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Detection of plant stress responses in aphid-infested lettuce using non-invasive detection methods

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Abstract: Lettuce cultures are prone to aphid infestations, but consumer tolerance for aphids in fresh lettuce is close to zero. To avoid losses of harvest due to aphids, lettuce plants are routinely sprayed with pesticides. Broadcast spraying of pesticides are costly, pollute the environment and may lead to pesticide residues in lettuce. Typically, early aphid infestation of lettuce cultures is not uniform, but shows a heterogeneous distribution. An early identification of aphid-infested lettuce plants prior to widespread infestation of whole fields would allow for selective spraying which reduces the use of pesticides. Although aphids have been shown to trigger physiological adjustments in plants, optical detection methods for aphid-infested lettuce plants based on their biotic stress response have yet to be developed. For other crop plants, aphid infestation has been shown to change the optical properties of leaves and canopies. Spectral reflectance measurements of wheat and soybean revealed an increased reflectance in the visible spectrum, but decreased reflectance in the near-infrared (NIR) due to changed chlorophyll content and structural characteristics of leaves. In our first experimental approach, we compared spectral reflectance of field-grown plants with and without aphid infestation. Here, we used aphid-proof enclosures with control plants and plants subjected to manual infestation with the potato aphid *Macrosiphum euphorbia*, a generalist aphid commonly found on lettuce plants. Three weeks after initial infestation, we counted an average of 130 aphids per plant, which were mainly found on the abaxial side of outer lettuce leaves, while control plants were mainly uninfested. A comparison of leaf-level spectral reflectance of control and aphid-infested plants revealed minor variation in spectral reflectance patterns. We conclude that despite high infestation levels, plants did not exhibit a strong systemic stress response to aphid infestation. This experiment is only the beginning of a row of field and laboratory experiments to find a non-invasive detection technique suitable to indicate the biotic stress response of lettuce plants, and will be followed by experimental approaches using plant- and canopy-level spectral reflectance measurements, chlorophyll fluorescence measurements and thermal imaging.

Key words: Aphid, lettuce cultivation, plant biotic stress response, precision agriculture, spectral reflectance

Introduction

Aphids are a major pest in lettuce cultivation, not because of direct damage of plants or reduction of yield, but due to a very low tolerance of consumers for insects in fresh produce (Morales et al., 2013). Aphids pierce plants with their long stylet to suck phloem and feed on photoassimilates and amino acids (Tjallingii, 1995; Will et al., 2013). Although plant damage is low due to movement of the stylet in the apoplast, plants sense aphid secretions and respond by various defense mechanisms (Smith & Chuang, 2014). Induced plant defences include increased levels of insecticidal phloem constituents including toxic and/or growth-inhibiting alkaloids, phenolics and proteins (Cardoza et al., 2005; Smith & Chuang, 2014). Furthermore, increased acitivities of enzymes involved in the biosynthesis of insecticidal compounds have been observed in response to aphid sucking (Ni et al., 2001).

Plants show early changes in gene expression upon aphid sucking, but early plant infestation with aphids is often not accompanied by visible differences in plant phenotype (Guerrieri & Digilio, 2008; Smith & Chuang, 2014). Prolonged aphid sucking leads to reduced photosynthetic carbon assimilation rates and reduced foliar chlorophyll content in susceptible plants, while resistant lines of tobacco, wheat and sorghum have been shown to compensate aphid sucking by increased photosynthesis rates (Franzen et al., 2007; Smith & Boyko, 2007). In addition, dispersed chloroplasts with fewer grana stacks and more starch granules have been observed in Johnson grass (Gonzáles et al., 2002). Ultimately, aphid infestation may result in stunted growth (soy plants, Ragsdale et al., 2007), foliar damage and rolling of leaves (wheat, Yang et al., 2009) or necrotic damage (Johnson grass, Gonzáles et al., 2002). In addition, aphids are vector for the transmission of plant viruses (Gray et al., 2014).

