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COMPARITIVE STUDIES ON THE BIOCONTROL EFFICIENCIES OF WET BIOMASS, DRY BIOMASS AND SECONDARY METABOLITES OF *PSEUDOMONAS FLUORESCENCE* ON *Cnaphalocrocis medinalis*

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Abstract:

An investigation was carried out to assess the efficiency of Pseudomonas fluorescence against Rice (PAPTATLA) leaf folder pest (Cnaphalocrocis medinalis) and the samples were obtained from the area of Thiruporur, Chennai. Pseudomonas fluorescence possess a variety of promising properties, which makes it a better bio control agent. A comparative study was made using formulated, wet biomass and a broth containing secondary metabolites of Pseudomonas fluorescence. The efficiency of secondary metabolites containing broth was very effective when compared to other preparations, which killed the rice leaf folder pest larvae within 7 hours. Histopathological examination of the pest larvae treated with formulated biomass disintegrated the epithelial cells of internal organs. The result revealed that there were significant histological changes in the treated pests when compared to the control. Production of secondary metabolites of Pseudomonas fluorescence was carried out and the compounds were characterized by TLC, SDS-PAGE, and GC-MS. There were 7 peak compounds found out, they are 1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane (13,69%) , 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione(10.11%), Pyrrolo[1,2-a] pyrazine 1,4dione,hexahydro-3-(2-methylpropyl(17.79%) l-Leucine,N-cyclopylcarbonyl-pentadecyl ester(7.09%), 3,6diisobutyl-2,5-piperazinedione (37.62%), 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione(8.52%), L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy(5.19%). Currently dry biomass formulations incorporated in talcum powder have been used to control pests but our research revealed that the secondary metabolites containing aqueous solution can very effectively control the pest Cnaphalocrocis medinalis.

Key Words: ***Cnaphalocrocis medinalis, Pseudomonas fluorescence*, Paddy crop, GC-MS, Histology,**

1. INTRODUCTION

The rice leaf folder, *Cnaphalocrocis medinalis* (Gurnee) (Lepidoptera: Pyralidae), one of the most destructive insect pests on rice, is distributed widely in rice-growing regions of Asia, This insect damages rice crops during its larval stage. The larva folds a leaf blade longitudinally with silk strands and feeds on mesophyll tissue inside the folded leaf, thereby creating longitudinal white and transparent streaks on the blade, disturbing photosynthesis and growth and ultimately reducing rice yield. Currently, the rice leaf folder populations are principally managed with chemical insecticides. However, in addition to causing the so-called '3R' problems, these chemicals have not achieved the desired control, largely due to the insect's shelter inside a folded leaf blade and its migratory behaviour, the latter necessitating precise timing and repeated insecticide applications. Therefore, it is imperative to develop an alternative approach to control this pest. Cultivar resistance and crop management are currently the dominant tactics being developed. (Yangquan Han et.al, 2015) (J. S. Bentur2006) *C.medinalis* is the dominant and wide spread species. The first two genera can be distinguished in adult stage by the markings on the wings. Adult female moth lays oval, flat eggs on leaf surface or on sheath which hatch on 4th day. Neonate larvae move to the tip of the leaf or into the whorl of an unopened leaf a scrape the surface. Second instar and older larvae fold the leaf and feed inside the fold. This results in white stripes on the leaf surface. In cases of severe infestation, the leaf margins and tips are dried up entirely and the crop gives a whitish appearance. Larvae pupate within the leaf fold and emerge as adults.

Insect pests damage rice crop at different stages of its growth. Among that leaf feeding insect pests are of major importance because of their ability to defoliate or to remove the chlorophyll content of the leaves leading to considerable

reduction in yield. Rice leaf folder, (*Cnaphalocrocis medinalis)* (Guen. Pyralidae Lepidoptera) was considered as pests of minor importance have increased in abundance in late 1980's and have become major pests in many parts world (Ahmed et al., 2010). Paddy leaf folder is one of the most important insect pests (Gunathilangaraj et al., 1986). Out of the eight species of leaf folder, the most widespread and important one is C.medinalis (Gunee) (Bhatti et al., 1995*). C. medinalis* (LF) has been reported to attain the major pest status in some important paddy growing areas (Madagascan et al., 1987). Second instars Leaf folder larvae glues the growing paddy leaves longitudinally for accommodation and feeds on green foliage voraciously which results in papery dry leaves (Khan et al., 1989). Loss incurred to the growing paddy crop is insurmountable (Ahmed et al., 2010). Feeding often results in stunting, curling or yellowing of plant green foliage (Alvi et al., 2003). The extent of loss may extend up to 63 to 80 percent depending on agro-ecological situations as reported by (Rajendran et al., 1986). The control of rice insect pests has often relied on extensive use of insecticides, which disrupt the beneficial insects and other insect fauna and also cause environmental contamination (Heong, 2005). The heavy use of insecticides and high fertilizer rates seem to favour leaf-folder population outbreaks (Gottfried and Fallil 1986].

