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Leaf antioxidant machinery stimulation by *Meloidogyne javanica* infestation: A case study on *Cucumis melo* seedlings

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ABSTRACT

Following nematode infestation, leaf-localized responses are vital not only as early infection signs but also as an indication of actions required to preserve crop productivity. In this context, the leaf-level physiological responses of cantaloupe seedlings were evaluated after 40 consecutive days of cultivation under different *Meloidogyne javanica* juveniles (J2s) inoculation regimes (0, 100, and 850 J2s plant^{-1}). Leaf growth parameters and photosynthetic pigments (chlorophyll, carotenoids) content were not affected by J2s infestation, while foliar Potassium concentration significantly declined. The *RuBIsCo activase* gene expression was negatively associated with the J2s inoculation level. Total flavonoids, total phenolics, lipid peroxidation indexes, phenylalanine ammonia-lyase activity, as well as, ascorbate peroxidase gene transcription, were higher in the inoculated plants (regardless of the J2s inoculant level). Taken together, present data indicate that J2s infestation impacts primarily the Potassium levels in leaves. Additionally, a substantial stimulation of the plant antioxidant machinery (independent of the nematode infestation intensity) is triggered. Finally, it was established that the transcriptional regulation of the *RuBIsCo activase 1* gene under biotic stress has the capacity to be employed as a potential stress indicator marker.

1. Introduction

The root-knot nematode (RKN) *Meloidogyne javanica* is a globally-distributed obligatory endoparasite. It is a generalist species, capable of infesting a great variety of crops including the cucurbits, melon, cucumber, zucchini, and watermelon (López-Gómez and Verdejo-Lucas 2014; López-Gómez et al., 2015). RKNs parasitization can cause severe growth defects, as well as, mineral deficiencies on infected plants. Moreover, nematodes can alter the host's physiological and hormonal homeostasis (having an impact on root functionality) and may also act synergistically with other soil-borne pathogens causing significant yield losses; thus, it is considered as a major economic importance factor, especially in mild climate areas (López-Gómez et al., 2015; Tzortzakakis et al., 2016; Bernard et al., 2017). In general, yield losses strongly depend on the RKNs (soil) population density and the average temperature (Seinhorst, 1970; Kim and Ferris, 2002; López-Gómez et al., 2014; Ishida et al. 2020).

Meloidogyne javanica juveniles (J2s) initially infest the host plant roots and then gradually migrate to the vascular system. The functionality of parenchyma cells is progressively modulated, creating a feeding site. This site is comprised by "giant cells" and a vast network of xylem and phloem cells, providing the pathogen essential nourishing sources (e.g., water and nutrients) at the expense of the host. The pathogen and its feeding site are localized in characteristic galls, which soon become macroscopically visible on the infected roots, following infestation (Bartlem et al., 2014; Liu et al., 2016; Bernard et al., 2017; Aydinli et al., 2019).

While RKNs directly stimulate profound alterations on root physiology and morphology, via the redifferentiation of root cells into specialized giant cells, the effect on the aerial organs mostly relies on

Abbreviations: APX, Ascorbate Peroxidase; AsA, Ascorbate; Car, Carotenoids; Chl, Chlorophyll; DW, Dry Weight; J2, Juvenile; PAL, Phenylalanine Ammonia-Lyase; RCA, RuBIsCo Activase; RGI, Root Galling Index; RKN, Root-Knot Nematode; ROS, Reactive Oxygen Species; TARS, Thiobarbituric Acid Reactive Substances; TCA, Trichloroacetic Acid; TF, Total Flavonoids; TP, Total Phenolics.

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an indirect procedure, mediated by the reduced root functionality and restriction of nutrient availability and the accumulation of Reactive Oxygen Species (ROS). However, the hormonal disarrangement triggered by RKN activity can directly effectuate molecular and biochemical equilibria even at distant aerial parts of the plants; that are regularly manifested as organ deformities (Olmo et al. 2017; Molinari and Leonetti, 2019; Sato et al., 2019). Since RKNs and cyst nematodes can induce a series of plant resistant responses via pathogen-associated pattern (PAMP) molecules and effector-triggered immunity (Arce et al., 2017; Sato et al., 2019; Molinari and Leonetti, 2019; Przybylska and Obrępalska-Stęplowska, 2020; Nikoloudakis et al. 2020), the effects on leaves' functionality can be multidimensional.

The timely evaluation of leaves-localized physiological responses to nematode infestation can be extremely useful not only as a sign of early infestation recognition, but also as a mark for a course of actions (e.g., genotype selection, fertilization, or specific cultivation practices) in order to sustain an economically feasible level of crop productivity. Under this background, several leaf-level assessments were conducted on cantaloupe plants infected by J2s of *M. javanica* under two population densities. These analyses included the estimation of macro- and micro-nutrients levels, photosynthetic pigments [i.e., Chlorophyll (Chl), carotenoids (Car)], total phenolics (TP), total flavonoids (TF), and thiobarbituric acid reactive substances (TARS; as an indication of lipid peroxidation). Besides the assessment of metabolites, we also evaluated the transcriptional responses of infected plants; focusing on the RuBIsCo activase (*RCA*) gene expression, as well as, the transcriptional regulation and enzymatic activities of Phenylalanine ammonia-lyase (PAL) and ascorbate peroxidase (APX).

2. Materials and methods

2.1. Plant Material and growing conditions

Seeds of cantaloupes (*Cucumis melo* var *cantalupensis* cv. Retato Degli Ortolani) (Hortus, Longiano-Italy) were pre-germinated between moist filter paper in Petri dishes. Germinated seedlings then were transferred in 0.5 L pots containing a commercial nutrient-enriched substrate (Compo Sana, Compo, Münster, Germany), and placed in a climate-controlled growth chamber at the Laboratory of Vegetable Crops of Institute of Olive Tree, Subtropical Crops and Viticulture (IOSV; Heraklion, Crete, Greece). Environmental conditions were as follows: air temperature of 26.1 ± 1,5 °C, relative air humidity of 60.2 ± 1,2%, and a light intensity of 300 μmol m^{-2} s^{-1} photosynthetic photon flux density at the plant level for 12 h per day provided by fluorescence lamps (HQI-BT 400 W/D pro, Osram, Munich, Germany). Cantaloupe plants were grown for 28 d prior to nematode inoculation (the procedure is described in detail below), and 40 d thereafter.

