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NOTE

Spectral indices to rapidly monitor chloroplast distribution patterns of three cecidomyiid galls

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ABSTRACT: The objective of this research was to study altered distributions of chloroplasts in cecidomyiid galls derived from the leaves of *Machilus thunbergii* Sieb. & Zucc. and *Litsea acuminata* (Blume) Kurata using confocal laser-scanning microscopy and transmittance spectra. Chloroplasts in all gall tissues gradually decreased in the direction from the exterior toward the inside larval chamber based on Integrated Optical Density (IOD) and absorptivity indices, and chloroplast distributions in various gall tissues were species-specific. Different species of herbivorous insect dramatically altered chloroplasts in their host leaves. The spectral index was significantly and positively correlated with the IOD, and therefore, the transmittance spectra could be utilized as a tool to easily and quickly study the ecophysiology of galls.

KEY WORDS: Cecidomyiid gall, Chloroplast, Photosynthesis, Confocal microscopy, Transmittance spectra.

INTRODUCTION

Numerous insect groups induce plant galls, which are structures composed of plant tissues with insects feeding inside, and they are distinguished from other insect-generated shelters, such as rolled leaves or leaf mines, by the active differentiation and growth of plant tissues (Stone and Schönrogge, 2003). Multiple changes in response to gall inducers were found in host plant tissues, including changes in morphology, anatomy, and chemical composition. The physiological changes of insects were listed as if it was plant response to gall inducers (Mani, 1992; Rohfritsch, 1992). Relatively little work has been done on chloroplasts of cecidomyiid galls and their photosynthesis. Sun *et al.* (2005) reported that the influences of plant tissues on incident light in leaves and etiolated seedlings, and tissues of both leaves and seedlings can transform the quality, quantity, and conduction direction of incident light to form a characteristic internal light environment of stems, which is of crucial physiological significance for light-related metabolic activities. The net photosynthesis rate in galls is usually much lower than that in normal leaf tissues (Dorchin *et al.*, 2006). Mature stems, twigs, and branches of many plant species contain fluorescence activity of photosystem II (PSII) in all internal stem tissues under the periderm and after pigment extraction, and a decreasing gradient of chlorophyll (Chl) levels from the cortex to the pith (Dima *et al.*, 2006). Insect-induced galls induce the infected leaf to synthesize fewer photosynthetic pigments to save energy so it can produce more compounds to protect the plant system against insects in the gall (Nabity *et al.*, 2013). Previously, we found that herbivorous insects caused a deficiency of pigment-protein complexes of PSI and PSII in an oval-pointed cecidomyiid gall of a *Machilus thunbergii* Sieb. & Zucc. leaf at a very early stage of development. Insect-induced galls contained low amounts of light-harvesting Chl, but still possessed normal grana stacking and thylakoid morphology (Yang *et al.*, 2003). The insect gall's tissues and stem tissues were similar to all non-leaf green tissues by containing chloroplasts in the epidermis (Yang *et al.*, 2007). Moreover, leaf-derived cecidomyiid galls are sinks in *Machilus thunbergii* leaves (Huang *et al.*, 2014). Insect-induced galls may be a new organ that is mostly heterotrophic but still retains an autotrophic function (Huang *et al.*, 2015).

Ecophysiological studies of leaf spectra have become popular because of their simplicity, rapidity, and non-destructive nature for leaves. The photosynthetic rate, PSII efficiency, pigment content, and water content of plants can be estimated by the reflectance spectra of leaves (Weng *et al.*, 2006). Nevertheless, insect gall infections reduce the photosynthetic efficiency that is detected using both reflectance spectra and fluorescence (Huang *et al.*, 2011). Spectral transmittance indices were successfully used to study ecosystem structure and function, and estimate physiological processes that occurred on temporal (seasonal) and spatial scales in an oak forest (Serrano and Peñuelas, 2005). Red (R) light (650 nm) is strongly absorbed by Chl; therefore, the transmittance of red light increases due a decrease in the Chl content. Near-

infrared (NIR) light (780 nm) is a 'reference wavelength' used to adjust for differences in tissue structures. A transmittance-based index, absorptivity (Abs), is correlated to the Chl content and can be used by the instrument in conjunction with Chl fluorescent measurements to assess the fraction of incident light used in photosynthesis (Dima *et al.*, 2006). The aims of this research were to evaluate the Chl fluorescence of chloroplasts in gall tissues by confocal microscopy, and determine the quantitative distribution of Chl in gall tissues by transmittance spectra at specific spectral wavelengths. The hypotheses of this study were that leaf-derived cecidomyiid galls induced by different insects can alter the distribution of chloroplasts, and there is a positive correlation between confocal images and transmittance spectra of chloroplast in cecidomyiid galls.

