is defined broadly as the use of natural or modified organisms, gene products to reduce the effects of undesirable organisms and support desirable beneficial organisms. Biological control of plant-parasitic nematodes can be accomplished either by application of antagonistic organisms, conservation and enhancement of indigenous antagonists or a combination of both strategies. It depends on the knowledge of biological interactions at the ecosystem, organism, cellular and molecular levels and is often a more complicated management strategy than physical and chemical methods.

The antagonists or biological control agents against nematodes are belongs to fungi, bacteria, VAM and predatory nematodes. Out of these, few potential biocontrol agents belonging to fungi and bacteria are more extensively used against nematodes.

1. *Trichoderma* spp.

Trichoderma are one of the small but potential group of beneficial fungi that are present nearly all soils and diverse habitats. This microorganism is registered as a biopesticides in many countries including India and its different formulations are available in markets at different names. There are many species of *Trichoderma* but *T.viride* and *T. harzianum* are most commonly used against nematodes.

Mechanisms of action against phytonematodes

The following are the mechanisms by which *Trichoderma*spp.functions:

a) Mycoparasitism

b) Antibiosis

c) Competition for nutrients or space

d) Tolerance to stress through enhanced root and plant development

e) Induced resistance

f) Solubilization and sequestration of inorganic nutrients

a. Mycoparasitism

Trichoderma hyphae penetrate the cell wall of the target nematodes and suck the cell contents. These fungi have the ability to secrete chitinases which dissolve the cell wall of nematodes. Nematode cell wall as well as the egg shells is provided with chitin which makes them impermeable to foreign materials. So these fungi can dissolve the chitin of the cell wall (Chet, 1987). Some species of Trichoderma can produce acetic acids which has nematicidal properties (Dijian*et al*., 1991). Moreover, they can penetrate the nematodes through the natural openings like mouth, vulva and anus.

b. Antibiosis

Antibiotics are low molecular weight secondary metabolites produced during nutrient limiting conditions. About 43 substances produced by *Trichoderma* spp. which have antibiotic activities have been recorded. It produces antibiotics like trichodermin, deramatin, trichoviridin, gliotoxin, gliovirin, trichotoxin etc. These antibiotics interact with

phospholipid membrane of nematodes and induce membrane permeability. Many of these antibiotics are synergistic with cell wall degrading enzymes.

c. Competition

Competition for space and nutrients has long been considered as one of the classical mechanisms of biocontrol by *Trichoderma* spp. (Chet, 1987). They have high rhizosphere competency and can easily colonize the roots. They reduce the feeding sites for nematodes.

d. Tolerance to stress through enhanced root and plant development

Another possible mechanism gaining credence is tolerance to stress through enhanced root and plant growth. The enhanced rooting due to application of Trichoderma probably also induces tolerance to pest that it does directly control. The larger root systems of plant colonized by Trichoderma are better able to withstand the damaging effect of nematodes.

e. Induced resistance

Trichoderma spp. elicits resistance in plants. *T. viride* was found to induce systemic resistance against root knot nematode, *Meloidogyne incognita* in green gram (Umamaheswari *et al*., 2002). Enzymes such as peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase, catalase and chitinase are induced in *T. viride* treated plants. Induction of these defence mechanisms determine the ability of a plant to survive any pathogen attack.

f. Solubilisation and sequestration of inorganic nutrients

In soil, various plant nutrients undergo complex transitions from insoluble to soluble forms that influence their accessibility and absorption by roots. Enhanced nitrogen use efficiency was documented in corn by Piekielek and Fox (1992) due to application of *T. harzianum* as seed treatment. A number of poorly soluble nutrients like rock phosphate, zinc, manganese, iron, copper etc. are made soluble to plants due to application of *T. harzianum* (Altomare *et al*., 1999). *Trichoderma* spp. are found to be effective against sedentary nematodes like *Meloidogyne, Heterodera, Globodera*. They can be used as seed treatment or as soil application. Seeds of different vegetables like tomato, brinjal, okra, cowpea and pulses like black gram, green gram, pea etc. are treated with *T.viride* @ 10g/kg seed. It is also effective as soil application. *T. viride* or *T.harzianum* should be applied in soil before sowing or transplanting @ 2.5 kg/ha (Talc formulation). The spore load should be

$2x10^8$cfu. For effective use, it should be multiplied in FYM or vermicompost. The biocontrol agent should be mixed with these materials and incubated for 15 days.

2. *Paecilomyces lilacinus* (= *Purpureocillium lilacinum*)

It is an opportunistic fungi which is a very good egg parasite of nematodes. Jatala *et al*., (1979) were the first to report *P. lilacinum* as a parasite of eggs of *M. incognita* on potato roots in Peru. In India, it was first isolated from *M. incognita* eggs during a survey in 1993-94 (Goswami and Uma Rao, 1997).

Mode of action

The fungus has been reported to parasitize eggs of many sedentary endoparasitic nematodes. The infection process starts with growth of fungal hyphae in the gelatinous matrix and eventually the eggs of nematodes are engulfed by the mycelial hyphae. The proliferated hyphal branches penetrate the eggs. In cyst nematodes, the fungus penetrates through vulva or the broken and exposed neck region. After entering the cyst, the fungus grows saprophytically on the body content surrounding the eggs during or before its parasitism of the eggs. In all cases, eggs in the early embryonic developmental stages prior to gastrulation process are more vulnerable to infection. Once the hyphae are in contact with the eggs a series of ultrastructural changes occurs in the eggs due to effects of exogenous metabolites and chitinolytic activities of the fungus. Once inside, the fungal mycelium radiated profusely in the eggs of early embryonic development and the entire embryo is replaced by the mycelial biomass.

Occasionally, *P.lilacinum* may penetrate the egg laying female through the anus or vulva. In such cases, the infected female body cavity filled with the fungal biomass and the nematode die. *P.lilacinum*is compatible with organic amendments like neem cake, castor cake and green manures. It can also be combined with *Trichoderma viridi, Verticilium chlam ydosporium* (=*Pochonia chlamydosporia*) against root knot nematode and best as seed treatment. It can also be applied in soil. In that case, its culture filtrate should be used to enrich the vermicompost and this should be used before sowing or transplanting (Anonymous, 2017). Pre planting soil treatment of *P.lilacinum* was found effective in reducing root gall and soil population of *M. incognita* in tomato (Kiewnick and Sikora, 2006)

3. Pseudomonas fluorescens

Among the different PGPR strains, *P. flourescens*has been proved to be an efficient bio control agent against plant parasitic nematodes.

Mechanisms of action by PGPR strains

a. Plant growth promotion

Mechanisms of growth promotion mays be direct, to be mediated by production of plant hormones such as auxins, cytokinins or gibberellic acid (Arshad and Berger,1991) or indirect due to control of minor pathogens such as deleterious rhizobacteria (Schippers *et al*.,1987).

b. Parasitism and lysis

The *Pseudomonas* spp. produce lytic enzymes such as chitinase and glucanase which are involved in degradation of chitin layer of nematode eggs (Loganathan *et al*.,2001). Moreover, some other toxic substances are also produce by PGPR for which the juveniles of *Meloidogyne* and *Heterodera* become vulnerable.

c. Induced systemic resistance

PGPR produced several defence related proteins and chemicals viz., peroxidase, phenylalanine ammonia lyase, polyphenol oxidase, phenols, chitinase, glucanase etc. *P. flourescens* can effectively be used against sedentary endoparasitic nematodes like *Meloidogyne, Heterodera* and *Globodera*. It is very effective as seed treatment. It can also be used in combination with other biocontrol agent like *Trichoderma viride*. It is effective as soil application also (Anonymous, 2016).

4. *Pasteuria penetrans*

This is an obligate parasitic bacterium of phytoparasitic nematodes and has attracted the attention of nematologists and bacteriologists since this has great potential as biocontrol agent against plant parasitic nematodes. It is gram positive endospore forming bacterial parasite. The bacterial parasite produces endospore that adheres to the cuticle of plant parasitic nematodes and interface with the life cycle of the nematodes resulting in the suppression of nematode population.

Life cycle

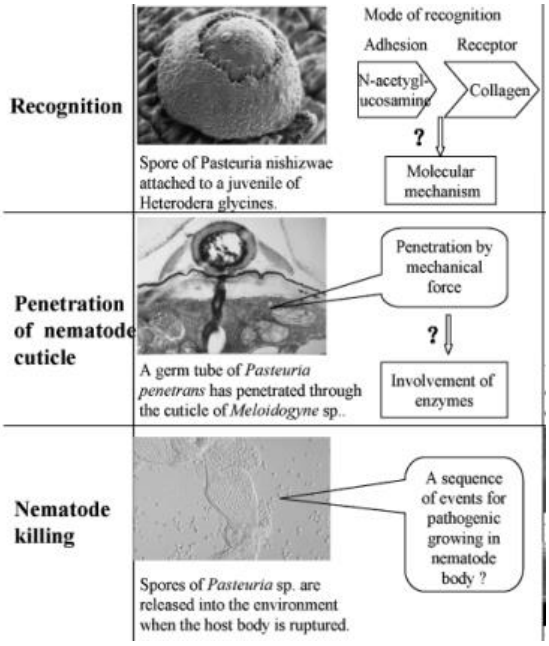
Life cycle of *P.penetrans* starts with endospore, the infective stage of the bacterium that perpetuates year after year in the soil and are released on decomposition of nematode

body of adult females and males or in plant root tissues. The spores of *P.penetrans* attach to the cuticle of second stage juvenile and germinate within a few days after the juvenile starts feeding within the root. The germination of spores occurs only after the encumbered second stage juveniles invade the plant root and begin to feed. The germ tube emerges through a central opening in the basal attachment layer and penetrates the cuticle of the nematodes and finally reaches the pseudocoelom.

The germ tube in the pseudocoelom develops into vegetative micro colony consisting of dichotomously branched septate mycelium. The intercalary cells in the microcolony lyse and give rise to daughter colonies. The process continues resulting in a large number of daughter colonies containing fewer but large vegetative cells. Eventually, quartets of developing sporangia predominate in the nematode body cavity. These quartets separate into doublets of sporangia and finally separate into a single sporangium that will eventually contain an endospore. Endospores of *P.penetrans*are released into the soil when the plant roots with parasitized root knot nematode female decompose. The life cycle completes within 20 to 30 days at 30°C.

Biocontrol potential

Dried root powder containing spores of *P.penetrans* when incorporated in the soil @ 100mg/kg soil, 99 per cent second stage juveniles of *Meloidogyne javanica* were infected. Kokalis (2015) effectively controlled *M.incognita* in tomato and cucumber and *M. arenaria* in snapdragon by seed treatment. It can be used as seed treatment as well as soil application with known spore load. Moreover, it is compatible with nematicide like carbofuran and other biocontrol agents.



5. Arbuscular mycorrhiza

Mycorrhizal fungi are also used against plant parasitic nematodes. Mycorrhizal fungi colonize the roots of more than 90% plant species which are mutualistic. These symbiotic fungi are present in almost all terrestrial ecosystems and play a major role in plant growth and development of plants. AM symbiosis is often associated with improved plant growth. This enhanced growth has been attributed to nutritional and non-nutritional effects of AM fungi. It has been reported to benefit plants by increasing uptake of nutrients such as P, Zn, Cu and N. The non-nutritional effects of AM fungi would be due increased tolerance to saline conditions, improved water relations, increased survival rates of transplanted seedlings, control of root diseases and increased soil aggregation by the external hyphal network (Dodd and Thompson, 1994).

The AM are also known to produce wide array of plant growth promoting substances like IAA, IBA, GA. Out of all these attributes, the increased uptake of P is considered as the primary factor responsible for improved plant growth. So AM has indirect effect on plant parasitic nematodes. Due to the enhanced plant growth, the host plants may develop resistance to the nematode pests.

Physical Methods

There are two heat based techniques will efficiently kill root knot nematodes (and other nematodes, pests or pathogens), first, steam sterilization and second soil solarization.

Steam sterilization

Steam sterilization is an effective curative physical measure that can be used to mitigate the severe incidence of root knot nematode under protected cultivation. However, this method is expensive to practice in open field condition.

Soil solarization

Soil solarization is a method of heating moist soil by covering it with transparent plastic sheets to trap solar radiation during hottest period or summer season of the year. This is technology of thermal disinfestations. Linear Low Density Polyethylene (LLDPE) clear films were efficient to manage root knot nematode incidence. The principle involved in this mechanism is (1) accumulation of heat due to transmission of short wave solar radiation and prevents loss of long wave radiation in solarized soil; (2) increase in temperature due to greenhouse effect; (3) soil moisture helps in solarization process by conducting heat energy;

(4) increase in microbial and physico-chemical reactions in the soil resulting in to accumulations of gases, some being toxic pathogens and others acting as a nutrient source or induce resistance to subsequent crop and (5) prolonged exposure to higher temperature resulting in increased mortality of nematodes and also making them susceptible to antagonists.

Questions

0.5 mark

1.	Size of the plant parasitic nematodes ranged from			
	A) 0.5 to 2.0 mm	B) 0.5 to 1.0 mm	C) 0.5 to 1.5 mm	D) 0.1 to 0.5 mm
2.	---------- is the diagnostic key character for differentiating plant parasitic nematodes from others			
	A) Cone	B) shaft	C) knob	**D) stylet**
3.	A single release of large no. of a natural enemy to manage plant parasitic nematodes is called			
	A) Inundative release	B)Inoculative release	C) Conservation	D) **I**mportation
4.	---------- is the nematode cell wall degrading enzymes secreted by egg parasitic fungus			
	A) Chitinase	B) Polyphenol oxidase	C) Peroxidase	D) Catalase
5.	Secondary metabolites secreted by *Pochonia chlamydosporia* is			
	A) Gliovirin	**B) Pochonin**	C) Peroxidase	D) Catalase
6.	**The following is a** gram positive endospore forming bacterial antagonist against nematodes are			
	A) *Pochonia chlamydosporia*	**B) *Pasteuria penetrans***	C) *Pupureocilium lilacinum*	D) *Pseudomonas fluorescens*

One mark

1. Define foliar nematodes
2. Biological control

3. Trap Crop
4. Bio fumigation

Two marks

1. Define soil solarisation
2. Semi endoparasite
3. List out tomato resistant variety against root knot nematode
4. Antagonistic crop
5. Summer Ploughing
6. Migratory endoparasite

Three marks

1. Classify endo parasitic nematodes based on their feeing habit.
2. Write the concepts of Bio control agents
3. Illustrate the mode of action of *Pasteuria penetrans* against plant nematodes

Five marks

1. Classify the plant parasitic nematodes based on their parasitism
2. Write the desirable attributes of Bio control agents
3. Briefly explain the components of eco friendly nematode management

Lecture 2	**Types of Bio agents – Fungal antagonistic organisms – applications – dosage - commercial formulations available in India – Merits and demerits**

Types of Bio agents

The antagonists or biological control agents against nematodes are belongs to: Fungi, Bacteria, VAM and Predatory nematodes. Out of these, few potential biocontrol agents belonging to fungi and bacteria are more extensively used against nematodes. Linford *et al.* (1938) who first laid out classical pot and field experiments on management of plant parasitic nematodes by biocontrol agents, lots of efforts have been made to control many important nematode pests by using different groups of antagonists.

Fungal biocontrol agents

1. Saprophagous fungi (*Trichoderma* spp)

Trichoderma are one of the small but potential group of beneficial fungi that are present nearly all soils and diverse habitats. They have proven commercially viable as a biological control agent. This microorganism is registered as a biopesticides in many countries including India and its different formulations are available in markets at different names. There are many species of *Trichoderma* but *T.viride* and *T.harzianum* are most commonly used against nematodes.

Mechanisms of action against phytonematodes

The following are the mechanisms by Trichoderma spp. against phytonematodes

a) Mycoparasitism b) Antibiosis c) Competition for nutrients or space d) Tolerance to stress through enhanced root and plant development e) Induced resistance f) Solubilization and sequestration of inorganic nutrients

a. Mycoparasitism

This is complex process that involves trophic growth of the biocontrol agent towards target pathogen. Trichoderma hyphae penetrate the cell wall of the target nematodes and suck the cell contents. These fungi have the ability to secrete chitinases which dissolve the

cell wall of nematodes. Nematode cell wall as well as the egg shells is provided with chitin which makes them impermeable to foreign materials. So these fungi can dissolve the chitin of the cell wall (Chet, 1987). Some species of Trichoderma can produce acetic acids which has nematicidal properties (Dijian *et al*.,1991). Moreover, they can penetrate the nematodes through the natural openings like mouth, vulva and anus.

b. Antibiosis

Antibiotics are low molecular weight secondery metabolites produced during nutrient limiting conditions. About 43 substances produced by *Trichoderma* spp. which have antibiotic activities have been recorded. It produes antibiotics like trichodermin, deramatin, trichoviridin, gliotoxin, gliovirin, trichotoxin etc. These antibiotics interact with phospholipid membrane of nematodes and induce membrane permeability. Many of these antibiotics are synergistic with cell wall degrading enzymes.

c. Competition

Competition for space and nutrients has long been considered as one of the classical mechanisms of biocontrol by *Trichoderma* spp. (Chet, 1987). They have high rhizosphere competency and can easily colonize the roots. They reduce the feeding cites for nematodes.

d. Tolerance to stress through enhanced root and plant development

Another possible mechanism gaining credence is tolerance to stress through enhanced root and plant growth. The enhanced rooting due to application of *Trichoderma* probably also induces tolerance to pest that it does directly control. The larger root systems of plant colonized by *Trichoderma* are better able to withstand the damaging effect of nematodes.

e. Induced resistance

Trichoderma spp. elicits resistance in plants. *T.viride* was found to induce systemic resistance against root knot nematode *Meloidogyne incognita* in green gram (Umamaheswari *et al*., 2002). Enzymes such as peroxidase, polyphenol oxidase, phenyl alanine ammonia lyase, catalase and chitinase are induced in *T.viride* treated plants. Induction of these defence mechanisms determine the ability of a plant to survive any pathogen attack.

f. Solubilization and sequestration of inorganic nutrients

In soil, various plant nutrients undergo complex transitions from unsoluble to soluble forms that influence their accessibility and absorption by roots. Enhanced nitrogen use efficiency was documented in corn by Piekielek and Fox (1992) due to application of *T.harzianum* as seed treatment. A number of poorly soluble nutrients like rock phosphate, zinc, manganese, iron, copper etc. are made soluble to plants due to application of *T.harzianum* (Altomare *et al.,* 1999).

Trichoderma spp. are found to be effective against sedentary nematodes like *Meloidogyne, Heterodera, Globodera*. They can be used as seed treatment or as soil application. Seeds of different vegetables like tomato, brinjal, okra, cowpea and pulses like black gram, green gram, pea etc. are treated with *T.viride* @ 10g/kg seed. It is also effective as soil application. *T.viride* or *T.harzianum* should be applied in soil before sowing or transplanting @ 2.5 kg/ha (Talc formulation). The spore load should be $2x10^8$ cfu. For effective use, it should be multiplied in FYM or vermicompost. The biocontrol agent should be mixed with these materials and incubated for 15 days. Before application in the field it is advised to assess the spore load (Anonymous, 2017). Trichoderma spp. is compatible with many insectides, nematicides, fungicides etc.

2. ***Paecilomyces lilacinus* (= *Purpureocillium lilacinum*)**

It is an opportunistic fungi which is a very good egg parasite of nematodes. Jatala *et al.*,(1979) were the first to report *P.lilacinum* as a parasite of eggs of *M. incognita* on potato roots in Peru. In India, it was first isolated from *M. incognita* eggs during a survey in 1993-94 (Goswami and Uma Rao, 1997).

Mode of action

The fungus has been reported to parasitize eggs of many sedentary endoparasitic nematodes. The infection process starts with growth of fungal hyphae in the gelatinous matrix and eventually the eggs of nematodes are engulfed by the mycelial hyphae. The proliferated hyphal branches penetrate the eggs. In cyst nematodes, the fungus penetrate through vulva or the broken and exposed neck region. After entering the cyst, the fungus grows saprophytically on the body content surrounding the eggs during or before its parasitism of the eggs. In all cases, eggs in the early embryonic developmental stages prior to gastrulation process are more vulnerable to infection.

