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Title : Interrelated responses of tomato and the leafminer *Tuta absoluta* to nitrogen supply

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Short title: **Interrelated responses of tomato and *Tuta absoluta* to nitrogen**

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Abbreviations

CHAx: isomer x of caffeoyl hexaric acid

CP: caffeoylputrescine

FLA: flavonoids

FQA: feruloyl quinic acid

Hx: Harvest x

HCAD: hydroxycinnamic acid derivatives

HN: high nitrogen

KR: kaemperol rutinoside

LN: low nitrogen

N: nitrogen

NFT: nutrient film technique

pCoQA: p-coumaroyl quinic acid

PHE: phenolamide

PPO: polyphenol oxidase

PVPP: polyvinylpolypyrolidone

QAR: quercetin apiosyl-rutinoside

R: rutin

x-CGA: isomers of chlorogenic acid

ABSTRACT

Plant-insect interactions are strongly modified by environmental factors. This study evaluates, for the first time, the means by which nitrogen fertilisation affects the interaction between tomato plants (*Solanum lycopersicum* L. cv. Santa clara) and the leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae).

Greenhouse grown tomato plants were fed hydroponically on a complete nutrient solution containing either a low nitrogen concentration (LN) limiting plant growth or a high nitrogen concentration (HN) sustaining maximum growth. Insect-free plants were compared with plants infested by *T. absoluta*. Seven and 14 days after an artificial oviposition leading to efficacious hatching and larvae development, we measured vegetative tissue composition in primary insect resources (total carbon, nitrogen, protein) together with defencive compounds (phenolics, glycoalkaloids, polyphenol oxidase activity) in HN vs. LN plants.

It was only in the HN treatment that *T. absoluta* infestation slightly impaired leaf growth and induced polyphenol oxidase (PPO) activity in the foliage. The concentration of phenolic compounds and proteins, together with the total N distribution within the plant, were not affected by *T. absoluta* infestation. LN nutrition impaired the *T. absoluta*-induced PPO activity. It decreased protein and total nitrogen plant organ concentrations and enhanced the accumulation of constitutive phenolics and tomatine. Moreover LN nutrition impaired *T. absoluta* development by notably decreasing pupal weight and increasing the development time from egg to adult. Nitrogen nutrition may thus be a means of altering the life cycle of *T. absoluta*.

These results confirm for tomato, the existence of several cross-responses of plant composition and *T. absoluta* development to nitrogen nutrition.

INTRODUCTION

Plants respond to herbivorous insect feeding by means of a set of resistance mechanisms. These mechanisms involve localised and systemic synthesis/emission of secondary metabolites (Zangerl *et al.* 2002), induction of defencive enzymes (Stout *et al.* 1994) and tolerance mechanisms such as resource and metabolite remobilisation within plant organs (Tiffin 2000). It is now established that plant responses are highly specific to the insect feeding guild, this specificity being driven by the complex interaction of, at least, three major phytohormone signalling pathways *i.e.* jasmonic acid, ethylene and salicylic acid (Erb *et al.* 2012, Pieterse *et al.* 2009). Indeed, plants submitted to either phloem-feeding or chewing insects have been shown to exhibit differences in defencive enzyme induction (Felton *et al.* 1994), phenolic compound accumulation (Olson & Roseland 1991), regulation of primary metabolism (Schmidt *et al.* 2009) and transcriptomic responses (Kempema *et al.* 2007). By contrast, plant responses to leaf miners have received less interest (Stout et al., 1994, Cardoso *et al.* 2014, Zhang *et al.* 2012). Leaf mining results from the ability of insect larvae to feed and develop within plant tissues, mostly leaves and stems. From an ecological viewpoint, this feeding strategy confers protection against natural enemies and allows larvae to avoid the defence barriers (trichomes, spines…) on the leaf surface (Connor & Taverner 1997). Furthermore, it is acknowledged that leaf miners provide a valuable model to study plant responses to insect damage due to the intimate interactions created by larvae developing within plant tissues (Han *et al.* 2014, Inbar *et al.* 2001).

Plant-herbivorous insect interactions are highly dependent on environmental factors. Of these, plant nutrition and particularly nitrogen (N) fertilisation, has been widely studied (Bentz *et al.* 1995, Cates *et al.* 1987, Chen *et al.* 2010, Fischer & Fiedler 2000, Mattson 1980, Han et al., 2014). N is an important macronutrient for both plants and herbivores. Plants require a great deal of N to attain maximum growth and N concentration in the insect body tissues is more concentrated than in their foodstuff (Schoonhoven *et al.* 2005). N deficiency (or limitation) at the root level reduces tomato plant growth and N tissue concentration (Adamowicz & Le Bot 2008) whereas the concentrations of constitutive secondary compounds such as phenolic acids and flavonoids are increased (Fritz *et al.* 2006, Larbat *et al.* 2012a, Larbat *et al.* 2012b, Larbat *et al.* 2014, Le Bot *et al.* 2009) and also glycoalkaloids (Royer *et al.* 2013) in Solanaceae. From the viewpoint of the plant, N fertilisation affects the inducible defence in a complex way that depends on the pathway considered. Indeed, N limitation reduces the induction of trypsin inhibitor and the accumulation of nicotine in infected tobacco, whereas it has no effect on the induction of volatile terpenes (Lou & Baldwin 2004). From the insect viewpoint, plant N fertilisation influences the development of various herbivores, especially lepidopterans, through either (i) the plant nutritional value linked to tissue N concentration (Cates *et al.* 1987, Estiarte *et al.* 1994, Grundel *et al.* 1998, Han *et al.* 2014, Hunter & McNeil 1997, Inbar *et al.* 2001, Schoonhoven *et al.* 2005), or (ii) the content of constitutive or induced chemical compounds and mechanical plant defence (Gutbrodt *et al.* 2011, Koricheva 2002). The relative importance of both effects on herbivore performance, however, is difficult to assess and is likely pathosystem-specific.

The objective of this study was to evaluate the impact of N fertilisation on the ability of tomato to resist to the leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). This pest originating, from South America (Guillemaud *et al.* 2015), is now well established in Europe, Africa and the middle East since its first appearance in Spain in 2006 (Desneux *et al.* 2011, Desneux *et al.* 2010). *Tuta absoluta* larvae feed exclusively on Solanaceae, tomato plants being the major host. Leaf miners bring about serious leaf injuries, leading notably to hydraulic damage and reduction in C acquisition (Aldea *et al.* 2005, Tang *et al.* 2006), which eventually result in plant death. In tomato, *T. absoluta* is considered as a serious pest causing large production losses. Because it is an emergent pest in Europe, data in the literature remain insufficient to fully characterise the response patterns of tomato to *T. absoluta*, although these are necessary to develop pest control strategies, especially connected to management practice. A recent study from our group identified that water and N availabilities can modulate the tomato susceptibility to *T. absoluta* infestation and the *T. absoluta* development (Han et al., 2014). In this study, the physiological clues underlying these effects were not assessed. The present study was thus designed to assess the impact of N availability on the tomato plant response to *T. absoluta*. Our experimental strategy was to grow tomato plants hydroponically in a greenhouse under two regimes of nitrogen fertilisation, one limiting plant growth (low nitrogen: LN) and the other adequate for maximum growth (high nitrogen: HN). We measured tissue composition in primary insect resources (total carbon, nitrogen, protein) together with defencive compounds (phenolic compounds, glycoalkaloids, polyphenol oxidase activity) in the vegetative parts of HN vs. LN tomato plants subjected to *T. absoluta* infestation or maintained insect-free. The consequences of N fertilisation on leaf miner life traits were determined and analysed in relation to the composition of plant tissues.

MATERIALS AND METHODS

Plant growth, Tuta absoluta infestation and harvests

This experiment was carried out under glasshouse conditions in Avignon (43°56’58” N, 4°48’32” E). Tomato seeds (*Solanum lycopersicum* L. var. Santa Clara) were sown in an NFT (nutrient film technique) system set up in a growth room as described in Larbat *et al.* (2012a). Twelve-day-old plantlets were then transferred to the glasshouse and grown from May 30th to June 21th 2012 under the following conditions: heating when air temperature ≤ 18°C, ridge opening when ≥ 25°C, mist spraying when humidity ≤ 55%. The glasshouse was whitewashed to ease temperature control. Plants were grown hydroponically in six fully randomised blocks, each providing a complete nutrient solution at two regimes of N concentration, respectively representing high N (HN, 1.5 mM NO_3^-) and low N (LN). In the latter, [NO_3^-] was modified once per day (from 10 µM to 30 µM) in order to maintain the daily NO_3^- uptake of LN plants around 1/3rd of the value measured in the HN plants, using the Totomatix system (Adamowicz *et al.* 2012). During the experiment, [NO_3^-] and pH were corrected hourly in the nutrient solutions. In both solutions the sum ($NO_3^- + SO_4^{2-}$) = 12 eq m^{-3}, inferring constant concentrations of other ionic species in all treatments (Le Bot *et al.* 2009).

