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Nitrogen Metabolism Components as a Tool To Discriminate between Organic and Conventional Citrus Fruits

PAOLO RAPISARDA,* MARIA LUISA CALABRETTA, GABRIELLA ROMANO, AND FRANCESCO INTRIGLIOLO

Istituto Sperimentale per l'Agrumicoltura, Corso Savoia 190, 95024 Acireale (CT), Italy

The aim of this work was to develop a method for authenticity control of organically grown orange fruits. Due to the different kinds of nitrogen fertilization of the soil in organically and conventionally managed farms, the study tried to verify the possibility to differentiate Navelina and Tarocco orange fruits obtained by these production systems through the detection of markers linked to nitrogen metabolism. In addition to the classic quality parameters, total nitrogen (N) and synephrine contents in juice and $^{15}N/^{14}N$ isotope ratio (expressed as $\delta^{15}N$‰) in proteins of pulp and amino acids of juice were determined. The results obtained indicated that total N and synephrine contents were significantly higher in conventional fruits, whereas the $\delta^{15}N$‰ values were higher in the organic ones. The new markers identified in this research by linear discriminant analysis of the data may constitute a useful tool to differentiate organic citrus fruits or juices from conventional ones.

KEYWORDS: Orange fruits; markers; synephrine; N isotope ratio; vitamin C

INTRODUCTION

During the past decade, there has been increasing consumer interest in organic products because they are considered to be safer for health owing to the absence of pesticide residues and because they are produced in a more environmentally compatible manner (*1*, *2*). In fact, the organic cultivation system excludes the use of synthetic fertilizers, pesticides, and insecticides. This method is based on frequent crop rotations, the use of soil amendments such as compost or organic waste, and usually biological pest control (*3*).

In line with the EC Regulation (No. 2092/91) on organic farming, organic products are subject to controls by an accreditation and certification system. In particular, they must be recorded at each step of production, thereby ensuring the complete traceability at all stages of production, processing, and marketing. In addition, every year, one or two inspections are made in the farms and, on these occasions, samples can be taken for analysis.

Generally, the analytical controls performed on organic fruits and vegetables consist of the search for pesticide residues (*1*, *4*). Thus, the absence or presence of these substances is used as a criterion to discriminate between organically and conventionally grown products.

Citrus grown in the Mediterranean climate, unlike other fruit species, such as peach, apricot, and cherry, does not entail notable problems regarding pests and disease, and therefore the frequent use of pesticides is not necessary. Consequently, it is not easy to distinguish organically from conventionally grown citrus fruits by pesticide residues analysis. To guarantee the consumer that the purchased citrus fruit is really organic, it is not enough to prove the absence of pesticides, because a product without pesticides does not always come from an organically managed farm.

With regard to soil fertility management, in organic production systems the input of nutrients is based on organic fertilizers and amendments such as composted or uncomposted cattle manure or other organic waste, whereas in conventional production systems, synthetic fertilizers are used. In the former, the nitrogen content of the organic fertilizers must be mineralized before being absorbed by the plants and the mineralization rate depends on many factors such as climate, soil type, and microbiological activity (*5*). In addition, the chemical composition of the organic material applied will affect its decomposition and, hence, the nutrients available for crops (*6*). Thus, a risk for plants in organically managed farms is a low nitrogen availability that leads to negative effects on yield and fruit quality. To avoid this problem, unscrupulous farmers could apply synthetic nitrogen to the soil, for example, by fertigation. In this case, the fraud is difficult to detect.

At present, only limited research has been conducted to distinguish fruits produced by the two cultivation practices through the determination of components linked to different fertilization systems. Some studies have demonstrated that compost used as soil supplement significantly enhanced levels of ascorbic acid and glutathione in fruits of two strawberry cultivars (*7*), and a parallel increase was found in the polyphenol content and polyphenol oxidase activity of organic peaches and pears, as compared with the corresponding conventional samples (*8*). Furthermore, no differences emerged between Ca, Mg, Fe,

* Author to whom correspondence should be addressed [telephone 0039-95-7653134; fax 0039-95-7653113; e-mail isa@mail.gte.it].

