Distribution of miliacin (olean-18-en-3β-ol methyl ether) and related compounds in broomcorn millet (Panicum miliaceum) and other reputed sources: Implications for the use of sedimentary miliacin as a tracer of millet

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ABSTRACT

Using sedimentary miliacin (olean-18-en-3β-ol methyl ether) as a molecular tracer of the history of *Panicum miliaceum* (broomcorn millet) cultivation depends upon broomcorn millet being sedimentary miliacin's dominant source. It also requires knowledge of the variability in miliacin concentration in broomcorn millet. Finally, it is affected by the presence of other pentacyclic triterpene methyl ethers (PTMEs) that may exist in conjunction with miliacin in other sources but not in broomcorn millet.

Miliacin biosynthesis has been proposed for other *Panicum* species, *Setaria italica* (Italian or foxtail millet), *Pennisetum* sp., and *Chaetomium olivaceum* (an olive green mold). We found miliacin concentrations in seeds of different varieties of *P. miliaceum* to be similarly high (with trace amounts of β- and α-amyrin methyl ethers). It was absent from hulls and roots, and nominally present in leaves and stems. The transfer of miliacin from plant to sediments is therefore mostly from seeds. Miliacin was abundant (often with larger amounts of β- and α-amyrin methyl ethers) in all other *Panicum* species studied but only in some species of the genus *Pennisetum* and absent in

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Setaria italica. Neither *C. olivaceum* nor its growth medium (rice) showed any trace of miliacin. Our results of no miliacin from *S. italica* and *C. olivaceum* , high miliacin concentrations in seed of *P. miliaceum* relative to other PTMEs and to other grasses, and considering the high biomass that cultivated broomcorn millet has relative to other potential plant sources, support the use of sedimentary records of miliacin in some contexts, to track past millet agricultural dynamics.

Keywords

Pentacyclic triterpenes, *Panicum miliaceum*, millet, miliacin, agriculture

1. Introduction

Molecular biomarkers archived in geological materials such as sediments are preserved or modified compounds whose source is restricted to a limited number of taxa. Temporal records of such compounds can provide information about the history of the source organisms. The combination of advances in understanding the distributions of given phytochemicals within organisms and of evolutionary trends in biosynthesis provides key information for identifying new biomarkers that can help discern past trends of vegetation. C-3 oxygenated pentacyclic triterpenes are such compounds. Their potential use in medicine (Das and Mahato, 1983; Mors et al., 2000; Lin et al., 2003; Siddique and Saleem, 2011) motivated phytochemical studies that increased our understanding of their distributions in plants (Xu et al., 2004). Although compounds such as lupeol, β- and α-amyrin are found in most angiosperms (i.e. Das and Mahato, 1983), several pentacyclic triterpenes bearing original functional groups can be more taxon specific. Examples from multiple sedimentary sources (Jacob et al., 2005; Oyo-Ita et al., 2010) include those having an acetate function at C-3, that are restricted predominantly to Asteraceae (Lavrieux et al., 2011) and those bearing a methyl ether (ME) function at C-3 that are constrained to *Poaceae* (Ohmoto et al., 1970a).

The ideal biomarker has a structure unique to one species source. In practice, a useful biomarker has an overwhelmingly dominant source in a given environmental context. This is what we hypothesized when miliacin (olean-18-en-3β-ol methyl ether; Fig. 1) was detected in sediments from Lake Bourget in the French Alps. Coupling of a carpological inventory of a Bronze Age settlement on the lake shore with a literature search of the taxonomic occurrence of miliacin (at the exception of any other PTME) led Jacob et al. (2008a) to propose that *Panicum miliaceum* (broomcorn millet) was the only source of that compound in those sediments. The hypothesis was supported by $\delta^{13}C$ measurements demonstrating that, although miliacin can be produced by C_4 and C_3 plants, the compound in Lake le Bourget sediments was exclusively of C_4 origin (Jacob et al., 2008b) and *P. miliaceum* is the only C4 plant likely to have produced that isotopic signal. Because miliacin is resistant to degradation, changes in its content in sediments were then used to infer when

millet cultivation began and subsequent fluctuation in its production in the catchment (Jacob et al., 2009). Since these pioneering studies, miliacin has also been detected in the sediments from other lakes (Simonneau et al., 2013) and in paleosols (Motuzaite-Matuzeviciute et al., 2013) where it was interpreted as a tracer of former millet cultivation.