Bio-pesticides are eco-friendly pesticides which are obtained from naturally occurring substances microbes and plants. Not all natural products bio pesticides. Some are chemical pesticides if they acts on nervous system of the pest. Through the use of bio pesticides in a wides way, agriculture and health programmes can be beneficially affected. There are many disadvantages associated with the use of chemical pesticides like genetic variations in plant population, reduction of beneficial species, damage to the environment or water bodies, poisoning of food and health problems such as cancer. Their usage reduces risk of exposure to chemical, reduces water pollution through fertilizer runoff, reduces number of application, causes less harm to beneficial pests, biodegradable, and provides better nutritional quality. Bio pesticide currently being developed may be excellent alternatives to chemical pesticides. The interest in bio pesticide is based on the advantages associated with such products which are:

- Inherently less harmful add less environmental load.
- Designed to affect only one specific pest or in some cases, a few target organism.
- Often effective in very small quantities and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problem

When used as a component of Integrated Pest Management (IPM) programs, bio pesticides can contribute greatly. (Vaishali kandpal (2014)).

2.MATERIALS & METHODS

2.1 Collection of sample

The commercially available bio pesticide *Pseudomonas fluorescence* was collected from the farmers of Wayanadu, Kerala and which was used for the further study to find out the similar biocontrol efficiency of the bacteria against the predominat pests infesting the crops of Tiruporur agriculture area of Tamil Nadu.

2.2 Isolation of *Pseudomonas fluorescence*

Pseudomonas fluorescence biopesticide powder was collected from Wayanadu, kerala was further reviwed using nutrient agar and King's B medium.

2.3 Production of biomass & secondary metabolites

Pseudomonas fluorescence isolated using the solid media was inoculated in two conical flasks containing 100 ml of sterile Nutrient broth and two conical flasks containing 100ml of sterile King's B broth. The media were incubated at 37ºC and Biomass was collected after 48 hours by centrifugation at 10,000rpm for 30 minutes. Secondary metabolite extraction was done after 72 hours of incubation period.

2.4 Extraction of secondary metabolites

The 72 hours old culture was used for this extraction. Culture was centrifuged at 10,000 rpm for 30 minutes and the supernatant was collected. Solvent extraction procedure was carried out using ethyl acetate (1:1). Middle layer was collected and samples were air dried and used for further characterization.

2.5 Bio control Efficacy to rice leaf folder pest

Two set of 4 containers with paddy rice leaf folder pest was taken marked as pest with supernatant, biomass, bio pesticide powder and control. In first set of 4 containers, the pest was treated with supernatant collected from king's B broth, biomass produced from King's B medium, pesticide powder and control. In second set of 4 containers, the pest was treated with supernatant collected from nutrient broth, biomass produced from nutrient broth, pesticide powder and control. Then the mobility & mortality of the pests were observed.

2.6 Reisolation of *Pseudomonas fluorescence* from treated rice leaf folder pest

The treated pests were surface sterilized using 50% ethanol and crushed using sterile mortar and pestle. The exudate was serially diluted using sterile distilled water and spread plate method was followed using King's B agar. After 24 hours of incubation period at 37ºC, the plates were examined under UV. The presence fluorescing colonies indicates *Pseudomonas fluorescence.*

2.7 Histopathological examination

K'B broth supernatant treated rice leaf folder pest and control pest treated and untreated leaf folder larvae were fixed in 37% formaldehyde. The fixed larvae were processed using 70%, 90% and absolute alcohol for 30 minutes in each respectively. Then the alcohol processed larvae were transferred in to different grades of Xyol (70%, 80% and 90%)] for 30 minutes in each concentration. The processed larvae were embedded in a molten paraffin vax. The blocks were placed in a microtome and thin sections were prepared. In thin sections containing the larvae segments were stained using haematoxylin and eosin. The stained slides were mounted using DPX mountant and observed under high power objective.

