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Survival of *Bemisia tabaci* and activity of plant defense-related enzymes in genotypes of *Capsicum annuum* L.

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The whitefly *Bemisia tabaci* (Gennadius, 1889) is a major plant pest of horticultural crops from the families Solanaceae, Fabaceae and Cucurbitaceae in Neotropical areas. The exploration of host plant resistance and their biochemical mechanisms offers an excellent alternative to better understand factors affecting the interaction between phytophagous insect and host plant. We evaluated the survival of *B. tabaci* in landrace genotypes of *Capsicum annuum* L., and the activity of plant defense-related enzymes (chitinase, polyphenoloxidase, and peroxidase). The landrace genotypes Amaxito, Tabaquero, and Simojovel showed resistance to *B. tabaci*, as we observed more than 50% nymphal mortality, while in the commercial susceptible genotype Jalapeño mortality of *B. tabaci* nymphs was not higher than 20%. The activities of plant defense-related enzymes were significantly different among pepper genotypes ($P < 0.05$). Basal activities of chitinase, polyphenoloxidase and peroxidase were significantly lower or equal in landrace genotypes than that of the commercial genotype Jalapeño. The activity of plant enzymes was differential among pepper genotypes ($P < 0.05$). For example, the activity of chitinase enzyme generally was higher in non-infested plants with *B. tabaci* than those infested. Instead polyphenoloxidase ('Amaxito' and 'Simojovel') and peroxidase enzymes activities ('Tabaquero') increased in infested plants ($P < 0.05$). We conclude that basal activities of plant defense-related enzymes could be act through other mechanism plant induction, since plant defense-related enzymes showed a different induction response to *B. tabaci*. We underlined the role of polyphenoloxidase as plant defense in the pepper genotype Simojovel related to *B. tabaci*.

Key words: Host plant resistance, pepper germplasm, plant defense-related enzymes, whitefly.

INTRODUCTION

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a phytophagous insect that has caused big losses in vegetable, ornamental, and horticultural crops worldwide. This species is particularly harmful in tropical and subtropical areas, where currently *B. tabaci* is known as a complex composed of cryptic species (Wang et al., 2013; Marubayashi et al., 2013; Boykin, 2014; Barbosa et al., 2014). *Bemisia tabaci* damages plants by direct feeding and transmission of *Begomoviruses* and *Criniviruses* that cause serious problems due to viral epidemics (Morales, 2007; Marubayashi et al., 2013). Worldwide there is enormous interest in the study of this phytophagous insect due to the negative impact on productivity of various crops; in this regard, the first international meeting (International Whitefly Symposium) has taken place in Crete, Greece, in 2013, where a platform for discussing aspects of molecular biology, physiology, ecology, behavior, and management has been offered to the international scientific community in an attempt to synergize knowledge and experience to strategically undertake actions in a wide arrange of topics related to *B. tabaci* (Boykin, 2014). In the last decade, *B. tabaci* populations have developed high levels of resistance to synthetic insecticides (Roditakis et al., 2009). To cope with potential damage caused by *B. tabaci* in horticultural agroecosystems, the exploration of host plant resistance has been considered a promising alternative in sustainable agriculture (Sharma and Ortiz, 2002).

Studies on plant resistance to *B. tabaci* have shown that host resistance is mediated by the external/physical characteristics of the leaf surface, such as hairiness, glandular trichomes, and leaf thickness (Jindal et al., 2008). For example, the cuticle thickness of leaf may prevent the insect stylet from reaching the phloem (Firdaus et al., 2011). While the presence of high density of glandular

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trichomes may cause high mortality of whiteflies, due to the secretion of chemical compounds such as acylsugars, which act as a glued trap for the whiteflies and attractant for natural enemies (Rodríguez et al., 2012). Resistance to *B. tabaci* and its relation to plant morphological traits has been well documented in tomato, cotton, and cassava (Bellotti and Arias, 2001; Boiça et al., 2007; Oriani and Vendramim, 2010).

