

**COMPARATIVE PERSISTENCE OF PESTICIDES
IN Bt-TRANSGENIC CABBAGE
(*Brassica oleracea* L. var. capitata) CULTIVATION**

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NEW DELHI-110 012**

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TRANSGENIC CABBAGE (*Brassica oleracea* L. var.
capitata) CULTIVATION**

By

DEBASHIS DUTTA

A thesis submitted to the faculty of Post-Graduate School,
Indian Agricultural Research Institute, New Delhi
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of
DOCTOR OF PHILOSOPHY

In

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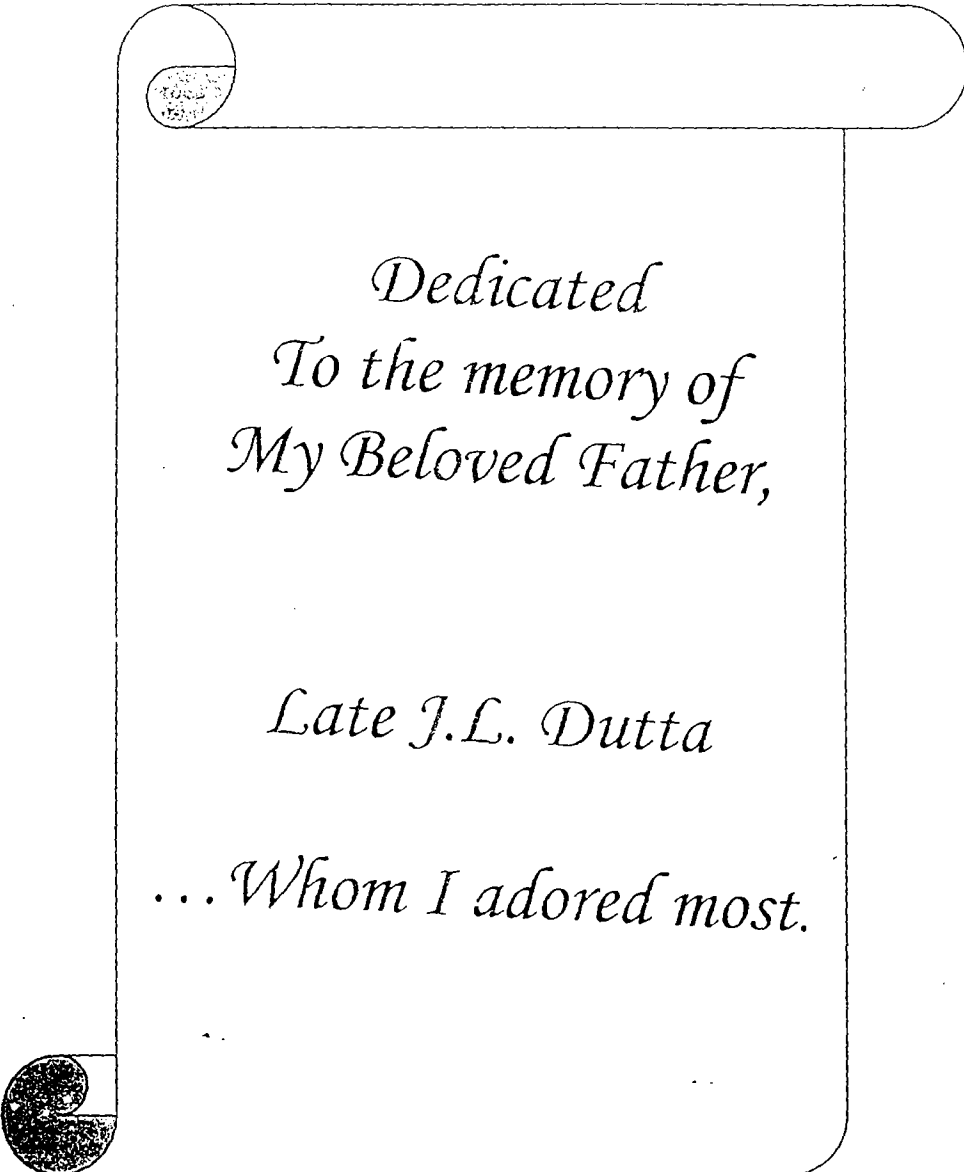
CERTIFICATE

This is to certify that the thesis entitled “**Comparative persistence of pesticide in Bt-Transgenic Cabbage (*Brassica oleracea* L. var. capitata) cultivation**” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Agricultural Chemicals** embodies the results of *bona fide* research work carried out by **Debashis Dutta**, under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information that has been availed of in this connection has been duly acknowledged by him.

Place : New Delhi
Date : 12th December, 2003


(**Madhuban Gopal**)
Chairman
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*Dedicated
To the memory of
My Beloved Father,*

Late J.L. Dutta

... Whom I adored most.

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Chapter - I

Introduction

1. INTRODUCTION

The introduction of transgenic crop plants containing insect resistance genes has launched a new era in agriculture. Transgenic insecticidal crops expressing *Bacillus thuringiensis* (Bt) toxins, deliver a remarkable level of insect control unsurpassed by any other insecticidal treatment. There are significant environmental hazards due to pesticide drift, residual pesticides in soil and adverse effects on non target species also. Bt-microbes have a long history of effectiveness against target pest. When expressed in plants, Bt-proteins are much more persistent and effective even against insects that feed at sites difficult or impossible to reach with sprays (Roush, 1997). This contributed to the development of Bt-crops, which are among the first plant biotechnology products with commercial relevance (Estruch *et al.*, 1997).

Transgenic crops often referred to as genetically modified crops, represent promising technologies that can make a vital contribution to global food, feed and fiber security. It is estimated that about 13 per cent of 37 per cent of pre harvest crop production, which is lost due to pests and diseases, is accounted to insect pests (Gatehouse *et al.*, 1992). The scale of both the economic and environmental cost of insect control in agriculture and of the losses incurred (in spite of such degree of measures) is extremely high. In order to control insect pests various pesticides are being used and the cost of pesticide application is valued at US \$10 billion (Oerke *et al.*, 1994). In India, crop losses due to all insect pests may range from 10 to 30% annually, depending on the crop and the environment. Annual crop losses due to pest in India have been estimated at Rs. 60-70 billion (Jayaraj, 1990; Nagarajan, 1990). Massive applications of pesticide

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not only leave behind harmful residues in the food, but also cause adverse effects on non target organism and the environment. Having realized the necessity of developing insect resistance genotypes of various crop species, the scientists and breeders have been trying to explore natural gene pool/genetic diversity and identify the wild genotypes of crop plant species that have potential to show resistance to infestation by insect pests and subsequently transfer the conserved traits through breeding into the susceptible cultivars of crop plant species. Secondly, in isolating genes those are considered to have potential to confer resistance to crop plants against infestation by insect pest from plants or organism and in introducing these into cultivars of susceptible crops through genetic engineering technology. Global adaptation rates for transgenic crops were unprecedented and reflect grower satisfaction. With the products that offer significant benefits ranging from more convenient and flexible crop management, highest productivity or net returns per hectare and a safer environment through decreased use of conventional pesticides, which collectively contribute to a more sustainable agriculture. If the rising adoption of genetically modified crops the world over is any indication, their role is likely to widen appreciably in ensuring sharply higher productivity and sustainable agriculture despite their certain much debated negative consequences on biosafety, which are being addressed in the onward researches to improve their acceptance.

Among the vegetable Brassica in India, cabbage (*Brassica oleracea* cv. *capitata*) is grown in large scale providing vegetable. According to the Food and Agriculture Organization of United Nations in 2001, cabbage is grown on 2.3×10^6 ha globally with 10% of the production occurring in India.

Cabbage is third largest vegetable grown with yield amounting to three million metric tones annually. One of the major problems in cabbage cultivation is insect pest, particularly Diamond Back moth (DBM) and aphid.

The insects have become the most destructive enemies of cruciferous plants throughout the world. In India, DBM was first recorded in 1914 on cruciferous vegetables and is distributed all over India, wherever cruciferous are grown. According to an estimation based on path coefficient analysis, 52% loss in marketable yield occurs due to DBM attack on cabbage (Chelliah and Srinivasan, 1985).

Our limitation in addressing crop loss due to pests and diseases is the almost exclusive reliance on chemical pesticides. Prolonged and intensive selection pressure in large monocultures has brought about evolution of insect resistance against a variety of commonly used insecticide. The DBM was the first crop pest in the world to develop resistance to DDT in 1953. It has now become resistant to almost all traditional insecticide used against it in the field. In this aspect fipronil, an insecticide discovered and developed by Poulenc between 1985-87, can effectively control the DBM in the experimental field conditions (Arora, 2002). It is member of a relatively new and small class of pesticide, the phenyl pyrazoles that were principally considered chemicals with herbicidal effects. Fipronil however acts as a broad spectrum low rate insecticide with contact and stomach action. It is an extremely active molecule and is a potent disruptor of the insect central nervous system via interference with the passage of chloride ion through the gamma-amino butyric acid (GABA) regulated chloride channel. This results in uncontrolled central nervous system activity and subsequent death of the insect. Thiacloprid on the other hand is a systemic insecticide with broad spectrum activity and

particularly effective against sucking insects such as aphid, thrips, whiteflies etc. It was discovered by Bayer. It comes under a new group of active ingredients, the chloronicotinyl insecticide. The action of this compound is based on interference with the transmission of impulses in the nerve system of insect. Similar to the naturally occurring signal transmitting acetylcholine, this compound acts by exciting certain nerve cells by acting on a receptor protein. The lasting effect of the product results, in a disorder of the nervous system of the insect and as a consequence of that in the death of the treated insects. It shows a low toxicity to warm blooded animals, excellent systemic properties and a unique activity. Both these compounds can effectively control the two major pests of cabbage. But through the use of transgenic crop cultivation, the load of pesticide on this crop can be reduced in order to obtain higher yield of residue free cabbage. From biosafety point of view, the comparison of the residue behaviour in both the crop i.e., traditional and transgenic variety of cabbage are not available. It is not known how much pesticide persist in transgenic crop as compared to ordinary (non-transgenic) cabbage variety to be grown. So before releasing the Bt-transgenic cabbage in market, it is necessary to check whether there is any difference in the uptake of plant nutrient and nutritional quality in transgenic cabbage. The transgenic had one gene, which is specific for controlling a specific lepidopteran pest. Whenever insecticides are registered or transgenic crop having insect controlling property is to be released, there is a need for its evaluation from safety point of view. Will such transgenic actually affect the microorganism or nematode population in the rhizospheric soil? Moreover the residue data of these two compounds on transgenic cabbage are not available in any part of the world. It was of interest to see whether inserting one gene in a pool of genes, will there be any difference in transgenic and isogenic line of

cabbage from pest attack or number of application of pesticides and dissipation of pesticides. Keeping these points in mind, the following objectives were formulated.

1. Preparation of analytical standard of main metabolite and to compare the dissipation behaviour and rate of metabolism of pesticides in the two isogenic lines namely Bt-transgenic and normal cabbage.
2. To quantify the variation in key nutrients and total pesticide residues in two types of cabbage at the time of harvesting.
3. To carry out concomitant studies such as (a) seed vigour (b) preparation of balance sheet of pesticide application in transgenic vs. normal cabbage.

Beside these, other parameters such as microbial population, dehydrogenase activity, nematode population in the rhizospheric soil of the two line of cabbage and phosphorus uptake pattern of both the isogenic as well as transgenic cabbage were also carried out.

2. REVIEW OF LITERATURE

2.1 *Bacillus thuringiensis*

Bacillus thuringiensis, commonly known as Bt is a species of aerobic, gram-positive, rod-shaped and spore forming bacterium. During sporulation, Bt produces both endospore and crystalline inclusions or parasporal crystal (Cry) protein within a sporangium during the dormant stage of bacterial life cycle. The parasporal bodies are also called δ -endotoxins that have insecticidal properties affecting a selective range of insect orders.

2.1.1. History

Bt was first isolated in Japan from diseased silkworm larvae (Ishiwata, 1901). It was later isolated from Mediterranean flour moths and named *Bacillus thuringiensis* by the German biologist, E. Berliner (1911). The earliest commercial production of Bt began in France in approximately 1938 under the trade name Sporeine (Lambert and Peferoen, 1992). A resurgence of interest in Bt has been attributed to Edward Steinhaus, the "father of insect pathology", who obtained a culture in 1942 and attracted attention to the potential of Bt through his subsequent studies (Tanada and Kaya, 1993). During the 1960s, several industrial formulations of Bt with different insecticidal spectra have been manufactured in the United States, Soviet Union, France and Germany and used with various degree of success. A major advance occurred when the first collection of Bt strains was assembled by Howard Dulmage and Clayton Beegle. They had isolated the HD-1 strain, which is still used as a reference in many studies and is included in many Bt-based products (Dulmage, 1970). By 1989, Bt products had captured 90-95 per cent of the world total biopesticide market

(Feitelson *et al.*, 1992). As early as 1998 there were nearly 200 registered Bt products in the United States (Schnepf *et al.*, 1998). In the 1970s it was believed that Bt was active only against lepidopteran insects. However, the discovery of *B. thuringiensis* sub sp. *israelensis* with activity against mosquitoes and blackflies (Goldberg and Margalit, 1977), followed by the isolation of *B. thuringiensis* subsp. *tenebrionis* in 1983 with activity against some coleopteran insects (Krieg *et al.*, 1983), showed the broad potential of Bt strains. There are at least 34 subspecies of Bt (de Barjac and Frachon, 1990) also called serotypes or varieties and probably over 60,000 Bt isolates are being held in collections throughout the world (Boucias and Pendland, 1998). While the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favour the future development of Bt. Bt products thus far studied are not toxic to mammals, birds, amphibians, or reptiles, but are specific to the groups of insects and invertebrates pests against which they have activity. And therefore, for over 30 years, this insect pathogen has been the most successful microbial pesticide with application for protecting crops, forests and a sanitary human environment (Beegle and Yamamoto, 1992; Navon, 1993). Significantly each new strain demonstrated a certain level for specificity, suggesting that Bt was ideal for integrated pest management (IPM) and environmentally sensitive uses. Moreover, the mode of action of the Cry proteins, the active insecticidal product of Bt, differs completely from the mode of action of known synthetic chemical pesticides making Cry proteins key components of integrated pest management strategies aimed at preserving natural enemies of pests and managing insect resistance.

2.1.4 Mode of action

The parasporal crystals consist of one or more δ -endotoxins or crystal

(Cry) proteins of 120-140 kDa size. The efficiency of crystal proteins as toxic component of Bt involves solubilization of the crystal in the alkaline environment of insect midgut. Proteolytic processing of the protoxin by midgut proteases results in a protease-resistance toxic fragment. The 'toxin' (about 60 kDa in size) binds to the midgut receptors. Following receptor binding, the toxin forms a pore or ion channel in the epithelial cell membrane as a consequence of insertion of the toxin in the membrane eventually kills the epithelial cells (eventually insect death) by a colloid osmotic-type lytic mechanism (Knowles, 1994; Schnepf *et al.*, 1998).

The efficiency of Cry protein solubilization depends on the alkaline pH prevailing in insect midgut and the composition of the parasporal inclusion bodies (Hofmann *et al.*, 1988). After solubilization, many protoxins must be processed by insect midgut to become activated toxins. The major proteases of lepidopteran insect midgut are trypsin-like (Milne and Kaplan, 1993) or chymotrypsin-like (Peterson *et al.*, 1995). The protoxins are subsequently trimmed by gut proteases to an N-terminal, 65-70 kDa-truncated form of the activated toxin. The activated toxin binds readily to specific receptors on the midgut columnar cells (Hofmann *et al.*, 1988; Wolfersberger *et al.*, 1986). Some of the toxins with potential activity against Coleoptera are toxic only after *in vitro* solubilization, probably because the protoxin is insoluble at the neutral to weakly acidic gut pH of Coleoptera; for example, Cry 1Ba for some species (Bradely *et al.*, 1995) and Cry 7Aa (Lambert *et al.*, 1992). The main digestive proteases of Lepidoptera and Diptera are serine proteases, whereas those of Coleoptera are mainly cysteine and aspartic proteases (Tera and Ferreira, 1994). In addition to strains active against Lepidoptera, Diptera and Coleoptera, Bt strains active against Hymenoptera, Mallophaga,

Nematoda and Protozoa have been discovered (Rajamohan *et al.*, 1998). A few toxins have an activity spectrum that spans two or three insect orders-most notably Cry 1Ba, which is active against larvae of moths, flies and beetles (Zhong *et al.*, 2000). The activated toxins can be divided into three structural regions; a N-terminal region, the toxin binding domain (amino acids 1-279), consisting of several conserved hydrophobic regions and conserved C-terminal region (amino acid 461-695); and variable region between these two regions that contains most of the amino acids differences (Gill *et al.*, 1992). The long hydrophobic and amphipathic helices of domain I suggest that this domain might be responsible for the formation of the lytic pores in the intestinal epithelium of the target organism. Modification of one of the domain I helices by site directed mutagenesis resulted in a change in the net charge and caused a decrease in toxicity (Wu and Aronson, 1992) suggesting that α -helices of domain I plays a significant role in Cry protein toxicity. It was also demonstrated that synthetic peptides corresponding to α -helix 5 of Cry 3A and Cry1Aa were found to be able to penetrate phospholipid membranes, to form ion channels in planar lipid bilayers and to exert toxicity against insect cells (Slatin *et al.*, 1990; Schwartz *et al.*, 1993; Grochulski *et al.*, 1995). A synthetic α -helix 7 did not show similar membrane penetration itself, but it formed ion channels when combined with a peptide of an α -helix 5 (Gazit and Shai, 1993a, b; Cummings *et al.*, 1994). The surface-exposed loops at the spices of the three β sheets of domain II and its similarities to immunoglobulin antigen-binding sites (Li *et al.*, 1991; Grochulski *et al.*, 1995), were proposed as sites for involvement in receptor bindings. Recently, a site-directed mutagenesis study of substitution of loop residues in this domain in Cry3A, Cry1A and Cry1C has affected the toxin binding affinity

to midgut membrane receptors and toxicity (Rajamohan *et al.*, 1998). Domain III has been implicated in binding, but individual role of loops or residues has not been proved. However, involvement of domain III in the stabilization of the toxin by protecting it against proteases (Li *et al.*, 1991; Grolchulski *et al.*, 1995), in receptor binding and insect specificity has been reported (Lee *et al.*, 1995; de Maagd *et al.*, 1996).

2.2.1 The conventional Bt biopesticide

In 1995, the market volumes of Bt preparations were at an estimated 90 million US dollars and 67 preparations were registered worldwide (Kumar *et al.*, 1996) and Bt biopesticides account for 90-95 per cent of biopesticide market (Feitelson *et al.*, 1992). By contrast, their share in the whole insecticide market is not more than 1-2 per cent though there is upward tendency since early nineties. Forecasts made in 1991 predicted that by the year 2000, Bt biopesticide would account for 5-10 per cent of world insecticide market (Bernhard and Utz, 1993). Commercial Bt based bioinsecticides, used worldwide for controlling the larval forms of economically important pests, are usually formulations of the spores and crystalline inclusions that are released upon lysis of Bt during its stationary phase of growth. The products are applied at 10-50 g per acre. The molecular potency of Bt is high compared to that of other pesticides. For instance, Bt is 300x higher than synthetic pyrethroids and 80,000x higher than organophosphates (Feitelson *et al.*, 1992). Most of the Bt biopesticides available on the market are mostly derived from the highly potent strain *B. thuringiensis* subsp. *kurstaki* HD 1 that was isolated in the sixties (Dulmage, 1970; Nakamura and Dulmage, 1988). Common brand names of Bt biopesticides include Dipel, Foray, Thuricide (all from *B. thuringiensis* subsp. *kurstaki*), Mosquito Attack (from *B. thuringiensis* subsp. *israelensis*) and M-track

(*B. thuringiensis* subsp. *tenebrionis*) (Entwistle *et al.*, 1993). Bt has been used against a number of forest pests. As forests usually require aerial, broadcast application, Bt has been the pesticide of choice to replace synthetic chemical pesticides because of non-target and health concern. Aerial application of *B. thuringiensis* subsp. *kurstaki* from 1990-1995 resulted in total eradication of gypsy moth in Utah Forest Mountains, USA (Smith and Barry, 1998). *B. thuringiensis* subsp. *kurstaki* is also used widely for crop and horticultural pest control. *B. thuringiensis* subsp. *tenebrionis* has been used for control of Colorado potato beetle in the USA and elsewhere (Keller and Langenbruch, 1993). In Tasmania, Australia, *B. thuringiensis* subsp. *tenebrionis* has become the control product of choice for the chrysomelid beetle (*Chrysopharta bimaculata*) on *Eucalyptus* plantations (Elek, 1997).

In India, Delfin WG and BioBit WP (products of *B. thuringiensis* subsp. *kurstaki* H3a, 3b1 and HD7) are commercially available to control DBM and semiloopers. Bt biopesticide is now widely acknowledged as an environmentally compatible promising source of insecticide, an alternative strategy of insect pest management to control insect pests.

In spite of their environmental friendly reputation, Bt formulations barely occupy any share of insecticide market. The major pitfalls of conventional Bt biopesticides are:

1. Lack of systemic activity and presence of viable spores
2. Narrow specificity
3. Short-shelf life due to environmental factors like ultraviolet radiation and rain (Lambert and Peferoen, 1992).

These constraints are largely overcome by development of improved Bt biopesticides by recombinant DNA technology and genetic exchange mechanism

(Bernhard and Utz, 1993) and also, by other conventional approaches especially to achieve photoprotection that includes encapsulation (Dunkle and Shasha, 1989), granular formulations (Ahmed *et al.*, 1973) and the addition of a variety of photostabilizers (Cohen *et al.*, 1991). Significant developments are made by genetic engineering and many large agrochemical companies are involved in this method for development of new strains with high potency and wider specificity by combining different toxin genes. Genetic engineering method has made it feasible to transfer Cry protein genes into genome of *Pseudomonas fluorescens* that does not undergo sporulation. After fermentation and chemical treatment active toxic compounds that reside within bacteria and the formulations are produced free of Bt spores (Gelertner, 1990). Gawron-Burke and Baum (1991). Another development resulted in improved Bt formulations by conjugation method to transferring one Bt toxin gene to another to yield new combination of gene products. Other improved Bt delivery systems include engineering Bt toxins into endophytic bacteria like *Clavibacter xyli* subsp. *cynodontis* (Dimock *et al.*, 1989), Baculovirus, naturally occurring pathogens, that infect some lepidopteran and hymenopteran insects, cyanobacteria like *Azospirillum* (Udayasuriyan *et al.*, 1995) that colonize plant tissues or that live in the same ecological niches as the target insects which will then ingest the bacteria.

In spite of various strategies devised to improve the Bt biopesticides there are many other drawbacks, which cannot be addressed by conventional Bt products. Since Bt sprays lack systemic activity and do not remain active longer during a crop season, it is necessary to make multiple application throughout the growing season, which raises the costs of pesticide treatment. The half-life of Bt spores in the phyllosphere is much shorter than that in soil. Hansen *et al.* (1996) reported that experimentally determined half-lives were

usually in the range of less than one to three days and these estimates are supported by results of field studies. Pederson *et al.* (1995) showed that initial half-life (1st week) was 16 h for *B. thuringiensis* subsp. *kurstaki* when applied to cabbage foliage. The numbers declined by five log units during the first four weeks after spraying. Bt biopesticides are ineffective against sap-sucking and piercing insects or larvae that after hatching rapidly burrow or bore into plant tissues. Also, there are many pests that cannot be controlled by a single Bt products.

These problems have been effectively addressed by elegant delivery systems for Bt toxin such as the transgenic plant that expressed Cry proteins. Expressing genes for Cry proteins with different specificity (gene stacking or pyramiding) may overcome the problem of narrow specificity and might effectively curtail resistance development (Caprio, 1998; Gould, 1998),

2.2.3 Bt-transgenic plants

Expression of Cry proteins of Bt in plants to protect them from insect attack is a revolution in insect pest management. Of the US \$ 8.1 billion spent annually on insecticides worldwide, it is estimated that nearly US \$ 2.7 billion could be substituted with Bt biotechnology application (Krattiger, 1997). In India, crop losses due to all pests may range from 10 to 30% annually, depending on the crop and the environment. Annual crop losses due to pests in India have been estimated at Rs. 60-70 billion (Jayaraj, 1990; Nagarajan, 1990). Therefore, it is not surprising that at least 16 companies are presently developing transgenic crops with systemic Bt genes and the US Department of Agriculture (USDA) has approved at least 18 BT-transgenic crops for field testing (Mellon and Rissler, 1998). The long history of safe use of Bt Cry protein as an insecticide, the

availability of several Bt genes encoding specific insecticidal activity (Schnepf *et al.*, 1998; Crickmore *et al.*, 1998, 2001) and advances in the transformation of various crop species (Birch, 1997; Hansen and Martha, 1999) have all made remarkable progress in Bt-transgenic plant development. The first genetically engineered Bt-transgenic plant was tobacco (Barton *et al.*, 1987; Vaeck *et al.*, 1987). It was transformed with *cry 1A* gene for insecticidal activity against tobacco pest, *Manduca sexta*. Several Bt-transgenic plants expressing Cry proteins have been developed, including many major crop species such as corn, potato, cotton, rice, sugarcane etc. (Peferoen, 1997; Schuler *et al.*, 1998). Cotton varieties containing Bt toxin genes have been on sale since 1996 (Roush, 1997a), and a number of other crops with Bt genes are undergoing field trials or at an early commercial stage. Furthermore, only four Bt toxins, that is, Cry1Aa, Cry1Ab, Cry1Ac and Cry3Aa are currently produced in commercialized transgenic plants (Frutos, 1999). Corn rootworm is one of the principal insect pests of corn. Currently the only alternative to pesticides for controlling rootworm is crop rotation. Moellenbeck *et al.* (2001) have demonstrated through laboratory and green house studies that novel binary proteins in *B. thuringiensis* strain PS 149B1 successfully control western corn rootworm (*Diabrotica virgifera virgifera* LeConte) in bioassays and in transgenic corn as effectively as, or better than, soil insecticides. The two proteins with molecular masses approximately 14 kDa and 44 kDa have no similarity to other Cry proteins from Bt. Transgenic corn expressing Cry 3Bb protein also provide resistance to corn rootworms (Gray, 2000). The protection from rootworms conferred in transgenic plants expressing these proteins is superior to the control provided by chemical insecticides on wild type plants in the field tests. Among the vegetable Brassicas, cauliflower, cabbage and broccoli have been transformed

with different Bt genes (Metz *et al.*, 1995a, b, Shelton, 2000).

McBride *et al.* (1995) transformed tobacco chloroplast with an unmodified, full-length *cry1Ac* gene by particle bombardment, which resulted in 3-5% buffer extractable protein, which could provide resistance even against Cry1Ac resistant insect pests. Since transcriptional and translational machinery of plastids is similar to that of prokaryotes, a modification of native Bt gene sequence is not necessary. Transgenic tobacco leaves overexpressing the Cry2Aa2 protoxin in chloroplasts when fed to susceptible, Cry1A toxins-resistant (20,000 to 40,000-fold resistant) and Cry2Aa2-resistant (330 to 400-fold) insects like *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua*, 100% mortality was observed in all insect species and strains (Kota *et al.*, 1999). Recently, De Cosa *et al.* (2001) demonstrated *cry2Aa2* operon expression and crystal formation via the chloroplast genome using a universal chloroplast expression vector pLD CtV2. This vector contains a 16S ribosomal (rRNA) promoter (*Prn*) driving the aminoglycoside 3'-adenyltransferase (*aadA*) gene for spectinomycin selection and the three genes of the strong *cry2Aa2* operon. This construct integrates both genes into the spacer region between the chloroplast transfer RNA genes coding for isoleucine and alanine within the inverted repeat (IR) region of the chloroplast genome by homologous recombination. The integration into these transcribe spacer regions allows the gene to be inserted without interfering with gene coding regions. Foreign protein accumulated at 45.3% of the buffer extractable protein in mature leaves and remained stable even in old bleached leaves. This represents the highest level of foreign gene expression reported in transgenic plants to date. Insects (10-day old cotton bollworm, beet armyworm) that are normally difficult to control were killed 100% after consuming transgenic leaves.

2.3 Fipronil

Fipronil, ($\pm$) -5-amino-1-(2,6-dichloro- α,α,α trifluoro-p-tolyl-4-[(trifluoromethyl) sulfinyl] pyrazole-3-carbonitril, is a phenyl pyrazole insecticide. It was discovered in 1987, by the French company Rhone-Poulenc Agro. It is applied at low doses, as compared to conventional insecticides and is highly effective against wide range of insect pests infesting various crops, notably among them are rice insects, thrips (citrus, cotton, mango etc.), termites (sugarcane, corn) and click beetles (cereals, corn, sunflower, beets etc.) (Gauiliard, 1996). It is effectiveness against those insects that have developed resistance or tolerance to pyrethroids, cyclodiene, organophosphorus and carbamate insecticides, (Nagaim, 1997). It can be used as a bait, as a foliar or soil application or as a seed treatment (Hamon,1996). It has outstanding activity in the areas of public and animal health also (Blagburn *et al.*, 1994 and Hunter, 1994).

Its wide range of action (locust and grass hoppers, ready to hatch eggs, larvae, and imagos) and multiple uses (total cover or barrier treatment, possible use in preventive or curative control, in agricultural, forestry or pastoral zones) seem to give it an advantage over growth regulators in chemical locust control (Bobe, 1998).

Fipronil interferes with the passage of chloride ion through the γ -amino butyric acid (GABA) regulating channel, thereby disrupting central nervous system activity and at sufficient doses, causes death (Cole *et al.*, 1993).

Salient features of fipronil

- a. Common name : Fipronil
- b. IUPAC name : 5-amino-3-cyano-1- (2,6-dicholoro-4-trifluoromethyl)

phenyl-[4- trifluoromethyl) sulfinyl] pyrazole.

c. Commercial name : Regent 5 SC

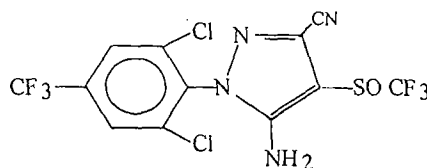
d. Formulation types : SC (suspension concentrate), GR (granular) and flowable suspension.

e. Molecular formulae : $C_{12}H_4$

$Cl_2F_6N_4OS$

f. Molecular weight : 437.2

g. Structural formulae :



h. Appearance : Creamish yellow, odourless, non-hygroscopic powder.

i. Melting point : 200-201⁰C (Technical-200⁰C)

j. Vapour pressure : 370 nPa (20⁰C).

k. Solubility : 2 mg/l in water, in acetone->50% and in corn oil ->10g/l

l. Mode of action : Broad-spectrum systemic insecticide with contact and stomach poison activity. It blocks the GABA regulated chloride channel in a hitherto unknown novel way.

m. Toxicology : Acute oral LD₅₀ for rats 100 mg/kg. Acute percutaneous LD₅₀ > 200mg/kg. Non-irritating to skin, slightly irritating to eyes.

n. Dosage : Generally 10-50 g a.i./ha as a foliar treatment

o. Uses : Generally used for control of many soil and foliar insects (e.g. corn rootworm beetle larvae, Colorado potato beetles and rice water weevils) on a variety of crops and non crops.

2.3.1 Bioefficacy of fipronil

2.3.1.1 Cereals

Carbofuran was used earlier as seed treatment for control of rice water

weevil. Bernhardt (1996) tested efficacy of fipronil to replace carbofuran. Fipronil was applied 0.49, 0.99 and 1.48 g a.i./kg seed as a seed treatment; @ 0.022, 0.045 and 0.089 lb a.i. acre⁻¹ (24.97, 51.075 and 101.025 g a.i. ha⁻¹, respectively) as a pre planting treatment; and @ 0.022, 0.045 lb a.i. acre⁻¹ (24.97 and 51.075 g a.i. ha⁻¹ respectively) as a post planting flood treatment one day after permanent flood for control of rice water weevil. A significantly lower number of weevils were observed in plots treated with fipronil.

Fipronil @ 80, 200 and 400 ml 667 m⁻², Dime hypo (18%) with same rate and triazophos (EC 20%) @ 100 and 200 ml/ 667 m² were screened against *Bombyx mori* and for controlling *Chilo suppressalis* in rice. Liu *et al.* (1999) observed fipronil as the least toxic treatment to *B. mori*. The dead sheath rate was almost zero when fipronil was applied @ 80 ml 667 m⁻².

Fipronil (Regent 25%FS) was applied as seed dressing to control pests of late sown rice. Zhan *et al.* (1998) observed that after 14 days of sowing Regent 25% FS @ 40, 100 or 200 ml ha⁻¹ controlled 98.8% thrips oryzae (*Stenchaeto thrips biformis*). On 58th day after dressing at 200 ml ha⁻¹, 87.3 % control of *Cnaphalocrocis medinalis* and 61th day after dressing at 200 or 100 ml/ha controlled *Chilo suppressalis* over 61.7%. In addition, fipronil treatment could promote rice growth and tillering and had little effect on natural enemies in field.

Liu *et al.* (1999) reported low dosage of fipronil @ 4 and 8 g a.i. ha⁻¹ as an effective contact and stomach insecticide against *Oxya* sp. (*Cantantopidae*). The action as a contact insecticide was quicker than via the diet. One day after treatment, the density of *Oxya* sp. in quadrates treated with fipronil was significantly lower than in quadrates treated with other conventional insecticides.

Regent (fipronil) as seed treatment enhanced the formation of roots of rice

seedlings and increased number of tillers. Leaf senescence was inhibited and leaf chlorophyll content increased resulting in increase in grain yield (Lin *et al.*, 2000).

Leite *et al.* (1998) reported that fipronil 20G @ 60 and 80 g a.i. ha⁻¹, fipronil 800 WG @ 60 and 80 g a.i./ha and carbofuran 50 G @ 750 g a.i./ha⁻¹ afforded similar levels of control of larvae of rice water weevils (*Oryzophagus oryzae*) in rice.

Fipronil was found to be highly toxic against final instar larvae of *Chironomus tepperi skuse* in aerially sown rice crops. Stevens *et al.* (1998) observed that fipronil @ 0.005 mg l⁻¹ gave superior residual control than malathion at concentration of upto 0.6 mg l⁻¹. Raising the application rate to 50 or 100 g a.i. ha⁻¹ increased residual control to between 14 and 19 days and provided significantly better control than malathion. Fipronil, when applied to pre-germinated seed at 12.5 g a.i. ha⁻¹ proved to be more efficacious seed treatment than malathion @ 300 g a.i. ha⁻¹ and provided protection against *Chironominae* for 9-14 days after sowing in rice crops.

Efficacies of fipronil and mineral oil with *Beauvaria bassiana* for control of *Oryzophagus oryzae* on irrigated rice were compared. Fipronil 2G was applied @ 12.5, 25, 35.5, 50 and 100 kg ha⁻¹; fipronil SC 200 @ 250-500 ml/ha; mineral oil @ 15 l ha⁻¹; mineral oil plus *B. bassiana*; *B. bassiana* plus water; fungus @ 1012 spores ha⁻¹. Leite *et al.* (1995) observed fipronil SC 200 at 250 and 500 ml ha⁻¹ gave 87, 93 and fipronil 2G at 100 kg ha⁻¹ gave 82 % larval control.

2.3.1.2 Cotton

Fipronil (Regent 2.5 EC) was applied @ 0.05 lbs a.i. acre⁻¹ (56.75 g a.i. ha⁻¹) in cotton fields using ULV aerial application method. Fipronil was shown to be superior to malathion for boll weevil control as

measured by leaf disk bioassays as well as field observations. Mortality was consistently greater with fipronil than malathion at 24 h evolution. Reed and Jones (1998) reported fipronil to be an ideal alternative to malathion for boll weevil eradication program, provide superior mortality of the target insect, and helps in preserving natural enemies. It was extremely easy on lady bird beetles and softer on orius than malathion.

Regent 80G @ 0.05 a.i. acre⁻¹ and 2.5 EC @ 0.05 and 0.038 a.i acre⁻¹ was evaluated for the control of cotton fleahoppers and compared to a grower standard and untreated check over three years. Tommy (1998) observed that Regent provided effective control of fleahoppers without causing any delay in the build up of the beneficial population or secondary pest problems.

Three water dispersible fipronil formulations, namely 800 WG, 200 SC and 300 EC were tested for toxicity against second instar tobacco budworm, *Heliothis virescens* (F) in cotton crop. Pedibhotla *et al.* (1999) observed that topical bioassay EC (0.07 µg a.i.) was the most toxic followed by WG (0.54 µg a.i.) and SC (1.75 µg a.i.).

Mulrooney and Goli (1999) reported fipronil to be effective for control of *Anthonomus grandis grandis* (Coleoptera) in cotton crop. A comparison of fipronil, applied at a conventional application rate of 93.4 lit ha⁻¹ (with water as diluent) was made with an ULV application applied at 1.17 lit ha⁻¹ (with cotton seed oil as diluent). The difference in mortality between treatments was observed on day three, when ULV application produced higher mortality (98%) as compared to conventional application (85% mortality).

Treatments of 25, 50 and 100 g a.i. ha⁻¹ of fipronil (800 WG) were compared to the commercial standard azinphos methyl at 500 g a.i. ha⁻¹ for boll weevil control in cotton in Mexico. Martinez and

Pacheco (1997) reported that re-infestation was observed seven days after application and mortality readings taken two days later indicated that fipronil was significantly different from azinphos methyl. The percentage mortality observed for fipronil and azinphos methyl were 16.1 % and 5%, respectively.

Shaw *et al.* (1997) conducted trials by foliar application of fipronil @ 0.038 to 0.05 lbs a.i. acre⁻¹ (43.125 to 56.75 g a.i. ha⁻¹) in cotton for control of *Lygus hesperus* and *L. lineolaris* (plant bugs) and confirmed fipronil activity equal to or superior to commercial standards in both initial and residual control of *Lygus* sp.

Leaf disk bioassay method was used for studying the efficacy of fipronil against boll weevils on cotton crop. Mortality was observed when boll weevils were exposed for 6, 12, 24, 48 and 72 h to fipronil, azinphos methyl, two rates of endosulfan, oxamyl and low rate of ULV application of malathion. Spurgeon *et al.* (1997) studied that fipronil, azinphos methyl and high rates of endosulfan caused high levels of mortality immediately after treatment but fipronil showed greater activity than others when it was exposed for 24 h.

Foliar application of fipronil 80 WG @ 0.025 and 0.038 lbs a.i. acre⁻¹ (28.375 and 43.125 g a.i. ha⁻¹) provided effective and competitive thrips (Thysanoptera) control in cotton crop, across all trial locations as compared to acephate @ 454-113.5 g a.i. acre⁻¹ (1135-283.75 g a.i. ha⁻¹), dicotophos @ 90.8 g a.i. acre⁻¹ (227 g a.i. ha⁻¹) and dimethoate @ 90.8 g a.i. acre⁻¹ (Christian *et al.*, 1997).

Samples of tobacco budworm (*Heliothis* sp.) and cotton bollworm (*Helicoverpa* sp.) from cotton fields were bioassayed against different insecticides using a treated diet. Hasty *et al.* (1997) found that the activities of

insecticides followed an order spinosad > pirate R > fipronil > cypermethrin > MVP IIR.

Tillman and Mulrooney (1997) reported fipronil as the only insecticide which had continued toxicity to *Anthonomus grandis* at 48 HAT but has lower toxicity for the natural enemies, *Geocoris punctipes* and *Cardiochiles nigriceps*, as compared to malathion and cyfluthrin.

Shaw and Yang (1996) reported foliar sprays of fipronil on cotton to be equal to or better than commercial standards for control of thrips, boll weevils and *Lygus* sp. at rates as low as 10 times less than the commercial standard.

Two or three applications of fipronil 80 WG (56.75 g a.i. ha⁻¹), 2.5 EC (56.75 g a.i. ha⁻¹) and imidacloprid 1.6 F (53.35 g a.i. ha⁻¹) were needed to control a large insecticide resistant population of *L.lineolarus* on cotton in Mississippi (Scott *et al.*, 1996).

Fipronil (Regent 2.5 EC) @ 43.125 g a.i./ha significantly reduced plant bug infestation on cotton and was considered a promising new product (Robbins *et al.*, 1998).

Kirk *et al.*, (2000) conducted experiment with aerial application of electrostatic fipronil, fipronil in ULV oil and ULV malathion for control of boll weevils. Electrostatic fipronil had higher boll weevils mortalities in leaf bioassays three days after application than the other treatments.

2.3.1.3 Sugarcane

Soil incorporation of Padan (cartap) and fipronil were effective in keeping down the pest infestation of sugarcane with early shoot borers, *Chilo infuscatellus* Snellen, which is difficult to control after appearance of the dead hearts. Nagalakshmi *et al.* (1999) investigated these insecticides to be effective

even in improving cane production.

Melo and Veiga (1998) reported that fipronil (800 WG) @ 0.32 g a.i. mound⁻¹ and 20 G @ 0.20 g a.i. mound⁻¹ as the most efficient treatment causing 100% mortality of termite (*Nasutitermes* sp.) in sugarcane.

2.3.1.4 Vegetables

Polyphagous and highly mobile insect, onion thrips (*Thrips tabaci luid*) was effectively controlled by seed coating with fipronil. Ester *et al.* (1997) reported this insecticide as the most effective insecticide, which did not cause phytotoxicity, where as imidacloprid reduced and slowed germination. Fipronil @ 50 g and 75 g a.i. per unit of seed gave excellent protection of leeks against the immature thrips for at least 16 weeks after sowing.

Field experiments were carried out with fipronil and other insecticides, applied to leek seed, for the control of onion fly (*Delia antiqua*). Fipronil and teflubenzuron as seed coating were most effective insecticides and were phytotoxic to this plant (Ester and Brunel, 1999).

Mau *et al.* (1999) evaluated fipronil and lambda cyhalothrin and found them to be highly effective against *Thrips tabaci* in field experiments on onion.

In vegetables, fipronil, *Btk* and avermectin were tested for field application and gave 100% mortality of 3rd instar larvae of diamond back moth (*P. xylostella*). Diafenthiuron and chlorfluazuron gave 95% mortality while fenvalerate and methomyl gave low rate of mortality (Shi *et al.*, 1998).

Foliar applications of fipronil @ 25, 40 or 50 g a.i. ha⁻¹ is effective in reducing the incidence of DBM (*P. xylostella*) on cabbages by 67.8-84.8 %, when compared to an untreated control. Panda *et al.* (1999) reported fipronil to be more effective than cartap and endosulfan and provided 46.8 and 26%

reduction in the population of this pest, respectively.

The insecticides fipronil (Regent 5%, diluted 2000 times), 5% flufenoxuron (Cascade, diluted 1000 times) were applied to infected radish (*Raphanus sativus* cv. Aiye) seedlings at the 2.5 leaf stage. Wu and Wu. (2000) observed that after 1, 3 and 7 days, fipronil had reduced live pests number by 98.13, 88.13 and 81.55 % and Cascade by 22.88, 39.62 and 90.06 %, respectively.

Rushtapakornchai *et al.* (1996) investigated fipronil (0.03 % G) as a foliar insecticide (5% SC) for controlling tobacco whitefly *Bemisia tabaci* and leaf minor *Liriomyza trifolii* on tomato. Two applications of fipronil, bifenthrin, fenpropathrin and acephate gave significant control of *L. trifolii*.

The transmission rate of tomato spotted wilt virus (TSWV) by its main vector *Frankliniella occidentalis* has been reduced by insecticide fipronil solution (5 g a.i. ha⁻¹). Garzo *et al.* (2001) in his efficacy studies, applied insecticide on the leaves of capsicum and tomato and found that incidence of TSWV was reduced as compared to untreated control.

2.3.1.5 Fruits

Bioefficacies of fipronil 20GR, fipronil 8000 WG, fipronil + mineral oil, mineral oil, isozofos, carbofuran and control were tested against banana borer (*Cosmopolites sordidus*) with concentrations 0.02 g of fipronil, 0.25 g of isazofos, 0.25 g of carbofuran and 1.2 ml of mineral oil. Raga *et al.* (1996) reported fipronil, isozofos and carbofuran as more efficient against *C. sordidus*.

Sekkat *et al.* (1997) reported granular formulation of fipronil to be the best insecticide for controlling young capnodis larvae in orchards. It ensured an efficient protection that lasted over two months.

One gram of bait consisting of 0.2% fipronil on dried citrus meal per nest entrance provided satisfactory control of *Acromyrmex octospinosus* and 50 g of bait @ 2.0 % a.i./nest entrance provided satisfactory control of *Atta cephalotes*.

2.3.1.6 Efficacy of fipronil against grasshoppers

Fipronil was more effective and persistent against grasshoppers as compared to in relation to organophosphorus insecticides. At 13.4 g a.i. ha⁻¹, it eliminated all desert locust hoppers and 80% of adults in one day. Five days after the treatment, the mortality of adults reached 93%. Balanca and Visscher (1997) reported that fipronil, at 4.2 g a.i. ha⁻¹, reduced the initial desert locust hopper population, by 93% in five days.

A 0.6 g a.i. ha⁻¹ spray treatment of fipronil was effective against grasshopper outbreaks. Forty seven per cent mortality was obtained in two days and 91% in 10 days. Cage trial with grasshoppers showed that 1 and 2 g a.i. ha⁻¹ of fipronil were active for at least 23 days post treatment. Balanca and Visscher (1997) reported that acaricidal effect of fipronil remained high during the first eight days after spraying.

Experimental plots with hopper bands (*Rhammatocerus schistocercoids*, 8th instar) in natural vegetation were sprayed with fipronil (Adonis 4UL) in a shorter time. It gave preventive control in natural vegetation with 4 g a.i. ha⁻¹ within 2-4 days or curative control in crop with 8 g a.i. ha⁻¹ doses gave excellent results. Lecoq and Balanca (1998) reported mean mortality as 82% after one day and 98% after two days in curative control.

Liu *et al.* (1999) observed fipronil to be a powerful contact and stomach poison at very low doses of 4 and 8 g a.i. ha⁻¹, for the management of pasture grasshoppers. The persistence of fipronil on the 5th instar

nymphs of *Chorthippus dubius* was over two weeks. The immediate effect of fipronil was stronger than that of Shamieling (a mixture of organophosphorous and pyrethroid insecticides). After 26 days the densities of grasshoppers remained at very low level when treated by fipronil @ 3 g a.i. ha⁻¹ or more.

Fipronil, an ULV formulation (Adonis, 12.5 µ L) was applied @ 15.7 and 16.6 lit km⁻¹ by aerial spraying over grass lands to control bands of hoppers of *Schistocerca gregaria* (Rachadi and Foucart, 1999).

2.3.1.7 Efficacy of fipronil against ants, termites and cockroaches

The ant *Lasius neoniger* Emery, a dominant insect in turf-grass can be a serious problem, when their nesting and mound building occur on putting greens, tees and close cut fairways of golf courses. Lopez *et al.*, (2000) reported that broadcast application of fipronil (0.028 kg a.i. ha⁻¹) on heavily infested, sand based creeping ventgrass putting green turf gave 80 % reduction of ant mounds at 7 days and 86 % at 60 days after treatment.

Costa and Rust, (1999) studied mortality and foraging rates of Argentine ants (Hymenoptera : Formicidae) colonies exposed to potted plants treated with fipronil. Soil mix treatments of fipronil killed > 90% of worker ants in one week, whereas broadcast treatments of fipronil and diazinon killed <50% of workers. The soil mix treatments of fipronil killed all queens in four weeks, whereas the diazinon did not kill any queen.

Granular baits containing 3.0 to 30 µg a.i. mg⁻¹ of fipronil eliminated colonies of workers of Red imported fire ants (*Solenopsis invicta*), 8-11 weeks after treatment. Collins and Callcott , (1998) showed in a field trial that a 15 µg mg⁻¹ granular bait provided over 80% colony mortality 6 and 12 weeks after broadcast application in non grazed pastures.

Sucrose water baits containing $1 \times 10^{-5}\%$ fipronil and 0.1% hydramethylnon provided complete mortality of Argentine ant (*Linepithema humile*) workers baited for 24 h. Hooper and Rust, (2000) studied that baits containing $1 \times 10^{-4}\%$ and $1 \times 10^{-5}\%$ fipronil provided 100% kill of queens.

2.3.1.8 Termites

Raetano *et al.* (1997) evaluated efficiency of fipronil in subterranean termite (*Heterotermes tenuis* and *Cornitermes* sp.) in two year old eucalyptus grandis plantation. Regent 20 G was applied @ 2.5, 5.0 and 7.5 g plant⁻¹ on the soil surface around the tree stems. The best termite control was observed with with atio⁻¹; the observed control was 84.0- 92.9 % and 96.0-100.06 %, respectively. Aldrin (10G) in plant showed lowest average efficiency of termite control of 67.6 %.

The insecticides (quantity of a.i. per termite mound) were tested against mound building termite sp. (*Cornitermes cumulens* n pastures). Valerio *et al.* (1998) reported that fipronil @ 100 mg a.i. per mound gave 100% mortality for *C. cumulans* and *C. bequaerti* and 50% for *Syntermes* sp. after 30 days.

2.3.1.9 Cockroaches

Fipronil baits without glucose provided slightly faster mortality rates of glucose adverse T-164 strains of *Blattella germanica* L. (cockroaches) than did hydramethylnon baits without glucose. Silverman and Liang (1999) observed that hydramethylnon and chlorpyriphos baits with glucose produced little T-164 mortality till 14 days, however the fipronil-glucose bait matrix caused T-164 male mortality beginning on day 7 and reached 60% on day 14.

Gahlhoff *et al.* (1999) reported that fipronil baits with LT₅₀ values of 1.22 days killed 1st and 2nd instar cockroaches significantly faster than

hydramethylnon, boric acid or abamectin baits with LT_{50} values of 3.18, 2.48 and 2.66 days, respectively.

Fipronil caused higher nymphal mortality of cockroaches than chlorpyrifos, 48-72h after exposure in both feeding assay (continuous and abbreviated). In large population chamber tests fipronil bait was more effective and faster in killing *Periplaneta americana* than Raid (0.528% chlorpyrifos), as reported by Kaakeh *et al.* (1997).

2.3.1.10 Miscellaneous

Fipronil gave outstanding control of vine weevil (*Otiorhynchus sulcatus*) in container grown ornamentals. Parsons *et al.* (1998) observed that with fipronil @ 1g a.i. m^{-3} of compost, control was consistent, persistent, and clearly better than that given by the standard insecticides.

Tripathy *et al.* (2000) studied residual toxicity of fenobucarb, carbosulfan (each at 20, 40, 60, 80 ppm) and fipronil (10, 20, 30, and 40 ppm) against seed beetle, *Caryedon serratus* infesting stored groundnut. The residual toxicity was in the order- fipronil > carbosulfan > fenobucarb. Fipronil successfully checked the growth and multiplication of the beetle after one year of treatment.

Doses of 0.2 mg of fipronil active (0.2 g of bait when used at 0.1%) were sufficient to reduce social wasps (*Vespula* sp.) colony activity by 90%. It was faster acting and equally toxic at concentrations over 1000 times lower than sulfuramid. Harris and Etheridge (2001) observed that use of fipronil reduced colony activity within the treated area by 99.7%, whereas sulfuramid had minimal effect on colony activity.

Kaakeh *et al.* (1996) reported fipronil to be the least toxic to *Hippodamia convergens* (coleoptera:coccinellidae) in a laboratory topical bioassay test. The

activity in decreasing order of LT_{50} was : fipronil > diazinon>chlorpyrifos, propoxur > carbaryl > fenvalerate and imidacloprid.

2.3.2 Residue analysis and persistence of fipronil on crops, soil and water

Bobe *et al.* (1998) studied behaviour of fipronil formulation (Adonis 4-UL), applied @ 4 g l⁻¹ for locust control, as well as the persistence of the same in soil. Air dried soil samples were extracted using acetonitrile + acetone (70+30, v/v⁻¹), filtered through anhydrous sodium sulfate on a glass micro fibre filter. After evaporation of solvent, the sample was cleaned on silica cartridge using petroleum ether + acetone (70 + 30, by volume). The analysis was carried out in toluene using GLC with ECD. The mean recovery for fipronil was 85 (±5)% and limit of detection for fipronil was 0.0001 mg kg⁻¹. Fipronil dissipated from 0.001 to 0.0002 mg/kg and it never leached below 10 cm layer of soil. Moreover its residue level was noticeably higher in upper layer (0-5 cm) than in 5-10 cm layer. The half life was calculated as 36 h. After 3 days, it dissipated by 75 %.

Stevens *et al.* (1998) studied persistence of fipronil (suspensible concentrate), applied @ 500 g l⁻¹ as seed treatment in water samples in aerially sown rice crops for control of *Chironomid* larvae. The samples were buffered at pH 6.9 and extracted using C₁₈ cartridge at 5 ml min⁻¹. Acetonitrile was used for elution. The recovery rates for control water spiked with fipronil at 5.24 µg l⁻¹ was 91.4 ± 1.4 % and the limit of detection was 0.005 µg l⁻¹. The sample was analyzed using GLC with capillary column. The GLC conditions were -

Column temperature

50° (1 min) $\xrightarrow{35^{\circ}\text{C min}^{-1}}$ 245° C (10 min) $\xrightarrow{50^{\circ}\text{min}^{-1}}$ 300° C (10 min)
 Injector temperature 250°C

Detector temperature 300°C

Fipronil level was below detection limit ($0.005\ \mu\text{g l}^{-1}$) 20 days after sowing.

