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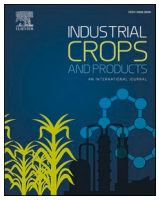

Metaproteomics identifies key cell wall degrading enzymes and proteins potentially related to inter-field variability in fiber quality during flax dew retting

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ABSTRACT

In this study, metaproteomics and biochemical analyses were used to identify for the first time specific proteins and associated micro-organisms responsible for cell wall degrading enzyme activity during dew retting of flax in two adjacent fields in northern France. This approach identified 6032 non-redundant proteins present at 4 key retting stages (R0/day 1, R2/day 6, R4/day 13, and R7/day 25), of which 75 contained CAZy (Carbohydrate Active Enzyme) motifs belonging to 31 different families from all 5 CAZy classes. 19 families were putatively related to the degradation of different plant cell wall polymers including lignin (AA1), pectins (CE13, CE8, PL1, PL3, GH28, GH35), hemicellulose (GH2, GH10, GH26, GH35, GH55, GH3, GH5, GH17) and cellulose (GH5, GH7, GH3, GH94, AA3). Taxonomy of identified proteins indicated that 85 % come from bacteria, 13 % from fungi, and 2 % from plants; however, ~60 % of CAZymes involved in the degradation of plant wall polymers are of fungal origin. Although 88 % of total proteins and almost 65 % of cell wall degrading CAZymes were similar between the two investigated fields, certain differences in the abundance and dynamics of certain CAZymes might be related to observed inter-field variability in cell wall degrading enzyme activities, stem/fiber yield and industrial qualities of fibers harvested from the two fields. Overall, these results highlight the interest of using metaproteomics for improving our biological understanding of how retting impacts fiber quality. In addition, the identification of several new bacterial and fungal species in this study demonstrates that such an approach is also extremely powerful for generating novel taxonomic data.

1. Introduction

Flax (*Linum usitatissimum* L) is an herbaceous, annual, self-pollinating dicot plant belonging to the family *Linaceae*. It is cultivated for both oil, and for its cellulose-rich bast fibers that are classically used in textiles (linen) and, more recently, composite materials (Mohanty et al., 2000; Chand and Fahim, 2020). Currently, quality variability between different fiber batches is an important factor holding back the widespread use of natural fibers in a range of different industrial applications (Müssig, 2010; Summerscales et al., 2010).

The qualities of both flax textiles and natural fiber composites (NFCs) are influenced by two main factors: firstly, the intrinsic mechanical and chemical attributes of individual fibers and/or fiber bundles, and secondly, the processing techniques used during fiber extraction and industrial manufacturing (Bourmaud et al., 2018). Variability in the quality of different fiber batches can be attributed to several different factors, some directly related to the plant (varieties, cultivation practices, soil type, meteorological conditions during growth etc.) (Chabi et al., 2023), while others concern post-harvest treatments (e.g., retting, scutching, combing, etc.) (Morgillo et al., 2023). In this article, we will

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look at field retting, which is considered to be one of the sources of variability (Martin et al., 2013; Baley et al., 2020).

Retting is a biological process used for the first stage of fiber extraction from the flax stem. In this organ, the individual fibers (elementary fibers) are elongated cells (reaching ~30 mm in length, 15–20 μm in diameter) organized into bundles of 30–40 cells embedded in the cortical parenchyma and located between the phloem and epidermis (Akin et al., 1996; Day et al., 2005; Salmon-minotte and Franck, 2005; Gorshkova et al., 2018). During retting, enzymes produced by microorganisms progressively degrade the cell wall polymers of fiber cells and surrounding tissues (Fig. 1**a**) thereby reducing inter-cell cohesion and facilitating fiber extraction (Sharma et al., 1992; Meijer et al., 1995). This operation cannot be achieved directly by a mechanical process as this would damage the fiber cell wall resulting in poor-quality fibers (Chabbert et al., 2020).

Historically, retting was often performed by placing flax stems in water tanks (water retting), however for both economic and ecological reasons, another type of retting - dew retting - is now the preferred process in Europe. The difference between these two processes is that in water retting, the flax stems are fermented anaerobically in a water tank, whereas in dew retting the stems lie on the field for several weeks, organized in swaths after being up-rooted (Paridah et al., 2011). Although similar to the natural microbial process associated with biomass degradation into forest litter (Voříšková and Baldrian, 2013; Liu et al., 2015; Tláskal et al., 2021), retting distinguishes itself by its "controlled" nature. Specifically, this means that cell wall degradation should be sufficient to facilitate fiber separation but must be stopped before excessive degradation of the cellulose cell wall of fibers leads to a loss of mechanical properties (Akin et al., 1998; Goodman et al., 2002). In the light of such an observation, it is clear that retting is a major factor affecting fiber quality and homogeneity.

To understand the biology behind this complex process, researchers have used different approaches to both identify the different microorganisms involved and to characterize the cell wall degrading enzyme activities involved in retting. Historically, conventional (culture-based) strategies allowed researchers to establish partial inventories of the bacterial and fungal consortia involved in dew retting (Brown and Sharma, 1984; Sharma, 1986; Henriksson et al., 1997; Fila et al., 2001). More recently, the composition and dynamics of the flax-retting microbial community have been studied with high throughput sequencing methods (targeted metagenomics on 16S rDNA and ITS markers) (Djemiel et al., 2017; Chabbert et al., 2020). However, while such studies have undoubtedly contributed to a better understanding about what microorganisms are present, they do not allow a precise identification of which cell-wall degrading enzymes are actually produced by these microorganisms during retting (Herbst et al., 2016; Djemiel et al., 2020). Although biochemical measurements (enzyme assays) have previously been used to evaluate the activities and dynamics of cell wall degrading enzymes during retting in flax (Sharma et al., 1992; Chabbert et al., 2020) and hemp (Liu et al., 2017; Bleuze et al., 2018), such approaches do not provide information about the individual enzymes contributing to such activity. Indeed, when biochemical approaches are used to measure enzyme activity during retting, what is actually being determined is a "global activity" resulting from the activities of a number of different individual enzymes, all showing the same type of activity, but differing in structure and originating from different microorganisms. Nevertheless, recent studies on the degradation of forest litter and lignocellulosic biomass (Hori et al., 2018; Chirania et al., 2022) have shown that a metaproteomic approach represents an interesting strategy that can be used to precisely identify individual enzymes as well as the microorganisms producing them.

In this paper, we have attempted to meet two objectives. The first is to identify and establish the dynamics of the key retting enzymes using a metaproteomic approach, the second is to investigate whether differences in retting microbial communities and/or enzyme dynamics might be related to inter-field variability in fiber quality. It is well established that the Northern French climate is favorable for growing and retting flax, nevertheless, field-to-field variability in fiber quality still occurs even when the same variety is grown by the same farmer in the same geographical region. In an attempt to see whether such variability might be related to differences in retting, we sampled three adjacent retting fields and selected the two with the most contrasting fiber properties for further analysis.

2. Material and methods

2.1. Experimental design, environmental data and fiber characterization

Flax plants (*Linum usitatissimum* L., cultivar Evasion, Terre de Lin) were sown by flax farmers contracted to the Van Robaeys Frères company on the 30th March 2021, in a typical silty-clay soil field (50°47'05.5"N 2°27'07.4"E) near Bavinchove, Hauts-de-France, France (Fig. 1**b**). Dew retting started at uprooting (23rd July, stage R0), swathes were turned on day 12 and then retting continued until the stem harvesting (15 ^{h} August, stage R7) (Fig. 1**c**). Swath lines were marked at 5 plots (150×100 cm) for 5 biological replicates (T1-T5-per time point) (Fig. 1**d**) samples (30 cm-long sections from the middle regions of flax swathes) within each replicate were collected during retting on the following days (R0/day 1, R1/day 4, R2/day 6, R3/day 11, R4/day 13, R5/day 18, R6/day 20 and R7/day 25) and kept at −80 C° for further analysis. Climatic data during field retting was obtained from the closest 'Infoclimat' weather station (https://www.infoclimat.fr/observations-meteo/temps-reel/mazinghem/000RL.html) (Supplementary Fig. 1a; Supplementary Data 1). Soil samples were also collected from the three fields for all replicate plots and sent to the "Laboratoire d'Analyses des Sols d'Arras, ***INRAE*** (https://las.hautsdefrance.hub.inrae.fr/prestations)" to analyze soil composition (Supplementary Fig. 1b, Supplementary Data 1). At the end of retting, flax samples (4–5 kg) from all three experimental fields were collected and fibers extracted (scutching) by the company Van Robaeys Frères (Killem, Hauts-de-France) for industrial classification according to USTRL (Union Syndicale des Rouisseurs-Teilleurs de Lin) standards (Supplementary Fig. 2).

2.2. Enzymatic activities measurements

Procedures were mainly adapted from (Chabbert et al., 2020). Two grams of fresh flax stem outer tissue (comprising epidermis, parenchyma, bast fibers and phloem) were blended in 100 mL of 50 mM Tris-HCl buffer (pH7) for 1 min and filtered with a GF/A filter (pore size 1.6 μm). The freshly prepared soluble fraction was used for enzymatic assays. Cellobiohydrolase, β-D-glucosidase, β-D-galactosidase and β-D-xylosidase enzyme activities were assayed by measuring fluorescence release from cleaved 4-methylumbelliferone-ester substrates (Sauvadet et al., 2016). Endo-polygalacturonase enzymatic activity was measured following the DNS method (Miller, 2002; Bleuze et al., 2018). Peroxidase and phenol-oxidase activities were measured with L-3, 4-hydroxyphenylalanine as a substrate. All measurements were done in technical triplicate for five biological replicates (T1-T5) per retting stage (R0-R7) and average enzymatic activities were expressed by μmol or nmol g^{-1} dry weight h^{-1}.

