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Leaf Surface Bacterial Colonization in Response to *Brevicoryne brassicae* (L.) Infestation of *Brassica chinensis* L.

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Abstract: Problem statement: Epiphytic population is an intrinsic part of the leaf surface. The effect of stress like insect infestation is not well understood. The aim of the study was to determine possible effects of insect infestation on bacteria community. **Approach:** The leaf surface microbial profile of *Brassica rapa ssp. chinensis* (L.) was investigated using a culture-dependent approach under normal and infested condition. **Results:** The presence of mostly novel Gram-negative bacilli on the leaf surface of *B. chinensis* was evident, with maximum identity with members of the phyla Firmicutes and Actinobacteria at the 16S rDNA level, as evident from GenBank accession numbers FJ231349 to FJ231355. The specialist aphid *Brevicoryne brassicae* (L.) was used as a model to study the effect of insect infestation on the epiphytic leaf surface microbial populations in *B. chinensis*. Plants infected with aphids had significantly higher bacteria counts than non-infested plants. **Conclusion:** These differences were up to 10,000-fold within three days post infestation. This increase is probably due to the secretion of honeydew, which acts as an additional carbon source for the pre-existing microbes. However, aphid infestation had no effect on the qualitative variation of microbes on the plants, which rules out the abundance of microbial population on the leaf as a contribution of the aphids alone.

Key words: Epiphytic microbial, *Brassica chinensis*, aphid infested group, *Brevicoryne brassicae*, phyllospheric bacterial, Genomic DNA, scanning electron microscope, statistical analysis

INTRODUCTION

Brassica rapa ssp. chinensis (L.), pak choi, is a very popular tropical leafy vegetable. It is a non-heading Chinese cabbage with fleshy petioles and upstanding glabrous leaves that forms a loose rosette. Plants in the Brassicaceae family are susceptible to various aphid infestations, including the specialist cabbage aphids, *Brevicoryne brassicae* (L.). Aphids, often acting as a vector for plant diseases, cause minimal direct plant tissue damage while inserting their slender stylets intercellularly to feed on the phloem sap. In order to survive, the plant needs to identify and respond to such unusual feeding strategies. *Arabidopsis thaliana* L., a member of the Brassicaceae family, is a well-understood genetic and genomic system that has been the subject of investigation by different groups (De Vos *et al*., 2007; Kusnierczyk *et al*., 2008). Insects feeding on plants induce a series of highly complex, well-coordinated defense responses, which is evident within the first six hours of infestation in the form of gene expression changes in *A. thaliana*. The insect-specific defense response induction in plants is mediated by salivary components (Reymond *et al*., 2004). Although plant resistance mechanisms have been investigated in several studies, there have not yet been any studies in which the effect of insect infestations on the epiphytic microbial profile is analyzed.

It is known that aphids secrete a shiny, sticky film of honeydew - a carbon-rich waste product - on the host's leaves. Honeydew attracts ants, encourages black sooty mold growth and also contributes to increased phyllospheric bacterial populations (Stadler and Muller, 1996). Phyllospheric microbes grow in unique micro-scale habitats on the plant surface, depending on

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specific metabolites secreted by the host plants. The phylloplane environment determines both the morphology and the primary metabolism of microbes. These bacteria are an integral part of any plant (originating primarily from aerosol-based spreading) (Hashidoko, 2005). Some plant surface bacteria are capable of detoxifying xenobiotics producing growth hormones. They can also be beneficial in seed germination, as it has been shown for soybean (Kutschera *et al*., 2002). The differential inhibition of organ development surface bacteria has been demonstrated in sunflower. Phyllospheric bacterial abundance varies within plant species, according to growth condition, leaf age, leaf position, physiological status, composition and quality of nutrition, leaf cuticle characteristics and a multitude of other factors (Ruppel *et al*., 2008; Yang *et al*., 2001; Mercier and Lindow, 2000). Phyllospheric bacteria are thought to be subjected to immense stress from profuse UV radiation, intense light, high temperatures and daily fluctuations in temperature and humidity. Their ability to withstand or avoid repeated stress is necessary for their survival in such an environment (Wilson *et al*., 1999; Beattie and Lindow, 1994; Leben, 1981). For this reason, it is thought that studying these microbes in greater detail may shed light on unrevealed mechanisms of adaptation that have so far remained concealed.

To date, no studies have been conducted on either the epiphytic micro flora of pak choi leaves or the role of aphid infestation on the leaf surface bacterial population in pak choi. Therefore, the focus of the present study was to determine aphid-mediated changes on the leaf surface micro flora in pak choi, whereby the specialist aphid *B. brassicae* was used as the model insect.

