STUDIES ON THE ECOLOGICAL DISTRIBUTION OF *BACILLUS THURINGIENSIS* BERLINER AND ITS EFFECT ON DIFFERENT TARGETED PESTS AND BENEFICIAL SOIL MICROORGANISMS

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CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON THE ECOLOGICAL DISTRIBUTION OF *BACILLUS THURINGIENSIS* BERLINER AND ITS EFFECT ON DIFFERENT TARGETED PESTS AND BENEFICIAL SOIL MICROORGANISMS**" submitted in part fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE) in AGRICULTURAL MICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a *bonafide* record of research work carried out by **Mr. G. PRASAD** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

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Native isolates of *Bacillus thuringiensis* were tested for efficiency against *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera*. Selected 7 isolates that effected more than 65 per cent mortality on the three targeted pests. LC_{50} values were assessed. Tested for safety against non targeted organisms like silkworm, honeybees *Trichogramma, Chrysoperla* and fish(common carp).The isolates effected a higher degree of mortality in the case of silkworms. The other organisms were not affected by these isolates.

The interactions with biofertilizer agents like *Azospirillum brasilense Azotobacter chroococcum, Bacillus megaterium, Pseudomonas striata* and *Rhizobium leguminosarum* was also studied under sterile conditions. There was no antagonism or ammensalism observed. The absence of antagonism

between biocontrol agent and biofertilizer agents was confirmed by cross streak assay.

Unsuccessful attempts were made to increase the efficiency by chemical mutagenesis with NMNG. The effect of these selected *Bacillus thuringiensis* on phyllosphere was also studied.

The most effective strain on all the three targeted pests (MS24B1) was test grown on three different media to find out an economic alternative, at the same time with out compromising the quality or potency of the formulation. Defatted soy flour was identified as the best substrate for the medium for mass multiplication of potent *Bacillus thuringiensis* isolate.

A formulation was made with the help of easily available substances and tested for pathogenicity and residual toxicity. The effect of storage was also studied. The test formulation was comparable with a popular brand of commercial product

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The global agrochemical market in the early nineties was $ 26800 million, yet biological products were reported to account for only $ 120 million per annum, less than 0.5 per cent of the total. The majority of these sales were attributed to bio insecticide of which *Bacillus thuringiensis* accounted for over 90 per cent. The biological products have technical limitations, including extreme specificity, sensitivity to environmental factors and problems with robustness of the formulations, but these limitations which also give the biological control an image of environmental acceptability (Powell and Jutsum. 1993).

The biological control field now needs clear, well-defined goals for the current successful position to become basis for the future success rather than a limited experiment in alternative technology. For which generation of data regarding safety, compatibility, fate of the biocontrol agents in the environment etc. is very important.

Cannon (1993) reported that *Bacillus thuringiensis* based insecticide products constitute overwhelming majority of biopesticides but, they have made little impact (< 1%) on the crop protection market as a whole. This is due to poor cost - performance factors relative to synthetic organic pesticides. However recent biotechnological advances including genetic modification - combined with a variety of emerging opportunities, have created the prospect of the commercialization of a new range of highly effective *Bacillus thuringiensis* based biopesticides. Genetically modified biocontrol products offer advantages in terms of efficacy, flexibility and safety but public concerns regarding perceived risks need to be

answered. Hence, there is a need to carry out the ecological safety assessments for the effective and environment friendly use of these biopesticides in agriculture.

Several multinational companies have developed *Bacillus thuringiensis* strains suitable for the control of noctuids in India, which are normally less susceptible to *Bacillus thuringiensis* products (Sundara Babu, 1998). Now, that the Govt. of India has cleared *Bacillus thuringiensis* for commercial production in India though on a limited scale, the need to build up data on the safety of non targeted organisms (both macro and micro) is also very important at this juncture.

The genetically engineered plants (with *Bacillus thuringiensis* toxin genes) offer economic and environmental benefits to agriculture and society. Based on experiences gained over 50 years with pest resistant crop varieties developed through conventional genetic selection, the scientists suggest that, unless effective strategies are developed and implemented the pests will adapt to transgenic crops with in short time. When resistance becomes wide spread, alternative insecticidal toxin genes will be required to maintain the benefits of agricultural biotechnology. However issues related to sustaining the effectiveness of transgenic crops are more complex when compared to environmentally benign foliar spray of *Bacillus thuringiensis* toxin (McGaughey and Whalon 1992). One simple strategy could be searching for newer native strains of pathogens with broad-spectrum activity for safe and effective biological control.

The selection of efficient, broad spectrum, native *Bacillus thuringiensis* isolates and development of simple mass production technology may help ultimately India to save foreign exchange on imports. With these in mind the present study was undertaken with the following objectives.

- Isolation and characterization of *Bacillus thuringiensis* strains from soil samples collected from sericulture areas in Coimbatore and Erode districts.
- Comparison of the selected isolates against a standard strain for efficacy in pest control and for safety towards silkworm.
- To find out the biological safety towards non targeted macro organism *viz.*, silk worms, honeybees, parasites and predators
- To find out the biological safety towards non targeted microorganism *viz., Azospirillum, Azotobacter*, Phosphobacteria and *Rhizobium* .
- To develop a cheaper mass multiplication medium for *Bacillus thuringiensis* using agricultural bye products and biological wastes
- To test the shelf life of the developed product.

REVIEW OF LITERATURE

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2.1. History

The most widely used bacterium in microbial control of insect pests on agricultural crops is *Bacillus thuringiensis.* Louis Pasteur found this bacterium in dusts from silk worm rearing menageries in late 60's of the nineteenth century. Ishiwata in 1901 isolated a bacillus from diseased silk worm larvae and named it as "sotto disease bacillus" which was highly toxic. He attributed the toxin to the spore and something lying close to the spore. In 1911, Berliner isolated from Mediterranean flour moth (*Anagasta kuehniella*) a bacillus that he named in 1915 as *Bacillus thuringiensis* after the province Thuringia in Germany.

Mattes in 1927 reisolated the bacterium and found the high pathogenicity of this bacillus for the flour moth larvae. Both Berliner and Mattes noted the parasporal bodies in the sporangia but did not consider them as being a source of toxin. Hannay (1953) described the properties of the parasporal body of *Bacillus thuringiensis,* such as its structure, staining properties, solubility in dilute alkali (not in organic solvents).

Angus (1954 and 1956) demonstrated that the parasporal body was the source of the toxin for silk worms. A large number of sub species of *Bacillus thuringiensis* have been isolated from insects. Scientists also refer them as strains, varieties, serotypes, serovars, biovars, pathovars and crystovars.

The subspecies have been differentiated by many different methods such as antibiotics production (Toumanoff and Lapied 1954), biochemical tests (Heimpel and Angus 1958), H - serotypes (de Barjac and Bonnefoi 1962), esterase

production (Norris and Burges 1963), parasporal (crystal) antigen (Pendleton and Morrison 1967; Lynch and Bauman 1985), enzymes (de Barjac and Bonnefoi 1973), phages (Rautenstein *et al.,* 1975; Varatharajan and Munirathnamma 1980; Jones *et al.,* 1983) and lectin grouping (De Lucca 1984). Genes responsible for the synthesis of crystal protein are encoded in mega plasmids (Gonzalez *et al.*, 1981) and according to Carlton and Gonzalez (1985) the plasmid number varies from 2-12 with sizes from 1.5 to 150 mega daltons. Profiles of plasmids are identical with in subspecies level which also help in the identification.

2. 2. Distribution of *Bacillus thuringiensis*

This bacillus is not as common in soil as any other bacillus group and may represent about 35 percent of the *Bacillus cereus – Bacillus thuringiensis* group (Ohba and Aizawa 1986). Travers *et al.,* (1987) used acetate selection technique and obtained over a thousand isolates of *Bacillus thuringiensis* from soils of five continents About two thirds of the isolates were found to be toxic for lepidopterons and dipterons. Martin and Travers (1989) screened *Bacillus thuringiensis* from 1115 samples obtained from 30 countries including United States of America. Seven hundred and eighty five samples contained about 9,000 isolates, of which only 48 per cent fit in to described *Bacillus thuringiensis* types.

Nataraju *et al.* (1991) reported that only 25 per cent of the total *Bacillus thuringiensis* isolates from sericulture areas of Karnataka (India) were toxic to silk worm. Chilcott and Wigley (1993) obtained 6909 isolates of *Bacillus thuringiensis* form 455 samples of soil, insect larvae and insect habitats from throughout New Zealand. They were found to be toxic to insects of three orders *viz.*, lepidoptera, diptera and coleoptera. The scientists used crystal diversity based on SDS-PAGE to accommodate the isolates in different groups. Johnson and Bishop (1996)

described a method for isolating *Bacillus thuringiensis* enrichment method. The diversity of the strains isolated by this method suggested that the method does not preferentially select one strain or subspecies of *Bacillus thuringiensis* over others.

Ohba (1996) reported the presence of *Bacillus thuringiensis* on mulberry leaf surfaces (both adaxial and abaxial). About 186 colonies from 96 per cent of the mulberry shrubs screened were found to be *Bacillus thuringiensis*. Of which only 10 per cent were toxic to silkworm.

Bernhard *et al.* (1997) isolated *Bacillus thuringiensis* from natural samples collected from 80 countries. Twenty five percent of the 5303 isolates originated from soil. Analysis of insecticidal properties revealed about 50 per cent of the total strains has less than 25 per cent insecticidal activity. These scientists also focused presence of different types in relation to geographical origin.

2. 3. Characterization of *Bacillus thuringiensis*

The bacterium *Bacillus thuringiensis* is aerobic, Gram positive, motile rod, produces catalase, spore and parasporal body. It produces different types of toxins *viz.*, alpha-exotoxin (heat labile lecithinase-c) beta-exotoxin (heat stable adenine containing compound), delta - endotoxin (crystal protein) and the louse factor. It varies in shapes and sizes, and in the mode of its formation depending on the sub species of the bacillus (Norris 1971; Dulmage 1978; Mikkola *et al.*, 1982).

There is considerable agreement in characterizing the sub species based on crystal antigens (Smith 1987) which have been used to differentiate sub species of *Bacillus thuringiensis*. The parasporal crystals also characterize it, which is often referred to as the crystal inclusion, crystal endo toxin or crystalloid.

The spores and crystals can be differentiated by employing an improved staining method developed by Chilcott and Wigley (1988) which involves steaming and staining with amido black or naphthalein black and Giemsa stains. Use of bio chemical tests viz., catalase production, utilization of different carbon sources, esterase production etc. and flagellar antigens are also being followed throughout the world to characterize *Bacillus thuringiensis* (de Barjac and Frachon 1990).

2.4. Survival of *Bacillus thuringiensis* in soil

The experiments conducted by Saleh *et al.* (1970 a and b) revealed that the *Bacillus thuringiensis* could be recovered from the soil even after 40 days after inoculation. They have also found out that the inoculated spores of *Bacillus thuringiensis* survived, germinated, multiplied and resporulated even in uncropped soils.

The situation for spore germination is not alike is soil and in larval gut, where there is very little competition from other organism. The ability of spores to germinate rapidly confers *B, thuringiensis* the advantage for survival. The relative ability of the *Bacillus thuringiensis* spores to germinate and proliferate under favourable conditions poses a distinct ecological significance. The parameter optimum for *B. subtilis* spore germination is not responsive for *Bacillus thuringiensis* spores. The germination is slow or rapid depending up on the strain (Stahly *et. al.*, 1978).

There are reports that testify the presence of viable spores even 6 months after inoculation. The laboratory trials with many soil isolates confirmed the positive presence of antagonism towards vegetative cells of *Bacillus thuringiensis* but not

against spores. There is no knowledge of what ecological role they might play when the spores and crystals accumulate in soil (Pruett *et al.* 1980).

Petras and Casida (1985) have studied the survival of *Bacillus thuringiensis* spores in soil. They also found that the crystals seemed to survive intact in the soil but made no attempt to assess the toxicity. They concluded that the ability of *Bacillus thuringiensis* to produce crystals was also not lost during its residence in soil i.e., all colonies of *Bacillus thuringiensis* recovered from soil were capable of producing crystals even after 12 months.

Most of the commercial preparations of *Bacillus thuringiensis* contain both spores and crystals suspended in a menstruum for spraying. Thus, any ecological or other considerations of the commercial preparation should take in to account the spores also along with the crystals of the suspending menstruum. The glycoprotein protoxin component of the crystal is found in the spore coat also, thus relates to insecticidal power of the formulation to spores (Bulla *et al.*, 1990).

Addison (1993) reported that the *Bacillus thuringiensis* spores survive for several years after spraying in forest ecosystem. He also stated that there is an urgent need for further research to elucidate the relationship between and natural microflora and fauna as it is not even known where and under what conditions the *Bacillus thuringiensis* in nature.

Akiba (1996) observed that the *Bacillus thuringiensis* germinated in sterilized soil but not in natural soil. *Bacillus thuringiensis* vegetative cells were not able to produce spores in natural soil and suggested that the spore is the only state in which *Bacillus thuringiensis* persists in soil.

2.5. Pathogenicity of *Bacillus thuringiensis* isolates

Heimpel and Angus (1959) classified the lepidopteron susceptible to *Bacillus thuringiensis* in to three types. Type I species are lethally stricken by general paralysis caused by delta-endotoxin alone. There is an increase in blood pH of 1.0 to 1.5 units from the leakage of alkaline midgut contents in to the poorly buffered haemolymph e.g. silk worm, tobacco horn worm, tomato horn worm, mosquito larvae, black flies, etc.

Type II insects are also like type I, suffer midgut paralysis a few minutes after ingesting the crystal but type II insects do not develop general paralysis and die without either blood pH change or paralysis. Most of the lepidopterons belong to type II. Type III insects do not develop general paralysis alone and require the presence of spores e.g. *Anagasta kuehniella* and *Galleria mellonella.* In the case of *Plodia interpunctella* and *Ephestia cantella* larvae the gut pH is just above neutral and crystals do not dissolve but the spores are able to germinate and produce conditions suitable for the delta - endo toxin and other enzymes (Mc Gaughey 1978). In some cases the spores especially in the presence of delta-endotoxin are responsible for the death of the insect through septicemia (Luthy and Ebersold, 1981).

2.5.1. Pathogenicity of *Bacillus thuringiensis* against *Plutella xylostella*

Justin *et al.* (1988 and 1990) reported *Bacillus thuringiensis* effected better control of the pyralid on cauliflower. They reported that the suspension containing 10^7 spores per ml of *Bacillus thuringiensis* sub sp. *kurstaki* was most toxic to 3rd instar larvae of *Plutella xylostella* by leaf dip method and diet incorporated diagnostic assay. Perez and Shelton (1996) reported that both bioassays *viz.*, leaf

dip method and diet incorporated diagnostic assay were perfect representation for field evaluation studies of *Bacillus thuringiensis* sub sp. *kurstaki* (Berliner).

2.5.2. Pathogenicity *of Bacillus thuringiensis* to *Cnaphalocrocis medinalis*

Srivastava and Nayak (1978) tested four commercial formulation of *Bacillus thuringiensis* against *Cnaphalocrocis medinalis* and found significant control. At the same time Narayanasamy and Baskaran (1979) in their study to determine the effectiveness of *Bacillus thuringiensis* sub sp. *kurstaki* alone or in combination with *Verticillium lecanii* and other organochlorine and organophosphate pesticides for controlling rice pests, reported that the application of *Bacillus thuringiensis* alone was less effective against *Cnaphalocrocis medinalis* under the field conditions. Joshi *et al.* (1987) reviewed the role of *Bacillus thuringiensis* as one of the biological control agents of the rice pest *Cnaphalocrocis medinalis*

Padua and Juanillo (1996) screened 201 and 177 local *Bacillus thuringiensis* isolates against *Cnaphalocrocis* sp. and *Marasmia* sp. and selected three isolates based on LD_{50} values. Their results showed that the growth conditions of these isolates determined the yield and toxicity.

2.5.3. Pathogenicity of *Bacillus thuringiensis* against *Helicoverpa armigera*

Bell and Romnie (1985) reported that the application of *Bacillus thuringiensis* alone and in combination with NPV, effceted identical control on cotton bollworm. Also they observed that the damage was only 0.5 per cent in the bacteria treated fields.

Kulkarni and Amonkar (1988) characterized a new isolate (ISPC-7) as *B thuringiensis* sub sp. *kenyae* and compared it with ISPC-4 (*Bacillus thuringiensis* sub sp. *Kurstaki)* in the range of 10^5 to 10^9 spores per ml against 1st to 4th instar larvae of *Helicoverpa armigera*. The isolates were toxic (99 per cent mortality recorded on the 2nd day after treatment), to first instar larvae. Older larvae had a slower response. The best LC_{50} value for the *Bacillus thuringiensis sub sp. kurstaki* was 2.85×10^7 cells per ml. Ali and Young (1993) reported that survival of 2 species of cotton bollworms generally decreased as the rate of *Bacillus thuringiensis* dose increased.

Jyoti *et al.* (1996) evaluated *Bacillus thuringiensis* sub sp. *kurstaki* based formulation on cotton against *Helicoverpa* spp.. through bioassays. They not only recorded best results of *Bacillus thuringiensis* on *Helicoverpa zea*, but also reported that the spraying made the larvae move from the location where they were devastating to locations where they were less damaging.

Domo and Solsoloy (1997) compared pesticides along with *Bacillus thuringiensis* formulation for cotton bollworm control. From their experiments they found that the reduced rate of chemical insecticides with *Bacillus thuringiensis* application was next only to deltamethrin which effected a superior control over cotton bollworm. The least effective treatment was *Bacillus thuringiensis* as an individual application, indicating resistance development.

2.5.4. Bioassay

The evaluation of pathogenicity of *Bacillus thuringiensis* is done by leaf dip and diet incorporation bioassays. Van Frankenhuyzen and Nystrom (1989) used

leaf dip and diet incorporation assay techniques to evaluate residual toxicity of a high potency formulation of *Bacillus thuringiensis* to spruceworms.

The bio assays involve, whole cells, crystals and spores, crystals alone and spores alone mixed with diet or sprayed on leaves for assessing pathogenicity, persistence and residual toxicity. Zhu *et al.* (1989) described a method for separating *Bacillus thuringiensis* protein crystals by Ludox gradient centrifugation, which was simple, inexpensive, fast, and efficient, compared to other techniques. It has been successfully used to purify and characterize the protein crystals from several *Bacillus thuringiensis* strains. Murthy *et al.* (1994) described another simple and rapid technique to separate intact crystals from spores and cell debris of *Bacillus thuringiensis* using a carboxy methyl cellulose column with Tris- EDTA buffer as eluent. The SDS-PAGE and microscopic study revealed that the purity was more than 98 per cent. One of the above-mentioned techniques was used regularly for obtaining crystal proteins for bioassays.

Perez *et al.* (1997) compared *Plutella xylostella* for resistance to *Bacillus thuringiensis* sub sp. *kurstaki* by leaf dip and diet incorporation assays. They also suggested that leaf dip assay could be used to evaluate persistence of *Bacillus thuringiensis* in phyllosphere and diet incorporation of *Bacillus thuringiensis* could be used as resistance test kit.

2.6. Occurrence of *Bacillus thuringiensis* in phyllosphere and rhizosphere of crops plants

Brown, (1977) made a note of sporostasis of some kind limiting the growth of bacteria in the rhizosphere that was confirmed by Petras and Casida (1985). Nambiar *et al.* (1990) released in to the rhizosphere of pigeon pea, the *Bradyrhizobium sp.* cloned with DNA responsible for crystal protein production.

Smith and Couch (1991) isolated novel variants of *Bacillus thuringiensis* from the phylloplane of deciduous and conifer trees as well as from phylloplane of other plants. These isolates displayed a wide range of toxicity towards larvae of lepidoptera, diptera and coleoptera. They also proposed *Bacillus thuringiensis* to be considered as a part of the common leaf microflora of many plants.

Sundaram and Sundaram (1992) studied the persistence of *Bacillus thuringiensis* sub sp. *kurstaki* in the phyllosphere of oak foliage following the application of commercial formulations using special bioassay techniques. The constructed strain provided protection against root nodule damage by the *Rivellia angulata* larvae. Though there are many reports available with reference to *Bacillus thuringiensis* in soil, literature on its survival in the rhizosphere regions of crop plants is scanty (Addison 1993).

