A metabolomic approach to thrips resistance in tomato

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Chapter 6

Effect of chlorogenic acid on thrips performance in artificial diet bioassays

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Abstract

With the separation of CQAs, FQAs and diCQAs from green coffee beans it has been proven that the manipulation of electrostriction in an aqueous solution by changing the concentration of one or more salts can constitute an effective method of performing a reversed-phase-like gradient elution in CCS. A major contribution of these salting-out gradients lies on the fast single-column elution of compounds largely differing on their distribution constants without experiencing significant column bleeding. Although this achievement is also possible with a conventional normal-phase gradient in CCC, very few biphasic systems meet the conditions to actually do it. In addition, notwithstanding the fact that this proposed method may suffer from some impracticability for routine application when compared to other powerful ones like the dual (Agnely and Thiebaut, 1997; Delannay et al., 2006), the elution-extrusion (Berthod, 2007; 2003; Lu et al., 2008) and the cocurrent (Berthod and Hassoun, 2006) modes, these ionic gradients represent a proof and a reminder of how salting effects could open new possibilities in poor resolution cases limited by solvent system stability.

Introduction

Phenolics are a very diverse and prominent group of secondary metabolites spread throughout the entire plant kingdom. They play various key roles in plants, behaving as antioxidants, building blocks of secondary cell walls, allelochemicals, UV-protectants, antimicrobials, signal molecules and insect defenses (Treutter, 2006). Many plant scientists have unquestionably accepted the defensive role of phenolics, to such extent that these compounds are by default a variable in almost every single study on fitness costs of plant defense and represent a pillar in the resource availability theory (Coley et al., 1985). However, still after several decades of intense research no consensus about their efficacy in providing protection against attackers, in particular against herbivores, has been reached. Part of this controversy originates in the fact that phenolics have multiple modes of action, which can have positive and negative effects on pathogens and insects. The following have been proposed as some of the main modes of action of phenolics: radical scavenging, pro-oxidation, covalent and hydrogen bonding to proteins and free amino acids, enzyme inhibition and crosslinking, chelation of enzyme metal cofactors and wound sealing (Fig. 1). Due to these chemical properties phenolics have been shown to act as feeding deterrents, phagostimulants, digestion inhibitors, digestion stimulants, toxins, toxicity reducers, signal inhibitors and signal transducers (Appel, 1993; Crozier et al., 2009). In addition, their activity is also dose-dependent, structure-sensitive and organism-specific.

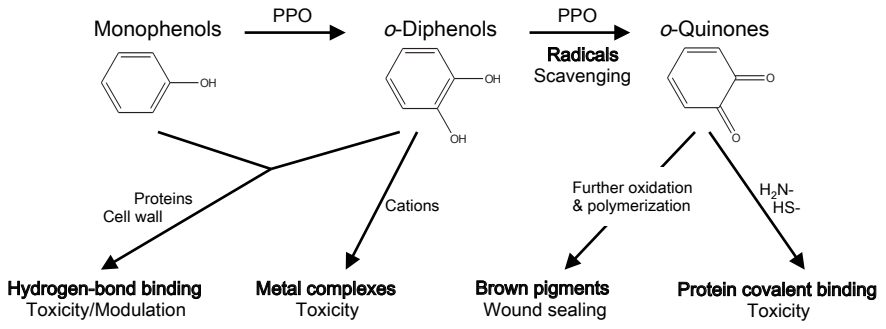

Figure 1.Main modes of action of simple phenolics. PPO: oxidase.

