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Research article

Excessive nitrogen impairs hydraulics, limits photosynthesis, and alters the metabolic composition of almond trees

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ABSTRACT

Horticulture nitrogen (N) runoffs are major environmental and health concerns, but current farming practices cannot detect ineffective N applications. Hence, we set to recognize high N conditions and characterize their effects on the physiology of almond trees grown in drainage lysimeters. Water and nutrients mass balances exhibited that N benefitted almond trees in a limited range (below 60 mg N L^{-1} in irrigation), while higher N conditions (over a 100 mg N L^{-1}) reduced evapotranspiration (ET) by 50% and inherently constrained N uptake. Respectively, whole-tree hydraulic conductance reduced by 37%, and photosynthesis by 17%, which implied that high N concentrations could damage trees. Through gas-chromatography, we realized that high N conditions also affected components of the citric acid cycle (TCA) and carbohydrates availability. Such changes in the metabolic composition of roots and leaves probably interfered with N assimilation and respiration. It also determined the proportions between N and starch in almond leaves, which formed a new index (N:ST) that starts at 0.4 in N deficiency and reaches 0.6–0.8 in optimal N conditions. Importantly, this index continues to increase in higher N conditions (as starch reduces) and essentially indicates to excessive N applications when it exceeds 1.1.

1. Introduction

Nitrogen is critical for intensive farming, but its application is often excessive and damaging to the environment. As the use of synthetic N fertilizers continues to increase, there is renewed controversy concerning their effects on food quality or dietary benefits (Albornoz, 2016; Stefanelli et al., 2010) and rising environmental concerns for nitrate (NO_3^-) leaching or gaseous N (N_2O) emissions (Jordan et al., 2011). It is especially critical in arid and semi-arid environments, where the shift to intensive fertilization through irrigation (i.e., fertigation) due to low soil organic matter and inherent infertility, alters nutrient utilization and demands specific considerations (He et al., 2015). Potentially, fertigation systems deliver precise quantities of minerals and support the plant requirements at maximum efficiency. However, as fertigation protocols are not supported by extensive research or agricultural know-how, fertigation could lead to significant nutrient runoffs and low fertilization efficiency (Clark et al., 2010). In fact, 47% of global synthetic N fertilization will not transform to increased yield (Lassaletta et al., 2014) due to numerous parameters that affect its effectivity, e.g., crop species and variety, the N form (nitrate or ammonium), soil type, water availability, and application method (El-Sharkawy et al., 1998; Ospina et al., 2014). Such inefficient N applications raise worldwide concerns and involve national regulations (Moffat, 1998) as they contaminate ground-water and increase crop production costs. Thus, N fertigation should be studied within the conditions and limitations of the agricultural industry to ensure its application promotes yields and minimizes damage to the environment.

Farmers can readily diagnose and mitigate N deficiencies during the growing season, while excess N is difficult to diagnose. N deficient plants stop leaf elongation (Marschner, 2011), inhibit photosynthesis (Gregoriou et al., 2007), reduce the size of chloroplasts (Li et al., 2013), and overall lack vigor. Practically, in N sufficient plants, N accumulates in leaves to specific concentrations, leading extension researchers to develop protocols to diagnose N sufficiency. This approach is somewhat arbitrary, changing between species and referring to particular times in the year while overlooking the rest of the growing season. However, it is widely used in multiple tree crops, including olives (Fernández-Escobar et al., 2009), almonds (Saa et al., 2014), and citrus (Chapman, 1949). On the other hand, it is also essential to assess N overfertilization. Unfortunately, N overfertilization cannot be diagnosed by most current methods, which are based on mineral leaf analysis (Muñoz-Huerta et al., 2013) or its derivatives [root N compounds (Taylor and May 1967), amino acids (Påhlsson, 1992), or chlorophyll content (Tschaplinski and Norby, 1991)]. Once N

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Abbreviations

C	Carbon
TCA	Citric acid cycle
C:N	C to N ratio
D	Drainage
DI	Deionized water
DW	Dry weight
ET	Evapotranspiration
N	Nitrogen
NSC	Non-structural Carbohydrates
N:ST	N to starch ratio
P	Phosphorus
A	Photosynthesis
K	Potassium
SC	Soluble Carbohydrates
ST	Starch
g_s	Stomatal Conductance
T	Transpiration
gplant	Whole-tree Hydraulic Conductivity

reaches an upper threshold (a couple of percent of total dry weight), it will not accumulate in leaves (Rubio-Covarrubias et al., 2009). For this reason, excessive N concentrations can reduce rice yields (Peng et al., 2009) or olive oil quality (Erel et al., 2013) without being detected throughout the growing season. Nevertheless, as high N concentrations affect numerous biological processes, it should induce detectable physiological changes.

Nitrogen, constituting core components in plants, determines the composition and allocation of photosynthetic products. Generally, N availability dictates whether the newly assimilated carbon (C) forms proteins and amino acids (that include N), or carbohydrates for structure and metabolism (Marschner et al., 1996). Nitrogen composes C sinks in the chloroplasts (e.g., amino acids, chlorophyll, and proteins) and therefore often has a positive effect on photosynthesis and transpiration (Bar-Tal et al., 2001; Leuning et al., 1995; Sinclair and Horie, 1989). Consequently, N promotes vegetative growth (Kang et al., 2004). Yet N might promote above-ground growth at the expense of roots development (Marschner, 2011), decrease the root-to-shoot ratio (Grechi et al., 2007), eventually limit transpiration, and force plants to utilize their residual photosynthates (Bi et al., 2004). Low N, on the other hand, promotes root growth and supports high transpiration demands (Marschner, 2011; Thornley, 1972). Nevertheless, due to limited growth, photosynthates often accumulate in the canopy (Boussadia et al., 2010; Hermans et al., 2006; Rufty et al., 1988). In turn, high levels of photosynthates could suppress the canopy's capacity for non-structural carbohydrates (NSC) and down-regulate photosynthesis (Goldschmidt and Huber, 1992). In fact, sugars are often considered to signal for N deficiencies (Paul and Driscoll, 1997). Therefore, allocation and abundance of photosynthates can indicate if N concentrations are favorable to plants' physiological performances. Consequently, identifying these metabolic changes could be vital in detecting excessive N applications.

We hypothesized that high N concentrations compromise plants because they need to excessively invest in N assimilation and N uptake at the expense of carbohydrate synthesis and storage. Hence, we set to study N excesses in a major horticulture crop, almond (*Prunus dulcis*), and aimed to 1) **recognize excessive N applications for tree crops**, 2) **characterize high N effects on tree physiology**, and 3) **derive metabolic indices for diagnosing N over-fertilization**. We selected almonds as a model crop for its economic role in intensively irrigated horticultural regions (e.g., California's Central Valley, southern Spain, and south-east Australia) where agricultural N runoffs are threatening both urban and natural environments (Baram et al., 2016). We used a unique setup of maturing (nonproductive) trees planted in large lysimeters that enabled complete mass-balances of water and minerals. We also monitored the trees' photosynthesis, growth, and photosynthates allocation.