Mulrooney and Goli (1999) carried out efficacy as well as dissipation studies of fipronil, applied @ $112\ \text{g a.i. ha}^{-1}$ against *A. grandis grandis* in cotton crop. The residues were collected from the upper surface of leaves by using dual-side leaf washer (Carlton, 1992) on the same day as petridish bioassays. The residues were removed with EtOH and were analysed using HPLC at wavelength of 280 nm, with reverse phase column. Methanol: water ($70:30, \text{vv}^{-1}$) at a flow rate of $1.0\ \text{ml min}^{-1}$ was used as mobile phase. Fipronil dissipated from 187 ± 10 to $3 \pm 1\ \text{ng cm}^{-2}$ on cotton leaves 7 DAT.

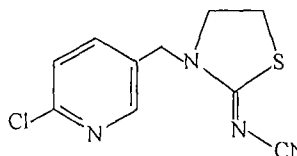
2.4 Thiacloprid

Thiacloprid, N-[3-(6-Chloro-pyridin-3-ylmethyl)-thiazolidin-2-ylidene]-cyanamide, is a neonicotinoid insecticide. The German Company Bayer Agro discovered it. Thiacloprid (Bay YRC 2894) is a highly active chloronicotinyl insecticide for foliar application, with systemic properties and broad-spectrum efficacy against sucking and biting insects. Like all chloronicotinyl insecticides, thiacloprid selectively acts on the insect nervous system as an agonist of the nicotinic acetylcholine receptor (nAChR). It shows good plant compatibility in all relevant crops. (Jeschke *et al.*, 2001). Thiacloprid is mainly used for control of insects pest in pome and stone fruits, tomatoes, melons and ornamentals. From comparisons of exposure and toxicity data and findings of higher Tier studies, it can be concluded that thiacloprid will pose a negligible risk to terrestrial microflora, terrestrial plants, earthworms, birds, mammals, vascular/non-vascular

aquatic plants and fish under normal conditions of practical use (Schmuck, 2001). A high biological effect of thiacloprid, against sucking insects and excellent control of the chewing insects *Cydia pomonella* and *Lissorhoptrus oryzophilus* were detected. This compound also showed excellent performance against the first, second and fourth instar larvae of *C. pomonella* in a diet incorporation bioassay, and ovicidal activity against 2-day-old eggs of the same insect on another bioassay. A positive temperature coefficient and good light stability were found. In the greenhouse, thiacloprid showed no phytotoxicity to any of the crops tested even at high concentrations (Elbert *et al.*, 2001). Due to its broad spectrum of action, thiacloprid (Calypso) is an excellent insecticide for combating important, difficult-to-control insect pests encountered in fruit cultivation. Calypso is harmless to bees and important beneficial organisms. It can therefore be used before, during and after flowering in integrated crop management in fruit (Erdelen 2001).

Salient feature of thiacloprid

- (a) Common name : Calypso
- (b) Commercial name : Thiacloprid
- (c) IUPAC name : N-[3-(6-Chloro-pyridin-3-ylmethyl)- thiazolidinylidene]-cyanamide.
- (d) Formulation : SC (suspension concentrate)
- (e) Molecular formulae : $C_{10}H_9ClN_4S$
- (f) Molecular weight : 252.8 g /mol
- (g) Density : 1.46g/cm³ at 20°C
- (h) Structural formulae :
- (i) Appearance : Powder of crystals
- (j) Melting point : 136⁰ C
- (k) Vapour pressure : 3×10^{-12} h Pa at 20°C
- (l) Solubility : 0.185 g /l in water, 160g/l in dichloromethane, <0.1g l⁻¹ in n-heptane. 3.0 g l⁻¹ in 2-propanol.



- (m) Mode of action : The action of this compound based on an interference with the transmission of impulses in the nerve system of insect. It acts by exciting certain nerve cell by acting on a receptor protein. The lasting effect of the product results in a disorder of the nervous system of the insect and as a consequence of that leads to the death of the treated insects.
- (n) Toxicology : Acute oral LD₅₀ for male rat ~ 836 mg /kg body weight. Acute dermal toxicity for male rat ~ 0.2000g/kg body weight. Slightly irritation to skin and eyes.
- (o) Dosage : Generally 48-180 g a.i. ha⁻¹ as a foliar treatment
- (p) Uses : Generally used for control of various sucking insect such as aphid, thrips, *Empoasca* sp. White fly and also against various beetles on a variety of crop and non crops.

2.4.1 Bioefficacy of thiacloprid

Albuquerque *et al.* (1999) evaluated the effect of carbosulfan (Marshal @ 120 g a.i. ha⁻¹), oxydemeton-methyl (Metasystox R @ 200 g a.i. ha⁻¹), prothiofos (Tokuthion @ 500 and 750 g a.i. ha⁻¹), imidacloprid (Provado @ 50 g a.i. ha⁻¹) and thiacloprid (Calypso @ 34 and 48 g a.i. ha⁻¹) against *Frankliniella schultzei* on cotton. They reported that imidacloprid and thiacloprid were the most effective for, controlling the pest up to 7 days after treatment as compared to other insecticides. Albuquerque *et al.* (1999) evaluated the efficacy of carbosulfan, oxydemeton-methyl, prothiofos, imidacloprid and thiacloprid for the control of *Aphis gossypii* on cotton. Among these insecticide, imidacloprid and thiacloprid gave the best control of *A. gossypii*, 3 days after application, with at least 96% control. Eight days after application, control with these insecticides was at least 80%. Erdelen (2001)

applied Calypso as a 480 SC formulation @ 0.02% and showed outstanding efficacy against apple blossom weevil (*Anthonomus pomorum*), rosy leaf-curling aphid (*Dysaphis devector*), rosy apple aphid (*D. plantaginea*), apple sawfly (*Hoplocampa testudinea*), black cherry aphid (*Myzus cerasi*) and raspberry beetle (*Byturus tomentosus*). Application rate of 0.025% gave good control of codling moth (*Cydia pomonella*). Alves *et al.* (2001) evaluated the efficacy of *Beauveria bassiana* (strain PL63), associated or not with the insecticides Calypso 480SC (thiacloprid), Gaucho 700PM and Provado 200SC (imidacloprid) for control of *Bemisia tabaci* (B biotype) and the incidence of the bean gold mosaic virus (BGMV). They concluded from their experiment that the use of *Beauveria bassiana* associated with insecticide is an important strategy for the prevention of resistance development by the *Bemisia tabaci* pest population to these insecticides. Ciglar and Baric (2002) tried to control the apple sawfly (*Hoplocampa testudinea*). The investigation of effective control strategies with insecticides showed high efficacy of thiamethoxam (Actara), imidacloprid (Confidor), thiacloprid (Calypso) and betacyfluthrin + oxydemeton-methyl (Enduro) and lower efficacy of phosalone (Zolone), chlorpyrifos-methyl (Reldan), and lambda-cyhalothrin (Karate). Yanovskii (2001) reported that Confidor [imidacloprid], Calypso (thiacloprid) and Actara (thiamethoxam) effectively control insect on apple cv. Melba. Artyukhov *et al.* (2001) tested calypso on apple cultivars Pamyat' Michurina and Spartan for the control of apple curculio, leaf roll insects and apple psylla (*Cacopsylla mali*). It was found that calypso provided effective control agent of *C. mali*, apple aphids and San Jose scale (*Diaspidiotus perniciosus*). Akey *et al.* (2001) evaluated the effectiveness of some Insecticides (endosulfan, methomyl, dimethoate, phosmet, bifenthrin, cyfluthrin, fenpropathrin, thiamethoxam,

acetamiprid, thiacloprid, and imidacloprid) for use against the glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*), a vector of *Xylella fastidiosa* that causes Pierce's disease. It was found that bifenthrin, fenpropathrin, cyfluthrin, thiamethoxam, acetamiprid, thiacloprid and imidacloprid were the most promising control agents for GWSS infestation. Yanovskii and Larcheva (2000) reported that Confidor (imidacloprid) and Calypso (thiacloprid) were successfully used in apple orchards (cultivars *Slava Pobeditelyam* and *Renet Simirenko* grafted on *Antonovka obyknovennaya*) to control San Jose scale (*Diaspidiotus perniciosus*), aphids (including apple green aphid [*Aphis pomi*]), apple psyllid (*Cacopsylla mali*) and red mites (*Panonychus ulmi*).

Schaub *et al.* (2001) evaluated the efficacy of imidacloprid (Confidor 70%), acetamiprid (Gazelle 20%) and thiacloprid (Alanto 40%) against *Dysaphis plantaginea* in the laboratory. The LC_{50} s of acetamiprid and thiacloprid were 4.24 and 2.79 ppm. Castelo and Pontes (2001) studied the effects of thiacloprid on adults, female fertility, egg hatch and nymph development of *Bemisia argentifolii*. Their results indicated that thiacloprid had good effect on adults. Egg viability was not affected when females had contact with leaves treated with the insecticide as more than 97% of the eggs hatched. More than 97% of the eggs treated with thiacloprid did not hatch, indicating that inhibition of egg hatch caused by thiacloprid has no connection with the age of egg. Thiacloprid affected nymph development. Only 1.2% of the second instar nymphs treated with the insecticide developed to the third stage. Schuld and Schmuck (2000) reported that thiacloprid, a new chloronicotinyl insecticide, is targeted chiefly to control aphid pest species in orchards and vegetables. They concluded from their study that a spray treatment with thiacloprid (SC 480) in the field would not significantly interfere with the pest control function of a

parasitoid fauna in the target crops. Boselli and Vergnani (2001) evaluated the efficacy of some insecticides for the control of first generation of *Cydia pomonella* (a pest of apples and pears) and found that among larvicidal insecticides, spinosad, thiacloprid and CpGV-based products (Carpovirusine and Madex [granulosis virus of *Cydia pomonella*]) gave the best results. Elbert *et al.* (2000) concluded from their field study that thiacloprid can effectively control, important pests in pome fruit, cotton, vegetables, and potatoes. Besides aphids and whiteflies it was also active against various species of beetles (e.g. *Leptinotarsa decemlineata*, *Anthonomus pomorum*, *Lissorhoptrus oryzophilus*) and Lepidoptera such as leaf miners and *Cydia pomonella* in apples and shows good plant compatibility in all relevant crops. Like imidacloprid, thiacloprid acts agonistically on the nicotinic acetylcholine receptor. As a result there is no cross-resistance to conventional insecticides such as pyrethroids, organophosphates, and carbamates and consequently it will fit well into resistance management strategies. Visigalli *et al.* (2000) reported that Calypso SC and Fenitrocap (fenitrothion) were most effective on the shoots and Calypso WG on the fruits. Ciglar and Baric (2001) studied the efficacy of insecticides Calypso (thiacloprid), Confidor (imidacloprid), Actara (thiamethoxam), Enduro (cyfluthrin + oxydemeton-methyl) and Aztec (triazamate). The efficacy was investigated on rosy apple aphid *Dysaphis plantaginea*, *D. devector* and green apple aphid *Aphis pomi*. All the insecticides were very effective in controlling aphids on apples. Pasqualini and Civolani (2002) reported that thiacloprid WG provided good control. Wegorek (2002) reported that Colorado Potato beetle (CPB) strains are tolerant to nereistoxin analogue pyrethroids (bensultap) and organophosphorus insecticides (chlorfenvinphos). However, resistance to imidacloprid, thiamethoxam, acetamiprid, thiacloprid and fipronil was

not recorded. Results also showed no indication of resistance or cross-resistance of tested CPB populations to the new classes of insecticides for CPB control. It is suggested that the compound be included for general resistance management guidelines. Premalatha *et al.* (2003) found that thiacloprid 240 SC at 125 ml/ha gave excellent protection to cotton plants from leafhoppers (*Amrasca biguttula biguttula* Ishida). They also reported that that treatment gave complete control of leafhoppers up to seven days after transplanting and even after four weeks after treatment the population was found to be very low. Thiacloprid 240SC at 75 and 100 ml/ha was superior to oxydemeton methyl 25 EC at 500ml ha⁻¹ but on par with imidacloprid 200SL at 100 ml ha⁻¹.

2.4.2 Persistence of thiacloprid on crop, soil and water

Klein (2001) investigated the metabolism of YRC 2894 (thiacloprid) in apples (cv. James Grieve), tomatoes and cotton as well as in rats, goats and poultry. In plants, a clear and consistent picture was obtained. The metabolic degradation in qualitative and also quantitative terms was similar in all crops investigated. The parent compound is always the major component. No metabolite occurred in quantities above 3% of the radioactive residue. The high level of recovery and characterization achieved in the studies strongly supports the residue definition proposed as parent compound only. The presence of high amounts of 6-chloronicotinic acid in cottonseed was due to accumulation in the seed as a phloem sink after being secreted from the apoplasm into the phloem as a trap compartment for weak acids. It originated from leaf metabolism where also it was identified. The studies with YRC 2894 in rats showed a high degree of absorption of radioactivity followed by fast elimination from the body. After oral administration more than 92% of the totally recovered radioactivity was

excreted in almost all dose groups within 48 h independent of the labeling position. The major route of elimination was renal, with 60% or more of the recovered radioactivity in the urine. Faecal excretion accounted for 10-37% of the recovered radioactivity. Although YRC 2894 was quickly eliminated from the body, it was intensively metabolized. By using two different labeling positions, besides the unchanged parent compound, 25 metabolites were isolated and identified in the excreta. The identification rate ranged from 55 to 79% of the administered dose. In goats and hen, the residue levels in tissues and organs as well as in milk and eggs were low due to the fast elimination. There was no indication for bioaccumulation of residues in any of the tissues. The residues were extracted with high recoveries. YRC 2894 was metabolized by goats and hen in close similarity to the rat. The main residue in milk, eggs and edible tissues was unchanged parent compound. The total identification rate was very high in most of the matrices ranging from 62.5 to 95.4%. Only in hen liver (40.2%) and muscle (50.2%) a lower rate was obtained. Since all main metabolites identified in plants and livestock were also detected in the rat metabolism study, the toxicological studies with the parent compound also cover plant and livestock metabolites. Generally, the metabolism of YRC 2894 proceeded via the following pathways: hydroxylation of the parent compound at the thiazolidine ring and cleavage at the methylene bridge leading to the partially and fully oxidized products 6-chloropicolyl alcohol and 6-chloronicotinic acid followed by conjugation. The corresponding thiazolidine-ring metabolites mainly comprised the free thiazolidine and its hydroxylated and olefinic derivatives as well as conjugates. Furthermore, the N-nitrile moiety of YRC 2894 was hydrolysed to the corresponding amide derivative, which was then N-hydroxylated. Additionally, the thiazolidine ring was

also opened and the sulfur oxidized and methylated. Krohn (2001) reported that thiacloprid disappeared rapidly from the environment after having been applied as a crop protection chemical. Half-lives in soil measured under field conditions of northern Europe ranged from 9 to 27 days, and in southern Europe from 10 to 16 days. The major metabolites formed are only intermediates before complete mineralization to carbon dioxide occurs. Thiacloprid must be classified as being only slightly mobile in soil and hence it has no potential for leaching into groundwater. The metabolites occurring in groundwater are of no concern as toxicological, ecotoxicological and biological testing has shown. In the aquatic environment, thiacloprid will also undergo rapid biotic degradation with half-life in the total system of 12-20 days.

2.4.3 Residue analysis of thiacloprid

Placke and Schoning (2001) described the determination of insecticide residues of YRC 2894 (thiacloprid) in plant materials. YRC 2894 was extracted from plant materials with acetone/water. After filtration, an aliquot of the extract is concentrated to the aqueous remainder. The residues are dissolved in water and partitioned against cyclohexane/ethyl acetate using a ChemElut column. Further clean up was performed by column chromatography on Florisil and elution with acetonitrile. The residues are quantified by reversed phase HPLC with UV-detection. The method was validated by conducting recovery experiments with commodities relevant for the intended use of YRC 2894. Control samples were amended with YRC 2894 at fortification levels of 0.02 and 0.2 mg kg⁻¹. The lowest fortification level corresponding to the limit of quantitation (LOQ) and was 0.02 mg kg⁻¹ for all matrices tested. Recoveries of YRC 2894 ranged from 72-105% (mean: 95%, relative standard deviation (RSD): 5.1%, n=165). The

recoveries were not corrected for interferences. Matrix interference was minimum as illustrated in representative control sample chromatograms ($< 10\%$ of LOQ). Well resolved peaks were obtained with all sample commodities at all fortification levels. This shows the specificity of the method for the compound of interest. The repeatability was determined for apple, cotton, aubergine, melon and potato running five recoveries at 0.02 and 0.2 mg kg^{-1} each, simultaneously. The RSD of the repeatability determination for each recovery set ranged from 1.5 to 6.2% . These results clearly demonstrated the excellent precision of the method. The analytical solutions were stable for at least 2-4 weeks when stored in a refrigerator. Schoning and Placke (2001) described another method of determination of residues of the insecticide YRC 2894 (thiacloprid) in/on plant materials. YRC 2894 was extracted from plant material using a mixture of acetonitrile/water. After filtration the extracts were concentrated to the aqueous remainder. The residues were diluted with water and partitioned against cyclohexane/ethyl acetate on a ChemElut column. The residues are quantified by Reversed Phase HPLC with Electrospray Ionization and MS/MS-detection. The instrumental response was linear over the range $0.0005\text{-}0.2 \text{ mg l}^{-1}$. The method was validated by conducting recovery experiments. For that purpose, the control sample material was spiked with YRC 2894 at concentrations of $0.02\text{-}2.0 \text{ mg/kg}$. The lowest fortification level corresponding to the limit of quantitation (LOQ) was 0.02 mg/kg for all matrices (except: tobacco green leaf 0.05 mg kg^{-1} and tobacco dry leaf 0.2 mg kg^{-1}). In the control samples, no residues or interferences were observed. Recoveries of YRC 2894 ranged from 66 to 114% (mean: 91% , relative standard deviation (RSD): 7.2% , $n=191$). The repeatability was determined for cherry juice, cotton seed, potato tuber, potato pommes and strawberry fruit analysing five recoveries at

concentrations of 0.02 and 0.2 mg kg⁻¹ and two control samples. The RSD was between 2.5 and 9.5%. Lee *et al.*, (2001) standardized a competitive enzyme-linked immunosorbent assay (ELISA) for the chloronicotinyl insecticide imidacloprid, using a polyclonal antibody produced against a hapten conjugated through the imidazolidine to keyhole limpet hemocyanin. In the standard curve of imidacloprid, an IC₅₀ of 17.3 ng ml⁻¹ was obtained using a competitive heterologous system at pH 10. Very low cross-reactivity was found for some structurally related compounds including the insecticide thiacloprid. The high cross-reactivity with a metabolite containing the carbonyl group in the imidazolidine moiety suggests the involvement of its polarity and stereochemical fitness in forming the antibody-antigen complex. The effects of various assay conditions, including organic solvents, detergent content, salt concentration, and pH on the sensitivity were evaluated. High-performance liquid chromatography was run for comparison to validate the ELISA with fortified water samples, the correlation being 0.997-0.998 (n = 15) with a slope of 1.10-1.38. The ELISA turned out to be a convenient tool for monitoring imidacloprid residues in agricultural and environmental samples.

2.5 Phosphorus uptake

Staud (1974) conducted an experiment with mono-, di- and tri-calcium phosphate at 3 levels. They were applied in pot trials of pea to an acid soil of low P status. Dicalcium phosphate was the most effective P source for peas and monocalcium phosphate the least effective. It was found that different P levels had no effect on the plant morphological characteristics or yield components (vine, pod, seed). Malajczuk *et al.* (1975) experimented to determine whether

ectotrophic mycorrhizae had any effect on the P absorption and growth of *E. calophylla* seedlings in soil with various concentrations of phosphorus. They concluded that mycorrhiza have an important role in P uptake by Eucalyptus. In a series of experiments with lettuce, radish and foxtail millet (*Setaria italica*), seeds of *S. italica* were sown in vermiculite containing 0, 0.25, 0.5 or 1 g P kg⁻¹ substrate. Avnimelech and Scherzer (1971) concluded that at least one of the mechanisms controlling the growth of some plants was due to a reaction, which occurred during the early stages of development and was affected by the supply of P at that time. Application of P during later stages of growth had little effect on restoring growth. The role of some specific biochemical reactions involved in phosphate assimilation was studied in young maize roots. The role of some specific biochemical reactions involved in phosphate assimilation, using ³²P was studied by Michalik (1974) in young maize roots. The results obtained indicated the essential role of metabolism, especially oxidation (glycolysis and respiration) and phosphorylation, in phosphate accumulation in root cells. In pot trials a mixture of wheat cv. Giza 144 and flax cv. Giza 4 were grown on 2 different soils with different doses of nitrogen and phosphorus. El Sherif and El Sherif (1973) concluded from this experiment that drymatter yield and phosphorus uptake increased with increasing soil moisture and increasing rates of applied phosphorus and nitrogen. Pettersson (1975) suggested from an experiment that at low ionic strength of the nutrient solution the initial passive step of ion transport through the root free space could limit the overall phosphorus uptake rate. Clark and Brown (1974) carried out an experiment to evaluate the phosphorus uptake pattern of two inbred line of maize, Pa 36 and WH to evaluate the phosphorus uptake pattern. It was found that intact Pa 36 roots exhibited higher phosphatase activity than WH roots at various levels of P. In a field

experiment of rice, conducted by Mohamed-Ali and Morachan (1973) to study the uptake pattern of nitrogen and phosphorus by rice plant, it was found that uptake of N and P by rice plants was higher at the early stages of growth, whereas uptake of K was higher at the harvesting stage. Throughout crop growth total uptake of N, P, and K was greater under submerged conditions. Ivanov (1973) found that phosphorus uptake by Italian ryegrass was most closely correlated with the fluoride-soluble P and its isotopically exchangeable fraction and with the isotopically-nonexchangeable fraction of the Ca-phosphates. In typical and leached chernozems, P uptake by plants best correlates with the total Al-phosphates and their labile fractions and with the isotopically exchangeable Fe- and Ca-phosphates. The Uptake in podzolized and grey forest soils also correlated best with Al-phosphates. The correlation between P uptake and total and isotopically exchangeable Fe-phosphates was poor. Bulaev and Bulaeva (1974) studied the effect of nitrogen fertilizers on the movement of superphosphate labelled with ^{32}P and, on its uptake by the potato. It was found that the movement of P was decreased somewhat by urea but not by ammonium nitrate or ammonium sulfate (NH_4NO_3 or $(\text{NH}_4)_2\text{SO}_4$). The lower rate of N increased P uptake by the plants but the higher rate prevented the roots from penetrating the band and decreased P uptake. Jones (1975) concluded, from the results of an experiment to study the uptake of phosphorus by dune and dune slack plants, that phosphorus uptake was greater from slack sand than dune sand even though the latter had higher total phosphorus content. A field experiment was undertaken during the third crop season of 1971 to study the effect of different levels of nitrogen and phosphorus on the uptake of phosphorus by the rice variety 'Triveni'. Alexander *et al.* (1974) concluded from this experiment that with increasing levels of nitrogen, the total uptake of

phosphorus increases during all the growth stages of the crop. Similarly nitrogen phosphorus interaction significantly increased the phosphorus uptake at harvest. The various rates of phosphorus had no significant effect on the uptake of phosphorus at any growth stages. Joshi *et al.* (1973) reported that phosphorus uptake by mustard increases with increasing rate of P_2O_5 and/or S. S uptake increased with increasing rates of P_2O_5 or S up to 50 kg ha⁻¹ and decreased when both P and S were applied. S: P_2O_5 ratios between 0.9 and 1.4 were the best combinations for increasing yields and nutrient uptake. Chandra *et al.* (1973) found the highest correlations between available phosphorus content and plant P uptake and also with drymatter yield of rice. Herrera (1973) reported that at the lowest P level, a sharp decrease in drymatter yield with increasing moisture stress was noted; this effect was even more marked in terms of P uptake by Italian ryegrass. Stryker *et al.* (1974) reported that the growth rate of roots dependent on an internal supply of P via translocation (roots in no-P zones), as compared with that of roots with an external supply, was limited by the rate at which inorganic P was supplied to the growing areas. It was postulated that these non-uniform effects on leaves were the result of non-uniform transport of P from the roots to the leaves. Kogan *et al.* (1973) conducted an experiment to study the effect of 2,4-D on phosphorus uptake, using ³²P as tracer. It was found that marked decrease in the absorption of P by roots was observed in the presence of 2,4-D. Sheard (1974) conducted an experiment to study the nitrogen enhancement of surface applied fertilizer phosphorus uptake by forage species using ³²P as a tracer. The proportion of P in timothy derived from a single surface application of 60 kg P ha⁻¹ increased from 16-21% without N to 38-43% with 60 kg N ha⁻¹ applied before spring growth and after the 1st and 2nd harvests. It was suggested that the enhancement of fertilizer P utilization

was in part the result of an increase in the accumulated N in the plant. It was concluded that adequate N should be provided for efficient utilization of surface-applied P by perennial forage grasses. Jungk and Barber (1974) found that phosphorus levels in the shoot of maize, had influence on the rate of P uptake. It was concluded that P in soil must be in contact with a large proportion of the roots for the supply to the plant to be adequate. It appeared that P levels in the shoot influenced the rate of P uptake. It was concluded that P in soil must be in contact with a large proportion of the roots for the supply to the plant to be adequate. Studies by Gopalachari *et al.* (1973) with ^{32}P -labelled superphosphate, showed that applications of P fertilizer to soils low in available P increased total uptake and percentage recovery. Application of $50 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$ increased the yield of cured leaf of tobacco significantly in these soils. The addition of P reduced yield and phosphorus uptake in soils already rich in available P. Ivanov and Shidlovskii (1973) reported that the percentage utilization of soil P and superphosphate P by barley increased with increasing rate of nitrogen and potassium. Anter *et al.* (1973) conducted experiments in pots with mixtures of soil and sand containing 1 to 50% CaCO_3 , dry matter production, uptake of ^{32}P from labelled phosphate and uptake of Fe by test plants decreased when the substrate had a CaCO_3 content of 8% or more. Fageria (1974) reported that uptake of potassium and calcium but not of phosphorus was suppressed by magnesium. Taber and McFee (1972) reported that the depressing effect of $\text{NH}_4^+\text{-N}$ in Pine may be due to excessive accumulation of NH_4^+ , since low concentrations of $\text{NH}_4^+\text{-N}$ (<12 ppm) increased P uptake. Blair *et al.*, (1972) experimented to study the effect of nitrogen status on short-term phosphorus uptake. It was found that the uptake of ^{32}P in the presence of 20 ppm nitrogen by 13-day-old maize plants which had been grown in

nutrient solution containing 0.1, 5 or 20 ppm N increased with increasing nitrogen contents in the nutrient solution. The uptake of ^{32}P was higher if the 20 ppm nitrogen in the solution from which the phosphorus absorbed was in the form of calcium nitrate than if it was in the form of ammonium sulphate. Thien (1971) reported that pre treatment with N significantly increased P uptake and translocation in 10 day old maize plants, the stimulatory effect on P uptake lasted for 12 h after removal from the N pre-treatment. Maximum stimulation of P uptake occurred with 56 ppm N, whereas the stimulation of P translocation increased with increasing N concentrations. The protein-synthesis inhibitors cycloheximide and chloramphenicol altered the subsequent stimulation of P-transport systems when included in the pre-treatment. This stimulation of P transport was not ubiquitous among species including wheat soyabeans and sunflower. Evidence supported the existence of a proteinaceous compound involved in P-absorption reactions, and observations suggested that systems moving P across the plasmalemma were not identical to those governing the radial movement of P to the xylem for translocation. Sokolova (1972) found that application of wild mustard (*Sinapis arvensis*) root exudates to vetch and vetch root exudates to mustard markedly increased uptake of labelled P uptake in the 2 species. The uptake was highest at different growth stages. It was concluded that root exudates of mustard and vetch grown in mixtures promote growth of each other because the 2 species do not compete for highest P uptake at the same time. Khasawneh and Copeland (1973) reported that rate of root growth and rate of P uptake were controlled by a feedback mechanism that caused the two rates to vary concurrently and in the same direction. White (1973) reported that the demand for P associated with the rate of plant growth, or the level of metabolic activity within the tissues. It appeared to have a marked

effect on the rate of P uptake at deficient to optimum levels of supply. It was suggested that the rate of influx of orthophosphate into the root cortical cells was regulated by the turnover rate of the pool of inorganic P in the cytoplasm and by the rate of transport of inorganic P to the shoot. The turnover rate of this pool was considered to depend on inherent factors such as the RGR of the species and on environmental factors including nutrient supply. Dombovari (1973) concluded from an experiment that soil moisture content had no significant effect on ^{15}N uptake from the solution, but P uptake from the solution increased with decreasing soil moisture. In treatments where N and P were supplied to soil and water, ^{15}N uptake was not significantly affected by soil moisture, whereas ^{32}P uptake was higher at the lower soil moisture. Niemann *et al.* (1972) reported that uptake and transport of ^{32}P were similar in the air and O_2 treatments, but N_2 markedly reduced P uptake. There was evidence that a decrease in the rate of P uptake at higher temperatures and longer treatments was due to depletion of energy-supplying carbohydrates in the roots. Koyama and Snitwongse (1971) conducted experiments to study the growth and P uptake of two varieties of rice and compared these two parameters in pot and water culture using ^{32}P labelled fertilizer. In the culture experiments no significant varietal differences were observed, but in the pot experiments varietal differences were observed particularly in the ability of the varieties to utilize soil P. Tewari and Mandal (1972) reported that there was a positive correlation between the P and Mg contents of the seed. The seed P content was inversely related to the P contents of the leaf and stem. The latter were positively correlated with the Mg content before the stage of maturity. Glass (1973) carried out an experiment with thirteen naturally occurring phenolic acids to study the inhibition effect to phosphorus uptake. These tested acid inhibited, P uptake by barley

from ^{32}P -labelled phosphate solution. This result supports the hypothesis of inhibition through altered membrane properties. Cathey and Sabbe (1972) reported that P uptake was decreased when fertilizer and trifluralin were located in the same soil zone, whereas, in some cases a stimulation of P uptake from the fertilizer zone occurred when the herbicide was confined to a separate soil zone. The fertilizer P uptake was affected least when the herbicide was placed in the top 5 cm of soil. Meelu *et al.* (1977) found that P uptake by maize was greater with the wholly water-soluble sources, indicating luxury consumption and also reported that yields of succeeding crops were significantly correlated with soil test values for phosphorus. Hanker and Kudelova (1977) studied the effect of pesticide on the uptake of radioactive phosphorus and it was found that the uptake of radioactive phosphorus (^{32}P) by the roots of plants of broad bean (*Vicia faba*) and cucumber was significantly decreased on the second day after the application of dimethoate, pirimicarb or methomyl (Lannate) to the roots. Diminished uptake of ^{32}P was also noted when the aerial parts of the bean plants had been treated with pirimicarb. However, on the second day after the fungicide cypendazole had been applied to the roots of cucumber, ^{32}P uptake was found to have been significantly stimulated. Uptake of ^{32}P was significantly reduced during treatment of the roots of intact plants of horse bean (*Vicia faba*) cv. Pluto with Pirimor (pirimicarb) and on the 2nd day after application of dimethoate, pirimicarb or methomyl. Foliar applications of pirimicarb also reduced plant uptake of ^{32}P . Soon and Miller (1977) suggested from the result that NH_4^+ and NO_3^- enhanced P absorption. It was as a result of increased physiological capacity to absorb P and/or increased root growth. Newman and Miller (1977) reported that presence of exudates caused significant differences in P uptake compared with the controls, in some cases increases and

in other cases decreases and also gave strong evidence that root exudates from one species could influence the P uptake of another. Edwards and Barber (1976) conducted experiments with soyabean plants ranging in age from 18 to 74 days. They showed that the average net rate of influx of P decreased with increasing plant age, but that the rate of decrease was less than that previously observed in maize. Removing 50% of the root mass 2 days before measurement increased the rate of influx to an extent that left the uptake pattern/plant unchanged. It was concluded that optimum phosphorus fertilization for soyabean and maize are likely to differ. Idris *et al.* (1976) reported that N, P, NP and NPK applications increased plant P uptake by maize at all stages of growth; K application alone did not affect P uptake. Lin *et al.* (1977) conducted a study in 20 field and 4 soils in pot trials using soil from these sites, correlations between soil P contents and uptake of P by rice indicated that soil Fe-P was the main source of P absorbed by the plant. Soil Ca-P content was negatively correlated with uptake in both field and pot trials and in field trials, soil organic P content was negatively correlated with P uptake. In both field and pot trials a close negative relationship was found between soil pH and P uptake. In the field, soil clay and OM contents were negatively correlated with uptake. Inhibition of uptake by Ca-P and organic P was attributed to pH and clay content effects. Basu *et al.* (1974) reported that a higher per cent utilization of fertilizer phosphorus was observed at lower rates of application. Densitometric study of the plant autoradiographs showed maximum accumulation of fertilizer phosphorus in the cu'm, followed by the leaf sheath; accumulation was lowest in the leaf blades of paddy. Marais and Wiersma reported that moisture stress affected P uptake largely through its influence on diffusion rather than mass-flow. Somawanshi and Goswami (1980) reported that drymatter yield and P uptake were increased with

increasing levels of either soil moisture or applied P or both. Singarum and Manickam (1978) found that application of aldicarb, carbofuran, di-syston (disulfoton) or phorate, had no effect on the availability of soil P. Sinha *et al.* (1980) conducted a trial to study utilization of fertilizer P by rice cv. Jaya, Ratna and Sona using ^{32}P -labelled single superphosphate, the uptake of total P and fertilizer P increased with increasing rates of applied P from 0 to 90 kg ha⁻¹ while utilization percentage of the applied P decreased. Increase in P rates increased paddy and straw yields and decreased uptake of soil P. Mahajan and Bisen (1980) conducted an experiment, using ^{32}P -labelled superphosphate to study the uptake and utilization of soil and fertilizer P by wheat. It was found that the uptake of fertilizer P increased with increasing P rates and decreased with increasing soil fertility. The utilization percentage of applied P decreased with its increasing levels and with increasing soil fertility. The uptake of soil P increased with increasing soil fertility. Wassif *et al.* (1980) reported that total phosphorus uptake decreased with increasing salinity and was not affected either by the level or by the source of phosphorus. Stoyanova *et al.* (1978) conducted an experiment to determine the ^{32}P of phosphate uptake by the tomato cultivar Khebros in relation to soil nutrient supply. They reported that the coefficient of phosphate utilization by tomato plants was highest (19.15%) on soil, which had received 240 kg ha⁻¹ each of N, P₂O₅ and K₂O. Long term fertilization with higher rates of 1:1:1 increased the soil nutrient status but reduced the coefficient of phosphate utilization. Mego (1978) conducted an experiments with ^{32}P . He showed that most P was transported in the stem and that the leaf was the centre of active P metabolism. Suseelan *et al.* (1978) found that P absorption increased up to the primordia initiation stage and gradually declined thereafter. Ca enhanced the uptake and translocation of P by increasing its solubility.

Negi (1979) conducted an experiment to study the phosphorus uptake by maize and wheat at two stages of growth by using radio labeled phosphorus. It was found that total P uptake, P uptake from the soil and P uptake from fertilizer were positively correlated with dry matter yield and plant P content at both 1 month after sowing and at the time of heading in wheat. In maize, dry matter yield was positively correlated with total P uptake from soil at 1 month after sowing and with P uptake from soil at heading in wheat and ear emergence in maize; plant P content was positively correlated with P uptake from fertilizer at 1 month after sowing and at heading in wheat and ear emergence in maize and with total P uptake at heading in wheat and ear emergence in maize. Total P uptake, P uptake from the soil and P uptake from fertilizer were positively correlated with drymatter (DM) yield and plant P content at both 1 month after sowing and at heading in wheat and ear emergence in maize in wheat. In maize, DM yield was positively correlated with total P uptake from soil at 1 month after sowing and with P uptake from soil at heading in wheat and ear emergence in maize; plant phosphorus content was positively correlated with P uptake from fertilizer at 1 month after sowing and at heading in wheat and ear emergence in maize and with total P uptake at heading in wheat and ear emergence in maize. Joginder *et al.* (1979) reported that application of N together with P increased the P uptake. The initial build-up of nutrients in plants determined the amount of nutrients which translocated from the vegetative parts to the ears. Temple-Smith and Menary (1977) studied the phosphate depletion zones around roots of cabbage and lettuce grown in two soils by autoradiography using ^{32}P . It was concluded from the experiment that in podzolic soil the depletion zones were equivalent to the diameter of the root hair cylinder, but in krasnozem the zones were only slightly greater than the root diameter, although root diameters

and root hair lengths were similar. Fayeze and El-Nennah (1978) reported that under adequate nutrient supply, plants were limited in growth by relatively low or high levels of soil moisture (30 or 90% of the water holding capacity) and had a lower P-content in both soil and fertilizer than plants under comparable fertility at 50 or 70% W.H.C. However, P-uptake at 70% was higher than at 50% W.H.C. Varying soil moisture had a greater effect on the absorption of phosphorus from the fertilizer than from the soil. Moreover, the effect of soil moisture levels on P-uptake was more pronounced in the roots than in the tops. Bezdek and Zenisceva (1975) reported that the variability of the P uptake capacity at individual stages was markedly affected by genotype, locality and level of N fertilization. Genotypic differences in the P uptake capacity of below-ground parts were manifested especially in the period of maximum growth in length. Genotypes with a massive root system and high tillering capacity showed distinctly higher P uptake during ontogenesis by the belowground parts and also greater capacity for adaptation to a high level of N fertilization. Increased P uptake, especially in the initial growth stages 3-6 leaf of winter wheat, was directly related to soil fertility, total biomass formation and grain yield. Schilier *et al.* (1978) found that super phosphate markedly increased P uptake and P content of the plants. P uptake was inversely correlated with soil pH, organic matter, clay and carbonates. Rajoo and Ghonsikar (1978) found that phosphorus uptake is significantly reduced in paddy and maize by the application of atrazine, propanil, EPTC and 2, 4-D when applied at quarter of normal to twice the normal levels, except 2 kg/ha of 2, 4-D which increased phosphorus absorption in maize. Absorption of ^{32}P by paddy and maize has also been reduced significantly by the application of above mentioned herbicides with the exception of 1 kg EPTC ha^{-1} which slightly increased it in maize. Negi (1979) reported that the dry matter yield and

P content of wheat were positively correlated with the uptake of P from soil and from fertilizers at both growth stages; the importance of fertilizer P increased, and that of soil P decreased at the later growth stage. At both growth stages the dry matter yield of maize was positively correlated with P uptake from soil and the P content was particularly correlated with P uptake from fertilizer. Mitra and Majumdar (1981) reported that no difference in the ^{32}P uptake by healthy and little leaf (MLO) affected eggplant tissues, but absorption was much higher in infected than in healthy tobacco tissues. Chaudhary *et al.* (1984) conducted an experiment on loamy sand and sandy loam soils having low, medium and high categories of available phosphorus, results of three levels of phosphorus application indicated a significant rise in dry matter yield and P uptake of forage sorghum with increasing P levels at the three stages of harvest. Under low P status of the soil, the effect of added phosphorus was more pronounced than where available P was high, irrespective of soil texture or stages of crop growth. Smith and Jackson (1981) found that when the seedling of maize was treated with nitrogen, uptake of phosphorus was increased. It was also found that enhanced P uptake was characterized by an increase in the maximum velocity of the uptake system. Azetidine-2-carboxylic acid, an analogue of proline, did not affect the rate of P uptake but eliminated P transport through the xylem. It is suggested that treatment with N affects the mechanism of P uptake rather than subsequent translocation via the activation of pre-existing proteins. Souza *et al.* (1983) concluded from an experiment that there was a positive interaction between P and Ca which significantly increased dry matter yield and P uptake. It was concluded that the main effect of liming was through elimination of phytotoxicity due to exchangeable Al ions. Deshpande *et al.* (1983) concluded that P availability was increased by FYM particularly in

the early stages of low land rice growth. Fontes and Wilcox (1984) found that the P uptake by tomato, increased over the entire P concentration range in soil solution. The plants utilized P more efficiently with a higher dry weight accumulation per mg P absorbed at low than at high P concentration in soil solution with no differences among the cultivars of tomato. Zalawadia and Patel (1983) studied the effect of soil moisture on the growth parameter of groundnut and concluded that the 75% average soil moisture (ASM) significantly out yielded the other levels with respect to growth, kernel production, shelling percentage, oil content and P uptake by the crop; its effect over 25% ASM was more pronounced at later growth stages. Application of P up to 50 kg P_2O_5 ha⁻¹ significantly increased the above parameters with the exception of shelling percentage. Available P increased significantly in surface and sub-surface soils with level of irrigation, but only in the surface layer was it increased by rate of P application. Irrespective of treatments, the highest level of available P was noted during pod development. Available P in surface soil was significantly correlated with dry matter yield and P uptake at all growth stages, but with subsoil it showed a significant relationship with haulm and pod yields and P uptake by the haulms.

Ghosh and Ghosh (1988) reported that the phosphorus supplying capacity of a soil can be determined by combining the concentration (C) in the soil, quantity (q) parameter and capacity (b) factor. A term, called supply parameter (SP), is defined combining these three parameters. SP was determined in four soils at three levels of P application. The SP values were compared with the quantity parameter and isotopically exchangeable phosphorus (Et) values of the soils. The magnitude of SP, q and Et values did not show the same trend in all four soils. The statistically significant positive

correlation obtained between fertilizer P and SP values suggests that SP may be a suitable index for predicting fertilizer P uptake by plants. Aghimien (1987) found that nitrogen (N) fertilization significantly increased nitrogen and phosphorus (P) uptake, while P fertilization only increased P uptake. In the waterlogged soils there was a significantly greater uptake of added N but not of soil N, whereas uptake of both soil P and added P was increased. This was accompanied by an increase in seedling dry matter yield, a waterlogged regime in the nursery is therefore recommended for *Raphia vinifera*. Morel and Fardeau (1989) found in a greenhouse experiment where ^{32}P labelled fertilizers were used to measure native soil and fresh fertilizer P uptake by *Lolium perenne* grown in soils containing low and medium P levels. At the low soil P level, the native P uptake increase was independent of P-DAP applied and represented 19% of the nil P uptake. At the medium soil P level, the variability of the native soil as a nutrient P source depended on the source and rate of application of phosphate fertilizer. It was therefore suggested that the amount of total P uptake could conceal differences in P fertilizer evaluations as the nutrient P source. Fresh P uptake increased linearly with the rates of P applied as standard or tested P fertilizer. The comparison of various P sources by means of fresh P uptake ratio (i.e. fresh P uptake from tested phosphate: fresh P uptake from standard phosphate) was independent of the rate of P uptake for a single rate of application. It was suggested that various P sources be evaluated by measuring the fresh P uptake for a single rate of application. Holford (1989) reported that uptake of soil phosphate was increased by lime application. This was associated with higher levels of exchangeable and soluble phosphate but not of sodium bicarbonate-extractable phosphate. Lime had much more variable effects on the apparent uptake of fertilizer phosphate which were unrelated to

lime-induced changes in soil phosphate sorptivity. It increased the uptake of previously applied phosphate in all but two soils, and it had a more beneficial effect on the uptake of old phosphate than of fresh phosphate. Because lime usually increases the uptake of soil phosphate but often depresses the uptake and effectiveness of fertilizer phosphate, the more beneficial effect of lime on phosphate residues is consistent with the partial transformation into composition resembling native soil phosphates. Rao *et al.* (1986) reported that the P uptake at various growth stages of *Vigna radiata* was decreased with increases in soil moisture stress (SMS) and was increased with increasing P rates. The increasing P rates increased P uptake at low to medium levels of SMS but not at the highest SMS level. Jupp and Newman (1987) conducted an experiment where mature *Lolium* plants grown in pots of P deficient soil were subjected to gradually increasing drought, and uptake of P was determined during and after the drought. Relatively mild drought (soil matric potentials -0.6 to -0.8 MPa) stopped P uptake, probably because P in the drying soil became unavailable. After drought, the rates of uptake of P and of applied ^{32}P from rewetted soil were reduced, although some recovery in rate of P uptake occurred 2 and 3 weeks after rewetting. Reduction in ^{32}P uptake following drought was approximately the same whether the plants were mycorrhizal or not. Control of the reduced uptake was located in the root. Staud (1985) reported that the amount of ^{32}P taken up from the fertilizer was independent of application rate. The ^{32}P :total P uptake ratio was 30-37% in the period from the 3rd true leaf to the beginning of stem elongation. At the beginning of green ripeness, utilization of P from fertilizer was 2.7-5.7%. Haynes *et al.* (1991) found that wild oats accumulated P more rapidly, had higher root DW and produced significantly more DM for a given accumulation of P than cv. Pennlo at both low and high P

concentration. Pennlo showed higher P concn in all plant parts than wild oats, while wild oats had significantly higher nitrogen concentration in vegetative organs but not in seeds. It was concluded that due to greater allocation of drymatter to the roots and higher P use efficiency wild oats were likely to be competitively superior to cultivated oats. Patiram and Prasad (1991) reported that the adsorption maximum and energy of adsorption were inversely related to soil native P uptake. Uptake of P by maize was negatively correlated with differential P buffering capacity. Percentage adsorption saturation and adsorption saturation index were positively correlated with P uptake. Westermann (1992) reported that plant P uptake was increased by P fertilization and decreased by increasing lime concentration. Phosphorus uptake was curvilinearly related to solution P (extracted by 0.01M CaCl_2). Solution P concentration increased linearly as the resin-extractable P:equilibrium buffer capacity (EBC) ratio increased, where EBC is the slope of the P-absorption isotherm at the indigenous equilibrium P concentration. The EBC increased as the lime concentration increased. Phosphorus applications increased solution P and resin-extractable P and decreased EBC within a given lime concentration. These data indicate that the soil-test P concentration or P fertilization rate should increase as the lime concentration increases to provide the same degree of P availability and plant P uptake in this calcareous soil.

Watanabe *et al.* (1997) reported that phosphorus availability was improved for cabbage by applying phosphorus before transplanting. When potassium, ammonium or sodium phosphate-monobasic solution was applied to cabbage seedlings before transplanting, the weight of the cabbage head was the same as that when using the usual fertilizer system. Phosphorus uptake increased when 40% or 20% P was applied before transplanting,

especially during the early stage after transplanting. Consequently, 30-60% less of the phosphatic fertilizer was required, and phosphatic availability was improved 2.5 times. However, no effect was observed when controlled-release fertilizer was applied. This high phosphorus availability was due to high phosphorus absorption resulting in a high growth rate during the early stage, as well as less phosphorus fixation in the soil. However, excess phosphorus often induced salt injury such as chlorosis and leaf burn. Patel *et al.* (1996) reported that number of pods per soybean plant, test weight, grain and straw yield, nitrogen and phosphorus uptake were significantly increased with the increasing nitrogen and phosphorus levels. Nitrogen and phosphorus uptake was significantly increased with increasing nitrogen and phosphorus levels. Liu and Liu (1996) found that the amount of P absorbed by hybrid rice during the middle growth stage was over half of the total P absorbed during the whole growth period. However, less P was supplied by soil at the middle growth stage. P deficiency decreased plant height and delayed growth. Phosphorus application resulted in normal growth, early ripening and increased yield. With increasing P application, the contents of P in leaves, seeds and shoots increased. The contents of protein and nitrogen in rice leaves decreased when insufficient P was applied. P enhanced the photosynthetic efficiency of leaves, increased the grain yield and shortened the growth period of rice. Decreased P content in rice leaves was accompanied by an increase of starch accumulation and a decrease in sucrose. It is suggested that P application could enhance the absorption and use of nitrogen and potassium by hybrid rice. Choluj (1996) reported that high temperature pre-treatment (40°C for 24 h) strongly stimulated ^{45}Ca uptake and drastically inhibited its export to the shoot, independently of K^+ supply. The net uptake of ^{32}P increased only after long-term heat stress in plants

with lower K^+ content. This seemed to be the result of preventing P efflux from roots in plants with higher Ca^{2+}/K^+ ratio, which was observed after the 2 h heat treatment. Nirale (1995) found that Moisture stress inhibited growth in both sorghum cv. M35-1 (drought tolerant) and CSH-1 (drought susceptible), but less so in M35-1. ^{32}P uptake, absorption and translocation were inhibited by moisture stress, with most effect on translocation. The inhibition of ^{32}P uptake was less in M35-1 than in CSH-1 in intact seedlings as well as in excised root tissue. It is suggested that this radiotracer technique could be used for assessing drought tolerance. Natale *et al.* (1999) reported that about 12% of the applied P was taken up by guava plants after 20 days. Zhang *et al.* (1998) reported that when $CaCO_3$ was present, the rate of phosphorus uptake by the seedlings decreased significantly, but as the solution pH increased, the difference between P uptake in the two treatments (with $CaCO_3$ and without $CaCO_3$) was reduced. Under pH 5.5, 6.5, 7.5 and 8.5, the effects of pH on the availability of phosphate in calcareous aquent soil was greater than that in non-calcareous aquent soil. Song *et al.* (1998) reported that phosphate starvation also decreased the dry weight accumulation of the tomato seedlings but had little effect on the rate of the accumulation. After 7 days of phosphate starvation, tomato seedlings distinctly increased their P uptake rates at much higher levels than the controls. The kinetic analysis of phosphorus uptake by the seedlings showed that phosphate starvation enhanced the affinity of the permease to phosphorus, and that resupply of phosphorus to the seedlings distinctly decreased this high affinity of the permease but the decrease process became slower as phosphate starvation was enhanced. Covacevich *et al.* (1998) reported that Phosphorus uptake and root length of wheat were increased by phosphorus and nitrogen supply. Root:shoot ratio decreased with phosphorus addition because of

increased shoot growth relative to root. Gutierrez-Boem and Thomas (1998) concluded from an experiment that the ability of plants to cope with mild water stress was enhanced by adequate P nutrition. Abichequer and Bohnen reported that there was no significant difference between wheat cv. Toropi and IAC 5 (P efficient) and IAS 20 and CNT 8 (P inefficient) in P uptake, but P translocation to the shoots and P use efficiency were greater in the P-efficient cultivars. Jha *et al.* (2000) studied the P uptake and utilization pattern of onion and suggested that it demands the constant supply of P at all stages of growth.

Szlovak and Almasi (2001) reported that the total phosphorus uptake by maize increased in all aboveground plant parts up to 80 days after emergence and then, with the exception of the grain, decreased until the final harvest. At the end of the growing season, the grain stored most of the absorbed phosphorus (84.85% on the average). Zhang *et al.* (2000) reported that flooding strongly increased phosphorus (P) adsorption and decreased P desorption in the paddy soil while simulated O₂ secretion from rice root significantly reduced P adsorption and enhanced both P desorption in rhizosphere and P uptake by ion-exchanger. It is suggested that the effect of oxygen (O₂) secreting from rice root should be one of the important mechanisms for rice take up of P, normally under the conditions of P availability reduction by flooding. Phosphorus availability in the soil reduced by flooding should be related to the transformation of P fractions because the Al-P fraction increased by P amendment in the soil was almost transformed into the Fe-P fraction by a 2-week flooding. Thiyageshwari *et al.* (2000) found that growth of black gram and P uptake was slow in the vegetative phase but rapid during the reproductive phase. Vermicompost application significantly enhanced grain yield followed by phosphobacteria above 100% P as Tunisia rock phosphate. Phosphorus uptake by black

gram was higher in the combined application of rock phosphate with vermicompost and phosphobacteria. Available phosphorus was higher in the vegetative stage and later decreased at harvest due to P utilization by black gram. Bates and Lynch (2000) concluded from an experiment that the response of root hairs to low-phosphorus availability is an efficient strategy for phosphorus acquisition. Gahoonia *et al.* (2000) conducted an experiment to study the phosphorus uptake and organic acids released from root of two barley cultivars Marinka and Sonate. From the experiment it has been concluded that the total P uptake per plant did not differ between the cultivars. Marinka retained more P in roots. In sterile nutrient solution culture, the cultivars differed mainly in release of organic acids from the roots, with Marinka releasing 3 times more citric acid and nearly 2 times more acetic acid than Sonate. The cultivars had similar root hair lengths and they did not differ in depletion of available soil P fraction in the rhizosphere. Marinka absorbed nearly twice as much P from the strongly adsorbed soil P fraction as Sonate. Under field conditions, Marinka absorbed more P and produced more shoot dry matter. The higher P uptake by Marinka than Sonate can be attributed to its ability to acquire P from strongly adsorbed soil P by releasing more organic acids, especially citric acid, from its roots. Lopez *et al.* (2000) reported that the ability to use insoluble P compounds can be significantly enhanced by engineering plants to produce more organic acids. The results show that when compared to the controls, citrate-overproducing plants yield more leaf and fruit biomass when grown under P-limiting conditions and require less P fertilizer to achieve optimal growth. Poustini and Aboutalebian (2001) suggested that the uptake and distribution of phosphorus could be considered as a parameter closely related to salinity resistance in wheat. Kovacova *et al.* (2002) reported that

traditional fertilizer regimes (a combination of organic and artificial fertilizers) maximized phosphorus uptake of an irrigated sugar beet crop, and achieved the best yields. Subbaswamy *et al.* (2001) reported that application of ammonium sulfate increased mulberry leaf yield. P content (0.35%) in mulberry leaves was also higher when N was applied in the form of ammonium sulfate as compared to the other treatments. In addition, arginine content was low (10.8 mg g^{-1}) in the ammonium sulfate-treated plots where P uptake was higher compared to calcium ammonium nitrate- and urea-treated plots.