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Table 1. Cultural Parameters of Organic and Conventional Farms Relating to the 3 Year Study

cultural parameter		Navelina		Tarocco	
		organic	conventional	organic	conventional
nutrient input (kg/ha)	N	138	146	130	137
	P	103	115	127	113
	K	107	113	99	96
pruning (%)	yes	91	75	89	91
	no	9	25	11	9
pruning residual recycling (%)	yes	80	25	89	75
	no	20	75	11	25
yield (t/ha)		18	17	19	18
weed control		mechanical	mechanical and herbicides	mechanical	mechanical and herbicides
insect control		biocontrol	chemical–biological	biocontrol	chemical–biological
type of fertilization		organic	chemical	organic	chemical
time of fertilization		early spring			
type of irrigation		sprinkler localized			

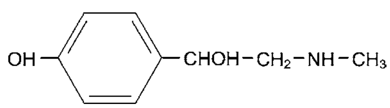

Figure 1. Structure of synephrine.

Mn, Zn, and Cu contents of conventional and organic strawberry fruits (*9*). Finally, Gundersen (*10*) has patented a method for determining if an agricultural or horticultural product has been organically produced, using elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS).

No study has been made in recent years on the N-containing compounds level in organically and conventionally grown citrus fruits as consequence of different kinds of nitrogen fertilization.

The present work focused on the following parameters linked to nitrogen metabolism: total N and synephrine (Syn) content in juice and the ratio of stable isotopes of nitrogen $^{15}N/^{14}N$ (expressed as $\delta^{15}N$‰) determined in proteins of pulp ($\delta^{15}N$ PP) and amino acids ($\delta^{15}N$ AA) of juice.

Synephrine (**Figure 1**) is an N-containing alkaloid belonging to the group of ephedrine present in citrus leaves and fruits (*11*, *12*). It represents 3−4% of the total soluble nitrogen in the juice and constitutes a final product of the metabolism of this element. The biosynthesis of the synephrine is linked to metabolism of the amino acids and in particular to tyrosine, which undergoes a decarboxylation reaction, methylation of the amino group and, last, hydroxylation of the carbon in the α position to the phenol group (*12*).

The $^{15}N/^{14}N$ ratio represents the ratio between the less abundant and more abundant stable isotopes. Many studies used this parameter, in combination with ratios of stable isotopes of other elements, for origin assignment and the authenticity control of various products such as fruit juices (*13*), essential oils (*14*), Pecorino Sardo cheese (*15*), and French, Italian, and Spanish cheeses (*16*).

Other studies on nitrogen stable isotope ratio analysis have demonstrated that this parameter is affected by many factors such as climate (*17*), soil characteristics (*18*), and primary nitrogen sources (*19*). Consequently, the $^{15}N/^{14}N$ ratio in the biomolecules of the plants depends also on the kind of fertilizer used on the crops (*19*, *20*). The use of organic or synthetic nitrogen fertilizers, respectively, in organically and conventionally managed farms, therefore, is reflected in the $\delta^{15}N$ value of N-containing compounds, including proteins and amino acids present in fruit juices.

The objective of the present work was to examine the differences in the fruit quality between organically and conventionally grown Navelina and Tarocco orange fruits. In addition, taking into account some specific aspects of the two cultivation methods, such as the different kinds of nitrogen soil fertilization, a study was carried out to verify the possibility to differentiate between organically and conventionally grown Navelina and Tarocco orange fruits through the detection of markers linked to nitrogen metabolism.

MATERIALS AND METHODS

Plant Material. The study was conducted over a 3 year period (1999−2001) on orange fruits of Navelina and Tarocco cultivars, sampled from 28 farms (7 organic and 7 conventional for each cultivar), situated in the citrus area of eastern Sicily. Farms were chosen to obtain similar pairs in the same environmental conditions. Moreover, orchard pairs were homogeneous for age and rootstock to reduce effects not linked to fertilization. Details of crop fertilization, management practices adopted, and yields (3 year average) are reported in **Table 1**. Organic farming systems were managed according to EC Regulation 2092/91. Main N inputs derived from organic fertilizers consisting of uncomposted cattle or poultry manure and plant residues, whereas P and K inputs derived mainly from soft ground rock phosphate and potassium sulfate containing magnesium salt, respectively. In addition, on all organic farms legumes and green manures were cultivated in one of the 3 years of the trial. Conventional farming systems were based on the Best Agricultural Practices of the region, according to Integrated Pest Management (IPM) approach. Nutrient inputs were made with granular synthetic fertilizers both in simple (N, P, K) and in complex (NPK) forms.