Once the hyphae are in contact with the eggs a series of ultrastructural changes occurs in the eggs due to effects of exogenous metabolites and chitinolytic activities of the fungus. Once inside, the fungal mycelium radiated profusely in the eggs of early embryonic development and the entire embryo is replaced by the mycelial biomass. Occasionally, *P.lilacinum* may penetrate the egg laying female through the anus or vulva. In such cases, the infected female body cavity filled with the fungal biomass and the nematode die.

P.lilacinum is compatible with organic amendments like neem cake, castor cake and green manures. It can also be combined with *Trichoderma viride, Verticilium chlamydosporium* (= *Pochonia chlamydosporia*) against root knot nematode and best as seed treatment. It can also be applied in soil. In that case, its culture filtrate should be used to enrich the vermicompost and this should be used before sowing or transplanting (Anonymous, 2017).Its liquid and powder formulations are available in different names. Pre planting soil treatment of *P.lilacinum* was found effective in reducing root gall and soil population of *M.incognita* in tomato (Kiewnick and Sikora, 2006)

3. Nematophagous fungi *(P. chlamydosporia)*

Species of Pochonia are widely distributed in agricultural soils and infect eggs of plant parasitic nematodes. Within the genus *Pochonia*, *P. chlamydosporia* appears the most effective in infecting nematode eggs. This species is one of the major facultative antagonistic fungi that can parasitize egg and female stages of root-knot nematodes and female cyst nematodes. Parasitism of this fungus is based on appressorial formation developed from undifferentiated hyphae, which allows the colonization of the egg surface and penetration

through both mechanical and enzymatic actions.

Observations during the infection process have shown that the penetration of the eggshell occurs from both the appressorium and the lateral branch of the mycelium, and leads to the disintegration and the dissolution of three layers composing the eggshell. Formulations based on *P. chlamydosporia* have been developed and are currently being commercialized (e.g., KlamiC ® based on *P. chlamydosporia* var. *catenulate* RES 392 from Cuba).

4. Nematode-Trapping Fungi (NTF)

Fungi capturing nematodes are called nematode-trapping fungi. The first nematode-trapping fungus to be described was *Arthrobotrys oligospora* Fresen. in 1852. They play an important ecological role in regulating nematode dynamics in soil.

Nematode-trapping fungi are a unique and intriguing group of carnivorous microorganisms that can trap and digest nematodes by means of specialized trapping structures. They can develop diverse trapping devices, such as adhesive hyphae, adhesive branches, adhesive knobs, adhesive networks, constricting rings and non-constricting rings.

(a) Adhesive Hyphae

The fungal hyphae form adhesive which capture nematodes. These hyphae produce adhesive at any point in response to nematode contact or the hyphae are coated with adhesive along their entire surface. At the point of hyphae where contact is made for capture, a thick and yellowish chemical material is secreted for example, *Stylopagehadra*. Thereafter, an outgrowth of hyphae similar to appressorium develops. When the nematode is trapped, it becomes inactive first and killed after penetration of hyphae. After penetration, elongate, unbranched absorptive hyphae grow along the nematode body and completely exploit the contents.

(b) Adhesive Branches

The nematode trapping fungi produce the most primitive and simple organ of capture, the adhesive branches, which are a few cells in height. From the main prostrate hyphae short laterals grows as erect branches on or below the substrate. Over the whole surface of branch a thin film of adhesive material is coated. Examples for adhesive branch producing fungi are *Dactylella cionopaga* and *D. gephyropaga* (Fig.1).

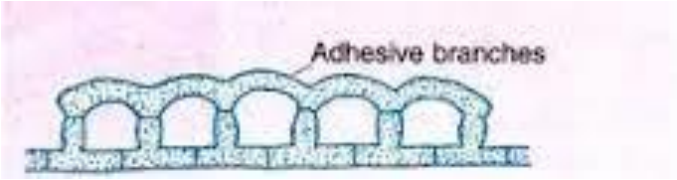


Fig.1 Adhesive branches of *Dactylariagephytopaga*

(c) Adhesive Knobs

Morphologically a distinct adhesive cell, globose to sub-globose in structure, is produced at the apex of a slender non-adhesive stalk containing 1-3 cells. A thin film of adhesive material is produced over the surface of knob. If a nematode is caught by a knob, soon it is attacked by several knobs with subsequent penetration. The immobilized

nematode is destroyed thereafter. Examples of adhesive knob-producing fungi are *Dactyleria Candida*, *Dactylella* and *Nematoctonus*.

(d) Adhesive Networks

Nets are formed by fungal hyphae which are adhesive in nature. Nets may be in the form of a single hoop-like loop (e.g. *Arthrobotrys musiformis*) to a complex multi-branched networks (e.g. *A. oligospora*). Upon observation with electron microscope it appears that the hyphae are coated with adhesive material. As the nematode comes in contact of hyphae it is attached at many points resulting in penetration by infectious hyphae. Initially penetration is accompanied by the formation of infectious bulb which leads to form hyphae which grow inside nematode. Hyphal growth exploits nutrients and results death of the prey.

(e) Constricting Rings

The constricting rings are produced similar to non-constricting rings but the supporting stalk is shorter and stouter (Fig. 2). In this case also a three celled ring is formed. It is a most sophisticated ring formed by predaceous fungi such as *Arthrobotrys anchonia*, *Dactylaria brachopaga* and *Dactylella* which are abundant in soil.

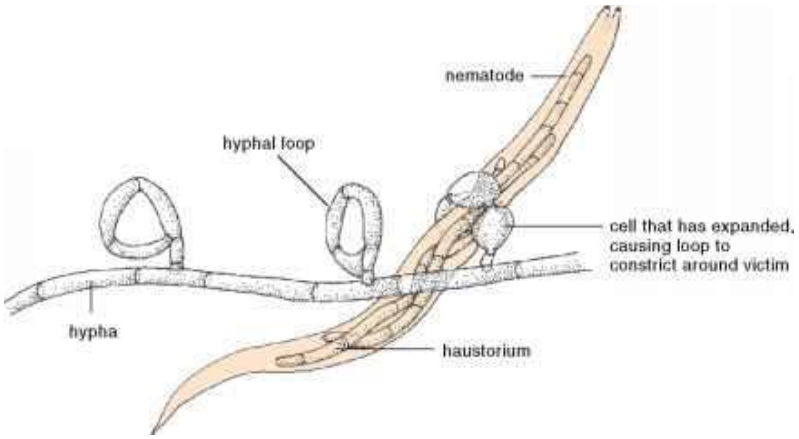


Fig. 2Constructing rings of *Arthrobotrys dactyloides*

(f) Non-Constricting Rings

From the prostrate creeping septate hyphae which arise erect and lateral branches and form non-constricting rings, initially the branch is slender but widens subsequently and being curved to form a circular structure. At the point where tip of branch makes contact with supporting stalk, cell walls get fused. Thus, it results in formation of three-celled ring with a stalk. A nematode enters the ring and moves forward.

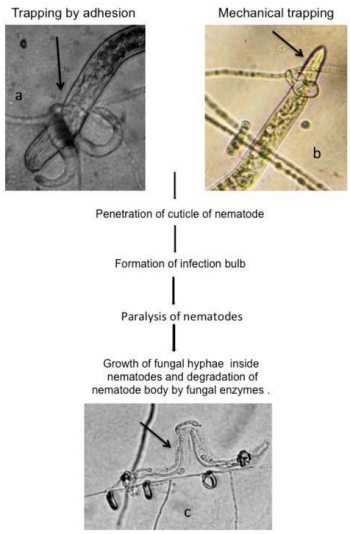
This results in marked constriction in cuticle. Generally rings are impossible to dislodge. During struggle the rings break from the weak point. Therefore, nematodes containing rings can move. Initially rings do not have any harmful effect on nematode but eventually nematode is penetrated and its body content is consumed. *Dactylaria Candida* and *D. lysipage* produce non-constricting rings, in spite of producing the adhesive knobs.

The nematode is captured by garrotting action of the ring cell. By swallowing the ring cell grasps the nematode in a single hold when a nematode enters into the ring; friction created by its body induces to swell the rings soon. The cells swell inwardly by three times greater than the original one within 1/10 of a second resulting in body of nematode deeply constricted.Struggle between nematode and fungi go on for a few minutes. Thereafter nematode becomes still and hyphae from ring cell penetrate the body and exploit the nutrients of nematode with the consequences of death. The most potential predatory fungi are the species of *Dactylaria*.

Mechanism of interaction

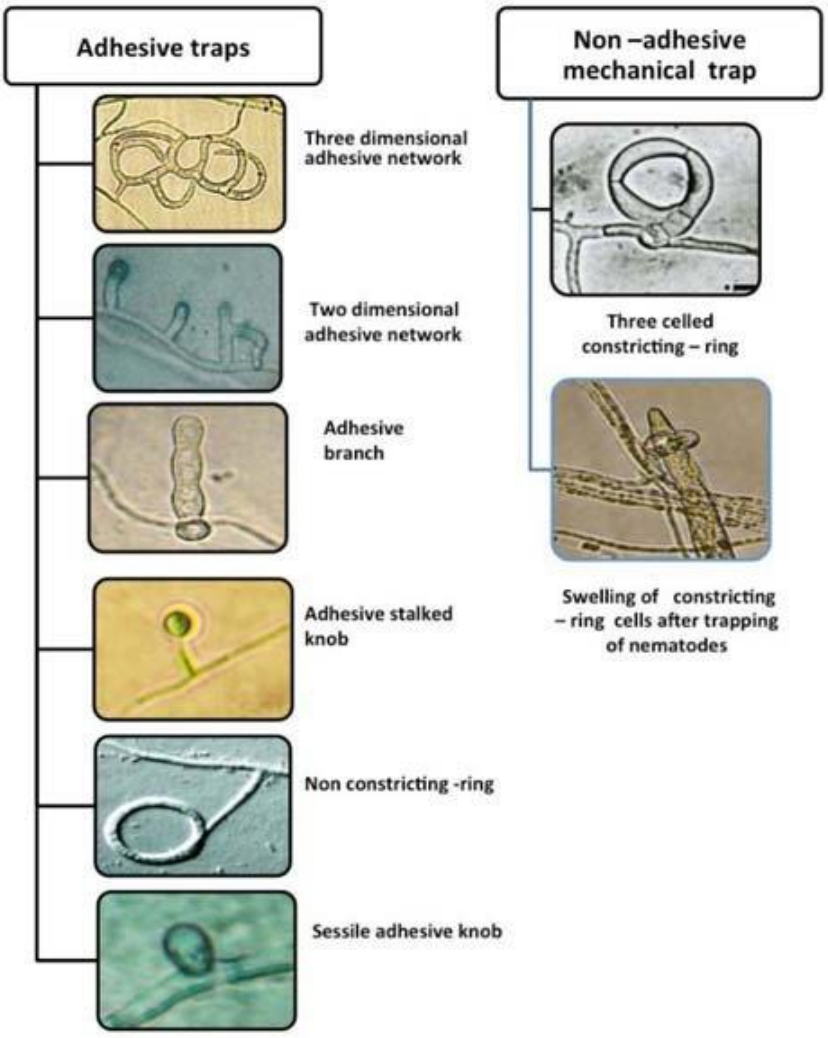
The body of nematode consists of a low molecular weight peptide (or possibly a single amino acid) which is called nemin. Nemin is water soluble and potential stimulant for trap-formation. It causes morphological changes in nematophagous fungi. The process of nematode-fungus interaction is accomplished through a series of molecular events resulting in nematode death. Pramer and Kuyama (1963) identified on the trap of *A. oligospora* the initiator of prey-predator recognition as lectin. The lectin of fungus binds especially to the sugar, N-acetyl-D-galactosamine, present on nematode cuticle. The nematode cuticle is lysed at the point where lectin combines with N-acetyl-D-glucosamine. Within an hour fungal hyphae penetrate the prey. The enzyme collagenase is secreted by the fungus which dissolves collagen protein of nematode cuticle. The hyphae which have penetrated the nematode digest the body content and translocate to rest of the parts of hyphae for fungal growth and reproduction.

Mode of action of nematode-trapping fungi



(a) Capturing of free-living nematode by adhesive trap of nematode trapping fungus, (b) Capturing of *Meloidogynehapla* by constricting ring of *A. dactyloides*, (c) Growth of fungal hyphae of *A. dactyloides* in the nematode by after consuming the content of nematode body

Trapping organs of nematode-trapping fungi



5. Endophytic fungi (Arbuscular mycorrhiza fungi (AMF)

Mycorrhizal fungi are also used against plant parasitic nematodes. Mycorrhizal fungi colonize the roots of more than 90% plant species which are mutualistic. These symbiotic fungi are present in almost all terrestrial ecosystems and play a major role in plant growth and development of plants. AM symbiosis is often associated with improved plant growth. This enhanced growth has been attributed to nutritional and non-nutritional effects of AM fungi. It has been reported to benefit plants by increasing uptake of nutrients such as P, Zn, Cu and N. The non-nutritional effects of AM fungi would be due increased tolerance to saline conditions, improved water relations, increased survival rates of transplanted seedlings, control of root diseases and increased soil aggregation by the external hyphal network (Dodd and Thompson, 1994).The AM are also known to produce wide array of plant growth promoting substances like IAA, IBA, GA. Out of all these attributes, the increased uptake of P is considered as the primary factor responsible for improved plant

growth. So AM has indirect effect on plant parasitic nematodes. Due to the enhanced plant growth, the host plants may develop resistance to the nematode pests.

Bacterial biocontrol agents

1. *Pseudomonas fluorescens*

Among the different PGPR strains, *P.flourescens* has been proved to be an efficient biocontrol agent against plant parasitic nematodes.

Mechanisms of action by PGPR strains

a. Plant growth promotion

Mechanisms of growth promotion mays be direct, to be mediated by production of plant hormones such as auxins, cytokinins or gibberellic acid (Arshad and Berger,1991) or indirect due to control of minor pathogens such as deleterious rhizobacteria (Schippers *et al*.,1987).

b. Parasitism and lysis

The *Pseudomonas* spp. produce lytic enzymes such as chitinase and glucanase which are involved in degradation of chitin layer of nematode eggs (Loganathan *et al.,*2001). Moreover, some other toxic substances are also produce by PGPR for which the juveniles of *Meloidogyne* and *Heterodera* become vulnerable

c. Induced systemic resistance

The defensive capacity of the host plants increases due to the introduction of PGPR. The resulting elevated resistance due to biotic agent like Pseudomonas is referred to as ISR In PGPR treated plants, several defence related proteins and chemicals *viz*., peroxidase, phenylalanine ammonia lyase, polyphenol oxidase, phenols, chitinase, glucanase etc. are reported (Loganathan *et al*.,2001). *P. flourescens* can effectively be used against sedentary endoparasitic nematodes like *Meloidogyne, Heterodera* and *Globodera*. It is very effective as seed treatment. It can also be used in combination with other biocontrol agent like *Trichoderma viride*. It is effective as soil application also (Anonymous, 2016). The spore load should be $2x10^8$ cfu. Different liquid and powder formulations of *Psedumonas flourescens* are available in market now a day. Moreover, in most of the Agricultural universities these common biocontrol agents are available. It is always advisable to use the local isolates of biocontrol agents not only against nematodes but against all pest and diseases.

2. *Pasteuria penetrans*

This is an obligate parasitic bacterium of phytoparasitic nematodes and has attracted the attention of nematologists and bacteriologists since this has great potential as biocontrol agent against plant parasitic nematodes. It is mycelial, gram positive endospore forming bacterial parasite. The bacterial parasite produces endospore that adheres to the cuticle of plant parasitic nematodes and interface with the life cycle of the nematodes resulting in the suppression of nematode population.

Life cycle of *P.penetrans* starts with endospore, the infective stage of the bacterium that perpetuates year after year in the soil and is released on decomposition of nematode body of adult females and males or in plant root tissues. The spores of *P.penetrans* attach to the cuticle of second stage juvenile and germinate within a few days after the juvenile starts feeding within the root. The germination of spores occurs only after the encumbered second stage juveniles invade the plant root and begin to feed. The germ tube emerges through a central opening in the basal attachment layer and penetrates the cuticle of the nematodes and finally reaches the pseudocoelom.

The germ tube in the pseudocoelom develops into vegetative microcolony consisting of dichotomously branched septate mycelium. The intercalary cells in the microcolony lyse and give rise to daughter colonies. The process continues resulting in a large number of daughter colonies containing fewer but large vegetative cells. Eventually, quartets of developing sporangia predominate in the nematode body cavity. These quartets separate into doublets of sporangia and finally separate into a single sporangium that will eventually contain an endospore. Endospores of *P.penetrans* are released into the soil when the plant roots with parasitized root knot nematode female decompose. The life cycle completes within 20 30 days at 30^{o} C.

Biocontrol potential

There are many strains of *P.penetrans* and the strains are host specific. *P. penetrans* exerts various degrees of nematode biocontrol under greenhouse and field condition. Dried root powder containing spores of *P.penetrans* when incorporated in the soil @ 100mg/kg soil, 99 per cent second stage juveniles of *Meloidogyne javanica* were infected. Kokalis (2015) effectively controlled *M. incognita* in tomato and cucumber and *M. arenaria* in snapdragon by seed treatment. It can be used as seed treatment as well as soil application

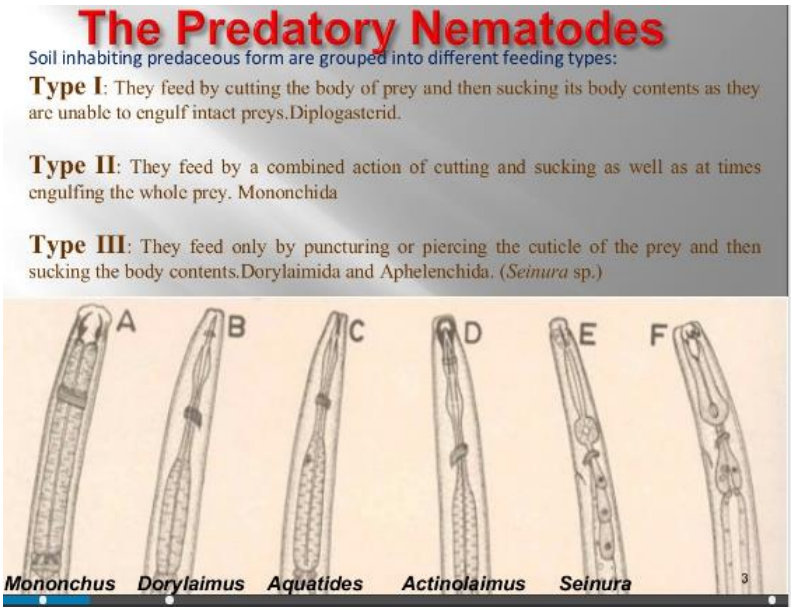
with known spore load. Moreover, it is compatible with nematode like carbofuran and other biocontrol agents.

Nematode as Biocontrol agents

1. Predatory nematodes

This is another group of biocontrol agent which has been given minimum attention. In soil ecosystem along with plant parasitic and free living nematodes, numerous predatory nematodes are also encountered. These nematodes belong to different groups. Most of them are found under the order Mononchida. Others are Dorylaims, Nygolaims, Aphelenchus, Actinolaims etc.

Mononchus have large and strong buccal cavity provided with teeth and denticles. They can swallow the prey nematodes. Others cannot swallow their prey. They rupture the cuticle of the prey nematodes by the needle like feeding apparatus and kill the prey nematodes. The biocontrol potential of predatory nematodes is very limited. They are difficult to mass multiply due to their low fecundity. Moreover, the predatory nematodes hardly search for the prey nematodes. It is mostly chance encounter only. The predatory nematodes cannot kill and swallow the large sized nematodes (Choudhury, 1996).



Methods of application

Seed treatment

Pseudomonas fluorescens and/ or *Trichoderma harzianum* can be used as seed treatment. Dosage – 15 to 20 grams of talc formulation of *P. fluorescens* and/ or *T. harzianum*/kg of seed.

Substrate treatment

Treatment of coco-peat (substrate) in which seedlings are grown under shade net or protected conditions. Dosage – 5 to 10g of formulation/kg of coco-peat (substrate).

For 1 ton of Coco-peat or any other substrate prepared by mixing Neem cake @ 50kg + phorate @ 5Kg + *Trichoderma harzianum* + *Pseudomonas fluorescens* each at the rate of 2 kg, can be used for producing the seedlings of horticultural crops especially vegetable crops such as tomato, egg plant, capsicum, cauliflower, cabbage, chilies, onion.