2.6 Soil microbial population

Gupta and Bajpai (1974) studied the diversity of microbial population in salt affected soil and found that maximum numbers of bacteria, fungi, Azotobacter and nitrifiers were observed at neutral pH and normal salinity. Proportion of actinomycetes to bacteria was higher in moderately to highly salt affected than normal soils. Nitrite formers were higher and were possibly less salt sensitive than nitrate formers. A considerable decrease in the population of bacteria, fungi and Azotobacter was noticed with increase in EC and pH. Cheshire *et al.* (1974) reported that the soil microbial population showed a marked increase in response to added polysaccharide material but only slight qualitative changes were detected. It is concluded that the persistence of naturally occurring polysaccharide in soil is related to inaccessibility caused by chemical combination, complexing or insolubility but not to a biologically stable molecular structure. Makawi *et al.* (1972) evaluated the effect of dalapon-sodium (85%) on the microbial population in soil. From the result it was found that counts of bacteria and of spore-forming bacteria decreased with the increased rate of the herbicide application but inhibition decreased with time;

actinomycetes and fungi counts suffered no marked changes. Gowda and Patil (1972) studied the effect of simazine and nitrofen on the biological activity in sandy soil and it was found that simazine depressed the actinomycetes population during the first 2 weeks and stimulated it during the following 2 weeks. Fungal populations were increased by nitrofen. Simazine decreased soil respiration initially and thereafter it had no effect. Mathur *et al.* (1980) reported that the changes in soil microbial activities and populations caused by disulfoton and prometryne were temporary and did not influence crop growth in organic soil except where permethrin was applied to carrots. Permethrin suppressed the soil microbial population and biochemical activity rather consistently though not markedly because of its persistence in this organic soil. Misra and Gaur (1977) reported that gamma HCH at 1 and 10 ppm, was toxic to bacteria and, to a lesser extent to actinomycetes but stimulated the growth of *Azotobacter*. At 100 ppm gamma HCH was toxic to all the organisms tested. Application of farmyard manure to the soil reduced the toxicity of this insecticide when applied at low concentrations to bacteria but increased the toxicity to actinomycetes. At a high temperature (45°C), toxicity to actinomycetes and *Azotobacter* was greatly increased. The toxicity to fungi and *Azotobacter* was higher with acidic or alkaline conditions than under neutral conditions, whereas it was lower to bacteria and actinomycetes. High moisture content (60%) in soil increased the toxicity of the highest concentration to fungi. Azad and Saleem (1976) conducted an experiment to find out the effect of metasystox and folidol on microbial process in soil and reported that folidol stimulated *Azotobacter* populations, the effect being highly significant at higher rates. Metasystox showed a stimulating effect on *Azotobacter* initially, but after the 14th day an adverse effect was observed, which increased with increasing concentration

and with time. The lower rates (0.000025%) of both chemicals and the medium rate (0.005%) of Folidol, slightly stimulated the development of the fungal flora. Metasystox at all rates and Folidol at the higher rate (0.05%) reduced total bacterial counts in soil for 28 days. Lower rates of Folidol stimulated the bacterial population. Shivashankar *et al.* (1981) conducted an experiment to study the effects of fertilizer and herbicidal interactions on soil microbial population and nutrient uptake by soybean and weeds under recommended and 75% of the recommended fertilizer rates in combination with 4 weed control treatments. The bacterial, fungal and actinomycetes populations were affected considerably up to 45 days after spraying alachlor and metribuzin; they showed no adverse effect in the subsequent period. The microbial population with recommended and 75% of the recommended rate of fertilizer was similar up to the 30th day; on the 45th day, the lower rate gave higher populations of bacteria and actinomycetes. Fungal population was inhibited up to 45 days with both fertilizer levels and recovered at harvest. Rai and Tripathi (1984) experimented in Meghalaya to find out the allelopathic effects of *Eupatorium riparium* and reported that the soil microbial population and growth of the *Galinsoga* spp. declined in soil which had earlier received leachate of different plant parts of *E. riparium*. Partly decomposed litter of *E. riparium* reduced the soil microbial population and the growth of both *Galinsoga* species much more strongly than litter in advanced stages of decomposition. Patel *et al.* (1985) found that the microbial population of the soil estimated at 1-month intervals decreased initially but later developed rapidly during maize growth period after applying FYM and atrazine and fertilizer application. Chopra and Magu (1985) reported that the use of city compost was made to mitigate the inhibitory effect, if any, caused by agrochemicals. Soil microbial population was not

adversely affected by any herbicide, except on applying isoproturon at high rates (1.40 ppm) at early growth stages of maize. Famoso and Bautista (1983) reported that mulching also reduced the maximum daily soil temperatures and (investigated only for bagasse) increased the soil microbial population. Lang and Beese (1985) reported that liming caused pronounced increases in microbial activity in the humic layers but had little effect in the mineral soil, even in the top 5 cm. The oxygen consumption after addition of glucose indicated that the microbial biomass had increased. Diaz-Ravina *et al.* (1989) reported that composted urban refuses had a high microbial population. Bacteria, a small proportion of which were spore-forming, predominated, but actinomycetes and fungi were also abundant; algae were absent, except in CaCO_3 -amended compost. Most of the population had proteolytic, ammonificant, amylolytic and aerobic cellulolytic capacities, followed by denitrifiers, sulphate reducers and elementary sulphur oxidizers. The urban refuse composts did not substantially affect the soil microbial population and had a short-term, slightly-positive, effect over nitrite oxidizers. Composting with CaCO_3 notably improved the nitrite oxidizer population of the soil. Acea and Carballas (1988) found that cattle-slurry treatment brought about a great rise in total microbial population but affected different groups differently, though without changing the order of their population sizes. Bacteria were favoured, while the numbers of actinomycetes, fungi and algae were reduced, especially the last. Kumari *et al.* (1987) reported that populations of bacteria and actinomycetes were reduced when SBP (stable bleaching powder) was applied at depths of 3 and 6 inch. Applications to the soil surface caused no significant inhibition. Total populations of soil fungi were not changed significantly but individual fungi were affected. Tripathi and Upadhyay (1988) found that the soil microbial population was

increased by applying N, P, K, Zn and especially N + P + K + Zn, and was decreased by applying Machete [butachlor], Cuman-L [ziram], malathion and BHC. Walker and Suett (1986) observed that repeated use of the same pesticide at the same site could occasionally induce changes in the soil microbial population such that their rate of degradation was increased. Cattelan and Vidor (1990) evaluated the effects of seven cropping systems on the soil microbial population. The systems that produced a large quantity of crop residues showed the highest counts of bacteria. Populations of fungi and actinomycetes were not significantly affected by different cropping systems. The counts of bacteria and fungi were twice as small in the 5-15 cm than the surface soil layer, but this effect was less pronounced for actinomycetes.

Phosphate-solubilizing microorganisms were not significantly affected by cropping systems, soil depth or by levels of available phosphorus. Triger *et al.* (1990) suggested from an experiment that the regulation of soil microbial population depends largely on inter, rather than intra-population processes. Alterations of the environmental conditions changed the type of interaction and the type of interaction between a given pair of populations altered during the course of the succession. Chakravarty and Chatarpaul (1990) reported that glyphosate at 0.54 and 3.23 kg ha⁻¹ and hexazinone at 1-8 kg did not reduce soil microbial population or carbon dioxide evolution in the long term (6 months). However, there was a significant short-term (2 months) effect of glyphosate on both fungal and bacterial counts at 0.54 kg. Odeyemi *et al.* (1988) studied the effect of 11 pesticides on the soil microorganisms (bacteria, actinomycetes, fungi and protozoa). The fungicides Agrosan (phenyl mercury acetate) (50 µg g⁻¹ soil) and PCNB (quintozene) (240 mg g⁻¹ soil⁻¹) reduced bacterial numbers. Vetox-85 (1-naphthylmethylcarbamate) (100 µg g⁻¹ soil) and

Gammalin 20 (lindane) ($1300 \mu\text{g g}^{-1}$ soil) reduced bacterial numbers 100 times. PCNB reduced the actinomycete density from 360 to 340 g^{-1} soil and eliminated all fungal and protozoan propagules. Gammalin 20 removed all fungi whereas Agrosan removed all protozoa and reduced the fungal population. In general, protozoa and fungi appeared to be more susceptible to fungicides than bacteria and actinomycetes. whereas the herbicides Dacthal (chorthal-dimethyl), Preforan (fluorodifen) and Dual (metolachlor) did not cause any detrimental effects to soil microorganisms at concentration of 0.1, 0.08 and $0.04 \mu\text{g g}^{-1}$ soil, respectively. Benlate (benomyl) and Brestan (fentin acetate + maneb) appeared to stimulate rather than inhibit the soil fungal population. Gramoxone (paraquat) decreased the number of fungi 10 times. Acea and Carballas (1990) found that the factor influencing the density and functional diversity of the microbial population most is depth within the profile, both density and diversity decreasing with increasing depth. In the surface horizons the distribution of microflora is controlled by the time of year and the associated changes in climatic conditions, with lack of humidity limiting the microbial population more than temperature. Stearman and Matocha (1992) studied the impact of tillage on microbial populations by studying soil properties after 11 years of a tillage management experiment with continuous sorghum on sandy clay loam. Soil microbial populations differed with soil depth among all tillage treatments before the growing season, but were similar among tillage treatments when counts by soil depth were composited, indicating that, although microbial distribution differed, total populations were similar. The actinomycetes were lower in no tillage treatments, while Heterotrophic bacteria counts were not significantly different between treatments. Soil nitrifier populations were usually greater in minimal tillage and no tillage treatments. Soil nitrate was significantly

correlated with nitrogen-transforming bacteria in several instances, although there was no predictable effect from soil depth or sampling date. Heterotrophic bacteria and actinomycete population levels were not significantly correlated with soil physical or chemical properties. Hiroki (1992) reported that the number of actinomycetes and bacteria decreased significantly with increasing heavy metal content, there was no significant correlation between the number of fungi and heavy metal content. Actinomycetes were affected by heavy metals most strongly. These results suggest that the relatively low concentration of heavy metals in the research area affected the soil microbial population. Jha *et al.* (1992) found that dehydrogenase activity showed a positive correlation with fungal population numbers in all of the forest stands. However, the bacterial population number only showed a significant correlation ($r = 0.639$) with dehydrogenase enzyme in the less degraded forest at the lower altitude. A significant positive correlation was established between urease activity and fungal and bacterial population numbers at the lower altitude, but only with the fungal population numbers at the higher altitude. At the higher altitude no significant correlation could be established with phosphatase and any of these traits. At the lower altitude, however, phosphatase showed a positive correlation with organic C and fungal population numbers. These enzymes showed a marked seasonality. The results of the investigations indicated that disturbance of soil and vegetation has an adverse effect on microbial population numbers and microbial enzyme activities. Rai (1992) studied the effect of fifteen years application of 2,4-D on soil microbial population and found that fungal, bacterial and actinomycetes populations were reduced by 2,4-D treatment, especially by its ester. Soil microbial respiration were temporarily increased by the amine, but were reduced substantially by the ester, indicating that

the ester probably interfered with nutrient cycling. Yu *et al.* (1993) reported that the reproductive capacity and concentration of soil nitrogen-fixing bacteria was significantly inhibited by butachlor-fertilizer (BF) applications of 112.5 kg ha^{-1} . Effects on the soil microbial population were greatest when BF was applied 7-14 days after transplantation. Bernat (1993) concluded from a study that the composition of soil micromycetes was shown to be a good indicator of changes in soil formation process. Hiroki (1993) found that fungi appeared to be much more tolerant to As (III) than bacteria and actinomycetes in paddy field. The ratio of the As (III)-tolerant fungi to the whole fungal population was greater in the polluted paddy plot than in the other plots. It was concluded that the continuous pollution of soil with arsenic promoted the dominance of As-tolerant fungi, and that the effects were more marked in paddy fields than upland fields. Joshi *et al.* (1991) reported that undisturbed grassland soil had the most bacteria and actinomyces, barren soil had the fewest microbes and symbionts, while the greatest number of microbes was observed in forest and barren soils. Organic matter, moisture content, CO_2 evolution, and enzyme activities were positively correlated with the microbial population. Vento *et al.* (1991) reported that soil microbial population increased by taking up molecules from the biodegrading herbicides, so that herbicides did not persist long in the soil. This should be considered when programming the timing of herbicide application, in order to avoid weed incidence earlier than anticipated. The fungi population increased more than others and mesoranyl caused the greatest increase in microorganisms. Ragab and AlMasoom. (1993) reported that counts of total bacteria were negatively correlated with total soluble salts and positively correlated with organic carbon in surface layers. No correlations were obtained between total soluble salts or organic carbon and nitrogen fixing

bacteria. Nitrifiers were inversely correlated to salt in surface layers and directly correlated with the organic carbon in both soil layers. Similar results were obtained for the effects of total soluble salts and organic carbon on counts of cellulose-decomposers. Halophilic bacteria were positively correlated with total soluble salts in both soil layers and positively correlated with organic carbon only in soil surface layers. Kloepper and Bowen (1991) reported that G/S (no. geocarposphere microorganisms/no. soil microorganisms) and R/S (no. rhizosphere microorganisms/no. soil microorganisms) ratios were calculated for total fungi, *Aspergillus flavus*, spore-forming bacilli, coryneform bacteria, fluorescent pseudomonads and total bacteria isolated on low- and high-nutrient media. G/S and R/S ratios were generally >1.0 for all groups of microorganisms except bacilli. G/S ratios were greater for total bacteria than for total fungi at 2 of the 3 sample times, suggesting that bacteria were stimulated more than fungi in the zone around developing pods. In contrast, R/S ratios were higher for total fungi than for total bacteria at 2 sample times. The preferential association of fungi and bacteria with early developmental stages of the pod suggested that some microorganisms are particularly well adapted for colonization of the groundnut geocarposphere. It is concluded that these microorganisms are logical candidates for evaluation as biological control candidates for *A. flavus*. Chauhan *et al.* (1994) studied the effect of pendimethalin and butachlor pesticides on soil microbial population. Small concentration of these pesticides did not alter the activity of the organisms, but at higher concentration, most bacterial and fungal species were affected. Pakale and Alagawadi (1993) found that the highest population of ammonium oxidizers and nitrite oxidizers and the highest nitrification potential were observed in the red soil. In all soils there was a positive correlation between nitrification potential and

ammonium oxidizers and nitrite oxidizers. There was a negative correlation between total bacteria and nitrification potential. John and Abraham (1996) studied the seasonal effect on the population of soil microflora, bacteria, fungi and actinomycetes. The study showed that there was considerable variation in the population of soil microflora between treatments. A significantly higher population was observed as a result of the interaction effect of seasons and treatments and hence the extent of availability of nutrients. Donkova and Nikolova (1996) found that soil compaction led to an increase in the total biological activity and biomass; however, there were no differences due to the type of previous crop grown. Soil scarification to a depth of 10-12 cm had a positive effect on soil microorganisms. Gaikwad *et al.* (1996) reported that rhizosphere microbial population was enhanced by application of pressmud cake. Miltner *et al.* (1992) reported that soil microbial population is higher due to sufficient water supply throughout the year in two calcareous soils under mediterranean and temperate climate. Chen *et al.* (1998) showed that after long-term coverage with plastic sheet, the numbers of bacteria, actinomyces and fungi in the top layer of soil (0-15cm) increased 22.6%, 29.3%, and 19.7%, respectively. At seedling stage, bacteria increased by 21.4%; at the elongating and ripening stages, actinomyces increased by 22.4% and 24.4%, respectively; and within the whole growing period, fungi increased by 12.2-14.6%. In addition, the dominant facultative anaerobial populations of bacteria tended to be increased, but those of fungi were not changed. Sundaravarathan and Kannaiyan (1998) reported that green manuring with prilled urea significantly increased the population of bacteria, actinomycetes and fungi in soil than green manuring without prilled urea. However, neem cake application along with green manure incorporation significantly reduced the soil microbial

population on day 10, while it increased significantly thereafter. Xia *et al.* (1998) found that the overlying vegetation on soil can change surface soil environment, and affect some ecological factors for soil microbial living. The soil environment with plant activity is more suitable for soil microbial population. It's better for microorganisms to reproduce and grow in the surface soil environment with overlying plants. Diversity is higher in surface soil with overlying plants than that in the soil environment without overlying plants, if the original subsurface soil was not affected by vegetation. Vegetation can affect the activity of DNA from soil microbial community. Patil and Patil (2001) concluded from an experiment that fly ash with or without organic matter (FYM) promote good microbial growth. Shetty and Magu (2000) reported that low concentration of metalaxyl stimulated the bacterial and actinomycete population, but its higher levels significantly inhibited the population. However, different doses of metalaxyl inhibited fungal and *Azotobacter* population. Interestingly, 0.5 ppm and 1 ppm of metalaxyl did not affect *Nitrosomonas* and *Nitrobacter* population; however, at higher concentration it significantly inhibited the nitrification process. Arunachalam *et al.* (1997) found that soil microbial population was less during periods when temperature and moisture conditions are low, while it peaked during rainy season when the litter decomposition rate is at its peak on the forest floor. Kennedy *et al.* (1999) reported that the carbofuran application at a field dose of 0.5 kg a.i. ha⁻¹ showed no effect on the fungal and bacterial population whereas higher levels are found to reduce the fungal and bacterial population up to 30 days after application. The higher levels, 1.0, 1.5 kg a.i. ha⁻¹ had no significant effect on the actinomycete population. Patel and Patel (1998) reported that bacterial and fungal populations in the soil were significantly reduced when compared to the untreated control due to

fluchloralin application at both the recommended (1.5 kg ha^{-1}) and double the recommended (3.0 kg ha^{-1}) concentration. Addition of farmyard manure @ 5.0 t ha^{-1} significantly increased the bacterial and fungal populations. The interaction effect of fluchloralin concentration and farmyard manure on the soil microbial population 3 h after application was significant. Naidu *et al.* (2000) evaluated the effect of manures, bio and chemical fertilizers on the soil microbial population and growth and yield of okra. Application of manures and vermicompost with biofertilizers harboured significantly more microbes in soil than the control. Bacterial, fungal and actinomycetes count were all maximum under $90 \text{ kg K}_2\text{O} + 7.5 \text{ tonnes poultry manure} + 1.0 \text{ tonne vermicompost per ha}$ application.

2.7 Dehydrogenase activity:

Moore and Russell (1972) reported that there is a lack of useful correlations between dehydrogenase activity and either soil properties known to influence plant growth or plant yields. Dehydrogenase activity does not respond to addition of nutrients to deficient soils. Muller *et al.* (1972) found that dehydrogenase activity was affected by all factors except fertility level, and crumb structure was affected most by texture grade and moisture level. Malkomes (1977) conducted an experiment to find out the effect of herbicide Venzar (lenacil) on the soil microflora in sugar beet growing in sand, peat and clay soil and found that dehydrogenase activity slightly reduced only in peat soil. Boguslawski *et al.* (1976) reported that the estimation of dehydrogenase activity in gamma -triphenylformazan was a good measure of soil biological activity, in comparison with CO_2 measurement, and there was extensive agreement between dehydrogenase activity and soil productivity (cereal yields). Malkomes *et al.* (1977) conducted an experiment to find out the long term application of Temik 10

G (aldicarb) on soil micro-organisms in sugar beet monoculture for twelve years. It was found that after the last application, soil dehydrogenase activity decreased for a few weeks and then recovered. Later in the season the level declined again, possibly due to indirect effects and the application of other pesticides. Miskovic *et al.* (1977) reported that soil dehydrogenase activity in the rhizosphere was higher than in the soil. It was increased by applied N, especially with ploughed-in straw and was negatively correlated with grain yield. Malkomes (1979) found that in dichlorprop-treated soil, dehydrogenase activity was increased by about 10% during the whole season of winter wheat but Methabenzthiazuron reduced dehydrogenase activity during the dry month of June. Soil respiration was reduced 1 and 3 months after methabenzthiazuron application. Malkomes (1979) reported that in the lower organic matter containing soil, dehydrogenase activity in the 0- to 5-cm layer was depressed by chlortoluron, methabenzthiazuron and pendimethalin, whereas only pendimethalin affected dehydrogenase in the higher organic matter (OM) soil. Pavlyuchuk *et al.* (1982) tried to find out relationship between dehydrogenase activity and soil moisture tension. In the chernozem and the derno-podzolic soil, highest dehydrogenase activity was recorded at a tension of -3.8 atm; in the zheltzem, highest activity occurred at - 2.8 atm. No enzyme activity was detected at tensions less than - 70 atm in the chernozem and derno-podzolic soil, and less than - 165 atm in the zheltzem. Balasubramanya and Patil (1981) reported that soil dehydrogenase activity have a good correlation with total microbial numbers. Pedrazzini and McKee (1984) reported that immediate decrease in soil redox potential and rapid increase in soil dehydrogenase activity upon flooding indicated a shift of the microflora from aerobic to predominantly anaerobic microorganisms. Soil dehydrogenase activity remained elevated as long as the soil continued to be

flooded, but was negligible in soils allowed free drainage. Rogers and Li (1985) reported that the measurement of soil dehydrogenase activity is a good tool to determine the effect of a number of potential toxic inorganic ions on soil microbial activity. Trevors (1984) reported that there were high positive correlations between dehydrogenase activity and substrate concentration, incubation temperature and soil pH. Dehydrogenase activity also displayed a high negative correlation with O_2 concentration. Ammonium sulfate at concentrations ranging from 40 to 120 $\mu\text{g g}^{-1}$ soil had no significant effect on dehydrogenase activity. However, at concentrations of 160 and 200 $\mu\text{g g}^{-1}$, dehydrogenase activity was significantly reduced. Potassium nitrate at concentrations ranging from 40 to 200 $\mu\text{g g}^{-1}$ had no significant effect on soil dehydrogenase activity, whereas sodium nitrate significantly inhibited activity at concentrations of 120 and 160 $\mu\text{g g}^{-1}$ soil. Gopalaswamy and Vidhyasekaran (1987) found that with the application of fresh leaves of *Glyricidia maculata*, *Azadirachta indica*, *Pongamia glabra* as green manure the soil dehydrogenase activity became 100.0, 82.5, 62.5, 22.5 unit and in control 17.5 units relative to an optical density scale, respiration. It was concluded that green leaf manures increased the soil fertility status. Lumsden *et al.* (1987) reported that biological factors associated with disease suppression in the traditional system were an increased amount of biological activity (measured by soil dehydrogenase activity), reduced rate of germination of *P. aphanidermatum* oospores, and elevated populations of *Pseudomonas* sp. and saprophytic *Fusarium* spp. Dynamic biological processes in the traditional agroecosystem may be responsible for the suppression of the pathogen, *P. aphanidermatum*, through increased biological activity brought about by the high content of organic matter and elevated mineral nutrient status of the soils. Bolton

et al. (1991) found that stimulation of soil dehydrogenase activity by the addition of lucerne was greater in the microcosms than in the field in an experiment where soil-core microcosms were compared with the field in terms of ecological response to the introduction of a large inoculum of a rifampicin-resistant rhizobacterium, *Pseudomonas* sp. Sethi *et al.* (1990) reported that soil characteristics, the associated vegetation and soil biological activities were found to be closely related. Soil dehydrogenase activity (DHA) and total nitrifier population (TNP) were greatest in the soil planted with wheat and lowest in barren virgin soil with a high salt content. Soil biological activities were significantly and positively correlated with soil organic carbon but negatively correlated with soil pH and EC. Geethakumari and Shivashankar (1991) reported that intercropping and organic amendment increased dehydrogenase activity; CO₂ enrichment had no effect. Pavlyukova and Dolgova (1993) found that soil enzyme activity could be used as an index for diagnostic indicators of the degree and spatial extent of nitrate pollution. Additions of 0.5, 1.5 and 2.5 g cm⁻³ nitrogen peroxide reduced soil dehydrogenase activity by 82, 71, and 59%, catalase activity by 85, 74, and 53% and invertase activity by 81, 55 and 28%, respectively. Klubek *et al.* (1992) observed that dehydrogenase activity and soil respiration increased when ash or soil samples were treated with glucose or glucose plus ammonium nitrate, indicating that the availability of simple organic compounds and N are limiting factors for microbial activity. Cumulative CO₂ evolution indicated that a priming effect occurred by day 15 of incubation after glucose or glucose plus ammonium nitrate had been applied. The results suggest that the abundance, diversity and activity of microorganisms increase as the ash basin ages. The land-disposal of fly ash will require the addition of low C:N ratio residues to stimulate microbial community development.

Bolton *et al.* (1993) found that soil microbial biomass C and N, soil respiration and soil dehydrogenase activity were higher in the top 5 cm of the soil than in the 5-15 cm layer, regardless of plant type. Malik *et al.* (1995) found that soil dehydrogenase activity in unamended soils fluctuated from 0.089-0.047 mg g⁻¹ per day. These values were 3-4 times higher in soils amended with Brassica residues and Salsola-Heliotropium biomass. Dehydrogenase activity was maximum in Salsola-Heliotropium amended soils. Dehydrogenase activity and carbon dioxide evolution were negatively correlated with soil salinity. Kumari and Devi (1994) used dehydrogenase activity to monitor the degradation of subabul (*Leucaena leucocephala*), *Acacia auriculiformis*, silver oak (*Grevillea robusta*), and Eucalyptus sp forest litters in a red sandy loam soil in India. Dehydrogenase activity was measured after 120 d incubation and expressed as mg triphenyl formazan (TPF) formed/g soil per h. The TPF formed in soils with forest leaf litter decreased in the order: subabul < silver oak < acacia < eucalyptus irrespective of organic matter level. Kumar and Kapoor (1995) reported that soil dehydrogenase activity declined with increasing soil pH. Felsot and Dzantor (1995) reported that microbial bioactivity in terms of dehydrogenase activity was stimulated by addition of organic amendments that may enhance co-metabolism of high concentrations of pesticides (alachlor) in soil. Cooper and Warman (1997) reported that compost produced higher dehydrogenase activity than manure or fertilizer treatments to the silty clay soil. Sabra *et al.* (1997) conducted an experiment to find the effects of oxadiazon, butachlor and thiobencarb on clay soil enzyme activity. They found that soil dehydrogenase activity was enhanced by low rates of oxadiazon (10 µg g⁻¹ soil) and decreased by 55% at rates of 50 µg g⁻¹ soil. Sinclair *et al.* (1997) measured soil dehydrogenase activity on both ryes meal-supplemented

and unsupplemented soil samples. Irrespective of supplementation, significant reductions in dehydrogenase activity were associated with increased soil concentrations of leached preservative (chromated fluoride) constituents, which were found within 5 cm of the treated pole sections. These reductions were enhanced by the lower organic matter content of the sand-amended soil. Supplementation with rye meal resulted in raised levels of dehydrogenase activity in all soil samples, which highlighted the negative effects of preservative soil contamination on microbial activity. Abul *et al.* (1996) reported that soil dehydrogenase activity was moderately inhibited by application of calixin. Inhibition increased both with concentration and incubation period. Liu *et al.* (1996) found that soil dehydrogenase activity significantly increases up to 18.8 times with 20% and 40% compost. The soil was inhibitory to microbial activity in all rates of compost addition, with no stimulation in microbial activity at less than 20% compost. Zaghloul *et al.* (1996) reported that sewage sludge gave the highest soil dehydrogenase activity as compared to biogas slurry, composted municipal waste, *Azospirillum brasilense* and ammonium sulphate treatment. Marzadori *et al.* (1996) reported that soil dehydrogenase activity was influenced by both lead (Pb) additions and variations in soil moisture content. Only the addition of 5000 $\mu\text{g Pb g}^{-1}$ soil led to a significant decrease in dehydrogenase activity as compared to the controls, while the other doses of Pb did not always result in a clear reduction in enzyme activity. Drying the soil lead to a considerable reduction in dehydrogenase activity, sometimes so far as to render the differences between the various treatments not statistically significant. Mueller *et al.* (1998) found that acephate, benomyl, cartap, chlorothalonil, deltamethrin, mancozeb, metalaxyl, oxadixyl, metribuzin, dimethoate, methamidophos, cyromazine, abamectin and oxamyl

have very low inhibitory effects on soil respiration but significant effects on dehydrogenase activity and nitrification capacity which increased with time. Park *et al.* (1998) reported that soil dehydrogenase activity showed an increasing trend with liming while fertilizing had a negative effect. It is concluded that fertilizing may not always be advantageous to the soil ecosystem. Herrero *et al.* (1998) reported that a high negative correlation between NH_4^+ -N content in soil and dehydrogenase activity. Alkaline phosphatase and dehydrogenase activities were generally enhanced after organic treatments, but application rate made no difference. Park and Park (1998) reported that the soil dehydrogenase activity was more sensitive to soil chemical properties than the denitrifying bacteria or sulfur-reducing bacteria, since the enzyme activity was significantly different between regions and seasons while the two bacterial biomasses were not. In addition, the dehydrogenase activity showed relatively high correlation coefficients with organic matter, total nitrogen and C/available P ratio, which were thought to be closely related to microbial activity. Thus, the dehydrogenase activity was thought to be a useful index of soil ecosystem dynamics (as long as it is applied to soils with the same texture). Steinberge *et al.* (1998) found that drying of the soil during the long summer period resulted in a reduction in dehydrogenase activity, while increased soil moisture resulted in a marked increase in dehydrogenase activity. Brzezinska *et al.* (1998) reported that the combined effect of flooding and temperature increase to 30°C increased dehydrogenase activity by on average 129-times as compared with the 15.9 kPa at 10°C treatment. Flooding and temperature increases caused a decrease in soil redox potential and O_2 diffusion rates. Soil dehydrogenase activity was highly correlated with redox potential and it is suggested that soil water content and temperature influence the dehydrogenase activity

indirectly by affecting the soil oxidation-reduction status. Jarak *et al.* (1997) reported that the addition of heavy metals reduced soil dehydrogenase activity. Koper and Piotrowska (2001) reported that the soil dehydrogenase activity was higher in soil samples collected from loamy soil than from sandy soil both with cattle slurry. It is suggested that high amount of slurries applied (460 kg ha^{-1}) usually decreased the enzyme activity. Brzezinska *et al.* (2001) reported that irrigation with municipal wastewater elevated soil dehydrogenase activity on average by 44% and 27% for the low and high irrigation doses, respectively, but reduced catalase activity at the high irrigation dose on average by 12.4% (significant at $P < 0.001$). Plant cover significantly influenced soil dehydrogenase and catalase activities. Chen *et al.* (2001) observed that soil dehydrogenase activity was stimulated by benomyl (18-21%) and chlorothalonil (8-15%) except in the alfalfa amended soil, but was decreased by captan (40-58%) in both the straw-amended and unamended soils. Chen and Edwards (2001) found that when benomyl was applied in its recommended dose and ten times of this dose, both the concentration of benomyl increased peak soil microbial activity and biomass rates and soil dehydrogenase activity (DHA). Captan and chlorothalonil decreased both peak substrate induce respiration (SIR) rates and DHA activity. Brzezinska *et al.* (2001) found a significant correlation between dehydrogenase activity and aeration indexes (Eg, ODR, Eh, Fe^{+2}). Sneh *et al.* (1999) found that the soil dehydrogenase activity increased significantly with Bokashi or a combination of inorganic fertilizer with Bokashi. Carpenter *et al.* (2000) found that both biodynamic and non-biodynamic composts increased soil microbial biomass, respiration, dehydrogenase activity, soil C mineralized in 10 d (MinC), earthworm (*Lumbricus terrestris*) population and biomass. Shukla *et al.* (2000) reported that soil solarization caused soil dehydrogenase

activity to decrease more than that of alkaline phosphatase or acid phosphatase activity. Kara and Penezoglu (2000) found that green manuring had a significant effect on soil CO₂ production and soil DHA activity. McGrath and Singleton (2000) found that soil dehydrogenase activity remained very low in all soils containing pentachloro phenol (PCP) and did not recover throughout the experiment (6 weeks) despite the decrease in PCP. Soil dehydrogenase activity remained very low in all soils containing PCP and did not recover throughout the experiment (6 weeks) despite the decrease in PCP. Rames *et al.* (2000) reported that anilofos showed the maximum inhibition of soil dehydrogenase and urease activity. Soyabean seed yield increased most with the application of propaquizafop (1490 kg ha⁻¹) i) in st year and with imazethapyr (2150 kg ha⁻¹) in second year. Seed yield had positive association with dehydrogenase and urease activity.

2.8 Nematode population:

Kurten and Kemper (1974) studied the nematode population in the following crop rotation of fodder-beet, spring barley (2X), horse bean, winter rye (2X), grain maize, spring wheat and reported that the soil nematode population was slightly reduced after the application of anhydrous ammonia. There was no long-term difference between nematode populations in the fields when calcium ammonium nitrate and anhydrous ammonia treatments were given. Kozłowska and Domurat (1975) reported that nitrogen-increased the total number of nematodes in soil under potato and sunflowers towards the end of the vegetative period and also increased the percentage of true parasites in the total population. Saly and Matulikova (1978) reported that fertilizers (240 kg ha⁻¹ NPK) reduced the density and biomass of nematode populations to a greater extent in the

autumn than in the spring soil samples. Castillo *et al.* (1978) reported that flooding of soil have negative correlation with *Rotylenchulus reniformis* and *Meloidogyne incognita* population in soil and in root also. Yeates (1981) found that total soil nematode population was reduced by 37-66% in field, which was inoculated with earthworm. Kiknadze and Eliavab (1984) studied the soil nematode fauna of a beech forest on the Saguramsk mountains, eastern Georgia, USSR in 1969-1970. The 135 species recorded were grouped according to their nutrition. 37.2% of all nematodes were bacteriophages, 18.6% were mycophages and non-specific plant parasites, 9.3% were species with mixed nutrition and algophages, 9.3% were specific plant parasites and 4.6% were carnivorous. *Tylencholaimus minimus*, present in 93% of all samples, was the dominant species. *Lelenchus discrepans*, *Anaplectus granuiosus*, *Plectus elongatus* and *Clarkus papillatus* were subdominant. Mani *et al.* (1981) found that application of carbofuran 3G at 4 kg a.i. ha⁻¹ to a 4-month-old turmeric crop had reduced the soil nematode population by 81.6% 4 in months after treatment compared with a 45.2% increase observed in the control plot. Hota *et al.* (1988) studied the population dynamic of nematode in low land and upland rice field and found that plant feeders constituted about 74% of the total population and all the tropic groups were more frequent in the 10 cm soil layer at both sites. Nematode population diversity was higher in upland than lowland. Mohammed (1987) found in an experiment that fluchloralin markedly stimulated the growth of the plant parasitic nematode population per 100 ml of soil compared with that of an untreated crop from flowering until after harvesting. Alachlor stimulated the soil nematode population at crop branching but this effect subsequently declined. At the later stages of crop development, flurodifen had a similar effect on phytoparasitic nematodes as alachlor. In particular,

alachlor increased *Longidorus* spp. until crop maturity as well as *Aphelenchus* and *Hoplolaimus* during crop branching. Fluchloralin markedly increased the numbers of *Tylenchorhynchus* spp., especially towards the end of the growing season. Fluorodifen eliminated *Tylenchus* and *Aphelenchoides* spp. throughout the study period. Moorhead *et al.* (1987) studied the effect of moisture and temperature on the dynamic of nematode population in northern Chihuahuan desert and found that nematodes enter a cryptobiotic state (anhydrobiosis) when soil moisture is limiting, a condition, which is quickly reversible when adequate moisture, becomes available. Therefore, anhydrobiotes persist during dry periods, insuring the immediate activity of a nematode population following rainfall. Eggs and larvae develop quickly at warm summer temperatures, providing a high potential growth rate for the population when moisture is not limiting. Most annual precipitation in the northern Chihuahuan desert falls in frequent summer convection storms when temperatures are high and soils dry quickly. Model results suggest that rainfall frequency is a major determinant of nematode activity patterns in surface and shallow soil layers. Predicted activity patterns were consistent with field observations of nematode-related decomposition processes. Bhattacharyya *et al.* (1984) evaluated the effect of soil cover soil moisture regimes on nematode population in soil and root of banana and reported that soil covers significantly reduced soil nematode populations and among them black polyethylene was the most effective followed by sugarcane leaf trash and banana trash. The highest soil nematode population (1051) count was under no cover treatment at 20% average soil moisture depletion level while the lowest of 151 was recorded under black polyethylene at 20% average soil moisture depletion. The highest root nematode population count of 325 was recorded under no cover treatment at 60% average soil

moisture depletion level as compared to the lowest of 26 under black polyethylene at 60% average soil moisture depletion level. Total nematode populations in soil and roots were significantly reduced by the soil covers and among them black polyethylene was the most effective, followed by sugarcane leaf trash and banana trash. The population comprised of *Radopholus* and *Pratylenchus*. Heald and Robinson (1987) found that soil solarization significantly reduced soil nematode population densities 0-15 cm deep and increased yields of lettuce and cowpea (*Vigna unguiculata*). The length of time required for 90% mortality of nematodes in soil heated under controlled conditions in the laboratory varied from 25 hours to less than 1 hour between 41 and 47°C. Daily exposures of nematode-infested soil to lethal temperatures for sub lethal time periods had a cumulative lethal effect. In water, vermiform stages required up to 10 days to recover from sub lethal thermal stress. Mishra and Dash (1987) reported that soil amendments suppressed the population of *Helicotylenchus* sp., with apparently little effect on other trophic groups of nematodes. Mandal and Mishra (1988) studied the effect of intensive cropping (jute-rice-wheat) and manuring on the nematode population in soil and found that Application of NPK (0, 50, 100 or 150%) did not give a statistically significant reduction in nematode populations. Reddy *et al.* (1991) found that maximum reduction in soil nematode population of citrus was obtained with neem cake + *Paecilomyces lilacinus*, while castor cake + *Paecilomyces lilacinus* gave the least nematode population in the roots. Cho and Kim (1991) reported the results of the effects of different cropping systems. They showed that soil nematodes were not found in the *Cassia tora* mono-cropped plot from the mid growing season of *C. tora*, while many nematodes were found in the soyabean mono-cropped plot in upland fields. Khan (1991) conducted a survey of 23

sugarcane growing localities in the states of Kaduna, Kano and Katsina in Nigeria and revealed 29 plant parasitic nematode genera associated with this crop in low to high populations. Of these, *Meloidogyne incognita*, *Helicotylenchus* spp., *Pratylenchus* spp., *Tylenchorhynchus* spp., *Telotylenchus* spp., *Xiphinema* spp., *Rotylenchulus reniformis*, *Scutellonema* spp. and *Hoplolaimus* spp., were considered the most frequent and pathogenic species. Optimum moisture conditions and the sandy soil favoured nematode population build-up. El-Nagar *et al.* (1994) conducted an experiment to study the impact of organic soil supplement on *Rotylenchulus reniformis* infecting cowpea, *Vigna sinensis*. The results indicated that the highest reduction in the numbers of egg masses were obtained by Sincocin 5% (76.7%) followed by pigeon manure and sewage water + pigeon manure treatments (74.9%). The highest reduction in soil nematode population (79.5%) was obtained by pigeon manure. The highest plant weight was obtained by sewage water and Sincocin 5% treatments, while plant length increased 51% when pigeon pea manure was added. Ramakrishnan *et al.* (1997) conducted experiment with okra in greenhouse and found that maximum reduction in number of *Meloidogyne incognita* females, number of egg masses, number of eggs per egg mass and soil nematode population were also recorded in soil amended with neem. Ramakrishnan *et al.* (1996) reported that when okra plants were treated with dry *Azolla pinnata* at 3% w w⁻¹ in pot experiments, maximum reduction in number of adult *M. incognita* females, egg masses, eggs per egg mass and soil nematode population was recorded when compared with the treatment with *Azospirillum brasilense* at 1.5% w w⁻¹. Ugbaja (1997) reported that the introduction of castor reduced the nematode population (*Pratylenchus* sp. was identified) by 90% and increased the yields of cassava and cocoyam by 29% and 28% respectively. It also reduced

the severity of nematode infestation of the tubers and corms. Patel and Patel (1993) reported that application of carbofuran at 3 kg ha⁻¹ a day prior to sowing significantly reduced the root knot index and soil nematode population 53.9 and 61.4% of controls, respectively. Youssef (1999) conducted an experiment to study the population dynamics of *Hirschmanniella oryzae*, in relation to rice cultivar, soil temperature and nematode control. It was found that there was a positive correlation ($r = 0.9$) between the root population of *H. oryzae* and prevalent soil temperature, whereas, negative correlation ($r = -0.07$) was observed for the soil nematode population. There was a positive correlation ($r = 0.9$) between the root population of *H. oryzae* and prevalent soil temperature, whereas, negative correlation ($r = -0.07$) was observed for the soil nematode population. Ali and Kamal (1998) found that under greenhouse conditions the *Serratia marcescens* and *Streptomyces avermitilis* agents considerably reduced nematode soil population and gall formation on grape root systems. The average reduction in nematode population was 61, 54.3 and 35% in treatments with *S. marcescens*, *S. avermitilis* and fenamiphos respectively. Westphal and Becker (2001) reported that *H. schachtii* population densities under wheat and the resistant cultivars of sugar beet and oilseed radish did not differ significantly from the fallow treatment. Ricci *et al.* (2000) concluded from an experiment that soil solarization reduced purple nutsedge population by 59%. There was a significant increase in yields of carrot (28%), cabbage (34%), beet (37%), and greenbean (32%) when compared to unsolarized plots. Biological nitrogen fixation, as judged by greenbean nodulation, was not affected by solarization but the soil nematode population was considerably reduced. Rajeswari *et al.* (2001) reported that although carbofuran caused a sudden and large reduction in the soil nematode population immediately after application, it did

not give sustained suppression of nematodes, as did *Glomus mosseae*. Williamson *et al.* (2000) reported that nematodes are a greater problem in sandy soils. Khan *et al.* (2001) conducted a survey to detect and identify the nematodes associated with Chir (*Pinus roxburghii*) pine seedlings. Results showed that four groups of nematodes were recorded and the highest incidence was found by *Tylenchidae* (55.5%) followed by *Mononchidae* (42.0%), *Dorylaimidae* (37.9%) and *Aphelenchidae* (16.0%).

Chapter - 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 Plant materials

The seeds of six transgenic lines of tropical cabbage (*Brassica oleracea* var. *capitata*) were provided by Dr. P. Anand Kumar, (Department of Molecular Biology and Biotechnology, NRC on plant Biotechnology), Indian Agricultural Research Institute, New Delhi, India. These pines are able to form head at higher temperature and produce the seed under tropical condition. Weather condition during the period of experiment, has been presented in Appendix-I.

3.1 Tissue culture protocol for cabbage

3.1.1 Raising of seedlings

Although, the optimum temperature required is around 20°C, it can grow and form head when the temperature is between 14-28°C. Seeds of six transgenic cabbage lines ($^{22}T_0$, $^{22.6}T_0$, $^{22.2}T_0$, $^3T_1$, $^4T_1$, $^{11}T_1$) and Golden Acre variety seed were surface sterilized in 0.1% (w v⁻¹) mercuric chloride solution for 10 min and washed thoroughly in sterile distilled water four time. Seeds were germinated in Borosil™ test tube (150 mm × 25 mm) with polypropylene plastic caps, containing 10 ml of half strength Murashige and Skoog (MS) (Murashige and Skoog 1962) medium (Himedia, India) with B₅ vitamins (Gamborg *et al.*, 1968) (Sigma USA) (Table1) and solidified with 0.8% agar (Qualigens, Glaxo India). About five seeds were inoculated in each test tube and incubated at 25°C with a 16 h photophase.

Table 1: Compositions of Murashige and Skoog (1962) medium with Gamborg (1968) B₅ Vitamins.

Constituents	Amount (mg ⁻¹)
Macro nutrients	
NH ₄ NO ₃	1650.00
KNO ₃	1900.00
MgSO ₄ · 7 H ₂ O	370.00
KH ₂ PO ₄	170.00
CaCl ₂ · 2H ₂ O.	440.00
Micronutrient	
H ₃ BO ₃	6.20
MnSO ₄ H ₂ O.	16.90
ZnSO ₄ · 7 H ₂ O.	8.60
Na ₂ MoO ₄ · 2H ₂ O.	0.25
CuSO ₄ · 5H ₂ O	0.025
CaCl ₂ · 6H ₂ O	0.025
KI	0.830
Iron source	
FeSO ₄ · 7 H ₂ O	27.80
Na ₂ EDTA · 2H ₂ O	37.30
Meso-inositol	
Myo-inositol	100.00
Organics (with B₅ vitamins)	
Glycine	2.00
Nicotinic acid	1.00
Pyridoxin HCl	1.00
Thiamine HCl	10.00
Carbon source	
Sucrose	20000.00

3.1.2 Preparation of culture media

The pH of MS medium was adjusted between 5.7 to 5.8 and for the

preparation of semi solid medium 8.0 g l⁻¹ or 7.0 g l⁻¹ phyto gel was added. Then the medium was autoclaved at 121°C under a pressure of 1.06 kg cm⁻² for 20 min either directly in 1 litre Erlenmeyer flask or after dispensing into culture vessels. Depending on the experiment the culture vessels were either Borosil test tubes (150 mm x 25 mm) or glass bottles (135 mm x 30 mm) test tubes and glass bottles were covered with plastic caps (Figure1). The amount of medium dispensed in these vessels was varied from 10 ml (test tubes) to 40 ml (glass bottles).

3.1.3 Maintenance of culture

All the cultures were incubated at 25 ± 2°C using 16 h photophase provided by cool white fluorescent lights (Trulite, Philips, India) producing an intensity of 120 µ mol m⁻²s⁻¹ (100 Lux) at the culture level.

3.2 Molecular analysis

Total genomic DNA from cabbage plant was isolated following the method documented by Doyle and Doyle (1990). About 5 gm of young leaf tissue was homogenized in liquid nitrogen and mixed with 20 ml of preheated (65°C) CTAB buffer [0.1 M Tris-HCl; 0.02 M Na₂EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% hexa decyltrimethyl ammonium bromide (CTAB), pH 8.0] in 50 ml polypropylene centrifuge tube and incubated at 65°C water bath for 1h with occasional gentle swirling. After incubation, fixed volume of chloroform : isoamyl alcohol (24:1) was added to the mixture and mixed carefully for 10 minutes. Then the mixture was centrifuged at 1500 XG (Sorvall RC 5C) for 15 min at room temperature and the aqueous phase was transferred to a fresh centrifuge tube and mixed with 2/3 volume of isopropanol by gently inverting the tube at least six times. This mixture was kept at 4°C for two hours to allow

complete precipitation of DNA and then centrifuged at 1500 XG for 10 min to pellet the DNA. The pellet was washed with wash buffer (70 v^v-¹ ethanol, 10 mM ammonium acetate) for 20 min and washed twice with 70% ethanol. Finally DNA was dissolved in 1 ml TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and the content was transferred into 2 ml microfuge tube. The dissolved DNA was treated with 10 mg ml⁻¹ RNase A at 37°C for 2h and the DNA was further purified to remove proteins and RNase A by extracting with equal volume of phenol : chloroform and followed chloroform : isoamyl alcohol (24 : 1) extraction. The DNA was pelleted by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol and then the mixture was kept at -20°C for 30 min. The pellet was recovered by centrifugation at 10,000 XG (Microfuge E, Beckman) at 4°C for 15 min. The DNA pellet was desalted by washing twice with 70% ethanol for 20 min. The pellet was air dried and dissolved in 500 µl of TE. Spectrophotometer (Beckman, U.K.) readings at A₂₆₀ and A₂₈₀ were taken to determine the concentration and purity of DNA samples. The following formula was used to calculate the concentration of DNA : $[A_{260} \times 50 \times \text{dilution factor}] / 1000 \mu\text{g ml}^{-1}$.

3.2.1 Polymerase chain reaction

Plant DNA isolated from the leaf tissue was used as template to amplify *nptII*, *cry1B*, *cry1Ab* genes by using corresponding primers. The sequences of the primers used are

npt II primers

Forward primer : 5' - CAATCGCTGCTCTGATGCC - 3'

Reverse primer : 5' - GGCGATAGAAGGCGATGCG - 3'

cry 1B primers

Forward primer : 5'GGTTACCTCCAA CCGTAAG - 3'

Reverse primer : 5'-CAACTGGGCAGAGGTGTTG - 3'

cry 1Ab primers

Forward primer : 5'-ACAACCCAAACATCAACG- 3'

Reverse primer : 5'-CTTCTGGGAAGTTATAGCC - 3'

1 unit Taq DNA polymerase (Gen Taq. Singapore) with 10x buffer containing 1.5 mM MgCl₂ and 0.1% TritonX-100 was used for amplification. The reaction volume was made up to 50 µl. The annealing temperature for *npt II*, *cry 1Ab*, *cry 1B* were 59, 52 and 54°C, respectively. Cycle conditions were (1) Initial denaturation at 94°C for 5 min, (2) Cycle denaturation at 94°C for 1 min, (3) Annealing at 59, 52 and 54°C for 1 min, (4) Polymerization at 72°C for 2 min. The polymerization for 2 min at 72°C was kept for all three primers and a final complete polymerization step was kept at 72°C for 7 min. The melting temperature (T_m) was obtained from manufacturer (Genset Oligos, Singapore) on the basis of the base composition of the primer. The following simple rule was followed to calculate the T_M (Rychlik and Rhoads, 1989) : 4X(C+G) + 2X(A+T)°C. However, final annealing temperature was experimentally determined for a particular primer/template combination. A positive control for *npt II*, *cry 1Ab* *cry 1B*, genes were also included. After completion of PCR process, PCR products were cleared up with QIAquick PCR kit and stored at -20°C until used.

3.3.1 Buffer extractable protein estimation

Leaf extracts were prepared from fresh young leaves by homogenizing leaf segments (1 gm) in liquid nitrogen with pestle and mortar in the presence of 1 ml phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄ ; pH 7.4) containing 0.05% Tween-20 (PSB/T). The extract was centrifuged at 1000 XG for 10 min in microfuge and the supernatant was taken as buffer extractable protein. The buffer extractable protein was

estimated by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA, Fraction V; Sigma, USA) as standard protein.

Aliquots of 0.5 mg /ml BSA (5, 10, 15 and 20 μ l) were prepared and the volume in each tube was made upto 100 μ l with 0.15 M NaCl. A blank tube containing only 100 μ l of 0.15 M NaCl was also made. 1 ml of Bradford reagent was added to each tube, vortexed and left at room temperatures for 5 min. The same method was followed for buffer extractable protein extracted from leaf tissue. Absorbance was measured spectrophotometrically (Beckman, UK) at A_{595} and the protein concentration was determined from the BSA standard curve.

3.3.2 Enzyme-linked immunosorbant assay (ELISA)

The Cry fusion protein expression in plant leaf tissue of transgenic plant was quantified by double antibody sandwich ELISA analysis (Clark *et al.*, 1986). Paranitrophenyl phosphate disodium is the substrate of choice for use with alkaline phosphatase in ELISA procedures. This substrate produces a soluble products that are yellow in colour and can be read spectrophotometrically at 405 nm. The pNPP reaction may be stopped with 0.5 M NaOH and read at 405 nm.

The buffer extractable protein solution was centrifuged in microfuge at 10,000 XG for 10 min. 100 μ l of 1 μ g polyclonal antibodies of Cry 1B and Cry 1Ab protein was loaded in alternate well and the first well was kept as blank, which contained only 150 μ l PBS/T. This was incubated at 37°C for one hour. The wells were blocked with 100 μ l blocking buffer containing a non-specific protein solution (5% fat free milk and 50 mM CDTA in PBS/T) in order to keep the background levels low. 100 μ l of buffer extractable protein samples in PBS/T solution were then added to the wells to bind to the antibody immobilized on the plate and in parallel wells. This was incubated at 4°C overnight. ELISA plate wells were thoroughly washed with jet of water from a plastic squirt bottle three

times to remove any unbound reagents and gently flicked face down on to several paper towels to remove residual liquid. This step was followed between every successive step. Following this, 100 μ l of anti-Ig G - AP conjugate diluted 1:2000 in PSB/T was added, followed by 2 h incubation at room temperature. This second antibody conjugate binds to the immobilized antigen completing the sandwich. Microwells were washed with a jet of water three times. As a final step 150 μ l of enzyme substrate pNPP was added and the reaction was stopped after 10 min by adding 25 μ l of 0.5 M NaOH. For quantification, ELISA reader (MR 710 microplate Reader, Dyanatech, Great Britain) was used to read the spectrophotometric measurement of absorbance at 405 nm.

