Impact of insecticide cartap hydrochloride on bacterial biofertilizers

DISSERTATION

Submitted by

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In partial fulfilment for the award of the degree of

Master of Science in Biotechnology

Under the Guidance of

Prof. Dinesh Goyal

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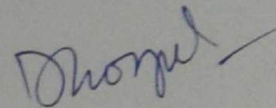
JULY, 2017

CERTIFICATE

Certified that the thesis entitled '**Impact of insecticide cartap hydrochloride on bacterial biofertilizers**' submitted by Ms. **Loveleen Kaur** (301501009) in partial fulfilment of the requirement for the award of the degree of **Master's of Science** in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, Punjab is the record of candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for award of any degree.



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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Impact of insecticide cartap hydrochloride on bacterial biofertilizers**" submitted by me for the award of the degree of **Masters in Science** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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ACKNOWLEDGEMENT

I owe my endless gratitude and regards to my supervisor, **Dr. Dinesh Goyal,** Professor, Department of Biotechnology for his guidance, co-operation, supervision & timely help throughout this project without which this project would not have been possible to reach its goal.

I wish to express my deep sense of humbleness to **Dr. Moushumi Ghosh,** Head of Departament of Biotechnology, Thapar University, Patiala (Punjab) for her support.

Though it was an independent project but without my lab seniors' advice and guidance it was impossible to make out. I would like to thanks **Mrs. Ishtpreet Kaur** and **Mrs. Simarpreet Kaur**. They were always there to support me and guide me by sharing their knowledge and skills. I am also very thankful to STEP for providing me all the logistic and other instrumental assistance. I am thankful to **Mr. Radhe Shyam** for his help during lab work.

Heart–felt thanks to all my friends and lab mates, **Tavinder Kaur**, **Aakriti, Karamjeet Kaur, Jenia** and **Saruchi** for their unforgettable moral support, affection and company.

How can I forget to pen down my deepest sense of indebtedness towards my parents who left no stone unturned to fulfil my cherished dream of pursuing higher studies in this esteemed university. I express my gratitude to all my family members who soulfully provided me with their constant support to undertake these challenges. No words are enough to describe their unconditional love, blessings and co-operation which enabled me to achieve every goal of my life. I am thankful to my lovely siblings **Kirandeep Kaur**, **Jasjeet Singh** for always being on my side. I consider myself the luckiest in the world to have such a supportive family.

Last but not least, it has been a privilege to spend memorable two years in Thapar University. I wish to acknowledge all those, whose names have not been figured here, but who helped me in any form during the period of my research work.

Loveleen

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Place: Patiala

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Dedicated

To

My parents

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LIST OF SYMBOLS

%	Percentage
˚C	Degree Celsius
N	Nitrogen
P	Phosphorous
Al	Aluminum
Ca	Calcium
Fe	Iron
µg	Microgram
µgN/ml	Microgram normality/millilitre
µm	Micrometer

LIST OF ABRREVATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ATCC	American type culture collection
BIS	Bureau of Indian Standards
BNF	Biological nitrogen fixation
cfu	Colony forming unit
cm	Centimetre
DNA	Deoxyribonucleic acid
DTNB	5,5-dithiobis (2-nitrobenzoic acid)
eg.	Example
Fig.	Figure
g	Gram
g/ha	Gram/hectare
g/L	Gram/litre
GA	Gibberllic acid
hr	Hour
i.e	that is
IAA	Indole-3-acetic acid
kg	Kilogram
LB	Luria-Bertani broth
M	Molarity
mg	Miligarm
mg/L	Miligrm/litre
mg/ml	mili-gram/mili-litre
min	Minutes
mL	Millilitre
mm	Milimeter
MM	Minimal media
N	Normality
NB	Nutrient broth
NBF	Nitrogen fixing fungus
nm	Nanometer
OD	Optical density

OMD	Organic matter decomposer
PGPR	Plant growth promoting rhizobacteria
PKV	Pikovaskaya broth
ppm	Parts per millions
PSB	Phosphate solubilizer bacteria
PSM	Phosphate solubilising microorganism
rpm	Revolutions per minute
TCP	Tri Calcium Phosphate
w.r.t	With respect to

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ABSTRACT

Soil microorganisms play an important role in maintaining ecological balance through active participation in carbon, nitrogen, sulphur and phosphorous cycles. Agricultural microbials such as *Pseudomonas striata* and *Azotobacter sp.*, used as biofertilizers, play a significant role in plant nutrition by increasing phosphate uptake and nitrogen fixation respectively. Indiscriminate use of insecticides to control crop losses from insect pests has increased considerably. Cartap hydrochloride is one of the widely applied insecticide to control a wide range of sucking and chewing insect pests of paddy crop and vegetables. Present study reports the uptake of cartap hydrochloride by bacterial inoculants (*Pseudomonas striata* and *Azotobacter* CBD15) using enrichment technique. Bacterial biofertilizers were grown in minimal medium containing different concentration of cartap hydrochloride (0-100 ppm). There was a stimulatory effect on the growth of both the strains, which indicates that bacteria were utilizing insecticide as carbon and nitrogen source. LD_{50} was 50 ppm for *Azotobacter* CBD15 and 90 ppm for *Pseudomonas striata*. Effect of cartap hydrochloride on growth, IAA production and phosphate solubilisation by these bacterial inoculants was carried out along with determination of its residual concentration. 50 ppm cartap hydrochloride had stimulatory effect on phosphate solubilisation by *Azotobacter* CBD15 (42.8%) and *Pseudomonas striata* (52.9%) as compared to control. 6.5mg/ml of IAA was produced by *Pseudomonas striata* at 100 ppm and 3.3mg/ml of IAA was produced by *Azotobacter* CBD15 at 50 ppm of cartap hydrochloride. The study revealed that cartap hydrochloride can be safely used with no deleterious effect on the growth and desirable biochemical activity of bacterial biofertilizers at its recommended dose (10 ppm) of field application.

INTRODUCTION

Worldwide use of pesticide has increased dramatically during the last two decades. But, the extensive use of these agrochemicals leads to an aggregation of an enormous expanse of residues in the environment which causes a significant threat to human health due to the uptake and accretion of these lethal compounds in the food chain and drinking water (Mohammed, 2009). Rice is one of the most vital primary foods for large part of world population (Barah and Pandey, 2005). In India, rice continues to remain as the chief food for more than 65% of population (Subbaiah *et al.,* 2001). A study described that one of the 20 major challenges in rice production included biotic stress triggered by insect pests and diseases. Among these, insect pests cause about 10–15% yield loss in rice production. In India, stem borers and plant hoppers account for a loss of 30% and 20%, respectively, while 15% and 10% losses are caused by gall midge and leaf folder, respectively. To accomplish maximum yield level, farmers are using a large number of insecticides, fungicides and herbicides. Indian economy is agricultural based and the major challenge is due to crop pests to maintain good economy.

In India 76% of the pesticide used is insecticide, as against 44% globally (Mathur, 1999). The herbicides and fungicides application is consistently less hefty. Cotton accounts for the maximum share of pesticide consumption i.e. around 37% followed by paddy (20%). In India together they account for around 57% of the total pesticide consumption. While wheat and pulses contribute about 4 %, vegetable 9 % and the other plantation crops 7 % (Ministry of Agriculture, 2009). Andhra Pradesh is the highest pesticides consuming state (23%) followed by Punjab and Maharashtra (Bhardwaj and Sharma, 2013).

Indiscriminate use of pesticides on large scale poses a great threat to non target soil microflora and ecology including bacterial and cyanobacterial inoculants. The several classes of pesticides comprise the organophosphates, carbamates, pyrethroids, organochlorine etc. (Zerrouk and Fessi, 2000). Pesticides are used to control the harms triggered by the pest to commercially essential crops in agricultural field (Rokade and Mali, 2013). Modern effectively managed agricultural structure has an essential reliance on the use of chemical pesticides. One of the major problems besides toxicity and carcinogenicity of

pesticides is their long constancy in nature that increases the toxicity and health risk problems (Murugesan *et al.,* 2009).

Some of the well-known health effects of pesticide exposure in human beings include acute poisoning, cancer, neurological effects and reproductive and developmental harm (Bhardwaj and Sharma, 2013). Several species are known to tolerate many of pesticides and some of them are known to degrade several pesticides in different situations (Tejera *et al.,* 2005). Pesticide resistant and degrading bacterial group includes many species such as *Pseudomonas, Flavobacteria, Azotobacter, Arthrobacter, Bacillus, Enterobacter, Alcaligenes* and *Klebsiella* (Chennappa *et al.,* 2014).

A large number of insecticides are recommended for pest control in the rice fields. Indiscriminate use of these insecticides pose great threat to environment and beneficial soil microflora (Singh *et al.,* 2015). Cartap hydrochloride is a broad spectrum insecticide which is widely used in fields to control stem borer leaf roller of rice crop and diamond back moth of vegetable and tea plantation insects and also used for sugarcane, banana, tomato, potatoes, cabbage, soyabeans, peanuts, sunflowers, maize, sugar beets, wheat, citrus, cotton and cauliflower cultivation.

Present study was aimed at investigating the effect of cartap hydrochloride commonly used insecticide in agriculture on the growth of bacterial biofertilizers such as *Pseudomonas striata* and *Azotobacter sp.*

2. REVIEW OF LITERATURE

The usage of fertilizers, biofertilizers and pesticides to improve the crop yield by farmers has increased considerably. Biofertilizers used to improve the crop yield and pesticides are used to protect the crop from insect pests and check crop losses. Use of biofertilizers along with pesticides being beneficial or harmful for the soil fertility, crop production and field salinity is an important area of investigation.

2.1 Pesticides and their classification

Pesticides include diverse products with dissimilar functions (Uqab *et al.,* 2016). The term pesticide is combination of range of altered types of chemicals including herbicides, insecticides, fungicides, and rodenticides, among others. The pesticides are categorized depending upon target species and mode of action. According to the application the main classes of pesticides include herbicides (that kill weeds), fungicides (that kill fungi) and insecticides (that kill insects). The major classification is based on the chemical nature i.e. organochlorine, organophosphorous, thiocarbamates, pyrethroids and neonicotinoids (Tiwana *et al.*, 2007) (Fig. 1).

2.1.1 Organochlorines

An organochlorine, organochloride, chlorinated hydrocarbon, chlorocarbon, or Chlorinated solvent is an organic compound comprising at least one covalently bonded chlorine atom. Organochlorine insecticides composed mainly of carbon, hydrogen and chlorine. Most of them collapse slowly and can persist in the environment long after application and remains in organisms long after exposure. Some of the examples of organochlorine pesticides include alpha-BHC, beta-BHC, delta-BHC, gamma-BHC (Lindane), heptachlor, endosulfan, methoxychlor, aroclor and dichlorodiphenyltrichloroethane (Tiwana *et al.*, 2007).

2.1.2 Organophosphates

Organophosphates bind to acetylcholinesterase and other cholinesterases leading to disturbance of nerve impulses, killing the insects or inability to carry out normal function. Organophosphorous compounds includes Acephate, azinphos-methyl, bensulide, chlorethoxyfos, chlorpyrifos, diazinon, dicrotophos, dimethoate, disulfoton, ethoprop, fenamiphos, fenitrothion, fenthion, fosthiazate, methamidophos, monocrotophos and malathion (Tiwana *et al.*, 2007).

2.1.3 Carbamates

Carbamate is derived from carbamic acid, closely related to organoposphorus compound in mode of action and resistance development. Bendiocarb, carbofuran, carbaryl, dioxacarb, fenobucarb, fenoxycarb, isoprocarb and methomyl are some of the examples of carbamate insecticides (Tiwana *et al.*, 2007).

2.1.4 Pyrethroids

Cypermethrin, cyfluthrin, deltamethrin, etofenprox, fenvalerate, permethrin, phenothrin, prallethrin, resmethrin and tetramethrin are some examples of pyrethroids. These are group of pesticides which are applied against household pests. These are not persistent, which is a sodium channel modulators, and are less toxic than organophosphates and carbamates (Tiwana *et al.*, 2007).

2.1.5 Neonicotinoids

These are artificial analogues of natural compound nicotine. They are functional as sprays, drenches, seed and soil treatments, and also used as alternates for organophosphates and carbamates. Treated insects show leg tremors, rapid wing motion, stylet withdrawal (aphids), disoriented movement, paralysis and death. Acetamiprid, clothianidin, imidacloprid, nitenpyram, nithiazine, thiacloprid and thiamethoxam are some of neonicotiniods (Tiwana *et al.*, 2007).

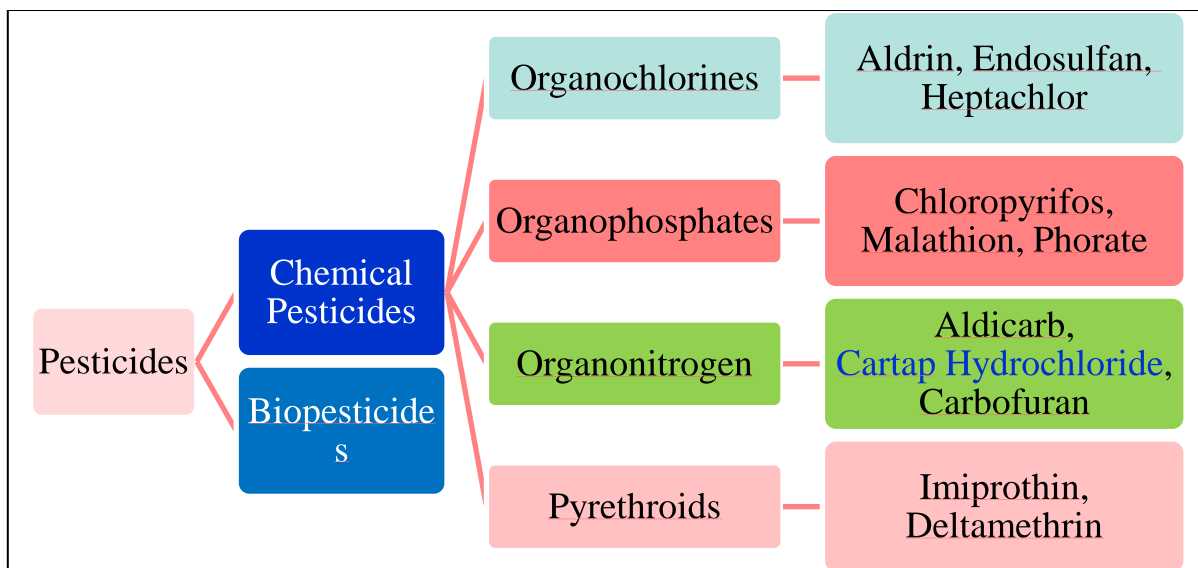

Fig.1 Classification of pesticides (Tiwana *et al.*, 2007)

2.2 Insecticides

Central Insecticides Board and Registration Committee had given list of insecticides (Table 1) and their dosage used in paddy fields.

