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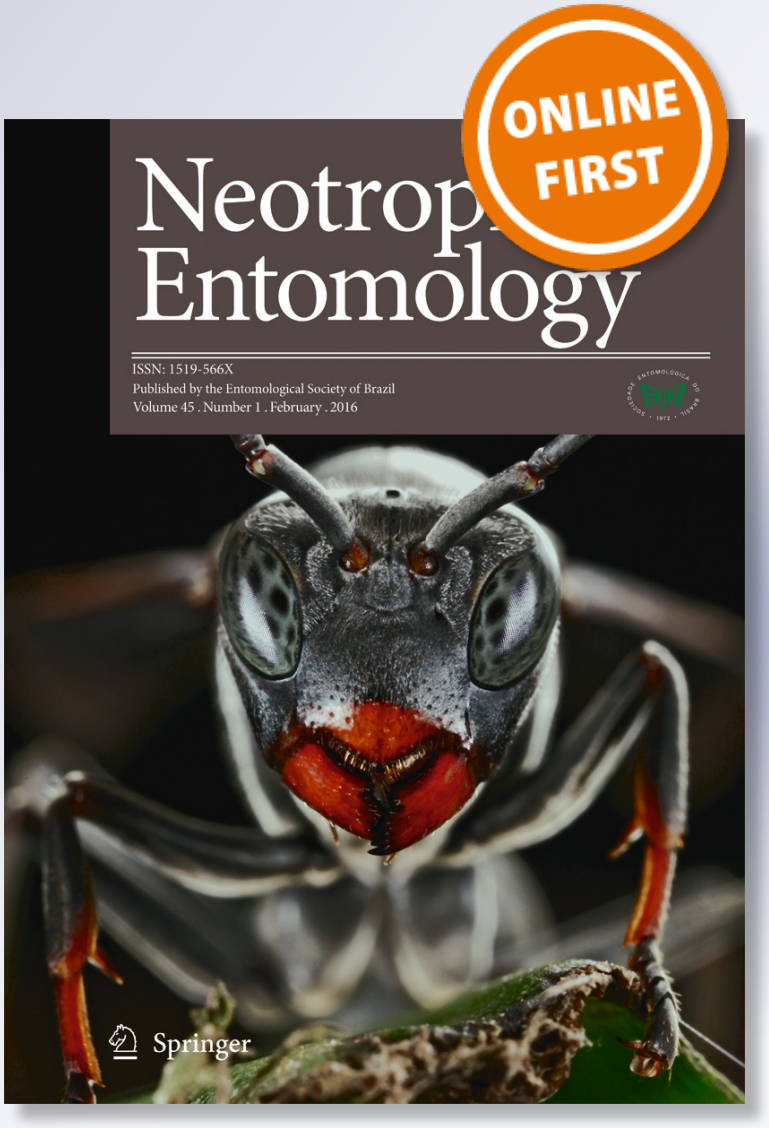

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PEST MANAGEMENT

Characterization of Feeding Injuries Caused by *Ceresa nigripectus* Remes Lenicov (Hemiptera: Membracidae) on Alfalfa Stems

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Abstract

Piercing-sucking insects cause mechanical and physiological injury to plants. *Ceresa nigripectus* Remes Lenicov is a pest of alfalfa in subtropical regions of South America and a carrier of the ArAWB phytoplasma. The aim of this study was to determine the feeding habits of this treehopper and to describe the effects of the feeding injuries on stem vascular tissues in alfalfa. Adults and nymphs of *C. nigripectus* inserted their stylets repeatedly girdling the stem. One week after feeding, alfalfa stems exhibited numerous feeding canals with salivary deposits, most of which reached the phloem. Two weeks after feeding, cortex and phloem cells next to the salivary sheath collapsed, mature tracheal elements became sparse and appeared with an increased cross-section area, and phenolic compounds increased in cells and cell walls compared to undamaged plants. Three weeks after feeding, an annular callus, formed by abnormal cell division and hypertrophy of preexisting cortex and vascular cambium cells, appeared immediately above the stem girdle. Parenchyma cells from the outer layers of the callus differentiated to form secondary anomalous amphicribal bundles in the wound. The aerial parts above the stem girdle eventually withered and died.

