Host plant resistance of tomato plants to western flower thrips

Mirnezhad, M.

Chlorogenic acid does not affect damage of western flower thrips to transgenic tomato plants

Abstract

Chlorogenic acid (CGA) is a phenol with antioxidant activity that accumulates in many vegetables and fruits. It is known to have negative effects on insect herbivores including thrips. In this study we used transgenic (TG) tomato plants with increased amounts of CGA to test its effect on western flower thrips (WFT), *Frankliniella occidentalis*, a serious pest of tomato plants worldwide. Whole plant and leaf bioassays were used to compare silver damage of WFT to TG and control plants. Chlorogenic acid content in leaves and fruits of tomatoes was determined by HPLC. Survival and growth rate of thrips larvae were tested with *in-vitro* bioassays consisting in the administration of a CGA-enriched artificial diet and one of with the juice of green and red tomatoes of TG and control plants. The possibility of CGA induction resulting from wounding or thrips damage was also investigated. In contrast to our expectations, increased CGA levels in the TG tomato plants did not affect thrips nor was any significant difference observed in silver damage neither in whole plant nor in leaf bioassays. Similarly, survival and relative growth rate of thrips were unaffected by CGA, as observed in both the CGA supplemented diet and high-CGA fruit juice *in-vitro* bioassay. Interestingly, a significantly higher larval growth rate was observed with red tomatoes as compared to green ones. Neither wounding nor thrips infestation was observed to induce CGA production.

Introduction

The rich chemical diversity of plants is the result of ongoing evolutionary processes. Enzymes involved in natural product biosynthesis arise through mutation and gene duplication leading to the continued elaboration of new chemical structures that will be selected if they impart an adaptive advantage to the plant. It is assumed that most secondary plant metabolites play a role in resistance to abiotic or biotic stresses. Concurrently, they could have a positive impact on human health, providing a scope for a molecular approach leading to more resistant food crops that are, at the same time, more beneficial to human health. Secondary metabolites with

antioxidant properties are compounds with potentially negative effects on pests but potentially positive effects on human health. Therefore, we chose the tomato plant known for its high levels of antioxidants for this study. S*olanum lycopersicum* is grown around the world, both in fields and greenhouses, for fresh market use and for processing, being an exceptional source of nutrients. Cultivated tomatoes, however, are hosts to many kinds of insects and are attacked by a number of serious pests (Lange & Bronson, 1981) one of which is the western flower thrips (WFT), *Frankliniella occidentalis* (Mirnezhad et al., 2010). Wild tomato plants are rich in phenolics. Phenolics are the most widespread plant dietary antioxidants, and among these, chlorogenic acid (5-caffeoylquinic acid) (CGA) can accumulate in high levels in plants. Chlorogenic acid occurs in many vegetables and fruits such as apples, pears, peaches, plums, cherries, apricots (Risch & Herrmann, 1988) and artichoke (Sonnante et al., 2010). In particular, CGA is the major water-soluble phenolic in *Solanaceous* species such as potato, eggplant and tomato (Isman & Duffey, 1982), constituting the highest mean concentration as an antioxidant in the latter (Hernandez et al., 2007). The CGA content in tomato leaves was found to range from 0.1 to 1.5% dry weight (Jansen & Stamp, 1997) and 2-20 µmol/g fresh weight (Wilkens et al., 1996). In the leaves of a cultivated tomato (var. Moneymaker), the CGA content was 0.90-1.00 ± 0.08 µg $mgfwt^{-1}$ (Niggeweg et al., 2004).

In plants, it is possible to find two other isomers of chlorogenic acid, neo- and cryptochlorogenic acid and all three have an equally strong antioxidant activity (Nakatani et al., 2000). Chlorogenic acid is claimed to have a series of health benefits such as the reduction of the relative risk of cancer and cardiovascular disease (Ranheim & Halvorsen, 2005), diabetes type 2 (van Dam & Feskens, 2002), alzheimer (Lindsay et al., 2002) and antibacterial properties (Almeida et al., 2006).

There is increasing evidence that CGA is involved in host plant resistance to herbivores, having been observed to inhibit feeding, reduce growth and retard the developmental stages of herbivorous insects (Stamp, 1994; Stamp & Yang, 1996). Its activity against insects is based on its oxidation to chlorogenoquinone (Felton et al., 1989), a quinone that acting as a highly reactive electrophile reacts with the nucleophilic –SH and $–NH_2$ moieties in proteins (Matheis & Whitaker, 1984). The resulting cross-linking of proteins with chlorogenoquinone reduces the availability of free amino acids and proteins to insects (Felton et al., 1989). While CGA mainly acts as a constitutive defence, there are some indications that it is inducible. The concentration of CGA in tomato plants, for example, increased after infection with the fungus, *Fusarium*

oxysporum (Matta et al., 1969; Mendez & Brown, 1971) and the same effect was observed when *Spodoptera littoralis* fed on corn (Erb et al., 2009a,b). Conversely, feeding by *Diabrotica virigifera* on the roots of corn did not induce CGA, but when this infestation was followed by an attack of *S. littoralis*, plants showed an even higher CGA production compared to the control. This indicates that underground herbivory may prime CGA production aboveground.