Insect preparation and infestation

To obtain the *T. absoluta* eggs the method of Chailleux *et al.* (2013) was used. Ten couples of adult insects were maintained for 24 hours in a double-cup system containing a fresh tomato leaf and honey provided as foodstuff, under the following conditions: air temperature 25°C, 65% relative humidity, 12/12h photoperiod. The adults laid eggs on this leaf. On the following day, an artificial oviposition was carried out whereby the eggs were gently transferred with a wet brush, to the terminal leaflet of the third leaf (counting from the base) of nineteen-day-old plantlets growing in the greenhouse. A load of two eggs was placed on each leaflet to maximise the chances of *T. absoluta* development. The infested leaves were bagged *i.e.* enclosed using a nylon mesh (0.2 mm, 30 × 24 cm). The third leaf of the non-infested plants (controls) was similarly bagged to take into account any possible effect of the mesh on leaf growth and metabolism.

Harvests and sample preparation

Three harvests were taken. The first (H1) was made on June 6th 2012 prior to *T. absoluta* oviposition, to characterise plant morphology and biochemical composition before infestation. The second (H2) and the third (H3) were taken 7 and 14 days respectively after *T. absoluta* oviposition. At each harvest, leaves, stems and roots were separated. Leaves were sub-sampled within the bulk foliage to separate the infested leaf (*i.e.* 3rd leaf from the base) and its opposite leaf (*i.e.* 4th leaf), which served to test for systemic plant responses. Roots were rinsed in deionised water and spin-dried (2 min at 2800 g). Plant parts were weighed, frozen in liquid N_2 and stored at -80 °C until freeze-drying. Dried samples were weighed, ground to a fine powder and stored under dry air in a desiccator at room temperature. In addition, at harvests H2 and H3, the leaflets containing the larvae were weighed and a digital picture was taken in order to calculate, by image processing, the number and the surface of mines dug by the larvae.

Tuta absoluta survival and development

For each infested leaf harvested at H3, larvae survival was recorded. Each infested leaf was thus kept in a double-cup system containing HN or LN nutrient solution as in the initial treatment, until the larvae of *T. absoluta* pupated and the adult emerged. The pupae were counted and weighed individually. The development time from egg to pupa or to adult was recorded for all individuals.

Standards and chemicals

Chlorogenic acid (5-CGA), rutin, kaempferol rutinoside, ferulic, p-coumaric and caffeic acids were purchased from Sigma (Steinheim, Germany). Solanine and tomatine were obtained from Extrasynthese (Genay, France). Caffeoylputrescine was kindly provided by Dr. Werck-Reichhart (IBMP, Strasbourg, France).

Analyses of plant tissues

Total C and N concentrations were determined using an elemental auto-analyser (Flash EA 1112 series, Thermo Fisher Scientific, Courtaboeuf, France), on 3 mg of dry powder, according to the Dumas method.

Phenolics and tomatine were extracted from 20 mg dry powder of the infested and opposite leaves, stems and roots as described in Royer *et al.* (2013). For tomatine quantification, the extract was diluted fiftyfold in 70% MeOH containing 2 µM solanine as internal standard. The compounds, from undiluted and diluted extracts respectively, were separated on a U-HPLC system (Prominence, Shimadzu, Japan) consisting in a binary solvent delivery pump connected to a diode array detector. Two microliters of extract were separated on a C18 Zorbax Eclipse Plus (150 mm × 2.1 mm, 1.8 µm) column (Agilent, USA) by using a gradient elution from 1 to 50% MeOH 0.1% formic acid (FA) in 7.1 min, then 99% MeOH 0.1% FA in 0.8 min with a flow rate of 430 µl min^{-1}. The column was rinsed during 2 min with 99% MeOH 0.1% FA and re-equilibrated to the initial conditions for 2 min prior to the next run. Phenolic quantification was based on the area under peak determined at 320 nm and expressed relative to calibration curves with ferulic acid (for FQA), chlorogenic acid (for 5-CGA, 1-CGA, 4-CGA), coumaric acid (for pCoQA), caffeic acid (for CHA1-6), caffeoylputrescine (for CP). Regarding flavonoids, quantification was determined at 350 nm and expressed relative to calibration curves with rutin (for R and QAR) and kaempferol rutinoside (for KR). Tomatine was detected by mass analysis carried out in ESI positive ion mode (ESI+) by following the major ion at m/z 529. The internal standard, solanine, was followed at m/z 868. Tomatine was quantified relative to a tomatine calibration curve (0.2-10 µM). Mass spectrometric conditions were previously described in Royer *et al.* (2013).

Polyphenol oxidase and total protein assays

Polyphenol oxidase (PPO) and total protein assays were made on H3 leaf extracts. All the leaves of each individual plant were pooled, frozen in liquid nitrogen and crushed in a mortar. Then, 50 mg of fresh weight (FW) were macerated, in a 2 ml tube, with 500 µl cold extraction buffer (sodium phosphate 0.1 M pH 7 with 3% polyvinylpolypyrolidone (PVPP) and 1% Triton X-100). This extract was mixed with a vortex for 1 min, then centrifuged at 10000 g for 10 min. The supernatant was used for both the polyphenol oxidase assay and the total protein assay.

PPO assay was carried out by mixing 20 µl of leaf extract with 200 µl of pre-warmed reaction buffer (sodium phosphate 0.1 M pH 7 containing 3 mM caffeic acid). PPO activity was determined by monitoring the appearance of quinone products from caffeic acid at 470 nm at 25°C. PPO global activity was then expressed as the rate of absorbance change per mg of FW and PPO specific activity as the rate of absorbance per mg of total protein.

Protein quantification followed the Bradford procedure using bovine serum albumin as standard. Concentration was expressed as mg protein per g of leaf FW.

Data processing

Whole leaf and damaged areas, perimeters and mine numbers were determined by image processing with Adobe Photoshop CS4 extended (Adobe systems Software, Ireland Ltd.). Computations were performed using the R software (R project for statistical computing, available at http://www.r-project.org) and statistical significance was set at $p < 0.05$. Analyses of variance were performed using the aov procedure, with nutrition, and infestation as fixed factors and blocks as random. Box-plots, Normal Q-Q plots and correlation between variance and mean, assessed the data distribution and homoscedasticity. Square root transformation was necessary for homoscedasticity of third leaf weight data. *Tuta absoluta* survival on tomato plants subjected to the nitrogen treatments was analysed using a log-linear model. Proportions of individuals alive at each developmental stage were compared by pairwise Fisher’s exact tests (with the Dunn–Sidak adjustment method). The effects of the nitrogen treatment on development time from egg to pupa stage and from egg to adult stage were tested, as well as on pupal weight using a generalised linear model with a log-link function.

RESULTS

Impact of N and T. absoluta on plant growth

In the LN treatment, the insect-free plants showed no visual symptom of N deficiency throughout the entire experiment. However, they were markedly reduced in shoot FW as compared with the HN plants (Table 1). At the first harvest, shoot FW was significantly decreased ($p = 0.04$) by 21%, the difference between the two N regimes in favour of the HN treatment increasing at harvests H2 (42%, $p = 2\ 10^{-8}$) and H3 (66%, $p = 8\ 10^{-18}$). For both N treatments, infestation by *T. absoluta* did not significantly alter the shoot FW at H2 and H3. However, the infestation specifically decreased the FW of the infested leaves at H2 and H3 in the HN treatment ($p < 0.003$).

Tissue N concentration and C/N ratio

At H3, tissue N concentration and C/N ratios were significantly altered by N nutrition (Fig 1. A-B). Indeed, N limitation significantly reduced N concentrations ($p < 0.001$) and thus, increased C/N ratios ($p < 0.001$) in all tissues. The effects were organ dependent ($p < 0.001$), being more pronounced on stems. Insect feeding did not affect the N concentration or C/N ratio of any tissue ($p = 0.13$ and 0.20 respectively).