3.3.3 Precipitation of buffer extractable protein for analysis by SDS PAGE

The buffer extractable protein of transgenic and wild type plants was suspended in 10% trichloroacetic acid (TCA) and 20 mM DTT. This mixture was incubated on ice for 45 min and then centrifuged in microfuge at 4°C for 10 min. The pellet was washed with 80% acetone containing 20 mM DTT. Finally the pellet was air dried and resuspended in 100 μ l 1 x SDS (sodium dodecyl sulphate) gel loading buffer [50 mM Tris-Cl (pH 6.8); 150 mM DTT, 2% SDS (Electrophoresis grade), 0.1% bromophenol blue 10% glycerol].

3.3.4 SDS-polyacrylamide gel electrophoresis

Approximately 5 μ g of buffer extractable proteins extracted from wild type, Bt- transgenic cabbage plants and the purified Bt-fusion protein were subjected to 10% SDS-PAGE according to a standard protocol (Sambrook *et al.*, 1989). Gel cassettes were placed in the assembly according to manufactures instruction (Hoefer, Mighty Smail II, Pharmacia Biotech, USA). In an Erlenmeyer flask 25 ml of 10% resolving gel was prepared by mixing 9.9 ml

H₂O, 8.3 ml 30% acrylamide mix, 6.3 ml 1.5 M Tris (pH 8.8), 0.25 ml 10% SDS, 0.25 ml ammonium persulfate and 0.01 ml TEMED.

Without delay the solution was swirled and poured between the glass plates and overlaid with 0.1% SDS. After polymerization was complete in 30 min the top of the gel was washed with double distilled water to remove any unpolymerized acrylamide. The 5% stacking gel (5 ml) was prepared as follows. In a disposable plastic tube, 3.4 ml H₂O, 8.3 ml 30% acrylamide mix, 0.63 ml 1.0 M Tris (pH 6.8), 0.05 ml 10% SDS, 0.05 ml 10% ammonium persulfate and finally 0.005 ml TEMED was added. The solution was swirled immediately and poured directly to the surface of the polymerized resolving gel. A Teflon comb was inserted immediately into stacking gel. The protein sample in 1 x SDS gel loading buffer was heated to 100°C for 3 min to denature the proteins. Forty µl of the proteins sample was loaded in each well. Protein standards ranging from 10 to 250 kDa (Precision Protein Standard, Bio-Rad) was loaded to serve as a reference band. Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine (electrophoresis grade) (pH 8.3), 0.1% SDS (electrophoresis grade) was used as tank buffer. The gel was run at 8 vcm⁻¹ till the dye front moved into the resolving gel and the voltage was subsequently increased to 15 vcm⁻¹ until the bromophenol blue reached the bottom of the resolving gel.

The gel was carefully removed from the glass plate and was fixed in fixing solution [50% (vv⁻¹) methanol ; 10% (vv⁻¹) acetic acid, 40% double distilled water] for 2 h. The gel was stained with - Coomassie Brilliant Blue R-250 (Biosafe, Coomassie Blue, BIO-RAD) for one hour and left in destaining solution (7% acetic acid, 5% methanol and 88% double distilled water) with gentle agitation (30 rpm) for 2h or until blue band and clear background were obtained. Finally the gel was ringed twice in double distilled water for one hour

and left in 200 ml double distilled water at room temperature for overnight before photography.

3.4 Insect bioassay

Insect bioassays were carried out to study the efficacy of fusion protein expressed in Bt-transgenic cabbage plants against neonate larvae of *P. xylostella*.

3.4.1 Rearing and maintenance of Diamond back moth

Pupae of *Plutella xylostella* were obtained from the Division of Entomology, IARI and maintained in insect cage at $27 \pm 2^{\circ}\text{C}$, $60 \pm 2\%$ relative humidity under a photophore for emergence of adult moths. The pupal period was found to be 10 days. Adult moths were maintained in separate insect cage with cabbage leaves and a sterile cotton swab moistened in 10% honey. Female moths started laying eggs soon after mating, and the oviposition lasted 4 days, during which the female laid 10-150 eggs. Majority of eggs were laid in concavities of upper and lower surface rather than smooth surfaces. The incubation period lasted for 5 days. After 5 days, fresh leaves were laid over leaves with eggs and neonate larvae. After 3 days, leaves with neonate larvae were transferred to a new insect cage and every 3 days fresh leaves were added until pupation. The pupae were carefully picked up with drawing brush No. 3 and transferred to another cage for emergence. For experiment purpose, neonate larvae were used.

3.4.2 Leaf disc bioassay

Leaf disc bioassay was performed in a 6 well macroplate (Cellstar, Greiner Labor technik, Germany). Whatman No. 1 paper was cut to the exact size of macroplate well, moistened in distilled water and was laid inside the well. Leaf discs of 3 cm diameter cut from transgenic and wild type plants were

a. Cabbage on half strength MS medium.



b. Cabbage during hardening stage.



c. Hardened cabbage ready for transplanting into earthen pots.



Figure 1: Raising of Transgenic cabbage under laboratory condition.

laid with abaxial side down over distilled water moistened Whatman No. 1 paper.

About 10 neonate larvae, carefully picked up with sable hair no. 3 brush, were laid on leaf discs. The macro plate was wrapped with Saran Wrap and experiment was conducted at $26 \pm 2^{\circ}\text{C}$, under 16 h photophase. These insect bioassay were performed in triplicates and count on mortality of larvae were made every day.

3.4.3 Detached leaf bioassay

Young leaves with petiole from transgenic plants and wild type were used. The leaf area of each leaf was approximately 25 cm^2 . Detached leaf bioassay were performed using second instar larvae of DBM. Each leaf with petiole was placed in a Jam bottle containing moistened cotton swab and maintained at $26 \pm 2^{\circ}\text{C}$ under a 16 h photophase. Each leaf was bioassayed with 20 larvae of second instar stage. Leaf damage, insect mortality were evaluated after 24 h interval. This bioassay was performed in triplicated.

3.4.4 Whole plant bioassay

For whole plant bioassay, 6 (six) separate plants of transgenic plants and normal type plant, grown in agro-eco chamber were infested with 10 (ten) third instar larvae of DBM. The larvae were allowed to feed on plant and observation was taken after 24 h interval.

3.5 Chemicals

A sample of analytical grade fipronil (99.8% purity) was supplied by Rhone-Poulenc Agro, France and sample of analytical grade thiacloprid (98.8% purity) was supplied by Bayer, Germany. The analytical grade fipronil and thiacloprid were found to be chromatographically (TLC and HPLC) pure and had a mp 200°C and 136°C , respectively. Structure was further confirmed spectroscopically ($^1\text{H-NMR}$). The laboratory grade solvents were purified prior to

use by distillation. All the chemicals and reagents were of analytical grade. HPLC grade solvents were used.

3.5.1 Formulation analysis of pesticides for estimation of active ingredient

Fipronil in reagent 5 SC formulation

A glass column (50 x 1.5 cm) was packed with silica gel (60-120 mesh size) using hexane. Five gram of fipronil formulation, Regent 5 SC, weighed in a weighing bottle was placed over the silica gel layer packed in the column. The column was first eluted with 100 ml hexane and 25 ml fractions were collected. The polarity was increased slowly by adding acetone to hexane solvent so 5, 10, 15, 20, 25, 30, and 50% (vv⁻¹) acetone in hexane solvent system. Four 25 ml fractions were collected for each solvent system. The solvent was evaporated from each fraction to around 10 ml and was monitored by TLC using hexane : acetone (70-30) solvent system. The fraction showing same R_f values were collected together in one conical flask. Finally the solvent was evaporated to dryness and the solid product (fipronil) was collected and weighed after drying. It was recrystallized using hexane as solvent. The compound was identified and confirmed by its melting point and spectroscopic analysis.

3.5.2 Thiacloprid in Calypso 240 SC formulation

A glass column (50 x 1.5 cm) was packed with silica gel (60-120 mesh size) in hexane. Five grams of thiacloprid formulation Calypso 240 SC, weighed in a weighing bottle, was placed over the silica gel layer packed in the column. The column was first eluted with 100 ml hexane and 25 ml fractions were collected. The polarity was increased slowly by adding acetone to hexane solvent to obtain 10, 15, 20, 25, 30, 35, and 50% acetone in hexane solvent system taking 100 ml of the solvent for eluting and four fraction of 25 ml size were collected

for each solvent system. The solvent was evaporated from each fraction to around 10 ml and the contents were separated by TLC in hexane : acetone (60 : 40) solvent system. The fractions showing same R_f values were collected together in one conical flask. It was crystallized by adding hexane. The structure of compound was identified and confirmed by its melting point and spectroscopic analysis.

3.6 Residue analysis of pesticides

3.6.1 Recovery studies

3.6.1.1 Fipronil from cabbage

Recovery experiments were carried with the aim to determine the accuracy and precision of the analytical method, which was adopted during the experiment.

3.6.1.2 Preparation of fipronil standard solution

Accurately weighed 25.05 mg of analytical grade fipronil was dissolved in HPLC grade methanol in 25 ml volumetric flask. Volume was made up to the mark to obtain exactly 1000 $\mu\text{g ml}^{-1}$ solution. 1 ml of the stock solution was diluted to 10 ml with methanol to obtain about 100 $\mu\text{g ml}^{-1}$ solution. It was further diluted appropriately to get about 10 $\mu\text{g ml}^{-1}$ solution. These stock solutions were kept in a refrigerator for further use and appropriately diluted, when required.

3.6.1.3 Calibration of fipronil by HPLC

3.6.1.3.1 Determination of λ_{max}

The wavelength in the region 200 to 300 nm was scanned to determine the λ_{max} . Different values of absorbance were recorded against different wave length and from the available data λ_{max} was determined.

3.6.1.3.2 Preparation of standard curve

From the stock solution of fipronil, standard curve was prepared. The stock solution containing $10 \mu\text{g ml}^{-1}$ was diluted serially to obtain 0.5, 1, 1.5, 2.5, 5 and $10 \mu\text{g ml}^{-1}$ solution, respectively.

A $10 \mu\text{l}$ aliquot of each solution containing 0.5, 1, 1.5, 2.5, 5, $10 \mu\text{g ml}^{-1}$ fipronil was injected into HPLC. Each run was repeated 3 times and the detector response was measured in terms of peak area. Calibration curve was prepared by plotting concentration of fipronil in μg on X-axis against average peak area on Y axis.

3.6.1.4 High performance liquid chromatography (HPLC)

Reverse phase high performance liquid chromatography was used for quantitative analysis. A Lachrom HPLC instrument equipped with quaternary pump, ultraviolet detector connected with autoinjector and a computer (model HITACHI) was used for analysis. The stationary phase consisted of Lichrosphere RP-18 packed stainless steel column (250 mm x 5 mm id). Chromatogram was recorded in a window' 95 NT based programme.

The chromatographic conditions were :

- 1) Mobile phase : Methanol : water (75 : 25)
- 2) Flow rate : 0.8 ml min^{-1}
- 3) λ_{max} : 242 nm
- 4) Injection volume : $10 \mu\text{l}$
- 5) Column and solvent temperature : Ambient

3.6.1.5 Fortification of cabbage with fipronil

Cabbage head was first cut into small pieces and 25 g of samples were kept in 250 ml conical flasks in triplicate and spiked with 0.1 ml and 1 ml of 100

$\mu\text{g ml}^{-1}$ to obtain deposits of 0.4 μg and 4 μg of fipronil per gram of cabbage. The flasks containing spiked heads were shaken for 2 minutes. Small amount of acetone was added in the flask for uniform deposit of fipronil in the cabbage and left overnight. Control samples were treated only with the solvent. Each treatment was replicated thrice including control.

3.6.1.6 Extraction of fipronil form cabbage heads

The fortified samples (25 g) were homogenized in a blender (Remi mixie). The extract was treated with 25 ml acetone and was filtered through a Buchner funnel fitted with a Whatman No. 1 filter paper. For easy and quick filtration, a vacuum pump was used. The residual pieces of cabbage head on the filter paper were again transferred to the blender. This process was repeated to three more times with 25 ml acetone. After extraction, the blender jar was rinsed with 25 ml acetone and rinsate was also filtered the same way and the final volume of the extract, around 100 ml, was collected.

3.6.1.7 Cleanup of the extracts

This is the most important step in pesticide residue analysis. Clean up of the extract was done by liquid-liquid partitioning followed by adsorption column chromatography.

3.6.1.7.1 Liquid-liquid partitioning

Solvent from the extract was evaporated off with the help of a rotary vacuum evaporator to around 10 ml and then transferred to a 250 ml separately funnel. 30 ml of saturated sodium chloride solution and then 15 ml distilled hexane were added to it. Separatory funnel was thoroughly shaken for 1 minute by slowly releasing pressure through stop cork and then allowed to stand until the two layers separated. The bottom aqueous layer was collected and organic

layer was discarded. This aqueous layer was further subjected to partitioning with chloroform. 25 ml of distilled chloroform were added to it. Separatory funnel were thoroughly shaken for 1 minute by slowly releasing pressure through stop cork and then allowed to stand (for about 5 minutes) until the two layers separated. The bottom layer was collected in a conical flask after passing through anhydrous sodium sulphate (15 g). The aqueous solution in separator funnel was shaken two more times with 25 ml chloroform. The anhydrous sodium sulphate layer was given additional washing with 10 ml chloroform and the filtrate was collected in the same flask.

3.6.1.7.2 Column chromatography

Clean up by neutral alumina, basic alumina, and Florisil separately and in a combination was carried out. Glass column (50 cm x 1.5 cm) was plugged with cotton at the bottom, then dry packed with 5 g of anhydrous sodium sulphate followed by 5 g of adsorbent and finally again with 5 g of sodium sulfate. It was prewashed with 20 ml hexane. The extract in chloroform was evaporated to dryness. The extract was taken in 20 ml distilled hexane and was added to the adsorbent column. The column was first eluted with distilled hexane. Then 25 ml hexane : acetone (7:3 v/v⁻¹) was taken four time for elution of the column. This eluate was collected in a conical flask. For optimizing elution, different solvent systems were tried earlier.

3.6.1.7. 2.1 Clean up by neutral alumina and Florisil

The column was plugged with cotton at the bottom and then filled with 5 g sodium sulfate; 4 g of neutral alumina followed by 4 g of Florisil and finally by 5 g of sodium sulfate were loaded in the column. It was then pre washed with 20 ml hexane. The extract in the chloroform was evaporated to dryness. Then the

extract was taken in 10 ml distilled hexane and transferred to the column. The column was then eluted with 50 ml distilled hexane. The hexane was discarded. The column was then sequentially eluted with 100 ml of hexane : acetone (7:3; v/v¹). The eluted solvent collected from the column was completely evaporated to dryness in a rotary vacuum evaporator. The final volume was made up with methanol. The cleaned samples obtained were analyzed using HPLC (Model HITACHI) equipped with UV detector (UVD) and autoinjector system connected to a Samtron computer monitor (Model 75E).

3.6.1.7.2.2 Clean up by basic alumina and Florisil

Same procedure was adopted as mentioned in section 3.6.1.7.1. It was used by putting 4 g of basic alumina followed by 4 g Florisil.

3.6.2 Recovery studies

3.6.2.1 Thiacloprid from cabbage head

3.6.2.2. Preparation of Thiacloprid standard solution

Accurately weighed 25.30 mg of thiacloprid (analytical grade 99.7%) was dissolved in HPLC grade acetonitrile in 25 ml volumetric flask. Volume was made up to the mark to obtain exactly 1000 µg/ml solution. One ml of this stock solution was diluted to 10 ml of acetonitrile in 10 ml volumetric flask to obtain 100 µg/ml solution. It was further diluted to get about 10 µg/ml solution. These stock solutions were kept in a refrigerator for further use and diluted when required.

3.6.2.3 Calibration of Thadoprid by HPLC

3.6.2.3.1 Determination of $\lambda_{\max}$

The wavelength in the region 200 to 300 nm was scanned to determine the $\lambda_{\max}$. Different values of absorbance were recorded against different

wavelength and from the available data $\lambda_{\max}$ was determined.

3.6.2.3.2 Preparation of standard curve

From the stock solution of thiacloprid, standard curve was prepared. The stock solution containing 10 μg /ml was diluted serially to obtain 0.5, 1, 1.5, 2.5, 5 and 10 $\mu\text{g ml}^{-1}$ solution respectively.

A 10 μl aliquot of each solution containing 0.5, 1, 1.5, 2.5 , 5 and 10 $\mu\text{g ml}^{-1}$ thiacloprid was injected into HPLC. Each run was repeated 3 times and the detector response was measured in terms of peak area. Calibration curve was prepared by plotting concentration of thiacloprid in μg on X axis against average peak area on Y axis.

3.6.2.4 High performance liquid chromatography (HPLC)

Reverse phase high performance liquid chromatography was used for quantitative analysis. a Lachrom HPLC (Model HITACHI) instrument equipped with quaternary pump UV detector (UVD) connected with autoinjector and a Samtron Computer monitor (Model 75E) was used for analysis. The stationary phase consisted of Lichrosphere RP-18 packed stainless steel column (250 mm x 5 mm id). Chromatogram was recorded in a window '95 NT based programme.

The chromatographic conditions were :

1. Mobile phase : Acetonitrile : water (60 : 40)
2. Flow rate : 0.5 ml min^{-1}
3. $\lambda_{\max}$: 242 nm
4. Injection volume : 10 μl
5. Column and solvent temperature : Ambient

3.6.2.5 Fortification of cabbage with thiacloprid

Cabbage was first cut into small pieces and 25 g samples were kept in 250 ml conical flasks in triplicate and spiked with 0.1 ml and 1 ml of 100 µg/ml solution to obtain 0.4 and 4 µg thiacloprid per gram of cabbage. The flask containing spiked heads were shaken for 2 minutes. Enough of acetone was added in the flask to submerge the pieces of spiked cabbage head and left overnight. Control samples were treated only with the solvent. Each treatment were replicated thrice including control.

3.6.2.6 Mechanical extraction

The fortified samples (25 g) were homogenized in a blender (Remi mixie). The extract was treated with 25 ml acetone and was filtered through Buchner funnel fitted with a Whatman No. 1 filter paper. For easy and quick filtration, vacuum pump was used. The residual pieces of cabbage on the filter paper were again transferred to the blender. This process was repeated two more times with 25 ml acetone. After extraction, the blender jar was rinsed with 25 ml acetone and rinsate was also filtered the same way and the final volume of the extract (around 100 ml) was collected.

3.6.2.7 Clean up of the extracts

Clean up of the extract was done by liquid-liquid partitioning followed by adsorption column chromatography.

3.6.2.7.1 Liquid-liquid partitioning

Solvent from the extract was evaporated with the help of a rotary vacuum evaporator to around 10 ml and then transferred to a 250 ml separatory funnel. Thirty ml of saturated sodium chloride solution followed by 20 ml of distilled hexane were added to it. Separatory funnel was thoroughly shaken for one minute by slowly releasing pressure through stop cork and then allowed to stand until the two layers were separated. Hexane layer was discarded. Aqueous layer

was again partitioned with 25 ml distilled dichloromethane. Bottom layer (dichloromethane) was collected in a conical flask after passing through anhydrous sodium sulphate (15 g). The aqueous solution present in the separatory funnel was shaken two more times with 25 ml dichloromethane. The anhydrous sodium sulphate layer was given additional washing with 10 ml dichloromethane and the filtrate was collected in the same flask.

3.6.2.7.2 Column chromatography

Glass column (50 cm x 1.5 cm) was plugged with cotton at the bottom, then dry packed with 5 g of anhydrous sodium sulfate followed by 6 g of Florisil and finally again with 5 gm of sodium sulfate. The extract in dichloromethane was evaporated in a rotary vacuum evaporator before column chromatographic clean up. The extract was taken in 10 ml ethyl acetate and cyclohexane (1:1) solvent system and was added to the adsorbent column. The column was eluted with 100 ml ethyl acetate : cyclohexane (1:1 vv^{-1}) and the eluate was discarded. Finally the column was eluted with 40 ml acetonitrile. The acetonitrile was collected in a round bottomed flask and it was evaporated to dryness by rotary vacuum evaporator. Total 10 ml HPLC grade acetonitrile in two split portion was added to the round bottomed flask to rinse out the pesticide residue for estimation of the pesticide by HPLC.

3.6.3 Recovery of Fipronil residue from soil

Seventy ml of a mixture of acetone : acetonitrile (70 : 30 vv^{-1}) was added to the soil sample (50 g) taken in an Erlenmeyer flask (150 ml). The flask was stoppered and shaken on a mechanical shaker for 35 minutes. The contents were allowed to settle and supernatant phase was passed through anhydrous sodium sulphate and filtered through Whatman No. 1. The filtration was done twice more

with the same solvent system by taking it 70 ml each time and filtered in the same way. The combined filtrate was concentrated by evaporating the solvent on a rotary vacuum evaporator to dryness. The concentrated extract was passed through a column containing silica gel (10 g). The column was first eluted with 30 ml distilled hexane, followed by 100 ml hexane : acetone (70 : 30) solvent mixture. This eluant was collected and evaporated to dryness. Finally, the extract was taken in 5 ml HPLC grade methanol.

3.6.4 Sample analysis

The retention time (Rt) and peak areas of standard sample extracts were recorded. The quality of pesticide present in the extract was calculated using following equation.

$$y = \frac{\alpha \times C}{\beta} \times \frac{v}{w}$$

Where, y = concentration of pesticide residue in soil ($\mu\text{g g}^{-1}$)

α = peak areas of sample aliquot

β = peak area of standard solution

C = concentration of standard solution ($\mu\text{g ml}^{-1}$)

v = volume of the sample extract (ml)

w = weight of the sample (g)

3.7 Decontamination of thiacloprid by different chemicals

Cabbage was treated with thiacloprid by dipping it in a thiacloprid solution of known strength. The Cabbage was kept on this thiacloprid solution for 30 minutes. The solution of 0.5% NaOH, 0.5% KMnO_4 , 0.5% NaHCO_3 was prepared in different containers, one container was kept for control in which simple water was taken. One sample of cabbage was directly taken from the thiacloprid solution for determining the thiacloprid content at zero time. Other

replicates of the cabbages were kept in different containers, for 30 minutes and one hour. The thiacloprid contaminated cabbage was treated with different solutions and then the remaining pesticide was extracted, cleaned up and estimated again to calculate the effectiveness of the treatment.

3.8 Preparation of metabolites

3.8.1 Preparation of sulfone derivative of fipronil

Fipronil sulfone was prepared according to the procedure described by Trost and Curran (1981). Potassium hydrogen persulfate in aqueous methanol is a highly chemoselective oxidising agent for the oxidation of sulfides to sulfones. 10 m mol of fipronil was dissolved in methanol (40 ml) and cooled to 0°C. To this solution of 49.5% KHSO₅ (30 m moles) in water (40 ml) was added. The resulting cloudy slurry was stirred for 4 h at room temperature; diluted with water, and extracted with chloroform. The combined organic layer was washed with water and brine, dried over sodium sulfate (Na₂SO₄) and concentrated to give a quantitative recovery of sulfone derivative of fipronil as a white solid. Solid sulfone was purified by recrystallization using hexane solvent.

3.9 Determination of nutrient content in plant sample

Plant samples were dried in oven after sun drying for two days. The samples were kept in an oven at 60° ± 5°C, until they dried and reached a constant weight. The dried plant sample was ground. One gram of grinded plant sample was taken in a 100 ml conical flask and 30 ml diacid mixture (HNO₃ : HOCl; 5:1 vv⁻¹). It was heated in hot plate for digestion until it become colourless. It was diluted with 6 NHCl and the volume was made up to 100 ml

in volumetric flask. Aliquot was taken from the volumetric flask for estimation of different nutrient contents.

3.9.1 Determination of calcium and magnesium

One ml aliquot was taken in a conical flask and 30 ml distilled water was added. The pH of solution was adjusted to 10 by the addition of 15 ml buffer containing (NH₄Cl + NH₄OH). Ten drops of potassium ferrocyanide and 10 drops of Eriochrome Black T indicator was added.

The solution was titrated with 0.01N EDTA solution till the colour changed from red to bright blue. A blank sample, which was distilled water was titrated in exactly the same manner. The Ca and Mg content was calculated using the following formula :

$$\text{meq. of (Ca + Mg) / gm of sample} = (V_2 - V_3) \times \frac{N}{w} \times \frac{V}{V_1}$$

W = weight of sample taken

V = volume of extract made

V₁ = volume of aliquot taken for analysis

V₂ = volume of EDTA used for the sample

V₃ = volume of EDTA used for blank

N = Normality of EDTA

3.9.2 Determination of calcium

One ml of sample solution was taken in a conical flask and it was diluted by adding 50 ml of water. Ten drops of potassium ferrocyanide followed 5 ml NaOH solution were added. 50 mg Murexide indicator powder was added and the solution was titrated with 0.01 N EDTA solution till the colour changed from pink to purple. A blank sample was also run for incorporating correction of sample reading. The amount of Calcium was determined by following the

formula as used for calcium and magnesium. The magnesium content was determined by subtracting the value of calcium from the total amount of calcium and magnesium.

3.9.3 Determination of sulphur

One ml of sample solution was taken in a 25 ml volumetric flask. One gram of crystalline BaCl_2 was added and shaken for 1 minute. 1 ml of 0.25% solution of gum acacia was also added to stabilize the turbidity and volume was made up to 25 with water. The turbidity was measured spectrophotometrically at 420 nm wave length using blue filter. The readings were plotted in a standard curve for the quantitative estimation of sulphur.

3.9.4 Determination of potassium

One ml sample was taken in a 50 ml volumetric flask and the volume was made up with distilled water. One ml solution was taken separately in a 25 ml volumetric flasksk a the volume was made up with distilled water. This solution was fed into the atomizer of flame photometer. The readings were plotted on the standard curve which gave the K-concentration in the solution.

3.9.5 Determination of phosphorus

One ml solution was taken in a 25 ml volumetric flask. 5 ml of vanadomolybdate solution was added and the phosphorus content was determined using a standard curve. It was plotted on a graph paper taking concentration on x axis and absorbance recorded from spectrophotometric reading on y axis.

3.10 Determination of phosphorus uptake pattern

A brief outline of the nature and characteristics of the materials used and

a short description of the methods followed during the course of the phosphorus uptake study, are described below. This experiment was conducted during the month of November 2002.

3.10.1 Soil

The bulk of uncontaminated surface soil was collected from the Indo-Israel project area of Indian Agricultural Research Institute (IARI) farm. The soil was dried, ground and sieved through 2 mm sieve. Ten kg of the processed soil thoroughly mixed with the required fertilizer solution tagged with ^{32}P radioactivity were filled in the glazed pots of twelve kg capacity.

3.10.2 Soil analysis

The soil sample was analysed for important physico-chemical characteristics as per the standard procedures (Table 2).

3.10.3 Treatment

Crop	:	Cabbage
Cultivar	:	2 (Golden acre, Transgenic variety of Golden Acre)
Phosphorus levels	:	3 (0, 20 and 40 mg P_2O_5 /kg soil)
Replication	:	3
Design	:	CRD

The phosphorus was supplied through the aqueous solution of diammonium phosphate. A uniform dose of nitrogen (30 mg N/kg) and potassium (20 mg K_2O /mg) was also given. Nitrogen was supplied through urea and potassium was through potassium sulphate.

Since the population of phosphate solubilizing bacteria was found to be significantly different in rhizospheric soil of transgenic cabbage, it was of interest to see whether phosphorus uptake is different in the rhizospheric soil of

transgenic vs. its isogenic (normal) counterpart. Radioactive phosphorus is an active tool to test whether there is any difference in the uptake of phosphates in the two types of plants (Transgenic & normal cabbage).

To study the phosphorus derived from soil and fertilizer separately, aqueous solutions of diammonium phosphate was tagged with ^{32}P .

The amount of fertilizer solution needed for each pot as per treatment specifications was added to the soil and mixed thoroughly before the soil was filled in the pot.

3.10.4 Preparation of labelled fertilizer solution

3.10.4.1 Di-ammonium phosphate solution (DAP)

Labeled DAP was prepared by dissolving the required amount of DAP (Laboratory reagent) in minimum quantity of distilled water in a volumetric flask to which carrier free $\text{H}_3\text{P}^{32}\text{O}_4$ in dilute HCl, supplied by BARC, Trombay, was added to give a specific activity of $0.4 \text{ mci g}^{-1} \text{ P}_2\text{O}_5$ and mixed well and the volume was made up.

Table 2 : Physico-chemical properties of experimental soil

Soil texture		
% sand	:	71.84
% silt	:	11.99
% clay	:	16.16
Textural class	:	Sandy loam
pH (1:2.5) (Soil : Water)	:	8.4
CEC (C mol (p ⁺) kg ⁻¹)	:	13.06
Available nitrogen content	:	211.12
Available phosphorus content	:	17.92
Exchangeable potassium content	:	246.40
Organic carbon content	:	0.22

3.10.5 Sowing and after care

Good quality seeds of the two cultivars were selected for sowing in the nursery bed during the month of November 2002. When the seedling became 5 to 6 cm tall, these were transplanted in pots. Healthy plants were selected for transplanting. Sufficient amount of water was applied to maintain the soil moisture at the field capacity.

Sampling and processing of plant sample for analysis of cabbage samples were collected randomly from 18 pots in 3 replications, at 25, 50, 75 days after transplanting from both the varieties. The plant material was air dried in open air for 3 days and then oven dried at 70°C till constant weight was obtained. Dry weight of plants from each pot was recorded.

The plant samples were ground thoroughly in a willy millr and mixed well. A representative sample of 1g was taken for digestion. The plant samples were taken in conical flasks and soaked over night with 10 ml of diacid mixture containing nitric acid and perchloric acid in the ratio of 5 : 1 (by volume) and digested on the hot plate as per the method described by Piper (1967). The samples were made up to unknown volume and filtered through Whatman No. 1 filter paper to get rid of silica.

3.10.6 Estimation of phosphorus

The total phosphorus content of the aliquot was determined calorimetrically by vanado molybdate method in nitric acid (HNO₃) medium (Koenig and Johnson, 1942).

3.10.7 Estimation of radioactive phosphorus

The radio assay method followed was the same as described by Mackenzie and Dean (1948). Counting measurements were made in an end window Geiger counter (Window thickness of 2.5 mg per cm²). From the counts

of the sample, specific activity of the plant material was calculated.

3.10.8 Calculation of per cent Pdf, fertilizer phosphorus uptake and per cent utilization of applied fertilizer

Corrections for radioactive decay were noted and applied to calculate the specific activity of the sample using the formula given below :

$$\begin{aligned} \text{a. specific activity} &= \frac{P^{32}}{P^{31} + P^{32}} \\ &= \frac{\text{CPM of the sample- CPM of the background}}{\text{mg of P in the plant sample}} \end{aligned}$$

b. Per cent phosphorus in the plant derived from tagged fertilizer (% Pdf)

$$= \frac{\text{Specific activity of plant sample}}{\text{Specific activity of the tagged source}} \times 100$$

c. Fertilizer phosphorus uptake by plant (mg/plant)

$$= \frac{\% \text{ Pdf} \times \text{total P uptake (mg plant}^{-1})}{100}$$

d. Per cent utilization of applied fertilizer phosphorus

$$= \frac{\text{Fertilizer P uptake (mg plant}^{-1})}{\text{Fertilizer P applied to the soil (mg pot}^{-1})} \times 100$$

3.11 Microbiological analysis

On 25, 50 and 75 days after transplanting the cabbage, 10 g sample of the soil from each treatments of both the varieties was drawn, weighed, air dried and suspended in 90 ml of sterile water in 250 ml conical flasks. The flasks were shaken well and ten fold serial dilutions were made. One ml of sample from

suitable dilutions was plated with appropriate media. The plates were incubated at 30°C for 3 days in case of bacteria and fungi and for 7 days in case of actinomycetes.

3.11.1 Medium for bacteria

3.11.1.1 Nutrient agar (Subba Rao, 1977)

Brief extract	:	3.0 g
Peptone	:	5.0 g
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	6.6-7.0

3.11.1.2 Medium for phosphate solubilizing bacteria

Pikovskaya medium

Glucose	:	10.0 g
Tricalcium phosphate	:	5.0 g
(NH ₄) ₂ SO ₄	:	0.5 g
KCl	:	0.2 g
MgSO ₄ .7H ₂ O	:	0.1 g
MnSO ₄	:	Trace
FeSO ₄	:	Trace
Yeast extract	:	0.5 g
Agar	:	15.0 g
Distilled water	:	1000 ml

3.11.2. Medium for actinomycetes

Ken Knight's medium (Subba Rao, 1977)

Glucose	:	1.0 g
K ₂ HPO ₄	:	0.1 g
NaNO ₃	:	0.1 g
MgSO ₄ .7H ₂ O	:	0.1 g
KCl	:	0.1 g

Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	7.0

3.11.3 Medium for fungi

Martins Rose Bengal medium

KH ₂ PO ₄	:	1.0 g
MgSO ₄	:	0.5 g
Peptone	:	5.0 g
Dextrose	:	10.0 g
Rose Bengal (1%)	:	3.3 ml
Streptomycin sulphate	:	1 g per 100 ml
Agar	:	15.0 g
pH	:	5.0

The antibiotics was sterilized and added aseptically to sterilized medium.

3.11.4 Microbial population counting

On 25, 50 and 75 days after transplanting, 10 g soil sample from each treatment of both the varieties was drawn, weighed, air dried and suspended in 90 ml of sterilized water in 150 ml conical flask. Serial dilution of the starting suspension was done according to the dilution needed for the determination of bacterial, actinomycetes, fungi and phosphorus solubilizing bacterial (PSB) population. For bacterial 10^{-8} , for actinomycetes 10^{-5} , for fungi 10^{-4} and for PSB 10^{-5} dilution was needed for their population counting.

3.12 Determination of dehydrogenase activity

Dehydrogenase, which are respiratory enzymes and integral part of all soil organisms, provides a measure of biological activity of soil at a given time.

Biological oxidation of reduced compounds occur during respiration which is catalyzed by dehydrogenase. Under anaerobic condition compound like 2, 3, 4-triphenyl tetrazolium chloride (TTC) can act an electron acceptor. In the process TTC gets reduced to a pink coloured compound triphenyl formazan (TPF) which can be quantitatively extracted by methanol and measured colorimetrically. Higher the biological activity faster will be the formation of TPF. Dehydrogenase activity can be determined by putting 1 g air dried representative soil sample in an air tight screw capped test tube (15 ml capacity) 0.2 ml of 3% TTC solution in each of the tubes was added to saturated soil. 0.5 ml of 1% glucose solution in each tube was added. The bottom of tube was gently tapped to drive out all trapped oxygen so that a water seal is formed above the soil. The tubes were kept in an incubator at $28 \pm 0.5^\circ\text{C}$ for 24 hours. After incubation 10 ml of methanol was added. The tubes were shaken vigorously and was allowed to stand for 6 hours. Clear pink coloured supernatant was withdrawn and reading was taken with a spectrophotometer at a wave length of 485 nm (blue filter).

The amount of triphenyl formazon was calculated from the standard curve of triphenyl formazon. Results were expressed in μl of H_2 formed per gram of soil per 24 hrs because 1 μg of TPF is equivalent to 0.150 μl of H_2 .

3.13 Assessing nematode population in rhizospheric soil by Cobb Decanting and sieving method

Nematodes are filamentous. They can be separated from finer components through a series of sieving and decanting techniques. Their gregarious nature is utilized in separating them from the soil debris of equal size fractions. Soil samples were collected from the rhizosphere zone and the sample was kept moist without allowing the soil to dry. Hundred g of soil was kept in a pan and was soaked in one litre of water for 5 minutes. Soil crumbs was broken by hand. Big

stones or granules was separated by hand and suspension was thoroughly mixed. The pan was filled and allowed to stand for 1 sec. for settling of heavier particles. The muddy mixture was poured through 20 mesh sieve into another pan. To obtain optimum yield of nematodes, more water was added to the residue in pan I and residues on 20 mesh sieve was discarded and the content in pan II was mixed thoroughly. The suspension was poured in the same way through 60 mesh sieve to pan I. The process was repeated two to three times and the sieve was rinsed. The process of decanting and sieving with the 100, 200 and 325 sieves with the only additional precaution to keep the sieves at 45° angle from the ground while sieving. This prevented the filamentous nematodes to pass easily through sieve. The material coming through 325 mesh was discarded. This is supposed not to carry any nematode. The suspension on the 325 mesh sieve was collected in a beaker. The content in the beaker was allowed to settle for 2 hours. The suspension was poured on a tissue paper in the Bearmann funnel assembly and was left for 24 to 84 h to allow the nematodes to swim across the tissue paper in the water below leaving behind the other debris. The volume of contents was measured in the Petridish or collection vial. Five to ten per cent of the content was spread over a graduated dish for counting the nematodes under microscope of 40-60X magnification.

3.14 Germination vigour

Germination test was conducted Top of the Paper (TP) in 10 replicate of 25 seed each. In this method seeds were placed on top of two moist filter paper kept in a petri plate. Care was taken that the seeds were equidistant from each other. The lid of the Petri plate was placed and all of the samples were kept in 25°C at 95% RH germination chamber for 10 days. On the 10th day the germinated seeds were evaluated into normal, abnormal, hard, fresh-

ungerminated, and dead seeds. The percentage of germination was calculated as per ISTA rules (1993). Vigour index was computed adopting the method of Abdul-Baki and Anderson (1973) by using the following formula.

1. Vigour index I = (Root length in cm + shoot length in cm) x Germination %
2. Vigour index II = (Seedling dry weight in gm) x Germination %

3.15 Monitoring of aphid population

Aphid population in transgenic cabbage and normal cabbage was monitored during January to February 2001 under field condition and February to March 2002 under net house condition. The aphid population was counted for 6 weeks in both the cases.

3.16 Preparation of balance sheet

All the cost inputs incurred for the cultivation of transgenic and normal cabbage was recorded during these experiments. Yield data of cabbage were also recorded for the preparation of balance sheet of comparative cabbage cultivation.

Chapter - 4

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Polymerase chain reaction (PCR) analysis for npt II, cry 1B and cry 1Ab genes

In order to ensure that the seeds used are truly of transgenic crops, following tests were carried out. cry 1B and cry 1Ab genes are responsible for the translation of the Bt toxin (or cry 1B and cry 1Ab proteins) which induce mortality among DBM insects.

PCR analysis was carried out for the confirmation of the presence of npt II, cry 1B and cry 1Ab genes in *Brassica oleracea* var. *capitata*. Specific primers for npt II gene were designed to yield a 700 bp amplification products. Specific primers for cry 1B and cry 1Ab genes were designed to yield 850 bp and 1 kb amplification product, respectively. In order to confirm the presence of full length DNA in gene modified cabbage plant, all these primers mentioned above were used. The DNA isolated from the leaves of transgenic cabbage and non-transgenic cabbage were multiplied and amplified by using these three primers. From the results of PCR as shown in the fig. 2, no amplification was observed in the DNA isolated from the leaves of non transgenic plant, whereas six selected line of transgenic plant exhibited PCR positive result for npt II, cry 1B and cry 1Ab genes.

4.2 SDS-PAGE for Bt fusion protein in cabbage

Approximately 5 µg of the purified Bt-fusion protein (Cry 1B, Cry 1Ab fusion protein) from over expression *E. coli* cultures were taken. The soluble protein isolated from non transgenic and Bt-transgenic cabbage plants, were resolved by SDS-PAGE as shown in fig 3. The lane 3 clearly shows the molecular weight of the

Table 3. Quantification of Cry1B and Cry1Ab fusion protein in leaf tissue of Bt-Transgenic Cabbage.

Plants	Buffer extractable protein (mg)	Fusion protein content (%)	Amount of fusion Protein content (μg / g fresh leaf weight)
Wild type	15.277	-	0
$^{22}\text{T}_0$	7.961	0.13	10.35
$^{22.2}\text{T}_0$	15.00	0.10	15.00
$^{22.6}\text{T}_0$	9.916	0.12	11.90
$^3\text{T}_1$	11.80	0.15	17.70
$^4\text{T}_1$	18.618	0.11	20.48
$^{11}\text{T}_1$	18.15	0.10	18.15

Table 4. Toxicity effect of Cry1B-Cry1Ab fusion protein present in Bt-Transgenic Cabbage expressed on DBM larvae

Type of bioassay	Larval stage released	Number of larvae released	Infestation	Mortality (%)	Time(h) to cause mortality
Leaf disc	Neonate	10	Nil	100	24
Detached leaf	2nd	20	Nil	100	48
Whole plant	3rd	40	Nil	100	48

fusion protein, 135 kDa. Lanes 4-9 clearly shows the presence of fusion protein in individuals of transgenic plant that were PCR positive. Lane 2 containing protein from non transgenic plant demonstrated the absence of bands corresponding to 135 kDa using protein band. Lane 1 shows standard marker for determination of the molecular weight of Bt protein.

4.3 Expression of the Bt protein gene, cry 1B - cry 1Ab

The expression of the cry 1B cry 1Ab fusion gene was confirmed through Double Antibody Sandwiched Enzyme Linked Immunosorbant Assay (DAS-ELISA). DAS-ELISA was performed using immunoaffinity purified polyclonal rabbit antibodies specific for cry 1B and cry 1Ab protein to detect the levels of fusion protein expression in the six lines of transgenic plants. The fusion protein levels as measured by ELISA were converted to μg toxin per gram of fresh leaf. The levels of Cry 1B and Cry 1Ab protein in the leaf extracts were determined by plotting the ELISA data on a BSA standard curve.

The amount of Bt fusion protein in transgenic plant ranged from 0.072% of buffer extractable protein to 0.13% of buffer extractable protein. It was found in the buffer extract from young leaves of transgenic plants (Table 3). As expected, no Bt fusion protein. It was found in the buffer extracted protein from non-transgenic plant. Bt transgenic cabbage plant protein resolved by SDS-PAGE also supports these findings Kumar and Sharma (1995) analyzed 20 Brassica genes for codon usage preference in comparison with those of other plant genes and found that the avoidance of CG and TA doublets in Brassica genes is less than that of other plants.

4.4 Insect bioassay

Insect bioassay was performed to find the resistance of the transgenic plants against Diamond back moth (DBM) larvae due to expression of cry 1B cry 1Ab fusion gene (Table 4). Three insect bioassays were conducted with three replicates. Larvae of DBM were tested for their susceptibility to the fusion protein expressed by Bt-transgenic cabbage plants in each experiment. A control non transgenic cabbage leaves was also kept. In all the experiments, data were collected at 24 hr after releasing DBM larvae on Bt-transgenic cabbage and non-transgenic plants.

4.4.1 Leaf disc bioassay

Ten neonate larvae of DBM were released on the leaf discs (3-5 cm diameter) in culture plates. It was observed that all lines of transgenic plants were completely resistant to infestation by DBM larvae. Larval mortality was 100% on the leaves of all lines of transgenic plants after 24 hr of releasing.

In non transgenic plant the leaf discs were severely infested by DBM (Fig. 4a) which attained second instar stage on 4 day. The present study demonstrated that all six lines of transgenic cabbage plants caused 100% mortality to all larval stages of DBM. DBM larvae at neonate stage are considered to be the most vulnerable stages of the insect (Takkar and Shelton, 1933). The larval mortality observed due to its feeding nature. The neonate larvae mine in the spongy mesophyll tissue thereby consuming more fusion which protein actually caused their mortality. There was 100% mortality of DBM larvae in all the six experiments.

4.4.2 Detached leaf bioassay

Twenty larvae of DBM in 2nd instar stage were released in Petri plate containing petiolated leaf of transgenic cabbage plant and normal cabbage leaf as control. It was observed that all lines of transgenic leaf were completely resistance

to the infestation by DBM larvae. The larval mortality was 100% on the leaf of all lines of transgenic plant after 48 h of releasing whereas normal cabbage leaf was severely infested by DBM larvae (Fig 4b).

4.4.3 Whole plant bioassay

For whole plant bioassay forty 3rd instar larvae of DBM were released at a time on transgenic plants. All transgenic plants exhibited high resistance to the 3rd instar stages of DBM and caused 100% mortality. Complete mortality was observed within 48 hrs, whereas the non transgenic plant was severely infested due to DBM (Fig. 4c).

4.5 Standardization of analytical methodology

Two important pre-requisite in adoption of any analytical method for determination of concentration of any pesticides present in samples are:

- I. Development and/or standardization of the analytical technique and
- II. Evaluation of efficiency of extraction and clean up steps for a particular substrate.

4.5.1 Fipronil

Fipronil can be estimated by HPLC technique using methanol : water (75:25) mixture as moving phase. Therefore, HPLC technique using a Reverse phase (RP) C₁₈ (5 μ M) packed stainless steel column (250 mm $\times$ 4 mm id), UV detector and flow rate of methanol : water (75:25) mixture 0.8 ml/min. was examined for resolution of fipronil. Wave length of the detector was 242 nm. Retention time of fipronil was 10.05 min and retention time of two metabolites in photo degradation and oxidation were 9.12 min and 12.41 min, respectively (Fig 5a, 6a & 6b). The calibration graph of concentration versus peak areas for fipronil has

Table 5. Standardisation of Solvent System for Recovery of Fipronil from Cabbage Head

Solvent system (Hexane: Acetone)	Amount added ($\mu\text{g/g}$)	Amount recovered (μg)				Recovery (%)
		R ₁	R ₂	R ₃	Avg.	
1:1	0.4	0.179	0.191	0.179	0.183	45.750
	4.0	1.960	1.818	2.006	1.928	48.200
7:3	0.4	0.269	0.273	0.285	0.276	68.917
	4.0	2.709	2.731	2.852	2.764	69.100
9:1	0.4	0.181	0.179	0.184	0.181	45.333
	4.0	1.764	1.796	1.840	1.800	45.000

Table 6. Recovery of Fipronil from Cabbage Head

Absorbent used	Amount added ($\mu\text{g/g}$)	Amount recovered (μg)				Recovery (%)
		R ₁	R ₂	R ₃	Avg.	
Neutral alumina	0.4	0.246	0.271	0.227	0.248	62.0
	4.0	2.420	2.503	2.637	2.520	63.0
Basic alumina	0.4	0.310	0.331	0.283	0.308	77.0
	4.0	3.200	3.180	3.100	3.160	79.0
Basic alumina + Florisil	0.4	0.360	0.390	0.270	0.340	85.0
	4.0	3.321	3.169	3.846	3.445	86.1
Neutral alumina + Florisil	0.4	0.381	0.351	0.264	0.332	83.0
	4.0	3.422	3.281	3.412	3.372	84.3

been shown in figure 5b. The curve shows linearity over the range of 0.5 to 10 $\mu\text{g ml}^{-1}$ concentration of compound.

4.5.2 Thiacloprid

Thiacloprid can be estimated by HPLC technique using acetonitrile: water (40:60) mixture on moving phase. Therefore, HPLC technique using reverse phase (RP) C_{18} (5 μM) packed stainless steel column (250 mm $\times$ 4 mm id), UV detector and flow rate of acetonitrile: water (60:40) mixture 0.5 ml/min. was examined for resolution of thiacloprid. Wavelength of the detector was 242 nm retention time of thiacloprid was 6.8 min (Fig.7a). The calibration graph of concentration versus peak area for thiacloprid has been shown in figure 7b. The curve shows linearity over the range of 0.5 to 10 $\mu\text{g ml}^{-1}$ concentration of thiacloprid.

4.6 Recovery of fipronil from cabbage head

Recovery data of fipronil from spiked cabbage head are presented in table 5 and table 6. The insecticide was extracted with acetone from cabbage head and was partitioned into chloroform. It was then cleaned up separately using neutral alumina, basic alumina, Florisil.

For recovery experiment, the pesticide was spiked at two levels and different solvent combinations were tried using neutral alumina as the adsorbent initially. For clean up the solvent system hexane : acetone (7:3 v v^{-1}) in table 5, was found to be the best solvent system to afford highest recovery (over 69%) with neutral alumina as adsorbent. This may be due to less solubility of the compound (fipronil) in hexane. Hexane : acetone (1:1) is more polar as compared to 7:3 and 9:1, so additional unwanted co-extractives of the sample also eluted besides extraction of

Table 7. Persistence of Fipronil Residue on Transgenic Cabbage

Time (days)	Treatments (kg. a.i. ha)	Residue (μ g/g)				% Dissipation
		R ₁	R ₂	R ₃	Mean $\pm$ SD	
0	0.05	1.227	1.068	0.975	1.090 $\pm$ 0.127	0
	0.10	2.513	1.899	2.053	2.155 $\pm$ 0.319	0
1	0.05	1.059	0.929	0.922	0.970 $\pm$ 0.077	11.009
	0.01	2.349	1.943	1.873	2.055 $\pm$ 0.257	13.086
3	0.05	0.893	0.995	0.956	0.948 $\pm$ 0.051	13.028
	0.10	1.862	1.532	1.619	1.671 $\pm$ 0.171	24.872
7	0.05	0.598	0.538	0.460	0.532 $\pm$ 0.069	51.193
	0.10	1.032	1.072	1.240	1.115 $\pm$ 0.110	42.459
10	0.05	0.501	0.493	0.440	0.478 $\pm$ 0.033	56.147
	0.10	0.956	0.723	0.932	0.870 $\pm$ 0.128	56.752
15	0.05	0.372	0.350	0.232	0.318 $\pm$ 0.075	70.826
	0.10	0.832	0.431	0.592	0.618 $\pm$ 0.202	72.529

Table 8. Persistence of Fipronil Residue on Normal Cabbage

Time (days)	Treatments (kg. a.i. /ha)	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Mean $\pm$ SD	
0	0.05	0.946	1.024	0.943	0.971 $\pm$ 0.046	0
	0.1	1.89	2.194	1.928	2.004 $\pm$ 0.166	0
1	0.05	0.707	0.753	0.769	0.743 $\pm$ 0.032	23.481
	0.1	1.412	1.283	1.118	1.271 $\pm$ 0.147	36.577
3	0.05	0.724	0.648	0.665	0.679 $\pm$ 0.040	30.072
	0.1	1.213	0.923	1.308	1.148 $\pm$ 0.201	42.715
7	0.05	0.385	0.429	0.434	0.416 $\pm$ 0.027	57.158
	0.1	0.938	0.913	0.948	0.933 $\pm$ 0.018	53.443
10	0.05	0.356	0.341	0.371	0.356 $\pm$ 0.015	63.337
	0.1	0.759	0.831	0.732	0.776 $\pm$ 0.051	61.277
15	0.05	0.190	0.282	0.215	0.229 $\pm$ 0.048	76.416
	0.1	0.424	0.346	0.397	0.389 $\pm$ 0.040	80.589

Table 9. Photoproduct of Fipronil Content of Transgenic Cabbage

Time (days)	Single dose of Fipronil				Double dose of Fipronil			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.262	0.196	0.204	0.221 ± 0.036	0.308	0.246	0.301	0.285 ± 0.034
3	0.139	0.261	0.214	0.205 ± 0.062	0.298	0.243	0.267	0.269 ± 0.028
7	0.224	0.167	0.194	0.195 ± 0.029	0.316	0.273	0.268	0.286 ± 0.026
10	0.215	0.253	0.246	0.238 ± 0.020	0.196	0.316	0.279	0.264 ± 0.061
15	0.189	0.229	0.214	0.211 ± 0.020	0.249	0.308	0.319	0.292 ± 0.038

Table 10. Photoproduct of Fipronil Content of Normal Cabbage

Time (days)	Single dose of Fipronil				Double dose of Fipronil			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.182	0.216	0.149	0.182 ± 0.034	0.298	0.285	0.274	0.286 ± 0.012
3	0.241	0.196	0.162	0.200 ± 0.040	0.317	0.276	0.257	0.283 ± 0.031
7	0.196	0.187	0.169	0.184 ± 0.014	0.287	0.301	0.252	0.280 ± 0.025
10	0.195	0.179	0.159	0.178 ± 0.018	0.245	0.261	0.283	0.263 ± 0.019
15	0.186	0.201	0.128	0.172 ± 0.039	0.264	0.298	0.210	0.257 ± 0.044

the desired compound, which was evident from HPLC analysis. It showed several unwanted peaks, other than that of fipronil, in the chromatogram of samples eluted with more polar combination (1:1) of hexane : acetone confirmed it. Solvent system 9:1 was the least polar among these three solvents and it offered lowest percentage (45%) of recovery of fipronil. So for valid reasons, solvent combination of 7:3 (hexane : acetone) was chosen for this experiment. Among the individual adsorbents tried, (Table 6) basic alumina gave the highest recovery (over 86%) followed by neutral alumina (over 62% to 63%). The recovery experiment was repeated with these two adsorbents along with florisil, by layering method. With florisil, neutral alumina layering gave (83% to 84.292%) recovery. The use of basic alumina with florisil also yielded equivalent recovery over 85% to 86.133%, but greenish yellow color was observed in the cleaned samples. When basic alumina was not used for clean up either alone or along with Florisil, no such color was observed in cleaned samples (including control) when neutral alumina was use as adsorbent. This many be due to the elution of lesser amount of co-extractives from the column in the absence of basic alumina. So neutral alumina plus florisil combination was found to be the most suitable adsorbent mixture to remove the co extractives of cabbage sample. The limit of quantification has been found to be 0.05 µg/g.

