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Mining the Biomolecules of *Pseudomonas aeruginosa* Reveals the Anti-oomycetes Nature Against *Phytophthora infestans,* the incitant of Late Blight of Potato

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ABSTRACT: Late blight of potato caused by the *Phytophthora infestans;* is well known across the world for its Irish famine in 1845. Whilst chemical pesticides are better in management of late blight, but they are also burden for environment and native microbes. However, unless pathogen co-evolution occurs, resistant varieties play little role in minimising damage caused by late blight. The present study aims at addressing these two issues by isolation of native endophytic bacteria that can provide a new strategy in management of late blight of potato. The dual culture assay revealed that endophytic *P. aeruginosa* NM314 had strong anti-oomycete activity against *P. infestans*. Phenazine-1-carboxylic acid was produced by *P. aeruginosa* NM314 in the zone of inhibition might have a key role in anti-oomycete action. Further, phylogenetic analysis revealed the evolutionary relationship of *P. aeruginosa* NM314 with 31 strains of *P. aeruginosa*. Based on these results, the indigenous potato endophyte *P. aeruginosa* NM314 can be explored an alternative for chemical pesticides to control the late blight of potato.

Keywords: *Phytophthora infestans*, Phenazine-1-carboxylic acid, biocontrol, *Pseudomonas aeruginosa*, NM314, endophyte.

INTRODUCTION

Chemical pesticides have been widely employed in recent decades to reduce yield losses caused by plant diseases and pests (Hillocks, 2012). However, their continued and abusive use has been related to adverse side effects, causing environmental and human health concerns. Oomycetes that are phytopathogenic, such as *P. infestans*, are responsible for some of the most severe plant diseases known to man. *P. infestans*, the causative agent of potato and tomato late blight, currently has a nearly global distribution (Birch *et al*., 2012) and responsible for both economic and food loss. Hence, it is necessary to control this deadly menace with the potential biocontrol agents in eco-friendly manner.

The term endophyte refers to any organism that grows within plant tissues, although it is now more specifically defined in terms of their types (fungal and bacterial) and relations (obligate or facultative with the host plant) (Rosenblueth and Martínez-Romero, 2006). Every plant that has been studied so far has had one or more types of endophytes (Strobel and Daisy, 2003).Besides, their interaction with other pathogenic microbes and their biomolecules haven't exploited completely. Islam *et al*. (2022) used potential biocontrol agents *Pseudomonas putida* (BDISO64RanP) and *Bacillus subtilis* (BDISO36ThaR) against late blight of potato in growth chamber and field conditions, which played a key role in reduction of late blight incidence up to 99% until 60 days after planting. These bioagents helped to regulate the late blight disease after 70 days of planting. The usage of fungicides, however, was reduced, as was the damage caused by late blight. On the other hand, the race development against resistant varieties also problematic in controlling the late blight. Rakosy-Tican *et al*. (2020) explored *Solanum bulbocastanum* Dun Plus for race specific resistant genes and identified *Rpi-Blb1* and *Rpi-Blb3* genes by studying the *Avr* effectors of *P. infestans*.

Understanding the biology of both the pathogen and biocontrol agent is very important to manage any plant disease. Being an endophyte, which is already well accomplished with the plant system, has an added advantage to compete with pathogen under the same environments. Considering the same, in this study, we isolated the endophytes from potato plants and screened against *P. infestans*. Further, the mechanism of action

involved in anti-oomycete nature of *P. aeruginosa* NM314 has been investigated.

MATERIALS AND METHODS

Source of microorganisms and Isolation: The late blight pathogen, *P. infestans* SKM (MW285753) was obtained from the Culture Collection Centre, Department of Plant Pathology, TNAU, Coimbatore. For isolation of endophytic bacteria, plant samples were collected from potato growing areas in Nilgiris, Tamil Nadu. The healthy leaves were washed thoroughly and surface sterilized according to the protocol given by Yan *et al*. (2018) with minor changes.Briefly, the samples were cut into small pieces and sterilized in the following order: a 30-s soaking in 70% ethanol, followed by a 1-minsoaking in 1.5% sodium hypochlorite, a 30-s soaking in 70% ethanol, and a rinse by sterile water for five times. About 2 g of surface sterilized leaf tissues were homogenized using sterile pestle and mortar with sterile water. Further, the aliquot of one ml was transferred up to 10^{-3} dilution for pour plate method using nutrient agar (NA) medium. The NA plates were then incubated at 28°C for 48 h in orbital shaker cum incubator. The bacterial colonies developed in NA plates were further re-streaked continuously for obtaining pure cultures.

