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Effects of tomato leaves allelochemicals on tomato borer (*Tuta absoluta* Meyrick) in Tlemcen region, Algeria

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ABSTRACT

We investigated the qualitative and quantitative composition of phenolic compounds in tomato (*Lycopersicon esculentum* Mill L.) leaves with and without infestation of tomato borer (*Tuta absoluta* Meyrick). Infested and healthy leaves of tomato were extracted with aqueous methanol, which was partitioned with ethyl acetate and *n*-butanol. Infested leaves contained higher levels of total phenolics, flavonoids, flavonols and tannins. The HPLC analysis of the *n*-butanol fraction indicated that the leaves contained the catechin and two unknown compounds, which are likely to be phytoalexins. The protective role of these molecules need to be investigated, to incorporate this finding in the tomato breeding programmes against the tomato borer.

Key words: Algeria, allelochemicals, biochemical defence, HPLC, *Lycopersicon esculentum*, phenolics compounds, tomato, tomato borer, *Tuta absoluta*.

INTRODUCTION

The tomato borer (*Tuta absoluta* Meyrick, Lepidoptera: Gelechiidae) is a devastating insect pest in tomato (*Lycopersicon esculentum*) originated in South America and recently appeared in the Mediterranean region. It attacks the leaves of tomato at all growth stages, which reduces the yields and quality of tomato (9) and has become major insect pest in tomato (14). Insecticides are used to control it by growers, however, their effectiveness is limited due to the nature of insect damage and the development of resistant tomato borer biotypes (21). Another strategy to control this pest, is the development of resistant varieties, however, the resistance traits against *Tuta absoluta* are not yet identified in local tomato germplasm grown in Algeria. Tomato leaves produces several bioactive metabolites [steroidal alkaloids (11), phenolic compounds and flavonoids (10,25)]. These substances are involved in the host-plant defences and also have several pharmacological and nutritional functions in humans (11). They act as deterrents and toxins, especially the phenolic compounds to specialist herbivores such as *Tuta absoluta* (16). They can be produced in large quantity during an insect pest attack (13). In tomato, their accumulation is induced after insect attack (7). This research aimed to investigate the role of phenolic compounds in the biochemical defence of Algerian tomato against *Tuta absoluta*.

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MATERIAL AND METHODS

Collection of leaf samples

This research was conducted at Tlemcen province, northwest Algeria (34° and 35°30' north latitude and 1°20' and 2° 30' west longitude). It has temperate winter (Annual precipitation : 311.72 mm and maximum temperature : 33.68 °C, minimum temperature : 5.4 °C). Leaf samples were collected from a commercial crop of tomato (*Lycopersicon esculentum* Mill L.) of the Tafna variety from March to May 2015, during the infestation period of tomato borer. Samples (100 g, fresh weight) of infested or uninfested leaves of the same age (2-4 months) were harvested, washed, dried with a paper towel and dried in an oven at 60°C for 24 h. Then, they were powdered in a mortar with a pestle before use. Three samples were collected per treatment.

PREPARATION OF CRUDE EXTRACT AND ORGANIC FRACTIONS

(i). Crude extracts: Leaves (1 g) were powdered and extracted for 24 h with 20 ml of 80% aqueous methanol at room temperature, filtered through 0.45-µm-pore-size filter paper. The filtrates were evaporated to dryness under vacuum at 60 °C using a Buchi Rotavapor R-200 (3).

(ii). Ethyl acetate and *n*-butanol fractions of crude extract: The aqueous extract was partitioned first with ethyl acetate and then with *n*-butanol to extract the different classes of flavonoids. The extraction was done according to Bekkara *et al.* (2). The dry residues obtained from the crude extracts were dissolved in 10 ml of boiling water to dissolve the flavonoids. The aqueous solution was then filtered through 0.45-µm-pore-size filter paper. The filtered solution was first partitioned with 10 ml ethyl acetate and then with 10 ml of *n*-butanol. The two extracts were evaporated, weighed and finally dissolved in 3 ml methanol.

(iii). **Tannin fraction:** The tannins were extracted from the infested and uninfested tomato leaves as per the method of Zhang *et al.* (31). Five g of infested or uninfested leaves were dried in shade and milled into powder in a mortar with a pestle**.** This powder was extracted with 100 ml acetone-water (70:30, v/v) and the mixture was continuously stirred for 72 h at room temperature. Then, the mixture was filtered and evaporated under vacuum at 40 °C to remove the acetone. The remaining solution was washed with 30 ml dichloromethane to remove the lipid soluble substances. After separation of the organic phase, the aqueous layer was extracted with 15 ml of ethyl acetate. Then, the resulting water layer was evaporated to dryness and the dry residue was weighed and dissolved in methanol.