4.7 Persistence of fipronil on transgenic and non-transgenic line of cabbage

Data on the persistence of fipronil residues on cabbage heads of transgenic and isogenic line, after application of the insecticide @ 50 g a.i. /ha (Normal dose) and 100 g a.i. /ha (Double dose), are presented in table 7 and table 8, respectively. Persistence of fipronil residues on transgenic and non transgenic cabbage, with time, are presented in fig. 8a, 8b and 9a, 9b, respectively. The residue data was analysed

Table 11. Sulfone Metabolite of Fipronil Content in Transgenic Cabbage

Time (days)	Single dose of Fipronil				Double dose of Fipronil			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.296	0.246	0.318	0.287 ± 0.037	0.219	0.401	0.315	0.312 ± 0.091
3	0.379	0.498	0.496	0.458 ± 0.068	0.416	0.506	0.462	0.461 ± 0.045
7	0.218	0.264	0.312	0.265 ± 0.047	0.318	0.214	0.312	0.281 ± 0.058
10	0.186	0.256	0.261	0.234 ± 0.042	0.342	0.249	0.298	0.296 ± 0.047
15	0.127	0.272	0.214	0.204 ± 0.073	0.244	0.216	0.316	0.259 ± 0.052

Table 12. Sulfone Metabolite of Fipronil Content in Normal Cabbage

Time (days)	Single dose of Fipronil				Double dose of Fipronil			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.256	0.296	0.218	0.257 ± 0.039	0.262	0.314	0.295	0.290 ± 0.026
3	0.309	0.364	0.296	0.323 ± 0.036	0.368	0.396	0.364	0.376 ± 0.017
7	0.237	0.267	0.241	0.248 ± 0.016	0.268	0.271	0.281	0.273 ± 0.007
10	0.265	0.186	0.242	0.231 ± 0.041	0.268	0.263	0.247	0.259 ± 0.011
15	0.126	0.198	0.157	0.160 ± 0.036	0.222	0.221	0.261	0.235 ± 0.023

by micro stat programme for calculation of half life of fipronil. The half life of fipronil residues was found to be 8.3 & 8.0 days for transgenic cabbage in single & double dose of fipronil application, respectively and 7.5 and 7.6 days for isogenic line of cabbage in single & double dose, respectively. This implies that the dissipation of insecticide did not vary significantly with the passage of time. Log of residue data when plotted against time (days) gave almost straight line, implying that dissipation of the insecticides on the cabbage followed first order kinetics.

During the determination of fipronil residue, metabolites of fipronil were also detected. The metabolites of photo product of fipronil (5-amino-1-(2,6-dichloro-4-trifluoromethylphenyl)-4-trifluoromethyl pyrazole-3-carbonitrile) was observed during 1st day sampling. The amount of these metabolites was 0.221 $\mu\text{g/g}$ for single dose application and 0.285 $\mu\text{g/g}$ for double dose application, in case of transgenic cabbage (Table 9). In case of normal cabbage the amount was 0.182 $\mu\text{g/g}$ and 0.286 $\mu\text{g/g}$ in single and double dose application respectively (Table 10).

When single dose of fipronil was applied to transgenic plant, highest amount (0.238 $\mu\text{g/g}$) of metabolite was detected in 10 days sampling though the amount was not significantly higher (Fig. 10a). In case of double dose of fipronil application to transgenic cabbage, higher amount (0.292 $\mu\text{g/g}$) photo product (desulfonyl fipronil) metabolite was detected in 15 days sampling (Fig 10b).

In case of normal cabbage or isogenic line of transgenic cabbage when single dose of fipronil was applied higher amount of photo metabolite (desulfonyl fipronil) was found in 3rd sampling then the amount gradually decreased (Table 10 and Fig. 11a). When the double dose of fipronil was applied, higher amount (0.283

µg/g) of photo metabolites of fipronil was detected in 3rd day sampling. Though the amount was not varying significantly from each other (Fig. 11b).

Another metabolites of fipronil is sulfone derivative (5-amino-1-(2,6-dichloro-4-trifluoromethyl phenyl)-4-trifluoro methyl sulfonyl pyrazole-3-carbonitrile). The sulfone metabolite in both transgenic and isogenic line of cabbage was detected in 1st day sampling. The amount of sulfone metabolite was higher in case of 3rd day sampling in case of transgenic and isogenic line of cabbage. This pattern of sulfone metabolite formation as found in single dose and double dose application of fipronil on both type of cabbage (Table 11 and 12). After 3rd days of sampling, the amount of sulfone metabolite was gradually decreased with the passage of time in both the cabbages. The formation of metabolite in the two lines of cabbage followed the same trend. Bobe *et al.* (1998) found that these two metabolites rapidly formed after application under field condition.

The initial concentration of residue on the transgenic and isogenic lines were 1.09 and 0.971 µg/g for normal dose respectively. This amount of fipronil residue dissipated to 0.318 and 0.229 µg/g respectively after 15 days of fipronil application. The rates of dissipation in 15 days, on transgenic and isogenic line were 70.83% and 76.42%, respectively. It has been found from the dissipation curve (Fig 12a & 12b) that it was not smooth some regions or depression were observed for both the lines of cabbage. This may be due to the formation of metabolite or their variable rate of dissipation during these periods.

4.8 Persistence of fipronil residues in soil

For recovery experiments the pesticides was spiked at two levels in soil. The fipronil was extracted from soil by using acetone : acetonitrile (3:1) mixture. The extract was then passed through silica gel column using (7 : 3), hexane : acetone

Table 13. Persistence of Fipronil in Transgenic Cabbage Rhizosphere

Time (days)	Treatments	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Mean $\pm$ SD	
0	Single Dose	1.231	1.956	0.953	1.380 $\pm$ 0.518	0
	Double Dose	1.970	3.014	2.052	2.345 $\pm$ 2.345	0
1	Single Dose	1.160	1.341	1.449	1.317 $\pm$ 0.146	4.589
	Double Dose	1.355	2.194	1.546	1.698 $\pm$ 1.698	27.576
3	Single Dose	0.934	0.621	0.846	0.800 $\pm$ 0.161	42.005
	Double Dose	1.06	1.364	1.237	1.220 $\pm$ 1.220	47.960
7	Single Dose	0.512	0.241	0.647	0.467 $\pm$ 0.207	66.184
	Double Dose	0.983	0.429	0.613	0.675 $\pm$ 0.675	71.215
10	Single Dose	0.394	0.216	0.613	0.408 $\pm$ 0.199	70.459
	Double Dose	0.946	0.854	0.513	0.771 $\pm$ 0.771	67.122
15	Single Dose	0.146	0.214	0.133	0.164 $\pm$ 0.044	88.092
	Double Dose	0.216	0.295	0.189	0.233 $\pm$ 0.233	90.050

Table 14. Persistence of Fipronil in Normal Cabbage Rhizosphere

Time (days)	Treatments	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Average $\pm$ SD	
0	Single Dose	1.601	1.031	1.082	1.238 $\pm$ 0.315	0
	Double Dose	2.103	1.623	2.414	2.047 $\pm$ 0.398	0
1	Single Dose	0.849	0.946	1.024	0.940 $\pm$ 0.088	24.098
	Double Dose	1.987	1.024	1.031	1.347 $\pm$ 0.554	34.180
3	Single Dose	0.816	0.513	0.864	0.731 $\pm$ 0.190	57.539
	Double Dose	1.523	0.916	0.964	1.134 $\pm$ 0.337	44.586
7	Single Dose	0.612	0.316	0.649	0.526 $\pm$ 0.183	40.953
	Double Dose	0.638	0.548	0.581	0.589 $\pm$ 0.046	71.226
10	Single Dose	0.598	0.308	0.219	0.375 $\pm$ 0.198	69.709
	Double Dose	0.487	0.561	0.491	0.513 $\pm$ 0.042	74.939
15	Single Dose	0.193	0.105	0.156	0.151 $\pm$ 0.044	87.776
	Double Dose	0.302	0.199	0.216	0.239 $\pm$ 0.055	88.324

Table15. Photoproduct of Fipronil Content in the Rhizosphere of Transgenic and Normal Cabbage

Time (days)	Transgenic Cabbage				Normal Cabbage			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.093	0.131	0.126	0.117 $\pm$ 0.021	0.086	0.120	0.012	0.073 $\pm$ 0.055
3	0.153	0.099	0.103	0.118 $\pm$ 0.030	0.127	0.134	0.124	0.128 $\pm$ 0.005
7	0.124	0.146	0.132	0.134 $\pm$ 0.011	0.127	0.138	0.096	0.120 $\pm$ 0.022
10	0.106	0.128	0.123	0.119 $\pm$ 0.012	0.113	0.143	0.121	0.126 $\pm$ 0.016
15	0.101	0.090	0.118	0.103 $\pm$ 0.014	0.101	0.093	0.098	0.097 $\pm$ 0.004

Table 16. Sulfone Metabolite of Fipronil Content in the Rhizosphere of Transgenic and Normal Cabbage

Time (days)	Transgenic Cabbage				Normal Cabbage			
	Concentration ($\mu\text{g/g}$)				Concentration ($\mu\text{g/g}$)			
	R ₁	R ₂	R ₃	Avg. $\pm$ SD	R ₁	R ₂	R ₃	Avg. $\pm$ SD
0	0	0	0	0	0	0	0	0
1	0.213	0.146	0.218	0.192 $\pm$ 0.036	0.254	0.164	0.098	0.172 $\pm$ 0.041
3	0.613	0.714	0.427	0.585 $\pm$ 0.144	0.207	0.625	0.349	0.394 $\pm$ 0.148
7	0.413	0.301	0.152	0.289 $\pm$ 0.083	0.109	0.156	0.134	0.133 $\pm$ 0.013
10	0.421	0.174	0.146	0.247 $\pm$ 0.052	0.148	0.098	0.124	0.123 $\pm$ 0.015
15	0.124	0.176	0.261	0.187 $\pm$ 0.046	0.142	0.114	0.085	0.114 $\pm$ 0.017

mixture as eluant. The fipronil concentration in the extract was determined by HPLC. The recovery data shows that 82% of applied fipronil concentration was recovered in the final solution. This method was applied for the determination of fipronil concentration in soil. The fipronil was not applied in soil separately. Recommended amount of fipronil for pest control on foliage was applied on transgenic and isogenic line of cabbage and during spraying whatever amount of fipronil fell on the soil during application was used to study the behaviour of the fipronil in soil. The data on the persistence of fipronil residues on rhizospheric soil of transgenic and isogenic line after application of the insecticide in single dose and double dose are presented in the table 13 and 14, respectively. The degradation of the insecticide in the rhizospheric soil of transgenic and isogenic line is presented in the figure 13a, 13b and 14a, 14b, respectively. The per cent dissipation of fipronil is presented in 15a and 15b, respectively. The half life of fipronil residues was found to be 5.0 and 5.3 days respectively in transgenic and isogenic line of cabbage for normal dose and 5.1 days for double dose in both the rhizosphere of transgenic and normal cabbage. It was found that half life of fipronil was the same for both the lines of cabbage and for both dosages of fipronil application. The initial concentration of fipronil in the rhizospheric soil of transgenic and normal cabbage in zero days sampling were 1.380 $\mu\text{g/g}$ and 1.238 $\mu\text{g/g}$ respectively. These amounts of fipronil dissipated or degraded in transgenic & non-transgenic cabbage of 15 days sampling to 0.164 $\mu\text{g/g}$ and 0.151 $\mu\text{g/g}$ in normal dose respectively. From the fig. 15a and fig. 15b of fipronil dissipation, it was found that the rate of dissipation was not smooth with time. The fipronil residue dissipated 88.00% and 87.78% up to 15 days of soil sampling in the transgenic and isogenic line of cabbage. Same depression zone in the dissipation curve of both cabbages was observed. It may be due to the formation of fipronil metabolite. Two major metabolites were found in

the soil where double dose of fipronil was applied on plant. One (Table 15, 16 & Fig. 16, 17) is the photo product and another is the sulfone metabolite of fipronil. The formation of photo metabolite did not vary significantly with time but the sulfone metabolites of fipronil was found to be formed in maximum amount on 3rd day of soil sampling. The amount of sulfone metabolite gradually declined with time. The same pattern of metabolite formation in soil was observed in the rhizospheric soil of both types of cabbage.

4.9 Recovery of thiacloprid from cabbage

According to literature, pesticide thiacloprid was extracted with acetone from cabbage head and was partitioned into dichloromethane. It was then cleaned up separately using Florisil as absorbent. The column was first eluted with ethyl acetate : cyclohexane (1:1) and then eluted with acetonitrile to collect the thiacloprid. For recovery experiment, the pesticide was spiked at two levels and analysed by HPLC. From the data of recovery it was found that 92% of treated thiacloprid was present in the final solvent for analysis. The limit of detection of the instrument was found to be 0.02 µg/g.

4.10 Persistence of thiacloprid on transgenic and isogenic line of cabbage

The data of the persistence of thiacloprid residues on cabbage heads of transgenic and isogenic line, after application of insecticide @ 72 g a.i./ha Normal dose) and 144 g a.i./ha (Double dose) are presented in the table 17 and table 18, respectively.

Degradation of thiacloprid residues on transgenic and non transgenic cabbage plants, with time are presented in fig. 18a, 18b and 19a, 19b, respectively for single and double dose application. The per cent dissipation pattern of

Table 17. Persistence of Thiacloprid in Transgenic Cabbage

Time (days)	Treatments (kg. a.i. /ha)	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Mean $\pm$ SD	
0	0.072	1.84	2.242	2.041	2.041 $\pm$ 0.201	0
	0.144	2.875	2.959	3.652	3.162 $\pm$ 0.426	0
1	0.072	1.822	1.684	1.819	1.775 $\pm$ 0.079	13.049
	0.144	3.239	3.016	2.598	2.951 $\pm$ 0.325	6.666
3	0.072	1.421	1.337	1.493	1.445 $\pm$ 0.078	29.180
	0.144	3.185	2.515	2.01	2.570 $\pm$ 0.589	18.710
7	0.072	1.066	1.269	1.271	1.202 $\pm$ 0.118	41.094
	0.144	2.332	2.346	2.195	2.291 $\pm$ 0.083	27.550
10	0.072	0.885	1.095	1.237	1.072 $\pm$ 0.176	47.500
	0.144	1.844	1.386	1.864	1.698 $\pm$ 0.270	46.292
15	0.072	0.785	0.835	0.876	0.832 $\pm$ 0.046	59.247
	0.144	1.605	1.089	1.641	1.445 $\pm$ 0.309	54.28717

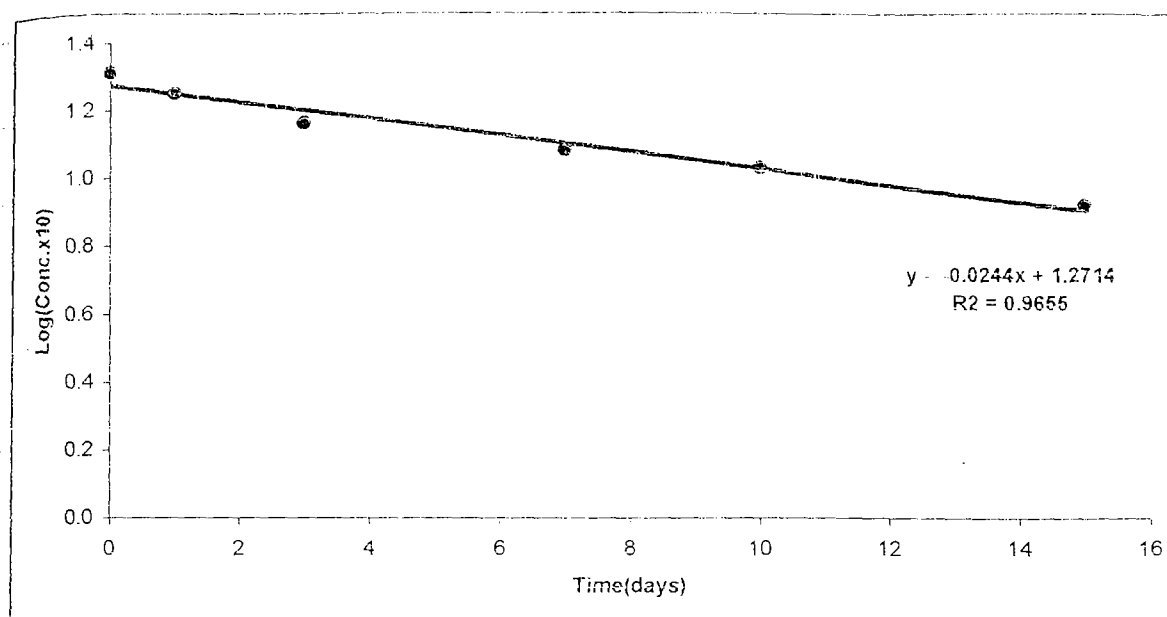


Figure 18a. Logarithmic Plot of Single Dose of Thiacloprid in Transgenic Cabbage

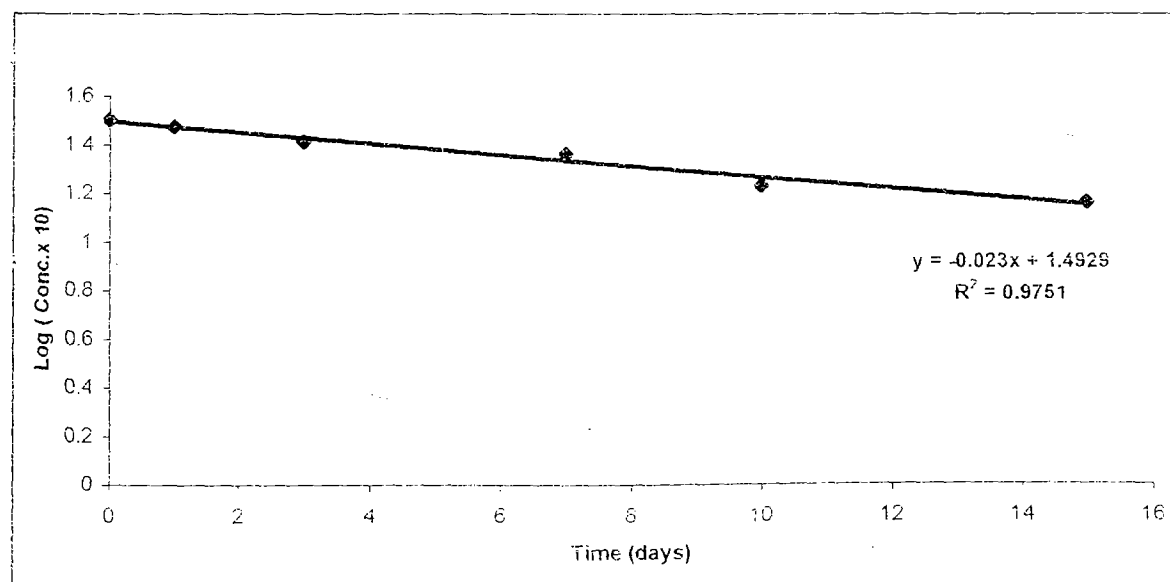


Figure 18b. Logarithmic Plot of DoubleDose of Thiacloprid in Transgenic Cabbage

Table 18. Persistence of Thiacloprid in Normal Cabbage

Time (days)	Treatments (kg. a.i. /ha)	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Mean $\pm$ SD	
0	0.072	2.287	2.0146	1.879	2.060 $\pm$ 0.208	0
	0.142	3.013	3.671	3.213	3.299 $\pm$ 0.337	0
1	0.072	1.7017	1.825	1.629	1.719 $\pm$ 0.099	16.574
	0.142	2.765	2.649	3.04	2.818 $\pm$ 0.201	14.569
3	0.072	1.615	1.598	1.61	1.608 $\pm$ 0.009	21.958
	0.142	2.967	2.438	2.671	2.692 $\pm$ 0.265	18.414
7	0.072	1.217	1.474	1.325	1.339 $\pm$ 0.129	35.016
	0.142	2.164	1.954	2.149	2.089 $\pm$ 0.117	36.669
10	0.072	1.062	0.947	0.985	0.998 $\pm$ 0.059	51.553
	0.142	1.848	1.537	1.829	1.738 $\pm$ 0.174	47.323
15	0.072	0.842	0.942	0.842	0.875 $\pm$ 0.058	57.508
	0.142	1.525	1.260	1.261	1.349 $\pm$ 0.152	59.110

thiacloprid is presented in the fig. 20a and fig. 20b respectively. The half life of thiacloprid residues was found to be 12.3 days and 12.4 days for transgenic and isogenic line of cabbage, respectively for normal dose of thiacloprid. It was found that the half life of thiacloprid was same for both lines of cabbage and for both doses of thiacloprid. This implies that the dissipation of insecticide did not vary significantly with the passage of time. Plotting log of residue with time gave almost a straight line. During the determination of thiacloprid residue no metabolite was found. The initial concentration of residues on transgenic and isogenic line were 2.041 and 2.060 $\mu\text{g/g}$, respectively for normal dose thiacloprid. These amount of thiacloprid residue dissipated to 0.832 and 0.875 $\mu\text{g/g}$ respectively after 15 days of thiacloprid application in normal dose. The rate of dissipation for transgenic and isogenic line were 59.25% and 57.51% respectively for normal dose. The dissipation curve of thiacloprid and data showed that the thiacloprid persisted for a longer time in cabbage plant in both lines of cabbage than fipronil.

4.11 Persistence of thiacloprid on normal cabbage under field condition

Thiacloprid was applied to cabbage crop under field condition @ 72 g a.i./ha (Normal dose) and @ 144 g a.i./ha (Double dose). The data of thiacloprid persistence with time under field condition are presented in the table 19 and in the fig. 21a and 21b The dissipation of thiacloprid with time under field condition are presented in the figure 22. The initial concentration of thiacloprid on cabbage were 0.869 $\mu\text{g/g}$ for normal dose and 1.816 $\mu\text{g/g}$ for double dose. These amounts of thiacloprid dissipated to 0.313 $\mu\text{g/g}$ and 0.678 $\mu\text{g/g}$ for normal and double dose respectively after 15 days of thiacloprid application. Half life of thiacloprid under field condition is 10.8 days for single dose. The dissipation curve and data showed

Table 19. Persistence of Thiacloprid on Normal Cabbage under Field condition

Time (days)	Treatments (kg. a.i. /ha)	Residue ($\mu\text{g/g}$)				% Dissipation
		R ₁	R ₂	R ₃	Average $\pm$ SD	
0	0.072	0.859	0.916	0.832	0.869 $\pm$ 0.043	0
	0.144	1.637	2.162	1.649	1.816 $\pm$ 0.300	0
1	0.072	0.665	0.825	0.586	0.692 $\pm$ 0.122	20.388
	0.144	1.732	1.626	1.561	1.640 $\pm$ 0.086	9.692
3	0.072	0.541	0.629	0.537	0.569 $\pm$ 0.052	34.539
	0.144	1.516	1.502	1.248	1.422 $\pm$ 0.151	21.678
7	0.072	0.350	0.412	0.464	0.426 $\pm$ 0.057	51.024
	0.144	1.195	1.294	1.084	1.191 $\pm$ 0.105	34.403
10	0.072	0.397	0.348	0.419	0.388 $\pm$ 0.036	55.334
	0.144	0.897	0.851	0.913	0.887 $\pm$ 0.032	51.148
15	0.072	0.273	0.305	0.361	0.313 $\pm$ 0.045	63.982
	0.144	0.801	0.592	0.641	0.678 $\pm$ 0.109	62.685

Table 20. Decontamination of Thiacloprid contaminated Cabbage by different Chemical Treatments

Treatments	Time (Min.)	Concentration of Thiacloprid ($\mu\text{g/g}$)			% Decontamination
		R ₁	R ₂	Average	
NaOH	30	2.392	1.726	2.059	61.828
	60	1.907	1.929	1.918	64.442
NaHCO ₃	30	3.486	3.084	3.285	39.099
	60	2.613	1.663	2.138	60.363
KMnO ₄	30	3.701	3.164	3.4325	36.364
	60	3.061	3.389	3.225	40.211
Water	30	5.159	4.135	4.647	13.849
	60	4.032	3.91	3.971	26.381

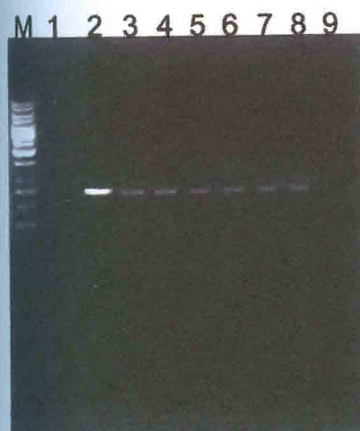
that the thiacloprid persisted for a long time in the cabbage even under field condition. Even 15 days after the application, 36.02% and 37.31% thiacloprid persisted on cabbage for single and double dose respectively. This may be due to the systemic nature of the compound, which entered the plant system and degraded slowly. This indicated that this compound is very effective for controlling the sucking pest like aphid on the plant for a long time.

4.12 Decontamination of thiacloprid in cabbage

Decontamination studies of thiacloprid with water, NaHCO_3 , KMnO_4 , NaOH was carried out at 30 minutes intervals up to 60 minutes. The recovery data of thiacloprid with different treatments is presented in table 20. It was envisaged to see the effect of the above treatments on the residues of thiacloprid. After following standardized extraction and clean up process, the treated samples were analyzed. The data of decontamination study showed that simple washing with water was least effective for decontaminating the thiacloprid residue. By use of water, only 13.784% of the initial concentration of the pesticide was decontaminated (Fig.23). When the pesticide contaminated cabbage was treated with 0.5% NaHCO_3 , and 0.5% KMnO_4 , only 39.02% and 40.2% of initial concentration respectively was decontaminated in 30 minutes of dipping in these solutions. When 0.5% NaOH treatment was given to thiacloprid treated cabbage for 30 minutes, 61.8% of initial concentration of pesticide was decontaminated that was nearly similar to the rate of decontamination (60.36%) by 0.5% NaHCO_3 treatment for one hour. Among these treatments the decontamination of pesticides, was found to be maximum by the treatment with 0.5% NaOH for 1 h which decontaminated 64.44% of initial concentration of pesticide. This implies that the pesticide can be detoxified by alkali.

4.13 Nutrient content

The nutrient content in the transgenic and non transgenic cabbage was measured. Total six lines of transgenic cabbage were taken. The nutrient content in



→ 700 bp

a. PCR amplification of *npt II* fragment

Lane M: 1 kb DNA ladder

Lane 1: Wild type cabbage plant negative for *npt II* gene

Lane 2: Amplification of *npt II* gene from pBinBt6

Lane 3-8: Transformants positive for *npt II* gene

Lane 9: Without template DNA



→ 850 bp

b. PCR amplification of *cry1B* fragment

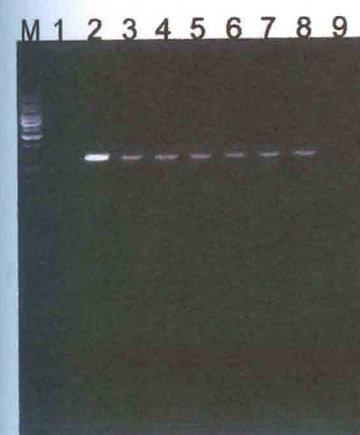
Lane M: 1 kb DNA ladder

Lane 1: Wild type cabbage plant negative for *cry1B* gene

Lane 2: Amplification of *cry1B* gene from pBinBt6

Lane 3-8: Transformants positive for *cry1B* gene

Lane 9: Without template DNA



→ 1 kb

c. PCR amplification of *cry1Ab* fragment

Lane M: 1 kb DNA ladder

Lane 1: Wild type cabbage plant negative for *cry1Ab* gene

Lane 2: Amplification of *cry1Ab* gene from pBinBt6

Lane 3-8: Transformants positive for *cry1Ab* gene

Lane 9: Without template DNA

Fig 2. PCR analysis of Bt-transgenic cabbage plant.

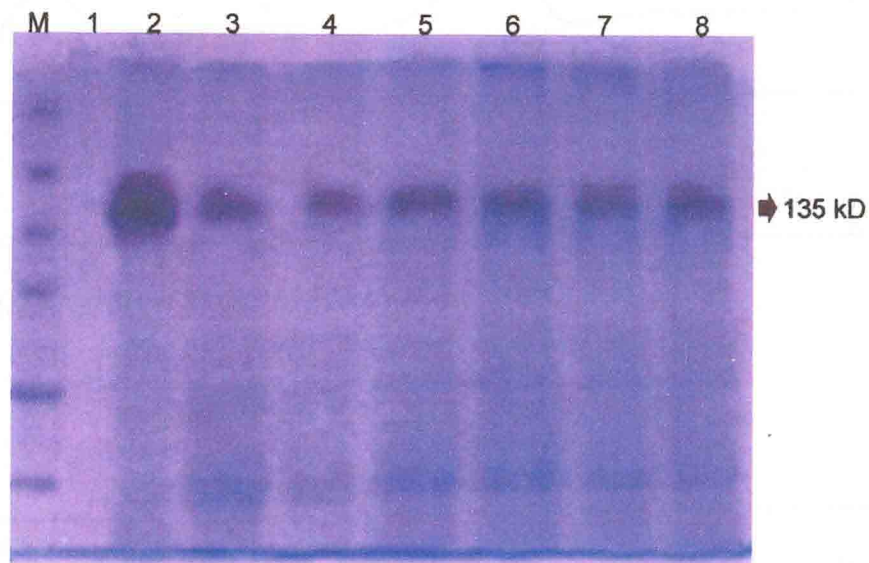


Fig. 3. Resolving Bt fusion protein expressed in Bt-transgenic cabbage plants by 10% SDS-PAGE.

Lane M: Protein standard

Lane 1: Buffer extractable protein from wild type cabbage plant

Lane 2: Purified Cry1B-Cry1Ab fusion protein

Lane 3-8: Buffer extractable protein from Bt-transgenic cabbage plants expressing the fusion protein

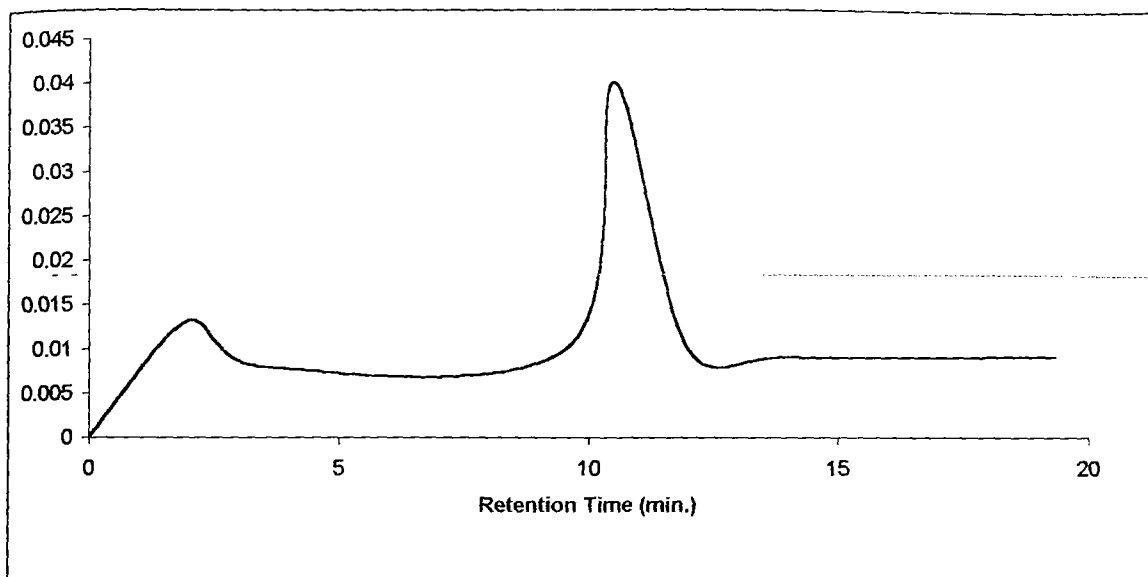


Figure 5a. Retention Time of Fipronil in HPLC

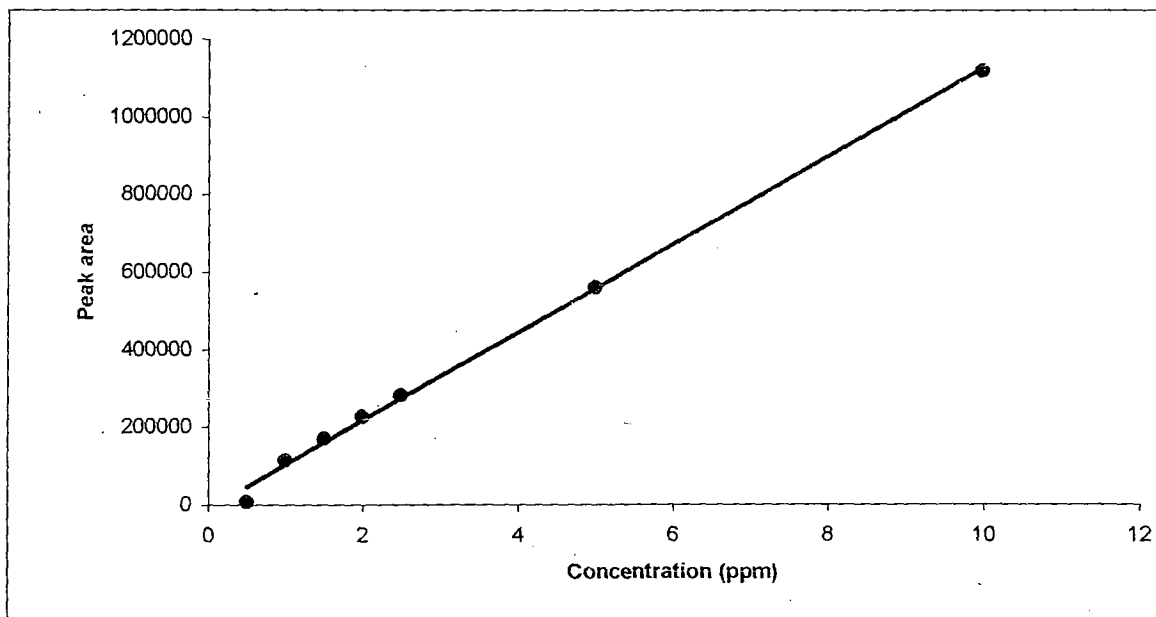


Figure 5b. Standard Curve Of Fipronil

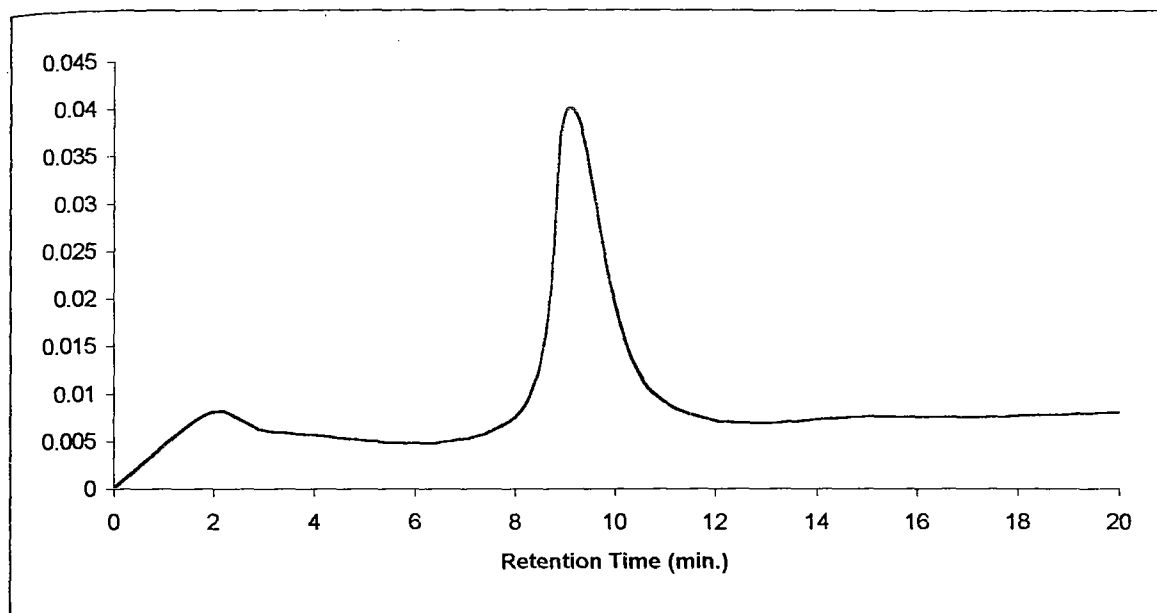


Figure 6a. Retention Time of Photo Metabolite of Fipronil

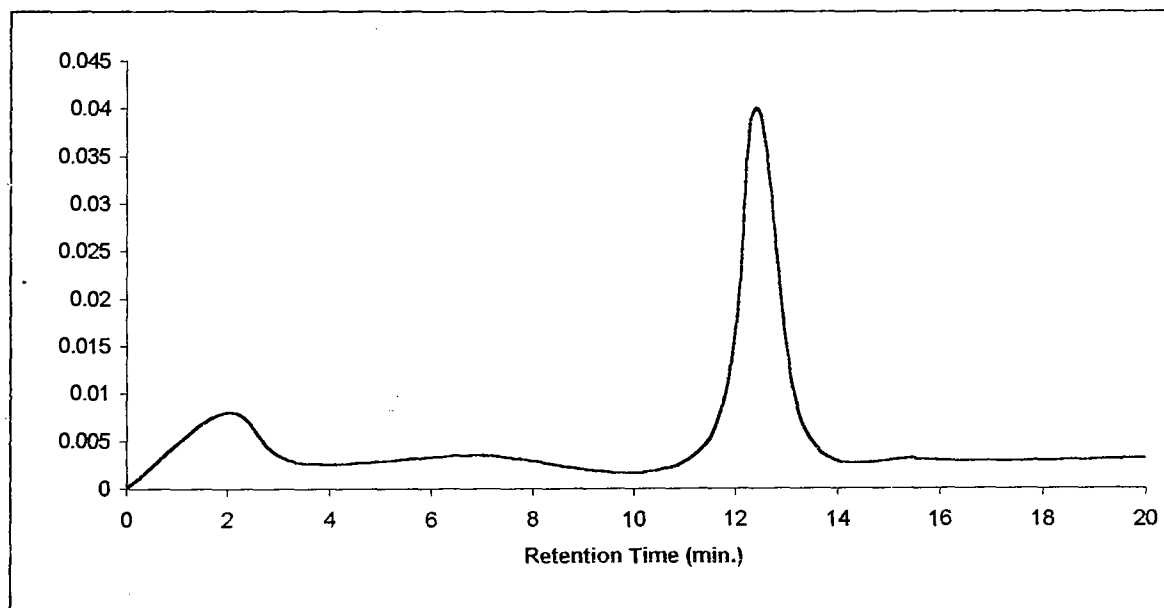


Figure 6b. Retention Time of Sulfone Metabolite of Fipronil

Table 21. Primary Nutrient Content in Different Lines of Transgenic Cabbage

Lines	Protein Content (mg/g)				Phosphorus Content (mg/g)				Potassium Content (mg/g)			
	R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average
CT ₀	10.850	10.020	12.290	11.053	2.898	3.244	3.002	3.048	14.770	1.090	13.440	9.767
²² T ₀	9.850	10.020	12.290	10.720	2.761	2.105	2.673	2.513	13.610	1.610	13.430	9.550
²² ² T ₀	10.500	10.800	12.010	11.103	2.623	2.899	3.037	2.853	11.940	15.760	12.560	13.420
²² ⁶ T ₀	11.090	11.290	11.160	11.180	2.112	2.139	2.725	2.325	17.590	14.270	12.440	14.767
CT ₁	9.760	11.830	10.780	10.790	2.898	3.485	3.175	3.186	14.600	15.930	15.430	15.320
³ T ₁	10.660	11.210	10.590	10.820	3.209	2.415	3.244	2.956	11.780	17.590	15.050	14.807
⁴ T ₁	12.040	11.630	11.880	11.850	3.002	2.346	2.835	2.728	14.270	17.420	14.920	15.537
¹¹ T ₁	10.850	11.920	11.100	11.290	3.148	3.313	2.623	3.028	12.780	14.430	16.920	14.710

Table 22. Secondary Nutrient Content in Different Lines of Transgenic Cabbage

Lines	Calcium Content (mg/g)				Magnesium Content (mg/g)				Sulphur Content (mg/g)			
	R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average
CT ₀	6.023	5.713	5.227	5.654	2.011	1.895	2.287	2.064	8.2430	8.890	6.570	7.901
²² T ₀	5.293	5.607	5.060	5.320	1.923	2.144	2.189	2.085	8.0600	7.840	7.860	7.920
^{22,2} T ₀	5.019	5.340	5.503	5.287	1.735	1.918	1.985	1.879	5.4400	7.236	5.576	6.084
^{22,6} T ₀	5.723	5.490	5.286	5.500	2.056	1.957	2.013	2.009	7.4000	9.150	8.017	8.189
CT ₁	6.219	6.043	5.523	5.928	2.149	1.926	1.997	2.024	6.5960	5.707	6.830	6.378
³ T ₁	6.316	6.316	6.029	6.220	2.215	2.431	2.012	2.219	5.4530	5.183	7.200	5.945
⁴ T ₁	6.223	6.890	6.015	6.376	2.133	2.315	2.187	2.212	6.7900	5.293	5.483	5.855
¹¹ T ₁	6.516	5.810	6.440	6.255	2.345	2.119	1.997	2.154	4.6630	4.937	3.860	4.487

the six lines was compared with the non transgenic cabbage plant. Calcium, magnesium, potassium, sulfur, phosphorus were estimated along with the total protein content in the plant. It was found that average calcium content in the transgenic line was 6.015 mg/g (Table 22) whereas the average calcium content in the non transgenic plant was 5.791 mg/g. The results of the calcium content in transgenic and non transgenic cabbage were statistically analysed and it was found that the calcium content in transgenic cabbage was significantly higher in comparison to non transgenic plant. Another important nutrient element magnesium was also analysed. The magnesium content in transgenic plant ranges from 1.879 mg/g to 2.219 mg/g where as the magnesium content in non transgenic plant on an average was 2.014 mg/gm of dry plant sample. It was also found that in two lines of transgenic (T_0 and T_1), differ significantly based on their nutrient content also. T_1 line of transgenic plant contained more magnesium as compared to the T_0 line of transgenic line. These data were statistically analysed and the difference in the nutrient content was also found to be statistically significant from each other. *Brassica* crop is very susceptible to the sulfur nutrient content, so sulfur nutrient was also chemically analysed. The sulfur nutrient content was comparatively higher in T_0 line of transgenic plant than T_1 line of transgenic cabbage. On an average the sulfur content in non transgenic plant was 7.139 mg/g but the average sulfur content in transgenic cabbage was 6.653 mg/g of dry plant sample. This difference was found to be statistically significant. Phosphorus content, potassium content and protein content were also estimated (Table 21). The phosphorus content in transgenic crop plant varied from 2.325 mg/g to 3.028 mg/g of dry plant sample. The phosphorus content in non transgenic plant was 3.117 mg/g of dry sample. Though the phosphorus content in transgenic plant varied but the difference in phosphorus content was not statistically significant from each other. The phosphorus content of transgenic cabbage was also not statistically different from that of non transgenic cabbage plant. The potassium content of transgenic plant

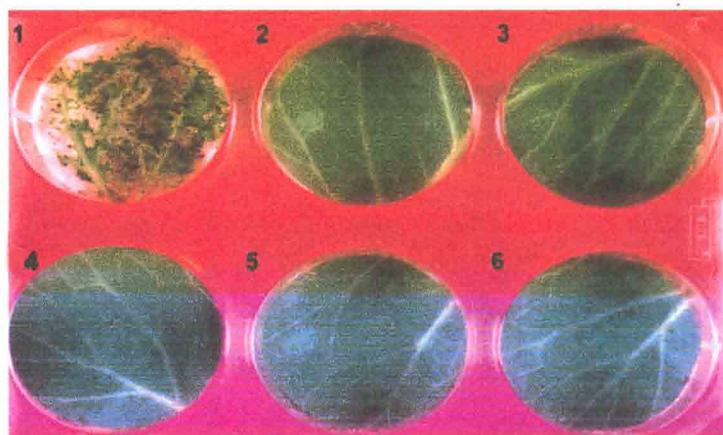
varied from 9.550 mg/g to 15.537 mg/g whereas the potassium content in non transgenic cabbage was 12.543 mg/g on an average. The potassium content in the transgenic and non transgenic cabbage was not statistically significant. The potassium content in the two lines of cabbage was not different statistically. The protein content in the transgenic line varied from 10.72 mg/g to 11.85 mg/g. The average protein content in the non transgenic plant was 10.92 mg/g of fresh plant sample. The protein content more or less equal, there is no statistically significant difference between them. So among the nutrient content, only Ca, Mg, S content in transgenic plant is statistically different in between the line and variety.

4.14 Dehydrogenase activity

Dehydrogenase activity in the rhizosphere soil of transgenic and non transgenic soil was determined by triphenyl tetrazolium chloride (TCC) method. The soil was treated with radio labelled phosphorus at the rate of 40 kg, P_2O_5 and 80 kg P_2O_5 per ha. The soil was collected at 25, 50 and 75 days after transplanting (DAT). It was found that dehydrogenase activity causes hydrogen evolution. The hydrogen evolution varied with the treatment. Over all amount hydrogen evolved or dehydrogenase activity was found to be higher in case of transgenic plant when it was compared with non transgenic plant at control and 40 kg P_2O_5 ha^{-1} level, but dehydrogenase activity was found to be higher in case of non transgenic plant where 80 kg P_2O_5 ha^{-1} was applied per pot (Table 23). The amount of dehydrogenase activities were analysed statistically observed in various pots. It was found that it was statistically insignificant in case of 25 DAT. When dehydrogenase activity was measured for 50 DAT rhizospheric soil, the initial activity is less in case of transgenic plant as compared to non-transgenic plant. In other two case of activity the dehydrogenase activity was higher in comparison to non transgenic rhizospheric soil. But the variation of dehydrogenase activity was not statistically significant.

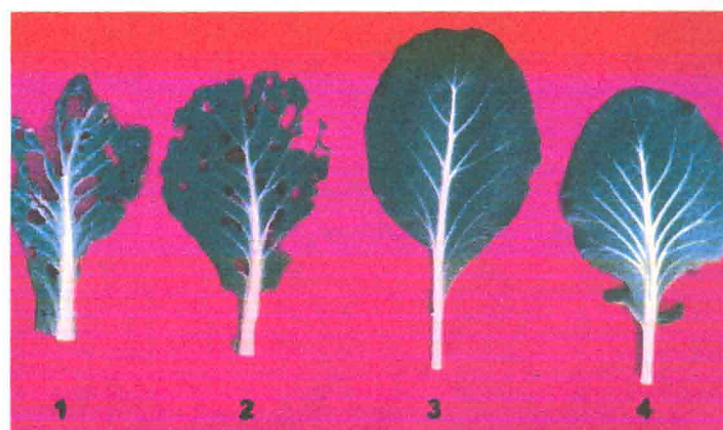
4a. Leaf disc bioassay.

1: Wild type cabbage plant.
2-6: Bt-Transgenic cabbage plant.



4b. Detached leaf bioassay.

1&2: Wild type plant.
3&4: Bt-transgenic cabbage plant.



4c. Whole plant bioassay.

1: Wild type cabbage plant.
2: Bt-transgenic cabbage plant.



Figure 4 : Insect bioassay of Bt-transgenic cabbage plants by using DBM larvae.

Table 23. Dehydrogenase Activity in the Rhizosphere of Transgenic and Normal Cabbage

25 Days After Transplanting (DAT)				
Transgenic Cabbage	$\mu\text{l of H}_2 \text{ evolved h}^{-1} \text{g}^{-1} \text{ of soil}$			
Treatments	R ₁	R ₂	R ₃	Average
Control	0.028	0.025	0.026	0.026
40 KgP ₂ O ₅	0.028	0.025	0.029	0.027
80 KgP ₂ O ₅	0.026	0.022	0.026	0.024
Normal Cabbage				
Treatments				
Control	0.015	0.027	0.024	0.022
40 KgP ₂ O ₅	0.025	0.029	0.026	0.027
80 KgP ₂ O ₅	0.030	0.018	0.028	0.025

50 Days After Transplanting (DAT)				
Transgenic Cabbage	$\mu\text{l of H}_2 \text{ evolved h}^{-1} \text{g}^{-1} \text{ of soil}$			
Treatments	R ₁	R ₂	R ₃	Average
Control	0.026	0.028	0.026	0.027
40 KgP ₂ O ₅	0.044	0.028	0.032	0.035
80 KgP ₂ O ₅	0.030	0.029	0.023	0.027
Normal Cabbage				
Treatments				
Control	0.035	0.026	0.026	0.029
40 KgP ₂ O ₅	0.031	0.028	0.026	0.028
80 KgP ₂ O ₅	0.024	0.027	0.027	0.026

75 Days After Transplanting (DAT)				
Transgenic Cabbage	$\mu\text{l of H}_2 \text{ evolved h}^{-1} \text{g}^{-1} \text{ of soil}$			
Treatments	R ₁	R ₂	R ₃	Average
Control	0.029	0.028	0.028	0.028
40 KgP ₂ O ₅	0.048	0.045	0.044	0.045
80 KgP ₂ O ₅	0.032	0.035	0.048	0.035
Normal Cabbage				
Treatments				
Control	0.041	0.028	0.032	0.034
40 KgP ₂ O ₅	0.028	0.038	0.044	0.037
80 KgP ₂ O ₅	0.046	0.029	0.034	0.036

when the dehydrogenase activity was measured in the soil samples of 75 DAT. It was found that dehydrogenase activity was less in case of control and 80 kg P_2O_5 treated pot in comparison to non transgenic rhizospheric soil. But when the pot was treated with 40 kg P_2O_5 treated, the activity of dehydrogenase became higher in case of transgenic cabbage plant. When all the data were analysed statistically it was found that the dehydrogenase activity did not vary significantly when it was compared between these two types cabbage. So it can be concluded that the dehydrogenase activity did not vary significantly in the radioactive phosphorus treated rhizospheric soil of transgenic and non transgenic cabbage.

4.15 Phosphorus uptake

Phosphorus uptake by transgenic and non transgenic cabbage was carried out. The experimental soil was treated at the rate of 40 kg and 80 kg P_2O_5 ha^{-1} by using radio labelled phosphate fertilizer. The sample was taken at 25, 50 and 70 days after transplanting (DAT) to study, whether there is any difference between these two types of cabbage plant. Dry matter yield (Table 24) at 25 DAT of the transgenic plant and non transgenic plant were significantly different from each other and they were no significant difference based on phosphorus content in these two types of cabbage. It was also found that the response due to fertilizer treatment was also statistically significant. The phosphorus uptake pattern was same for the transgenic and isogenic cabbage with the increase in phosphorus dose. The dry matter yield of transgenic plant was higher than the dry matter yield of non transgenic plant. Due to this fact, the total phosphorus uptake was also high. At 40 kg P_2O_5 ha^{-1} the total phosphorus uptake by transgenic cabbage was 1.874 mg plant⁻¹ whereas 1.366 mg plant⁻¹ by non transgenic plant. The same case was also found at 80 kg P_2O_5 ha^{-1} treated pot, where the total phosphorus uptake was 2.457 mg

Table 24. Phosphorus Utilization Pattern by 25 days Old Transgenic and Normal Cabbage

Transgenic Cabbage		Dry matter yield (g/Plant)		Average (g/plant)		Phosphorus Content (mg/plant)			Average (mg/plant)		Total Phosphorus uptake (mg/plant)			Average (mg/plant)	
Treatments		R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average		R ₁	R ₂	R ₃	Average	
Control		0.222	0.201	0.252	0.225	3.176	5.089	4.488	4.251		0.705	1.023	1.131	0.953	
40 Kg P ₂ O ₅		0.331	0.298	0.357	0.329	5.519	6.157	5.487	5.721		1.827	1.835	1.959	1.874	
80 Kg P ₂ O ₅		0.381	0.397	0.453	0.410	6.552	5.861	5.623	6.012		2.496	2.327	2.547	2.457	
Normal cabbage															
Treatments															
Control		0.201	0.186	0.192	0.193	4.625	4.015	3.123	3.921		0.929	0.747	0.599	0.758	
40 Kg P ₂ O ₅		0.279	0.251	0.225	0.252	5.852	4.812	5.59	5.418		1.633	1.208	1.258	1.366	
80 Kg P ₂ O ₅		0.277	0.308	0.246	0.277	5.959	6.145	5.692	5.932		1.651	1.893	1.4	1.648	

Transgenic Cabbage		% Phosphorus derived from fertilizer			Average (%)		Fertilizer Phosphorus uptake (mg/plant)			Average (mg/plant)		% Phosphorus utilization			Average (%)	
Treatments		R ₁	R ₂	R ₃	Average		R ₁	R ₂	R ₃	Average		R ₁	R ₂	R ₃	Average	
Control		0	0	0	0		0	0	0	0		0	0	0	0	
40 Kg P ₂ O ₅		47.85	44.91	51.78	48.18		0.874	0.824	1.014	0.904		1.952	1.961	2.093	2.002	
80 Kg P ₂ O ₅		52.995	63.128	52.897	56.34		1.323	1.469	1.347	1.380		1.333	1.243	1.361	1.312	
Normal cabbage																
Treatments																
Control		0	0	0	0		0	0	0	0		0	0	0	0	
40 Kg P ₂ O ₅		37.513	45.164	43.803	42.160		0.613	0.546	0.551	0.57		1.745	1.291	1.344	1.460	
80 Kg P ₂ O ₅		52.18	42.482	48.889	47.850		0.861	0.804	0.684	0.783		0.882	1.011	0.748	0.880	

plant⁻¹ and 1.648 mg plant⁻¹ by transgenic and non transgenic cabbage, respectively. At 25 days sampling % phosphorus derived from fertilizer by transgenic and non transgenic cabbage though appeared to vary but it was not statistically significant. Fertilizer phosphorus uptake by transgenic and non-transgenic cabbage was statistically significant. Fertilizer phosphorus uptake by transgenic and non transgenic cabbage was 0.904 mg plant⁻¹ and 0.570 mg plant⁻¹, when the soil was treated at the rate of 40 kg P₂O₅ ha⁻¹. The fertilizer phosphorus uptake were 1.014 mg plant⁻¹ and 0.551 mg plant⁻¹ by transgenic and non transgenic cabbage crop respectively, when the pot soil was treated at the rate of 80 kg P₂O₅ ha⁻¹, it was found that % of phosphorus utilization pattern was comparatively higher in 25 days sample. It was 2.002% and 1.460% by transgenic and non transgenic plant respectively in pot treated @ 40 kg P₂O₅ ha⁻¹. But it was also found that at higher dose of phosphate application, the % phosphorus utilization pattern was reduced in both the cases namely transgenic and non transgenic plant, implying that higher application of P is not useful and is rather waste. The results are suggestive in nature and is needed to be validated by field trials.