Anti-oomycete activity of bacterial endophytes against *P. infestans*: The pure cultures of bacterial endophytes were assessed for their anti-oomycete activity against *P. infestans* in dual culture assay. The actively growing bacteria of 48h old cultures were streaked at 2 cm away from one side of the Petri plate containing pea sucrose agar (PSA). A 5 mm *P. infestans* disc from 14 days old culture was kept 2 cm away from the end of Petri plate exactly opposite to the bacteria-streaked area. Then the PSA plates were kept for incubation at 18° in incubator for 16 days. The effective bacterial endophyte was identified using the following formula; Percent inhibition of pathogen radial mycelial growth= (MP-MT/MP) x100; where, MP= Mycelia growth of pathogen in control, MT= Mycelia growth of pathogen in bacteria treated plates.

Molecular characterization of effective bacterial endophyte:

The genomic DNA (gDNA) of effective bacterial endophyte was subjected to PCR using 16S rRNA primers; 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) (Frank *et al*., 2005). The PCR reaction was prepared as follows: 40µl reaction mixture containing 20µl of Taq DNA Polymerase Master Mix RED (AMPLIQON), 8µl of sterile water, 4µl each of forward and reverse primers with 0.5 mM concentration and 4µl of gDNA (50-100 ng/µl). The amplifications were carried out in Eppendorf Mastercycler Nexus Thermal Cycler (Eppendorf SE, Germany) with following protocol; initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 58° for 1 min, and extension at 72° C for 1 min, followed by 15 mins of final extension at 72° C. The resulted PCR amplicon of ~1500bp was sent for Sanger sequencing. The identification of aligned sequence was confirmed by comparing it in two databases; NCBI and EzBio cloud for sequence similarity. Further, to know the evolutionary relationship of our bacterial endophyte, we performed neighbor-joining method with 1000 bootstrap replicates in MEGA11 (Tamura *et al.,* 2021).

Biomolecules extraction and identification: To identify the anti-oomycete mechanism of effective bacterial endophyte, we extracted the biomolecules from zone of inhibition in dual culture assay as described by Cawoy *et al.* (2015). The extracted samples were passed through a vacuum flash evaporator (Rotrva Equitron Make) to remove eluent. The HPLC grade methanol (one ml) was used as a solvent and the samples were sent for GC-MS analysis (GC Clarus 500 Perkin Elmer Analysis using NIST version 2005 MS data library). The biomolecules from control (pea sucrose agar alone) were normalized in all other treatments like *P. infestans* alone, *P. aeruginosa* NM314 alone and in combination with *P. infestans*. The biomolecules identified by GC-MS were compared using heatmap analysis (Metsalu and Vilo, 2015). And the chemical groups of different biomolecules released in *P. infestans* alone, *P. aeruginosa* NM314 alone and in combination with *P. infestans* were represented using Pie charts.

Statistical analysis: The treatment means were compared with ANOVA by using Duncan's multiple range test at a 5% level of significance. All data were statistically analysed and interpreted using the Web Agri Stat Package 2.0 (WASP 2.0).

RESULTS AND DISCUSSION

Identification of effective bacterial endophyte against *P. infestans*: A total of five bacterial endophytes; NM220, NM314, NM351, NM377 and NM390 were obtained in pure cultures. Dual culture assay revealed that the isolate NM314 has showed the highest percent reduction of *P. infestans* mycelial growth over control *i.e*., up to 77.39% (Fig. 1) with 46.00 mm inhibition zone (Fig. 2, Table 1) followed by NM220 (45.15%), NM351 (40.42%), NM390 (24.43%) and NM377 (18.71%). The isolate NM314 has been identified as *P. aeruginosa* through molecular confirmation by 16S rRNA amplification and the sequence has been submitted in NCBI- GenBank (Accession no. ON411323). The *P. aeruginosa* FG106 has been isolated from the root portions of tomato and explored its biocontrol potential against various pathogens of potato, tomato and taro crops (Ghadamgahi *et al*., 2022). Among the pathogens tested, *P. aeruginosa* FG106 showed inhibition of *P. infestans* up to 83.40% in dual culture assay. Similarly, *P. aeruginosa* B18, a plant growth promoting rhizobacterium significantly reduced the mycelial growth of sugarcane pathogens; *Sporisorium scitamineum*, *Ceratocystis paradoxa*, and *Fusarium verticillioides* (Singh *et al*., 2021). The phylogenetic tree was constructed by 32 strains of *P. aeruginosa* from various geographical locations (data obtained from NCBI). The dendrogram revealed that all isolates were clustered majorly in to five groups namely, A, B, C, D, and E (Fig. 3). Our study isolate *P. aeruginosa*

