



Effects of nymphal density (*Bactericera trigonica*) and feeding on photosynthetic pigments, proline content and phenolic compounds in carrot plants

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Accepted: 22 December 2021 / Published online: 29 January 2022
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Abstract Insect feeding induces physiological and biochemical changes in host plants. Indeed, symptom severity often depends on insect density. Recently, the carrot psyllid, *Bactericera trigonica*, has negatively affected carrot production in Mediterranean countries. The present study was conducted to evaluate effects of psyllid nymphal density on photosynthetic pigments and biochemical parameters of carrot leaves. Also, changes to physico-chemical parameters of carrot juice in response to insect feeding were quantified. A significant decrease in leaf weight, total number of leaves, root weight, chlorophyll (a + b) and carotenoid content was recorded in response to elevated infestations of *B. trigonica*. A positive correlation was observed between psyllid feeding and proline, total phenolic and flavonoid contents of leaves. A significant increase of titratable acidity was also observed in carrot juice obtained from infested plants,

while total soluble solids decreased as number of attacking insects increased. Results indicate that the stress response of carrot plants to psyllid infestation depended on nymphal density.

Keywords *Bactericera trigonica* · *Daucus carota* subsp. *Sativus* · Insect density · Chlorophyll · Carotenoid · Biochemical parameters

Introduction

Psyllid species are small insects that feed on plants by ingesting phloem sap. Psyllid species are vectors of economically important plant diseases, including many newly emerging ones. The three ‘*Candidatus Liberibacter*’ species associated with huanglongbing (HLB) citrus disease are transmitted by *Diaphorina citri* Kuwayama, *Trioza erytreae* Del Guercio, and *Cacopsylla citrisuga*. *Candidatus Liberibacter solanacearum* (CaLsol) is transmitted by *Bactericera cockereli* (Šulc) to potato and tomato plants (Secor et al., 2009). In northern Europe this disease was observed in carrot fields and transmitted by *Trioza apicalis* Foerster (Nissinen et al., 2014). Recently, a ‘*Candidatus Liberibacter*’ species was associated with vegetative disorders in carrot fields in Tunisia and transmitted by *Bactericera trigonica* Hodgkinson (Ben Othmen et al., 2018; Ben et al., 2019). This psyllid causes considerable damage to carrots (*Daucus carota* L) through injection of toxic saliva during

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feeding. Severe carrot psyllid attacks can lead to serious yield loss. Because effective management of psyllids is lacking worldwide, it is necessary to determine the impact of psyllid feeding on the physiology of their host plants.

The chlorophyll content of plant tissues is one of the main parameters impacted by interactions between host plants and insects. Indeed, chlorophyll levels change in response to a wide variety of stresses, including biotic stresses such as insect feeding and pathogen infection (Goławska et al., 2010; Heng-Moss et al., 2003; Ni et al., 2001, 2002). However, previous studies on *Bactericera trigonica* on carrots mainly focused on the control and biology of the pest. Thus, little is known about how psyllid damage may affect quantity and quality of carrot yields. Previous studies emphasized that carrot psyllid feeding significantly affects total sugars and phenolic content of tap-roots of carrot plants attacked by *T. apicalis* (Nissinen et al., 2012). The aims of the present study are to: i) investigate how nymphal density affects carrot yield; ii) quantify variation in foliar pigments, phenolic, flavonoids and proline levels in leaf tissues in response to increasing nymphal density; and iii) assess whether psyllid feeding affects the sugar content of carrot roots.

Materials and methods

Plant material and sample processing

The study was carried out in a carrot field located in the South Kairouan delegation “Zaafrana area” (35°32′31.21″N, 10°04′30.48″E). The field was planted with “Arbi Zaafrana” hybrids. The following symptoms were frequently observed in this field: general stunting, yellowing and curling of leaves. These symptoms were associated with the presence of a *B. trigonica* population. After collecting symptomatic plants, every five plants with the same nymph density were grouped together; in total, six groups of different psyllid nymph density were collected. The plant sampling was done according to the degree of damage sustained by each plant. Plant samples were immediately transported to the laboratory. The control group was represented by five plants not colonized by psyllid nymphs. To assess leaf damage, leaf pigments

contents were analyzed using a spectrophotometer (ThermoSpectronic Helios γ , Cambridge, England).