With the increase in the number of days, the dry matter of the plant increased for both types of plants. For control pot, the dry matter of the cabbage plant became 3.212 g which was earlier 0.225 g in case of transgenic plant (Table 25). In case of non transgenic plant of control pot, the dry matter yield was 1.906 g plant⁻¹ in 50 DAT. The phosphorus content was more or less equal as it was observed in the earlier case, although there was a slight decline. Statistically the dry matter content in these two variety was not similar but there was no significant difference in their phosphorus content. Due to the higher dry matter yield the total phosphorus uptake by transgenic plant was also higher than that of non transgenic

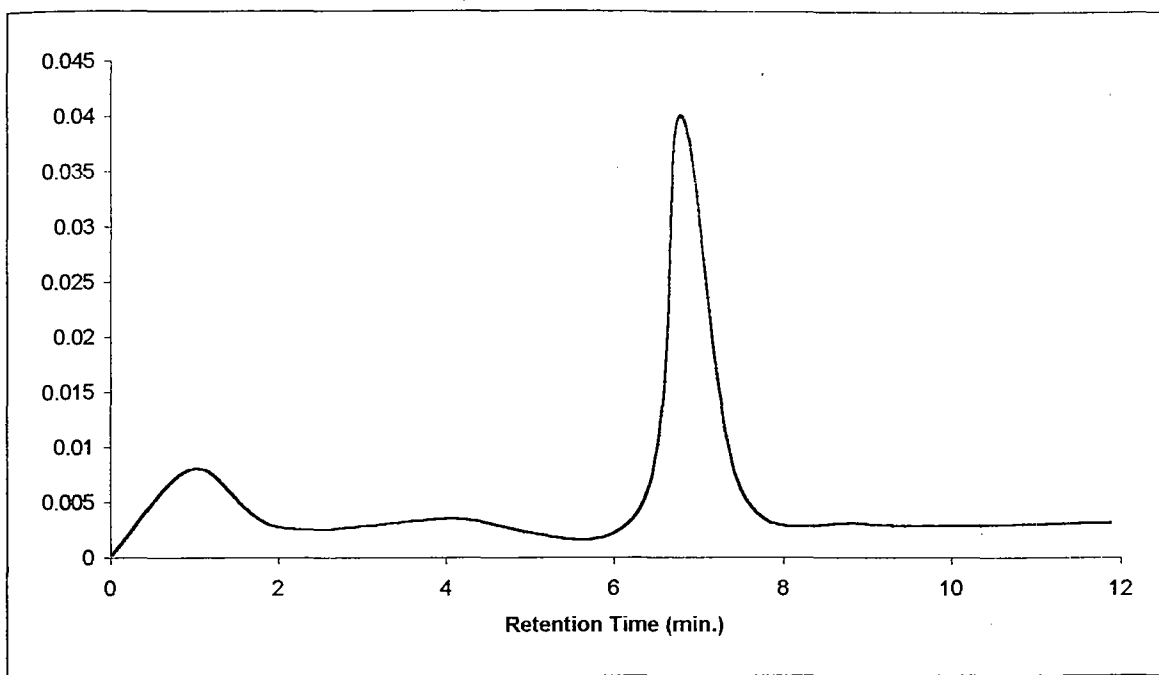


Figure 7a. Retention Time of Thiachloprid in HPLC

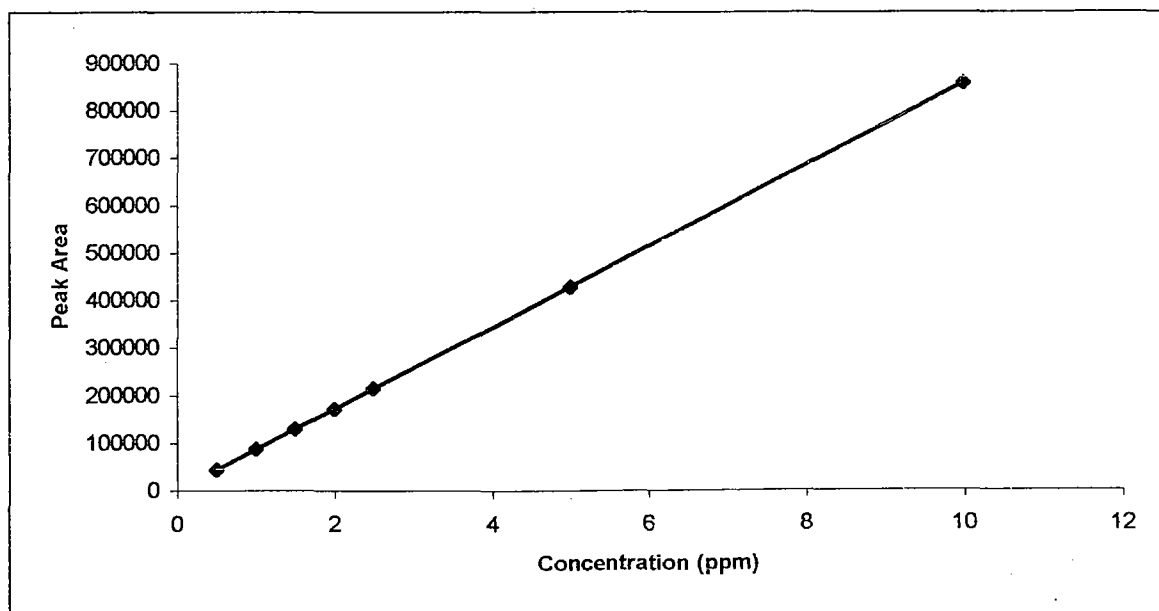


Figure 7b. Standard Curve of Thiachloprid

Table 25. Phosphorus Utilization Pattern by 50 days Old Transgenic and Normal Cabbage

Transgenic Cabbage	Dry matter yield (g/Plant)			Average (g/plant)		Phosphorus Content (mg/plant)			Average (mg/plant)		Total Phosphorus uptake (mg/plant)			Average (mg/plant)
	R ₁	R ₂	R ₃	R ₁	R ₃	R ₁	R ₂	R ₃	R ₁	R ₃	R ₁	R ₂	R ₃	
Control	2.891	3.752	2.993	3.212	3.796	4.865	3.185	3.796	3.949	11.361	14.065	11.95	11.361	12.459
40 Kg P ₂ O ₅	6.638	5.251	5.642	5.844	5.025	5.475	5.195	5.025	5.232	28.351	36.343	27.279	28.351	30.658
80 Kg P ₂ O ₅	5.271	6.387	6.849	6.169	5.972	5.525	5.645	5.972	5.714	40.902	29.122	36.055	40.902	35.360
Normal cabbage														
Treatments														
Control	1.496	1.927	2.296	1.906	3.125	3.869	4.264	3.125	3.753	7.175	5.788	8.271	7.175	7.078
40 Kg P ₂ O ₅	3.921	2.574	3.537	3.344	4.815	5.264	5.005	4.815	5.028	17.031	20.64	12.883	17.031	16.851
80 Kg P ₂ O ₅	5.81	4.059	4.678	4.849	5.892	5.411	5.205	5.892	5.503	27.563	31.438	21.127	27.563	26.709

Transgenic Cabbage	% Phosphorus derived from fertilizer			Average (%)		Fertilizer Phosphorus uptake (mg/plant)			Average (mg/plant)		% Phosphorus utilization			Average (%)
	R ₁	R ₂	R ₃	R ₁	R ₃	R ₁	R ₂	R ₃	R ₁	R ₃	R ₁	R ₂	R ₃	
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40 Kg P ₂ O ₅	37.254	41.105	44.921	41.093	5.103	5.239	4.912	5.103	5.085	30.293	38.832	29.148	30.293	32.758
80 Kg P ₂ O ₅	39.62	47.867	44.031	43.839	18.009	14.399	17.258	18.009	16.555	21.852	15.558	19.262	21.852	18.891
Normal Cabbage														
Treatments														
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40Kg P ₂ O ₅	36.721	30.773	39.197	35.564	2.812	2.125	2.529	2.812	2.489	18.198	22.054	13.766	18.198	18.006
80 Kg P ₂ O ₅	37.921	40.184	35.188	37.764	9.699	11.92	8.489	9.699	10.036	14.726	16.796	11.287	14.726	14.270

plant and it was statistically significant in 50 DAT. Phosphorus derived from fertilizer was higher by transgenic plant as compared to the non transgenic plant. At 50 DAT, the phosphorus derived from fertilizer was statistically significant when it was compared with the non transgenic plant. It indicates that there was statistically significant difference due to variety but not due to treatment. Fertilizer phosphorus uptake by both the type of plant was also statistically significant and fertilizer phosphorus uptake was higher in case of transgenic plant when compared to the non-transgenic plant. It was also found that there was significant difference due to P treatment and variety both at this stage. Per cent (%) phosphorus utilization pattern was also statistically significant due to transgenic and non-transgenic factor (Table 25). In every case higher value was obtained in case of transgenic plant. But one important thing was found that with the increase of the fertilizer dose the utilization pattern decreased gradually. At the final stage of observation, the dry matter gradually increased and the dry matter produced by transgenic plants was higher when compared to the non transgenic plant and it was statistically significant and the dry matter increase due to treatment was also statistically significant (Table 26). The phosphorus content at 75 DAT was statistically significant due to the treatment but the difference due to variety was not statistically significant. The total phosphorus uptake by both the plant was statistically significant due to treatment and variety both. Phosphorus derived from fertilizer by transgenic cabbage was significantly different from the non transgenic cabbage. The difference in phosphorus derived from fertilizer by both the cabbage was not statistically different due to variety and treatment, but the fertilizer phosphorus uptake and % phosphorus utilization was statistically different due to both variety and treatment. The fertilizer phosphorus uptake and % phosphorus utilization was higher in case of transgenic

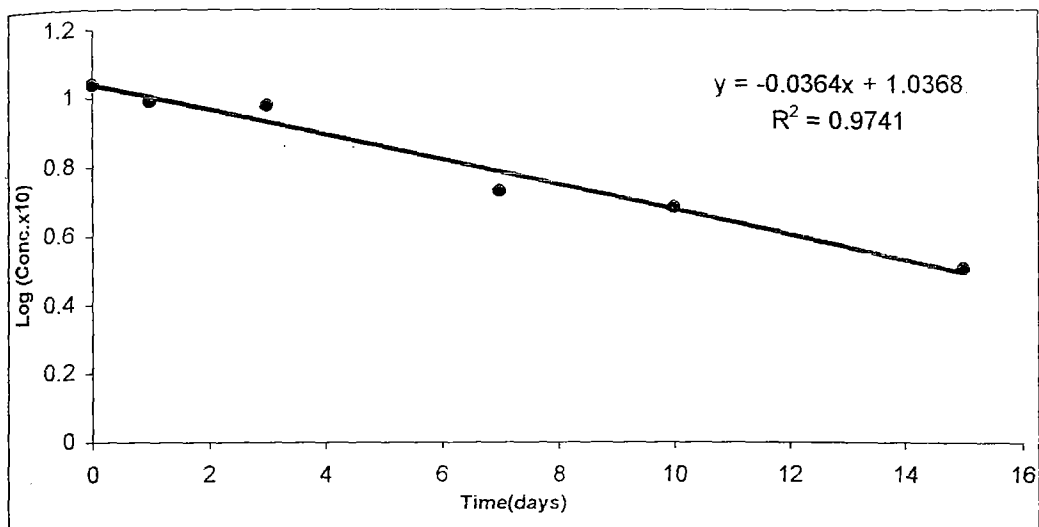


Figure 8a. Logarithmic Plot of Single Dose of Fipronil in Transgenic Cabbage

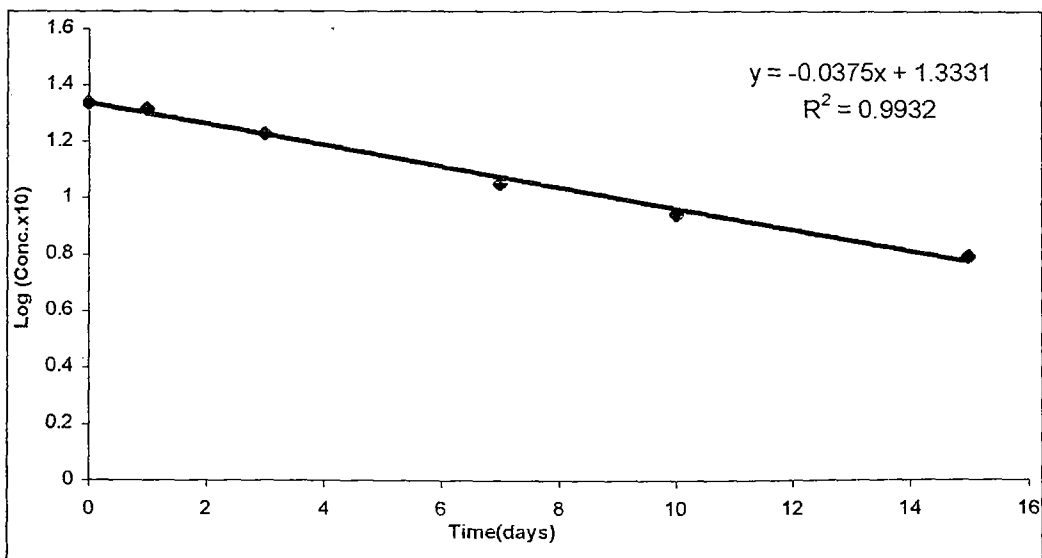


Figure 8b. Logarithmic Plot of Double Dose of Fipronil in Transgenic Cabbage

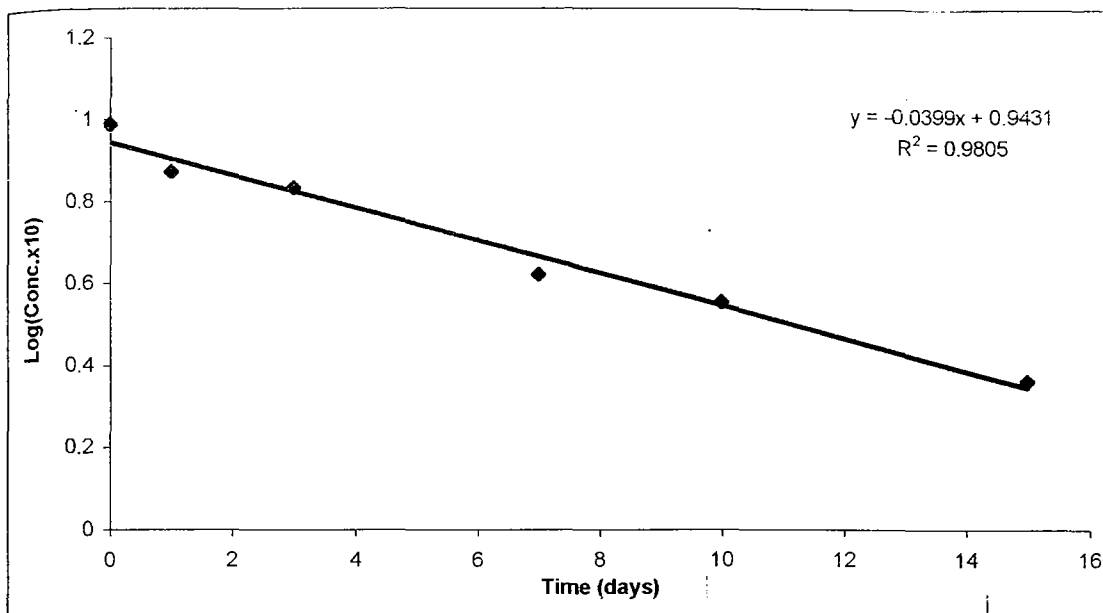


Figure 9a. Logarithmic Plot of Single Dose of Fipronil in Normal Cabbage

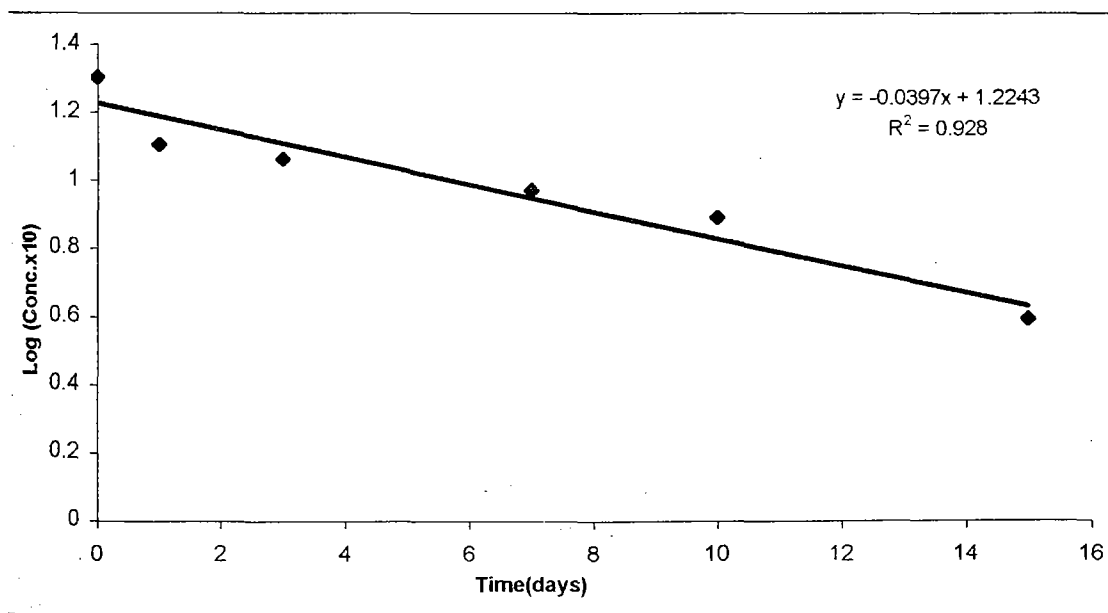


Figure 9b. Logarithmic Plot of Double Dose of Fipronil in Normal Cabbage

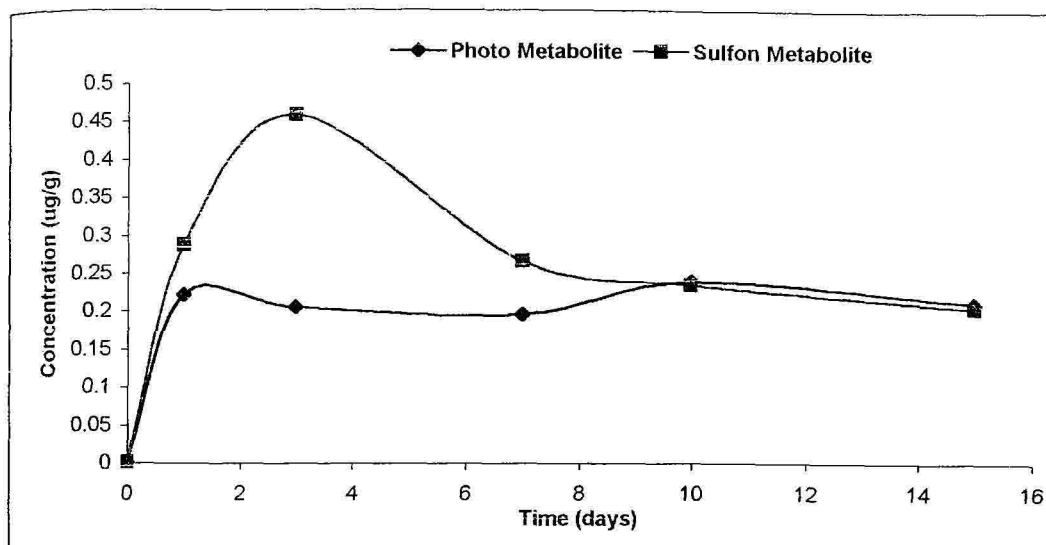


Figure 10a. Pattern of Fipronil Metabolite formation in Transgenic Cabbage after Single Dose Application

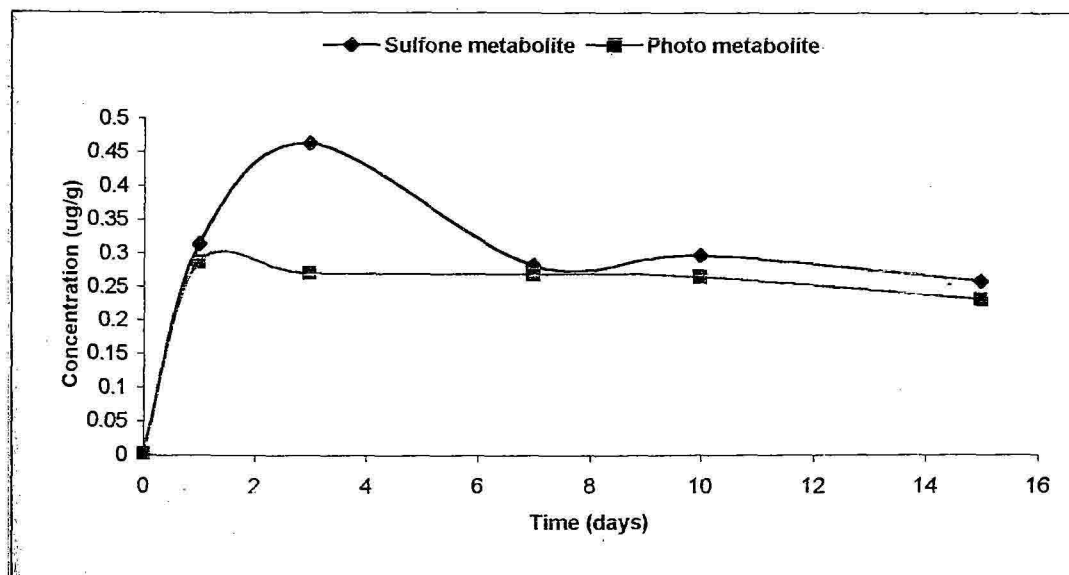


Figure 10b. Pattern of Fipronil Metabolite formation in Transgenic Cabbage after Double Dose Application

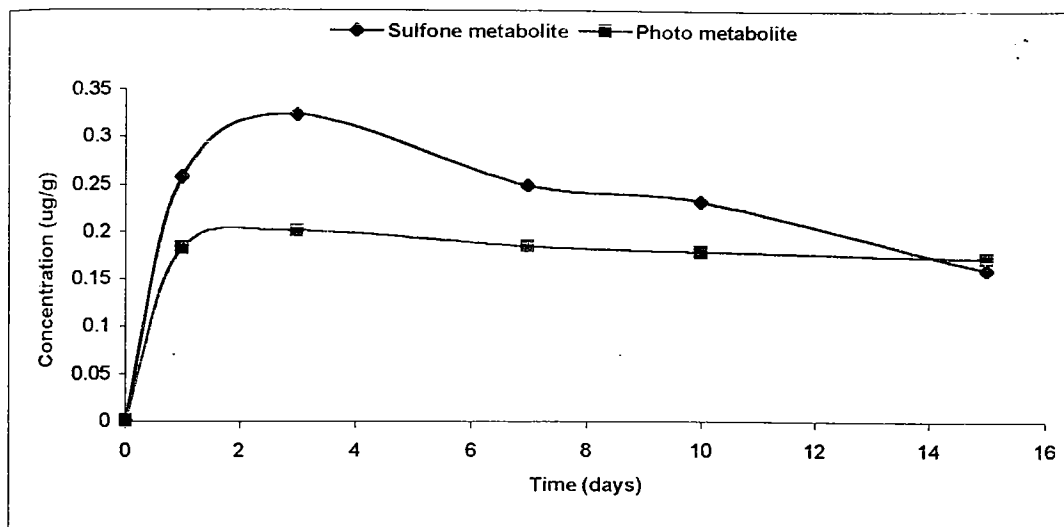


Figure 11a. Pattern of Fipronil Metabolite formation in Normal Cabbage after Single Dose Application

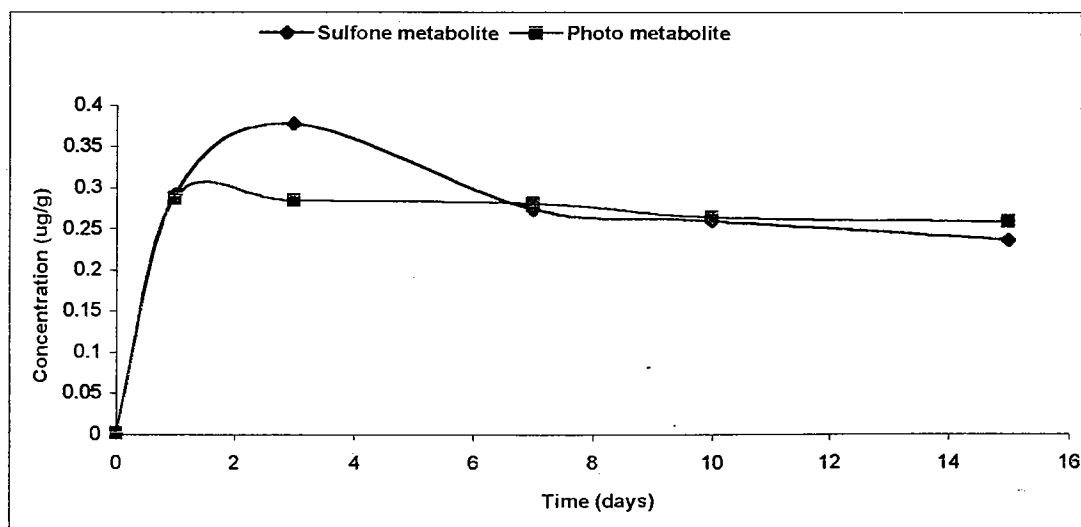


Figure 11b. Pattern of Fipronil Metabolite formation in Normal Cabbage after Double Dose Application

Table 26. Phosphorus Utilization Pattern by 75 days Old Transgenic and Normal Cabbage

Transgenic Cabbage	Dry matter yield (g/Plant)			Average (g/plant)	Phosphorus Content (mg/plant)			Average (mg/plant)	Total Phosphorus uptake (mg/plant)			Average (mg/plant)
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	13.62	17.5	15.96	15.693	2.665	2.145	2.659	2.490	36.297	37.537	42.438	38.757
40 Kg P ₂ O ₅	20.62	18.713	21.55	20.294	3.76	3.875	3.375	3.670	77.531	72.513	72.731	74.258
80 Kg P ₂ O ₅	27.75	25.197	25.013	25.987	3.42	4.595	4.215	4.077	94.905	115.78	105.429	105.371
Normal cabbage												
Treatments												
Control	13.106	16.92	10.672	13.566	2.613	2.008	2.512	2.378	34.246	33.975	26.808	31.676
40 Kg P ₂ O ₅	17.89	15.139	15.832	16.287	2.501	3.875	3.76	3.379	44.743	58.664	59.528	54.312
80 Kg P ₂ O ₅	23.04	20.011	18.121	20.391	2.94	3.815	3.913	3.556	67.74	76.342	70.907	71.663

Transgenic Cabbage	% Phosphorus derived from fertilizer			Average (%)	Fertilizer Phosphorus uptake (mg/plant)			Average (mg/plant)	% Phosphorus utilization			Average (%)
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	0	0	0	0	0	0	0	0	0	0	0	0
40 Kg P ₂ O ₅	24.595	18.685	20.173	21.151	24.617	17.893	16.034	19.515	82.842	77.48	77.713	79.345
80 Kg P ₂ O ₅	28.1	24.774	30.985	27.953	24.746	32.098	39.695	32.180	50.703	61.855	56.325	56.294
Normal cabbage												
Treatments												
Control	0	0	0	0	0	0	0	0	0	0	0	0
40 Kg P ₂ O ₅	15.18	11.087	17.549	14.605	5.368	5.967	9.919	7.085	47.8	62.68	63.606	58.029
80 Kg P ₂ O ₅	26.261	30.239	22.027	26.176	17.789	23.085	15.619	18.831	36.19	40.785	37.882	38.286

plant as compared to the non transgenic plant. From the above result, it may be concluded that phosphorus utilization is better by the cabbage plant when it is applied at recommended dose. It was also found that comparatively the transgenic plant utilized more phosphorus in other words the phosphorus utilization efficiencies of transgenic is higher as compared to the non transgenic plant. Though the transgenic plant was made from the same non transgenic cabbage i.e. isogenic line but when one foreign (Bt) gene was inserted into the transgenic cabbage plant, the gene may have got inserted in the vicinity of a gene which may be responsible for the phosphorus uptake by the cabbage plant. This Bt gene in this vicinity of uptake gene might have enhance the activity of latter gene. So, the transgenic cabbage utilizes phosphorus more efficiently than isogenic line of the cabbage plant. It was also found that this transgenic plant is very much responsive to the fertilizer phosphate than the native one. Example of hybrid vigour is known but the Bt gene may act as a marker for locating line to unknown P uptake gene for future exploitation.

4.16 Microbial population in the rhizosphere of cabbage

Microbial population of the rhizosphere soil where transgenic and non transgenic cabbage crop were grown, was monitor 25, 50 and 75 days after transplanting (DAT) of the crops. The population of bacteria, actinomycetes, fungi and phosphate solublizing bacteria was studied to compare the soil microbial activity in transgenic and non transgenic cabbage rhizosphere.

4.16.1 Bacterial population

In the radio labeled phosphorus treated pots, bacterial population was higher than the other micro organisms. The pots were treated with 0, 40 and 80 kg P_2O_5 ha⁻¹ with radio labeled phosphatic fertilizer. At 25 DAT the bacterial population in the

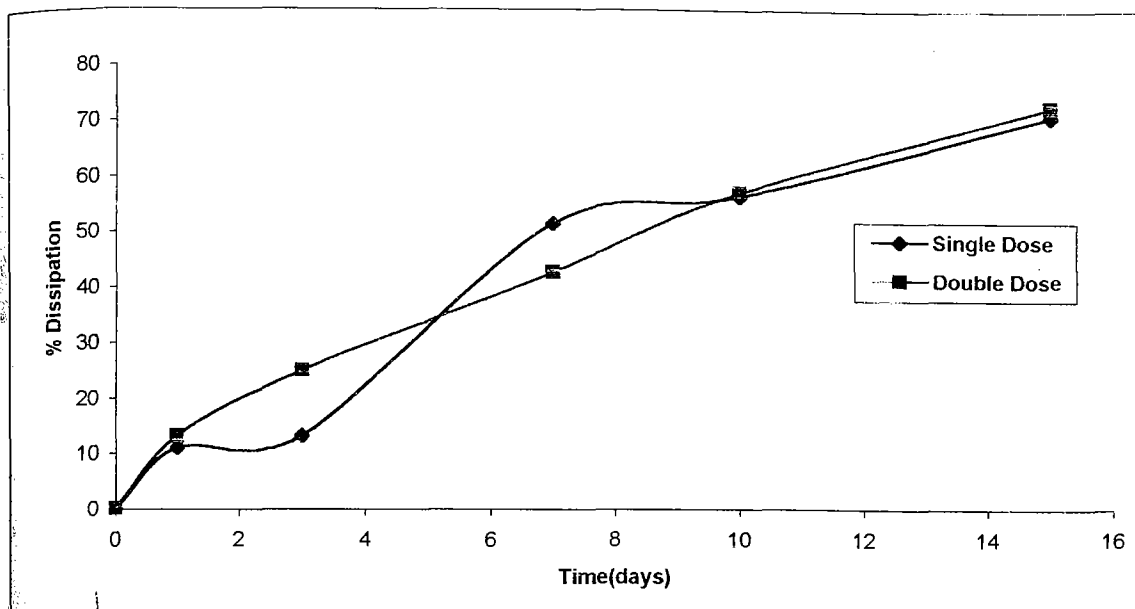


Figure 12a. Dissipation of Fipronil in Transgenic Cabbage

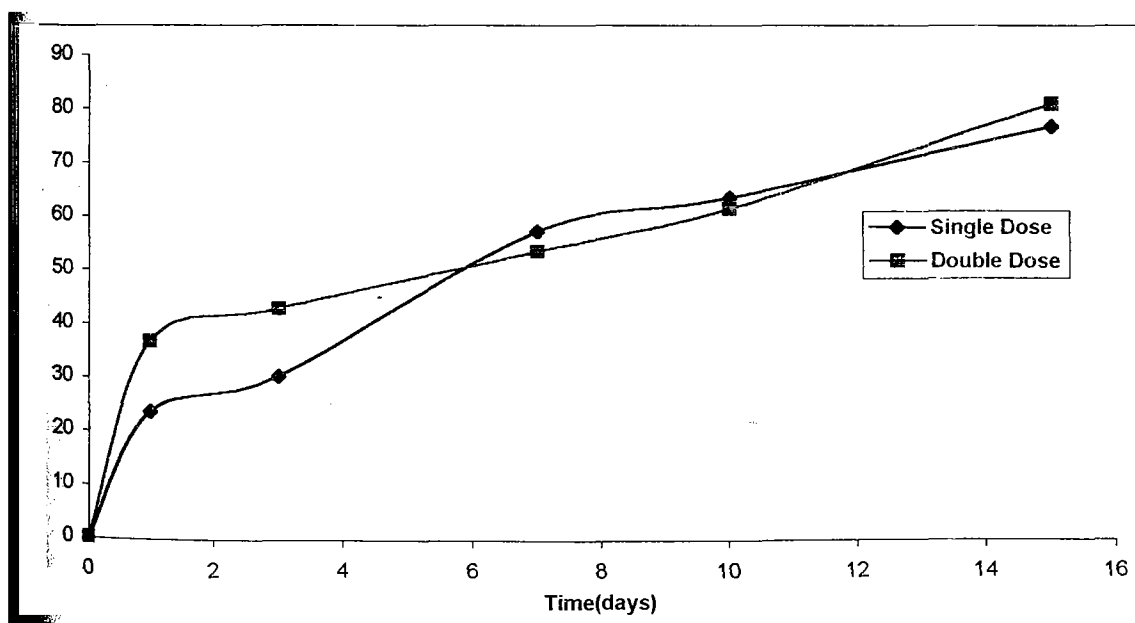


Figure 12b. Dissipation of Fipronil in Normal Cabbage

Table 27. Bacterial Population in the Rhizosphere of Transgenic and Normal Cabbage

Transgenic Cabbage	25 DAT			Average	50 DAT			Average	75 DAT			Average
	cfu x 10 ⁸				cfu x 10 ⁸				cfu x 10 ⁸			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Treatments												
Control	17	11	15	14.333	17	29	23	23	12	29	21	20.667
40 kg P ₂ O ₅ /ha	6	13	10	9.667	11	27	20	19.333	18	23	21	20.667
80 kg P ₂ O ₅ /ha	16	9	13	12.667	29	17	23	23	17	16	17	16.667
Normal Cabbage	cfu x 10 ⁸			Average	cfu x 10 ⁸			Average	cfu x 10 ⁸			Average
	cfu x 10 ⁸				cfu x 10 ⁸				cfu x 10 ⁸			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Treatments												
Control	12	27	15	18	32	43	38	37.667	21	32	16	23
40 kg P ₂ O ₅ /ha	33	52	41	42	29	22	26	25.667	32	17	24	24.333
80 kg P ₂ O ₅ /ha	18	2	10	10	12	23	18	17.667	11	7	10	9.333

rhizosphere of transgenic cabbage was comparatively lower than that of the non transgenic cabbage crop. It was found that the pot containing transgenic plant and 40 kg P_2O_5 ha⁻¹ treated pot had low bacterial population as compared to the control pot and 80 μ g P_2O_5 ha⁻¹ treated pot. When the bacterial population around the soil of the transgenic and non transgenic plant was compared, it was found that the rhizospheric bacterial population was less in case of transgenic plant at 25 DAT. But the bacterial population was lower in the 80 kg, P_2O_5 ha⁻¹ treated pot in case of non transgenic cabbage plant (Table 27). When the data were statistically analysed, it was found that the variation of the bacterial population in the rhizosphere variation of transgenic and non transgenic was statistically significant at 25 DAT. When bacterial population was counted in 50 DAT soil sample, it was found that variation of bacterial population in between the two types of cabbage plant were statistically not significant due to varietal and treatment difference. From the table 27, it was found that bacterial population around the rhizosphere of transgenic plant, which was treated with 40 kg P_2O_5 ha⁻¹ radio labeled phosphorus, have comparatively low bacterial population in comparison to the control sample and the pot treated with 80 kg P_2O_5 ha⁻¹ radio labeled phosphatic fertilizer. In case of non transgenic plants, the bacterial population was less in the rhizosphere which were treated with radio labeled phosphatic fertilizer at the rate of 80kg P_2O_5 ha⁻¹. In case of 75 DAT sampling the over all population of bacteria in the rhizosphere reduced as compared to the earlier sampling. In this sampling the bacterial population was equal in the rhizosphere of the control and 40kg P_2O_5 treated pot of transgenic cabbage plant. But in case of non transgenic cabbage crop, the bacterial population was low when pot was treated with 80kg P_2O_5 ha⁻¹. When the bacterial populations were compared in between the transgenic and non transgenic pot with different dose of phosphatic

Table 28. Actinomycetes Population in the Rhizosphere of Transgenic and Normal Cabbage

Transgenic Cabbage	25 DAT			Average	50 DAT			Average	75 DAT			Average
	cfu x 10 ⁵				cfu x 10 ⁵				cfu x 10 ⁵			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	5	8	7	6.667	16	7	10	11	23	15	20	19.333
40 kg P ₂ O ₅ /ha	9	5	7	7.000	8	3	6	5.667	13	6	9	9.333
80 kg P ₂ O ₅ /ha	12	14	13	13.000	4	12	6	7.333	18	10	14	14.000
Normal cabbage	cfu x 10 ⁵			Average	cfu x 10 ⁵			Average	cfu x 10 ⁵			Average
	cfu x 10 ⁵				cfu x 10 ⁵				cfu x 10 ⁵			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	6	2	5	4.333	19	12	16	15.667	11	17	16	14.667
40 kg P ₂ O ₅ /ha	9	4	6	6.333	33	39	30	34.000	14	10	16	13.333
80 kg P ₂ O ₅ /ha	2	5	4	3.667	8	10	9	9.000	16	23	21	20.000

fertilizer, little variation was observed. But when it was statistically analyzed it was found that the variation was statistically significant in between the rhizosphere of these two plants. Overall it may be concluded that the variation of bacterial population was not significant but there are some effects of transgenic plant's roots exudates on the bacterial population for which the bacterial population was little bit lower as compared to non transgenic cabbage plant.

4.16.2 Actinomycetes

In 25 DAT soil samples, rhizosphere actinomycetes population recorded in transgenic and non transgenic cabbage was not found to be statistically significant due to varietal and treatment difference. The overall population of actinomycetes in the rhizosphere of transgenic plant was higher in 25 DAT soil sample (Table 28). In 50 DAT soil samples, overall rhizosphere population of the actinomycetes was higher in non transgenic plant compared to the transgenic plant. When these data were statistically analyzed, by using SAS, the results showed that the variation in actinomycetes population in between transgenic and non transgenic plant was not statistically significant. The same trend was also found in the soil collected on 75 DAT. But the overall populations of actinomycetes were less in case of transgenic cabbage plant in 40 kg and 80 kg P_2O_5 ha^{-1} treated pot. From the above result it can be concluded that the transgenic and non transgenic plant have same effect on the rhizosphere actinomycetes population. Microbial population is dynamic in nature and these results should be validated by further field experiments.

4.16.3 Fungi

The experiments were done to study the effect of transgenic cabbage on soil fungi population at 25, 50, 75 days after transplanting. The fungal population was found less in case of non transgenic plant but higher in case of transgenic plant.

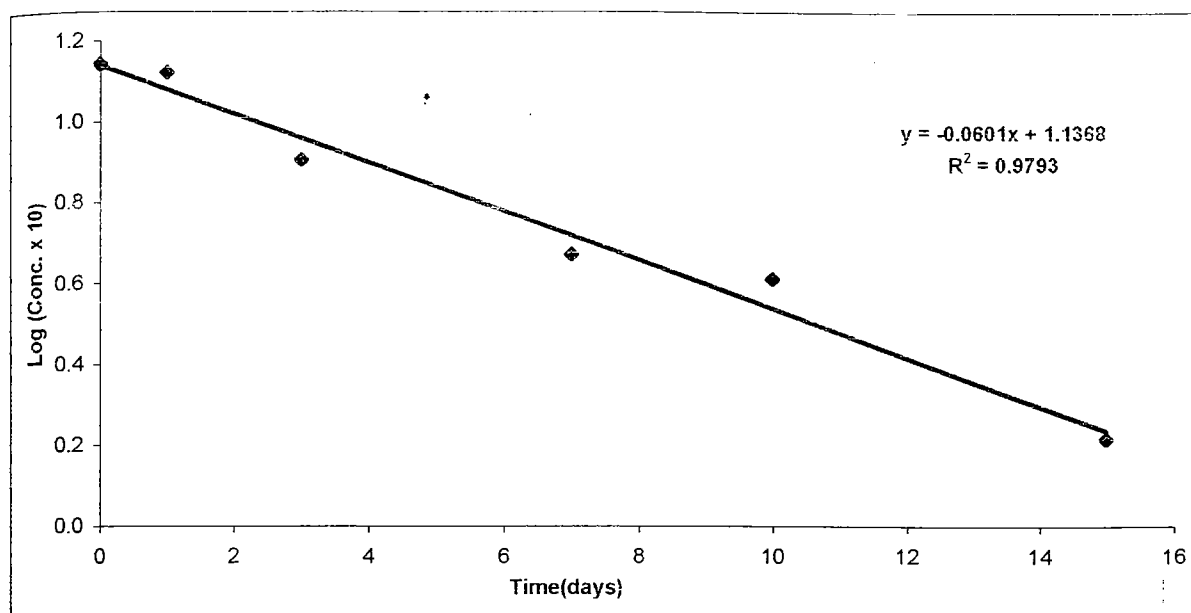


Figure 13a. Logarithmic Plot of Single Dose of Fipronil in the Rhizosphere of Transgenic Cabbage

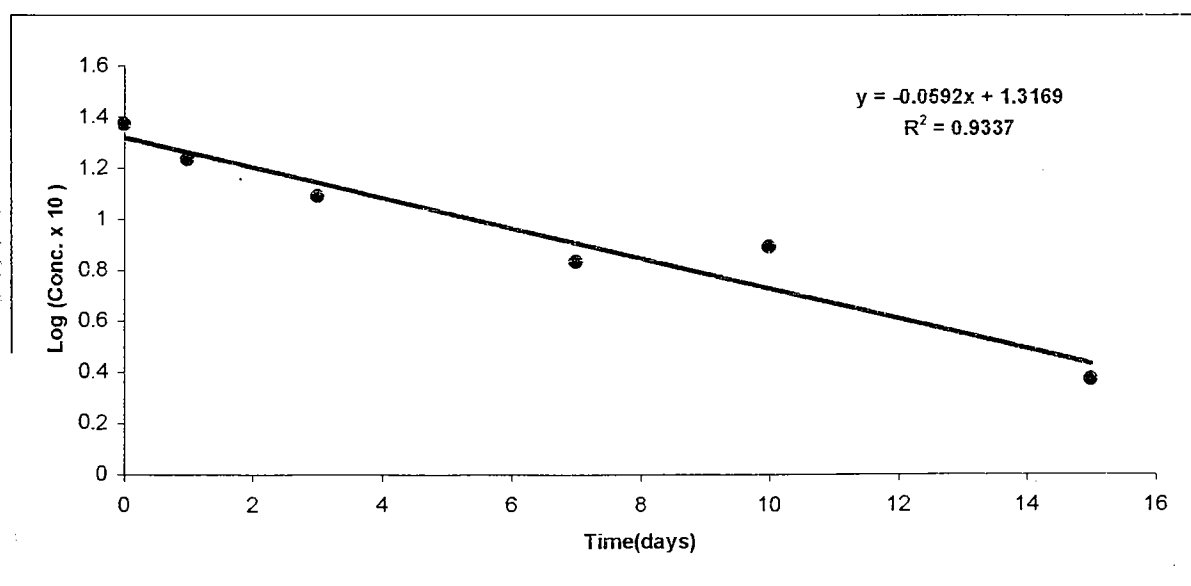


Figure 13b. Logarithmic Plot of Double Dose of Fipronil in the Rhizosphere of Transgenic Cabbage

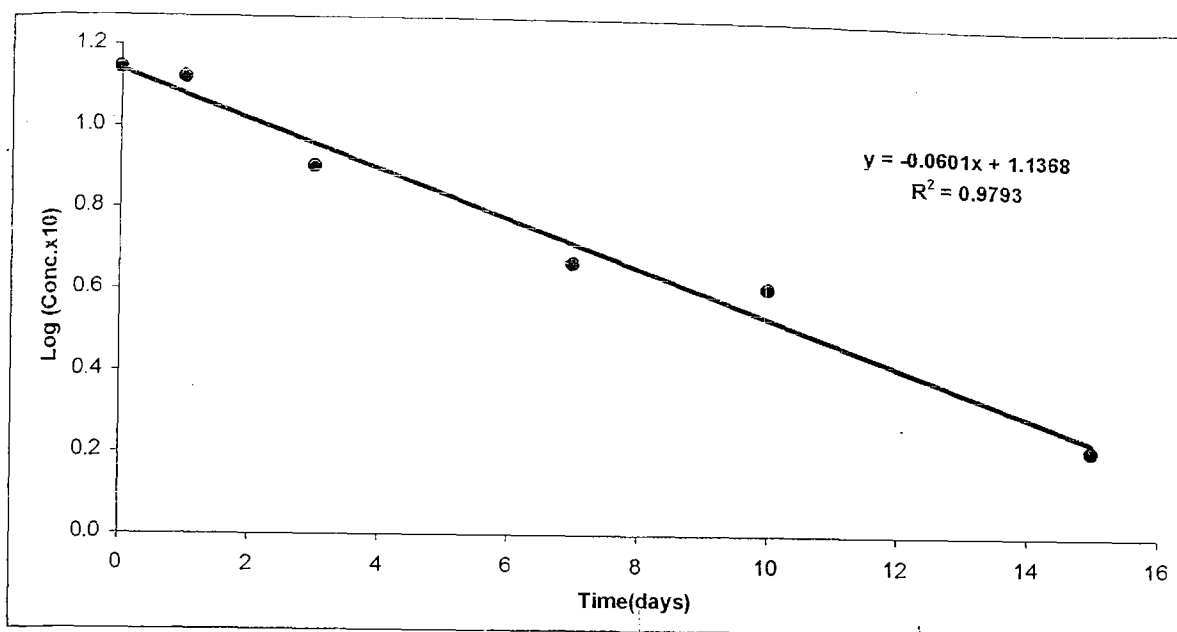


Figure 14a. Logarithmic Plot of Single Dose of Fipronil in the Rhizosphere of Normal Cabbage

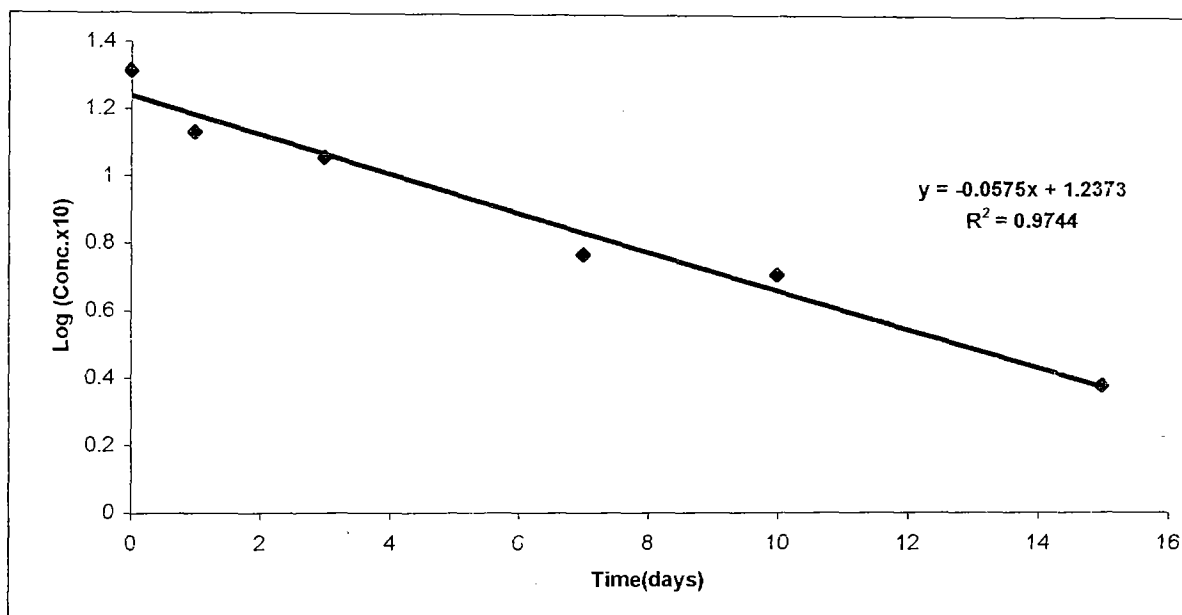


Figure 14b. Logarithmic Plot of Double Dose of Fipronil in the Rhizosphere of Normal Cabbage

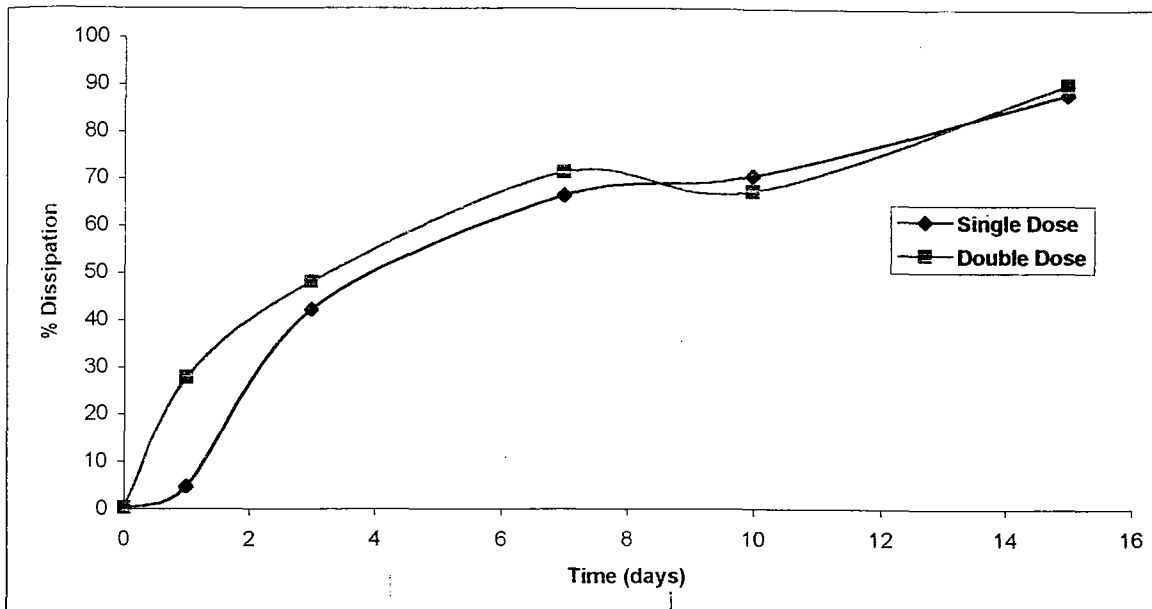


Figure 15a. Dissipation of Fipronil in the Rhizosphere of Transgenic Cabbage

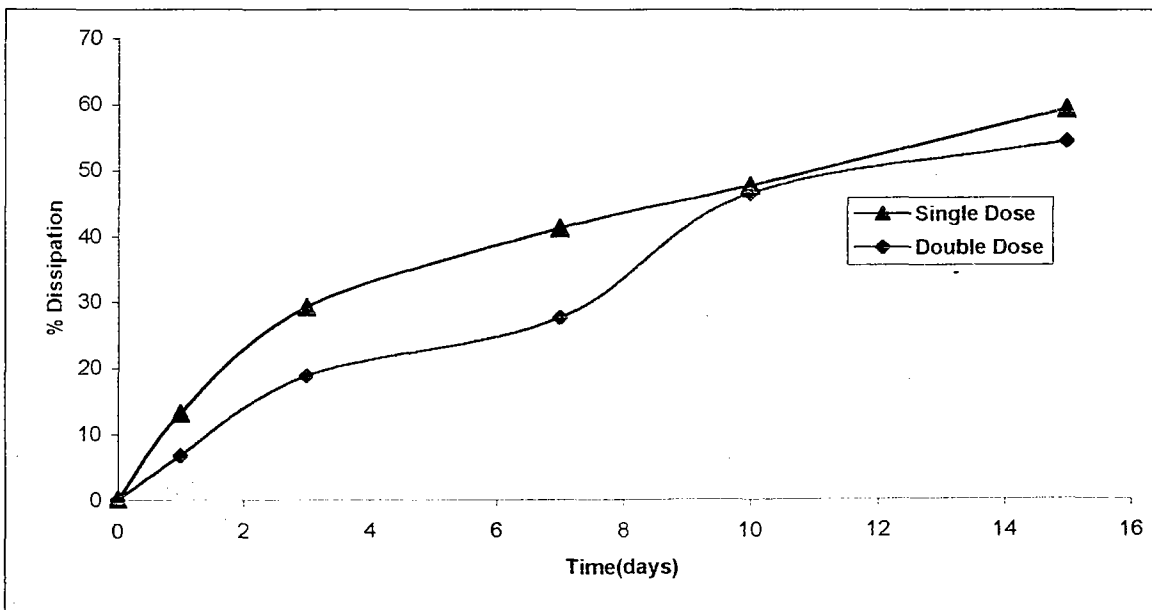


Figure 15b. Dissipation of Fipronil in the Rhizosphere of Normal Cabbage

Table 29. Fungi Population in the Rhizosphere of Transgenic and Normal Cabbage

Transgenic Cabbage	25 DAT			50 DAT			75 DAT		
	cfu x 10 ⁴			cfu x 10 ⁴			cfu x 10 ⁴		
	R ₁	R ₂	R ₃	Average	R ₁	R ₂	R ₃	Average	Average
Control	3	6	5	4.667	8	2	6	5.333	10
40 kg P ₂ O ₅ /ha	5	8	8	7.000	8	12	11	10.333	8.667
80 kg P ₂ O ₅ /ha	4	3	5	4.000	5	7	5	5.667	22.333
Normal Cabbage	cfu x 10 ⁴			Average			cfu x 10 ⁴		
	R ₁	R ₂	R ₃	Average			R ₁	R ₂	R ₃
	Average	Average	Average	Average	Average	Average	Average	Average	Average
Control	2	5	3	3.333	7	2	5	4.667	19.333
40 kg P ₂ O ₅ /ha	4	6	6	5.333	8	25	16	16.333	33.333
80 kg P ₂ O ₅ /ha	6	7	7	6.667	2	10	6	6.000	43.333

Though there was some difference in the fungal population but the variation was not found to be statistically significant due to transgenic and non transgenic factor and treatment difference in 25 DAT soil samples. Comparatively higher fungal population in 25 DAT soil sample was observed in case of transgenic plants. Besides this variation in transgenic rhizosphere, higher fungi population was observed in the soil, treated with 40 kg P_2O_5 ha⁻¹ (Table 29) in comparison to control and 80 kg P_2O_5 treated soil. But in case of non transgenic plant, higher population was found in case of 80 kg P_2O_5 ha⁻¹ applied per pot. In case of 50 DAT soil sample showed the same trend. Fungi population was comparatively higher in the rhizosphere of non transgenic plants. Highest population was observed in the soil containing phosphatic fertilizer at the rate of 40 kg P_2O_5 ha⁻¹. The variation of fungi population in the rhizosphere of transgenic and non transgenic plant was there; but the variation was not statistically significant. But the variation of fungi population in the rhizosphere was significant for the 75 DAT soil sample. Comparatively higher fungi population was recorded in the rhizospheric soil of non transgenic plant. The fungal population in the rhizospheric soil of non transgenic cabbage was recorded and it was found that the population was lower in case of transgenic plant and the fungi population significantly lower in case of transgenic plants.

