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Characterization of Gut Bacterial Diversity and its Functional Role of Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

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Authors Contribution

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Abstract

The invasive insect pest known as the coconut rugose spiraling whitefly was discovered. Recently in 2018, the research was conducted to examine the variation of gut microbiota in Rugose Spiraling Whiteflies collected from three distinct locations in Karnataka where coconut is produced. Nine culturable bacteria were identified using molecular methods after being found in the gut of three populations of Rugose Spiraling Whiteflies. The nine isolates were found to produce chitinase, siderophores and proteases. The activity of chitinase, siderophore and protease ranged in the gut bacterial isolates of coconut rugose spiraling whitefly 0.023 to 0.44 $nmol^{-1}$ min^{-1} mL^{-1}, 8.27 to 35.63 per cent and 19.10 to 84.71 per cent, respectively. They have analysed for the presence of enzymase activity. An analysis of the 16S rRNA gene revealed that the gut of *Aleurodicus rugioperculatus* has a number of bacterial species. Includes *Bacillus cereus*, *Lederbergia*. Sp. *Proteus vulgaris*, *Lysinibacillus fusiformis*, *Pseudomonas helleri*, *Pseudomonas fragi*, *Pseudomonas psychrophila*, *Hafinia paralvei*.

Keywords : Gut bacterial diversity, Chitinase, Siderophores, Proteases, 16S rRNA

Invasive species threaten the ecological and economic wellbeing of a country (Pimentel *et al*., 2001). In the last decade, several soft scale insects and fall army worm have been accidentally introduced to India of which some have become serious pests others are widening their host ranges and spreading rapidly (Joshi, 2017). A recent study revealed that around 1300 species of invasive insect pests have been introduced into 124 different countries (Paini *et al.,* 2016).

The Rugose Spiraling Whitefly is an invasive insect pest that poses a significant threat to coconut trees and other plant species. This insect, scientifically known as *Aleurodicus rugioperculatus,* belongs to the family Aleyrodidae and is native to Central America. It has rapidly spread to various parts of the world, causing considerable damage to coconut plantations and posing a significant challenge to agricultural and horticultural industries (Prasanna *et al*., 2022). The Rugose Spiraling Whitefly gets its name from the distinctive spiraling pattern that its nymphs create on the undersides of coconut tree leaves. These nymphs, commonly referred to as 'spirals', secrete wax and form dense clusters on the leaves, which can lead to reduced photosynthetic activity and overall weakening of the tree. The infestation causes leaf yellowing, premature defoliation and reduced coconut production, leading to economic losses for farmers and coconut growers (Saranya *et al*., 2022).

The Rugose Spiraling Whitefly has been documented in several countries, including India, the Philippines, Sri Lanka and parts of Africa, among others. Its ability to spread quickly and establish populations in new areas has made it a major concern for coconut cultivation and plant health authorities worldwide. Efforts to control and manage this invasive pest involve integrated pest management strategies, including cultural practices, biological control

methods and judicious use of insecticides (Sivakumar *et al*., 2017). The life cycle and behavior of the Rugose Spiraling Whitefly to develop effective control measures and minimize the impact on coconut crops.

Whiteflies, including the Rugose Spiraling Whitefly, feed on plant sap, which is nutrient-poor (Saranya *et al*., 2022). To supplement their nutritional requirements, they rely on symbiotic relationships with microorganisms present in their guts. These microorganisms help in the digestion and breakdown of complex sugars and amino acids present in the plant sap, providing the whiteflies with essential nutrients. Jones *et al*. (2019).

There are no studies on cultivable gut bacterial diversity in rugose spiraling whitefies of host plant. This study was designed to reveal the cultivable gut bacterial diversity of rugose spiraling whitefy collected from different locations of host plants along with their functional significance.

