The Journal of Agricultural Sciences - Sri Lanka
Vol. 21 No 2, May 2026. pp 356-365
https://doi.org/10.4038/jas.v21i2.11282

RESEARCH

Metagenomics Insight into Plant-ant-fungi Mycobiome Using *Anoplolepis gracilipes* (Smith F., 1857) (Hymenoptera, Formicidae) Crawled on *Solanum melongena* (L.)

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Received: 30th October 2024 / Accepted: 02nd February 2026

ABSTRACT

Purpose: *Ants are commonly observed crawling on crop plants in Sri Lanka, where fungal diseases are frequently reported. This pilot study aimed to identify plant-ant-fungi associations on Solanum melongena (L., 1753) and to determine whether A. gracilipes workers carry the inoculated plant pathogenic fungus, Lasiodiplodia theobromae (Pat.) Griffon & Maubl, in their gut contents.*

Research Method: *Worker ants observed on S. melongena plants at three selected localities in the Gampaha District were identified, and their frequency of occurrence was recorded. A. gracilipes workers, the ant species with the highest frequency of occurrence in the field, was collected from the same localities. In each trial, 200 workers were starved for 48 hours, and then allowed to crawl on brinjal plants inoculated with L. theobromae. Genomic fungal DNA was extracted from pooled heads and digestive tracts, followed by metagenomic sequencing and bioinformatics analysis.*

Findings and Values: *The inoculated plant-pathogenic fungus L. theobromae was detected in the gut contents of A. gracilipes workers. Metagenomic analysis revealed a diverse gut mycobiome comprising 78 fungal species, including plant pathogens, endophytes, epiphytes, and insect gut symbionts. These findings suggest the potential ingestion and transport of plant-pathogenic fungi by A. gracilipes, conclusive evidence requires further research.*

Keywords: *Anoplolepis gracilipes, Ant-plant mycobiome, Internal transcribed spacer region (ITS), Metagenomic sequencing, Plant-pathogenic fungi, Solanum melongena.*

INTRODUCTION

Sri Lanka is a tropical agricultural country with a high diversity of ant fauna (Dias *et al*. 2020) and foraging worker ants are very common on various vegetable crop plants in Sri Lanka (Dias *et al*. 2019). A mutualistic interaction between ants and fungi has been reported in ant fungal gardens, in which ants provide nutrient sources and space for fungal growth in their nests, and the fungi, in turn, serve as a primary food source for the ants (Blatrix *et al*. 2012, Mayer *et al*. 2013, Offenberg & Damgaard 2019).

Studies on the gut content analysis of worker ants for plant-pathogenic fungi present on a crop plant are scarce in Sri Lanka and elsewhere. Hence, this pilot study was focused on the dominant ant species that crawled on *S. melongena* and analysis of its head and gut contents for the detection of *L. theobromae*,

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a pathogen causing leaf necrosis in *S. melongena* (Koshila *et al*. 2022).

Solanum melongena (L.) of the family Solanaceae, commonly known as brinjal or eggplant, is a popular and commonly grown vegetable in the country (Wijesooriya *et al*. 2015). Field observations revealed that worker ants crawled regularly on visually healthy and unhealthy *S. melongena* plants (Dias *et al*. 2019). Although seen as healthy plants, invisible, unknown pathogenic fungal growths could be on the leaf surfaces and unhealthy appearance may have been caused by various pathogens. Hence, analysis of head (buccal cavity) and gut contents of the ants that crawled on the leaf surfaces for the identification of plant-pathogenic fungi is of research interest.

L. theobromae, *Pseudopestalotiopsis theae* (Sawada) Maharachch, K.D. Hyde & Crous and *Diaporte eugeniae* (Punith.) R.R. Gomes, Glienke & Crous were recently reported as fungal pathogens that cause leaf diseases of *S. melongena* in Sri Lanka (Koshila *et al*. 2020). Many other pathogenic fungal species of *S. melongena* were recorded in Sri Lanka and other countries (Mahendranathan *et al*. 2010, Amienyo & GuGu 2014, Weeraratne & De Costa 2018, Adikaram & Yakandawala 2020, Jinal *et al*. 2021, Aumentado & Balendres 2022).

Molecular identification techniques are considered as more advanced methods for the identification of fungi up to the species level (Alsohaili & Bani-Hansa 2018) and to overcome the difficulty of identifying fungal species, associated with ant communities (Blatrix *et al*. 2012). Culture-independent metagenomic analysis of the ITS region provides a comprehensive understanding of the total composition of gut fungal communities (Forin *et al*. 2018, Hariharan & Prasannath 2021).