The internal characteristics of leaves, such as chemical composition of leaf sap, nutritional value of leaves, and activity of plant defense-related enzymes has been less studied. Specifically, plant defense-related enzymes are presumably induced defenses that protect plants against the attack of phytophagous and herbivore insects (Fürstenberg-Hägg et al., 2013). The increased activity of plant defense-related enzymes can induce synthesis of toxic metabolite in leaves, which in turn can affect negatively the survivorship and physiology of herbivore insects (Kahl et al., 2000). In particular, phenylalanine ammonia lyase, polyphenoloxidase, chitinases, β-1,3-glucanases, peroxidases, lipoxygenases, and protease inhibitors have been documented as plant defense-related enzymes involved in response to phytophagous insects (Felton et al., 1994; Zhang et al., 2008). Other enzymes that may also affect feeding of herbivore insects are those that alter the digestive process, such as the arginases and the threonine deaminases that may act in insect gut by using the amino acids required for insect development (Falco et al., 2001; Mello and Silva, 2002; Chen et al., 2005). Particularly, studies on the interaction between *B. tabaci* and its host plant have been carried out in tomato and cassava, where resistant genotypes respond increasing the activity of various plant defense-related enzymes, such as β-1,3-glucanases, peroxidases, and chitinases when plants are exposed to *B. tabaci* (McKenzie et al., 2002; Binu and Palaniswami, 2006).

The high genetic diversity of peppers in southern Mexico offers an excellent alternative to study various aspects of germplasm potentiation, such as those related to host plant resistance to pests (Castañón-Nájera et al., 2008; Aguilar et al., 2009). To contribute to the knowledge on the biochemical mechanisms of host plant resistance to *B. tabaci*, the present work was carried out to evaluate the activity of plant defense-related enzymes and survivorship of *B. tabaci* in landrace genotypes of *C. annuum*.

MATERIALS AND METHODS

Pepper germplasm, seedling, and plant growth conditions

Twenty *C. annuum* landrace genotypes were initially pre-screened for resistance to *B. tabaci*. In this work, we used only one wild ('Amaxito' var. *glabriusculum*) and two semicultivated genotypes ('Simojovel' var. *glabriusculum*, and 'Tabaquero' var. *annuum*) that caused high mortality of *B. tabaci* nymphs. As susceptible reference, the commercial genotype Jalapeño (var. *annuum*) was used. Seeds of all genotypes, except 'Jalapeño', were obtained from dried fruits collected in Tabasco ('Amaxito', 'Tabaquero') and Chiapas ('Simojovel'), México. The susceptible genotype 'Jalapeño' was obtained from a commercial store HomeDepot, at Mérida, México.

Seeds of *C. annuum* genotypes were germinated in polystyrene trays containing peat moss (Acadian Peat Moss, Canada) as substrate. Thirty days after sowing, seedlings were transplanted into 1 L plastic pots containing (v/v) Luvisol soil (50%), peat moss (30%), and gravel (20%). Seedlings were maintained with 80% moisture in the substrate and fertilized twice a week in the irrigation water with 1 g L^{-1} of Poly-Feed Drip (Hayfa, México) that contained NPK at 19% each. Plants were used for bioassays when they reached 45-d-old.

Bemisia tabaci colony and nymphal survival bioassays

Adult whiteflies were obtained from a stock rearing colony maintained on 'Habanero' chili pepper in entomological metal cages (1.2 × 1.2 × 1.0 m) covered with anti-aphid mesh at 25-35 ºC, 55-75% RH and natural light at the Instituto Tecnológico de Conkal, Yucatán, México. The stock colony corresponds to *B. tabaci*, which has been previously used for other related works (Ballina et al., 2013).

Small confinement cages were made from transparent plastic tubes (1.5 cm diameter and 2.5 cm high). The distal end of the tube was covered with a plastic mesh to allow air circulation, while the end attached to the underside of the leaf was lined with thin piece of foam to avoid leaf damage. Ten female adults of *B. tabaci* were released into confinement cages and 24 h later adults were removed from leaves of *C. annuum* (Muñiz and Nombela, 2001). The infested plants were placed in 1.2 × 1.2 × 1.0 m cages and survival rates were measured daily and reported as percent mortality of nymphs.