Carrot yield

For each carrot plant, roots were lifted, washed, and weighed individually.

Damage estimate

The number of symptomatic leaves and the total number of leaves of each sampled carrot plant were determined. If the oldest leaves were yellowish in color these were not considered as damaged but a result of normal senescence. The fresh weight of total carrot leaves was measured for each plant.

RT-PCR analysis

To confirm that psyllids were responsible for changes observed in carrot plants, a real-time PCR assay was performed according to Li et al. (2006) using a Light Cycler 480 (Roche, Switzerland) to detect the presence of CaLsol. The reaction mix consisted of 1X Quantimix (Biotools, Spain), 0.24 μ M of each primer, 0.12 μ M of TaqMan probe and 3 μ l of the template (purified DNA or direct extraction from the spot) in a final volume of 12 μ l. The real-time PCR amplification protocol included the following steps: 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 58 °C for 40 s. Primers and probe sequences were, respectively: LsoF, 5′-GTTCGAGCGCTTATTTTATA AGGA-3′; HLBr, 5′-GCGTTATCCCGTAGA AAAAGG TAG-3′; and HLBp, 5′FAM-AGACGG GTGAGTAA CGCG-3′BHQ. The positive results (Ct values \leq 40 and exponential curve) and the negative results were also checked by the real-time PCR protocol of Teresani et al. (2014). In this case, the reaction mix consisted of 1X Quantmix (Biotools, Spain), 0.5 μ M of each primer, 0.16 μ M TaqMan probe and 3 μ l of the template. The amplification protocol consisted of 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers and probe sequences were the following: CaLsppF, 5′GCAGGC CTAACACATGCAAGT3′; CaLsppR, 5′GCACAC GTTTCCATGCGTTAT-3′, and the specific TaqMan probe CaLsolP, 5′ FAM-AGCGCTTA TTTTAAATA GGAGCGGCAGACG-3′ TAMRA (Teresani et al., 2014).

Titrateable acidity (TA), pH, total soluble solids (TSS)

Carrot roots were crushed using a juicer. Juice pH was measured using a pH-meter (Jenway). The titrateable acidity was determined by acid-based potentiometry (0.1 mol L⁻¹ NaOH up to pH 8.1), expressed as g L⁻¹. The TSS content was recorded using a refractometer (Atago) at 20 °C with values being expressed as °Brix.

Extraction and measurement of chlorophylls a, b and carotenoids

The determination of chlorophyll was made according to Holden (1965). 0.1 g of leaves (± 5 leaves) were ground in a mortar with 10 ml of 80% acetone (CH₃COCH₃). The extract was then filtered through a 5 μ m diameter millipore filter. After 72 h in the dark at a temperature of 4 °C, the obtained filtrate was used to measure chlorophyll a, chlorophyll b and carotenoids using a spectrophotometer (ThermoSpectronic Helios γ , Cambridge, England). Chlorophyll a and chlorophyll b were measured at 645 and 663 nm and carotenoids at 470 nm. Each sample measurement was performed in three replicates and the pigment content was calculated from the following equations:

$$Chla(\text{mg/gFW}) = [(12.7 \times A663) - (2.69 \times A645)](V/1000W) \times e$$

$$Chlb(\text{mg/gFW}) = [(22.9 \times A645) - (4.68 \times A663)](V/1000W) \times e$$

$$Car(\text{mg/gFW}) = (1000 \times A470 - 1.82 \times Chla - 85.02 \times Chlb/198 \times (v/1000W) \times e$$

With: A: absorbance, V: extraction volume (10 ml), W: sample mass, e: dilution factor.