Lettuce plants only exhibit morphological changes like deformed lettuce heads and changed leaf color in response to prolonged infestation with aphids (McCreight & Liu, 2012). Nevertheless, induced plant defenses upon initial aphid sucking may affect leaf spectral reflectance. Measurements of spectral reflectance of wheat plants on the leaf and canopy level have revealed differences between healthy and aphid-infested plants (Mirik et al., 2012; Yang et al., 2009; Zhao et al., 2012). An increased reflectance in the visible wavelength spectrum has been attributed to reduced content of chlorophylls, while reduced reflectance within the near-infrared spectral range is likely affected by changes in cell structure and water content (Carter & Knapp, 2001; Junker & Ensminger, 2016; Zhao et al., 2011). Similar changes of spectral reflectance have been observed in soy and sorghum plants infested with aphids (Alves et al., 2015; Elliott et al., 2015). Despite the importance of sensor-based detection of plant pests for precision farming and phenotyping of plants for the selection of resistant cultivars (Goggin et al., 2015), little is known about changes in spectral reflectance of major crop plants in response to insect pests (Alves et al., 2015).

The aim of our study is therefore the identification of specific changes in the spectral reflectance of lettuce plants in response to aphid infestation. We hypothesize, that aphid infestation triggers systemic biotic stress responses, which lead to characteristic changes of spectral reflectance on the leaf level. To measure spectral reflectance of field-grown plants with and without aphid infestation, we have compared leaf-level spectral reflectance of plants grown in aphid-proof enclosures without and with infestation with the potato aphid *Macrosiphum euphorbiae*. Thereby, we contribute to the development of precision farming techniques for lettuce cultures and phenotyping of lettuce cultivars for breeding.

Material and methods

Plant cultivation

Mini-Romaine lettuce plants (*Lactuca sativa* 'Thimble') were grown in aphid-proof enclosures to modify aphid density by targeted infestation. Six custom-build enclosures, consisting of a wooden frame sized 1.5 by 0.3 m and 0.25 m high, tightly covered with 0.4,mm insect netting, were placed in a garden of Research Centre Jülich (50° 54' 36" N, 6° 24' 36" E). Cages were placed block-wise, with cage 1, 2, and 3 in a row south of cages 4,5, and 6. Three days prior to planting, soil was fertilized with 20 g/m^2 NPK+Mg/B/Zn (12/12/17/2) and watered generously. We chose the mini-romaine lettuce variety 'Thimble', because it is commonly grown at the time of the experiment by major growers in the area of North Rhine-Westphalia, and has a high resistance to downy mildew, *Bremia lactucae*, Bl 16-33EU, and currant-lettuce aphid, *Nasonovia ribisnigri*, Nr: 0. Lettuce seedlings were obtained from a commercial grower (Jungpflanzen Rudolf Klein, Bonn, Germany) at an age of about two weeks on August 14th, 2017. On the same day, five plants were equally distributed in each cage. Plants were generously watered throughout the experimental procedure. At the end of the experiment, plants were cut above the soil for the determination of fresh weight.

Aphid culture and infestation

The aphid infestation experiment was carried out with the potato aphid *Macrosiphum euphorbiae*, which is a common pest in lettuce cultures. This generalist aphid is commonly found on the abaxial side of intermediate and outer lettuce leaves. A laboratory culture of *Macrosiphum euphorbiae* was estabilished based on a single apterous exule. The culture was reared on young mini-romaine plants and maintained at room temperature. Three days after planting of lettuce seedlings, two aphids were placed on every lettuce plant in three randomly distributed cages, namely cage 2, 4, and 6 (d = 0). After 4,7, and 11 days, plants were visually inspected, and an average of 0.8, 3.6, and 9.1 aphids per plant were found. To ensure similar levels of infestation, additional aphids were placed when less than two aphids were found per plant. On September 7th, three weeks after the initial infestation, plants were sampled and total number of aphids per plant counted. While we did not observe any *Macrosiphum euphorbiae* on control plants, we noted in average less than one *Uroleucon sonchi* aphid per control and aphid-infested plant.