Soil samples from the 28 farms were collected and analyzed (*21*) in March 1999 to determine the soil status at the baseline of the study. These data are shown in **Table 2**.

Fruit Quality. Samples of 80−100 fruits were harvested each year (from each of the 28 farms) at commercial maturity (TSS/TA ratio, 8−10) from 20 randomly selected trees grown in a plot of 0.25 ha of both organic and conventional managed farms. The fruits were collected from the outer part of the canopy and from the four cardinal points. Each sample was divided into three subsamples, and on each of these physical parameters (firmness, fruit weight, width of the central axis, peel thickness) were measured using standard methods (*22*). Each subsample of fruits was squeezed, and juice content, total acidity (TA), and total soluble solids (TSS) were determined (*23*). Vitamin C was analyzed by high-performance liquid chromatography (HPLC) (*24*). The HPLC system consisted of a Waters (Milford, MA) 600E pump and a PDA Waters 996 detector, both run by Millennim 32 Waters software. Separation was performed on a 5 μm Hypersil ODS column (250 × 4.6 mm i.d.) (Phenomenex, Torrence, CA). The eluting solvent was KH_2PO_4/H_3PO_4 buffer at pH 2.3, the flow rate in the column was 1 mL/min, and the wavelength was set at 260 nm.

Mineral Elements Analysis. The determination of mineral elements was carried out on the centrifuged juice, after heat-drying at 105 °C and ashing at 550 °C. The ashes were dissolved in 10% hydrochloric acid; Ca and Mg were determined by atomic absorption spectropho-

Table 2. Main Physicochemical Parameters of Soil at Organic and Conventional Farms

		clay (%)	silt (%)	sand (%)	pH	organic matter (%)	N (‰)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	CSC (mequiv/100 g)
Navelina	organic	39.03	22.86	38.11	7.92	2.37	1.30	21.12	395.50	3448.21	487.47	22.94
	conventional	33.60	25.50	40.90	7.91	2.08	1.34	26.21	439.05	4125.00	337.47	25.35
		ns[a]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Tarocco	organic	39.60	22.29	38.11	8.01	2.29	1.24	11.29	445.51	4292.86	479.43	27.71
	conventional	36.17	22.86	40.97	8.09	1.78	0.89	14.13	488.36	5044.64	673.93	36.82
		ns	ns	ns	ns	ns	*	ns	ns	ns	*	ns

[a] Significant differences: *, $p \leq 0.05$; ns, not significant.

tometry and K by emission spectrophotometry (Perkin-Elmer model 2380). Phosphorus was measured according to the vanadate−molybdate−yellow colorimetric method (*25*). Total N was determined in the juice with Kjeldahl's method.

Extraction and Determination of Synephrine. Ten milliliters of centrifuged juice was passed through a strong cationic exchange (SCX) cartridge (Varian Mega Bond Elut SCX) preconditioned with methanol. Synephrine was eluted by methanol containing concentrated NH_4OH (85:15 v/v) and the solution evaporated (35 °C, low pressure). The extract was diluted with a solution containing 0.1% of H_3PO_4 and injected into the HPLC, after filtering with a 0.45 μm membrane.

Separation was performed on an analytical Symmetry C-18 5 μm column (150 × 4.6 mm i.d.) (Waters), using the same HPLC equipment described above. The mobile phase consisted of CH_3CN/H_2O (35:65) containing 0.01% H_3PO_4 and 0.3% sodium dodecyl sulfate (SDS). The flow rate in the column was of 1 mL/min, and the detection wavelength was set at 230 nm.

Sample Preparation and $^{15}N/^{14}N$ Isotope Ratio Determination. The determination of the $^{15}N/^{14}N$ ratio present in the proteins of pulp and in the amino acids of juice was done following the methods described respectively by Bricout and Koziet (*26*) and by Kornexl et al. (*13*), with slight modifications.