2. Methods

2.1. Experimental setup

On February 2017, 24 almond (*Prunus dulcis*) saplings (Um-El-Fahem grafted on an almond and peach Spanish hybrid, GN) were planted in open field conditions at Gilat Research Center, ARO, Israel (31° 20′ N and 34° 39′ E) in 1 m^3 containers filled with an inert soilless media (perlite, type 4, 4–6 mm in diameter) and fertigated with 60, 10, and 60 $mg\,L^{-1}$ of N, P, and K (respectively). The remaining nutrients concentrations were Ca (60 $mg\,L^{-1}$) and Mg (30 $mg\,L^{-1}$). Microelements were supplied in EDTH chelates at concentration of: 0.8 mg Fe L^{-1}, 0.4 mg Mn L^{-1}, 0.2 mg Zn L^{-1}, 0.4 mg B L^{-1}, 0.3 mg Cu L^{-1}, and 0.22 mg Mo L^{-1}. In May 2017 the experiment began, and irrigation N concentrations changed to 0, 10, 30, 60, 100, or 150 $mg\,L^{-1}$ (i.e., 6 treatments in 4 repetitions and a randomized design). The irrigation solutions were prepared by dissolving commercial-grade salts (NH_4NO_3, $NH_4H_2PO_4$, KH_2PO_4, K_2SO_4, KNO_3, $NaNO_3$, and $MgCl_2$) in tap water (electrical conductivity, EC, of 0.3–0.4 $dS\,m^{-1}$) to 100 L of concentrated stock solutions with a 9:1 NO_3^- to NH_4^+ ratio. Then, 5 L aliquots of the stock solutions were mixed in 1250 L of tap water to produce the final irrigation solutions. These solutions were sampled monthly to ensure that pH and EC are similar and constant (6.9 ± 0.3 and 0.95 ± 0.1 $dS\,m^{-1}$, respectively) and that N is within 5% of the anticipated concentrations. Trees were irrigated 3 times a day (because perlite has low water retention) by automated vacuum pumps and drainage was quantified and collected for mineral analysis every 14 days. Finally, irrigation amounts were set regularly and independently for every treatment to ensure a 0.3 (± 0.05) leaching fraction, favorable water conditions, and sufficient root-zone washing.

2.2. Field measurements

During the 2018 growing season (May through November, i.e., a full year since the treatments began) evapotranspiration (ET) was quantified bi-weekly for each tree by subtracting 24 h of drainage (D) collection from the daily irrigation (I) quota (EQ. 1). Concurrently, 50 mL liquid samples were analyzed to determine irrigation and drainage N concentrations ($[N]_I$ and $[N]_d$ respectively), and to compute the seasonal nutrient uptake (N Uptake, EQ. 2). Photosynthesis rate (A_n) and stomatal conductance (g_s) were measured by leaf CO_2 and H_2O gas exchange (CIRAS-3, PP Systems) for the 10, 30, 60, 100, and 150 mg N L^{-1} treatments on August 6th, September 1st, and October 20th. The measurements were conducted on matured and sun-lit leaves between 10:30 and 11:30 a.m. at ambient light, temperature, and humidity (Table 1) and with a reference CO_2 of 400 ppm. Chlorophyll (SPAD-502, Minolta) content was also determined in similar leaves for all the trees on August 6th. On August 2nd, transpiration (T) was measured

Table 1
Photosynthetic radiation (PAR, $\mu mol\,m^{-2}\,s^{-1}$) and vapor pressure deficit (VPD, kPa) at the time of gas exchange measurements in August, September, and October.

MONTH	PAR	VPD
8	1900 ± 60	5.2 ± 0.7
9	1850 ± 27	3.2 ± 0.3
10	1590 ± 70	4.5 ± 0.4

(H_2O efflux, CIRAS-3) and divided by the midday (12:00) water potential (WP, PMS-1000) to compute the whole tree hydraulic conductance (g_{plant}, EQ. 3). To ensure that the leaves WP represented the stem WP, they were covered with an impermeable aluminum bag for 30 min before they were excised and sealed, and the WP reading occurred within 1 h. Predawn (05:00 a.m.) WP was also measured on August 2nd in trees irrigated with 30, 60, and 150 mg N L^{-1}. This time leaves were not covered (presuming there was no transpiration at night) and the measurement represented the system's WP. Stem circumference was manually measured monthly (average for 10 and 40 cm from the soil surface) to compute the daily growth while the leaf area was indexed (LAI) by light penetration (LP-80, Decagon). Finally, diagnostic leaves (young and fully developed) and roots (5 mm thick) were excised monthly for carbohydrate analysis. The July leaves and root samples were also analyzed for metabolic and mineral composition. These samples were washed in DI, oven-dried at 70 °C for 48 h and ground to a fine powder by a ball mill (MiniBeadbeater, BioSpec) before the laboratory analysis.

$$ET = I - D \tag{1}$$

$$N\ \ Uptake = [N]_I \times I - [N]_D \times D \tag{2}$$

$$g_{plant} = {}^{T}\!/_{WP} \tag{3}$$

2.3. Laboratory analysis

Powdered leaf material was digested with sulfuric acid and hydrogen peroxide (Snell and Snell, 1950). Then, N, P, and K were detected in liquid samples (irrigation, drainage, or digested tissues) by an automated photometric analyzer (Thermo Scientific Gallery152 Plus). Starch analysis followed an updated version of enzymatic digestion and colorimetric readings (Akman et al., 2012; Leyva et al., 2008) where soluble carbohydrates (SC) are first removed by suspension of 25 mg of dried tissue in 1 mL deionized water (DI), 15 min incubation at 72 °C, 10 min centrifugation at 17,000 g, and liquid removal (3 times). Then, the washed samples were incubated at 37 °C for 4 h in 500 μL sodium acetate buffer (0.2 M, pH 5.6), while 100 μL amyloglucosidase (70 units ml^{-1}, Sigma-Aldrich) and 100 μL amylase (7 units ml^{-1}, Sigma-Aldrich) were added to digest the starch. Afterwards, 50 μL of supernatant was extracted, diluted (by 21), mixed in a 96 well plate with 150 μL sulfuric acid and anthrone solution (0.1%), incubated at 96 °C for 10 min, and cooled to 22 °C. Finally, light absorbance at 620 nm was detected by spectrophotometry (Multiskan, Thermo Scientific) and glucose equivalent starch concentrations were determined.

Dried samples (leaves and roots) were also prepared for metabolite analysis according to a modified method that is described by (Roessner-tunali et al., 2003) where a 100 mg of powdered tissue is extracted in 700 μL cold methanol with 60 μL internal standard (ribitol, 0.2 mg in 1 mL of water). The samples were shaken 20 min at 4 °C, centrifuged at 20,000 g, mixed with 750 μL DI, vortexed, and centrifuged again. A 400 μL aliquot was dried by a SpeedVac (Alpha RVC, Christ), re-dissolved and derivatized for 90 min at 37 °C (in 40 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine), and treated for 30 min with 70 μL N-methyl-N-[trimethylsilyl]trifluoroacetamide at 37 °C and centrifugation. The metabolites were detected by a mass-spectrometer (Agilent 6850 GC/5795C, Agilent Technology) where one μL is injected in split-less mode at 230 °C to a helium carrier gas at a flow rate of 1 mL min^{-1}. Chromatography was performed by an HP-5MS capillary column (30 m × 0.250 mm × 0.25 μm) and the spectrum was scanned for m/z 50–550 at 2.4 Hz. Finally, the ion chromatograms and mass-spectra were evaluated by the MSD ChemStation E.02.00.493 software that identifies sugars and amino acids by comparison of retention times and mass-spectra with authentic standards (Sigma), while characterizing other metabolites through the NIST05 and Wiley libraries.

