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Charring effects on stable carbon and nitrogen isotope values on C_4 plants: Inferences for archaeological investigations

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ABSTRACT

Experimental studies demonstrated that charring affects stable isotope values of plant remains. Therefore, it is necessary to consider the impact of charring to reliably interpret $\delta^{13}C$ and $\delta^{15}N$ values in archaeobotanical remains before using this approach to reconstruct past water management, paleoclimatic changes, and infer paleodietary patterns. Research so far has focused mostly on C_3 plants while the charring effect on C_4 plants is less understood. This study explored the effects of charring on $\delta^{13}C$, $\delta^{15}N$, %C, %N, and C:N in grains of two C_4 species, *Sorghum bicolor* (L.) Moench (NADP-ME) and *Cenchrus americanus* (L.) Morrone (heterotypic synonym *Pennisetum glaucum* (L.) R.Br.) (NAD-ME), grown under the same controlled environmental conditions (watering, light, atmospheric humidity). Sorghum and pearl millet grains were charred from 1 to 3 h at 200–300 °C. Comparing first the uncharred grains, the results show that sorghum has lower $\delta^{15}N$ and higher $\delta^{13}C$ values than pearl millet. This evidence is also recorded in the charred grains. The charring experiments indicate that the temperature to which the grains are exposed has a higher impact than time on the preservation, mass loss, %C, %N, C:N, and $\delta^{13}C$ and $\delta^{15}N$ values. Every 50 °C of increase resulted in significant increases of $\delta^{15}N$ (+0.37‰) and of $\delta^{13}C$ (+0.06‰) values. Increasing the duration of charring to 3 h resulted in significant changes of $\delta^{15}N$ (+0.17‰) and no significant changes for $\delta^{13}C$ (−0.04‰) values. The average charring effects estimated in our experiment is 0.27‰ (95% CI between −0.02 and 0.56) for $\delta^{15}N$ and −0.18‰ (95% CI between −0.30 and −0.06‰) for $\delta^{13}C$. Considering the average values, our data show that pearl millet is more affected by charring than sorghum; however, according to the standard deviations, sorghum shows a greater variability charring effect than pearl millet. This study provides new information to correctly assessing the isotopic values obtained from ancient C_4 crops, providing a charring offset specific for C_4 plants. Furthermore, it suggests that NAD-ME and NADP-ME species present isotopic differences under the same growing conditions and this must be taken into account in analytical works on ancient C_4 crops.

1. Introduction

Charring has a significant impact on preservation, morphology and size estimations of macrobotanical remains (Araus et al., 2003; Ferrio et al., 2004; Hubbard and al Azm, 1990; Walsh, 2017; Yang et al., 2011b) as well as the molecular structure and the isotopic composition (Aguilera et al., 2008; Bogaard et al., 2007; Fiorentino et al., 2012; Fraser et al., 2013; Hartman et al., 2020; Kanstrup et al., 2012; Nitsch et al., 2015; Poole et al., 2002; Styring et al., 2013; Yang et al., 2011a, 2011b). For this reason, it is necessary to understand the effects of charring on isotopic composition for applications related to the reconstruction of past crop management practices (e.g. irrigation, manuring, land use), paleoenvironment and, indirectly, past dietary habits (Aguilera et al., 2008; Araus et al., 1999; Bogaard et al., 2013; Ferrio et al., 2005; Gron et al., 2021; Knipper et al., 2020; Larsson et al., 2019; Masi et al., 2014; Riehl et al., 2014; Varalli et al., 2021, 2023; Wallace et al., 2013). Several studies on the effects of charring have been carried out on pulses (Caracuta et al., 2015; Hartman et al., 2020; Nitsch et al., 2015; Poole et al., 2002; Stroud et al., 2023) and cereals (Charles et al., 2015; Fiorentino et al., 2012; Fraser et al., 2013; Nitsch et al., 2015; Styring et al., 2013; Stroud et al., 2023), and the results showed significant, albeit minimal, $\delta^{13}C$ and $\delta^{15}N$ offsets between uncharred

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and charred grains. These estimated offsets affect isotopic interpretations as they are often applied to correct the isotopic results from archaeobotanical grains used to infer past agricultural activities such as the introduction of manuring practices or the irrigation (e.g. Styring et al., 2017; Varalli et al., 2021). Therefore, their application need to be carefully assessed.

C_3 crops have been the focus of much archaeobotanical and isotopic work, especially in Europe and the Near East, because represent a key component of the human diet since the Neolithic (e.g. Gron et al., 2021; Stroud et al., 2021; Styring et al., 2018). Current studies, however, have been able to highlight also the importance of C_4 crops in past human diet (Filipović et al., 2020; Lightfoot et al., 2013; Varalli et al., 2016, 2021, Varalli, A. et al., 2022, Varalli et al., 2023). C_4 crops were present in protohistoric Europe, most likely used as staple foods during arid periods. They were also cultivated in the driest regions across Eurasia (Martin et al., 2021; Miller et al., 2016; Pokharia et al., 2017), often becoming the primary crops (D'Andrea et al., 2001). Indeed, recent work shows that C_4 plants played (and play) a crucial role in human diet in many drylands (Biagetti et al., 2022; Lancelotti and Biagetti, 2021) because their high resistance to drought and to grow well in poor soils (Rachie, 1975; Rao, 1989; Sage and Monson, 1999).

Despite the significant role of C_4 plants in historical and contemporary agricultural systems, their isotopic variability and the impact of charring on their caryopses remains relatively unexplored. However, stable isotope research is growing, especially in China, Central Asia, and Eastern Europe, due to the abundant findings in the archaeological record (Liu et al., 2022; Martin et al., 2021; Yang et al., 2022).

Given the limited availability of isotopic data from C_4 crops, the primary objective of this study was to address key physiological and methodological inquiries:

1) How charring affects C_4 crops isotopic values, especially under different charring conditions (temperature and duration)?
2) Do species belonging to different C_4 pathways (NAD-ME and NADP-ME) respond differently to charring?
3) Are charring effects in C_4 crops similar to those observed in C_3 crops?

In order to answer these questions, we selected two C_4 species: *Sorghum bicolor* (L.) Moench with NADP-ME pathway (NADP-dependent malic enzyme) and *Cenchrus americanus* (L.) Morrone (heterotypic synonym *Pennisetum glaucum* (L.) R.Br. – this binomial will be used in this paper) with NAD-ME pathway (NAD-dependent-malic-enzyme) (Cousins et al., 2008). The plants were cultivated in central India in lysimeters under outdoor semi-arid environmental conditions. Several physiological parameters (transpiration rate, biomass production, etc.) were regularly monitored (see D'Agostini et al., 2022; Vadez et al., 2011b, 2011a). The grains from these plants were charred at different temperatures and for different time-length.

1.1. C_4 photosynthesis: NADP-ME versus NAD-ME

C_4 plants have the ability to withstand high temperatures and scarce and erratic rainfall (Slack and Hatch, 1967). This is achieved through a structural arrangement called Kranz anatomy, where CO_2 fixation occurs in the mesophyll and its decarboxylation around RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) in the bundle sheath cells. This anatomical configuration compensates for CO_2 reduction caused by decreased stomatal opening, that the plants set in place to prevents water loss during drought (Bräutigam et al., 2014). Concentrating CO_2 in the bundle sheath cells allows RuBisCO to operate close to its saturation point and suppressing photorespiration by >80% (Sage and Zhu, 2011). However, the carbon fixation efficiency of C_4 plants is reduced by a leakage (Φ) of CO_2 and HCO_3^- from the bundle sheath into the mesophyll cells, impacting carbon discrimination and fractionation (Farquhar 1983).

Different evolutionary lineages developed distinct C_4 anatomical features for decarboxylating CO_2 (Pyankov et al., 2010). The two more common subtypes are NADP-ME and NAD-ME (Sage and Monson, 1999). In the NADP-ME subtype malate is decarboxylated to pyruvate by NADP-dependent malic enzyme in the chloroplasts, and a layer of suberin is deposited around the bundle sheath cell wall (Rao and Dixon, 2016). The NAD-ME subtype decarboxylate malate to pyruvate in mitochondria using a NAD-dependent malic enzyme (Rao and Dixon, 2016). In addition, NAD-ME present an outer bundle sheath (lacking of suberin lamella) and an inner non-photosynthetic mestome sheath covered by suberin (Lundgren et al., 2014; Fouracre et al., 2014; Lundgren et al., 2014; Mertz and Brutnell, 2014). Because of this anatomical arrangement, leakage (Φ) is probably lower in NADP-ME than in NAD-ME (Ghannoum et al., 2002). The quantification of this phenomenon, as well as its associated carbon fractionation, is currently not well understood. However, Schulze et al. (1996) estimated that NADP-ME plants have higher $\delta^{13}C$ values (−11.7‰) than NAD-ME plants (−13.4‰).

Thus, the two plants chosen for this study, sorghum (NADP-ME) and pearl millet (NAD-ME), exhibit contrasting features in terms of their Kranz anatomy, metabolite flow through bundle sheath cells, and CO_2 leakage from the bundle sheath (Dengler et al., 1994; Dengler and Nelson, 1999; Ghannoum et al., 2002; Rao and Dixon, 2016). These differences suggest that they may also display divergent carbon discrimination and fractionation abilities. Moreover, the biochemical variances between the two pathways impact water requirements, photosynthetic efficiency, and leaf structure (Sage and Monson, 1999), consequently leading to distinct environmental adaptations (Ghannoum et al., 2002). This distinction is evident in the geographical distribution of the plants and the prevalence of NADP-ME in regions with higher average annual rainfall (Hattersley, 1983; Teeri and Stowe, 1976) .

1.2. The charring process and previous research on C_4 crops

Charring allows organic plant materials to survive in archaeological contexts. During this process, the chemical composition of the organic matter is modified, preventing further degradation and microbial attack (Maillard, 1912; Moreira, 2012). The organic matter becomes progressively enriched in carbon (C) and depleted in hydrogen (H) and oxygen (O), due to the formation of condensed aromatic structures (Bird and Ascough, 2012). Therefore, the carbon and nitrogen isotope ratios of seeds, fruits and wood are affected by charring: the losses or changes in a specific biochemical component over another during charring may significantly alter the final isotopic value of the material. For grains, the magnitude of the isotopic difference between charred and uncharred grains depends on the stable isotope compositions of the original biochemical components (e.g. lipids, carbohydrates and proteins) and on how the length and level of heating affects such components (Ascough et al., 2008, 2010; Czimczik et al., 2002). For example, lipids and other volatiles such as water are lost at moderately low temperatures (<150 °C) (Czimczik et al., 2002). Since lipids have relatively negative $\delta^{13}C$ values compared to the rest of the grain components, their loss will lead to an increase of $\delta^{13}C$ ratios in the charred material (Benner et al., 1987; Czimczik et al., 2002). At higher temperatures (220°C–300 °C), cellulose and starches also break down and start to volatilize (>300 °C). Cellulose is isotopically heavier relative to the bulk, thus a loss of cellulose and starch will lead to a decrease of the $\delta^{13}C$ values of the charred material (Czimczik et al., 2002). Therefore, several factors, including the initial isotopic composition and the influence of the heating treatment, require further investigation to understand their impact on the biochemical composition and physical structure of charred cereal grains.