This pilot study was focused on the metagenomic-directed pathogenic fungi analysis of the genomic DNA, which was extracted from the head and gut contents of *A. gracilipes* workers that crawled on the *L. theobromae* inoculated *S. melongena* plants.

MATERIALS AND METHODS

Diversity and frequency of occurrence of ant species that crawled on S. melongena

Worker ants on randomly selected 10 *S. melongena* plants were hand-collected on three occasions between the months of August and September, 2019 (Table 1) from each of the three *S. melongena* cultivations at Yonganmulla (Locality1), Amithirigala (Locality 2) and Hiswella (Locality 3) in *Gampaha* District. Collected workers of ant species were preserved immediately in the bottles filled with 70% ethanol, with appropriate labels. In the laboratory, ants were identified to the species level using a Low Power (x45) stereomicroscope (OLYMPUS-SZ61) with reference to Dias (2014), Dias *et al*. (2018) and AntWeb.org (2019). At each time point, the frequency of occurrence of each ant species (=Number of plants with the species/ 10) was calculated and presented in percentage values (Table 1). Significant differences among observed frequency values of each ant species were tested using the chi-square homogeneity test. Based on the considerably higher frequency of occurrence of *A. gracilipes*, it was selected for the subsequent ITS metagenomic analysis to study its gut mycobiome.

Inoculation of the pathogenic fungus to S. melongena plants and preservation of the starved workers and those crawled on symptomatic plants

Approximately, more than 400 *A. gracilipes* workers were collected and starved in a sealed beaker for 48 hours at 28 - 30 ºC (room temperature) for the gut evacuation while supplying water in a piece of cotton wool.

Ten healthy *S. melongena* plants purchased from the plant nursery at the Department of Agriculture in Mahara were grown for nearly two months in the garden of the Department of Plant and Molecular Biology, University of Kelaniya, Sri Lanka. A pathogenic fungus, *L. theobromae* was inoculated to several selected healthy leaves of 10 *S. melongena* plants, maintained at the Kelaniya University

garden. Two hundred starved *A. gracilipes* were introduced to 10 *S. melongena* plants with leaf necrosis symptoms, workers were allowed to forage for 2 hours and preserved immediately in absolute ethanol. Another 200 starved ants were also preserved in absolute ethanol at the same time to prepare the pooled, control fungal DNA sample of the head and the digestive tract.

Dissection of digestive system and buccal pouch containing head of A. gracilipes and genomic DNA extraction

Each worker ant was cleansed with sterilized distilled water for 30 seconds in a watch glass to remove ethanol residue on its surface. Moisture on the external surface of each ant was removed by transferring it to a sterilized filter paper kept in a Petri dish. Assuming that collected items are kept in the buccal pouch in the head of an ant for a period and as the rest of the ant digestive tract mostly lies in the gaster region, head and the digestive tract in gaster region were dissected for further analysis.

Worker ants allowed to crawl on the plants and the starved ants in the control sample were subject to the following procedure, separately. The digestive system in the gaster section of each ant was carefully dissected out under a low-power stereomicroscope (OLYMPUS-SZ61), using two sterilized entomological pins. Next, dissected parts were then transferred to a sterilized microcentrifuge tube filled with 180 µL of tissue lysis buffer provided in DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). In addition, the head, which contains the buccal pouch, was severed, crushed and kept with the dissected digestive tract. Each sample was stored at -20 ºC until DNA extraction.

The dissected digestive system part of each worker ant and the head containing buccal pouch was homogenized and DNA extraction was performed using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's standard animal tissue protocol to extract the fungal DNA. DNA was eluted into 20 μL of AE buffer (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0). Extracted fungal DNA was quantified using nanodrop (Nabi, South Korea) and the DNA quality was evaluated by 1% agarose gel electrophoresis (90 V and 45 min). Genomic DNA extracted from treatment samples, consisting of pooled heads and digestive tracts of ants that crawled on *L. theobromae*-inoculated *S. melongena* plants, along with respective control samples, was sent to Macrogen Inc., a commercial sequencing facility in South Korea, for the ITS metagenomics sequencing.

Metagenomic analysis conducted by Macrogen Inc., South Korea

Sample preparation and quality control: The composite DNA sample was checked for initial quality control and quantity assessment prior to the library construction. The DNA sample of the treatment was prepared according to the Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit (genomic DNA concentration of $\geq 2ngin50$ μL volume of DNA).