Sudarsan *et al.* (1994) studied on the colonizing ability of the transcipient strain of *Bacillus megaterium* carrying *Bacillus thuringiensis* cry IA gene along with *Bacillus thuringiensis* sub sp. *kurstaki* (HD-I) strain as reference. They have reported that the persistence of transcipient was upto 28 days on the phyllosphere of bhendi and cotton. The constructed strain provided protection against root nodule damage by the *Rivellia angulata* larvae. Though there are many reports available with reference to *Bacillus thuringiensis* in soil, literature on its survival in the rhizosphere regions of crop plants is scanty (Addison 1993).

Maciel Rosas *et al.* (1994) reported to have isolated a new strain of highly toxic *Bacillus thuringiensis* (near non-motile) from the grain dust of silo which contained maize from central Mexico. *Bacillus thuringiensis* was isolated from the phylloplane of organically grown cabbage in one field during two growth seasons.

Majority (64 per cent) of the isolates belonged to *Bacillus thuringiensis* sub sp. *kurstaki* (Damgaard *et al.* 1997.).

2.7. Safety of *Bacillus thuringiensis* to non targeted organisms

Babrikova and Kuzmanova (1984) have experimented on toxicity of biological preparations made on the basis of *Bacillus thuringiensis* for the predator *Chrysopaerla carnea* and recorded varying levels of toxicity at very high concentrations when fed to different stages of *Chrysopa semptempunctata, C. formosa* and *C. perla.*

Vandenberg (1990) tested 4 entomopathogens on caged adult honeybees. *Bacillus thuringiensis* was found to cause no disease to honey bees even at a concentration of 10^6 cells per ml of sucrose syrup. They affirmed that under field conditions this bacterium would probably not cause undesirable effects on honeybees.

Vandenberg and Shimanuki (1990) and Mc Killup and Brown (1991) suggested the use of *Bacillus thuringiensis* formulation to control greater wax moth (*Galleria mellonella*), which infest and damage beeswax combs, without any harmful effect on honeybees. Also, they recommended that the *Bacillus thuringiensis* spraying as the best-suited control measure for beekeepers as the persistent nature of *Bacillus thuringiensis* gave protection for longer period (10-20 weeks). Only limited number of viable cells and spores were recorded in the honey stored in treated combs.

Kallapur *et al.* (1992) reported that the solubilized crystalline proteins of *Bacillus thuringiensis* sub sp. *israelensis* when administered to Japanese quails by intra abdominal injection were toxic with an LD_{50} at 24h of 22.8 mg per kg of body

weight. These toxins were hemolytic in nature. This reduced serum lipid levels and alkaline phosphatase, and increased serum glucose, creatinine, phosphokinase and lactate dehydrogenase levels.

James *et al.* (1993) observed the toxic nature of *Bacillus thuringiensis* to late instar larvae of cinnabar moth when it was applied to control spruce bud worm and gypsy moth. Cinnabar moth (*Tyria jacobar*) is an effective biocontrol agent on tansy ragwort weed in the forest ecosystems of United States of America.

Mertz *et al.* (1995) conducted field studies for testing commercially available *Trichogramma brassicae* and *Bacillus thuringiensis* for the control of *Ostrina nubilalis* (Hubner) in sweet corn. The integration of both biocontrol agents did not result in additional insect control, effected on par results with other treatments. This suggested the non-toxic nature of *Bacillus thuringiensis* towards the beneficial *Trichogramma* sp., which is widely used as an egg parasite

Addison and Holmes (1996) studied the effect of two commercial formulation of *Bacillus thuringiensis* on the survival, growth and reproduction of forest earthworm. *Dendrobaena octaedra* was not adversely affected by the formulation at 1000 per cent expected environmental concentration. However survival, growth and cocoon production were significantly reduced at 10 times the recommended dosage (mandatory testing level in Canada to study the effect of naturally occurring microbial pest control agents before registration).

There are reports about the production of 'bacteriocin' by *Bacillus thuringiensis* an antibacterial substance in closely related species, for example tochicin from *Bacillus thuringiensis* sub sp. *tochigiensis* (Paik *et al.*, 1997). Partially purified tochicin showed a narrow antibacterial spectrum against *Bacillus*

thuringiensis serotypes and *Bacillus cereus* but not against other bacteria and yeasts tested.

2.8. Antibiotic sensitivity and mutagenesis in *Bacillus thuringiensis*

Chugtai and Shakoori (1994) tested *Bacillus thuringiensis* sub sp. *kurstaki* for sensitivity towards antibiotics. The bacterium resisted 40 ppm penicillin, 78 ppm tetracycline, 20 ppm ciclacillin, ampicillin, streptomycin and chloramphenicol. The growth of *Bacillus thuringiensis* was significantly retarded in the presence of antibiotics (extended lagphase, short log and abrupt stationary phases).

Crawford and Streips (1990) have developed separate methods for mutagenesis for *Bacillus thuringiensis*. Kim *et al.* (1994) obtained several mutants of *Bacillus thuringiensis* sub sp. *israelensis* HD-500 after treatment with *N'nitro-N'methyl-nitrosoguanidine*. On the basis of production or absence of spores and crystals the mutant strains obtained by them were grouped in to three categories. First group contained spores only; second only crystals and the third had both spores and multiple crystals. Aronson *et al.* (1995) reported that random mutagenesis of two 30-nucleotide regions of cry 14^0 C gene resulted in about 50 mutants. While the mutants obtained from cells with randomly mutagensied first sequence (responsible for the formation of ion channels) showed no loss of toxicity, the mutants obtained from cells with randomly mutagensied second sequence (which encode for amino acids unique for toxins) were with low or no toxicity.

2.9. Formulations from *Bacillus thuringiensis*

Mummigatti and Raghunathan (1988) studied the relative toxicity of the entomopathogenic bacterium *Bacillus thuringiensis* sub sp. *kurstaki* produced in submerged culture, surface culture and *in vivo* culture (on *Bombyx mori).* Their results revealed that the products from submerged cultures and surface cultures were 29.9 and 13.4 times as potent as those were from the *in vivo* method. The maximum biomass was obtained from *in vivo* production. On the basis of toxicity units submerged cultures produced $272.5x10^3$ followed by surface cultures ($121.6x10^3$) and *in vivo* cultures ($9.1x10^3$) toxicity units per kg medium.

El-moursy *et al.* (1992) developed some biochemical approaches to enhance the potency of *Bacillus thuringiensis* against *Plodia interpunctella.* They tested different classes of chemical compounds to make a safe, nontoxic to human beings and cheap product and recommended zinc sulphate, serine, EDTA and formic acid as additives to *Bacillus thuringiensis* formulation for enhanced potency

Gangurde and Sethna (1995) studied sporulation and toxin production by *Bacillus thuringiensis* sub sp. *israelensis* and *B. sphaericus* in media based on mustard seed meal. Gibson *et al.* (1995) identified tannic acid as an inexpensive additive that increased the efficacy of sub lethal concentration of *Bacillus thuringiensis* sub sp. *kurstaki.* Tannic acid mimicked the active constituents contained in an aqueous, tannin rich extract of *Taxus baccata* bark that retarded *H. virescence* development at 10,000 ppm. Twentyfive - 500 ppm tannic acid with *Bacillus thuringiensis* (16.3µg per ml diet) yielded 55-75 per cent mortality in *Trichoplusia ni.*

Mc Guire and Shasha (1995) suggested that microbial insecticide formulations were more efficacious and had longer residual activity than commercial formulations. Mc Guire *et al.* (1996) reported that tank mixing of pregelatinized starch with equal quantity of sucrose at 4 per cent solids level provided protection of *Bacillus thuringiensis* on cabbage leaves for 5 days under sunny field conditions.

Morris *et al.* (1996) studied the effect of aeration, initial pH, l-cystine concentration, supplemental sodium chloride and polyoxyethylene sorbitan monostearate in culture media on biomass production and potency of *Bacillus thuringiensis* sub sp. *aizawai* HD-133. They observed that HD -133 had a high oxygen requirement and a starting pH par of 7.0 for optimal biomass production and spore crystal toxicity they found out that HD- 133 had a low tolerance for per cent L-cystine. The addition of 0.5 per cent sodium chloride and 0.1 per cent Tween 60 to a culture medium containing cotton seed meal and glucose as the main nitrogen and carbohydrate sources respectively enhanced the potency of the spore crystal product.

Behle *et al.* (1996) reported that by formulating caesin based *Bacillus thuringiensis* products the residual insecticidal activity can be extended. The casein formulation also provided some protection from light induced degradation compared with unformulated *Bacillus thuringiensis.* However, the amount of protection was less than that provided by other experimental formulation.

Tamez-Guerra *et al.* (1996), used spray dried *Bacillus thuringiensis* sub sp. *kurstaki* formulation composed of citric acid or lactic acid, pregelatinized cornflour, cornstarch, isopropyl alcohol, sugar and corn oil to determine the effects of solar radiation and rain on insect populations by leaf dip and diet incorporation bio assay

methods. They recorded that *Bacillus thuringiensis* activity did not decrease and also the effect of solar radiation was minimum on the formulation suggesting that the formulation provided protection against solar radiation also.

Bacillus thuringiensis sub sp. *aizawai* HD –133 was grown in culture media in which dextrose was a common carbon source and 30 different agricultural products were tested as the main nitrogen sources. These products included legumes, cereals, animal proteins, leaf protein, yeasts, oilseeds, tubers, and casamino acids. Of the 30 products tested, cotton seed meal, defatted soyflour and corn gluten meal was the most efficient substrate for the production of spore crystal biomass with higher endotoxin potency. The carbohydrate: nitrogen ratio for these additives ranged from 0.3 to 0.5 (Morris *et al.*, 1997).

2.9.1. Photo protection against UV-inactivation

Pusztai *et al.* (1991) carried out detailed photo stability studies using purified delta-endotoxin of B. *thuringiensis* sub sp.*kurstaki* H.D-1 and HD-73. The mechanism and course of sunlight inactivation was investigated by three different methods. Their results demonstrated that 300-380 nm range spectrum was largely responsible for bringing about crystal damage and consequent loss of toxicity. The results concerning photo degradation supported a photo sensitization mechanism involving the presence of exogenous/endogenous chromophores which created singlet oxygen species upon exposure to sunlight.

Cohen *et al.* (1991) suggested photo protection of *Bacillus thuringiensis* from UV irradiation and subsequent loss of toxicity. Addition of acriflavin, methyl green and such cationic chromophores gave photoprotection of *Bacillus*

thuringiensis especially acriflavin was the best at 0.42 m mol dye adsorbed to 1g of *Bacillus thuringiensis* level.

Jones *et al.* (1991) demonstrated how to get mutant with increased resistance to UV- irradiation by successive exposure to UV-rays. In addition to that they also successfully isolated mutants with specific pathogenicity.

2.9.2. Effect of storage on cell viability and pathogenicity

Balaraman and Hoti (1984) studied the impact of storage period and temperature on the larvicidal activity of *Bacillus thuringiensis* sub sp. *israelensis* and *Bacillus sphaericus* formulation. In their laboratory studies, they prepared a water-soluble formulation and stored at -40°C, 8°C and 30°C and analysed the activity against *Culex quinquefasciatus.* The *Bacillus thuringiensis* sub sp. *israelensis* showed no significant reduction in the activity in all storage treatments over a 30-week period of observation.

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Collection of soil samples

Twenty sericulture areas from Erode and Coimbatore districts were randomly selected. Mulberry soil samples from 0 – 5 cm depth were collected and were used for the isolation of native *Bacillus thuringiensis* culture.

3.1.1. Isolation of *Bacillus thuringiensis* from soil samples and characterization

Modified technique developed by Travers *et al.* (1987) was used for isolation. One-gram soil sample was thoroughly mixed with 50 ml Luria broth (in 0.25-M sodium acetate) (composition presented in Appendix - 1) contained in 250 ml Erlenmeyer flask and heated for 15 minutes at 65°C. Again after 4 hours of incubation at 31°C heat shock was given. Transferred one ml of the content to a petridish and plated with T_3 medium (composition presented in Appendix - 1). The acetate delayed the germination of *Bacillus thuringiensis* spores. The second heat treatment at the end of 4 hours incubation killed other species of *Bacillus* that germinated ahead of *Bacillus thuringiensis* spores.

The colonies that formed on T_3 medium were of *Bacillus thuringiensis*, which were confirmed by, Gram staining (Gerhardt *et al.*1981), cell morphology, presence of spores and crystals (Chilcot and Wigley,1988), catalase activity, Voges-Proskauer test (for acetyl methyl carbinol production) and growth on D-mannitol (Stahly *et al.* 1991) agar medium.

3.1.1.1. Endospore and crystal staining

Sporulated cultures were used for the structural staining. Air-dried smear was heat fixed and incubated at 100°C for ten minutes. Placed the hot slide in to Amido black 10 B (1.5 g in 100 ml 35% glacial acetic acid) solution for ten minutes. Washed excess stains thoroughly in tap water, air-dried, flooded with

Giemsa stain for 2-5 min. Washed excess stains thoroughly in tap water, air-dried and observed under oil immersion objective. Crystals stain dark blue and spores pale to light blue with dark margin.

3.1.2. Authentication of the collected *Bacillus thringiensis* isolates

Apart from Gram staining, cell morphology and presence of spores and crystals, the following experiments were done to authenticate the cultures. (Reagents and media composition are presented in Appendix-I).

3.1.2.1. Catalase activity (Gerhardt *et al.*, 1981)

A loop full of 24 h old culture from slant was transferred to a glass test tube containing 0.5 ml distilled water and mixed thoroughly. Hydrogen peroxide 3 per cent solution (0.5 ml) was added. Oxygen effervescence indicated the presence of catalase.

3.1.2.2. Voges-Proskauer test (Gerhardt *et al.*, 1981)

Inoculated MR - VP medium with a loop full of 24 h old culture. Incubated at 31°C on rotary shaker (100 rpm) for 48 h. Mixed 1 ml culture with 0.6 ml VP reagent A and 0.2 ml VP reagent B. Placed the open tube in a slanting position to increase the contact with air. The change of surface colour to pink in 10-15 minutes indicated the positive acetyl methyl carbinol production.

3.1.2.3. Growth and acid production from D-mannitol as carbohydrate source: (Holt, 1984 and Lacey, 1997)

Added D-mannitol (filter sterilized using 0.22 μM filter) to make 1 per cent final concentration of pre-sterilized ammonium salts and sugar medium contained in test tubes before slants were made. Incubated for 15 days at 31°C after inoculation of the 24 h old test culture. No change in colour of the medium indicated negative test for the fermentation of D-mannitol. *Bacillus thuringiensis* seldom utilizes D-mannitol.

3.2. Culturing *Bacillus thuringiensis* (Liansky *et al.,1993*)

Inoculated a tube containing UG medium with a loop full of 24-h old *Bacillus thuringiensis* culture (10 ml) to serve as a pre culture. After incubation on a rotary shaker (100-rpm) for 48 h at 31°C and after observation under microscope, the sporulated preculture was heat shocked at 75 - 80°C for 10 minutes to kill all vegetative forms. This enabled a better homogeneity of growth of the new culture to be inoculated with the pre culture. Flasks (500 ml) containing 100 ml of sterilized UG medium was mixed with 1 per cent final concentration of glucose (filter sterilized) and inoculated with heat shocked pre culture. Incubated with orbital agitation (100 rpm) for 48 - 72 h at 31°C untill cell lysis was completed. After checking under phase contrast microscope for lysis of the cells to release spores and crystals, centrifuged at 7000 rpm for 20 min to pelletize spore and crystals. Resuspended and washed thrice with 0.5 M NaCl (once) and distilled or demineralized water (twice) and kept the spores and crystals frozen at -20°C.

3.2.1. Method of analysis for spore count (Postgate, 1969)

The viable spores were counted by the following method. The spore suspension was kept in a hot water bath at 80°c for 10 min. for killing of vegetative cells. Then the samples were serially diluted in water (1:10) to get up to 10^{-15} dilution. A volume of 1-ml suspension from 10^{01} - 10^{15} dilutions was plated on nutrient agar medium. The plates were incubated for 24h at 31°c. The number of colonies were counted and documented. The number of spores per unit volume was calculated by multiplying the number of colonies formed by the dilution factor. Plating was done before heat shock for total cells and after heat shock for spore count.

3.3. Toxicity of the isolates of *Bacillus thuringiensis* against target pests

A preliminary study for screening toxicity of the 80 isolates (that were found to have both spores and crystals against the three targeted pests *viz., Plutella xylostella*; *Helicoverpa armigera* and *Cnaphalocrocis medinalis*) had been carried out using a bioassay procedure demonstrated by Dulmage (1971) and Beagle *et al.* (1986)). Leaf dip method for *Plutella xylostella* and *Cnaphalocrocis medinalis* and diet incorporation method for *Helicoverpa armigera*. The procedures for the mass culturing of target pests are presented in Appendix-II.

3.3.1. Leaf dip method (Beagle *et al.*,1986)

Centrifuged, washed and lysed cells were resuspended in 0.85 per cent NaCl with 0.6 per cent K_2HPO_4 saline buffer (pH 6.5). Fresh cauliflower leaves and cut leaf blades of rice were dipped in the lysed cell suspension (10^7spores per ml), air-dried at room temperature. The treated leaves were transferred to petridishes lined with moistened filter paper (Plates1 and 2). Third instar larvae of *Plutella xylostella* (on cauliflower leaves) and *Cnaphalocrocis medinalis* (on leaf blades of rice) were allowed to feed on. Care was taken not to injure the larvae. Ten larvae per replication, 3 replication for each isolate and 1 control for every ten isolates tested (leaves dipped in saline buffer) was set up. Observations on mortality was recorded at upto 72 h.

3.3.2. Diet incorporated bioassay

Artificial diet (Appendix-II) for *Helicoverpa armigera* (Dulmage *et al.*, 1971) was prepared and before solidification lysed cell suspensions containing spores and crystals of selected *Bacillus thuringiensis* cultures were mixed separately in sufficient quantities to make final concentrations of 10^7 spores per ml of diet. The

Plate 1. Bioassay of *Bacillus thuringiensis* isolates against *Plutella xylostella* to assess LC_{50}

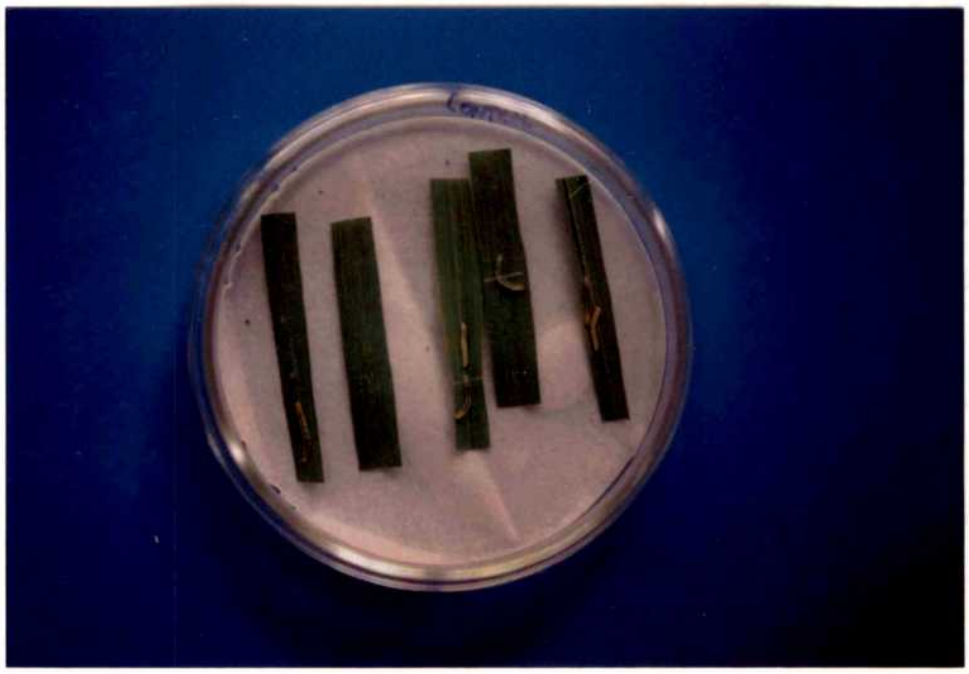


Plate 2. Bioassay of *Bacillus thuringiensis* isolates against *Cnaphalocrocis medinalis* to assess LC_{50}

mixtures were dispensed into small glass vials, allowed to cool and larvae of *Helicoverpa armigera* (III instar) were released into the vials (1 per vial) and plugged with cotton (Plate.3). The operation was carried out in a contamination free environment. Ten larvae per replication and three replications (30 vials) per isolate were kept. One control for every 10 isolates tested was set up. Observed for mortality upto 72 hours after release into the vials.