Many studies claiming adverse effects of phenolics on herbivores rely on correlations between insect performance and any phenolic content as the single factor responsible for the observed variation in the dependent variable (Bi et al., 1997a; Felton, 1989; Ikonen et al., 2001; Isman and Duffey, 1982a; Johnson and Felton, 2001). These studies overlook in this way covariables that could have a stronger and hence overriding influence on the performance of the insect. Moreover, correlation analysis does not prove causation, requiring additional evidence to invoke a cause-effect relationship. Results from other studies in which the number of factors has been minimized, e.g. artificial diet bioassays, were inconsistent or contradictory. Most research on the role of phenolics in host-plant resistance has been conducted on chewing insects. Along the course of more than a decade researchers collected partial evidence implicating phenolic compounds in host-plant resistance against caterpillars. A lot of these studies focused on chlorogenic acid (5-caffeoylquinic acid, CQA) as the most common and abundant representative compound among simple phenolics in plants. Isman and Duffey (1982a) observed that semi-purified extracts of phenolics from tomato leaves inhibited growth of the fruitworm, *Heliothis zea*, but could not detect a correlation between foliar phenolic content and larval growth among several tomato cultivars. Authors underlined the potential danger of comparing biological activities from artificial diet bioassays and *in situ* experiments. Rutin and chlorogenic acid inhibited early larval growth of *H. zea* but had no adverse effect on third and fifth-instar larvae at concentrations of up to 1% in artificial diets (Isman and Duffey, 1982b). Felton et al. (1989) showed that polyphenol oxidase (PPO) was required for CQA to exhibit anti-insect activity against *Spodoptera exigua*. Performance of larval *H. zea* barely correlated with PPO activity (R=0.55) but did not correlate with the content of either rutin or chlorogenic acid. Polyphenol oxidase catalyzes the conversion of *o*-diphenols into *o*-quinones (Fig. 1). These quinones are very reactive electrophiles and oxidizing agents that can covalently bond more than one amino or thio group, causing protein cross-linkage and indigestibility (Kalyanaraman et al., 1987). In a later study using proteins from different sources Felton et al. (1992) not only confirmed the enzymatic requisite but also observed that CQA had a beneficial impact on the performance of *S. exigua* in the absence of PPO. Although CQA induced oxidative stress in midgut tissue of the generalist *Helicoverpa zea* no deleterious effect was observed on the performance of larvae acutely exposed to this phenolic. Chronic exposure to CQA did cause a significant reduction of *H. zea* growth but had no effect on survival (Summers and Felton, 1994). Differences in activity were observed among different phenolic compounds but no explanations were offered. Using transgenic tobacco with significant differential expression of both CQA and PPO Bi et al. (1997a) showed that phenolics are not involved in host-plant resistance to either the generalist *Heliothis virescens* or the specialist *Manduca sexta*. In a choice setup *M. sexta* did not discriminate between tobacco leaves with a differential CQA content of more than ten times (Eichenseer et al., 1998). Likewise, neither phenolic content, including CQA, nor PPO activity correlated with resistance to the coffee leaf miner, *Leucoptera coffeella*, across 15 genotypes of coffee (Melo et al., 2006). In a study involving 13 Finnish willow species and four willow-feeding beetles only one of the coleopteran species was apparently affected by CQA (Ikonen et al., 2001). Measuring the total Trolox equivalent antioxidant capacity in hemolymph of *H. virescens* larvae fed foliage with high phenolic content Johnson and Felton (2001) showed that phenolics may actually serve as antioxidants for herbivorous insects. Although these studies constitute only a small fraction of the vast literature on phenolics the sample shows how in the course of about a decade the image of these metabolites could diametrically change from "anti-herbivore" to "insect-beneficial", proving how complex and debatable still is the manifold role of phenolic compounds in plant-insect interactions.

In contrast to chewing insects very few experiments have been performed on sucking herbivores. In artificial diet bioassays chlorogenic acid significantly deterred the apple aphid, *Aphis pomi*, only in combination with ascorbic acid (Miles and Oertli, 1993), while catechin significantly reduced feeding of the rose aphid, *Macrosiphum rosae* (Peng and Miles, 1991), and the spotted alfalfa aphid, *Theoriaphis trifolii maculata* (Miles and Oertli, 1993). However, phenolics were phagostimulant to aphids at very low concentrations (Peng and Miles, 1991). Aphids polymerize phenolic compounds with salivary polyphenol oxidase to strengthen the stylet and facilitate feeding (Miles and Peng, 1989; Peng and Miles, 1988a; Peng and Miles, 1988b). A similar dual behavior was observed for quercetin and chlorogenic acid against the redlegged earth mite, *Halotydeus destructor* (Ridsdill-Smith et al., 1995).