MATERIALS AND METHODS

Three different mature cecidomyiid galls (Fig. 1A) residing on the lower epidermis of *Machilus thunbergii* Sieb**.** & Zucc. (Lauraceae) and *Litsea acuminata* (Blume) Kurata (Lauraceae) mature leaves (Huang *et al.*, 2011; 2015) were collected from 700 m in elevation at Erhtzupin of Yangmingshan National Park (total area 11,455 ha, while the elevation ranges from 200–1120 m) located in the northwestern part of Taipei, Taiwan. Mature galls (cup-shaped galls of *Bruggmanniella* sp., obovate galls of *Daphnephila sueyenae* (Tokuda, Yang & Yukawa), and hairy ovoid galls of *D. taiwanensis* (Tokuda, Yang & Yukawa) were detached from an infected mature leaf, and the surrounding healthy leaf tissue was trimmed away to avoid contamination.

Hand-cut sections of leaves and galls were placed on slides in distilled water. Images were captured using a Zeiss LSM510 Meta confocal microscope (Oberkochen, Germany) with a Plan-Apochromat 20x/0.6 objective. Specimens were excited with an 8% argon 488 nm laser, and emission signals were detected with a photon multiplier tube (main beam splitter: HFT 488; beam splitter 1: mirror; beam splitter 2: NFT 545; BP 650-710 IR, Zeiss). Chl red auto-fluorescence indicated chloroplast localization. Confocal images were analyzed with Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). Color cube-based segmentation from the software was used to select shades of red in the area of interest, and the integrated optical density (IOD) of red staining was measured after standard OD calibration. The IOD was indicated as a positive correlation to chlorophyll fluorescence intensity. Localization of the considered sample area (400–2800 μm in depth) in the direction of the larval chamber was detected.

Hand-cut sections of leaves and galls were placed on slides in distilled water and then mounted on a microscope (B1-223A, Motic, Groisy, France) so that they could be irradiated with light. The light was from a 20-W halogen lamp with interference filters (IEC-578, Isuzu, Tokyo, Japan). Transmittance spectra of the sections were recorded (labeled as Abs-trans) using a spectrophotometer (V8EQE, Spectral Imaging, Helsinki, Finland), and remitted photons were captured (labeled as Abs-rem) with a CCD-detector (C8484-05G, Hamamatsu Photonics, Tokyo, Japan). The absorptivity (Abs) was calculated as 1 - (R/NIR), where R and NIR are remissions at corresponding spectral bands (Dima *et al.*, 2006). In this study, the transmittance-based spectral index was calculated by the following equation: Abs = 1 - ($\lambda1/\lambda2$), where $\lambda1$= transmitance of light at 650 nm and $\lambda2$ = transmitance of light at 780 nm. The instrument was pre-calibrated for zero absorptivity on a white background (slides). R and NIR are remissions at corresponding spectral bands. Red (R) light (650 nm) is strongly absorbed by chlorophyll (Chl); therefore, the transmittance of red light increases due a decrease in the Chl content. NIR light (780 nm) is a 'reference wavelength' used to adjust for differences in tissue structures.

Galls and leaves were sampled from three plants, with three replicates from each of the plants. Each shoot of the plant was measured three times with leaves and leaf galls, and results are expressed as the mean ± standard deviation (SD). The experiment was performed with a randomized design for the physiological analyses. An analysis of variance (ANOVA) was calculated separately for spectral index and IOD. For significant values, means were separated by the least significant difference (LSD) test at $p \leq 0.05$. The relationship between spectral index and IOD was examined using simple linear regression model. All statistical analyses were conducted using JMP software, version 5.01 (SAS Institute; Cary, NC, USA). Regression analyses were performed with Sigma Plot 8.0 (Jandel Scientific, San Rafael, CA, USA).