2.3. Analysis of plant cell wall monosaccharides

Alcohol insoluble residues (AIR) were prepared from 2 g of starch-degraded samples (flax stem external tissues) for all retting points R0 (day 1) to R7 (day 25), following (Yeats et al., 2016). Hydrolysis of non-crystal matrix polysaccharides was done with mild 72 % sulfuric acid (Matrix hydrolysis), while crystalline cellulose was targeted with concentrated 4 % sulfuric acid (Saeman hydrolysis), followed by autoclaving for 1 h at 121°C. Monosaccharide compositions were determined by High-Pressure Anion Exchange Chromatography equipped with Pulsed Amperometric Detection (HPAEC-PAD) as described by

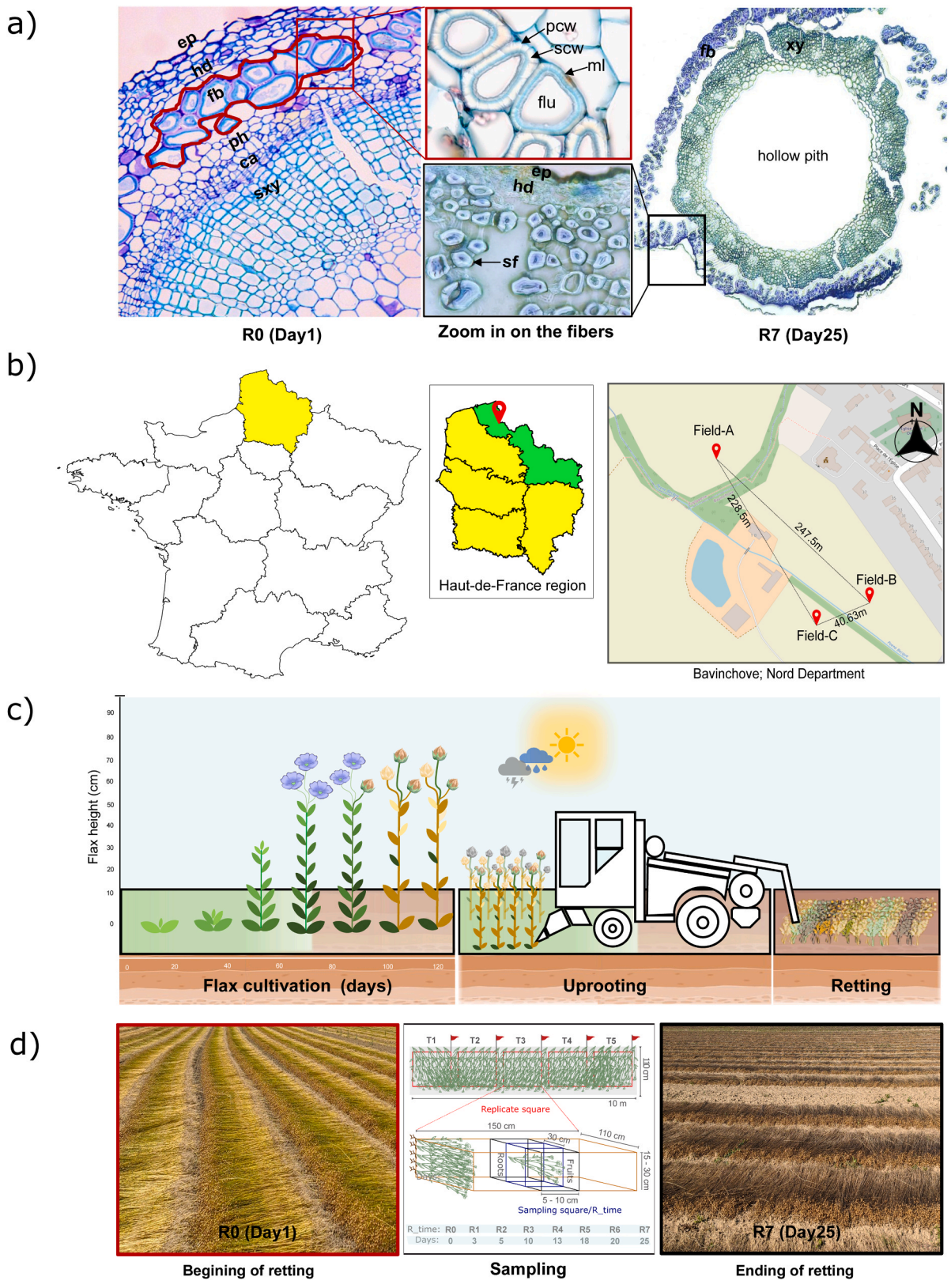

Fig. 1. Flax retting and experimental design. a) Cross sections of flax stem, included in Technovitt 7100, sectioned at 5 μm then stained with TBO (Toluidine blue O, 0.1 % w/mL) at the beginning (left, R0) and at the end (right, R7) of retting. Zoom-in sections of elementary fiber cells joined together in the fiber bundle (middle, upper) and separated because of retting (middle, lower). [ep = epidermis, hd = hypodermis, fb = fiber bundle, ph = phloem, ca = cambium, sxy = secondary xylem, pcw = primary cell wall, scw = secondary cell wall, ml = middle lamella, flu = fiber lumen, xy = xylem]. b) Geographical localization and mapping of the three experimental fields (A to C). c) Schematic representation of the main steps of flax fiber production from germination to retting. d) Flax stem sampling during the retting phase from day 1 to day 25 (R0 to R7).

(Yeats et al., 2016; Menna et al., 2020).

2.4. Preparation, and measurements for nano-LC-MS/MS-based metaproteomics

Choice of samples for metaproteomics — Four protein samples [day 1 (R0), day 6 (R2), day 13 (R4), and day 25 (R7), (fields A and B)] corresponding to key points during the retting process were chosen for metaproteomics: Points R0 and R7 correspond to the start (R0), and end (R7) of the retting process. The point R2 was chosen because this sample showed statistically significant modifications in the activities of five out of the seven enzymes evaluated (increased xylosidase, galactosidase and glucosidase activities; decreased peroxidase and phenoloxidase activities) compared to R0. The point R4 was chosen as it was the first point after swath turning that is generally considered by flax farmers to be an essential step during retting. This point would also be a good point for analysis by metaproteomics.

Extraction, quantification and preparation of protein samples — 100 mL of soluble fractions (prepared as described above, M&M 2.2) from three biological replicates (T1, T2 and T3) for the retting points R0, R2, R4, and R7 (i.e. 12 samples) were processed as described in (Goulas et al., 2001), with the exceptions that, prior to acidic precipitation, i) soluble fractions were concentrated into a final volume of roughly 2 mL with vivaspin-20centrifugal concentrator (10 kDa MWCO, Merck) and ii) protein quantification was performed according to Bradford using BSA as a standard (Bradford, 1976). Pellets were resuspended in 65 mM Tris-HCL buffer (pH 6.8) containing 20 % (v/v) glycerol, 2.3 % (w/v) sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT) and 0.04 % (w/v) bromophenol blue, boiled for 5 min to denature the proteins, and then cooled until required for electrophoresis. SDS-PAGE was denatured as described by Laemmli (1970), using a 5.5 % stacking gel and a 12 % separation gel in a Mini-PROTEAN Tetra Cell system (BIO-RAD). Equal amounts of 20 micrograms of protein were loaded per lane, according to the protein quantification results. After a 2 mm migration into the separation gel, migration was stopped and the gel was stained by using Quick Coomassie Stain (Generon, Slough, UK) according to manufacturer's instructions. The gel slice containing the concentrated proteins was excised, transferred into clean microtubes and stored at 4°C until further LC-MS/MS analysis.

Nano LC-MS/MS — Trypsin digestion was performed on each sample. Then peptides were extracted with 0.1 % formic acid in acetonitrile, evaporated to reduce volume at 8 µl and injected on an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific). Peptides were automatically fractionated onto a commercial C18 reversed phase column (75 µm × 500 mm, 2-µm particle, PepMap100 RSLC column, Thermo Fisher Scientific, temperature 55 °C). Trapping was performed during 4 min at 5 µL/min, with solvent A (98 % H2O, 2 % acetonitrile and 0.1 % formic acid). The peptides were eluted using two solvents A (0,1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) at a flow rate of 300 nL/min. Gradient separation was 3 min at 3 % B, 170 min from 3 % to 20 % B, 20 min from 20 % B to 80 % B and maintained for 15 min at 80 % B. The column was equilibrated for 17 min with 3 % buffer B prior to the next sample analysis. The eluted peptides from the C18 column were analyzed by Q-Exactive instruments (Thermo Fisher Scientific). The electrospray voltage was 1.9 kV, and the capillary temperature was 275 °C. Full MS scans were acquired in the Orbitrap mass analyzer over the m/z 400–1200 range with a 70,000 (m/z 200) resolution. The target value was 3.00E+06. Fifteen most intense peaks with charge state between 2 and 5 were fragmented in the higher-energy collision-activated dissociation cell with a normalized collision energy of 27 %, and the tandem mass spectrum was acquired in the Orbitrap mass analyzer with a 17,500 (m/z 200) resolution. The target value was 1.00E+05. The ion selection threshold was 5.0E+04 counts, and the maximum allowed ion accumulation times were 250 ms for full MS scans and 100 ms for tandem mass spectrum. Dynamic exclusion was set to 30 seconds.

2.5. Proteomic data analysis

Spectra analysis and identification of proteins — Raw data collected during nanoLC–MS/MS analyses were processed and converted into a *.mgf peak list format with Proteome Discoverer 1.4 (Thermo Fisher Scientific). MS/MS data were analyzed using search engine Mascot (version 2.4.0, Matrix Science, London, UK) installed on a local server. Searches were performed with a tolerance on mass measurement of 10 ppm for precursor and 0.02 Da for fragment ions, against a composite target-decoy database (372988*2 total entries) built with a Linum Uniprot database (taxonomy 4505, june 2022, 1404 entries), a Bacteria Swissprot database (taxonomy 2, june 2022, 335515 entries) and a Fungi Swissprot database (taxonomy 4751, june 2022, 35951 entries) fused with the sequences of recombinant trypsin and a list of classical contaminants (118 entries). Cysteine carbamidomethylation, methionine oxidation, protein N-terminal acetylation, and cysteine propionamidation were searched as variable modifications. Up to one missed trypsin cleavage was allowed. The identification results were imported into Proline software (http://proline.profiproteomics.fr) for validation. Peptide spectrum matches taller than 7 residues and ion scores >15 were retained. The false discovery rate was then optimized to be below 1 % at the protein level using the Mascot Modified Mudpit score. Protein abundance was measured by Extracted Ion Current based quantification with Proline 2.0. The data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD055634 and 10.6019/PXD055634.

Statistical analysis of resulting metaproteomic dataset — Protein abundances were log2 transformed and distributions were checked for normality. Since R0 sample group showed particular distributions compared to other groups of samples, this group was removed from the global dataset. Nevertheless, presence information for each protein was collected according to the following rule: a protein is considered present in R0 if, in at least two of the replicates, it shows an abundance value higher than the first quartile of the abundances of the whole dataset. Then distributions across samples of the remaining dataset (groups R2, R4 and R7) were normalized by the method of Quantiles using LIMMA procedure under R (https://www.bioconductor.org/packages/devel/bioc/vignettes/limma/inst/doc/usersguide.pdf). Proteins were then filtered for contaminants and assigned to field A and/or B according to the rule: a protein is considered present in a field if its abundance is higher than the first quartile of the dataset in at least two replicates of one of the retting points. Missing values at that stage represented less than 10 % of the total values and so imputed to simulate the mass spectrometer's limit of detection according to the Perseus method (Tyanova et al., 2016; Yu et al., 2020) with a mean minus 1.8 times the standard deviation and a width of 0.4 times the standard deviation as parameters. For each field, significant differences in protein abundances between time points were assessed by pairwise Welsh's t-test followed by a Benjamini-Hochberg FDR correction (p-value $\leq$0.05, cut off: 0.66 $\geq$ mean ratio $\geq$ 1.5).

