Article

Callose and reactive oxygen species expressed in sugar cane leaves by mechanical damage of spittlebugs

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Abstract

Spittlebugs is considered the main pest insect in sugar cane, this due to mechanical damage caused by the insertion of its stylet in the leaves. This research is focused on the response of the plant to the presence of said hemiptera, which involves the production of callose expressing β-glucans and high presence of reactive oxygen species (ERO) with the reaction of peroxides. These cause cell deaths in the damaged area and limit cell damage. In this document, the presence of callose and ERO is expressed in sugarcane seedlings subjected to spittlebugs infestations. The tissues of the damaged leaves were treated with fluorescent dyes such as aniline blue fluorochrome that adheres to the (1-3) β-glucans of the callose and the CM-H2DCFDA that binds to the intracellular glutathione of ERO by the presence of peroxides. This expression was measured in nanometers based on the intensity of fluorescence expressed by the presence of β-glucans (700 to 7 000 nm) and peroxides expressed between 1 000 to 6 400 nm. These results reveal the real damage caused by the insect's stylet on the leaf and its effects on the surface and inside the leaf.

Keywords: cell death, ERO, fluorescence, hemipterans, wavelength.

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Introduction

Sugarcane (*Saccaharum officinarum*) is a crop affected by several species of insect pests, among which spittlebug or 'mosca pinta' (*Aeneolamia albofasciata*) (Lallemand, 1939) (Hemiptera: Cercopidae) that feeds on leaf blades and causes a phytotoxemia called burning of leaves with the consequent decrease in photosynthetic capacity (Badilla 2002; Alatorre-Rosas and Hernández-Rosas, 2015).

The response to these insect attacks requires an integrated mechanism where the internal and external signals of the plant detect and cause an appropriate reaction in the plant (Stepanova *et al*., 2005). The insect in the nymph and adult state, is characterized by having a stylet that inserts into the leaf blade to feed, which causes cell damage with inter and intracellular fracture (mechanical damage) that induces biotic stress (Ammar *et al.*, 2013).

When the mechanical damage is caused by the insect, the plant is able to set in motion different defense mechanisms, whose objective is to stop, reduce or counteract the infection caused by it. There is structural barrier defense which consists of the deposition of lignin as a reinforcement of the tissues that suffer physical damage and the formation of papillae in the cells of the epidermis, which are composed of callose (β-1,3-glucan), that seek to prevent the penetration of pathogens (Skalamera *et al*., 1997).

The callose is expressed in response to mechanical damage or invasion of pathogens in the leaf of the plants. It is a polysaccharide of plant origin and an extremely important element to repair sites with mechanical wounds in plants. The callose is produced in the cell wall from enzymes called callose synthase and this can be degraded by the action of β-1,3-glucanases (Arcos and Narro, 2009).

The fastest plant defense reactions to pathogen attack is the so-called oxidative burst, which induces the production of ERO, mainly superoxide and hydrogen peroxide (H_2O_2) at the site of invasion (Apostol *et al.*, 1989). Therefore, when the hemiptera produce a mechanical wound by the insertion of their stylet during their feeding, they induce an increase in the levels of hydrogen peroxide inside the plant, which is associated with the oxygen burst caused by pathogens at the moment of penetrate by said cellular fracture (Bi and Felton, 1995; Bradley *et al*., 1992).

The ERO levels affect various processes including development, the hypersensitive response to pathogen attack and the response to stress (Mittler and Berkowitz, 2001; Tsukagoshi *et al*., 2010). Changes in ERO levels are recognized as specific plant signatures in response to biotic and abiotic stress (Swanson and Gilroy, 2010; Wolf *et al*., 2014). This response of the plant fortifies its cell wall and protects against mechanical damage of the insect, where the main function is to transduce the extracellular stimuli recognized by the cell receptors to a large number of target molecules (Ichimura *et al*., 2002).

This can serve as a switch that regulates the function of proteins and redox signaling pathways in response to stress by damage or invasion of microorganisms (Corcoran and Cotter, 2013). Therefore, the response of the plant is not sufficient and the mechanical damage continues due

to the constant suction of the sap by means of the stylet that can extend during long periods of part of the adult of the spittlebugs, this can cause malnutrition of the leaf of the plant and the invasion of opportunists when colonizing the affected area of the crop (Gutiérrez, 2001).

These defense processes have been studied in several models, some in citrus where they study the damage caused by the stylet of the nymphs of *Diaphorina citri*, which is the feeding site (Ammar *et al*., 2013). In the case of grasses, there are studies in corn where the accumulation of callose due to the presence of aluminum was observed due to the exposure of the roots to said element that induces the expression of damage (Arcos and Narro, 2009).