Using an artificial diet setup developed to monitor thrips larvae (de Jager et al., 1996) we intended hereby to assess the impact of phenolic compounds in general and CQA in particular on the performance of one of the most economically important pests nowadays, the western flower thrips, *Frankliniella occidentalis* (Pergande; Thysanoptera: Thripidae). Isomers of CQA were tested as well to explore the potential influence of structural features on the activity of CQA against larvae of this piercing-sucking generalist herbivore.

Methods

Reagents

All reagents were of analytical grade. Mushroom tyrosinase (polyphenol oxidase, PPO), casein, Vanderzant vitamin mixture, chlorogenic acid (97%), linoleic acid, sucrose, sodium citrate and hydrochloric acid were purchased from Sigma (St. Louis, MO, USA). Cholesterol, casein hydrolysate, potassium phosphate salts and pH-indicator strips were purchased from Merck KGaA (Darmstadt, Germany). Potassium carbonate salts were purchased from J.T.Baker (Deventer, Netherlands) and Wesson's salt was purchased from ICN Biochemicals (Cleveland, USA). Regioisomers of chlorogenic acid were obtained through base-catalyzed isomerization of chlorogenic acid followed by centrifugal partition chromatography as described in chapter 5. Ultra-pure deionized water was used to prepare all solutions. A Hanna PH20 pH-meter (Hanna Instruments, Ann Arbor, Michigan, USA) was used to measure the pH of all buffers.

Artificial diet

The thrips diet was based on a general dietary mixture for insects formulated by Singh (1983). We adapted the original formula to obtain a liquid diet on which piercing-sucking insects, like WFT, could feed (Table 1). All ingredients were mixed with a specific volume of either ultra-pure deionized water or buffer solution. The mixture was vortexed (1 min), sonicated (5 min, 20 °C) and centrifuged to remove remaining insoluble residues. A solution of chlorogenic acid (5-caffeoylquinic acid, 5-CQA) was added at this point to obtain a concentration of 1%. The solubility of 5-CQA in water is ca. 2% at 20 °C. However, amorphous crystals of 5-CQA do not disintegrate easily at room temperature and the solution must be heated up to 70 °C to achieve complete dissolution. The concentration of PPO used was 450 units mL^{-1}. Fresh diets were prepared right before each bioassay and their final pH values were measured with pH-indicator strips of 0.5 units of precision.

Table 1. Composition of the artificial diet for larval thrips based on the generalist insect formula by Singh (1983).

Ingredient		Concentration (%)
Casein		3.50
Cholesterol		0.05
Linoleic acid		0.25
Sucrose		3.00
Vanderzant vitamins		2.00
α-Tocopherol	2.26	
Ascorbic Acid	76.43	
Biotin	0.01	
Calcium Pantothenate	0.28	
Choline Chloride	14.15	
Folic Acid	0.07	
Inositol	5.66	
Vitamin B3 amide	0.28	
Vitamin B6 HCl	0.07	
Vitamin B2	0.14	
Vitamin B1 HCl	0.07	
Vitamin B12	0.57	
Wesson's salt		1.00

Deionized water was used as solvent. Mixture was vortexed, sonicated and centrifuged

