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Amino acid metabolism and expression of genes involved in nitrogen assimilation in common oranges cv. Valencia Late

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Abstract

Biochemical and transcriptional approaches can provide crucial evidence about the physiological changes which can occur in organic and conventional cultivated common orange [*Citrus sinensis* (L.) Osbeck]. This study aimed to investigate the change in physicochemical parameters, the concentrations of free amino acids and other N-containing compounds, and the expressions of key genes coding for enzymes linked to N assimilation in fruits of common orange cv. "Valencia Late". Two enzymes involved in different ways in N assimilation were considered: nitrate reductase (NR), catalyzing the conversion of nitrate into nitrite, and glutamate dehydrogenase (GDH), operating in the assimilation of ammonium (interacting with glutamate synthase), and in ammonium re-assimilation through glutamate deamination. Results showed that the different fertilizers did not affect the physicochemical characteristics of fruits but induced the different accumulation of free amino acids, with higher concentrations of proline and contemporarily lower concentrations of glutamate, in addition to upregulated the expression of *GDH* gene in fruits from organically managed tress. This study identified a possible adaptive response of common orange plants to organic or conventional fertilizers. The present work is intended as a first step to make the mechanisms underlying plant responses to N supply clearer by comparing organic and conventional cultivation. It also can support breeders to select the best citrus cultivars and agronomists to improve crop fertilization and production management.

Keywords: *Citrus sinensis*, glutamate dehydrogenase, nitrate reductase, proline, real-time PCR.

Introduction

Numerous studies have been conducted to evaluate the impact of organic and conventional production systems on nutrients, antioxidant substances, and N-containing components in fruits and vegetables (Brandt and Molgaard 2001, Carbonaro and Mattera 2001, Rapisarda *et al.* 2005 and 2010, Del Amor *et al.* 2008, Esch *et al.* 2010, Rosen 2010, Camin *et al.* 2011, Chebrolu *et al.* 2012). Ecological and agronomic studies on the influence of fertilization on plant metabolism have shown that there is an inverse relationship in plants between the available N and the accumulation of defence-related secondary metabolites such as vitamin C (Brandt *et al.* 2011). It has been shown that changes in N status play an important role in the secondary metabolism of plants, in which nitrogen deficiency leads to a marked shift from the N-containing compounds to the production of carbon-rich defence-related phenylpropanoids (Leser and Treutter 2005, Toor *et al.* 2006, Benbrook *et al.* 2008, Nguyen and Niemeyer

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Abbreviations: AA - ascorbic acid; CB - citrus by-products compost; EC - electrical conductivity; EF - elongation factor; GABA - γ-aminobutyric acid; GDH - glutamate dehydrogenase; LW - livestock waste compost; MF - mineral fertilizer; NiR - nitrite reductase; NR - nitrate reductase; PM - poultry manure; TA - total acidity; TCA - tricarboxylic acid cycle; TSS - total soluble solids.
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2008, Baranski *et al.* 2014). N usage by plants occurs *via* a succession of different steps, starting with uptake and translocation, and ending with remobilization when the plant has aged. Organic N supplied to the soil in organic cultivations is not immediately available for plant nutrition and must be previously converted by soil microflora into inorganic forms, such as ammonium (NH_4^+) and nitrate (NO_3^-), to be exploitable by plants (Lambers *et al.* 2009). Synthetic fertilizers, usually employed in conventional practices, contain variable percentages of nitrogen in these inorganic forms; therefore, it is possible to suggest that the nitrogen metabolism in conventional plants takes place at different times with respect to organically grown plants.