All the bioassays were carried out in phased manner, *i.e.* ten isolates were tested at a time. When mortality observed in control, the data were corrected using Abbot's formula (Padua *et al.*,1987).

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Test mortality} - \text{percent control mortality})}{(100 - \% \text{ Control mortality})}$$

Those isolates which effected more than 65 per cent mortality were taken up for further studies.

3.3.3. Assessment of LC_{50} of the selected isolates on target pests

An experiment was conducted to assess the LC_{50} value of the isolates against all the three targeted pests. Cell concentrations (with spores and crystals) from 10^5 to 10^9 per ml were taken up for this study. For each replication 10 larvae (III instar) were kept and 5 replication for each treatment were kept for this experiment. The bioassays were carried out as mentioned in section 3.3.1 for *Plutella xylostella* and *Cnaphalocrocis medinalis* and for *Helicoverpa armigera* as mentioned in the section 3.3.2.Per cent mortallity (Plates 6,7 and 8) was observed up to 72 h and recorded. The results were corrected by using Abbot's formula

Plate 3. Bioassay of *Bacillus thuringiensis* isolates against *Helicoverpa armigera* to assess LC_{50}

Plate 4. Bioassay of *Bacillus thuringiensis* isolates against *Helicoverpa armigera* for residual toxicity on phyllosphere

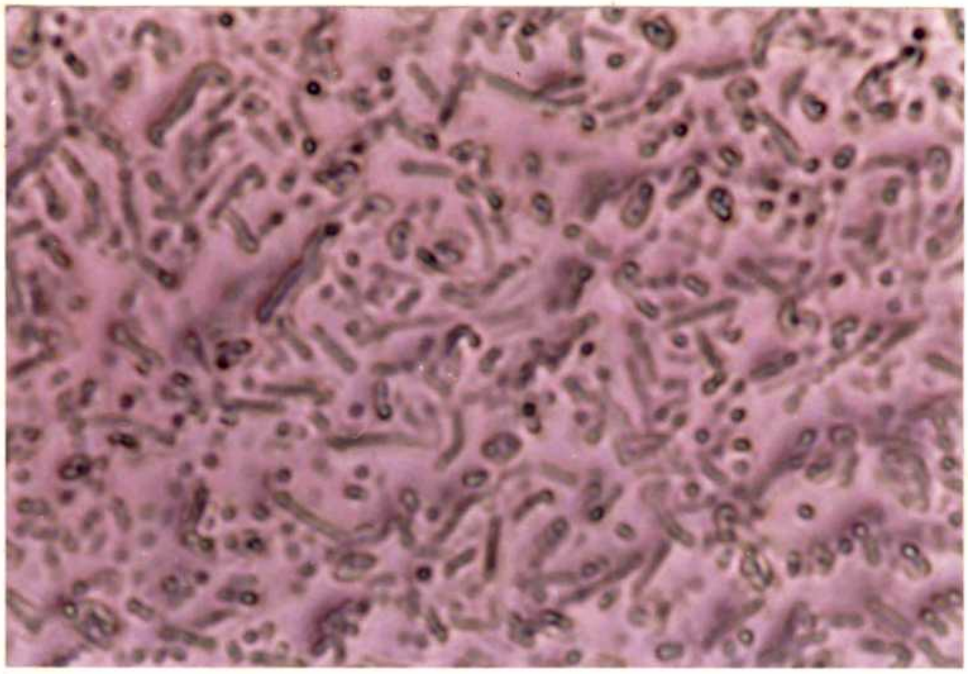
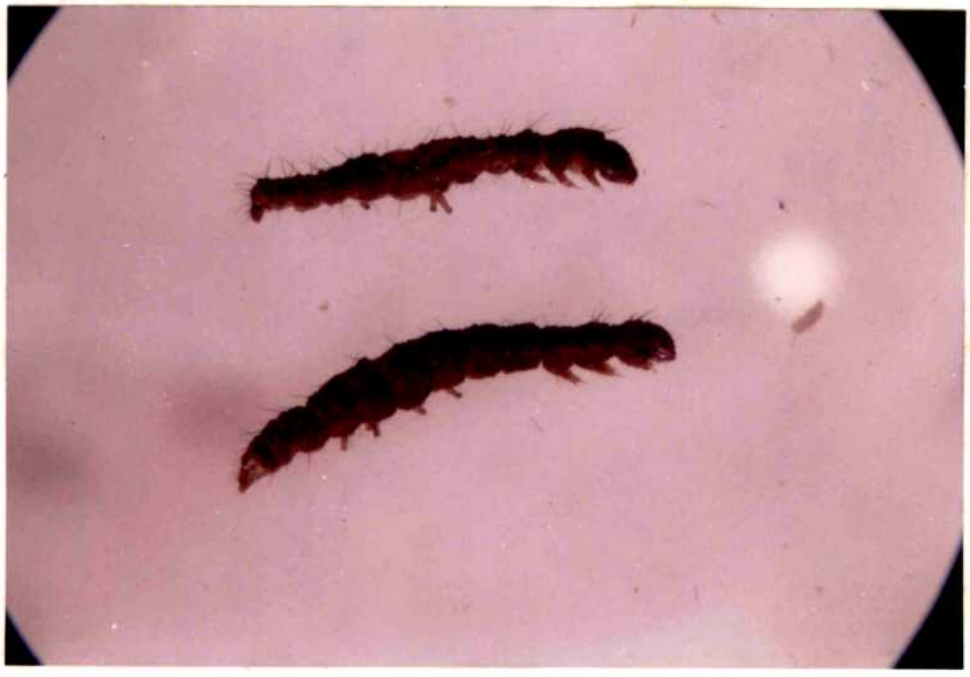


Plate 5. *Bacillus thuringiensis* cells showing spores and crystals

(X 1000)

Plate 6. III instar larvae of *Plutella xylostella* (Live)



III instar larvae of *Plutella xylostella* (Infected cadaver)

(MS24B1)

Plate 7. III instar larvae of *Cnaphalocrocis medinalis* (Live)

III instar larvae of *Cnaphalocrocis medinalis* (Infected cadaver)

(MS24B1)

Plate 8. III instar larvae of *Helicoverpa armigera* (Live)

III instar larvae of *Helicoverpa armigera* (Infected cadaver)

(MS24B1)

when mortality was observed in control Probit analysis was done to find out LC_{50} along with the fiducial limit (Finney, 1962)

3.4. Safety of selected *Bacillus thuringiensis* isolates on non-targeted organisms

The selected *Bacillus thuringiensis* isolates were tested for safety to some of the non targeted beneficial organism like silkworms, honey bees, *Chrysoperla carnea* (Lacewing – a predator of insect pests on agricultural crops) *Trichogramma chilonis* (egg parasite of many lepidopteron crop pests) and fish (common carp- an aquatic inhabitant) The bioassays were carried out as demonstrated by Lacey (1997)

3.4.1. On silkworms (*Bombyx mori)*

A bioassay experiment was conducted to find out the effect of selected *Bacillus thuringiensis* isolates on silk worm The third instar larvae of *Bombyx mori* were fed with mulberry leaves dipped in the suspensions selected *Bacillus thuringiensis* isolates (10^7 cells/ml) The treated mulberry leaves were airdried and cut before feeding the silk worms The treatments included seven isolates of *Bacillus thuringiensis* and the standard *Bacillus thuringiensis* sub sp *Kurstaki* H D-1 for reference, apart from untreated control Ten larvae per replication and three replications per treatment were maintained The observation was made and mortality was recorded at 8-h intervals The final mortality rate was assessed at the end of 72 h The silk worm larvae were reared on mulberry leaves The egg cards were obtained from the Department of Sericulture, TNAU, Coimbatore-3 to carry out this experiment

3.4.2. On Honey bees (*Apis mellifera*)

Neonates of *Apis mellifera* were tested for any susceptibility to the selected *Bacillus thuringiensis* isolates The treatment details were as in 3 4 1 and observed for any lethal effect The young workers (10 per replication) were released in to a petridish along with cotton wads dipped in sugar syrup inoculated with the isolates of *Bacillus thuringiensis* (10^7 cells per ml) as feed Mortality was recorded at 8h intervals for 72 h Three replications for each treatment along with control (uninoculated sugar syrup) were maintained for this experiment The neonates were obtained from the apiary, Department of Agricultural Entomology, TNAU, Coimbatore – 3

3.4.3. On *Trichogramma chilonis* (Egg Parasite)

The *Corcyra cephalonica* eggs pasted cards were sprayed with test cultures of *Bacillus thuringiensis* before and after parasitization by *Trichogramma chilonis* separately and observed for any change in the per cent emergence over untreated control The *Bacillus thuringiensis* cells harvested at the end of late log phase were washed and resuspended in sterile distilled water Cell concentration was adjusted to 10^7 per ml and sprayed on egg cards that were exposed to parasitization and egg cards (250-300 eggs per square cm) that were not exposed to parasitization by *Trichogramma chilonis* (and later exposed to parasitization) The per cent emergence from parasitized cards were recorded and compared with controls *Corcyra* eggs and *Trichogramma chilonis* for this experiment were obtained from the Bio control laboratory, Department of Agricultural Entomology, TNAU, Coimbatore

3.4.4. On *Chrysoperla carnea* (Predator)

The effect of *Bacillus thuringiensis* isolates on the predator of crop pests was assayed (the young larvae of *Chrysoperla* feed on eggs of lepidopteron crop pests) The adults (ten per replication and three replications for each treatment) were fed with artificial diet impregnated with the *Bacillus thuringiensis* isolates (10^7 cells per ml of diet) and observed for mortality Observations were recorded at 8 h interval for 72 hrs The *Chrysoperla* adults for this experiment were obtained from the Bio-control laboratory of the Department of Agricultural Entomology, TNAU, Coimbatore

3.4.5. On fish (*Cyprimus carpio*) (Aquatic Environment)

Even sized fishlings (5-6cm long) of common carp (*Cyprimus carpio*) obtained from the Department of Fisheries, Coimbatore were used in this study Commercially available granulated feed was mixed with each isolate of selected *Bacillus thuringiensis* separately to make a final cell concentration of 10^7 per g and used as feed Aquaria (plastic tubs) were filled with clear pond water (20 lit), fishlings were released (20 per treatment) and fed with *Bacillus thuringiensis* mixed artificial feed (1g per tub) three times on 0, 3rd and 6th day and later on fed with normal commercial feed The aquaria were aerated regularly, observed for 30 days for any mortality

3.5. Effect of *Bacillus thuringiensis* isolates on beneficial soil microorganisms

Safety of the isolates, which were selected for further studies were assessed against nontargeted beneficial microorganisms The microorganisms chosen were *viz*, *Azospirillum brasilense*, *Azotobacter chroococcum*, *Bacillus*

megaterium, *Pseudomonas striata* and *Rhizobium leguminosarum* The cultures were obtained from the microbial type culture collections of the Department of Agricultural Microbiology, TNAU, Coimbatore -3 The composition of all the culture media used in this study are presented in Appendix-I

Two different methods *viz* , 1) cross streak assay and 2) Gnotobiotic assay were employed for assessing the effect of selected *Bacillus thuringiensis* isolates on the bacterial bio fertilizers

3.5.1. Cross streak assay (Smibert and Kreig,1981)

A special medium – CSA medium was developed to facilitate even growth of all the cultures in the same petriplate The 24 h old bacterial cultures of *Azospirillum brasilense*, *Azotobacter chroococcum*, *Bacillus megaterium*, *Pseudomonas striata* and *Rhizobium leguminosarum* were streaked perpendicular to the *Bacillus thuringiensis* isolate which was already streaked as a short chord on the plated CSA medium After 48 h of incubation at 31^{0}C, observed for any inhibitory effect of *Bacillus thuringiensis* and other test cultures *vis-a-vis*

3.5.2. Gnotobiotic assay (Smibert and Kreig, 1981)

Sieved and air dried field soil rich in organic matter was taken up for this study Autoclavable polyurethane cups (10-cm ht 8-cm dia) were filled with 200 g of soil, covered and sterilized at 121°C for 20 min

Five experiments were carried out for the five biofertilizer agents in a phased manner Each experiment contained nine treatments, which included seven most efficient *Bacillus thuringiensis* isolates in combination with one of the

bio fertilizer agents The individual treatment of the bio fertilizer agent and *Bacillus thuringiensis* sub sp *kurstaki* H D-1 served as controls Five replications were maintained for each treatment The cultures were grown in respective mass multiplication media up to late log phase, harvested at 5000 rpm, washed, pellatized and resuspended in distilled water to get desired cell concentration The inocula contained 10^9 cells per ml The quantity of inoculum added in to each cup containing sterilized soil was 5 ml The moisture level was maintained at 65-70 per cent of maximum water holding capacity of the soil by adding sterile distilled water at regular intervals

Soil samples were drawn periodically at 20 days interval to enumerate the bacterial population for the assessment of saprophytic competence (survival) and compatibility The media used for enumerating *Azospirillum* was Dobereiner's malate medium, Waksmann-77 for *Azotobacter*, Pikovskaya's for *Bacillus megaterium* and *Pseudomonas striata* and congo red yeast extract mannitol agar for *Rhizobium* The population of *Azospirillum* was estimated by MPN technique (Cochran, 1950) Only the bacterial colonies with clear zones around in the Pikovskaya medium were counted as phosphobacteria

3 6 **Intrinsic antibiotic resistance of the *Bacillus thuringiensis* isolates** (Chugtai and Shakoori 1994)

All the selected *Bacillus thuringiensis* isolates were studied for their intrinsic resistance towards the following antibiotics *viz*, penicillin, erythromycin, tetracycline, chloramphenicol, gentamycin and streptomycin

Freshly prepared antibiotic solutions were filter sterilized, added to molten LB agar medium in sufficient quantities so as to get the following final concentrations *viz* , 10, 20, 30, 40, 80, 120, 160 and 200 ppm of all the antibiotics and plated in sterilized petridishes Selected isolates of *Bacillus thuringiensis* in the

mid log phase were inoculated with the help of a multiple inoculator and incubated at 31°C for 48 h The growth indicated the maximum tolerance limit The maximum tolerance levels were considered as the intrinsic resistance of that particular isolate to a particular antibiotic

3.7. Mutation studies on the selected *Bacillus thuringiensis* isolates

N-methyl-N'-nitro-N-nitroso guanidine (NMNG) was used to obtain a mutant from the isolates that were selected based on the effectiveness on the three target pests (seven isolates) A procedure devised by Adelberg *et al* (1965) was followed The methylating compound N-methyl-N'-nitro-N-nitrosoguanidine was made into a solution of 1000 ppm concentration and one ml of which was transferred to 9 ml mid log cultures of the *Bacillus thuringiensis* isolates and incubated at 31°C for half an hour without shaking Centrifuged and resuspended in UG medium, repeated the above process and plated

3.7.1. Assessment of pathogenicity and residual toxicity of the mutant strain of *Bacillus thuringiensis*:

Spraying of the parent strain of MS24B1 mutant and standard culture along with the mutant was carried out (10^7 spores per ml) on the potted plants (cauliflower, rice and cotton) Leaf samples were collected aseptically at 0h 48h 96h and 144h intervals and assayed for pathogenicity and residual toxicity against respective target pests Third instar larvae were used A total of 10 larvae per replication and three replications per treatment were kept Larvae of *Helicoverpa armigera* were confined individually to petridishes to avoid cannibalism The leaf samples were kept in petridishes with moistened filter paper to prevent drying Observations were made at the end of 72 h

3.8. Enumeration of phyllosphere micro organisms (Gardner and Hornby 1987)

Leaf samples were collected aseptically from the plants, transferred to conical flasks (500ml) containing 100 ml of sterile distilled water Vigorously agitated for 30 min at 31°C Serial dilutions was made up to 10^{-6} Dilutions 10^{-2} and 10^{-3} were plated in Ken Knight's agar and rose bengal agar media for enumerating actinomycetes and fungi respectively The dilution 10^{-6} was used for enumerating bacteria on nutrient agar medium The samplings were done i) before spraying *Bacillus thuringiensis* (MS24B1), ii) 0 day after spraying, iii) 10 days after spraying and iv) 20 days after spraying

The *Bacillus thuringiensis* (MS24B1) colonies were enumerated on streptomycin (160 ppm) impregnated nutrient agar medium The total colonies were enumerated and the results were expressed as cells (or) colony forming units per cm^2 after assessing the surface area of the leaf samples used Samples were collected from potted plants on 0 day (before and after treatmentat), 10^{th} day, 20^{th} day and 30^{th} day after spraying The samples were collected from the middle portion of the plants to minimize the dilution effect due to plant growth

3.9. Effect of two agricultural products and one biological waste on the growth of *Bacillus thuringiensis* under submerged conditions

Three different culture media were tested for mass multiplication of *Bacillus thuringiensis* with an aim of utilizing cheaper agricultural products and biological wastes as sources of carbon and nitrogen for minimizing the cost of inputs Comparison was made with UG broth for production capacity and for potency The following subsstsnces were tested *viz* , tapioca starch, defatted soy flour and basin water

3.9.1. Tapioca starch broth medium

Ten grams of tapioca starch 5 g of skimmed milk powder and 5 ml each of the three stock solutions of UG-medium constituted 1 litre of this medium in water. The pH was adjusted to 7.0 and sterilized at 121° c for 20 min. Composition of the stock solutions are presented in Appendix-I.

3.9.2. Defatted soy flour broth medium

This broth was made of 10g defatted soy flour with 5 ml each of the three stock solutions of UG-medium in one litre of water . The pH was adjusted to 7.0. Sterilized at 121°C for 20 min.

3.9.3. Basin water broth medium

From the silk reeling units, bave water, otherwise known as basin water rich in proteinaceous substances like serecin and fibroin was obtained from Sericulture Board reeling unit and made use of for the formulation of this medium. This culture broth contained 10 ml of bave water, 5 g of glucose and 5 ml each of the three stock solutions used for UG medium in one litre of water. The pH adjusted to 7.0 and sterilized at 121°C for 20 min. (The thick yellowish viscous fluid after the removal of silk and pupae will usually be disposed into drainage).

3.9.4. Growth conditions

Above media were prepared in 100-ml quantities along with 100 ml of UG broth. Inoculated with 1 ml of preculture of MS24BI having a cell concentration of 10^9 cells per ml. Five replications were maintained and incubated on a temperature controlled rotary shaker (100 rpm at 31°C) for 72 h. Samples of 1ml

quantity were drawn at 8h interval, serially diluted and plated for assessing the population growth Sporulation was monitored under phase contrast microscope Assessment of pathogenicity was carried out as described earlier in section 3 3 of this chapter

3.10. Development of a suitable formulation for *Bacillus thuringiensis*

Attempts were made to develop a suitable formulation using cheaper and easily available substances The materials used were compatible and were selected based on the findings of Heimpel (1967)

The best alternate culture medium *viz* , defatted soy flour medium was used to mass multiply *Bacillus thuringiensis* MS24BI isolate The cells (spore and crystals) were harvested after 12h in to the stationary phase by centrifuging at 10,000 rpm for 30 min Washed and resuspended the pellet in two volumes of sterile distilled water By using this suspension, 100 ml of vortexed mixture of 1 per cent carboxy methyl cellulose (sticking agent), 1 per cent Tween 20 (dispersing agent) and 1 per cent charcoal powder (UV-protectant), having 10^{12} spores per ml was prepared and thoroughly mixed with 500 g kaolinite clay (micronized, pharmaceutical grade) The final spore concentration of the formulation was 1 66 x 10^{11} per g This formulation was used for pathogenicity, residual toxicity and storage studies Compared with a popular commercial *Bacillus thuringiensis* formulation for pathogenicity and residual toxicity using the protocol described in sections 3 3 and 3 7 1 of this chapter A concentration of 2 5g formulation per litre was used

3.11. Storage studies

Portions of 100 g each of the formulation were stored at ambient temperature 31° C ± 5 ° C and at 4°C to evaluate the effect of storage on toxicity to target pests Samples from the stored formulations were drawn at 30 days interval and viable cell count was carried out as mentioned in the section 3 2 1 of this chapter Bioassays were done on silk worms to assess the effect of storage on toxicity of the formulation

3.12. Statistical analysis

Appropriate statistical designs like probit analysis (Finney,1962), factorial completely randomized block design and randomized block design (SPSS and IRRISTAT computer soft ware) were used.