Spraying

Pseudomonas fluorescens & Trichoderma harzianum has to be sprayed on the plants at regular intervals of 30 days at a dosage of 5g/ lit or 5ml/ lit.

Drenching or application through drip irrigation system

Pseudomonas fluorescens & Trichoderma harzianum has to be given through drip/ by drenching @ 5g/ lit or 5ml/ lit at regular interval of 30 days.

Soil application

Land should be thoroughly ploughed and soil should be brought to fine tilth. Beds are to be prepared after bringing the soil as fine tilth. one ton of well decomposed FYM enriched with *Pseudomonas fluorescens* + *Trichoderma harzianum* + *Paecilomyces lilacinus* during the land preparation or on the beds 5 – 10 days before sowing seeds or transplanting the seedlings.

Process of Enrichment of FYM

One ton of well decomposed FYM has to be enriched by mixing with 2 kg each of *Pseudomonas fluorescens + Trichoderma harzianum + Paecilomyces lilacinus* formulation under shade. It has to be covered with mulch and optimum moisture of 25 - 30% has to be maintained for a period of 15 days. Once in a week thoroughly mix the FYM for maximum multiplication of and homogenous spread of the microorganisms in the entire lot of FYM.

Process of Enrichment of Neem cake

One ton of neem cake has to be enriched by mixing with 2 kg each of *Pseudomonas fluorescens + Trichoderma harzianum + Paecilomyces lilacinus.* It has to be covered with mulch and optimum moisture of 25 - 30% has to be maintained for a period of 15 days. Once in a week thoroughly mix the neem cake for maximum multiplication and homogenous spread of the microorganisms in the entire lot of neem cake.

Process of Enrichment of vermicompost

One ton of vermicompost has to be enriched by mixing with 2 kg each of *Pseudomonas luorescens + Trichoderma harzianum + Paecilomyces lilacinus*. It has to be covered with mulch and optimum moisture of 25 - 30% has to be maintained for a period of 15 days. Once in a week thoroughly mix the vermicompost for maximum multiplication and homogenous spread of the microorganisms in the entire lot of vermicompost.

- Soil application with *Paecilomyces lilacinus* @ 2.5 kg/ha at sowing significantly reduced the population of reniform nematode infecting soybean.
- *Trichoderma harzianum* @ 2.5 kg/ha along with *Pochonia chlamydosporia* @ 10 kg/ha one week prior to sowing effectively managed lesion nematode, *Pratylenchus thornei* and *Fusarium* species in chickpea.
- The effectiveness of *Pseudomonas fluorescens* in castor as soil application @ 2.5 kg/ha in the management of *Rotylenchulus reniformis* was confirmed.
- Combined application of Neem cake @ 100 g/m^2 + *Paecilomyces lilacinus* @ 50 g/m^2 has been found most effective in reducing the *Meloidogyne incognita* population in soil and also increasing the yield of cucumber grown in polyhouses.
- Soil application of *Pseudomonas fluorescens* @ 20 g/m^2 and paring with hot water treatment at 55°C + Carbofuran @ 0.5g a.i./plant + neem cake @ 1 kg/plant proved effective in reducing the root-knot nematode population and increasing the yield of banana cv. Grand Naine.
- Use of *Paecilomyces lilacinus* @ 20kg/tree + Castor cake @ 2 ton/ha in root zone of pomegranate at regular interval of six months reduced the root-knot nematode population below economic threshold level.

Application of the bio-pesticides to a standing crop

It is possible that farmers would not have prepared beds or main field initially as mentioned above and still observe the infestation of nematodes, soil borne pathogenic fungi and bacteria on the crops. Then these following steps for the management of nematodes, soil borne pathogenic fungi and bacteria need to be taken.

Soil application: Apply one ton of vermicompost enriched with *Pseudomonas fluorescens + Trichoderma harzianum + Paecilomyces lilacinus* (each 2kg)around the rhizosphere of the plants.

Commercial formulations available in India

S.No.	Antagonist	Name of the commercial product	Pathogen controlled	Country
1.	*Pseudomonas fluorescens*	SHEATHGUARD	RKN, Cyst and Citrus nematode	Agri Life, Hyderabad,India
2.	B.armus	Bionem – WP Biosafe – WP Chancellor - WP	Root knot nematode	Agro Green, Multinational
3.	B. subtilis	Biostart	Root knot nematode	USA
4.	Bacillus sp., Pseudomonas sp., Rhizobacterium sp., Rhizobium sp	Micronema	Root knot nematode and other phytonematodes	Agricultural Research Centre Giza, Egypt
5.	Pasteuria penetrans	Econem	Root knot and sting nematode	Bayer Crop Science Multinational

Table 12. Some important biopesticides being currently formulated in India and abroad.

Products	Biocontrol agents	Name of the manufacturer
Pant Biocontrol Agent 1 and 3	*Trichoderma harzianum* *Pseudomonas fluorescens*	Deptt. of Plant Pathology, G.B.P.U., Pantnagar
Biowilt-X	*T. harzianum*	Deptt. of Plant Protection, AMU, Aligarh
Bionem-X	*Pochonia chlamydosporia*	Deptt. of Plant Protection, AMU, Aligarh
Biocomp-X	*P. fluorescens*	Deptt. of Plant Protection, AMU, Aligarh
Biocure-X	*B. subtilis*	Deptt. of Plant Protection, AMU, Aligarh
Actiguard	Acibenzolar-S-methyl	Syngenta Crop Protection, Greensboro
Actinovate	*Streptomyces lydicus*	Natural Industries, Houston, USA
AQ10	*Ampelomyces quisqualis*	Ecogen, Inc. Israel
Anti-Fungus	*Trichoderma* spp.	Grondortsmettingen De Cuester, Belgium
Aspire		*Candida oleophola* Ecogen, Inc. Israel
Bactophyte	*Bacillus subtilis*	Russia
Biofungus	*Trichoderma* spp.	Grondortsmettingen De Cuester n.v., Belgium
Bas-derma	*T. viride*	Basarass Biocontrol Res.Lab., India
Binab-T	*T. harzianum, T. polysporum*	Bio-Innovation AB, UK
Bioderma	*T. viride/T. harzianum*	Biotech International Ltd., India
Biofox C	*Fusarium oxysporum*	S .I. A. P. A., Italy

Bioject Spot-Less	*Pseudomonas aureofaciens*	Eco Soil Systems, USA
Bio-Save 10LP	*P. syringae*	CTT Corp., USA
Bio-Save11	*P. syringae*	EcoScience Corp., USA
Blight Ban A506	*P. fluorescens*	Plant Health Technologies, USA
Blue Circle	*Burkholderia cepacia*	EcoScience Corp., USA
Cedomon	*P. chloraphis*	Bio Agri AB, Sweden
Coniothyrin	*Coniotyrium minitans*	Russia
Companion	*Bacillus subtilis* GB03	Growth Products, USA
	B. lichniformis, B. megaterium	
Contans, Intercept	*Coniothyrium minitans*	Prophyta Biolog. Pflanzenschutz, Germany
Conquer	*P. fluorescens*	Mauri Foods, Australia and Sylvan Lab, USA
Deny	*B. cepacia, P. cepacia*	Stine Microbial Products, Shwanee, KS
DiTera	*Myrothecium verrucaria*	Valent Biosciences, USA
Ecofit	*T. viride*	Hoechst Schering Afgro Evo Ltd., India
Epic	*Bacillus subtilis*	Gustafson Inc., USA
Fusaclean	*F. oxysporum*	Natural Plant Protection, France
Kalisena	*Aspergillus niger*	Cadilla Pharma., India
Galtrol-A	*Agrobacterium radiobacter*-84	AgBioChem Inc., USA
Intercept	*P. cepacia, B. cepacia*	Soil Technologies Corp., USA
HiStick N/T	*B. subtilis*	MicroBio Group, UK
Kodiak, Kodiak	*B. subtilis*	Gustaffson Inc., USA
Koni	*Coniothyrium minitans*	BIOVED Ltd., Hungary
Messenger	*Erwinia amylovora* HrpN	BIOVED Ltd., Hungary
Mycostop	*Streptomyces griseoviridis*	Kemira Agro. Oy, finland
Nogall, Diegall	*Agrobacterium tumefaciens*	Bio-Care Technology Pvt. Ltd., Australia
Norbac 84 C	*A. radiobacter* strain 84	New BioProducts Inc., USA
Paecil	*Paecilomyces lilacinus*	Tech. Innov. Corp. Pvt. Ltd., Australia
Phagus	Bacteriophage	Natural Plant Protection, France
Polyversum	*Pythium oligandrum*	Biopreparatory Ltd., Czech Republic
PSSOL	*P. solanacearum*	Natural Plant Protection, France
Funginil	*T. viride*	Crop Health Bioproduct Res. Centre, India
Ecoderma	*T. viride* + *T. harzianum*	Margo Biocontrol Pvt. Ltd. Banglore
Defence	*T. viride*	Wockhardt Life Science Ltd. Mumbai
Trichoguard	*T. viride*	Anu Biotech Int. Ltd. Faridabad

Merits of Biocontrol agents

- Biological control is less costly and cheaper than any other methods
- Biocontrol agents give protection throughout the crop period
- They are highly effective against specific pest
- They do not cause toxicity to the plant
- Application of biocontrol agent is safer to the environment and to the person who applies them
- They multiply easily in soil and leave no residual problem
- Biocontrol agents can eliminate pathogens from the site of infection

- Biocontrol agents also enhance the root and plant growth by way of encouraging the beneficial soil microflora
- Agents are easy to handle and apply
- It can be combined with biofertilizer

Demerits of Biocontrol agents

- Efficiency depends on environmental conditions.
- Shelf life is short
- Specific to particular pathogen
- Incompatibility with conventional pesticides is also a major problem
- Biological control is a slow process , It takes a lot of time
- The required amount of population should be checked periodically
- Although it is cheap in the long run, the process of setting up the biological control system is a costly endeavour.

Questions

0.5 mark

1.	Dichotomously branched septate mycelium is produced by the following nematode antagonist			
	A) *Pochonia chlamydosporia*	B) *Pupureocilium lilacinum*	**C) *Pasteuria penetrans***	D) *Pseudomonas fluorescens*
2.	A fungal antagonist which increasing the uptake of Phosphorus is			
	A) Pochonia	B) Trichoderma	C) Pseudomonas	**D) VAM**
3.	Predatory nematodes belongs to the order			
	A) Nematoda	**B) Mononchida**	C) Tylenchida	D) Dorylaimida
4.	Commercial product of nematode antagonist belongs to Pseudomonas for the management of root knot nematode is			
	A) Sheathguard	B) Biosafe	C) Econem	D) Micronema
5.	*Pochonia chlamydosporia* was first reported as a parasite of nematode eggs by			
	A) Wilcox and Tribe	B) Lopez	C) Gams	D) Morton

One mark

1. Induced systemic resistance
2. Antibiosis
3. Mononchus
4. Adhesive branches

Two marks

1. Define Mycoparasitism
2. Adhesive Network
3. Endophytic Fungi (AMF)

Three marks

1. Classify trapping organs produced by NTF
2. Write about nematophagus fungi *Pochonia chlamydosporia*
3. Illustrate the morphological description of *Purpureocilium lilacinum*

Five marks

1. Write about Predatory nematodes with neat sketch
2. Role of nematode trapping fungi for the management of plant nematodes
3. Briefly explain the bio control potential of bacterial antagonist, *Pasteuria penetra*
4. Different application methods of bio control agents

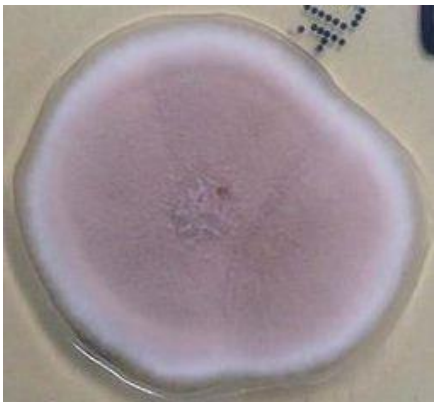
Lecture 3	***Purpureocillium lilacinum (=Paecilomyces lilacinus)*** **– Morphological identification of colony, phialids and conidial spore**

Purpureocillium lilacinum (=Paecilomyces lilacinus)

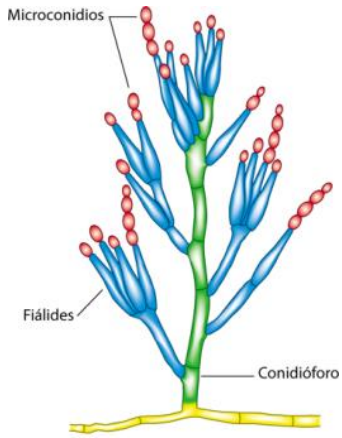
Purpureocillium lilacinum is commonly isolated from soil, decaying vegetation, insects, nematodes and as a laboratory contaminant.*P. lilacinum*morphological identification Colony characteristics of the fungal isolate were observed after the cultures were grown on PDA for 7- 14 days. Morphological features (e.g., conidiophores, phialides) of the isolate were examined by a light microscope.

Morphological Description

Colonies are fast growing, pink to violet-coloured. Conidiophores are erect 400-600 µm in length, bearing branches with densely clustered phialides. They are swollen at their bases, gradually tapering into a slender neck. Conidia are ellipsoidal to fusiform, smooth-walled to slightly roughened, hyaline to purple in mass, 2.5-3.0 x 2-2.2 µm and are produced in divergent chains. Conidiophore stipes are 3-4 µm wide, yellow to purple and rough-walled. Chlamydospores are absent.



Purpureocillium lilacinum mycelia colony



Expected Questions

1. Write about morphological description of *Purpureocillium lilacinum*

Lecture 4	***Pochonia chlamydosporia* – Morphological identification of colony, conidia spore and chlamydospores identification**

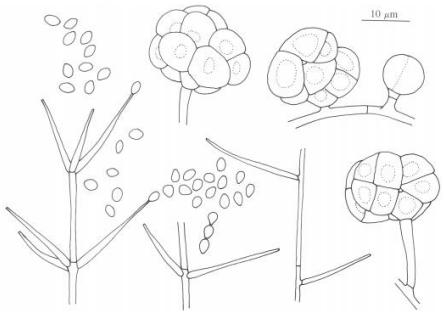
Pochonia chlamydosporia

Species of *Pochonia* are widely distributed in agricultural soils and infect eggs of plant parasitic nematodes. Within the genus *Pochonia*, *P. chlamydosporia* appears the most effective in infecting nematode eggs. This species is one of the major facultative antagonistic fungi that can parasitize egg and female stages of root-knot nematodes and female cyst nematodes.

Morphological Description

The colonies of *Phochonia chlamydosporia* are white in colour, which later becomes creamy, pale yellow to orange. It produces vegetative hyaline hyphae which are septate, and fine aerial mycelium. The conidiophores are usually prostrate and formed in ramified hyphae where conidia generating cells are produced. These cells are termed as phialides and can be borne singly or in pairs frequently in whorls of three to five. The conidia have an oval to ellipsoidal shape, depending on the sub species of fungus, and they are borne terminally, either singly or in small clusters or chains.

P. chlamydosporia produces two types of asexual spores, conidia and dictyochlamydospores. The dictyochlamydospores are hyaline and multicellular spores produced abundantly on aerial mycelium or submerged in agar, with thick cell walls. These spores are always produced terminally on more or less well-developed pedicels.



Mycelium, Conidiophores, conidia and dictyochlamydospores (from ZARE *et al*., 2001)

Questions

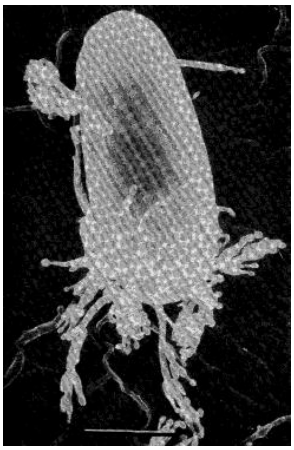
1. Write about morphological description of *Pochonia chlamydosporia*

Lecture 5	**Isolation of *P. lilacinum* and *Pochonia chlamydosporia* from nematode eggs and mode of action**

Isolation of *P. lilacinus* from *Meloidogyne* spp. eggs

Root samples were collected from root knot nematode infested plants. Galled roots were washed with running tap water and the egg masses was carefully removed from the roots with a dissecting needle. Eggs were extracted from the egg masses following the method of Hussey and Barker (1973) with some modifications. Egg masses were treated with 1% sodium hypochlorite (NaOCl) for 1 min, after treatment, eggs was rinsed with sterile-distilled water.

About 100 eggs were spread onto each of Petri dish (9 cm diameter) containing potato dextrose agar (PDA), and at least, five plates were applied for each sample. All plates were incubated at 25°C and routinely examined with an inverted microscope. The fungal hyphae growing from eggs were transferred to PDA plates for isolation and identification of *Paecilomyces lilacinus* (Domsch, Gams and Anderson 1980; Barnett and Hunter 1998).



Conidial production of *Paecilomyces lilacinus* emerging from egg of *Meloidogyne incognita*. Bar =10 micron meter

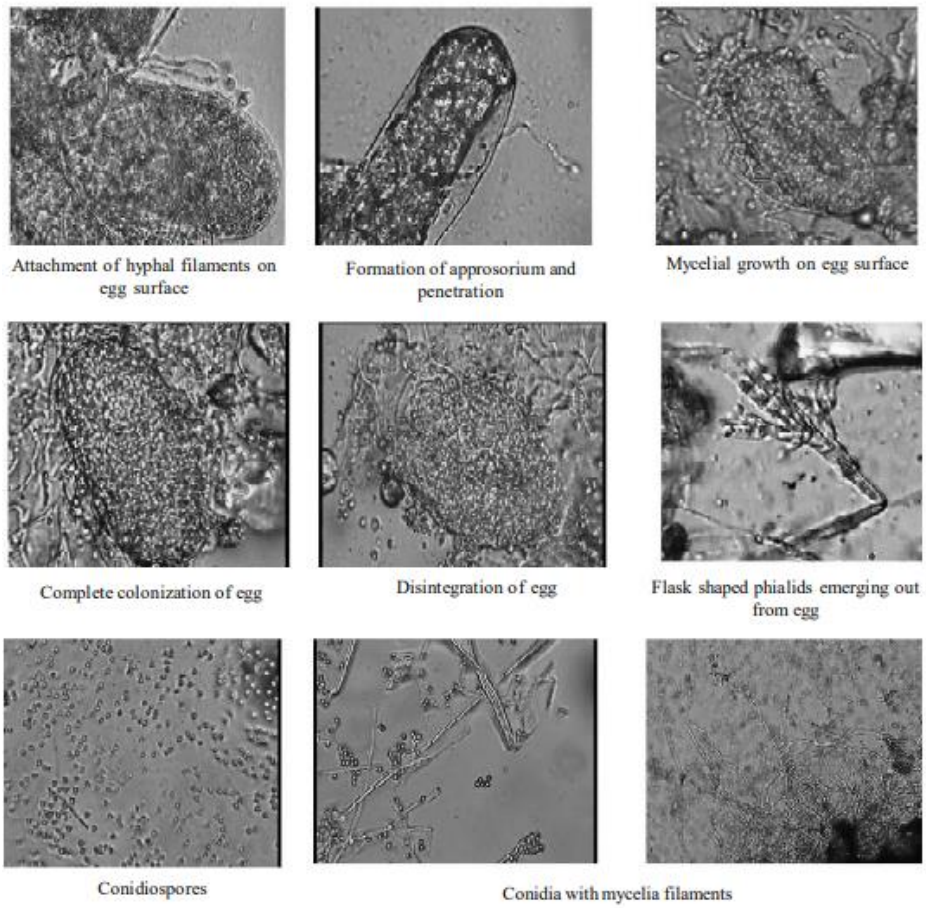
Mode of action of *P. lilacinum*

The fungus has been reported to parasitize eggs of many sedentary endoparasitic nematodes. The infection process starts with growth of fungal hyphae in the gelatinous

matrix and eventually the eggs of nematodes are engulfed by the mycelial hyphae. The proliferated hyphal branches penetrate the eggs. In cyst nematodes, the fungus penetrate through vulva or the broken and exposed neck region. After entering the cyst, the fungus grows saprophytically on the body content surrounding the eggs during or before its parasitism of the eggs. In all cases, eggs in the early embryonic developmental stages prior to gastrulation process are more vulnerable to infection.