2.4. Data processing and statistical analysis

Data were processed in an open-source R (version 3.5.2) RStudio environment (version 1.1.463) with the *ggplot2*, *plyr*, *reshape2*, *lubridate*, *cowplot*, and the *agricole* packages. Variances due to N treatments were analyzed by one-way ANOVA, and the means were compared by Tukey HSD at 95% confidence (denoted by lowercase letters in figures). In the case of linear regressions, the confidence parameters were presented in tables. The average MS readings for leaf and root samples from the 60 mg N L^{-1} treatment (Mt_{60N}) were used as metabolic references. The proportional metabolic changes (Rm) in the 10, 30, 100, and 150 mg N L^{-1} treatments were computed by dividing the metabolic reading of each sample (Mt) by the relevant reference (considering N treatment and plant part, EQ. 4). Then, significant differences between the 10, 30, 100, and 150 mg N L^{-1} treatments and the 60 mg N L^{-1} reference were confirmed for each metabolite in leaves and root by a two-way, equal variance, *t*-test ($p < 0.05$).

$$R_m = {}^{Mt}\!/_{Mt_{60N}} \tag{4}$$

3. Results

3.1. Irrigation N effects on physiological performances

Almond trees increased water uptake during spring and reached peak ET levels of 200 L^{-1} $tree^{-1}$ day^{-1} in trees irrigated with 60 mg N L^{-1} by mid-July (Fig. 1A). By August ET temporarily decreased in all the trees and was down to 177 L $tree^{-1}$ day^{-1} in the 60 mg N L^{-1} treatment. Then, in late September, ET increased again to 188 L $tree^{-1}$ day^{-1} at the 60 mg N L^{-1} treatment before it finally decreased towards October. This seasonal trend of ET repeated in the 30, 100, and 150 mg N L^{-1} with changes in magnitude. ET in the 0 and 10 mg N L^{-1} treatments was low (below 50 L $tree^{-1}$ day^{-1} in August) and did not change considerably during the summer. Interestingly, high N concentrations lowered average ET to 140 L $tree^{-1}$ day^{-1} (100 mg L^{-1}) and 69 L $tree^{-1}$ day^{-1} (150 mg L^{-1}) in August. In turn, the more almond trees transpired the more N they extracted from the irrigation solution, extracting only 1 g $tree^{-1}$ day^{-1} in very low ET conditions (~40 L $tree^{-1}$ day^{-1}) and peaking at 14 g $tree^{-1}$ day^{-1} in high ET rates (Fig. 1B). N uptake was factored by irrigation N concentrations and each treatment presented and independent linear correlation to ET (Table 2). Low N concentrations (0 and 10 mg L^{-1}) resulted in stunted trees that barely transpired or extracted N. 30 mg N L^{-1} enabled trees to extract up to 7 g $tree^{-1}$ day^{-1} at peak ET rates of 210 L $tree^{-1}$ day^{-1}. At 60 mg N L^{-1} trees nearly doubled their N uptake, to 13 g $tree^{-1}$ day^{-1}, in similar ET rates of 210 L $tree^{-1}$ day^{-1}. Finally, 100 mg N L^{-1} did not increase N uptake but reduced ET below 154 L $tree^{-1}$ day^{-1}, while 150 mg N L^{-1} limited ET to ~80 L $tree^{-1}$ day^{-1} and N uptake to 10 g $tree^{-1}$ day^{-1}.

Almond trees developed full canopy (LAI reached 5.8 in the 60 mg N L^{-1} treatment) and reached maximum stem growth (0.35 mm day^{-1} of stem cross-axial growth in the 60 mg N L^{-1} treatment) by June (Fig. 2). They continued to grow steadily as their canopy matured and by October their LAI weathered below 2 and their growth rate fell under 0.2 mm day^{-1}. N concentrations determined if almond trees reached their full growth potential. Between 0 and 30 mg N L^{-1} in irrigation, trees increased stem growth during August from 0.015 to 0.27 mm day^{-1} and LAI from 0.66 to 4. Nevertheless, in 60 and 100 mg N L^{-1} almond trees maintained a similar growth rates and canopy cover. Finally, trees irrigated with 150 mg N L^{-1} decreased growth to 0.15 mm day^{-1} and canopy cover to 3.

Consistent with canopy maturation and reduced LAI, almond trees reduced maximum photosynthesis (A_n) from ~19 μmol CO_2 m^{-2} s^{-1} in August to 13.5 μmol CO_2 m^{-2} s^{-1} in October (Fig. 3A). While N concentrations did not affect photosynthesis in August, by September trees

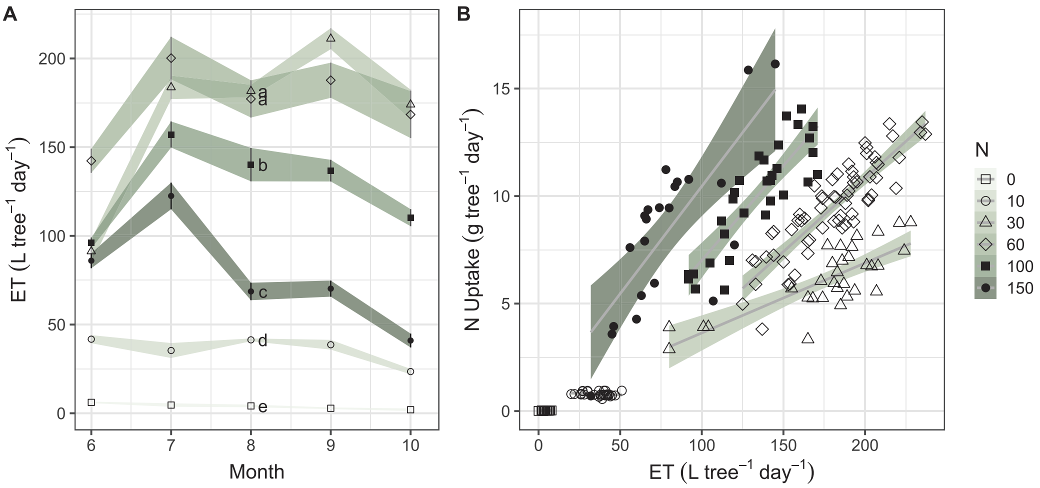

Fig. 1. Interactions between nitrogen (N) concentration, water use, and nitrogen uptake in almond trees. (A) Evapotranspiration (ET) rates for almond trees irrigated with 0 (empty squares), 10 (empty circles), 30 (empty triangles), 60 (empty diamonds), 100 (full squares), or 150 (full circles) mg N L^{-1} in June through October 2018. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments in August (Tukey HSD, $p < 0.05$, df = 18), and ribbons illustrate the continuity of the measurements (shades of green represent N concentrations). (B) The effect of ET on N uptake by almond trees irrigated with 0–150 mg N L^{-1} in June through October 2018. Symbols represent single measurements, lines illustrate linear regression, and ribbons denote 95% confidence intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Parameters for the linear regressions between transpiration and N uptake, as illustrated in Fig. 1B.