Young, fully expanded, grown under direct light leaves were sampled. Tissues were collected and stored at -80 °C for further analyses. Dry weight (DW) was evaluated after drying the tissues (75 °C) in an UM200 oven (Memmert GmbH, Schwabach, Germany) for 48 h (Hassanvand et al., 2019). For each treatment, 10 plants were used. The main experiment was conducted after preliminary experimental optimization.

2.2. Nematode inoculation

A population of *M. javanica*, maintained in potted tomato plants at the Laboratory of Nematology of IOSV (Heraklion, Crete, Greece), was employed for inoculation. In brief, eggs were extracted from infected roots with sodium hypochlorite and incubated (25°C) in an extraction dish (Hussey and Barker, 1973). J2s hatched within 24 h were discarded, and the ones collected after 72 h were used for the seedlings' inoculation under three regimes (0, 100, and 850 J2s per plant). After the cultivation period (40 d), plants were uprooted, and the remaining soil from the root was carefully removed under running tap water to assess the root galling index (RGI) (Bridge and Page, 1980). At this stage, nematodes inside the galls had reached the adult stage.

2.3. Foliar macro and micro nutrient concentrations

All leaves were ground into a fine powder and passed through a 30-mesh screen. Each sample (circa 0.5 g) was dry-ashed in a muffle furnace, at 515°C for 5 h. Then, the ash was dissolved in 3 mL of 6N HCl and diluted with double distilled water up to 50 mL. The concentrations of P, K, Ca, Mg, Fe, Mn, Zn, and Cu were determined by ICP (Perkin Elmer-Optical Emission Spectrometer, OPTIMA 2100 DV, Waltham, MA, USA) as reported (Chyla and Zyrnicki, 2000). Nitrogen was determined by the Kjeldahl method (Chapman and Pratt, 1961). Macronutrient concentrations were expressed as a percentage (%) of dry weight and micronutrients were expressed in mg kg^{-1}.

2.4. Photosynthetic pigment composition

Chl a, Chl b, as well as, Car contents were determined as previously described in Sotiras et al., (2019). Fresh leaf samples (75 mg) were ground in a mortar using 10 ml of cold 80% acetone (v/v), transferred in falcon tubes kept in the dark for 1 h, and vortexed at 15 min intervals. A centrifugation (4400 g for 5 min) at 4°C was then performed. Pigments concentrations were spectrophotometrically determined by measuring the absorbance at 470, 647, and 663 nm. The followings equations (Lichtenthaler and Buschmann, 2001) were employed:

$$\left[\mathrm{Chl_a}\right] = 12.25\,A_{663} - -2.79\,A_{647} \quad (1)$$

$$\left[\mathrm{Chl_b}\right] = 21.5\,A_{647} - -5.1\,A_{663} \quad (2)$$

$$[\mathrm{Car}] = \left\{1000\,A_{470} - -1.82\left[\mathrm{Chl_a}\right] - -85.02\left[\mathrm{Chl_b}\right]\right\}/198 \quad (3)$$

2.5. Total Phenolics (TP) and Total Flavonoids (TF) content

The extraction of TP and TF was performed by homogenizing frozen leaves with 80% acetone (v/v) in deionized water (3 mL g^{-1} tissue) with mortar and pestle. The mixture was placed in a super-sonic ice bath at 4°C under darkness for 15 min, and then centrifuged (4000 g for 5 min). The procedure was repeated three times, for TP and TF analyses (Kafkaletou et al., 2018).

Leaf TP concentration was estimated using the Folin-Ciocalteu colorimetric method (Singleton et al., 1999) with minor modifications as described in Kafkaletou et al. (2018). Briefly, 0.2 mL of the diluted extract with distilled water (1:2) was added into a tube containing 0.2 mL Folin-Ciocalteu reagent (Sigma-Aldrich, Saint Lewis, MI, USA) and 2.6 mL deionized water. After stirring, the tube was stored at room temperature (25°C) for 6 min. Then, 2 mL Na_2CO_3 (7%, w/v) were added to the mixture and the absorbance was measured at 750 nm, after 90 min incubation at room temperature (25°C). The results were expressed as gallic acid equivalents on a DW basis.

Foliar TF were assayed using the aluminum chloride colorimetric method. Quercetin was used as a standard to make the calibration curve according to Aryal et al. (2019). One mL of leaf extract was mixed with 0.2 mL of 10% (w/v) $AlCl_3$ (Merck, Darmstadt, Germany) solution in acetone, 0.2 mL potassium acetate (1 M) and 5.6 mL water. The mixture was incubated for 30 min at room temperature (25°C), followed by the measurement of absorbance at 415 nm against a blank.

2.6. Thiobarbituric acid reactive substances' (TARS) content

TARS' determination was performed as an indication of lipid peroxidation. Leaf fresh tissue (0.5 g) was homogenized in 10 ml 0.1% trichloroacetic acid (TCA) (Sigma-Aldrich, Saint Lewis, MI, USA) at 4°C. After centrifugation (4400 g at 4°C) for 10 min, the supernatant was

used for the assay. The content of TARS was determined after the reaction with 0.5% 2-thiobarbituric acid in 20% TCA (w/v), as calculated from the subtraction of the absorbances at 532 and 600 nm using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Heath and Packer, 1968; Papadakis et al., 2018).

2.7. Protein extraction

The extraction for enzyme activities was performed according to Tsaniklidis et al. (2014) with minor modifications. Five hundred mg of leaf tissue were grounded in liquid nitrogen using a sterile mortar and a pestle. The proteins were extracted with 50 mM potassium phosphate (pH 7.4) buffer containing 0.25 mM EDTA, 2% w/v soluble PVP-40, 10% (w/v) glycerol, and protease inhibitor cocktail (all chemicals from Sigma-Aldrich, Saint Lewis, MI, USA) according to the manufacturer's instructions. The extracts were centrifuged (10000 g for 10 min), and then filtered using a syringe filter prior to the estimation of enzyme activity. The extract protein content was estimated using the Bradford protein assay (Bradford, 1976), using bovine serum albumin as a standard.

2.8. Ascorbate peroxidase (APX) activity

The estimation of APX activity with a reaction solution containing 25 mM Na-phosphate (pH 7.4), 0.1 mM EDTA, 0.2 mM H_2O_2 and 4 mM L-ascorbate (AsA) (all chemicals from Sigma-Aldrich, Saint Lewis, MI, USA). Protein extract of 200 μl mixed with 850 μl of the reaction solution was used to perform the assay. The oxidation rate of L-AsA was photometrically assayed at 265 nm in 10 min intervals at room temperature (25°C). Pre-defined concentrations of AsA were used for the reference curve (Tsaniklidis et al., 2014).

