Turkish Journal of Agriculture - Food Science and Technology, 12(s4): 2817-2822, 2024
DOI: https://doi.org/10.24925/turjaf.v12is4.2817-2822.7301

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

Effect of Honeydew Secreted by *Aphis gossypii* Glover (Hemiptera: Aphididae) on Fungal Growth

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ARTICLE INFO

Research Article

Received : 05.11.2024
Accepted : 30.11.2024

Keywords:
Aphid
Beauveria
Biological control
Honeydew
Trichoderma

ABSTRACT

Honeydew is a sugar-rich, sticky substance secreted by many plant-feeding insect species from the order of Hemiptera and Lepidoptera. Aphids (Hemiptera: Aphididae), on the other hand, feed on nitrogen-poor, carbohydrate-rich phloem sap and excrete excess carbohydrate as honeydew from their anus. The aphids, constituting the main material of the study were sampled from the *Catalpa bungei* C. A. Mey (Bignoniaceae) tree located in the central campus of Niğde Ömer Halisdemir University and then preparation procedures were carried out for species identification under laboratory conditions. According to the identification key organized according to the host plant, the samples were identified as *Aphis gossypii* Glover. The honeydew of *A. gossypii* Glover, known as the cotton aphid, was collected from the host plant and the effects of two different concentrations of the honeydew (10 and 20 g/L) on fungal growth were determined using both solid and liquid media. Different *Trichoderma* strains and *Beauveria bassiana* were used to examine fungal growth. Fungal growth in the prepared nutrient media was determined as the amount of biomass (gram). The honeydew content (phenolic substance, sugar and amino acid amounts) was determined and supported by FT-IR analyses. The growth of fungal species in the PDA medium, which was preferred as the control medium, and the medium containing honeydew was compared. It has been determined that fungal growth is better in the medium containing honeydew, and therefore honeydew increases fungal growth. With this study, it is predicted that aphid honeydew can support the growth of both fungal agents used in biological control and plant pathogens.

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Introduction

The honeydew is a sweet watery waste product of the aphid's diet of phloem sap, which is rich in sugar but poor in amino acids (Fischer et al., 2005). It is released by insects, mainly aphids, leafhoppers, mealybugs, planthoppers, psyllids, scale insects, treehoppers and whiteflies. Often overlooked, the honeydew plays an important mediator role affecting the dynamics between insects and plants (Dyer et al., 2018; Álvarez Pérez et al., 2023; Zhang et al., 2023; Fernández de Bobadilla et al., 2024). Honeydew constitutes the food source of many insect groups, especially ants. Aphids (Aphididae), which feed on phloem sap, have developed mutualistic relationships with ants thanks to the honeydew they produce (Dixon, 1998; Ali et al., 2024). While ants meet their carbohydrate needs with the honeydew produced by aphids (Saha et al., 2018), aphids also benefit from this relationship by reducing predation, parasitism and the risk of fungal infection (Völkl et al., 1999). The composition and quantity of honeydew may vary depending on aphid species, host plant species and phloem quality, and biotic and abiotic environmental factors (Fischer et al., 2005; Blanchard et al., 2022). Fischer and Shingleton (2001) demonstrated that while feeding on *Populus tremula* L. (Salicaceae) as compared with *Populus alba* L. (Salicaceae), the honeydew of *Chaitophorus populialbae* (Boyer de Fonscolombe) and *Chaitophorus populeti* (Panzer) contained a higher amount of the trisaccharide melezitose. In insects, gastrointestinal enzymes convert plant-derived sucrose into the sugars found in honeydew, such as melezitose, erlose, raffinose, trehalose, and

trehalulose (Hendrix et al., 1992; Wäckers, 2000). On the other hand, some aphid species release more honeydew than their own body weight on an hourly basis; the process of producing honeydew is intricate and impacted by many variables including insect age, size, species, seasonal and geographical location of the host plant, diurnal shifts, and climate (Ali et al., 2024). The quantity and quality of honeydew, as well as the sugar demand of the ants, are often correlated positively with the intensity of ant-aphid mutualism (Bonser et al., 1998; Fischer et al., 2001).