Soluble phenolics

The phenolic composition was highly dependent on plant organs (Sup. data 1). Fourteen phenolic compounds *i.e.* six isomers of caffeoyl hexaric acid (CHA1-6), three isomers of chlorogenic acid (5-CGA, 3-CGA and 1-CGA), feruloyl quinic acid (FQA), coumaroyl quinic acid (pCoQA), rutin (R), quercetin apiosyl-rutinoside (QAR), kaempferol rutinoside (KR) and caffoylputrescine (CP) were investigated (12 in infested and opposite leaves, 10 in stems and 3 in roots). Analyses were carried out for the three harvest periods (H1 to H3, all data are detailed in Sup. data 2), but for the benefit of the reader, the 14 phenolic compounds were pooled into 3 groups *i.e.* hydroxycinnamic acid derivatives (HCAD, comprising CHA1-6, 1-

CGA, 3-CGA, 5-CGA, pCoQA and FQA), flavonoids (FLA, comprising R, QAR and KR) and phenolamides (PHE, comprising CP).

HCAD, FLA and PHE were distributed differently within the plant. HCAD and PHE were detected in all plant organs (Sup. data 1-2) with the highest HCAD concentration in leaves (infested and opposite, $p < 0.001$) and the highest PHE concentration in stems ($p < 0.001$). FLA were detected only in shoots (Sup. data 1). The concentration of all phenolic groups varied between harvests in all organs, but not in the same way (Sup. data 2). HCAD concentrations were highest at H1 ($p < 0.001$) but did not differ significantly between H2 and H3. PHE concentrations were highest at H1 and H2 then dropped markedly at H3 to reach undetectable levels in opposite leaves. By contrast, FLA concentrations increased significantly at H3 ($p < 0.001$). Nitrogen limitation did not affect HCAD and FLA concentration at H1. However, LN clearly increased HCAD and FLA concentrations in all organs at H2 and H3 ($p < 0.001$) (Fig. 2). LN reduced PHE concentrations in stems and roots at H3 only. Insect feeding did not affect HCAD, FLA and PHE concentrations at any harvest nor in any organ.

Tomatine

The concentration of tomatine, the major tomato glycoalkaloid involved in plant defence, was determined in all plant organs at H2 and H3 (Fig. 3). Tomatine responded significantly to N nutrition only in the stems, where its concentration increased under LN (H2 $p < 0.001$, H3 $p < 0.05$). Insect feeding significantly affected tomatine concentration in stems and roots at H2 with opposite effects. Indeed, tomatine concentration decreased in the roots of infested plants ($p < 0.01$) while it increased in stems ($p < 0.01$). These effects disappeared at H3. Insect feeding also brought about a small, non-significant ($p = 0.06831$) decrease in tomatine concentration of infested leaves at H3.

Inducible responses of proteinaceous defence and total protein at H3

The effects of N limitation and insect infestation on polyphenol oxidase (PPO) activity and total protein content were assessed in infested and control leaves at H3 (Fig. 4 A, B, C). The global PPO activity expressed on a leaf FW basis (Fig. 4-A) increased under *T. absoluta* infestation. The effect was significant under HN ($p < 0.01$), but not under LN ($p = 0.32$). The global PPO activity did not respond to N nutrition. By contrast, total protein concentration (Fig. 4-B) responded significantly to N nutrition (infested and control leaves, $p < 0.001$) but not to *T. absoluta* feeding ($p = 0.29$). The specific PPO activity expressed on a total protein basis (Fig. 4-C) increased significantly in response to insect feeding in the HN treatment only ($p < 0.05$). In addition, the specific PPO activity was higher under LN ($p < 0.001$) than under HN.

Tuta absoluta traits

The survival of *T. absoluta* (Fig. 5) decreased significantly in response to low N nutrition ($\chi^2 = 4.8$, df = 1, $p = 0.028$) and varied with the insect's developmental stage ($\chi^2 = 11.9$, df = 3, $p = 0.008$). The interaction between both factors was not significant ($\chi^2 = 2.5$, df = 3, $p = 0.47$). Under LN, the survival rate was mainly reduced during the larval stage while under HN, the survival rate did not differ significantly between egg and larva or egg and pupa stages. It is only between egg and adult stages that it was possible to observe a significant decrease in the survival rate of *T. absoluta*.

Tuta absoluta development

Overall, the LN treatment significantly depressed pupal weight at H3 (Fig. 6-A; $\chi^2 = 6.4$, df = 1, $p = 0.011$). Low N nutrition significantly increased the duration of insect development from egg to pupa ($\chi^2 = 9.9$, df = 1, p = 0.002, Fig. 6-B), as well as from egg to adult ($\chi^2 = 4.6$, df = 1, p = 0.032, Fig. 6-C).

DISCUSSION

Tuta absoluta infestation slightly impaired plant growth and modified tissue composition in a N-dependent manner

Tuta absoluta infestation generated moderate plant responses. It decreased FW accumulation in the infested leaves, it enhanced PPO activity in HN leaves and brought about discrete and transient modification of stem and root tomatine concentrations. Besides, as expected at this short-term time scale, *T. absoluta* infestation did not alter the C/N ratio, or the concentrations of N, proteins and phenolics in the vegetative organs. The small egg load during oviposition (only two per plant) may not be the main reason for this moderate plant response. Indeed, in a recent study, Mouttet *et al.* (2013) reported that the pre-infestation of tomato with three *T. absoluta* larvae was enough to expand plant susceptibility to oïdium infection at a systemic level, implying that plant physiology can respond to a small load of *T. absoluta* individuals. Moreover, our recent unpublished data also confirm induction of response at a very low larval density. The design of the experiment and harvest procedure may also have hindered an existing stronger local response at the leaflet scale, however the study form Mouttet et al. (2013) demonstrated the existence of a systemic tomato response to *T. absoluta*.

The low defensive response of tomato to *T. absoluta* could thus be explained by the insect feeding mode. The plant response to *T. absoluta* has not been described previously neither on tomato nor on other Solanaceous plants. But leaf mining effects on tomato have been previously assessed in several studies on the serpentine leaf miner *Liriomyza sp* (Stout et al., 1994, Inbar et al., 1999, Kawazu et al., 2012). Indeed, leaf mining by *Liriomyza trifolii* led, also, to a smaller induction of defensive proteins in tomato plants (peroxidase, lysozyme) than did other insects with other feeding guilds including leaf-chewing and sap-sucking (Stout et al., 1994, Inbar et al., 1999). This stealthy effect may result from the signalling pathways activated in response to the leaf miner infestation. A recent study highlighted that the jasmonic acid (JA) pathway, which activates the expression of a large range of defensive proteins in tomato, was induced only moderately and transiently before the leaf miners enter the tissue, but not once the larvaes were inside. On the contrary, once inside, the larvaes activated the salicylic acid (SA) pathway which acts antagonistically to the JA pathway (Kawazu et al., 2012). The authors postulated that this SA activation pathway may be a strategy of the leaf miner to decrease the JA-induced tomato defence. Since the low response of tomato to *L. trifolii* and *T. absoluta* compared, a more in depth study should be conducted to identify possible similarities in the plant response to these two leaf miners.

Tomato plants responded to *T. absoluta* infestation by activating some resistance mechanisms. We observed in particular an increase in PPO activity in the leaves of the high N treatment. Such an increase in PPO activity is a well-known response to chewing insects, and pathogens (Mayer 2006, Stout *et al.* 1998) but, to our knowledge, this paper is the first to report such a response for the tomato-*T. absoluta* pathosystem. The enzyme PPO catalyses the oxidation of phenolic compounds into quinones, which can bind to amino acids. This accumulation of by-products alters the plant nutritional quality and may also be toxic to the larvae (Constabel & Barbehenn 2008). Additionally, the soluble phenolic compound concentrations in leaves and other vegetative organs were not affected by *T. absoluta* infestation. A similar pattern (induction of PPO with no impact on soluble phenolics) was previously shown in tomato infested by the chewing insect *Helicoverpa zea* (Stout *et al.* 1998), indicating common tomato response traits to different insect feeding guilds.

Although tomatine and more generally glycoalkaloids were previously described as toxic compounds for many insect larvae (for review, see Friedman 2002), the effect of herbivory on these compounds is scarcely documented and seems to depend on the insect feeding guild (Fragoyannis *et al.* 2001, Hlywka *et al.* 1994). Our data show that *T. absoluta* infestation had no significant effect on the foliar tomatine concentration but it induced limited and opposite responses on stem (increase) and root (decrease) concentration at the second harvest, 7 days after *T. absoluta* oviposition. This observation suggests the transport of tomatine from roots to stems. However, a specific experiment is needed to confirm this hypothesis.

Leaf FW accumulation and PPO activity responded significantly to *T. absoluta* infestation only under the HN treatment. Our results for PPO activity differ from other studies dealing with leaf response to damage, which show that inductions of PPO and proteinase inhibitor, another protein-based plant defence, were not affected by N availability in tomato (Stout *et al.* 1998, Tan *et al.* 2012). Our data, however, are in agreement with those of Lou & Baldwin (2004), who showed that low nitrogen fertilisation reduced the magnitude of damage-induced signalling pathways together with the accumulation of nicotine and trypsin inhibitor in *Nicotiana attenuata* infested with the chewing insect *Manduca sexta*.