In the case of sugarcane, the defense response of the plant to the mechanical damage caused by the stiletto of the spittlebugs had not been observed. In this work, the response at the site of infection during the insect attack, the triggering of ERO and callose from the damage caused by adults of spittlebug on the leaf blade of the sugarcane leaf was evaluated at the tissue level. For this, techniques of transmitted light, fluorescence and confocal microscopy were used to visualize the affected areas and the reaction of ERO and callose expressed in fluorescent rounds.

Materials and methods

Insects, plant material, infection conditions

The species of spittlebugs *Aeneolamia albofasciata* and sugarcane seedlings variety CP 72-2086 was used. The insects were collected in the supply area of El Potrero Central Sugar Mill in the state of Veracruz. The plant material was obtained from vitroplants of VitroMotz, with an average age of 4 months, free of pests and diseases. The plant material was placed together with the adults of the spittlebugs, for 5 days, inside transparent plastic boxes with anti-aphid mesh windows, to prevent the introduction of non-objective insects and promote feeding of the insect, causing mechanical damage to the leaf blade.

Identification of physical damage

For the selection of samples, the areas affected by mechanical damage were observed with a dissecting microscope (Nikon® Brand) and the tissue was cut, delimiting the damaged area. These zones presented the characteristic symptomatology by affectation of adults of spittlebugs.

Histological sections

To know the physical damage caused by the stiletto of the spittlebugs on the cane leaf, the methodology described by Vázquez and Echeverria (2000) was followed. This describes the stages of preparation of plant biological material for the study of optical microscopy; fixation, dehydration, inclusion, cut and contrast.

Coal stain

The plant material exposed to insect feeding was checked and parts of the damaged tissue were selected to be treated with a Biosupplies® fluorochrome blue aniline solution that reacts with the (1-3) β-glucans of the callose. The dye solution was prepared in distilled water (1 mg in 10 ml) (w/v). The sections of tissue to be analyzed (fresh tissue) were incubated with the fluorochrome aniline solution (50 µL/section) for 45 min at 20 °C. The samples were washed with distilled water and examined by fluorescence microscopy with a TE300 inverted microscope (Nikon®, Japan) coupled to a xenon illumination source (DG-4, Sutter Instruments®, Novato, CA, USA) and a Uniblitz® shutter (Vincent Associates, Rochester, New York, USA). Image analysis and quantification of the fluorescence level were performed with the ImageJ software.

ERO stain

The plant material exposed to insect feeding was checked and selected by parts of the damaged tissue to be treated with a solution of CM-H2DCFDA Molecular Probes® that diffuses passively into the cells.

For the preparation of the dye, 25 µl of CM-H2DCFDA dye plus 25 µl of DMSO (Dimetthyl Sulfoxide, anhydrous, 99.9%) were added and 80 µl of Fahraeus medium (Fahraeus, 1957) was added, the dye solution was homogenized. In a Petri dish, 25 µl of prepared dye was placed in each tissue sample and left exposed for 30 min, washed with distilled water and placed in modified Petri dishes, to be observed by epifluorescence microscopy (inverted microscope TE300 (Nikon®, Japan) coupled to a xenon lighting source (DG-4, Sutter Instruments®, Novato, CA, USA). A Uniblitz® shutter (Vincent Associates, Rochester, New York, USA) and Confocal microscopy (Metalaser Zeiss LSM 510). Image analysis and quantification of the fluorescence level were performed with the ImageJ software.

Acquisition of images and their processing

Both fluorophores were visualized by means of fluorescence microscopy and images were obtained with a CCD camera (Sensys®, Scientific Roper, Tucson, AZ, USA) connected to a TE300 inverted microscope (Nikon®, Japan) coupled to a source of xenon illumination (DG-4, Sutter Instruments®, Novato, CA, USA). A Uniblitz® shutter (Vincent Associates, Rochester, New York, USA), which allowed the acquisition of transmitted light for each image. The filter with excitation wavelength of 390 nm that emits at 480 nm was used for both dyes, so that the emission spectra were taken using a emission filter at 440/10 nm. All these systems were operated by the MetaMorph®/MetaFluor® software (Universal Imaging-Molecular Devices, Downingtown, PA, USA).