The presence of a high concentration of miliacin in *P. miliaceum* has long been known (Ito, 1934; Abe, 1960). However, miliacin is not unique to *P. miliaceum.* Its pharmaceutical potential and role as an anti-microbial/anti-fungal agent led to search for its presence in other plant species (Panfilova et al., 2003; 2006; Hoeller Obrigkeit et al., 2006; Zheleznova et al., 2007). Most reputed miliacin producers are also in the Poaceae family, including *Eragrostis ferruginea*, *Miscanthus floridulus*, *Panicum dichotomiflorum*, *Paspalum dilatatum*, *Microstegium vimineum*, *Syntherisma sanguinalis*, *Glyceria acutiflora* (Ohmoto et al., 1970a), several *Chionochloa* spp (Connor and Purdie (1976; 1981) and, most recently, *Setaria italia* (Lu et al., 2009). Of these, only *S. italica* (foxtail or Italian millet) was abundantly cultivated by Bronze Age populations around Lake Bourget (Bouby and Billaud, 2001) and thus it is the only species likely to confound an interpretation of the agricultural history of *P. miliaceum* from the sedimentary record of miliacin there. Consequently, if *S. italica* synthesizes significant quantities of miliacin, this biomarker could be used only as a general tracer of the two millets (broomcorn and foxtail) in the Lake Bourget area. Finally, in one study (Smetanina et al., 2001 cited also by Volkman, 2005 and Lebar et al., 2007) miliacin was detected in *Chaetomium olivaceum*, a fungus that can develop on cereal seeds and in sediments. If *C. olivaceum* can synthesize miliacin in sufficient quantities, then the use of miliacin as a biomarker would be questionable in many situations.

One objective of this study was to explore the occurrence of miliacin in *P. miliaceum* and related plants (e.g. *Setaria* sp., *Pennisetum* sp., *Panicum* sp.) of significance to archaeological contexts such as those surrounding Lake le Bourget. We have also examined the distribution of miliacin in the different organs of *P. miliaceum* in order to identify the biomass that can be responsible for its dissemination in soils and sediments. Because the hypothesis of Jacob et al. (2008a; c) to propose miliacin as a tracer of *P. miliaceum* relies on the absence of other PTMEs in the sediment [only miliacin was detected in *P. miliaceum* by Ito (1934) and Ohmoto et al. (1970a; b)], we also have screen other PTMEs in all sources analysed. In addition, we have reproduced the experiment of Smetanina et al. (2001) in order to test their findings of miliacin in *C. olivaceum*. Finally, we evaluate the transfer of miliacin and other pentacyclic triterpenes from plant to soil in a field cultivated for broomcorn millet, discuss eventual transformations that could affect miliacin and other PTMEs during early diagenesis and, at the view of these results, provide guidelines for using sedimentary miliacin as a tracer of *P. miliaceum* cultivation.

2. Material and methods

2.1. Plant, seed and soil collection

The list of samples analysed is given in Table 1 for *P. miliaceum* and in Table 2 for other species. *P. miliaceum* seeds of the "sunrise" subspecies were collected from 76 plants at maturity from a cultivated field in Mézières-lez-Cléry, France (Bossard et al., 2011). For two of them, leaves, roots and stems were also analysed. From the same field, we collected 20 top soil (0-2 cm) samples. *P. miliaceum* subsp. *ruderal* seeds were collected from plants in the Vendée region of France, some being described by Poissonnier (1994). These plants are considered to originate from formerly cultivated millet that survives today as an adventive weed. Seeds from the "sunrise" strain and from a variety with black seeds collected at Saint Hilaire la Forêt (France; Table 1) were allowed to germinate and were then grown in a greenhouse at the BioEMCo laboratory (Thiverval-Grignon, France). Leaves, roots and seeds of the "sunrise" strain and seeds of the variety with black seeds from Saint Hilaire la Forêt were collected at maturity. Several *Panicum* sp. and *Pennisetum* sp. seeds were acquired from the Kew Seed Database (http://www.kew.org – Table 2). We only report the presence or absence of specific pentacyclic triterpenes in the Kew samples as they were too small to permit quantification of concentration. *Setaria italica* seeds from 2 different varieties were purchased from a local pet shop.

2.2. Cultivation of Chaetomium olivaceum

The *C. olivaceum* strain was provided by the French Museum of Natural History and isolated from immersed wood. Cultivation followed the protocol described by Smetanina et al. (2001). Briefly, it was grown in a 500 ml flask at room temperature for three weeks on a medium consisting of *Oriza sativa* (rice) seeds (commercial) and water with the following proportions (g): sodium tartrate, 0.01; rice, 20; KH_2PO_4, 0.01; sea water, 40. The only difference with the protocol of Smetanina et al. (2001) was that they used yeast extract in their experiments and we did not since enzymes contained in yeast extracts could biotransform pentacyclic triterpenes (Bastos et al., 2007; Muffler et al., 2011). The possibility that yeast and not *C. olivaceum* resulted in miliacin being present in their experiment but not ours cannot be discounted. Smetanina et al. (2001) did not mention whether seeds or leaves of *Oryza sativa* were used as a substrate for their experiment. We assumed that Smetanina et al. (2001) used seeds and not leaves as a substrate for the following reasons. First, 20 g of leaves will not readily fit into a 500 mL flask. Second, seeds have the highest sugar contents and can thus produce the greatest quantities of fungi.