RESULTS

RESULTS

4.1. Isolation of *Bacillus thuringiensis* from soil

Ninety-six isolates of *Bacillus thuringiensis* were isolated from the 25 soil samples by employing the isolation method demonstrated by Travers *et al* (1984) The isolates were designated as MS1B1 to MS25B7 - MS for mulberry soil (place code) and B for bacterial isolate The soil types were distinctly different, ranging from black to red and clayey loam to alluvial The pH of the soil samples ranged from 6 3 to 7 9 (Table 1)

4.2. Authentication the isolates

Parameters like shape, motility, Grams reaction, presence of spores and crystals, catalase activity, utilization of D-mannitol and Voges-Proskauer test for acetyl methyl carbinol (AMC) production were considered as criteria for authentication of *Bacillus thuringiensis* isolates Eighty isolates were confirmed as *Bacillus thuringiensis* (Table 2)

4.3. Screening for pathogenicity against target pests

The preliminary study on three targeted pests, *viz*, *Pluttella xylostella*, *Cnaphalocrocis medinalis* and *Helicoverpa armigera* with regard to pathogenicity revealed that, at 10^7 spores per ml concentration the isolates MS10B2, MS18B6, MS22BB and MS24B1 effected more than 65 per cent mortality on *Plutella xylostella* MS10B2, MS19B1, MS23B3 and MS24B1 effected 65 percent and more mortality on *Cnaphalocrocis medinalis* The isolates MS16B2, MS22B3, MS23B3 and MS24B1 effected 65 per cent and more mortality on *Helicoverpa*

Table 1. *Bacillus thuringiensis* isolates collected from mulberry grown areas of Coimbatore and Erode districts.

Sl No	Name of the place	code	Soil type	pH	No of isolates
1	Aappakudal	MS 1	Black	7 2	3
2	Darmaapuri	MS 2	Clayey loam	7 4	4
3	Dharapuram	MS 3	Red	6 6	3
4	Kavundapadi	MS 4	Alluvial	6 8	3
5	Komangalam	MS 5	Alluvial	7 1	3
6	Kothamangalam	MS 6	Red	6 4	7
7	Kovanur(site-1)	MS 7	Black soil	7 6	3
8	Kovanur(site-2)	MS 8	Black soil	7 6	2
9	Kudalur Kavundampalayam(site-1)	MS 9	Alluvial	6 8	5
10	Kudalur Kavundampalayam(site-2)	MS 10	Alluvial	6 8	4
11	Kundadam	MS 11	Clay	6 9	2
12	Mekinar	MS 12	Black	7 4	3
13	Metrathi	MS 13	Alluvial	7 9	3
14	Narasimmanaickenpalayam(site-1)	MS 14	Clayey loam	7 6	3
15	Narasimmanaickenpalayam(site-2)	MS 15	Clayey loam	7 2	4
16	Poorandapalayam	MS 16	Alluvial	6 9	5
17	Ramakrishna foundary(site-1)	MS 17	Black	6 8	3
18	Ramakrishna foundary(site-2)	MS 18	Black	6 7	6
19	Senjerimalai	MS 19	Red	6 4	3
20	Sulthanpet	MS 20	Red	6 4	5
21	TNAU(site-1)	MS 21	Clayey loam	6 3	4
22	TNAU(site-1)	MS 22	Alluvial	6 8	4
23	Thali	MS 23	Red	6 9	3
24	Thamaraikulam	MS 24	Alluvial	7 2	4
25	Thottipalayam	MS 25	Red	7 1	7
	TOTAL				**96**

Table 2. Authentication of *Bacillus thuringiensis* isolates

Sl No	Culture code	Shape	Motility	Gram's	Spores	Crystals	Catalase activity	Growth on mannitol	V-P Test for AMC
1	MS1 B1	Short rod	Motile	+	+	-	+	-	+
2	MS1 B2	Rod	Motile	+	+	-	-	+	-
3	MS1 B3	Rod	Motile	+	+	+	+	-	+
4	MS2 B1	Rod	Motile	+	+	+	+	-	+
5	MS2 B2	Rod	Motile	+	+	+	+	-	+
6	MS2 B3	Rod	Motile	+	+	+	+	-	+
7	MS2 B4	Rod	Motile	+	+	+	+	-	+
8	MS3 B1	Short rod	Motile	+	+	+	+	-	+
9	MS3 B2	Rod	Motile	+	+	+	+	-	+
10	MS3 B3	Rod	Motile	+	+	+	+	-	+
11	MS4 B1	Rod	Motile	+	+	+	+	-	+
12	MS4 B2	Rod	Motile	+	+	+	+	-	+
13	MS4 B3	Rod	Motile	+	+	+	+	-	+
14	MS5 B1	Rod	Motile	+	+	+	+	-	+
15	MS5 B2	Rod	Motile	+	+	+	+	-	+
16	MS5 B3	Short rod	Non-motile	+	+	-	-	+	-
17	MS6 B1	Rod	Motile	+	+	+	+	-	+
18	MS6 B2	Rod	Motile	+	+	+	+	-	+
19	MS6 B3	Rod	Motile	+	+	+	+	-	+
20	MS6 B4	Rod	Motile	+	+	+	+	-	+
21	MS6 B5	Rod	Motile	+	+	+	+	-	+
22	MS6 B6	Rod	Motile	+	+	+	+	-	+
23	MS6 B7	Short rod	Motile	+	+	+	+	-	+
24	MS7 B1	Rod	Motile	+	+	+	+	-	+
25	MS7 B2	Rod	Motile	+	+	-	-	-	+
26	MS7 B3	Rod	Motile	+	+	+	+	-	+
27	MS8 B1	Rod	Motile	+	+	+	+	-	+
28	MS8 B2	Rod	Motile	+	+	+	+	-	+
29	MS9 B1	Rod	Motile	+	+	+	+	-	+
30	MS9 B2	Rod	Motile	+	+	+	+	-	+
31	MS9 B3	Rod	Motile	+	+	+	+	-	+
32	MS9 B4	Rod	Motile	+	+	+	+	-	+

Table 2. (Contd.)

Sl No	Culture code	Shape	Motility	Gram's	Spores	Crystals	Catalase activity	Growth on mannitol	V-P Test for AMC
33	MS9 B5	Rod	Motile	+	+	+	+	-	+
34	MS10 B1	Short rod	Non-motile	-	-	-	-	+	-
35	MS10 B2	Rod	Motile	+	+	+	+	-	+
36	MS10 B3	Rod	Motile	+	+	+	+	-	+
37	MS10 B4	Rod	Motile	+	+	-	+	+	-
38	MS11 B1	Rod	Motile	+	+	-	+	-	-
39	MS11 B2	Rod	Motile	+	+	+	+	-	+
40	MS12 B1	Rod	Motile	+	+	+	+	-	+
41	MS12 B2	Rod	Motile	+	+	-	+	-	+
42	MS12 B3	Rod	Motile	+	+	+	+	-	+
43	MS13 B1	Rod	Motile	+	+	+	+	-	+
44	MS13 B2	Rod	Motile	+	+	+	+	-	+
45	MS13 B3	Rod	Motile	+	+	+	+	-	+
46	MS14 B1	Rod	Motile	+	+	+	+	-	+
47	MS14 B2	Rod	Motile	+	+	+	+	-	+
48	MS14 B3	Rod	Motile	+	+	+	+	-	+
49	MS15 B1	Rod	Motile	+	+	+	+	-	+
50	MS15 B2	Rod	Motile	+	+	+	+	-	+
51	MS15 B3	Rod	Motile	+	+	+	+	-	+
52	MS15 B4	Rod	Motile	+	+	+	+	-	+
53	MS16 B1	Rod	Motile	+	+	+	+	-	+
54	MS16 B2	Rod	Motile	+	+	+	+	-	+
55	MS16 B3	Rod	Motile	+	+	+	+	-	+
56	MS16 B1	Rod	Motile	+	+	+	+	-	+
57	MS16 B2	Rod	Motile	+	+	+	+	-	+
58	MS17 B1	Rod	Motile	+	+	+	+	-	+
59	MS17 B2	Rod	Motile	+	+	+	+	-	+
60	MS17 B3	Long rod	Motile	+	+	-	+	+	+
61	MS18 B1	Rod	Motile	+	+	+	+	-	+
62	MS18 B2	Rod	Motile	+	+	+	+	-	+
63	MS18 B2	Rod	Motile	+	+	+	+	-	+
64	MS18 B4	Rod	Motile	+	+	+	+	-	+

Table 2. (Contd.)

Sl No	Culture code	Shape	Motility	Gram's	Spores	Crystals	Catalase activity	Growth on mannitol	V-P Test for AMC
65	MS18 B5	Rod	Motile	+	+	+	+	-	+
66	MS18 B6	Rod	Motile	+	+	+	+	-	+
67	MS19 B1	Rod	Motile	+	+	+	+	-	+
68	MS19 B2	Rod	Motile	+	+	+	+	-	+
69	MS19 B3	Short rod	Non motile	+	+	-	+	-	+
70	MS20 B1	Rod	Motile	+	+	+	+	-	+
71	MS20 B2	Long rod	Motile	+	+	-	-	+	-
72	MS20 B3	Short rod	Motile	+	+	-	-	+	-
73	MS20 B4	Rod	Motile	+	+	+	+	-	+
74	MS20 B5	Rod	Motile	+	+	+	+	-	+
75	MS21 B1	Rod	Motile	+	+	+	+	-	+
76	MS21 B2	Rod	Motile	+	+	-	+	-	+
77	MS21 B3	Rod	Motile	+	+	+	+	-	+
78	MS21 B4	Rod	Motile	+	+	+	+	-	+
79	MS22 B1	Rod	Motile	+	+	+	+	-	+
80	MS22 B2	Rod	Motile	+	+	+	+	-	+
81	MS22 B3	Rod	Motile	+	+	+	+	-	+
82	MS22 B4	Rod	Motile	+	+	+	+	-	+
83	MS23 B1	Rod	Motile	+	+	+	+	-	+
84	MS23 B2	Short rod	Non-motile	+	+	-	-	+	-
85	MS23 B3	Rod	Motile	+	+	+	+	-	+
86	MS24 B1	Rod	Motile	+	+	+	+	-	+
87	MS24 B2	Rod	Motile	+	+	+	+	-	+
88	MS24 B3	Rod	Motile	+	+	+	+	-	+
89	MS24 B4	Rod	Motile	+	+	+	+	-	+
90	MS25 B1	Rod	Motile	+	+	+	+	-	+
91	MS25 B2	Rod	Motile	+	+	+	+	-	+
92	MS25 B3	Long rod	Motile	-	-	-	-	+	-
93	MS25 B4	Rod	Motile	+	+	+	+	-	+
94	MS25 B5	Rod	Motile	+	+	+	+	+	+
95	MS25 B6	Long rod	Motile	+	+	-	+	+	-
96	MS25 B7	Rod	Motile	+	+	+	+	+	+

armigera These isolates were taken up for further studies In all seven isolates were selected in such a way that, they were grouped as i the most effective isolate on all the three target pests (1), ii most effective isolates with host spectrum for any two of the targeted pests (3) and iii most effective isolates on individual target pests (3)

The isolate MS24B1 was found to be the most effective on all the three target pests, *i e*, it effected 68 9 per cent, 65 5 per cent and 67 9 per cent on *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* respectively Isolate MS10B2 was found to have effective control over *Plutella xylostella* and *Cnaphalocrocis medinalis* The isolate MS22B3 was very effective on *Plutella xylostella* and *Helicoverpa armigera* where as MS23B3 was effective against *Cnaphalocrocis medinalis* and *Helicoverpa armigera* The MS10B2 effected 65 5 per cent and 67 9 mortality on *Cnaphalocrocis medinalis* and *Plutella xylostella* respectively The isolate MS22B3 was found to effect 70 3 per cent and 67 9 per cent mortality on *Plutella xylostella* and *Helicoverpa armigera* respectively The mortality percentage effected by MS23B3 was 68 9 per cent on *Cnaphalocrocis medinalis* and 67 9 per cent on *Helicoverpa armigera* The isolate MS18B6 effected 71 4 per cent mortality on *Plutella xylostella* The most effective isolate on *Cnaphalocrocis medinalis* was MS19B1, which effected 68 9 per cent mortality MS16B2 effected 67 9 per cent mortality on *Helicoverpa armigera* (Table 3)

4.4. Assessment of Lethal concentration (LC_{50}) of the selected isolates

The LC_{50} of the selected isolates on the target pests was analysed by bioassay techniques The probit analysis revealed that the lethal concentration (LC_{50}) of all the selected isolates for *Plutella xylostella* was biologically more or

Table 3. Effect of *Bacillus thuringiensis* isolates on the target pests

Sl. No	Culture code	Per cent mortality		
		Plutella xylostella (10^7 spores perml)	*Cnaphalocrocis medinalis* (10^7 spores per ml)	*Helicoverpa armigera* (10^7spores per ml)
1	MS1 B1	26 6	28 6	27 6
2	MS2 B1	30 4	28 6	44 8
3	MS2 B2	34 2	35 8	51 7
4	MS2 B3	26 6	28 6	44 8
5	MS3 B1	30 4	42 8	44 8
6	MS3 B2	41 8	57 2	37 9
7	MS3 B3	57 0	35 8	27 6
8	MS3 B4	38 0	28 6	44 8
9	MS4 B1	30 4	28 6	55 1
10	MS4 B2	26 6	35 8	37 9
11	MS5 B1	51 8	48 3	28 6
12	MS5 B2	40 7	44 8	28 6
13	MS6 B1	37 0	27.6	35 8
14	MS6 B2	51 8	44 8	42 8
15	MS6 B3	40 7	44 8	28 6
16	MS6 B4	55 6	27 6	42 8
17	MS6 B5	48 1	27 6	35 8
18	MS6 B6	37 0	58 6	28 6
19	MS6 B7	40 7	51 7	28 6
20	MS7 B1	44 4	27 6	35 8
21	MS7 B3	53 5	48 3	55 1
22	MS8 B1	57 2	44 8	58 6
23	MS8 B2	35 8	37 9	37 9
24	MS9 B1	50 0	37 9	37 9
25	MS9 B2	39 2	27 6	58 6
26	MS9 B3	28 6	37 9	27 6
27	MS9 B4	25 1	27 6	27 6
28	MS9 B5	25 1	27 6	34 4
29	MS10 B1	53 5	48 3	44 8
30	**MS10 B2**	**67.9**	**65.5**	48 3

Table 3. (Contd.)

Sl. No.	Culture code	Per cent mortality		
		Plutella xylostella 10^7 spores perml	*Cnaphalocrocis medinalis* 10^7 spores per ml	*Helicoverpa armigera* 10^7 spores per ml
31	MS10 B3	44.4	41.4	31.0
32	MS11 B2	40.7	41.4	37.9
33	MS12 B1	37.0	34.4	55.1
34	MS12 B3	55.6	27.6	31.0
35	MS13 B1	44.4	34.4	31.0
36	MS13 B2	37.0	34.4	44.8
37	MS13 B3	33.3	27.6	44.8
38	MS14 B1	37.0	41.4	37.9
39	MS14 B2	40.7	48.3	31.0
40	MS14 B3	51.8	55.1	37.9
41	MS15 B1	51.7	42.8	39.2
42	MS15 B2	58.6	42.8	32.1
43	MS15 B3	44.8	28.6	46.5
44	MS15 B4	51.7	50.0	53.5
45	MS16 B1	54.4	28.6	53.5
46	**MS16 B2**	48.3	42.8	**67.9**
47	MS16 B3	27.6	28.6	46.5
48	MS16 B4	41.4	28.6	53.5
49	MS16 B5	48.3	35.8	46.5
50	MS17 B1	51.7	42.8	35.8
51	MS17 B2	42.8	27.6	41.4
52	MS18 B1	32.1	27.6	31.0
53	MS18 B3	46.5	37.9	31.0
54	MS18 B4	50.0	44.8	24.1
55	MS18 B5	46.5	37.9	24.1
56	**MS18 B6**	**71.4**	51.7	51.7
57	**MS19 B1**	53.3	**68.9**	51.7
58	MS19 B2	46.5	55.1	51.7
59	MS20 B1	42.8	55.1	41.4
60	MS20 B4	35.8	31.0	31.0

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Table 3. (Contd.)

Sl No.	Culture code	Per cent mortality		
		Plutella xylostella 10^7 spores perml	*Cnaphalocrocis medinalis* 10^7 spores per ml	*Helicoverpa armigera* 10^7 spores per ml
61	MS20 B5	44 4	48 3	42 8
62	MS21 B1	37 0	41 4	35 8
63	MS21 B3	48 1	34 4	50 0
64	MS21 B4	40 7	48 3	42 8
65	MS22 B1	40 7	34 4	57 2
66	MS22 B2	48 1	27 6	35 8
67	**MS22 B3**	**70.3**	58 6	**67.9**
68	MS22B4	37 0	27 6	28 6
69	MS23 B1	59 2	48 3	42 8
70	**MS23 B3**	55 6	**68.9**	**67.9**
71	**MS24 B1**	**68.9**	**65.5**	**67.9**
72	MS24 B2	27 6	48 3	46 5
73	MS24 B3	48 3	48 3	46 5
74	MS24 B4	58 6	48 3	46 5
75	MS25 B2	34 4	41 4	39 2
76	MS25 B3	55 1	41 4	39 2
77	MS25 B4	44 8	48 3	46 5
78	MS25 B5	48 3	31 0	28 6
79	MS25 B6	31 0	41 4	39 2
80	MS25 B7	44 8	24 1	25 1

less equal though statistically significant The best isolate based on the LC_{50} value for controlling *Plutella xylostella* was MS18B6 (1 374 x 10^6 spores per ml) but was slightly inferior to the reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D-1 (1 30 x 10^6 spores per ml) The isolate MS16B2 had the highest LC_{50} value towards *Plutella xylostella* (Table 4)

The best isolate for the control of *Cnaphalocrocis medinalis* was found out to be MS 23B3 as its LC_{50} was 1 382 x 10^6 spores per ml of spray fluid The second best isolate was MS19B1 which had a LC_{50} value of 1 527 x 10^6 spores per ml The standard *Bacillus thuringiensis* sub sp *Kurstaki* H D-1 culture was found to be superior to the above isolates statistically, the least effective culture was MS16B2 (Table 5)

Of the 7 isolates tested against *Helicoverpa armigera*, the isolate MS16B2 (1 085 x 10^6 spores per ml) was found out to be the best isolate The isolate MS10B2 had a LC_{50} value of 3 223 spores per ml that was the highest recorded LC_{50} value in this particular experiment The reference culture *Bacillus thuringiensis* sub sp *kurstaki* HD-1 was superior because of the least LC_{50} value (1 074 x 10^6 spores per ml) (Table 6)

4.5. Safety and compatibility of the selected *Bacillus thuringiensis* isolates

The selected isolates were studied for safety towards some of the nontargeted beneficial organisms

TABLE. 4. Assessment of LC_{50} of the selected *Bacillus thuringiensis* isolates for *Plutella xylostella* by leaf dip method

Sl. No	*B.t.* Isolates	No. of Larvae	$X^2_{(N-2)}$	b ± SE	LC_{50}	Fiducial limit	
						Lower	upper
1	MS10B2	150	0 0456[3]	0 403 ± 0 044	1 437x10^6	6 14X10^5	2 89X10^6
2	MS16B2	150	0 661[3]	0 439 ± 0 0468	1 595x10^6	7 45X10^5	3 03X10^6
3	MS18B6	150	1 636[3]	0 356 ± 0 0437	1 374x10^6	5 15X10^5	3 00X10^6
4	MS19B1	150	1 289[3]	0 393 ± 0.445	1 560x10^6	6 59X10^5	3 16X10^6
5	MS22B3	150	4 668[3]	0 359 ± 0 044	1 400x10^6	5 40X10^5	3 02X10^6
6	MS23B3	150	2 702[3]	0 380 ± 0 0442	1 500x10^6	6 11X10^5	3 12X10^6
7	MS24B1	150	0 211[3]	0 427 ± 0 0456	1 419x10^6	6 37X10^5	2 76X10^6
8	*B t-k* (HD-1)	150	1 033[3]	0 408 ± 0 045	1 300x10^6	5 55X10^5	2 61X10^6

TABLE 5. Assessment of LC_{50} of the selected *Bacillus thuringiensis* isolates for *Cnaphalocrocis medinalis* by leaf dip assay