Results and discussion

Evidence suggests that oxidative stress is a key detrimental factor in plants exposed to a variety of stress conditions and that they must resist oxidative stress by inducing antioxidant enzymes (Cuypers *et al*., 2002; Verma and Dubey, 2003). In this work, the effect of the damage caused by

the stiletto of the adult fly during the attack of the cells of the sugarcane leaf was addressed. In particular, the accumulation of callose and the formation of ERO in order to better understand the physiological relationship between the presence of the adult fly and its damage by the insertion of the stylet on the leaf blade. This insertion of the stylet fractures the cells of the tissue of the leaf through the vascular zone until it crosses the xylem and phloem, consequently, the oxidative stress is stimulated, and the antioxidant defense system is expressed (Kombrink and Schmelzer, 2001).

Histological sections

The damage caused by feeding the insect into the cell tissue was observed; through cross-sectional histological sections (Figure 1). It was observed how the stylet almost manages to cross the leaf reaching the vascular zone through a stoma damaging the xylem and phloem. This causes the crushing of the cells including the epidermis, buliform cells and perennial sheath surrounding the damaged area. According to Hagley and Blackman (1996) they observed that *Aeneolamia varia saccharina* introduces its stiletto using the stomata of sugarcane leaves, traverses the tissues until reaching the cells at the edge of the parenchyma leaf (underside). In the same way García *et al*. (2007) showed that the adult insects of *Mahanarva fimbriolata* introduce their stilettos preferably in the stomata of the leaf blade, these passes through the chloroplasts and from the support of the cells of the parenchyma to the metaxilema of the vascular bundles.

Like the works mentioned above, this work refers to adults who spittlebugs (*A. albofasciata*), introduce their stiletto looking for stomata by perching on the surface of the leaf blade, thus causing cell rupture at the chamber level cellular subestomatic of the parenchyma and the bundle sheaths, even to the limits of the lower epidermis (lower). With the above, considerable mechanical damage was observed, with laceration and cell rupture, causing cell death of the leaf blade of the cane leaf.

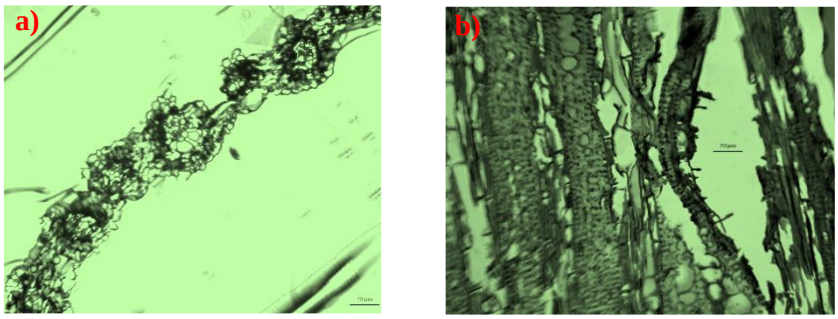

Figure 1. Cuts of the leaf blade of the cane leaf exposed to the stiletto of the spittlebugs; a) cross section of the cane leaf tissue (20X); b) frontal cut of the cane leaf tissue (20X).

After 5 days of exposure of the plants to the adults of the spittlebugs, new samples of tissue were taken and the same treatments were carried out for the microscopic observation of transmitted light, resulting in photographs showing the damage that is extending to the interior of the tissue cells of the leaf (Figure 2).

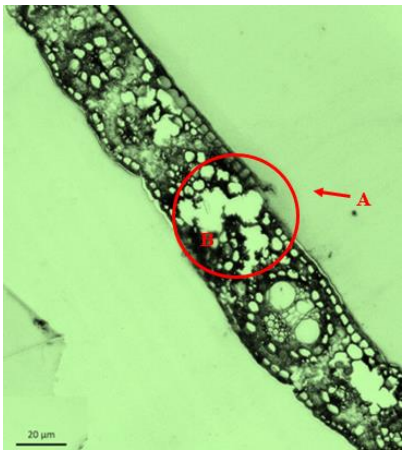

Figure 2. Cross section of the leaf blade of the cane leaf exposed to the spittlebug stylet. A) damage to the tissue caused by the insertion of the stylet; B) damage of the cells inside the tissue (20X).

Fluorescent treatments

Stain for callose

Photographs were obtained through the epifluorescence microscope that allowed us to visualize the presence of callose in the damaged tissues by insertion of the stylet of the adults of the spittlebugs. Figure 3 shows the presence of callose, the fluorescence values in the damaged cells vary with respect to the presence of callose, this is because the fluorescent dye shows the expression of β-glucans. In the zone of mechanical damage, it can be observed that there is a higher concentration of callose and as it moves away from the area of damage there is less presence of it. This happens because as we move away from the site of damage, the cells are less damaged until they reach healthy cells. These data allowed to demonstrate that there is a high accumulation of callose in the sites around the damage caused by the spittlebugs.

