Chlorophyll and its degradation products in the two-spotted spider mite, Tetranychus urticae: observations using epifluorescence and confocal laser scanning microscopy

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Chlorophyll and its degradation products in the two-spotted spider mite, *Tetranychus urticae*: observations using epifluorescence and confocal laser scanning microscopy

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Abstract

Chlorophyll and chlorophyll degradation products were observed in the two-spotted spider mite (*Tetranychus urticae*) using epifluorescence microscopy (EFM) and confocal laser scanning microscopy (CLSM). A clear red fluorescence (EFM) and a fluorescence induced by a laser wavelength of 650 nm (CLSM) were observed. In the lateral caeca, in the ventriculus and in the excretory organ, a bright light blue fluorescence was observed in close association with chlorophyll by using EFM. The same material can be localized with CLSM by using a laser with a wavelength of 488 nm. By comparison with synthetic guanine, this bright fluorescence is supposed to be guanine. The presence of guanine fluorescence in the mite pellets confirms this hypothesis. A possible mechanism for guanine formation is discussed.

Keywords

Tetranychus urticae Epifluorescence microscopy Confocal laser scanning microscopy Chlorophyll Guanine

Electronic supplementary material

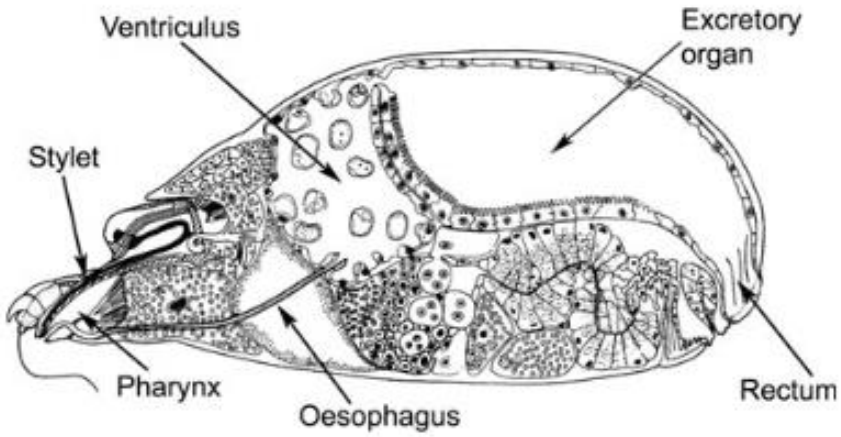
The online version of this article (doi:10.1007/s10493-013-9686-6) contains supplementary material, which is available to authorized users.

Introduction

The disposal of chlorophylls during arthropod herbivory is still poorly documented. Some insect herbivores of economic significance have been examined in detail. For instance, silkworm excretes up to four different chlorophyll derivatives, of which two were identified as 13^2-HO phaeophytin *a* and *b* (Nakatani et al. 1981). Pigment concentrations from guts of nematodes feeding on microphytobenthic organisms (Majdi et al. 2012) and caterpillars feeding on plants (Shao et al. 2011) show the presence of chlorophyll and chlorophyll breakdown products (e.g., pheophorbide *a* and pheophytin *a*) (Hendry et al. 1987). The two-spotted spider mite, a polyphagous species that feeds on more than 1,100 host plants, sucks the plant cell content of leaf mesophyll (Van der Geest 1985). The digestive system of spider mites consists of the foregut (mouth, pharynx, oesophagus), midgut (ventriculus and coeca) and hindgut (excretory organ, rectum and anus) (Alberti and Crooker 1985) (Fig. 1). Processed food is eventually compacted in the excretory organ and excreted

