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ORIGINAL PAPER

Effect of Nitrogen Sources on Photosynthesis and Biosynthesis of Alkaloids and Leaf Volatile Compounds in *Annona sylvatica* A. St.-Hil

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Abstract

Nitrogen (N) has been reported to act on both primary and specialized metabolism of plants. However, it is not clear how different N sources affect metabolism in species of the genus *Annona*. Thus, the aim of the work was to analyze how nitrate (NO_3^-) and ammonium (NH_4^+) influence photosynthesis and the production of alkaloids and leaf volatile compounds in *Annona sylvatica* A. St.-Hil. Plants were submitted to four treatments [NH_4^+, NO_3^-, NO_3^-:NH_4^+, and no N (W/N)], in hydroponic cultivation, and collected at 30, 60, and 90 days after the beginning of treatments. Plants maintained in NH_4^+ showed greater photosynthetic activity, high production of total alkaloids, in particular liriodenine, and increased production of leaf volatile compounds commonly related to stress situations. On the other hand, plants cultivated with NO_3^- showed lower photosynthetic activity and higher production of leaf volatile compounds related to plant resistance and defense. *A. sylvatica* seedlings are adapted to NH_4^+ with energy resources used to increase both primary and specialized metabolism, while using NO_3^-, the lower energy availability leads *A. sylvatica* plants to invest in leaf defense and not in photosynthesis. The individual use of NH_4^+ and NO_3^- increases the phytochemical potential of the species by stimulating the production of different groups of specialized metabolites.

Keywords Ammonium · Annonaceae · Liriodenine · Primary metabolism · Specialized metabolism · Nitrate

Abbreviations

N	Nitrogen
NO_2^-	Nitrite
NO_3^-	Nitrate
NH_4^+	Ammonium
NO_3^-:NH_4^+	Nitrate plus ammonium
W/N	Without nitrogen
C	Carbon
DAT	Days after the start of treatments

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1 Introduction

N is widely reported in plants for maintaining metabolism (Kusano et al., 2011), being used in the synthesis of numerous molecules, such as amino acids, proteins, nitrogenous compounds, chlorophylls (Stitt et al., 2009), and the rubisco enzyme (Lawlor et al., 1989). For plants to be able to assimilate it, N must be available in the form of NO_3^- or NH_4^+ (Mur et al., 2016). N assimilation is energetically costly, requiring reducing agents, ATP and carbonic skeletons, especially when NO_3^- is the main N source. When absorbed as NO_3^-, the ion needs to be reduced into nitrite (NO_2^-) and then into NH_4^+ to be assimilated into a carbonic skeleton and, if absorption occurs in the form of NH_4^+, there is less energy expenditure because there is no reduction process involved (Mur et al., 2016). Thus, N metabolism is related to carbon (C) metabolism, being that both compete for reducing agents, since the reduction of NO_3^- into NO_2^- uses electrons from NAD (P) $H + H^+$ and the reduction of NO_2^- into NH_4^+ uses electrons from ferredoxin, thus interfering with the flow of electrons to reduce C during the photosynthetic process (Nunes-Nesi et al., 2010).

In addition to competition for reducing agents, N and C show codependence related to assimilation, where CO_2 assimilation depends on adequate N supply to compose the photosynthetic apparatus, since N is present, for example, in the composition of chlorophyll (Nunes-Nesi et al., 2010) and the rubisco enzyme (Lawlor et al., 1989), and carbonic skeletons are necessary for N incorporation (Fritz et al., 2006), acting during its assimilation (Nunes-Nesi et al., 2010).

In relation to specialized metabolism, N is present in the structure of molecules such as alkaloids, which are nitrogenous compounds derived from amino acids (Evans, 2009; Wink, 2010). Among alkaloids, benzylisoquinolines (BIA) stand out, which are some of the most specialized metabolites found in the Annonaceae family (González-Esquinca et al., 2014; Lucio et al., 2015). BIA include aporphine and oxoaporphine alkaloids such as liriodenine, considered a chemotaxonomic marker in the Annonaceae family (González-Esquinca et al., 2014, Lucio et al., 2015). Liriodenine is an alkaloid of interest because it has medicinal properties, such as cytotoxic action against cancer cells (Chen et al., 2013; Lucio et al., 2015 Suresh et al., 2012) and properties against phytopathogens (De-la-Cruz-Chacón et al., 2019).

In addition to alkaloids, N also influences the biosynthesis of volatile compounds, which depends on C and N availability, as well as on energy provided by the primary metabolism (Dudareva et al., 2013). Volatile compounds act in the plant defense system (Alcântara et al., 2017; Erb, 2018) and in the medicinal field (Formagio et al., 2013), and are divided into classes including terpenoids, phenylpropanoids, benzenoids, fatty acids, and amino acids derivatives (Dudareva et al., 2013).

Based on the above, the effects of N concentrations and sources can be observed on the primary metabolism of several species (Cechin and Fumis, 2004; Imran et al., 2019; Liu et al., 2017; Zhao et al., 2005; Zhou et al., 2011). However, in the genus *Annona*, no studies were found assessing how different N sources (NH_4^+ and NO_3^-) affect photosynthetic variables and specialized metabolism. The literature only reports the effect of NO_3^- concentrations on primary and specialized metabolisms (leaf volatile compounds) in *A. emarginata*, indicating that at low NO_3^- concentrations (1.87 mM of N), there is low activity of the NO_3^- reductase enzyme and use of reducing agents and carbonic skeletons to increase volatile compounds and carbohydrates (Campos et al., 2019). In *A. diversifolia*, there are reports of increased production of alkaloids with the use of 30 mM of N in treatment with two N sources used together (NO_3^-:NH_4^+) (Orozco-Castillo et al., 2016). Thus, in both works, the effects of different N sources were not evaluated in isolation, which highlights the gap to be explored in relation to the individual supply of each N source (NH_4^+ and NO_3^-) on the primary and specialized metabolism of species of the genus *Annona*.

A. sylvatica (≡ *Rollinia sylvatica*) is an endemic and native species from Brazil, known as "araticum," "araticum-do-mato," "embira," "cortiça," and "cortiça-amarela" and inhabits the phytogeographic domains of Mata Atlântica and Pantanal (Lorenzi et al., 2005; Maas et al., 2015; Flora do Brasil, 2020). In addition, the species produces specialized metabolites of high pharmacological interest such as acetogenins, terpenes, and alkaloids (Andrade-Silva et al., 2020; Mikolajczak et al., 1990; Formagio et al., 2013; Gonçalves et al., 2015).

The work aimed to analyze how N sources (NH_4^+ and NO_3^-) impact the photosynthetic process and the production of specialized metabolites used to expand the phytochemical potential of *A. sylvatica*. The hypothesis is that the supply of NH_4^+ will increase the synthesis of alkaloids and volatiles with less energy expenditure. The results also provided physiological and chemical arguments for the conservation of this plant endemic and native to Brazil since the detection of an N source that increases the production and/or diversity of metabolites can be of great importance for the pharmaceutical area and future studies.

2 Material and Methods

The experiment was carried out using 180 *A. sylvatica* seedlings in hydroponic cultivation conducted in a paddy fan greenhouse with temperature maintained at ± 25.50 °C, air humidity at ± 50.05%, photosynthetic photon flux density (PPFD) at ± 300.00 µmol m^{-2} s^{-1}, and CO_2 environment at ± 420 PPM, located at 48° 240′ 3500″ W, 22° 49′ 1000″ S, and 800 m above sea level at the Biosciences Institute of the São Paulo State University, Campus of Botucatu, São Paulo, Brazil.