In non transgenic plants, rhizosphere soil showed highest fungal population which was treated with 80 kg P_2O_5 ha⁻¹. The same pattern of population dynamics was found in case of rhizosphere transgenic plants.

4.16.4 Phosphate Solubilizing Bacteria (PSB)

Phosphate solubilizing bacteria play an important role in phosphorus uptake. To study the effect of PSB on transgenic and non transgenic plants, rhizospheric soil

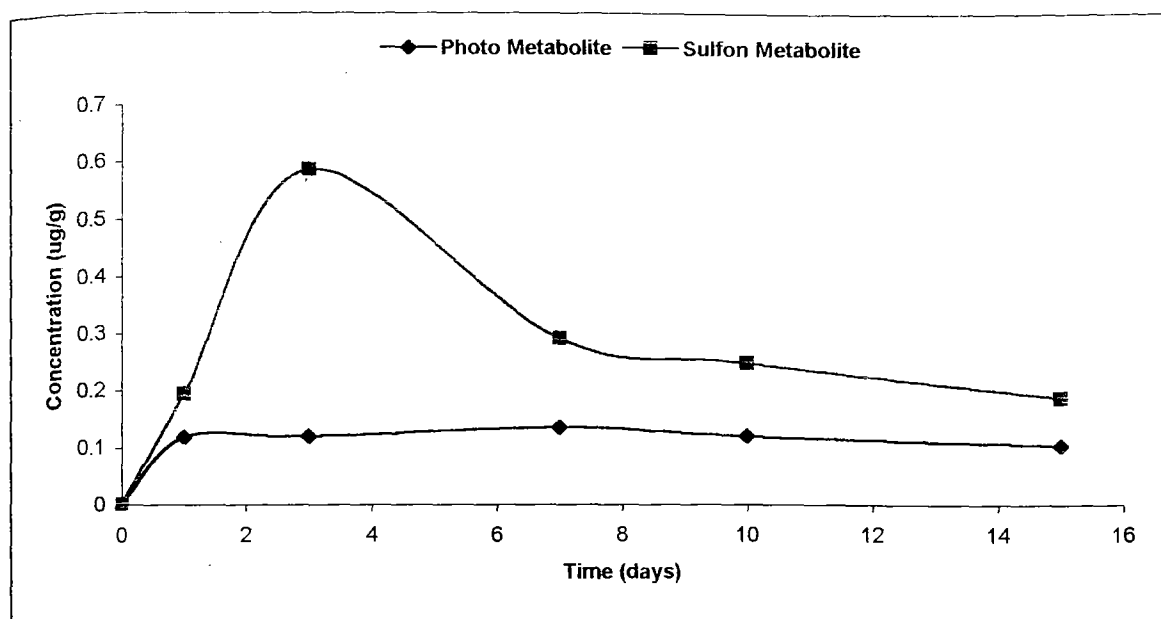


Figure 16. Pattern of Fipronil Metabolite formation in the Rhizosphere of Transgenic Cabbage

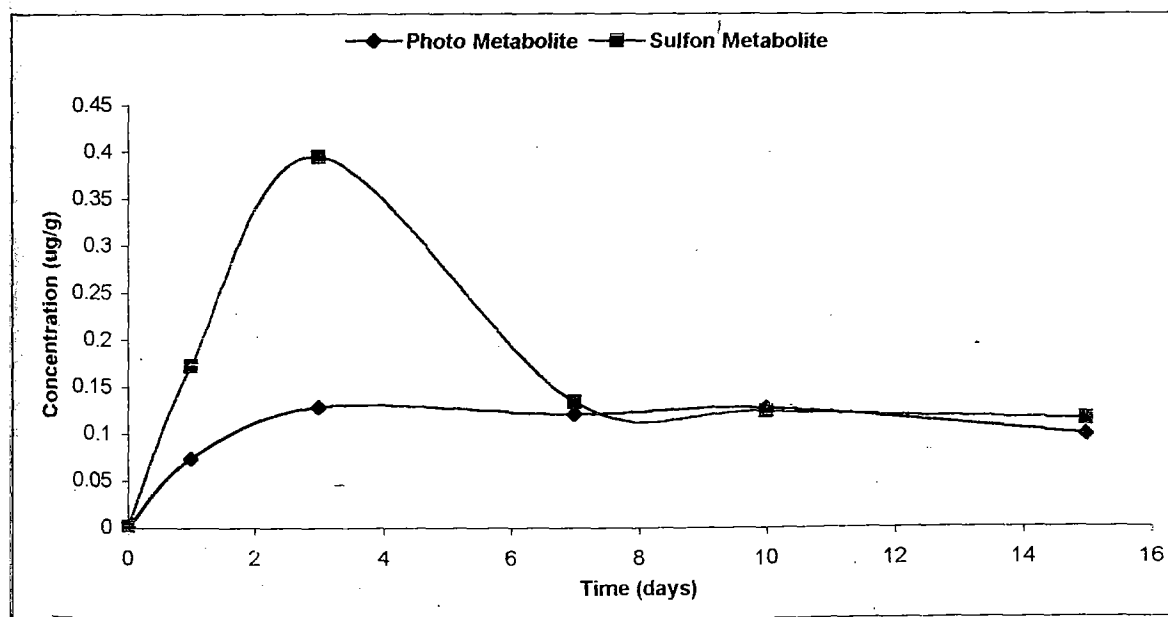


Figure 17. Pattern of Fipronil Metabolite formation in the Rhizosphere of Normal Cabbage

Table 30. Phosphate Solubilizing Bacterial Population in the Rhizosphere of Trans and Normal Cabbage

Transgenic Cabbage	25 DAT			Average	50 DAT			Average	75 DAT			Average
	cfu x 10 ⁴				cfu x 10 ⁴				cfu x 10 ⁴			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	20	12	16	16	6	3	5	4.667	40	45	42	42.333
40 kg P ₂ O ₅ /ha	9	3	7	6.333	5	5	6	5.333	22	26	17	21.667
80 kg P ₂ O ₅ /ha	9	5	6	6.667	33	23	27	27.667	60	69	58	62.333
Normal Cabbage	cfu x 10 ⁴			Average	cfu x 10 ⁴			Average	cfu x 10 ⁴			Average
	cfu x 10 ⁴				cfu x 10 ⁴				cfu x 10 ⁴			
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	15	11	12	12.667	10	17	3	10	50	57	51	52.667
40 kg P ₂ O ₅ /ha	7	5	7	6.333	15	11	16	14	16	14	14	14.667
80 kg P ₂ O ₅ /ha	13	4	9	8.667	18	16	16	16.667	19	18	15	17.333

was collected at 25, 50 and 75 days after transplanting of transgenic (4T1) line and non transgenic cabbage. Rhizospheric soil was treated with 0, 40 and 80 kg P_2O_5 ha⁻¹ radio labeled phosphatic fertilizer. In soil sample collected after 25 days highest number of phosphate solubilizing bacteria (PSB) colony were recorded in control (non-transgenic) sample for both type of cabbage plant. There was variation of PSB colony number but in both cases of cabbage rhizospheric, the variation was not statistically significant due to varietal (transgenic and non-transgenic) and treatment difference (Table 30). When rhizospheric soil sample was collected 50 days after transplanting significant difference in bacterial colony was observed. Highest number of bacterial colony was recorded in the soil, treated with 80 kg P_2O_5 ha⁻¹ for both type of rhizospheric soil. The variation PSB in the rhizosphere was statistically significant due to variety and treatment. In the soil sample collected on 75 days after transplanting the bacterial colonies were recorded. Highest bacterial colony was recorded in the rhizospheric of transgenic plant, treated @ 80 kg P_2O_5 ha⁻¹ by phosphatic fertilizer. In case of the non transgenic plant, higher bacterial population was recorded in the control soil sample (Table 30). The data of bacterial population was statistically analyzed. It was found that variation of bacterial population in this two types of rhizosphere was statistically significant due to variety only. More study will be required to confirm the observation made for the transgenic line taken for this two year study. The transgenic cabbage taken for study favors the multiplication of this bacteria, which is too early to offer the reason for the increase of the population of PSB.

4.17 Nematode population

In order to know whether there is a difference in the nematode population in the rhizospheric soil of two type of cabbage plant, soil samples were collected from

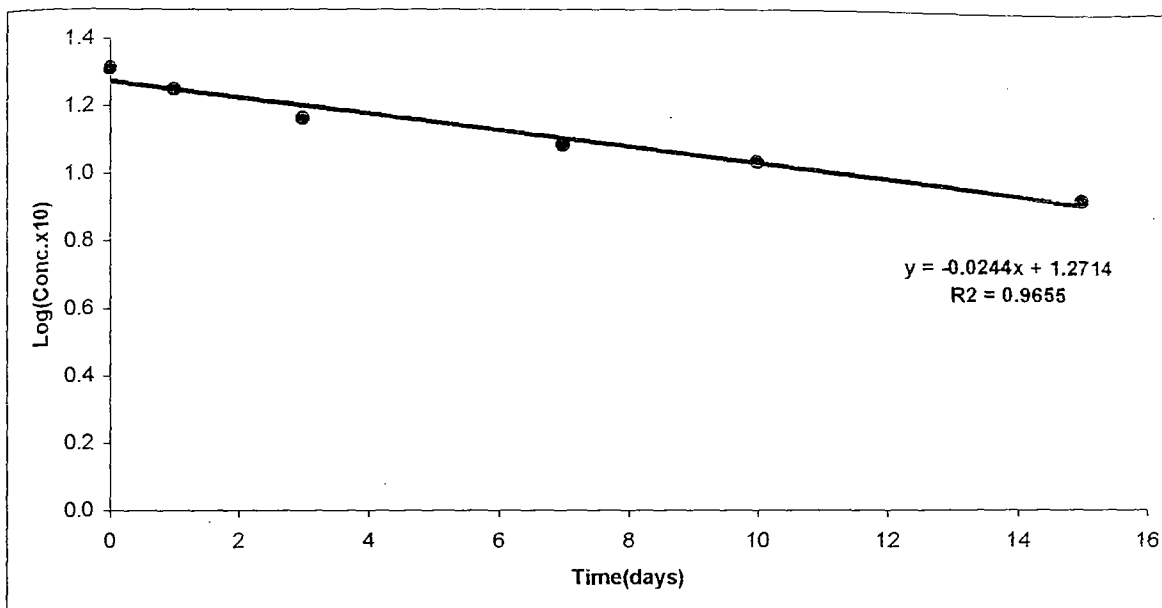


Figure 18a. Logarithmic Plot of Single Dose of Thiacloprid in Transgenic Cabbage

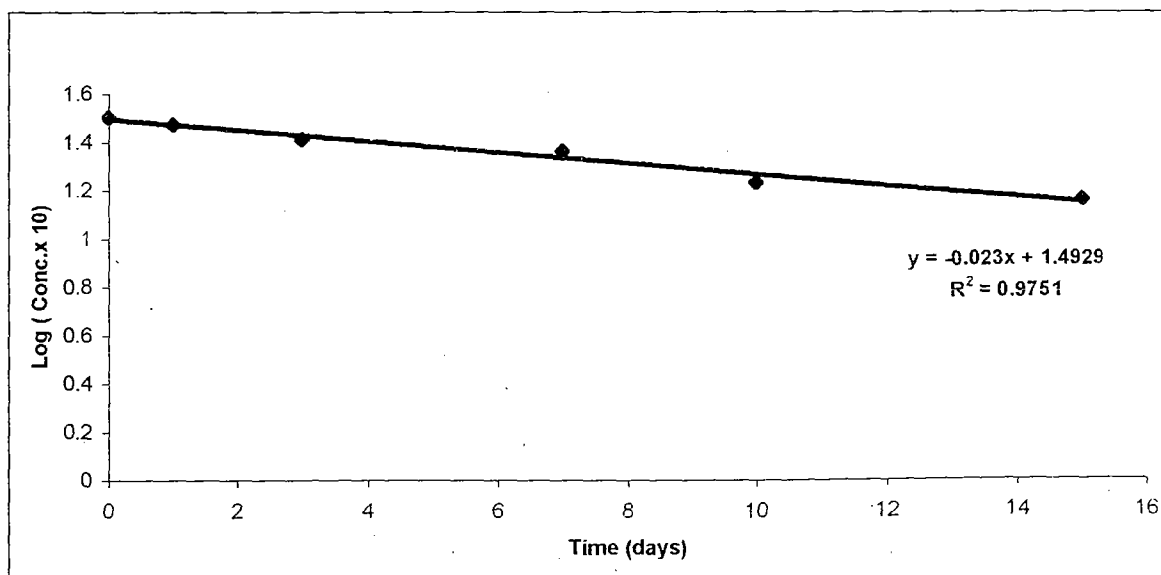


Figure 18b. Logarithmic Plot of DoubleDose of Thiacloprid in Transgenic Cabbage

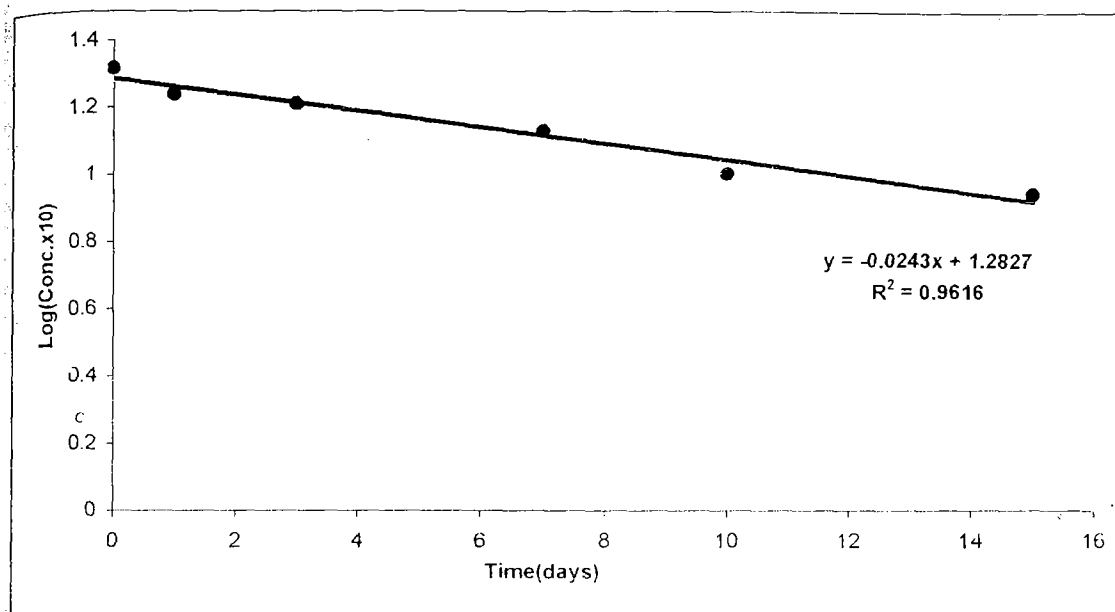


Figure 19a. Logarithmic Plot of Single Dose of Thiacloprid in Normal Cabbage

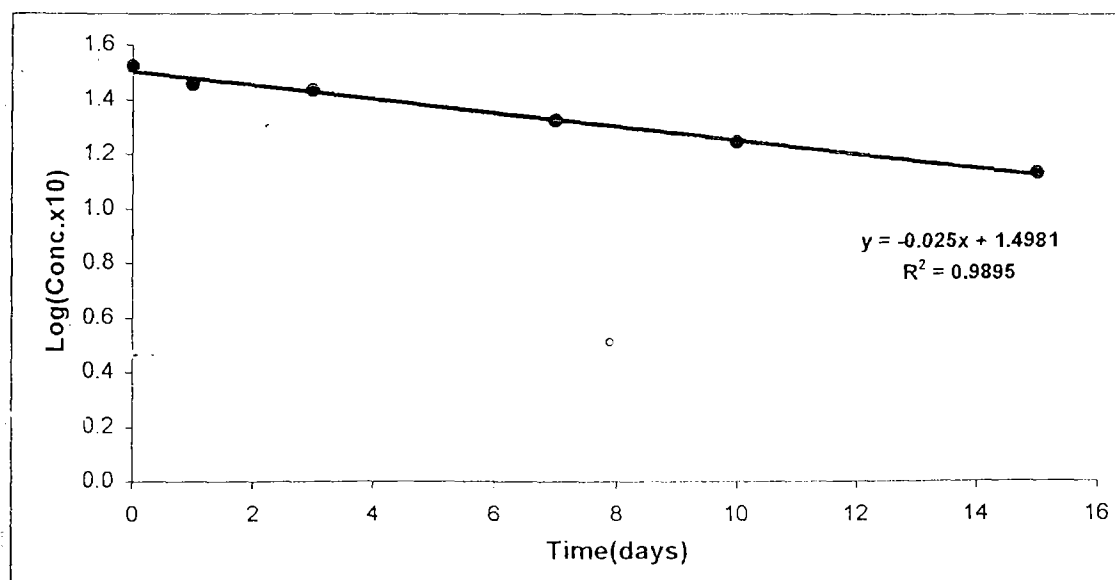


Figure 19b. Logarithmic Plot of Double Dose of Thiacloprid in Normal Cabbage

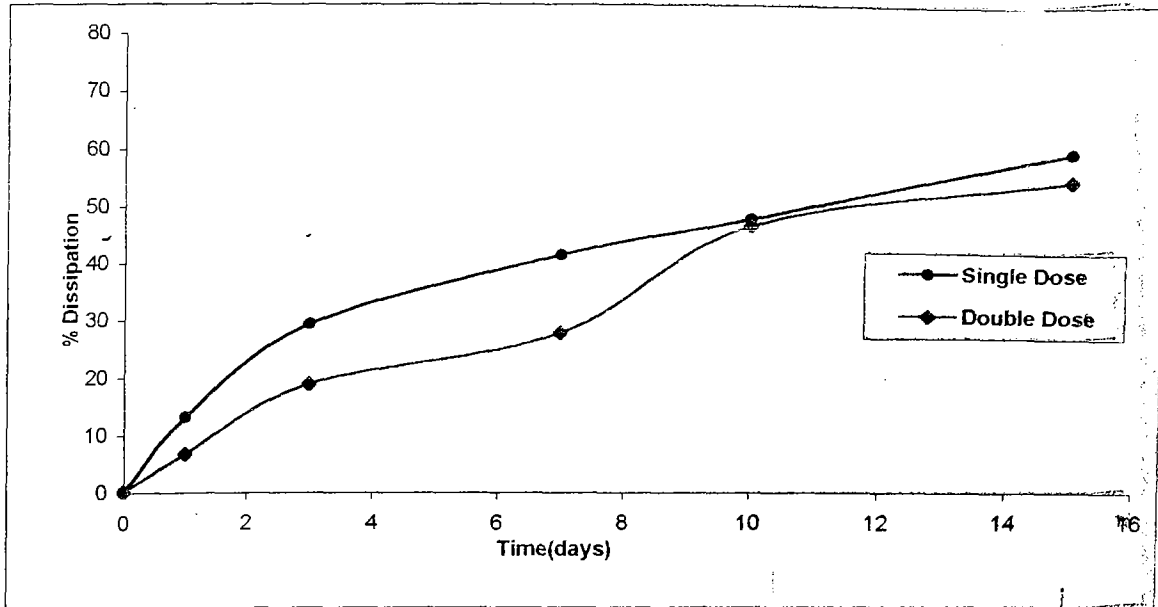


Figure 20a. Dissipation of Thiacloprid in Transgenic Cabbage

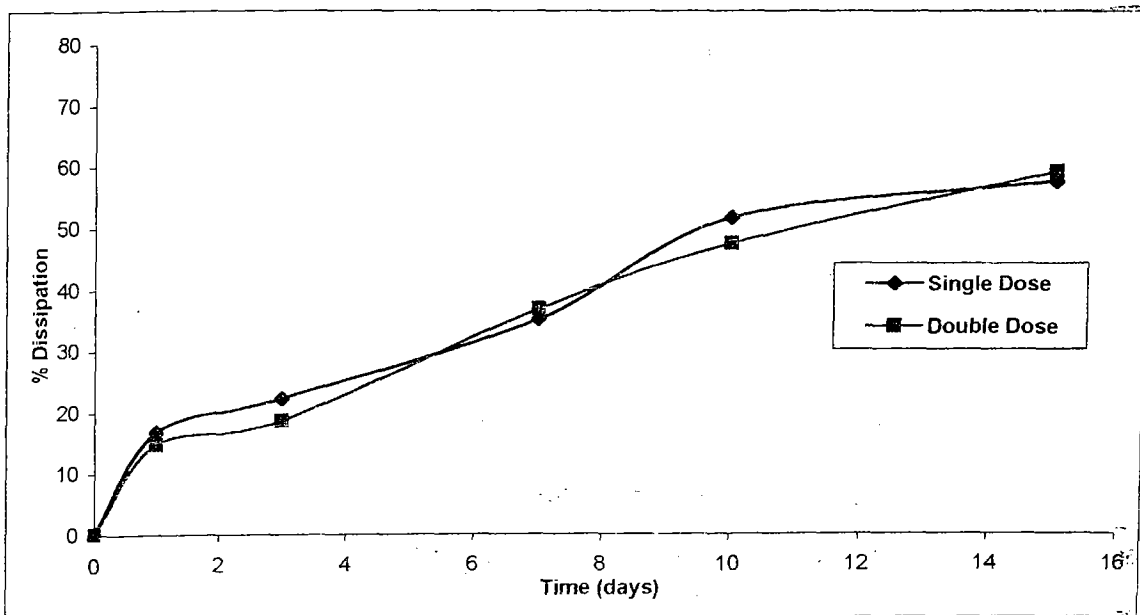


Figure 20b. Dissipation of Thiacloprid in Normal Cabbage

Table 31. Nematode population in the Rhizosphere of Transgenic and Normal Cabbage after 25 days of Transplanting

Transgenic Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	10	6	8	8	5	2	3	3.333	6	5	10	7
40 kg P ₂ O ₅ /ha	5	12	8	8.333	1	1	3	1.667	15	9	6	10
80 kg P ₂ O ₅ /ha	2	4	7	4.333	6	1	2	3	3	5	8	5.333
Normal Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	13	10	17	13.333	3	1	1	1.667	6	11	8	8.333
40 kg P ₂ O ₅ /ha	14	8	6	9.333	2	1	3	2	6	11	3	6.667
80 kg P ₂ O ₅ /ha	4	6	9	6.333	2	1	1	1.333	2	13	5	6.667

Table 32. Nematode Population in the Rhizosphere of Transgenic and Normal Cabbage after 50 days of Transplanting

Transgenic Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	9	4	2	5.00	1	1	2	1.33	10	2	6	6.00
40 kg P ₂ O ₅ /ha	4	8	2	4.66	1	1	2	1.33	5	9	3	5.66
80 kg P ₂ O ₅ /ha	7	6	5	6.00	4	2	1	2.33	11	12	6	9.66
Normal Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Control	7	5	3	5.00	2	1	2	1.66	2	6	5	4.33
40 kg P ₂ O ₅ /ha	6	6	4	5.33	1	3	1	1.66	5	9	6	6.66
80 kg P ₂ O ₅ /ha	13	2	1	5.33	3	2	6	3.66	12	4	4	6.66

Table 33. Nematode Population in the Rhizosphere of Transgenic and Normal Cabbage after 75 days of Transplanting

Transgenic Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Treatments												
Control	4	7	1	4.00	1	0	1	0.66	6	3	17	8.33
40 kg P ₂ O ₅ /ha	2	2	1	1.66	2	1	1	1.33	18	6	3	9.33
80 kg P ₂ O ₅ /ha	3	6	2	3.66	3	1	0	1.33	6	11	4	7.33
Normal Cabbage	Rotylenchulus X10			Average	Tylenchulus X10			Average	Saprophyte X10			Average
	R ₁	R ₂	R ₃		R ₁	R ₂	R ₃		R ₁	R ₂	R ₃	
Treatments												
Control	2	6	7	5.00	5	3	1	3.00	20	4	10	11.33
40 kg P ₂ O ₅ /ha	3	2	2	2.33	1	4	2	2.33	23	10	11	14.66
80 kg P ₂ O ₅ /ha	4	4	7	5.00	3	3	1	2.33	29	11	10	16.33

rhizosphere on 25, 50 and 70 days after transplanting. Three types of nematodes were identified namely, *Rotylenchulus*, *Tylenchulus* and Saprophyte. In 25 days sample, it was found that *Rotylenchulus* population was higher than *Tylenchulus* sp. Soil was treated with tagged ^{32}P @ 0, 40 and 80 kg P_2O_5 ha $^{-1}$ and nematode population was compared treatment wise between these two types of cabbage plant. In 25 days sample, highest *Rotylenchulus* population was found in control sample (Table 31). Whereas highest *Rotylenchulus* and Saprophyte population was recorded in 25 days soil sample. When nematode population was compared treatment wise, i.e., nematode population in transgenic soil sample treated with 0, 40 and 80 kg P_2O_5 , with non transgenic cabbage soil sample treated with 0, 40 and 80 kg P_2O_5 , respectively, it was found that the variation in nematode population in two rhizospheric soil was statistically insignificant. In 50 days control soil sample, highest number of *Rhotylenchulus* population was found in rhizosphere of transgenic cabbage among the other type of soil having different quantity of phosphorus. Highest number of saprophyte nematode was found in the rhizosphere of transgenic plant treated with 80 kg P_2O_5 ha $^{-1}$. In case of non transgenic plant, highest number of *Rotylenchulus* nematodes was found in the soil sample treated with 80 kg P_2O_5 ha $^{-1}$ and also highest number of saprophyte population was found. Regarding *Tylenchulus* nematode, highest number of nematode was found in the soil sample treated with 80 kg P_2O_5 ha $^{-1}$ for both type of rhizospheric soil sample (Table 32). When nematode population in these two type of rhizospheric soil was statistically analyzed, it was found that variation of nematode population in the rhizospheric soil was statistically insignificant. In 75 days soil sample of transgenic cabbage, highest number of saprophyte nematode was found in 40 kg P_2O_5 ha $^{-1}$ treated soil sample where as highest number of *Rotylenchulus* population was found

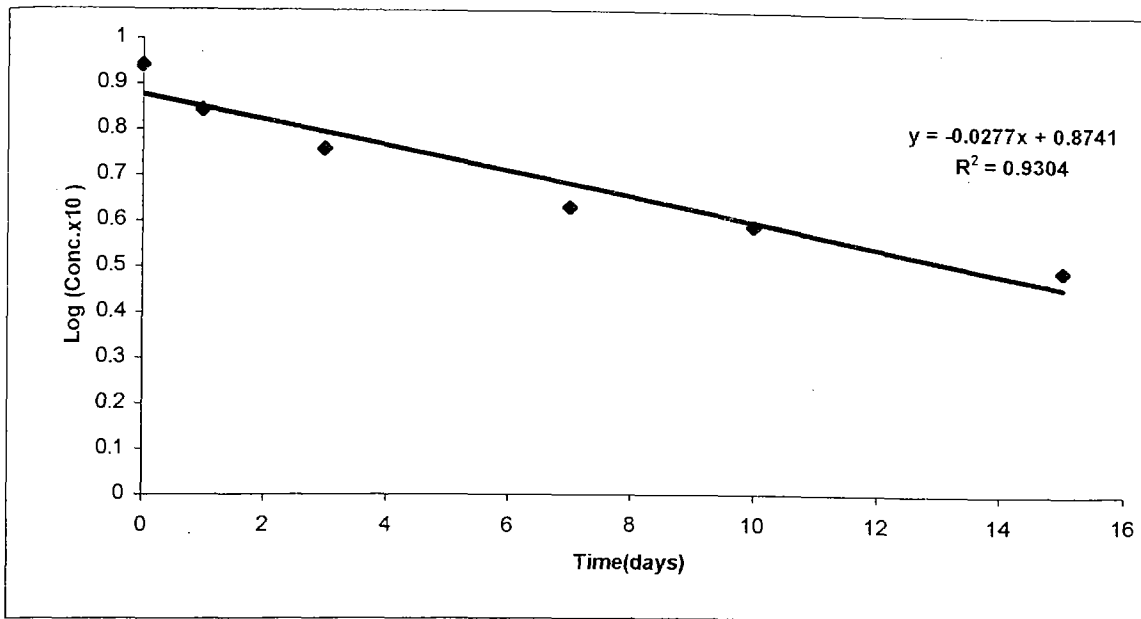


Figure 21a. Logarithmic Plot of Single Dose of Thiacloprid under Field Condition

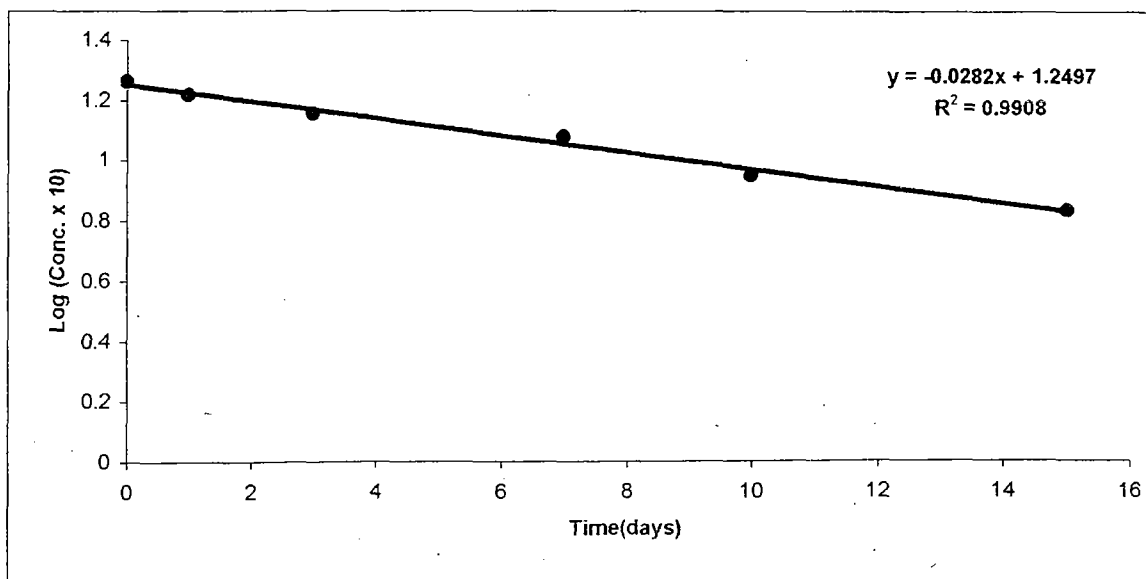


Figure 21b. Logarithmic Plot of Double Dose of Thiacloprid under Field Condition

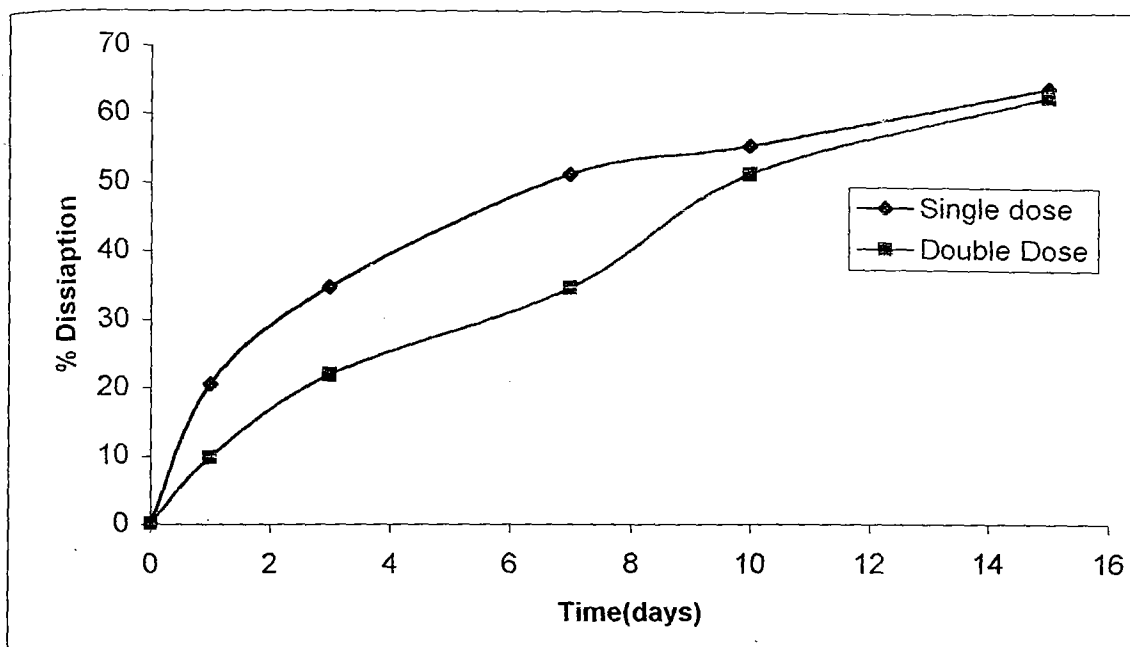


Figure 22. Dissipation of Thiacloprid Under Field Condition

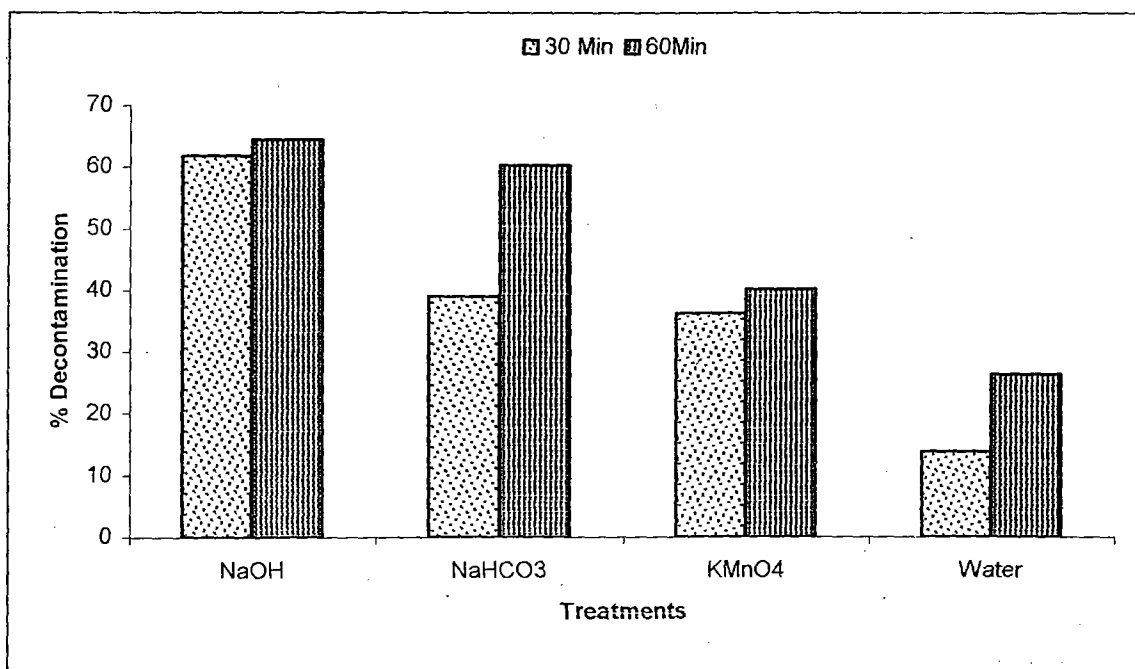


Figure 23. Decontamination of Thiacloprid under Different Chemical Treatments



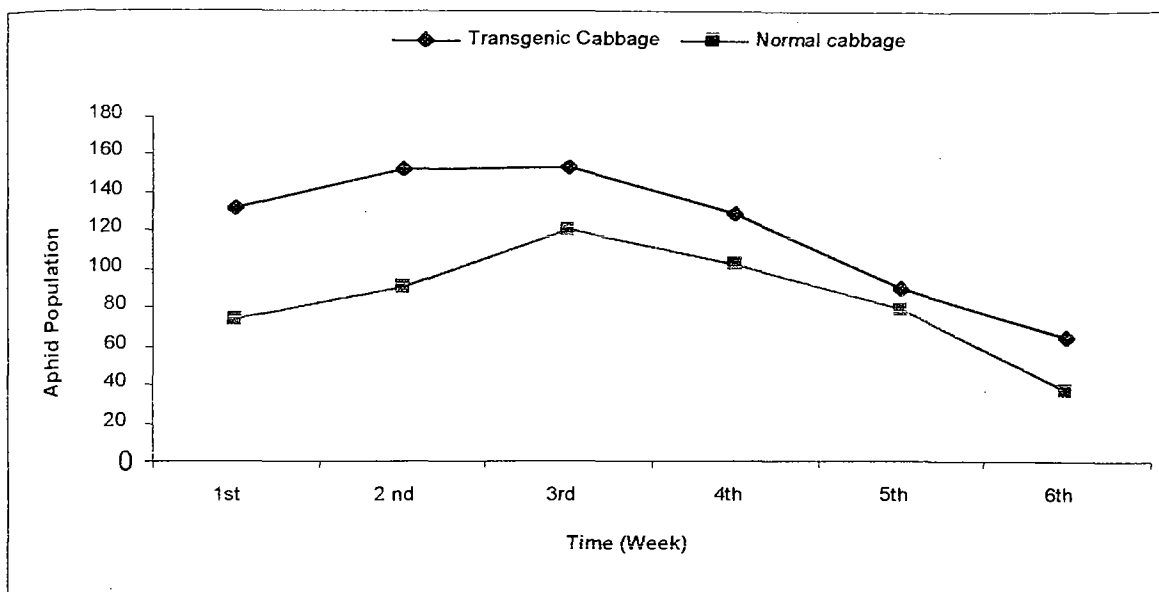
Fig. 24 a. Aphid infestation in Bt-transgenic cabbage

Effect of *Bt* on Aphid in Transgenic and Normal Cabbage during
Summer - February, 2004

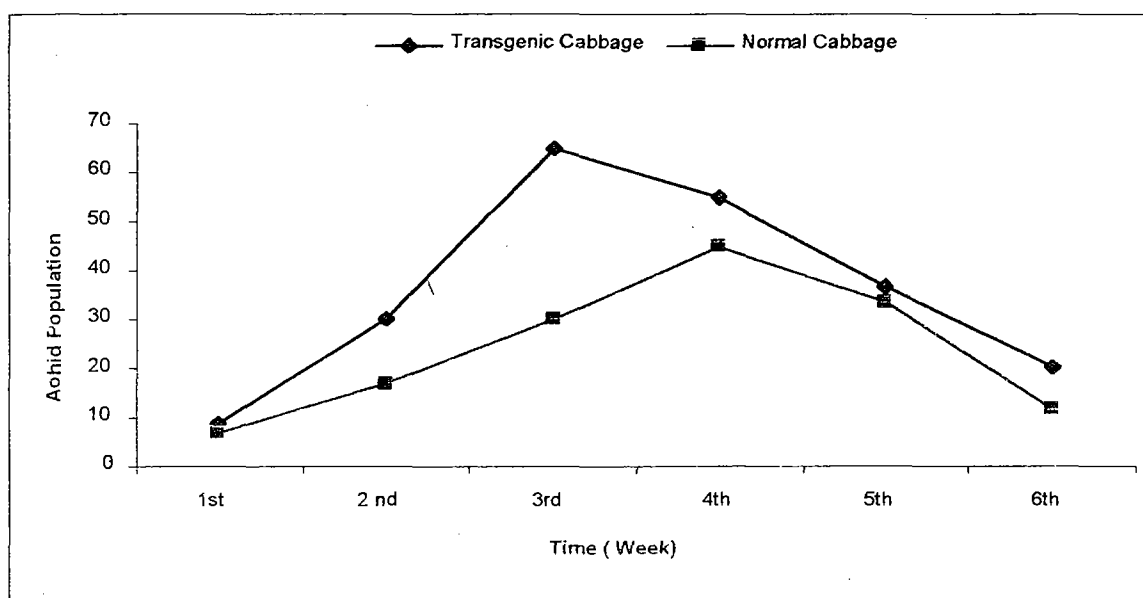


Fig. 24 b. Aphid infestation in normal cabbage

Effect of *Bt* on Aphid in Transgenic and Normal Cabbage during
Winter - March 2004



25 a. Aphid Population in Transgenic and Normal Cabbage during January - February 2001



25 b. Aphid Population in Transgenic and Normal Cabbage during February - March 2002

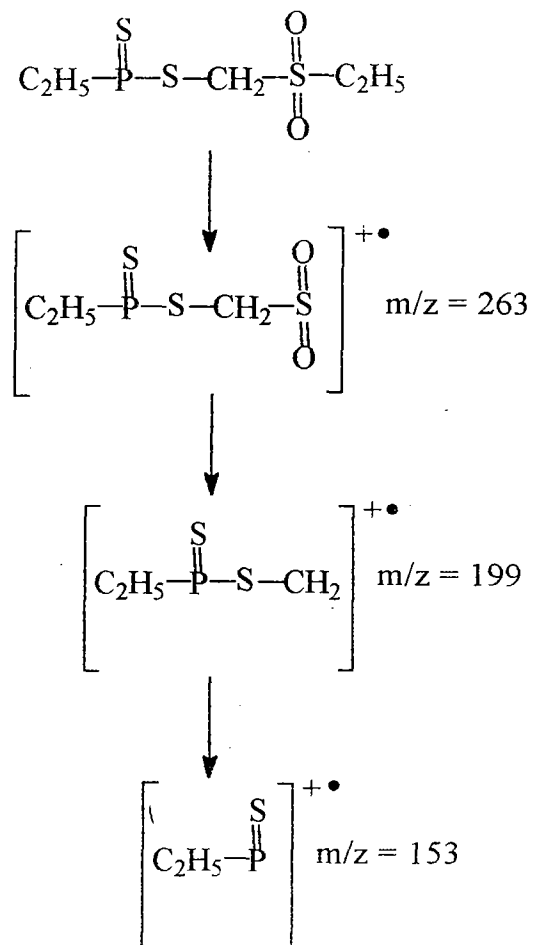
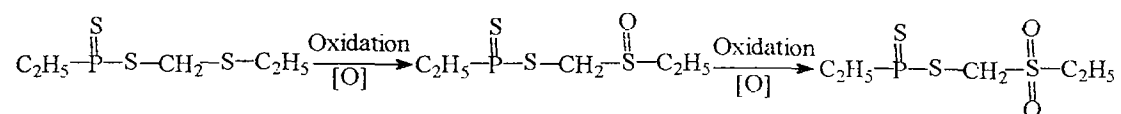


Fig.27. Fragmentation pattern of sulfonmetabolites of phorate

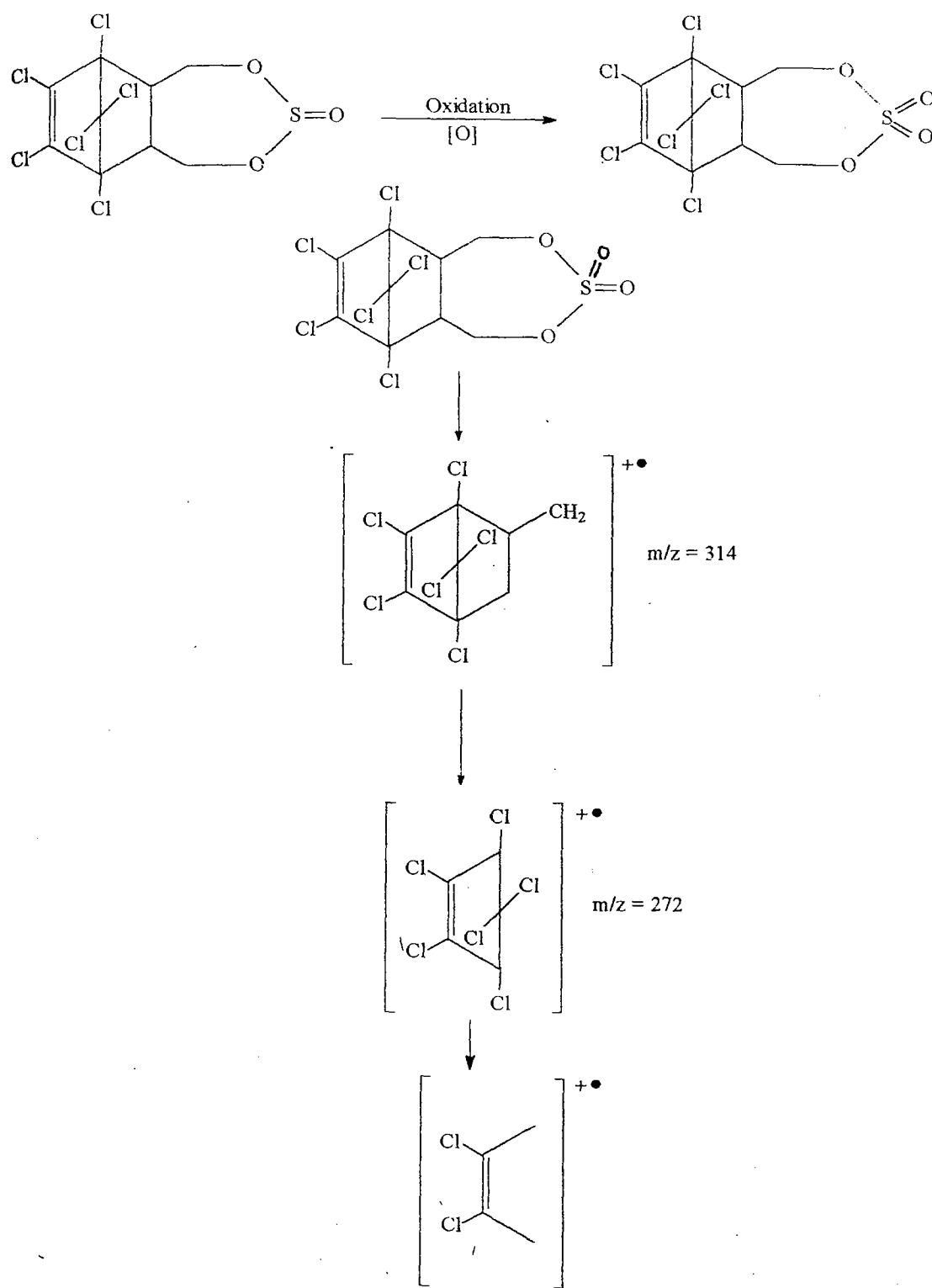


Fig.28. Fragmentation pattern of endosulphan sulphate

Table 34a. Aphid population in Transgenic and Normal Cabbage under field condition during January – February 2001

Aphid Population in Transgenic Cabbage					Aphid Population in Normal Cabbage				
Time	R ₁	R ₂	R ₃	Average	Time	R ₁	R ₂	R ₃	Average
1 st Week	125	150	120	131.67	1 st Week	80	75	65	73.333
2 nd Week	120	175	160	151.67	2 nd Week	70	80	120	90.000
3 rd Week	145	125	100	123.33	3 rd Week	130	120	120	123.33
4 th Week	160	80	145	128.33	4 th Week	90	95	120	101.67
5 th Week	120	70	80	90.000	5 th Week	90	78	70	79.333
6 th Week	60	60	75	65.000	6 th Week	40	30	40	36.667

Table 34b. Aphid population in Transgenic and Normal Cabbage under net house condition during February-March 2002

Aphid Population in Transgenic Cabbage					Aphid Population in Normal Cabbage				
Time	R ₁	R ₂	R ₃	Average	Time	R ₁	R ₂	R ₃	Average
1 st Week	10	10	5	8.3333	1 st Week	10	5	5	6.6667
2 nd Week	30	35	25	30.000	2 nd Week	15	20	15	16.667
3 rd Week	60	75	60	65.000	3 rd Week	25	30	35	30.000
4 th Week	60	55	50	55.000	4 th Week	55	40	40	45.000
5 th Week	30	50	30	36.667	5 th Week	30	45	25	33.333
6 th Week	25	15	20	20.000	6 th Week	10	15	10	11.667

in control soil sample. Among the *Tylenchulus* nematode population, highest number of *Tylenchulus* population was observed in the soil, treated with 40 kg P_2O_5 ha^{-1} . In case of non-transgenic soil sample, higher number of saprophyte nematode was observed in the soil sample treated with 80 kg P_2O_5 ha^{-1} by phosphatic fertilizer (Table 33). The same was also true in case of *Tylenchulus*. Higher number of *Tylenchulus* nematode was found in the control soil sample. When the nematode population was compared with treatmentwise among these two type of rhizospheric soil, it was found that variation of nematode was statistically non significant. Among these three types of nematodes, *Rotylenchulus* and *Tylenchulus* nematode were parasites to the cabbage plant. Though there was non significant variation but the nematode was less in case of transgenic plant as compared to non transgenic plant. It may be due to the root exudates which may have some effect on nematode population in the rhizosphere. Some insecticides of higher dose are known to act as nematicides. Bt-transgenic crop is having mainly insecticidal activity and the amount of Bt toxin present in it over 15 μ g/g. it was worth explaining whether the root exudates of transgenic plant will have some insecticidal activity. Since the transgenic plant has *Cry 1Ab* which is specific for controlling lepidopteran insects, so the variation in the nematode population in the rhizospheric soil of transgenic and non-transgenic cabbage plant is therefore insignificant.