RESULTS AND DISCUSSION

Stronger IOD of the fluorescence intensity, indicated by the Chl fluorescence intensity of chloroplasts were found in host leaves (*M. thunbergii* = 37429.96 ± 14680.26, and *L. acuminate* = 23580.87±4518.73) than in leaf-derived galls for all types of gall tested. As the shape and size of the cells that build the gall tissue may influence the values of the optical parameters observed from the bright field microscopic images of gall sections (Figs. 1B, C). While the characteristics of chloroplasts were homogeneous throughout tissues of all host leaves, in contrast, these features in all gall tissues gradually decreased in the direction of the exterior toward the larval chamber, as indicated by either the spectral index or IOD (Figs. 1B, C). The values of spectral index or

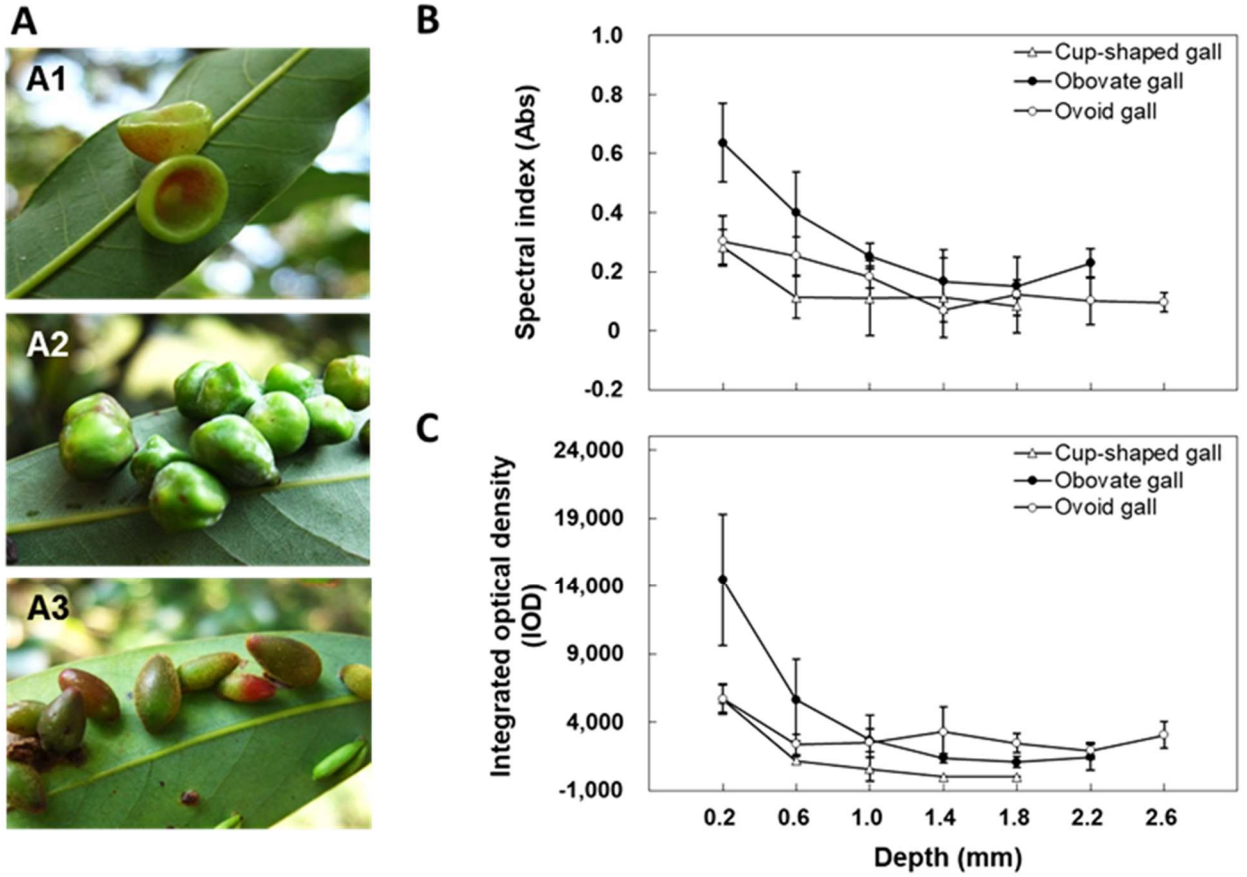

Fig. 1. Morphology of galls derived from leaves of host plants of *Litsea acuminata* (A1, cup-shaped galls induced by *Bruggmanniella* sp.) and *Machilus thunbergii* (A2, obovate galls induced by *Daphnephila sueyenae* and A3, ovoid galls induced by *D. taiwanensis*). The spectral index (B) and integrated optical density (C) of gall tissues were determined. Data are the average of three samples for each species. Error bars show the SD.