Insecticides and Formulation	Common name of the pest	Dosage (g/ha)
Azadirachtin 0.03%	Leaf roller, Stem borer	0.03
Azadirachtin 0.15%	Stem borer, Brown Plant hopper, Leaf folder	10
Benfuracarb 3%	Stem borer, Leaf folder	1000
Bifenthrin 10%	Stem borer, leaf folder & Green leaf hopper	50
Carbaryl 5%	Leaf roller/folder, Brown plant hopper	1250
Carbaryl 10%	Blue Jassid, Case worm	2500
Carbaryl 50%	Brown Plant Hopper, Stem Borer	1000
Carbofuran 3%	Brown plant hopper Gall midge, Stem borer, Nematodes	750
Carbosulfan 25%	Green leaf hopper, White plant hopper, Brown plant hopper	200-250
Cartap hydrochloride 4%	Stem borer, Leaf folder, Whorl Maggot	750-1000
Cartap hydrochloride 50%	Stem borer, Leaf folder	500

Table 1. Commonly used insecticide (Source: Central Insecticides Board & Registration Committee MAJOR USES OF PESTICIDES (Registered under the Insecticides Act, 1968) up to 31.08.2015

2.3 Cartap hydrochloride

Cartap hydrochloride (Fig.2) selected for this study (Table 2) is one of the most commonly used insecticides in paddy fields of Punjab for the control of stem borer.

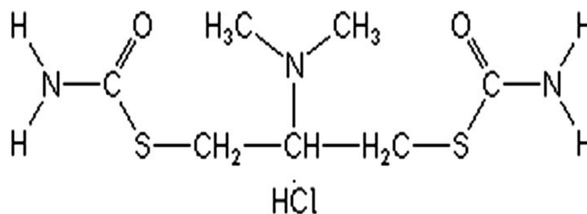

Fig.2. Structure of cartap hydrochloride

Cartap, an organonitrogen insecticide, has been extensively perceived as an analogue of nereistoxin since it was first introduced in Japan in 1967 (Liao *et al.*, 2006). The insecticide is used against pests of rice, sugarcane, fruit tree, vegetable and tea plants and acts in multiple ways including contact, stomach and gaseous intoxication. The insecticide has been widely used throughout the world due to less toxicity and high insecticidal activity (Nagawa, 1971). Cartap accounts for 19% of insecticides used in rice and rice-fish farms in Vietnam (Berg, 2001). The insecticide has also been recommended for use in rice fields of Punjab (Gill and Bajwa, 2012) and is one of the most commonly used insecticides. The large scale use of cartap could lead to accumulation of its residues at dangerous levels, which may affect the non-target microorganism including beneficial soil microbes such as bacterial and cyanobacterial biofertilizers.

Molecular Weight:	273.80384 g/mole
Molecular Formula:	$C_7H_{16}ClN_3O_2S_2$
IUPAC Name	S-[3-carbamoylsulfanyl-2-(dimethyl amino)propyl] carbamothioate; hydrochloride
Colour	Colourless, crystalline , slightly hygroscopic
Odour	Slight odour
Melting point	179° to 181° C
Solubility	In water at 25° C , approx. 200g/l; Slightly soluble in ethanol, methanol; Insoluble in acetone, chloroform

Table 2: Physical characteristics of cartap hydrochloride

2.4 Biofertilizers

Biofertilizers are living organisms, when applied to seed, plant surface, or soil, colonize the rhizosphere and promote plant growth by increasing the availability of primary nutrients to the host plant (Isfahani and Besharati 2012). Biofertilizers are living cells of different microorganisms, which have the ability to convert unavailable form of nutrients into available form to plants through various biological processes (Hegde *et al.,* 1999). Biofertilizers are substitute to chemical fertilizers to reduce the ecological disturbance (Kulandaivel and Nagarajan, 2014).

Microbial biofertilizers and biopesticides would lead to a decline in the use of chemicals leading to reduction in pollution and environmental hazards apart from enhancing organic agriculture (Morrissey *et al.,* 2004). Soil microorganisms, specifically plant growth promoting rhizobacteria (PGPR) facilitate plant growth by solubilizing insoluble phosphates, fixing atmospheric N and transferring it to plants and promoting uptake of plant nutrients.

Plant growth promoting rhizobacteria are also described to enhance root development in plants (Mantelin and Touraine, 2004) and modify root architecture (Kloepper *et al.,* 2007). Many phytohormones such as IAA, ethylene, cytokines and gibberellins are produced by plants itself and or in association with the microbes.

Wheat seeds inoculated with *Azotobacter* exhibited 50 % increase in leaves and 55 % increase in height of seedling in 7 days of germination with respect to control (Shaukat *et al.,* 2006). Most of the bacteria used as biofertilizers have close relationship with plant roots. Symbiotic interaction of Rhizobium with legume roots, and rhizobacteria inhabit on root surface or in rhizosphere soil.

Biofertilizers are of three types based on their functions, 1) Nitrogen fixing (NBF) 2) Phosphate solubilizer (PSB) 3) Organic matter decomposer (OMD). Nitrogen fixing biofertilizers are *Rhizobium, Azotobacter, Azospirillum, Azolla* and *BGA*. Phosphate solubilizer biofertilizers are *Bacillus, Pseudomonas* and *Aspergillus*. Organic matter decomposer biofertilizers are *Cellulomonas* and *Arthrobacter* (ICAR Research Complex For Goa, 1999).

2.5 Phosphate solubilizing microorganisms

The most preventive nutrient for plant growth after nitrogen (N) is Phosphorus (P). It exists in both forms inorganic and organic. P availability to plants is influenced by pH,

compaction, aeration, moisture, temperature, texture and organic matter of soil, crop residues, extent of plant root systems and root exudate secretions and available soil microbes (Gopalakrishnan *et al.,* 2015). Phosphorus occupies for about 0.2%-0.8% of the plant dry weight, but only 0.1% of the P is accessible for plants from soil (Zhou *et al.,* 1992). Although the P fertilizer delivers the plants with available from of phosphate solubilizing activity of the microbes has a major role (Sharma *et al.,* 2013).

Microorganisms ability of P-solubilisation is considered to be one of the most important qualities associated with plant nutrition (Chen *et al.,* 2006). From the dates back to 1903 occurrence of rhizospheric phosphorus solubilizing microorganism (PSM) has great evidence (Khan *et al.,* 2014). Bacteria are more active in phosphorus solubilisation than fungi (Alam *et al.,* 2002). From whole microbial population in soil, phosphate solubilisation bacteria (PSB) constitute 1 to 50%, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5% in P solubilisation potential (Chen *et al.,* 2006). Strains from bacterial genera *Pseudomonas, Bacillus, Rhizobium* and *Enterobacter* along with Penicillium and *Aspergillus* fungi are the most powerful P solubilizes (Whitelaw, 2000).

The ability of microbes to discharge metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms was determined by their phosphorus solubilizing activity (Sagoe *et al.,* 1998). Phosphate solubilisation takes place through various microbial processes/ mechanisms including organic acid production and proton extrusion (Surange, 1995; Dutton and Evans, 1996; Nahas, 1996). The action of PSB by secreting the organic and inorganic acids in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils which solubilize the inorganic P (Kpomblekou and Tabatabai, 1994; Stevenson, 2005).

2.5.1 Effect of pesticides on Phosphate solubilisation

Chennappa *et al* (2014) treated microorganism with 5% of pesticides (pendimethalin, chloropyrifos, glyphosate and phorate) and observed 7 to 9.8cm of halo zones after 7days. But, when *A. slinetris* (GYT-1) was treated with 5% phorate concentration 9.8cm 0f halo zones were observed. Therefore, he concluded that 3% concentration of pesticides showed adverse effect on bacterial growth whereas 1% concentration did not affect bacterial activity

as 13 to 14.4 cm of halo zones were observed in case of control i.e, which does not contain pesticides.

Tripti *et al* (2012) studied the effect of chemical pesticides on phosphate solubilisation by *Pseudomonas spp*., *Bacillus spp*. and *Azotobacter spp*. strains. After 3 days of incubation, zone of solubilisation was observed from 3 to 6.3mm. In case of *Bacillus* and *Pseudomonas* phosphate solubilisation zone was observed from 4.6 to 10.67mm after 5 days of incubation (Jarak *et al.,* 2012).

Ahmed and Khan (2011) observed effect of pesticides (fipronil, pyriproxyfen, imidacloprid and thiamethoxam) on phosphate solubilisation of *Pseudomonas putida* by treating it with normal to three times higher recommended concentration rate and observed that range from normal to two times showed minor decrease on halo zones whereas highest concentration (3X) showed adverse effect on zone formation. The toxicity of insecticides on halo zone was observed as: pyriproxyfen>fipronil>imidacloprid>thiamethoxam.

2.5.2 Pseudomonas

The genus *Pseudomonas* encompasses a group of ubiquitous microorganisms found in diverse ecological habitats such as water, soil, sediments, and plant surfaces maybe just because of their simple nutritional requirements (Jain and Das, 2015).The rhizosphere is a microbial hot spot zone in which root associated pseudomonads play a promising role as plant health managers (Bossis *et al.,* 2010; Dowling and O'Gara, 1994). Beneficial pseudomonads are rapid root colonizers, directly and indirectly improving plant growth and yield (Shippers *et al.,* 1987; Couillerot *et al.,* 2009). *Pseudomonas* is common gram-negative, chemoheterotrophic, motile, rod shaped bacterium with polar flagella (Fig. 3).

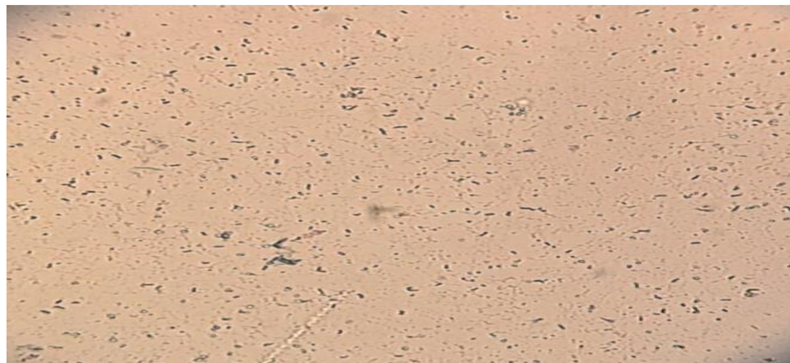

Fig. 3: ***Pseudomonas striata***

2.5.3 Effect of pesticides on *Pseudomonas*

Effect of pesticides cypermethrin, fenvalerate, deltamethrin and alpha cyhalothrin on *Pseudomonas florenseces, Bacillus subtilius* and *Serratia marcescrens* was observed resulted in phosphate activity in *Bacillus subtilius*, increased in case of *Pseudomonas* and more reduced pronounced in *Serratia* (Sethi *et al.,* 2013). Shanthala and Hiremath (2012) studied the effect of carbaryl on *E. coli* and *Pseudomonas aeruginosa* observed that the decrease in DNA content for both strains, while immobilized cells show tolerance toward the pesticide carbaryl.

Pseudomonas aeruginosa when grown in presence of endosulfan shows less toxic effect on the growth of bacteria (Mani *et al.,* 2011). Effect of pesticides- beta-cypermethrin, bensuluron-methyl and prometryne on *Pseudomonas putida* showed inhibited growth at 80ppm (Chen *et al.,* 2009). Kopytko *et al* (2001) studied the effect of gramoxone and matancha pesticide on *Pseudomonas putida* observed that the bacteria grow faster and to higher number of cells, when the Gramoxone (herbicide) concentration is highest. The growth of *P. putida* in the medium modified with 54.4 ppm matancha is the best of for its growth.

2.6 Nitrogen fixing microorganisms

Nitrogen (N) is necessary for synthesis of nucleic acids, enzymes, proteins and chlorophyll and vital element for plant growth. 78% of the atmospheric air is nitrogen, but it is in gaseous form which is unavailable for direct assimilation by plants. A variety of chemical N fertilizers is used for enhancing agricultural productivity, but they are non-renewable, non-economic and cause pollution (Gopalakrishnan *et al.,* 2015). Nitrification is an important process in nitrogen cycle in which ammonia is converted to nitrite and nitrate by nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* (Gopalakrishnan *et al.,* 2015). Plant growth promoting rhizobacteria (PGPR) can helps to replace chemical fertilizer nitrogen for the sustainable cultivation by fixing the atmospheric N_2 and producing growth promoting substances (Ahmad *et al.,* 2005). Biological Nitrogen Fixation (BNF) is a process of converting atmospheric N into plant assimilable N such as ammonia through a cascade of reactions between prokaryotes and plants with the use of complex enzyme systems (Wilson and Burris, 1947). Crops like wheat, rice, sugarcane and woody species have also the capacity to fix atmospheric N using free living (Gopalakrishnan *et al.,* 2015) rhizobacteria.

The symbiotic N contribution is also reported to benefit the cereal crops, such as maize, rice, wheat and sorghum with a relative yield increase of 11-35% (Peoples and Cranswell, 1992).

2.6.1 Effect of pesticides on Nitrogen (N_2) fixation

Chennappa *et al* (2014) studied the effect of pesticides on nitrogen fixation observed that GVT-1 strain fixed maximum amount of N_2 (30µgN/ml day) augment with 5% phorate pursue by pendimethalin (17.5µN/ml day). All 5 strains of *Azotobacter* fixed maximum of N_2 at 5% phorate, but at 5% glyphosate they showed negative effect.

Pesticides	**Strains**	**Results**	**References**
Cypermethrin Fenvalerate Deltamethrin Alphacyhalothrin	*Pseudomonas florenseces* *Bacillus subtilius* *Serratia marcescrens*	Phosphate activity reduced in *Bacillus sub*., increased in case of *Pseudomonas* and more pronounced in *Serratia*	Sethi *et al.,* 2013
Carbaryl	*E. coli* *Pseudomonas aeruginosa*	Pesticide shows decrease in DNA content in both strains, while immobilized cells shows tolerance	Shanthala and Hiremath., 2012
Endosulfan	*Pseudomonas aeruginosa*	Pesticide showed very less toxic effect on bacteria	Mani *et al.,* 2011
Beta Cypermethrin Bensulfuron methyl Prometryne	*Pseudomonas putida*	Growth was completely inhibited at 80µg/ml	Chen *et al.,* 2009
Gramoxone Matancha	*Pseudomonas putida*	Bacteria grow faster to highest concentration of Gramoxone but, growth was modified with 54.4ppm.	Kopytko *et al.,* 2002

Table 3: Effect of pesticides on *Pseudomonas*

Khudhur and Askar (2013) studied the effect of pesticides on nitrogen fixation observed that herbicide Imazetapir had no negative effect on nitrogen fixation, while dimethoate and Bayleton had.