To prepare juice and pulp samples, 100 mL of juice was centrifuged at 2000 rpm for 20 min to separate the supernatant from the pulp. Pulp was washed twice with 50 mL of water (to remove sugars) and 50 mL of acetone (to reduce lipids and pigments). Each washing was followed by centrifugation at 2000 rpm for 10 min. The pulp residue was freeze-dried and stored until $^{15}N/^{14}N$ ratio determination.

The supernatant was decolored with 2−3 g of polyvinylpyrrolidone, filtered, and brought up to a total volume of 100 mL with water; after that, the pH was adjusted to 1.7−2.0 with 0.5 N HCl. This solution was passed through a column containing strong cationic resin Dowex 50 WX8 (Sigma Aldrich, Milan, Italy). Amino acids were retained on the column, whereas other soluble compounds were eluted with 300 mL of an HCl solution at pH 1.7. Amino acids were eluted with 300 mL of 0.1 N NaOH. The eluate was collected in 10 mL test tubes, and the pH was measured for each tube. The basic fractions were mixed and neutralized with 0.5 N HCl. The solution obtained was freeze-dried and used for $^{15}N/^{14}N$ ratio determination.

Freeze-dried samples of pulp and amino acid extracts were weighed in tin containers and introduced by means of an autosampler into the elemental analyzer (Nitrogen Analyzer 1500, Carlo Erba, Rodano, Italy), where in the presence of O_2 and CuO, they were burnt quantitatively to CO_2 and NO_x; the latter was then reduced to N_2 with copper. The formed gases were separated on a gas chromatographic column and analyzed in an isotopic mass spectrometer (Sira II-VG Fisons, Rodano, Italy).

The values of the isotope ratio are expressed in δ‰ according to the formula

$$\delta‰ = (R_{\text{sample}} - R_{\text{standard}}/R_{\text{standard}}) \times 1000$$

where R_{sample} and R_{standard} are the $^{15}N/^{14}N$ ratios of the sample and the standard, respectively. By international convention, the standard used for the analysis was the nitrogen isotope ratio in air.

Data Set and Statistical Analysis. The number of samples analyzed, in the 3 year trial, for each cultivation method was lower with respect to the samples expected in the experimental plan (31 organic and 33 conventional samples instead of 42 for each one) because of seasonal variations or sampling problems.

Table 3. Physicochemical Parameters of Organically and Conventionally Grown Navelina and Tarocco Orange Fruits

parameter	Navelina		Tarocco	
	organic	conventional	organic	conventional
firmness (kg)	7.21	6.66 ns[a]	4.45	4.19 ns
fruit weight (g)	193.13	188.80 ns	224.32	226.81 ns
central axis (mm)	9.71	7.89 *	10.27	11.31 ns
peel thickness (mm)	5.19	5.00 ns	5.39	5.47 ns
juice yield (%)	41.50	39.28 ns	40.72	40.52 ns
total acidity (g/100 mL)	1.25	1.12 ns	1.33	1.35 ns
total soluble solids (%)	11.82	11.77 ns	11.17	10.90 ns
vitamin C (mg/100 mL)	65.50	58.47 ***	65.96	58.95 ***

[a] Significant differences: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, not significant.

The data were elaborated by two-factor ANOVA (farming systems/year). Because no statistically significant differences, for all variables considered, were found among the years of the trial (data not reported), to compare organically and conventionally grown orange fruit parameters, only means of the 3 years were reported. Linear discriminant analysis (LDA) has been performed using the data sets composed by all citrus samples (64 organic and conventional samples) and 11 variables (TA, TSS, vitamin C, Ca, K, Mg, P, N, Syn, $\delta^{15}N$ AA, $\delta^{15}N$ PP) of the two studied varieties. The year has not been inserted among the variables of the LDA analysis because ANOVA showed that it had no influence on the results.

Experimental data were processed with the aid of the SPSS-95 statistical package.

RESULTS AND DISCUSSION

Table 3 shows the results of the physical and chemical parameters determined in the organic and conventional Navelina and Tarocco orange fruits. For most of the parameters, no statistically significant differences emerged among the fruits obtained with the two cultivation systems, confirming the results obtained by Rapisarda et al. (*27*). Only the central axis of the Navelina organically grown orange fruits was statistically higher ($p \leq 0.05$). This difference was not found in the Tarocco cultivar.