Plant utilization of nitrate starts with its absorption from external media. Nitrate reductase (NR, EC 1.6.6.1) catalyzes the conversion of nitrate into nitrite followed by nitrite reductase (NiR), which turns nitrite into ammonium. Due to its toxicity if in high concentrations, plants have to quickly assimilate ammonium into organic compounds (Forde 2000, Wickert *et al.* 2007). This last step requires free carbon skeletons to allow ammonium assimilation to occur, thus indirectly promoting carbon flow in the tricarboxylic acid cycle (TCA) (McAllister *et al.* 2012). There is a discordant thesis regarding the main catalyzing activity in higher plants exerted by the enzyme glutamate dehydrogenase (GDH, EC 1.4.1.2). Indeed, GDH could operate in the assimilation of ammonium through the amination of α- ketoglutaric acid to obtain glutamate (*e.g.*, aminating or synthetic reaction). In this way, GDH could cooperate with the glutamate synthase cycle in the synthesis of glutamate, which finally enters the transamination cycle. Alternatively, GDH could catalyze the oxidation of glutamate ensuring carbon skeletons flux for the operation of the TCA (*e.g.*, deamination or catabolic reaction) (Loulakakis *et al* 1994). Although some authors suggest that it might have a role to play in N assimilation (Melo-Oliveira *et al* 1996, Kisaka and Kida 2003, Skopelitis *et al.* 2006, Magadlela *et al.* 2019), it has been suggested that GDH has its main function in the catabolism of glutamate (Robinson *et al.* 1991, Fox *et al.* 1995, Lea and Miflin 2003, Purnell and Botella 2007, Limami *et al.* 2008, Miyashita and Good 2008, Kishorehumar *et al.* 2020), ensuring that N metabolism does not negatively affect mitochondrial operations. Moreover, its activity allows the synthesis and transport of N-rich compounds during N remobilization. Indeed, GDH is prominently localized in mitochondria (Dubois *et al.* 2003) associated with low N availability (Tercé-Laforgue *et al.* 2015) and represents a stress/senescence-associated enzyme involved in the production of 2-oxoglutarate from glutamate (Fontaine *et al.* 2012, Foyer *et al.* 2011). Furthermore, GDH activity depends on specific environmental conditions and under excessive ammonium stress, this enzyme also occurs in the cytosol, indicating an involvement in the process of plant detoxification. GDH thus plays a detoxification role by acting in a catabolic manner to recover 2-oxoglutarate, because excessive ammonia is most likely to be assimilated by glutamine synthetase causing toxicity by sequestering carbon (Lea and Miflin 2003, Kishorehumar *et al.* 2020).

An in-depth review (Vidal *et al.* 2010) showed that nitrate, in addition to other forms of N, can also act as a signal and consequently as a key factor for the regulation of the expression of hundreds of genes linked to plant metabolism, physiology, and growth. The role of genes involved in the N metabolism, specifically in response to organic and conventional cultivation, has also been demonstrated (Lu *et al.* 2005, Bowles *et al.* 2015, Vita *et al.* 2018). The effect induced by external factors, such as the environmental conditions, as well as cultivation, causes modifications also in the panorama of gene expression. Nevertheless, the use of a transcriptomic approach to study the response of plants to organic and conventional systems of cultivation has been poorly reported (Pacifico *et al.* 2017, Fess and Benedito 2018, Sharpe *et al.* 2020), in part because the role of differently expressed genes is widely variable, and heavily dependent on the long-term cultivation (Delate *et al.* 2015), and also because the choice of the tissues to be considered could be difficult to be selected. Moreover, to date, most studies have been performed on wheat (Lu *et al.* 2005, Vita *et al.* 2018), potato (Pacifico *et al.* 2017), and tomato (Sharpe *et al.* 2020), but nothing regarding fruit trees including citrus is available.

This study aimed to evaluate the different adaptations of citrus plants grown under organic and conventional systems through a biochemical and a gene-based transcriptomic approach. In fact, we hypothesize that nutrition of the soil, if organic or conventional, could induce some changes in the nitrogen assimilation in fruits. Indeed, fruits and vegetables represent the main plant products that are considered by consumers and competent institutions in the framework of so-called Organic Agriculture.