Callose is an amorphous high molecular weight β-1,3-glucan that is deposited in the form of papillae by the development of fungi and other pathogens in leaf tissue (Aist, 1976). It acts as a physical barrier against the fungal colonization of the intercellular space. Two research groups have independently found that mutations in the *Arabidopsis thaliana* callus synthase gene paradoxically confer resistance to fungi (Jacobs *et al*., 2003; Nishimura *et al*., 2003). Also, Nishimura *et al*. (2003) mention that the rapid deposition of callose during the first stages of damage, can inhibit the defense response that the plant may have and that potentially can be harmful for it.

If a pathogen has developed mechanisms to overcome the defense barrier against callose, the conditions become favorable for subsequent colonization. On the other hand, Jacobs *et al*. (2003) confirmed that the deposition of callus plugs at sites of fungal penetration is an early response widely recognized by host plants against microbial attack; therefore, callose plugs are involved in preventing the entry of the fungus or the confinement of its development. In this investigation, the expression of callose caused as a defense of the plant against mechanical damage was examined by fluorescence.

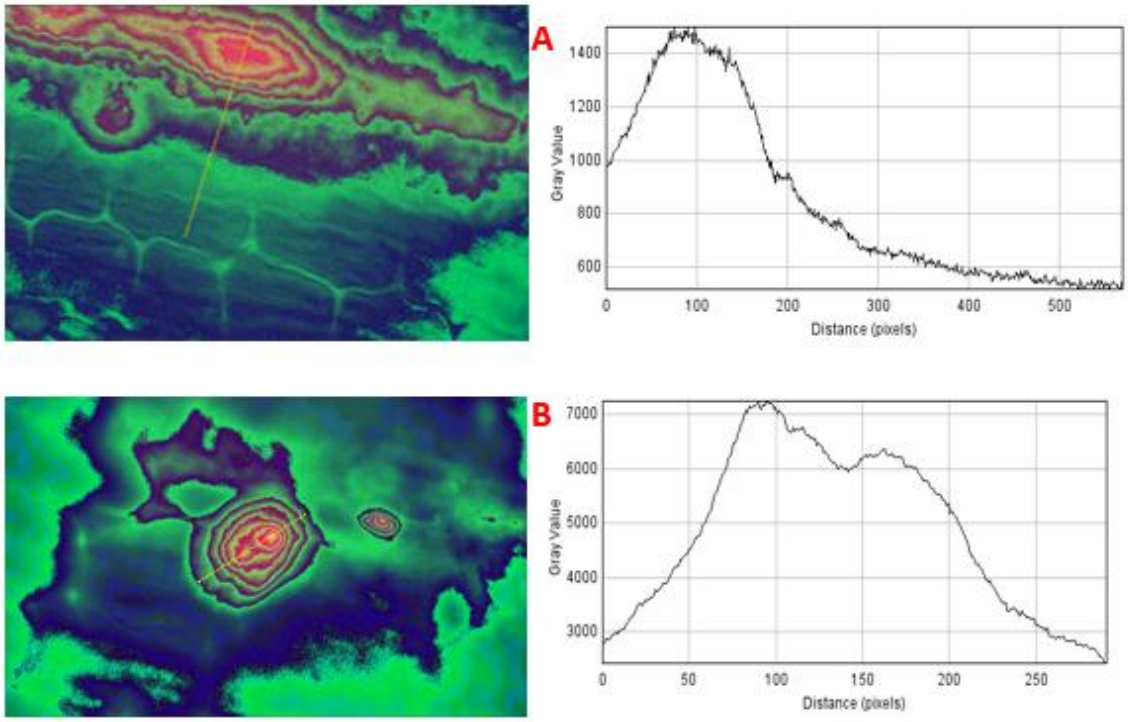

Figure 3. Expression of callose in the leaf blade of the cane leaf by fluorescence; A) expression of β-1,3-glucan according to the extent of the mechanical damage, extends from 1 000 nm to 500 nm from the damage zone; B) expression of β-1,3-glucan according to the extent of mechanical damage, extends from 3 000 - 7 000 - 2 500 nm from the central zone of damage.