2.6.2 Azotobacter

Azotobacter spp. are Gram negative, free-living, aerobic soil dwelling (Gandora *et al.,* 1998), oval or spherical bacteria that form thick-walled cysts (means of asexual reproduction under favourable condition) (Salhia, 2013).They are normally polymorphic and their size ranges from 2-10 µm long and 1-2 µm wide (Salhia, 2013). Bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. This cell protein is then mineralized in soil after the death of *Azotobacter* cells thereby contributing towards the nitrogen availability of the crop plants (Jnawali *et al.,* 2015) (Fig. 4).

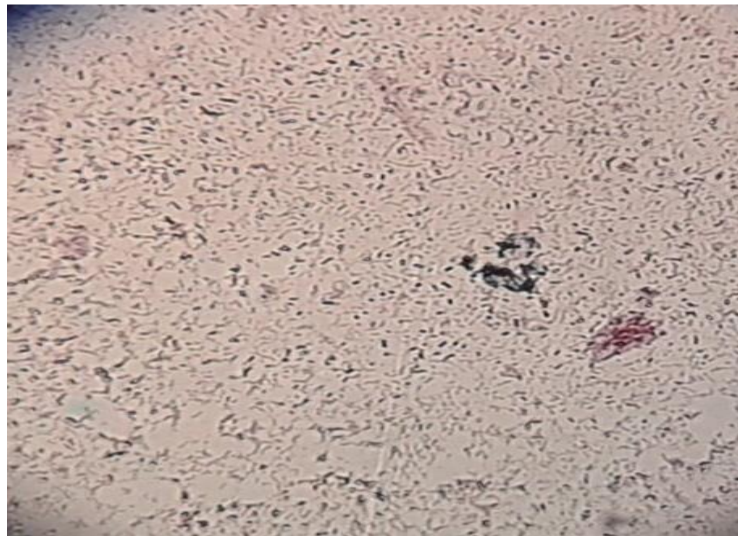

Fig. 4: *Azotobacter* CBD15

Azotobacter sp. has beneficial effects on plants when present in soil, but the abundance of these bacteria is related to many factors, soil physico-chemical (e.g. organic matter, pH, temperature, soil moisture) and microbiological properties (Kizilkaya, 2009). *Azotobacter* is much more abundant in the rhizosphere of plants than in the surrounding soil and that this abundance depends on the crop species (Sariv and Ragoviv, 1963a and b).

2.6.3 Effect of pesticides on *Azotobacter*

Hilcyperil and nuvan showed negative effect on growth as well as nitrogen fixation of *Azotobacter spp.* (Gulhane *et al.,* 2015). The herbicide- imazetapir has no negative effect on the growth and nitrogenase activity of *A. chroococcum* and *A. vinelandii*, but the insecticide-

dimethoate and fungicide- bayleton 50 had negative effect on the as well as enzyme nitrogenase activity on strains (Khudhur and Askar, 2013). *A. chroococcum* and *A. vinelandii* shows tolerance when incubated with pesticide Phorate (Kadam and Gangawane, 2005).

Effect of different pesticides was studied on *Azotobacter chroococcum*. It observed that the herbicide- Pyramin and insecticide- Lindan had no negative effect on the growth and activity of *A. chroococcum*, but the fungicide-Mankogal shows inhibitory effect on growth (Mrkovacki *et al.,* 2002). Sudhakar *et al* (2000) studied the effect of different insecticides (Methyl parathion, Dimethoate, Endosulfan, Carbendazim and Mancozeb) on *A. chroococcum, Azospirillum brasilense* and *Beijerinckia indica* strains. They observed that insecticide carbendazin shows maximum inhibitory effect followed by other insecticides on nitrogen fixation by bacteria. Simazine (fungicide) effect on *A. chroococcum* and *A. vinelandii* was studied and resulted in increased production of thiamin (Murica *et al.,* 1997).

Pesticides	**Strains**	**Results**	**References**
Hilcyperil Nuvan	*Azotobacter spp.*	Both the pesticides had negative effect on nitrogen fixation	Gulhane *et al.,* 2015
Imazetapir(herbicide) Dimethoate(insectide) Bayleton50 (fungicide)	*A. chroococcum* *A. vinelandii*	Herbicide had no negative effect, insecticide and fungicide had negative effect on growth as well as nitrogenase activity	Khudhur and Askar, 2013
Phorate	*A. chroococcum* *A. vinelandii*	*Azotobacter* shows tolerance against pesticide	Kadam and Gangawane, 2005
Ro-neet, Pyramin (herbicide), Lindan (insecticide) Mankogal (fungicide)	*Azotobacter chroococcum*	Herbicide and insecticide had no effect on growth, while fungicide showed inhibitory effect	Nastasija *et al*., 2002
Methyl parathion, Dimethoate, Endosulfan, Carbendazim, Mancozeb, Wettable sulfar	*A. chroococcum* *Azospirillum brasilense* *Beijerinckia indica*	Bacteria exhibits maximum inhibitory effect on nitrogen fixation by carbendazin as compared with other pesticides	Sudhakar *et al.,* 2000
Simazine	*A. chroococcum* *A. vinelandii*	No effect on growth, thiamin production increased, no change in niacin production	Murcia *et al.,* 1997

Table 4: Effect of pesticides on *Azotobacter*

2.7 Indole-3 Acetic Acid

Indole-3 acetic acid (IAA) is the most abundant endogenous auxin which has roles in stem elongation and root growth. The auxin level is usually higher in the rhizosphere, where high percentage of rhizosphere bacteria is likely to synthesize auxin as secondary metabolites because of the rich supplies of root exudates. The production of auxin (IAA) has been known as an important factor in direct plant-growth-promoting abilities of rhizosphere bacteria.

IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and presence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Spaepen and Vanderleyden, 2011; Tsavkelova *et al.,* 2006). The bacterial IAA having the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, would result in increased synthesis of ACC (Jackson, 1991), and a subsequent rise in ethylene that inhibited root elongation (Riov and Yang 1989). Bacterial IAA increases root surface area and length, and thereby provides the plant has greater contact to soil nutrients (Ano *et al.,* 2012).

Plant growth promoting rhizobacteria accounts for about 2-5% of total rhizobacteria involved in plant growth promotion (Antoun and Kloepper, 2001). *Rhizobium spp.* for IAA production and effect of tryptophan concentration, carbon sources and pH on IAA production was assessed (Madhuri and Sahasrabudhe, 2011). *Azotobacter* produces indole-3 acetic acid (IAA), gibberllic acid (GA) which are important plant growth hormones and these hormones will help in seed germination and plant growth considerably (Asma *et al.,* 2012).

2.7.1 Effect of pesticides on Indole-3-acetic acid (IAA) production

Chennappa *et al* (2014) studied the impact of pesticides on plant growth promoting rhizobacteria (PGPR) activity of *Azotobacter sp.* observed that strain GVT-1 produced highest amount of indole acetic acid (31.8µg/ml) in 5% pendimethalin. *Pseudomonas spp*. and *Azospirillum spp*. were producing IAA up to 1.25% concentration of pesticides Endosulfan, Monocrotophos, Lambda and Mancozeb. *Klebsiella spp*. did not produce IAA because of inhibition by pesticide endosulfan or change IAA producing metabolic pathway.

It was found that the concentration of IAA decreased with increase in concentration of pesticides. If the concentration was further increased the IAA production was completely inhibited (Kulandaivel and Nagarajan, 2014).

Pseudomonas putida strain PS9 possessed insecticide fipronil, pyriproxyfen, imidacloprid and thiamethoxam tolerance and indole acetic acid production observed in control was 34μg/ml which decreased with increase concentration of each insecticide (Ahemad and Khan, 2011).

3. MATERIAL AND METHOD

Bacterial cultures *Azotobacter* CBD15 and *Pseudomonas striata* procured from Division of Microbiology, IARI, New Delhi were grown in Jensen and PKV respectively at 26°C for 3 days. Bacterial cultures were grown in test tube with 16ml of Nutrient broth (NB) containing different concentration of insecticide cartap hydrochloride (0-700 ppm) for 72 hrs. Growth was estimated in terms of absorbance at 600 nm and plate count. All the experiments were set up in triplicates along with control without any pesticide.

Ingredients	**g/L**
Yeast extract	0.05
Dextrose	10
Calcium phosphate	5
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate.7H_20	0.1
Manganese sulphate	0.0001
Ferrous sulphate.7H_20	0.0001
Sodium chloride	0.2

Table 5: Composition of Pikovskaya (PKV) medium

Ingredients	**g/L**
Sucrose	20
Di-hydrogen potassium phosphate	1
Magnesium sulphate.7H_20	0.5
Sodium chloride	0.5
Ferrous sulphate.7H_20	0.1
Sodium molybdate	0.005
Calcium carbonate	2

Table 6: Composition of Jensen medium

Ingredients	g/L
Di-hydrogen potassium phosphate	1
Di-potassium hydrogen phosphate	1
Ammonium nitrate	1
Magnesium sulphate.7H_2O	0.2
Calcium chloride.2H_2O	0.02
Ferrous sulphate.7H_20	0.01

Table 7: Composition of Minimal media (MM)

Ingredients	g/L
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride	5

Table 8: Composition of Nutrient broth (NB)

3.1 Cartap uptake

Cartap uptake experiments were conducted in 250mL Erlenmeyer flasks containing 20mL of with 10 ppm to 100 ppm of cartap. Exponential growing (48 hr) cultures were inoculated to get initial absorbance 0.5 at 600nm. At regular intervals, a known volume of culture was withdrawn and centrifuged at 5000g the supernatant was used for the estimation of cartap as per the method given below.

3.1.2 Cartap estimation

Cartap was estimated as per Indian standards cartap hydrochloride technical- specification, published by Bureau of Indian Standards, New Delhi (1994).

Bureau of Indian Standards (BIS) method: Standard curve

Reagent

1. Cartap hydrochloride standard solution:- Weigh (0.101gm) accurately of cartap hydrochloride and dissolve in 100ml of methanol. The stock of 1000 ppm was prepared,

from the stock working cartap hydrochloride concentration was prepared 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ppm for standard curve.

2. 5,5-dithiobis (2-nitrobenzoic acid (DTNB)) analytical- reagent grade:- DTNB Solution- Weigh accurately 50mg DTNB into 100ml volumetric flask. Dissolve and make up the volume to the mark with methanol.

3. Buffer Solution:- 80ml of each phosphoric acid, boric acid and acetic acid into 1000ml volumetric flask and dilute with distilled water to the mark. Add sodium hydroxide solution and adjust the pH of solution at 9.0 with pH meter.

4. Methanol- analytical reagent grade
5. Phosphoric acid- 0.5M (49.0g/l)
6. Boric acid- 0.5M (30.915g/l)
7. Acetic acid- 0.5M (30.025g/l)
8. Sodium hydroxide solution- 0.2N

Procedure

Two mL of each DTNB and buffer solution was added in 2mL cartap solution and incubated for 20 min at 35° C. The absorbance of yellow colour developed was measured at 412nm. Standard curve was prepared by using different concentrations of cartap.

3.2 Standard curve of Indole-3-acetic acid (IAA) (Gordon and Weber method, 1951)

Reagent

1. Salkowski's reagent:- 1ml of 0.5M ferric chloride solution and 50ml of 35% perchloric acid was added and mixed properly. Store in brown bottle and use for 6months.

2. 0.5M ferric chloride solution:- 13.5gm of ferric chloride ($Fe(II)Cl_3$)was dissolved in distilled water and volume make up to 100ml.

3. 35% perchloric acid solution:- 50ml of 70% perchloric acid was diluted with distilled water and volume make up to 100ml.

Procedure

1. Stock of 1mg/ml (1000 ppm) IAA was dissolved in acetone. Working concentration of 100 ppm was prepared from stock in Luria Bertanii broth (LB).

2. Working concentrations of 0, 5, 10, 20, 30, 40, 50 and 100 ppm were prepared volume make up to 3ml with LB medium.

3. 1ml of each concentration was taken in fresh test tubes and 2ml of salkowski's reagent was added.

4. Incubate it for 25min in dark.

5. Absorbance was noted at 530nm with in 50min after adding the reagent.

6. Graph was plotted against concentration and OD.

Estimation of IAA

Procedure

1. 500µl of culture was inoculated in Luria Bertanii broth (LB) (appendix 1) with tryptophan (0.1mg/ml) for 72hrs at 25˚C at 100rpm with cartap hydrochloride (*Pseudomonas striata* (0-100 ppm) and *Azotobacter* CBD15 (0-50 ppm)).

2. After 72 hours of incubation 5ml of sample was taken out, centrifuged at 6000rpm for 5min.

3. 1ml of supernatant was taken and 2ml of Salkowski's reagent was added.

4. Incubated in dark for 25min, absorbance was noted at 530nm.

5. Amount of IAA (mg/ml) was estimated from the standard graph equation.

3. 3 Phosphate solubilisation: Qualitative method

Phosphate solubilisation was measured qualitatively by method given by Chennappa *et al.,* 2014.

1. *Azotobacter* CBD15 and *Pseudomonas striata* were inoculated onto a Pikovskaya agar plates containing tri calcium phosphate (TCP) as phosphate source.

2. Supplemented with LD_{50} concentration (0 to 50 ppm for *Azotobacter* CBD15 and 0 to 100 ppm for *Pseudomonas striata*) of pesticide i.e, cartap hydrochloride.

3. Incubated at 27±2˚ C for 7days whereas inoculated plate without pesticide taken as control.

4. Observed for halo zone formation around the discrete colonies.

4. RESULTS AND DISCUSSION

Pesticide application in agriculture can be acutely toxic to bacteria which are used as biofertilizer for improving plant growth. In the present study, effect of cartap hydrochloride on *Pseudomonas striata* and *Azotobacter* CBD15 was studied. The selection of insecticide was done on the basis of its current usage in Punjab. *Pseudomonas striata* and *Azotobacter* CBD15 were grown in the presence of different concentration of insecticide.