The experimental design was in randomized blocks in a 4 × 3 factorial scheme, with treatments consisting of four N sources (NO_3^-, NH_4^+, NO_3^-:NH_4^+, W/N) and three collection times (30, 60, and 90 days after the start of treatments (DAT)) with five replicates of three plants. The beginning of treatments was the moment in which plants were placed under different N conditions. Solutions were periodically changed and monitored as a function of pH and electrical conductivity, in order to keep nutrients as tools throughout the experiment. Plants were grown with Hoagland and Arnon (1950) nutrient solutions 1 and 2, with modifications, to supply the desired N source in amount equal to 0.105 g of N per liter, such as NO_3^-, NH_4^+, NO_3^-:NH_4^+, and W/N (Supplementary Fig. S1).

2.1 Gas Exchange and Chlorophyll a Fluorescence

Gas exchange and chlorophyll *a* fluorescence were simultaneously evaluated in one plant of each replicate for each treatment at 30, 60, and 90 days after the beginning of the experiment, between 09:00 am and 11:00 am, in leaves with fully expanded limbus. Open photosynthesis system equipment with CO_2 and water vapor analyzer by infrared radiation and a coupled fluorometer (infrared gas analyzer (IRGA), model GSF 3000 FL, with Array/PAM-Fluorometer 3055-FL, Walz) was used.

For gas exchanges, saturating light of 1200 µmol m^{-2} s^{-1} was used, and net CO_2 assimilation rate (A_{net}, µmol CO_2 m^{-2} s^{-1}), transpiration rate (*E*, mmol water vapor m^{-2} s^{-1}), stomatal conductance (*gs*, mmol m^{-2} s^{-1}), and internal CO_2 concentration in the substomatal chamber (*Ci*, µmol CO_2 mol^{-1}) were evaluated. Water use efficiency (WUE, µmol CO_2 (mmol $H_2O)^{-1}$) was calculated using the relationship between net CO_2 assimilation rate (A_{net}, µmol CO_2 m^{-2} s^{-1}) and transpiration rate (*E*, mmol water vapor m^{-2} s^{-1}); the instant carboxylation efficiency of the rubisco enzyme (A_{net}/*Ci*, mmol m^{-2} s^{-1} Pa^{-1}) was calculated using the relationship between net CO_2 assimilation rate (A_{net}, µmol CO_2 m^{-2} s^{-1}) and internal C concentration in the substomatal chamber (*Ci*, µmol CO_2 mol^{-1}) (Zhang et al., 2001).

For chlorophyll *a* fluorescence, actinic light pulse of 4,500 µmol m^{-2} s^{-1} was used, and maximum fluorescence (Fm '), potential quantum efficiency of photosystem II (Fv'/Fm '), effective quantum efficiency of photosystem II (ΦFSII), electron transport rate (ETR), photochemical dissipation (qP), fraction of light absorbed by the photosystem II antenna dissipated as heat (*D*), and fraction of excitation energy not dissipated in the antenna that cannot be used in photochemical reactions (*Ex*), calculated according to Demmig-Adams et al. (1996), were evaluated in light.

2.2 Extraction and Quantification of Alkaloids

To obtain the alkaloid extract, 5 replicates with a pool of 3 plants were used for each treatment.

Alkaloids were extracted from roots previously dried in a forced aeration oven at 30 °C, using the acid–base method. After thorough grinding, the plant material was moistened with a saturated sodium carbonate (Na_2CO_3) solution and left to dry for 48 h at room temperature. Alkaloids were extracted with chloroform ($CHCl_3$) by constant stirring for 1 h and then filtered and washed with distilled water. The $CHCl_3$ phases were extracted into a 1 M hydrochloric acid (HCl) solution before being alkalinized to pH 9.5 with a saturated solution of Na_2CO_3. The alkaline solution was then re-extracted with $CHCl_3$, dried with anhydrous sodium sulfate (Na_2SO_4), filtered, and evaporated at approximately 25 °C to obtain total alkaloids (De-la-Cruz-Chacón and González-Esquinca, 2012; Sousa et al., 2019; Vinche et al., 2020).

The same extract was used for quantification of total alkaloid, HPLC alkaloids analysis, and liriodenine quantification. The quantification of total alkaloids was performed by spectrophotometry at 254 nm using liriodenine as reference for the elaboration of the standard curve ($y = 0.0881x$—0.0112, $R^2 = 0.9949$). Alkaloid detection analysis and liriodenine quantification were performed by ultra-high-performance liquid chromatograph (UHPLC—Thermo Fisher-Scientific®, Waltham, MA, USA) with gradient pump and UVVIS detector using the C18 reverse phase column (150 × 4.6 mm and 5 µm particle diameter). The mobile phase was water (pH 3.5 with trifluoroacetic acid) and 30:70 gradient methanol with flow rate of 1 mm/min maintaining column temperature at 30 °C; detection was carried out in UV at 280 nm.

For liriodenine quantification, calibration curve was performed by analyzing the series of 100 mg mL^{-1} stock solutions ($y = 0.3658x + 1.114$; $R^2 = 0.9999$), and for the alkaloid detection analysis, standards of reticulin, norpredicentrine, N-methyl-laurotetanine, norglaucine, discretine, xylopine, xylopinin, assimilobin, laurotetanine, liriodenine, oxoglaucine, and lanulinusin alkaloids were used (adapted from Sousa et al., 2019). Liriodenine was provided by Iván De-la-Cruz-Chacón. The methods for isolation and data identification of liriodenine were reported by De-la-Cruz-Chacón and González-Esquinca (2012). The presence identification of alkaloids norpredicentrine, reticuline, and discretine was also carried out by comparison to standards provided by Emmanoel Vilaça Costa and Jackson Roberto Guedes da Silva Almeida (spectrometric data were previously reported in Sousa et al., 2019).

2.3 Leaf Volatile Compounds

Leaf volatile compounds were captured from leaves dried in forced aeration oven at 30 °C until constant mass was obtained. Three replicates of 3 plants were used for each treatment, totaling 108 plants.

For each sample, 0.05 g of macerated dry leaves was placed in glass flask added of 10 mL of distilled water. The flask was sealed and placed in water bath for 1 h at temperature of 90 °C. After that period, the flask was removed from the water bath and the capture of volatiles was performed by headspace solid-phase microextraction (HS-SPME) using SPME Fiber Assembly 75, CarboxenTM-PDMS for Manual Holder-SUPELCO, for 30 min. Then, the chemical composition of volatiles was determined by gas chromatography coupled to mass spectrometry (GC–MS) in Shimadzu equipment model QP-5000 equipped with fused silica capillary column DB-5 (30 m × 0.25 mm × 0.25 µm); electron ionization (EI) was used at 70 eV, helium as carrier gas (flow rate

of 1.0 mL min^{-1}), injector at 220 °C, transfer line at 230 °C, and split ratio of 1:20, using the 60 °C to 240 °C, 3 °C min^{-1} temperature program.

To identify substances present in leaf volatile compounds, mass spectra of compounds were compared with those in the GC–MS system database (Nist. 62 Libr.) and linear retention indexes (LRI) found in literature (Adams, 2017). Linear retention indices were obtained from the injection of a standard mixture of n-alkanes (C9-C24), applying the equation of Van den Dool and Kratz (Van Den Dool, Kratz, 1963).

2.4 Statistical Analyses

Data obtained (gas exchange, fluorescence, total alkaloids, liriodenine, and relative percentage of leaf volatiles) were submitted to two-way analysis of variance (ANOVA). Two-way ANOVA was conducted to determine the effects of treatments (NO_3^-, NH_4^+, NO_3^-:NH_4^+, W/N) with time (30, 60, 90 DAT) and their interaction. Means were compared by the Tukey test at 5% significance level ($p < 0.05$), as mean with standard error (M ± SE) (Gomes, 1990).

For the heat map, hierarchical cluster analysis was performed, using the relative percentage of the leaf volatile profile after transforming data into log, distance measure using Euclidean, and clustering algorithm using ward.D and the MetaboAnalyst 4.0 statistical software (Chong et al., 2019).