2.3. Lipid extraction and purification

Each of the following parts of *P. miliaceum* subsp. *sunrise* was analyzed separately: seeds, hulls (lemmas, paleas, and glumes), leaves and stems. The seed (bran, endosperm and embryo) was the only plant part analyzed for the other taxa. Plant and soil materials were dried at 40 °C in an oven and ground to powder with a mortar and pestle. *C. olivaceum* (C.o) was manually separated from the rice on which it developed (rice-C.o). C.o, rice-C.o and a control consisting of untreated rice were dried for 48 h in an oven at 40 °C and ground to powder. Lipids were ultrasonically extracted (3 x) with dichloromethane (DCM):isopropanol (2:1) and the extract combined. The total lipid extract was separated into neutral, acidic and polar fractions on a Pasteur pipette filled with aminopropyl bonded silica. The neutral fraction was collected after elution with DCM:isopropanol 2:1.

For plant material, the neutral fraction was separated using flash chromatography on silica (activated at 120 °C for 24 h, then deactivated with H_2O at 5% by wt.) into aliphatic and aromatic hydrocarbons, ethers, esters and ketones, and alcohols by sequentially elution with solvent of increasing polarity as described in Jacob et al. (2008b). All fractions were dried under a stream of N_2 and stored at -18 °C until analysis using gas chromatography-mass spectrometry (GC-MS). 5α-Cholestane was added as internal standard prior to GC-MS, for quantification purposes. The fraction that contained sterols and triterpenols was silylated with N,O-*bis*(trimethylsilyl)trifluoroacetamide in pyridine. For soil samples, the neutral fraction was not separated and was not derivatised prior to GC-MS analyses.

2.3. Lipid identification and quantification

Lipids were identified and quantified using GC-MS with a Trace GC Ultra gas chromatograph coupled to a TSQ Quantum XLS mass spectrometer equipped with an AS 3000 autosampler (both Thermo-Scientific, Bremen, Germany). The GC instrument was fitted with a TG-5 MS column (60 m, 0.25 mm i.d., 0.25 µm film thickness; Thermo, Bellefonte, PA, USA). The temperature programme was: 40 °C (1 min) to 120 °C at 30 °C min^{-1} and then to 300 °C (held 70 min) at 3 °C.min^{-1}. The sample was dissolved in toluene and 2 µl were injected in splitless mode at 280 °C. The carrier gas was He at 1.0 mL min^{-1}. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV and scanned from *m/z* 50 to 600. Compounds were identified on the basis of their mass spectral data, and, when available (miliacin, olean-12-en-3β-ol ME, urs-12-en-3β-ol ME, germanicol), by comparison with authentic standards. Key mass spectral data are provided in Table 1.

3. Results and discussion

3.1. Miliacin and other pentacyclic triterpenes in P. miliaceum

We have screened for pentacyclic triterpenes in different organs of *P. miliaceum* var. sunrise in order to identify the plant parts that are the most susceptible of contributing for miliacin to the soil or sediment. Table 1 summarizes the distribution of pentacyclic triterpenes in the different samples of *P. miliaceum*. The compound structures are depicted in Fig. 1. The ether/ester/ketone fraction of the seed extract yielded miliacin as the most abundant compound, and minor amounts of β-amyrin ME (isosawamilletin or olean-12-en-3β-ol ME), α-amyrin ME (urs-12-en-3β-ol ME; Fig. 2) and friedelin (friedelan-3-one; D:A-friedo-olean-3-one). Apart from phytosterols and *n*-alcohols that are not discussed further, the alcohol fraction contained several triterpenols: α-amyrin (urs-12-en-3β-ol), β-amyrin (olean-12-en-3β-ol), germanicol (olean-18-en-3β-ol) and glutinol (glut-5-en-3β-ol; D:B-friedo-olean-5-en-3β-ol) (Figs. 1 and 2).