Functional and taxonomic analysis of proteomes — For functional analysis, the metaproteome database was first annotated with Gene Ontology (GO) terms, using their UniProt ID and an in-house python script written to use the UniProt QuickGO API (https://www.ebi.ac.uk/QuickGO/api/index.htm). Information about protein, gene name, organism name and ID were retrieved from the UniProt database using the R package UniProt.ws. For identification of CAZymes in the metaproteome, a docker version of the dbCAN3 pipeline (https://bcb.unl.edu/dbCAN2/) was run and proteins were categorized as "CAZymes" when it was annotated by the three dbCAN3 tools HMMER, DIAMOND, and E-CAMI. HMMER annotations took priority over DIAMOND and E-CAMI tools. Enzymatic functions and target polymers (plant cell wall polymers) were assigned manually integrating CAZy information about families (http://www.cazy.org/), as well as the functional assignment of CAZy families reported in the literature (Chirania et al., 2022; Djemiel et al., 2017; Tláskal et al., 2021). The taxonomic information concerning

the organisms was reconstructed from the NCBI taxonomy (https://www.ncbi.nlm.nih.gov/guide/taxonomy/) using the "Taxizedb" package in an in-house script under R software. To examine statistical significance of categories of proteins (GO, CAZymes, by taxon) in retting time points, pairwise Welch's t-tests were performed between the considered R point and R2 (used as reference) followed by a Benjamini-Hochberg FDR correction. The log2 values of mean ratios are calculated and represented on heatmaps. All data representations were drawn using R software and specific packages: "ggplot2", heatmaps were constructed and drawn with "pheatmap" packages, Venn diagrams using the "ggVennDiagram" package, pie donuts with "webr" package, chord diagrams with "Circlize" package.

2.6. Other statistical analysis

For all quantitative data (except proteomic), data normality was assessed with the Shapiro-Wilk test and checked for equal variances among groups using Levene's test. Outliers identified by interquartile range (IQR) were removed, and any missing values (if present), were replaced with the mean of the group. A one-way analysis of variance (ANOVA) along with Tukey's HSD (Honestly Significant Difference) test was performed to assess the effect of the degree of retting on the stem's chemical composition and enzyme activity. To compare the retting dynamics between two fields, a pairwise T-test is performed with FDR (False Discovery Rate) correction of p-value by multiple comparisons.

3. Results

3.1. Inter-field variability exists in both fiber yield and industrial qualities

Measurements showed that differences in the stem and fiber contents existed between the three fields indicating the existence of inter-field variability in terms fiber extraction yield (Supplementary Data 1). Field A showed the highest fiber extraction yield compared to fields B and C which showed similar values (Fig. 2a). Industrial evaluation of fiber qualities using the USTRL (Union Syndicale des Rouisseurs-Teilleurs de Lin, https://www.usrtl-ifl.fr/) standard (Fig. 2b, Supplementary Data 1) indicated that fibers from field A show the highest values (6,6,4,3,3) in all evaluated categories (nature, color, resistance, fineness and homogeneity) (see Supplementary Fig. 2). The lowest values were obtained for field B (6,4,3,2,2), except for the 'nature' category where fibers from all 3 fields show the same value, and the 'homogeneity' and 'strength' categories where fibers from fields B and C have the same value (lower than field A) (see Supplementary Data 1). Overall, field C fibers show intermediate values. In the light of these results, it was decided to continue subsequent analyses of the retting process by comparing fields A and B which showed the most inter-field variability in terms of fiber yield and quality.

3.2. Small differences are observed in cell wall degrading enzyme activities between both fields

The activities of seven cell wall degrading enzymes were determined at several time points (R0 – R7) throughout retting in fields A (Fig. 3) and B (Fig. 4). For all enzymes, except for glucosidases, no obvious difference in terms of dynamics between the two fields is observed. For field A, Endo-polygalacturonase activity shows a high level at the beginning of retting before decreasing about 10-fold at day 11 (R3) (Fig. 3**a**). A similar pattern is observed for phenol-oxidase and peroxidase activities (Fig. 3**c**), although the decrease in peroxidase activity is more marked. The activities of xylosidase and galactosidase are initially low but increase at R2 until R4 before decreasing at R5 (Fig. 3**b**). Similarly, both glucosidase and cellobiohydrolase activities initially show low levels of activities before increasing to a peak at R2 (glucosidase) and R4 (cellobiohydrolase) (Fig. 3**d**). Comparing the two studied fields, the peak of glucosidase activity in field A seems to be reached as early as R2, whereas it is only attained at R3 in field B (Fig. 4). For certain enzymes (xylosidase, galactosidase, cellobiohydrolase and glucosidase), activity levels are significantly higher in field B compared to field A.

3.3. Cell wall polymer composition evolution in stem outer tissues is similar in both fields

In order to follow the degradation of cell wall polymers during retting, monosaccharide analyses were performed on hydrolyzed cell wall preparations (AIR) prepared from flax outer-stem samples. Our results (Supplementary Fig. 3 & 4) indicated that sample monosaccharide profiles are very similar in both fields. Statistical analyses (ANOVA test, p.value<0.05,) identified 5 monosaccharides (fucose, galactose, xylose, galacturonic acid, and arabinose) that showed statistically significant changes between retting points during the process of dew retting for both fields. The quantities of galactose, xylose, and galacturonic acid decreased gradually and smoothly by roughly 50 % during retting. In contrast, the amount of arabinose decreased more abruptly (by approx. 70 %) between R0 and R2. Finally, fucose decreased by 55 % between R4 and R7 (Supplementary Data 1).

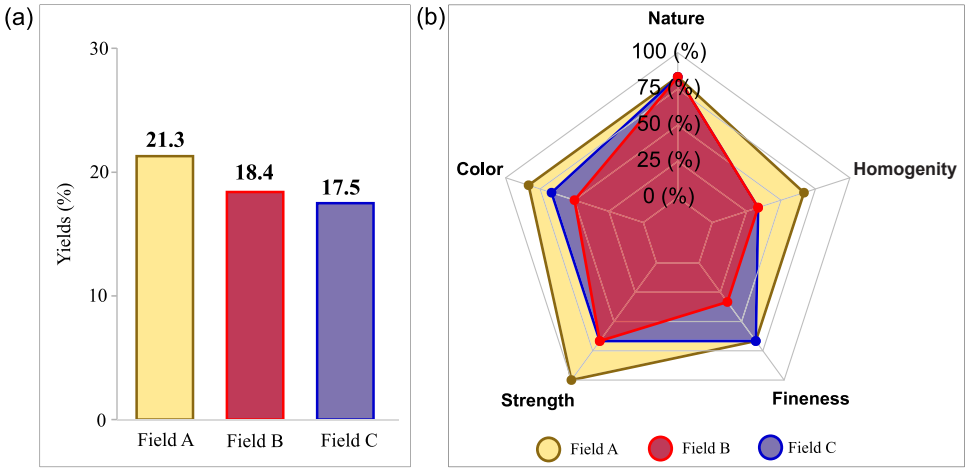

Fig. 2. Fiber yield and industrial quality assessments. a) Comparison of scutched fiber yield (in %) between three experimental fields (A to C). b) Comparison of industrially assessed retted fiber quality obtained from the three experimental fields (A to C). Individual notation for each parameter, i.e. nature, homogeneity, fineness, strength and color, is represented as a % of the possible maximum quotation of the USTRL notation grid (supp-Fig. 2).

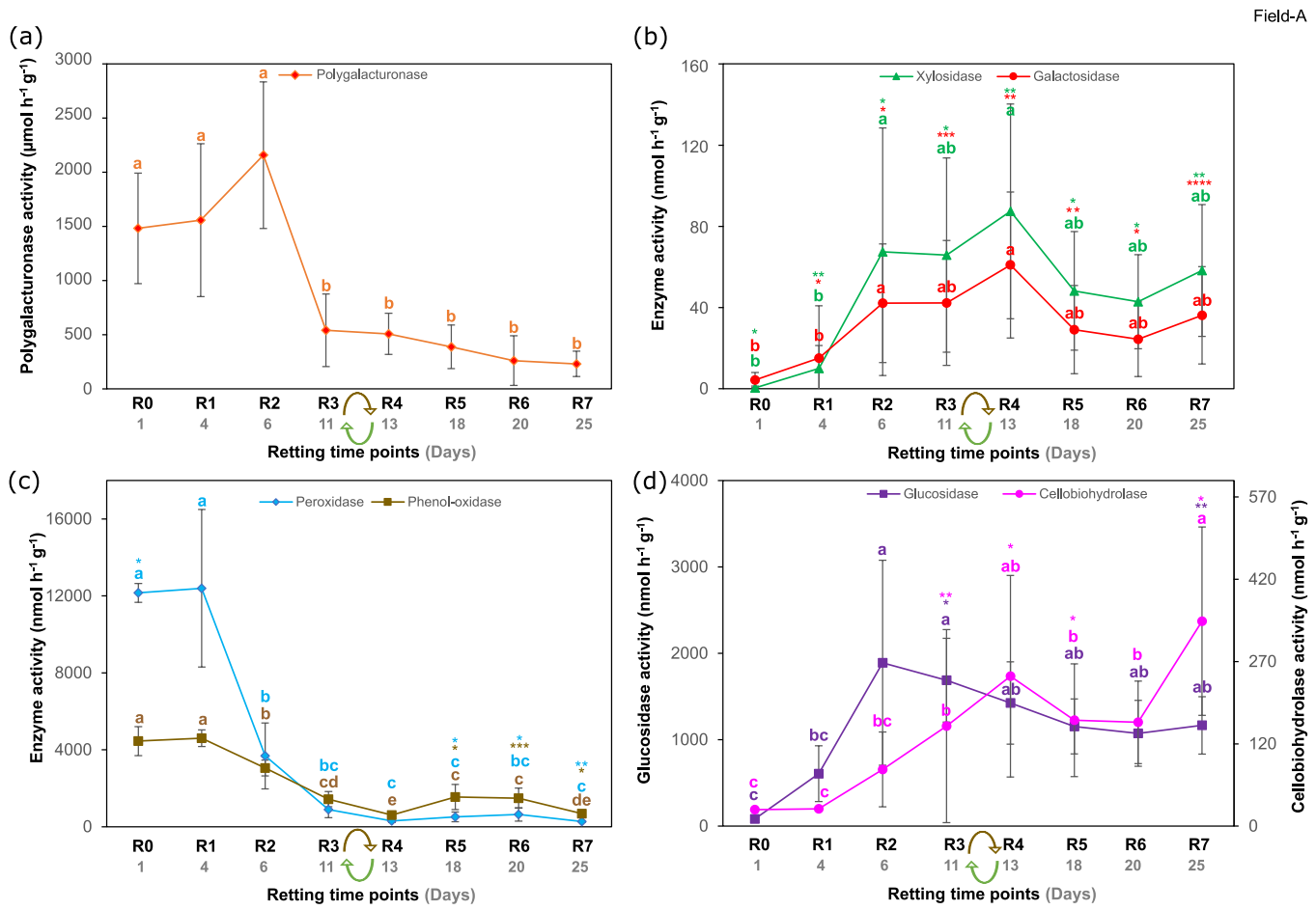

Fig. 3. Enzyme activity dynamics in outer tissues of flax stems during dew retting in field A. a) Polygalacturonase activity; b) Xylosidase and Galactosidase activities; c) Peroxidase and Phenol-oxidase activities; d) Cellobiohydrolase and Glucosidase activities. All enzyme activities are expressed in nmol.h^{-1}g^{-1} dry matter except polygalacturonase (µmol.h^{-1}g^{-1} dry matter); n = 5, with n being the mean of 3 technical replicates, Anova (Tukey's HSD) test between retting points are shown with different letter marking p-Value < 0.05; point to point comparison between two fields (A and B), tested with Pairwise T-Test (p.adjust: Fdr), p-Value < 0.05 are indicated with * (data and tests results are shown in supp-data 1). Brown/green arrows indicate swath turning.