The caryopses of our two species, sorghum and pearl millet, are mainly composed by endosperm (Abdelrahman et al., 1984; Serna-Saldivar and Rooney, 1995), which consists primarily of starch (51%–78% of the grain weight) and proteins. The charring process is responsible for Maillard reactions, which result in the conversion of starch and

Table 1
Summary of growing conditions for pearl millet and sorghum grains used for experimental charring.

Taxon	Genotype *(ICRISAT genebank abbreviations)*	Experiment
Pear millet (*Pennisetum glaucum*)	IP9859	Plants have been irrigated weekly to maintain 80% of the soil field capacity (Well-watered plants)
Sorghum (*Sorghum bicolor*)	IS35215	

Table 2
Summary of the sampling and charring experiments for pearl millet and sorghum grains.

Species	N of replicates for each experiment	Time (h)	Temperature (°C)	TOT number of samples
Pear millet (*Pennisetum glaucum*)	3	0	0	3
	9	1, 2, 3	200, 250, 300	81
Sorghum (*Sorghum bicolor*)	3	0	0	3
	9	1, 2, 3	200, 250, 300	81

amino acids into high-molecular-weight polymeric compounds called melanoidins. These compounds possess a complex structure that exhibits resistance to microbial activity (Braadbaart et al., 2004a, 2004b; Styring et al., 2013). Although the amino acids and monosaccharides reactions early in the process are well characterised, the final formation of the melanoidins remains relatively poorly understood (Machiels and Istasse, 2002). However, it is clear that the reactions forming melanoidins release volatile heterocyclic compounds of low molecular weight (e.g. pyrazines and furans) and therefore it is likely that the isotopic composition of the carbonized organic matter may be affected (Styring et al., 2013). Maillard reactions have the potential to continue even at room temperature after charring, suggesting that plant remains may undergo additional changes (Charles et al., 2015; Styring et al., 2013). However, despite these post-charring reactions, the isotopic compositions of the plant remains is preserved following burial (Fraser et al., 2013; Styring et al., 2013).

Research conducted so far on C_4 plants produced a diversity of findings. Yang et al. (2011a, 2011b) charred foxtail (*Setaria italica* (L.) P. Beauv.) and broomcorn (*Panicum miliaceum* L.) millet grains at 50–300 °C for 2–3 h and observing positive and negative changes of 0.2‰ for $\delta^{13}C$ values between the charred and uncharred samples. Similarly, Fraser et al. (2013) charred broomcorn millet grains at 230 °C for 0–24 h, which resulted on the average $\delta^{13}C$ and $\delta^{15}N$ offsets between charred and uncharred grains of 0.0‰ for $\delta^{13}C$ values and 1.0‰ for $\delta^{15}N$ values. Styring et al. (2019) analyzed $\delta^{15}N$ values of pearl millet (*Pennisetum glaucum*) charring the seeds at 215–260 °C for 4–24 h. They calculated a 'worst-case' $\delta^{15}N$ value offset based on a predicted increase in $\delta^{15}N$ value where the grains were still identifiable to species and, according to this, they estimated an offset of 0.34‰ between the values of charred and uncharred grains. Dong et al. (2022) charred broomcorn and foxtail millets grains at 215–260 °C for 4–24 h. The average $\delta^{13}C$ and $\delta^{15}N$ offsets between charred and uncharred grains were 0.24‰ for $\delta^{13}C$ values and 1.0‰ for $\delta^{15}N$ values for foxtail millet and 0.16‰ for $\delta^{13}C$ values and 1.3‰ for $\delta^{15}N$ values for broomcorn millet. In the case of this experiment, the grains were obtained from a supermarket in China. Consequently, the specific growing conditions under which these grains were produced remain unknown. As a result, it becomes difficult to assess how environmental and/or human factors might have influenced the initial isotopic values of the grains.

Considering the limited literature concerning the effects of charring on C_4 grains and the observed variations in behavior across different analyzed species, there is a clear need to enhance our understanding and estimation of the fractionation of both $\delta^{13}C$ and $\delta^{15}N$ values that occur during the charring process. In this respect, the current investigation brings new data on the effect of charring on grains from different C_4 plant subtypes that were cultivated under strictly controlled environmental conditions mimicking traditional, pre-industrial agricultural settings. The values of charring-induced fractionation presented here allow the estimation of the corresponding original, non-charred values of archaeobotanical grains.

2. Material and methods

2.1. The experimental cultivation

Traditional landraces (genotypes) of sorghum (*Sorghum bicolor* L. Moench) and pearl millet (*Pennisetum glaucum* L. R. Br), selected from the ICRISAT gene bank, were cultivated in experimental fields at ICRISAT (Hyderabad, India; 17°31′N 78°16′E) between February and May 2019 (Table 1). The plants were grown in lysimeters filled with a soil mixture of 1:1 Alfisol-Vertisol without the addition of manure or chemical fertilizers. The plants were cultivated under controlled watering conditions, ensuring that their water needs were met based on their transpiration rate. This approach provides precise information on the actual amount of water that passed through the plant and allows to directly quantify the level of hydric stress. Transpiration rate and a set of physiological parameters were measured during the entire life cycle, to obtain an accurate measurement of the plants water consumption during growth (for a full description of the experimental settings, see D'Agostini et al., 2022). Following harvest at physiological maturity, all panicles were dried for 1 week at 60–70 °C and then grains and chaff were mechanically separated from the panicles.

2.2. Sampling and charring experimental settings

For each species, we selected grains from one plant to avoid any inter-plant variability. Based on previous experiments (e.g. Yang et al., 2011a, 2011b) and the total amount of grains available for the selected plants, we identified a set of charring conditions to produce undistorted and identifiable charred samples. Nine different charring scenarios at 200, 250, 300 °C for 1, 2, 3 h, were set up under controlled laboratory conditions at the Laboratory of Environmental Archaeology (Pompeu Fabra University, CASEs Research Group). For each charring scenario and species, we considered 9 replicates with 3–10 grains each in order to reach the minimum weight required for analyses. The difference in the number of grains depended on the weight of each species grains (Table 2). This choice was due to the need to maximize the number of replicates for each charring experiment. The weight of each sample was noted before and after charring to evaluate mass loss. The grains were wrapped in aluminium foil and covered with clean dried coarse sand (1–2 mm, SiO_2>98.5%) to avoid oxygen exchange. Samples were charred in a digitally controlled chamber furnace (Nabertherm C450 of 5 l capacity) allowing for the sand to reach the experimental temperature and then keeping the samples for each established experimental time length (e.g. 1, 2, 3 h). The temperature was monitored using a thermocouple buried in the sand. Samples were extracted from the furnace after the required time-period and allowed to cool. Morphological changes and preservation after the charring process were assessed using the Hubbard and al Azm (1990) scale. Afterwards, the grains from each sample were ground by hand in an agate mortar and homogenized.

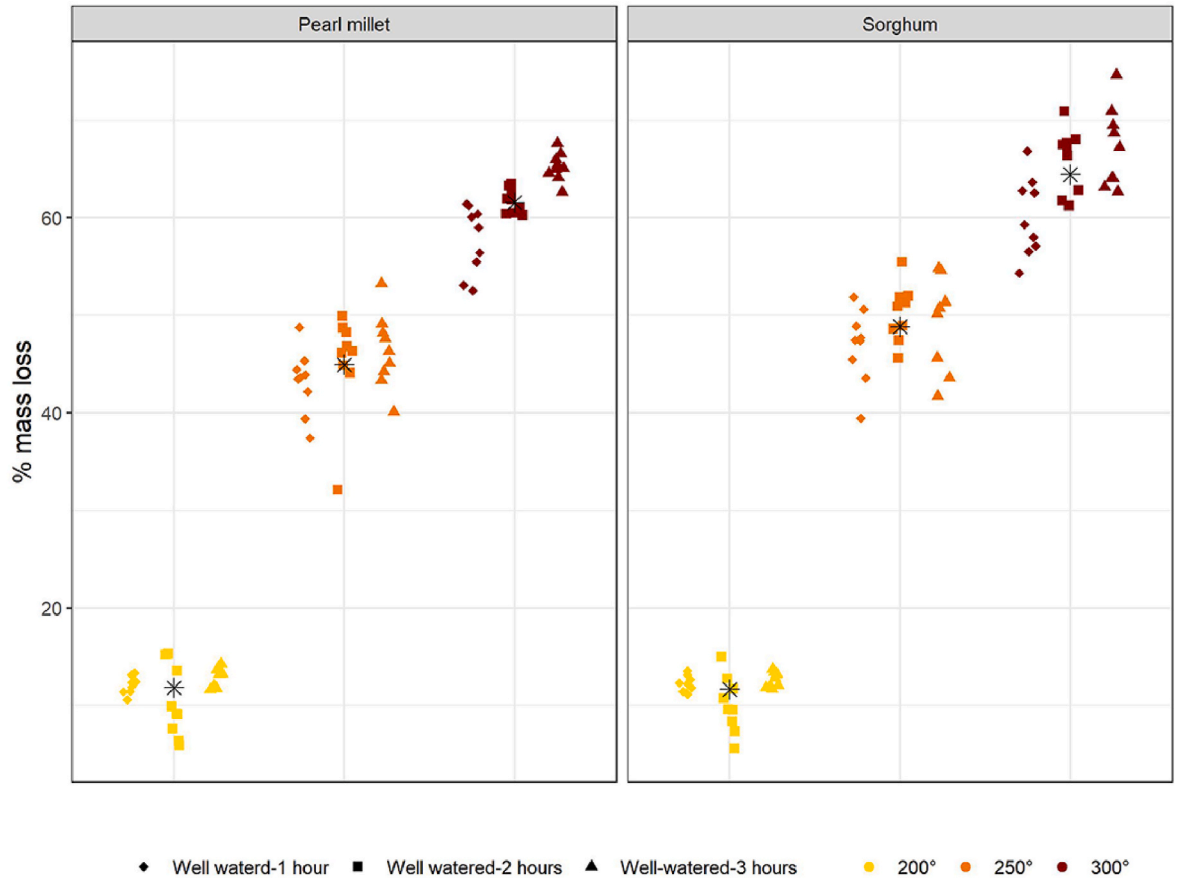

Fig. 1. Scatter plot of mass loss (%) for pearl millet and sorghum samples charred from 1 to 3 h at 200, 250 and 300 °C. Stars are the mean values calculated for all the samples charred at 200, 250 and 300 °C.