Miliacin was detected in the roots, stems, leaves and seeds of *P. miliaceum* var. sunrise grown in the field. Concentration that averages 306 μg g^{-1} seeds, is far higher than in leaves (7.5 μg g^{-1}), roots (23.9 μg g^{-1}) or stems (171 μg g^{-1}). If the weight of each organ relative to the total plant biomass is considered, seeds constitute by far the main contributor. This high abundance in the seeds is in agreement with results of the first detection of miliacin in *P. miliaceum* (Ito, 1934). Although Ito (1934) does not specify which organs he analysed, the weight of material used (133.8 kg) suggests that he analysed seeds, in which case the miliacin concentration of 270 μg g^{-1} seeds found by Ito (1934) is very close to that determined for millet seeds from Mézières-lez-Cléry (306 μg g^{-1}, Table 1). Since pentacyclic triterpenes are reputed to be anti-microbial compounds, the exceptionally high concentration of miliacin could explain the high preservation potential of *P. miliaceum* seeds that has been known since antiquity (Varro, De Re Rustica).

Our results (Table 1) indicate that miliacin is not present in the hull but only in the seed (albumen and embryo). Its absence from millet hull (i.e. glumes and lemma) contradicts previous results that erroneously showed miliacin in millet hull due to incomplete separation between fruit and glumes/lemma before extraction (Jacob et al., 2008c). Its presumed presence in millet hull led us to propose an eolian transport to the sediment after winnowing and threshing of millet seeds (Jacob et al., 2008c). In the light of the new results, and considering that seeds are the major miliacin contributing organ in *P. miliaceum*, a high level of miliacin in Lake le Bourget sediments could result from its transportation via consumption by people (or animals). A lower level could be interpreted as a geochemical background resulting from runoff from soils formerly cultivated for millet.

The low concentration in leaves and stems may be because the compound is synthesized in the leaves but then translocated to the seeds through the stems. We also found it in the roots of

millet cultivated in the open field but not in the roots of plants grown in a controlled environment chamber (Table 1). At Mézières-lez-Cléry, we found significant level of miliacin in the soil in which *P. miliaceum* grew (cf. infra), probably due to seeds falling on to the soil during years of cultivation (Bossard, unpublished results). In contrast, we did not detect any in a hydroponic solution on which millet was grown in controlled environment chambers. Therefore, roots of plants sampled in the open field were likely contaminated by miliacin already present in the soil (cf. infra).

The presence of friedelin, germanicol, α-amyrin, β-amyrin and glutinol in the leaves of *P. miliaceum* (Table 1) is consistent with findings for millet leaf wax (Tulloch, 1981). Tulloch (1981) did not detect miliacin, β-amyrin ME or α-amyrin ME in leaf wax, in agreement with our findings of little to none of these compounds in tissue other than seeds.We found the distribution of friedelin to differ from that of miliacin since it is 10 x more concentrated in leaves (111.2 μg g^{-1}) than in seeds (11 μg g^{-1}). The ratio of friedelin to miliacin reaches 14.8 in leaves whereas it is only of 0.036 in the seeds of the samples collected from the Mézières-lez-Cléry field. Germanicol, similar in structure to miliacin, is more abundant than β- and α-amyrins or glutinol in leaves, stems and seeds of *P. miliaceum* and is more abundant in leaves than in seeds; α-amyrin, β-amyrin and glutinol concentrations do not differ between seeds and leaves.

3.2. Variation in miliacin and other PTMEs among P. miliaceum varieties

Because the strains of *P. miliaceum* evidently differ from those cultivated during ancient times, we have explored the variability of distribution of miliacin and other PTME in several strains of *P. miliaceum*. The distributions of pentacyclic triterpenes in seeds of *P. miliaceum* var. *sunrise* collected from the open field are similar to those of the same variety cultivated in controlled-environment chambers and to those of all the *P. miliaceum* taxa (Table 1). Miliacin was, by far, the most abundant pentacyclic triterpene in any sample, ranging from 296 to 476 μg g^{-1}. This accounts not only for the variety presently cultivated in an open field but also for the same variety cultivated in a climate chamber or for the ruderal varieties thought to be derived from millet cultivated in ancient times (Poissonnier, 1994). Therefore, the very large abundance of miliacin in *P. miliaceum* does not appear to be dependent on cultivation conditions or affected by the strain/degree of selection.

Although a similar concentration of miliacin was found in all *P. miliaceum* seed samples, some of the other pentacyclic triterpene methyl ethers (β-amyrin ME and α-amyrin ME) were only detected in the sunrise variety and hardly reached 1 % of miliacin concentrations. Considering this variability within *P. miliaceum* varieties, we cannot support the conclusion by Lu et al. (2009) that millet species (i.e. foxtail and broomcorn millet) can be distinguished based on their respective

abundance of miliacin, β-amyrin ME and α-amyrin ME as proposed. In addition, chemical and geochemical transformations could slightly affect the proportions of Δ^{12} and Δ^{18} oleanene-type pentacyclic triterpenes (Rullkötter et al., 1994), which reinforces the idea that caution that must be taken when interpreting subtle differences in the proportion of these compounds (see section 3.6.). The second most abundant pentacyclic triterpene is friedelin, the concentration of which varies from 5 to 40 μg g^{-1}. Scarce data on other compounds could result from detection limits, or to the size of seed that could be related to maturity at harvest (Hunt et al., 2011).