2.9. Phenylalanine ammonia lyase (PAL) activity

For PAL activity, the protein extract (200 μl) was added to 2 ml of 0.01 M borate buffer (pH 8.7) and 1 ml of 0.02 M L-phenylalanine (Sigma-Aldrich, Saint Lewis, MI, USA) dissolved in 0.01 M borate buffer (pH 8.7). Absorbance (290 nm) was measured at 0, 30, and 60 min following incubation at room temperature (Kim and Huang, 2014).

2.10. RNA extraction and qPCR

Total RNA was extracted from leaves using the method described in Tsaniklidis et al. (2020). In Brief, one g of grounded tissue was mixed with 10 ml of "lysis buffer" (8 M GuHCl, 25 mM EDTA, 1% Sarcosyl, 2% Triton X-100, 25 mM sodium citrate, 0.2 M sodium acetate; pH adjusted to 5.2 with acetic acid; all chemicals from Sigma-Aldrich, Saint Lewis, MI, USA). The lysate was incubated at 65 °C for 10 min and then centrifuged (16000 g for 10 min). Half ml of the supernatant was transferred to a fresh tube and 625 μl of absolute ethanol was added (to obtain 55.5% final concentration). Subsequently, the mixture flowed through a silica column (FT-2.0 Filter-Tube Spin-ColumnSystem, G. Kisker GbR, Steinfurt, Germany) by centrifugation (1500 g for 10 min). The column was washed once with 700 μl of "wash buffer 1" (4 M GuHCl, 25 mM Tris–HCl pH 6.6, and 60% ethanol) and twice (700 and 400 μl, respectively) with "wash buffer 2" (2 mM Tris–HCl pH 7.0, 20 mM NaCl, and 80% ethanol) by centrifugation (8000 g for 1 min). RNA was finally recovered in 50 μl of preheated (80 °C) nuclease-free elution buffer (10 mM Tris–HCl, pH 8.0). Total RNA was treated with DNAase I (ThermoFisher, Waltham, MA, USA) and inactivated via heating (74 °C). cDNA synthesis was performed with the Superscript II cDNA synthesis kit (ThermoFisher, Waltham, MA, USA) according to the manufacturer's instructions.

Cantaloupe nucleotide sequences were retrieved from the public database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/); *Cucumis melo RCA1* (XM_008465876) one of the two *RCA* isoforms characterized in Cucumis melo, a cytosolic *APX1* (NM_001297449), a plastidial *APX2* (XM_008468507) (of the at least six existing *APX* genes in *Cucumis melo*) and *PAL* (XM_008451014). The selection of genes studied here was performed after prior experimentation. Primers for the above-mentioned nucleotide sequences were designed using Beacon designer software suite. Quantitative PCR amplifications were performed using the PowerUp™ SYBR® Green Master Mix (ThermoFisher, Waltham, MA, USA) and a QuantStudio 3 Real-Time PCR System (ThermoFisher, Waltham, MA, USA). The relative quantification of gene expression was performed as previously described (Tsaniklidis et al., 2020), while cantaloupe cytochrome oxidase (EU069547) was used for normalization (Müller et al. 2015).

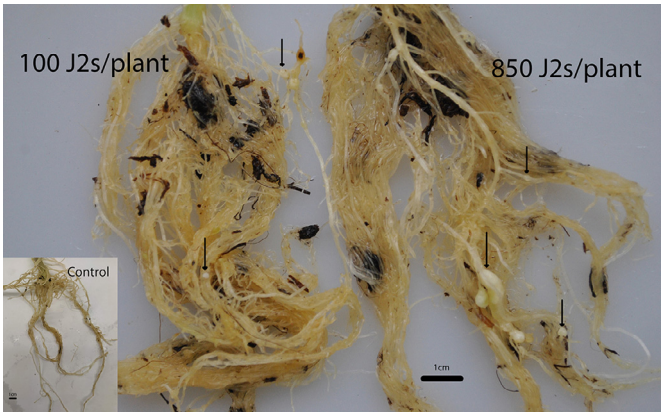

Fig. 1. Images of representative roots following 40 d cultivation under different inoculation rates, 100 (left) and 850 (right) *M. javanica* juveniles (J2s) per plant. Arrows indicate visible galls produced by nematode adults on melon roots.

2.11. Statistical analysis

The experimental study design consisted of three separate biological replicates (across all measurements). Each biological replicate (5 to ten bulked plants) was analyzed three times (qPCR was repeated twice). The statistical significance across treatments (control, 100 J2s/plant, and 850 J2s/plant) was inferred employing a parametric One-Way Analysis of variance (ANOVA) and the 'ggpubr' Package as implemented in the RStudio Version 1.1.463/R 3.6.1., p values for statistic testing against the control were depicted as: non-significant (ns): $p > 0.05$;: $p <= 0.05$; *: $p <= 0.01$; *: $p <= 0.001$ and **: $p <= 0.0001$. Tests for normality are appended as a supplementary file.

3. Results

3.1. Root gall formation by nematodes on Cucumis melo roots

Plants inoculated with *M. javanica* J2s exhibited macroscopically visible nodes on their secondary roots (Fig. 1). The RGI (Root Galling Index) was 2.2 and 3.9 for 100 and 850 J2s per plant, respectively. The nematodes inside the galls have reached the adult stage, however, no egg sacks were detected.

3.2. Leaf fresh and dry weight, leaf area

The leaf weight (fresh and dry) was not affected by the nematode inoculation nor its intensity since no statistical differences were found (Fig. 2). The average weight per plant (FW/DW) was 6.41g/0.69 g for control, 5.84 g/0.64 g for 100 J2s/plant initial inoculant, and 5.61 g/0.62 g for 850 J2s/plant initial inoculant. Correspondingly, the total leaf area was also reduced in the 850 J2s/plant infestation (290.5 cm^2) in comparison to the Control and the 100 J2s/plant treatment (326.4 cm^2 and 344.7 cm^2 respectively).