3.4. Metaproteomics of key retting points identifies 6032 unique proteins

Protein identification and quantification was performed on selected samples [day 1 (R0), day 6 (R2), day 13 (R4), and day 25 (R7), (fields A and B)] corresponding to key points during the retting process (see Materials and methods). Analyses of all samples identified 7373 non-redundant proteins of which 102 were filtered as contaminants (mainly animal proteins; Supplementary Data 2). After normalization, proteins were further filtered to determine the effective metaproteome for field A (5702 proteins) and field B (5610 proteins). Overall, combined metaproteomes from field A and B led to the identification of 6032 unique proteins (Supplementary Table 1). The list of proteins, normalized values and information on whether proteins are present/absent at R0 is provided in Supplementary Data 2. 5280 proteins (88 %) are shared between the two fields, 422 proteins (7 %) are specific to field A and 330 proteins (5 %) to field B (Supplementary Fig. 5a).

3.5. Basic metabolism proteins are dominant in the metaproteome

Functional gene ontology (GO) assignation (https://geneontology.org/) was undertaken to provide an initial overview of the functional composition of the retting metaproteome. Full GO annotations by protein, counts of proteins by GO terms and filtering with GO slims metagenomic are presented in Supplementary Data 3. A comparison of the GO profiles for fields A and B shows that they are very similar (Fig. 5; Supplementary Fig. 6). Among the molecular functions identified, approximately 33 % are related to nucleic acid metabolism and 6 % are ribosomal components. Functions related to degradation activities, such as peptidase, lyase, and hydrolase represent only 1 %, 3.2 % and 2.7 %, respectively. In terms of processes, protein and nucleic acid metabolism dominate. Processes related to carbohydrate metabolism, which probably include enzymes involved in the degradation of plant polymers, account for about 7 % of the GO annotations. Interestingly, the photosynthesis process is still represented within both metaproteomes indicating the presence of plant proteins (mainly from flax) but also cyanobacteria proteins. About half of all proteins are associated with the cytoplasm, while 3.7 % and 1.4 % of proteins are associated with the extracellular and periplasmic compartments, respectively.

3.6. The metaproteome is mainly composed of bacterial proteins

A taxonomic analysis of the metaproteome based on the NCBI organism database (https://www.ncbi.nlm.nih.gov/taxonomy) (Supplementary Fig. 7, Supplementary Data 4) showed that the majority of proteins come from the bacteria kingdom (85 % for field A, 86 % for field B). Most of these proteins correspond to bacteria belonging to the *Pseudomonadota phylum* (71.6 % for both fields), which includes *Alpha, Beta and Gammaproteobacteria*, classes already identified in other studies on flax retting. Other phyla identified include *Bacillota* (around 7 % in both fields), *Actinomycetota* (5–6 %) and *Cyanobacteria* (around 3.2 %). Thirteen percent (field A) and 12 % (field B) of identified proteins are of fungal origin. Fungal proteins come mainly from the *Ascomycetes*

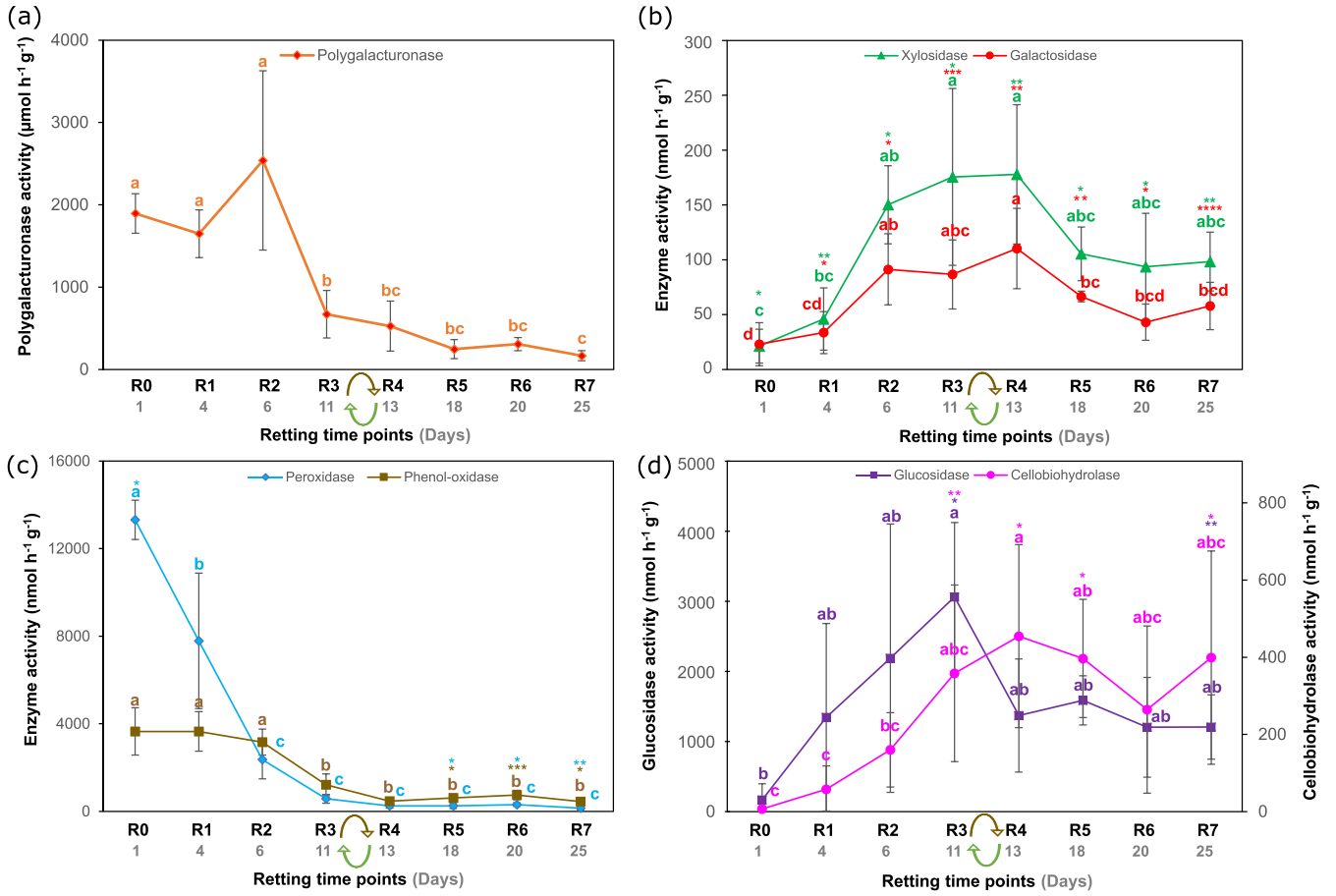

Fig. 4. Enzyme activity dynamics in outer tissues of flax stems during dew retting in field B. a) Polygalacturonase activity; b) Xylosidase and Galactosidase activities; c) Peroxidase and Phenol-oxidase activities; d) Cellobiohydrolase and Glucosidase activities. All enzyme activities are expressed in nmol.h^{-1}g^{-1} dry matter except polygalacturonase (µmol.h^{-1}g^{-1} dry matter); n = 5, with n being the mean of 3 technical replicates, Anova (Tukey's HSD) test between retting points are shown with different letter marking p-Value < 0.05; point to point comparison between two fields (A and B), tested with Pairwise T-Test (p.adjust: Fdr), p-Value < 0.05 are indicated with * (data and tests results are shown in supp-data 1). Brown/green arrows indicate swath turning.

phylum (around 91 %), and to a lesser extent from the *Basidiomycetes* phylum (around 7 %). Two percent of proteins come from plants (*Streptophyta*), mainly flax (Supplementary Fig. 7).

3.7. The abundance of about one-third of total proteins varies significantly during retting

Pairwise differential analysis was performed on three different combinations of protein groups (R2 vs R4, R2 vs R7 and R4 vs R7). R0 proteins were not included in the pairwise analysis due to their particular distribution as previously explained. 1913 and 1431 proteins showing significant differences (FDR p-values ≤ 0.05, 0.66 ≥ mean ratio ≥ 1.5) in abundance between the compared retting points were identified for fields A and B, respectively (Supplementary Data 2). Among these differentially abundant proteins, 711 (27 %) were identified in both fields A and B (Supplementary Fig. 5b). For each field, a list of the top 30 most over-expressed and most repressed proteins between the R2 and R7 retting points were generated (Supplementary Data 2). In the most over-expressed protein category for field A, the number one protein corresponds to an exoglucanase (cellobiohydrolase, Cel1) from an ascomycete parasite of *Poaceae* (*Cochliobolus carbonum*). A second exoglucanase (cellobiohydrolase, Cel2) from a basidiomycete (*Agaricus bisporus*), and a Beta-mannosidase (bmann9) from an ascomycete known for its cellulolytic activity (*Myceliophthora thermophila*) are also present in the over-expressed category. None of these proteins were present at the start of the retting process (R0). The repressed protein category for field A is dominated by flax proteins, including proteins related to pectin metabolism (pectinesterases pme5, pme3), and peroxidases (PER3, PER4) that are already present at R0. Regarding field B, it is surprising to observe that the top 2 proteins in the over-expressed category correspond to flax proteins encoding lignin/monolignol metabolism enzymes (CAD7 and CAD8). Nevertheless, two cellobiohydrolases, Cel1 and Cel2 from *Cochliobolus carbonum* and *Agaricus bisporus* are also present. In field B the repressed proteins are dominated by the same flax proteins (except for pme3) found in field A.