Once the hyphae are in contact with the eggs a series of ultrastructural changes occurs in the eggs due to effects of exogenous metabolites and chitinolytic activities of the fungus. Once inside, the fungal mycelium radiated profusely in the eggs of early embryonic development and the entire embryo is replaced by the mycelial biomass. Occasionally, *P.lilacinum* may penetrate the egg laying female through the anus or vulva. In such cases, the infected female body cavity filled with the fungal biomass and the nematode die.

Fig. 1. Egg parasitization process of *P. lilacinum* on root knot nematode, *M. incognita*



Isolation of *Pochonia chlamydosporia* from egg masses of *Meloidogyne* spp.

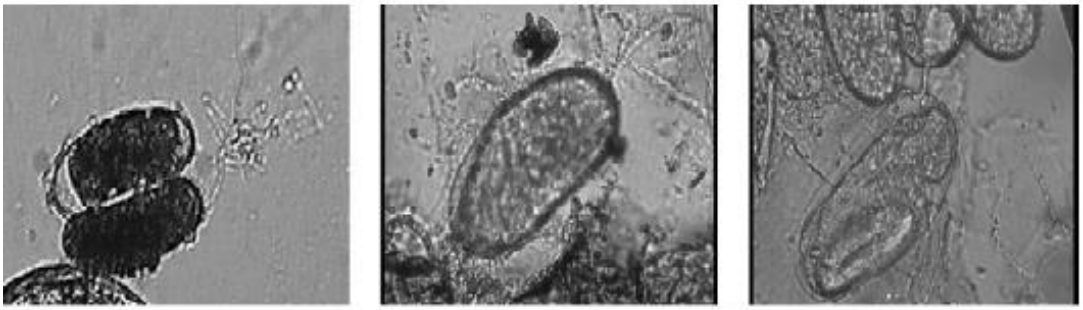
Fifty nematode galls were taken from a subsample of roots of each field-collected sample and carefully washed under running water. From each sample of galls, 50 egg masses were removed using fine needles under a stereoscopic microscope at 4× magnification. Egg masses were placed into sterile Petridishes, washed twice with sterile distilled water and the eggs released from the mass by direct maceration with sterile mortar and pestle.

Aliquots of 0.5 ml (1000 eggs) were plated onto sterile potato dextrose agar (PDA) supplemented with antibiotics (50 mg/l of streptomycin sulphate, chloramphenicol and chlortetracycline hydrochloride in 9 cm Petri-dishes and incubated at 27°C for 10 days in darkness. Based on colony morphology (Kerry & Bourne, 2002), selected putative colonies of *P. chlamydosporia* and subcultured them onto PDA in sterile 9 cm triple vented Petri-dishes, which were incubated at 27°C in darkness for a further 7 days. One sample of the aerial mycelium from each putative *P. chlamydosporia* colony was sampled under sterile conditions, stained with cotton blue lactophenol (MartínezRodríguez, Alba, & Garza, 2004) and observed under the microscope at 40× and 60× magnification to confirm species identity based on morphology and morphometric characters (Zare, Gams, & Evans, 2001).

Mode of action of *Pochonia chlamydosporia*

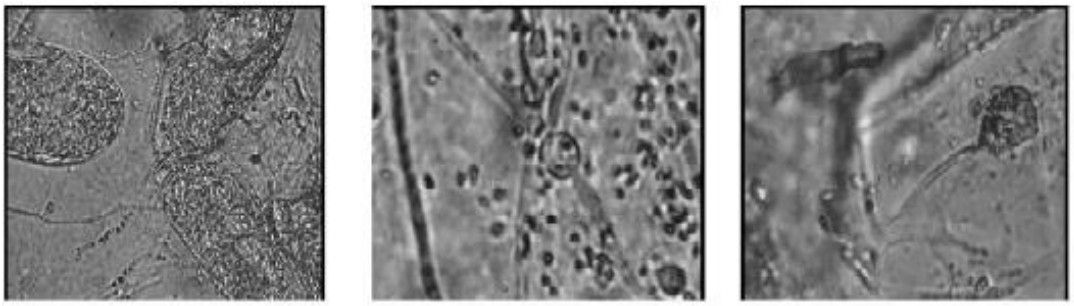
Parasitism of this fungus is based on appressorial formation developed from undifferentiated hyphae, which allows the colonization of the egg surface and penetration through both mechanical and enzymatic actions. Observations during the infection process have shown that the penetration of the eggshell occurs from both the appressorium and the lateral branch of the mycelium, and leads to the disintegration and the dissolution of three layers composing the eggshell.

Fig. 2. Infection and chlamydospore formation of *P. chlamydosporia* on root knot nematode, *M. incognita*



Attachment of hyphal filaments on egg surface

Penetration of fungal hyphae



Egg colonization with terminal chlamydospore

Intercalary formation of chamydospores

Thick walled chlamydospores at hyphal tip

Questions

1. Write about mode of action of *Pochonia chlamydosporia* on root knot nematodes
2. Illustrate the procedure for isolation of *P. lilacinum* from root knot nematode eggs?

Lecture 6	**Host range of *P. lilacinum* and *P. chlamydosporia* – virulence and effect of biotic and abiotic factors on growth – compatability with chemical inputs**

Host range

Paecilomyces lilacinus (Thom.) Samson 1 974 (Deuteromycotina: Hyphomycetes) is a nematode egg pathogenic fungus, which has been shown to be effective against a wide spectrum of plant parasitic nematodes and is considered one of the most promising biological control agents for the management of PPNs (Jatala, 1986; Kerry and Evans 1996; Siddiqui and Mahmood, 1996; Cannayane and Sivakumar, 2001). The fungus is a common soil hyphomycete, reported from numerous parts of the world, but more frequently from warm regions (Samson, 1974; Domsch *et al*., 1980). It has been isolated from a wide range of habitats including cultivated and uncultivated soils, forests, grassland, desert, estuarinesediments and sewage sludge.

Paecilomyces lilacinus attacks mainly sedentary stages and to a lesser extent juveniles of root knot and cyst nematodes. There are also reports of control of other nematode species by the fungus (Walters and Barker; 1994). The first report of *P. lilacinus* as an effective parasite of *Meloidogyne incognita* and *Globodera pallida* was by Jatala *et al*. (1979). Since then, many greenhouse and field studies have been conducted in a number of countries with different isolates of *P. lilacinus* that have demonstrated the potential of the fungus to suppress populations of root-knot and cyst nematodes (Dube and Smart, 1987; Cabanillas and Barker, 1989; Gomes Carneiro and Cayrol, 1991; Mittal *et al*., 1995). The fungus is applied as spores in large quantities to the soil, where it parasitizes the nematode eggs reducing the nematode multiplication and providing population control.

Pochonia chlamydosporia (previously *Verticillium chlamydosporium*) (Goddard), is a wide-spread facultative parasite of root-knot nematodes (Bourne and Kerry, 2000; Moosavi *et al*., 2010). It exists in soil as saprophyte, but can shift to parasite to root-knot nematodes. *P. chlamydosporia* has also proved effective against potato cyst nematode (Tobin *et al*., 2008). The fungus colonizes the root system of several species. It is confined to the rhizosphere and the rhizoplane.

Virulence

Hydrolytic enzymes, such as proteases and chitinases produced by *P. lilacinus*, have been implicated in the variation of its ovicidal and/or parasitic activities against nematodes (Kunert *et al*. 1985, 1987; Dackman *et al*. 1989). The production of secondary metabolites by fungi, or induced in fungi by the presence of nematodes, can also be a mechanism of nematode control or/and suppression (Stirling 1991; Anke *et al*. 1995).

Pochonia chlamydosporia (Goddard) Zare and Gams is a nematophagous fungus which infects females and eggs of cyst or root-knot nematodes (RKN). Extracellular enzymes secreted by *P. chlamydosporia* have been related with nematode egg infection. Chitinases and especially proteases are considered potential virulence factors for degradation of egg-shell components. *P. chlamydosporia* Pc_2566 encodes for an active chitin deacetylase (*Pc*CDA) potentially involved in nematode egg infection. Phospholipases are key enzymes in pathogenic fungi that cleave host phospholipids, resulting in membrane destabilization and host cell penetration.

Physiological and molecular mechanisms in colonization and penetration

The serine protease VCP1 of *P. chlamydosporia* is involved in the early stages of the infection process (Morton *et al*., 2003a, 2003b). *P. chlamydosporia* produces antifungal and toxic compounds include pochonins and antiviral and antiparasitic resorcylic acid lactones. Aurovertins are among the major constituents produced by *P. chlamydosporia*. Aurovertin type metabolites (A3 and A4), obtained from the fungus, have proved to be toxic to *Panagrellus redivivusin vitro* assays (Niu *et al*., 2010). The chemical structures of aurovertin like metabolites have been shown to be partly similar to phomalactone, a compound with nematicidal properties. Aurovertin 1 (A1) is the first natural aurovertin identified in *P. chlamydosporia* (Niu *et al*., 2010).

Females of root knot nematodes and cyst nematodes can be parasitized by vegetative hyphae in the rhizosphere with the production of an appressorium but without further specialized infective structures. Appressorium formation and egg infection must be preceded by environmental signaling, a process not yet understood (Lopez-Llorca *et al*., 2002b). Thigmotropic responses (i.e., toward hydrophobic and hydrophilic surfaces) and adhesion process mediated by glycoproteins, and enzymes (e.g., serine proteases) are

involved in pre penetration events (Lopez-Llorca *et al*., 2002b). Appressoria formation is thought to be a nutritional response in many plant and entomopathogenic fungi.

Effect of biotic and abiotic factors on growth

Pochonia chlamydosporia (Goddard) Zare & W. Gams 2001 (Hypocreales, Clavicipitaceae), was first reported in 1974 as a parasite of nematode eggs by Wilcox and Tribe in the UK. Ithas a worldwide distribution and found in nematode suppressive soils to parasitize eggs. The fungus can remain saprophytic in soil in the absence of both plant and nematode hosts. In the rhizosphere, the fungus can colonize the roots of host plants, and several *Pochonia* species have even been reported to show endophytic behavior in some Gramineae and Solanaceae species - a growth habit that may result in benefits to the host plant defence against soil-borne pathogens (Lopez-Llorca *et al*., 2002a; Macia Vicente *et al*., 2009a, 2009b). The fungus is also a facultative parasite of nematode and mollusk eggs, and a hyperparasite of other fungi (Lumsden *et al*., 1982; Zare *et al*., 2001).

Several factors have contributed to target this fungus for nematode biocontrol, including easy laboratory culturing, access to strains (from fungal collections), and effectiveness in nematode control. The success as BCA depends on the method of mass culturing of *P. chlamydosporia*, its formulation, addition of nutrient sources to increase sporulation, shelf life, and storage conditions (Jacobs *et al*., 2003; Duan *et al*., 2008; Montes de Oca *et al*., 2009).

The efficacy of *Pochonia chlamydosporia* as a biological control agent for root-knot nematodes is influenced by three key factors: the inoculum load of fungus in the rhizosphere, the age of eggs in the egg masses and the size of the galls (Bourne, 1995). In large galls female root-knot nematodes may produce egg masses which remain within the gall and are not exposed to parasitism by *P. chlamydosporium*. Hence, *P. chlamydosporium* is less effective in controlling root-knot nematodes in heavily infested soils and also on highly susceptible crops due to larger gall formation leading to deposition of egg masses with in roots and many eggs escape parasitism (Bourne, 1995). Sundararaju and Cannayane (2004) have also observed above phenomenon in the root-knot nematode infested banana roots, where the egg masses remain within the cortical tissues, thus preventing the eggs from parasitism by the antagonist. Further, most of the eggs in the egg masses are not

colonized and the egg masses near epidermis are partially parasitized by the fungus and also only the developing eggs or those in embryonic stages are engulfed by *P. chlamydosporium*.

Colonize the rhizosphere of plants and cultivars

The efficacy of the BCA depends on fungal rhizosphere colonization to facilitate colonization of galls, egg masses of root-knot nematodes (RKN), as well as eggs within cysts. Crop selection in a rotation plan can enhance the effective numbers of propagules available in soil for rhizosphere colonization during the next crop, in a plant-fungus compatible interaction. Significant levels of nematode control can be achieved only if fungal isolates are capable of proliferating in the rhizosphere.

Plant species and their varieties and cultivars differ in root exudates and rhizo deposits which support *P. chlamydosporia* growth in the rhizosphere. Crops and plant species that can support more than 200 colony forming units (CFU)/ cm^2 of root are considered to be good hosts of the fungus. Good hosts for *P. chlamydosporia* include beans, cabbage, crotalaria, kale, pigeon pea, potato, pumpkin and tomato. Chili, sweet potato, cowpea, rye, tobacco and cotton are moderate hosts (100 – 200 CFU/cm^2 of root) whereas poor hosts (less than 100cfu/cm^2 of root) including okra, soybean, sorghum and wheat (de Leij, 1992; Bourne *et al*., 1996).

Chlamydospores

Conidia and hyphal fragments have low survival rates in soil and are subjected to fungistasis when added to soil without a supplementary energy source. Chlamydospores have sufficient food reserves to enable the fungus to establish without the addition of other energy sources (Kerry *et al*., 1993; Mauchline *et al*., 2002). Therefore, *in vitro* chlamydospore production has become one of the criteria to select fungus strains. An application rate of 5,000 chlamydospores/cm3 soil is common for experimental purposes but it may vary according to strain and target nematode (de Leij *et al*., 1992; Kerry *et al*., 1993; Stirling and Smith, 1998; Viane and Abawi, 2000)

Tri-trophic interactions

Plants may or may not be compatible with nematodes, and this interaction will determine nematode reproduction, as well as the extent of damage and yield loss caused depending on its host status to nematodes (i.e., resistant, susceptible, tolerant). Good hosts for *P. chlamydosporia* can be either good or poor hosts for the plant-parasitic nematode. In

general, fungal control of the nematode can be greater in plants that are poor hosts for the nematode but which also support extensive rhizosphere fungal growth.

Plant susceptibility or resistance to RKN infection affects root gall size and this may result in eggs being retained inside large galls, thereby preventing them from being exposed in the rhizosphere to *P. chlamydosporia* parasitism (Atkins *et al*., 2003), thus "escaping" fungal infection. Therefore, application of the fungus to a relatively poor host for the nematode, producing smaller galls so that most egg masses are exposed on the root surface, might provide a more effective control (Kerry, 1997). Thus increasing *P. chlamydosporia* application rate in soil does not necessarily increase the colonization of the rhizosphere, a situation observed in a poor host where the fungal growth in the rhizosphere does not respond to increased soil application doses (de Leij *et al*., 1992).

Nematode root invasion

It is also important to note that *P. chlamydosporia* does not reduce initial plant invasion by juvenile stages, the main target being eggs, although second-stage juveniles of *Meloidogyne* within egg masses can be colonized and parasitized (Manzanilla-Lopez,unpub.data)

Egg infection

Anatomical and physiological features affect the infection process of the eggs, including enzymatic activity within fungal isolates and nutrient availability for the fungus. Eggs of RKN and cyst nematodes (CN) also differ in their susceptibility to fungal strains, which show a nematode host preference. Eggs in early stages of embryogenesis are more susceptible to parasitism than those containing a second-stage juvenile.

Conducive conditions are provided by nutrient depletion, low levels of complex nitrogen sources, and low C:N ratios (Blakeman and Parbery, 1977; St. Leger *et al*., 1989; Jackson and Schisler, 1992). Low C:N ratios influence egg pathogenicity by *P. chlamydosporia* (Segers, 1996), whereas production of conidia seems to be favored by media with relatively high C:N (Mo *et al*., 2005). The change, or ''switch,'' from the saprophytic to the parasitic phase of the fungus may therefore be related to nutrients (including C and N) that are either released by plants into the rhizosphere or available in eggs, egg masses, and galls.

VCP1 is an important enzyme in the early stages of parasitism, and the effect of various nutrients and pH on its expression was analyzed, by enzyme assays and qPCR, in the presence and absence of nematode eggs (Ward *et al*., 2011, 2012). Glucose repressed VCP1 production by *P. chlamydosporia* and this repression could not be overcome by addition of eggs of the host (*M. incognita*). The effect of ammonium chloride was more complicated; at early stages after transfer to ammonium chloride-containing medium (4 hr), VCP1 levels were repressed but in most cases they were increased after 24 hr. For most strains, VCP1 expression was higher when the fungus was grown in more alkaline pH conditions (E. Ward *et al*., unpub. data).

***Pochonia chlamydosporia* gene expression under different nutrient regimes**

Gene expression profiles were recently identified by Rosso *et al*. (2011) through a c-DNA amplified fragment length polymorphism (AFLP) approach. Two isolates (RKN and potato CN biotypes) were assayed at four different times under conditions of saprophytic to parasitic transition under different nutritional stresses (i.e., starvation in presence/absence of eggs or growth media). Expression of a number of genes was compared using electrophoretic profiles of the transcript derived fragments (TDF) of the two isolates. Annotation of basic local alignment search tool (BLAST) analyses showed that most TDFs could be included in functional groups related to metabolic functions, or were involved in mechanisms of cellular

signal regulation, cellular transport, regulation of gene expression, as well as DNA repair.

Comparison of treatments ranging from saprophytism to true parasitism showed significant transcriptional reprogramming between treatments (i.e., a ‘‘switch’’ from saprophytic to parasitic phase). Some genes were induced/expressed or repressed thus suggesting a concerted regulation, especially when activated after exposure to eggs. Common amplification profiles reflected expression of basic metabolic genes (constitutive), and were not affected by assay conditions. Statistical analyses applied to the TDFs showed that genes involved in parasitism or other metabolic pathways clustered together (Rosso *et al*., 2011).

Effect of abiotic factors on growth of Biocontrol agents

Environmental parameters such as abiotic (soil type, soil temperature, soil pH, water potential and such like) and biotic (plant species and variety, microbial activity of the soil)

factors as well as other factors such as method and timing of applications may have influence on the biological control efficacy of nematophagous fungi.

Effect of temperature

Different isolates of fungi responded differently to various temperature regimes. Growth of three isolates of *Trichoderma harzianum* and *Arthrobotrys oligospora* was maximum at 20-25°C. *Pochonia chlamydosporia* var. *chlamydosporia* had better growth on 15-20°C. Temperature of 10-15°C found to be not suitable for the growth of those isolates. Among the external factors which influence the growth of fungi, temperature plays an extremely important role.

Effect of pH

The mycelial growth was different among isolates and different pH levels. At all pH levels tested, Trichoderma isolates grew significantly better in 7-8. In *A. oligospora* recorded maximum growth at pH 5-6 while pH 4-5 supported the maximum growth of *P.chlamydosporia*. In *Trichoderma harzianum* best growth occurred in PH 6-7.

Different isolates varied significantly in their survival ability and also in proliferation in different soils. For example the fungus proliferates in calcareous loams and organic soil in England and could survive at least 3 month after application. The fungus prefers peaty sand soil rather than loamy sand or sand in tomato plots infected with *M. incognita*. However in sandy loam micro plots a 90% control of *M. hapla* could be achieved, only if the temperature did not exceed 25°C. Its optimal pH for growth was 6, but some could grow even at pH 3. As optimum growth temperature for this variety is measured (18–21°C) and can be a good candidate for temperate and cool regions. The fungus showed optimal growth at pH 6, but generates red pigments on acidic media that when was extracted in chloroform/methanol had nematicidal effect on potato cyst nematode, *Globodera rostochiensis*.

Effect of light intensity

Light has profound effect on the mycelial growth of nematophagus fungi. The exposure of the fungus to alternate cycles of 12 h light and 12 h darkness for 10 days resulted in the maximum mycelial growth of *Trichoderma harzianum*. The maximum growth of *A. oligospora* was one that exposed to continuous light resulted and *Trichoderma harzianum* and *P. chlamydosporia* gerw best in, continuous darkness.

Effect of carbon source

Sorbitol was the least utilized carbon compound by *Trichoderma* isolates. The fungi grown on Arabinose and Fructose recorded significantly higher dry mycelial weight followed by manitol. *Arthrobotris oligospora* had significantly higher mycelial growth on sorbitol followed by Arabinose, while in manitol the least mycelial growth occurred. There was minimum growth observed when those isolates were grown on a medium without any carbon source.