4.18 Aphid population

Aphid population on transgenic and normal cabbage was monitored during January-February 2001 under field condition at 7 days interval (Table 34a). Next year aphid population under net house condition was carried out during February-March 2002 at 7 days interval (Table 34b). From the observation it was found that, higher infestation of aphid occurred in case of transgenic cabbage compared to the

normal cabbage. The aphid population data was statistically analysed it was found that the variation of aphid population was statistically significant in both the years (Fig. 25a and 25b). With the available information the reason for higher aphid infestation in transgenic cabbage may be the following: Since major pest of cabbage, DBM, is controlled by Bt-transgenic plant, aphids may be finding less competition for the same space in the transgenic cabbage as compared to the non-transgenic cabbage. This is the result of this two year study, three location data is needed for finding the exact cause.

4.19 Seed vigour of transgenic & normal cabbage seeds

Different parameters of seed vigour were recorded for transgenic and normal cabbage seeds (Table 35 & 36). From this table it was found that the vigour index-I and vigour index-II for both the transgenic and non transgenic cabbage seed did not vary significantly. So it may be concluded that seed vigour of both transgenic and non transgenic cabbage were more or less same as there was no difference in their vigour indices.

4.20 Synthesis of metabolites

4.20.1 Oxidative product of fipronil

The oxidation reaction of fipronil was monitored by TLC periodically after every 4 hrs. After 12 hours of continuous stirring at 54°C temperature the starting material disappeared and got transformed into a less polar product. The reaction of oxone^R with fipronil gave almost 100% yield. HPLC analysis of the reaction showed a new peak whose Rt tallied with one of the authentic standard of a fipronil metabolite procured from Rhone and Poulenc. This product was found to be sulfone metabolite. The product however, could not be separated using preparatory TLC due to poor resolution of the parent compound and the metabolites. The reaction mixture

Table 35. Germination Test of Transgenic and Normal Cabbage seeds.

	Normal	Abnormal	Dead	Fresh Ungerminated	Hard	Germination %
Transgenic cabbage						93.6
R ₁	21	4	0	0	0	
R ₂	24	0	1	0	0	
R ₃	25	0	0	0	0	
R ₄	22	3	0	0	0	
R ₅	25	0	0	0	0	
R ₆	23	0	2	0	0	
R ₇	25	0	0	0	0	
R ₈	23	2	0	0	0	
R ₉	23	2	0	0	0	
R ₁₀	23	0	2	0	0	
Non Transgenic Cabbage						90.8
R1	25	0	0	0	0	
R2	22	3	0	0	0	
R3	24	0	1	0	0	
R4	23	2	0	0	0	
F5	23	1	1	0	0	
R6	23	2	0	0	0	
R7	19	6	0	0	0	
R8	23	0	2	0	0	
R9	23	2	0	0	0	
R10	22	2	1	0	0	

Table 36. Estimation of Seedling Vigour of Transgenic and Non Transgenic Cabbage

Replications	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Seedling dry-weights (g)	Average seedling length (cm)	Average seedling dry-weight (g)	Germi- nation (%)	Vigour index-I	Vigour index-II
Transgenic Cabbage									
R ₁	4.5	6.8	11.3	0.0513	11.7	0.04928	93.6	1095.12	4.612608
R ₂	3.6	5.1	8.7	0.0393					
R ₃	5.6	6.8	12.4	0.0453					
R ₄	4.3	3.9	8.2	0.0498					
R ₅	5.1	8.6	13.7	0.0463					
R ₆	6.1	8.1	14.2	0.0633					
R ₇	2.8	5.7	8.5	0.0485					
R ₈	3.7	6.7	10.4	0.0410					
R ₉	5.4	10.5	15.9	0.0683					
R ₁₀	4.8	8.9	13.7	0.0400					
Non Transgenic Cabbage									
R ₁	8.5	7.8	16.3	0.0533	12.19	0.04865	90.8	1106.852	4.41742
R ₂	5.4	6.4	11.8	0.0470					
R ₃	6.1	8.4	14.5	0.0570					
R ₄	5.8	4.1	9.9	0.0435					
F ₂	7.6	6.4	14	0.0495					
R ₆	2.8	4.1	6.9	0.0460					
R ₇	6.1	8.5	14.6	0.0463					
R ₈	4.4	3.8	8.2	0.0505					
R ₉	6.1	6.8	12.9	0.0473					
R ₁₀	5.5	7.3	12.8	0.0463					

on TLC plate was run using acetone : Benzene (10:90). The R_f value of sulfone was 0.7143 and for fipronil it was 0.5986. The fractions were therefore separated and collected by preparatory HPLC and then injected in GLC for confirmation of the same. Its identify was further confirmed by NMR and finally by Mass spectroscopy (Molecular ion peak at 452, Appendix II). The compound was identified as $\pm$ -5-amino-1-(2,6 dichloro- $\alpha\alpha\alpha$ trifluoro-p-tolyl)-4-trifluoromethyl-sulfonyl pyrazole-3-carbonitrile. Same method was also applied for the preparation of sulfone metabolite of phorate and sulfate of endosulfan. It was found that this method effectively converted 65% phorate to its sulfone metabolite and 36% endosulfan parent compared into its sulfate metabolite. Beside sulfate metabolite, other metabolite of endosulfan like ether and lactone was also formed during this reaction, so the total amount of sulfate production became lesser in amount. Ether and sulfate metabolite of endosulfan and sulfone metabolite of phorate were identified by GCMS. The fragmentation pattern of these metabolites are presented in the fig.25, 26, 27. Chromatograph and Mass fragmentation of these pesticides are presented in Appendix II.

4.21 Balance sheet of transgenic and normal cabbage cultivation

Input use and return for the cultivation of normal as well as transgenic cabbage were calculated to compare their relative profitability and the summary is reported in table 37. Total cost of cultivation was found to be (nearly Rs. 32,000/ha.) higher for transgenic cabbage as compared to normal cabbage (nearly Rs. 28,000/ha.). The major cost component was observed to be the use of human labour, irrespective of the type of cabbage. Similar trend was also observed for the other cost components, such as transplanting of seedling (nearly 10%), fertilizer use (nearly 11%), irrigation applied (nearly 5%), machine labour used (nearly 5%) and

Table 37. Input Use and Return of Normal vis-à-vis Transgenic Cabbage Cultivation

	Normal Cabbage	Transgenic Cabbage
1 Share of Input Use (%)		
a. Human Labour	36.17	31.53
b. Machine Labour	5.42	4.73
c. Seed	1.01	18.92
d. Transplanting	10.85	9.46
e. Fertiliser (N+P+K)	11.57	10.09
f. Irrigation	5.42	4.73
g. Pesticides		
(i) Pendimethalin	7.16	6.24
(ii) Iprodione	2.15	1.87
(iii) Fipronil	4.02	0.00
(iv) Imidacloprid	1.03	0.00
(v) Bt formulation	4.34	0.00
(vi) Thiacloprid	0.00	1.40
(vii) Harvesting	10.85	11.03
2 Total Cost	100	100
	(27650)	(31719)
3 Yield (q / ha)	320	365
4 Gross Return (Rs. / ha)	64000	77000
5 Net Return (Rs. / ha)	36350	45281
6 Output-Input Ratio	2.31	2.43

Figures in parentheses indicate total cost in Rs.

for harvesting (nearly 11%). but the expenditure on seed was found to be substantially higher for transgenic cabbage (19%) as compared to normal cabbage (1%). Regarding the use of pesticides, pendimethalin accounted for nearly 7% of total cost under normal cabbage cultivation, while the same was nearly 6% for transgenic cabbage. The level of iprodione used was quite similar for both the cabbages and was accounted for nearly 2% of total cost of cultivation. The other pesticides, fipronil, imidacloprid, Bt. formulation were applied only on normal cabbage. These three pesticides were not applied on transgenic cabbage due to less pest infestation. However, eco-friendly pesticide thiacloprid was used for transgenic cabbage cultivation and accounted for nearly 1% of total cost.

Substantial higher yield (365 q ha^{-1}) was obtained under transgenic cabbage cultivation as compared to normal cabbage (320 q ha^{-1}). Similarly gross return was also found to be higher for transgenic cabbage (Rs. 77,000 ha^{-1}) than normal cabbage (Rs. 64,000 ha^{-1}). So the higher cost of cultivation of transgenic cabbage was compensated through realizing higher net return (Rs. 45,281) for the same as compared to the net return (Rs. 36,350) from normal cabbage. The output ratio was calculated to be higher for transgenic cabbage cultivation (2.43) than normal cabbage (2.31). The cost of transgenic cotton seed was compared with that of non-transgenic cotton seed and the same factor of the cost of cotton seeds has been used to calculate the real cost of transgenic cabbage seed. This is the first attempt of this kind. Some of the results are preliminary. More work will have to be done to validate these results by field studies.

Chapter - 5

Summary and Conclusions

5. SUMMARY AND CONCLUSION

Behaviour, usage and need of pesticides were compared in transgenic and normal cabbage; a study was carried out to assess the similarity and dissimilarity in these two types of cabbage plants. The persistence study of fipronil, applied at the rate of 50 g and 100 g a.i. ha⁻¹ and thiacloprid, applied at the rate of 72 g and 144 g a.i. ha⁻¹ were done. Residues of both the compound were detectable up to 15 days on the treated cabbage. The metabolites of the pesticides found on the leaves and in the soil were identified and quantified. Besides these, comparison of plant nutrient content, uptake of phosphorus, aphid population, rhizospheric population of nematode, bacteria, actinomycetes, fungi, phosphate solubilizing bacteria, dehydrogenase activity and finally input output cost of both type of cabbage cultivation were carried out. Results are summarized below.

A. Confirmation of Bt-transgenic cabbage crop

- i. Plant DNA was isolated from both the transgenic and normal cabbage and amplified with npt-II, cry1B, and cry 1Ab primer. PCR analysis of transgenic plants showed positive results for the presence of Bt-gene in it.
- ii. Protein in the transgenic and normal cabbage was extracted and analysed through SDS-PAGE. Protein from transgenic plant gave band at 135 kDa, which was absent in case of normal cabbage.
- iii. Protein extracted from transgenic and normal cabbage, were analysed by DAS-ELISA technique for quantification of Bt- protein present in it. The Bt- protein content varied from 15 µg/g to 20.48µg/g fresh leaf weight in different lines of transgenic cabbage plant.

- iv. DBM larvae in neonate, 2nd and 3rd instar stage died within 48 hrs. in leaf disc, detached leaf, and whole plant bioassay respectively.

B. Preparation of metabolite, recovery and Standardization of HPLC Technique

- i. A new method for the synthesis of sulfone metabolite was developed the product was characterized by IR, NMR & GCMS.
- ii. A HPLC technique was standardized for quantification of residue of fipronil and its metabolites in plant extract.
- iii. Recovery of fipronil residues from fortified cabbage were quantified using chloroform as extraction solvent and neutral alumina with florisil as adsorbent for clean up. The recovery was 83.0 to 86.1 % from plant.
- iv. Recovery of thiacloprid residues from fortified cabbage was carried out using dichloromethane as partitioning solvent and Florisil as adsorbent for clean up. Acetonitrile was used as final eluting solvent of thiacloprid from column. The recovery of the method was 92%.
- v. Recovery of fipronil from soil was 82%.

C. Persistence of pesticides

- i. The half life of fipronil applied on transgenic cabbage was found to be 8.3 days. The half life of fipronil in normal cabbage was 7.5 and 7.4 days for the two rates of application.
- ii. Two metabolites of fipronil desulfonyl fipronil and sulfone metabolite of fipronil were identified by chromatographic and spectroscopic methods in both types of cabbage crops. The highest amount of oxidative product was recorded after 3 days of application, on transgenic and normal cabbage as well as in their rhizospheric soil. The photoproduct of fipronil was observed

in lesser amount in the two types of cabbages but present even after 15 days of application.

3. Persistence of pesticides in rhizosphere of transgenic and normal cabbage.

- i) Dissipation of fipronil in transgenic as well as normal cabbage rhizosphere was 87 to 90% of fipronil application. The half life of fipronil in rhizospheres of both normal and transgenic cabbage was 5 days.
- ii) Photo product and oxidative metabolite of fipronil was identified in both the rhizosphere. Maximum amount of oxidative metabolite of fipronil was detected in 3 day after application.

4. Persistence of thiacloprid in transgenic and normal cabbage

- i) The half life of thiacloprid in transgenic as well as in normal cabbage were 12.3 days for single dose of application. 59.25% and 57.51 % dissipation of thiacloprid occurred in 15 days at low and higher rates of application.
- ii) Under field condition thiacloprid dissipated 63.98 and 62.68% for single dose and double dose application respectively on normal cabbage. The half life of thiacloprid was 10.75 days. No metabolite of thiacloprid was detected in cabbage.
- iii) Decontamination of thiacloprid contaminated cabbage was carried out by different chemical treatments. NaOH, NaHCO₃, KMnO₄ and water tried to decontaminate thiacloprid contaminated cabbaged. 0.5% NaOH treatment decontaminated 64.44% thiacloprid for one hour soaking and it was the highest as compared other chemical treatments.

D. Nutrient content in transgenic and normal cabbage.

- i) Variation in primary nutrient, namely phosphorus, potassium and protein content in transgenic and normal cabbage was found to be statistically insignificant.

- ii) The variation in secondary nutrient content namely calcium, magnesium and sulfur in transgenic plants were higher as compared to normal cabbage and these variations were statistically significant.

E. Dehydrogenase activity in the rhizosphere of transgenic and normal cabbage.

The difference in dehydrogenase activities in the rhizosphere of transgenic and normal cabbage was statistically insignificant on all the three days 25, 50 and 75 days of soil sampling.

F. Phosphorus utilization pattern of transgenic and normal cabbage.

- i) Phosphorus content in these two types of cabbage plant was more or less equal i.e. the variation was statistically insignificant. The variation in % phosphorus derived from fertilizer and percent of phosphorus utilization by transgenic cabbage was statistically higher as compared to normal cabbage and it was statistically significant.
- ii) In 50 and 75 days samplings, the phosphorus content in transgenic and normal cabbage was same i.e. this variation was not statistically significant. The dry matter yield, total phosphorus uptake, percent phosphorus derived from fertilizer and percent phosphorus utilization was higher in transgenic cabbage and all these were statistically significant.

G. Microbial population in the rhizospheric soil of transgenic and normal cabbage

- i) The bacterial, fungal population was comparatively less in transgenic cabbage rhizosphere as compared to that normal cabbage of 25 days soil sampling. In 50 and 75 days of soil sampling, the populations of both organisms were similar.

- ii) Variation in fungal population was statistically significant due to variety (transgenic and non-transgenic) in the soil sample of 75 days after transplanting.
- iii) The variation in actinomycetes population was not statistically significant.
- iv) Phosphorus solubilizing bacteria population in the rhizosphere of transgenic cabbage plant upto 25 DAT was more or less same as compared to the normal cabbage rhizosphere. The population was higher in case of transgenic plant rhizosphere after 50 and 75 days after transplanting as compared to normal cabbage and this variation was statistically significant.

H. Nematode population in the rhizosphere of transgenic and normal cabbage

Nematode population of *Rotylenchulus*, *Tylenchulus* and Saprophyte was less in transgenic cabbage rhizosphere of 25, 50, 75 days after transplaining as compared to normal cabbage rhizosphere. But the variation of these nematode population was not statistically significant.

I. Aphid population on transgenic and normal cabbage

The aphid population was higher in case of transgenic cabbage as compared to normal cabbage plant during January-February 2001 under field condition and also under net house condition during February - March 2002. This variation of aphid population was statistically significant.

J. Seed vigour of transgenic and normal cabbage seeds

Seed vigour indices for both the transgenic and normal cabbage was more or less same.

K. Balance sheet of transgenic and normal cabbage cultivation

Only one application of systemic insecticide thiacloprid and need base application of contact fungicide, iprodione was enough for cultivation of transgenic cabbage as compared to 4-5 applications of insecticide and one application of fungicides in normal cabbage.

There by concluding that transgenic plants require less number of application of synthetic pesticides, so the produce has less pesticides residues in comparison to that of non-transgenic cabbage plant and consequently less problems of toxic residue. Though the cost of cultivation of transgenic cabbage was higher but the net profit from transgenic cabbage cultivation was higher as compared to normal cabbage.

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* Original not seen

Appendix - I

Jan-Mar2003

Date (Jan)	Temperature			Relative Humidity			Rainfall (mm)	Sunshine (hours)	Evaporation (mm)	Avg Wind Velocity (km/hr)
	Maximum	Minimum	Average	Forenoon	Afternoon	Mean				
1	11.6	9.8	10.7	90.8	35.0	62.9	25.2	6.9	3.9	3.8
2	15.5	7.5	11.5	86.3	29.0	57.7	0.0	7.9	1.1	3.2
3	13.9	6.6	10.2	90.4	31.0	60.7	0.3	7.1	1.9	5.5
4	14.1	9.0	11.5	90.6	27.0	58.8	0.0	6.9	1.6	5.1
5	13.6	7.8	10.7	84.2	32.0	58.1	0.0	6.9	2.2	2.5
6	13.4	5.1	9.3	87.9	29.0	58.5	0.0	8.2	4.0	5.7
7	13.6	4.7	9.1	87.7	51.0	69.4	0.3	8.3	3.4	5.9
8	9.6	6.7	8.1	90.6	41.0	65.8	0.0	1.1	2.2	4.5
9	12.3	4.5	8.4	83.1	62.0	72.6	0.0	5.9	2.0	2.5
10	14.1	4.2	9.1	82.7	46.0	64.4	0.0	0.8	1.8	5.1
11	11.1	2.7	6.9	89.0	54.0	71.5	0.0	4.9	1.6	6.2
12	12.0	3.7	7.9	88.4	46.0	67.2	0.0	4.8	2.2	6.4
13	14.1	3.1	8.6	84.9	32.0	58.5	0.0	6.3	2.6	5.2
14	16.2	4.5	10.3	81.8	33.0	57.4	0.0	4.2	2.0	4.2
15	15.2	4.8	10.0	81.9	43.0	62.5	0.0	5.7	2.2	3.8
16	15.7	2.9	9.3	84.5	37.0	60.8	0.3	6.0	2.0	4.4
17	14.4	3.3	8.8	87.5	31.0	59.3	0.0	6.8	1.6	5.1
18	15.0	6.4	10.7	88.0	37.0	62.5	0.3	7.4	2.0	4.3
19	15.0	4.5	9.7	88.8	34.0	61.4	0.0	3.2	2.2	4.5
20	14.0	4.5	9.3	90.0	31.0	60.5	0.3	7.3	2.0	4.4
21	13.0	3.3	8.2	88.5	38.0	63.3	0.0	7.0	1.8	3.9
22	15.5	3.6	9.6	84.3	31.0	57.7	0.3	6.3	1.6	3.5
23	20.5	2.8	11.6	72.5	34.0	53.3	0.0	5.5	1.8	2.4
24	22.4	5.2	13.8	74.2	28.0	51.1	0.0	7.3	2.0	2.9
25	22.6	7.1	14.9	71.7	32.0	51.9	0.0	7.2	2.0	3.2
26	23.3	5.7	14.5	69.3	32.0	50.7	0.0	7.8	3.5	3.3
27	25.1	7.3	16.2	67.5	65.0	66.3	0.0	6.1	3.2	2.9

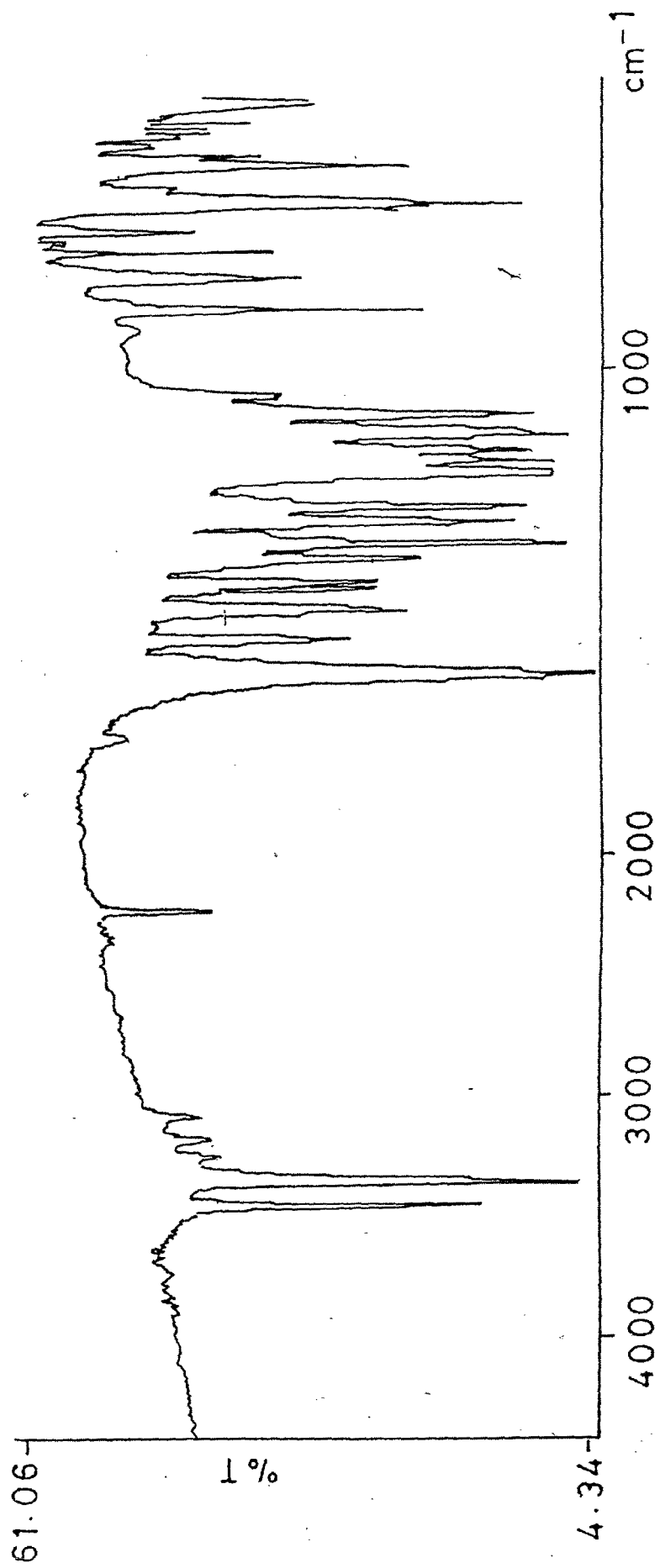
2	22.1	11.6	16.9	69.7	32.0	50.9	0.0	8.6	2.6	1.8
24	25.6	10.9	18.2	71.9	37.0	54.5	0.0	8.4	0.6	2.2
25	25.5	13.3	19.4	71.5	28.0	49.8	0.0	8.4	1.1	1.8
26	25.6	13.6	19.6	69.0	23.0	46.0	0.0	8.6	1.9	1.8
27	27.5	13.8	20.6	67.8	25.0	46.4	0.0	6.8	0.9	1.8
28	27.6	14.8	21.2	71.0	24.0	47.5	0.0	7.8	0.9	1.9

Jan-Mar2003

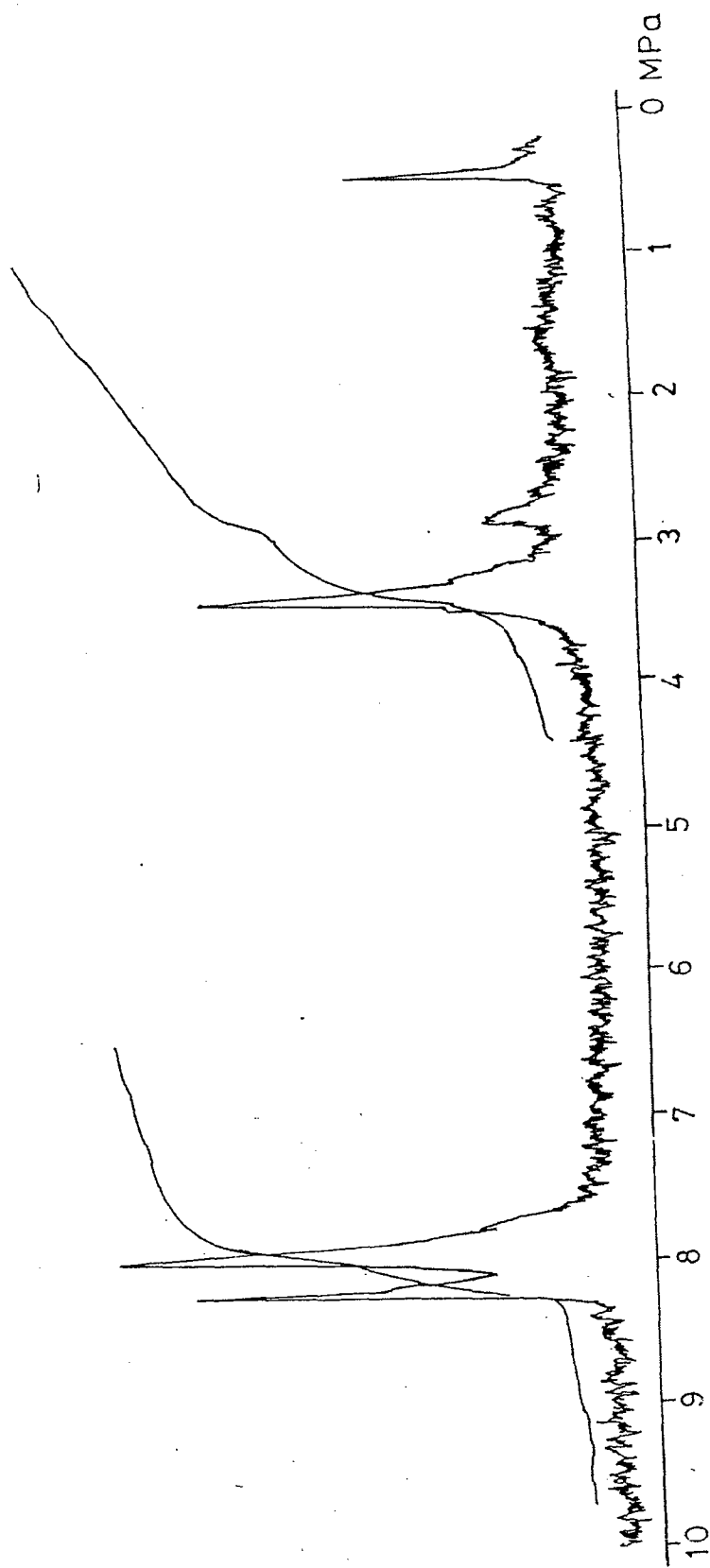
Date (Mar)	Temperature			Relative Humidity			Rainfall (mm)	Sunshine (hours)	Evaporation (mm)	Avg Wind Velocity (km/hr)
	Maximum	Minimum	Average	Forenoon	Afternoon	Mean				
1	26.8	16.5	21.6	77.7	24.0	50.9	15.2	5.0	1.3	3.3
2	26.1	17.2	21.7	74.2	18.0	46.1	0.0	8.2	3.0	1.8
3	28.1	16.8	22.5	70.7	21.0	45.9	0.0	9.0	2.4	1.8
4	23.1	14.5	18.8	40.5	15.0	27.8	0.0	8.9	2.7	1.8
5	17.8	10.7	14.2	43.3	30.0	36.6	0.0	8.5	4.1	1.8
6	21.4	8.0	14.7	47.7	24.0	35.8	0.0	6.9	4.3	1.8
7	24.0	7.6	15.8	50.9	27.0	38.9	0.0	8.5	1.8	1.8
8	23.2	9.6	16.4	51.6	22.0	36.8	0.0	9.5	3.0	1.8
9	24.3	12.2	18.2	46.7	22.0	34.4	0.0	9.2	3.3	1.8
10	29.0	8.9	19.0	54.6	20.0	37.3	0.0	9.0	1.1	1.8
11	27.9	14.7	21.3	52.9	27.0	40.0	0.0	8.2	2.3	1.8
12	25.1	12.7	18.9	64.6	36.0	50.3	0.0	7.3	1.0	1.8
13	27.7	11.3	19.5	60.7	41.0	50.8	0.0	1.2	0.7	1.8
14	27.0	12.5	19.7	64.1	29.0	46.5	0.0	1.8	0.6	1.8
15	24.6	12.9	18.7	66.5	19.0	42.8	0.0	7.1	1.9	1.8
16	29.1	13.0	21.0	59.9	20.0	40.0	0.0	5.8	0.7	1.8
17	30.7	16.6	23.6	51.7	19.0	35.4	0.0	8.8	1.9	1.8
18	31.3	14.8	23.0	50.6	21.0	35.8	0.0	7.5	0.8	1.8
19	31.0	14.9	22.9	39.7	22.0	30.9	0.0	6.2	0.9	1.8
20	29.1	16.7	22.9	44.0	43.0	43.5	0.0	9.6	1.7	1.8

21	29.8	17.0	23.4	46.8	44.0	45.4	0.0	2.7	2.9	1.8
22	31.1	16.1	23.6	52.7	52.0	52.3	0.0	0.1	1.5	1.8
23	32.9	15.3	24.1	59.4	45.0	52.2	0.0	0.4	0.8	2.0
24	32.9	15.9	24.4	56.6	42.0	49.3	0.0	3.4	0.7	1.9
25	31.4	18.6	25.0	56.9	64.0	60.4	0.0	2.4	1.5	2.1
26	32.0	16.2	24.1	58.3	34.0	46.1	1.3	0.0	0.7	2.6
27	31.1	16.9	24.0	55.3	36.0	45.7	0.0	4.1	1.0	1.7
28	31.1	16.8	23.9	58.7	25.0	41.8	0.0	6.0	0.9	1.7
29	32.6	15.7	24.1	52.7	29.0	40.9	0.0	8.1	1.1	1.8
30	31.8	20.4	26.1	47.8	22.0	34.9	0.0	6.4	1.8	1.8
31	31.6	19.8	25.7	62.0	19.0	40.5	2.0	7.4	1.2	2.3

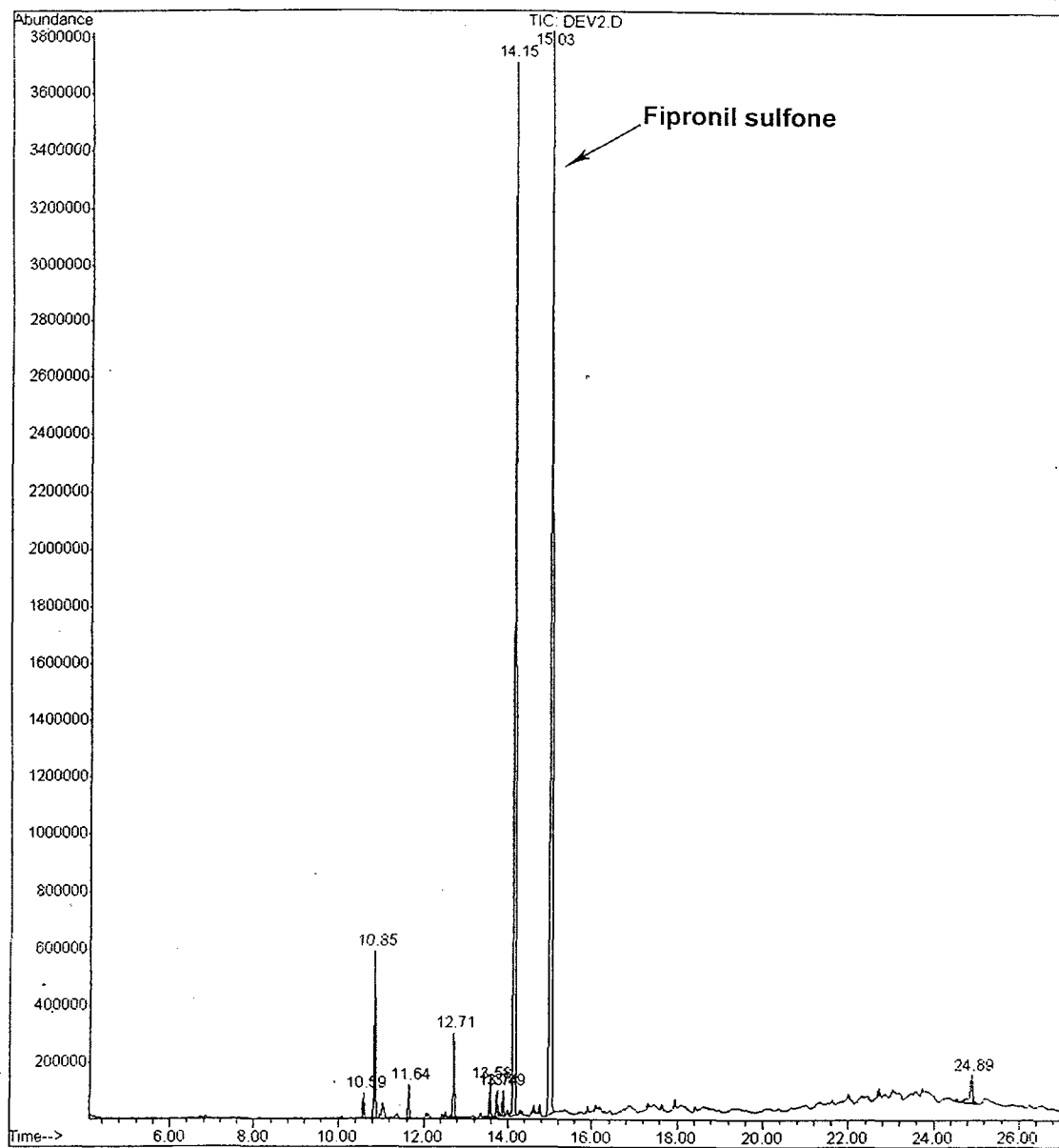
Appendix - II



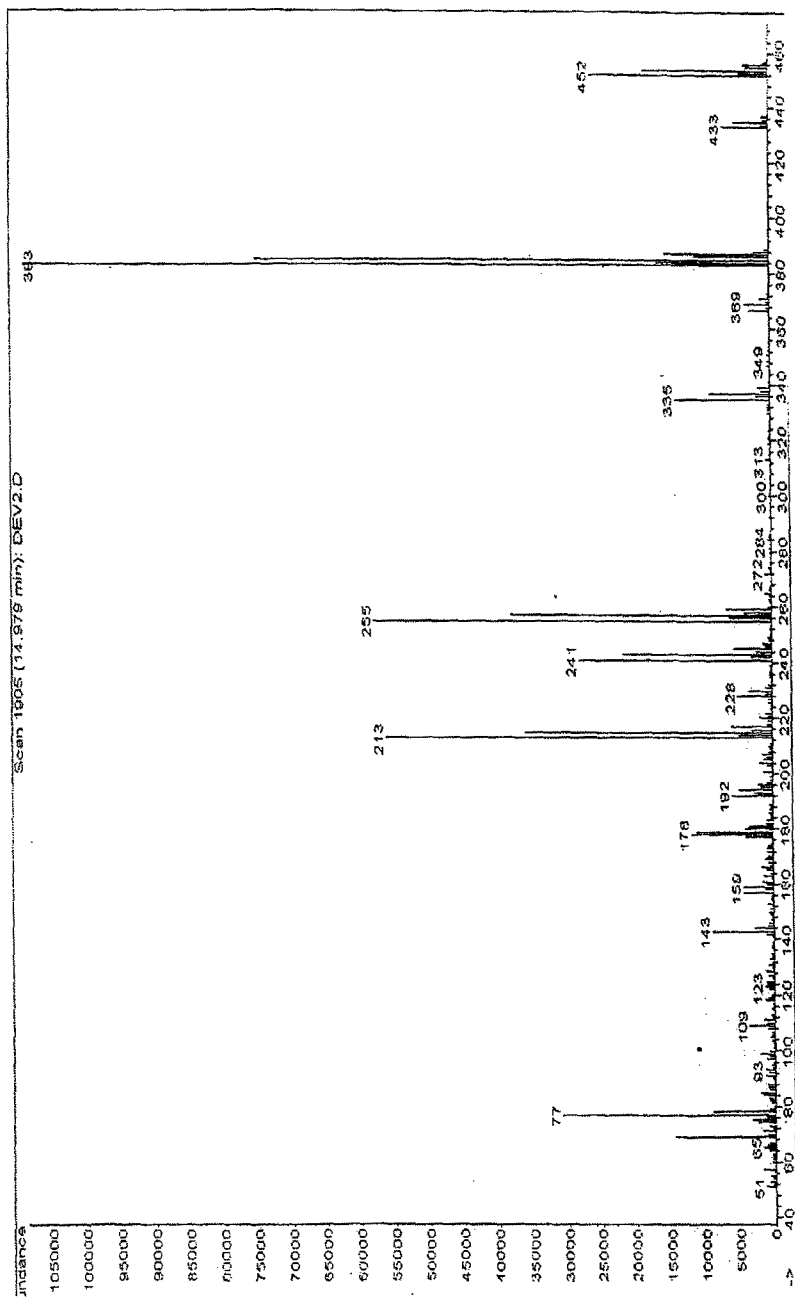
IR spectra of fipronil sulfone metabolite



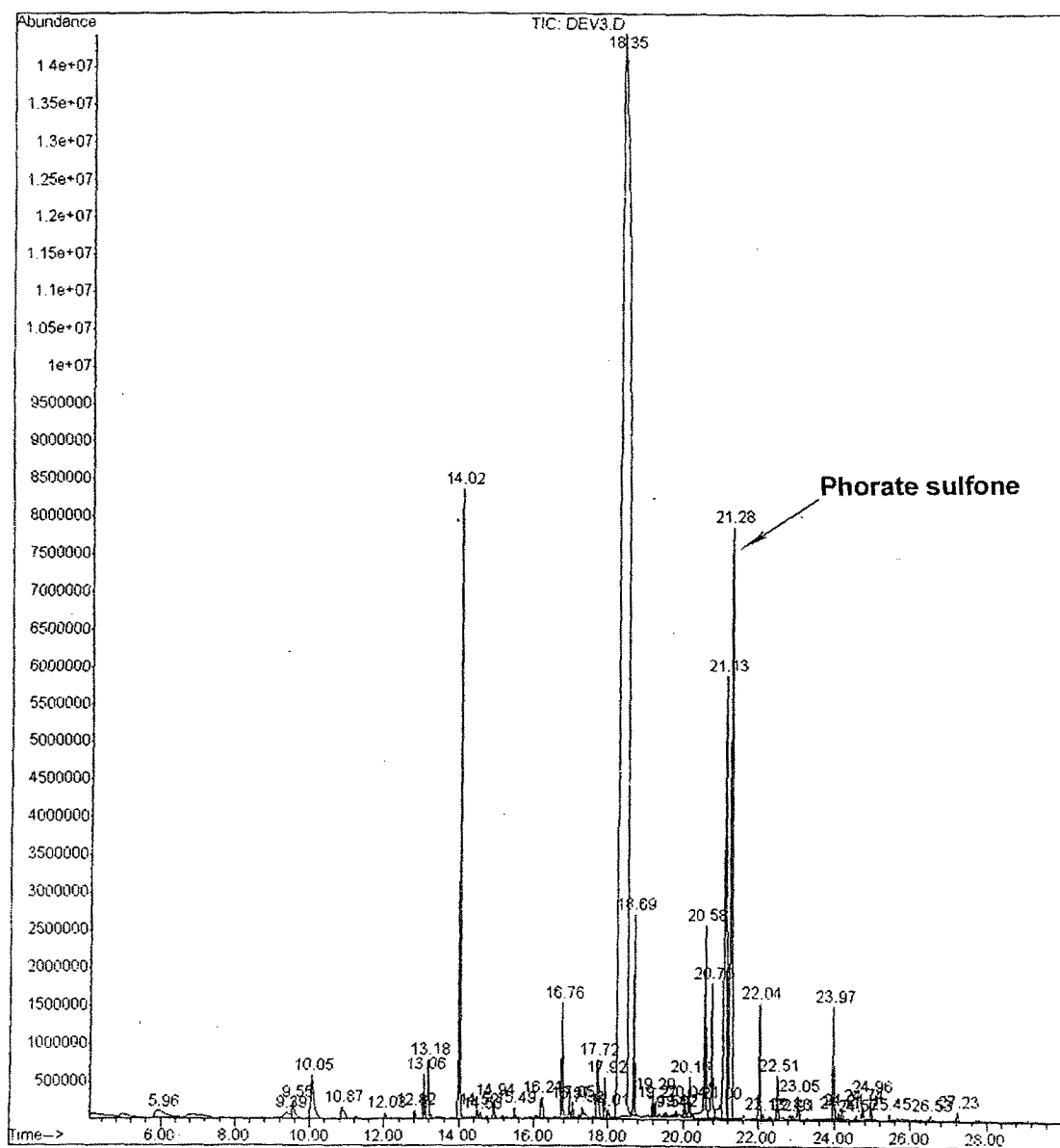
NMR spectra of fipronil sulfone metabolite.



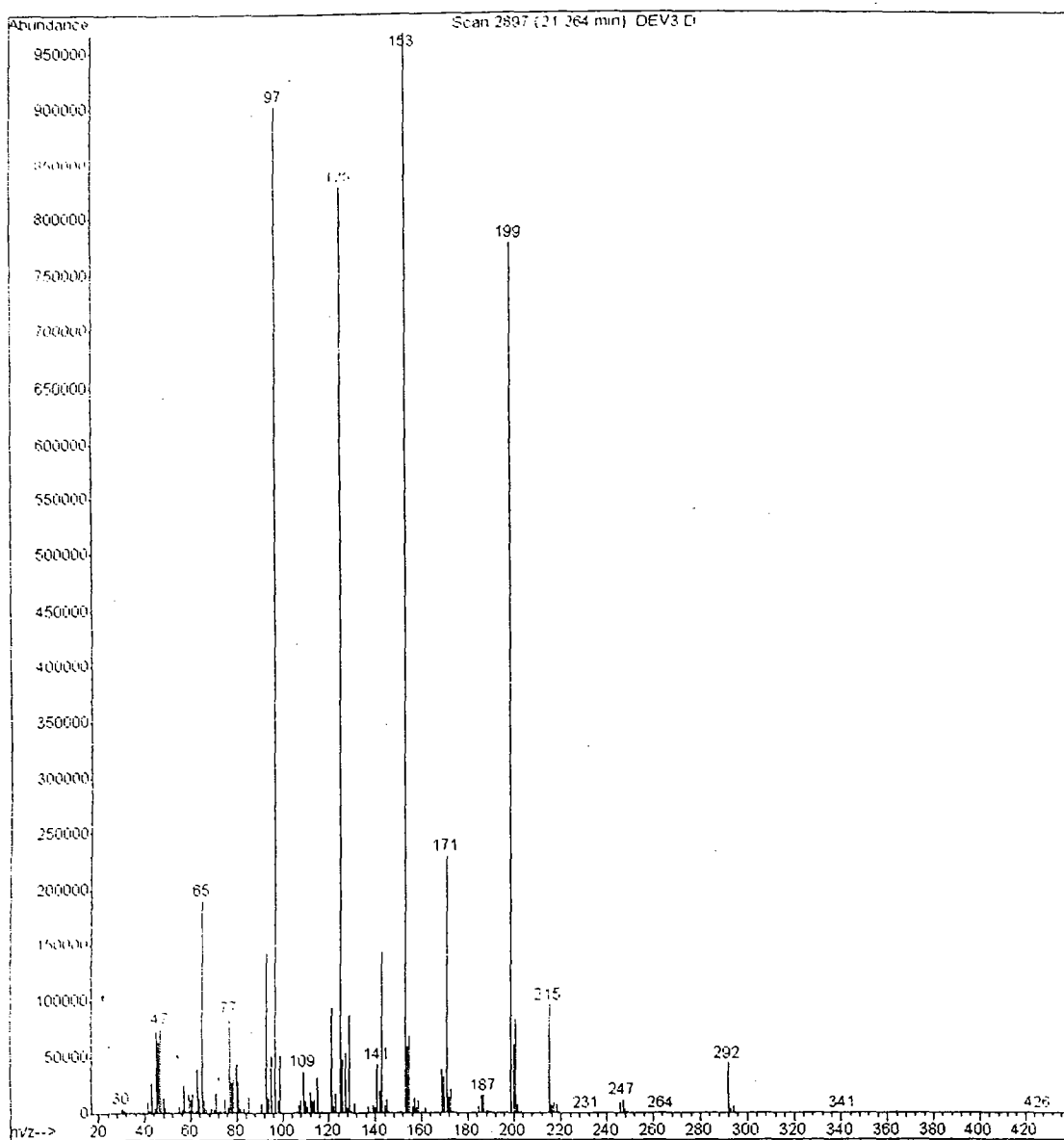
GLC chromatograph of fipronil sulfone metabolite



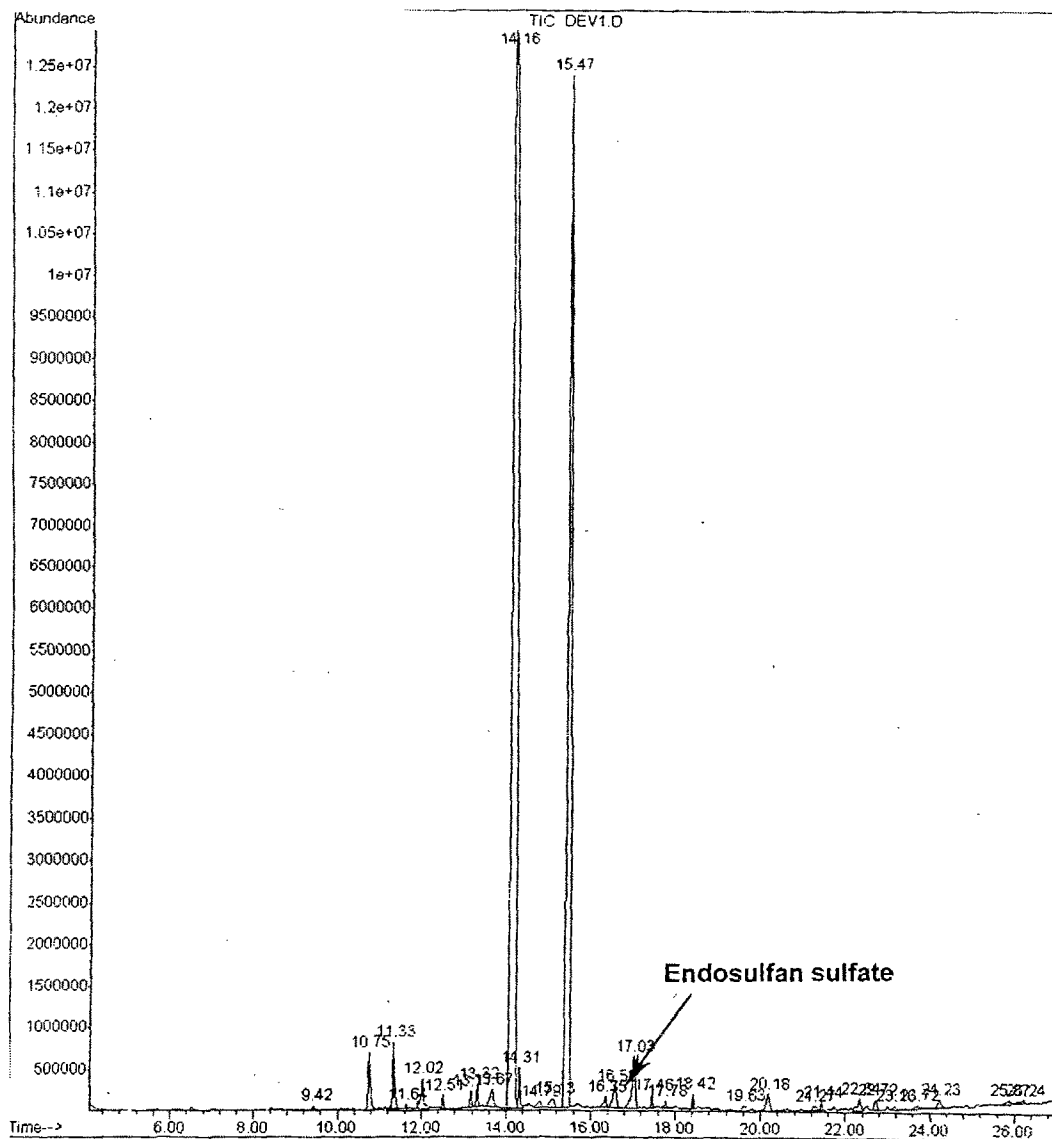
Chromatogram of fipronil sulfone mass fragments



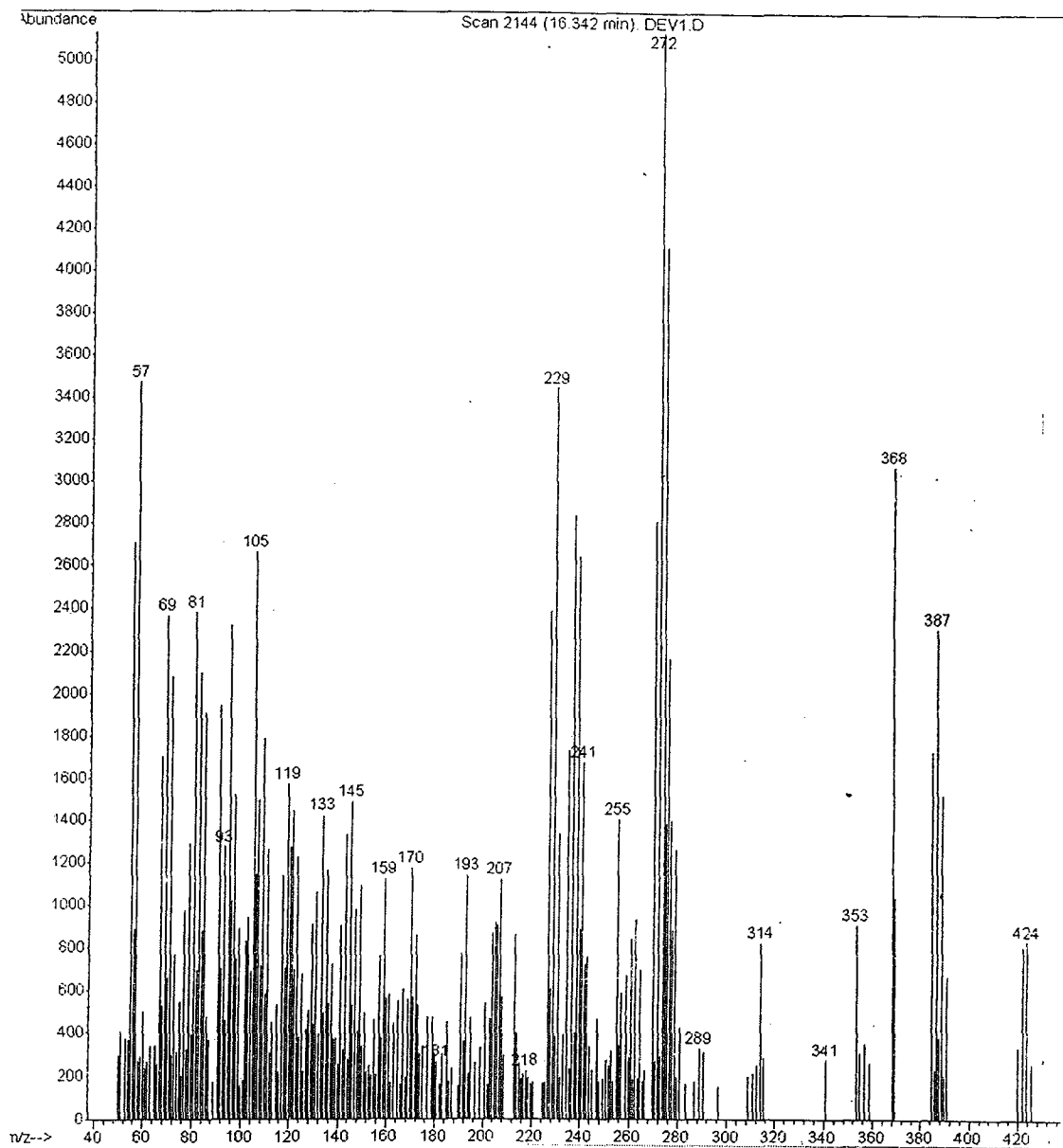
GLC chromatogram of phorate sulfone metabolite



Chromatogram of phorate sulfone mass fragments



GLC chromatograph of endosulfan sulfate



Chromatogram of endosulfan sulfate mass fragments.

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