Low N nutrition altered T. absoluta development, plant growth and tissue composition

LN nutrition significantly impaired the development of *T. absoluta*, by decreasing the survival rate from egg to larvae, pupa and adult and by increasing the development time from egg to adult. LN also reduced the pupa weight. These results accord fully with the observations of Han *et al.* (2014) for the same pathosystem, and raise the question of how LN nutrition is able to impair *T. absoluta* development. Our analyses of plant tissue composition (and particularly the leaves) clearly indicate two possible explanations. Firstly, LN nutrition lowered the plant nutritional value for *T. absoluta* by decreasing total N and protein concentrations and increasing the C/N ratio. Since N concentration is higher in the herbivore than in plant tissues, and because N-based compounds (notably proteins) are essential for larval growth, LN conditions necessarily lower the grazing efficiency of the insect for biomass production, thus impairing its development. The second explanation is that LN increased the level of tomato plant constitutive defence, by increasing the concentration of glycoalkaloids and phenolic compounds but not phenolamides. All these compounds are known to be toxic or repellant to a large array of organisms including insects. Thus, we hypothesise that reduction in plant nutritional quality and increase in constitutive defence both contribute to the observed effect of LN on *T. absoluta* development. Our finding that PPO activity is not induced under LN reinforces this view, and might also indicate low plant responsiveness to *T. absoluta* under N limitation. To confirm these assumptions, however, it will be necessary to determine the impact of LN on other tomato inducible responses. These include the induction of the methyl-jasmonate pathway, the activity of other defencive enzymes and the emission of volatile organic compounds, which have recently been shown to increase in *T. absoluta* infested tomato plants (Strapasson *et al.* 2014).

From a practical viewpoint, lower N fertilisation input induced a lower survival and a sub-optimal development status in *T. absoluta*, which may offer a possible means of pest management strategy via manipulation of fertilisation regimes in managed cropping systems (*i.e.* glasshouse tomato production). However, the efficiency of such a strategy should be tested at population scales, by integrating the impact of *T. absoluta* infestation and the impact of N itself on tomato yield, using different levels of N supply, since N supply is a detrimental elemental governing tomato yield (Warner et al., 2004). Furthermore, the induction of higher chemical defence by *T. absoluta* may influence the fitness of other pest insects (*i.e.* aphids and whiteflies), or the infection of plant pathogens, often coexisting in tomato crops (Mouttet *et al.* 2013). This might be even more complex when organisms from the higher trophic level are involved (*i.e.* predators) (Bompard *et al.* 2013). Overall, the net effect of N fertilisation on tomato plant health (or yield) depends on the interactions of various factors: the occurrence of insect pests (single or multiple species), plant pathogens and natural enemies introduced into the system.

The present study provided some clues concerning cross-responses of tomato plants and the leaf miner *T. absoluta* to N nutrition. *T. absoluta* infestation led to a slight plant response, restricted to induction of PPO activity and reduction of fresh weight accumulation in HN plants. LN nutrition impeded PPO induction in infested plants but also impaired the development of *T. absoluta*. This effect of LN nutrition on *T. absoluta* development may be explained by the tissue composition of the LN-fed plants, which were depleted in primary resources (total N and protein) and enriched in constitutive soluble defence molecules (phenolic compounds and tomatine). Further investigation is necessary to assess the relative contribution of the primary resource depletion and the constitutive defence accumulation on *T. absoluta* development.

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FIGURE LEGENDS

Figure 1: Effects of N supply and *T. absoluta* infestation on C/N ratio (A) and N concentrations (B) in tomato vegetative organs at the third harvest (H3). Inf L: Infested leaf, Opp L: Leaf opposite to the infestation site, S: Stem, R: Root. Error bars are standard error of means (± SEM, n=6).

Figure 2: Effects of N supply and *T. absoluta* infestation on HCAD (A), FLA (B) and PHE (C) concentrations in tomato vegetative organs at the third harvest (H3). Inf L: Infested leaf, Opp L: Leaf opposite of the infestation site, S: Stem, R: Root. Error bars are standard error of means (± SEM, n=6)

Figure 3: Effects of N supply and *T. absoluta* infestation on tomatine concentrations at the second (H2) and third (H3) harvests in infested leaf (A), leaf opposite to the infestation site (B), stem (C) and root (D). Error bars are standard error of means (± SEM, n=6).

Figure 4: Effects of N supply and *T. absoluta* infestation on global PPO activity (A), total protein content (B) and specific PPO activity (C) in leaves at the third harvest (H3). Error bars are standard error of means (± SEM, n=6).

Figure 5: Survival rate of *T. absoluta* individual eggs reaching larva, pupa or adult stage feeding on HN or LN tomato plants (HN: high nitrogen; LN: low nitrogen; n=24) For each nitrogen treatment, bars followed by the same letter are not significantly different (pairwise Fisher's exact tests with Dunn–Sidak adjustment method).

Figure 6: (A) Mean pupal weight (mg, ± SEM, n = 9-16); Mean development time (B) from egg to pupa (days, ± SEM, n = 9-16) and (C) from egg to adult (days, ± SEM, n = 7-15) of *T. absoluta* individuals feeding on HN or LN tomato plants (HN: high nitrogen; LN: low nitrogen). * $p < 0.05$, ** $p < 0.01$ GLM analysis).

Supplemental data 1: U-HPLC chromatograms of soluble phenolics from vegetative organs of tomato (cv. Santa Clara) grown under HN (high nitrogen) nutrition. The profiles were recorded at 300 nm. A: leaf; B: stem; C: root. IS: Internal Standard.

Supplemental data 2: Concentration of phenolic compounds and tomatine in different tomato organs in Control (= insect-free) or *T. absoluta* infested (= Tuta) plants harvested immediately prior to infestation (H1), 7 days (H2) or 14 days (H3) after the infestation. Plants were grown hydroponically in the greenhouse and supplied with a complete nutrient solution with either high nitrogen (HN) or low nitrogen (LN) concentration. Inf L: Infested leaves, Opp L: opposite leaves. All concentrations are given in µg g^{-1} DW except for tomatine (in mg g^{-1} DW). Molecules abbreviations as follows: CHA1-6 = six isomers of caffeoyl hexaric acid; 5-CGA, 4-CGA and 1-CGA = three isomers of chlorogenic acid; FQA = feruloyl quinic acid; pCoQA = coumaroyl quinic acid; R = rutin; QAR = quercetin apiosyl-rutinoside; KR = kaemperol rutinoside; CP = caffoylputrescine; HCAD = hydroxycinnamic acid derivatives (CHA1-6 + 1,4,5 CGA + FQA + pCoQA); FLA = flavonoids (R + KR + QAR). Values are means of 6 replicates and are given ± SE. nd: not defined.