Effect of Nitrogen source

Mycelial growth of the fungus was influenced by all the nitrogen sources. Nitrate amonium supported the maximum growth followed by sodium nitrate and Nitrate potassium. L-prolin was the least efficiently utilized nitrogen source by all fungi isolates.

Compatibility of Biocontrol agents with chemical inputs

In the agricultural practices, application of various agrochemicals such as fungicides, antibiotics and fertilizers were applied for crop improvement management of number of crop plant diseases. However, these agrochemicals are chemical based materials and are potential for residual toxic effects on the crops and soil microflora including beneficial microorganisms. These materials also affect the soil characteristics causing pollution like hazards. The excessive use of chemical based agrochemicals may affect the biocontrol agents and their survival was reduced. Metabolic activities were retarded and the population may be decreased. But majority of crop plant diseases were controlled by number of fungicides because these diseases controlled effectively.

In the integrated disease management system, application of biocontrol agents like *Trichoderma* along with controlled dose of chemical based pesticides and fungicides were undertaken. Therefore the combined use of biocontrol agent and agrochemicals has attracted much attention in the control of soil borne diseases (Locke *et al*., 1985). Reduced amount of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by antagonist (Hjeljord and Tronsmo, 1998). Integration of biocontrol agents and commonly used fungicides showed positive association by reducing the seed infection compared to fungicide and the antagonist individually (Srinivas and Ramkrishnan, 2002). Hence, knowledge of compatibility of *T.viride* with important pesticides may help opt for better plant protection measures. Tolerance to commonly used pesticides

enhances the efficacy and expands the scope of application of biocontrol agents such as *T.viride*.

Trichoderma species were not resistant to commonly used selective fungicide Carbendazim; however, Trichoderma species exhibited significant resistance to other common fungicides like Mancozeb and Captan at lower concentration. Moity et al, (1982) reported that Benomyl was found to be strongly inhibitory to Trichoderma in culture at 0.5mg/ml. while Captan and Pentachloro nitro benzene (PCNB) were not inhibitory to Trichoderma species. It was reported that *T. viride* and *T. harzianum* were highly sensitive to Carbendazim (Pandey and Upadhyay, 1998). Bagwan (2010) conducted compatibility tests under in vitro condition for finding safer fungicides and insecticides against *Trichoderma*. He found that Thiram (0.2%), Copper oxychloride (0.2%) and Mancozeb (0.2%) were safer against *T. harzianum* and *T. viride*. These *Trichoderma* species were not sensitive to Captan, Tebuconazole, Vitavax, Propiconazole and Chlorothalonil. But *Trichoderma* was tolerant to all pesticides and weedicides and none of them inhibited the growth of *Trichoderma*. Mycelial growth of *T. harzianum* was totally inhibited under the treatment of Bordeaux mixture (1%) followed by Quinolphos. The fungus, *T.viride* was highly compatible with Imazathafir followed by 2,4 – D sodium salt and Oxyfluoforen while being totally incompatible with systemic fungicides like Carbendazim, Hexaconazole, Tebuconazole and Propiconazole.

Pochonia chlamydosporia can be effectively integrated with the nematicide Aldicarb. The nematicide mostly prevents initial nematode injury, while the fungus part causes a long-term protection. Aldicarb did not influence the activity of the fungus and resulted in a better control of *M. hapla* than treatment with Aldicarb or *P. chlamydosporia* alone.

Questions

1. Virulence factors of *P. lilacinum* which involved in nematode parasitisam
2. Write the effect of abiotic factors on growth of Biocontrol agents
3. Tri-trophic interactions of *P. chlamydosporia*
4. Define Chlamydospores

Lecture 7	Sterilization – Types and Preparation of sterilizing agents – Equipments used for production of bioagents

Sterilization refers to any process that eliminates, removes, kills, or deactivates all forms of life and other biological agents (such as fungi, bacteria, viruses, spore forms, present in a specified region, such as a surface, a volume of fluid or in a compound such as biological culture media.

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 methods 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. Dry heat denatures the proteins in any organism, causes oxidative free radical damage, causes drying of cells, and can even burn them to ashes, as in incineration.

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 then the dry heat.

B. Moist Heat

Moist heat is usually provided by saturated steam under pressure in an autoclave or pressure cooker and it's 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 hydrolysed 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°C. For most purposes 15 min at 121°C or 30min at 115°C are recommended.

C. Flame sterilization

Flame sterilization is used for metal objects such as inoculation needles and the tips of forceps and glass objects such as the neck of flask 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° angle in the upper portion of the flame from a Bunsen burner or 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.

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.

Chemical method

Disinfectants are chemical agents (usually liquids), which are used on the surface of non-living 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.

Preparation of sterilizing agents

1. 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 pathogens. 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.

2. 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.

3. Dettol

Normally one per cent or 2% Dettol is used for sterilization. Take 10ml of Dettol and add 90ml of distilled water to make up one litre to get 1% Dettol. This is an antiseptic solution to disinfect the culture room and working places like the table tops.

Equipment's used for production of bioagents

Bright field microscope

Bright-field microscopy is one of the simplest optical microscopy. In bright-field microscopy, illumination light is transmitted through the sample and the contrast is generated by the absorption of light in dense areas of the specimen. The limitations of

bright-field microscopy include low contrast for weakly absorbing samples and low resolution due to the blurry appearance of out-of-focus material.

Pressure cooker

It is 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°C.

Microwave oven

It is mainly used for boiling and melting of culture media very quickly. Boiling potato, Agar agar can be done efficiently within a short time. Care should be taken that only glass/ oven proof vessels alone must be used. Avoid metal vessels.

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°C - 180°C for 20 min to 1 h. An oven consists of an insulated cabinet, which is held at a constant temperature by means of 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°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 below in Table 2.

Table 2. Schedule of time and temperature used in hot air oven

Temperature (°C)	Time
120	8h
140	3h
160	1h
180	20min

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.

Other equipment's/ glassware required for culturing work

1. Petridish holder
2. Inoculation needle
3. Spirit lamp/ Bunsen burner
4. Haemocytometer
5. Balance
6. Test tube stand
7. Pipette
8. Petri plates
9. Test tubes
10. Conical flasks

Questions

1. Define Sterilization
2. Write the procedure for preparation of different sterilizing agents
3. List out different methods of sterilization
4. Explain about the equipment's used for production of bio agents

Lecture 8	Principle of Autoclave, Laminar Air Flow Chamber and Fermentor

Principle of autoclave

The autoclave is an apparatus, used for the sterilization of culture media (autoclaving). The increased pressure increases the boiling point of water and produces steam with a high temperature which destroys the cells of microbes. Autoclave and pressure cooker works on the same principle (Boyle's law).

Most of the microorganisms are killed at 121°C (i.e., 15lbs/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. The relationship between pressure and temperature of steam in an autoclave is furnished in table 1.

Table 1. Relationship between pressure and temperature of steam in an autoclave

Pressure in pounds per square inch (psi)	Temperature (°C)
Free flowing steam i.e., 0	100
5	109
10	115
15	121
20	126
25	130
30	135

Principle of Laminar Air Flow Chamber

Laminar air flow chamber is used for the culturing of microorganisms under aseptic conditions. It works by passing sterile air into the chamber using the fibrous filter. Before the usage of the equipment clean the work bench with surface disinfectant and then close the doors of the chamber and switch on the UV lamp for a period of 20 minutes. Then

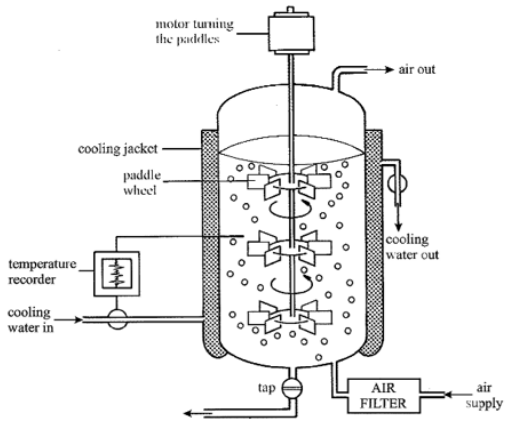
switch off the UV lamp and switch on the air flow and the lights. Then open the chamber for use.

It is used for reducing the danger of infection while working with infectious microorganisms and for preventing contamination of sterile materials. It is an alternative to inoculation chamber or culture room. Laminar air flow chamber is most suitable, convenient and reliable instrument for aseptic works. It allows one to work for longer period, which is not possible inside the inoculation chamber. Long hours of work inside the inoculation chamber may also cause suffocation and needs the interruption of work. 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 work station 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 0.3mm. The ultra-clean air, which is free from fungal and bacterial contaminants flows at the velocity of about 27± 3m/ 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 the sprit lamp. Therefore, a sprit lamp can be used conveniently during the work.

Principle of Fermentor

The device that is able to perform the process of fermentation is known as fermenter. A metabolic process in which the conversion takes place from sugar to acids, gases or alcohol is called fermentation. The systems which are utilized for the prime purpose of enhancing the growth and maintenance of a population of bacterial or fungal cells in a controlled mode are given by the fermentors. A fermentor must control temperature and keep the chamber anaerobic (free of oxygen) to optimize conditions for desired microbial fermentation.



Large scale multiplication of microorganisms often requires large vessels, commonly called fermenters. Industrial fermenters 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 fermenters have a total volume of one to two litres of medium with a maximum of about 12 to 15 lit pilot plant fermenters, which are

used in large scale studies of fermentations. Pure culture fermentation usually requires that the medium be sterilized. In small laboratory fermenters, 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.

Questions

1. Write the Principle of autoclave
2. Write the Principle of Laminar Air Flow Chamber
3. Write the Principle of Fermentor

Lecture 9	**Laboratory up-keep – Preparation of cleaning solutions – preparation of stock solutions**

For getting successful results from the experiments performed in a laboratory, it is essential that the lab and glassware gets perfectly cleaned. However cleaning laboratory and glassware is not a very easy task. Laboratory and glassware cleaning has to be done physically as well as chemically so that they become free of any germs.

Laboratory Cleaning

- All surfaces and equipment within the laboratory should be regarded as potentially infectious and should be cleaned on a daily basis. Exterior surfaces of equipment should be wiped with surface disinfectant and then wiped with water to remove any residue.
- Floors should not be swept or waxed, but should be mopped weekly with soap solution followed by an appropriate mycobactericidal disinfectant.
- Floors and work surfaces should be kept as free of clutter as possible. Materials should be stored in closed cupboards where possible. Excess reagents should be boxed, labelled and stored in an appropriate store room.
- Check spill kit contents on a monthly basis. Fresh disinfectant must be prepared each week. Record all cleaning and maintenance in the Laboratory Cleaning and Maintenance Logbook.

Laboratory Glassware Cleaning

1. Always clean your apparatus immediately after each use. It is much easier to clean the glassware before residues in them become dry and hard. If the glassware is dirty and cannot be washed immediately, put it in water to soak.
2. Handle glassware carefully while cleaning it. Most breakage occurs at this time.
3. Quality water produced by water purification system may be used for laboratory work and rinsing of glassware.
4. Rinse off all soap or detergent residue after washing glassware to prevent any possible contamination later. Most pieces of laboratory glassware can be cleaned by washing and brushing with a detergent or a special laboratory cleaning product called teepol. After they have been thoroughly cleaned, they are rinsed with tap water and finally with deionised

water. If the surface is clean, the water will wet the surface uniformly. On soiled glass the water stands in droplets.

5. Most new glass is slightly alkaline and should be washed upon receipt and generally can be soaked in a 1% HCL or HNO_3 solution before wash and deionised water rinse.

6. Never soak for long periods in strong alkaline solutions as it will damage the glass. Always follow up a soap or acid wash with a good deionised water rinse.

7. Always use soft brushes with a wooden or soft plastic handle to avoid abrasion. Do not use wire brushes or brushes with a wire core as it can abrade the glass.

Chromic Acid

A great cleaner and also removes organic residues. Use gloves and well ventilate the area when using chromic acid as it is a carcinogen and very corrosive. Make sure metal clamps or flanges are removed. It is best to fill the vessel or soak the item in the solution for a short time in a plastic tub so that you can contain the wash material, then rinse immediately several times before proceeding to a detergent wash. Make sure the residual chromic acid is diluted after use and disposed of properly and according to your local or company regulations.

Occasionally stronger acid washes are necessary for certain types of precipitates or residues. It is best to keep these very dilute and be used in an area where there is good ventilation and make sure you contain the residual acid and dissolved material for proper disposal. This method should only be used when absolutely necessary. One other caution is that strong acid washes may damage the graduation markings.

Removal of Grease

Grease is best removed by boiling the glass in a weak solution of sodium carbonate. Acetone or any other organic solvent can be used also, followed by several water and deionised water rinses.

Other stains

For permanganate stains use a mixture of equal 3% sulfuric acid and 3% hydrogen peroxide. For Iron stains use a solution containing one part hydrochloric acid and one part water. For bacteriological contamination glassware should be soaked in a disinfectant solution and then steam autoclaved then followed by a suitable washing and rinsing.

Rinsing

Glassware should always have a water rinse after any cleaning procedure followed by a deionised water rinse. It is best to give smaller pieces such as test tubes a soaking rinse followed by a deionised water soaking rinse. Glass pipettes are best soaked in a suitable pipette washer and washed and given both a water rinse and deionised water soaking rinse.

Drying

Oven drying at 100° C is best for all glassware. If not convenient, rack drying will work.

Steam Autoclaving or Sterilizing

Proper protocol for steam autoclaving of borosilicate glassware is 15-20 minutes at 100-120°C. Always leave closures off or loose during autoclaving.

Inspection after cleaning

Always inspect all glassware before steam autoclaving for cracks, chips or damage. The autoclave procedure will cause glassware to break if already damaged.

Culture Tubes

Used culture tubes need to be sterilized prior to cleaning. The most dependable technique for sterilizing culture tubes is to autoclave it for half an hour at around 121°C (15 psi). As media gets solidified on cooling, it should be poured out at the time the tubes are still hot. The tubes when emptied must be brushed with water and detergent and then rinsed with running tap water. After this it should be rinsed with distilled water, and placed properly for drying. When tubes are filled with media that is sterilized by autoclaving, there is no need to plug until the addition of the media. Media and tubes are both sterilized with one step autoclaving.

Test Tubes

Test tubes if new should be filed up with the cleaning solution and put up in a wire basket. It should then be heated up for approximately 15 minutes in an autoclave. After removing from the autoclave they should be thoroughly rinsed using a brush under running water. Then the test tubes are also rinsed using distilled water and then drained. Sometimes alcohols are also used to facilitate drying before draining is done.

Slides and Cover Glass

Slides should first be washed, and then placed in solution containing glacial acetic acid for around 10 minutes. Afterwards the slides and cover glass are thoroughly rinsed with distilled water and then wiped perfectly dry using paper towels or clean cloth. As soon as this is done they should be placed in a wide jar containing alcohol. Whenever the slides are needed they are to be removed from the jar and then wiped dry. In case the slides are stored dry then they should be washed with alcohol prior to use.

Glass Disposal

Prior to disposal of used and/or broken glassware it should be made sure that they are free from any chemical and biological hazards. The glass should be placed in a puncture-resistant box, labelled as "BROKEN GLASS," and then secured properly using tape. The box should be placed in a trash dumpster.

Preparation of cleaning solutions

Cleaning agents are substances (usually liquids, powders, sprays, or granules) used to remove dirt, including dust, stains, bad smells, and clutter on surfaces. Purposes of cleaning agents include removing offensive odour and avoiding the spread of contaminants.

List of cleaning agents

- Water, the most common cleaning agent, which is a very powerful polar solvent
- Soap or detergent
- Calcium hypochlorite (powdered bleach)
- Citric acid
- Sodium hypochlorite (liquid bleach)
- Sodium hydroxide
- Acetic acid (vinegar)
- Various forms of alcohol - like isopropyl alcohol
- Sodium bicarbonate (baking soda)
- Tetrachloroethylene (dry cleaning)
- Chromic acid

Chromic acid is a commonly used glassware cleaning reagent. It is prepared in a one litre container by dissolving 60 grams of potassium dichromate in approximately 150ml of

warm distilled water and then slowly adding concentrated sulphuric acid to produce a total volume of one litre chromic acid solution.

Preparation of stock solutions

A stock solution is a concentrated solution that will be diluted to some lower concentration for actual use. Stock solutions are used to save preparation time, conserve materials, reduce storage space, and improve the accuracy with which working lower concentration solutions are prepared. It can be stored at specific conditions in laboratory for long time and used as a standard reference material for analysis of the target analyte(s) in the daily use.

A stock solution is prepared by weighing out an appropriate portion of a pure solid or by measuring out an appropriate volume of a pure liquid and diluting to a known volume. Exactly how this is done depends on the required concentration unit. For example, to prepare a solution with a desired molarity you weigh out an appropriate mass of the reagent, dissolve it in a portion of solvent, and bring to the desired volume. To prepare a solution where the solute's concentration is a volume per cent, you measure out an appropriate volume of solute and add sufficient solvent to obtain the desired total volume.

Per cent (%) solution

One gram of solute dissolved in small quantity of water and the final volume is made up to 100ml with water. When the solute is a solid and the solvent a liquid, a per cent solution is prepared on a W/V basis, i.e., a known weight of a substance (solute) is added to 100ml of the solvent.

e.g. To prepare a 10 per cent $CuSO_4$ solution, add 10 g of $CuSO_4$ to 100ml of water.

If both the solute and the solvent are liquids, per cent solution is prepared on volume basis, and here, a known volume of the solute is taken and the final volume is made up to 100ml with the solvent.

e.g. To make a 35 per cent solution of perchloric acid, 35 ml of perchloric acid is taken in a measuring cylinder and the volume is made up to 100 ml with water.

This method of expression does not show the relative number of molecules of the solute that are present in the solution. Although per cent solutions are used quite frequently in the laboratory, they are generally found inadequate for precise work. When

we use salts while preparing a per cent solution, based on weight by volume, it will give per cent of salts but not different ions in the salts.

e.g. To prepare percentage solution of an ion present in the salt (for *e.g.*, Na in NaOH), the ratio of the ion to the total salt has to be determined. This is calculated based on the molecular weight of the salt to the molecular weight of the ion.

Mol. wt. of NaOH = 40, Na = 23, O = 16, H = 1

23 g of Na is present in 40 g of NaOH. To prepare 10 per cent Na solution, 10 g of Na has to be dissolved in 100 ml H2O. 23 g Na is present in 40 g NaOH, 17.4 g of NaOH contains 10 g of Na, and therefore dissolving 17.4 g of NaOH in 100 ml equals to 10 g of Na gives 10% Na.

Normal (N) solution

One gram equivalent weight of the substance dissolved in one litre of solution i.e., the final volume made up to 1 litre with water.

$$1\ g\ equivalent\ wt.of\ a\ substance = \frac{1\text{ g mol. wt. of the substance}}{\text{No. of replaceable hydrogen atoms or hydrogen equivalents}}$$

Hydrochloric acid (HCl) with molecular weight of 36.47 has one replaceable hydrogen atom, hence 1 litre of 1N HCl contains 36.47 g of the acid. On the other hand, sulfuric acid (H2SO4) having a mol. wt. of 98, has 2 replaceable hydrogen atoms. Therefore a normal solution of H2SO4 contains 49 g (98/2) of the acid per liter. Similarly, 1N NaOH will contain 40 g of the base per litre (as one hydrogen ion is equivalent to one acid hydrogen atom), while 1N Ca(OH)2 contains 74.096/2 = 37.048 g/litre.

Salts are also considered in the same terms. 1N K2SO4 contains 174.26/2 = 87.13 g/litre (K2SO4 contains 2 hydrogen equivalents).

e.g. To prepare 1N NaOH, dissolve 1 g eq. wt. (40 × 1 = 40) of NaOH in a small quantity of water and make up the volume of 1 litre.

Molar (M) solution

$$\text{Molarity} = \frac{\text{moles of solute}}{\text{litre of solution}} \text{ or } \frac{\text{grams of solute}}{\text{molar mass solute x litres of solution}}$$

A molar solution is one containing as many grams of the solute per litre of the solution as the molecular weight of the dissolved substance, i.e., one gram molecular weight of a substance dissolved in 1 litre of the solution.

e.g. 1M sucrose: 342.2 g sucrose dissolved in a small quantity of water and the volume is made up to 1 litre.