4.1 Impact of cartap hydrochloride on *Azotobacter* CBD15: *Azotobacter* CBD15 was grown in different concentration of insecticide ranging from 0 to 700 ppm in nutrient broth. Growth of *Azotobacter* CBD15 was decreased with an increase in the concentration of cartap hydrochloride w.r.t control after 48hr of incubation in nutrient broth (Fig. 5; Table 9). At 10 ppm which is recommended field application dose, the growth of *Azotobacter* CBD15 decreased to 5.65% (appendix 2.1). At 100 ppm which is the 10x of the recommended field application dose, the growth of *Azotobacter* CBD15 decreased to 59.3% as compared to control.

Azotobacter CBD15 was grown in minimal media and minimal media supplemented with 0.5% glucose and cartap hydrochloride (0 to 100 ppm). Growth of *Azotobacter* CBD15 was observed to be inhibited to some extent w.r.t control after 48hr of incubation (Fig. 6; Table 10). At 10 ppm, 6.6% decrease and at 100 ppm, 32.3% decrease was observed in minimal media w.r.t control. There was no growth stimulation in minimal media, however after supplementation with 0.5% glucose growth was observed and it was stimulated up to 90 ppm of cartap hydrochloride. It was observed that the growth of *Azotobacter* CBD15 was stimulated at 10 ppm (6.86% increase) and inhibited at 100 ppm (8.96% decrease) as compared to control after 48hr of incubation in minimal media supplemented with 0.5% glucose (Fig. 6; Table 10).

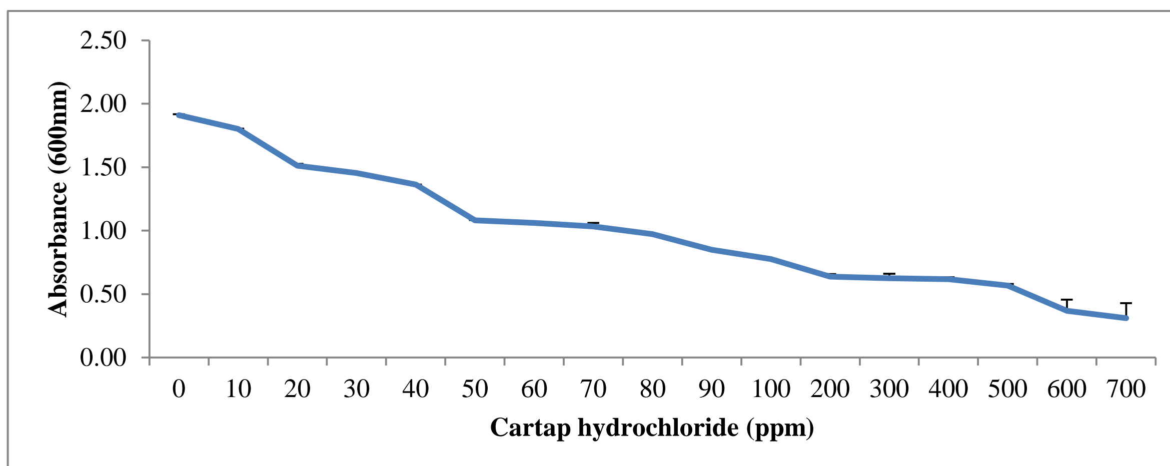

Fig. 5: Growth (absorbance at 600nm) of *Azotobacter* CBD15 in graded concentration (0-700 ppm) of cartap hydrochloride in nutrient broth after 48hr incubation at 28°C

Cartap hydrochloride (ppm)	Absorbance (600nm)
0	1.91±0.008
10	1.80±0.004
20	1.51±0.014
30	1.45±0.003
40	1.36±0.002
50	1.08±0.001
60	1.06±0.002
70	1.03±0.029
80	0.97±0.002
90	0.85±0.003
100	0.78±0.003
200	0.64±0.020
300	0.63±0.034
400	0.62±0.016
500	0.57±0.012
600	0.37±0.087
700	0.31±0.119

Table 9: Growth (absorbance at 600nm) of *Azotobacter* CBD15 in graded concentration (0-700 ppm) of cartap hydrochloride in nutrient broth after 48hr of incubation at 28°C

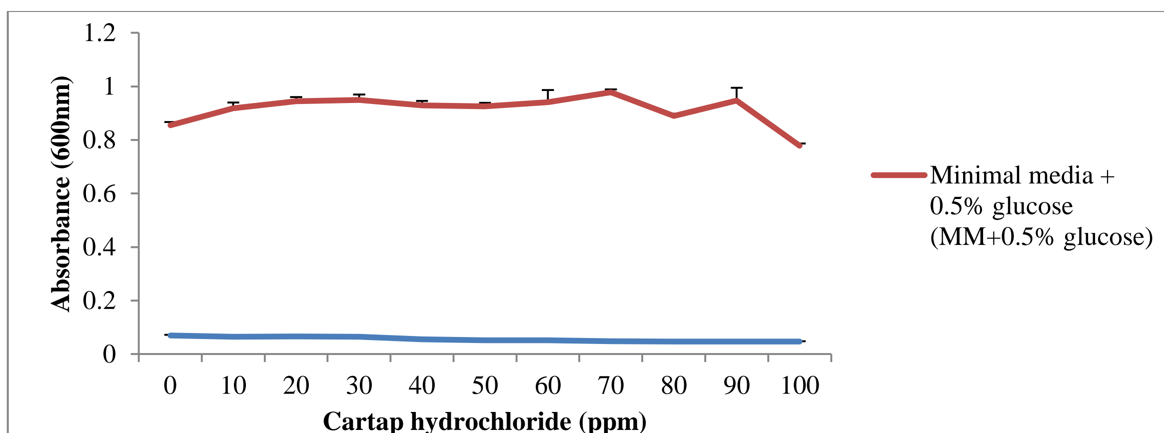

Fig. 6: Growth (absorbance at 600nm) of *Azotobacter* CBD15 in graded concentration of cartap hydrochloride in minimal media (MM) and minimal media supplemented with 0.5% glucose after 48hr incubation at 28°C

Cartap hydrochloride (ppm)	Absorbance (600nm) MM	Absorbance (600nm) MM +0.5% Glucose
0	0.070±0.002	0.855±0.012
10	0.065±0.002	0.918±0.021
20	0.067±0.001	0.945±0.015
30	0.065±0.001	0.949±0.021
40	0.055±0.001	0.929±0.017
50	0.052±0.001	0.925±0.014
60	0.052±0.001	0.941±0.045
70	0.048±0.004	0.978±0.012
80	0.047±0.001	0.889±0.009
90	0.047±0.001	0.947±0.048
100	0.047±0.001	0.778±.008

Table 10: Growth (absorbance at 600nm) of *Azotobacter* CBD15 in graded concentration of cartap hydrochloride in minimal media (MM) and minimal media supplemented with 0.5% glucose after 48hr of incubation at 28°C

Mrkovacki *et al* (2002) studied the effect of three concentrations of four different pesticides (two herbicides: ro-neet and pyramin (41/ha), one insecticide: lindane (51/ha) and one fungicide: mankogal (600-850g/100kg)) on the growth of *Azotobacter chroococcum*. The

lowest and highest concentrations used in the study were ten times lower and ten times higher than recommended dose used in actual agricultural practice (in the field). *Azotobacter chroococcum* grew unimpeded regardless of the pyramin, ro-neet-a and lindane concentration, whereas all three mankogal concentrations caused growth inhibition.

Khudhur and Aakar (2013) observed that herbicide imazetapir (10g/L) had no negative effect on the nitrogen fixation of *Azotobacter spp.*, but herbicide- dimethoate (3g/L) and bayleton (2g/L) inhibited growth by 99.9% and 95.2% respectively.

Murcia *et al* (1996) studied the effect of herbicide simazine on vitamin production in two *Azotobacter spp*. The herbicide simazine added to culture media at concentrations of 10, 50 or 100 μg ml^{-1} affected the quantitative production of B-group vitamins (thiamin, niacin, pantothenic acid, cyanocobalamin and biotin) by *Azotobacter vinelandii* ATCC 12837 and *A. chroococcum* H23. The response was different for each strain and conditioned by culture media composition. Effect was more pronounced when the strains were grown in dialysed-soil medium, similar to the natural habitat of the organisms.

Moneke *et al* (2010) studied the effect of pesticide glyphosate on *Azotobacter* and *Pseudomonas fluorescens*. As the concentration of glyphosate increased, decrease in the growth of both the microorganisms was seen.

4.2 Impact of cartap hydrochloride on *Pseudomonas striata*

Pseudomonas striata at different concentration of pesticide showed slight variation in growth at low concentration but at 90 ppm and above there was decreasing trend in dose dependent manner after 48 hr of incubation (Fig. 7; Table 11). At 100 ppm 59.2% decrease in growth and at 800 ppm the growth was decreased to 83.8%.

Pseudomonas striata was grown in minimal media and minimal media supplemented with 0.5% glucose and cartap hydrochloride (0 to 100 ppm). It was observed that the growth of *Pseudomonas striata* was inhibited after 48hr in minimal media (Fig. 8; Table 12). At 10 ppm 6.02% and at 100 ppm 10.1% inhibition was observed. There is stimulation up to 20 ppm thereafter even up to 100 ppm growth was higher than control. It was observed that the growth of *P. striata* was inhibited to some extent when compared to the control after 48hr in minimal media supplemented with 0.5% glucose (Fig. 8; Table 12).

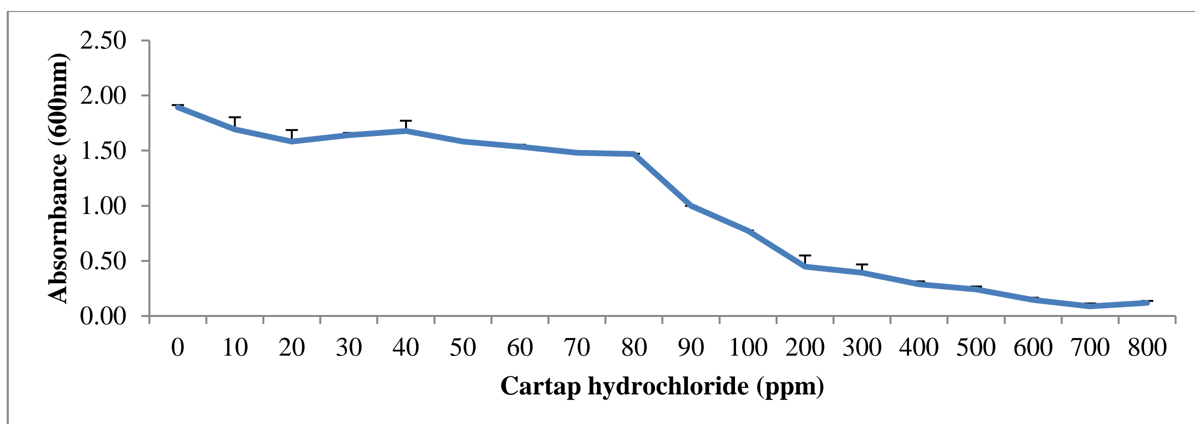

Fig. 7: Growth (absorbance at 600nm) of *Pseudomonas striata* in graded concentration (0-800 ppm) of cartap hydrochloride in nutrient broth after 48hr incubation at 28°C

Cartap hydrochloride (ppm)	Absorbance (600nm)
0	1.89±0.020
10	1.69±0.111
20	1.58±0.106
30	1.64±0.019
40	1.68±0.094
50	1.58±0.002
60	1.54±0.016
70	1.48±0.002
80	1.47±0.003
90	1.00±0.001
100	0.77±0.003
200	0.45±0.102
300	0.39±0.073
400	0.29±0.026
500	0.24±0.027
600	0.15±0.019
700	0.09±0.027
800	0.12±0.019

Table 11: Growth (absorbance at 600nm) of *Pseudomonas striata* in graded concentration of cartap hydrochloride in nutrient broth after 48hr of incubation at 28°C

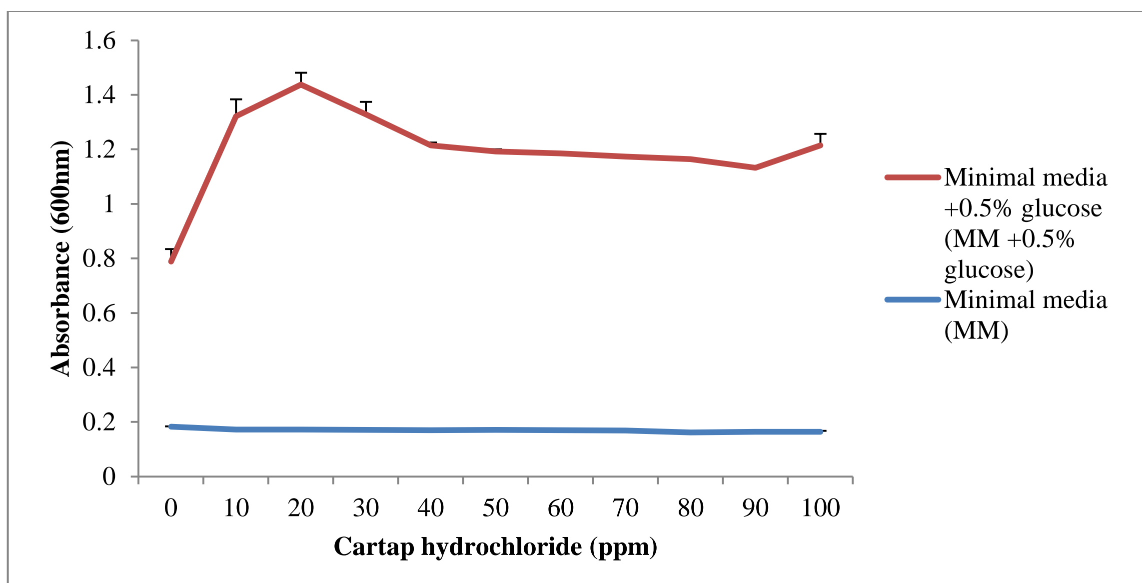

Fig. 8: Growth (absorbance at 600nm) of *Pseudomonas striata* in graded concentration of cartap hydrochloride in minimal media (MM) and minimal media supplemented with 0.5% glucose after 48hr of incubation at 28°C

Cartap hydrochloride (ppm)	Absorbance (600nm) MM	Absorbance (600nm) MM +0.5% Glucose
0	0.18±0.001	0.788±0.0463
10	0.17±0.001	1.321±0.0623
20	0.17±0.000	1.438±0.0433
30	0.17±0.002	1.328±0.0460
40	0.17±0.001	1.215±0.0101
50	0.17±0.002	1.192±0.0087
60	0.17±0.002	1.185±0.0027
70	0.17±0.002	1.173±0.0024
80	0.16±0.002	1.164±0.0024
90	0.16±0.001	1.132±0.0012
100	0.16±0.003	1.214±0.0428

Table 12: Growth (absorbance at 600nm) of *Pseudomonas striata* in graded concentration of cartap hydrochloride in minimal media (MM) and minimal media supplemented with 0.5% glucose after 48hr of incubation at 28°C

Murugesan *et al* (2009) studied the effect of cypermethrin on five different bacterial isolates (*Pseudomonas aeruginosa, Corynebacterium*, *Bacillus sp., Klebsiella sp.* and *E. coli*). Positive growth was observed in *Pseudomonas aeruginosa* at 0.01 – 1 % of cypermethrin. *Corynebacterium* and *Bacillus sp*. were found to grow up to 0.3% of cypermethrin, but as the pesticide concentration increased, growth was inhibited. Similarly growth of *Klebsiella sp.* and *E. coli* was observed up to 1% cypermethrin.