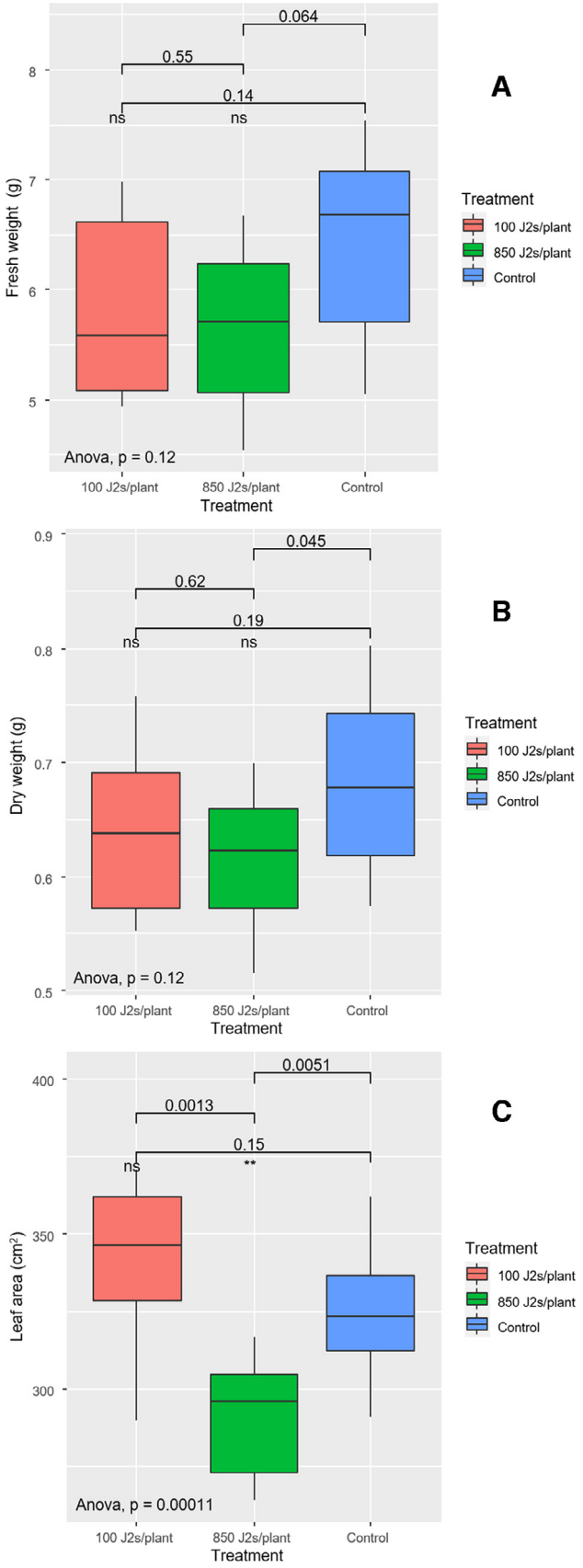

Fig. 2. Effect of the nematode inoculation intensity (0, 100 and 850 J2s plant^{-1}) on the Fresh weight (A), dry weight (B) and leaf area (C) for *C. melo* plants.

3.3. Nutrient concentrations

Nutrient levels in *Cucumis melo* leaves showed little variation across treatments; hence manifesting that nematode infestation does not holistically affect the nutrient equilibrium; at least in leaves. Nonetheless, statistically significant differences were found at potassium levels, where the 850 J2s/plant initial inoculant resulted in a significant reduction of the macronutrient in leaves; thus, signifying that the transfer of potassium forms is severely affected by RKNs infestation (Table 1). Furthermore, it seems that the magnitude of potassium deregulation correlates to the infestation rates (3.11 ± 0.26 %K for 100 J2s/plant as opposed to 3.07 ± 0.14 %K for 850 J2s/plant). A general gradual reduction of Ca and Zn levels in leaves was also observed in nematode inoculated plants; however, it was established that nutrient down-regulation was not significant at the $p < 0.05$ level.

3.4. Photosynthetic pigment content, and RuBIsCo activase1 (RCA1) expression

Nematode inoculation and its intensity did not drastically affect the accumulation of photosynthetic pigments in the leaves of the plant in this experiment (Fig 3). The analysis of variance revealed that the probability values were not significant for $Chlr_a$ and Chl_b levels ($p = 0.086$ and $p = 0.074$ respectively). Nonetheless, it was established that carotenoids were significantly affected across treatments ($p = 0.00029$), stressing the discrete roles and biochemical pathways among these two types of leaf pigments. It seems that despite the stress imposed by nematode infection and the lack of potassium in leaves, still the levels of chlorophylls are rather maintained constant. This observation corresponds at a molecular level since an observable effect was recorded for *RCA1* gene transcription. It was established that nematode inoculation regardless of the severity, resulted in a significant increase of transcripts accumulation (Fig. 3).

3.5. TBRS detection and lipid peroxidation

Nematode inoculation resulted in a progressive increase of the lipid peroxidation levels, suggesting that RKNs can elevate the oxidative damage rates in leaves. Moreover, it seems that lipid peroxidation is proportional to the infestation stress (Fig. 4), since TBRS levels were increased after the low (12.78 nmole/g FW for 100 J2s/plant) and higher severity inoculant (13.36 nmole/g FW for 850 J2s/plant), compared to the uninfected *Cucumis melo* plants (10.60 nmole/g FW).

3.6. Antioxidant Components (TP, TF, PAL enzyme activity and PAL transcription)

The infestation of *Cucumis melo* plants with nematodes initiated a general antioxidant components response both at the metabolic and molecular level. As a result, the total phenolics and flavonoids content was significantly enhanced under biotic stress; still did not follow a proportional to intensity manner (Fig. 5). For both TP and TF levels significant deviations from the Control were established. Total Phenolic levels were 21.8 mg GAE (gallic acid equivalent)/ g FW for the Control, while biotic stress had an upregulating effect for infected plants (25.7 mg GAE (gallic acid equivalent)/ g FW and 26.8 mg GAE (gallic acid equivalent)/ g FW respectively for 100 J2s/plant and 850 J2s/plant treatments). Total Flavonoids levels were 12.31; 16.75 and 15.32 mg GAE (gallic acid equivalent)/ g FW for Control, 100 J2s/plant and 850 J2s/plant groups correspondingly. However, Student's t-test revealed that treatments (among 100 J2s/plant and 850 J2s/plant) did not significantly differ. Extensive discrepancies were also detected across treatments for PAL enzyme activity and *PAL* gene expression that were also induced as a response to nematode infection, regardless of the initial inoculant. Relative *PAL* gene expression peaked at the 100 J2s/plant

Table 1
Leaf macronutrient (P, K, Ca, Mg) and micronutrient (Mn, Zn, Fe, Cu) content following 40 d cultivation under different *M. javanica* inoculation rates. Different letters at each day of measurement indicate a significant difference between treatments at ($p \leq 0.05$).
Table 1. Foliar macronutrient (N, P, K, Ca, Mg) and micronutrient (Mn, Zn, Fe, Cu) concentrations following 40 d cultivation under different M. javanica inoculation rates. Different letters at each day of measurement indicate a significant difference between treatments at ($p \leq 0.05$).