QUANTITATIVE DETERMINATION OF PHENOLICS

(i). Total phenolics content: These were spectrophotometrically determined by adding in a tube 1 ml of the Folin-Ciocalteu reagent to 200 µl of extract or fraction (24). Then, the mixture was diluted 10-times with water and 0.8 ml of 7.5% sodium carbonate solution. After stirring, the tube was left for 30 min. Then, the absorbance was measured at 765 nm on Jenway 6405 UVVIS spectrophotometer. Gallic acid was used as standard to build a calibration curve. The total phenolics content was expressed as mg of gallic acid equivalents per g in dry weight of an extract or organic fraction (mg GAE/g DW).

(ii). Total flavonoids content: These were determined according to Zishen *et al.* (32). Five hundred µl of an extract or fraction was dissolved in 4 ml methanol and evaporated to dryness. Then, the residue was dissolved in 1500 µl distilled water and 150 µl of 5% $NaNO_2$ was added. After 5 min, 150 µl of $AlCl_3$ (10 %, w/v) was added and the mixture was incubated for 6 min at room temperature. After adding 500 µl of 1 M NaOH, the mixture was fully homogenized by stirring. The absorbance of the solution was determined at 510 nm against the blank. The total flavonoids content of the extracts was expressed in mg catechin equivalents per g dry weight of extract or fraction (mg CE/ g DW).

(iii). Total flavonols content: These were determined according to Lee *et al.* (24). Aliquots (0.25 ml) of extract or fraction were mixed with 0.25 ml $AlCl_3$ (2 mg/ml) and 1.5 ml sodium acetate (50 mg/ml). The absorbance was recorded at 440 nm after 2.5 h. The content of flavonols was expressed as mg of quercetin equivalents per g in dry weight of an extract or a fraction (mg QE/ g DW).

HPLC analysis

The composition of *n*-butanol fraction from each leaf sample was determined using an HPLC (Agilent HPLC) system consisting of a Prostart11000 pump, a Hypersil C18 column (4.6 m x 250 mm, 5 µm). The mobile phase was water and acetonitrile. The sample was dissolved in 80% aqueous methanol and then filtered through a 0.45 µm-millipore filter. A 20 µL aliquot of the sample solution was injected. Elution of the phenolic compounds was monitored at 280 nm. Standards of gallic acid, catechin hydrate, ferulic acid, rutine hydrate, naringenin, coumarin, quercetin, vanillic acid, caffeic acid were purchased from Sigma (St. Louis, MO, USA). Peaks of the phenolic compounds in the samples were identified by comparing their retention times with those of standards and by co-injection of the *n*-butanol fraction with standards of phenolic compounds.

Statistical analysis

The mean values of total contents of phenolics, flavonoids and flavonols were subjected to one way ANOVA. The differences among means were tested by the LSD (least significant difference) at 0.05 level.

RESULTS

Total phenols, flavonoids, flavonols and tannin contents

The total phenolics, flavonoids and flavonols contents were measured in the crude extracts and its organic fractions obtained from the healthy and tomato-borer infested leaves (Figure 1). In the crude extract and the *n*-butanol fraction, leaves infested with *T. absoluta* showed significant higher level (6.88 ± 0.01 mg/g, 14% more; 13.04 ±0.82 mg/g, 27% more) of phenolic compounds than the undamaged leaves. The ethyl acetate fractions did not differ from each other in the total phenolics content. Total flavonoids content was same in the crude extracts (Figure 2). The ethyl acetate fraction contained the highest amount of these substances in healthy leaves (0.46 ± 0.01 mg/g, 18.5% more), while the *n*-butanol fraction showed the highest content in infested leaves (0.74 ± 0.01 mg/g, 90% more). Flavonols were more abundant in the crude extract (2.23 ± 0.12, 39% more) and the *n*-butanol fraction (1.01± 0.03, 71% more) of leaves damaged by the tomato borer (Figure

3) and in the ethyl acetate fraction of healthy leaves (1.01± 0.01 mg/g, 300% more). Infested leaves showed a 6-folds higher level of tannins than the undamaged leaves.

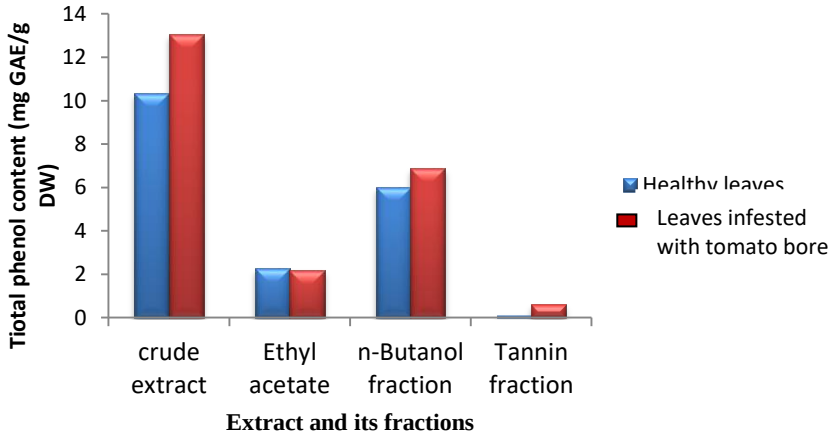

Figure 1. Total phenolics content of the crude extract and its organic fractions, and tannin fraction obtained from healthy and infested tomato leaves with tomato borer.