3.8. The metaproteome contains key cell wall degrading enzymes belonging to carbohydrate active enzymes (CAZy) families

All identified protein sequences were subjected to CAZy (Carbohydrate-Active enZymes) motif searches using dbCAN3. A total of 60 and 58 proteins presenting a CAZy motif were identified in fields A and B, respectively. Forty-four proteins (59 %) are common to both fields. The identified CAZymes are distributed in 31 families from all 5 different CAZy classes: GTs = glycosyl transferases, GHs = glycosyl hydrolases, CEs = carbohydrate esterases, PLs = polysaccharide lyases and AAs = auxiliary activities (Fig. 6 for field A and Supplementary Fig. 8 for field B, Supplementary Data 5). The percentage distribution of proteins in the different CAZy classes is quite similar between the two fields with GH representing the largest class (58 %) in both fields. Within the GH class,

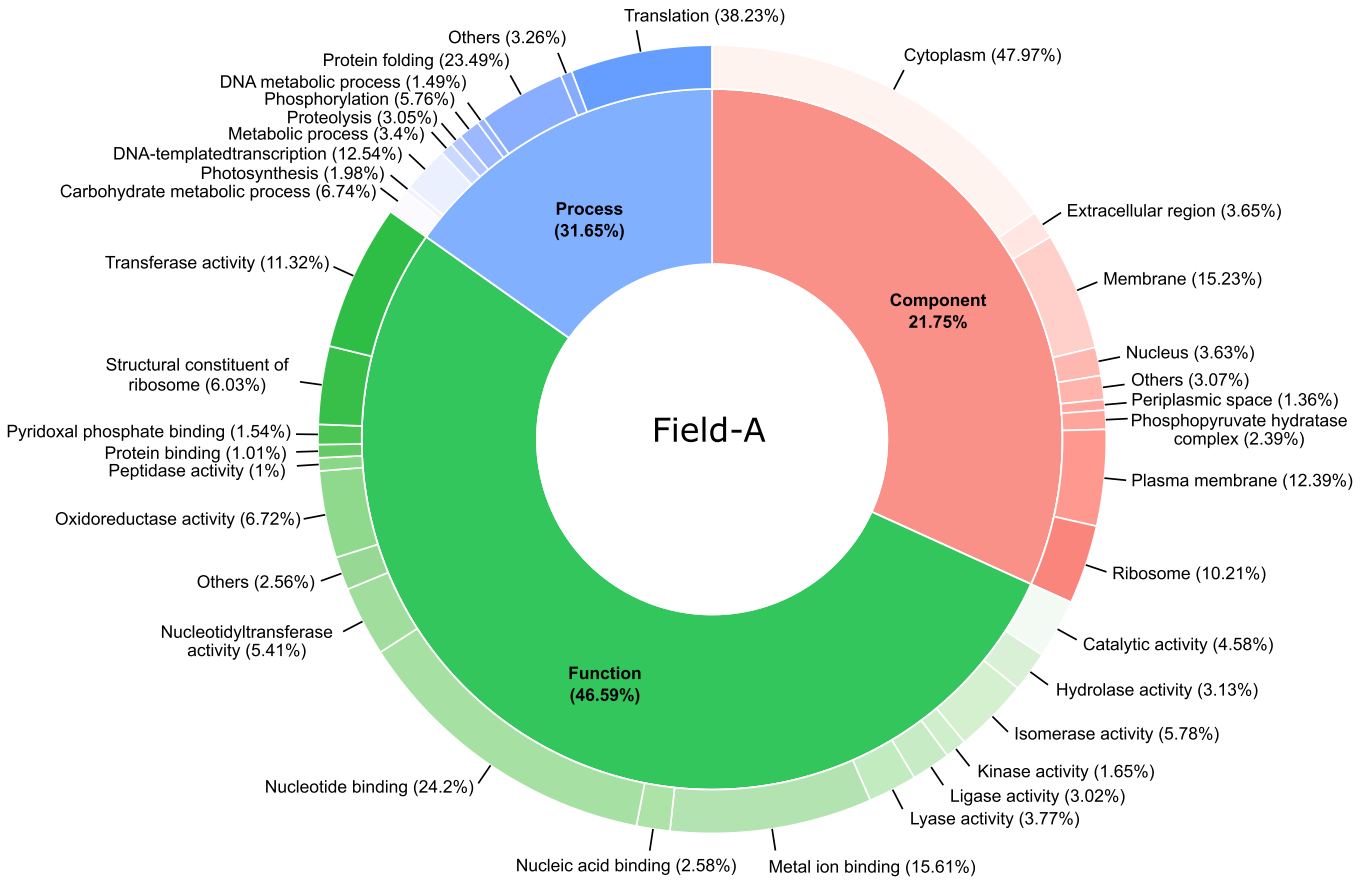

Fig. 5. Distribution of proteins by GO category according to GO aspects for field A. Identified proteins were annotated for GO aspects, sorted and counted by GO categories (Biological process in blue, Cellular components in red and Molecular function in green), and filtered according to GOslims metagenomic (https://www.ebi.ac.uk/QuickGO/). Percentages are presented in imbricated pie donuts chart.

family GH13 proteins generally associated with starch degradation represent the largest category (37 % field A, 44 % field B). The second most represented CAZy family is GH7 (around 17 % for both fields), whose activity is often associated with cellobiohydrolase-type exoglucanase activity. Other GH families associated with the degradation of plant wall polymers can also be identified for each field. It is interesting to note that the GH10, GH37, and GH31 families are only present in field B samples while the GH94, GH54, GH55, GH17 and GH18 families are only present for field A.

Polysaccharide lyases account for 8.3 % of CAZymes (field A) and 12 % (field B), while carbohydrate esterases represent 8.5 % CAZy proteins for both fields. These classes include enzyme families that degrade pectins. The GT (Glycosyl transferase) class accounts for a similar percentage value in both fields (18.3 % field A; 15.5 % field B) with the GT1 family representing over 60 % of identified GTs.

In order to further characterize the enzymes potentially involved in the degradation of plant cell walls, a manual annotation was carried out by integrating the UniProt annotation of each protein, the description of the CAZy families (http://www.cazy.org/) and the functional assignment of CAZy families according to the literature (Djemiel et al., 2017; Tláskal et al., 2021; Chirania et al., 2022). This approach allowed the selection of 31 proteins (field A) and 30 proteins (field B), each associated with an enzymatic activity and target polymer(s) (Fig. 7, Supplementary Data 5, Supplementary Table 2). Taken together, 36 non-redundant proteins belonging to 19 families putatively involved in the degradation of different plant cell wall polymers were identified including lignin (AA1), pectins (CE13_8, PL1_3, GH35, GH28), hemicelluloses (GH2, GH26, GH35, GH55, GH3, GH5, GH17) and cellulose (GH7, GH3, GH35, GH94).

Analysis of how the relative abundance of these enzymes evolved during retting revealed a number of different profiles according to the CAZy family/enzyme activity and target polymer (Fig. 7, Supplementary Data 5). The results firstly show that enzymes belonging to some of the CAZy families (GH5, GH17, CE8, CE13) were present from the very beginning (R0) of retting while others appeared later.

The total abundance of cellulose-degrading enzymes increases significantly from R2 to the end of the retting process for both fields. In this group, the most important CAZy family is GH7 (exo-glucanase/cellobiohydrolase), corresponding to six identified proteins, followed by GH3 (beta-glucosidase) with 3 enzymes targeting both cellulose and the xyloglucan hemicellulose backbone. In contrast to the GH7 group, the abundance of the GH3 group does not vary between R2 and R7. The only GH5 (endoglucanase) enzyme identified was already present at R0 in both fields, but only increased in abundance after point R2 in field A, but not field B. It is also noteworthy that the abundance of certain proteins involved in cellobiose degradation (AA3 - fields A and B; GH94 - field A only) increases sharply at the end of retting, most likely reflecting an increase in the abundance of cellulose-derived oligosaccharides.

With regards to hemicellulose metabolism, the mannosidase/mannanase group is the most represented, with 1 mannanase enzyme (GH26) and 3 beta-mannosidase enzymes (GH2). The abundance of proteins in the latter group increased significantly during retting in both fields. Two other enzymes classified in the GH17 (endoglucanase) and GH55 (beta-glucosidase) families are only found in field A. The abundance of both of these proteins decreased between R2 and R7. In contrast, 2 xylanase enzymes belonging to the GH10 family were only found in field B. The abundance of these proteins remained constant throughout retting. Finally, one enzyme belonging to the GH35 family (galactosidase) potentially targeting either hemicellulose or pectin was identified in both fields. The abundance of this protein decreased during

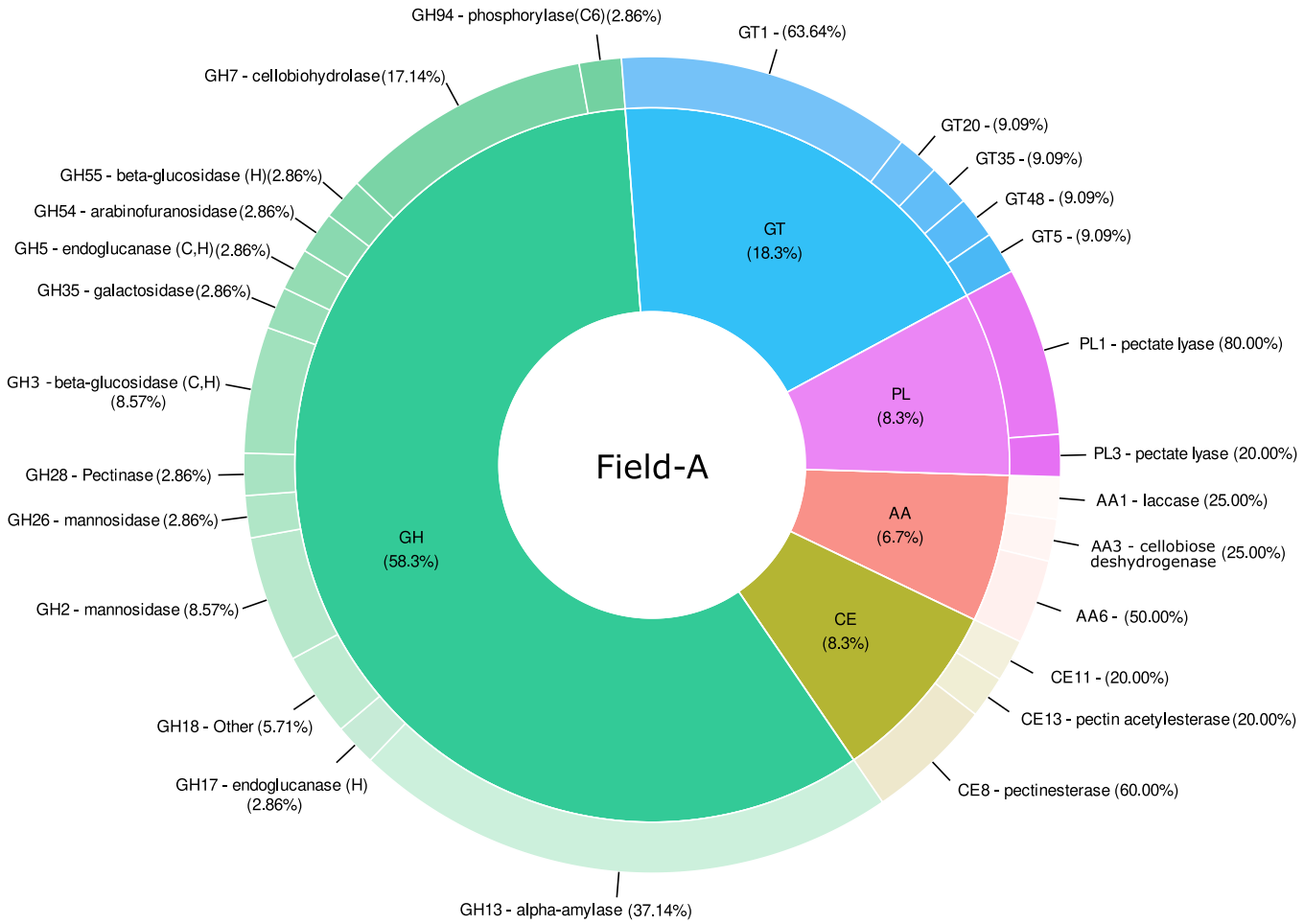

Fig. 6. Distribution of CAZymes identified in the metaproteome of Field A presented by class and families. Proteins within the flax metaproteome were sorted by the dbCAN3 pipeline (https://bcb.unl.edu/dbCAN2/) and are listed in Supp._Data 5. Percentages are presented in imbricated pie donuts chart.

retting in field A, but remained stable in field B.