Even small changes, which may seem insignificant, in the content of the honeydew can affect the feeding behavior of insects in different ways. For instance, aphids that produce melezitose-rich honeydew, such as *Metopeurum fuscoviride* Stroyan and *Cinara* spp., are known to attract ants, but aphids that produce minimal melezitose, such as *Macrosiphoniella tanacetaria* (Kaltenbach) and *Macrosiphum euphorbiae* (Thomas), have little or no impact on ant behaviour (Hendrix et al., 1992; Völkl et al., 1999). In addition, although the sugar and amino acid contents of flower, stem nectars and honeydew are not very different, repellent plant substances play an important role in the feeding preferences of ants (Blüthgen et al., 2004). Moreover, it has been reported that the honeydew of many insects can have positive effects on herbivore population dynamics by increasing the fitness of parasitoids (Evans & England, 1996; Tena et al., 2016). It also has an effect on the initiation and maintenance of seeking behavior of hyperparasitoids of aphids (Buitenhuis et al., 2004). This adds a different dimension to the ecological importance of honeydew.

The honeydew, which is the carbohydrate source of ants, is not only a sugary waste product of aphids, but also has an ecological importance that extends to various living groups in the ecosystem. For example, aphids like *Sitobion avenae* (Fabricius) and *Metopolophium dirhodum* (Walker) are individually impacted by the microbial influence on honeydew dynamics. When compared to healthy plants, these aphids significantly reduce the amount of honeydew excreted by plants infected with the Barley Yellow Dwarf Virus-BYDV (Ajayi & Dewar, 1982; Ali et al., 2024; Fiebig et al., 2004). The complex interactions between sap-feeding insects, plants, and microbial communities that shape honeydew dynamics and its ecological implications are further highlighted by this finding. The honeydew, rich in sugar and other components, also serves as a food source for various microorganisms and fungi i.g. pathogenic fungi (Tena et al., 2016). Thus, the microbial communities that develop in honeydew, in turn, support the larger ecological environment (Owen & Wiegert, 1976; Leroy et al., 2011; van Neerbos et al., 2020). It is known that, the fungi produce volatile organic compounds (VOCs), including aldehydes, alcohols, benzene derivatives, phenols, heterocycles, hydrocarbons, ketones, cyclohexanes, thioesters and thioalcohols (Karslı & Şahin, 2021). The volatile organic compounds (VOCs) can affect plant health, insect behaviour, and even attract natural enemies of the honeydew producers. Exploring honeydew's ecological effects reveals that this seemingly unremarkable material has significant effects on pest management, nutrient cycling, and biodiversity (Álvarez-Pérez et al., 2024).

In this study, the effect of honeydew content on fungal growth was investigated. It was investigated whether aphid honeydew has an effect on the development and activity of *Bauveria bassiana* and some *Trichoderma* species, which are fungi that can be applied in agricultural fields.

Materials and Methods

Aphid Material and Honeydew Collection

Aphids forming dense colonies and a large amount of honeydew were observed on *Catalpa bungei* C. A. Mey (Bignoniaceae) plants at the Niğde Ömer Halisdemir University Central Campus, on September 28, 2022. It has been determined that the wingless individuals of the aphid population are mostly yellow, some are light green and dark green, and they prefer the lower parts of the plant's leaves.

Firstly, the aphids taken from the host plant were transferred to eppendorf tubes containing 96% alcohol and transferred to the laboratory for identification. Preparations of the samples were made according to the principles specified by Martin (1983). Then, aphids were identified as *Aphis gossypii* Glover according to the identification key provided by Blackman & Eastop (2024). *Aphis gossypii* Glover, known as the cotton aphid, is a cosmopolitan species (Holman, 2009; Kök & Özdemir, 2021; Blackman & Eastop, 2024; Görür et al., 2024). It can use a variety of plants as hosts, including cotton, cucurbits, eggplant, pepper, potato, many ornamental plants, and *Catalpa* spp. (Holman, 2009; Blackman & Eastop, 2024). Later on, nylon material was stretched over the undersides of the leaves of the plant where honeydew, was detected. The honeydew was collected twice a week from these nylon to-glass tubes (Figure 1). After collection, samples were capped with parafilm and stored at -20°C until analysis.