as faecal pellets (Alberti and Crooker 1985; Van der Geest 1985). Besides excreting chlorophyll-degradation byproducts, the excretory organ is known to function in the excretion and removing of nitrogenous metabolic wastes, presumably guanine in mites (Gasser 1951; McEnroe 1961; Wiesmann 1968; Alberti et al. 1999). The process of guanine pellet production remains in many respects enigmatic because there are a few direct evidences of formation, transport and secretion of the residuals by the epithelial cells of the excretory organ (Van der Geest 1985; Alberti et al. 1999). Recently, the lumen of the excretory organ from different terrestrial mites was found to be filled with yellowish double-refractive crystals of excretory wastes (probably guanine) and single electron-dense globules (Shatrov 2010). Although the catabolic fate of food products and the development of spider mites has been investigated in numerous occasions, most of the studies have been performed using bright field microscopy or scanning/transmission electron microscopy (Crooker 1985; Helle and Sabelis 1985). To our knowledge, there are no reports on the autofluorescence features of spider mite food products and excreta. The aim of this work was to use epifluorescence (EFM) and confocal laser scanning microscopy (CLSM) to better describe the catabolic fate of chlorophyll and the formation of guanine in *T. urticae*. Different developmental stages were analyzed as well as the fluorescence features of white and black pellets excreted by the mites.

Fig. 1



Schematic representation of a longitudinal section through a female *Tetranychus urticae*. Adapted from Alberti and Crooker (1985)

Materials and methods

Mite strains and rearing

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on Lima bean (*Phaseolus lunatus* L.) plants. These plants were grown in a greenhouse at a temperature of 22 ± 3 °C, relative humidity of 60–80 % and a photoperiod of L16:D8 h. The spider mites used in the experiments were all well-fed. Observations were performed on hundreds of spider mites from several generations during a two-year period.

Bright field microscopy (BFM) and epifluorescence microscopy (EFM)

Spider mite-infected Lima bean leaf sections of about 100 mm^2 were placed on a glass slide. A drop of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (50 mM, pH 6.5) (Fluka) was placed on the leaf and covered with a glass coverslip. Additional buffer was gently perfused in order to get rid of air bubbles.

Slides were placed on the slide holder of a Nikon Eclipse 90i epifluorescence microscope and different developmental stages of spider mites were observed by using bright field light (BF) and UV light. In the latter case, a Nikon UV-2A filter was used (Excitation 325–375 nm; Emission 420–800 nm). Photographs were taken by using a Nikon DS-Fi1C Peltier-cooled 3CCD camera.

Confocal laser scanning miscroscopy (CLSM)

Mites mounted as described above were also observed with a Nikon D-Eclipse C1 spectral confocal laser scanner microscope. Chlorophyll and fluorescent chlorophyll degradation products were observed with a He–Ne laser by using an excitation wavelength of 637 nm and a LP of 650 nm. A red false-color was used to indicate the chlorophyll fluorescence. Other autofluorescent molecules were observed with an argon neon laser by using an excitation wavelength of 488 nm and an emission wavelength of 590 ± 50 nm. A green false-color was used to indicate all other autofluorescent material. Images generated by the Nikon EZ-C1 3.80 software were elaborated by using the layers option of Adobe Photoshop®.