Materials and methods

Experimental design: *Citrus sinensis* (L.) Osbeck cv. Valencia Late, fruits were collected at maturity from the experimental farm of the Council for Agricultural Research and Economics (CREA) - Research Centre for Olive, Citrus and Tree Fruit, located in Palazzelli (Siracusa, Italy; 37° 20’ N, 14° 53’ E). The orchard soil had a high cation-exchange capacity [0.65 meq. g^{-1}(dry soil)] and subalkaline pH (7.8). Four treatments (1 conventional and 3 organic plots in which total amounts of applied nitrogen were the same) were carried out on uniform 35-year-old Valencia Late cultivar grafted onto the sour orange *C. auranthium* L. rootstock and distributed in a randomized-block design with three replicates (12 plots, each consisting of 30 trees). Within each plot, four replicate samples were collected from four plants (10 fruits from each plant). Four fertilizer treatments were applied: citrus by-products compost (CB), poultry manure (PM), livestock waste compost (LW), and mineral fertilizer (MF) as control. The trees received every year the same amount of N input. In particular: CB (N, 1.4 %; P, 1.8 %; K, 0.5 %; SO, 0.8 %); PM (N, 3.5 %; P, 3.0 %; K, 3 %; SO, 3.5 %); LW (N, 1.3 %; P, 1.2 %; K, 1.2 %; SO, 29.0 %) and MF (NPK (20-10-10) 2.35 kg/tree; superphosphate 0.73 kg/tree; K_2SO_4 0.55 kg/tree). The soil composition of the four different plots was evaluated before starting the experimental trial with respect to N content (0 - 40 cm depth) and the values obtained did not show any statistically significant differences. Indeed, N content was 2060.79 ± 267.55 mg kg^{-1} in LW plot, 1963.24 ± 272.04 mg kg^{-1} in PM plot, 2069.90 ± 367.52 mg kg^{-1} in CB plot and 1760.39 ± 233.31 mg kg^{-1} in MF plot. The average content of the organic matter of the plots was equal to 1.8 % ± 0.08. The fertilization managements of the organic and conventional plots were the same for almost two decades. Total soil organic carbon content in plots treated with the different organic and conventional fertilizers has previously shown an increasing improvement in soil quality (Intrigliolo *et al.* 2003). The electrical conductivity (EC, mS m^{-1}; soil:water ratio, 1:2.5) was measured after fertilization of the four plots using an *HI 9813* portable EC meter (*Hanna Instruments*, Woonsocket, RI, USA) to be sure that livestock waste manure could not influence sodium chloride concentration. Values for the four plots did not show significant differences (MF: 1.05 ± 0.02 mS m^{-1}; CB: 1.34 ± 0.03 mS m^{-1}; PM: 1.01 ± 0.02 mS m^{-1}; LW: 1.27 ± 0.04 mS m^{-1}).

Physicochemical analysis, ascorbic acid, and total N in orange juice: Fruits were squeezed by a domestic juicer and the juice obtained was used for further physicochemical and analytical determinations. Total acidity (TA), pH and total soluble solids (TSS) were measured using standard methods (Kimbal 1999). Ascorbic acid (AA) content was measured using an HPLC system (*Waters*, Milford, MA, USA) equipped with a *Waters 484* UV detector (Rapisarda and Intelisano 1996). Total N in fruit juice was determined by Kjeldahl's method using an *AutoKjeldahl Unit K-370* apparatus (*Buchi*, Flawil, Switzerland).

Amino acid metabolism: Free amino acids were determined by HPLC (*Waters*) equipped with a *Waters 996* PDA (photo-diode array) detector employing pre-column derivatization with 9-fluorenylmethyl-chloroformate according to the method developed by Fabiani *et al.* (2002). The column (*Hypersil ODS*, 5 µm, 250 mm × 4.6 mm i.d., *Phenomenex*, Torrance, CA, USA) was operated at 25 °C with a flow rate of 1.0 cm^3 min^{-1}. Separation of amino acids was obtained using linear gradient elution conditions (min/A%): 0 min/72 % eluent A, 3 min/72 % eluent A, 27 min/55 % eluent A, 32 min/0 % eluent A, 37 min/0 % eluent A, 39 min/72 % eluent A, and 47 min/72 % eluent A. Eluent A was represented by 50 mM acetate buffer (pH 4.2) and eluent B was 100 % acetonitrile.