Adding CGA to an artificial diet significantly reduced the growth of different caterpillar species, such as *Trichoplusia ni* (Beninger et al., 2004), *Manduca sexta* (Sphingidae) (Stamp, 1994) and *Heliothis zea* (Isman & Duffey, 1982; Farrar & Kennedy, 1987; Felton & Duffey, 1990). Experiments with fresh foliage showed CGA to be a deterrent to various *Lepidoptera* larvae such as *Pieris rapae* (Huang & Renwick, 1995) and *Spodoptera litturalis* (Mallikarjuna et al., 2004). Similarly, CGA significantly reduced the feeding of the corn leafhopper *Dalbulus maidis* (Dowd & Vega, 1996) and various leaf beetles (Fulcher et al., 1998; Ikonen et al., 2002; Jassbi, 2003). Moreover, the willow leaf beetle *Lochmaea capreaea*, preferred the willow species with the lowest CGA contents (Ikonen et al., 2001).

Chlorogenic acid exhibited negative effects on aphids (Miles & Oertli, 1993) and WFT (Leiss et al, 2009a), both sucking insects. A comparison of the metabolomic profiles of thrips resistant and susceptible chrysanthemum varities identified CGA as a candidate compound for thrips resistance. Consequently, the effect of CGA on thrips was investigated *in-vitro*. Rearing first instar larvae on an artificial medium containing 5% CGA significantly reduced their growth rates initially and then inhibited their growth altogether. Furthermore, in choice tests, they showed a significant preference for a medium without any CGA rather than media with concentrations as low as 1% of CGA.

Chlorogenic acid has also been reported to exhibit negative effects on fungi and bacteria such as *Phytophtora capsicii* in sweet pepper (Lizzi et al., 1995), and *Pseudomonas syringae* in tomato plants (Niggeweg et al., 2004). Tobacco plants that overexpressed phenylalanine ammonia-lyase (PAL) producing thus, elevated levels of CGA, exhibited an increased systemic resistance to tobacco mosaic virus infection (Kiraly et al., 2002). Chlorogenic acid also significantly inhibited the infectivity of nuclear polyhydrosis viruses in tomato plants (Felton & Duffey, 1990).

As a pest, WFT is one of the most serious problems in cultivated crops worldwide (Jensen, 2000). Thrips have a number of traits that combine to ensure their success as horticultural crop pests: polyphagy, small size, affinity for enclosed spaces, short generation time, a high

reproductive potential (Morse & Hoddle, 2006). They also have piercing-sucking mouthparts (Hunter & Ullman, 1989) that allow them to feed efficiently. Feeding on plants' actively growing tissue leads to distortion, reduction in growth and eventually to yield loss. Feeding on expanded tissue, on the other hand, results in the characteristic silver leaf scars, which affect product appearance and reduces market quality (de Jager et al., 1995a). Additionally, WFT causes indirect damage as the primary vector of tospoviruses, among which tomato spotted wilt virus is the economically most important (Maris et al., 2003).

Control of thrips relies mainly on pesticides. Thrips, however, are difficult to control because they feed in the inner whorls of flowers and buds (Immaraju et al., 1992; Brødsgaard, 1994), which are quite inaccessible to insecticides. Consequently, repeated and frequent spraying is necessary , often leading to the appearance of resistance to various insecticides (Jensen, 2000). At the same time, excessive use of pesticides involves risks to human health, toxicity towards non-target organisms and pollution of the environment. An important option to control WFT, thus, may be the use of host plant resistance as part of an integrated pest management approach.

Because of the unique combination of reported negative effects on thrips with positive effects on human health, CGA seemed to qualify as the substance of choice to use for the development of host plant resistance to thrips. Inducing the overexpression of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT), Niggeweg et al. (2004) produced tomatoes with double the amount of CGA for dietary purposes, without influencing other phenylpropanoids. Sienkiewicz-Porzucek et al. (2008) obtained transgenic tomato plants in which the amount of CGA was also doubled as a side effect of the use of an antisense citrate synthase. In this study, we used these transgenic plants to determine whether increased chlorogenic acid (CGA) levels affected thrips resistance. The effects of CGA were assayed under both *in-vitro* and *in-vivo* conditions. Furthermore, we investigated the induction of CGA by thrips infestation.

Our investigation was aimed specifically at answering the following questions:

- Do transgenic tomato plants with higher foliar contents of CGA show increased resistance to thrips?
- Is CGA content positively related to thrips resistance in tomato plants?
- Do leaf bioassays provide a reliable estimate for thrips resistance of whole plants?

- Does CGA added to artificial diets have a negative effect on growth rate and mortality of first instar larvae of thrips and if so at which concentrations?
- Does fruit juice of TG tomato plants with higher CGA contents lead to lower growth rates and higher mortality of first instar larvae of thrips?
- Does wounding and thrips infestation lead to induction of CGA?

Material and methods

Plants

1) Two-fold CGA tomato plants (UK). The seeds of transgenic tomatoes (TG) lines (T6 and T9) with 2- fold CGA and of one line in which CGA production was silenced (T8) were obtained from the *John Innes Center* at Norwich (UK) in 2008 (Niggeweg et al., 2004). The background of these lines was the cultivated tomato var. Moneymaker. The seeds were placed on 1% water agar medium containing 50 µg /ml kanamycin. Kanamycin resistance is used in molecular biology as a selective trait to identify transgenic seeds (Taniguchi et al., 1997; Misumi & Tanaka, 1980). The best germination was obtained by placing the medium with the seeds on the soil surface of 13 cm diameter pots filled with potting soil and covering them slightly with soil. Germination of seeds in Petri dishes did not succeed. The seeds of the control plants were directly sown into the soil. Seeds were thinned to one plant per pot after one week. Twenty replicates of each line were grown in a randomized fashion in a climate chamber (L16: D8, 25 °C, 70% RH & 11.36 µmol m^{-2} s^{-1} light intensity) for 5 weeks. Ten replicates were used for the whole plant thrips bioassay and another ten replicates were used for the leaf bioassay and measurement of CGA with HPLC.