Leaf-level spectral reflectance measurements

Spectral reflectance of two intermediate leaves per lettuce plant were measured after the three-week infestation period using a PolyPen RP 400 (Photon Systems Instruments, Drasov, Czech Republic) and averaged per plant. The internal light source was used to measure spectral reflectance in the visual and near-infrared wavelength range (325 to 793 nm) in comparison to a Spectralon® Reflectance standard (Labsphere Inc., North Sutton, US). Normalised Difference Vegetation index (NDVI) was calculated as NDVI = $(R_{780} - R_{670})/(R_{780} + R_{670})$ (Rascher et al., 2011). For visualization of differences across the full spectral reflectance range, a principal component analysis (PCA) was conducted based on fundamental bands (Galvao & Vitorello, 1995; Köksal, 2011). Fourteen bands with a bandwidth of 10 nm were equally distributed across the spectral range from 450 to 775 nm, equaling one band every 25 nm.

Statistics

All statistical analyses were carried out using R 3.0.3 (R Core Team, 2014). Differences in NDVI between control and aphid infested plants were assessed by comparison of cages using a one-way ANOVA (function *aov*), after homogeneity of variance and normality of distribution were ensured by Levene's test and Shapiro-Wilk-Test, respectively (function *levene* from the library *car* and *shapiro.test*). Principal component analysis was conducted with the function *prcomp* and displayed using the function *autoplot* (libraries *ggfortify* and *ggplot2*).

Results

Aphid infestation levels and plant growth

The experimental setup to excude naturally occurring aphids in three control cages and guide infestation with *Macrosiphum euphorbiae* in three cages worked well. After three weeks, there were in average only 0.3 aphids per control plant, while infested plants had in average 138.0 ± 87.2 aphids. Aphid infestation did not result in visual changes or differences in plant growth between control and aphid-infested plants in terms of biomass at harvest. Instead, we observed generally better growth of plants in cage 4 to 6 compared to plants in cage 1 to 3, likely due to differences in crop rotation on the field prior to the start of the experiment.

Spectral reflectance measurements

After three weeks of aphid infestation, leaf-level spectral reflectance in the visible and near-infrared wavelength regions did not vary between control and aphid-infested plants. Instead, Figure 1 shows that reflectance spectra of all plants were typical for healty green vegetation. In accordance with this observation, Figure 2 shows that values of the Normalised Difference Vegetation Index (NDVI) for all plants ranged between 0.66 and 0.69.

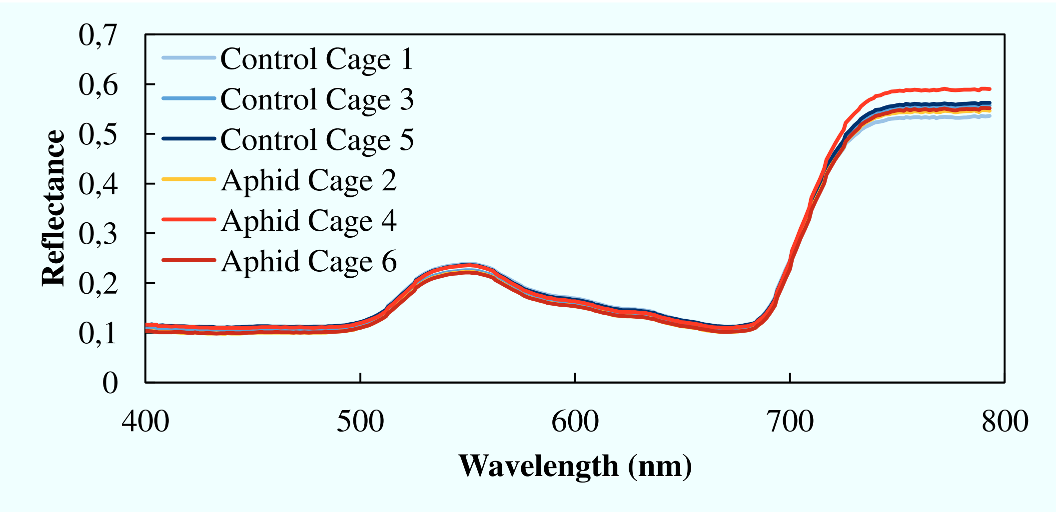

Figure 1. Leaf-level spectral reflectance of lettuce plants. Plants in cage 1, 3, and 5 are control plants, while plants in cage 2, 4, and 6 were infested with 130 ± 90 *Macrosiphum euphorbiae* aphids. Each cage averages spectral reflectance of N = 4-5 plants.