Initial inoculation rate (J2s plant^{-1})	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Fe (ppm)	Cu (ppm)
0	4.51 ± 0.54 ^{a}	3.38 ± 0.32^{a}	3.46 ± 0.17^{a}	6.12 ± 0.54^{a}	0.99 ± 0.14^{a}	287 ± 0.56^{a}	97 ± 11^{a}	297 ± 36^{a}	19 ± 4^{a}
100	4.63 ± 0.62^{a}	3.12 ± 0.33^{a}	3.11 ± 0.26^{ab}	6.14 ± 0.51^{a}	0.92 ± 0.08^{a}	218 ± 0.54^{a}	86 ± 8^{a}	289 ± 45^{a}	18 ± 3^{a}
850	4.39 ± 0.59^{a}	3.20 ± 0.25^{a}	3.07 ± 0.14^{b}	5.36 ± 0.44^{a}	0.89 ± .017^{a}	259 ± 0.37^{a}	81 ± 9^{a}	295 ± 27^{a}	18 ± 2^{a}

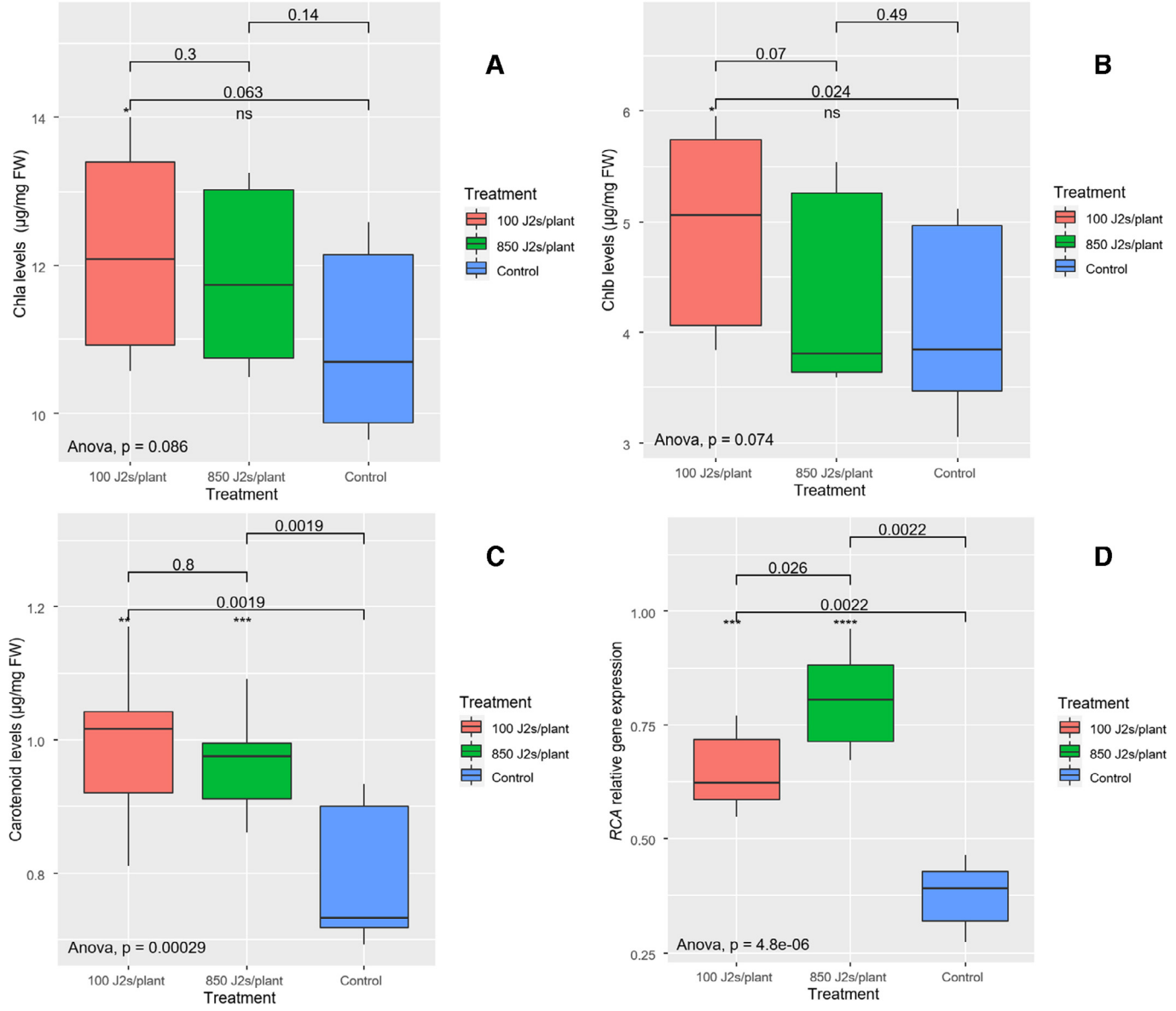

Fig. 3. Photosynthetic pigment [i.e., Chlorophyll a (A), chlorophyll b (B), total carotenoid content (C), RuBIsCo activase (*RCA*) gene expression (D), following 40 d cultivation under different *M. javanica* juveniles (J2s) initial inoculation rates (0, 100 and 850 J2s plant^{-1}).

treatment (0.821) but was somewhat lower at the 850 J2s/plant treatment (0.664); but still significantly higher than the Control (0.467). Correspondingly, PAL enzymatic activity was also upregulated and ranged from 70.75 nmole cinnamic acid/mg protein*h (Control) to 82.42 and 86.98 nmole cinnamic acid/mg protein*h for 100 J2s/plant and 850 J2s/plant respectively (Fig. 5).