2.3. Carbon and nitrogen isotope analysis

Carbon and nitrogen isotope analysis was undertaken using a Europa Scientific ANCA-SL elemental analyser (EA), with zero-blank autosampler, coupled to a Europa Scientific 20-20 isotope ratio mass spectrometer (IRMS) at Iso-Analytical (Sercon Ltd., Crewe, UK). The powdered and homogenized grains were weighted (1.5 ± 0.1 mg) in tin capsules (8 × 5 mm), sealed, and loaded together with standards into the Europa Scientific elemental analyser. A subset representing 20% of the samples was analyzed in duplicate. Carbon and nitrogen isotope values are calibrated to V-PDB and AIR using IA-R001 standard (wheat flour, $\delta^{13}C_{V\text{-}PDB} = -26.43‰ \pm 0.08$, $\delta^{15}N_{AIR} = 2.55‰ \pm 0.22$). Carbon and nitrogen measurements uncertainty was monitored using two check samples: IA-R045/IA-R005 (mixture of ammonium sulphate and beet sugar, $\delta^{13}C_{V\text{-}PDB} = -26.03‰ \pm 0.11$, $\delta^{15}N_{AIR} = -4.71‰ \pm 0.07$) and IA-R046/IA-R006 (mixture of ammonium sulphate and cane sugar, $\delta^{13}C_{V\text{-}PDB} = -11.64‰ \pm 0.03$, $\delta^{15}N_{AIR} = 22.04‰ \pm 0.06$). IA-R001 is calibrated against and traceable to IAEA-CH-6 (sucrose, $\delta^{13}C_{V\text{-}PDB} = -10.449‰$) and IAEA-N-1 (ammonium sulphate, $\delta^{15}N_{AIR} = 0.40‰$). IA-R005 and IA-R006 are calibrated against and traceable to IAEA-CH-6. IA-R045 and IA-R046 are calibrated against and traceable to IAEA-N-1. IAEA-CH-6 and IAEA-N-1 are inter-laboratory comparison standards distributed by the International Atomic Energy Agency, Vienna.

The isotope ratios are reported as "delta" defined according to IUPAC (International Union of Pure and Applied Chemistry):

$$\delta = (Rs/Rst) - 1 = 10^3[(Rs/Rst) - 1]\ ‰$$

where Rs and Rst are the isotope ratios $^{15}N/^{14}N$ and $^{13}C/^{12}C$ of the isotopic abundances of ^{15}N, ^{14}N and ^{13}C, ^{12}C and $‰ = 10^{-3}$. Using the calculation provided by Szpak et al. (2017) based on repeated measurements of calibration standards, check standards and sample replicates, the precision ($u(R_W)$) of the measurements are estimated to be ± 0.042‰ for $\delta^{13}C$ and for ± 0.074‰ for $\delta^{15}N$ values. Accuracy or systematic errors ($U(bias)$) are determined to be ± 0.083‰ for $\delta^{13}C$ and ± 0.21‰ for $\delta^{15}N$ values, on the basis of the difference between the observed and known δ values of the check standards and the long–term standard deviations of these check standards. The total analytical uncertainties ($(u(c)$) are estimated to be ± 0.093‰ for $\delta^{13}C$, ± 0.22‰ for $\delta^{15}N$ values. All details and measured standards are provided in S1.

3. Results

3.1. The effects of heating on morphology, grain mass, %C and %N content, C/N

The results from pearl millet and sorghum charred samples are listed in Table 3 and S2 and the charred samples are summarized, together with the statistic for both species according to water treatment, temperature and time of charring, in Table 4.

Pearl millet and sorghum grains charred at 200 °C for up to 3 h result morphologically intact, showing almost no changes in shape or in other physical characteristics, except for colour. The colour changes from brown (1 h), to dark brown (2 h), to nearly black (3 h). Since no physical distortion is observed, the samples result in a good preservation (score 1). At 250 and 300 °C all samples show major morphological changes and the colour is always black, with sorghum presenting greater distortion than pearl millet. At a temperature of 250 °C, the seeds of both species exhibit swelling and after 1 h of heating the epidermis remains mostly intact. However, for longer heating durations, especially those lasting 3 h, the epidermis occasionally appears incomplete or

Table 3
Values of uncharred pearl millet and sorghum grains (mean and SD).

Taxon	ID	N seeds	%N	δ^{15}N (‰AIR)	%C	δ^{13}C (‰V-PDB)	C/N
Sorghum bicolor	SWW 1	9	1.91	−2.41	40.40	−12.08	24.5
	SWW 2	9	2.24	−2.13	42.28	−12.15	21.9
	SWW 3	9	2.15	−2.24	40.12	−12.16	21.7
	Mean (sd) SWW		2.10 (0.17)	−2.26 (0.14)	40.93 (1.18)	−12.13 (0.04)	22.72 (1.58)
Pennisetum glaucum	PMWW 1	9	2.43	−0.73	41.34	−12.56	19.8
	PMWW 2	9	2.72	−1.10	41.50	−12.42	17.7
	PMWW 3	9	2.46	−1.07	42.04	−12.65	19.8
	Mean (sd) PMWW		2.53 (0.16)	−0.97 (0.21)	41.63 (0.37)	−12.55 (0.12)	19.11 (1.20)

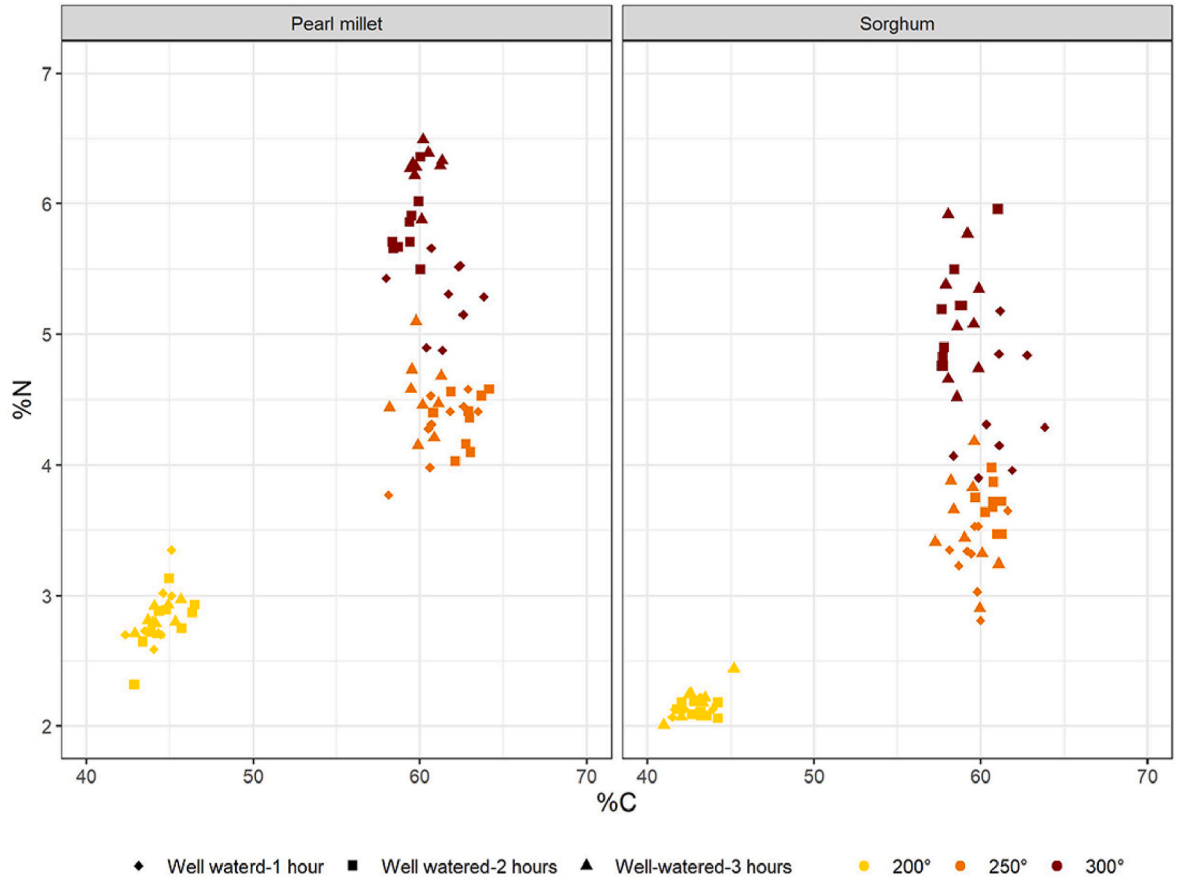

Fig. 2. Scatter plot of nitrogen and carbon content for pearl millet and sorghum samples charred from 1 to 3 h at 200, 250 and 300 °C.

fragmented (score 3 or 4 on the evaluation scale). At 300 °C most of the grains presents significant distortion and rarely preserving physical characteristics for their identification. This was also observed in other C_4 species charring experiments (Yang et al., 2011a, 2011b), albeit a recent study demonstrated that domesticated varieties of sorghum can in some cases tolerate temperatures up to 350 °C, with variance according to seed size (Beldados and Ruiz-Giralt in review).

Significant mass loss is seen in all experimental settings. Considering both species, mass loss at 200 °C ranges between 5.57% and 15.27% (mean and SD = 11.70% ± 2.24). At 250 °C mass loss ranges between 32.13% and 55.50% (mean and SD = 46.89% ± 4.52) and at 300 °C is between 52.56% and 74.66% (mean and SD = 63.01% ± 4.55) (Fig. 1 and Table 4).

In respect to content, uncharred grains show lower %N and %C than the charred grains for both sorghum and pearl millet. This is particularly evident for %N, while only marginally detectable for %C. For charred grains, temperature has a more noticeable impact than time in both the %N and %C of species, supporting the results from previous charring experiments (Fraser et al., 2013; Nitsch et al., 2015). At 200 °C there are almost no differences between charred and uncharred grains for nitrogen content (Tables 3 and 4) but major differences occur at 250 °C and 300 °C. The %N gradually increases with higher temperatures and longer duration in both species (Fig. 2 and Table 4). Similarly, the difference in %C is minimal between the charred and uncharred grains at 200 °C (Tables 3 and 4) but considerably increases at 250 °C and at 300 °C, showing similar values at these temperatures (Fig. 2 and Table 4).