Introduction

Alfalfa is an essential crop in temperate regions in the USA, Canada, Italy, France, China, and southern Russia in the Northern Hemisphere, and in Argentina, Chile, South Africa, Australia, and New Zealand in the Southern Hemisphere. It represents the best source of hay for domestic use, silage, and it is used for conservation and recovery of productive soils (Yuegao & Cash 2009).

The feeding behavior of Hemiptera-Auchenorrhyncha has been extensively studied because of two reasons: the direct damages caused on their hosts and transmission of pathogens to plants (Backus 1985, Miles 1989, Nault 1997, Andersen *et al* 2002, Godoy *et al* 2006). Piercing-sucking feeding causes mechanical damage, facilitating the entry of opportunistic microorganisms, and physiological effects by the deposited saliva (Ecale & Backus 1995a, Shackel *et al* 2005, Savatin *et al* 2014).

Membracidae (Hemiptera: Auchenorrhyncha: Cicadomorpha) is a large family that includes about 3500 species worldwide (Deitz *et al* 2011). Remes Lenicov (2014) listed 140 species from 55 genera of occurrence in Argentina, where the subfamily Smiliinae is the most diverse with 63 species. Membracidae are typically phloem sap feeders (Jordan 1952, Tjallingii 1994, Gajalakshmi & Jayakumar 2011). Similar to other Hemiptera, these insects produce at least two types of salivary secretions. The first one is a watery type of saliva, a vehicle primarily for digestive enzymes secreted during ingestion, accomplishing the digestion of sap and liquefying plant cellular contents and cell walls (Frati *et al* 2006). The second type of saliva is lipoproteinaceous and is secreted, while the stylets are penetrating the plant tissues.

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It solidifies rapidly on ejection forming sheaths to prevent breakage of the stylets (Backus 1985, Backus *et al* 1988).

Treehoppers of the genus *Ceresa* are widely distributed in South America with representatives in Venezuela, Colombia, Ecuador, Peru, Bolivia, Paraguay, Brazil, Uruguay, and Argentina (Kopp & Yonke 1979). In Argentina, only three species have been associated with alfalfa crops: *Ceresa brunnicornis* (Germ.), *Ceresa extensa* (Fairm.) (Remes Lenicov 1973), and recently *Ceresa nigripectus* Remes Lenicov, a carrier of Argentine alfalfa witches' broom (*ArAWB*) phytoplasma (Meneguzzi 2009). Among these species, *C. nigripectus* has a wide distribution in northern and central Argentina, Bolivia, and Brazil (Andrade 2004), and it has been collected from alfalfa, potato, wheat, and surrounding vegetation (Remes Lenicov 1973, Meneguzzi 2009). Field studies have demonstrated that this treehopper is one of the most frequent and abundant species associated with alfalfa in Argentina (Meneguzzi 2009). Recently, Perez Grosso *et al* (2014) studied the life cycle of this treehopper, establishing that *C. nigripectus* is a solitary species with long living adult stage that completes its life cycle entirely on alfalfa, producing visible damage to the plants. Newly emerged nymphs move to the basal portion of the plant and third instar nymphs begin to climb the stem. At this moment, *C. nigripectus* begins its injury to the plants, and later, instars and adults become more active and more voracious feeders (Perez Grosso *et al* 2014).

Considering the abundance of *C. nigripectus* in alfalfa crops in subtropical and semiarid areas of South America, and the evident injuries caused to this crop, the aim of this work was to characterize the feeding habits of this treehopper and describe the effects of feeding injuries caused on stems and vascular tissues of alfalfa.

Material and Methods

Insect rearing and plant growing

A colony of *C. nigripectus* was established with adults collected during the summer of 2012 from alfalfa fields in Manfredi, Córdoba, Argentina (31°25′S, 64°11′W; at 390-m altitude).