The different production methods affect the levels of vitamin C in orange fruits of both varieties. The vitamin C content of the organically grown fruits was higher than that of the conventional ones. Nagy (*28*) reported that this component, in different citrus species, is inversely correlated with the nitrogen supply. Therefore, the lower vitamin C content in conventional fruits might be due to the application of immediately available synthetic fertilizers in these crops (**Table 1**).

With regard to the concentration of mineral elements in juices of the two studied cultivars (**Table 4**), significant differences were recorded for Mg ($p \leq 0.05$) and K ($p \leq 0.001$) in Navelina cultivar, with higher values of these elements in conventional

Table 4. Levels of Mineral Elements in Juice of Organically and Conventionally Grown Navelina and Tarocco Orange Fruits

		N (mg/L)	P (mg/L)	Ca (mg/L)	Mg (mg/L)	K (mg/L)
Navelina	organic	748.15	125.55	71.70	101.36	1299.90
	conventional	972.74	135.44	68.92	108.41	1611.64
		***[a]	ns	ns	*	***
Tarocco	organic	695.18	121.47	61.62	88.56	1445.36
	conventional	777.33	116.81	54.81	84.44	1496.67
		***	ns	***	**	ns

[a] Significant differences: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, not significant.

Table 5. Parameters Linked to Nitrogen Metabolism in Organically and Conventionally Grown Navelina and Tarocco Orange Fruits

		synephrine (mg/L)	δ^{15}N PP (‰)	δ^{15}N AA (‰)
Navelina	organic	19.13	6.21	5.67
	conventional	23.07	4.43	4.39
		**[a]	**	**
Tarocco	organic	27.37	6.76	6.15
	conventional	33.33	5.53	5.09
		**	**	**

[a] Significant differences: **, $p \leq 0.01$.

fruits. In contrast, in Tarocco cultivar Ca ($p \leq 0.001$) and Mg ($p \leq 0.01$) were significantly higher in organic ones. However, mineral elements of the two studied varieties were not significantly correlated with the level of nutrients present in the soil (**Table 2**). In fact, correlation coefficient (r) values ranged between 0.002 for K in organic Tarocco and 0.354 for Mg in conventional Navelina.

Total N present in the juice of conventionally grown fruits was significantly higher than in organically grown ones, even though the concentrations found in the organic juices proved to be optimal and comprised in the mean values of nitrogen measured in the juice of oranges of the Navel group and Tarocco (*29*, *30*).

With regard to the individuation of new markers to differentiate organic and conventional orange fruits, two parameters linked to the nitrogen metabolism in the plants were determined: synephrine, determined in juice, and the ratio of stable isotopes of nitrogen ^{15}N/^{14}N (expressed as δ^{15}N‰) determined in proteins of pulp (δ^{15}N PP) and amino acids (δ^{15}N AA) of the juice.

The results (**Table 5**) showed that synephrine was statistically higher in fruits obtained with the conventional method in both of the cultivars. The presence of higher concentrations of total N and, consequently, of synephrine in the conventional fruit shows that citrus trees fertilized with synthetic nitrogen accumulate this element in their tissue, also exceeding the real nutritional needs. A similar trend was observed in N leaf content of conventionally grown Clementine (*Citrus clementina* Hort. Ex Tan.) with respect to organically grown ones (*31*).

The different N contents between organic and conventional fruits do not certainly depend on the amount of N application in the two farming systems, which is not different (**Table 1**), but may be due to the lower N mineralization rate that occurs in the organic farming system (*32*), which implies a lower amount of soil N available to crop plants.

For the ^{15}N/^{14}N isotope ratio present in N-containing compounds of organic and conventional fruits, opposing values with respect to N and synephrine contents were found (**Table 5**). Fruits from organically managed farms had statistically higher δ^{15}N PP and δ^{15}N AA values compared to the conventional ones. Therefore, the application of organic fertilizers, which notoriously increases the level of ^{15}N in the soil (*20*), also determines an increase in the δ^{15}N values in the organic fruit.