Guanine autofluorescence

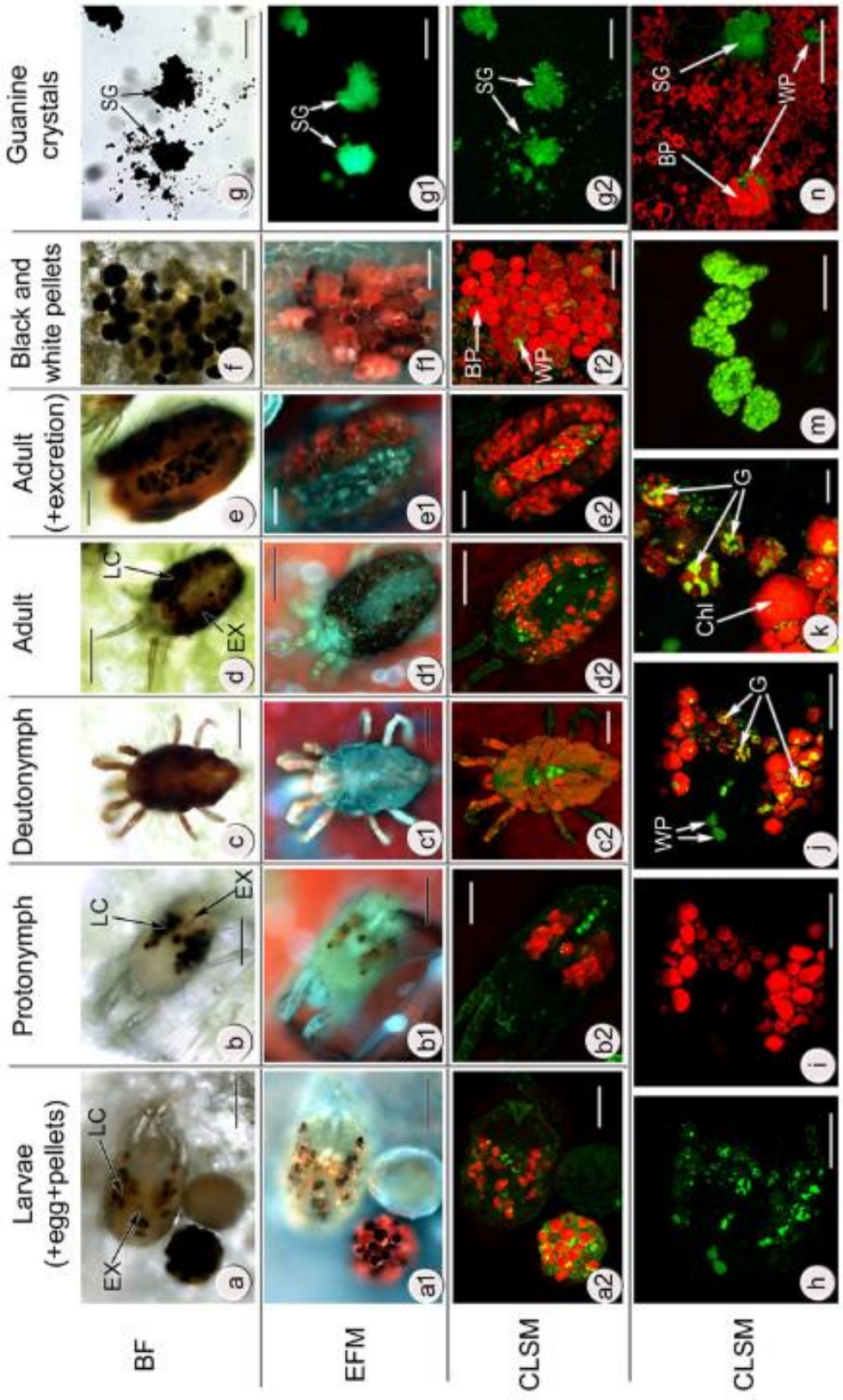
Crystals of synthetic guanine (Fluka) were placed on a microscopic slide and observed using BFM, EFM and CLSM, as described above. Guanine crystals were also placed on a leaf infested by spider mites and observed using CLSM.

Results

Bright field observation of eggs shows a light blue fluorescence when observed with the UV-2A filter in EFM; however, no chlorophyll fluorescence could be observed with either EFM or CLSM. Moreover, a clear autofluorescence was observed in the outer egg layers, when observed with EFM (Fig. 2).

When *T. urticae* larvae were observed with BFM, they show the characteristic two red pigment spots and several other aligned spots in the two lateral caeca and central ventriculus. When observed with EFM these spots turned to different fluorescent colors. In particular, two classes of fluorescent spots could be distinguished: red-brown spots, corresponding to chlorophyll and chlorophyll degradation products, and white bright spots, which were not related to chlorophyll. A CLSM observation of larvae confirms the distinction between chlorophyll-related spots (visible with a red false-color) and non-chlorophyll-related spots (indicated by a green false-color) (Fig. 2). Excreted pellets show the same pattern of EFM and CLSM as observed in larvae lateral caeca, central ventriculus and excretory organ.