Total RNA extraction: Total RNA was extracted from 3 cm^3 of juice by adapting the extraction procedure described by Ancillo *et al.* (2007). One volume of extraction buffer containing 0.2 M TRIS, pH 8.0, 0.2 M NaCl, 50 mM EDTA, 2 % (m/v) sodium dodecyl sulphate (SDS), one volume of phenol, and 0.02 volume of β-mercaptoethanol was added to the sample. After incubation at 50 °C for 5 min, samples were centrifuged at 1 700 g and 4 °C for 15 min. Two cycles of centrifugation were carried out, adding to the upper aqueous phase one volume of chloroform:isoamyl alcohol (24:1, v/v). RNA was precipitated by adding the one-half volume of 6 M LiCl to the upper phase at -20 °C overnight. After centrifugation at 7 600 g for 40 min, the precipitated RNA was washed with 70 % (v/v) ethanol and centrifuged at 6 000 g for 20 min. The total RNA was resuspended in 0.05 cm^3 of RNase-free water. The qualities and the quantities were evaluated using a *Nanodrop 1000* spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA) and by gel electrophoresis using 0.8 % (m/v) agarose in TAE buffer (Tris-acetate-EDTA). The quality was considered optimal for values of 260/280 between 1.80 and 2.0. The total RNA was treated, purified, and qualitatively and quantitatively estimated according to Bernardi *et al.* (2010). DNAse treatment was carried out by adding to 0.04 cm^3 of RNA and 1× *RNaseOUT* recombinant ribonuclease inhibitor (*Invitrogen*, Carlsbad, CA), 0.1 M dithiothreitol (DTT; *Invitrogen*), 5× buffer, 1× DNAse in a final volume of 0.05 cm^3. Samples were incubated at 37 °C for 30 min and purified using *RNA Cleanup* (*Qiagen*, Hilden, Germany), according to the manufacturer's protocol.

Expression analysis through real-time PCR: The relative quantification using real-time PCR analysis was executed on *NR* (Cs3g19060) and *GDH* (Cs7g19160). The *Elongation factor 1 alpha* (Genbank accession number AY498567) was used as a housekeeping gene, as previously reported (Licciardello *et al.* 2014). Oligonucleotides and probes sequences were drawn using *ABI PRISM* primer express software *v. 3.0* (*Applied Biosystems*, Foster City, CA, USA) and are listed in Table 1. At least 500 ng of total RNA was retro-transcribed into cDNA using the *High-Capacity cDNA Archive* kit (*Applied Biosystems*). Due to the extremely low expression of the genes of interest, it was necessary to use the preamplification *TaqMan PreAmp Master Mix* kit (*Applied Biosystems*). The final volume of the *PreAmp Mix* was 0.5 cm^3 and included 1× *TaqMan PreAmp Master Mix*, 0.18 µM of forward and reverse oligonucleotides, and 200 ng of cDNA. Thermocycler conditions consisted of denaturation at 95 °C for 10 min, 14 cycles at 95 °C for 10 s, and annealing at 60 °C for 4 min. Expression analysis was conducted on a *7300 Real-Time* PCR system (*Applied Biosystems*) using the *TaqMan Gene Expression Master Mix* kit (*Applied Biosystems*). The 0.5 cm^3 of final volume consisted of a 1× *Master mix*, 0.90 µM of each forward and reverse oligonucleotide, 0.20 µM of the relative probe, and 100 ng mm^{-3} of respective sample diluted 1:20 according to the manufacturer's instructions. Amplification conditions comprised preheating at 50 °C for 2 min, followed by denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was replicated three times (technical replicate) and a no-template sample (H_2O control) was also considered. For relative quantification, the standard curve was used.

Statistical analysis: Data were statistically analyzed using *StatSoft 6.0* software (*Vigonza*, Padova, Italy). The estimation of the statistical differences was carried out by *ANOVA* and means partitioning was carried out by the Tukey test.

Table 1. Primers and probes for *GDH*, *NR,* and *EF* housekeeping gene used for real-time PCR expression analysis.

Primer/probe name	Gene locus	Sequence 5’-3’	Amplicon length [bp]
*GDH*1-130_for	Cs7g19160	GGACAGGCAATTAGGGATTTGA	63
*GDH*1-173_rev		AGCCCATGATCCCACATTTC	
*GDH*_probeVIC		TTTGTAATCCAGGGCTTT	
*NR*1-597_for	Cs3g19060	TTGCCACGGCCAGTGTATT	60
*NR*1-536_rev		GGGATCCATACTGGACGTGAA	
*NR*_probeVIC		TGTGACCCAATGGAC	
*EF*344_fw	Cs8g16990	AAGCTGGTATCTCCAAGGATGGT	61

Table 2. Quality parameters of juice from organically and conventionally grown common orange cv. Valencia late. Means ± SD, $n = 3$. MF - mineral fertilizer (conventional plot), CB - citrus by-product, PM - poultry manure, LW - livestock waste (organic plots).