3.7. Ascorbate peroxidases gene expression and enzyme activity

Inoculation of *Cucumis melo* plants with NPKs had a diverse consequence on the *APX* genes expression and enzyme APX activity. The transcription of the *APX1* isoform was not severely affected by nematode infestation; nonetheless, the 850 J2s/plant treatment presented a pattern

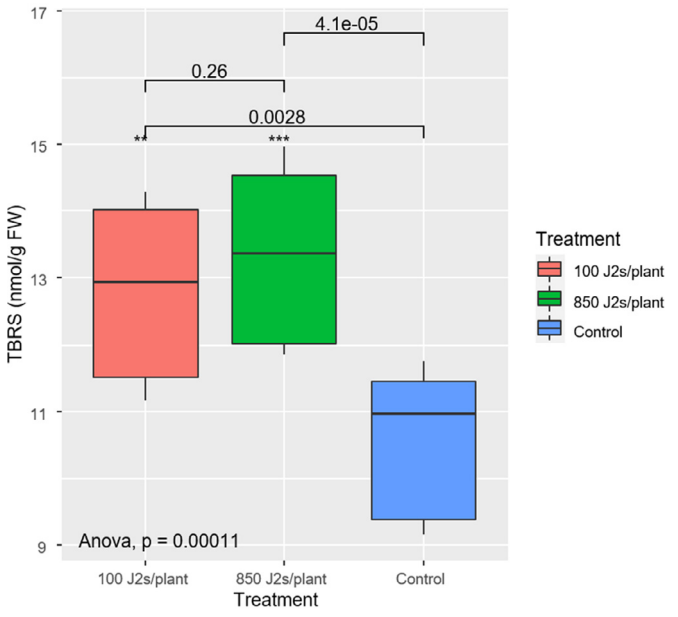

Fig. 4. Thiobarbituric acid reactive substances (TARS; as an indication of lipid peroxidation) following 40 d cultivation under different *M. javanica* juveniles (J2s). Initial inoculation rates (0, 100 and 850 J2s plant^{-1}).

of general upregulation. Analysis of variance revealed a clear demarcation for the *APX2* isoform, since for both the 100 J2s/plant and 850 J2s/plant treatments, a significant p value was established ($p = 3e^{-9}$; Fig. 6) and a remarkable induction of the transcription after nematode inoculation regardless of its initial size was observed. In terms of enzymatic activity however, the intensity of the inoculum was a defining factor since enzymatic APX activity peaked at the 850 J2s/plant treatment, while no statistically significant differences were recorded between the other two treatments (Fig. 6).

4. Discussion

*4.1. Root galling, nutrient concentrations, photosynthetic pigment content, and RuBIsCo activase (*RCA1*) expression*

The values of RGI of this experiment were dependent on the level of the initial inoculant and are correlated to a rather limited effect on the biomass accumulation of the aerial organs. A similar effect was observed in other cucurbits (Ploeg and Phillips 2001); our results are in accordance with these conclusions as fresh/dry leaf weight were not significantly affected by J2s inoculation rate; nonetheless, leaf area significantly differed in the case of 850 J2s/plant treatment (Fig. 2). Ploeg and Phillips (2001) reported that a rather high RKNs density (GI ≥ 5) is required for an observable effect on the growth of the aerial organs of cantaloupes and other cucurbits. Foliar K concentration was significantly decreased, related to the higher initial inoculant J2s rate (Table 1). Comparably to our findings, Mashela et al. (2008) reported that suppression of the RKNs population resulted in a significant increase of K levels in tomato leaves. Similarly, in other species (banana), reduced K accumulation after RKN infestation (Devrajan et al., 2003). Given the patterns and the dynamics of the mineral concentrations of the present study, we suggest that prolonged interaction with increasing RKN populations could lead to the development of K deficiency, which might be harmful to a number of critical physiological processes (Du et al., 2019). Indeed, the reduced availability of K, which is associated with the nematode populations in soil were indicated by Kayani et al. (2017) as critical factors for the reduced growth and yield of cucumbers. Thus, we believe that more attention should be paid by the farmers to combat of RKNs infections in cucurbit crops, to prevent nutritional disorders and to expand as much as possible the productive life of an already infected crop.

Moreover, a close correlation of potassium levels in the soil/roots and an induced plant resistance against nematodes has been established. Gao et al. (2018) indicated that higher K levels can induce multiple mechanisms and improve the resistance of soybean against Soybean cyst nematodes via elevated Phenylalanine Ammonia Lyase (PAL) and Polyphenol Oxidase (PPO) expression and root exudation of phenolic acids. Also, in tomato plants, the disease index of root-knot nematode was significantly decreased with K application, compared to the control plants (XiuJuan et al. 2016). Hence the possibility of deliberate K constraint or relocation in the roots could have an effect on the potassium levels across plant organs.

No significant differences were found across the treatments in the leaf photosynthetic pigment (Chl) accumulation (Fig. 3a; b). The RKN-induced root functionality reduction attenuates water and mineral uptake (manifested here as a statistically important reduction of K in plants infested with 850 nematode J2s plant^{-1}), which leads to a series of symptoms in the aerial organs such as stunting, yellowing, wilting (Moens et al., 2009). However, the level of the infestation employed in the current study did not result in obvious symptoms (such as leaf discoloration or wilting) in the aerial organs nor changes in chlorophyll photosynthetic pigment accumulation in the plants of the present experiment. Still, significant deviations for Car were detected among the uninfected and inoculated plants (Fig. 3c). An interplay between Carotenoids and Rice-*Meloidogyne graminicola* interaction has also been established by Kyndt et al. (2017), while carotenoids are known to participate in ROS detoxification schemes (Shen et al., 2018).

The initial RKN inoculant level proportionally affected the *RCA1* gene transcript accumulation (Fig. 3d). *RCA* has been proven to respond to a variety of stressful conditions (Chen et al., 2015; Perdomo et al., 2017).

Nematodes alter the plant's hormonal homeostasis during the formation of their feeding sites; still, this is not a root restricted phenomenon since critical metabolic processes throughout the plant can be affected (McAdam et al., 2017; Gheysen and Mitchum, 2019). RCA is fundamental for the photosynthetic process, regulating the RuBisCO activity, and is critical for the acclimation to a variety of abiotic stresses, counterbalancing the effects on the photosynthetic activity under the influence of stressors (Chen et al., 2015; Perdomo et al., 2017). In this study, for the first time a positive correlation between *RCA* transcription and the levels of initial nematode inoculation was reported (Fig. 3d), indicating that apart from abiotic stresses, RCA is also a biotic stress responder. Indeed, Bali et al. (2018) also observed a substantial effect upon nematode infestation in photosynthetic parameters of tomato seedlings cultivated in Petri plates. A more moderate effect of RKN inoculation on leaf morphology and the photosynthetic mechanism was reported for cotton under greenhouse conditions (Lu et al., 2014).