2) Twenty-fold CGA tomato plants (UK). In 2009 we received 2 transgenic lines that allegedly had a 20-fold content of foliar CGA, from the *John Innes Center* at Norwich (UK). The background of these lines was the cultivated tomato var. Microtom. One of the TG lines did not germinate at all and only 2 seedlings of the lines could be germinated and grown for further seed production. The seeds from these plants were sown as described above and grown for 5 weeks. Twenty replicates were used: ten replicates for the whole plant thrips bioassay and ten for the leaf bioassay and determination of CGA.

3) Two-fold CGA tomato plants (Germany). We obtained tomato plants in tissue culture with double CGA contents (lines CS25, CS40 and CS45) and a control line (WT) from the *Max Planck Institute of Molecular Plant Physiology in Potsdam, Golm* (Germany) (Sienkiewicz-Porzucek et al., 2008). The background of these lines was the cultivated tomato var. Moneymaker. Seven

replicates of each line were transplanted into 13 cm diameter pots filled with potting soil. The pots were transferred to a growth chamber and grown for 5 weeks as described in the previous paragraph.

In-vivo thrips bioassay

1) Whole Plant Bioassay. All 3 tomato lines, the two fold and twenty fold lines from John Innes and the two fold lines from Max Planck were all were subjected to the non-choice whole plant bioassay as described by Leiss et al., (2009a). For the two fold CGA lines this bioassay was performed twice. Whole plants were placed in individual thrips-proof cages consisting of Perspex cylinders (60 cm height, 20 cm diameter) closed on top with 120 µm mesh size nylon gauze. Ten replicates were used for each line. The cages were arranged in a completely randomized design in a climate chamber (L16: D8, 25°C: 20 °C, 70% & 11.36 µmol.m^{-2}.s^{-1} PAR). Twenty WFT adults, reared on flowering chrysanthemum, were added per plant and left for one week. Silver damage, expressed as the leaf area damaged per mm^2, was visually scored for each leaf. Plants were dried for 3 days in an oven at 50 °C, whereupon plant dry mass was measured. Differences in silver damage were analyzed with ANOVA using plant dry mass as a co-variate (Sokal & Rohlf, 1995).

2) Non-choice leaf bioassay. Leaves from TG tomatoes with two fold CGA contents were tested with this bioassay as described by de Kogel et al. (1997) in addition the whole plant bioassay**.** Compared to a whole plant bioassay, a leaf bioassay is cheaper, faster and takes up less space. Furthermore, it allows the measurement of thrips damage and analysis of metabolomic profiles of parts of the same plant. One leaflet of the third oldest leaf of tomato plants was collected. Leaflets of one leaf do not significantly differ in CGA content according to a pre-test. The leaflet was placed in a Petri dish (9 cm diameter) filled with 1% water agar and left for 5 days. A photograph of each leaf was taken before the experiment and the leaf area measured using ImageJ software (Rasband, 1997-2006). Twenty female thrips were placed in the Petri dish that was then closed with a lid with a perforation (Ø 3 cm) covered with fine thrips-proof mesh to prevent accumulation of condensed water. The Petri dish was sealed with Parafilm to prevent thrips from escaping and placed in a climate chamber (L16: D8, 25 °C, 70% RH & 11.36 µmol m^{-2} s^{-1} light intensity). Ten replicates were used for each line. After 5 days the leaves were inspected for silver damage and it was expressed as the damaged leaf area (mm^2). Data were analysed by ANOVA.

3) Choice leaf bioassay. No correlation was detected between the results of the whole-plant and leaf bioassay described in the previous paragraph. Therefore, an alternative choice leaf bioassay described by Kumar et al. (1995) was used for the TG tomatoes with 20-fold CGA content. In this case, the fifth oldest leaf of the tomato plants was used. The entire leaflets, except the lateral ones, were cut to form a long petiole to absorb enough water throughout the 5 days of the experiment. A lateral leaflet of the TG plant with 20- fold CGA was placed in a jar and the same was done with a control leaflet. Both were then placed in one cage. Five such cages were used as replicates. The cages were placed in a completely randomized design in a climate chamber (L16: D8, 25°C, 70% RH & 11.36 µmol m^{-2} s^{-1} light intensity) with 10 adult WFTs reared on flowering chrysanthemums per leaflet and left for five days. Silver damage, expressed as the damaged leaf area (mm^2), was visually scored for each leaf. Differences in silver damage were analyzed with ANOVA (Sokal & Rohlf, 1995).