MATERIALS AND METHODS

Leaf collection: Plants were allowed to grow in the greenhouse until they were 25 days old. There were two treatment groups: one control group with non-infested plants and one aphid infested group. Plants for the two treatment groups were kept at separate locations within the same cubical of the green house, to avoid aphids from contaminating the control plants. With the infested group, aphids were present on the leaves for five days before the bacteria count was monitored for the subsequent three days. Leaves were collected from both groups with gloved hands and sterilized scissors. Two randomly selected leaves were used for each observation. The experiment lasted for 3 days, with 5 replicates for each group.

Epiphytic bacterial count: The leaves were taken to the laboratory and weighed. The leaf surface bacterial count was monitored according to the modified protocol of Mercier and Lindow (2000). Two leaves each were placed in a sterile falcon tube with 20 mL of 0.1 M potassium phosphate buffer, pH 7.0. They were sonicated twice for 7 min each using Bandelin Sonorex RK510S Ultrasonic Bath at room temperature to remove the bacteria from the leaf surface and to place them into the suspension. They were subsequently vortexed at maximum speed for 20 s before being plated at desired dilutions on Luria Bertani Agar plates at pH 7.5. Plates were kept overnight at 37°C under inverted condition. The colony counts were taken after twelve hours and the bacterial count for identical weight was calculated for each sample.

Characterization of microbial isolates: The resulting unique colonies from the control group were streaked on LB agar plate three subsequent times to finally obtain pure isolates. The pure isolates were maintained at 4°C and characterized with light microscopy after standard methylene blue staining. Gram staining was also conducted using Axioplan by Zeiss. The images were captured using MC100 attachment; they were also visualized under phase contrast (Ph3) using Nikon ECLIPSE E400 at 1000x magnification. Three of the isolates were selected for scanning electron microscopy to analyze their detailed structure. The overnight cultures were fixed according to reported protocols (Adarsh *et al*., 2007) and visualized under a Scanning Electron Microscope (LEO 435VP) at a pressure of 005 mbar. Samples were sputtered with palladium inside an Edwards Sputter Coater S150B at 3 mbar pressure 20 mA current and a 1.5 kV voltage, after the chamber was flushed three times with argon.

Molecular characterization: Genomic DNA was isolated from overnight cultures that were grown in LB broth using Wizard Genomic DNA Purification kit from Promega, according to the manufacturer's protocol. The DNA was quantified using the NanoDrop 3.0.1. The samples were loaded on a 1% agarose gel, along with 1 Kb ladder from Fermentas and subjected to electrophoresis at 5 V cm^{-1} in standard TBE buffer. The gel was stained with ethidium bromide (1 μg mL^{-1} of TBE) and observed over the transilluminator and photographed using the BioDocAnalyze 2.0 gel documentation system by Biometra. Four of the isolates were selected for molecular characterization. Genomic DNA was used as a template for PCR amplification of a 1300bp long 16S rDNA fragment. The bacterial 16S rDNA primers reported by Chaudhuri and Thakur (2006) were used for this purpose. The PCR was performed using PCR Master Mix by Promega,

50 pmol of each primer and 100 ng of template in a total of 50 µl reaction volumes in a Biometra TGradient thermocycler. The PCR program included a denaturation temperature of 92°C for 2 min for one cycle, followed by 40 cycles at 92°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. It ended with a single final extension at 72°C for 2 min. The PCR product was analyzed on a 1% agarose gel at 5 V/cm with a 1 Kb ladder by Fermentas as marker. The PCR product was purified using peqGold Cycle-Pure kit by peQlab Biotechnologie GmbH and sent for sequencing to the DNA Sequencing Facility at the Faculty of Agriculture and Horticulture, Humboldt University Berlin. Sequencing was performed using the BigDye Terminator v1.1 Ready Reaction Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analyzer by Applied Biosystems. The sequences were subjected to BLAST-N analysis against the GenBank nucleotide sequence database. The novel sequences were submitted to the GenBank.

Statistical analysis: One-way ANOVA was performed to analyze the data of leaf surface bacterial count variation between normal and infested pak choi plants using the statistics software Statgraphics Centurion XV. The data was analyzed for statistical significance at 95% confidence level.

RESULTS

Epiphytic bacterial count: The bacterial count for the control group for 3 subsequent days varied from 1.06-1.8×10^{-3} on day one, 2.5-4.6×10^{-4} on day 2 and 1.25-3.738×10^{-4} on day 3. In case of infested plants, the count varied from 1.7-4.332×10^{-5} on day one, 2-5×10^{-8} on day two and 5×10^{-8}-1×10^{-9} on day three. The p-value at 95% confidence level of bacterial count between normal and infested plants was 0.025 for day one, 0.018 for day two and 0.007 for day three. These highly significant results are summarized in Table 1 and show an enormous increase in bacterial count in infested leaves compared to non-infested leaves.