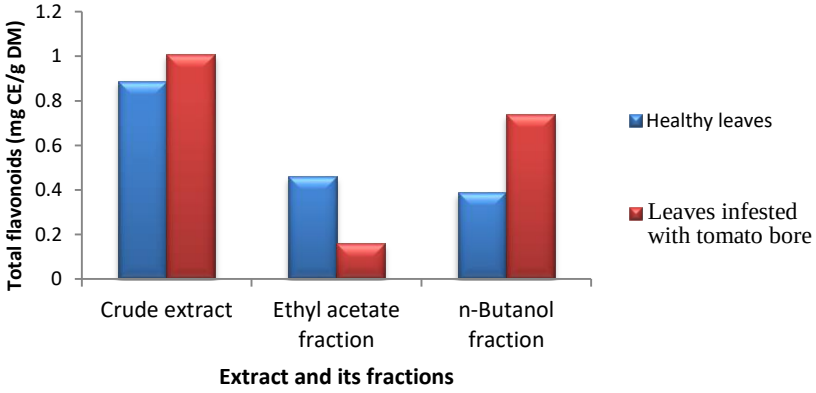

Figure 2. Total flavonoids content in the crude extract and the ethyl acetate and the butanol fractions obtained from healthy and infested leaves with tomato borer.

Identification of phenolic compounds

The four phenolic acids (Gallic, ferulic, vanillic and caffeic acids), four flavonoids (Catechin hydrate, rutine hydrate, naringenin and quercetin) and coumarin were determined in the crude extract and its organic fractions by HPLC coupled to a UV-VIS detector. The retention times obtained for these compounds were 3.67 min (gallic acid), 4.24 min (catechin hydrate), 5.56 min (caffeic acid), 6.05 min 16.87 min (ferulic acid), 16.75 min (rutine hydrate), 17.68 min (naringenin), 18.22 min (coumarin) and 18.46 min (quercetin). Chromatograms of crude extracts from healthy leaves and leaves infested with

T. absoluta showed same peak pattern. A similar situation was observed in the ethyl acetate fraction, but not in the *n*-butanol fraction, where peaks 1, 2 and 4 were only found in infested leaves (Fig. 4A) and peaks 5 and 6 were unique of undamaged leaves (Fig 4B). Peaks 3 and 6 were in both chromatograms. Peak 1 was identified as catechin hydrate which was absent in the healthy leaves.

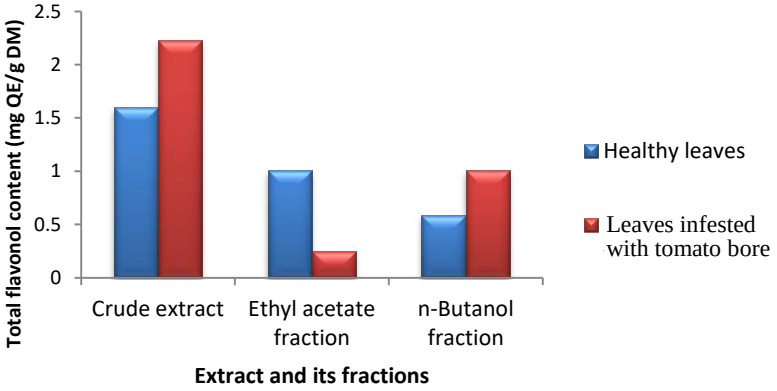

Figure 3. Total flavonols content in the crude extract and the ethyl acetate and the butanol fractions obtained from healthy and infested leaves with the tomato borer.