3 Results

In the first 30 days of maintaining plants in different N sources, it was possible to observe mortality of some plants maintained in NH_4^+ and W/N. However, over time, plants adapted to NH_4^+, unlike those maintained in W/N (Table 1). This fact represents an important adaptive characteristic of the species as will be observed in the gas exchange and fluorescence results as well as in the production of alkaloids and leaf volatiles.

Table 1 Mortality rate determined at 30, 60, and 90 days after the beginning of treatments (DAT) in *Annona sylvatica* plants grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-:NH_4^+), and without nitrogen supply (W/N)

	30 DAT	60 DAT	90 DAT
NO_3^-	0 ± 0 bA	0.2 ± 0.2 bA	0 ± 0 bA
NH_4^+	1.4 ± 0.2 aA	1.2 ± 0.2 aAB	0.4 ± 0.4 abB
NO_3^-:NH_4^+	0 ± 0 bA	0.2 ± 0.2 bA	0.6 ± 0.4 abA
W/N	1.2 ± 0.2 aA	0.2 ± 0.2 bB	1.2 ± 0.3 aA

Averages followed by the same letter, lower case letters compare the 4 treatments in the same collection period (column) and upper case letters compare collection times in each treatment (row), do not differ by the Tukey test at 5% probability. Data are presented as mean ± SE ($n = 5$)

3.1 Gas Exchange

During the 90 days of plant cultivation, no variations in stomatal conductance (*gs*) and transpiration (*E*) were observed according to the different N sources (Fig. 1E, F). However, the instant carboxylation efficiency of the rubisco enzyme (A_{net}/Ci) was influenced by NO_3^- and NH_4^+ at different times (60 and 90 DAT, respectively).

When NO_3^- was used, plants showed high net CO_2 assimilation (A_{net}) at 60 DAT (Fig. 1a) and the lowest internal CO_2 concentration in the substomatal chamber (*Ci*) (Fig. 1b), which resulted in the greatest instant carboxylation efficiency of the rubisco enzyme (A_{net}/Ci) (Fig. 1d). However, at 90 DAT, internal C accumulation (*Ci*) and reduction in CO_2 assimilation (A_{net}) and consequently less carboxylation efficiency (A_{net}/Ci) were observed.

On the other hand, for plants maintained in NH_4^+, the greatest carboxylation efficiency was observed at 90 DAT (30 days later than plants maintained in NO_3^-), at which time there was high net CO_2 assimilation (A_{net}) and low internal C concentration (*Ci*).

Treatment with combined N sources (NO_3^-:NH_4^+) decreased C assimilation (A_{net}) and the carboxylation efficiency of the rubisco enzyme (A_{net}/Ci). Likewise, the absence of N was also harmful to gas exchange, causing low efficiency in the photosynthetic process with lower carboxylation efficiency values (A_{net}/Ci) due to C assimilation values (A_{net}) and high internal C accumulation (*Ci*).

3.2 Chlorophyll a Fluorescence

At 60 and 90 days after the beginning of the experiment (DAT), no differences were observed in plants maintained in the various conditions in relation to the maximum efficiency of photosystem II (Fv'/Fm') (Fig. 2a).

At the end of the experiment (90 DAT), plants cultivated with NH_4^+ showed increase in photochemical dissipation (qP) (Fig. 2d) and in the electron transport rate (ETR) (Fig. 2e), resulting in higher effective quantum efficiency of photosystem II (ΦFSII) (Fig. 2f). The supply of NH_4^+ led plants to present low fraction of accumulated energy, which cannot be used in photochemical reactions (*Ex*), regardless of collection time (Fig. 2c).

On the other hand, plants cultivated with NO_3^- showed low photochemical dissipation (qP) (Fig. 2d), low electron transport rate (ETR), and effective quantum efficiency of photosystem II (ΦFSII) at the end of the experiment. The response of plants to NO_3^- in relation to energy deviated and not used in photochemical reactions (*Ex*) was intermediate in the other treatments.

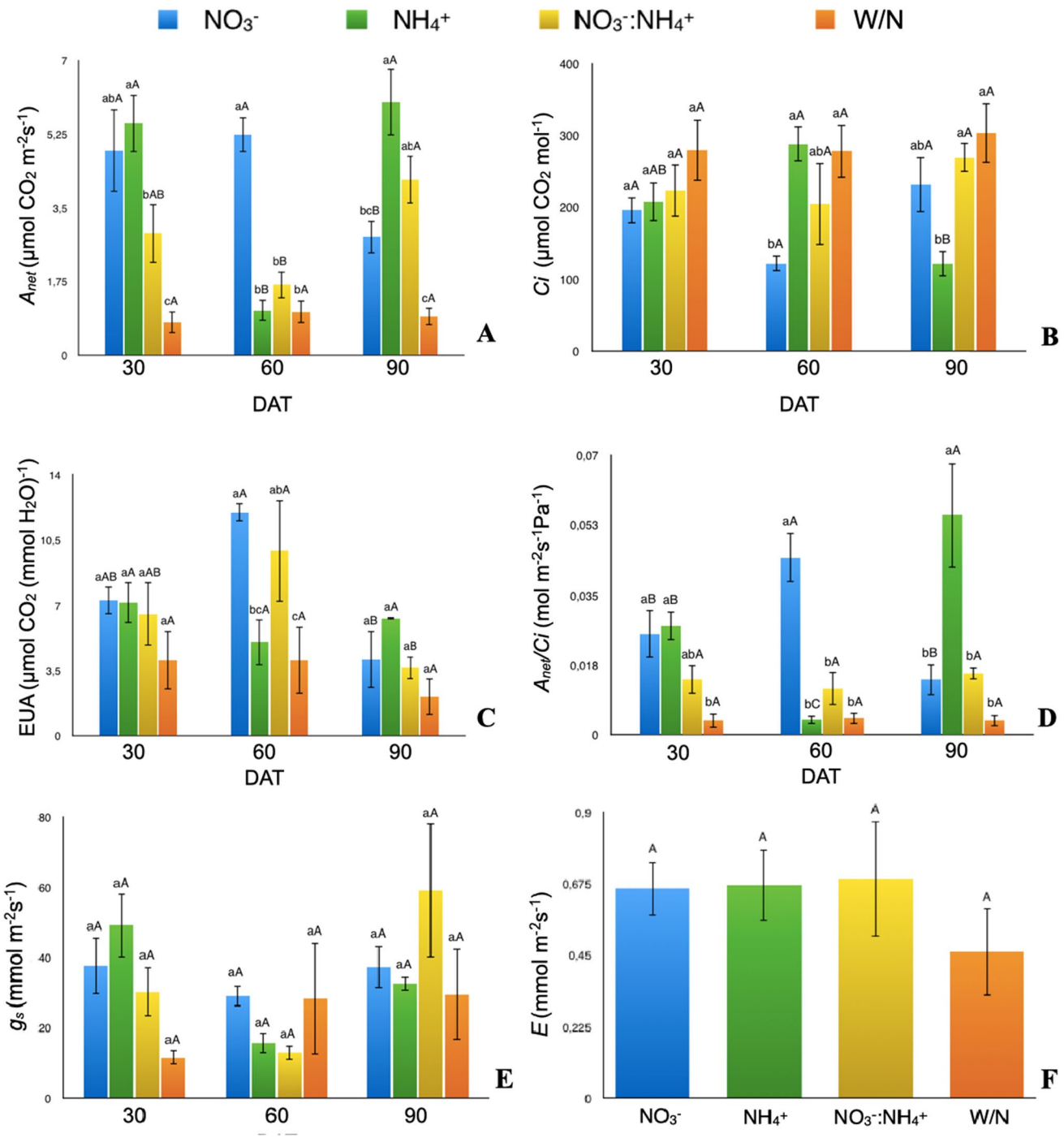

Fig. 1 Gas exchanges—net CO_2 assimilation rate (A_{net}, µmol CO_2 m^{-2} s^{-1}) (**a**); internal CO_2 concentration in the substomatal chamber (*Ci*, µmol CO_2 mol^{-1} air) (**b**); water use efficiency (WUE, µmol CO_2 (mmol $H_2O)^{-1}$) (**c**); instant carboxylation efficiency of the rubisco enzyme (A_{net}/Ci, µmol m^{-2} s^{-1} Pa^{-1}) (**d**); stomatal conductance (g_s, mmol m^{-2} s^{-1}) (**e**); transpiration rate (*E*, mmol m^{-2} s^{-1}) (**f**)—determined at 30, 60, and 90 days after the beginning of treatments (DAT) in *Annona sylvatica* plants grown with nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N). Averages followed by the same letter, lower case letters compare the 4 treatments in the same collection period and upper case letters compare collection times for each treatment, do not differ by the Tukey test at 5% probability. Data are presented as mean ± SE ($n = 5$)