N	INTERCEPT	SLOPE	R^2	DF	P VALUE
0	0.02	0.03	0.02	29	0.4
10	0.9	−0.12	0.05	30	0.2
30	0.38	1.35	0.62	30	< 0.001
60	−2.84	2.85	0.77	30	< 0.001
100	−1.77	3.66	0.74	29	< 0.001
150	0.46	4.16	0.58	20	< 0.001

irrigated with 10 or 150 mg N L^{-1} reduced photosynthesis to ~16.5, versus ~19.8 μmol CO$_2$ m^{-2} s^{-1} for N concentrations of 30, 60, and 100 mg L^{-1}. These differences amplified in October, as A_n dropped to 14, 15, 13, 12, and 10 μmol CO$_2$ m^{-2} s^{-1} for 10, 30, 60, 100, and 150 mg N L^{-1}, respectively. This variability in photosynthetic rates corresponded to changes in stomatal conductance (g_s) in all the trees without diverging from the linear regression (Fig. 3B). In fact, except for trees irrigated with 10 mg N L^{-1}, the regression lines of all the N treatments nearly converged to a linear slope of 0.05 μmol CO$_2$ mmol^{-1} H$_2$O with an intercept at 3.8 μmol CO$_2$ m^{-2} s^{-1} (Table 3). Neither did high N concentrations affect the leaf chlorophyll content (SPAD index) which increased from 25 in 0 mg N L^{-1} to ~41 at 100 and 150 mg N L^{-1} (Fig. 3C).

Irrigation N did not affect almond trees WP at predawn which was ~-7 bar for 30, 100, and 150 mg N L^{-1} (Fig. 4A). Neither did it significantly alter the stem WP (at midday) that ranged between −15.5 bar at 0 mg N L^{-1} to −17.7 bar at 150 mg N L^{-1}. Yet, irrigation N concentrations did profoundly affect the whole-plant conductance of almond trees which dropped from 0.43 mmol H$_2$O m^{-2} s^{-1} kPa^{-1} at 10 mg N L^{-1} to 0.37, 0.35, 0.31, and finally to 0.27 mmol H$_2$O m^{-2} s^{-1} kPa^{-1} (a 37% reduction) for 30, 60, 100, and 150 mg N L^{-1}, respectively (Fig. 4B).

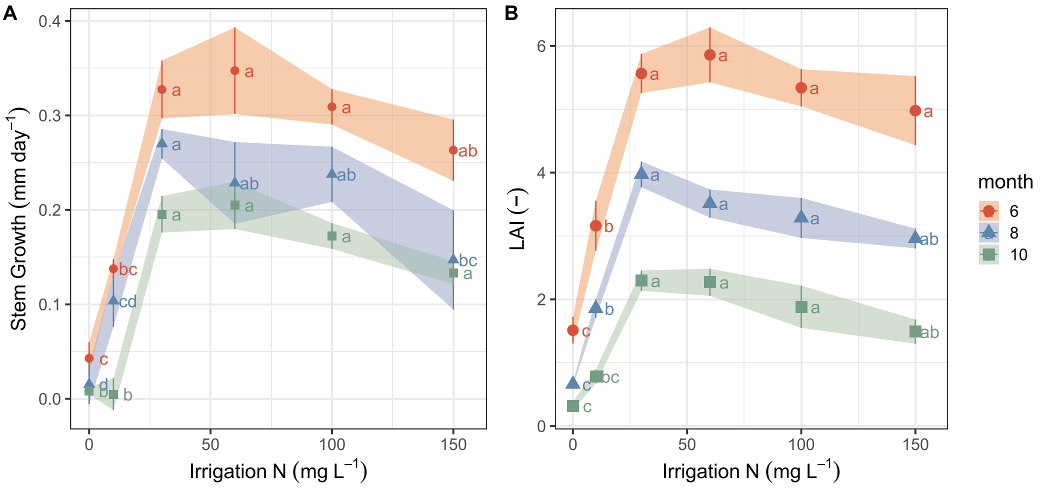

Fig. 2. Nitrogen effects on vegetative growth of almond trees. Stem growth (left panel) and leaf area index (LAI, right panel) of almond trees irrigated with 0–150 mg N L^{-1} in June (red circles), August (blue triangles), and October (green squares) 2018. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments (independent for each month and distinguished by colors, Tukey HSD, $p < 0.05$, df = 18), and ribbons illustrate the continuity of the N treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