Evaluation of activity of plant defense-related enzymes

Activity of plant defense-related enzymes in *C. annuum* leaves was evaluated 1-d prior (basal) and 7 d after *B. tabaci* infestation. Forty-five day old plants of each genotype were used for the bioassay. For infestation, confinement cages were set in two upper fully expanded leaves per plant as previously described. Twenty female adults of *B. tabaci* were confined in each leaf cage and kept them permanently. Young and old leaves of the plants were randomly taken for enzyme bioassay. Activities of the plant defense-related enzymes chitinase (CHI), polyphenoloxidase (PPO), and peroxidase (POD) were evaluated from leaf samples as described below.

Chitinase. Excised leaf tissue was washed under running tap water, dried and immediately ground in liquid nitrogen. One gram of ground sample was homogenized in 0.5

mL 0.1 M sodium acetate buffer pH 5. The homogenate was centrifuged at 14 000 rpm for 5 min at 4 °C, the supernatant was collected and kept at -20 °C until use. For determination of CHI activity, commercial deacetyl glycol chitin (DGC) was used as substrate. To start enzyme reaction, 5 μL of enzymatic extract was added to 300 μL 0.5% DGC in 0.1 M sodium acetate buffer (pH 5.0) pre-incubated previously at 37 °C for 10 min. The reaction mixture was incubated at 37 °C in water bath for 30 min. The reaction was stopped by adding 1 M NaOH (250 μL) and immediately centrifuged at 10 000 rpm for 10 min. The supernatant was collected, mixed with 1 mL of Schales' reagent (0.5 M sodium carbonate + 1.5 mM potassium ferricyanide) and heated in boiling water for 15 min. After removal from boiling water, the mixture was cooled at 4 °C for 5 min (Jung et al., 2005). The absorbance was immediately measured at 420 nm using a spectrophotometer Genesys 10 UV-VIS. The activity was calculated from a standard curve obtained from known concentrations of N-acetyl-b-D-glucosamine [$r^2 = 0.998$, y = absorbance-0.9435/0.002]. One unit of CHI activity (U) was defined as the amount of enzyme that released 1 mol of N-acetylglucosamine (GlcNAc) per min.

Polyphenoloxidase. Leaf tissue was prepared as described for chitinase; 100 mg of ground sampled was homogenized in 1 mL buffer [0.1 M Tris-HCl, pH 7.0, 0.1 M KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg mL^{-1} leupeptin, Triton X-100 (1% v/v), polyvinylpyrrolidone (PVP) (3% v/v) and 1 mM Na_2EDTA]. The homogenate was centrifuged 10 000 rpm at 4 °C for 20 min, and the supernatant was used for PPOs activity assay. PPOs activity was determined spectrophotometrically at 400 nm at room temperature as described by Saiedian et al. (2007) using 2 mM pyrogallol (ε_{400} = 4.226 M^{-1} cm^{-1}) as substrate. The reaction mixture (2 mL) contained phosphate buffer (1.92 mL, 0.1 M), enzymatic extract (30 μL) and pyrogallol (50 μL). A unit of specific activity (U) was expressed as micromole of quinone formed per minute per milligram of protein.

Peroxidase. Leaf tissue was prepared as described for chitinase. Enzyme extraction was carried out at 4 °C. One gram of ground sample was homogenized in 0.5 mL 0.1 M sodium acetate buffer (pH 6.8) containing 3 g PVP. The mixture was filtered through filter paper Whatman nr 4 and then centrifuged at 10 000 rpm for 30 min. The supernatant was collected for enzyme activity assay. POD activity was measured as described by Furumo and Furutani (2008), the assay mixture contained 1.8 mL sodium phosphate buffer, 60 μL H_2O_2, 100 μL 4% guaiacol as substrate, and 40 μL enzyme extract (diluted 1:10). The assay mixture was incubated for 3 min at room temperature and POD activity was monitored for 2 min at 30 s intervals. POD activity was monitored at 470 nm. Activity was expressed as units of enzyme activity (U). Units were calculated using a molar absorptivity of 26 600 M^{-1} cm^{-1} for tetraguaiacol (Maehly and Chance, 1954).