Material and Methods

Isolation of Gut Bacterial Isolates from Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

Five population of Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin collected from three different location, Rashmi *et al.* (2019), According to the usual approach outlined by Feng *et al*. (2011), gut microbiota was extracted. The body was then extensively surface sterilised with 0.1% NaCl for 60s, followed by 70 per cent ethanol twice for 1 min. With the aid of a sterilised little pestle and mortar, the body was macerated. The macerated contents were placed in a 10 ml sterile water blank, stirred, and diluted up to a 10^{-3} concentration. Four growth media, namely Potato Dextrose Agar (PDA), Nutrient Agar (NA), Luria Bertani Agar (LB) and Yeast Peptone Dextrose Agar (YPDA), were each given an aliquot of 100 L of the diluted content. Every 24 hours, the plates were checked to see if any microbial colonies had formed during the 48 hours of incubation at 30°C.

Molecular Characterization of Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

DNA extraction of all the twelve isolated bacterial strains was carried out with the help of HiPurATM Bacterial and Yeast Genomic DNA Purification Spin Kit. The isolated genomic DNA was amplified using the forward primer pA5'AGAGTTTGAT CCTGGCTCAG3' and reverse primer pH-5'AAGGAGGTGATCCAGCCGCA3'. PCR products were sequenced directly with the Taq-mediated dideoxy chain terminator cycle sequencing in ABI 3130xl automated genetic analyser as per manufacturer's instructions. The sequences obtained were submitted to NCBI data base and accession numbers were obtained. The contiguous sequences were formed from forward and reverse sequences using online CAP3 programme. The contiguous sequences were used for homology search of the 16S rDNA sequences using the Blast N with the sequences deposited in public databases (GenBank). The identification were based on percentage similarity (>97% compared with public database sequences, NCBI) by BLAST homology. (Sivakumar *et al*., 2016).

Siderophore Assay of Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

LB broth were prepared and 4.5 ml of the broth were aliquoted in sterile falcon tubes. The broth was inoculated with bacterial culture and incubated for 24 hours at 37°C and 3000 rpm. The Chrome Azurol S (CAS) dye was prepared as per Schwyn and Neilands (1987). The dye was added to the bacterial culture and incubated for 1 hour (Arora and Verma, 2017). The samples were then centrifuged for 10 minutes at 8000 rpm and 200 µl of the supernatant was pipetted into a 96 well titer plate (Bio Rad). The absorbance of the sample in the plate was measured at 630 nm using BioRad Laboratories spectro-photometer (Himpsl *et al*., 2019).

The Siderophore production was calculated using the formula = (Ar-As) x 100/ Ar

Ar - Absorbance of reference (CAS solution and uninoculated broth).

As - Absorbance of the sample (CAS solution and supernatant)

Detection of Protease Activity of Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

The Protease activity was determined by using a skim milk agar plate. The medium was prepared by suspending Skim Milk Agar (HiMedia) in distilled water and sterilized by autoclaving. The bacterial culture was spotted on the Skim Milk Agar plate and incubated at 30°C for 24 hours. The zone of clearance around the colony indicates that the culture is positive for protease. The protease activity was calculated by the below formula Saranya *et al*., 2022.

Screening for Chitinase of Coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin.

The bacterial isolates were inoculated in Davis minimal media supplemented with 3 per cent colloidal chitin and incubated for 48 hrs at 35 C at 200 rpm. Colloidal chitin was prepared as described by Rodriguez-Kabana *et al*. (1983). The culture was centrifuged at 8000 rpm for 10 minutes and 1 ml of supernatant was transferred to a microfuge tube. The chitinolytic activity was quantified by performing DNS assay, DNS was prepared as described by Narendra *et al*. (2020) 1.6 g of NaOH was added to 75 ml of distilled water and 1 g of 3,5-Dinitrosalicylic acid was added. Finally 3 g of sodium potassium tartrate was added and the volume was made up to 100 ml. N-acetyl glucosamine (NAG) of known concentration (40, 112, 184, 256, 328 and 400 µg/ml) were used as standard to calculate the enzymatic activity. One unit of enzyme activity is the amount of enzyme that liberates 1 µmol of NAG per minute (Wiwat *et al*., 2000). The sample absorbance was taken using a 96 well plate with known standards and samples using BioRad 96 well titer plate; Spectrophotometer absorbance at 490 nm.