Shanthala and Hiremath (2012) observed the effect of carbaryl on growth kinetics, DNA, RNA and protein content of *Pseudomonas aeruginosa.* Carbaryl had negative effect on all these parameters. At concentration of 10^{-8}M carbaryl, DNA content was reduced to 65.0µg/ml, RNA content to 24.2µg/ml and protein content to 25.4µ/ml after 24 hr of incubation.

14 different strains of *Pseudomonas* were screened for their tolerance level against various concentrations of Quinalphos (5mg/L, 10mg/l, 15mg/L and 20mg/L). Results indicated that only one strain could tolerate and degrade the highest concentration of Quinalphos. This strain was subjected to degradation of insecticide at the level of 15mg/L and 20mg/L. The results showed that in the presence of glucose, isolated strain could degrade Quinalphos up to 90.4%, whereas up to 38.2% in the absence of glucose, which may be due to the role of glucose as an inducer for the growth of organism (Pawar and Mali, 2014).

Aziz *et al* (2014) studied degradation of malathion by *Pseudomonas aeruginosa.* It was found that *Pseudomonas aeruginosa* was capable of degrading malathion at different concentration (6841 ppm, 1453 ppm, 28506 ppm and 42759 ppm). Maximum degradation was observed at 42759 ppm, which reveals that the bacterium can survive at high concentration. Ahemad and khan (2011) studied effect of Fipronil, Pyriproxyfen, Imidacloprid and Thiame on *Pseudomonas putida* and observed that the bacterium could degrade when different grown at concentrations (0 to 2400 ppm) of insecticide.

Jilani and khan (2004) studied effect of different insecticides- malathion, methamidophos, cartap and cypermethrin on *Pseudomonas*. They observed that the viable cell number was reduced at 300 ppm of malathion. In presence of methamidophos (80-200 ppm), growth was stimulated but inhibited at 1200 ppm. In case of cartap, growth of bacterium was increased at 60 to 80 ppm, but limited growth was observed at 400 ppm as compared to control. In

case of cypermethrin, growth was increased up to 40 ppm and 60 ppm, but decreased at 80 to 125 ppm.

4.3 Viable cell enumeration (cfu/ml) of *Azotobacter* CBD15: The culture was first grown in nutrient broth at different concentration of pesticide and then grown on nutrient agar plates. The cfu count of *Azotobacter* CBD15 decreased in dose dependent manner.

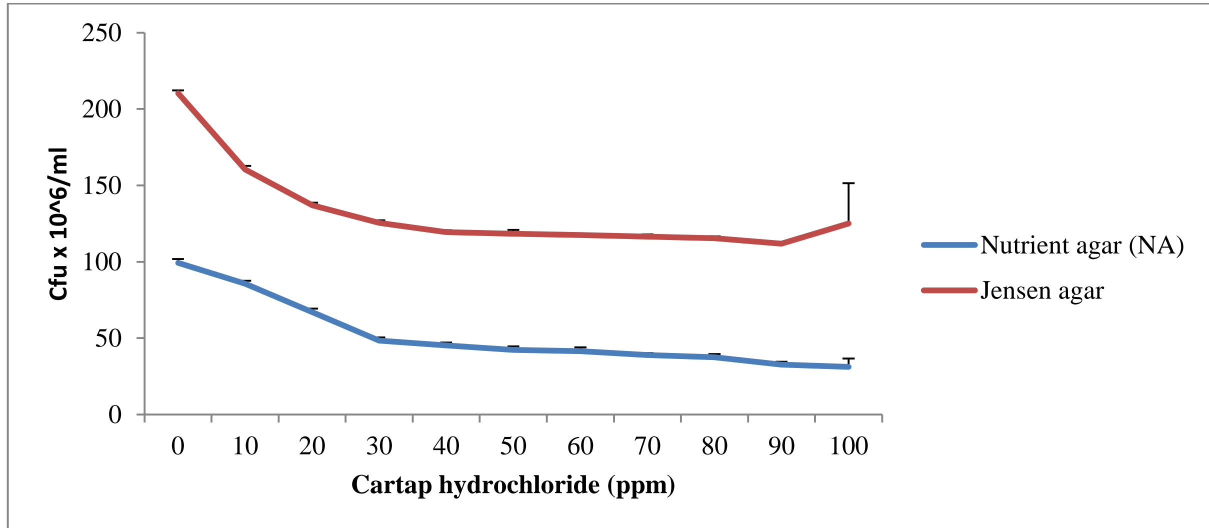

Fig. 9: Viable cell count (cfu x 10^6/ml) of *Azotobacter* CBD15 grown on nutrient agar plates and Jensen agar plates after growth in presence of different concentration of cartap hydrochloride (0-100 ppm) after 48hr of incubation at 28˚ C

At 100 ppm there was 68.6% decrease in cfu (appendix 2.2) count as compared to control. LD_{50} value of *Azotobacter* CBD15 was found to be 50 ppm.

In case of *Azotobacter* CBD15 grown in Jensen's medium, cfu count decreased as the concentration increased but not much difference was seen (Fig. 9; Table 13). At 10 ppm 23.7 % and at 100 ppm 47.5% decrease was observed after 48hr of incubation.

4.4 Viable cell enumeration (cfu/ml) of *Pseudomonas striata*: Cfu count of *Pseudomonas striata* was studied by growing *Pseudomonas striata* in nutrient broth supplemented with cartap hydrochloride (0-100 ppm) for 48 hr and then plating on nutrient agar plates. Cfu count of *Pseudomonas striata* decreased as the concentration of insecticide increases (in dose dependent manner (Fig. 10; Table 14). At 100 ppm growth was inhibited to 44.2% and at 10 ppm to 10.6%. LD_{50} value of *Pseudomonas striata* was calculated from graph was found to be 100 ppm.

Cartap hydrochloride (ppm)	Viable cell count (cfu x 10^6/ml) on nutrient agar	Viable cell count (cfu x 10^6/ml) on jensen's agar
0	99.3±2.60	210.4±1.86
10	85.6±2.03	160.4±2.40
20	67.0±2.31	137.1±1.53
30	48.4±2.03	125.5±1.73
40	45.3±1.73	119.4±1.20
50	42.3±2.40	118.4±2.40
60	41.5±2.52	117.6±0.58
70	39.0±1.20	116.4±1.53
80	37.4±2.08	115.5±1.20
90	32.7±1.76	111.9±0.88
100	31.1±5.51	125±26.40

Table 13: Viable cell count (cfu x 10^6/ml) of *Azotobacter* CBD15 grown on nutrient agar plates and Jensen agar plates after growth in presence of different concentration of cartap hydrochloride (0-100 ppm) after 48hr of incubation at 28° C

Pseudomonas striata was first grown in pikovskaya's medium supplemented with different concentration of cartap hydrochloride (0-100 ppm). Results showed decrease in cfu count in dose dependent manner (Fig. 10; Table 14). At 10 ppm 30.4% and at 100 ppm 66.3% inhibition was observed. In case of pikovskaya's broth, LD_{50} value comes out to be 90 ppm.

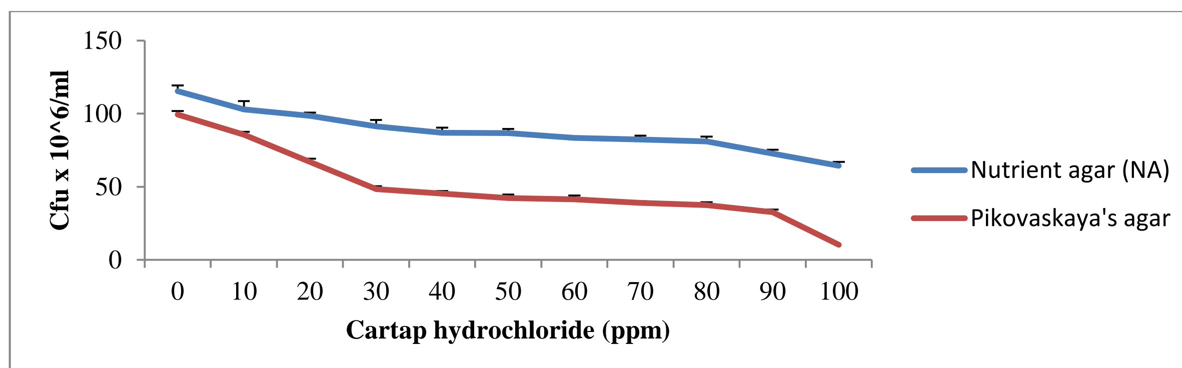

Fig. 10: Viable cell count (cfu x 10^6/ml) of *Pseudomonas striata* grown on nutrient agar plates and pikovskaya's agar plates after growth in presence of different concentration of cartap hydrochloride (0-100 ppm) after 48hr of incubation at 28° C

Cartap hydrochloride (ppm)	Viable cell count (cfu x 10^6/ml) on nutrient agar	Viable cell count (cfu x 10^6/ml) on pikovaskaya's agar
0	115.3±4.041	99.3±2.60
10	103.0±5.508	85.6±2.03
20	98.5±2.309	67.0±2.31
30	91.2±4.485	48.4±2.03
40	87.0±3.464	45.3±1.73
50	86.7±2.906	42.3±2.40
60	83.4±0.882	41.5±2.52
70	82.4±2.603	39.0±1.20
80	81.1±3.283	37.4±2.08
90	72.7±2.603	32.7±1.76
100	64.3±2.667	10.3±48.77

Table 14: Viable cell count (cfu x 10^6 / ml) of *Pseudomonas striata* grown on nutrient agar plates and pikovskaya's agar plates after growth in presence of different concentration of cartap (0-100 ppm) after 48hr of incubation at 28°C

4.5 Growth curve of *Pseudomonas striata* and *Azotobacter* CBD15 in nutrient broth: Growth curve of *Pseudomonas striata* was studied in Nutrient broth in 0, 10 and 100 ppm cartap hydrochloride. A series of growth curve experiments was performed with specific doses of cartap hydrochloride in order to determine the generation time of *Pseudomonas striata* and *Azotobacter* CBD15 to verify whether they could utilize these compounds for their growth. Growth was observed after every hour till 7hr and then two hour interval after 24hr day to the 48hr. In all the case, generation time was 10.8hr and specific growth rate in was 0.088 in case of *Pseudomonas striata* (Fig. 11; Table 15). In case of *Azotobacter* CBD15 generation time (appendix 2.3) for all was same i.e, 17.12hr and specific growth rate (appendix A2.4) was 0.098 (Fig. 12; Table 16). Growth curve in presence and absence of cartap hydrochloride was almost similar. There was no change in growth pattern in presence

of either 10 ppm the recommended dose or 100 ppm the 10x higher dose of cartap hydrochloride.

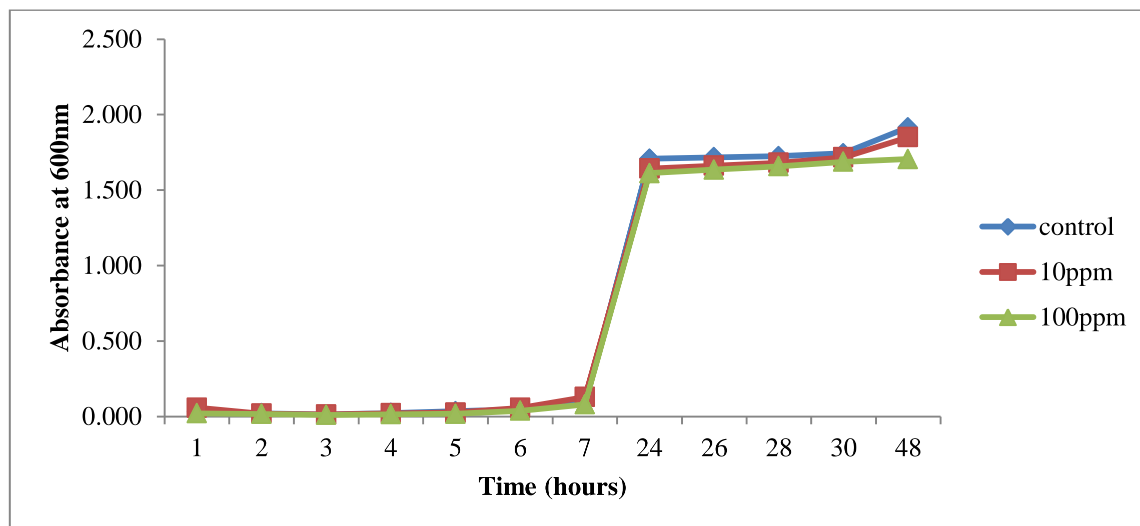

Fig. 11: Growth curve of *Pseudomonas striata* in Nutrient broth (NB) at different intervals

Time(hr)	Absorbance (600nm)		
	Control	10 ppm cartap hydrochloride	100 ppm cartap hydrochloride
1	0.031±0.0040	0.058±0.032	0.021±0.008
2	0.021±0.0012	0.019±0.001	0.017±0.001
3	0.014±0.0021	0.013±0.001	0.010±0.001
4	0.023±0.0007	0.021±0.001	0.015±0.0
5	0.035±0.0055	0.026±0.001	0.018±0.002
6	0.049±0.0044	0.057±0.002	0.038±0.002
7	0.090±0.0137	0.129±0.008	0.081±0.003
24	1.708±0.0295	1.643±0.034	1.612±0.041
26	1.717±0.0289	1.663±0.035	1.635±0.037
28	1.725±0.0295	1.681±0.031	1.658±0.034
30	1.744±0.0304	1.717±0.035	1.688±0.026
48	1.915±o.0135	1.851±0.031	1.705±0.020