3.3. Miliacin and other pentacyclic triterpenes in Panicum spp.

We have explored whether the distribution of miliacin and other compounds found in *P. miliaceum* is a common traits in other species of the *Panicum* genus. Miliacin was detected in each *Panicum* species (Table 2), even those for which we had very small amounts of seeds. Seed sample size also had no apparent effect on the detection of other compounds. For example, β-amyrin ME, α-amyrin ME and friedelin were detected in *P. humile* but were absent from *P. halii*, for which we extracted 10 x the weight of *P. humile*.

The widespread occurrence of miliacin among our *Panicum* species is in agreement with the report of this compound in *P. dichotomiflorum* (in culms and blades; Ohmoto et al., 1970b) and implies that it is very common in the *Panicum* genus. Although the fraction containing miliacin was not explored and his study focused on leaf waxes, Tulloch (1981) found germanicol in *P. texanum*. Considering the genetic relationship between miliacin and germanicol and that we always detected germanicol together with miliacin (Tables 1, 2), miliacin is probably present in *P. texanum*. Concerning other triterpenoids, β-amyrin ME and α-amyrin ME were found in six and five species among the eleven analyzed (Table 2). As for *P. miliaceum*, some *Panicum* species (*P. capilare, P. coloratum, P. kalaharense, P. halii, P. aldabrense, P. laxum* and *P. pseudowoeltzkowii*) are characterized by the dominance or exclusive presence of miliacin among PTMEs. Other species (*P. nervatum, P. phragmitoides* and *P. turgidum*) display significant proportions of β-amyrin and α-amyrin MEs. Friedelin was only detected in two species (*P. humile* and *P. phragmitoides*) and β-amyrin, α-amyrin, germanicol and glutinol were rarely found, probably due to detection limits. Considering the much lower concentration of miliacin in *Panicum* spp. other than *P. miliaceum*, and the much smaller contribution of seeds from these species to their biomass, a significant input of miliacin from other *Panicum* species (that are not crops) is unlikely.

3.4. Miliacin and other pentacyclic triterpenes in other Poaceae

Ohmoto et al. (1970a) concluded that miliacin is common throughout the Poaceae after detecting it in *Microstegium vimineum, Paspalum dilatatum* (culms and blades) and *Eragrostis*

ferruginea (culms and blades). It was also found in 6 out of > 20 *Chionochloa* spp (Connor and Purdie, 1976). In the *Pennisetum* genus, it was only detected in *P. hordeoides* (associated to β-amyrin and α-amyrin MEs) and *P. polystachion* (where it is the sole PTME), but not *P. divisum*, *P. sieberianum*, or *P. unisetum.* Similarly, Ohmoto et al. (1970a) did not detect it in *P. alopecuroides* seeds. Therefore, and in contrast to the *Panicum* genus where it is present in every species we analyzed, miliacin is not ubiquitous in *Pennisetum* or *Chionochloa* species.

Miliacin was not detected in any of the two varieties of *Setaria italica* we analyzed, despite the fact that we performed extractions on large samples of seeds. Our results are consistent with those of Ohmoto et al. (1970a), who did not detect miliacin in the seeds of *S. chondrachne*, *S. faberi* and *S. italica* but disagree with its detection in *S. italica* by Lu et al. (2009). Information about seed sample weight was not given in the last study and perhaps Lu et al. (2009) used even larger samples than ours. Miliacin peaks on their chromatograms are of low amplitude for *S. italica* and off the chart for *P. miliaceum*, suggesting at most, a much lower concentration in the former species than in the latter, or, more likely, a contamination of *S. italica* sample by *P. miliaceum* sample (memory effect). *Pennisetum* and *Panicum* are biochemically (Giussani et al., 2001) and genetically (Benetzen et al., 2012) more similar to one another than they are to *Setaria*, so this could explain why miliacin is present in both *Pennisetum* and *Panicum* spp. but absent from *Setaria* spp.