The C/N molar ratio of the charred grains at 200 °C is similar to the C/N molar ratio range observed for the uncharred grains in both sorghum and pearl millet (Tables 3 and 4). However, the C/N molar ratio of the charred grains decreases with the raising of charring time and temperature (Fig. 3), showing an opposite trend to the %N and %C described above. This means that the increase in %N is proportionally higher than the increase in %C, as it has been observed in other studies (Fraser et al., 2013; Nitsch et al., 2015; Styring et al., 2013).

Table 4
Summary statistics of pearl millet and sorghum according to water treatment, temperature and times of charring.

Taxon	Time (hours)	Temperature (°C)	N sample	Values	% mass loss	%N	δ^{15}N (‰ AIR)	%C	δ^{13}C (‰V-PDB)	C/N	Δ^{15}N(‰) offset	Δ^{13}C(‰) offset
Sorghum bicolor	1	200	9	average	12.16	2.09	−2.65	42.36	−12.31	23.58	−0.39	−0.18
				sd	*0.84*	*0.11*	*0.13*	*1.20*	*0.11*	*0.96*	0.13	0.11
		250	9	average	46.94	3.31	−2.36	59.61	−12.20	21.01	−0.10	−0.07
				sd	*3.75*	*0.26*	*0.22*	*0.96*	*0.10*	*1.74*	0.22	0.10
		300	9	average	60.13	4.40	−2.12	61.16	−12.26	16.27	0.14	−0.13
				sd	*4.05*	*0.45*	*0.31*	*1.60*	*0.12*	*1.52*	0.31	0.12
	2	200	9	average	10.05	2.12	−2.51	43.06	−12.47	23.55	−0.25	−0.34
				sd	*2.87*	*0.05*	*0.13*	*0.86*	*0.12*	*0.83*	0.13	0.12
		250	9	average	50.26	3.70	−2.19	60.70	−12.29	19.05	0.07	−0.16
				sd	*2.93*	*0.17*	*0.13*	*0.50*	*0.16*	*0.93*	0.13	0.16
		300	9	average	65.96	5.15	−1.75	58.43	−12.13	13.22	0.51	0.00
				sd	*3.26*	*0.40*	*0.22*	*1.08*	*0.12*	*0.78*	0.22	0.12
	3	200	9	average	12.63	2.20	−2.65	42.68	−12.47	22.55	−0.39	−0.34
				sd	*0.79*	*0.12*	*0.21*	*1.21*	*0.11*	*0.77*	0.21	0.11
		250	9	average	49.33	3.54	−2.30	59.25	−12.17	19.64	−0.04	−0.04
				sd	*4.66*	*0.39*	*0.35*	*1.13*	*0.15*	*2.34*	0.35	0.15
		300	9	average	67.23	5.16	−1.68	58.78	−12.23	13.31	0.58	−0.10
				sd	*4.08*	*0.49*	*0.18*	*0.81*	*0.11*	*1.25*	0.18	0.11
Pennisetum glaucum	1	200	9	average	12.18	2.85	−0.76	44.13	−12.65	18.04	0.21	−0.11
				sd	*0.95*	*0.23*	*0.12*	*0.84*	*0.16*	*1.19*	0.12	0.16
		250	9	average	43.17	4.30	−0.40	61.26	−12.74	16.56	0.57	−0.20
				sd	*3.30*	*0.27*	*0.12*	*1.63*	*0.06*	*0.77*	0.12	0.06
		300	9	average	57.75	5.30	−0.23	61.48	−12.77	13.50	0.74	−0.22
				sd	*3.45*	*0.27*	*0.23*	*1.68*	*0.16*	*0.80*	0.23	0.16
	2	200	9	average	10.22	2.80	−0.90	44.73	−12.78	18.65	0.07	−0.24
				sd	*3.61*	*0.23*	*0.11*	*1.29*	*0.14*	*1.30*	0.11	0.14
		250	9	average	45.26	4.35	−0.51	62.70	−12.81	16.76	0.46	−0.27
				sd	*5.26*	*0.20*	*0.19*	*0.99*	*0.05*	*0.79*	0.19	0.05
		300	9	average	61.75	5.82	−0.01	59.32	−12.80	11.84	0.96	−0.26
				sd	*1.26*	*0.25*	*0.24*	*0.67*	*0.22*	*0.46*	0.24	0.22
	3	200	9	average	12.99	2.82	−0.79	44.33	−12.87	18.22	−0.18	0.32
				sd	*0.97*	*0.10*	*0.11*	*0.85*	*0.08*	*0.49*	0.11	0.08
		250	9	average	46.37	4.53	−0.39	60.02	−12.69	15.41	0.58	−0.15
				sd	*3.79*	*0.29*	*0.10*	*1.00*	*0.09*	*1.02*	0.10	0.09
		300	9	average	65.23	6.27	−0.01	60.18	−12.65	11.14	0.96	−0.10
				sd	*1.43*	*0.17*	*0.11*	*0.66*	*0.06*	*0.31*	0.11	0.06

3.2. Carbon isotopes results

The uncharred δ^{13}C values for both species range between −12.65‰ and −12.08‰, with an average of −12.54‰ ± 0.31. The sorghum uncharred grains average is −12.13‰ ± 0.04, while the pearl millet uncharred grains average is −12.55‰ ± 0.12. The overall charred δ^{13}C values range between −13.36‰ and −11.96‰, with an average of −12.52‰ ± 0.28. The sorghum charred grains average is −12.28‰ ± 0.16, while the pearl millet charred grains average is −12.75‰ ± 0.14 (Fig. 4).

Considering the overall δ^{13}C values of sorghum and pearl millet charred grains, a multiple linear regression with coefficients for temperature, time and taxon shows no significant differences when increasing the charring duration (Beta = −0.012, SE = 0.014, t = −0.889, p = 0.375) and every 3 h the δ^{13}C values show a decrease of −0.04‰. On the contrary, significant differences are observed with increasing temperature (Beta = 0.0012, SE = 0.00028, t = 4.3, p < 0.000), with δ^{13}C increasing 0.06‰ every 50 °C. Significant difference exists also for taxon, with sorghum that increases 0.47‰ (Beta = 0.47, SE = 0.023, t = 20.654, p < 0.01).

When sorghum values are tested alone, a multiple linear regression with coefficients for temperature and time shows that this species displays no significant differences in respect to time (Beta = −0.018, SE = 0.019, t = −0.950, p = 0.345), with δ^{13}C values decrease every 3 h equivalent to −0.06‰. Significant differences are recorded with increasing temperature (Beta = 0.002, SE = 0.0003, t = 5.5, p < 0.001), namely an increase of 0.11‰ every 50 °C (Figs. 5 and 6).

When pearl millet is tested, a multiple linear regression with coefficients for temperature and time shows that this species displays no significant differences in respect to time (Beta = −0.006, SE = 0.019, t = −0.347, p = 0.73), with δ^{13}C values decrease every 3 h equivalent to −0.02‰. Similarly, there are no significant differences observed with increasing temperature (beta = 0.0002, SE = 0.0003, t = 0.74, p = 0.46). This indicates an increase of 0.01‰ per 50 °C, as shown in Fig. 5.

3.3. Nitrogen isotopes results

The overall uncharred δ^{15}N values range between −2.41‰ and −0.73‰, with an average of −1.23‰ ± 0.69. The average for sorghum uncharred grains is −2.26‰ ± 0.14, while for pearl millet the average is −0.97‰ ± 0.21. The overall charred δ^{15}N values range between −2.92‰ and 0.45‰, with an average of −1.34‰ ± 0.98. The average for sorghum charred grains is −2.24‰ ± 0.40, while for pearl millet is −0.44‰ ± 0.34 (Fig. 4).

Considering the overall δ^{15}N values of charred sorghum and pearl millet grains, a multiple linear regression with coefficients for temperature, time and taxon shows significant changes related to the length of charring (Beta = 0.057, SE = 0.019, t = 2.89, p < 0.005) and every 3 h the δ^{15}N value increases of 0.17‰. Significant differences are also observed with increasing temperature (Beta = 0.0074, SE = 0.00039, t = 18.7, p < 0.001), with an increase of 0.37‰ every 50 °C. For taxon as well significant difference are observed (Beta = −1.8, SE = 0.032, t = −55.42, p < 001), with sorghum showing a decrease of −1.8‰.

When sorghum values are tested alone, a multiple linear regression with coefficients for temperature and time shows significant differences related to the length of charring (Beta = 0.08, SE = 0.03, t = 2.47, p < 0.01), corresponding to an increase of 0.24‰ every 3 h. Significant differences are also recorded with rising temperature (Beta = 0.0075, SE = 0.0006, t = 11.49, p < 0.000), corresponding to an increase of 0.38‰ every 50 °C (Figs. 5 and 7).