Table 15: Growth curve of *Pseudomonas striata* in Nutrient broth (NB) at different intervals

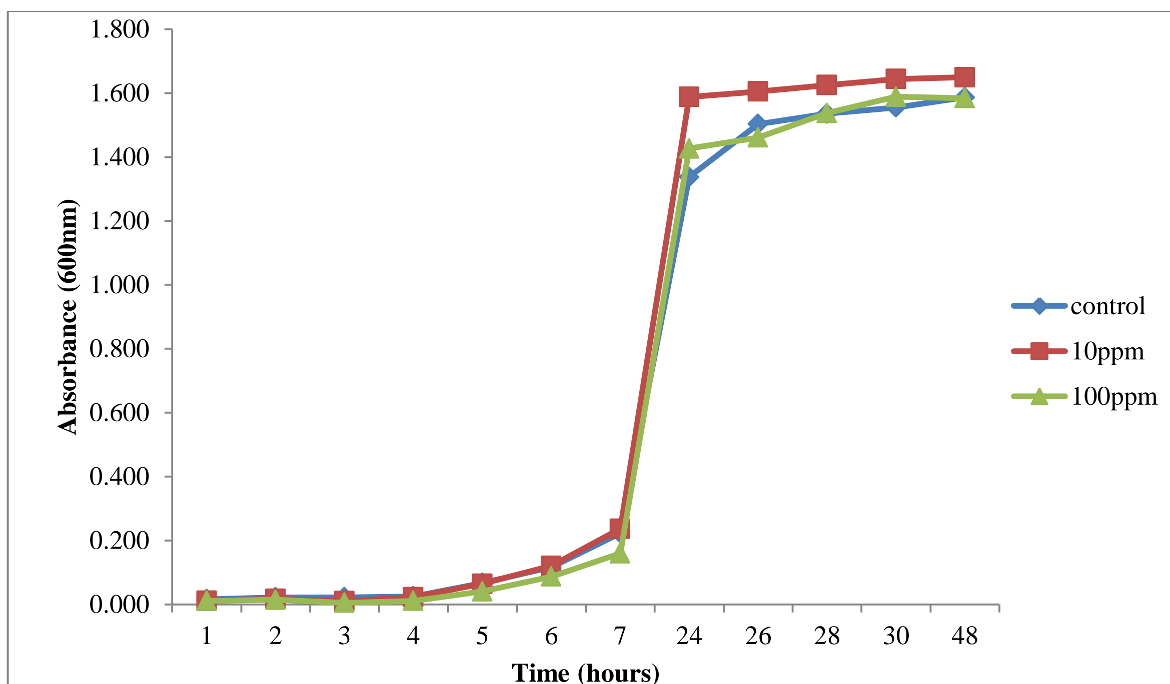

Fig. 12: Growth curve of *Azotobacter* CBD15 in Nutrient broth at different intervals

Time (hr)	Absorbance (600nm)		
	Control	10 ppm cartap hydrochloride	100 ppm cartap hydrochloride
1	0.016±0.001	0.012±0.002	0.011±0.001
2	0.022±0.001	0.018±0.002	0.015±0.004
3	0.022±0.001	0.010±0.004	0.005±0.002
4	0.025±0.002	0.023±0.006	0.011±0.002
5	0.067±0.004	0.065±0.012	0.041±0.001
6	0.117±0.008	0.121±0.017	0.087±0.006
7	0.223±0.009	0.237±0.039	0.160±0.009
24	1.337±0.080	1.588±0.018	1.427±0.040
26	1.503±0.020	1.605±0.016	1.461±0.017
28	1.536±0.015	1.625±0.010	1.538±0.001
30	1.555±0.006	1.644±0.002	1.589±0.002
48	1.587±0.007	1.650±0.000	1.584±0.000

Table 16: Growth curve of *Azotobacter* CBD15 in Nutrient broth at different intervals

Moneke *et al* (2010) studied the growth kinetics of *Pseudomonas fluorescens* and *Azotobacter* in presence of glyphosate and observed that the growth of *Pseudomonas fluorescens* was higher than *Azotobacter*. Growth kinetics of both follow similar pattern with a lag phase of about 12hr. Jilani and Khan (2004) studied the growth response of pesticides malathion, methamidophos, cartap and cypermethrin on *Pseudomonas sp*. In nutrient broth, the growth rate was 0.014 and generation time of 69min was observed. Reduction in growth of *Pseudomonas* was observed after 6 hr of incubation at 0.3 ppm. At 0.8 ppm methamidophos, the growth of organism was inhibited and significant death was observed. 0.06 to 0.08 ppm cartap hydrochloride increased the bacterial growth, but limited growth was observed after 24 hr in culture having 0.16 ppm cartap and reduction in viable cell count was observed at 0.4 ppm. 40 and 60 ppm Cypermethrin, showed increase in growth after 24 hr and at concentration of 80 to 125 ppm the growth was significantly decreased.

4.6Removal of cartap hydrochloride by *Azotobacter* CBD15:

Residual analysis of concentration of cartap hydrochloride was done to test if the *Azotobacter* CBD15 was resistant to cartap hydrochloride or whether there is an intracellular uptake. Nutrient broth supplemented with different concentration of cartap (10-100 ppm) was inoculated with *Azotobacter* CBD15 for 48 hr. The results revealed that the concentration of cartap hydrochloride decreased from the inoculated concentration, which indicates that the bacterium *Azotobacter* CBD15 was utilizing the insecticide from the medium as a source of Carbon and Nitrogen. Decrease in residual concentration was observed higher at low concentrations of cartap hydrochloride, when compared to that at high concentrations.

Residual concentration of cartap hydrochloride in Nutrient broth for *Azotobacter* CBD15 after 48hr of incubation was found to be 5.48 ppm (Fig. 14a) from the inoculated 100 ppm which indicates that the 94.52 ppm of cartap hydrochloride was utilized by the microorganism.

Residual concentration of cartap hydrochloride in Jensen's broth after 48hr of inoculation with *Azotobacter* CBD15 was found to be 2.38 ppm (Fig. 14b). This indicates that 97.52 ppm of cartap hydrochloride was utilized by *Azotobacter* CBD15. Amount of cartap consumed in *Azotobacter* CBD15 in nutrient broth media was less as compared to the

Jensen's broth which inferred that the culture was utilizing or degrading the cartap hydrochloride more in selective media.

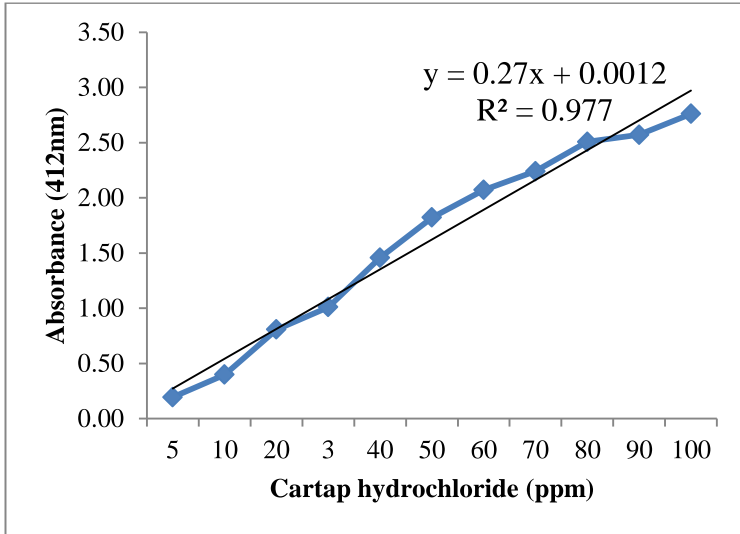

Fig. 13: Standard curve of cartap hydrochloride as per Bureau of Indian Standards (BIS) method

Cartap hydrochloride (ppm)	Absorbance (412nm)
5	0.19±0.012
10	0.40±0.001
20	0.81±0.030
30	1.01±0.005
40	1.46±0.127
50	1.82±0.096
60	2.07±0.000
70	2.24±0.067
80	2.51±0.063
90	2.57±0.000
100	2.76±0.111

Table 17: Standard curve of cartap hydrochloride as per Bureau of Indian Standards (BIS) method

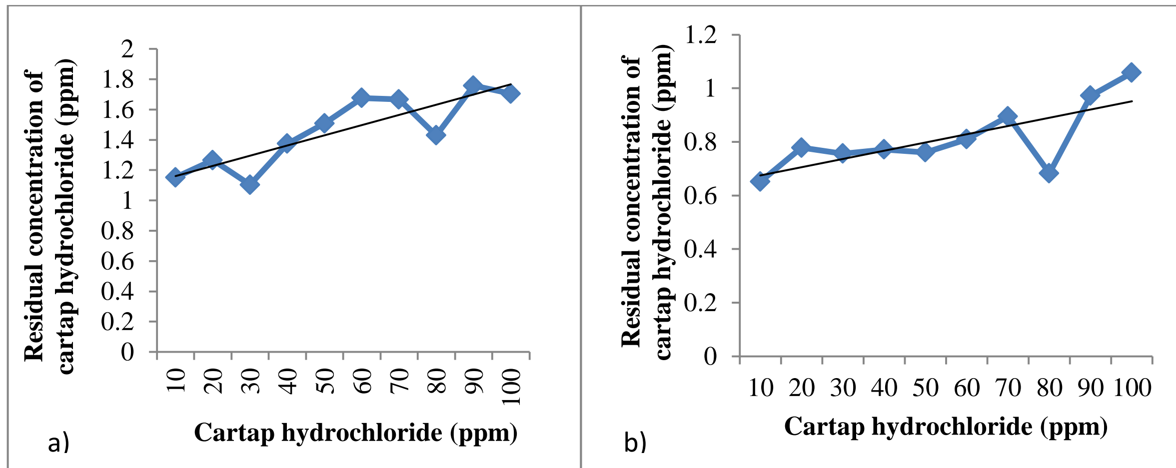

Fig. 14: Residual concentration of cartap hydrochloride in (a) nutrient broth and (b) jensen broth of *Azotobacter* CBD15 after 48hr of incubation at 28°C

4.7 Removal of cartap hydrochloride by *Pseudomonas striata*: *Pseudomonas striata* was grown in nutrient broth supplemented with different concentrations of cartap (10-100 ppm). Results revealed that the residual concentration of cartap hydrochloride was decreased with time thus indicating that *Pseudomonas striata* was able to grow in the presence of insecticide and utilizes it as an energy source for its growth. Residual concentration of cartap hydrochloride in nutrient broth for *Pseudomonas striata* after 48hr of incubation was 4.56 ppm (Fig.15a), which indicates that the 95.44 ppm of cartap hydrochloride was utilized by *Pseudomonas striata*.

Residual concentration of cartap hydrochloride in pikovskaya's broth for *Pseudomonas striata* after 48hr of incubation was 0.59 ppm (Fig. 15b) in 100 ppm cartap hydrochloride which indicates that the 99.41 ppm of cartap hydrochloride was utilized by *Pseudomonas striata*. Amount of cartap utilized by *Pseudomonas striata* in nutrient broth was less as compared to the pikovaskaya's broth, which inferred that the culture was utilizing or degrading cartap hydrochloride more in selective media. Cartap hydrochloride utilization or its degradation was higher by *Pseudomonas striata* than *Azotobacter* CBD15.

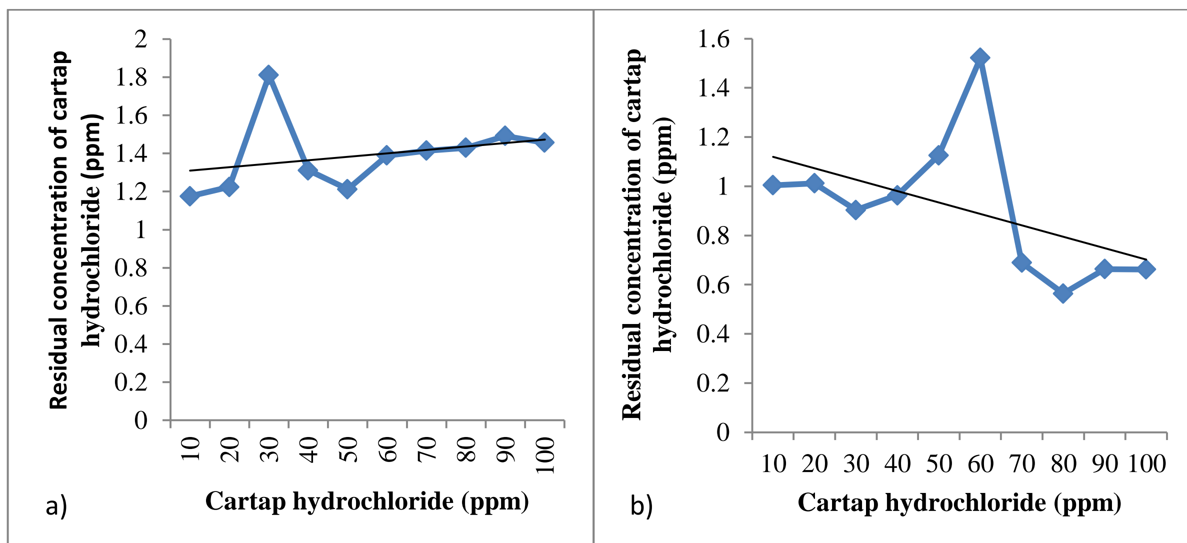

Fig. 15: Residual concentration of cartap hydrochloride in (a) nutrient broth and (b) pikovaskaya's broth of *Pseudomonas striata* after 48hr of incubation at 28°C

4.8 Residual concentration of cartap hydrochloride in minimal media and minimal supplemented with 0.5% glucose in *Pseudomonas striata*: Concentration of insecticide was decreased from the initial concentration after 48hr of incubation (Fig. 16; Table 18). It was observed that the initial 10 ppm concentration of cartap hydrochloride was reduced to

0.07 ppm after incubation of 48 hr. Concentration of insecticide decreased from the initial concentration after 1:30 hr to 48 hr (Fig. 17; Table 19). There was a gradual decline in residual concentration of cartap hydrochloride with increase in time of incubation at all concentration of cartap hydrochloride (10-100 ppm). This indicates that *P. striata* is able to grow well in presence of insecticide as well as is capable of degrading the insecticide.