Library construction: The sequencing library was prepared by random fragmentation of the genomic DNA, followed by 5' and 3' adapter ligation. Adapter-ligated fragments were then subjected to PCR amplification using internal transcribed spacer regions (nrDNA ITS 1-5.8S-ITS 2) of fungi using universal primer pair, ITS 3: 5'-GCATCGATGAAGAACGCAGC-3' and ITS 4: 5'-TCCTCCGCTTATTGATATGC-3'. Amplicons were gel purified prior to sequencing.

Cluster generation and sequencing: The library was loaded into a flow cell. Each fragment was then amplified into distinct, clonal clusters through bridge amplification. After the cluster generation, the templates were subjected to sequencing. Sequencing was performed on Miseq 300 bp PE; 30 % Phi X (Illumina, USA), following the manufacturer's protocol at Macrogen Inc., South Korea. Library construction, amplification, cluster generation and sequencing were performed by Macrogen Inc., South Korea.

Pre-processing (de-noising) and clustering: High-quality reads were then clustered into operational taxonomic units (OTUs) based on sequence similarity (97 %) using the CD-HIT clustering method (Macrogen, Inc. 2022) to

produce OTU cluster.

Taxonomy and diversity analysis: A representative read was picked from each OTU and representative reads were used for taxonomy assignment using QIIME (Quantitative Insights into Microbial Ecology, (Macrogen, Inc. 2022) program. Blastn search was performed against NCBI fungal database to identify fungal species in the digestive system of *A. gracilipes*, which crawled on the plants.

Alpha diversity and compositional Analysis

A variety of alpha diversity metrics, Chao 1, Shannon, Simpson and Good's coverage, were calculated for the fungal DNA in the same sample prepared from the crawled workers. The taxonomic fungal composition of that sample was explained by grouping fungal identities to taxonomical levels from phylum to species level and their relative abundance based on the OTUs counts.

RESULTS AND DISCUSSION

Frequency of occurrence and diversity of ant species on S. melongena plants

The diversity of Sri Lankan ant species was thoroughly documented in the past two decades (Dias *et al*. 2020) but the species that crawl on *S. melongena* plants and the importance of their crawling on the plants have not documented, so far. For the first time, ant species crawled on *S. melongena* plants, *A. gracilipes*, *Camponotus opaciventris* Mayr, 1879, Mayr, 1879, *Camponotus parius* Emery, 1889, *Technomyrmex albipes* (Smith, F. 1861), *Meranoplus bicolor* (Guérin-Méneville, 1844) and *Oecophylla smaragdina* (Fabricius, 1775) were identified among the workers crawled on *S. melongena* plants that had been cultivated in the three localities. *A. gracilipes*, the species observed with a higher frequency of occurrence (Table 1), based on the data collected at the three selected study sites, located in *Gampaha* District, Sri Lanka, was selected to investigate the plant-ant-fungi association. The frequency of occurrence observed for each ant species was significantly different on three sampling occasions (chi-square test, $p < 0.05$). Gut mycobiome of starved *A. gracilipes* workers after allowing them to crawl on symptomatic *S. melongena* plants has been reported after subjecting the sample for metagenomic-directed mycobiota analysis.

Metagenomic analysis of A. gracilipes gut mycobiome after crawling on S. melongena

Starved *A. gracilipes* workers were allowed to crawl on *S. melongena* plants inoculated with *L. theobromae*, the pathogenic fungus responsible for leaf necrosis in this crop. Starvation of workers for two days was determined from three preliminary experiments, in which all starved ants died after 2 days. Hence, assuming that 2-day starvation removed the contents in buccal pouch and the digestive tract, starved worker ants were introduced to the *S. melongena* plants in the field. Ants keep the gathered content in the buccal pouch, which lies in the head (Richter & Economo 2023), so genomic DNA was extracted from the pooled heads containing buccal pouch and digestive system of *A. gracilipes*, which were introduced to 10 *S. melongena* plants. Extraction of sufficient concentration of genomic DNA from those of *A. gracilipes* was a challenging task in this study due to the smaller size. Hence, a composite sample of 200 heads and digestive tracts in the gaster region was used for the preparation of each genomic DNA sample of starved workers (the control) and those crawled on the plants after the starvation (the treatment).