Sl. No.	*B. t* Isolates	No. of Larvae	$X^2_{(N-2)}$	b ± SE	$LC_{50} \times 10^6$	Fiducial limit	
						Lower	Upper
1	MS10B2	150	0 316	0 394 ± 0 044	1 956	$8\ 540 \times 10^5$	$3\ 92 \times 10^6$
2	MS16B2	150	1 215	0 408 ± 0 044	2 858	$13\ 40 \times 10^5$	$5\ 55 \times 10^6$
3	MS18B6	150	0 537	0 393 ± 0 044	2 381	$10\ 06 \times 10^5$	$4\ 76 \times 10^6$
4	MS19B1	150	0 402	0 401 ± 0 045	1 527	$6\ 544 \times 10^5$	$3\ 07 \times 10^6$
5	MS22B3	150	1 270	0 401 ± 0 044	1 968	$8\ 721 \times 10^5$	$3\ 91 \times 10^6$
6	MS23B3	150	0 154	0 386 ± 0 044	1 382	$5\ 642 \times 10^5$	$2\ 86 \times 10^6$
7	MS24B1	150	0 436	0 386 ± 0 044	1.956	$8\ 560 \times 10^5$	$3\ 92 \times 10^6$
8	*Bt-k*(HD-1)	150	0 201	0 362 ± 0 044	1 332	$5\ 056 \times 10^5$	$2\ 88 \times 10^{65}$

TABLE 6. Assessment of LC_{50} of the selected *Bacillus thuringiensis* isolates for *Helicoverpa armigera* by diet incorporation method

Sl. No.	*B. t.* Isolates	No. of Larvae	$X^2_{(N-2)}$	b ± SE	LC_{50} x10^6	Fiducial limit	
						Lower	Upper
1	MS 10 B2	150	1 076	0 411 ± 0 044	3 223	15 36 x 10^5	6 23 x 10^6
2	MS16B2	150	1 500	0 400 ± 0 045	1 085	4 40 x 10^5	2 23 x 10^6
3	MS 18 B6	150	1 163	0 417 ± 0 044	2 800	13 32 x 10^5	5 38 x 10^6
4	MS 19 B1	150	1 972	0 387 ± 0 044	1 548	6 42 x 10^5	3 18 x 10^6
5	MS 22 B3	150	2 009	0 394 ± 0 045	1 222	4 978 x 10^5	2 51 x 10^6
6	MS23 B3	150	2 967	0 356 ± 0 044	1 331	4 93 x 10^5	2 91 x 10^6
7	MS 24 B1	150	1 175	0 389 ± 0 044	1 373	5 62 x 10^5	2 83 x 10^6
8	*Bt-k* (HD-1)	150	4 101	0 377 ± 0 044	1 074	4 08 x 10^5	2 29 x 10^6

4.5.1. On Silk worms (*Bombyx mori*)

The isolate MS23B3 was found to be less toxic even though all the isolates tested including references *Bacillus thuringiensis* sub sp *kurstaki* H D-1 culture were highly toxic The per cent mortality ranged from 86 95 per cent to 100 per cent at 10^7 spores per ml MS10B2 and *Bacillus thuringiensis* sub sp *kurstaki* H D-1 effected 100 per cent mortality within hours of treatment (Table 7, Fig 1) The values were corrected according to Abbot's formula as mortality was observed in controls

4.5.2. On Honey bees (*Apis mellifera*)

Selected *Bacillus thuringiensis* isolates were studied for safely towards honey bees (newly hatched adult workers) The isolates at a concentration of 10^7 spores per ml of feed (sugar syrup) effected very low mortality which ranged from 0 00 to 7 96 per cent The isolate MS19B1 effected 7 96 per cent, where as MS10B2 and MS24B1 effected no mortality at all confirming its absolute safety towards honey bees The standard reference *Bacillus thuringiensis* sub sp *kurstaki* H D-1 effected 6 25 per cent at the end of 72 h (Table 8, Fig 2) The values were corrected according to Abbot's formula as mortality was observed in control also

4.5.3. On *Trichogramma chilonis* (Egg parasite)

The experiment revealed that there was no deleterious effect on the emergence of egg parasite from the *Corcyra* eggs that were sprayed with the selected *Bacillus thuringiensis* isolates before and after parasitization The percentage emergence of *Trichogramma chilonis* was between 86 4 to 92 0 and 89 6 to 94 6 in the treatments where spraying was done before parasitization and

Table 7. Effect of selected *Bacillus thuringiensis* isolates on silk worm larvae *Bombyx mori*

Sl No	Treatment	Per cent mortality at the end of 72 h	
1	MS10B2	100 00	
2	MS16B2	95 65	
3	MS18B6	91 30	
4	MS19B1	95 65	
5	MS22B3	91 30	
6	MS23B3	86 95	
7	MS24B1	95 65	
8	*B t k* HD-1	100 00	
		S E D	CD (P=0 05)
		1 0000	2.1789

Conc of *B thuringiensis* in the suspension = 10^7 spores per ml
Abbot's correction made

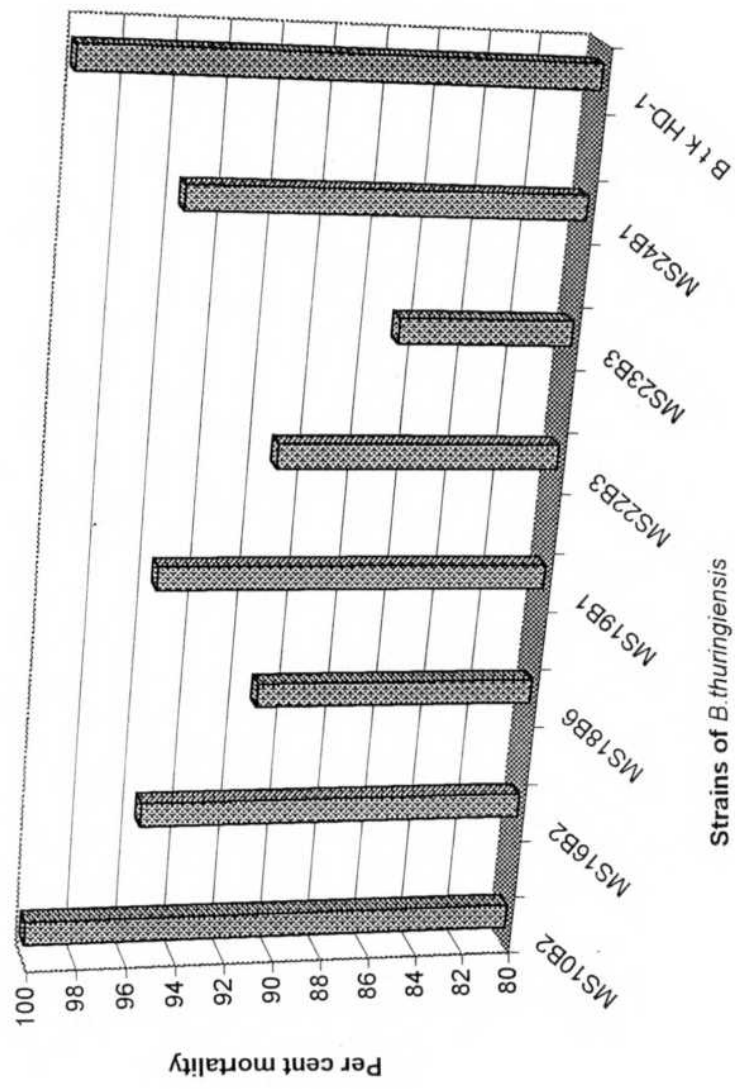


Fig 1 . Effect of selected *Bacillus thuringiensis* isolates on silkworm larvae (*Bombyx mori*)

Table 8. Effect of selected *Bacillus thuringiensis* isolates on newly hatched honey bees (workers):*Apis mellifera*

Sl No	Treatment	Per cent mortality at the end of 72 h	
1	MS10B2	0 00	
2	MS16B2	2 08	
3	MS18B6	6 25	
4	MS19B1	7 96	
5	MS22B3	3 08	
6	MS23B3	6 25	
7	MS24B1	0 00	
8	B t k (H D-1)	6 25	
		S E D=0 0760	CD=0 1722

Conc of *B thuringiensis* in feed (sugar syrup) 10^7 spores per ml
Abbot's correction carried out

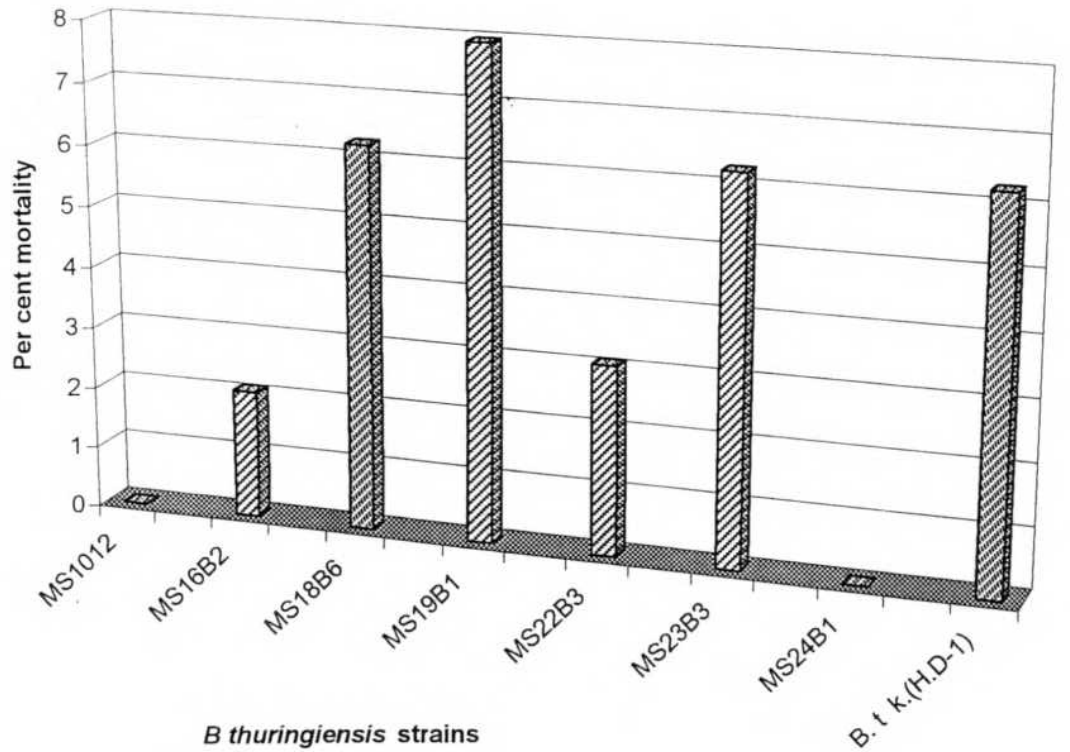


Fig 2. Effect of selected *Bacillus thuringiensis* **isolates on honey bees (***Apis mellifera* **)**

after parasitization respectively The isolate MS24B1 effected the least mortality where the percentage of emergence recorded was 92 0 A slight increase of 0-5 4 percent in the emergence of *Trichogramma chilonis* was observed in all the treatments where spraying was given after parasitization when compared to the treatments where spraying was given prior to parasitization (Table 9, Fig 3)

4.5.4. *Chrysoperla carnea* (Predator on eggs and adults of insect pests)

The selected isolates were tested for safety towards the insect predator *Chrysoperla* that is commonly referred as green lacewing Larvae of this insect feed on the eggs of most of the lepidopteran crop pests The selected isolates of *Bacillus thuringiensis* effected 0 to 10 37 percent mortality in the case of larvae fed with treated *Corcyra* eggs (10^7 spores per CC of eggs) The standard reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D-1 and MS22B3 effected 10 37 per cent mortality MS19B1 and MS24B1 found to be safest strains, as they effected no mortality

The adult lacewings were comparatively unaffected by most of the strains The isolated MS10B2 effected 6 66 percent and MS22B3 effected 3 33 per cent mortality All other tested isolates including the reference culture effected no mortality at the end of 72 h at a final concentration of 10^7 spores per ml of the artificial diet (Table 10, Fig 4) The results were corrected according to Abbot's formula as the controls recorded death of a few test organisms

4.5.5. Fish (*Cyprinus carpio* - commom carp)

The high efficiency isolates were tested for safety in aquatic environment The freshwater fish, common carp (*Cyprinus carpio*), was taken as a representative of aquatic environment and studied for any effects of the *Bacillus*

Table 9. Effect of selected *Bacillus thuringiensis* isolates on egg parasite *Trichogramma chilonis*

Sl. No.	Treatment	Per cent emergence at the end of 72h (Spraying before parasitization)		Per cent emergence Spraying before (Spraying after parasitization)	
1	MS10B2	87 0		89.6	
2	MS16B2	88 6		94.0	
3	MS18B6	88 0		91.6	
4	MS19B1	89.0		91.2	
5	MS22B3	86.4		89.4	
6	MS23B3	89.6		89.6	
7	MS24 B1	92.0		94.0	
8	B.t k (H D 1)	92.0		92 0	
		S E D	CD (P=0 05)	S E D	CD (P=0 05)
		0 9789	2 1330	0 9789	2 1330

Conc of *B thuringiensis* in suspension 10^7 spores per ml
Abbot,s correction carried out

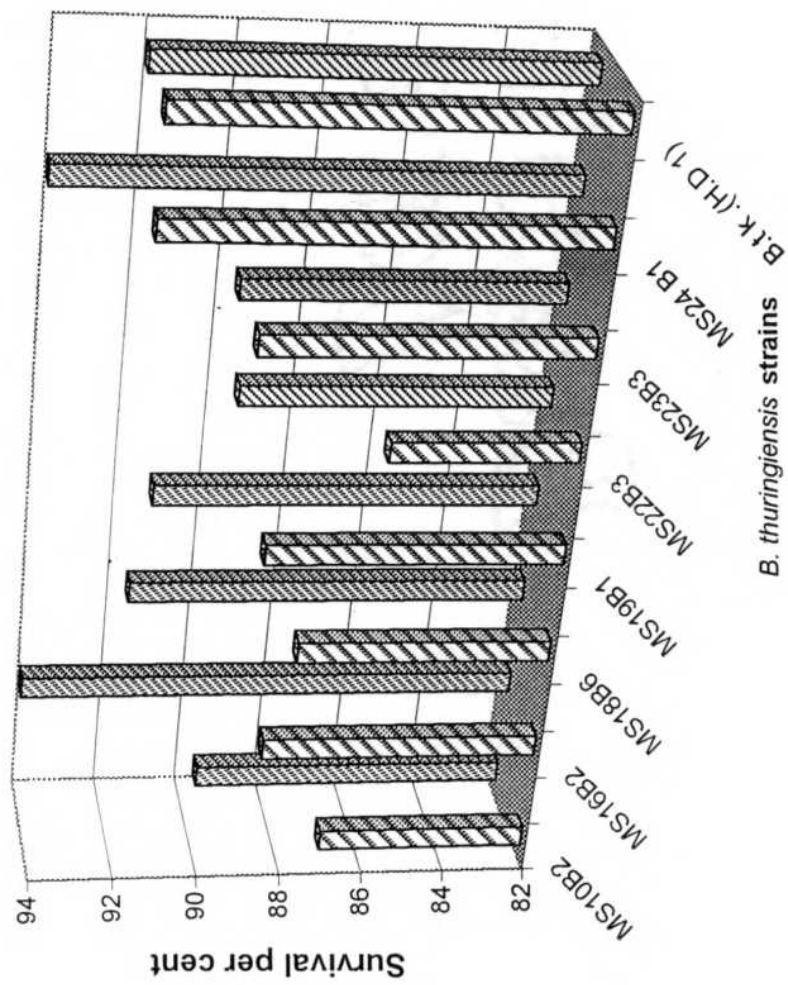


Fig 3. Effect of selected *Bacillus thurigiensis* isolates on *Trichogramma chilonis*

Table 10. Effect of selected *Bacillus thuringiensis* isolates on the inset pest predator *Chrysoperla carnea*

Sl No	Treatment	Per cent mortality at the end of 72 h (Larvae)		Per cent mortality at the end of 72 h (Adults)	
1	MS10B2	4 36		6 66	
2	MS16B2	6 92		0 00	
3	MS18B6	4 36		0 00	
4	MS19B1	0 00		0 00	
5	MS22B3	10 37		3 33	
6	MS23B3	6 92		0 00	
7	MS24B1	0 00		0 00	
8	*B t k*(HD-1)	10 37		0 00	
		S E D	CD (P=0 05)	S E D	CD (P=0 05)
		0 2338	0 5203	0 2082	0 4536

Final larval feed conc of *B thuringiensis* 10^7 spores per cc of *Corcyra* eggs
Adult feed Conc of *B thuringiensis* 10^7 spores per ml of artificial diet
Abbot's correction carried out

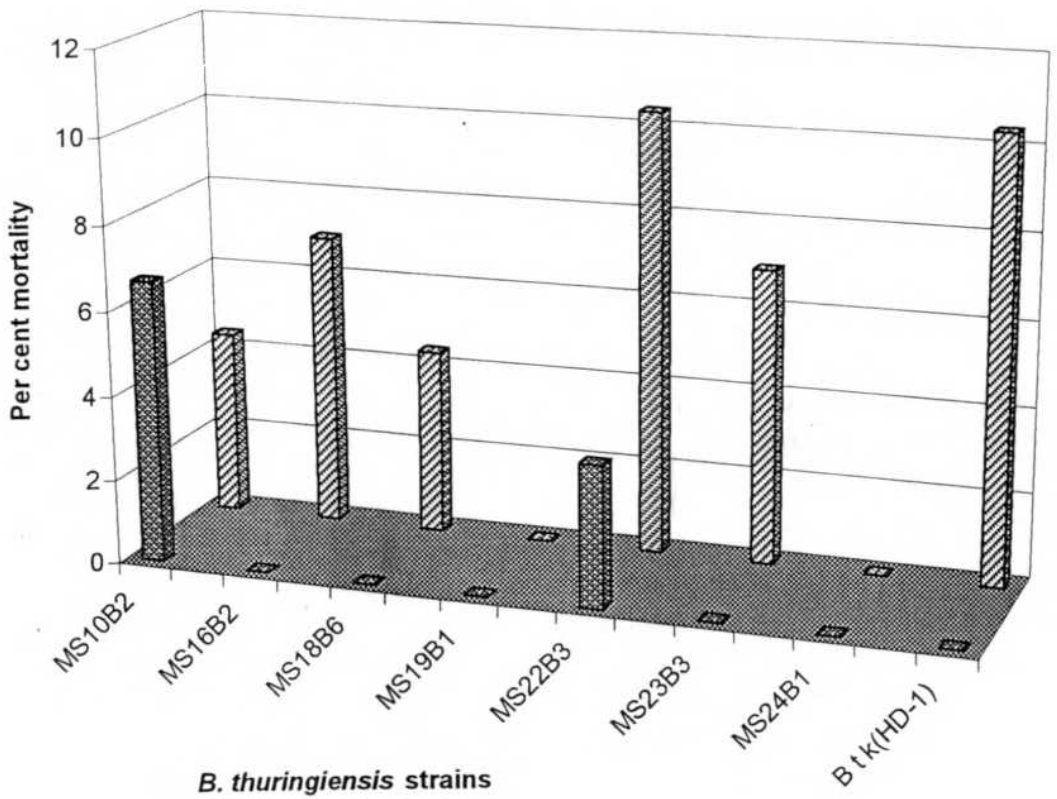


Fig 4. Effect of selected *Bacillus thuringiensis* isolates on *Chrysoperla carnea*

thuringiensis isolates with the standard reference culture Exposure to contaminated feed (10^7 spores per gram of artificial feed) did not affect the survival of the fish in aquaria for 30 days Though there was 0 to 8 percent mortality (after making corrections according to Abbot's formula) the isolates were not alarmingly toxic to fish Five of the 8 cultures tested effected less than 5 percent mortality MS24B1 effected no mortality where as the reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D -1 effected 8 percent mortality (Table 11) Data corrected according to Abbot's formula

4.6. Interaction of selected *Bacillus thuringiensis* with microbial biofertilizer agents

The experimental results revealed that there was no antagonistic effect between the isolates and test cultures (Plate.9) The interaction between selected *Bacillus thuringiensis* isolates and the biofertilizer inoculants was studied by cross streak assay.