2.8 Extraction of secondary metabolites by ethyl acetate solvent extraction method

The K'B broth was centrifuged and the 40ml of supernatant was separated.50 ml of ethyl acetate and 50ml of supernatant are transfer in separating funnel and is mixed for 30mins.Then the funnel was kept in stand at room temperature for three hours. After 3 hours 3 layers were separated out. The three layers were carefully collected out. The middle layer was used for further analysis.

2.9 Thin layer chromatography

The ethyl acetate extract samples were applied on the bottom of the activated TLC plate using capillary tubes. It was then kept in TLC tank containing the mixture of chloroform: ethyl acetate: acetic acid in the ratio of 100:80:20 ml .The top of the TLC tank was closed with a thick glass plate to avoid the evaporation of solvents. The mobile phase moved up by capillary action and thus the active constituents were separated based on their solubility. The TLC plate was take out when the solvent front reaches the top of the plate. Then the 2% ferric chloride solution was sprayed on plate and dried in 10 minutes in hot air oven. Then the TLC plate viewed under UV light. All the sample have fluorescent nature, thus fluoresced under UV light. The TLC plates showed fluorescent bands/compounds.

2.10 Thin layer chromatography: amino acid

Thin layer chromatography was used to check the active constituents present in *Pseudomonas fluorescence* (ethyl acetate)

2.11 SDS PAGE [Sodium Dodicylsulphate Polyacrylamide Gel Electrophoresis

10%Stock acrylamide-13.3ml, Tris HCL-8ml [ph-8.8], Water-18.1ml, APS-200µl, 10%SDS-400µl, TEMED-20µl.Mixed well and poured the gel solution into the chamber and distilled water was added to top of the gel and left for 30 minutes. 4 % Stock acrylamide-1.35ml, Tris HCL-1ml [PH-6.8], Water-7.5ml, APS-50µl, 10%SDS-100µl, TEMED-10µl. The stacking gel mixture was poured into top of the chamber and comb was placed and allowed the gel to set 30mins.After stacking polymerized, the comb was removed carefully without damaging the wells. Clip was removed and bottom site of the spacers was removed. Electrode buffer was filled with electrophoresis apparatus. The gel plate inserted in to the electrophoresis apparatus .A 200µl of sample buffer and sample was mixed well and heated it for 2 minutes, cool it, 50µl of sample was loaded in to the each well and run the sample. After electrophoresis the chamber was removed from the electrophoresis apparatus. The side spaces were removed and gel was removed carefully from the chamber. The gel was transferred in to clean plastic container .Then the gel was kept inside the staining solution comassive blue solution overnight. Stain was discarded and detainer was done.

2.12 GC-MS (Gas Chromatography and Mass Spectrometry):

A 200µl sample was taken in beaker and ethyl acetate was added to it and is mixed by pipetting. Sample mixer was taken in syringe, 0.2 PTFF FILTER was inserted in syringe and test samples were filtered in 1.5ml of vial tube. In 4ml vial tube ethyl acetate was added. This 2 vial tubes were kept in injector, in injector the samples was washed and 1µl sample was injected in to inlet. After 43minutes the result peaks was observed in monitor.

3.RESULTS

3.1 Isolation of *P. fluorescence*

Pseudomonas fluorescence biopesticide powder was collected from wayanadu kerala,steak plate method were done . Colonies were examined under UV transilluminator. Fluorescing colonies were picked up and subcultured on Nutrient agar and King's – B medium. Kings – B media was found to be more selective for the cultivation of *P. fluorescence*

3.2 Biochemical characterization

The fluorescing colonies were identified by phenotypic methods of Bergy's Manual of systemic bacteriology. The results were tabulated. (table no: 1).

Table-1

S.NO	NAME OF TEST	RESULT
1.	Gram's staining	Gram negative
2.	Motility test	Motile
3.	Flourescence under UV	Positive
4.	Indole test	Negative
5.	Methyl red test	Negative
6.	Vp test	Negative
7.	Gelatin plate test	Positive
8.	Gelatin tube test	Positive
9.	Nitrate test	Positive

the results were confirmed the presence of *Pseudomonas fluorescence.*

3.3 Pest infestation in study area

Pest was collected from Thiruporur paddy field and it effect the leaf of rice plant. It effect was seen more in the larva stages it goes inside the leaf and fold itself and consume the leave changing its colour from green to white or cuts the leave and damage the whole rice plant.

