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In-silico and *In-vitro* Analysis of Targets of Bio-control Compounds Produced from *Pseudomonas fluorescens* Isolated from Vermicompost

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A B S T R A C T

The predominant pest infestation during the period of February to March 2019 was analyzed in the village Melkalvai, Kanchipuram district. They were identified as Brinjal Pest: Hadda / spotted beetle: *H. Vigintioitopunctate* and *Maconellicoccus hirsutus*. They were highly dangerous which affect the brinjal plant yield and Malvaceae family plants respectively. The crude extract of the secondary metabolites produced by *Pseudomonas fluorescens* from Kings B broth was found to be very effective against the two important pests studied. The treatment was given to find out the pesticidal effects and the mortality was found out in 24 hours incubation period with 1ml of secondary metabolite. The compounds present in the secondary metabolites were identified by GC/MS and they are2,3-butanediol (37.4 2%), 1,2-propanediol (29.69%), 1,2,3-Propanetriol, 1-acetate (1.46%), L-Proline, N-(hexanoyl)-, hexadecyl ester (2.46%), 3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-d(1.87%), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-(18.09%), n-Hexadecanoic acid (3.32%), 3-isobutyl hexahydropyrrolo[1,(4.03%) and (1-tert-Butoxypropan-2-yloxy) trimethylsilane (1.65%). The secondary metabolites of the same bacteria was reported previously were compared with this present results and we found that only two compounds were similar. They are Pyrrolo[1,2-a] pyrazine 1,4dione, hexahydro-3-(2-methylpropyl) and 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione. Many of the compounds have been reported with therapeutic effects and some are having pesticidal effects.

***Key words*:** *H. Vigintioitopunctate, Maconellicoccus hirsutus, Pseudomonas fluorescens,* Bio-pesticide

Agriculture plays a vital role in India. Apart from fulfilling the food requirement of the growing Indian population, it also plays a role in improving the economy of the country. The green revolution technology adoption from 1960 onwards has introduced wide varieties of rice crop in agriculture leading to an increase of 12-13% crop yield per hectare. Southeast Asia and India were the first developing countries to show the impact of GR on varieties of rice yields. Inputs like fertilizers and pesticides helped a lot in this regard. But in spite of this fact, food insecurity and poverty still prevail prominently in India [1].

Cultivar resistance and crop management are currently the dominant tactics being developed [2-3]. *C. medinalis*is the dominant and wide spread species in Tamil Nadu specifically Kanchipuram district [4]. 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 the fourth day. Neonate larvae move to the tip of the leaf or into the whorl of an unopened leaf and 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 [4].

Insect pests damage rice crop at different stages of its growth. Among that leaves 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 (LF) *Cnaphalocrocis medinalis* and Guen. Pyralidae Lepidopteraonce considered as pests of minor importance have increased in abundance in the late 1980's and have become major pests in many parts of the world [5]. Paddy leaf folder is one of the most important insect pests [6]. Out of the eight species of leaf folder, the most widespread and important one is *C. medinalis* (Gunee) [7].

Cnaphalocrocis medinalis (LF) has been reported to attain the major pest status in some important paddy growing areas [8]. 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 [9]. Loss incurred to the growing paddy crop is insurmountable [10]. Feeding often results in stunting, curling or yellowing of

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plant green foliage [11]. The extent of loss may extend up to 63 to 80 percent depending on agro-ecological situations [12]. 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 [13]. The heavy use of insecticides and high fertilizer rates seem to favour leaf-folder population outbreaks [14].

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; ultimately reducing rice yield. Currently, the rice leaf folder populations are principally managed with chemical insecticides. Use of chemical insecticides is harmful to rice which is used for human consumption and the hay a byproduct of the rice plant which is fodder for the cattle. Besides these chemicals do not achieve 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.

Our recent studies indicated that of the compounds produced by *Pseudomonas fluorescence* could kill the pest larvae in 7 hrs of treatment and the same biomass and secondary metabolites were very effective against a plant pathogen Pythiumsps. Beneficial paddy soil bacteria *Pseudomonas fluorescence* had a larvicidal effect on leaf folder pest, widely 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 [4]. The ethyl acetate extract of the secondary metabolites incorporated Saborauds dextrose agar was prepared and Pythiumsps was inoculated and the growth pattern was studied and the results showed the fungi static ability of the compounds [15]. We have identified seven peak compounds namely 1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane, 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl), l-Leucine,N-cyclopylcarbonyl-pentadecyl ester, 3,6-diisobutyl-2,5-piperazinedione, 3,6-diisobutyl-2,5-piperazinedione, 3,6-diisobutyl-2,5-piperazinedione, 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione and l-Prolinamide, 5-oxo-l-prolyl-l-phenylalanyl-4-hydroxy in the ethyl acetate extract of secondary metabolites produced by *Pseudomonas fluorescence* and these compounds were used for docking analysis which showed a complementarity of all the seven compounds against the cell wall receptor of *Pythiumsps.* The present work is intended to study the target of all the seven compounds in infesting larvae of *C. Medinalis* because our previous histopathological examinations of infected larvae showed disintegrated tissues in digestive tracts of the larvae [4] and study the new strains of *Pseudomonas fluorescens* present in vermicompost and produce secondary metabolites from the bacteria and to investigate the new pest targets.