Treehopper colonies were maintained in 50×25×50 cm high aluminum cages containing six healthy alfalfa plants var. Monarca, cultured in 6.3-L pots. Pots were closed with a nylon mesh cloth for aeration and kept in a climatic chamber under the following conditions: 27±2°C, 60–80% RH and a 16:8 h L/D artificial light photoperiod. The cages had two openings for insect manipulation, one at the front and another at the back of the cage.

Alfalfa plants were grown from seeds in a greenhouse, and plants used for each assay were at least 30-day old. New potted alfalfa plants were introduced in the breeding chambers when necessary.

Insect exposure to host plants and observations of feeding habits

Groups of five individuals in fourth and fifth instars or five adults of *C. nigripectus* (Fig 1a) were brought in contact with alfalfa plants for 72 h. Treehoppers were placed in 10×10×2-cm cages. Each cage wall was made of 10×10-cm transparent plastic plate surrounded by adhesive weather-strip borders (1 cm) allowing holding the plant stem between plates without damaging them. The two plates were placed to embrace two alfalfa stems containing the third internode and leaves (a rubber band was used to attach the two walls forming the cage). A wooden stick was used as a guide to hold the cages and thus prevent the breakage of the stems. Samples of the third internode of each plant exposed to insect feeding were collected 7, 14, 21, and 30 days after caging the insects on the plants, and the observations were run using three replications. Stems of plants kept without insect infestation were used as control.

During the assay, daily observations were made to determine the feeding habits exhibited by each of the different life stages.

Light microscopy

Samples consisting of 1 cm long of each internode containing the entire feeding region plus at least 2 mm from each side of health were fixed in FAA (formalin, acetic acid, 50% ethanol, 5:5:90 *v/v/v*) during 1 week after processing at room temperature. Further, 25-µm sections were obtained with a rotation microtome, which were subsequently stained with astra blue-safranin and mounted in 50% glycerol (Zarlavsky 2014).

In order to detect salivary secretion and other chemical compounds, sections were stained with Sudan IV for identification of lipids, acid fuchsine for proteins, and ferric chloride for phenolic compounds (O'Brien & McCully 1981, Backus *et al* 1988, Zarlavsky 2014). These sections were visualized with a Zeiss Axiolab optic microscope equipped with a Zeiss Axiocam ERc 5s digital camera.

Results and Discussion

Insect exposure to host plants and observations of feeding habits

Nymphs and adults of *C. nigripectus* feed at one side of the stem for 5–10 min and then move around the stem to a new position at the same level. The insect begins to feed again inserting its stylets repeatedly, thus making a complete