Determination of proline content

From each sample, 100 mg of leaves cut into small pieces were placed in test tubes containing 2 ml Methanol (40%). The mixture was heated to boiling in a water bath at 85 °C for 60 min. After cooling samples, 1 ml of the extract was removed from each sample and placed into a new tube to which was added: 1 ml of acetic acid, 1 ml of a solution containing 120 ml H₂O, 300 ml acetic acid, 80 ml ortho-phosphoric acid "H₃PO₄, density 1.7" and 25 mg of ninhydrin. The mixture was heated

to boiling for 30 min, till the solution turned to red. After cooling, 5 ml of toluene was added to each tube with stirring for two phases, then the upper phase was removed to which a pinch of Na₂SO₄ was added to remove water and the optical density was measured by a spectrophotometer at 528 nm (Bagues et al., 2017).

Determination of total phenolic content

The total phenolic content was determined using the Folin–Ciocalteu method (Aryal et al., 2019). Briefly, 1 mL of methanolic extract solution was mixed with 2.5 mL of 10% (w/v) Folin–Ciocalteu reagent. After 5 min, 2.0 mL of Na₂CO₃ (75%) were subsequently added to the mixture and incubated at 50 °C for 10 min with intermittent agitation. The absorbance was measured utilizing a UV spectrophotometer at 765 nm. The results (mean \pm standard error) were expressed as mg/g of gallic acid equivalents (mg GAE/g).

Determination of flavonoid content

The flavonoid contents were measured as described by Aryal et al. (2019). An aliquot of 1 mL of extract solution was mixed with 0.2 mL of 10% (w/v) AlCl₃ solution in methanol, 0.2 mL 1 M potassium acetate and 5.6 mL distilled water. The mixture was incubated for 30 min and the absorbance was measured at 415 nm. The results were expressed as mg quercetin equivalent/g.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA), and the significant differences between means were determined by Duncan's multiple range test using SPSS 20 software. Significance was defined at $P < 0.05$.

Results

CaLsol incidence

The real-time PCR assays were not able to confirm presence of CaLsol bacteria in any of the tested carrot plants.

Leaf weight, total number of leaves, number of curled leaves, root weight

The weight of fresh leaves decreased with nymphal density. A decrease of over 62% of leaf weight was observed when psyllid density exceeded eight nymphs per plant compared to controls. Results also showed that total number of leaves was significantly affected by psyllid nymph feeding and decreased with nymphal density. Also, the number of leaves with curling symptoms significantly increased with nymphal density. However, when the number of nymphs exceeded 8 nymphs per plant, there was no additional increase in number of symptomatic leaves with nymphal density (Table 1).

The root weight of the psyllid-exposed carrots significantly decreased as psyllid density increased. The psyllid nymphs greatly reduced the root fresh weight when their density exceeded eight per plant. A density of 15 nymphs per plant caused a decrease of over 85% of taproot weight (Table 1).

Changes in foliar pigments

Significant differences were recorded for pigment contents of carrot leaves infested with different densities of *B. trigonica* nymphs (Table 2). The chlorophyll a (Ch a) content was significantly lower in infested carrot leaves than that in control plants, with Ch a content decreasing as nymphal density increased. Also, a decrease of chlorophyll b (Ch b) content was recorded as psyllid nymph

density increased. Chlorophyll b content varied from 10.04 mg/g FW in controls to 6.62 mg/g FW in plants infested by eight nymphs and 3.43 mg/g FW in plants infested with 15 nymphs. Psyllid nymph density also had a significant effect on total carotenoid content. The highest content (891.37 mg/g FW) was registered in control plants. However, a decrease of 3.46-fold of total carotenoid content was observed for plants infested with 15 nymphs compared to controls.

Total phenolic content, total flavonoid content, proline content

Significant differences in total phenolic, flavonoid and proline contents were recorded between infested and non-infested leaves (Table 3).

Total phenolic contents (TPC) ranged from 13.97 mg/100 g F.W for control leaves to 70.60 mg/100 g FW in leaves infested with 15 *B. trigonica* nymphs. The TPC content showed a consistent increase in response to insect damage. Also, the total flavonoid content increased with the rate of *B. trigonica* leaf infection. The TFC content varied from 1.06 mg/100 g for control leaves to 21.44 mg/100 g for leaves infested with 15 nymphs. Regarding the proline content, a higher concentration was observed in infested leaves than in control leaves, with the increase proportional to nymphal density. It varied from 104.04 mg/100 g in control leaves to 521.87 mg/100 g in leaves infested with eight nymphs and 1274.18 mg/100 g in leaves infested with 15 nymphs.