Fig. 2



Bright field (BF), epifluorescence microscopy (EFM) and confocal laser scanning miscroscopy (CLSM) of different developmental stages of the two-spotted spider mite (*Tetranychus urticae*).

Crystals of synthetic guanine are shown under BF, EFM and CLSM. Larvae (*a*, *a1*, *a2*), protonymph (*b*, *b1*, *b2*), deutonymph (*c*, *c1*, *c2*), adult (*d*, *d1*, *d2*), adult and excretory organ filled with fecal pellets (*e*, *e1*, *e2*), *black* and *white* pellets (*f*, *f1*, *f2*), synthetic guanine crystals (*g*, *g1*, *g2*). In protonymph, *h* the green fluorescence is evenly distributed in the lateral caeca and the central ventriculus; *i* the red fluorescence derives from the chlorophyll and chlorophyll degradation products ingested and catabolized by the spider mite. *j* Merging of green and red fluorescence. *k* Closer view of the central ventriculus showing a higher proportion of green fluorescent particles with respect to chlorophyll fluorescent particles. *m* Particular of guanine pellets inside the excretory organ. *n* Synthetic guanine crystals placed near to white pellets on a leaf. *LC* lateral caecum, *EX* excretory organ, *BP* black pellets, *WP* white pellets, *G* guanine, *Chl* chlorophyll degradation product, *SG* synthetic guanine. *Metric bar* scales: *a*, *a1* = 50 μm; *a2* = 35 μm; *b*, *b1* = 80 μm; *b2* = 40 μm; *c*, *c1* = 135 μm; *c2* = 100 μm; *d*–*d2* = 125 μm; *e*–*e2* = 100 μm; *f*–*f2* = 70 μm; *g*–*g2* = 80 μm; *h*–*j* = 30 μm; *k* = 9 μm; *m* = 25 μm; *n* = 70 μm

Protonymphs show a clearer separation and organization of the two fluorescent spots. Both EFM and CLSM show the presence of bright (EFM)/green (CLSM) spots in the excretory organ and red/brown (EFM)/red (CLSM) spots in the lateral caeca and the central ventriculus. In CLSM images, red particles are surrounded by smaller, green particles.

Deutonymphs and adult spider mites are often characterized by an intense coloration, which is caused mainly by the presence of carotenoids (Van der Geest 1985). These subcuticular deposits make it more difficult to observe the distribution of pellets inside the mite body with BFM. EFM is also affected by the autofluorescence of cuticles and yields little information. The use of CLSM allows the specific characterization of autofluorescent material inside both deutonymphs and adult spider mites.

In deutonymphs, as observed in protonymphs, the lateral caeca and the central ventriculus are filled with chlorophyll fluorescent compounds surrounded by particles of a non-chlorophyll nature, whereas the excretory organ shows distinct spots characterized by a brilliant fluorescence (as evidenced by the green color) (Fig. 2 deutonymph and adult). In adults, the excretory organ is filled with both red and green fluorescent material (Fig. 2, adults + excretion). These materials represent the black and white pellets that can be observed on leaf surfaces of mite-infested Lima bean plants.

Black and white pellets are observed under BFM as clusters of varying colors, ranging from green to dark-brown. When observed with the EFM, most of these particles turn reddish, indicating the presence of chlorophyll degradation compounds, and bright white spots. When observed with CLSM, a clear association between red (chlorophyll degradation) and green (non-chlorophyll) pellets can be observed (Fig. 2, black and white pellets).