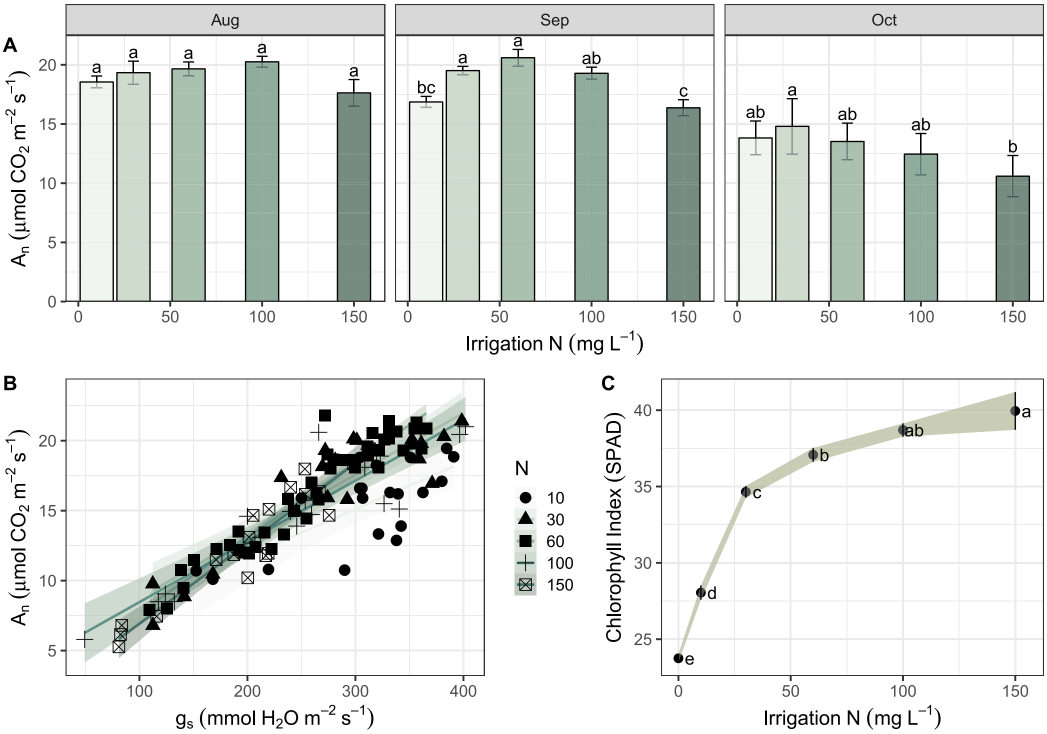

Fig. 3. Nitrogen effects on the photosynthetic and stomata performances of almond trees. (A) Photosynthesis of almond trees irrigated with 10, 30, 60, 100, or 150 mg N L^{-1} in August through October 2018. Columns represent averages (± SE), letters denote significant differences between N treatments (independent for each month, Tukey HSD, $p < 0.05$, df = 18), and shades of green symbolize N concentrations. (B) Linear relationships between stomatal conductance (g_s) and photosynthesis (A_n) for August through October 2018 in almond trees irrigated with 10 (full circles), 30 (full triangles), 60 (full squares), 100 (crosses), and 150 (empty squares) mg N L^{-1}. Symbols represent single measurements, lines illustrate linear regression ($An = 0.05gs + 3.8$, $R^2 = 0.77$), shades of green symbolize N concentrations, and ribbons denote 95% confidence intervals. (C) Chlorophyll index in August 2018 for almond trees irrigated with 0–150 mg N L^{-1}. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments (Tukey HSD, $p < 0.05$, df = 18), and ribbons illustrate the continuity of the N treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Parameters for the linear regressions between stomatal conductance and photosynthesis, as illustrated in Fig. 3B.

N	INTERCEPT	SLOPE	R^2	DF	P VALUE
10	5.04	0.03	0.53	18	< 0.001
30	4.38	0.04	0.81	18	< 0.001
60	2.35	0.05	0.9	18	< 0.001
100	4.13	0.04	0.84	18	< 0.001
150	1.19	0.06	0.9	18	< 0.001

3.2. *Metabolic responses to N concentrations*

Low N concentrations induced root starch reserves as they peaked in September at 120 mg g^{-1} of dry weight (DW) for 10 mg N L^{-1}, and at 78 mg g^{-1} DW for the stunted trees irrigated with 0 mg N L^{-1} (Fig. 5A). Correspondingly, low N markedly increased reducing-sugars (Fig. 5B) in roots (Glucose by 1529%, Galactinol by 249%, and Fructose by 1227%) despite some reductions of alcohol sugars (Sorbitol 6P by 62% and Mannitol by 20%). Low N downregulated the presence of amino acids in roots (by ~90% and nearly always significant) and components of the TCA (Fumaric acid by 70%, Citric acid by 55%, and Aconitic acid by 84%). In leaves, low N (10 and 30 mg L^{-1}) reduced the level of amino acids (Phenylalanine by 51%, Glutamic acid by 43%, and Asparagine by 79%) and components of the TCA (Malic acid by 29%) relative to their levels at the 60 N L^{-1} treatment (Fig. 5B). Low N also reduced the levels of Sorbitol by 34% and Mannitol by 28%, while it increased the level of Glucose by 197%. Similar to Glucose, starch concentrations peaked in August at 49 mg g^{-1} DW in leaves of trees irrigated with 10 mg N L^{-1} treatment. These trees exhausted their starch reserves fast, and by September trees irrigated with 30 mg N L^{-1} had maximal starch concentrations (47 mg g^{-1} DW).

High N concentrations (150 mg N L^{-1}) increased the amino acids in the leaves (Threonine by 210% and Asparagine by 380%) compared to the 60 mg N L^{-1} treatment but had no significant effect on their reducing-sugars (although it seemingly decreased Glucose and Fructose, Fig. 5B). High N did limit starch in leaves, and although these differences were still insignificant in June and July, by August leaves starch reserves dropped significantly to 18 mg g^{-1} DW at 150 mg N L^{-1} (Fig. 5A). Root starch reserves were not affected by N treatments above 30 mg N L^{-1}, averaging at 52 mg g^{-1} DW in trees irrigated with 30–150 mg N L^{-1}. Neither did 100 or 150 mg N L^{-1} have a prominent effect on roots metabolism, compared to the 60 mg N L^{-1}, except for a 181% increase in Citric acid (part of the TCA).

3.3. *Possible indices to excessive N applications*

On July 25th (2018) leaf N concentrations ranged between 13 mg N g^{-1} DW in severely deficient almond trees (0 mg N L^{-1}) and ~22 mg N g^{-1} DW in trees irrigated with 30–100 mg N L^{-1} (Fig. 6A). Then, even a 5 folds increase in irrigation N (from 30 to 150 mg L^{-1}) increased leaf N concentration by only 10% (to 23 mg N g^{-1} DW). Phosphorus (P), on the other hand, dropped from 4 to 2.1 mg P g^{-1} DW, when irrigation N increased from 0 to 30 mg N L^{-1} and remained constant for 30–150 mg N L^{-1}. Roots supplied a much sharper indication to N deficiency, having practically negligible N concentrations (< 5 mg N g^{-1} DW) in almond trees irrigated with 0 or 10 mg N L^{-1}, and accumulating ~24 mg N g^{-1} DW for 30–150 mg N L^{-1}. Phosphorus accumulation tightly corresponded to root N concentrations, rising from 1.3 to

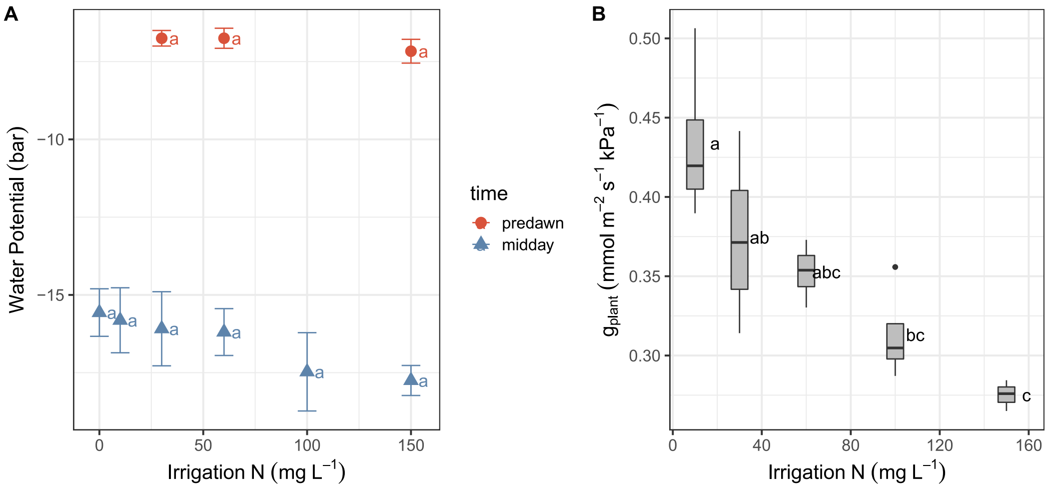

Fig. 4. Nitrogen effects on water status and hydraulic capacity of almond trees. (A) Predawn (green boxes) and midday (blue) water potentials for almond trees irrigated with 0–150 mg N L^{-1} in August. Boxes enclose 1st and 3rd quartile, horizontal lines point to the median, vertical lines mark the minimum and maximum values, single points show outliers, and letters denote significant differences between N treatments (independent for each time and distinguished by colors, Tukey HSD, $p < 0.05$, df = 18). (B) Whole plant hydraulic conductance (g_{plant}) in August of almond trees irrigated with 10–150 mg N L^{-1} (boxes enclose 1st and 3rd quartile). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.6 mg P g^{-1} DW between 0 and 30 mg N L^{-1}, and averaging 4.7 mg P g^{-1} DW in trees irrigated with 30–150 mg N L^{-1}. Finally, K did not accumulate (~5 mg K g^{-1} DW) in the roots of almond trees, independent of irrigation N concentrations. Coupling the mineral and the metabolic analysis for July 25th, the leaf N to starch ratio (N:ST) increased from 0.4 to 0.6 as irrigation N increased from 0 to 10 mg N