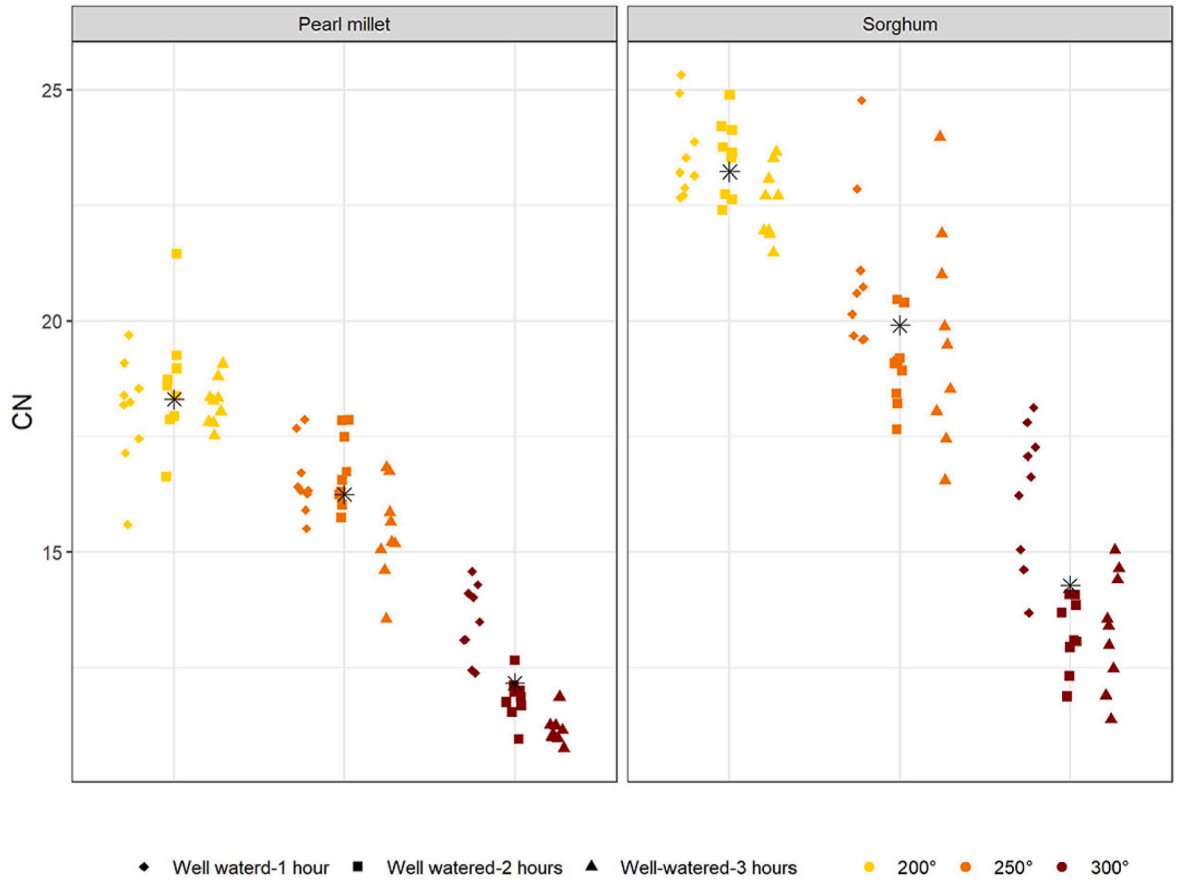

Fig. 3. Scatter plot of C/N ratio of pearl millet and sorghum samples charred from 1 to 3 h at 200, 250 and 300 °C. Stars are the mean values calculated for all the samples charred at 200, 250 and 300 °C.

Considering pearl millet, a multiple linear regression with coefficients for temperature and time shows no significant differences due to increasing time (Beta = 0.33, SE = 0.0226, t = 1.496, p = 0.139). Similar to sorghum, significant differences are recorded due to increasing temperature (Beta = 0.0073, SE = 0.0004, t = 16.20, p < 0.000), which corresponds to an increase of 0.37‰ every 50 °C (Figs. 5 and 7).

3.4. Pear millet vs. sorghum

The results from the uncharred material show that sorghum grains have higher $\delta^{13}C$ values than pearl millet and the difference between the two means is 0.42‰. Pearl millet has greater $\delta^{13}C$ intra-specific variability than sorghum, as suggested by the standard deviations (Table 3). The trends observed in the uncharred samples are consistent with those observed in the charred samples. However, sorghum grains subjected to different charring temperatures consistently exhibit higher values compared to pearl millet grains at the same temperatures (Fig. 4). The $\Delta^{13}C$ offsets has been calculated using the method published by Nitsch et al. (2015) and Stroud et al. (2023) comparing the isotopic values of all the charred samples to uncharred samples. The average value is −0.15‰ ± 0.16 (min = −0.54‰ max = 0.17‰) for sorghum and −0.21‰ ± 0.14 (min = −0.81‰ max = 0.16‰) for pearl millet. Significant statistical differences occur between the calculated offsets of the two species (Wilcoxon Mann-Whitney test, W = 2514.5, p = 0.01032). These results show that charring affects carbon values more in pearl millet than in sorghum. A multiple linear regression models was calculated for $\delta^{13}C$, comparing the effect of charring (all charred samples vs. all uncharred samples) with different coefficients for each taxon. The model produced a good fit, with an adjusted R^2 of 0.71 indicating that the effect of charring is significant (Beta = −0.17823‰, SE = 0.06278‰, t = −2.839, p < 0.01). The 95% CI for the estimated effect of charring was between −0.30 and −0.06‰, with an average of −0.18‰.

The results obtained from the uncharred material reveal an interesting contrast in the trends of $\delta^{15}N$ values compared to the $\delta^{13}C$ values. Specifically, pearl millet exhibits higher $\delta^{15}N$ values than sorghum, and the mean difference in $\delta^{15}N$ values between the two species is −1.29‰, indicating a significant disparity in the $\delta^{15}N$ values based on the species (Table 3). Moreover, pearl millet has greater $\delta^{15}N$ intra-species variability than sorghum, as indicated by the standard deviations. The trends observed for the uncharred material are also observed in the charred samples. Pearl millet grains charred at different temperatures have higher values than sorghum grains (Fig. 4). The $\Delta^{15}N$ offsets between the $\delta^{15}N$ values of the uncharred and charred grains, similarly calculated to the $\Delta^{13}C$ offset, have an average value of 0.02‰ ± 0.40 (min = −0.66‰, max = 0.9‰) for sorghum and 0.52‰ ± 0.34 (min = −0.08‰, max = 1.41‰) for pearl millet (Table 5), and significant statistical differences exist between the two species (Wilcoxon Mann-Whitney test, W = 5450, p < 0.000). In summary, the results show that charring affects pearl millet more than sorghum; however, as highlighted by the standard deviations, sorghum has slightly greater charring effect variability than pearl millet. A multiple linear regression models was calculated for $\delta^{15}N$, comparing the effect of charring (all charred samples vs. all uncharred samples) with different coefficients for each taxon. The model produced a good fit, with an adjusted R^2 of 0.85 indicating that the effect of charring is only moderately significant (Beta = 0.269‰, SE = 0.153‰, t = 1.675, p = 0.081). The 95% CI for the estimated effect of charring is between −0.02 and 0.56‰, with an average of 0.27‰.

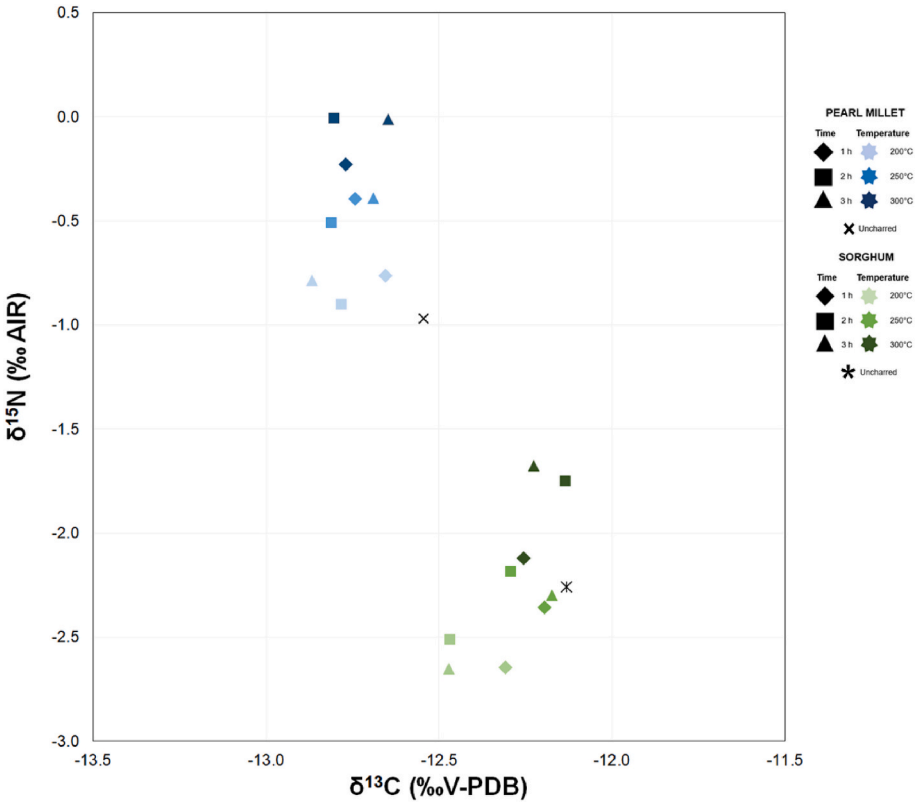

Fig. 4. Mean $\delta^{13}C$ and $\delta^{15}N$ values of the uncharred and charred series according to the time and temperature of the charring process.

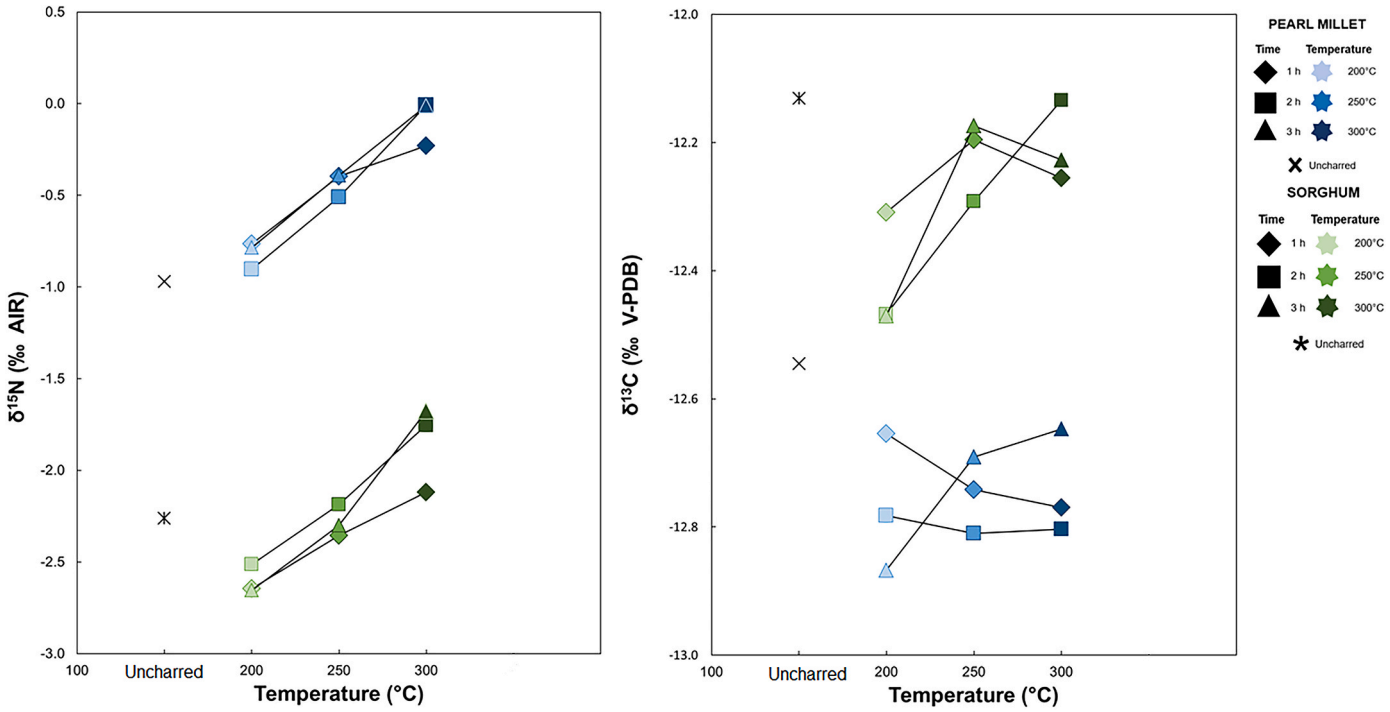

Fig. 5. Changes in the $\delta^{15}N$and $\delta^{13}C$ mean values of sorghum and pearl millet grain samples when heated at different temperatures and times.