Cartap hydrochloride (ppm)	Time (hr)				
	1:30hr	3:00hr	4:30hr	24hr	48hr
10	0.29	0.26	0.20	0.17	0.07
20	0.44	0.45	0.12	0.10	0.10
30	0.63	0.42	0.27	0.25	0.19
40	0.65	0.26	0.27	0.19	0.18
50	0.69	0.24	0.26	0.215	0.19
60	0.60	0.48	0.43	0.194	0.20
70	0.79	0.71	0.37	0.300	0.27
80	0.96	0.58	0.54	0.417	0.31
90	1.23	0.43	0.72	0.43	0.31
100	0.82	0.49	0.37	0.323	0.34

Table 18: Residual concentration of cartap hydrochloride in minimal media (MM) at different interval

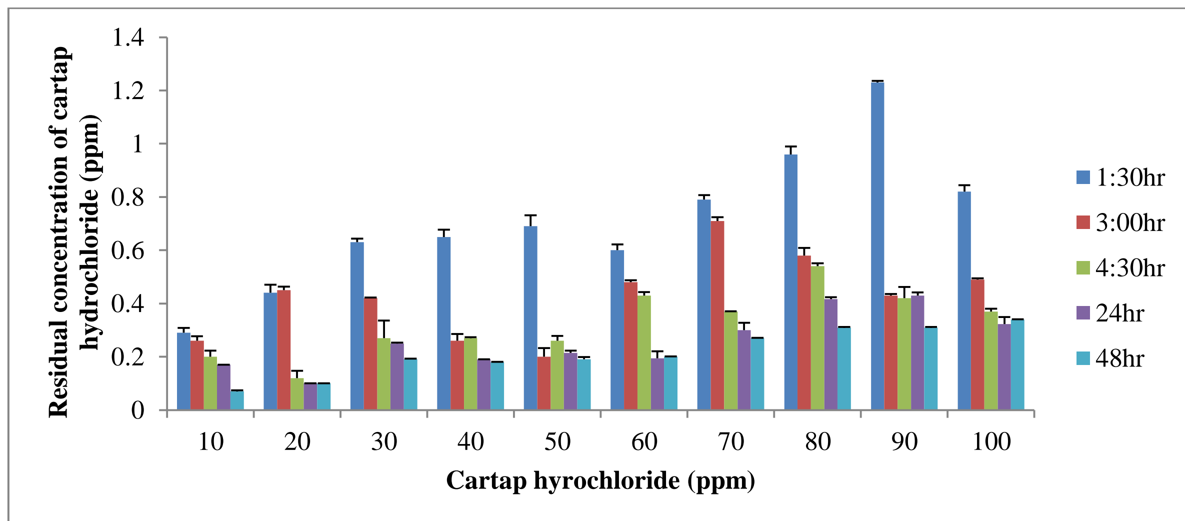

Fig. 16: Residual concentration of cartap hydrochloride in minimal media at different interval

Cartap hydrochloride (ppm)	Time (hr)				
	1:30hr	3:00hr	4:30hr	24hr	48hr
10	0.963	0.926	1.926	1.889	1.222
20	1.998	1.944	1.778	1.130	0.667
30	1.685	0.741	0.715	0.630	1.296
40	2.204	2.000	0.833	2.259	1.815
50	2.815	1.648	4.852	2.481	2.407
60	2.481	1.833	0.296	1.907	0.630
70	2.352	1.537	0.667	3.352	2.704
80	3.370	2.648	1.611	1.741	3.111
90	3.630	0.833	0.778	0.444	4.259
100	2.500	2.019	1.167	1.019	4.593

Table 19: Residual concentration of cartap hydrochloride in minimal media supplemented with 0.5% glucose at different interval

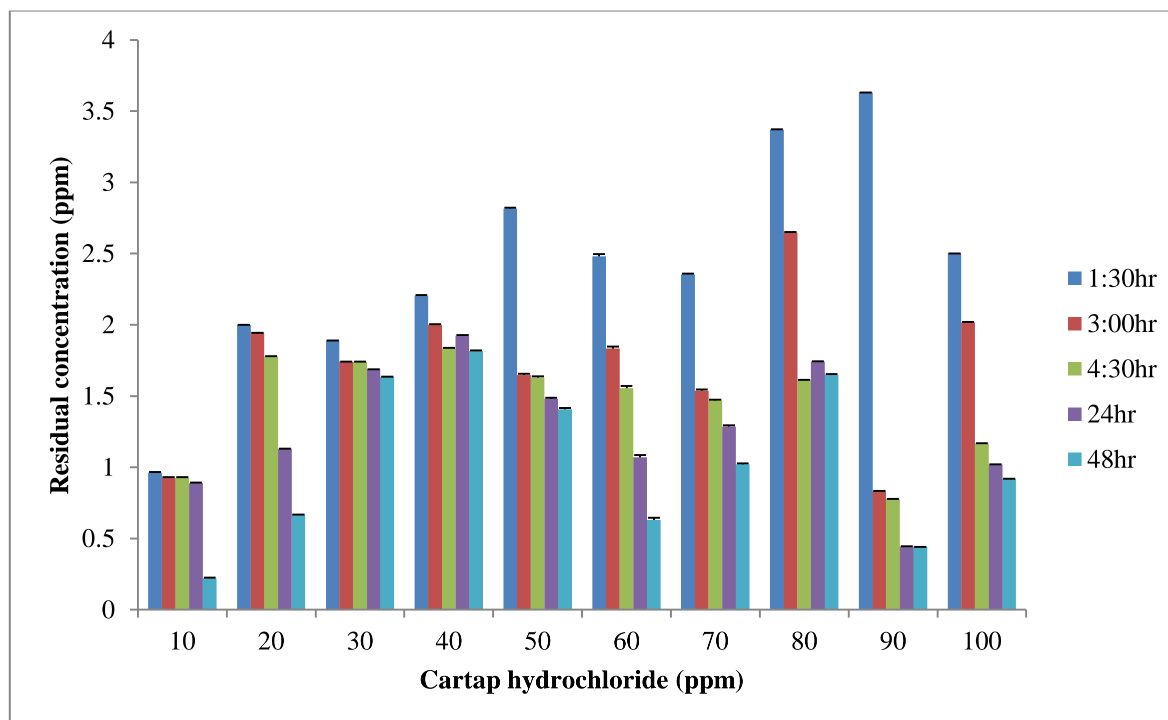

Fig. 17: Residual concentration of cartap hydrochloride in minimal media (MM) supplemented with 0.5% glucose at different interval

When we compare the utilization of cartap hydrochloride by the bacterium in minimal medium to that of nutrient broth and pikovskaya's broth, it was observed that the bacterium consumed the insecticide as the energy source for its growth in minimal medium. Cartap hydrochloride contains carbon, nitrogen and sulphur. It's degradation by bacterium in minimal media without any carbon source indicates that the bacteria are effectively utilizing cartap hydrochloride as the energy source for its growth.

4.9 Residual concentration of cartap hydrochloride in minimal and minimal supplemented with 0.5% glucose in *Azotobacter*: Insecticide concentration was observed to decrease from the initial inoculation concentration after the 48hr of estimation in minimal medium (Fig. 18; Table 20). After 24hr of incubation, the initial concentration of cartap hydrochloride observed to decreased 0.17 ppm from 10 ppm. Concentration of insecticide was observed to decrease from the initial inoculation concentration after the 1:30hr to 48hr of estimation in minimal supplemented with 0.5% glucose medium (Fig. 19; Table 21). This indicates that the culture were able to grow well in insecticide conditions as well as degrading the insecticide from the medium.

Cartap hydrochloride (ppm)	Time (hr)			
	1:30hr	3:00hr	4:30hr	24hr
10	0.12	0.08	0.08	0.17
20	0.13	0.12	0.13	0.12
30	0.20	0.16	0.04	0.15
40	0.18	0.17	0.12	0.11
50	0.25	0.23	0.20	0.18
60	0.25	0.20	0.20	0.18
70	0.33	0.20	0.18	0.13
80	0.36	0.34	0.33	0.19
90	0.32	0.15	0.16	0.10
100	0.30	0.15	0.015	0.05

Table 20: Residual concentration of cartap hydrochloride in minimal medium at different interval

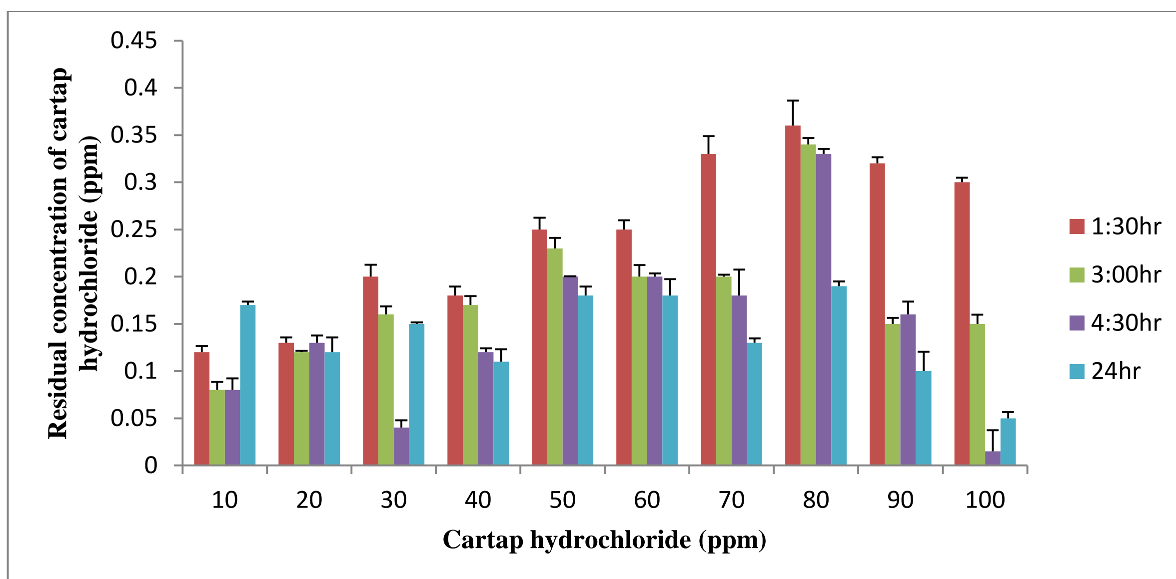

Fig. 18: Residual concentration of cartap hydrochloride in minimal medium at different interval

Cartap hydrochloride (ppm)	Time (hr)				
	1:30hr	3:00hr	4:30hr	24hr	48hr
10	7.58	3.483	3.354	0.637	1.063
20	7.32	2.956	2.928	0.664	0.835
30	7.18	3.388	0.820	0.681	1.146
40	6.90	2.784	0.853	0.822	0.768
50	6.81	2.000	0.722	0.496	0.643
60	7.14	1.683	0.646	0.62	0.759
70	7.32	2.069	0.589	0.541	0.493
80	6.50	1.877	0.575	0.53	0.474
90	6.81	1.83	0.600	0.377	0.519
100	6.97	1.507	0.626	0.496	0.485

Table 21: Residual concentration of cartap hydrochloride in minimal medium supplemented with 0.5%glucose medium at different interval

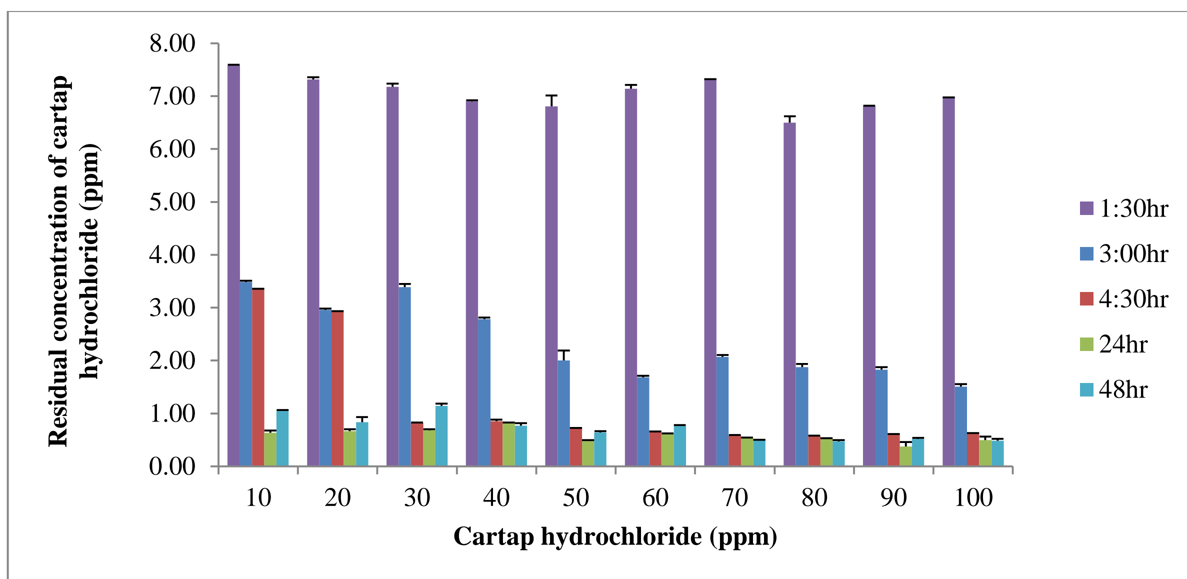

Fig. 19: Residual concentration of cartap hydrochloride in minimal medium supplemented with 0.5% glucose medium at different interval

Azotobacter CBD15 grows effectively in the enriched nutrient broth and Jensen's medium (Fig.18 and 19) at different concentration of cartap hydrochloride. Also, when the growth of cartap hydrochloride was studied on minimal media and minimal media supplemented with 0.5% glucose contains media, the *Azotobacter* CBD15 showed an efficient growth. Thus, it can be inferred that the *Azotobacter* CBD15 was utilizing cartap hydrochloride as its energy source and degradation of cartap hydrochloride is indicative of efficient growth of *Azotobacter* CBD15.

4.10 Indole-3 acetic acid (IAA) production by biofertilizers in LB broth medium

IAA production by *Pseudomonas striata* and *Azotobacter* CBD15 in the Luria-Bertani (LB) broth was estimated by using Salkowski's reagent for IAA estimation. Cultures were inoculated in LB medium with tryptophan (1mg/ml) as IAA precursor. Cartap hydrochloride was added at its LD_{50} concentration of 100 ppm for *P. striata* and 50 ppm for *Azotobacter* CBD15.