Treatment	pH	Total acidity [% citric acid]	Total soluble solids [%]	Ascorbic acid [mg cm^{-3}]	Nitrogen [mg dm^{-3}]
MF	3.55 ± 0.15	1.23 ± 0.17	10.45 ± 0.97	0.524 ± 0.029	882.00 ± 105.61
CB	3.60 ± 0.18	1.32 ± 0.19	10.52 ± 0.85	0.538 ± 0.029	852.69 ± 55.54
PM	3.66 ± 0.10	1.30 ± 0.16	10.28 ± 0.89	0.525 ± 0.036	812.00 ± 75.53
LW	3.64 ± 0.14	1.28 ± 0.15	9.86 ± 1.06	0.506 ± 0.031	916.85 ± 191.32

Results and discussion

Data reporting physicochemical parameters showed no statistically significant differences among the fruits obtained from trees using the four types of fertilizers (Table 2). TA, pH, and TSS highlighted that the different fertilizers did not affect the physicochemical characteristics of the fruit juice. Fruits from the four different treatments reached a balanced TSS/TA ratio, showing no influence of the kind of fertilizer on ripened fruit quality as concern content of sugars and acids. The type of fertilizers did not produce any impact on fruit production and yield as previously reported (Rapisarda *et al.* 2010).

Similarly, no significant differences were found in AA content (Table 2). It is known that there is a strict correlation between vitamin C content of fruits or vegetables and soil N availability (Nagy 1980, Brandt and Molgaard 2001). Indeed, more specifically, it has been demonstrated that, in many fruits and vegetables, high rates of N fertilization could cause a decline of the vitamin C content (Lee and Kader 2000). Our study demonstrated that when the amount of N applied to the soil is the same, no difference in vitamin C content occurs in juice from organically and conventionally grown orange trees. Also, no significant differences between N content in orange juice from trees treated with different fertilizers were observed (Table 2). Therefore, because the amount of N applied in the four plots was the same, our study confirmed that the choice of cultivation method does not result in a significant difference in the total N content in the juice.

The response of cv. Valencia Late orange trees to the different types of fertilization was also evaluated by measuring the free amino acids in the fruit juice. The free amino acid profile has been determined following the main aim of the study of some different physiological traits linked to N assimilation and further metabolism in organically *vs*. conventionally grown orange trees. Indeed, the synthesis of amino acids and the further involved transamination reactions represent the main metabolic route of N utilization in plants. Thus, the amino acid profile is of relevant importance. Furthermore, the amino acid profile has been previously proposed as a potential fingerprint for geographical origin or varietal assessment of fruit trees (Licciardello *et al.* 2011, Botoran *et al.* 2019) showing their intrinsic influence on fruit quality. The mean values and standard deviation of free amino acid content in orange juice from organic and conventional grown trees are shown in Table 3. Lysine and γ-aminobutyric acid (GABA) occurred in low unmeasurable amounts. Especially GABA was present in negligible concentration so that it was impossible to reliably detect it and quantify it. Maybe it could be due to the fact that GABA in oranges may vary across cultivars and may depend on many factors, such as the plant developmental stage, environmental conditions, and response to biotic and abiotic stresses. Proline, arginine, asparagine, serine, aspartic acid, glutamic acid, and alanine predominated in mature fruits, as widely reported by other authors (Gomez-Ariza *et al.* 2005, Cerdan-Calero *et al.* 2012), representing on average approximately 90 % of the total amino acid content. Arginine content ranged from 703.37 ± 99.32 mg dm^{-3} in orange juice from the MF plot to 909.93 ± 100.24 mg dm^{-3} in PM plot. Furthermore, statistically significant differences in arginine content were recorded with higher values for the samples collected from the two plots fertilized with organic fertilizers of animal origin (PM and LW) with respect to MF and CB plots. This could presumably happen since arginine, being an essential amino acid for chicken and cattle farm animals, is widely employed as a food supplement on farms. Thus, fertilization by organic poultry manure

Table 3. Free amino acid concentration and quantitative expression of *NR* and *GDH* genes in juice from organically and conventionally grown orange cv. Valencia late. Means ± SD, $n = 3$, different letters indicate significant differences based on Tukey's method. MF - mineral fertilizer (conventional plot), CB - citrus by-product, PM - poultry manure, LW - livestock waste (organic plots).