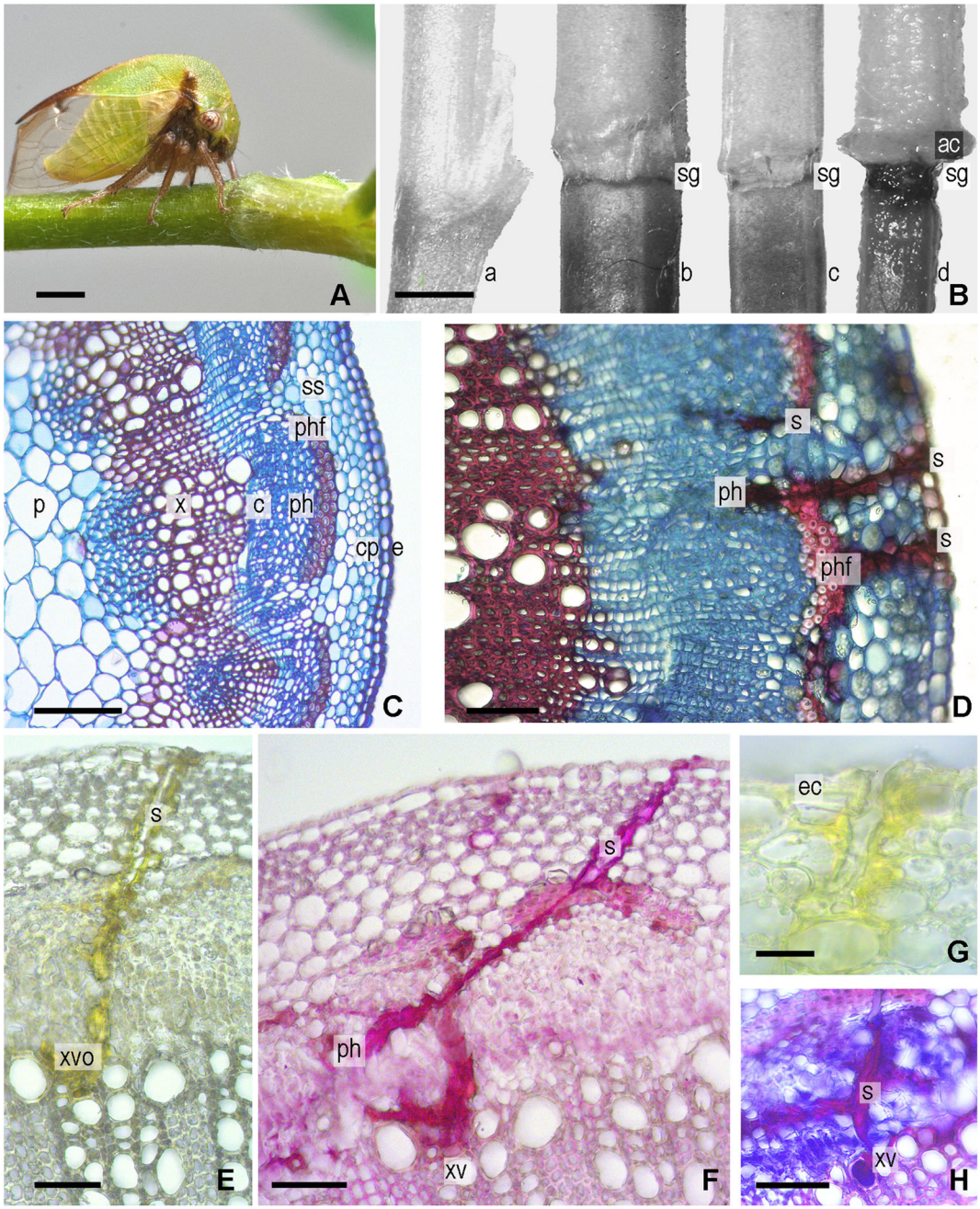

Fig 1 Internodes of alfalfa stems control (uninfested) and exposed to feeding injury by *Ceresa nigripectus*. **a** Adult of *Ceresa nigripectus. Scale bar* at 1 mm. **b** Third internode of alfalfa plants exposed to feeding injury by *C. nigripectus*. Alfalfa stem 1 week after feeding, normal aspect (*a*), 2 weeks after feeding with evident stem girdling (*sg*) (*b*), **c** 3 weeks after feeding with evident stem girdling (*sg*) (*c*), **d** 4 weeks after feeding with evident stem girdling (*sg*) and annular callus (*ac*) (*d*). *Scale bar* at 1 mm. **c** Cross-sectional view of uninfested alfalfa stem. Epidermis (*e*), cortical parenchyma (*cp*), starch sheath (*ss*), phloem fibers (*phf*), phloem (*ph*), cambium (*c*), xylem (*x*), and pith (*p*). *Scale bar* at 100 µm. **d–h** Cross-sectional view of alfalfa stem 1 week after feeding of *Ceresa nigripectus*. **d** Astra blue-safranin staining: salivary sheath (*s*) reaching the phloem (*ph*) or in phloem fibers (*phf*). *Scale bar* at 50 µm. **e** Without staining: salivary sheath (*s*) reaching xylem vessels; some xylem vessels are partially occluded with sheath material (*xvo*). *Scale bar* at 50 µm. **f** Cross-sectional view of alfalfa stem 1 week after feeding of *Ceresa nigripectus*; staining with acid fuchsin shows salivary sheath (*s*) with branched pattern, reaching phloem (*ph*) and xylem vessels (*xv*). *Scale bar* at 50 µm. **g** Cross-sectional view of alfalfa stem 1 week after feeding of *Ceresa nigripectus*: penetration through epidermal cell (*ec*) without staining. *Scale bar* at 10 µm. **h** Cross-sectional view of alfalfa stem 1 week after feeding of *Ceresa nigripectus*: Astra blue-safranin stained salivary sheath (*s*) with branched pattern reaching xylem vessels (*xv*). *Scale bar* at 50 µm.