IOD obtained with Abs-trans and Abs-rem were similar. Although the above features of the studied species showed a gradient of diminishing Chl from the exterior to the larval chamber, the Chl distribution patterns of various species differed. Chl distributions of cup-shaped galls and obovate galls obviously decreased from the exterior to larval chamber tissues, but that of hairy ovoid galls gradually declined and remained at a low level. The results indicated that the profile of Chl distribution in the various gall tissues was species-specific among these three galls.

The altered gall chloroplast distribution was poorly understood in the leaf physiology. Chloroplasts were almost not found in deeper central tissues of cup-shaped galls, and the number and density of chloroplasts in obovate gall tissues were greater than those of other gall tissues. Our previous data showed that some of the photosynthetic apparatus, such as pigment-protein complexes of PSI and PSII, of galls were deficient over the lifetime of the gall (Yang *et al.*, 2007). Compared to infected leaves, Chl-related pigments related to photosynthesis in the gall, such as Chl and carotenoid, drastically decreased (Yang *et al.*, 2003). Those results can be explained by a stronger fluorescence intensity of chloroplasts being found in host leaves than in leaf-derived galls.

Much research on chlorophyll-deficient mutants was reported in higher plants. These mutants have a lower Chl content and higher Chl a/b ratio than do normal plants. The contents of pigment-protein complexes decrease throughout their lifetimes. They are sensitive to temperature, irradiation, and photoperiod (Lu *et al.*, 1995). The observed similarities between the optical properties of insect-induced galls and chlorophyll-deficient mutants suggest that galls might also be sensitive to temperature, light intensity, and photoperiod. There is much literature on the optical properties of leaves, with much of it relating to physiological studies and large-scale remote sensing of vegetation (Gay *et al.*, 2008). Traditional methods of pigment analysis, through extraction and spectrophotometric or HPLC measurements, require destruction of the measured leaves and are time-consuming and expensive. In contrast, measurement of optical properties is simple, rapid, and can be applied across spatial scales (Sims and Gamon, 2002). A positive and significant ($p < 0.01$ and

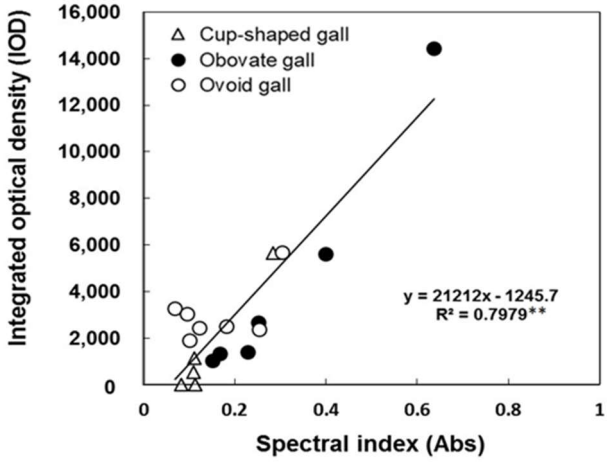

Fig. 2. Relationship between the spectral index (Abs) and integrated optical density (IOD). The regression line was calculated from the pooled results for all types of galls, assuming that the type of gall does not affect the slope and the intercept of the regression line. Multiple depths for all types of galls were pooled for the calculation of relationship between spectral index and IOD. (**, p<0.01)

R^2 = 0.7979) correlation was found between the spectral index (Abs) and integrated optical density (IOD) of gall tissues (Fig. 2). The results indicated that different species of herbivorous insect dramatically altered the photosynthetic characteristics of chloroplasts in their host leaves, and it is possible that transmittance spectra can be utilized as a tool for studying the ecophysiology of galls in large-scale remote sensing of vegetation.

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