In order to better assess the nature of the two autofluorescent spots inside the spider mite caeca and central ventriculus we examined by CLSM a protonymph by dissecting the green and the red fluorescence. The green fluorescence (Fig. 2h) is evenly distributed in the lateral caeca and the central ventriculus whereas an average number of 4–5 spots of larger size accumulate in the excretory organ. The red fluorescence derives from the chlorophyll and chlorophyll degradation products ingested and catabolized by the spider mite. In the lateral caeca chlorophyll degradation takes place in big spherical particles, whereas in the central ventriculus these particles are of smaller size (Fig. 2i). The merging of green and red fluorescence (Fig. 2j) shows a close association between red and green particles in the lateral caeca, whereas a closer view of the central ventriculus shows a higher proportion of green fluorescent particles with respect to chlorophyll fluorescent particles (Fig. 2k). Crystals of synthetic guanine were also observed and their autofluorescence was found to be identical to that observed inside the spider mites and in mite white pellets (Fig. 2n).

A higher magnification of the green fluorescent pellets inside the excretory organ shows that these big pellets arise from clustering of smaller green particles (Fig. 2m).

Discussion

Spider mites are leaf parenchyma cell feeders and during feeding they suck the full content of a plant cell, largely composed of chloroplasts containing chlorophyll (Helle and Sabelis 1985). Our results show that most of this chlorophyll is catabolized and that a large portion of still fluorescent chlorophyll byproducts are excreted in the form of (black) pellets. Other waste products are also excreted along with chlorophyll degradation compounds. It is well known that two purines are the main nitrogenous waste products in arthropods, namely uric acid in insects and guanine in spiders, and the lack of certain enzymes in spiders was offered as a possible reason to explain this difference (Prosser 1950; Boudreaux 1963; Anderson 1966). Both guanine and uric acid are insoluble and may be stored within Malpighian tubules as crystals prior to excretion (Evans 1992; Alberti et al. 1999). In 1951 Gasser, on the basis of solubility tests, described the concretions in the excretory organ and part of the black fecal pellets as containing guanine (Gasser 1951). Later, by using Gomori's methylamine silver stain, McEnroe (1961) was able to localize the guanine in the concretions of the mite excretory organ. Anderson (1966) found that guanine was the only purine detected in the chromatographic analysis of excreta obtained from 34 species representing 17 families of spiders. Spectral analysis of individual extracts of excreta from 10 species has confirmed chromatographic evidence and indicated that guanine made up 34–76 % of the excreta by weight, with guanine containing most of the excreted nitrogen (Anderson 1966). More recently Entrekin and Oliver (1982) showed that guanine might act as a general attractant for *Dermanyssus gallinae* mites. However, guanine may exert also other roles; for instance it is stored in intestinal cells and is responsible for the white coloration of some spiders (Insausti and Casas 2008).

The green fluorescence evidenced in CLSM analyses and the white bright fluorescence shown in EFM images of *T. urticae* agreed with the presence of guanine, as demonstrated by the comparative analysis with synthetic guanine. Moreover, this fluorescence is visible in the excretory organ pellets and in the white pellets excreted by the mites as described earlier (McEnroe 1961).

By considering the above assumptions and based on several microscopic and morphological observations, we hypothesize that guanine might be formed in the lateral caeca during catabolic degradation of chlorophyll and then, as soon as the guanine particles merge, the guanine is moved toward the central ventriculus. Peristaltic movements allow the aggregation of several guanine particles into clusters that are concentrated in the excretory organ and eventually excreted (see Supplementary movie S1).

Further studies by using transmission electron microscopy and chemical analyses by liquid chromatography/mass spectrometry, are under way to better assess the formation of guanine inside the lateral caeca and its processing up to the excretory organ.

Supplementary material

Supplementary movie S1. This movie clip describes the pattern of guanine formation in spider mites. Guanine is formed in the lateral caeca (white circles) and is moved with peristaltic movements towards the central ventriculus. Here guanine accumulates and is merged in big guanine clusters that are stored in the excretory organ. Peristaltic movements inside the excretory organ aggregate more and more guanine pellets (yellow circles). Eventually 4-5 guanine clusters are formed and excreted. (MP4 5543 kb)

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