Insects and bioassay

Thrips larvae were reared on fresh Italian string beans, *Phaseolus vulgaris*, in a climate room (12/12 hr photoperiod, 20 °C). Adults of a virus-free WFT biotype, reared for several months on chrysanthemum flowers, were let to feed and oviposit on the beans for 24 hrs. After 5-6 days the larvae emerged. For the artificial diet bioassay first-instar larvae were transferred to special growth plates made of clear plastic. These plates consisted of three detachable pieces. The first piece had a cylindrical well (1mm, 4 mm i.d.) where the liquid dietary treatment was poured. The well was covered each time with maximally stretched parafilm, through which thrips can feed (Teulon, 1992). The second block had a cylindrical orifice (3 mm, 6 mm i.d.) that constituted the chamber for the larva. A 3 mm-thick lid was used as the top layer. All pieces were tightly kept together with strong clips. A total of 30 replicates were prepared for each treatment. Larvae were let to feed for 72 hrs in the climate room. At the beginning and at the end of the bioassay pictures of each larva were taken under an Optika SZM-45B2 stereomicroscope (Optica Microscopes, Ponteranica, Italy) equipped with an Optikam 3 digital camera. Initial and final larval lengths were measured with the aid of the public-domain software ImageJ 1.44 (Rasband, 1997) and growth rates were calculated. Mortality was also recorded.

Statistical analysis

The significance of differences in growth rates between treatments was investigated with ANOVA followed by Duncan's multiple range test, whereas mortality rates were analyzed with Fisher exact tests. ANOVA was performed using SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA) while the Fisher tests were conducted through the online service SISA (Uitenbroek, 1997).

Results and discussion

Chlorogenic acid had a clear negative effect on the performance of thrips larvae when tested at a concentration of 1% in the artificial diet (Fig. 2). The addition of 5-CQA to the liquid dietary solution significantly reduced larval growth (F=8.0, df=3, p=0.0001) and increased mortality compared to the plain diet and to the polyphenol oxidase (PPO) used as controls. The presence of PPO did not improve the deleterious effect of 5-CQA on larval thrips, suggesting that its mode of action does not involve the formation of reactive quinones or other reactive oxygen species. However, at the concentration of 1% 5-CQA caused a decrease of more than 2 units on the pH of the dietary solution, from 5.5 to 3.0. This raised the question of a possible influence of pH on the observed negative effect. Such pH changes may be sufficient to disrupt the digestion of thrips and significantly reduce nutrient bioavailability. Thus a buffered artificial diet was introduced.

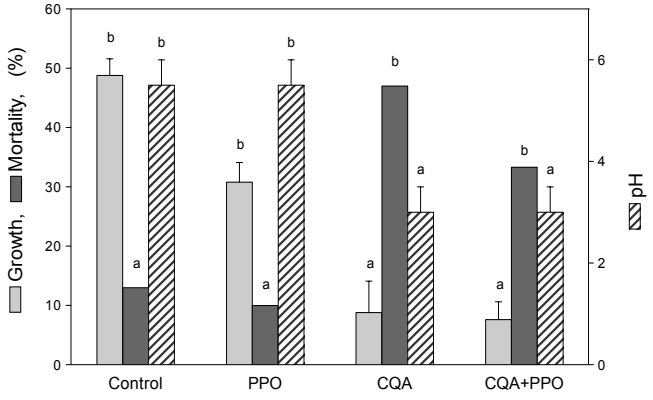

Figure 2. Effect of chlorogenic acid on the performance of first-instar larvae of *Frankliniella occidentalis* in un-buffered artificial diet bioassays. The concentration of chlorogenic acid and polyphenol oxidase (PPO) were 1% and 450 units mL^{-1} respectively. Letters refer to significant differences at the 0.05 level as analyzed with ANOVA.

The ideal buffer concentration had to be high enough to maintain the pH of the diet after the addition of 1% 5-CQA but could not exceed the electrolyte tolerance limit of thrips larvae. To determine this threshold value we tested a series of diets with increasing concentrations of potassium phosphate buffer pH 7.0 in the range of 25-900 mM. Buffer toxicity to larval thrips was detected above 200 mM (Fig. 3). A sharp decline in growth and a concomitant increase in mortality were observed between 200 and 500 mM, beyond which insect survival was zero. Therefore, the buffer concentration for the following bioassays was cautiously set at 150 mM.