Figure 1. Honeydew collection from Catalpa bungei plants

Preparation of Medium

Ten (10) grams of dry honeydew samples collected from the leaves and stems of about four trees, were used for the experimental studies. Two grams of honeydew were dissolved with 10 ml of distilled water and filtered to remove any physical material. The culture medium was prepared by adding 5.0 ml of honeydew solution to 100 ml of distilled water and 1.5 grams of agar medium and sterilized by autoclaving at 121°C for 15 minutes. The pH of the medium was 7.3 ±0.2 at room temperature before autoclaving. This solid medium containing honeydew is called HDA (Honeydew Agar) medium. Honeydew broth medium was prepared similar to HDA solid medium without agar. Mineral medium (MM) was prepared as a negative control for fungal growth in Petri dishes containing % 0.9 g (w/v) NaCl. In addition to the mineral medium, Potato Dextrose Agar (Merck 110130 Potato Dextrose Agar (PDA), 500 gr) used for the growth of *Trichoderma* species and *Beauveria bassiana* was also prepared as a positive control.

Fungus

Eight fungal strains isolated in a previous study (Project no: FMT 2022/15-LUTEP supported by Niğde Ömer Halisdemir University) were used in this study. First, fungal strains were grown on PDA (Potato Dextrose Agar) medium. Then, fungal disks of 6.0 mm diameter were taken from one-week cultures and placed in the center of petri dishes containing HDA medium. All plates were incubated at 27±2°C for 7 days. Test media, negative and positive control medium were prepared as three parallels, and averaged the measurements. Abbreviations for fungal strains used are as follows: *Trichoderma koningiopsis* (TK), *Trichoderma citrinoviride* (TC), *Trichoderma saturnisporum* (TS), *Trichoderma harzianum* (TH), *Trichoderma pleuroticola* (TP), *Beauveria bassiana* (BB), *Trichoderma asperellum* isolated from banana plant root (TA), *Trichoderma longibrachiatum* isolated from soil (TL).

Analysis of Sugars and Amino Acids in Aphid Honeydew

Sugar and organic acid analysis were performed using AGILENT 6460 Triple Quadrupole System (ESI+Agilent Jet Stream) and AGILENT 1200 Series HPLC at METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center, Mass Spectroscopy Laboratory, Ankara, Turkey.

For organic acid analysis; Instrument: Agilent 1260 HPLC system, Column: Metacarb 87H column and Detector: PDA-210nm, Mobile Phase: 0.008N H2SO4, Flow Rate: 0.6mL/min.

For Sugar Analysis; Instrument: Agilent 1260 HPLC system, Column: Hiplex H column and Detector: RID, Mobile Phase: Water Flow Rate: 0.6mL/min.

Amino acid analysis was performed at TUBITAK-MAM.

In addition, FT-IR study was (Central Laboratory, Niğde Ömer Halisdemir University) also conducted for phenolic substance content and other contents. In the FT-IR study; gallic acid (MERCK) was used as a control in the investigation of phenolic substance content.

Results and Discussion

The comparison of the growth of fungal isolates on HDA medium with the growth on mineral medium and PDA medium used as a negative and positive control is shown in Figure 2. As shown in Figure 2, all fungal strains showed very well growth in HDA medium than in the negative control. In order to confirm the growth of fungal strains in HDA medium, liquid media containing different volumes of honeydew were also prepared in three parallels. When their growth on liquid medium was measured; the growth rate of each species varied. In addition, it was shown in Figure 3 that there were differences in mean biomass production of fungal growth in liquid media containing 1.0 % and 0.5 % (w/v) of honeydew.