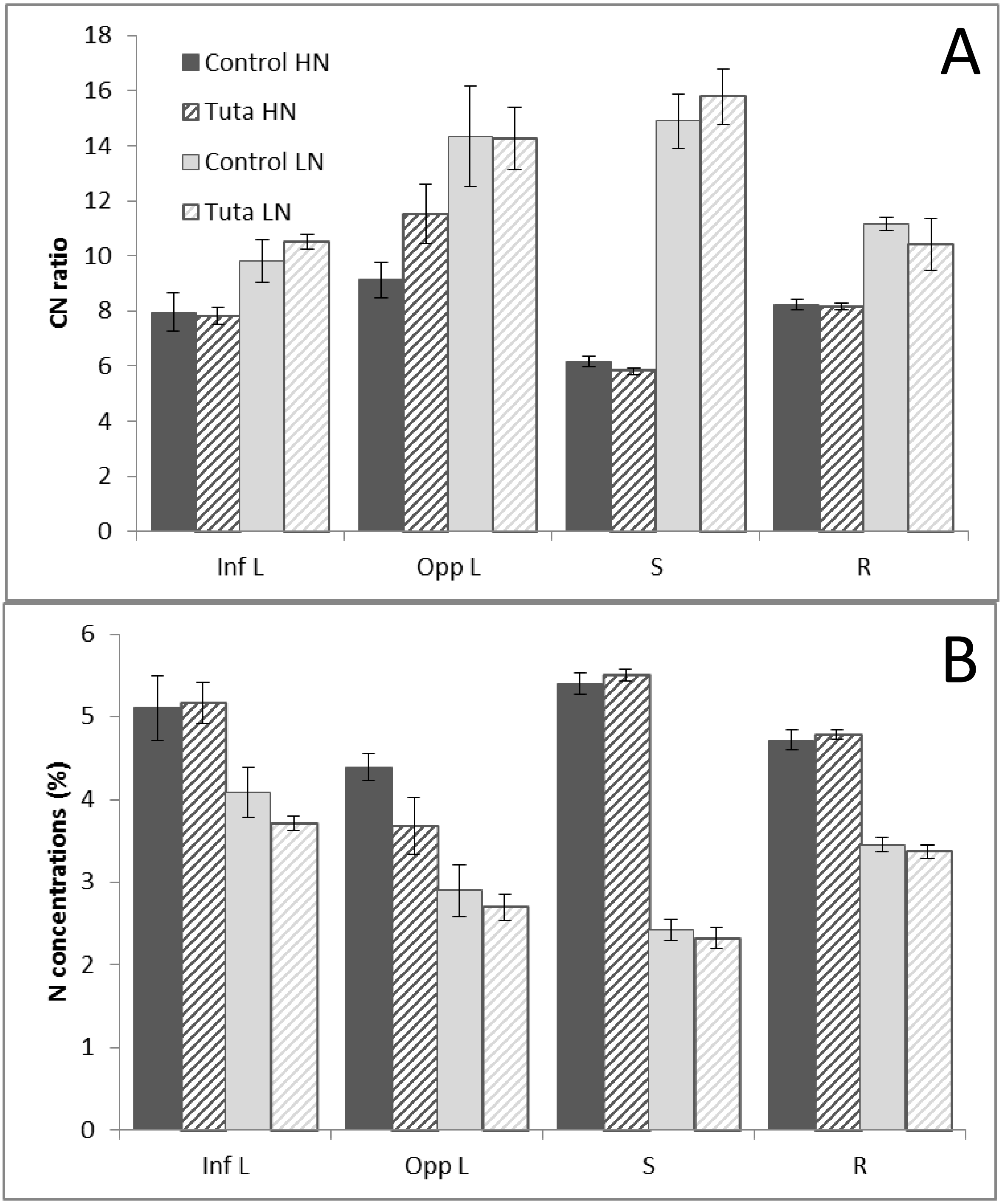
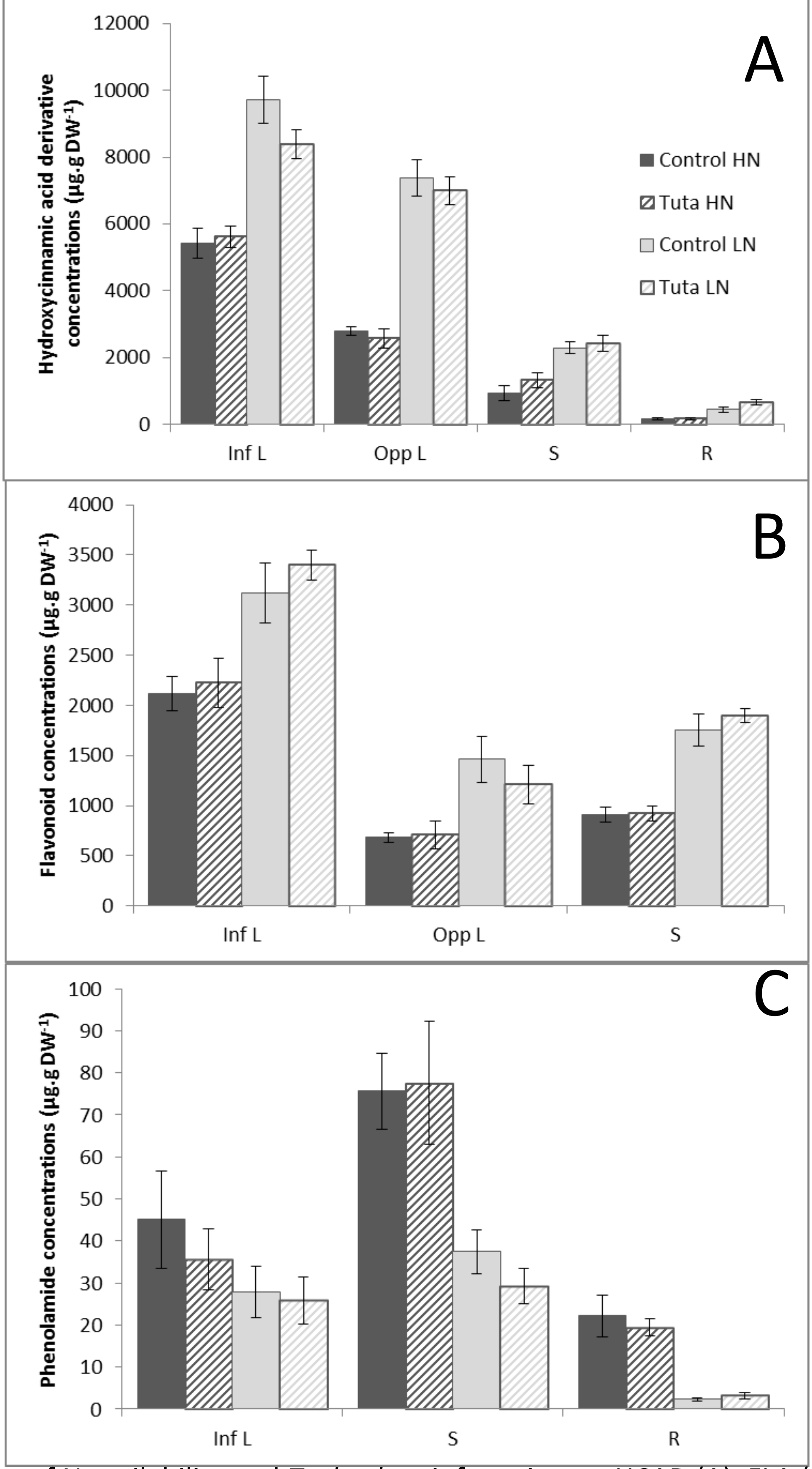


Figure 1: Effects of N availability and *T. absoluta* infestation on CN ratio (A) and N concentrations (B) in tomato vegetative organs at the third harvest (H3). Inf L: Infested leaf, Opp L: Leaf opposite to the infestation site, S: Stem, R: Root. Error bars are standard error of means (± SEM, n=6).



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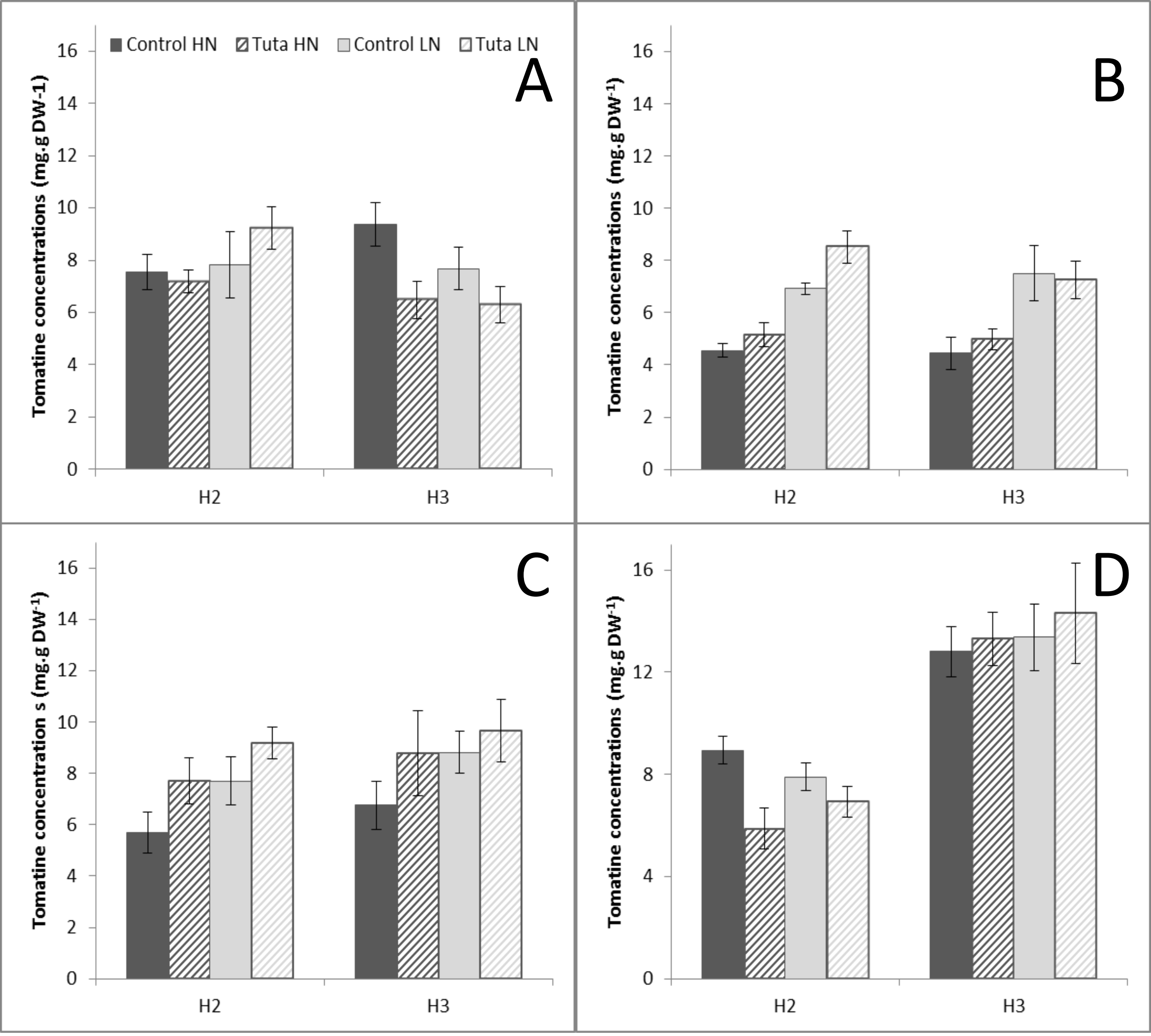