Table 1 Effects of psyllid nymphal density on carrot root weight, leaf fresh weight, total number of leaves and number of curled leaves

Number of nymphs	Leaf weight (g)	Total number of leaves	Number of curled leaves	Root weight (g)
Control	30.66 ± 1.45 a	14.33 ± 0.47 a	0.00 d	212.76 ± 7.05 a
3	26.31 ± 0.95 b	10.00 ± 0.81 b	1.33 ± 0.47c	141.39 ± 3.10 b
5	20.97 ± 0.94 c	9.33 ± 1.24bc	1.67 ± 0.47bc	131.40 ± 5.06b
8	18.75 ± 0.48 c	7.67 ± 0.94 cd	2.33 ± 0.47 b	105.30 ± 7.57 c
10	16.00 ± 0.99 d	6.33 ± 0.47 d	3.33 ± 0.47 a	81.63 ± 8.10 d
12	14.17 ± 0.44 d	6.00 ± 0.81 d	3.33 ± 0.47 a	62.93 ± 4.00 e
15	11.60 ± 1.77 e	4.00 ± 0.81 e	3.67 ± 0.47 a	31.10 ± 7.47 f
Significance	**	**	**	**

Values are means of fifteen replicates ± standard error. Means followed by the different letter within a same column indicate significant differences according to Duncan test. Significance of F ratio: ** $P < 0.017$

Table 2 Changes of Chlorophyll contents (Ch a, Ch b) and total carotenoid content in carrot leaves according to psyllid nymphal density

Number of nymphs	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total carotenoids (mg/g FW)
Control	0.10 ± 0.01a	10.04 ± 0.51a	891.37 ± 14.65a
3	0.09 ± 0.01b	9.02 ± 0.21ab	830.50 ± 13.46ab
5	0.01 ± 0.01c	7.84 ± 1.26bc	756.36 ± 17.49b
8	0.01 ± 0.00c	6.62 ± 0.40 cd	597.81 ± 34.90c
10	0.00 ± 0.01c	5.39 ± 1.07de	539.30 ± 63.81c
12	0.00 ± 0.01c	4.73 ± 0.20de	431.03 ± 17.46d
15	0.00 ± 0.00d	3.43 ± 0.59 ^e	257.36 ± 6.24e
Significance	**	**	**

Values are means of fifteen replicates ± standard error. Means followed by the different letter within a same column indicate significant differences according to Duncan test. Significance of F ratio: ** $P < 0.01$

Table 3 Changes of total phenolic content, total flavonoid content, and proline content according to psyllid nymphal density. Values are means of fifteen replicates ± standard error. Means followed by the different letter within a same column indicate significant differences according to Duncan test. Significance of F ratio: ** $P < 0.01$

Number of nymphs	Total phenolic content (mg GAE.g ⁻¹)	Total flavonoid content (mg QE. g ⁻¹)	Proline (mg/100 g)
Control	13.97 ± 0.36f	1.06 ± 0.06f	104.04 ± 6. 68 g
3	17.73 ± 0.54f	1.73 ± 0.14f	245.66 ± 16.31f
5	24.17 ± 2.20e	4.20 ± 0.29e	426.76 ± 5.34e
8	30.08 ± 0.39d	6.52 ± 0.54d	521.87 ± 2.08d
10	47.90 ± 2.82c	8.40 ± 0.67c	687.17 ± 10.01c
12	61.58 ± 0.41b	10.55 ± 0.04b	1074.54 ± 24.05b
15	70.60 ± 3.88a	21.44 ± 1.32a	1274.18 ± 28.15a
Significance	**	**	**

pH, titratable acidity (TA), total soluble solids (TSS)

The pH value of carrot juices made from infested and control plants ranged from 6.36 to 6.93. The juice pH decreased significantly with psyllid density. The lowest pH value was observed in carrot juice from plants infested with 15 nymphs. The titratable acidity showed a significant variation between infested and non-infested plants (Table 4). A significant increase of TA values was associated with the increase of psyllid nymph density. The values ranged from 0.03 for controls to 0.19 in the most infested plants (15 nymphs). *Bactericera trigonica* infestation also significantly affected total soluble solids (TSS) values in carrot juice (Table 4). The TSS values ranged from 3.67°Brix to 7.90°Brix. The control plants had higher total soluble solids content than infested plants. A decrease of TSS values was noted with the increase of insect damage. The most exposed carrot plants (15 nymphs) had the lowest total soluble solids content.