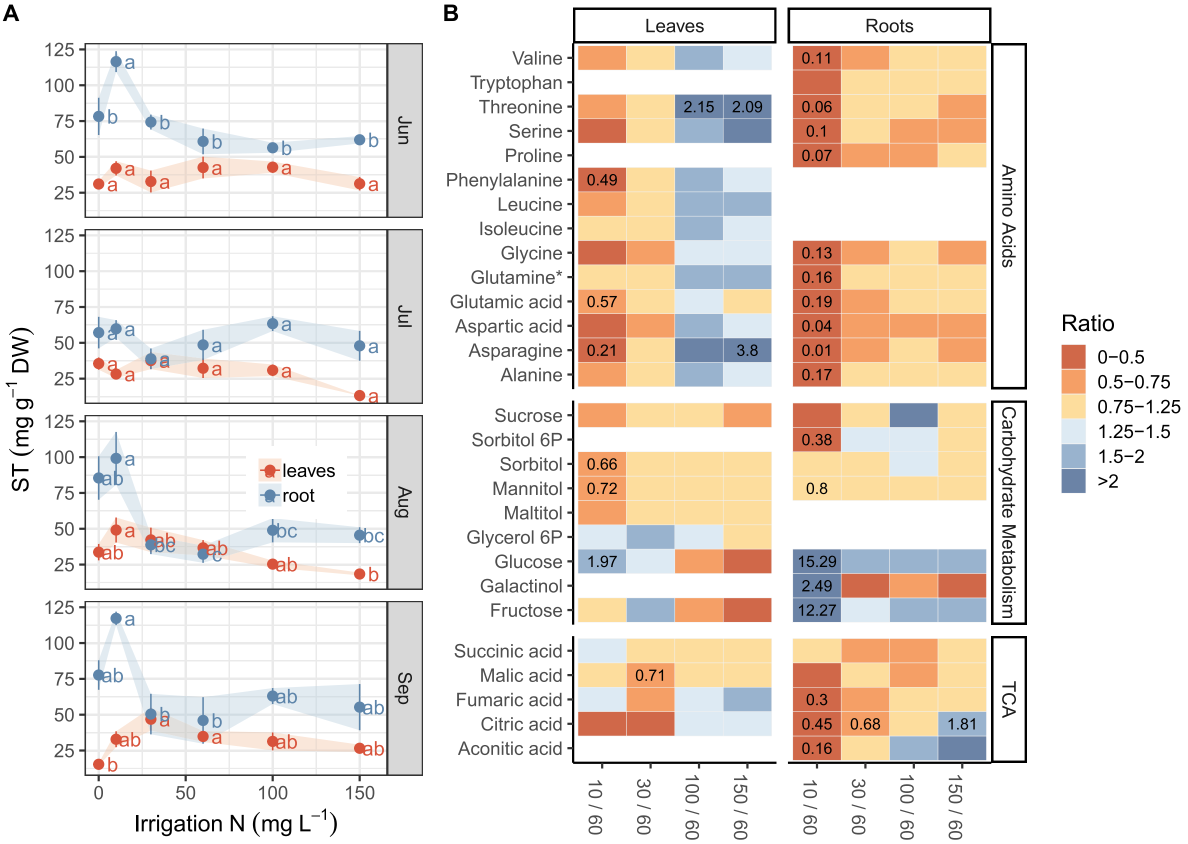

Fig. 5. Nitrogen effects on the metabolic composition of almond trees. (A) Starch (ST) concentrations in leaves (red symbols) and root (blue symbols) of almond trees irrigated with 0–150 mg N L^{-1} in June, July, August, and September 2018. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments (independent for each part and distinguished by colors, Tukey HSD, $p < 0.05$, df = 18), and ribbons illustrate the continuity of the N treatments. (B) A heatmap of primary metabolites associated to the amino acids, carbohydrate metabolism, or the TCA pathways in leaves and roots of almond trees irrigated with 10, 30, 100, and 150 mg N L^{-1} in July 2018. Colors represent the proportions between the means of each treatment and the reference group (60 mg N L^{-1}), and text denotes significant changes (*t*-test, $p < 0.05$). Asterisks denote that during the derivatization process most Glutamine, and some Glutamic Acid, cyclized to Pyroglutamic Acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

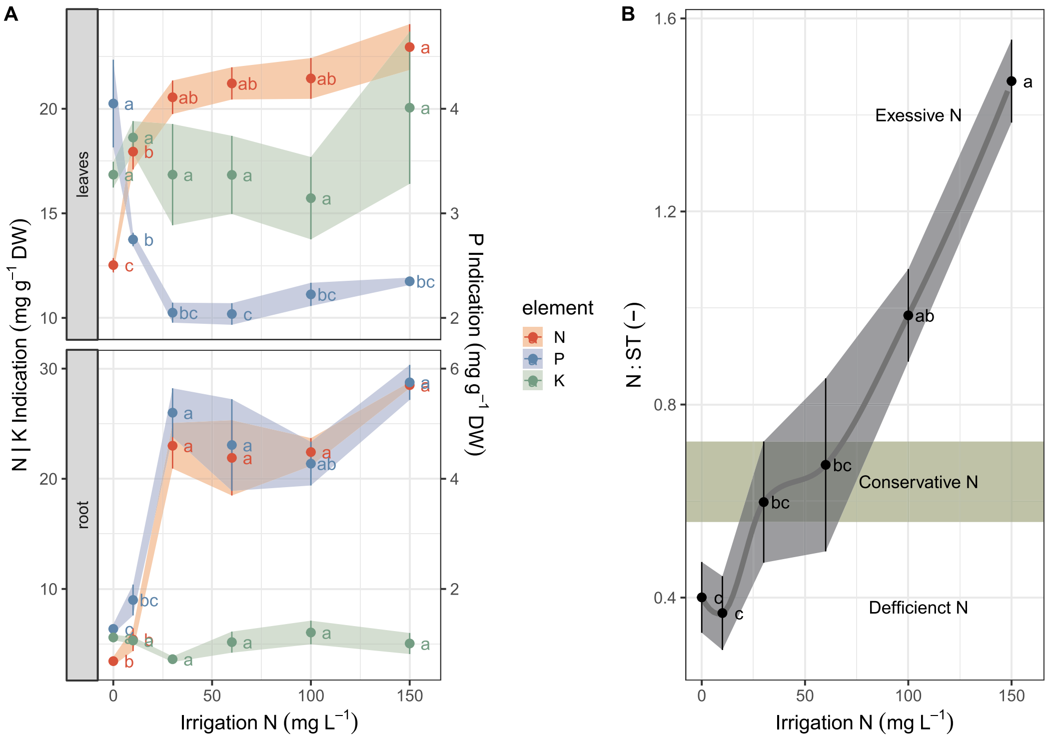

Fig. 6. Mineral and metabolic indices to the nutritional status of almond trees in July. (A) Nitrogen (N, red symbols, left axis), potassium (K, green, left axis), and phosphorus (P, blue, right axis) concentrations in leaves (upper panel) and root (lower panel) of almond trees irrigated with 0–150 mg N L^{-1}. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments (independent for each nutrient and distinguished by colors, Tukey HSD, $p < 0.05$, df = 18), and ribbons illustrate the continuity of the N treatments. (B) Nitrogen to starch ratio (N:ST) on July 25th for almond trees irrigated with 0–150 mg N L^{-1}. Symbols represent averages, error-bars denote ± SE, letters denote significant differences between N treatments (Tukey HSD, $p < 0.05$, df = 18), ribbons illustrate the continuity of the N treatments, line marks polynomial (1st order) regression, and green zone encompasses a range of optimal levels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