Protein assay

The protein extract was made as in POD assay. The protein content was determined as described by Peterson (1977) with some modifications. Briefly, the mixture reaction contained protein extract (15 μL), distilled water (985 μL), and 0.15% sodium deoxycholate (100 μL). This mixture was incubated at room temperature for 10 min and then 72% Trichloroacetic acid (100 μL) was added. The mixture was agitated gently and incubated on ice for 15 min and centrifuged at 10 000 rpm for 30 min at 4 °C. The supernatant was discharged and the pellet was suspended in 1 mL distilled water and added with 1 mL of a mixture of copper-tartrate-carbonate solution ($CuSO_4$ $5H_2O$ 0.1%, $KNaC_4H_4O_2$ 0.2%, Na_2OH_3 10%)-sodium dodecyl sulfate 10% and 0.8 M NaOH (1:1:1.1). The resulting mixture was gently agitated, added to 500 μL Folin Ciocalteu phenol reagent (Sigma Aldrich, Madison, Wisconsin, USA) diluted 5x, and incubated at room temperature for 30 min. The absorbance was measured at 750 nm in a spectrophotometer (Genesys 10 UV). Total protein was calculated by interpolating the absorbances of samples in a standard curve obtained from solutions containing bovine serum albumin fraction V (BSA) as protein standard.

Data analyses

Prior to analysis, data in percent were transformed to y = arcsin(sqrt(x/100)). For data on basal activity of plant defense-related enzymes, data analyses, ANOVA, and Tukey mean comparison were performed. For comparison of enzyme activity between *B. tabaci*-infested plants and their own control (non-infested plants), t-test was performed. All statistical analyses were carried out using the software Statistical Analysis System version 8.1 for Windows (SAS Institute, Cary, North Carolina, USA).

RESULTS AND DISCUSSION

Survival of *B. tabaci* on pepper genotypes

Mortality of *B. tabaci* nymphs was significantly ($P < 0.05$) higher in the wild and semi-cultivated pepper genotypes compared to that of the commercial genotype ('Jalapeño'). Mortality of nymphs was 63%, 59%, and 51% in 'Tabaquero', 'Amaxito', and 'Simojovel', respectively; while in 'Jalapeño' mortality of nymphs was only 19% (Figure 1). The *Capsicum* genus of the Solanaceae family is a common host for *B. tabaci* (Morales and Cermeli, 2001; Khan et al., 2011). Thus, *B. tabaci* is able to develop successfully on commercial genotypes of *C. annuum*. However there is a small percentage of nymphs that does not survive even though when the host plant is susceptible. In this sense, mortalities of nymphs in the range of 20%

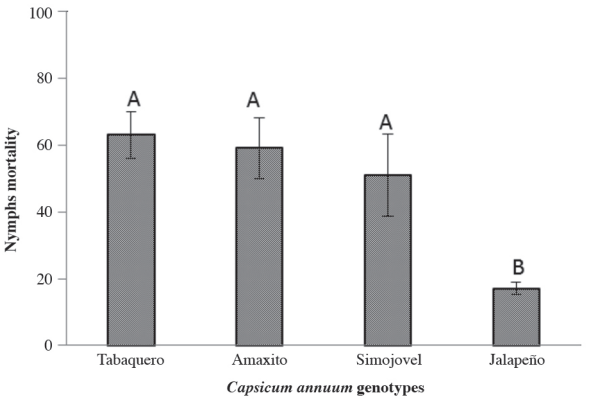

Figure 1. Mortality of *Bemisia tabaci* nymphs on different genotypes of *Capsicum annuum*. Each bar represents the mean ± standard error. Distinct letters indicate significant differences according to Tukey's test (P ≤ 0.05).