Parts per million (ppm) Solution

One mg of a substance dissolved in 1 liter of a solution give 1 ppm solution. This is a way of expressing very dilute concentrations of substances. As per cent means out of a hundred, parts per million or ppm means out of a million.

The quantity of substances necessary to prepare 10 ppm solution is same for substances differing in molecular weight

Example: GA: mol wt. = 342

10 mg of GA in a litre - 10 ppm

IAA : mol wt. = 175

10 mg of IAA in a litre - 10 ppm

Though the molecular weight is different, the amount necessary to prepare 10 ppm is 10 mg per litre.

Examples

1. Preparation of 50 ml of 0.2M of sodium di-hydrogen phosphate

Molecular Weight (MW) of NaH_2PO_4 is 156.

Solute required (g) = Molecular weight × Molarity required × Volume required in litre

= 156 × 0.2 × 0.05

= 1.56 g

Weigh 1.56 g of NaH_2PO_4 and dissolve in some amount of water and make up the volume to 50 ml.

2. Preparation of 150 ml of 0.1N NaOH (MW of NaOH = 40)

Solute required (g) = Equivalent weight × Normality required × Volume required in litre

= 40 × 0.1 × 0.15

= 0.6 g

3. Preparation of 50 ml of 1% sulphanilamide in 1.5N HCl

(MW of HCl = 36.46; Specific gravity = 1.18; Minimum Assay or purity = 35.4 %)

Equivalent weight of HCl = 36.46 / 1 = 36.46

Solute required (g) = Equivalent weight × Normality required × Volume required in litre.

= 36.46 × 1.5 × 0.05

= 2.735 g

HCl is in the form of liquid, hence, it is necessary to convert weight into volume basis using purity and specific gravity

$$\text{Weight into Volume} = \frac{\text{Solute required (g)}}{\text{Specific gravity}} \times \frac{100}{\text{Minimum Assay}}$$

$$\text{Weight into Volume} = \frac{2.74}{1.18} \times \frac{100}{35.4} = 6.5 \text{ ml}$$

Take 6.5 ml of HCl from the container and add 0.5 g of sulphanilamide and make up the volume to 50 ml.

Questions

1. How to prepare the following solutions

i. Per cent (%) solution

ii. Normal (N) solution

iii. Parts per million (ppm) Solution

2. How to prepare 50 ml of 0.2M of sodium di-hydrogen phosphate

Lecture 10	Hemocytometer – Assessment of spore load – colony forming units

Hemocytometer

A hemocytometer is a device used to count number of cells/spores present in a given sample solution. Invented by Louis-Charles Malassez, a hemocytometer consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. Each chamber is engraved with a laser-etched grid of perpendicular lines. The area bounded by the lines and depth of the chamber is known. It is therefore possible to count the number of cells in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

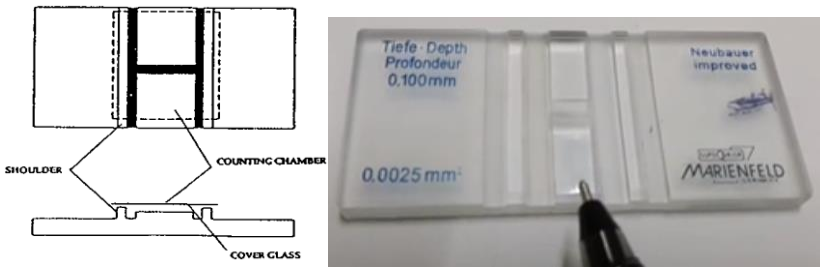


Fig. 1 A sketch of hemocytometer representing various parts

There are several kinds of hemocytometers. The size of counting chambers can vary with model and manufacturer. In general, a typical chamber depth is 0.1mm. A cover glass closes up the top of the cavity, determining a specific chamber volume.

Procedure to count spores using hemocytometer:

1. Carefully clean hemocytometer and cover glass with 70% ethanol to avoid contamination and counting errors
2. Dry it with sterilized tissue paper
3. Moisten the shoulders of the hemocytometer and fix the cover-slip using gentle pressure. Make sure that the cover-slip is properly positioned on the surface of the counting chamber. When the two glass surfaces are in proper contact, Newton's rings can be observed.
4. Now, the conidial suspension (filtered through one or more layers of muslin cloth depending on the size and morphology of conidia) can be applied to the edge of cover-slip and be sucked into the void by capillary action which completely fills the chamber with the sample.

5. Make sure the suspension is well mixed either by gentle agitation of the flask containing the suspension. The chamber should neither be overfilled nor under-filled.
6. Place hemocytometer under microscope under 10X objective to facilitate localization of the grid.

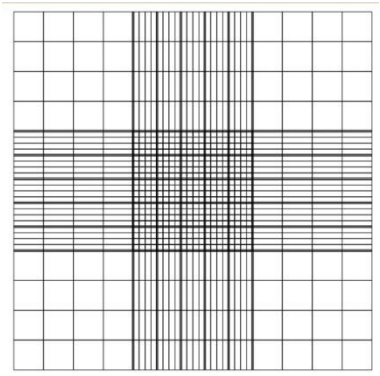


Fig. 2 counting chamber of Hemocytometer

7. Allow 2 minutes for the spores to settle in the chamber before counting.
8. Now focus on large square consisting of 16 small squares at each corner.
9. Count the number of spores on each large square. Count the spores within each small square and any positioned on right hand or bottom boundary line while skip the ones on left border and upper boundary lines.
10. Follow this process on each of the 4 large corner squares.
11. Each large square in the chamber has space to accommodate 0.1µl of suspension. Using unitary method we can thus calculate average number of spores per ml of the suspension.

Determining spore count:

1. Count the number of spores on each large square at the corners.
2. Calculate total number of spores.
3. Calculate spore count as follows:

   Spores per ml of suspension = Average spore count per large square x 10^4/ 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

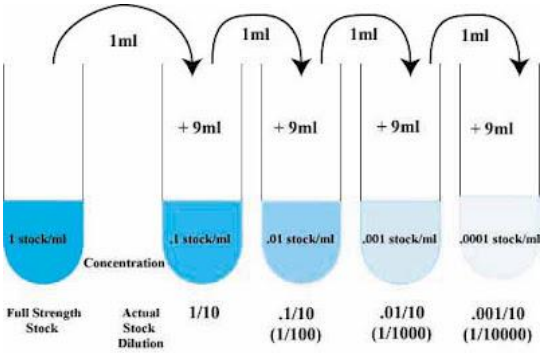
Although counting of cells or spores by this method are rapid but disadvantages lie in counting both dead and living cells. More over this method is not sensitive to populations of fewer than one million cells or spores.

A **colony-forming unit** (**Cfu**) is a unit used to estimate the number of viable bacteria or fungal cells in a sample. Viable is defined as the ability to multiply under the controlled conditions. Counting with colony-forming units requires culturing the microbes and counts only viable cells, in contrast with microscopic examination which counts all cells, living or dead. The visual appearance of a colony in a cell culture requires significant growth, and when counting colonies it is uncertain if the colony arose from one cell or a group of cells. Expressing results as colony-forming units reflects this uncertainty.

For moulds, a colony is a group of hyphae (filaments) of the same mould growing together. Colony forming units are used as a measure of the number of microorganisms present in or on surface of a sample. Colony forming units may be reported as CFU per unit weight, CFU per unit area, or CFU per unit volume depending on the type of sample tested. To determine the number of colony forming units, a sample is prepared and spread or poured uniformly on a surface of an agar plate and then incubated at some suitable temperature for a number of days. The colonies that form are counted. CFU is not a measure for individual cells or spores as a colony may be formed from a single or a mass of cells or spores.

Example:

Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor The number of colonies per ml reported should reflect the precision of the method and should not include more than two significant figures.



Serial Dilution of Bacterial Culture

The CFU/ml can be calculated using the formula:

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

For example, suppose the plate of the 10^6 dilution yielded a count of 130 colonies. Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:

Bacteria/ml = (130) x (10^6) = 1.3×10^8 or 130,000,000.

CFU/mL Practice Problems - CFU/mL Calculation Examples

Problem 1:

Five ml of Bacterial Culture is added to 45 ml of sterile diluent. From this suspension, two serial, 1/100 dilutions are made, and 0.1 ml is plated onto Plate Count Agar from the last dilution. After incubation, 137 colonies are counted on the plate. Calculate CFU/mL of the original Sample?

Answer:

First thing we need to know is the Dilution Factor, or how much the original sample is diluted:

Here Initially 5mL in 45mL = Final Volume / Sample volume = 50/5 = 10.

Then two serial dilutions of 1/100.

Total Dilution Factor = 10 * 100 *100 = $\mathbf{10^5}$

CFU/mL = cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

= (137 * $\mathbf{10^5}$)/0.1

=$1.37*10^8$

So Total colony forming units = **$1.37*10^8$ CFU/mL**

Questions

1. Write the procedure to count spores using haemocytometer
2. Clonoy forming Unit (CFU)

Lecture 11	**Preparation of different culture media – Maintenance of pure culture - Mother culture – Sub culturing**

Culture media

Growth medium or **culture medium** is a solid, liquid or semi-solid designed to support the growth of microorganisms or cells.

Types of Culture media

- Cultural media
- Minimal media
- Selective media
- Differential media
- Transport media
- Indicator media

Culture media

Culture media contain all the elements that most bacteria need for growth and are not selective, so they are used for the general cultivation and maintenance of bacteria kept in laboratory culture collections.

An **undefined medium** (**also known as a basal or complex medium**) contains:

- a carbon source such as glucose
- water
- various salts
- a source of amino acids and nitrogen (e.g., beef, yeast extract)

A **defined medium** (**also known as chemically defined medium or synthetic medium**) is a medium in which

- all the chemicals used are known
- no yeast, animal, or plant tissue is present

Minimal media

Minimal media are those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids, and are often used by microbiologists and geneticists to grow "wild-type" microorganisms. Minimal media can also be used to select for or against recombinants or exconjugants.

Minimal medium typically contains:

- a carbon source, which may be a sugar such as glucose, or a less energy-rich source such as succinate
- various salts, which may vary among bacteria species and growing conditions; these generally provide essential elements such as magnesium, nitrogen, phosphorus, and sulfurto allow the bacteria to synthesize protein and nucleic acids
- water

Supplementary minimal media are minimal media that also contains a single selected agent, usually an amino acid or a sugar. This supplementation allows for the culturing of specific lines of auxotrophic recombinants.

Selective media

Selective media are used for the growth of only selected microorganisms. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium to prevent other cells, which do not possess the resistance, from growing. Selective growth media are also used in cell culture to ensure the survival or proliferation of cells with certain properties, such as antibiotic resistance or the ability to synthesize a certain metabolite.

Examples of selective media include:

- YM (yeast extract, malt extract agar) has a low pH, deterring bacterial growth.

Differential media

Differential or indicator media distinguish one microorganism type from another growing on the same medium. This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosin y, or methylene blue) added to the medium to visibly indicate the

defining characteristics of a microorganism. These media are used for the detection of microorganisms and by molecular biologists to detect recombinant strains of bacteria.

Examples of differential media include:

- Eosin methylene blue is differential for lactose fermentation.
- Mannitol salt agar is differential for mannitol fermentation.

Transport media

Transport media should fulfill these criteria:

- Temporary storage of specimens being transported to the laboratory for cultivation
- Maintain the viability of all organisms in the specimen without altering their concentration
- Contain only buffers and salt
- Lack of carbon, nitrogen, and organic growth factors so as to prevent microbial multiplication
- Transport media used in the isolation of anaerobes must be free of molecular oxygen.

Examples of transport media include:

- Thioglycolate broth is for strict anaerobes.
- Stuart transport medium is a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralize.

Enriched Media

Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones. They are commonly used to harvest as many different types of microbes as are present in the specimen. Blood agar is an enriched medium in which nutritionally rich whole blood supplements the basic nutrients.

Maintenance of pure culture

The following points highlight the top four methods used for maintenance and preservation of pure cultures. The methods are: 1. Refrigeration 2. Paraffin Method 3. Cryopreservation 4. Lyophilisation

1. Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for

fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

2. Paraffin Method

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium. This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

3. Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at-196°C) helps survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol, that prevent the formation of ice crystals and promote cell survival.

4. Lyophilisation (Freeze-Drying)

In this method, the culture is rapidly frozen at a very low temperature (-70°C) and then dehydrated by vacuum. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators. Freeze- drying method is the most frequently used technique by culture collection centres.

Mother culture

Subculture is a new cell or microbiological culture made by transferring some or all cells from a previous culture to fresh growth medium. This action is called sub culturing or passaging the cells. Subculture is used to prolong the life and/or expand the number of cells or microorganisms in the culture.

Cell lines and microorganisms cannot be held in culture indefinitely due to the gradual rise in toxic metabolites, use of nutrients and increase in cell number due to growth. Subculture is therefore used to produce a new culture with a lower density of cells than the originating culture, fresh nutrients and no toxic metabolites allowing continued growth of the cells

without risk of cell death. Subculture is important for both proliferating (e.g. a microorganism like *E. coli*) and non-proliferating (e.g. terminally differentiated white blood cells) cells. Typically, subculture is from a culture of a certain volume into fresh growth medium of equal volume, this allows long-term maintenance of the cell line. Subculture into a larger volume of growth medium is used when wanting to increase the number of cells for, for example, use in an industrial process or scientific experiment.

Questions

1. Define cultural media
2. Define selective media
3. Different methods used for maintenance of pure culture
4. Types of sub culturing

Lecture 12	Mass culturing Techniques – Solid and liquid formulations – Shelf life

Efficacy of the biocontrol agent largely depends on the formulation of that particular bioagent. Most of the biocontrol agents are formulated in solid form (contain only conidia) and in liquid form with shelf life of 6 months at 5°C and 3 months at 25-35°C.

Mass culturing of biocontrol fungi, *Pochonia chlamydosporia*

Bagasse-soil-molasses (BSM) was used to prepare mass culture of *P. chlamydosporia*. The bagasse, soil and molasses were mixed in the ratio of 4:1:2 (5% solution in water) and filled in conical flasks of 500 ml capacity. The flasks were sealed with cotton plugs and butter paper and autoclaved two times at 15 kg/cm^ pressure at 121°C for 15-20 minutes. The flasks were inoculated with *P. chlamydosporia* separately and incubated in a BOD incubator at 27±2°C for 8-10 days.

Large-scale production

BSM medium was filled in heat resistant plastic bags and autoclaved twice at 15 kg/cm^ pressure at 121 °C for 15-20 minutes. The medium in polybags was inoculated with the colonized medium from conical flasks. After inoculation, the bags were sealed and kept in an incubator at 27±2°C for 8-10 days and shaken periodically. During this period the BSM mixture was fully colonized by the antagonistic fungi and ready for field application. Before application, colony forming unit (CFU) count of the bio control agents in the BSM was determined using dilution plate method.

Production of mass/stock culture of bioagents, *P. chlamydosporia*

Various agricultural and waste materials *viz*., seed husk-soil-molasses, saw dust-soil molasses, bagasse-soil-molasses, leaf litter-molasses, sorghum meal-molasses and sorghum seeds were tested for mass production of bio control fungi. Based on relative performance of the materials tested, a mixture of saw dust-soil 5% molasses (15:5:1) was selected to develop mass (stock) culture of bioagents.

One kg of the mixture was filled in heat resistant polybags, sealed and steam sterilized at 121 °C for 15 minutes. For *P. chlamydosporia*, chloramphenicol 10 mg/kg material was added to the 1 kg material. Thereafter, the bags containing 1 kg autoclaved saw dust soil-molasses mixture were inoculated with homogenized pure culture of the biocontrol agents (5 ml/bag) by sterilized needle and syringe. The puncture made in the

polybag to insert the needle was resealed with cello tape. A pure culture of *P. chlamydosporia* was prepared in potato dextrose broth supplemented with 10 mg chloramphenicol/litre. The bags were resealed and incubated at room temperature (25-30°C) or at 25±2°C in an incubator for 10-15 days (fungi). During incubation the bags were shaken daily for a few minutes to achieve uniform colonization by the biocontrol agents on the material. Luxuriant and uniform colonization by the microorganisms occurred within the incubation duration of 5- 15 days.

Talc based formulation

In India, talc based formulations of *Pochonia* and *Pupureocilium* was developed at Tamil Nadu Agricultural University, Coimbatore for seed treatment of pulse crops and rice (Jeyarajan *et al*., 1994). *Pochonia* and *Pupureocilium* was grown in the liquid medium is mixed with talc powder in the ratio of 1:2 and dried to 8% moisture under shade. The talc formulations of *Pochonia* and *Pupureocilium* has shelf life of 3 to 4 months. It has become quite popular in India for management of several soil-borne diseases of various crops through seed treatment at 5 g/kg seed.

Vermiculite-wheat bran based formulation

Trichoderma is multiplied in molasses-yeast medium for 10 days. 100 g vermiculite and 33 g wheat bran are sterilized in an oven at 70°C for 3 days. Then, 20 g of fermentor biomass, 0.05 N medium and concentrated or entire biomass with HCl are added, mixed well and dried in shade (Lewis, 1991). Pesta granules based formulation 52 ml Fermentor biomass is added to wheat flour (100 g) and mixed by gloved hands to form cohesive dough. The dough is kneaded, pressed and folded by hand several times. Then one mm thick sheets (pesta) is prepared and air-dried till it breaks crisply. After drying, dough sheet was ground and passed through an eighteen mesh and granules were collected (Connick et al., 1991).

Alginate prills based formulation

Sodium alginate is dissolved in one portion and distilled water (25 g/750 ml) and food base is suspended in another portion (50 g/250 ml). These preparations are autoclaved and when cool are blended together with biomass. The mixture is added drop wise into $CaCl_2$ solution to form spherical beads, which are air-dried and stored at 5°C (Fravel et al., 1985).

Press mud based formulation

Press mud is available as a byproduct of the sugar factory and this can be used as a substrate for mass multiplication of *Trichoderma*. The method involved uniformly mixing of 9 days old culture of *T. viride* prepared in potato dextrose broth into 120 kg press mud. Water was sprinkled intermittently in to keep it moisten. This was covered by gunny bags to permit air movement and trap moisture under shade. Within 25 days, nucleus culture for further multiplication becomes ready. The same was added to 8 tons of press mud, mixed thoroughly and incubated for 8 days under shade condition before being applied in the field. By this we added 8000 times more inoculums in the soil than the recommended doses of biopesticides which rapidly get established showing rapid and visible effect.

Oil-based formulations

It was prepared by mixing the conidia harvested from the solid state/liquid state fermentation with a combination of vegetable/mineral oils in stable emulsion formulation. In such formulations, microbial agents are suspended in a water immiscible solvent such as a petroleum fraction (diesel, mineral oils), and vegetable oils (groundnut etc.) with the aid of a surface active agent. This can be dispersed in water to form a stable emulsion. Emulsifiable concentrates require a high concentration of an oil soluble emulsifying agent, to give an instantaneous formation of a homogenous emulsion on dilution in water. The oils used should not have toxicity to the fungal spores, plants, humans and animals. Such formulation of Trichoderma is now being used as foliar sprays. Oil-based formulations are supposed to be suitable for foliar sprays under dry weather and to have prolonged shelf life.

The spores can survive for longer time in the plant surface even during the dry weather as the spores are covered by oil that protects them 5°C from drying. Batta (2005) developed an emulsion formulation of *T. harzianum* for the control of post-harvest decay of apple caused by *Botrytis cinerea*.

Banana waste based formulations

The mass multiplication protocol of *Trichoderma* sp. in banana waste was proposed by Balasubramanian *et al*. (2008). For the same banana waste, urea, rock phosphate, culture of *Bacillus polymixa*, *P. sajor caju* and *T. viride* area used. A pit of different banana waste *viz.*, sheath pseudo stem and core is chopped in the length of 5 to 8 cm. A pit is prepared and different ingredients are placed in five different layers. Each layer contains one ton

banana waste, 5 kg urea, 125 kg rock phosphate and one litre broth culture of *B. polymixa, P. sajor caju* and *T. viride*. Five different layers are prepared similarly and mixed thoroughly Banana. Banana waste is decomposed within 45 days and enriched culture is mass available for field application.