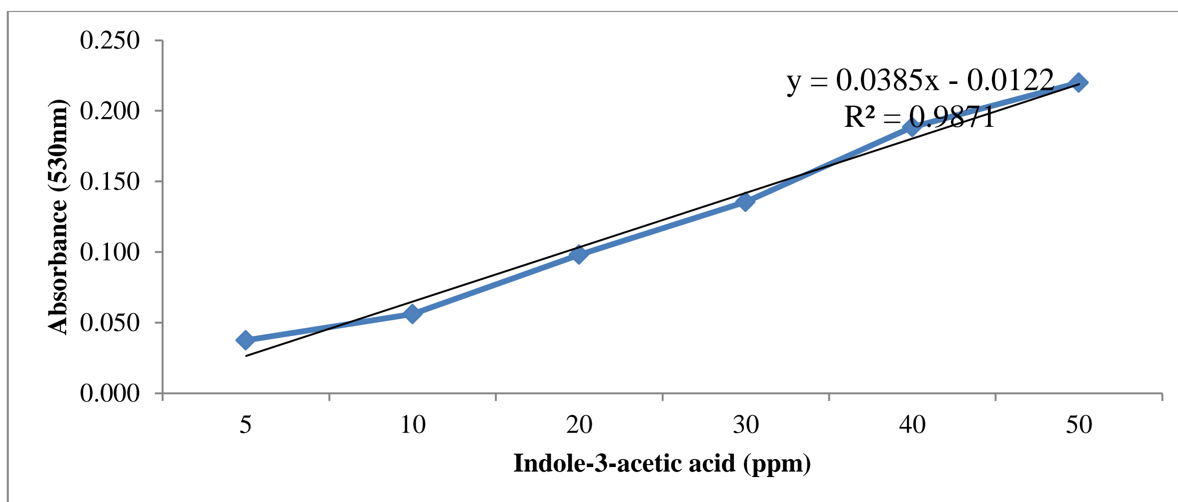

Fig. 20: Standard curve of Indole-3-acetic acid (IAA)

4.11 IAA (mg/ml) production in the LB medium by *Pseudomonas striata* and *Azotobacter* CBD15

Amount of IAA produced (mg/ml) by the *Pseudomonas striata* in LB medium was estimated from the standard graph. Amount of IAA produced was maximum (6.5mg/ml) at LD_{50} (100 ppm) concentration of cartap hydrochloride (Fig. 21; Table 22), which was as even higher as compared to control. This indicates that the presence of cartap hydrochloride had no effect on IAA production rather it stimulates IAA production by *Pseudomonas striata. Pseudomonas striata* shows decline at low concentration and thereafter increase at high concentration.

It was observed that IAA production by *Azotobacter* CBD15 was decreased at low concentration and increased at higher concentration but was less than control in dose dependent manner and at 50 ppm IAA production was 3.3mg/ml as compared to control (Fig. 22; Table 23), IAA produced at 50 ppm was inhibited to 22.9%.

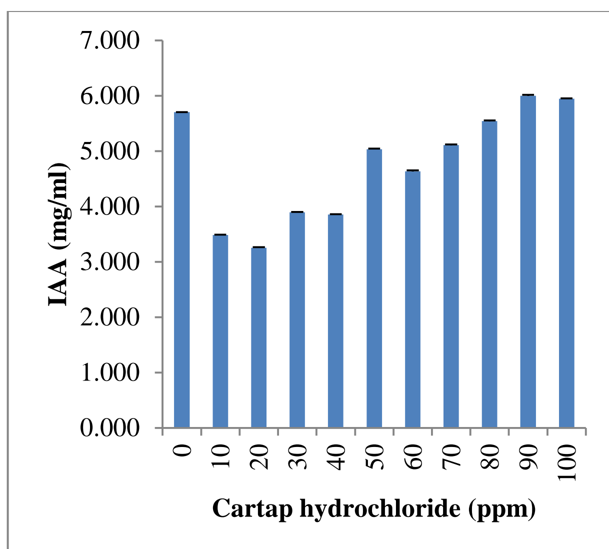

Fig. 21: IAA production by *Pseudomonas striata* in graded concentration of cartap hydrochloride

Cartap hydrochloride (ppm)	IAA (mg/ml)
0	5.702±0.0012
10	3.482±0.0041
20	3.254±0.0070
30	3.895±0.0021
40	3.851±0.0077
50	5.035±0.0068
60	4.632±0.0170
70	5.105±0.0132
80	5.544±0.0080
90	6.000±0.0145
100	5.947±0.0029

Table 22: IAA production by *Pseudomonas striata* in graded concentration of cartap hydrochloride

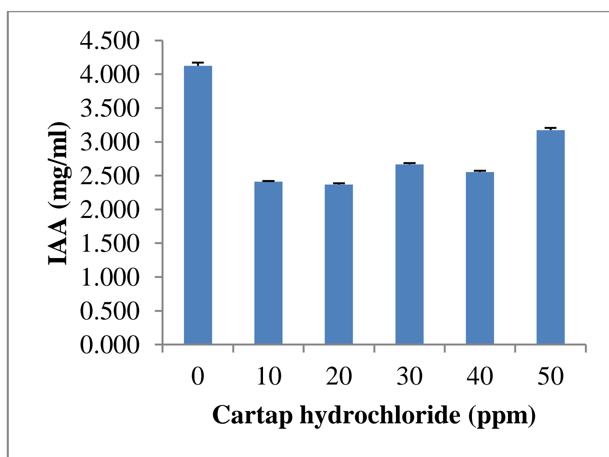

Fig. 22: IAA production by *Azotobacter* CBD15 in graded concentration of cartap hydrochloride

Cartap hydrochloride (ppm)	IAA (mg/ml)
0	4.123±0.049
10	2.412±0.008
20	2.368±0.019
30	2.667±0.018
40	2.553±0.018
50	3.175±.0330

Table 23: IAA production by *Azotobacter* CBD15 in graded concentration of cartap hydrochloride

Kulandaivel and Nagarajan (2014) studied the impact of insecticides endosulfan, monocrotophos, lambda and macnozeb on IAA production at 2.5-12.5 ppm. *Pseudomonas sp*. and *Azospirillum sp.* showed higher resistance to endosulfan than other pesticides, these

strains produced IAA (29µg/ml) up to 12.5 ppm concentration of pesticide. Amount of IAA produced by these strains in presence of other pesticide such as monocrotophos, lamba and macnozeb was decreased with increase in concentration of pesticide.

Ahemad and Khan (2011) studied the effect of four insecticides fipronil, pyriproxyfen, imidacloprid and thiamethoxam on *Pseudomonas striata*. It was observed that the amount of IAA decreased with increase in the concentration of insecticides. Among these 4 insecticides, thiamethoxam had more inhibitory effect on IAA biosynthesis and at 3 times higher concentration than recommended dose, IAA production was decreased by 84%.

Chennappa *et al* (2014) studied the effect of pendimethalin on two different *Azotobacter* strains (GVT-1 and Azt-16). It was observed strain GVT-1 produced maximum quantity of IAA (31.8µg/ml) when supplemented with 5% insecticide (pendimethalin) w.r.t control (33µg/ml). Other isolates produce IAA in range of 10-31.8µg/ml at 5% of different pesticide concentration. *Azotobacter* Azt-16 was sensitive towards IAA production in presence of 5% glyphosate. Pendimethalin did not affect the IAA production *Azotobacter* and it was resistant to 5% of pendimethalin as compared to other strains. 3% pesticide reduced IAA production and was lethal to bacterial respiration.

4.12 Effect of pesticide on phosphate solubilisation

Effect of cartap hydrochloride on phosphate solubilisation by both *Azotobacter* CBD15 and *Pseudomonas striata* was studied by the method given by Chennappa *et al* (2014). In case of control *Azotobacter* CBD15, there was 46.6% solubilisation (appendix 2.5) and in pesticide treated sample, 42.8% solubilisation was observed which shows that there is slight effect of increase in pesticide concentration on phosphate solubilisation (Fig. 23; Table 24). In case of *Pseudomonas striata* as the concentration of pesticide increases phosphate solubilisation also increased (Fig. 24; Table 25). At 10 ppm 23.8% and at 100 ppm 52.9% of phosphate solubilisation was observed in *Pseudomonas striata*. But solubilisation was less as compared to control, which showed 40.7% phosphate solubilisation. This indicated that in case of *Pseudomonas striata*, phosphate solubilisation was increased at higher concentration.

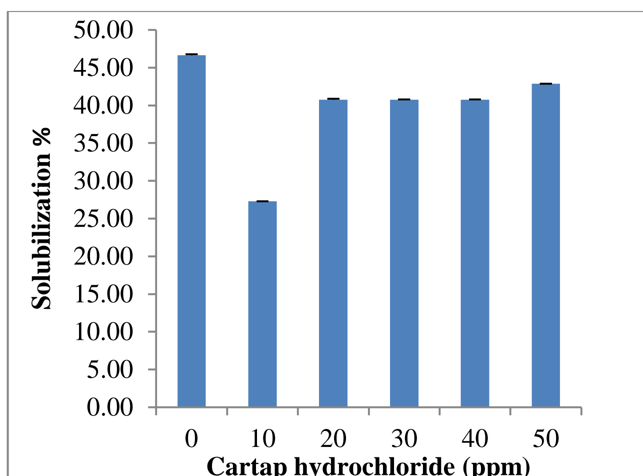

Fig. 23: Phosphate solubilisation % by *Azotobacter* CBD15 on plates after 7 days of incubation

Cartap hydrochloride (ppm)	Solubilisation %
0	46.67±0.010
10	27.27±0.00
20	40.74±0.015
30	40.74±0.05
40	40.74±0.05
50	42.86±0.00

Table 24: Phosphate solubilisation % by *Azotobacter* CBD15 on plates after 7 days of incubation

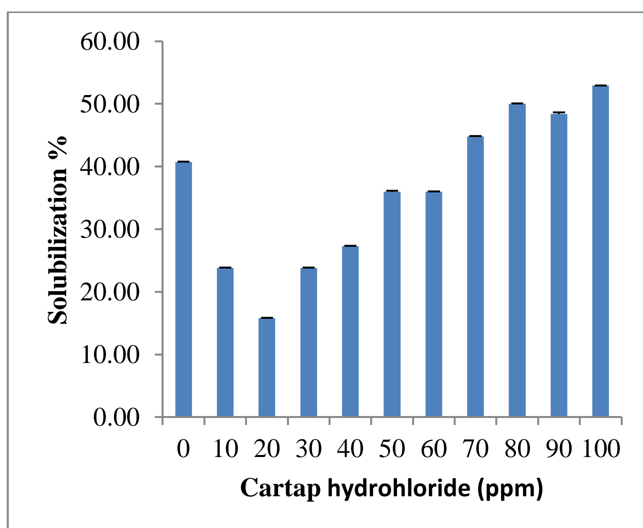

Fig. 24: Phosphate solubilisation % by *Pseudomonas striata* on plates after 7 days of incubation

Cartap hydrochloride (ppm)	Solubilisation %
0	40.74±0.05
10	23.81±0.05
20	15.79±0.05
30	23.81±0.05
40	27.27±0.01
50	36.00±0.15
60	36.00±0.05
70	44.83±0.05
80	50.00±0.10
90	48.39±0.25
100	52.94±0.00

Table 25: Phosphate solubilisation % by *Pseudomonas striata* on plates after 7 days of incubation

When microorganisms treated with pesticides pendimethalin, chloropyrifos, glyphosate and phorate observed the halo zone of 7 to 9.8cm in 5% pesticide concentration. Maximum halo

zone of 9.8cm was observed in 5% phorate. In case of control, halo zone between 13cm - 14.2cm was observed. All isolates of *Azotobacter* were equal in phosphate solubilisation and 3% of all the pesticides showed negative effect on bacterial growth (Chennapa *et al.,* 2014).

Tripti *et al* (2012) investigated phosphate solubilisation on selected isolates of *Pseudomonas*, *Azotobacter* and *Bacillus.* The plates were incubated for 3 days at 28±2°C and it was observed that zone of solubilisation ranged from 3 to 6.3cm. Strains S_2 and S_{30} showed maximum phosphate solubilisation showing halo zone 3.1 and 3.0cm respectively.

Jarak *et al* (2012) studied the phosphate solubilisation on *Pseudomonas* sp. Q4b and *Bacillus* sp.. Q5a showed good phosphate solubilisation ability and halo zone of 4.6mm to 10.67mm was observed respectively after 5days of incubation on pikovskaya's agar plates supplemented with tri-calcium phosphate.

Ahemad and Khan (2011) studied insecticide stress on plant growth promoting bacteria *Pseudomonas putida*, observed that with increase in insecticide concentration ranging from normal to three times recommended rate, the halo zone was decreased with minor fraction while highest concentration (3x) had most hostile effect on halo zone formation.

5. CONCLUSION

1. There was stimulatory effect on growth of both strains, which indicates that bacteria were utilizing insecticide as carbon and nitrogen source. LD_{50} was 50 ppm for *Azotobacter* CBD15 and 90 ppm for *Pseudomonas striata*.
2. 50 ppm cartap hydrochloride had stimulatory effect on phosphate solubilisation by *Azotobacter* CBD15 (42.8%) and *Pseudomonas striata* (52.9%) as compared to control.
3. There was no change in growth pattern in presence of either 10 ppm the recommended dose or 100 ppm the 10x higher dose of cartap hydrochloride in nutrient broth.
4. *Azotobacter* CBD15 besides nitrogen fixation can also solubilize phosphate in presence of insecticide cartap hydrochloride.
5. The study revealed that cartap hydrochloride can be safely used with no deleterious effect on the growth and desirable biochemical activity of bacterial biofertilizers at its recommended dose (10 ppm) of field application.

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APPENDIX

1. Composition of Luria-Bertani

Ingredients	g/L
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	10
Final pH (at 25°C)	7.5±0.2

2. FORMULA

2.1 **Percentage (%) decrease** = C-V/C * 100

where, C = control value

V = value of concentration whose percentage to be calculated

2.2 **Cfu/ml** = N x D x S/ml

where, N = number of viable cell count on plate

D = dilution amount

S = amount of sample to be spreaded

2.3 **G (generation time)** = (time, in minutes or hours)/n (number of generations)

G = t/n

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

$b = B \times 2^n$ (This equation is an expression of growth by binary fission)

Solve for n:

logb = logB + nlog2

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

$n = 3.3 \log b/B$

$G = t/n$

Solve for G

$G = t / 3.3 \log b/B$

2.4 **Specific growth rate (k)** = $2.32 (\log N_2 - \log N_1)/ T_2 - T_1$

where, N_1 = initial absorbance

N_2 = final absorbance

$T_2 - T_1$ = time interval

2.5 **Phosphate solubilisation percentage** (%) = $Z - C / C \times 100$

where, Z = solubilisation zone (cm)

C = colony diameter (cm)