3.4 Collection Of Data

The data was collected in five different area and the same pest with same effect was observed. On this basis the pest was identified as rice leaffolder. (Table 2)

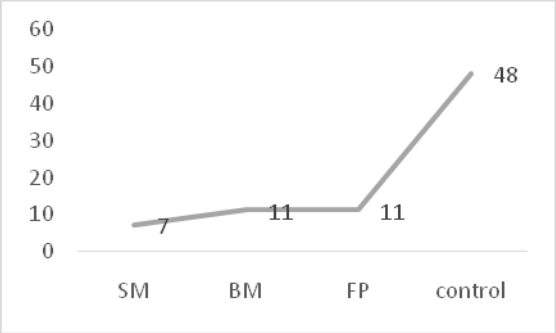
Table – 2: Prevalence Of Pest Kind In Study Area

S.no	Name of crop	Name of pest	Area	Season
1.	Rice (NLR)	Rice leaf folder	Thaiyur	Winter
2.	Rice (papatla)	Rice leaf folder	Thiruporur	Winter
3.	Rice (papatla)	Rice leaf folder	Pungery	Winter
4.	Rice (NLR)	Rice leaf folder	Mullibakkam	Winter
5.	Rice (NLR)	Rice leaf folder	Thandalam	Winter

3.5 Production of biomass & secondary metabolites

King's B broth which emitted the fluorescence under UV trans illuminator which confirmed that production of *Pseudomonas fluorescence* in the King's B m *Pseudomonas fluorescence* culture containing Nutrient broth emitting fluorescence under UV trans illuminator which confirm that production of *Pseudomonas fluorescence* in the nutrient media.

3.6 Bio control of rice leaf folder pest by *Pseudomonas fluorescence*



SM: Secondary metabolite broth

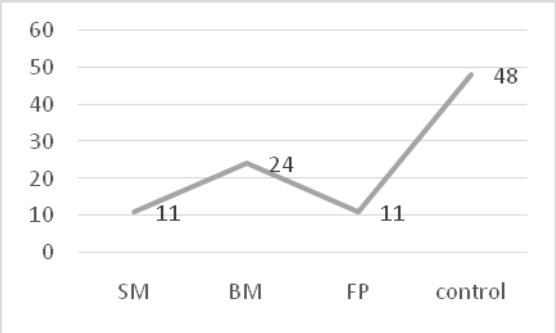
BM: Biomass

FP: Formulated pesticide

Chart-1: **Mortality rates (in hours) of *Pseudomonas fluorescence* produced in King's B medium.**

King's B broth treatment

Shows the treatment of *Pseudomonas fluorescence* on Rice leaffolder pest (*Cnaphalocrocis medinalis)*. The experiment revealed that the efficiency of King's B broth supernatant containing secondary metabolites were very effective when compared to the freshly prepared biomass and commercial biomass. The effect was very rapid and the mortality of the pest was achieved within 7 hours.



SM: Secondary metabolite broth

BM: Biomass

FP: Formulated pesticide

Chart-2: Mortality rates (in hours) of *Pseudomonas fluorescence* produced in Nutrient medium

Nutrient broth treatment

Shows the effect of *Pseudomonas fluorescence grown in Nutrient broth* on Rice leaffolder pest: (Cnaphalocrocis medinalis). The result shows that the supernatant containing secondary metabolite of P. fluorescence has more efficiency than biomass same as the previous result but the duration taken to kill the pest in this experiment was 11 hours. Hence the King's B 10^{-5} dilution sample emitted fluorescence under UV trans illuminator, presences of *Pseudomonas fluorescence*s in the treated rice leaf folder larvae, which confirmed the effects of *Pseudomonas fluorescens* on rice leaf folder pest broth can serve as a best choice for the production of the secondary metabolites when compared to nutrient broth.