Liquid bioformulation

Steps involved in preparationof liquid bioformulation of fungal biocontrol agents

1. Preparation of culture media

Fungal biocontrol agents can be mass multiplied in Potato dextrose broth medium. Preparation of Potato Dextrose Broth (PDB)

Materials required

Readymade PDB (HIMEDIA): 24.0 g, Distilled water: 1000ml, Beaker, Measuring cylinder, conical flasks, Non- absorbent cotton, Brown paper, Rubber band etc

Procedure

- Suspend 24.0 grams of PDB media in 1000 ml distilled water
- Heat to boiling to dissolve the medium completely
- Dispense about 250ml of medium into each of the four 500 ml conical flasks
- Plug the mouth of the flasks with non-absorbent cotton
- Sterilize the medium by autoclaving at 15 lbs pressure (121 ^{o}C) for 15 minutes and mix well before dispensing.

2. Mass multiplication of bio control agents

Materials required

Pure culture of antagonistic organism, Suitable liquid medium, Inoculating needles, Alcohol, Spirit lamp, Non-absorbent cotton, Brown paper, Thread etc.

Procedure

- Inoculate the PDB with mycelial disc of 5 mm size cut out from 3-4 days old culture of the fungal antagonist
- Concentration of the antagonist should be 1×10^{8} cfu/ml of water
- Incubate the inoculated flasks at 28±1^{o}C for 7 days
- Count the spore concentration and colony forming unit (cfu) per ml of water after 7 and 14 days of inoculation.

- After that the liquid bioformulation need to be applied in field by appropriate method of application.

Advantages of liquid bioformulation

- Stabilize the organism during production, distribution and storage.
- Easy handling and application of the product so that it is easily delivered to the target in the most appropriate manner and form.
- Protect the agent from harmful environmental factor at the target site, thereby increasing persistence.
- Enhance the activity of the organism at the site of application by increasing its activity, reproduction, contact and interaction with the target pest or disease organism.

Shelf life of bioformulations

Shelf life of the formulated product of a biocontrol agent plays a significant role in successful commercialization. In general, the antagonists multiplied in an organic food base have longer shelf life than the inert or inorganic food bases.

Questions

1. Write the methodology for mass culturing of biocontrol fungi, *Pochonia chlamydosporia*
2. What are all the steps involved in preparation of liquid bioformulation of fungal biocontrol agents

Lecture 13	Commercial formulations – Carrier materials – Packing – Quality control and shelf life

Commercial formulations

The various solid substrates used for the mass multiplication of fungal bio control agents are given below:

1. Diatomaceous earth granules

Diatomaceous earth granules are added into a broth consisting of 100ml black strap molasses (pH 5.0), 900ml of water, 3g each of KNO_3 and KH_2PO_4 till the level of saturation. It is autoclaved at 121°C for 15min and spread in shallow pans to a height of 3 – 5cm 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°C 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.

2. Wheat bran: saw dust formulation

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

3. Wheat bran: peat formulations

Wheat bran: peat mixture (1:1 v/v) is autoclaved for 1 hr. Substrate moisture is adjusted to 50 % (w/w) with sterile water, medium was inoculated with 0.1 ml of a conidial suspensions containing $2x10^4$ conidia/ml and incubated for 7 days at 30°C and used at 10% (v/v) level.

4. Vermiculite – wheat bran formulation

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

5. Alginate pellets

Sodium alginate (20g) is dissolved in 750ml water at 40°C on a stirring hot plate. Wheat bran ground to pass through a 0.425mm mesh screen was placed in a glass blender container with distilled water (50g/ 250ml). Kaolin is also used in place of bran at the same rate. It is autoclaved for 30min and cooled. Depending upon the extent of fungal growth, wet fermentor biomass (FB) is added at 16-21 g and dried at 2.8-4.2g/lit. The fungal biomass containing mycelium and spores are added to provide $7X10^6$ propagules /lit. The mixture containing fungus, alginate and bran or kaolin is added drop wise into 500ml gallant solution (0.25 M Cacl2, pH 5.4). As it enters, each droplet gelled and a distinct spherical bead is formed. After 20 min in the gallant, beads are separated from the solution by gentle filtration, washed and dried for 24h in a stream of air at 25°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 popular trend of formulating bio pesticides is mainly in solid carriers like talc, peat, lignite, clay, etc. However, these solid formulations suffer from major setbacks like shorter shelf life, high contamination and low field performance (Hedge, 2002). The carrier should be carefully chosen in such a way to enhance the inherent potential of the formulated organism. Liquid formulations offer longer shelf life, with high purity, carrier-free activity, easiness in handling and application, convenience in storage and transport, better quality parameters and enhanced export potential (Pindi and Satyanarayana, 2012).

The carrier material and other constituents used in a bioformulation should be non-inhibitory to the infective propagule i.e., conidia, inert on the target crop plant, at the same time should maintain viability during storage. Groundnut oil was proved as a better carrier for Verticillium lecanii compared to sunflower oil, as germination in the former was found to be excellent (Verhaaret al., 1999).

Carrier materials

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, sugar 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.

Packing

The inoculum suspension is mixed with the carrier material and it should be inert and not 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. Moisture content of the product should not be more than 20% in the final product. The pH of the talc before mixing should be 7.0 (This can be achieved by addition of calcium carbonate @ 150g / kg of talc). The resulting fine powder formulation is immediately packed in white polythene/ polypropylene bags in required quantities and sealed carefully. The cell or spore count of the sample of the formulation should be at least 108/ 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.

1. Name of the biocontrol agent
2. Strain/ Species
3. Spore or Cell count
4. Net weight
5. Date of manufacture
6. Date of expiry
7. Batch number
8. Address of the manufacturer
9. Other details about the method of inoculation, dosage, crops etc., may also be printed.

Quality control

Quality control is the most essential aspect of bio pesticide production. A good quality of the preparation is necessarily required to retain the confidence of fanners on the efficacy of biocontrol formulations. Being living agents their population in a product may be influenced by storage. The other contaminating microorganisms in the product should also be within permissible limits. 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 mentioned below.

1. Assessment of population (cfu) in the product

Take one gram of the product and make it up to 10ml with sterile distilled water and shake well (1:10). Take one ml from 1:10 dilution and transfer 9ml 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 petriplate and add 15ml of molten and cooled medium, which is selective for the concerned antagonist in the same petri plate. Rotate the petriplate 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 gram of the product. The product should contain the required minimum population.

2. Dual culture bioassay method

Mix 15 mg of the product with 15ml of molten and cooled agar medium and pour in a sterilized petri plate. After solidification, transfer one 8mm 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 15ml 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 @ 2IJ/g of soil. After a week, sow the antagonist treated seeds in the nematode inoculated 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 no. of galls and gall index.

5. Field testing of bio control agents

Apply the bio control agents by any of the method suitable (seed, soil, root, foliar etc.) and record the gall index 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.

Shelf life

The biggest obstruction in commercialization of a biocontrol agents preparation is its loss of viability of the biocontrol agents over time. Considerable efforts have been made in India itself to determine the viability of biocontrol agents in their preparations when stored at room temperature and in refrigerator. Most of the results are variable and therefore it appears that shelf life is also dependent upon species/ isolate/strain. In general the antagonist multiplied in an organic food base has greater shelf life than that on an inert or inorganic food base (Jeyarajan and Nakkeeran, 1996). Talc based fomiulations ofT. harzianum can retained more than 10^ viable propagules per g upto 90 days (Prasad and Rangeshwaran, 2000). A talc based preparation of T. virens conidia retained 82% viability at 5°C in refrigerator after 6 months, while at room temperature (25-35°C) same level of viability was observed only up to 3 months. Lewis et al. (1995) reported that among the different carriers tested; the shelf life of 5. subtilis in soybean flour was increased upto three months. Storage at 5°C increased the shelf life of *T.virens* and *T. hamatum* in granular formulations of pre gelatinizing starch flour upto 6 months. Chlamydospores based formulations of r virens and T. harzianum exhibited longer shelf life (80% viability for 7 months) than conidia based formulations (80% viability after 4 months) at room temperature (Mishrae^a/., 2001). Jeyarajane/a/. (1994) developed talc, peat, lignite and kaolin based fomiulations oiT. viride, which had a shelf-life of 4 months. Ranganathan et al. (1995) also reported 4 month shelf Hfe of T viride in gypsum based formulations. Studies on storage temperature revealed that 20-30°C was optimum to store vemiiculite fermentor biomass of *Trichoderma* upto 75 days without losing the viability (Nakkeeran *et al*., 1997).

Questions

1. Explain about various solid substrates used for the mass multiplication of fungal bio control agents

2. Explain about different quality control test for evaluating bio formulations

Lecture 14	Market demand analysis – Economics of Bio nematicides

Nematicides are class of pesticides that inhibit or kill plant parasitic nematodes. Rise in global demand for food has increased the pressure on farmers to enhance their crop yield. Hence, the farmers are increasingly adopting nematicides to prevent loss of crops from parasitic attack. The commonly used nematicides include fumigants, organophosphates and carbamates.

Technavio's analysts forecast the global nematicide market to grow at a CAGR of 3.48 % over the period of 2014 – 2019. Harmful effects are caused by nematodes to roots of crops and other plants thereby hampering their growth. This may lead to farmers bearing financial losses, this requiring implementation of particular measures to reverse this scenario. Such a dire consequence has led towards increasing implementation of nematicides to get rid of the nematode worms, thus driving the relevant market. In the last few years, rapid industrialization and urbanization has lessened the availability of arable and fertile lands, which has caused nematodes to thrive in such conditions. This had caused a rapid uptake of nematicides by farmers and agriculturist all over the globe, thus boosting the market. With an ever growing population, a rapid surge in demand of quality foodstuff has been noted. This has caused farmers to use fertilizers, nematicides, pesticides and other such substances to bring about proper growth, thus improving the chances of receiving a good yield.

Market Landscape

- Market is expected to grow at a steady pace during the forecast period owing to an increase in food requirements.
- Large quantities of nematicides are being used to inhibit or kill insects and enhance crop yield and profitability owing to the decrease in the availability of cultivable land to grow crops because of urbanisation, sanitation and erosions.
- Stringent regulations imposed on insecticides to lesson toxicity are hindering market growth.

Market Analysis

1. Market analysis by product type
2. Market analysis by application
3. Market analysis by geography

Key buying criteria

Parameters	Details
Availability	It should be readily available
Cost	It should be affordable prices
Ease of use	Easy to use
Eco - friendliness	Should have minimal negative impact on environment

Economics of nematode biocontrol agent *P. lilacinum*

Fixed cost

BUILDING COST:

- Construction area needed = 1200 sq.ft
- Closed shed area needed = 1300.ft.
- Total area needed = 2500 sq.ft.
- Construction cost = Rs. 1000 x 1200 sq.ft.
  = Rs. 12, 00,000
- Open land cost = Rs. 300 x 400 sq.ft
  = Rs. 1, 20,000
- Closed shed construction cost = Rs. 1000 x 1300 sq.ft.
  = Rs. 13, 00,000
- Total cost of building = Rs. 26,20,000
- Life period = 50 years
- **Total Building cost** = Cost of building/Life period
  = Rs.26, 20,000/50
  = Rs. 52,400/year

Equipment Cost:

S.No	Particulars	Quantity	Capacity	Price (in Rs.)
1.	Autoclave	1	500 L vetical	36,000
2.	Fermentor	3	300 L	11,94,400
3.	Laminar air flow chamber	2	Vertical laminar	1,44,000
4.	Pan balance	1	10 kg	4000
5.	Pan balance	1	120 kg	48,000
6.	pH and EC meter	1		25,000
7.	Water distillation unit	1	20 liters/hr	29,000
8.	Air connditioners	3	1.5 tonnes	35,000
9.	Trinocular microscope	1	4x, 10x, 40x and 100x	40,000
10.	Hot air oven	1	50^0-120^0C	15,000
11.	Rotary shaker	3		9,000
12.	Deep freezer and refrigerator	1		14,000
13.	Glass wares			35,000
14.	Colony counter			5,000
15.	Office furniture			20,000
16.	Centrifuge	1		17,400
17.	Tube lights and fans	10+5		20,000
	TOTAL			15,61,230

- Life period = 10 years
- Total = Equipment cost / Life period
  = 15, 61,230 / 10
  = Rs. 1, 56,123
- **Total Equipment cost** = Rs. 1,56,123/year

- **Total fixed cost** = Building cost + Equipment cost
  = 52,400+1, 56,123
  = Rs. 2, 08,523

Variable cost:

a) Electricity charges:

The plant will require power of 160 kwh per day and the total power cost around one year is Rs.5 lakhs.

b) Package charges:

Biocontrol agent canes:

No. of canes (per cane – Rs.25) : 1 liter cane – 50,000

Cost of canes : Rs. 12, 50,000

Cost of stickers : Rs. 3, 00,000

Total cost for package : Rs. 15, 50,000

c) Cost for labours:

Scientist	Rs.3,00,000
Marketing officer	Rs.1,50,000
Sales manager	Rs.2,00,000
Skilled labour	Rs.1,48,000
Daily wages labour	Rs.8,00,000
Total cost for labour	**15,98,000**

d) Chemical cost **: Rs.2,26,153**

Total variable cost = Electricity charges + Chemical cost + Package charges + Labour cost

= Rs.38, 74,153

TOTAL VARIABLE COST = Rs. 38, 74,153

TOTAL EXPENTITURE **= Total fixed cost + Total variable cost**

= Rs.2, 08,523 + Rs.38, 74,153

= Rs.40, 82,676

GROSS INCOME:

Bio control agent	Cost/kg(Rs.)	Quantity produced	Total (Rs.)
P. lilacinum	250	20000 kg	50,00,000

NET INCOME

Net income = Gross income + Total expenditure

= 50, 00,000-40, 82,676

= Rs.9, 17,324

NET INCOME = Rs.9, 17,324

BENEFIT COST RATIO

Benefit cost ratio = Gross income / Total expenditure

= 50, 00,000 / 4082676

= 1.22 per rupee invested

NET PROFITABLE RATIO

Net profitable ratio = Net income / Net sale

= 9, 17,324/50, 00,000

Net profitable ratio = 0.18

Questions

1. Explain about market demand analysis for bio control agents
2. Write the economics for nematode antagonistic production

Lecture 15	Establishment of pilot plant – Infrastructure requirement – cost analysis

MARKETING & COST BENEFIT ANALYSIS OF COMMERCIAL BIOCONTROL AGENTS

ROOT KNOT NEMATODE IN TOMATO

In tomato crop, Root knot nematode is caused by *Meloidogyne incognita.*

Yield of Tomato fruit in acre = 16000kg/acre

Yield losses in tomato due to root knot nematode = 40%

Therefore, Yield of tomato is reduced = 16000*40/100

= 6400kg/acre

Cost of 1 kg tomato = Rs.25

6400 Kg of tomato = 6400*25

= Rs.1, 60,000

MANAGEMENT

1. Seed treatment

Pseudomonas fluroscens @ 10g/kg seed

2. Nursery bed treatment

Pupureocilium lilacinum @ 50 g/m^2

3. Main field application

Pupureocilium lilacinum @ 2 kg along with 1 tons of FYM/acre in main field

COST OF BIOCONTROL AGENTS (1 kg)

1. *Pseudomonas fluroscens = Rs*. 120

2. Pupureocilium lilacinum = Rs. 250

Seed rate of tomato = 150g/acre

Nursery area for raising one acre tomato field = 60sq.m

MATERIAL AND APPLICATION COST OF BIOCONTROL AGENTS FOR ONE ACRE

1. Material cost

i) Seed treatment

Pseudomonas fluroscens = 1.5g/150g of seed

Cost = Rs.15

ii) Nursery bed treatment

Paecilomyces lilacinus = 3kg/60 sq.m

Cost = Rs.750

iii) Main field application

Paecilomyces lilacinus = 2kg/acre

Cost = Rs.500

1 ton of FYM Cost = Rs.500

Total material cost = Rs.1765

APPLICATION COST (LABOUR COST)

1. Seed treatment (1) = Rs.150
2. Nursery bed treatment (2+1) = Rs.800
3. Main field application (10) = Rs.3000

Total application cost = Rs. 3950

Total cost for application of nematode bio control agent = Rs.5715

BENEFIT COST RATIO

BCR = Benefit/cost

= 1, 60,000/5715

BCR = 27.99

INFERENCE

- Due to root knot nematode infection in tomato field, Rs.1, 60,000 amount is lost.
- To meet out the amount, we apply bio control agents to control root knot nematode.
- Total cost for application of nematode bio control agent is Rs.5715.
- Benefit Cost Ratio is 27.99

MARKETING OF COMMERCIAL BIOCONTROL AGENTS

- The practical aspects of marketing such a live, perishable product are reviewed.
- The normal demands of the marketplace for an effective, reliable, user-friendly and readily available product apply.
- Marketing strategies such as advertising, packaging, and labelling are important, but they are often not sufficient to ensure customer acceptance and successful usage.

- Problems relating to the nature of the product point to the need for a team approach.
- The marketing team should consist of producers, researchers, sales people, distributors, advertisers, and extension agents familiar with, and committed to, the concept of biological pest management.

MARKETING OF PRODUCT

- Marketing of biological control agents is done either directly by the producer or through a distributor.
- The decision how to market a product depends on several issues, including the geographical area, the customer's location, the presence of a greenhouse supply company in the area, and customer relations.

MARKETING THROUGH A DISTRIBUTOR

- Marketing through a distributor has several advantages for a natural enemy producer.
- The most important is that supplying products through a distributor often broadens your customer base.

DISADVANTAGES

- The distributor may also sell chemical pesticides as well as biological pest control products.
- At times, selling both products may have a negative effect on the biological control agents.
- Distributors that are only interested in sales often have more failures with their biological control programs.
- The goal must be a successful biological control program, not just product sales.

MARKETING BY THE PRODUCER

- When a biological control producer decides to market their biological control products directly, a complete product line must be available to ensure that the biological pest control "system" is complete.
- The advantage of this marketing system is that the technical support staff is focused only on selling biological control products and providing technical support to the grower.

- A biological control system becomes more reliable if growers work with one producer or supplier and one technical support person.

Questions

1. What are all the basic requirements for establishment of bio pesticides unit

Lecture 16	Budget preparation – marketing and cost – benefit analysis – risk analysis

***Pseudomonas*:**

Initial investment cost = 50.00lakhs

S.No	Particulars	Amount (Rs. In lakhs)
A	**Capital investment (fixed cost)**	
1	Building including cost of site (app.1200 sq.ft)	12.00
2	Equipment and apparatus	40.55
B	**Operational cost (variable cost)**	
1	Working capital	10.00
2	Staff salary	2.04
3	Labour	2.5
4	Electricity	0.5
5	Travelling expenses	0.5
6	Administrative expenses	0.5
7	Interest on loan and depreciation	0.7
8	Miscellaneous expenses	0.26
	Total variable cost	**17.00**
	Total investment	**69.55**
	Actual initial investment	**50.00**

A) **Expenditure details** :

S.No	Equipment & apparatus	Quantity (Nos)	Amount (Rs in lakhs)
1	Fermentor (200 lit capacity)	4	26.00
2	Shaker	2	1.50
3	Laminar air flow chamber	1	0.60
4	Autoclave	2	0.30
5	Hot air oven	1	0.10
6	Incubator	1	0.10
7	Refrigerator	1	0.30
8	Microscope	1	0.75
9	pH meter	1	0.15
10	Physical balance	1	0.10
11	Electronic balance	1	0.75
12	Sealing machine	5	0.25
13	Work benches	4	0.30
14	Plastic trays	50	0.25
15	Trolley	1	0.10
16	Automatic packing machine	1	9.00
	Total cost		**40.55**

Working capital:

Particulars	Amount in lakhs
Cost of mother culture	0.05
Glass wares	0.70
Chemicals	2.50
Polythene bags	3.50
Carrier materials	3.00
Miscellaneous items	0.25
Total cost	**10.00**

Staff salary:

Technical staff (1)	9000*12 month	1,08,000
Laboratory staff (2)	4000*2*12month	96,000
	Total	**2,04,000**

Production:

Capacity	Production /year
60% capacity	90 MT
75% capacity	112.5 MT
90% capacity	135 MT
100% capacity	150 MT

Receipts:

Cost of 1kg of pseudomonas – Rs 100

Cost	Amount in lakhs
Cost of 90 MT (60% capacity)	90
Cost of 112.5 MT (75% capacity)	112.5
Cost of 135 MT (90% capacity)	135
Cost of 150MT (100%capacity)	150

Profitability:

Year	Production	Receipts (lakhs Rs)	Expenditure (lakhs Rs)	Gain (lakhs Rs)
1	60%	90	50.000	40
2	75%	112.5	18.700	93.8
3	90%	135	20.570	114.43
4	100%	150	22.630	127.37

Profit anticipated after 4 years = 375.6 lakhs

Every year 10% increase in the expenditure

Purpureocilium lilacinum

Receipt:

Cost of 1kg of *Purpureocilium* = Rs 250/kg

Cost	**Amount in lakhs**
Cost of 90 MT (60% capacity)	180
Cost of 112.5 MT (75% capacity)	225.0
Cost of 135 MT (90% capacity)	270.0
Cost of 150MT (100%capacity)	300.0

Profitability:

Year	**Production**	**Receipts (lakhs Rs)**	**Expenditure (lakhs Rs)**	**Gain (lakhs Rs)**
1	60%	180	50.000	130
2	75%	225	18.700	206.3
3	90%	270	20.570	249.43
4	100%	300	22.630	277.37

Market demand analysis

- In the tropical and sub-tropical climates, crop production losses attributable to nematodes were estimated at 14.6% compared with 8.8% in developed countries.
- Overall average annual loss of the world's major crops due to damage by plant parasitic nematodes was estimated to be 12.3% which is one third of the losses attributed to pests and diseases in general.
- For the 20 major life sustaining crops that serve as man's primary food source, annual yield loss of l0.7% was estimated.
- For another group of 20 crops mainly of commercial importance, a 14% annual yield loss was assessed.
- Developing countries suffer a crop loss of 14.6% compared to 8.8% in developed countries, when estimated losses for all the 40 crops were considered.
- Monetary losses due to nematodes on 21 crops were estimated at US $ 77 billion annually based on 1984 production figures and prices.