NM314 has clustered in group A and had close relationship with *P. aeruginosa* YB01 (China), *P. aeruginosa* BP C2 (India), *P. aeruginosa* 9 (China), *P. aeruginosa* ZNW (China) and *P. aeruginosa* 1213-2 (China). Further, phylogenetic analysis confirmed the distribution of *P. aeruginosa* in various countries starting from India, China, Peru, Serbia, Republic of Korea, Sudan and South Korea.

Table 1: Anti-oomycete activity of endophytic *P. aeruginosa* NM314 against *P. infestansin vitro.*

Isolates	Radial growth of the pathogen (mm)	Inhibition zone diameter (mm)	Percentradial growth inhibition of *Phytophthora infestans* SKM over control* (%)
NM220	48.00	23.00	45.15^{b} (42.21)
NM314	21.33	46.00	77.39^{a} (61.61)
NM351	53.66	16.00	40.42^{c} (39.47)
NM377	73.00	9.00	18.71^{e} (25.62)
NM390	66.33	12.00	24.43^{d} (29.60)
Control	90.00	0.00	0.00
SEm= 0.9048			
CD (0.05)= 1.791			

*Mean of three replications

Note: Means in a column followed by same subscript are not significantly different according to DMRT at P ≤0.05. Arcsin values are shown in the parentheses.

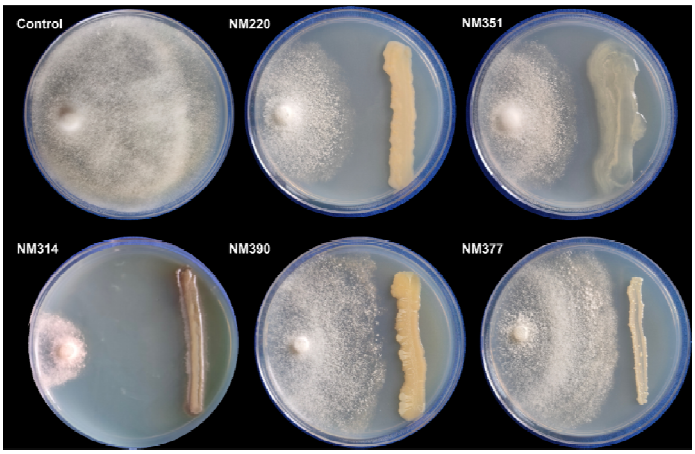

Fig. 1. Assessment of anti-oomycete activity of potato bacterial endophytes against *P. infestans* using dual culture assay.

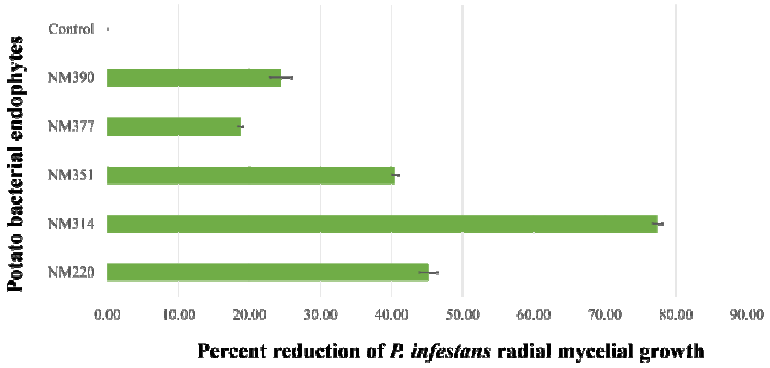

Fig. 2. Percent reduction of *P. infestans* mycelial growth over control by potato bacterial endophytes