L^{-1} to 30 mg N L^{-1} (Fig. 6B). The N to starch ratio remained ~0.6 at 60 mg N L^{-1} irrigation, and then increased to 0.98 at 100 mg N L^{-1}, and to 1.47 at 150 mg N L^{-1}.

4. Discussion

We set to identify, study, and find tools to mitigate excessive nitrogen applications in horticulture. To this end, studying 2-year-old almond trees planted in large scale drainage lysimeters that monitor whole-tree water and nutrients mass balances proved useful. We supported our original perception that ***high N concentrations could compromise essential physiological processes in almond trees***. Specifically, we showed that high N concentrations (over a 100 mg N L^{-1} in irrigation) could hinder transpiration and photosynthesis. We tracked this back to limited whole-tree hydraulic conductance and found that it changed the metabolic composition of almond leaves. Subsequently, coupling these novel findings with current leaf mineral indices, we proposed a new index to N nutrition in horticulture.

Excessive N concentrations strain trees' water uptake and could inhibit their development and growth. This concurs with recent reports concerning olive (Erel et al., 2013; Haberman et al., 2019), citrus (Alva et al., 2006), and cherry (Neilsen et al., 2007) that high N concentrations have adverse effects on production. Specifically, we report that while N is essential for the productivity of almond trees, its benefits are exhausted at relatively low concentrations of 30-60 mg N L^{-1} in irrigation water (Fig. 1A). Our findings coincide with previous studies concerning the N requirements of almond trees (Muhammad et al., 2018), and yet most studies focused on field applications and did not extend the investigation to higher N concentrations. We did study high N concentrations and found that irrigation of 100–150 mg N L^{-1} would lower trees' annual ET by over 50%, from 27,000 L to less than 12,500 L. On the other hand, N uptake is a rigid property of plants, determined by physiological constraints (Glass, 2003), that ranged between 5 and 10 g tree^{-1} day^{-1} in our research case, and would not exceed 13 g tree^{-1} day^{-1} at extremely high N concentrations (Fig. 1B). Considering an average uptake of 8 g tree^{-1} day^{-1}, 240 trees in a hectare, and continuous N uptake for May through September (150 days), this adds to ~290 kg N hectare^{-1}, which fits the current fertilizing recommendations in California (Doll, 1996). Currently, farmers apply N in doses during March through May to meet the trees' reproductive demands (they apply a final dose after harvest). Our findings suggest that N fertilization should follow ET for almond trees to extract nitrogen continuously throughout the growing season (Fig. 1B). Moreover, as N uptake is tightly coupled to the transpiration stream (Fig. 1B), high N concentrations that lower ET (Fig. 1A) could limit N uptake, and intensify N runoffs to the environment. Hence, identifying the actual mechanisms affected by high N is pivotal for a precise N application.

High N concentrations limit trees' photosynthetic productivity by lowering the whole-tree hydraulic conductivity. High N concentrations affected ET by early summer, and reduced midday photosynthesis by 30% in September (Fig. 3A). Nakaji et al. (2002) observed this in Japanese pines and concluded that N amassed during summer till it interfered with carboxylation in the 2nd half of the growing season. In our research case, considering that photosynthesis was tightly coupled with stomatal conductance (Fig. 3B) and that chlorophyll was not affected by high N (Fig. 3C), it seems that the limitation was hydraulic. Hence, as N deficiencies are considered to cause photochemical changes (Erel et al., 2014), it appears that high N concentrations should be studied independently. Since the pressure gradients between soil, plant, and atmosphere were similar in all the trees (independent of N concentrations, Fig. 4A), these were not external, but intrinsic, constraints to photosynthesis and transpiration. Accordingly, irrigation N concentration was inversely correlated to almonds whole-tree hydraulic conductance (Fig. 4B). It did not affect ET in irrigation N concentrations between 10 and 60 mg N L^{-1} because canopy size increased (Fig. 2B), but the hydraulic loss became a dominating factor in the 100 and 150 mg N L^{-1} treatments. Hydraulic constraints at high N concentrations could be explained by the *Optimal Partitioning* theory and the Thornley model (Thornley, 1972), as N is easily transported to the canopy. There, photosynthates abundancy promotes vegetative development at the expense of root growth and water extraction from the soil [see the case of nectarine trees (Sauge et al., 2010)]. In our research case, this could be the hydraulic limitation that inhibited photosynthesis. Alternatively, transpiration limitations due to high N concentrations were previously attributed to low nitrate to ammonium ratio in peppers (Bar-Tal et al., 2001). In our case, the NO_3^- to NH_4^+ ratio in the irrigation was 9:1, although the NH_4^+ concentrations in the 100 and 150 mg N L^{-1} treatments could still lead to ammonium toxicity. Moreover, molecular studies demonstrated that high N concentrations could cause negative feedback to N transporters in roots, and lead to ammonium effluxes and even higher NH_4^+ at the root zone (Glass, 2003).

Nitrogen concentration in trees determines their partitioning of photosynthates, the composition of biomolecules in their tissues, and their disposition to grow canopy and roots. Carbon enters trees' canopy through photochemical phosphorylation to glyceraldehyde 3-phosphate (G3P) that could immobilize as structure and starch or oxidize for the composition of macromolecules through the citric acid cycle. We drew a conceptual model to this (Fig. 7) which illustrates that, overall, almond trees assimilated most of their nitrogen in their leaves and transported N complexes to the roots according to their needs. In N deficiency, leaves assimilated the majority of the available N and then transported their limited resources down to the roots (Fig. 5B), probably to acquire additional N (Rufty et al., 1988). Such leaves produce less chlorophyll for photosynthesis (Boussadia et al., 2010), or proteins for cell elongation (MacAdam et al., 1989), and immobilize much of their carbohydrates to starch. Consequently, N deficient roots received sugars, but with limited N assimilation, they could not produce ample amino acids and proteins for cell elongation or growth and also accumulated it as starch (Fig. 5A). Hence, in low N conditions, photosynthates were primarily C substrates (sugars and structure), and both leaves and roots had a high C to N ratio. In N abundance, on the other hand, as N substrates tend to increase and carbohydrates tend to decrease (Bi et al., 2004), almond leaves accumulated amino acids with supplementary N assimilates (Fig. 5B). Such leaves would have a low C to N index and promote vegetative growth (Taiz and Zeiger, 2002). With limited cellular resources for metabolism, these leaves would also limit respiration, which could limit their energy for the demanding N assimilation (Marschner, 2011). In this case, high N concentrations could ultimately act as a feedback inhibition to N assimilation (King et al., 1993). In the roots, a corresponding increase of major TCA components (mainly Citrate, Fig. 5B) could have also inhibited Pyruvate synthesis and limited further carbohydrate oxidation to available energy (Taiz and Zeiger, 2002). Such roots, with a limited influx of nitrogen complexes and probable respiratory restrictions that would affect N assimilation (Reich et al., 2008), are known for downregulating root branching and development (Walch-Liu et al., 2006). Hence, while it's difficult to identify high N conditions by leaf mineral analysis (Sanchez and Righetti, 1990), detecting shifts between sugar substrates and N rich composites is evidently feasible.

