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RESEARCH PAPER

Carbon and nitrogen partitioning during the post-anthesis period is conditioned by N fertilisation and sink strength in three cereals

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Keywords

^{13}C and ^{15}N isotopic labelling; grain filling; N use efficiency; N/C partitioning; wheat.

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ABSTRACT

Further knowledge of the processes conditioning nitrogen use efficiency (NUE) is of great relevance to crop productivity. The aim of this paper was characterise C and N partitioning during grain filling and their implications for NUE. Cereals such as bread wheat (*Triticum aestivum* L. cv Califa sur), triticale (× *Triticosecale* Wittmack cv. Imperioso) and tritordeum (× *Tritordeum* Asch. & Graebn line HT 621) were grown under low (LN, 5 mm NH_4NO_3) and high (HN, 15 mm NH_4NO_3) N conditions. We conducted simultaneous double labelling ($^{12}CO_2$ and $^{15}NH_4{}^{15}NO_3$) in order to characterise C and N partitioning during grain filling. Although triticale plants showed the largest total and ear dry matter values in HN conditions, the large investment in shoot and root biomass negatively affected ear NUE. Tritordeum was the only genotype that increased NUE in both N treatments (NUE_{total}), whereas in wheat, no significant effect was detected. N labelling revealed that N fertilisation during post-anthesis was more relevant for wheat and tritordeum grain filling than for triticale. The study also revealed that the investments of C and N in flag leaves and shoots, together with the 'waste' of photoassimilates in respiration, conditioned the NUE of plants, and especially under LN. These results suggest that C and N use by these plants needs to be improved in order to increase ear C and N sinks, especially under LN. It is also remarkable that even though tritordeum shows the largest increase in NUE, the low yield of this cereal limits its agronomic value.

INTRODUCTION

It is estimated that in the Mediterranean environment, which comprises 10% of the world's total wheat growing area, N availability is the main environmental factor, alongside drought stress, that limits productivity (Passioura 2002; Cossani *et al.* 2007). The fact that cereals require large amounts of N to increase maximum yield has led to a worldwide increase in fertiliser application from 1.3 million tonnes in 1930 to 85–90 million tonnes by 2004. Furthermore, it is predicted that by 2050, fertiliser application will reach 240 million tonnes (Masclaux-Daubresse *et al.* 2008). However, in developed economies, nitrogen use efficiency (NUE) is very low and estimated to be ca. 33% (Raun & Johnson 1999). The abuse of N fertilisers can lead to contamination of ground and surface water (Matson *et al.* 2002). To cope with this problem, European policies encourage an increase in the NUE of agriculture through crop management and plant breeding in order to reduce the excessive input of fertilisers, while maintaining acceptable yields (Lea & Azevedo 2006, 2007).

Improving wheat performance and NUE under low N availability conditions requires improved knowledge of yield determining factors (Masclaux-Daubresse *et al.* 2008, 2010). Grain filling, and by extension grain yield, are mainly sustained by assimilation and management of C and N compounds. In wheat, grain N content is the result of the N taken up after anthesis, together with the amount of remobilised N originating from pre-anthesis uptake (Dupont & Altenbach 2003). On the other hand, C required for grain filling is mostly provided by flag leaf photosynthesis (Evans *et al.* 1975), translocation of C assimilated before anthesis (mainly stored in the internodes; Gebbing *et al.* 1999) and ear photosynthesis (Tambussi *et al.* 2007). Contrasting results concerning the origin of C and N contributing to grain filling are reported in the literature (Palta & Fillery 1995; Ercoli *et al.* 2008) because the relative contribution of those sources varies with growth conditions and the cereal studied (Dupont & Altenbach 2003; Aranjuelo *et al.* 2009). The relative contribution of remobilisation to grain yield depends mainly on C/N source/sink interaction during grain filling (Asseng & Van Herwaarden 2003). These authors observed that when crops are exposed to water stress during grain filling, pre-anthesis reserves of carbohydrate and N contribute to grain filling to a greater extent under high N status than under low N. Among other factors, grain filling has been described as being affected by the rate and timing of N application and on the level and form of soil N (Spiertz & De Vos 1983; Borghi *et al.* 1997). Furthermore, as observed by Papakosta & Gagianas (1991), high soil N availability during grain filling

favours post-anthesis N uptake and reduces the remobilisation of pre-anthesis N.

Improvements conducted in plant breeding programmes derived from the application of genetic studies appear to be reaching a plateau. Approaches that complement traditional breeding methodologies with new approaches for more efficient phenotyping, along with a deeper understanding of the metabolic mechanisms that modulate plant responses to environmental conditions, are a central goal of breeding programmes (Araus *et al.* 2008). In this context, the use of stable isotopes has provided key information for the identification of new traits to assess plant performance (Aranjuelo *et al.* 2009, 2011a,b; Masclaux-Daubresse & Chardon 2011). Previous studies conducted in wheat have revealed that the 'classical' assumptions concerning C partitioning during grain filling were, in some cases, not totally accurate. The low number of studies analysing the loss of photoassimilates by respiration during wheat grain filling underscores the relevancy of this process, a topic that has been observed to require a large amount of carbohydrate (Aranjuelo *et al.* 2009, 2011b). These studies on canopy-level C labelling conducted with wheat plants revealed that 2 weeks after anthesis, nearly half of the CO_2 respired by the plants came from C fixed in the previous week. These studies also revealed that at the same phenological stage, 21% of C present in root total organic matter (TOM) was labelled (*i.e.* C assimilated in the previous 7 days). Overall, these results suggest that a substantial part of the C assimilated during grain filling effectively does not contribute to this important process. It appears reasonable to speculate that this may be due to low sink strength.