Results and Discussion

Most of the bacterial colonies were sperical, out of nine isolated microorganisms six were found to be Gram negative and three were Gram positive (Table 2). The cultivable gut microflora of *Aleurodicus rugioperculatus* samples were collected from three different locations of Karnataka (Table 1). And were identified using 16S rRNA sequences in conjunction with the closest representatives of the bacterial sequences that are available in public databases (GenBank, NCBI). Homology searches in DNA databases were performed on the collected bacterial

Table 1

Identification of gut bacteria associated with coconut coconut rugose spiraling whitefly *Aleurodicus rugioperculatus* Martin.

Bacterial isolates Strain code	Geographical location	Bacterial isolates	Accession number	Match %
MRSW01	Mandya12.569 ^{0}N76.800 ^{0}E	*Bacillus cereus*	OR195779	099.18
MRSW03		*Lederbergia* sp.	OR195780	094.92
MRSW05		*Proteus vulgaris*	OR195781	100.00
CHRSW02	Chamarajnagar11.893 ^{0}N76.952^{0}E	*Proteus vulgaris*	OR195782	100.00
CHRSW026		*Lysinibacillus fusiformis*	OR065053	100.00
CHRSW028		*Pseudomonas helleri*	OR065055	100.00
CHRSW030		*Pseudomonas fragi*	OR065056	100.00
CRSW024	Kolar13.357 ^{0}N78.079 ^{0}E	*Pseudomonas psychrophila*	OR065051	099.93
CRSW08		*Hafnia paralvei*	OR195783	099.92

TABLE 2

Morphological characteristic of gut bacteria associated with coconut coconut rugose spiraling whitefly *Aleurodicus rugioperculatus* Martin.

Bacterial isolates Strain code	Form	Colour	Gram's reaction	Shape
MRSW01	Circular	White	Gram positive	Rod
MRSW03	Circular	White	Gram positive	Rod
MRSW05	Circular	White	Gram negative	Rod
CHRSW026	Circular	Dry white	Gram positive	Rod
CHRSW028	Circular	yellow	Gram negative	Rod
CHRSW030	Circular	yellow	Gram negative	Rod
CRSW024	Circular	yellow	Gram negative	Rod
CRSW08	Irregular	Dry white	Gram negative	Rod

strain nucleotide sequences, revealing that the sequences were found between the 16S rRNA gene sequences of the corresponding identified organism, and the field-captured population of identified bacterial isolates from different geographical locations in Karnataka, along with their respective accession numbers and geographical coordinates. *Bacillus cereus* (MRSW01) was isolated from Mandya (12.569°N, 76.800°E) with an accession number OR195779, showing a 99.18 per cent match. *Lederbergia* sp. (MRSW03) was found in the same location with OR195780 (94.92% match). *Proteus vulgaris* (MRSW05) and *Proteus vulgaris* (CHRSW02) were both isolated from Chamarajnagar (11.893°N, 76.9520°E) with OR195781 and OR195782, respectively, exhibiting a 100 per cent match. *Lysinibacillus fusiformis* (CHRSW026), *Pseudomonas helleri* (CHRSW028) and *Pseudomonas fragi* (CHRSW030) were also found in Chamarajnagar with OR065053, OR065055 and OR065056, respectively, all showing 100 per cent matches. *Pseudomonas psychrophila* (CRSW024) was isolated from Kolar (13.357°N, 78.079°E) with accession number OR065051 (99.93% match) and *Hafnia paralvei* (CRSW08) was found in Kolar with OR195783, demonstrating a 99.92 per cent match (Table 1).

A variety of bacteria from various genus and species were examined in the current study and their 16S rRNA sequences were used to characterize them. Published a study using 16S rRNA sequences to examine the diversity and distribution of bacteria in several insects. A good diversity of microflora was recorded across the location and is unique with respect to the locations (Sivakumar *et al*., 2016). The common inhabitants, *viz*., *Bacillus* spp. *Serratia* spp. and *Pseudomonas* spp. in many insect guts were reported by Broderick *et al*. (2004), and was reported that *Bacillus* spp. play an important role in the growth and development of insects.