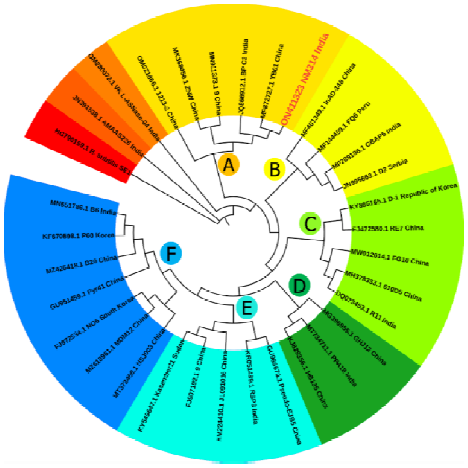

Fig. 3. Phylogenetic tree of *P. aeruginosa* based on 16S rRNA. Evolutionary analysis was conducted using MEGA11 by neighbor-joining method with 1000 bootstrap replicates. The tree is rooted to *B. subtilis* SE1 16S rRNA.

Understanding the mechanism of *P. aeruginosa* NM314 against *P. infestans*: The biomolecules identified by GC-MS analysis (Fig. 4) in various treatments has been represented in Pie chart (Fig. 5). The *P. infestans* alone produced biomolecules of organic oxygen compounds (43%), lipids and lipid like molecules (57%). The *P. aeruginosa* NM314 alone produced organic oxygen compounds (15%), benzenoids (14%), organo-heterocyclic compounds (14%), organic acids (43%), lipids and lipid like molecules (14%). Di-trophic interaction of *P. aeruginosa* NM314 and *P. infestans* produced the biomolecules of organic oxygen compounds (50%), organo-heterocyclic compounds (13%), organic acids (12%), lipids and lipid like molecules (25%). The heatmap analysis (Fig. 6) clearly showed the differential expression of biomolecules in all treatments. In *P. infestans*, the biomolecules; dl-Glyceraldehyde dimer; Dihydroartemisinin; D-Galactose, diethyl mercaptal, pentaacetate; Glycodeoxycholic acid; ç-Sitosterol; à-D-Galactopyranoside showed the higher abundance when compared to other treatments. Similarly, *P. aeruginosa* NM314 produced 5-Hydroxymethylfurfural; Aziridinone, 1,3-bis(1,1-dimethylethyl)-; 9-Octadecenamide; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; Deoxyspergualin; 10-Undecenoic acid; Cyclopentanecarboxylic acid, 2-hydroxy-, when cultured alone and in di-trophic interaction, Melezitose; Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester; 3-Deoxy-d-mannoic lactone; Oleic Acid; Stigmasterol and 1-Phenazinecarboxylic acid (PCA) biomolecules were produced. The PCA biomolecule has been reported to be involved in various functions related with antifungal, anti-oomycete, anti-bacterial and induction of plant defense genes (Wang *et al.*, 2021). Interestingly, the presence of PCA from the di-trophic interaction confirms the anti-oomycete nature of *P. aeruginosa* NM314, which resulted in significant reduction in mycelial growth of *P. infestans*. In agreement with this finding, transcriptome studies of *P. infestans* in response to PCA indicated that PCA involved in damage of *P. infestans* mycelium (Roquigny *et al.*, 2018). Similarly, Léger *et al.* (2021) studied the anti-oomycete effect of PCA producing 23 *Pseudomonas* spp. in potato tuber bioassays.Conversely, results indicated as there is no correlation of PCA production and biocontrol activity of *Pseudomonas* spp. against *P. infestans*, which indicated apart from PCA, other determinants like side rophores, cyclic lipopeptides and NRPS (non-ribosomal peptide synthase) could play important role in biocontrol of *P. infestans*. Further, Perneel *et al.* (2008) reported that PCA produced by *P. aeruginosa* PNA1 found to have antifungal effect. For instance, *P. aeruginosa* GC-B26 has showed both antifungal and anti-oomycete activity against *Colletotrichum orbiculare*, *Phytophthora capsici* and *Pythium ultimum*by producing PCA (Lee *et al.*, 2003). Consistent with these results, in this study, we observed anti-oomycete activity of *P. aeruginosa* NM314 against *P. infestans*. On the other hand, PCA has been reported to induce the plant defense in potato against *P. infestans* by induction of genes related ethylene and jasmonic acid signalling pathways in potato plants (Morrison *et al.*, 2017). So, our study strain, endophytic *P. aeruginosa* NM314 producing PCA, could be a new strategy for the management of late blight of potato.