Traditional breeding strategies are limited by the low genetic variability of yield components under stress conditions (Cook 1999). Plant genetic resources for agriculture might be one of the biological bases of future agriculture. *Tritordeum* is a doubled haploid hexaploid (× *Tritordeum* Asch. & Graebn) that resulted from an interspecific cross between the diploid *Hordeum chilensis* and the tetraploid wheat, *Triticum turgidum* L. ssp. *durum*. *Tritordeum* is considered as a new crop having great potential (Martin 1988) as well as a bridge species for transferring useful barley traits, *e.g.* adaptation to Mediterranean conditions, to wheat (Martin *et al.* 1999; Ballesteros *et al.* 2005). Studies carried out in tritordeum (Barro *et al.* 1991, 1996) have highlighted an increased capacity for N absorption. Maximising the NUE of N fertilisers is a matter of great concern, especially in the context of sustainable agriculture.

Ensuring an adequate C and N balance in the sink and source tissues is an essential target point for maximising the response of cereals to growth under low N availability conditions. The main goal of this paper was to determine the C and N partitioning during grain filling and its implications in the NUE of two contrasting genotypes (wheat and triticale) together with a new crop (tritordeum) with potential interest for introgression (*i.e.* bridging traits) in wheat breeding programmes for Mediterranean conditions. Moreover, these three cereals show a clear range in harvest index (the ratio of grain weight to total shoot biomass), with bread wheat and tritordeum exhibiting the highest and the lowest values, respectively (Villegas *et al.* 2010). Therefore, the three cereals show different constitutive (*i.e.* in the absence of any stress) sink strengths. $^{12}CO_2$ labelling was conducted at the whole plant level to better understand C partitioning of these plants during the post-anthesis period.

Preliminary studies carried out previously in tritordeum plants suggested that they have higher NUE, which means that their performance should be better in low N conditions than other cereals. This hypothesis was tested in low N and compared with high yielding cultivars. The sampling and subsequent analyses of respired CO_2 isotopic composition through GC-C-IRMS enabled the characterisation of labelled C invested in respiration processes. Respiration represents an important C sink that has not been considered in classical approaches studying the contribution of C assimilated during pre/post-anthesis in grain filling. In parallel to C labelling, N labelling was applied to the plants in a $^{15}NH_4{}^{15}NO_3$ enriched solution in order to test N uptake and partitioning to different plant organs.

MATERIAL AND METHODS

Experimental design

Seeds of bread wheat (*Titicum aestivum* L. cv. Califa Sur) and its two amphiploids, triticale (× *Triticosecale* Wittm. cv. Imperioso) and tritordeum (× *Tritordeum* Asch & Graeb, HT 621) were sown (eight plants per pot) in 4-l pots filled with sand. Being amphidiploids, the latter two genotypes contain the diploid chromosome sets of both parents. Triticale is the result of a cross between wheat (*Triticum turgidum*) and rye (*Secale cereale*), whereas tritordeum is a fertile amphiploid between a wild barley (*Hordeum chilense*) and a durum wheat (*Triticum turgidum*). The plants were grown in two controlled environment chambers (Conviron E15; Controlled Environments Ltd, Winnipeg, MB, Canada) throughout the experiment. Inside the growth chambers, the growth conditions were 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD), 70% relative humidity, 25/15 °C day/night temperature and a 14-h photoperiod. The air of each module was sampled every 5 min using an industrial programmer (PLS C20K; Omron, Kyoto, Japan) and the CO_2 concentration was analysed through an infrared gas analyser (IRGA; Lira 3600; MSA, Barcelona, Spain). Air probes were placed at the centre of each module. The data were continuously recorded with a computer through analogue–digital converters (Microlink 751; Biodata Ltd, Manchester, UK) and Windmill software with a TEST-SEQ programming tool (Biodata Ltd).

The plants were watered with a modified Hoagland nutrient solution (Arnon & Hoagland 1939). A random selection of half of the pots was watered with 5 mM NH_4NO_3 [low N treatment (LN)] and the other half with 15 mM NH_4NO_3 [high N treatment (HN)]. The two levels of N were selected according to observations of previous studies conducted by our group with wheat (Cabrera-Bosquet *et al.* 2009) and barley (Yousfi *et al.* 2010) plants exposed to different nutritional levels.

For each species we carried out double (C and N) labelling over 1 week, starting 1 week after anthesis (defined when at least 50% of the spikes in a pot showed protruding anthers). This period was selected because growth conditions between 20 days pre- and 10 post-anthesis are considered a critical

period for grain yield (Schnyder 1992). The labelling was conducted in a random selection of half of the pot plants that were placed in separated growth chambers. The pots were rotated regularly within the corresponding chamber to avoid chamber effects. All the determinations were conducted in samples collected immediately after the end of the labelling period.

N and C isotope labelling procedures

One week after anthesis, coinciding with the period of largest photoassimilate contribution to grain filling, we undertook simultaneous N and C labelling (for 1 week). The N labelling was conducted by replacing the $^{14}NH_4{}^{14}NO_3$ in the Hoagland solution with $^{15}NH_4{}^{15}NO_3$ that had a $\delta^{15}N$ enriched at 5%. For C labelling, $^{12}CO_2$-enriched gas was used in one of the growth chambers, whereas the other chamber was maintained at ambient conditions. Bottles of CO_2 required for the C labelling were provided by Carburos Metálicos S.A. (Barcelona, Spain). Mixing the commercial CO_2 ($\delta^{13}C$ ca. $-32.0 \pm 0.1‰$) with the ambient air ($\delta^{13}C$ ca. $-11.3‰$) resulted in a $^{13}CO_2$ isotopic composition of $\delta^{13}C$ -23.3 ‰ in the labelling chamber. Air isotopic composition inside the corresponding growth chamber was collected daily using 50-ml syringes (SGE International PTY Ltd, Ringwood, Vic., Australia) and kept in 10-ml vacutainers (BD Vacutainers, Plymouth, UK). To avoid contamination with the air present in the syringe and the needle, both were purged with N_2 prior to each sampling. The vacutainers were also over-pressurised with the same N_2 gas so that the pressure inside the vacutainer was above ambient.