    - 11% in vegetables ($267 per year fruit)
    - 6% in field crops ($110 million/year)
    - 12% in fruits and nuts ($225 million/year) and
    - 10% loss in ornamentals ($60 million/year).

Conclusion

The important nematode that wants to be control in current situation is RKN. Root knot nematode is a major problem in current situation. That causes the severe yield losses in Tube rose, Guava, Tomato, Chillies etc., and also burrowing nematode causes a severe yield loss in Banana. so these nematode should be control for reduce the economic loss. And also many crops like Rice, Wheat, Citrus, Potato, Crucifers and Coconut are affected by different types of nematodes. Hence, nematode control is important to reduce the economic loss. Now a day's farmers are very well aware about the nematode biocontrol agents. So there is a huge demand for nematode bio control agents.

The total cultivable area in Tamilnadu is 4 lakhs ha. So if one farmer having 1 acre can buy one Kg of our product *P. lilacinum* means, the target can be easily achieved.

Questions

1. Write about the budget preparation for the production of *Pochonia chlamydosporia*

Lecture 17	Environmental impact test with vertebrate and invertebrate organisms

Land use is a major cause of human ecological change in an ecosystem. Changes in land use and intensity play a major role on the destruction of habitat and biodiversity decline. Destruction of habitat and decline in biodiversity affect the ecosystem heath and functions. Therefore easy detection mechanism that rapidly identifies changes in ecosystem conditions must be made.

The rationale of the existence of a bioindicator is the close relationship between the presence of these indicator organisms with biotic and abiotic parameters of an ecosystem. Soil organisms are essential for the functioning of natural and managed ecosystems and the productivity of land. They maintain soil conditions favorable to plant growth. Termites, in particular, are ecosystem engineers, altering soil composition and hydrology. Their associations with symbionts play a significant role in the digestion and decomposition of organic matter. Overall, tropical termites may consume up to half of the annual litter production. A diversity of taxa also feed on a wide range of mammalian dung.

HONEYBEES

Pollinators like honey bees may be exposed to nematode biocontrol agents for a prolonged period of time, either *via* contaminated food, stored and consumed by the bees in the hive, or by foraging on contaminated areas. To address this potential risk, a chronic toxicity study can be conducted in the laboratory by exposing young adult bees to treated food (sucrose solution) over a period of 10 days.

Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

INITIAL CONSIDERATIONS

Test chemicals can either be tested as active substances or as formulations.

The bees used in this test should be young worker bees (max. 2 days old) in order to start the test with bees of a similar age. A reference substance should be used to verify the sensitivity of the bees and the reliability of the test system.

Young bees (max. 2 days old) are exposed to 50 % (w/v) aqueous sucrose solution containing the biocontrol agent by continuous and *ad libitum* feeding over a period of 10 days. Mortality and behavioural abnormalities are observed and recorded daily during the 10 day test period. The chronic effects of the test chemical are evaluated by comparing the results of the test chemical treated group to those of the respective control group. The test is designed for the determination of the following endpoints:

 LC50 (median Lethal Concentration) and the LDD50 (median Lethal Dietary Dose) values after 10 days of exposure.

- NOEC (No Observed Effect Concentration) and NOEDD (No Observed Effect Dietary Dose).

In some cases (*e.g.* when a test chemical is expected to be of low toxicity or when a test chemical is poorly soluble) a limit test may be performed, in order to demonstrate that the NOEDD is greater than or equal to the limit dose tested, and the LDD50 is greater than the limit dose tested, if no effects are observed in the study.

VALIDITY OF THE TEST

For the test to be valid, the following criteria apply:

- The average mortality across replicates for the untreated control and solvent control groups is ≤ 15 % at the end of the test (10 days following start of exposure); when a solvent control is included, the average mortality across replicates for the solvent control should also be ≤ 15 %.
- The average mortality in the reference substance treated group is ≥ 50 % at the end of the test (10 days following start of exposure).

DESCRIPTION OF THE METHOD

Collection of the bees

Young bees (max. 2 days old) reared out from brood combs taken from queen-right colonies that have no symptoms of diseases and that have a known maintenance and physiological status history should be used for the test. No chemical substances (such as antibiotics, anti *varroa* treatments, *etc.*) should have been used in the hive for at least one month prior to the test. If one colony cannot provide the appropriate number of bees, comb(s) from several colonies may be used. In this case, it is ensured that the bees are equitably distributed across the treatments.

Brood frames with capped cells that are expected to hatch on the same day can either be incubated in a climatic chamber or be kept without nurse bees in a worker excluder box within the hive until hatch. In the first case sufficient food supply should be ensured either by honey and pollen which is on the same brood comb or by an additional comb containing food.

One day before the test starts, the bees can be collected from the combs and distributed into the test cages. Anesthetisation should be avoided during collection. Bees should be acclimated to test conditions for about one day (after a hatching period of one day). Bees are to be fed with sucrose solution *ad libitum* but no additional feeding of pollen or water is necessary during the acclimation and test period. No starvation period is necessary before test start.

Test cages

Easy to clean or disposable and passively ventilated cages are used. Any appropriate material can be used, *e.g.* stainless steel, cardboard, wire mesh, plastic, disposable wooden cages, *etc.* Groups of 10 bees per cage are used, since this number allows a precise assessment of affected *vs.* non affected bees. The size of the cages should provide adequate space for 10 bees (minimum 200 cm3).

Feeding Solutions

The feeding solutions for the control, test chemical and reference substance treatments are prepared with 50 % (w/v) aqueous sucrose solution. All feeding solutions have to be homogeneous without obvious signs of precipitation throughout one feeding interval (about 24 hours).

PROCEDURE

Test and control groups

The number of concentrations and replicates tested should meet the statistical requirements for the determination of NOEC/NOEDD values, the LC50/LDD50 (or LCx/LDDx where applicable) with 95 % confidence limits at the end of the test period.

Normally at least five test concentrations with a factor not exceeding 2.5 covering the range for the LC50 are required for the test (in specific cases with *e.g.* a flat dose-response relationship a larger spacing factor may be applicable). In case of unknown

toxicity, a range-finding test can be performed to derive appropriate concentrations in the final test.

In case of a dose-response test a minimum of three replicates (cages), each containing 10 bees should be used per treatment. Limit tests should be performed with five replicates (cages) for the control and the test chemical treatment groups, and at least 3 replicates for the reference substance group.

Exposure (feeding)

The feeding solutions are offered *ad libitum* to the honey bees via feeders (*e.g.* plastic syringes, minimum content of 2 mL; tip removed). The bees in one replicate share the feeding solution (trophallaxis) and thus can be expected to all be exposed. The feeding solution is replaced daily by changing the feeders. Each feeding interval is 24 h (± 2h). The amount of feeding solution(s) consumed is determined daily by initially weighing the feeders before and after feeding using a calibrated balance.

Evaporation

It is necessary to adjust for possible evaporation of test solutions from the feeders with additional test cages which are set up at the main test. These cages contain no bees, only pre-weighed feeders containing diet of untreated control and/or solvent control (each tested with min. 3 replicates).

These should be placed in the test environment alongside the test units. At the daily feeder exchange the feeders are re-weighed and replaced with new feeders. This evaporation figure can then be subtracted from the calculated food consumption to give the corrected food consumption accounting the loss by evaporation.

Test conditions

The bees should be kept in constant darkness (except during observation) under controlled climatic conditions at a target temperature of 33°C with maximum deviations of ± 2°C and a relative humidity of 50 – 70 %. Short-term deviations (≤ 2 hours per day) from the recommended test conditions are unavoidable and should not affect the integrity or outcome of the test.

Temperature and humidity should be recorded continuously with appropriate and calibrated equipment.

Duration

Bees are continuously exposed to the feeding solutions over a period of 10 days.

Observations

Mortality should be recorded daily at about the same time of the day (every 24 ± 2 hours), starting 24 ± 2 hours after start of the test period (initial feeding).

Additionally, behavioural abnormalities should be recorded daily at the same time as the assessments of mortality.

Behavioural abnormalities should be quantitatively observed according to the following categories:

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation; *e.g*. light or blowing; bees may recover but usually die),

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination; hyperactivity; aggressiveness; increased self-cleaning behaviour; rotations; shivering),

c = cramps (bees contracting abdomen or entire body),

ap = apathy (bees show only low or delayed reactions to stimulation *e.g.* light or puff of air; bees are sitting motionless in the unit).

v = vomiting

Any behavioural abnormalities which are not included in the list should be noted and clearly described.

After 10 days of exposure the final assessments of mortality and food consumption are done and thereafter the test is terminated by freezing the test cages including the bees at ≤ -10 °C (preferably lower) or by using other humane methods.

DATA AND REPORTING

Data

The data should be summarized in tabular form, showing the number of bees tested, mortality and number of bees with adverse behaviour assessed at each observation time. Data on mortality are analysed by appropriate statistical methods (*e.g.* regression analyses, moving-average interpolation, binominal probability) in order to calculate the LC50 (expressed in mg/kg) and LDD50 (and LCx if applicable(expressed in µg or ng/bee/day)

values with 95 % confidence limits and the NOEC/NOEDD at 245 OECD/OCD the end of the test. Correction for control mortality could be made using standard procedures (e.g. Abbott, [4]):

Data on food consumption should be calculated and displayed as:

- mean consumption of feeding solution per bee for each day (mg/bee); the number of living bees at the beginning of each feeding interval is taken for this calculation;
- overall mean daily consumption of feeding solution per treatment over the test period (mg/bee/day);
- overall mean daily consumption of feeding solution per replicate over the test period (mg/bee/day);
- mean uptake of test chemical per bee per day (µg or ng a.i./bee/day);
- Accumulated uptake of test chemical per bee over the test period (µg or ng a.i. /bee).

It is necessary to adjust for possible evaporation of test solutions from the feeders. In case the subtraction of the evaporation figure from the calculated food consumption leads to a negative value, the food consumption of the respective day will be considered to be “0” (no food consumption).

Test report

The test report includes the following information:

Test and reference substance

- Mono-constituent substance:

physical appearance, water solubility, and additional relevant physico-chemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

- Multi-constituent substance, UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) and mixtures:

characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents;

- source, lot number, expiration date for use;
- stability of the test chemical itself, if known;

- solubility and stability of the test chemical in water and solvent (if used);

Test system

- Details on the test species (scientific name, race, age, incubation and collection method, and information on the colonies used like health status, pre-treatment etc.);

Test conditions:

- Conditions during incubation, acclimatization (if applicable) and test period;
- Description of the test cages (type, material, size);
- Method and frequency of the preparation of the stock solution and the feeding solutions;
- Test design (number of treatment groups (control(s), test chemical, reference substance), number of replicates, number of bees per cage);
- Date of the start and the end of the test;

Results

- Mortality at each observation time for all treatments tested;
- Consumption of feeding solution at each observation time for all treatments tested;
- Nominal test concentrations used and measured concentrations of the test chemical in the feeding solutions, and analytical method used;
- Evaporation figures;
- LC50/LDD50, NOEC/NOEDD and/or LCx values if some of them are applicable with 95 % confidence limits for the test chemical at the end of the test; Description of all statistical procedures used in the study;
- Any other biological effects observed *e.g.* behavioural abnormalities, anti-feeding effects;
- Deviations from the guideline and any other relevant information.

TERMITES

Termite mounds are rich in different plant nutrients and shape many environmental properties. Rather, mound-field landscapes are more robust toward the impact of aridity, suggesting that termites may help stabilizing ecosystems, under global change. Termites are a good bioindicator of perturbation and restoration processes. They can modify degraded environments and their biogenic structures, modifying the availability of resources for other organisms. During their feeding and nesting activities, termites improve soil aeration, enhance absorption and storage of water in soils, and facilitate carbon fluxes and storage. These processes are crucial for longterm soil fertility, plant growth, and soil formation.

These insects may also emit large quantities of greenhouse gases like methane, carbon dioxide, and nitrous oxide into the atmosphere. Production of gases is, however, species specific and varies, depending on the soil environment and food quality. Termites have a key role in tropical ecosystems function. They are one of the main decomposer in tropical terrestrial ecosystems. It also shows high sensitive to environmental conditions. The colonies of soil eating termites which are most sensitive to habitat disturbance.

Methodology

Transect Sampling Method

Standardized transect method (Jones and Eggleton, 2000) was used for sampling termites. The area to be sampled was divided into no. of sites, which was further zoned. A belt transect of 100m was laid making use of stick and rope. In order to standardize sampling effort, each transect was divided into 20 contiguous sections of 5m x 2m and in each section 20 minutes was spent searching for termites. Within each section the following microhabitats were searched; surface soil , leaf litter and humus on the forest floor (at the base of trees, between buttress roots etc.), inside dead logs, tree stumps, branches, twigs, subterranean nests, mounds, runaway on vegetation and visible arboreal nests up to a height of 2 m above ground. The number of encounters with termites (hits) of a given species within a transect was taken as the relative abundance of that species within that transect. An encounter is the presence of a species in one transects section (5 x 2 m). In each site, termite nests were searched for on the ground, in live and dead trees, rotten wood, wooden objects and walls of buildings.

Dead wood with a diameter ≥ 1 cm found in every part of transect were dismantled and the termites that are found there were also collected. The collected termites were inserted in a tube containing 70% alcohol and labelled. The next step was specimen storing and identifying. Initial identification was done until the level of morphological genus. Relative abundance was compared with other locations. Relative abundance is the per cent composition of an organism of a particular kind relative to the total number of organisms in the area. Termite biomass was measured by wet weight of 20 termites.

Preparation of dust and bait mixture

About 10 g of harvested spores was mixed in 100 g of mixture containing sugarcane bagasse (50 g), cardboard (20 g) and sawdust (30 g). To make the bait mixture, 30 ml of

starch water was added and mixed in a Speed Mixer at 3000 rpm for 5–10 min to obtain a semi-solid paste. The mixture was poured into the ice-cube trays for the preparation of bait cubes.

HORIZONTAL TRANSMISSION OF FUNGAL INFECTION

The horizontal transfer of fungal infection among the termite *O. obesus* was studied for five isolates of *M. anisopliae*, where twenty workers and twenty soldiers were allowed to walk over the partially dried fungal suspensions of 108 conidia ml−1 separately for 1 min. The treated twenty workers and twenty soldiers were transferred separately to plastic trays of size 60 cm (l)×30 cm (b)×15 cm (h) containing eighty workers (untreated) and eighty soldiers (untreated) with small pieces of rubber wood (Hevea brasiliensis) and pieces of fresh fungal combs.

The trays were covered with a dark moist cloth and incubated at 26±0.3 °C in darkness. For each isolate, five replicates, each with twenty (treated) and eighty (untreated) individual workers and soldiers were maintained separately, and mortality was recorded at 24 h intervals for ten days. A batch of 100 workers and soldiers of infected termites was maintained separately as the control.

Dead insects were incubated in a humid chamber to confirm the growth of the fungus on cadavers. Foraging activity of termites to dry conidia and conidia mixed with attractant Repellency of the workers and soldiers of O. obesus to the five isolates of M. anisopliae was evaluated

Plastic trays (size: 90 cm (l)×60 cm (b)×30 cm (h)) were filled with mound soil (up to 15 cm). Each tray was separated equally into two
compartments A and B with nylon gauze (60-mesh size) where compartment
A served as the releasing site for the termites. In the treatments, the first set contains (A — termite comb and rubber wood; B — dry conidia 0.1 g, termite comb and rubber wood), the second set contains (A — termite comb and rubber wood; B — bait without conidia, termite comb and rubber wood) and the third set contains (A — termites comb and rubber wood; B — bait with conidia, termite comb and rubber wood). About 100 workers and 50 soldiers were released in compartment A, where the releasing site was illuminated with a florescent bulb (220 V, 13 A, AC) for 3 min. The remaining portion of the trays was shielded with a dark moistened cotton cloth.

The combination of brightness and darkness acted as a “push–pull” set of visual stimuli to induce the termites to move away from the release area towards the treated compartments. The number of termites foraging in the treated and control compartments together with those in their respective arms was recorded at an interval of 10 min up to 90 min to give nine readings for each replicate and mortality and mycosis was evaluated after 10 days of incubation at 26±0.3 °C.

TREE TREATMENTS

The sandal tree bark covered with mud galleries of O. obesus was selected around the plots. The experiments were set up as follows; the mud galleries were gently removed up to a diameter of 30 cm in the middle of the infected stem (from base two 60 cm height) leaving the other part intact.

The gunny bags (30 cm) dusted with dry conidia, conidia mixed with attractant and only attractant were tied separately over the 30 cm area between the mud plasters on the stem. The mortality, mycosis and width of galleries roofed over the gunny bags were observed at an interval of (4, 7, 14, 28, 42, 56, 70, 84 and 98 days) for a period of three months. The control trees were treated with gunny bags containing attractants only. Five replicates of twenty-five trees each for each treatment were maintained.

SOIL TREATMENTS

The soils were removed up to a depth of 15 cm around 30 cm away from the centre of the stem of sandal tree. The mean average height of the mud galleries on each branch of the stem was measured and marked before starting the experiments. A quantity of eight bait cubes was buried around the stem of each plant. The width of the mud plaster covering the branches, mortality and mycosis was observed in an interval of (4, 7, 14, 28, 42, 56, 70, 84 and 98 days) for a period of three months. The control trees were treated with only baits without conidia. Five replicates of twenty-five trees each for each treatment were maintained.

MOUND TREATMENTS

Two to three year old active colonies of O. obesus were selected fifteen days before the experiment by asking the farmers/forest guards about the age of each mound in the sandal plots. The activity of each mound was determined at the time of selection using a “hole repair method” where, rectangular holes on each side of the active mounds were dug

down to the level of the termite comb. The volume of the mound material removed was measured and the size of hole was measured after 24 and 48 h.

Mounds with holes sealed within 48 h were considered as active mounds. Conidial dust (100 g) mixed with the attractant was applied by blowing them through ventilation holes at the center (top) of the mound, with a small container connected to a bicycle pump and rubber hose. During the operation, cardboard was used to cover the hole to prevent the spore dust from puffing out of the artificial hole.

After applications, the holes were covered with plastic sheets to prevent rainwater fromentering the holes. The activity of termite colonies in the mound was measured by an AED-2000L (Acoustic Emission Consulting) Insect pest detection kit at an interval of 0, 1st, 3rd and end of 5th months. About twenty five readings per mound were measured and the average was graphically represented as the RMS value. The mounds were cut open to observe mortality and mycosis after five months of treatments.

EARTHWORMS

The nematode bio control agents will be mixed with un decomposed organic wastes and then known number of earthworms will be inoculated. Mortality and behavioural change in the earthworms will be monitored periodically.

Questions

1. Write the protocol for environmental impact test by using honey bees.
2. Define bait cubes