MATERIALS AND METHODS

Melkalvai, is a one of the thaluk of Kanchipuram district; encompass 600 acres of cultivation land. So, in this place farming is the foremost profession of the people with 47% of the inhabitants involved in it. Paddy is the principal crop cultivated in this thaluk and edible vegetables, groundnuts, sugarcane, cereals and millets and pulses are the other major crops cultivated.

Collection of samples

Vermicompost was collected from our college vermicopost plant and *Pseudomonas fluorescens*was isolated 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 Melkalvai, agriculture area of Tamil Nadu.

Isolation and identification of Pseudomonas fluorescens

Nutrient agar and King's B medium (Hi Media, India) was prepared and pour plate method was used to isolate the bacteria present in the diluted samples of vermicompost. Yellowish green fluorescing baceria was selected for further study. Bergys manual of systemic bacteriology was used for the phenotypic characterization of the unkwon bacteria and idenfied as *Pseudomonas fluorescens.*

Production of biomass and secondary metabolites

Production of Secondary metabolites from *Pseudomonas fluorescence* was optimized under various parameters such as temperature, pH, incubation period and agitation speed. Secondary metabolite extraction was extracted using ethyl acetate (1:1).

Characterization of the extracted secondary metabolites

Thin layer chromatography [4]

Thin layer chromatography was used to check the active constituents present in *Pseudomonas fluorescence* (ethyl acetate extract). 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 taken 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. The entire sample has fluorescent nature, thus fluoresced under UV light. The TLC plates showed fluorescent bands/compounds.

*GC-MS [*4]

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 and 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.

Molecular docking: Autodock 2.0

AutoDock is an automated procedure for predicting the interaction of ligands with biomacromolecular targets. The motivation for this work arises from problems in the design of bioactive compounds, and in particular the field of computer-aided drug design. Progress in biomolecular x-ray crystallography continues to provide important protein and nucleic acid structures. These structures could be targets for bioactive agents in the control of animal and plant diseases, or

simply key to the understanding of fundamental aspects of biology. The precise interaction of such agents or candidate molecules with their targets is important in the development process.

Step 1—Coordinate file preparation

AutoDock4.2 is parameterized to use a model of the protein and ligand that includes polar hydrogen atoms, but not hydrogen atoms bonded to carbon atoms. An extended PDB format, termed PDBQT, is used for coordinate files, which includes atomic partial charges and atom types. The current AutoDock force field uses several atom types for the most common atoms, including separate types for aliphatic and aromatic carbon atoms, and separate types for polar atoms that form hydrogen bonds and those that do not. PDBQT files also include information on the torsional degrees of freedom. In cases where specific sidechains in the protein are treated as flexible, a separate PDBQT file is also created for the sidechain coordinates. AutoDock tools, the graphical user interface for AutoDock, may be used for creating PDBQT files from traditional PDB files.

Step 2—AutoGrid calculation

Rapid energy evaluation is achieved by pre calculating atomic affinity potentials for each atom type in the ligand molecule being docked. In the AutoGrid procedure the protein is embedded in a three-dimensional grid and a probe atom is placed at each grid point. The energy of interaction of this single atom with the protein is assigned to the grid point. AutoGrid affinity grids are calculated for each type of atom in the ligand, typically carbon, oxygen, nitrogen and hydrogen, as well as grids of electrostatic and desolvation potentials. Then, during the AutoDock calculation, the energetics of a particular ligand configuration is evaluated using the values from the grids.

Step 3—Docking using AutoDock

Docking is carried out using one of several search methods. The most efficient method is a Lamarckian genetic algorithm (LGA), but traditional genetic algorithms and simulated annealing are also available. For typical systems, AutoDock is run several times to give several docked conformations, and analysis of the predicted energy and the consistency of results is combined to identify the best solution.

Step 4—Analysis using AutoDockTools

AutoDockTools includes a number of methods for analyzing the results of docking simulations, including tools for clustering results by conformational similarity, visualizing conformations, visualizing interactions between ligands and proteins, and visualizing the affinity potentials created by AutoGrid.