When chlorogenic acid was retested in a pH-controlled diet, with all other variables remaining unchanged, its previously observed anti-insect effect was abolished (Fig. 4). No significant differences were detected in neither growth nor mortality of thrips compared to control groups. In this experiment the final pH values of the CQA-containing diet was 6.0, only one unit more acidic than the controls. These results indicate that the observed underperformance of thrips larvae was connected to the strong acidification of the diet and not to the reactive *o*-diphenol moiety on CQA. Most digestive enzymes have been selected to work optimally in a relatively narrow pH range. Out of this range enzyme denaturation or inactivation takes place, precluding the insect from extracting nutrients out

of the food source. Increased vacuolar accumulation of organic acids, including CQA, in otherwise susceptible foliar tissue may therefore represent a contributing factor to host-plant resistance against herbivores with neutral or alkaline digestive tracts.

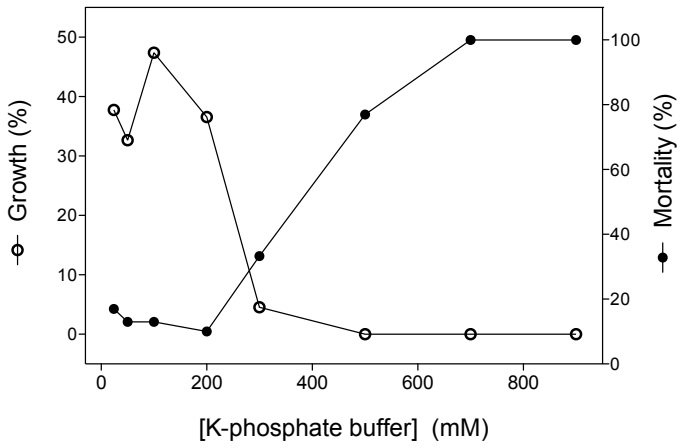

Figure 3. Influence of the concentration of phosphate buffer pH 7.0 on the performance of first-instar larvae of *Frankliniella occidentalis*.

Polyphenol oxidase has its activity maximum at a pH of 7.0 (Felton, 1989). Yet, the addition of this enzyme to the neutral dietary treatment did not trigger any anti-insect effect in CQA. The browning of the liquid diet upon addition of PPO evidenced the oxidation of CQA. It has been shown that activation of phenolics by PPO is necessary for these metabolites to exhibit anti-insect activity in artificial diet setups (Felton, 1989; 1992). However, both PPO activity and phenolic levels have failed to correlate with host-plant resistance in different plant-insect systems (Bi et al., 1997a; Isman and Duffey, 1982a; Melo et al., 2006). Considering our results we wonder whether insufficient control of diet pH could account in some cases for such discrepancy between *in situ* and *in vitro* herbivory tests.

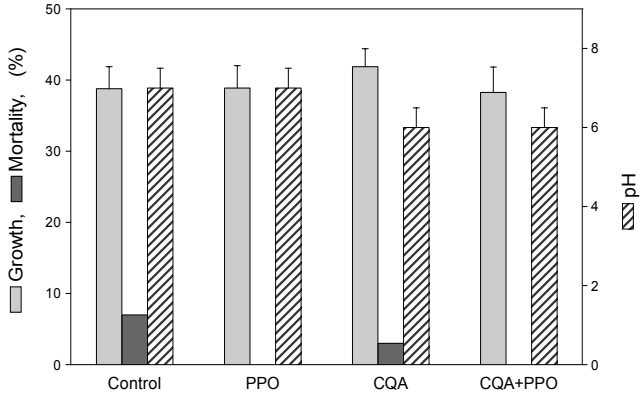

Figure 4. Effect of chlorogenic acid on the performance of first-instar larvae of *Frankliniella occidentalis* in pH-controlled artificial diet bioassays. The concentration of chlorogenic acid and polyphenol oxidase (PPO) were 1% and 450 units mL^{-1} respectively.