Overview of metagenomic data and mycobiota composition of digestive tract of A. gracilipes

According to the raw data statistics, 112,610 sequence reads were generated by the DNA libraries created with genomic DNA extracted with pooled head and digestive system of *A. gracilipes* that crawled on *S. melongena* plants. Total read bases were 40.3M bp, with GC content of 55.79% and 96.17% of reads were above the Q30 quality score. After removing low quality, chimera and primer mismatched reads, in total, 68,339 reads (60.6%) were retained for downstream analysis. However, metagenomic data for the control sample (corresponding to starved ants not exposed to plants) were

Table 1. Sampling occasion and percentage frequency of occurrence of each ant species on brinjal plants at each locality (Locality 1 - Yonganmulla; Locality 2 - Amithirigala; Locality 3 – Hiswella)

Date of sampling 2019	Locality	Ant species	% frequency of occurrence
15 August	1	*Anoplolepis gracilipes*	100
		Camponotus opaciventris	70
	2	*Anoplolepis gracilipes*	100
		Camponotus parius	30
	3	*Technomyrmex albipes*	100
		Meranoplus bicolor	20
02 September	1	*Anoplolepis gracilipes*	100
		Camponotus opaciventris	50
		Technomyrmex albipes	10
	2	Anoplolepis gracilipes	100
		Technomyrmex albipes	20
		Camponotus parius	30
		Camponotus opaciventris	20
	3	*Technomyrmex albipes*	100
		Meranoplus bicolor	20
27 September	1	*Anoplolepis gracilipes*	90
		Camponotus opaciventris	80
		Oecophylla smaragdina	40
	2	*Anoplolepis gracilipes*	100
		Camponotus opaciventris	10
		Camponotus parius	30
		Technomyrmex albipes	20
	3	*Technomyrmex albipes*	100
		Meranoplus bicolor	40

unavailable, as the library preparation step could not be completed due to insufficient genomic DNA concentration. This limitation is consistent with challenges documented in microbiome research involving low-biomass samples (Hornung *et al*. 2019).

Based on the total number of high quality reads (68,339), 179 OTUs represented a total of 4 fungal phyla of 16 classes, 33 orders, 59 families, 83 genera, and 78 fungal species, including plant pathogens, endophytes, epiphytes and insect gut inhabitants. Ascomycota was dominant (79.91%) while lower proportions of Basidiomycota (4.70%), Mucoromycota (0.03%), Chytridiomycota (0.02%) and unclassified (15.34 %) were also identified. Within Ascomycota, Eurotiomycetes (48%) and Dothideomycetes (23.94%) were predominant classes. Aspergillaceae (47.65%), Teratosphaeriaceae (14.99%), and Cladosporiaceae (3.33%) were dominant families (Fig. 1). The observed high fungal diversity in the gut mycobiome of *A. gracilipes* was supported by diversity index values. Specifically, the elevated Shannon diversity index indicates a rich and evenly distributed fungal community, while the high Chao1 index reflects a substantial abundance of fungal species in the pooled genomic DNA sample derived from the heads and digestive tracts of the ants (Table 2).

Detection of plant pathogens and other associated fungal species

Intentionally inoculated pathogenic fungus, *L. theobromae* was reported from the pooled head and digestive system sample of starved *A. gracilipes* workers. Additional fungal genera detected *Diaporthe*, *Fusarium*, *Cercospora*, *Colletotrichum*, *Penicillium*, *Corynespora* are also known foliar or fruit pathogens of *S. melongena* in Sri Lanka and other countries