In-vitro thrips bioassay

Artificial diet. The larval performance in liquid media with different CGA concentrations was studied using special observation plates of clear plastic, as described by de Jager (1995b). A general insect diet developed by Singh (1983) was modified, including only the soluble ingredients, and used as a liquid medium. The medium was buffered with 200 mM of potassium salt (K_2HPO_4/KH_2PO_4). Adequate amounts of chlorogenic acid were added to reach concentrations of 0%, 0.06%, 0.13%, 0.5% and 2%, the maximum solubility in these conditions. The medium was placed in small cups of a bottom plate and covered with stretched Parafilm, through which thrips were able to feed. A middle plate with six holes was placed on top of the Parafilm to keep the thrips larvae separated and covered with a top plate. Thirty first instar WFT larvae were introduced in each treatment. A photograph of each thrips larva was taken with a binocular microscope and their length was measured with the ImageJ software (Rasband, 1997-2006). After three days, at the end of the first larval instar period, their length was measured again and their relative growth rate calculated. The percentage of surviving larvae after 3 days was also determined. The *in-vitro* test was performed in a climate chamber (L12: D12, 25°C, 70% RH & 11.36 µmol.m^{-2}.s^{-1} PAR). Thirty replicates were used for each concentration and each bioassay was performed by duplicate. An ANOVA was performed to

analyse relative growth rates, while survival of larvae was analyzed by a chi-square test (Sokal & Rohlf, 1995).

Fruit juice. To study larval performance on tomato fruits, the juice of ripe red and unripe, green tomatoes of transgenic lines with a 20-fold content of CGA and control plants was used as a medium for an *in-vitro* bioassay. The fleshy part of the tomato, known to contain the highest amount of CGA in tomato fruit (Takayuki, personal communication), was separated from the seeds and skin. A two-way ANOVA was performed to analyse the relative growth rates of larvae as well as the CGA content of fruits. Larval survival was analyzed by a chi-square test (Sokal & Rohlf, 1995). Thirty replicates were used for each treatment and each bioassay was performed thrice.

Induction of CGA

To test for induction of CGA in tomato plants by wounding and thrips infestation, cultivated tomato var. Moneymaker was grown in a climate chamber (L12: D12, 25°C, 70% RH & 11.36 µmol m^{-2} s^{-1} light intensity). Four replicates were used for each treatment. At the sixth leaf age, one leaflet of the third oldest leaf was cut to simulate wounding. The leaflet was shock-frozen in liquid nitrogen and stored at -80 °C until CGA determination. After one hour the opposite leaflet, as well as, the two leaflets below the one that had been cut were also harvested for CGA determination by HPLC. Equivalent leaflets were harvested at the same time from unwounded control plants.

To evaluate thrips-induced CGA production, 20 adult WFTs were placed on a leaflet of the third oldest leaf of 5 week old plants that was then wrapped in a net of thrips-proof gauze. Five replicates were used for each treatment. After 5 days, when the first symptoms of silver damage appeared, the thrips infested leaflet and its opposite leaflet were harvested for CGA determination by HPLC. Equivalent leaflets were harvested at the same time from uninfested plants control plants. Data were analysed by ANOVA.

HPLC (High performance liquid chromatography) determination of CGA

All plants used in the experiment were grown under standard conditions. Half of them were used for the bioassay and the other half for CGA determination by HPLC using the method published by Shao & Zhuang (2004). From each individual plant the third leaf from the bottom

was taken for analysis. Immediately after collection, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction. The CGA content of fruits was determined using the freeze-dried fleshy part of tomato fruits. Samples were ground to a fine powder in a Retsch mill and 50 mg of each sample was transferred to a 2 ml eppendorf tube.

The samples were sonicated with 1.5 ml of 50% methanol during 25 min for CGA extraction. A preliminary experiment showed this method to be more efficient than tissue lysation with a Retsch mill for 10 minutes at 30 rps or shaking for 72 hours. After centrifugation (13 krpm, 15 min), an aliquot of 500 µL of each sample was transferred to vials and analyzed by HPLC. An ODS- column (Phenomenex, 3 µm, 150 mm × 4.6 mm) was used. Gradient elution was used for the mobile phase from 10:88:2 to 48: 50: 2 (v:v:v, methanol: water: acetic acid) within 23 minutes.

Results

In-vivo bioassay

1) 2-fold CGA tomato plants (UK). In the first experiment the CGA concentration in the high CGA tomato lines T6 and T9 was greater than in the control plants (Figure 1), but this difference was not significant (F = 1.929, df = 3, P = 0.168). However, in line T6 seed germination was low and only 2 plants were tested. In the second experiment, CGA content exhibited a three fold increase and was thus significantly higher in lines T6 and T9 as compared to the control and silenced line T8 (F = 18.38, df = 3, P < 0.001). The concentration of CGA in the control was also 6.8 times higher than T8 line. Bioassays with plants from experiment 1 and 2 both showed no significant differences in silver damage between the different lines, (F = 0.406, df = 3, P = 0.75 and F = 3.673, df = 3, P = 0.104, first and second experiment respectively) (Figure 1). Additionally, no significant differences in thrips damage between lines were detected in the non-choice leaf bioassay (F= 2.805, df = 3, P = 0.51, and F = 0.871, df = 3, P = 0.463, for the first and second repetition of the experiment, respectively) (Figure 1). Thrips damage in the *in–vivo* and the *in-vitro* bioassays was not correlated with CGA content in the leaves (r = - 0.107, n = 40, P = 0.513, and r = - 0.191, n = 40, P = 0.239, first and second experiment respectively).