To further examine the influence of hydrogen ion concentration on thrips performance we tested dietary solutions prepared with phosphate buffers of eight different pH values, ranging from 2 to 12, at a total phosphate concentration of 150 mM. As evidence by the growth and mortality profiles thrips larvae performed best at pHs between 5 and 7 (Fig. 5), suggesting that the digestive tract of larval thrips is in the acidic-neutral range. To the best of our knowledge this represents the first indirect determination of the physiological midgut pH of *F. occidentalis*. Using a series of indicators fed to larvae and adults Day and Irzykiewicz (1954) determined a narrow range of possible midgut pH values for two other thrips species, the onion thrips, *Thrips tabaci*, and the apple blossom thrips, *T. imaginis*. Larval and adult thrips of both species had a digestive pH between 5.0 and 5.6. Acidic to neutral digestive tracts are also characteristic of species in the orders Orthoptera, Diptera, Hymenoptera, and in some cases Coleoptera. Whereas alkaline digestive conditions are typical in Lepidopteran larvae (Johnson and Felton, 1996).

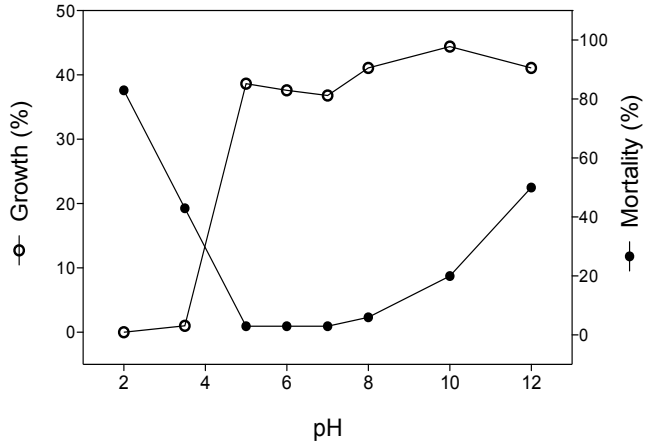

Figure 5. Influence of diet pH on the performance of first-instar larvae of *Frankliniella occidentalis*. All buffer systems are based on potassium phosphate salts with a total phosphate concentration of 150 mM.

Other diet parameters, such as high nutrient levels, may partially counteract potential adverse physiological effects of CQA. For instance, the oxidation of CQA into reactive *o*-quinone would be inhibited by other metabolites with higher reducing potentials, such as the ascorbic acid present in the vitamin mixture (Table 1). To investigate this hypothesis the relative concentrations of protein and vitamins in the liquid diet were reduced. As expected, thrips larvae grew significantly less (F=8.3, df=8, p=0.0001) when the contents of protein and vitamins were cut down to a fifth and an eighth of the full-diet values respectively (Fig. 6). Growth did not decrease much more when the concentrations of protein and vitamins were reduced further to a tenth and a fortieth of the full diet content, respectively. Larval performance was not affected by CQA in any of the low-nutrient diets, confirming thus that CQA does not have acute negative effects on larval thrips. The potential antioxidant benefits of phenolics, claimed in some cited literature, did not reflect on thrips performance either.

Concentrations of CQA higher than 1% could have been tested with the aid of suitable ratios of organic solvents. However, such values are usually considered physiologically unrealistic and lack in evident ecological meaning. The highest foliar concentrations of CQA reported to date are 2% of dry weight for tomato, *Solanum lycopersicum* (Jansen and Stamp, 1997) and 3.3% of dry weight for

tobacco, *Nicotiana tabacum* (Camacho-Cristobal et al., 2004). Metabolic engineering of the phenylpropanoid biosynthetic pathway in tomato increased the foliar accumulation of CQA from 0.9% to 1.85% of dry weight (Niggeweg et al., 2004). The engineered plants showed improved resistance to the bacterial pathogen *Pseudomonas syringae* but were not challenged with herbivores.