While TK and BB showed similar growth in both ratios, the growth in TC, TS, TA was better in medium with 0.5% honeydew. It is estimated that the better growth in these strains in the medium containing 0.5% honeydew is related to the phenolic substance content of the honeydew. On the other hand, it was determined that the TH, TP, TL strains had better growth in the medium containing 1.0% honeydew.

In the analysis of the honeydew substance content, butyric acid and formic acid were found, while trehalose, sucrose, maltose and raffinose were detected as sugars. Lactose, a disaccharide, was not detected. It was thought that all these components provided suitable conditions as a carbon source for fungal growth in both liquid and solid media, however, nitrogen content should also be in the components, so the amino acid content was examined. However, it could not be detected in the present solution. The presence of amino acids in the solution is indisputable, but it is thought that it could not be detected due to the very low amount in the solvent. In addition, FT-IR study of the honey was also performed to examine the phenolic content and the total content (Figure 4).

FT-IR analyses showed that the phenolic content of the honeydew was higher than that of the gallic acid standard (Figure 4). It has been determined that some fungal isolates grow better in a medium with 0.5% honeydew content than in a medium with 1.0% honeydew content. It is thought that the reason why fungal growth is lower in 1.0% liquid medium is due to the toxic effect of the phenolic compounds in the honeydew. It has been shown in the literature that high phenolic contents (especially gallic acid, which is common in plants) have a toxic effect on some fungi and yeast species (Dix, 1979; Li et al., 2017). Additionally, the FT-IR spectrum of standard gallic acid shows distinct peaks around 3300 (C-OH peak), 2900 (C-H-H peak), 1600 (C=0 peak), 1400 (C=C peak), 1200, and 1000 cm^{-1} and many peaks overlap with the honeydew. It is noteworthy that the FT-IR spectrum also overlaps with the extract of *Catalpa ovata* G. Don (Bignoniaceae) plant (Yang et al., 2020). Similarly, it is reported by Anjos et al. (2015) that various sugars have peaks at 3200, 2900, 1600, 1300, 1200 and 1000 cm^{-1}.