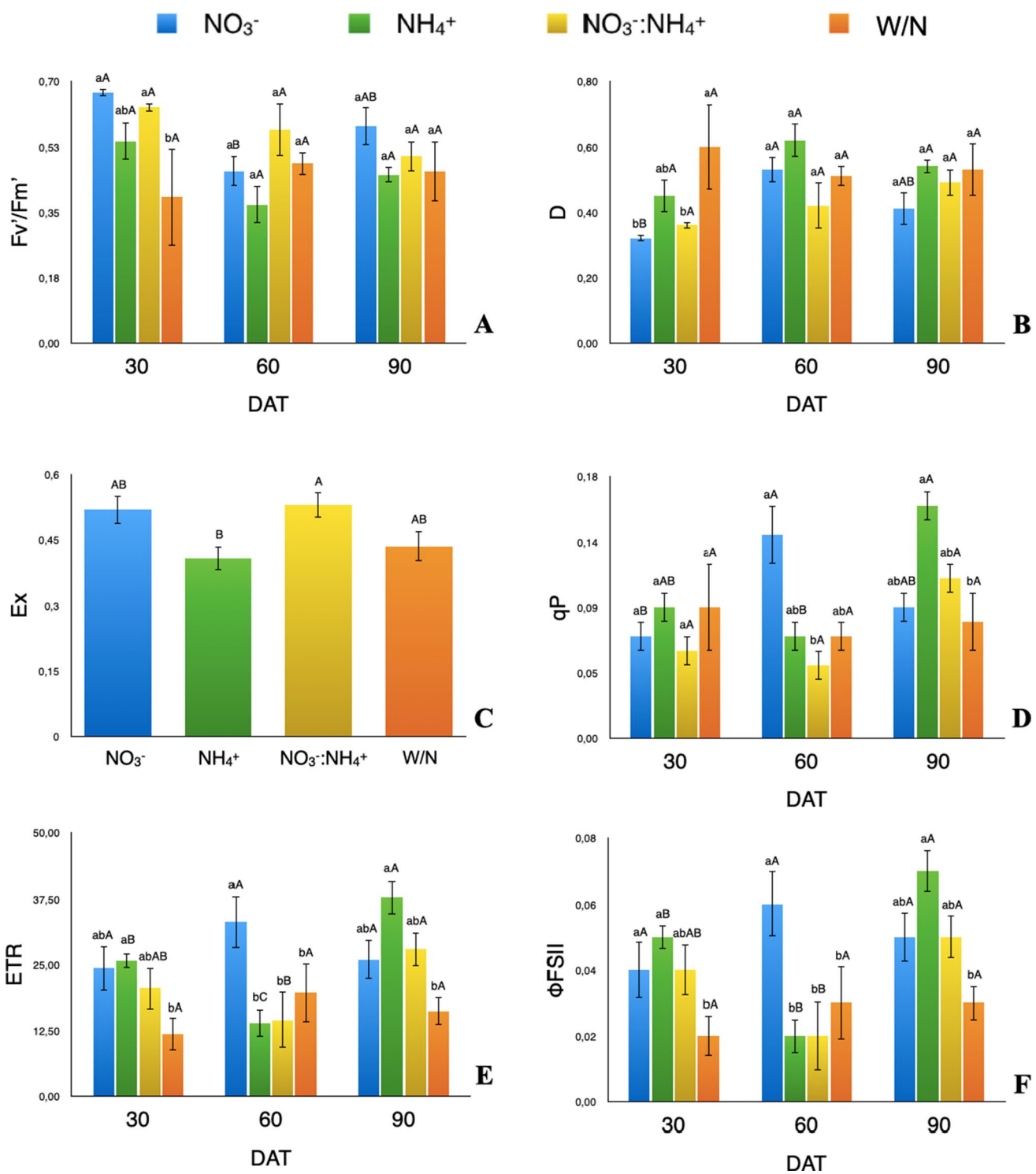

Fig. 2 Chlorophyll *a* fluorescence—potential quantum efficiency of photosystem II (Fv'/Fm') (**a**), fraction of light absorbed by photosystem II antenna dissipated as heat (*D*) (**b**), fraction of excitation energy not dissipated in the antenna that cannot be used in photochemical reactions (*Ex*) (**c**), photochemical dissipation (qP) (**d**), electron transport rate (ETR) (**e**), and potential quantum efficiency of photosystem II (ΦFSII) (**f**)—determined at 30, 60, and 90 days after the beginning of treatments (DAT) in A*nnona sylvatica* plants grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N). Averages followed by the same letter, lower case letters compare the 4 treatments in the same collection period and upper case letters compare collection times for each treatment, do not differ by the Tukey test at 5% probability. Data are presented as mean ± SE ($n = 5$)

In the absence of N, plants did not show fluorescence variations throughout the experiment. The use of NO_3^-:NH_4^+ led to increase in ETR and ΦFSII at 90 DAT.

3.3 Production of Total Alkaloids

The production of total alkaloids in *A. sylvatica* seedlings remained stable over time in plants submitted to treatment with mixture of N sources (NO_3^-:NH_4^+) and in those maintained in W/N (Fig. 3).

Plants cultivated with NH_4^+ showed significant increase (DF: 6; *f*: 7.017; $p < 0.001$) in the concentration of alkaloids roots, being responsible for the highest production of total alkaloids at the end of the experiment (90 DAT) and the largest amount of the entire experiment. In plants cultivated with NO_3^-, increase in the concentration of alkaloids was also observed at 60 DAT, which did not differ from 90 DAT; however, concentration was lower in plants cultivated with NH_4^+ compared to those with NO_3^-:NH_4^+ (Fig. 3).

3.4 Alkaloid Detection and Liriodenine Production

Eight alkaloids were identified with the standard alkaloids used, and six of them are present in all treatments. Furthermore, discretine was not detected in plants grown with NO_3^-. Liriodenine appears as the main alkaloid, regardless of N sources used, and this main metabolite increased gradually over time in plants grown with NH_4^+, resulting in higher concentration at 90 DAT and throughout the experiment,

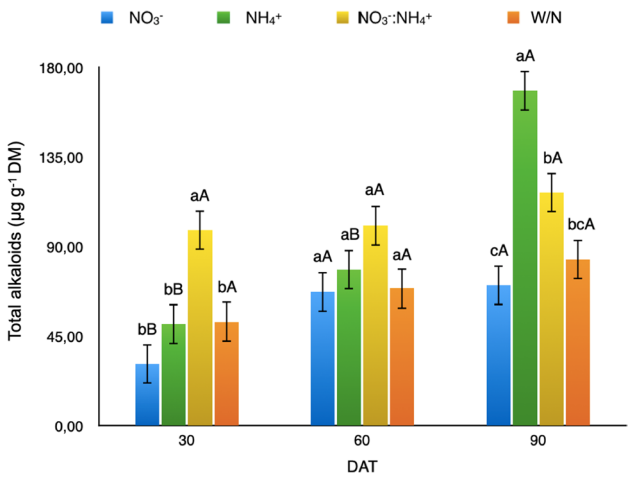

Fig. 3 Concentration of total alkaloids (µg g^{-1} DM) determined at 30, 60, and 90 days after the beginning of treatments (DAT) in *Annona sylvatica* roots grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N). Averages followed by the same letter, lower case letters compare the 4 treatments in the same collection period and upper case letters compare collection times for each treatment, do not differ by the Tukey test at 5% probability. Data are presented as mean ± SE ($n = 5$)