3.7 Re isolation of *Pseudomonas fluorescence* in treated pest sample

Formation of fluorescent yellow colour colonies reconfirms the presence *Pseudomonas fluorescence* in treated rice leaf folder larvae

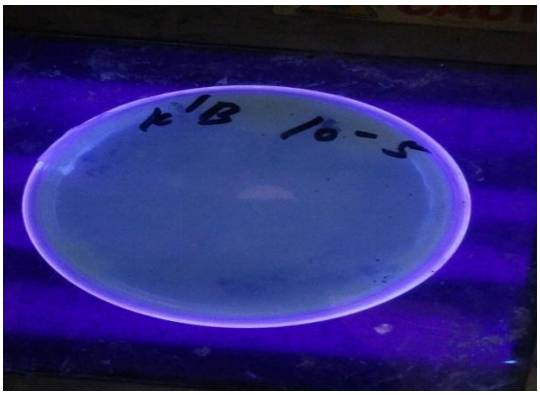


Fig-1: Reconfirmation of P.fluorescence from treated pest

10^{-5} dilution sample emitted fluorescence under UV Tran illuminator, presences of *Pseudomonas fluorescence*s in the treated rice leaf folder larvae, which confirmed the effects of *Pseudomonas fluorescens* on rice leaf folder pest.

3.8 Histopathological examination

After the pest was treated with the two different broth the pest was subjected under the Histopathological examination to view the changes between the control pest and normal pest. After the Histopathological examination organelle damage has been seen in the treated pest, against the normal pest.

Normal pest internal organs

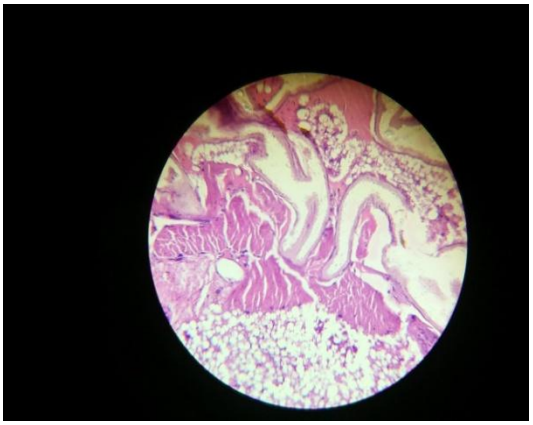


Fig-2: Histopathological examination of untreated leaf folder pest intestinal epithelial cell linings

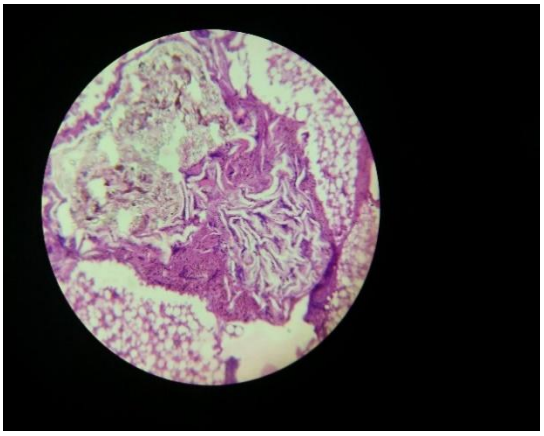


Fig-3: Intact epithelial cells in untreated leaf folder pest.

Histopathological examination of intestinal tract of that normal pest.

***Pseudomonas fluorescence* treated rice leaf folder pest internal organs structure**

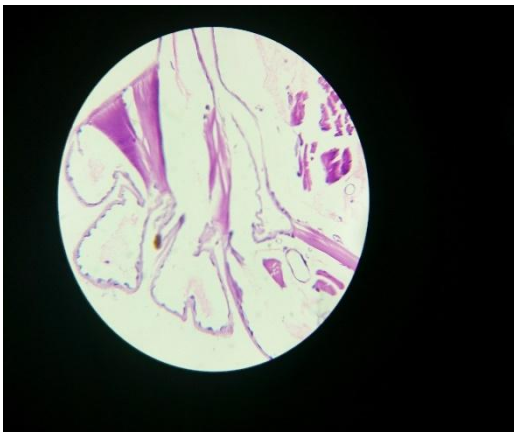


Fig-4: *Pseudomonas fluorescence* treated rice leaf folder pest shows disintegration of epithelial cells.