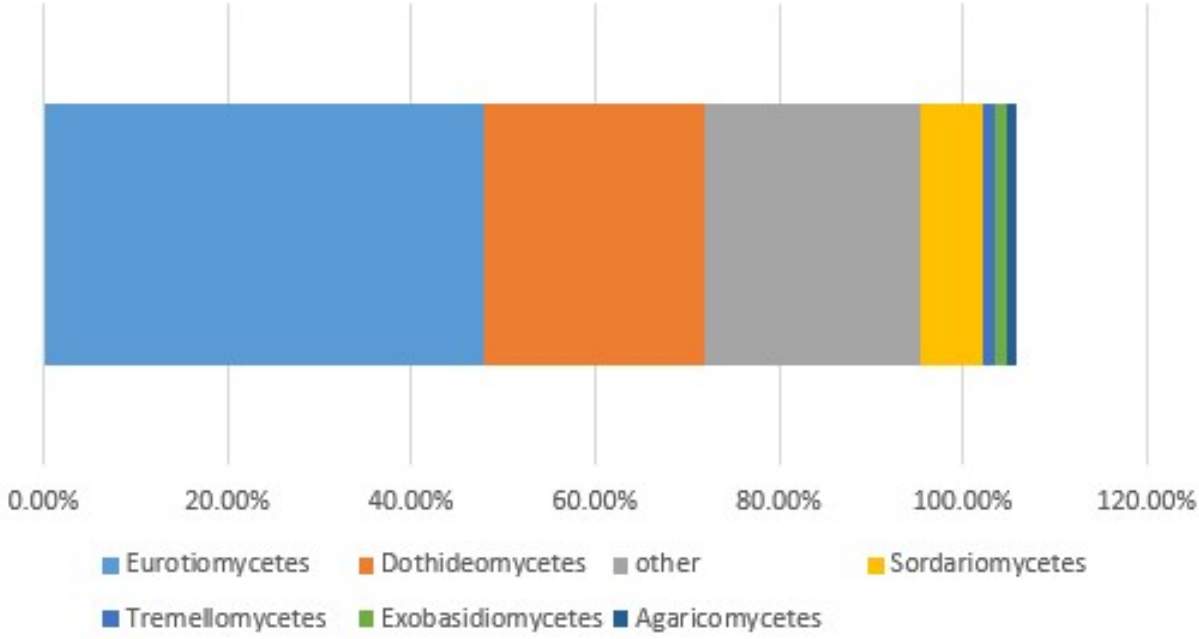

Figure 1. Graphical representation of the percentage abundance of dominant fungal families (more than 1% relative abundance) in the pooled heads and digestive tracts of starved *A. gracilipes* workers that crawled on *L. theobromae*-inoculated *S. melongena* plants.

Table 2. Diversity indices for mycobiota diversity and richness in the genomic DNA extracted from pooled head and digestive system of *A. gracilipes* (from Macrogen, South Korea)

Chao1	Shannon	Gini-Simpson	Good's coverage
179.0	4.87	0.92	1.0

(Mahendranathan *et al*. 2010, Amienyo & GuGu 2014, Weeraratne & De Costa 2018, Adikaram & Yakandawala 2020, Koshila *et al*. 2020, Jinal *et al*. 2021, Aumentado & Balendres 2022).

Approximately 23% of those recorded species out of the total number are recorded as plant pathogenic fungi according to the findings of previous studies (Fig. 2) (Mahendranathan *et al*. 2010, Weeraratne & De Costa 2018, Adikaram & Yakandawala 2020, Koshila *et al*. 2020). Also, 19% of fungi had been recorded as endophytes, while a few of them were recorded as plant epiphytes and insect gut inhabitants.

For example, fungal species such as *Aspergillus allahabadii*, *A. flavus*, *Penicillium cataractarum*, and *Pestalotiopsis microspora* have been documented as endophytes on various plants including crops like tomato, species of the genus *Ocimum*, and plants belonging to the families Orchidaceae, Fabaceae, and Combretaceae (Sadorn *et al*. 2016, Abdel-Motaal *et al*. 2020, Karthika 2022), while *Hanseniaspora opuntiae* was recorded as an epiphyte on grape berry (Zhang *et al*. 2021).

Beyond endophytes and epiphytes, various fungi recognized as gut inhabitants of insects were also identified in the ant gut mycobiome; notably, *Cyberlindnera fabianii*, a known gut inhabitant fungus of *Spodoptera litura*, was detected from the head and digestive tract DNA samples of *A. gracilipes* (Kumar *et al*. 2017, Pandey & Rajagopal 2019). This highlights the complex fungal community within the ant's gut, including plant-associated symbionts and insect gut symbionts, while demonstrating diverse fungal associations. Knowledge of ant–fungi associations remains limited; however, several studies worldwide have documented mutualistic relationships primarily in ant fungal gardens,