Staining for ROS

For the determination of the ERO (ROS), the cells were treated with the ERO-sensitive fluorescent probe and visualized by means of epifluorescence microscopy. The data obtained allowed to demonstrate that there is a high presence of ERO in the tissue that is being damaged by insertion of the stylet of the adult of the spittlebugs. In Figure 4 are shown, as it is similar to ERO in damaged cells before mechanical damage. The specific dye for ERO is only expressed in the presence of peroxides present in the area with mechanical damage caused by the insect, so in the images the zone of invasion of the insect in the tissue is observed and how the cells surrounding the damage begin to be invaded with the formation of these free radicals, which are most likely involved in the cell death of the damage site cells.

Reactive oxygen species (ERO) in plants play a predominant role in the response to all types of pathogens. However, a growing number of reports describe the possible role of reactive oxygen species (especially hydrogen peroxide) as a cellular messenger in signal transduction pathways, in particular in signaling the response of plants to pathogens (Bolwell, 1999; Bowler and Fluhr, 2000; Grant and Loake, 2000). A large number of biochemical pathways are involved in the response of the plant to the attack of pathogens, but the response of the plant begins with the recognition of the elicitor or signal of the pathogen at the time of contact of the hyphal structures on the tissue and in particular, in the contact for inter and intracellular damage. This recognition is given by the presence of a receptor for the elicitor in the plant cell.

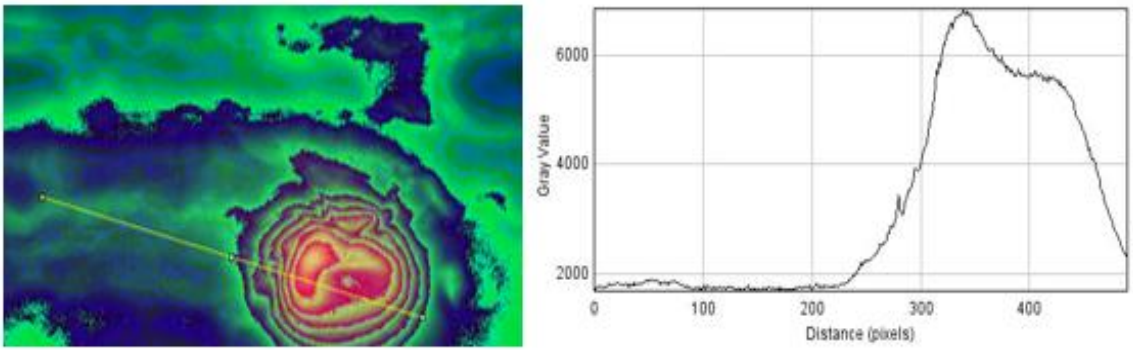

Figure 4. Expression of ERO according to the extent of mechanical damage, extends from 2 000-6 500-2 500 nm from the damage zone.

The activation of this receptor activates various local processes such as the release of reactive oxygen species (the oxidative burst) to the extra-cellular environment. This often results in response to hypersensitivity (HR), a type of programmed cell death located at the site where the pathogen-initiated development to invade the plant (Bowler and Fuhr, 2000). A delayed response is produced by a long-range signaling mechanism that later causes acquired systemic resistance (SAR), in which the exposure to the pathogen is located and gives rise to the resistance of the whole plant to the Non-related pathogens, this type of resistance can last several months (Bowler and Fuhr, 2000). Although it has been observed in the leaves of sugarcane, that the incidence of adult fly paints above 20 adults on average per trap in 24 h, causes damage that is expressed in spots or lines along the length of the leaf blade of reddish color to ocher color.

After this damage, the cells become dry due to cell death and in the distance the coffee or reddish crop is observed. It becomes 'burned' and consequently this can cause the inhibition or even the blockade of sucrose synthesis. Therefore, it has not been possible to measure the real damage with quantitative methods but qualitative rather than the damage severity references established by Campbell and Madden (1990). In contrast, fluorescence is a numerical reference in nanometers where the severity of damage can be associated by the expression of peroxides and clumps of callose, by the insertion of the stylet and time of absorption of the sap, as well as the incidence of adults on the leaf blade greatly affects such damage.

Conclusion

The fluorescent staining methods established for the recognition of ERO and callose in the tissues of the sugarcane leaves, allow to demonstrate the effect by cellular damage by the spittlebugs for its exploration and with this, it was possible to identify the mechanically damaged cellular structures. In addition, it was possible to obtain a numerical data of the damage that fluctuated for the tissues observed in a range of 1 000 to 6 400 nm and of 600 to 7 000 nm and not qualitative of cell damage, by the expression of peroxides and β-glucans, respectively, that delimit the real damage to the interior of the tissue and not in a superficial way of the tissue of the leaf blade.

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