Parameter	MF	CB	PM	LW
Proline [mg dm^{-3}]	920.97 ± 53.98b	1152.76 ± 143.41a	1118.56 ± 217.64a	1250.50 ± 194.10a
Arginine [mg dm^{-3}]	703.37 ± 99.32b	778.75 ± 128.96b	909.93 ± 100.24a	894.12 ± 195.35a
Asparagine [mg dm^{-3}]	475.03 ± 111.35	508.63 ± 120.31	591.46 ± 175.31	538.15 ± 168.50
Serine [mg dm^{-3}]	63.09 ± 8.78	73.65 ± 7.46	76.20 ± 8.39	68.59 ± 15.54
Aspartic acid [mg dm^{-3}]	44.53 ± 7.06	50.34 ± 6.08	51.74 ± 7.58	49.05 ± 8.47
Glutamic acid [mg dm^{-3}]	38.69 ± 3.66a	33.39 ± 3.77b	33.92 ± 2.81b	30.04 ± 4.75b
Alanine [mg dm^{-3}]	3.56 ± 1.00	4.10 ± 1.50	4.04 ± 1.08	3.61 ± 1.04
Nitrate reductase mRNA fold increase	1.74 ± 0.41	1.82 ± 0.43	1.73 ± 0.41	1.59 ± 0.68
Glutamate dehydrogenase mRNA fold increase	1.68 ± 0.59b	2.11± 0.47a	2.16 ± 0.45a	1.84 ± 0.70a

or livestock wastes could produce an accumulation of this amino acid in fruits. Proline was the most abundant amino acid, reaching 1250.50 ± 194.10 mg dm^{-3} in orange juice from the LW plot, whereas alanine reached the lowest concentration, 3.56 ± 1.00 mg dm^{-3}, in orange juice from the MF plot. Results highlighted that proline concentration was significantly ($P \leq 0.01$) lower in orange fruit from the conventional plot compared to the orange fruit from the three different organic plots. It has been hypothesized that an increase in proline acts as a key signal able to activate multiple responses in the adaptation process or as a regulator of the accumulation of useable nitrogen (Maggio *et al.* 2002). In addition, proline has been proposed to be a compatible solute that can store carbon and nitrogen (Verbruggen and Hermans 2008). Finally, proline accumulation can be responsible for the buffering of cytosolic pH while balancing the redox status of cells and might be involved in the stress responses (Maggio *et al.* 2002). Based on our results, we suppose that the high content of this amino acid in fruit organic plots is due to the onset of an adaptive metabolism caused by the different environmental conditions in which plants were grown. Glutamic acid content was significantly ($P \leq 0.01$) lower in fruit collected from organic plots with respect to MF plot, suggesting the onset of catabolic reactions in organic plots. Glutamate catabolism in fruits can be explained by two metabolic reaction chains. The first is linked to the fact that the main pathway through which proline is synthesized is from glutamate. Indeed, this compound is converted to proline by two sequential reductions (Hu *et al.* 1992). Once formed, proline can be a well-suited component to transfer reducing potential, nitrogen, and carbon to fruit. Secondly, glutamate can be deaminated through an oxidation reaction catalyzed by GDH, thus providing enough carbon skeletons to ensure the operation of the TCA cycle.