4.6.1. Azospirillum brasilense (Bio fertilizer- associative symbiont)

The effect of *Bacillus thuringiensis* isolates on the population dynamics of *Azospirillum brasilense* and *vice versa* was studied under sterile soil conditions There was a statistically significant reduction in population of *Azosipirillum brasilense* and the *Bacillus thuringiensis* isolates over a period of time (Table 12) There was also significant variation in population dynamics between treatments A significant decrease in the population dynamics of *Bacillus thuringiensis* sub sp *kurstaki* H D–1 and *Azospirillum brasilense* was observed individually as in the case of combined inoculation over a period of time In all the treatments there was a 15 to 30 fold decrease from initial population at the end of 60 days

Table 11. Effect of selected *Bacillus thuringiensis* isolates on fish *Cyprimus carpio* (Common carp)

Sl No	Treatment	Per cent survival of fish fed with contaminated feed at the end of 30 days	
1	MS10B2	96	
2	MS16B2	92	
3	MS18B6	92	
4	MS19B1	96	
5	MS22B3	96	
6	MS23B3	100	
7	MS24B1	98	
8	*B t k* (H D-1)	92	
		S E D	CD (P=0 05)
		1 0911	2 3773

Concentration of *B thuringiensis* in feed 10^7 spores per g feed
Abbot's correction carried out

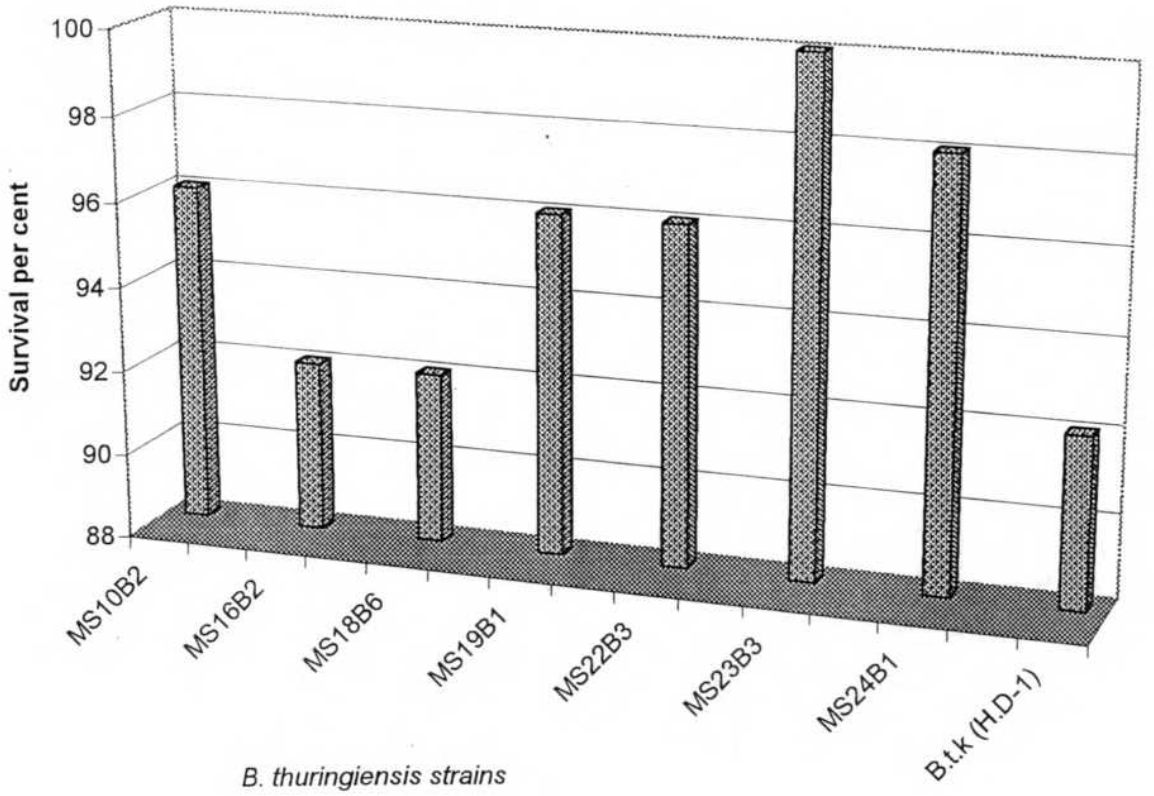


Fig 5. Effect of selected *Bacillus thuringiensis* isolates on common carp (*Cyprimus carpio*)

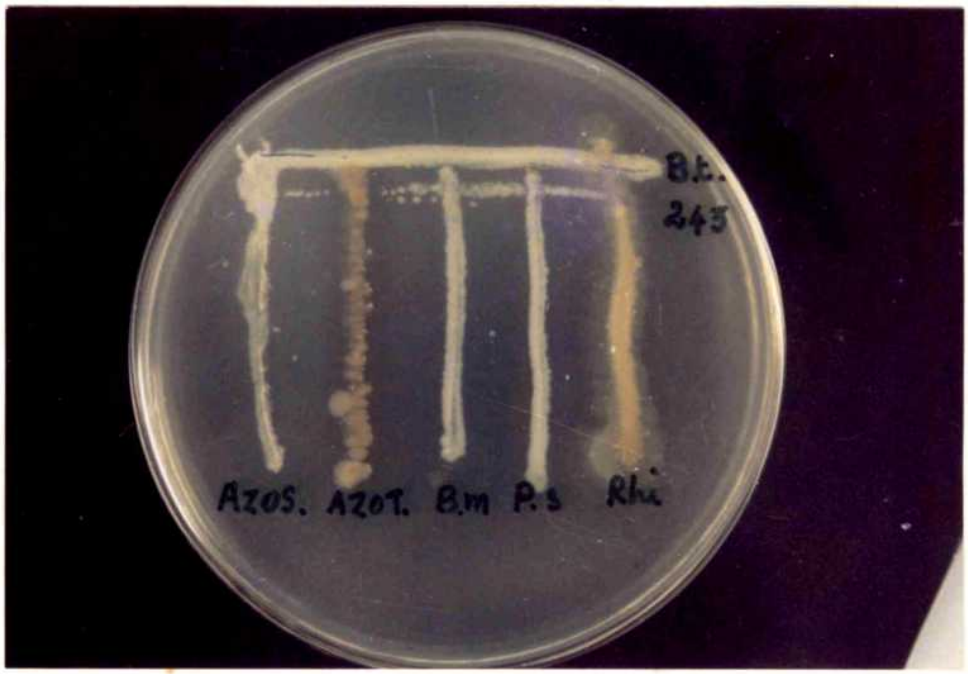


Plate 9. Cross streak assay *Bacillus thuringiensis* x microbial biofertilizers

Table 12. Effect of selected *Bacillus thuringiensis* isolates on the survival of *Azospirillum brasilense* in sterilized field soil.

Sl No	Treatment	Population (cells per g of oven dry soil)									
		B thuringiensis					*A brasilense*				
		Initial x 10^6	20 DAT x 10^6	40 DAT x 10^5	60 DAT x 10^5	Mean x10^6	Initial x 10^6	20 DAT x 10^6	40 DAT x 10^5	60 DAT x 10^5	Mean x10^6
1	MS10 B2+*Azospirillum*	22 27	16 74	64 40	12.51	11 65	27 71	13 50	55 2	9 38	11 90
2	MS16 B2+ *Azospirillum*	23 80	16 20	56 20	11 04	11 67	26 35	14 04	46 92	10 30	11 47
3	MS18 B6+*Azospirillum*	23 97	15 66	63 84	11 78	11 73	24 82	14 22	54 64	14 72	11 45
4	MS19 B1+*Azospirillum*	24 31	13 86	57 76	9 38	11 17	25 16	13 68	47 84	11 96	11 15
5	MS22 B3+*Azospirillum*	25 33	16 38	59 43	10 67	12 12	24 14	14 58	49 68	13 80	11 20
6	MS23 B3+*Azospirillum*	27 20	15 12	63 29	9 75	12 37	23 80	15 48	48 94	11 41	11 32
7	MS24 B1+*Azospirillum*	28 05	15 84	62 56	14 17	12 85	26 35	13 86	47 28	13 43	11 52
8	*Azospirillum* alone	0 00	0 00	0 00	0 00	0 00	27 20	14 22	48 94	10 48	11 82
9	Standard (HD-1)	25 05	16 20	61 72	15 46	12 20	0 00	0 00	0 00	0 00	0 00
	Mean (DAT) x 10^6	22 20	13 97	53 82	10 11	10 64	22 81	12 57	44 11	10 22	10 21

Initial inoculum *A brasilense* 5x10^9cells
B thuringiensis 5x10^9cells

	B thuringiensis		*A brasilense*	
	S E D	CD	S E D	CD
DAT	0 02	0 04	0.02	0 04
Treatment	0 03	0 06	0 03	0 06
D X T	0 06	0 11	0 06	0 12

4.6.2. *Azotobacter chroococcum* (Free living diazotroph)

The population of the bio fertilizer agent in the presence of *Bacillus thuringiensis* isolates showed a significant (at 1 per cent level) reduction in population over a period of 60 days Likewise the *Bacillus thuringiensis* isolates also recorded a significant decrease in the population under sterile conditions in the presence of *Azotobacter chroococcum* The reduction in population was highly significant that 30 to 50 fold decreases were observed over a period of time (at the end of 60 days after treatment) The individual application of standard culture of *Bacillus thuringiensis* sub sp *Kurstaki* H D-1 and *Azotobacter chroococcum* recorded a slightly better survival rate even though the decrease in population was significant statistically (Table 13)

4.6.3. Bacillus megaterium (Phosphobacterium)

The phosphobacterium - belonging to the same genus of the bio control agent was studied for any effect on the population dynamics in the presence of *Bacillus thuringiensis* isolates The decrease in population of *Bacillus thuringiensis* isolates and *Bacillus megaterium* in the presence of each other was highly significant The rate of decrease was also highly significant over a period of time The populations of *Bacillus megaterium* and the standard reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D-1 also were found decreased over a time of 60 days from the date of application (Table 14)

Table 13. Effect of selected *Bacillus thuringiensis* isolates on the survival of *Azotobacter chroococcum* in sterilized field soil

Sl No	Treatment	Population (cells per of oven dry soil)									
		B thuringiensis					*A chroococcum*				
		Initial x 10^6	20DAT x10^5	40DAT x10^4	60DAT x10^4	Mean x10^6	Initial x 10^6	20DAT x10^5	40DAT x10^4	60DAT x10^4	Mean x10^6
1	MS10 B2+*Azoto*	24 99	14 58	91 08	66 24	6 99	22 10	10 62	69 92	37 72	6 08
2	MS16 B2+*Azoto*	27 20	15 12	89 24	65 32	7 57	22 61	11 16	72 62	41 40	6 21
3	MS18 B6+*Azoto*	24 31	15 30	90 16	62 56	6 84	22 78	9 72	56 12	39 56	6 18
4	MS19 B6+*Azoto*	22 95	15 48	79 12	63 48	6 41	24 99	9 54	59 80	39 19	6 73
5	MS22 B3+*Azoto*	23 46	16 02	81 88	58 88	6 62	24 65	12 24	64 40	38 46	6 72
6	MS23 B3+*Azoto*	24 14	14 94	87 84	46 92	6 74	25 33	15 12	75 44	33 67	6 98
7	MS24 B1+*Azoto*	22 78	13 86	84 64	48 76	6 37	22 27	11 70	56 12	32 94	6 08
8	*Azotobacter* alone	0 00	0 00	0 00	0 00	0 00	22 95	87 30	239 20	184 55	8 98
9	Standard (HD-1)	25 33	167 40	235 52	173 88	11 54	0 00	0 00	0 00	0 00	0 00
	Mean (DAT) x 10^6	21 67	3 03	0 92	0 63	6 56	20 86	1 86	0 77	0 49	5 99

Initial inoculum *A chroococcum* 5x10^9
B thuringiensis 5x10^9

	B thuringiensis		*A chroococcum*	
	S E D	CD	S E D	CD
DAT	0 17	0 03	0 01	0 02
Treatment	0 02	0 05	0 01	0 03
D X T	0 05	0 10	0 03	0 06

Table 14. Effect of selected *Bacillus thuringiensis* isolates on the survival of *Bacillus megaterium* in sterilized field soil

Sl No	Treatment	Population (cells per g of oven dry soil)									
		B thuringiensis					*B megaterium*				
		Initial x 10^6	20DAT x10^6	40DAT x10^6	60DAT x10^5	Mean **x10^6**	Initial x 10^6	20DAT x10^6	40DAT x10^6	60DAT x10^5	Mean **x10^6**
1	MS10 B2+*B megaterium*	21 75	8 90	1 78	8 19	8 31	24 19	12 46	3 56	18 20	10 51
2	MS16 B2+*B megaterium*	20 75	10 68	1 95	8 37	8 55	24.52	13 17	3 74	19 11	10 84
3	MS18 B6+*B megaterium*	21 58	9 07	2 67	8 55	8 54	23 86	12 64	3 38	17 29	14 29
4	MS19 B1+*B megaterium*	24 07	10 32	1.60	9 10	9 22	25 37	13 35	3 20	14 56	10 86
5	MS22 B3+*B megaterium*	23 24	9 61	1 95	9 83	8 71	24 19	14 06	3 56	16 38	10 86
6	MS23 B3+*B megeterium*	20 58	9 97	2 49	10 01	8 51	25 03	12 28	4 45	20 02	10 94
7	MS24 B1+*B megaterium*	22 91	10 68	2 31	11 10	9 25	23 35	13 52	4 63	22 75	10 94
8	*Bacillus megaterium* alone	0 00	0 00	0 00	0 00	0 00	23 24	17 80	4 27	20 93	1 88
9	Standard (HD-1) alone	25 07	9 43	5 34	28 21	10 66	0 00	0 00	0 00	0 00	0 00
	Mean (DAT) x 10^6	19 88	8 75	2 23	1 04	7 98	21 53	12 14	3 43	3.39	10 12

Initial inoculum *B thuringiensis* 5x10^9 cells
B megaterium 5x10^9 cells

	B thuringiensis		*B megaterium*	
	S E D	CD	S E D	CD
DAT	0 01	0 01	0 01	0 01
Treatment	0 01	0 02	0 01	0 02
D X T	0.02	0 03	0 02	0.03

4.6.4. *Pseudomonas striata* (Phosphobacterium)

The pseudomonad phosphorus solubilizer was inoculated along with the selected isolates of *Bacillus thuringiensis* in sterile soil to evaluate the compatibility The results revealed that there was significant reduction in the population of *Bacillus thuringiensis* isolates as well as the pseudomonads over a period of time The standard reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D-1 and *Pseudomonas striata*, when inoculated alone in sterilized soil, also showed a corresponding reduction in population over a period of time The reduction in population of *Pseudomonas striata* was about 25 times over a period of 60 days under sterilized soil conditions (Table 15)

4.6.5. *Rhizobium leguminosarum* (Root nodule bacterium)

An experiment was conducted to study the survival and compatibility of *Rhhizobium leguminosarum* in the presence of *Bacillus thuringiensis* isolates The sterile soil experiment showed a significant reduction in the population of both bio control agents and the diazotroph The individual application of *Rhizobium leguminosarum* also suffered a comparatively enormous decrease in its population, even in the absence of *Bacillus thuringiensis* isolates with in 60 days under sterile condition (Table 16)

4.7. Intrinsic antibiotic resistance (IAR) of the selected *Bacillus thuringiensis*

All the selected *Bacillus thuringiensis* isolates were tested for IAR and each one exhibited a different pattern of tolerance to various antibiotics like penicillin,

Table 15. Effect of selected *Bacillus thuringiensis*. isolates on the survival of *Pseudomonas striata* in sterilized field soil.

Sl No	Treatments	Population(cells per g of over dry soil									
		B thuringiensis					*Ps striata*				
		Initial x 10^6	20 DAT x 10^5	40 DAT x 10^5	60 DAT x 10^5	Mean x 10^6	Initial x 10^6	20 DAT x 10^5	40 DAT x 10^5	60 DAT x 10^5	Mean x 10^6
1	MS10B2+*Ps striata*	24 90	77 40	68 25	59 15	11 34	24 57	84 55	64 79	54 60	11 24
2	MS16B2+*Ps striata*	25 73	79 21	68 06	58 24	11 57	25 89	80 99	66 43	53 59	11 49
3	MS18B6+*Ps striata*	24 73	80 99	68 98	61 88	11 48	26 56	81 70	67 52	55 51	11 76
4	MS19B1+*Ps striata*	22 41	76 54	63 70	60 96	10 63	23 07	83 66	69 16	52 78	10 70
5	MS22B3+*Ps striata*	25 73	81 88	67 34	57 33	11 59	23 90	85 08	70 98	54 78	11 24
6	MS23B3+*Ps striata*	23 90	77 43	59 15	55 51	10 78	24 57	80 10	63 01	55 51	11 11
7	MS24B1+*Ps striata*	24 73	80 63	62 79	56 24	11 17	23 24	84 55	65 86	54 24	10 92
8	*Ps striata* alone	0 00	0 00	0 00	0 00	0 00	24 73	79 50-	67 8	56 20	11 27
9	Standard(HD-1) alone	26 56	112 85	89 18	81 35	13 72	0 00	0 00	0 00	0 00	0 00
	Mean (DAT) x 10^6	22 08	74 10	60 77	54 49	10 25	21 84	73 3	59 47	48 54	9 99

Initial inoculum *B thuringiensis* $5x10^9$ cells
Ps Striata $5x10^9$ cells

	B thuringiensis		*Ps striata*	
	S E D	CD	S E D	CD
DAT	0 001	0 002	0 002	0 003
Treatment	0 002	0 003	0 003	0 004
D X T	0 004	0 007	0 005	0 010

Table 16. Effect of selected *B.thuringiensis* isolates on the survival of *Rhizobium leguminosarum* in sterilized field soil.

Sl No.	Treatments	Population (cells per g of over dry soil)									
		B thuringiensis					*R leguminosarum*				
		Initial x 10^6	20 DAT x 10^6	40 DAT x 10^5	60 DAT x 10^6	Mean x 10^6	Initial x 10^6	20 DAT x 10^6	40 DAT x 10^6	60 DAT x 10^6	Mean x 10^6
1	MS10 B2 +*R leguminosarum*	25 56	12 10	5 58	5 07	12 08	23 24	0 20	0 18	0 16	5 95
2	MS16 B2 +*R leguminosarum*	25 06	12 28	5 04	4 58	11 74	24 07	0 21	0 19	0 16	6 16
3	MS18 B6 +*R leguminosarum*	24 73	13 52	5 49	5 00	12 19	23 74	0 21	0 19	0 13	6 07
4	MS19 B1 +*R leguminosarum*	24 56	12 46	5 67	5 16	11 96	26 56	0 23	0 21	0 13	6 78
5	MS22 B3 +*R leguminosarum*	23 90	12 64	5 61	5 11	11 82	25 89	0 22	0 20	0 16	6 62
6	MS23 B3 +*R leguminosarum*	26 39	13 17	6 06	5 51	12 78	23 41	0 23	0 22	0 13	6 00
7	MS24 B1 +*R leguminosarum*	24 07	10 86	5 38	4 89	11 30	24 73	0 22	0 19	0 15	6 32
8	*R leguminosarum* alone	0 00	0 00	0 00	0 00	0 00	23 90	0 21	0 19	0 14	6 11
9	Standard (HD 1) alone	23 41	20 75	11 26	10 26	16 42	0 00	0 00	0 00	0 00	0 00
	Mean (DAT) x 10^6	29 96	11.97	5 57	5 06	11 14	21 73	0 19	0 17	0 13	5 56

Initial inoculum *B thuringiensis* 5x10^9 cells
R leguminosarum 5x10^9 cells

	B.thuringiensis		*R leguminosarum*	
	S E D	CD	S E D	CD
DAT	0 001	0 003	0 002	0 004
Treatment	0 002	0 004	0 003	0 006
D X T	0 004	0 009	0 006	0 013

erythromycin, tetracycline, chloramphenicol, gentamycin and streptomycin (Table 17) No appreciable resistance to any of the antibiotics tested was observed except in the case of streptomycin to which the isolates MS24B1 and MS22B3 showed resistance upto 160 ppm The intrinsic antibiotic resistance of the isolates to streptomycin, penicillin ranged from 10 to 60 ppm For erythromycin it ranged from 10 to 30 ppm, for tetracycline it was from 10 to 20 ppm, for chloramphenicol and gentamycin it ranged from 10 to 30 ppm IAR of isolates towards streptomycin ranged from 80 to 160 ppm The MS24B1 and MS22B3 recorded the maximum resistance to anyone of the antibiotics tested, which was 160 ppm to streptomycin

4.8. Effect of *Bacillus thuringiensis* (MS24B1) on the phyllosphere microflora of the crop plants

A study on the effect of *Bacillus thuringiensis* (MS24B1) on phyllosphere microflora of the respective crop plants was carried out The experiment revealed that though there was an initial suppression of the fungal and actinomycetes population the natural flora regained the equilibrium within 20 days after spraying

The population of total bacteria was still without any change over the entire duration of the study period There was a remarkable reduction in the population of *Bacillus thuringiensis* isolate MS24B1 per unit area on all the three plants leaf surfaces (Table 18)