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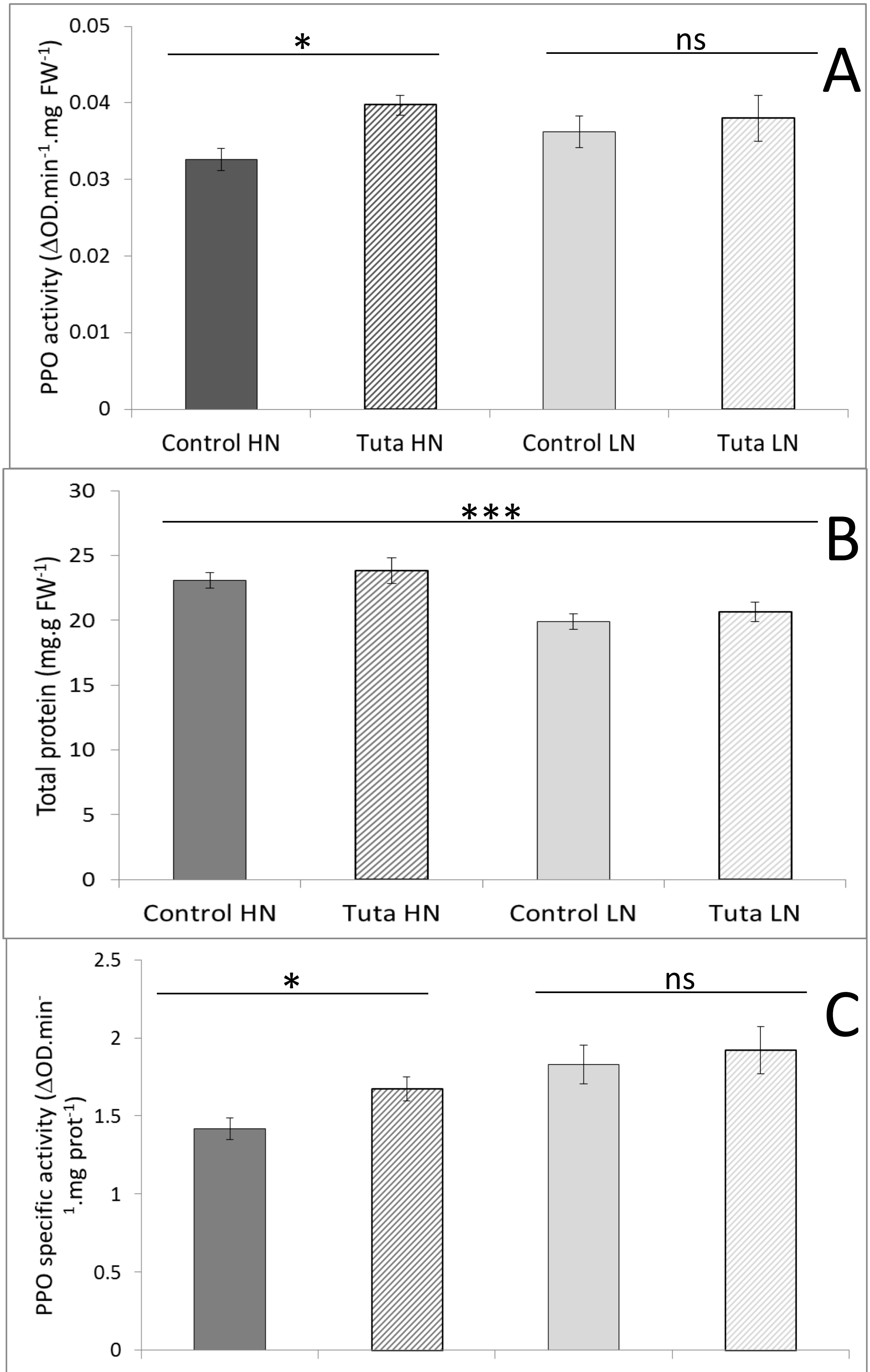

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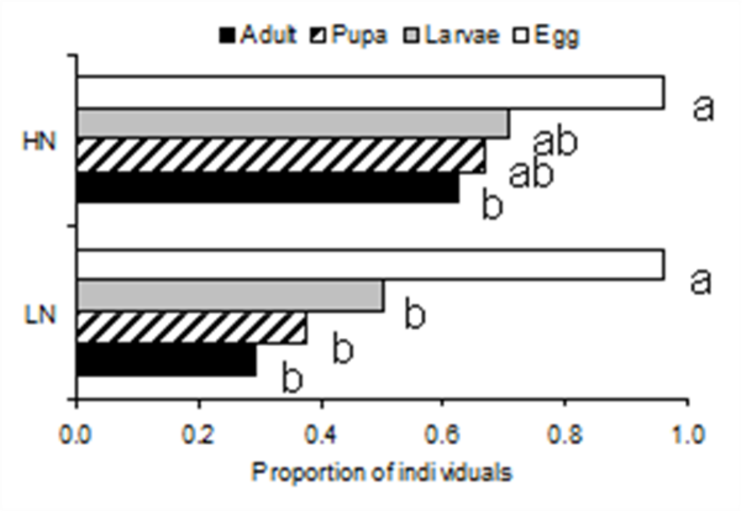