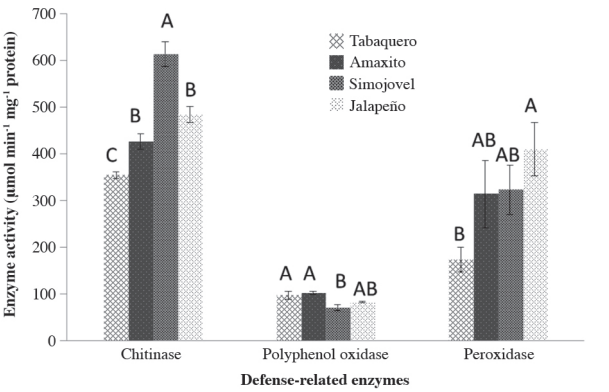

Figure 2. Basal activity of three plant defense-related enzymes in leaves of *Capsicum annuum* genotypes. Each bar represents the mean ± standard error. Distinct letters indicate significant differences according to Tukey's test (P ≤ 0.05).

or lower have been reported for commercial genotypes of *C. annuum* (González and Gallardo, 1999). On the other hand, Baldin and Beneduzzi (2010) considered a host plant as resistant when about 60% of *B. tabaci* nymphs did not survived. This suggests that, in the present work, particularly 'Tabaquero' and 'Amaxito' are considered resistant to *B. tabaci*.

Successful exploration of host resistance to phytophagous insects has been particularly carried out in land race genotypes. In this regard, various studies have shown that wild and semi-wild relatives of cropped species are resistant to phytophagous insects by antibiosis, antixenosis, or both (Korgan and Ortman, 1978). In the present work we may suggest that 'Tabaquero', 'Amaxito', and 'Simojovel' are resistant to *B. tabaci* by antibiosis. To the best of our knowledge no detailed study on resistant to *B. tabaci* has been carried out on pepper. In the close relative species tomato, resistance to *B. tabaci* has been studied at some degree. Moreover, the exploration of mechanisms of resistance has been also studied in this case (Baldin et al., 2005; Oriani et al., 2011).

Basal activity of plant defense-related enzymes in leaves of pepper genotypes

Before infestation with *B. tabaci* the basal activity of plant defense-related enzymes in the genotypes was variable (Figure 2). Chitinase activity (CHI) was significantly different ($P < 0.05$) among pepper genotypes, 'Simojovel' showed the highest CHI activity (613.1 U) while 'Tabaquero' showed the lowest (357.3 U). There was no difference in activity of CHI (426.0 and 483.6 U, respectively) in genotypes 'Amaxito' and 'Jalapeño'. Basal activity of PPO was also evaluated in all genotypes (Figure 2). The PPO activity in 'Tabaquero' (96.5 U) and 'Amaxito' (101.7 U) was significantly higher than that of 'Simojovel' (70.5 U). There was significant difference ($P < 0.05$) in POD activity among commercial and wild-relative genotypes of *C. annuum*. 'Jalapeño' showed higher POD activity (410.2 U) when compared with 'Tabaquero' (172.8 U). POD activity of 'Jalapeño' was not significantly different than those of 'Amaxito' (313.7 U) and 'Simojovel' (322.5 U).

The enzyme CHI is an enzyme system that catalyzes hydrolysis of chitin, the major component of insect exoskeletons. Chitin is also an important component of the peritrophic matrix lining the gut epithelium (Merzendorfer and Zimoch, 2003); and it is considered a protein that can act to decrease plant susceptibility to pest (Sharma et al., 2011). This is important to note that the high basal activity of CHI, as observed in 'Simojovel', was not closely related to the high levels of resistance to *B. tabaci*.

The enzyme PPO is an oxidoreductase that catalyzes distinct reactions involving phenolic compounds and molecular oxygen. In plants, PPO enhances the plant defense system (Saiedian et al., 2007). In this study, we found no general trend of high basal activity of PPO on *B. tabaci*-resistant pepper genotypes.

The enzyme POD is an oxidoreductase that is involved in many functions in plant physiology, such as defense mechanisms and lignin biosynthesis. In our study, higher activity of POD on *B. tabaci*-resistant genotypes was expected; however, data showed otherwise, the highest activity of POD was observed in the susceptible genotype 'Jalapeño'.

Induced activity of plant defense-related enzymes by *B. tabaci* infestation

To evaluate possible variations in induction of the plant defense-related enzymes in *B. tabaci*-resistant and in *B. tabaci*-susceptible genotypes, the activity of evaluated enzymes was compared between *B. tabaci*-infested and non-infested plants (control plants) for each genotype.