2) 20-fold CGA tomato plants. The CGA content of this line was significantly increased (F = 68.17, df =1, P < 0.001). This increase, however, was below the expected 20-fold increase. The TG line contained only 3 times more CGA than the control (Figure 2). In contrast to our expectations,

thrips damage was even slightly greater in the TG line than in the control plants, although this was not significant (F= 3.163, df =1, P = 0.084). Similarly, the choice leaf bioassay did not show any significant differences between tomato lines (F = 0.66, df =1, P =0.429) (Figure 2).

3) 2- fold CGA tomato plants (Germany). The whole plant bioassays of transgenic tomato plants from the *Max Planck Institute of Molecular Plant Physiology in Potsdam, Golm* (Germany) did not reveal any significant differences in silver damage between lines (F = 0.934, df = 3, P = 0.444). Damage to line number "CS40" was very low as compared to the control but the other lines showed levels of thrips damage comparable to the control. The CGA content of leaves of all lines did not differ significantly (F = 1.902, df = 3, P = 0.164). No correlation between silver damage and CGA content was observed in these lines (r = 0.052, n = 23, P = 0.813).

In-vitro bioassay

Artificial diet. The growth rates of first instar thrips larvae in the medium containing different concentrations of CGA were neither significantly different in the first (F = 1.43, df = 4, P = 0.23) nor in the second experiment (F = 2.23, df = 4, P = 0.67) (Figure 3A). Thrips survival, did not vary significantly with the different treatments (χ^2= 4.08, df = 4, P = 0.666 and χ^2= 6.56, P = 0.16, first and second experiment respectively) (Figure 3B).

Fruit juice. There was no significant difference in the growth rates of thrips larvae on tomato fruit from the alleged 20 -fold CGA content tomato and control plants (F= 1.475, df = 1, P = 0.226) (Figure 4). Conversely, growth rates of larvae on red fruit, both of TG or control plants, were significantly greater than on green fruit (F= 17.991, df = 1, P <0.001), while there was no significant interaction between the fruit type and plant line (F= 0.054, df = 1, P = 0.816). There was no significant difference in thrips survival in TG and control fruits (χ^2= 1.56, df = 1, P = 0.21) nor between green and red fruits (χ^2= 0.28, df = 1, P = 0.598). The average concentrations of CGA in red and green TG fruits was double that of the respective control fruits, which contained low levels of CGA in general (F = 4.874, df = 1, P = 0.058) and practically none in the case of the red control fruits. Green fruits, from both TG and control plants, contained 5 times more CGA than red fruit (F = 16.065, df =1, P = 0.004) (Figure 4).

Induction of CGA. Neither wounding nor thrips infestation induced CGA. The CGA content of the different leaflets harvested after wounding was the same as that of leaflets that had been cut to simulate wounding (F = 2.074, df =3, P = 0.126) and the same occurred with the amount of CGA in wounded and control plants (F = 2.531, df = 1, P = 0.122). Infestation with thrips led to

similar results: no significant differences in CGA content were observed between thrips infested leaflets and the opposite non- infested leaflets (F = 0.012, df = 1, P = 0.916) nor between infested and control plants (F = 0.31, df = 1, P = 0.585).

Discussion

In this study we did not detect a negative effect of chlorogenic acid on thrips in tomato plants, neither *in-vivo* nor *in-vitro.* These results contrast with those obtained in similar studies performed with chrysanthemums (Leiss et al., 2009a). Resistant chrysanthemum varieties contained significantly higher amounts of CGA than susceptible varieties. In that case, authors also reported that in the *in -vitro* test, the relative growth rate of first instar thrips larvae was significantly reduced by greater concentrations of CGA. The reasons for the difference with our experimental results may be attributed to the following:

1. The amount of CGA applied was insufficient to cause a negative effect on thrips. Tomato plants with an alleged 20- fold content of CGA unfortunately only had three times the amount and did not inhibit thrips feeding either. On average, however, they contained 0.075% CGA, which is comparable to the 0.05% CGA present in the thrips-resistant chrysanthemums (Leiss et al., 2009a).

2. In accordance with our findings, a six-fold increase in CGA in phenylalanine ammonia-lyase (PAL) modified tobacco lines, did not cause a remarkable reduction in herbivory by tobacco hornworm, *M. sexta,* or tobacco budworm, *Heliothis virescens,* (Eichenseer et al., 1998; Johnson & Felton, 2001). Since PAL occurs relatively at the beginning of the phenyl propanoid pathway, these lines showed elevated levels of other phenolic acids besides CGA. Therefore, the negative effect of CGA on herbivores in these lines might have depended on the predominant types of phenolics present. In contrast, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) occurs relatively at the end of the phenylpropanoid pathway and Niggeweg et al. (2004), therefore, reported that their tomato lines with two-fold increased CGA did not show elevated levels of other phenolic acids.

3. The negative effect of CGA on herbivores may depend on additive or synergistic effects with other defence compounds. Miles & Oertli (1993) showed that the addition of the antioxidant ascorbate enhanced the negative effect of CGA on apple aphid, *Aphis pomi,* Similarly, the effect of CGA may be enhanced by a synergistic effect with feruloyl quinic acid, which was also significantly increased in thrips resistant chrysanthemum varieties (Leiss et al.,

2009a). Initial oxidation of CGA to chlorogenoquinone increases toxicity, but further oxidation may decrease toxicity due to formation of phenolic oligomers (Felton & Duffey, 1991). This, however, is unlikely because the amount of CGA in the thrips resistant chrysanthemum varieties is comparable to that of the modified tomatoes. Thus detoxification of high CGA amounts via oligomer formation is improbable.