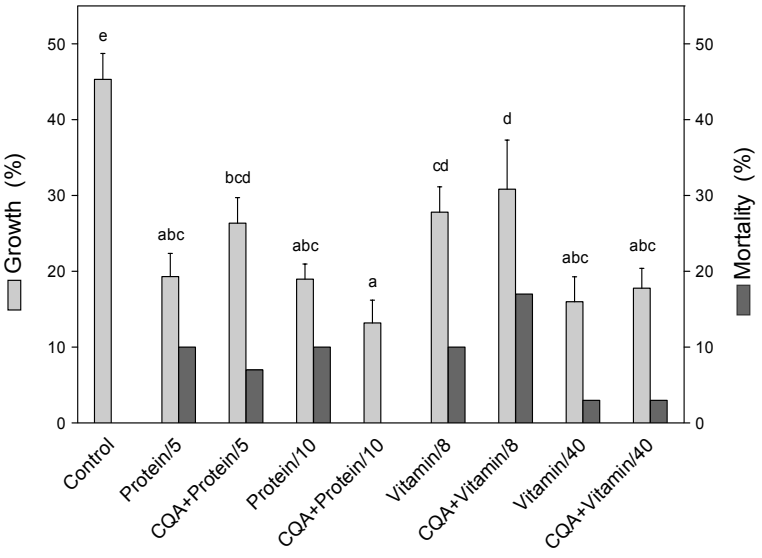

Figure 6. Effect of the nutrient/chlorogenic acid ratio on the performance of first-instar larvae of *Frankliniella occidentalis* in pH-controlled artificial diet bioassays. Concentrations of protein and vitamin in the control diet were 3.5 and 2 % respectively. For the treatments protein content was reduced to 1/5 and 1/10 whereas the vitamin content was reduced to 1/8 and 1/40. The concentration of chlorogenic acid was 1%.

When thrips larvae were fed diets containing either one of CQA regioisomers, 3-CQA (neochlorogenic acid) and 4-CQA (cryptochlorogenic acid), no significant changes in their performance were observed (Fig. 7). This result indicates that the differences in physical properties between these molecules are irrelevant as well to the potential anti-insect activity of CQA.

Researchers often draw the inference that phenolic compounds are involved in host-plant resistance against herbivores on the ground of their induction or increase upon herbivory or wounding. This connection is in many cases a misinterpretation of incomplete evidence, especially when polyphenol oxidases are concomitantly induced (Bi et al., 1997b). One of the most essential roles of phenolics and oxidases in plants is to seal off damaged tissue and reduce thereby the plant vulnerability to pathogen attack. The induced accumulation of phenolics after injury by pathogens or pests has been extensively reviewed (Freucht and Treutter, 1999). Our results suggest that in the course of plant-insect coevolution thrips may have indeed evolved adaptations similar to those in mammals to circumvent the predicted adverse effects of simple phenolics like chlorogenic acid. In addition, our study joins the mounting body of evidence compelling us to abandon the assumption that phenolics are in general effective defenses against herbivores. Interactions between insects and phenolic compounds should be evaluated on a case-to-case basis as long as the specific molecular modes of action of these secondary metabolites are not elucidated and fully understood.

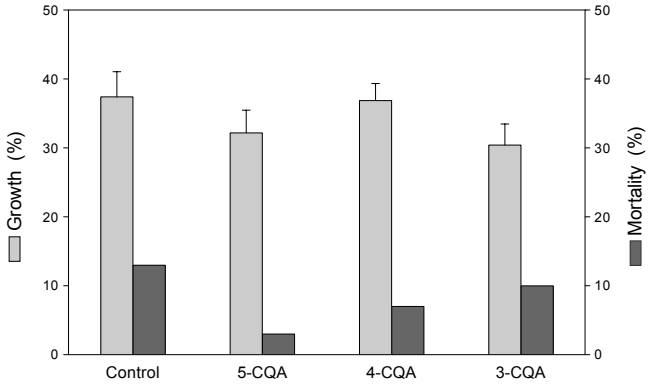

Figure 7. Effect of the chlorogenic acid regioisomers on the performance of first-instar larvae of *Frankliniella occidentalis* pH-controlled artificial diet bioassays. The concentration of chlorogenic acids was 1%. Letters refer to significant differences at the 0.05 level as analyzed with ANOVA.