With the aim of obtaining a better differentiation between organically and conventionally grown orange fruits, some chemical parameters of fruit juice were investigated by multivariate analysis, applying, in particular, an explorative LDA. This statistical method allows classification within groups of a sample (certainly belonging to one of the groups) of unknown origin. Eleven chemical parameters determined in the juice of each sample of both varieties, some of which are linked to soil fertility, were chosen as original variables of the statistical analysis.

Table 6 shows the canonical discriminant function, obtained by linear combination of original variables. Because there are only two groups (organic and conventional groups), one discriminant function is possible. **Table 7** reports the standardized canonical discriminant function coefficients of each variable. According to the values of these coefficients, one can deduce that the most relevant variables to discriminate between the two groups were δ^{15}N PP, vitamin C, and synephrine.

A histogram showing the clustering within the two "known" groups of the 64 cases is shown in **Figure 2**. Organic and conventional samples were well separated in two distinct groupings or clusters. With regard to classification results (**Table 8**), 87.1% of 31 cases were classified as organic fruits and almost 94% of 33 cases were classified as conventional fruits. Finally, the percentage of correctly classified grouped cases was 90.63%. The four organic samples (one Navelina and three Tarocco) incorrectly classified as conventional come from fruits with low levels of vitamin C (range = 54.00−58.00 mg/L) and δ^{15}N PP (4.62‰ for Navelina samples and 4.75−5.64‰ for Tarocco samples), whereas the two conventional samples (Navelina only) incorrectly classified as organic come from fruits with high δ^{15}N PP (6.28 and 5.54‰) levels.

In conclusion, the results obtained in this research showed that the production methods do not determine differences between organically and conventionally grown fruits in the classic quality parameters, with the exception of the vitamin C content.

Parameters linked to the nitrogen metabolism (N, Syn, δ^{15}N PP, δ^{15}N AA) showed differences in fruits obtained with the two cultivation methods. In addition, by choosing some potential discriminating variables such as δ^{15}N PP, Syn, and vitamin C it is possible, by LDA, to classify a product the origin of which (be it organic or conventional) is unknown. Therefore, these parameters can be considered new markers to differentiate organically from conventionally grown orange fruits.

This is a first tentative step to provide a method for authenticity control of organically grown citrus fruits based on

Table 6. Canonical Discriminant Function

discriminant function	eigenvalue	relative percentage	canonical correlation	function derived	Wilks λ	χ^2	DF	significance level
1	1.5273	100.00	0.7774	0	0.3957	52.385	11	0.0000

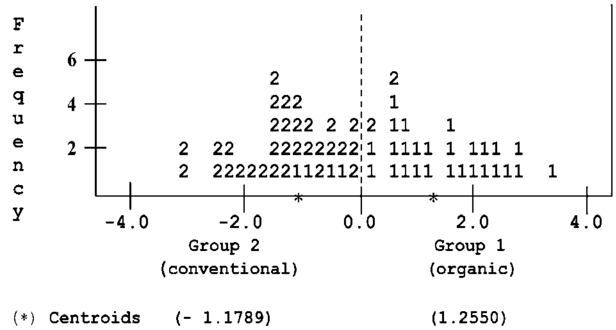

Figure 2. Plot of discriminant scores of the 64 organic and conventional samples.

Table 7. Standardized Canonical Discriminant Function Coefficients

variable	function 1	variable	function 1
total acidity	−0.43829	P	0.09216
total soluble solids	−0.06134	N	0.18149
vitamin C	0.72658	synephrine	−0.56880
Ca	0.03617	δ^{15}N AA	−0.16607
K	−0.16228	δ^{15}N PP	0.98725
Mg	−0.34958		

Table 8. Classification Results Obtained by LDA of the Organic and Conventional Groups and Percentage of Grouped Cases

actual group		no. of cases	predicted group membership 1	predicted group membership 2
organic	1	31	27 (87.1%)	4 (12.9%)
conventional	2	33	2 (6.1%)	31 (93.9%)

“grouped” cases correctly classified: 90.63%

analysis of some juice components, a few of them linked to different nitrogen fertilization between organic and conventional production systems.

Because the ^{15}N/^{14}N ratio in N-containing compounds depends on the ^{15}N content in organic or inorganic nitrogen present in the soil as well as soil characteristics, climate, and water stress, further studies are necessary to evaluate the influence of soil and climate conditions on the newly identified marker δ^{15}N PP.

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