4. The concentration of CGA may vary among plant organs. Young tomato leaves exhibited higher concentrations of soluble phenolics than intermediate and mature ones (Stamp & Horwath, 1992; Wilkens et al., 1996). Older tomato leaves proved, indeed, to be more susceptible to thrips damage than younger ones (Mirnezhad et al., 2010). The amount of CGA was highest in green tomato fruits as compared to leaves, stems, roots and red fruits (Tohge, personal communication). In our study, however CGA content did not differ between leaves and fruits but thrips larvae did grow better on ripe red fruit. This is probably due to the decrease in the concentration of malic and citric acids and an increase in monosaccharides resulting from starch breakdown (Goodenough et al., 1982).

5. The concentration of CGA in plants may be influenced by environmental factors. It is known that temperature can have an effect on the interaction between CGA and Lepidoptera larvae (Stamp & Yang, 1996, Stamp & Osier, 1998). Drought stress can increase CGA levels affecting the growth of herbivores (English Loeb et al., 1997). Another factor influencing CGA is the nutrient level. In tomato plants, CGA was high using intermediate levels of a potassium nitrate fertilizer (Wilkens et al., 1996). Elevated carbon dioxide treatment increased CGA in birch (Kuokkanen et al., 2003). In our study, environmental influences on CGA contents are unlikely, since the plants were grown under controlled conditions in a growth chamber. But the interaction with other factors that are influenced by growing conditions may cause a different relationship between CGA content and resistance to thrips if plants were grown in different conditions.

6. CGA is known to affect development stages of herbivores differently. Isman & Duffey (1982) reported no effect on the growth of the third or fifth instar of *H. zea* fed with a CGA enriched - artificial diet, but the compound did inhibit growth of neonate larvae. In our study, however, neither adults nor larvae were affected by CGA.

7. Non-chemical factors may affect CGA. This has been shown for willow, *Salix* spp.,, in which the response of different leaf beetles to foliar CGA was dependant on the hairyness of the willow species (Ikonen et al., 2001; 2002). However, in our investigation, the tomato variety

Moneymaker, used as a genetic background, is highly thrips susceptible. Thus mechanic resistance does not play a role in our study system.

In our study, no induction of CGA neither by wounding nor by thrips damage was detected. This coincides with the findings of Broadway et al. (1986), who did not detect induction of phenols, including chlorogenic acid, after 24-hour feeding of the beet armyworm, *Spodoptera* exigua, on tomato plants. On the other hand, Erb et al. (2009a,b) reported induction of CGA in corn due to the feeding of *S. littoralis* after 3 days. It is known that induction can be influenced by many factors such as the kind of wounding (Ohgushi, 2005; 2008), the measured leaf (Karban & Myers, 1989), the time elapsed until measuring (Agrawal, 1998), and environmental conditions (Karban & Myers, 1989).

In conclusion: A three-fold increase of CGA in transgenic plants did not lead to any evidence that these plants were better protected against thrips. This contradicts the results obtained with other plants species. At this point we have no indication about the reasons for this. Although perhaps higher increases in CGA may affect resistance against thrips, results in tomato are not very promising so far. The result of this experiment and our previous research showed that the best way to increase resistance against thrips in tomato plants is therefore, not through CGA, but through acylsugars, which have been proven to be an important factor in host plant resistance of thrips in tomato (Mirnezhad et al., 2010).

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Figure. 1. Means and SE of silver damage and CGA content in transgenic tomato lines with doubled CGA (T6, T9), silenced CGA (T8) and control (var. Moneymaker) in whole plant and non-choice leaf bioassay. Plants under - or overexpressed HQT. Plants were obtained from the *John Innes Center* at Norwich (UK). With A) First experiment, and B) second experiment. Ten replicates were used for each line. Different letters indicate significant differences in CGA content at the 0.001 level.