4.2. Antioxidant-related responses

In the present study, a general response of the antioxidant mechanisms was recorded at the molecular, as well as the metabolic level. Lipid peroxidation indexes (TARS) were elevated in infected plants (Fig. 4), while TF and TP levels were significantly induced as a reaction to nematode infestation (Fig. 5). An equivalent response was also found at the transcriptional level of related genes (*APX*) and enzymatic activity (APX). Moreover, PAL enzyme activity and gene expression (Fig. 5), as well as *APX2* gene expression (Fig. 6b) were higher in the inoculated plants regardless of the initial level of the inoculant. A delicate and complex network involving ROS, plant hormones, signaling molecules, and defense-related genes and proteins are initiated during pathogen attack (Vellosillo et al., 2010; Nikoloudakis et al. 2020). Our data suggest that even at the lower level of initial RKN inoculation, the stimulation of the antioxidant machinery was significant but partially unaffected by the intensity of the nematode infestation. Moreover, the increased ROS production in leaves resulted in increased peroxidation of cellular membranes as indicated by TARS levels (Fig. 4). This phenomenon can

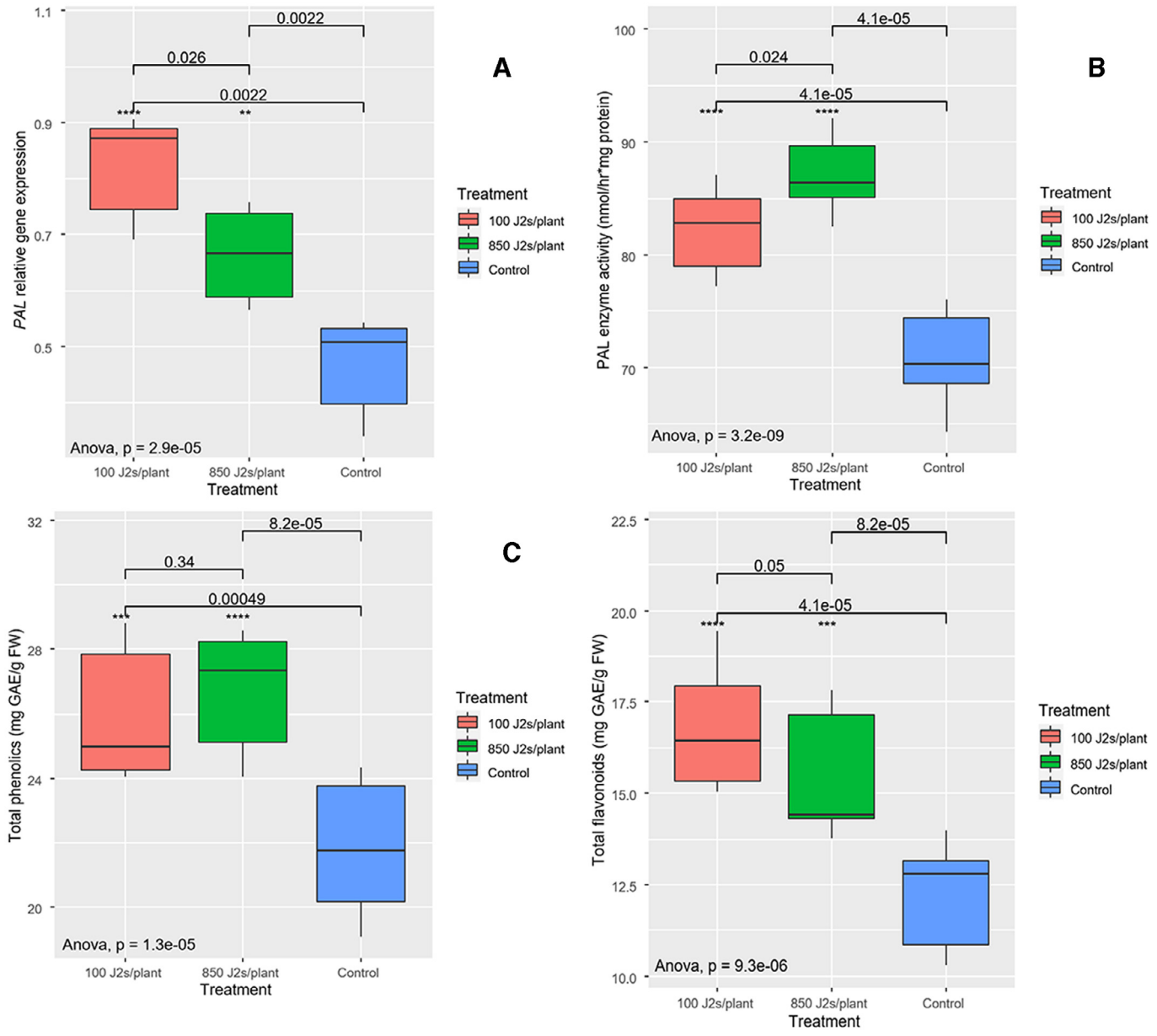

Fig. 5. *PAL* transcriptional regulation (A), PAL enzymatic activity (B), total phenolics (C) and total flavonoids (D), as affected by *M. javanica* infestation under different intensity rates (0, 100 and 850 J2s plant^{-1}).

be attributed to the combined effect of events emanated from the infestation of the root by pathogens. The establishment of nematodes is known to initiate both localized and systemic stimulation of immune responses and of the antioxidant machinery (Ali et al., 2018; Sato et al., 2019). This was also observed in the present study with a comparable intensity of the phenomenon between the two treatments with different levels of initial infestation. Moreover, the reduced provision of leaves with macro and micronutrients (Table 1) can also trigger ROS accumulation and antioxidant responses in leaves (Tewari et al., 2013; Hauer-Jákli and Tränkner, 2019). In conjunction, the systemic responses and the lower levels of nutrients can possibly explain the triggering of the antioxidant responses by the nematode infestation. Cytosolic APXs are at a higher degree responsible for the total APX enzyme activity (Tsaniklidis et al. 2014). In the current study, indeed *APX1* gene transcriptional activation and APX activity followed a similar pattern in inoculated and non-inoculated plants. However, as chloroplasts are a major site of ROS production (Galvez-Valdivieso and Mullineaux 2010), the transcript accumulation of *APX2* indicates a higher stress level in RKN inoculated plants regardless of the level of the initial inoculant (Fig. 6b).