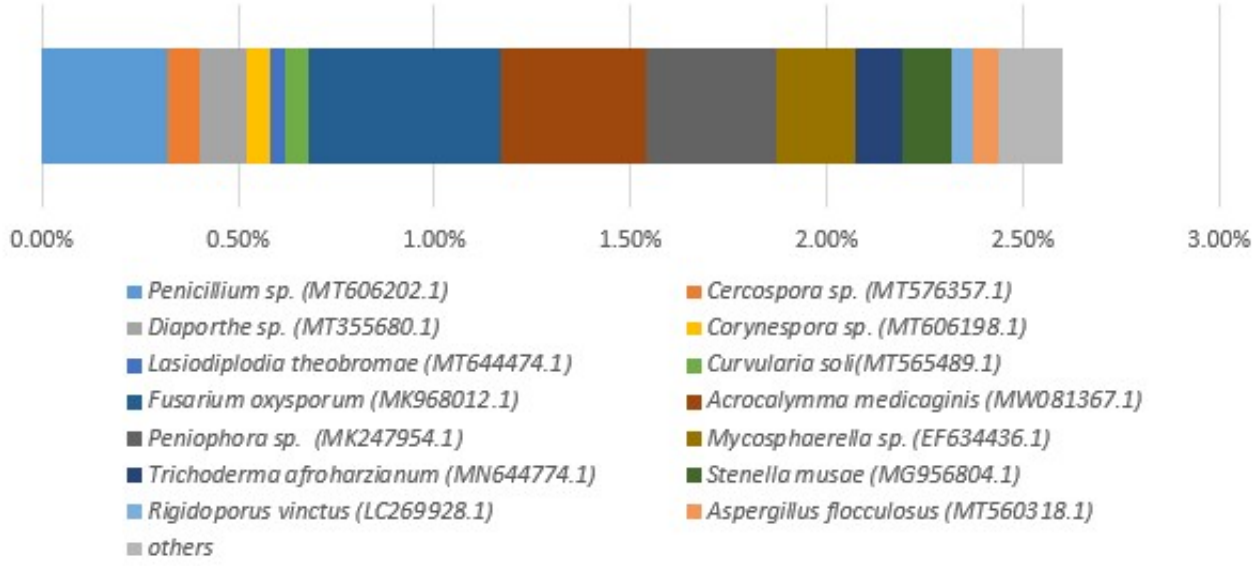

Figure 2. Graphical representation of the dominant plant pathogenic fungal species (more than 0.04 % relative abundance) in the pooled heads and digestive tracts of starved A. gracilipes workers that crawled on *L. theobromae*-inoculated *S. melongena* plants.

where ants provide a stable nesting environment by cultivating fungal gardens and supplying substrates such as plant material or detritus to promote fungal growth. In return, the fungi serve as a primary food source for the ants and are dominantly observed in underground chambers, rotten logs, and under rocks and branches.

This study explains the involvement of ants in transporting fungal inoculum and associated bacteria to establish these symbiotic colonies (Biedermann & Vega 2020, Mayer *et al*. 2023). While ant involvement in fungal garden formation is well documented, ant-mediated microbial dispersal beyond this remains poorly understood.

However, a recent study by Lash *et al*. (2024) showed that ant-handled seeds harbor different fungal communities compared to unhandled controls, demonstrating that ants not only disperse seeds but also influence the microbial communities associated with them.

Nevertheless, the association of ants with plant pathogenic fungi has not been explored in previous studies. This study indicates the presence of inoculated *L. theobromae* in the gut content of *A. gracilipes* workers. This is the first report to describe the potential role of the ant species *A. gracilipes* crawling on *S. melongena* in carrying the inoculated plant pathogenic fungus *L. theobromae* in their gut contents to suppress the growth of the pathogen *L. theobromae*.

However, the findings are not yet conclusive due to the failure of the sequenced control sample. Future research should include successfully sequenced control samples with biological replicates to confirm robustly the observation. Additionally, further investigations are recommended to elucidate the potential roles of other dominant ant species that crawl on *S. melongena* and other crop plants.

CONCLUSION

This study provides the first comprehensive documentation of ant species crawling on *S. melongena* plants across selected localities in Sri Lanka, identifying six species with *A. gracilipes* as the most frequent. The intentionally inoculated fungi, *L. theobromae* was detected in the pooled head and digestive system sample of starved *A. gracilipes* workers. Diversity metrics indicated high fungal diversity in the ant gut, comprising a total of 78 fungal species, including plant pathogenic fungi, endophytes, epiphytes, and insect gut symbionts.

Further investigations with properly sequenced control samples and biological replicates are essentially required. Overall, these findings provide new insights into the complex nature of plant-ant-fungi interactions and suggest promising avenues for future research into the mutualistic roles of ants in crop protection.

Acknowledgements

Authors thank the World Bank "Accelerating Higher Education Expansion and Development (AHEAD) Operation-Development Oriented Research (DOR)" fund for the provision of financial support.

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