3.5. *Variability and comparability*

To minimize inter-plant variability, we specifically selected and analyzed grains from a single sorghum and a single pearl millet plant. This approach ensured a more controlled comparison between the two species. For each charring experiment, a sufficient quantity of material was ensured by homogenizing six grains of pearl millet and three grains of sorghum per measurement. This approach guaranteed an appropriate sample size for isotopic analysis in each experiment. Similarly, to Nitsch et al. (2015) and Stroud et al. (2023), we estimated the average

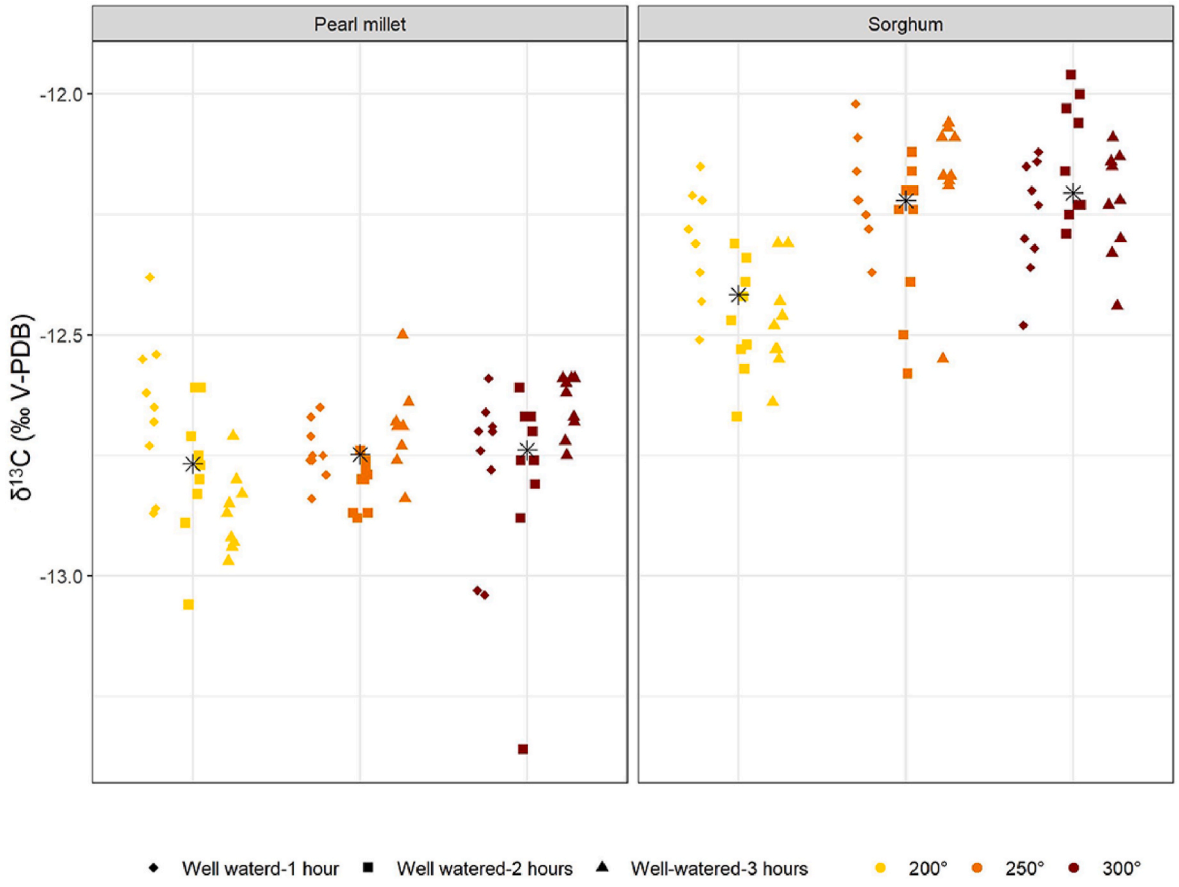

Fig. 6. Scatter plot of $\delta^{13}C$ of pearl millet and sorghum samples charred from 1 to 3 h at 200, 250 and 300 °C. Stars are the mean values calculated for all the samples charred at 200, 250 and 300 °C.

variability for $\delta^{13}C$ and $\delta^{15}N$ by examining the residual SE of a multiple regression model that accounted for the effect of temperature and time. Albeit the homogenization of a different number of grains, the taxon-specific residual SEs are similar, both for $\delta^{13}C$ and $\delta^{15}N$ values. In fact, for $\delta^{13}C$ the SE is 0.1412 (95% CI = ±0.276752) for pearl millet and 0.1404 (95% CI = ±0.275184) for sorghum. While for $\delta^{15}N$ the SE is 0.1664 (95% CI = ±0.326144) for pearl millet and 0.241 (95% CI = ±0.47236) for sorghum (Table 6). Our results are in accordance with the residual SE estimated by Nitsch et al. (2015) and Stroud et al. (2023) that analyzed different C_3 taxa (cereals and legumes) homogenizing a higher number of grains (n = 10). Nitsch et al. (2015) calculated that 0.25‰ for $\delta^{13}C$ and 0.75‰ for $\delta^{15}N$ could provide a rough estimate of the expected population standard error of a given growing condition. Thus, extrapolating from that 1.96xSE, they estimated that a 95% CI of ± ~0.5‰ for $\delta^{13}C$ and ± ~1‰ for $\delta^{15}N$ would account for the variability within a single growing condition. In our study, both species exhibit standard errors below these values, indicating that the limited number of sampled seeds from the same plants does not significantly impact the variability of the isotopic results.

4. Discussion

Sorghum and pearl millets are two millet crops with different anatomical characteristics (which make them easily identifiable in the archaeobotanical record; e.g. Jacomet 2006) but also with different physiological characteristics that have not been sufficiently investigated in respect to their significance for past agricultural dynamics. Effectively, it is expected that these two species, due to such different physiologies (different photosynthetic pathways), should respond differently to similar growing environmental conditions. Although isotopic differences between the NAD-ME and NADP-ME have been observed in previous studies, isotopic variability between sorghum and pearl millet has never been explored before. In our study, the direct comparison between these two species enables us to evaluate potential isotopic variations in the uncharred grains of each species. This comparison helps us understand the magnitude and significance of these differences and, when carbonized, how they are manifested in the charred samples. Furthermore, the watering schedule of the field growing environment of our experiment was set according to the transpiration rate of the plants to avoid any effect related to variations in water availability. Such experimental design provides precise information about the amount of water that actually passed through the plant, assuring ideal hydric conditions based on the plant transpiration rather than on a standard quantity of water that is arbitrarily provided. We consider this experimental control an essential pre-requisite for analyzing isotopic values in plant species because such control allows for the plants to be grown under the same environmental conditions of humidity, soil, temperature, etc., but more importantly, also under no hydric stress according to physiological parameters. Using physiological parameters to control the hydric conditions of the plants assure comparability beyond the single experiment and minimize physiologically driven isotopic variability.

Our results show that the $\delta^{13}C$ values of the uncharred grains are lower in pearl millet than in sorghum. A similar $\delta^{13}C$ difference has been observed in other experiments on NAD-ME and NADP-ME leaves and seeds (An et al., 2015; Cousins et al., 2008; Dong et al., 2022; Yang et al., 2011a) as well as in plants grown in natural environment (Schulze et al., 1996). It is likely that the lack of photosynthetic carbon reduction in the suberized lamella of the NAD-ME subtypes is responsible for the faster leakage of CO_2 and HCO_3^-. This implies that more of the carbon released during C_4 acid decarboxylation leaks back into the mesophyll tissue in

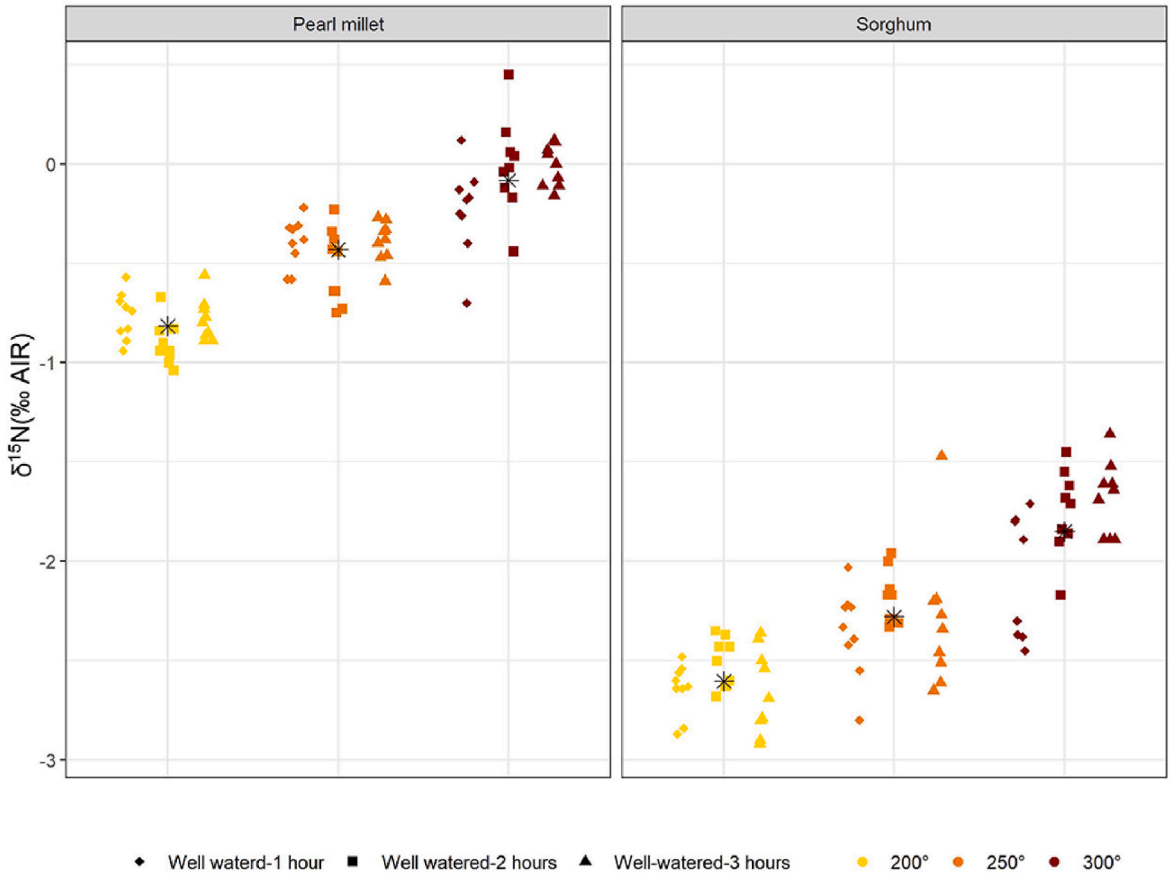

Fig. 7. Scatter plot of $\delta^{15}N$ values of pearl millet and sorghum samples charred from 1 to 3 h at 200, 250 and 300 °C. Stars are the mean values calculated for all the samples charred at 200, 250 and 300 °C.