Discussion

Bactericera trigonica is the most abundant insect species in carrot fields (occurring from crop emergence to harvest). This psyllid causes direct damage to carrot (Ben Othmen et al., 2018).

In this study, a significant decrease of root fresh weight was observed in response to increasing psyllid density. Feeding also significantly affected leaf fresh weight. Number of curled leaves increased with number of nymphs suggesting that leaf-curling is caused by psyllid feeding. Previous studies reported also that nymphs feeding at high density leads to a dramatic reduction in yield and leaf curling (Nissinen et al., 2007, 2012).

Changes in chlorophyll (Cha and Chb) and carotenoid contents in carrot leaves were associated with psyllid density. These contents decreased as number of nymphs increased. Similar results from other studies have revealed a significant reduction in total

Table 4 Changes of pH, titratable acidity and total soluble solids (TSS) in carrot juice according to nymphal density. Values are means of fifteen replicates \pm standard error. Means followed by the different letter within a same column indicate significant differences according to Duncan test. Significance of F ratio: ** $P < 0.01$

Number of nymphs	pH	Titratable acidity (g.l ⁻¹)	TSS (°Brix)
Control	6.93 \pm 0.15a	0.03 \pm 0.002d	7.90 \pm 0.12a
3	6.82 \pm 0.02ab	0.04 \pm 0.01d	6.00 \pm 0.080b
5	6.52 \pm 0.02bc	0.08 \pm 0.001c	5.50 \pm 0.0.15c
8	6.50 \pm 0.10bc	0.10 \pm 0.005b	5.13 \pm 0.06d
10	6.45 \pm 0.29c	0.12 \pm 0.01b	4.80 \pm 0.1e
12	6.41 \pm 0.25c	0.18 \pm 0.01a	4.20 \pm 0.01f
15	6.36 \pm 0.24c	0.19 \pm 0.01a	3.66 \pm 0.13 g
Significance	**	**	**

chlorophyll and carotenoid content in response to herbivore attack (Blanchfield et al., 2005, 2007; Kumar & Sharma, 2014). Grape leaves infested by phylloxera (*Daktulospharia vitifoliae* Fitch) had significantly lower contents of total chlorophyll and carotenoid than those in the control (Blanchfield et al., 2005, 2007). Huang et al. (2014) also reported that relative chlorophyll loss on Brassicaceous plant species was related to the feeding damage caused by *Bagrada hilaris* Burmeister. However, as psyllids are phloem feeders they are often thought to cause less severe damage to leaves than other groups of herbivores. Nevertheless, it was shown that *Diaphorina citri* (Kuwayama) and *Aonidella orientalis* (Newstead) are able to decrease the total chlorophyll and carotenoid contents in citrus and guava leaves, respectively (Killiny and Nehela, 2017; Kumar & Sharm, 2014). Dai et al. (2009) also reported that *Hypericum sampsoni* Hance plants synthesized less chlorophyll pigment in leaves infested with *Thrips tabaci* (Linderman) compared to control leaves. In fact, chlorophyll degradation is a complex phenomenon that is often linked to insect feeding damage to plants (Ni et al., 2001). Therefore, these changes have often been regarded as a relatively late mechanism of photosynthetic adaptation (Anderson et al., 1995). Golan et al. (2014) reported that *Coccus hesperidum* L. feeding induced a stress response in host plants, represented by a decrease in chlorophyll and carotenoid content and photosynthesis in response to increasing insect density.