Plant growth

Plant biomass was determined by harvesting just after the end of labelling (14 days after anthesis). Ears, flag leaves, shoots (including the rest of the leaves) and roots were collected separately. After drying at 60 °C for 48 h, dry mass (DM) was determined. Total nitrogen use efficiency (NUE_{total}) was determined as the ratio between total dry matter and total N content. Ear nitrogen use efficiency (NUE_{ear}) was calculated as the ratio between ear DM and total N content. The shoot/-root ratio was determined as the total shoot DM (sum of ear, stem, including leaf sheaths, and leaf blades DM) divided by the root DM.

$\delta^{13}C$ of dark-respired CO_2 determination

Prior to the dark respiration determinations, the plants were dark-adapted for 45 min in a dark room. Flag leaf blades, ears and roots were placed separately in a plastic gas analysis chamber to determine the $\delta^{13}C$ of dark respired CO_2 ($\delta^{13}C_{DR}$). In the case of root respiration analysis, root samples were gently cleaned and immediately placed on damp paper inside the chamber; determinations were conducted during the first 4 min. Due to methodological constraints associated with shoot structure, shoot respiration was not determined. The plastic chamber ($20 \times 12 \times 6 \times 10^{-6}$ m^3) included two fans and was connected to a LI-6400 photosynthesis system (Li-Cor, Lincoln, NE, USA). Temperature in the chamber was maintained at ca. 25 °C by cool-watering the jacket around the leaf chamber. Ingoing air was passed through the chamber at a rate of 1 l·min^{-1}, monitored by the LI-6400.

Molar fractions of CO_2 and humidity were measured with the IRGA of the LI-6400. The PPFD inside the chamber was maintained at 0 μmol·m^{-2}·s^{-1} by covering the entire system with a black cover. The gas analysis chamber, included in the closed system, was first flushed with CO_2-free air to ensure that only the CO_2 respired in the chamber was accumulated. The CO_2 concentration inside the chamber was measured by the LI-6400. When the CO_2 inside reached 300 μmol·mol^{-1}, CO_2 samples were collected and analysed as described in more detail below.

N and C isotope composition ($\delta^{15}N$ and $\delta^{13}C$) and total C and N content

Ear, flag leaf blade, shoot (including the rest of leaves) and root samples were collected on the last day of labelling (*i.e.* last day of the experiment), dried at 60 °C for 48 h, and analysed for the C isotopic composition of total organic matter ($\delta^{13}C$; TOM). A ground sample of 1 mg was used for each determination. The $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios (R) of plant material were determined using an elemental analyser (EA1108, Series 1; Carlo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C; Finnigan, Mat., Bremen, Germany) operating in continuous flow mode.

The $^{13}C/^{12}C$ ratios (R) of air samples were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Briefly, water vapour and oxygen from gas samples were removed and the CO_2, argon, and N gases were separated by gas chromatography (Agilent 6890 Gas Chromatograph) coupled to an isotope ratio mass spectrometer Delta^{plus} *via* a GC-C Combustion III interphase (ThermoFinnigan). The column used was 30 m × 0.32 mm i.d. GS-GASPRO (J. and W. Scientific, USA). The carrier gas was helium at a flow rate of 1.2 ml·min^{-1}. The injection port temperature was 220 °C. The oven temperature was kept at 60 °C during the entire run. Injection was conducted in the split mode (injected volume 0.3 ml, split flow 20 ml·min^{-1}).

The $^{13}C/^{12}C$ ratios (R) of plant material and air samples were expressed as $\delta^{13}C$ values using international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 l-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision of 0.1‰:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}}\right) - 1$$

The $^{15}N/^{14}N$ ratios of plant material were also expressed in δ notation ($\delta^{15}N$) using international secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) referred to N_2 in air, with analytical precision at about 0.2‰:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}}\right) - 1$$

The N isotope discrimination ($\Delta^{15}N$, ‰), calculated in non-labelled plants was expressed following Evans (2001):

$$\Delta^{15}N = (\delta^{15}N_f - \delta^{15}N_p)/[1 + (\delta^{15}N_p/1000)]$$

where $\delta^{15}N_f$ and $\delta^{15}N_p$ refer to the fertiliser and plant N isotope composition, respectively.

The N and C isotopic composition analyses of TOM and air samples (in case of C) were conducted at the Serveis Científico-Tècnics of the University of Barcelona.

Statistical analyses

The effect of N fertilisation and plant species was tested by two-way analysis of variance (ANOVA). The analysis was conducted with the SPSS 12.0 statistical software package (SPSS Inc., Chicago, IL, USA). When there were differences between means, we made comparisons using a Tukey b multiple comparison test. The results were accepted as significant at $P < 0.05$.

RESULTS

The N treatment ($F = 26.41$, $P = 0.00$) had more effect on total DM than on analysed genotype ($F = 4.38$, $P = 0.03$). Triticale was the genotype that produced the most biomass under HN conditions, while tritordeum was the least productive, with wheat in between (Table 1). Low N availability decreased biomass production by 42% (wheat), 58% (triticale) and 25% (tritordeum), and no differences among cereals were observed (Tables 1 and S1). The more specific analyses of growth parameters revealed that the larger DM of triticale at HN was related to its larger ears ($F = 8.32$, $P = 0.00$) and shoots ($F = 5.38$, $P = 0.02$) DM. The effect of LN availability on plant growth was highest on the DM of shoots ($F = 48.56$, $P = 0.00$) and ears ($F = 26.24$, $P = 0.00$; Table S1). LN had no effect on wheat root DM, whereas root DM increased in triticale and tritordeum. Low N availability reduced ($F = 98.52$, $P = 0.00$) the shoot/root ratio (Table 1). Under HN conditions, the largest shoot/root ratio was observed in triticale plants and the lowest in wheat. The ear DM/total DM ratio (Table 1) highlighted that LN negatively affected DM values in triticale and wheat, whereas no effect was observed in tritordeum.