3.5. Miliacin in Chaetomium olivaceum

The rice seeds that we used as a cultivation medium for *C. olivaceum* contained several phytosterols such as campesterol, stigmasterol and β-sitosterol (in agreement with Nasu et al., 2000; Macias et al., 2006) but no pentacyclic triterpene ME (PTME). We know of no study that found PTMEs in rice seeds but arundoin (fern-9(11)-en-3β-ol ME) and cylindrin (arbor-9(11)-en-3β-ol ME) have been detected in rice leaves (Ohmoto et al., 1970b). We did not detect any miliacin in the rice on which *C. olivaceum* developed but ergosterol, which is known to be synthetized de novo by fungi (Fryberg et al., 1973; Alcazar-Fuoli et al., 2008) and is therefore classically used as a fungal biomarker (Grant and West, 1986), was detected in addition to the sterols found in the control rice. Neither miliacin nor any other pentacyclic triterpene was detected in our extract of *C. olivaceum*. This is consistent with current knowledge of C-3 oxygenated pentacyclic triterpenes, which are exclusively reported in higher plants (i.e. Das and Mahato, 1983; Connolly and Hill, 1989; 1996; 2001; 2007; Mahato et al., 1992; Connolly et al., 1994a; b; Mahato and Sen, 1997; Xu et al., 2004) due to distinct oxidosqualene cyclases between fungi and plants (i.e. Abe et al., 1993). The strong contrast between the results of Smetanina et al. (2001) who extracted sufficient amounts of miliacin from their samples (> 12 mg) to perform NMR identification and the absence of any miliacin in our samples cannot be explained by the absence of yeast extract in our experiment. To our knowledge,

the results from Smetanina et al. (2001) constitute the sole report on a pentacyclic triterpene in a fungus. We know of no other efforts to reproduce their results except ours and we did not find miliacin or any other pentacyclic triterpene in *C. olivaceum*.

3.6. Guidelines for using sedimentary miliacin as a tracer of P. miliaceum

Based on previous studies, we hypothesized that where sedimentary archives of miliacin are found in conjunction with high concentrations of other PTMEs (e.g., for a Brazilian lake, Jacob et al., 2005; for Niger Delta, Oyo-Ita et al., 2010), this compound cannot be used as a biomarker for *P. miliaceum*. In contrast, miliacin was the sole detectable PTME in the sediments of lake Le Bourget (Jacob et al., 2008a; 2008b; 2009) and lakes Ledro and Paladru (Simonneau et al., 2013), and in Ukrainian paleosols that were thought to have been cultivated for *P. miliaceum* (Motuzaite-Matuzeviciute et al., 2013). In conjunction with other evidence, the presence of miliacin in these cases was interpreted as a tracer for the cultivation of *P. miliaceum* in the catchment area.

In order to determine whether miliacin also predominates over other PTMEs found in *P. miliaceum* in soils, we first analyzed seeds in 76 millet plants collected in the field of Mézières-lez-Cléry. We found that miliacin constitutes an average of 94.9% of the three PTMEs (max. 100; min. 86.5), α-amyrin ME 4.4 % (max. 10.5; min. 0) and β-amyrin ME 0.7 % (max. 7.2; min. 0). In soil (20 top soils analysed), PTMEs also mainly consist of miliacin (average 97.4 %, max. 100 %; min. 85 %), α-amyrin ME (average 0.4 %, max. 5.4 %; min. 0 %) and β-amyrin ME (average 2.2 %, max. 15 %; min. 0 %). Therefore, the overwhelming predominance of miliacin over other minor PTMEs in *P. miliaceum* seeds is, at least, preserved during the transfer to soil. Further work is underway to test whether this assertion is robust during the transfer to aquatic sedimentary archives. C-3 oxygenated pentacyclic triterpenes are reputed to undergo double bond migrations during early diagenesis (Rullkötter et al., 1994). Depending on the physical conditions that prevail during transportation from the plant source to the soil or sediment archive, one can expect minor to moderate changes in the relative proportions of miliacin and, for example, β-amyrin ME.

Miliacin can, therefore, be synthetized by a large array of plants but only *P. miliaceum* produces large amounts of miliacin that is, in addition, overexpressed when compared to other PTMEs. Therefore, miliacin should only be used as a tracer of *P. miliaceum* when it constitutes the exclusive or, at least, predominant PTME in a geological archive. Of importance is the large biomass constituted by cultivated millet that, added to the elevated concentrations of miliacin in the seeds can explain miliacin from *P. miliaceum* is detectable in sediments whereas miliacin and other PTMEs from weeds or grasses of minor importance might not reach the threshold of GC-MS detection. As for any molecular biomarker, future work involving miliacin should take into account

the geological, ecological and archaeological context in order to ascertain the relationship between soil or sedimentary miliacin and cultivated *P. miliaceum*.