girdling on the stem (Fig 1b (b)). After 1 week or sometimes two, a series of feeding scars encircle the stem. This structure is called stem girdling (Fig 1b (c)) and has previously been described for *Spissistilus festinus* (Say) (Membracidae), the

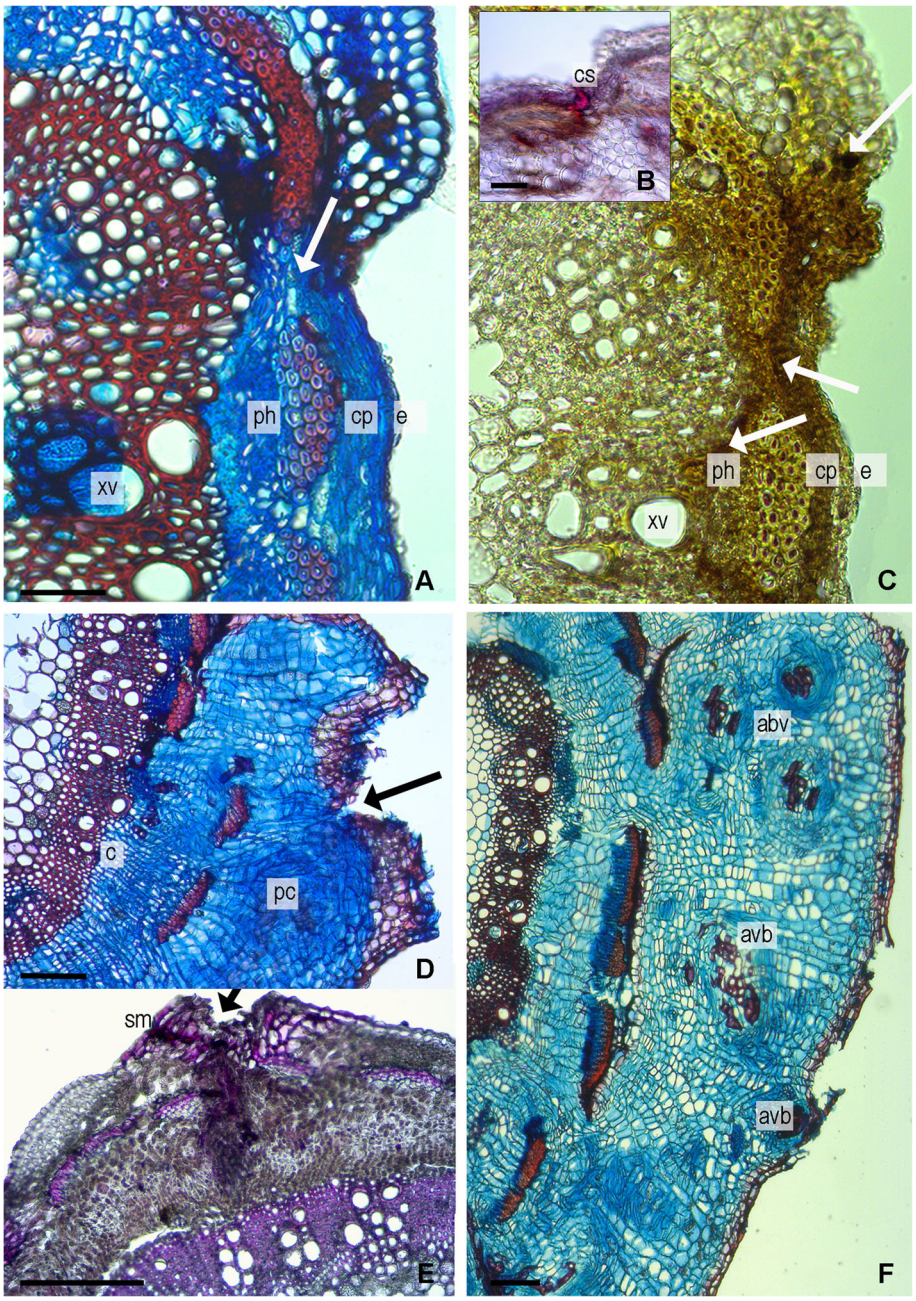

Fig 2 Internodes of alfalfa stems 2, 3, and 4 weeks after feeding of *Ceresa nigripectus*. **a–c** Cross-sectional view of alfalfa stem 2 weeks after feeding of *Ceresa nigripectus*. Girdle zone. **a** Astra blue-safranin staining: *arrows* show the collapse of cortical parenchyma (*cp*), epidermal (*e*), and phloem (*ph*) cells. Tracheal element (*xv*) with increased cross-section. *Scale bar* at 50 µm. **b** Collapsed salivary sheath (*cs*) stained with acid fuchsin. *Scale bar* at 50 µm. **c** Positive reaction of phenolic compounds (*arrow*) with ferric chloride. Collapsed cortical parenchyma (*cp*), epidermal (*e*), and phloem (*ph*) cells. Tracheal element (*xv*) with increased cross section is shown. *Scale bar* at 50 µm. **d, e** Cross-sectional view of alfalfa stem 3 weeks after feeding: section of annular callus. **d** Abnormal cell division and hypertrophy of parenchymatous cortex (*pc*) and vascular cambium (*c*) cells. Stylet pathways (*arrow*) are evidenced by expansion of adjacent cells. *Scale bar* at 100 µm. **e** Acid fuchsin staining: salivary sheath material (*sm*) is hardly observed in the callus tissues next to previous stylet pathways (*arrow*). *Scale bar* at 100 µm. **f** Cross-sectional view of alfalfa stem 4 weeks after feeding. Parenchyma cells from the outer layers of the callus differentiated to form secondary anomalous amphicribal vascular bundles (*avb*) in the wound. *Scale bar* at 100 µm.