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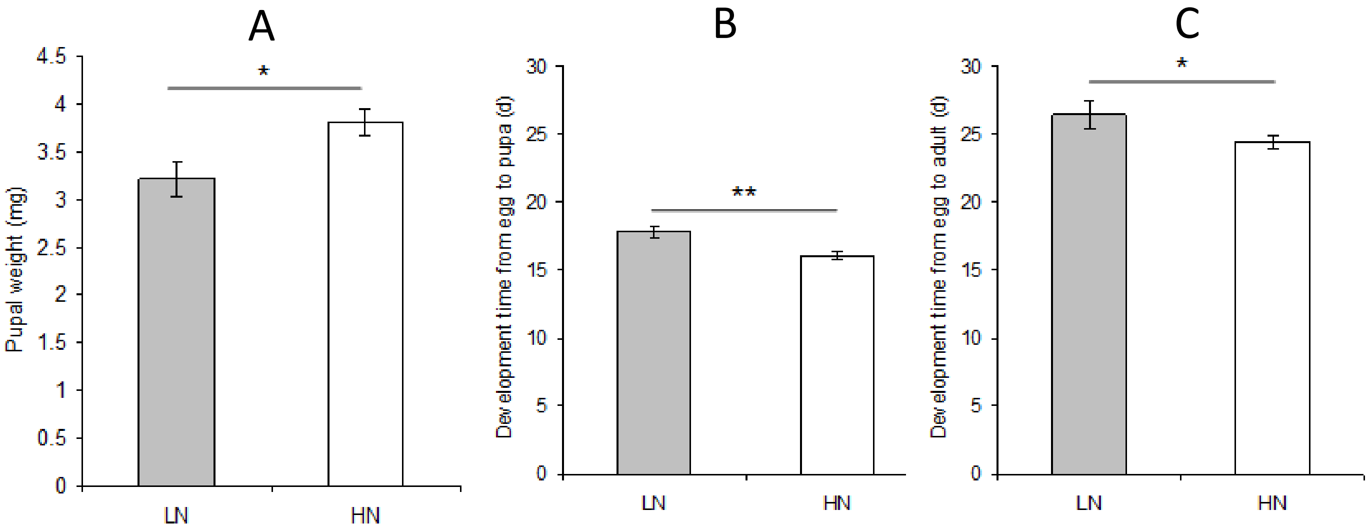
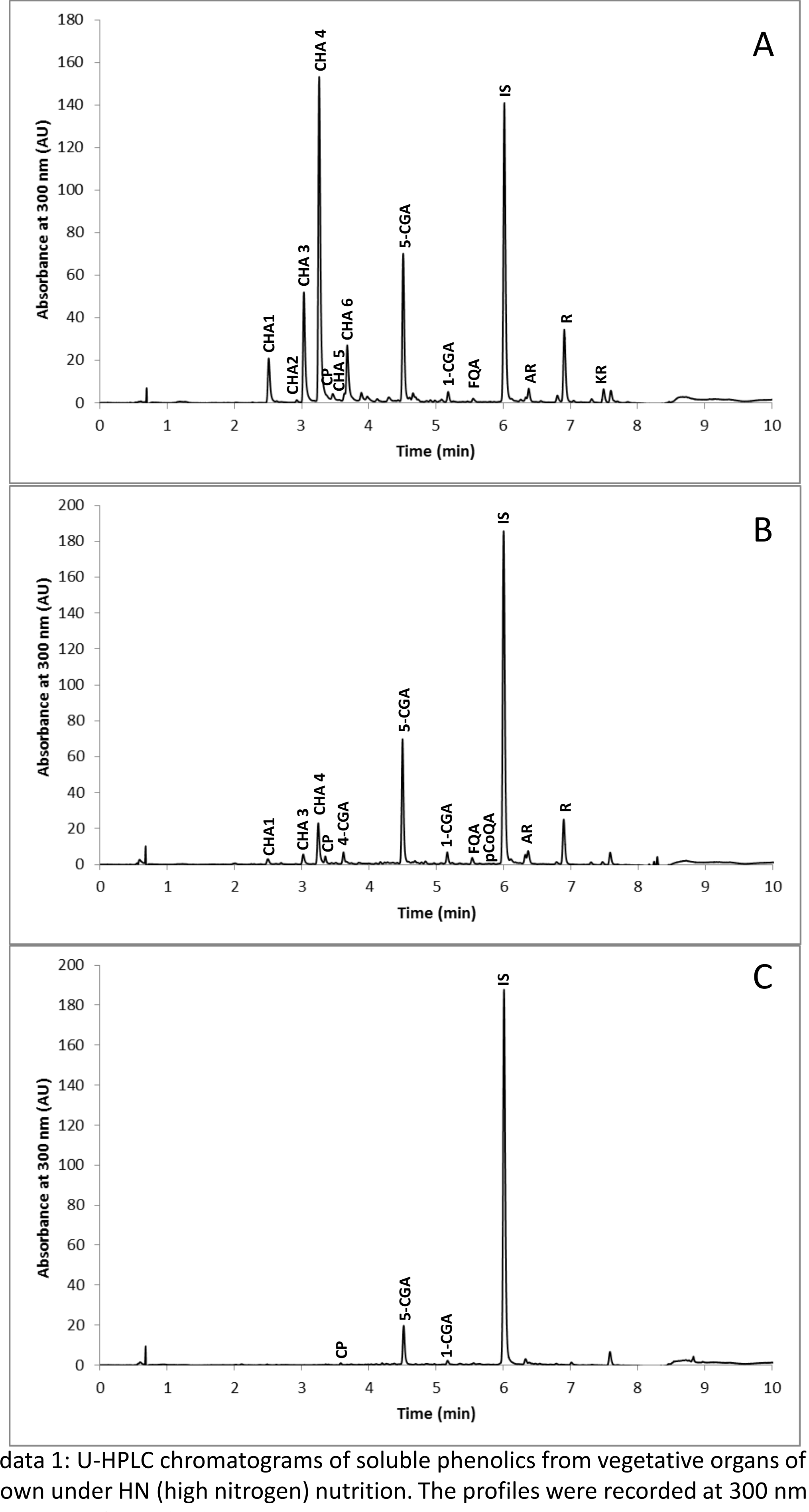


Figure 6: (A) Pupal weight (mean ± SEM, n = 9-16); Development time from (B) egg to pupa (mean ± SEM, n = 9-16) and(**C**) from egg to adult (mean ± SEM, n = 7-15) of *T. absoluta* feeding on high nitrogen (HN) vs. low nitrogen (LN) tomato plants. (* $p< 0.05$, ** $p < 0.01$ GLM analysis).

		Insect-free		**Infested**	
	Harvests	HN	LN	HN	LN
Shoot	1	4.1 (0.4)	3.2 (0.3)	-	-
	2	38.4 (2.2)	22.3 (1.6)	34.3 (1.9)	23.6 (2.1)
	3	227.0 (13.1)	77.3 (3.9)	183.6 (12.3)	81.0 (5.5)
3rd Leaf	2	5.3 (0.4)	3.4 (0.4)	4.6 (0.4)	3.1 (0.2)
	3	9.8 (0.6)	4.4 (0.3)	6.6 (0.7)	4.4 (0.3)

Table 1 : Impact of N availability and *T. absoluta* feeding on the plant fresh weight (g/plant). n=12, standard errors are between brackets



Supplemental data 1: U-HPLC chromatograms of soluble phenolics from vegetative organs of tomato (cv. Santa Clara) grown under HN (high nitrogen) nutrition. The profiles were recorded at 300 nm. A: leaf; B: stem; C: root. IS: Internal Standard.

Supplemental data 2: Concentration of phenolic compounds and tomatine in different tomato organs in Control (= insect-free) or T. absoluta-infested (= Tuta) plants harvested immediately prior to infestation (H1), 7 days (H2) or 14 days (H3) after the infestation. Plants were grown hydroponically in the greenhouse and supplied with a complete nutrient solution with either high nitrogen (HN) or low nitrogen (LN) concentration. Inf L: Infested leaves, Opp L: opposite leaves. All concentrations are given in µg g-1 DW except for tomatine (in mg g-1 DW). Molecules abbreviations as follows: CHA1-6 = six isomers of caffeoyl hexaric acid; 5-CGA, 4-CGA and 1-CGA = three isomers of chlorogenic acid; FQA = feruloyl quinic acid; pCoQA = coumaroyl quinic acid; R = rutin; QAR = quercetin apiosyl-rutinoside; KR = kaemperol rutinoside; CP = caffoylputrescine; HCAD = hydroxycinnamic acid derivatives (CHA1-6 + 1,4,5 CGA + FQA + pCoQA); FLA = flavonoids (R + KR + QAR). Values are means of 6 replicates and are given ± SE. nd: not defined.

Inf L

Harvests	Nutr	Treat	CHA1	CHA2	CHA3	CHA4	CHA5	CHA6	5CGA	1CGA	AR	R	KR	FQA	CP	Tomatin (mg/g DW ^{1})	HCAD	FLA
H1	HN	Control	514 ± 91	37 ± 7	739 ± 136	5070 ± 588	137 ± 25	420 ± 58	3000 ± 228	133 ± 34	109 ± 7	636 ± 20	241 ± 10	36 ± 2	80 ± 13	nd	10088 ± 1114	986 ± 25
	LN	Control	788 ± 65	54 ± 4	1206 ± 110	6422 ± 667	108 ± 25	575 ± 69	2949 ± 288	135 ± 29	112 ± 5	694 ± 70	250 ± 9	34 ± 2	67 ± 10	nd	12273 ± 1194	1057 ± 69
H2	HN	Control	293 ± 20	18 ± 2	541 ± 44	2875 ± 175	29 ± 2	340 ± 17	1601 ± 109	70 ± 4	59 ± 16	651 ± 42	99 ± 5	28 ± 2	107 ± 14	7.5 ± 1.0	5796 ± 324	810 ± 52
	HN	Tuta	281 ± 32	20 ± 2	518 ± 62	2814 ± 253	27 ± 2	340 ± 26	1589 ± 131	75 ± 7	63 ± 6	695 ± 55	104 ± 12	30 ± 2	113 ± 11	7.2 ± 0.6	5691 ± 490	862 ± 72
	LN	Control	478 ± 19	27 ± 4	951 ± 51	4171 ± 153	46 ± 2	574 ± 39	1848 ± 197	81 ± 9	88 ± 5	1024 ± 73	165 ± 10	32 ± 1	118 ±8	7.8 ± 1.2	8208 ± 242	1277 ± 85
	LN	Tuta	422 ± 31	27 ± 2	836 ± 73	3714 ± 234	44 ± 5	506 ± 22	1638 ± 144	73 ± 7	79 ± 9	914 ± 80	152 ± 12	29 ± 2	113 ± 16	9.2 ± 0.8	7289 ± 404	1145 ± 99
H3	HN	Control	385 ± 44	16 ± 4	805 ± 95	2574 ± 300	42 ± 9	535 ± 36	967 ± 158	54 ± 16	326 ± 26	1606 ± 150	185 ± 13	43 ± 4	45 ± 11	9.4 ± 0.7	5421 ± 462	2117 ± 167
	HN	Tuta	405 ± 31	18 ± 3	860 ± 70	2563 ± 155	41 ± 6	634 ± 64	1004 ± 142	52 ± 13	431 ± 129	1617 ± 227	179 ± 21	40 ± 7	36 ± 7	6.5 ± 1.0	5619 ± 309	2226 ± 245
	LN	Control	660 ± 89	25 ± 3	1534 ± 225	3947 ± 392	61 ± 9	981 ± 127	1710 ± 228	48 ± 2	409 ± 49	2195 ± 7287	263 ± 37	67 ± 10	28 ± 6	7.7 ± 0.4	9723 ± 709	3117 ± 299
	LN	Tuta	539 ± 38	22 ± 3	1280 ± 85	3618 ± 248	52 ± 6	855 ± 50	1868 ± 115	65 ± 16	487 ± 22	2607 ±111	304 ± 19	86 ± 9	26 ± 5	6.3 ± 1.0	8388 ± 420	3398 ± 149