In this study, psyllid infestation affected the total phenolic content of carrot leaves, which increased in response to *B. trigonica* feeding. These results are consistent with those reported by Talcott and Howard (1999) and Nissinen et al. (2012). Often, plants may

respond to pathogen and insect infestations by producing greater levels of secondary metabolites such as certain phenolics and terpenoids (Wallis et al., 2008). Plant phenols constitute one of the most common and widespread groups of defensive compounds, which play a major role in host plant resistance (HPR) against herbivores including insects (Sharma, 2008; Usha et al., 2010; War et al., 2011). Previous studies also reported that increased levels of polyphenolic molecules in the leaves increase defense response or resistance in several plants (Felton & Duffey, 1990; Miles & Oertli, 1993). Lattanzio et al. (2009) revealed that polyphenolic molecules in plants have been reported to fluctuate in response to insect damage. A defense mechanism occurs after pathogen or insect damage and may involve the activation of phenylalanine ammonia-lyase (PAL) (Felton et al., 1999). This enzyme acts as a catalyst in phenylpropanoid biosynthesis. Similar results revealed that PAL activity correlated with elevated concentration of phenols was strongly elevated in *Chrysanthemum* during the early period (0.5 to 6 h) after aphid infestation (He et al., 2011) and in kale after *P. brassicae* herbivory (Ibrahim et al., 2018).

Total flavonoid contents were higher in infested leaves compared to non-infested ones. Beyond their well-known antioxidant properties, flavonoids also play an important role in insect–plant interactions (Kovalikova et al., 2019). They are generally involved in plant resistance to insects. In the present study, carrot plants responded to *B. trigonica* feeding by increasing flavonoid production. The onset of heavy infestations was observed in conjunction with higher levels of flavonoids.

An increase of proline content was also noted under the stress of psyllid feeding. A positive

correlation was recorded between proline content and number of psyllid nymphs.

This may indicate that proline accumulation is a defensive response to insect herbivory as proline is a tissue-repairing metabolite. Similar results have been reported in eucalypt leaves infested with xylem-feeding insects (Khattab, 2005). Proline is a universal osmolyte accumulated in response to several stresses (Öncel et al., 1996) and may have a role in plant defense reactions (Ding et al., 2000; Stevenson et al., 2009). The excessive proline accumulation in proportion to number of attacking insects suggest that proline estimates may be used to determine the extent of herbivory in carrot and other plants. Such increases in total phenols, flavonoids and proline contents are considered elements of induced resistance in hosts against herbivory.

Changes of physico-chemical properties in carrot juices under insect stress were also determined. A decrease of juice pH was observed.

However, increasing psyllid density led to an increase in titratable acidity. Similarly, psyllid feeding was shown to increase orange juice acidity as reported by Plotto et al. (2010).

Total soluble solids (TSS) decreased as nymph density increased. This decrease may be due to a reduction in the surface area of the leaf exposed to light due to the damage associated with increased insect pest populations (Zubair et al., 2015). Zubair et al. (2017) reported that carbohydrates and total soluble solids also decreased due to stress induced by insect attack, which led to the reduction of prospective growth and ultimate juice content in citrus fruits. Thus, the reduction in sugar content caused by psyllid attack indicates increased respiration and carbohydrate consumption due to stress and wound-healing activity by the plant. This is confirmed by results from other studies of psyllid-exposed carrots (Nissinen et al., 2012).

Conclusion

Psyllid infestation causes physiological and biochemical changes to carrot plants. A decrease in the photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid content) was associated with psyllid nymph feeding. Proline content increased in response

to *B. trigonica* infestation. Infested leaves also showed higher phenol and flavonoid contents. Feeding by nymphs of *B. trigonica* also caused changes to parameters associated with carrot juice quality such as pH, TSS and titratable acidity. These changes could be considered as a result of the host's defense response.

All the tested carrot samples were negative for the presence of "*Ca. L. solanacearum*". Thus, the obtained results were mainly caused by nymph feeding. However, further studies are required to examine how carrot host chemistry can be affected by '*Ca. L. solanacearum*' which may contribute to developing a clear picture of carrot host responses to '*Ca. L. solanacearum*' or psyllids.

Acknowledgements We gratefully acknowledge the Tunisian Union of Agriculture and Fisheries (UTAP) for facilitating the fieldwork in the governorate of Kairouan.

The authors are grateful to Joshua Gearing for English editing that improved the quality of the manuscript.

Declarations

Competing of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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