Low N availability (LN) decreased the plant N content in all three cereals by 43% (wheat), 61% (triticale) and 56% (tritordeum) ($F = 52.75$, $P = 0.00$; Table 2). LN affected ear N content (N_{ear}) negatively in the three genotypes ($F = 17.44$, $P = 0.00$) but this decrease was more marked in tritordeum, where its content decreased by 50% (Table S2). Flag and shoot N content (N_{flag} and N_{shoot}, respectively) highlighted that these values were negatively affected by LN in wheat (79% and 32%, respectively) tritordeum (78% and 70%, respectively) and in triticale (74% and 83%, respectively). The effect of N availability on total and ear N use efficiencies (NUE_{total} and NUE_{ear}, respectively) was different depending on the analysed genotype ($F = 27.74$, $P = 0.00$ and $F = 5.82$, $P = 0.01$, respectively; Table S2). Although no effect was observed in wheat, NUE_{total} increased in triticale (13%) and especially in tritordeum (73%) plants. LN negatively affected NUE_{ear} in wheat and triticale (24% and 25%, respectively) but in the case of tritordeum plants, NUE_{ear} increased by 146%.

The $\delta^{13}C_{TOM}$ of labelled plants was more ^{13}C-depleted than the corresponding non-labelled plants in wheat ($F = 58.92$, $P = 0.00$), triticale ($F = 395.85$, $P = 0.00$) and tritordeum ($F = 138.75$, $P = 0.00$; Fig. 1). Under HN conditions, the ears were the main C sink in wheat and triticale plants, whereas in tritordeum plants more labelled C was detected in flag leaves and shoots. However, in LN, the

Table 1. Low (LN) *versus* high (HN) N fertilisation effect on total dry matter (DM, g·plant^{-1}), ear DM (g·plant^{-1}), flag DM (g·plant^{-1}), shoot DM, (g·plant^{-1}), root (g·plant^{-1}), and ear DM/total DM and shoot/root of wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants. Each data represents the mean of, at least, five replicates ± SD.

ecotype	treatment	total DM	ear DM	flag DM	shoot DM	root DM	ear DM/total DM	shoot/root
wheat	HN	6.78 ± 0.04	3.23 ± 0.45	0.34 ± 0.03	2.00 ± 0.16	1.21 ± 0.30	0.48 ± 0.07	4.85 ± 1.42
	LN	3.91 ± 0.83	1.38 ± 0.20	0.08 ± 0.02	1.56 ± 0.58	0.98 ± 0.34	0.37 ± 0.13	3.25 ± 0.95
triticale	HN	9.23 ± 1.25	4.20 ± 0.65	0.26 ± 0.03	4.07 ± 0.78	0.69 ± 0.20	0.46 ± 0.04	12.92 ± 3.24
	LN	3.90 ± 1.26	1.18 ± 0.41	0.08 ± 0.03	1.23 ± 0.09	1.42 ± 0.37	0.30 ± 0.01	2.12 ± 1.06
tritordeum	HN	4.94 ± 1.99	1.06 ± 0.45	0.20 ± 0.11	3.20 ± 0.71	0.48 ± 0.12	0.19 ± 0.05	9.31 ± 0.52
	LN	3.71 ± 0.88	1.19 ± 0.13	0.06 ± 0.03	1.56 ± 0.46	0.91 ± 0.35	0.28 ± 0.12	3.35 ± 1.40

Each value represents the mean of four replicates ± SD.

Table 2. Low (LN) *versus* high (HN) N fertilisation effect on total N (N_{total}, mg·plant^{-1}), ear N (N_{ear}, mg·plant^{-1}), flag leaf N ($N_{flagleaf}$, mg·plant^{-1}), shoot N (N_{shoot}, mg·plant^{-1}), root N (N_{root}, mg·plant^{-1}), total N use efficiency (NUE_{total}, mg·g^{-1} plant) and ear N use efficiency (NUE_{ear}, mg·g^{-1} plant) of wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants.

ecotype	treatment	N_{total}	N_{ear}	$N_{flagleaf}$	N_{shoot}	N_{root}	NUE_{total}	NUE_{ear}
wheat	HN	162.24 ± 2.20	76.12 ± 10.48	16.97 ± 1.64	51.34 ± 4.17	17.79 ± 4.33	41.83 ± 0.52	19.92 ± 2.62
	LN	91.89 ± 11.03	43.14 ± 6.30	3.41 ± 0.99	34.52 ± 12.95	10.81 ± 8.83	41.42 ± 3.03	15.20 ± 4.33
triticale	HN	188.24 ± 29.01	81.29 ± 15.01	9.81 ± 1.48	82.01 ± 19.07	15.11 ± 1.78	48.11 ± 0.38	21.85 ± 2.44
	LN	72.61 ± 16.70	27.36 ± 9.48	2.51 ± 0.88	13.51 ± 1.01	19.22 ± 5.85	54.57 ± 3.32	16.33 ± 0.47
tritordeum	HN	152.27 ± 45.95	24.38 ± 11.92	10.79 ± 4.16	108.23 ± 23.89	8.85 ± 3.97	32.24 ± 1.21	6.21 ± 2.96
	LN	66.96 ± 24.49	24.39 ± 13.12	2.34 ± 1.12	32.04 ± 9.56	8.19 ± 4.04	55.93 ± 4.55	15.29 ± 6.47

Each value represents the mean of four replicates ± SD.