Opp L

Harvests	Nutr	Treat	CHA1	CHA2	CHA3	CHA4	CHA5	CHA6	5CGA	1CGA	AR	R	KR	FQA	CP	Tomatin (mg/g DW	HCAD	FLA
H2	HN	Control	329 ± 52	22 ± 6	637 ± 146	2889 ± 567	38 ± 9	360 ± 43	1141 ± 253	56 ± 13	50 ± 8	510 ± 98	83 ± 16	24 ± 4	8 ± 3	5.7 ± 0.8	5497 ± 1100	644 ± 120
	HN	Tuta	363 ± 40	24 ± 5	708 ± 80	3126 ± 312	43 ± 5	358 ± 22	1197 ± 185	63 ± 8	59 ± 9	681 ± 93	99 ± 14	25 ± 3	16 ± 5	7.7 ± 0.9	5908 ± 634	839 ± 115
	LN	Control	651 ± 20	29 ± 7	1316 ± 40	5372 ± 268	62 ± 2	550 ± 18	1539 ± 79	66 ± 5	98 ± 8	968 ± 64	157 ± 13	31 ± 2	14 ± 4	7.7 ± 0.9	9617 ± 307	1223 ± 81
	LN	Tuta	508 ± 53	33 ± 4	1050 ± 110	4209 ± 498	52 ± 6	464 ± 48	1234 ± 222	54 ± 11	78 ± 12	767 ± 132	136 ± 21	24 ± 3	11 ± 4	9.2 ± 0.6	7629 ± 858	980 ± 164
H3	HN	Control	185 ± 11	10 ± 1	440 ± 27	1322 ± 66	23 ± 2	542 ± 16	236 ± 417	25 ± 4	95 ± 7	532 ± 39	58 ± 4	13 ± 2	0	6.7 ± 0.9	2797 ± 125	685 ± 47
	HN	Tuta	170 ± 22	8 ± 2	407 ± 49	1223 ± 145	21 ± 3	488 ± 57	224 ± 30	18 ± 2	78 ± 13	574 ± 113	57 ± 10	11 ± 2	0	8.8 ± 1.6	2570 ± 300	709 ± 135
	LN	Control	560 ± 38	27 ± 1	1398 ± 77	3237 ± 237	61 ± 4	1355 ± 54	659 ± 86	56 ± 9	214 ± 27	1100 ± 184	147 ± 24	23 ± 3	0	8.8 ± 0.8	7377 ± 541	1461 ± 233
	LN	Tuta	534 ± 38	25 ± 2	1357 ± 104	3075 ± 195	58 ± 6	1361 ± 91	525 ± 37	38 ± 2	185 ± 21	905 ± 146	120 ± 21	18 ± 2	0	9.7 ± 1.2	6991 ± 423	1210 ± 187

Stems

Harvests	Nutr	Treat	CHA1	CHA3	CHA4	4CGA	5CGA	1CGA	AR	R	FQA	*p* CoQA	CP	Tomatin (mg/g DW $^{-1}$)	HCAD	FLA
H1	HN	Control	228 ± 31	301 ± 44	1614 ± 89	34 ± 6	2392 ± 165	55 ± 4	nd	594 ± 43	89 ± 5	21 ± 2	188 ± 23	nd	4733 ± 298	594 ± 43
	LN	Control	291 ± 24	375 ± 34	1438 ± 131	39 ± 9	2173 ± 162	52 ± 2	nd	485 ± 58	94 ± 5	19 ± 2	1689 ± 16	nd	4482 ± 303	485 ± 59
H2	HN	Control	39 ± 6	67 ± 9	403 ± 60	49 ± 8	847 ± 139	56 ± 11	42 ± 4	351 ± 28	45 ± 5	11 ± 1	197 ± 33	4.6 ± 0.3	1734 ± 96	407 ± 31
	HN	Tuta	46 ± 4	81 ± 8	436 ± 34	66 ± 8	967 ± 53	66 ± 7	47 ± 8	367 ± 24	51 ± 3	12 ± 1	201 ± 12	5.2 ± 0.4	1725 ± 98	413 ± 32
	LN	Control	98 ± 10	167 ± 18	616 ± 36	70 ± 5	1112 ± 66	57 ± 5	74 ± 7	527 ± 27	64 ± 5	13 ± 1	153 ± 12	6.9 ± 0.2	2198 ± 119	600 ± 33
	LN	Tuta	69 ± 11	120 ± 19	501 ± 85	46 ± 9	908 ± 163	52 ± 11	59 ± 7	484 ± 43	53 ± 7	11 ± 1	145 ± 23	8.5 ± 0.6	2052 ± 68	549 ± 54
H3	HN	Control	31 ± 6	50 ± 10	175 ± 36	104 ± 21	513 ± 157	13 ± 3	216 ± 15	695 ± 62	27 ± 6	29 ± 5	32 ± 13	4.4 ± 0.6	943 ± 225	911 ± 73
	HN	Tuta	33 ± 4	56 ± 6	237 ± 30	132 ± 8	769 ± 154	17 ± 2	232 ± 30	689 ± 47	47 ± 7	33 ± 5	59 ± 19	5.0 ± 0.4	1324 ± 211	921 ± 72
	LN	Control	79 ± 9	146 ± 17	423 ± 37	185 ± 17	1311 ± 110	36 ± 2	484 ± 36	1270 ± 13	47 ± 11	59 ± 8	38 ± 9	7.5 ± 1.1	2287 ± 176	1754 ± 165
	LN	Tuta	81 ± 6	144 ± 10	429 ± 48	231 ± 16	1393 ± 166	38 ± 2	566 ± 24	1332 ± 49	61 ± 12	58 ± 5	23 ± 3	7.2 ± 0.7	2435 ± 239	1898 ± 69

Roots

Harvests	Nutr	Treat	5CGA	1CGA	CP	Tomatin (mg/g DW^{-1})	HCAD
H1	HN	Control	670 ± 109	5.4 ± 0.6	72 ± 17	nd	675 ± 110
	LN	Control	557 ± 73	5.5 ± 1.1	51 ± 12	nd	562 ± 74
H2	HN	Control	265 ± 41	29 ± 6.5	27 ± 7	8.9 ± 0.5	294 ± 44
	HN	Tuta	272 ± 49	23 ± 5.3	34 ± 5	5.9 ± 0.8	295 ± 51
	LN	Control	363 ± 46	35 ± 4.9	22 ± 7	7.9 ± 0.5	399 ± 46
	LN	Tuta	449 ± 74	37 ± 6.1	19 ± 3	6.9 ± 0.6	486 ± 69
H3	HN	Control	154 ± 39	9.9 ± 1.7	22 ± 5	13 ± 0.8	163 ± 39
	HN	Tuta	162 ± 20	5.6 ± 0.7	19 ± 2	13 ± 0.8	167 ± 20
	LN	Control	439 ± 71	9.0 ± 1.0	2.3 ± 0.4	13 ± 1.2	448 ± 72
	LN	Tuta	652 ± 82	12 ± 0.8	3.2 ± 0.7	14 ± 2.0	664 ± 84