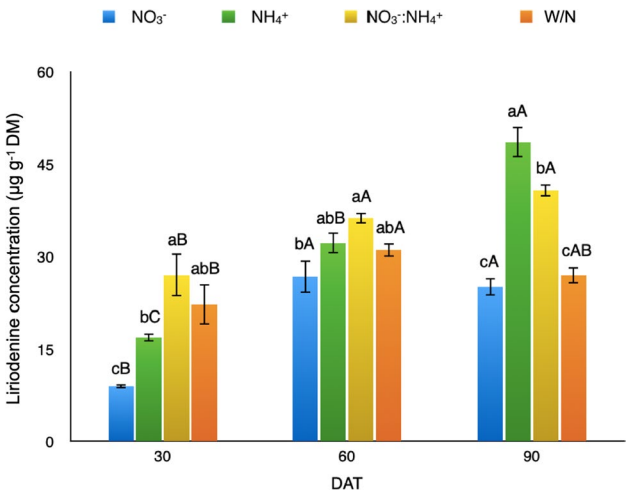

Fig. 4 Liriodenine concentration (µg g^{-1} DM) determined at 30, 60, and 90 days after the beginning of treatments (DAT) in *Annona sylvatica* roots grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N). Averages followed by the same letter, lower case letters compare the 4 treatments in the same collection period and upper case letters compare collection times for each treatment, do not differ by the Tukey test at 5% probability. Data are presented as mean ± SE ($n = 5$)

which differed from plants grown with NO_3^- and W/N (Fig. 4, Table 2).

3.5 Leaf Volatile Compounds

Forty-six volatile compounds were identified in *A. sylvatica* leaves grown with the different N sources, which correspond to approximately 69–99% of substances that make up the profile. Volatile profile is mainly composed of sesquiterpene (29 metabolites), followed by monoterpenes and fatty acids derivatives (13 and 4 compounds, respectively) (Tables 3, 4, and 5). The major volatile compounds were sesquiterpenes β-selinene with relative abundance varying from 13.74 to 40.4%, *E*-caryophyllene (3.23 to

Table 2 Alkaloids identified in *Annona sylvatica* roots grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N)

Alkaloids	NO_3^-	NH_4^+	NO_3^-:NH_4^+	W/N
Liriodenine	x	x	x	x
Xylopinin	x	x	x	x
Assimilobin	x	x	x	x
Laurotenanine	x	x	x	x
Lanulinusin	x	x	x	x
Norglaucine	x	x	x	x
N-Methyl-laurotenanine	x	x	x	
Discretine		x	x	

Table 3 Relative percentage of leaf volatile compounds identified in *Annona sylvatica* plants grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-:NH_4^+), and without nitrogen supply (W/N) at 30 days after the beginning of treatments (DAT)

Substances	IRC	IRL	30 DAT			
			NO_3^-	NH_4^+	NO_3^-:NH_4^+	W/N
Hexanal	800	801	1.43	0.8	1.74	0.19
2-*E*-Hexenal	849	855	1.72 a	0.15 b	0.18 b	0.26 b
Heptanal	901	902	0.03	0.03	0.04	0.02
α-Thujene	925	930	18.85 a	3.37 b	2.31 b	8.32 b
α-Pinene	930	939	7.77	5.67	2.86	7.73
Camphene	944	954	0.02	0.03	0.03	0.03
Sabinene	970	975	0.01	0.04	0.17	0.55
β-Pinene	972	979	3.34 a	0.64 b	0.24 b	0.65 b
6-Methyl-5-hepten-2-ona	986	985	0.03	0.03	0.04	0.04
Myrcene	990	990	1.06 ab	0.46 bc	0.14 c	1.31 a
α-Phellandrene	1002	1002	0.15	0.08	0.83	2.73
α-Terpinene	1014	1017	0.01	0.02	0.03	0.03
ρ-Cymene	1021	1024	0.94	0.67	0.26	0.74
Limonene	1025	1029	0.1	0.08	0.1	0.55
(*E*)-β-Ocimene	1047	1050	0.6 a	0.03 b	0.04 b	0.03 b
γ-Terpinene	1056	1059	0.03	0.01	0.02	0.09
n-Nonanal	1102	1100	0.14	0.54	0.2	0.12
δ-Elemene	1334	1338	0.53	0.08	0.58	0.53
α-Cubebene	1346	1351	0.14	0.62	0.54	0.8
Cyclosativene	1361	1371	0.04	0.34	0.04	0.12
Isoledene	1364	1376	0.04	0.34	0.18	0.53
α-Copaene	1372	1376	2.65	4.59	3.35	4.32
Daucene	1376	1381	0.83	0.59	0.4	0.12
β-Bourbonene	1380	1388	0.04 b	0.2 b	1.4 a	0.13 b
β-Elemene	1388	1390	1.53	0.93	1.67	1.76
cis-Caryophyllene	1392	1408	0.04 b	0.09 b	0.1 b	0.6 a
α-Gurjunene	1395	1409	1.58	1.15	1.89	1.32
E-Caryophyllene	1414	1419	4.04 c	14.64 a	11.96 ab	6.51 bc
β-Copaene	1423	1432	0.04 b	1.3 a	1.13 a	0.76 ab
β-Gurjunene	1430	1433	0.1 b	0.08 b	0.04 b	0.62 a
γ-Elemene	1432	1436	0.02	0.03	0.17	0.01
Aromadendrene	1434	1441	3.29 ab	1.64 c	2.25 bc	4.61 a
cis-Prenyl limonene	1439	1445	0.83 a	0.12 b	0.7 a	0.85 a
α-Humulene	1449	1454	1.2 ab	2.07 a	1.6 ab	0.93 b
trans-Prenyl limonene	1454	1459	0.02	0.03	0.03	0.01
allo-Aromadendrene	1456	1460	2.59	1.3	1.66	2.56
γ-Gurjunene	1472	1477	0.04 c	0.92 a	0.53 b	0.04 c
Germacrene D	1476	1481	0.86	0.99	1.58	0.88
γ-Himachalene	1478	1482	0.04	0.04	0.04	0.17
β-Selinene	1492	1490	40.4 a	18.61 c	24.72 bc	30.34 ab
α-Muurolene	1496	1500	1.82	0.77	1.67	0.85
γ-Cadinene	1509	1513	0.81 a	1.34 a	0.99 a	0.04 b
δ-Cadinene	1519	1523	1.11 ab	1.61 a	1.63 a	0.83 b
Dauca-4(11),8-diene	1523	1531	0.39	0.18	0.26	0.1
Spathulenol	1571	1577	0.89	0.66	1.15	0.59
Caryophyllene oxide	1571	1583	0.87	1.22	1.47	0.61
Total identified			99.79	69.13	72.92	84.93

Averages followed by the same letter, comparing 4 treatments in the same collection period, do not differ by the Tukey test 5% probability. *CRI* calculated retention index; *LRI* literature retention index

Table 4 Relative percentage of leaf volatile compounds identified in *Annona sylvatica* plants grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-: NH_4^+), and without nitrogen supply (W/N) at 60 days after the beginning of treatments (DAT)