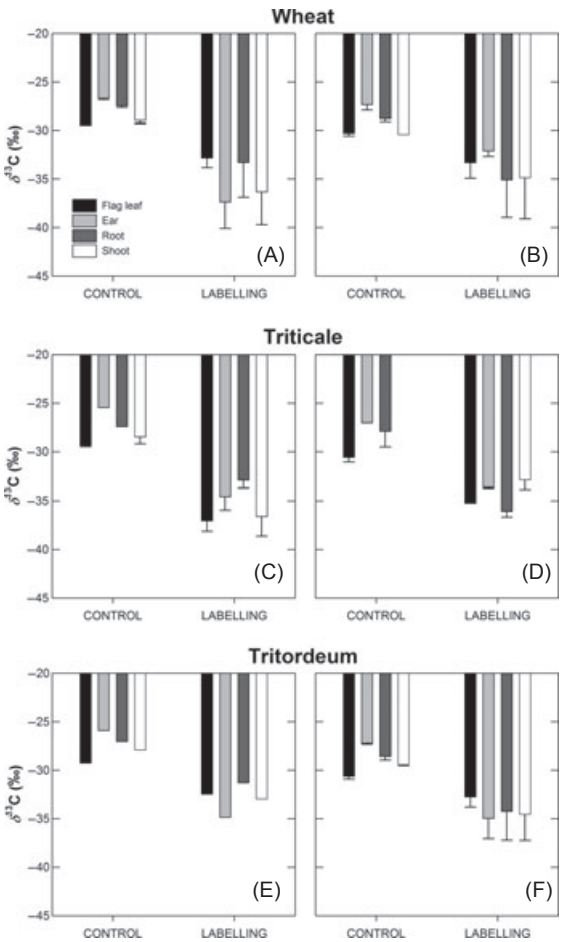

Fig. 1. ^{13}C isotopic composition ($\delta^{13}C$) of Control (non-labelled) and labelled total organic matter (TOM) of ears, flag leaf, shoot and roots in wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants. A, C and E are high N treatments, whereas B, D and F are low N treatments. Each value represents the mean of three replicates ± SD.

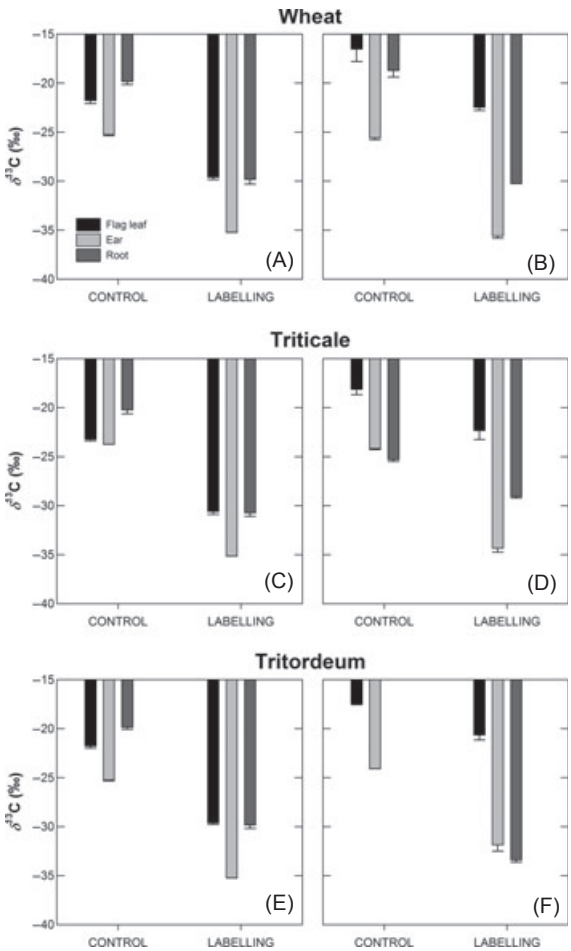

Fig. 2. ^{13}C isotopic composition ($\delta^{13}C$) of Control (non-labelled) and labelled dark respired CO_2 (DR) of ears, flag leaf, shoot and roots of wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants. A, C and E are high N treatments whereas B, D and F are low N treatments. Each value represents the mean of three replicates ± SD.

$\delta^{13}C_{TOM}$ was more homogeneous among the different organs (Fig. 1). By contrast, in unlabelled plants the flag leaf had the lowest $\delta^{13}C$ (Fig. 1). On the other hand, the $\delta^{13}C_{DR}$ also revealed significant differences (Fig. 2, Table S4) between organs for wheat ($F = 543.72$, $P = 0.00$), triticale ($F = 443.17$, $P = 0.00$) and tritordeum ($F = 779.37$, $P = 0.00$). Regardless of the genotype analysed and the N treatment, the ears respired the largest amount of labelled C (Fig. 2). In triticale and tritordeum plants, higher $\delta^{13}C$ values (*i.e.* ^{13}C-enriched) for respired CO_2 were observed in LN plants. In the case of wheat, although labelled C decreased in flag leaves in LN treatments, in ears and roots no significant differences were detected (Fig. 2). The most depleted $^{13}CO_2$ was respired by ears. Interestingly, the $\delta^{13}C_{DR}$ was similar in ears of the three genotypes exposed to HN. Although the ear $\delta^{13}C_{DR}$ values were similar in LN and HN plants of wheat and triticale, in tritordeum plants the $\delta^{13}C_{DR}$ was lower (−34‰ *versus* −32‰).

Although no N treatment effect was observed on the $\delta^{15}N$ of wheat ($F = 0.82$, $P = 0.38$), triticale ($F = 2.78$, $P = 0.11$) and tritordeum ($F = 0.80$, $P = 0.38$), the ^{15}N labelling showed that ears were the main N sink in wheat and tritordeum plants subjected to HN, whereas in triticale, more labelled N was detected in flag leaves and shoots (Fig. 3). In LN treatments, with the exception of wheat (where the $\delta^{15}N$ was lower), the $\delta^{15}N$ of ears was similar to the values detected in flag leaves, shoots and roots.

In non-labelled plants, with the exception of wheat flag leaves, triticale ears and tritordeum roots, the $\delta^{15}N$ values obtained under LN were lower than those recorded in the corresponding HN plants (Fig. 3). As a consequence, N isotope discrimination ($\Delta^{15}N$) values recorded in LN treatments were higher for wheat ($F = 144.35$, $P = 0.00$), triticale ($F = 92.52$, $P = 0.00$) and tritordeum ($F = 31.77$, $P = 0.00$) than those in the corresponding HN treatments (Tables 3 and S3).