The expressions of *NR* and *GDH* genes represent additional components of amino acid determination to better understand the physiological adaptation of citrus trees grown under organic or conventional conditions (Table 3). Until now, few studies have used a transcriptomic approach to explain the role of genes and their modulation in plants grown under conventional or organic conditions (Pacifico *et al.* 2017, Fess and Benedito 2018, Sharpe *et al.* 2020). Some studies have aimed to understand the molecular basis of plant responses to nitrate and other forms of N (NH_4^+, organic-N) and identify N-responsive genes (Wang *et al.* 2000, Krouk *et al.* 2010, Liao *et al.* 2019). Among all genes involved in the N metabolism, *NR* and *GDH* have been reported to be among the key genes responsible for N accumulation in crops (particularly tomato) grown using organic and conventional fertilizers (Sharpe *et al.* 2020). For this reason, in this study, a gene-specific transcriptomic approach was used to evaluate if expressions of *NR* and *GDH* could be indicative of different nitrogen assimilation metabolisms. Table 3 compares the *NR* and *GDH* expressions. Our results showed no difference in the expression of *NR* gene in fruits from organic plots compared to those from the conventional plot, inducing us to hypothesize that a slower N assimilation cannot be associated with organic fertilization. Indeed, our results do not validate the putative hypothesis that a delay in N assimilation in organically cultivated plants can occur due to the time needed for the conversion of organic N into inorganic forms available for plant nutrition. In fact, it must be noted that the experimental field in which the plots are located has been managed according to an organic regime for two decades, thus suggesting that plants could have activated adaptive strategy aimed at optimizing nitrate recovery in the case of deficiency (*i.e.*, during nitrification of N by soil microflora). To confirm this, Remans *et al.* (2006) demonstrated that, in *Arabidopsis* N limitation in the growth medium does not produce a deficiency of nitrate translocation to vegetative tissues, because high-affinity nitrate transporters underwent up-regulation as a physiological response of the root system to nitrogen limitation.

Regarding *GDH* gene expression, real-time PCR data showed significant differences ($P \leq 0.01$) among orange fruits grown in organic and conventional plots (Table 3). Specifically, *GDH* was expressed by approximately 1.5 fold more in fruits collected from the three organic plots, compared to those collected from the conventional plot. It has been suggested that under stress conditions *GDH* is involved in the ammonium re-assimilation through glutamate deamination (Srivastava and Singh 1987). Based on nuclear magnetic resonance (NMR) analysis, Robinson *et al.* (1991) demonstrated that in higher plants under stress conditions, *GDH* is active in the *in vivo* catabolism of glutamate and, specifically, it is catabolized to ammonium and 2-oxoglutarate, thus ensuring carbon skeletons for the TCA cycle. McIntosh *et al.* (1998) confirmed that, under many different environmental conditions, higher plants modify their respiratory metabolism to ensure the turnover of the TCA cycle to continuous supply carbon skeletons for biosynthetic demands, as a regulatory phenomenon, allowing a metabolic response to stresses. As also previously reported (Brandt and Molgaard 2001, Wang *et al.* 2008), our results suggest that the plants grown in organically managed plots modified their metabolism, not only in terms of glutamate content (significantly lower in the three different organic plots compared to the conventional plot) but also in the higher expression of *GDH* gene. The main pathway of ammonium assimilation in plants is represented by the GOGAT cycle. Our results suggest that GDH significantly contributes to secondary ammonium assimilation, playing a complementary role to that of the GS/GOGAT cycle. As previously reported in wheat (Vita *et al.* 2018), *GDH* genes show a higher expression in plants from organic plots compared to those grown using mineral fertilizers. It has also been recently demonstrated that GDH alleviates ammonium toxicity and suppresses photorespiration by assimilating excess NH_4^+ and disturbing the delicate balance of carbon and nitrogen metabolism, thereby improving drought tolerance in rice (Yan *et al.* 2021).

Conclusions

According to our hypothesis, we have observed for the first time, a tangible change in the plant physiology that occurs in citrus trees cultivated in organically managed plots in comparison with a conventional control plot. Results showed that the different fertilizers did not affect the physicochemical characteristics of fruit juice but induced the differential accumulation of free amino acids. In addition, an upregulated *GDH* expression in organic plots was probably due to a different ammonium re-assimilation in plants. These data can be assumed as a direct and adaptive response of citrus plants grown in organic plots. Although the use of either mineral or organic fertilizers did not affect the fruits' physicochemical characteristics, including ascorbic acid content, the presence of higher concentrations of proline and contemporarily lower concentrations of glutamate in fruits from trees treated by organic fertilizers suggest an adaptive offset of the plant metabolism. Furthermore, our results highlighted that, in citrus plants grown in organically managed plots, there was an over-expression of the *GDH* gene, putatively linked to its role in ammonium re-assimilation through glutamate deamination. Further studies are needed to evaluate the feasibility of using *GDH* as a potential marker of the organic cultivation method, not only in fresh orange fruits and processed juice but also in tissues such as leaves and roots.

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