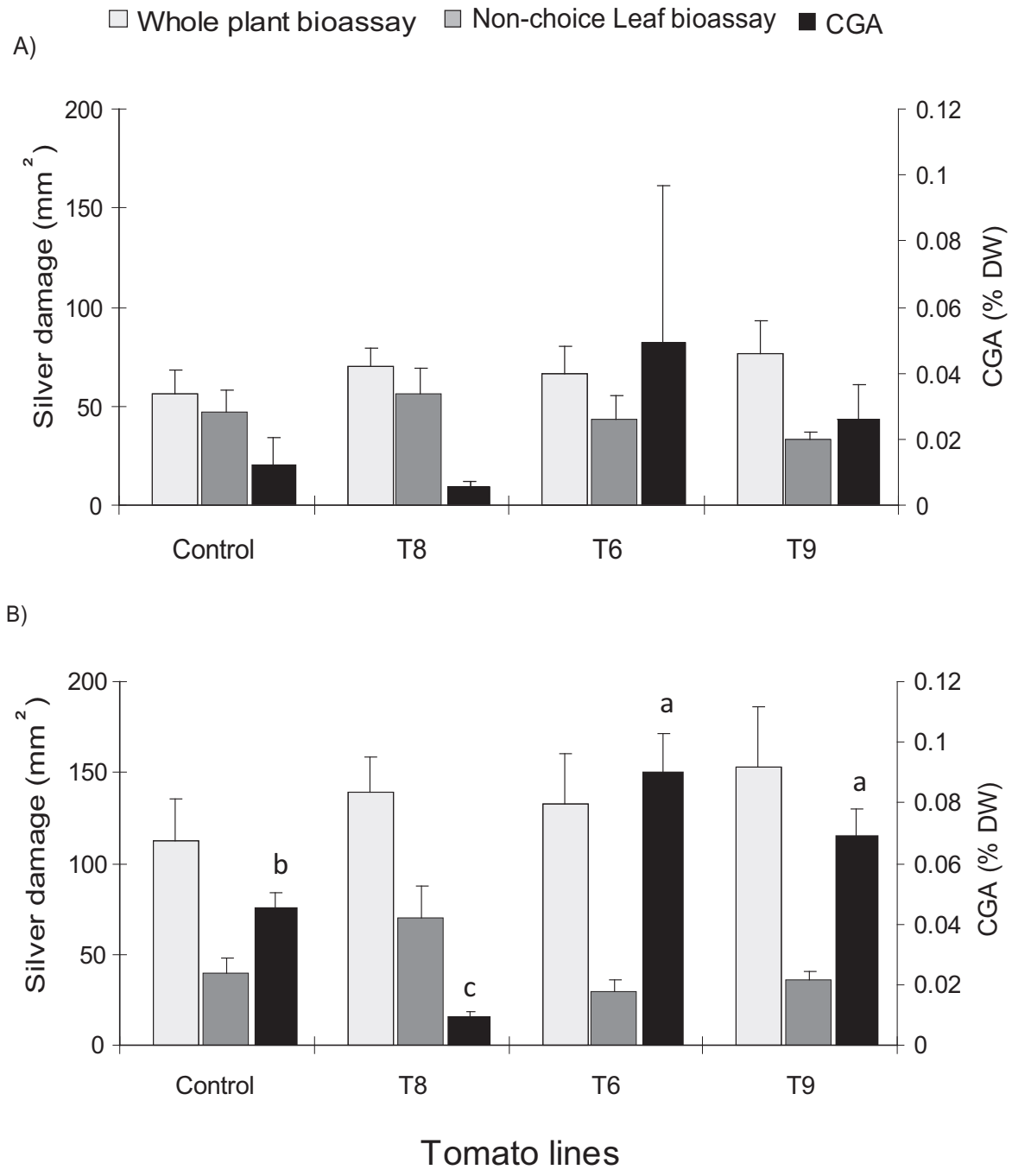

Figure. 2. Means and SE of silver damage and CGA content of transgenic tomato lines (TG) with alleged 20 fold increased CGA and control lines (var. Microtom) in whole plant and choice leaf bioassay. Plants were obtained from the *John Innes Center* at Norwich (UK). Plants overexpressed HQT. Ten replicates were used for each line. Asterisks indicate significant differences in CGA content between lines at the 0.001 level.