Table 5
Summary statistic of $\delta^{15}N$ and $\delta^{13}C$ of all the charred samples of pearl millet and sorghum and $\Delta^{15}N$(‰) and $\Delta^{13}C$(‰) offsets.

Taxon	Values	n	$\delta^{15}N$ (‰ AIR)	$\Delta^{15}N$ offset	$\delta^{13}C$ (‰V-PDB)	$\Delta^{13}C$ offset
Sorghum bicolor	Mean	81	−2.24	0.02	−12.28	−0.15
	(sd)		(0.40)	(0.40)	(0.16)	(0.16)
Pennisetum glaucum	Mean	81	−0.44	0.52	−12.75	−0.21
	(sd)		(0.34)	(0.34)	(0.14)	(0.14)

Table 6
The residual standard error of a multiple regression model accounting for the effect of time and temperature (as per Nitsch et al., 2015 and Stroud et al., 2023).

Taxon	$\delta^{15}N$ (‰AIR)	$\delta^{13}C$ (‰V-PDB)
Sorghum bicolor	0.24	0.14
Pennisetum glaucum	0.16	0.14
All taxa	0.20	0.14

NAD-ME than in NADP-ME. This difference would be reflected in the $\delta^{13}C$ values, with higher $\delta^{13}C$ values in NADP-ME subtypes than in NAD-ME subtypes. The measured difference between uncharred grains of pearl millet and sorghum is 0.42‰. A similar difference is also observed in the charred samples (0.47‰) and therefore it cannot be disregarded in archaeological isotopic investigations.

When the $\delta^{15}N$ values of uncharred grains is assessed, we notice an opposite trend in respect to the $\delta^{13}C$ values because pearl millet shows higher values than sorghum. Research on nitrogen isotopes and charring effects is less common than that on carbon because the focus in studying nitrogen isotopes is mainly related to understanding the effect of manuring. Also, isotopic studies have rarely compared $\delta^{15}N$ values of different C_4 subtypes. To our knowledge, only Dong et al. (2022) compared the $\delta^{15}N$ values of uncharred and charred NAD-ME and NADP-ME grains of broomcorn millet and foxtail millet. In such study, the effects of manure and charring are discussed, but we observed differences between the two species that are in line with our results showing that NAD-ME species have higher $\delta^{15}N$ values than NADP-ME. In our research, the difference between the uncharred pearl millet and sorghum grains is 1.29‰, while that between the charred samples is 1.80‰, widening the gap between the two species.

Therefore, considering the carbon and nitrogen isotopic compositions of the uncharred samples, the first important outcome of this study is that NADP-ME and NAD-ME species must not be directly compared when working on archaeological remains because an average offset of approximately 0.5‰ for $\delta^{13}C$ and 1.8‰ for $\delta^{15}N$ between the two subgroups must be considered, especially when grains are found in the same context and presumably have been cultivated under the same environmental conditions. Particularly for nitrogen, the difference between the two subtypes is higher than that recorded for carbon, and it should not be neglected when analysing different C_4 subgroup grains from archaeological contexts. This is particularly important to take into consideration, especially when agricultural strategies such as the use of manure are discussed. Thus, to estimate the level of manure used in the past, different $\delta^{15}N$ ranges should be used, according to the species.

Considering the charred grains, we notice that the $\delta^{13}C$ values are not influenced by the length of heat exposure. With increasing temperature, even if differences are recorded for $\delta^{13}C$ values every 50 °C, variability can be considered negligible (less than 0.1‰). In order to evaluate a general charring offset for the two C_4 subtypes, which would be suitable

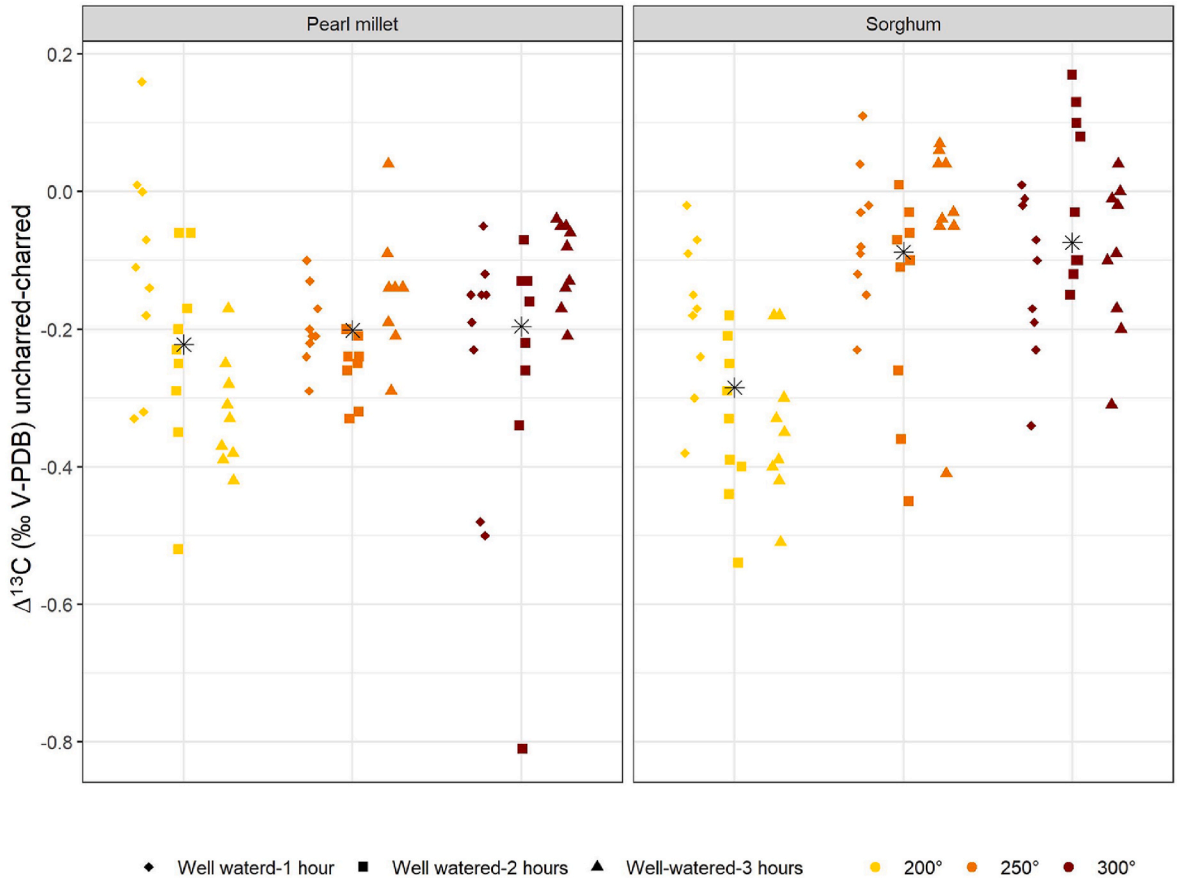

Fig. 8. Charred $\delta^{13}C$ for each pearl millet and sorghum samples charred from 1 to 3 h at 200–300 °C compared to the mean $\delta^{13}C$ of the uncharred replicates respectively.

for applying to archaeobotanical samples, we compared the $\delta^{13}C$ and $\delta^{15}N$ values of the charred to the uncharred grains (all charred samples vs. all uncharred samples) by looking at the combined data from both sorghum and pearl millet and all charring times and duration. The model to predict the charring effect to carbon indicate an offset of −0.18‰. Thus, we consider that charring negatively affects the $\delta^{13}C$ values (Fig. 8). According to our results, the lower $\delta^{13}C$ values reflect the negative effect recorded in each species (pearl millet: 0.21‰ and sorghum: 0.15‰); thus, these data demonstrate that different C_4 subpathways (NAD-ME and NADP-ME) respond similarly to charring. The decrease in $\delta^{13}C$ values grains could be mainly caused by changes in starch composition, and pearl millet is more affected by charring than sorghum. Yang et al. (2011) also observed a negative charring effect on broomcorn millet (−0.2‰). Nevertheless, a positive charring effect has been observed on foxtail millet. Indeed, Fraser et al. (2013) and Yang et al. (2011), working with longer charring durations (up to 24 h), observed a small but positive charring offset, generally lower than 1.0‰, which they considered negligible. Similarly, Dong et al. (2022) observed a positive increase of 0.24‰ for foxtail millet and 0.16‰ for broomcorn millet charred until 24 h and between 215 °C and 245 °C. Increases in $\delta^{13}C$ values of crop remains are usually attributed to the preferential loss of ^{13}C-depleted lipids during charring. Unfortunately, some of these experiments were carried out with too small a sample to be statistically significant.

When C_3 plants are observed, charring experiments mainly recorded a positive effect on $\delta^{13}C$ values in species like emmer, bread wheat and hulled barley (Fraser et al., 2013; Nitsch et al., 2015). Only einkorn showed a small negative charring effect of −0.1‰ (Nitsch et al., 2015). Albeit the average estimated effect of charring we measured on $\delta^{13}C$ is generally lower than the $\Delta^{13}C$ variability recorded by each charring test, the $\Delta^{13}C$ we obtained is higher than the variability recorded in most of the individual sample measurements in the experiments we performed. The $\Delta^{13}C$ we obtained is also higher than the calculated total analytical uncertainties ($(u(c))$ for the $\delta^{13}C$ values. This is worth considering when dealing with samples from archaeological contexts, especially after having noticed that C_4 species like pearl millet and sorghum show opposite charring effects compared to most C_3 plants.