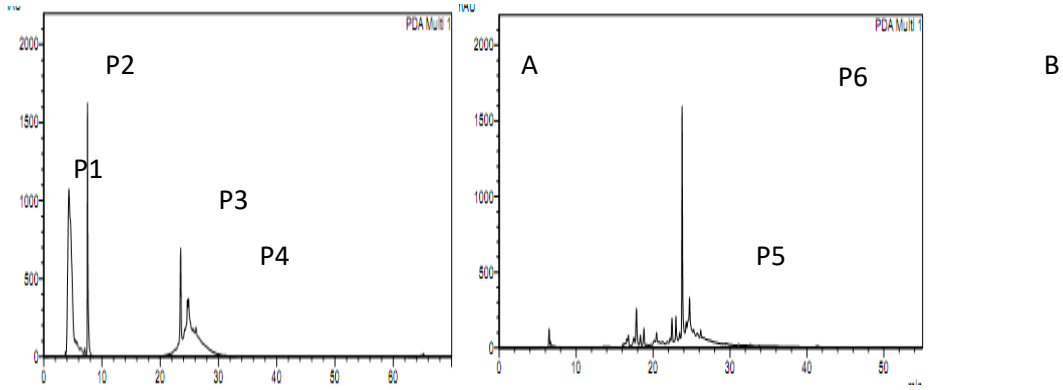

Figure 4. HPLC chromatograms showing the phenolic compounds present in the *n*-butanol fraction of (A) leaves infested with tomato borer and (B) undamaged tomato leaves. The peaks showed retentions times of 4.24 min (P1), 7.80 min (P2), 23.20 min (P3), 25.2 min (P4), 8 min (P5) and 24 min (P6). Peak one was identified as catechin hydrate. The remaining peaks are unknown compounds.

DISCUSSION

Phenolic compounds are structurally diverse metabolites with an ubiquituous distribution in higher plants. They have very diverse roles including the protection of plants against pests and diseases (15). They can be accumulated as structural components offering a physical barrier to the entrance of phytophagous and phytopathogenic organisms into the plant (Sampietro *et al*., 2009). Some plant species also increase their phenolics

contents after a pathogen or pest attack (1). This defence response often occurs together with a change in the carbon and energy flows, which are oriented to synthesize phenolic compounds with antimicrobial and/or antifeedant activities, instead of phenolic molecules with other physiological roles. Both situations occurred in leaves infested with *T. absoluta*. The increase in phenolic compounds synthesized in the damaged leaves was accompanied by a higher accumulation of tannins and *n*-butanol soluble phenolic compounds, including flavonol and flavonoids, not observed in healthy leaves. A similar result was observed in soybean plants (18) and in infested olives compared to non-infested olives (12). Usha Rani and Ravibabu (27) also showed that the content of phenolic compounds in plants infested with *Achaea janata* was higher than in those infested with *Spodoptera litura* and *Dichocrocis punctiferalis* (26). The reason for this differences may be that the defence response can be pest specific. In this work, the chemical response triggered by the tomato borer could not be associated with a brief incidence of *T. absoluta* on tomato leaves. Similar situations were previously reported (29) and are due to the fact that several natural plant pesticides often act as retardants of insect development without the immediate killing effect, usually generated by commercial insecticides (30). Hence, these phenolic compounds are likely to reduce the insect damage without completely stopping it. The synthesis of phenolic compounds reduces the chances of insect adaptations (20).

Regarding the HPLC analysis, the different patterns of peaks observed between the *n*-butanol leaf fractions confirmed the synthesis *de novo* of new compounds in leaves infested with *T. absoluta*, which were not found in the undamaged leaves. Catechin was identified but other unknown constituents were also induced. Catechin acts as the deterrent to the European corn borer (*Ostrinia nubilalis*) (20). This kind of phenolic molecules whose *de novo* biosynthesis is induced in response to insect attack are known as phytoalexins (30). Several phenolic compounds are identified in tomato leaves by HPLC-MS including the *trans* and *cis*-*p*-coumaric, caffeic, *trans*-ferulic, sinapic, protocatechuic and vanillic acids (4,5,22,28). Gallic acid, chlorogenic acid, rutin and quercetin were found as predominant phenolic compounds in tomato leaves (23,25). The inducible or constitutive expression of these phenolic compounds was not investigated in the tomato leaves. Nevertheless, they are likely to function as feeding deterrents or insect growth retardants. For example, ferulic acid incorporated into artificial diets decreased the survival and reduced the reproductive index of green bug (*Schizaphis graminum*) (6). The induced accumulation of phenyl propanoids such as ferulic and *p*-coumaric acids were reported in response to insect feeding in wheat tissues exposed to the wheat fly (*Sitodiplosis nonagriodies*) (8). Chlorogenic acid and rutin inhibited the early larval growth of the fruit worm (*Heliothis zea*) when added to artificial diets (17).

CONCLUSIONS

The leaves of tomato under attack of tomato borer (*T. absoluta*) accumulated higher contents of total phenolics, flavonoids, flavonols and tannin contents. The infested leaves contained the phenolic compounds different from the healthy leaves. Some phenolic compounds, including catechin, were *de novo* biosynthesized due to the insect attack. They are likely phytoalexins. Their protective role should be investigated to incorporate this finding in the tomato breeding programmes against the tomato borer.

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