4.9. Mutation studies on the selected *Bacillus thuringiensis* (MS24B1)

The isolate MS24B1 alone when subjected to mutation agents lost its intrinsic antibiotic resistance This was confirmed by test growing the mutant strain on streptomycin (160 ppm) impregnated medium The mutant was unable to grow on the streptomycin-impregnated medium

Table 17. Intrinsic antibiotic resistance of the selected *Bacillus thuringiensis* isolates to various antibiotics (ppm)

Sl. No	Isolates	Penicillin	Erythromycin	Tetracycline	Chloramphenicol	Gentamycin	Streptomycin
1	MS10B2	10	10	10	10	20	120
2	MS16B1	20	10	10	10	10	120
3	MS18B6	10	20	10	10	20	80
4	MS19B1	10	10	20	20	30	120
5	MS22B3	20	30	10	20	20	160
6	MS23B2	10	10	20	10	10	80
7	MS24B1	60	20	10	30	20	160

Table 18. Persistence of *Bacillus thuringiensis* on the phyllosphere and its effect on other phyllosphere microflora

Sl No	Observation made on	Total bacteria x 10^6/cm^2	*B t* x 10^6/cm^2	Fungi x 10^2/cm^2	Actinomycetes x 10^2/cm^2
			Rice		
A	Before *B t* spraying	0 03	-	26 81	3 41
B	0 day after spraying	2 42	2 38	24 33	2 61
C	10 day after spraying	1 29	1 27	13 22	2 83
D	20 day after spraying	0 31	0 28	28 27	3 21
			Cotton		
A	Before *B t* spraying	0 02	-	13 7	11 13
B	0 day after spraying	2 63	2 61	12 6	9 84
C	10 day after spraying	1 93	1 92	4 8	6 76
D	20 day after spraying	1 11	1 09	14 3	8 61
			Cauliflower		
A	Before *B t* spraying	0 03	-	2 41	4 62
B	0 day after spraying	2 26	2 24	1 98	3 16
C	10 day after spraying	0 98	0 96	0 87	4 32
D	20 day after spraying	0 42	0 39	1 12	4 51
	SED	0 0071	0 0070	0 0069	0 0071
	CD	0 0154	0 0153	0 0154	0 151

An experiment was conducted to assess the effect of the mutants on target pests and to assess residual toxicity in the phyllosphere of respective crop plants The mutants of MS24B1 could effect only less than 15 per cent mortality on the target pests where as the parent strain effected 75 8 per cent, 70 per cent and 66 6 per cent mortality on *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* respectively The standard reference culture *Bacillus thuringiensis* sub sp *kurstaki* H D -1 performed better than the isolated strain MS24B1 A consistent decrease in pathogenicity against all targeted pests over a period of time was observed when leaf samples were collected and conducted bioassays at different time intervals *viz*, 0, 48, 96 and 144 h after spraying (Table 19) The data were corrected according to Abbots' formula

4.10. Growth of selected *Bacillus thuringiensis* isolate MS24B1on different culture media

The *Bacillus thuringiensis* isolate MS24B1 was tested for biomass production using different culture media Basin water broth, tapioca starch broth and defatted soy flour broth were compared with UG medium for *Bacillus thuringiensis* for the biomass production efficiency The UG medium supported maximum growth of *Bacillus thuringiensis* isolate MS24B1 at the end of 72 h followed by basin water, tapioca starch and defatted soy flour enriched media in descending order The logarithmic growth phase lasted from 12h to 40 h after inoculation (28 h) The sporulation of the isolate MS24B1 grown in UG medium, started at the end of 32 h whereas, it started only at the end of 40 h of incubation in the case of other three media More than 90 per cent of the cells in all the four culture media sporulated at the end of 56 h (Table 20, Fig 6)

Table 19. Assessment of Residual toxicity of unformulated strain of *Bacillus thuringiensis* (MS24B1) and its mutant against the target pests (% mortality)

Sl No	Isolates	*P xylostella* III instar larvae allowed to feed after (h)				*C medinalis* III instar larvae allowed to feed after (h)				*H armigera* III instar larvae allowed to feed after (h)			
		0	48	96	144	0	48	96	144	0	48	96	144
1	MS 24 B1	75 8	57 2	46 6	30 0	70 0	57 2	36 6	24 1	66 6	51 7	28 6	20 0
2	MS 24 1(M)	10 7	7 2	3 1	0 0	10 0	10 71	3 3	0 0	13 3	13 8	10 71	3 3
3	*B tk* (H D-1)	82 7	60 7	46 6	43 3	76 6	60 7	43 3	37 9	76 6	62 0	46 5	26 6

Conc of *B thuringiensis* suspension = 10^7/ml (spores)

Abbot's correction carried out

	P xylostella		*C medinalis*		*H armigera*	
	SED	CD (P=0 05)	SED	CD (P=0 05)	SED	CD (P=0 05)
H A T	0 12	0 25	0 22	0 47	0 21	0 44
Isolates	0 10	0 22	0 19	0 40	0 18	0 38
H A T x Isolates	0 21	0 44	0 39	0 81	0 37	0 76

HAT - Hours after treatment

TABLE 20. Growth of *Bacillus thuringiensis* MS24B on different media (log values of cell conc. per ml)

Sl No	Culture medium	No of cells at the end of h (log values)								
		8	16	24	32	40	48	56	64	72
1	UG broth	9 54	11 05	11 86	13 39	13 91	13 94	13 91	13 90	13 89
2	Basin water broth	9 24	10 91	11 56	12 91	13 74	13 76	13 73	13 73	13 72
3	Tapioca flour broth	9 09	9 70	11 05	12 83	13 57	13 59	13 56	13 56	13 53
4	Defatted soy flour broth	9 06	9 57	10 19	12 71	13 39	13 44	13 38	13 37	13 36

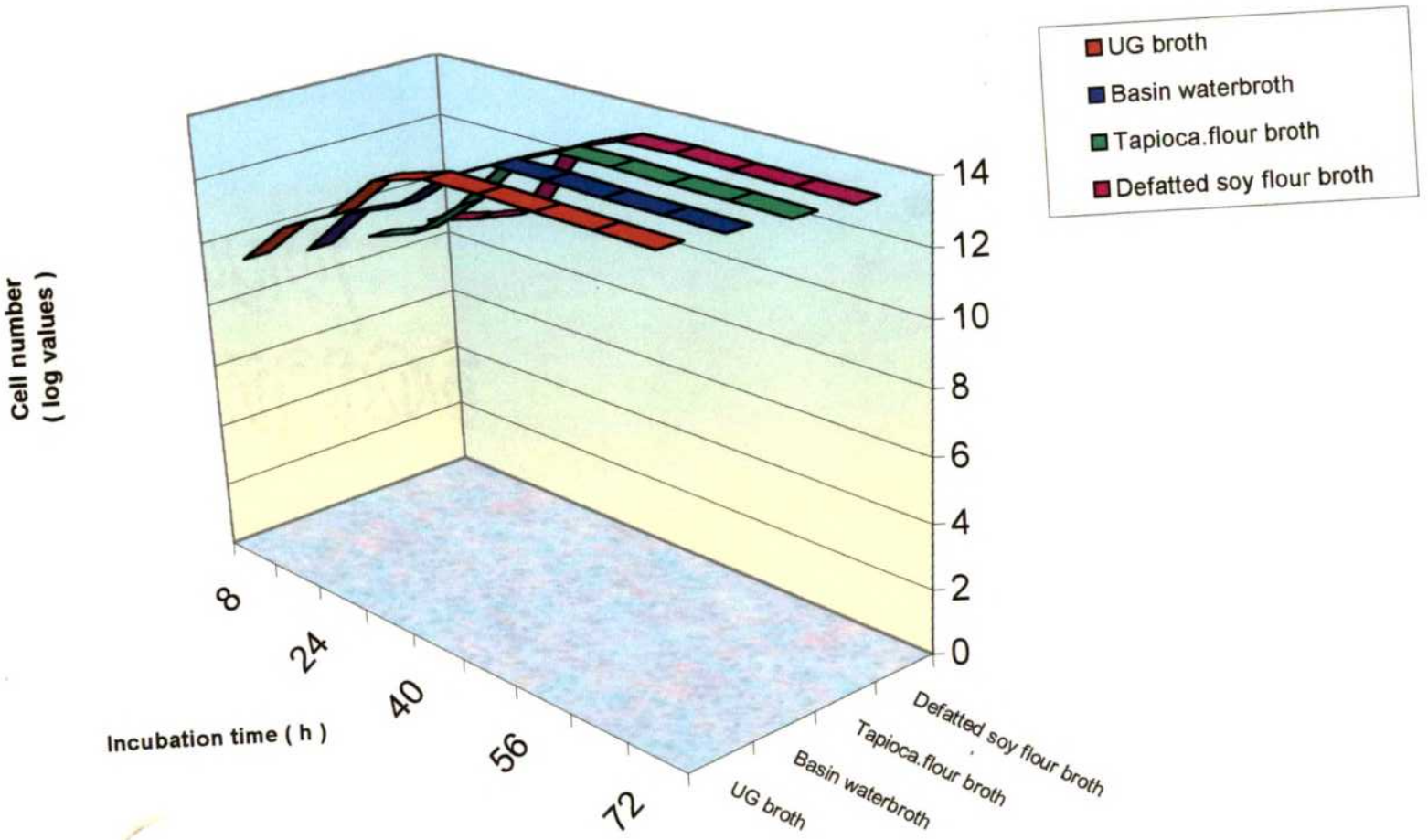


Fig 6. Effect of different growth media on *B.thuringiensis* MS24B1

4.10.1. Effect of culture media on pathogenicity of the selected *Bacillus thuringiensis* isolate MS24B1to targeted pests

The cultures of *Bacillus thuringiensis* isolate MS24B1 grown on different media were tested for the pathogenicity The best control on all the three target pests was effected by the culture grown in UG broth The percent mortality recorded was 71 4 in the case of *Plutella xylostella*, 68 1 in the case of *Cnaphalocrocis medinalis* and 62 9 in the case of *Helicoverpa armigera* The second best control was effected by the culture grown on defatted soy flour broth The per cent mortality recorded by *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* were 68 9, 65 5 and 61 9 respectively (Table 21) The cultures grown on tapioca starch medium and basin water broth were able to effect less than 60 per cent mortality in all target pests

4.10.2. Effect of formulation on pathogeneicity and residual toxicity

An experiment was conducted to study the effect of MS24B1 culture after formulation on the target pests The residual toxicity was assayed at 2 days interval after spraying, upto 6 days after treatment Compared with that of a popular commercial formulation The MS24B1 formulation effected 68 9, 62 0 and 65 5 per cent mortality at the end of 72h on *Plutella xylostella, Cnaphalocrocis medinalis* and *H armigera* respectively when compared to the commercial formulation which recorded more than 70 per cent mortality on all the three target pests The effect of sprayed formulation lasted 6 days, but the gradual reduction in mortality rate was noticeable At the end of six days the MS24B1, formulation effected only 37 0, 36 6 and 33 3 per cent mortality on *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* respectively The commercial formulation effected 42 8, 39 2 and 35 8 per cent mortality respectively on the

TABLE 21. Evaluating *Bacillus thuringiensis* MS24B1 cultured on different media for pathogenicity to targeted pests

Sl No	Test organisms	Per cent mortality at the end of 72 h			
		Tapioca starch broth	Defatted soy flour broth	Basin water broth	UG broth
1	*P xylostella*	55 6	68 9	53 3	71 4
2	*C medinalis*	58 6	65 5	55 1	68 1
3	*H armigera*	57 2	61 9	55 1	62 9

Spore conc 10^7 per ml
Abbot's correction carried out

three targeted pests mentioned above (Table 22) Data corrected according to Abbots' formula

4.10.3. Effect of storage on the viability and pathogenicity of the most efficient *Bacillus thuringiensis* isolate MS24B1

The results of cell viability and pathogenicity studies revealed that the formulation when stored at 4°C tend to sustain viable spore count level at 10^{10} per gram of the formulation up to 90 days The spore counts fell below 10^{10} per gram from 75th day onwards in the case of formulation stored at ambient temperature At the end of five months viable spore counts of the formulations stored at 4°C and at ambient temperature recorded a tenfold reduction The pathogenicity was also found to be influenced up on by storage At the end of 150 days a reduction of 12 03 per cent and 16 37 per cent was recorded in the pathogenicity of the formulations stored at 4°C and at ambient temperatures respectively (Table 23)

Table 22. Effect of formulation on pathogenicity and residual toxicity (% mortality)

Sl No	Test Organisms	Test formulation					Commercial formulation				
		0 day	2DAT	4DAT	6DAT	Mean	0 Day	2DAT	4DAT	6DAT	Mean
1	*Plutella xylostella*	68 9	55 1	40 0	37 0	59 33	73 3	66 6	51 7	42 8	58 33
2	*Cnaphalocrocis medinalis*	62 0	58 6	46 6	36 6	59 33	70 0	63 6	48 3	39 2	55 30
3	*Helicoverpa armigera*	65 5	51 7	43 3	33 3	57 63	70 0	63 6	44 8	35 8	53 63
	D Mean	65 47	55 13	43 30	35 60		71 10	64 6	48 3	39 3	

Dose 2 5 g formulation per lit of spray
Abbot's correction carried out

	Test formulation		Commercial formulation	
	S E D.	CD (P=0 05)	S E D.	CD (P=0.05)
DAT	0 36	0 74	0 27	0 58
Treatment	0.31	0 64	0 24	0 50
D X T	0 42	1 29	0 48	1 00

Table 23. Effect of storage on cell viability and pathogenicity on the formulation

Sl No	Sampling (Days)	Cold storage		At ambient temperature	
		Viable spores per gram X 10^{10}	Pathogenicity (percent mortality)	Viable spores per gram X 10^{10}	Pathogenicity (percent mortality)
1	0	49 2	96 40	56 7	96 53
2	15	43 7	96 25	41 3	96 08
3	30	39 8	96 08	32 1	93 44
4	45	28 6	93 44	21 9	90 00
5	60	17 6	90 00	16 8	86 66
6	75	16 1	89 30	12 7	85 64
7	90	12 8	88 55	9 1	85 10
8	105	8 9	86 66	8 7	85 33
9	120	8 1	86 14	7 4	82 70
10	135	7 4	85 10	6 8	81 10
11	150	6 9	84 50	5 8	80 70

Concentration of formulation in suspension 2 5 g per l

Abbot's correction carried out

DISCUSSION

DISCUSSION

The present study was aimed at selecting native, efficient bacterial pathogens with special reference to *Bacillus thuringiensis* and succeeded in identifying a few native isolates active against the three target pests *viz*, *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* The selection of newer strains help in building up of genetic pool which is always necessary to keep in check the pests that are fast adapting themselves to biological pest control agents

In the present study the safety aspects of the selected isolates towards some of the non-targeted organisms were also looked into The effect of storage of formulation on pathogenicity and residual toxicity was studied Testing of cheaper alternative nutrient sources for mass multiplication media was also attempted

5.1. Isolation of *Bacillus thuringiensis*

Twenty-five soil samples from 20 different locations of Coimbatore and Erode districts yielded 96 isolates resembling *Bacillus thuringiensis* Martin and Travers (1987) were able to get about 4500 isolates from 800 samples of soil collected from 30 countries, a ratio of about 1 5 6, when compared to the present study where the soil sample to *Bacillus thuringiensis* isolates ratio was only 1 3 2 This may be due to the fact that the soil samples were restricted to mulberry plantations only The method described by Johnson and Bishop (1996) would have yielded more number of isolates as it resulted in the enrichment of *Bacillus thuringiensis* compared to other members of the genus *Bacillus*

5.2. Authentication of *Bacillus thuringiensis*

Out of the 96 isolates, 80 were confirmed to be *Bacillus thuringiensis* based on cell morphology, Gram's staining reaction, positive presence of spores and crystals, positive catalase activity, positive AMC production (V-P test) and negative growth on D–mannitol medium (Stahly *et al*, 1991, Holt, 1984 and Lacey 1997) The isolates (16) that were with out crystals, that utilized D-mannitol and that lacked catalase activity were not used for further studies

5.3. Pathogenicity to target pests and LC_{50} of selected isolates

By performing leaf dip and diet incorporation bioassays the isolates MS10B2, MS16B2, MS18B6, MS19B1, MS22B3, MS23B3 and MS24B1 were identified based on their efficiency in effecting more than 65 percent mortality to one or the other or on all the three targeted pests *viz*, *Plutella xylostella, Cnaphalocrocis medinalis* and *Helicoverpa armigera* As there were 80 isolates to be screened the preliminary bioassay was conducted with one level of spore concentration *viz*, 10^7 spores per ml The LC_{50} values for the seven selected isolates were between 1 30 to 1 595 x 10^6 spores per ml for *Plutella xylostella* The fiducial limits (lower and upper) were ranging from 5 15 to 7 45 x 10^5 and from 2 61 to 3 168 x 10^6 spores per ml respectively

LC_{50} for *Cnaphalocrocis medinalis* was recorded as 1 33 to 2 858 x 10^6 spores per ml The fiducial limits were 5 056 to 13 40 x 10^5 spores per ml (lower) and 2 85 to 5 553 x 10^6 spores per ml (upper)

The LC_{50} of the selected isolates for *Helicoverpa armigera* ranged from 1 074 to 3 223 x 10^6 spores per ml The fiducial lower and upper limit were 4 087

to 15 364 x 10^5 spores per ml and 2 231 to 6 231 x 10^6 spores per ml respectively

Kulkarni and Amonker (1988) in their study used different levels of cell concentrations ranging from 10^5 to 10^9 spores per ml for assessing LC_{50} and LT_{50} values for I to IV instar larvae of *Helicoverpa armigera* The LC_{50} values obtained by him were 2 57 x 10^7 to 7 04 x 10^8 spores per ml were about 10 to 100 times higher than that of the present study results Also the LT_{50} values 4 5 days to 6 7 days In the present study the per cent mortality effected was at the end of 72 h This may be because of the sub species used by them, which was *Bacillus thuringiensis* sub sp *kenyae*

Sarag and Satpute (1988) reported that *Bacillus thuringiensis* sub sp *kenyae*.(6 5 x 10^8 spores per gram) even in the presence of Acephate (0 1 per cent) did not reduce the pest population in a field trial conducted by them in Maharashtra, India in 1985 The LT_{50} values obtained by Justin *et al* , (1988) in their experiment with III instar larvae of *Plutella xylostella* were between 21 9 h to 22 6 h at a concentration of 10^7 spores per ml This was in conformity with the present study as about 85 percent mortality was effected within 48 h after treatment and maximum mortality was recorded at the end of 72h Srivastava and Nayak (1978) reported that the mortality of *Cnaphalocrocis medinalis* decreased with decreasing concentration

5.4. Safety of the *Bacillus thuringiensis* isolates on non-targrted organisms

As there are chances of the *Bacillus thuringiensis* formulations reaching environments where they may pose very sensitive problems and unwarranted risks, safety to non targeted organisms, both macro and micro organisms

(beneficial only) was analyzed in this course of study Melin and Cozzi (1990) and Lacey and Mulla (1990) studied the safety to non-targeted invertebrates of lepidopteron strains of *Bacillus thuringiensis* and their beta-endotoxins Lacey and Mulla (1990) also studied the effect of mosquitocidal *Bacillus thuringiensis* formulations in the aquatic environment

The effect of *Bacillus thuringiensis* on an egg parasite – *Trichogramma chilonis*, an insect predator – *Chrysoperla carnea,* silk worms, honey bees and fish- *Cyprinus carpio* along with soil inhabiting biofertilizer agents *viz*, *Azospirillum brasilense*, *Azotobacter chroococcum*, *Bacillus megaterium*, *Pseudomonas striata* and *Rhizobium leguminosarum* Except silk worms, which showed a great degree of susceptibility, all other organisms showed a relatively high degree of resistance In other words the honeybees, *Trichogramma, Chrysoperla* and fish were not affected by *Bacillus thuringiensis* isolates These were in conformity with the observations made by, Babrikova and Kuzmanova (1984), Vandenberg (1990) and Mertz *et al*, (1995) with regard to *Chrysopa* spp , honeybees and *Trichogramma* spp respectively

The *Bacillus thuringiensis* isolates were not inhibitory to other microorganisms tested The cross streak and gnotobiotic assays were carried out to assess the compatibility of *Bacillus thuringiensis* with biofertilizer agents

The cross streak assays revealed no antagonism by *Bacillus thuringiensis* isolates *Vis-a-Vis* biofertilizer agents There were no references available to support or oppose this fact but there were reports of *Bacillus thuringiensis*'s possible role in biological control of diseases too Amer *et al* (1997) studied the interaction of *Bacillus thuringiensis* with *Pythium ultimatum* and *Fusarium*

oxysporum f sp *lycopersici* and found that the *Bacillus thuringiensis* was able to suppress the growth of both the plant pathogenic fungi