Regarding the $\delta^{15}N$ values of the charred grains, the length of charring significantly influence the $\delta^{15}N$ values for sorghum but not for pearl millet, while the changes in temperature significantly affect both species, leading to a mean increase of 0.37‰ every 50 °C. This implies that regardless of the starting isotopic values of the uncharred grains, temperature affects both species equally. The variability between charred and uncharred grains is higher than that recorded for carbon values. The model to predict the charring effect obtained comparing the charred to the uncharred grains (all charred samples vs. all uncharred samples) combining both sorghum and pearl millet and all charring times and duration is estimated to be 0.27‰ (95% CI between −0.02 and 0.56‰). Thus, charring positively affects the $\delta^{15}N$ ratios under most experimental conditions, as observed by the mean $\Delta^{15}N$ offset for pearl millet that is 0.52‰, whereas it is more variable for sorghum, with a mean $\Delta^{15}N$ offset of 0.02‰. Even though the mean $\Delta^{15}N$ offset is positive, there are cases in which sorghum $\delta^{15}N$ values decrease compared to their uncharred counterparts (e.g. 1 h charring for all durations) (Fig. 9). Nevertheless, the $\Delta^{15}N$ offset of 0.27‰ that we estimated supports the results by Fraser et al. (2013) in broomcorn millet (ca. 1‰) and by Dong et al. (2022) in broomcorn and foxtail millet (1–2‰). This result is also consistent with the 'worst-case' 0.34‰ $\delta^{15}N$ value offset between the charred and uncharred grains of pearl millet calculated by Styring et al. (2019) and based on a predicted increase in $\delta^{15}N$ value with charring at

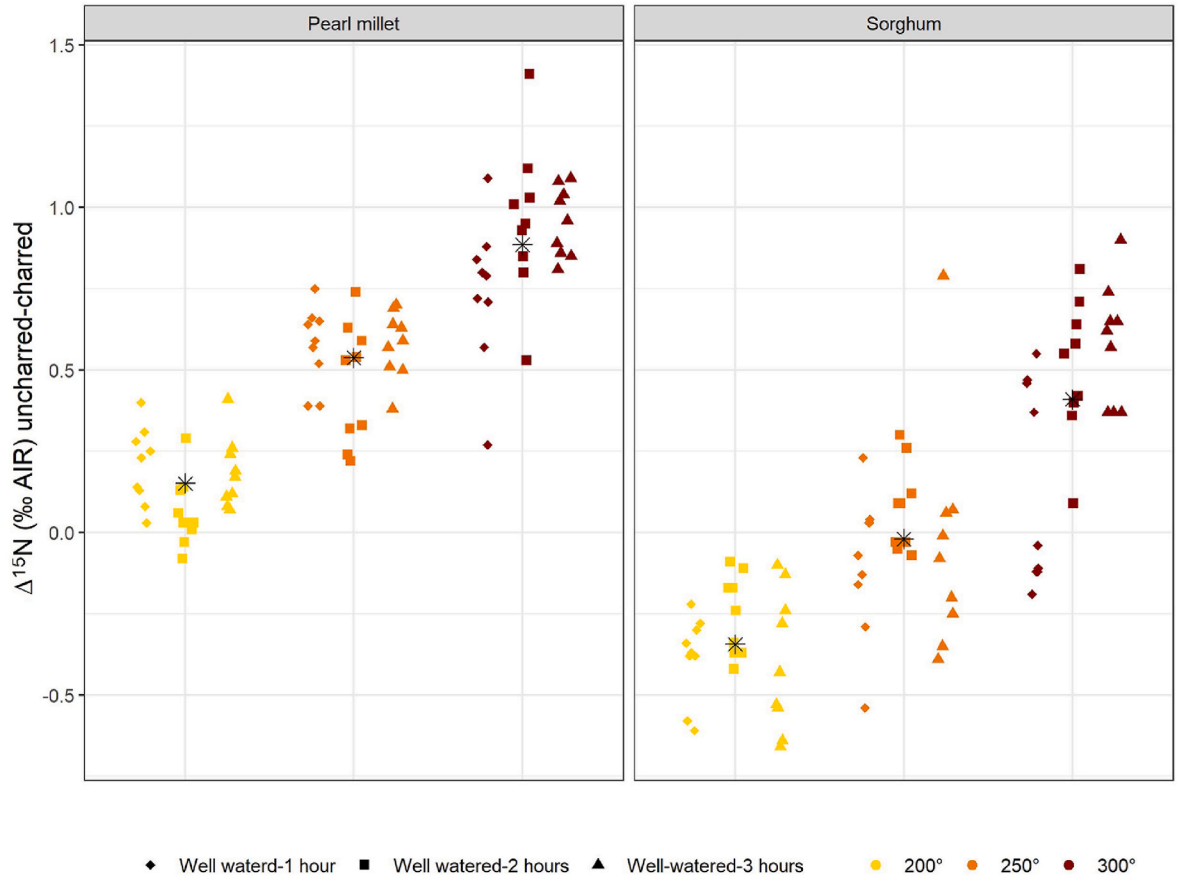

Fig. 9. Charred $\delta^{15}N$ values for each pearl millet and sorghum samples charred from 1 to 3 h at 200–300 °C compared to the mean $\delta^{15}N$ values of the uncharred replicates respectively.

245 °C for 24 h. Thus, all research are consistent in detecting a positive effect due to charring on $\delta^{15}N$ values in most of C_4 species and subtypes analyzed.

Charring experiments conducted on C_3 cereals and pulses like bread wheat, emmer, barley and lentil heated at different temperatures and durations observed similar enrichments. For example, Nitsch et al. (2015) reported an offset of 0.31‰ (4–24 h at 215–260 °C) and Stroud et al. (2023), increasing the charring temperature to 300 °C, report a similar offset (0.32‰). Other experiments on C_3 plants do not supply the average offset but the authors claim that charring did not systematically or substantially modify $\delta^{15}N$ (Fiorentino et al., 2012; Kanstrup et al., 2012). Therefore, we can suggest that, according to previous and current research, C_4 plants are affected by charring in a comparable way to C_3 plants. Based on our experiments where we considered two C_4 subtypes and a variety of charring conditions aiming to consider an heterogeneous assemblage, the charring offset of 0.27‰ we estimated is not negligible. For nitrogen, other factors can undoubtedly affect the $\delta^{15}N$ values more than charring (such as manuring, see e.g. Bogaard et al., 2007; Fraser et al., 2011; Varalli et al., 2021, Varalli et al., 2023), but charring cannot be ignored because its positive effect on $\delta^{15}N$ values can lead to an overestimation of the practice of manuring, especially for C_4 plants for which the impact of manuring is still poorly explored (Christensen et al., 2022; Styring et al., 2019). Moreover, as highlighted for carbon, the estimated nitrogen offset is higher than the variability recorded in most of the individual sample measurements of this experiment, and it is also higher than the calculated total analytical uncertainties (*(u(c))* for the $\delta^{15}N$ values. This makes the charred offset relevant when analysing the $\delta^{15}N$ values of charred plants and, more specifically, for assessments made on archaeobotanical material. For example, the application of the charring offset correction can improve interpretations of palaeodietary reconstruction. Indeed, Fraser et al. (2013) in their study at Vahingen, and Nitsch et al. (2015) applying the adjustment of 0.31‰ to the same dataset have demonstrated through different model comparisons that this correction has a significant impact because it allows the refinement of the contribution of different foodstuffs (animal and vegetal resources). This was possible thanks to a study realized considering isotopic data obtained by the whole trophic chain components. Other similar studies support the importance of considering the charring offsets for archaeobotanical remains when investigating the human diet, particularly when making inferences using Bayesian models (Varalli et al., 2021, Varalli et al., 2023).

5. Conclusion

The effects of charring on the nitrogen and carbon stable isotope values of sorghum and pearl millet were studied by analyzing grains heated at 200, 250 and 300 °C for 1, 2 and 3 h. The uncharred grains show that sorghum has lower $\delta^{15}N$ and higher $\delta^{13}C$ values than pearl millet when grown under well-watered conditions, according to plant physiology, and this evidence is also recorded in the charred grains. This means that the diverse physiological features based on differences in bundle sheath permeability in NAD-ME and NADP-ME plants affect the isotopic values more than the charring effect. Therefore, this difference between subtypes must be considered when analyzing the isotopic values from different C_4 species from archaeobotanical contexts because a direct comparison of the isotopic data could be misleading for past agricultural practice discussions.

The charring experiment results indicate that isotopic values are more sensitive to temperature than charring duration, causing a significant increase of 0.37‰ for $\delta^{15}N$ and 0.06‰ for $\delta^{13}C$ values every 50 °C

and no systematic significant changes for both carbon and nitrogen according to the duration of the charring. The offsets between the charred and the uncharred samples are 0.27‰ for $\delta^{15}N$ and −0.18‰ for $\delta^{13}C$ values. For nitrogen, our study is in accordance with previous research and, indeed, a subtraction of 0.27‰ need to be applied to the charred remains. Regarding carbon, we have observed a higher degree of variability in the results, indicating the need for additional charring experiments. These experiments should encompass a broader range of NAD-ME and NADP-ME species, as well as different growing conditions. Regardless of the specific findings, we strongly advocate for the inclusion of these offsets when analyzing archaeobotanical data from C_4 plants. Incorporating the corrected values provides a more accurate representation of the original isotopic signatures, enabling us to better understand the environmental conditions under which these crops were cultivated. By considering these offsets, we can enhance the reliability and validity of our interpretations and draw more accurate conclusions from the data. This is particularly important for $\delta^{15}N$ when making inferences regarding animal and human dietary patterns as well as the use of manure.

In conclusion, our study underscores the importance of considering multiple factors when analyzing C_4 plants from archaeological contexts. The effects of charring, along with other crucial elements such as interspecies variability, significantly impact the carbon and nitrogen isotope values. These findings have implications for our understanding of paleoclimate, paleoecology, and changes in agricultural practices. From an archaeobotanical standpoint, our research highlights the need for further investigations involving other NAD-ME and NADP-ME species. Additional data from such studies will contribute to a better understanding of the variability within C_4 plants and provide insights into their utilization and consumption by past societies in arid environments. Currently, we are engaged in ongoing research to gain a better understanding of the isotopic variability within and between pearl millet and sorghum, especially in respect to water availability. These ongoing efforts aim to provide valuable insights into these specific species and further enhance our understanding of C_4 plants. Overall, our study underscores the complexity of analyzing C_4 plants in archaeological contexts and highlights the importance of continued research to deepen our knowledge in this area.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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