Substances	IRC	IRL	60 DAT			
			NO_3^-	NH_4^+	NO_3^-:NH_4^+	W/N
Hexanal	800	801	0.83 a	0.83 a	0.76 a	0.12 b
2-*E*-Hexenal	849	855	2.07 ab	3.42 a	2.42 ab	1.53 b
Heptanal	901	902	0.04	0.04	0.04	0.04
α-Thujene	925	930	16.87 a	9.96 ab	12.22 ab	3.78 b
α-Pinene	930	939	6.18	4	4.2	2.01
Camphene	944	954	0.02	0.02	0.02	0.02
Sabinene	970	975	1.64 a	0.88 ab	1.27 a	0.1 b
β-Pinene	972	979	0.57	0.04	0.01	0.04
6-Methyl-5-hepten-2-ona	986	985	0.04	0.04	0.04	0.03
Myrcene	990	990	1.08	1.08	0.63	0.16
α-Phellandrene	1002	1002	1.91 b	2.7 ab	6.31 a	2.28 b
α-Terpinene	1014	1017	0.04	0.09	0.03	0.03
ρ-Cymene	1021	1024	0.19	0.72	0.6	1
Limonene	1025	1029	0.8	0.61	0.9	0.62
(*E*)-β-Ocimene	1047	1050	0.13	0.12	0.04	0.7
γ-Terpinene	1056	1059	0.89 a	1 a	0.81 ab	0.03 b
n-Nonanal	1102	1100	0.04	0.09	0.13	0.04
δ-Elemene	1334	1338	0.04 b	0.1 b	0.58 a	0.68 a
α-Cubebene	1346	1351	0.87 a	0.1 b	0.04 b	0.04 b
Cyclosativene	1361	1371	0.14 b	0.58 a	0.04 b	0.04 b
Isoledene	1364	1376	0.8 a	0.78 a	0.04 b	0.04 b
α-Copaene	1372	1376	6.1 a	4.87 a	1.56 b	1.66 b
Daucene	1376	1381	1.02 ab	1.3 a	0.75 b	0.12 c
β-Bourbonene	1380	1388	0.04	0.04	0.3	0.04
β-Elemene	1388	1390	0.8 b	0.78 b	1.87 a	1.22 ab
cis-Caryophyllene	1392	1408	0.1 b	0.61 a	0.64 a	0.93 a
α-Gurjunene	1395	1409	1.3 ab	1.74 a	1.26 ab	0.7 b
E-Caryophyllene	1414	1419	7.68 a	3.23 b	8.11 a	4.41 b
β-Copaene	1423	1432	0.73	0.71	0.85	1.29
β-Gurjunene	1430	1433	0.12 b	0.74 ab	0.86 a	1.28 a
γ-Elemene	1432	1436	0.04 b	0.63 a	0.04 b	0.01 b
Aromadendrene	1434	1441	4.05 b	4.8 b	6.35 b	9.69 a
cis-Prenyl limonene	1439	1445	0.91	0.97	1.11	1.56
α-Humulene	1449	1454	1.63 a	0.68 b	1.36 a	1.03 ab
trans-Prenyl limonene	1454	1459	0.02	0.01	0.02	0.01
allo-Aromadendrene	1456	1460	1.66 b	2.54 ab	2.43 ab	2.86 a
γ-Gurjunene	1472	1477	0.68	0.59	0.63	0.6
Germacrene D	1476	1481	1.03 a	0.79 ab	0.41 b	0.85 ab
γ-Himachalene	1478	1482	0.04	0.04	0.04	0.12
β-Selinene	1492	1490	21.94 b	25.79 b	32.62 ab	39.96 a
α-Muurolene	1496	1500	2.22	0.86	1.01	1.62
γ-Cadinene	1509	1513	0.93	0.64	0.52	0.97
δ-Cadinene	1519	1523	1.93	1.53	1.46	2.01
Dauca-4(11),8-diene	1523	1531	0.09 b	0.6 a	0.08 b	0.04 b
Spathulenol	1571	1577	0.04	0.04	0.33	0.1
Caryophyllene oxide	1571	1583	0.11	0.48	0.72	0.73
Total identified			90.4	82.21	96.46	87.14

Averages followed by the same letter, comparing 4 treatments in the same collection period, do not differ by the Tukey test 5% probability. *CRI* calculated retention index; *LRI* literature retention index

Table 5 Relative percentage of leaf volatile compounds identified in *Annona sylvatica* plants grown in nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-:NH_4^+), and without nitrogen supply (W/N) at 90 days after the beginning of treatments (DAT)

Substances	IRC	IRL	90 DAT			
			NO_3^-	NH_4^+	NO_3^-:NH_4^+	W/N
Hexanal	800	801	1.22 b	4.63 a	0.66 b	1.12 b
2-*E*-Hexenal	849	855	5.07 b	10 a	3.01 b	3.17 b
Heptanal	901	902	0.04	0.12	0.03	0.04
α-Thujene	925	930	2.43	5.83	7.13	10.19
α-Pinene	930	939	5.31 ab	3.59 b	7.09 a	3.06 b
Camphene	944	954	0.03	0.32	0.02	0.02
Sabinene	970	975	0.14 b	0.11 b	0.66 ab	1.02 a
β-Pinene	972	979	0.04	0.04	0.04	0.02
6-Methyl-5-hepten-2-ona	986	985	0.12	0.13	0.04	0.24
Myrcene	990	990	0.81	1.04	1.38	0.53
α-Phellandrene	1002	1002	2.87	6.1	5.58	0.59
α-Terpinene	1014	1017	0.08	0.02	0.28	0.03
ρ-Cymene	1021	1024	0.38	1.03	0.85	0.18
Limonene	1025	1029	0.21 b	0.11 b	1.07 a	0.47 ab
(*E*)-β-Ocimene	1047	1050	0.14 b	0.04 b	0.63 a	0.06 b
γ-Terpinene	1056	1059	0.1	0.03	0.2	0.03
n-Nonanal	1102	1100	0.32	0.54	0.1	0.13
δ-Elemene	1334	1338	0.51 ab	0.13 b	0.51 ab	0.61 a
α-Cubebene	1346	1351	0.14	0.1	0.1	0.04
Cyclosativene	1361	1371	0.69 a	0.66 a	0.04 b	0.51 a
Isoledene	1364	1376	1.39 a	1.19 a	0.15 b	0.62 ab
α-Copaene	1372	1376	5.13 a	4.45 a	2.37 b	4.18 ab
Daucene	1376	1381	2.06 ab	2.15 a	1.6 ab	0.84 b
β-Bourbonene	1380	1388	0.04	0.04	0.04	0.04
β-Elemene	1388	1390	0.99	0.81	1.52	1.22
cis-Caryophyllene	1392	1408	0.63 a	0.13 b	0.56 a	0.86 a
α-Gurjunene	1395	1409	2.89 ab	3.88 a	1.46 b	0.77 b
E-Caryophyllene	1414	1419	5.71	4.7	3.3	3.52
β-Copaene	1423	1432	0.91	0.83	0.82	1.04
β-Gurjunene	1430	1433	0.76 a	0.15 b	0.64 a	0.82 a
γ-Elemene	1432	1436	1.48 a	0.41 ab	0.59 ab	0.07 b
Aromadendrene	1434	1441	3.59 b	2.34 b	4.57 ab	6.36 a
cis-Prenyl limonene	1439	1445	0.94	1.06	0.85	1.06
α-Humulene	1449	1454	1.73 a	1.1 ab	0.97 ab	0.88 b
trans-Prenyl limonene	1454	1459	0.17 b	0.88 a	0.01 b	0.01 b
allo-Aromadendrene	1456	1460	2.14 ab	1.13 b	2.61 a	2.75 a
γ-Gurjunene	1472	1477	0.54	0.73	0.63	0.5
Germacrene D	1476	1481	0.6	0.91	0.68	0.75
γ-Himachalene	1478	1482	0.11	0.04	0.1	0.04
β-Selinene	1492	1490	26.92 ab	13.74 b	24.84 ab	35.65 a
α-Muurolene	1496	1500	1.27	1.43	1.31	2.15
γ-Cadinene	1509	1513	0.77	0.81	0.73	0.78
δ-Cadinene	1519	1523	1.61	1.66	1.41	1.82
Dauca-4(11),8-diene	1523	1531	0.69 a	0.86 a	0.73 a	0.04 b
Spathulenol	1571	1577	0.04	0.04	0.1	0.04
Caryophyllene oxide	1571	1583	0.7 a	0.11 b	0.12 b	0.58 a
Total identified			84.46	80.15	82.3	89.45