The degradation of middle lamella and primary cell wall pectins is an important phase during retting. The results (Fig. 7, Supplementary Data 5) showing that the relative abundance of different proteins belonging to pectin-targeting CAZy families progressively decreases would suggest that pectin degradation takes place at the start of retting. The same pectin-targeting CAZy families are represented in both field A and B samples, and include CE13 (pectin acetylesterases), CE8 (pectin-esterases), PL1 and PL3 (pectate lysases), as well as GH28 (pectinases).

Metaproteomics also revealed other enzymes that potentially target components of the plant cell wall. Examples include arabinogalactan-proteins (AGPs), which are targeted by arabinofuranosidase (GH54), and lignin which could be targeted by laccases (AA1). These two enzymes are only found in the A-field samples. In contrast, a cutin hydrolase enzyme (CE5) was present in field B, but not field A samples. This enzyme might play a role in promoting flax stem penetration for certain fungi and bacteria at the beginning of the retting by degrading cutin in epidermal cell walls.

3.9. CAZymes involved in the degradation of plant wall polymers come mainly from fungi

As stated above, analysis of the total metaproteome for both fields showed that the majority of proteins (85 %) were of bacterial origin. In contrast, analysis of the CAZy proteins acting on cell wall polymers suggested that a little more than half of proteins were of fungal origin. Nineteen (61 %) out of 31 proteins in field A and 17 (57 %) out of 30 proteins in field B come from fungi (Supplementary Table 2).

All of the fungal CAZymes identified are produced by fungi belonging to the sub-kingdom *Dikarya*. Of these, the majority are *Ascomycota* (classes *Sordariomycetes*, *Saccharomycetes*, *Leotiomycetes*, *Eurotiomycetes*, and *Dothideomycetes*). The *Basidiomycota* are only weakly represented with a single GH7 CAZyme from *Agaricus bisporus* (class *Agaricomycetes*) (Fig. 8 and Supplementary Table 2). Among the fungi identified, some are classified as plant pathogens (*Fusarium oxysporum, Cochliobolus carbonum, Botryotinia fuckeliana*), while others are saprophytic (*Neurospora crassa, Myceliophthora thermophila, Agaricus bisporus*) or endo/epiphytes (*Aureobasidium pullulans*). It is a little more surprising to find species that are pathogenic for humans, even if they are known to be telluric (*Neosartorya fischeri, Aspergillus clavatus, Aspergillus Niger*).

Bacterial CAZymes acting on cell wall polymers are less represented with only 6 and 8 proteins identified for fields A and B, respectively. These proteins are mainly produced by two genera (*Pseudomonas* and *Pectobacterium*), that contain plant pathogens and/or saprophytes that are known to produce pectinolytic enzymes. The genus *Paenobacillus* is also present and is represented by the species *lautus* that is a ubiquitous bacillus living on both plants and animals, and well known for producing polysaccharide degrading enzymes. A little more surprising is the presence of the species *E. Coli* as a GH3 beta-glucosidase cell wall degrading enzyme producer, since this species is commonly associated with the lower intestine habitat. Nonetheless, *E. coli* has been reported to be able to survive in different types of habitats including soil, manure and water (van Elsas et al., 2011).

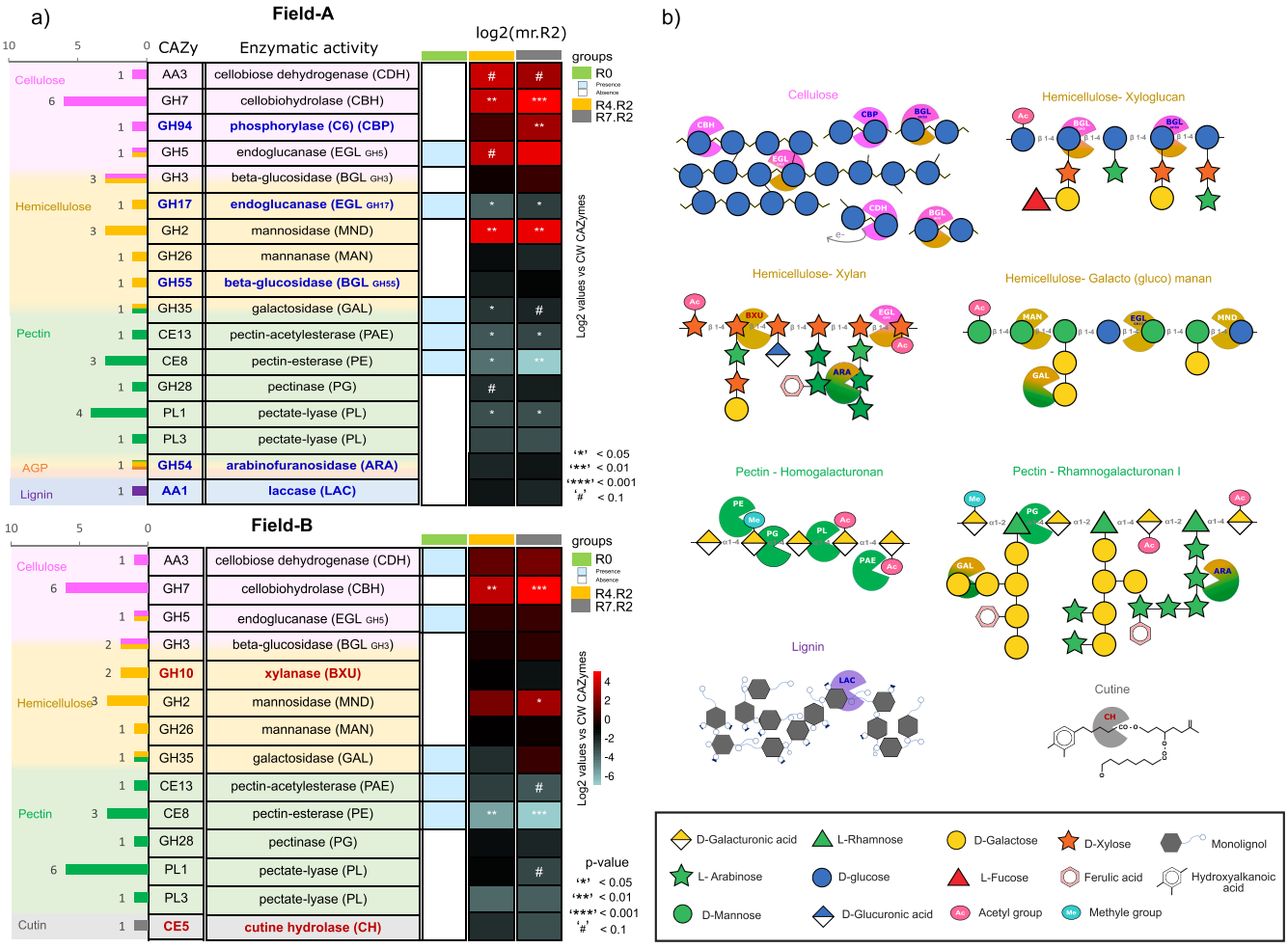

Fig. 7. Dynamic changes in CAZyme abundances during flax retting in fields A and B. a) CAZymes were identified within the metaproteome of field A and B and then manually assigned to an enzymatic function and polymer target. CAZymes were then grouped by polymer and enzymatic activities. Numbers in the left barplot diagram indicate the number of unique proteins grouped in each CAZy family. Enzymatic activities specific to field A are shown in blue and enzymatic activities specific to field B are shown in red. The first heatmap column shows presence (light blue) or absence (white) of CAZymes on day 1 (R0); the second and third columns show, respectively, the log2 mean ratio of cumulative abundance values of CAZymes (area under peak) of day 13 (R4) and day 25 (R7) compared to day 6 (R2); n =3, Welsch T-test with FDR. b) schematic representations of plant cell wall polymers and associated degrading enzymes identified in metaproteomes, enzyme abbreviations correspond to those indicated within brackets in (a).

4. Discussion

Northern France is known to be a favorable region for the growth and retting of fiber flax. Nevertheless, field-to-field variability in fiber quality can still occur even when the same biological variety is cultivated and processed in the same geographical location. In such a situation, plants from different fields are exposed to the same meteorological conditions and it is therefore unlikely that the observed variability is due to differences in plant physiology and fiber formation during growth. In an attempt to see whether such variability might be related to differences in the retting process, we used a biochemical approach combined with a novel metaproteomics strategy to characterize and compare the cell-wall-degrading arsenal in two adjacent fields (A and B) showing variability in both fiber yield and quality.

4.1. Biochemical analyses identify small differences in retting between fields A and B

A detailed biochemical comparison of cell wall degrading enzyme activities between the two fields during retting revealed that the dynamics were basically similar, although differences in overall activity levels were observed. In both cases, different dynamic profiles could be discerned, broadly according to the cell wall polymer targeted. Endo-polygalacturonase (targeting pectins) and phenol-oxidase/laccase (targeting lignin/polyphenols) activities showed peak activity at the beginning of retting while xylosidase (hemicelluloses) and galactosidase (hemicelluloses and pectins) activities peaked later (R2) before decreasing. Glucosidase (cellulose and hemicellulose) and cellobiohydrolase (cellulose) activities peaked afterward (R3-R4). Overall, these results are similar to the findings of other studies on both flax and hemp retting (Bleuze et al., 2018; Chabbert et al., 2020). The observed activity profiles correspond well with the timing of changes in flax stem anatomy that are known to occur during retting where degradation of the pectin-rich middle lamella is followed by the destruction of the primary cell walls in the cortex leading to the liberation of fiber bundles from surrounding tissues together with the progressive separation of elementary fibers from each other. The observed lignin/phenol-oxidase activity might be related to the degradation of phenolics in the cuticle of the epidermis and/or low amounts of lignin that can be present in the common compound middle lamella between neighboring bast fibers (Day et al., 2005; Lion et al., 2017). Swathe turning has previously been shown to significantly affect the microbiological consortium during retting (Djemiel et al., 2017), however global enzymatic profiling performed during this study did not reveal any significative effect of this