4. Conclusions

The exploration of several varieties of *Panicum miliaceum* for miliacin and other pentacyclic triterpenes reveals that miliacin is very abundant (much more than any other pentacyclic triterpene) in the seeds of this plant, with a similar concentration regardless of the variety considered (cultivated or ruderal). High amounts of miliacin, largely exceeding other PTMEs, are also noted in soils cultivated for millet. Miliacin is also found in all species belonging to the *Panicum* genus, either as the unique PTME or accompanied by low to significant levels of β-amyrin ME and α-amyrin ME. Miliacin is only sparsely detected in *Pennisetum* species and was not found in *Setaria* two varieties of *Setaria italica* we analyzed. It thus can help distinguish between the two millets. The absence of miliacin in *Chaetomium olivaceum* strongly contradicts the results of the one other study on this topic but is consistent with the global scheme of the C-3 pentacyclic triterpene distribution in organisms. Our results also provide guidelines for interpreting miliacin which, when found in abundance and as the sole/dominant PTME in soils and sediments, can confidently be used as a tracer for unravelling the history of millet cultivation.

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Table captions

Table 1

Concentration (µg g^{-1}) of pentacyclic triterpenes in parts of *Panicum miliaceum* (n.d., not detected).

Species	Variety	Origin	Organ	Sampling date	Olean-18-en-3β-ol ME (miliacin)	Olean-12-en-3β-ol ME (Isosawamilletin)	Urs-12-en-3β-ol ME	DA-Friedo-oleanan-3-one (friedelin)	Olean-12-en-3β-ol (β-amyrin)	Urs-12-en-3β-ol (α-amyrin)	Olean-18-en-3β-ol (germanicol)	D:B-Friedo-olean-5-en-3β-ol (glutinol)
Panicum miliaceum	Sunrise	Mézières-lez-Cléry, France	Leaves	2010	7.5	n.d.	n.d.	111.2	2.6	7.1	30.5	2.2
	Sunrise	Mézières-lez-Cléry, France	Roots	2010	23.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Sunrise	Mézières-lez-Cléry, France	Seeds	2010	306	3.2	4.2	11	6.9	4.1	6.2	2.8
	Sunrise	Mézières-lez-Cléry, France	Stems	2010	171	1.8	2.9	n.d.	7.2	5.3	8.3	n.d.
	Sunrise	Mézières-lez-Cléry, France	Glumes	2010	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Sunrise	Greenhouse / Bioemco[a]	Seeds	2011	424	4.5	5.7	41.2	n.d.	n.d.	n.d.	n.d.
	Sunrise	Greenhouse / Bioemco[a]	Roots	2011	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Black seeds[b]	St Hilaire la Forêt, France	Seeds	1990	399	n.d.	n.d.	17.1	n.d.	n.d.	0.9	n.d.
	Black seeds[c]	St Hilaire la Forêt, France[b]	Seeds	2011	413	n.d.	n.d.	40.7	n.d.	n.d.	n.d.	n.d.
	Yellow seeds[c]	St Hilaire la Forêt, France	Seeds	1990	368	n.d.	n.d.	26.2	1.9	0.2	0.9	n.d.
	Black seeds[c]	St Vincent sur Graon, France	Seeds	1991	448	n.d.	n.d.	12.3	n.d.	n.d.	1.6	n.d.
	A[c]	CAIRN[d]	Seeds	1990	323	n.d.	n.d.	23.8	n.d.	n.d.	1.2	n.d.
	B[c]	CAIRN[d]	Seeds	1990	436	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C1[c]Small spiklets	CAIRN[d]	Seeds	1991	408	n.d.	n.d.	20.7	n.d.	n.d.	n.d.	n.d.
	C2[c]	CAIRN[d]	Seeds	1992	476	n.d.	n.d.	21.6	n.d.	n.d.	0.5	n.d.
	-	Bougon, France	Seeds	2004	372	n.d.	n.d.	11.2	n.d.	n.d.	0.8	n.d.
	-	Aubigny, Le Baillargeau, France	Seeds	2006	331	n.d.	n.d.	4.7	n.d.	n.d.	1.3	n.d.

-	La Fembretière, France	Seeds	2006	302	n.d.	n.d.	13.8	n.d.	n.d.	1.0	n.d.
-	Aubigny, La Livraie, France	Seeds	2006	297	n.d.	n.d.	5.5	n.d.	n.d.	1.3	n.d.

[a] Seeds from Mézières-lez-Cléry grown in a greenhouse at Bioemco; [b] Seeds from St Hilaire la Forêt grown in a greenhouse at Bioemco; [c] for a description, see Poissonnier (1994); [d] Centre Archéologique d'Initiation et de Recherche sur le Néolithique, St Hilaire la Forêt, France.