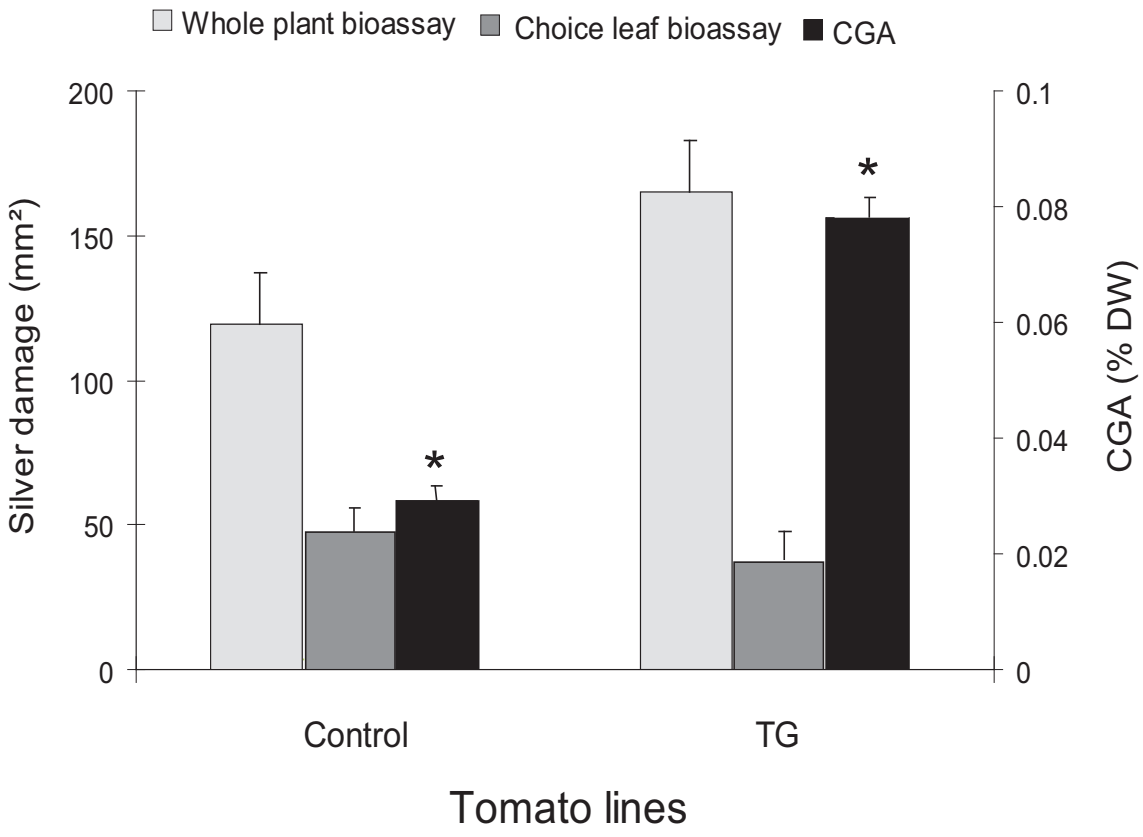

Figure 3. *In vitro* bioassay with first instar larvae of WFT on artificial diets with 0%, 0.06%, 0.13%, 0.5% and 2% chlorogenic acid (CGA). Means and standard errors of relative growth rate (A) and survival (B) are presented. For each diet 30 larvae were tested. The bioassay was performed twice.

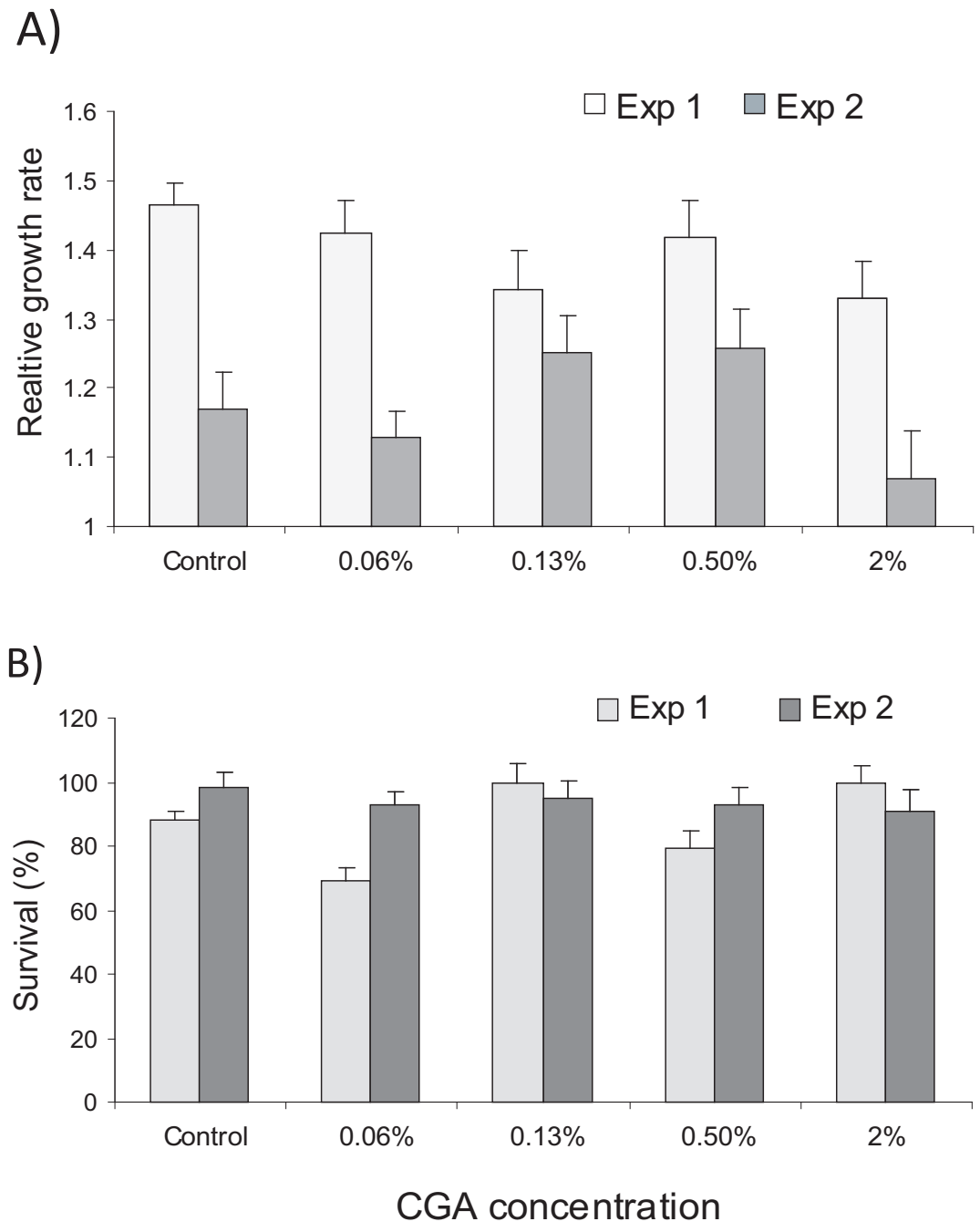

Figure 4. *In vitro* bioassay with first instar larvae of WFT fed on artificial diets with tomato fruit juice of transgenic tomatoes with an alleged 20 fold content of CGA. Means and standard errors of relative growth rate and amounts of CGA, expressed as % dry-weight content are presented. For each diet 30 larvae were tested.