three-cornered alfalfa hopper (Kopp & Yonke 1973). After 3 to 4 weeks, an annular callus is formed above the girdle (Fig 1d); the aerial parts above the stem girdle eventually wither and, when two or more girdles are present, more than 50% of the attacked stems dies (personal observation). This feeding behavior not only reduces the plant nutrients but also physically weakens the stem, showing signals of chlorosis and leaf burn, called “hopper-burn” by Ecale & Backus (1995b).

The behavior of *C. nigripectus* is very similar to that previously reported for *S. festinus*, a well-known alfalfa and soybean pest in North America (Levin Mitchel & Newsom 1984). Jordan (1952) stated that the typical damage caused by the three-cornered alfalfa hopper is complete girdling of stems. These girdles were produced by the feeding punctures of the insect that are apparently made side by side in a ring around the stem of the plant. Girdles on cowpea and soybean stems usually result in a slightly swollen area as observed during the fourth week on an alfalfa stem exposed to *C. nigripectus*. The author also mentioned that nymphs were responsible for most of the stem girdling, whereas our studies showed production of annular calluses both by fourth and fifth instar nymphs and adults. Similar feeding habits and injuries have been described for *Ceresa brunnicornis* and *Ceresa*

fasciatithorax Remes Lenicov (Membracidae: Ceresini) affecting soybean in Brazil (Hoffman-Campo *et al* 2012).

Plant morpho-anatomical responses to treehopper feeding

Internodes and tissues from distal regions of the uninfected plants did not present evidence of anatomical changes (Fig 1c), in comparison to those observation made on internodes subjected to treehopper feeding.