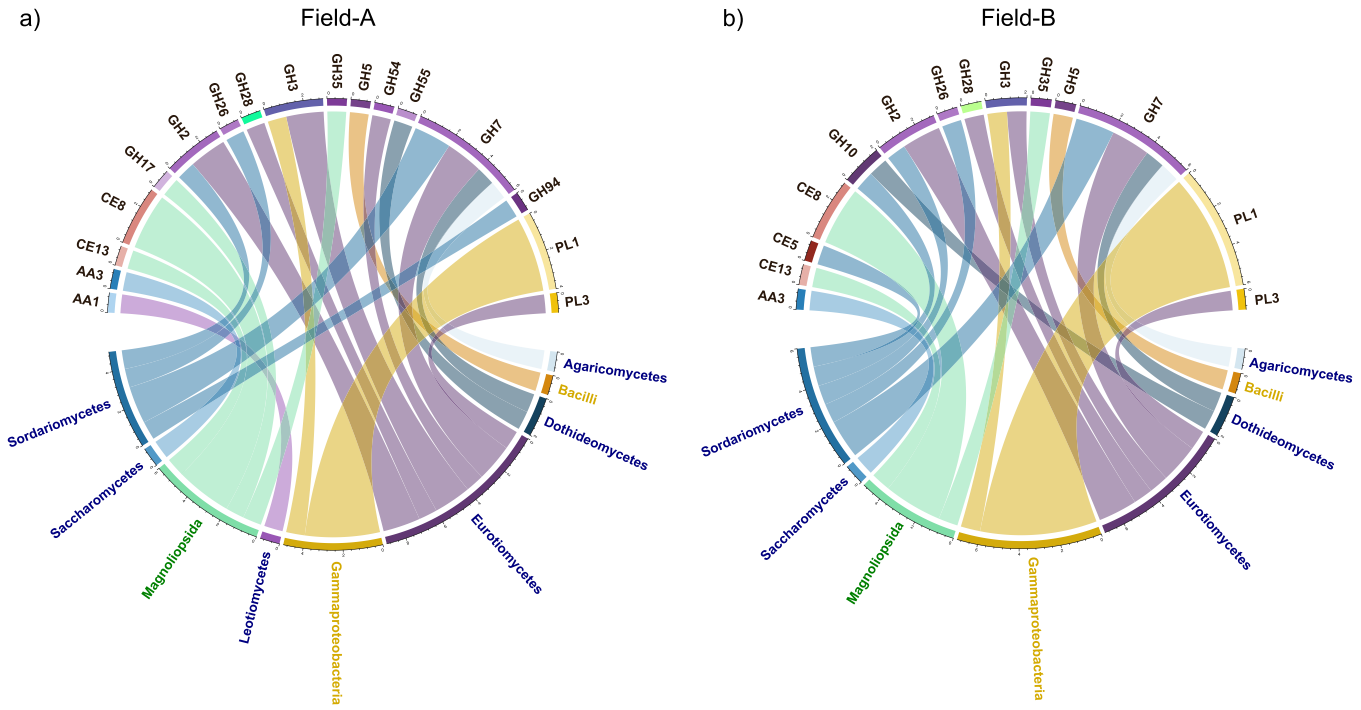

Fig. 8. Taxonomic origins of CAZymes associated with plant cell wall degradation identified in the metaproteomes of fields A and B. Chord diagrams show the microbial origin (at the class level) of CAZy families associated with flax cell wall polymer modification during retting in field A (a) and field B (b). CAZy families (in black) are connected to their microbial origin by color chords: Fungal classes are indicated in blue and are connected to their related CAZymes by blue or purple chords; bacterial classes are indicated in yellow and are connected to their related CAZymes by yellow or orange chords; plant (Magnoliopsida) CAZymes are connected with green chords.

practice.

Comparative compositional analyses of monosaccharides released from hydrolyzed cell wall polymer samples during retting showed very similar patterns for both fields as previously observed during flax or even hemp retting (Bleuze et al., 2018; Chabbert et al., 2020).

Taken together, these results would suggest that the process of retting is basically very similar between the two fields. Such a conclusion is logical given that the fiber quality and yield from both fields is comparable, but not identical. In the light of these observations, it is possible that the biochemical analyses classically utilized in this paper and by other workers to study retting are not sufficiently discriminatory and/or appropriate to detect the underlying parameters responsible for the observed differences. Indeed, the different enzymatic activities measured during retting in this work and previous studies correspond to the sum of the activities of the individual enzymes of a given type extracted from the samples. As an example, the "beta-glucosidase activity" shown in Figs. 3 and 4, is the sum of the activities of all of the different beta-glucosidase enzymes present in the sample extract. Such an approach therefore does not provide precise enough information about either the identity of the individual enzymes or the (micro) organisms producing such proteins during retting. In an attempt to improve our understanding of these points we previously reported the use of a meta-barcoding approach coupled with the use of the bioinformatic tool PICRUSt (Djemiel et al., 2017). This work identified a high number of new bacterial and fungal species associated with retting as well as predicting what bacterial cell wall degrading CAZymes *might* be present based upon the presence of certain species in the retting microbiome. However, as previously discussed (Djemiel et al., 2020), PICRUSt and similar approaches cannot confirm whether a protein is really present or not in the field.

Several recent studies have demonstrated the value of a metaproteomic approach in providing precise information on the different proteins responsible for enzymatic functions observed in a given habitat (Herbst et al., 2016), including those associated with the degradation of lignocellulosic materials (Hori et al., 2018; Chirania et al., 2022). We therefore decided to implement a metaproteomics approach to - i) identify specific cell wall degrading enzymes and associated microorganisms and, ii) evaluate whether such an approach could be powerful enough to detect any differences in the cell wall degrading arsenal of the two fields.

4.2. *Metaproteomics identifies specific proteins present during retting*

This approach identified 6032 non-redundant proteins (5702 proteins in field A and 5610 proteins in field B). Compared to other studies involving metaproteomics performed with the same technology, the number of proteins identified in this study is relatively high [(Callister et al., 2018): 4000, (Kipping et al., 2020): 1600, (Hori et al., 2018): 1964]. Nevertheless, other studies based on the analyses of a large number of samples, geographical locations and habitat types (soil, litter, rhizosphere…) have led to the identification of a much higher number of proteins [(Starke et al., 2021), 139127]. Depending upon the samples analyzed in this latter study, it was reported that the percentage of CAZymes present varied between 0.2 % and 1.4 %. These values are comparable to the 1.23 % (75 proteins) observed in our analyses. A much higher value (14 %) was recently reported in a metaproteomic study linked to the decomposition of plant matter, but in this case, the degradation was performed in fermenters where all fractions (liquid, planktonic and solid) were analyzed (Chirania et al., 2022). In our study we focused on the identification of proteins in the liquid extracts used for enzyme activity measurements. Although such an approach probably leads to the identification of a lower number of overall proteins, it offers the advantage of targeting those enzymes that are secreted and therefore most likely to be active in the stem tissues during retting.

Given that both bacteria and fungi are generally considered to be involved in retting, our results showing that - i) the great majority (85 %) of proteins identified in the metaproteome come from the bacterial kingdom and, ii) bacteria represent 80 % identified organisms are

somewhat surprising. Nevertheless, this latter figure is similar to the results of Starke and colleagues (Starke et al., 2021), who used a metaproteomic approach to study plant litter degradation and showed that bacteria made up between 65 % and 75 % of identified organisms. It is possible that such high values might be related to the overrepresentation of bacteria in the databases, which are based on the most studied organisms (Starke et al., 2019).

Despite such an apparent over-representation of bacterial proteins in the metaproteome, the strategy used in this paper is viable for a "functional" investigation of retting (i.e., identification of specific enzymes potentially involved in retting). This is clearly demonstrated by the fact that protein distribution between the different kingdoms becomes much more balanced when only CAZymes are considered with fungal, bacterial and *Streptophyta* proteins representing 41 %, 36 % and 21 % of the total protein content, respectively. When only cell-wall degrading CAZymes are considered (i.e., excluding starch-/glycogen-degrading enzymes) the percentage of fungal CAZymes increases to 61 % versus 19.5 % for bacteria. A similar preponderance of fungal CAZymes was also observed in a metaproteomic study of plant litter decomposition (Starke et al., 2021). In this case, fungal CAZymes represented more than 75 % of identified CAZymes. Although both situations involve the degradation of lignocellulosic biomass, the higher percentage value observed for fungal CAZymes in the latter case might be related to their different time-frames: a few months for plant litter and only a few weeks for retting. The shorter time involved for retting is unlikely to allow a complete colonization of the stalks by fungi, whose growth is slower than that of bacteria (Hicks et al., 2019).

Examination of the polymers targeted by the different identified CAZymes indicated that most of the enzymes involved in the degradation of cellulose and hemicelluloses come from fungi. Only 2 proteins (a GH5 endoglucanase and a GH3 beta-glucosidase) are of bacterial origin and 2 enzymes (a GH17 endoglucanase and a GH35 galactosidase) are flax proteins. In contrast, the majority (10 out of 13) of enzymes involved in the degradation of pectins belong to bacteria or flax. These results are similar to those obtained by Starke and colleagues (Starke et al., 2021), who also observed that fungal CAZymes are more associated with the degradation of recalcitrant polymers such as hemicellulose and cellulose, whereas bacterial CAZymes are more associated with the degradation of more accessible polymers such as pectins.

Overall, the bacterial CAZymes identified in this work correspond to those previously predicted to be present based on the use of the bioinformatic tool PICRUSt (Djemiel et al., 2017). Nevertheless, our metaproteomics approach offers two main advantages over PICRUSt. Firstly, and most importantly, PICRUSt is based on the use of rRNA16s sequences and it therefore does not consider fungal CAZymes (Langille et al., 2013). As shown in our study and others (Starke et al., 2021), plant cell wall degrading enzymes are mainly derived from fungi, and therefore predictions based on 16 s rRNA using PICRUSt-type tools can only provide a fragmented view of the CAZyme and its dynamics. Secondly, the predictive approach is based on the relative abundance of organisms as determined by OTU (Operational Taxonomic Units) counts. As such, it can only indicate whether organisms containing CAZyme genes in their genomes are present in the retting population. It does not provide any information about the level of expression of these genes, or indeed whether they are expressed. In contrast, the metaproteomics approach provides "real" information about both the presence of a given protein and its relative amount. It therefore becomes possible to establish much more realistic dynamic profiles of the different CAZymes during retting complementary to the analysis of enzymatic activity profiles.