The negative correlation across organs and species ($R^2 = 0.58$) observed between TOM $\delta^{15}N$ and $\delta^{13}C$ revealed that, in general terms, the sink strength of the organs was similar for the C and N assimilated during the previous 7 days of the post-anthesis period (Fig. 4). The organ correlation analyses (Fig. 5) highlighted that ears ($R^2 = 0.69$), followed by shoots ($R^2 = 0.55$) and flag leaves ($R^2 = 0.54$) had

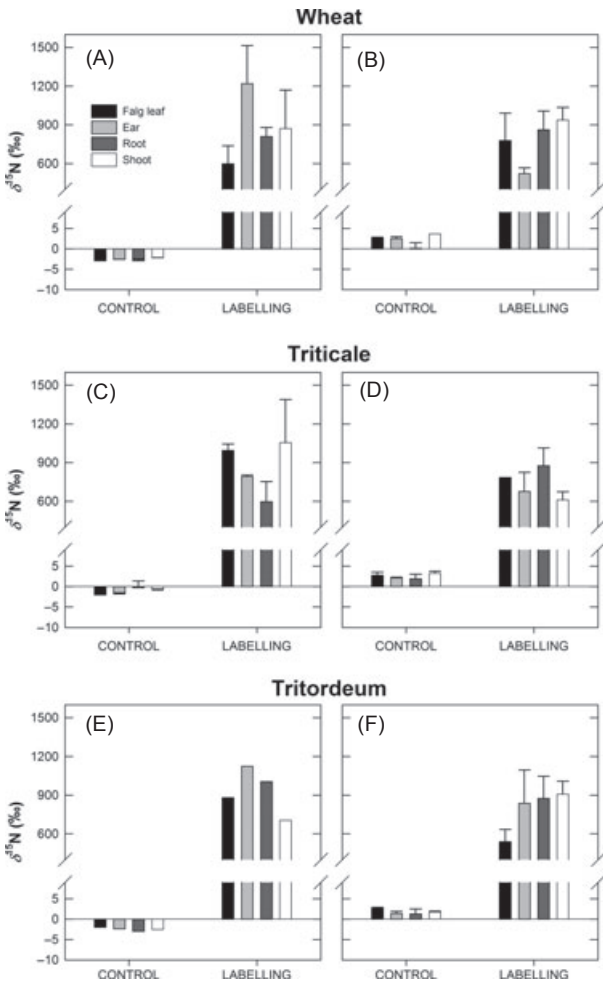

Fig. 3. ^{15}N isotopic composition ($\delta^{15}N$) of Control (non-labelled) and labelled total organic matter (TOM) of ears, flag leaf, shoot and roots of wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants. A, C and E are high N treatments whereas B, D and F correspond to low N treatments. Each value represents the mean of three replicates ± SD.

similar C and N sinks. However, in the case of roots, no correlation ($R^2 = 0.06$) was detected.

DISCUSSION

In this study the NUE, together with C and N partitioning, of three cereals fertilised with HN and LN solutions were characterised. Similar to observations in a previous study (Aranjuelo *et al.* 2009), triticale, followed by wheat, was the

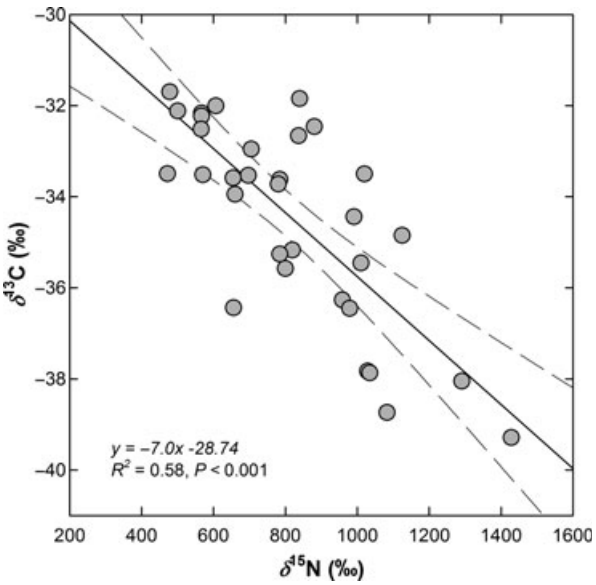

Fig. 4. The relationship between ^{15}N isotopic composition ($\delta^{15}N$) and ^{13}C isotopic composition ($\delta^{13}C$) wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants.

genotype that produced the most biomass under optimal growth conditions. Similar results were described in Ehdaie & Waines (2001), who also observed that fertilisation with low N affected wheat and triticale production similarly. However, in this study, the data obtained showed that the production of triticale was the most N availability-dependent of the three genotypes (Table S1). Total DM and ear DM decreased in triticale by 58% and 72%, respectively, under LN and revealed that cultivation of the selected triticale cultivar (Imperioso) in soils with low N availability might be of little interest. Apparent discrepancies between our findings and the results obtained by Ehdaie & Waines (2001) could be explained by the fact the cultivars selected in these studies were derived from different breeding programmes. On the other hand, although total DM decreased by 25% and ear production was not affected by low N availability in tritordeum, the low DM (75% and 52% lower than triticale and wheat respectively) in HN conditions constrains its agronomic interest.

The negative effect of LN treatment (compared with the corresponding HN treatment) on harvest index (expressed as ear DM per total DM; Table 1) observed in wheat and triticale, revealed that these species had lower ear sink strength as N availability decreased. This result is in agreement with

ecotype	treatment	ear	flag leaf	shoot	root
wheat	HN	2.52 ± 0.03	2.83 ± 0.14	2.09 ± 0.01	2.85 ± 0.31
	LN	−2.57 ± 0.44	−2.67 ± 0.23	−3.72 ± 0.31	−0.21 ± 0.11
triticale	HN	1.71 ± 0.19	1.95 ± 0.01	0.76 ± 0.13	0.18 ± 0.15
	LN	−2.23 ± 0.09	−2.87 ± 0.53	−3.33 ± 0.35	−1.99 ± 0.80
tritordeum	HN	2.29 ± 0.18	1.90 ± 0.01	2.47 ± 0.21	2.89 ± 0.05
	LN	−1.45 ± 0.38	−2.67 ± 0.13	−1.82 ± 0.14	−1.38 ± 0.85

Each value represents the mean of three replicates ± SD.

Table 3. Low (LN) *versus* high (HN) N fertilisation effect on the ear, flag leaf, shoot and root ^{15}N isotopic discrimination ($\Delta^{15}N$, ‰) of non-labelled wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants. Each data represents the mean of, at least, three replicates ± SD.