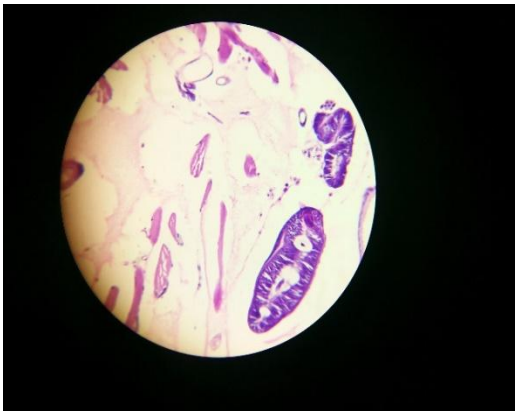


Fig-5: ***Pseudomonas fluorescence*** **treated rice leaf folder pest shows disintegration of epithelial cells.**

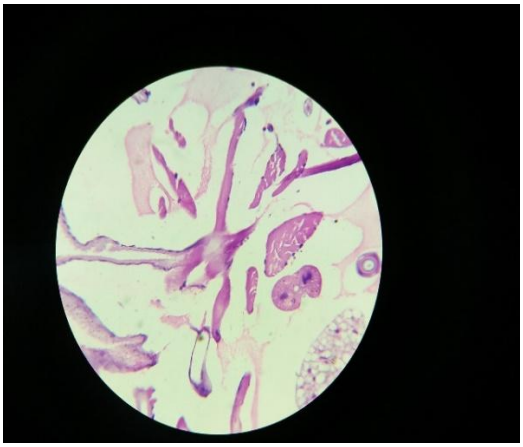


Fig-6: ***Pseudomonas fluorescence*** **treated rice leaf folder pest shows disintegration of epithelial cells**.

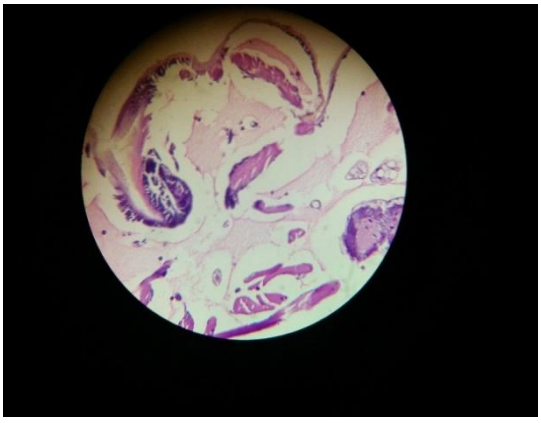


Fig-7: ***Pseudomonas fluorescence*** **treated rice leaf folder pest shows disintegration of epithelial cells**.

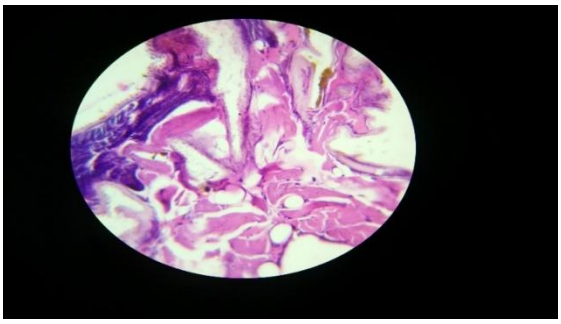


Fig-8: *Pseudomonas fluorescence* treated rice leaf folder pest shows disintegration of epithelial cells.

Histopathological examination of intestinal tract of pre-treated with *Pseudomonas fluorescence* and there is a disintegration of epithelial cells.

3.9 Characterization of secondary metabolites secondary metabolites of *Pseudomonas fluorescence*

Solvent extraction:

Crude metabolites were extracted from the effective growth medium (King's B) by partitioning with organic solvent (ethyl acetate). The extracted metabolites were tested for their efficiency against pathogens.

Thin layer chromatography

TLC - phenolic compound

Extracted metabolites, the phenolic compounds were identified by TLC and the compounds were fluorescing under UV Tran illuminator.

SDS PAGE

No proteins were identified in the extracted metabolites by comparing with protein standard marker.

GC-MS Analysis:

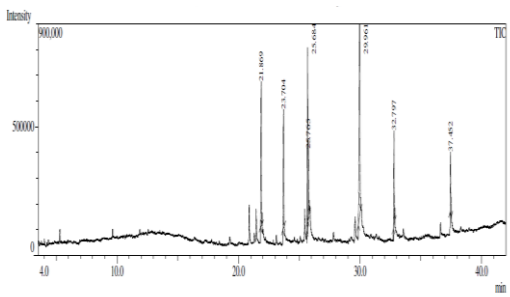


Fig-9: GC-MS analysis peaks compounds

The Gas chromatography and Mass Spectometry of *P.fluorescence* ethyl acetate crude extract shows the appearance of the seven major compounds at different retention times, , they are 1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane (13,69%) , 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione(10.11%), Pyrrolo[1,2-a] pyrazine 1,4dione,hexahydro-3-(2-methylpropyl(17.79%) l-Leucine,N-cyclopylcarbonyl-pentadecyl ester(7.09%), 3,6diisobutyl-2,5-piperazinedione (37.62%), 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione(8.52%), L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy(5.19%).