Activity of CHI in 'Jalapeño' and 'Amaxito' showed significantly ($P < 0.05$) higher activity in non-infested plants (control) than in *B. tabaci*-infested plants, while in

'Tabaquero' and 'Simojovel', no difference in CHI activity was found between plants under both conditions (Figure 3). Previous studies have shown that CHI may be induced when resistant plants are infested by phytophagous insects, creating a protective barrier against the mechanic damage caused by the introduction of the stylet (Falco et al., 2001). CHI might also act on chitin, which suggests that chitinases might interfere with insect digestion and feeding (Jouanin et al., 1998). In this study we found no evidence in *B. tabaci*-resistance genotypes of an increase in CHI activity when plants were exposed to *B. tabaci*. Thus, CHI might not be involved in the response to *B. tabaci* in the evaluated *C. annuum* genotypes.

In two of *B. tabaci*-resistant genotypes ('Amaxito' and 'Simojovel') PPO activities increased significantly ($P < 0.05$) when plants were exposed to *B. tabaci* (Figure 4); PPO activity in 'Amaxito' and 'Simojovel' was 133.8 and 128.9 U in *B. tabaci*-infested plants, while in non-infested plants was 98.3 U in 'Amaxito' and 95.0 U in 'Simojovel'. In 'Tabaquero' no increase in PPO activity was observed

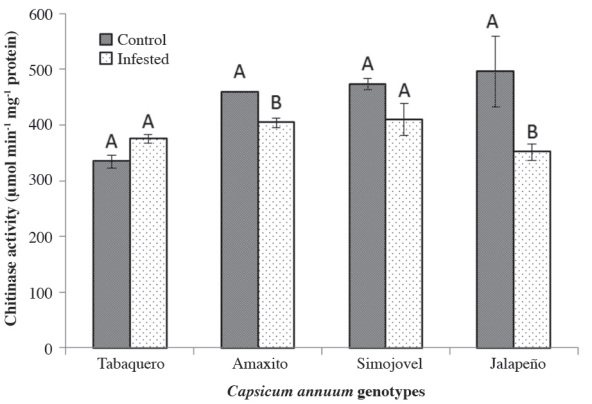

Figure 3. Chitinase activity in leaves of *Bemisia tabaci*-infested and non-infested plants of *Capsicum annuum* genotypes. Each bar represents the mean ± standard error. Distinct letters within the same genotype indicate significant differences according to Tukey's test (P ≤ 0.05).

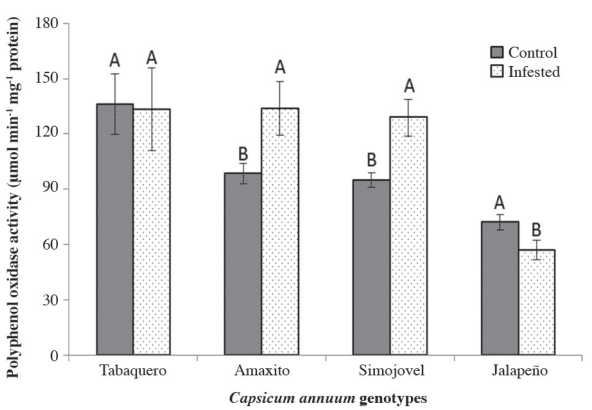

Figure 4. Polyphenoloxidase activity in leaves of *Bemisia tabaci*-infested and non-infested plants of *Capsicum annuum* genotypes. Each bar represents the mean ± standard error. Distinct letters within the same genotype indicate significant differences according to Tukey's test (P ≤ 0.05).

when plants were exposed to *B. tabaci*. In contrast, PPO activity in the commercial genotype 'Jalapeño' decreased significantly when plants were exposed to *B. tabaci*.