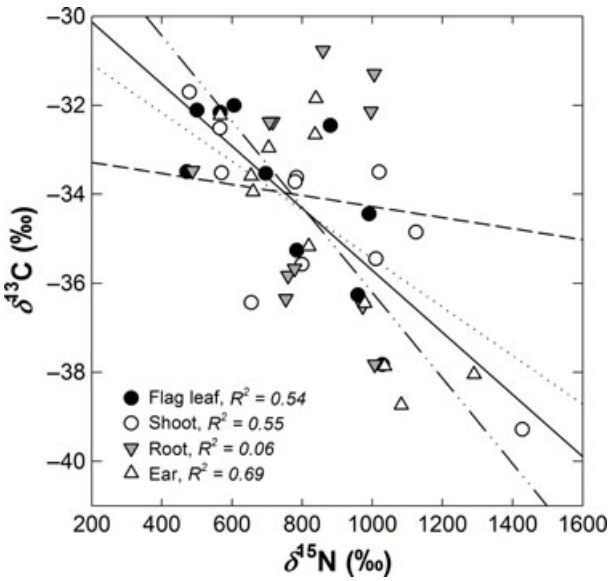

Fig. 5. The relationship between $\delta^{15}N$ and $\delta^{13}C$ of flag leaf (●) shoot (○), root (▲) and the ear (△) of wheat (Califa sur), triticale (Imperioso) and tritordeum (HT 621) plants.

previous studies (Ehdaie & Waines 2001; Mergoum *et al.* 2004) that describe how triticale yield is strongly conditioned by N fertilisation levels. It can also be highlighted that under HN conditions, tritordeum had an ear DM/total DM ratio lower than the other two cereals. Similar results have been described in a multi-local field study conducted with the same genotypes (Villegas *et al.* 2010). According to the observations of these authors, tritordeum had fewer grains per spike and the kernel weight was also the lowest compared with wheat and triticale plants, The fact that in tritordeum plants the relative investment in the shoots was higher than in the ears (Aranjuelo *et al.* 2009) reflects a lack of agronomic improvement of this amphiploid compared with wheat and triticale. Since tritordeum is a quite a new species, its present genotypes have not been subjected to extensive plant breeding programmes with the consequent limitations on yield of those plants. Our data highlight the need to increase breeding efforts directed towards increasing ear sink strength in tritordeum.

Grain filling is conditioned by the size and availability of C and N pools (Fuertes-Mendizábal *et al.* 2010). As mentioned before, the main C sources for grain filling are the flag leaf, the stem internodes and the C assimilated by the ears. The fact that the flag leaves had the lowest labelled C in wheat and tritordeum is indicative that these organs acted as a C source for grain filling, with grain having more labelled C. Interestingly, the large labelled C values of flag leaves and shoots in triticale suggest two possibilities: (i) shoots represented major labelled C sinks that competed with the ears as C sinks; or (ii) shoots acted as major labelled C sinks due to the poor sink strength of triticale ears. In fact, triticale is characterised by constitutively (*i.e.* in the absence of stress) wrinkled grains. Despite the large investment of C compounds in respiration, little attention has been given to this point in previous studies in cereals (Bort *et al.* 1996; Dupont & Altenbach 2003; Aranjuelo *et al.* 2009). The fact that in wheat and tritordeum the $\delta^{13}C_{DR}$ was less labelled than the corresponding TOM revealed that those plants used respiratory substrates formed before the period when labelling was performed (*i.e.* pre-labelling C), which means that more labelled C was available for grain filling. However, in the case of triticale plants, $\delta^{13}C_{DR}$ respired by the ears was composed of more labelled C that could not be directed toward grain filling. Since, under LN conditions, a similar $\delta^{13}C$ was observed in TOM and respired CO_2, it can be expected that triticale plants invested more labelled C in respiration processes to the detriment of grain filling. This study highlights that in spite of species and N treatment, the plants 'invested' a large amount of labelled C in respiratory processes, which means that less labelled C was available for grain filling.

The stable N isotope signature ($\delta^{15}N$) is a useful screening tool for plant N metabolism (Coque *et al.* 2006; Yousfi *et al.* 2010). $\delta^{15}N$ is conditioned by the N uptake from the medium into the root cells or by the subsequent enzymatic assimilation into other N forms (Kolb & Evans 2003; Tcherkez & Hodges 2008) and by the discrimination of enzymes involved in N uptake (Tcherkez & Hodges 2008). Futhermore, soil N concentration levels have been described (Serret *et al.* 2008; Yousfi *et al.* 2010) to condition ^{15}N discrimination ($\Delta^{15}N$). In agreement with these authors, our data revealed that $\Delta^{15}N$ is conditioned by the plant N demand/availability ratio. The fact that (under non-labelled conditions) LN plants had higher $\delta^{15}N$ values highlighted that when N availability is limiting, the $\Delta^{15}N$ in LN plants was reduced.

Our study showed different NUE response patterns depending on the genotype analysed and the N treatment. Under HN conditions, tritordeum plants had the lowest total and ear N use efficiency (NUE_{total} and NUE_{ear}). The fact that N_{shoot} represented 71% of the total N, whereas N_{ear} only represented 16% of it, highlights the low ear N sink strength, and this explained the low NUE in tritordeum. However, when N availability was limiting, tritordeum was the only genotype that increased NUE_{total} and NUE_{ear}. Compared with the corresponding HN, under LN tritordeum increased in ear sink strength (ear contained 36% of total N and shoots 48%; Tables 1 and 2). However, in the case of triticale, NUE_{total} increased under HN, and the large shoot and root biomass in LN treatments decreased ear sink strength and, consequently, grain filling. In this study, wheat plants proved to be the only genotype where N treatment had no effect on NUE_{total} and NUE_{ear}. The stability of NUE under both N fertilisation regimes is explained by the fact that the present wheat cultivars are the consequence of improved plant breeding programmes.