4.DISCUSSION:

Secondary metabolites produced by *P. fluorescence* in King's B broth, the supernatant of the broth was treated with the rice leaf folder pest (Cnaphalocrocis medinalis) which killed at 7 hours treatment by changing the internal organ structure or damaged internal organs . When compare to the control. (Siddiqui et.al, 2006) hydrogen cyanide, secondary metabolite produced by P. *fluorescence* may inhibit cytochrome c oxidase (CCO) of the termite respiratory chain and actually killed the insect by cyanide poisoning.

Misra et al. (2012) and Kaushik and Deb (2011) who have noted that the population suppression capacity of monocrotophos and cypermethrin was essentially prudent in some regions of India. The rice leaf folder population before and after application of insecticides were averagely counted. When the rice leaf folder was appeared on the rice field sprays with different chemical insecticides against rice leaf folder. The rice field was sprayed till sizeable reduction was brought by the different chemical products which were testes against rice leaf folder. In the same patteren Ramasubbaiah et al. (1980) noted that fenthion, phosphamidin, fenitrothion, endosulphon dimethoate, quinalphos, diazinon and carbaryl could effectively suppress LF menace. The rice field was sprayed six times since the appearance of rice leaf folder onto them the 1st spray was done. The data reveal that maximum reduction numbers in rice leaf folder population was recorded with one week after spray in all treatments during all six sprays. Saroja and Raju (1982) have viewed that cypermethrin and fanvalerate are best suitable pesticide to suppress rice leaf folder population and accordingly to maximize paddy yield. Our findings are in agreement with their of Bhanu et al. (2008) who have noted considerable variations in the efficacy of pesticides in field condition. Wakil et al. (2001) from Pakistan have reported that not all the pesticides were equally effective to check leaf folder attack.

Data further revelled that the maximum reduction percentage of rice leaf folder population was recorded as (73.22%) in the plots treated with Deltaphos followed by in the plots treated with Tracer (64.17%). However, the minimum reduction %age was recorded in the plots treated with Thioluxan (43.80%) during all for applications of insecticides on rice crop. The plots treated with Deltaphos gave the highest reduction in the population of rice leaf folder followed by Tracer and Thioluxan. The reduction in the population caused by these insecticides was statistically at par. Thioluxan significantly lower mortality than the former pesticides. Our results are in partial agreement with those of Mishra et al. (1998) and Kaushik and Deb (2011) who observed that monocrotophos and cypermethrin gave good control of rice leaf-folder and were at par statistically. All insecticidal treatments significantly out yielded the untreated plots. The highest average paddy yield was obtained in plots treated with the application of Deltaphos followed by Tracer, Thioluxan and Control. Our results are almost similar to the Saroja and Raju (1982) who obtained similar increase in yield by controlling C. medinalis damage by the application of synthetic pyrethroids.

[Sajjad Ali khuhro et.al. 2014]The result on reduction in the population of C. medinalis was recorded after one, three and seven days after 1st spray of insecticides. Different pesticides reduce C. medinalis population progressively. All insecticides showed maximum reduction %age after 7th day of 1st sprays except Thioluxan, which reduced maximum population on 3rd day. The maximum reduction percentage was recorded in the plots treated with Deltophos (76.19%), followed by the plots treated with Tracer (65.93%). The maximum reduction (46.92%) brought by Thioluxan 3rd day of spray after that it reduced its efficacy and rice leaf folder enhanced it activates on the crop.

But in ours investigation *P.fluorescence* killed the paddy rice leaf folder pest (Cnaphalocrocis medinalis) within 7 hours treatment comparing to others investigation which shows better bio control efficiency.

5. CONCLUSIONS

Beneficial paddy soil bacteria *Pseudomonas fluorescence* was able to kill the rice leaf folder pest, predominately present in the study area Thiruporur, Chennai. In the previous research, the biomass was used and even commercial preparations have only viable bacteria but the present study revealed that the cell free supernatant containing secondary metabolites of *Pseudomonas fluorescence* has more effect than the biomass

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