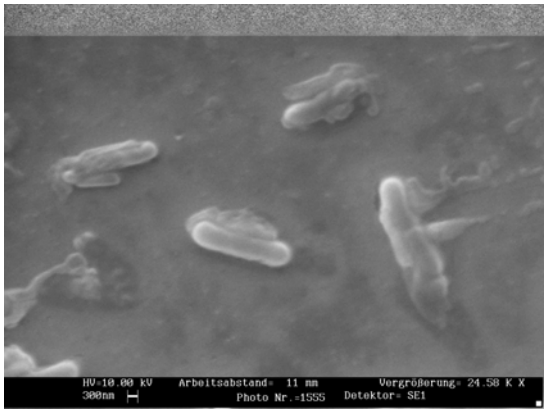
Table 1: Leaf surface bacterial count in pak choi in control plants and plants infested with *B. brassicae* during the experiment (statistical analysis: one-way ANOVA)

Sample	Mean	Standard deviation	Coefficient of variation	F- quotient	P-value
DAY 1					
Control	1400	373.631	26.69%	12.24	0.0249
Infested	278867	137364	49.26%		
DAY 2					
Control	34000	10816.7	31.82%	15.21	0.0175
Infested	3.40E+08	1.51E+08	44.41%		
DAY 3					
Control	25460	12472.6	48.99%	25.47	0.0072
Infested	7.33E+08	2.52E+08	34.32%		

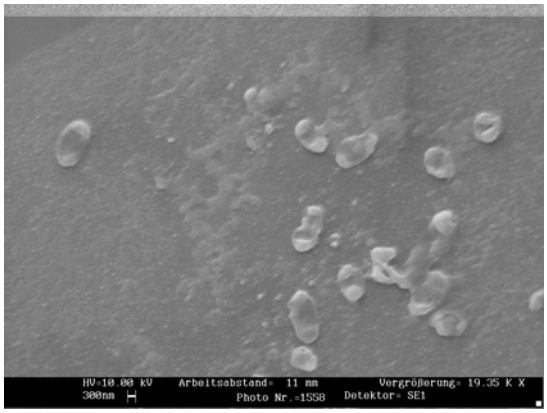
Microbial characterization: Eight isolates were obtained from the control samples. Genomic DNA was isolated from these strains and 1300nt long PCR product of partial 16S rDNA was obtained and further sequenced for four isolates. Isolate BC1 with bright orange colored colonies, were found to be a Gram-negative bacilli which, at the molecular level, demonstrated 99% identity with *Bacillus* sp. (FJ231351) (Fig. 1a). Isolates BC2 and BC3 (Fig. 1b) were yellow colored colonies, which were assigned to Gram-negative bacilli. Cream colored colonies of isolate BC4 were identified as novel Gram-negative short bacilli, GenBank accession numbers FJ231352 and FJ231353. Isolate BC5 (FJ231349 and FJ231355) contained dark yellow colonies. These were found to be Gram-positive bacilli which, at the molecular level, showed 99% identity with *Arthrobacter* sp. (Actinobacteria). Isolate BC6 had colonies that were yellow with an orange tinge. This Gram-positive bacillus showed an unusual structure on SEM imaging. At the molecular level, it showed just 84% identity with gamma Proteobacteria. This indicates that the isolate is a novel organism, with almost no resemblance to the reported members of the domain bacteria (FJ231350 and FJ231354) (Fig. 1c). Isolate BC7 had cream colored colonies of Gram-negative bacilli, whereas isolate BC8 contained cream colored colonies of Gram-negative short bacilli. The isolates obtained from infested leaves were the same as those of the non-infested control samples as far as the diversity was concerned. However, there was variation in total count.