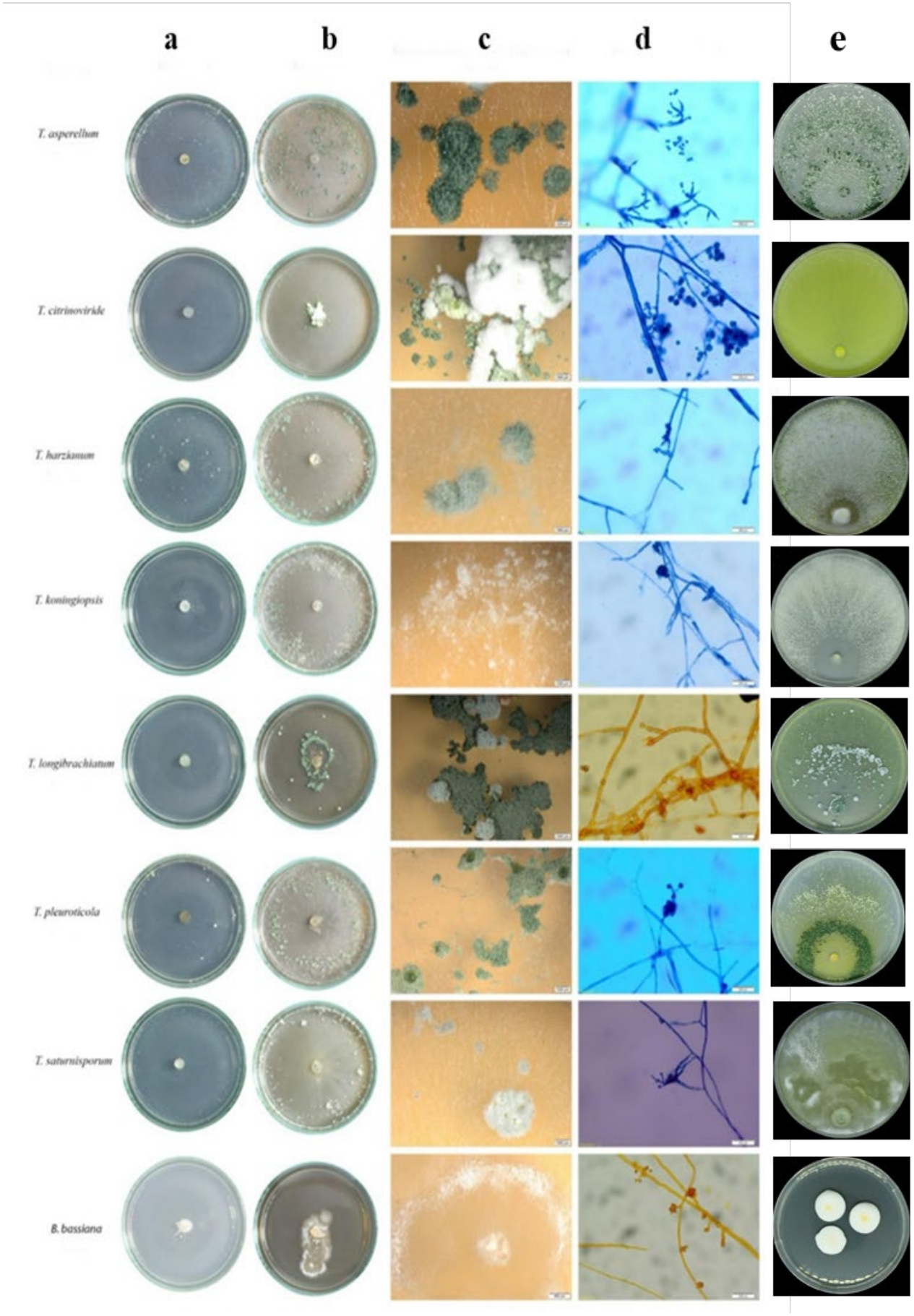


Figure 2. Growth of fungal species

a) fungal growth on MM medium as negative control, b) fungal growth on HDA medium as test media, c) mycelial growth and d) hyphal morphology, e) fungal growth on PDA medium as positive control

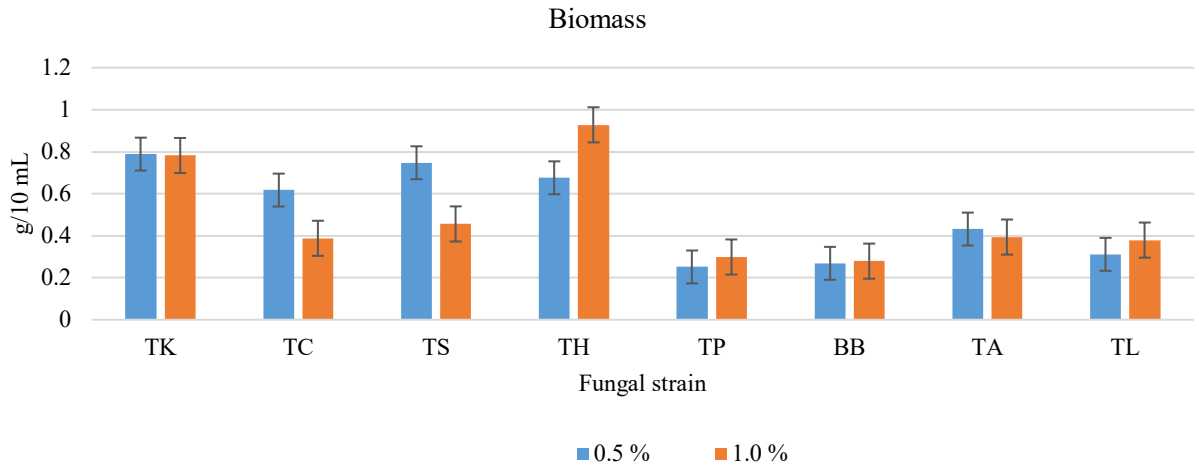
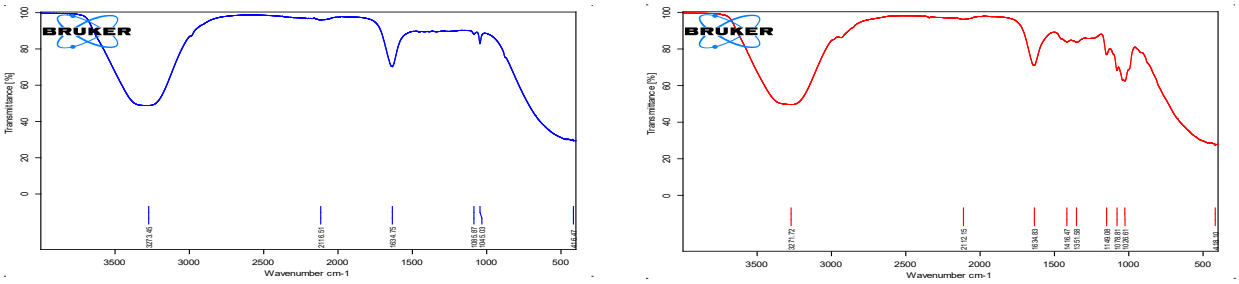