4.3. Metaproteomics identifies plant enzymes potentially impacting cell wall structure

Of major interest is the fact that plant proteins, mostly from flax, represent 21 % total identified CAZymes. Such an observation raises the possibility that such (plant) enzymes might impact the structure of cell walls thereby contributing to the retting process and/or modifying fiber quality as previously suggested (Chabbert et al., 2020). This idea is highly plausible since flax plants are still alive when they are uprooted at the start of the retting process. Following uprooting, the plants (still living) will be exposed to abiotic and biotic stresses and will therefore react via the activation of multiple defense mechanisms including modifications to cell wall metabolism and structure (Houston et al., 2016). Among the plant CAZymes identified, certain are related to pectin metabolism, in particular pectin methylesterases (CE8) and pectin acetylesterases (CE13). These proteins are present at time point R0, still abundant at R2, and then decrease significantly at R4. Pectin-methylesterases (PMEs) catalyse the demethylesterification of homogalacturonan pectins (HGs) in the cell wall. Under blockwise action of PMEs, the blocks of demethylesterified HGs can interact with Ca^{2+}, promoting the formation of the so-called "eggs-box" structure and thus stiffening the cell wall (Shin et al., 2021). However, uncoordinated PME action can result in partially demethylesterified HGs that can become a target for pectin-degrading enzymes, such as polygalacturonases, affecting the texture and stiffness of the cell wall and increasing pathogen/saprotroph susceptibility (Levesque-Tremblay et al., 2015). Another flax enzyme involved in pectin metabolism and present in the metaproteome is the Beta-galactosidase BGal1 (GH35). This enzyme is present at the beginning of retting (R0 and R2) and decreases in abundance during the middle and end of retting. A previous study showed that BGal1 β-galactosidase activity is necessary for the dynamic remodeling of polysaccharides that occurs during normal secondary wall development in flax fibers, facilitating the formation of crystalline cellulose in developing fiber cell walls (Roach et al., 2011). The fact that this protein is present throughout retting raises the possibility that it may also play a role in modifying fiber structure post-harvest, modulating cellulose crystallinity and mechanical properties.

Our results also identified two other cell-wall related (non-CAZy) proteins that showed increasing abundance in field B during retting: cinnamyl alcohol dehydrogenase 7 (CAD7) and CAD8. These enzymes catalyse the last step in the formation of monolignols which are the monomers for lignin and lignans (Wang et al., 2013). Overexpression of *CAD* genes in flax has been associated with an increase in the presence of lignin precursor oligolignols, and lignans (Huis et al., 2012; Chantreau et al., 2014) known for their antimicrobial activities (Chhillar et al., 2021). Their expression is affected by both injury and stress and it is therefore not surprising to find these proteins in flax stems that have been uprooted, and subjected to the onslaught of pathogenic or saprophytic microorganisms. What is surprising, however, is the presence of these plant proteins at the end of the retting process. While care should be taken since protein presence does not necessarily mean enzyme activity (Campbell et al., 2016), the presence of these plant proteins during retting raises the question of whether retting can be considered as a "simple" degradation process, or whether it also involves plant cell wall remodeling that occurs after uprooting. Similarly, the presence of enzymes associated with the production of anti-microbial compounds (e.g., lignans) would suggest that plants have the ability to modify, post-harvest, the bacterial and fungal communities. In this context, metaproteomics is a powerful tool to gain a better understanding of these interactions, and it would be interesting to combine this approach with meta-metabolomic analyses (Wallenstein et al., 2010; Zhao et al., 2016).

4.4. Metaproteomics identifies new retting micro-organisms

Specific identification of individual proteins also allowed us to determine what microorganisms were present during flax dew-retting. Many of the CAZyme-producing bacteria identified in this study have already been identified in other studies, either based on a culture approach (Rosemberg, 1965; Sharma, 1986; Tamburini et al., 2003), or

by metabarcoding (Liu et al., 2015; Djemiel et al., 2017; Chabbert et al., 2020). Nevertheless, our results also identified, for the first time, CAZyme-producing bacterial species that have been previously associated with field composting such as *Pectobacterium carotovorum* (Suárez-Estrella et al., 2013) and *Paenibacillus lautus* (Vaz-Moreira et al., 2010) (Supplementary Table 3). For fungi, only five species out of the 15 identified were previously identified in other retting studies, including *Aspergillus niger* and *Aspergillus flavus* (Fila et al., 2001; Nykter et al., 2008; Ribeiro et al., 2015), *Fusarium oxysporum* (Henriksson et al., 1997; Koivula et al., 2004), *Bipolaris zeicola*, and *Botrytis cinerea* (Nykter et al., 2008; Djemiel et al., 2017; Chabbert et al., 2020) (Supplementary Table 3).

The taxonomic study of retting has greatly benefited these last years from metagenomic approaches such as 16S/18 S rDNA sequencing (Ribeiro et al., 2015), and even more from high throughput sequencing metabarcoding (Djemiel et al., 2017; Chabbert et al., 2020; Xu et al., 2022), although this latter is sometimes limited by the relative poorness of databases, especially for fungi (Starke et al., 2019). The identification of several new bacterial and fungal species in this study demonstrates that the metaproteomic approach, in addition to providing functional information, is also extremely powerful for generating novel taxonomic data. Additional functional studies (e.g., culture and addition of the microorganisms to a model retting system) would be necessary to be able to determine the extent to which these species contribute to retting. If these microorganisms are found to play a significant role, it may be possible to develop an antibody-based test to monitor their presence and levels during dew retting.

4.5. Metaproteomics potentially identifies the reasons for inter-field variability

One of the aims of this work was to assess the ability of metaproteomics to provide biological explanations for the differences in fiber quality observed at the end of retting in two contiguous fields A and B. Our results had indicated that the two characteristics that mainly differentiate fibers are fineness (field A > field B) and strength (field A > field B). Fineness is a parameter that depends directly on the ability of the elementary fibers to be separated, both from each other and from the surrounding tissue (Bourmaud et al., 2010; Chabbert et al., 2020). Proper decohesion of fiber cells requires degradation of the pectins and hemicelluloses, followed by the cellulose present in the primary walls of cortical parenchyma. Comparison of enzyme activity dynamics showed that polygalacturonase, beta-glucosidase and xylosidase activities peaked earlier in field A compared to field B. This pattern could be correlated with the abundances of certain CAZYmes abundances identified in the metaproteome: GH26, GH35, GH55 and GH3 showing maximum abundance before R4. An identified GH2 mannosidase also showed a more significant increase in abundance in field A compared to field B. Taken together, these results could suggest that hemicellulose degradation occurs more slowly in field B compared to field A. Although further investigation would be required it is plausible that the observed difference in fiber bundle fineness between the two fields might be related to differences in hemicellulose breakdown. Similarly, the general increase in the abundance of CAZymes involved in cellulose degradation (GH7, GH5, GH3) that occurs at the end of the decomposition process is more pronounced in field A than in field B. Such a difference would be expected to favor the degradation of primary cell walls in the stem cortex surrounding the fiber bundles, thereby accelerating fiber (bundle) release and dissociation and leading to increased fineness.

The observed differences in fiber bundle strength between the two fields may also be related to differences in fiber separation resulting from differential degradation of cell wall polymers. Better separated fibers would require less force to extract them from the remaining stem tissues during the subsequent mechanical extraction (scutching) thereby reducing physical damage that could negatively impact their strength (Andersons et al., 2011; Baley et al., 2020).

Metaproteomics also revealed the presence of field-specific CAZymes targeting different cell wall polymers. Proteins targeting cellulose (GH94), hemicellulose (GH17, GH55), arabinogalactan proteins (GH54) and lignin (AAI) were found in field A but not field B. In contrast, other proteins targeting hemicellulose (GH10) and cutin (CE5) were only found in field B. It is plausible that such field-specific differences may also contribute to the observed inter-field variability in fiber quality (Jeannin et al., 2024). Since fields A and B are geographically very close to each other (< 250 m), it is unlikely that major differences in meteorological conditions are responsible for the observed specificities (even though differences in microclimate could still play a role). Similarly, the history of field use was the same for both fields. It is possible that the observed variability might be related to small differences in soil composition between the two fields (Supplementary Fig. 1b, Supplementary Data 1). Samples from field B showed higher organic, clay and limestone levels that could modify the water-retaining capacity of the soil. These differences might have an influence on the composition of the microbial populations and associated enzyme activities in field B compared to field A.

Interestingly, further examination of the CAZyme-producing organisms in the two fields highlighted a number of "functional redundancies" (i.e., synthesis of the same CAZyme family protein by different organisms). For example, the polygalacturonase GH28 in field A was produced by *Aspergillus flavus* whereas in field B the GH28 CAZyme came from *Neosartorya fischeri*. These results would suggest that certain functions are universal during the retting process, but do not necessarily come from the same microbial consortium, even in very close fields under the same meteorology. Nevertheless, without an in-depth functional study of each enzyme, we cannot rule out the possibility that enzymes produced by different organisms belonging to the same CAZy family and performing the same enzymatic function may differ in their efficiency (Km, Vmax).

5. Conclusion and perspectives

In conclusion, this study has demonstrated that metaproteomics is a powerful tool for the identification of specific cell-wall degrading CAZymes underlying measured enzymatic activities during retting. This technique also allows the identification of organisms whose enzymatic functions are really involved in retting as compared to other approaches such as meta-barcoding that will identify any organisms present in the microbial population. The implementation of the metaproteomic approach as part of a multi-omic strategy including metatranscriptomics, meta-metabolomics and metabarcoding would lead to a much more complete understanding of the biology of retting as previously suggested (Djemiel et al., 2020). Such a strategy would also be useful for the study of retting in other fiber plant species (Aktar et al., 2024; Bou Orm et al., 2023; Datta et al., 2020; Ribeiro et al., 2015; Xu et al., 2022), as demonstrated in a recent study of the degradation of plant matter in forest litter (Starke et al., 2021).

As well as identifying fungal and bacterial CAZymes, this work also highlighted the possible contribution of plant enzymes to the overall process of cell wall modifications and polymer degradation that occur during retting. The comparative investigation of retting CAZymes in two adjacent fields also allowed us to address the question of inter-field variability in fiber quality. Our results pinpointed a number of key differences that may be responsible for such variability. Although further investigation is obviously necessary this work provides a credible path towards understanding the underlying reasons of the observed differences in fiber quality that are currently limiting the wide-scale utilization of plant fibers in diverse applications.

Finally, the use of metaproteomics in this study made it possible to identify certain CAZymes that are more specific to certain stages of retting regardless of their organism of origin. Such information should prove invaluable for the development of new efficient retting enzyme mixes to improve fiber quality as part of a biotechnological strategy (see

Akin, 2013 for a detailed discussion of enzymatic retting in flax). Nevertheless, although such approaches have yielded good quality fibers in extensive pilot schemes, they have yet to be adopted on an industrial scale, primarily because of the high cost of recombinant enzyme production (Manian et al., 2021). Alternatively, the identified CAZymes could be used as reliable markers for the development of enzymatic and/or antigenic tests embedded on "lab on a chip" type sensors thereby allowing flax farmers to apply agriculture 4.0. practices to flax growth and retting (Griesche and Baeumner, 2020; Fuller et al., 2022).

Author contributions

SM, SG and SH conceived the project and decided on the scientific strategy. PdA and the Van "Robaeys Frères" company provided fields for sampling and industrial fiber qualifications. SM and ASB made microscopy observations, SM and AC performed enzymatic activities. SM, AM and FK performed cell wall monosaccharides analysis. EG, SR, ASL JMS and SM performed metaproteomic analysis and peptide identifications. SG, DG and SM made bioinformatic and statistical analysis. This article was written by SM, SH and SG with substantial contributions from all co-authors.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alycia Menuge reports financial support was provided by Federation Lilloise de Mécanique - FED 4130. Dmitry Galinousky reports financial support was provided by College de France. Suvajit Mukherjee reports financial support was provided by European Union. Suvajit Mukherjee reports financial support was provided by I-SITE ULNE foundation (ANR). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.119907.

Data Availability

Data will be made available on request.

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