Coupling photosynthates portioning with the traditional mineral analysis is a better measure of trees' nutritional status. The trees we studied often transpired 200 L day^{-1} (Fig. 1A). According to the 1 C (sequestration) to 500 H_2O (efflux) molar ratio we measured, which also fits the literature concerning almonds (Egea et al., 2011), it implies they could have metabolized 260 g C day^{-1}. Considering that respiration requires 30% of sequestered C (Lambers, 1985) and that C accounts to 40% of plant dry matter (Abiven et al., 2005), this would amount to ~455 g of biomass. If 20 mg N g^{-1} DW (as we measured in leaves and root) applies to stem and branches, 455 g DW consist ~9 g N. This quantity of N fits the uptake we measured in almond trees.

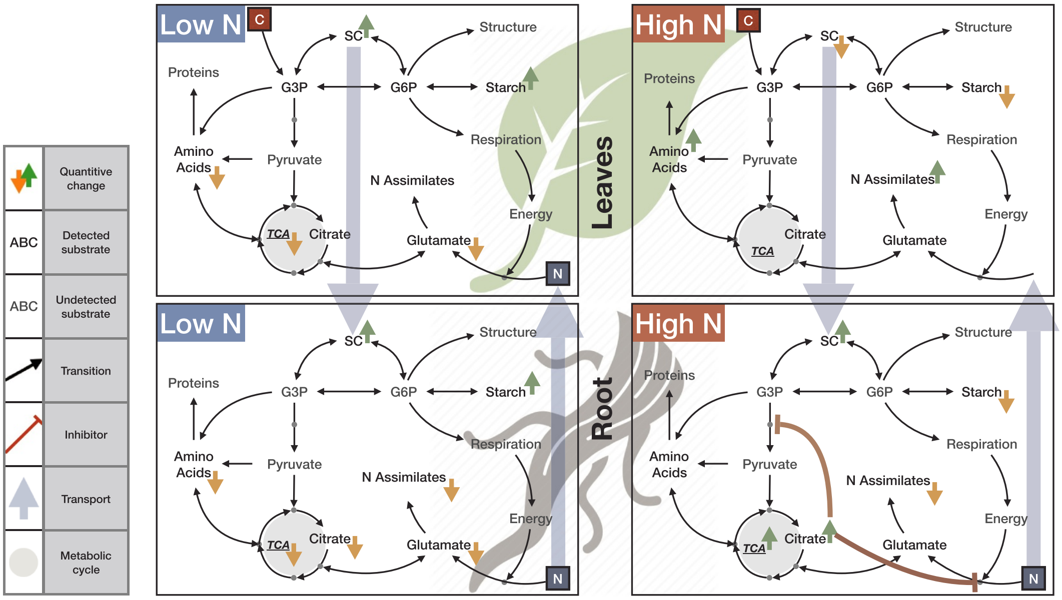

Fig. 7. A schematic interpretation of changes in carbon (C) and nitrogen (N) metabolism in almond trees due to nitrogen applications. Upper left panel represents metabolic differences (green for induction and yellow for reduction) between the leaves of trees irrigated with 10 mg N L^{-1} to the reference group (60 mg N L^{-1}) and the lower left panel illustrates a similar comparison in roots. Right panels repeat the comparison in trees irrigated with 150 mg N L^{-1} (to the reference group). Transport is illustrated by thick blue arrows, the citric acid cycle (TCA) by a grey circle, and generic biomolecules by plain grey text. The black text highlights biomolecules that were detected by chromatography, black arrows mark state transitions, and bar-headed lines mark biological inhibitions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Nevertheless, in N constituents (e.g., proteins or amino acids), N comprises 10–50% of the total biomass. In this case, only 100 g of biomass attributes to N metabolism, which leaves another 350 g C to structure (cellulose) and essential sugar metabolites (storage starch or soluble sugars). As starch constituted ~4% of the dry biomass, it means that any shift in N metabolism would have a significant effect on sugar storage status. While leaves do not hold the critical sugar reserves in trees, they do act as intermediate storages and give a fair proxy to the affinity of woody tissues to utilize or immobilize carbohydrates (Li et al., 2003). Although we initially suggested that leaves' mineral analysis cannot detect high N concentrations, our extreme treatments (150 mg N L^{-1}) and proficient sampling did exhibit a significant increase of N concentrations in leaves (Fig. 6A). Nevertheless, repeating this in farms is insurmountable. Alternatively, higher P levels in leaves did concur with N deficiencies (Fig. 6A), but such analyses are useless because N measurements would remain critical for confirmation. Interestingly, we found that N and P did not accumulate in the roots until canopy N was sufficient (at 30 mg N L^{-1} in irrigation), implying that roots are good proxies to mineral deficiencies. Yet neither N or P accumulated further in roots of trees irrigated with 100 or 150 mg N L^{-1}. What did change is the ratio between N constitutes and sugar storages (Fig. 6B). This ratio also changed at low N conditions (increasing from 0.4 to 0.6), but as the denominator (starch content) did not change, N concentrations sufficed to represent these conditions. In high N conditions, abundance of free amino acids and proteins at the expense of sugar metabolites, and a constant nominator (N), induced the N:ST index in a detectable manner. Under appropriate N applications the N:ST ratio ranged between 0.5 and 0.9, while in N concentrations that inhibited transpiration and photosynthesis of almond trees it exceeded 1.1. Hence, the N:ST ratio is a physiological index that represents the proportions between development and conservation, and we should integrate it into farming applications.

5. Conclusions

We found that high N applications in tree crops compromised their ET and photosynthetic productivity. The notion that orchards might be underperforming, and using unnecessary resources due to poor nitrogen management, augments our environmental concerns. The physiological repression caused by high N, which appears to be hydraulic, promotes photosynthates portioning to N constitutes over sugars. It could exhaust the trees' energetic resources and render them vulnerable alternative stresses. Such shifts in the metabolic composition are easily detected, and they could be combined with the current mineral analysis to identify excessive N conditions in crops. Hence, with further measurements, integrations and modifications, we could substantiate the N:ST index and apply our findings to promote precise N fertilization.

Authors' contributions

OS and UY conceptualized the research and established the methods to approach it. RK and OS carried the field work, physiological measurements, and plant tissue collections. OS, UY, RE, HY, and LK established the analytical procedures for laboratory analyses and data processing. Finally, OS and RK wrote the initial draft of the paper, and then all the corresponding authors revised it.

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