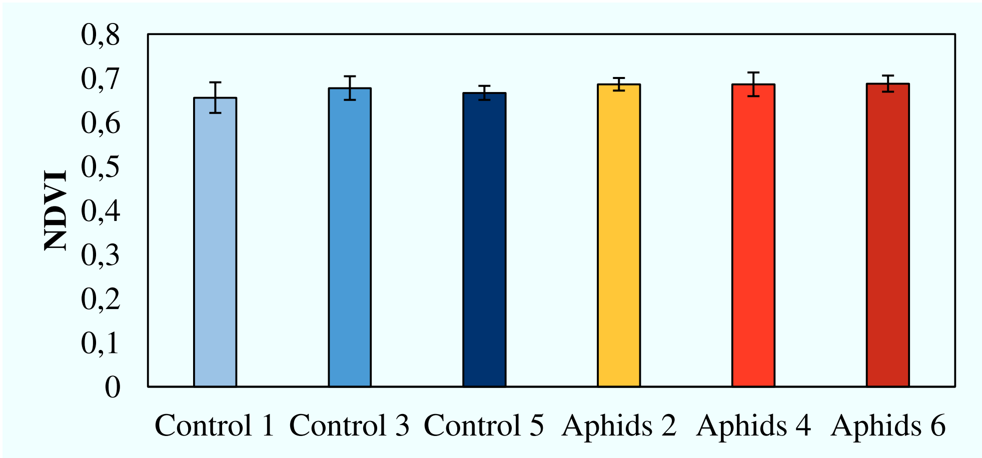

Figure 2. Normalised difference vegetation index (NDVI) calculated from leaf-level spectral reflectance of lettuce plants. Control 1, 3, and 5 denote cages with control plants, while Aphids 2, 4, and 6 denote cages in which plants were infested with 130 ± 90 *Macrosiphum euphorbiae* aphids. Each cage averages spectral reflectance of N = 4-5 plants ± SD.

To further discriminate between differences in leaf-level spectral reflectance of control and aphid-infested lettuce plants, we conducted a principal component analysis of fundamental wavelengths across the visible and near-infrared spectrum. PC1 explained 78.2% of the variation across spectra, mainly based on wavelength bands in the visible wavelength region, while PC2 explained 18.4% of the observed variation, mainly based on differences in spectral reflectance in the near-infrared wavelength region. Figure 3 reveals minor variation in clustering between control and aphid-infested plants.