Phenolics are considered as major contributors in plant defense, while their production is stimulated under a plethora of biotic stress scenarios. Similar to the results of this study that indicated a distinguishable stimulation of the Phenylpropanoid biosynthetic pathway by nematode infection (Fig. 5), Pegard et al. (2005) reported a substantial increase of the biosynthesis of phenolics in RKNs infested roots of pepper. Our data suggest that a systemic acceleration of the phenylpropanoid biosynthetic pathway takes place under the influence of RKNs infestation. Yang et al. (2018) reported an increase of both hydrogen peroxide levels and transcripts' accumulation of genes coding for critical antioxidant enzymes (including APX) in RKN infested watermelon seedlings. Moreover, Veronico et al. (2017) studied the effect of RKNs on the antioxidant machinery of tomato roots, and also reported a remarkable stimulation. Similarly, Bali et al. (2018) revealed extensive activation of the antioxidant detoxification responses in tomato seedlings as a result of RKN infestation.

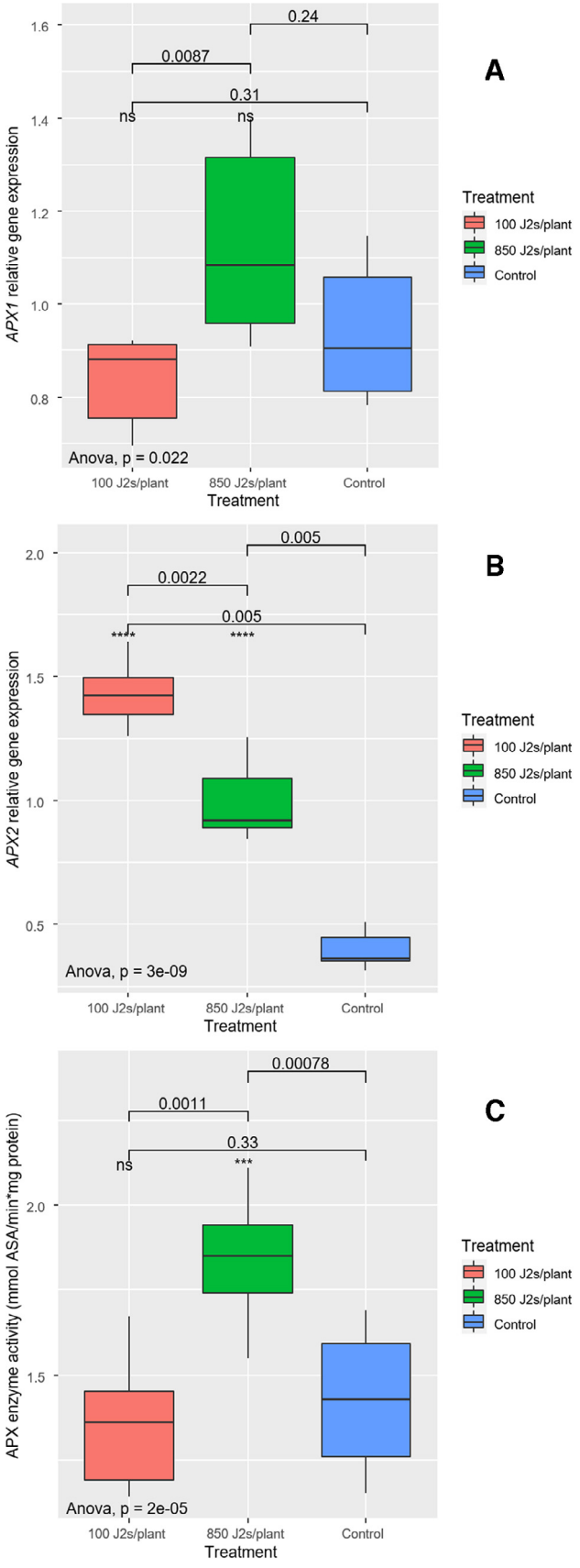

Fig. 6. APX activity (A) and gene expression (B, C) of ascorbate peroxidase (*APX*) following 40 d cultivation under different *M. javanica* juveniles (J2s) inoculation rates (0, 100 and 850 J2s plant^{-1}).

5. Concluding remarks

The leaf-localized physiological response of cantaloupe seedlings was evaluated following 40 d of cultivation under different *Meloidogyne javanica* juveniles' (J2s) inoculation rates (0, 100, and 850 J2s plant^{-1}). Leaf growth and photosynthetic pigment (chlorophyll) content remained unchanged upon J2' infestation, while carotenoid levels were induced as a response. The *RCA* gene was however transcriptionally activated, in a manner that depended on the level of nematode inoculation rate; suggesting that a proportional with the infestation effect on photosynthesis exist prior to the development of visible symptoms. Regarding nutrient concentrations, macro and micro elements remained rather unaffected. Still in the case of Potassium statistically significant deviations were detected. Thus, increased attention should be paid when combating *Meloidogyne javanica* nematodes, in order to avoid K deficiency in *Cucumis melo* with careful monitoring and appropriate re-scheduling of the fertilization plan. Several findings of this research converge to the conclusion that the antioxidant machinery by the nematode infestation is activated; regardless of the initial inoculant. These factors include the total flavonoid content, the total phenolic levels, lipid peroxidation indexes, phenylalanine ammonia-lyase (PAL) activity and gene expression, as well as ascorbate peroxidase (*APX*) gene expression. The relevant disassociation of the antioxidant machinery stimulation from the infestation intensity indicates that the boundary inoculation rate line might be extremely low. Still, the determination of the nematode infestation intensity, especially in asymptomatic plants, by using aerial plant organs is beyond any doubt elemental for both plant breeding industries and agricultural production systems.

Moreover, the effects on plant physiology can be evaluated for minimizing the nematode related losses of the production, without the use of toxic chemicals. Such effects alongside the innate immunity triggering mechanisms can be critical for the timely identification of the problem and can provide critical data for the control and amelioration of the problem. Still, the use of biological markers as an indication of an infestation is a very complex subject that can vary across environments, species or even at the developmental stage.

Declaration of Competing 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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2021.100002.

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