Figure 3. Microbial mean biomass in liquid honeydew media
(From left to right; *Trichoderma koningiopsis* (TK), *Trichoderma citrinoviride* (TC), *Trichoderma saturnisporum* (TS), *Trichoderma harzianum* (TH), *Trichoderma pleuroticola* (TP), *Beauveria bassiana* (BB), *Trichoderma asperellum* (TA), *Trichoderma longibrachiatum* (TL)



a b

Figure 4. FT-IR analyses (a) Gallic acid, (b) Honeydew

Table 1. Sugar and organic acid content of honeydew (mg/mL)

	Butyric acid	Formic acid	Raffinose	Trehalose+Sucrose+Maltose	Lactose
Honeydew	0.5 ±0.0	6.1±0.3	62±3	26±3	not detected

Conclusion

The aphid species *A. gossypii* Glover, which has a high population density on the *Catalpa bungei* C. A. Mey (Bignoniaceae) plant, produces a large amount of honeydew. It is seen that the content of the honeydew produced depends on the host plant and the sugars in the plant sap can be dominant. In addition, it appears that the content of the honeydew produced by aphids may positively or negatively affect the development of various fungal species. It is thought that the inhibitory effect may be due to the various phenolic substances contained in the honeydew, which was found to be consistent with the literature.

In biological control studies, focus should be placed on the honeydew produced by insects, in addition to nectar and honey (resin), which are carbohydrate sources for each living group in the food chain. In this study, it was determined that *A. gossypii* Glover honeydew had a positive effect on the development and activity of some *Trichoderma* species and *B. bassiana* fungus species, which can be applied in agricultural fields. In light of the research conducted, this study highlights the multifaceted role of honeydew in shaping the complex dynamics of insect-plant-microorganism interactions, highlighting its importance in both pest management and conservation strategies. Moreover, as one of the first studies on whether it supports agriculturally beneficial or harmful fungal developments, it has shown that honeydew should be focused on.

Declarations

Author Contribution Statement

G.O.E.: Investigation, formal analysis, conceptualization, writing the original draft, supervision, review and editing

Y.B., G.B. and E.Y.C.: Data collection, investigation and methodology

G.G.: Investigation, writing the original draft, supervision, review and editing

A.Ö.: Data collection, Investigation, writing the original draft, supervision, review and editing

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

A part of this study was presented as "Presentations Online Abstract" at the 7th International Anatolian Agriculture, Food, Environment, and Biology Congress-TARGID 2024 which is being held 26-29 September 2024 at Kastamonu/Türkiye.

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