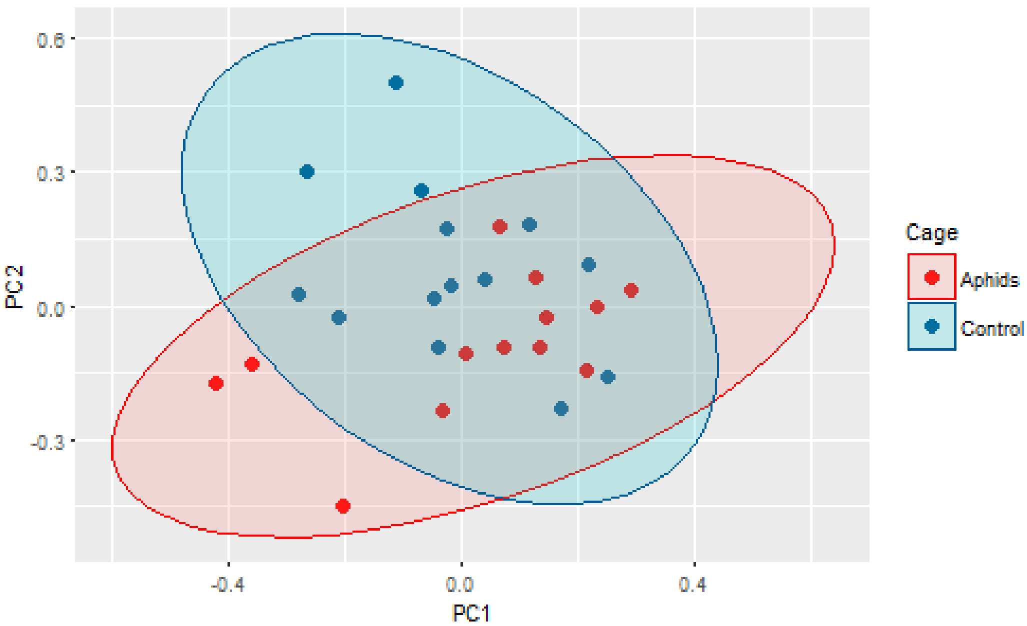

Figure 3. Principal Component Analysis of leaf-level spectral reflectance of lettuce plants. Control comprises 3 cages with N = 5 uninfested plants each, while Aphids comprises 3 cages with N = 5 plants infested with an average of 130 ± 90 *Macrosiphum euphorbiae* aphids.

Discussion

Aphid infestation of lettuce plants had only a minor influence on leaf-level spectral reflectance

The experimental setup effectively excluded natural aphid infestation and led to high differences in infestation levels with the generalist aphid *Macrosiphum euphorbiae*, but did not lead to visually different spectral reflectance in the visible and near-infrared wavelengths regions or the commonly used vegetation index NDVI, as shown in Figures 1 and 2. Therefore, we conducted an additional principal component analysis (PCA), which reduces the complexity of data and visualizes qualitative differences between spectra (Galvao & Vitorello, 1995; Thomas et al., 2016). The PCA analysis shown in Figure 3 revealed, that there is minor variation between control and aphid-infested plants, which are mainly explained by differences in the visible wavelength region. We have expected larger variation between control and aphid-infested plants, and consider several aspects, which may have hampered the detection of differences in spectral reflectance.

First, aphid infestation may have not been severe enough to trigger strong systemic plant responses (see Yang et al., 2005). Leaf-level spectral reflectance measurements with the handheld leaf spectrometer can be only made at the outer edge of leaves. Aphids have been found mainly at the abaxial side of leaves towards the leaf base. Therefore, localized stress responses to aphids may not be assessed using a handheld spectrometer, as has been previously observed for a fungal pathogen with localized occurrence (almond red leaf blotch, López-López et al., 2016). Second, plants may show a stronger response to different aphid species, for example the specialist aphid *Nasonovia ribisnigri* (Ali & Agrawal, 2012; McCreight, 2008). Differences in biotic stress responses to two different aphid species have been shown for wheat (Yang et al., 2009). Third, aphid-resistant lettuce cultivars as used in this experiment might be able to compensate for losses of photosynthates by increased photosynthetic efficiency, as has been previously oberved in tobacco, wheat and sorghum (Smith & Boyko, 2007). This might rather infer an increased chlorophyll content over time which would be expressed by a higher NDVI (Rascher et al., 2010). Nevertheless, changes in photosynthetic pigment levels can be only expected after days to weeks and upon strong infestation (Franzen et al., 2007), and the duration and aphid infestation levels in this experiment may have been insufficient to reaveal such a response.

Therefore, we will repeat the experiment with susceptible and resistant lettuce cultivars and a longer aphid infestation period using *N. ribisnigri*, to trigger stronger biotic stress responeses in lettuce plants. Furthermore, we will conduct spectral reflectance measurements on the leaf-level, plant, and canopy level, and apply advanced statistical methods to further assess the effect of aphid infestation on spectral reflectance of lettuce plants.

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