One week after treehopper exposure, alfalfa stems presented a normal aspect (Fig 1b (a)), but sections obtained from these samples presented numerous feeding canals (Fig 1c–h). Stylet pathways through cortical cells were mainly intracellular causing cellular damage (Fig 1d, e) differently from an occasional plant feeder species (Torres *et al* 2010). Salivary deposits remained within the plant tissue even after removing the stylets (Fig 1e, g). The salivary sheaths were single (Fig 1d, e, g), rarely branched (Fig 1f, h), straight, or slightly curved, and in general, they finished in the phloem (Fig 1d–h). However, some of the sheaths did not reach the phloem, but instead ended in the cortex, or even near the xylem vessels (Fig 1e, f). Sheaths provided a coherent lining of the stylet tracks from the plant surface to the feeding site (Pollard 1973, Miles 1989).

A positive reaction of the salivary sheath material with safranin, acid fuchsine, and Sudan IV indicated that it was mainly made of lipoproteinaceous material (Fig 1f, h). The strong staining reactions observed are consistent with numerous reports that state that salivary sheaths of plant feeding Hemiptera are largely composed of gelled lipoproteins (Pollard 1973, Miles 1989). In the vascular bundle, shrinkage of phloem cells suggested that they had been punctured by the stylets (Fig 1h). Little or no injury was observed in other tissues.

Two weeks after the feeding event, the stem girdle was readily apparent (Fig 1b (b)). Cortex and phloem cells next to the salivary sheath had collapsed, while mature tracheal elements had become sparse, with an increased cross-section area (Fig 2a–c). Cells and cell walls appeared amber-yellow without staining, showing a positive reaction for phenolic compounds (Fig 2c). It is well known that phenolic compounds act as phytoalexins (Nicholson & Hammerschmidt 1992). Sheath saliva deposits were rare (Fig 2b).

During the third and fourth week after insect feeding, an annular callus was formed immediately above the stem girdle through abnormal cell division and hypertrophy of preexisting parenchymatous cortex and vascular cambium cells (Fig 2d). In the cambial region, some of the dedifferentiating cells served as callus initials, producing abundant parenchymatic cells. In some cases, stylet pathways were more evident through expansion of adjacent cells and dissolution of necrotic cell walls (Fig 2d). Little or no salivary sheath material was found in the callus tissues; the sheaths observed were probably the result of new feeding attempts by the treehopper (Fig 2e). In the late phase of wound response (fourth week), parenchyma cells from the outer layers of the callus differentiated to form secondary anomalous amphicribal bundles in the wound (Fig 2f). Similar responses have previously been reported for other plant species like *Jazminum fluminens* (Oleaceae), *Serjania polyphylla* (Sapindaceae), and *Bougainvillea* sp. (Nyctaginaceae), after girdling injuries (Fisher & Ewers 1989).

Both salivation and stylet movements induced a plant response; cortex and phloem cells collapsed, synthesis of phenolic compounds occurred, and a callus was formed through posterior hyperplasia and cellular division. These alterations are similar to those produced by other phloem feeders such as *Empoasca fabae* Harris (Cicadellidae) (Kabric & Backus 1990) and *Spissistilus festinus* (Say) (Membracidae) (Levin Mitchel & Newsom 1984) on alfalfa, *Nilaparvata lugens* (Stål) (Delphacidae) in rice (Spiller 1990), and *Delphacodes kuscheli* (Fennah) (Delphacidae) in corn (Brentassi & Maldonado 2002).

According to Jordan (1952), salivary juice injected by the three-cornered alfalfa hopper into the phloem results in the breakdown and necrosis of phloem tissues, preventing carbohydrate transport from the green parts of the plant to the roots. This effect might not be important in annual plants, but in perennials such as alfalfa, starvation of the roots seriously affects the ability of the plants to overwinter. *S. festinus* nymphs on alfalfa plants; Moellenbeck & Quinsenberry (1991) observed that nymphs were found feeding near the girdle site because it disrupts translocation, creating a nutrient sink from which the insects feed.

Our results are the first full examination about the feeding behavior and injuries caused by *C. nigripectus* on alfalfa stems. The feeding activities of the treehopper on alfalfa exhibited particular signals (stem girdling) caused by phloem collapse, which induced plant anatomical alterations and physiological responses. Further research is currently being carried out in order to determine yield losses caused by this treehopper and its ability to transmit the *ArAWB* phytoplasma.

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