RESULTS AND DISCUSSTION

Pseudomonas fluorescence biopesticide powder was collected from wayanadu kerala,streak plate method were carried on Nurtient agar and Kings B agar as per the previous procedure [4]. Colonies were examined under UV transilluminator. Fluorescing colonies were picked up and subcultured (Fig 1). Kings – B media was found to be more selective for the cultivation of *P. fluorescenc and the growth was confirmed based on the* production of yellowish pigment on King's B Medium. Further the same bacteria was characterized by biochemical tests. They were gelatin liquefiers, catalase positive, oxidase positive and nitrate reductase positive and were identified as *Pseudomonas fluorescence*.

Maximum production of active crude extract was achieved at 37C, PH7.4, tryptophan as nitrogen source, glycerol as carbon source in a modified King's B broth under submerged fermentation process.

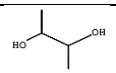
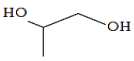
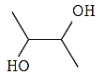
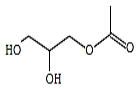
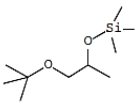
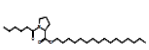
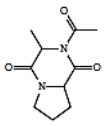
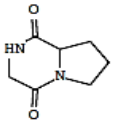
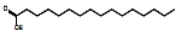
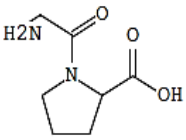
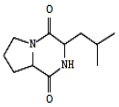
Fig 1 Growth of *P. fluorescence* produced yellow colour colonies in Nutrient Agar, King's B media

Pseudomonas fluorescence was effectively inhibited (57%) (Fig 2) the mycelial growth *Pythium spp*. The antifungal activity of *P. fluorescence* had reported against the *P. oryzae* and *R. solani* [16]. *Pseudomonas fluorescence* was shown to effectively inhibit *P. oryzae* and *R. solani* by agar plating method reported that *P. fluorescence* inhibit a maximum of (50-85%) growth of 2 pathogens when compare to the control. The antifungal activity of *P. fluorescence* R21, F113 had reported against the *Pythium spp* [17] *P. fluorescence*metabolites 2, 4-diacetylphloroglucinol (DAPG) was shown to effectively inhibit *Pythiumspp* By agar plating method reported that *P. fluorescence* R21 effectively inhibit a maximum of (58.3%) growth of *Pythium spp* and *P. fluorescence F113* effectively inhibit a maximum of (66.7%) growth of *Pythiumspp* when compare to control. In ours *Pseudomonas fluorescence* was effectively inhibit (5cm).

Dead pest | Skeletonized - Grub | *Maconellicoccus hirsutus*

Fig 2 The mycelial growth *Pythium spp*.

Table 1 Identification of secondary metabolite in the *Pseudomonas fluorescence*

S. No.	Retention time	Compound name	Molecular formula	Molecular weight	Structure
1	3.161	2,3-BUTANEDIOL	$C_4H_{10}O_2$	90	
2	3.345	1,2-PROPANEDIOL	$C_3H_8O_2$	76	
3	4.027	2,3-BUTANEDIOL	$C_4H_{10}O_2$	90	
4	4.701	1,3-Propanediol	$C_3H_8O_2$	76	
5	11.037	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	134	
6	13.268	(1-tert-Butoxypropan-2-yloxy)trimethylsilane	$C_{10}H_{24}O_2Si$	204	
7	27.062	L-Proline, N-(hexanoyl)-, hexadecyl ester	$C_{27}H_{51}NO_3$	437	
8	27.611	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl	$C_{10}H_{14}N_2O_3$	210	
9	28.088	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	
10	32.225	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	
11	35.303	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	
12	36.292	3-ISOBUTYLHEXAHYDROPYRROLO[1,2-A]PYRAZINE-1,4-DIONE	$C_{11}H_{18}N_2O_2$	210	

The antifungal activity of *Pseudomonas fluorescence* EBC5, EBC6, EBC7 had reported that *P. fluorescence* against the *Pythium aphanidermatum* [18] *P. fluorescence* EBC 5, EBC 7 and EBC 6 recorded that inhibit a minimum mycelial growth (28.00, 30.66 and33.33 mm, respectively) with maximum inhibition zone of (12.33, 11.66 and 11.08 mm, respectively) of *Pythium aphanidermatum* over control. In our studies *Pseudomonas fluorescence* was effectively inhibit (5cm) the mycelial growth *Pythium spp*. The *in vitro* action of *P. Fluorescens* against *P. aphanidermatum* obvious by the inhibition zones shows fungal activity of the bacterium [19]. Most of the authors showed antimicrobial activity of bacteria, particularly *Pseudomonas* species on fungal pathogens. Antifungal metabolite of *Pseudomonas antimicrobial* inhibited efficiently showed the establishment of inhibition zone due to *in vitro* antifungal activity of. Microscopic observations of mycelium tissue inside inhibition zone showed the absence of spore and the denaturation of the mycelium. The culture of a portion of this zone on the new PDA could not allow the mycelium development. This result could be suggested that compounds released by the bacterium are fungicides.