The PPO activity is induced as defense mechanism during insect attack in wounds or adjacent tissues (Antony and Palaniswami, 2006). In other plant species, like cucumber, Avdiushko et al. (1993) found an increase in PPO activity in leaves damaged by insect feed. In our study, PPO activity significantly increased after *B. tabaci* infestation only in 'Amaxito' and 'Simojovel', while no difference was observed in 'Tabaquero'. This suggests that the increase in PPO activity is not a general defensive mechanism in *C. annuum* against *B. tabaci*, which contrast with other studies, such that of Meena et al. (2008) that documented an increase in PPO activity in *C. annuum* leaves and augmentation in the oxidation rate of phenolics substrates, which participate in defense reaction of host plant in general (Bhonwong et al., 2009).

When genotypes were exposed to *B. tabaci*, only in the resistant genotype 'Tabaquero' the POD activity increased significantly from 285.1 U to 394.9 U (Figure 5). In contrast, in 'Amaxito' the POD activity decreased significantly (from 1215.4 U to 357.9 U) when exposed to *B. tabaci*. In the rest of the genotypes there were no significant differences in POD activity when plants were infested with *B. tabaci*.

Various works have documented that the induction of POD in plants play an important role in defense against insect attacks (Heng et al., 2004). In the present work, however, no clear trend of an increase in POD activity in *B. tabaci*-resistant genotypes as response to *B. tabaci* infestation was observed. We only observed induction of POD activity in the *B. tabaci*-resistant genotype 'Tabaquero'.

In summary, in an attempt to find possible relations among three plant defense-related enzymes in pepper genotypes and their resistance to *B. tabaci*, we evaluated

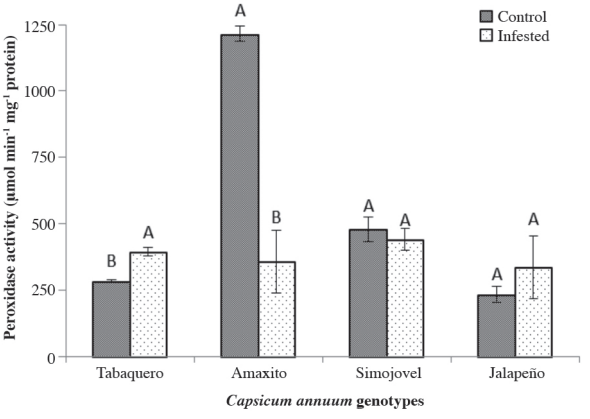

Figure 5. Peroxidase activity in leaves of *Bemisia tabaci*-infested and non-infested plants of *Capsicum annuum* genotypes. Each bar represents the mean ± standard error. Distinct letters within the same genotype indicate significant differences according to Tukey's test (P ≤ 0.05).

mortality of nymphs and the activity of CHI, PPO, and POD in leaves of pepper genotypes. We observed significant differences in basal activity of enzymes and differential response of enzyme activity when genotypes were exposed to *B. tabaci*. The increase in activity of plant defense-related enzymes in the genotypes exposed to *B. tabaci* depended on the genotype and enzyme. Enhanced activity of plant defense-related enzymes may contribute to bioprotection of *C. annuum* against *B. tabaci*, this mechanism may only occur in some resistant genotypes. We do not rule out the importance of three enzymes CHI, PPO, and POD in plant defense of *C. annuum* against *B. tabaci*; however, other possible mechanisms like activation of other related enzyme systems (lipooxygenase or arginase) or the influence of morphological traits of leaves may also be part of the defense system of pepper against *B. tabaci*.

CONCLUSIONS

The survival of *Bemisia tabaci* varied significantly in the evaluated genotypes of *Capsicum annuum*. The results clearly demonstrated that wild and semicultivated genotypes of *C. annuum* caused higher nymph mortalities than commercial genotype 'Jalapeño'. The activity of plant defense-related enzymes in wild and semi-cultivated pepper genotypes showed no consistent trend when correlated with the resistance to *B. tabaci*. Thus, there was no clear evidence for an increase in activity of chitinase as response to *B. tabaci* feeding in resistant genotypes. Only polyphenoloxidase activities in the genotypes 'Amaxito' and 'Simojovel', and peroxidase activity in 'Tabaquero' were found to increase when plants were exposed to *B. tabaci*.

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