Visser *et al* (1994) studied the effect of *Bacillus thuringiensis* sub sp *kurstaki* formulations on the soil microflora mediated carbon and nitrogen mineralization processes An experiment was conducted by them where litter and fermentation humus were exposed to *Bacillus thuringiensis* (at field rate and 1000 times field rate or left untreated) The scientists observed the following *viz* , respiration, substrate induced respiration, microbial biomass, ammonaical nitrogen, nitrate nitrogen, cellulose decay and *Bacillus thuringiensis* sub sp *kurstaki* viability regularly for over 8 weeks The field application rate had no significant impact on soil processes in both substrates The 1000 times field application rate treatment increased substrate-induced respiration and biomass and decreased metabolic quotients consistently in both substrates The 1000 times field rate stimulated respiration initially, then reduced it below control levels It enhanced cellulose decay and inhibited ammonification and nitrification

At the same time the *Bacillus thuringiensis* sub sp *kurstaki* viability was not lost in this period This proved that at field application rates the *Bacillus thuringiensis* does not inhibit or adversely interact with soil microflora In the present study the population dynamics were closely studied for over two months Though there was significant reduction in population of both organisms (*Bacillus thuringiensis* isolates x biofertilizer agent) the trend was as in any other soil microbial dynamics

The reduction in population over time was mainly due to the nutrient exhaustion This was evident from the trend of reduction in the *Bacillus thuringiensis* and biofertilizer agents even when they were inoculated separately

Meikle and Jarret (1994) reported that under field conditions (non-sterile) the rate decrease of *Bacillus thuringiensis* population was even faster When applied at a rate of 2 kg per ha the population gradually declined in the size and with in 14 days of application went below the population of native *Bacillus cereus* They also confirmed that there was no evidence of spore germination

5.5. Phyllosphere microflora and *Bacillus thuringiensis*

The phyllosphere and the phylloplane of plants harbour thousands of microorganisms Mostly fungi and bacteria dominate the microflora that colonize the phyllosphere and phylloplane, especially more of bacteria belonging to the genera *viz*, *Beijerinckia*, *Aerobacter, Arthrobacter* and *Pseudomonas* Fungi were dominated by yeasts (Ruinen, 1961)

Devenport (1970) showed that cultural procedures could have a greater effect on the enumeration of phyllosphere microorganisms He also reported that the dormant buds and leaf blades might have internal microflora that cannot be removed by washing These situations warrant the need for assessing the *Bacillus thuringiensis* population in places where the microbial pest control agents were being used

The presence of *Bacillus thuringiensis* in the phyllosphere organically grown cabbage was confirmed by Damgaard *et al* (1997) This may be due to the wide spread use of commercial formulations in agriculture The presence of *Bacillus thuringiensis* cells spores or crystals may interfere with microbial interaction with plant, if not with the colonizers on the leaves of crop plants

This prompted the present investigation The initial native microflora *viz* , bacteria, fungi and actinomycetes were slightly affected by the *Bacillus thuringiensis* spray This may be because of excessive washing off of the surface microflora by the spray fluid or may be due to antibiosis But the native microflora populations regained the original population limits with in 20 days of time There was considerable reduction in the *Bacillus thuringiensis* population after each enumeration done at an interval of 10 days The dilution effect by plant growth was one of the reasons for the decrease The trend is almost equal on all three crops studied Klausner (1984) also observed a similar trend in his studies

5.6. Antibiotic sensitivity of *Bacillus thuringiensis* isolates

Most of the antibiotics used for controlling Gram positive bacteria were tested and found to inhibit all the isolates at 10 to 60 ppm except streptomycin, which at 160 ppm had not inhibited the *Bacillus thuringiensis* isolates screened to be efficient against all the three target pests Chugtai and Shakoori (1994) presented a not very contradicting result from their experiment The isolates had only about 20 ppm IAR to streptomycin and about 40 ppm level of penicillin and 80 ppm level of tetracycline had no impact on the cultures tested by them The IAR of 160 ppm towards the *Bacillus thuringiensis* isolates helped to fix an identifiable criterion for mutation studies

5.7. Mutation studies on the selected isolates of *Bacillus thuringiensis*

From the seven cultures tested only one isolate MS24B1 had lost its IAR (160 ppm) owing to the use of methylating, mutagenic NMNG This was confirmed by growing by isolate on streptomycin impregnated medium Quite unexpectedly, it was found out to be a nontoxic mutant against any of the target

pests The loss of pathogenicity (below 15 percent mortality) may be attributed to many reasons Basically, change of even a single amino acid in the domain-II of *Bacillus thuringiensis* delta-endo toxin resulted in irreversible binding to *Manduca sexta* mid gut vesicles (Francis, 1995)

The residual toxicity in the phyllosphere of the isolate MS24B1 (unformulated) was effective only up to 96 h after which the efficacy was lost and the percent mortality it could effect was only 30 The standard reference strain fared slightly better than the isolate The loss of toxic nature may be attributed to some changes in the amino acid sequence of the crystal protein due to methylation as demonstrated by Francis (1995) Aronson *et al* (1995) reported seven amphipathic helices, that help the formation of ion channels during protein synthesis (delta-endotoxin) and that, only mutation within a region encoding the central helix resulted in substantial number of mutants with low or no toxicity

5.8. Growth of *Bacillus thuringiensis* on different culture media

The *Bacillus thuringiensis* – MS24B1 isolate was selected for this study only because it had a broader spectrum of activity This isolate was effective and could effect more than 65 percent kill on all the three targeted pests No specific modalities were kept for choosing the three substrates *viz*, tapioca starch, defatted soyflour and basin water The three commodities cost less when compared to the nutritional value they provided The basin water cost nothing for it had no economic value so far The culture media were compared with UG medium, which was generally used for growing *Bacillus thuringiensis*

The biomass produced was at the highest in the UG culture medium at the end of 72 h followed by basin water broth and tapioca starch broth Defatted soy

flour broth supported the least number of cells per unit volume at the end of 72 h There are instances, where such alternative nutrient sources were tried and evaluated Balaraman *et al*, (1986) set up pilot plants for producing *Bacillus thuringiensis* sub sp *israelensis* using jaggery as the major source of carbon Singh and Rana (1988) reported that jaggery medium supported more growth of *Bacillus thuringiensis* H-14 7 serotype

The tapioca starch was selected instead of costlier jaggery as *Bacillus thuringiensis* (diastatic) can hydrolyse starchy substrate The growth curve was almost alike for *Bacillus thuringiensis* on all the four media This showed the possibility of substituting the tested alternative substrates *in lieu* of costly synthetic chemicals The control effected upon the targeted pests by defatted soy flour broth culture to that of UG broth culture was also almost identical though statistically not on par

Alves *et al* (1997) observed that cheese whey, soy milk and molasses yielded maximum spores and crystals The potency of the formulation was highly influenced by yeast extract in nutrient broth The medium prepared by using molasses and *Bombyx mori* pupae was the cheapest and supported more growth of *Bacillus thuringiensis* with high potency From this experiment it was understood that the increased population or biomass production not necessarily enhance the efficacy

5.9. Pathogenicity and residual toxicity of selected *Bacillus thuringiensis* MS24B1 formulation

The pathogenicity and residual toxicity were analysed for the *Bacillus thuringiensis* MS24B1 formulation against all the three target pests and compared with a popular commercial brand The formulation made was a

wettable powder The components chosen for the preparation were compatible to each other (Heimpel, 1967) The residual effect of *Bacillus thuringiensis* formulation, which lasted for only six days under open conditions exposed to sunlight, proved that the use of charcoal (activated) did not help in photo protection

The effect of storage on the formulation revealed that the viability of spores and pathogenicity tend to decrease after 3 months of storage Which was not in conformity with the study conducted by Balaraman and Hoti (1984) This may be because the sub species of *Bacillus thuringiensis* used by them was *israelensis* effective against mosquito larvae In the present study the strain MS24B1 *Bacillus thuringiensis* isolated from mulberry plantations was yet to be identified at the subspecies level

SUMMARY

SUMMARY

The present study was aimed at obtaining native *Bacillus thuringiensis* isolates that could be used to control *Plutella xylostella*, *Cnaphalocrocis medinalis* and *Helicoverpa armigera* - most serious pests of rice, cauliflower and cotton respectively Further, to study the biological safety to non-targeted organisms like silk worms, honey bees, biofertilizer agents etc , and to identify cheaper mass production medium and formulation technology without compromising the pathogenicity To summarize,

Eighty *Bacillus thuringiensis* isolates were obtained from 25 soil samples from mulberry areas of Coimbatore and Erode districts

Screened for pathogenicity and identified seven isolates which effected more than 65 percent mortality on targeted pests They were grouped in to three categories I) the most effective on all the three target pests (1 isolate), ii) the most effective isolates with a host spectrum of any two targeted pests (3 isolates) and iii) most effective isolates on individual target pests (3 isolates)

Lethal concentration (LC_{50}) was assessed to find out the efficiency of selected isolates on the targeted pests The most efficient isolate based on LC_{50} value for the control of *Plutella xylostella* was MS23B1, for *Cnaphalocrocis medinalis* it was MS19B1 and MS16B2 for *Helicoverpa armigera*

The selected isolates were found to be safe for honey bees, *Trichogramma*, *Chrysoperla* and common carp The silk worms were highly susceptible to the selected *Bacillus thuringiensis* isolates

The effect of *Bacillus thuringiensis* isolates on five biofertilizer agents was studied. The cross streak assay revealed no antagonistic effect on each other, assuring compatibility.

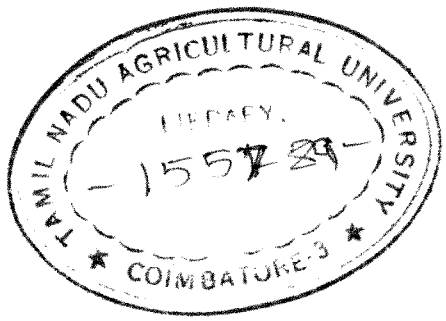
The gnotobiotic studies though revealed a significant reduction in the population of biofertilizer agents and the *Bacillus thuringiensis* isolates over a period of time under sterilized conditions, there was no antagonism. The population reduction was attributed to the exhaustion of nutrients in the substrate.

The intrinsic antibiotic resistance for the selected *Bacillus thuringiensis* isolates was ascertained. The MS24B1 recorded maximum resistance (160 ppm) for streptomycin sulphate.

Mutation with NMNG of the isolates was tried with an idea of getting mutant strains with high efficiency and spectrum of activity. One mutant of the isolate MS24B1 was identified that had lost the streptomycin resistance. Unfortunately the pathogenicity was also lost.

The effect of *Bacillus thuringiensis* isolate MS24B1 on phyllosphere microflora of the three crop plants was analysed. There was a slight reduction initially in the case of fungi and actinomycetes. At the end of three weeks the leaf surface colonizers reached the initial population. The *Bacillus thuringiensis* population was found decreasing over a period of 20 days.

Different culture media were tried and compared with regular UG broth. The cultures grown on defatted soy flour broth was most effective of the three substrates tested with regard to pathogenicity.



Effect of formulation on the storage and pathogenicity was studied The viable spore count was observed to be on the decline after 75 days At the end of 150 days there was a tenfold reduction The pathogenicity recorded 12 to 16 per cent reductions over a period of 5 months storage

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APPENDICES

APPENDIX-I

Stains and media composition

1. Gram stains

Cristal Violet 1g in 100 ml distilled watrer(D W)

Potassium iodide 2g, Iodine 1g in 20 ml D W (make up to 100 ml)

Safranin / Basic Fuchsin 0 1 g in 100 ml D W

Ethanol 50 ml

2. MR-VP medium

Polypeptone/ Trypsic peptone	5g
Glucose	5g
Na Cl	5g
D W	1000 ml
pH	7

VP Reagent A 40 g KOH in 100ml D W

VP Reagent B Alpha napthol 6g in ethonol (absolute) 100ml

3. Ammonium salts and sugar medium (base)

$(NH_4)_2 HPO_4$	1 0 g
K Cl	0 2g
Mg SO_4 , 7 H_2O	0 2g
Yeast Extract	0 2g
Agar	15 0g
D W	1000 ml

4. Lurias' broth

Tryptone	10 0 g
Yeast extract	5 0 g
NaCl	5 0 g
D W	1000 ml
pH	7

5. T_3 medium (Travers *et al* , 1987)

Tryptone	3 0g
Tryptose	2 0 g
Yeast extract	1 5 g
Sodium phosphate	0 05 m (PH 6 8)
Manganous chloride	0 005 g
D W	1000 ml
Agar agar	15 0g (For solid medium)

6. UG usual medium

Bactopeptone	7 5 g
Glucose	5 0 g
Potassium phosphate solution	100 ml
(KH_2PO_4 68 g in 1000ml DW)	
Stock solution I	10 ml
Stock solution II	10 ml
Stock solution III	10 ml
Distilled water	870 ml
pH	7 4

Sterlized at 121^0 C for 20 minutes

a. **Stock solution-I**

$MgSO_4 \cdot 7H_2O$	12 3 g
$MnSO_4 \cdot 1H_2O$	0 17 g
$ZnSO_4 \cdot 7H_2O$	1 4 g

Dissolved gently by heating in distilled water and make up to one litre

b **Stock solution-II**

$Fe_2(SO_4)_3$	2 0 g
D W	10 5 ml
Conc H_2SO_4 →	3 ml

Heated for 4 min then filtered Adjusted to one litre with distilled water

c. **Stock solution-III**

$CaCl_2 \cdot 2H_2O$	14 7 g
D W	1000 0 ml

7. **Rose bengal agar medium**

1 Glucose	10 0 g
2 Peptone -	5 0 g
3 $MgSO_4 \cdot 7H_2O$ -	0 5 g
4 $K_2H_2PO_4$	1 0 g
Rose bengal	0 33 mg
Streptomycin –	30 mg
(added after sterilization)	
Agar	15 – 20 g
D W	1000 ml
pH	6 0

8. Nutrient agar medium

Glucose	5 0g
Peptone	5 0g
Beef extract	3 0 g
Sodium chloride	5 0g
Agar	15 0 – 20 0 g
D W	1000 ml
pH	6 8 - 7 2
Agar agar	15-20 g

9. Kenknight's agar medium

Glucose	10 0g
K_2HPO_4	0 1 g
$NaNO_2$	0 1g
KCl	0 1 g
$MgSO_4$	0 1 g
Agar	15 – 20 g
D W	1000 ml
pH	7 2

10. Waksmann '77 medium

Mannitol	10 0 g
$CaCO_3$	5 0g
K_2HPO_4	0 5 g
$MgSO_4$	0 2 g
NaCl	0 2 g
$FeCl_2$	Trace
$MnSo_4$	Trace

D W	1000 ml
pH	7 0

11. Congored yeast extract medium (YEMA)

Mannitol	10 0 g
K_2HPO_4	0 2 g
NaCl	0 1
Yeast extract	1 0 g
Distilled water	1000 ml
Congo red solution 1%	2 5 ml
Agar agar	15 - 20 g

12. Pikovskaya's medium

Glucose	10 0 g
$Ca_3(PO_4)_2$	5 0g
$(NH_4)_2 SO_4$	0 5 g
KCl	0 2 g
Mg SO_4	Trace
Mn SO_4	Trace
Yeast extract	0 5 g
D W	1000 ml

13. N-FREE SEMISOLID MALATE MEDIUM (Dobereiner and Day , 1975)

Malic acid	5 0 g
Dipotassium hydrogen Orthophosphate	0 5g
Magnesium sulphate	0 2 g

Magnesium sulphate	0 2 g
Sodium chloride	0 1 g
Calcium chloride	2.0 g
Fe-EDTA (1 64 percent w/v aqueous)	4 0 ml
Trace element solution	2 0 ml
Alcoholic solution of	
Bromothymol blue (0 5 per cent)	2 0 ml
Vitamin solution	1 0 ml
Potassium hydroxide	4 0 g
Agar	1 75 g
Distilled water	1000 ml
pH	6.8
Trace element solution	
Sodium molybdate	200 mg
Manganous sulphate	235 mg
Boric acid	280 mg
Copper sulphate	8 0 mg
Zinc sulphate	24 0 mg
Distilled water	200 ml
Vitamin solution	
Biotin (Sigma, USA)	10 0 mg
Pyridoxin (Sigma, USA)	20 0 mg
Distilled water	1000 ml

14. CSA Medium (Cross Streak Assay medium)

Luria's agar medium Nutrient agar medium Waksmann 77 agar medium @ 1 . 1 · 1

APPENDIX-II

Mass culturing of test organisms (Target pests)

1. Diamond back moth: *Plutella xylostella*

Diamond back moth larvae were collected from crucifers grown in the Oddanchathiram area of Dindigul district Allowed to pupate, disinfected with 15 per cent sodium hypochlorite,rinsed with three changes of distilled water before they were placed in dishes and allowed to emerge inside the rearing cages Cotton wads soaked in honey solution served as feed for the adults Plastic cups with 2-3 days old seedlings of mustard were placed inside the cages for oviposition and upon hatching the seedlings were pulled out and placed on cauliflower leaves, for migration, that were kept supported on small water bottles to keep the rigidity intact (Plates 10 and 10a) The leaves were changed regularly and supplemented with more leaves as and when required The third instar larvae were used for conducting bioassay studies (Padua *et al* , 1987)

2. Rice leaf folder: *Cnaphalocrocis medinalis*

The adult moths were collected from paddy breeding station rice fields of TNAU, Coimbatore-3 using insect collection net and released for oviposition on 30-35 days old potted rice seedlings of the variety Taichung Native-1 Kept in GI trays filled with water inside wooden case having wire mesh all over the sides (Plate 11) The plotted plants were fertilized with urea at the rate of 1 g per pot

Plate 10. Mass culturing of *Plutella xylostella*
i. Mustard seedlings for oviposition

ii. I instar larvae migrate to cauliflower leaf

Plate 10a. Mass culturing of *Plutella xylostella*
iii. Culturing on cauliflower leaf

Plate 11. Mass culturing of *Cnaphalocrocis medinalis*

twice a week. First instar larvae emerged from the eggs in a period of six days. The third instar larvae were collected and used for bioassays (Dandapani, 1998).

3. Cotton boll worm: *Helicoverpa armigera*

The colony of *H.armigera* was started from the wild populations from cotton grown in the garden lands of TNAU farm, Coimbatore. Healthy adults were transferred to oviposition chamber. The male moths have slight greenish tinge. The oviposition chamber was a 30 x 20 cm plastic containers covered with a muslin cloth kept in a cool place. The adults lay eggs on the muslin cloth. The collected egg cloths were labeled and incubated in closed plastic containers. Humidity maintained by moist cotton wads. The eggs were sterilized using 0.025% sodium hypochlorite treatment that resulted in the release of eggs from the muslin cloth. Eggs were made free of the chemical sterilant by repeated washings in distilled water, dried and distributed evenly on to diet surface directly for reclusion (Plate 12). Semi synthetic diet was used for rearing the larvae. Third instar larvae were used for bioassays (Rabindra , 1998).

4.Diet for *Helicoverpa armigera*

Group-I	Materials	Quantity
	1. D .W.	1200 ml
	2. 4 N KOH	6.0 ml
	3. Soy bean flour	242.0 g
	4. Sucrose	43.8 g
	5. Wessen Salt mixture	36.0 g
	6. Wheat germ	108.0 g
	7. Ascorbic acid	1.92 g

Plate 12. Mass culturing of *Helicoverpa armigera* - Oviposition chamber

Feeder trays for I and II instar larvae

	8. Sorbic acid	3.4 g
	9. Chlor tetra cycline HCl.	0.17 g
	10. 15 percent methyl – p- hydroxy benzoate soln.	5.0 g
	11. 15 percent choline chloride W/V soln.	24.9 ml
	12. 10 per cent formaldehyde W/V soln.	15.0 ml
Group-II	13. Agar 34.0 g in 2 lit. Boiling D.W.	
Group-III	14. Vitamin solution*	4 ml.

*(Nicotinic acid amide 12.0 g ; calcium pantothenate 12.0 g ; thiamine HCl. 3.0 g Pyridoxine HCl. 30 g; Biotin 0.24 g ; Vit. B12 0.024 g ; D.W 1000 ml).

Prepared **group-I** using a blender (variable speed) mixed one by one in the order given above at slow speed. Added **group-II** cool to about 60– 65°C and added **group-III.** Blended for 2 minutes slowly. pH (final) would be 5.2.