Broderick *et al*. (2004) reported the presence of common gut bacterial species *Enterococcus*, *Serratia*, *Enterobacter*, *Staphylococcus*, *Paenibacillus*, *Pantoea* and *Bacillus* in insects of different crop types and their contribution to host fitness features. The current investigation also demonstrated the presence of these genera in the leafhopper's gut; the precise function they served will be elucidated in subsequent research. *Pantoea* spp., which is frequently detected in *Dendroctonus frontalis* larvae, engaged in nitrogen fixation and detoxification to allow the release of protective chemicals that are known to be metabolized by bacteria, according to Archana *et al*. (2006).

Out of the 9 isolates, 4 showed evidence of protease activity, while 6 showed evidence of chitinase and 9 siderophore synthesis. *Proteus vulgaris* CHRSW02 had the highest chitinolytic activity, followed by *Proteus vulgaris* CHRSW05 and *Lysinibacillus*

TABLE 3

Functional activity of gut bacteria isolated from coconut rugose spiraling whitefly *Aleurodicus rugioperculatus* Martin.

Organism	Strain code	Protease
Bacillus cereus	MRSW01	+
Lederbergia sp.	MRSW03	-
Proteus vulgaris	MRSW05	-
Proteus vulgaris	CHRSW02	-
Lysinibacillus fusiformis	CHRSW026	+
Pseudomonas helleri	CHRSW028	+
Pseudomonas fragi	CHRSW030	-
Pseudomonas psychrophila	CRSW024	-
Hafnia paralvei	CRSW08	+

Note : These positive indicate the formation of halozones, which indicates the production of protease enzymes, negative indicate the absence of protease enzymes '+' : positive; '-' : Negative

TABLE 4

Chitinase activity of gut bacteria isolated from coconut rugose spiraling whitefly *Aleurodicus rugioperculatus* Martin.

Organism	Strain code	Chitinase (nmol/min/mL)
Proteus vulgaris	MRSW05	0.430 [a]
Proteus vulgaris	CHRSW02	0.440 [a]
Lysinibacillus fusiformis	CHRSW026	0.270 [b]
Pseudomonas helleri	CHRSW028	0.023 [d]
Pseudomonas fragi	CHRSW030	0.079 [c]
Hafnia paralvei	CRSW08	0.110 [c]

Note : The values represented in the table are averages of three replications. Means superscribed by the different letters differ significantly at 1% level of significance by DMRT

fusiformis CHRSW026, both of which were isolated from the *Aleurodicus rugioperculatus* of coconut. *Pseudomonas helleri* CHRSW02828, which was isolated from the *Aleurodicus rugioperculatus* of coconut of location *chamarajanagar*, had the lowest chitinolytic activity (Table 4). *Bacillus cereus* MRSW01 had the highest levels of protease activity (84.71%), followed by *Pseudomonas helleri* CHRSW028 (34.1%) *Hafnia paralvei.* CRSW08 (19.1%) from *Aleurodicus rugioperculatus* of coconut plants, had the lowest level of protease activity (Fig.1).

The tested bacterial strains exhibited varying levels of siderophore synthesis. *Pseudomonas helleri* (CHRSW028) displayed the highest siderophore production at 35.63 per cent, followed by *Lysinibacillus fusiformis* (CHRSW026) with 32.92 per cent. Other strains, such as Proteus vulgaris (CHRSW02), *Lederbergia* sp. (MRSW03) and *Pseudomonas fragi* (CHRSW030), also showed significant siderophore synthesis at 26.49, 25.60 and 24.96 per cent, respectively. *Bacillus cereus*