This result was also suggested hypothesis depending that *P. fluorescence* produced antibiotics responsible of the bio protection. Indeed, it was isolated more secondary substances produce by *P. fluorescence* which were fungicides antioomycete properties. In our studies *Pseudomonas fluorescence* was effectively inhibit (5cm) the mycelial growth *Pythium spp*. [20] *P. fluorescence* strains (PSB-1, PUR-46, R1, R2 and R3) are significantly effective against all the Phyto pathogenic fungi. Out of the five strains studied, the best result was shown by PUR-46, which showed almost complete inhibition and maximum activity against *Fusarium sp*. (96.30%), *Curvularia lunata* (96.07%) and *Bipolaris sp*. (95.08%) followed by R1 that showed inhibition percentage of 94.55% against Helminthosporium. In our studies Pseudomonas *fluorescence* was effectively inhibit (57%) the mycelial growth *Pythium spp*.

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 a larval stage of the pest within 7 hours treatment [4]. Siddiqui *et al*. [21] reported that hydrogen cyanide, secondary

metabolite produced by *Pseudomonas fluorescence* may inhibit cytochrome c oxidase (CCO) of the termite respiratory chain and actually kill an insect by cyanide poisoning.

Investigation on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new pesticide products. Several such products have been developed and used as insecticide, bactericide, fungicide, acaricide, insecticide or acaricide in agriculture. However, research on new metabolites and its development, as agrochemical by fermentation technology has not been addressed adequately in India. Therefore, it is considered worthwhile to generate reliable data on the isolation, production, productivity and bio-efficacy of antifungal metabolites from some native *P. fluorescence*. Kraft [22] invented that the diketopiperazine is E)-3,6-bis[4-(N-carboxyl-2-alkyl)amidoalkyl]-2,5-diketopiperazine (which may also be referred to as diketopiperazine or DKP), their use in the preparation of such drugs comprising healing, prophylactic and analytical agents, stabilizing agents and structures for their transport is unveiled.

The piperazine framework has been classified as an advantaged assembly and is regularly establish in biologically dynamic compounds through a number of diverse therapeutic areas. Certain of these therapeutic areas include antifungals, antidepressants, antiviral, and serotonin receptor (5-HT) antagonists/agonists [23].

Our previous study revealed that the inhibitory effect of the Pythium spp. Growth was due to the compounds present in the ethyl acetate extract of the secondary metabolites produced by *P. fluorescence* and they are reported as 1,4-diaza-2,5-dioxobicyclo[4.3.0] nonane, 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione, Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl), l-Leucine,N-cyclopylcarbonyl-pentadecyl ester, 3,6-diisobutyl-2,5-piperazinedione, 3,6-diisobutyl-2,5-piperazinedione, 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione and l-Prolinamide, 5-oxo-l-prolyl-l-phenylalanyl-4-hydroxy [4].

Our present investigation using molecular docking of the same compounds produced many novel findings. The secondary metabolite crude extract could able to kill *Cnaphalocrocis medinalis* quickly when compared to wet and dry biomass whereas the green clover worm (chewing larvae), *Hypenascabra* was found to be ineffective one of the pest effect the rice plant in starting growth stage.

There were seven peak compounds were used for autodock – molecular docking software and the receptor chosen was Arylphorin. It is a larval storage protein (LSP) which may serve as a storage protein used primarily as a source of aromatic amino acids for protein synthesis during metamorphosis. It is a constituent of the sclerotizing system of the cuticle, and serves as a carrier for ecdysteroid hormone. Among the seven compounds studied, four compounds had good binding energy and inhibition constant. 3,6-diisobutyl-2,5-piperazinedione was found to be a more suitable compounds which docked on the selected receptor and gave an acceptable inhibition constant (Table 1). This may be the reason for the larvicidal effect of the compounds produced by *Pseudomonas fluorescence* [4]. New compounds were identified in the new strain of *Pseudomonas fluorescens* isolated from vermicompost and used against other pests.

CONCLUSION

Beneficial paddy soil bacteria *Pseudomonas fluorescence* was able to produce maximum concentration L-prolinamide, 5-oxo-l-prolyl-l-phenylanyl-4-hydroxy which possess antifungal activity against *Pythiumspp MTCC 10247*, which can be extended with various phytopathogens for further application. *Pseudomonas fluorescence* which also having a potential to kill the Rice leaf folder pest (*Cnaphalocrocis medinalis*). The presented data exhibit the antifungal activity of *Pseudomonas fluorescence* and indicate the possibility of using *Pseudomonas fluorescence* as a biological control agent of some plant pathogenic fungi and leaf folder pest. However, this requires further screening of a large number of *Pseudomonas fluorescence* from different regions of India. The antimicrobial activity of *Pseudomonas fluorescence* may be attributed to the various phytochemical constituents have even more potency with respect to the inhibition of microbes and insects.

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