Averages followed by the same letter, comparing 4 treatments in the same collection period, do not differ by the Tukey test 5% probability. *CRI* calculated retention index; *LRI* literature retention index

14.64%), aromadendrene (2.25 to 9.69%), the monoterpenes α-thujene (2.31 to 18.85%), α-pinene (2.01 to 7.77%), and the fatty acid derivative 2-*E*-hexenal (0.15 to 10.00%). Correlations between leaf volatile compounds of *A. sylvatica* and treatments were evaluated using the heat map shown in Fig. 5.

In plants cultivated with NO_3^-, α-thujene showed high relative percentage and positive correlation at 30 and 60 DAT (Tables 3, 4, and 5, Supplementary Fig. S3), as well as β-pinene, α-cubebene, γ-elemene, and germacrene D had higher relative percentage at 30 and/or 60 and 90 DAT. Among major compounds, β-selinene showed irregular

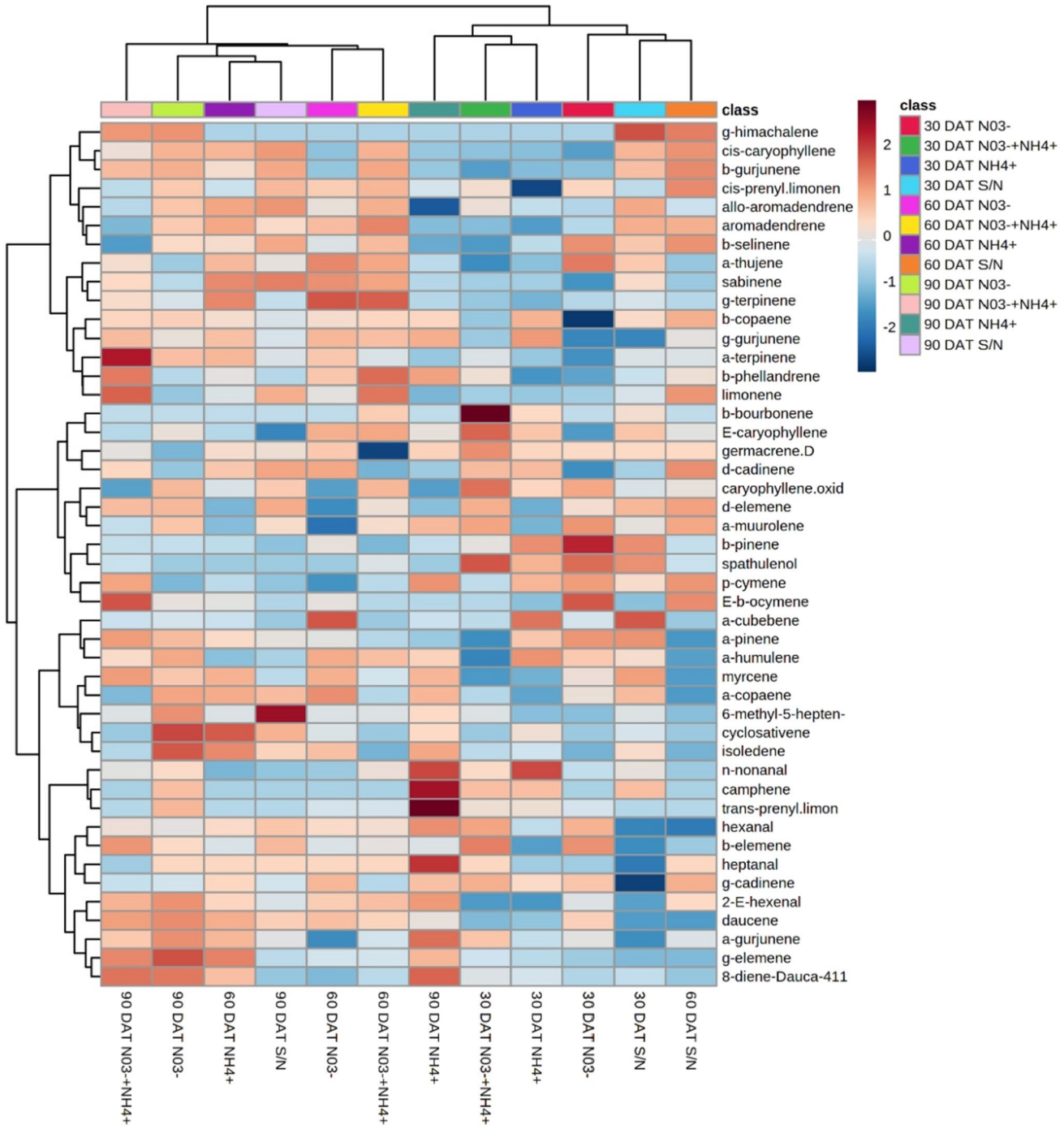

Fig. 5 Heat map indicating positive (red) and negative (blue) correlation between volatile compounds extracted from *Annona sylvatica* leaves and treatments with nitrate (NO_3^-), ammonium (NH_4^+), nitrate plus ammonium (NO_3^-:NH_4^+), and without nitrogen supply (W/N) at 30, 60, and 90 days after the beginning of treatments (DAT)

responses to NO_3^- treatments with respect to recollection time, high relative percentage, and positive correlation compared to those grown with NH_4^+ at 30 DAT, while at 60 and 90 DAT, plants showed lower proportions (Tables 3, 4, and 5, Supplementary Fig. S1).

Hexanal and 2-*E*-hexenal were the main compounds that showed increase in the relative percentage when plants were grown with NH_4^+ 60 and 90 DAT, being 2-*E*-hexenal the treatment with the highest relative percentage and positive correlation at 90 DAT (Table 5, Supplementary Fig. S5). *E*-caryophyllene showed its highest proportion at 30 DAT but at 90 days, it decreased with respect to the other treatments. *Trans*-prenyl limonene and γ-gurgujene were other volatiles that showed their highest proportions with the NH_4^+ treatment.

In plants cultivated with NO_3^-:NH_4^+, limonene, α-phellandrene, β-bourbonene, and β-elemene showed higher relative percentage (Supplementary Fig. S4, Table 5).

Finally, in W/N treatments, β-gurjunene and aromandendrene showed their most outstanding proportions.

4 Discussion

N source or absence of this element supplied to *A. sylvatica* generated different responses in the primary and specialized metabolism, which indicated the capacity of this species to adapt and survive to different nutritional conditions. Other *Annona* species, like *A. macrophyllata* (*A. diversifolia*) and *A. emarginata*, were able to grow in crops with various N concentrations either in NO_3^-:NH_4^+ combination or only in NO_3^-, respectively (Orozco et al., 2016; Campos et al., 2019). In *A. sylvatica* plants, NH_4^+ increased the photosynthesis, production of alkaloids, and specific leaf volatile compounds.