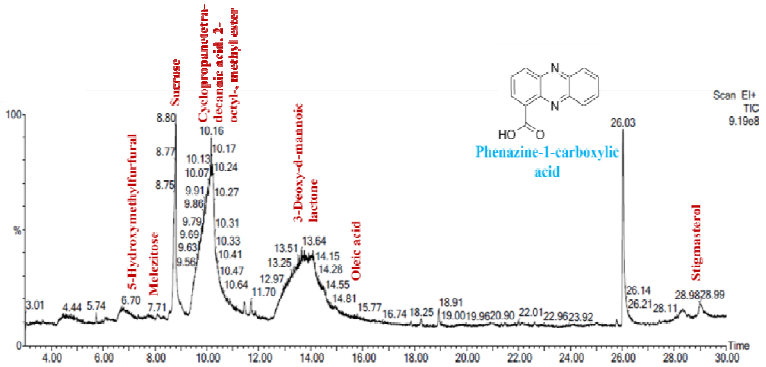

Fig. 4. GC-MS chromatogram of biomolecules produced during di-trophic interaction of *P. aeruginosa* NM314 and *P. infestans*.

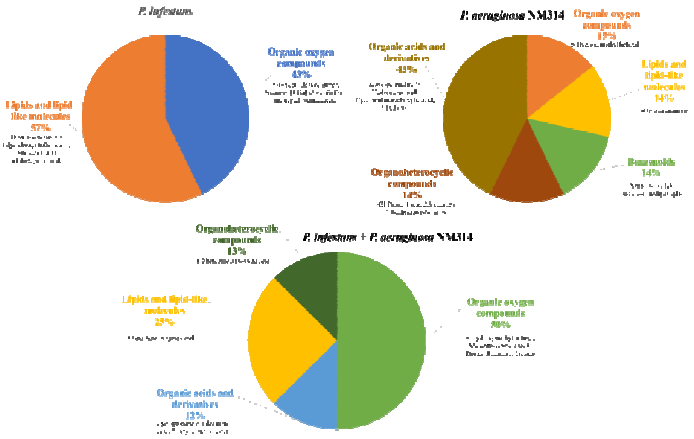

Fig. 5. Pie chart representing the different chemical groups of biomolecules identified from *P. infestans* alone, *P. aeruginosa* NM314 alone and *P. infestans* + *P. aeruginosa* NM314.

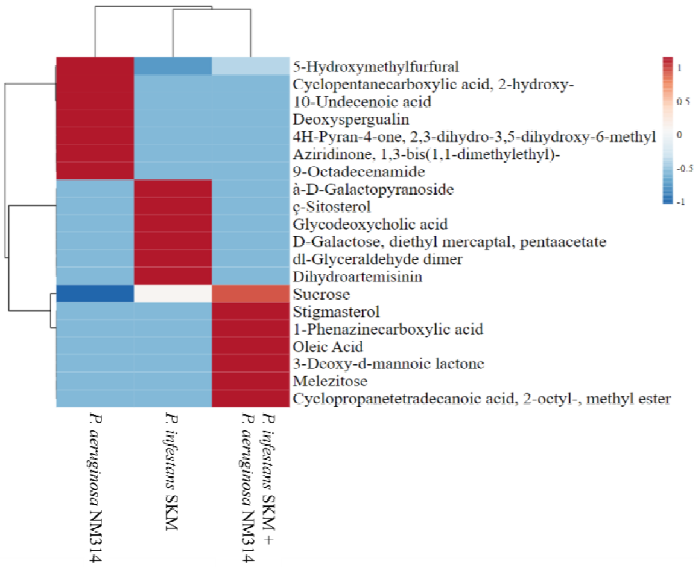

Fig. 6. Heat map analysis of differential expression of biomolecules identified from *P. infestans* alone, *P. aeruginosa* NM314 alone and *P. infestans* + *P. aeruginosa* NM314.

CONCLUSION

In dual culture assay, *P. aeruginosa* NM314 exhibited various biomolecules when cultured alone and in combination with *P. infestans*. During di-trophic interaction, it produced PCA, which is a very important antibiotic plays important role in anti-oomycete action. Additionally, the phylogenetic tree construction using neighbor-joining method revealed its close relationship with other *P. aeruginosa* strains like *P. aeruginosa* YB01 (China) and formed a separate cluster. At this stage, little is known about the complete mechanisms exhibited by *P. aeruginosa* NM314 against *P. infestans*. Evaluation of *P. aeruginosa* NM314 under field conditions is further required for its effective deployment as a potential biocontrol agent against *P. infestans*.

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