DISCUSSION

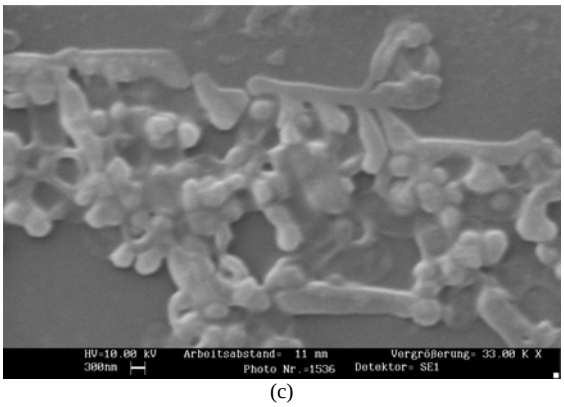
Bacterial diversity in the phyllosphere can mainly be classified into four phyla namely Cyanobacteria, Actinobacteria, Firmicutes and Proteobacteria (Beattie, 2006). The first three phyla along with two others possess important adaptations that make them ideal for their leaf surface existence. These adaptations include oxygenic photosynthesis and resistance to environmental hazards, such as desiccation and ultraviolet radiation (Battistuzzi and Hedges, 2009). Actinobacteria and Firmicutes are a highly diverse group of bacteria. Initially, these bacteria were found to be strictly Gram-positive and are ancestral to Gram-negative microbes. Further investigations also reveal Gram-negative members among these groups, representing gradual evolution. Comparative genomic analysis among their members indicates the presence of horizontal gene transfer, but an absence of lateral gene transfer in case of acquisition of virulence genes.



(a)



(b)



(c)

Fig. 1: SEM photographs of three of the isolates obtained from pak choi leaf surface. 1a: Image of Isolate BC1 which is a bacilli of around 2 µm size. At the molecular level it appears as a new member of the genus *Bacillus*. 1b: Image of isolate BC3 of less than 1 µm in size. 1c: Image of Isolate BC6 with an atypical structure. The molecular analysis identifies it as a novel isolate with little similarity with the members of the domain bacteria

Gram-positive bacterial plant pathogens have been reported to adapt to a wide variety of virulence-causing strategies for host invasion. Research into plant pathogenic members of the phyla Actinobacteria have revealed novel toxins, plant hormones and proteins that could regulate plant metabolic and defense pathway through largely unknown mechanisms (Hogenhout and Loria, 2008; Stavrinides *et al*., 2008; Abramovitch *et al*., 2006). Actinobacteria and Firmicutes have evolved strategies for plant pathogenecity independent of the Gram-negative Proteobacteria (Hogenhout and Loria, 2008). Microbial pathogenesis is usually mediated by the secreted proteins that are transported inside the plant cell through type III protein secretion system (TTSS) in case of Gram-negative bacteria. This is lacking, however, in Actinobacteria and Firmicutes (TTSS).

Gram-positive phytopathogens also utilize and manipulate the sap-feeding insects for entering into the phloem sieve cells (Hogenhout and Loria, 2008). Their study indicates an association of Actinobacteria with a vast number of insects in a symbiotic relation. Mutualistic bacteria are important for protecting the host and its nutritional resource. The latter is generally due to the extraordinary ability of members of this group to use a wide variety of carbon and nitrogen sources, while the former is due to its ability to produce an expanded repertoire of metabolites, including antibiotics, predisposing them to engaging in protective symbioses (Kaltenpoth, 2009).

In this study, the abundance and profile of an epiphytic microbial population on partially aphid-infested pak choi plants is reported for the first time. Bacteria present on the leaf surface of pak choi are primarily Gram-negative bacilli that show a closer identity to the phyla Firmicutes and Actinobacteria, generally indicating the recently evolved (newer) members of these phyla. Some of the novel isolates were also Gram-positive in nature, indicating the diversity of Gram nature among the microbial population. The presented results indicate the presence of novel members of three out of four phyla of Terrabacteria (Battistuzzi and Hedges, 2009) on the pak choi leaf surface, pointing towards a rich microbial diversity. This study indicates a much lower ($\sim 10^{-3}$) leaf surface microbial population in pak choi (in terms of count) compared to other plants (10^{-5}-10^{-7}), as previously reported by Yang *et al*. (2001). Aphid infestation causes a 100- to 10,000-fold increase in *B. chinensis* leaf surface microbial count (within 8 days of infestation) with no variation in diversity. This enormous increase in bacterial count in infested leaves could be explained in two ways. The first and most

likely reason is the presence of honeydew on the leaves. Honeydew has been reported to increase counts of phyllospheric bacterial, yeast and filamentous fungi in conifer trees (Stadler and Muller, 1996). Another possibility is that this increase could be partially due to bacteria present on the aphid themselves. When leaves were placed into the falcon tubes for analysis, aphids were not removed in order to avoid contamination from handling the sample. Since there was no variation in the bacterial profile at the qualitative level among the two groups, the latter explanation (aphid introduction) was unlikely.

CONCLUSION

This study could be expanded further by characterizing the novel microbes in detail. It could be beneficial to understand their role in the normal functioning of the plant and understanding their role in terms of aphid-microbe interaction.

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