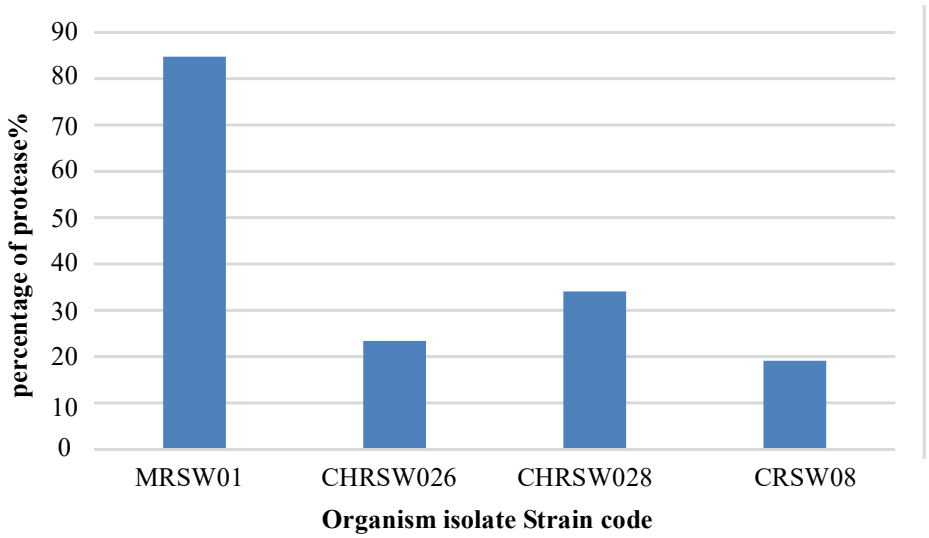

Fig. 1 : Protease activity of gut bacteria isolated from coconut Rugose Spiraling Whitefly *Aleurodicus rugioperculatus* Martin

(MRSW01), *Proteus vulgaris* (MRSW05), *Pseudomonas psychrophila* (CRSW024) and *Hafnia paralvei* (CRSW08) had lower siderophore levels at 8.27% 18.51, 18.24 and 24.27 per cent, respectively (Table 5).

TABLE 5

Functional significance of siderophore in gut bacteria isolated from coconut rugose spiraling whitefly *Aleurodicus rugioperculatus* Martin.

Organism	Strain code	Siderophore (psu)
Bacillus cereus	MRSW01	8.27^{f}
Lederbergia sp.	MRSW03	25.60^{cd}
Proteus vulgaris	MRSW05	18.51^{e}
Proteus vulgaris	CHRSW02	26.49^{c}
Lysinibacillus fusiformis	CHRSW026	32.92^{b}
Pseudomonas helleri	CHRSW028	35.63^{a}
Pseudomonas fragi	CHRSW030	24.96^{cd}
Pseudomonas psychrophila	CRSW024	18.24^{e}
Hafnia paralvei	CRSW08	24.27^{d}

Note : The values represented in the table are averages of three replications. Means superscribed by the different letters differ significantly at 1% level of significance by DMRT

Based on their ability to produce chitinase, potential bacterial isolates were screened and the screened isolates were investigated to determine their functional importance. Insect midgut lined with the peritrophic membrane (PM) supports digestion and nutrient absorption, these gut bacteria producing siderophore, chitinase and protease are providing host protection and enhances the growth of the insects host (Indiragandhi *et al*., 2007).

According to a pest management method, gut bacteria that produce chitinase and protease have been shown to enter the host insect's gut by feeding and damage the peritrophic membrane's thickness, which results in nutrient imbalances and insect mortality (Krishnamoorthy *et al*., 2020 and Okongo *et al*., 2019).

In this study, the cultivable gut microflora of *Aleurodicus rugioperculatus* from different locations in Karnataka were examined, revealing a diverse microbial community with a significant presence of Gram-negative and Gram-positive bacteria. The identified isolates demonstrated various enzymatic activities, such as chitinase, protease and siderophore synthesis, which can play a vital role in insect host protection and growth enhancement. These findings highlight the potential functional importance of gut bacteria in supporting insect digestion, nutrient absorption and defense mechanisms. The ability of certain bacterial isolates to disrupt the peritrophic membrane may offer a promising avenue for pest management strategies. Overall, this research underscores the significance of gut microbiota in insect biology and agriculture.

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