The ^{15}N labelling enabled the further characterisation of N assimilation and partitioning during this post-anthesis phenological stage. During grain filling, shoot proteins are hydrolysed to amino acids to enable the remobilisation of N content toward grains (Gebbing *et al.* 1998, 1999; Masclaux-Daubresse *et al.* 2008). As described in previous studies (Palta & Fillery 1995), 60–95% of N present in grains comes from the remobilisation of N stored in shoots and roots before anthesis. The lower amount of labelled N present in triticale ears (compared with wheat and tritordeum) higlights that pre-anthesis N remobilisation contributed toward grain filling in this genotype (Table S4). In tritordeum and wheat plants the redistribution of labelled N from source organs (flag

leaves and shoots) toward sink organs (ears) was more marked in HN, especially in HN tritordeum (Gebbing *et al.* 1999). Such differences between HN and LN were caused by the fact that N remobilisation starts earlier in plants under LN fertilisation conditions compared to HN fertilisation conditions. These results suggest that N fertilisation during this period was more relevant for grain filling of wheat and tritordeum plants compared with triticale.

The double $^{13}CO_2$ and ^{15}N labelling also highlighted that C and N assimilated post-anthesis were similarly partitioned (Fig. 3), regardless of the genotype and N treatment (Gebbing & Schnyder 1999). The $\delta^{13}C$ and $\delta^{15}N$ correlation analyses (Fig. 4) confirmed that the distribution among organs of the labelled C and N was similar. The more specific analyses at the level of the different organs (Fig. 5) revealed that the ears showed the highest correlation between $\delta^{15}N$ and $\delta^{13}C$ ($R^2 = 0.69$), indicating their major roles as C and N sinks. Negative correlations between $\delta^{15}N$ and $\delta^{13}C$ were also found in shoots ($R^2 = 0.55$) and to a lesser extent in flag leaves ($R^2 = 0.54$). These results are in agreement with the finding of Gebbing *et al.* (1998), who observed that post-anthesis C was mainly accumulated in glumes and to a lesser extent in the stem.

Although the role of ears as a major sink has been widely described (Schnyder 1992; Gebbing *et al.* 1998, 1999; Gebbing & Schnyder 1999), the C and N sink strength during post-anthesis has been related to the synthesis of structural compounds and elongation of the peduncle (Borrell *et al.* 1991; Bonnett & Incoll 1992). Such expansion is associated with the synthesis of cell wall material, which requires the utilisation of post-anthesis photosynthate (Gebbing *et al.* 1998). On the other hand, no such correlation was observed in roots (Fig. 5). The use of N and C compounds by plants involves, in part, resource remobilisation (Masclaux-Daubresse *et al.* 2008). Furthermore, as observed by Kichey *et al.* (2007), grain yield is based on N uptake before flowering and on the remobilisation of N during grain filling. The redistribution of labelled N from source organs (flag leaves and shoots) toward sink organs (ears) of N assimilated during the post-anthesis period was more marked in HN triticale and in HN tritordeum (Gebbing *et al.* 1999). Such differences between HN and LN were caused by the fact that N remobilisation starts earlier when plants are under low N fertilisation conditions compared to high N fertilisation conditions.

CONCLUSIONS

As mentioned above, the challenge for breeders is to select plants with high yields and high NUE. This study revealed that even if triticale had the largest total and ear DM values in HN conditions, it proved to be the genotype that was the most agronomically negatively affected by LN. The largest allocation of labelled C and N in shoots was involved in the decreased ear N use efficiency (NUE_{ear}) of triticale. Furthermore, N fertilisation during post-anthesis was least relevant in triticale. Although wheat NUE was similar to values in triticale, it did not improve under LN conditions. Tritordeum plants were the only ones where NUE (NUE_{total} and NUE_{ear}) values increased under LN treatment. The remobilisation of pre-labelled N in these plants was shown to be more relevant to the increase in grain filling and NUE in LN.

In summary, this study has shown that ear sink and NUE strength need to be improved in wheat and triticale LN plants, whereas in the case of tritordeum, although NUE was enhanced, the low yield in HN conditions reduced the potential of this genotype. The 'competition' for photoassimilates between ear DM and respiration processes, together with the investment of C and N resources in leaves and shoots also limited the NUE of plants. The study also highlights the potential of breeding for improved NUE in LN, especially in triticale plants that were revealed to be more sensitive to fertiliser levels. Furthermore, the promising results for tritordeum plants highlight the need to improve their yield under LN conditions and more especially under HN.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Mean squares explained by ANOVA for the main effects (genotype and N treatments and their corresponding interactions) for total dry matter (DM), ear DM, flag leaf DM, shoot DM, root DM, ear DM/total DM and shoot/root. Percentage of the sum of squares is expressed in brackets. The different symbols indicate significant differences at 5% (*) and 1% (**), whereas n.s. refers to no significant difference.

Table S2. Mean squares explained by ANOVA for the main effects (genotype and N treatment and their corresponding interactions) for total N (N_{total}), ear N (N_{ear}), flag leaf N ($N_{flagleaf}$), shoot N (N_{shoot}), root N (N_{root}), total N use efficiency (NUE_{total}) and ear N use efficiency (NUE_{ear}). Percentage of the sum of squares is in brackets. The different symbols indicate significant differences at 5% (*) and 1% (**), whereas n.s. refers to no significant difference.

Table S3. Mean squares explained by ANOVA for the main effects (genotype and N treatments and their corresponding interactions) for ^{15}N isotopic discrimination ($\Delta^{15}N$, ‰). Percentage of the sum of squares is in brackets. The different symbols indicate significant differences at 5% (*) and 1% (**), whereas n.s. refers to no significant difference.

Table S4. Mean squares explained by ANOVA for the main effects (labelling, N treatments, organ and their corresponding interactions) for total organic matter (TOM) ^{13}C and ^{15}N isotopic composition and dark respired (DR) CO_2 $\delta^{13}C$. Percentage of the sum of squares is in brackets. The different

symbols indicate significant differences at 5% (*) and 1% (**), whereas n.s. refers to no significant difference.

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