Key mass spectral data:

- Miliacin: M^+ 440, $[M^+-15]$ 425, significant ions at m/z 177+189+204 +218
- Isosawamilletin: M^+ 440, $[M^+-15]$ 425, significant ions at m/z 218+203+189
- Urs-12-en-3β-ol ME: M^+ 440, $[M^+-15]$ 425, significant ions at m/z 189+218 +203
- Friedelin: M^+ 426, $[M^+-15]$ 411, significant ions at m/z 163+231+273
- β-amyrin (as TMS derivative): M^+ 498, $[M^+-15]$ 483, significant ions at m/z 218+203+189
- α-amyrin (as TMS derivative): M^+ 498, $[M^+-15]$ 483, significant ions at m/z 189+218 +203
- Germanicol (as TMS derivative): M^+ 498, $[M^+-15]$ 483, significant ions at m/z 177+189+204 +218-
- Glutinol (as TMS derivative): M^+ 498, $[M^+-15]$ 483, significant ions at m/z 259+274

Table 2

Distribution of pentacyclic triterpenes in seeds of *Panicum*, *Pennisetum* and *Setaria* species. Percentages are calculated from the total pentacyclic triterpenes identified and quantified.

Genus	Species	Number of extracted seeds	Extracted seeds weight (mg)	Olean-18-en-3β-ol ME (miliacin)		Olean-12-en-3β-ol ME (isosawamilletin)		Urs-12-en-3β- ol ME		DA-Friedooleanan-3-one (friedelin)		Olean-12-en-3β ol (β-amyrin)		Urs-12-en-3β- ol (α-amyrin)		Olean-18-en-3β-ol (germanicol)		D:B-Friedo-olean-5-en-3β-ol (glutinol)	
				ng/g seed	%	ng/g seed	%	ng/g seed	%	ng/g seed	%	ng/g seed	%	ng/g seed	%	ng/g seed	%	ng/g seed	%
Panicum	*capilare*	50	67	365.3	95	-	-	-	-	-	-	-	-	-	-	7.4	2	10.4	3
	*coloratum**	37	2	100.6	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*humile**	39	3	228.6	43	148.8	28	135.6	26	13.6	3	-	-	-	-	-	-	-	-
	kalaharense	50	89	3.2	72	0.1	2	-	-	-	-	0.6	13	0.5	12	-	-	-	-
	*miliaceum***	20	117	306	89	3.2	1	4.2	1	11	3	6.9	2	4.1	1	6.2	2	2.8	1
	*nervatum**	50	8	35.6	42	12.8	15	36.2	43	-	-	-	-	-	-	-	-	-	-
	phragmitoides	50	48	79.9	23	105.6	30	135.5	39	11.2	3	-	-	5.1	1	-	-	11.7	3
	turgidum	50	101	77.2	54	35.3	25	29.5	21	-	-	-	-	-	-	-	-	-	-
	halii	49	52	64.0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*aldabrense**	50	9	356.8	88	26.7	7	12.3	3	-	-	-	-	-	-	10.4	3	-	-
	*laxum**	50	5	108.4	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*pseudowoeltzkowii**	49	12	577.0	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pennisetum	*divisum*	50	101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	*hordeoides**	50	4	53.1	59	19.3	22	17.0	19	-	-	-	-	-	-	-	-	-	-
	*polystachion**	50	18	22.4	73	-	-	-	-	-	-	-	-	-	-	8.2	27	-	-
	sieberianum	50	158	-	-	-	-	2.5	100	-	-	-	-	-	-	-	-	-	-
	*unisetum**	49	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Setaria	*italica* (N=2 var.)		1056	-	-	-	-	0.2	19	0.2	24	0.3	34	0.2	23	-	-	-	-

* Error margins in concentration are likely to be high due to low sample weight.

** Corresponds to line 3 in Table 1 (*P. miliaceum* var. Sunrise seeds).

Figure captions

Fig. 1. Structure of pentacyclic triterpenes found in *Panicum miliaceum*.

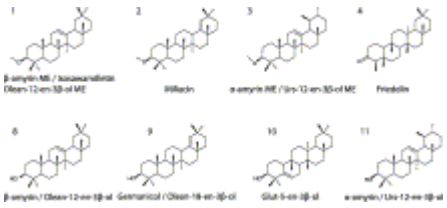

Fig. 2. Distribution of pentacyclic triterpenes and other compounds on the partial chromatograms of the (a) ether/ester and ketone, and (b) alcohol fractions of lipids extracted from seeds of *Panicum miliaceum* var. *sunrise* collected from the field at Mézières-lez-Cléry (France). 1, β-amyrin ME; 2, miliacin; 3, α-amyrin ME; 4, friedelin; 5, cholesterol; 6, ergosterol; 7, stigmastadienol; 8, β-amyrin; 9, germanicol; 10, glutinol; 11, α-amyrin; *n*-C_x, alcohols; *, unknowns.