The supply of NH_4^+ resulted in higher photosynthetic efficiency (high instant carboxylation efficiency of the rubisco enzyme and high effective quantum efficiency of photosystem II—ΦFSII) and also the higher production of alkaloids. This fact may be related to the ready N availability for use in metabolic processes, which corroborates reports by Mur et al. (2016). Besides, the low *Ex* values suggest that the energy was destined for photosynthesis, since such N source is readily available, and there is no need to deviate electrons for reduction processes, as would occur with NO_3^- (Nunes-Nesi et al., 2010). The high photosynthetic efficiency with the use of NH_4^+ in *A. sylvatica* seedlings also shows that there was no excess energy dissipated in the form of heat or re-emitted as light, since according to Maxwell and Johnson (2000), these three processes occur in competition.

The results observed in *A. sylvatica* maintained in NH_4^+ suggest tolerance or acclimatization of this species to NH_4^+, since this ion is considered toxic to vegetables, as observed in species such as *Cucumis sativus*, where plants cultivated in NH_4^+ showed lower gas exchange rates and photosynthetic rate than plants cultivated in NO_3^- (Zhou et al., 2011), and in *Catharanthus roseus* plants, which showed low photosynthetic rate when submitted to high NH_4^+ concentrations (Guo et al., 2012). According to Marino and Moran (2019), tolerance to NH_4^+ is the way plant responds to this stress before suffering serious damage or even cell death. In this context, one of the ways to tolerate stress is NH_4^+ assimilation into organic compounds. Such organic compounds include amino acids and precursor alkaloid molecules (Wink, 2010). Thus, *A. sylvatica* plants cultivated in NH_4^+ showed high photosynthetic rate and directed possible NH_4^+ excess to produce alkaloids, such as the increase in total alkaloids and particularly that of oxoaporphine liriodenine (the main alkaloid of this species) (Table 2).

Treatment with NO_3^- led to decrease in photosynthetic efficiency, which can be seen by the reduction in the instant carboxylation efficiency of the rubisco enzyme (A_{net}/Ci) at the end of the experiment, reflecting in high internal C concentration and decreased net CO_2 assimilation rate (A_{net}), and in lower photochemical dissipation (qP) and effective quantum efficiency of photosystem II values. This decrease in the photosynthetic process seems to be related to the energy deviation of this process to reduce NO_3^-. There is need to reduce NO_3^- into NO_2^-, which occurs in the cytosol using electrons from NAD (P) $H+H^+$ and then NO_2^- is transported to chloroplasts where it will be reduced into NH_4^+ by transfer of ferredoxin electrons (Miller and Cramer, 2004; Nunes-Nesi et al, 2010). Thus, *A. sylvatica* plants deviate the electron flow from photosynthesis to reduce NO_3^- when cultivated in the presence of this N source (alone or in combination), which can be proven by the increase in the accumulated energy fraction not used in photochemical reactions (*Ex*). Besides, in *A. sylvatica*, the deviation of electron flow from photosynthesis to reduce NO_3^- reduces not only photosynthesis, but also the synthesis of alkaloids.

The reduced photosynthetic efficiency observed by the gas exchange and fluorescence variables in *A. sylvatica* plants maintained without N supply confirms what is expected in relation to the importance of N in the various metabolic processes of plants (Lawlor et al., 1989; Stitt et al., 2009; Wang et al, 2014; Zhao et al., 2005).

In addition to the relationships between N sources in primary metabolism and alkaloids, the supply of different sources led to the differential synthesis of major leaf volatile compounds. In this context, NH_4^+ increased the synthesis of leaf volatile compounds related to plant responses to stress situations, such as 2-*E*-hexenal, emitted by plants under biotic stress caused by pathogens (Scala et al., 2013) and induced by herbivory (Goldberg et al., 2019). These components should also be highlighted because they have

antimicrobial (Patrignani et al., 2008) and antifungal properties (Zhang et al., 2016).

The supply of NO_3^- led to higher production of leaf volatile compounds related to plant resistance and defense. Studies have shown that NO_3^- can cause changes in defense mechanisms related to pathogens, with increase in nitric oxide and polyamines and decrease in gamma-aminobutyric acid (GABA) (Mur et al., 2016). In *A. emarginata* (Campos et al., 2019), the application of NO_3^- at concentrations similar to those provided for *A. sylvatica* also induced plant defense mechanisms, with synthesis of mono- and sesquiterpenes. In *A. sylvatica*, NO_3^- promoted high relative percentage of β-selinene, which has antifungal properties (Ding et al., 2017), and of α-thujene, a compound that has cytotoxic properties (Blowman et al., 2018).

Treatment with NO_3^-:NH_4^+ resulted in high relative percentage of α-pinene in *A. sylvatica*, which may be interesting since this compound has antimicrobial (Leite et al., 2007), anti-inflammatory (Kim et al., 2015), and cytotoxic properties (Blowman et al., 2018). In addition, several other compounds, such as ρ-cymene, β-pinene, myrcene, limonene, 6-methyl-5-hepten-2-one, spathulenol, and α-humulene, also have cytotoxic properties (Blowman et al., 2018), which can also be explored from the pharmaceutical point of view.

A. sylvatica seems to adjust aspects of its specialized metabolism according to the N source, while NH_4^+ causes increases in alkaloids and to a lesser degree volatile derivatives of fatty acids (hexanal, 2-*E*-hexenal) and NO_3^- increases some important terpenic volatiles (β-selinene, α-thujene, β-pinene) and secondarily alkaloids, the combination of both salts expresses an intermediate behavior. Another aspect to be highlighted is that with the W/N treatment, the amount of total alkaloids and liriodenine is stable over the 3 months of experiment, which may be an indication of the relevance of these N molecules for *A. sylvatica*.

In summary, the main advances obtained with this research refer to the ability of different N sources to influence the primary and specialized metabolism of *A. sylvatica*, in addition to the fact that the species is adapted to possible toxicity caused by NH_4^+. Thus, although the different N sources present similar responses at the beginning of treatments (30 DAT), over time, NH_4^+ (source more readily available and with the lowest energy expenditure for the plant) leads *A. sylvatica* to a stress condition with reduction in photosynthesis, but without altering the synthesis of alkaloids (60 DAT), and later, the maintenance of plants in NH_4^+ (90 DAT) leads to acclimatization, with increase in photosynthesis and production of alkaloids, transferring to the specialized metabolism, the probable excess that would be toxic to the primary metabolism (Britto and Kronzucker, 2002). The N availability can influence the dynamics of specialized metabolite expression and the release of defense compounds, an important condition in plant-animal relationships (Nagegowda, 2010; Campos et al., 2019). In this context, variations in leaf volatile profiles were also observed depending on the source used, with increase in compounds related to plant defense (antifungal and cytotoxic substances) when using NO_3^-, while with NH_4^+, there is production of volatiles related to biotic stress (pathogens and herbivory). Such characteristics of *A. sylvatica* also suggest that the species can be used as an alternative to obtain bioactive molecules of interest, whose production can be modulated with specific treatments such as the N sources evaluated in this study.

5 Conclusion

Variations of N sources have distinct impact on the primary and specialized metabolism of *A. sylvatica*. Photosynthesis reduction caused by the deviation of the electronic flow for NO_3^- reduction also leads to reduction in the synthesis of alkaloids, while there is increase in the production of volatile defense compounds. On the other hand, *A. sylvatica* plants show acclimation to NH_4^+, with increase in both photosynthesis and alkaloids and leaf volatiles compounds related to stress situations. It could be concluded that plants maintained in NO_3^- mobilize their energy more towards specialized metabolism, while plants maintained in NH_4^+ invest in both primary and specialized metabolism, which increases the phytochemical potential of the species by stimulating the production of different groups of metabolites.

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Author Contribution PLCC and GF contributed equally to this manuscript. CSFB assisted in the elaboration and development of the work, in addition to making laboratory resources available. IDCC carried out the extraction and identification of total alkaloids and liriodenine. MOMM and MARV contributed with the analysis of leaf volatiles compounds. MCS and FGC contributed with all the work processes. All authors read and approved the final manuscript.

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Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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